



Paternal Grandmother Age Affects the Strength of *Wolbachia*-Induced Cytoplasmic Incompatibility in *Drosophila melanogaster*

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ABSTRACT *Wolbachia* are obligate intracellular bacteria that are globally distributed in half of all arthropod species. As the most abundant maternally inherited microbe in animals, *Wolbachia* manipulate host reproduction via reproductive parasitism strategies, including cytoplasmic incompatibility (CI). CI manifests as embryonic death when *Wolbachia*-modified sperm fertilize uninfected eggs but not maternally infected eggs. Thus, CI can provide a relative fitness advantage to *Wolbachia*-infected females and drive the infection through a population. In the genetic model *Drosophila melanogaster*, the *Wolbachia* strain *wMel* induces variable CI, making mechanistic studies in *D. melanogaster* cumbersome. Here, we demonstrate that sons of older paternal *D. melanogaster* grandmothers induce stronger CI than sons of younger paternal grandmothers, and we term this relationship the “paternal grandmother age effect” (PGAE). Moreover, the embryos and adult sons of older *D. melanogaster* grandmothers have higher *Wolbachia* densities, correlating with their ability to induce stronger CI. In addition, we report that *Wolbachia* density positively correlates with female age and decreases after mating, suggesting that females transmit *Wolbachia* loads that are proportional to their own titers. These findings reveal a transgenerational impact of age on *wMel*-induced CI, elucidate *Wolbachia* density dynamics in *D. melanogaster*, and provide a methodological advance to studies aimed at understanding *wMel*-induced CI in the *D. melanogaster* model.

IMPORTANCE Unidirectional cytoplasmic incompatibility (CI) results in a postfertilization incompatibility between *Wolbachia*-infected males and uninfected females. CI contributes to reproductive isolation between closely related species and is used in worldwide vector control programs to drastically lower arboviral vector population sizes or to replace populations that transmit arboviruses with those resistant to transmission. Despite decades of research on the factors that influence CI, penetrance is often variable under controlled laboratory conditions in various arthropods, suggesting that additional variables influence CI strength. Here, we demonstrate that paternal *D. melanogaster* grandmother age influences the strength of CI induced by their sons. Older *D. melanogaster* females have higher *Wolbachia* densities and produce offspring with higher *Wolbachia* densities that associate with stronger CI. This work reveals a multigenerational impact of age on CI and expands our understanding of host-*Wolbachia* interactions and the biology of CI induced by the *Wolbachia* strain infecting the most widely used arthropod model, *D. melanogaster*.

KEYWORDS *Wolbachia*, *Drosophila melanogaster*, cytoplasmic incompatibility, maternal transmission

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W*olbachia* are obligate intracellular bacteria that infect 40% to 65% of arthropod species (1–3) and 37% of the members of the Onchocercidae family of filarial nematodes (4). These bacteria are maternally transmitted from ova to offspring (5) and often cause cytoplasmic incompatibility (CI) to selfishly increase their transmission through the matriline (6–10). CI manifests as embryonic death when *Wolbachia*-modified sperm fertilize uninfected eggs but not when they fertilize infected eggs (11–13). Thus, infected transmitting females have a fitness advantage relative to their uninfected counterparts that leads to the spread of *Wolbachia* through host populations (6–10). Additionally, since CI reduces gene flow between *Wolbachia*-infected and uninfected populations or populations with different *Wolbachia* strains, it is associated with reproductive isolation and incipient speciation (14, 15).

Global vector control efforts have successfully leveraged CI to either suppress native populations (16–19) or promote the spread of disease-resistant *Wolbachia* strains (20–22) specifically through release of mosquitoes transinfected with the *wMel* *Wolbachia* strain of *Drosophila melanogaster*. *wMel*'s success in these efforts is partially due to the strong CI that it induces in mosquito hosts (23, 24); however, in the native host *D. melanogaster*, *wMel*'s CI strength can range from an average of nearly 0% (no CI) to 100% (complete CI) (25–32). There are numerous factors reported to impact the penetrance of *wMel*-induced CI: *Wolbachia* density in the testes (25, 33), expression level of the CI genes *cifA* and *cifB* (29, 34), male age (30), male mating rate (30, 35), time of male emergence (32), fly rearing density (32), and temperature (30). However, these factors are not independent, and they have likely hampered the researcher's ability to use the vast resources of *D. melanogaster* for the study of reproductive parasitism and endosymbiosis. For example, CI strength rapidly decreases with male age (30), which also cocorrelates with *cifA* and *cifB* gene expression (29) and *Wolbachia* density in the testes (33).

Despite control of male age, time of emergence, rearing density, and temperature, we continued to see various levels of CI strength in our laboratory, suggesting that additional factors are involved. This variation in phenotype makes *wMel* in *D. melanogaster* difficult to study despite the fly's extensive history as a powerful animal model. However, anecdotal observations in our laboratory suggested that stronger CI was induced in embryos when their infected paternal grandmothers were significantly aged before mating. Here, we used hatch rate analyses to formally test the hypothesis that paternal grandmother age influences the strength of CI induced by her sons. We also measured the effect of age and virginity on female *Wolbachia* titers and assessed whether females with higher *Wolbachia* titers deposited more *Wolbachia* into their progeny. Our results reveal a "paternal grandmother age effect" (PGAE) on CI strength, where older grandmothers produce males that induce stronger CI. We also characterize transgenerational *Wolbachia* density dynamics that correlate with CI penetrance. This work enhances our understanding of *Wolbachia*-host dynamics and provides methodological techniques of importance to studies of *wMel*-induced CI in *D. melanogaster*.

RESULTS

To test the hypothesis that *D. melanogaster* paternal grandmother age influences the strength of CI, we measured the percentage of surviving offspring produced by sons of differentially aged, infected y^1w^* grandmothers. CI strength increased with grandmother age when uninfected females were mated to infected sons of 2-, 5-, 11-, 14-, and 18-day-old grandmothers (Fig. 1). Sons of 2-day-old grandmothers produced statistically weaker CI than those of either 14-day-old ($P = 0.0031$) or 18-day-old ($P = 0.0005$) grandmothers, and the same was true for sons of 5-day-old grandmothers compared to those of either 14-day-old ($P = 0.0095$) or 18-day-old ($P = 0.0018$) grandmothers. Importantly, sons of 11-day-old uninfected grandmothers produced high hatch rates (Fig. 1), suggesting that the reduction in hatch rate in the remaining crosses was not associated with further aging of the flies. Together, these data suggest that CI is strongest in sons of older grandmothers (Fig. 1).

Next, we tested whether the increase in embryonic death with *D. melanogaster*

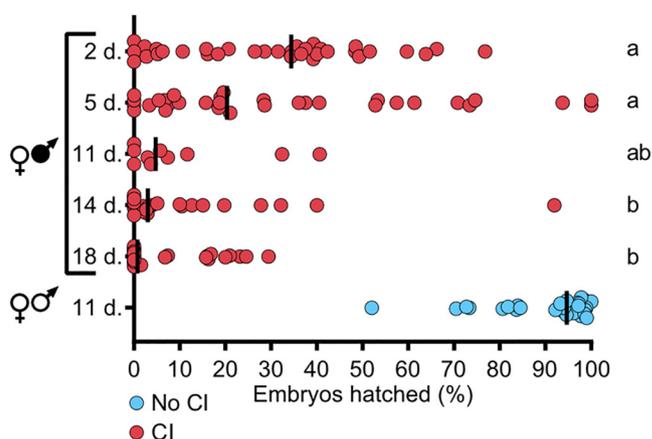


FIG 1 Paternal grandmother age effect impacts CI strength. Hatch rate assays were conducted with either uninfected y^1w^* males derived from uninfected females aged 11 days (d.) before mating or infected y^1w^* males derived from infected females aged 2, 5, 11, 14, or 18 days. *Wolbachia* infections are represented by filled sex symbols, and the age of the paternal grandmother is shown immediately to the left of the y axis. Each dot represents a replicate of offspring from single-pair matings. Vertical bars represent medians, and letters to the right indicate significant differences based on $\alpha = 0.05$ calculated by a Kruskal-Wallis test followed by a Dunn's multiple-comparison test performed between all CI crosses. All statistical values are presented in Table S1 in the supplemental material.

grandmother age indeed represented CI and not some other transgenerational embryonic defect. In accordance with prior results (Fig. 1), there was an overall trend indicating that older grandmothers produced sons that induced stronger CI. Indeed, sons derived from 2-day-old infected grandmothers induced statistically weaker CI than sons of 11-day-old ($P = 0.0008$) and 14-day-old ($P = 0.0110$) grandmothers (see Fig. S1 in the supplemental material). Sons of 11-day-old grandmothers produced a lower median hatch rate than sons of 14-day-old grandmothers; however, the differences were not statistically significant ($P > 0.9999$). As expected for CI rescue, high rates of embryonic hatching were observed when infected females were mated to sons of infected 2-, 5-, 11-, and 14-day-old grandmothers and the rates did not differ significantly between groups (Fig. S1; $P = 0.3705$). Together, these results suggest that the PGAE is not attributable to other transgenerational, age-associated defects.

To test if the PGAE is specific to the y^1w^* strain, these experiments were repeated in a *nos-GAL4-tubulin* genetic background. The *nos-GAL4-tubulin* line was chosen because it was previously used to identify the *cifA* and *cifB* genes that underpin *wMel*-induced CI (29). The 2-, 5-, and 11-day time points were selected because they had demonstrated the greatest differences in hatch rate in the previous experiments. As predicted, CI strength correlated with the age of paternal grandmothers when uninfected *nos-GAL4-tubulin* females were mated to infected sons of 2-, 5-, and 11-day-old *nos-GAL4-tubulin* grandmothers (Fig. S2). Sons of 11-day-old grandmothers induced significantly stronger CI than sons of 2-day-old grandmothers ($P = 0.0033$; Fig. S2), suggesting that the PGAE is not specific to y^1w^* flies. When sons of uninfected grandmothers aged 2, 5, or 11 days were mated to uninfected females, there were no statistically significant differences in hatching rates across all three groups ($P = 0.3907$; Fig. S2), indicating that the PGAE is CI associated in *nos-GAL4-tubulin* flies as seen with y^1w^* flies.

Since *Wolbachia* densities are positively associated with CI strength (25, 36–38), we then tested the hypothesis that infected sons derived from older *D. melanogaster* grandmothers have higher *Wolbachia* densities than infected sons from younger grandmothers. We did so by measuring the abundance of the single-copy *Wolbachia groEL* gene relative to that of the *Drosophila rp49* housekeeping gene. Abdomen samples were taken from virgin male siblings of those used in the hatch rate experiment represented in Fig. 1. As predicted, *Wolbachia* densities in male abdomens positively correlated with paternal grandmother age, and sons of 18-day-old grand-

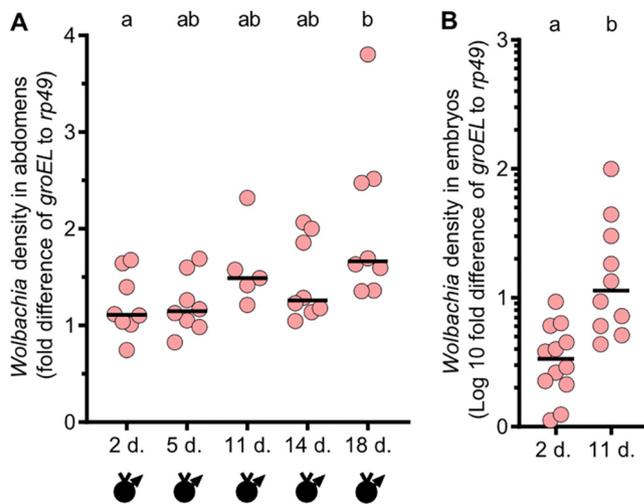


FIG 2 *Wolbachia* densities are highest in sons and embryos of older *D. melanogaster* grandmothers. (A) *Wolbachia* density assays were conducted with virgin females (indicated by a “v” above a sex symbol) and with infected $y'w^*$ males derived from grandmothers aged 2, 5, 11, 14, or 18 days (d.). *Wolbachia* infections are represented by filled sex symbols, and the age of the grandmother is shown immediately below the x axis. The samples analyzed were from abdomens of siblings of fathers corresponding to the hatch rate data in Fig. 1. (B) *Wolbachia* density assays were conducted with pools of 50 1-to-2-h-old embryos collected from 2-and-11-day-old grandmothers. The sex of the embryos was unknown since it cannot be determined visually. *Wolbachia* titers were lower in adults, requiring a standard linear scale (A), but higher in embryos, requiring a common logarithmic scale (B). Each dot represents the average of results from triplicate technical replicates for panel A and duplicates for panel B. Horizontal bars indicate medians, and the letters above the bars indicate significant differences based on $\alpha = 0.05$ calculated by Kruskal-Wallis test followed by a Dunn's multiple-comparison test performed between all groups (A) or by a Mann Whitney *U* test (B). All statistical values are presented in Table S1. Fold differences in *Wolbachia* densities (*groEL*) relative to *D. melanogaster* reference gene *rp49* were determined with $2^{-\Delta\Delta CT}$.

mothers had significantly higher *Wolbachia* densities than sons of 2-day-old grandmothers ($P = 0.0450$) (Fig. 2A). However, no significant differences were observed between sons of 5-, 11-, or 14-day-old grandmothers relative to any other group, presumably due to the variable penetrance of CI, low sample sizes, or biological reasons proposed in the Discussion. Taken together, these data suggest that older grandmothers produced sons with higher *Wolbachia* titers, which allowed the sons to induce stronger CI, though this density effect was weak relative to the effect that we see for CI.

Next, we tested the hypothesis that embryos from older *D. melanogaster* grandmothers had higher *Wolbachia* titers than those from younger grandmothers. *Wolbachia* densities were measured in 0-to-1-h-old embryos produced by both 2-day-old and 11-day-old grandmothers (Fig. 2B). The 2-day and 11-day time points were chosen because they exhibited the greatest differences in CI strength over the shortest time interval. Here, embryos produced by 11-day-old grandmothers had significantly higher *Wolbachia* densities than embryos from 2-day-old grandmothers ($P = 0.0006$) (Fig. 2B). Thus, these data indicate that older females produce embryos with higher *Wolbachia* titers.

Finally, this led to the hypothesis that older *D. melanogaster* grandmothers have higher *Wolbachia* densities than younger grandmothers and that they transfer more *Wolbachia* to their offspring. Supporting this hypothesis, *Wolbachia* densities were significantly higher in the ovaries of 11-day-old virgin females than in those of 2-day-old virgin females ($P = 0.0045$) (Fig. 3). Additionally, we predicted that *Wolbachia* densities would decrease in ovaries after egg-laying if grandmothers loaded *Wolbachia* into their offspring. As such, we measured *Wolbachia* densities in ovaries of mated grandmothers that laid eggs in the embryo density study described previously. We found that ovaries from mated 11-day-old females had significantly less *Wolbachia* than virgin 11-day-old females ($P = 0.0240$) (Fig. 3). Likewise, mated 2-day-old females had

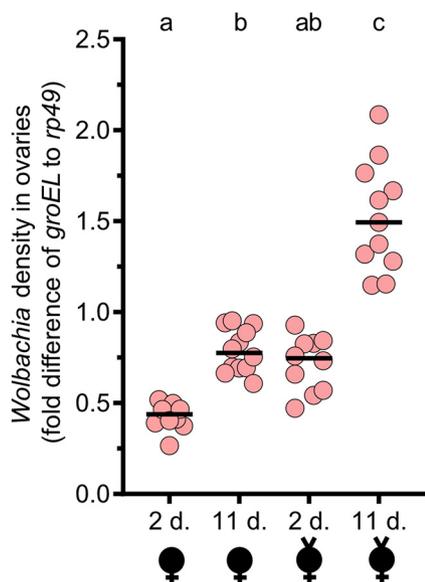


FIG 3 *Wolbachia* densities increase with female age in ovaries and decrease after mating. *Wolbachia* density assays were conducted with pools of 4 ovaries from virgin females (indicated by a “v” above a sex symbol) and nonvirgin females aged 2 or 11 days (d.). *Wolbachia* infections are represented by filled sex symbols, and the age of the sample is shown immediately below the x axis. Virgin and nonvirgin females were siblings. The nonvirgin females produced the embryos whose results are shown in Fig. 2B. Nonvirgin females were allowed to mate and lay for 48 h before ovary dissections. Nonvirgin and virgin females were incubated for that same period of time and dissected in parallel. Each dot represents the average of duplicate values. Horizontal bars indicate medians, and the letters above the bars indicate significant differences based on $\alpha = 0.05$ calculated by a Kruskal-Wallis test followed by a Dunn’s multiple-comparison test performed between all groups. All statistical values are presented in Table S1. Fold differences in *Wolbachia* density (*groEL*) relative to *D. melanogaster* reference gene *rp49* were determined with $2^{-\Delta\Delta CT}$.

lower *Wolbachia* titers than virgin 2-day-old females, though the differences were not significant ($P = 0.0882$) (Fig. 3). Despite the overall decrease in relative *Wolbachia* densities after mating, ovaries from 11-day-old mated grandmothers had significantly higher densities than ovaries from 2-day-old mated grandmothers ($P = 0.0087$). Importantly, threshold cycle (C_T) values remained consistent across age and virginity states for the *Drosophila rp49* gene, suggesting that changes in the *Wolbachia groEL* gene, rather than in *rp49* copy number, were responsible for the density dynamics that we report here (Fig. S3).

Similar results can be observed in measuring *Wolbachia* densities in abdomens instead of in ovaries (Fig. S4). Measuring abdominal titers, 11-day-old virgin females had statistically higher *Wolbachia* densities than 2-day-old virgin females ($P < 0.0001$). There was a detectable trend indicating that the mated females had less *Wolbachia*, though neither mated 11-day-old females ($P = 0.2291$) nor mated 2-day-old females ($P > 0.9999$) had titers significantly different from those of their virgin counterparts (Fig. S4). The titers in 11-day-old and 2-day-old mated females were not significantly different ($P > 0.9999$). Taken together, these data suggest that females accumulate *Wolbachia* as they age, that older females transfer more *Wolbachia* to their offspring, and that sons of older females induce stronger CI. Moreover, laying eggs appears to quickly reduce the amount of *Wolbachia* contained in the ovaries, suggesting that the PGAE is strongest soon after initial mating.

DISCUSSION

D. melanogaster is a valued model system in studies of *Wolbachia*-host interactions due to its genetic tractability and the importance of its native *Wolbachia* strain, *wMel*, in vector control (39). However, the study of *wMel*-induced CI in *D. melanogaster* is inhibited by its variable penetrance, ranging from nearly complete embryonic death to

none at all (26–32). Some phenotypic variation persists despite control of known variables of CI strength, leading to the hypothesis that as-yet-unknown factors contribute to CI variability. Anecdotal observations in our laboratory suggested that stronger CI may be induced by offspring of older virgin females, leading to the formal hypothesis that variation in CI penetrance is partly controlled by a paternal grandmother age effect (PGAE).

Here, we report evidence in support of the PGAE, namely, that sons of older *D. melanogaster* grandmothers induce stronger CI than sons of younger grandmothers. Paternal grandmother age did not influence the ability of CI to be rescued, suggesting that no other age-associated transgenerational deficiencies contributed to the increased embryonic death. Additionally, we found that embryos of older grandmothers had higher *Wolbachia* densities than the offspring of younger grandmothers. Likewise, older virgin females had more *Wolbachia* than younger virgin females. As such, the data support a model whereby PGAE is caused by an accumulation of *Wolbachia* in a virgin as she ages, leading to an increase in levels of *Wolbachia* passed on to her sons, who induce stronger CI in their offspring than sons of younger grandmothers.

In this study, we measured *Wolbachia* densities by comparing the number of *Wolbachia groEL* gene copies to the number of *Drosophila rp49* gene copies. Note that we cannot make direct claims about the density of *Wolbachia* per host cell based on these analyses, since doing so would assume that the number of host cells and host ploidy remain constant. Recent work has highlighted that a protein-enriched diet can influence relative estimates of *Wolbachia* density analysis in *D. melanogaster* by increasing ovary size and *rp49* copy number (40). While age and mating state may be hypothesized to influence *rp49* copy number, *rp49* C_T values remained constant across female age and mating states whereas *groEL* C_T values changed (see Fig. S3 in the supplemental material). These data suggest that despite possible fluctuations in *rp49* copy number across cell types within ovaries, the average *rp49* copy number remains consistent across the extracted tissue samples. As such, we conclude that changes in *Wolbachia groEL* copy number, not *rp49* copy number, underpin the results. However, future work will be necessary to describe how these density estimates explicitly relate to *Wolbachia* titers per host cell and across cell types in these tissues.

In addition to the PGAE, CI variation has previously been attributed to a “younger-brother effect” where the slowest-developing males, from a clutch of embryos within the 0-to-5-h age range, induced the weakest CI (32). If embryo deposition order correlates with maturation rate, then the younger-brother effect is at least in part explained by our findings that (i) *Wolbachia* densities in ovaries quickly decrease after mating and egg laying, (ii) the *Wolbachia* density in embryos correlates with ovary densities, and (iii) sons from eggs laid by mothers with lower *Wolbachia* densities induce weaker CI. As such, when a *D. melanogaster* female lays eggs, the amount of *Wolbachia* in her ovaries may be sequentially depleted after each embryo is produced. Thus, younger brothers that take longer to develop may receive fewer *Wolbachia* and then induce a weaker CI than their older counterparts that originally had received more *Wolbachia*. Therefore, the dynamics of the interaction that we observed between CI induction and *Wolbachia* densities across generations may explain the younger-brother effect, although this remains to be precisely established in future research.

Additionally, this paper adds to a growing body of literature reporting an influence of female insect age on *Wolbachia* densities. Indeed, older females harbor higher *Wolbachia* titers in *wAlbA*- and *wAlbB*-infected *Aedes albopictus* (41, 42), *wVulC*-infected *Armadillidium vulgare* (43), and *wStri*-infected *Laodelphax striatellus* (44). The relationship between paternal grandmother age and the strength of *wMel*-induced CI was explored once before; however, no relationship was found (32). Crucially, the virginity status of the grandmothers differs between the cited study and the one presented here and may in part explain the discrepancy. Our study maintained the virginity of all grandmothers as they aged, and grandmothers were allowed only 24 h of mating prior to egg deposition for hatch rate analysis. In contrast, the grandmothers in the prior study remained virgin until 3 days old and were then allowed to continuously mate

until they were 11 days old, and the CI levels from sons produced at each of the two time points were compared (32). Our results suggest that mating has a detectable impact on *Wolbachia* densities and may explain why the PGAE was not observed in the earlier study. Additionally, we predict that the PGAE most strongly applies to aged virgins, since mating significantly reduced *Wolbachia* densities in our study.

The depletion of *Wolbachia* found in females following egg laying supports the hypothesis that the PGAE is caused by an effect of maternal loading of *Wolbachia* into her sons. However, the source of that loading is still unclear. In *D. melanogaster*, the following four sources of *Wolbachia* transfer to progeny are known: bacteriocyte-like cells (BLCs), germ line stem cells (GSCs), the somatic stem cell niche (SSCN), and late-stage oogenesis (5, 38, 45–48). BLCs found at the tip of the ovarioles are densely packed with *Wolbachia* and are predicted to transfer *Wolbachia* to GSCs (47). When a GSC asymmetrically undergoes mitosis (49, 50), its population of *Wolbachia* is divided between two daughter cells, one of which is an identical GSC that remains in the ovaries and the other a differentiating cytotblast that develops into the egg (5). Therefore, it is possible that the levels of *Wolbachia* allocated to the daughter cytotblast (and thus the offspring) are proportional to the densities in the parent GSC or the surrounding BLCs. Additionally, as the cytotblast develops into a germ line cyst, it comes into contact with the highly infected SSCN, acquiring additional *Wolbachia* (45, 46, 48). Finally, while *Wolbachia* replication in the oocyte occurs primarily at the beginning of oogenesis in *wMel*-infected *D. melanogaster* and halts at the onset of vitellogenesis, it can resume at a lower rate before egg laying in late-stage oogenesis (38). As such, prolonged retention of eggs in aged virgins may lead to an accumulation of *Wolbachia* in these developed oocytes. We hypothesize that *Wolbachia* replicate in the BLCs, GSCs, SSCNs, or late-stage oocytes as a mother ages, resulting in eggs with relatively high titers. Since eggs account for the greatest proportion of *Wolbachia* cells in the ovaries, this hypothesis could explain why titers are depleted after mating and egg laying.

Intriguingly, differences in CI strength more closely correlated with *Wolbachia* densities in embryos than with densities in adult males. CI is hypothesized to be caused by *cif* gene modifications of sperm-associated host products (51–58) or to be a consequence of loading of toxins into the sperm (52, 53, 59, 60); however, *Wolbachia* are stripped from the sperm during individualization (37, 61, 62). Therefore, *Wolbachia* titers are likely more important during a specific stage of spermatogenesis than at the time of CI induction. In *D. melanogaster*, spermatogenesis is a continuous process lasting approximately 11 days (63). As such, there may be a lag of several days between the time that sperm are subjected to the actions of *cifA* and *cifB* gene products and the time of CI induction. Spermatogenesis begins during larval development (63) and continues throughout the adult life span (64), though the first batches of mature sperm are produced soon after adult hatching (65). Since the males in our study were mated shortly after adult hatching, the majority of their sperm would have started spermatogenesis at a time closer to embryonic deposition than adult hatching, which may explain why CI strength correlates better with *Wolbachia* densities in embryos than in adult males. Additionally, spermatogenesis may incorporate and eliminate *Wolbachia* faster than they can multiply, resulting in the reduction and equalization of titers in adults (37). This may explain why some studies, including studies analyzing the younger-brother effect, found that CI strength did not always correlate with *Wolbachia* densities in adults (25, 32, 66). As such, we predict that the PGAE is the result of the presence of high *Wolbachia* densities during a critical time point in spermatogenesis when CI-defining changes occur, which may become the subject of future research.

It remains unclear if the association between female age and *Wolbachia* densities would be the case in wild populations. Since wild *D. melanogaster* females are estimated to mate, on average, every 27 h (67), it would seem unlikely that the *Wolbachia* accumulation reported here would occur in nature. However, infection status has been reported to influence mate choice behaviors in numerous animals, including *D. subquinaria*, *D. paulistorum*, *Nasonia vitripennis*, and *Tetranychus urticae* (68–71). For example, male mating rate affects CI strength (30), so *wMel*-infected males mate more

frequently to reduce the impact of CI strength and therefore improve their lifetime reproductive success (35). Additionally, females infected with *Wolbachia* have a higher reproductive fitness when their daughters can sufficiently rescue CI and when their sons induce weak CI. Thus, it is plausible that the latency to copulation could be either lengthened in instances where a higher *Wolbachia* titer would be preferable (rescue efficiency) or, conversely, shortened in populations where a lower density is preferred (weakened CI). While it is unlikely that a fly in nature will remain virgin for as long as reported in this study, it is notable that CI strength increased substantially with every time point measured. As such, even small changes in mating latency may influence CI strength sufficiently to change the rate of spread through a population. Field studies measuring the latency toward copulation in sites with different infection rates would help determine if insects can modulate their mating latency, and thus *Wolbachia* titers, to increase their fitness and the fitness of their offspring.

While this work reports a PGAE for *wMel* in *D. melanogaster*, it is unknown if these dynamics occur for *wMel* in mosquito hosts. In *wMel*-infected *Aedes aegypti* mosquitoes, CI is consistently strong (23, 24). However, some factors such as *Wolbachia* densities and temperature were shown previously to correlate with CI penetrance (72). It is possible that other as-yet-unstudied factors in mosquitoes, such as the PGAE, can contribute to changes in CI strength. Since strong CI is crucial for rapid spread of *wMel*-infected mosquitoes through populations for successful vector control applications (73), understanding the factors that contribute to variation in CI strength would further inform the efficacy of population replacement and rearing strategies. Moreover, comparative studies exploring *wMel*-induced CI in *D. melanogaster* and *A. aegypti* could clarify the *Wolbachia*-host dynamics that govern the penetrance of CI.

Finally, there is a striking range of CI penetrance across *Wolbachia* and hosts, and more work is necessary to determine if the PGAE applies to other CI or reproductive parasite systems. For example, *wRi* in *D. simulans* consistently induces strong CI (7, 10, 74) and *wYak* and *wTei* in the *D. yakuba* clade cause weak and variable levels of CI similar to those seen with *wMel* (75). Intriguingly, *wMel* and *wTei* were initially thought not to cause CI until factors such as male age and host genotype were found to have a significant impact on CI strength (30, 75–79). Since it is clear that some *Wolbachia* cause CI only under strictly limited conditions, it remains possible that other weak-CI-inducing *Wolbachia* are mislabeled as non-CI strains because factors such as the PGAE had not been controlled for during initial testing. Indeed, while this work presents the first reported case of transgenerational *Wolbachia* titers influencing CI, it is not the first case of transgenerational *Wolbachia* titers influencing reproductive parasitism. In *D. innubila*, male-killing *Wolbachia* frequently kill all male offspring, but females with lower *Wolbachia* titers are known to produce some viable sons (80). The surviving female offspring inherit lower-than-average *Wolbachia* titers, leading to a greater-than-average chance that those infected females would also produce sons (80). Together, our results and those in *D. innubila* suggest that a transgenerational effect of titers may be common and consequential with respect to the expression of reproductive parasitism traits.

In conclusion, we characterize *Wolbachia* density dynamics in females in relation to age and mating, and we link a transgenerational influence of grandmother age to CI penetrance. This work highlights the importance of controlling grandparent age in future studies of *wMel*-induced CI in *D. melanogaster* and has implications for laboratory experiments where precise control over levels of CI would be valuable for dissecting the genetic and functional basis of CI. Additionally, it expands our understanding of *Wolbachia*-host interactions in relation to CI penetrance and titer dynamics and should motivate additional studies exploring these interactions in *wMel*-infected mosquitoes.

MATERIALS AND METHODS

Fly strains and maintenance. The following *D. melanogaster* strains were used in this study: *wMel*-infected and uninfected variants of *y¹w^{*}* (BDSC 1495) and *nos-GAL4-tubulin* (BDSC 4442). Unin-

fect lines were generated through three generations of tetracycline treatment as previously described (29). All stocks were reared on 50 ml of a standard medium containing cornmeal, molasses, and yeast and were maintained at 25°C with a 12-h/12-h light:dark cycle and at 70% relative humidity (RH). All virgin flies were collected using CO₂ anesthetization per standard procedures. Briefly, virgin flies were collected in the morning based on the presence of a meconium, bottles were subsequently cleared of adult flies, and flies collected in the evening were assumed virgin due to the standard time of latency until mating. All virgin flies were kept at room temperature prior to experimentation.

Hatch rate assays. Hatch rate assays were used to assess the impact of *D. melanogaster* paternal grandmother age on the strength of CI induced by their sons. We conducted 3 variant hatch rate assays to test (i) whether paternal grandmother age influences CI hatch rates, (ii) whether this effect is specific to the *y¹w^{*}* genetic background, and (iii) whether the transgenerational impact of age on hatching is indeed caused by CI.

First, we assessed if *D. melanogaster* paternal grandmother age influences CI hatch rates in the *y¹w^{*}* genetic background. Paternal *y¹w^{*}* grandmothers were collected as virgins and allowed to reach 2, 5, 11, 14, or 18 days of age before mating in parallel with paternal grandfathers aged 0 to 2 days. Paternal grandparents from each age cohort were crossed in single-pair matings in standard vials of media. Since rearing density influences CI strength (32), paternal grandparents were allowed 24 h to mate and to deposit eggs before the grandfathers were discarded and the grandmothers were flash frozen and stored at -80°C for *Wolbachia* titer analysis. To control for the younger-brother effect and the effect of male age on the strength of CI (30, 32), the earliest eclosing fathers were collected as virgins and left to age 1 day at room temperature before being used in hatch rate assays.

Maternal *y¹w^{*}* grandparents were crossed in standard medium bottles and allowed to mate for 4 days before flies were cleared, as described above for the paternal grandparents. Mothers were collected as virgins and allowed to reach 6 to 8 days of age at room temperature to maximize fertility (81).

Parental *y¹w^{*}* mating pairs were placed in 8-oz *Drosophila* stock bottles (Genesee Scientific) with a grape juice agar plate covered in yeast affixed to the top to collect embryos for hatch rate analysis as previously described (29, 34). Parents were allowed two back-to-back 24-h mating and laying periods, each with separate freshly yeasted grape juice agar plates. The plates from the first mating period were discarded due to the typically low levels of egg laying in the first 24 h. The embryos from the second mating period were immediately counted after 24 h of additional laying. Embryos were then incubated for 30 h at 25°C to allow time to hatch. The unhatched embryos were counted, and the percentage of embryonic hatching was determined by dividing the number of unhatched embryos by the total number of embryos laid during the second mating period.

To minimize the effect of female fecundity on embryo viability (81), any plate with fewer than 25 embryos was excluded. We measured the hatch rates of offspring produced by two sons of each paternal grandmother. If both sons from the same family produced 25 or more embryos, one was randomly selected and used in analysis.

Next, to assess if the PGAE was specific to the *y¹w^{*}* genetic background, a separate hatch rate assay was conducted using *nos-GAL4-tubulin*-infected and uninfected flies. This experiment was conducted similarly to the hatch rate experiment described above, with the following adjustments: age and virginity of paternal grandfathers were not controlled. Paternal *nos-GAL4-tubulin*-infected and uninfected grandmothers were collected as virgins and allowed to reach 2, 5, or 11 days of age before they were allowed to mate in standard medium bottles, and these bottles were cleared of flies after 4 days of laying to control rearing density (32).

Finally, to determine if the PGAE was in fact due to *Wolbachia* and not to other forms of inviability induced by a transgenerational impact on age, we conducted compatible rescue crosses with males derived from 2-, 5-, 11-, or 14-day-old females. This experiment was conducted similarly to the hatch rate experiment described above, with the following adjustments: both infected and uninfected males were produced from virgin females aged 2, 4, 11, or 14 days; the uninfected males were mated to uninfected females; and infected males were mated to infected females. Paternal grandparents were paired in 8-oz *Drosophila* stock bottles (Genesee Scientific) with a grape juice agar plate (29) covered in yeast affixed to the top for a 24-h mating and laying period, and then grandparents were collected from the bottles. The plates were maintained for 24 h, and then 20 of the largest larvae were transferred from each plate to a standard medium vial to control rearing density (32).

***Wolbachia* titer assays.** To assess the relationship between the PGAE and *Wolbachia* titers, the following tissues were collected: ovaries, female abdomens, embryos, and male abdomens. Since the low biomass of *Drosophila* testes requires them to be pooled, abdomens were used instead of testes so that samples could be taken directly from the males used in hatch rate assays. To test if virginity and age impact female *Wolbachia* titers, virgin and nonvirgin females 2 and 11 days of age were reared in parallel, ovaries were dissected in phosphate-buffered saline (PBS), and samples were frozen in liquid nitrogen followed by storage at -80°C. Samples consisted of 4 pairs of ovaries. Nonvirgin females were mated in cohorts of 60 females to 12 males, provided grape juice plates, and allowed 48 h to mate and lay eggs before dissection. Additionally, full bodies from 2-or-11-day-old paternal grandmothers from a hatch rate assay were collected alongside virgin paternal grandaunts (siblings to the paternal grandmothers), frozen in liquid nitrogen, and stored at -80°C. To determine if embryos derived from older females had higher *Wolbachia* titers, 0-to-1-h-old embryos were collected from grape plates in batches of 50, frozen in liquid nitrogen, and stored at -80°C. Finally, to assess whether the sons of aged paternal grandmothers had higher *Wolbachia* titers, full bodies from virgin uncles (siblings of fathers used in a hatch rate assay) derived from 2-, 5-, 11-, 14-, or 18-day-old grandmothers were collected and aged 48 h at room temperature in a standard medium vial. *Wolbachia* titers were measured in virgin uncles rather than the

fathers used in the hatch rate assay because of the relationship between CI strength and male mating rate (35).

Upon removal from -80°C conditions, abdomens were immediately dissected from full-body tissues, homogenized in liquid nitrogen, and mixed with $40\ \mu\text{l}$ ice-cold RNase-free PBS. Each sample was split, and 30% ($12\ \mu\text{l}$) was flash frozen and stored at -80°C for DNA extractions. The DNA was extracted from all tissue types using a Genra PureGene tissue kit (Qiagen). Forty cycles of quantitative PCR (qPCR) were performed using *rp49* and *groEL* primers (Table S2) for all DNA samples as well as positive controls (infected DNA), negative controls (uninfected DNA), no-reverse-transcription controls (RNA), and no-tissue controls (water). Male and female abdomen samples were tested in triplicate and ovaries and embryos in duplicate under the following qPCR conditions: 50°C for 10 min; 95°C for 5 min; 40 cycles of 95°C for 10 s and 55°C for 30 s; and 95°C for 30 s. Samples were excluded from analysis if the standard deviation of results of comparisons between replicates was >0.3 . Fold difference between *Wolbachia* (*groEL*) density and that of the *D. melanogaster* *rp49* reference gene was determined with $2^{-\Delta\Delta\text{CT}}$.

Statistical analyses. All statistical analyses were conducted using GraphPad Prism 7. *Wolbachia* titers of embryos were analyzed using a Mann-Whitney *U* test. All other data (including data from hatch rate assays and ovary *Wolbachia* titer comparisons) were analyzed using the Kruskal-Wallis test followed by a Dunn's multiple-comparison test. Figures were created in GraphPad Prism 7 and 8. All data used in these analyses have been made publicly available (see Data Set S1 in the supplemental material).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.01879-19>.

FIG S1, TIF file, 0.2 MB.

FIG S2, TIF file, 0.2 MB.

FIG S3, TIF file, 0.2 MB.

FIG S4, TIF file, 0.2 MB.

TABLE S1, DOCX file, 0.01 MB.

TABLE S2, DOCX file, 0.01 MB.

DATA SET S1, XLSX file, 0.7 MB.

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We declare that we have no competing interests.

All of us contributed to the design of the study. E.M.L. and J.O. conducted hatch rate assays with J.I.P.'s assistance. E.M.L. and J.D.S. conducted titer studies. E.M.L. and J.D.S. analyzed data. E.M.L., J.D.S., and S.R.B. wrote the paper. J.D.S. and J.I.P. provided technical assistance throughout the study. All of us contributed to the final manuscript edits and revision.

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