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Expanding the canon: Non-classical mosquito genes at the interface of arboviral infection

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ABSTRACT

Mosquito transmitted viruses cause significant morbidity and mortality in human populations. Despite the use of insecticides and other measures of vector control, arboviral diseases are on the rise. One potential solution for limiting disease transmission to humans is to render mosquitoes refractory to viral infection through genetic modification. Substantial research effort in *Drosophila*, *Aedes* and *Anopheles* has helped to define the major innate immune pathways, including Toll, IMD, Jak/Stat and RNAi, however we still have an incomplete picture of the mosquito antiviral response. Transcriptional profiles of virus-infected insects reveal a much wider range of pathways activated by the process of infection. Within these lists of genes are unexplored mosquito candidates of viral defense. *Wolbachia* species are endosymbiotic bacteria that naturally limit arboviral infection in mosquitoes. Our understanding of the *Wolbachia*-mediated viral blocking mechanism is poor, but it does not appear to operate via the classical immune pathways. Herein, we reviewed the transcriptomic response of mosquitoes to multiple viral species and put forth consensus gene types/families outside the immune canon whose expression responds to infection, including cytoskeleton and cellular trafficking, the heat shock response, cytochromes P450, cell proliferation, chitin and small RNAs. We then examine emerging evidence for their functional role in viral resistance in diverse insect and mammalian hosts and their potential role in *Wolbachia*-mediated viral blocking. These candidate gene families offer novel avenues for research into the nature of insect viral defense.

1. Introduction

Mosquitoes transmit a range of viruses to humans, many with serious health consequences (Gould et al., 2017), including dengue, Zika, chikungunya, West Nile, yellow fever and Japanese encephalitis, among others. Despite decades of mosquito control programs focusing primarily on the use of insecticides and the reduction of mosquito breeding habitat, arboviral disease rates continue to rise (Bhatt et al., 2013). Expanded geographic ranges for vectors, human travel, growing urbanization and climate change are assisting with disease spread (Fauci and Morens, 2016). The production of genetically modified mosquitoes that are refractory to viral transmission offers one potential strategy for reducing the incidence of human disease. As methods for modification advance (Chaverra-Rodriguez et al., 2018), there is a growing need to identify optimal targets for modification. Genes involved with the insect's natural antiviral response are ideal candidates.

Insects have evolved innate immune responses to combat the pathogens they encounter in their environment. Recognition of pathogens as non-self induces immune signaling cascades that initiate pathogen defense mechanisms. The Toll, IMD and Jak/Stat signaling pathways have received the most focus and functional characterization in the presence of different pathogens. Activation of these pathways leads to translocation of transcription factors to the nucleus and induces transcription of antimicrobial peptides and other immune factors. Bacteria, fungi, and *Plasmodium* sp. can activate one or more of these pathways in mosquitoes (Simões et al., 2018). The Toll, IMD and Jak/Stat pathways also respond to arboviral infections, but their viral defense mechanisms are unknown. The RNA interference (RNAi) pathway is viewed as the main viral defense pathway in mosquitoes. This general response to viral infection recognizes small RNAs produced by viral genomes and targets complementary sequences for destruction (Olson and Blair, 2015).

In addition to the microorganisms that cause human disease, mosquitoes harbor native bacteria, fungi, protozoans and insect-specific viruses that can shape their life-history traits and immune responses (Bartholomay and Michel, 2018). *Wolbachia* sp., endosymbiotic bacteria that naturally infect arthropods and are maternally transmitted (Zug and Hammerstein, 2012), are one such example. While present in

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Abbreviations

RNAi	RNA interference
DENV	dengue virus
WNV	West Nile virus
CHIKV	chikungunya virus
ZIKV	Zika virus
YFV	yellow fever virus
SINV	Sindbis virus
DCV	Drosophila C virus
CYPs	cytochromes P450
Hsp	heat shock protein
Ahr	aryl hydrocarbon receptor
miRNA	microRNA
siRNA	small interfering RNA pathway
piRNAs	PIWI-interacting RNA
tRF	tRNA-fragments
ROS	reactive oxygen species

a range of mosquitoes including many vector species, *Wolbachia* are absent in the primary vector of dengue virus (DENV), *Aedes aegypti* (Gloria-Soria et al., 2018; Kittayapong et al., 2000; Shaw et al., 2016). A strain of *Wolbachia* native to *Drosophila melanogaster*, however, has been introduced into *Ae. aegypti* where it forms a stably inherited infection (McMeniman et al., 2009; Xi et al., 2005). The presence of *Wolbachia*, through unknown mechanisms, reduces DENV viral loads, decreases the total number of infected mosquitoes in a population, and lengthens the extrinsic incubation period; an effect known as *Wolbachia*-mediated 'pathogen-blocking' (Moreira et al., 2009; Walker et al., 2011; Ye et al., 2015). Consequently, mosquitoes infected with *Wolbachia* are being released into wild populations as a biocontrol tool and are showing potential to decrease DENV transmission to humans (Carrington et al., 2018; Frentiu et al., 2014). In other *Wolbachia*-infected mosquito vectors the degree of blocking for a range of viruses is known to vary and in one instance even exhibits enhanced infection (Johnson, 2015). Although the mechanisms of *Wolbachia*-mediated pathogen-blocking are unknown, multiple studies suggest the involvement of host immunity (Pan et al., 2012; Terradas et al., 2017). Such a hypothesis is appealing because it could broadly explain the diverse nature of *Wolbachia's* blocking effect in *Ae. aegypti* against chikungunya virus (CHIKV), Zika virus (ZIKV), yellow fever virus (YFV), West Nile virus (WNV), filarial nematodes and malaria parasites (Joubert and O'Neill, 2017; Kambris et al., 2009; Moreira et al., 2009; van den Hurk et al., 2012). Recent evidence suggests that immune signaling pathways actually help maintain *Wolbachia* infection in mosquitoes (Pan et al., 2018). Additionally, activation of immune pathways is not as wide ranging or as strong in insects that naturally harbor *Wolbachia* (Herbert and McGraw, 2018; Molloy and Sinkins, 2015). These data suggest that while the enhanced immune response in *Ae. aegypti* may help to strengthen pathogen blocking in this species, there is an alternate primary mechanism shared across these insects.

Though the Toll, Jak/Stat and RNAi pathways respond to DENV (Sánchez-Vargas et al., 2009; Sim et al., 2012; Souza-Neto et al., 2009; Xi et al., 2008), many genes outside of these immune pathways are also transcriptionally altered in response to DENV infection (Bonizzoni et al., 2012). These additional genes may respond to infection or the associated stress, and may either directly or indirectly modulate antiviral mechanisms. How viral infection induces changes in transcription for the majority of these genes, as well as their functional roles in infection are largely unknown. *Wolbachia* infection also triggers the transcription of genes that are not members of the classical innate immune pathways (Herbert and McGraw, 2018; Terradas et al., 2017; Y. H. Ye et al., 2013).

This review is motivated by our incomplete picture of the insect antiviral response and the substantial number of genes outside of the classical immune pathways that are transcriptionally altered in response to arboviral infections in *Ae. aegypti*. Here we have examined transcriptomic data and report consensus gene types/families (identified by gene names, GO terms and descriptions), that are differentially expressed in *Ae. aegypti* across DENV and other viral infections (Table 1), and that may represent novel or underappreciated aspects of the host immune response. We identified 6 families of interest: cytoskeleton and cellular trafficking, the heat shock response, cytochromes P450, cell proliferation, chitin and small RNAs. We then reviewed available functional data for the role of these proteins in viral defense across the insects, and examined whether there was any evidence that these proteins play a specific role in *Wolbachia*-mediated viral blocking.

2. Cytoskeleton and cellular trafficking

Many viruses interact with the host cytoskeletal network at the plasma membrane and in the cytoplasm. The cytoskeletal network facilitates cellular trafficking, the spatial organization of organelles and other cellular processes. The main structures of the cytoskeleton are composed of filamentous actin and microtubules formed by tubulin. Along the microtubules, motor proteins move cargo and are stabilized by filaments and microtubule-associating proteins. Viral pathogens use host microtubules and microtubule-associated proteins to facilitate cell-entry, transport and egress (Naghavi and Walsh, 2017). During infection, changes in the cytoskeleton often benefit the pathogen and can even be pathogen-driven (Walsh and Naghavi, 2018). However, cytoskeletal changes have also been shown to serve as host-driven immune responses during bacterial infection (Mostowy and Shenoy, 2016). In mammalian cells; the clathrin-mediated endocytosis of WNV, DENV and ZIKV are microtubule-dependent processes (Hackett and Cherry,

Table 1
***Ae. aegypti* transcriptome studies.** N/A, not applicable; IT, intrathoracic; d, day; inf., infection; WB, whole body; Mg, midgut; SG, salivary gland; Car, carcass; RNA-seq, RNA-sequencing.

Reference	Mosquito Strain	Cell line	Virus	Inf. Route	*Wolbachia*	Time post-Inf.	Tissue	Technology
Colpitts et al. (2011b)	Rockefeller	CCL-125	DENV(2), WNV, YFV	IT	N/A	1 d, 2 d, 7 d	WB	Microarray
Bonizzoni et al. (2012)	Chetumal	N/A	DENV(2)	Oral	N/A	1 d, 4 d, 14 d	Mg, SG, Car	RNA-seq
Behura et al. (2011)	Moyo-S, Moyo-R	N/A	DENV(2)	Oral	N/A	3 h, 18 h	WB	Microarray
Shrinet et al. (2017)	–	N/A	DENV(2), CHIKV	IT	N/A	1 d	WB	RNA-seq
Sim et al. (2012)	Rockefeller/UGAL	N/A	DENV2	Oral	N/A	14 d	SG, Car	Microarray
Angleró-Rodríguez et al. (2017)	Rockefeller	N/A	DENV(2), ZIKV	Oral	N/A	7 d	Mg	RNA-seq
Etebari et al. (2017)	Galveston	N/A	ZIKV	Oral	N/A	2 d, 7 d, 14 d	WB	RNA-seq
Sanders et al. (2005)	Higgs' white eye	N/A	SINV	Oral	N/A	1 d, 4 d, 8 d	Mg	Microarray
Ye et al. (2013)	PGYP1	N/A	N/A	N/A	*w*MelPop	N/A	Head, Muscle	Microarray
Rancès et al., (2012)	PGYP1	N/A	N/A	N/A	*w*Mel, *w*MelPop	N/A	WB	Microarray
Kambris et al. (2009)	PGYP1	N/A	N/A	N/A	*w*MelPop	N/A	WB	Microarray

2018); intracellular trafficking of WNV and Semliki Forest virus occurs along the microtubules (Chu and Ng, 2002; Vonderheit and Helenius, 2005); and the cytoskeleton is involved in the endocytosis and replication of CHIKV (Bernard et al., 2010; Issac et al., 2014). In mosquitoes, DENV entry into the midgut epithelia cells occurs through clathrin-mediated endocytosis, however, the role of the cytoskeleton during the process is unknown (Acosta et al., 2008; Mosso et al., 2008).

Mosquito transcriptomic studies have shown that arbovirus infections alter the expression of genes in the cytoskeletal network (or predicted to be) during infection with WNV, CHIKV, Sindbis virus (SINV) and DENV (Bonizzoni et al., 2012; Colpitts et al., 2011b; Sanders et al., 2005; Shrinet et al., 2017; Sim et al., 2012). For example, infection with DENV affects expression of actin, tubulin, microtubule-associating and motor proteins, most often decreasing expression (Bonizzoni et al., 2012; Shrinet et al., 2017). Additionally, a proteomics study has revealed decreased production of actin and actin-binding proteins during DENV and CHIKV infection in the midgut, the initial tissue of infection in the vector (Tchankouo-Nguetcheu et al., 2010). Across studies, the same genes are not always affected, but there are consistencies within gene families. For instance, β-tubulin expression is decreased in the midgut and salivary glands at 14 days post-infection (dpi) with DENV (Bonizzoni et al., 2012), and protein levels of another β-tubulin are decreased in the salivary glands at 5 dpi with CHIKV (Tchankouo-Nguetcheu et al., 2012). Furthermore, protein binding assays have demonstrated that WNV and DENV proteins can bind tubulin, as well as actin and myosin proteins in *Aedes albopictus* C6/36 cells (Chee and Abubakar, 2004; Colpitts et al., 2011a). Together these data suggest that arboviruses may interact with the cytoskeleton in mosquito tissues.

It is unknown if interactions with the cytoskeleton are essential for infection; however, chemical inhibition of actin and myosin decreases WNV and DENV infection (Colpitts et al., 2011a), indicating the cytoskeleton is involved. Modulation of the mosquito cytoskeletal network may also result from host-mediated responses. Though the intracellular cytoskeletal network has not been shown to have immune function in mosquitoes, cytoplasmic actin has been identified as an extracellular immune factor in *Anopheles gambiae* (Sandiford et al., 2015). Upon bacterial challenge, secreted actin 5C promotes phagocytosis and has direct antibacterial activity. In addition actin 5C is involved in the antiparasitic response to *Plasmodium falciparum*, although the mechanisms are unknown.

Obligate intracellular bacteria also interact with the actin cytoskeleton of host cells (Colonne et al., 2016). In the germline and embryo of *D. melanogaster, Wolbachia pipientis* uses host microtubules for cellular localization in the oocyte and uses actin to maintain *Wolbachia* titer and maternal transmission (Ferree et al., 2005; Newton et al., 2015). Recently, the *Wolbachia* protein WALE1, predicted to be secreted, was shown to interact with and bundle filamentous actin *in vitro*, suggesting this protein may modify the host cytoskeleton (Rice et al., 2017; Sheehan et al., 2016). Cell to cell transfer of *Wolbachia* in *Drosophila* occurs through phagocytic-like engulfment and clathrin- and dynamin-mediated endocytosis and appears to occur similarly when *Wolbachia* is transferred from *Ae. albopictus* C6/36 cells to *Drosophila* cells (White et al., 2017). In general, dynamin binds microtubules and actin and is involved in the formation of endocytic vesicles (Menon and Schafer, 2013), suggesting that *Wolbachia* uses actin dependent processes to spread to other cells. In *Ae. aegypti* infected with *Wolbachia*, the expression of genes annotated as encoding actin, actin-binding, clathrin and microtubule-binding proteins increase in the muscle tissues (Y. H. Ye et al., 2013). *Wolbachia* interactions with the mosquito cytoskeleton may overlap or conflict with DENV-directed regulation of the cytoskeletal network.

In mammalian cells, pathogen interactions with host cytoskeletal networks can be viral-mediated or host-mediated, and may result in stabilizing or de-stabilizing the network. Viral pathogens have also been shown to benefit from disruption of microtubule organization in mammalian systems (Martin et al., 2010; Zan et al., 2017). Therefore, directional changes in transcription are difficult to interpret in terms of potential effects on infection. Although current evidence demonstrates that arboviral infections result in altered transcription of mosquito cytoskeletal network genes, *in vivo* experiments are needed to identify any associated structural changes. It is not clear if these differences are host- or viral-mediated and if they affect the progression of infection.

3. Heat shock response

The heat-shock response can be induced as a result of stress from temperature, injury and other damaging processes and results in the upregulation of heat shock proteins (Hsps). These proteins manage and prevent damage by mediating protein folding, aggregation and degradation (King and MacRae, 2015). Once a mosquito has ingested a warm blood meal, the heat shock response is induced and heat shock protein 70 (Hsp70) is expressed in the midgut, providing protection from rapid thermal changes (Benoit et al., 2011). In an infectious blood meal, the interactions between Hsps and viruses may add another dimension to Hsp responses.

Mosquito transcriptomic studies have identified differential expression of Hsps and/or heat shock factor binding genes during DENV, WNV, YFV, ZIKV, SINV and CHIKV infection (Angleró-Rodríguez et al., 2017; Bonizzoni et al., 2012; Colpitts et al., 2011b; Sanders et al., 2005; Shrinet et al., 2017; Sim et al., 2012). Although the specific genes and their direction of transcriptional regulation varies across studies, their expression is consistently affected by the presence of arboviruses. Hsp70, in particular, has been shown to facilitate DENV replication in both human and mosquito cell culture (Taguwa et al., 2015). In mammalian cells, inducing heat shock during ZIKV infection increases Hsp70 and correlates with an increase in the number of ZIKV plaque-forming units, suggesting that Hsp70 promotes infection (Pujhari et al., 2017). Infection with Japanese encephalitis virus (JEV) also increases Hsp70 in mammalian cell culture, and the co-precipitation of Hsp70 with JEV proteins (J. Ye et al., 2013).

Hsps have generally been shown to facilitate viral infection, however one study in mosquitoes has demonstrated the opposite. In *An. gambiae*, heat shock protein cognate 70B (Hsc70B) restricted infection when mosquitoes were injected with o'nyong-nyong virus (Sim et al., 2007). RNAi based knockdown (KD) of Hsc70B increased the number of plaque-forming units per mosquito at 6 dpi and decreased survival rates from 6 to 12 dpi, suggesting Hsc70B may have a role in viral defense in this mosquito-virus interaction. One possible explanation is that an increase in Hsc70 B at the cellular level may result in systemic signaling that increases immunity globally. A recent study in *Drosophila* demonstrated that Drosophila C Virus (DCV) infection induces the heat shock response, and Hsp70 has a role in mediating this response (Merkling et al., 2015). Heat shock deficient adult flies have decreased survival during infection, and survival improved upon overexpression of *Hsp70*. This was the first indication that the heat shock response limits viral infection in *Drosophila*, but the mechanism remains unknown. Hsp effects may be direct or mediated through induced signaling and activation of other pathways. However, in honey bees there appears to be an antagonistic relationship between the heat shock response and factors in the humoral immune response (McKinstry et al., 2017), suggesting possible resource trade-offs between these pathways. Finally, *Drosophila* studies indicate that the heat shock pathway is linked to the endogenous siRNA pathway components Dicer2 and Ago-2, as they are also part of the complex in the nucleus that regulates heat shock-mediated transcriptional repression, and their KD increases *Hsp70* transcription (Swevers et al., 2018).

Temperature can change the density of *Wolbachia* in *Ae. aegypti* and the vectorial capacity for DENV in the presence of *Wolbachia* (Ross et al., 2017; Ross and Hoffmann, 2018; Ulrich et al., 2016; Ye et al., 2016). In *Ae. aegypti*, a putative Hsp gene is upregulated in the presence of *Wolbachia* (Rancès et al., 2012), suggesting that mosquito-*Wolbachia* interactions may induce Hsps. Finally, there is also evidence that genes

involved in the heat shock response have been acted upon by natural selection in *w*Mel (Brownlie et al., 2007), indicating that *Wolbachia* heat shock factors are important for symbiont establishment and maintenance in its host.

4. Cytochromes P450

Cytochromes P450 (CYPs) are enzymes located on the endoplasmic reticulum and mitochondria that metabolize hormones, chemicals, fatty acids and detoxify xenobiotic substances (Feyereisen, 1999). In mosquitoes, infection with DENV, WNV and YFV results in the altered expression of CYP transcripts (Behura et al., 2011; Bonizzoni et al., 2012; Colpitts et al., 2011b; Sim et al., 2012). The genes encoding these enzymes are consistently identified in these differential expression studies. Experimental work in mammals also highlights a role for CYPs in viral infection, acting through oxidative stress and immune pathways (Stavropoulou et al., 2018). Insect genomes can encode a range of tens to hundreds of CYPs across taxa (Zhu et al., 2013), with over 100 present in *An. gambiae* and *Ae. aegypti* and over 200 in *Culex quinquefasciatus* (Yang and Liu, 2011). Although some CYPs and their functions are conserved, such as metabolism of hormones, there are rapidly evolving paralogs that may have non-canonical functions (Feyereisen, 2006). In mosquitoes, CYPs are expressed across tissues including the midgut, fat body and the Malpighian tubules. Expression can also be tissue specific, and CYPs are differently expressed between life stages and after a blood meal (Esquivel et al., 2016; Félix et al., 2010; Li et al., 2017).

In mammals, inflammation leads to oxidative stress and this has been linked to the downregulation of CYP transcription and activity (Stavropoulou et al., 2018). In *Drosophila*, knockdown of a CYP4 gene in the Malpighian tubules resulted in the upregulation of Reactive Oxygen Species (ROS) and increased expression of *Diptericin* in the NF-ƙβ pathway and *Turandot* in the JAK/STAT pathway in whole bodies (Terhzaz et al., 2015). This indicates that CYP activity is linked to oxidative stress and immune pathways. If a similar interaction exists in mosquitoes, CYPs may regulate the metabolism of ROS and the crosstalk between immune and stress pathways. A particular CYP gene, CYP307A1, upregulated during DENV infection and subsequently targeted by RNAi knockdown in *Ae. aegypti* cells resulted in decreased DENV infection (Londono-Renteria et al., 2015). CYP307A1 may directly facilitate DENV infection, or its activity may cause downstream decreases in ROS or immune factors. In support of either hypothesis, an analysis of the transcriptome from the midguts of refractory and susceptible *Ae. aegypti* identified expressed sequence tags of CYPs exclusively in the midguts of susceptible mosquitoes at 48 h following DENV infection (Barón et al., 2010). In summary, decreasing CYP expression is associated with reduced DENV infection and this may be due to enhancement of ROS and immune factors, as is observed in mammals and *Drosophila*.

The expression of CYPs during infection could be mediated by a transcription factor. One candidate is the aryl hydrocarbon receptor (AhR), a transcription factor in mammals that mediates antiviral responses and promotes the expression of CYPs and cytokines (Head and Lawrence, 2009). AhR activity during viral infections has only recently begun to be elucidated. During bacterial infection in the intestine, where AhR regulates epithelial homeostasis (Lamas et al., 2018), mice deficient for AhR have increased bacterial penetration of colon cells compared to wild type mice, where bacteria remained attached to the luminal surface (Schiering et al., 2017). Additionally, AhR interacts with the transcription factor NF-ƙβ, a regulator of immune responses, which results in suppression of AhR activities (Tian, 2009). In insect cells, AhR has been demonstrated to enhance promoter activity of a CYP *in vitro* (Brown et al., 2005), but the possible role of AhR in insect immunity has not been explored. The potential roles that CYPs could have in insect immunity and intestinal barriers, along with the transcriptional data presented above, make CYPs interesting candidates in arboviral interactions.

Wolbachia infection may also alter CYP function. This is not surprising, since *Wolbachia* can induce stress-like responses in mosquitoes (Lindsey et al., 2018), and it has been suggested that *Wolbachia* may regulate host factors to balance redox homeostasis (Zug and Hammerstein, 2015). In the head and muscle tissues of *Ae. aegypti* infected with the *w*MelPop strain, the expression of 14 CYPs increased (Y. H. Ye et al., 2013). A comparison of genes differentially expressed during *w*MelPop and *w*Mel infections in whole mosquito bodies identified 9 CYPs in *w*Mel infected mosquitoes and 65 CYPs in *w*MelPop infected mosquitoes, 5 of which overlapped (Rancès et al., 2012). The *w*MelPop strain is more virulent in mosquitoes and induces more effective blocking, therefore, more CYP gene expression changes may reflect an increase in stress, which could also be associated with increased blocking. Crosstalk between stress responses and immune pathways indicate that feedback and balance is crucial; therefore, disturbances in detoxification caused by symbionts or pathogens could have far-reaching effects that may modulate immunity.

5. Cell proliferation

The mechanisms of viral dissemination from the gut epithelium across the basal lamina and into the hemocoel are poorly understood (Simões et al., 2018). The pathways and genes regulating gut homeostasis in mosquitoes are just being elucidated, and recent studies suggest that DNA synthesis and cell proliferation in the gut have roles in limiting viral infection (Serrato-Salas et al., 2018; Taracena et al., 2018). So, it is important to understand how the gut deals with physical changes incurred in response to blood feeding and infection, and whether there are influences on pathogen dissemination. The expression of genes involved in cell proliferation are altered during DENV, WNV and YFV infection (Behura et al., 2011; Colpitts et al., 2011b; Serrato-Salas et al., 2018). Only a few genes with annotated roles in cell proliferation are differentially expressed, but this may reflect the generally poor annotation of cell proliferation genes in mosquitoes.

In *Drosophila*, intestinal stem cells in the midgut regularly undergo mitosis to generate newly differentiated cells, and multiple signaling pathways regulate midgut cell differentiation, including the Notch and Jak/Stat pathways (Nászai et al., 2015). The Notch pathway is activated by binding of the ligand Delta to the Notch receptor, and this activates the transcription factor Hindsight. In adults, *Notch* transcription is altered during YFV and DENV infections (Colpitts et al., 2011b; Serrato-Salas et al., 2018), albeit the directional changes of *Notch* transcription are not congruent across studies and in some instances are not significant (Bonizzoni et al., 2012). A single study identified an increase in the expression of *Delta*, *Notch* and *Hindsight* transcripts during DENV infection at 5 and 7 dpi (Serrato-Salas et al., 2018), indicating that multiple genes in the Notch pathway can be altered by viral infection. The discrepancies between these studies indicate that Notch may be affected by other unknown factors, however additional studies correlate Notch function with viral defense (Taracena et al., 2018). Knockdown of *Delta*, the Notch ligand, decreases the number of proliferative cells in the *Ae. aegypti* midgut and correlates with increased DENV plaque forming units in the midgut at 5 dpi in a refractory strain but not in a susceptible strain (Taracena et al., 2018). In *Ae. aegypti* adults, proliferative cells have been identified in the midgut using a marker for mitosis (Janeh et al., 2017; Taracena et al., 2018). A method has recently been developed in larvae to distinguish intestinal stem cells and recently divided cells from differentiated cells in the midgut (Valzania et al., 2018). Ongoing characterization of midgut homoeostasis will further our understanding of this tissue and the effects of cell proliferation on viral infection.

Wolbachia pathogen blocking has also been associated with cell growth signaling. In *Drosophila*, the extracellular signal-regulated kinase (ERK) pathway also regulates intestinal stem cell proliferation, and ERK is required for *Wolbachia* protection against oral infection by DCV

(Wong et al., 2016). Further data supports that ERK signaling may have a role in viral defense, as knockdown of factors in the ERK signaling pathway results in increased SINV infection in Aag2 cells (Xu et al., 2013). The presence of *Wolbachia* in adult *Ae. aegypti* does not alter the expression of *ERK* (Rancès et al., 2012; Y. H. Ye et al., 2013), and the role of ERK in *Wolbachia*-mediated pathogen blocking in mosquitoes is unknown.

6. Chitin

Chitin is a primary component of the cuticle along with cuticular proteins and lipids (Klowden, 2013). Chitin is also a major component of the peritrophic matrix, a lining in the lumen of the gut that is secreted by the epithelium (Zhu et al., 2016). During DENV infection, genes with annotations of chitin metabolism, chitin-binding and cuticle structure are differentially expressed across studies (Bonizzoni et al., 2012; Colpitts et al., 2011b; Shrinet et al., 2017). These transcriptional changes may be particular responses to viral infection or part of a more generalized stress response. In *Ae. aegypti*, infection with DENV, WNV or YFV decreased expression of a cuticle protein 1 dpi, and overexpression of the protein in mosquito cells decreased WNV infection (Colpitts et al., 2011b). Incubation of WNV with the recombinant cuticle protein prior to injection in mice decreased WNV infection and increased mouse survival (Colpitts et al., 2011b). This demonstrates that cuticle proteins can be antagonists of viral infection, and similar mechanisms may occur in mosquito tissues. Interestingly, ZIKV, CHIKV, YFV, SINV and WNV also induce transcriptional changes in genes that encode for cuticular proteins, the majority of which decrease (Colpitts et al., 2011b; Etebari et al., 2017; Sanders et al., 2005; Shrinet et al., 2017).

One potential role of chitin-binding proteins in the gut is mediating peritrophic matrix formation. The peritrophic matrix is formed in response to an ingested blood meal and is a physical barrier from which *Plasmodium* must escape to reach the midgut epithelium, however, evidence suggests the presence or absence of the peritrophic matrix does not limit DENV dispersal (Kato et al., 2008). Peritrophic matrix proteins (also called peritrophins) contain chitin-binding domains and provide structure and support to the peritrophic matrix. Of the hundreds of proteins identified in a recent proteomic analysis of the *Aedes aegypti* peritrophic matrix, 2 are characterized peritrophins, AeIMUC1 and AeAper50, and 2 are putative peritrophins (Whiten et al., 2018). Additionally, some of these peritrophin genes are altered transcriptionally in the carcass and salivary glands during DENV infection, suggesting that function may not be specific to the peritrophic matrix (Bonizzoni et al., 2012). Genes associated with the cuticle and chitin may also have roles within other tissues with cuticular linings, including the trachea of the respiratory system and the salivary ducts. DENV, SINV, western equine encephalomyelitis virus and La Crosse virus can be detected in the tracheal tissues, though recent evidence suggests this may not be common for CHIKV (Franz et al., 2015; Kantor et al., 2018). The salivary ducts, that transport saliva from the glands to the mouth parts and therefore also transport pathogens within the saliva, have a cuticular lining. Transcription of *AeIMUC1* and 2 putative peritrophin genes increases during DENV infection in salivary glands at 14 dpi (Sim et al., 2012). The impact of this on salivary gland infection and virus transmission is unknown.

The decreased expression of cuticle genes with chitin-binding domains is also observed in lepidopterans infected with baculoviruses (McTaggart et al., 2015; Noland et al., 2013), suggesting that this is a generalizable aspect of insect responses to a virus. Altered expression of chitin association genes is also not limited to viral infection, as systemic bacterial infection in *Aedes aegypti* increases expression of a gene predicted to be involved in chitin metabolism in the hemolymph (Bartholomay et al., 2007). In *Drosophila* too, expression of chitin metabolism and chitin-binding genes increases in the gut during bacterial infection (Buchon et al., 2009).

Wolbachia independently affects the expression of chitin and cuticle associated genes of its mosquito hosts (Kambris et al., 2009; Rancès et al., 2012; Y. H. Ye et al., 2013). In *Ae. aegypti*, *w*Melpop infection induces the expression of a peritrophin (Kambris et al., 2009) and of multiple cuticle proteins in the head (Y. H. Ye et al., 2013). DENV infection in *Ae. albopictus* naturally infected with *Wolbachia* increases expression of a gene containing a chitin-binding domain in the midgut at 5 dpi (Tsujimoto et al., 2017). The increase in expression of chitin associated genes in the presence of *Wolbachia* suggests these genes are candidates for pathogen interference. One could hypothesize that viral-induced mechanisms decrease chitin and cuticle proteins to evade host responses, and *Wolbachia*-induced expression of these genes may interfere with infection. Contrary to this idea, the presence of *Wolbachia* also results in decreased transcript levels of some cuticle proteins (Rancès et al., 2012), and the expression of only some of the genes identified across studies are affected in the presence of both DENV and *Wolbachia*.

7. Small RNAs

In insects there are three main pathways that generate small RNAs: the small interfering RNA (siRNA) pathway, the microRNA (miRNA) pathway and the PIWI-interacting RNA (piRNA) pathway. The siRNA pathway targets exogenous molecules by recognizing dsRNA and processing it into siRNAs, which then bind and silence complimentary sequences (Gammon and Mello, 2015). The miRNA pathway generates endogenous small RNAs that regulate the expression of transcripts (Lucas and Raikhel, 2013). piRNAs are generated through mechanisms distinct from the siRNA pathway, and these small RNAs target and silence transposable elements in the genome (Miesen et al., 2016b). The siRNA pathway appears to play a substantial role in viral defense (Olson and Blair, 2015). Below we review the literature on the regulation of small RNAs outside of the siRNA pathway that may also respond to viral infection: miRNAs, tRNA-fragments (tRFs) and piRNAs.

7.1. miRNAs

In mosquitoes, miRNAs have been shown to regulate processes involved in development, digestion and reproduction (Hussain et al., 2016)). Multiple studies have demonstrated that miRNAs are transcriptionally regulated in response to viral infection (Campbell et al., 2014; Dubey et al., 2017; Liu et al., 2015; Maharaj et al., 2015; Zhou et al., 2014). Transcriptional regulation of miRNAs occurs in *Ae. aegypti* in response to ZIKV (Saldaña et al., 2017), in *Ae. aegypti* and *Ae. albopictus* in response to DENV (Campbell et al., 2014; Liu et al., 2015; Yan et al., 2014; Zhou et al., 2014) and CHIKV (Dubey et al., 2017; Maharaj et al., 2015), in *C. quinquefasciatus* in response to WNV (Skalsky et al., 2010), and in *Anopheles coluzzii* in response to ONNV (Carissimo et al., 2018). Predictions of miRNA targets can be made bioinformatically and often result in hundreds of potential target genes, making functional characterization complex. Additionally, a single miRNA may target multiple genes, and a single gene may be regulated by multiple miRNAs (Asgari, 2014).

Though the majority of miRNAs are yet to be investigated, studies using miRNA mimics and inhibitors have shown that some miRNAs decrease viral infection (Dubey et al., 2017; Slonchak et al., 2014; Yan et al., 2014) and some facilitate infection (Zhou et al., 2014). Many of the predicted mosquito targets have known roles in other biological processes; however, their functional mechanisms in viral interactions are unknown. Whether miRNAs directly facilitate immune activities or respond to changes incurred during infection, their altered levels indicate a potential role in responses to infection in mosquitoes; therefore, further annotation is required to assess the role of miRNAs during viral infection.

Wolbachia infection also affects the production of mosquito miRNAs (Hussain et al., 2011; Mayoral et al., 2014; Osei-Amo et al., 2012), and

a subset of these miRNAs have been further investigated for potential effects on viral infection (Mayoral et al., 2014; Zhang et al., 2014, 2013). The miRNA aae-miR-2940 increases in abundance during *Wolbachia* infection and has been shown to target multiple genes (Hussain et al., 2011; Zhang et al., 2014, 2013). This miRNA enhances the expression of *metalloprotease m41* ftsh and decreases the expression of the *DNA methyltransferase* (*Dnmt2)*, and both result in increases in *Wolbachia* densities (Hussain et al., 2011; Zhang et al., 2013). *Dnmt2* overexpression in Aag2 cells correlates to increases in DENV infection (Zhang et al., 2013), suggesting that *Dnmt2* may facilitate DENV infection, and this activity is disrupted by aae-miR-2940. In contrast, expression of *Dnmt2* in *D. melanogaster* increases in the presence of its native *Wolbachia* sp. and has been shown to have viral defense activity, though the role of miRNAs in this process is unknown (Bhattacharya et al., 2017; Durdevic et al., 2013).

7.2. tRNA-fragments (tRFs)

The transfer RNAs (tRNAs) are another source of small RNA molecules (Shigematsu et al., 2014), and small RNAs originating from tRNAs are referred to as tRNA-fragments (tRFs) (Keam and Hutvagner, 2015; Luo et al., 2018; Miyoshi et al., 2010). Only recently, small RNA deep-sequencing has identified tRFs in *Ae. aegypti* (Eng et al., 2018). Expression of tRFs varied by sex, developmental stage and blood-feeding. Additionally, in the DENV refractory strain, Moyo-R, the expression of an individual tRF increased upon infection (Eng et al., 2018), suggesting for a role in host immunity and potentially vector competence. In *An. gambiae*, a study using deep-sequencing to identify small RNAs in association with Argonaute, a unit of the silencing complex, identified small RNAs from tRNA sources along with miRNAs and other small RNAs (Fu et al., 2017). These initial studies suggest that tRFs regulate gene expression in mosquitoes, and that they may be a novel class of small RNAs affecting host-viral interactions. Whether *Wolbachia* has any effects on the expression of tRFs is unknown.

7.3. PIWI-interacting RNAs (piRNAs)

The piRNA pathway is well-studied in germ cells, where piRNAs originate from the genome and protect the germline from transposable elements (Ishizu et al., 2012), and most of what we know about piRNA function and biogenesis has been described in *Drosophila* (Miesen et al., 2016b). Outside of the germline, piRNAs are also present in cells derived from *Drosophila* ovaries, but have not otherwise been detected in flies (Mussabekova et al., 2017). In mosquitoes, piRNAs are found in somatic tissues (Hussain et al., 2016), and multiple studies have shown that during infection virus-specific piRNAs (vpiRNAs) are produced from viral RNA in both mosquito cell lines and adults (Goic et al., 2016; Hess et al., 2011; Morazzani et al., 2012; Varjak et al., 2017a; Vodovar et al., 2012). Viral-derived piRNAs have not been shown to affect viral infection, and their role is unknown. The purported role in the viral immune response is intriguing and supported by sequence data and functional studies in cell lines, indicating that vpiRNAs are generated during infection and that they associate with proteins in the piRNA pathway (Miesen et al., 2015).

Building on the elucidation of the piRNA pathway in *Drosophila*, orthologs in the piRNA pathway are being characterized in mosquitoes. PIWI proteins associate with piRNAs and regulate biogenesis (Samuel et al., 2018). In *Ae. aegypti*, PIWI proteins have undergone an expansion as there are now 8 proteins (Campbell et al., 2008). Evidence suggests that piRNA production is independent of Dicer2, the main enzyme in the siRNA pathway, and essential proteins in the *Drosophila* piRNA pathway are non-essential to piRNA biogenesis in mosquitoes (Miesen et al., 2016a; Varjak et al., 2018, 2017b). In *Dicer2* knockout cells infected with Semliki Forest virus, no virus-specific siRNAs produced by the siRNA pathway were detected, yet piRNAs were produced (Varjak et al., 2017b). Knockdown of *Piwi4* in these cells did not change vpiRNA levels but did lead to increased viral replication (Varjak et al., 2017b). That Piwi4 is not required for vpiRNA production and has viral defense activity is in agreement with other studies (Kang et al., 2018; Miesen et al., 2016a, 2015; Varjak et al., 2017a). Interestingly, this suggests that a Piwi4-mediated response separate from the canonical piRNA and siRNA pathways may exist in Aag2 cells (Varjak et al., 2017b). The helicase Spindle-E is another example of a piRNA pathway protein with noncanonical activities in a mosquito. In *Drosophila*, Spindle-E is required for transposon silencing by piRNAs, however, in *Ae. aegypti* cells it negatively impacts viral replication and does so independent of the piRNA and the siRNA pathways (Varjak et al., 2018). *Spindle-E* knockdown increased replication of the alphaviruses CHIKV and SFV but had no effect on Bunyamwera virus or ZIKV (Varjak et al., 2018). Together these studies indicate novel roles of piRNA pathway proteins in viral defense.

Sequence data from *Ae. aegypti* has shown that some piRNAs are located within clusters on the genome, and piRNAs align to transposable elements, virus-derived sequences, gene-containing regions, and repetitive sequences in the genome (Arensburger et al., 2011). Viral genes can integrate into host genomes and are referred to as Endogenous viral elements (EVEs). Most EVEs result from retroviral integrations, however, non-retroviral virus integrations are also present in the genome of many eukaryotes (Katzourakis and Gifford, 2010). In mosquitoes, EVEs are enriched within piRNA clusters and produce piRNA-like small RNAs (Palatini et al., 2017; Whitfield et al., 2017). The possibility that these piRNAs may feed into the piRNA pathway is intriguing and highlights the potential for past viral infections to contribute to the formation of an immune response. Just as the role of the piRNA pathway during viral infection in mosquitoes is still being elucidated, efforts to assess the effects of *Wolbachia* on piRNAs are ongoing (Mayoral et al., 2014; Rainey et al., 2016).

8. Conclusions

The natural variation observed in mosquito susceptibility to viral infections highlights the underlying complexity of mosquito-arbovirus interactions (Palmer et al., 2018). The genotypes of vector, virus and other microbiota, as well as interactions with the environment all contribute to vector competence, and recent work is advancing our understanding at the intersection of these influential parameters (Raquin et al., 2017; Ross et al., 2017; Ye et al., 2016). Furthermore, evidence of immunity conferred through prior exposure, referred to as 'immune priming' or 'trained immunity' (Hillyer, 2016; Shaw et al., 2018), is just emerging from the study of repeated viral infections. Genes that are transcriptionally altered during infection across tissues may be induced through either host or viral mechanisms and may functionally be agonist, antagonist or neutral in terms of viral defense. Although major advances in our understandings of the traditional immune pathways have occurred, these responses do not entirely explain observed complexities in infection outcomes (Cheng et al., 2016). Increasing numbers of RNA-sequencing studies in mosquitoes are revealing additional candidate genes in the viral defense response. In parallel, in the *Wolbachia* system, viral blocking appears to be multifaceted and unlikely to be driven by known immune pathways (Molloy and Sinkins, 2015; Terradas et al., 2017; Terradas and McGraw, 2017). Expanding our search beyond the canonical pathways and using genetics to do so may uncover novel antiviral mechanisms. In particular, genetic approaches that probe natural variation and that utilize artificial selection can provide unbiased means of identifying essential factors involved in a specific trait. These approaches were used recently in *Drosophila* and identified the genes *pastrel*, decreases DCV load (Magwire et al., 2012), and *adar*, targets Sigma virus RNA (Piontkivska et al., 2016). Similar approaches should be used to uncover novel mosquito-virus interactions.

Here we identify a candidate set of gene families encoding broad spectrum viral defense factors in mosquitoes. Recent advances in

CRISPR-Cas9 techniques for *Ae. aegypti* will assist with testing the functional roles of these candidates and others in response to viral infection (Chaverra-Rodriguez et al., 2018). *Ae. aegypti* has immense global impact on human disease incidence through its transmission of DENV, ZIKA, CHIKV and YFV. Genes exhibiting clear antiviral effects may be harnessed to create refractory mosquitoes and further developed for biocontrol in the field. Expanding the search for antiviral effector molecules and revealing novel responses in the mosquito may also provide insight into the mechanisms of *Wolbachia*-mediated viral blocking. As this trait underpins a massive effort to roll out *Wolbachia* around the globe for disease control (Flores and O'Neill, 2018), understanding its mode of action is paramount to protecting its long-term efficacy in the field.

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Appendix A. Supplementary data

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