Diverse wMel 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,* Paula Fernandez-Begne, Cynthia K. S. Ulbing, and Charles F. Aquadro

Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14850, USA
*Corresponding author: Department of Molecular Biology and Genetics, 233 Biotechnology Building, 526 Campus Rd. Ithaca, NY 14853, USA. Email: jeb468@cornell.edu

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<sup>bw</sup> hypomorphic mutant as a transheterozygote over a bam null. However, bam<sup>bw</sup> was generated more than 20 years ago in an uncontrolled genetic background and maintained over a balancer chromosome. Consequently, the chromosome carrying bam<sup>bw</sup> 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<sup>L255F</sup>) and a new bam null disruption mutation into the W. pipientis isogenic background. We assess the fertility of wildtype bam<sup>1w1118</sup>, bam<sup>L255F</sup>/bam<sup>1w1118</sup> hypomorphic, and bam<sup>L255F</sup>/bam<sup>L255F</sup> mutant females, each infected individually with 10<sup>4</sup> 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. In addition, 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.

Keywords: Wolbachia; germline stem cell; bam; oogenesis; differentiation

Introduction

Wolbachia pipientis is a maternally inherited endosymbiotic bacteria that infects over 65% of insect species and manipulates reproduction in a myriad of ways in order to ensure its transmission through the female germline (Werren et al. 2008; Lindsey 2020; Ote and Yamamoto 2020). The phenotypes W. pipientis 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 (GSC) differentiation in order to increase female fertility (Werren et al. 2008). While W. pipientis manipulates its host to increase its own transmission, some W. pipientis also provide the host protection against viruses, increase fecundity, modulate thermal preference, and increase longevity (Dedelne et al. 2001; Chrostek et al. 2013; Arnold et al. 2019; Truit et al. 2019; Hague et al. 2020; López-Madrigal and Duarte 2020).

Understanding the genetic mechanisms that W. pipientis uses to manipulate its host has been of immense interest to both basic and applied fields of study. W. pipientis is of particular interest as a control for disease vectors such as mosquitoes due to its ability to sweep through a population (due to CI) and then protect the insect from viruses that can also cause human illness such as Dengue, Zika, and Chikungunya (Moreira et al. 2009; Hoffmann et al. 2011; Dutra et al. 2016; Utarini et al. 2021). However, it has been difficult to perform genetic studies of W. pipientis function to understand the mechanisms of these host-microbe interactions since W. pipientis is an obligate endosymbiont and cannot be cultured. Over the past few years, multiple groups have utilized bioinformatic and in vitro screens to identify candidate W. pipientis loci that modulate Drosophila phenotypes (Ote et al. 2016; Le Page et al. 2017). These loci have then been expressed in Drosophila as transgenes. Through these methods, the W. pipientis genes cifA and cifB (orthologs in wMel and wPip) that cause CI have been identified and functionally validated in D. melanogaster (Beckmann et al. 2017; Le Page et al. 2017). These transgenic tools have been used to further define the CI phenotype (Shropshire et al. 2018), investigate the consequences of genetic variation at
cifA and cifB on CI (Shropshire et al. 2021), and identify other host phenotypes affected by cifA and cifB (Deehan et al. 2021). Another group has identified the W. pipiens TorM locus which is one of likely multiple W. pipiens loci that interact with Sex lethal, a GSC gene that controls GSC maintenance and sex determination (Ote et al. 2016).

Previously, we reported that the W. pipiens variant uMel genetically interacts with another D. melanogaster GSC gene, bag of marbles (bam). Infection with the uMel variant rescues the fertility defect of females transheterozygous for the classic bam hypomorph allele (bam<sup>W</sup>) and a classic bam null allele (bam<sup>W595</sup>) (Flores et al. 2015). Bam is the switch for GSC daughter differentiation in D. melanogaster females and the switch for terminal spermatocyte differentiation in males (McKearin and Spradling 1990, Insco et al. 2012, Ting 2013). Although bam function is essential for gametogenesis, and bam is both necessary and sufficient for GSC daughter differentiation, we observe that it is evolving under positive selection for amino acid divergence in an episodic manner across the Drosophila genus (Bauer DuMont et al. 2007; Choi and Aquadro 2014). We see particularly strong bursts of amino acid changes at bam in the D. melanogaster and D. simulans lineages (Bauer DuMont et al. 2007). This episodic pattern of positive selection is consistent with selective pressures that are present in 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. pipiens 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. pipiens and bam genetically interact led us to hypothesize that W. pipiens may drive the adaptive evolution of bam (Flores et al. 2015). Since W. pipiens is a known manipulator of reproduction, W. pipiens may be in conflict with bam and other GSC genes for control of oogenesis. While it may seem favorable to host fitness if W. pipiens manipulates GSC 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. pipiens. For example, W. pipiens may promote oogenesis when it is not favorable for the host to reproduce due to environmental or physiological factors. To further understand if W. pipiens 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. pipiens.

Fully sequenced variants of W. pipiens that infect D. melanogaster have been found to cluster phenotypically and phylogenetically into two distinct groups of clades: uMel-like variants (including uMel and uMel12) and MelCS-like variants (including uMelCS, uMelCS2, and uMelPop) that are estimated to have diverged 80,000 fly generations before present (Richardson et al. 2012; Chrostek et al. 2013). The uMelCS-like variants predominated in D. melanogaster originally but have largely (although not completely) been replaced by uMel-like variants world-wide in the late 20th century (Riegler et al. 2005). The uMelCS-like variants provide stronger viral protection, reach higher intracellular bacterial titers in males, and often shorten lifespan compared to the more benign uMel-like variants that now predominate. In addition, uninfected D. melanogaster and those infected with uMel-like variants have a higher temperature preference than D. melanogaster infected with uMelCS-like variants (including uMelPop) (Truit et al. 2019).

To assess the specificity of our initial observation that uMel rescues the female fertility phenotype of the bam<sup>W</sup>/bam<sup>W595</sup> hypomorphic mutant, we made use of a genetic tool for functionally assessing W. pipiens variation generated by Chrostek et al. (2013), a set of u<sup>1118</sup> isogenic lines individually infected with 10 diverse uMel W. pipiens variants from two of the uMel-like clades and the uMelCS-like clade defined by Richardson et al. (2012). The 10 W. pipiens variants that we analyze thus include both phenotypic classes of uMel variants: the uMelCS-like variants which are higher titer, reduce host lifespan, and confer higher viral resistance and lower thermal preference in contrast to the more recent uMel-like variants.

Since fertility is a phenotype affected by genome-wide variation and both the bam<sup>W</sup> and bam<sup>W595</sup> alleles were generated in nonisogenic backgrounds, we sought to control the Drosophila genetic background to better isolate the effect of W. pipiens variation and bam genotype on fertility. We used CRISPR/Cas9 to edit the single amino acid change of the original bam<sup>W</sup> hypomorph (Ohielstein et al. 2000) into the u<sup>1118</sup> isogenic background, and also to create a new bam null allele in the same genetic background. In addition, we had previously not been able to determine the phenotype of bam<sup>W</sup>/bam<sup>W</sup> females, as the bam<sup>W</sup> 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 wild-type females. We then assessed the effect of infection by the individual W. pipiens variants by crossing the cytoplasmic maternal background infected with each W. pipiens variant into our u<sup>1118</sup> bam hypomorph line thereby maintaining the same nuclear background (except for the bam locus).

We find that all uMel W. pipiens variants tested here do not increase fertility in the wildtype bam background, but all uMel W. pipiens variants rescue the fertility of bam mutant females, with uMelCS-like W. pipiens showing the highest rescue effects. Therefore, the fertility rescue of the bam hypomorph by W. pipiens is not due to an overall increase in fertility modulated by W. pipiens, but a specific interaction between bam and W. pipiens 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-h light–dark cycle. The lines carrying the classic bam alleles, bam<sup>W595</sup> (null) and bam<sup>W</sup> (hypomorph) are described on Flybase (Thurmond et al. 2019) and in Flores et al. (2015). The W. pipiens infected u<sup>1218</sup> 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 u<sup>1118</sup>, bam<sup>W5557f</sup>/bam<sup>W595</sup> and u<sup>1118</sup>, bam<sup>W1</sup>/bam<sup>Wf</sup> fertility assays. We used u<sup>1118</sup> isogenic males for the u<sup>1118</sup>, bam<sup>W1</sup>/bam<sup>W5557f</sup>/bam<sup>W595</sup> fertility assays. We verified that males were uninfected with W. pipiens by endpoint PCR and qPCR using primers described below. To generate the bam hypomorphic lines infected with the 10 different W. pipiens variants, we crossed females from the u<sup>1118</sup>, TM2/TM6 stock infected with each variant to u<sup>1118</sup>, bam<sup>W5557f</sup>/TM6 males.
Generation of bam alleles with CRISPR/Cas9
We engineered the new bam<sup>L255F</sup> hypomorph and bam null alleles described in this study in the w<sup>1118</sup> isogenic background using synthetic gRNAs and Cas9 protein as follows.

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

**Cloning**
We generated all PCR products for cloning with NEB High Fidelity qPCR, and cloning from IDTDNA (Supplementary Table S2). We generated all PCR products for cloning with NEB High Fidelity Cloning mini-prep kit and sequenced plasmids with Sanger sequencing (Cornell BRC Genomics Core). We ordered primers for PCR, sequencing, and cloning from IDTDNA (Supplementary Table S2).

**Donor sequences**
To generate the hypomorphic bam<sup>L255F</sup> allele we designed a single stranded oligo donor (ssODN) that contained the single amino acid mutation of the original bam<sup>BW</sup> 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 80bp of homology on each side of our targeted change (Supplementary Table S1).

To generate the null bam<sup>null-In2-3xP3-DsRed</sup> allele, we amplified 1.5 kb homology arms from the w<sup>1118</sup> isogenic line and cloned them into the pHd-attp-DsRed vector from flyCRISPR (Supplementary 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.

**Injections**
All CRISPR/Cas9 injections were sourced to Genetivision and were done in the w<sup>1118</sup> isogenic line. The injection mix contained plasmid or ssODN donor, sgRNAs, Cas9 protein (Synthego), and an siRNA for Lg4 (IDT DNA, Supplementary Table S1).

For the bam<sup>null-In2-3xP3-DsRed</sup> allele, we screened for the eye color cassette in F1’s in house using a Nightsea fluorescent system (DsRed filters). For the bam<sup>L255F</sup> allele, the edited nucleotide change disrupts an AflII restriction site, allowing us to screen F1’s by PCR (Promega GoTaq mastermix) followed by restriction digests with AflII (NEB). We prepared genomic DNA using the Qiagen PureGene kit.

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

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

**Immunostaining**
We performed immunostaining as described in Aruna et al. (2009) and 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 2 h then washed and mounted in ProLong Glass NucBlue for imaging (Thermo Fisher). We used an anti-Vasa antibody from Santa Cruz Biotechnology at 1:200 (Cat# sc-30210, RRID: AB_793874, anti-rabbit) and a goat anti-rabbit secondary antibody from Thermo Fisher Scientific at 1:500 (Alexa Fluor Plus 488, Cat# A32731, RRID: AB_2633280). 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).

**Assays for nurse cell positive egg chambers**
We used the same assay we previously described to assess the rescue of the original bam<sup>BW</sup>/bam<sup>A59</sup> hypomorphic cytological phenotype by W. pipientis (Flores et al. 2015). We dissected ovaries from mated 2-4 days old bam<sup>L255F</sup>/bam<sup>null-In2-3xP3-DsRed</sup> females for all W. pipientis variants and the uninfected control in PBS. Ovaries were fixed in 4% paraformaldehyde (EMS), washed with PBST (1X PBS, 0.1% Triton-X 100), and then mounted in ProLong Glass with NucBlue (Thermo Fisher). We imaged ovaries on a Zeiss i880 confocal microscope with a 405 nm laser line at 10X (C-Apochromat 0.45 NA, water) and 40X (described above). We analyzed and edited images using Fiji (ImageJ). Developing ovaries consist of cysts containing differentiated nurse cells which are polyploid and feature large, easily identifiable nuclei. In contrast, GSC daughter differentiation is blocked in bam loss-of-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<sup>1118</sup> isogenic bam genotypes for the fertility assays: w<sup>1118</sup>, bam<sup>*</sup>/bam<sup>*</sup>, w<sup>1118</sup>, bam<sup>L255F</sup>/bam<sup>null-In2-3xP3-DsRed</sup> and w<sup>1118</sup>, bam<sup>L255F</sup>/bam<sup>L255F</sup>. In addition, we performed a small control fertility assay using combinations of the alleles described above and the classic bam<sup>BW</sup> hypomorph and bam<sup>A59</sup> alleles.

We performed the fertility assays for w<sup>1118</sup>, bam<sup>*</sup>/bam<sup>*</sup> and w<sup>1118</sup>, bam<sup>L255F</sup>/bam<sup>null-In2-3xP3-DsRed</sup> 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<sup>BW</sup> and bam<sup>A59</sup> analysis) as follows (described by Flores et al. 2015):
We collected virgin females and aged them 2-3 days, only using flies that eclosed within 48 h 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 was allowed to mate for 8 days, and then flipped to new vials. After the second 8 days, the trio was cleared. The progeny for each trio was counted every other day to get the total adult progeny per female. Progeny per female is reported in increments by day that reflect the days after the first progeny eclosed. For example, we report total progeny for days 1–9 which are the progeny counted on day 1 of eclosion to day 9 of eclosion.

For the control fertility assay containing the bam<sup>BW</sup> hypomorph and bam<sup>A59</sup> alleles, we collected and aged flies as described above, except the progeny per trio was counted only once at the end of the experiment.

**Egg laying assay**

We collected virgin females for each genotype and *W. pipientis* variant and CantonS virgin males and aged them as described above for the fertility assays. We allowed each trio to mate for 24 h on grape juice agar supplemented with yeast before flipping them to a new grape juice agar vial. We counted the eggs laid within 24 h and repeated this for 3 days.

**Statistics**

For the nurse cell assay, egg laying assay, and fertility assays we used estimation statistics to assess the mean difference (effect size) of nurse cells, eggs, and adult progeny between infected and uninfected lines. All counts for nurse cells, eggs, and progeny from these assays are reported in Supplementary File S1. All estimation statistics were done using the dabest package in Python (v. 0.3.1) with 5000 bootstrap resamples (Ho et al. 2019). Estimation statistics provide a nonparametric alternative to other statistical tests of the difference of the mean (e.g., ANOVA), and allow us to understand the magnitude of the effect of *W. pipientis* variation on the bam phenotype. We display the data with a swarm plot that shows all of the data points and either a Cumming estimation plot (for more than two sample comparisons) or a Gardner-Altman plot (two sample comparisons) 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.

**Results**

bam<sup>L255F</sup> is a hypomorphic bam allele

We used CRISPR/Cas9 to recreate the same bam hypomorphic mutation as the original bam<sup>BW</sup> allele (Ohlstein et al. 2000) and a new bam null allele in the isogenic *w<sup>1118</sup>* background (Figure 1). Therefore, we have two new bam mutant alleles, bam<sup>L255F</sup> and bam<sup>null-In2-3xP3-DsRed</sup> in the same genetic background so we can compare wildtype bam fertility, the transheterozygous hypomorph/null fertility (as we did previously with bam<sup>BW/bam</sup><sup>A59</sup>) as well as assess the phenotype of the homozygous bam hypomorphic genotype which has never been assessed before.

We evaluated if the *w<sup>1118</sup>*, bam<sup>L255F</sup> allele recapitulated the bam<sup>BW/bam</sup><sup>A59</sup> mutant phenotypes by assessing the phenotype of a transheterozygous *w<sup>1118</sup>*; bam<sup>L255F</sup> 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<sup>1118</sup>* 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<sup>1118</sup>* background marked with a trackable eye marker (*w<sup>1118</sup>*; bam<sup>null-In2-3xP3-DsRed</sup>, Figure 1B). Females homozygous for this bam<sup/null-In2-3xP3-DsRed</sup> allele exhibit the expected tumorous ovary phenotype with no developing nurse cell-positive cysts and are sterile (Figure 2A, fertility data not shown).

We then assessed the phenotype of the bam<sup>L255F</sup> allele as a transheterozygous mutant over our new bam<sup)null-In2-3xP3-DsRed</sup> in the same *w<sup>1118</sup>* isogenic background. We observed a large fertility defect, with some sterile and some weakly fertile females with only 1–5 total progeny (Figure 3). The bam<sup>L255F/bam</sup><sup>null-In2-3xP3-DsRed</sup> ovaries exhibited the expected tumorous ovary phenotype, with the presence of some differentiating, nurse cell positive cysts indicating that bam is partially functional, and thus that this single amino acid change fully explains the original bam<sup>BW</sup> hypomorphic phenotype (Figure 2B). To further verify the nature of the bam<sup>L255F</sup> allele, we assessed its phenotype over one of the classic bam null alleles that we previously used to study the interaction between bam and *W. pipientis*, bam<sup>A59</sup>. The bam<sup>A59</sup> null allele is a nearly full deletion of the bam coding sequence. We examined ovaries from bam<sup>L255F/bam</sup><sup>A59</sup> females and observed the tumorous ovary phenotype with some developing cysts as has been previously documented for bam<sup>BW/bam</sup><sup>A59</sup> and additionally shown here (Figure 2, C and D). We measured the fertility of bam<sup>L255F/bam</sup><sup>A59</sup> females and observed a large fertility defect, consistent with the fertility of the bam<sup>L255F/bam</sup><sup>null-In2-3xP3-DsRed</sup> genotype (Figure 3). Therefore, the bam<sup>L255F/bam</sup><sup>null-In2-3xP3-DsRed</sup> cytological and fertility phenotypes are consistent with the previously documented bam hypomorph phenotypes.

As the bam<sup>BW</sup> allele is homozygous lethal due to accumulated recessive lethal mutations on the third chromosome, we used our bam<sup>L255F</sup> allele to ask if the homozygous bam<sup>L255F</sup> females also had a hypomorphic phenotype. We asked if bam<sup>L255F/bam</sup><sup>L255F</sup> females also exhibited the classic bag of marbles cytological phenotype and reduced fertility. In contrast to the tumorous ovary phenotype of bam<sup>BW/bam</sup><sup>A59</sup> and bam<sup>L255F/bam</sup><sup>null-In2-3xP3-DsRed</sup>, a
result of a defect in bam’s differentiation function which causes GSC-like cells to over-proliferate, we found no evidence of tumorous cysts in ovaries of bamL255F/bamL255F females (Figure 2F). These ovaries resembled those of a wildtype bam background, with all developing egg chambers featuring nurse cells (Figure 2E). Previously we were unable to compare the fertility of the bamL255F hypomorph to wildtype bam fertility as fertility is greatly affected by different genetic backgrounds. We compared the fertility of the w1118, bamL255F/bamL255F females to w1118, bam+/bam+ females and found that the mean difference in total progeny per female between the two genotypes was not significantly different (Figure 2G). While we did not measure all aspects of bam function of this genotype, the fertility and cytological data here indicate that the bamL255F/bamL255F genotype does not cause a severe bam mutant phenotype, as we observed previously for the transheterozygous bamL255F/bamL255F hypomorphic (bamL255F/bamA59) genotype. Therefore, two copies of the bamL255F allele are sufficient for GSC 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 bamL255F hypomorphic allele over the classic bamL255F hypomorphic allele also show similar fertility to wildtype females as we observed for bamL255F/bamL255F females. We observed that bamL255F/bamL255F females and homozygous bamL255F females did not show significantly different fertility from w1118, bam+/bam+ females (Figure 3). Further, we asked if our new bamL255F/bamL255F genotype showed a similar fertility defect to bamL255F/bamA59 (classic null) and found that both showed severely reduced fertility compared to wildtype (Figure 3). These data further confirm that our new bamL255F and bamL255F/bamL255F alleles behave similarly to alleles we have used in the past to study the interaction between bam and W. pipientis.

Since we were able to successfully recapitulate the partial-loss of function bam cytological and fertility phenotypes with the bamL255F/bamL255F genotype as we have previously described (Flores et al. 2015), but we did not observe the bam partial loss of function ovarian cytological phenotype in the bamL255F/bamL255F females, we focused primarily on the effect of W. pipientis variation on the bamL255F/bamL255F female fertility and cytological phenotypes.

For convenience and readability, we will frequently refer to the w1118, bamnull-In2-3xP3-DsRed allele as bamnull and the w1118, bamL255F allele as bamL255F.

**Infection with the 10 different wMel variants we assayed does not increase w1118, bam+/bam+ isofemale fertility**

First, to determine if the wMel bam hypomorph rescue is a consequence of a general increase in female fertility induced by
W. pipientis, we assessed the effect of W. pipientis variation on the fertility of w^{1118}, bam*/bam^+ females of which we have lines infected with 10 different wMel variants of W. pipientis as described by Chrostek et al. (2013) (Figure 4A).

Here, fertility measured over multiple days reflects progeny produced cumulatively by the female over time, although there could also be variation in development time of the progeny. We first assessed the effect of W. pipientis variation on wildtype bam female fertility (w^{1118}, bam*/bam^+). Over 17 days, we counted the progeny produced by each test female infected individually for each of the 10 W. pipientis variants and assessed the mean difference of adult progeny from uninfected control females. We found that all of the W. pipientis variants increased female fertility in the wildtype bam background, and some lines showed a significant decrease in total progeny per female across all days measured (wMelPop8X, and wMelCS2b_60) (Figure 4, B and C). Of the lines that showed a negative impact of W. pipientis on fertility, this effect was only significant as the female fly aged. On days 1–9 of progeny eclosion, no W. pipientis variant had a significantly negative effect on progeny counts, which is notable as these days represent progeny from eggs laid earlier in the test female’s life and thus are more likely to reflect effects of W. pipientis variation on fertility in nature (Figure 4B). The variants that showed a significant and large negative effect of W. pipientis infection on fertility were both in the wMelCS clade (mean difference between −32 and −46 progeny per female for days 1–17 Figure 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).

**Figure 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, bootstrap 95% confidence interval, effect size), as do bam^{L255F}/bam^{W1118} females (mean difference = −56.7, bootstrap 95% confidence interval, effect size).

All wMel variants rescue bam hypomorphic 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 (Figure 4). 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 10 W. pipientis variants. Females of the bam^{L255F}/bam^{null} genotype exhibit tumors ovaries and are only weakly fertile (Figures 2B and 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 (Figure 5, A and 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, and wMelPop8X) (Figure 5, A and 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 (Figure 5, A and 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, and wMelCS2b) there were outlier females who had large rescue of fertility, some in the range of what we observe for wildtype bam fertility (Figure 4, B and C). However, for most of the individuals tested, fertility was not fully rescued, e.g., restored to wildtype levels (Figures 4, B and C; 5, A and 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 hypomorphic 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 (Figure 6A). Properly developing egg chambers contain polyploid nurse cells, which feature large,
easily identifiable nuclei (Figure 6A, arrowheads). We counted the number of nurse cell positive egg chambers as a measure of GSC daughter differentiation and thus \( \text{bam} \) function. We found that all of the \( \text{W. pipientis} \) variants tested significantly increased the number of nurse cell positive egg chambers, and that \( \text{W. pipientis} \) variants in the \( \text{wMelCS-like} \) clade showed highest numbers of nurse cell positive egg chambers (Figure 7B). We then pooled together the data individually for \( \text{wMel-like} \), \( \text{wMel2-like} \), and \( \text{wMelCS-like} \) and asked if there was a difference in mean number of cysts containing nurse cells per ovary between each clade (Figure 6, C and D). We found that \( \text{wMelCS-like} \) variants had a significantly higher effect size than \( \text{wMel-like} \) and \( \text{wMel2-like} \) (Figure 6, C and D), and that \( \text{wMel2-like} \) had a higher effect than \( \text{wMel-like} \) (Figure 6E). These results are consistent with the fertility assay results for the \( \text{bam}^{1255F}/\text{bam}^{+} \) hypomorph genotype, however we observed a more obvious trend of higher \( \text{bam} \) rescue by the \( \text{wMelCS-like} \) variants in this assay. All of the \( \text{W. pipientis} \) variants with the highest effect size of increased \( \text{bam} \) function are in the \( \text{wMelCS-like} \) 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 \( \text{W. pipientis} \) impacts the \( \text{bam} \) 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 \( \text{W. pipientis} \) variants of high and low rescue and counted the total number of eggs laid per female over days 1–3. In \( \text{w1118}, \text{bam}^{+}/\text{bam}^{+} \) females, neither \( \text{W. pipientis} \) variant affected the number of eggs laid per female (Supplementary Figure S1A). In contrast to the \( \text{w1118}, \text{bam}^{+}/\text{bam}^{+} \) females, neither \( \text{W. pipientis} \) variant affected the number of eggs laid per female over days 1–17 post eclosion (Supplementary Figure S1A). In contrast to the \( \text{w1118}, \text{bam}^{+}/\text{bam}^{+} \) females, neither \( \text{W. pipientis} \) variant affected the number of eggs laid per female over days 1–17 post eclosion. As the fertility assays were performed in batches, each batch is compared to its own uninfected control. No \( \text{W. pipientis} \) variant was associated with a significant difference in fertility for wildtype \( \text{bam} \) 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 \( \text{W. pipientis} \) 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. \( \text{W. pipientis} \) variants \( \text{wMelPop8X} \) and \( \text{wMelCS2b} \) 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).
Figure 5 Total progeny per female and mean difference of progeny per female for the w1118; bam\textsuperscript{L255F}/bam\textsuperscript{null} genotype infected with 10 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.
by the lower rescuing W. pipientis wMel59, and the highest number of eggs laid by females infected with the high rescuing line, wMelCSa_66 (Supplementary Figure S1B).

Homozygous bam\textsuperscript{L255F} females exhibit more variable fertility which is not negatively affected by W. pipientis

In light of the variable effect of infection by some W. pipientis variants on wildtype bam fertility after day 9, and that the uninfected bam\textsuperscript{L255F}/bam\textsuperscript{L255F} females did not exhibit a statistically significant fertility defect (Figures 2G; 4, B and C), we asked if infection by a subset of the W. pipientis variants we assessed in the wildtype bam background had any effect on the fertility of bam\textsuperscript{L255F}/bam\textsuperscript{L255F} females. We chose three W. pipientis variants that did not have a significant effect on wildtype bam female fertility for the entire time frame (wMel2a_63, wMelCSa_66, and wMelCSb_577 Figure 7, A and B), and one that had a significant negative effect on female fertility in the second half of the experiment (wMelCS2b, Figure 7B). We measured fertility of homozygous females infected with W. pipientis variants as previously described. In both bam\textsuperscript{-}/bam\textsuperscript{-} and bam\textsuperscript{L255F}/bam\textsuperscript{L255F} backgrounds, no wMel variants affected fertility during the first half of the experiment (Figures 4B and 7A). However, in contrast to the bam\textsuperscript{-}/bam\textsuperscript{-} background where wMelCS2b negatively affected female fertility across all days measured, we found that in the bam\textsuperscript{L255F}/bam\textsuperscript{L255F} background wMelCS2b did not have a significant effect on female fertility (Figure 7B). In addition, in contrast to our findings in the bam\textsuperscript{-}/bam\textsuperscript{-} background that wMel2a_63...
Overall, we observed a more positive effect of *W. pipientis* on female fertility in the bam$^{255F}$/bam$^{255F}$ background than in the bam$^+$/bam$^+$ background. As the differential effects of *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$^{255F}$/bam$^{255F}$ 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. In addition, 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$^{255F}$ females (Figure 7A). We also observed that the total progeny per female of the homozygous bam$^{255F}$/bam$^{255F}$ genotype had a wider range than wildtype, which was not impacted by *W. pipientis* and is therefore likely a subtle bam mutant phenotype (Figure 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 uMel rescued the fertility defect of bam$^{RW}$/bam$^{AS9}$ 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. However, in order to understand if and how this interaction could be adaptive, we need to understand the nature of the manipulation of bam function by *W. pipientis*. Second, *W. pipientis* is of immense interest as a potential means to control vectors of some human diseases (Moreira et al. 2009).

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

Chrostek et al. (2013) generated isogenic *Drosophila* lines infected with previously described and genetically distinct uMel variants of *W. pipientis*. The original bam$^{RW}$ mutant was not in this host genetic background and thus we would not be able to conclude that any differential interaction between bam and these variants was due to *W. pipientis* variation and not host genetic variation. Therefore, we used CRISPR/Cas9 to create the same amino acid mutant present in the original bam$^{RW}$ hypomorph allele in the w$^{118}$ isogenic background. To ensure we had the tools to assess the *W. pipientis* rescue phenotype as previously

![Figure 7](image-url)
described, we also generated a new bam null allele using CRISPR/Cas9 in the same w^{118} genetic background.

We have now confirmed that these two new alleles behave similarly to the alleles we previously used to document the interaction between W. pipientis 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^{RW/bam^{AS9}} 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 (Figures 5 and 6). For the bam^{RW/bam^{AS9}} 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 ovariole 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^{RW/bam^{AS9}} 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, 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.

**wMel W. pipientis variants do not broadly increase fertility in the w^{118} genetic background**

Due to the shared w^{118} 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 misregulating 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). In addition, Sergy 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.

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

We previously showed that a single wMel variant partially rescued the fertility and GSC daughter differentiation defect of the bam^{RW/bam^{AS9}} hypomorph. Here, we asked if there was variation in the bam rescue phenotype among W. pipientis variants. The 10 variants we used have been fully sequenced and differ by varying degrees of sequence divergence. All 10 W. pipientis variants of the three clades tested (III, VI, and VIII; see Figure 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 (Figures 5 and 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.

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 highest levels of viral resistance to D. melanogaster males (Chrostek et al. 2013). In addition, wMelCS-like
variants have a stronger effect on the thermal preference of the *D. melanogaster* host, with uMelCS-like variants conferring preference for cooler temperatures compared to uMel-like and uninfection *D. melanogaster* (Truitt et al. 2019). Another study showed that uMelCS-like variants increased stress resistance in *D. melanogaster*, and no observed effect from uMel-like variants (Gruntenko et al. 2017). uMelPop is a virulent derivative of uMelCS and characterized by uncontrolled proliferation and titer caused by increased copy number of the Octomom locus. uMelPop2X and uMelPop8X exhibit 2X copies of the Octomom locus and anywhere from 4X to 8X copies, respectively (Chrostek and Teixeira 2018). The high rescue of both the cytological and fertility defect of the *bam*<sup>−255F</sup>*bamnull* by uMelPop2X is suggestive that titer and possibly other phenotypes that increase interactions with the host are contributing to the rescue of *bam*. We observed lower rescue for uMelPop8X, indicating that the increasing copy number of the Octomom locus that causes apoplosis and early death likely negatively affects host fertility (Zhukova and Kiseleva 2012; Chrostek and Teixeira 2018). uMelPop2X is closely related to uMelCSb, with the only variation between them being a synonymous SNP and the amplification of the octomom locus which contains *W. pipientis* loci predicted to be involved with nucleic acid binding, and thus likely how it increases its titer, as well as proteins with predicted homology to eukaryotic domains (Chrostek and Teixeira 2018). These loci may then increase the interaction of *W. pipientis* and its host (López-Madrigal and Duarte 2020). A natural next step would be to determine the *W. pipientis* factors that manipulate GSC daughter differentiation.

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

It is well established that disrupting *bam* function negatively affects fertility (McKearin and Spradling 1990; Ohlstein and McKearin 1997; Lavioie et al. 1999; Flores et al. 2015), and *bam<sup>−255F</sup>bamnull* females are almost completely sterile. However, we do not predict such deleterious alleles to reach high frequency in natural populations. In fact, we have not found this nucleotide variant segregating in any of the natural populations of *D. melanogaster* that have been sampled in the Drosophila genome Nexus (Lack et al. 2015). Thus, while studying this mutant is effective in further refining how *bam* and *W. pipientis* interact, we cannot conclude that this type of interaction occurs in natural populations. However, given the severity of the mutant and strength of *W. pipientis*’s rescue, one hypothesis is that if *W. pipientis* is increasing GSC daughter differentiation when it is not favorable for the host, the host would evolve a way to evade *W. pipientis*’s manipulation of this pathway. In the case of our lab generated mutant, it is possible that when *bam* is not fully functional that reproduction is more sensitive to manipulation by *W. pipientis*. *W. pipientis* have been documented to respond to changes in the host environment, as *W. pipientis* gene expression is affected by host age and sex, and as *W. pipientis* transmission requires functional oogenesis, it is reasonable to hypothesize that *W. pipientis* is sensitive to changes in gametogenesis (Rice et al. 2017; Newton and Sheehan 2018; Russell et al. 2020).

An additional observation we made is that *bam<sup>−255F</sup>bam<sup>−255F</sup>* females show a broader range of adult progeny per female compared to wildtype *bam* females regardless of *W. pipientis* infection status. The individual *bam<sup>−255F</sup>bam<sup>−255F</sup>* females exhibit both higher and lower than average fertility, indicating that misregulating *bam*’s differentiation function could both increase and decrease fertility. This phenotype is worth further investigation, since although *bam* shows a signature of positive selection, we do not know specifically what aspect of *bam* function is adaptive. If variation in *bam* function can both increase and decrease mean fertility, and *W. pipientis* has a generally positive effect on fertility of *bam<sup>−255F</sup>bam<sup>−255F</sup>* females, this is further evidence that *W. pipientis* may be able to manipulate *bam* in order to increase oogenesis for its own benefit, and that genetic variation at *bam* could affect the regulation of oogenesis. Therefore, if *W. pipientis* increased the rate of oogenesis to ensure its own transmission and this was not favorable for the host, *bam* may be in conflict with *W. pipientis* to regulate oogenesis in a favorable manner for the host. Here we see that although the average female fertility of uninfected *bam<sup>−255F</sup>bam<sup>−255F</sup>* mutants is not significantly different from wildtype, there is a wider distribution of individual female fertility, indicating that the *bam* hypomorphic phenotype is an increased variance in fertility (Figure 7). Since these females are in a common genetic background and we do not expect genetic variation between individual females, the *bam*-mediated mis-regulation of differentiation may set off a cascade of other genetic mis-regulation that results in higher or lower fertility over time. We see that infection by some *W. pipientis* variants in this background increases female fertility, indicating that perhaps *W. pipientis* can restore the mis-regulation of GSC daughter differentiation, and even increase reproductive output through this mechanism. It would be interesting to use this genotype for future experiments to explore the possibility that this *bam* mutation may disrupt the mechanism *D. melanogaster* evolved to evade *W. pipientis*’s effect on differentiation.

An interesting question that remains is how *W. pipientis* have evolved in their interaction with GSC genes, including *bam*. While uMelCS-like and uMel-like variants share a most recent common ancestor about 8000 years ago, uMelCS-like variants were recently (~2000 years ago) replaced by uMel-like variants in natural populations of *D. melanogaster* (Riegler et al. 2005; Richardson et al. 2012). However, this replacement has not been complete, and there are global populations still infected with uMelCS variants (Riegler et al. 2005; Nunes et al. 2008). Interestingly we find that the uMelCS variants we assayed show higher rescue of *bam<sup>−255F</sup>bamnull* female fertility and nurse cells. If *bam* and *W. pipientis* are evolving in an arms race, it could be that the evolutionarily more recent uMel-like variants have not evolved the same level of interaction with *bam* as uMelCS has. Future work to investigate these dynamics should include sampling populations that are still infected with uMelCS as well as those infected with uMel and asking if there is any evidence of genetic differentiation at *bam*. Some populations have been identified that are still infected with uMelCS, such as a natural population of *D. melanogaster* from Uman, Ukraine that has been infected with both uMel and uMelCS and has been monitored yearly for infection frequency, some additional Paleartic populations, and a population in the Netherlands (Early and Clark 2013; Bykov et al. 2019; Serga et al. 2021).

In addition, we could utilize existing *W. pipientis* sequence variation from natural populations to ask if there is any evidence of associations between *bam* variation and *W. pipientis* variation. If we were to sample populations that are differentially infected with uMel and uMelCS to assess genetic differentiation at *bam* as discussed above, we could also do the same for *W. pipientis* variants. One caveat being that if we did not observe genetic signatures of adaptive evolution in *W. pipientis* loci, this does not mean that *W. pipientis* and *bam* are not 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. In addition, 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. (2013), between the uMel clades and uMelCS 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 uMel variants [for example, uAu 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.

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

Supplementary material is available at G3 online.

Acknowledgments
The authors 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. They also would like to thank Kristen Rose Baxter for her contributions to our early fertility experiments. They are also grateful to Mariana F. Wolfner, Andrew G, Clark, Miwa Wenzel, and Catherine Kagemann for experiments. They are also grateful to Mariana F. Wolfner, Kristen Rose Baxter for her contributions to our early fertility experiments. They affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Funding
This work was supported by National Institutes of Health grant R01-GM095793 to C.F.A. and by the National Science Foundation Graduate Research Fellowship Program DGE-114415 to J.E.B.

Conflicts of interest
The authors declare that there is no conflict of interest.

Literature cited
Arnold PA, Levin SC, Stevanovic AL, Johnson KN. 2019. Drosophila melanogaster infected with Wolbachia strain wMelCS prefer cooler temperatures. Ecol Entomol. 44:287–290. 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. 181:1437–1450. 10.1534/genetics.108.100057 19153254.
Bailly-Bechet M, Martins-Simões P, Széllosi GJ, Mialdéa G, Sagot MF, et al. 2017. How long does Wolbachia remain on board? Mol Biol Evol. 34:1183–1193. doi:10.1093/molbev/msx073.
Bauer DuMont VL, Flores HA, Wright MH, Aquadro CF. 2007. Recurrent positive selection at lryn, a key determinant of germ line differentiation, does not appear to be driven by simple coevolution with its partner protein Bam. Mol Biol Evol. 24:182–191. doi:10.1093/molbev/msi141.
Beckmann JF, Ronau JA, Hochstrasser M. 2017. A Wolbachia debiquitylating enzyme induces cytoplasmic incompatibility. Nat Microbiol. 2:17007. doi:10.1038/s41564-017-0021-z.
Bykov RA, Yudina MA, Gruntenko NE, Zakharov IK, Voloshina MA, et al. 2019. Prevalence and genetic diversity of Wolbachia endosymbiont and mtDNA in Palearctic populations of Drosophila melanogaster. BMC Evol Biol. 19:48. 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. 31:2457–2471. doi:10.1093/molbev/msu204.
Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, et al. 2013. Wolbachia variants induce differential protection to viruses in Drosophila melanogaster: a phenotypic and phylogenomic analysis. PLoS Genet. 9:e1003896. doi:10.1371/journal.pgen.1003896.
Chrostek E, Marialva MSP, Yamada R, O’Neill SL, Teixeira L. 2014. High anti-viral protection without immune upregulation after interspecies Wolbachia transfer. PLoS One. 9:e99025. doi:10.1371/journal.pone.0099025.
Chrostek E, Teixeira L. 2018. Within host selection for faster replicating bacterial symbionts. PLoS One. 13:e0191530. doi:10.1371/journal.pone.0191530.
Dedeine F, Vavre F, Fleury F, Loppin B, Hochberg ME, et al. 2001. Removing symbiotic Wolbachia bacteria specifically inhibits oogenesis in a parasitic wasp. Proc Natl Acad Sci USA. 98: 6247–6252. doi:10.1073/pnas.1013042.
Deehan M, Lin W, Blum B, Emili A, Frydman H. 2021. Intracellular density of Wolbachia is mediated by host autophagy and the bacterial cytoplasmic incompatibility gene cifB in a cell type-dependent manner in Drosophila melanogaster. mBio. 12:1–19. doi:10.1128/mBio.02025–20.
Dutra HLC, Rocha MN, Dias FBS, Caragata EP, et al. 2016. Wolbachia blocks currently circulating zika virus isolates in Brazilian Aedes aegypti mosquitoes. Cell Host Microbe. 19: 771–774. doi:10.1016/j.chom.2016.04.021.
Early AM, Clark AG. 2013. Monophyly of Wolbachia pipientis genomes within Drosophila melanogaster: geographic structuring, titre variation and host effects across five populations. Mol Ecol. 22: 5765–5778. doi:10.1111/mec.12530.
Flores HA, Bubnell JE, Aquadro CF, Barbash DA. 2015. The Drosophila bag of marbles gene interacts genetically with Wolbachia and shows female-specific effects of divergence. PLoS Genet. 11:e1005453. doi:10.1371/journal.pgen.1005453.
Gratz SJ, Ukken FP, Rubinstein CD, Thiede G, Donohue KL, et al. 2014. Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in Drosophila. Genetics. 196:961–971. doi:10.1534/genetics.113.160713.
Gruntenko NE, Ilinsky YY, Adonyeva NV, Burdina EV, Bykov RA, et al. 2017. Various Wolbachia genotypes differently influence host Drosophila dopamine metabolism and survival under heat stress conditions. BMC Evol Biol. 17:252. doi:10.1186/s12862-017-0110-y.
Gruntenko NE, Karpova EK, Adonyeva NV, Andreekova OV, Burdina EV, Ilinsky YY, et al. 2019. Drosophila female fertility and juvenile hormone metabolism depends on the type of Wolbachia infection. J Exp Biol. 222:1e195347. doi:10.1242/jeb.195347.
Hague MTJ, Caldwell CN, Cooper BS. 2020. Pervasive effects of Wolbachia on host temperature preference. MBio. 11:e01768-20. doi:10.1128/mBio.01768-20.
Ho J, Tumkaya T, Aryal S, Choi H, Claridge-Chang A. 2019. Moving beyond P values: data analysis with estimation graphics. Nat Methods. 16:565–566. doi:10.1038/s41592-019-0470-3.

Hoffmann AA, Montgomery BL, Popovic J, Iturbe-Ormaetxe I, Johnson PH, et al. 2011. Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. Nature. 476:454–457. doi:10.1038/nature10356.

Inasco ML, Bailey AS, Kim J, Olivares GH, Wapinski OL, et al. 2012. A self-limiting switch based on translational control regulates the transition from proliferation to differentiation in an adult stem cell lineage. Cell Stem Cell. 11:689–700. doi:10.1016/j.stem.2012.08.012.

Lack JB, Cardeno CM, Crepeau MW, Taylor W, Corbett-Detig RB, et al. 2015. The Drosophila genome nexus: a population genomic resource of 623 Drosophila melanogaster genomes, including 197 from a single ancestral range population. Genetics. 1229–1241. doi:10.1534/genetics.111.174664.

Lavoie CA, Ohlstein B, Mckearin DM. 1999. Localization and function of Barn protein require the benign gonial cell neoplasm gene product. Dev Biol. 405:413. doi:10.1016/diabio.1999.9346.

Lindsey ARI. 2020. Sensing, signaling, and secretion: a review and update21391. Nat Biotechnol. 16:565–566. doi:10.1038/s41592-019-0470-3.

Lavoie CA, Metcalf JA, Bordenstein SR, On J, Perlmutter JI, et al. 2017. Prophage WO genes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility. Nature. 543:243–247. doi:10.1038/nature23191.

Rice DW, Sheehan KB, Newton ILG. 2017. Large-scale identification of Wolbachia pipientis effectors. Genome Biol Evol. 9:1925–1937. doi:10.1093/gbe/evx139.

Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, et al. 2012. Population genomics of the Wolbachia endosymbiont in Drosophila melanogaster. PLoS Genet. 8:e1003129. doi:10.1371/journal.pgen.1003129.

Riegler M, Sidhu M, Miller WJ, O’Neill SL. 2005. Evidence for a global Wolbachia replacement in Drosophila melanogaster. Curr Biol. 15:1428–1433. doi:10.1016/j.cub.2005.06.069.

Russell SL, Ruelas Cj, Russell SL, Castillo JR. 2020. Chapter 5 trends in symbiont-induced host cellular differentiation. Results Probl Cell Differ. 69:137–176. doi:10.1007/978-3-030-51849-3_5.

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

Serga SV, Maistrenko OM, Rozhok A, Mousseau T, Kozerskia IA. 2014. Fecundity as one of possible factors contributing to the dominance of the wMel genotype of Wolbachia in natural populations of Drosophila melanogaster. Symbiosis. 63:11–17. doi:10.1007/s13199-014-0283-1.

Serga SV, Maistrenko OM, Matityvs NV, Vaiserman AM, Kozerskia IA. 2021. Effects of Wolbachia infection on fitness-related traits in Drosophila melanogaster. Symbiosis. 83:163–172. doi:10.1007/s13199-020-00743-3.

Shrophshire, JD Orj Layton, EM Zhou, H Bordenstein, SR. 2018. One prophage WO gene rescues cytoplasmic incompatibility in Drosophila melanogaster. Proc Natl Acad Sci USA. 115:4987–4991. doi:10.1073/pnas.1800650115.

Shrophshire JD, Rosenberg R, Bordenstein SR. 2021. The impacts of cytoplasmic incompatibility factor (cifA and cifB) genetic variation on phenotypes. Genetics. 217:1–8. doi:10.1093/genetics/iyaa007.

Starr DJ, Cline TW. 2002. A host-parasite interaction rescues Drosophila oogenesis defects. Nature. 418:76–79. doi:10.1038/nature00843.

Thurmond J, Goodman JL, Strelets VB, Attrill H, Marygold SJ, et al.; FlyBase Consortium. 2019. FlyBase 2.0: the next generation. Nucleic Acids Res. 47:D759–D765. doi:10.1093/nar/gky1003.

Ting X. 2013. Control of germline stem cell self-renewal and differentiation in the Drosophila ovary: concerted actions of niche signals and intrinsic factors. Wiley Interdiscip Rev Dev Biol. 2:261–273. doi:10.1002/wdev.60.

Truitt AM, Kapun M, Kaur R, Miller WJ. 2019. Wolbachia modifies thermal preference in Drosophila melanogaster. Environ Microbiol. 21:3259–3268. doi:10.1111/1462-2920.14347.

Utarini A, Indriani C, Ahmad RA, Tantowijoyo W, Arguni E, et al. 2021, Vol. 11, No. 12. Wolbachia pipientis abundance in natural populations. PLoS Genet. 8:e1003129. doi:10.1371/journal.pgen.1003129.