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 infection with the *w*Mel strain of *Wolbachia* rescued the fertility and cytological ovarian defect of a *bam* hypomorphic mutant. However, this mutant was generated over 20 years ago in an uncontrolled and variable genetic background, and thus we have been unable to perform controlled experiments to further assess the interaction. Here, we used CRISPR/Cas9 to engineer the same single amino acid *bam* mutation into the w^{1118} isogenic background as well as generated a *bam* null allele in the same background. We confirm that the amino acid replacement results in the *bam* hypomorphic phenotype previously described when expressed over a *bam* null allele. We assess the female fertility of wildtype *bam*, a *bam* transheterozygous 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 variants on female fertility.

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 [1–3]. 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 [1]. While *Wolbachia* manipulates its host to increase its own transmission, some *Wolbachia* also provide the host protection against viruses, increase fecundity, and increase longevity [4–6].

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 [7–9]. 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 [10,11]. These loci have then been expressed in *Drosophila* as transgenes. Through these methods, the *Wolbachia* genes (orthologs in *w*Mel and *w*Pip) that cause CI have been identified and functionally validated in *Drosophila* [11,12]. Another group has identified the *Wolbachia* TomO locus which is one of

67 likely multiple *Wolbachia* loci that interact with *Sex lethal*, a germline stem cell (GCS) gene that
68 controls GSC maintenance and sex determination [10]. However, it is unclear how *Wolbachia*
69 genetic variation affects these phenotypes.
70
71 Previously, we reported that the *W. pipientis* variant *w*Mel genetically interacts with a
72 *Drosophila melanogaster* GSC gene, *bag of marbles* (*bam*), to rescue the fertility defect
73 caused by a single amino acid mutation [13]. *Bam* is the switch for GSC daughter
74 differentiation in *Drosophila melanogaster* females and the switch for terminal spermatocyte
75 differentiation in males [14–16]. Although *bam* function is essential for gametogenesis, and
76 *bam* is both necessary and sufficient for GSC daughter differentiation, we observe that it is
77 evolving under positive selection for amino acid divergence in an episodic manner across the
78 *Drosophila* genus [17,18]. We see a particularly strong burst of amino acid changes at *bam* in
79 the *Drosophila melanogaster* lineage [17]. This episodic pattern of positive selection is
80 consistent with selective pressures that are present in some lineages and not others and that
81 may come and go over time. This is similar to the nature of infections in natural populations,
82 where *Wolbachia* variants are known to infect populations, but also can be lost and replaced
83 fairly often and on both long and short time scales [19].
84
85 Our observation that *Wolbachia* and *bam* genetically interact led us to hypothesize that
86 *Wolbachia* may drive the adaptive evolution of *bam* [13]. Since *Wolbachia* is a known
87 manipulator of reproduction, *Wolbachia* may be in conflict with *bam* and other GSC genes for
88 control of oogenesis. While it may seem favorable to host fitness if *Wolbachia* manipulates
89 germline stem cell regulation to increase reproduction, and thus not present an evolutionary

90 conflict, it may not be beneficial for the host if oogenesis is regulated by *Wolbachia*. For
91 example, *Wolbachia* may promote oogenesis when it is not favorable for the host to reproduce
92 due to environmental or physiological factors. To further understand if *Wolbachia* could be in
93 genetic conflict with *bam* and drive its adaptation, we believe it is necessary to define the
94 genetic mechanism of the interaction between *bam* and *Wolbachia*.
95
96 Since we cannot culture *Wolbachia*, we made use of a genetic tool for functionally assessing
97 *Wolbachia* variation generated by Chrostek et al. [4], a set of w^{1118} isogenic lines individually
98 infected with 10 diverse *w*Mel *Wolbachia* variants from three clades defined by Richardson et
99 al [20]. These *Wolbachia* variants are documented to differentially protect the *Drosophila*
100 *melanogaster* host from viruses and increase cold tolerance [4,21]. Additionally, these
101 *Wolbachia* variants exhibit different titer phenotypes in *Drosophila melanogaster* males [4]. To
102 utilize this tool to further analyze our initial observation that *w*Mel rescues a *bam* hypomorphic
103 mutant, we used CRISPR/Cas9 to edit the original hypomorph amino acid change [22] into the
104 w^{1118} isogenic background, and to create a *bam* null allele in the same genetic background.
105 We confirmed that the amino acid replacement results in the *bam* hypomorphic phenotype
106 previously described when expressed over a *bam* null allele in uninfected females [13]. We
107 also show that uninfected females homozygous for the *bam* hypomorphic allele do not exhibit
108 GSC tumors or show reduced fertility compared to wildtype females. We then assessed the
109 effect of infection by the individual *Wolbachia* variants by crossing the cytoplasmic maternal
110 backgrounds infected with each *Wolbachia* variant into our w^{1118} *bam* hypomorph line thereby
111 maintaining the same nuclear background (except for the *bam* locus).
112

113 We find that all *w*Mel *Wolbachia* variants tested here generally increase the fertility of *bam*
114 mutant females, with *w*MelCS-like *Wolbachia* showing the highest effects. In contrast, in
115 wildtype *bam* backgrounds *w*Mel variants do not increase fertility, and some even decrease
116 fertility.
117

118 # Materials and Methods

119 **Fly stocks and rearing:**

120 Prior to experiments, we raised fly stocks on standard cornmeal molasses food at room
121 temperature. We used yeast glucose food for fertility assays. During experiments, we
122 maintained crosses and stocks in an incubator at 25°C with a 12-hour light-dark cycle. The
123 $bam^{\Delta 59}$ line and bam^{BW} line are described on Flybase [23] and in Flores et. al [13]. The
124 *Wolbachia* infected w^{1118} isogenic lines used in this study were generous gifts from Luis
125 Texiera and described in [4]. We used CantonS males for the w^{1118}; bam^{L255F}/bam^{null} and w^{1118};
126 bam^{+}/bam^{+} fertility assays. We used w^{1118} isogenic males for the w^{1118}; bam^{L255F}/bam^{L255F}
127 fertility assays. We verified that males were uninfected with *Wolbachia* by endpoint PCR and
128 qPCR using primers described below. To generate the *bam* hypomorphic lines infected with
129 the 10 different *Wolbachia* variants, we crossed females from the w^{1118}; TM2/TM6 stock
130 infected with each variant to w^{1118}; bam^{L255F}/TM6 males.
131

132 **Generation of *bam* alleles with CRISPR/Cas9:**

133 We engineered the two alleles described in this study in the w^{1118} isogenic background using
134 synthetic gRNAs and Cas9 protein as follows.
135

136 gRNAs:

137 We used the flyCRISPR target finder to choose gRNAs with zero predicted off-targets in the *D.*
138 *melanogaster* genome (Supplement Table S1). We then ordered synthetic gRNAs (sgRNA)
139 from Synthego.

140

141 Cloning:

142 We generated all PCR products for cloning with NEB High Fidelity Q5 master mix, and gel
143 extracted and purified PCR products using the Qiagen MinElute gel extraction kit. We created
144 the donor plasmid for homology directed repair using the NEB HiFi assembly cloning kit and
145 the pHD-attP-DsRed vector from flyCRISPR [24]. For plasmid prep, we used Qiagen plasmid
146 plus midi-prep kit and sequenced plasmids with Sanger sequencing (Cornell BRC Genomics
147 Core). We ordered primers for PCR, sequencing, and cloning from IDTDNA (Supplement
148 Table S2).

149

150 Donor sequences:

151 To generate the hypomorphic bam^{L255F} allele we designed a single stranded oligo donor
152 (ssODN) that contained the single amino acid mutation of the previously described *bam*
153 hypomorphic allele (CTT->TTT) as well as a single synonymous change to the closest
154 preferred codon in order to kill the gRNA site upon homology directed repair. We used 80 bp of
155 homology on each side of our targeted change (Supplement Table S1).

156

157 To generate the null $bam^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$ allele, we amplified 1.5 kb homology arms from the
158 w^{1118} isogenic line and cloned them into the pHD-attP-DsRed vector from flyCRISPR

159 (Supplement TableS3). As we did not need the attP site in our line, we amplified the 3xP3-
160 DsRed cassette from the plasmid and assembled the homology arms, 3xP3-DsRed, and the
161 original vector backbone with the NEB HiFi assembly kit, thereby removing the attP site.
162
163 Injections:
164 All CRISPR/Cas9 injections were sourced to Genetivision and were done in the w^{1118} isogenic
165 line. The injection mix contained plasmid or ssODN donor, sgRNAs, Cas9 protein (Synthego),
166 and an siRNA for Lig4 (IDT DNA, Supplement Table S1).
167
168 For the $bam^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$ allele, we screened for the eye color cassette in F1's in house using
169 a Nightsea fluorescent system (DsRed filters). For the bam^{L255F} allele, the edited nucleotide
170 change disrupts an AflII restriction site, allowing us to screen F1's by PCR (Promega GoTaq
171 mastermix) followed by restriction digests with AflII (NEB). We prepared genomic DNA using
172 the Qiagen PureGene kit.
173
174 We backcrossed females of all CRISPR/Cas9 mutants to w^{1118} isogenic males for three
175 generations, and then crossed the mutants to the w^{1118}; TM2/TM6 line to maintain the *bam*
176 mutants over the TM6 balancer. All CRISPR/Cas9 edits in the lines were confirmed by Sanger
177 sequencing (Cornell BRC Genomics Facility).
178
179 **PCR assays to detect *Wolbachia*:**
180 To test for the presence of *Wolbachia*, we used the Zymo quick DNA miniprep kit to prepare
181 DNA from three replicate samples each with three female flies. For each sample, we used

182 three different primers for endpoint or qPCR (Supplement Table S1). We used the common
183 *wsp* primers [13], a primer pair targeting *DprA*, and a highly sensitive primer pair to the ARM
184 repeat ([25] for endpoint PCR, modified here for qPCR) (Supplement Table S2).
185
186 **Immunostaining:**
187 We performed immunostaining as described in Aruna et al. [26] and Flores et al. [13]. Briefly,
188 we dissected ovaries in ice cold 1X PBS and pipetted the tissue up and down to improve
189 antibody permeability. We fixed ovaries in 4% paraformaldehyde (EMS), washed with PBST
190 (1X PBS, 0.1% Triton-X 100), blocked in PBTA (1X PBS, 0.1% Triton-X 100, 3% BSA) (Alfa
191 Aesar), and then incubated in the appropriate primary antibody overnight. We then washed,
192 blocked, and incubated in the appropriate secondary antibody for two hours, then washed and
193 mounted in ProLong Glass NucBlue for imaging (ThermoFisher). We used an anti-Vasa
194 antibody from Santa Cruz biologicals (anti-rabbit, 1:200). We imaged ovaries on a Zeiss i880
195 confocal microscope with 405 and 488 nm laser lines at 40X (Plan-Apochromat 1.4 NA, oil)
196 (Cornell BRC Imaging Core). We analyzed and edited images using Fiji (ImageJ).
197
198 **Assays for nurse cell positive egg chambers:**
199 We dissected ovaries from mated 2-4 day old $bam^{L255F}/bam^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$ females for all
200 *Wolbachia* variants and the uninfected control in PBS. Ovaries were fixed in 4%
201 paraformaldehyde (EMS), washed with PBST (1X PBS, 0.1% Triton-X 100), and then mounted
202 in ProLong Glass with NucBlue (ThermoFisher). We imaged ovaries on a Zeiss i880 confocal
203 microscope with a 405 nm laser line at 10X (C-Apochromat 0.45 NA, water) and 40X
204 (described above). We analyzed and edited images using Fiji (ImageJ). We counted the

205 number of nurse cell positive egg chambers (cysts) per ovary for uninfected females and
206 females infected with each *Wolbachia* genotype (as these ovaries are small and
207 underdeveloped, they stay intact during the fixation and washing steps).
208
209 **Fertility Assays:**
210 We used the following w^{1118} isogenic *bam* genotypes for the fertility assays, w^{1118}; bam^{+}/bam^{+},
211 w^{1118}; $bam^{L255F}/bam^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$, and w^{1118}; bam^{L255F}/bam^{L255F}. Additionally, we performed a
212 small control fertility assay using combinations of the alleles described above and the classic
213 bam^{BW} hypomorph and $bam^{\Delta 59}$ alleles.
214
215 We performed the fertility assays for w^{1118}; bam^{+}/bam^{+} and w^{1118}; $bam^{L255F}/bam^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$
216 in three batches to reduce technical error from too large of an experiment. We included
217 separate uninfected controls in each batch of *Wolbachia* variants and used these to make
218 statistical comparisons of mean progeny per female.
219
220 We performed all fertility assays (except for the small bam^{BW} and $bam^{\Delta 59}$ analysis) as follows
221 (and described by Flores et al. [13]):
222 We collected virgin females and aged them 2-3 days, only using flies that eclosed within 48
223 hours of each other to reduce developmental variation. We collected virgin males uninfected
224 with *Wolbachia* and aged them for 2-3 days. We distributed males from different bottles across
225 the female genotypes to control for any bottle effects. We individually crossed virgin females to
226 two virgin males. The trio were allowed to mate for 8 days, and then flipped to new vials. After
227 the second 8 days the trio was cleared. The progeny for each trio was counted every other day

228 to get the total adult progeny per female. Progeny per female is reported in increments by day
229 that reflect the days after the first progeny eclosed. For example, we report total progeny for
230 days 1-9 which are the progeny counted on day 1 of eclosion to day 9 of eclosion.
231
232 For the control fertility assay containing the bam^{BW} hypomorph and $bam^{\Delta 59}$ alleles, we
233 collected and aged flies as described above, except the progeny per trio was counted only
234 once at the end of the experiment.
235
236 **Egg Laying Assay:**
237 We collected virgin females for each genotype and *Wolbachia* infection status and CantonS
238 virgin males and aged them as described above for the fertility assays. Then, we allowed each
239 trio to mate for 24 hours on grape juice agar supplemented with yeast before flipping them to a
240 new grape juice agar vial. We counted the eggs laid within 24 hours and repeated this for three
241 days.
242
243 **Statistics:**
244 For the nurse cell assay, egg laying assay, and fertility assays we used estimation statistics to
245 assess the mean difference (effect size) of nurse cells, eggs, and adult progeny between
246 infected and uninfected lines. All estimation statistics were done using the dabest package in
247 Python (v. 0.3.1) with 5000 bootstrap resamples [27]. Estimation statistics provide a non-
248 parametric alternative to other statistical tests of the difference of the mean (for example,
249 ANOVA), and allow us to understand the magnitude of the effect of *Wolbachia* variation on the
250 *bam* phenotype. We display the data with a swarm plot that shows all of the data points and a

Cumming estimation plot that shows the effect size for each sample compared to the control and 95% bootstrap confidence interval. In text, we report significance as a mean difference (effect size) outside the 95% bootstrap confidence interval. While one benefit of these statistical methods is that they are less biased than reporting traditional P-values, we also report P-values for an approximate permutation test with 5,000 permutations.

258 # Results

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

260 We used CRISPR/Cas9 to recreate the same *bam* hypomorphic mutation as the original
261 *bam*^{BW} allele [22] in the isogenic *w*^{1118} background, of which we have infected with 10 different
262 variants of *Wolbachia* as described by Chrostek et al. [4] (Fig 1A&B, Fig 2A).

263

264 We asked if the *w*^{1118}; *bam*^{L255F} allele recapitulated the *bam*^{BW}/*bam*$^{\Delta 59}$ mutant phenotypes by
265 assessing the phenotype of a transheterozygous *w*^{1118}; *bam*^{L255F} hypomorph over a *bam* null
266 allele. Since we wanted all of our alleles in the same genetic background and there were no
267 existing *bam* null alleles in the *w*^{1118} isogenic background, we used CRISPR/Cas9 to knock in
268 a 3xP3-DsRed cassette into the second intron of *bam*, which resulted in a *bam* null allele in the
269 same *w*^{1118} background marked with a trackable eye marker (*w*^{1118}; *bam*$^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$, Fig
270 2B). For convenience and readability, we will refer to the *w*^{1118}; *bam*$^{null\text{-}In2\text{-}3xP3\text{-}DsRed}$ allele as
271 *bam*^{null} and the *w*^{1118}; *bam*^{L255F} allele as *bam*^{L255F}. Females homozygous for this *bam*^{null} allele
272 exhibit the expected tumorous ovary phenotype with no developing nurse cell-positive cysts
273 and are sterile (Fig 2C, fertility data not shown).

274

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

281 classic *bam* null alleles that we previously used to study the interaction between *bam* and
282 *Wolbachia, bam*$^{\Delta 59}$. The *bam*$^{\Delta 59}$ null allele is a nearly full deletion of the *bam* coding sequence.
283 We examined ovaries from *bam*^{L255F}/ *bam*$^{\Delta 59}$ females and observed the tumorous ovary
284 phenotype with some developing cysts as has been previously documented for *bam*^{BW}/*bam*$^{\Delta 59}$
285 and additionally shown here (Fig 2E&F). We measured the fertility of *bam*^{L255F}/ *bam*$^{\Delta 59}$ females
286 and observed a large fertility defect, consistent with the fertility of the *bam*^{L255F}/*bam*^{null}
287 genotype (Fig 3). Therefore, the *bam*^{L255F}/ *bam*^{null} cytological and fertility phenotypes are
288 consistent with the previously documented *bam* hypomorph phenotypes.
289
290 As we previously were unable to assess the phenotype of the *bam*^{BW} allele as a homozygote
291 due to recessive lethal mutations on the chromosome, we used our *bam*^{L255F} allele to ask if the
292 homozygous *bam*^{L255F} females also had a hypomorphic phenotype. We asked if *bam*^{L255F}
293 /*bam*^{L255F} females also exhibited the classic *bag of marbles* cytological phenotype and reduced
294 fertility. In contrast to the tumorous ovary phenotype of *bam*^{BW}/*bam*$^{\Delta 59}$ and *bam*^{L255F}/*bam*^{null}, a
295 result of a defect in *bam*'s differentiation function which causes GSC-like cells to over-
296 proliferate, we found no evidence of tumorous cysts in ovaries of *bam*^{L255F} /*bam*^{L255F} females
297 (Fig 2H). These ovaries resembled those of a wildtype *bam* background, with all developing
298 egg chambers featuring nurse cells (Fig 2H). Previously we were unable to compare the
299 fertility of the *bam*^{BW} hypomorph to wildtype *bam* fertility as fertility is greatly affected by
300 different genetic backgrounds. We compared the fertility of the *bam*^{L255F} /*bam*^{L255F} females to
301 *w*^{1118}; *bam*$^{+}$/*bam*$^{+}$ females and found that the total progeny per female was similar between the
302 two genotypes (Fig 2I). While we did not measure all aspects of *bam* function of this genotype,
303 the fertility and cytological data here indicate that the *bam*^{L255F} /*bam*^{L255F} genotype does not

304 cause a severe *bam* mutant phenotype, as we observed previously for the transheterozygous
305 bam^{BW} hypomorph ($bam^{BW}/bam^{\Delta 59}$).
306
307 Although we cannot make strong claims about the effect of *bam* genotype on fertility between
308 lines with different genetic backgrounds, we did perform a small control experiment to ask if
309 our new bam^{L255F} hypomorphic allele over the classic bam^{BW} hypomorphic allele also show
310 similar fertility to wildtype females as we observed for bam^{L255F}/bam^{L255F} females. We
311 observed that bam^{L255F}/bam^{BW} females and homozygous bam^{L255F} females did not show
312 significantly different fertility from w^{1118} bam^{+}/bam^{+} females (Fig 3). Further, we asked if our
313 new $bam^{L255F}/bam^{\mathrm{null}}$ genotype showed a similar fertility defect to $bam^{L255F}/bam^{\Delta 59}$ (classic null)
314 and found that both showed severely reduced fertility compared to wildtype (Fig 3).
315
316 These data further confirm that our new bam^{L255F} and bam^{null} alleles behave similarly to alleles
317 we have used in the past to study the interaction between *bam* and *Wolbachia.* Since we did
318 not observe a phenotype in the bam^{L255F}/bam^{L255F} females that would be sufficient to measure
319 the *Wolbachia* rescue of both a fertility defect and an ovarian cytological defect as we have
320 previously described [13], but we were able to successfully recapitulate the partial-loss of
321 function *bam* phenotype with the bam^{L255F}/bam^{null} genotype, we focused primarily on the effect
322 of *Wolbachia* variation on the bam^{L255F}/bam^{null} female fertility and cytological phenotype and
323 the effect of *Wolbachia* variation on fertility of w^{1118}; bam^{+}/bam^{+} females, which has not been
324 previously described.

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

326 Fertility measured over multiple days reflects progeny produced cumulatively by the female
327 over time, although there could also be variation in development time of the progeny. We first
328 assessed the effect of *Wolbachia* variation on wildtype *bam* female fertility (w^{1118}; bam^{+}/bam^{+}).
329 Over 17 days, we counted the progeny produced by each test female infected individually for
330 each of the 10 *Wolbachia* variants and assessed the mean difference of adult progeny from
331 uninfected control females. We found that none of the *Wolbachia* variants increased fertility in
332 the wildtype *bam* background, and some lines showed a significant decrease in total progeny
333 per female across all days measured (*w*MelPop8X, and *w*MelCS2b_60) (Fig 4A&B,
334 Supplement Fig S1). Of the lines that showed a negative impact of *Wolbachia* on fertility, this
335 effect was more pronounced as the female fly aged. On days 1-9 of progeny eclosion, no
336 *Wolbachia* variant had a significantly negative effect on progeny counts, which is notable as
337 these days represent progeny from eggs laid earlier in the test female's life and thus are more
338 likely to reflect effects of *Wolbachia* variation on fertility in nature. The strains that showed a
339 significant and large negative effect of *Wolbachia* infection on fertility were both in the *w*MelCS
340 clade (mean difference between -32 and -46 progeny per female for days 1-17 Fig 4A&B).
341 *w*MelCS variants exhibit higher *Wolbachia* titer in males [4], which if also true in females may
342 negatively impact female fertility if titer gets too high [28].

343

344

345

346

347 **All *w*Mel variants rescue *bam* hypomorph female fertility but show a range of effect**
348 **sizes.**

349 Our results from the w^{1118}; bam^{+}/bam^{+} fertility assay indicated that none of the *Wolbachia*
350 variants tested have an overall increase in fertility in females. We performed the same fertility
351 assay described above for the bam^{L255F}/bam^{null} genotype infected with the same 10 *Wolbachia*
352 variants. In contrast to the wildtype *bam* lines, in the bam^{L255F}/bam^{null} lines we found that all
353 variants increased female fertility, and some variants had a notably large effect on female
354 fertility. The variants with the largest effect sizes were in the *w*MelCS-like clade (*w*MelCS2b,
355 *w*MelPop2x, *w*MelPop8x) (Fig 5A&B). This fertility rescue is in contrast to the decrease in
356 fertility by the same *Wolbachia* variants in the wildtype *bam* background, especially for
357 *w*MelCS2b which had the largest negative effect on wildtype *bam* fertility, but the largest
358 positive effect on bam^{L255F}/bam^{null} fertility (Fig 5A&B). In all of the lines tested there were many
359 individual females that had no progeny as well as females with varying distributions of
360 progeny. In some of the lines (*w*Mel2a, *w*MelCS2a, *w*MelCS2b) there were outlier females who
361 had large rescue of fertility, some in the range of what we observe for wildtype *bam* fertility (Fig
362 4A). However, for most of the individuals tested, fertility was not fully rescued, e.g. restored to
363 wildtype levels (Fig 4A, Fig 5A). Although the largest effect of *Wolbachia* on bam^{L255F}/bam^{null}
364 rescue was amongst the *w*MelCS-like variants, there were other notable variants outside of the
365 *w*MelCS clade that showed high increase in female fertility, particularly *w*Mel2a. Overall these
366 data further indicate a genetic interaction between *bam* and *Wolbachia* that is different from
367 the overall effect of *Wolbachia* on female fertility.

368 **All but one *w*Mel variant rescue the ovarian *bam* hypomorph cytological defect but to**
369 **varying degrees**

370 Although we wanted to assess the rescue of the *bam* hypomorphic fertility phenotype by
371 *Wolbachia* because we can also compare this to the effect of *Wolbachia* on wildtype *bam*
372 fertility, we also wanted to know if genetic variation in *Wolbachia* affected the rescue of the
373 *bam* hypomorphic cytological phenotype. Because fertility is affected by environmental and
374 genetic differences that act in later developmental stages outside of *bam*'s function early in
375 gametogenesis, we also measured the effect of *Wolbachia* variation on the rescue of the
376 cytological *bam* mutant phenotype. Since the partial loss of function of *bam* results in over-
377 proliferation of GSCs and reduced differentiation, we can quantify *bam* function by assaying for
378 differentiated germ cells. The cytological *bam* hypomorphic phenotype in females consists of
379 tumorous ovarioles filled with GSC-like cells, with few properly developing egg chambers (Fig
380 6B). Properly developing egg chambers contain polyploid nurse cells, which feature large,
381 easily identifiable nuclei (Fig 6B, arrowheads). We counted the number of nurse cell positive
382 egg chambers as a measure of GSC daughter differentiation and thus *bam* function. We found
383 that all but one of the *Wolbachia* variants tested significantly increased the number of nurse
384 cell positive egg chambers, and that *Wolbachia* variants in the *w*MelCS-like clade showed
385 highest numbers of nurse cell positive egg chambers (Fig 6A&B). The one variant that did not
386 result in a significant increase in nurse-cell positive egg chambers was *w*Mel2b, although we
387 did observe a significant increase in fertility in females infected with this variant. One possible
388 explanation for this observation is that the nurse cell assay as we performed it here assesses
389 differentiating germ cells from 2-3 day old females only. We assume that the counts of progeny
390 eclosion over time reflect oogenesis of the parental female over time (although variation in

391 development of the progeny also adds additional variation to the distribution of adult progeny
392 over time). Given this assumption, as day 6 of progeny eclosion shows the highest progeny
393 counts, parental female fertility is likely highest later than 2-3 days old (data not shown).
394 Therefore assaying 2-3 day old females may have been too early to detect many nurse cell
395 positive cysts and it would be informative to perform the nurse cell assay at additional time
396 points. Aside from *w*Mel2b, these results are consistent with the fertility assay results for the
397 bam^{L255F}/bam^{null} hypomorph genotype, however we observed a more obvious trend of higher
398 *bam* rescue by the *w*MelCS-like variants in this assay. All of the *Wolbachia* variants with the
399 highest effect size in increased *bam* function are in the *w*MelCS-like clade. It is unclear
400 whether this higher rescue is due to titer or another genetic difference between these clades,
401 but these data indicate that genetic variation in *Wolbachia* impacts the *bam* rescue phenotype.
402
403 To ask if the nurse cell positive egg chambers resulted in oocytes capable of development if
404 fertilized, and therefore represent properly differentiated GSC daughters, we picked two
405 representative *Wolbachia* variants of high and low rescue and counted the total number of
406 eggs laid per female over days 1-3. In w^{1118}; bam^{+}/bam^{+} females, neither *Wolbachia* variant
407 affected the number of eggs laid per female (Fig 7A). In contrast to the w^{1118}; bam^{+}/bam^{+}
408 females and consistent with the nurse cell assay, we observed few eggs laid by uninfected
409 w^{1118}; bam^{L255F}/bam^{null} females, with significantly more eggs produced by the lower rescuing
410 *Wolbachia* *w*Mel59, and the highest number of eggs laid by females infected with the high
411 rescuing line, *w*MelCSa_66 (Fig 7B).
412

413 **Homozygous *bam*^{L255F} females exhibit more variable fertility which is not negatively**
414 **affected by *Wolbachia***

415 In light of the negative effect of infection by some *Wolbachia* variants on wildtype *bam* fertility
416 and that the uninfected *bam*^{L255F}/*bam*^{L255F} females did not exhibit a fertility defect (Fig 2I, Fig
417 8A&B), we asked if infection by a subset of the *Wolbachia* variants we assessed in the
418 wildtype *bam* background had any effect on the fertility of *bam*^{L255F}/*bam*^{L255F} females. We
419 chose three *Wolbachia* variants that did not have a significant effect on wildtype *bam* female
420 fertility (*w*Mel2a_63, *w*MelCSa_66, and *w*MelCSb_577 Fig 4), and one that had a significant
421 negative effect on female fertility (*w*MelCS2b, Fig 4). We measured fertility of homozygous
422 females infected with *Wolbachia* variants as previously described. In contrast to our findings in
423 the *bam*$^{+}$/*bam*$^{+}$ background that *w*MelCS2b negatively affected female fertility, we found that
424 *w*MelCS2b did not have a significant effect on female fertility in the *bam*^{L255F}/*bam*^{L255F}
425 background (Fig 8A&B). Additionally, in contrast to our findings in the *bam*$^{+}$/*bam*$^{+}$ background
426 that *w*Mel2a_63 and *w*MelCSa_66 did not significantly affect female fertility, we found that in
427 the *bam*^{L255F}/*bam*^{L255F} background both of these variants had a significantly positive effect on
428 female fertility (Fig 8A&B). In both *bam*$^{+}$/*bam*$^{+}$ and *bam*^{L255F}/*bam*^{L255F} backgrounds,
429 *w*MelCSb_577 did not have a significant effect on female fertility (Fig 4, Fig 8A&B).
430

431 Interestingly, during days 1-6 uninfected wildtype *bam* females had significantly fewer progeny
432 than uninfected homozygous *bam*^{L255F} females (Fig 8B, Supplement Fig S2). We also
433 observed that the total progeny per female of the homozygous *bam*^{L255F} genotype had a wider
434 range than wildtype, which was not impacted by *Wolbachia* and is therefore likely a subtle *bam*
435 mutant phenotype (Fig 8A). As *bam* regulates GSC daughter differentiation, its mis-regulation

436 may increase or decrease fertility. These data further indicate that there is a genetic interaction
437 between *bam* and *Wolbachia* that is resulting in the increase in fertility, not simply an overall
438 increase in fertility that is more sensitive in a *bam* mutant background.
439

440 ## Discussion

441

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

444 We previously reported that infection with *w*Mel rescued the fertility defect of $bam^{BW}/bam^{\Delta 59}$
445 hypomorphic mutant females [13]. We sought to better understand this interaction for multiple
446 reasons. The first reason is that we hypothesize that an interaction between *Wolbachia* and
447 *bam* may be driving the adaptive sequence divergence of *bam*. However, in order to
448 understand if and how this interaction could be adaptive, we need to understand the nature of
449 the manipulation of *bam* function by *Wolbachia*. Second, *Wolbachia* is of immense interest as
450 a potential means to control vectors of some human diseases [8]. However, we do not know
451 the phenotypic range of host adaptations to new *Wolbachia* infections. Defining the genetic
452 mechanisms between the host and *Wolbachia* that are necessary for its reproductive
453 manipulations may aid in monitoring efforts to control vectors with *Wolbachia*.
454

455 One such way to find the *Wolbachia* loci necessary for the interaction between bacteria and
456 the host would be to systemically mutagenize the bacteria and then screen for variants that
457 enhance or reduce the phenotype of interest. However, *Wolbachia* are obligate endosymbionts
458 that cannot be cultured, and so are currently not amenable to such genetic manipulation. While

459 some studies have identified *Wolbachia* loci that interact with *D. melanogaster* by
460 transgenically expressing *Wolbachia* loci in *D. melanogaster*, this requires identifying
461 candidate *Wolbachia* loci that affect the host phenotype of interest [11,29]. One method to
462 identify candidate *Wolbachia* loci that affect the host phenotype is to use genetic variation in
463 *Wolbachia* to test for differences in the effect of *Wolbachia* variants on the host phenotype.
464
465 Chrostek et al [4] generated isogenic *Drosophila* lines infected with previously described and
466 genetically distinct *w*Mel strains of *Wolbachia*. As our current *bam* mutant was not in this host
467 genetic background, and thus we would not be able to conclude that any differential interaction
468 between *bam* and these variants was due to *Wolbachia* variation and not host genetic
469 variation, we used CRISPR/Cas9 to create the same amino acid mutant present in the original
470 bam^{BW} hypomorph allele in the w^{1118} isogenic background. To ensure we had the tools to
471 assess the *Wolbachia* rescue phenotype as previously described, we also generated a *bam*
472 null allele using CRISPR/Cas9 in the same genetic background.
473
474 We have now confirmed that these two new alleles behave similarly to the alleles we
475 previously used to document the interaction between *w*Mel and *bam*. There have been a
476 handful of studies assessing the phenotypes of diverse *Wolbachia* in isogenic backgrounds
477 [4,28,30,31], however there have been no documented studies of interactions between
478 *Drosophila* mutants and *Wolbachia* in these backgrounds. Recent work on the interaction
479 between *Wolbachia* and *Sex lethal* in the female germline showed that *Wolbachia* rescues the
480 loss of GSCs in some *Sxl* mutants through a *nanos* dependent interaction with the *Wolbachia*
481 protein TomO [10]. However, when researchers transgenically expressed *TomO* in *Sxl*

482 hypomorphic ovaries, the GSC number was rescued, but fertility was not. This result indicated
483 that there are other mechanisms *Wolbachia* is using to fully rescue GSC number and fertility in
484 *Sxl* mutants. We believe the strategy we describe here could be used to further define genetic
485 interactions such as the *Sxl* and *Wolbachia* interaction [32]. As we and others have also had
486 success with CRISPR/Cas9 in non-*melanogaster* species, we believe this type of systematic
487 analysis could be extended to assess interactions of host genes and *Wolbachia* in other
488 species outside of *Drosophila melanogaster*.
489
490 ***w*Mel *Wolbachia* variants do not broadly increase fertility**
491 Due to the isogenic nature of the *Wolbachia* infected lines, we were able to rigorously assess
492 the effect of different *Wolbachia* variants on wildtype *bam* female fertility. In addressing if and
493 how *Wolbachia* may drive the adaptive evolution of *bam*, it was important for us to know if
494 *Wolbachia* has an effect on fertility in a wildtype *bam* background. Here, we are not measuring
495 specifically how *Wolbachia* is modulating a functional *bam* allele, as fertility is affected by many
496 loci, but if we observed a large effect of a particular *Wolbachia* variant on fertility, this would
497 motivate further experiments to assess functional *bam* activity in lines infected with that
498 *Wolbachia* variant. We observed no line with increased fertility for any of the *Wolbachia*
499 variants, indicating that *Wolbachia* is not generally upregulating fertility through *bam* or another
500 pathway in this genetic background. However, we also observed that some *Wolbachia* variants
501 had a negative effect on female fertility. We cannot distinguish whether this effect is due to
502 *Wolbachia* mis-regulating the germline or some other developmental consequence of its
503 infection. In fact, the lines with the highest reported titers in males showed the largest negative
504 impact on female fertility [4]. This observation highlights the complexity of the interaction

505 between *Wolbachia* and *Drosophila*, where depending on the genetic background of the host,
506 the fitness effect of *Wolbachia* on a phenotype may vary. This effect could further vary based
507 on aspects of the host's environment where the manipulation of fertility by *Wolbachia* may not
508 always be beneficial. Here we may expect the host and microbe to evolve ways of evading
509 each other, which may become more or less apparent in different genetic backgrounds. Of
510 note, our observations are under laboratory conditions in a highly inbred line, and so it is
511 unclear what effect these *Wolbachia* variants have on female fertility in natural populations.
512

513 **_w_Mel _Wolbachia_ variants across three clades genetically interact with _bam_**

514 We previously showed that a single *w*Mel strain partially rescued the fertility and GSC
515 daughter differentiation defect of the $bam^{BW}/bam^{\Delta 59}$ hypomorph. Here we asked if there was
516 variation in the *bam* rescue phenotype due to strain variation in *Wolbachia*. The strains we
517 used have been fully sequenced and differ by varying degrees of sequence divergence. Of the
518 three clades tested, all 10 *Wolbachia* variants rescued the fertility defect of the bam^{L255F}/bam^{null}
519 hypomorph. Therefore, none of the variants tested contained genetic variation that suppressed
520 the interaction between *bam* and *Wolbachia*. We did find that in both the counts of nurse cell
521 positive egg chambers and the fertility assays, *w*MelCS-like *Wolbachia* variants showed the
522 highest rescue (Fig 5, Fig 6). This pattern was the clearest in the counts of nurse cell positive
523 egg chambers as expected, since the presence of nurse cell positive egg chambers reflects
524 GSC daughter differentiation, a phenotype that is a more direct output of *bam* activity. It is
525 likely that the higher level of variability in the fertility assays is also due to the variable effect
526 the *Wolbachia* variants have on other stages of development post GSC daughter
527 differentiation. However, we feel it is important to also measure the effect of *Wolbachia* on

528 fertility, since we cannot assess the count of nurse cell positive egg chambers for *bam* alleles
529 that do not show a tumorous mutant phenotype, and it gives us insight into how *Wolbachia*
530 infection could be adaptive. For example, would there be a fitness tradeoff between a high
531 fecundity (GSC daughter differentiation) rescue and a low fertility (adult progeny) rescue?
532 Measuring the impact of *Wolbachia* on multiple stages of oogenesis and reproduction therefore
533 gives us insight into any further complexities in the interaction between GSC genes and
534 *Wolbachia*.
535
536 *w*MelPop is a virulent strain of *w*MelCSb characterized by uncontrolled proliferation and titer
537 caused by increased copy number of the Octomom locus. wMelPop2X and *w*MelPop8X exhibit
538 2X copies of the Octomom locus and anywhere from 4X-8X copies, respectively [30].The high
539 rescue of both the cytological and fertility defect of the bam^{L255F}/bam^{null} by *w*MelPop2X is
540 suggestive that titer and possibly other phenotypes that increase interactions with the host are
541 contributing to the rescue of *bam*. We observed lower rescue for *w*MelPop8X, indicating that
542 the increasing copy number of the Octomom locus that causes apoptosis and early death likely
543 negatively affects host fertility [30,33]. *w*MelPop2X is closely related to *w*MelCSb, with the only
544 variation between them being a synonymous SNP and the amplification of the octomom locus
545 which contains *Wolbachia* loci predicted to be involved with nucleic acid binding, and thus
546 likely how it increases its titer, as well as proteins with predicted homology to eukaryotic
547 domains [30]. These loci may then increase the interaction of *Wolbachia* and its host [6]. A
548 natural next step would be to determine the *Wolbachia* factors that manipulate GSC daughter
549 differentiation.
550

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

552 It is well established that disrupting *bam* function negatively effects fertility [13,15,34,35], and
553 bam^{L255F}/bam^{null} females are almost completely sterile. However, we do not predict such
554 deleterious alleles to reach high frequency in natural populations. In fact, we have not found
555 this variant segregating in any of the natural populations of *D. melanogaster* that have been
556 sampled in the *Drosophila* genome Nexus [36]. Thus, while studying this mutant is effective in
557 further refining how *bam* and *Wolbachia* interact, we cannot conclude that this type of
558 interaction occurs in natural populations. However, given the severity of the mutant and
559 strength of *Wolbachia*'s rescue, one hypothesis is that if *Wolbachia* is increasing GSC
560 daughter differentiation when it is not favorable for the host, the host would evolve a way to
561 evade *Wolbachia*'s manipulation of this pathway. In the case of our lab generated mutant, it is
562 possible that when *bam* is not fully functional, the background is more sensitive to
563 manipulation by *Wolbachia*. *Wolbachia* have been documented to respond to changes in the
564 host environment, as *Wolbachia* gene expression is affected by host age and sex, and as
565 *Wolbachia* transmission requires functional oogenesis, it reasonable to hypothesize that
566 *Wolbachia* is sensitive to changes in gametogenesis [37,38].
567

568 An additional observation we made is that bam^{L255F}/bam^{L255F} females show a broader range of
569 adult progeny per female compared to wildtype *bam* females regardless of *Wolbachia* infection
570 status. The individual bam^{L255F}/bam^{L255F} females exhibit both higher and lower than average
571 fertility, indicating that mis-regulating *bam's* differentiation function could both increase and
572 decrease fertility. This phenotype is worth further investigation, since although *bam* shows a
573 signature of positive selection, we do not know specifically what aspect of *bam* function is

574 adaptive. If variation in *bam* function can both increase and decrease mean fertility, and
575 *Wolbachia* has a generally positive effect on fertility of bam^{L255F}/bam^{L255F} females, this is further
576 evidence that *Wolbachia* may be able to manipulate *bam* in order to increase oogenesis for its
577 own benefit, and that genetic variation at *bam* could affect the regulation of oogenesis.
578 Therefore, if *Wolbachia* increased the rate of oogenesis to ensure its own transmission and
579 this was not favorable for the host, *bam* may be in conflict with *Wolbachia* to regulate
580 oogenesis in a favorable manner for the host. Here we see that although the average female
581 fertility of uninfected bam^{L255F}/bam^{L255F} mutants is not significantly different from wildtype, there
582 is a wider distribution of individual female fertility, indicating that the *bam* hypomorphic
583 phenotype is both an increase and decrease in fertility (Fig 8A). Since these females are in an
584 isogenic background and we do not expect genetic variation between individual females, the
585 *bam*-mediated mis-regulation of differentiation may set off a cascade of other genetic mis-
586 regulation that results in higher or lower fertility over time. We see that infection by some
587 *Wolbachia* variants in this background increases female fertility, indicating that perhaps
588 *Wolbachia* can restore the mis-regulation of GSC daughter differentiation, and even increase
589 reproductive output through this mechanism. It would be interesting to use this genotype for
590 future experiments to explore the possibility that this *bam* mutation may disrupt the mechanism
591 *D. melanogaster* evolved to evade *Wolbachia*'s effect on differentiation.
592
593 An interesting question that remains is how *Wolbachia* have evolved in their interaction with
594 GSC genes, including *bam*. While *w*MelCS-like and *w*Mel-like variants share a most recent
595 common ancestor about 8000 years ago, *w*MelCS-like variants were recently (~2000 years
596 ago) replaced by *w*Mel-like variants in natural populations of *D. melanogaster* [20,39].

597 However, this replacement has not been complete, and there are global populations still
598 infected with *w*MelCS variants [39,40]. Interestingly we find that these variants show higher
599 rescue of bam^{L255F}/bam^{null} female fertility and nurse cells. If *bam* and *Wolbachia* are evolving in
600 an arms race, it could be that *w*Mel has not evolved the same level of interaction with *bam* as
601 *w*MelCS has. Future work to investigate these dynamics should include sampling populations
602 that are still infected with *w*MelCS as well as those infected with *w*Mel and asking if there is
603 any evidence of genetic differentiation at *bam*. Additionally, we could utilize existing *Wolbachia*
604 sequence variation from natural populations to ask if there is any evidence of associations
605 between *bam* variation and *Wolbachia* variation. If we were to sample populations that are
606 differentially infected with *w*Mel and *w*MelCS to assess genetic differentiation at *bam* as
607 discussed above, we could also do the same for *Wolbachia* variants. One caveat being that if
608 we did not observe genetic signatures of adaptive evolution in *Wolbachia* loci, this does not
609 mean that *Wolbachia* and *bam* are not coevolving, as we do not know the true infection history
610 of a *Drosophila* population with *Wolbachia* and thus which *Wolbachia* variant may have been in
611 conflict with *bam.* Additionally, there may be general *Wolbachia* functions that are interacting
612 with *bam* and not a single locus (e.g. loci that regulate titer).
613
614 Further work should focus on determining the *Wolbachia* loci contributing to the different
615 magnitude of *bam* rescue. However, as has been previously pointed out by Chrostek et al. [4],
616 between the *w*Mel clades and *w*MelCS clades there are eight indels, 108 SNPs, and of those,
617 polymorphisms in coding sequence of 58 genes. So further work would have to be done to
618 narrow down which variants contribute to the degree of rescue. A next step could be moving
619 out to more divergent *Wolbachia* (for example, *w*Au that infects *D. simulans* [41]) to ask how

recently the interaction with *bam* evolved. To complement this, it would be beneficial to perform these same rescue experiments with *bam* hypomorphs in *D. simulans*.

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633

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Figures

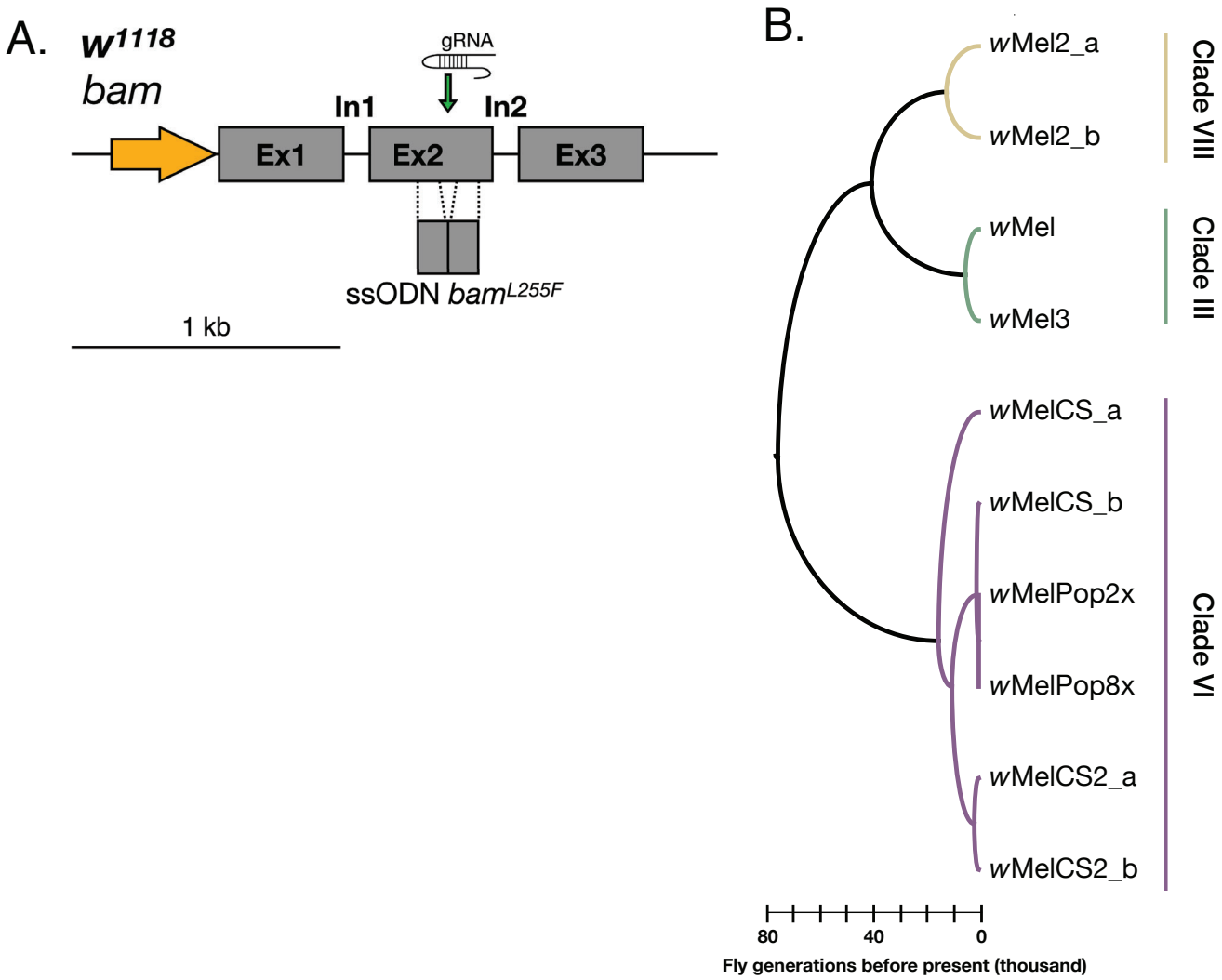


Fig 1. Design for recreating the classic *bam*^{BW} hypomorph allele in the *w*^{1118} isogenic background with CRISPR/Cas9 to test for differential rescue by diverse *Wolbachia.* (A) Schematic of the *bam* gene region showing the single gRNA target site in the second exon and the single stranded oligo donor containing the hypomorphic missense mutation. (B) A cladogram adapted from Chrostek et al. [4] showing the relationships and clade assignments for the *Wolbachia* variants tested in this study.

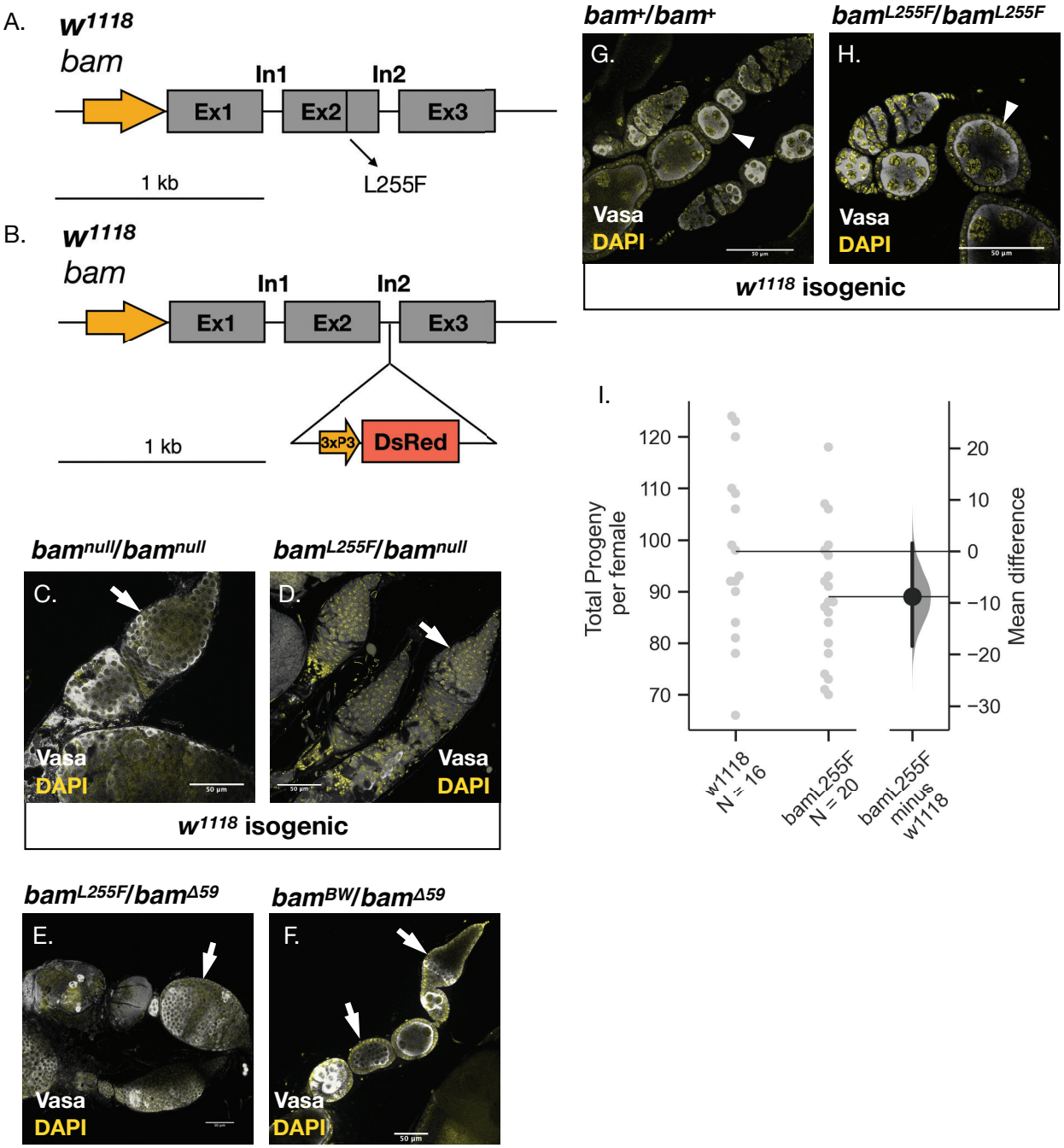


Fig 2. The *bam*^{BW} hypomorphic allele and a novel *bam* null allele in the *w*^{1118} isogenic background recapitulates the classic *bam* phenotypes. (A) Schematic of the *bam* gene region with the single amino acid change from *bam*^{BW}. (B) Schematic of the *bam* gene region with the 3xP3-DsRed 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.

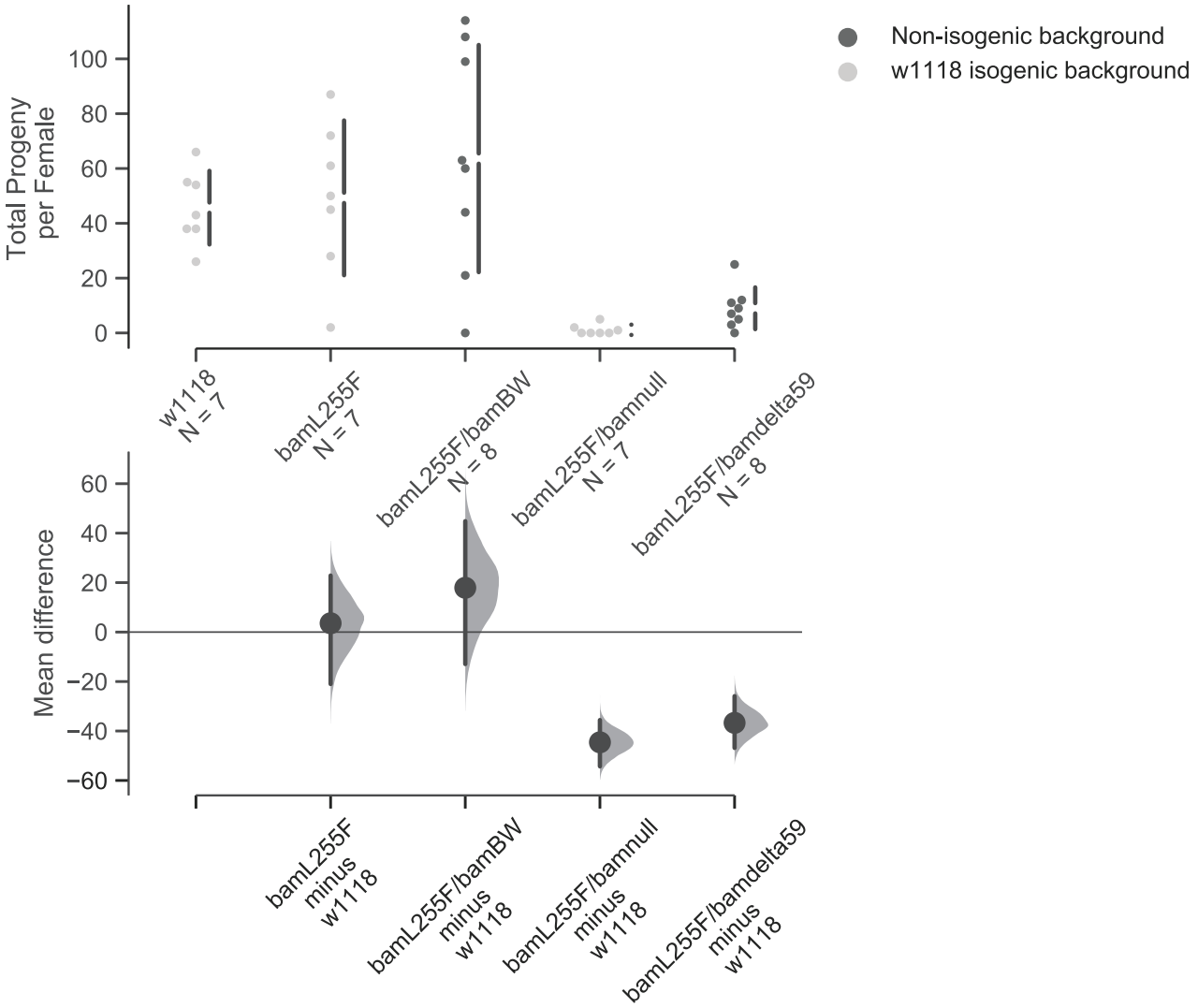


Fig 3. Female fertility of genotypes containing the w^{1118}; bam^{L255F} hypomorphic allele are consistent with those of classic alleles. Swarm and Cumming estimation plots of a control fertility experiment showing that the fertility of bam^{L255F}/ bam^{L255F} females is not significantly different 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).

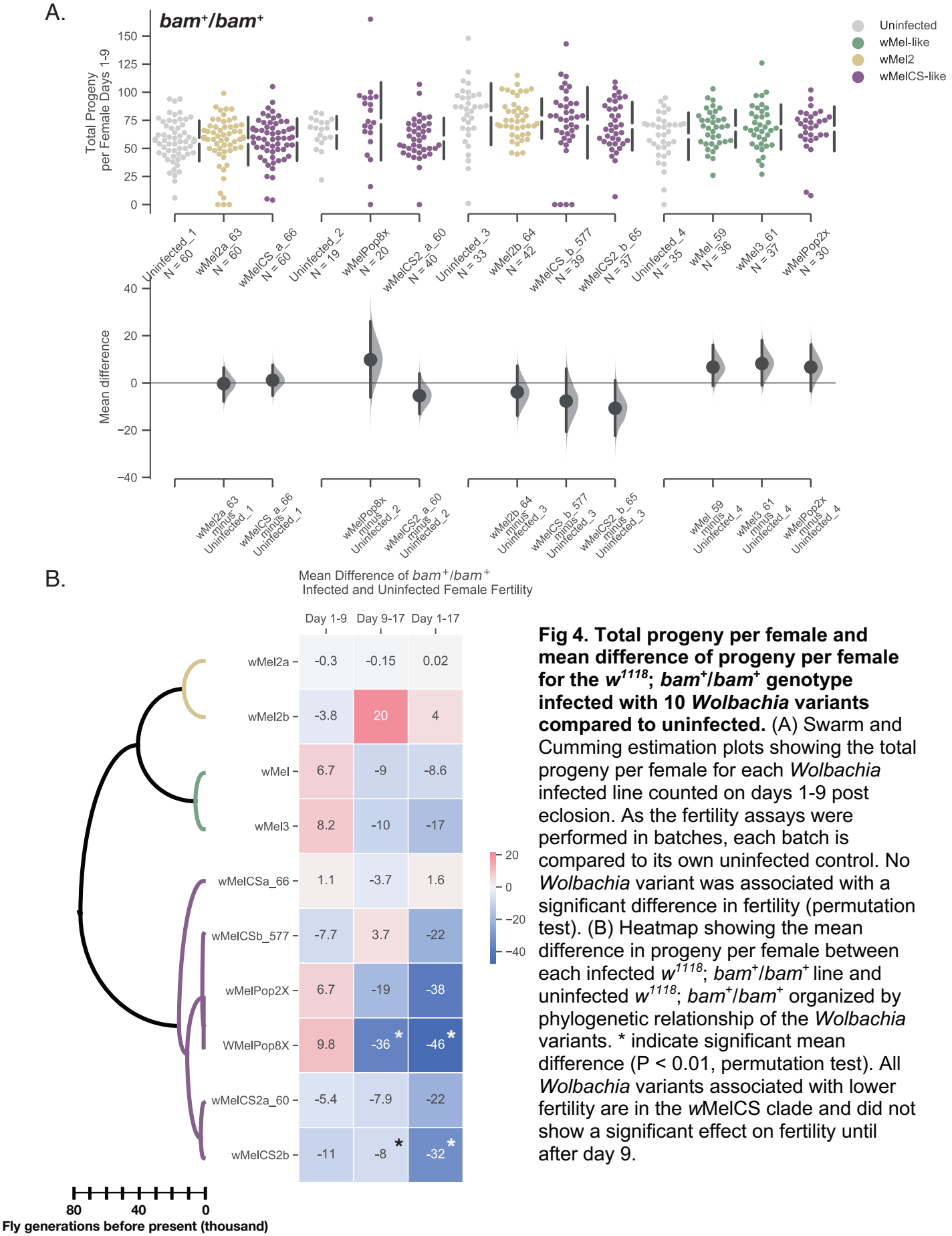
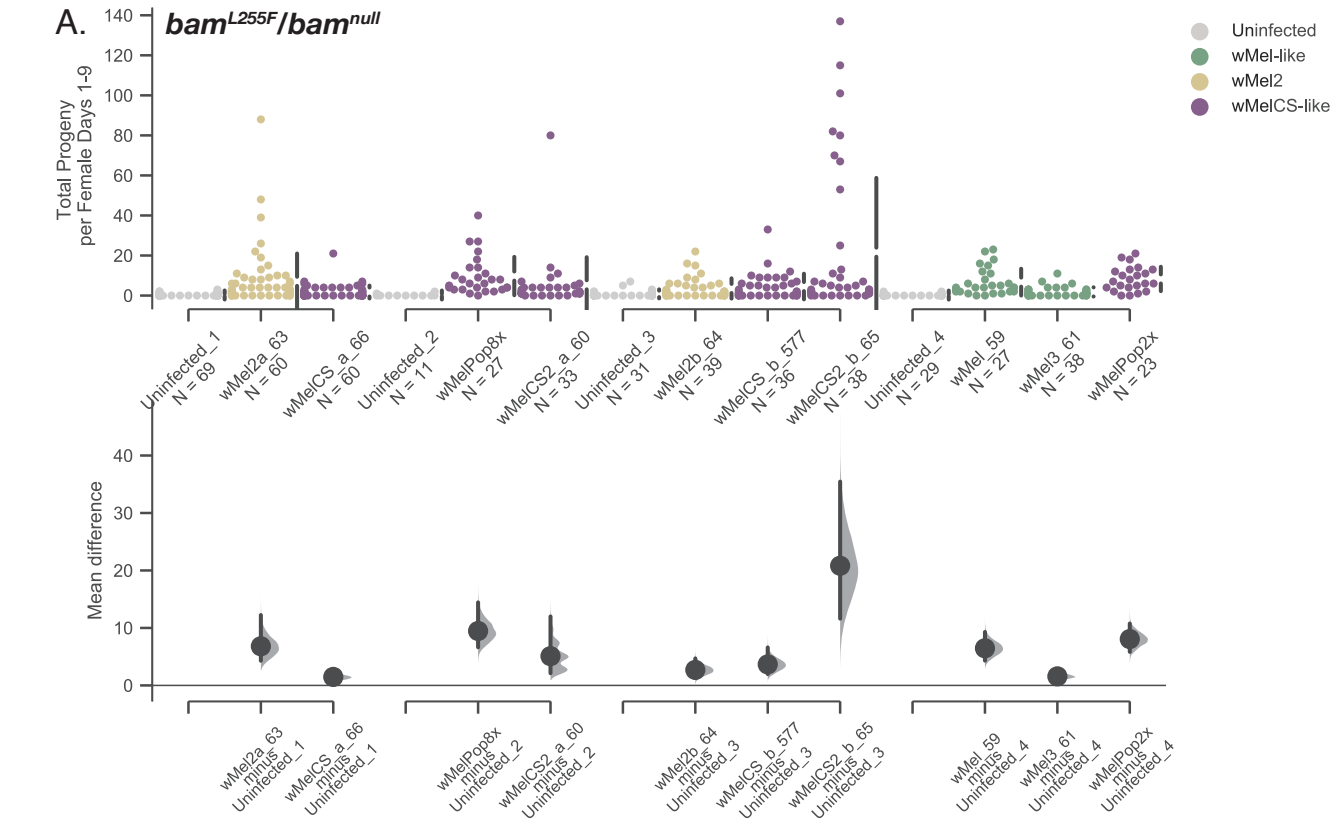


Fig 4. Total progeny per female and mean difference of progeny per female for the w^{1118}; bam^{+}/bam^{+} genotype infected with 10 *Wolbachia* variants compared to uninfected. (A) Swarm and Cumming estimation plots showing the total progeny per female for each *Wolbachia* infected line counted on 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.



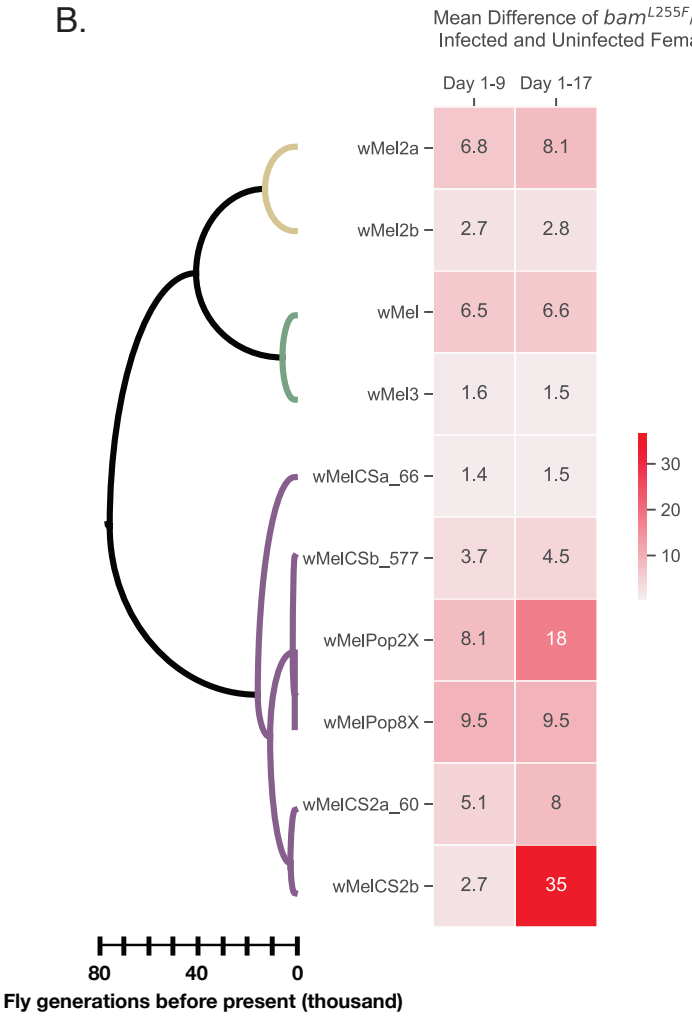
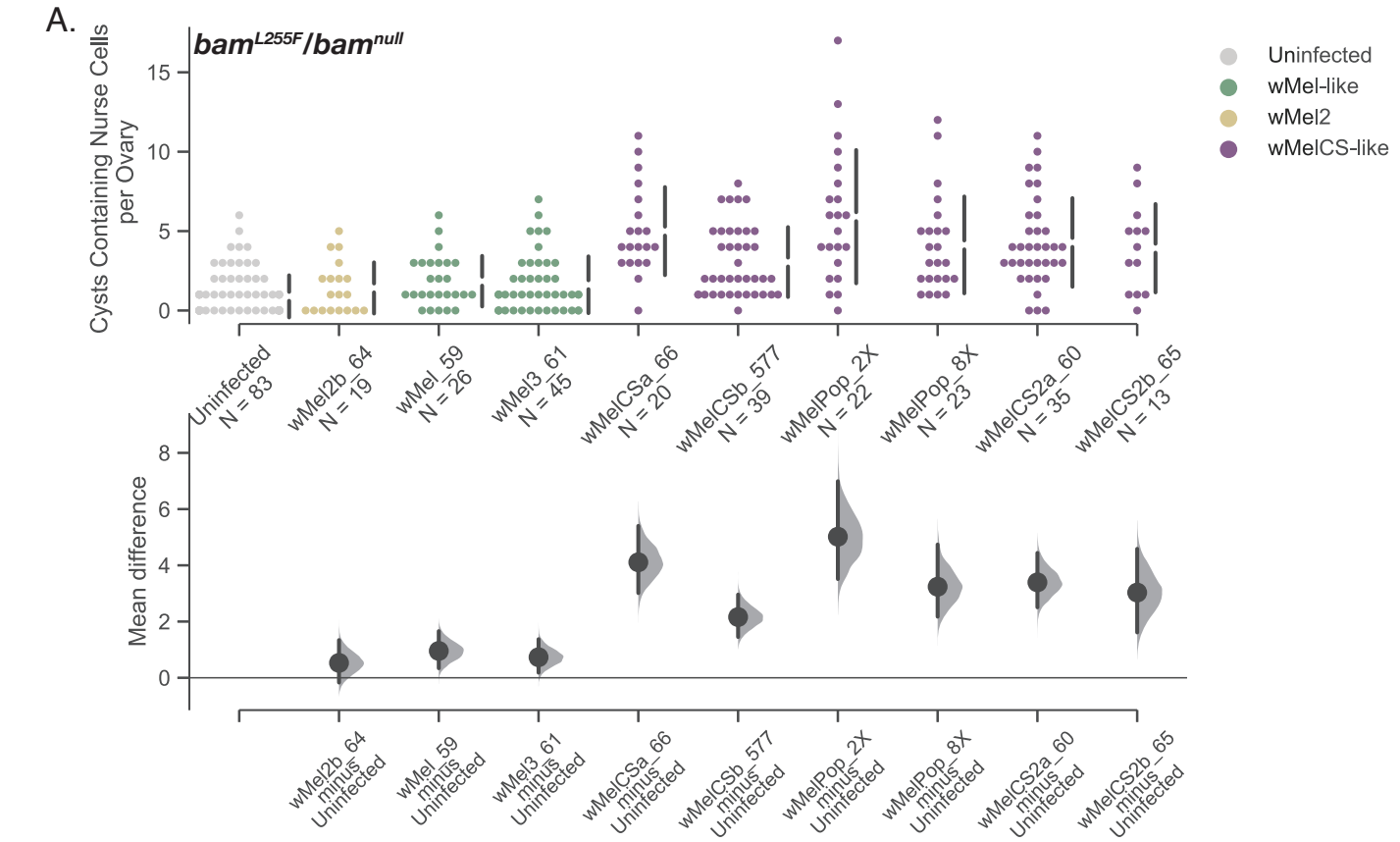


Fig 5. Total progeny per female and mean difference of progeny per female for the *w*^{1118}; *bam*^{L255F}/*bam*^{null} genotype infected with 10 *Wolbachia* variants compared to uninfected. (A) Swarm and Cumming estimation plots showing the total progeny per female for each *Wolbachia* infected line 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.



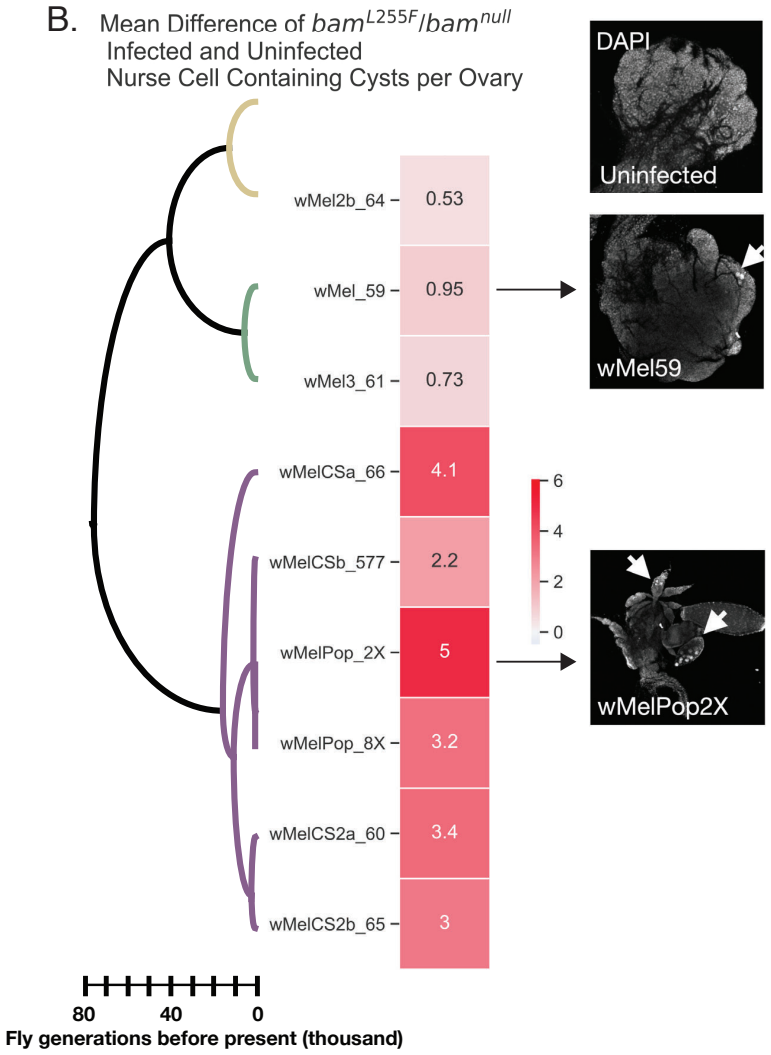


Fig 6. Total cysts containing nurse cells per ovary and mean difference of nurse cell positive cysts for the *w*^{1118}; *bam*^{L255F}/*bam*^{null} genotype infected with 9 *Wolbachia* variants compared to uninfected. (A) Swarm and Cumming estimation plots showing the total cysts containing nurse cells per ovary for each *Wolbachia* infected line assayed on 2-3 day old females. All *Wolbachia* variants except *w*Mel2b_64 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). The *w*MelCS-like variants are all associated with a mean difference of nurse cell containing cysts greater than two, while *w*Mel2 and *w*Mel-like variants do not exhibit mean differences greater than one.

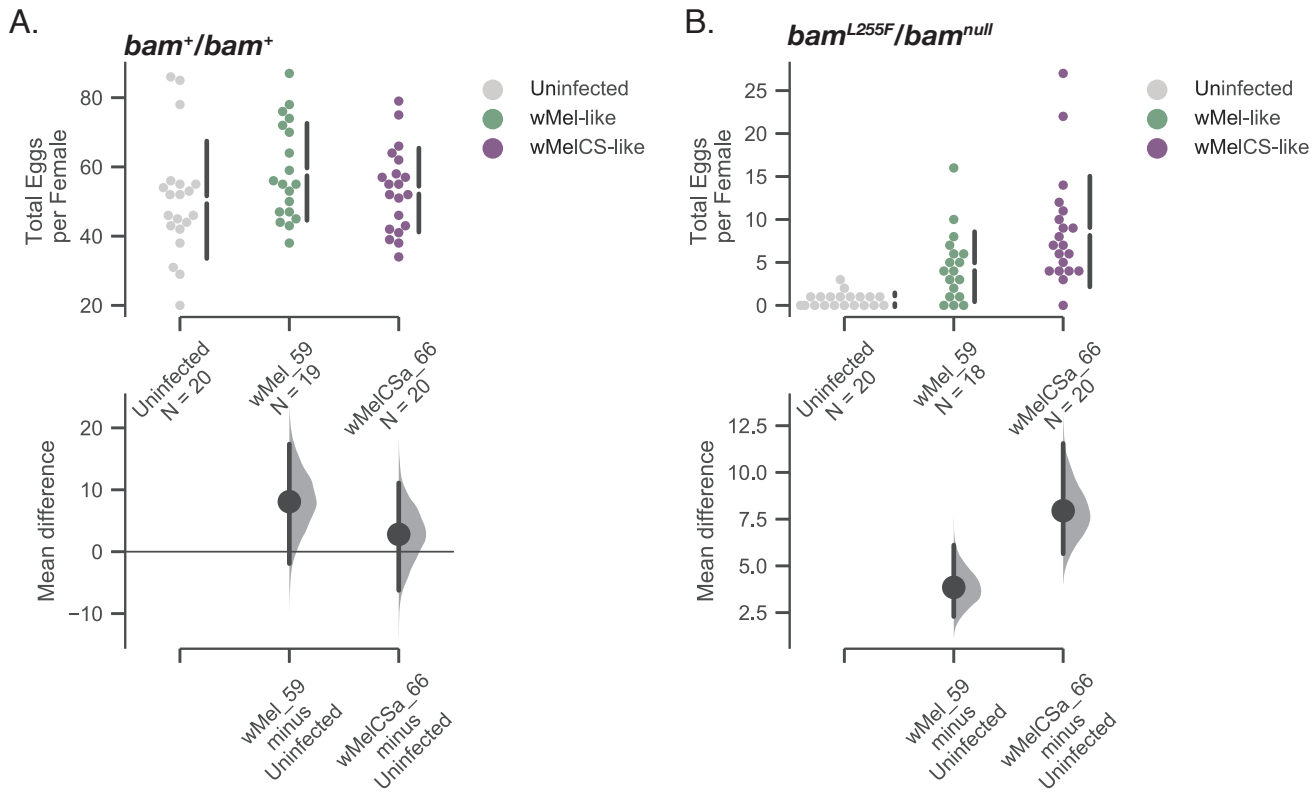
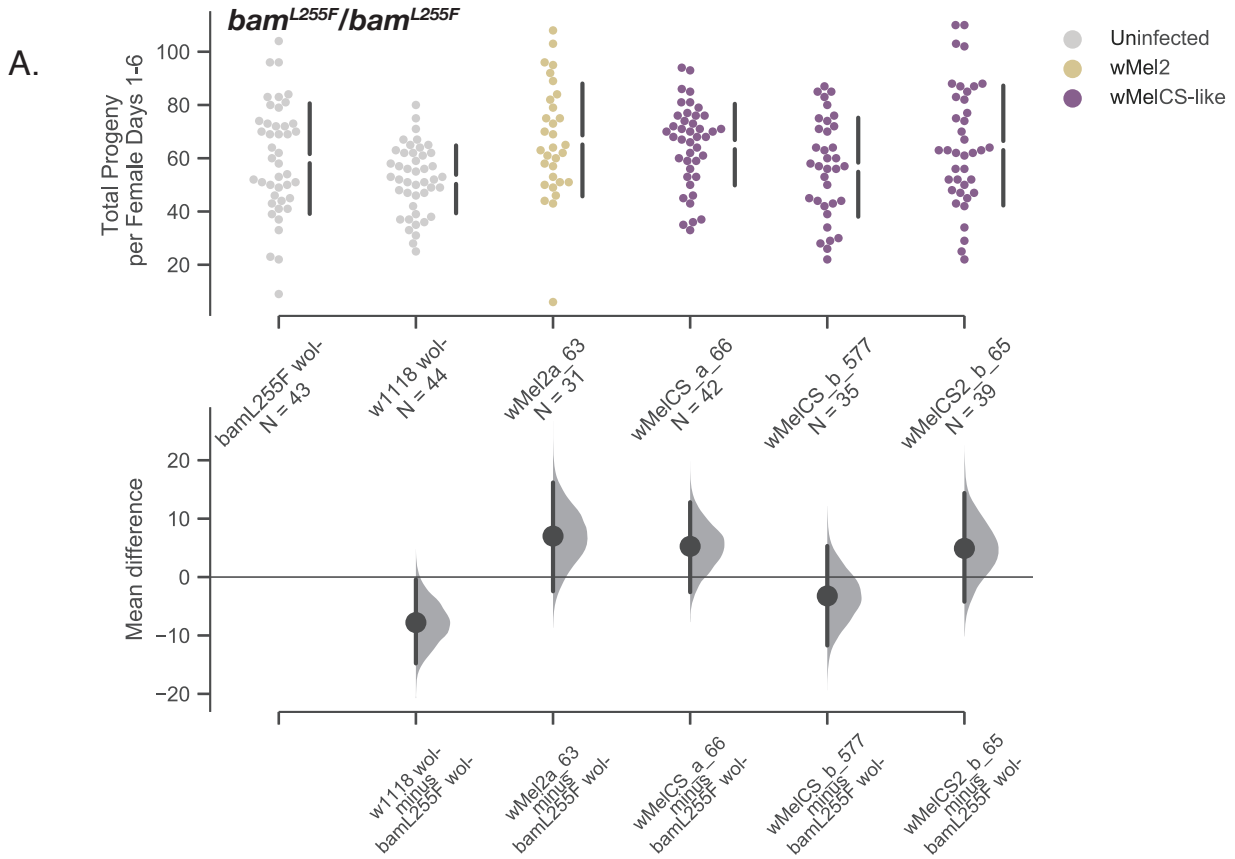


Fig 7. Total eggs per female counted over 3 days for the *w*^{1118}; *bam*$^{+}$/*bam*$^{+}$ genotype and the *w*^{1118}; *bam*^{L255F}/*bam*^{null} genotype infected with representative *Wolbachia* variants from the *w*Mel and *w*MelCS-like clades. (A) Swarm and Cumming estimation plots showing the 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.



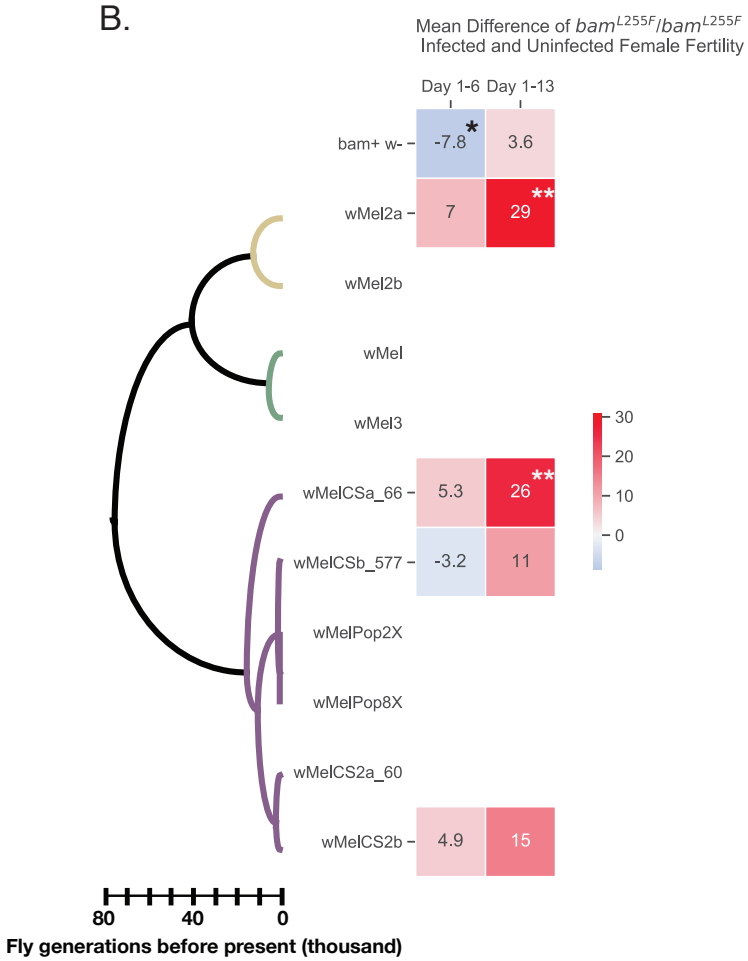
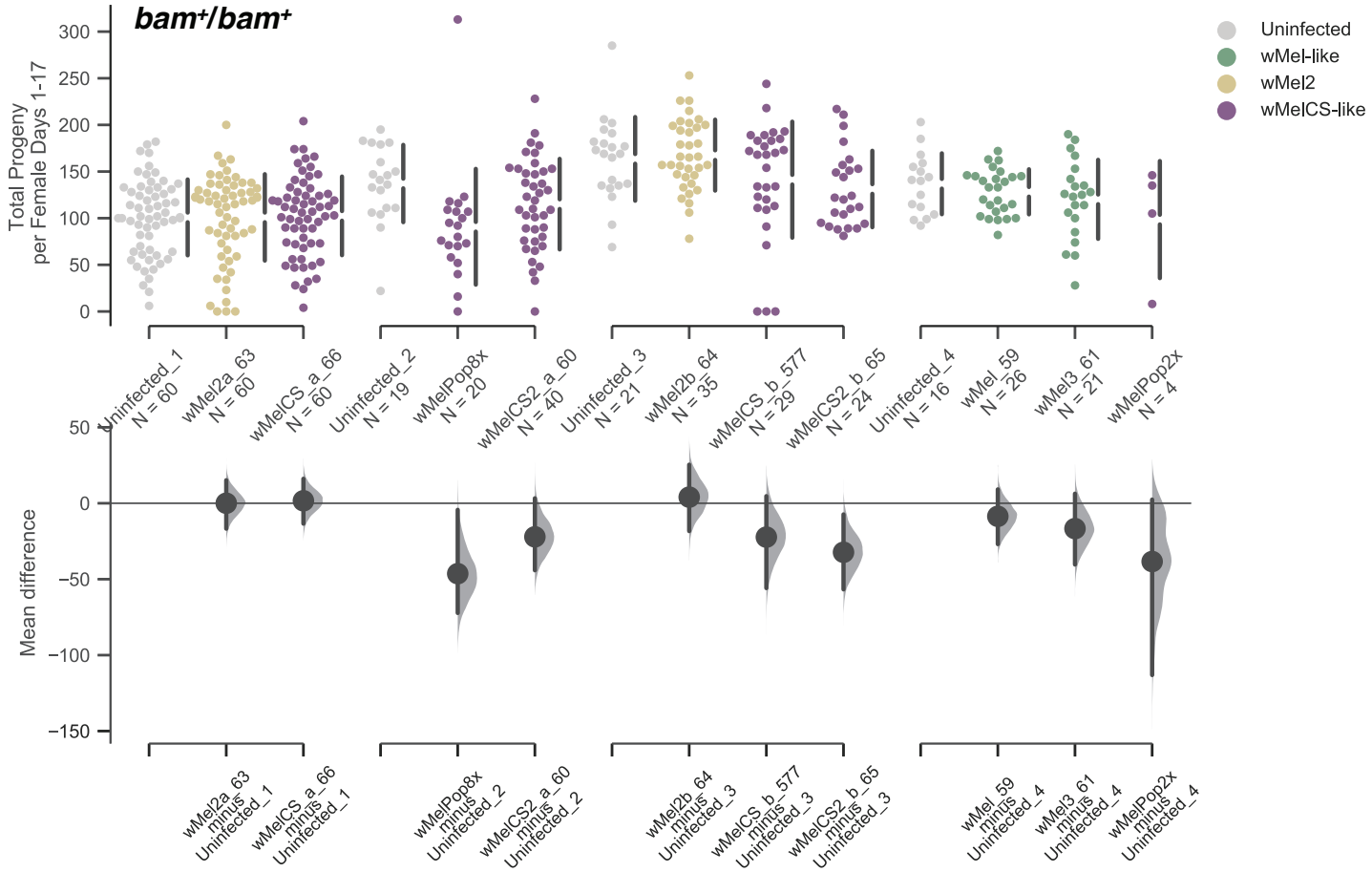
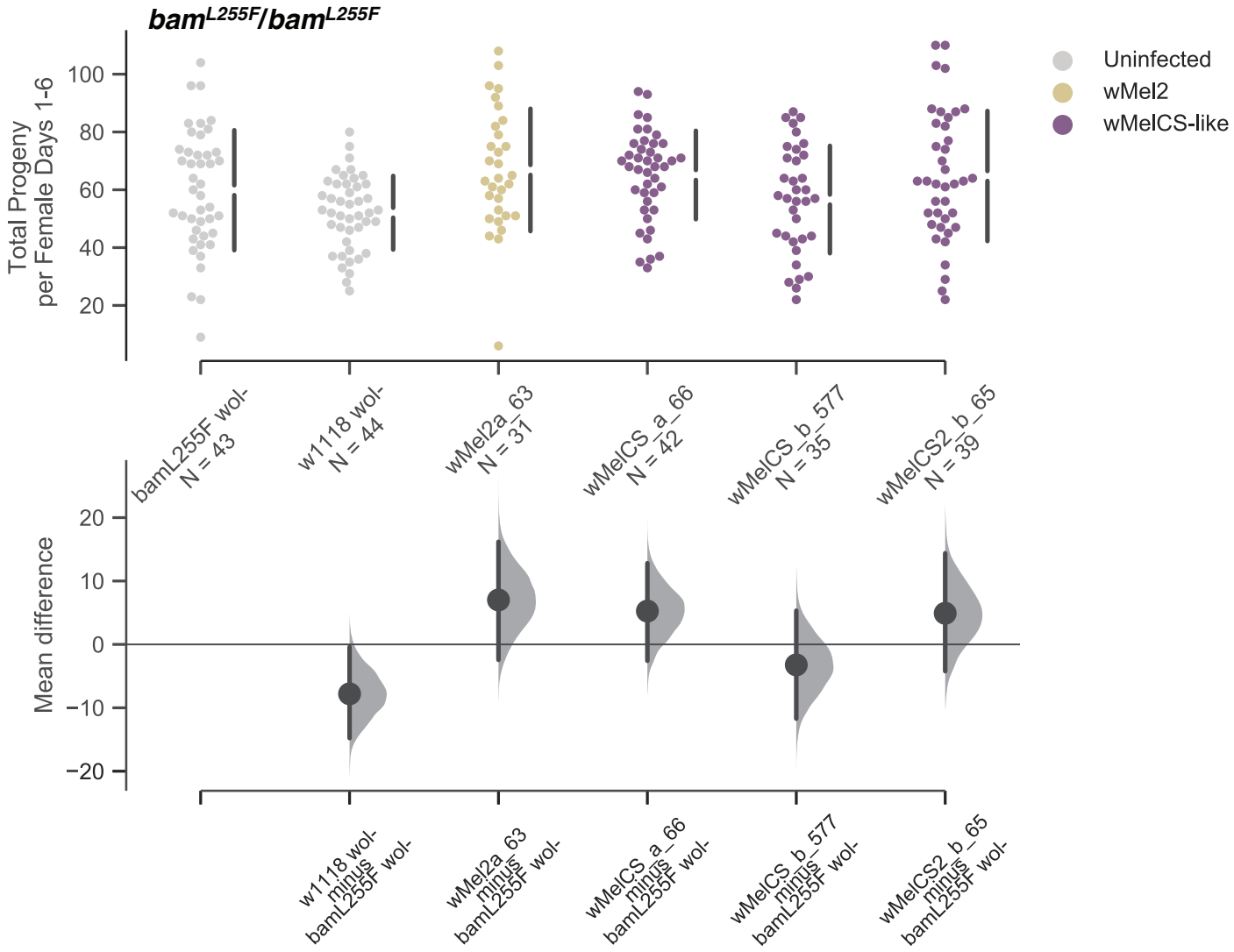


Fig 8. Total progeny per female and mean difference of progeny per female for the *w*^{1118}; *bam*^{L255F}/*bam*^{L255F} genotype infected with four representative *Wolbachia* variants compared to the uninfected control. (A) Swarm and Cumming estimation plots showing the effect of *Wolbachia* variants 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).