Diverse *w*Mel variants of *Wolbachia pipientis* differentially rescue fertility and cytological defects of the *bag of marbles* partial loss of function mutation in *Drosophila melanogaster*

Jaclyn E. Bubnell^{1*}, Paula Fernandez-Begne¹, Cynthia K.S. Ulbing¹, and Charles F. Aquadro^{1*}

¹ Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, USA

*Correspondence: Jaclyn E. Bubnell, jeb486@cornell.edu and Charles F. Aquadro, cfa1@cornell.edu

Keywords: *Wolbachia*, germline stem cell, *bam*, oogenesis, differentiation, CRISPR/Cas9

Authors performed the following: JEB - conceptualization, investigation, validation, formal analysis, and writing. PFB and CKSU - investigation, analysis, and validation, CFA - conceptualization, funding acquisition, supervision, and writing.

Abstract

In *Drosophila melanogaster*, the maternally inherited endosymbiont *Wolbachia pipientis* interacts with germline stem cell genes during oogenesis. One such gene, *bag of marbles* (*bam*) is the key switch for differentiation and also shows signals of adaptive evolution for protein diversification. These observations have led us to hypothesize that *W. pipientis* could be driving the adaptive evolution of *bam* for control of oogenesis. To test this hypothesis, we must understand the specificity of the genetic interaction between *bam* and *W. pipientis*. Previously, we documented that the *W. pipientis* variant, wMel, rescued the fertility of the *bam^{BW}* hypomorphic mutant as a transheterozygote over a *bam* null. However, *bam^{BW}* was generated more than 20 years ago in an uncontrolled genetic background and maintained over a balancer chromosome. Consequently, the chromosome carrying *bam^{BW}* accumulated mutations that have prevented controlled experiments to further assess the interaction. Here, we used CRISPR/Cas9 to engineer the same single amino acid *bam* hypomorphic mutation (*bam^{L255F}*) and a new *bam* null disruption mutation into the *w¹¹¹⁸* isogenic background. We assess the fertility of wildtype *bam*, *bam^{L255F}/bam^{null}* hypomorphic, and *bam^{L255F}/bam^{L255F}* mutant females, each infected individually with ten *W. pipientis* wMel variants representing three phylogenetic clades. Overall, we find that all of the *W. pipientis* variants tested here rescue *bam* hypomorphic fertility defects with wMelCS-like variants exhibiting the strongest rescue effects. Additionally, these variants did not increase wildtype *bam* female fertility. Therefore, both *bam* and *W. pipientis* interact in genotype-specific ways to modulate female fertility, a critical fitness phenotype.

45 Introduction

46 *Wolbachia pipientis* is a maternally inherited endosymbiotic bacteria that infects over 65% of
 47 insect species and manipulates reproduction in a myriad of ways in order to ensure its
 48 transmission through the female germline (Werren et al. 2008; Lindsey 2020; Ote and
 49 Yamamoto 2020). The phenotypes *W. pipientis* induces in its hosts include cytoplasmic
 50 incompatibility (CI), in which embryos of matings between infected males and uninfected
 51 females die; male killing, in which male embryos die; feminization of embryos; and
 52 manipulation of germline stem cell differentiation in order to increase female fertility (Werren et
 53 al. 2008). While *W. pipientis* manipulates its host to increase its own transmission, some *W.*
 54 *pipientis* also provide the host protection against viruses, increase fecundity, modulate thermal
 55 preference, and increase longevity (Dedeine et al. 2001; Chrostek et al. 2013; Arnold et al.
 56 2019; Truitt et al. 2019; López-Madrugal and Duarte 2020; Hague et al. 2020).

57
 58 Understanding the genetic mechanisms that *W. pipientis* uses to manipulate its host has been
 59 of immense interest to both basic and applied fields of study. *W. pipientis* is of particular
 60 interest as a control for disease vectors such as mosquitoes due to its ability to sweep through
 61 a population (due to CI) and then protect the insect from viruses that can also cause human
 62 illness such as Dengue, Zika, and Chikungunya (Moreira et al. 2009; Hoffmann et al. 2011;
 63 Dutra et al. 2016; Utarini et al. 2021). However, it has been difficult to perform genetic studies
 64 of *W. pipientis* function to understand the mechanisms of these host-microbe interactions since
 65 *W. pipientis* is an obligate endosymbiont and cannot be cultured. Over the past few years,
 66 multiple groups have utilized bioinformatic and in vitro screens to identify candidate *W.*
 67 *pipientis* loci that modulate *Drosophila* phenotypes (Ote et al. 2016; Le Page et al. 2017).

68 These loci have then been expressed in *Drosophila* as transgenes. Through these methods,
69 the *W. pipientis* genes *cifA* and *cifB* (orthologs in *wMel* and *wPip*) that cause CI have been
70 identified and functionally validated in *D. melanogaster* (Beckmann et al. 2017; Le Page et al.
71 2017). These transgenic tools have been used to further define the CI phenotype (Shropshire
72 et al. 2018), investigate the consequences of genetic variation at *cifA* and *cifB* on CI
73 (Shropshire et al. 2021), and identify other host phenotypes affected by *cifA* and *cifB* (Deehan
74 et al. 2021). Another group has identified the *W. pipientis* TomO locus which is one of likely
75 multiple *W. pipientis* loci that interact with *Sex lethal*, a germline stem cell (GSC) gene that
76 controls GSC maintenance and sex determination (Ote et al. 2016).

77
78 Previously, we reported that the *W. pipientis* variant *wMel* genetically interacts with another *D.*
79 *melanogaster* GSC gene, *bag of marbles* (*bam*). Infection with the *wMel* variant rescues the
80 fertility defect of females transheterozygous for the classic *bam* hypomorphic allele (*bam^{BW}*)
81 and a classic *bam* null allele (*bam^{Δ59}*) (Flores et al. 2015). *Bam* is the switch for GSC daughter
82 differentiation in *D. melanogaster* females and the switch for terminal spermatocyte
83 differentiation in males (McKearin and Spradling 1990; Insco et al. 2012; Ting 2013). Although
84 *bam* function is essential for gametogenesis, and *bam* is both necessary and sufficient for
85 GSC daughter differentiation, we observe that it is evolving under positive selection for amino
86 acid divergence in an episodic manner across the *Drosophila* genus (Bauer DuMont et al.
87 2007; Choi and Aquadro 2014). We see particularly strong bursts of amino acid changes at
88 *bam* in the *D. melanogaster* and *D. simulans* lineages (Bauer DuMont et al. 2007). This
89 episodic pattern of positive selection is consistent with selective pressures that are present in
90 some lineages and not others and that may come and go over time. This is similar to the

nature of infections in natural populations, where *W. pipientis* variants are known to infect populations, but also can be lost and replaced fairly often and on both long and short time scales (Bailly-Bechet et al. 2017).

Our observation that *W. pipientis* and *bam* genetically interact led us to hypothesize that *W. pipientis* may drive the adaptive evolution of *bam* (Flores et al. 2015). Since *W. pipientis* is a known manipulator of reproduction, *W. pipientis* may be in conflict with *bam* and other GSC genes for control of oogenesis. While it may seem favorable to host fitness if *W. pipientis* manipulates germline stem cell regulation to increase reproduction, and thus not present an evolutionary conflict, it may not be beneficial for the host if oogenesis is regulated by *W. pipientis*. For example, *W. pipientis* may promote oogenesis when it is not favorable for the host to reproduce due to environmental or physiological factors. To further understand if *W. pipientis* could be in genetic conflict with *bam* and drive its adaptation, we believe it is necessary to better understand the genetic interaction between *bam* and *W. pipientis*.

Fully sequenced variants of *W. pipientis* that infect *D. melanogaster* have been found to cluster phenotypically and phylogenetically into two distinct groups of clades: wMel-like variants (including wMel and wMel2) and wMelCS-like variants (including wMelCS, wMelCS2 and wMelPop) that are estimated to have diverged 80,000 fly generations before present (Richardson et al. 2012; Chrostek et al. 2013). The wMelCS-like variants predominated in *D. melanogaster* originally but have largely (although not completely) been replaced by wMel-like variants world-wide in the late 20th century (Riegler et al. 2005). The wMelCS-like variants provide stronger viral protection, reach higher intracellular bacterial titers in males, and often

114 shorten lifespan compared to the more benign wMel-like variants that now predominate.
 115 Additionally, uninfected *D. melanogaster* and those infected with wMel-like variants have a
 116 higher temperature preference than *D. melanogaster* infected with wMelCS-like variants
 117 (including wMelPop) (Truitt et al. 2019).
 118
 119 To assess the specificity of our initial observation that wMel rescues the female fertility
 120 phenotype of the *bam^{BW}/bam^{Δ59}* hypomorphic mutant, we made use of a genetic tool for
 121 functionally assessing *W. pipientis* variation generated by Chrostek et al. (Chrostek et al.
 122 2013), a set of *w¹¹¹⁸* isogenic lines individually infected with ten diverse wMel *W. pipientis*
 123 variants from two of the wMel-like clades and the wMelCS-like clade defined by Richardson et
 124 al (Richardson et al. 2012). The ten *W. pipientis* variants that we analyze thus include both
 125 phenotypic classes of wMel variants: the wMelCS-like variants which are higher titer, reduce
 126 host lifespan, and confer higher viral resistance and lower thermal preference in contrast to the
 127 more recent wMel-like variants.
 128
 129 Since fertility is a phenotype affected by genome-wide variation and both the *bam^{BW}* and
 130 *bam^{Δ59}* alleles were generated in non-isogenic backgrounds, we sought to control the
 131 *Drosophila* genetic background to better isolate the effect of *W. pipientis* variation and *bam*
 132 genotype on fertility. We used CRISPR/Cas9 to edit the single amino acid change of the
 133 original *bam^{BW}* hypomorph (Ohlstein et al. 2000) into the *w¹¹¹⁸* isogenic background, and also
 134 to create a new *bam* null allele in the same genetic background. Additionally, we had
 135 previously not been able to determine the phenotype of *bam^{BW}/bam^{BW}* females, as the *bam^{BW}*
 136 allele was isolated over 20 years ago and then maintained over a balancer chromosome which

allowed the mutation carrying chromosome to accumulated recessive lethal mutations. Thus, we also used these new lines to analyze *bam* hypomorph and null alleles as homozygotes and transheterozygotes.

We confirmed that the amino acid replacement results in the *bam* hypomorphic phenotype previously described when expressed over a *bam* null allele in uninfected females (Flores et al. 2015). Interestingly, we further show that uninfected females homozygous for the *bam* hypomorphic allele do not exhibit GSC tumors or show reduced fertility compared to wildtype females. We then assessed the effect of infection by the individual *W. pipientis* variants by crossing the cytoplasmic maternal backgrounds infected with each *W. pipientis* variant into our *w¹¹¹⁸ bam* hypomorph line thereby maintaining the same nuclear background (except for the *bam* locus).

We find that all *wMel W. pipientis* variants tested here do not increase fertility in the wildtype *bam* background, but all *wMel W. pipientis* variants rescue the fertility of *bam* mutant females, with *wMelCS-like W. pipientis* showing the highest rescue effects. Therefore, the fertility rescue of the *bam* hypomorph by *W. pipientis* is not due to an overall increase in fertility modulated by *W. pipientis*, but a specific interaction between *bam* and *W. pipientis* genotypes.

Materials and Methods

Fly stocks and rearing:

Prior to experiments, we raised fly stocks on standard cornmeal molasses food at room temperature. We used yeast glucose food for fertility assays. During experiments, we

maintained crosses and stocks in an incubator at 25°C with a 12-hour light-dark cycle. The lines carrying the classic *bam* alleles, *bam*^{A59} (null) and *bam*^{BW} (hypomorph) are described on Flybase (Thurmond et al. 2019) and in Flores et. al (Flores et al. 2015). The *W. pipientis* infected *w*¹¹¹⁸ isogenic lines used in this study were generous gifts from Luis Texiera and described in (Chrostek et al. 2013). We used CantonS males for the *w*¹¹¹⁸; *bam*^{L255F}/*bam*^{null} and *w*¹¹¹⁸; *bam*⁺/*bam*⁺ fertility assays. We used *w*¹¹¹⁸ isogenic males for the *w*¹¹¹⁸; *bam*^{L255F}/*bam*^{L255F} fertility assays. We verified that males were uninfected with *W. pipientis* by endpoint PCR and qPCR using primers described below. To generate the *bam* hypomorphic lines infected with the ten different *W. pipientis* variants, we crossed females from the *w*¹¹¹⁸; TM2/TM6 stock infected with each variant to *w*¹¹¹⁸; *bam*^{L255F}/TM6 males.

170

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

172 We engineered the new *bam*^{L255F} hypomorph and *bam* null alleles described in this study in the
173 *w*¹¹¹⁸ isogenic background using synthetic gRNAs and Cas9 protein as follows.

174

175 gRNAs:

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

179

180 Cloning:

181 We generated all PCR products for cloning with NEB High Fidelity Q5 master mix, and gel
182 extracted and purified PCR products using the Qiagen MinElute gel extraction kit. We created

the donor plasmid for homology directed repair using the NEB HiFi assembly cloning kit and the pHD-attP-DsRed vector from flyCRISPR (Gratz et al. 2014). For plasmid prep, we used Qiagen plasmid plus midi-prep kit and sequenced plasmids with Sanger sequencing (Cornell BRC Genomics Core). We ordered primers for PCR, sequencing, and cloning from IDTDNA (Supplement Table S2).

188

189 Donor sequences:

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

195

To generate the null *bam*^{null-In2-3xP3-DsRed} allele, we amplified 1.5 kb homology arms from the *w*¹¹¹⁸ isogenic line and cloned them into the pHD-attP-DsRed vector from flyCRISPR (Supplement Table S3). As we did not need the attP site in our line, we amplified the 3xP3-DsRed cassette from the plasmid and assembled the homology arms, 3xP3-DsRed, and the original vector backbone with the NEB HiFi assembly kit, thereby removing the attP site.

201

202 Injections:

All CRISPR/Cas9 injections were sourced to Genetivision and were done in the *w*¹¹¹⁸ isogenic line. The injection mix contained plasmid or ssODN donor, sgRNAs, Cas9 protein (Synthego), and an siRNA for Lig4 (IDT DNA, Supplement Table S1).

206

207 For the *bam*^{null-In2-3xP3-DsRed} allele, we screened for the eye color cassette in F1's in house using
208 a Nightsea fluorescent system (DsRed filters). For the *bam*^{L255F} allele, the edited nucleotide
209 change disrupts an AflII restriction site, allowing us to screen F1's by PCR (Promega GoTaq
210 mastermix) followed by restriction digests with AflII (NEB). We prepared genomic DNA using
211 the Qiagen PureGene kit.

212

213 We backcrossed females of all CRISPR/Cas9 mutants to *w*¹¹¹⁸ isogenic males for three
214 generations, and then crossed the mutants to the *w*¹¹¹⁸; TM2/TM6 line to maintain the *bam*
215 mutants over the TM6 balancer. All CRISPR/Cas9 edits in the lines were confirmed by Sanger
216 sequencing (Cornell BRC Genomics Facility).

217

218 PCR assays to detect *W. pipientis*:

219 To test for the presence of *W. pipientis*, we used the Zymo quick DNA miniprep kit to prepare
220 DNA from three replicate samples each with three female flies. For each sample, we used
221 three different primers for endpoint or qPCR (Supplement Table S1). We used the common
222 *wsp* primers (Flores et al. 2015), a primer pair targeting *DprA*, and a highly sensitive primer
223 pair to the ARM repeat (Schneider et al. 2014) for endpoint PCR, modified here for qPCR)
224 (Supplement Table S2).

225

226 Immunostaining:

227 We performed immunostaining as described in Aruna et al. (Aruna et al. 2009) and Flores et
228 al. (Flores et al. 2015). Briefly, we dissected ovaries in ice cold 1X PBS and pipetted the tissue

up and down to improve antibody permeability. We fixed ovaries in 4% paraformaldehyde (EMS), washed with PBST (1X PBS, 0.1% Triton-X 100), blocked in PBTA (1X PBS, 0.1% Triton-X 100, 3% BSA) (Alfa Aesar), and then incubated in the appropriate primary antibody overnight. We then washed, blocked, and incubated in the appropriate secondary antibody for two hours then washed and mounted in ProLong Glass NucBlue for imaging (ThermoFisher). We used an anti-Vasa antibody from Santa Cruz biologicals (anti-rabbit, 1:200) and a goat anti-rabbit secondary antibody from Invitrogen (Alexa Fluor Plus 488 1:500). We imaged ovaries on a Zeiss i880 confocal microscope with 405 and 488 nm laser lines at 40X (Plan-Apochromat 1.4 NA, oil) (Cornell BRC Imaging Core). We analyzed and edited images using Fiji (ImageJ).

239

240 **Assays for nurse cell positive egg chambers:**

241 We used the same assay we previously described to assess the rescue of the original
242 *bam^{BW}/bam^{Δ59}* hypomorphic cytological phenotype by *W. pipientis* (Flores et al. 2015). We
243 dissected ovaries from mated 2-4 day old *bam^{L255F}/bam^{null-In2-3xP3-DsRed}* females for all *W.*
244 *pipientis* variants and the uninfected control in PBS. Ovaries were fixed in 4%
245 paraformaldehyde (EMS), washed with PBST (1X PBS, 0.1% Triton-X 100), and then mounted
246 in ProLong Glass with NucBlue (ThermoFisher). We imaged ovaries on a Zeiss i880 confocal
247 microscope with a 405 nm laser line at 10X (C-Apochromat 0.45 NA, water) and 40X
248 (described above). We analyzed and edited images using Fiji (ImageJ). Developing ovaries
249 consist of cysts containing differentiated nurse cells which are polyploid and feature large,
250 easily identifiable nuclei. In contrast, GSC daughter differentiation is blocked in *bam* loss-of-
251 function ovaries resulting in cysts filled with small GSC-like cells with small nuclei. To quantify

the rescue of *bam*'s differentiation function, we counted the number of nurse cell positive egg chambers (cysts) per ovary for uninfected females and females infected with each *W. pipientis* genotype (as these ovaries are small and underdeveloped, they stay intact during the fixation and washing steps).

Fertility Assays:

We used the following w^{1118} isogenic *bam* genotypes for the fertility assays: $w^{1118}; bam^+/bam^+$, $w^{1118}; bam^{L255F}/bam^{null-In2-3xP3-DsRed}$, and $w^{1118}; bam^{L255F}/bam^{L255F}$. Additionally, we performed a small control fertility assay using combinations of the alleles described above and the classic *bam^{BW}* hypomorph and *bam^{Δ59}* alleles.

We performed the fertility assays for $w^{1118}; bam^+/bam^+$ and $w^{1118}; bam^{L255F}/bam^{null-In2-3xP3-DsRed}$ in three batches to reduce technical error from too large of an experiment. We included separate uninfected controls in each batch of *W. pipientis* variants and used these to make statistical comparisons of mean progeny per female.

We performed all fertility assays (except for the small *bam^{BW}* and *bam^{Δ59}* analysis) as follows (and described by Flores et al. 2015):

We collected virgin females and aged them 2-3 days, only using flies that eclosed within 48 hours of each other to reduce developmental variation. We collected virgin males uninfected with *W. pipientis* and aged them for 2-3 days. We distributed males from different bottles across the female genotypes to control for any bottle effects. We individually crossed virgin females to two virgin males. The trio were allowed to mate for 8 days, and then flipped to new

275 vials. After the second 8 days the trio was cleared. The progeny for each trio was counted
276 every other day to get the total adult progeny per female. Progeny per female is reported in
277 increments by day that reflect the days after the first progeny eclosed. For example, we report
278 total progeny for days 1-9 which are the progeny counted on day 1 of eclosion to day 9 of
279 eclosion.

280

281 For the control fertility assay containing the *bam*^{BW} hypomorph and *bam*^{Δ59} alleles, we
282 collected and aged flies as described above, except the progeny per trio was counted only
283 once at the end of the experiment.

284

285 **Egg Laying Assay:**

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

290

291 **Statistics:**

292 For the nurse cell assay, egg laying assay, and fertility assays we used estimation statistics to
293 assess the mean difference (effect size) of nurse cells, eggs, and adult progeny between
294 infected and uninfected lines. All estimation statistics were done using the dabest package in
295 Python (v. 0.3.1) with 5000 bootstrap resamples (Ho et al. 2019). Estimation statistics provide
296 a non-parametric alternative to other statistical tests of the difference of the mean (for
297 example, ANOVA), and allow us to understand the magnitude of the effect of *W. pipientis*

298 variation on the *bam* phenotype. We display the data with a swarm plot that shows all of the
 299 data points and either a Cumming estimation plot (for more than two sample comparisons) or a
 300 Gardner-Altman plot (two sample comparisons) that shows the effect size for each sample
 301 compared to the control and 95% bootstrap confidence interval. In text, we report significance
 302 as a mean difference (effect size) outside the 95% bootstrap confidence interval.

303

304 **Data Availability:**

305 Fly lines and plasmids used in this study are available upon request. The authors affirm that all
 306 data necessary for confirming the conclusions of the article are present within the article,
 307 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) and a new *bam* null allele in the isogenic *w*¹¹¹⁸ background. Therefore, we have two new *bam* mutant alleles, *bam*^{L255F} and *bam*^{null-In2-3xP3-DsRed} in the same genetic background so we can compare wildtype *bam* fertility, the transheterozygous hypomorph/null fertility (as we did previously with *bam*^{BW}/*bam*^{Δ59}) as well as assess the phenotype of a the homozygous *bam* hypomorphic genotype which has never been assessed before.

We evaluated if the *w*¹¹¹⁸; *bam*^{L255F} allele recapitulated the *bam*^{BW}/*bam*^{Δ59} 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-In2-3xP3-DsRed}, Fig 1B). Females homozygous for this *bam*^{null-In2-3xP3-DsRed} allele exhibit the expected tumorous ovary phenotype with no developing nurse cell-positive cysts and are sterile (Fig 2A, fertility data not shown).

We then assessed the phenotype of the *bam*^{L255F} allele as a transheterozygous mutant over our new *bam*^{null-In2-3xP3-DsRed} 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-In2-3xP3-DsRed}

331 ovaries exhibited the expected tumorous ovary phenotype, with the presence of some
 332 differentiating, nurse cell positive cysts indicating that *bam* is partially functional, and thus that
 333 this single amino acid change fully explains the original *bam^{BW}* hypomorphic phenotype (Fig
 334 2B). To further verify the nature of the *bam^{L255F}* allele, we assessed its phenotype over one of
 335 the classic *bam* null alleles that we previously used to study the interaction between *bam* and
 336 *W. pipientis*, *bam^{Δ59}*. The *bam^{Δ59}* null allele is a nearly full deletion of the *bam* coding
 337 sequence. We examined ovaries from *bam^{L255F}/bam^{Δ59}* females and observed the tumorous
 338 ovary phenotype with some developing cysts as has been previously documented for
 339 *bam^{BW}/bam^{Δ59}* and additionally shown here (Fig 2C&D). We measured the fertility of *bam^{L255F}/*
 340 *bam^{Δ59}* females and observed a large fertility defect, consistent with the fertility of the *bam^{L255F}/*
 341 *bam^{null-In2-3xP3-DsRed}* genotype (Fig 3). Therefore, the *bam^{L255F}/bam^{null-In2-3xP3-DsRed}* cytological
 342 and fertility phenotypes are consistent with the previously documented *bam* hypomorph
 343 phenotypes.

344
 345 As the *bam^{BW}* allele is homozygous lethal due to accumulated recessive lethal mutations on
 346 the third chromosome, we used our *bam^{L255F}* allele to ask if the homozygous *bam^{L255F}* females
 347 also had a hypomorphic phenotype. We asked if *bam^{L255F}/bam^{L255F}* females also exhibited the
 348 classic *bag of marbles* cytological phenotype and reduced fertility. In contrast to the tumorous
 349 ovary phenotype of *bam^{BW}/bam^{Δ59}* and *bam^{L255F}/bam^{null-In2-3xP3-DsRed}*, a result of a defect in
 350 *bam*'s differentiation function which causes GSC-like cells to over-proliferate, we found no
 351 evidence of tumorous cysts in ovaries of *bam^{L255F}/bam^{L255F}* females (Fig 2F). These ovaries
 352 resembled those of a wildtype *bam* background, with all developing egg chambers featuring
 353 nurse cells (Fig 2E). Previously we were unable to compare the fertility of the *bam^{BW}*

hypomorph to wildtype *bam* fertility as fertility is greatly affected by different genetic backgrounds. We compared the fertility of the $w^{1118}; bam^{L255F}/bam^{L255F}$ females to $w^{1118}; bam^{+}/bam^{+}$ females and found that the mean difference in total progeny per female between the two genotypes was not significantly different (Fig 2G). While we did not measure all aspects of *bam* function of this genotype, the fertility and cytological data here indicate that the bam^{L255F}/bam^{L255F} genotype does not cause a severe *bam* mutant phenotype, as we observed previously for the transheterozygous *bam*^{BW} hypomorph ($bam^{BW}/bam^{\Delta 59}$). Therefore, two copies of the bam^{L255F} allele are sufficient for germline stem cell daughter differentiation and fertility.

Although we cannot make strong claims about the effect of *bam* genotype on fertility between lines with different genetic backgrounds, we did perform a small control experiment to ask if our new bam^{L255F} hypomorphic allele over the classic *bam*^{BW} hypomorphic allele also show similar fertility to wildtype females as we observed for bam^{L255F}/bam^{L255F} females. We observed that bam^{L255F}/bam^{BW} females and homozygous bam^{L255F} females did not show significantly different fertility from $w^{1118} bam^{+}/bam^{+}$ females (Fig 3). Further, we asked if our new $bam^{L255F}/bam^{null-In2-3xP3-DsRed}$ genotype showed a similar fertility defect to $bam^{L255F}/bam^{\Delta 59}$ (classic null) and found that both showed severely reduced fertility compared to wildtype (Fig 3). These data further confirm that our new bam^{L255F} and $bam^{null-In2-3xP3-DsRed}$ alleles behave similarly to alleles we have used in the past to study the interaction between *bam* and *W. pipientis*.

376 Since we were able to successfully recapitulate the partial-loss of function *bam* cytological and
 377 fertility phenotypes with the *bam*^{L255F}/*bam*^{null-In2-3xP3-DsRed} genotype as we have previously
 378 described (Flores et al. 2015), but we did not observe the *bam* partial loss of function ovarian
 379 cytological phenotype in the *bam*^{L255F}/*bam*^{L255F} females, we focused primarily on the effect of
 380 *W. pipientis* variation on the *bam*^{L255F}/*bam*^{null-In2-3xP3-DsRed} female fertility and cytological
 381 phenotypes.

382

383 For convenience and readability, we will frequently refer to the *w*¹¹¹⁸; *bam*^{null-In2-3xP3-DsRed} allele
 384 as *bam*^{null} and the *w*¹¹¹⁸; *bam*^{L255F} allele as *bam*^{L255F}.

385 **Infection with the ten different wMel variants we assayed does not increase *w*¹¹¹⁸;** 386 ***bam*⁺/*bam*⁺ isofemale fertility**

387 First, to determine if the wMel *bam* hypomorph rescue is a consequence of a general increase
388 in female fertility induced by *W. pipientis*, we assessed the effect of *W. pipientis* variation on
389 the fertility of *w*¹¹¹⁸; *bam*⁺/*bam*⁺ females of which we have lines infected with ten different wMel
390 variants of *W. pipientis* as described by Chrostek et al. (Chrostek et al. 2013) (Fig 4A).

391
392 Here, fertility measured over multiple days reflects progeny produced cumulatively by the
393 female over time, although there could also be variation in development time of the progeny.
394 We first assessed the effect of *W. pipientis* variation on wildtype *bam* female fertility (*w*¹¹¹⁸;
395 *bam*⁺/*bam*⁺). Over 17 days, we counted the progeny produced by each test female infected
396 individually for each of the ten *W. pipientis* variants and assessed the mean difference of adult
397 progeny from uninfected control females. We found that none of the *W. pipientis* variants
398 increased female fertility in the wildtype *bam* background, and some lines showed a significant
399 decrease in total progeny per female across all days measured (wMelPop8X, and
400 wMelCS2b_60) (Fig 4B&C). Of the lines that showed a negative impact of *W. pipientis* on
401 fertility, this effect was only significant as the female fly aged. On days 1-9 of progeny eclosion,
402 no *W. pipientis* variant had a significantly negative effect on progeny counts, which is notable
403 as these days represent progeny from eggs laid earlier in the test female's life and thus are
404 more likely to reflect effects of *W. pipientis* variation on fertility in nature (Fig 4B). The variants
405 that showed a significant and large negative effect of *W. pipientis* infection on fertility were both
406 in the wMelCS clade (mean difference between -32 and -46 progeny per female for days 1-17
407 Fig 4C). wMelCS variants exhibit higher *W. pipientis* titer in males (Chrostek et al. 2013), which

if also true in females may negatively impact female fertility if titer gets too high, as this leads apoptosis of infected cells (Chrostek et al. 2014).

All wMel variants rescue *bam* hypomorph female fertility but show a range of effect sizes.

Our results from the $w^{1118}; bam^+/bam^+$ fertility assay indicated that none of the *W. pipientis* variants tested have an overall increase in fertility in females (Fig4). We performed the same fertility assay as described above for the $w^{1118}; bam^+/bam^+$ genotype for the bam^{L255F}/bam^{null} genotype infected with the same ten *W. pipientis* variants. Females of the bam^{L255F}/bam^{null} genotype exhibit tumorous ovaries and are only weakly fertile (Fig 2B, Fig 3). In contrast to the wildtype *bam* lines, in the bam^{L255F}/bam^{null} lines we found that all variants increased female fertility both in the first week of progeny eclosion and throughout the entire measured timeframe with the exception of wMelPop2X, as infected females died before the end of the experiment, thus we only report the first week of progeny production for this variant (Fig 5A&B). Notably, and some variants had a large effect on female fertility. The variants with the largest effect sizes were in the wMelCS-like clade (wMelCS2b, wMelPop2x, wMelPop8x) (Fig 5A&B). This fertility rescue is in contrast to the decrease in fertility by the same *W. pipientis* variants in the wildtype *bam* background, especially for wMelCS2b which had the largest negative effect on wildtype *bam* fertility, but the largest positive effect on bam^{L255F}/bam^{null} fertility (Fig 5A&B). In all of the lines tested there were many individual females that had no progeny as well as females with varying distributions of progeny. In some of the lines (wMel2a, wMelCS2a, wMelCS2b) there were outlier females who had large rescue of fertility, some in the range of what we observe for wildtype *bam* fertility (Fig 4B&C). However, for most of the

individuals tested, fertility was not fully rescued, e.g., restored to wildtype levels (Fig 4B&C, Fig 5A&B). Although the largest effect of *W. pipientis* on *bam*^{L255F}/*bam*^{null} rescue was amongst the wMelCS-like variants, there were other notable variants outside of the wMelCS clade that showed high increase in female fertility, particularly wMel2a. Overall these data further indicate a genetic interaction between *bam* and *W. pipientis* that is specific from the overall effect of *W. pipientis* on female fertility.

All wMel variants rescue the ovarian *bam* hypomorph cytological defect but to varying degrees

Although we wanted to assess the rescue of the *bam* hypomorphic fertility phenotype by *W. pipientis* because we can also compare this to the effect of *W. pipientis* on wildtype *bam* fertility, we also wanted to know if genetic variation in *W. pipientis* affected the rescue of the *bam* hypomorphic cytological phenotype. Because fertility is affected by environmental and genetic differences that act in later developmental stages outside of *bam*'s function early in gametogenesis, we also measured the effect of *W. pipientis* variation on the rescue of the cytological *bam* mutant phenotype. Since the partial loss of function of *bam* results in over-proliferation of GSCs and reduced differentiation, we can quantify *bam* function by assaying for differentiated germ cells. The cytological *bam* hypomorphic phenotype in females consists of tumorous ovarioles filled with GSC-like cells, with few properly developing egg chambers (Fig 6A). Properly developing egg chambers contain polyploid nurse cells, which feature large, easily identifiable nuclei (Fig 6A, arrowheads). We counted the number of nurse cell positive egg chambers as a measure of GSC daughter differentiation and thus *bam* function. We found that all of the *W. pipientis* variants tested significantly increased the number of nurse cell

positive egg chambers, and that *W. pipientis* variants in the wMelCS-like clade showed highest numbers of nurse cell positive egg chambers (Fig 7B). We then pooled together the data individually for wMel-like, wMel2-like, and wMelCS-like and asked if there was a difference in mean number of cysts containing nurse cells per ovary between each clade (Fig 6C-D). We found that wMelCS-like variants had a significantly higher effect size than wMel-like and wMel2-like (Fig 6C&D), and that wMel2-like had a higher effect than wMel-like (Fig 6E). These results are consistent with the fertility assay results for the *bam*^{L255F}/*bam*^{null} hypomorph genotype, however we observed a more obvious trend of higher *bam* rescue by the wMelCS-like variants in this assay. All of the *W. pipientis* variants with the highest effect size of increased *bam* function are in the wMelCS-like clade. It is unclear whether this higher rescue is due to titer or another genetic difference between these clades, but these data indicate that genetic variation in *W. pipientis* impacts the *bam* rescue phenotype.

To ask if the nurse cell positive egg chambers resulted in oocytes capable of development if fertilized, and therefore represent properly differentiated GSC daughters, we picked two representative *W. pipientis* variants of high and low rescue and counted the total number of eggs laid per female over days 1-3. In *w*¹¹¹⁸; *bam*⁺/*bam*⁺ females, neither *W. pipientis* variant affected the number of eggs laid per female (Fig S1A). In contrast to the *w*¹¹¹⁸; *bam*⁺/*bam*⁺ females and consistent with the nurse cell assay, we observed few eggs laid by uninfected *w*¹¹¹⁸; *bam*^{L255F}/*bam*^{null} females, with significantly more eggs produced by the lower rescuing *W. pipientis* wMel59, and the highest number of eggs laid by females infected with the high rescuing line, wMelCSa_66 (Fig S1B).

477 **Homozygous *bam*^{L255F} females exhibit more variable fertility which is not negatively**
 478 **affected by *W. pipientis***

479 In light of the variable effect of infection by some *W. pipientis* variants on wildtype *bam* fertility
 480 after day 9, and that the uninfected *bam*^{L255F}/*bam*^{L255F} females did not exhibit a statistically
 481 significant fertility defect (Fig 2G, Fig 4B&C), we asked if infection by a subset of the *W.*
 482 *pipientis* variants we assessed in the wildtype *bam* background had any effect on the fertility of
 483 *bam*^{L255F}/*bam*^{L255F} females. We chose three *W. pipientis* variants that did not have a significant
 484 effect on wildtype *bam* female fertility for the entire time frame (wMel2a_63, wMelCSa_66, and
 485 wMelCSb_577 Fig 7A&B), and one that had a significant negative effect on female fertility in
 486 the second half of the experiment (wMelCS2b, Fig 7B). We measured fertility of homozygous
 487 females infected with *W. pipientis* variants as previously described. In both *bam*⁺/*bam*⁺ and
 488 *bam*^{L255F}/*bam*^{L255F} backgrounds, no wMel variants affected fertility during the first half of the
 489 experiment (Fig 4B, Fig 7A). However, in contrast to the *bam*⁺/*bam*⁺ background where
 490 wMelCS2b negatively affected female fertility across all days measured, we found that in the
 491 *bam*^{L255F}/*bam*^{L255F} background wMelCS2b did not have a significant effect on female fertility
 492 (Fig 7B). Additionally, in contrast to our findings in the *bam*⁺/*bam*⁺ background that wMel2a_63
 493 and wMelCSa_66 did not significantly affect female fertility, we found that in the
 494 *bam*^{L255F}/*bam*^{L255F} background both of these variants had a significantly positive effect on
 495 female fertility across all days measured (Fig 7B).

496
 497 Overall, we observed a more positive effect of *W. pipientis* on female fertility in the
 498 *bam*^{L255F}/*bam*^{L255F} background than in the *bam*⁺/*bam*⁺ background. As the differential effects of
 499 *W. pipientis* on female fertility in both *bam* backgrounds were only in older females, this may

be related to the general decline in fecundity as the female ages. Although we did not observe a tumorous ovarian phenotype in the *bam*^{L255F}/*bam*^{L255F} females, it is possible that *W. pipientis* is interacting with a subtle *bam* phenotype that becomes more apparent as the fly ages and oogenesis declines. This further indicates that there is an effect of *bam* genotype and *W. pipientis* genotype on the interaction between *W. pipientis* and female fertility, and that *W. pipientis* does not just generally increase female fertility in a wildtype *bam* background. Additionally, this interaction does not require the *bam* tumor phenotype.

Interestingly, during days 1-6 uninfected wildtype *bam* females had significantly fewer progeny than uninfected homozygous *bam*^{L255F} females (Fig 7A). We also observed that the total progeny per female of the homozygous *bam*^{L255F} genotype had a wider range than wildtype, which was not impacted by *W. pipientis* and is therefore likely a subtle *bam* mutant phenotype (Fig 7A). As *bam* regulates GSC daughter differentiation, its mis-regulation may increase or decrease fertility.

Discussion

Revisiting the phenotypes of classic *bam* alleles using CRISPR/Cas9 in a controlled genetic background

We previously reported that infection with *wMel* rescued the fertility defect of *bam*^{BW}/*bam*^{Δ59} 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 *W. pipientis* and *bam* may be driving the adaptive sequence divergence of *bam*.

523 However, in order to understand if and how this interaction could be adaptive, we need to
 524 understand the nature of the manipulation of *bam* function by *W. pipientis*. Second, *W.*
 525 *pipientis* is of immense interest as a potential means to control vectors of some human
 526 diseases (Moreira et al. 2009).

527
 528 One such way to find the *W. pipientis* loci necessary for the interaction between bacteria and
 529 the host would be to systemically mutagenize the bacteria and then screen for variants that
 530 enhance or reduce the phenotype of interest. However, *W. pipientis* are obligate
 531 endosymbionts that cannot be cultured, and so are currently not amenable to such genetic
 532 manipulation. While some studies have identified *W. pipientis* loci that interact with *D.*
 533 *melanogaster* by transgenically expressing *W. pipientis* loci in *D. melanogaster*, this requires
 534 identifying candidate *W. pipientis* loci that affect the host phenotype of interest (Ote et al. 2016;
 535 Le Page et al. 2017). An alternative method to identify candidate *W. pipientis* loci that affect
 536 the host phenotype is to use genetic variation in *W. pipientis* to test for differences in the effect
 537 of *W. pipientis* variants on the host phenotype.

538
 539 Chrostek et al. (Chrostek et al. 2013) generated isogenic *Drosophila* lines infected with
 540 previously described and genetically distinct wMel variants of *W. pipientis*. The original *bam^{BW}*
 541 mutant was not in this host genetic background and thus we would not be able to conclude that
 542 any differential interaction between *bam* and these variants was due to *W. pipientis* variation
 543 and not host genetic variation. Therefore, we used CRISPR/Cas9 to create the same amino
 544 acid mutant present in the original *bam^{BW}* hypomorph allele in the *w¹¹¹⁸* isogenic background.
 545 To ensure we had the tools to assess the *W. pipientis* rescue phenotype as previously

described, we also generated a new *bam* null allele using CRISPR/Cas9 in the same *w*¹¹¹⁸ genetic background.

We have now confirmed that these two new alleles behave similarly to the alleles we previously used to document the interaction between *wMel* and *bam*. Of note, our *bam*^{L255F}/*bam*^{null} hypomorph exhibits a stronger fertility defect and GSC daughter differentiation defect in comparison to our previous findings in the *bam*^{BW}/*bam*^{Δ59} hypomorph (Flores et al. 2015). Here, for uninfected *bam*^{L255F}/*bam*^{null} females, we observed a mean progeny per female of ~2 and we observed a mean of ~2 cysts containing nurse cells per ovary (Fig 5, Fig 6). For the *bam*^{BW}/*bam*^{Δ59} hypomorph, we previously observed a mean progeny per female of ~40 and ~2 nurse cell containing cysts per ovariole (Flores et al. 2015). Notably, each ovary contains 15-20 ovarioles, so 2 nurse cell containing cysts per ovariole would correspond to ~30-40 nurse cell containing cysts per ovary. Therefore, *wMel* not only rescues the mild GSC daughter differentiation defect in the original *bam*^{BW}/*bam*^{Δ59} hypomorph, but *wMel* and the other nine variants tested here also rescue the stronger *bam* GSC daughter differentiation defect we observe in the *bam*^{L255F}/*bam*^{null} hypomorph. This is especially notable, since *wMel* does not rescue fertility or GSC daughter differentiation in *bam* null mutants studied by Flores et al. (2015). Therefore, the *wMel* rescue of the *bam* mutant phenotype requires some functional *bam* gene product, but is also robust to a more severe fertility and differentiation defect. The difference in severity between the two *bam* hypomorphic genotypes also highlights the importance in controlling genetic background to rigorously assess fecundity phenotypes.

There have been a handful of studies assessing the phenotypes of diverse *W. pipientis* in isogenic backgrounds (Chrostek et al. 2013; Chrostek et al. 2014; Chrostek and Teixeira 2018; Gruntenko et al. 2019), however there have been no documented studies of interactions between *Drosophila* mutants and *W. pipientis* in these backgrounds. Recent work on the interaction between *W. pipientis* and *Sex lethal* in the female germline showed that *W. pipientis* rescues the loss of GSCs in some *Sxl* mutants through a *nanos* dependent interaction with the *W. pipientis* protein TomO (Ote et al. 2016). However, when researchers transgenically expressed *TomO* in *Sxl* hypomorphic ovaries, the GSC number was rescued, but fertility was not. This result indicated that there are other mechanisms *W. pipientis* is using to fully rescue GSC number and fertility in *Sxl* mutants. We believe the strategy we describe here could be used to further define genetic interactions such as the *Sxl* and *W. pipientis* interaction (Starr and Cline 2002). As we and others have also had success with CRISPR/Cas9 in non-*melanogaster* species, we believe this type of systematic analysis could be extended to assess interactions of host genes and *W. pipientis* in other species outside of *Drosophila melanogaster*.

***w*¹¹¹⁸ *W. pipientis* variants do not broadly increase fertility in the *w*¹¹¹⁸ genetic background**

Due to the shared *w*¹¹¹⁸ genetic background of the *W. pipientis* infected lines, we were able to rigorously assess the effect of different *W. pipientis* variants on wildtype *bam* female fertility. In addressing if and how *W. pipientis* may drive the adaptive evolution of *bam*, it was important for us to know if *W. pipientis* has an effect on fertility in a wildtype *bam* background. Here, we are not measuring specifically how *W. pipientis* is modulating a functional *bam* allele, as fertility

is affected by many loci, but if we observed a large effect of a particular *W. pipientis* variant on fertility, this would motivate further experiments to assess functional *bam* activity in lines infected with that *W. pipientis* variant. We observed no line with increased fertility for any of the *W. pipientis* variants, indicating that *W. pipientis* is not generally increasing fertility through *bam* or another pathway in this genetic background. However, we also observed that some *W. pipientis* variants had a negative effect on female fertility as the females aged. We cannot distinguish whether this effect is due to *W. pipientis* mis-regulating the germline or some other developmental consequence of its infection. In fact, the lines with the highest reported titers in males showed the largest negative impact on female fertility (Chrostek et al. 2013). Additionally, Serga et. al 2014 reported lower fecundity for females from a natural population in Uman, Ukraine infected with wMelCS compared to females infected with wMel. These observations highlight the complexity of the interaction between *W. pipientis* and *Drosophila*, where depending on the genetic background of the host, the fitness effect of *W. pipientis* on a phenotype may vary. This effect could further vary based on aspects of the host's environment where the manipulation of fertility by *W. pipientis* may not always be beneficial. Here we may expect the host and microbe to evolve ways of evading each other, which may become more or less apparent in different genetic backgrounds. Of note, our observations were made under laboratory conditions in a highly inbred line, and so we cannot be sure these *W. pipientis* variants impart the same effects on female fertility in natural populations.

611

wMel *W. pipientis* variants across three clades genetically interact with *bam*

We previously showed that a single wMel variants partially rescued the fertility and GSC daughter differentiation defect of the *bam*^{BW}/*bam*^{Δ59} hypomorph. Here we asked if there was

variation in the *bam* rescue phenotype among *W. pipientis* variants. The ten variants we used have been fully sequenced and differ by varying degrees of sequence divergence. All ten *W. pipientis* variants of the three clades tested (III, VI and VIII; see Fig 4A) rescued the fertility defect and the cytological defect of the *bam*^{L255F}/*bam*^{null} hypomorph. Therefore, none of the variants tested contained genetic variation that suppressed the interaction between *bam* and *W. pipientis*. We did find that in both the counts of nurse cell positive egg chambers and the fertility assays, wMelCS-like *W. pipientis* variants (clade VI) showed the highest rescue (Fig 5, Fig 6). This pattern was the clearest in the counts of nurse cell positive egg chambers as expected, since the presence of nurse cell positive egg chambers reflects GSC daughter differentiation, a phenotype that is a more direct output of *bam* activity. It is likely that the higher level of variability in the fertility assays is also due to the variable effect the *W. pipientis* variants have on other stages of development post GSC daughter differentiation. However, we feel it is important to also measure the effect of *W. pipientis* on fertility, since we cannot assess the count of nurse cell positive egg chambers for *bam* alleles that do not show a tumorous mutant phenotype, and it gives us insight into how *W. pipientis* infection could be adaptive. For example, would there be a fitness tradeoff between a high fecundity (GSC daughter differentiation) rescue and a low fertility (adult progeny) rescue? Measuring the impact of *W. pipientis* on multiple stages of oogenesis and reproduction therefore gives us insight into any further complexities in the interaction between GSC genes and *W. pipientis*.

634

We found that the wMelCS-like variants had the highest rescue effect on *bam* function, another example of a *W. pipientis* induced *D. melanogaster* fitness phenotype of which wMelCS-like variants exhibit the highest effects. The wMelCS-like variants tested here also confer the

638 highest levels of viral resistance to *D. melanogaster* males (Chrostek et al. 2013). Additionally,
 639 wMelCS-like variants have a stronger effect on the thermal preference of the *D. melanogaster*
 640 host, with wMelCS-like variants conferring preference for cooler temperatures compared to
 641 wMel-like and uninfected *D. melanogaster* (Truitt et al. 2019). Another study showed that
 642 wMelCS-like variants increased stress resistance in *D. melanogaster*, and no observed effect
 643 from wMel-like variants (Gruntenko et al. 2017).

644

645 wMelPop is a virulent derivative of wMelCSb and characterized by uncontrolled proliferation
 646 and titer caused by increased copy number of the Octomom locus. wMelPop2X and
 647 wMelPop8X exhibit 2X copies of the Octomom locus and anywhere from 4X-8X copies,
 648 respectively (Chrostek and Teixeira 2018). The high rescue of both the cytological and fertility
 649 defect of the *bam*^{L255F}/*bam*^{null} by wMelPop2X is suggestive that titer and possibly other
 650 phenotypes that increase interactions with the host are contributing to the rescue of *bam*. We
 651 observed lower rescue for wMelPop8X, indicating that the increasing copy number of the
 652 Octomom locus that causes apoptosis and early death likely negatively affects host fertility
 653 (Zhukova and Kiseleva 2012; Chrostek and Teixeira 2018). wMelPop2X is closely related to
 654 wMelCSb, with the only variation between them being a synonymous SNP and the
 655 amplification of the octomom locus which contains *W. pipientis* loci predicted to be involved
 656 with nucleic acid binding, and thus likely how it increases its titer, as well as proteins with
 657 predicted homology to eukaryotic domains (Chrostek and Teixeira 2018). These loci may then
 658 increase the interaction of *W. pipientis* and its host (López-Madrigal and Duarte 2020). A
 659 natural next step would be to determine the *W. pipientis* factors that manipulate GSC daughter
 660 differentiation.

661 **Possible adaptive interactions between *W. pipientis* and *bam***

662 It is well established that disrupting *bam* function negatively effects fertility (McKearin and
 663 Spradling 1990; Ohlstein and McKearin 1997; Lavoie et al. 1999; Flores et al. 2015), and
 664 *bam*^{L255F}/*bam*^{null} females are almost completely sterile. However, we do not predict such
 665 deleterious alleles to reach high frequency in natural populations. In fact, we have not found
 666 this nucleotide variant segregating in any of the natural populations of *D. melanogaster* that
 667 have been sampled in the *Drosophila* genome Nexus (Lack et al. 2015). Thus, while studying
 668 this mutant is effective in further refining how *bam* and *W. pipientis* interact, we cannot
 669 conclude that this type of interaction occurs in natural populations. However, given the severity
 670 of the mutant and strength of *W. pipientis*'s rescue, one hypothesis is that if *W. pipientis* is
 671 increasing GSC daughter differentiation when it is not favorable for the host, the host would
 672 evolve a way to evade *W. pipientis*'s manipulation of this pathway. In the case of our lab
 673 generated mutant, it is possible that when *bam* is not fully functional that reproduction is more
 674 sensitive to manipulation by *W. pipientis*. *W. pipientis* have been documented to respond to
 675 changes in the host environment, as *W. pipientis* gene expression is affected by host age and
 676 sex, and as *W. pipientis* transmission requires functional oogenesis, it reasonable to
 677 hypothesize that *W. pipientis* is sensitive to changes in gametogenesis (Rice et al. 2017;
 678 Newton and Sheehan 2018; Russell et al. 2020).

679

680 An additional observation we made is that *bam*^{L255F}/*bam*^{L255F} females show a broader range of
 681 adult progeny per female compared to wildtype *bam* females regardless of *W. pipientis*
 682 infection status. The individual *bam*^{L255F}/*bam*^{L255F} females exhibit both higher and lower than
 683 average fertility, indicating that mis-regulating *bam*'s differentiation function could both increase

684 and decrease fertility. This phenotype is worth further investigation, since although *bam* shows
685 a signature of positive selection, we do not know specifically what aspect of *bam* function is
686 adaptive. If variation in *bam* function can both increase and decrease mean fertility, and *W.*
687 *pipientis* has a generally positive effect on fertility of *bam*^{L255F}/*bam*^{L255F} females, this is further
688 evidence that *W. pipientis* may be able to manipulate *bam* in order to increase oogenesis for
689 its own benefit, and that genetic variation at *bam* could affect the regulation of oogenesis.
690 Therefore, if *W. pipientis* increased the rate of oogenesis to ensure its own transmission and
691 this was not favorable for the host, *bam* may be in conflict with *W. pipientis* to regulate
692 oogenesis in a favorable manner for the host. Here we see that although the average female
693 fertility of uninfected *bam*^{L255F}/*bam*^{L255F} mutants is not significantly different from wildtype, there
694 is a wider distribution of individual female fertility, indicating that the *bam* hypomorphic
695 phenotype is an increased variance in fertility (Fig 7). Since these females are in a common
696 genetic background and we do not expect genetic variation between individual females, the
697 *bam*-mediated mis-regulation of differentiation may set off a cascade of other genetic mis-
698 regulation that results in higher or lower fertility over time. We see that infection by some *W.*
699 *pipientis* variants in this background increases female fertility, indicating that perhaps *W.*
700 *pipientis* can restore the mis-regulation of GSC daughter differentiation, and even increase
701 reproductive output through this mechanism. It would be interesting to use this genotype for
702 future experiments to explore the possibility that this *bam* mutation may disrupt the mechanism
703 *D. melanogaster* evolved to evade *W. pipientis*'s effect on differentiation.

704

705 An interesting question that remains is how *W. pipientis* have evolved in their interaction with
706 GSC genes, including *bam*. While *wMelCS*-like and *wMel*-like variants share a most recent

707 common ancestor about 8000 years ago, wMelCS-like variants were recently (~2000 years
 708 ago) replaced by wMel-like variants in natural populations of *D. melanogaster* (Riegler et al.
 709 2005; Richardson et al. 2012). However, this replacement has not been complete, and there
 710 are global populations still infected with wMelCS variants (Riegler et al. 2005; Nunes et al.
 711 2008). Interestingly we find that the wMelCS variants we assayed show higher rescue of
 712 *bam*^{L255F}/*bam*^{null} female fertility and nurse cells. If *bam* and *W. pipientis* are evolving in an arms
 713 race, it could be that the evolutionarily more recent wMel-like variants have not evolved the
 714 same level of interaction with *bam* as wMelCS has. Future work to investigate these dynamics
 715 should include sampling populations that are still infected with wMelCS as well as those
 716 infected with wMel and asking if there is any evidence of genetic differentiation at *bam*. Some
 717 populations have been identified that are still infected with wMelCS, such as a natural
 718 population of *D. melanogaster* from Uman, Ukraine that has been infected with both wMel and
 719 wMelCS and has been monitored yearly for infection frequency, some additional Palearctic
 720 populations, and a population in the Netherlands (Early and Clark 2013; Bykov et al. 2019;
 721 Serga et al. 2021).

722

723 Additionally, we could utilize existing *W. pipientis* sequence variation from natural populations
 724 to ask if there is any evidence of associations between *bam* variation and *W. pipientis*
 725 variation. If we were to sample populations that are differentially infected with wMel and
 726 wMelCS to assess genetic differentiation at *bam* as discussed above, we could also do the
 727 same for *W. pipientis* variants. One caveat being that if we did not observe genetic signatures
 728 of adaptive evolution in *W. pipientis* loci, this does not mean that *W. pipientis* and *bam* are not
 729 coevolving, as we do not know the true infection history of a *Drosophila* population with *W.*

pipientis and thus which *W. pipientis* variant may have been in conflict with *bam*. Additionally, there may be general *W. pipientis* functions that are interacting with *bam* and not a single locus (e.g., loci that regulate titer).

Further work should focus on determining the *W. pipientis* loci contributing to the different magnitude of *bam* rescue. However, as has been previously pointed out by Chrostek et al. (Chrostek et al. 2013), between the *wMel* clades and *wMelCS* clades there are eight indels and 108 SNPs, including differences in the coding sequence of 58 genes. So further work would have to be done to narrow down which variants contribute to the degree of rescue. A next step could be moving out to more divergent *W. pipientis wMel* variants (for example, *wAu* that infects *D. simulans* (Miller and Riegler 2006)) 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*.

Acknowledgements

This work was supported by National Institutes of Health grant R01-GM095793 to C.F.A. We would like to thank Luis Teixeira for his advice on assessing the effect of *W. pipientis* variation on *Drosophila* fertility and for the gifts of the *w¹¹¹⁸* isogenic lines infected with *W. pipientis* variants used in this study. We also would like to thank Kristen Rose Baxter for her contributions to our early fertility experiments. We are also grateful to Mariana F. Wolfner, Andrew G Clark, Miwa Wenzel and Catherine Kagemann for valuable input and discussion of these experiments and results and helpful comments on this manuscript.

References

- Arnold PA, Levin SC, Stevanovic AL, Johnson KN. 2019. *Drosophila melanogaster* infected with *Wolbachia* strain wMelCS prefer cooler temperatures. *Ecol Entomol*. doi:10.1111/een.12696.
- Aruna S, Flores HA, Barbash DA. 2009. Reduced fertility of *Drosophila melanogaster* Hybrid male rescue (Hmr) mutant females is partially complemented by Hmr orthologs from sibling species. *Genetics*. doi:10.1534/genetics.108.100057.
- Bailly-Bechet M, Martins-Simões P, Szölloosi GJ, Mialdea G, Sagot MF, Charlat S. 2017. How Long Does *Wolbachia* Remain on Board? *Mol Biol Evol*. doi:10.1093/molbev/msx073.
- Bauer DuMont VL, Flores HA, Wright MH, Aquadro CF. 2007. Recurrent positive selection at Bgcn, a key determinant of germ line differentiation, does not appear to be driven by simple coevolution with its partner protein bam. *Mol Biol Evol*. 24(1):182–191. doi:10.1093/molbev/msl141.
- Beckmann JF, Ronau JA, Hochstrasser M. 2017. A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nat Microbiol*. doi:10.1038/nmicrobiol.2017.7.
- Bykov RA, Yudina MA, Gruntenko NE, Zakharov IK, Voloshina MA, Melashchenko ES, Danilova M V., Mazunin IO, Ilinsky YY. 2019. Prevalence and genetic diversity of *Wolbachia* endosymbiont and mtDNA in Palearctic populations of *Drosophila melanogaster*. *BMC Evol Biol*. doi:10.1186/s12862-019-1372-9.
- Choi JY, Aquadro CF. 2014. The coevolutionary period of *Wolbachia pipientis* infecting *Drosophila ananassae* and its impact on the evolution of the host germline stem cell regulating genes. *Mol Biol Evol*. doi:10.1093/molbev/msu204.
- Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins FM, Teixeira L. 2013.

777 Wolbachia Variants Induce Differential Protection to Viruses in *Drosophila melanogaster*:
778 A Phenotypic and Phylogenomic Analysis. *PLoS Genet.* 9(12):1003896.
779 doi:10.1371/journal.pgen.1003896. [accessed 2020 Oct 6]. <http://www.fct.pt>.

780 Chrostek E, Marialva MSP, Yamada R, O'Neill SL, Teixeira L. 2014. High anti-viral protection
781 without immune upregulation after interspecies Wolbachia transfer. *PLoS One*.
782 9(6):99025. doi:10.1371/journal.pone.0099025. [accessed 2020 Oct 6].
783 /pmc/articles/PMC4049622/?report=abstract.

784 Chrostek E, Teixeira L. 2018. Within host selection for faster replicating bacterial symbionts.
785 *PLoS One*. doi:10.1371/journal.pone.0191530.

786 Dedeine F, Vavre F, Fleury F, Loppin B, Hochberg ME, Boulétreau M. 2001. Removing
787 symbiotic Wolbachia bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc Natl*
788 *Acad Sci U S A*. doi:10.1073/pnas.101304298.

789 Deehan M, Lin W, Blum B, Emili A, Frydman H. 2021. Intracellular density of wolbachia is
790 mediated by host autophagy and the bacterial cytoplasmic incompatibility gene cifb in a
791 cell type-dependent manner in *drosophila melanogaster*. *MBio*. 12(1):1–19.
792 doi:10.1128/mBio.02205-20.

793 Dutra HLC, Rocha MN, Dias FBS, Mansur SB, Caragata EP, Moreira LA. 2016. Wolbachia
794 Blocks Currently Circulating Zika Virus Isolates in Brazilian *Aedes aegypti* Mosquitoes.
795 *Cell Host Microbe*. doi:10.1016/j.chom.2016.04.021.

796 Dylan Shropshire J, On J, Layton EM, Zhou H, Bordenstein SR. 2018. One prophage WO
797 gene rescues cytoplasmic incompatibility in *Drosophila melanogaster*. *Proc Natl Acad Sci*
798 *U S A*. doi:10.1073/pnas.1800650115.

799 Early AM, Clark AG. 2013. Monophyly of Wolbachia pipientis genomes within *Drosophila*

800 melanogaster: Geographic structuring, titre variation and host effects across five
801 populations. Mol Ecol. doi:10.1111/mec.12530.

802 Flores HA, Bubnell JE, Aquadro CF, Barbash DA. 2015. The Drosophila bag of marbles Gene
803 Interacts Genetically with Wolbachia and Shows Female-Specific Effects of Divergence.
804 doi:10.1371/journal.pgen.1005453.

805 Gratz SJ, Ukken FP, Rubinstein CD, Thiede G, Donohue LK, Cummings AM, Oconnor-Giles
806 KM. 2014. Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair
807 in Drosophila. Genetics. 196(4):961–971. doi:10.1534/genetics.113.160713.

808 Gruntenko NE, Ilinsky YY, Adonyeva N V., Burdina E V., Bykov RA, Menshanov PN,
809 Rauschenbach IY. 2017. Various Wolbachia genotypes differently influence host
810 Drosophila dopamine metabolism and survival under heat stress conditions. BMC Evol
811 Biol. doi:10.1186/s12862-017-1104-y.

812 Gruntenko NE, Karpova EK, Adonyeva N V., Andreenkova O V., Burdina E V., Ilinsky YY,
813 Bykov RA, Menshanov PN, Rauschenbach IY. 2019. Drosophila female fertility and
814 juvenile hormone metabolism depends on the type of Wolbachia infection. J Exp Biol.
815 222(4). doi:10.1242/jeb.195347. [accessed 2020 Oct 7].
816 <https://jeb.biologists.org/content/222/4/jeb195347>.

817 Hague MTJ, Caldwell CN, Cooper BS. 2020. Pervasive effects of wolbachia on host
818 temperature preference. MBio. doi:10.1128/mBio.01768-20.

819 Ho J, Tumkaya T, Aryal S, Choi H, Claridge-Chang A. 2019. Moving beyond P values: data
820 analysis with estimation graphics. Nat Methods. doi:10.1038/s41592-019-0470-3.

821 Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F,
822 Greenfield M, Durkan M, Leong YS, Dong Y, et al. 2011. Successful establishment of

823 Wolbachia in Aedes populations to suppress dengue transmission. Nature.
824 doi:10.1038/nature10356.

825 Insko ML, Bailey AS, Kim J, Olivares GH, Wapinski OL, Tam CH, Fuller MT. 2012. A self-
826 limiting switch based on translational control regulates the transition from proliferation to
827 differentiation in an adult stem cell lineage. Cell Stem Cell. 11(5):689–700.
828 doi:10.1016/j.stem.2012.08.012.

829 Lack JB, Cardeno CM, Crepeau MW, Taylor W, Corbett-Detig RB, Stevens KA, Langley CH,
830 Pool JE. 2015. The drosophila genome nexus: A population genomic resource of 623
831 Drosophila melanogaster genomes, including 197 from a single ancestral range
832 population. Genetics. doi:10.1534/genetics.115.174664.

833 Lavoie CA, Ohlstein B, McKearin DM. 1999. Localization and function of bam protein require
834 the benign gonial cell neoplasm gene product. Dev Biol. doi:10.1006/dbio.1999.9346.

835 Lindsey ARI. 2020. Sensing, signaling, and secretion: A review and analysis of systems for
836 regulating host interaction in wolbachia. Genes (Basel). 11(7):1–21.
837 doi:10.3390/genes11070813. [accessed 2020 Nov 17].
838 /pmc/articles/PMC7397232/?report=abstract.

839 López-Madrigal S, Duarte EH. 2020. Titer regulation in arthropod-Wolbachia symbioses.
840 FEMS Microbiol Lett. doi:10.1093/femsle/fnz232.

841 McKearin DM, Spradling AC. 1990. Bag-of-marbles: A Drosophila gene required to initiate both
842 male and female gametogenesis. Genes Dev. 4(12 B):2242–2251.
843 doi:10.1101/gad.4.12b.2242.

844 Miller WJ, Riegler M. 2006. Evolutionary dynamics of wAu-like Wolbachia variants in
845 neotropical Drosophila spp. Appl Environ Microbiol. doi:10.1128/AEM.72.1.826-835.2006.

846 Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-
847 Mendelin S, Day A, Riegler M, et al. 2009. A Wolbachia Symbiont in *Aedes aegypti* Limits
848 Infection with Dengue, Chikungunya, and Plasmodium. *Cell*.
849 doi:10.1016/j.cell.2009.11.042.

850 Newton ILG, Sheehan KB. 2018. Gateway Entry Vector Library of *Wolbachia pipientis*
851 Candidate Effectors from Strain wMel. *Microbiol Resour Announc*.
852 doi:10.1128/mra.00806-18.

853 Nunes MDS, Nolte V, Schlötterer C. 2008. Nonrandom *Wolbachia* infection status of
854 *Drosophila melanogaster* strains with different mtDNA haplotypes. *Mol Biol Evol*.
855 25(11):2493–2498. doi:10.1093/molbev/msn199. [accessed 2020 Oct 6].
856 <https://academic.oup.com/mbe/article/25/11/2493/1094848>.

857 Ohlstein B, Lavoie CA, Vef O, Gateff E, McKearin DM. 2000. The *Drosophila* Cystoblast
858 Differentiation Factor, benign gonial cell neoplasm, Is Related to DExH-box Proteins and
859 Interacts Genetically With bag-of-marbles. [accessed 2020 Apr 14]. <http://www.fruitfly.org>.

860 Ohlstein B, McKearin D. 1997. Ectopic expression of the *Drosophila* Bam protein eliminates
861 oogenic germline stem cells. *Development*.

862 Ote M, Ueyama M, Yamamoto D. 2016. *Wolbachia* Protein TomO Targets nanos mRNA and
863 Restores Germ Stem Cells in *Drosophila* Sex-lethal Mutants. *Curr Biol*. 26(17):2223–
864 2232. doi:10.1016/j.cub.2016.06.054.

865 Ote M, Yamamoto D. 2020. Impact of *Wolbachia* infection on *Drosophila* female germline stem
866 cells. Elsevier Inc. [accessed 2020 Apr 10]. <https://doi.org/10.1016/j.cois.2019.10.001>.

867 Le Page DP, Metcalf JA, Bordenstein Sarah R., On J, Perlmutter JI, Shropshire JD, Layton
868 EM, Funkhouser-Jones LJ, Beckmann JF, Bordenstein Seth R. 2017. Prophage WO

869 genes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility. *Nature*.
870 doi:10.1038/nature21391.

871 Rice DW, Sheehan KB, Newton ILG. 2017. Large-scale identification of Wolbachia pipientis
872 effectors. *Genome Biol Evol.* doi:10.1093/gbe/evx139.

873 Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, Jiggins FM, Bergman CM.
874 2012. Population Genomics of the Wolbachia Endosymbiont in *Drosophila melanogaster*.
875 Kopp A, editor. *PLoS Genet.* 8(12):e1003129. doi:10.1371/journal.pgen.1003129.
876 [accessed 2020 Oct 6]. <https://dx.plos.org/10.1371/journal.pgen.1003129>.

877 Riegler M, Sidhu M, Miller WJ, O'Neill SL. 2005. Evidence for a global Wolbachia replacement
878 in *Drosophila melanogaster*. *Curr Biol.* doi:10.1016/j.cub.2005.06.069.

879 Russell Shelbi L, Castillo Jennie Ruelas, Russell S L, Castillo J R. 2020. Chapter 5 Trends in
880 Symbiont-Induced Host Cellular Differentiation. doi:10.1007/978-3-030-51849-3_5.
881 https://doi.org/10.1007/978-3-030-51849-3_5.

882 Schneider DI, Klasson L, Lind AE, Miller WJ. 2014. More than fishing in the dark: PCR of a
883 dispersed sequence produces simple but ultrasensitive Wolbachia detection. *BMC*
884 *Microbiol.* doi:10.1186/1471-2180-14-121.

885 Serga S V., Maistrenko OM, Matiytsiv NP, Vaiserman AM, Kozeretska IA. 2021. Effects of
886 Wolbachia infection on fitness-related traits in *Drosophila melanogaster*. *Symbiosis*.
887 doi:10.1007/s13199-020-00743-3.

888 Shropshire JD, Rosenberg R, Bordenstein SR. 2021. The impacts of cytoplasmic
889 incompatibility factor (cifA and cifB) genetic variation on phenotypes.
890 *Genetics.*(iiyaa007):1–8. doi:10.1093/genetics/iiyaa007.

891 Starr DJ, Cline TW. 2002. A host-parasite interaction rescues *Drosophila* oogenesis defects.

892 Nature. 418(6893):76–79. doi:10.1038/nature00843.

893 Thurmond J, Goodman JL, Strelets VB, Attrill H, Gramates LS, Marygold SJ, Matthews BB,
894 Millburn G, Antonazzo G, Trovisco V, et al. 2019. FlyBase 2.0: The next generation.
895 Nucleic Acids Res. doi:10.1093/nar/gky1003.

896 Ting X. 2013. Control of germline stem cell self-renewal and differentiation in the *Drosophila*
897 ovary: concerted actions of niche signals and intrinsic factors. Wiley Interdiscip Rev Dev
898 Biol. 2(2):261–273. doi:10.1002/wdev.60. [accessed 2020 May 4].
899 <http://doi.wiley.com/10.1002/wdev.60>.

900 Truitt AM, Kapun M, Kaur R, Miller WJ. 2019. Wolbachia modifies thermal preference in
901 *Drosophila melanogaster*. Environ Microbiol. doi:10.1111/1462-2920.14347.

902 Utarini A, Indriani C, Ahmad RA, Tantowijoyo W, Arguni E, Ansari MR, Supriyati E, Wardana
903 DS, Meitika Y, Ernesia I, et al. 2021. Efficacy of Wolbachia-Infected Mosquito
904 Deployments for the Control of Dengue. N Engl J Med. 384(23):2177–2186.
905 doi:10.1056/NEJMoa2030243. [accessed 2021 Jun 16].
906 <http://www.nejm.org/doi/10.1056/NEJMoa2030243>.

907 Werren JH, Baldo L, Clark ME. 2008. Wolbachia: Master manipulators of invertebrate biology.
908 Nat Rev Microbiol. doi:10.1038/nrmicro1969.

909 Zhukova M V., Kiseleva E. 2012. The virulent Wolbachia strain wMelPop increases the
910 frequency of apoptosis in the female germline cells of *Drosophila melanogaster*. BMC
911 Microbiol. 12(SUPPL. 1):S15. doi:10.1186/1471-2180-12-S1-S15.
912 <http://www.biomedcentral.com/1471-2180/12/S1/S15>.
913

Figure Legends

Fig 1. Design for recreating the classic *bam*^{BW} hypomorph allele and a new *bam* null allele in the *w*¹¹¹⁸ isogenic background with CRISPR/Cas9 (A) Schematic of the *bam* gene region showing the single gRNA target site in the second exon and the hypomorphic missense mutation. (B) Schematic of the *bam* gene region showing the single gRNA target site in the second intron with the 3xP3-DsRed eye marker to create a *bam* null allele.

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) The newly generated *bam*^{null-In2-3xP3-DsRed}/*bam*^{null-In2-3xP3-DsRed} genotype results in tumorous ovaries (arrow). (B) The recreated *bam*^{L255F} mutation over *bam*^{null-In2-3xP3-DsRed} exhibits tumorous ovaries in the *w*¹¹¹⁸ isogenic background (arrow). (C) The recreated *bam*^{L255F} mutation over the classic *bam*^{A59} null allele exhibits tumorous ovaries (arrow). (D) The classic *bam*^{BW}/*bam*^{A59} hypomorphic genotype exhibits tumorous ovaries (arrow). (E) 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). (F) *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. (G) *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). $bam^{L255F}/bam^{null-In2-3xP3-DsRed}$ females have significantly lower fertility compared to wildtype (mean difference = -44.6, bootstrap 95% confidence interval, effect size), as do $bam^{L255F}/bam^{\Delta 59}$ females (mean difference = -36.7, bootstrap 95% confidence interval, effect size).

Fig 4. Total progeny per female and mean difference of progeny per female for the $w^{1118}; bam^+/bam^+$ genotype infected with ten *W. pipientis* wMel variants compared to uninfected. (A) Cladogram adapted from Chrostek et al. 2013 showing the relationships and clade assignments for the *W. pipientis* variants tested in this study. (B) Swarm and Cumming estimation plots showing the total progeny per female for each *W. pipientis* 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 *W. pipientis* variant was associated with a significant difference in fertility for wildtype *bam* females over days 1-9 post eclosion (bootstrap 95% confidence interval, effect size). (C) Swarm and Cumming estimation plots showing the total progeny per female for each *W. pipientis* 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. *W. pipientis* variants wMelPop8X, and wMelCS2b were associated with significantly lower fertility over the longer period of days 1-17 post eclosion compared to uninfected controls (bootstrap 95% confidence interval, effect size)

Fig 5. Total progeny per female and mean difference of progeny per female for the $w^{1118}; bam^{L255F}/bam^{null}$ genotype infected with ten *W. pipientis* wMel variants compared to uninfected. (A) Swarm and Cumming estimation plots showing the total progeny per female for each *W. pipientis* 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 *W. pipientis* variants were associated with significant increases in female fertility compared to uninfected controls (bootstrap 95% confidence interval, effect size). (B) Swarm and Cumming estimation plots showing the total progeny per female for each *W. pipientis* 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. All *W. pipientis* variants were associated with significant increases in female fertility compared to uninfected controls (bootstrap 95% confidence interval, effect size). wMelPop2X is not reported for this time frame, as most females died after day 9.

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 ten *W. pipientis* wMel variants compared to uninfected. (A) Representative images of bam^{L255F}/bam^{null} ovaries from uninfected, wMel59, and wMelpop2X infected females. Nurse cell positive cysts labeled with arrowheads. (B) Swarm and Cumming estimation plots showing the total cysts containing nurse cells per ovary for each *W. pipientis* infected line assayed on 2-3 day old females. All *W. pipientis* variants are associated with a significant increase in nurse cell positive cysts per ovary (bootstrap 95% confidence interval, effect size). *W. pipientis* variants in the wMelCS-like clade have the highest effect on nurse cell positive cysts. (C-E) Swarm and Gardner-Altman plots showing pairwise comparisons of nurse cell positive cyst counts pooled together for each wMel clade. wMelCS-like variants exhibit higher effects on nurse cell positive cysts compared to wMel2 and wMel-like variants (95% confidence interval, effect size), and wMel2 variants exhibit a higher effect on nurse cell positive cysts than wMel-like (95% confidence interval, effect size).

Fig 7. Total progeny per female and mean difference of progeny per female for the w^{1118} ; bam^{L255F}/bam^{L255F} genotype infected with four representative *W. pipientis* 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. *W. pipientis* 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 *W. pipientis* 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). (B) Swarm and Cumming estimation plots showing the effect of *W. pipientis* variants on bam^{L255F}/bam^{L255F} female fertility over days 1-13 of eclosion. *W. pipientis* 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 *W. pipientis* 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.

Fig S1. 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 *W. pipientis* variants from the wMel-like and wMelCS-like clades. (A) Swarm and Cumming estimation plots showing the total eggs per female for w^{1118} ; bam^+/bam^+ lines infected with wMel59 or wMelCSa66. 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 *W. pipientis* variants. Infection with either wMel_59 or wMelCSa_66 significantly rescues the number of eggs laid per female compared to the uninfected control (95% confidence interval, effect size). Consistent with the results of the nurse cell assay, wMelCSa_66 shows a higher rescue effect than wMel_59.

A.

w*¹¹¹⁸bam*

B.

w*¹¹¹⁸bam*

bam^{null-In2-3xP3-DsRed}/*bam*^{null-In2-3xP3-DsRed}

*w*¹¹¹⁸ isogenic

bam^{L255F}/*bam*^{null-In2-3xP3-DsRed}

bam^{L255F}/*bam*^{Δ59}

bam^{BW}/*bam*^{Δ59}

Non-isogenic

bam⁺/*bam*⁺

*w*¹¹¹⁸ isogenic

bam^{L255F}/*bam*^{L255F}

G.

● w^{1118} isogenic background ● Non-isogenic background

A.

B.

C.

A.

B.

A. *bam*^{L255F}/*bam*^{null}

B.

C.

D.

E.

A.

● Uninfected ● wMel2 ● wMelCS-like

B.

A.

bam⁺/*bam*⁺

B.

bam^{L255F}/*bam*^{null}