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**Review**

# Intracellular Interactions between Arboviruses and *Wolbachia* in *Aedes aegypti*

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## Abstract

*Aedes aegypti* is inherently susceptible to arboviruses. The geographical expansion of this vector host species has led to the persistence of Dengue, Zika and Chikungunya human infections. These viruses take advantage of the mosquito's cell to create an environment conducive for their growth. Arboviral infection triggers transcriptomic and protein dysregulation in *Ae. aegypti* and in effect, host antiviral mechanisms are compromised. Currently, there are no existing vaccines able to protect human hosts from these infections and thus, vector control strategies such as *Wolbachia* mass release program is regarded as a viable option. Considerable evidence demonstrates how the presence of *Wolbachia* interferes with arboviruses by decreasing cellular components vital for the pathogen and strengthening antiviral host responses. However, variation in the magnitude of *Wolbachia's* viral inhibition that is neither due to strain nor density has been observed. Furthermore, the cellular mechanisms involved in the endosymbiont's pathogen-blocking differs among hosts. This prompts the need to explore the cellular interactions between *Ae. aegypti*-arboviruses-*Wolbachia* and how these interactions overall affect the mosquito's cell. Understanding what happens at the cellular and molecular level will provide evidence on the sustainability of *Wolbachia* vector control.

Keywords: Aedes aegypti; Dengue; Zika; Chikungunya; Wolbachia; Vector control

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# 1 INTRODUCTION

*Aedes aegypti* is a target of vector control owing to its complete susceptibility to Dengue (DENV), Zika (ZIKV) and Chikungunya (CHIKV) viruses. Substantively, these arboviruses are detectable from mosquito populations worldwide resulting in geographical spread of human infections (Espinal et al., 2019). To curb increasing prevalence of arboviral diseases, vector control has been at the forefront with *Wolbachia* drawing considerable attention this decade (Indriani et al., 2020). The use of *Wolbachia* to control DENV, ZIKV and CHIKV transmission to humans first stems from its ability to establish a symbiotic relationship with *Ae. aegypti* (Moreira et al., 2009). *Wolbachia* modifies the host's gametes that results in cytoplasmic incompatibility or sperm-egg mismatch unable to form a viable offspring (Johnson, 2015). Other than reproductive manipulation, multiple evidences prove that *Wolbachia* significantly inhibits viral replication yet little information is known on the cellular mechanisms underlying this antiviral impact.

Previous studies have reported that in general, *Wolbachia* confers a pathogen blocking phenotype in arthropods either by priming the host's immunity and/or fighting for scarce host cellular resources (Caragata et al., 2016; Lindsey et al., 2018). For instance, *Wolbachia* transinfection in *Ae. aegypti* leads to the activation of antiviral mechanisms along with an elevated production of antimicrobial peptides (Terradas and McGraw, 2017). Similarly, *Anopheles gambiae* transiently infected with the endosymbiont displays an upregulated expression of malaria-related immune genes and reduction in *Plasmodium* infection, confirming *Wolbachia's* direct influence on host immunity (Kambris et al., 2010). Other studies have also shown that host cellular resources mainly cholesterol is an essential determinant of *Wolbachia's* viral inhibition in *Drosophila* (Caragata et al., 2013) whereas gene network analysis involving the same insect species and other RNA viruses reveal strong interactions between metabolism pathways related to host nutrient production and viral replication (Lindsey et al., 2021).

Existing review articles on *Wolbachia's* pathogen blocking effect present a general discussion in various arthropod species (Caragata et al., 2016; Kamtchum-Tatuene et al., 2017; Lindsey et al., 2018; Pimentel et al., 2021). Two of these papers have enumerated different *Wolbachia* strains that inhibit viruses and have focused on how factors e.g. density, tissue tropism and type of virus, affect such interference (Caragata et al., 2016; Pimentel et al., 2021). Although others have presented cellular mechanisms responsible for pathogen blocking, these papers only covered competition for cholesterol and lipids, cellular stress and immunity (Kamtchum-Tatuene et al., 2017; Lindsey et al., 2018). Therefore, we expand the coverage of these cellular mechanisms by adding direct viral inhibition via host cytoskeleton (Lu et al., 2020), antagonistic lipid modulation (Koh et al., 2020) and cellular regeneration (Ford et al., 2019) based on recent reports. We also incorporate new information on how *Wolbachia's* density/strain may not be a contributing factor to immunity contrary to previous findings. Furthermore, we also present the corresponding host gene and protein changes linked to each cellular mechanism.

This review aims to explore *Ae. aegypti's* cellular mechanisms which *Wolbachia* regulates to block arboviruses. We begin by illustrating how these medically important viruses change the

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gene/protein expression patterns of the host cell and exploit corresponding cellular mechanisms to strengthen viral infection. Subsequently, we will discuss how *Wolbachia* directly or antagonistically interferes with *Ae. aegypti*-arbovirus interactions through a) inhibition of viral entry and replication, b) reduction of specific nutrients required in arboviral infection, c) increase in ROS production and immunity, d) cellular regeneration to enhance midgut barrier and e) regulation of genes with various cellular functions (Hussain et al., 2011, 2011; Ford et al., 2019; Koh et al., 2020; Lu et al., 2020). Finally, we summarize the effects on *Ae. aegypti* when infected with either virus or *Wolbachia* or both. Given that *Ae. aegypti* is a target of *Wolbachia* release programs, focusing on mechanisms specifically linked to this mosquito specie will provide significant information on the sustainability of vector control strategy.

## *2* MOLECULAR INTERACTIONS BETWEEN ARBOVIRUSES AND *AE. AEGYPTI*

*Ae. aegypti's* intrinsic ability to act as a vector for disease is supported by its genome ingrained with genes that regulate cellular mechanisms for arboviral infection and defense. The latest annotated reference genome (AaegL5) of this mosquito portrays a wider coverage of gene families like chemosensory receptors, glutathione S-transferase and C-type lectin with some unique quantitative trait loci (chromosome 2), all of which are paramount to viral susceptibility (Adelman and Myles, 2018; Matthews et al., 2018). DENV, ZIKV and CHIKV, to some extent, impose differential transcriptomic changes and alter the function of translated proteins in *Ae. aegypti* (Mukherjee et al., 2019). Specifically, these arboviruses cause differential expression of transcripts under cytoskeletal, replication/transcription, immunity, reactive oxygen species (ROS) and metabolism to control *Ae. aegypti's* intracellular environment (Angleró-Rodríguez et al., 2017; Carvalho-Leandro et al., 2012; Colpitts et al., 2011; Mukherjee et al., 2019; Ramirez and Dimopoulos, 2010; Sim et al., 2013; Xi et al., 2008; Zhao et al., 2019) DENV, ZIKV and CHIKV induce these changes in the mosquito in order to thrive without being pathogenic, allowing them to complete their life cycle (Mukherjee et al., 2019). In this section, we concentrate on the effects of arboviral infection on *Ae. aegypti's* transcriptome, with only a brief discussion of proteomic and metabolomic expression patterns. These changes will be discussed together with the cellular mechanisms they affect to demonstrate host-virus interactions. Altogether, we explore the mechanisms on how arboviruses enforce viral infection and *Ae. aegypti's* responses at the cellular level.

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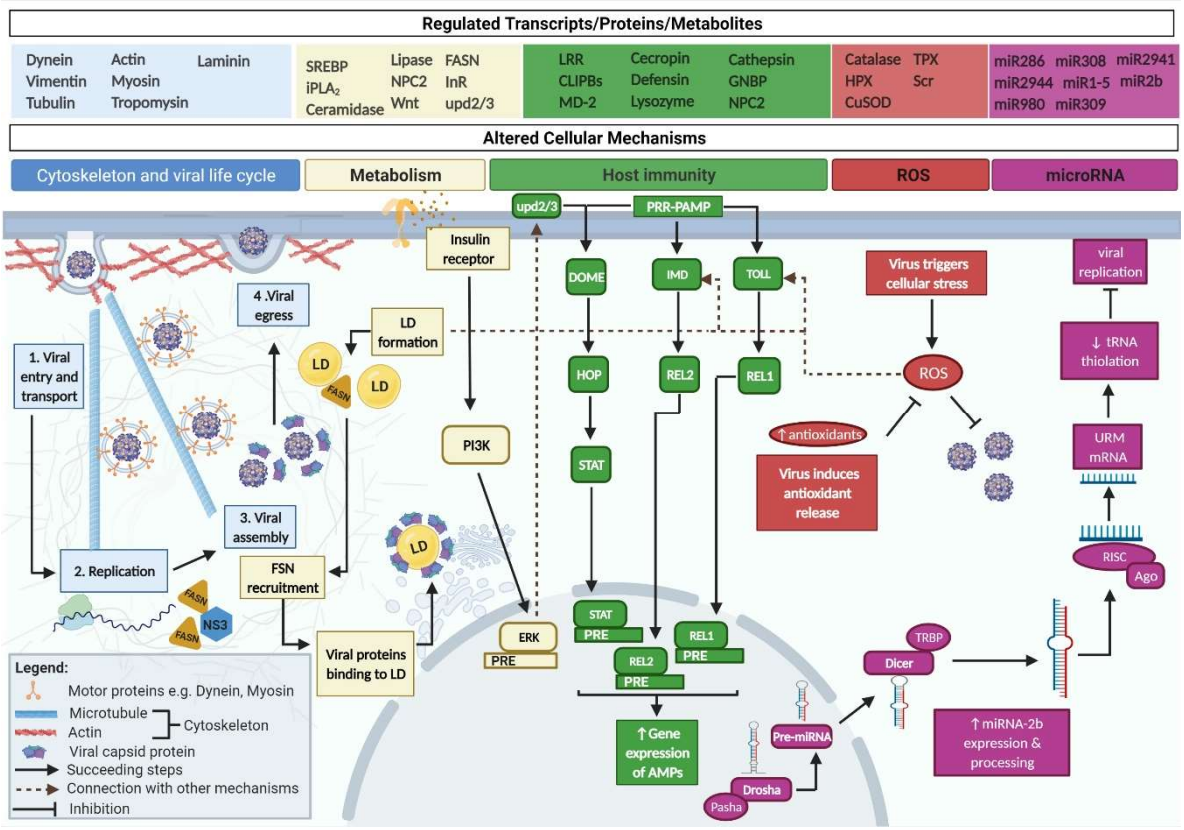


**Figure 1.** ***Ae. aegypti*** **cellular mechanisms are affected by** ***Arboviral infection.*** Arboviral infections alter *Ae. aegypti's* genes, proteins and metabolites to control the host's cellular mechanisms. First, arboviruses utilize host cytoskeletal proteins (e.g. dynein and myosin) to facilitate their intracellular transport. Second, the need for host cell nutrients allows the virus to alter host lipid/cholesterol and also triggers the formation of LD. LD can enhance host immunity via Toll/Imd, can aid viral replication through fatty acid synthase (FASN) recruitment by DENV NS3 and can be integrated into viral capsids. The activation of insulin receptor is also said to signal antiviral mechanisms i.e. ERK and JAK/STAT via upd2/3. Following activation of host immunity, antimicrobial peptides are produced. Third, arboviruses cause *Ae. aegypti* to produce elevated ROS. ROS can either directly harm invading arboviruses or activate the Toll immune mechanism. Arboviruses respond to the effects of ROS by upregulating antioxidant release which functions to lower down ROS in the cell. Fourth, miRNA-2b is initially processed inside the nucleus and brought out to the cytoplasm for further cleaving. A seed sequence produced from the RISC complex binds to URM, its target mRNA leading to the suppression of tRNA thiolation and inhibition of viral replication.

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## 2.1 Completion of Arboviral Life Cycle and Evasion of *Ae. aegypti's* Midgut Barriers Strengthen Infection

Arboviral infection in *Ae. aegypti* begins with the ingestion of a viremic blood meal followed by an Extrinsic Incubation Period (EIP). EIP is a period of viral incubation within the host where virus enters the midgut, passes through the hemolymph to reach other organs and culminates in the salivary glands for subsequent infection (Chan and Johansson, 2012). Throughout the EIP, arbovirus tries to take over the host cell by using the cytoskeleton and overcoming midgut barrier.

*Ae. aegypti's* cytoskeleton, composed of a network of microtubules and actin filaments, is required for an infecting arbovirus to successfully traverse viral entry, replication, assembly and egress **(Figure 1)** (Foo and Chee, 2015). During infection, arboviruses cause differential expression of *Ae. aegypti's* cytoskeletal transcripts and proteins which are cellular components essential for viral life cycle. Genes like dynein, vimentin, tubulin, actin, myosin, tropomyosin and laminin are highly expressed in DENV-infected *Ae. aegypti* (Bonizzoni et al., 2012; Sim et al., 2012) whereas CHIKV infection has shown marked cytoskeletal protein expression (Cui et al., 2020) as opposed to the uninfected. These gene/protein modifications within the host may represent the way arboviruses' take advantage of the mosquito's cytoskeleton by rearranging it into tracks to actively transport endosomes containing viral particles (Walsh and Naghavi, 2019). To facilitate transport at a later phase, direct interaction between arboviral proteins and host cytoskeletal motor proteins (e.g., dynein and myosin) occur (Paingankar et al., 2010; Mairiang et al., 2013; Foo and Chee, 2015; Walsh and Naghavi, 2019). In the case of DENV infection in *Ae. aegypti*, link between nonstructural (NS) protein 5 and myosin has been reported (Mairiang et al., 2013). Similarly, other cytoskeletal structures like actin and tubulin is said to interact with DENV to facilitate infection *in vitro* (Paingankar et al., 2010). Protein interaction network prediction in *Ae. aegypti* also suggests that tubulin is highly associated with DENV infection in the mosquito host with roles in transport and assembly (Guo et al., 2010). In CHIKV infection, impaired viral delivery from the cell surface to the cytoplasm occurred when microtubule network is disrupted (Hoornweg et al., 2020). While ZIKV's effect in *Ae. aegypti's* cytoskeleton has not been fully explored, differential expression of cytoskeletal-related transcripts signifies that ZIKV likely uses the cytoskeleton for its entry, replication, assembly and egress as other arboviruses (Etebari et al., 2017).

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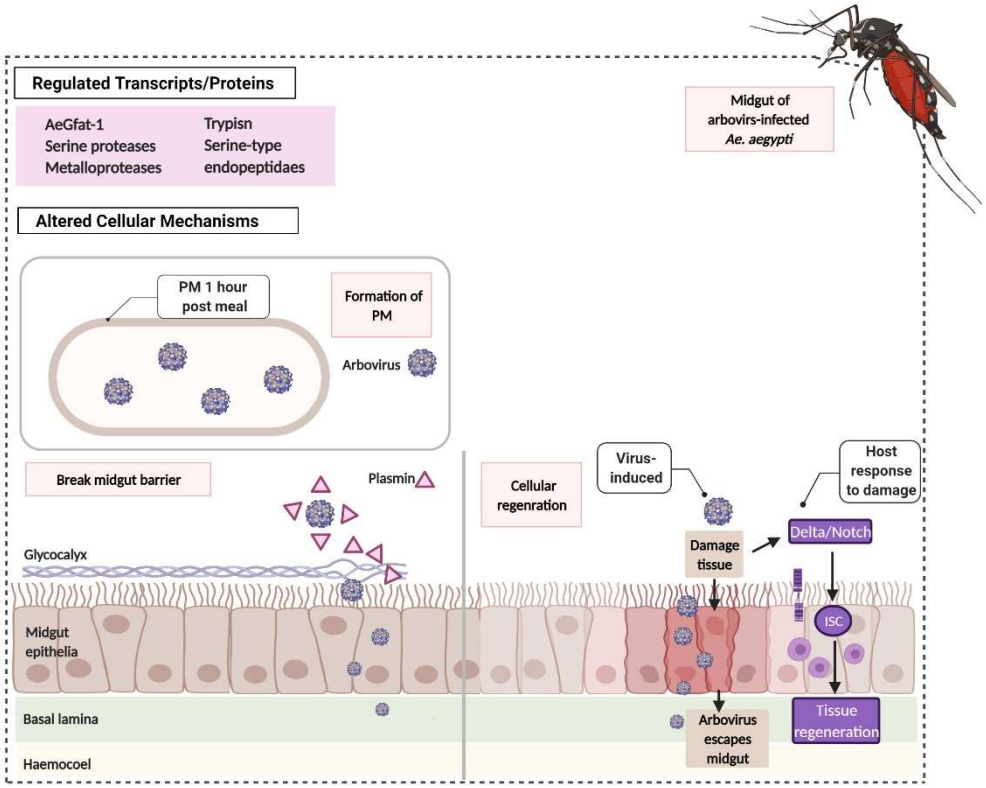


**Figure 2. Arboviruses induce the formation of PM, cleaving of protective layer and Delta/Notch in *Ae. aegypti's* midgut.** An hour from the ingestion of virus-infected blood meal, *Ae. aegypti* forms a thick peritrophic membrane (PM) that encloses the virus separating it from the midgut epithelia. This PM prevents the virus from escaping the midgut and spreading the infection. Arboviruses may utilize proteolytic enzymes e.g. plasmin to break the glycocalyx, a protective layer at the surface of the midgut epithelia. Arboviruses may also directly damage the midgut tissue. *Ae. aegypti* can reverse this damage by activating the Delta/Notch resulting in the proliferation of Intestinal Stem Cells (ISC) for cellular regeneration.

Following a blood meal, *Ae. aegypti* transcribes the glucosamine fructose-6 phosphate aminotransferase (*AeGfAt-1*) gene involved in the formation of a chitinous sac called peritrophic membrane (PM) that encloses the ingested blood (**Figure 2**). PM formation happens in the midgut within 6 to 12 hours post feeding and is a physiological response for digestion (Kato et al., 2002; Suwanmanee et al., 2009). However, intake of blood with DENV forms a PM earlier, showing clear visibility just an hour after meal and is otherwise thicker (Suwanmanee et al., 2009). It has been speculated that PM could function as part of the Midgut Infection Barrier (MIB) by preventing the virus from penetrating the midgut epithelium and reaching other mosquito organs. This is consistent with the reports that demonstrate how PM hinders systemic infection in other vector mosquitoes

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(Rodgers et al., 2017). Aside from the upregulation of *AeGfAt-1*, DENV-, ZIKV- and CHIKV-infected *Ae. aegypti* markedly express proteolytic transcripts (e.g. serine proteases, metalloproteases, trypsin, serine type endopeptidases) concurrent with specific abundance in protein composition of serine type proteases (Cui et al., 2020; Etebari et al., 2017; Sim et al., 2013; Whiten et al., 2018). These proteases can breakdown other proteins that strengthen the midgut barrier and can therefore be utilized by arboviruses to assert systemic infection. Recent evidence demonstrates that in *Ae. aegypti* mosquitoes, a protease called plasmin enhances DENV infection by breaking glycocalyx, a layer covering midgut epithelia cells whereas inhibition of plasmin's activity resulted in low infection **(Figure 2)** (Ramesh et al., 2019).

Although arboviruses exploit different host cellular structures, viral replication and dissemination inside the host can be reversed by *Ae. aegypti's* ability to regenerate the midgut epithelium (**Figure 2**). *Taracena et al.* describes how the exposure of the midgut epithelium to stress i.e. presence of arbovirus can result in the damage of the tissue. To counteract this, the host stimulates the proliferation of Intestinal Stem Cells (ISCs) responsible for cellular regeneration via a signaling pathway referred to as the Delta/Notch. Delta/Notch is able to increase DENV susceptibility of a refractory strain of *Ae. aegypti* when inhibited (Taracena et al., 2018). Hence, cellular regeneration via the Delta/Notch pathway is another important cellular response for arboviral blockade in *Ae. aegypti*.

## 2.2 Metabolic Changes Facilitating Viral Replication and Inhibition

Arboviruses take over *Ae. aegypti's* metabolism by disrupting lipid homeostasis intracellularly as demonstrated in the mosquito's altered transcriptome and metabolome. *Ae. aegypti* infected with either of these arboviruses demonstrates high abundance of differentially expressed genes/proteins for lipid biosynthesis **(Figure 1)** (Tchankouo-Nguetcheu et al., 2010; Raquin et al., 2017; Royle et al., 2017; Shrinet et al., 2017; Chotiwan et al., 2018; Fukutani et al., 2018). DENV infection in particular upregulates sterol regulatory element-binding protein *(SREBP)*, calcium independent phospholipase A2 *(iPLA$_2$)*, ceramidase, lipase, Niemann pick-type C2 protein *(NPC2)* and Wnt pathway regulator genes significantly relative to uninfected control (Raquin et al., 2017). Moreover, elevated glycerophospholipids, glycerolipids, sphingolipids and fatty acids are found not just in DENV-infected *Ae. aegypti* but also those infected with ZIKV and CHIKV. All in all, these represent a significant increase in lipids during infection (Chotiwan et al., 2018; Royle et al., 2017; Tchankouo-Nguetcheu et al., 2010). The exact cellular pathway that links these genes and metabolites to virus infection remain undiscovered. *SREBP* is the only gene whose function has been directly attributed to promote DEN infection whereas its knockdown decreases the viral load significantly (Raquin et al., 2017).

Further reports have associated high lipids with the viral replication and formation of lipid rich structures called lipid droplets (LD; **Figure 1)** (Heaton et al., 2010; Barletta et al., 2016). Initially, LD is regarded as a reservoir for cholesterol but has later been discovered to have a dynamic role. LD formation is accompanied by an increase in fatty acid synthase enzyme that catalyzes LD metabolism during DENV and CHIKV infection (Heaton et al., 2010; Tchankouo-Nguetcheu et al., 2010). Fatty

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acid synthase is said to be recruited by DENV NS3 at the site of replication in the absence of other NS proteins. Alongside its role in viral replication, LD is incorporated in the viral capsid during assembly. LD has also been linked to antiviral immunity in *Ae. aegypti* given that its accumulation strengthens the host's immunity by activating Toll and Immune deficiency (Imd) cellular mechanisms (Barletta et al., 2016).

Interestingly, recent finding suggests that insulin and its receptor strengthen immunity against DENV and ZIKV infection in *Ae. aegypti* (Ahlers et al., 2019) by activating Janus kinase/signal transducers and activators of transcription (JAK/STAT). When mosquito cells are treated with insulin, the insulin receptor (InR) activates a cellular mechanism called the phosphatidylinositol 3'-kinase (PI3K) signaling pathway that send signals to the extracellular signal-regulated kinases (ERK). Once ERK is activated, it is said to prompt the release of molecules under the unpaired (upd2/3) family. Upd2/3 then engages the Domeless receptor (Dome), that sends signals to downstream proteins under the JAK/STAT leading to arboviral inhibition (Ahlers et al., 2019).

## 2.3 The Tug of War between Host's Immune System and Viral Infection

The involvement of the host's immunity as a key player in regulating arboviral infection has been corroborated in several studies (Angleró-Rodríguez et al., 2017; Caicedo et al., 2019; Carvalho-Leandro et al., 2012; Colpitts et al., 2011; Mukherjee et al., 2019; Sim et al., 2013; Xi et al., 2008; Zhao et al., 2019). Arboviruses cause *Ae. aegypti* to actively respond to infection by switching immune-related genes on/off and utilizing the same genes to dictate the extent of viral susceptibility. This manifests at the transcriptomic level where high expression of leucine rich repeat (LRR) – containing proteins, Clip-domain serine proteases (CLIPBs) and Myeloid differentiation 2 - related lipid recognition protein (MD-2) receptors are noted (**Figure 1**) (Angleró-Rodríguez et al., 2017; Caicedo et al., 2019; Xi et al., 2008; Zhao et al., 2019). LRR, CLIPBs and MD-2 initiates *Ae. aegypti's* immunity by functioning as Pattern Recognition Receptors (PRRs). PRRs recognize viral particles as Pathogen-Associated Molecular Patterns (PAMPs) at the surface of the host cell (Mukherjee et al., 2019; Xi et al., 2008). Consequently, the PRR-PAMPs binding causes Upd ligands to associate with Dome stimulating JAK tyrosine kinase Hopscotch (Hop) to phosphorylate. In turn, STAT proteins are activated and translocated to the nucleus where it is able to bind to the palindromic response element (PRE) to induce gene expression. The same signaling cascade applies to the Imd and Toll pathways only that different ligands and receptors are activated. Downstream of these pathways are proteins referred to as Relish (REL2, REL1) that binds to the PRE in the nucleus and triggers the expression anti-microbial peptides (AMPs) i.e. cecropin, defensin, lysozyme and cathepsin (Hoffmann, 2003; Jang et al., 2006; Mukherjee et al., 2019). AMPs can directly kill microbes or further enhance the immunity. In both midgut and carcass tissue of DENV-, ZIKV- and CHIKV-infected *Ae. aegypti*, AMP transcripts are highly evident (Angleró-Rodríguez et al., 2017; Sim et al., 2013; Xi et al., 2008). More so, silencing Cactus and Caspar that are inhibitory to Toll and Imd resulted in enhanced antimicrobial peptide gene expression (Xi et al., 2008). Indeed, these studies provide the basis for *Ae. aegypti's* natural ability to resist viral infection. Contrary to this, viruses can increase host arboviral susceptibility by downregulating the same AMP genes as seen in

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transcriptomic and *in vitro* studies of DENV-, ZIKV- and CHIKV-infected mosquitoes (Carvalho-Leandro et al., 2012; Colpitts et al., 2011; Sim and Dimopoulos, 2010). Some immune-related genes have also been directly linked to susceptibility. In a recent study by *Caicedo et al.,* genes such as Gram negative binding protein – GNBP *(AAEL009176)*, NPC2 *(AAEL015136),* Keratinocyte lectin *(AAEL009842)* and Cathepsin-b *(AAEL007585)* when inhibited in a susceptible strain of *Ae. aegypti* led to a significant reduction in DENV dissemination (Caicedo et al., 2019). This proves the importance of these genes and their corresponding functions in DENV infection.

## 2.4 Inhibition of Reactive Oxygen Species (ROS) Promotes Arboviral Infection

Arboviral infection results in production of ROS as part of the physiological response of *Ae. aegypti* to cellular stress (**Figure 1**). Excess ROS production act as secondary messengers that signals the innate immune response and can directly damage invading microbes (Tchankouo-Nguetcheu et al., 2010; Bottino-Rojas et al., 2018). To ensure infection, these arboviruses can neutralize the upregulated activity of ROS antiviral defense by modifying the gene expression profile of *Ae. aegypti*. Transcripts classified as antioxidants are markedly increased in arbovirus susceptible mosquito host. These antioxidants scavenge the ROS to decrease its harmful effects (Oliveira et al., 2017; Shrinet et al., 2018; Wang et al., 2019). During DENV infection, the antioxidant catalase functions to balance ROS and increase DENV concentration in mosquito's midgut epithelia (Oliveira et al., 2017). Antioxidant hemopexin (HPX) exerts the same effect and enhances not just DENV infection but also ZIKV in *Ae. aegytpi* (Wang et al., 2019). Concomitantly, antioxidant transcripts like superoxide dismutase (CuSOD), thioredoxin peroxidase (TPX) and scavenger reporter (Scr) in *Ae. aegypti* infected with individual DENV/CHIKV as well as co-infection have demonstrated significant abundance (Shrinet et al., 2018). Downregulating the expression of antioxidants results in the reduction of DENV, ZIKV and CHIKV titer (Oliveira et al., 2017; Wang et al., 2019). Similar to the manner by which virus directly exploits host immunity to their advantage, arboviruses must also ensure that oxidative stress represented by an increase in ROS is circumvented by adequate antioxidant release.

## 2.5 Micro-RNAs Regulate *Ae. aegypti* Genes that affect Viral Infection

Arboviruses utilize *Ae. aegypti's* micro-RNAs (miRNAs) for infection albeit the host can also use these for viral inhibition. miRNA is a type of non-coding RNA (ncRNA) that bind to DNA/RNA to either enhance or suppress the function of genes under various functional categories (Campbell et al., 2014). Initially, pre-miRNAs are composed of ~22 nt base-paired strands that undergo cleaving by Drosha and Pasha within the nucleus (**Figure 1**). Subsequently, it goes through additional modifications in the cytoplasm via RISC-loading complex composed of Dicer, Argonaute and RNA binding protein where selection of one strand (aka guide strand) occurs. This guide strand constitutes 2-6 nucleotides and is also referred to as the seed sequence that directly binds to the target mRNA and regulate it. In general, arboviral infection give rise to a significant decline in *Ae. aegypti* global miRNA expression (Dubey et al., 2017, 2019; Saldaña et al., 2017). *Saldaña et al.* reported 17 miRNAs that are significantly regulated in three time points during ZIKV infection. Maximum

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negative fold change has been observed in *aae-miR-286a, aae-miR-2944b-3p*, *aae-miR-980-3p* at 2 days post infection (dpi) and *aae-miR-308-3p* (7 dpi) whilst *aae-miR-2940-3p* and *aae-miR-1-5p* are enriched at 14 dpi. In addition, the abundance of some of the miRNAs particularly *aae-miR-309-a* and *aae-miR-2941* are altered (Saldaña et al., 2017). A comparable observation has been reported in DENV2-infected *Ae. aegypti* (Campbell et al., 2014) but further studies investigating their target genes and effects are warranted. To illustrate the role of miRNA during virus infection, miRNAs and its potential targets in *Ae. aegypti* infected with CHIKV have been analyzed. *miR-2b* is one of the most significantly upregulated miRNA said to regulate ubiquitin-related modifier (URM), a cellular factor that promotes CHIKV replication (Dubey et al., 2017). This miRNA binds to the URM gene thereby exerting a suppressive effect on tRNA thiolation, a process required for gene translation. By inhibiting tRNA thiolation, CHIKV viral load is reduced (Dubey et al., 2017). Additionally, *miR-2944b-5p* has been said to enhance vps-13 expression which in turn stabilizes CHIKV in *Ae. aegypti* cells by maintaining mitochondrial stability (Dubey et al., 2019).

## 3 *WOLBACHIA* AS AN ARBOVIRAL INHIBITOR

Mass release programs have been implemented to deploy *Wolbachia* in different communities that so far led to a reduction in dengue cases (O'Neill et al., 2019). Studies demonstrate that *Wolbachia* strains from other species when transinfected into *Ae. aegypti* induces cytoplasmic incompatibility suppressing the mosquito population and are inhibitory to arboviruses (Lindsey et al., 2018). However, there is growing evidence that different *Wolbachia* strains carry varying blocking effect in *Ae. aegypti* with some strains failing to reduce viral replication and transmission despite being present at high density (Fraser et al., 2020).

*Wolbachia* must use the same cellular mechanisms that DENV, ZIKV and CHIKV exploit to potently inhibit arboviral infections in *Ae. aegypti.* The previous sections explained how arboviruses exploit *Ae. aegypti* cellular machinery by utilizing the mosquito's cytoskeletal elements for viral infection and proteolytic enzymes to break the midgut barrier. In addition, arboviral infections take advantage of the host nutrients for their propagation. Counteracting ROS through antioxidant release and miRNA generation are also means for creating a conducive intracellular environment for these arboviruses to thrive. In the following sections, we present how *Wolbachia* adds another layer to *Ae. aegypti*-arbovirus interaction by interfering with the same molecular mechanisms and inducing cellular perturbations that are detrimental to the pathogen.

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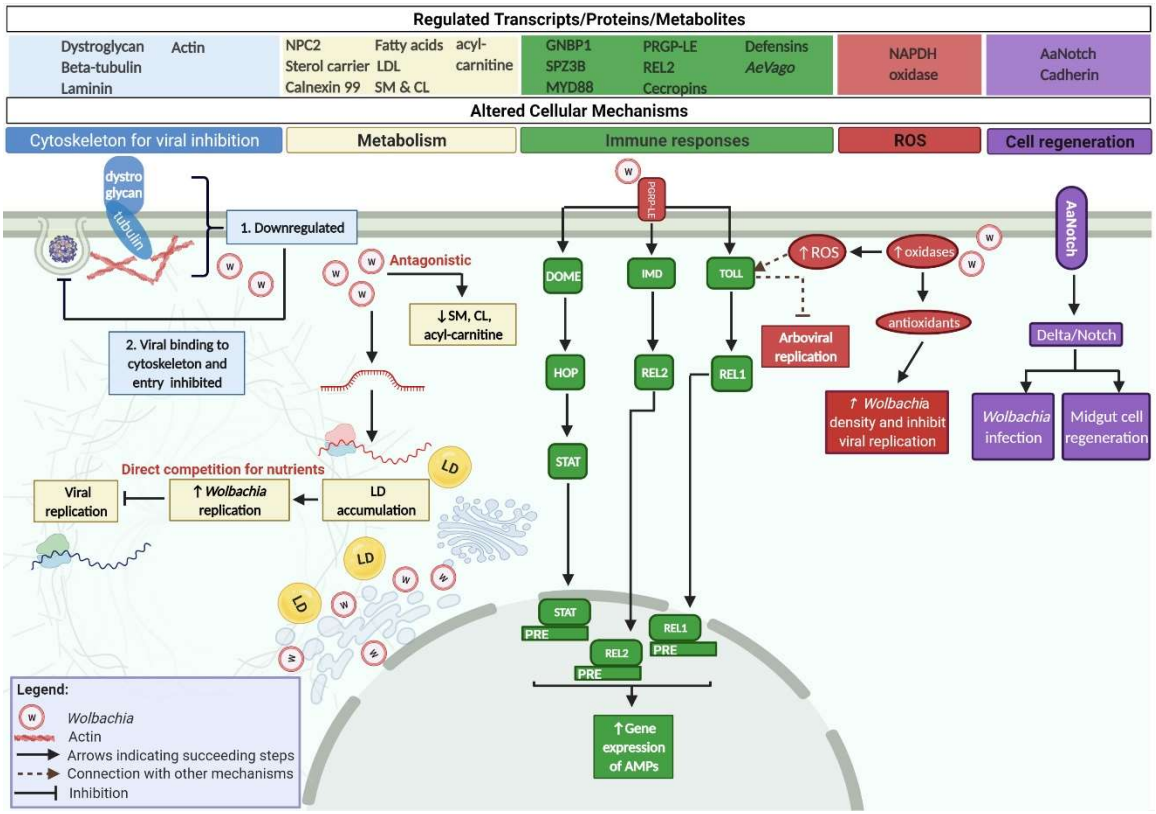


**Figure 3. *Wolbachia* interferes with Arboviral life cycle by interfering with host factors and strengthening host antiviral mechanisms.** *Wolbachia* blocks DENV, ZIKV and CHIKV by interfering with the same cellular mechanisms arboviruses control in *Ae. aegypti.* First, *Wolbachia* downregulates cytoskeletal structures that directly bind to arboval proteins for host cell infection. Next, *Wolbachia* competes for host's lipid/cholesterol for its replication making this nutrient insufficient for the virus. *Wolbachia* can also deplete lipids namely spingomyelins (SM), cardiolipins (CL) and acyl-carnitine that are essential for viruses. *Wolbachia* also strengthens the host's immunity by activating Toll, IMD and JAK/STAT via PGRP-LE. The endosymbiont further induces ROS release in the presence of an arbovirus by increasing oxidases in the cell. This inhibits viral replication and in turn, stimulates the Toll pathway. *Wolbachia* then counteracts the release of antioxidants to sustain its infection within the host. Lastly, *Wolbachia* upregulates receptor gene, *AaNotch* in *Ae. aegypti*. This receptor gene signals the Delta/Notch pathway. Consequently, the adhesion molecule Cadherin is increased strengthening the host's reproductive ability and ensures maintenance of *Wolbachia.* Delta/Notch also promotes the regeneration of the midgut epithelia to prevent systemic viral infection.

## 3.1 *Wolbachia* Uses *Ae. aegypti's* Cytoskeleton to Inhibit Viral Binding and Entry

Studies have attributed *Wolbachia's* pathogen blocking effect on its ability to decrease viral load. The mechanism by which this effect is accomplished, as well as the point in the virus' life cycle at which such interference occurs, are previously unknown. Present study reveals that transinfected

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*wAlbB* strain in *Ae. aegypti* (Aag2) cell line infected with either DENV or ZIKV caused the downregulation of cytoskeletal membrane proteins, dystroglycan and beta-tubulin (**Figure 3**) (Lu et al., 2020). Concurrently, viral binding assays display significant reduction in viral RNA copy number as early as 2 hours post infection suggestive of an early viral interference in DENV and ZIKV binding as well as entry. Further validation done by silencing both cytoskeletal membrane proteins inhibited DENV binding to Aag2 cells (Lu et al., 2020). This is the first demonstration to confirm the direct involvement of *Wolbachia* in arbovirus binding and entry by taking advantage of the same host cytoskeletal proteins DENV and ZIKV utilize. Although there is a lack of data that demonstrates the same mechanism in CHIKV, this arbovirus necessitates an intact microtubule network consisting of alpha-tubulin (same family as beta-tubulin) for efficient viral entry and genome delivery to replication sites (Hoornweg et al., 2020).

In other host species that harbor *Wolbachia* infection, cytoskeletal proteins are vital for maintaining the endosymbiont's density (Baldridge et al., 2014; Sheehan et al., 2016). Native *Wolbachia* in *Drosophila* expresses a type IV secretion system (T4SS) that releases an effector molecule called *Wolbachia* actin-localizing effector 1 protein (WalE1) (Sheehan et al., 2016). WalE1 directly binds to actin aiding in *Wolbachia's* localization within the host (Sheehan et al., 2016). This is consistent with the report on significant reduction of the endosymbiont's density coupled with inefficient maternal transmission when mutations in the actin gene are present (Newton et al., 2015). In like manner, *Ae. albopictus* expresses T4SS and a corresponding WalE1 homolog which may suggest contribution to the maintenance of *Wolbachia* infection in this specie (Baldridge et al., 2014).

Based on the evidence, *Wolbachia* interacts with the host cytoskeleton in two ways. First, its ability to secrete effector molecules that bind to cytoskeletal structures ensures that *Wolbachia* resides in the host cell at an optimal density and guarantees transmission to other hosts. Second, *Wolbachia* regulates the expression of cytoskeletal proteins like laminin, actin, dystroglycan and tubulin, required for arboviral infection (Lu et al., 2020). It is remarkable how arboviruses and *Wolbachia* have contrasting effects on the host cytoskeleton. While the former upregulates cytoskeletal structures for virus entry, replication, assemble and egress, the latter downregulates the same structures to block arboviral binding/entry (Sim et al., 2012, 2013; Lu et al., 2020).

### 3.2 Lipid Perturbations Caused by *Wolbachia* are antagonistic to Arboviruses

*Wolbachia* localizes within golgi-related vesicles in close proximity to the endoplasmic reticulum, the site of cell membrane biogenesis (Cho et al., 2011). Provided that membrane biogenesis relies on host lipids, this position allows *Wolbachia* to acquire nutrients for itself (Fattouh et al., 2019). Presence of *Wolbachia* strains, *wMelpop* and *wMel,* in *Ae. aegypti* result in an increase in stored cholesterol with an accumulation of LD (**Figure 3**) (Geoghegan et al., 2017) This is represented by an upregulation of Niemann-Pick type C2 (*NPC2*), sterol carrier protein 2, calnexin 99 and a simultaneous downregulation of fatty acid synthase and LDL receptor proteins corresponding to a perturbed intracellular cholesterol transport (Geoghegan et al., 2017). Several studies have pointed out that these metabolic changes induced by *Wolbachia* could be responsible for the pathogen-blocking mechanism in transinfected *Ae. aegypti*. Significant increase in the host's

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cholesterol content takes place due to *Wolbachia's* sequestration of cholesterol and lipid droplets, the same nutrients crucial for DENV, ZIKV and CHIKV replication (Frentiu, 2017; Cui et al., 2020; Leier et al., 2020). *Wolbachia* and arboviruses' need for the same resources become the theoretical basis for competition between the two microbes with *Wolbachia* surpassing viral infection for its self-preservation (Frentiu, 2017). Conversely, recent studies propose that instead of competition for lipids, antagonistic lipid modulation occurs (Koh et al., 2020; Manokaran et al., 2020). In the study by *Koh et al.,* mono-infection of DENV results in lipid abundance whereas *w*Mel causes mild depletion. However, co-infection of DENV and *w*Mel in *Ae. aegypti* displays a lipid profile that resembles DENV induced perturbations signifying arbovirus control while certain lipids have been reported to be indirectly antagonistic (Koh et al., 2020). Take for example spingomyelins (SM) and cardiolipins (CL) which were enriched in DENV3-infected *Ae. aegypti* but depleted in the presence of the same virus and the endosymbiont. CL knockdown also decreased DENV load regardless of *w*Mel's presence but replication of *w*Mel is impaired only in the absence of DENV (Koh et al., 2020). This antagonistic interaction also applies in another study where another class of lipids called acyl-carnitine is elevated during DENV and ZIKV infection but significantly diminished in *wMel*-infected Aag2 cells (Manokaran et al., 2020). Reduction in acyl-carnitine enhanced *wMel* density yet addition of the said lipid in Aag2 containing *wMel* boosts DENV and ZIKV infection (Manokaran et al., 2020).

*Wolbachia* is similar with arboviruses in a sense that both are unable to synthesize lipids making them dependent on the host cell. While this competition means that one must utilize host nutrients at the expense of the other, recent findings show us that co-infection of these microbes within *Ae. aegypti* may also entail an antagonistic interaction. Perhaps the virus tries to establish an intracellular environment high in lipids for its replication which *Wolbachia* hampers.

Meanwhile, other factors that play a role in lipid homeostasis is insulin which promotes fat storage in cells. Latest study reveals that DENV and ZIKV suppression is mediated by the downregulation of insulin receptor in *wMel*-transinfected *Ae. aegytpi* (Haqshenas et al., 2019). In the previous section, insulin has been linked to the activation of host immune system. The underlying interaction between *Wolbachia* and arboviruses in the context of this mechanism warrants further investigation.

### 3.3 *Wolbachia* Induces Variable Immunity in *Ae. aegypti* and the Role of Reactive Oxygen Species (ROS)

Existing view posits that trainsinfected *Wolbachia* infection in *Ae. aegypti* primes the mosquito's immune system prior arboviral infection (Pan et al., 2018) yet the extent of immune activation differs among *Wolbachia* strains. Similar with the host's response to virus, *wAlbB*-transinfected *Ae. aegypti* upregulates genes under the Toll (GNBP1, SPZ3B, MYD88) and Imd (PRGP-LE, REL2) pathways leading to antimicrobial peptide (e.g. cecropins, defensins) release during arboviral infecton (**Figure 3**) (Angleró-Rodríguez et al., 2017; Pan et al., 2018; Xi et al., 2008; Zhao et al., 2019). Furthermore, innate immunity can be activated by ROS indicating cross-talk between immune and redox mechanisms (Pan et al., 2012). With DENV infection, *wAlbB* stimulates the production of NAPDH oxidases which are key producers of ROS. ROS then activates the Toll

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pathway to reduce DENV load. These oxidases, when silenced, deactivate the host's immunity leading to an increased DENV titer (Pan et al., 2012). Simultaneously, Toll controls antioxidant expression that helps *wAlbB* maintain its infection in *Ae. aegypti* (Pan et al., 2012). It is interesting to note that ROS-dependent immune pathway activation does not occur in *Wolbachia's* natural host, *Ae. albopictus* (Molloy and Sinkins, 2015). Adult mosquito lines carrying transinfected *w*Alb and *w*Mel that varies in density show neither an upregulation in ROS-related genes nor innate immune activity (Molloy and Sinkins, 2015). In the same study, this effect has been observed *in vitro* using *w*MelPop, *w*Mel and *w*AlbB transinfection suggesting that ROS-dependent immune activation could be unique to *Ae. aegypti* (Molloy and Sinkins, 2015).

Meanwhile, *wMelpop-CLA* blocked DENV and CHIKV via JAK/STAT in *Ae. aegypti* (Moreira et al., 2009; Asad et al., 2018). *wMelpop-CLA* inhibits DENV through an upregulation of a Vago protein homolog (*AeVago*) that activates JAK/STAT (Asad et al., 2018). In CHIKV, this strain has been observed to cause an upregulation of AMPs but displayed unparalleled results in two independent experiments in terms of transcriptional modulation of genes involved in immunity (Moreira et al., 2009). All in all, multiple strains of *Wolbachia* demonstrate arboviral blocking that is partly mediated by the host's immunity yet there remains an underlying issue on the magnitude of this effect. And whether this immunity merely depends on the strain or density is still debatable.

A recent study that compared 8 *Wolbachia* strains in a consistent *Ae. aegypti* line provided deeper understanding of viral blocking based on the endosymbiont's strain and density (Fraser et al., 2020). Here, transinfected *wPip* at high density in *Ae. aegypti,* failed to block DENV replication and dissemination (Fraser et al., 2020). Notably, *wPip* together with DENV-inhibitory *wAlbB* and *wMel* only induce very small to no increase in expression of genes under the Toll pathway. Marked upregulation in immune-related genes have only been observed in *wMelpop* (Fraser et al., 2020). Contrary to other hypotheses, this study demonstrates that there is no association between *Wolbachia* strain/density and the extent of immune activation to block DENV.

*Wolbachia* strains when traced back to their natural hosts may suggest potential pathogen blocking activity but it does not automatically lead to the same effect when transinfected in *Ae. aegypti* and in the case that it does, the immune pathways *Wolbachia* strains activate may not be conserved. Other than the strain or density per se, the regulation of innate immunity in *Ae. aegypti* seems more complex and influenced by intracellular responses that have yet to be elucidated.

### 3.4 Cellular Regeneration – a feature of *Ae. aegypti* with High *Wolbachia*-mediated Viral Blocking

New inferences on the molecular mechanisms of *Wolbachia*-mediated viral blocking in *Ae. aegypti* involves the Notch signaling pathway and cell-cell adhesion (**Figure 3**). Generally, Notch is a conserved mechanism that enhances the host's fitness (Kopan and Ilagan, 2009; Chang et al., 2018). In fact, *Ae. aegypti* carries a Notch receptor gene (*AaNotch)* involve in maintaining micropyle pores and fecundity, all of which contributes to overall reproductive ability (Chang et al., 2018). Notably, this high fitness advantage in *Ae. aegypti* has been recently associated with stronger *Wolbachia*-

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mediated viral blocking (Ford et al., 2019, 2020). In the study by *Ford et al., wMel*-infected *Ae. aegypti* artificially selected for high *wMel*-mediated viral inhibition displays a gene profile in favor of Notch activation which significantly differs from mosquitos classified with low *wMel*-mediated DENV blocking. Notch activation coupled with high viral blocking has been linked to elevated cadherin expression (Ford et al., 2019, 2020). Although the mechanistic relationship between Notch and cadherin in *Ae. aegypti* has not been explicitly defined, a study in *Drosophila* shows how these two combines as a single complex to form cell-cell junctions implicated in Delta/Notch activation. Moreover, depletion of cadherin results in a downregulated activity of the said mechanism (Sasaki et al., 2007).

Consistently, studies on host-virus interactions also provided more context to this potential mechanism by proving that Notch has a regulatory role on midgut cell proliferation in *Ae. aegypti* during DENV infection. This proliferative response is referred to as endoreplication, a process that promotes tissue regeneration by producing cells with excess copies of genomic DNA (Serrato-Salas et al., 2018; Taracena et al., 2018). In a susceptible *Ae. aegypti* strain, delayed cellular regeneration has been observed upon DENV infection whereas induction of the midgut cell proliferation made this mosquito more resistant to the virus. Alternatively, *Ae. aegypti* refractory strain demonstrates higher susceptibility to DENV upon inhibition of Notch (Taracena et al., 2018). These findings therefore suggest that in *Ae. aegypti* the Notch specifically regulates *Wolbachia's* viral blocking activity by increasing both host fitness and midgut cellular regeneration.

### 3.5 miRNAs Solely Expressed During *Wolbachia* Infection Blocks Arboviruses

miRNA functions as a post-transcriptional regulator of gene expression and has a wide-ranged effect given its control over multiple genes. Some miRNAs that usually exist within the host are dysregulated while others become exclusively expressed in the presence of microbes. Successively, microbes can drive these miRNAs to alter the mosquito host's responses as they persist inside the cell (Feng et al., 2018) (**Table 1**).

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**Table 1. miRNAs induced by *Wolbachia* in *Ae. aegypti***

The presence of *Wolbachia* in *Ae. aegypti* can trigger the release of miRNAs derived from the host (miR-2940, aae-mir-12, aae-miR-981) or the endosymbiont (WsRNA-46). These miRNAs can regulate different host cellular mechanisms to maintain *Wolbachia's* density, facilitate transport and strengthen host antiviral responses.

| miRNAs | Target genes | Activity on target gene | Proposed effect on arbovirus-infected *Ae. aegypti* |
|---|---|---|---|
| miR-2940 | m41 ftsh<br>AaArgM3 | enhance | *wMelpop-CLA* density |
| | AaDnmt2 | suppress | *wMelpop-CLA* density |
| aae-miR-12 | MCT1 | suppress | downregulates autophagy controlled by the virus |
| | MCM6 | suppress | wMelpop-CLA density |
| aae-miR-981 | Importin β-4 | suppress | AGO1 translocation<br>cellular regeneration by Notch activation |
| WsRNA-46 | dynein | enhance | *wMelpop* replication and migration<br>mRNA localization<br>lipid droplet movement |

Studies have substantiated the impact of *Wolbachia* on *Ae. aegypti's* miRNA profile (Hussain et al., 2011; Mayoral et al., 2014). *wMelpop-CLA* induces differential expression of miRNAs with exclusive induction of *miR-2940* and *miR-309a-2* in *Wolbachia* positive mosquitoes (Hussain et al., 2011). Between the two, *miR-2940* is well-known to target genes that regulate *Wolbachia* density (Hussain et al., 2011; Zhang et al., 2013, 2014). In particular, this miRNA increases and stabilizes the expression of metalloprotease m41 ftsh gene and arginine methyl transferase 3 *(AaArgM3)* further enhancing *wMelpop-CLA* replication in both *Ae. aegypti* cells and mosquitoes. Inhibiting miR-2940 only led to a significant reduction of the target genes and endosymbiont (Hussain et al., 2011; Zhang et al., 2014). The specific role of this *miR-2940* in arbovirus infection remains undiscovered although this miRNA is downregulated in mosquito cells to limit West Nile virus replication (Slonchak et al., 2014). Regardless, metalloprotease genes like m41 ftsh are upregulated in DENV and ZIKV-infected *Ae. Aegypti* (Sim et al., 2012; Etebari et al., 2017) suggesting *Wolbachia* may be utilizing host miRNAs to control a specific host gene essential for its growth which arboviruses also need. Conversely, *miR-2940* suppresses DNA methyltransferase (*AaDnmt2*) in *wMelpop-CLA* transinfected mosquitoes (Zhang et al., 2013). Some of the biological functions of *AaDnmt2* are host defense, genome stability and lifespan regulation. Presence of DENV in *Ae. aegypti* without the endosymbiont produces higher levels of this gene. In this case, *Wolbachia* creates a cellular environment unconducive or antagonistic to the virus (Zhang et al., 2013).

Meanwhile, miRNAs can also have an effect on host autophagy, viral replication and cellular transport. As an example, *wMelPop-CLA* in Aag2 cells triggers marked expression of *aae-miR-12* capable of suppressing two genes namely monocarboxylate transporter (*MCT1*) and DNA replication

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licensing factor (*MCM6*) (Osei-Amo et al., 2012). The exact role of these genes in *Ae. Aegypti* is unknown but existing studies in other insects reveal that *MCT1* is a key player in autophagy (Velentzas et al., 2018) whereas in DENV and ZIKV infection, these viruses take over the host's autophagy response to evade the host immune defenses (Choi et al., 2018). One possibility that requires further investigations is that *Wolbachia* produces a miRNA that can suppress the activity of MCT1 and therefore, autophagy.

*Wolbachia* infection also induces the expression of *aae-miR-981* and in effect, downregulates importin β-4 in *wMelPop-CLA* infected Aag2. Reducing the activity of importin β-4 blocks AGO1 translocation to the nucleus (Hussain et al., 2013). There is no existing data that answers why hindering AGO1 translocation to the nucleus is advantageous for *Wolbachia's* viral blocking. Nevertheless, importin β serves a different role in arboviral infection. Importin β binds to DENV and ZIKV nonstructural proteins to assist in their nuclear migration for optimal replication as observed in *Ae. Albopictus* (Pryor et al., 2007; Ji and Luo, 2019). If this effect is the same for *Ae. aegypti* then the downregulation of importin β during *Wolbachia* infection may not only impair AGO1 translocation but also potentially block viral transcription.

Finally, *Wolbachia*-derived miRNAs that may contribute to viral blocking have also been reported. For instance, *WsRNA-46* found in *Wolbachia*-infected Ae. *aegypti* promotes dynein expression, a cytoskeletal protein important in cellular transport, mRNA localization and movement of lipid droplets (Mayoral et al., 2014). In both *Wolbachia* and arboviruses, dynein is crucial for maintaining density and infection (Baldridge et al., 2014; Mayoral et al., 2014). Given that arboviruses also use the same cytoskeletal structure for their benefit, this represents an overlapping need for the same host cellular factors.

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## 4. DISCUSSION

In the recent years, *Wolbachia*-infected *Ae. aegypti* are being released to control medically important arboviruses (Indriani et al., 2020, O'Neill et al., 2019). The impact of *Wolbachia* on arboviral inhibition has been validated by looking into disease prevalence after mass release programs have been implemented (Indriani et al., 2020) and by simulating its effect computationally (Zhang and Lui, 2020). However, elucidating the exact cellular mechanisms that block off these viruses inside the mosquito cell is still underway. The two well-established hypotheses suggest that the transinfection of *Wolbachia* into *Ae. aegypti* induces an immune reaction that fights off the invading viruses and that the endosymbiont wins the competition for scarce resources (Caragata et al., 2016; Lindsey et al., 2018). Based on the facts presented in this review, there could be another potential explanation as to how *Wolbachia* blocks a pathogen. It is likely that *Wolbachia* directly interferes with arboviruses. As an example, *Wolbachia* has been discovered to reduce host proteins that help viruses enter the cell (Lu et al., 2020). *Koh et al.* also demonstrated this antagonistic effect when dual infection of *Wolbachia* and DENV in *Ae. aegypti* resulted in a decrease in specific types of lipids that are normally upregulated when only the virus is present (Koh et al., 2020). More so, the endosymbiont enhances ROS production (Pan et al., 2012) and cellular regeneration (Taracena et al., 2018) that can directly harm an invading pathogen as well as prevent systemic viral infection, respectively. More interestingly, these antagonistic effects are consistent with the contrasting patterns in gene expression when either microbes are present or both (**Table 2**).

**Table 2. Summary of interactions between *Ae. aegypti*-Arboviruses-*Wolbachia***
*Wolbachia's* pathogen blocking is mediated by cellular and molecular changes that occur in different host cell processes. Individual infection of arboviruses compromises *Ae. aegypti* antiviral responses whereas *Wolbachia* infection in the same host strengthens it. Presence of both arbovirus and *Wolbachia* demonstrate antagonistic interference by the endosymbiont to block the invading pathogen.

| Host cellular factor/mechanism | *Ae. aegypti* + virus | Ae. aegypti + *Wolbachia* | *Ae. aegypti* + virus + *Wolbachia* |
|---|---|---|---|
| Cytoskeleton | Upregulated to aid in cell transport and viral life cycle | Upregulated to maintain localization and density within the host | Downregulated to inhibit virus infection |
| Midgut barrier/Cellular regeneration | Earlier formation of a thicker PM.<br>Delayed regeneration of midgut epithelium via Delta/Notch.<br>Upregulated proteolytic transcripts to break | Activation of Delta/Notch and Cadherin to increase host fitness. | Delta/Notch activation both for host fitness and regeneration of midgut epithelium. |

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| | | | |
|---|---|---|---|
| | host's midgut epithelium. | | |
| Metabolism | Increased cellular lipids/cholesterol for viral replication. Insulin/LD activate immunity | Increase stored cholesterol with LD accumulation for maintaining density. | Depletion of specific lipids that are needed by arboviruses. |
| Immunity/ROS | Downregulated immune-related transcripts. Decrease ROS with antioxidant production. | Upregulates immunity. | Increase ROS to activate immunity. Releases antioxidants to maintain density. |

The challenge of discovering these mechanisms comes from the complex relationship between three factors, namely the host, arboviruses and *Wolbachia*. *Ae. aegypti* and both microbes equally contribute to the overall pathogen blocking effect. This is also why the extent to which *Wolbachia* blocks a pathogen can be heterogeneous. Moving forward, using next-generation sequencing of *in vivo* samples for determining genes/proteins related to viral susceptibility while combining it with *in vitro* models to examine each cellular process may be a good starting point for future research. By identifying host factors that promotes viral infection and exploring how *Wolbachia* could affect the same factors may confirm the antagonistic interference occurring between the two microbes in *Ae. aegypti* host.

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## Author Contributions

J.I.L.R and K.W. conceptualized the review article. J.I.L.R wrote and revised the manuscript with K.W. supervision. J.I.L.R created the figures. Y.S. and T.C. added concepts. K.W., Y.S., T.C. and M.N.M. reviewed and revised the initial drafts. All authors contributed to this work and agreed to the published version of the manuscript.

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