

The Toll and Imd Pathways Are Not Required for *Wolbachia*-Mediated Dengue Virus Interference

Edwige Rancès,^{a*} Travis K. Johnson,^{a,b} Jean Popovici,^{a*} Iñaki Iturbe-Ormaetxe,^a Tasnim Zakir,^a Coral G. Warr,^a Scott L. O'Neill^{a,c}

School of Biological Sciences, Monash University, Clayton, Victoria, Australia^a; Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia^b; Institute of Molecular Biosciences, University of Queensland, St Lucia, Queensland, Australia^c

***Wolbachia* blocks dengue virus replication in *Drosophila melanogaster* as well as in *Aedes aegypti*. Using the *Drosophila* model and mutations in the Toll and Imd pathways, we showed that neither pathway is required for expression of the dengue virus-blocking phenotype in the *Drosophila* host. This provides additional evidence that the mechanistic basis of *Wolbachia*-mediated dengue virus blocking in insects is more complex than simple priming of the host insect innate immune system.**

The common intracellular bacterium *Wolbachia pipiensis* (1) is maternally inherited through the eggs of its insect hosts. It is able to successfully invade host populations through a range of reproductive manipulations that either directly or indirectly favor its transmission between insect generations (2–8). In its natural host, it has recently been shown that the presence of *Wolbachia* can block the replication of RNA viruses (9–13). This effect is the basis for the recent development of *Wolbachia* as a biocontrol approach to block dengue virus (DENV) transmission by the mosquito *Aedes aegypti* (14, 15).

The main vector of DENV, the mosquito *Aedes aegypti*, is not naturally infected with *Wolbachia*. However, different strains of *Wolbachia* have recently been artificially introduced from *Drosophila melanogaster* (*Wolbachia* strains *wMel* and *wMelPop*) or *Aedes albopictus* (*Wolbachia* strain *wAlbB*) into *A. aegypti* and are stably maintained in laboratory and wild mosquito populations (8, 16–18). The expectation is that the negative impact that *Wolbachia* has on DENV replication in the insect will reduce virus transmission to humans and subsequent disease (18–20).

The mechanism(s) that underlies the ability of *Wolbachia* to affect the replication of DENV appears complex. Using the heterologous association of *Wolbachia*-infected *A. aegypti*, transcriptomic and biochemical studies have demonstrated that *Wolbachia* induces the production of reactive oxygen species (ROS); primes the innate immune system of the mosquito, especially the Toll signaling pathway; and induces the production of various antimicrobial effectors (19–23). In addition, the use of RNA interference (RNAi) depletion to partially knock down defensin and cecropin genes in *Wolbachia*-infected *A. aegypti* lowered resistance to DENV and suggested a role for the innate immune system in mediating virus resistance (22). In contrast, in their natural host *D. melanogaster*, the same *Wolbachia* strains do not induce overexpression of immune genes, including the Toll pathway and cecropin- and defensin-encoding genes, yet RNA virus interference, including DENV interference, occurs (23, 24). These results demonstrated that induction of the Toll pathway by *Wolbachia* is not the exclusive mechanism mediating resistance. However, gene expression studies are not sufficient to make a link between a phenotype and a genetic pathway. Since previous studies confirmed the ability of DENV to replicate, and of *Wolbachia* to block its replication in *Drosophila* (23), we took advantage of preexisting and well-characterized mutant fly strains lacking functional Toll

or Imd pathways to determine a precise role for these pathways in the *Wolbachia*-mediated viral blocking phenotype.

Role of the Toll and Imd pathways in controlling *Wolbachia wMelPop* density and tissue tropism. In order to characterize the role of the Toll and Imd pathways in the phenotype of *Wolbachia*-mediated viral blocking, we introduced the *Wolbachia* strain *wMelPop* into *Drosophila* lines carrying loss-of-function mutations in genes essential to the two pathways. For the Toll pathway, strong hypomorphic mutations of *spätzle* (*spz*) were tested (*spz*² and *spz*⁴). For the Imd pathway, a null allele of *relish* (*rel*^{E20}) was used (25, 26). These particular alleles of *spz* and *rel* were used as they have been well documented to cause pathogen susceptibility due to reduced production of antimicrobial effector peptides such as cecropins and defensins (25, 26). Prior to introduction of *wMelPop* into the different mutant strains, we tested each for the presence of *Wolbachia* using *Wolbachia*-specific PCR, targeting the *wsp* gene that encodes the major surface protein of the bacterium (27). All strains were determined to be uninfected with the exception of the original *spz*⁴ strain, which was positive for *Wolbachia* infection. Further characterization by sequencing the *wsp* amplicon showed that it was identical to the *wsp* sequence of *wMel*, a *Wolbachia* strain known to commonly infect *D. melanogaster* (7). Since *Wolbachia* is only maternally transmitted, we could use simple crosses to establish *wMelPop* and *wMel* single-infected lines in different host genetic backgrounds. To generate *wMelPop*-infected balancer lines, *D. melanogaster* *w*¹¹¹⁸ *wMelPop* virgin females were crossed with virgin TM3/TM6B males (*w*¹¹¹⁸ background). *wMelPop*-infected TM6B/+ virgin females were crossed with *wMelPop*-infected TM3/+ males to generate the *wMelPop*-infected TM3/TM6B balancer line. The *Wolbachia*-infected mutant lines were established by crossing *wMelPop*-infected TM3/TM6B virgin females with males of the Toll and Imd mutant lines. The *spz*²*wMelPop*, *spz*⁴*wMelPop*, *spz*⁴*wMel*, and

Received 5 June 2013 Accepted 19 August 2013

Published ahead of print 28 August 2013

Address correspondence to Scott L. O'Neill, scott.oneill@monash.edu.

* Present address: Edwige Rancès, Institut Pasteur de la Guyane, Cayenne, French Guiana; Jean Popovici, Institut Pasteur de la Guyane, Cayenne, French Guiana.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.01522-13

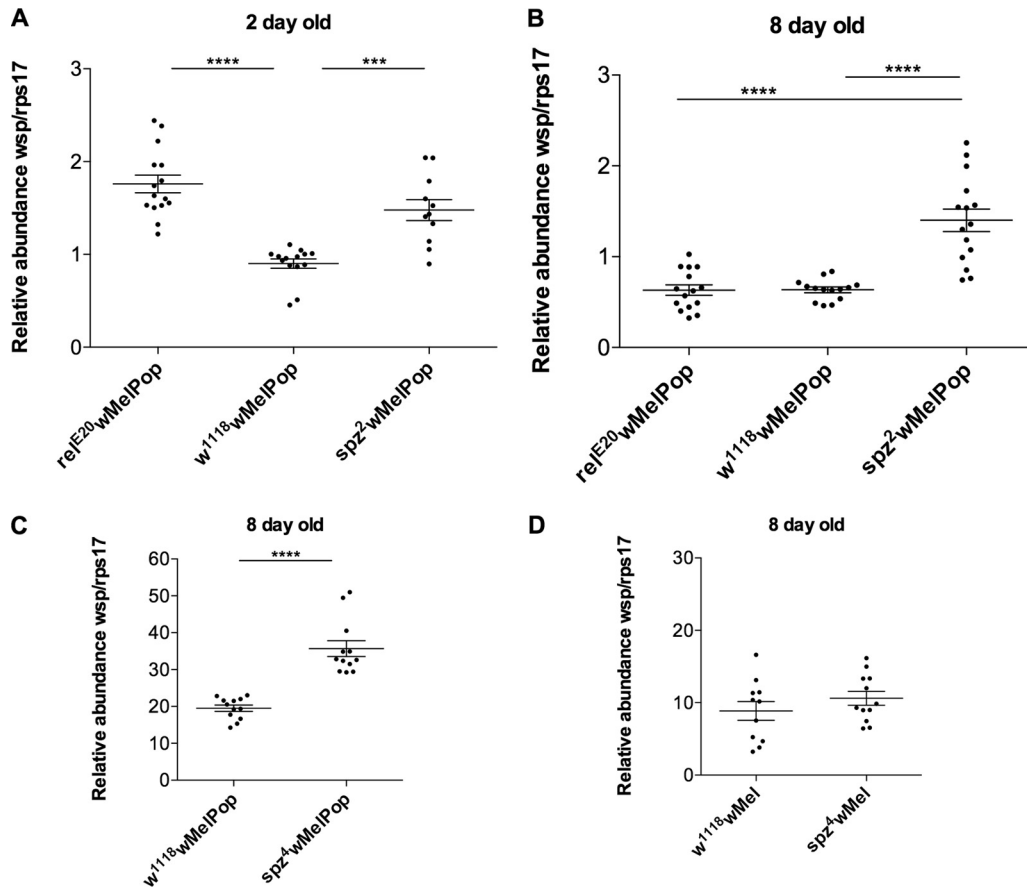


FIG 1 *Wolbachia* density in *Drosophila* lines deficient for Toll and Imd pathways. (A and B) *wMelPop* relative density in 2 (A)- and 8 (B)-day-old virgin females deficient for Imd (*rel^{E20}wMelPop*) and Toll (*spz²wMelPop*) pathways and the control line (*w¹¹¹⁸wMelPop*). (C and D) *wMelPop* (C) and *wMel* (D) relative density in 8-day-old virgin females deficient for Toll pathway (allele *spz⁴*). $n = 11$ to 15 ; Mann-Whitney U test; ***, $P < 0.001$; ****, $P < 0.0001$.

rel^{E20}wMelPop flies were maintained as heterozygotes balanced and as homozygotes for each experiment. The tetracycline-cured lines were derived by the addition of tetracycline (0.3 mg/ml) to the larval diet for two generations and confirmed to be free of *Wolbachia* by PCR (28). The *wMelPop*-infected mutant lines and their uninfected counterparts were confirmed to be homozygous mutant for the different Toll and Imd pathway alleles by bacterial challenge using appropriate pathogens (data not shown; see also reference 29).

Previous studies suggest that the magnitude of virus blocking and *Wolbachia* density are positively correlated (30, 31). Moreover, the *wMelPop* strain of *Wolbachia* is known to overreplicate in host tissues, causing pathology and ultimately reducing the life span of its host (17, 32). Given these observations, we first examined the impact of both the Toll and Imd pathways on *Wolbachia* density and tissue tropism before testing DENV replication in *wMelPop*-infected mutant backgrounds. To assay *Wolbachia* density, 15 virgin females from each line were maintained in vials under controlled conditions, at 26°C with 60% relative humidity and a 12-h light/dark cycle. DNA was extracted from 9 to 15 individual females either 2 or 8 days posteclosion, using the ReliaPrep gDNA Tissue Miniprep system (Promega), according to the manufacturer's instructions. *Wolbachia* density was then determined by relative quantitative PCR (qPCR) by comparing the abundance of the single-copy *Wolbachia* surface protein gene (*wsp*) to that of

the single-copy *D. melanogaster rps17* gene as previously described (18). Data were analyzed using Mann-Whitney U tests (GraphPad Prism 5). Three independent experimental replicates were performed to confirm the results obtained with *rel^{E20}* and *spz²* mutants. Surprisingly, we found a significant increase in *wMelPop* density (approximately 2-fold) in both mutants (*rel^{E20}* and *spz²*) in 2-day-old flies compared to the wild-type line *w¹¹¹⁸wMelPop* (Fig. 1A). However, in 8-day-old flies only the Toll signaling pathway mutant *spz²* exhibited a significant difference in *wMelPop* density for all three replicates (Fig. 1B). Similar results were obtained with another genetic background for loss of function of Toll pathway, using 2- and 8-day-old *spz⁴* mutants (Fig. 1C). This suggests that Toll and Imd pathways both have a role in modulating *wMelPop* density.

We took advantage of having *spz⁴* mutants infected with a different strain of *Wolbachia* to test whether the impact of the Toll signaling pathway on *wMelPop* density extended to other strains of *Wolbachia*. No significant difference in *wMel* density was observed between 8-day-old virgin females deficient for Toll pathway and the wild-type line *w¹¹¹⁸wMel* (Fig. 1D). This suggests that only the overreplicative strain *wMelPop* is affected.

We then verified whether the observations made with *wMelPop* were linked with its tissue tropism in *Drosophila* strains deficient for Toll and Imd pathways. Fluorescence *in situ* hybridizations (FISH) to detect *Wolbachia* in tissues were made on paraffin sec-

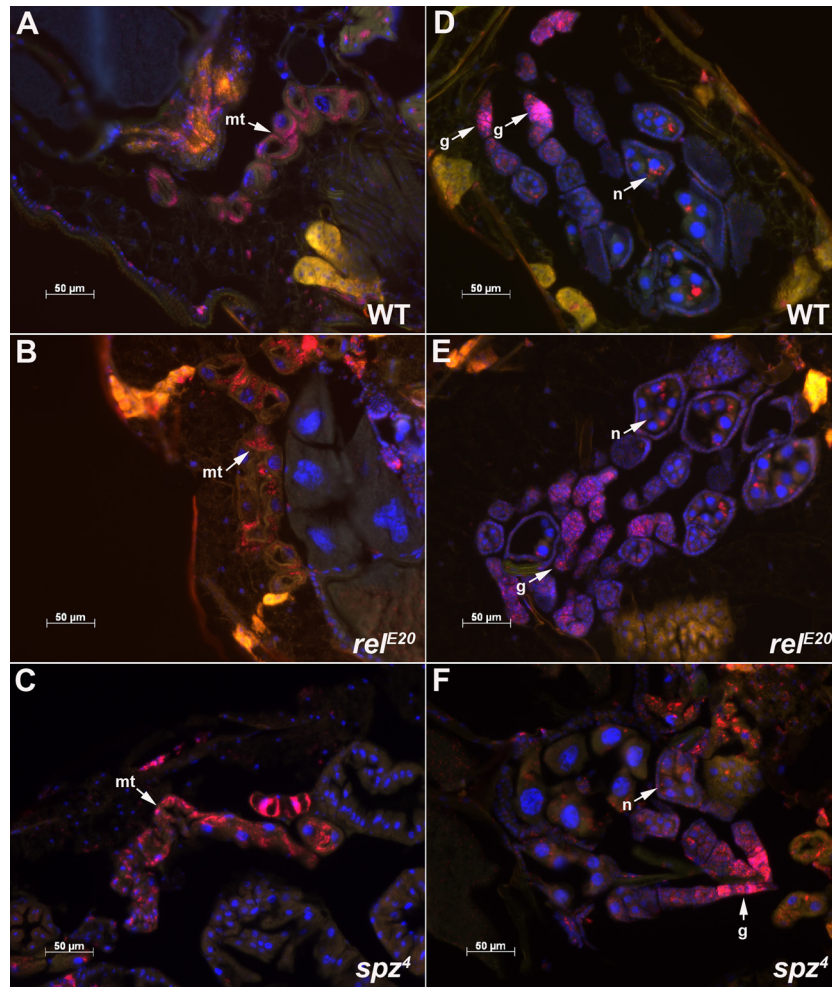


FIG 2 *Wolbachia* tissue tropism in *Drosophila* lines deficient for Toll and Imd pathways. *Wolbachia* is shown in red and DNA in blue. (A to C) *Wolbachia* localization in Malpighian tubules (mt) from w^{1118} wMelPop (WT), rel^{E20} wMelPop, and spz^4 wMelPop flies. (D to F) Ovary sections, showing the localization of *Wolbachia* in the germarium (g) and in nurse cells (n) in developing oocytes.

tions of spz^2 wMelPop, spz^4 wMelPop, rel^{E20} wMelPop, and wild-type w^{1118} wMelPop females, as described previously (19). No difference in *Wolbachia* localization was observed between the different lines in 8-day-old flies (Fig. 2).

Role of the Toll and Imd pathways in the *Wolbachia*-mediated DENV-blocking phenotype. *D. melanogaster* has already been shown to be a good model for studying interactions between human viruses or parasites (including DENV) and innate immunity (33, 34). In a previous study, we artificially infected *D. melanogaster* with DENV, showing that the virus can replicate in *Drosophila* and, secondly, that *Wolbachia* interferes with this replication in flies (23). Using *Drosophila* as a model for DENV infection allows both utilization of the natural *Wolbachia* host and access to the genetic tools of this model species to gain a deeper understanding of the complexity of the *Wolbachia*-induced phenotype. After confirmation that the different *Drosophila* mutants used for this study retained *Wolbachia* infection with a cellular tropism similar to that for the wild type, we measured the impact of *spätzle* and *relish* loss of function on the DENV-*Wolbachia* interaction.

Two-day-old *Drosophila* females, lines spz^2 wMelPop and

rel^{E20} wMelPop and their uninfected counterparts, spz^2 tet and rel^{E20} tet, were intrathoracically injected with 69 nl of DENV-2 suspension (strain ET300, 2.7×10^7 PFU/ml). Virus propagation and injection were performed as described previously (23, 35). Each experiment was repeated independently 2 to 3 times with 15 females injected per line. After injection, flies were maintained under identical controlled conditions; low density (15 females per vial), 26°C, 60% relative humidity, and 12-h light/dark cycle. Insects were collected 6 days postinjection and kept at -80°C until RNA extraction. Accumulation of genomic (+RNA) RNA strands was assessed by quantitative real-time PCR (qRT-PCR) using hydrolysis probes (TaqMan) specific to the 3' untranslated region (UTR) of the four serotypes of DENV (36). Only individuals with detectable levels of DENV infection were used to examine the effect of wMelPop on virus titer using Mann-Whitney *U* tests (GraphPad Prism 5). Regardless of the loss of function of Toll (spz^2) and Imd (rel^{E20}) pathways, *Wolbachia* still dramatically reduced DENV replication in flies (Fig. 3A). The Toll pathway result was further confirmed with a second mutant allele, spz^4 (Fig. 3B). The number of DENV copies is approximately 6 times higher in spz^4 tet than in w^{1118} tet, in which the Toll pathway is intact

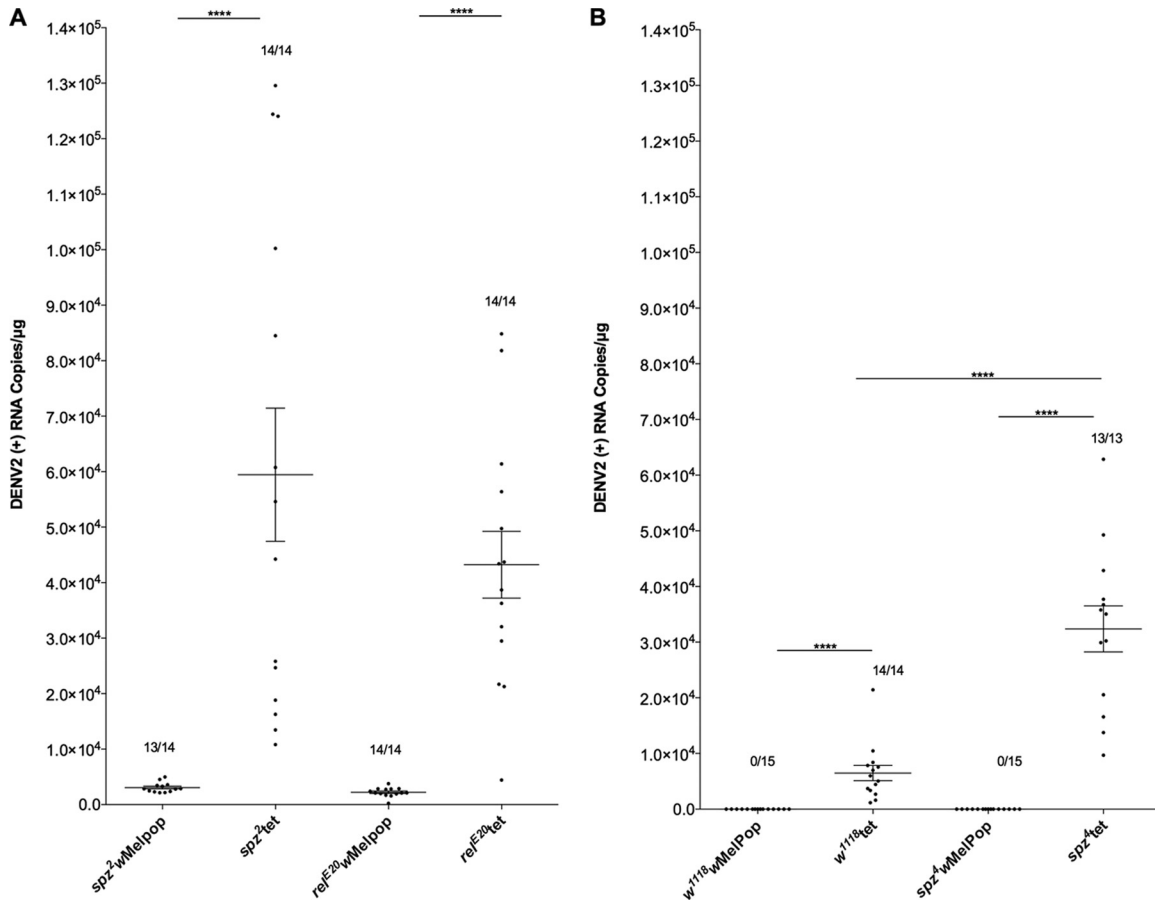


FIG 3 Dengue virus blocking in *Wolbachia*-infected *Drosophila* lines deficient for Toll and Imd pathways. (A) DENV-2 was injected into 2-day-old female flies, *spz*²*wMelPop* and *rel*^{E20}*wMelPop*, and their tetracycline-treated uninfected counterparts, *spz*²*tet* and *rel*^{E20}*tet*. (B) DENV-2 was injected into 2-day-old females, *spz*⁴*wMelPop* and *w*¹¹¹⁸*wMelPop* control flies, and their tetracycline-treated counterparts, *spz*⁴*tet* and *w*¹¹¹⁸*tet*. Total DENV-2 in whole female *Drosophila* ($n = 13$ to 15) was measured 6 days postinjection by qRT-PCR using a TaqMan assay specific to DENV in 1 μ g of total RNA. The fraction of flies that had detectable DENV-2 infections is shown above each set of data points. Mann-Whitney U test; ****, $P < 0.0001$.

(Fig. 3B). These results indicate that the Toll pathway, independently of *Wolbachia* infection status, influences DENV replication in *Drosophila*, as has been described previously in *A. aegypti* (37), reinforcing the relevance of this model to studying DENV-*Wolbachia* interactions. This study clearly demonstrates that both Toll and Imd pathways are not required for viral replication blocking by *Wolbachia*.

Conclusion. Utilizing *Drosophila* mutants for key regulatory genes of both the Toll and Imd pathways, we showed a clear interaction with infection density of the pathogenic *Wolbachia* strain *wMelPop*. Considering that this effect was not shared with the nonpathogenic *wMel* strain that grows to lower densities within the fly, it is possible that this effect is a response of the fly to the pathology and overreplication associated with this infecting *Wolbachia* strain. This is the first evidence to suggest an active role by the host insect in regulating *Wolbachia* densities. Our results show that the mutant genetic backgrounds do not negatively impact on *Wolbachia* densities in the host, by decreasing *Wolbachia* infection, which could confound interpretation of virus-blocking effects. Our results clearly demonstrate that functional Toll and Imd pathways are not required for the DENV interference phenotype to be expressed in *wMelPop*-infected flies. Other work done in *Drosophila* has shown that the antiviral small interfering RNA

pathway is not involved either (38). It would be interesting to look at the impact of *Wolbachia* on dengue virus replication using *Drosophila* mutants for other immune pathways such as the JAK-STAT pathway (39).

This study provides further evidence that the mechanism of DENV blocking is likely to be more complicated than a simple priming of the insect innate immune system (23). Two recent studies support this notion: *Wolbachia* and *Drosophila* C virus compete for cholesterol, resulting in a delay in virus-induced mortality for *Wolbachia*-infected flies (40), and upregulation of an *A. aegypti* methyltransferase gene by *Wolbachia* contributes to dengue virus inhibition (41). These results are important in an applied context of utilizing *Wolbachia* infections for dengue virus control. If the mechanism of interference has a complex basis, then there is a reasonable expectation that the development of resistance by the virus may be slower than if a single interference mechanism is involved.

ACKNOWLEDGMENTS

We thank members of the O'Neill, McGraw, Warr, and Burke labs for helpful discussions and Tova Crossman and Lauren Forbes-Beadle for their help with bacterial infections. We also thank Bruno Lemaitre (Global Health Institute, EPFL, Lausanne, Switzerland), the Bloomington *Dro-*

sophila Stock Centre, and the Australian *Drosophila* Biomedical Research Support Facility (OzDros) for supplying *Drosophila* strains.

This study was supported by a grant from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health initiative of the Bill and Melinda Gates Foundation.

REFERENCES

- Werren JH, Baldo L, Clark ME. 2008. *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* 6:741–751.
- Hurst GDD, Jiggins FM, von der Schulenburg JHG, Bertrand D, West SA, Goriacheva II, Zakharov IA, Werren JH, Stouthamer R, Majerus MEN. 1999. Male-killing *Wolbachia* in two species of insect. *Proc. Biol. Sci.* 266:735–740.
- Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. Biol. Sci.* 250:91–98.
- Stouthamer R, Breeuwer JA, Luck RF, Werren JH. 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature* 361:66–68.
- O'Neill SL, Karr TL. 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature* 348:178–180.
- Turelli M, Hoffmann AA. 1991. Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature* 353:440–442.
- Riegler M, Sidhu M, Miller WJ, O'Neill SL. 2005. Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. *Curr. Biol.* 15:1428–1433.
- Hoffmann A, Montgomery B, Popovici J, Iturbe-Ormaetxe I, Johnson P, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, O'Neill SL. 2011. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 476:454–457.
- Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. 2008. *Wolbachia* and virus protection in insects. *Science* 322:702. doi:10.1126/science.1162418.
- Teixeira L, Ferreira A, Ashburner M. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol.* 6:e2. doi:10.1371/journal.pbio.1000002.
- Osborne SE, San Leong Y, O'Neill SL, Johnson KN. 2009. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathog.* 5:e1000656. doi:10.1371/journal.ppat.1000656.
- Glaser RL, Meola MA. 2010. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. *PLoS One* 5:e11977. doi:10.1371/journal.pone.0011977.
- Mousson L, Zouache K, Arias-Goeta C, Raquin V, Mavingui P, Failloux AB. 2012. The native *Wolbachia* symbionts limit transmission of dengue virus in *Aedes albopictus*. *PLoS Negl. Trop. Dis.* 6:e1989. doi:10.1371/journal.pntd.0001989.
- Iturbe-Ormaetxe I, Walker T, O'Neill SL. 2011. *Wolbachia* and the biological control of mosquito-borne disease. *EMBO Rep.* 12:508–518.
- McGraw EA, O'Neill SL. 2013. Beyond insecticides: new thinking on an ancient problem. *Nat. Rev. Microbiol.* 11:181–193.
- Xi ZY, Khoo CCH, Dobson SL. 2005. *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science* 310:326–328.
- McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang YF, O'Neill SL. 2009. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* 323:141–144.
- Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, Leong YS, Dong Y, Axford J, Kriesner P, Lloyd AL, Ritchie SA, O'Neill SL, Hoffmann AA. 2011. A non-virulent *Wolbachia* infection blocks dengue transmission and rapidly invades *Aedes aegypti* populations. *Nature* 476:450–453.
- Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL. 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell* 139:1268–1278.
- Bian G, Xu Y, Lu P, Xie Y, Xi Z. 2010. The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathog.* 6:e1000833. doi:10.1371/journal.ppat.1000833.
- Kambris Z, Cook PE, Phuc HK, Sinkins SP. 2009. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* 326:134–136.
- Pan X, Zhou G, Wu J, Bian G, Lu P, Raikhel AS, Xi Z. 2012. *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. *Proc. Natl. Acad. Sci. U. S. A.* 109:E23–E31.
- Rancès E, Yixin HY, Woolfit M, McGraw EA, O'Neill SL. 2012. The relative importance of innate immune priming in *Wolbachia*-mediated dengue interference. *PLoS Pathog.* 8:e1002548. doi:10.1371/journal.ppat.1002548.
- Bourtzis K, Pettigrew MM, O'Neill SL. 2000. *Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides. *Insect Mol. Biol.* 9:635–639.
- Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette *spätzle*/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 86:973–983.
- Hedengren M, Dushay MS, Ando I, Ekengren S, Wihlborg M, Hultmark D. 1999. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Mol. Cell* 4:827–837.
- Braig HR, Zhou W, Dobson SL, O'Neill SL. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipiensis*. *J. Bacteriol.* 180:2373–2378.
- Hoffmann AA, Turelli M, Simmons GM. 1986. Unidirectional incompatibility between populations of *Drosophila simulans*. *Evolution* 40:692–701.
- Romeo Y, Lemaitre B. 2008. *Drosophila* immunity: methods for monitoring the activity of Toll and Imd signaling pathways. *Methods Mol. Biol.* 415:379–394.
- Lu P, Bian G, Pan X, Xi Z. 2012. *Wolbachia* induces density-dependent inhibition to dengue virus in mosquito cells. *PLoS Negl. Trop. Dis.* 6:e1754. doi:10.1371/journal.pntd.0001754.
- Osborne SE, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL, Johnson KN. 2012. Antiviral protection and the importance of *Wolbachia* density and tissue tropism in *Drosophila simulans*. *Appl. Environ. Microbiol.* 78:6922–6929.
- Min KT, Benzer S. 1997. *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc. Natl. Acad. Sci. U. S. A.* 94:10792–10796.
- Hughes TT, Allen AL, Bardin JE, Christian MN, Daimon K, Dozier KL, Hansen CL, Holcomb LM, Ahlander J. 2012. *Drosophila* as a genetic model for studying pathogenic human viruses. *Virology* 423:1–5.
- Cherry S, Silverman N. 2006. Host-pathogen interactions in *Drosophila*: new tricks from an old friend. *Nat. Immunol.* 7:911–917.
- Frentiu FD, Robinson J, Young PR, McGraw EA, O'Neill SL. 2010. *Wolbachia*-mediated resistance to dengue virus infection and death at the cellular level. *PLoS One* 5:e13398. doi:10.1371/journal.pone.0013398.
- Warrilow D, Northill JA, Pyke A, Smith GA. 2002. Single rapid TaqMan fluorogenic probe based PCR assay that detects all four dengue serotypes. *J. Med. Virol.* 66:524–528.
- Xi Z, Ramirez JL, Dimopoulos G. 2008. The *Aedes aegypti* toll pathway controls dengue virus infection. *PLoS Pathog.* 4:e1000098. doi:10.1371/journal.ppat.1000098.
- Hedges LM, Yamada R, O'Neill SL, Johnson KN. 2012. The small interfering RNA pathway is not essential for *Wolbachia*-mediated antiviral protection in *Drosophila melanogaster*. *Appl. Environ. Microbiol.* 8:6773–6776.
- Souza-Neto JA, Sim S, Dimopoulos G. 2009. An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proc. Natl. Acad. Sci. U. S. A.* 106:17841–17846.
- Caragata EP, Rancès E, Hedges LM, Gofton AW, Johnson KN, O'Neill SL, McGraw EA. 2013. Dietary cholesterol modulates pathogen blocking by *Wolbachia*. *PLoS Pathog.* 9:e1003459. doi:10.1371/journal.ppat.1003459.
- Zhang G, Hussain M, O'Neill SL, Asgari S. 2013. *Wolbachia* uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*. *Proc. Natl. Acad. Sci. U. S. A.* 110:10276–10281.