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Mechanisms of DENV control and genetic variation in *Aedes aegypti*

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Publications during enrolment

1. **Terradas G**, McGraw EA (2017) *Wolbachia*-mediated virus blocking in the mosquito vector *Aedes aegypti.* Curr Opin Insect Sci **22**, 37-44.

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ABSTRACT

Mosquito-borne diseases such as malaria, Zika and dengue fevers represent a global socioeconomic and health burden. Annually, more than 2.5 billion people are at risk of becoming infected with dengue virus (DENV), most of them living in tropical and subtropical areas. DENV is transmitted to humans through the bite of a DENV-infected female mosquito. So far, no antiviral drugs or vaccines have been approved for general use against DENV. An alternate strategy being developed for control involves the use of an endosymbiotic bacterium, *Wolbachia*. Despite being present in 40-60% of the insect species, *Wolbachia* is not naturally present in the primary vector of DENV, *Aedes aegypti*. However, a strain of *Wolbachia* (*w*Mel) was successfully introduced into *Ae. aegypti* via embryonic microinjection where it forms a stably vertically inherited infection. Two characteristics make *Wolbachia* very suitable to be deployed in the field as means of vector control. First, *Wolbachia* is able to reduce the replication of a vast range of pathogens in insect hosts, including DENV, Zika and Chikungunya viruses and the malaria parasite. Second, *Wolbachia* also drives its own spread through wild populations via manipulation of female reproduction. The most common means is via cytoplasmic incompatibility, an effect that gives infected females a reproductive advantage and allows for exponential expansion of *Wolbachia* through uninfected populations.

The mechanism of *Wolbachia's* pathogen blocking ability is unclear, although several different hypotheses have emerged. There is evidence that *Wolbachia* increases the insect's basal immune response, especially in novel associations. Basal immune activation would likely reduce the success of any subsequent pathogen infection. However, some native associations do not show immune priming but do exhibit pathogen blocking, although often a weaker. Another set of hypotheses posit competition between *Wolbachia* and infecting viruses for host resources, including space, lipids and nitrogen sources. There is also some evidence that *Wolbachia* may manipulate expression of key antiviral genes via microRNAs. Lastly, there is some evidence that *Wolbachia*-induced disruption of the endoplasmic reticulum and secretion pathways might hamper effective pathogen survival and reproduction, as they are key spatial components for viral replication and exit.

This thesis tests the involvement of several aspects of the host response in cellular DENV control in mosquitoes. In Chapter 2, we evaluated the importance of different insect immune pathways to the *Wolbachia*-mediated pathogen blocking in *Ae. aegypti* cells. The results show that, while immune priming does not fully explain the blocking phenotype, the RNAi pathway still has an important role in viral control and is the main driver of the insect immune response.

Regardless of the mechanism by which *Wolbachia* confers antiviral protection to the host, the strength of blocking in any association appears to correlate to the levels at which *Wolbachia* is able to infect host cells, with highly infected associations displaying complete viral inhibition. This however comes with evolutionary trade-offs. Natural selection for high *Wolbachia* infections is unlikely to occur in the field, as harbouring these infection levels cause critical fitness costs to the host. In terms of long-term applicability in the field, the ability of *Wolbachia* to spread to wild populations is as important as maintaining a consistent expression of pathogen blocking. In Chapter 3, we assessed the variation present for the DENV blocking trait in *Wolbachia*-infected mosquitoes using a modified full sib breeding design. We show presence of family level variation for the blocking trait, as well as its correlation to *Wolbachia* loads and expression of immune genes. We predict that blocking may be able to evolve or be differentially expressed in diverse environments.

In Chapter 4, we also used mosquito genetic variation to test a set of gene candidates produced by transcriptomic studies for their relevance in DENV control in wildtype *Ae. aegypti*. We found that around half the putative genes were necessary for host antiviral activities, and were associated with immunity, metabolism and adhesion/transport. This project has helped to identify a set of promising genes that could be genetically modified with emerging CRISPR techniques to produce virus refractory mosquitoes.

Overall, the thesis reveals the complexity of the genetic response of the vector to dengue virus infection and the potential interaction of *Wolbachia* with these pathways. More broadly, it contributes to the dialog on emerging approaches for dengue fever control by the use of symbionts, as well as through genetic modification of mosquitoes.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer review journals and two submitted publications. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Professor Elizabeth A McGraw.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
1	*Wolbachia*-mediated virus blocking in the mosquito vector *Aedes aegypti*	Published	60% - Wrote review	McGraw EA (40%) Wrote review	N
2	The RNAi pathway plays a small part in Wolbachia-mediated blocking of dengue virus in mosquito cells	Published	60% - Conceptualization, experimental work, data collection, analysis and wrote paper	1) Joubert DA (10%) Conceptualization, revised paper 2) McGraw EA (30%) Conceptualization, analysis and wrote paper	N
3	Genetic architecture of *Wolbachia*-mediated dengue virus blocking in *Aedes aegypti*	Submitted	60% - Experimental work, data collection, analysis and wrote paper	1) Allen SL (5%) Data analysis 2) Chenoweth SL (5%) Data analysis 3) McGraw EA (30%) Conceptualization, analysis and wrote paper	N
4	From transcriptomics to functionality: What can be learned using genetic variation in dengue virus vector competence in *Aedes aegypti*	Submitted	60% - Experimental work, data collection, analysis and wrote paper	McGraw EA (40%) Conceptualization, wrote paper	N

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date:** July 10, 2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature [redacted] **Date:** July 10, 2017

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CHAPTER ONE

Introduction

A short version of this chapter was published as a Review article in *Current Opinion in Insect Science* under Terradas G and McGraw EA (2017) "*Wolbachia*-mediated virus blocking in the mosquito vector *Aedes aegypti".*
This chapter contains additions throughout and in particular a section on microbiota.

The shorter, published version of the chapter can be found in Appendix 1.

Abstract

Viruses transmitted by mosquitoes such as dengue, Zika and West Nile cause a threat to global health due to increased geographical range and frequency of outbreaks. The bacterium *Wolbachia pipientis* may be the solution reducing disease transmission. Though commonly missing in vector species, the bacterium was artificially and stably introduced into *Aedes aegypti* to assess its potential for biocontrol. When infected with *Wolbachia*, mosquitoes become refractory to infection by a range of pathogens, including the aforementioned viruses. How the bacterium is conferring this phenotype remains unknown. Here we discuss current hypotheses in the field for the mechanistic basis of pathogen blocking and evaluate the evidence from mosquitoes and related insects.

Wolbachia and pathogen blocking

Wolbachia pipientis was first discovered in 1924 in the ovaries of the mosquito *Culex pipiens* [1]. *Wolbachia* is a maternally transmitted endosymbiotic bacterium estimated to chronically infect 40% of all known arthropod species [2]. The symbiont's success has been credited to its ability to spread through uninfected populations by altering the reproductive biology of its hosts and providing a fitness advantage to infected females. *Wolbachia*'s manipulations, which include feminization of males, parthenogenesis, male killing and cytoplasmic incompatibility (CI), are all female biased given its maternal inheritance [3,4]. The latter is the best-studied manipulation and is witnessed by embryonic death of offspring generated from the cross of an infected male with an uninfected female, or in crosses where two different *Wolbachia* strains are involved [3,5,6]. Infected females therefore have greater relative reproductive success, leading to increased numbers of *Wolbachia*-infected progeny in the subsequent generation. Recently, the effect of two prophage *WO*-induced genes encoded in an operon and acting in the germline was able to explain the mechanism of CI [6,7], where each gene additively increases the embryonic lethality of the cross between an infected male and uninfected female. However, *Wolbachia*-infected females are able to rescue the lethal phenotype, producing *Wolbachia*-infected viable offspring and thus giving them a reproductive advantage over uninfected females. *Wolbachia* infection also has other physiological effects that can be exploited for biological control: it can inhibit the replication of many pathogens [8-11] and shorten the lifespan of its host [12]. Though little is known about the mechanisms that underlie the 'pathogen blocking' trait, these unusual properties have made *Wolbachia* extremely attractive as potential means of vector-borne disease control [13].

Wolbachia limits the replication of viruses such as dengue, yellow fever, Zika, West Nile and chikungunya, as well as filarial nematodes and the malaria parasite *Plasmodium* in their associated mosquito vectors [8-11,14,15]. Of these, the field-testing of *Wolbachia* for biocontrol is most advanced in the case of dengue virus (DENV) [16]. Dengue fever is a human disease affecting an estimated of one-third of the world's population [17].

Some mosquitoes are naturally infected with *Wolbachia* strains, however, the primary vector of DENV, *Aedes aegypti,* is naturally *Wolbachia*-free. A virulent *Wolbachia* strain (named *w*MelPop) from a laboratory line of *Drosophila melanogaster* was initially introduced into *Ae. aegypti* in order to reduce transmission of DENV by significantly shortening the lifespan of female mosquitoes [12]. This would be effective as the probability of transmitting DENV increases with mosquito age. Unfortunately, the *w*MelPop strain was also found to have severe negative effects on fecundity [18] and was predicted not to spread in field populations [16,19]. Other strains of *Wolbachia* also have the capacity to interfere with viruses in insects, blocking transmission of the agent while conferring only mild or no fitness costs to the vector [18]. A second such *Wolbachia* strain from *D. melanogaster*, *w*Mel, was introduced into *Ae. aegypti* and tested in mosquito populations in northern Queensland, Australia, where DENV is epidemic upon introduction by travellers [16]. Current field trials in Indonesia are testing whether the anti-DENV effects of *w*Mel seen in laboratory lead to reductions in the incidence of dengue fever in humans, following release of *Wolbachia* in wild mosquito populations. *Wolbachia* is being also field tested for its potential use against other important arboviruses including Zika [20,21]. Other associations of *Wolbachia* strains and mosquitoes have been explored as a consequence of the potential of the blocking phenotype seen in novel associations such as *Ae. aegypti*-*w*Mel [14,22,23].

This review explores possible mechanisms behind *Wolbachia's* pathogen blocking phenotype (Fig. 1). To date, two main theories have prevailed: host immune priming and competition for host resources. In addition we examine the differential expression of pathogen blocking in native and novelly created host associations. These differences may help to discern mechanism and also predict the future trajectory of the blocking phenotype in novel hosts.

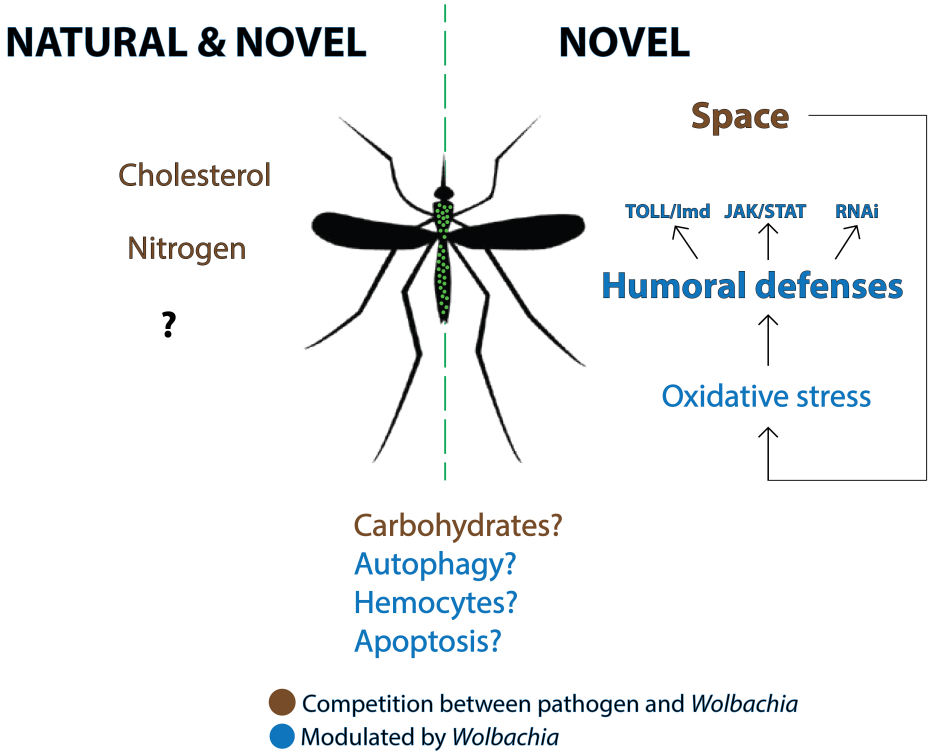

Figure 1. – Suggested mechanisms behind *Wolbachia's* pathogen blocking phenotype in *Ae. aegypti*. Competition events are shown in brown, with modulation events depicted in blue.

Mechanisms of pathogen blocking

The successful transmission of a virus is dictated by contributions from both viral and vector genomes [24] and in some cases from *Wolbachia* or other insect associated microbes [25]. Nevertheless, the exact mechanism that underpins *Wolbachia*-mediated blocking is not well understood as teasing apart the contribution of the three partners in the association can be difficult, particularly without the capacity to genetically modify *Wolbachia*. Most of what is known has come from comparing the strength and expression of blocking in different combinations of vector species, virus genotypes and *Wolbachia* strains [26]. Variation in behaviour of different *Wolbachia* strains infecting the same host also demonstrates the contribution of the symbiont genome to the association. In particular, the expression of blocking appears to differ between natively or novelly infected vectors [27]. In *Ae. albopictus,* which is natively infected with two *Wolbachia* strains (*w*AlbA and *w*AlbB), viral blocking is weaker or non existent [28] compared to *Ae. aegypti* novelly infected with *w*Mel or wAlbB [18,29]. Additionally, blocking is strong when *Ae. albopictus* is novelly infected with the *w*Mel strain [30], indicating that the degree of familiarity between partners can also dictate strength of blocking, rather than just history of infection in the host.

The amount of *Wolbachia* inside host cells and tissues, usually referred to as "density", also appears to correlate with the strength of *Wolbachia*-mediated blocking [26,28]. This relationship is seen in several contexts. Novel host/*Wolbachia* strain combinations tend to have higher densities and more widespread tissue distributions [27,31]. Comparisons between a range of *Wolbachia* strains that naturally infect *Drosophila simulans* reveal differences in blocking that are predicted by their individual densities and tissue distributions [32]. Lastly, the highly replicative *Wolbachia* strain called *w*MelPop, that exhibits greater cellular loads and causes tissue damage, induces near perfect blocking in *Ae. aegypti* [8] compared to the more moderate blocking of *w*Mel in the same host [18]. This difference in load between *w*Mel and *w*MelPop [8,18] and the associated virulence is thought to underpin the failure of the latter strain to spread in field [33].

Immune priming

Upregulation of mosquito antimicrobial encoding genes was first seen in response to *w*MelPop infections [34]. This finding led to the theory of 'innate immune priming', where pre activation of the immune response could then theoretically protect the insect from a range of pathogens. Subsequently, support for a complementary set of theories based on resource competition emerged [35-37], where *Wolbachia* and pathogens would compete for limited host resources.

Wolbachia-induced changes in immunity gene expression are largely seen in mosquitoes with artificially introduced strains (i.e. *Ae. aegypti* (*w*MelPop [9], *w*Mel [38], *w*AlbB [29])), where more genes are affected and to a greater degree than in natively-infected hosts (i.e. *D. melanogaster* (*w*Mel) [38], *Ae. albopictus* (*w*AlbA and B) [28]). The occurrence of blocking in both of these native *Wolbachia* hosts therefore indicates that immune activation must not be essential for pathogen blocking [38,39]. Although it may not be essential, the greater strength of blocking seen in *Ae. aegypti* may stem from the additive effects from immune activation [9,40,41]. A detailed examination of the functional role of immunity in blocking has been confined to a subset of insect immunity pathways that may not be the most relevant for DENV control [42,43].

Animals possess diverse systems of defense to protect themselves against invasion by foreign substances and pathogens. The first line of defense, known as innate immunity (humoral or cellular), is the sole immune response in invertebrates. Humoral responses are based on the rapid production of non-constitutively expressed antimicrobial peptides (AMP) in the fat body, that are released into the hemolymph to act against the pathogen [44,45]. AMP also activate different enzymatic cascades and the production of reactive oxygen and nitrogen species [46]. Simultaneously, cellular responses are also triggered, mainly in the hemolymph. This response is driven by differentation of hemocytes and includes the processes of phagocytosis, encapsulation and melanization [47,48].

Humoral innate immunity

The humoral innate immune response activation begins following recognition of the pathogen through different receptors. These receptors detect non-self molecular conserved motifs such as double-stranded RNA in viruses or cell wall components in bacteria. The most important signalling cascades that are already known to be involved in insect humoral immunity are the Toll, the Immune Deficiency (Imd), the Janus Kinase-Signal Transducer and Activator of Transcription (JAK/STAT) and the different RNA interference pathways [49-55]. Successful pathogens are able to evade or suppress these innate defense mechanisms [56,57].

The systemic immune response in insects relies on the production of antimicrobial peptides (AMPs) [58] and antiviral molecules in the fat body and subsequent release into the hemolymph. AMP production is mainly due to the antibacterial pathways Toll and the Imd, which have also been the most studied on the *Wolbachia*-insect interaction due to the bacterial nature of the symbiont. The Toll pathway is triggered by gram-positive bacteria and fungi whereas the Imd pathway responds mainly against gram-negative invaders [59]. Both pathways have been well described and characterised in Diptera [60-62]. Although primarily antibacterial, Toll is also required for the mosquito's response to DENV [60,63]. *Wolbachia* does not induce upregulation in Toll or Imd effectors in *Drosophila* and yet *Wolbachia* is able to limit DENV replication, suggesting that these pathways are not required for the protective phenotype to occur [38]. It has been demonstrated that *Wolbachia* does not activate the antimicrobials cecropin or diptericin in *D. simulans*, even though the species is heavily infected with the bacteria [64]. This does not seem the case for *Ae. aegypti*, where both are upregulated following *Wolbachia* infection [37]. Not only are more genes activated by *Wolbachia* and expression changes generally higher in *Ae. aegypti*, the breadth of pathogen targeting is also wider, including bacteria [65]. These differences may also provide stronger antiviral effects [55,65].

Other humoral immune responses comprise the JAK/STAT and the RNA interference pathways, which lead to antiviral production or to cleavage of targeted foreign double-stranded nucleic sequences. The JAK/STAT signal transduction cascade was discovered in mammals and soon identified in almost all species as an innate immunity process, a role player in antiviral defense including protection against dengue [66,67]. Activation of JAK/STAT pathway is initiated by the binding of a cytokine to the Unpaired-D (1-3) ligand and the receptor domeless (DOME) [68]. It would then cause the self-phosphorylation of associated JAKs (known as HOP), which would recruit STAT, phosphorylating it and allowing its translocation into the nucleus as a homodimer. The accumulation of nuclear STAT molecules then promotes gene transcription. In mammalian cells, the JAK/STAT pathway is activated by different cytokines, including interferon, interleukins and growth factors. In insects though, no interferon has yet been discovered. Despite that, a recent study showed that Vago acts as an interferon-like molecule, activating the JAK/STAT pathway and upregulating different effectors including the virus induced RNA-1 (*vir-1*) gene [43]. JAK/STAT's transcriptional profiling and regulation is complex and its specific effect on pathogens remains still unknown. The effector *vir-1* has been shown to be specifically upregulated in response to virus [50]. Also, studies have shown restriction of malaria and West Nile virus replication after the induction of JAK/STAT in *Culex spp* [43]. In *Ae. aegypti*, the function of the pathway is conserved and high activation levels of JAK/STAT limit DENV replication [52]. It is clear that the pathway has a crucial role in insect immunity.

The RNA interference (RNAi) pathway also plays a key role in insect immunity [69]. Two types of RNA molecules are essential for RNAi, microRNA (miRNA) and small interfering RNA (siRNA), both of which have been hypothesised as being modulated by *Wolbachia* infection [70,71]. The RNAi pathway mediates specific cleavage of targeted dsRNA molecules via Dicer into 20-25 bp long double-stranded fragments called siRNAs. Then, the siRNA are separated into single strands and loaded into the RISC complex, which contains an argonaute (AGO) protein. The siRNA-loaded RISC complex would base pair the siRNA to its target mRNA cleaving it and impeding its translation.

In contrast, a miRNA-loaded RISC blocks ribosomal access to the mRNA, stopping translation. In nature, RNAi acts as a potent defense mechanism against viral infections and aberrant transcription due to its gene-silencing activity [72-74]. Considered the major insect antiviral pathway, RNAi has been shown to limit DENV, chikungunya and Sindbis viruses in *Ae. aegypti* [51,55,73] and is regarded as a key contributor to control of arboviruses in mosquitoes [74]. There are studies showing that RNAi is not essential for *Wolbachia*-mediated viral blocking in *Drosophila* [75,76] or in an *Ae. albopictus* cell line where a non functional Dicer 2 did not hamper blocking [31]. However, a recent study using an *Ae. aegypti* cell line showed that *Wolbachia* may be upregulating *AGO2* intracellular levels and providing the host with an increased basal expression of a key component for the control of DENV infection [77]. Upregulation of *AGO2* and in turn the activity of the siRNA pathway would allow for a greater protection from the arboviral infection.

In addition to the exogenous siRNA pathway, the miRNA pathway has also been suggested to be involved in the pathogen blocking effect as part of *Wolbachia'*s modulation of host components [70,78]. Modulation and function of miRNAs in insect:pathogen interactions has been reviewed previously [79] and the manipulations are highly specific, with two closely related pathogens having the opposite effect on a particular miRNA [80]. Infection with *w*MelPop also has an effect on the miRNA profile of *Ae. aegypti* [70]. Moreover, the inhibition of certain miRNAs leads to reduction of *Wolbachia* densities. This suggests that the symbiont facilitates its maintenance in the host by manipulation of gene expression using host miRNAs. In terms of the conferred protective phenotype, *Wolbachia* is thought to be altering the intracellular localisation of AGO1 [81]. This is a principle component of the RNA interference cascades, which would affect the trafficking of miRNAs into the nucleus leading to differential gene expression and methylation. A recent study [82] in *Drosophila* cells shows that blocking of Semliki Forest virus (SFV) by *Wolbachia* occurs early in infection and without the activation of host transcriptional responses or microRNAs. It suggests that blocking is reliant on an intrinsic mechanism that is already in place when the pathogen comes into contact with the host.

Although *Drosophila* is not a natural carrier of SFV, this is a crucial finding. A new study [83] is in keeping with the notion that *Wolbachia* may have fundamental effects on cells via modulation of the morphology and composition of the endoplasmic reticulum. This disruption may allow the symbiont to access nitrogen, but could also prevent viral replication. If these effects are consistent across host species and pathogens, the upregulation of immune responses may only be a by-product of *Wolbachia's* infection and secondary to blocking.

Hemocytes

Hemocytes, along with the fat body, are major immune tissues within mosquitoes since immune effectors are released into the hemocoel. Hemocytes are also regarded as essential replication sites for *Wolbachia* due to the need for the bacteria to modulate these responses in order to propagate and maintain a systemic infection [15,84]. Interestingly, immune upregulation is related to function of hemocytes through cellular signalling [50,52,85]. Hemocytes are also crucial for the insect's phagocytic activity [86]. A single bloodmeal is enough to stimulate the proliferation of hemocytes in *Ae. aegypti* [87], as they provide the first defense mechanism of any pathogen entering the hemocoel. Hemocytes are also thought to be important for bacterial survival in the population. At least one study has provided evidence of *Wolbachia* infection directly affecting hemocyte counts [88]. The ability to spread to the ovaries is key for proper transmission, where hemocytes that have phagocytized *Wolbachia* would be serving as shuttle carriers from a primary infected organ to the rest of the body [89,90].

Autophagy

Autophagy is a highly conserved biological process responsible for the degradation of self-proteins and damaged organelles through autophagosomes, but also involved in many host-pathogen interactions as part of the host cellular response. The first evidence that autolytic vesicles were able to degrade pathogens dates back to 1965 when poliovirus degradation was described to take place in the vesicles [91].

It is now well known that pathogens have the ability to manipulate autophagy responses in order to enhance their replication and establish infection. One example is DENV, known to modulate the autophagosomal membrane content to adapt the vesicles to its own benefit as replication sites in mammalian cells [92] as well as upregulating genes known to promote DENV replication [93]. The deletion of diverse autophagy genes in *Drosophila* affects survival and decrease refractoriness to a vesicular stomatitis virus (VSV) infection [94]. Autophagy can also be activated through PAMPs via Toll-7 receptors in the plasma membrane and independent of a humoral Toll activation [95]. A species related to *Wolbachia*, *Anaplasma phagocytophilum*, hijacks autophagy initiators probably as means to obtain host nutrients [96]. High levels of autophagy act as a host response in tissues where *Wolbachia* levels are also high and it has been shown that activation of autophagy can lead to limited *Wolbachia* replication in *Ae. albopictus* cells [97]. Also, DENV seems to promote a specific type of autophagy that alters the metabolism of the cell causing release of fatty acids, which are required for its proper viral replication [98]. This is an interesting finding since *Wolbachia* also requires unsaturated fatty acids from host cells and competition for host nutrients between *Wolbachia* and viruses has been hypothesised to explain the *Wolbachia*-mediated viral blocking phenotype [8].

Apoptosis

Apoptosis is a programmed cell death mechanism with diverse biological functions in multicellular organisms, from balancing homeostasis to lysis of viral particles. In dipterans, the only apoptotic pathway known affects the mitochondria, also called intrinsic pathway of apoptosis [99]. Many viruses contain sequences encoding for inhibitors of apoptosis, which lead to the idea of apoptosis having a role in immunity. Infection of Flock House virus (FHV) induced pro-apoptotic genes in *Drosophila*, even though apoptosis did not seem to have an effect on FHV replication [100]. The role of apoptosis in arboviral infections was examined by different studies, finding that apoptotic cell death can be detected in mosquitoes' midgut and salivary glands after infection with a range of viruses [101-104]. Pro-apoptotic genes were upregulated

as part of the response of *Ae. aegypti* to DENV by a refractory DENV-2 strain [105]. While suppression of autophagy seems to be important for infecting viruses, no studies have tested the apoptotic relevance in *Wolbachia*-infected mosquitoes. Nevertheless, *Wolbachia* strains have been found to downregulate apoptotic responses in parasitic wasp ovaries as part of mutualistic relationships between organisms. If *Wolbachia* is removed from its host, wasp ovaries fail to develop as apoptotic death occurs on the ovarian cells during formation of the tissue [106,107]. The apoptotic pathway plays a complex role in host-pathogen interactions. A decrease in host apoptosis levels would give the virus an advantage to propagate, whereas an increase in the apoptotic activity would lead to a host self-destruction. Either outcome in response to modulation by *Wolbachia* would have negative consequences.

Iron metabolism and Oxidative stress

Reactive oxygen species (ROS) when overproduced cause cell damage but, when properly regulated provide a beneficial role as part of immune defenses and intracellular signalling. ROS are by-products of the metabolism of oxygen, mostly as part of the aerobic respiration in the mitochondria for energy production. During ATP synthesis, electrons are transferred along the mitochondrial respiratory chain where the final electron acceptor is an oxygen molecule that gets reduced to produce water. Sometimes, oxygen is incompletely reduced producing superoxide. Superoxide anions (O_2^-) and other oxygen-derived molecules such as hydrogen peroxide (H_2O_2) or hydroxyl radicals (OH^-) oxidize other molecules and create what is known as oxidative stress. Generation of ROS is at its peak at infection sites as part of the insect's cellular immune responses and immune-derived mechanisms such as encapsulation or melanisation. ROS are known to be an immune alternative to the production of antimicrobial peptides and autophagy-related proteins.

The maintenance of redox homeostasis is crucial for gut immunity following a bloodmeal in *Ae. aegypti*. Ingestion of a bloodmeal causes a decrease in ROS in the mosquito midgut [108]. The decrease is in part due to a heme-mediated activation of protein kinase C as part of host counteractive measures to a pro-oxidative bloodmeal. However, lowered ROS levels probably correlate to a higher susceptibility to infection and increased mortality. Moreover, a downregulation of antioxidant production following *Wolbachia* infection was shown in novelly infected cell lines [109,110]. Nevertheless, an increase in ROS production has also been shown in *Ae. aegypti* [41], *Ae. polynesiensis* [111] and *Anopheles stephensi* [14] transinfected with the *w*AlbB strain from *Ae. albopictus*, making the oxidative stress' role in non-native *Wolbachia* associations inconclusive.

Wolbachia have recently been shown to influence ROS production and modulate the oxidative environment in natural hosts. Direct evidence of the interaction between *Wolbachia* and oxidative stress was first shown in a naturally-infected *Ae. albopictus* cell line, where the presence of *Wolbachia* was correlated to high levels of ROS using a *Wolbachia*-free population as control [112]. Moreover, upregulation of various antioxidant genes was reported (copper-zinc superoxide dismutase 1, Prx5, GPx). This probably shows a host response to high ROS levels and associated negative effects. Conversely, it could also be a *Wolbachia*-induced effect to protect itself against host immune responses based on increased ROS levels.

Effectors that go through the bacterial type IV secretion system (T4SS) likely determine the induction of antioxidant activity. *Wolbachia*'s own system may be involved in inducing host phenotypes after the transfer of effectors into the cytoplasm [113]. *Ehrlichia,* a species closely related to *Wolbachia*, was shown to be able to translocate T4SS to the mitochondria and upregulate a host superoxide dismutase (the enzyme that eliminates superoxide molecules from the cell), leading to reduced oxidative stress and apoptosis [114]. *Wolbachia* also seems to be able to produce its own antioxidants for self-defense.

The same study [112] detected the presence of a couple of bacterial antioxidant proteins, a bacterial iron SOD (Fe-SOD) and bacterioferritin (Bfr). A similar result was seen in *D. simulans*, where upregulation of *Wolbachia*-Bfr expression was detected under iron-induced stress conditions [115]. Iron is an essential element in most organisms but also causes great amounts of oxidative stress through the production of hydroxyl radicals [116]. Therefore, the production of bacterioferritin, not commonly known as an antioxidant but an iron storage molecule, helps reduce these oxidative stress levels [117]. Since iron has a crucial role in immunity, infection and host-pathogen interactions [118,119], the ability of *Wolbachia* to interfere with and modulate iron metabolism in the host can be a key factor contributing to its success [120].

In natural hosts, and despite studies showing modulation of ROS and antioxidants in cell lines, a recent study in adult mosquitoes [121] showed no differences between *Wolbachia*-free and infected *Ae. albopictus* in either ROS or antioxidant production. This result is in keeping with the hypothesis that native associations lean towards an attenuation of immune responses due to coevolution between *Wolbachia* and the host. It also suggests that the ROS-mediated Toll activation seen in *Ae. aegypti* [41] does not apply for other associations and this immune upregulation may not be a common causal factor of pathogen blocking.

Microbiota

Several factors are important contributors in any host-pathogen interaction. In order to succeed, *Wolbachia* needs to establish infection and have the capacity to be horizontally and vertically transmitted through a naïve host population. Diverse mechanisms to explain horizontal transmission have appeared, such as hemolymph transfer or cohabitation [89,122]. Most studies have treated insects as holobionts [123], even when the specific microbiome of individual organs is relevant in associations soon to be applied in the field. For example, *Wolbachia* residing in salivary glands of *Aedes spp*. impedes DENV transmission [124,125]. Bloodfeeding drastically modulates the insect microbiota [108] and *Wolbachia* levels are suppressed after the intake of the blood meal [126].

Native microbiota plays a crucial role in controlling *Wolbachia* infection levels in non-gut tissues and therefore it has potential to affect vertical transmission [126] and vector competence in the mosquito [25,127]. Bacterial gut microbiota in *Wolbachia*-infected insects is largely dominated by *Wolbachia*; however, a decrease in bacterial diversity does not affect the dengue blocking phenotype [128]. Nevertheless, competitive inter and intraspecific microbial interactions with *Wolbachia* have been shown to occur in insects [129,130], suggesting that incompatibility between host microbiota and *Wolbachia* may explain the refractoriness of some insect species to carry *Wolbachia* infections in nature. Overall, microbiome research in different insect associations with native or novel *Wolbachia* infections is just beginning.

Competition

The other widespread theory to explain pathogen blocking proposes that pathogens and *Wolbachia* are in competition for essential resources from their vector host that are in short supply and essential for living success. Several points of tension have been suggested including physical space, macronutrients and lipids.

Space

Different studies have shown a positive correlation between the density of *Wolbachia* in tissues and the strength of pathogen blocking [8,29,32]. Similarly to protective phenotypes, *Wolbachia* densities also appear to be greater in novel hosts as part of the initial colonisation of host tissue with associated fitness costs that decrease in generations after the establishment of infection [131]. The density of *Wolbachia* infection can be important at all levels; cellular [31], tissue [8] and within the whole organism [132]. *Wolbachia* infection levels have to be sufficiently high to allow the symbiont to be transmitted vertically but low enough not to cause host pathology and mortality. Bacterial densities are often lower in native hosts compared to those seen in transinfected species, which could explain the blocking phenotype being often more severe in the latter [131]. One scenario for blocking is that both an invading pathogen and bacteria are competing to use the same tissue or cellular location, regardless

of other factors. Additionally, the importance of bacterial densities may just be a competition by-product between *Wolbachia* and viruses for available space. This is supported by the observation of a non-existent *Wolbachia*-virus co-localization in *Ae. aegypti*, demonstrating that the virus does not replicate in those sites where *Wolbachia* is highly present [133].

Macronutrients: Carbohydrates and Nitrogen

Lifetime fitness of mosquitoes is highly influenced by environmental and nutritional conditions during development. It comes as no surprise that specific nutritional components have been shown to also have an effect on infection dynamics, especially determining bacterial composition and abundance [134]. Dietary balance between protein intake (P) and carbohydrates (C) is strongly regulated by insects, with P-C balance thought to be impacting lifespan, reproduction and immunity [135]. In a recent study assessing different dietary conditions in *Ae. aegypti*, a high carbohydrate intake was found to be essential to mosquito longevity whist both extremes of carbohydrate levels lead to higher pathogen prevalence and intensity of infection [37]. Nutrition and diet are primary factors contributing to the insect's resistance to different pathogens [136-138] that in turn are also dependent on resource availability and can manipulate host metabolism in order to facilitate infection [139-141].

In addition, *Wolbachia* is dependent on some of these same nutrients, including nitrogen and carbohydrates, so competition for host resources between *Wolbachia* and pathogens has been raised as a possibility to explain the pathogen interference phenotype seen in *Wolbachia*-infected hosts [35,36,142]. The supplementation with a diet high in amino acids affects both fecundity and egg viability in those mosquitoes infected with the bacterium [36]. It has also been found that the ratio of P-C modulates *Wolbachia*'s abundance in the gut of *Drosophila* [135]. A high nitrogen supply supports maximum viral replication, whereas *Wolbachia* competes for and uses nitrogen abundantly, depleting the cellular amino acid pool. This decrease in available nitrogen would hamper intracellular viral propagation and likely contribute to *Wolbachia*'s pathogen blocking effect.

Cholesterol

Sterols are essential components in insects, known to be vital components for membrane stability, control the regulation of different hormones (i.e. ecdysteroids) and regulate development. Cholesterol is the dominant sterol in most insects and DENV and many other infecting viruses are dependent on it to successfully infect hosts [143]. DENV infection perturbs lipid homeostasis altering host lipid profiles to meet the required criteria for efficient infection [144]. DENV induces the upregulation of genes involved in fatty acid biosynthesis and relocates the machinery into its own replication complexes [98]. Some studies have found that host immune responses include sterol downregulation as means to limit viral propagation [145]. *Wolbachia* has very limited lipid biosynthesis capabilities and therefore bets heavily on host cell production to meet its requirements [146]. The infection and effective replication of *Wolbachia* inside the cells depends on cholesterol-rich membranes [147]. Similar to DENV, *Wolbachia* also increases lipid production in the cell via the upregulation of fatty acid synthase [38]. It has been suggested that *Wolbachia*'s usage of host cholesterol could impact on the ability of viruses to replicate. Besides cholesterol, *Wolbachia* may also be competing for other key lipids that underpin pathogen blocking. Moreover, cholesterol and other lipids are present in host membranes but also as part of the Golgi apparatus, which corresponds to *Wolbachia's* and viruses' main replication site [147-149]. This organelle also accumulates excess cellular cholesterol in *Drosophila*, a feature that could explain the preference to replicate in the Golgi for both bacterium and virus [150]. *Wolbachia* has also been shown to affect the lipid profiles of inbred *Drosophila* flies and has been positively correlated to odd-chain lipid abundance [151].

Conclusion

To date the mechanism that underpins *Wolbachia*-mediated pathogen blocking is unknown, but two main theories have arisen: immune priming and competition between symbiont and pathogen for host resources. Current evidence suggests that *Wolbachia* increases immune gene expression levels in transinfected vectors, where pathogen protection is greater [40,65]. However, it seems that immune activation is not essential since some native *Wolbachia* strains do not induce those responses and yet confer pathogen protection [38]. There is support for competition theories since *Wolbachia* and arboviruses do not seem to coexist in the same tissues when *Wolbachia* densities are high [8,31] and because manipulation of nutrients can suppress or assist pathogen replication respectively in the presence of *Wolbachia* [35,37].

Additionally, there is the complexity of host history. *Wolbachia*-mediated viral blocking is expressed differentially in some natural associations versus artificially infected mosquitoes. Many natural *Wolbachia* infections lead to little or no reduction in arbovirus transmission [27,152], whereas others seem to confer protection [124]. This is not surprising given that many of the proposed mechanisms appear to be unique for certain mosquito-symbiont associations. In contrast to natural infections, *Wolbachia* seems to cause changes at genetic and cellular levels that trigger host responses in novelly infected vectors, probably as part of colonization and maintenance of infection.

Consequently, pathogen blocking is stronger and broader, providing greater antiviral effects against a range of arboviruses like DENV, ZIKV or CHIKV [8,11,29] as well as other pathogens like *Plasmodium* [8]. A recent study provided some clarity on differences of blocking in novel associations, comparing transient to stable *Wolbachia* infections in *Aedes aegypti*. Even though both conferred refractoriness to pathogen infection, small differences were detected on DENV and WNV replication and transmission between the compared mosquito lines [153]. These detected differences could be due to the variation in *Wolbachia* densities between the two models.

What is emerging is a sense that there are fundamental mechanisms that may confer blocking in native and novel hosts as well as additional mechanisms that may act in novel hosts and increase the efficacy and breadth of blocking. In these novel infections, upregulation of immune regulators or effectors is quite prevalent, giving rise to crosstalk among different humoral pathways as well as increased iron metabolism and ROS production. Dissecting the degree to which the fundamental versus the novel host specific responses contribute to the overall effect of blocking is challenging because they cut across diverse physiological and cellular processes and stem from the contribution of at least three organisms' genomes.

A recent study in flies suggests that *Wolbachia*-mediated pathogen blocking occurs early in infection [82], before inducible immune responses would have a chance to act. In this study, the fly is a natural host for *Wolbachia*, but the virus used is native to mosquitoes and so this may affect the generality of the findings. Regardless, the interpretation is that *Wolbachia* is modifying the host cell environment or interface in an intrinsic way that renders the cell inhospitable for virus [83]. For example, *Wolbachia* may affect host protein structure or trafficking, decreasing replication times and virion release, alter lipid membrane structures or endoplasmic reticulum [82] required for viral replication or even cause stress conditions that may lead to reduced host cell activity. Each of these avenues would also lead to the observed correlations between density of *Wolbachia* and strength of blocking. Future research will need to focus on these fundamental and conserved components of cellular change due to *Wolbachia* infection.

When these fundamental mechanism(s) have been elucidated their individual contributions can be assessed in isolation and then in the context of novel hosts where additional host responses may enhance the trait. The pattern of stronger pathogen blocking in novel hosts than native hosts, predicts that the effect is likely to decline with coadaptation of the artificially created associations. The question remaining is whether the fundamental mechanisms remaining will be sufficient to limit virus transmission in vectors.

As such, strategies for counteracting this potential problem are already being considered, including the creation of stable double-infected mosquito lines (consisting of two *Wolbachia* strains infecting one individual) [154] as means of boosting the immune system and prolonging pathogen blocking as a biocontrol strategy.

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CHAPTER TWO

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SCIENTIFIC REPORTS

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The RNAi pathway plays a small part in *Wolbachia*-mediated blocking of dengue virus in mosquito cells

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Wolbachia pipientis **is an insect endosymbiont known to limit the replication of viruses including dengue and Zika in their primary mosquito vector, *Aedes aegypti*. *Wolbachia* is being released into mosquito populations globally in a bid to control the diseases caused by these viruses. It is theorized that *Wolbachia's* priming of the insect immune system may confer protection against subsequent viral infection. Other hypotheses posit a role for competition between *Wolbachia* and viruses for host cellular resources. Using an *A. aegypti* cell line infected with *Wolbachia*, we tested the effects of targeting siRNAs against the major innate immune pathways on dengue virus loads. We show that while *Wolbachia* infection induces genes in the Toll, JAK/STAT and RNAi pathways, only reduced expression of RNAi leads to a rebound of dengue virus loads in *Wolbachia*-infected cells. The magnitude of the effect explained less than 10% of the total DENV load, demonstrating that blocking must be dependent on other factors in addition to the expression of RNAi. The findings bode well for the long-term stability of blocking given that immunity gene expression would likely be highly plastic and susceptible to rapid evolution.**

Arthropod-borne diseases, mainly those transmitted by mosquitoes, are one of the leading causes of mortality in humans, especially in tropical and subtropical areas[1]. Dengue virus (DENV, serotypes 1–4) is a positive single stranded RNA virus of the family *Flaviviridae* and the causative agent of dengue fever, a debilitating illness and the most prevalent of all arthropod-borne diseases worldwide[2,3]. Current estimates suggest upwards of 400 million people are at risk of becoming infected annually[4,5]. DENVs are transmitted to humans during blood feeding by female *Aedes* mosquitoes: *Aedes aegypti* is the main vector and, to a lesser extent, *Aedes albopictus*[2,6,7].

The virus is spreading quickly due to globalization[8] and climate change[9], which is allowing *Aedes spp.* to colonise traditionally colder regions. Current vaccines are imperfect[10,11] and there are no effective antivirals. Additionally, the severity of outbreaks appears to be increasing[12]. More recently, Zika virus, also vectored by *A. aegypti*, has re-emerged with devastating health and socioeconomic impact worldwide[13,14]. Current strategies for limiting arthropod-borne diseases are heavily dependent on effective vector control[4]. The most novel of these type of approaches relies on the use of an insect bacterial endosymbiont, *Wolbachia*, that has the capacity to limit the replication of arboviruses inside mosquito vectors[15].

Wolbachia pipientis is vertically transmitted by females to their offspring and drives its own spread through insect populations by manipulating host reproductive success to its own advantage[16]. The ability to invade a population and be self-sustaining is hugely appealing with respect to its potential use for biological control. Not native to most of the major insect vectors, *Wolbachia* had to be transinfected from *Drosophila melanogaster* into *A. aegypti*, where it then formed a stably inherited infection[17]. The ability of *Wolbachia* to spread into native *A. aegypti* populations and remain at high frequencies was first demonstrated in Cairns, Australia[18]. *Wolbachia* also has been shown to reduce the replication of a range of pathogens inside insects including viruses, bacteria, nematodes and the malaria parasite[19–22]. Subsequently, release programs have begun in multiple locations throughout the tropics.

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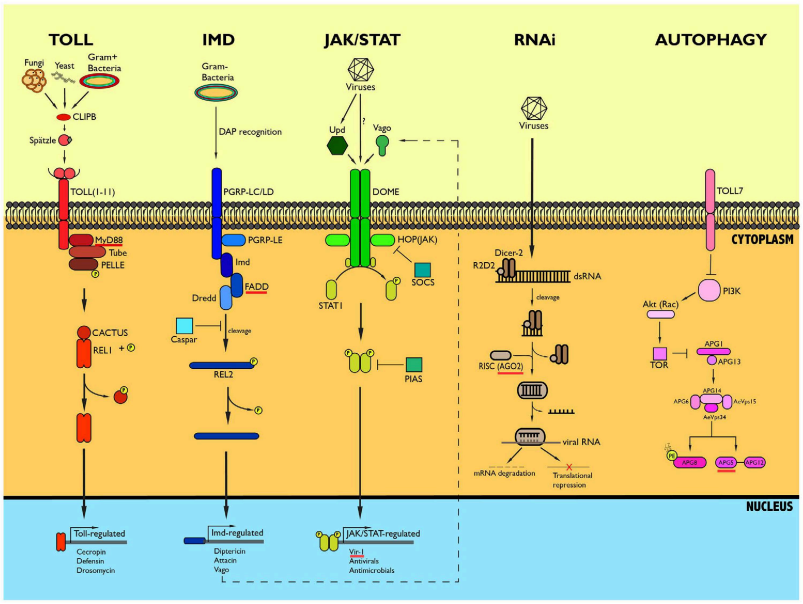

Figure 1. The main *Aedes aegypti* innate immune pathways, The Toll, Imd, JAK/STAT, RNAi and Autophagy. All genes shown correspond to the *A. aegypti* relationships and nomenclature. Underlined genes are targeted with siRNA in this study.

Despite the global scale of *Wolbachia's* release, the mechanism of *Wolbachia*-based 'pathogen blocking' is not well understood. Modulations of essential cellular components such as cholesterol[23] and host microRNAs[24], as well as competition between pathogens and *Wolbachia* for limited host resources[21] have been theorized to underpin blocking. It has also been suggested that the diversity of pathogens blocked by *Wolbachia* could be explained by a *Wolbachia*-mediated host gene modulation leading to an increase in the basal immune activity of the host[21,22]. Any subsequent exposure to a pathogen would therefore lead to greater ability to control the assault, in a theory deemed 'innate immune priming'[25,26].

The primary humoral pathways of the insect innate immune response include Toll, Immune Deficiency (Imd), Janus Kinase-Signal Transducer Activator of Transcription (JAK/STAT) and the exogenous siRNA pathway as part of the RNAi response (Fig. 1)[27–33]. Additionally, there are cellular responses including the Toll-induced autophagy pathway and melanization cascades[34,35]. Each of the pathways has some specificity with respect to type of pathogen targeted, i.e. bacteria, viruses or fungi but several of the pathways are not yet completely defined and there is a growing evidence of overlap between pathways classified as antimicrobial or antiviral[36,37]. For example, the Toll and Imd pathways are primarily antibacterial in effect, but Toll is also required for the mosquito's response to DENV[38,39].

Multiple studies have demonstrated that *Wolbachia* infection increases the basal expression of innate immunity genes[22,25,26]. In *D. melanogaster*, *Wolbachia* does not induce the Toll and Imd pathways. Regardless, there is some evidence of *Wolbachia*-mediated blocking when DENV is injected into the fly, suggesting these pathways are not required[40]. Immune activation by *Wolbachia* in the mosquito, however, is stronger and more widespread in terms of genes and pathways affected[26,41,42]. This difference in the nature of immune activation may also explain the much broader spectrum of pathogen blocking seen in the mosquito including antibacterial and antiviral effects[42]. Few studies have addressed the role of the primary antiviral pathways JAK/STAT and RNAi, or cellular responses like autophagy in regards to *Wolbachia*-mediated blocking in mosquitoes[43].

The induction of the JAK/STAT pathway has been shown to restrict infection of another flavivirus, West Nile virus, in *Culex* mosquitoes[44] and the malaria parasite in *Anopheles gambiae*[45]. In *A. aegypti*, the antiviral function of the pathway is conserved with high activation levels of JAK/STAT limiting DENV replication. The effectors of this pathway, however, are poorly characterized[32]. RNAi is considered one of the major antiviral pathways, shown to limit DENV, chikungunya and Sindbis viruses in *A. aegypti*[29,46,47] but seems less important for pathogen blocking in insects with native *Wolbachia* infections[48,49]. The RNAi pathway is initiated with the recognition and cleavage of viral double stranded RNA (dsRNA) into siRNAs that then operate through cellular machinery to degrade viral ssRNA. Lastly, the cellular responses of autophagy[33,50] and apoptosis also have some antiviral relevance[51,52].

In this study we investigated which components of the mosquito innate immune response are both primed by *Wolbachia* and are essential to induce DENV blocking. We focused on each of the above mentioned major pathways, selecting genes to represent pathways that were either starting points in a signaling cascade, like *MyD88* (Toll) and *FADD* (Imd), or because they serve as effectors, like *vir-1* (JAK/STAT), argonaute-*2* (*AGO2*, RNAi)

and *APG5* (autophagy). We then examined the functional role of each associated pathway in DENV control in *A. aegypti* cells by manipulating gene expression via targeted RNAi techniques in both *Wolbachia*-infected and *Wolbachia*-free cells. We predicted that if genes were uninvolved in *Wolbachia*-mediated effects, reducing their expression via siRNA treatment should have little impact on DENV loads in *Wolbachia*-infected cells. In contrast, if the *Wolbachia*-mediated blocking was reliant on the activity of particular genes, DENV loads should rebound after siRNA treatment. We were particularly interested in genes exhibiting this pattern, whose basal expression was also enhanced by *Wolbachia*.

Results

To gauge the relative contribution of each of the immune pathways to *Wolbachia*-mediated DENV blocking, candidate genes for each pathway were knocked down in an *A. aegypti* embryonic cell line infected with *Wolbachia* and a tetracycline treated version of the same line (Aag2 ± *w*Mel) that served as a *Wolbachia*-free control. Reductions in gene expression were confirmed 18 h post-transfection. Cell lines were then challenged with a DENV-2 strain to assess whether reduced activity of each immune pathway lead to a corresponding increase in DENV load at 5 days post-infection. In two cases (*MyD88* and *FADD*) where selected genes were early in the pathway, we also assessed whether expression changes were carried through to effectors. To assess whether the different pathways have an additive effect on inhibiting DENV replication, we performed consecutive treatments with siRNA for pairs of genes prior to the challenge with DENV-2. We first showed that transfection with a non-*Aedes* targeted siRNA did not alter gene transcription levels so it could be used as a transfection control across samples (Fig. S1). We also demonstrated that single and successive paired siRNA treatment had no effect on *Wolbachia* densities (Fig. S2).

Toll and Imd. Both *Wolbachia* infection ($F = 10.23$, $df = 1$, $p = 0.003$) and siRNA treatment ($F = 611.94$, $df = 1$, $p < 0.0001$) had significant effects on *MyD88* expression. Posthoc comparisons demonstrated reductions in *MyD88* expression (Fig. 2a) between the treatment and scrambled control for both Aag2*w*Mel.tet ($t = 20.03$, $df = 20$, $p < 0.0001$) and Aag2*w*Mel ($t = 14.52$, $df = 20$, $p < 0.0001$). Concurrent reductions in expression of genes downstream in the Toll pathway demonstrate the generality of the effect (Fig. S3). A direct comparison of the expression levels in the scrambled treatment across lines revealed differences ($t = 4.65$, $df = 20$, $p = 0.0002$) that may be explained by *Wolbachia* infection as well as effects of the antibiotic treatment or drift in the post antibiotic passaging period. This reinforces the need to examine fold changes in expression after siRNA treatment relative to the scrambled control to correct for line effects. This difference may also highlight up-regulation by *Wolbachia* of immunity genes in keeping with the immune priming hypothesis[25]. In comparison, relative to scrambled controls, the magnitude of the reduction in *MyD88* expression was roughly 4.5-fold in the Aag2*w*Mel.tet and 9-fold in Aag2*w*Mel. In the case of FADD expression, siRNA treatment ($F = 86.73$, $df = 1$, $p < 0.0001$) had a significant effect on expression but not *Wolbachia* infection ($F = 1.07$, $df = 1$, $p = 0.308$). *FADD* expression levels were similarly reduced in Aag2*w*Mel.tet ($t = 6.46$, $df = 21$, $p < 0.0001$) and Aag2*w*Mel ($t = 6.32$, $df = 19$, $p < 0.0001$) after siRNA treatment (Fig. 2b). The knock down was also conferred to other genes downstream in the Imd pathway (Fig. S3). The achieved reduction of *FADD* expression in both cell lines relative to scrambled controls was ~2.3-fold.

After 18 h of targeted gene knockdown, infection with a DENV-2 strain was performed and cells were then collected at 5 days post infection (dpi). After Toll modulation, there was a significant effect of both the siRNA treatment ($F = 75.66$, $df = 1$, $p < 0.0001$) and *Wolbachia* infection ($F = 1606.62$, $df = 1$, $p < 0.0001$) on DENV load (Fig. 2c). The results show that the siRNA treatment leads to increases in DENV loads and *Wolbachia* infection leads to reductions in DENV load. There was also a slight significant interaction between *Wolbachia* status and siRNA treatment on DENV load ($F = 5.1$, $df = 1$, $p = 0.03$) as the magnitude of impact on DENV load was greater in Aag2*w*Mel than in Aag2*w*Mel.tet. These represent only 3.0 and 7.4% increase in the DENV load relative to scrambled controls for *Wolbachia*-free and *Wolbachia*-infected, respectively. It is unclear whether these differences are large enough to be biologically meaningful. When challenging *FADD*-knocked down cells (Fig. 2d), DENV loads were affected by *Wolbachia* infection status ($F = 360.25$, $df = 1$, $p < 0.0001$) as expected. There was however no effect of siRNA treatment ($F = 1.69$, $df = 1$, $p = 0.873$).

Our results point to Toll having an important role in *A. aegypti's* immunity against DENV, but the pathway is not essential to explain the protective phenotype conferred by *Wolbachia,* as reported previously[40] in *Drosophila*. We also do not see evidence of Imd pathway involvement in DENV protection, since inactivation of the pathway does not lead to an increase in DENV loads and *Wolbachia* infection does not affect its expression.

JAK/STAT. Both *Wolbachia* infection ($F = 80.31$, $df = 1$, $p < 0.0001$) and siRNA treatment ($F = 355.13$, $df = 1$, $p < 0.0001$) had significant effects on *vir-1* expression (Fig 3a). A direct comparison of the expression levels in the scrambled treatment across lines revealed *Wolbachia*-associated increases in expression of *vir-1* ($t = 8.63$, $df = 19$, $p < 0.0001$). The siRNA treatment produced a significant decrease in expression of *vir-1* for both Aag2*w*Mel.tet ($t = 15.26$, $df = 20$, $p < 0.0001$) and Aag2*w*Mel ($t = 12.15$, $df = 20$, $p < 0.0001$). In comparison, relative to scrambled controls, the magnitude of the reduction in *vir-1* expression was roughly 4.2-fold in the Aag2*w*Mel.tet and 6-fold in Aag2*w*Mel. We then tested if DENV levels were affected by the siRNA treatment and the *Wolbachia* infection status (Fig. 3b). There was a significant effect of the siRNA treatment ($F = 18.88$, $df = 1$, $p < 0.0001$) and of the *Wolbachia* infection ($F = 337.75$, $df = 1$, $p < 0.0001$). According to the results, the siRNA treatment increases DENV loads, whereas the presence of *Wolbachia* leads to a reduction in DENV, as expected. These represent only 5.8 and 7.9% increases in the DENV load relative to scrambled controls for *Wolbachia*-free and *Wolbachia*-infected, respectively. Importantly, the reduction of *vir-1* expression in Aag2*w*Mel does not lead to a recovery of DENV toward loads seen in *Wolbachia* uninfected untreated cells.

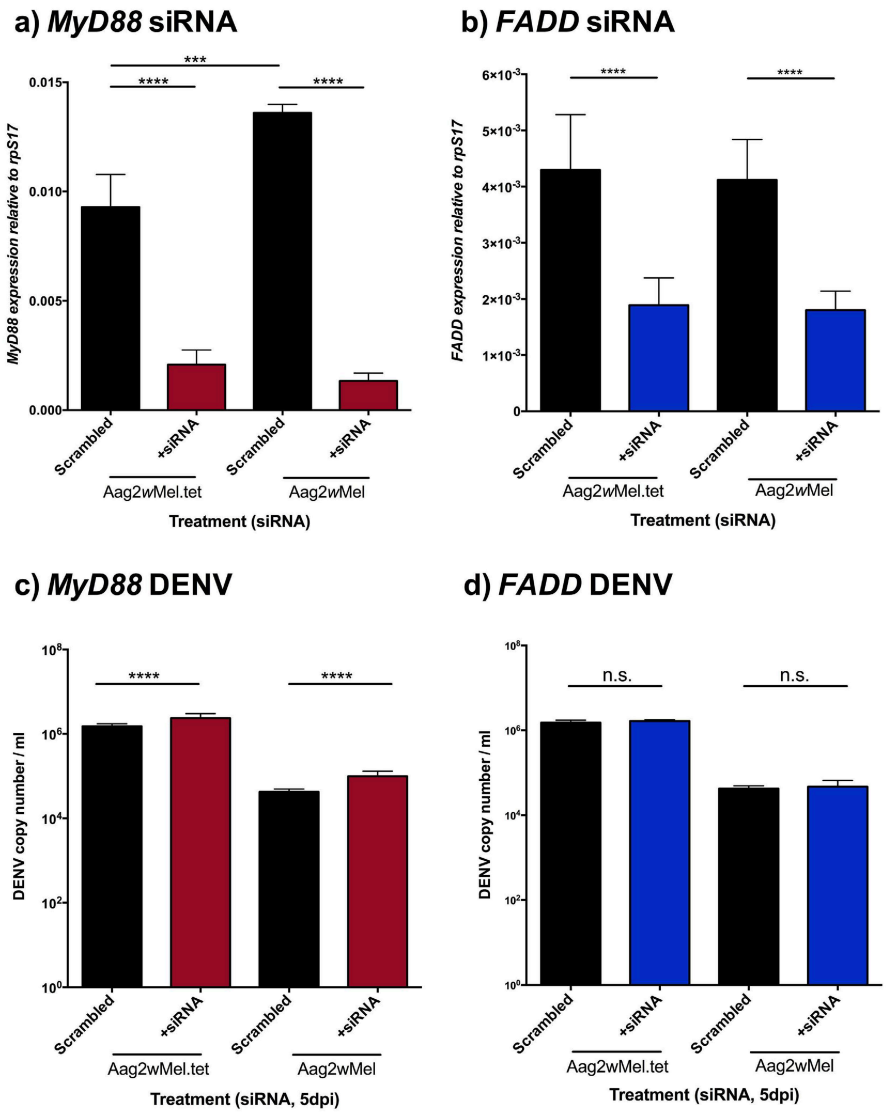

Figure 2. Knockdown of antibacterial pathways. Cell knockdown in *Wolbachia*-infected or tetracycline-treated Aag2 for (**a**) *MyD88* (Toll) and (**b**) *FADD* (Imd). Gene expression was normalized to *A. aegypti* housekeeping gene rpS17. Graphs (**c,d**) correspond to DENV loads after cells were challenged with DENV-2 and collected at 5dpi. All graphs show medians with interquartile ranges (n = 12 per treatment). Black columns depict scrambled controls. Significance is based on post-hoc comparisons following ANOVAs on logarithmic transformed data. ***$p < 0.001$; ****$p < 0.0001$.

RNAi (Exogenous siRNA pathway). Both *Wolbachia* infection ($F = 33.88$, $df = 1$, $p < 0.0001$) and siRNA treatment ($F = 264.75$, $df = 1$, $p < 0.0001$) had significant effects on *AGO2* expression (Fig. 4a). A direct comparison of the expression levels in the scrambled treatment across lines revealed *Wolbachia*-associated increases in expression of *AGO2* ($t = 6.29$, $df = 19$, $p < 0.0001$). Targeted siRNA caused a decrease in *AGO2* expression levels on both Aag2*w*Mel.tet ($t = 11.68$, $df = 19$, $p = 0.0033$) and *w*Mel-infected ($t = 14.62$, $df = 19$, $p < 0.0001$) lines. In comparison, relative to scrambled controls, the magnitude of the reduction in *AGO2* expression was roughly 4.2-fold in the Aag2*w*Mel.tet and 5.3-fold in Aag2*w*Mel. When testing for effects on DENV loads after gene knockdown (Fig. 4b), both the effects of siRNA treatment ($F = 1506$, $df = 1$, $p < 0.0001$) and *Wolbachia* infection ($F = 1667$, $df = 1$, $p < 0.0001$) were significant. Similar to other genes tested, siRNA treatment caused DENV loads to increase whereas *Wolbachia* infection limited DENV replication. These

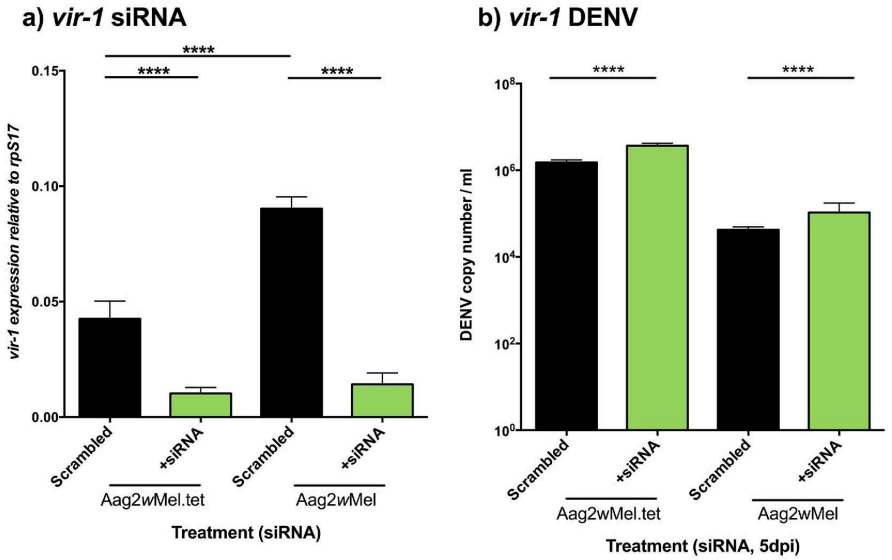

Figure 3. Knockdown of the JAK/STAT pathway. (**a**) *vir-1* gene (JAK/STAT) knockdown in *Wolbachia*-infected or tetracycline treated Aag2 cells. Gene expression was normalized to *A. aegypti* housekeeping gene rpS17. (**b**) DENV loads after knock down and challenge with DENV-2. Graphs show medians with interquartile ranges (n = 12 per treatment). Black columns depict scrambled controls. Significance is based on post-hoc comparisons following ANOVAs on logarithmic transformed data. ****$p < 0.0001$.

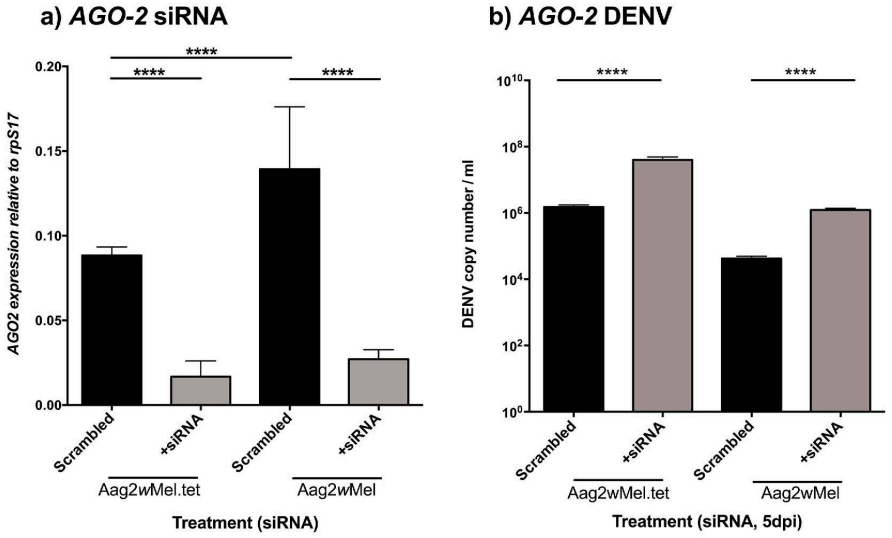

Figure 4. Knockdown of RNAi. (**a**) *AGO2* gene (RNAi) knockdown in *Wolbachia*-infected or tetracycline treated Aag2 cells. Gene expression was normalized to *A. aegypti* housekeeping gene rpS17. (**b**) DENV loads after knock down and challenge with DENV-2. Graphs show medians with interquartile ranges (n = 12 per treatment). Black columns depict scrambled controls. Significance is based on post-hoc comparisons following ANOVAs on logarithmic transformed data. ****$p < 0.0001$.

increases in DENV load are on the order of 18.0 and 24.0% relative to scrambled controls for *Wolbachia*-free and *Wolbachia*-infected, respectively. These higher fold changes than previous genes demonstrate the

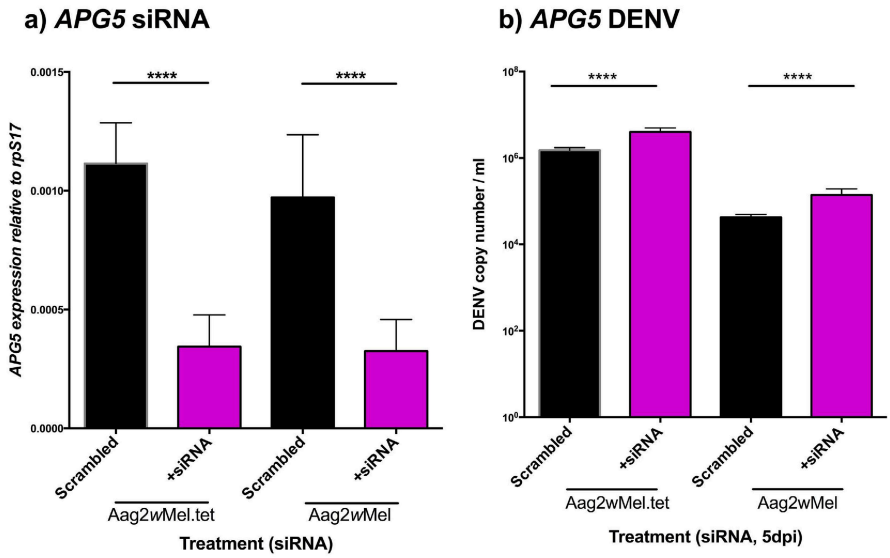

Figure 5. Knockdown of autophagy. (**a**) *APG5* gene (autophagy) knockdown in *Wolbachia*-infected or tetracycline treated Aag2 cells. Gene expression was normalized to *A. aegypti* housekeeping gene rpS17. (**b**) DENV loads after knock down and challenge with DENV-2. Graphs show medians with interquartile ranges (n = 12 per treatment). Black columns depict scrambled controls. Significance is based on post-hoc comparisons following ANOVAs on logarithmic transformed data. ****$p < 0.0001$.

importance in general of AGO2 for DENV control. The differential between the two lines (6%) represents the extra increase in DENV load that is due to *Wolbachia* via RNAi interactions.

Our results suggest that the exogenous siRNA pathway of those examined in the humoral response is the main controller of DENV replication in *Aedes aegypti*. When comparing specific antiviral pathways, the suppression of JAK/STAT through knockdown of *vir-1* doesn't affect DENV loads as much as inactivation of the exogenous siRNA pathway through knockdown of *AGO2*. The presence of *Wolbachia* increases basal *AGO2* gene levels, which have been proven crucial for DENV control. We hypothesize that *Wolbachia*-mediated blocking of DENV is in part utilizing the exogenous siRNA pathway through up-regulation of its components to control DENV replication. This effect however explains less than 10% of *Wolbachia's* ability to limit DENV.

Autophagy. There was a significant effect of siRNA treatment ($F = 132.18$, $df = 1$, $p < 0.0001$) but not *Wolbachia* infection ($F = 0.60$, $df = 1$, $p = 0.44$) on APG5 expression (Fig. 5a). Successful knockdown of *APG5* was achieved in Aag2*w*Mel.tet ($t = 8.14$, $df = 21$, $p < 0.0001$) and Aag2*w*Mel ($t = 8.61$, $df = 18$, $p < 0.0001$), reducing *APG5* levels ~2.8-fold in both cell lines compared to scrambled control. A direct comparison of the expression levels in the scrambled treatment across lines also revealed no effect of *Wolbachia* infection on expression of *APG5* ($t = 0.96$, $df = 19$, $p < 0.34$). There was a significant effect of siRNA treatment ($F = 142.55$, $df = 1$, $p < 0.0001$) and *Wolbachia* status ($F = 1365$, $df = 1$, $p = 2.75e^{-7}$) on DENV load (Fig. 5b). Our results show that siRNA treatment leads to an increase in DENV load and *Wolbachia* presence reduces DENV. In comparison, relative to scrambled controls, the magnitude of the reduction in *APG5* expression was roughly 6.3-fold in the Aag2*w*Mel.tet and 10.0-fold in Aag2*w*Mel.

Even though DENV loads are increased with the suppression of *APG5*, the presence of *Wolbachia* does not modulate *APG5* levels and the lines respond similarly to siRNA treatment. Altogether, it allows us to conclude that *Wolbachia*-based protection is not reliant on autophagy. This non-modulation of its expression levels indicates that autophagy is acting independently of the presence of a *Wolbachia* infection, but is probably an important factor in the *A. aegypti* general immune response against dengue virus.

Stacking of Toll, JAK/STAT and RNAi. Sequential delivery of siRNAs for individual genes followed by *AGO2* was necessary to test combined effects of pathways, as the efficacy of the siRNA treatment itself relies on a functional RNAi response. We first determined whether the knockdown of each individual gene was affected by the presence of the second siRNA. In all cases, save *vir-1* in Aag2*w*Mel only, the knockdown for each gene was the same between the single gene and stacked approach (Fig. S4), indicating a lack of interference or overlap between siRNAs. As previously reported, we found a *Wolbachia*-mediated up-regulation for three genes belonging to three different innate immune pathways. The genes were *MyD88* (Toll; $t = 3.461$, $df = 22$, $p = 0.0022$), *vir-1* (JAK/STAT; $t = 4.257$, $df = 22$, $p = 0.0003$) and *AGO2* (exogenous siRNA; $t = 4.495$, $df = 22$, $p = 0.0002$).

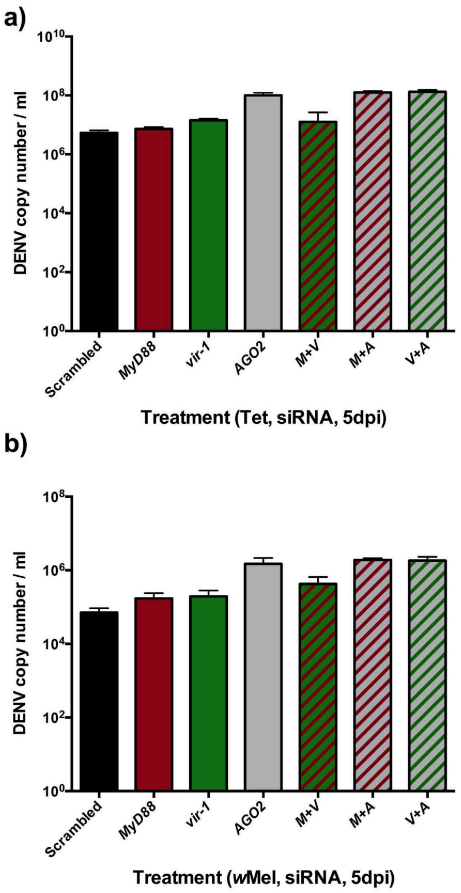

Figure 6. Knockdown of stacked *MyD88*, *vir-1* and *AGO-2*. DENV load in response to single gene (solid bars) and stacked gene knockdowns (hatched bars) for (**a**) Aag2*w*Mel.tet and (**b**) Aag2*w*Mel. Black columns depict scrambled controls. Graphs show medians with interquartile ranges (n = 12 per treatment). Statistical significance reported in Table 1.

Also shown previously in the single gene siRNA assays, the greatest increase in DENV load was for *AGO2*-treated samples (Fig. 6) for both *Wolbachia*-free ($t = 28.04$, $df = 22$, $p < 0.0001$) and infected line ($t = 13.98$, $df = 21$, $p < 0.0001$). In each case the control comparisons of single gene siRNA treatments versus the scrambled controls recapitulated the findings in the single gene assays. There was no effect of sequential treatment of siRNAs for *vir-1* and *MyD88* together compared to *vir-1* alone on DENV load (Fig. 6, Table 1). In contrast, the stacking of *MyD88* and *vir-1* siRNAs each with *AGO2* produced greater DENV loads than for *AGO2* alone, demonstrating the contributory role of these pathways in DENV control. A marginally significant interaction for both of these comparisons *AGO2* resulted from the slightly larger effect of siRNA treatment in *Wolbachia* infected cells compared to *Wolbachia*-free. The results of the stacking assays demonstrate the greater importance of *AGO2* than *MyD88* and *vir-1* in DENV control but also in *Wolbachia*-mediated blocking. The contributions from the latter two pathways, while small, are additive.

Discussion

In this study we aimed to determine if any of the major innate immunity pathways in mosquitoes plays a role in *Wolbachia*-mediated DENV blocking. Using siRNA against single and pairs of genes, we reduced the transcription of key genes representing each of 5 immunity pathways in mosquito cells and assessed the impact of gene knockdown on DENV load. By comparing the responses of *Wolbachia*-infected to *Wolbachia*-free cells, we were able to specifically determine which genes were involved not only with anti-DENV responses, but contributing specifically to *Wolbachia*-mediated effects. The Toll, JAK/STAT and RNAi pathways all demonstrated increased basal expression in response to *Wolbachia* infection. When expression of the RNAi pathway was reduced, however, DENV loads in *Wolbachia*-infected cells rebounded, suggesting that *Wolbachia*-mediated blocking was in

Comparison	Factors	F value	df	p
Scrambled vs *MyD88*	siRNA treatment	12.31	1	0.001
	Wolbachia (+/−)	526.62	1	<0.001
	siRNA* *Wolbachia*	9.46	1	0.004
Scrambled vs *vir-1*	siRNA treatment	97.82	1	<0.001
	Wolbachia (+/−)	467.72	1	<0.001
	siRNA* *Wolbachia*	91.26	1	<0.001
Scrambled vs *AGO2*	siRNA treatment	154.73	1	<0.001
	Wolbachia (+/−)	180.91	1	<0.001
	siRNA* *Wolbachia*	145.59	1	<0.001
vir-1 vs *MyD88* + *vir-1*	siRNA treatment	0.898	1	0.35
	Wolbachia (+/−)	113.28	1	<0.001
	siRNA* *Wolbachia*	0.527	1	0.47
AGO2 vs *AGO2* + *MyD88*	siRNA treatment	4.84	1	0.033
	Wolbachia (+/−)	478.75	1	<0.001
	siRNA* *Wolbachia*	4.28	1	0.045
AGO2 vs *AGO2* + *vir1*	siRNA treatment	7.12	1	0.011
	Wolbachia (+/−)	353.1	1	<0.001
	siRNA* *Wolbachia*	6.72	1	0.013

Table 1. Analysis of Variance table investigating single gene siRNA vs scrambled control and sequential treatment siRNA versus single gene treatments on DENV for *Wolbachia*-infected and *Wolbachia*-free lines.

part reliant on the action of RNAi. The magnitude of this effect was small however, explaining less than 10% of the total DENV load. The differential involvement of genes in DENV control across the two lines was also seen for Toll and JAK/STAT, with greater DENV load increases seen in the Aag2*w*Mel cell line compared to the tetracycline-treated line. These additional pathways therefore provide a small but significant contribution to the blocking conferred by RNAi. The additive nature of these gene contributions was further confirmed with the stacking assays.

In insects, RNAi plays a key role in antiviral defense. We focused on components of the exogenous siRNA pathway, whose primary function is the cleavage of dsRNA[53]. Briefly, exogenous dsRNA molecules are recognized and cleaved into smaller fragments (siRNAs) by a dsRNA-specific RNAse (Dicer). Then, siRNAs destroy their complementary mRNA targets by binding and guiding the complex to the argonaute protein that carries out endoribonucleic cleavage[54]. Because the silencing activity of siRNAs is responsive to the particular agent infecting the cell at any one time, it has broad efficacy against diverse viruses. Even though DENV is a (+) ssRNA virus, detectable amounts of dsRNA are created as replicative intermediates, similar to other flaviviruses[55]. Several studies have demonstrated the involvement of RNAi responses in regulating arboviruses including chikungunya, DENV, yellow fever or o'nyong-nyong inside the vector[46,47,56,57].

Interestingly, in *Drosophila*, RNAi is not essential to achieve *Wolbachia*-mediated blocking from Drosophila C Virus[48] and nor is it needed for control of Semliki Forest Virus in *Drosophila* cells infected with *Wolbachia*[43]. The fly and the mosquito may differ, however, with clear evidence of a much stronger and more far reaching immune response to *Wolbachia* in that latter[42,58]. Studies across a range of insect species suggest that older and more established *Wolbachia*:host associations may have lower *Wolbachia* densities, contracted tissue distributions and fewer effects on the immune response[59–62]. That *Wolbachia* may be causing a greater reaction in the recently infected *A. aegypti* is in keeping with co-evolutionary theories for novel host:pathogen pairings[63]. Additionally, mosquitoes/mosquito cells themselves may have histories of adaptation to native viruses that are not seen when non-native hosts like *Drosophila* are infected.

JAK/STAT has also been proposed as a major pathway involved in antiviral protection[28,32]. Our data supports previous studies suggesting that RNAi is more important for DENV control than JAK/STAT[46]. This difference between the two main antiviral pathways may stem from their mode-of-action. RNAi pathways are triggered by intracellular exogenous dsRNA[64] whereas the activation of JAK/STAT is dependent on the binding of secreted ligands following pathogen recognition[36]. JAK/STAT's antiviral mode of action remains unknown, though it is thought to be complex and versatile[65]. Moreover, DENV also suppress many signaling pathways. For example, the viral protein NS4B causes inhibition of the IFN pathway (JAK/STAT is involved in mammal type I interferon signaling[66]) by preventing STAT1 phosphorylation and activation, as well as its transport to the nucleus[67]. Similarly, NS5 has been shown to inhibit human STAT2 phosphorylation[68]. In mosquitoes, inhibition of immune signaling pathways Toll, Imd and JAK/STAT has been shown in the DENV-related Semliki Forest Virus[69].

It is unclear how *Wolbachia* may be modulating the expression of either the RNAi or JAK/STAT pathways. Because RNAi acts intracellularly, there may be greater opportunities for the symbiont to manipulate its expression than for pathways like JAK/STAT. However, while cross talk has been demonstrated between JAK/STAT and Toll[70], such interactions have not yet been shown between RNAi and other pathways that may respond directly to bacterial effectors. A previous study has shown that *Wolbachia* has the capacity to affect intracellular localization of AGO-1, a member of the miRNA pathway within RNAi[71]. It is not clear how this change is mediated, but it has capacity to affect the host's immune response to pathogens.

Several aspects of the study's design may limit the scope of its interpretation. First, as with any siRNA approaches, the ability to detect phenotypic effects is dependent on the strength of silencing. In almost all cases transcription was reduced by ~75% but we cannot rule out that additional effects might have emerged if we had reduced the gene expression further. Next, we have utilized the standard approach of creating a *Wolbachia*-free line by tetracycline treatment. Although few passages were allowed for treatment with antibiotics and for recovery, it is possible that some genetic drift occurred between the treatment and control lines. Regardless, the comparisons via a scrambled control intermediate should mitigate such issues. Creation of a newly *Wolbachia*-infected line where the original recipient line serves as the wildtype is also likely to lead to drift given the number of reinfection events and also selection that must commonly be employed to get a highly infected cell line[72]. Last, the approach taken here is highly reductionist. Blocking in adult mosquitoes is likely to be more complicated given the diversity of cell and tissue types and their potential to vary with respect to immune activity[73]. There is also likely, yet to be discovered, avenues of cross talk between immune pathways. More generally, these are complex interactions involving three organisms and their genomes. While the results can speak directly to the involvement of immune priming, our ability to estimate its proportional involvement relative to other mechanisms is limited.

Wolbachia is being released in a number of sites throughout the tropics as a possible biocontrol agent against viruses vectored by *A. aegypti*, including DENV and Zika[15,74]. Understanding mechanistically how *Wolbachia* restricts pathogen replication is key for assessing the long-term evolutionary stability of pathogen blocking in vector populations. Our findings show that increased expression of a gene in the mosquito's antiviral response confers a small amount of DENV blocking in cells. Given the breadth of involvement of RNAi in protection against a range of arboviruses, this is likely to be true for Zika and other viruses vectored by *A. aegypti*. The concern, if blocking were heavily reliant on this immune reaction and not other factors, is the plasticity of gene expression as well as its capacity to evolve in response to pathogens[75]. In the field, mosquitoes may evolve tolerance to *Wolbachia* and limit the need to mount a costly immune response to the symbiont[76]. The vector may also evolve resistance, limiting *Wolbachia* densities or tissue distributions, as tends to be seen in native hosts such as *Drosophila*[42,61,62,73,77]. Field release populations of *A. aegypti* are being monitored to assess the stability of blocking and strategies are being developed for dealing with emerging resistance[78]. This study suggests at least that the risk reduced blocking efficacy due to a rapidly evolving immune response is low.

Materials and Methods

Cell line maintenance. The *w*Mel strain was transinfected from *D. melanogaster* into the immune-competent *A. aegypti* cell line Aag2[79,80] using the shell vial technique, as previously performed for other mosquito cell lines[72,81]. The Aag2*w*Mel cell line was serially passaged and checked for *Wolbachia* infection using quantitative PCR (qPCR)[18] and fluorescent *in situ* hybridization (FISH) against *Wolbachia*-specific 16S rRNA probe[82]. The control *w*Mel uninfected line (Aag2*w*Mel.tet) was obtained after three successive passages in the presence of tetracycline treatment at 10 mg/ml. The complete absence of *Wolbachia* was also confirmed using FISH and qPCR. Both cell types were routinely passaged in filtered complete media: a 1:1 mixture of Schneider's media (Life Technologies) and Mitsuhashi-Maramorosch (MM), supplemented with 10% heat-inactivated FBS (Life Technologies) and 1% Penicillin/Streptomycin (Life Technologies). Cells were reared in an incubator at 25 °C.

Virus. Dengue virus serotype-2 strain (ET-300; GenBank: EF440433.1) was isolated from a patient in East Timor-Leste in 2000 and passaged in C6/36 cells prior to experimental use. C6/36 cells were continuously kept in RPMI 1640 media (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies), 1% Glutamax (Life Technologies), and 25 mM HEPES buffer (Sigma-Aldrich, St. Louis, MO). Cells were kept in a non-humidified incubator at 25 °C for optimal growth. Cells were allowed to grow to an 80% confluent monolayer prior to virus inoculation for 2 h and then maintained in 2% FBS media. Virus was collected at 7 days post-infection by harvesting the cell culture supernatant and centrifuged at max speed for 15′ at 4 °C. Virus was aliquoted and stored at −80 °C until use. Viral stocks were titrated using plaque assays and dengue copies quantified via qPCR. ET300 viral stocks were diluted in serum-free RPMI media to a concentration of 4×10^5 plaque forming units per milliliter before experimental use.

***Wolbachia* density.** *Wolbachia* densities were measured in Aag2*w*Mel after every passage and Aag2*w*Mel.tet lines were assessed in parallel to confirm their uninfected status. We also assessed the effect of siRNA treatment on *Wolbachia* densities. Taqman® multiplex qPCR was performed to detect the *w*Mel strain levels using primers for the *Wolbachia WD0513* gene[83] relative to the mosquito housekeeping mosquito gene *rpS17*[84] in a LightCycler480 instrument (Roche Applied Science, Switzerland). The primers used are listed in Table 2. Each multiplexed qPCR was run in triplicate and consisted of a 2x LightCycler480 Probes Master reaction mix, 10 μM *rpS17* and *w*Mel-IS5 primers, probes and 1.5 μl of DNA template in a total volume of 10 μl, as stated in the manufacturer's protocol. Ratios of *w*Mel-IS5 to *rpS17* were obtained following the ΔΔCt method[85].

siRNA transfection. Aag2*w*Mel and Aag2*w*Mel.tet cells were seeded the day before transfection in a flat bottom Greiner 96-well plate (Sigma-Aldrich) at a 70–80% confluence. The following day, three different treatments were applied to the cells in a serum-free environment: Mock transfected, Scrambled siRNA at 10 μM and *gene of interest* (GOI) siRNA at 10 μM. Custom siRNAs targeting *A. aegypti* immune genes were manufactured by Sigma-Aldrich. All siRNA treatments were performed with the addition of Lipofectamine RNAiMAX (Sigma-Aldrich) reagent, and transfected altogether according to manufacturer's protocol. The single gene assays (n = 6 replicate wells per treatment) were replicated a second time and pooled (n = 12). For stacking experiments, (n = 12 replicate wells per treatment) the second GOI siRNA was applied to the sample 18 h after the first siRNA treatment, performed as stated above.

Gene ID	Pathway	*Aedes* gene name	Direction	Sequence (5′-3′)	Tm	Gene function
AAEL017251	RNAi	*argonaute-2*	Fw	ACAACAGCAACAATCCCAGA	60	Catalytic compound of RISC, mRNA cleavage
			Rv	GTGGACGTTGATCTTGTTGG	60	
AAEL002286	Autophagy	*APG5*	Fw	CCAGGACTTGTTGGAGGACT	56	Autophagosome elongation
			Rv	GTCCGGATAGCTGAGGTGTT	56	
AAEL000627	Toll	*cecropin-A*	Fw	CCATGGCTGTTCTTCTCCTGA	60	Antimicrobial peptide
			Rv	GGCGGCATTGAAAACTCGTT	60	
AAEL004833	Imd	*diptericin-A*	Fw	CCAATTCAGGAAGTGGAACC	56	Antimicrobial peptide
			Rv	TGTTGATGGGTAGCTCCAAA	56	
AAEL014148	Imd	*dredd*	Fw	GTGGCTGTTATGCGAGAAGA	60	Initiatior caspase, cleavage of REL2
			Rv	AGCGTAGTTCTGCCTGAGGT	60	
AAEL001932	Imd	*FADD*	Fw	GGGACCGTCGAACACTTCTT	60	Imd signal transducer
			Rv	CACTCAGCTGCATTAACCGC	60	
AAEL007768	Toll	*MyD88*	Fw	GGACTACAAGCGCTCGAACA	60	TOLL signaling cascade starter
			Rv	CTGGTTTGGTTTGCGTTCGA	60	
AAEL006571	Toll	*PELLE*	Fw	ACAACCGACGAAAACTCCGA	56	Signaling molecule - Kinase
			Rv	GCGAAGTTCTTCCCCACTGA	56	
AAEL000718	JAK/STAT	*vir-1*	Fw	GCCAAAGTCCGGTATTCTTC	60	Antiviral effector
			Rv	TTCACGAGATCGTCAAGGTAA	60	
AAEL004175	—	*rpS17*	Fw	TCCGTGGTATCTCCATCAAGCT	60	Ribosomal small subunit assembly
			Rv	CACTTCCGGCACGTAGTTGTC	60	
AE017196	—	*WD0513*	Fw	GTATCCAACAGATCTAAGC	60	
			Rv	ATAACCCTACTCATAGCTAG	60	
NC_001474.2	—	DENV	Fw	AAGGACTAGAGGTTAGAGGAGACCC	60	
			Rv	CGTTCTGTGCCTGGAATGATG	60	
			Pr	HEX-AACAGCATATTGACGCTGGGAGAGACCAGA-BHQ1		

Table 2. Candidate genes and associated primers for each pathway. Gene function from Flybase and Swiss-Prot.

RNA extraction and cDNA synthesis. Cell RNA was extracted from cells 18 to 36 h hours post transfection using the Nucleospin 96 RNA kit (Machery-Nagel, Germany), following modified manufacturer's protocol. cDNA synthesis reactions were performed using SuperScript® III Reverse Transcriptase (Invitrogen) and contained 12.5 μl of RNA template, 1 μl of random primers (RP, 125 ng/μl), 1 μl of deoxynucleotides (dNTPs, 2.5 mM), dithiothreitol (DTT), 5X buffer and enzyme as per kit instructions, in a total volume of 20 μl. Reactions were carried out in a C1000™Thermal Cycler (Bio-Rad) with the cycling regime: 65 °C for 5 min followed by 10 min at 25 °C, 50 min at 50 °C, 10 min at 75 °C.

Selection of candidate immunity genes. Candidate genes were selected for each pathway of interest that met the following criteria: they had to be required for the function of the pathway, be sufficiently transcribed and the ability to be knocked down efficiently in our cell model. Refer to Table 2 for chosen candidates and associated primer sequences with gene IDs and function. All primers were designed using the open-source Primer3 software[86]. For candidates involved upstream we confirmed that expression of downstream genes (Toll and Imd) was reduced after targeted knockdown to assure complete inactivation of the pathway.

Immunity gene quantitative PCR analysis. Gene expression was measured using SYBR® Green I Master (Roche) according to manufacturer's protocol. All runs were performed in duplicate using a 10 times dilution of the cDNA. The temperature profile used is as follows: one cycle at 95 °C for 5 min followed by 45 amplification cycles of 95 °C for 10 sec, 60 °C for 10 sec and 72 °C for 10 sec, followed by a melting curve analysis after the last cycle. In all qPCR analyses, GOI were normalized to the housekeeping gene *rpS17,* run in parallel. GOI to housekeeping gene ratios were obtained for each biological sample using the aforementioned ΔΔCt method[85].

DENV infection and quantification. DENV infections were performed 18 h post-transfection with siRNA. Cells were washed with PBS before and after the virus inoculation at a DENV-2 multiplicity of infection (MOI) of 0.5. At this MOI DENV-blocking in *w*Mel is most clearly seen as per pilot studies (data not shown). The viral inoculum was removed 2 h post-infection and cells were grown in complete media containing 2% FBS. DENV was quantified 5 days post-infection by collection of 20 μl supernatant and mixed 1:1 with 20 μl squash buffer (10 mM Tris base, 1 mM EDTA, 50 mM NaCl and 0.25 μl proteinase K). They were then incubated in a C1000™Thermal Cycler (Bio-Rad, California USA) at 56 °C for 5 min, then 98 °C for 5 min for the simultaneous isolation of RNA and DNA. The RNA/DNA was subsequently used for the absolute quantification of DENV-2 via qPCR using a standard curve, as described previously[87].

One-step quantitative PCR was performed using TaqMan® Fast Virus 1-step Master Mix (Roche) in a total 10 μl, following manufacturer's instructions.

The primer sequences used for the detection of DENV were as described previously[87]. The thermal profile was as stated previously for qPCR analysis, with the addition of 10 min incubation retrotranscription step at 50 °C followed by 20 sec at 95 °C for RT inactivation at the start of the run.

Data analysis. qPCR reactions were run in duplicate and samples that failed to amplify for at least one replicate were removed. Statistics were carried out using IBM SPSS Statistics (v23) and R software (R Development Core Team (2008), Vienna, Austria). Gene expression ratios and dengue loads were both log transformed prior to analysis. Two-way ANOVAs were performed testing for the effects of *Wolbachia* infection (presence/absence), siRNA treatment (+/−) and replicate (1, 2) as factors on gene expression or DENV load. At no point was 'replicate' significant and so reported statistics focus only on main effects. Interactions are reported only when significant. Post hoc comparisons were employed multiple tests accounted for using a Bonferroni correction, leading to adjusted p-values of 0.025 and 0.017, when 2 or 3 comparisons were made, respectively. All DENV loads were reported on a log scale given the spread of values.

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Author Contributions

E.A.M., G.T. and D.A.J. designed the experiments. G.T. performed the experiments. G.T. and E.A.M. analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

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CHAPTER THREE

Family level variation in *Wolbachia*-mediated dengue virus blocking in *Aedes aegypti*

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Abstract

The mosquito vector *Aedes aegypti* is responsible for transmitting a range of arboviruses including dengue (DENV) and Zika (ZIKV). The global reach of these viruses is increasing due to an expansion of the mosquito's geographic range and increasing urbanization and human travel. Vector control remains the primary means for limiting these diseases. *Wolbachia pipientis* is an endosymbiotic bacterium of insects that has the ability to block the replication of pathogens, including flaviviruses such as DENV or ZIKV, inside the body of the vector. A strain of *Wolbachia* called *w*Mel is currently being released into wild mosquito populations to test its potential to limit virus transmission to humans. The mechanism that underpins the virus blocking effect, however, remains elusive. Using a modified full-sib breeding design in conjunction with vector competence assays, we show the first evidence of family level variation in *Wolbachia*-mediated blocking. This variation may stem from either genetic contributions from the mosquito and *Wolbachia* genomes or environmental influences on *Wolbachia*. If genetic variation exists, it would suggest that the blocking trait has the potential to evolve. In these families we also tested for correlations between strength of blocking and expression level for several insect immunity genes with possible roles in blocking, identifying two genes of interest.

Author Summary

Wolbachia is a bacterium that lives inside insects. The presence of *Wolbachia* can prevent insects like mosquitoes from becoming infected with viruses and parasites they transmit. *Wolbachia* bacteria are currently being released into wild mosquito populations to see if they can prevent transmission of dengue and Zika viruses to humans. There are two key questions that remain to be answered; what is the genetic basis of the blocking trait and is blocking likely to evolve? The latter could lead to reduced efficacy of *Wolbachia* against pathogens. Here for the first time we reveal evidence of family level variation in *Wolbachia*-mediated blocking of dengue virus. This variation may stem from differences in the mosquito or *Wolbachia* genomes or from environmental effects on *Wolbachia* loads. We use these differences to test for correlations between strength of blocking and gene expression for several candidate genes that mediate blocking. These findings suggest that *Wolbachia*-mediated blocking may vary across different mosquito populations and may have the capacity to evolve or change with time.

Introduction

Wolbachia pipientis is an insect endosymbiont capable of manipulating host reproductive success via different mechanisms, the primary and most studied being cytoplasmic incompatibility (CI) [1]. CI gives *Wolbachia*-infected females a reproductive advantage and because the symbiont is maternally transmitted, the bacterium spreads rapidly through uninfected populations. *Wolbachia* also reduces susceptibility of their hosts to a range of pathogens, including viruses, other bacteria, nematodes, fungi and the malaria parasite [2-6]. The traits of CI and *Wolbachia*-mediated pathogen blocking together form the basis of emerging strategies to use *Wolbachia* as an agent of biocontrol against vector-borne diseases [7]. Though present in an estimated 40% of all insect species [8], *Wolbachia* is naturally absent in the main dengue vector, *Ae. aegypti*. However, stably inherited *Wolbachia* infections with a range of strains (*w*Mel & *w*MelPop originally from *Drosophila melanogaster* and *w*AlbB from *Ae. albopictus*) have been created in the mosquito using microinjection techniques [9-11]. Adult *Ae. aegypti* mosquitoes infected with *w*Mel [12] and an alternate strain, *w*AlB [9], are currently being released into the wild to test the ability of *Wolbachia* to spread and to limit human disease [13].

Natural *Ae. aegypti* populations vary in their susceptibilities to dengue virus (DENV) [14-18] and laboratory-based breeding experiments have demonstrated substantial contribution of the mosquito genome to variation in susceptibility often through the innate immune response [17,19-21]. When *Wolbachia* infection is present, pathogen blocking is exhibited by reductions in viral infection rates, loads and transmissibility [5,22-25] beyond the wildtype host's natural antiviral mechanisms. *Wolbachia's* presence throughout the body of the mosquito [5,11] provides numerous opportunities for the symbiont to interfere with the successful colonization and replication of viruses. Inside cells, *Wolbachia* lives within a vacuole of host origin [26,27] utilizing transporters to feed off host resources like amino acids that its incomplete genome cannot synthesize [28,29], and communicating with the extracellular environment using a Type IV secretion system [30,31]. *Wolbachia*-mediated phenotypes including pathogen blocking must therefore, by necessity, be enacted via host physiologies and

across host membranes. We would therefore predict that variation in the mosquito genome is likely to play a role in *Wolbachia*-mediated blocking.

It is unclear whether the *Wolbachia* genome evolves fast enough to be a substantial contributor to variation in the trait. Each generation the population of inherited symbiont experiences a bottleneck at the point of transmission via the embryo [32,33] and there is little opportunity to exchange genes with diverse *Wolbachia* strains in the intracellular environment [34]. In the case of stable transinfection of the *w*MelPop strain into *Ae. aegypti* no new substitutions were witnessed in the symbiont genome in the 4-year period post introduction [35]. Changes have been demonstrated however in a *Wolbachia* strain's effects on *Drosophila simulans* over a longer timeframe [36,37].

Understanding the mechanistic underpinning of the blocking trait, and in particular its complexity, is necessary to assess the role that genetic variation and evolution may play in shaping the trait's expression in the field. Various theories have arisen with regard to mechanism [38]. The first theory suggested that *Wolbachia* may "prime" or activate the host immune response, leading to a heightened ability to limit the growth and replication of subsequent infections with pathogens [4,22,39-41]. While there is growing evidence that immune priming may provide blocking against bacterial pathogens [42], innate immunity may only offer a small boost in viral blocking [43,44]. A second set of theories relate to competition for resources between *Wolbachia* and incoming pathogens. The resources have included intracellular space [5,45], lipids [26,46,47] and nitrogen [48]. Nitrogen may serve as a primary source of energy for *Wolbachia* [48] and *Wolbachia's* modulation of lipid profiles in insect cells may create an environment that is antagonistic toward viral replication [47]. A third set of studies suggests that *Wolbachia* may manipulate expression of host genes that control viruses via microRNAs [49-51]. Most recently, several studies have indicated that *Wolbachia* infection may alter fundamental structures [52] or environments in the host cell [53] that prevent viral replication immediately after entry into cells. A trend that is compatible with all of the above mechanistic explanations for blocking is that higher *Wolbachia* loads are associated with stronger blocking [11,54-56].

As the *Wolbachia* genome is intimately tied to that of the host through maternal inheritance, it is difficult to tease apart the independent genetic contributions of the partners to the trait [43]. In the ideal experimental scenario, we could partition the relative contribution of the mosquito and *Wolbachia* genomes as well as the role of the environment in determining variation in DENV blocking. Such traditional quantitative breeding approaches would require the same mosquito families to be studied with and without *Wolbachia* infection. As transinfection of mosquitoes often requires injection of thousands of individuals to achieve success [10] and removal of *Wolbachia* by antibiotic treatment takes multiple generations [57], the ideal experiment cannot be accomplished. Instead, we have used a modified full sib breeding design approach to assess family level variation in *Wolbachia*-mediated blocking in a population of Australian *Ae. aegypti*. By examining the same trait in parallel in *Wolbachia*-free mosquito families we were also able to demonstrate the additional contribution (both genotypic and environmental) of *Wolbachia* infection to the variance of DENV load. We then used families exhibiting the phenotypic extremes in DENV blocking to screen candidate mosquito genes for correlations in expression that would be suggestive of a functional role in blocking.

Results

DENV load in head tissue by family

Breeding in a modified full-sib [58,59] framework yielded 25 wild type and 33 *w*Mel-infected *Ae. aegypti* families with sufficient offspring for injections. For each family 5 to 30 females were injected with DENV-2 and then their midgut, head and carcass (representing the rest of the body) were dissected at 7-8 days post infection (dpi). After RNA extraction of 5+ individual heads per family, DENV-2 load was quantified via RT-qPCR. Head DENV loads have been commonly used as a proxy for dissemination of the virus [11,60,61] and so we used them to rank families (Figure 1). Carcasses from the selected individuals were then used to test for *Wolbachia* loads and gene expression analyses. All individuals for both WT and *w*Mel lines were infected given the use of intrathoracic injection that bypasses the MIB and allows the virus to disseminate freely. As expected, due to the action of blocking, DENV loads were lower in *w*Mel families compared to WT ($t=31.94$, $df=340$, $p<0.0001$). Heritabililties for DENV load were high and significantly greater than zero for each line; WT ($H^2 = 0.99$, LRT: $\chi^2= 38.4$, $p= 5.76 \times 10^{-10}$) and *w*Mel ($H^2=0.87$, LRT: $\chi^2= 70.0$, $p=1.11 \times 10^{-6}$). Given the maternal inheritance of *Wolbachia*, the latter estimate will be highly inflated, suggesting greater similarity across families due to shared environmental variation and linkage of host and *Wolbachia* genomes. The lower heritability suggests *Wolbachia* infection and its interaction with the host is introducing additional variation compared to the simple system involving the vector and virus alone.

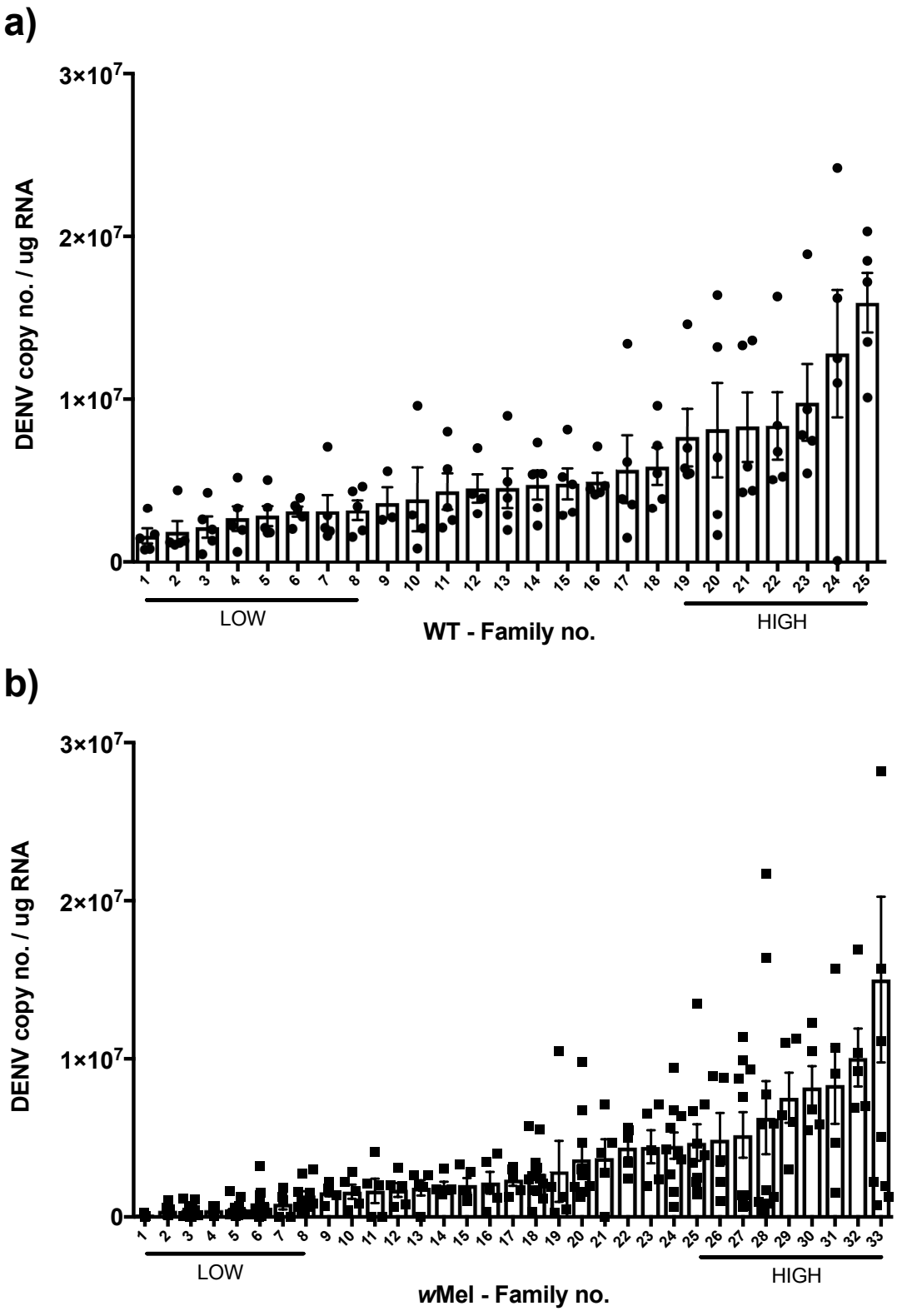

Figure 1. – Head DENV load. WT (a) and *w*Mel-infected (b) families with mean and SEM depicted.

DENV load in carcass tissue by family

To determine if the differences seen in DENV loads for the heads correspond to similar differences in carcasses, RNA extractions were performed on carcasses from individuals previously classified as extreme families (Low and High, Figure 1). Carcass DENV loads mostly recapitulated the patterns seen in heads (Figure 2, Figure S1) and for each treatment we selected 6 families that were most concordant for subsequent analysis (Figure 2). A generalized nested mixed model was used to test for differences between low and high clusters. *Wolbachia* infection status ($F=15.32$, $df=1$, $p=0.001$), DENV load ($F=26.39$, $df=1$, $p<0.001$) as well as the interaction between these two main factors and family ($F=9.47$, $df=21$, $p<0.001$) were significant. The significant interaction is due to the higher range of DENV loads in WT families, given pathogen blocking in the *w*Mel line.

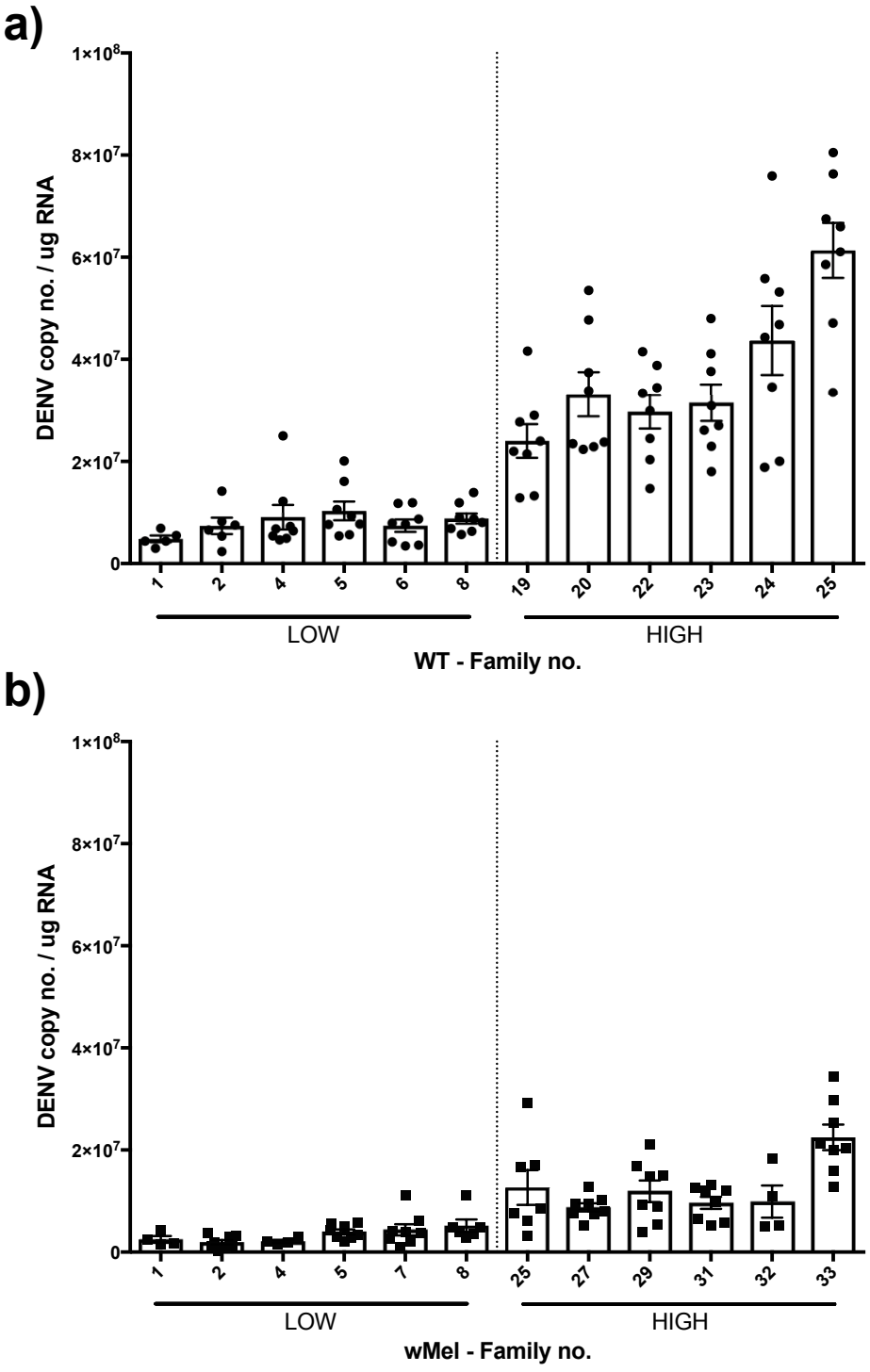

Figure 2. – Carcass DENV load. Differences in DENV load in carcass of families previously classified as High and Low by head tissue (a) WT and (b) *w*Mel-infected individuals, mean and SEM.

Wolbachia correlation to DENV titres

To assess the variability of *Wolbachia* densities amongst families as well as a possible *Wolbachia*-based determination of DENV loads, gDNA was extracted from 3 individual carcasses per family and *Wolbachia* levels were checked using qPCR. As mean *Wolbachia* densities rise in families, DENV loads decline (Figure 3). This negative correlation was significant (Figure S2; $r= 0.546$, $p<0.0001$) and may indicate greater protection against DENV dissemination in the carcass in response to *Wolbachia*. Virus infection did not have an effect on *Wolbachia* loads (Figure S3, $p=0.16$).

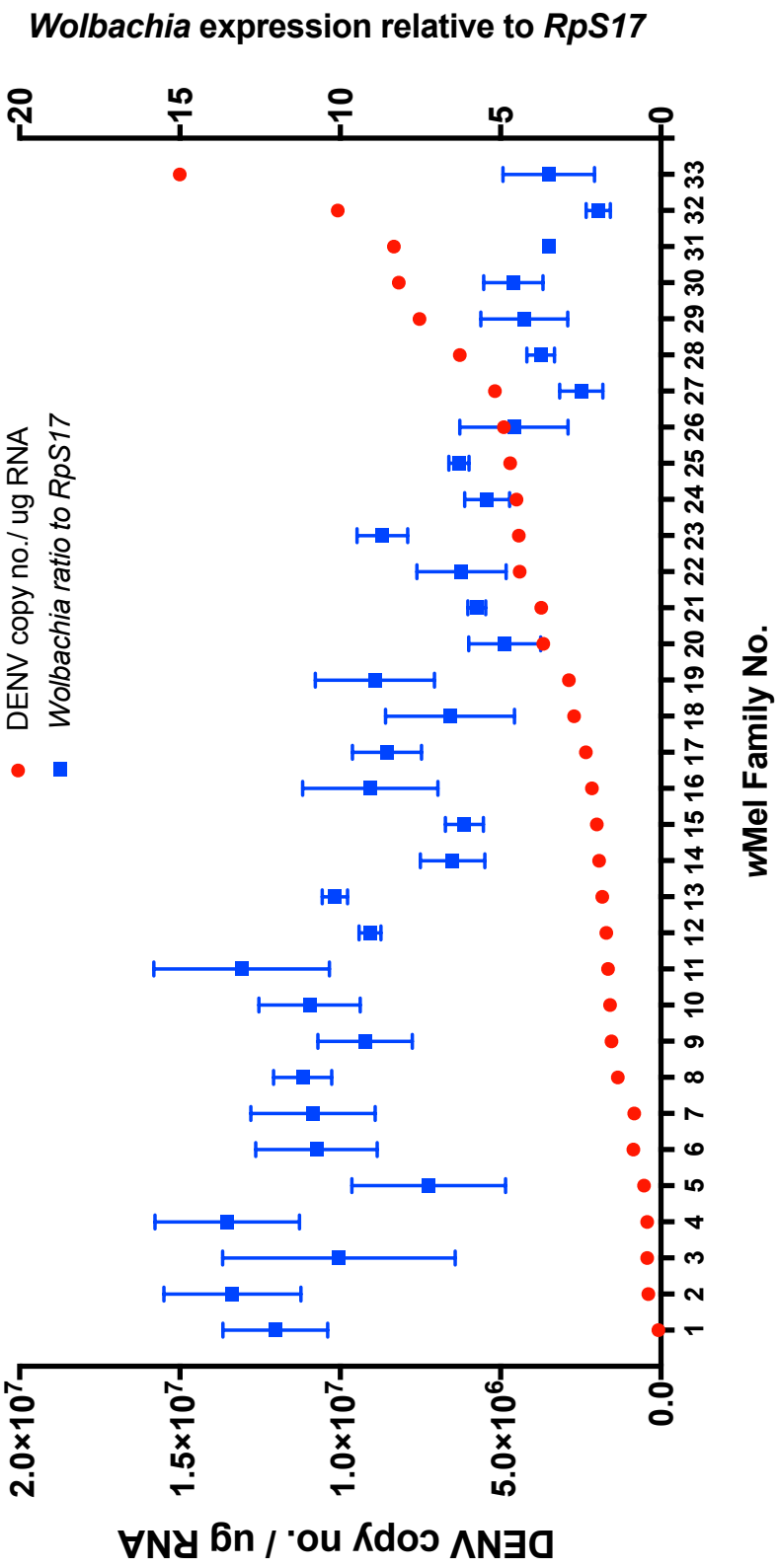

Figure 3. –*Wolbachia* loads determine pathogen protection. Red circles show family means for DENV load (left axis). Blue squares depict mean *Wolbachia* counts relative to *RpS17,* with SEM (right axis).

Candidate gene expression – Immunity

Having confirmed that the *w*Mel strain reduces DENV replication at an individual and population level, we then used our families with extreme blocking phenotypes to test for associations with expression of immunity genes with potential roles in blocking (Figures 4 & 5). We focused on *vir-1* and *AGO2*, genes that represent the two major antiviral pathways in mosquitoes, JAK/STAT and RNAi, respectively [62]. The latter gene has been shown to play a minor role in DENV blocking in mosquito cells [44]. Gene expression was analysed using a generalized mixed model with the random variable Family nested with *Wolbachia**DENV load, with *Wolbachia* and DENV load as fixed factors. The effect of *Wolbachia* infection was significant (Figure 4; F=12.83, df=1, p=0.002), causing upregulation in the expression of *vir*-1. However, *vir-1* expression was not associated with DENV load/family (Figure 4; F=3.1, df=1, p=0.091). There was also no significant interaction between the two main factors (F=1.05, df=21, p=0.412, Figure S4a). These results suggest that while *vir-1* levels may be important for DENV control in the mosquito they do not explain variation in the blocking trait in *Wolbachia*-infected mosquitoes at least at the time point surveyed post infection.

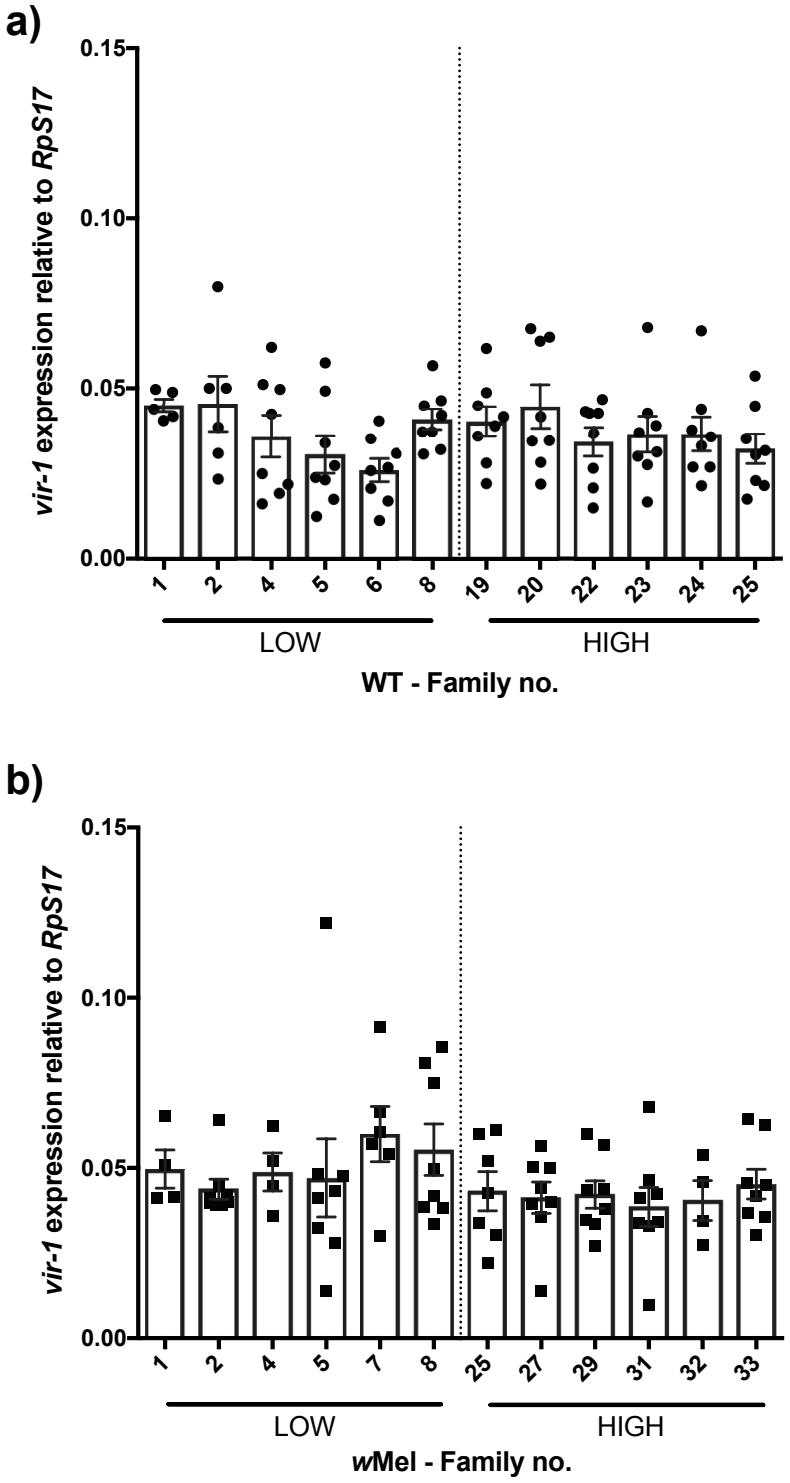

Figure 4. – *vir-1* expression in families classified as High and Low DENV. Graphs show the expression of *vir-1* relative to the housekeeping gene *RpS17* in **(a)** WT individuals, filled circle and **(b)** *w*Mel-infected individuals, filled square. Means with SEM (n=8).

The same mixed effects model was applied to test for differences in *AGO2* gene expression levels. The effect of *Wolbachia* was significant (Figure 5; $F=16.72$, $df=1$, $p=0.001$), leading to heightened expression of the gene. We also detected a significant effect of DENV load/family (Figure 5; $F=27.62$, $df=1$, $p<0.001$), demonstrating higher expression of the gene in Low DENV load families. The interaction was also significant ($F=5.26$, $df=21$, $p<0.001$), showing that the differences between High and Low DENV loads in *AGO2* expression are greater in *w*Mel-infected mosquitoes than in WT (Figure S4b). In WT families, gene expression decreases as DENV titres increase. The same is true for *w*Mel-infected families, but with an even greater disparity between Low and High families.

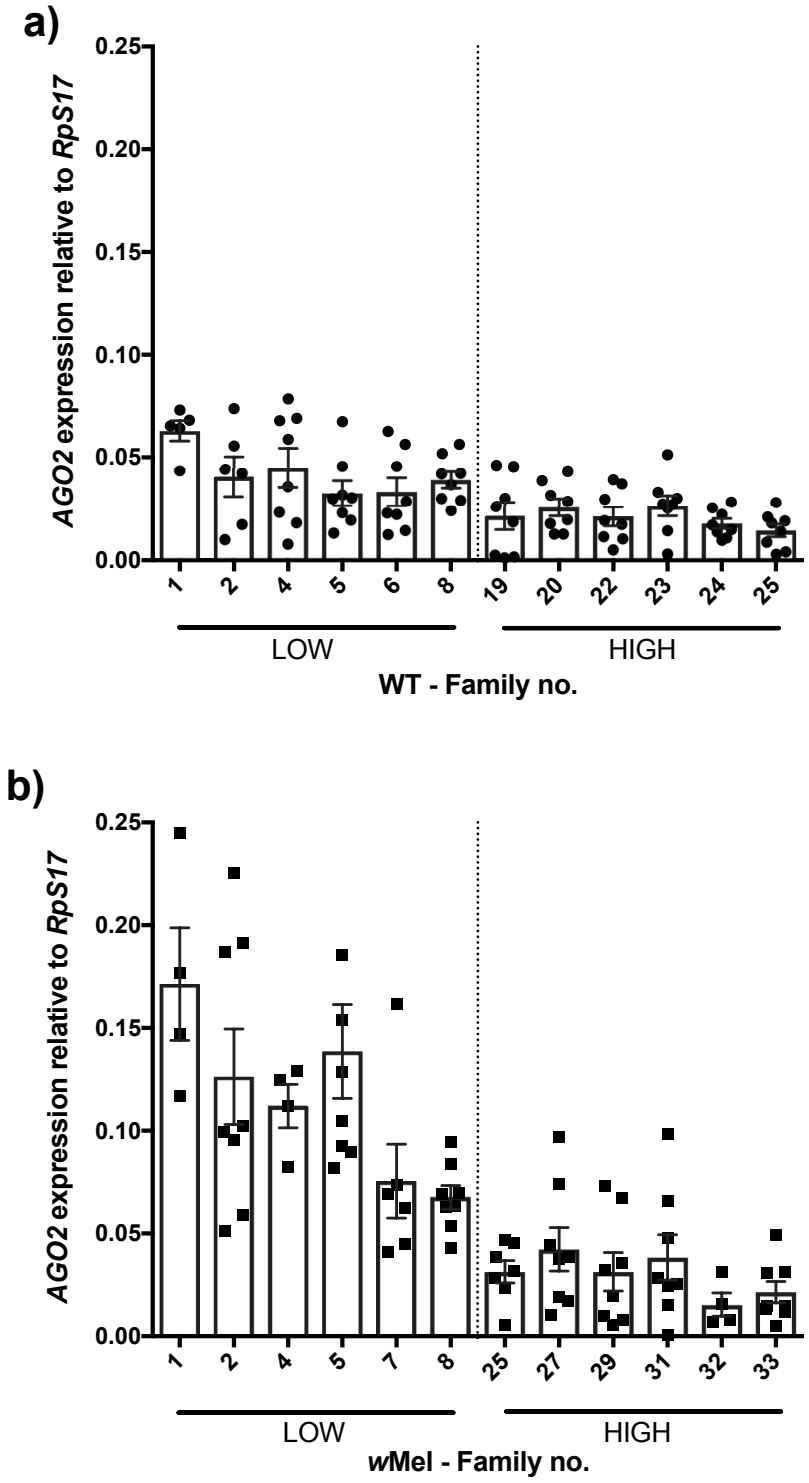

Figure 5. – *AGO2* expression in families classified as High and Low DENV. Graphs show the expression of *AGO2* relative to the housekeeping gene *RpS17* in **(a)** WT individuals, filled circle and **(b)** *w*Mel-infected individuals, filled square. Means with SEM (n=8).

Candidate gene expression - host factor competition

We also examined how genes involved in intracellular lipid transport (*Sterol carrier protein 2*, *SCP-2*) and nitric oxide biosynthesis (*Nitric oxide synthase*, *NOS*) are differentially expressed for each cell line and cluster. These genes have previously been proposed as not only important for lipid distribution or nitrogen production but also to be critical for DENV infection in *Ae. aegypti* [63,64]. The bacterium and the virus are hypothetically competing for host nutrients and thus providing the host with a *Wolbachia*-mediated blocking phenotype.

The effect of *Wolbachia* infection on *SCP-2* expression was significant (Figure 6; F=5.01, df=1, p=0.035), with *SCP-2* expression slightly downregulated in *w*Mel mosquitoes relative to WT. We also see a significant DENV load effect on gene expression (Figure 6; F=64.91, df=1, p<0.001). In this case, contrary to what we see in *AGO2* expression, *SCP-2* levels are higher in those individuals clustered into High DENV Load for both WT and *w*Mel-infected mosquitoes and hence the interaction was not significant (Figure S4c; F=1.5, df=21, p=0.087). This suggests that while *SCP-2* may be a contributing factor to viral success in mosquitoes, its expression is not associated with variation in *w*Mel-mediated blocking.

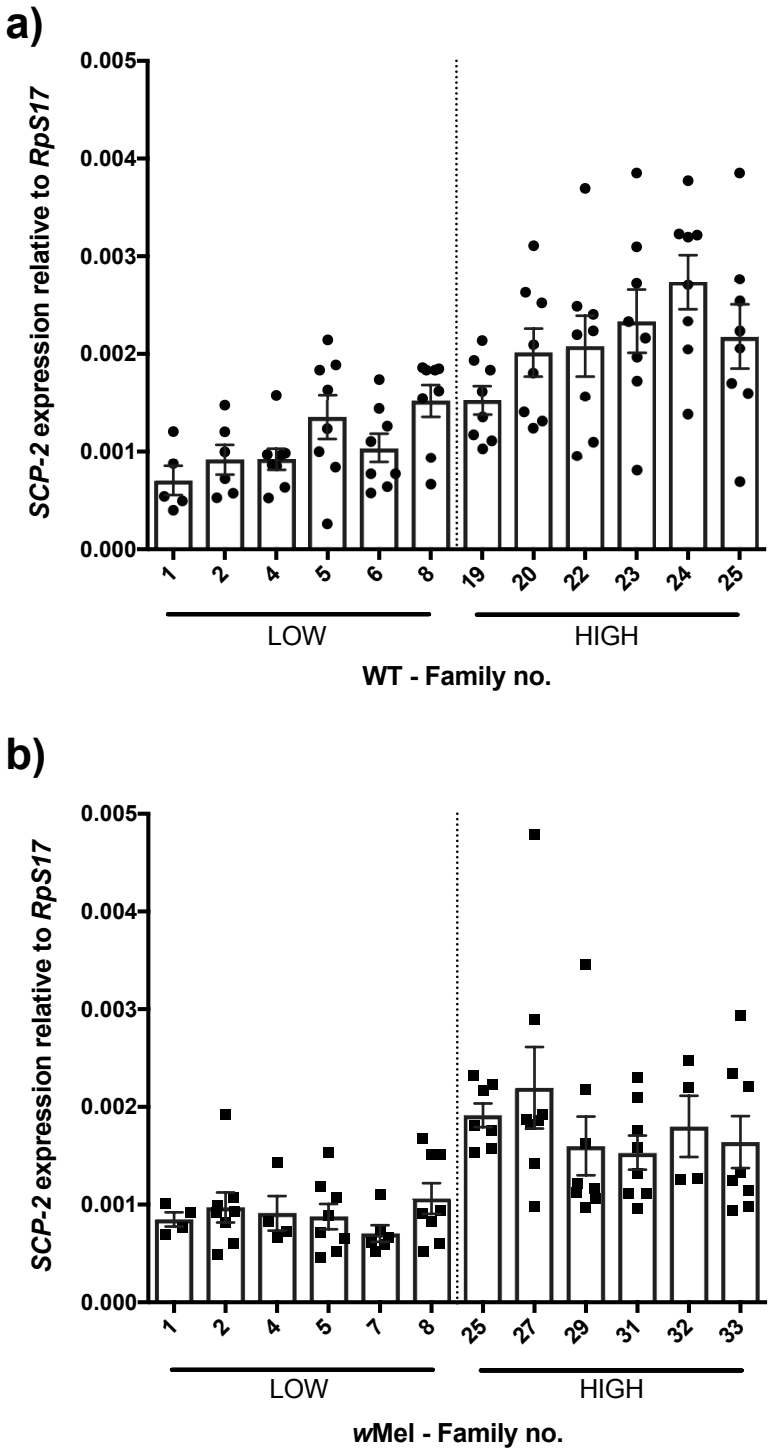

Figure 6. – *SCP-2* expression in families classified as High and Low DENV. Graphs show the expression of *SCP-2* relative to the housekeeping gene *RpS17* in **(a)** WT individuals, filled circle and **(b)** *w*Mel-infected individuals, filled square. Means with SEM (n=8).

For *NOS*, neither *Wolbachia* infection (Figure 7; F=0.48, df=1, p=0.491) nor DENV load (Figure 7, F=1.3, df=1, p=0.267) had an effect on the gene's expression. However, the interaction was significant (Figure 7, F=3.73, df=21, p<0.001). The nature of the interaction is difficult to interpret given the high level of variation in expression between families particularly for the WT line (Figure 7, Figure S4d). These data would suggest that *NOS* expression is unlikely to be associated with *Wolbachia*-mediated blocking.

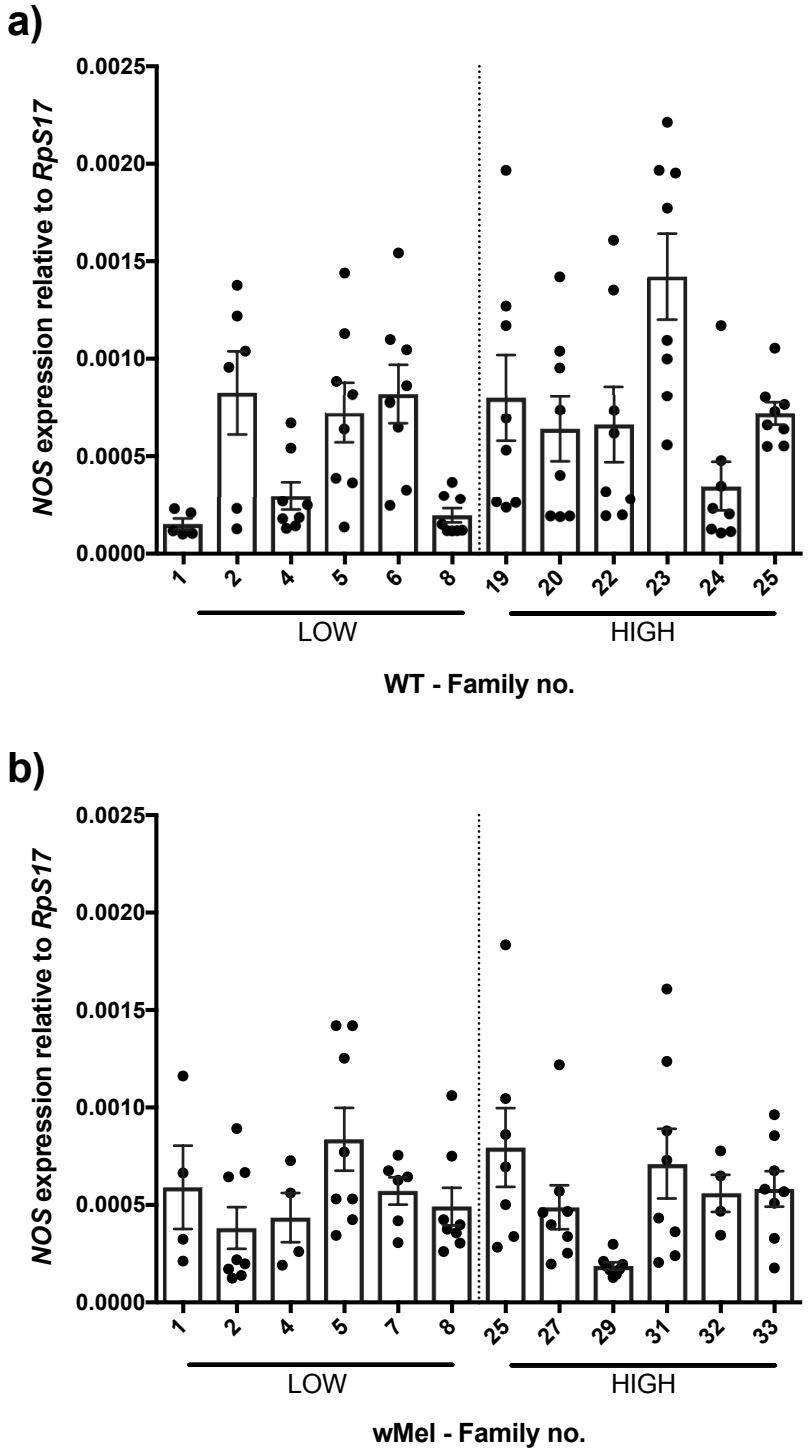

Figure 7. – *NOS* expression in families classified as High and Low DENV. Graphs show the expression of *NOS* relative to the housekeeping gene *RpS17* in **(a)** WT individuals, filled circles and **(b)** *w*Mel-infected individuals, filled squares. Means with SEM (n=8).

Discussion

In this study we aimed to measure family level variation present in the *Wolbachia*-mediated pathogen blocking trait in mosquitoes infected with the *w*Mel strain. To do so, we performed a modified full-sib breeding design that allowed us limit the contribution of environmental variation to the trait but not completely remove it given *Wolbachia's* maternal inheritance. We were then able to use families representing the phenotypic extremes in blocking to test for correlations in gene expression for a number of candidate genes for the basis of the trait.

The experiments demonstrate that there is greater variation in DENV loads in the *w*Mel-infected mosquitoes compared to wildtype mosquitoes. The DENV loads in extreme families of *w*Mel mosquitoes spanned 45-fold compared to the 5-fold difference seen for WT. DENV infection success in WT mosquitoes is highly influenced by genotype:genotype interactions between mosquito and virus [17,60,65]. The greater variation in *w*Mel mosquitoes may stem from contributions from the *Wolbachia* genome, as well environmental influences on the symbiont, confounded with family. Variation in pathogen blocking due to differences in *Wolbachia* strains has been demonstrated previously in *Drosophila* [66].

Studies that have examined phenotypic variation in blocking in both *Ae. aegypti* and *Drosophila* also show correlations between *Wolbachia* density and the strength of pathogen blocking [5,11,54,66,67]. Therefore, after determining the blocking phenotype in the families, we also examined the variation in *Wolbachia* load for the *w*Mel-infected population. We observed a high degree of variability in *Wolbachia* levels among families. Within families this measure will be confounded or inflated by *Wolbachia's* near perfect mode of vertical transmission. *Wolbachia* loads in the carcass also correlated with pathogen-blocking ability as predicted. While recent work from our group suggested that *Wolbachia* loads in particular tissues may not determine blocking strength [68], our study reaffirms the relationship for total *Wolbachia* loads.

Wolbachia is currently being assessed for its capacity to limit dengue virus transmission from mosquitoes to humans in the field [12,69,70]. The long-term efficacy of *Wolbachia* is not only reliant on the effective spread of the symbiont in the population but also dependent on the stability of expression of the blocking trait. Understanding how much variation and in particular genetic variation there is for blocking and *Wolbachia* load is critical. This is because populations can only adapt and change if there is genetic variation present for the trait of interest [71]. Blocking may be expected to vary across genetically diverse mosquito populations, in response to diverse viruses, over a range of environmental conditions and with sufficient coevolutionary time in response to diverging *Wolbachia* strains. Given genetic variation in both host and symbiont we may be able to predict the outcome of coevolutionary pressures. Interestingly, during a two-year period surveyed after release of the *Wolbachia* strain *w*Mel into wild populations, neither host longevity or DENV blocking showed evidence of change [23,72].

First, if high densities of *Wolbachia* confer better blocking but those densities are detrimental to the host, we may expect selection for reduced loads or lowered maternal transmission rates. The detriment to the host may come from the costs of producing an immune response [73] or supporting a symbiont with complex metabolic needs [46,74]. Additionally, there may be direct effects of damage on infected cells and tissues. The extreme form of this is demonstrated by the *w*MelPop strain [75] that overgrows inside host cells and causes cell lysis, the result being shortened lifespan. While the other strains of *Wolbachia* being developed for biocontrol, *w*Mel and *w*AlB, do not appear to cause cellular destruction, they still induce an immune response and spend a portion of their cellular resources on *Wolbachia* [22,39]. In the laboratory, these effects do not appear to have substantial impacts on the insect's reproductive output [72,76]. Lastly, modelling has demonstrated that even with some negative fitness costs, the high maternal transmission and CI of *Wolbachia* will help it remain in populations [72].

Second, the impact of viral and other infectious agents on the insect may select for stronger blocking. Flaviviral infections can result in fitness costs for the mosquito; in the case of DENV, both reduced fertility and lifespan are affected [77]. *Wolbachia*-mediated blocking would attenuate these potential fitness costs associated to a high viral infection, as infection rates are lower in *Wolbachia*-infected mosquitoes and for those that become infected, severity is reduced [23]. Therefore, selection pressure for the blocking trait would be greater in areas with a high incidence of DENV and other flaviviruses. Additionally, there may be protection of native viruses [78] although it is unclear what impact these viruses have on host fitness if any. In *D. melanogaster*, the symbiont does not appear to affect native viral diversity at all [79]. Lastly, *Wolbachia* could protect against systemic bacterial, fungal or other parasitic infections, encountered by insects in the field, the nature of which are very poorly understood.

Using our extreme families with respect to DENV blocking, we were able to test for correlations for several candidate genes for the mechanism of blocking. Gene expression is highly plastic and if the blocking trait was reliant on *Wolbachia*-mediated modulation of some genes, the phenotype of the trait could vary rapidly due to coevolution between *Wolbachia* and the mosquito [80]. Gene modulation in response to the symbiont is likely to be reduced greatly over time if the differences between novelly and natively infected hosts are predictive. For example, in *Drosophila* with long standing *Wolbachia* associations, the immune response is negligible [81]. We assessed genes involved in the humoral responses (*vir-1* and *AGO2*), intracellular lipid transport (*SCP-2*) and nitrogen production (*NOS*). Interestingly, *AGO2* and *SCP2* showed a correlation between their levels of expression and DENV load, which reaffirms that they play a role in the viral infection. However, neither are sufficient to explain *Wolbachia*-mediated blocking of DENV infection [44]. The JAK/STAT effector *vir-1* and *NOS* however, did not have patterns of expression related to strength of pathogen blocking trait. These data are in keeping with other studies [39,53], suggesting that the immune response to *Wolbachia*, particularly present in novelly infected hosts, cannot explain a significant portion of blocking.

Several aspects of the study may limit its interpretation. As detailed above the inheritance pattern of *Wolbachia* limits our ability to fully partition environmental and genetic variances. It also leads to correlations between DENV and *Wolbachia* loads in families. Regardless, the approach was able to limit the contribution of environmental influences by controlled breeding and infection of mosquitoes. Additionally, the approach used viral microinjection to infect mosquitoes due to the constraints of bloodfeeding compliance and difficulties with obtaining disseminated infections in *w*Mel-infected mosquitoes due to pathogen blocking. This method will not capture any of the variation in the trait associated with the midgut as it is bypassed by injection. However there is little evidence of strong W*olbachia* loads in the midgut [68] and it is not clear if this tissue contributes heavily to blocking. Also, we tested for DENV load at a single time point post injection. Blocking phenotypes may vary with time, as would gene expression profiles [82]. It is plausible, for example, that gene expression levels for the candidate genes peak immediately after bloodfeeding or exposure to the virus but decrease as soon as infection is established and viral replication promoted.

In this study we demonstrated substantial variation in *Wolbachia*-mediated DENV blocking in mosquitoes that may spring from genetic contributions from both partners and environmental influences on *Wolbachia*, not controlled by family breeding. This suggests that the *Wolbachia*-mediated blocking may have the opportunity to evolve through time or be expressed differentially across environments. The long-term efficacy of *Wolbachia* as a biocontrol tool will be dependent on the stability of blocking. We suggest the use of genome wide association studies to identify candidate genes that affect blocking. While the confounding of *Wolbachia* inheritance and environmental factors may lead to higher numbers of false positives, further functional testing using genetic modification would allow the isolation of key loci. Such broad genomic approaches offer the best means for identifying candidate pathways in the mosquito and *Wolbachia* without any *a priori* assumptions about how blocking might work. Understanding the mechanism of blocking will be necessary for the successful development of strategies [83] to counter the emergence of evolved resistance or variation in its expression under diverse conditions.

Materials and Methods

Ethics statement

The ET300 DENV strain used in the study was received from researchers associated to both University of Queensland and Queensland Health, Australia. IRB approval was obtained from UQ. Patient data was anonymised by QH.

The Monash University Human Research Ethics Committee gave ethical approval (permit CF11/0766-2011000387) for the experimental research. Human volunteer bloodfeeders were provided and agreed upon written informed consent prior to the study.

Mosquito collection

All *Ae. aegypti* mosquitoes collected from the field were identified by morphology and later checked by qPCR primer detection [12]. Two *Ae. aegypti* mosquito lines were used in this study: wildtype (WT) and *Wolbachia*-infected (*w*Mel). WT are naturally *Wolbachia* free and their eggs were collected outside the Eliminate Dengue *Wolbachia* release zone [12] in greater Cairns, Australia; whereas eggs from the transinfected line *w*Mel were collected from inside the same *Wolbachia* release zone and reared in the lab for 13 generations prior to the start of this study. Both lines were screened for presence/absence of *Wolbachia* infection using the same qPCR methods. At every generation, *w*Mel females were backcrossed to 20% uninfected WT males within 3 generations of the field to limit differences in genetic background while maintaining *Wolbachia* infection [24].

Mosquito rearing and family design

A modified full-sib [58,59] breeding design was performed independently in WT and *w*Mel *Ae. aegypti* mosquitoes. After synchronized egg hatching, mosquitoes were reared at a density of ~150 larvae in 30 x 40 x 8cm trays containing 3L of RO water. Rearing was performed under controlled conditions of temperature (26±2°C), humidity (~70%) and photoperiod (12:12, light:dark). Larvae were fed fish food (Tetramin®, Melle, Germany).

After pupation, males and females were sexed and transferred separately to 30 x 30 x 30cm cages to allow eclosion at a density of ~450 individuals/cage. Adult mosquitoes were fed a 10% sucrose water diet. Six to eight day old adult females (P1) were group fed on human volunteers. A total of 250 isofemale pairs containing a male and a bloodfed virgin female were placed in small housings. Eggs laid by isofemales on moist filter paper were collected every two days and dried uniformly for short-term storage. We chose families that produced more than 25 eggs that did not suffer from desiccation. F1 individuals from each family were hatched in deoxygenated water and interbred to increase the population number in F2. The experiment was performed using 25 WT and 33 *w*Mel independent families that produced sufficient numbers of eggs.

Virus

All experiments were carried out with a dengue virus serotype 2 strain (DENV-2, ET300) isolated from human serum collected from patients from East Timor in 2000. Virus was propagated in cell culture as described previously [84] before any experimental use. C6/36 cells were grown in RPMI 1640 media (Life Technologies, Carlsbad, CA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies), 1% Glutamax (Life Technologies) and 25 mM HEPES (Sigma-Aldrich, St. Louis, MO). Cells were maintained in a non-humidified incubator at 25ºC. Prior to injection, C6/36 cells were grown to 70-80% confluence and ET300 infective virions were allowed to attach to the cells for 2h, washed and then maintained in 2% FBS media. Virus was harvested at 7 dpi by collecting the cell culture supernatant before centrifugation at 3200 rpm for 15 minutes at 4ºC. Viral stocks were stored in individual aliquots at -80ºC until further use and titrated after using plaque assays.

Intrathoracic microinjections

DENV infected blood was injected to ensure uniformity of dosage that cannot be obtained by blood feeding. *Ae. aegypti* females were briefly anesthetized with CO_2 and DENV was injected under a microscope using a pulled glass

capillary with a manual microinjector (Nanoject II, Drummond Sci.). 69 µl of diluted virus stock (~70 DENV pfu) were delivered intrathoracically into every *Ae. aegypti* female. After injection, mosquitoes were maintained under identical initial controlled conditions at 25ºC with 60% relative humidity, 12h light/dark cycle and feeding on a 10% sucrose solution.

Dissection of tissues

At 7-8 dpi, females were knocked down via CO_2 and dissected in 1x phosphate buffered saline (PBS). Head, midguts and carcasses were dissected for 5-15 females per family. Dissecting needles were soaked in 80% ethanol between individual dissections to limit contamination. Different sets of needles were used for WT and *w*Mel dissections. Dissected tissues were immersed in 200 µl of TRIzol (Invitrogen) in a 1.5ml tube containing a 3-mm glass bead (Merck KGaA, Darmstadt, Germany). Dissected samples were immediately placed on ice, lysed using a mini-beadbeater (BioSpec Products, Bartlesville, OK, USA), snap frozen and stored at -80°C until further processing. Any remaining injected mosquitoes per family were collected, frozen and stored at -80°C as whole insects.

RNA/DNA extractions

Head and carcass samples were extracted using the manufacturer's protocol for TRIzol reagent (Invitrogen). Both DNA and RNA phases were collected. RNA was quantified using a Synergy™ MX microplate reader (Biotek, Winooski, VT, USA). All RNA samples were normalized by diluting to an even concentration of 10ng/µl prior to analysis. Genomic DNA was stored at -80ºC, until subsequent extraction with back extraction buffer (4M guanidine thiocyanate + 50mM sodium citrate + 1M Tris pH=8) according to the manufacter's guidelines for Trizol (Invitrogen).

DENV qRT-PCR and analysis

All qPCR assays were run on a LightCycler480 Instrument (Roche Applied Science, Switzerland). One-step quantitative RT-PCR (qRT-PCR) to detect DENV titres was performed using TaqMan® Fast Virus 1-step Master Mix

(Roche Applied Science, Switzerland) in a total of 10 µl, following manufacturer's instructions. Standards and samples were run in duplicate. Primer sequences used for DENV detection can be found in Table S1. DENV qRT-PCR reactions were performed and run as described previously [44]. The number of viral copies present in each sample was evaluated using known standards [5]. The used standards ranged from 10^8 to 10 DENV fragment copies. The limit of detection was set at 100 copies as virus was consistently detected at this level. Concentration of DENV in each sample was extrapolated from the standard curve and back calculated to DENV copies/µg of total RNA.

Analysis of genetic variance

Genetic variance and subsequent broad-sense heritabilities (H^2) for the focal traits (DENV and *Wolbachia* load) were estimated using a modified full-sib breeding design and the following random effects linear model;

$$z_{ij} = f_i + \varepsilon_{ij} \qquad (1)$$

where z_{ij} is the trait value for the jth female from the ith family, f_i is the random effect of the ith family and ε_{ij} is the unexplained error. To test whether genetic variance was greater than zero, model (1) was compared to a reduced model that had the family term omitted. A likelihood ratio test was constructed where twice the difference in log likelihood between the full and reduced models was contrasted with a Chi Squared distribution with one degree of freedom [85]. All models were fit using SAS version 9.3 (SAS Institute, Cary, NC) separately on the wildtype and *w*Mel-infected groups. Broad-sense heritability was calculated as twice the genetic variance (σ_{family}) divided by the total phenotypic variance ($\sigma_{family} + \sigma_{error}$).

Candidate gene expression

All carcass samples were reverse transcribed from RNA to cDNA using the SuperScript® III Reverse Transcriptase kit (Invitrogen) containing 12.5µl of RNA template, 1µl of random primers (RP, 125ng/µl), 1µl of deoxynucleotides (dNTPs, 2.5mM), dithiothreitol (DTT), 5X buffer and enzyme as per kit instructions, totaling a volume of 20µl. cDNA synthesis was performed in a

C1000™ Thermal Cycler (Bio-Rad) on the following temperature profile: 5' at 65°C followed by 10' at 25°C, 50' at 50°C, 10' at 75°C and kept at 4ºC. Gene expression levels were estimated using the SYBR® Green I Master (Roche) with 1μl of the previously synthesized cDNA, following manufacturer's instructions. All CT values were normalized to the housekeeping *Ae. aegypti RpS17* gene [86], whose expression was consistent in different samples and mosquito lines. Expression ratios were obtained using the ΔΔCt method [87]. All primers for candidates genes are listed in Table S1.

Wolbachia quantification

Wolbachia carcass densities were quantified after DNA extraction using a set of *w*Mel-specific primers amplifying for the IS5 repeat element [88]. TaqMan® multiplex qPCR was carried out following manufacturer's protocol (Roche Applied Science, Switzerland). The primers used can be found in Table S1. *Wolbachia* to *RpS17* housekeeping ratios were calculated using the ΔΔCt method [87].

Statistics and Data analysis

All qPCR reactions throughout the study were run in duplicate and samples that failed to amplify both times were discarded as negative. Gene expression data were analysed using a generalized mixed model with a random factor 'Family' nested with *Wolbachia**DENV load, with both '*Wolbachia'* and 'DENV load' set as fixed factors. Statistics were performed using IBM SPSS Statistics (v23) and GraphPad Prism 6.

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Supplementary Information (in order of appearance)

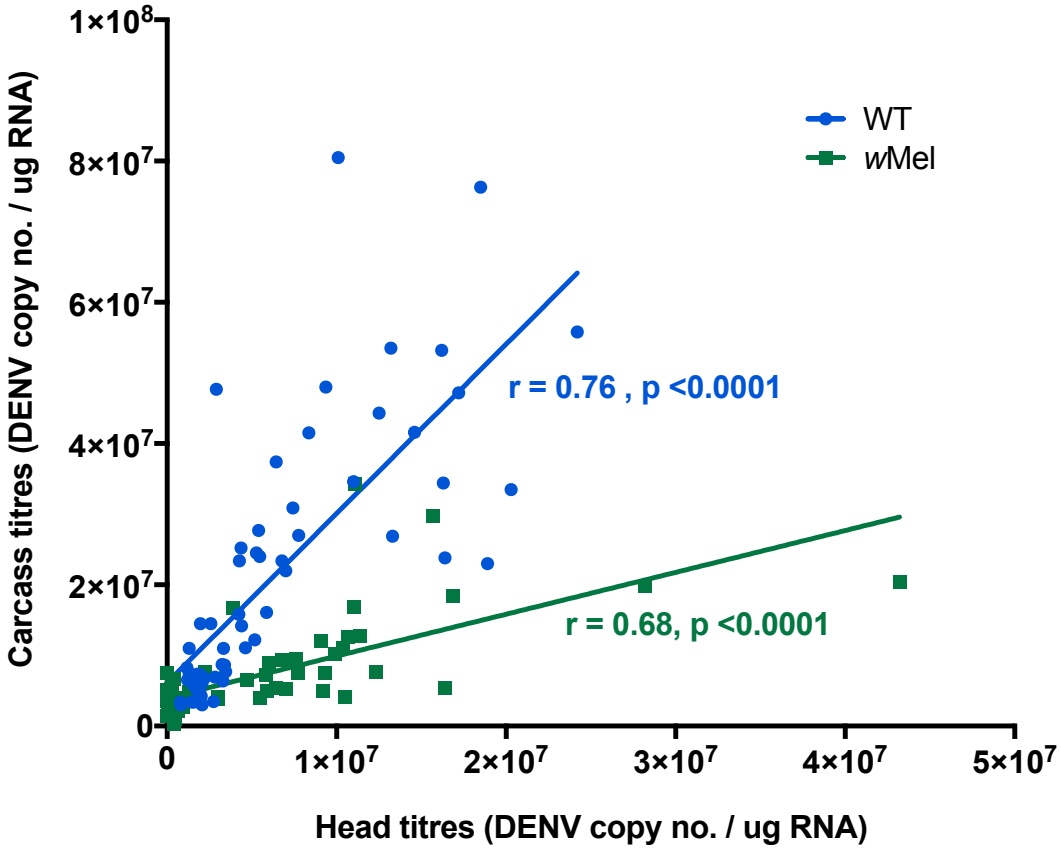

Figure S1. – Head DENV loads correlate with Carcass DENV loads. DENV loads in the head were directly correlated to the same individual's DENV loads in the carcass using Pearson's correlation. Each dot depicts an individual either WT (blue, filled circles) or *w*Mel-infected (green, filled squares).

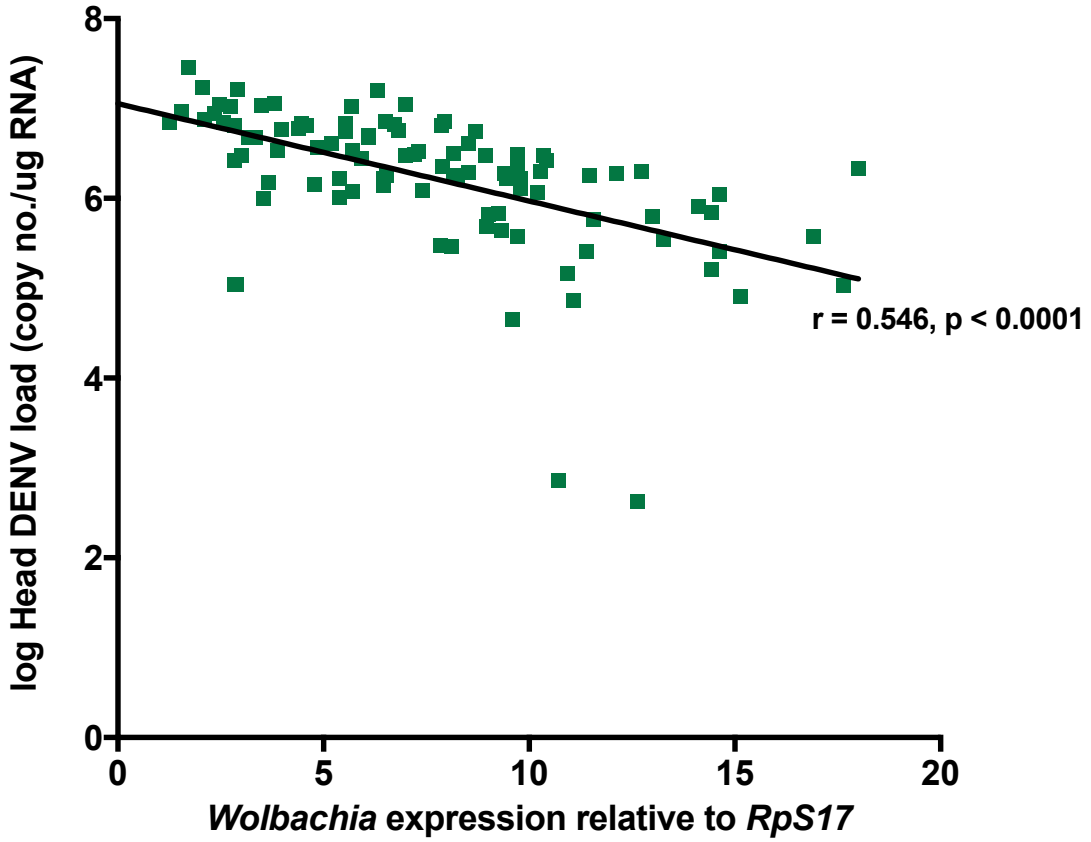

Figure S2. – Head DENV loads negatively correlate with *Wolbachia* loads. Individual DENV loads were directly correlated to the same individual's *Wolbachia* loads using Pearson's correlation. Each square depicts an individual.

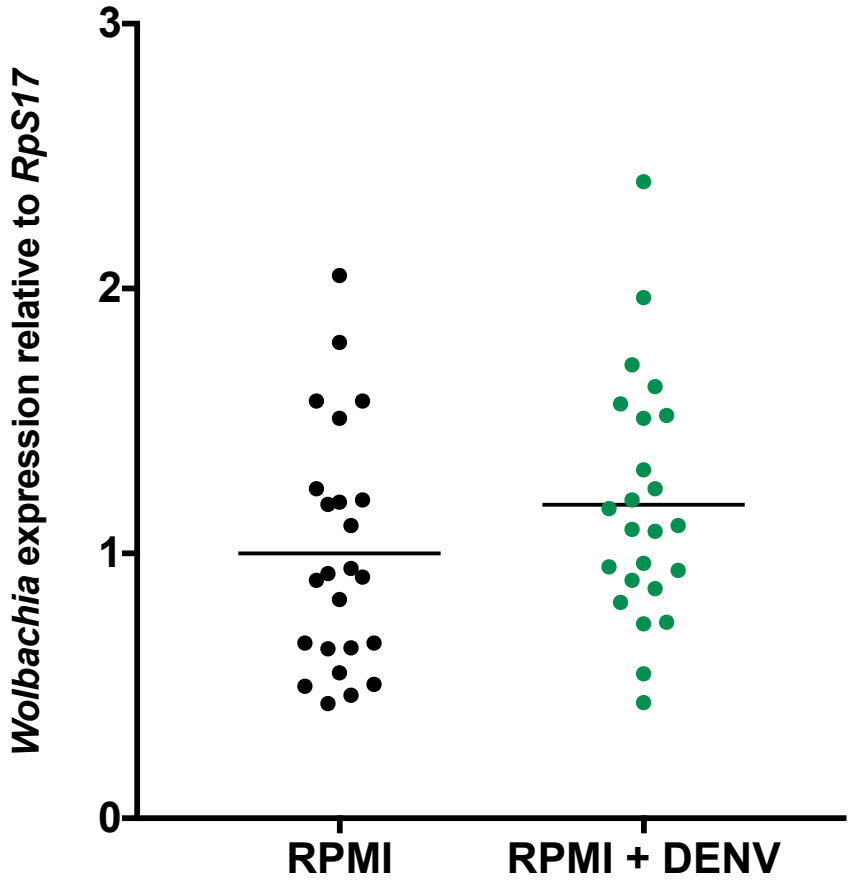

Figure S3. – *Wolbachia* loads after viral injection. No significant differences in *Wolbachia* levels were observed between media-injected mosquitoes (black) and virus-injected mosquitoes (green).

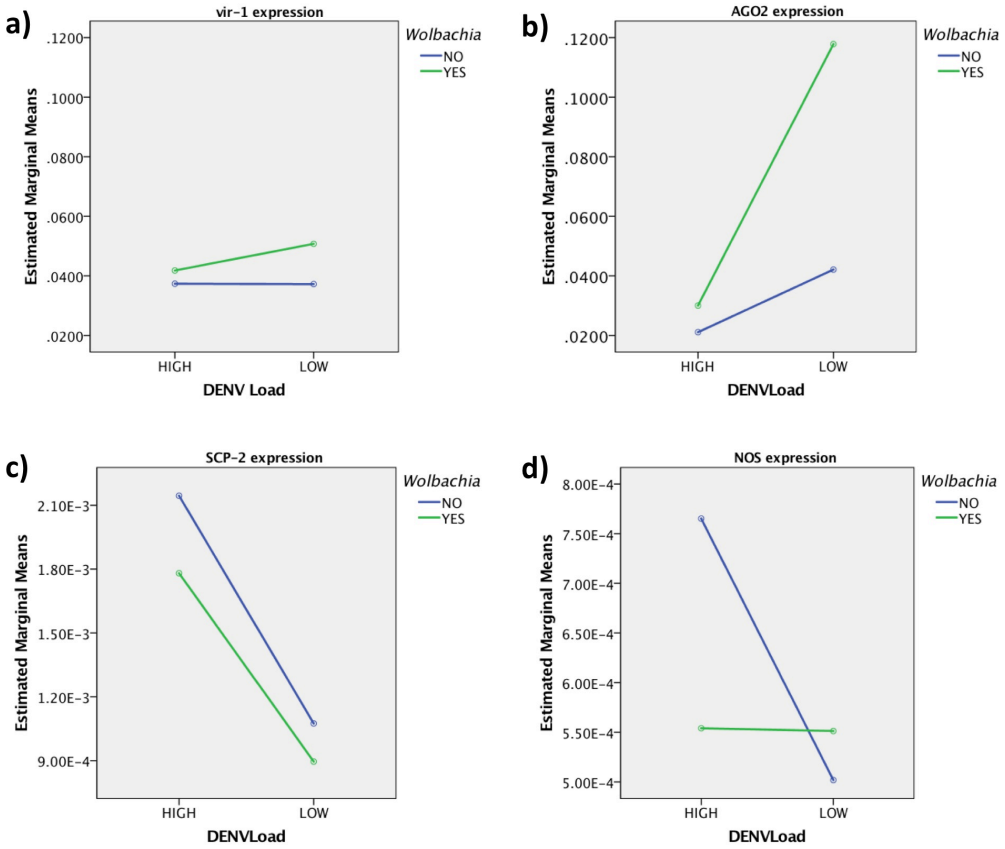

Figure S4. – Interaction plots on the expression of the four tested genes. Parallel lines show no-interaction occurring between main effects DENV Load (High/Low) and *Wolbachia* (+/-) **(a,c)**. Non-parallel lines show strong interaction **(b,d)**.

Table S1. – Primers and probes used for qPCR gene detection. Gene IDs taken from VectorBase and UniProt.

Gene ID	*Aedes* gene name	Direction	Sequence (5'-3')	Gene function
AAEL000718	*vir-1*	Fw	GCCAAAGTCCGGTATTCTTC	Antiviral effector
		Rv	TTCACGAGATCGTCAAGGTAA	
AAEL017251	*argonaute-2*	Fw	CCGTTCTGGACATGACTTGC	Catalytic compound of RISC, cleavage of mRNA
		Rv	CACAGCTCATGGTTGCTTCC	
AY190283.1	*Sterol Carrier Protein 2*	Fw	AAGTTTTCGCCAAGATCGCT	Non-specific lipid absorption and mobilization
		Rv	TTCTTGACAACCTTGCCACC	
AAEL009745	*Nitric oxide synthase*	Fw	AGGTTCGCAGTGTTTGCTCT	Nitric oxide synthesis / immune signalling
		Rv	GGTGCCCATTTCCTAAAAGC	
AAEL004175	*RpS17*	Fw	TCCGTGGTATCTCCATCAAGCT	Ribosomal small subunit assembly
		Rv	CACTTCCGGCACGTAGTTGTC	
		Pr	FAM-CAGGAGGAGGAACGTGAGCGCAG-BHQ1	
NC_001474.2	DENV	Fw	AAGGACTAGAGGTTAGAGGAGACCC	
		Rv	CGTTCTGTGCCTGGAATGATG	
		Pr	FAM-AACAGCATATTGACGCTGGGAGAGACCAGA-BHQ1	
AE017196	*Wolbachia*	Fw	GTATCCAACAGATCTAAGC	*w*Mel IS5 repeat
		Rv	ATAACCCTACTCATAGCTAG	
		Pr	LC640-TGAAATGGAAAAATTGGCGAGGTGTAGG-BHQ2	

CHAPTER FOUR

From transcriptomics to functionality: What can be learned using genetic variation in dengue virus vector competence in *Aedes aegypti*

This thesis chapter has been submitted for publication to PLoS Neglected Tropical Diseases in July 2017.

Abstract

Genetic modification techniques such as CRISPR/Cas9 or TALENs have been developed in the recent years for a range of insect vectors. These approaches could theoretically be used to engineer mosquitoes that are resistant to infection with a range of vector-borne pathogens. Prior to genetic modification, however, promising gene candidates have to be identified in the insect. In the primary vector of dengue virus, *Aedes aegypti*, traditional quantitative genetics approaches have been used to reveal a handful of genetic variants required for pathogen infection, particularly associated with the insect's midgut barrier. More recently, transcriptomic profiling has generated extensive lists of mosquito genes that respond to viral infection across diverse insect tissues and over a time course post infection. These gene lists contain two types of genes; those that are responsible for the insect's natural antiviral defense mechanisms, including some known innate immunity genes, as well as genes whose change in expression may occur as a by-product of infection. Candidates in the former category are of most interest for genetic modification. Here we have utilized natural genetic variation in *Ae. aegypti* refractoriness for dengue infection to test whether candidate genes from transcriptional studies are likely to be of functional importance for viral control. Using a modified full sib breeding design we were able to categorize mosquito families for their level of refractoriness. In families representing the phenotypic extremes in terms of viral load we tested for correlations in gene expression with 25 candidate genes.

Our approach was able to exclude roughly half of the candidate genes studied and hence provide a focused set of candidates worthy of progression to functional testing and potentially to genetic modification.

Author Summary

New techniques are emerging to allow the genetic modification of mosquito vectors of human disease with the aim of limiting their ability to transmit pathogens. To do so, researchers must identify key genes in the mosquito genome that control the insect's susceptibility to viruses like dengue and Zika. Gene candidates are commonly identified by taking a snapshot of all genes expressed in the mosquito in response to viral infection. However not all of these genes will be important for viral control, some will just represent the physiological response of the mosquito. Here we use mosquitoes that exhibit genetic variation in susceptibility to dengue virus to test whether a range of candidate genes are important for dengue control. Our approach excluded approximately half of the candidates tested. The remaining list of genes represent good candidates to be progressed for genetic modification with the goal of producing dengue refractory mosquitoes.

Introduction

In the last decade, technologies have been developed that improve the speed and specificity of genome editing including zinc finger nucleases and TALENS [1,2]. More recently, the emergence of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) approaches has further improved both the speed of production and the stability of genetic constructs [3-5]. CRISPR/Cas9 is a system native to prokaryotes that confers adaptive immunity to bacteria against foreign elements. It operates through the production of small RNA guides that promote cleavage of foreign DNA by Cas nucleases (reviewed in [6]). By introducing a construct consisting of Cas9 and a guide RNA complementary to the sequence of interest in any species, genes can be targeted and the Cas9 enzyme will create breaks in both strands. One of the system's main strengths is this ability to direct Cas' endonuclease activity to a specific DNA sequence, allowing for suppression of a gene or introduction of desired mutations through the host's DNA repair machinery.

CRISPR/Cas9-mediated gene approaches are currently being developed for a range of insect vectors [7-9]. In these insects, CRISPR is being used as a tool to test candidate gene function [10,11], but also as possible means for engineering pathogen refractory vectors [7,12]. Theoretically, genetic modification of species such as *Ae. aegypti* could produce mosquitoes that are resistant or with a reduced ability to transmit viruses like dengue (DENV) and Zika (ZIKV). Obtaining short lists of promising mosquito candidate genes that control vector competence of pathogens has become increasingly important. This is challenging, however, in non-model species where the genome is sequenced but not fully annotated, as is the case with *Ae. aegypti* [13]. The identification of genes associated with refractoriness is made more difficult by the potential for GxG interactions between the pathogen and mosquito genotypes [14,15], leading to variation between studies.

Early approaches involved the detection of quantitative trait loci (QTL) that underpin the vector's response to arboviral infection primarily by comparing mosquito lines that differ for their DENV susceptibility [16-18]. Despite *Ae. aegypti* containing a low abundance of microsatellites [19], a large number of QTL have been detected that correspond to polymorphisms in regions important for DENV infections [16,20,21]. However, the experimental approaches involved are labor intensive, require large samples sizes and commonly focused on testing for mosquitoes that limited or prevented arboviruses from infecting or escaping the midgut for the ease of execution. Therefore, most of the loci identified via this approach relate to midgut function [16,21,22].

Transcriptomic studies, in contrast, provide a snapshot of the entirety of genes being expressed in an organism at any one time. Past transcriptomic profiles for *Ae. aegypti* in response to DENV infection have examined responses in particular tissues [23,24] as well as the whole animal [25] and over a range of time points post infection [23-25]. These approaches have been instrumental in characterizing the nature of the insect's inducible immune response [26]. In addition to immunity related genes and large numbers of genes with unknown functions, the profiles tend to include genes in the following classes; energy metabolism, cellular degradation, signal transduction and intracellular transport, as well as transcription regulators and other minor classes. It is difficult to disentangle whether these transcriptional changes relate to the host antiviral response, the physiological response of the vector to infection or direct modulation of host pathways by the virus. While many insect immunity pathways have been mapped it is also clear that large numbers of genes outside of these pathways function in immunity in unknown ways. It is controversial whether DENV infection harms the mosquito in terms of lifespan or reproductive success [27,28], but at the cellular level viral infection is in the very least consuming host energies and inducing host immune responses if not causing other effects [29]. Lastly, as DENV creates a cellular environment that is ideal to promote its own viral replication [30], it will be affecting host cellular physiology. Transcriptional profiles in response to DENV infection typically include genes involved in signal transduction,

apoptosis and metabolism. A small number of these genes have been further examined in more functional studies [31-33].

The number of genes responding to pathogen infection can be in the thousands [25], producing a large number of hypothetical genes that must be confirmed by more functional tests. Candidate gene testing is both time-consuming and expensive however. While the identification of genes that behave in a similar fashion across studies can assist with narrowing potential candidates, some study-based differences may limit this capacity such as tissues examined, insect age, collection point post infection, reproductive states and other environmental factors [24,34,35]. In addition, gene expression induced by infection is expected to vary by mosquito genotype [35,36]. Natural variation and its interaction with the environment drive the process of evolution and adaption [37,38]. Hence mosquito variation is a key factor in understanding vector competence through time and over geographic landscapes as well as in interactions with diverse pathogen genotypes. While genetic variability may introduce “error” into cross study comparisons, it can also be used powerfully within a study to look for relationships between infection phenotypes and expression of candidate genes.

Here we utilised natural genetic variation in *Ae. aegypti* vector competence to assess the potential involvement of candidate genes in the process of DENV infection. We first determined a key set of mosquito genes in response to DENV infection from a range of transcriptional studies [23-25]. Then using a quantitative genetic breeding design we produced mosquito families that varied in their susceptibility to DENV. Lastly, we were able to examine expression of the candidate genes in these families and ask if their expression correlated with DENV load. Some of the genes we tested already had known roles in immunity or other functions such as lipid metabolism and cell adhesion, whereas others lacked complete annotation. From these tests we were able to determine whether genes were likely to play a role in the antiviral response or if they were simply part of the insect’s response to infection. This study demonstrates a useful approach for screening potential candidate genes prior to subsequent manipulation via genetic modification approaches.

Results

DENV load classification

We performed a half-sib breeding design on an Australian population of *Ae. aegypti* to determine the nature of the genetic variation for DENV susceptibility. For approximately 25 families of mosquitoes we assessed the load of DENV serotype 2 in the head tissue (disseminated) of females 7 days post intrathoracic injection of virus (Figure 1a). We then selected a range of families representing the extremes in DENV load (4 each) and confirmed that these differences were also seen in whole body measures of DENV load in sisters from the same families (Figure 1b). We used a nested generalized linear model (GLiM) to assess differences in total DENV loads between our extreme families, where 'Family number' was nested within 'DENV load' (High or Low, in heads). We observed a significant effect of 'DENV load' (Wald=104.08, df=1, $p<0.0001$), supporting our designation of families as high or low. We also observed a 'Family within DENV load' effect (Wald=81.97, df=6, $p<0.0001$) that relates to the presence of interfamily variation for the trait, especially in the High DENV group.

After demonstrating that DENV load varied between groups, we selected a subset of 4 families each representing the phenotypic extremes of DENV load to test for associations with expression of candidate antiviral genes. Genes tested (Table 1) stem from previous transcriptomic studies, but have yet to be confirmed by further functional studies. A range of genes (roughly half of those tested) representing diverse functional classes did not exhibit patterns of expression across families that would explain differences in DENV load. Other genes while exhibiting mean expression patterns consistent with DENV control also exhibited a large amount of variation between families within a phenotypic class and hence could not be interpreted (data not shown). These genes may be highly influenced by environmental or epistatic effects. Neither of these classes of genes would represent good candidates for subsequent genetic modification. Below we present the data from genes exhibiting uniformity of response across families within the phenotypic extremes and that differed with respect to DENV load.

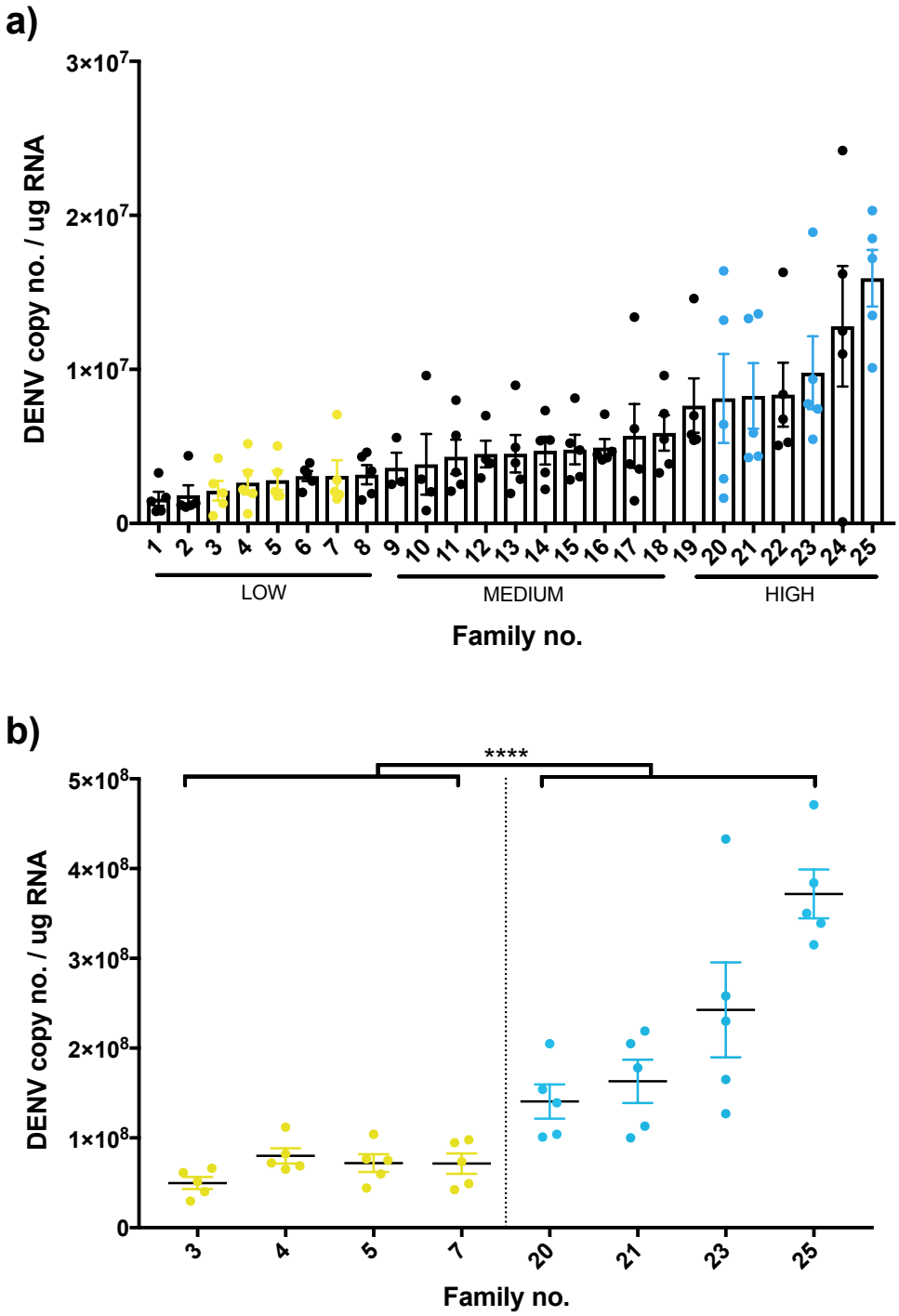

Fig.1 – Disseminated DENV loads. Wildtype DENV-infected families were classified based on head DENV loads; progressed families are highlighted in yellow or blue **(a)**. DENV phenotype was later confirmed with whole body load **(b)**. Bars depict family DENV mean and SEM.

Table 1 – Candidate genes tested and their relevance in DENV control. Accession numbers, gene names, function and patterns of expression across families with high and low DENV loads for all genes tested.

Accession number	Gene name	Transcriptomic study	Function	Direction	Differentially expressed	Interfamily variation
AAEL001022	*smp-30*	Behura 2011, Colpitts 2011	Ca^{2+} binding domain	Down	Yes	-
AAEL001156	*CG5280*	Colpitts 2011, Bonizzoni 2012	-	Up	No	Yes
AAEL001392	*defensin A*	Multiple	Immune effector	Down/Up	Yes	-
AAEL002413	*sphingomyelin*	Colpitts 2011	Cellular membrane	Down	Yes	-
AAEL002585	*CLIPA11*	Colpitts 2011, Zou 2011	Serine protease	Down	No	Yes
AAEL003619	-	Behura 2011, Colpitts 2011	Na/Cl transporter	Down	Yes	-
AAEL003787	*Nopo*	Colpitts 2011	Zinc finger	Up	Yes	-
AAEL004361	*alpha-glucosidase*	Colpitts 2011	Glycolysis	Down	Yes	-
AAEL004861	*dearingolade*	Colpitts 2011, Bonizzoni 2012	Peroxisomal integral protein	Up	No	Yes
AAEL005064	*CLIPB5*	Behura 2011, Colpitts 2011	Serine protease	Down	No	Yes
AAEL005527	*Nbr/mut-7*	Behura 2011, Colpitts 2011, Bonizzoni 2012	miRNA maturation	Down	No	Yes
AAEL006995	*CG9657*	Behura 2011, Colpitts 2011	-	Down	No	No
AAEL007495	*phosphoglycerate mutase*	Behura 2011, Colpitts 2011, Bonizzoni 2012	Glycolysis	Down	No	No
AAEL007845	*Rab5*	-	Receptor	-	Yes	-
AAEL008013	*Obp83b*	Behura 2011, Colpitts 2011	Odorant	Down	No	Yes
AAEL008108	*GB76c*	Behura 2011, Colpitts 2011, Bonizzoni 2012	Transmembrane signalling	Down	Yes	-
AAEL009317	*Rab11*	Colpitts 2011	GTPase, cellular trafficking	Up	No	No
AAEL009602	*Gdap1*	Behura 2011, Colpitts 2011, Dissanayake 2010	Mitochondrial membrane	Down	No	No
AAEL009770	*SUMOE2*	Colpitts 2011	Sumoylation	Up	Yes	-
AAEL011375	*trypsin*	Behura 2011, Colpitts 2011, Bonizzoni 2012	Serine protease	Down	Yes	-
AAEL011566	-	Behura 2011, Colpitts 2011, Dissanayake 2010	Adhesion	Down	Yes	-
AAEL011817	*rent1*	Colpitts 2011	mRNA decay	Down	No	Yes
AAEL012089	*xport-A*	Behura 2011, Colpitts 2011, Dissanayake 2010	Phototransduction	Down	No	No
AAEL013712	*Trypsin 5G1 precursor*	Brackney 2008, Colpitts 2011, Bonizzoni 2012	Serine protease	Up	No	No
AAEL014108	*aquaporin*	Behura 2011, Colpitts 2011	Transporter	Down	Yes	-

Immune genes and signalling

Host immune responses are one of the main contributors to mosquito pathogen control [26]. Successful bacteria and viruses are able to promote transcription of self-proteins that help supress key host immune responses, in order for the pathogen to replicate and proliferate freely. We evaluated a range of immune responses to elicit their involvement in viral control and how their expression differs between families. We classified immune responses in three main types; molecules that the virus use as cofactors to replicate (1), molecules involved in cell signalling (2) that in turn activate immune pathways to promote transcription of immune effectors (3).

From the first group, SUMOE2 is a protein with a range of effects on the host, whose high levels have also been linked to increased DENV loads in human cells, as the virus uses sumoylation to tag its NS5 and regulate replication via the suppression of antiviral responses [39]. In our study, the expression of the gene *AeSUMOE2* (*AAEL009770*) had a significant effect of DENV load (Figure 2a; Wald=5.34, df=1, p=0.021) and no significant difference was seen between families in each DENV group (Wald=5.68, df=6, p=0.46), suggesting that *AeSUMOE2* plays a role in DENV control. This is in keeping with the observations from previous transcriptomic studies, where a slight increase in *AeSUMOE2* expression was seen in hosts infected with DENV.

We also evaluated the contributions of two proteins that act as signalling molecules, AeGβ76C (*AAEL008108*) and a serine protease (*AAEL011375*), and one effector, defensin (*AAEL001392*). Little is known about *AeGβ76C* expression, involved in rhodopsin and signal transduction, but we detected a significant effect based on DENV load (Figure 2b; Wald=11.4, df=1, p=0.001) but not differences within families of each group (Wald=1.7, df=6, p=0.945). Similar effects are seen for *AAEL011375*, where DENV load group effect was significant (Figure 2c; Wald=9.69, df=1, p=0.002) but no effect of family within DENV load was detected (Wald=10.17, df=6, p=0.118). Both expression levels correlate with the downregulation seen in previous transcriptomic studies. The expression of *defensin*, however, does not match the modulation observed in transcriptomic profiles. We observed a significant DENV load effect (Figure 2d; Wald=21.42, df=1, p<0.0001) and no effect of Family within DENV

load (Wald=11.22, df=6, p=0.082), but the direction of the main effect is the opposite as that observed in transcriptomic studies, which suggest that *defensin* expression is downregulated by the virus, despite other functional studies showing upregulation of the immune effector in response to the viral infection [29].

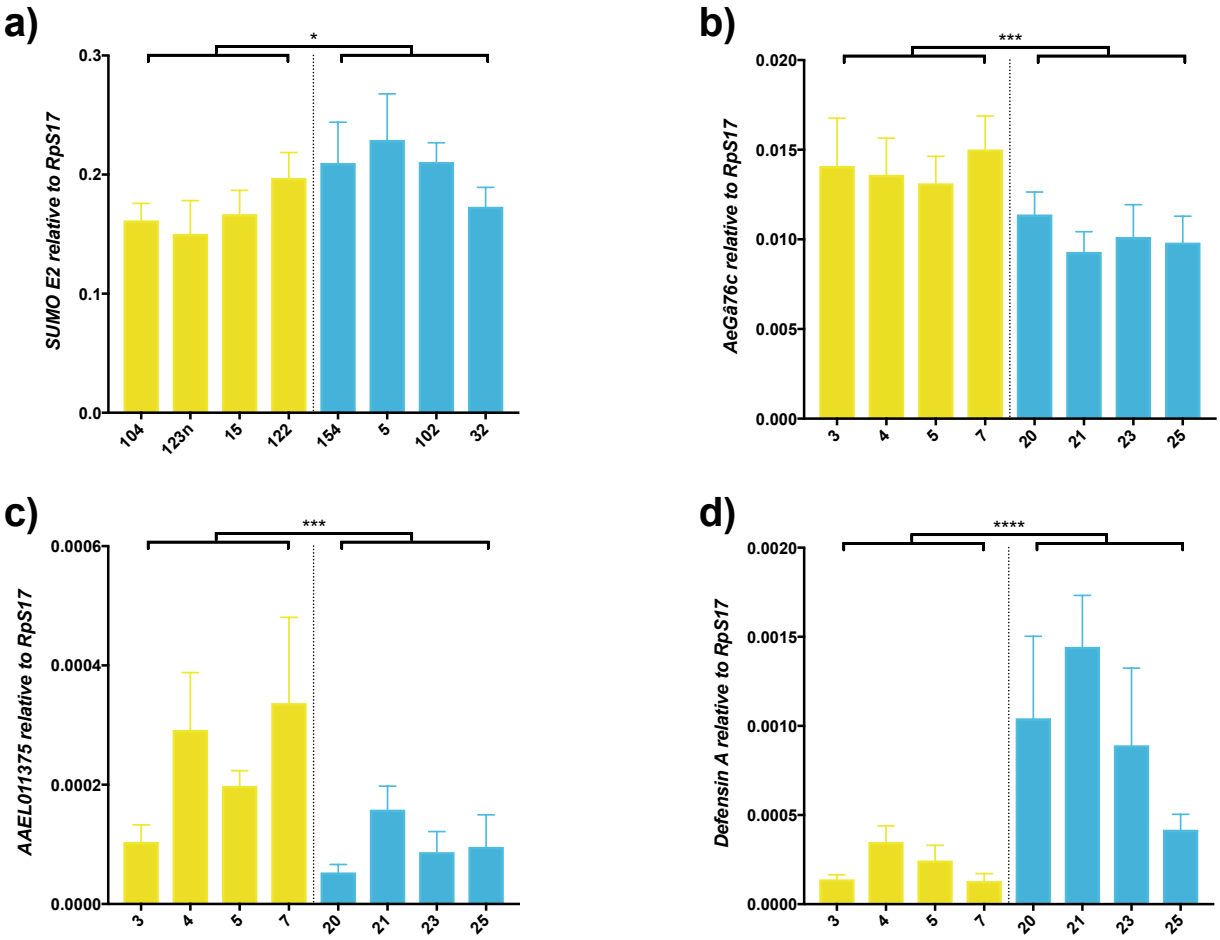

Fig.2 – Immune gene and signalling. Graphs show the expression of **(a)** *SUMOE2*, **(b)** *AeGα76C,* **(c)** *AAEL011375* and **(d)** *defensin A* relative to *RpS17* in DENV-infected individuals. Yellow bars represent refractory families, blue bars represent susceptible families. Bars depict family mean and SEM (n=5). * 0.05<p<0.01, ***0.001<0.0001, **** p<0.0001.

Apoptosis genes

Classic signalling immune pathways are not the only responses that the host mount against an incoming pathogen. Different immune pathways usually act synergistically with apoptotic responses to determine infection outcomes [25,40]. There have been previous studies that focus on the role of apoptosis-related genes and their relevance to viral control [32,41,42], where increased cellular death promotes replication. We evaluated two genes involved in the regulation of apoptosis, *AeNopo* (*AAEL003787*) and the *senescence marker protein 30* (*smp-30*, *AAEL001022*). *AeNopo* is a zinc finger domain that directly regulates caspase activity and thus its upregulation promotes cell death via activation of pro-apoptotic genes [43]. We observed a significant upregulation of *AeNopo* in highly infected families (Figure 3a; Wald=27.34, df=1, p<0.0001). The variation of the expression in families of the same DENV group was also significant (Wald=34.76, df=6, p<0.0001), suggesting that levels can vary greatly between genotypes. *smp-30* regulates cellular Ca^{2+} homeostasis and has a role in cellular protection against oxidative stress, which has been linked to DENV infection status [44]. We observed a significant downregulation of *smp-30* between DENV groups (Figure 3b; Wald=22.89, df=1, p<0.0001). The variation of the expression in families of the same DENV group was also significant (Wald=20.37, df=6, p<0.002). Both *AeNopo* and *SMP-30* data bode well with the transcriptomic patterns seen in previous studies.

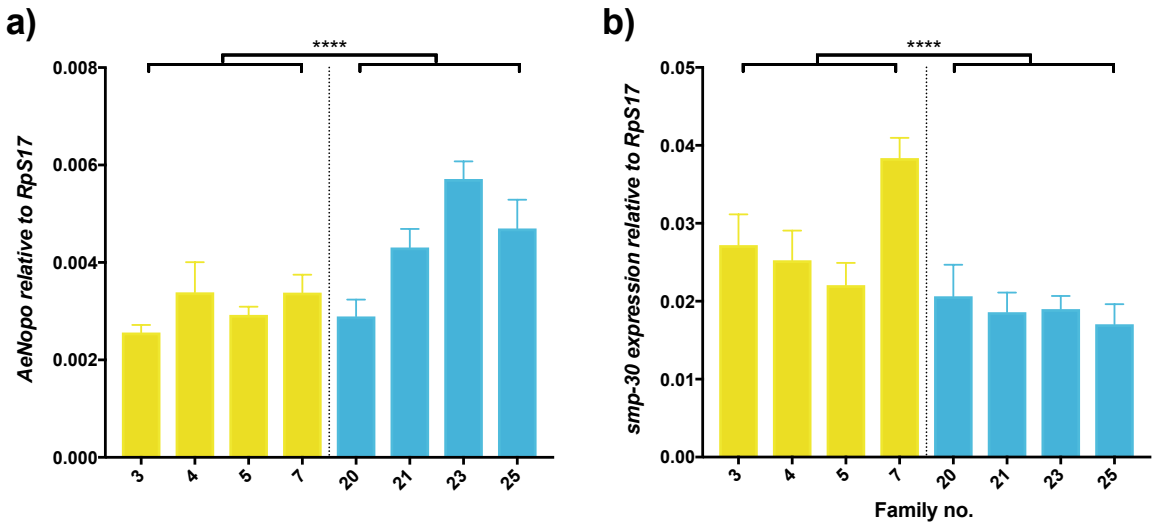

Fig.3 – Apoptosis. Graphs show the expression of **(a)** *AeNopo* and **(b)** *smp-30* relative to *RpS17* in DENV-infected individuals. Yellow bars represent refractory families, blue bars represent susceptible families. Bars depict family mean and SEM (n=5). **** p<0.0001

Metabolism genes

Another effect that DENV has on host cells is the modulation of lipid metabolism and its cellular homeostasis. This may be caused by the reliance of the virus on host structures to assemble its own replication machinery, a required modulation of membranes to facilitate viral infection or a mechanism to promote intracellular virion trafficking [30,45-47]. We analysed two different molecules involved in metabolism of lipids and sugars that were identified as downregulated in response to a DENV-infected blood meal. In concordance with transcriptomic studies, phosphoglycerate mutase (*Pglym*, *AAEL007495*) was observed to be downregulated in highly infected families (Figure 4a, Wald=17.47, df=1, p<0.0001) and so was α-glucosidase *(α-gluc*, *AAEL004361*) (Figure 4b, Wald=38.31, df=1, p<0.0001). Both genes' expression was also significant when analysing the variation between families of the same group (*Pglym*: Wald=24.27, df=6, p<0.0001; *α-gluc*: Wald=15.13, df=6, p<0.019).

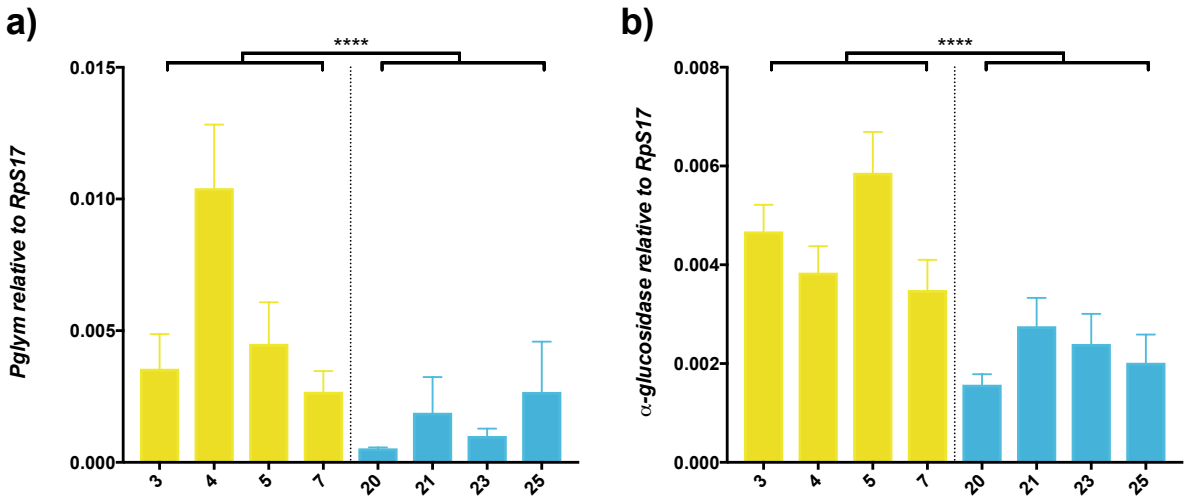

Fig.4 – Metabolism. Graphs show the expression of **(a)** *Pglym* and **(b)** *α-glucosidase* relative to *RpS17* in DENV-infected individuals. Yellow bars represent refractory families, blue bars represent susceptible families. Bars depict family mean and SEM (n=5). **** p<0.0001.

Transporter and adhesion genes

As mentioned previously, an essential component of the viral success is the attachment of the virion to the cell. After that, membrane fusion can occur and virus can start replicating inside the cytoplasm. We evaluated differences in expression for a range of intracellular transporters and molecules involved in cellular adhesion. From the latter, an uncharacterized adhesion molecule (*AAEL011566*) was highly downregulated in both transcriptomic studies on DENV infection and in the effect of bloodmeals in mosquitoes [48]. Our data supports its effects on DENV load, as its expression is downregulated in the high DENV load families (Figure 5a; Wald=10.95, df=1, p<0.0001). Variability among grouped families is also present, as expression differences within families from the same DENV load group are significant (Wald=15.9, df=6, p=0.014). Due to its relevance to viral success, a broad range of molecules involved in adhesion and endocytosis has been characterized in functional studies. Despite no modulation was seen in transcriptomic studies for *Rab5* (*AAEL007845*), an endocytic molecule, it has been previously labelled as a required component for cellular entry of arboviruses [49-51]. We investigated whether differences in expression between low and highly infected families were present at a late infection timepoint. *Rab5* expression was significantly upregulated in families belonging to the high DENV load group (Figure 5b; Wald=16.34, df=1, p<0.0001) and no differences were found among families from the same DENV load group (Wald=3, df=6, p=0.808).

We also analysed two cellular transporters, *aquaporin* (*AAEL014108*) and a putative Na/Cl-dependent amino acid transporter (*AAEL003619*). Not a lot has been investigated on the aquaporin family, transmembrane molecules that transport water and other small solutes in and out of the cell, besides its seasonal relevance and bloodmeal-induced diuresis [52,53]. Aquaporin was one of the main candidates that arose from transcriptomic studies [23,25], showing downregulation of its expression at all sampled timepoints after arboviral challenge. Similarly, we observed a significant difference in expression for the main effect of DENV load (Figure 5c; Wald=29.83, df=1, p<0.0001) but no effect of family within DENV group was present (Wald=9.46, df=6, p=0.149).

Downregulation of expression of *AAEL003619*, an amino acid transporter, may be due to the intracellular amino acid pool being used by the virus to replicate. The effect was significant for DENV load (Figure 5d; Wald=16.69, df=1, p<0.0001) and so was variation within each group, shown by the significance of the effect of family within DENV load (Wald=33.54, df=6, p<0.0001).

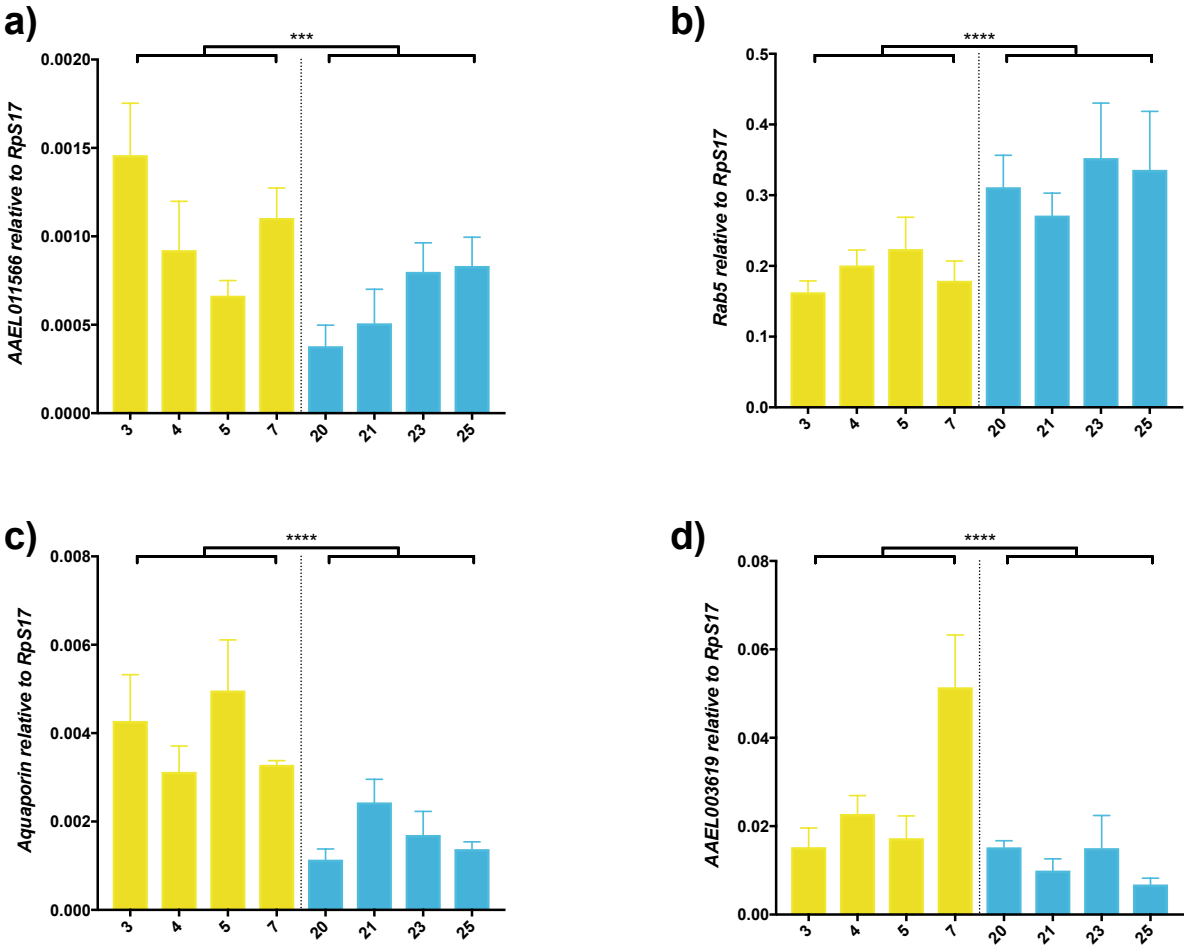

Fig.5 – Adhesion and transport. Graphs show the expression of **(a)** *AAEL011566*, **(b)** *Rab5,* **(c)** *aquaporin* and **(d)** *AAEL003619* relative to *RpS17* in DENV-infected individuals. Yellow bars represent refractory families, blue bars represent susceptible families. Bars depict family mean and SEM (n=5). *** 0.001<p<0.0001, **** p<0.0001.

Discussion

Transcriptomic studies and other novel approaches that reveal differentially transcribed genes produce long lists of candidates that can number in the thousands. When studying the response of a vector to viral infection, some of the gene candidates will be directly involved in viral control, whereas others may simply exhibit change in expression due to the cellular state of the vector. We know that many factors affect gene expression in an infection, not only those with antiviral functions. For example, expression of many genes change due to blood intake [54,55], and it is likely that other genes respond to cellular damage caused by the virus [56]. In this study we aimed to use natural genetic variation for dengue vector competence in a mosquito population to specifically test whether candidate genes from previous transcriptomic studies represented virus 'controlling' genes or virus 'responding' genes. We focused on genes where further experimental work has yet to confirm their functional roles in infection. By mapping the expression of candidate genes across families with extremes in vector competence we were able to reduce the list of candidate virus controller genes by half. This study revealed genes corresponding to three major clusters of functional classes involved in viral control: immunity, adhesion and intracellular transport and metabolism.

Immunity

DENV actively modulates host cellular processes to establish infection and propagate [57,58]. The host in turn responds with a range of known antiviral effects mostly via activation of the innate immune system [29,59,60]. Previously, Toll pathway activity has been shown to be required for DENV control in mosquitoes [29], but interestingly, transcriptomic studies demonstrate that one of the pathway's effector genes, *defensin,* is routinely downregulated in response to DENV infection [23,25]. Our results, however, show an upregulation of the gene's expression in families with high DENV loads. This discrepancy may be due to differences in mosquito genotypes, sample time post infection or other factors that vary across studies. Expression of immune effectors varies highly depending on the time post infection [40,61].

The genes, *SUMOE2* and *AeNopo* and *smp-30*, are thought to be involved in modification processes and apoptotic responses. The former plays a role in sumoylation, a process that stabilizes non-structural DENV proteins for proper replication [39] as well as modification of host proteins [62,63]. The latter two genes are involved in apoptosis, a cellular death process that promotes DENV replication [32,64]. *Nopo* has been shown to drive an interferon-mediated cell death process in *Drosophila* [43], whose upregulation correlates with higher DENV replication [41]. In addition to modulating host responses, DENV also must hijack host machinery to replicate efficiently [58,65], but we did not find any difference in expression for genes involved in transcription such as the *Ae. mut-7* homolog or *rent1*. Both of these genes are involved in splicing complexes and RNA processing and control [66,67].

Adhesion and intracellular transport

In our study, we revealed the differential expression of a variety of adhesion molecules and intracellular transporters that DENV may utilise for entry and replication [68,69]. *Rab5*, which has already been shown as required for flaviviral cell entry in humans [49], is a gene encoding a protein involved in vesicle formation and regulation of intracellular trafficking. We detected an increase in *Rab5* expression in families that harbour greater DENV loads, suggesting it may play a similar role for DENV entry in insects. Studies based on other vector-borne pathogens including chikungunya and Venezuelan Equine Encephalitis viruses, have demonstrated the role of the protein in promoting viral infection [50,51]. The *aquaporin* gene is a member of a large family of transporters of water, with known roles in mitigating desiccation [70] and managing bloodmeal-induced diuresis [53]. The expression of *aquaporin* is commonly downregulated in a range of transcriptomic studies of host response to DENV, Yellow Fever and West Nile viruses [23,25]. Similar to transcriptomic studies, we find that it is lowly expressed in families with high DENV loads. In *Drosophila*, *aquaporin* is primarily expressed in the carcass of the insect [71]. If the expression pattern is similar in *Ae. aegypti*, downregulation of *aquaporin* may promote viral replication in the body cavity.

Metabolism

DENV uses host receptors and intracellular transporters to achieve infection, but it also relies on lipid rafts and modulation of the cell membrane composition to match that of the viral membrane and therefore facilitate viral entry to host cells [30,72]. Our approach detected differential expression for genes involved in metabolism of lipids and sugars and possibly in the redistribution of such host resources. Among these metabolic genes, we detected the downregulation of *α-glucosidase* and *Pglym* in highly infected families. Studies suggest that α-glucosidase is proviral in humans [73,74] and, as such, the downregulation of its expression is likely a host-induced anti-viral response. The downregulation detected for *Pglym* expression may not be due to its antiviral activity, but its position in the glycolysis pathway. Other genes involved on the breakdown of glucose have been reported to be key for viral control, such as aldolase [75]. However, in the same study, *Pglym* did not show antiviral properties. The modulation of metabolic genes may be caused by DENV-mediated redirection of resources inside the host [30]. Despite the importance of metabolic pathways to viral replication, other genes involved in metabolism were also found to be irrelevant for viral control, such as *sphingomyelin phosphodiesterase (SMase)*. *SMase* is a gene that specifically degrades sphingomyelin (SM), but also acts in response to cellular stresses through production of ceramide, which is linked to DENV infection responses [30]. This suggests that SMase may be acting early in infection, altering the cell outer membrane to produce a more curved membrane that favours DENV infection [76,77]. However, we would not detect modulation of SMase given the late timepoint post infection we surveyed if SMase was not directly affecting viral replication.

Caveats

The design of our study presents some caveats that may limit its interpretation. The experimental conditions across the transcriptomic studies we surveyed and in our experiments. Since some host responses are highly plastic and modulated rapidly, comparisons across differ collection points may not be valid. Also, genotypes (both mosquito and viral) may dictate the nature of the viral infection process and the host response. In addition, our families were phenotyped based on load of DENV in

disseminated tissues following injection of virus. This approach would have missed the midgut response that is captured in transcriptomic studies from whole insects following oral feeding.

Conclusion

In conclusion, we found that natural genetic variation in vector competence can be used to further identify viral controlling genes from the long lists of gene candidates produced in transcriptomic and other genome wide expression studies. Our data support the use of genetic variation as a stepping-stone to test for relevance of gene candidates prior to experimental confirmation of function by more labor-intensive gene modification approaches. Here we have generated a list of 12 candidate genes that should be further examined as potential targets of gene modification to produce DENV refractory mosquitoes. Future research should exploit such phenotypic variation to confirm the involvement of genes in particular phenotypes, not limited to pathogen infection.

Materials and Methods

Ethics

The ET300 DENV strain was received from researchers associated with the University of Queensland (UQ) and Queensland Health, Australia. Patient data were anonymised by QH while IRB approval was obtained from UQ. Ethical approval for the research was obtained via The Monash University Human Research Ethics Committee (permit CF11/0766-2011000387). Adult human volunteer blood feeders agreed upon written informed consent prior to the study. No data were collected on these individuals.

Mosquito collection and rearing

Mosquitoes were collected by the Eliminate Dengue team associated with James Cook University from private properties with permission from the residents. Wildtype *Ae. aegypti* were identified by morphology and later checked by *Ae. aegypti*-specific qPCR primer detection. Mosquitoes were hatched and reared at a density of ~150 larvae in 30 x 40 x 8cm trays containing 3L of RO water in controlled conditions of temperature (26±2°C), humidity (~70%) and photoperiod (12:12, light:dark). Larvae were fed fish food (Tetramin®, Melle, Germany). Males and females were sexed after pupation and transferred separately to 30 x 30 x 30cm cages to allow eclosion at a density of ~450 individuals/cage. Adult mosquitoes were kept on a 10% sucrose water diet. Six to eight day old adult females (P1) were group fed on human volunteers. A modified full sib breeding design was performed as depicted in a previous paper [78] and yielded 25 independent WT families. In brief, parental single pair crosses (male with a virgin female) were set up and those that exhibited sufficient egg production were selected for F_1 intercrossing and progressed to F_2. DENV serotype 2 (DENV-2) was then injected intrathoracically into 6-7 day old F_2 mosquitoes and either tissues (head, ovaries, midgut and rest of the body) or whole mosquitoes were collected at 7 days post infection to evaluate both DENV-2 loads and candidate gene expression.

Virus intrathoracic injections

A dengue virus serotype 2 strain (DENV-2, ET300) isolated from human serum collected from patients from East Timor in 2000 was used for intrathoracic injections. Virus was propagated and collected in cell culture as described previously [79]. Viral stocks were stored at -80ºC until further use and titrated using plaque assays. *Ae. aegypti* females were anesthetized with CO_2 and DENV was injected using a pulled glass capillary with a manual microinjector (Nanoject II, Drummond Sci.). Intrathoracic injections were used to ensure that the same amount of virus was delivered into the mosquitoes and to prevent bloodmeal-induced responses to be mounted. Diluted virus stock (~70 DENV-2 pfu) was injected intrathoracically into every *Ae. aegypti* female. After injection, mosquitoes were maintained under identical initial controlled conditions as per above.

RNA extractions

Whole mosquitoes were collected at 7 days post injection and extracted using TRIzol (Invitrogen). RNA was extracted immediately following the manufacturer's instructions. RNA yield was quantified using a Nanodrop™ Lite Spectophotometer (ThermoFisher Scientific, Waltham, MA, USA). RNA samples were stored at -80ºC.
Heads were used initially as a proxy for DENV dissemination, but whole bodies were preferred for gene expression analyses as they are likely to capture a broader suite of genes involved with the infection response across the diverse tissues.

DENV analysis

RNA samples were diluted to a concentration of 10ng/μl prior to DENV qPCR analysis. One-step quantitative RT-PCR (qRT-PCR) to detect DENV titres was performed using TaqMan® Fast Virus 1-step Master Mix (Roche Applied Science, Switzerland) in a total volume of 10 μl and following manufacturer's instructions on a LightCycler480 (Roche Applied Science, Switzerland). DENV qRT-PCR reactions were performed as described previously [80]. The number of viral genome copies present in each sample was evaluated using known standards [81]. The used standards ranged from 10^8 to 10 DENV fragment copies. The limit of detection was set at 100 DENV copies as virus.

Concentration of DENV in each sample was adjusted to DENV copies/μg of total RNA using the standard curve. Standards and samples were run in duplicate.

Candidate gene expression

SuperScript® III Reverse Transcriptase kit (Invitrogen) was used to convert RNA to cDNA in all carcass samples. The reaction contained 12.5μl of RNA undiluted template, 1μl of random primers (RP, 125ng/μl), 1μl of deoxynucleotides (dNTPs, 2.5mM), dithiothreitol (DTT), 5X buffer and enzyme as per kit instructions, with a total volume of 20μl. cDNA synthesis was performed in a C1000™ Thermal Cycler (Bio-Rad) on the following temperature profile: 5' at 65°C followed by 10' at 25°C, 50' at 50°C, 10' at 75°C and kept at 4ºC. Gene expression levels were detected with SYBR® Green I Master (Roche) using 1.5μl of a 1:5 dilution from the previously synthesized cDNA on a LightCycler480 (Roche Applied Science, Switzerland). Corresponding Ct values were normalized to the housekeeping *Ae. aegypti RpS17* gene [34] and expression ratios obtained using the ΔΔCt method [82]. Primer sequences for candidate genes can be found in Table S1.

Statistical analysis

DENV loads and gene expression data were analysed using a generalized mixed model with a random factor 'Family' nested with 'disseminated' DENV load, with the latter also set as a fixed factor. Statistics were performed using IBM SPSS Statistics (v23) and graphs created using Prism 7 (GraphPad Software Inc.).

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CHAPTER FIVE

General discussion and Final remarks

Wolbachia pipientis is an insect endosymbiont capable of manipulating its host's reproductive success, giving an advantage to infected females and promoting its spread through an uninfected population. The presence of *Wolbachia* can also limit the replication of a range of co-infecting pathogens [1-3]. These features coupled with the ability of the symbiont to spread relatively quickly through populations have made *Wolbachia* an ideal target in a bid to control mosquito-borne diseases, especially dengue virus (DENV) [4]. Biocontrol strategies aiming towards pathogen control using *Wolbachia* strains have been suggested via either population suppression [5,6] or producing a pathogen blocking phenotype that increases mosquito's refractoriness to pathogen infections [7,8]. The degree of pathogen blocking is dependent on a range of factors including the association of host mosquito genotype to a specific *Wolbachia* strain [3], pathogen type [1] and environmental conditions [9]. For example, the effect of the pathogen-blocking trait is greater when the association between *Wolbachia* strain and the host is novel [1,10] as opposed to the minimal protection that is present in native hosts [11]. In a mosquito species such as *Aedes albopictus*, which carries native *Wolbachia* *w*AlbA and *w*AlbB strains, little to no protection of pathogen infections has been observed [11]. However, in a naturally non-infected species such as *Aedes aegypti*, the stable transinfection of the *Drosophila* *w*Mel or *w*MelPop strains via embryonic microinjections conferred the host with pathogen blocking abilities against a range of pathogens including viruses [3,12,13], parasites [1] and filarial nematodes [14]. Similar effects have been observed in other novelly infected mosquito species [15-17]. Currently, *Ae. aegypti* infected with *Wolbachia* are being released in a range of field sites globally to test the efficacy and stability of the association [18,19]. However, and despite its applicability in the field, the mechanism underlying the *Wolbachia*-mediated pathogen blocking is not well understood.

There are some suggested mechanisms that would explain part of the *Wolbachia*-mediated pathogen blocking. There is evidence that the transinfection of *Wolbachia* into a novel host modulates and promotes immune gene activation [1,14], which in turn would be already be prepared to suppress any incoming pathogen that comes in

contact with the host. As mentioned previously, the ability to limit pathogen replication is greater in novel infections compared to native. Similarly, immune activation has been shown to be prominent in a range of novel *Wolbachia* infections [2,14,20], but it is predominantly milder or absent in native associations [11,20].

Other hypotheses have been proposed for the blocking phenotype that are based around competition between *Wolbachia* and the pathogen for limited but essential host resources such as lipids [21,22] or nitrogen [23]. Both *Wolbachia* and viruses are thought to require cholesterol and nitrogen sources to infect and replicate, so *Wolbachia* hijacking certain lipids and amino acids would likely cause an antagonistic environment for viral replication. *Wolbachia* and the virus have also been hypothesized to be competing for intracellular space [1]. In turn, *Wolbachia* densities appear to be of relevant importance for the strength of pathogen blocking [11,24]. This is seen clearly with *Wolbachia* infections that are able to colonize cells and tissues at a high rate cause fully protective phenotypes [3,25], whereas milder *Wolbachia* infections correlate with those strains that are less protective [26]. This hypothesis would suggest that all *Wolbachia* strains are able to protect the host from pathogens if a certain threshold is reached, but some of them are unable to do so due to evolution constraints through time or ability to compete for space. Most recently, independent studies [27,28] proposed a mechanism where competition and immune upregulation would be by-products of *Wolbachia*'s fundamental effects on the morphology of the infected cells. Disruption of organelles such as the endoplasmic reticulum would therefore hamper successful replication and also explain some of the fitness costs present in highly infected individuals. Understanding how *Wolbachia* is able to provide the host with protection against pathogens is not only important for basic research but also to predict the coevolution between host and symbiont, which could potentially cause a shift in the trait to a less protective phenotype. The causal hypotheses for *Wolbachia*-mediated pathogen blocking, differences between native and novel associations and implications of a constant coevolving partnership between host and symbiont are reviewed in more detail in **Chapter 1**.

This thesis focuses on the identification of DENV control mechanisms in *Ae. aegypti,* either determined by mosquito genotype or by the involvement of a third party, *Wolbachia*. Using cell lines and a family breeding design, we evaluated the contribution of specific genes involved in immunity or in different cellular functions to DENV control. My research also focuses in the genetic variation and heritability present for the pathogen blocking phenotype in *Wolbachia*-infected mosquitoes, since both parameters are essential for the coevolution between host and symbiont and the capacity of the latter to limit pathogen replication.

Gene modulation in response to DENV infection

Higher susceptibility or refractoriness of *Ae. aegypti* to DENV infection is in part controlled by the activation of specific genes that defend the insect from the pathogen. Genetic studies have shown that the ability to overcome a DENV infection is determined by multiple quantitative trait loci (QTL) in the mosquito genome [29-32], mainly affecting genes involved in midgut infection barriers (MIB). For a DENV infection to succeed, the colonization of the midgut is essential, as the virus will establish infection, replicate and spread through the haemolymph after surpassing the MIB. Transcriptomic studies [33-35] have also examined patterns of expression over the time course of the infection in the mosquito. Most of the gene candidates that emerge from transcriptomic profiles however have not been tested functionally. The list of affected genes in long and their role in biological processes is diverse ranging from immune genes to energy metabolites to ion intracellular transporters or to others with unknown functions. Most of the immune-related genes have already been tested functionally in mosquitoes and some of them have been identified as important for DENV control [36-39]. However, eliciting other key genes involved in DENV control is crucial for potential future development of long-term therapeutic vaccines or mosquito genetic modification to decrease DENV incidence in humans. In **Chapter 4**, I reported the importance for DENV control of different non-immune gene candidates that had previously been revealed in one or more transcriptomic studies for significant change in their RNA expression levels. I used the genetic variation for the DENV phenotype in the breeding design performed in **Chapter 3** for the wildtype population to compare the expression of previously annotated genes between families infected with high and low DENV loads.

Transcriptomic profiles have gleaned lists of genes involved in DENV control; however, this type of study only provides information about differences in gene expression for two or more conditions. Without functional testing, the differential expression of genes may be due to the state of organism after DENV infection rather than a unique response of the host to control such infection. My data show it is not only immune-associated genes that are responsible for DENV control in *Ae. aegypti*. This suggests that the host response phenotype is highly multifactorial. To successfully infect the host, the virus not only has to alter immune responses but also recruit the host replication machinery to promote its own replication or alter cellular structures such as membranes or cellular receptors to facilitate the intracellular transport of new virions, their exit and successive reinfection events [40,41].

The interaction between host (*Ae. aegypti*) and pathogen (DENV) genomes is not the only determinant of the infection outcome, as in the case of three-way interactions such as *Ae. aegypti*-*Wolbachia*-DENV. In this association, the symbiont (*Wolbachia*) provides the host with protection against the pathogen, altering the host-pathogen natural interaction and controlling the outcome of infection. *Wolbachia* has a range of effects at both individual and cellular levels in infected hosts [42,43] that promote the growth of the bacterium intracellularly. *Wolbachia* also causes reproductive manipulations in the host that allow the symbiont to be transmitted efficiently, either vertically [44] or horizontally [45]. In addition to population spread, *Wolbachia*-mediated pathogen blocking is the standout feature that makes *Wolbachia* highly suitable for biocontrol purposes. Although it is not clear how *Wolbachia*-mediated blocking occurs, innate immune "priming" has been hypothesized as a mechanism that would lead to the restriction of subsequent viral infections in *Wolbachia*-infected insects. Despite there is evidence of insect immune modulation and functional studies highlight the independent contribution of different genes and immune pathways to DENV blocking in a range of associations [1,2,46], no study has looked at the relative contribution of all innate immune pathways in *Ae. aegypti*. In **Chapter 2**, specifically, I demonstrated that some of the pathways are modulated by the infection of *Wolbachia* and have a role to play in the mosquito's immunity against DENV. In a natural setting, immune pathways that respond to a specific viral infection tend not to act independently but as a network [47].

My work [48] **(Chapter 2, Chapter 3)** establishes the RNAi pathway as the main immune response to DENV infection in *Wolbachia*-infected cells, but fails to explain the totality of the blocking phenotype. The Toll [36] and the JAK/STAT [49] pathways have also been shown to be important for DENV control but they are likely *Ae. aegypti* immune responses rather than specific to *Wolbachia*-mediated blocking. These findings demonstrate that the effects of *Wolbachia* on the host are multifactorial and not explained by a single mechanism such as immune priming, thus likely to be more conserved and basic to the nature of the symbiont. Besides immunity and as hypothesized previously, my data suggests that part of the pathogen-blocking trait is also due to the competition for lipids between *Wolbachia* and the virus (**Chapter 3**).

Wolbachia densities affect strength of pathogen blocking

Different *Wolbachia* strains commonly display a variety of somatic tissue distributions [50,51], leading to variable densities in the infected hosts. The degree at which *Wolbachia* is able to infect insect cells is thought to directly dictate the degree of pathogen blocking in both *Wolbachia*-infected insect cultured cells [11] or adults [3,24,52], as highly invasive strains produce a greater blocking phenotype but they also cause more detrimental fitness costs to the host [3,9,53]. Similar to the blocking phenotype, densities are also greater in novel hosts as part of the colonisation of host tissues, causing fitness costs that usually decrease in some generations after infection establishment [54]. In **Chapter 2** we indirectly evaluated whether the alteration of certain immune responses affect the densities of the *Wolbachia* strain *w*Mel in *Ae. aegypti* cell lines. A strong reduction in the expression of different immune effectors does not affect the intracellular levels of the symbiont. My work shows that the capacity of *Wolbachia* to replicate freely and invade host tissues is not explained only by the immunity of the host controlling the bacterial infection but instead may be dependent on a variety of factors in the host-symbiont relationship that are unique for each insect association with a particular *Wolbachia* strain. This bodes well with the observation that strains produce different phenotypes depending on the host they infect [11] and that the same strain also has different effects on different hosts [54], especially when one association is natural and the other is novel.

However, these experiments were performed in a cell line, which may be differing from the adult mosquito. In cell lines, *Wolbachia* is able to grow to greater levels due to the lack of nutritional impediments.
In adults, *Wolbachia* grows to different densities depending on the tissue it infects, whereas some genes are also transcribed differently [55]. Consequently, we aimed to unravel how *Wolbachia* densities and pathogen blocking interact in adult *Ae. aegypti* mosquitoes.

In **Chapter 3**, families were classified as good, medium or bad DENV blockers after challenge with DENV and were further tested for differences in *Wolbachia* densities. This experimental design allowed us to evaluate the relationship between *Wolbachia* and the DENV blocking phenotype. Genetic variation was detected for DENV loads as well as *Wolbachia* densities across families. Variation in DENV infections has previously been studied thoroughly using vector competence assays [56,57], but it is quite interesting that genetic variation for DENV blocking is also present in *Wolbachia*-infected mosquitoes. Even though the trait is likely to be multifactorial, this observation highlights the potential for the trait to be affected by natural selection in the field, as genetic variation is essential for natural selection to occur to the genotypic variants already present in the population [58]. We also detected a negative correlation between DENV loads and *Wolbachia* densities, as individuals with high *Wolbachia* loads also result to harbour low DENV infections. The latter is supported by previous studies that show density dependence of the pathogen-blocking trait in cells or other associations [11,59].

Wolbachia infection has the potential to evolve

As mentioned above, *Wolbachia*-based biocontrol strategies may be affected by the evolvability of *Wolbachia* inside hosts [60]. An association between *Ae. aegypti* and *Wolbachia* that can evolve quickly may act in favour or against the conferred blocking ability and therefore have big impacts on the long-term application of the symbiont. After detecting variation in DENV blocking and correlation between pathogen and symbiont (**Chapter 3**), we tried to understand the trait's capacity to evolve in two different ways: (1) whether *Wolbachia* loads and the pathogen blocking

trait are heritable and (2) whether there is variation in the expression of different genes involved in DENV control of *Wolbachia*-infected mosquitoes.

In **Chapter 3** we detected a variation in DENV blocking phenotypes among families from the breeding design. This variation informed us of the capacity for the trait to evolve through generations, as phenotypic variability is a premise for evolution [58]. However, the pathogen blocking phenotype does not evolve on its own, since it is somehow dependent on *Wolbachia* cellular and tissue levels [10,11]. *Wolbachia* is transmitted through the maternal germline, but it is unclear if *Wolbachia* densities are also vertically transmitted. Using *Wolbachia*-infected isofemale *Ae. aegypti* families from the full-sib breeding design (**Chapter 3**), we evaluated the heritability of *Wolbachia* loads. Our data shows that the variation in *Wolbachia* loads is greater between families than within individuals from the same family, suggesting that *Wolbachia* densities are also inherited through generations. Showing that *Wolbachia* is heritable to the offspring is a novel finding in the field; the association between *Wolbachia* and the host can coevolve when selection pressures act on extreme phenotypes and select for the fittest. However, predicting the direction of coevolution is complicated. Not only the selection for *Wolbachia* has to occur, but also selection for DENV loads, since the latter is somehow dependent on the former due to the *Wolbachia*-conferred blocking phenotype. Understanding the contribution of immunity to DENV blocking is essential because immune responses rapidly evolve, especially when the mosquito faces high selective pressures. If immune upregulation explained part of the mechanism behind *Wolbachia*-mediated pathogen blocking, coevolution could veer towards a more mutualistic partnership where the mosquito immune system would not respond to the presence of the symbiont and therefore would lack in pathogen protection. Innate immunity, however, is important to explain only a small amount of the *Wolbachia*-mediated blocking against arboviruses (**Chapter 2, Chapter 3**) so immune attenuation may not shift the blocking phenotype. Expression levels for other non-immune genes (**Chapter 4**) also correlate to DENV infections in wildtype mosquitoes, as they function in a pro or antiviral capacity during infection. Empirical evidence shows that the density at which *Wolbachia* is able to infect the host tissues is highly relevant for the pathogen-blocking trait [11,24,61].

Our take is that evolution of *Wolbachia*-mosquito relationship is going to be determined by densities that consequently alter the expression of host genes essential for DENV control.

Future of genetics

My findings provide evidence on part of the *Wolbachia*-mediated pathogen blocking mechanism, where *Wolbachia* relies on the host's innate immunity to mount responses against pathogens but does not explain the full extent of the blocking trait. My data also provides insights on the capacity of the blocking trait to evolve in conjunction with *Wolbachia* in the field on long-term associations. There are some questions that arise from my thesis, as it is difficult to elicit the genetic basis of the *Wolbachia*-mediated blocking trait since every *Wolbachia* to host association is unique and a myriad of factors have to be taken into account. Differences in pathogen blocking could be understood through genetic association studies and extensive sequencing on different mosquito genotypes paired with different *Wolbachia* strains. This would not only have benefits from a research standpoint but also for *Wolbachia's* application in the field as a biocontrol tool. The detection of the ideal set of genetic variants important for DENV blocking appears a requirement to select for the best candidate in future deployments of the symbiont. In **Chapter 3** it was observed that *Wolbachia* varies between individuals from a population and that the conferred pathogen blocking can evolve, but some questions still need to be answered in that regard. *Wolbachia* densities determine pathogen blocking, but high *Wolbachia* levels also cause severe fitness costs to the host. Are high *Wolbachia* levels selected for in a population with high DENV incidence, or instead are they selected against due to fitness costs? Given the importance of the phenotype to biocontrol, not only evolutionary experiments should address such question but also look at the coevolution between *Wolbachia* genome and the virus, as coadaptation is likely to occur in the field following successive encounters with the pathogen.

Implications for biocontrol

Wolbachia demonstrates high potential for its use in biocontrol strategies. Its success in the field, however, may be limited after the initial release of *Wolbachia*-infected mosquitoes. If *Wolbachia*'s negative effects on host fitness are too high, it may be unable to spread through wildtype populations and require constant re-release of infected mosquitoes [19]. If in contrast *Wolbachia* establishes stable infection through native populations, a drop of the pathogen-blocking trait could occur after some time due to coevolution between *Wolbachia* and host.

My data shows differences in *Wolbachia* load and pathogen blocking between individuals with different genotypic backgrounds and consequently, there is some evolvability to the blocking trait (**Chapter 3**). The distribution and density of *Wolbachia* inside hosts is key for pathogen blocking and it can be altered due to co-evolution between the host and *Wolbachia*, especially if there is no evolution towards obligate symbiosis. After some generations in the field and coevolution between host, symbiont and virus, individuals with high *Wolbachia* loads could be selected for or against and affect the host's pathogen-blocking trait phenotype. We estimate the direction of evolutionary change to be driven towards a mild *Wolbachia* infection but invasive enough to still protect the mosquito from incoming pathogens. Fully protective *Wolbachia* strains are harboured in high densities and that also causes severe associated fitness costs, decreasing host's lifespan and fecundity [3,53,62]. Thus, anything beyond a mild infection would be likely selected against. On the other hand, evolution towards lesser *Wolbachia* would increase host's susceptibility to arboviruses, which can also affect host fitness decreasing its fecundity [63].

It is therefore necessary to have an understanding of the mechanistic basis of the anti-pathogen effects of *Wolbachia,* as well as estimate how evolution of the trait and the symbiont will occur in the field. Successful strategies to be developed are likely aiming towards reducing or mitigating the emergence of resistance. This thesis has added to our emerging understanding of the *Ae. aegypti*'s *Wolbachia*-mediated DENV blocking mechanism, shedding light on how the immune pathways interact to boost DENV blocking and the amount of variation present for the blocking trait in the population.

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APPENDIX 1

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Current Opinion in Insect Science

Wolbachia-mediated virus blocking in the mosquito vector *Aedes aegypti*

Gerard Terradas and Elizabeth A McGraw

Viruses transmitted by mosquitoes such as dengue, Zika and West Nile cause a threat to global health due to increased geographical range and frequency of outbreaks. The bacterium *Wolbachia pipientis* may be the solution reducing disease transmission. Though commonly missing in vector species, the bacterium was artificially and stably introduced into *Aedes aegypti* to assess its potential for biocontrol. When infected with *Wolbachia*, mosquitoes become refractory to infection by a range of pathogens, including the aforementioned viruses. How the bacterium is conferring this phenotype remains unknown. Here we discuss current hypotheses in the field for the mechanistic basis of pathogen blocking and evaluate the evidence from mosquitoes and related insects.

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Wolbachia and pathogen blocking

Wolbachia pipientis is a maternally transmitted endosymbiotic bacterium estimated to chronically infect 40% of all known arthropod species [1]. The symbiont's success has been credited to its ability to spread through uninfected populations by altering the reproductive biology of its hosts and providing a fitness advantage to infected females. *Wolbachia*'s manipulations, which include feminization of males, parthenogenesis, male killing and cytoplasmic incompatibility (CI), are all female biased given its maternal inheritance [2]. *Wolbachia* infection also has other physiological effects that can be exploited for biological control: it can inhibit the replication of many pathogens [3••,4,5,6••] and shorten the lifespan of its host [7]. Though little is known about the mechanisms that underlie this 'pathogen blocking' trait, these unusual properties have made *Wolbachia* extremely attractive as a potential means of vector-borne disease control [8].

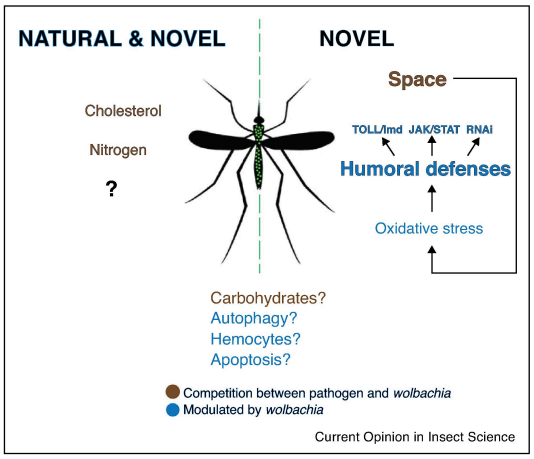
Wolbachia limits the replication of viruses such as dengue, yellow fever, Zika, West Nile and Chikungunya, as well as filarial nematodes and the malaria parasite *Plasmodium* in their associated mosquito vectors [3••,4,5,6••,9–11]. Of these, the field-testing of *Wolbachia* for biocontrol is most advanced in the case of dengue virus (DENV) [12•]. Dengue fever is a disease affecting an estimated of one-third of the world's population. Some mosquitoes are naturally infected with *Wolbachia* strains, but the primary vector of DENV, *Aedes aegypti*, is naturally *Wolbachia*-free. A virulent *Wolbachia* strain (named *w*Mel.Pop) from a laboratory line of *Drosophila melanogaster* was initially introduced into *Ae. aegypti* to reduce transmission of DENV by significantly shortening the lifespan of female mosquitoes [7]. Unfortunately, the *w*MelPop strain was also found to have severe negative effects on fecundity [13•] and was shown not to spread in field populations [14]. Other strains of *Wolbachia* also have the capacity to interfere with viruses in insects, blocking transmission of the agent while conferring only mild or no fitness costs to the vector [13•]. A second such *Wolbachia* strain from *D. melanogaster*, *w*Mel, was introduced into *Ae. aegypti* and tested in mosquito populations of northern Queensland, Australia, where DENV is epidemic upon introduction by travellers [12•]. Current field trials in Indonesia are testing whether the anti-DENV effects of *w*Mel seen in the laboratory lead to reductions in the incidence of dengue fever in humans, following release of *Wolbachia* in wild mosquito populations. *Wolbachia* is being also field tested for its potential use against other important arboviruses including Zika.

This review explores possible mechanisms behind *Wolbachia's* pathogen blocking phenotype (Figure 1). To date, two main theories have prevailed: host immune priming and competition for host resources. In addition we examine the differential expression of pathogen blocking in native and novelly created host associations. These differences may help to discern mechanism and also predict the future trajectory of the blocking phenotype in novel hosts.

Mechanisms of pathogen blocking

The successful transmission of a virus is dictated by contributions from both viral and vector genomes [15] and in some cases from *Wolbachia* or other insect associated microbes [16]. Nevertheless, the exact mechanism

Figure 1



Current hypotheses for the mechanism of *Wolbachia*-mediated pathogen blocking in both native and novel hosts.

that underpins *Wolbachia*-mediated blocking is not well understood as teasing apart the contribution of the three partners in the association can be difficult, particularly without the capacity to genetically modify *Wolbachia*. Most of what is known has come from comparing the strength and expression of blocking in different combinations of vector species, virus genotypes and *Wolbachia* strains [17•]. Variation in behaviour of different *Wolbachia* strains infecting the same host also demonstrates the contribution of the symbiont genome to the association. In particular, the expression of blocking appears to differ between natively or novelly infected vectors [18]. In *Ae. albopictus*, which is natively infected with two *Wolbachia* strains (*w*AlbA and *w*AlbB), viral blocking is weaker or non existent [19] compared to *Ae. aegypti* novelly infected with *w*Mel or wAlbB [13•,20]. Additionally, blocking is strong when *Ae. albopictus* is novelly infected with the *w*Mel strain [21], indicating that the degree of familiarity between partners can also dictate strength of blocking, rather than just history of infection in the host.

The amount of *Wolbachia* inside host cells and tissues, usually referred to as 'density', also appears to correlate with the strength of *Wolbachia*-mediated blocking [17•,19]. This relationship is seen in several contexts. Novel host/*Wolbachia* strain combinations tend to have higher densities and more widespread tissue distributions [18,22]. Comparisons between a range of *Wolbachia* strains that naturally infect *Drosophila simulans* reveal differences in blocking that are predicted by their individual densities and tissue distributions [23]. Lastly, the highly replicative *Wolbachia* strain called *w*MelPop, that exhibits greater cellular loads and causes tissue damage, induces near perfect blocking in *Ae. aegypti* [3••] compared to the more moderate blocking of *w*Mel in the same host [13•]. This difference in load between *w*Mel and *w*MelPop [3,13•] and the associated virulence is thought to underpin the failure of the latter strain to spread in field [14].

Immune priming

Upregulation of mosquito antimicrobial encoding genes was first seen in response to *w*MelPop infections [24]. This finding led to the theory of 'innate immune priming', where pre activation of the immune response could then theoretically protect the insect from a range of pathogens. Subsequently, support for a complementary set of theories based on resource competition emerged [25•,26,27], where *Wolbachia* and pathogens would compete for limited host resources.

Wolbachia-induced changes in immunity gene expression are largely seen in mosquitoes with artificially introduced strains (*i.e.*, *Ae. aegypti* (*w*MelPop [4], *w*Mel [28], *w*AlbB [20])), where more genes are affected and to a greater degree than in natively-infected hosts (*i.e.*, *D. melanogaster* (*w*Mel) [28], *Ae. albopictus* (*w*AlbA and B) [19]). The occurrence of blocking in both of these native *Wolbachia* hosts therefore indicates that immune activation must not be essential for pathogen blocking [28,29]. Although it may not be essential, the greater strength of blocking seen in *Ae. aegypti* may stem from the additive effects from immune activation [4,30,31]. A detailed examination of the functional role of immunity in blocking has been confined to a subset of insect immunity pathways that may not be the most relevant for DENV control [32,33].

Humoral innate immunity

The humoral innate immune response activation begins following recognition of the pathogen through different receptors. These receptors detect non-self molecular conserved motifs, such as double-stranded RNA in viruses or cell wall components in bacteria. The most important signalling cascades are the Toll, the Immune Deficiency (Imd), the Janus Kinase-Signal Transducer and Activator of Transcription (JAK/STAT) and the different RNA interference pathways [34]. Successful pathogens are able to evade or suppress these innate defense mechanisms [35].

The systemic immune response in insects relies on the production of antimicrobial peptides (AMP) and antiviral molecules in the fat body that are subsequently released into the hemolymph. AMP production is mainly due to the antibacterial pathways Toll and the Imd, which have also been the most studied in the *Wolbachia*-insect system due to the bacterial nature of the symbiont. Toll is triggered by gram-positive bacteria and fungi whereas Imd responds mainly to gram-negative invaders [34]. Both pathways have been well described and characterised in diptera. Although primarily antibacterial, Toll is also required for the mosquito's response to DENV

[36]. *Wolbachia* does not induce upregulation in Toll or Imd effectors in *Drosophila* and yet *Wolbachia* is able to block DENV replication, suggesting that these pathways are not required for the protective phenotype to occur [28]. It has been demonstrated that *Wolbachia* does not activate the antimicrobials cecropin or diptericin in *D. simulans*, even though the species is heavily infected with the bacteria [37]. This does not appear to be the case for *Ae. aegypti*, where both AMPs are upregulated following *Wolbachia* infection [27]. Not only are more genes activated by *Wolbachia* and expression changes generally higher in *Ae. aegypti*, the breadth of pathogen targeting is also wider, including bacteria [38]. These differences may also provide stronger antiviral effects [38,39].

Other humoral immune responses comprise the JAK/STAT and the RNA interference pathways, which lead to antiviral production or to cleavage of targeted foreign double-stranded nucleic sequences. The JAK/STAT signal transduction cascade was discovered in mammals and soon identified in almost all species as an innate immunity process, a role player in antiviral defense including blocking against dengue. In mammalian cells, the JAK/STAT pathway is activated by different cytokines, including interferon, interleukins and growth factors. In insects though, no interferon has yet been discovered. Despite that, a recent study showed that Vago acts as an interferon-like molecule, activating the JAK/STAT pathway and upregulating different effectors including the virus induced RNA-1 (*vir-1*) gene [33]. JAK/STAT's transcriptional profiling and regulation is complex and its specific effect on pathogens still unknown. The effector *vir-1* has been shown to be specifically upregulated in response to virus [40]. Also, studies have shown restriction of West Nile virus replication after the induction of JAK/STAT in *Culex spp* [33]. In *Ae. aegypti*, the function of the pathway is conserved and high activation levels of JAK/STAT limit DENV replication [41]. It is clear that the pathway has a crucial role in insect immunity.

The RNA interference (RNAi) pathway also plays a key role in insect immunity. Two types of RNA molecules are essential for RNAi, microRNA (miRNA) and small interfering RNA (siRNA), both of which have been hypothesised as being modulated by *Wolbachia* infection. In nature, RNAi acts as a potent defense mechanism against viral infections and aberrant transcription due to its gene-silencing activity [42,43]. Considered the major insect antiviral pathway, RNAi has been shown to limit DENV, Chikungunya and Sindbis viruses in *A. aegypti* [39,42,44] and is regarded as a key contributor to control of arboviruses in mosquitoes [43]. There are studies showing that RNAi is not essential for *Wolbachia*-mediated viral blocking in *Drosophila* [45] or in an *Ae. albopictus* cell line where a non functional Dicer 2 did not hamper blocking [22]. However, a recent study using an *Ae. aegypti* cell line showed that *Wolbachia* may be upregulating *AGO2* intracellular levels, providing the host with an increased basal expression of a key component for the control of DENV infection [46•]. Upregulation of *AGO2* and in turn the activity of the siRNA pathway would allow for a greater protection from the arboviral infection.

In addition to the exogenous siRNA pathway, the miRNA pathway has also been suggested to be involved in the pathogen blocking effect as part of *Wolbachia*'s modulation of host components [47,48•]. Infection with *w*MelPop also has an effect on the miRNA profile of *Ae. aegypti* [47]. Moreover, the inhibition of certain miRNAs leads to reduction of *Wolbachia* densities. This suggests that the symbiont facilitates its maintenance in the host by manipulation of gene expression using host miRNAs. In terms of the conferred protective phenotype, *Wolbachia* is thought to be altering the intracellular localisation of AGO1 [49]. This is a principle component of the miRNA cascade, which in turn would affect trafficking of miRNAs into the nucleus leading to differential gene expression and methylation. A recent study [50••] in *Drosophila* cells however shows that blocking of Semliki Forest Virus (SFV) by *Wolbachia* occurs early in infection and without the activation of host transcriptional responses or microRNAs. It suggests that blocking is reliant on an intrinsic mechanism that is already in place when the pathogen comes into contact with the host. Although *Drosophila* is not a natural carrier of SFV, this is a crucial finding. A recent study [51•] is in keeping with the notion that *Wolbachia* may have fundamental effects on cells via modulation of the morphology and composition of the endoplasmic reticulum. This disruption may allow the symbiont to access nitrogen, but could also prevent viral replication. If these effects are consistent across host species and pathogens, the upregulation of immune responses may only be a by-product of *Wolbachia*'s infection and secondary to blocking.

Hemocytes

Hemocytes, along with the fat body, are major immune tissues within mosquitoes since immune effectors are released into the hemocoel. Hemocytes are also regarded as essential replication sites for *Wolbachia* due to the need for the bacteria to modulate these responses to propagate and maintain a systemic infection [11,52]. Interestingly, immune upregulation is related to function of hemocytes through cellular signalling [41,53,54]. At least one study has provided evidence of *Wolbachia* infection directly affecting hemocyte counts [55]. The ability to spread to the ovaries is key for proper transmission, where hemocytes that have phagocytosed *Wolbachia* would be serving as shuttle carriers from a primary infected organ to the rest of the body.

Autophagy

Autophagy is a highly conserved biological process responsible for the degradation of self-proteins and

damaged organelles through autophagosomes, but also involved in many host-pathogen interactions as part of the host cellular response. It is well known that pathogens have the ability to manipulate autophagy responses in order to enhance their replication and establish infection. The deletion of diverse autophagy genes in *Drosophila* seems to affect survival and decrease refractoriness to a vesicular stomatitis virus (VSV) infection [56]. Autophagy can also be activated through PAMPs via Toll-7 receptors in the plasma membrane and independent of a humoral Toll activation [56]. High levels of autophagy probably act as a host response in those tissues where *Wolbachia* levels are high, as activation leads to limited *Wolbachia* replication in *Ae. albopictus* cells [57]. Also, DENV seems to promote a specific type of autophagy that alters the metabolism of the cell causing release of fatty acids, which are required for its proper viral replication [58]. This is an interesting finding since *Wolbachia* also requires unsaturated fatty acids from host cells and competition for host nutrients has been hypothesised to explain the *Wolbachia*-mediated viral blocking phenotype [3].

Apoptosis

Apoptosis is a programmed cell death mechanism with diverse biological functions in multicellular organisms, from balancing homeostasis to lysis of viral particles. Many viruses contain sequences encoding for inhibitors of apoptosis, which lead to the idea of apoptosis having a role in immunity. The role of apoptosis in arboviral infections has been examined, finding that apoptotic cell death can be detected in mosquitoes' midgut and salivary glands after infection with a range of viruses. Pro-apoptotic genes were upregulated as part of the response of *Ae. aegypti* to DENV by a refractory DENV-2 strain [59]. While suppression of autophagy seems to be important for infecting viruses, no studies have tested the apoptotic relevance in *Wolbachia*-infected mosquitoes. Nevertheless, *Wolbachia* strains have been found to down-regulate apoptotic responses in parasitic wasp ovaries as part of mutualistic relationships between organisms [60]. The apoptotic pathway plays a complex role in host-pathogen interactions. A decrease in host apoptosis levels would give the virus an advantage to propagate, whereas an increase in the apoptotic activity would lead to a host self-destruction. Either outcome in response to modulation by *Wolbachia* would have negative consequences.

Iron metabolism and oxidative stress

Reactive oxygen species (ROS) when over produced cause cell damage but, when properly regulated provide a beneficial role as part of immune defenses and intracellular signaling. ROS are by-products of the metabolism of oxygen. ROS are known to be an immune alternative to the production of antimicrobial peptides and autophagy-related proteins. Ingestion of a bloodmeal causes a decrease in ROS in the mosquito midgut [61]. The decrease is in part due to a heme-mediated activation of protein kinase C as part of host counteractive measures to a pro-oxidative bloodmeal. However, lowered ROS levels probably correlate with a higher susceptibility to infection and increased mortality. Moreover, downregulation of antioxidant production following *Wolbachia* infection was shown in novelly infected cell lines [62,63]. Nevertheless, an increase in ROS production has also been shown in *Ae. aegypti* [31] and *Anopheles stephensi* [10] transinfected with the *w*AlbB strain from *Ae. albopictus*, making the oxidative stress' role in non-native *Wolbachia* associations inconclusive.

In natural hosts, and despite studies showing modulation of ROS and antioxidants in cell lines, a recent study in adult mosquitoes [64•] showed no differences between *Wolbachia*-free and infected *Ae. albopictus* in either ROS or antioxidant production. This result is in keeping with the hypothesis that native associations lean towards an attenuation of immune responses due to coevolution between *Wolbachia* and the host. It also suggests that the ROS-mediated Toll activation seen in *Ae. aegypti* [31] does not apply for other associations and this immune upregulation may not be a common causal factor of pathogen blocking.

Competition

The other widespread theory to explain pathogen blocking proposes that pathogens and *Wolbachia* are in competition for essential resources from their vector host that are in short supply and essential for living success. Several points of tension have been suggested including physical space, macronutrients and lipids.

Space

Different studies have shown a positive correlation between the density of *Wolbachia* in tissues and the strength of pathogen blocking [3,20,23]. Similarly to protective phenotypes, *Wolbachia* densities also appear to be greater in novel hosts as part of the initial colonisation of host tissue with associated fitness costs that decrease in generations after the establishment of infection [65]. The density of *Wolbachia* infection can be important at all levels; cellular [22], tissue [3] and within the whole organism [66]. *Wolbachia* infection levels have to be sufficiently high to allow the symbiont to be transmitted vertically but low enough not to cause host pathology and mortality. Bacterial densities are often lower in native hosts compared to those seen in transinfected species, which could explain the blocking phenotype being often more severe in the latter [65]. One scenario to explain blocking is that both an invading pathogen and bacteria are competing to use the same tissue or cellular location, regardless of other factors. Additionally, the importance of bacterial densities may just be a competition by-product between *Wolbachia* and viruses for available space. This is supported by the

exclusion of virus from cells and tissues where *Wolbachia* is highly present.

Macronutrients: carbohydrates and nitrogen

Lifetime fitness of mosquitoes is highly influenced by environmental and nutritional conditions during development. It comes as no surprise that specific nutritional components have been shown to also have an effect on infection dynamics, especially determining bacterial composition and abundance [16]. Dietary balance between protein intake (P) and carbohydrates (C) is strongly regulated by insects, with P–C balance thought to be impacting lifespan, reproduction and immunity [67]. In a recent study assessing different dietary conditions in *Ae. aegypti*, a high carbohydrate intake was found to be essential to mosquito longevity whist both extremes of carbohydrate levels lead to higher pathogen prevalence and intensity of infection [27]. Nutrition and diet are primary factors contributing to the insect's resistance to different pathogens that in turn are also dependent on resource availability and can manipulate host metabolism in order to facilitate infection.

In addition, *Wolbachia* is dependent on some of these same nutrients, including nitrogen and carbohydrates, so competition for host resources between *Wolbachia* and pathogens has been raised as a possibility to explain the pathogen interference phenotype seen in *Wolbachia*-infected hosts [25,26,68•]. The supplementation with a diet high in amino acids affects both fecundity and egg viability in those mosquitoes infected with the bacterium [26]. It has also been found that the ratio of P–C modulates *Wolbachia*'s abundance in the gut of *Drosophila* [67]. High nitrogen supply supports maximum viral replication, whereas *Wolbachia* competes for and uses nitrogen abundantly. This decrease in available nitrogen would hamper intracellular viral propagation and likely contribute to *Wolbachia*'s pathogen blocking effect.

Cholesterol

Sterols are essential components in insects, known to be vital components for membrane stability, control the regulation of different hormones (*i.e.*, ecdysteroids) and regulate development. Cholesterol is the dominant sterol in most insects and DENV and many other infecting viruses are dependent on it to successfully infect hosts [69]. DENV induces the upregulation of genes involved in fatty acid biosynthesis and relocates the machinery into its own replication complexes [58]. Some studies have found that host immune responses include sterol down regulation as means to limit viral propagation [70]. *Wolbachia* has very limited lipid biosynthesis capabilities and therefore relies heavily on host cell production to meet its requirements. The infection and effective replication of *Wolbachia* inside the cells depends on cholesterol-rich membranes [71]. Similar to DENV, *Wolbachia* also increases lipid production in the cell via the upregulation of fatty acid synthase [28]. It has been suggested that *Wolbachia*'s usage of host cholesterol could impact on the ability of viruses to replicate. Besides cholesterol, *Wolbachia* may also be competing for other key lipids that underpin pathogen blocking. Moreover, cholesterol and other lipids are present in host membranes but also as part of the Golgi apparatus, which corresponds to *Wolbachia*'s and viruses' main replication site [71]. *Wolbachia* has also been shown to affect the lipid profiles of inbred *Drosophila* and has been positively correlated to odd-chain lipid abundance [72].

Conclusion

To date the mechanism that underpins *Wolbachia*-mediated pathogen blocking is unknown, but two main theories have arisen: immune priming and competition between symbiont and pathogen for host resources. Current evidence suggests that *Wolbachia* increases immune gene expression levels in transinfected vectors, where pathogen blocking is stronger [30,38]. However, it seems that immune activation is not essential since some native *Wolbachia* strains do not induce those responses and yet they still confer some level of pathogen blocking [28]. There is also support for competition theories since *Wolbachia* and arboviruses do not often coexist in tissues when *Wolbachia* densities are high [3,22] and because manipulation of nutrients can suppress or assist pathogen replication in the presence of *Wolbachia* [25•,27]. What is emerging is a sense that there are fundamental mechanisms that may confer blocking in native and novel hosts as well as additional mechanisms that may act in novel hosts and increase the efficacy and breadth of blocking. In these novel infections, upregulation of immune regulators or effectors is quite prevalent, giving rise to crosstalk with humoral pathways as well as iron metabolism and ROS production. Dissecting the degree to which the fundamental versus the novel host specific responses contribute to the overall effect of blocking is challenging because they cut across diverse physiological and cellular processes and stem from the contribution of at least three organisms' genomes.

A recent study in flies suggests that *Wolbachia*-mediated pathogen blocking occurs early in infection [50••], before inducible immune responses would have a chance to act. In this study the fly is a natural host for *Wolbachia*, but the virus used is native to mosquitoes and so this may affect the generality of the findings. Regardless, the interpretation is that *Wolbachia* is modifying the host cell environment or interface in an intrinsic way that renders the cell inhospitable for virus [51•]. For example, *Wolbachia* may affect host protein structure or trafficking, decreasing replication times and virion release, alter lipid membrane structures or the endoplasmic reticulum [50••,51•] required for viral replication or even cause stress conditions that may lead to reduced host cell activity. Each of these avenues would also lead to the observed

correlations between density of *Wolbachia* and strength of blocking. Future research will need to focus on these fundamental and conserved components of cellular change due to *Wolbachia* infection.

When these fundamental mechanism(s) have been elucidated their individual contributions can be assessed in native hosts and then in the context of novel hosts where additional host responses may enhance the trait. The pattern of stronger pathogen blocking in novel hosts predicts that the effect is likely to decline with time and coadaptation in the artificially created associations. The question remaining is whether the fundamental mechanisms remaining will be sufficient to limit virus transmission in vectors. As such, strategies for counteracting this potential problem are already being considered, including the creation of stable double-infected mosquito lines (consisting of two *Wolbachia* strains infecting one individual) [73•] as means of boosting the immune response and prolonging pathogen blocking as a biocontrol strategy.

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