MOSQUITO CONTROL

Changes in the microbiota cause genetically modified *Anopheles* to spread in a population

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The mosquito's innate immune system controls both *Plasmodium* and bacterial infections. We investigated the competitiveness of mosquitoes genetically modified to alter expression of their own anti-*Plasmodium* immune genes in a mixed-cage population with wild-type mosquitoes. We observed that genetically modified mosquitoes with increased immune activity in the midgut tissue did not have an observed fitness disadvantage and showed reduced microbial loads in both the midgut and reproductive organs. These changes result in a mating preference of genetically modified males for wild-type females, whereas wild-type males prefer genetically modified females. These changes foster the spread of the genetic modification in a mosquito cage population.

Mosquitoes act as vectors for numerous human diseases, notably malaria. Insecticides, medications, bed nets, and environmental modification have substantially reduced malaria transmission. However, resistance of mosquitoes to insecticides and of the parasite to drugs, coupled with the cost and complexity of deploying all interventions, has hampered malaria control efforts. One increasingly attractive complementary approach is the self-propagating replacement of natural mosquito populations with *Plasmodium*-refractory genetically modified (GM) mosquitoes. If implemented optimally, replacement could facilitate the removal of malaria with a limited need for participation by the local human population and no adverse ecological effects. Numerous GM mosquito lines with differing resistance to *Plasmodium* have been developed (*1*–*4*) but have yet to be deployed as part of a malaria-control program due to incomplete resistance, concerns about community acceptance of GM mosquitoes, and fears of negative fitness effects caused by the genetic modification. Release strategies and genetic drive systems have been developed to overcome possible negative fitness effects caused by the genetic modification (*5*, *6*), but the creation of GM mosquitoes that can compete with wild-type (WT) mosquitoes without a gene driver would greatly ease the replacement of natural populations. Therefore, we investigated the potential for driving parasite-resistant mosquitoes into the population by changing their mating preferences.

The mosquito innate immune system can combat a variety of human and mosquito pathogens. We have previously created multiple GM *Anopheles stephensi* lines that are resistant to *Plasmodium falciparum* due to up-regulation of mosquito immune genes in the midgut or fatbody after a blood meal, using the carboxypeptidase (Cp) or vitellogenin (Vg) promoter, respectively (*3*, *4*). These strains possess elevated anti-*Plasmodium* and antibacterial activities through either the immune-deficiency pathway–associated NF-κB transcription factor Rel2 (*3*) or the Down syndrome cell-adhesion molecule (AgDscam) splice form AgDsPf (*4*). The GM lines were backcrossed with the original WT stock for five generations (*3*, *4*) and have been continually reared under the same conditions to ensure the same genetic and environmental background (*7*). As with earlier studies of similar GM mosquitoes (*3*, *4*, *8*), four of the lines tested showed no effect of the genetic modification on mosquito life span under various conditions, the number of eggs laid, the egg hatch rate, or the sex ratio, though pupation time was delayed in GM mosquitoes (Fig. 1 and figs. S1 and S2). One line ($CpDsPfs_{11}$) exhibited some fitness costs (Fig. 1 and fig. S1), but these effects are the result of a position effect, as another line ($CpDsPfs_3$) carrying the same transgene in a different chromosomal location lacked adverse effects (Fig. 1, fig. S1, and table S1). Finally, there was no difference between any of the five GM strains and WT mosquitoes in terms of size or the amount of protein consumed by a female during her first blood meal (fig. S3). Together, these data suggest that transient activation of specific anti–*P. falciparum* and antibacterial immune mechanisms has little, if any, effect on life history traits under laboratory conditions.

We then tested the competitiveness of the two lines overexpressing transgenes in the midgut tissue with the highest *Plasmodium* resistance—$CpRel2_{15}$ and $CpDsPfs_3$—along with the fatbody-specific line, $VgRel2_1$ (*3*, *4*). We combined equal numbers of WT and GM larvae or adults and maintained a constant population for 10 generations, measuring the GM frequency of each generation. GM prevalence rose to ~90% during the first generation and remained at that level for all 10 generations (Fig. 2, A to D). The proportion of GM offspring was higher than expected under the assumptions of Hardy-Weinberg equilibrium (75%) (*9*), indicating that GM mosquitoes have an advantage (Fig. 2, C and D). These lines have been established as homozygous for the genetic insert, and our insert location sequencing indicated that each line had only a single transgene insertion (table S1). This phenomenon was observed only in strains expressing the transgene in the midgut tissue, not in the strain expressing *Rel2* in the fatbody (Fig. 2, C and D, and fig. S4). Our mosquitoes were not exposed to *Plasmodium*-infected blood, indicating that some other effect (distinct from parasite infection) of the midgut-specific immune overexpression was responsible for the GM advantage (*8*, *10*).

The two anti-*Plasmodium* transgenes, *Rel2* and *DsPfs*, also increase resistance to bacteria (*3*, *4*). Changes in the microbiota of *Drosophila melanogaster* alter their mating preference (*11*–*14*). To test whether changes in the mosquito microbiota could have influenced mating, we crossed WT and GM mosquitoes reared with or without antibiotics (*15*, *16*). Upon antibiotic treatment, the offspring exhibited nearly 75% transgene prevalence, as expected by a Hardy-Weinberg equilibrium (Fig. 2, C and D, and fig. S4). However, this effect was reduced over multiple generations, partly because the bacterial population was reestablished during the larval stage, despite treating the adults continuously with antibiotics (fig. S5). The mosquitoes may also have adapted to the changes in their microbiota and altered their mating behavior. When septic adult mosquitoes were mixed at a higher initial WT:GM ratio, the percent of GM mosquitoes similarly rose to >75% over multiple generations (fig. S6).

Using the $CpRel2_{15}$ line, we crossed virgin GM males with virgin WT or GM females under septic or aseptic conditions. Insemination rates for non–egg-laying females did not differ between crosses, and the number of multiple matings was low, so the number of egg-laying females was used as a proxy for insemination (tables S2 and S3). When the female mosquito microbiota was intact, $CpRel2_{15}$ males preferred to mate with WT females (Fig. 2E). This was not a 100% preference, which explains the remaining 10% of WT mosquitoes in mixed-cage populations. The slight increase in pupation time of GM mosquitoes could favor the persistence of a small number of WT mosquitoes (fig. S2C). Microbial removal by antibiotic treatment abolished this preference (Fig. 2F). WT males showed

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a mating preference for GM females, and this preference was also weaker in the absence of the microbiota (Fig. 2, G and H). With an intact microbiota, both WT and GM males prefer to mate with females of the opposite type, which explains the rapid spread of the transgene into the population. With antibiotic treatment, the reduction of male preference leads to the normalization of population dynamics (Fig. 2, C and D). Opposite crosses showed that females have no mating preference between WT and GM males (Fig. 2I). Because all offspring of GM females carried the transgene, we were unable to measure their mating preferences.

Cultivable bacteria in the midguts of sugar- and blood-fed female mosquitoes were measured, and both $CpRel2_{15}$ and $CpDsPfs_3$ females had significantly reduced numbers of bacteria compared with WT females. By contrast, the fatbody-specific Rel2-overexpressing mosquitoes showed no difference (Fig. 3, A and B, and fig. S7). The differential effects of midgut- and fatbody-specific immune activation on the GM mosquito prevalence in mixed populations, mating preference, and microbiota corroborate a link between the GM-mediated bacterial suppression and changes in mating preference.

Deep sequencing of the 16*s* ribosomal RNA (rRNA) gene from the midguts and ovaries of $CpRel2_{15}$, $VgRel2_1$ and WT females yielded a non-significant increase in alpha diversity in $CpRel2_{15}$ midguts and reduced diversity in $VgRel2_1$ midguts relative to WT mosquitoes (fig. S8), though the distribution of the top 40 bacterial genera changed (fig. S9). By contrast, $CpRel2_{15}$ midguts had significantly higher Shannon diversity than did $VgRel2_1$ midguts (fig. S8). $CpRel2_{15}$ ovaries showed a significant decrease in Shannon diversity and observed bacterial diversity (fig. S10) and changes in the distribution of the top 40 bacterial genera (fig. S11), indicating that the genetic modification substantially alters the populations of bacteria in the mosquitoes. Experimental colonization of $CpRel2_{15}$ mosquitoes with a cocktail of the three most common cultivable bacteria (from WT midguts) through exposure in the larval rearing water and adult sugar meals resulted in a level of total cultivable midgut bacteria similar to that of WT midguts and removal of the mating advantage (fig. S12).

Ovaries of non–blood-fed female GM mosquitoes had significantly lower bacterial 16*S* rRNA levels as compared with WT mosquito ovaries, whereas the testes of $CpRel2_{15}$ males had slightly higher levels of bacterial rRNA than did WT male testes (Fig. 3C). Expression of the inserted gene, *Rel2*, was higher in the reproductive tracts of both male and female GM mosquitoes versus WT mosquitoes, and there was measurable endogenous CP expression in WT mosquito reproductive organs, suggesting that the CP promoter is

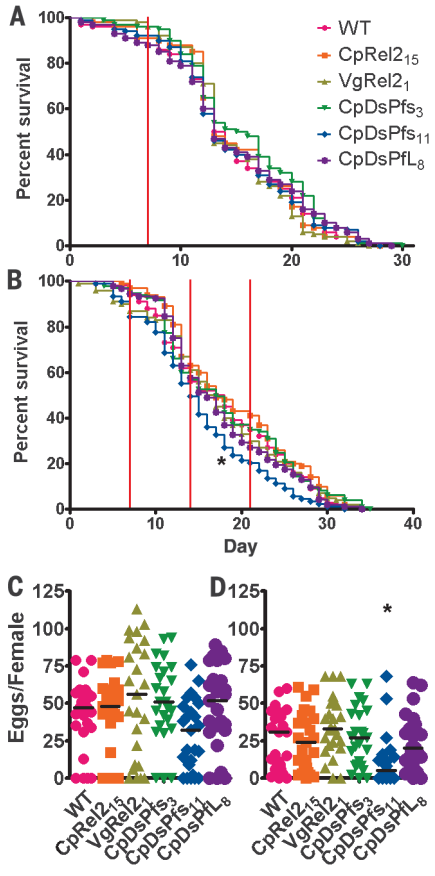

Fig. 1. GM mosquitoes have similar life span and fecundity to WT mosquitoes. (**A**) No female mosquitoes showed a significant difference in life span under standard rearing conditions when provided one blood meal, whereas (**B**) $CpDsPfs_{11}$ females had a reduced life span when provided three blood meals. The asterisk indicates a significant difference in life span compared with WT mosquitoes, measured by a log-rank test with $\alpha = 0.05$. (**C**) During the first gonotrophic cycle, there was no difference in the number of eggs laid by any of the tested strains. (**D**) However, during the second gonotrophic cycle, $CpDsPfs_{11}$ females laid significantly fewer eggs than did WT mosquitoes. The asterisk indicates a significant difference from the wild type, as determined by a Kruskal-Wallis test followed by Dunn's multiple comparison test, $\alpha < 0.05$. A schematic of the experimental design is provided in fig. S13.

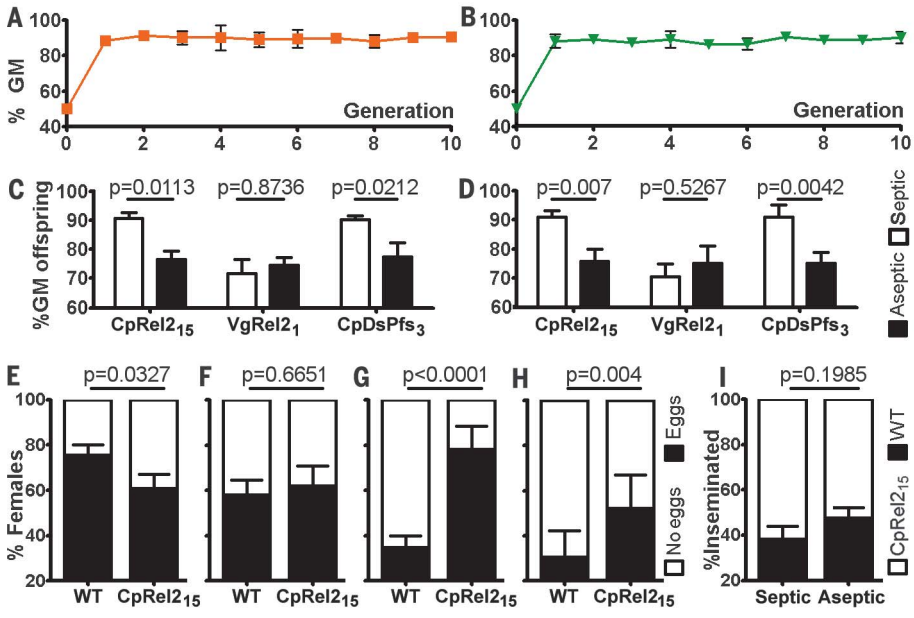

Fig. 2. GM males prefer mating with WT females with an intact microbiota. When started with equal numbers of WT and GM larvae, a 90% GM mosquito ratio arose and persisted for at least 10 generations in populations of WT mosquitoes crossed with either the (**A**) $CpRel2_{15}$ or (**B**) $CpDsPfs_3$ lines. $CpRel2_{15}$ and $CpDsPfs_3$, but not $VgRel2_1$, mosquitoes crossed with WT mosquitoes have >75% GM offspring, whether crosses were started with (**C**) larvae or (**D**) adults. When $CpRel2_{15}$ GM males were given a choice between mating with WT and GM females, a higher percentage of WT than GM females laid eggs when the mosquito (**E**) microbiota was intact, but not when (**F**) the microbiota was removed by antibiotic treatment. When WT males were given a choice between mating with WT and GM females, a higher percentage of $CpRel2_{15}$ than WT females laid eggs, and this preference was stronger (**G**) in the presence than (**H**) in the absence of the microbiota. (**I**) When WT females were given a choice between mating with WT or $CpRel2_{15}$ GM males, there was no significant difference in the proportion of WT females inseminated by WT or GM males under either septic or aseptic conditions. *P* values from Fischer's exact test were used to compare proportions. Error bars indicate SEM. Schematics of the experimental design are provided in figs. S14 and S15.

active in these organs and resulting in a reduced bacterial load in GM ovaries (Fig. 3, D and E). These data show a complex interrelation between the mosquito immune system, microbiota, and mating behavior. Many factors, such as the size and age of the mosquito (*17*, *18*) and olfactory cues, affect mosquito mating, and a relationship between an insect's immune system and its microbiota has been established in other insects (*11*, *19*).

Finally, GM mosquitoes from the 10th generation of the mixed-cage populations were significantly more resistant to *P. falciparum* infection than the 10% of WT mosquitoes remaining in the crossed cages (Fig. 4, A and B). This result, along with the fact that $CpRel2_{15}$ and $VgRel2_1$ mosquitoes have been cultured for more than 50 generations without losing their resistance, indicates that the resistance afforded by the genetic modification is very stable, even when the GM mosquitoes repeatedly mate with WT mosquitoes.

We have shown that modulation of the microbiota of *P. falciparum*–resistant immune-enhanced GM mosquitoes renders them more competitive than WT mosquitoes through mating preference in mixed-cage populations (Fig. 4C). A recent study identified distinct bacterial populations in the male and female reproductive organs, indicating that differences in the microbiome may influence mating in nature (*19*). Multiple studies have also shown that the *D. melanogaster* microbiota alters fly mating patterns (*11*–*14*). The microbiota of cage-reared mosquitoes differs from that of wild mosquitoes, meaning that this mating preference needs to be studied in semi-field and field conditions to ensure that it applies in nature. The stability of the mixed-cage populations at 90% GM may reflect WT alleles that modulate microbial load or other processes that influence mating. Though the incomplete *P. falciparum* resistance of these GM mosquitoes may warrant improvement, laboratory and modeling studies suggest that a 35% transmission-blocking effect would eliminate hypoendemic malaria (*20*). For example, expressing multiple or stronger anti-*Plasmodium* transgenes could maximize their epidemiological impact, and we could then select for GM mosquitoes without delayed pupation. Although our experiments were performed in laboratory cages, the study shows that the use of proper promoter-transgene combinations to modulate the mosquito microbiota, while conferring resistance to the malaria parasite, can facilitate the spread of GM mosquitoes in a population.

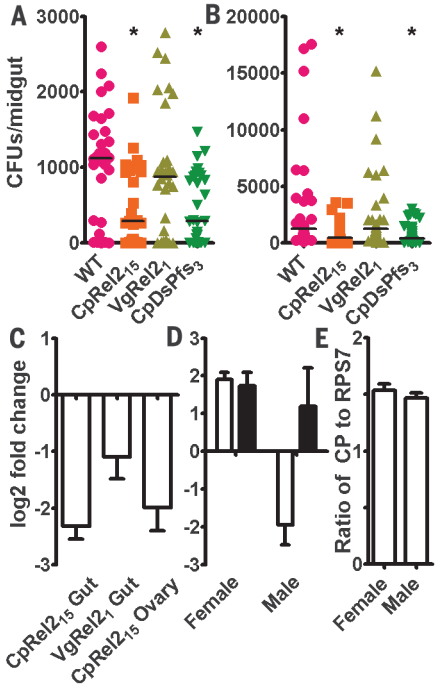

Fig. 3. The mosquito microbiota is disrupted by genetic modification. $CpRel2_{15}$ and $CpDsPfs_3$, but not $VgRel2_1$, GM mosquitoes have reduced numbers of cultivable bacteria in the midgut when compared with WT mosquitoes, both (**A**) before and (**B**) after a blood meal. The asterisk indicates a significant difference from the wild type, as determined by a Kruskal-Wallis test followed by Dunn's multiple comparison test, $\alpha < 0.05$ (table S4). CFUs, colony-forming units. (**C**) This reduction in bacterial levels relative to WT mosquitoes also occurs in non–blood-fed female $CpRel2_{15}$ GM gut and reproductive organs, as shown by an approximately 2 $\log_2$ decrease in 16S rRNA levels, whereas $VgRel2_1$ had a much lower decrease in 16s rRNA. (**D**) *Rel2* expression was also slightly elevated in the ovaries of non–blood-fed female $CpRel2_{15}$ mosquitoes, regardless of the presence of bacteria, and was slightly reduced in the testes of septic (open bars) but not aseptic (filled bars) males. (**E**) We detected low but measurable expression of carboxypeptidase in the reproductive organs of WT mosquitoes, indicating that genes under the control of this promoter can be active in these organs. CP, carboxypeptidase. Error bars indicate SEM. A schematic of the experimental design is provided in fig. S13.

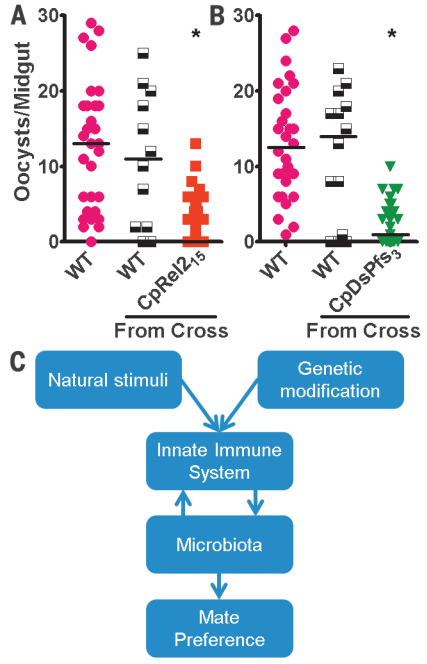

Fig. 4. GM mosquitoes maintain resistance to *Plasmodium*. After 10 generations of crossing, both the $CpRel2_{15}$ (**A**) and $CpDsPfs_3$ (**B**) strains maintain their *P. falciparum* resistance. The asterisk indicates a significant difference from the wild type, as determined by a Kruskal-Wallis test followed by Dunn's multiple comparison test, $\alpha < 0.05$. A schematic of the experimental design is provided in fig. S14. (**C**) Model of the interactions between the mosquito immune system, bacteria, and mate choice. The mosquito immune system decreases the mosquito microbiota, whether regulated by genetic modification or occurring naturally. This microbiota, in turn, affects both the insect innate immune system and mosquitoes' mate choices. These mate preferences lead to an altered prevalence of GM mosquitoes with increased immune activity. Arrows indicate interactions that may be positive or negative.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/357/6358/1396/suppl/DC1
Materials and Methods
Figures S1 to S16
Tables S1 to S5
References (*21–23*)

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Getting to the guts of mosquito control

Malaria persistently evades our best efforts to eliminate it. Pike *et al.* genetically modified malaria vector mosquitoes to be more immune-resistant to infection by the parasite, which altered the composition of the mosquitoes' gut bacteria. Genetically modified male (female) mosquitoes preferentially mated with wild-type females (males). Ten generations later, the genetically modified mosquitoes constituted 90% of a caged population without losing resistance to the malaria parasite. In an alternative strategy, Wang *et al.* engineered mosquitoes' gut bacteria. A strain of nonpathogenic bacteria, AS1, was both sexually and transgenerationally transmitted. The strain infected a laboratory population of mosquitoes and persisted for at least three generations. AS1 engineered to inhibit malaria parasite development in the midgut could do so without handicapping the mosquitoes.

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