Wolbachia uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector Aedes aegypti

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The obligate endosymbiont Wolbachia pipiditis is found in a wide range of invertebrates where they are best known for manipulating host reproduction. Recent studies have shown that Wolbachia also can modulate the life span of host insects and interfere with the development of human pathogens in mosquito vectors. Despite considerable study, very little is known about the molecular interactions between Wolbachia and its hosts that might mediate these effects. Using microarrays, we show that the microRNA (miRNA) profile of the mosquito, Aedes aegypti, is significantly altered by the wMelPop-CLA strain of W. pipiditis. We found that a host miRNA (aae-miR-2940) is induced after Wolbachia infection in both mosquitoes and cell lines. One target of aae-miR-2940 is the Ae. aegypti metalloprotease gene. Interestingly, expression of the target gene was induced after Wolbachia infection, ectopic expression of the miRNA independent of Wolbachia, or transfection of an artificial mimic of the miRNA into mosquito cells. We also confirmed the interaction of aae-miR-2940 with the target sequences using GFP as a reporter gene. Silencing of the metalloprotease gene in both Wolbachia-infected cells and adult mosquitoes led to a significant reduction in Wolbachia density, as did inhibition of the miRNA in cells. These results indicate that manipulation of the mosquito metalloprotease gene via aae-miR-2940 is crucial for efficient maintenance of the endosymbiont. This report shows how Wolbachia alters the host miRNA profile and provides insight into the mechanisms of host manipulation used by this widespread endosymbiont.

Estimated to infect more than 65% of all insect species, Wolbachia pipiditis are maternally inherited, Gram-negative endosymbiotic bacteria (1, 2). They manipulate host reproductive systems through a variety of strategies, including cytoplasmic incompatibility, male killing, feminization, and parthenogenesis (3), and also provide direct mutualistic benefits to hosts in certain contexts (4–6). Recently, a life-shortening W. pipiditis strain (wMelPop-CLA) was successfully introduced into Aedes aegypti (7), the main mosquito vector of dengue viruses. Mosquitoes carrying this Wolbachia strain exhibit an ~50% reduction in adult female lifespan (7), as well as altered feeding success (8, 9) and activity levels (10). Interestingly, A. aegypti infected with Wolbachia show significantly reduced replication of arboviruses, such as dengue (8, 11) and Chikungunya (8), as well as filarial nematodes (12) and Plasmodium (8). Despite considerable advances over the last 20 years in understanding the descriptive phenomenology of Wolbachia infections and their almost ubiquitous distribution in insects, relatively little is known about the underlying mechanisms that the bacteria use to mediate their diverse effects on hosts.

MicroRNAs (miRNAs) are nonprotein coding 18- to 25-nucleotide RNAs that play significant roles in regulating a range of cellular processes, including development, differentiation, apoptosis, and immunity (13, 14). Within the past decade, more than 10,000 miRNA sequences from plants, animals, insects, protozoans, and viruses have been deposited in miRNA databases (15). Dramatic changes in the expression levels of cellular miRNAs have been documented recently in response to bacterial and viral infections in animals and plants. For example, infection with the bacterium Helicobacter pylori induces expression of miR-155, which regulates the cAMP pathway in T cells both in vitro and in vivo (16). H. pylori infection also down-regulates miR-218, resulting in an increase in gastric carcinogenesis (17). Bacterial lipopolysaccharides (Salmonella and Escherichia coli) lead to the induction of miR-155, miR-132, and miR-146a expression in immune cells (18). miR-218 directly targets NF-κB, and overexpression of miR-218 has been shown to inhibit cell proliferation and apoptosis. In human cells, the Epstein–Barr virus (EBV) has been shown to trigger miR-21, miR-155, and miR-146a expression, which might be involved in the development of EBV-associated Burkitt’s lymphoma (19). A liver-specific miRNA, miR-122, targets the 5′ UTR of hepatitis C virus and enhances virus replication (20). Similarly, HIV uses host miRNAs that target its genes to its own advantage (21).

Recently, 86 distinct miRNAs were reported from Ae. aegypti after large-scale deep sequencing of small RNAs (22). The role of these miRNAs in the mosquito’s biology is largely unknown, however. Here we investigated differential expression of cellular miRNAs in Ae. aegypti infected with the Wolbachia strain wMelPop-CLA. Microarray and Northern blot analyses indicated differential expression of a number of cellular miRNAs. We analyzed one of the induced miRNAs, aae-miR-2940, at the functional level and found that it regulates expression of a mosquito host gene, metalloprotease m41fsh. Silencing of the target gene and inhibition of aae-miR-2940 using a synthetic inhibitor lead to reduced Wolbachia replication and density in cells and mosquitoes, suggesting that the endosymbiont manipulates the host gene via induction of this cellular miRNA to replicate efficiently in the host.

Results

Wolbachia Infection Leads to Differential Expression of Mosquito miRNAs. We tested the hypothesis that Wolbachia-infected Ae. aegypti would display differential expression of cellular miRNAs using miRNA microarrays covering 386 insect miRNAs (miRBase v.14) and 105 Ae. aegypti-specific custom miRNAs (22). miRNA expression levels were compared between wMelPop-CLA (+Wol) infected and tetracycline-treated (−Wol) females. miRNA expression and overexpression of miR-218 has been shown to inhibit cell proliferation and apoptosis. In human cells, the Epstein–Barr virus (EBV) has been shown to trigger miR-21, miR-155, and miR-146a expression, which might be involved in the development of EBV-associated Burkitt’s lymphoma (19). A liver-specific miRNA, miR-122, targets the 5’ UTR of hepatitis C virus and enhances virus replication (20). Similarly, HIV uses host miRNAs that target its genes to its own advantage (21).

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showed a significant change in expression level ($P < 0.01$; $t$ test) in +Wol mosquitoes compared with −Wol mosquitoes (Fig. 1), some being homologs represented from different species (e.g., miR-989). Northern blot analyses were used to validate the microarray results specifically for *Ae. aegypti* custom miRNAs (Fig. 2). The 13 *Ae. aegypti* miRNAs that showed significant differential expression in the microarray analysis were analyzed by Northern hybridization to confirm their expression status. Two of the *Ae. aegypti* miRNAs that did not show significant differential expression ($P > 0.05$; aae-miR-2943-1 and −275, not included in Fig. 1) were also analyzed by Northern hybridization to confirm their expression status. Let-7 was also included in the Northern blot analysis because it is a conserved miRNA. In addition, three *Ae. aegypti* miRNAs (aae-miR-2940, −309a-2, and −970) appeared on miRBase after the microarray analysis was carried out and were included in the hybridization analyses. Densitometry graphs comparing the intensity of Northern blot signals in +Wol and −Wol samples are shown in Fig. S1. Northern blot analyses were replicated only once for all miRNAs except aae-miR-2940, which was replicated at least five times as it was chosen for further analysis; therefore, the statistics presented in Fig. S1 are based on differences in the densitometry readings, not on differences between biological replicates. Two miRNAs, aae-miR-2940 and aae-miR-309a-2, showed exclusive induction in +Wol mosquitoes, whereas aae-miR-2943-1 and aae-miR-970 at 4 and 12 d after emergence and aae-miR-308* and aae-miR-2941-2 at 4 d after emergence were up-regulated in +Wol mosquitoes relative to −Wol mosquitoes (Fig. 2). Aae-miR-989, aae-miR-210, and aae-miR-988 expression was down-regulated in +Wol mosquitoes compared with −Wol mosquitoes at 12 d postemergence. Expression levels of the remaining miRNAs remained the same. Aae-miR-970 expression was down-regulated in both +Wol and −Wol mosquitoes at 12 d postemergence, suggesting that its expression might be age-related (Fig. 2). Aae-miR-286a-1 did not produce a signal on Northern hybridization even after several attempts, perhaps due to a low expression level not detectable by this technique. Putative targets of miRNAs analyzed by Northern hybridization were identified by bioinformatic analysis (Table S1).

**Fig. 1.** *Wolbachia* infection alters adult mosquito cellular miRNA expression profile, as determined using microarrays. Expression profiles of miRNAs that were significantly up- or down-regulated in 4-d-old (A) and 12-d-old (B) *Wolbachia*-infected female *Ae. aegypti* (+Wol) compared with uninfected (−Wol) female mosquitoes are shown. MFI, mean fluorescent intensity. The corresponding miRNA microarray color map shows expression levels of miRNAs in −Wol and +Wol samples. Each miRNA was replicated seven times on each chip. Red and green indicate up-regulation and down-regulation of expression, respectively.

**Fig. 2.** Validation of expression profile of miRNAs differentially expressed in mosquitoes. Aae-miRs that were significantly up-regulated or down-regulated in *Wolbachia*-infected (+Wol) and uninfected (−Wol) 4-d-old and 12 d-old females were analyzed by Northern hybridization with probes specific to each miRNA. The same blot was used multiple times after removal of probes. U6 was used as a control to demonstrate equal loading of samples.

**Aae-miR-2940 Regulates Expression of a Mosquito Metalloprotease Gene.** Induction of aae-miR-2940 only in *Wolbachia*-infected mosquitoes led us to investigate its possible function in the host-bacterium interaction. Using BLAST searches of the *Ae. aegypti* genome, we identified the candidate target gene, metalloprotease m41 ftsh (GenBank ID: XM_001660643), and subsequently confirmed it with the RNAHybrid and RNA22 software. Target sequences with complete complementarity to the aae-miR-2940 seed region were predicted in the 3′ UTR of the metalloprotease gene at nucleotides 2123–2145 (Fig. 3A). Using Northern blots with specific probes binding to the target gene, we found that transcript levels of the metalloprotease were induced in +Wol mosquitoes compared with −Wol mosquitoes (Fig. 3B). The difference was consistent with the up-regulation of aae-miR-2940 in +Wol mosquitoes compared with −Wol mosquitoes (Fig. 2), suggesting that by binding to the 3′ UTR of the target gene, the miRNA could enhance mRNA transcript levels and/or the stability of the metalloprotease mRNA.

We performed two additional independent experiments to confirm the specific interaction of aae-miR-2940 with the metalloprotease m41 ftsh target sequences and resulting transcript up-regulation. In the first experiment, we initially confirmed the expression of aae-miR-2940 in an *Ae. albopictus* cell line previously infected with the wMelPop-CLA strain (C6/36-wMelPop-CLA; ref. 23) and confirmed its reduction in *Wolbachia*-uninfected C6/36 cells (Fig. 4A). Compared with whole −Wol mosquitoes, in which aae-miR-2940 was not present in detectable levels on Northern hybridization (Fig. 2), a low level of the miRNA was detected in C6/36 cells in the absence of *Wolbachia* (Fig. 4A). This could be related to the fact that cell lines are usually clones of a single type of cell from an organism; C6/36 cells are derived from the salivary glands. Differential expression of miRNAs in...
various tissues within an insect is well established (24). As a result, when whole mosquitoes are analyzed, a mixture of tissues is analyzed that might not contain detectable quantities of transcripts. However, up-regulation of the target gene was distinctly greater in C6/36.wMelPop-CLA cells than in control C6/36 cells (Fig. 4A). We cloned both target and mutated target sequences (in nucleotides complementary to the aae-miR-2940 seed region) from the 3′ UTR of the metalloprotease gene, along with their flanking nucleotides, downstream of the GFP gene in the pIZ expression vector (Fig. 4B), resulting in the constructs pIZ/GFP-target and pIZ/GFP-AΔtarget, respectively. In another control construct, pIZ/GFP-AΔ2target, the complementary sequences to the aae-miR-2940 seed region were deleted from the target sequences (Fig. 4B). The constructs were transfected into C6/36. wMelPop-CLA cells. After 48 h, significantly higher GFP transcript levels were detected in cells transfected with pIZ/GFP-target compared with those transfected with pIZ/GFP-AΔtarget or pIZ/GFP-AΔ2target (P < 0.0001, ANOVA) (Fig. 4B).

In a second, independent experiment, we transfected *Ae. aegypti* Aag2 cells with a specific synthetic inhibitor of aae-miR-2940; the sequence is provided in Materials and Methods. Control cells were transfected with an inhibitor of random miRNA sequence. After 48 h, we observed lower transcript levels of the mosquito metalloprotease gene in cells transfected with the miRNA inhibitor compared with cells transfected with the control inhibitor (Fig. 4C). These results confirm that aae-miR-2940 specifically targets the metalloprotease gene and induces its expression.

**RNAi-Mediated Silencing of Metalloprotease Reduces Wolbachia Density.** We tested whether the metalloprotease gene is critical to *Wolbachia*’s persistence in insect cells using in vitro synthesized dsRNA specific to the gene’s coding region in an RNAi experiment. Equal numbers of *Wolbachia*-infected Aag2 (Aag2. wMelPop-CLA) cells were transfected with dsRNA of metalloprotease and with dsRNA of GFP and mock infection as controls. RT-qPCR confirmed silencing of metalloprotease (Fig. 5A). Using live/dead cells staining with Trypan blue, we confirmed that the metalloprotease knockdown had no substantial effect on cell viability, with ~1% of cells dead across all treatments at the time of sampling. qPCR using the *Wolbachia*-specific wsp gene showed a significant reduction in density of the bacteria in the metalloprotease-silenced cells compared with control cells at 72 h posttransfection (P = 0.0415, t test) (Fig. 5B). To examine whether the same effect could be reproduced in mosquitoes, +Wol mosquitoes were mock-injected (with sterile water), injected with dsRNA specific to GFP (control), or

**Fig. 3.** Aae-miR-2940 target prediction and expression levels of the corresponding target. (A) The *Ae. aegypti* metalloprotease gene was predicted to be the best target with complete seed region (underlined) complementarities. The target sequence was identified in the 3′ UTR of the metalloprotease gene. (B) Northern blot analysis of total RNA extracted from *Wolbachia*-infected (+Wol) and uninfected (–Wol) female *Ae. aegypti* at 4 d and 12 d after emergence. The blot was hybridized with a probe specific to the metalloprotease (metallo) gene. rRNA is shown to indicate equal loading of samples.

**Fig. 4.** Target validation of aae-miR-2940. (A) Northern blot analysis of expression of aae-miR-2940 in *Wolbachia*-infected C6/36.wMelPop-CLA (+Wol/C6) and uninfected C6/36 (C6) cells. (B) Cloning strategy of the metalloprotease (metallo) target, mutated target, and target with deleted sequences complementary to the miRNA seed region from the metalloprotease 3′ UTR under the GFP reporter gene ORF in the pIZ vector, denoted as pIZ/GFP target, pIZ/GFP-AΔ1 target, and pIZ/GFP-AΔ2 target constructs, respectively. Constructs were transfected into C6/36.wMelPop-CLA cells that overexpress aae-miR-2940. RT-qPCR analysis indicated that GFP expression was significantly higher (P < 0.0001) in pIZ/GFP-target (metallo) transfected cells than in pIZ/GFP-AΔ1 target (mut) and pIZ/GFP-AΔ2 target (no seed) transfected cells. Target sequences are underlined, and seed region complementary sequences are shown in bold italic type. (C) RT-PCR analysis of metalloprotease gene expression in Aag2. wMelPop-CLA cells at 48 h posttransfection with a synthetic inhibitor of aae-miR-2940 (1) and a control inhibitor with random sequences (2). Actin gene expression was analyzed in both experiments as a loading control.
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We focused on aae-miR-2940 for functional analyses. We found

one potential target in the 3’ UTR of an Ae. aegypti gene, metallo-

protease 41 ftsh. Transcript levels of the target gene analyzed

by Northern hybridization showed significant up-regulation in

Wolbachia-infected mosquitoes. We confirmed the interaction of

aae-miR-2940 with the 3’ UTR target sequence construct was expressed in C6/36.wMelPop-CLA cells. Interestingly, RNAi-mediated silencing of the metalloprotease gene in both Aag2.wMelPop-CLA cells and +Wol mosquitoes led to a significant decline in Wolbachia density, suggesting a critical role for this protein in the maintenance of the Wolbachia infection in mosquito cells.

In most scenarios, interaction of miRNA with its target leads to the suppression of the target gene by either degradation of the target miRNA or inhibition of translation (25). However, in addition to the aae-miR-2940 reported in this study, there are a few examples in which up-regulation of the target gene has been documented. An miRNA discovered in mouse embryonic stem cells, miR-4661, up-regulates both mRNA and protein expression of its target gene IL-10 in Toll-like receptor–triggered macrophages (26). miR-4661 competitively binds to the 3’ end of IL-10 mRNA. In another example, involving a pathogen, hepatitis C virus uses a liver-specific miRNA, miR-122, to enhance viral replication (27, 28). miR-122 protects viral RNA from degradation or from inducing innate immune responses to the RNA terminus (29).

Metalloproteases are known to enable temporal control of many cellular processes by regulating the protein stability of specific and critical regulators (30, 31). Although the precise function of the metalloprotease gene in the Wolbachia–insect interaction is unclear, it appears to play a critical role. In the beetle Callosobruchus chinensis, a metalloprotease ftsh gene, presumably transferred from Wolbachia to the insect genome, was expressed at significantly higher levels compared with several other genes investigated in association with Wolbachia infection (32). Thus, metalloproteases may be critical for Wolbachia symbiosis in general. It will be interesting to examine whether the manipulation of these molecules might facilitate horizontal transfer of Wolbachia into naive hosts, a key objective of applied studies that seek to introduce Wolbachia into anopheles mos-

quitoes for malaria control.

In conclusion, we have shown that the miRNA profile of the dengue virus vector Ae. aegypti is altered by the endosymbiont W. pipiens. A host miRNA, aae-miR-2940, was found to be up-regulated in Wolbachia-infected mosquitoes and mosquito-derived cell lines. A target of the miRNA was determined to be the host’s metalloprotease 41 ftsh gene, which was up-regulated

injected with dsRNA specific to the metalloprotease gene. Gene

down-regulation was analyzed using qPCR. Consistent with cell culture results, Wolbachia density was significantly lower in mosquitoes injected with metalloprotease dsRNA compared with those injected with control dsRNA (GFP) or water (P < 0.0001, t test) (Fig. 5C). These results suggest that the metalloprotease gene is critical to Wolbachia’s maintenance in mosquitoes.

Fig. 5. RNAi-mediated silencing of the metalloprotease gene. (A) RT-qPCR analysis of the metalloprotease gene relative to actin in Aag2.wMelPop-CLA cells 48 h after transfection with mock, GFP, and metalloprotease (metal) dsRNAs. Three biological replicates were analyzed for each transfection. Asterisks indicate a significant difference between transfection with metalloprotease dsRNA and the other treatments (P < 0.0001; t test). (B) Differences in the density of Wolbachia in Aag2.wMelPop-CLA cells transfected with the metalloprotease (MP) and GFP dsRNAs examined by qPCR (P = 0.0415; t test). (C) Differences in the density of Wolbachia in +Wol adult mosquitoes injected with sterile water, GFP, and the metalloprotease (metal) dsRNAs, analyzed using qPCR. Five biological replicates were analyzed per injection type. Asterisks indicate significant difference between mosquitoes injected with metalloprotease dsRNA and the other treatments (P < 0.0001; t test). (D) A significant difference in the density of Wolbachia in Aag2.wMelPop-CLA cells transfected with aae-miR-2940 inhibitor versus control inhibitor (random sequence) (P < 0.0001; t test), analyzed by qPCR. Three biological replicates were analyzed for each transfection.

Aae-miR-2940 Is Required for Wolbachia Replication. We tested whether aae-miR-2940 plays a role in Wolbachia replication by transfecting Aag2.wMelPop-CLA cells with a synthetic inhibitor of the miRNA. At 72 h posttransfection, qPCR results using the wsp gene indicated significantly lower Wolbachia density was in cells transfected with the aae-miR-2940–specific inhibitor compared with cells transfected with a control inhibitor of random sequence (Fig. 5D; t = 4.898; df = 4; P = 0.008). These results suggest that the miRNA aae-miR-2940 is critical to communication between Wolbachia and host that allows the bacteria to persist in mosquito cells.

Discussion

Despite considerable research into the biology of Wolbachia, the mechanisms by which the bacteria manipulate host environments to ensure their own survival have proven elusive. Recently, several cellular miRNAs have been implicated in host–pathogen interactions in animals and plants. Here we have reported differential expression of cellular miRNAs in response to Wolbachia infection in the mosquito Ae. aegypti. Functional analysis of one of the induced miRNAs revealed a role in the regulation of cellular proteins that appears to be fundamental to the ability of Wolbachia to colonize and persist in mosquito host cells.

Based on microarray and Northern blot analyses, the expression profiles of several cellular miRNAs were either up-regulated or down-regulated in mosquitoes infected with Wolbachia. This is consistent with previous reports on the differential regulation of host miRNAs in response to bacterial infection in human cells (17, 18). We observed induction of aae-miR-2940, aae-miR-2906-2, aae-miR-2943-1, and aae-miR-970 at 4 d and 12 d after emergence and of aae-miR-308* and aae-miR-2941-2 at 4 d after emergence in Wolbachia-infected mosquitoes. Based on our target predictions in the Ae. aegypti genome for these miRNAs, we focused on aae-miR-2940 for functional analyses. We found one potential target in the 3’ UTR of an Ae. aegypti gene, metalloprotease 41 ftsh. Transcript levels of the target gene analyzed by Northern hybridization showed significant up-regulation in Wolbachia-infected mosquitoes. We confirmed the interaction of aae-miR-2940 with the 3’ UTR target sequence through the use of a synthetic inhibitor of aae-miR-2940 in the Ae. aegypti Aag2 cell line, as well as a GFP-based reporter construct. We found down-regulation in transcript levels of the target gene in Aag2.wMelPop-CLA cells transfected with aae-miR-2940 inhibitor, confirming a functional role of aae-miR-2940 in transcriptional regulation of the metalloprotease gene. We detected a similar induction of GFP transcripts when a GFP-metalloprotease target sequence construct was expressed in C6/36.wMelPop-CLA cells. Interestingly, RNAi-mediated silencing of the metalloprotease gene in both Aag2.wMelPop-CLA cells and +Wol mosquitoes led to a significant decline in Wolbachia density, suggesting a critical role for this protein in the maintenance of the Wolbachia infection in mosquito cells.

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in Wolbachia infection. Inhibition of the miRNA led to reduced expression levels of the target gene. In addition, silencing of the metalloprotease gene led to significant reductions in Wolbachia density both in vitro and in vivo. This report describes a fundamental role for miRNAs in the manipulation of the host intracellular environment to favor the Wolbachia endosymbiont. Manipulation of host miRNAs also might be responsible for other aspects of the Wolbachia–host interaction, such as various reproductive phenotypes, and thus merits further study.

Materials and Methods

Mosquitoes and Insect Cells. Ae. aegypti infected with the wMelPop-CLA strain of Wolbachia (+Wol) and a Wolbachia-free, tetracycline-cured line (−Wol) generated previously (7) were used for experiments. Insects were reared at 25 °C with 80% relative humidity and a 12-h light regime. Larvae were maintained with fish food pellets (TetraMin; Tetra) and adults were offered 10% sucrose solution ad libitum. Ae. aegypti Aag2 cells were infected with Wolbachia (denoted by Aag2. Aag2 cells were transfected with primers as described previously (23). Western blotting apparatus (Bio-Rad), and UV cross-linked. DNA oligonucleotides (21 mer) with reverse complementarity to specific miRNA sequences and a mutated construct with the complementary sequences to miRNA Target Studies. NCBi BLAST, RNAhybrid, and RNA22 software (8M) were used to find potential targets of aae-miR-2940 in the Ae. aegypti genome. Expression profiles of target genes were confirmed by Northern blot analyses with gene-specific probes. A 111-bp fragment from the metalloprotease 3’UTR (2039–2149; XM_001660643) containing the target sequences and a mutated construct with the complementary sequences to the aae-miR-2940 seed region mutated were cloned into pIZ/V5-His vector (Invitrogen) downstream of GFP using XbaI and SacII restriction sites, representing small RNAs from 4-d-old and 12-d-old female mosquitoes from the same generation (+Wol and −Wol) generated previously (7) were used for experiments. Insects were reared at 25 °C with 80% relative humidity and a 12-h light regime. Larvae were maintained with fish food pellets (TetraMin; Tetra) and adults were offered 10% sucrose solution ad libitum. To knock down the metalloprotease gene in +Wol mosquitoes, 500 ng dsRNA (metalloprotease) in 69 μL of sterile water was injected in the thorax of CO2-anesthetized mosquitoes at 4 d after emergence. Control mosquitoes were injected with either sterile water or 500 ng of dsRNA GFP gene were analyzed by RT-PCR and Northern blot analysis. Expression levels of the GFP gene were analyzed by RT-qPCR using three biological replicates, each with three technical replicates.

miRNA Mediated Gene Silencing and qPCR of Wolbachia Density. For RNAi-based experiments, dsRNA was synthesized in vitro using the Megascript transcription kit (Ambion). The T7 promoter sequence (5′-TAATACGACTCACTATAGGG-3′) was incorporated in both forward and reverse primers designed to amplify 500 bp of Ae. aegypti metalloprotease (forward: 5′-CCCCGAC- CAAAGCTCTAGTA-3′; reverse: 5′-CACATTACGGCGAGTGAAC-3′) of the target fish GFP genes. For dsRNA synthesis, 1 μg of PCR product was incubated for 4 h at 37 °C, DNase-treated, and precipitated by the lithium chloride method following the manufacturer’s instructions (Ambion). A total of 2 μg of dsRNA was used for transfection of Aag2.wMelPop-CLA cells. The cells were transfected again with the same reagents at 48 h after the first transfection. At 24 h after the second transfection, cells were collected for genomic DNA isolation. Gene silencing experiments were performed in replicates in the metalloprotease gene. Three biological replicates with three technical replicates were analyzed. Live/dead cell staining with Trypan blue was performed to confirm that silencing of the metalloprotease gene had no effect on cell viability. After staining, cells were counted with a hemocytometer. Cells were randomly sampled from three replicates of each transfection, and two independent hemocytometer counts were performed for each replicate.


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