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Effect of *Wolbachia* on replication of West Nile Virus in mosquito cell line and adult mosquitoes

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Abstract

Wolbachia as an endosymbiont is widespread in insects and other arthropods and is best known for reproductive manipulations of the host. Recently, it has been shown that *w*Melpop and *w*Mel strains of *Wolbachia* inhibit replication of several RNA viruses, including Dengue virus, and other vector-borne pathogens (e.g. *Plasmodium* and filarial nematodes) in mosquitoes providing an alternative approach to limit transmission of vector-borne pathogens. In this study, we tested the effect of *Wolbachia* on the replication of West Nile Virus (WNV). Surprisingly, accumulation of the genomic RNA of WNV for all the three strains of WNV tested (New York 99, Kunjin and New South Wales) was enhanced in *Wolbachia*-infected *Aedes aegypti* cells (Aag2). However, the amount of secreted virus was significantly reduced in the presence of *Wolbachia*. Intrathoracic injections showed that replication of WNV in *A. aegypti* mosquitoes infected with *w*Mel strain of *Wolbachia* was not inhibited; while *w*MelPop strain of *Wolbachia* significantly reduced replication of WNV in mosquitoes. Further, when *w*MelPop mosquitoes were orally inoculated with WNV, virus infection, transmission and dissemination rates were very low in *Wolbachia*-free mosquitoes and these were completely inhibited in the presence of *Wolbachia*. The results suggest that (i) despite enhancement of viral genomic RNA replication in the *Wolbachia*-infected cell line, production of secreted virus was significantly inhibited, (ii) the anti-viral effect in intrathoracically-infected mosquitoes depends on the strain of *Wolbachia*, and (iii) replication of the virus in orally infected mosquitoes was completely inhibited in *w*MelPop strain of *Wolbachia*.

Introduction

Wolbachia pipientis is an intracellular endosymbiotic bacterium that has been reported from several groups of invertebrates. The bacteria are widespread in insects; estimated to be present in about 65% of insect species (10). *Wolbachia* is mainly known for its effects on reproductive traits of hosts causing feminization, male-killing and most commonly cytoplasmic incompatibility (CI) (30). Due to the induced CI effect, production of unviable progeny when an uninfected male mates with a *Wolbachia*-infected female, the endosymbiotic bacteria rapidly invade and spread within the host population (11). In addition to the manipulations of reproduction, recent reports have shown that certain strains of *Wolbachia* cause life-shortening and behavioural changes in the host (22, 24). Most importantly, *Wolbachia* infection also inhibits replication of RNA viruses (e.g. Dengue virus [DENV], Chikungunya virus [CHIKV], *Drosophila* C virus) and other insect-transmitted pathogens (filarial nematode and *Plasmodium*) (8, 15, 23, 29). This provided a breakthrough to utilize *Wolbachia* for the control of vector-borne diseases by targeting the vector. The introduction of *w*Mel and *w*MelPop-CLA strains of *Wolbachia* into *Aedes aegypti*, which is the main vector of DENV, provided an opportunity to generate insects that do not support replication of the virus (33); hence, inhibiting transmission of the virus. *w*Mel-infected *A. aegypti* mosquitoes have recently been released in the wild in Australia and shown to successfully invade and establish in two natural populations of the mosquitoes (12). However, the mechanism of inhibition of virus replication by *Wolbachia* is still unknown.

Flaviviruses are the most common insect-transmitted viruses (arboviruses) and include viruses such as Dengue virus, West Nile virus, Japanese encephalitis virus, and yellow fever virus. We recently showed that a microRNA (miRNA) is encoded by the Kunjin strain of West Nile Virus (WNV_{KUN}), KUN-miR-1, from the terminal 3' stem loop (3'SL) located in the 3'UTR of the virus genome and that non-coding subgenomic flavivirus RNA (sfRNA) is likely to be the main source of KUN-miR-1 (14). miRNAs are small non-coding RNAs of 21-22 nucleotides that have been shown to play important roles in the regulation of gene expression and are involved in various biological processes

such as development, cancer and host-pathogen interactions. Interaction of miRNAs with target mRNAs leads either to degradation of mRNA, repression of translation or in certain instances up-regulation of transcript levels (2, 20). KUN-miR-1 miRNA was found to be essential for virus replication as inhibition of the miRNA by a sequence-specific synthetic inhibitor RNA reduced replication of the virus (14). The target of KUN-miR-1 was determined to be the host GATA4 transcription factor, which is induced following virus infection. GATA4 induction was also shown to be essential for replication of WNV_{KUN} since silencing of GATA4 by RNAi significantly reduced replication of the viral RNA.

In this study, we found that *Wolbachia* infection of mosquito cells enhances replication and accumulation of the genomic RNA (gRNA) of different WNV strains, i.e. highly pathogenic New York 99 (WNV_{NY99}), non-pathogenic Kunjin MRM61C (WNV_{KUN}) and a recently isolated virulent strain of Kunjin from a 2011 outbreak in horses in New South Wales (Australia) ($WNV_{NSW2011}$) (6). Interestingly, we found that GATA4, which enhances WNV replication, is also up-regulated in *Wolbachia*-infected cells which may have led to more efficient replication of the gRNA. However, titration of secreted virus showed that the amount of secreted virus was significantly reduced in the presence of *Wolbachia*, which is consistent with the previously published significant inhibition of DENV replication in *Wolbachia*-infected cells (23). In vivo experiments by intrathoracic injections showed that WNV replication was not inhibited in *w*Mel-infected *A. aegypti* mosquitoes, but its replication was significantly reduced in *w*MelPop-infected mosquitoes. In contrast, oral feeding of *A. aegypti* mosquitoes showed that firstly, *A. aegypti* is confirmed to be a poor vector of WNV and secondly, *w*MelPop completely inhibited infection of these mosquitoes with the virus.

MATERIALS AND METHODS

Mosquito cells and viral infection. *A. aegypti* Aag2 cells and *Wolbachia*-infected Aag2 cells (aag2.*w*MelPop-CLA) were maintained in Schneider's medium supplemented with 10% fetal

bovine serum (Life Technologies) as monolayers (13). Cells were infected at a MOI=1 with either wild type or mutant WN viruses defective in the generation of sfRNA/miR-1 (14, 27). Three strains of WNV were used in this study: New York 99 (WNV_{NY99}), Kunjin MRM61C (WNV_{KUN}) and Kunjin New South Wales ($WNV_{NSW2011}$). Cells were also infected with DENV type 2 as above. Virus titres in the supernatants of infected cells were determined by standard plaque assay on BHK cells.

Virus infection and transmission rates in mosquitoes. PGYP1.out mosquitoes (designated as *w*MelPop) derived from *A. aegypti* stably transinfected with *w*MelPop-CLA strain of *Wolbachia* (22) and MGYP2.OUT mosquitoes (designated as *w*Mel) derived from *A. aegypti* stably transinfected with *w*Mel strain of *Wolbachia* (33) and their tetracycline-treated, *Wolbachia*-free but genetically identical mosquito lines (designated as Tet-cured). Insects were reared at 27ºC with 70% relative humidity and a 12-hour light regime. Larvae were maintained with fish food pellets (Tetra,Melle, Germany) and adults were offered 10% sucrose solution.

Female mosquitoes of 3-5 days old were intrathoracically inoculated with $WNV_{NSW2011}$ virus stock (6.5×10^8 $TCID_{50}$/ml), at a maximal volume of 69 nl per mosquito, using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific). The inoculated mosquitoes were kept at 27^{o}C until sampling. Saliva and body samples were collected at 7 and 10 days post-inoculation. The saliva was sampled by inserting the proboscis into a pipette tip loaded with 20 µl of fetal bovine serum (FBS) and allowing the mosquito to salivate for 45 min. The saliva samples and the body parts were stored at -80^{o}C until testing.

The body and saliva samples were tested for the presence of $WNV_{NSW2011}$ by cell culture-ELISA (3) to determine infection and transmission rates, respectively. The body of each mosquito was homogenized in 500 µl of grinding media (RPMI 1640, supplemented with 2% FBS, 1% Pen-Strep, 1% Fungizone), followed by centrifugation at 9000 rpm for 5 min, at 4^{o}C. The supernatant (100 µl/well) was used to inoculate C6/36 *A. albopictus* cell monolayers in duplicate for virus detection in 96-well tissue culture plates. The saliva samples were each mixed with 50 µl of grinding media

and the entire mixture was inoculated onto a C6/36 monolayer. Five days after inoculation, $WNV_{NSW2011}$ in the monolayers was detected by a flavivirus specific monoclonal antibody 4G4 (5). The positive body samples were subjected to titration for $WNV_{NSW2011}$ load by cell culture ELISA (3).

For oral inoculations, +Wol and –Wol *A. aegypti* mosquitoes were fed with sheep blood containing $10^{7.05}$ TCID50/ml WNV_{KUN}. Mosquitoes were collected at 4, 7 and 10 days post-feeding, and infection, disseminated infection and transmission rates were determined as above.

Quantitative RT-PCR. GATA4 transcript levels were determined by qRT-PCR using specific primers to *GATA4* (forward: 5'-GGGACCGATTCTACGTATG-3'; reverse: 5'-CGTAGAATGTTCAATCTGC-3'). To analyze virus RNA replication with RT-qPCR, specific primers to the genomic RNA (gRNA) in the capsid gene region were used (For WNV_{KUN} and $WNV_{NSW2011}$: Forward 5'-GCGAGCTGTTTCTTAGCACGA-3'; Reverse 5'-CCGTGAACCTAAAAAACGCC-3'; For WNV_{NY99}: Forward 5'-GCGGCGGCAATATTCATG-3'; Reverse 5'-ACGTTGTAGGCAAAGGGCAA-3'). RPS17 was used as normalizing reference. The PCR conditions were: 50 °C for 2 min; 95 °C for 2 min; and 40 cycles of 95 °C for 15s, 60 °C for 30s, and 72 °C for 15s, followed by the melting curve (68 °C to 95 °C). In all the qPCR reactions, SYBR Premix ExTaq II (TaKaRa), which utilizes SYBR Green, was used. The *t*-test was used to compare differences in means between different treatments. Fold changes in gRNA and *GATA4* were calculated first by normalizing data against *RPS17* cellular gene followed by normalizing data against mock or control treatment.

RESULTS

***Wolbachia* infection induces expression of GATA4 in mosquito cells.** Following our investigations into differential expression of mosquito host miRNAs and mRNAs upon *Wolbachia* *w*MelPop-CLA infection, the expression of GATA4 (GenBank accession number XM_001654324) was significantly increased in *A. aegypti* Aag2 cells infected with *w*MelPop-CLA (aag2.*w*MelPop-

CLA) compared to non-infected Aag2 cells (**Fig. 1A**; $P < 0.05$). To find out if GATA4 is also up-regulated in *A. aegypti* mosquitoes infected with *w*MelPop-CLA (+Wol), we tested +Wol mosquitoes and those without *Wolbachia* (-Wol) by qRT-PCR. The results confirmed that GATA4 is also up-regulated in +Wol mosquitoes (**Fig. 1B**; $P < 0.001$).

***Wolbachia* infection enhances WNV gRNA replication in mosquito cell line but inhibits virus assembly and/or secretion.** Previous studies have shown that *Wolbachia* infection inhibits replication of a variety of RNA viruses (8, 15, 23, 29). Since we recently showed that KUN-miR-1 encoded by WNV up-regulates GATA4 transcript levels which in turn enhances replication of WNV_{KUN} (14), we investigated replication of the virus in *Wolbachia*-infected Aag2 cells considering that they have increased levels of GATA4 expression (**Fig. 1A**). When cells were analyzed 72 h after WNV_{KUN} infection by qRT-PCR using specific primers to the capsid-coding region of viral genomic RNA, we found 13 fold more virus RNA replication in aag2.*w*MelPop-CLA cells compared to Aag2 cells (**Fig. 2A**; compare Aag2-KUNV and pop-KUNV; $P < 0.0001$). A mutant of WNV_{KUN} (IRAΔCS3) which produces significantly less KUN-miR-1 replicated poorly (4 folds less RNA) in Aag2 cells in comparison to the wild-type virus (14, 27); also see **Fig. 2A**, compare Aag2-KUNVmut and Aag2-KUNV; $P < 0.0001$). Interestingly, we found that the RNA of this mutant virus replicated more efficiently (12 fold more) in aag2.*w*MelPop-CLA cells compared to Aag2 cells (**Fig. 2A**; compare Aag2-KUNVmut and pop-KUNVmut; $P < 0.0001$). In addition, qRT-PCR results confirmed that the wild type WNV_{KUN} induced GATA4 transcription significantly higher than the mutant virus in Aag2 cells (**Fig. 2B**; $P < 0.0001$). This further confirmed that GATA4 induced by KUN-miR-1 and/or by *Wolbachia* infection enhances WNV_{KUN} gRNA replication.

To find out whether another flavivirus, DENV, also induces expression of GATA4, we infected Aag2 cells with DENV (type 2) and analyzed total RNA extracted from cells at 3 and 5 days after infection. Interestingly, we found that in contrast to WNV infection, GATA4 transcription was reduced in DENV-infected cells (**Fig. 2C**; $P < 0.05$). Although the 3'SL from which KUN-miR-1 is

processed is conserved among flaviviruses (27), the miRNA sequence is different between WNV and DENV. Even if a miRNA is produced from DENV 3'SL, it would not have sufficient complementarity with the sequence targeted by KUN-miR-1 in the GATA4 mRNA. We also confirmed that under our experimental conditions *Wolbachia* inhibits DENV gRNA replication in aag2.*w*MelPop-CLA cells compared to Aag2 cells (**Fig. 2D**), which is consistent with previous findings (23).

The virulent WNV_{NY99} strain has 98% amino acid sequence identity with the non-pathogenic WNV_{KUN} strain (1). Since its emergence in USA in 1999 and until 2010, ~1.8 million people were infected, with ~360,000 illnesses, close to 13,000 reported cases of encephalitis, and 1308 deaths (16). We examined WNV_{NY99} replication in Aag2 and aag2.*w*MelPop-CLA cells by qRT-PCR and verified that significantly more viral gRNA was produced in aag2.*w*MelPop-CLA cells compared to Aag2 cells (**Fig. 3A**; $P = 0.0007$). In addition, significantly more viral gRNA was produced in aag2.*w*MelPop-CLA cells compared to Aag2 cells infected with a WNV_{NY99} IRAΔCS3 mutant defective in sfRNA production (NY99mut, will be described elsewhere) (**Fig. 3B**; $P = 0.0003$). A more virulent strain of WNV_{KUN} was recently isolated from a 2011 outbreak in horses in New South Wales, Australia ($WNV_{NSW2011}$) that has 99% amino acid sequence identity to WNV_{KUN} (6). We also confirmed that significantly more $WNV_{NSW2011}$ gRNA was produced in aag2.*w*MelPop-CLA cells compared to Aag2 cells (**Fig. 3C**; $P < 0.0001$). Subsequently, we also confirmed that GATA4 expression is significantly up-regulated in both WNV_{NY99}- and $WNV_{NSW2011}$-infected cells (**Fig. 4**; $P < 0.0001$). However, significantly less GATA4 was produced in Aag2 cells infected with a NY99mut (defective in sfRNA production, therefore defective in KUN-miR-1 homolog production) (**Fig. 4**; $P < 0.0001$). Overall, these results clearly demonstrate that GATA4 is induced by all WNV strains (KUN, NY99, NSW2011) examined. It is therefore likely that *Wolbachia* infection enhances replication of the WNV gRNA by having significantly up-regulated levels of GATA4 prior to infection.

Since WNV gRNA replication was enhanced in *Wolbachia*-infected cells, we explored if this translates into more virus production in the culture fluid of aag2.*w*MelPop-CLA cells. Aag2 and aag2.*w*MelPop-CLA cells were infected with WNV_{KUN} and subsequently cells and media were collected from the cells at days 2 and 3 post-infection. Interestingly, plaque assays revealed that significantly less virus particles were produced in the culture fluid of aag2.*w*MelPop-CLA cells compared to Aag2 cells (**Fig. 5A;** $P < 0.0001$). This experiment with three biological replicates was independently repeated twice with reproducible results. When RNA extracted from cells from the same experiment was analysed by qRT-PCR, significantly more viral gRNA was found in aag2.*w*MelPop-CLA cells compared to Aag2 cells (**Fig. 5B;** $P < 0.0001$), consistent with the results shown above. In addition, Western blot analysis of cells from the same experiment using antibodies to the WNV protein E revealed that more viral protein was produced in aag2-*w*MelPop-CLA cells at 3 days post-infection compared to Aag2 cells (**Fig. 5C**). This suggested that although viral gRNA replication and protein production is enhanced in *Wolbachia*-infected cells, virus assembly and/or secretion is conversely inhibited in the presence of *Wolbachia*.

Effect of *Wolbachia* infection on WNV replication in mosquitoes. Previous studies (23, 33), and the confirmation shown in Fig. 2D, have shown that *Wolbachia* inhibits replication of DENV in *A. aegypti* cells and mosquitoes. Consistent with these, we demonstrated that *Wolbachia* inhibits production of secreted WNV in aag2-*w*MelPop-CLA cells. To investigate the effect of *Wolbachia* on WNV replication in mosquitoes, *A. aegypti* mosquitoes infected with *w*Mel or *w*MelPop strains of *Wolbachia* were intrathoracically injected with $WNV_{NSW2011}$. Subsequently, rate of infection and dissemination were determined in injected mosquitoes (Table 1). Virus titers were determined in the saliva and body samples by cell culture-ELISA using a monoclonal antibody to 4G4 (α-non-structural protein 1). The infection rate of WNV in *w*Mel mosquitoes was 100% in both +Wol and –Wol mosquitoes (Table 1). Transmission rate was also 49% and 66% at 7 and 10 days after inoculation, respectively (Table 1). In contrast, the transmission rate for DENV in *w*Mel-infected *A. aegypti* mosquito lines MGYP2 and MGYP2.OUT were reported as 4.2% and 0%, respectively

(33). This suggested that *w*Mel does not have the same inhibitory effect on WNV as on DENV. However, in *w*MelPop mosquitoes, inhibition of WNV infection was observed as the infection rate was determined to be 42% and 50% at 7 and 10 days after infection, respectively, as compared to 100% in –Wol mosquitoes at both days (Table 1). In *w*MelPop mosquitoes, transmission rates for WNV were determined to be 0% for both 7 and 10 days after inoculation (**Table 1**).

When virus loads were determined in *w*Mel and *w*MelPop mosquitoes injected with $WNV_{NSW2011}$, significantly less viral loads were detected in *w*Mel mosquitoes at 7 days after inoculation compared to –Wol mosquitoes (**Fig. 6A**; $P < 0.0001$). However, at 10 days after inoculation there was no significant difference between +Wol and –Wol mosquitoes (**Fig. 6A**; $P = 0.0611$). In *w*Mel DENV-infected mosquitoes, virus levels were strikingly lower (1500-fold fewer) than that of –Wol mosquitoes at 14 days post-inoculation (33). This demonstrated that the *w*Mel strain of *Wolbachia* does not inhibit WNV replication in mosquitoes when they are injected intrathoracically with the virus. However, in *w*MelPop mosquitos significantly less WNV loads were detected both at 7 and 10 days after inoculation compared to –Wol mosquitoes (**Fig. 6B**; $P < 0.0001$).

To mimic the natural route of mosquito infection, *A. aegypti* +Wol (*w*MelPop) and –Wol mosquitoes were orally inoculated with WNV_{KUN}. Compared to intrathoracic inoculation (**Table 1**), the infection, disseminated infection and transmission rates were substantially lower in –Wol mosquitoes (**Table 2**), which confirms that *A. aegypti* has a very poor vector competency for WNV (9, 31, 32) and that the gut provides a strong barrier against WNV infection. In +Wol mosquitoes, the infection, disseminated infection and transmission rates were all negligible (**Table 2**).

DISCUSSION

Vector-borne viruses, mostly belonging to the family *Flaviviridae*, cause significant number of mortalities/morbidities around the world. Among mosquito-borne flaviviruses, DENV and WNV account for ~50 million of cases per year worldwide (16, 17). In regard to both viruses, control options for the diseases caused by the viruses are limited and there are no effective vaccines

253 available for either. Therefore, control measures have concentrated on reducing the vector
254 populations. With the development of resistance to chemical pesticides in mosquitoes,
255 environmental contaminations caused by chemicals and public awareness, alternative approaches to
256 chemical control to reduce mosquito vector populations or limit transmission of viruses are of
257 immense importance. *Wolbachia* as a widespread endosymbiont of insects have provided promise
258 in disease control by reducing the lifespan of mosquito vectors (22) and most importantly by
259 inhibiting replication of arboviruses such DENV and CHIKV in mosquitoes (23). Recently, a
260 *w*Mel-infected population of *A. aegypti* were tested under controlled field conditions and were
261 shown to block DENV transmission in the mosquito providing an approach to inhibit DENV spread
262 (33).

263 In this study, we showed that *Wolbachia* enhances replication of WNV gRNA and protein
264 production in an *A. aegypti* cell line (Aag2) infected with *w*MelPop, but inhibits virus assembly
265 and/or secretion with the latter being consistent with published data for other arboviruses, such as
266 DENV and CHIKV (7). We also showed that three different strains of WNV (NY99, KUNV,
267 NSW2011) had enhanced gRNA replication and accumulation in aag2-*w*MelPop-CLA cells. In
268 contrast, under the same conditions, DENV gRNA replication and accumulation was significantly
269 inhibited in aag2-*w*MelPop-CLA cells. This suggests that *Wolbachia* may inhibit WNV and DENV
270 production by different mechanisms. While *Wolbachia* clearly inhibits DENV viral gRNA
271 replication and consequently virus production, the effect of *Wolbachia* on WNV infection appears
272 to occur at the later stages of infection interfering with either viral RNA packaging, or virion
273 assembly or virus secretion from infected cells. This interesting observation clearly requires further
274 investigations.

275 At 7 and 10 days after intrathoracic injection of $WNV_{NSW2011}$ in *A. aegypti*, differences in virus
276 loads were greater in *w*MelPop compared to *w*Mel-infected mosquitoes in relation to uninfected
277 mosquitoes, but the difference at 10 days after infection in *w*Mel mosquitoes was not significant.
278 The *w*Mel strain is known to have more specific tissue tropisms than *w*MelPop and our processing

of whole bodies rather than legs could lead to masking of interference by *Wolbachia*, due to the presence of both positive and negative tissues in the body samples. This is a plausible explanation considering that the anti-viral protection of *Wolbachia* has been shown to strongly correlate with the density and the tissue tropism of *Wolbachia* (19, 25). In *w*MelPop mosquitoes, however, WNV replication was inhibited. Consistently, *w*MelPop inhibited WNV infection of *A. aegypti* mosquitoes when they were orally inoculated, although the infection rate of the mosquitoes was substantially lower in orally inoculated mosquitoes (15%) compared to intrathoracically inoculated mosquitoes (100%). Inhibition of WNV replication in *Drosophila melanogaster* flies and *Culex quinquefasciatus* mosquitoes harbouring native *Wolbachia* endosymbionts was also shown previously (7).

Furthermore, we showed that induction of the transcription factor GATA4 by *Wolbachia* is likely to be the mechanism of the enhancement of WNV gRNA replication. We previously showed that a WNV_{KUN} virus encoded miRNA, KUN-miR-1, up-regulates the expression of GATA4 upon infection of Aag2 cells (14). In this study, we showed that the more virulent strains WNW_{NY99} and $WNV_{NSW2011}$, which are closely related to WNV_{KUN}, also induce expression of GATA4. We hypothesise that increased expression of GATA4 mRNA directly increases GATA4 protein levels. Therefore, considering that *Wolbachia*-infected mosquito cells overexpress GATA4, it would make this protein readily available to the virus from the moment it enters the host. This may give WNV an advantage to establish RNA replication compared to cells without *Wolbachia*. Notably, GATA4 expression decreases in DENV-infected cells suggesting that DENV gRNA replication may not require GATA4. In animals, GATA transcription factors are ubiquitous and play important roles in various biological processes such as development, differentiation, and innate immunity (28). They all share one or two zinc finger DNA binding domains with the conserved CX2CX17CX2C motifs (18). In *A. aegypti*, members of the GATA family have been shown to regulate egg development by repressing or activating genes involved in the process. GATA4, specifically, is expressed after a blood meal and acts as a transcriptional activator of vitellogenin (*vg*), which is an important protein

in vitellogenesis and egg development (26). In addition, GATA4 in conjunction with NF-κB transcription factors were found to be required for induction of lipophorin receptor gene involved in *A. aegypti* systemic immune responses and lipid metabolism (4). In insects, lipophorin is the main lipid carrier protein transporting lipids to various tissues and is also involved in immune responses (4, 21). It is not clear at this stage how up-regulation of GATA4 by KUN-miR-1 or *Wolbachia* may facilitate WNV gRNA replication in mosquito cells and this requires further investigation.

In conclusion, we have shown that the *w*MelPop strain of *Wolbachia* enhances replication of WNVs gRNA in vitro, while it inhibits replication of DENV gRNA. However, similar to DENV infection, production of secreted WNV virions was inhibited by *Wolbachia*. In addition, in *w*Mel-carrying *A. aegypti* mosquitoes, replication of the WNV (NSW2011 strain) was not inhibited when injected intrathoracically with the virus. In *w*MelPop-carrying mosquitos, however, WNV replication was inhibited both when inoculated intrathoracically or orally fed with WNV. The enhancement of replication of the WNV gRNA in *Wolbachia*-infected *A. aegypti* cells appears to correlate with the up-regulation of GATA4, which had been shown to facilitate replication of the virus gRNA (14). *A. aegypti* is not considered as the primary vector of WNVs, but the virus has the potential to infect and be disseminated by these mosquitoes (9, 31, 32). Infection and dissemination rates of up to 86%, respectively, were reported for *A. aegypti* infected with WNV (32). In our study, we found very low infection rates of *A. aegypti* (15%) when mosquitoes free of *Wolbachia* were orally fed with WNV, and this rate was nil in *Wolbachia*-infected mosquitoes. In this context, the results from this report suggests that the *Wolbachia*-infected *A. aegypti* mosquitoes released in the field to control the transmission of DENV (33) are not likely to pose a threat in enhancing replication of various strains of WNV. Further studies should direct towards the mechanism(s) by which GATA4 in *Wolbachia*- or WNV-infected cells is induced and how does induction of the transcription factor facilitates replication of the virus gRNA. In addition, the mechanism by which WNV RNA packaging, and/or virion assembly/secretion, is inhibited concurrently with enhancement of viral RNA replication and accumulation merits further investigation.

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445 **Figure legends**

446 **Figure 1:** *Wolbachia w*MelPop-CLA induces GATA4 transcript levels both *in vitro* and *in vivo*. **(A)**
447 qRT-PCR analysis of RNA from Aag2 and aag2.*w*MelPop-CLA cells (Aag2-pop) using specific
448 primers to GATA4. **(B)** qRT-PCR analysis of *A. aegypti* mosquitoes without (tet; treated with
449 tetracycline) and with *w*MelPop-CLA (pop). Error bars indicate standard deviations of averages
450 from two biological and three technical replicates.

451 **Figure 2:** Viral gRNA and GATA4 levels in WNV_{KUN} and Dengue virus infected Aag2 cells. **(A)**
452 Fold changes of WNV_{KUN} and mutant (KUNVmut) WNV_{KUN} gRNA in Aag2 and aag2.*w*MelPop-
453 CLA cells (pop) 3 days after infection analyzed by qRT-PCR using specific primers to the viral
454 capsid protein gene. **(B)** Fold changes of GATA4 transcripts in Aag2 cells infected with WNV_{KUN}
455 for 3 days and its mutant (KUNVmut) analyzed by qRT-PCR. **(C)** Fold changes of GATA4
456 transcripts in aag2.*w*MelPop-CLA cells either mock-infected or infected with DENV at 3 (3D) and
457 5 (5D) days post-infection. **(D)** Relative gRNA levels of DENV in Aag2 and aag2.*w*MelPop-CLA
458 cells 5 days post-infection. Error bars indicate standard deviations of averages from two biological
459 and three technical replicates.

460 **Figure 3:** WNV_{NY99} and $WNV_{NSW2011}$ RNA replicates and accumulates more efficiently in
461 *w*MelPop-infected cells. **(A)** qRT-PCR analysis of RNA from Aag2 and aag2.*w*MelPop-CLA (pop)
462 cells infected with WNV_{NY99}. **(B)** qRT-PCR analysis of RNA from Aag2 and aag2.*w*MelPop-CLA
463 (pop) cells infected with a WNV_{NY99} mutant (NY99mut) defective in production of sfRNA. **(C)**
464 qRT-PCR analysis of RNA from Aag2 and aag2.*w*MelPop-CLA (pop) cells infected with
465 $WNV_{NSW2011}$. Specific primers to the viral capsid protein were used. Cells were collected 3 days
466 after infection. Error bars indicate standard deviations of averages from two biological and three
467 technical replicates.

468 **Figure 4:** WNV_{NY99} and $WNV_{NSW2011}$ both induce GATA4 transcription. Aag2 cells were infected
469 with WNV_{NY99} (NY99), WNV_{NY99} mutant (NY99mut) and $WNV_{NSW2011}$ (NSW2011) for 3 days and

their extracted RNA were analyzed by qRT-PCR using specific primers to their capsid protein genes. Error bars indicate standard deviations of averages from two biological and three technical replicates.

Figure 5: *Wolbachia* enhances WNV RNA accumulation but inhibits virus assembly/secretion in *w*MelPop-infected cells. **(A)** Plaque assay of media collected from Aag2 and aag2.*w*MelPop-CLA cells (Pop) infected with WNV_{KUN} at 2 and 3 days post-infection. Error bars indicate standard deviations of averages from three biological replicates. **(B)** qRT-PCR analysis of RNA extracted from cells in (A) using specific primers to the viral capsid protein. Error bars indicate standard deviations of averages from three biological and three technical replicates. **(C)** Western blot analysis of cell from (A) probed with antibodies to the WNV E protein (E) and hsp70 as a loading control. Each lane is a mixture of cells from three biological replicates.

Figure 6: Viral titers in $WNV_{NSW2011}$-positive *A. aegypti* mosquitoes. The first 20 positive body samples from each group of mosquitoes collected at 7 and 10 days after viral inoculation were selected for determining viral titers by ELISA using monoclonal antibody against NS1 protein, 4G4. Virus titer in **(A)** *w*Mel and Tet mosquitoes, and **(B)** *w*MelPop and Tet mosquitoes at 7 and 10 days after $WNV_{NSW2011}$ inoculations.

Table 1: WNV body infection rate (IR) and transmission rate (TR) following intrathoracic inoculation in *A. aegypti* mosquitoes.

		Body			Saliva		
		Total	**Positive**	**IR(%)**	**Total**	**Positive**	**TR(%)**
7 day	Tet-cured	43	43	100	43	43	100
	*w*Mel	37	37	100	37	18	49
10 day	Tet-cured	39	39	100	39	39	100
	*w*Mel	35	35	100	35	23	66
7 day	Tet-cured	29	29	100	29	24	82.8
	*w*MelPop	26	11	42	26	0	0
10 day	Tet-cured	26	26	100	26	24	92.3
	*w*MelPop	26	13	50	26	0	0

Table 2: WNV body infection rate (IR), disseminated infection rate (DIR) and transmission rate (TR) following oral inoculation in *A. aegypti* mosquitoes. Infection = % infection (n).

	Day 4			**Day 7**			**Day 10**		
	IR	DIR	TR	IR	DIR	TR	IR	DIR	TR
*w*MelPop	0(40)	0(40)	0(40)	0(40)	0(40)	0(40)	0(40)	0(40)	0(40)
Tet	21.9(41)	4.8(41)	0(41)	7.5(40)	5(40)	0(40)	15(40)	12.5(40)	2.5(40)

Figure 1

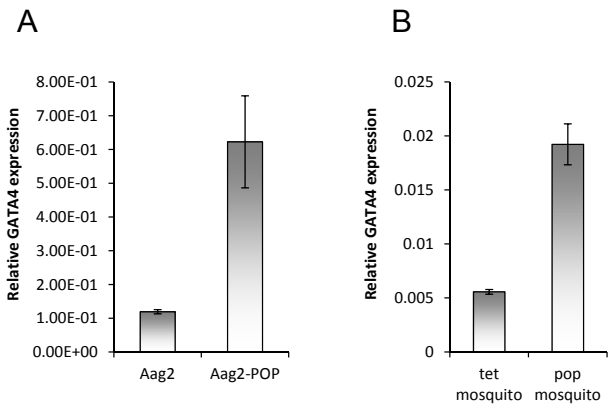
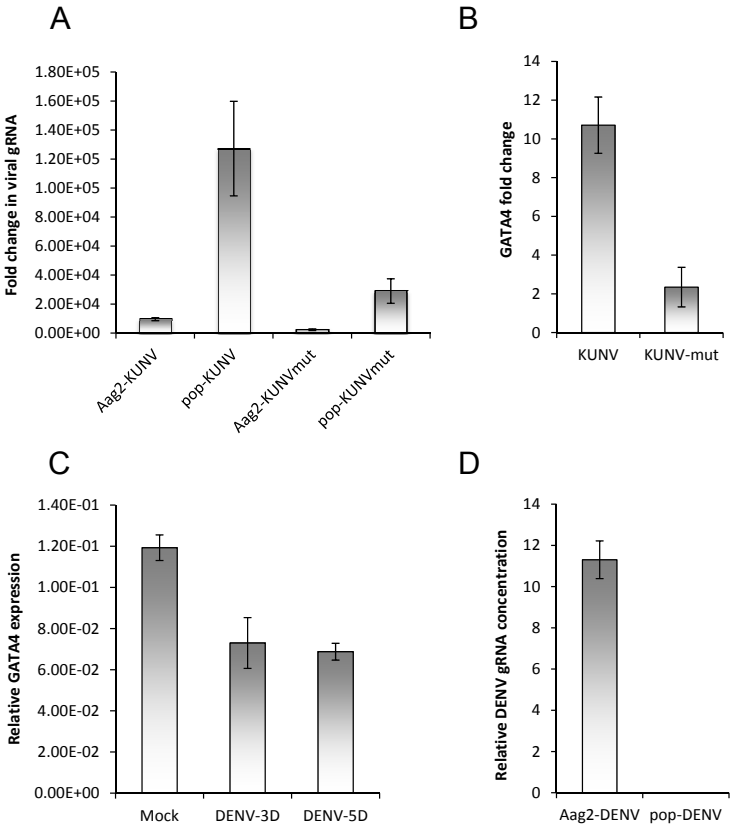


Figure 2



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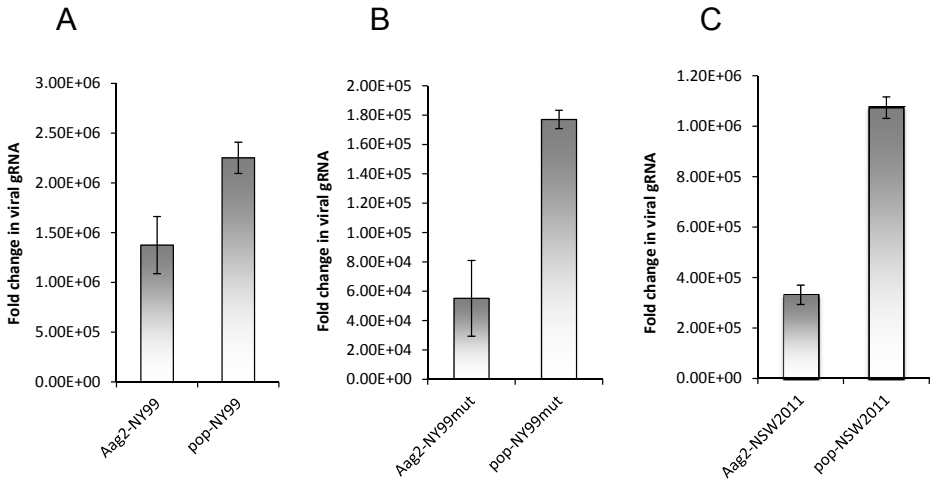

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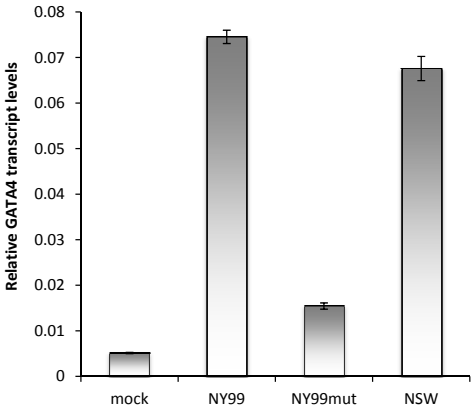

Figure 5

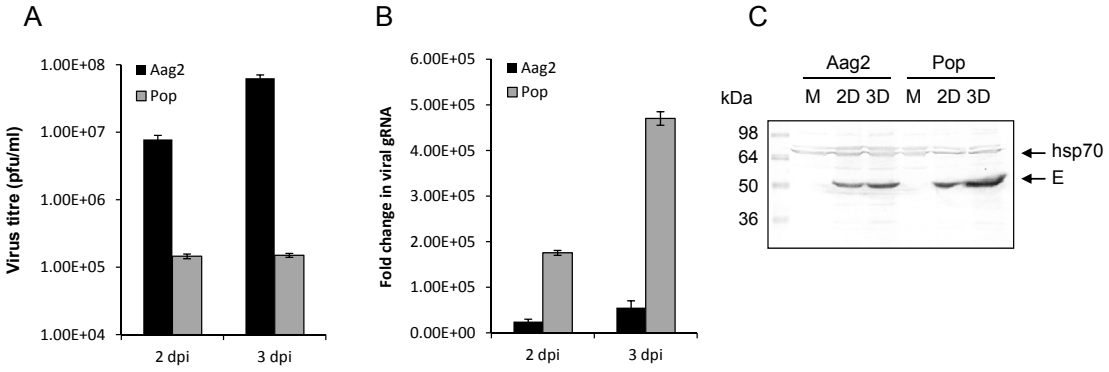

Figure 6