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Infection by wMel Wolbachia alters female post-mating behaviors and physiology in the dengue vector mosquito Aedes aegypti

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Abstract

Globally invasive *Aedes aegypti* mosquitoes disseminate numerous arboviruses that impact human health. One promising method to control *Ae. aegypti* populations is transinfection with the intracellular bacterium *Wolbachia pipiens*, a symbiont that naturally infects ~40-52% of insects but is normally absent from *Ae. aegypti*. Transinfection of *Ae. aegypti* with the wMel *Wolbachia* strain induces cytoplasmic incompatibility, allowing infected individuals to rapidly invade native populations. Further, wMel *Wolbachia*-infected females display refractoriness to medically relevant arboviruses. Thus, wMel *Wolbachia*-infected *Ae. aegypti* are being released in several areas to replace native populations, thereby suppressing disease transmission by this species. *Wolbachia* is reported to have minimal effects on *Ae. aegypti* fertility, but its influence on other reproductive processes is unknown. Female insects undergo several post-mating physiological and behavioral changes required for optimal fertility. Post-mating responses (PMRs) in female insects are typically elicited by receipt of male seminal fluid proteins (SFPs) transferred with sperm during mating, but can be modified by other factors, such as adult age, nutritional status, and microbiome composition. To assess how *Wolbachia* infection influences *Ae. aegypti* female PMRs, we collected wMel *Wolbachia*-infected *Ae. aegypti* from the field in Medellín, Colombia and introduced the bacterium into our laboratory strain. We found that *Wolbachia* influences female fecundity, fertility, and re-mating incidence. Further, we observed that *Wolbachia* significantly extends longevity of virgin females. Changes in female PMRs are not due to defects in sperm transfer by infected males, or sperm storage by infected females. Using proteomic methods to examine the seminal proteome of infected males, we found that *Wolbachia* infection has a moderate effect on SFP composition. However, we identified 125 *Wolbachia* proteins that are paternally transferred to females by infected males. Surprisingly, the CI factor proteins (Cifs), were not detected in the ejaculates of *Wolbachia*-infected males. Our findings indicate that *Wolbachia*
infection of *Ae. aegypti* alters female post-mating responses, potentially influencing control programs that utilize *Wolbachia*-infected individuals.

### Introduction

*Aedes aegypti* mosquitoes are a globally invasive species that have successfully colonized large portions of the tropics and sub tropics\(^1,2\). *Aedes aegypti* has a propensity for colonizing urban environments\(^3,4\), and females of this species have a preference for human hosts\(^5,6\), factors that have facilitated the transmission of viruses spread by this species, which include the dengue\(^7\), Zika\(^8\), chikungunya\(^9\) and yellow fever viruses\(^10\).

Habitable territory of *Ae. aegypti* is predicted to expand with rising global temperatures\(^2,11\) and increased urbanization\(^6,12\), making control of this species essential to mitigate its impact on human health.

Efforts to control *Ae. aegypti* have historically relied on insecticide use. However, increased insecticide resistance of *Ae. aegypti* populations have reduced the efficacy of chemical control\(^13\), necessitating development of novel control methods. One promising method is transinfection of *Ae. aegypti* with the obligate intracellular bacterium *Wolbachia pipientis*, a symbiont that naturally infects 40-52% of insect species\(^14,15\), but not *Ae. aegypti*. *Wolbachia* is maternally inherited and induces cytoplasmic incompatibility (CI) in transinfected *Ae. aegypti*\(^16,17\), a phenomenon where uninfected females that mate with infected males do not produce viable progeny, while infected females produce viable, *Wolbachia*-infected progeny regardless of the infection status of their mates. The induction of CI allows infected *Ae. aegypti* to rapidly spread into uninfected populations\(^16–18\), where they remain stable long-term\(^19\). *Wolbachia* infection also suppresses arbovirus transmission by *Ae. aegypti* females, including DENV, ZIKV and CHIKV\(^17,20–29\).
Given that *Wolbachia*-infected *Ae. aegypti* are able to quickly disseminate into mosquito populations, *Wolbachia*-infected populations—compromised for their ability to transmit disease—can effectively replace native populations upon the release of infected males and females into the environment\(^\text{30}\). Alternatively, the continuous release of *Wolbachia*-infected males can reduce native mosquito populations through the establishment of CI\(^\text{31,32}\). Population replacement programs utilizing *Ae. aegypti* infected with the wMel *Wolbachia* strain isolated from *Drosophila melanogaster* have been initiated in several areas where DENV transmission occurs\(^\text{33–36}\), including in Medellín, Colombia\(^\text{37}\). The successful establishment of infected populations is dependent on a minimal effect of *Wolbachia* on the reproductive parameters of liberated *Ae. aegypti* adults. wMel *Wolbachia* has been reported to have modest effects on male and female fertility\(^\text{26,38}\), but how *Wolbachia* might alter other reproductive processes in *Ae. aegypti* has not been explored.

Mating induces physiological and behavioral changes in female insects that facilitate the production of progeny\(^\text{39,40}\). Female *Ae. aegypti* post-mating responses (PMRs) include an inhibition to re-mating\(^\text{41}\), increased female longevity\(^\text{42}\), increased oviposition rates\(^\text{42}\), and changes in gene expression in female reproductive tissues\(^\text{43–45}\). The primary effectors of the *Ae. aegypti* female PMR are seminal fluid proteins (SFPs)\(^\text{42,46,47}\) transferred to the female reproductive tract along with sperm during mating. Female PMRs in insects are also influenced by other factors, such as male age\(^\text{48}\), adult nutrition\(^\text{49}\), and adult microbiome composition\(^\text{50,51}\). wMel *Wolbachia* has been shown to alter female PMRs in *D. melanogaster*\(^\text{62}\), which may be due to the observed modification of SFP composition in infected males\(^\text{53}\). However, *Wolbachia* infection alters protein secretion from the *D. melanogaster* female sperm storage organs\(^\text{53}\), which express genes essential for ovulation, oviposition, sperm motility, and sperm storage\(^\text{54,55}\). Thus, *Wolbachia* infection
could potentially influence female *Ae. aegypti* post-mating changes in a sex-specific manner.

In the present study, we examined how wMel *Wolbachia* influences *Ae. aegypti* female PMRs. We collected wMel *Wolbachia*-infected adults being released in Medellin, Colombia and backcrossed infected females to our laboratory strain for 7 generations to generate a *Wolbachia* infected colony in the same genetic background. We examined how *Wolbachia* influences fecundity, fertility, re-mating incidence, and female longevity. Although *Wolbachia* infection had no effect on sperm transfer during mating or the storage of sperm by mated *Ae. aegypti* females, fecundity, fertility, and re-mating incidence were impacted. Additionally, female longevity was altered in *Wolbachia* positive *Ae. aegypti* females independent of mating. We used proteomic methods to examine SFP levels transferred to females by *Wolbachia*-infected males, finding that *Wolbachia* has a modest effect on SFP composition. Our proteomic analysis also allowed us to identify *Wolbachia* proteins paternally transferred to females during mating. To our surprise, although 125 *Wolbachia* proteins were identified, our analysis did not reveal the presence of Cif proteins that modify sperm to establish CI. Our results show that the presence of *Wolbachia* in *Ae. aegypti* alters adult fertility and influences female post-mating behaviors and physiology. The effects we report here have potential implications for population replacement programs and population suppression programs that use *Wolbachia*-infected *Ae. aegypti* to control mosquito populations or suppress disease transmission.

**Results**

*Wolbachia* impacts fecundity and fertility of female *Aedes aegypti*.
To examine the effects of Wolbachia infection on the Ae. aegypti female PMR, we collected wMel Wolbachia-infected individuals from the field and backcrossed them to Thai strain Ae. aegypti, generating the ThaiWob strain. We examined the potential impact of Wolbachia on fecundity and fertility of female Ae. aegypti, as wMel Wolbachia has moderate sex-specific effects on fertility in this species. Given that female size influences fecundity, we first examined the size of the Thai and ThaiWob adults used in our assays, finding that Thai and ThaiWob adults were similarly sized when reared under the same conditions (Supplementary Figure 1). To assess possible male- or female-specific effects of Wolbachia infection on parameters of fertility, we performed our assays in all mating combinations (shown as female x male): Thai x Thai (control), Thai x ThaiWob, ThaiWob x Thai, ThaiWob x ThaiWob.

We found significant differences in fecundity between the different mating combinations (DF = 3, F = 17.78, p < 0.0001; Figure 1A). We did not detect an effect of male infection on female fecundity, as Thai females laid a similar quantity of eggs when mated to Thai or ThaiWob males (p = 0.95; Figure 1A). However, ThaiWob females laid significantly fewer eggs than Thai females when mated to Thai males (p = 0.007; Figure 1A) and suffered a further reduction in fecundity upon mating to ThaiWob males (p < 0.0001; Figure 1A).

Similarly, fertility (shown as hatch percentage) was significantly different between all mating combinations (DF = 3, F = 146.19, p < 2.2e-16; Figure 1B). The fertility of ThaiWob females mated to Thai males was significantly reduced compared to control matings (p < 0.0001; Figure 1B), with the largest suppression of fertility observed when both sexes were infected (p < 0.0001; Figure 1B). As expected, we observed a significant reduction in fertility when Thai females mated to ThaiWob males due to CI (p < 0.0001; Figure 1B).
Figure 1. Wolbachia infection impacts Aedes aegypti fertility. Fecundity (A) and hatch percentage (B) are shown for each mating combination. Crosses are shown as female x male. T = Thai, W = Thai\textsuperscript{Wolb}. Different letters above the box plots indicate a significant difference between mating combinations ($p < 0.05$) for a Tukey test.

Female remating incidence increases after mating with Wolbachia-infected males.

We next examined whether Wolbachia infection affects a male’s ability to inhibit remating by their mates. In our assays, 27% of Thai females re-mated when initially mated to Thai males (Table 1), similar to previous reports using this strain\textsuperscript{41,48}. When mated to Thai\textsuperscript{Wolb} males, however, we observed a significant increase in remating incidence ($\chi^2 = 4.5$, DF = 1, $p = 0.03$; Table 1). Thai\textsuperscript{Wolb} females also remated at significantly higher rates when first mated to a Thai\textsuperscript{Wolb} male compared to an initial mating with a Thai male ($\chi^2 = 3.2$, DF = 1, $p = 0.04$; Table 1). Given the increase in remating incidence observed after initially mating with Thai\textsuperscript{Wolb} males, we next evaluated if this effect is detectable upon mating with females of a different strain. We mated Thai and Thai\textsuperscript{Wolb} males to Ae. aegypti collected in Acacias, Colombia\textsuperscript{60} and to Rockefeller strain Ae. aegypti females. In both strains, we observed a similar trend: females initially mated to Thai\textsuperscript{Wolb} males remated at higher rates than those
initially mated to Thai males, although in each case the effect was not significant (Acacias: $\chi^2 = 1.3$, DF = 1, $p = 0.3$; Rockefeller: $\chi^2 = 0.5$, DF = 1, $p = 0.5$; Table 1).

<table>
<thead>
<tr>
<th>Female</th>
<th>1st mating male</th>
<th>N</th>
<th>% re-mated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thai</td>
<td>Thai</td>
<td>194</td>
<td>27.3%</td>
</tr>
<tr>
<td></td>
<td>Thai$^{Wolb}$</td>
<td>189</td>
<td>37.6%</td>
</tr>
<tr>
<td>Thai$^{Wolb}$</td>
<td>Thai</td>
<td>192</td>
<td>30.7%</td>
</tr>
<tr>
<td></td>
<td>Thai$^{Wolb}$</td>
<td>197</td>
<td>36.4%</td>
</tr>
<tr>
<td>Acacias</td>
<td>Thai</td>
<td>96</td>
<td>35.4%</td>
</tr>
<tr>
<td></td>
<td>Thai$^{Wolb}$</td>
<td>96</td>
<td>43.8%</td>
</tr>
<tr>
<td>Rockefeller</td>
<td>Thai</td>
<td>98</td>
<td>40.6%</td>
</tr>
<tr>
<td></td>
<td>Thai$^{Wolb}$</td>
<td>96</td>
<td>45.8%</td>
</tr>
</tbody>
</table>

Table 1. Remating incidence of the indicated strain of *Aedes aegypti* female after an initial mating to a Thai or Thai$^{Wolb}$ male.

*Wolbachia* increases the lifespan of virgin *Aedes aegypti* females.

As wMel *Wolbachia* increases female lifespan in *D. melanogaster* and in wMel transinfected *Ae. albopictus*, we asked if *Wolbachia* infection alters *Ae. aegypti* lifespan. Mated *Ae. aegypti* females have significantly longer lifespans than virgins due to the effect of transferred SFPs. We first examined matings between infected or uninfected individuals, observing that mated Thai females lived significantly longer than virgins ($p = 4e-08$; Figure 2A) as previously reported. However, lifespan of virgin and mated Thai$^{Wolb}$ females did not significantly differ ($p = 0.2$; Figure 2A), and the longevity of virgin Thai$^{Wolb}$ females was significantly greater than virgin Thai females ($p = 2e-12$; Figure 2A). When we examined all mating combinations, we again found that longevity of mated Thai females significantly differed from Thai virgins ($p = 2e-09$) and that virgin Thai$^{Wolb}$ females lived...
significantly longer than virgin Thai females ($p = 4e-05$; Figure 2B). Further, we found that the longevity of virgin Thai$^{Wolb}$ and Thai$^{Wolb}$ females mated to Thai males did not differ ($p = 0.8$; Figure 2B) but detected a difference in longevity between virgin Thai$^{Wolb}$ females and Thai$^{Wolb}$ females mated to Thai$^{Wolb}$ males ($p = 0.01$; Figure 2B). The observed increase in longevity was specific to females, as Wolbachia infection had no effect on male lifespan ($p = 0.86$; Supplementary Figure 2).

**Figure 2.** *Wolbachia* infection increases longevity of virgin females. (A) Longevity of virgin and females mated to males from the same colony (T x T and W x W). (B) Longevity of virgin and females mated in all combinations. Crosses are shown as female x male. T = Thai, W = Thai$^{Wolb}$, vT = virgin Thai, vW = virgin Thai$^{Wolb}$.

*Wolbachia* does not influence sperm quantity transferred by males, or sperm quantity stored by females.

*Wolbachia* impacts sperm production$^{64}$ and sperm quantity transferred$^{65}$ by *D. simulans* males, raising the possibility that *Wolbachia* infection impacts sperm production and/or sperm transfer by Thai$^{Wolb}$ males, which could impact the quantity of sperm stored by females$^{66}$. However, we did not detect differences in the quantity of sperm present in the male seminal vesicles—the organs that house mature sperm in the male reproductive
tract—of ThaiWolb males compared to Thai males (DF = 1, F = 0.752, p = 0.39; Figure 3A)
or the quantity of sperm transferred by ThaiWolb males during mating (DF = 3, F = 0.418, p
= 0.741; Figure 3B). Finally, we assessed the quantity of sperm females stored in their spermathecae, the long-term sperm storage organs. Sperm quantity in the spermathecae was similar in the spermathecae of Thai and ThaiWolb females 24 h after insemination (DF = 3, F = 2.039, p = 0.114; Figure 3C). Thus, Wolbachia infection does not appear to influence sperm production, sperm transfer during mating, or female sperm storage.

Figure 3. Wolbachia infection does not impact sperm production, sperm transfer during mating or female sperm storage. Sperm quantities in (A) the seminal vesicle of virgin males, (B) transferred to the bursa of the female reproductive tract during mating, and (C) stored in the spermathecae of mated females. Crosses are shown as female x male. T = Thai, W = ThaiWolb.

Wolbachia-dependent changes in sperm composition and SFP transfer

wMel Wolbachia alters the expression of genes that code for SFPs and changes SFP composition in naturally infected D. melanogaster males. Given that ThaiWolb males are
suboptimal in preventing subsequent copulations by their mates (Table 1), and re-mating inhibition is mediated by SFP receipt\textsuperscript{47}, we asked if SFP composition in \textit{Wolbachia}-infected males differs from uninfected males. To identify seminal proteins, we used a proteomic approach that allows for the identification of male proteins transferred to females during mating\textsuperscript{69,70}. Females labeled with the natural isotope \textsuperscript{15}N were mated to unlabeled, normally reared Thai and Thai\textsuperscript{Wolb} males and seminal proteins isolated from the bursae immediately after insemination were identified by tandem mass spectrometry (LC-MS/MS) and their abundances quantified (see Methods). To differentiate between sperm proteins and SFPs, we used the \textit{Ae. aegypti} sperm and SFP proteome reported in ref.\textsuperscript{70}.

We found that \textit{Wolbachia} infection had a modest effect on the composition of seminal proteins transferred during mating, as Thai\textsuperscript{Wolb} and Thai male ejaculates had similar abundances of SFPs, sperm proteins, and sperm/SFP overlapping proteins (i.e., proteins identified in both the sperm and SFP proteomes, but more abundant in the ejaculate relative to sperm\textsuperscript{70}) (Figure 4A-D). However, one seminal protein was significantly more abundant in Thai males compared to Thai\textsuperscript{wolb} males: the SFP trypsin-7 (AAEL006429) (Figure 4B). Further, although not statistically significant, three additional seminal proteins were found to be more than fourfold more abundant in Thai male ejaculates compared to Thai\textsuperscript{Wolb} males, the sperm protein AAEL024468 (Figure 4C), and two unclassified proteins, AAEL006115 and AAEL003365 (Figure 4A); both AAEL006115 and AAEL003365 are co-expressed in the \textit{Ae. aegypti} testes and male accessory gland\textsuperscript{70}. Finally, we identified seminal proteins that were only detected in Thai or Thai\textsuperscript{Wolb} male ejaculates but not detected in the other (Supplementary Table 1).
Figure 4. Abundance of seminal proteins transferred by *Wolbachia*-infected males compared to uninfected males. Volcano plots showing the abundance of all proteins identified in Thai and Thai*W*ob ejaculates (A), seminal proteins classified as SFPs (B), sperm proteins (C), and sperm/SFP overlapping proteins (D). Values below zero represent proteins with higher abundance in Thai*W*ob male ejaculates, and values above zero represent proteins with higher abundance in Thai male ejaculates.

Identification of paternally transferred *Wolbachia* proteins.

Our proteomic labeling method also allowed us to identify *Wolbachia* proteins present in the seminal fluid and transferred to females during mating. We identified 125 wMel *Wolbachia* proteins across all replicates that were present in Thai*W*ob male ejaculates (Supplementary dataset 1). However, only 20 of these proteins were consistently detected in all replicates (Table 2). Paternally transferred wMel *Wolbachia* proteins fell into several functional categories, including metabolic proteins, membrane proteins, chaperones, and others (Supplementary dataset 1). Although our analysis did not identify Cif proteins that recapitulate CI in a transgenic system\(^7\), we identified proteins that can modify the CI phenotype\(^7\) (Supplementary Table 2), including WD0462, a predicted *Wolbachia* effector molecule\(^7\). Several paternally transferred *Wolbachia* proteins are derived from phage WO
sequences integrated into the *Wolbachia* genome, or WO-like Islands that are associated with and/or derived from phage WO \(^74\) (Supplementary Table 2). Further, we detected transferred phage WO proteins that are associated with the Eukaryotic Association Module (Supplementary Table 2), a region of the phage genome that contains genes that code for proteins with eukaryotic-domains and are predicted to interact with the host\(^75\).

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPB_WOLPM</td>
<td>atpD</td>
<td>ATP synthase subunit beta</td>
</tr>
<tr>
<td>DNAK_WOLPM</td>
<td>dnaK</td>
<td>Chaperone protein DnaK</td>
</tr>
<tr>
<td>Q73H52_WOLPM</td>
<td>WD0722</td>
<td>Ammonium transporter</td>
</tr>
<tr>
<td>NUOD_WOLPM</td>
<td>nuoD</td>
<td>NADH-quinone oxidoreductase subunit D</td>
</tr>
<tr>
<td>Q73HV3_WOLPM</td>
<td>sdhA</td>
<td>Succinate dehydrogenase flavoprotein subunit</td>
</tr>
<tr>
<td>Q73GH8_WOLPM</td>
<td>WD0976</td>
<td>NADH-quinone oxidoreductase subunit F</td>
</tr>
<tr>
<td>ATPA_WOLPM</td>
<td>atpA</td>
<td>ATP synthase subunit alpha</td>
</tr>
<tr>
<td>RPOBC_WOLPM</td>
<td>rpoBC</td>
<td>Bifunctional DNA-directed RNA polymerase subunit beta-beta</td>
</tr>
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<td>HTPG_WOLPM</td>
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<td>Chaperone protein HtpG</td>
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<td>Q73GV1_WOLPM</td>
<td>cutA</td>
<td>Periplasmic divalent cation tolerance protein</td>
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<td>Fumarate hydratase class II</td>
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<td>Q73GU2_WOLPM</td>
<td>WD0838</td>
<td>Glycerolphosphoryl diester phosphodiesterase</td>
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</table>
Table 2. Paternally transferred *Wolbachia* proteins identified in all biological replicates.

**Discussion**

*Wolbachia* is a common insect symbiont that can modify host physiology and behaviors. Given that *Wolbachia* alters reproductive outcomes that favor its propagation, it is being used as a tool to reduce vector competency of *Ae. aegypti* females\(^57\), and offers an alternative to continued insecticide use and/or the release of genetically modified mosquitoes. Control programs are currently conducting large-scale releases of *Wolbachia*-infected male to suppress vector populations\(^31,32\) or both sexes in population replacement programs\(^30,57\). The success of these programs depends on *Wolbachia* having minimal effects on infected individuals to allow liberated *Wolbachia*-infected males to successfully mate with native females, or for liberated *Wolbachia*-infected adults to invade populations targeted for replacement. While studies have assessed the effects of wMel *Wolbachia* infection on *Ae. aegypti* fertility\(^26,38\), no studies have reported how *Wolbachia* influences other female post-mating responses in this important disease vector. We collected *Wolbachia*-infected *Ae. aegypti* from the field in Medellín, Colombia\(^37\), placed it into our laboratory strain and determined the effects of *Wolbachia* infection on *Ae. aegypti* female PMRs.

Previous studies have noted a suppression in fertility when comparing reproductive outcomes between uninfected or infected individuals (i.e., both sexes are infected or uninfected as in ref.\(^26\)). We found that *Wolbachia* lowers fecundity of *Ae. aegypti* females.
regardless of the infection status of their mates but is further suppressed after mating with an infected male. Mating\textsuperscript{43–45}, SFPs\textsuperscript{46} and blood-feeding\textsuperscript{44}, each modify gene expression in the \textit{Ae. aegypti} female reproductive tract, including genes expressed from the female sperm storage organs\textsuperscript{44,45} whose products are essential for fertility\textsuperscript{45,54,76}. Although \textit{Wolbachia} alters protein production from the sperm storage organs in \textit{Drosophila}\textsuperscript{53}, it is unknown if a similar effect occurs in \textit{Ae. aegypti}, which may account for the observed reduction in fertility. \textit{Wolbachia} also had a mating-independent effect on female, but not male, lifespan. The post-mating increase in female longevity was absent in wMel infected females, but longevity of virgin Thai\textsuperscript{Wolb} females was significantly increased. \textit{Wolbachia} increases longevity in other insects, including female \textit{D. melanogaster}\textsuperscript{61} and wMel transinfected \textit{Ae. albopictus}\textsuperscript{62}. The reasons for the increase in longevity are unclear. Insulin signaling is associated with lifespan in a number of organisms\textsuperscript{77}, including \textit{D. melanogaster}, where increased insulin-signaling reduces mated female lifespan\textsuperscript{78}. In \textit{Ae. aegypti}, insulin-like peptides (ILPs) have been implicated in altering female lifespan, with a reduction in ILPs increasing female longevity\textsuperscript{79}. Further analysis is required to determine how \textit{Wolbachia} might interact with insulin signaling, or other pathways, to modulate female lifespan.

We also observed male-specific effects of \textit{Wolbachia} infection. \textit{Wolbachia}-infected males were less successful than their non-infected counterparts at inhibiting remating by their mates. The suppression of re-mating is a key \textit{Ae. aegypti} female PMR. The probability of re-mating is highest within the first 2 h of an initial mating\textsuperscript{41}, but declines with increased time—females are completely refractory by ~20 h after an initial mating\textsuperscript{41} and are do not mate again after this time\textsuperscript{41,47}. Given that SFPs mediate this female PMR\textsuperscript{47}, and that wMel \textit{Wolbachia} changes SFP composition in naturally infected \textit{D, melanogaster} males\textsuperscript{53}, we hypothesized that \textit{Wolbachia} alters SFP composition in \textit{Ae. aegypti}. SFP quantification
detected moderate changes in SFP composition in *Ae. aegypti*. However, our analysis may not have identified proteolytically cleaved SFPs. Proteolysis of SFPs is common^80^ and is often required for, or enhances, SFP function^81^–^83^. Cleavage of SFPs occurs in transit to, or quickly after deposition into the female reproductive tract^83^. The identification of proteolytically cleaved SFPs using bioinformatic methods is difficult without knowledge of the resulting cleavage products. Additionally, post-translational modification of SFPs, which can be necessary for their proper function^84^, may be abnormal in *Wolbachia*-infected males. Further exploration is required to determine why *Wolbachia*-infected males are less able to prevent remating in their mates.

*Wolbachia* infection did not alter the quantity of sperm detected in the male reproductive tract or the quantity of sperm transferred during mating, suggesting that sperm production is unperturbed by *Wolbachia* in the testes. One aspect we did not assess is whether *Wolbachia* might affect sperm quality, as sperm function may be impacted by modifications made by *Wolbachia Cif* proteins during spermatogenesis^56^, or be affected by a potential increase in reactive oxygen species that occurs in the testes of *Wolbachia* infected *Drosophila*^85,86^. Sperm competitive ability is reduced in *Wolbachia*-infected *D. simulans* males^87^, suggesting that an intrinsic property of sperm may be affected by *Wolbachia*. Properties such as length and swim velocity influence sperm competitive outcomes in multiply mated females^88^. The competitive ability of sperm from *Wolbachia*-infected *Ae. aegypti* males needs to be further, examined which may identify subtle defects in sperm ability not detected in our assays.

Our proteomic experiment identified 125 *Wolbachia* proteins that are paternally transferred during mating. Although the wMel proteins responsible for CI establishment are known^56,71^, the molecular mechanism for the phenomenon has not been fully elucidated. Two models
have been proposed for the establishment of CI: host-modification and toxin-antidote. The host-modification model suggests that Cif proteins modify sperm, modifications that are rescued by infected females. The toxin-Antidote model suggests that Cifs are transported to the female via sperm but are inhibited by a rescue factor present in infected females that bind the Cifs and inhibit their toxicity. We did not detect Cif proteins in the ejaculates of Wolbachia-infected males, providing support for the host-modification model. However, we cannot rule out that Cif protein abundance might be low in artificially infected Ae. aegypti, have undergone modification, and/or started to degrade, thereby limiting our detection abilities. CifA and CifB have been detected in mature spermatozoa of wMel-infected D. melanogaster, while CidB has been detected in mature spermatozoa of Culex males naturally infected with wPip. Given that Ae. aegypti are artificially infected with Wolbachia, Cif proteins might not display the same properties observed in naturally infected insects. It would be interesting to examine wMel CifA and CifB localization patterns in developing and mature Ae. aegypti sperm to determine if they behave similarly to that what has been reported in naturally infected D. melanogaster.

Conclusions

Aedes aegypti is a major vector of arboviruses that impacts human health and has a large global territory, making it a target of numerous vector control interventions. One such effort is the release of wMel Wolbachia-infected Ae. aegypti, which has successfully suppressed dengue fever cases where population replacement has been successful. For the continued success of programs that utilize Wolbachia infected individuals, it is essential to understand how Wolbachia interacts with reproductive processes of Ae. aegypti, including the induction of female PMRs. Our results show that Wolbachia alters some female PMRs, with the decrease in male ability to prevent further copulations potentially complicating the efficiency of population suppression programs or the successful establishment of liberated
adults where population replacement is attempted. Continued investigation is necessary to
determine whether the effects of wMel Wolbachia on female PMRs are also observed in
Ae. aegypti transinfected with other Wolbachia strains used in control efforts\textsuperscript{91}, and to
determine the molecular pathways impacted by Wolbachia infection to modify post-mating
behaviors and physiology in female Ae. aegypti.

**Material and Methods**

**Mosquitoes**

Thai\textsuperscript{58}, DsRed\textsuperscript{92}, Acacias\textsuperscript{60}, and Rockefeller strain Ae. aegypti were used in our assays.
DsRed mosquitoes have a transgene that label sperm with the red fluorescent protein
DsRed (Aaβ2t::DsRed)\textsuperscript{92}. Mosquito eggs were hatched under vacuum pressure (-50 kPa)
for 30 min and larvae were reared at a density of 200/L in type II H\textsubscript{2}O supplemented with
four (7.2-8.2 mm) Hikari Gold Cichlid food pellets (Hikari, Himeji, Japan), a feeding
regimen that produces adults of similar size\textsuperscript{48,59}. \textsuperscript{15}N-labeled females were reared with a
yeast slurry (see below). Pupae were transferred to 5 ml tubes to ensure virginity, and
resulting adults were separated into sex-specific cages upon eclosion. Larvae and adults
were kept in incubators at 27°C, 70% relative humidity and a 12:12 h photoperiod. Adults
had access to 10% sucrose *ad libitum*. Four-to-six-day-old adults were used in our assays
except for \textsuperscript{15}N-labeled females, which were mated at 2-days-old. Wing lengths were
measured as in ref.\textsuperscript{93} to estimate individual size; wing lengths of the mosquito strains used
in our assays are shown in Supplementary Figure 1.

**Generation of Wolbachia infected Thai strain Aedes aegypti**

We collected Ae. aegypti infected with the wMel strain of Wolbachia being released in
Medellín, Colombia\textsuperscript{37} (we obtained permission to collect field specimens from the
Secretaria de Salud de Medellín). Ovitraps were placed in the neighborhood of Aranjuez, Medellín and egg-laying substrates were collected weekly. Eggs were hatched by submerging egg-laying substrates in water, and the species of emerging adults were identified using morphological characteristics. *Aedes aegypti* adults from individual ovitraps were allowed to mate and DNA extraction of female progeny was performed as follows: individuals were macerated in 50 μl STE (100 mM NaCl, 10 mM Tris-HCl, pH 8.0. 1 mM EDTA) and 1 μl of proteinase K (20mg/ml; Invitrogen, Waltham, USA) was subsequently added. Samples were incubated at 56°C for 1 h, followed by 95°C for 15 min. Isolated DNA was used to confirm the species using *Ae. aegypti* specific PCR primers (aegF 5′ – CTC TGC GTT GGA TGA ATG AT – 3'; aegR 5′ – ATA GCG TGG TAG CCG TAT G – 3'), and to determine *Wolbachia* infection status using primers specific to the IS5 repeat element (IS5F 5′– GTA TCC AAC AGA TCT AAG C-3'; IS5R 5′– ATA ACC CTA CTC ATA GCT AG – 3'). A *Wolbachia* positive colony was established, and we sequenced a portion of the *wsp* gene using primers reported in ref. (81F – TGG TCC AAT AAG TGA TGA AGA AAC; 691R – AAA AAT TAA ACG CTA CTC CA) and used the Basic Local Alignment Search Tool (BLAST) at https://blast.ncbi.nlm.nih.gov/Blast.cgi to verify that wMel was the infecting *Wolbachia* strain. *Wolbachia* positive females were backcrossed with Thai strain males for 7 generations to produce a *Wolbachia* infected strain in the Thai genetic background. Given the decline in *Wolbachia* density in eggs during storage, we hatched eggs monthly to maintain our colony, and tested 30-40 individuals by PCR to ensure infection status prior to our assays.

Fecundity and fertility assays
Males and females were mass mated in an 8 L cage in a 1:1 male: female ratio (25 females per cage); although a proportion of females remate when mass mated, multiple insemination does not significantly influence fecundity or total sperm stored in Thai
strain females. After 24 h, males were removed, and females were blood-fed on the arm of a volunteer. Blood feeding on human subjects was approved by the Comité de Bioética Sede de Investigación Universitaria (Universidad de Antioquia) and volunteers signed a consent form; all methods were performed in accordance with the relevant guidelines and regulations. Four days after blood-feeding, females were individually aspirated into 50 ml conical tubes with a 13 x 4 cm paper towel strip and 6.5 ml of type II H$_2$O. The strip was removed 48 h later, and oviposited eggs were counted using a ZEISS Stemi 508 stereo microscope (ZEISS, Oberkochen, Germany). Eggs were partially dried and stored in an incubator until hatching 5-7 days later. To hatch eggs, the paper towel strip was placed into a 40 ml cup, filled with type II H$_2$O, supplemented with a pinch of active yeast, and placed under a vacuum for 30 min. Resulting larvae were counted 4-6 days later. Hatch percentage was calculated as larvae/number of eggs; females that laid zero eggs were omitted from the analysis.

**Re-mating assays**

Females were first mated to Thai or Thai$^{\text{Wolb}}$ males in parallel, and then given the opportunity to re-mate with a DsRed male. We observed the first mating by placing a single male and female into an 8 L cage until a copulation occurred, defined as genitalia engagement for $\geq 10$ sec$^{58,59}$. After uncoupling, females were immediately aspirated into a separate 8 L cage with 25 DsRed males until a 1:1 male-female ratio was reached; the second mating opportunity lasted 4 h, after which males were removed. Females were frozen at -80°C until dissections commenced to determine female mating status.

Identification of multiply mated females was determined by dissection of the lower reproductive tract in 1X PBS to detect the presence (re-mated) or absence (not re-mated) of DsRed sperm$^{48,59}$ using a Nikon Eclipse Ti-U fluorescent microscope (Nikon Instruments Inc., Tokyo, Japan).
Sperm quantification

To determine if *Wolbachia* infection alters sperm production, we quantified sperm from the seminal vesicles of virgin males, the organs that store mature sperm transferred to females during mating. To assess total sperm transfer, we quantified sperm from the bursa (where males deposit the ejaculate) immediately after insemination. To assess total sperm stored by mated females, we quantified sperm from the spermathecae, the long-term sperm storage organs, 24 h after mating. Matings to determine sperm transfer were observed to ensure females only copulated once, while matings to determine sperm quantity in the spermathecae were performed as previously described. Our assays utilized adults from the same hatch and matings were performed on the same day. To quantify sperm transfer, females were flash frozen on dry ice immediately after uncoupling. To quantify spermathecal sperm quantity, females were mated and placed in the incubator for 24 h. Adults were stored at -80°C until dissections commenced. Sperm were isolated using a modified protocol reported in ref. Briefly, tissues were dissected in 1X PBS, placed into a 250 μl chamber containing 100 μl of 1X PBS, ruptured with minutiae pins to release sperm and mixed by pipetting up and down. An additional 100 μl of PBS was added and the solution re-mixed. Ten 5 μl aliquots were placed onto a glass slide and dried for 5 min at 50°C. Sperm were fixed in 70% ethanol and sperm nuclei stained with Giemsa dye (Merck, Kenilworth, USA). Sperm in each drop were counted under brightfield illumination at 200X magnification. This subsample was used to calculate total sperm.

Longevity

Females were mass mated in a 1:1 ratio as previously described, and males were removed prior to the start of our assays. Virgin and mated females were placed into separate 8L cages (50 individuals per cage) and kept in the incubator for the duration of
the experiment. Sugar solution was replaced weekly. Dead individuals were removed every three days until all individuals had perished.

**Labeling *Aedes aegypti* with $^{15}$N**

To examine differences in SFP quantities transferred to females by Thai and Thai$^{\text{Wolb}}$ males, we labeled females with the natural isotope heavy nitrogen ($^{15}$N) and identified male-derived proteins transferred at mating as in ref. To briefly, baker’s yeast ($Saccharomyces cerevisiae$; LEVAPAN, Sabaneta, Colombia) was inoculated in 200 mL of minimal medium—20 g/L D-glucose, 1.7 g/L yeast nitrogenous base without amino acids and 5 g/L ammonium sulfate with $^{15}$N (Cambridge Isotope Laboratories, Andover, USA) in sterile water—and incubated on a shaker at 190 rpm and 30°C for 24 h, after which an additional 800 ml of minimal media was added. Yeast was incubated until a density of $10^9$ cells/ml was reached. Yeast was harvested by centrifugation at 8000 rpm at 4°C for 10 min. The pellet was washed with type II H$_2$O, type I H$_2$O, and finally with 1X PBS. Yeast was resuspended in 20 ml of 1X PBS to generate a slurry to feed the mosquito larvae. The slurry was stored at 4°C and used shortly after its preparation.

Thai strain eggs were hatched as previously described, reared at a density of 200/L, and fed with 1 ml of the yeast slurry each day for 5 days. As previous experiments with $^{15}$N-labeled *Ae. aegypti* showed that the first cohort produced adults incapable of flight, larvae were grown in 200 ml of rearing water from a previous cohort and 800 ml type II H$_2$O. Pupae were transferred to 5 ml tubes and resulting adults were separated by sex into 8 L cages upon eclosion.

**Ejaculate collection**
N-labeled females were mated to unlabeled, normally reared Thai\textsuperscript{Wolb} or Thai males. A single male and female were placed into an 8 L cage until a copulation occurred. Mated females were flash frozen on dry ice immediately after uncoupling and stored at -80°C until dissections commenced. Bursae from experimental (mated to Thai\textsuperscript{Wolb} males) and control females (mated to Thai males) were dissected in 1X PBS with protease inhibitors (cOmplete Mini Protease Inhibitor Cocktail; Roche, Basel, Switzerland). Twenty bursae were collected from control and experimental females (three biological replicates each). After dissection, an equal volume of 2X Laemmli buffer + 5% β-mercaptoethanol was added to each sample. Proteins were solubilized by sonicating with a Elmasonic S30H sonicator (Elam, Singhem, Germany) for 30 sec, heated to 95°C for 15 min, and sonicated again for 30 sec. Samples were centrifuged for 10 min at 10,000 x g at 4°C, and the supernatant was placed into a fresh tube and stored at -80°C until shipment to the Cambridge Center for Proteomics (https://proteomics.bio.cam.ac.uk/) for mass spectrometry.

**Tandem Mass Spectrometry Proteomic Characterization**

Solubilized samples were separated by 1D SDS-PAGE to generate six fractions per sample, digested with trypsin, and analyzed by LC-MS/MS on an Orbitrap Lumos mass spectrometer. Peptides were loaded onto a PepMap 100 C18 pre-column (5 µm particle, 100 Å pore, 300 µm × 5 mm, Thermo Scientific) at 10 µL/min for 3 min with 0.1% formic acid. Peptides were separated on a reverse-phase nano EASY-spray C18 analytical column (2 µm particle, 100 Å pore, 75 µm × 500 mm; Thermo Fisher Scientific, Waltham, USA) with a gradient of 1.6% to 32% acetonitrile in 0.1% formic acid over 120 minutes at a flow rate of 300 nL/min. All m/z values of eluting ions (range between 375-1500 Da) were measured at a resolution of 120,000. The MS1 scan was followed by data-dependent MS2 scans (3 second cycle time) to isolate and fragment the most abundant precursor ions at
35% NCE. Fragment ions were measured at a resolution of 15,000. Ions with +1 or unassigned charge were excluded from the analysis, and dynamic exclusion of previously investigated precursor ions was 70s.

Raw spectral data were searched against the *Ae. aegypti* protein database (assembly AaegL5.0), appended with the cRAP v1.0 contaminant database (thegpm.org), using the standard workflow in PEAKS X+ (de novo + PEAKS DB + PEAKS PTM + SPIDER; Bioinformatics Solutions Inc.). Spectral data from all six samples (three control with Thai males and three with ThaiWolb males) were run together in a combined analysis using the following search parameters: mass tolerance of 15 ppm for parent ions and 0.5 Da for fragment ions, carbamidomethylation (C) as a fixed modification, oxidation (M) and deamidation (NQ) as variable modifications, and up to three missed tryptic cleavages. Peptide identifications were filtered to a false discovery rate (FDR) < 1% based on the decoy-fusion approach\(^9\). Protein identifications were filtered to a -10lgP score ≥ 20 and at least one unique peptide-spectrum matches (PSMs). Label free quantitative comparisons were based on the relative abundance of peptide features using the PEAKS Q module (Bioinformatics Solutions Inc., Waterloo, Canada). Additionally, raw spectra from the three samples from matings with ThaWolb males were searched directly against *Wolbachia pipiensis wMel* (UP000008215) resulting in 45,12 PSMs and 125 proteins (Supplementary dataset 1).

**Statistical analysis**

R statistical software version 3.6.1 coupled with R-Studio Version 1.2.1335 was used for all analyses\(^1\). The number of eggs laid by each female (fecundity), seminal vesicle and spermathecal sperm quantity data were first analyzed to determine the probability
distribution that fit the data including normal, negative binomial, and Poisson distributions. The Akaike information criterion (AIC) was used to compare the best distribution that fit the data, where the lowest AIC value corresponds to the best fitted distribution. We also evaluated if each replicate of our assays differed in the characteristics we were examining. Because no significant differences were found between replicates, data from each experiment was combined and replicate used as a random factor in the models. We analyzed fecundity, and seminal vesicle, bursa and spermathecal sperm quantity using a linear mixed model (LMM) using the mating combination as the fixed factor. For hatch percentage, a generalized linear mixed model (GLMM) with a binomial distribution was used with mating combination as a fixed factor.

Re-mating incidence was evaluated by performing the chi-square test of independence based on the contingency table of two variables—Wolbachia infection status and mating status (re-mated and not re-mated)—using the R statistical package and chi q test function. Adult longevity was analyzed using a Kaplan-Meier curve to illustrate the cumulative survival probability over time. Cox proportional hazards (PH) regressions and log-rank tests were used to evaluate differences between mating combinations. Wing sizes were analyzed using a LMM with wing size as the response variable, mosquito strain as a fixed factor and replicate as a random factor in the model.

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Rupinder Kaur for helpful discussion, the Cambridge Center for Proteomics for assistance in our sample preparations, and Ruta N Medellín for laboratory support.

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Author contributions
J.O., F.W.A., C.A.P. and S.D. conceived and designed the experiments; G.R.U. collected Wolbachia-infected individuals from the field; C.B. performed the genetic backcross; J.O. performed the fecundity, fertility, longevity, and receptivity assays; J.O., F.W.A., L.F.R.S. and L.B. performed the sperm quantification assays; S.V.A. labeled female Ae. aegypti with $^{15}$N; F.W.A., C.A.P. and S.V.A. mated $^{15}$N-labeled females with unlabeled males, performed the tissue dissections, and prepared protein extracts for mass spectrometry; J.O., C.C. and S.D. analyzed the data; J.O. and C.C. prepared the figures, F.W.A. wrote the manuscript, and all authors reviewed and approved the manuscript.

Data Availability
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository$^{101}$ with the dataset identifier PXDxxxxxx (submission pending).

Competing interests
The authors declare no competing interests.


58. Alfonso-Parra, C. et al. Synthesis, depletion and cell-type expression of a protein


76. Shaw, W. R. *et al.* Mating activates the heme peroxidase HPX15 in the sperm...


830 95. Tripet, F. *et al.* Competitive reduction by satyrization? Evidence for interspecific...


Supplementary Figure 1. Wing lengths of female (A) and male (B) mosquito strains used in our assays. Different letters above the box plots indicate a significant difference (p < 0.05) for a post hoc Tukey test. Females displayed significant differences in sizes (DF = 3, F = 19.83, p = 3.39e-11), with Rockefeller females significantly larger than Thai, \textit{Thai}^{Wolb}, and Acacias females. No differences were observed in sizes between \textit{Thai}^{Wolb} and Thai males or females (females: p = 0.1; males: p = 0.33).
Supplementary Figure 2. *Wolbachia* infection does not impact longevity of *Aedes aegypti* males. Longevity of Thai and Thai$^{\text{Wolb}}$ males.
### Seminal proteins identified only in Thai<sup>Wolb</sup> male ejaculates

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### Seminal proteins identified only in Thai male ejaculates

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**Supplementary Table 1.** Seminal proteins identified only in the ejaculate of *Wolbachia* infected males (top) or in the ejaculates of control males (bottom). Only proteins that had a PSM ≥ 5 were considered in this analysis.
### Cl-phenotype associated proteins

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### WO phage proteins

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**Supplementary Table 2.** Paternally transferred *Wolbachia* proteins that modify Cl-phenotypes and/or that are part of the phage WO sequences or WO-like Islands. Phage WO proteins that are associated with the Eukaryotic Association Module are shown in **bold.**
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DatasetS1.xlsx
- RS324.pdf