Genetic variation among *w*Mel strains of *Wolbachia pipientis* differentially rescues a *bag of marbles* partial loss of function mutant in *Drosophila melanogaster*

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Abstract

Wolbachia is an intracellular, maternally inherited endosymbiotic bacteria that infects over 65% of insects and manipulates their reproduction for its own transmission. In *Drosophila melanogaster, Wolbachia* genetically interacts with the adaptively evolving germline stem cell gene *bag of marbles* (*bam*). Since *Wolbachia* must enter the host female germline to propagate, one hypothesis is that *Wolbachia* and *bam* are in a genetic conflict for control of oogenesis. In order to understand if *Wolbachia* could be driving the adaptive evolution of *bam*, we must understand the nature of the genetic interaction between *bam* and *Wolbachia*. Previously, we documented that the *w*Mel strain of *Wolbachia* rescued the fertility and cytological ovarian defect of a *bam* hypomorphic mutant. However, this mutant was generated in a variable genetic background, and thus we have not been able to perform controlled experiments to further assess the interaction. Here, we used CRISPR/Cas9 to engineer the same single amino acid *bam* hypomorphic mutation and a *bam* null mutation into the w^{1118} isogenic background. We assess the female fertility of wildtype *bam*, a *bam* hypomorph/null mutant, and a homozygous *bam* hypomorphic mutant, each infected individually with 10 diverse *Wolbachia* variants. Overall, we find that the *Wolbachia* variants tested here do not generally increase bam^{+} female fertility, but they do rescue *bam* hypomorphic defects with variation in the effect size of some *w*Mel variants on female fertility. Therefore, both *bam* and *Wolbachia* interact in genotype-specific ways to modulate a critical fitness phenotype

Introduction

Wolbachia pipientis is a maternally inherited endosymbiotic bacteria that infects over 65% of insect species and manipulates reproduction in a myriad of ways in order to ensure its transmission through the female germline (Werren et al. 2008; Lindsey 2020; Ote and Yamamoto 2020). The phenotypes *Wolbachia* induces in its hosts include cytoplasmic incompatibility (CI), in which embryos of matings between infected males and uninfected females die; male killing, in which male embryos die; feminization of embryos; and manipulation of germline stem cell differentiation in order to increase female fertility (Werren et al. 2008). While *Wolbachia* manipulates its host to increase its own transmission, some *Wolbachia* also provide the host protection against viruses, increase fecundity, and increase longevity (Dedeine et al. 2001; Chrostek et al. 2013; López-Madrigal and Duarte 2020).

Understanding the genetic mechanisms that *Wolbachia* uses to manipulate its host has been of immense interest to both basic and applied fields of study. *Wolbachia* is of great interest as a control for disease vectors such as mosquitoes due to its ability to sweep through a population (due to CI) and then protect the insect from viruses that can also cause human illness such as Dengue, Zika, and Chikungunya (Moreira et al. 2009; Hoffmann et al. 2011; Dutra et al. 2016). However, since *Wolbachia* is an obligate endosymbiont and cannot be cultured, it has been difficult to perform genetic studies of *Wolbachia* function to understand the mechanisms of these host-microbe interactions. Over the past few years, multiple groups have utilized bioinformatic and in vitro screens to identify candidate *Wolbachia* loci that modulate *Drosophila* phenotypes (Ote et al. 2016; Le Page et al. 2017). These loci have then been expressed in *Drosophila* as transgenes. Through these methods, the *Wolbachia* genes

67 *cifA* and *cifB* (orthologs in *w*Mel and *w*Pip) that cause CI have been identified and functionally
68 validated in *Drosophila* (Beckmann et al. 2017; Le Page et al. 2017). These transgenic tools
69 have been used to further define the CI phenotype (Shropshire et al. 2018), investigate the
70 consequences of genetic variation at *cifA* and *cifB* on CI (Shropshire et al. 2021), and identify
71 other host phenotypes affected by *cifA* and *cifB* (Deehan et al. 2021). Another group has
72 identified the *Wolbachia* TomO locus which is one of likely multiple *Wolbachia* loci that interact
73 with *Sex lethal*, a germline stem cell (GCS) gene that controls GSC maintenance and sex
74 determination (Ote et al. 2016). However, it is unclear the extent to which *Wolbachia* genetic
75 variation affects host phenotypes.
76
77 Previously, we reported that the *Wolbachia* variant *w*Mel genetically interacts with a *Drosophila*
78 *melanogaster* GSC gene, *bag of marbles* (*bam*), to rescue the fertility defect caused by a
79 single amino acid mutation (Flores et al. 2015). *Bam* is the switch for GSC daughter
80 differentiation in *Drosophila melanogaster* females and the switch for terminal spermatocyte
81 differentiation in males (McKearin and Spradling 1990; Insco et al. 2012; Ting 2013). Although
82 *bam* function is essential for gametogenesis, and *bam* is both necessary and sufficient for
83 GSC daughter differentiation, we observe that it is evolving under positive selection for amino
84 acid divergence in an episodic manner across the *Drosophila* genus (Bauer DuMont et al.
85 2007; Choi and Aquadro 2014). We see a particularly strong burst of amino acid changes at
86 *bam* in the *Drosophila melanogaster* lineage (Bauer DuMont et al. 2007). This episodic pattern
87 of positive selection is consistent with selective pressures that are present in some lineages
88 and not others and that may come and go over time. This is similar to the nature of infections
89 in natural populations, where *Wolbachia* variants are known to infect populations, but also can

90 be lost and replaced fairly often and on both long and short time scales (Bailly-Bechet et al.
91 2017).
92
93 Our observation that *Wolbachia* and *bam* genetically interact led us to hypothesize that
94 *Wolbachia* may drive the adaptive evolution of *bam* (Flores et al. 2015). Since *Wolbachia* is a
95 known manipulator of reproduction, *Wolbachia* may be in conflict with *bam* and other GSC
96 genes for control of oogenesis. While it may seem favorable to host fitness if *Wolbachia*
97 manipulates germline stem cell regulation to increase reproduction, and thus not present an
98 evolutionary conflict, it may not be beneficial for the host if oogenesis is regulated by
99 *Wolbachia*. For example, *Wolbachia* may promote oogenesis when it is not favorable for the
100 host to reproduce due to environmental or physiological factors. To further understand if
101 *Wolbachia* could be in genetic conflict with *bam* and drive its adaptation, we believe it is
102 necessary to define the genetic mechanism of the interaction between *bam* and *Wolbachia*.
103
104 Since we cannot culture *Wolbachia*, we made use of a genetic tool for functionally assessing
105 *Wolbachia* variation generated by Chrostek et al. (Chrostek et al. 2013), a set of w^{1118} isogenic
106 lines individually infected with 10 diverse *w*Mel *Wolbachia* variants from three clades defined
107 by Richardson et al (Richardson et al. 2012). These *Wolbachia* variants are documented to
108 differentially protect the *Drosophila melanogaster* host from viruses and increase cold
109 tolerance (Chrostek et al. 2013; Truitt et al. 2019). Additionally, these *Wolbachia* variants
110 exhibit different titer phenotypes in *Drosophila melanogaster* males (Chrostek et al. 2013). To
111 utilize this tool to further analyze our initial observation that *w*Mel rescues a *bam* hypomorphic
112 mutant, we used CRISPR/Cas9 to edit the original hypomorph amino acid change (Ohlstein et

113 al. 2000) into the w^{1118} isogenic background, and to create a *bam* null allele in the same
114 genetic background. We confirmed that the amino acid replacement results in the *bam*
115 hypomorphic phenotype previously described when expressed over a *bam* null allele in
116 uninfected females (Flores et al. 2015). We also show that uninfected females homozygous for
117 the *bam* hypomorphic allele do not exhibit GSC tumors or show reduced fertility compared to
118 wildtype females. We then assessed the effect of infection by the individual *Wolbachia* variants
119 by crossing the cytoplasmic maternal backgrounds infected with each *Wolbachia* variant into
120 our w^{1118} *bam* hypomorph line thereby maintaining the same nuclear background (except for
121 the *bam* locus).
122
123 We find that all *w*Mel *Wolbachia* variants tested here generally increase the fertility of *bam*
124 mutant females, with *w*MelCS-like *Wolbachia* showing the highest effects. In contrast, in
125 wildtype *bam* backgrounds *w*Mel variants do not increase fertility, and some even decrease
126 fertility.
127

128 # Materials and Methods

129 **Fly stocks and rearing:**

130 Prior to experiments, we raised fly stocks on standard cornmeal molasses food at room
131 temperature. We used yeast glucose food for fertility assays. During experiments, we
132 maintained crosses and stocks in an incubator at 25°C with a 12-hour light-dark cycle. The
133 $bam^{\Delta 59}$ line and bam^{BW} line are described on Flybase (Thurmond et al. 2019) and in Flores et.
134 al (Flores et al. 2015). The *Wolbachia* infected w^{1118} isogenic lines used in this study were
135 generous gifts from Luis Texiera and described in (Chrostek et al. 2013). We used CantonS

136 males for the w^{1118}; bam^{L255F}/bam^{null} and w^{1118}; bam^{+}/bam^{+} fertility assays. We used w^{1118}
137 isogenic males for the w^{1118}; bam^{L255F}/bam^{L255F} fertility assays. We verified that males were
138 uninfected with *Wolbachia* by endpoint PCR and qPCR using primers described below. To
139 generate the *bam* hypomorphic lines infected with the 10 different *Wolbachia* variants, we
140 crossed females from the w^{1118}; TM2/TM6 stock infected with each variant to w^{1118};
141 bam^{L255F}/TM6 males.
142
143 **Generation of *bam* alleles with CRISPR/Cas9:**
144 We engineered the two alleles described in this study in the w^{1118} isogenic background using
145 synthetic gRNAs and Cas9 protein as follows.
146
147 gRNAs:
148 We used the flyCRISPR target finder to choose gRNAs with zero predicted off-targets in the *D.*
149 *melanogaster* genome (Supplement Table S1). We then ordered synthetic gRNAs (sgRNA)
150 from Synthego.
151
152 Cloning:
153 We generated all PCR products for cloning with NEB High Fidelity Q5 master mix, and gel
154 extracted and purified PCR products using the Qiagen MinElute gel extraction kit. We created
155 the donor plasmid for homology directed repair using the NEB HiFi assembly cloning kit and
156 the pHD-attP-DsRed vector from flyCRISPR (Gratz et al. 2014). For plasmid prep, we used
157 Qiagen plasmid plus midi-prep kit and sequenced plasmids with Sanger sequencing (Cornell

158 BRC Genomics Core). We ordered primers for PCR, sequencing, and cloning from IDTDNA
159 (Supplement Table S2).
160
161 Donor sequences:
162 To generate the hypomorphic *bam*^{L255F} allele we designed a single stranded oligo donor
163 (ssODN) that contained the single amino acid mutation of the previously described *bam*
164 hypomorphic allele (CTT->TTT) as well as a single synonymous change to the closest
165 preferred codon in order to kill the gRNA site upon homology directed repair. We used 80 bp of
166 homology on each side of our targeted change (Supplement Table S1).
167
168 To generate the null *bam*$^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$ allele, we amplified 1.5 kb homology arms from the
169 *w*^{1118} isogenic line and cloned them into the pHD-attP-DsRed vector from flyCRISPR
170 (Supplement TableS3). As we did not need the attP site in our line, we amplified the 3xP3-
171 DsRed cassette from the plasmid and assembled the homology arms, 3xP3-DsRed, and the
172 original vector backbone with the NEB HiFi assembly kit, thereby removing the attP site.
173
174 Injections:
175 All CRISPR/Cas9 injections were sourced to Genetivision and were done in the *w*^{1118} isogenic
176 line. The injection mix contained plasmid or ssODN donor, sgRNAs, Cas9 protein (Synthego),
177 and an siRNA for Lig4 (IDT DNA, Supplement Table S1).
178
179 For the *bam*$^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$ allele, we screened for the eye color cassette in F1's in house using
180 a Nightsea fluorescent system (DsRed filters). For the *bam*^{L255F} allele, the edited nucleotide

181 change disrupts an AflII restriction site, allowing us to screen F1's by PCR (Promega GoTaq
182 mastermix) followed by restriction digests with AflII (NEB). We prepared genomic DNA using
183 the Qiagen PureGene kit.
184
185 We backcrossed females of all CRISPR/Cas9 mutants to w^{1118} isogenic males for three
186 generations, and then crossed the mutants to the w^{1118}; TM2/TM6 line to maintain the *bam*
187 mutants over the TM6 balancer. All CRISPR/Cas9 edits in the lines were confirmed by Sanger
188 sequencing (Cornell BRC Genomics Facility).
189
190 **PCR assays to detect *Wolbachia*:**
191 To test for the presence of *Wolbachia*, we used the Zymo quick DNA miniprep kit to prepare
192 DNA from three replicate samples each with three female flies. For each sample, we used
193 three different primers for endpoint or qPCR (Supplement Table S1). We used the common
194 *wsp* primers (Flores et al. 2015), a primer pair targeting *DprA*, and a highly sensitive primer
195 pair to the ARM repeat ((Schneider et al. 2014) for endpoint PCR, modified here for qPCR)
196 (Supplement Table S2).
197
198 **Immunostaining:**
199 We performed immunostaining as described in Aruna et al. (Aruna et al. 2009) and Flores et
200 al. [13]. Briefly, we dissected ovaries in ice cold 1X PBS and pipetted the tissue up and down
201 to improve antibody permeability. We fixed ovaries in 4% paraformaldehyde (EMS), washed
202 with PBST (1X PBS, 0.1% Triton-X 100), blocked in PBTA (1X PBS, 0.1% Triton-X 100, 3%
203 BSA) (Alfa Aesar), and then incubated in the appropriate primary antibody overnight. We then

204 washed, blocked, and incubated in the appropriate secondary antibody for two hours, then
205 washed and mounted in ProLong Glass NucBlue for imaging (ThermoFisher). We used an
206 anti-Vasa antibody from Santa Cruz biologicals (anti-rabbit, 1:200). We imaged ovaries on a
207 Zeiss i880 confocal microscope with 405 and 488 nm laser lines at 40X (Plan-Apochromat 1.4
208 NA, oil) (Cornell BRC Imaging Core). We analyzed and edited images using Fiji (ImageJ).
209
210 **Assays for nurse cell positive egg chambers:**
211 We dissected ovaries from mated 2-4 day old $bam^{L255F}/bam^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$ females for all
212 *Wolbachia* variants and the uninfected control in PBS. Ovaries were fixed in 4%
213 paraformaldehyde (EMS), washed with PBST (1X PBS, 0.1% Triton-X 100), and then mounted
214 in ProLong Glass with NucBlue (ThermoFisher). We imaged ovaries on a Zeiss i880 confocal
215 microscope with a 405 nm laser line at 10X (C-Apochromat 0.45 NA, water) and 40X
216 (described above). We analyzed and edited images using Fiji (ImageJ). We counted the
217 number of nurse cell positive egg chambers (cysts) per ovary for uninfected females and
218 females infected with each *Wolbachia* genotype (as these ovaries are small and
219 underdeveloped, they stay intact during the fixation and washing steps).
220
221 **Fertility Assays:**
222 We used the following w^{1118} isogenic *bam* genotypes for the fertility assays, w^{1118}; bam^{+}/bam^{+},
223 w^{1118}; $bam^{L255F}/bam^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$, and w^{1118}; bam^{L255F}/bam^{L255F}. Additionally, we performed a
224 small control fertility assay using combinations of the alleles described above and the classic
225 bam^{BW} hypomorph and $bam^{\Delta 59}$ alleles.
226

227 We performed the fertility assays for w^{1118}; bam^{+}/bam^{+} and w^{1118}; $bam^{L255F}/bam^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$
228 in three batches to reduce technical error from too large of an experiment. We included
229 separate uninfected controls in each batch of *Wolbachia* variants and used these to make
230 statistical comparisons of mean progeny per female.
231
232 We performed all fertility assays (except for the small bam^{BW} and $bam^{\Delta 59}$ analysis) as follows
233 (and described by Flores et al. (Flores et al. 2015)):
234 We collected virgin females and aged them 2-3 days, only using flies that eclosed within 48
235 hours of each other to reduce developmental variation. We collected virgin males uninfected
236 with *Wolbachia* and aged them for 2-3 days. We distributed males from different bottles across
237 the female genotypes to control for any bottle effects. We individually crossed virgin females to
238 two virgin males. The trio were allowed to mate for 8 days, and then flipped to new vials. After
239 the second 8 days the trio was cleared. The progeny for each trio was counted every other day
240 to get the total adult progeny per female. Progeny per female is reported in increments by day
241 that reflect the days after the first progeny eclosed. For example, we report total progeny for
242 days 1-9 which are the progeny counted on day 1 of eclosion to day 9 of eclosion.
243
244 For the control fertility assay containing the bam^{BW} hypomorph and $bam^{\Delta 59}$ alleles, we
245 collected and aged flies as described above, except the progeny per trio was counted only
246 once at the end of the experiment.
247
248
249

250 **Egg Laying Assay:**

251 We collected virgin females for each genotype and *Wolbachia* variant and CantonS virgin
252 males and aged them as described above for the fertility assays. We allowed each trio to mate
253 for 24 hours on grape juice agar supplemented with yeast before flipping them to a new grape
254 juice agar vial. We counted the eggs laid within 24 hours and repeated this for three days.

255

256 **Statistics:**

257 For the nurse cell assay, egg laying assay, and fertility assays we used estimation statistics to
258 assess the mean difference (effect size) of nurse cells, eggs, and adult progeny between
259 infected and uninfected lines. All estimation statistics were done using the dabest package in
260 Python (v. 0.3.1) with 5000 bootstrap resamples (Ho et al. 2019). Estimation statistics provide
261 a non-parametric alternative to other statistical tests of the difference of the mean (for
262 example, ANOVA), and allow us to understand the magnitude of the effect of *Wolbachia*
263 variation on the *bam* phenotype. We display the data with a swarm plot that shows all of the
264 data points and a Cumming estimation plot that shows the effect size for each sample
265 compared to the control and 95% bootstrap confidence interval. In text, we report significance
266 as a mean difference (effect size) outside the 95% bootstrap confidence interval. While one
267 benefit of these statistical methods is that they are less biased than reporting traditional P-
268 values, we also report P-values for an approximate permutation test with 5,000 permutations.

269 **Data Availability:**

270 Fly lines and plasmids used in this study are available upon request. The authors affirm that all
271 data necessary for confirming the conclusions of the article are present within the article,
272 figures, and tables.

Results

bam^{L255F} is a hypomorphic *bam* allele

We used CRISPR/Cas9 to recreate the same *bam* hypomorphic mutation as the original *bam*^{BW} allele (Ohlstein et al. 2000) in the isogenic *w*^{1118} background, of which we have infected with 10 different variants of *Wolbachia* as described by Chrostek et al. (Chrostek et al. 2013) (Fig 1A&B, Fig 2A).

We asked if the *w*^{1118}; *bam*^{L255F} allele recapitulated the *bam*^{BW}/*bam*$^{\Delta 59}$ mutant phenotypes by assessing the phenotype of a transheterozygous *w*^{1118}; *bam*^{L255F} hypomorph over a *bam* null allele. Since we wanted all of our alleles in the same genetic background and there were no existing *bam* null alleles in the *w*^{1118} isogenic background, we used CRISPR/Cas9 to knock in a 3xP3-DsRed cassette into the second intron of *bam*, which resulted in a *bam* null allele in the same *w*^{1118} background marked with a trackable eye marker (*w*^{1118}; *bam*$^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$, Fig 2B). For convenience and readability, we will refer to the *w*^{1118}; *bam*$^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$ allele as *bam*^{null} and the *w*^{1118}; *bam*^{L255F} allele as *bam*^{L255F}*.* Females homozygous for this *bam*^{null} allele exhibit the expected tumorous ovary phenotype with no developing nurse cell-positive cysts and are sterile (Fig 2C, fertility data not shown).

We then assessed the phenotype of the *bam*^{L255F} allele as a transheterozygous mutant over the *bam*^{null} in the same *w*^{1118} isogenic background. We observed a large fertility defect, but the females were not sterile (Fig 3). The *bam*^{L255F}/*bam*^{null} ovaries exhibited the expected tumorous ovary phenotype, with the presence of some differentiating, nurse cell positive cysts indicating that *bam* is partially functional, and thus that this allele behaves as a hypomorph (Fig 2D). To

296 further verify the nature of the *bam*^{L255F} allele, we assessed its phenotype over one of the
297 classic *bam* null alleles that we previously used to study the interaction between *bam* and
298 *Wolbachia, bam*$^{\Delta 59}$. The *bam*$^{\Delta 59}$ null allele is a nearly full deletion of the *bam* coding sequence.
299 We examined ovaries from *bam*^{L255F}/ *bam*$^{\Delta 59}$ females and observed the tumorous ovary
300 phenotype with some developing cysts as has been previously documented for *bam*^{BW}/*bam*$^{\Delta 59}$
301 and additionally shown here (Fig 2E&F). We measured the fertility of *bam*^{L255F}/ *bam*$^{\Delta 59}$ females
302 and observed a large fertility defect, consistent with the fertility of the *bam*^{L255F}/*bam*^{null}
303 genotype (Fig 3). Therefore, the *bam*^{L255F}/ *bam*^{null} cytological and fertility phenotypes are
304 consistent with the previously documented *bam* hypomorph phenotypes.
305
306 As we previously were unable to assess the phenotype of the *bam*^{BW} allele as a homozygote
307 due to recessive lethal mutations on the chromosome, we used our *bam*^{L255F} allele to ask if the
308 homozygous *bam*^{L255F} females also had a hypomorphic phenotype. We asked if *bam*^{L255F}
309 /*bam*^{L255F} females also exhibited the classic *bag of marbles* cytological phenotype and reduced
310 fertility. In contrast to the tumorous ovary phenotype of *bam*^{BW}/*bam*$^{\Delta 59}$ and *bam*^{L255F}/*bam*^{null}, a
311 result of a defect in *bam*'s differentiation function which causes GSC-like cells to over-
312 proliferate, we found no evidence of tumorous cysts in ovaries of *bam*^{L255F} /*bam*^{L255F} females
313 (Fig 2H). These ovaries resembled those of a wildtype *bam* background, with all developing
314 egg chambers featuring nurse cells (Fig 2H). Previously we were unable to compare the
315 fertility of the *bam*^{BW} hypomorph to wildtype *bam* fertility as fertility is greatly affected by
316 different genetic backgrounds. We compared the fertility of the *bam*^{L255F} /*bam*^{L255F} females to
317 *w*^{1118}; *bam*$^{+}$/*bam*$^{+}$ females and found that the total progeny per female was similar between the
318 two genotypes (Fig 2I). While we did not measure all aspects of *bam* function of this genotype,

319 the fertility and cytological data here indicate that the *bam*^{L255F} /*bam*^{L255F} genotype does not
320 cause a severe *bam* mutant phenotype, as we observed previously for the transheterozygous
321 *bam*^{BW} hypomorph (*bam*^{BW}/*bam*$^{\Delta 59}$).
322
323 Although we cannot make strong claims about the effect of *bam* genotype on fertility between
324 lines with different genetic backgrounds, we did perform a small control experiment to ask if
325 our new *bam*^{L255F} hypomorphic allele over the classic *bam*^{BW} hypomorphic allele also show
326 similar fertility to wildtype females as we observed for *bam*^{L255F}/*bam*^{L255F} females. We
327 observed that *bam*^{L255F}/*bam*^{BW} females and homozygous *bam*^{L255F} females did not show
328 significantly different fertility from *w*^{1118} *bam*$^{+}$/*bam*$^{+}$ females (Fig 3). Further, we asked if our
329 new *bam*^{L255F}/*bam*^{null} genotype showed a similar fertility defect to *bam*^{L255F}/*bam*$^{\Delta 59}$ (classic null)
330 and found that both showed severely reduced fertility compared to wildtype (Fig 3).
331
332 These data further confirm that our new *bam*^{L255F} and *bam*^{null} alleles behave similarly to alleles
333 we have used in the past to study the interaction between *bam* and *Wolbachia.* Since we did
334 not observe a phenotype in the *bam*^{L255F}/*bam*^{L255F} females that would be sufficient to measure
335 the *Wolbachia* rescue of both a fertility defect and an ovarian cytological defect as we have
336 previously described (Flores et al. 2015), but we were able to successfully recapitulate the
337 partial-loss of function *bam* phenotype with the *bam*^{L255F}/*bam*^{null} genotype, we focused primarily
338 on the effect of *Wolbachia* variation on the *bam*^{L255F}/*bam*^{null} female fertility and cytological
339 phenotype and the effect of *Wolbachia* variation on fertility of *w*^{1118}; *bam*$^{+}$/*bam*$^{+}$ females, which
340 has not been previously described.

341 **Genetic variation among *w*Mel strains has variable effects on w^{1118} isofemale fertility**

342 Fertility measured over multiple days reflects progeny produced cumulatively by the female
343 over time, although there could also be variation in development time of the progeny. We first
344 assessed the effect of *Wolbachia* variation on wildtype *bam* female fertility (w^{1118}; bam^{+}/bam^{+}).
345 Over 17 days, we counted the progeny produced by each test female infected individually for
346 each of the 10 *Wolbachia* variants and assessed the mean difference of adult progeny from
347 uninfected control females. We found that none of the *Wolbachia* variants increased fertility in
348 the wildtype *bam* background, and some lines showed a significant decrease in total progeny
349 per female across all days measured (*w*MelPop8X, and *w*MelCS2b_60) (Fig 4A&B,
350 Supplement Fig S1). Of the lines that showed a negative impact of *Wolbachia* on fertility, this
351 effect was more pronounced as the female fly aged. On days 1-9 of progeny eclosion, no
352 *Wolbachia* variant had a significantly negative effect on progeny counts, which is notable as
353 these days represent progeny from eggs laid earlier in the test female's life and thus are more
354 likely to reflect effects of *Wolbachia* variation on fertility in nature. The strains that showed a
355 significant and large negative effect of *Wolbachia* infection on fertility were both in the *w*MelCS
356 clade (mean difference between -32 and -46 progeny per female for days 1-17 Fig 4A&B).
357 *w*MelCS variants exhibit higher *Wolbachia* titer in males (Chrostek et al. 2013), which if also
358 true in females may negatively impact female fertility if titer gets too high (Chrostek et al.
359 2014).
360
361
362
363

364 **All *w*Mel variants rescue *bam* hypomorph female fertility but show a range of effect**
365 **sizes.**
366 Our results from the w^{1118}; bam^{+}/bam^{+} fertility assay indicated that none of the *Wolbachia*
367 variants tested have an overall increase in fertility in females. We performed the same fertility
368 assay described above for the bam^{L255F}/bam^{null} genotype infected with the same 10 *Wolbachia*
369 variants. In contrast to the wildtype *bam* lines, in the bam^{L255F}/bam^{null} lines we found that all
370 variants increased female fertility, and some variants had a notably large effect on female
371 fertility. The variants with the largest effect sizes were in the *w*MelCS-like clade (*w*MelCS2b,
372 *w*MelPop2x, *w*MelPop8x) (Fig 5A&B). This fertility rescue is in contrast to the decrease in
373 fertility by the same *Wolbachia* variants in the wildtype *bam* background, especially for
374 *w*MelCS2b which had the largest negative effect on wildtype *bam* fertility, but the largest
375 positive effect on bam^{L255F}/bam^{null} fertility (Fig 5A&B). In all of the lines tested there were many
376 individual females that had no progeny as well as females with varying distributions of
377 progeny. In some of the lines (*w*Mel2a, *w*MelCS2a, *w*MelCS2b) there were outlier females who
378 had large rescue of fertility, some in the range of what we observe for wildtype *bam* fertility (Fig
379 4A). However, for most of the individuals tested, fertility was not fully rescued, e.g. restored to
380 wildtype levels (Fig 4A, Fig 5A). Although the largest effect of *Wolbachia* on bam^{L255F}/bam^{null}
381 rescue was amongst the *w*MelCS-like variants, there were other notable variants outside of the
382 *w*MelCS clade that showed high increase in female fertility, particularly *w*Mel2a. Overall these
383 data further indicate a genetic interaction between *bam* and *Wolbachia* that is different from
384 the overall effect of *Wolbachia* on female fertility.

385 **All *w*Mel variants rescue the ovarian *bam* hypomorph cytological defect but to varying**
386 **degrees**

387 Although we wanted to assess the rescue of the *bam* hypomorphic fertility phenotype by
388 *Wolbachia* because we can also compare this to the effect of *Wolbachia* on wildtype *bam*
389 fertility, we also wanted to know if genetic variation in *Wolbachia* affected the rescue of the
390 *bam* hypomorphic cytological phenotype. Because fertility is affected by environmental and
391 genetic differences that act in later developmental stages outside of *bam*'s function early in
392 gametogenesis, we also measured the effect of *Wolbachia* variation on the rescue of the
393 cytological *bam* mutant phenotype. Since the partial loss of function of *bam* results in over-
394 proliferation of GSCs and reduced differentiation, we can quantify *bam* function by assaying for
395 differentiated germ cells. The cytological *bam* hypomorphic phenotype in females consists of
396 tumorous ovarioles filled with GSC-like cells, with few properly developing egg chambers (Fig
397 6B). Properly developing egg chambers contain polyploid nurse cells, which feature large,
398 easily identifiable nuclei (Fig 6B, arrowheads). We counted the number of nurse cell positive
399 egg chambers as a measure of GSC daughter differentiation and thus *bam* function. We found
400 that all of the *Wolbachia* variants tested significantly increased the number of nurse cell
401 positive egg chambers, and that *Wolbachia* variants in the *w*MelCS-like clade showed highest
402 numbers of nurse cell positive egg chambers (Fig 6A&B). These results are consistent with the
403 fertility assay results for the bam^{L255F}/bam^{null} hypomorph genotype, however we observed a
404 more obvious trend of higher *bam* rescue by the *w*MelCS-like variants in this assay. All of the
405 *Wolbachia* variants with the highest effect size of increased *bam* function are in the *w*MelCS-
406 like clade. It is unclear whether this higher rescue is due to titer or another genetic difference

407 between these clades, but these data indicate that genetic variation in *Wolbachia* impacts the
408 *bam* rescue phenotype.
409
410 To ask if the nurse cell positive egg chambers resulted in oocytes capable of development if
411 fertilized, and therefore represent properly differentiated GSC daughters, we picked two
412 representative *Wolbachia* variants of high and low rescue and counted the total number of
413 eggs laid per female over days 1-3. In w^{1118}; bam^{+}/bam^{+} females, neither *Wolbachia* variant
414 affected the number of eggs laid per female (Fig 7A). In contrast to the w^{1118}; bam^{+}/bam^{+}
415 females and consistent with the nurse cell assay, we observed few eggs laid by uninfected
416 w^{1118}; bam^{L255F}/bam^{null} females, with significantly more eggs produced by the lower rescuing
417 *Wolbachia* *w*Mel59, and the highest number of eggs laid by females infected with the high
418 rescuing line, *w*MelCSa_66 (Fig 7B).
419
420 **Homozygous bam^{L255F} females exhibit more variable fertility which is not negatively**
421 **affected by *Wolbachia***
422 In light of the negative effect of infection by some *Wolbachia* variants on wildtype *bam* fertility
423 and that the uninfected bam^{L255F}/bam^{L255F} females did not exhibit a fertility defect (Fig 2I, Fig
424 8A&B), we asked if infection by a subset of the *Wolbachia* variants we assessed in the
425 wildtype *bam* background had any effect on the fertility of bam^{L255F}/bam^{L255F} females. We
426 chose three *Wolbachia* variants that did not have a significant effect on wildtype *bam* female
427 fertility (*w*Mel2a_63, *w*MelCSa_66, and *w*MelCSb_577 Fig 4), and one that had a significant
428 negative effect on female fertility (*w*MelCS2b, Fig 4). We measured fertility of homozygous
429 females infected with *Wolbachia* variants as previously described. In contrast to our findings in

430 the *bam*$^{+}$/*bam*$^{+}$ background that *w*MelCS2b negatively affected female fertility, we found that
431 *w*MelCS2b did not have a significant effect on female fertility in the *bam*^{L255F}/*bam*^{L255F}
432 background (Fig 8A&B). Additionally, in contrast to our findings in the *bam*$^{+}$/*bam*$^{+}$ background
433 that *w*Mel2a_63 and *w*MelCSa_66 did not significantly affect female fertility, we found that in
434 the *bam*^{L255F}/*bam*^{L255F} background both of these variants had a significantly positive effect on
435 female fertility (Fig 8A&B). In both *bam*$^{+}$/*bam*$^{+}$ and *bam*^{L255F}/*bam*^{L255F} backgrounds,
436 *w*MelCSb_577 did not have a significant effect on female fertility (Fig 4, Fig 8A&B).
437
438 Interestingly, during days 1-6 uninfected wildtype *bam* females had significantly fewer progeny
439 than uninfected homozygous *bam*^{L255F} females (Fig 8B, Supplement Fig S2). We also
440 observed that the total progeny per female of the homozygous *bam*^{L255F} genotype had a wider
441 range than wildtype, which was not impacted by *Wolbachia* and is therefore likely a subtle *bam*
442 mutant phenotype (Fig 8A). As *bam* regulates GSC daughter differentiation, its mis-regulation
443 may increase or decrease fertility. These data further indicate that there is a genetic interaction
444 between *bam* and *Wolbachia* that is resulting in the increase in fertility, not simply an overall
445 increase in fertility that is more sensitive in a *bam* mutant background.
446

447 ## Discussion

448

449 **Revisiting the phenotypes of classic *bam* alleles using CRISPR/Cas9 in controlled**
450 **genetic backgrounds**

451 We previously reported that infection with *w*Mel rescued the fertility defect of *bam*^{BW}/*bam*$^{\Delta 59}$
452 hypomorphic mutant females (Flores et al. 2015). We sought to better understand this

interaction for multiple reasons. The first reason is that we hypothesize that an interaction between *Wolbachia* and *bam* may be driving the adaptive sequence divergence of *bam*. However, in order to understand if and how this interaction could be adaptive, we need to understand the nature of the manipulation of *bam* function by *Wolbachia*. Second, *Wolbachia* is of immense interest as a potential means to control vectors of some human diseases (Moreira et al. 2009). However, we do not know the phenotypic range of host adaptations to new *Wolbachia* infections. Defining the genetic mechanisms between the host and *Wolbachia* that are necessary for its reproductive manipulations may aid in monitoring efforts to control vectors with *Wolbachia*.

One such way to find the *Wolbachia* loci necessary for the interaction between bacteria and the host would be to systemically mutagenize the bacteria and then screen for variants that enhance or reduce the phenotype of interest. However, *Wolbachia* are obligate endosymbionts that cannot be cultured, and so are currently not amenable to such genetic manipulation. While some studies have identified *Wolbachia* loci that interact with *D. melanogaster* by transgenically expressing *Wolbachia* loci in *D. melanogaster*, this requires identifying candidate *Wolbachia* loci that affect the host phenotype of interest (Ote et al. 2016; Le Page et al. 2017). One method to identify candidate *Wolbachia* loci that affect the host phenotype is to use genetic variation in *Wolbachia* to test for differences in the effect of *Wolbachia* variants on the host phenotype.

Chrostek et al. (Chrostek et al. 2013) generated isogenic *Drosophila* lines infected with previously described and genetically distinct *w*Mel strains of *Wolbachia*. As our current *bam*

476 mutant was not in this host genetic background, and thus we would not be able to conclude
477 that any differential interaction between *bam* and these variants was due to *Wolbachia*
478 variation and not host genetic variation, we used CRISPR/Cas9 to create the same amino acid
479 mutant present in the original bam^{BW} hypomorph allele in the w^{1118} isogenic background. To
480 ensure we had the tools to assess the *Wolbachia* rescue phenotype as previously described,
481 we also generated a *bam* null allele using CRISPR/Cas9 in the same genetic background.
482
483 We have now confirmed that these two new alleles behave similarly to the alleles we
484 previously used to document the interaction between *w*Mel and *bam*. There have been a
485 handful of studies assessing the phenotypes of diverse *Wolbachia* in isogenic backgrounds
486 (Chrostek et al. 2013; Chrostek et al. 2014; Chrostek and Teixeira 2018; Gruntenko et al.
487 2019), however there have been no documented studies of interactions between *Drosophila*
488 mutants and *Wolbachia* in these backgrounds. Recent work on the interaction between
489 *Wolbachia* and *Sex lethal* in the female germline showed that *Wolbachia* rescues the loss of
490 GSCs in some *Sxl* mutants through a *nanos* dependent interaction with the *Wolbachia* protein
491 TomO (Ote et al. 2016). However, when researchers transgenically expressed *TomO* in *Sxl*
492 hypomorphic ovaries, the GSC number was rescued, but fertility was not. This result indicated
493 that there are other mechanisms *Wolbachia* is using to fully rescue GSC number and fertility in
494 *Sxl* mutants. We believe the strategy we describe here could be used to further define genetic
495 interactions such as the *Sxl* and *Wolbachia* interaction (Starr and Cline 2002). As we and
496 others have also had success with CRISPR/Cas9 in non-*melanogaster* species, we believe
497 this type of systematic analysis could be extended to assess interactions of host genes and
498 *Wolbachia* in other species outside of *Drosophila melanogaster*.

499 ***w*Mel *Wolbachia* variants do not broadly increase fertility**

500 Due to the isogenic nature of the *Wolbachia* infected lines, we were able to rigorously assess
501 the effect of different *Wolbachia* variants on wildtype *bam* female fertility. In addressing if and
502 how *Wolbachia* may drive the adaptive evolution of *bam*, it was important for us to know if
503 *Wolbachia* has an effect on fertility in a wildtype *bam* background. Here, we are not measuring
504 specifically how *Wolbachia* is modulating a functional *bam* allele, as fertility is affected by many
505 loci, but if we observed a large effect of a particular *Wolbachia* variant on fertility, this would
506 motivate further experiments to assess functional *bam* activity in lines infected with that
507 *Wolbachia* variant. We observed no line with increased fertility for any of the *Wolbachia*
508 variants, indicating that *Wolbachia* is not generally upregulating fertility through *bam* or another
509 pathway in this genetic background. However, we also observed that some *Wolbachia* variants
510 had a negative effect on female fertility. We cannot distinguish whether this effect is due to
511 *Wolbachia* mis-regulating the germline or some other developmental consequence of its
512 infection. In fact, the lines with the highest reported titers in males showed the largest negative
513 impact on female fertility (Chrostek et al. 2013). This observation highlights the complexity of
514 the interaction between *Wolbachia* and *Drosophila*, where depending on the genetic
515 background of the host, the fitness effect of *Wolbachia* on a phenotype may vary. This effect
516 could further vary based on aspects of the host's environment where the manipulation of
517 fertility by *Wolbachia* may not always be beneficial. Here we may expect the host and microbe
518 to evolve ways of evading each other, which may become more or less apparent in different
519 genetic backgrounds. Of note, our observations are under laboratory conditions in a highly
520 inbred line, and so it is unclear what effect these *Wolbachia* variants have on female fertility in
521 natural populations.

522 ***w*Mel *Wolbachia* variants across three clades genetically interact with *bam***

523 We previously showed that a single *w*Mel strain partially rescued the fertility and GSC
524 daughter differentiation defect of the $bam^{BW}/bam^{\Delta 59}$ hypomorph. Here we asked if there was
525 variation in the *bam* rescue phenotype due to strain variation in *Wolbachia*. The strains we
526 used have been fully sequenced and differ by varying degrees of sequence divergence. Of the
527 three clades tested, all 10 *Wolbachia* variants rescued the fertility defect and the cytological
528 defect of the bam^{L255F}/bam^{null} hypomorph. Therefore, none of the variants tested contained
529 genetic variation that suppressed the interaction between *bam* and *Wolbachia*. We did find that
530 in both the counts of nurse cell positive egg chambers and the fertility assays, *w*MelCS-like
531 *Wolbachia* variants showed the highest rescue (Fig 5, Fig 6). This pattern was the clearest in
532 the counts of nurse cell positive egg chambers as expected, since the presence of nurse cell
533 positive egg chambers reflects GSC daughter differentiation, a phenotype that is a more direct
534 output of *bam* activity. It is likely that the higher level of variability in the fertility assays is also
535 due to the variable effect the *Wolbachia* variants have on other stages of development post
536 GSC daughter differentiation. However, we feel it is important to also measure the effect of
537 *Wolbachia* on fertility, since we cannot assess the count of nurse cell positive egg chambers
538 for *bam* alleles that do not show a tumorous mutant phenotype, and it gives us insight into how
539 *Wolbachia* infection could be adaptive. For example, would there be a fitness tradeoff between
540 a high fecundity (GSC daughter differentiation) rescue and a low fertility (adult progeny)
541 rescue? Measuring the impact of *Wolbachia* on multiple stages of oogenesis and reproduction
542 therefore gives us insight into any further complexities in the interaction between GSC genes
543 and *Wolbachia*.

544

545 *w*MelPop is a virulent strain of *w*MelCSb characterized by uncontrolled proliferation and titer
546 caused by increased copy number of the Octomom locus. wMelPop2X and *w*MelPop8X exhibit
547 2X copies of the Octomom locus and anywhere from 4X-8X copies, respectively (Chrostek and
548 Teixeira 2018).The high rescue of both the cytological and fertility defect of the
549 bam^{L255F}/bam^{null} by *w*MelPop2X is suggestive that titer and possibly other phenotypes that
550 increase interactions with the host are contributing to the rescue of *bam*. We observed lower
551 rescue for *w*MelPop8X, indicating that the increasing copy number of the Octomom locus that
552 causes apoptosis and early death likely negatively affects host fertility (Zhukova and Kiseleva
553 2012; Chrostek and Teixeira 2018). *w*MelPop2X is closely related to *w*MelCSb, with the only
554 variation between them being a synonymous SNP and the amplification of the octomom locus
555 which contains *Wolbachia* loci predicted to be involved with nucleic acid binding, and thus
556 likely how it increases its titer, as well as proteins with predicted homology to eukaryotic
557 domains (Chrostek and Teixeira 2018). These loci may then increase the interaction of
558 *Wolbachia* and its host (López-Madrigal and Duarte 2020). A natural next step would be to
559 determine the *Wolbachia* factors that manipulate GSC daughter differentiation.
560

561 **Possible adaptive interactions between *Wolbachia* and *bam***

562 It is well established that disrupting *bam* function negatively effects fertility (McKearin and
563 Spradling 1990; Ohlstein and McKearin 1997; Lavoie et al. 1999; Flores et al. 2015), and
564 bam^{L255F}/bam^{null} females are almost completely sterile. However, we do not predict such
565 deleterious alleles to reach high frequency in natural populations. In fact, we have not found
566 this variant segregating in any of the natural populations of *D. melanogaster* that have been
567 sampled in the *Drosophila* genome Nexus (Lack et al. 2015). Thus, while studying this mutant

568 is effective in further refining how *bam* and *Wolbachia* interact, we cannot conclude that this
569 type of interaction occurs in natural populations. However, given the severity of the mutant and
570 strength of *Wolbachia*'s rescue, one hypothesis is that if *Wolbachia* is increasing GSC
571 daughter differentiation when it is not favorable for the host, the host would evolve a way to
572 evade *Wolbachia*'s manipulation of this pathway. In the case of our lab generated mutant, it is
573 possible that when *bam* is not fully functional, the background is more sensitive to
574 manipulation by *Wolbachia*. *Wolbachia* have been documented to respond to changes in the
575 host environment, as *Wolbachia* gene expression is affected by host age and sex, and as
576 *Wolbachia* transmission requires functional oogenesis, it reasonable to hypothesize that
577 *Wolbachia* is sensitive to changes in gametogenesis (Rice et al. 2017; Newton and Sheehan
578 2018; Russell et al. 2020).
579
580 An additional observation we made is that bam^{L255F}/bam^{L255F} females show a broader range of
581 adult progeny per female compared to wildtype *bam* females regardless of *Wolbachia* infection
582 status. The individual bam^{L255F}/bam^{L255F} females exhibit both higher and lower than average
583 fertility, indicating that mis-regulating *bam's* differentiation function could both increase and
584 decrease fertility. This phenotype is worth further investigation, since although *bam* shows a
585 signature of positive selection, we do not know specifically what aspect of *bam* function is
586 adaptive. If variation in *bam* function can both increase and decrease mean fertility, and
587 *Wolbachia* has a generally positive effect on fertility of bam^{L255F}/bam^{L255F} females, this is further
588 evidence that *Wolbachia* may be able to manipulate *bam* in order to increase oogenesis for its
589 own benefit, and that genetic variation at *bam* could affect the regulation of oogenesis.
590 Therefore, if *Wolbachia* increased the rate of oogenesis to ensure its own transmission and

591 this was not favorable for the host, *bam* may be in conflict with *Wolbachia* to regulate
592 oogenesis in a favorable manner for the host. Here we see that although the average female
593 fertility of uninfected bam^{L255F}/bam^{L255F} mutants is not significantly different from wildtype, there
594 is a wider distribution of individual female fertility, indicating that the *bam* hypomorphic
595 phenotype is both an increase and decrease in fertility (Fig 8A). Since these females are in an
596 isogenic background and we do not expect genetic variation between individual females, the
597 *bam*-mediated mis-regulation of differentiation may set off a cascade of other genetic mis-
598 regulation that results in higher or lower fertility over time. We see that infection by some
599 *Wolbachia* variants in this background increases female fertility, indicating that perhaps
600 *Wolbachia* can restore the mis-regulation of GSC daughter differentiation, and even increase
601 reproductive output through this mechanism. It would be interesting to use this genotype for
602 future experiments to explore the possibility that this *bam* mutation may disrupt the mechanism
603 *D. melanogaster* evolved to evade *Wolbachia*'s effect on differentiation.
604
605 An interesting question that remains is how *Wolbachia* have evolved in their interaction with
606 GSC genes, including *bam*. While *w*MelCS-like and *w*Mel-like variants share a most recent
607 common ancestor about 8000 years ago, *w*MelCS-like variants were recently (~2000 years
608 ago) replaced by *w*Mel-like variants in natural populations of *D. melanogaster* (Riegler et al.
609 2005; Richardson et al. 2012). However, this replacement has not been complete, and there
610 are global populations still infected with *w*MelCS variants (Riegler et al. 2005; Nunes et al.
611 2008). Interestingly we find that these variants show higher rescue of bam^{L255F}/bam^{null} female
612 fertility and nurse cells. If *bam* and *Wolbachia* are evolving in an arms race, it could be that
613 *w*Mel has not evolved the same level of interaction with *bam* as *w*MelCS has. Future work to

614 investigate these dynamics should include sampling populations that are still infected with
615 *w*MelCS as well as those infected with *w*Mel and asking if there is any evidence of genetic
616 differentiation at *bam*. Additionally, we could utilize existing *Wolbachia* sequence variation from
617 natural populations to ask if there is any evidence of associations between *bam* variation and
618 *Wolbachia* variation. If we were to sample populations that are differentially infected with *w*Mel
619 and *w*MelCS to assess genetic differentiation at *bam* as discussed above, we could also do
620 the same for *Wolbachia* variants. One caveat being that if we did not observe genetic
621 signatures of adaptive evolution in *Wolbachia* loci*,* this does not mean that *Wolbachia* and *bam*
622 are not coevolving, as we do not know the true infection history of a *Drosophila* population with
623 *Wolbachia* and thus which *Wolbachia* variant may have been in conflict with *bam.* Additionally,
624 there may be general *Wolbachia* functions that are interacting with *bam* and not a single locus
625 (e.g. loci that regulate titer).
626
627 Further work should focus on determining the *Wolbachia* loci contributing to the different
628 magnitude of *bam* rescue. However, as has been previously pointed out by Chrostek et al.
629 (Chrostek et al. 2013), between the *w*Mel clades and *w*MelCS clades there are eight indels,
630 108 SNPs, and of those, polymorphisms in coding sequence of 58 genes. So further work
631 would have to be done to narrow down which variants contribute to the degree of rescue. A
632 next step could be moving out to more divergent *Wolbachia* (for example, *w*Au that infects *D.*
633 *simulans* (Miller and Riegler 2006)) to ask how recently the interaction with *bam* evolved. To
634 complement this, it would be beneficial to perform these same rescue experiments with *bam*
635 hypomorphs in *D. simulans*.
636

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645

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Figures

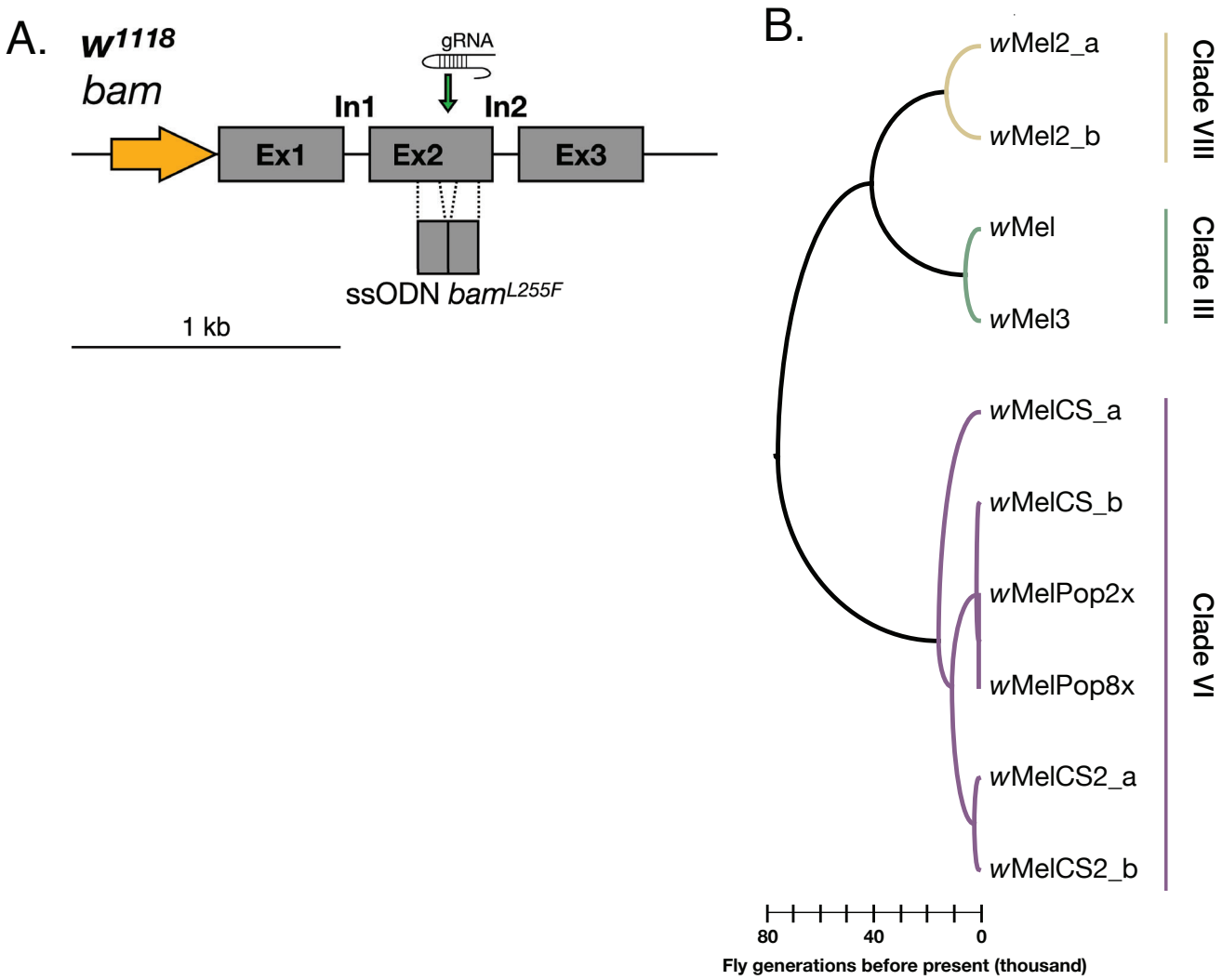


Fig 1. Design for recreating the classic *bam*^{BW} hypomorph allele in the *w*^{1118} isogenic background with CRISPR/Cas9 to test for differential rescue by diverse *Wolbachia.* (A) Schematic of the *bam* gene region showing the single gRNA target site in the second exon and the single stranded oligo donor containing the hypomorphic missense mutation. (B) A cladogram adapted from Chrostek et al. 2013 showing the relationships and clade assignments for the *Wolbachia* variants tested in this study.

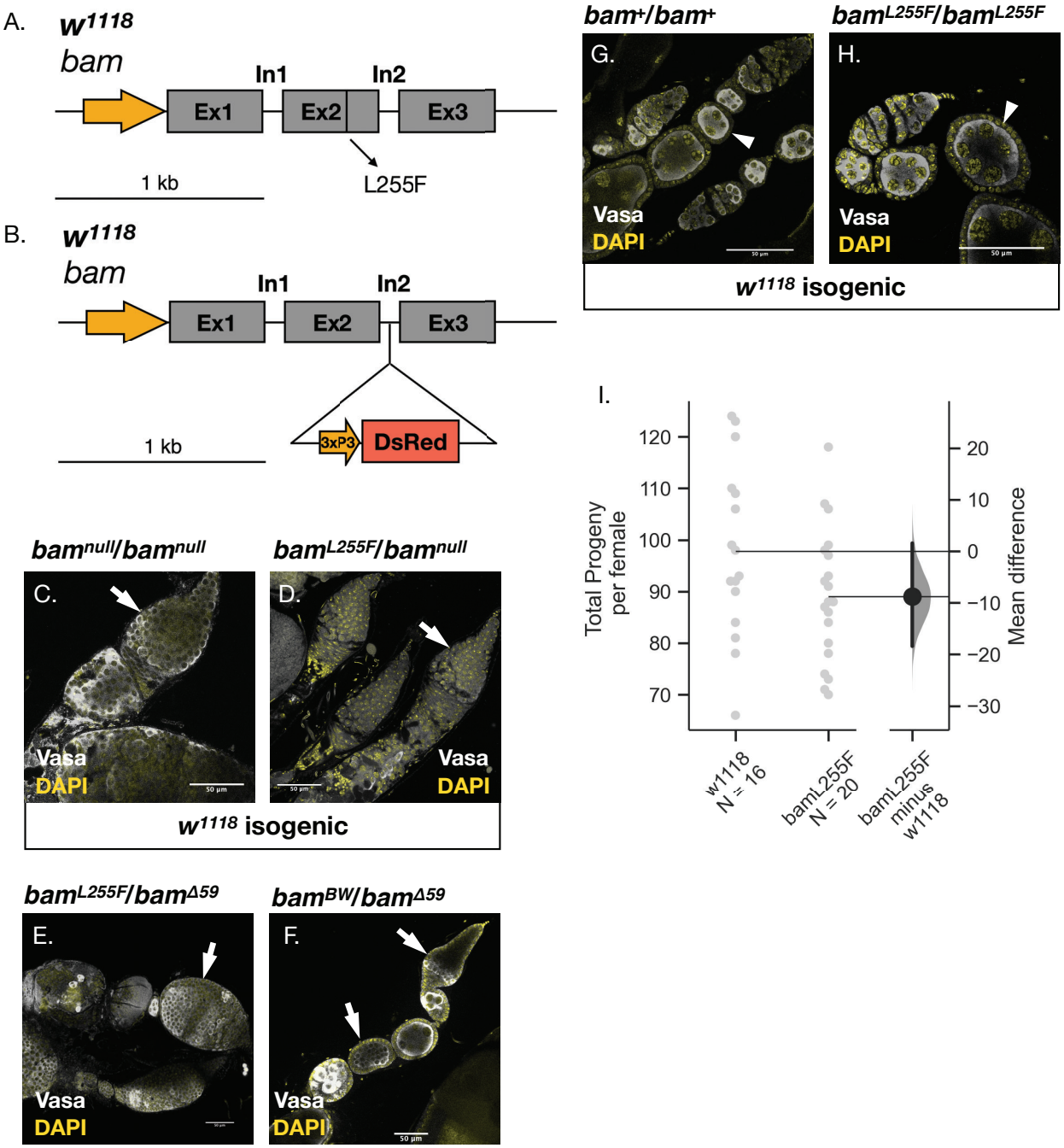


Fig 2. The *bam*^{BW} hypomorphic allele and a novel *bam* null allele in the *w*^{1118} isogenic background recapitulates the classic *bam* phenotypes. (A) Schematic of the *bam* gene region with the single amino acid change from *bam*^{BW}. (B) Schematic of the *bam* gene region with the 3xP3-DsRed eye marker cassette inserted into the second intron to create a *bam* null allele. (C) The newly generated *bam*^{null}/*bam*^{null} genotype results in tumorous ovaries (arrow). (D) The recreated *bam*^{L255F} mutation over the *bam*^{null} exhibits tumorous ovaries in the *w*^{1118} isogenic background. (E) The recreated *bam*^{L255F} mutation over the classic *bam*$^{\Delta 59}$ null allele exhibits tumorous ovaries. (F) The classic *bam*^{BW}/*bam*$^{\Delta 59}$ hypomorphic genotype exhibits tumorous ovaries. (G) Ovaries from the *w*^{1118} isogenic line wildtype for *bam* do not exhibit any germline tumors and show developing egg chambers with large nurse cell nuclei (arrowhead). (H) *bam*^{L255F}/*bam*^{L255F} homozygotes do not exhibit tumorous ovaries, indicating two copies of the partial loss of function *bam*^{L255F} mutation is sufficient for GSC daughter differentiation. (I) *w*^{1118} females and *bam*^{L255F}/ *bam*^{L255F} females do not show a significant difference in fertility (mean difference of progeny per female), further indicating that two copies of *bam*^{L255F} is sufficient for *bam* function.

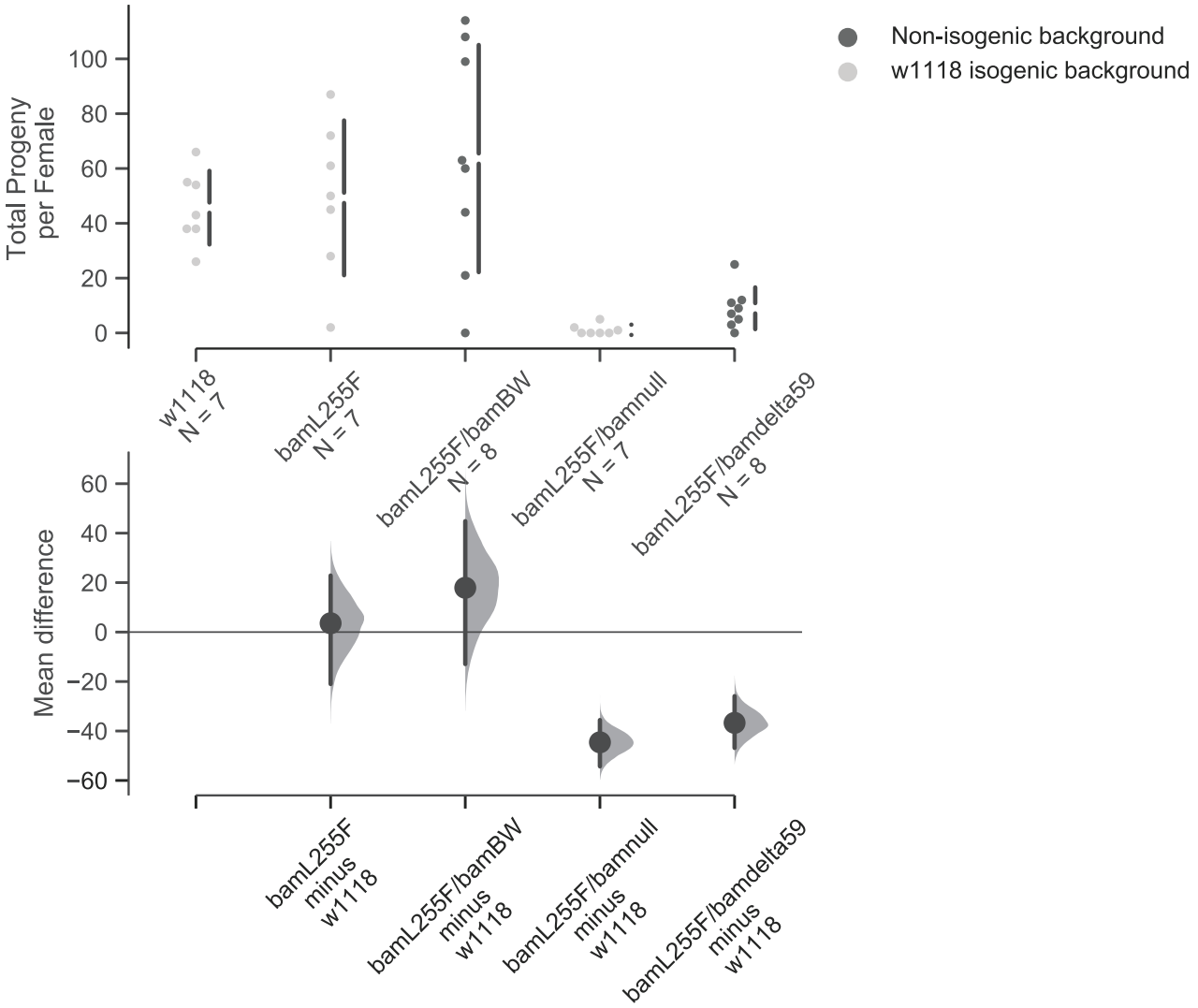


Fig 3. Female fertility of genotypes containing the w^{1118}; bam^{L255F} hypomorphic allele are consistent with those of classic alleles. Swarm and Cumming estimation plots of a control fertility experiment showing that the fertility of bam^{L255F}/ bam^{L255F} females is not significantly different from w^{1118} (mean difference = 3.6), nor are bam^{L255F}/bam^{BW} females (mean difference = 17.9). bam^{L255F}/bam^{null} females have significantly lower fertility compared to wildtype (mean difference = -44.6, $P < 0.01$ permutation test), as do bam^{L255F}/$bam^{\Delta 59}$ females (mean difference = -36.7, $P < 0.01$ permutation test).

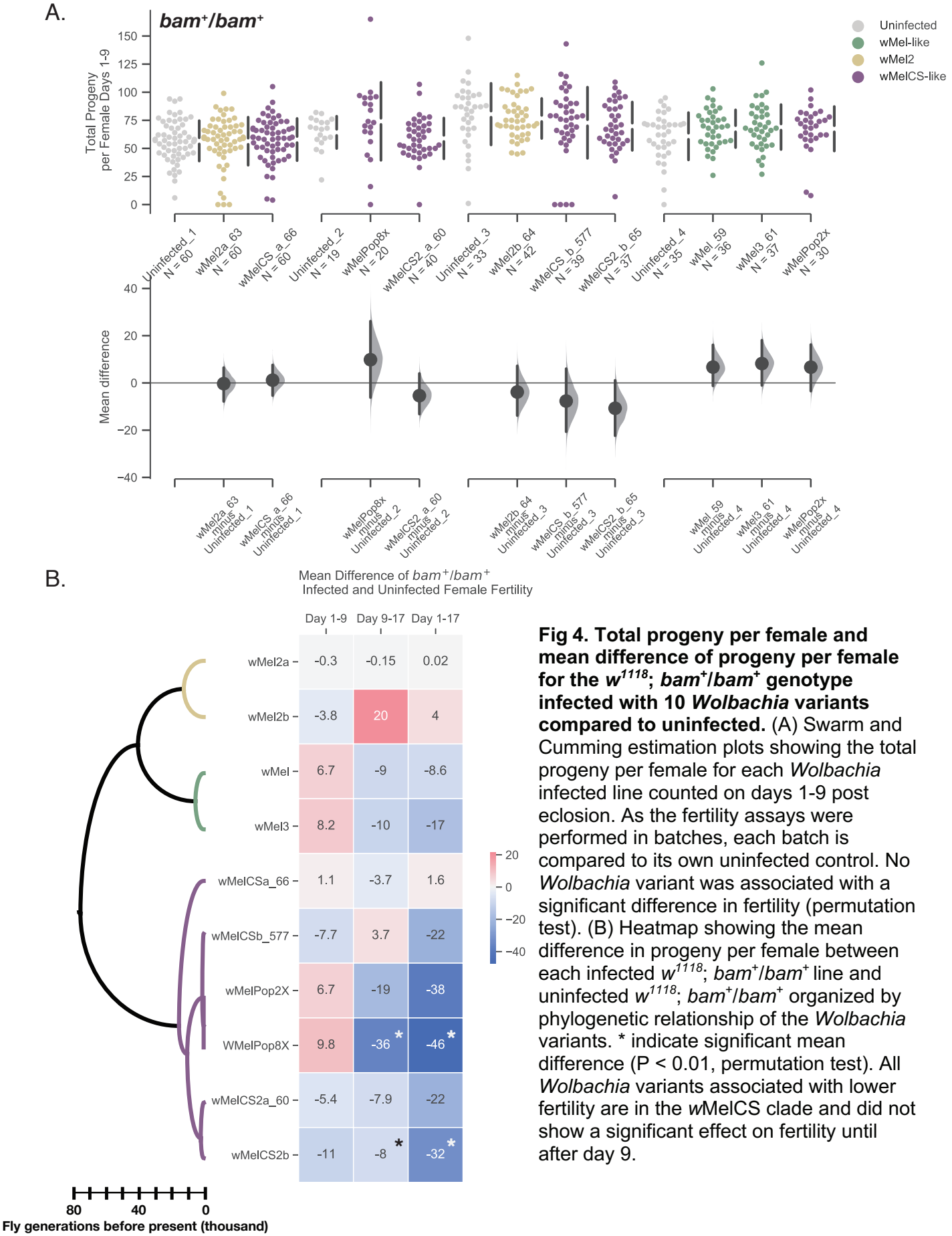
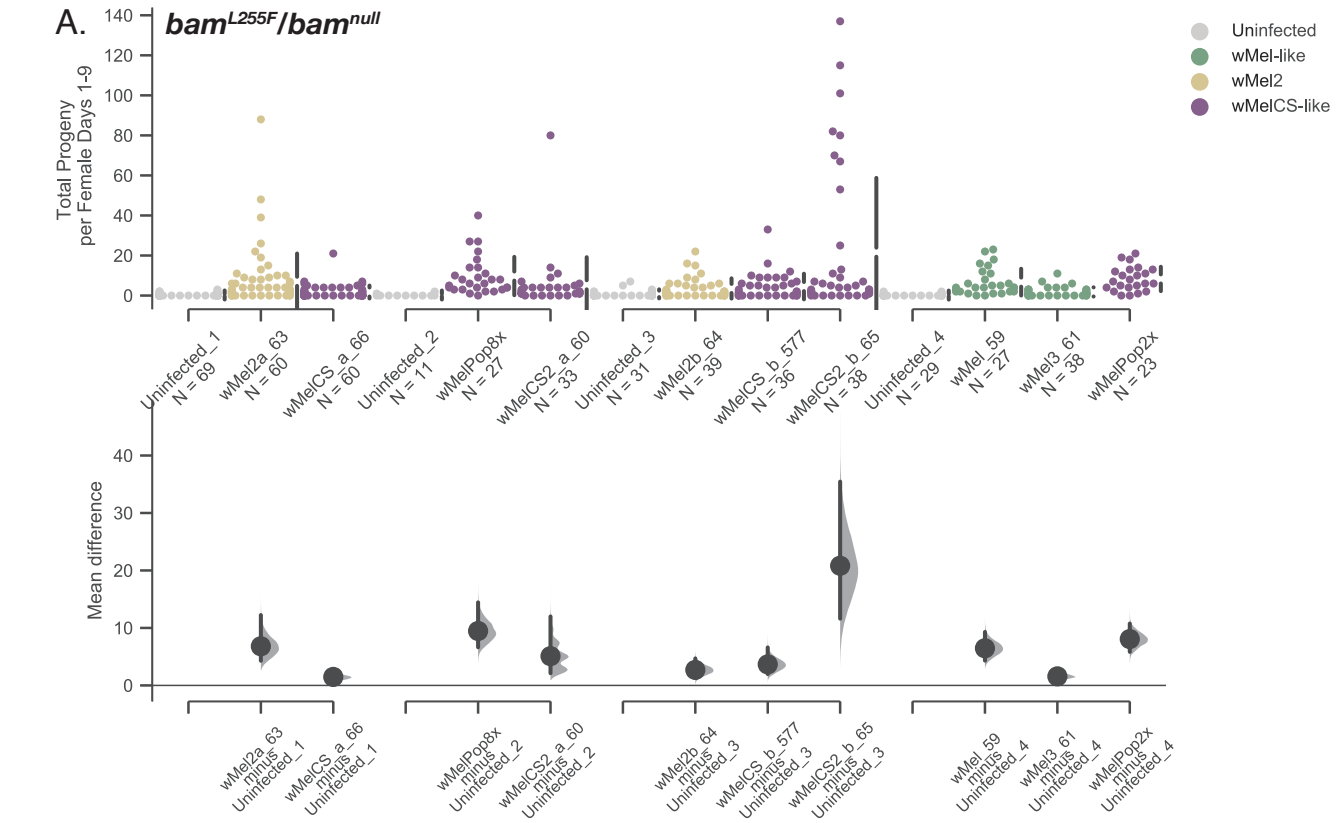


Fig 4. Total progeny per female and mean difference of progeny per female for the w^{1118}; bam^{+}/bam^{+} genotype infected with 10 *Wolbachia* variants compared to uninfected. (A) Swarm and Cumming estimation plots showing the total progeny per female for each *Wolbachia* infected line counted on days 1-9 post eclosion. As the fertility assays were performed in batches, each batch is compared to its own uninfected control. No *Wolbachia* variant was associated with a significant difference in fertility (permutation test). (B) Heatmap showing the mean difference in progeny per female between each infected w^{1118}; bam^{+}/bam^{+} line and uninfected w^{1118}; bam^{+}/bam^{+} organized by phylogenetic relationship of the *Wolbachia* variants. * indicate significant mean difference ($P < 0.01$, permutation test). All *Wolbachia* variants associated with lower fertility are in the *w*MelCS clade and did not show a significant effect on fertility until after day 9.



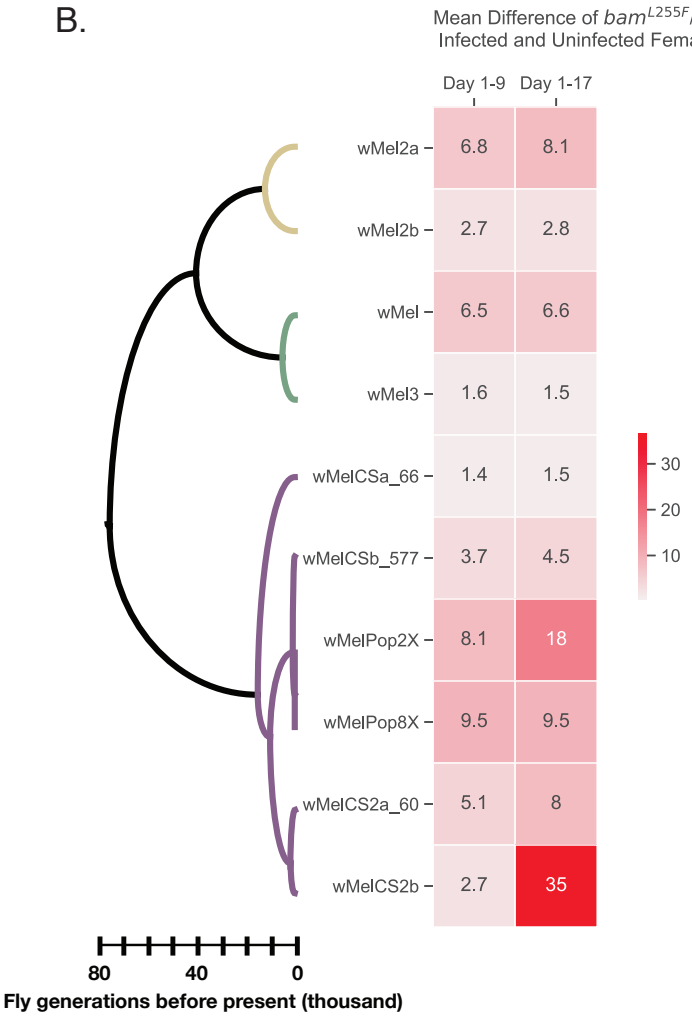
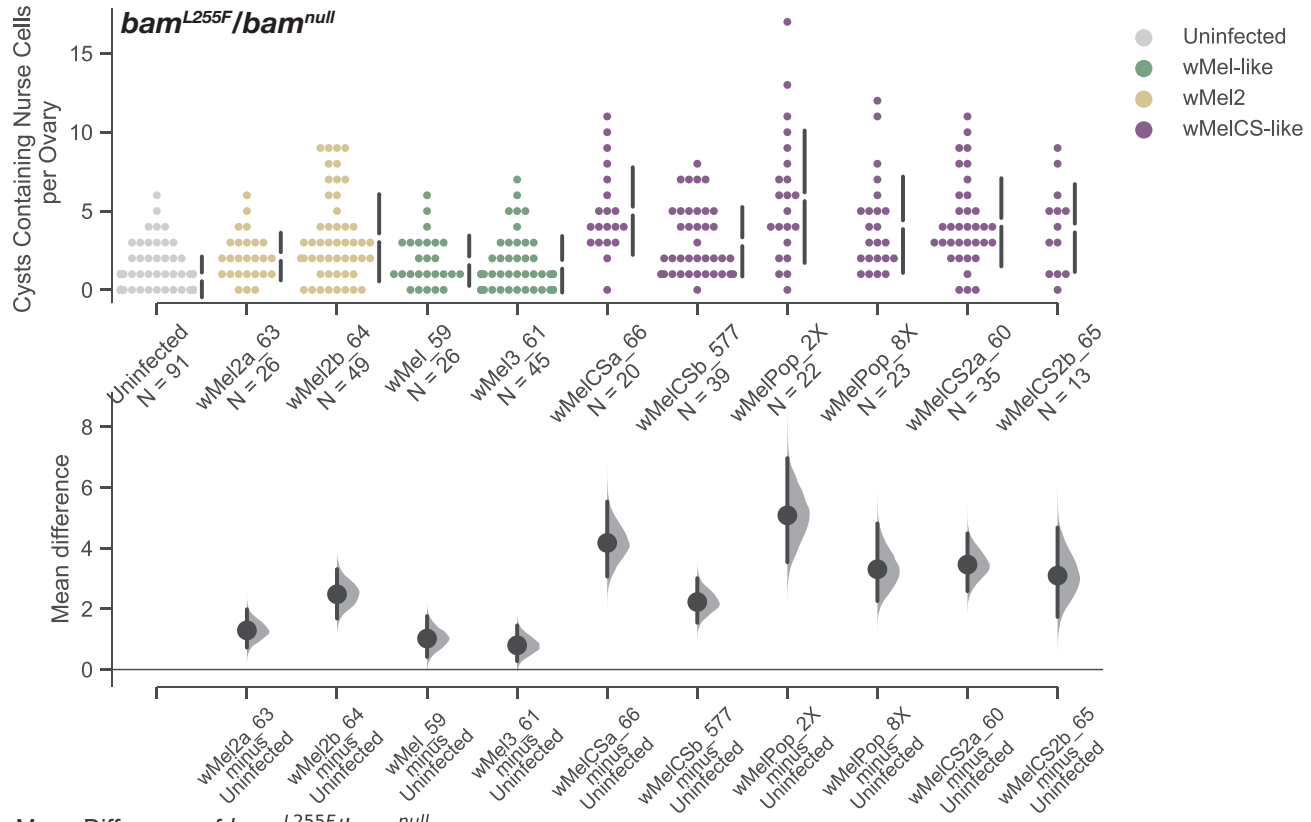


Fig 5. Total progeny per female and mean difference of progeny per female for the *w*^{1118}; *bam*^{L255F}/*bam*^{null} genotype infected with 10 *Wolbachia* variants compared to uninfected. (A) Swarm and Cumming estimation plots showing the total progeny per female for each *Wolbachia* infected line counted on days 1-9 post eclosion. As the fertility assays were performed in batches, each batch is compared to its own uninfected control. All *Wolbachia* variants were associated with significant increases in female fertility compared to uninfected controls ($P < 0.001$, permutation test). (C) Heatmap showing the mean difference in progeny per female between each infected *w*^{1118}; *bam*^{L255F}/*bam*^{null} line and uninfected *w*^{1118}; *bam*^{L255F}/*bam*^{null} controls organized by phylogenetic relationship of *Wolbachia*. All *Wolbachia* variants increased female fertility across the experiment. The *Wolbachia* variants associated with the two highest increases in fertility are in the *w*MelCS clade.

A.



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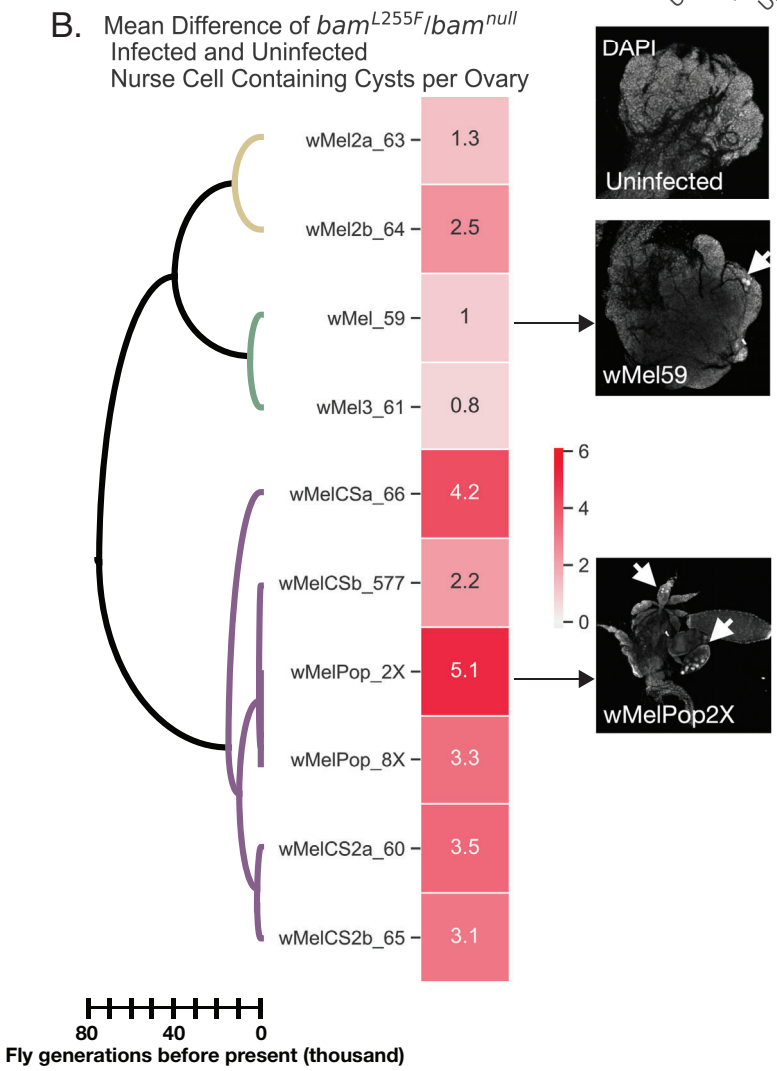


Fig 6. Total cysts containing nurse cells per ovary and mean difference of nurse cell positive cysts for the *w*^{1118}; *bam*^{L255F}/*bam*^{null} genotype infected with 10 *Wolbachia* variants compared to uninfected. (A) Swarm and Cumming estimation plots showing the total cysts containing nurse cells per ovary for each *Wolbachia* infected line assayed on 2-3 day old females. All *Wolbachia* variants are associated with a significant increase in nurse cell positive cysts per ovary ($P < 0.01$, permutation test). *Wolbachia* variants in the *w*MelCS-like clade have the highest effect on nurse cell positive cysts. (B) Heatmap illustrating the mean difference of nurse cell containing ovaries from (A) and images of ovaries from representative variants labeled with DAPI (nurse cell positive cysts marked with arrows). No *w*MelCS-like variants were associated with a mean difference of nurse cell containing cysts less than 2, but of the *w*Mel2 and *w*Mel-like variants, only *w*Mel2b exhibited a mean difference greater than 2.

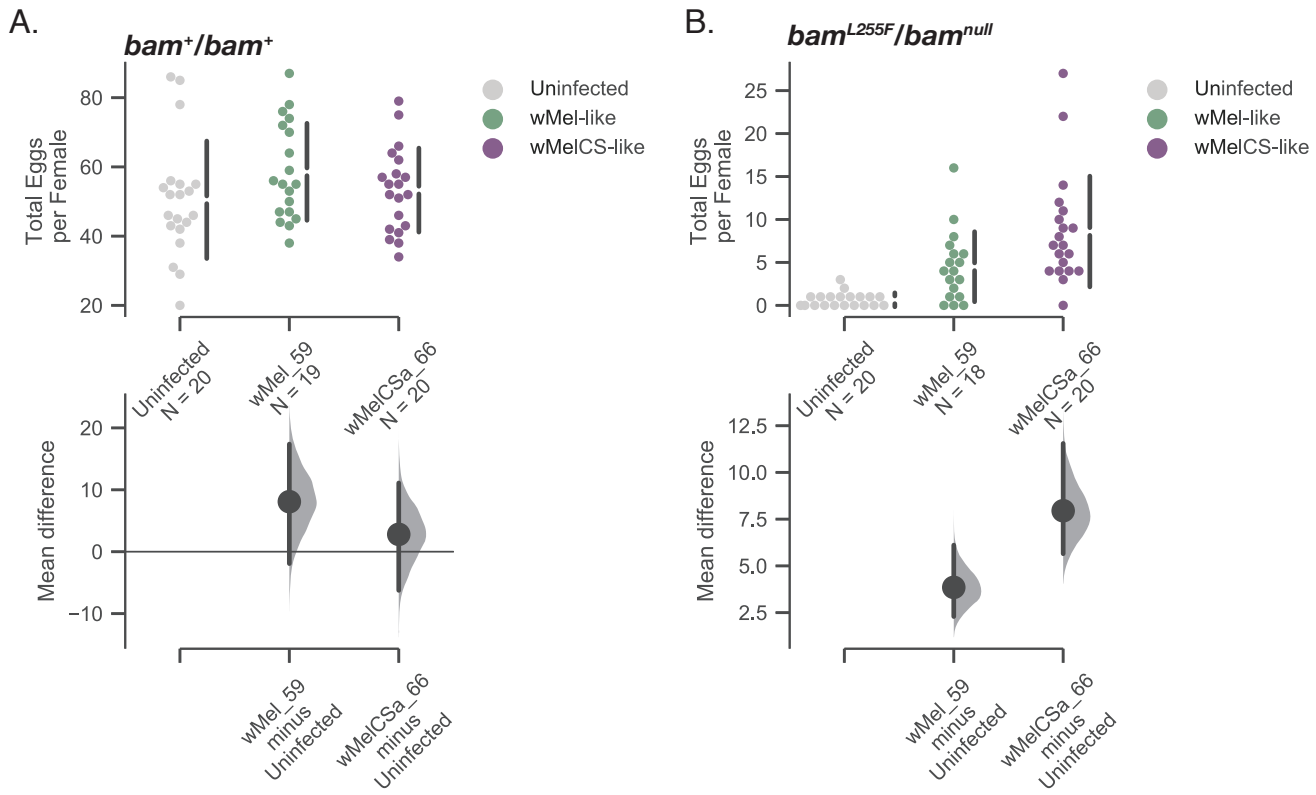
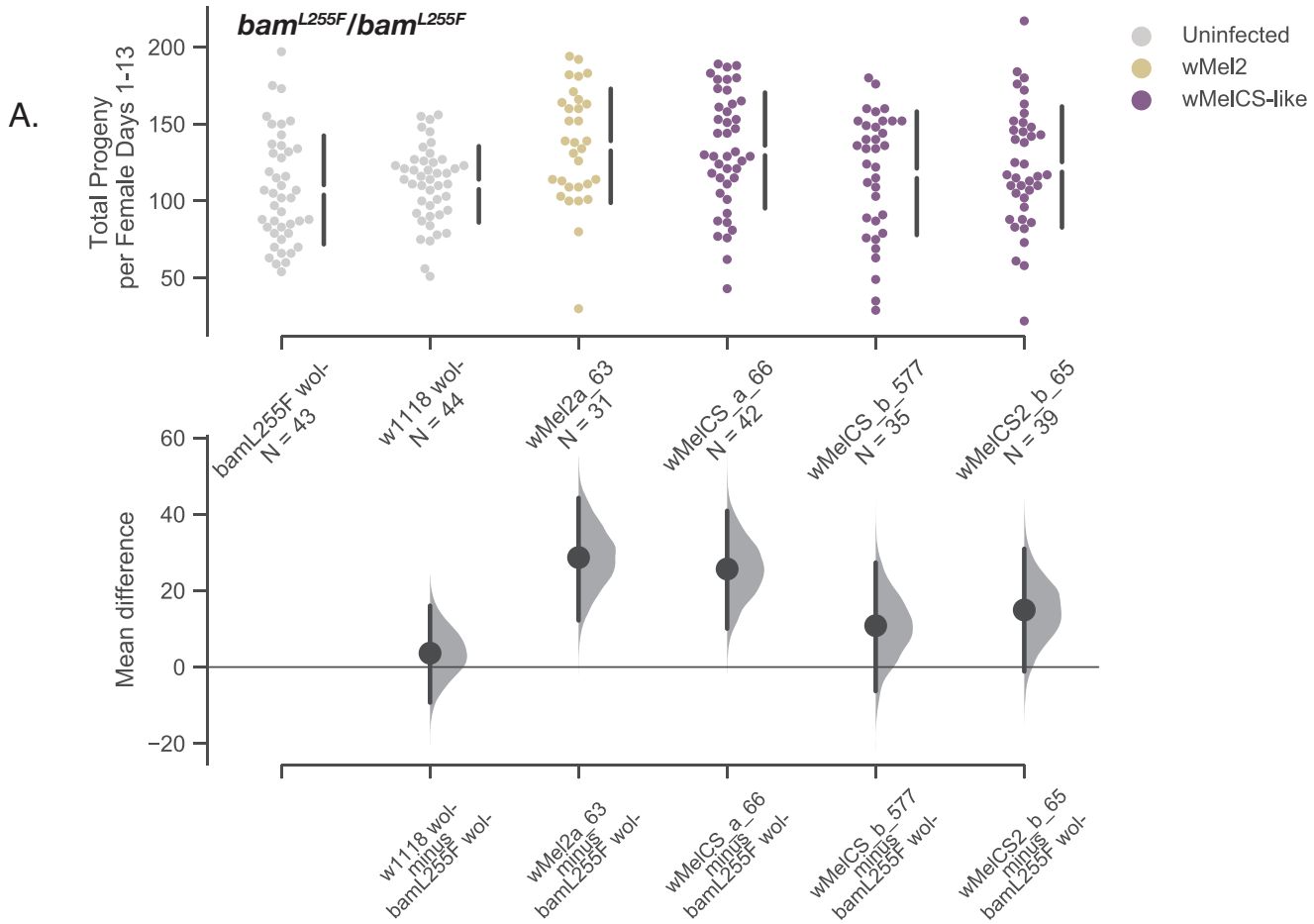


Fig 7. Total eggs per female counted over 3 days for the *w*^{1118}; *bam*$^{+}$/*bam*$^{+}$ genotype and the *w*^{1118}; *bam*^{L255F}/*bam*^{null} genotype infected with representative *Wolbachia* variants from the *w*Mel and *w*MelCS-like clades. (A) Swarm and Cumming estimation plots showing the total eggs per female for *w*^{1118}; *bam*$^{+}$/*bam*$^{+}$ lines infected with *w*Mel59 or *w*MelCSa66. Infection with either variant does not significantly affect total eggs per female. (B) Swarm and Cumming estimation plots showing the total eggs per female for *w*^{1118}; *bam*^{L255F}/*bam*^{null} females infected with the same two *Wolbachia* variants. Infection with either *w*Mel_59 or *w*MelCSa_66 rescues the number of eggs laid per female compared to the uninfected control (both $P < 0.001$, permutation test). Consistent with the results of the nurse cell assay, *w*MelCSa_66 shows a higher rescue effect than *w*Mel_59.



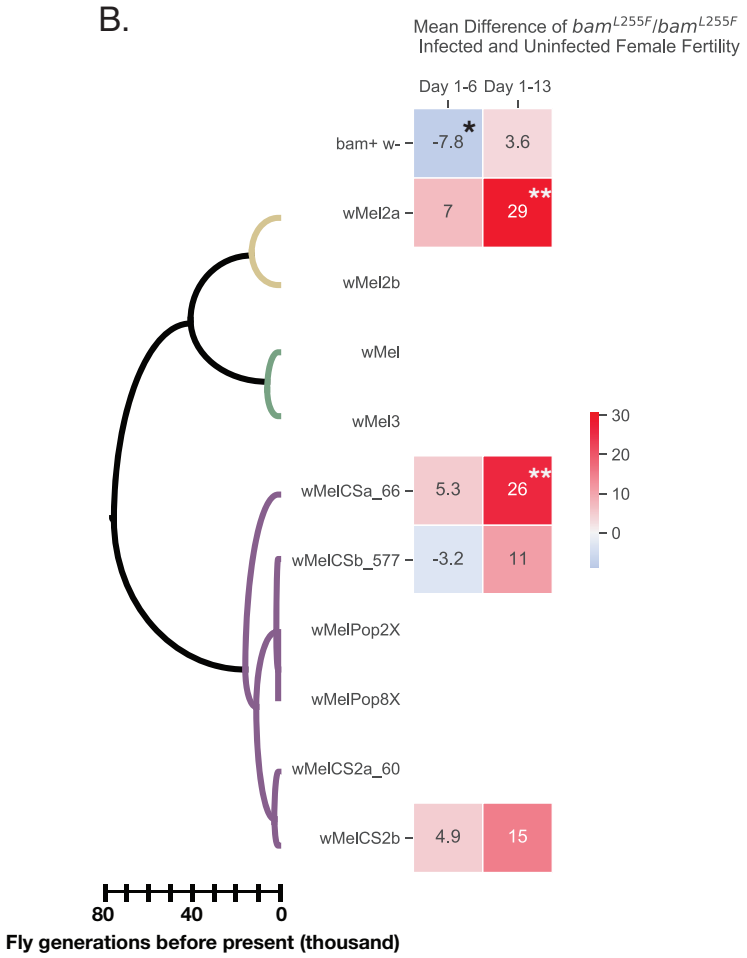
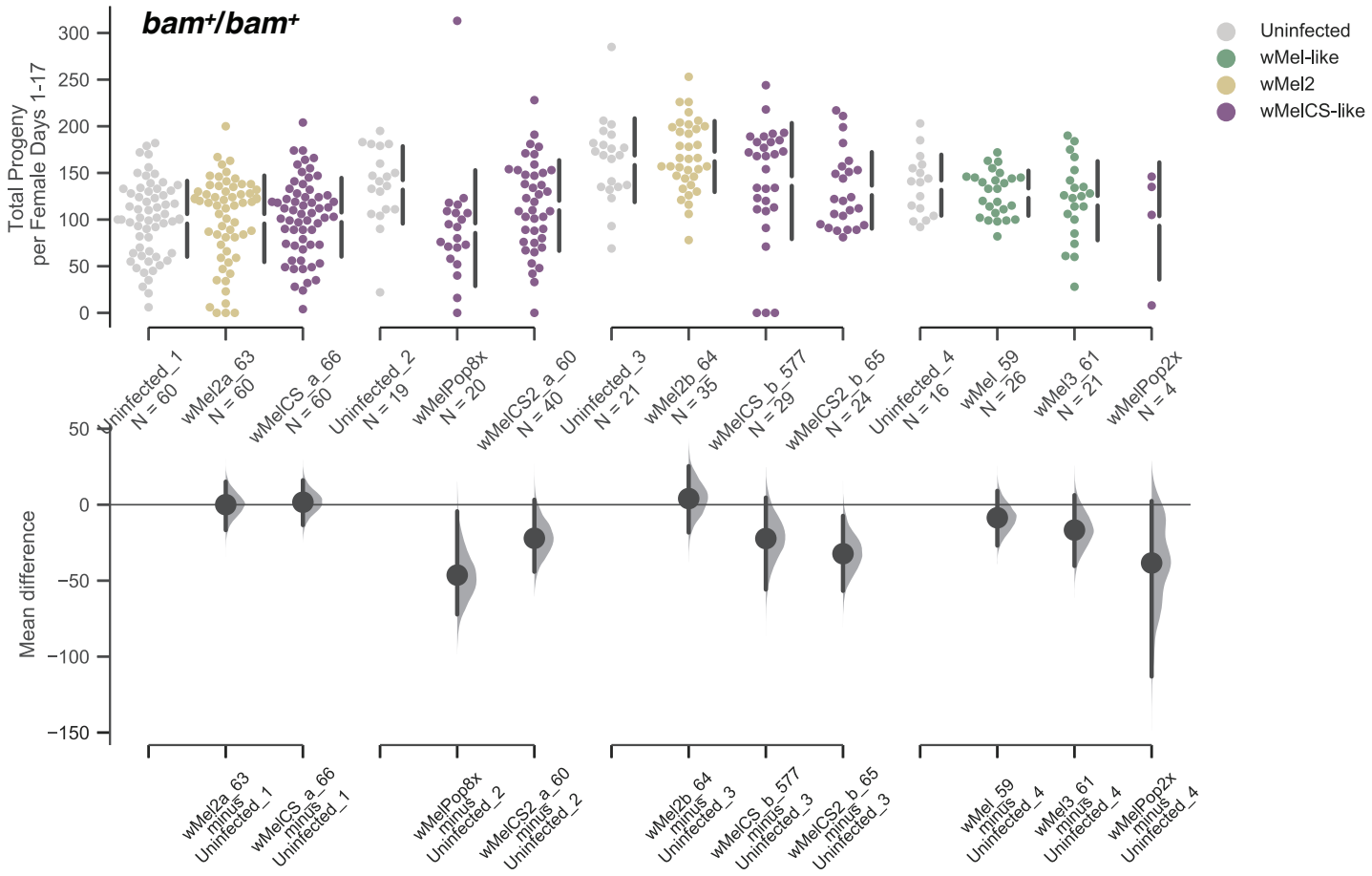
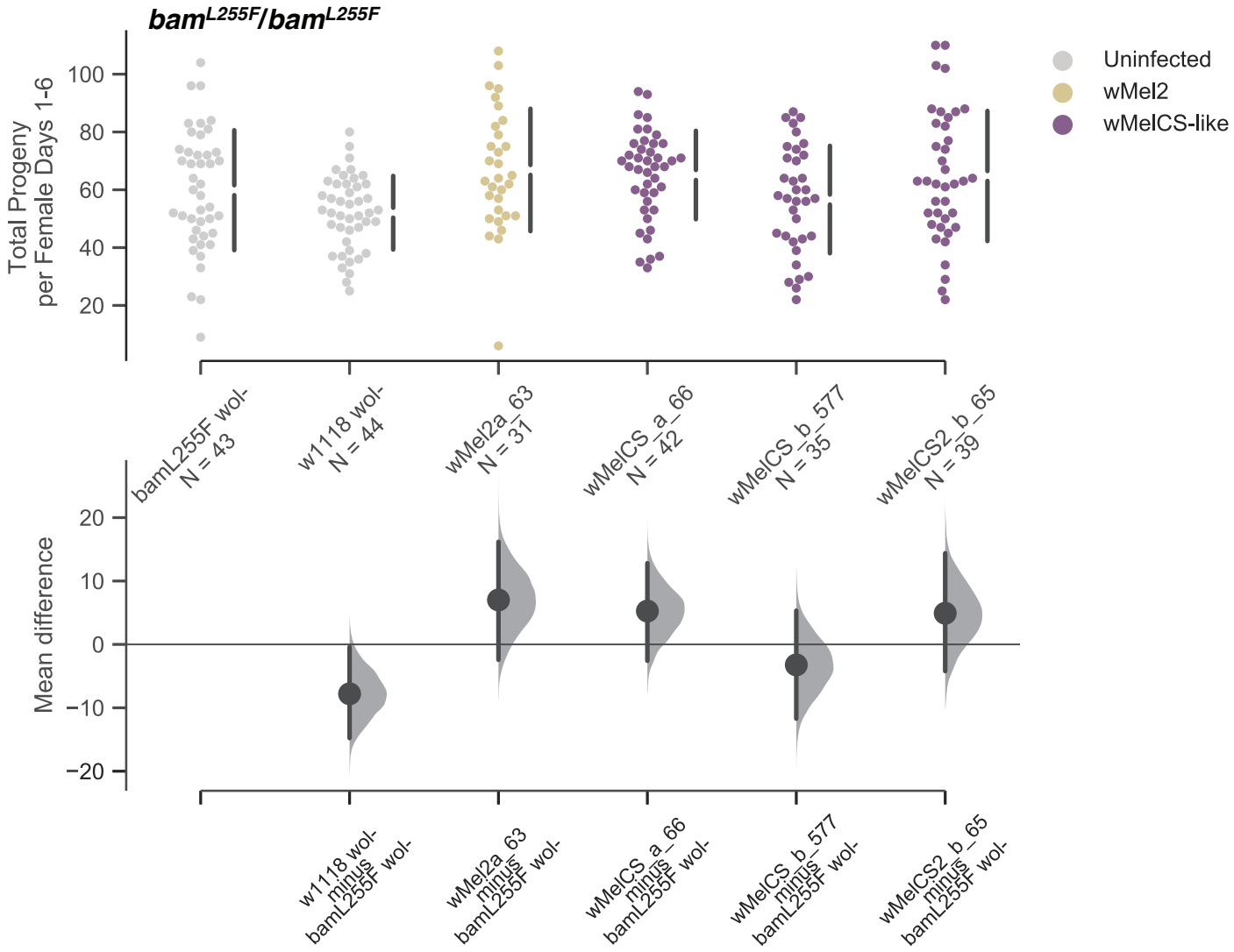


Fig 8. Total progeny per female and mean difference of progeny per female for the *w*^{1118}; *bam*^{L255F}/*bam*^{L255F} genotype infected with four representative *Wolbachia* variants compared to the uninfected control. (A) Swarm and Cumming estimation plots showing the effect of *Wolbachia* variants on *bam*^{L255F}/*bam*^{L255F} female fertility over days 1-13 of eclosion. We included uninfected *bam*$^{+}$/*bam*$^{+}$ females as well to compare *bam*^{L255F}/*bam*^{L255F} fertility to *bam*$^{+}$/*bam*$^{+}$ fertility. *Wolbachia* variants had differential effects on female *bam*^{L255F}/*bam*^{L255F} fertility. No variants had a negative effect on fertility. *Wolbachia* variants wMel2a_63, wMelCS_a_66 showed significant positive effects on fertility. The *bam*^{L255F}/*bam*^{L255F} genotype regardless of *Wolbachia* infection showed a wider range of progeny compared to *bam*$^{+}$/*bam*$^{+}$ females, but uninfected *bam*$^{+}$/*bam*$^{+}$ and uninfected *bam*^{L255F}/*bam*^{L255F} mean fertility was not significantly different. (B) Heatmap showing the mean difference in female fertility between females infected with each *Wolbachia* variant (or the *bam*$^{+}$/*bam*$^{+}$ genotype) and uninfected *bam*^{L255F}/*bam*^{L255F}. The significant positive effects of *w*Mel2a_63, and *w*MelCS_a_66 on fertility occurs after day 6 (**P < 0.01, permutation test). *bam*$^{+}$/*bam*$^{+}$ females exhibited marginally lower fertility than *bam*^{L255F}/*bam*^{L255F} females on days 1-6 (*P = 0.04), but the cumulative fertility across the whole experiment was not significantly different (days 1-13).

Supplementary Figures



FigS1. Total progeny per female and mean difference of progeny per female for the *w*^{1118}; *bam*$^{+}$/*bam*$^{+}$ genotype infected with 10 *Wolbachia* variants compared to uninfected. (A) Swarm and Cumming estimation plots showing the total progeny per female for each *Wolbachia* infected line counted on days 1-17 post eclosion. As the fertility assays were performed in batches, each batch is compared to its own uninfected control. *Wolbachia* variants *w*MelPop8X, and *w*MelCS2B were associated with significantly lower fertility compared to uninfected controls (95% confidence interval, effect size)



FigS2. Total progeny per female and mean difference of progeny per female for the *w*^{1118}; *bam*^{L255F}/*bam*^{L255F} genotype infected with four representative *Wolbachia* variants compared to the uninfected control. (A) Swarm and Cumming estimation plots showing the effect of *Wolbachia* variants on *bam*^{L255F}/*bam*^{L255F} female fertility over days 1-6 of eclosion. We included uninfected *bam*$^{+}$/*bam*$^{+}$ females as well to compare *bam*^{L255F}/*bam*^{L255F} fertility to *bam*$^{+}$/*bam*$^{+}$ fertility. *Wolbachia* variants did not have differential effects on female *bam*^{L255F}/*bam*^{L255F} fertility during this time frame. The *bam*^{L255F}/*bam*^{L255F} genotype regardless of *Wolbachia* infection showed a wider range of progeny compared to *bam*$^{+}$/*bam*$^{+}$ females, and uninfected *bam*$^{+}$/*bam*$^{+}$ mean fertility was significantly lower than uninfected *bam*^{L255F}/*bam*^{L255F} mean fertility (95% confidence interval, effect size).