The Relative Importance of Innate Immune Priming in Wolbachia-Mediated Dengue Interference

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Abstract

The non-virulent Wolbachia strain wMel and the life-shortening strain wMelPop-CLA, both originally from Drosophila melanogaster, have been stably introduced into the mosquito vector of dengue fever, Aedes aegypti. Each of these Wolbachia strains interferes with viral pathogenicity and/or dissemination in both their natural Drosophila host and in their new mosquito host, and it has been suggested that this virus interference may be due to host immune priming by Wolbachia. In order to identify aspects of the mosquito immune response that might underpin virus interference, we used whole-genome microarrays to analyse the transcriptional response of A. aegypti to the wMel and wMelPop-CLA Wolbachia strains. While wMel affected the transcription of far fewer host genes than wMelPop-CLA, both strains activated the expression of some immune genes including anti-microbial peptides, Toll pathway genes and genes involved in melanization. Because the induction of these immune genes might be associated with the very recent introduction of Wolbachia into the mosquito, we also examined the same Wolbachia strains in their original host D. melanogaster. First we demonstrated that when dengue viruses were injected into D. melanogaster, virus accumulation was significantly reduced in the presence of Wolbachia, just as in A. aegypti. Second, when we carried out transcriptional analyses of the same immune genes up-regulated in the new heterologous mosquito host in response to Wolbachia we found no over-expression of these genes in D. melanogaster, infected with either wMel or wMelPop. These results reinforce the idea that the fundamental mechanism involved in viral interference in Drosophila and Aedes is not dependent on the up-regulation of the immune effectors examined, although it cannot be excluded that innate priming in the heterologous mosquito host might enhance the virus interference trait.

Introduction

Wolbachia is a vertically transmitted endosymbiont that infects up to 70% of all insect species. The association is usually not obligatory for the insect and many Wolbachia strains assure their maintenance in populations by manipulating the reproduction of their host [1]. Interestingly, some strains interfere only weakly with host reproduction but still spread and are maintained in insect populations [2]. Their success may be explained by an additional positive selective advantage associated with Wolbachia infection. One possible advantage is the recently described pathogen blocking that the bacterium confers upon its host. This phenotype was first demonstrated in Drosophila, where Wolbachia induces resistance to different types of RNA viruses by reducing viral titer and/or making the host resistant to virus pathogenicity [3–5]. The extent and nature of blocking vary according to the virus and the Wolbachia strains tested. For example, Wolbachia reduces the titer of the closely related DCV and Nora viruses in Drosophila melanogaster and D. simulans [4,5] and as a consequence, the pathology associated with those two viruses is less intense in Wolbachia-infected flies [3–5]. In contrast, the bacterium does not affect FHV titer in Drosophila but still reduces the pathogenicity of the virus [3–5]. In D. simulans, the wAu Wolbachia strain has a strong effect against DCV pathogenicity, whereas the strains wHa and wNo do not [5]. This observation is thought to be related to the low infection density of wHa and wNo in Drosophila compared to that of the wAu strain [5].

Wolbachia does not naturally infect the main mosquito vector of dengue viruses, Aedes aegypti. However, two Wolbachia strains originally isolated from D. melanogaster (wMelPop-CLA and wMel) and one strain originally from A. albopictus (wAlbB) have been successfully trans-infected into A. aegypti and subsequently stably maintained [6–8]. All of these strains express cytoplasmic incompatibility in A. aegypti as they do in their original hosts, D. melanogaster and A. albopictus [6–8]. In addition, the virulent wMelPop-CLA strain that lacks normal replication control and reduces lifespan in D. melanogaster also does so in A. aegypti [7]. As observed in Drosophila, Wolbachia-infected A. aegypti are more resistant to RNA virus infection, including dengue and chikungunya [8,9], as well as bacteria, nematodes and Plasmodium [9,10]. Transient somatic infection of the main African vector of human malaria, Anopheles gambiae, by wMelPop also significantly decreased Plasmodium infection intensity [11].

The molecular mechanisms involved in Wolbachia-mediated pathogen protection are still not clear. One plausible hypothesis is that Wolbachia interferes with pathogens by pre-activating the immune response of its host. The virulent strain wMelPop-CLA activates a wide range of immune processes in A. aegypti, including
Author Summary

*Wolbachia pipientis* is an inherited intracellular bacterium that is widespread in insects. Because of its ability to interfere with various pathogens such as dengue viruses, nematodes and *Plasmodium* in insects, it has been proposed as a possible tool to control insect-transmitted disease. Recently, two strains of *Wolbachia* that interfere with RNA viruses in their natural host, *Drosophila melanogaster*, were introduced into the naturally uninfected mosquito vector of dengue fever, *Aedes aegypti*. As in their natural host, those two strains block the replication and the dissemination of viruses in the mosquito. Some studies suggest that pathogen blocking is due to *Wolbachia* priming the insect innate immune system. Here, we show that *Wolbachia* induces transcription of some immunity related genes only in its new host *A. aegypti*, and not in its natural host *D. melanogaster*, while *Wolbachia* reduces dengue replication in both hosts. These results suggest that immune priming by *Wolbachia* might not be the only mechanism responsible for viral interference.

the Toll and Imd signaling pathways, anti-microbial peptide synthesis, melanization, RNA interference and opsonisation [9,10] and the somatic infection of *A. aquasi* by *wMel* caused an increase in expression of opsonisation genes [11]. Evidence for the role of opsonisation in protection against *Plasmodium in this host was demonstrated by knocking down expression of the TEP1 gene [11]. Transcriptional analyses of *A. aegypti* immunity genes showed that *albH* increases expression of genes in the Toll pathway and in particular the anti-microbial peptide gene, defensin [12]. Activation of the Toll pathway has been shown previously to suppress dengue infection in mosquitoes [13]. Each of these previous studies was limited in that they examined *Wolbachia* strains that were either virulent and/or recently introduced into naturally uninfected host species. To our knowledge, only two previous studies have examined expression of innate immune genes in insect species naturally infected by *Wolbachia*, including *D. simulans*, *D. melanogaster* and *A. albopictus*. In these cases no differences in gene regulation were observed between *Wolbachia*-infected insects and their uninfected counterparts [14,15].

Since all previous studies that have shown evidence of immune activation have been based on recently established heterologous infections, it is unclear how generalizable the *Wolbachia* activation of the mosquito immune system is for all insects. To determine whether immune up-regulation by the bacterium is a general mechanism underlying *Wolbachia*-induced dengue interference, we performed transcriptional analyses on the two heterologous associations, *wMel* and *wMelPop-CLA* infected *A. aegypti*, and the two native associations, *wMel* and *wMel* infected *D. melanogaster*. We also tested if the non-virulent strain *wMel* blocks dengue replication in *Drosophila* as it does in mosquitoes. If the same strain of *Wolbachia* blocks the replication of the same virus in different hosts, we can make the parsimonious assumption that virus interference is likely to have a common mechanistic basis across different hosts. This cross-comparison with the two *Wolbachia* strains and dengue virus in both native and heterologous hosts allows us to remove extraneous effects, such as recent transfer to a heterologous host or virulence associated with the *wMelPop* infection, that might confound an understanding of the underlying mechanistic basis of *Wolbachia*-induced viral interference.

This study also contributes to our understanding of the physiological impact of *wMel* infection on *A. aegypti*. This is of particular relevance because *wMel*-infected *A. aegypti* have been released in north Queensland, Australia, in a field trial using *Wolbachia* as a biocontrol mechanism for dengue [16]. In the near future, this biological tool is also likely to be applied in dengue-endemic areas of Vietnam and Indonesia [17].

Results

Transcriptional response of *Aedes aegypti* to *Wolbachia* infection

We examined the global transcriptional response of mosquitoes to *Wolbachia* infection using microarrays. We compared the responses of 3 day old, non blood-fed *A. aegypti* females stably transfected with *wMelPop-CLA* (line PGYP1) or *wMel* (line MGYP2) to those of the corresponding tetracycline-cured lines PGYP1.tet and MGYP2.tet. The design of the microarray included 12,336 transcripts, which represented 12,270 of the 15,938 genes present in the *A. aegypti* genome. We considered a gene to be up- or down-regulated by *wMelPop-CLA* or *wMel* infection if the fold change in transcription relative to non-infected mosquitoes was significantly different from 1.0 and greater than 1.5. Because the *Drosophila* genome is better characterized, we identified *Drosophila* orthologs of each *A. aegypti* gene where possible to obtain additional functional annotations.

The *wMelPop-CLA* infection affected the transcription of far more genes (2723) than the *wMel* infection (327) (Figure 1). This is likely related to *wMelPop-CLA*’s higher density in its host, broader cellular tropism and pathogenicity [9,18]. Based on Gene Ontology (GO) annotations, *wMelPop-CLA* has an impact on a broader range of *A. aegypti* biological and molecular functions than *wMel* (Table 1, 2).

Many of the changes in gene regulation observed in mosquitoes infected with the virulent strain *wMelPop-CLA* are likely to be responses to the high physiological cost imposed by that strain. To identify mechanisms more likely to be involved in pathogen interference, we decided to focus on the 210 gene transcripts that showed significant changes in expression in both PGYP1 and MGYP2 compared to uninfected mosquitoes (Figure 1). Among those genes, 138 gene transcripts had functional annotations (Table S1).

Most of the 210 transcripts were either up-regulated in both PGYP1 and MGYP2 or down-regulated in both lines (Table S1). However, the magnitude of response was typically greater to *wMelPop-CLA* infection (Table S1). One of the few genes differentially expressed between PGYP1 and MGYP2 is AAL002487, which is up-regulated in MGYP2 and down-regulated in PGYP1. This gene encodes the protein P53 regulated 2A26 nuclear protein sestrin (dSesn in *Drosophila*) (Table S1). This protein is involved in the regulation of the target of rapamycin (TOR), a key protein in age-related pathologies like life-shortening or muscle degeneration [19], two phenotypes exclusively associated with *wMelPop-CLA* pathogenicity in *A. aegypti* [7,20]. Among the 210 genes, most of the genes showing the greatest up-regulation are immune genes (Table S1). Gene Ontology (GO) annotations also revealed enrichment in genes related to immunity and proteolysis for MGYP2 and PGYP1 (Table 1, 2). The results obtained for PGYP1 are in accordance with a previous study of *A. aegypti* infected by *wMelPop-CLA* [10].

Common immune pathways activated by *wMelPop-CLA* and *wMel* in *A. aegypti*

The virulent strain *wMelPop-CLA* significantly affected regulation of many characterized immune genes in the mosquito (Table S2, [10]). By comparison, many fewer of these genes were activated by *wMel* (Table 3, S1, S3). Those included genes...
encoding anti-microbial peptides, four cecropins (CECE, CECF, CECN, CECD), one defensin (DEFC) and one diptericin (DPT1). The magnitude of change in expression was substantial for some of these genes. The activation of these peptides is regulated by both Toll and Imd pathways, but we found up-regulation only of some Toll pathway genes, including the peptidoglycan recognition protein PGRP-SA and the Gram-negative binding proteins GNBP4 and GNBP1 (Drosophila homologs, Table 3). The Toll pathway effector defensin was the most highly up-regulated immune gene in A. aegypti infected by wMel (Table 3). This is consistent with the results of Bian et al [12], who examined immune gene expression in heterologous wAlbB infection in A. aegypti and found that among the immune genes tested defensin was also the most up-regulated.

Excluding anti-microbial peptides and the Toll pathway, the only other immune response activated by both wMel and wMelPop-CLA in A. aegypti was melanization. Four genes in this pathway were up-regulated: one pro-phenoloxidase (PPO4), one dopachrome-conversion enzyme (DCE) that converts dopachrome into 5,6-dihydroxyindole just before melanin production by phenoloxidase [21], one putative protease inducer sp7 and one protease inhibitor Srpn4 (Table 3). The activation of these genes suggests that production of melanin is induced in Wolbachia-infected mosquitoes.

**Effect of Wolbachia on dengue virus in Drosophila**

Since a comparative approach between Drosophila and Aedes to examine the effect of immune activation on virus interference is predicated on an assumption that dengue virus interference also occurs in Wolbachia-infected Drosophila, we then investigated the ability of dengue virus serotype 2 (DENV-2) to grow in Drosophila carrying the wMel Wolbachia strain. For both dengue virus strains, 92T and ET300, the total number of flies infected by dengue was lower in the presence of wMel, with only 40% of flies detected positive for the 92T strain compared with 93% for the Wolbachia-uninfected control. Similarly for the ET300 strain, 73% of Wolbachia-infected flies were positive for dengue compared to 93% for the Wolbachia-uninfected control (Figure 2). In addition, for the flies that did become infected with dengue the amount of DENV-2 RNA present was significantly reduced in the presence of wMel (Figure 2). It was unsurprising to note that dengue grew to higher levels when injected into its natural mosquito host compared to Drosophila but regardless of absolute virus levels significant Wolbachia interference effects were detected in both insect species. Dengue injection in flies did not have an effect on insect life span nor increased mortality compared to controls (data not shown).

**Effect of wMelPop and wMel on the Drosophila melanogaster immune system**

Considering that the Wolbachia strains wMelPop [22] and wMel in their original host interfere with natural Drosophila RNA viruses and also with dengue virus replication, we then investigated the
possibility that both Wolbachia strains boost Drosophila immunity as seen in the heterologous mosquito host. We examined by quantitative real time PCR the expression of the Drosophila homologs of the mosquito immune genes identified through microarray analysis to be up-regulated in the presence of Wolbachia.

| GO ID       | Term description                              | Adjusted P-values |
|-------------|-----------------------------------------------|-------------------|
| Biological process |                                               |                   |
| GO:0006508  | Proteolysis                                  | 5.87E-15          |
| GO:0009308  | Amine metabolic process                      | 9.22E-08          |
| GO:005114   | Oxidation reduction                          | 4.97E-07          |
| GO:0005975  | Carbohydrate metabolic process               | 7.41E-05          |
| GO:0009607  | Response to biotic stimulus                  | 2.16E-04          |
| GO:0055085  | Transmembrane transport                      | 8.08E-04          |
| GO:0044271  | Cellular nitrogen compound biosynthetic process | 2.72E-03          |
| GO:0006952  | Defense response                              | 3.19E-03          |
| GO:0022610  | Biological adhesion                           | 3.82E-03          |
| GO:0051704  | Multi-organism process                        | 7.77E-03          |
| GO:0051604  | Protein maturation                            | 9.19E-03          |
| GO:0019538  | Protein metabolic process                     | 1.18E-02          |
| GO:0002376  | Immune system process                         | 1.87E-02          |
| GO:0043565  | Chemical homeostasis                          | 2.27E-02          |
| GO:0051179  | Localization                                  | 3.08E-02          |
| GO:0071554  | Cell wall organization or biogenesis          | 3.50E-02          |
| GO:0044283  | Small molecule biosynthetic process           | 4.96E-02          |
| GO:0010876  | Lipid localization                            | 5.00E-02          |
| Molecular function |                                               |                   |
| GO:0005506  | Iron ion binding                              | 3.98E-16          |
| GO:0003824  | Catalytic activity                            | 6.62E-10          |
| GO:0046906  | Tetrapyrrole binding                          | 1.31E-09          |
| GO:0005215  | Transporter activity                          | 1.27E-06          |
| GO:0030246  | Carbohydrate binding                          | 4.29E-06          |
| GO:0009055  | Electron carrier activity                     | 6.98E-06          |
| GO:0004857  | Enzyme inhibitor activity                     | 3.31E-05          |
| GO:00164901 | Oxidoreductase activity                       | 4.12E-05          |
| GO:008233   | Peptidase activity                            | 7.56E-05          |
| GO:0017171  | Serine hydrolase activity                     | 1.08E-04          |
| GO:0061134  | Peptidase regulator activity                  | 2.23E-04          |
| GO:0005509  | Calcium ion binding                           | 3.99E-04          |
| GO:0005102  | Receptor binding                              | 3.82E-03          |
| GO:0005044  | Scavenger receptor activity                   | 4.70E-03          |
| GO:0005515  | Protein binding                               | 5.05E-03          |
| GO:0004047  | Aminomethyltransferase activity               | 1.08E-02          |
| GO:0043565  | Sequence-specific DNA binding                 | 1.64E-02          |
| Cellular component |                                              |                   |
| GO:0016020  | Membrane                                      | 5.79E-16          |
| GO:0005576  | Extracellular region                          | 4.32E-09          |
| GO:0043234  | Protein complex                               | 4.62E-03          |
| GO:0005856  | Cytoskeleton                                  | 7.77E-03          |

Adjusted P-values are the P-values generated by the Ontologizer program [38], using the Benjamini-Hochberg method. doi:10.1371/journal.ppat.1002548.t002

There have been multiple gene losses and gene duplications in immune gene families in both flies and mosquitoes [23], and we were therefore unable to reliably identify all orthologs for our anti-microbial peptide genes and pro-phenoloxidase genes of interest. Thus, we targeted all the cecropin, diptericin and pro-phenoloxidase
Table 3. *A. aegypti* putative immune transcripts significantly up-regulated in response to both wMelPop-CLA and wMel infections.

| Transcripts ID | wMelPop-CLA | wMel | Description | Dm Gene ID | H | Dm Symbol |
|----------------|-------------|-------|-------------|------------|---|-----------|
| **Anti-microbial peptides** | | | | | | |
| AAE0000598-RA | 10.44 | 1.83E-04 | 2.93 | 4.00E-03 | cecropin (CECD) | no homolog |
| AAE0060611-RA | 125.52 | 9.63E-06 | 12.62 | 6.41E-03 | cecropin (CECE) | no homolog |
| AAE006025-RA | 53.83 | 3.65E-05 | 6.07 | 9.84E-03 | cecropin (CECF) | no homolog |
| AAE006021-RA | 47.31 | 1.14E-05 | 10.11 | 4.10E-03 | cecropin (CECN) | no homolog |
| AAE003382-RA | 70.76 | 7.09E-06 | 22.99 | 2.89E-03 | defensin-C (DEFC) | FBgn0010385 | Def |
| AAE004833-RA | 2.72 | 6.72E-05 | 1.53 | 5.46E-03 | diptericin 1 (DPT1) | no homolog |
| **Toll pathway** | | | | | | |
| AAE077993-RA | 9.33 | 7.09E-06 | 1.90 | 4.81E-03 | clip-domain serine protease (CLIPB27) | FBgn0039494 | grass |
| AAE07626-RA | 3.04 | 2.68E-05 | 1.67 | 9.05E-03 | gram-negative binding protein (GNBPA1) | FBgn0040323 | GNPB1 |
| AAE091978-RA | 3.72 | 8.98E-04 | 7.50 | 6.19E-03 | gram-negative binding protein (GNBF4) | FBgn0040323 | GNPB1 |
| AAE011624-RA | 2.55 | 4.84E-04 | 2.35 | 2.89E-03 | gram-negative binding protein (GNBPA1) | FBgn0040323 | GNPB1 |
| AAE099474-RA | 6.76 | 5.10E-03 | 2.96 | 5.69E-03 | defensin-C (DEFC) | FBgn0030310 | PGRP-SA |
| AAE010867-RA | 4.27 | 1.15E-03 | 1.76 | 4.59E-03 | serine protease | FBgn003450 | snk |
| **Melanization** | | | | | | |
| AAE000024-RA | 2.18 | 1.72E-04 | 1.54 | 9.33E-03 | dopachrome-conversion enzyme (DCE) | FBgn0041710 | yellow-f |
| AAE03351-RA | 32.84 | 2.53E-05 | 2.71 | 4.81E-03 | pro-phenoloxidase (PPO4) | FBgn0000165 | |
| AAE013501-RA | 3.06 | 7.09E-06 | 3.46 | 1.91E-03 | serine protease | FBgn0037515 | Sp7 |
| AAE013936-RA | 1.65 | 6.22E-04 | 1.56 | 3.52E-03 | serine protease inhibitor (SRPN4) | FBgn0031973 | Spn28D |
| **Other putative immune related genes** | | | | | | |
| AAE005641-RA | 31.47 | 3.97E-05 | 5.27 | 2.68E-03 | C-type lectin - galactose binding (CTLGA5) | FBgn0033353 | lectin-37Db |
| AAE011621-RA | 5.84 | 2.50E-04 | 2.35 | 2.89E-03 | C-type lectin - mannose binding (CTLMA13) | FBgn0033353 | lectin-37Db |
| AAE011453-RA | 4.15 | 3.79E-05 | 1.89 | 8.54E-03 | C-type lectin (CTL14) | FBgn0033353 | lectin-37Db |
| AAE011408-RA | 3.06 | 2.16E-04 | 1.99 | 5.26E-03 | C-type lectin (CTL21) | no homolog |
| AAE002524-RA | 7.38 | 1.20E-04 | 4.10 | 9.78E-03 | C-type lectin (CTL24) | no homolog |
| AAE002601-RA | 7.31 | 6.12E-05 | 2.31 | 2.33E-03 | clip-domain serine protease (CLIPB1) | FBgn0033321 | CG8738 |
| AAE014349-RA | 6.74 | 3.97E-05 | 1.91 | 3.80E-03 | clip-domain serine protease (CLIPB1) | FBgn0033321 | CG8738 |
| AAE000059-RA | 2.10 | 4.14E-04 | 1.68 | 2.83E-03 | clip-domain serine protease, putative | FBgn0033321 | CG8738 |
| AAE001084-RA | 16.39 | 7.09E-06 | 4.25 | 3.80E-03 | clip-domain serine protease (CLIPB2) | no homolog |
| AAE008668-RA | 4.53 | 6.51E-05 | 2.00 | 7.73E-03 | clip-domain serine protease (CLIPB2) | no homolog |
| AAE006674-RA | 1.85 | 2.22E-04 | 1.53 | 4.82E-03 | clip-domain serine protease (CLIPB2) | no homolog |
| AAE000099-RA | 4.26 | 2.27E-05 | 2.11 | 2.83E-03 | clip-domain serine protease (CLIPB3) | no homolog |
| AAE005431-RA | 22.66 | 1.85E-05 | 3.95 | 2.68E-03 | cysteine-rich venom protein, putative | FBgn0031412 | CG16995 |
| AAE002022-RA | 5.15 | 3.40E-04 | 2.65 | 3.20E-03 | protein serine/threonine kinase, putative | FBgn0011695 | PebII/phk2 |
| AAE001964-RA | 4.45 | 6.57E-05 | 1.90 | 4.74E-03 | cysteine-rich venom protein, putative | FBgn0001695 | PebII/phk2 |
| AAE002585-RA | 8.05 | 2.19E-05 | 1.66 | 7.61E-03 | cysteine-rich venom protein, putative | FBgn0028864 | CG18477 |
| AAE002624-RA | 6.65 | 3.16E-05 | 1.89 | 2.74E-03 | cysteine-rich venom protein, putative | FBgn0028864 | CG18477 |
| AAE002610-RA | 6.93 | 1.14E-05 | 2.10 | 8.54E-03 | cysteine-rich venom protein, putative | FBgn0032638 | CG6639 |
| AAE003697-RA | 3.11 | 3.05E-05 | 1.77 | 6.42E-03 | serine protease inhibitor (SRPN17) | no homolog |
genes present in the genome of *D. melanogaster*. In total 13 immune genes were analyzed: seven anti-microbial peptide genes, two Toll pathway genes and four melanization genes (Table 4).

No significant changes in the expression of anti-microbial peptide genes were observed for *w*<sub>1118</sub> *w*<sub>MelPop</sub> or *w*<sub>1118</sub> *w*<sub>Mel</sub>, except for cecropin A1 (Table 4). The expression of cecropin A1 was two-fold higher in the presence of *w*<sub>MelPop</sub>, whereas no change was observed in the presence of *w*<sub>Mel</sub> (Table 4). No gene expression was detected for the cecropins B and C for either of the *Drosophila* lines tested. No significant changes in diptericin transcription were observed in *Wolbachia*-infected flies, which suggests that the Imd signaling pathway is not stimulated by

| Transcripts ID       | AFC | q-value | AFC | q-value | Description                      | Dm Gene ID H | Dm Symbol |
|----------------------|-----|---------|-----|---------|----------------------------------|--------------|-----------|
| AAEL006136-RA        | 4.83| 3.30E-05| 2.17| 3.66E-03| serine protease, putative         | FBgn0038211  | CG9649    |
| AAEL006434-RA        | 3.53| 4.42E-05| 1.80| 8.62E-03| serine protease, putative         | no homolog   |           |
| AAEL013033-RA        | 3.18| 1.52E-05| 2.32| 8.22E-03| serine protease, putative         | no homolog   |           |
| AAEL013432-RA        | 2.56| 6.78E-05| 3.84| 3.31E-03| serine protease, putative         | no homolog   |           |
| AAEL004761-RA        | 1.89| 3.12E-04| 1.67| 3.93E-03| serine/threonine-protein kinase MAK | FBgn0051711  |           |
| AAEL015458-RA        | 55.38| 7.09E-06| 12.23| 1.88E-05| transferrin                      | FBgn0022355  | Tsf1      |

Transcripts are ranked by biological process and/or molecular function. Transcript identifiers (Transcript ID) and Description were compiled from Vectorbase. *D. melanogaster* Gene Identifier Homolog (Dm Gene ID H) and Dm Symbol were compiled from Flybase. AFC, Absolute Fold Change.

doi:10.1371/journal.ppat.1002548.t003

Figure 2. Dengue blocking in *D. melanogaster* and *A. aegypti* infected by *Wolbachia* strain *w*<sub>Mel</sub>. 69 µl of 10<sup>7</sup> pfu/ml of DENV2 strain 92T (grey circles) and DENV2 strain ET300 (black circles) were injected into flies (*w*<sub>1118</sub>*w*<sub>MelPop</sub> and *w*<sub>1118</sub>*w*<sub>Mel</sub>) and mosquitoes (MGYP2) infected by *w*<sub>Mel</sub> and their tetracycline-treated uninfected counterparts (*w*<sub>1118</sub>*tet* and MGYP2<sub>tet</sub>). Dengue levels in individual insects were determined 8 days post-infection by RT-PCR using a TaqMan assay specific to dengue in 1 µg of total RNA. The fraction of flies that had detectable dengue infections is shown above each set of data points. (n = 15, Mann-Whitney U test, **: p<0.01, ***:p<0.001, ****:p<0.0001).

doi:10.1371/journal.ppat.1002548.g002
Table 4. Immune transcript analyses in D. melanogaster infected with wMelPop and wMel.

| Gene ID             | AFC     | q-value | AFC     | q-value | Description                          | Symbol |
|---------------------|---------|---------|---------|---------|--------------------------------------|--------|
| **Anti-microbial peptides** |         |         |         |         |                                      |        |
| FBgn0000276         | 2.24    | 0.030   | *       | −1.59   | 0.324 cecropin A1                   | CecA   |
| FBgn0000277         | 1.63    | 0.109   | 1.58    | 0.597   | cecropin A2                         | CecA2  |
| FBgn0000278         | ND      | ND      | ND      | ND      | cecropin B                          | CecB   |
| FBgn0000279         | ND      | ND      | ND      | ND      | cecropin C                          | CecC   |
| FBgn0004240         | 1.25    | 0.661   | −1.16   | 0.743   | diptericer                           | Dpt    |
| FBgn0034407         | 1.37    | 0.661   | −1.13   | 0.743   | diptericer B                        | DptB   |
| FBgn0010385         | 1.27    | 0.398   | 1.24    | 0.591   | defensin                            | Def    |
| **Toll pathway**    |         |         |         |         |                                      |        |
| FBgn0030310         | −1.49   | 0.030   | *       | 1.11    | 0.168 peptidoglycan recognition protein SA | PGRP-SA |
| FBgn0040323         | 1.05    | 0.631   | 1.29    | 0.002   | ** gram-negative binding protein 1   | GNB1   |
| **Melanization**    |         |         |         |         |                                      |        |
| FBgn0261363         | −2.6    | 0.008   | **      | −1.69   | 0.142 CG42640                        |        |
| FBgn0261362         | 1.67    | 0.011   | *       | −1.47   | 0.030 pro-phenoloxidase A1           | proPO-A1 |
| FBgn0033367         | 1.04    | 0.743   | −1.39   | 0.154   | CGB193                               |        |
| FBgn0041710         | 1.08    | 0.631   | −1.01   | 0.661   | yellow-f                             |        |
| **Other**           |         |         |         |         |                                      |        |
| FBgn0022355         | −2.25   | 0.008   | **      | −1.15   | 0.324 transferin 1                   | Tsf1   |
| FBgn0015221         | 1.99    | 0.109   | −1.22   | 0.661   | ferritin 2 light chain homologue     | Fer2lch|

Transcripts are ranked by biological process and/or molecular function. Gene identifiers (Gene ID), Description and Symbol were compiled from Flybase. AFC, Absolute Fold Change, ND, No Detection. Asterisks indicate a statistically significant difference (n = 10, Mann-Whitney U test with q-value adjustment, *: q<0.05, **: q<0.01). 
doi:10.1371/journal.ppat.1002548.t004

Wolbachia in Drosophila. The expression patterns of two major genes in the Toll pathway, PGRP-SA and GNB1, differed between flies infected with wMel and wMelPop. A slight inhibition of PGRP-SA was observed in flies infected with wMelPop, while in wMel-infected flies there was no effect. For GNB1, a minor but significant difference, 1.29-fold change, was observed for w1118/wMel but not for w1118/wMelPop (Table 4). The expression of only a single melanization gene was affected by wMel infection: proPO-A1 was down-regulated. In contrast, in flies infected with wMelPop, proPO-A1 was significantly up-regulated and another melanization gene, CG42640, was down-regulated (Table 4).

An enrichment of gene transcripts encoding the iron binding proteins transferrin and ferritin was detected in the data obtained from the A. aegypti transcriptome analysis in response to wMel and wMelPop-CLA infections (Table 1, 2, S1). These proteins have multiple functions in insects, including iron homeostasis and immunity [24], two potential mechanisms that could be involved in Wolbachia-mediated pathogen protection. The expression of the genes encoding transferrin 1 (Tsf1) and the light chain of ferritin (Fer2lch) was evaluated in w1118/wMel and w1118/wMelPop compared to w1118/wtet. However, no induction was found in Wolbachia-infected flies (Table 4) and wMelPop infection even significantly reduced the expression of transferrin.

The expression of immune genes was also tested in the same fly lines [w1118/wMel and w1118/wtet] infected with DENV-2, strain 92T. Even in the presence of dengue, wMel infection did not increase the expression of anti-microbial peptides and pro-phenoloxidases (Figure S1). No correlation was found between the amount of dengue detected and the level of expression for each of the anti-microbial peptide and pro-phenoloxidases genes tested in each fly line (Figure S2).

Discussion

Host immune priming by Wolbachia offers an appealing mechanistic explanation for pathogen blocking as it is conceivable that this single effect could lead to protection against a diversity of pathogens. The objective of this study was to compare the effect of two closely-related strains of Wolbachia on the immune system of hosts where the age of the Wolbachia association differs. By comparing wMelPop-CLA and wMel we could exclude any potential immune activation that may simply be due to the virulence of the wMelPop-CLA infection. By examining both D. melanogaster and A. aegypti, we were able to dissect aspects of the immune response that may be attributed solely to a host’s response to a recently acquired Wolbachia infection. This analysis depends on an assumption that the mechanism of virus interference is similar in the two insect hosts. Considering that Wolbachia infection in Drosophila interferes with dengue replication, as it does in A. aegypti, the assumption of a similar mechanism seems parsimonious. Moreover the success of maintaining dengue in Drosophila, even if viral replication is not as strong as in A. aegypti, provides a tractable genetic model for future studies into the mechanistic basis of Wolbachia-mediated dengue interference.

A previous analysis of A. aegypti whole genome transcription in response to wMelPop-CLA revealed strong immune induction by the bacterium [10]. In this present study, a similar approach was taken to analyze the impact of the non-virulent wMel strain on the immune system of A. aegypti, in comparison with the wMelPop-CLA strain. The results obtained revealed that wMel induces the activation of far fewer immunity genes in the mosquito. The comparative analysis between the different lines identified common responses only for genes encoding anti-microbial
peptides, the Toll pathway and melanization-associated proteins. Recent studies have provided important insights into *A. aegypti* immune response to dengue virus, showing that the Toll pathway and anti-microbial peptides are important for the mosquito’s defense against dengue infection [13,25]. Melanization is also a prominent immune response in insects against parasites like malaria and nematodes [26] but as far as we know it has never been demonstrated for dengue.

The main anti-viral pathway, RNA interference [27], seems to be activated exclusively by *w*MelPop-CLA. Several pieces of evidence also indicate that RNAi cannot explain virus blocking. First, Glaser et al [28] showed that even in Ago2 (a key gene in the RNAi pathway) mutant flies, *Wolbachia* infection increases resistance to viruses. Second, Frentiu et al [29] demonstrated that *w*MelPop-CLA induces complete inhibition of dengue virus replication in the C6/36 cell line that has been shown to be defective in the RNAi pathway [30].

This comparative analysis between *w*Mel and *w*MelPop-CLA infection within *A. aegypti* supports the potential implication of anti-microbial peptides and Toll pathway activation in dengue virus interference by the bacterium. If we assume that the fundamental mechanism involved in *Wolbachia*-mediated dengue interference is the same in mosquitoes and flies, and this mechanism is immune-based, then the same constitutive immune induction should also be observed in *D. melanogaster* infected by *w*Mel or *w*MelPop. We tested for transcriptional changes of the same immune genes identified through microarray analysis in *D. melanogaster* in response to *Wolbachia* infection, and identified a number of statistically significant changes. However, in no case were these changes consistent between *w*Mel and *w*MelPop infection. Furthermore, if we employed the same threshold for biological significance we used for our microarray data, that a gene is significantly up-regulated by *Wolbachia* infection only when its level is changed at least 1.5-fold compared with non-infected flies, we would conclude that *w*Mel did not constitutively prime any of the different immune genes tested in its natural host *D. melanogaster*. Those results are in accordance with previous data showing no pre-activation of different immune genes in *D. melanogaster*, *D. simulans* and *A. albopictus* by *Wolbachia* [14,15].

In summary, the only immune genes up-regulated by *w*MelPop-CLA and *w*Mel in *A. aegypti* are anti-microbial peptides, Toll pathway and melanization genes. However, the same *Wolbachia* strains did not up-regulate these genes in *Drosophila*, and yet dengue interference occurs in this host. This indicates that the up-regulation of these immune effector genes is not required to interfere with dengue virus replication, although it is likely that the immune up-regulation that occurs in mosquitoes, presumably due to the recent association with *Wolbachia*, might enhance this effect.

**Materials and Methods**

**Insect rearing**

All the mosquito strains used in this study were laboratory lines of *A. aegypti* infected with *w*Mel (MGYP2) or *w*MelPop-CLA (PGYP1), and their tetracycline-treated uninfected counterparts, MGYP2.tet and PGYP1.tet [7,8]. Adult mosquitoes were kept on 10% sucrose solution at 25°C and 60% humidity with a 12-h light/dark cycle. Larvae were maintained with fish food pellets (Tetramin, Tetra).

The fly experiments were performed with *w*1118 fly lines stably infected with *w*Mel (*w*1118;*w*Mel) [31] and *w*MelPop (*w*1118;*w*MelPop) [18] compared to the tetracycline-cured lines derived by the addition of tetracycline (0.3 mg/ml) to the adult diet for two generations. Those lines were confirmed to be free of *Wolbachia* by PCR, using primers specific for the *w*Mel and *w*MelPop IS5 repeat [22]. Females were kept under controlled conditions, low-density (30 females per vial), at 25°C with 60% relative humidity and a 12-h light/dark cycle.

**Sample collection and hybridization**

Three replicate pools of 20 female mosquitoes, 8 days post-eclosion were collected from each of the four lines (PGYP1, MGYP2, PGYP1.tet and MGYP2.tet), snap frozen in liquid nitrogen and extracted for total RNA using Trizol (Invitrogen). RNA was then purified using RNAeasy kits (Qiagen) according to manufacturer’s instructions. Whole-genome microarrays were then used to compare gene expression in the *Wolbachia*-infected lines relative to uninfected controls, using a dual-color reference design. All sample preparations and hybridizations were then carried out by the IMB Microarray Facility at the University of Queensland. Briefly, sample quality was examined using the Agilent 2100 Bioanalyzer (Agilent Technologies) and fluorescent cDNA was synthesized using Agilent Low RNA Input Linear Amplification Kit with Cy3 or Cy5. Each infected line and respective paired tetracycline-treated line were represented by 3 biological replicates (3 pools above). A total of 6 hybridizations were then carried out for each biological replicate, 3 labeled with Cy3 and three with Cy5 (dye swaps).

**Microarray design**

Microarrays were of the 4×44 K format (Agilent) each containing standard control features and 3 technical replicates of each 60 mer feature randomly distributed across the layout. The *A. aegypti* genomic sequence (Vectorbase genome build 1.1) was used for construction of oligonucleotide microarrays using eArray Version 3.0 (Agilent Technologies). After removing probes that cross hybridized, a total of 12,336 transcripts that represented 12,270 genes were spotted onto each microarray [32].

**Microarray data analyses**

For each transcript, raw data was extracted and analyzed using Genespring v.9.0 (Agilent Technologies). An intensity dependent (Lowess) normalization (Per Spot and Per Chip) was used to correct for non-linear rates of dye incorporation as well as irregularities in the relative fluorescence intensity between the dyes. Hybridizations from each mosquito line were used as replicate data to test for significance of expression changes using the cross-genome error model. The occurrence of false positives was corrected using the q-value [33,34]. All array data have been deposited in ArrayExpress (http://www.ebi.ac.uk/microarray-as/ ae/) under the accession number E-MEXP-2931.

Functional annotations of *A. aegypti* genes were retrieved from Biomart [35] in Vectorbase [36] and analyzed using the Ontologizer software with the parent child intersection method [37,38]. The over-expression of particular GO categories in the microarray data set was tested against the distribution of GO categories for the *A. aegypti* genome.

**Virus injection**

Dengue virus serotype 2 (DENV-2), strains 92T [9] and ET300 were isolated from human serum collected from patients from Townsville, Australia, in 1992 and East Timor in 2000, respectively. DENV-2 (strains 92T and ET300) was propagated and quantified as described by Frentiu et al [29]. For virus injection, 8 day old *D. melanogaster* females (*w*1118;*w*Mel and
and A. aegypti females (MGYP2 and MGYP2tet) were briefly anesthetized with CO₂ and injected under a dissecting scope into their thorax with a pulled glass capillary and a handheld microinjector (Nanoject II, Drummond Sci.). 69 μl of virus stock (10⁻⁷ pfu/ml) or sterile PBS 1X were injected. After injection flies and mosquitoes were maintained under identical controlled conditions, low-density (10 females per vial or cup), at 25°C with 60% relative humidity and 12:6 light/dark cycle. Insects were collected 8 days post-injection and kept at −80°C for RNA extraction.

Quantitative DENV-2 PCR analysis
RNA extraction was done on 15 individual 16 day old females per condition using Trizol (Invitrogen). 1 μg of total RNA was kept to quantify DENV-2 while the rest was used for immune gene expression analysis as described below.

Accumulation of genomic (+RNA) RNA strands was assessed by quantitative real time PCR using hydrolysis probes specific to the 3′ UTR region of the four dengue serotypes [39] with modifications (A.T. Pyke, unpublished data). The sequences of the primers were FWD: 5′-AAGGACATAGGGTGAGAGGACC-3′ and RWD: 5′-CGTTCTCTGTCGGATGAATGATG-3′ and the sequence of the probe was 5′-AAGCAGATATTGACGCTGGAGAGAAGACCA-3′. 1 μg of total RNA for each sample was mixed with 0.625 μM of the reverse primer plus 0.2 mM dNTPs. Samples were incubated at 86°C for 15 minutes and 5 minutes on ice, then 5X first strand buffer and 100 U of Superscript III (Invitrogen) was added to a total volume of 20 μl. Samples were incubated at 25°C for 10 minutes, followed by 42°C for 50 minutes and 10 minutes at 95°C to inactivate the transcriptase.

The qPCR reaction consisted of 2 μl of the synthesized cDNAs, 5 μl of 2X LightCycler 480 Probes Master (Roche), 0.5 μM of each primer (see above) and 0.5 μM of the probe (see above) in 10 μl total volume. Reactions were performed in duplicate in a LightCycler 480 Instrument (Roche) with the following conditions: 95°C for 5 minutes, and 45 cycles of 95°C for 10 s, 60°C for 15 s, 72°C for 1 s. A standard curve was created by cloning the DENV-2 3′UTR region fragment into pGEM T-Easy (Promega). After linearization with Pst I the plasmid was serially diluted into known concentrations and run in parallel, in order to determine the absolute number of DENV-2 copies in each 1 μg of total RNA. First, percentages of individuals infected with dengue were calculated for each treatment. Then only individuals with dengue infection (non zero quantification) were used to examine the effect of aMel on dengue titer using Mann-Whitney *U* tests (Graph Pad Prism 5).

Quantitative PCR analysis of immune genes
RNA extraction from flies was done using between 10 to 15 individual 8 day old females per condition using Trizol reagent (Invitrogen). To eliminate any contamination by DNA, samples were treated with DNase I recombinant (Roche), in accordance with the manufacturer’s instructions. cDNAs were synthesized from 1 μg of total RNA, using oligodT primers and the SuperScript III enzyme (Invitrogen), in accordance with manufacturer’s instructions. For each sample qRT-PCR was performed in triplicate on a 10 times dilution of the cDNAs using Platinum SYBR Green (Invitrogen) according to the manufacturer’s protocol. Primers are listed in Table S4. The temperature profile of the qPCR was 50°C for 2 minutes (UDG incubation), 95°C for 2 minutes, 45 cycles of 95°C for 5 s, 60°C for 5 s, 72°C for 10 s with fluorescence acquisition of 78°C at the end of each cycle, then a melting curve analysis after the final cycle. The housekeeping gene *rpS17* was used to normalize expression. Target gene to housekeeping gene ratios were obtained for each biological replicate using Q-Gene software [40]. Raw data were graphed as median ± interquartile range (IQR) and outliers beyond 1.5 IQR excluded. Treatment effects on expression ratios were then examined using the Mann-Whitney *U* tests (Graph Pad Prism 5).

The occurrence of false positives was corrected using the q-value [33,34].

Supporting Information
Figure S1 Immune gene expression in *Drosophila melanogaster* in response to aMel and DENV-2. The expression of immune genes was analyzed by qRT-PCR on individual females injected either with DENV-2 strain 92T (w1108wMel D+, w1108tet D+) or PBS (w1108wMel PBS, w1108tet PBS) in presence/absence of *Wolbachia* strain wMel. Flies were collected 8 days post-injection. Graphs show the target gene to house-keeping gene expression ratio (n = 15, Mann-Whitney *U* test with q-value adjustment, *: q<0.05, **: q<0.01, ***:q<0.001).

Figure S2 Correlation analysis between dengue titer and immune gene expression in *Drosophila melanogaster* in presence/absence of *Wolbachia* strain wMel (w1108wMel, w1108tet). The values were compared using Spearman correlation coefficients.

Table S1 *Aedes aegypti* transcriptional responses common to aMel and aMelPop-CLA infections. Transcripts are ranked by the magnitude of Absolute Fold Change (AFC). Transcript identifiers (Transcript ID) and Description were compiled from Vectorbase. *Drosophila melanogaster* Gene Identifier (Dm Gene ID) and Symbol were compiled from Flybase.

Table S2 *Aedes aegypti* transcriptional responses to aMelPop-CLA infection. Transcripts are ranked by the magnitude of Absolute Fold Change (AFC). Transcript identifiers (Transcript ID) and Description were compiled from Vectorbase. *Drosophila melanogaster* Gene Identifier (Dm Gene ID) and Symbol were compiled from Flybase.

Table S3 *Aedes aegypti* transcriptional responses to aMel infection. Transcripts are ranked by the magnitude of Absolute Fold Change (AFC). Transcript identifiers (Transcript ID) and Description were compiled from Vectorbase. *Drosophila melanogaster* Gene Identifier (Dm Gene ID) and Symbol were compiled from Flybase.

Table S4 Oligonucleotide primers used in Real-time qPCR experiments.

Acknowledgments
We thank members of the O’Neill and McGraw labs for helpful discussion, B. Wee for his help with the Gene Ontology program, J. Popovic for his help with the TaqMan assay, F. Frentiu for virus stock maintenance and virus injection advice, E. Caragata and T. Walker for their help with virus injection. We also thank the Queensland Institute of Medical Research and Queensland Health Forensic and Scientific Services for supplying viruses.

Author Contributions
Conceived and designed the experiments: ER EAM SLO. Performed the experiments: ER. Analyzed the data: ER YHY MW EAM. Wrote the paper: ER MW EAM SLO.
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