DISSERTATION

MEXICAN MOSQUITOES:

OVERCOMING BARRIERS FOR DENGUE AND ZIKA VIRUS INFECTION

Submitted by

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ABSTRACT

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The mosquito transmitted arboviruses cause an important burden of disease worldwide. In Latin America dengue disease is endemic with more than 1 million dengue fever cases reported yearly. In addition to dengue, chikungunya and Zika viruses have been also circulating since their introduction in 2014 and 2015 respectively. For a mosquito-borne infection to occur susceptible humans, the mosquito vector and the virus should coincide. This dissertation was focused in the mosquito vector and its ability to acquire, maintain and then transmit the virus, termed vector competence. The vector competence was a fundamental measure for the research chapters in which we studied different aspects on the interactions between *Aedes aegypti* and *Aedes albopictus* mosquitoes and Dengue-2 and Zika viruses. This dissertation includes three research chapters which were based on the following specific aims. Specific aim 1: Determine the patterns of gene flow and vector competence for DENV-2 of *Aedes aegypti* from around the Mexican Neovolcanic Axis.

It was previously reported that the intersection of the Neovolcanic axis (NVA) with the Gulf of Mexico coast in the state of Veracruz acts as a discrete barrier to gene flow among *Ae. aegypti* populations north and south of the NVA. These collections also differed in their vector competence (VC) for Dengue virus serotype 2 (DENV-2). Therefore, the goal of the present study was to determine if the same patterns remained 8 years later in collections from 2012. For which haplotype variation for the mitochondrial ND4 and the nuclear genes Dicer-2 and Argonaute-2 was analyzed for north and south of the NVA mosquito populations. Also, the VC of those populations for DENV-2 was determined (Chapter 2).

Specific aim 2: Profile the microRNA response of *Aedes aegypti* midguts to DENV-2 exposure and DENV-2 infection.

The microRNA pathway has been found to modulate important physiological mechanisms in mosquito vectors. Therefore in the context of DENV infection, miRNA modulation may provide information about key genes that are important for infection. Differential expression patterns of miRNAs from mosquito midguts upon infection have been unexplored. Therefore, we explored on the involvement of the miRNA pathway in persistently DENV-2 infected mosquitoes, for which DENV-2 virus was detected at 14 days post-infection (dpi). Two comparisons were included in the study. In the first group, DENV-2 infected midguts that produced a disseminated infection (did not have a midgut escape barrier) were contrasted with those that were given a non-infectious blood meal. Also, we included a comparison group from a subset of mosquitoes from the same cohort that were exposed to DENV-2 regardless of their midgut infection status contrasted to unexposed mosquitoes. Analysis of miRNA regulation in mosquitoes may help us to understand more about the intricate interactions between the virus and the vector host (Chapter 3).

Specific aim 3: Assess the variation in competence for Zika virus transmission by *Aedes aegypti* and *Aedes albopictus* from Mexico.

Previous studies have reported low Zika virus (ZIKV) transmission rates for the Asian lineage of ZIKV using mosquitoes from a wide geographical range from the Americas. Beside low transmission rates we hypothesized that VC is variable and is highly dependent upon the geographic origin of the mosquito populations. Hence, we analyzed the ZIKV transmission potential of recently colonized Aedes collections. Ten *Ae. aegypti* and three *Ae. albopictus* collections from different locations across Mexico were analyzed for ZIKV (strain PRVABC59 - Asian genotype) vector competence at 7 and 14 dpi. We calculated the additive contribution of each of the four transmission barriers to ZIKV infection. In addition, we

evaluated the contribution of both mosquito species to ZIKV transmission in areas where their distributions overlap (Chapter 4).

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CHAPTER 1: LITERATURE REVIEW

Arboviruses

Arthropod-borne viruses or arboviruses, is a term used to refer to the viruses transmitted to vertebrates by arthropod vectors[1]. The arthropod vectors are hematophagous insects like mosquitoes, sandflies, midges and cimicid bugs [2, 3], or ixodid and argasid ticks [3, 4]. The arboviruses belong mainly to seven viral families; *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, and *Asfarviridae* [3]. All, except for the *Asfarviridae* family with a double-stranded DNA genome, have RNA genomes[5]. Within the *Flaviviridae* family, the genus *Flavivirus* contains more than 70 viruses[6]. In this genus, the dengue viruses (DENV-1 to -4), Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), Yellow fever virus (YFV), West Nile virus (WNV) and Zika virus (ZIKV) are recognized etiological agents of disease in many areas of the world [6].

DENV

DENVs are mosquito-borne flaviviruses within the *Flaviviridae* family [6]. DENVs are mosquito-borne flaviviruses within the *Flaviviridae* family [6]. DENVs are traditionally classified into four serotypes (DENV-1 to DENV-4) based on their antigenic characteristics [7] hence each serotype generates a unique host immune response to infection [8, 9]. There is genetic variation within each serotype therefore; DENVs can be further classified based on their genetic similarity (using partial or complete genome sequences) into genetic groups or 'genotypes'. In addition, phylogenetically discrete groups of isolates can be found within each genotype, being considered as viral 'clades' [8, 9].

DENV genome

DENVs are flaviviruses that belong to the *Flaviviridae* family [6]. They have a single strand, positive sense RNA genome with a single open reading frame, which encodes three structural proteins (capsid [C], envelope [E], and prM proteins) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [10]. The genome is packaged in a spherical particle of around 40-50 nm in diameter, that is surrounded by a lipopolysaccharide envelope [10].

DENV transmission cycle

DENVs are transmitted to humans through the bite of an infectious mosquito. There are two DENV transmission cycles; a sylvatic enzootic cycle, between non-human primates and arboreal *Aedes* mosquitoes and an urban, endemic/epidemic cycle between peridomestic *Aedes* mosquitoes and human reservoir hosts [11]. Sylvatic cycles in Africa and Asia are ancestral to the endemic cycle that became established when urban populations became large enough to support continuous inter-human DENV transmission, which probably requires 10,000 to 1,000,000 people [11].

Clinical presentation

Upon infection with a DENV, an intrinsic incubation period (IIP) takes place in the human host, defined as the time between a human being infected and the onset of symptoms due to infection, this time allows for virus replication which leads subsequently to infection of the mosquito vector [12]. After the IIP, onset of fever lasts from 2-7 days and viremia lasts approximately 5-days [10, 13].

Infection with any of the four dengue serotypes can cause a spectrum of disease; from dengue fever (DF) a usually self-limited disease, characterized by flu-like symptoms to severe presentations like dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). However, the majority of infections are clinically inapparent [14].

The dengue clinical course is divided into three phases: febrile, critical and recovery. The febrile phase is characterized by the presence of nonspecific or flu-like symptoms, including fever, malaise, headache, body pains and rash. The critical phase, starts around defervescence or a drop and maintenance of temperature at or below 37.5-38°C [15]. During the critical phase severe symptoms may appear in a small proportion of patients. Severe symptoms include bleeding, thrombocytopenia <100,000 platelets mm^{-3}, ascites, pleural effusion, hematocrit >20% and clinical warning signs, including continuous abdominal pain, restlessness and/or somnolence, persisting vomiting and a sudden reduction in temperature associated with profuse perspiration, loss of strength and fainting, can be indicative of plasma extravasation, where the vascular permeability results in plasma leak into the pleural and abdominal cavities [16] and the occurrence of shock [10].Finally, the recovery phase lasts for 2-5 days, as symptoms resolve and clinical parameters return to normal [17].

ZIKV

ZIKV belongs to the *Flaviviridae family*, genus *Flavivirus*. It has a single-stranded, positive sense RNA genome of approximately 10.7 Kb, which encodes for a single polyprotein that is cleaved into three structural proteins and (C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [18]. There are three major ZIKV lineages: East African, West African, and Asian [19].

ZIKV was first isolated in Africa in 1957[20]. Causing sporadic cases in Africa and Asia until 2007 when it caused a large outbreak in the Yap Island, Micronesia [21]. It was then introduced into the Pacific Islands during 2013 and 2014 and finally arrived in the Americas being reported in Brazil in 2015 [22]. Analysis of ZIKV sequences from the Brazil outbreak revealed that American ZIKV isolates belonged to the Asian genotype with a common ancestor from the 2013 French Polynesia outbreak [22].

ZIKV is maintained in a sylvatic (enzootic cycle) and in an urban-epidemic transmission cycle [23]. For the sylvatic cycles, African [19, 20, 24, 25], Asian [26] and Brazilian [27] non-human primates have been found seroprevalent for ZIKV. The modes of transmission for ZIKV are vector-borne, mother-

to-child, sexual contact and by blood transfusion [22]. However, a mosquito bite is believed to be the most common way of dispersal in epidemic and endemic zones [18]. ZIKV clinical symptoms may be confused with those caused by DENV and CHIKV [28], which co-circulate and are transmitted by the same mosquito vectors.

DENV epidemiology

Dengue is the most prevalent arthropod transmitted disease in the world, being endemic in most tropical and subtropical countries [10].The World Health Organization (WHO) has estimated that 2.5 billion people are at risk for dengue and that 50 million dengue infections occur worldwide [15]. However, it was recently estimated that about 400 million infections occurred in 2010 [14].

Dengue infections are determined by the presence of the vectors, mainly *Aedes aegypti* (L.), in many areas of the world. However, the Americas have the majority of WHO-reported dengue cases worldwide [29].

Aedes aegypti was almost eradicated after a large scale program in Central and South America by using dichlorodiphenyl-trichloroethane (DDT), from 1947-1970, after which the mosquito resurged [30]. For almost 30 years (1950s-1970s) dengue was rare but the re-invasion of the vector returned dengue epidemics to these areas with an increasing number of cases [30, 31].

In the Americas, dengue is characterized with an endemic-epidemic pattern with outbreaks every 3 to 5 years [29, 30]. An epidemic in Cuba in 1981, caused by DENV-2 genotype III was the first major DHF epidemic in the Americas, where it caused 10,312 cases of DHF and 158 deaths [32]. A second DHF epidemic took place in Venezuela during 1989-1990 that was associated with multiple serotypes and also with DENV-2 genotype III [30]. In addition, to the more severe clinical presentations, fatal cases were associated with secondary infections in Brazil [30, 33]. After, 2000 a dramatic increase in the number of dengue cases with DENV hyper-endemicity was reported [30, 31]. From 1980s to 2007, a 4.6-fold increase in the total dengue and DHF cases was observed. During that period Brazil had the

higher number of dengue cases and Venezuela the highest number of cases with DHF. From 1995 to 1999, all four serotypes were reported and by 2007 all were circulating in the Americas [10, 29].

After the reappearance of *Ae. aegypti* in the 1970s, outbreaks with more than 130,000 dengue cases occurred in Mexico in 1980, 1997 and 2009 [29]. In addition, Mexico is considered a hyper-endemic country for DENV [31], with the presence of the four serotypes [34].

Just in 2016, the Pan American Health Organization (PAHO) reported more than 2 million infections of dengue in the Americas, including severe forms of dengue, of these, 17,795 confirmed DENV infections occurred in Mexico [35].

ZIKV epidemiology in Mexico

On October 21, 2015 an imported ZIKV infection was reported in the state of Queretaro from a returning individual from Colombia [36]. The first autochthonous cases or cases contracted in the area were reported simultaneously in the states of Nuevo Leon and Chiapas in November of 2015 [36].

At least two retrospective studies based on epidemiological data obtained from the Mexican health system have been published. One study analyzed 93 autochthonous confirmed ZIKV cases that occurred from November 25, 2015 to February 19, 2016 in Mexico. The other 84 confirmed ZIKV cases were reported between October 2015 and January 2016. It is important to note that some overlap of the results was expected since there was overlap during the periods when the data was obtained. Both studies found that the peak number of cases took place during the second and third epidemiological week of 2016, the majority of cases were females and the most common clinical symptoms were fever, rash, non-purulent conjunctivitis, headache and myalgia, others included polyarthralgias, exanthema, itchiness and shaking/chills. No deaths or severe outcomes (as Guillain –Barré Syndrome) of ZIKV infection were reported [36, 37]. Haque et al. (2016) mapped the 84 autochthonous ZIKV cases along the railways which may be important routes for migration of mosquitoes and infected persons.

In another study, early 2015 stored samples that were negative for the circulating arboviruses in Mexico (DENV and/or CHIKV); measles suspected cases and *Ae. aegypti* mosquito pools collected previous to the first ZIKV reported cases were reanalyzed. Fifty samples from the state of Veracruz (negative for CHIKV and DENV) and a second subset of 50 samples from the state of Yucatan (4 negative for DENV and CHIKV and 46 negative for DENV or CHIKV) were analyzed and seventy percent of them were positive for ZIKV by RT-PCR. This indicates that ZIKV has been in Mexico since July 2015. Eighty-six percent of the measles suspected cases were ZIKV positive indicating that ZIKV was present as early as May 2015. Mosquito pools from the state of Guerrero collected during January-March 2015 were also positive by RT-PCR, suggesting that ZIKV was circulating in this country since the beginning of 2015 [38]. The first microcephaly ZIKV-related case occurred on November 5, 2016 in the state of Oaxaca [39].

Dengue economic burden in the Americas and in Mexico

Dengue is the most prevalent arthropod transmitted disease in the world, with estimates of 400 million infections in 2010 [10, 14]. An elevated risk for dengue was associated with high precipitation, suitable temperature and low income urban /peri-urban settings [14]. Dengue is therefore a major public health concern in the Americas. Incidence has increased over the years with frequent epidemics and the co-circulation of multiple DENV serotypes [40].

Mexico as with many countries in Latin America is currently dealing with dengue and other mosquito-borne illnesses impacting public health and causing economic loss. Recent estimates for the annual economic costs and disease burden for dengue disease in Mexico, reported that 139,000 symptomatic and 119 fatal cases occurred during 2010-2011. During that period, estimates of US$170 million were spent from which US$87 million corresponded to illness cost. Surveillance and prevention measures accounted for an estimate of US$83 million. The annual disease burden averaged 65 disability-adjusted life years (DALYs) per million people [34]. Dengue is a reportable disease in Mexico through the country's Ministry of Health surveillance system [34, 41] which included the reporting of the

Chikungunya virus (CHIKV) and ZIKV infections upon their emergence in Mexico [41]. However, as with many surveillance systems, Mexico's system relies on passive case detection and reporting and therefore underestimates incidence [22].Despite efforts, surveillance systems have limitations including the low proportion of individuals with apparent infections seeking medical attention and misdiagnosis which is an even greater problem in regions where diseases with overlapping symptoms co-occur and usually results in underreporting [14, 23, 34, 36, 40]. Due the lack of effective vaccines or therapeutics against DENV, CHIKV and ZIKV, vector control remains the main measure to reduce disease [42]. In Mexico, the prevention program: "Wash, Cover, Flip and Throw out" is essential to fighting mosquito borne diseases through the reduction of larval breeding sites [43].

Flavivirus replication cycle / DENV replication cycle

The flavivirus replication cycle begins when a virus attaches to cell receptors. The proposed attachment molecules in mammalian cells are heparan sulfate, heat-shock proteins (HSP) 90 and 70, neolactotetraosylceramide, CD14, GRP78/BiP, 37–kDa/67-kDa laminin, and C-type lectins like the dendritic cell (DC)-specific intracellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN) and the C-type lectin domain family 5, member A and the mannose receptor; while in mosquito cells the HSP-90 related proteins, R80, R67 and a 45-kDa protein, the laminin-binding protein, prohibitin, tublin-like protein have been proposed [44-47]. However, a specific receptor for DENV has not been definitely identified [46].

Viral internalization occurs by clathrin-mediated endocytosis [48] alternative entrance by non-classical clathrin-mediated endocytosis, caveolae and lipid rafts have also been reported in cell culture studies [47, 49]. The low pH in the late endosomes causes conformational changes in the virion, resulting in trimerization of the envelope protein, exposing the fusion peptide inducing fusion of the viral and host cell membranes [50].

After fusion, viral RNA is released into the cytoplasm where it is translated into a single polyprotein that is inserted into the endoplasmic reticulum (ER) and is then processed by viral and host proteases into three structural (C, M and E) proteins and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins [51]. Following translation, a replication complex is assembled and associated with virus-induced membranes in the ER [52]. The replication complexes serve as a replicative niche for the virus in the cytoplasm but also protect the virus from immune detection [53]. Viral replication starts by transcription of the viral genome, a RNA (+) that then generates a negative strand (-) complementary RNA to act as template for the generation of new positive strand (+) RNA. This RNA can now initiate a new replication cycle or be used as a viral genome for new virions.
During the replication process double stranded RNA is generated that may trigger the innate immune response in mosquitoes. In addition the (-) RNA is recognized as evidence of viral replication in infected cells [54]. The DENV genome gets closely associated with the C protein forming the nucleocapsid. This then buds to the lumen of the ER containing the E and prM proteins, into immature particles [55]. The function of the prM protein is to prevent premature fusion of the virus during its transport out of the cell which occurs via the secretory pathway.

Viral maturation occurs during virus egress, where the trans-Golgi network acidification induces conformational changes and the host furin recognizes the Arg-X-Arg/Lys-Arg site and cleaves the prM protein into the pr peptide and M protein, generating mature viral particles [56, 57]. However, this process is inefficient since a mixture of mature, immature and partially mature viruses are secreted by the infected cell [44]. The viral particle is finally released by exocytosis [58-60].

Molecular evolution of DENV

DENV exists as four closely related serotypes; infection with any of them can cause dengue disease. The co-circulation of different serotypes may have an effect on disease outcome. For instance, a secondary infection with DENV-2 is more likely to result in severe disease compared with other

serotypes [61]. The association between clinical manifestations and the infecting serotype has been assessed. For instance, in a prospective study of adult febrile patients from Singapore (2005-2011), DENV-1 was associated with red eyes and higher risk for severe forms of dengue than infection with DENV-2 which was associated with joint pain and lower platelet count [62]. In another prospective study in children from Bangkok, Thailand (1994-2006), DENV-2 was associated with severe disease while secondary infections with DENV-2 and DENV-3 were twice as likely to result in DHF as DENV-4 [63].

There are many genotypes within each serotype. Obtaining the sequence of the DENV genome and its use for phylogenetic classification and correlation with clinical outcome and disease severity approach has been developed and has shown that certain genotypes are associated with severe disease. In the Americas some genotypes have been associated with higher virulence and disease outcome severity [64].

An example of genotype replacement was observed for the Southeast Asian (SEA) and the American genotypes of DENV-2. The SEA caused a shortened EIP [65] and more efficient dissemination in mosquitoes [66]. Likewise, there are lineages within each genotype. In Mexico, lineages are associated with frequent replacement and minimal serotype co-circulation even when the four serotypes are present [67]. A recent study used dengue molecular epidemiology data from Latin America (including the Caribbean) to describe the molecular epidemiological trends of dengue. This study found co-circulation of multiple serotypes, re-introductions of different genotypes within serotypes and coexistence, extinction and replacement of lineages [40].

Innate immunity against DENV

DENV infects monocytes, macrophages, B cells and DCs in the human host [68]. DENV infection elicits numerous intracellular innate immune responses. In general, the innate immune system recognizes pathogen –associated patterns (PAMPs) [69]. The most important DENV pathogen recognition receptors (PRR) are the membrane-bound Toll-like receptors (TLR3/TLR7/TLR8), the

cytosolic receptors retinoic acid-inducible gene I product (RIG-I) and the melanoma differentiation-associated antigen 5 (MDA-5) [70, 71]. Upon activation, signaling pathways activate downstream effectors in response to pathogens. This effector molecules are cytokines/chemokines, type I interferons (IFNs), and antimicrobial peptides [72, 73].

TLRs are transmembrane glycoprotein receptors with an N-terminal extracellular PAMP-binding region and a C-terminal intracellular signaling region. Upon extracellular ligand recognition, TLR dimerization is thought to be induced, bringing together the cytoplasmic TIR domains and subsequently recruiting adaptor molecules to initiate the signaling process.TLR3 is found in intracellular compartments in macrophages, B lymphocytes, DCs, natural killer (NK) cells, epithelial cells and fibroblasts recognizing dsRNA. TLR7 senses ssRNA oligonucleotide containing guanosine- and uridine-rich sequences from RNA viruses and short interfering RNAs (siRNA) [72, 73]. RIG-I –like receptors belong to the family of aspartate-glutamate-any amino acid-aspartate/histidine (DexD/H)-box helicases. Relevant members for DENV recognition are the RIG-I and MDA5, which recognizes long dsRNA fragments [69, 74].

Activation of PRR results in type I IFNs upregulation and cytokine production. IFN α and β are type I IFNs, both induce antiviral responses by the transcription of IFN inducible genes, promote intracellular antiviral responses influencing protein synthesis, growth regulation and apoptosis in the cell. They also, influence maturation of DCs, cytotoxicity of natural killer (NK) cells and the differentiation of virus-specific cytotoxic T lymphocytes [72, 73]. Nevertheless, DENV evades the immune response through the replication in ER membrane associated replication complexes [75] protecting the virus from the recognition by pathogen recognition receptors (PRR). And also, some of the DENV non-structural proteins block the innate immune pathways that produce type I IFN.

DENV NS2A, NS4A, NS4B and NS5 are antagonists of type I IFN signaling. NS4A induces autophagy to prevent cell death and promote viral replication [76]. NS4A and B can induce the unfolded

protein response and inhibit interferon signaling [77] and the NS5 antagonizes IFN signaling by binding and degrading STAT2 [78]. The subgenomic flavivirus RNA (sfRNA) acts as an RNA interference (RNAi) suppressor in both insect and mammalian cells by inhibiting the RNAse activity of Dicer [79, 80].

Humoral and cellular immune responses to DENV

The immune response upon DENV infection of the vertebrate host is unclear. Neutralizing antibodies (Nabs) and T-cell responses upon DENV infection have been associated either with protective or pathogenic roles.

Hyperendemicity or the circulation of multiple serotypes, has been associated to the emergence of DHF [31]. Evidence of secondary infection causing severe dengue was observed in Cuba where DENV-1 introduction was followed by introduction of DENV-2 in 1981 and 1997 with DHF presentation [81, 82].

After primary DENV infection, type specific antibodies [83] and T-cells are responsible for protection against re-infection with the same serotype [84]. So that secondary infections occur in the context of pre-existing heterotypic immunity that is serotype cross-reactive. Overall, it is recognized that previous immune response has the potential to increase the risk for developing severe clinical outcomes in a subsequent infection [85].

One of the mechanisms related with severity upon DENV secondary infections is known as antibody-dependent enhancement (ADE). Primary infection with a DENV confers long-term immunity against re-infection with the same serotype (homologous serotype) but not to other serotype (heterologous serotype); a temporal protection for the others serotypes is observed, but just lasts for a few months. In a subsequent infection with a heterologous DENV, protection is not complete [86], pre-existing sub-neutralizing antibodies from the primary infection do not clear the infection but help the virus to enhance its uptake by immune cells like macrophages through Fc receptors [87].

In other words, when not enough anti DENV E and prM antibody molecules bind to the virion, so that neutralization is not achieved, myeloid cells like monocytes and macrophages (which are the

principal site of DENV replication) that express receptors for immunoglobulins enhance viral entry [88], and cause higher viral loads.

The role of maternal antibodies in the presentation of severe disease in primary infections has been observed. ADE is supported by observations where young children are at higher risk to develop severe disease due the presence of antibodies from their mothers that protect the newborns from infection but such protection due maternal antibodies deteriorate reaching sub neutralizing levels enhancing primary DENV infection [89].

In a recent study, plasma from DENV-infected subjects after 6 months of infection, was able to promote ADE in the human myeloid cell line U937, antibodies bind to ZIKV but were not able to neutralize infection leading to ADE in a 12-fold [90]. Other studies have also shown that antibodies against DENV could enhance ZIKV infection [91, 92]. This has important implication for disease pathogenesis in places where DENV and ZIKV co-circulate and possibly compromise vaccine success.

The immune response upon DENV infection is still unclear, which has led to the description of multiple phenomena. Affinity maturation is a phenomenon where the affinity of antibodies that react with the antigen that triggered the response increase over time. In other words longer the time after primary infection, decreases neutralization and ADE may take place [93]. In contrast, recent analyses, showed that the quality of neutralizing antibodies depend on the number of previous exposures to different DENV serotypes. The antibody somatic hyper-mutation model where B-cell clones evolve during subsequent infections to secrete higher affinity and more broadly neutralizing antibodies [84].

Based on cell culture studies there are two ADE types, the extrinsic and intrinsic ADE. In the extrinsic ADE, high viremias result from a greater number of infected cells; while in the intrinsic ADE, antibody mediated infection, type I IFN and production of antiviral molecules like IL-10 are suppressed enhancing replication [85, 94, 95]. However, these results have been variable when other cells are studied [96, 97].

A second mechanism for severity upon secondary infection, known as Original Antigenic Sin (OAS), describes the response to a secondary infection which is dominated by the proliferation of cross-reacting B and T memory cells that were induced during primary DENV infection, over the following infecting serotype, leading to lower affinity antibodies for the secondary infecting DENV [87].

A rapid memory response upon infection may be good however. The production of lower affinity clones that do not appropriately clear the secondary infecting virus can instead lead to more severe pathogenesis. In addition, extensive T-cell activation leads to cytokine release and immune-mediated tissue damage. A protective role of the OAS has been suggested, with multifunctional CD8+ T cells responses during second infections [87, 98].

It has been observed that in secondary infection with DENV-1, T cells reacted to DENV-3, while in DENV-2 infection; the T cells reacted with DENV-1 and DENV-3. So, the cells were stimulated to proliferate by the currently infecting virus [99]. Also, titers of antibodies specific for the virus serotype that caused the first infection increase and often maintain higher levels relative to the currently infecting serotype during a secondary infection.

DENV pathogenesis is also associated with dendritic cells (DCs), macrophages and mast cells (MC). MCs can get activated upon DENV infection releasing immune mediators like the tryptase and chymase, histamine and heparin that are stored in its granules which can act directly on the vascular epithelium. So that degranulation has been associated with DENV pathogenesis. It was shown that the injection with similar levels of tryptase observed in DHF patients to mice was able to promote substantial vascular leakage [100]. As in other cells, cytokines and chemokines are also produced. High concentrations of cytokines and soluble immune factors like the tumor necrosis factor alpha (TNFα) and the vascular endothelial growth factor (VEGF) respectively, contribute to severe manifestations [87] by mediating the endothelial activation which may lead to vascular leakage. A better understanding of the pathogenesis of dengue may help to understand the implications and the development of vaccines.

Factors that influence DENV transmission

The vectors

Aedes (*Stegomyia*) *aegypti* (Linnaeous, 1762) and *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1894), are invasive vector species in the Americas with native ranges in Africa and Asia, respectively [101, 102]. Both species are associated with human dominated habitats, preference for human blood and breeding in artificial containers. The lifespan of a female *Ae. aegypti* mosquito under ideal conditions ranges from 30-50 days [103, 104] and the high association with humans enhances the transmission of arboviruses [105].

Aedes aegypti, is invasive and is mainly dispersed by human activities [102]. In an effort to control the mosquito vectors and to therefore limit the number of dengue cases the indiscriminate use of pesticides has led to the development and spread of insecticide resistance in mosquito populations [13, 30]. Specifically, *Ae. aegypti* feeds on humans during daylight hours and typically rest indoors [106] and may take several blood meals per gonotrophic cycle [107] all of which increase its potential as a vector.

Aedes albopictus, the Asian tiger mosquito, is an invasive mosquito with ancestral origins from China, Thailand and Japan. Now, the species is found in tropical, subtropical and temperate areas of the world and maintains relatively high genetic diversity [108]. In addition, this species is able to diapause, a process where the development of the organism is arrested due to unfavorable conditions. Diapause provides a mechanism that allows for temporal adaptation to the environment. After harsh conditions and in response to photoperiod, gradual reactivation of diapausing eggs is observed [109].

Aedes albopictus host-feeding patterns are opportunistic. They feed on a wide variety of hosts including mammals and birds [110]. Even though it has been considered as a mammal-feeding generalist, humans may be the preferred host [105, 111]. *Aedes albopictus* can be now found throughout the world except in Antarctica [112]. The species was first reported in the Americas in 1985,

in Houston, Texas [113] and in Mexico *Ae. albopictus* larvae were reported at border cities in Coahuila, in 1993 [114, 115]. Until recently it was only considered a secondary vector. However in the laboratory it has been reported to be competent to transmit more than 27 arboviruses, including dengue, chikungunya and Zika viruses [116]. The species has been involved in recent dengue [117] and chikungunya [118] outbreaks [102].

Human exposure to arboviruses depends on the mosquito contact which is increased in areas without access to potable water. In these locations water has to be stored in household containers such that people living under poor public sanitation may be at a greatest risk. In addition, anthropogenic factors like climate, land use, urbanization, social and political policies, poverty and human movements also have an influence [119]. As stated by Ali et al. 2016, “Poverty can create the ideal conditions for disease transmission, limiting access to running water, education and health care” [120] conditions that are found in many areas of Latin America.

To get infected with DENV, mosquitoes have to feed on a viremic human. It has been determined that for *Ae. aegypti*, the infectious dose to infect 50% (ID_{50})of the mosquitoes varies by serotype. The ID_{50} is higher for DENV-3 and DENV-4 than for DENV-1 and DENV-2. In addition, there is a dose-dependent effect [105]. Humans are infectious even before the onset of symptoms for approximately 5-6 days [105, 121]. Both, symptomatic and asymptomatic infections may be infectious to mosquitoes. However the majority of DENV infections are asymptomatic. These cases may have a role in keeping the virus in circulation [105].

Aedes albopictus is more susceptible to DENV infection in the midgut but dissemination is lower than observed for *Ae. aegypti* [117]. Such observation was confirmed in a study where both species were fed on viremic blood from dengue patients, the odds of *Ae. albopictus* becoming infectious were lower than those for *Ae. aegypti* [122].For ZIKV the viremia found in humans from the field was 4.4 lo $_{10}$

[123].In a study with Nicaraguan patients quantifiable viremia of 4.7 $\log_{10}$ for ZIKV and 5.84 $\log_{10}$ for DENV were reported [124].

Vector incrimination

It is recognized that *Ae. aegypti* is the main vector of dengue viruses. However, other *Aedine* mosquitoes like *Ae. albopictus* and *Ae. polynesiensis* may also act as vectors [31]. The first report of *Ae. albopictus* naturally infected with DENV in the Americas occurred in 1995, during a dengue outbreak in Reynosa, Tamulipas, Mexico [125]. Mainly *Ae. aegypti* and *Ae. albopictus* were collected, pooled and screened for DENV detection by cytopathic effect (CPE) in mosquito C6/36 cells [126] and in kidney epithelial cells from the African green monkey (Vero) and by haemagglutination assay and confirmed by immunofluorescence with monoclonal antibodies and RT-PCR [125]. Aiming to incriminate *Ae. aegypti* or *Ae. albopictus* as a vector during the 2014 CHIKV outbreak in Chiapas (a state in Southern Mexico) entomo-virological surveillance was performed. Mosquitoes were sampled from neighborhoods with suspected cases; the majority was identified as *Aedes aegypti* (1170), followed by *Culex quinquefasciatus* (453), *Cx. coronator* (4) and *Ae. albopictus* (3). At least 20% of the *Ae. aegypti* pools were positive for CHIKV and sequencing revealed that those belonged to the Asian lineage [127].

Some studies have tried to determine the natural vectors of ZIKV by screening wild-caught mosquitoes from inside and around homes of ZIKV suspected cases. In one study, 198 *Ae. aegypti*, 26 *Ae. albopictus* and 249 *Cx. quinquefasciatus* pools from Rio de Janeiro, Brazil were screened and three of the *Ae. aegypti* pools were positive for the ZIKV genome, including one pool with one male, raising the possibility of vertical/venereal transmission [18]. Using the same approach' the ZIKV genome was detected in 15 of *55 Ae. aegypti* pools from the Southern state of Chiapas, Mexico [128]. In addition, *Ae. albopictus* pools from the state of San Luis Potosi, Mexico were positive for ZIKV as reported by the Mexican Epidemiological Surveillance System [129]

Vectorial capacity

George Macdonald synthesized vector capacity into a mathematical model of the basic reproductive number (Ro) for malaria [130]. This was subsequently modified by Garrett-Jones in 1964, for case reproduction number. The model incorporates the main elements of the mosquito's life history that impact pathogen transmission.

Vectorial capacity (V) is a quantitative measure of the potential of an arthropod vector to transmit a pathogen. It is defined as the average number of potentially infective bites that will ultimately be delivered by all the vectors feeding on a single host in 1 day [131]. V is defined by the following equation:

$$V = \frac{mbp^n a^2}{-\ln p}$$

Where m=density of vectors (per host), p= survival rate of the vector (per day), a=biting rate (host preference index x feeding frequency), n= extrinsic incubation period (in days), and b= vector competence.

V is impacted by extrinsic factors like vector density, vector longevity, length of the extrinsic incubation period (EIP) and blood feeding behavior [132, 133] and also by intrinsic factors like vector competence (VC). Based on the equation, the factors that have a greater influence in V are the survival rate of the vector, the probability a vector feed on a host in 1 day (biting rate= host preference index x feeding frequency), and the duration of the EIP. However, virus transmission is highly dependent on other environmental conditions that affect *Ae. aegypti* survival and abundance, including temperature, humidity and availability of breeding sites [119].

Vector competence

VC is defined as the intrinsic ability of an arthropod vector to acquire, maintain and then transmit a pathogen [134]. Upon intake, the arbovirus has to replicate and be able to be transmitted to

a susceptible host in a subsequent feeding episode; however the virus has to first bypass a series of physiological barriers (Figure 1.1) [135]. Briefly, upon entry of the virus into the mosquito gut through an infectious blood meal, the virus has to establish an infection; if this does not occur the mosquito has a midgut infection barrier (MIB). Next, the virus has to replicate and disseminate to other mosquito tissues; if this does not occur the mosquito has a midgut escape barrier (MEB). The virus then may infect several mosquito tissues but especially the salivary glands where it again has to establish an infection. If this is prevented the mosquito has a salivary gland infection barrier (SGIB). Next, the virus has to replicate and disseminate into the saliva secretions from where it will be expectorated with the saliva into a susceptible vertebrate host. If this is limited, the mosquito has a salivary gland escape barrier (SGEB) [135, 136]. In conjunction, the MIB, MEB, SGIB and SGEB contribute to the overall VC phenotype.

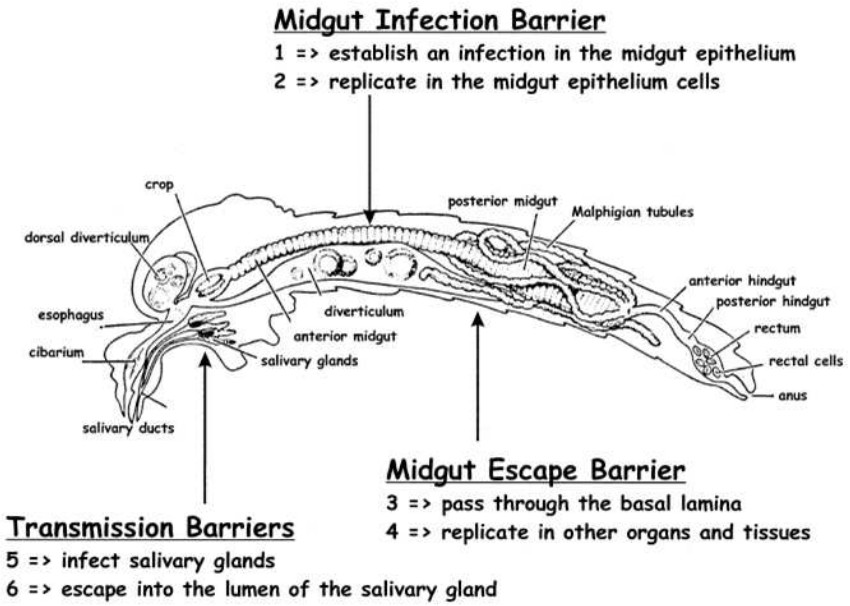

Figure 1.1. Barriers to arbovirus transmission. Diagram of a mosquito showing the barriers to biological transmission of arboviruses. Mosquito organs and the steps the arbovirus has to overcome in able to be transmitted are depicted. From Black, W.C., et al., 2002. Flavivirus susceptibility in *Aedes aegypti* [135]

Aedes aegypti vector competence publications are abundant and variation in VC is a constant theme. This variation may be due to interactions between genetic factors and complex environmental

conditions [108]. There is evidence that *Ae. aegypti* competence for DENV can vary in interactions between mosquito strains and viral genotype. In genetically diverse/natural populations [137]. Both, virus and vector genetics influence vector competence. An example of genotype x genotype interactions influencing VC was observed for DENV and YFV in Senegalese *Ae. aegypti* populations. Mosquitoes from Mont Rolland, Rufisque and PK10 were refractory to the infection by YFV but susceptible to DENV-2 from Senegal [138].

Populations of *Ae. aegypti* are highly variable in their competence for DENV [139]. Variation in the competence of Mexican mosquitoes to DENV-2 has been reported [140, 141]. In one study, 24 *Ae. aegypti* collections from Mexico and the US were orally infected with DENV-2 (JAM1409); variation in midgut infection was observed and VC ranged from 24 to 83%. In addition, the collections were assigned to three geographical regions [142]; Pacific, Yucatan, or Northeast. The Yucatan had greater VC (62%) when compared with the Northeast and Pacific with 53 and 57%, respectively. However, the VC was also variable within Northeast and Pacific collections so that geographic location was not correlated with VC [140].

Later, a more narrow geographical area was studied, 10 *Ae. aegypti* collections from Veracruz, a coastal state in the Gulf of Mexico, were assessed for DENV-2 (JAM1409). Mosquitoes from Southern Veracruz had low VC (20%) consistent with a high MIB while mosquitoes from the North were more competent (55%). The study concluded that the Neovolcanic Axis (NVA) acted as a barrier to gene flow and provided the observed pattern for VC [141].

In chapter 2 of this dissertation, we tested whether the NVA still impacts VC 8 years later. The VC of Mexican *Ae. aegypti* and *Ae. albopictus* for ZIKV is described in chapter 4.

Vector competence reports for ZIKV

The competence of *Culicidae* mosquitoes including the *Aedes*, *Culex* and *Anopheles* genera for ZIKV transmission has been explored using field-caught, recently colonized or laboratory strains. Both,

African and Asian ZIKV lineages and freshly grown or frozen virus have been used to infect mosquitoes through blood-meals or parenteral via, additionally infection of mosquito tissues or salivary secretions have been determined by a wide variety of detection methods. Therefore, comparisons of experimental vector competence outcomes should be evaluated with caution.

For this section, only studies where ZIKV exposure was through blood-meal and transmission was assessed were included in Table 1. ZIKV transmission was obtained at 14 dpi unless otherwise stated.

In summary, a great variation has been documented. *Anopheles* mosquitoes have not been demonstrated as a competent vector for ZIKV. Six *Culex* species have been tested and it has not been found a competent ZIKV vector with the exception of one study where Chinese *Cx. pipiens quinquefasciatus* were found extremely competent. For the *Aedes*, 11 species have been challenged, the species *triseriatus*, *polynesiensis*, *vigilax*, *procax* and *notoscriptus* have not been found competent while *vexans* competence was found to be low. *Aedes albopictus* showed low competence for ZIKV with the exception of mosquitoes from Singapore for which more than 75% of transmission was observed therefore extremely competent. Aedes aegypti from Senegal, Africa were not competent however other *Aedes* species, *vittatus* and *luteocephalus* competence was classified from low to moderate. In contrast, *Ae. aegypti* from the western hemisphere and the pacific islands have shown to have highly variable competence phenotypes.

The impact of the competence of the vector in the actual transmission or potential for transmission depends on the particular conditions where the vectors are present, vector densities, blood-feeding preferences and behavior.

Table 1.1. Vector competence reports for ZIKV.

Location	Mosquito species	Field/colonized	ZIKV lineage / strain	Dose	Detection method	Frozen	Fresh
Poza Rica, Ver. Mexico [143]	*Ae. aegypti*	Collected in 2012. ~F11-F13	West Africa: Senegal (41525) Asian: American (PRVABC59) and East Africa: Uganda (MR766)	1 x 10^6 pfu/mL	Plaque forming unit assay	+++ for PRVABC59, NT for 41525 and MR766	++++ for PRVABC59, +++ for 41525 and MR766.
Laboratory strains [143]	*Cx. quinquefasciatus*	>F20	Asian: American (PRVABC59)	1.6 x 10^7 pfu/mL (frozen) and 5 x 10^6 pfu/mL (fresh)	Plaque forming unit assay	NC	NC
	Cx. pipiens			5 x 10^6 pfu mL		NT	NC
	Cx. tarsalis			5 x 10^6 pfu/mL		NT	NC
Salvador, Brazil [144]	*Ae. aegypti*	F2	African: Senegal (DAK, AR 41525) Asian: Cambodia (FSS 13025) and 2015 Mexico (MEX 1-7).	10^6 ffu/mL	Focus forming assay (FFA)	+++ for DAK, no competent for FSS and MEX	++ for FSS
Republica Dominicana [144]	*Ae. aegypti*	F6				+++ for DAK, + for FSS and MEX	NT
Rio Grande Valley, Texas, USA [144]	*Ae. aegypti*	F4				++ for DAK, no competent for FSS and MEX	NT
Rio de Janeiro, Brazil [145]: Manguinhos, Copacabana,	*Cx. Quinquefasciatus*	F1	Asian: Rio-U1, Rio-S1	10^6 pfu mL	Titration by plaque forming unit assay and RT-qPCR RT-qPCR	NC	NT

Jacarepaguá							
Rio de Janeiro, Brazil [145]: Triagem	*Cx. Quinquefasciatus*	Laboratory strain				NC	NT
Rio de Janeiro, Brazil [145]:Urca	*Ae. aegypti*	F1				+++ for Rio-U1 and ++++ for Rio-S1	NT
Rio de Janeiro, Brazil [145]: Paquetá	*Ae. aegypti*	F2				++++ for Rio-S1	NT
Laboratory strains [146]	*Anopheles gambiae*	Laboratory strain	African: MR766	4.6 and 7 log_{10} pfu/mL	Plaque forming unit assay	NC	NT
	Anopheles stephensi	Laboratory strain	African: MR766	4.3 and 7.7 log_{10} pfu/mL		NC	NT
	Cx. quinquefasciatus	Laboratory strain	African: MR766 and Asian: PRVABC59	7.5 and 7.3 log_{10} pfu/mL		NC	NT
Laboratory strains [147]	*Cx. pipiens*	Iowa 2002	Asian: PRVABC59	6.02 log_{10}, 4.74 log_{10} and 6.83 log_{10} pfu/mL	Plaque forming unit assay	NT	NC
	Ae. triseriatus	Iowa 2002 and 2003				NT	NC
	Ae. albopictus	Missouri 2002				NT	+
	Ae. aegypti (Black-eye Liverpool strain)	Laboratory strain				NT	+
Urca, Rio de Janeiro, Brazil [148]	*Ae. Aegypti*	Collected in 2016	Asian: BRPE243/2015	5 x 10^6 pfu/mL	RT-qPCR	NT	++++
Hainan, China [149]	*Cx. pipiens quinquefasciatus*	Laboratory strain: collected in 2014	Asian: SZ01	8 dpi- 3 x 10^5 pfu/mL	RT-qPCR	++++	NT
Scalea, Italy [150]	*Ae. albopictus*	Late summer 2015	Asian: French Polynesia 2013 [151]	11 and 14 dpi- 6.46 log_{10} pfu/mL	qRT-PCR	+	NT
Reynosa,	*Ae. aegypti*	Laboratory				++	NT

Mexico. [150, 152]		strain: collected 1998					
Rome, Italy [152]	*Cx. pipiens*	Summer 2015	Asian: H/PF/2013	6.46 log_{10} pfu/mL	qRT-PCR	NC	NT
Laboratory strains [153]	*Cx. quinquefasciatus*	San Joaquin Valley, Ca. USA	Asian: NC-2014-5132	14 and 21 dpi. 7.2 log_{10} pfu/mL	Plaque forming unit assay	NC	NT
	Cx. pipiens	Tabarka, Tunisia 2010				NC	NT
Hamburg, Germany [154]	*Cx. pipiens pipiens* biotype *pipiens*	Collected in 2016. F0	FB-GWUH-2016	107 pfu/mL	Cytopathic effect (CPE) and qRT-PCR	NC	NT
	Cx. torrentium	Collected in 2016. F0				NC	
Freiburg, Germany [154]	*Ae. albopictus*	Collected in 2016. F7				+	
Laboratory strain [154]	*Cx. pipiens pipiens* biotype *molestus*	Heidelberg, Germany. 2011				NC	
	Ae. aegypti	Bayer company				++	
Calabria, Italy [154]	*Ae. albopictus*	Collected in 2016. F7				+	
Fort Collins, CO. USA [155]	*Ae. vexans*	Field caught in 2016.	Asian: American (PRVABC59)	6 and 7 log_{10} pfu/mL	Plaque forming unit assay	NT	+
Kedougou, Senegal [156]	*Ae. aegypti*	Collected in 2012. F1	ArD 128000, ArD 132912, ArD 157995, HD 7878 and MR766	15 dpi. 2.7 x 10^6 – 4 x 10^7 pfu/mL	RT-PCR	NC	NT
Dakar, Senegal [156]	*Ae. aegypti*					NC	
Kedougou, Senegal [156]	*Ae. unilineatus*					NC	
	Ae. vittatus					+ for HD 7878	
	Ae. luteocephalus					++ for MR 766	
Singapore [157]	*Ae. albopictus*	F3	African: Uganda MR766	7.5 log_{10} tissue culture	qRT-PCR	NT	++++

				infectious dose$_{50}$ (TCID$_{50}$)			
French Polynesia: Toahotu,Tahiti Island [158]	*Ae. aegypti*	F16- F18 Collected in 2014	PF13/251013-8	7.5 log$_{10}$ TCID$_{50}$	Indirect immunofluorescent assay	++	NT
French Polynesia: Atimaono,Tahiti Island [158]	*Ae. polynesiensis*			7.5 log$_{10}$ TCID$_{50}$		NC	NT
Brisbane, Queensland. Australia [159]	*Ae. vigilax*	F0	MR 766	6.7 log$_{10}$ TCID$_{50}$	Real time Taqman RT-PCR	NC	NT
	Ae.procax	F0				NC	NT
	Cx. annulirostris	F0				NC	NT
	Cx. sittiens	F0				NC	NT
Townsville, Queensland. Australia [159]	*Ae. aegypti*	F4				++	NT
Brisbane, Australia [159]	*Ae. notoscriptus*					NC	NT
	Cx. quinquefasciatus					NC	NT
Laboratory strains [160]	*Ae. aegypti*	Rockefeller	Asian: ZIKV^{BR}	2.2 x 10^{6} pfu/mL	qRT-PCR	++	NT
		Higgs white eyes				+	NT
		Rexville				+	NT
Cayenne, French Guiana [161]	*Ae. aegypti*	F1	Asian: NC-2014-5132	7 log$_{10}$ TCID$_{50}$		ND	NT
Baie-Mahault, Guadeloupe [161]		F2				ND	NT
Pointe Chaudiere, Martinique		F1				ND	NT

[161]							
Orlando, Florida, USA [161]		F10				ND	NT
Tubiacanga, Rio de Janeiro, Brazil. [161]		F1			Plaque forming unit assay	+	NT
Jurujuba, Rio de Janeiro, Brazil [161]	*Ae. albopictus*	F1				ND	NT
Vero Beach, Florida, USA [161]		F7			Plaque forming unit assay	+	NT

Competent: ZIKV found in saliva (out of the total mosquitoes analyzed) (++++) Extremely competent: >75% ZIKV, (+++) Highly competent: >50% transmission, (++) Moderately competent: 26-50% transmission, (+) Low competent: <25% transmission, (NC) No competent: ZIKV no found in saliva, (NT) No tested, (ND) No determined

Barriers to transmission

The main barriers to arbovirus transmission are the midgut infection barrier (MIB), the midgut escape barrier (MEB), the salivary gland infection barrier (SGIB) and the salivary gland escape barrier (SGEB). The barriers in the salivary glands are collectively considered a transmission barrier (Figure 1.1).

Midgut

The insect digestive tract consists of a tube of epithelial cells with three recognized developmental regions. The foregut (of ectodermal origin) is followed by the midgut (of mesodermal origin) and then the hindgut (of ectodermal origin) arranged from the mouth to the anus [162]. The midgut is the only part of the alimentary tract not protected by a chitinous cuticle and is where food digestion and absorption take place. However, in most insects, a peritrophic matrix (PM) occurs. It is a semipermeable extracellular layer consisting of proteins, glycoproteins, and chitin microfibrils in a matrix. It lines the digestive tract separating the ingested food from the absorptive/secretory intestinal epithelium, protecting the midgut epithelium from mechanical damage and insult from abrasive particles, pathogens and toxins [163].

There are two types of PM. Type I, is synthesized by the majority of the posterior midgut epithelial cells and forms a sac-like structure that contains the ingested meal. This is the most common type of PM in adult blood-sucking insects. Type II, is produced from the cardia, a specialized organ found between the foregut and the midgut. The cardia forms an open-ended sleeve like structure that lines the midgut and hindgut and is excreted with the feces through the anus [162].

In mosquitoes, the larvae produces a type II PM, while the adults produce a type I PM. After the ingestion of a blood meal, the type I PM formation starts within minutes but may take several hours to form, during which time the virus has direct access to the midgut epithelium [163].

The midgut is the first tissue of contact with the pathogen, being the first barrier that the pathogen must overcome to be transmitted. *Aedes aegypti* has a midgut composed of a single cell layer

surrounded by a basement membrane, muscle fibers, nerve fibers and tracheoles. Epithelial cells are initially squamoid before a blood meal. However,following a blood meal cells expand to become columnar and heavily microvillated. The main function of the midgut following a bloodmeal is to secrete digestive enzymes and absorb nutrients from the blood meal [164]. The establishment of an infection in the midgut is one of the most important intrinsic factors that define vector competence of the mosquito host [132]. It has been reported that the concentration of the virus in the bloodmeal may influence the outcome of infection in the midgut [140].

Salivary glands

The role of the salivary glands in arbovirus transmission is fundamental since the infection of the salivary glands is required for the completion of the transmission cycle. Physiologically, the salivary glands produce anesthetic, anticoagulant, anti-inflammatory and vasodilatory molecules that have a role during probing and blood meal acquisition [165]. Mosquitoes have tubular salivary glands. Salivary glands are paired organs located in the thorax of the mosquito and are composed of a single-cell epithelial layer. Each salivary gland consists of three cylindrical lobes (two lateral and one median). Each lobe consists of a basal lamina bounding a single layer of epithelial or acinar cells, which are distributed around a central salivary duct containing an apical cavity where saliva is stored [166]. Saliva is then secreted into the duct and delivered to the mouthparts. The anterior lobe secretes enzymes for sugar feeding while the posterior lobe secretes enzymes needed for blood feeding [167]. Slight structural differences have been observed among *Culex*, *Aedes* and *Anopheles* mosquitoes [168].

Salivary glands may get infected with high titers in the hemolymph and some sort of secondary viral replication may take place. For this, the virus has to pass through the basal membrane that surrounds the salivary gland to gain access to the plasma membrane of acinar cells. Generally, only a few cells from the lateral lobes of the salivary gland become infected [136].

The infection by JEV was characterized in *Cx. tritaeniorhynchus* and *Cx. pipiens*. For *Cx. tritaeniorhynchus* infected by membrane feeding, the formation of vacuoles filled with salivary secretions and virus particles were frequently found. The vacuolation process resulted in the deterioration of an adjacent part of the apical plasma membrane, resulting in shedding of virus particles and secretory products into the apical cavity. Mature virions were found individually or in arrays. A common sign of replication in salivary glands was the presence of spherical vesicles in the cytoplasm. JEV particles were seen in intracellular vacuoles and in the apical cavity of lateral acinar cells [169].

For CHIKV in *Ae. albopictus* salivary glands, evidence provided by transmission electron microscopy (TEM) showed the replication and storage of CHIKV in acinar cells, virions were observed in the apical cavities of cells where they were mixed with saliva [166]. In salivary glands of *Cx. pipiens quinquefasciatus* infected with WNV, cytopathologic changes including proliferation of membranes, endoplasmic reticulum vacuolization, phagolysosomal-like vacuoles and cell death were observed [170, 171].

For DENV-2, salivary glands infection occur as early as 4 dpi, viral antigen is found in the distal region of the lateral lobes, with subsequent infection in the medial and proximal lobes. Hypothesizing DENV receptors might be present at the distal regions of the lateral lobes [172]. DENV replication in salivary glands was demonstrated by strand-specific quantitative RT-PCR. DENV (+) RNA was accumulated and increased over time from 7 to 21 days post exposure [173].

The salivary glands are key organs for arbovirus transmission, the involvement of the salivary gland barriers to ZIKV infection will be described in chapter 4 of this dissertation: Variation in competence for ZIKV transmission by *Aedes aegypti* and *Aedes albopictus* is dependent on salivary gland infection and escape barriers.

VC as a way to understand mosquito role on arbovirus transmission

The study of viral transmission by mosquitoes has been difficult because there are no animal models for DENV transmission [174, 175].An alternative way to understand the potential for transmission led us to the use of VC studies. VC studies expose mosquitoes to the infectious agent (arbovirus) via an artificial infectious blood meal and transmission is measured. In this setting the virus grown in cell culture is mixed with a blood source which is then offered to naïve mosquitoes. Many authors do not believe that the artificial blood meal/injection systems mimic natural transmission [176]. Nevertheless these VC studies have helped us to understand the role of mosquito on arbovirus transmission.

The majority of VC studies have used intermediate to high infectious doses. It has been shown that below 10^3 viral RNA copies/mL limited transmission to mosquitoes occurs. Above 10^9 viral RNA copies/mL, almost 100% transmission is obtained [177]. The MIR has been correlated with virus dose, as titer increases a higher proportion of mosquito midguts get infected for DENV [140] and ZIKV [144]. One study used *Ae. aegypti* and low infectious doses of six DENV-2 isolates from Thailand; concluding the mosquito potential to get infected was variable also at low infectious doses [121, 177].

VC influence on arbovirus evolution

Even though, vector competence has a relatively small effect on vectorial capacity (V), VC may influence virus evolution. Some genotypes may be more virulent and become more easily transmitted to humans since one genotype might out-compete the other due a higher replication [178].

As an example, WNV was introduced to New York, USA in 1999. The rapid spread of WNV has been associated with adaptive mutations. First, the genotype WN02 had a shortened EIP relative to the introduced NY99. The WNV02 had a single amino acid change V159A in the envelope € protein [179, 180]. This probably increased viral fitness lead to the replacement of WN99 by WN02 [179, 180]. Subsequently, a mutation at the position 243 of the WNV helicase (NS3) caused high pathogenesis and

virulence in American crows [181]. A higher WNV diversification was observed when WNV passed through *Cx. quinquefasciatus* tissues, producing virus populations with higher fitness [182].

Another example of replacement was observed for DENV 2 Asian-American genotype NI-1 clade by the NI-2B in Nicaragua. NI-2B had a higher replicative index in the *Ae. aegypti* cell line (Aag2) and in Nicaraguan *Ae. aegypti* mosquitoes from 3 to 7 dpi, such advantage was lost at later times post infection, suggesting that a shortened EIP influenced clade replacement [183].

Viral adaptation to a mosquito vector is exemplified by the more efficient transmission of the East/Central/South African CHIKV by *Ae. albopictus*. Increased replication in this mosquito was due an amino acid change from alanine to valine at position 226 of the E1 glycoprotein of the virus [184]. Later, a second mutation in the E2 glycoprotein, L210Q caused increased dissemination in *Ae. albopictus* [185] but neither had an effect on *Ae. aegypti* transmission [186]. Complete CHIKV genome sequences isolated from the first imported and first autochthonous cases showed that those isolates belonged to the Asian genotype and that they did not contain the mutation E1 A226V that has been reported as a vector adaptation molecular marker for *Ae. albopictus* transmission [187].

Due the explosive expansion of ZIKV in the Americas, some studies have tried to elucidate if such behavior was associated with a higher replication in new world mosquito populations. However, the ZIKV lineage that circulates in the Americas (Asian) did not have a higher fitness in mosquitoes from this region when compared to the African lineages [143, 144]. New world *Ae. aegypti* populations do not have increased transmissionof the circulating ZIKV (Asian lineage), but an increased potential for introduction of the ZIKV African lineage into the Americas.

The apparently low rate of amino acid substitutions in arboviruses are due the adaptive constraints from the virus passing from vertebrate to invertebrate host during the arbovirus transmission cycle. In addition, arbovirus transmission requires that the virus pass through the vector, from the initial infection in the midgut, the virus has to disseminate and infect salivary glands, during

that process the virus undergoes several bottlenecks [135], where effective population size and genetic diversity are reduced [173]. For DENV, salivary glands contain a high diversity of genotypes [188]. Salivary glands from individual mosquitoes contain unique viral populations [189].For WNV, unique virus populations were found in *Cx. quinquefasciatus* [182].

Environmental determinants of vector competence.

Biotic and abiotic factors affect vector competence. Weather and climate shape mosquito geographical distribution, population abundance, lifespan and transmission potential. The effect of the temperature on the virus incubation rate has been examined. Mosquito inherent factors such as the rates of fecundity, development, survival and biting have also been examined. The effect of warmer temperatures may potentially expand the geographical range of *Ae. aegypti* and extend the length of transmission seasons.

Using mathematical modeling it has been estimated that ZIKV transmission may occur from 18-34 °C with a peak at 26-29 °C [190]. A long EIP may limit the time window for an infectious vector to transmit ZIKV to susceptible people [191]. Transmission permissive conditions are maintained year-round in some areas from tropical and subtropical regions.

Temperature

Temperature may affect critical factors of VC, such as mosquito developmental time, survivorship, blood feeding, fecundity and the EIP.

At high temperatures, without being detrimental for mosquito survivorship [103], the arboviruses replicate faster while at low temperatures the arbovirus takes longer to reach high enough titers to be transmitted to a new susceptible vertebrate host [103, 105]. Hence, environmental temperature influences the EIP in the mosquito.

The recognized EIP for DENV is from 8 to 12 days post-exposure. However, the EIP is highly dependent on temperature; at higher temperatures DENV replicates faster which shortness the EIP. From a literature review, natural EIP data (not from artificial infection) showed that 95% of EIPs are between 5 and 33 days at 25°C (mean 15 days), and 2 and 15 days at 30°C (mean 6.5 days)[12]. In *Ae. aegypti* the recognized EIP for DENV is 7-14 days at 25-30°C [192, 193] whilst for YFV is estimated from 12-16 days at 25 °C [103] and for ZIKV is 7-10 dpi at 26±1°C [144].

A shortened EIP for WNV (NY99 strain) in *Cx. quinquefasciatus* mosquitoes was consistent with an increase in environmental temperature from 10 -30°C [194]. While constant temperature may influence the duration of the EIP [103]; cycling temperatures did not affect the duration of the EIP for DENV [195], YFV [196] and eastern equine encephalitis viruses (EEEV) [197].

For instance, the impact of the diurnal temperature range (DTR) for DENV transmission by *Ae. aegypti* was assessed, using a mean of 26°C and different amplitudes of daily temperature variation; moderate (10°C), large (20°C) and constant/control (0°C). A study showed that at the largest deviation (20°C) 78.9% of the midguts were DENV-2 infected. That percentage increased at the moderate deviation (10°C) and control conditions with 94.9 % and 97.7% of the midguts infected with DENV-2, respectively. Those observations were reproducible for DENV-1. A lower percentage (88.4%) of mosquito midguts were infected at the largest deviation (20°C) compared to the control (constant at 26°C), (97%). This study concluded that the DTR did not have an impact on the duration of the EIP but had an effect on VC and mosquito survival, which influence vectorial capacity [195]. The role of the temperature in aspects of mosquito's physiology has been investigated. The reactions of *Cx. pipiens fatigans* or *Culex pipiens quinquefasciatus* [198, 199] to temperature were found to be more sensitive to high temperatures compared to low ones. Host-seeking females avoided high temperatures (25-30°C).

Humidity

Humidity may also affect the VC. The reactions to humidity were tested at constant temperature (25 °C), and showed that *Cx. pipiens quinquefasciatus* females avoided being under 40% relative humidity (RH) or above 95% RH. Low humidity is detrimental to survival causing desiccation and death [200].

Microbiome

Mosquito larvae feed on microorganisms present in their aquatic habitat, influencing directly the size of the mosquitoes, which might influence the adult mosquito VC, development and egg production. However, the microbiome may also be influenced by nectar feeding during the adult stage. The microbiome has roles in digestion, nutrition, reproduction and protection against pathogens [201].

Bacterial 16s ribosomal RNA screening and culturing of bacteria from the midgut of field mosquitoes are commonly used methods to characterize the microbiome of mosquitoes. Studies of the mosquito microbiome have mostly focused on the midgut where they may interact with pathogens that are acquired through a blood meal. Six bacterial genera have been isolated from mosquitoes, *Asaia spp*., *Aeromonas spp*., *Enterobacter spp*., *Paenibacillus spp*., *Proteus spp*., and *Comamonas spp*. [202-206]. Certain field derived bacterial isolates in the mosquito midgut correlated with resistance to DENV infection. For instance, the reintroduction of *Proteus sp*. and *Paenibacillus sp*. into aseptic mosquitoes led to lower levels of DENV infection [202]. Because DENV infection influences the mosquito immune system the midgut microbiota may also been affected. DENV infection decreased bacterial transcript levels [202]. *Chromobacterium* reduced DENV-2 replication [207].In contrast, an isolate of *Serratia odorifera* increased DENV [208]and CHIKV [209]replication. Bacteria influence arbovirus infection in mosquitoes through a variety of modes of action which include: immunity, production of anti-viral metabolites, resource competition and regulation of miRNAs [210].

A high bacterial abundance has been associated with resistance to DENV [211]. Also, bacteria diversity and abundance found in *Ae. aegypti's* midguts were higher than in midguts of *Ae. vittatus* and *Ae. albopictus*. At the same time, differences in diversity and abundance were found in DENV endemic areas compared to non-endemic areas for all the species [212].

Interestingly, analysis of other tissues and mosquito species revealed that the salivary glands of *Anopheles culicifacies* have a more diverse microbiome than the gut [213]. Yeast, fungus and insect-specific viruses also contribute to the mosquito microbiome and potentially influence vector competence [210, 214]. Eukaryotic microorganisms, *Candida*, *Yarrowia*, *Rhodotorula* and *Cryptococcus* and *Pichia* genera have been identified from *Aedes*, *Culex* and *Anopheles* mosquitoes [204, 215].

A case where microbiome studies can help us to control vector borne diseases is exemplified by *Wolbachia*, a maternally inherited intracellular bacterium that has been found in many insect species including mosquitoes. *Wolbachia* does not exist outside of mosquito cells. *Aedes aegypti* does not have native *Wolbachia* symbionts. However, the strain wMelPop has been introduced into *Ae. aegypti*, causing a shortening in the mosquito lifespan [216] and a reduction in vector competence. Recently, a reduced competence for ZIKV in wMel infected *Ae. aegypti* mosquitoes from Colombia [123] and Brazil [148] was observed. This has been seen for other viruses like DENV [217-221], CHIKV [217, 222, 223] and YFV [223]. It has been proposed as of potential use for the control of mosquito-borne diseases.

Another probable example of microbiome influence was observed during larvae competition between *Ae. aegypti* and *Ae. albopictus* larvae. This interaction enhances DENV-2 SEA genotype infection and dissemination in *Ae. albopictus*. The author hypothesize that competition causes a reduction in the barriers to infection and dissemination making *Ae. albopictus* more suitable for arbovirus transmission [224]. However, other factors like the microorganisms present at the breeding sites may also influence VC in natural settings.

Mosquito innate immune response

The insect immune system controls but does not clear arbovirus infections so that insects can be a vector for life. The Toll pathway, the immune deficiency (IMD) and the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathways are activated by DENV [71, 214, 225], resulting in melanization, encapsulation, apoptosis and production of anti-microbial peptides [226]. For instance, the cecropin-D and defensin-C peptides have anti-dengue activity [227, 228]. However, the RNA interference (RNAi) pathway is the major antiviral response against arboviral infections [225, 229].

RNAi pathway

The RNAi response involves three pathways to generate small RNAs (20-30 nt) that regulate gene expression: the small interfering RNA (siRNA), the microRNA (miRNA) and the Piwi-interacting RNA (piRNA) pathways [230]. The RNAi antiviral defense mechanism was initially observed in plants. Later, injection of dsRNA into *Caenorhabditis elegans* triggered a specific and robust silencing of the endogenous gene homologous to the injected RNA [231], which was also observed in *Drosophila melanogaster* embryos [232]. Nowadays, the exogenous (exo-) siRNA pathway is recognized as a major antiviral innate immune response in arthropods [229].

In general, siRNAs are induced through the presence of double-stranded RNA from replication intermediates or secondary structure, which are recognized and cleaved by the RNAse, dicer-2 or dicer-1, into 21-25 nt dsRNA fragments. The small dsRNA fragments generated are then loaded into the RNA-induced silencing complex (RISC) by the R2D2 protein. The RISC associated argonaute proteins (ago-) 1 and 2 (mainly 2), cleaves one of the strands, the “passenger strand” keeping the other as the “guide strand”, so that upon perfect complementary of the target ssRNA in the cytoplasm leads to its cleavage and degradation by the exosome and the XRN1 ribonuclease [233].

The exo-siRNA pathway is triggered by the detection of long dsRNA in the cytoplasm of an infected cell, which comes from replication intermediates of + RNA/ -RNA viral genomes, RNA secondary

structures can also act as a PAMP. The cellular nuclease Dicer-2 acts as a pathogen-recognition receptors (PRR), initiating the antiviral response in the infected cells.

The role of miRNA pathway in arthropods will be discussed in the chapter 3 of this dissertation: microRNA profiling by DENV-2 infection status of low vector competent *Ae. aegypti* isofemale lines.

The importance of the exo-si RNA pathway in the antiviral innate immune response in arthropods was demonstrated by knocking down dicer and argonaute genes, which lead to higher titers of O'nyong nyong virus (ONNV, *Alphavirus*, *Togaviridae*) [234] and DENV-2 [235]. In addition, the Dicer-2 defective mosquito cell line C6/36 allows for higher titers of DENV-2 relative to those in the RNAi competent mosquito line Aag2 [236]. Also, natural polymorphisms in Dicer-2 have been associated with susceptibility of *Ae. aegypti* to DENV-1 [237].

This antiviral defense brings to mind the concept of molecular "arms race". Arboviruses may be drive genome evolution in the invertebrate host while at the same time the vector genes are exerting selection on the arboviral genome [229]. Supporting evidence is that the genes involved in the RNAi pathway are among the fastest evolving genes in mosquitoes [238] however this is unlikely due arboviral infection in field mosquito populations [229]. On the other hand, given that the RNAi response is highly sequence specific, mutations that occur in the viral genome may provide an additional way to avoid the immune response [239].In addition, viral proteins antagonize this pathway; for instance, the DENV NS4B and the subgenomic flavivirus RNA (sfRNA) inhibit the RNAase Dicer blocking the RNA interference pathway [69, 80, 240].

CHAPTER 2: PATTERNS OF GENE FLOW AND VECTOR COMPETENCE AROUND THE MEXICAN NEOVOLCANIC AXIS

Introduction

Dengue is the most prevalent arthropod-transmitted diseases in the world. Recently, it was estimated that ~ 400 million infections occur per year [10, 14]. Disease is caused by infection with one of the four phylogenetically similar but antigenically different, dengue virus serotypes (DENV-1-4), (genus *flavivirus*, family *Flaviviridae*) [6]. Infection with DENV causes a wide spectrum of disease; from the self-limiting dengue fever (DF), characterized by flu-like symptoms, to severe presentations like dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). However, most of the infections are clinically inapparent [14].

DENV is transmitted to humans through the bite of an infectious mosquito, mainly *Aedes aegypti* (L.) [10].Due the presence of the vector, dengue is a major public health concern in the Americas where incidence has increased recently with frequent epidemics and co-circulation of multiple DENV serotypes and genotypes [40].In addition, the Americas report the majority of World Health Organization(WHO) dengue cases worldwide [29]. In 2016 alone, the Pan American Health Organization (PAHO) reported more than 2 million dengue infections, including severe forms of dengue, of which 17,795 confirmed DENV infections occurred in Mexico [35].

Many studies have focused on the ability of the mosquito vector to acquire, maintain and then transmit a pathogen. All stages of this process are collectively referred to as vector competence (VC) [134]. An arbovirus has to bypass several physiological barriers to be transmitted to a susceptible host. Upon the intake of an infectious blood meal from a viremic host, the virus is drawn into the mosquito gut, where it has to establish an infection; if this does not occur the mosquito has a midgut infection

barrier (MIB). Next, the virus has to disseminate to other tissues; if this does not happen the mosquito has a midgut escape barrier (MEB). Upon dissemination, the virus may infect other mosquito tissues, especially the salivary glands. If this fails it is said to have a salivary gland infection barrier (SGIB). In the salivary glands the virus has to replicate and disseminate into the saliva from where it will potentially be transmitted to a susceptible host in a subsequent blood meal. If this is blocked the mosquito has a salivary gland escape barrier (SGEB) [135, 136]. All of these barriers (MIB, MEB, SGIB and SGEB) contribute to the overall VC phenotype. Published reports have shown significant variation in VC and the four barriers to transmission. This has been especially well-documented for DENV-2 in *Ae. aegypti* [138-140, 241-246]. VC for DENV-2 is a variable quantitative genetic trait for which up to 60% of the variation has been associated with random or uncontrolled environmental effects [135, 247].

Early population genetic studies determined the genetic structure of 38 Mexican *Ae. aegypti* populations collected in the late 1990's by the mitochondrial gene ND4, separating them into three genetically different regions; Northeastern, Yucatan and Pacific. The Northeastern collections had moderate gene flow relative to the Yucatan and Pacific collections which had extensive gene flow albeit the analysis also determined Yucatan collections were isolated by distance [142]. Subsequent work documented considerable variation in VC among 24 Mexican *Ae. aegypti* collections for DENV-2 (JAM 1409 strain). Collections from the Northeastern region displayed the lowest VC and Yucatan the highest [140]. Nevertheless, the state of Veracruz was not thoroughly sampled, so a subsequent study focused on *Ae. aegypti* genetic analysis and VC assessment from this state. In that study the Neovolcanic Axis (NVA) appeared to act as a barrier to gene flow [141]. However, the mechanisms underlying this were not determined. In addition VC was greater in northern collections and appeared to diminish in collections from the south. The goal of the present study was to determine if the same patterns remained 8 years later in 2012.

Methods

Mosquito collections

As in the 2003-2004 study, mosquito collections were performed in late August of 2012 in the coastal state of Veracruz, Mexico (Figure 2.1). Collections were made primarily in municipal cemeteries after obtaining verbal permission from the local cemetery administration. Larvae and pupae were collected from at least four containers located at least 100 meters apart from each other. Immatures were transported to a temporary local laboratory in Orizaba, Veracruz at Universidad Veracruzana, reared to adults and identified as *Ae. aegypti* (L.) based on scale patterns on the thorax after adult eclosion [248]. *Aedes aegypti* were given a blood meal to generate F1 eggs which were brought back to Colorado State University to be colonized.

Figure 2.1. Map showing the collection sites for the *Ae. aegypti* populations used in this study. Black dots represent the collection sites.

Mosquito rearing

Upon arrival at CSU eggs were hatched in tap water. Larvae were fed *ad libitum* with finely ground fish food (TetraMin Tropical Flakes-Spectrum Brands, Inc). In order to prevent overcrowding, approximately 50 larvae per liter were reared per container. Adult mosquitoes were maintained on sucrose *ad libitum.* For egg production citrated sheep's blood (Colorado Serum Co., Boulder, CO) was provided once a week through water-jacketed glass feeders using hog gut as a membrane. Adults were kept in incubators maintained at 28°C, 70% relative humidity (RH) and a 12:12 light:dark diurnal cycle. The eggs were collected in filter papers and stored at 70% (RH) for up to four months.

Cells

Mosquito C6/36 and mammalian LLC-MK2 cells were cultured in modified Eagle's medium (MEM) supplemented with 8% heat-inactivated fetal bovine serum, L-glutamine, non-essential amino acids and penicillin/streptomycin and maintained at 28°C and 37°C respectively with 5% CO2. For DENV-2 infections, MEM was supplemented with 3% heat-inactivated fetal bovine serum (FBS), L-glutamine, non-essential amino acids and penicillin/streptomycin.

Mosquito infections

Aedes aegypti mosquitoes from the F_4 generation were used for VC studies. DENV-2 JAM1409 was used to infect monolayers of C6/36 cells at a MOI of 0.01. At 7 days post infection (dpi), media was replaced and at 11 dpi, supernatant was harvested and centrifuged at 3,000xg for 10 min. The supernatant was then mixed with defibrinated sheep blood (Colorado Serum Co., Boulder, CO) in a 1:1 proportion and 1 mM ATP for the mosquito infectious blood meals. Blood feeding was conducted under BSL-3 containment. The titer of the infectious blood meal was determined subsequently by titration on LLC-MK2 cells, estimated to be 10^6pfu/ml for DENV-2 [247]. Prior to feeding, 5-7 day old mosquitoes were deprived of sucrose and water for 8 hours. The infectious blood meal was provided through water-

jacketed glass feeders with a hog gut membrane. After up to one-hour of feeding, mosquitoes were cold-anesthetized at 4°C and engorged females were placed into new containers where a water and sugar source were provided. Mosquitoes were maintained for 14 days at insectary conditions (28°C, 70% relative humidity and 12:12 light:dark diurnal cycle).

Vector competence assessment

At 14 dpi, mosquitoes were cold anesthetized at 4°C and tissues (midgut, carcass, and the first segment of the thorax as a proxy for salivary glands and saliva) were dissected and placed into individual tubes. The mosquito proboscis was placed into a capillary tube that contained immersion oil (~5 µL) and allowed to expectorate saliva for 30 minutes. Following salivation, the tip of the capillary tube was broken into a tube containing 100 µL of MEM supplemented with 3% FBS. Forceps were dipped in 70% ethanol and cleaned after each tissue was dissected and between individual mosquitoes. Mosquito tissues were stored at -80°C until further processing.

Mosquito sample processing

Frozen mosquito tissues were thawed, grinded (except saliva samples) individually in 1 mL of MEM supplemented with 3%FBS and then centrifuged down at 14,000 rpm for 5 minutes at 4°C and kept on ice. The supernatant was filtered through a 0.22-m syringe filter to a new tube, from which 10-fold dilutions were made.

Plaque assays

Plaque assays were performed on LLC-MK2 cells. Briefly, confluent cell monolayers on 24-well plates were infected with 10-fold serial dilutions of the samples. After 1-hour incubation with continuous rocking, an agarose-nutrient overlay was added to each well and plates were incubated at 37°C with 5% CO_2. After 7 days, the plates were stained with a 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-

diphenyltetrazolium bromide (MTT) solution and incubated for 4 hours. Titers were determined by counting plaques that were visualized on a light box.

Mosquito genetic analysis

For the genetic analysis, DNA was extracted from individual adult mosquitoes by the salt extraction method [249] and suspended in 150 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

The Nicotinamide Adenine Dinucleotide Dehydrogenase subunit 4 mitochondrial gene (ND4) was amplified from the DNA from ten individual field caught (F_0) mosquitoes from each collection site. In addition, DNA from thirty F4 mosquitoes exposed to DENV-2 from each population was used to amplify Dicer-2, Argonaute-2 and ND4 genes. Using primers developed in an earlier study [238] (Table 2.1), the exon 10 of Dicer-2 (AAEL006794 –RA-E10 – 246,370 – 249,357) and exon 2 of Argonaute-2 (NCBI= ACR56327, VectorBase = AAEL017251-RA-E2 368,511 – 369,674). These regions were chosen based upon the variation present in these regions in our earlier study [238]. Products were purified using the MinElute PCR Purification Kit (Qiagen) and submitted for Sanger sequencing.

Table 2.1. List of primers for Dicer-2, Argonaute-2 and ND4 amplification.

Gene	Gene ID	Region	Orientation	Sequence 5' -3'	Product size
Dicer-2	AAEL006794	Exon 10	Sense	CGATCCCAAAAACGACTGGCA	933
			Antisense	AAAACCTGAGACCTTTTGCTGAAACG	
Argonaute-2	AAEL017251	Exon 2	Sense	CCATGAGCTGTGGTACGGTCTGTTC	911
			Antisense	AGCGGCACTTCCTAGACCTGT	
ND4	EU352212.1	Mit	Sense	GTTCATTTATGACTACCAAA	389
	ABY51631.1		Antisense	CTTATCCTTCTGCTTC	

Products from at least two mosquitoes representing each haplotype were sequenced. These 20 sequences were compared to sequences reported previously and assigned the same GenBank accession numbers [250, 251]. Phylogenetic relationships among haplotypes have been previously described [250, 251].

Statistical analysis of haplotype frequencies

Variation in haplotype frequencies between northern and southern collections and among collections was examined using Molecular Analysis of Variance (AMOVA) [252] in Arlequin3. The significance of the variance components associated with each level of genetic structure was evaluated by a nonparametric permutation test with 100,000 pseudo-replicates [253]

Data and statistical analysis for vector competence

We determined the proportion of midgut, disseminated and salivary gland infections and of transmission for each of the mosquito populations tested by plaque assay [143, 254]. The proportion of midgut infection (MI) was defined as the number of mosquitoes with infectious DENV in the midgut divided by the total number of mosquitoes that had blood fed. The proportion of disseminated infection (DI) was defined as the number of mosquitoes with infectious DENV in the carcass divided by the number of blood fed mosquitoes. The proportion of salivary gland infection (SGI) was defined as the number of mosquitoes with infectious DENV in the first segment of the thorax (proxy for salivary glands) divided by the total number of blood fed mosquitoes. The proportion of transmission (TR) was defined as the number of mosquitoes that expectorated saliva containing infectious DENV divided by the total number of blood fed mosquitoes. Since all the barriers contribute to the VC, TRs was used as a synonym for VC.

Additionally, the DENV-2 titers of each of the mosquito tissues were obtained by end-point dilution and plaque assay for each of the individual mosquitoes assessed for vector competence.

We calculated the additive contribution of each of the four barriers to transmission (equations 1-4) where all the equations sum to 100% by adjusting the MI, DI, SGI and TR proportions. Adjusted midgut infection (AMI) was the number of mosquitoes with infectious DENV in the midgut divided by the total number of mosquitoes that had blood fed. The adjusted dissemination (ADI) was the number of mosquitoes with infectious DENV in the carcass divided by the number of mosquitoes with infectious

DENV in the midgut. The salivary gland infection (ASGI) was defined as the number of mosquitoes with infectious DENV on the proximal thorax (proxy for salivary glands) divided by the total number of mosquitoes with infectious DENV in the carcass (without thorax). The adjusted transmission (ATR) was defined as the number of mosquitoes that expectorated saliva containing infectious DENV divided by the adjusted number of mosquitoes with infectious DENV in the first segment of the thorax.

$$\% MIB = \frac{\log (AMI)}{\log (Total)} \times 100 \qquad (1)$$

$$\% MEB = \frac{\log (ADI)}{\log (Total)} \times 100 \qquad (2)$$

$$\% SGIB = \frac{\log (ASGI)}{\log (Total)} \times 100 \qquad (3)$$

$$\% SGEB = \frac{\log (ATR)}{\log (Total)} \times 100 \qquad (4)$$

$$\log (Total) = \log (AMI) + \log (ADI) + \log (ASGI) + \log (ATR) \quad (5)$$

The midgut infection, disseminated infection, salivary gland infection and transmission proportions were estimated in WinBUGS [255] using a binomial distribution as a model. Where the number of infected mosquito tissues (e.g. midgut) was a binomial sample. Bayesian 95% Highest Density Intervals (HDI) were also obtained. The Bayesian analysis provides logical estimates for proportions where an uninformative prior distribution is assumed. Therefore, the prior distribution for the probability of a mosquito tissue to be infected was 0.0-1.0 [256].

Error bars represent 95% HDI credible intervals and non-overlapping error bars indicate statistical significance. Infection rates were compared by Fisher's exact tests, significance was determined at p=0.05. GraphPad PRISM version 7.03 (GraphPad Software, San Diego, CA, USA) was used for graph construction. The map was constructed with the QGIS (2.8.1) Wien software free access using the public access shapes (USA_adm0, NIC_adm0, HND_adm0, GTM_adm0, MEX_adm1).

Results

Table 2.2 shows the result of the AMOVA tests performed. Table 2.2A shows the proportion of the total variance in ND4 haplotypes frequencies in the field collection and Table 2.2B shows the proportion of the total variance in ND4 haplotypes frequencies in the F_4 generation. In the present study 22% of the variance arose between northern and southern collections and this was significant. This is very similar to the 24.5% reported in our 2003-2004 study which was also significant. After four generations in the laboratory this dropped to 14% and was no longer significant. Table 2.2C shows the proportion of the total variance in Dicer-2 haplotypes frequencies in the F_4 generation and Table 2.2D shows the same for Argonaute-2. In both cases virtually none of the variation arose between northern and southern collections. A negative percentage of variation was observed for the Argonaute-2 in North vs South populations which in other words was zero, since the majority of the variation was observed within collections. This suggested extensive gene flow between the northern and southern collections.

Table 2.2. AMOVAs for the mitochondrial ND4 and the nuclear Dicer-2 and Argonaute-2 genes.

A) Mitochondrial ND4 (Field collection)

Source of variation	D.F.	Variance components	F-statistic (Prob.)	% variation in 2012 (% variation in 2003)
North vs. South	1	0.1028	0.2243 (0.0303)	22.43 (24.50)
In North vs. South collections	5	0.0743	0.2090 (<0.0001)	16.21 (13.00)
Within collections	61	0.2811	0.3864 (<0.0001)	61.36 (62.50)
Total	67	0.4581		100.00

B) Mitochondrial ND4 (F_4)

Source of variation	D.F.	Variance components	F-statistic (Prob.)	% variation in 2012
North vs. South	1	0.062	0.1386 (0.0880)	13.86
In North vs. South collections	6	0.1134	0.1134 (<0.0001)	25.05
Within collections	230	0.2765	0.3892 (<0.0001)	61.09
Total	237	0.4527		100.00

C) Dicer-2 (F4)

Source of variation	D.F.	Variance components	F-statistic (Prob.)	% variation in 2012
North vs. South	1	0.0018	0.0043 (0.2854)	0.43
In North vs. South collections	6	0.0200	0.0476 (<0.0001)	4.74
Within collections	467	0.3990	0.0518 (<0.0001)	94.82
Total	474	0.4207		100.00

D) Argonaute-2 (F4)

Source of variation	D.F.	Variance components	F-statistic (Prob.)	% variation in 2012
North vs. South	1	0.0006	-0.0014 (0.42693)	-0.14
In North vs. South collections	6	0.0204	0.04707 (<0.0001)	4.71
Within collections	476	0.4134	0.04573 (<0.0001)	95.43
Total	483	0.4332		100.00

Northern collections are Tuxpan, Poza Rica and Martinez de la Torre and southern collections are Cardel/Zempoala, Veracruz, Alvarado, and Coatzacoalcos/Minatitlan. D.F. stands for degrees of freedom. Sample size was the total number of mosquitoes analyzed.

Vector competence of *Ae. aegypti* collected through Veracruz.

The number of DENV-2 infected tissues out of the total tested individuals from each collection is included in table 2.3. DENV-2 infection was determined by plaque assay at 14 dpi.

Table 2.3. Susceptibility of *Ae. aegypti* from Veracruz, Mexico to DENV-2 at 14 dpi

	Location	MI	DI	SGI	TR
North of NVA	Tuxpan	4/30	4/30	4/30	2/30
	Poza Rica	9/30	7/30	7/30	0/30
	Martinez de la Torre (G)	17/29	17/29	17/29	6/29
	Martinez de la Torre (H)	17/29	11/29	11/29	3/29
South of NVA	Cardel/Zempoala	17/29	14/29	14/29	7/29
	Veracruz	14/28	9/28	8/28	4/28
	Alvarado	12/30	8/30	5/30	1/30
	Coatzacoalcos/Minatitlan	11/30	8/30	5/30	1/30

Proportion of MI, DI, SGI and TR by location of the *Ae. aegypti* populations from Veracruz at 14 dpi are shown in Figure 2.2. Panel A shows the populations located at North of the NVA whilst panel B shows populations at South of the NVA. In which *Ae. aegypti* from Tuxpan were the most refractory to DENV-2 infection having the lowest while the mosquitoes from Martinez de la Torre and Cardel/Zempoala were the more susceptible with the highest MI proportion. TRs or VC ranged from 0 to 0.24, mosquitoes from Poza Rica showed the lowest competence and Cardel/Zempoala the highest. Interestingly, the *Ae. aegypti* G and H forms ,as designated by McClelland's abdominal tergite scale patterns [257] differed in the proportion of DI and SGI but this difference was not significant (Fisher's Exact Test *p*-value = 0.1885) nor were the difference in TR proportions (*p*-value = 1.0) (Fig. 2.2).

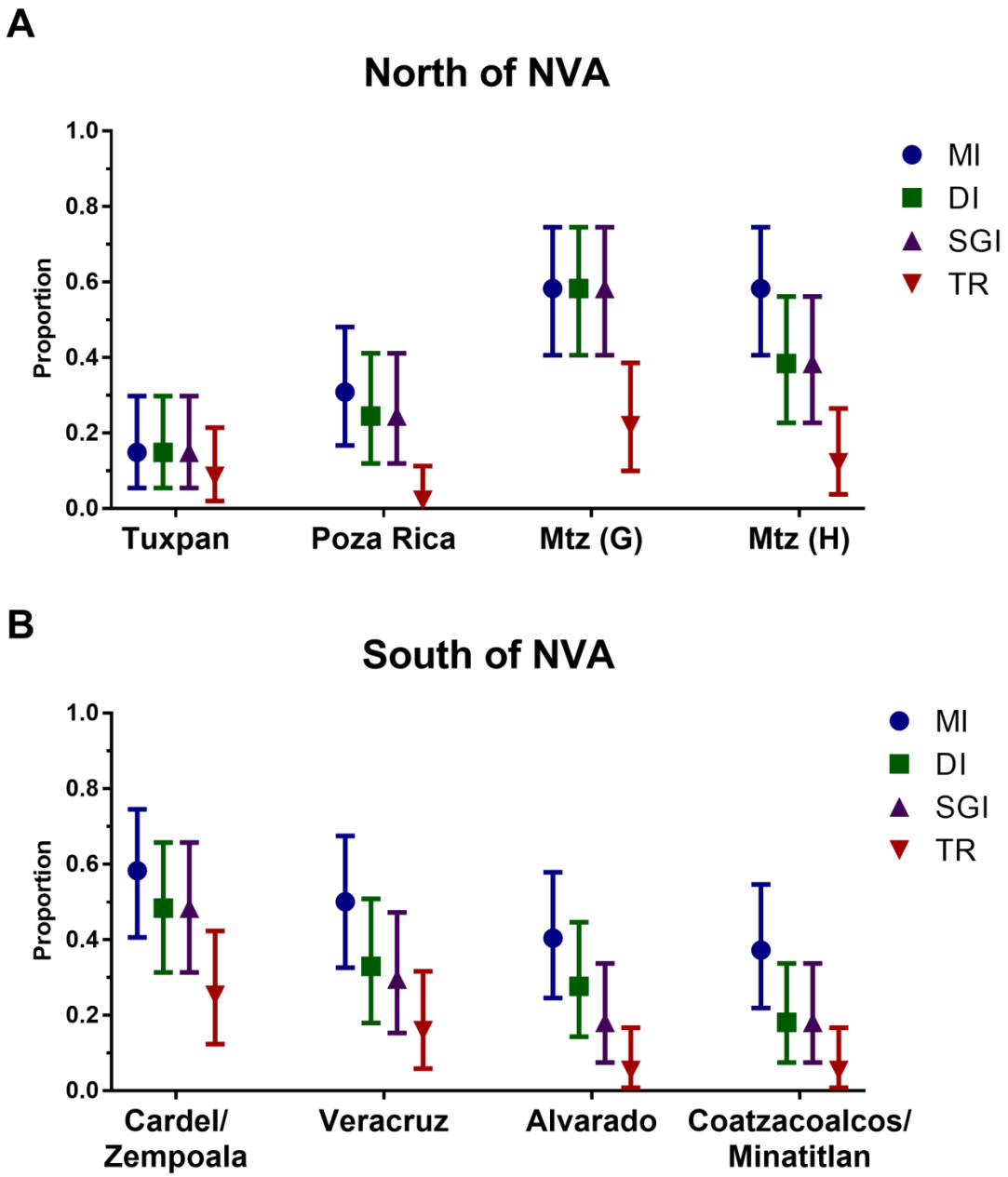

Figure 2.2. Midgut Infection (MI), disseminated infection (DI), salivary gland infection (SGI) and transmission (TR) proportions grouped by their location respect to the NVA. DENV-2 infections were determined by plaque assay. The furthest northern collections are to the left. Error bars represent 95% HDI credible intervals and non-overlapping error bars (e.g Cardel/Zempoala vs. Poza Rica) indicate statistical significance.

Overall, the MI determined the DI and SGI since they were not significantly different within any of the studied populations. A decrease from the initial MI to low TR was a recurrent observation for all the *Ae. aegypti* populations (Figure 2.2). In addition, the MI and DI proportions seem to have changed more in populations from the north (Fig 2.2 A) than from the south (Figure 2.2 B) of the NVA.

Additionally, we measured the DENV-2 titers in each of the mosquito tissues relevant for transmission. We found consistent titers in the midgut and carcass of the mosquitoes followed by an increase in titer in the thorax, a proxy for salivary glands. Even though a higher titer was found in the "salivary glands" a decrease in the DENV-2 titers was observed in all the mosquito populations analyzed (Figure 2.3). All the above was suggestive of the presence of a SGEB.

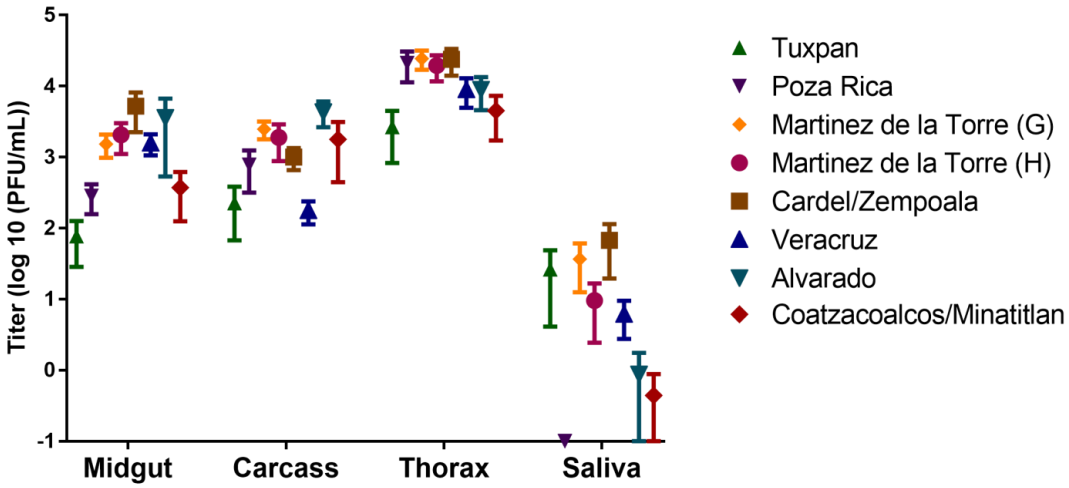

Figure 2.3. Mean titers in the analyzed tissues (midgut, carcass, thorax 'salivary glands' and saliva). Tissues were collected at 14 dpi. Titers were determined by plaque assay. Bars represent the standard error of the mean (SEM).

A comparison of the midgut infection proportions between northern and southern collections in 2003-2004 and 2012 is shown in Table 2.4. In 2003-2004 the MI was 0.21 in the north with a range from 0.13 - 0.35 and was 0.45 in the south with a range from 0.27 to 0.64. These differences were significant (*p*-value = 4.84E-10). In contrast, in 2012 the MI had increased to 0.40 in the north with a wide range

from 0.13 - 0.59 and was 0.46 in the south with a range from 0.37 to 0.59. These differences were not significant (*p*-value = 0.3577). Table 4 also shows that the MI proportion in the north was significantly greater in 2012 (0.40) than in 2003-2004 (0.21) and this difference was significant (*p*-value = 0.0003). However in the south the MI proportion did not change between 2003-2004 and 2012.

Table 2.4. Comparison of the midgut infection proportions between Northern and Southern collections in 2003-2004 and in the present study of mosquitoes collected in 2012.

	2003-2004			**2012**			
North	**DENV +**	**Total**	**MI**	**DENV +**	**Total**	**MI**	**Prob (FET)**
Panuco	21	60	0.35	-	-	-	-
Tantoyuca	17	77	0.22	-	-	-	-
Tuxpan	-	-	-	4	30	0.13	-
Poza Rica	6	47	0.13	9	30	0.30	0.0803
Martinez de la Torre	11	72	0.15	34	58	0.59	3.10E-07
Total North	55	256	0.21	47	118	0.40	0.0003
South							
Cardel/Zempoala	20	75	0.27	17	29	0.59	0.0031
Veracruz	-	-	-	14	28	0.50	-
Alvarado	36	56	0.64	12	30	0.40	0.0409
Coatzacoalcos	25	71	0.35	11	30	0.37	1.0000
Cosoleacaque	30	63	0.48	-	-	-	-
Minatitlan	31	60	0.52	-	-	-	-
Acayucan	38	73	0.52	-	-	-	-
Total South	180	398	0.45	54	117	0.46	0.9160
North versus South Prob. Fisher's Exact Test =		4.84E-10			0.3577		

A comparison of the proportions of disseminated infections between northern and southern collections in 2003-2004 and in the present study of mosquitoes collected in 2012 is presented in table 2.5. In 2003-2004 the DI was 0.60 in the north with a range from 0.38 to 0.74 and was 0.20 in the south with a range from 0.11 to 0.33. These differences were significant (Fisher's Exact Test p-value = 2.20E-16). In contrast, in 2012 the DI proportion had decreased to 0.33 in the north with a range from 0.13 - 0.48 and was 0.27 in the south with a range from 0.17 to 0.48. These differences were not significant (p-

value = 0.3945). Table 2.5 also shows that the DI proportions in the north was significantly greater in 2003-2004 (0.60) than in 2012 (0.33) and this difference was significant (p = 0.0188). However in the south the DI proportions increased from 0.20 in 2003-2004 to 0.27 in 2012 but this difference was not significant. Thus both, the DI and MI proportions seem to have changed more in the north than in the south (Figure 2.2).

Table 2.5. Comparison of the disseminated infection proportions between Northern and Southern collections in 2003-2004 and in the present study of mosquitoes collected in 2012.

	2003-2004			2012			
North	**DENV +**	**Total**	**DI**	**DENV +**	**Total**	**DI**	**Prob(FET)**
Panuco	23	60	0.38	-	-	-	-
Tantoyuca	45	77	0.58	-	-	-	-
Tuxpan	-	-	-	4	30	0.13	-
Poza Rica	35	47	0.74	7	30	0.23	0.0000
Martinez de la Torre	37	72	0.51	28	58	0.48	0.8600
Total North	140	256	0.54	39	118	0.33	0.0188
South							
Cardel/Zempoala	25	75	0.33	14	29	0.48	0.1802
Veracruz	-	-	-	8	28	0.29	-
Alvarado	6	56	0.11	5	30	0.17	0.5045
Coatzacoalcos	22	71	0.31	5	30	0.17	0.2178
Cosoleacaque	8	63	0.13	-	-	-	-
Minatitlan	12	60	0.20	-	-	-	-
Acayucan	8	73	0.11	-	-	-	-
Total South	81	398	0.20	32	117	0.27	0.1269
North versus South Prob. Fisher's Exact Test =		2.20E-16			0.3945		

We also calculated the additive contribution of each of the four barriers (MIB, MEB, SGIB and SGEB) to transmission (equations 1-4) where all the equations sum to 100% after adjusting the MI, DI, SGI and TR (Table 2.6). This analysis could not be compared to the 2003-2004 study because no data was collected on DENV in the salivary glands or in saliva in that study. Table 2.6 indicates that a MIB was the primary reason for the low VC in Tuxpan and Poza Rica in the North. In contrast a SGEB primarily controlled VC in both the northern and southern collections.

Table 2.6. Contribution of the barriers (MIB, MEB, SGIB, SGEB) to VC.

Location	VC	MIB	MEB	SGIB	SGEB
Tuxpan	7%	**74%**	0%	0%	26%
Poza Rica	0%	**83%**	17%	0%	0%
Martinez de la Torre (G)	21%	34%	0%	0%	**66%**
Martinez de la Torre (H)	10%	24%	19%	0%	**57%**
Cardel/Zempoala	24%	38%	14%	0%	**49%**
Veracruz	14%	**36%**	23%	6%	**36%**
Alvarado	3%	27%	12%	14%	**47%**
Coatzacoalcos/Minatitlan	3%	29%	23%	0%	**47%**

Major contributors to VC are shown in bold

Discussion

Gene flow is the movement of genes among populations, hence a major force for evolution. It happens through natural migration, movement of fertilized eggs, extinction and recolonization of entire populations, or transposition of nuclear segments of DNA [258]. Gene flow helps to maintain high diversity in natural populations providing higher opportunities to succeed in natural conditions. However, this gene flow may be disrupted by geographic and/or physical barriers and also by anthropogenic activities. One goal of the present study was to determine if these same genetic patterns surrounding the NVA remained after 8 years. The amount of the variation in the frequency of the mitochondrial ND4 haplotypes between north and south was 24.5% in 2003-2004 and remained large at 22.4% in 2012. But mitochondrial genes reflect historical rather than current gene flow patterns and so we analyzed variation in two nuclear genes (exon 10 of Dicer-2 and exon 2 of Argonaute-2) in the same mosquitoes. The amount of the variation in the frequency of nuclear genes between north and south was negligible and suggests abundant gene flow between north and south collections.

Additionally, we measured DENV-2 titers in different mosquito tissues, which allowed us to make inferences about replication and overcoming barriers to transmission. For instance, the previous study considered that infected salivary glands (inferred from positive heads) were able to transmit

DENV-2 [172]. In contrast, in this study we found a SGEB limiting transmission in the majority of the mosquito populations. It was previously hypothesized that an introduction of the sylvatic *Ae. aegypti* subspecies *formosus* (*Aaf*) from Africa into Veracruz may be a possible source of low competent mosquitoes since it has been reported as having a low competence for DENV-2 transmission [245]. However, recent studies have shown that this pattern is entirely dependent on the genotype of the flavivirus, observation made for DENV-2 and Yellow fever virus (YFV) [138].

Over the eight year period, collections at north and south of the NVA became phenotypically (VC, MIB) and genotypically similar even though mitochondrial markers continue to reflect historical gene flow or a phylogenetic relationship in the past. This study highlights the fact that components of vector competence (and gene flow) in *Ae. aegypti* are dynamic. This probably arises through vector control operations that reduce effective population size and founders' effects that arise as *Ae. aegypti* individuals are moved around through human commerce. Both factors cause excessive genetic drift and this affects genes that may condition VC randomly. For instance, heavier interventions aimed to control disease vector populations at the north of the state in comparison with the south may have caused the observed change in the VC.

Over the last 35 years phylogenetic relationships among global populations of *Ae. aegypti* have been derived using allozyme markers [259-261], microsatellites [251, 253, 262], nuclear SNP loci [245, 250, 263], mitochondrial DNA [253, 264] and most recently a SNP-Chip [263]. One of the justifications for understanding phylogenetic relationships is that this knowledge may enable us to predict important aspects of these populations including morphology, host preference, VC for arboviruses and insecticide resistance. However results such as those presented here suggest that few inferences can be predicted for phenotypic characters based on genetic similarity from neutral markers measured at one or a few points in time.

CHAPTER 3: MICRORNA MODULATION IN DENGUE VIRUS-2 EXPOSED AND INFECTED AEDES AEGYPTI MIDGUTS

Introduction

RNA interference (RNAi) is a mechanism in which gene silencing is mediated by small (20-30 nucleotides (nt)) RNAs. There are three pathways, small interfering RNA (siRNA), micro RNA (miRNA) and Piwi-interacting RNA (piRNA). The siRNA and miRNAs are mediated by 21-23 nt RNAs and the piRNAs by 24 -27 nt RNAs. (reviewed in [265]).

The siRNA pathway is triggered by long dsRNAs, usually replication intermediates in the cytoplasm of an infected cell which are recognized by the RNAse III Dicer-2, initiating the antiviral response that requires full complementarity with the target gene in order to be degraded [229]. Knocking down the important mediator genes Dicer and Argonaut resulted in higher titers of O'nyong-nyong virus (ONNV) [234] and dengue virus (DENV)-2 [235], highlighting the importance of this pathway in the antiviral response.

The piRNAs were previously thought to exclusively protect the germ-line cells of *Drosophila melanogaster* from retrotransposons, so that the genome of the next generation is protected [265, 266]. The piRNAs are dicer-independent generated small RNAs of 24-27 nt. For their generation long ssRNA precursors from piRNA clusters are transcribed in the nucleus. Long ssRNAs are subsequently transported to the cytoplasm where they are cleaved by the endonuclease Zucchini into 24-30 nt primary piRNAs (pri-piRNAs). PriRNAs have a bias for a 5' uridine at position one. The mature piRNA are then loaded into the piwi proteins: PIWI or Aubergine (Aub), and subsequently transported back into the nucleus where they interact with their targets causing transcriptional gene silencing [229, 230, 265].

Additionally, through a 'ping-pong' mechanism, pri-piRNAs can produce and amplify secondary piRNAs, which are recognized by an adenosine in the 10th residue from the 5' end [229, 230, 265].

Evidence of virus genome derived piRNAs has been found in C6/36 cells infected with DENV-2 [236] Sindbis virus (SINV) and La Crosse virus (LACV) [267]. piRNAs have been suggested as an antiviral mechanism synergistic with the siRNA antiviral pathway since both have been found upon chikungunya virus (CHIKV) [268], SINV [267] and Rift Valley Fever virus (RVFV) [269] infections in mosquitoes and mosquito derived cells.

Lastly, the miRNAs are also non-coding RNAs of ~22 nt, with roles in the regulation of gene expression at both transcription and post-transcriptional levels. The miRNAs are primarily generated by a canonical pathway in which the primary miRNA (pri-miRNA) is transcribed from nuclear genes by RNA polymerase II. The pri-miRNA has a 5' cap and a poly (A) tail, which is processed by the nuclear miRNA microprocessor RNase III type endonuclease Drosha and the double-stranded RNA binding protein Pasha, which generate a precursor miRNA (pre-miRNA). The pre-miRNA is excised by Drosha, generating a ~70 nt stem-loop structure that is transported into the cytoplasm by exportin 5-dependent transport. In the cytoplasm, Dicer-1 cleaves the hairpin head of the stem-loop releasing a ~22 nt miRNA 5p:3p duplex (miRNA:miRNA), which is then recruited in Ago-1 or Ago-2 proteins initiating the formation of the miRNA-RNA Induced Silencing Complex (miR-RISC). One of the strands, the passenger strand is discarded while the guide strand is kept and guides the miR-RISC to target sequences. The interaction between the miRNA-RISC and the target sequence is mediated by imperfect complementary between the miRNA and the target sequence, with complete complementary at the seed region, which consists of nucleotides 2-8 from 5' end of the mature miRNA [229, 230, 265, 270].

The miRNAs have been found to modulate important physiological mechanisms in mosquito vectors. For instance, in *Ae. aegypti* the miR-1174, miR-1890 and miR-275 are important for mosquito blood meal digestion [271-273], while miR-275 has also a role in egg development [271] and miR-8

regulates vitellogenesis [274]. In addition to this physiological mechanisms other important phenotypes in mosquitoes may be modulated by the miRNA pathway; for example, miR-71 and miR-278-3p, which have been suggested to play a role in the resistance of *Culex pipiens* to deltamethrin [275].

Differential expression patterns of host miRNAs upon infection have been relatively unexplored. In addition, some of the studies have not been experimentally validated; those include a study with *Ae. aegypti* mosquitoes infected with DENV-2 [276] and another of *Ae. albopictus* infected with CHIKV [277]. However, some miRNAs have been probed to have an effect on arbovirus infection. The miR-281 was shown to facilitate DENV replication by targeting the 5'untranslated region (UTR) in *Ae. albopictus* derived C6/36 cells and whole mosquitoes [278]. Based on an enhanced green fluorescent protein (EFGP) reporter system in C6/36 cells, the miR-281 interacted with the DENV viral genome at the 5'UTR. In contrast with the majority of cases where miRNA-target interaction leads to degradation of the target the authors suggested an increase of stability leading to an upregulation of mRNA type of result [278]. However, this needed to be further assessed. In this regard, the Hepatitis C virus (HCV) another flavivirus, encodes two binding sites in its 5'UTR region for the miRNA-122 [279] which also results in an enhanced viral replication [280, 281]. Even though the mechanism is not fully understood it may include, promoting cap independent viral translation, enhance viral stability or facilitate de novo initiation of viral RNA synthesis [282]. Other studies have shown that miR-252 increases upon DENV-2 infection in C6/36. miR-252 targets the E gene and therefore decreases DENV replication in C6/36 cells [283]. The miR-2940-5p is required for WNV replication in C6/36 cells through the regulation of the metalloprotease m41 ftsh (MEtP) [284].

There is another possibility, in which the arbovirus may produce functional miRNA like viral small RNAs. For instance, in DENV-2 infected mosquitoes, six miRNA-like viral small RNAs (vsRNAs) were identified and mapped to the 5' and 3' UTRs. Pre-treatment with a synthetic inhibitor of vsRNA-5, on C6/36 and RML-12 cells, lead to increased DENV-2 replication. This observation was validated by

northern blots in mosquito cells (Aag2 and C6/36); also dicer-2 silencing did not affect vsRNA-5 levels. When vsRNA-5 was added to the cells, the DENV-2 replication decreased. Considered together, these results led to the conclusion that vsRNA-5 functioned through the miRNA pathway. The target of vsRNA-5 was the DENV-2 genome itself in the NS1 region, which suggested an auto regulated mechanism during replication [285]. However, others have not found DENV-derived miRNAs or noticeable changes in the host miRNAs upon DENV-2 infection of Aag2 cells [286] and so results are controversial.

Many studies have focused on DENV since it causes the most prevalent arthropod transmitted disease in the world [10]. DENV (genus *flavivirus,* family *Flaviviridae*) has a single strand, positive sense RNA genome with a single open reading frame, which encodes three structural proteins (capsid [C], envelope [E], and precursor membrane [prM]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [6]. DENV replicates in the cytoplasm of the infected cells where the pathways aforementioned can take place. DENV is transmitted to a susceptible human host through the bite of an infectious vector, mainly *Ae. aegypti*. It has been estimated that about 400 million infections occur annually [14].

DENV transmission by *Ae. aegypti* is possible by the intrinsic ability of arthropod vector to acquire, maintain and then transmit a pathogen, which is known as vector competence (VC) [134]. Upon intake, the arbovirus has to replicate and be able to be transmitted to a susceptible host in a subsequent feeding episode; however the virus has to first bypass a series of physiological barriers (Figure 1) [135]. Briefly, upon entry of the virus into the mosquito gut through an infectious blood meal, the virus has to establish an infection; if this does not occur the mosquito has a midgut infection barrier (MIB). Next, the virus has to replicate and disseminate to other mosquito tissues; if this does not occur the mosquito has a midgut escape barrier (MEB). The virus then may infect several mosquito tissues but especially the salivary glands where it again has to establish an infection. If this is prevented the mosquito has a salivary gland infection barrier (SGIB). Next, the virus has to replicate and disseminate into the saliva

secretions from where it will be expectorated with the saliva into a susceptible vertebrate host. If this is limited, the mosquito has a salivary gland escape barrier (SGEB) [135, 136]. In conjunction, the MIB, MEB, SGIB and SGEB contribute to the overall VC phenotype.

Competent vectors develop persistent virus infections. In Chapter 2 of this dissertation we report variable transmission (TR) for DENV-2 in natural populations of *Ae. aegypti* from Mexico. For some of those populations the proportion of midgut infection (MI) was lower than 0.30. In this study we used mosquito isofemale lines with low MI phenotypes.

We wanted to further explore on the involvement of the miRNA pathway in persistently DENV-2 infected mosquitoes, for which DENV-2 virus was detected at 14 days post-infection (dpi) relative to unexposed mosquitoes. Also, we included a comparison from a subset of mosquitoes from the same cohort that were exposed to DENV-2 regardless of their midgut infection status in contrast to unexposed mosquitoes. Analysis of miRNA regulation in mosquitoes may help us to understand more about the intricate interactions between the virus and the vector host.

Methods

Generation of *Ae. aegypti* isofemale lines (AaeIL)

Parental *Ae. aegypti* collections were made in the state of Veracruz, Mexico in 2012. Approximately twenty-five individual *Ae. aegypti* females from the parental Cardel/Zempoala (C/Z) collection were placed individually into containers following a non-infectious blood meal. They were allowed to lay eggs. The offspring of individual females were challenged with DENV-2 New Guinea C strain (NGC) to corroborate their VC phenotype (data not shown). From the above, the AaeIL C/Z 2, C/Z 9, C/Z 11, C/Z 12 and C/Z 22 were selected for a low competency to DENV-2 NGC.

Mosquito Infections

For mosquito infections, the DENV-2 NGC strain was used to infect C6/36 cells at a multiplicity of infection (MOI) of 0.01. At 7 days post infection (dpi) Minimum Essential Medium Eagle (MEM) media supplemented with 3% fetal bovine serum (FBS) was changed. At 12 dpi, supernatant was harvested and centrifuged at 3,000xg for 10 min at 4°C. The supernatants were then mixed with defibrinated calf blood in a 1:1 proportion for the mosquito infectious blood meals, which were performed under biosafety level-3 containment.

Prior to feeding, 5-7 day old mosquitoes were deprived of sucrose and water for 8 hours. The infectious blood meal, with about 10^6 PFU/mL was provided through water-jacketed glass feeders with a hog gut membrane. After up to one-hour of feeding, mosquitoes were cold-anesthetized and engorged females were selected and placed into new containers, and water and a sugar source were provided. Mosquitoes were maintained for 14 days at insectary conditions (28°C, 70% relative humidity and 12:12 light:dark diurnal cycle).

Determination of DENV infection status for vector competence

At 14 dpi, mosquitoes were cold anesthetized at 4°C. Legs and wings were removed and placed into a tube containing 60 µL of TNA lysis buffer (Mag-Bind Viral DNA/RNA, Omega).The mosquito proboscis was then placed into a capillary tube that contained immersion oil (~5 µL) and allowed to expectorate saliva for 30 minutes. After salivation, the tip of the capillary tube was broken into a 1.5 mL centrifuge tube containing 100 µL of mosquito diluent (1X phosphate buffer saline (PBS) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 50 µg/mL penicillin/streptomycin, 50 µg/mL gentamycin, 2.5 µg/mL fungizone). Subsequently, the midgut was dissected, rinsed individually in PBS and placed into a tube containing 60 µL of TNA lysis buffer (Mag-Bind Viral DNA/RNA, Omega) Forceps were dipped in 70% ethanol and cleaned after each tissue was taken and between individual mosquitoes.

RNA was extracted from legs, midguts or saliva using the MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems) on the Thermo Scientific KingFisher Flex. RNA was then tested by RT-qPCR using the primers for DENV-2 reported by Johnson in 2005 to determine infection status [287]. DENV RNA standard dilutions from $1x10^7$ to $1x10^3$ genome equivalents (ge) and non-template control reactions were included in the assay. A sample was considered positive if the cycle threshold (C_T) value was ≤36 and the presence of a melting peak around 80.1-80.5°C.

Vector competence assessment

For the vector competence the proportion of midgut infection (MI) was the number of mosquitoes with DENV RNA in the midgut divided by the total number of mosquitoes that had blood fed. The proportion of disseminated infection (DI) was the number of mosquitoes with DENV RNA in the legs/wings divided by the number of mosquitoes that had blood fed. The transmission (TR) was defined as the number of mosquitoes that expectorated saliva containing DENV RNA divided by the total number of mosquitoes that had blood fed.

Small-RNA library preparation

A series of mosquito infections were performed in order to obtain DENV-2 infected midguts for the small-RNA library preparation. For which midguts from positive carcasses or legs at 14 dpi were pooled in groups of five from which RNA was extracted using the *mir*Vana™ miRNA Isolation Kit with an enrichment step for small RNAs.

Small RNA libraries were prepared using the TruSeq Small RNA library preparation kit. For each of the isofemale lines (n=5), 2 replicates of the unexposed, 2 replicates of the DENV-2 exposed and 4 replicates of the DENV-2 infected were included. Each of the replicates consisted of 5 midguts from which RNA was obtained and used for small RNA library preparation (table 3.1). Prepared libraries were

amplified using the KAPA Library amplification Kit (KAPABIOSYSTEMS). A total of 40 libraries were multiplexed to be sequenced in 2 lanes of 1x50 base pairs (bp) on the Illumina HiSeq2500 platform.

Table 3.1. Sample sizes for the small RNA libraries

Group	Replicates	Number of small RNA libraries
Unexposed	2 (5 isofemale lines)	10
Exposed	2 (5 isofemale lines)	10
Infected (MEB-)	4 (5 isofemale lines)	20
Total		40

Small RNA analysis

The reads obtained from the Next Generation Sequencing (NGS) were analyzed with a small RNA pipeline that uses Bowtie and SAMtools. A target selection of 19-23 nt, forward reads only and a single mismatch were allowed. As a reference, available sequences for known *Diptera* microRNAs from miRBase [288, 289] were used. The fold change analysis was performed using the package DESeq in R, which determines the differential gene expression analysis based on the negative binomial distribution [290]. For the analysis, >=200 counts of each miRNA were considered. Adjusted p-values or false discovery rates (FDR) were determined using a Benjamini-Hochment adjustment for the multiple comparisons. The significantly modulated miRNA were determined by a greater or equal to one or lower or equal to negative one $\log_2$ fold change with *p* values and FDR <0.05.

MicroRNA target prediction

For the identification of microRNA targets the 3' untranslated regions (UTRs) were used. As previously [276], the putative miRNA targets were identified from the *Aedes aegypti* transcriptome release 1.3 (Vectorbase.org) using the miRanda [291], PITA [292]and TargetScan [293]prediction algorithms.

Data and statistical analysis

The midgut infection, disseminated infection, salivary gland infection and transmission proportions were estimated in WinBUGS [255] using a binomial distribution as a model. Where the number of infected mosquito tissues (e.g. midgut) was a binomial sample. Bayesian 95% Highest Density Intervals (HDI) were also obtained. The Bayesian analysis provides logical estimates for proportions where an uninformative prior distribution is assumed. Therefore, the prior distribution for the probability of a mosquito tissue to be infected was 0.0-1.0 [256].

Error bars represent 95% HDI credible intervals and non-overlapping error bars indicate statistical significance. MIRs for the different C/Z lines were compared by Fisher's exact tests, significance was determined at $p<0.05$. GraphPad PRISM version 7.03 (GraphPad Software, San Diego, CA, USA) was used for graph construction. MIR for the different C/Z lines was analyzed by Fisher's exact test to determine significance at $p<0.05$.

Results

Vector competence characterization of the C/Z lines

For this study we used *Ae. aegypti* isofemale lines, inbred mosquito lines therefore with limited genetic variation. The parental C/Z line was chosen by their low competence for DENV-2 NGC. We corroborated this observation by assessing the vector competence of the individual C/Z lines (table 3.2).

Table 3.2. - MI, DI and TR for each of the isofemale lines

Isofemale line	MI	DI	TR
C/Z 2	16/80	10/80	3/80
C/Z 9	10/51	9/51	2/51
C/Z 11	23/132	13/132	1/132
C/Z 12	14/80	8/80	5/80
C/Z 22	10/50	5/50	1/50

We determined by infection experiments that the C/Z lines had a low vector competence phenotype with low MI, DI and TR proportions (Figure 3.1 and table 3.2). From the results, the low MI proportion was consistent with a strong midgut infection barrier. When compared, the MI, DI and TR proportions between the C/Z lines were not statistically significant.

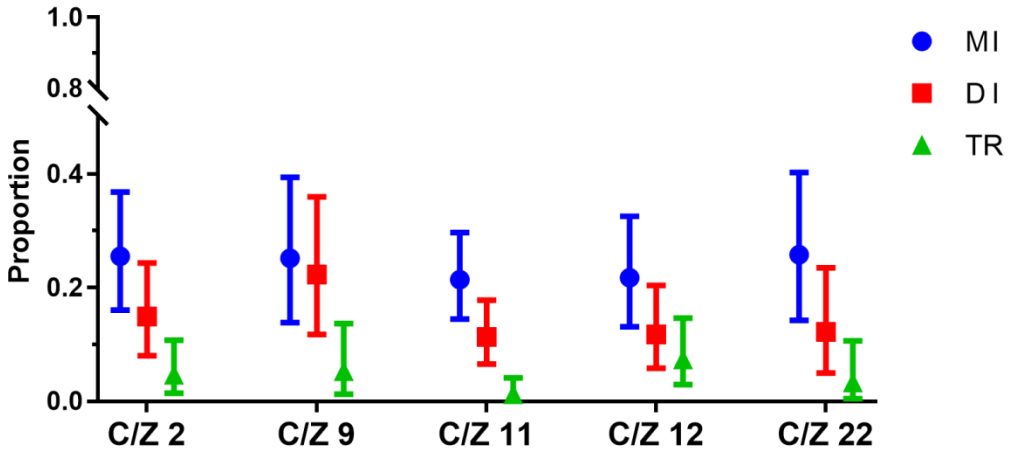

Figure 3.1. Midgut infection, disseminated infection and transmission proportions of the AaeIL for DENV-2. DENV-2 infection was determined by RT-qPCR at 14 dpi. The error bars represent 95% HDI credible intervals and non-overlapping error bars indicate statistical significance.

Modulation of miRNAs upon DENV-2 exposure and DENV-2 infection.

Since the MI proportions were uniform for all the C/Z lines, all of them were combined for the differential expression analysis. Our analysis was divided into two parts, 1) modulation upon DENV-2 exposure, those mosquitoes were given a DENV-2 blood meal relative to mosquitoes that were given a non-infectious blood meal, 'exposed group' and 2) mosquitoes with DENV-2 infected midguts and disseminated infection at 14 dpi relative to midguts of mosquitoes that received a non-infectious blood meal, 'infected group' (Fig 3.2).

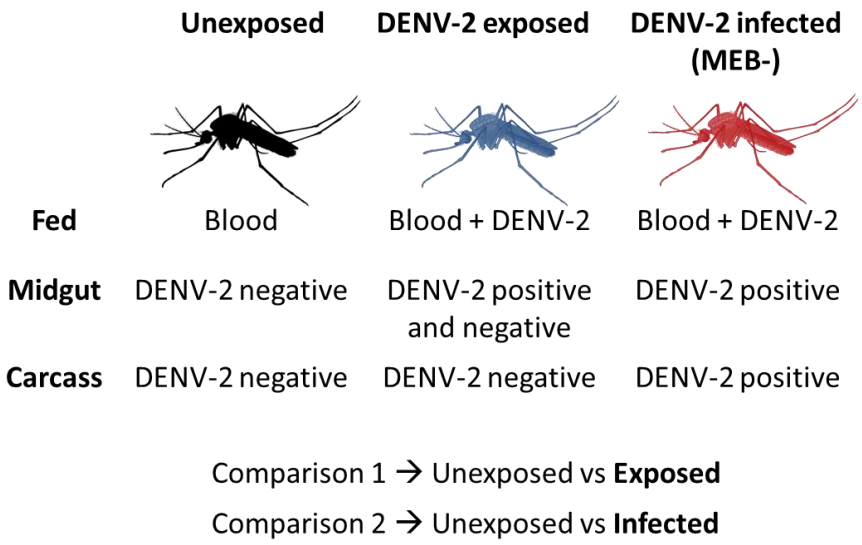

Figure 3.2 Schematic representation of the groups compared for the miRNA modulation analysis

We found a total of 18 differentially expressed miRNAs upon exposure to DENV-2 of which 11 were up-regulated and 7 were down-regulated. In addition 2 miRNAs were found to be present while absent in the unexposed midguts (Table 3.3).

The predicted targets of those are included in Table 3.4. Interestingly, multiple miRNAs targeted the same predicted genes. That was observed on both comparison groups, DENV-2 exposed and DENV-2 infected midguts. The multiple targeted genes for the DENV-2 exposed group are included in table 3.5.

Table 3.3. List of differentially expressed miRNAs in *Ae. aegypti* midguts exposed to DENV-2.

miRNA	log2FC	*p* value	FDR	miRNA sequence
miR-989	5.961916	2.79E-08	4.62E-07	TGTGATGTGACGTAGTGGTAC
miR-375	4.09341	6.52E-07	1.14E-05	TTTGTTCGTTTGGCTCGAGTTA
miR-210-3p	4.060982	1.08E-09	2.87E-08	CTTGTGCGTGTGACAACGGCTAT
miR-10-3p	3.912796	2.17E-07	4.16E-06	CAAATTCGGTTCTAGAGAGGTTT
miR-957	2.879338	1.63E-07	3.16E-06	TGAAACCGTCCAAAACTGAGGC
miR-252-5p	2.498544	3.34E-11	1.95E-09	CTAAGTACTAGTGCCGCAGGAG
miR-305-3p	2.367956	0.000276	0.043393	CGGCACATGTTGGAGTACACTTA
miR-2951-3p	2.350976	9.92E-08	2.52E-06	ACCCGCGTCTACCCGGTTCCGTGTACTGGAAAT
miR-281-1-5p	1.557874	6.22E-13	1.40E-11	AAAGAGAGCTGTCCGTCGACAGT
miR-276b	1.459636	0.003485	0.018867	AAAACCGAAGTCTTTTTACCATCAGCGAGGTATA

				GAGTTCCTACGTTCCTATATTCAGTCGTAGGAACT TAATACCGTGCTCTTGGAGGACTGTCGACC
miR-275	1.287198	0.001267	0.043393	ATCCTTTCGATTTCGCGCGCTAAGCAGGAACCGAG ACTTTGTCATTTGCTAGCAGTCAGGTACCTGAAGTA GCGCGCGTGATC
miR-2941	Present	3.03E-09	6.31E-08	TAGTACGGCTAGAACTCCACGG
miR-2951-5p	Present	7.71E-17	4.50E-14	AGAGCTCAGCACGCAGGGGTGGC
miR-998-3p	-1.13276	1.29E-15	5.18E-14	TAGCACCATGAGATTCAGCTC
miR-125-5p	-1.26072	8.78E-07	1.41E-05	TCCCTGAGACCCTAACTTGTGA
miR-308-5p	-1.43375	9.71E-14	3.65E-12	CGCGGTATATTCTTGTGGCTTG
miR-13-5p	-1.57858	7.87E-05	0.001022	TCGTAAAAATGGTTGTGCTGTG
bantam	-2.00666	0.000132	0.002177	TGAGATCACTTTGAAAGCTGATT
miR-100	-2.08388	2.00E-55	3.76E-53	AACCCGTAGATCCGAACTTGTG
miR-11-5p	-2.35726	2.34E-06	3.66E-05	CGAGAACTCCGGCTGTGACC

Up-regulated miRNAs are shown in red. Down-regulated miRNAs are shown in blue. Present miRNAs are shown in black. Unique miRNAs in the exposed group are shown in bold. miRNA modulation was obtained from midguts exposed to DENV-2 at 14 dpe. FDR stands for false discovery rate.

Table 3.4. Predicted targets of the differentially expressed microRNAs in midguts exposed to DENV-2

miRNA	**Gene ID**	**Description**
miR-989	AAEL000567	Tret1: facilitated trehalose transporter
miR-989	AAEL001612	dicer-1
miR-989	AAEL001935	CTL-like protein 1
miR-989	AAEL003505	jun
miR-989	AAEL001549	protein kinase c
miR-989	AAEL000188	elongase, putative
miR-989	AAEL002969	brain chitinase and chia
miR-989	AAEL002809	down syndrome critical region protein
miR-989	AAEL002692	beat protein
miR-989	AAEL002261	GTP cyclohydrolase i
miR-989	AAEL000088	brefeldin A-sensitive peripheral Golgi protein, putative
miR-989	AAEL000713	reticulon/nogo
miR-989	AAEL001946	four and a half lim domains
miR-989	AAEL002549	sosie: protein sosie
miR-989	AAEL001108	protein kinase c
miR-989	AAEL001715	chaperonin
miR-989	AAEL000599	wingless protein, putative
miR-989	AAEL001433	fgf receptor activating protein
miR-989	AAEL001126	rest corepressor (corest) protein
miR-375	AAEL001159	ribonuclease t2
miR-375	AAEL002550	polyA-binding protein interacting protein, putative
miR-375	AAEL001232	tubulointerstitial nephritis antigen

miR-375	AAEL001938	ATP-binding cassette sub-family A member 3, putative
miR-375	AAEL000540	fasciclin, putative
miR-375	AAEL002277	cAMP-dependent protein kinase type i-beta regulatory subunit
miR-375	AAEL000704	synaptotagmin
miR-375	AAEL002329	alpha-1,3-mannosyl-glycoprotein beta-1, 2-n-acetylglucosaminyltransferase
miR-375	AAEL002973	zinc finger protein
miR-375	AAEL001467	sdk-P1
miR-375	AAEL002594	P21-activated kinase, pak
miR-375	AAEL001239	cation efflux protein/ zinc transporter
miR-375	AAEL001952	28 kDa heat- and acid-stable phosphoprotein (PDGF-associated protein), putative
miR-375	AAEL002804	oligosaccharyl transferase
miR-375	AAEL000291	V-type proton ATPase 16 kDa proteolipid subunit
miR-375	AAEL000064	dopachrome-conversion enzyme (DCE) isoenzyme, putative
miR-375	AAEL002135	tubulin-specific chaperone b
miR-375	AAEL001698	charged multivesicular body protein 4b
miR-375	AAEL001933	membrane associated ring finger 1,8
miR-375	AAEL002280	ctl2
miR-375	AAEL001317	DEAD box ATP-dependent RNA helicase
miR-210-3p	AAEL000471	monocarboxylate transporter
miR-210-3p	AAEL001985	protein serine/threonine kinase
miR-210-3p	AAEL002550	polyA-binding protein interacting protein, putative
miR-210-3p	AAEL001518	zinc finger protein
miR-210-3p	AAEL002155	ras-related protein Rab-10, putative
miR-210-3p	AAEL000704	synaptotagmin
miR-210-3p	AAEL000175	eIF3-S8: eukaryotic translation initiation factor 3 subunit C
miR-210-3p	AAEL001673	actin
miR-210-3p	AAEL002918	centaurin beta
miR-210-3p	AAEL002789	26S proteasome non-ATPase regulatory subunit
miR-210-3p	AAEL000767	phospholipid-transporting ATPase 1 (aminophospholipid flippase 1)
miR-210-3p	AAEL000759	gamma-glutamylcysteine synthetase, putative
miR-210-3p	AAEL000856	germ cell-less protein
miR-210-3p	AAEL000839	O-fucosyltransferase, putative
miR-10-3p	AAEL001218	alanyl-tRNA synthetase
miR-10-3p	AAEL002879	heterogeneous nuclear ribonucleoprotein r
miR-10-3p	AAEL000193	histone-lysine n-methyltransferase
miR-10-3p	AAEL002493	short-chain dehydrogenase
miR-10-3p	AAEL003288	mitochondrial uncoupling protein, putative
miR-10-3p	AAEL000599	wingless protein, putative
miR-957	AAEL001935	CTL-like protein 1
miR-957	AAEL002049	circadian protein clock/arnt/bmal/pas

miR-957	AAEL002761	tropomyosin invertebrate
miR-957	AAEL001088	beta-1,3-galactosyltransferase
miR-957	AAEL000383	beta-1,3-galactosyltransferase brn
miR-957	AAEL001673	actin
miR-957	AAEL000720	Med11: mediator of RNA polymerase II transcription subunit 11
miR-252-5p	AAEL001856	adenosine kinase
miR-252-5p	AAEL000339	lim domain
miR-252-5p	AAEL001766	leucine-rich transmembrane proteins
miR-252-5p	AAEL000343	serine/threonine-protein kinase vrk
miR-252-5p	AAEL001916	eukaryotic translation initiation factor 4e
miR-252-5p	AAEL001796	Nuclear hormone receptor (HR78)
miR-252-5p	AAEL000577	DNA binding protein elf-1
miR-305-3p	AAEL000126	molybdopterin cofactor synthesis protein a
miR-305-3p	AAEL000558	neural stem cell-derived dendrite regulator
miR-305-3p	AAEL000925	leucine-zipper-like transcriptional regulator 1 (LZTR-1)
miR-305-3p	AAEL001016	zinc finger protein
miR-305-3p	AAEL000278	poly(p)/ATP NAD kinase
miR-305-3p	AAEL002594	P21-activated kinase, pak
miR-305-3p	AAEL001334	geranylgeranyl transferase type ii beta subunit
miR-305-3p	AAEL000770	platelet-activating factor acetylhydrolase isoform 1b alpha subunit
miR-305-3p	AAEL001108	protein kinase c
miR-305-3p	AAEL002372	RpS11: 40S ribosomal protein S11
miR-305-3p	AAEL002904	juvenile hormone-inducible protein, putative
miR-305-3p	AAEL001964	protein serine/threonine kinase, putative
miR-305-3p	AAEL000374	cysteine-rich venom protein, putative
miR-2951-3p	AAEL005008	aquaporin
miR-2951-3p	AAEL002511	ionotropic glutamate receptor subunit ia
miR-2951-3p	AAEL002412	monocarboxylate transporter
miR-2951-3p	AAEL000485	paramyosin, putative
miR-2951-3p	AAEL000420	cathepsin o
miR-2951-3p	AAEL000138	NADH dehydrogenase, putative
miR-2951-3p	AAEL001044	Aats-tyr: tyrosyl-tRNA synthetase
miR-2951-3p	AAEL000339	lim domain
miR-2951-3p	AAEL002145	gonadotropin inducible transcription factor
miR-2951-3p	AAEL001421	high density lipoprotien binding protein / vigilin
miR-2951-3p	AAEL002407	DNA repair protein xp-e
miR-2951-3p	AAEL002373	juvenile hormone-inducible protein, putative
miR-2951-3p	AAEL000641	protein disulfide isomerase
miR-2951-3p	AAEL000217	serine/threonine protein kinase
miR-2951-3p	AAEL002892	protein kinase c, mu
miR-2951-3p	AAEL002375	NBP2b protein, putative

miR-2951-3p	AAEL000321	acetyl-coa synthetase
miR-2951-3p	AAEL000773	kinesin heavy chain
miR-2951-3p	AAEL000715	zinc finger protein
miR-2951-3p	AAEL000080	phosphoenolpyruvate carboxykinase
miR-2951-3p	AAEL000343	serine/threonine-protein kinase vrk
miR-2951-3p	AAEL000746	NADP-specific isocitrate dehydrogenase
miR-2951-3p	AAEL000987	RpL8: 60S ribosomal protein L8
miR-2951-3p	AAEL001659	misexpression suppressor of ras, putative
miR-2951-3p	AAEL002802	WD-repeat protein
miR-2951-3p	AAEL002904	juvenile hormone-inducible protein, putative
miR-2951-3p	AAEL002672	matrix metalloproteinase
miR-2951-3p	AAEL002196	procathepsin L3, putative
miR-2951-3p	AAEL002818	splicing factor u2af large subunit
miR-2951-3p	AAEL002598	OBP15: odorant binding protein OBP15
miR-2951-3p	AAEL001830	geranylgeranyl transferase type i beta subunit
miR-2951-3p	AAEL001204	sterol o-acyltransferase
miR-2951-3p	AAEL000450	ras GTPase activating protein
miR-2951-3p	AAEL000800	microsomal dipeptidase
miR-2951-3p	AAEL000101	AMP dependent coa ligase
miR-281-5p	AAEL002422	cytoplasmic polyadenylation element binding protein (cpeb)
miR-281-5p	AAEL001963	protein serine/threonine kinase, putative
miR-281-5p	AAEL002587	OBP11: odorant binding protein OBP11
miR-281-5p	AAEL000034	meiotic recombination repair protein 11 (mre11)
miR-281-5p	AAEL000599	wingless protein, putative
miR-281-5p	AAEL002779	ribokinase
miR-281-5p	AAEL002723	peroxisomal membrane protein pmp34
miR-281-5p	AAEL001622	dual specificity mitogen-activated protein kinase kinase MAPKK
miR-281-5p	AAEL001641	deoxyribonuclease I, putative
miR-281-5p	AAEL002422	cytoplasmic polyadenylation element binding protein (cpeb)
miR-281-5p	AAEL001963	protein serine/threonine kinase, putative
miR-281-5p	AAEL002587	OBP11: odorant binding protein OBP11
miR-281-5p	AAEL000034	meiotic recombination repair protein 11 (mre11)
miR-281-5p	AAEL000599	wingless protein, putative
miR-281-5p	AAEL002779	ribokinase
miR-281-5p	AAEL002723	peroxisomal membrane protein pmp34
miR-281-5p	AAEL001622	dual specificity mitogen-activated protein kinase kinase MAPKK
miR-275	AAEL001159	ribonuclease t2
miR-275	AAEL000964	regulatory factor X-associated ankyrin-containing protein, putative
miR-275	AAEL000641	protein disulfide isomerase
miR-275	AAEL001467	sdk-P1
miR-275	AAEL000817	rhomboid

miR-275	AAEL000088	brefeldin A-sensitive peripheral Golgi protein, putative
miR-275	AAEL000813	dimethylaniline monooxygenase
miR-275	AAEL002194	uricase
miR-275	AAEL001933	membrane associated ring finger 1,8
miR-275	AAEL000101	AMP dependent coa ligase
miR-275	AAEL002714	kinesin-like protein KIF23 (mitotic kinesin-like protein 1)
miR-2941	AAEL001523	secretory Phospholipase A2, putative
miR-2941	AAEL001091	malic enzyme
miR-2941	AAEL000540	fasciclin, putative
miR-2941	AAEL002214	amino acid transporter
miR-2941	AAEL001963	protein serine/threonine kinase, putative
miR-2941	AAEL005730	cop9 signalosome complex subunit
miR-2941	AAEL002287	trans-prenyltransferase
miR-2941	AAEL001766	leucine-rich transmembrane proteins
miR-2941	AAEL001574	septin
miR-2941	AAEL002906	26S proteasome regulatory subunit rpn2
miR-2951-5p	AAEL001159	ribonuclease t2
miR-2951-5p	AAEL001919	protein tyrosine phosphatase, non-receptor type nt1
miR-2951-5p	AAEL001963	protein serine/threonine kinase, putative
miR-2951-5p	AAEL002853	ccaat/enhancer binding protein
miR-2951-5p	AAEL001549	protein kinase c
miR-2951-5p	AAEL001901	MRAS2, putative
miR-2951-5p	AAEL002587	OBP11: odorant binding protein
miR-2951-5p	AAEL001766	leucine-rich transmembrane proteins
miR-2951-5p	AAEL002478	double-stranded binding protein, putative
miR-2951-5p	AAEL000563	CTLMA15: C-Type Lectin (CTL) - mannose binding.
miR-2951-5p	AAEL000599	wingless protein, putative
miR-2951-5p	AAEL000666	pmp22 peroxisomal membrane protein, putative
miR-125-5p	AAEL000014	cyclic-nucleotide-gated cation channel
miR-125-5p	AAEL000037	CLIPB35: Clip-Domain Serine Protease family B.
miR-125-5p	AAEL000190	trypsin
miR-125-5p	AAEL000263	Zinc finger protein jing homolog
miR-125-5p	AAEL000389	brain chitinase and chia
miR-125-5p	AAEL000486	chaperonin
miR-125-5p	AAEL000656	pangolin
miR-125-5p	AAEL000661	t-cell specific transcription factor, tcf
miR-125-5p	AAEL000828	vitellogenin,, putative
miR-125-5p	AAEL000906	guanyl-nucleotide exchange factor
miR-125-5p	AAEL000971	smile protein
miR-125-5p	AAEL001046	phosphatase fragment
miR-125-5p	AAEL001069	histone deacetylase

miR-125-5p	AAEL001102	adenosine kinase
miR-125-5p	AAEL001165	ras GTP exchange factor, son of sevenless
miR-125-5p	AAEL001177	signal recognition particle, 14kD, putative
miR-125-5p	AAEL001196	cadherin
miR-125-5p	AAEL001218	alanyl-tRNA synthetase
miR-125-5p	AAEL001260	Med20: mediator of RNA polymerase II transcription subunit 20
miR-125-5p	AAEL001312	CYP9M6: cytochrome P450
miR-125-5p	AAEL001440	E1a binding protein P400
miR-125-5p	AAEL001548	glucosyl/glucuronosyl transferases
miR-125-5p	AAEL001586	glucosyl/glucuronosyl transferases
miR-125-5p	AAEL001629	cAMP-specific 3,5-cyclic phosphodiesterase
miR-125-5p	AAEL001662	kinesin family member 21A
miR-125-5p	AAEL001711	activin receptor type I, putative
miR-125-5p	AAEL001732	candidate tumor suppressor protein
miR-125-5p	AAEL001830	geranylgeranyl transferase type i beta subunit
miR-125-5p	AAEL001894	kek1
miR-125-5p	AAEL001896	UDP-N-acetylglucosamine: polypeptide-N-acetylglucosaminyl transferase, putative
miR-125-5p	AAEL001933	membrane associated ring finger 1,8
miR-125-5p	AAEL002204	fatty acid synthase
miR-125-5p	AAEL002227	fatty acid synthase
miR-125-5p	AAEL002266	p15-2b protein, putative
miR-125-5p	AAEL002280	ctl2
miR-125-5p	AAEL002295	leucine-rich transmembrane protein
miR-125-5p	AAEL002306	hect E3 ubiquitin ligase
miR-125-5p	AAEL002307	leucine-rich transmembrane protein
miR-125-5p	AAEL002441	larval cuticle protein, putative
miR-125-5p	AAEL002458	pupal cuticle protein, putative
miR-125-5p	AAEL002468	lipid a export ATP-binding/permease protein msba
miR-125-5p	AAEL002539	fimbrin/plastin
miR-125-5p	AAEL002603	triacylglycerol lipase, putative
miR-125-5p	AAEL002655	matrix metalloproteinase
miR-125-5p	AAEL002683	aldehyde oxidase
miR-125-5p	AAEL002769	homeobox protein prospero/prox-1
miR-125-5p	AAEL002911	lysosomal acid lipase, putative
miR-125-5p	AAEL002972	brain chitinase and chia
miR-125-5p	AAEL003123	deoxyribonuclease I, putative
miR-125-5p	AAEL003125	acyl-coa dehydrogenase
miR-125-5p	AAEL003129	neuroligin
miR-125-5p	AAEL003155	dynein heavy chain
miR-125-5p	AAEL003308	trypsin, putative
miR-125-5p	AAEL003632	CLIPB39: Clip-Domain Serine Protease family B.

miR-125-5p	AAEL003758	sorting nexin
miR-125-5p	AAEL003763	CYP329B1: cytochrome P450
miR-125-5p	AAEL003960	arylsulfatase b
miR-125-5p	AAEL003990	myeloid leukemia factor,
miR-125-5p	AAEL004009	glucose dehydrogenase
miR-125-5p	AAEL004124	mitochondrial glutamate carrier, putative
miR-125-5p	AAEL004291	translin
miR-125-5p	AAEL004573	delta(9)-desaturase 2, putative
miR-125-5p	AAEL004661	beta-hexosaminidase
miR-125-5p	AAEL004716	chromodomain helicase DNA binding protein
miR-125-5p	AAEL004750	nonmuscle myosin heavy chain-A, putative
miR-125-5p	AAEL004833	DPT1: diptericin anti-microbial peptide
miR-125-5p	AAEL004866	protein farnesyltransferase beta subunit
miR-125-5p	AAEL004925	lysosomal acid lipase, putative
miR-125-5p	AAEL005166	eyes absent
miR-125-5p	AAEL005175	lipin
miR-125-5p	AAEL005276	target of myb1 (tom1)
miR-125-5p	AAEL005321	neurexin iv
miR-125-5p	AAEL005324	titin
miR-125-5p	AAEL005341	hk: Protein hook
miR-125-5p	AAEL005387	WD-repeat protein
miR-125-5p	AAEL005533	synaptic vesicle protein
miR-125-5p	AAEL005732	acyl-coa dehydrogenase
miR-125-5p	AAEL005742	transcription initiation factor TFIID subunit 10, putative
miR-125-5p	AAEL005833	cytosolic purine 5-nucleotidase
miR-125-5p	AAEL005921	D-lactate dehydrognease 2
miR-125-5p	AAEL006157	aldehyde oxidase
miR-125-5p	AAEL006240	purple acid phosphatase, putative
miR-125-5p	AAEL006297	venom allergen
miR-125-5p	AAEL006457	asparagine synthetase
miR-125-5p	AAEL006640	DEAD box ATP-dependent RNA helicase
miR-125-5p	AAEL006824	cytochrome P450
miR-125-5p	AAEL007041	low-density lipoprotein receptor (ldl)
miR-125-5p	AAEL007060	lipase
miR-125-5p	AAEL007216	elongase, putative
miR-125-5p	AAEL007225	dynein heavy chain
miR-125-5p	AAEL007235	mitochondrial uncoupling protein
miR-125-5p	AAEL007322	phosphatidate phosphatase
miR-125-5p	AAEL007412	sphingomyelin phosphodiesterase
miR-125-5p	AAEL007542	glutamate decarboxylase
miR-125-5p	AAEL007597	CLIPC3: Clip-Domain Serine Protease family C

miR-125-5p	AAEL007656	receptor for activated C kinase, putative
miR-125-5p	AAEL007657	low-density lipoprotein receptor (ldl)
miR-125-5p	AAEL007658	partitioning defective 3, par-3
miR-125-5p	AAEL007689	phospholipid-transporting ATPase 1 (aminophospholipid flippase 1)
miR-125-5p	AAEL007760	mct-1 protein
miR-125-5p	AAEL007762	mRpL40: mitochondrial ribosomal protein, L40, putative
miR-125-5p	AAEL007898	calmin
miR-125-5p	AAEL007924	GPRNPY3: GPCR Neuropeptide Y Family
miR-125-5p	AAEL007925	histone H2A
miR-125-5p	AAEL007938	serine-type enodpeptidase,
miR-125-5p	AAEL008035	ATP-dependent RNA helicase
miR-125-5p	AAEL008114	p15-2b protein, putative
miR-125-5p	AAEL008345	CYP4G35: cytochrome P450
miR-125-5p	AAEL008508	translin
miR-125-5p	AAEL008595	Protein maelstrom homolog
miR-125-5p	AAEL008620	D7 protein, putative
miR-125-5p	AAEL008685	lim homeobox protein
miR-125-5p	AAEL008688	G-protein signalling modulator
miR-125-5p	AAEL008715	AAA ATPase
miR-125-5p	AAEL008773	laminin A chain, putative
miR-125-5p	AAEL008866	pupal cuticle protein 78E, putative
miR-125-5p	AAEL008873	pupal cuticle protein 78E, putative
miR-125-5p	AAEL008889	CYP6AL1: cytochrome P450
miR-125-5p	AAEL009149	kinectin, putative
miR-125-5p	AAEL009192	SCRASP1: Class A Scavenger Receptor (SRCR domain) with Serine Protease domain.
miR-125-5p	AAEL009200	Exo3: exocyst complex component 3
miR-125-5p	AAEL009353	ssm4 protein
miR-125-5p	AAEL009555	Niemann-Pick Type C-2, putative
miR-125-5p	AAEL009579	NBP2b protein, putative
miR-125-5p	AAEL009676	glyoxylate/hydroxypyruvate reductase
miR-125-5p	AAEL009806	low-density lipoprotein receptor (ldl)
miR-125-5p	AAEL009850	GALE14: galectin
miR-125-5p	AAEL009855	sodium/dicarboxylate cotransporter, putative
miR-125-5p	AAEL009886	CCC3: sodium-coupled cation-chloride cotransporter
miR-125-5p	AAEL010108	sex-determining protein fem-1
miR-125-5p	AAEL010210	neurogenic locus notch (notch)
miR-125-5p	AAEL010269	venom allergen
miR-125-5p	AAEL010336	zinc phosphodiesterase
miR-125-5p	AAEL010351	phosphopentothenoylcysteine decarboxylase
miR-125-5p	AAEL010354	homeobox protein nk-2
miR-125-5p	AAEL010356	phosphopentothenoylcysteine decarboxylase

miR-125-5p	AAEL010414	set domain protein
miR-125-5p	AAEL010502	transcriptional regulator ATRX (X-linked helicase II)
miR-125-5p	AAEL010513	class b basic helix-loop-helix protein (bhlhb)
miR-125-5p	AAEL010585	spermatogenesis associated factor
miR-125-5p	AAEL010606	down syndrome cell adhesion molecule
miR-125-5p	AAEL010630	xanthine dehydrogenase
miR-125-5p	AAEL010803	ncd
miR-125-5p	AAEL010939	group ii plp decarboxylase
miR-125-5p	AAEL011013	single-minded
miR-125-5p	AAEL011078	CTLGA1: C-Type Lectin (CTL) - galactose binding.
miR-125-5p	AAEL011247	arrowhead
miR-125-5p	AAEL011415	lysine-specific histone demethylase
miR-125-5p	AAEL011528	triacylglycerol lipase, putative
miR-125-5p	AAEL011566	cell adhesion molecule
miR-125-5p	AAEL011729	Molybdenum cofactor sulfurase 3
miR-125-5p	AAEL011755	integral membrane protein, Tmp21-I (p23), putative
miR-125-5p	AAEL012037	sulphate transporter
miR-125-5p	AAEL012045	gar2, putative
miR-125-5p	AAEL012083	receptor protein-tyrosine phosphatase 10d
miR-125-5p	AAEL012110	protease m1 zinc metalloprotease
miR-125-5p	AAEL012152	activin receptor type I, putative
miR-125-5p	AAEL012192	ATP-binding cassette transporter
miR-125-5p	AAEL012345	lipase 1 precursor
miR-125-5p	AAEL012364	spermatogenesis associated factor
miR-125-5p	AAEL012386	ATP-binding cassette transporter
miR-125-5p	AAEL012421	cadherin
miR-125-5p	AAEL012673	ubiquitin conjugating enzyme 7 interacting protein
miR-125-5p	AAEL012687	juvenile hormone-inducible protein, putative
miR-125-5p	AAEL012717	WD-repeat protein
miR-125-5p	AAEL012730	dbl
miR-125-5p	AAEL012826	replication factor a 1, rfa1
miR-125-5p	AAEL012956	elastase, putative
miR-125-5p	AAEL013089	proteasome subunit alpha type
miR-125-5p	AAEL013111	glutamate transporter
miR-125-5p	AAEL013112	PGRPLE: Peptidoglycan Recognition Protein (Long)
miR-125-5p	AAEL013274	n-acetylgalactosaminyltransferase
miR-125-5p	AAEL013284	LT1: late trypsin 1, serine-type enodpeptidase
miR-125-5p	AAEL013372	ABC transporter
miR-125-5p	AAEL013697	condensin, SMC5-subunit, putative
miR-125-5p	AAEL013752	rfx5
miR-125-5p	AAEL013765	arrowhead

miR-125-5p	AAEL013873	cadherin
miR-125-5p	AAEL013896	smad4
miR-125-5p	AAEL014021	Med23: mediator of RNA polymerase II transcription subunit 23
miR-125-5p	AAEL014069	catrin, putative
miR-125-5p	AAEL014110	sulfite reductase
miR-125-5p	AAEL014134	kinesin heavy chain
miR-125-5p	AAEL014139	proacrosin, putative
miR-125-5p	AAEL014195	G-protein signalling modulator
miR-125-5p	AAEL014222	low-density lipoprotein receptor (ldl)
miR-125-5p	AAEL014252	sulfite reductase
miR-125-5p	AAEL014303	neuroligin,
miR-125-5p	AAEL014381	Molybdenum cofactor sulfurase 1
miR-125-5p	AAEL014578	ssm4 protein
miR-125-5p	AAEL014594	CYP301A1: cytochrome P450
miR-125-5p	AAEL014906	LAP4 protein, putative (Scribble protein, putative)
miR-125-5p	AAEL014916	lipase 1 precursor
miR-125-5p	AAEL015151	acid phosphatase
miR-125-5p	AAEL015283	synaptic vesicle protein
miR-125-5p	AAEL015458	transferrin
miR-125-5p	AAEL015639	transferrin
miR-125-5p	AAEL018292	HPX4: heme peroxidase
miR-125-5p	AAEL018680	ND4: NADH dehydrogenase subunit 4
miR-308-5p	AAEL002972	brain chitinase and chia
miR-308-5p	AAEL001159	ribonuclease t2
miR-308-5p	AAEL000091	sumo-1-activating enzyme E1a
miR-308-5p	AAEL000126	molybdopterin cofactor synthesis protein a
miR-308-5p	AAEL002395	Cdk8: Cyclin-dependent kinase
miR-308-5p	AAEL001194	fatty acid synthase
miR-308-5p	AAEL001963	protein serine/threonine kinase, putative
miR-308-5p	AAEL000661	t-cell specific transcription factor, tcf
miR-13-5p	AAEL001935	CTL-like protein 1
miR-13-5p	AAEL001088	beta-1,3-galactosyltransferase
miR-13-5p	AAEL002077	UV excision repair protein rad23
miR-13-5p	AAEL001126	rest corepressor (corest) protein
Bantam	AAEL001279	merozoite surface protein, putative
Bantam	AAEL001963	protein serine/threonine kinase, putative
Bantam	AAEL000088	brefeldin A-sensitive peripheral Golgi protein, putative
Bantam	AAEL002478	double-stranded binding protein, putative
Bantam	AAEL001928	Act1: actin-1
miR-100	AAEL002412	monocarboxylate transporter
miR-100	AAEL000126	molybdopterin cofactor synthesis protein a

miR-100	AAEL001467	sdk-P1
miR-100	AAEL001930	pra1 protein
miR-100	AAEL000291	V-type proton ATPase 16 kDa proteolipid subunit
miR-100	AAEL002294	sulphate transporter
miR-100	AAEL001372	sentrin/sumo-specific protease senp7
miR-11-5p	AAEL001963	protein serine/threonine kinase, putative
miR-11-5p	AAEL002551	DNA topoisomerase type I
miR-11-5p	AAEL000219	lactoylglutathione lyase
miR-11-5p	AAEL002714	kinesin-like protein KIF23 (mitotic kinesin-like protein 1)

Differential expression was determined from *Ae. aegypti* midguts exposed to DENV-2 at 14 dpi. Targets with not known function, duplicate genes and multiple transcripts within each miRNA were eliminated. Targeted genes for each miRNA were obtained from miRanda.

Table 3.5. Multiple targeted genes by different miRNAs expressed in DENV-2 exposed midguts

miRNA expression	**miRNA**	**Gene ID**	**Description**
up-regulated	miR-2951-3p miR-275	AAEL000101	AMP dependent coa ligase
up-regulated	miR-252-5p miR-2951-3p	AAEL000339	lim domain
up-regulated	miR-252-5p miR-2951-3p	AAEL000343	serine/threonine-protein kinase vrk
up-regulated	miR-375	AAEL000540	fasciclin, putative
present	miR-2941		
up-regulated	miR-989 miR-10-3p miR-281-5p	AAEL000599	wingless protein, putative
present	miR-2951-5p		
up-regulated	miR-2951-3p miR-275	AAEL000641	protein disulfide isomerase
down-regulated	miR-125-5p miR-308-5p	AAEL000661	t-cell specific transcription factor, tcf
up-regulated	miR-375 miR-210-3p	AAEL000704	synaptotagmin
up-regulated	miR-989 miR-305-3p	AAEL001108	protein kinase c
up-regulated	miR-989	AAEL001549	protein kinase c
present	miR-2951-5p		
up-regulated	miR-210-3p miR-957	AAEL001673	actin
up-regulated	miR-252-5p	AAEL001766	leucine-rich transmembrane proteins
present	miR-2941 miR-2951-5p		

up-regulated	miR-375 miR-210-3p	AAEL002550	polyA-binding protein interacting protein, putative
up-regulated	miR-2951-5p miR-281-5p	AAEL002587	OBP11: odorant binding protein
up-regulated	miR-375 miR-305-3p	AAEL002594	P21-activated kinase, pak
up-regulated	miR-305-3p miR-2951-3p	AAEL002904	juvenile hormone-inducible protein, putative
down-regulated	miR-125-5p miR-308-5p	AAEL002972	brain chitinase and chia

We found 16 miRNAs that were differentially expressed from midguts that were DENV-2 infected relative to the unexposed midguts. Of the 16 miRNAs, 7 were up-regulated and 9 were down-regulated additionally 1 miRNA was present in the infected but not in the unexposed midguts (Table 3.6). The predicted targets of the miRNAs modulated by DENV-2 infection are listed in table 3.7. As above, some of the miRNAs targeted the same gene and those are included in table 3.8.

Some of the miRNAs were shared in both, the DENV-2 exposed and DENV-2 infected groups. We found 7 up-regulated and 6 down regulated miRNAs that were shared in the DENV-2 exposed and DENV-2 infected groups. Also one miRNA was found to be present in both of them. Therefore, 5 up-regulated, 1 down-regulated and 1 present miRNAs were uniquely modulated in the DENV-2 exposed midguts. In the DENV-2 infected midguts, 3 miRNAs were uniquely down-regulated (Fig. 3.3). The miRNAs 989, 252 and 281 have been reported to be differentially expressed in mosquito cells or whole mosquitoes upon DENV-2 exposure/infection and those were found in this study, this finding is discussed below.

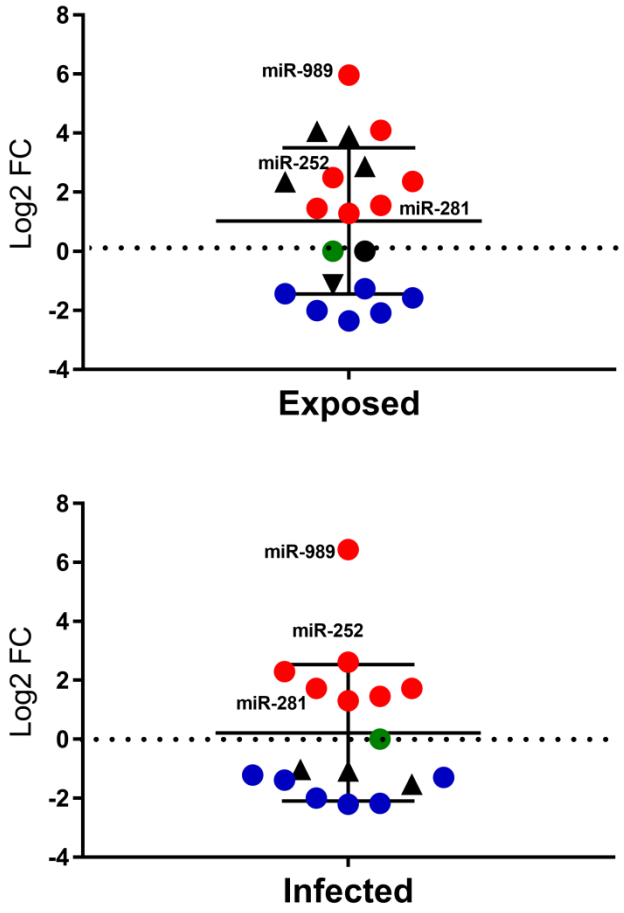

Figure 3.3. Differentially expressed miRNAs in the DENV-2 exposed and DENV-2 infected midguts. Black dots represent unique miRNAs. Red dots represent shared up-regulated miRNAs in, blue dots represent shared down-regulated and green dots represent shared present miRNAs in both groups.

The unique miRNAs found in the DENV-2 infected group were miR-137, miR-278-5p, miR-and miR-927 which were down-regulated. For the uniquely miRNAs found in the DENV-2 exposed group miR-210.-3p, miR-10-3p, miR-957 and miR-2951-3p were up-regulated, 2951-5p was present and miR-998 was down-regulated.

Table 3.6. List of differentially expressed miRNAs in *Ae. aegypti* midguts infected with DENV-2.

miRNA	**log2FC**	***p* value**	**FDR**	**miRNA sequence**
miR-989	6.434348	7.85E-10	1.39E-08	TGTGATGTGACGTAGTGGTAC
miR-252-5p	2.61148	8.34E-12	2.28E-10	CTAAGTACTAGTGCCGCAGGAG
miR-305-3p	2.297252	0.000291	0.010528	CGGCACATGTTGGAGTACACTTA

miR-281-1-5p	1.727969	7.10E-17	1.50E-15	AAAGAGAGCTGTCCGTCGACAGT
miR-375	1.726242	3.52E-07	5.52E-06	TTTGTTCGTTTGGCTCGAGTTA
miR-276b	1.457447	2.06E-05	0.000235	AAAACCGAAGTCTTTTTACCATC AGCGAGGTATAGAGTTCCTACGT TCCTATATTCAGTCGTAGGAACTTA ATACCGTGCTCTTGGAGGACTGTCGACC
miR-275-3p	1.308627	0.000211	0.008164	TCAGGTACCTGAAGTAGCGC
miR-2941	Present	6.95E-24	2.73E-22	TAGTACGGCTAGAACTCCACGG
miR-137	-1.02079	0.004782	0.022634	TTCATCGAGCAACTTGGTTGGCCA CGCGTATTCTTGGGTTATTAACACA CTGTTTATGTTGTTATTGCTTGAGAAT ACACGTAGTTGACAAGTGTTTCACATCG
miR-278-5p	-1.07118	4.38E-05	0.008164	ACGGACGATAGTCTTCAGCGGCC
miR-308-5p	-1.21508	4.62E-11	9.06E-10	CGCGGTATATTCTTGTGGCTTG
miR-13-5p	-1.29835	5.70E-06	8.69E-05	TCGTAAAAATGGTTGTGCTGTG
miR-125-5p	-1.38194	9.18E-09	1.57E-07	TCCCTGAGACCCTAACTTGTGA
miR-927	-1.52948	0.001409	0.038248	TTTAGAATTCCTACGCTTTACC
bantam	-1.9959	0.000579	0.007538	AGAACCGGTTTTCATTTTCGATCTGA CTTATTTGATTTAACAAGAGTGAGAT CATTTTGAAAGCTGATTT
miR-11-5p	-2.17188	4.51E-13	9.17E-12	CGAGAACTCCGGCTGTGACC
miR-100	-2.20453	7.99E-74	1.46E-71	AACCCGTAGATCCGAACTTGTG

Up-regulated miRNAs are shown in red. Down-regulated miRNAs are shown in blue. Present miRNAs are shown in black. Unique miRNAs in the exposed group are shown in bold. miRNA modulation was obtained from midguts exposed to DENV-2 at 14 dpe. FDR stands for false discovery rate.

Table 3.7. Predicted targets of the differentially expressed microRNAs in DENV-2 infected (MEB-) midguts.

miRNA	**Gene ID**	**Target description**
miR-989	AAEL000567	Tret1: facilitated trehalose transporter
miR-989	AAEL001612	dicer-1
miR-989	AAEL001935	CTL-like protein 1
miR-989	AAEL003505	jun
miR-989	AAEL001549	protein kinase c
miR-989	AAEL000188	elongase, putative
miR-989	AAEL002969	brain chitinase and chia
miR-989	AAEL002809	down syndrome critical region protein
miR-989	AAEL002692	beat protein
miR-989	AAEL002261	GTP cyclohydrolase i
miR-989	AAEL000088	brefeldin A-sensitive peripheral Golgi protein, putative
miR-989	AAEL000713	reticulon/nogo
miR-989	AAEL001946	four and a half lim domains
miR-989	AAEL002549	sosie: protein sosie

miR-989	AAEL001108	protein kinase c
miR-989	AAEL001715	chaperonin
miR-989	AAEL000599	wingless protein, putative
miR-989	AAEL001433	fgf receptor activating protein
miR-989	AAEL001126	rest corepressor (corest) protein
miR-252-5p	AAEL001856	adenosine kinase
miR-252-5p	AAEL000339	lim domain
miR-252-5p	AAEL001766	leucine-rich transmembrane proteins
miR-252-5p	AAEL000343	serine/threonine-protein kinase vrk
miR-252-5p	AAEL001916	eukaryotic translation initiation factor 4e
miR-252-5p	AAEL001796	Nuclear hormone receptor (HR78)
miR-252-5p	AAEL000577	DNA binding protein elf-1
miR-305-3p	AAEL000126	molybdopterin cofactor synthesis protein a
miR-305-3p	AAEL000558	neural stem cell-derived dendrite regulator
miR-305-3p	AAEL000925	leucine-zipper-like transcriptional regulator 1 (LZTR-1)
miR-305-3p	AAEL001016	zinc finger protein
miR-305-3p	AAEL000278	poly(p)/ATP NAD kinase
miR-305-3p	AAEL002594	P21-activated kinase, pak
miR-305-3p	AAEL001334	geranylgeranyl transferase type ii beta subunit
miR-305-3p	AAEL000770	platelet-activating factor acetylhydrolase isoform 1b alpha subunit
miR-305-3p	AAEL001108	protein kinase c
miR-305-3p	AAEL002372	RpS11: 40S ribosomal protein S11
miR-305-3p	AAEL002904	juvenile hormone-inducible protein, putative
miR-305-3p	AAEL001964	protein serine/threonine kinase, putative
miR-305-3p	AAEL000374	cysteine-rich venom protein, putative
miR-281-5p	AAEL002422	cytoplasmic polyadenylation element binding protein (cpeb)
miR-281-5p	AAEL001963	protein serine/threonine kinase, putative
miR-281-5p	AAEL002587	OBP11: odorant binding protein
miR-281-5p	AAEL000034	meiotic recombination repair protein 11 (mre11)
miR-281-5p	AAEL000599	wingless protein, putative
miR-281-5p	AAEL002779	ribokinase
miR-281-5p	AAEL002723	peroxisomal membrane protein pmp34
miR-281-5p	AAEL001622	dual specificity mitogen-activated protein kinase kinase MAPKK
miR-281-5p	AAEL001641	deoxyribonuclease I, putative
miR-281-5p	AAEL002422	cytoplasmic polyadenylation element binding protein (cpeb)
miR-281-5p	AAEL001963	protein serine/threonine kinase, putative
miR-281-5p	AAEL002587	OBP11: odorant binding protein
miR-281-5p	AAEL000034	meiotic recombination repair protein 11 (mre11)
miR-281-5p	AAEL000599	wingless protein, putative
miR-281-5p	AAEL002779	ribokinase
miR-281-5p	AAEL002723	peroxisomal membrane protein pmp34

miR-281-5p	AAEL001622	dual specificity mitogen-activated protein kinase kinase MAPKK
miR-375	AAEL001159	ribonuclease t2
miR-375	AAEL002550	polyA-binding protein interacting protein, putative
miR-375	AAEL001232	tubulointerstitial nephritis antigen
miR-375	AAEL001938	ATP-binding cassette sub-family A member 3, putative
miR-375	AAEL000540	fasciclin, putative
miR-375	AAEL002277	cAMP-dependent protein kinase type i-beta regulatory subunit
miR-375	AAEL000704	synaptotagmin
miR-375	AAEL002329	alpha-1,3-mannosyl-glycoprotein beta-1, 2-n-acetylglucosaminyltransferase
miR-375	AAEL002973	zinc finger protein
miR-375	AAEL001467	sdk-P1
miR-375	AAEL002594	P21-activated kinase, pak
miR-375	AAEL001239	cation efflux protein/ zinc transporter
miR-375	AAEL001952	28 kDa heat- and acid-stable phosphoprotein (PDGF-associated protein), putative
miR-375	AAEL002804	oligosaccharyl transferase
miR-375	AAEL000291	V-type proton ATPase 16 kDa proteolipid subunit
miR-375	AAEL000064	dopachrome-conversion enzyme (DCE) isoenzyme, putative
miR-375	AAEL002135	tubulin-specific chaperone b (tubulin folding cofactor b)
miR-375	AAEL001698	charged multivesicular body protein 4b
miR-375	AAEL001933	membrane associated ring finger 1,8
miR-375	AAEL002280	ctl2
miR-375	AAEL001317	DEAD box ATP-dependent RNA helicase
miR-275	AAEL001159	ribonuclease t2
miR-275	AAEL000964	regulatory factor X-associated ankyrin-containing protein, putative
miR-275	AAEL000641	protein disulfide isomerase
miR-275	AAEL001467	sdk-P1
miR-275	AAEL000817	rhomboid
miR-275	AAEL000088	brefeldin A-sensitive peripheral Golgi protein, putative
miR-275	AAEL000813	dimethylaniline monooxygenase
miR-275	AAEL002194	uricase
miR-275	AAEL001933	membrane associated ring finger 1,8
miR-275	AAEL000101	AMP dependent coa ligase
miR-275	AAEL002714	kinesin-like protein KIF23 (mitotic kinesin-like protein 1)
miR-2941	AAEL001523	secretory Phospholipase A2, putative
miR-2941	AAEL001091	malic enzyme
miR-2941	AAEL000540	fasciclin, putative
miR-2941	AAEL002214	amino acid transporter
miR-2941	AAEL001963	protein serine/threonine kinase, putative
miR-2941	AAEL005730	cop9 signalosome complex subunit
miR-2941	AAEL002287	trans-prenyltransferase

miR-2941	AAEL001766	leucine-rich transmembrane proteins
miR-2941	AAEL001574	septin
miR-2941	AAEL002906	26S proteasome regulatory subunit rpn2
miR-278-5p	AAEL000053	myotubularin
miR-278-5p	AAEL005552	succinyl-coa synthetase beta chain
miR-278-5p	AAEL002938	skd/vacuolar sorting
miR-278-5p	AAEL000679	NEDD8, putative
miR-278-5p	AAEL001963	protein serine/threonine kinase, putative
miR-278-5p	AAEL002973	zinc finger protein
miR-278-5p	AAEL001165	ras GTP exchange factor, son of sevenless
miR-278-5p	AAEL001046	phosphatase fragment
miR-278-5p	AAEL002282	zinc finger protein
miR-278-5p	AAEL001303	Or49: odorant receptor
miR-278-5p	AAEL002469	endophilin a
miR-278-5p	AAEL002135	tubulin-specific chaperone b
miR-278-5p	AAEL001433	fgf receptor activating protein
miR-278-5p	AAEL000435	THO complex, putative
miR-278-5p	AAEL001087	synaptic vesicle protein
miR-278-5p	AAEL002877	kinesin-like protein KIF17
miR-278-5p	AAEL000800	microsomal dipeptidase
miR-308-5p	AAEL002972	brain chitinase and chia
miR-308-5p	AAEL001159	ribonuclease t2
miR-308-5p	AAEL000091	sumo-1-activating enzyme E1a
miR-308-5p	AAEL000126	molybdopterin cofactor synthesis protein a
miR-308-5p	AAEL002395	Cdk8: Cyclin-dependent kinase
miR-308-5p	AAEL001194	fatty acid synthase
miR-308-5p	AAEL001963	protein serine/threonine kinase, putative
miR-308-5p	AAEL000661	t-cell specific transcription factor, tcf
miR-13-5p	AAEL001935	CTL-like protein 1
miR-13-5p	AAEL001088	beta-1,3-galactosyltransferase
miR-13-5p	AAEL002077	UV excision repair protein rad23
miR-13-5p	AAEL001126	rest corepressor (corest) protein
miR-125-5p	AAEL000014	cyclic-nucleotide-gated cation channel
miR-125-5p	AAEL000037	CLIPB35: Clip-Domain Serine Protease family B.
miR-125-5p	AAEL000190	trypsin
miR-125-5p	AAEL000263	Zinc finger protein jing homolog
miR-125-5p	AAEL000389	brain chitinase and chia
miR-125-5p	AAEL000486	chaperonin
miR-125-5p	AAEL000656	pangolin
miR-125-5p	AAEL000661	t-cell specific transcription factor, tcf
miR-125-5p	AAEL000828	vitellogenin,, putative

miR-125-5p	AAEL000906	guanyl-nucleotide exchange factor
miR-125-5p	AAEL000971	smile protein
miR-125-5p	AAEL001046	phosphatase fragment
miR-125-5p	AAEL001069	histone deacetylase
miR-125-5p	AAEL001102	adenosine kinase
miR-125-5p	AAEL001165	ras GTP exchange factor, son of sevenless
miR-125-5p	AAEL001177	signal recognition particle, 14kD, putative
miR-125-5p	AAEL001196	cadherin
miR-125-5p	AAEL001218	alanyl-tRNA synthetase
miR-125-5p	AAEL001260	Med20: mediator of RNA polymerase II transcription subunit 20
miR-125-5p	AAEL001312	CYP9M6: cytochrome P450
miR-125-5p	AAEL001440	E1a binding protein P400
miR-125-5p	AAEL001548	glucosyl/glucuronosyl transferases
miR-125-5p	AAEL001586	glucosyl/glucuronosyl transferases
miR-125-5p	AAEL001629	cAMP-specific 3,5-cyclic phosphodiesterase
miR-125-5p	AAEL001662	kinesin family member 21A
miR-125-5p	AAEL001711	activin receptor type I, putative
miR-125-5p	AAEL001732	candidate tumor suppressor protein
miR-125-5p	AAEL001830	geranylgeranyl transferase type i beta subunit
miR-125-5p	AAEL001894	kek1
miR-125-5p	AAEL001896	UDP-N-acetylglucosamine: polypeptide-N-acetylglucosaminyl transferase, putative
miR-125-5p	AAEL001933	membrane associated ring finger 1,8
miR-125-5p	AAEL002204	fatty acid synthase
miR-125-5p	AAEL002227	fatty acid synthase
miR-125-5p	AAEL002266	p15-2b protein, putative
miR-125-5p	AAEL002280	ctl2
miR-125-5p	AAEL002295	leucine-rich transmembrane protein
miR-125-5p	AAEL002306	hect E3 ubiquitin ligase
miR-125-5p	AAEL002307	leucine-rich transmembrane protein
miR-125-5p	AAEL002441	larval cuticle protein, putative
miR-125-5p	AAEL002458	pupal cuticle protein, putative
miR-125-5p	AAEL002468	lipid a export ATP-binding/permease protein msba
miR-125-5p	AAEL002539	fimbrin/plastin
miR-125-5p	AAEL002603	triacylglycerol lipase, putative
miR-125-5p	AAEL002655	matrix metalloproteinase
miR-125-5p	AAEL002683	aldehyde oxidase
miR-125-5p	AAEL002769	homeobox protein prospero/prox-1
miR-125-5p	AAEL002911	lysosomal acid lipase, putative
miR-125-5p	AAEL002972	brain chitinase and chia
miR-125-5p	AAEL003123	deoxyribonuclease I, putative
miR-125-5p	AAEL003125	acyl-coa dehydrogenase

miR-125-5p	AAEL003129	neuroligin
miR-125-5p	AAEL003155	dynein heavy chain
miR-125-5p	AAEL003308	trypsin, putative
miR-125-5p	AAEL003632	CLIPB39: Clip-Domain Serine Protease family B.
miR-125-5p	AAEL003758	sorting nexin
miR-125-5p	AAEL003763	CYP329B1: cytochrome P450
miR-125-5p	AAEL003960	arylsulfatase b
miR-125-5p	AAEL003990	myeloid leukemia factor
miR-125-5p	AAEL004009	glucose dehydrogenase
miR-125-5p	AAEL004124	mitochondrial glutamate carrier, putative
miR-125-5p	AAEL004291	translin
miR-125-5p	AAEL004573	delta(9)-desaturase 2, putative
miR-125-5p	AAEL004661	beta-hexosaminidase
miR-125-5p	AAEL004716	chromodomain helicase DNA binding protein
miR-125-5p	AAEL004750	nonmuscle myosin heavy chain-A, putative
miR-125-5p	AAEL004833	DPT1: diptericin anti-microbial peptide
miR-125-5p	AAEL004866	protein farnesyltransferase beta subunit
miR-125-5p	AAEL004925	lysosomal acid lipase, putative
miR-125-5p	AAEL005166	eyes absent
miR-125-5p	AAEL005175	lipin
miR-125-5p	AAEL005276	target of myb1 (tom1)
miR-125-5p	AAEL005321	neurexin iv
miR-125-5p	AAEL005324	titin
miR-125-5p	AAEL005341	hk: Protein hook
miR-125-5p	AAEL005387	WD-repeat protein
miR-125-5p	AAEL005533	synaptic vesicle protein
miR-125-5p	AAEL005732	acyl-coa dehydrogenase
miR-125-5p	AAEL005742	transcription initiation factor TFIID subunit 10, putative
miR-125-5p	AAEL005833	cytosolic purine 5-nucleotidase
miR-125-5p	AAEL005921	D-lactate dehydrognease 2,
miR-125-5p	AAEL006157	aldehyde oxidase
miR-125-5p	AAEL006240	purple acid phosphatase, putative
miR-125-5p	AAEL006297	venom allergen
miR-125-5p	AAEL006457	asparagine synthetase
miR-125-5p	AAEL006640	DEAD box ATP-dependent RNA helicase
miR-125-5p	AAEL006824	cytochrome P450
miR-125-5p	AAEL007041	low-density lipoprotein receptor (ldl)
miR-125-5p	AAEL007060	lipase
miR-125-5p	AAEL007216	elongase, putative
miR-125-5p	AAEL007225	dynein heavy chain
miR-125-5p	AAEL007235	mitochondrial uncoupling protein

miR-125-5p	AAEL007322	phosphatidate phosphatase
miR-125-5p	AAEL007412	sphingomyelin phosphodiesterase
miR-125-5p	AAEL007542	glutamate decarboxylase
miR-125-5p	AAEL007597	CLIPC3: Clip-Domain Serine Protease family C
miR-125-5p	AAEL007656	receptor for activated C kinase, putative
miR-125-5p	AAEL007657	low-density lipoprotein receptor (ldl)
miR-125-5p	AAEL007658	partitioning defective 3, par-3
miR-125-5p	AAEL007689	phospholipid-transporting ATPase 1 (aminophospholipid flippase 1)
miR-125-5p	AAEL007760	mct-1 protein
miR-125-5p	AAEL007762	mRpL40: mitochondrial ribosomal protein, L40, putative
miR-125-5p	AAEL007898	calmin
miR-125-5p	AAEL007924	GPRNPY3: GPCR Neuropeptide Y Family
miR-125-5p	AAEL007925	histone H2A
miR-125-5p	AAEL007938	serine-type enodpeptidase
miR-125-5p	AAEL008035	ATP-dependent RNA helicase
miR-125-5p	AAEL008114	p15-2b protein, putative
miR-125-5p	AAEL008345	CYP4G35: cytochrome P450
miR-125-5p	AAEL008508	translin
miR-125-5p	AAEL008595	Protein maelstrom homolog
miR-125-5p	AAEL008620	D7 protein, putative
miR-125-5p	AAEL008685	lim homeobox protein
miR-125-5p	AAEL008688	G-protein signalling modulator
miR-125-5p	AAEL008715	AAA ATPase
miR-125-5p	AAEL008773	laminin A chain, putative
miR-125-5p	AAEL008866	pupal cuticle protein 78E, putative
miR-125-5p	AAEL008873	pupal cuticle protein 78E, putative
miR-125-5p	AAEL008889	CYP6AL1: cytochrome P450
miR-125-5p	AAEL009149	kinectin, putative
miR-125-5p	AAEL009192	SCRASP1: Class A Scavenger Receptor (SRCR domain) with Serine Protease domain.
miR-125-5p	AAEL009200	Exo3: exocyst complex component 3
miR-125-5p	AAEL009353	ssm4 protein
miR-125-5p	AAEL009555	Niemann-Pick Type C-2, putative
miR-125-5p	AAEL009579	NBP2b protein, putative
miR-125-5p	AAEL009676	glyoxylate/hydroxypyruvate reductase
miR-125-5p	AAEL009806	low-density lipoprotein receptor (ldl)
miR-125-5p	AAEL009850	GALE14: galectin
miR-125-5p	AAEL009855	sodium/dicarboxylate cotransporter, putative
miR-125-5p	AAEL009886	CCC3: sodium-coupled cation-chloride cotransporter
miR-125-5p	AAEL010108	sex-determining protein fem-1
miR-125-5p	AAEL010210	neurogenic locus notch (notch)

miR-125-5p	AAEL010269	venom allergen
miR-125-5p	AAEL010336	zinc phosphodiesterase
miR-125-5p	AAEL010351	phosphopentothenoylcysteine decarboxylase
miR-125-5p	AAEL010354	homeobox protein nk-2
miR-125-5p	AAEL010356	phosphopentothenoylcysteine decarboxylase
miR-125-5p	AAEL010414	set domain protein
miR-125-5p	AAEL010502	transcriptional regulator ATRX (X-linked helicase II)
miR-125-5p	AAEL010513	class b basic helix-loop-helix protein (bhlhb) (differentially expressed in chondrocytes) (mdec) (sharp)
miR-125-5p	AAEL010585	spermatogenesis associated factor
miR-125-5p	AAEL010606	down syndrome cell adhesion molecule
miR-125-5p	AAEL010630	xanthine dehydrogenase
miR-125-5p	AAEL010803	ncd
miR-125-5p	AAEL010939	group ii plp decarboxylase
miR-125-5p	AAEL011013	single-minded
miR-125-5p	AAEL011078	CTLGA1: C-Type Lectin (CTL) - galactose binding.
miR-125-5p	AAEL011247	arrowhead
miR-125-5p	AAEL011415	lysine-specific histone demethylase
miR-125-5p	AAEL011528	triacylglycerol lipase, putative
miR-125-5p	AAEL011566	cell adhesion molecule
miR-125-5p	AAEL011729	Molybdenum cofactor sulfurase 3
miR-125-5p	AAEL011755	integral membrane protein, Tmp21-I (p23), putative
miR-125-5p	AAEL012037	sulphate transporter
miR-125-5p	AAEL012045	gar2, putative
miR-125-5p	AAEL012083	receptor protein-tyrosine phosphatase 10d
miR-125-5p	AAEL012110	protease m1 zinc metalloprotease
miR-125-5p	AAEL012152	activin receptor type I, putative
miR-125-5p	AAEL012192	ATP-binding cassette transporter
miR-125-5p	AAEL012345	lipase 1 precursor
miR-125-5p	AAEL012364	spermatogenesis associated factor
miR-125-5p	AAEL012386	ATP-binding cassette transporter
miR-125-5p	AAEL012421	cadherin
miR-125-5p	AAEL012673	ubiquitin conjugating enzyme 7 interacting protein
miR-125-5p	AAEL012687	juvenile hormone-inducible protein, putative
miR-125-5p	AAEL012717	WD-repeat protein
miR-125-5p	AAEL012730	dbl
miR-125-5p	AAEL012826	replication factor a 1, rfa1
miR-125-5p	AAEL012956	elastase, putative
miR-125-5p	AAEL013089	proteasome subunit alpha type
miR-125-5p	AAEL013111	glutamate transporter
miR-125-5p	AAEL013112	PGRPLE: Peptidoglycan Recognition Protein (Long)
miR-125-5p	AAEL013274	n-acetylgalactosaminyltransferase

miR-125-5p	AAEL013284	LT1: late trypsin 1, serine-type enodpeptidase
miR-125-5p	AAEL013372	ABC transporter
miR-125-5p	AAEL013697	condensin, SMC5-subunit, putative
miR-125-5p	AAEL013752	rfx5
miR-125-5p	AAEL013765	arrowhead
miR-125-5p	AAEL013873	cadherin
miR-125-5p	AAEL013896	smad4
miR-125-5p	AAEL014021	Med23: mediator of RNA polymerase II transcription subunit 23
miR-125-5p	AAEL014069	catrin, putative
miR-125-5p	AAEL014110	sulfite reductase
miR-125-5p	AAEL014134	kinesin heavy chain
miR-125-5p	AAEL014139	proacrosin, putative
miR-125-5p	AAEL014195	G-protein signalling modulator
miR-125-5p	AAEL014222	low-density lipoprotein receptor (ldl)
miR-125-5p	AAEL014252	sulfite reductase
miR-125-5p	AAEL014303	neuroligin,
miR-125-5p	AAEL014381	Molybdenum cofactor sulfurase 1
miR-125-5p	AAEL014578	ssm4 protein
miR-125-5p	AAEL014594	CYP301A1: cytochrome P450
miR-125-5p	AAEL014906	LAP4 protein, putative (Scribble protein, putative)
miR-125-5p	AAEL014916	lipase 1 precursor
miR-125-5p	AAEL015151	acid phosphatase
miR-125-5p	AAEL015283	synaptic vesicle protein
miR-125-5p	AAEL015458	transferrin
miR-125-5p	AAEL015639	transferrin
miR-125-5p	AAEL018292	HPX4: heme peroxidase
miR-125-5p	AAEL018680	ND4: NADH dehydrogenase subunit 4
miR-927	AAEL000060	Gr7: gustatory receptor
miR-927	AAEL000342	peroxidasin
miR-927	AAEL001518	zinc finger protein
miR-927	AAEL001705	odorant response protein ODR-4, putative
miR-927	AAEL001982	endoplasmic reticulum-resident kdel protein
miR-927	AAEL002401	proteasome subunit beta type
miR-927	AAEL002993	mRpL43: mitochondrial ribosomal protein L43
miR-927	AAEL003053	allergen, putative
miR-927	AAEL004064	meiotic checkpoint regulator cut4
miR-927	AAEL004200	DNA replication licensing factor MCM8
miR-927	AAEL004247	Sialin, Sodium/sialic acid cotransporter, putative
miR-927	AAEL005071	GTP binding protein
miR-927	AAEL005454	AAA ATPase
miR-927	AAEL005513	mothers against dpp protein

miR-927	AAEL005754	cgmp-dependent protein kinase
miR-927	AAEL005990	adrenodoxin reductase, putative
miR-927	AAEL006539	serine/threonine protein kinase
miR-927	AAEL006733	FAD NADPH dehydrogenase
miR-927	AAEL007061	cyclophilin
miR-927	AAEL007326	ccr4-not transcription complex
miR-927	AAEL007375	pyruvate dehydrogenase
miR-927	AAEL007796	CLIPD1: Clip-Domain Serine Protease family D
miR-927	AAEL007898	calmin
miR-927	AAEL008103	RpS8: 40S ribosomal protein S8
miR-927	AAEL008701	myoinositol oxygenase
miR-927	AAEL009723	fibrinogen and fibronectin
miR-927	AAEL009925	amidase
miR-927	AAEL011006	guanylate kinase
miR-927	AAEL012112	ubiquitin conjugating enzyme 7 interacting protein
miR-927	AAEL012607	fetal alzheimer antigen, falz
miR-927	AAEL013263	high affinity copper transporter, putative
miR-927	AAEL013572	N-acetylgalactosaminyltransferase I, putative
miR-927	AAEL013767	ccr4-not transcription complex
miR-927	AAEL014066	ubiquitin conjugating enzyme 7 interacting protein
miR-927	AAEL014510	sprouty
miR-927	AAEL014733	nuclear pore complex protein nup214
miR-927	AAEL014853	otoferlin
miR-927	AAEL015015	fetal alzheimer antigen, falz
miR-927	AAEL015305	sulfotransferase (sult)
miR-927	AAEL016210	tRNA-Lys
miR-927	AAEL016211	tRNA-Lys
miR-927	AAEL016212	tRNA-Lys
miR-927	AAEL016214	tRNA-Lys
miR-927	AAEL016217	tRNA-Lys
miR-927	AAEL016273	tRNA-Lys
miR-927	AAEL016354	tRNA-Lys
miR-927	AAEL016691	tRNA-Lys
miR-927	AAEL016892	tRNA-Lys
miR-927	AAEL016931	tRNA-Lys
miR-927	AAEL017879	mir-927: microRNA mir-927
miR-927	AAEL018292	HPX4: heme peroxidase
miR-927	AAEL018418	mir-927: microRNA mir-927
Bantam	AAEL001279	merozoite surface protein, putative
Bantam	AAEL001963	protein serine/threonine kinase, putative
Bantam	AAEL000088	brefeldin A-sensitive peripheral Golgi protein, putative

Bantam	AAEL002478	double-stranded binding protein, putative
Bantam	AAEL001928	Act1: actin-1
miR-11-5p	AAEL001963	protein serine/threonine kinase, putative
miR-11-5p	AAEL002551	DNA topoisomerase type I
miR-11-5p	AAEL000219	lactoylglutathione lyase
miR-11-5p	AAEL002714	kinesin-like protein KIF23 (mitotic kinesin-like protein 1)
miR-100	AAEL002412	monocarboxylate transporter
miR-100	AAEL000126	molybdopterin cofactor synthesis protein a
miR-100	AAEL001467	sdk-P1
miR-100	AAEL001930	pra1 protein
miR-100	AAEL000291	V-type proton ATPase 16 kDa proteolipid subunit
miR-100	AAEL002294	sulphate transporter
miR-100	AAEL001372	sentrin/sumo-specific protease senp7

Differential expression was determined from *Ae. aegypti* midguts infected with DENV-2 at 14 dpi. Targets with not known function, duplicate genes and multiple transcripts within each miRNA were eliminated. Target genes for each miRNA were obtained from miRanda.

Table 3.8. Multiple targeted genes by different miRNAs expressed in DENV-2 infected (MEB-) midguts

miRNA expression	**miRNA**	**Gene ID**	**Target description**
up-regulated	miR-375	AAEL000540	fasciclin, putative
present	miR-2941		
up-regulated	miR-989 miR-281-5p	AAEL000599	wingless protein, putative
down-regulated	miR-308-5p miR-125-5p	AAEL000661	t-cell specific transcription factor, tcf
down-regulated	miR-278-5p miR-125-5p	AAEL001046	phosphatase fragment
up-regulated	miR-989 miR-305-3p	AAEL001108	protein kinase c
down-regulated	miR-278-5p miR-125-5p	AAEL001165	ras GTP exchange factor, son of sevenless
up-regulated	miR-252-5p	AAEL001766	leucine-rich transmembrane proteins
present	miR-2941		
up-regulated	miR-305-3p miR-375	AAEL002594	P21-activated kinase, pak
down-regulated	miR-308-5p miR-125-5p	AAEL002972	brain chitinase and chia
down-regulated	miR-125-5p miR-927	AAEL007898	calmin
down-regulated	miR-125-5p miR-927	AAEL018292	HPX4: heme peroxidase

Discussion

The purpose of this analysis was to characterize those miRNAs that are associated with a persistent DENV-2 infection (MEB-) phenotype at 14 dpi. Our analysis included a DENV-2 exposed group and a DENV-2 infected group. The exposed group included exposed midguts regardless of their infection status (infected or uninfected) whilst the infected group consisted of only DENV-2 infected midguts. Since the infection status was determined from DENV- 2 disseminated mosquito tissues (legs), the infection group serves as a proxy for the lack of midgut escape barrier (MEB-).

The identification of 3 down-regulated miRNAs in DENV-2 infected midguts at 14 dpi is consistent with a scenario wherein the virus sets the expression of certain miRNAs that may help it to maintain a persistent DENV-2 infection in the midgut and to favor dissemination. While in the exposed group the miRNAs may be involved in limiting midgut infection and/or restricting the virus to the midgut of the mosquito.

Previous studies have reported miRNAs modulated by DENV infection in mosquito derived cells or whole mosquitoes earlier after exposure/infection. Variation in the modulation of the miRNAs is observed at different times post infection or exposure and in different species [277]. So, it is not surprising that the miRNAs found differ from what has been reported [230, 276, 294]. In addition, this study reports miRNAs modulated in the mosquito midgut, which is the first barrier for DENV infection [135].

In contrast with what was reported by Hess et al. 2011, we did not find DENV-2 viral RNAs (vi-RNAs)[295]. However, the absence of viRNAs has been observed in *Ae. albopictus* [277].

The previously reported miR-281-5p, miR-305-3p, [276] were found again in this study. Some of the differences between both studies included, that our samples were taken at a later time after infection, 14 days versus the 9 dpi from the previous report. Importantly, we determined infection by

RT-qPCR and used mosquito midguts instead of the whole mosquitoes with ~50% infection by plaque assay.

We found miR-375 and miR-275 to be up-regulated in the DENV-2 exposed and DENV-2 infected groups. The miR-375 was reported to be induced upon blood feeding and also its involvement enhancing DENV-2 infection in *Ae. aegypti* derived cells through the regulation of cactus and REL1 [296]. While miR-275 was reported to have an important role in blood digestion [271].

The miR-281 is an abundantly miRNA found in *Ae. albopictus* [278] and *Ae. aegypti* [297]. miR-281 was expressed in the midgut and was up-regulated upon DENV-2 infection [278] and was also found in *Culex quinquefasciatus* upon West Nile virus infection [298]. From the results of this study, miR-281 was up-regulated in the DENV-2 exposed and DENV-2 infected groups. Even when our miRNA analysis was from midguts after 14 days of infection and previous reports used a time course of less than 7 days [278, 297, 298].

We found miR-989 to be up-regulated in the DENV-2 exposed and DENV-2 infected groups. The miR-989-3p was reported as up-regulated in DENV-2 infected mosquitoes when compared with control mosquitoes [294]. The miR-989 was down-regulated in DENV-2 exposed *Ae. albopictus* midguts [277] and in *Cx. quinquefasciatus* mosquitoes infected with WNV [299]. In *Anopheles gambiae* the miRNA-989 was up-regulated during *Plasmodium* infection [300]. This might be suggestive of a pathogen dependent regulation.

Also, miR-210-3p was down-regulated in DENV-2 infected samples [294] and was found uniquely up-regulated in the DENV-2 exposed group but not in the DENV-2 infected group. Probably, miR-210-3p modulation has a role in restricting DENV-2 infection.

The miR-252 was reported to have an impact on DENV-2 replication by interacting with the E protein in a potential anti-viral manner [283]. We found an up-regulation of miR-252 in both infected

and exposed groups. Therefore, an anti-viral effect may have regulated DENV-2 infection even in persistent midgut infection after 14 dpi.

The dicer-1 was modulated by the up-regulated miR-989 in the exposed and infected groups. Dicer-1 is a key component of the miRNA pathway, so its targeting may be part of an auto-regulation mechanism.

Through the target prediction analysis, we found some genes that were targeted by multiple miRNAs. For example, synaptotagmin is a class of type-I membrane protein that regulates vesicle docking and fusion in processes such as exocytosis and phagocytosis [301, 302]. We found the miRNA that targets it to be down-regulated in the DENV-2 infected group, suggesting that synaptotagmin translation may be increased in persistently infected mosquitoes. High levels of synaptotagmins may be beneficial during virus infection by all the trafficking that occurs during virus infection.

From transcriptomic analysis it was found that the leucine rich transmembrane proteins (immune receptors) and cathepsin B were down-regulated in *Ae. albopictus* infected carcasses with DENV-2 [303]. We found up-regulated miRNAs that targeted leucine-rich transmembrane and cathepsin genes in *Ae. aegypti* DENV-2 exposed midguts indicating that their down-regulation of may occur also in *Ae. aegypti* and importantly mediated by the miRNA pathway.

This work had several limitations, the lines we used were low competent for DENV-2. So, the miRNAs found may have a role in the MIB. By comparing low and high competent lines this hypothesis could be further characterized. Specifically, lines with high and low MIRs may be useful to assess what is necessary to overcome the MIB. However, low to moderate competence is reported for DENV-2 and ZIKV in this dissertation, so the use of low competent lines may be similar to phenotypes of field populations.

The unique miRNAs differentially expressed upon DENV-2 exposure gave us information about antiviral responses or modulation required for DENV replication. While the miRNAs for the DENV-2

infected group, specifically give us information about requirements for the infection and dissemination of DENV-2 out of the midgut. However, the miRNAs found in this study have not been validated. In future studies the possible antiviral role of the up-regulation of miR-210-3p, miR-10-3p, miR-957, miR-2951-3p and down-regulation of miR-998 has to be assessed. In addition, the down-regulation of miR-137, miR-278-5p, and miR-927 in the DENV-2 replication and dissemination in mosquitoes that lack of a MEB should be further explored.

Based on the variability documented for the miRNA expression in different systems (mosquitoes and cell culture), times post infection/exposure and different host-pathogen pairs. The miRNA responses add a layer of complexity to the interaction between vector and pathogen. Specifically, we included miRNAs modulation results from *Ae. aegypti* midguts at 14 dpi, and also we suggest a number of miRNAs that may be implicated in the lack of MEB.

Recently, it was shown that DENV infection slightly affected miRNAs expression in Aag2 cells [286]. However, through the miRNA pathway many cellular processes are regulated, so future investigations may help us to find targets for mosquito control or blockage of DENV infection.

CHAPTER 4: VARIATION IN COMPETENCE FOR ZIKV TRANSMISSION BY *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* IS DEPENDENT ON SALIVARY GLAND INFECTION AND ESCAPE BARRIERS

Introduction

Zika virus (ZIKV, *Flavivirus*, *Flaviviridae*) was first isolated from a febrile sentinel rhesus macaque in the Zika forest of Uganda in 1947 and later in 1948 from *Aedes africanus* mosquitoes from the same area [20]. ZIKV circulated in Africa and Asia without much attention until 2007 when a major outbreak occurred in the Pacific Island of Yap in the Federate States of Micronesia [21, 304]. Outbreaks were later reported in other Pacific islands: French Polynesia, Easter Island, the Cook Islands and New Caledonia during 2013-2014 [23, 305, 306]. Making its arrival to the Americas in early 2015, ZIKV circulation was confirmed in Brazil in May and, as expected, ZIKV spread quickly to areas where the vectors were present. Mosquito-borne transmission has been reported in 48 countries of the Americas since its introduction [307]. In addition, ZIKV was associated with congenital abnormalities such as microcephaly and an increased incidence of Guillain-Barré syndrome, and was thus declared a Public Health Emergency of International Concern by the World Health Organization on February 1, 2016 [308], which ended nine months later [309]. Since its introduction, the Pan American Health Organization has reported more than 1 million cumulative Zika cases in the Americas and Mexico alone has a total of 128,624 cases [310], with its first case of congenital ZIKV syndrome in November of 2016 [39].

The main mechanism of ZIKV transmission in epidemic and endemic areas is through the bite of an infectious mosquito, with *Ae. aegypti* serving as the primary vector [18]. From the screening of wild-caught mosquitoes in Mexico, ZIKV RNA has been detected in *Ae. aegypti* pools collected in and around houses of suspected ZIKV cases [128]. *Aedes albopictus* [311] have also been confirmed to be infected with ZIKV.

Vectorial capacity is a quantitative measure of the potential of an arthropod vector to transmit a pathogen. It is defined as the average number of potentially infective bites that will ultimately be delivered by all the vectors feeding on a single host in 1 day [131]. Vectorial capacity is impacted by extrinsic factors like vector density, vector longevity, length of the extrinsic incubation period (EIP) and blood feeding behavior [132, 133] and also by intrinsic factors like VC. VC is defined as the intrinsic ability of an arthropod vector to acquire, maintain and then transmit a pathogen [134]. Upon intake, the arbovirus has to replicate and be able to be transmitted to a susceptible host in a subsequent feeding episode; however the virus has to first bypass a series of physiological barriers [135]. Briefly, upon entry of the virus into the mosquito gut through an infectious blood meal, the virus has to establish an infection; if this does not occur the mosquito has a midgut infection barrier (MIB). Next, the virus has to replicate and disseminate to other mosquito tissues; if this does not occur the mosquito has a midgut escape barrier (MEB). The virus then may infect several mosquito tissues but especially the salivary glands where it again has to establish an infection. If this is prevented the mosquito has a salivary gland infection barrier (SGIB). Next, the virus has to replicate and disseminate into the saliva secretions from where it will be expectorated with the saliva into a susceptible vertebrate host. If this is limited, the mosquito has a salivary gland escape barrier (SGEB) [135, 136]. In conjunction, the MIB, MEB, SGIB and SGEB contribute to the overall VC phenotype.

By harvesting mosquitoes at 7 and 14 days post infection (dpi) we can obtain potential indicators of establishment of infection and dissemination and/ or transmission respectively, for flaviviruses such as dengue virus (DENV) [172] and West Nile virus (WNV) [182]. Previous studies have reported low ZIKV transmission rates for the Asian lineage of ZIKV using mosquitoes from a wide geographical range from the Americas [144, 161]. Beside low transmission rates we hypothesized that VC is variable and is highly dependent upon the geographic origin of the mosquito populations. Hence, we analyzed the ZIKV transmission potential of recently colonized Aedes collections. A total of 13

collections, 10 of *Ae. aegypti* and 3 of *Ae. albopictus,* from different locations across Mexico were analyzed for ZIKV (strain PRVABC59 - Asian genotype) VC at 7 and 14 dpi. We found that both *Aedes* species were competent for ZIKV transmission and that VC varied by mosquito species, as well as by region and geographic location. A wide range of transmission rates, defined as ZIKV positive saliva out of the total bloodfed mosquitoes ranged from 2-51% at 7 dpi and from 8-51% at 14 dpi in *Ae. aegypti. Aedes albopictus* had from 0-8% transmission at 7 dpi and 2-26% at 14 dpi. We describe the contribution of each of the barriers for ZIKV transmission showing that a SGEB is the most important barrier to ZIKV transmission in *Ae. aegypti* populations while for *Ae. albopictus*, both SGIB and SGEB were important barriers to ZIKV transmission. In addition, we evaluated the contribution of *Ae. aegypti* and *Ae. albopictus* to ZIKV transmission in areas where their distributions overlap.

Methods

Mosquitoes

Aedes eggs were collected from ovitraps set at different locations in Mexico during 2016 with exception of the collections from the state of Chiapas (Huehuetan and Mazatan) where immature stages were obtained from at least 20 different containers (Figure 4.1 and table 4.1).

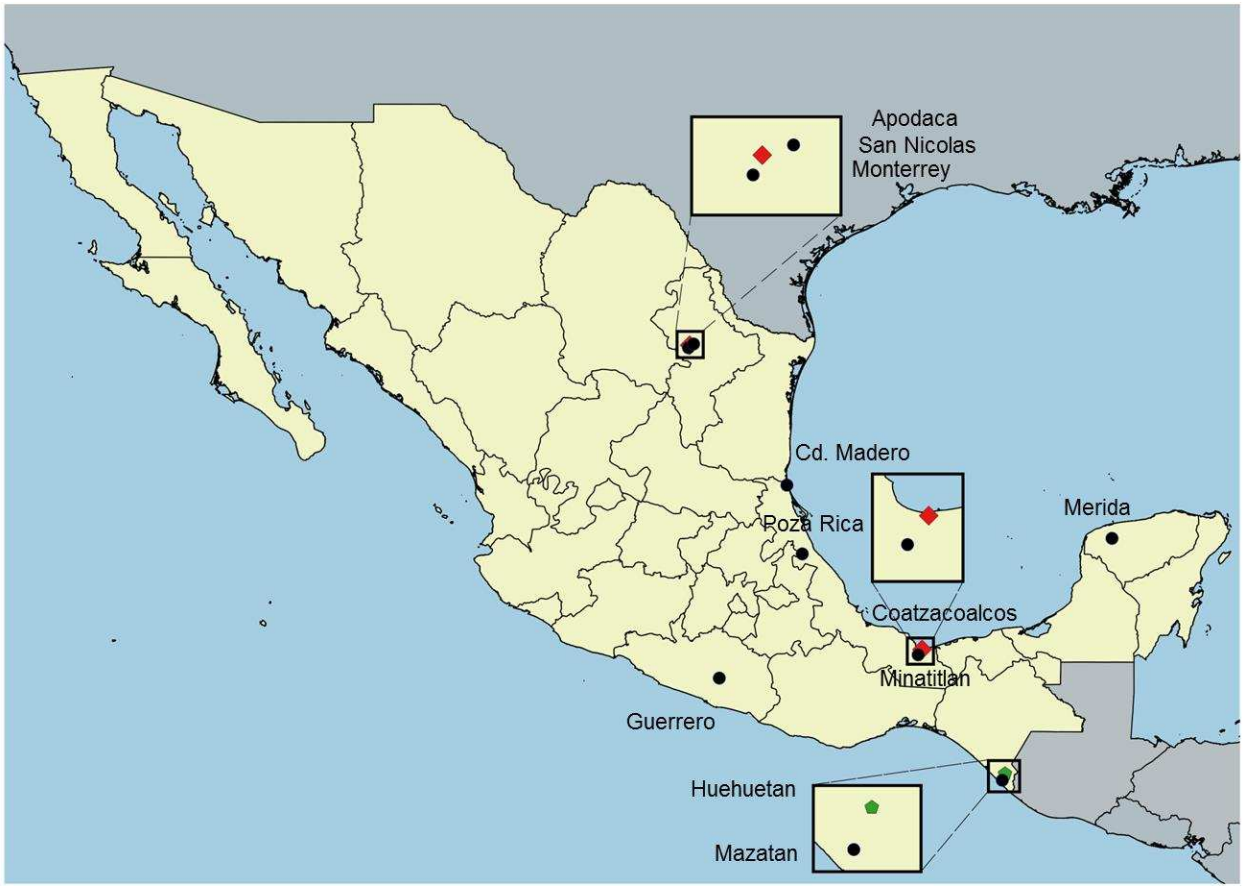

Figure 4.1. Map showing the collection sites of Aedes mosquitoes used in this study. Red diamonds indicate where both species were collected. Black dots depict *Ae. aegypti* collections and green dots indicates *Ae. albopictus* collections.

At each location where ovitraps were used, 4 to 5 were set and checked once a week. The eggs were dried and shipped to the laboratory at Colorado State University (PHS permit no. 2016-06-185), where they were hatched, reared to adults and then identified to species. Larvae were fed *ad libitum* with a 10% (w/v) liver powder solution. Adult mosquitoes were maintained on sucrose *ad libitum* and for egg production *citrated* sheep blood was given once a week through water-jacketed glass feeders using hog gut as a membrane. Adults were maintained at insectary conditions (28°C, 70% relative humidity and 12:12 light:dark diurnal cycle). Mosquitoes were identified as *Ae. aegypti* (L.) or *Ae. albopictus* (Skuse) based on scale patterns on the thorax after adult eclosion [248].

Table 4.1. Location, mosquito species, repetitions, generations and sample sizes used for the Aedes collections from Mexico.

State of collection	Location	Mosquito species	DPI	Repetitions	Generations used	n	SG n
Nuevo Leon	Monterrey	Aae	7 dpi	2	F0, F2	83	60
			14 dpi	2	F0, F2	92	41
	Apodaca	Aae	7 dpi	2	F1, F2	73	45
			14 dpi	2	F1, F2	82	49
	San Nicolas	Aae	7 dpi	2	F2, F3	47	41
			14 dpi	2	F2, F3	44	44
	San Nicolas	Aal	7 dpi	2	F4	88	74
			14 dpi	2	F3, F4	101	87
Tamaulipas	Ciudad Madero	Aae	7 dpi	2	F1	94	63
			14 dpi	2	F1	107	55
Veracruz	Poza Rica	Aae	7 dpi	2	F0, F1	73	25
			14 dpi	3	F0, F1	92	52
	Coatzacoalcos	Aae	7 dpi	2	F1, F2	95	64
			14 dpi	2	F1	106	19
	Coatzacoalcos	Aal	7 dpi	2	F2	88	46
			14 dpi	3	F2, F3	105	56
	Minatitlan	Aae	7 dpi	3	F0, F1	132	45
			14 dpi	3	F0, F1	69	50
Yucatan	Merida	Aae	7 dpi	2	F1	89	63
			14 dpi	3	F1	126	80
Chiapas	Mazatan	Aae	7 dpi	2	F2	81	75
			14 dpi	2	F2	78	74
	Huehuetan	Aal	7 dpi	2	F2, F3	72	53
			14 dpi	2	F2, F3	106	87
Guerrero	Guerrero	Aae	7 dpi	2	F4, F6	91	41
			14 dpi	2	F4, F6	86	49

Aae, *Aedes aegypti*. Aal, *Aedes albopictus*.

Mosquito infections

For mosquito infections, ZIKV strain PRVABC59 (passage 4) belonging to the Asian genotype [312] was used to infect Vero cells at a MOI of 0.01. After 4 days infection, the supernatant was harvested and centrifuged at 3,000xg for 10 min at 4°C. The supernatant was then transferred to a clean tube and a sample was taken to perform ZIKV quantification by quantitative-reverse transcriptase PCR

(RT-qPCR) with oligonucleotides for the ZIKV 3' untranslated region (Table 4.2) prior to the infection of mosquitoes. RNA was extracted from 50 µL of the clarified supernatant using the Direct-zol™ RNA MiniPrep Kit (Zymo Research Corp.) following manufacturer recommendations. Based on the result, the supernatant was supplemented with Dulbecco's modified Eagle's medium (DMEM) and 20% FBS and further mixed 1:1 with defibrinated calf blood to a final concentration of 1 x10^9 genome equivalents (GE)/mL. Viral titers were confirmed by plaque assays on Vero cells, averaging 10^6 PFU/mL.

Table 4.2. Primer and probe sequences for the ZIKV 3' UTR assay

	Sequence (5'→3')	Length	Start	Stop	TM (°C)
Forward primer	CCCAGGAGAAGCTGGGAAAC	20	10453	10472	60
Reverse primer	TCGCCACCTTCTTTTCCCAT	20	10581	10600	60
Probe_10,514	GCCATGCTGCCTGTGAGCCCCT	19	10514	10535	69.7

Prior to feeding, 5-6 day old mosquitoes were deprived of sucrose and water for 24 hours. Mosquito infections were performed under biosafety level-3 containment where they were offered a ZIKV infectious blood meal through water-jacketed glass feeders with hog gut as a membrane. After up to one-hour of feeding, engorged females were selected and placed into new cartons and water and a sugar source were provided.

Vector competence assessment

At 7 and 14 dpi, mosquitoes were cold anesthetized at 4°C. Legs and wings were removed and placed into a tube with 250 µL mosquito diluent (1X phosphate buffer saline (PBS) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 50 µg/mL penicillin/streptomycin, 50 µg/mL gentamycin, 2.5 µg/mL fungizone) and a stainless steel bead for homogenization. The mosquito proboscis was then placed into a capillary tube that contained immersion oil (~5 µL) and allowed to expectorate saliva for 30 minutes. Following salivation, the tip of the capillary tube was broken into a 1.5 mL centrifuge tube containing 100 µL of mosquito diluent. Subsequently, the midgut and salivary glands were dissected,

rinsed individually in PBS and placed in tubes with mosquito diluent and a stainless steel bead. Forceps were dipped in 70% ethanol and cleaned after each tissue was dissected and between individual mosquitoes. Mosquito tissues were stored at -80°C until further processing.

Mosquito tissues (midguts, legs/wings and salivary glands) were thawed and homogenized at 25 cycles/second for one minute using a Retsch Mixer Mill MM400 (Germany) and centrifuged at 20,000xg for 5 minutes at 4°C while saliva samples were centrifuged at 20,000 x g for 3 minutes at 4°C, mixed by vortexing and centrifuged for 3 additional minutes. Clarified supernatant was titrated by plaque assay on Vero cells to determine whether individual mosquito tissues contained infectious ZIKV.

Plaque assays

Plaque assays were performed on Vero cells which were maintained in DMEM containing 8% FBS, 50 µg/mL penicillin and streptomycin and 50 µg/mL gentamycin at 37°C with 5% CO_2.

Twelve-well plates were seeded with Vero cells and allowed to reach 90 to 95% confluency. At this point, media was removed and replaced with 250 µL of DMEM containing 1% FBS, 50 µg/mL penicillin and streptomycin, and 50 µg/mL gentamycin. Subsequently, each sample (30 µL for mosquito saliva or 70 µL for midgut, salivary glands and legs/wings) was added to a well of the plate. The plates were rocked for 90 minutes to allow absorption after which 1 mL of overlay (tragacanth gum (6 g/L) in 1X DMEM supplemented with 10% FBS, 50 µg/mL penicillin/streptomycin and 50 µg/mL gentamycin) was added to each well and plates were incubated at 37°C with 5% CO_2. After 5 days, the plates were fixed with a staining solution (1 g/L crystal violet in 20% ethanol solution); plaques were visualized on a light box and recorded as plaque positive or negative.

Data and statistical analysis

We determined the proportion of midgut infection, disseminated infection, salivary gland infection and transmission of each of the mosquito populations tested [143, 254]. The proportion of

midgut infection (MI) was defined as the number of mosquitoes with infectious ZIKV in the midgut divided by the total number of mosquitoes that had bloodfed. The proportion of disseminated infection (DI) was defined as the number of mosquitoes with infectious ZIKV in the legs/wings divided by the number of bloodfed mosquitoes. Salivary gland infection proportion (SGI) was defined as the number of mosquitoes with infectious ZIKV in the salivary glands divided by the total number of bloodfed mosquitoes. The transmission proportion (TR) was defined as the number of mosquitoes that expectorated saliva containing infectious ZIKV divided by the total number of bloodfed mosquitoes. In addition, transmission efficiency (TE) was defined as the number of mosquitoes which expectorated saliva containing infectious ZIKV divided by the number of mosquitoes with disseminated infection.

We calculated the additive contribution of each of the four transmission barriers to infection (equations 1-4) where all the equations sum to 100% by adjusting the MI, DI, SGI and TR proportions. Adjusted midgut infection (AMI) was the number of mosquitoes with infectious ZIKV in the midgut divided by the total number of mosquitoes that had bloodfed. The adjusted disseminated infection (ADI) was the number of mosquitoes with infectious ZIKV in the legs/wings divided by the number of mosquitoes with infectious ZIKV in the midgut. For the salivary glands we did not have the same number of samples as for the other tissues; therefore, they were adjusted proportionally. Hence the adjusted salivary gland infection (ASGI) was defined as the adjusted number of mosquitoes with infectious ZIKV on the salivary glands divided by the total number of mosquitoes with infectious ZIKV in the legs/wings. The adjusted transmission (ATR) was defined as the number of mosquitoes that expectorated saliva containing infectious ZIKV divided by the adjusted number of mosquitoes with infectious ZIKV in the salivary glands.

$$\% MIB = \frac{\log (AMI)}{\log (Total)} \times 100 \qquad (1)$$

$$\% MEB = \frac{\log (ADI)}{\log (Total)} \times 100 \qquad (2)$$

$$\% SGIB = \frac{\log (ASGI)}{\log (Total)} \times 100 \quad (3)$$

$$\% SGEB = \frac{\log (ATR)}{\log (Total)} \times 100 \quad (4)$$

$$\log (Total) = \log (AMI) + \log (ADI) + \log (ASGI) + \log (ATR) \quad (5)$$

The midgut infection, disseminated infection, salivary gland infection and transmission proportions were estimated in WinBUGS [255] using a binomial distribution as a model. Where the number of infected mosquito tissues (e.g. midgut) was a binomial sample. Bayesian 95% Highest Density Intervals (HDI) were also obtained. The Bayesian analysis provides logical estimates for proportions where an uninformative prior distribution is assumed. Therefore, the prior distribution for the probability of a mosquito tissue to be infected was 0.0-1.0 [256].

A two-tailed Fisher's exact test was used to compare MI, DI, SGI, TR and TE proportions. GraphPad PRISM version 7.03 (GraphPad Software, San Diego, CA, USA) was used for graph construction. Significance was defined as $p<0.05$.

Results

Mexican *Ae. aegypti* and *Ae. albopictus* vector competence for ZIKV

Both *Ae. aegypti* and *Ae. albopictus* were competent vectors for ZIKV transmission (Figure 4.2). For *Ae. aegypti* at 7 dpi, MI, DI and SGI proportions were statistically different which is consistent with the presence of barriers for ZIKV transmission. At 14 dpi DI and SGI proportions were not statistically different, suggesting the SGEB as the most important barrier limiting ZIKV transmission. In addition, an increase in TR was observed from 0.20 at 7 dpi to 0.39 at 14 dpi (Figure 4.2A and 4.2B). For *Ae. albopictus*, MI, DI and SGI proportions were statistically different at 7 and 14 dpi. As observed for *Ae. aegypti*, TR proportions also increased from 0.1 at 7 dpi to 0.25 at 14 dpi (Figure 4.2C and 4.2D). The TR

proportions were higher for *Ae. aegypti* than for *Ae. albopictus* at both 7 and 14 dpi. A summary of the observations at 7 and 14 dpi for *Ae. aegypti* and *Ae. albopictus* is included in table 4.3.

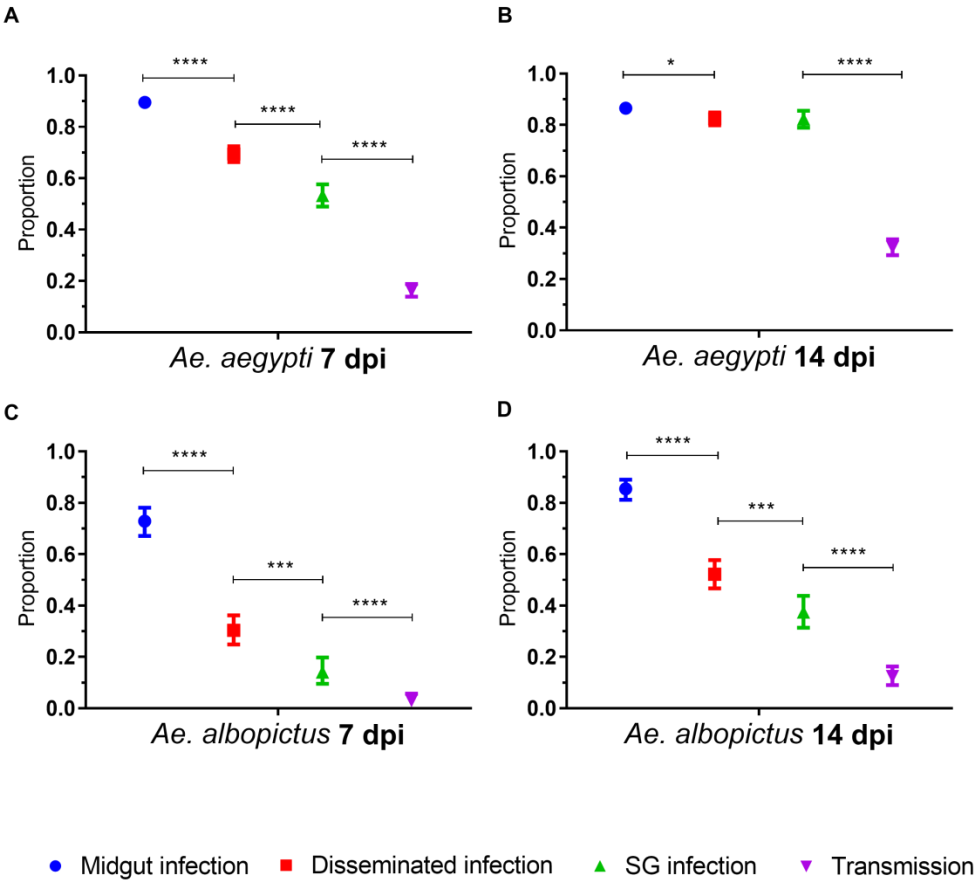

Figure 4.2. Proportion of ZIKV midgut infection, disseminated infection, salivary gland infection and transmission by Mexican Ae*. aegypti* and *Ae. albopictus.* Midgut infection, disseminated infection, salivary gland infection and transmission proportions for *Ae. aegypti* (A and B) and *Ae. albopictus* at 7 and 14 dpi (C and D). Error bars represent 95% HDI confidence intervals. Data from at least 2 independent replicates. Statistical significance is depicted as **** for p <0.0001, *** p<0.001, **p<0.01 and * p<0.05 by two-tailed Fisher's exact test.

Table 4.3. Vector competence of *Ae. aegypti* and *Ae. albopictus* from Mexico

Location	Mosquito species	DPI	ZIKV positive midguts /total midguts	ZIKV positive legs and wings /total legs and wings	ZIKV positive salivary glands /total salivary glands	ZIKV positive saliva /total saliva samples
Monterrey	Aae	7 dpi	69/83	16/83	5/60	1/83
		14 dpi	59/92	47/92	24/41	7/92
Apodaca	Aae	7 dpi	63/73	52/73	30/45	11/73

		14 dpi	69/82	66/82	41/49	27/82
San Nicolas	Aae	7 dpi	46/47	24/47	9/41	2/47
		14 dpi	41/44	39/44	39/44	12/44
San Nicolas	Aal	7 dpi	42/88	0/88	0/74	0/88
		14 dpi	75/101	19/101	9/87	2/101
Ciudad Madero	Aae	7 dpi	52/94	28/94	11/63	7/94
		14 dpi	64/107	56/107	28/55	18/107
Poza Rica	Aae	7 dpi	72/73	71/73	22/25	7/73
		14 dpi	92/92	91/92	50/52	38/92
Coatzacoalcos	Aae	7 dpi	87/95	69/95	41/64	23/95
		14 dpi	104/106	101/106	17/19	54/106
Coatzacoalcos	Aal	7 dpi	87/88	69/88	22/46	7/88
		14 dpi	102/105	93/105	46/56	28/105
Minatitlan	Aae	7 dpi	121/132	104/132	32/45	20/132
		14 dpi	56/69	54/69	36/50	20/69
Merida	Aae	7 dpi	88/89	68/89	38/63	9/89
		14 dpi	121/126	116/126	70/80	53/126
Mazatan	Aae	7 dpi	81/81	77/81	64/75	12/81
		14 dpi	78/78	78/78	73/74	19/78
Huehuetan	Aal	7 dpi	52/72	6/72	2/53	0/72
		14 dpi	90/106	51/106	31/87	8/106
Guerrero	Aae	7 dpi	90/91	87/91	35/41	47/91
		14 dpi	80/86	80/86	45/49	37/86

Aae, *Aedes aegypti*. Aal, *Aedes albopictus*.

Variation of *Ae. aegypti* MI, DI, SGI and TR proportions by region

MI and TR for ZIKV were dependent on the region of origin of the *Ae. aegypti* populations. Proportions were higher in southern populations. We observed that collections from the Northeast of Mexico had lower MI proportions relative to collections from Yucatan and Pacific regions which indicated the presence of a stronger MIB for the Northeast. The SGI proportions were not different between Northeast and Yucatan. MI and TR proportions were not different between Yucatan and Pacific collections at 14 dpi. As expected, TR proportions increased as time progressed following infection for most of the populations tested. However, TR proportions in the Pacific region were constant after 7 dpi (Figure 4.3).

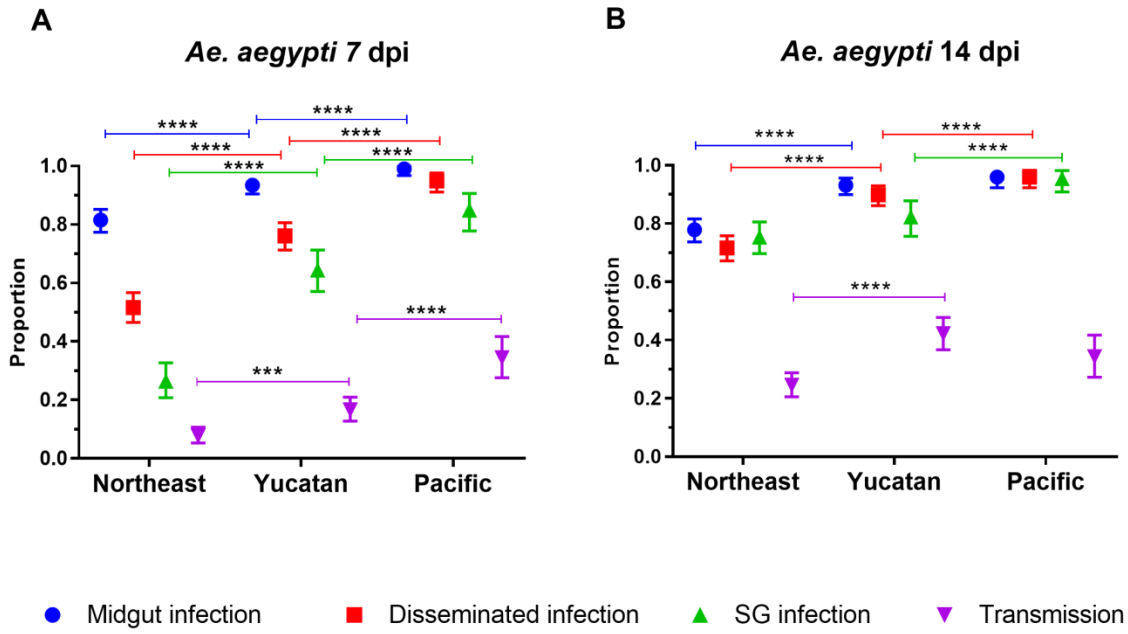

Figure 4.3. Proportion of ZIKV midgut infection, disseminated infection, salivary gland infection and transmission in *Ae. aegypti* by their region of origin. Midgut infection, disseminated infection, salivary gland infection and transmission proportions by region of origin of the *Ae. aegypti* populations (Northeast, Yucatan and Pacific) at 7 and 14 dpi (A and B). Error bars represent 95% HDI confidence intervals. Data from at least 2 independent replicates. Statistical significance is depicted as **** for p <0.0001, *** p<0.001, **p<0.01 and * p<0.05 by two-tailed Fisher's exact test.

Variation in *Ae. aegypti* and *Ae. albopictus* vector competence by location

We analyzed ten *Ae. aegypti* collections by individual location and determined the contribution of each of the barriers to transmission of ZIKV. At 7 dpi, multiple populations overlapped based on MIRs (Table 4.4), and just one population, Cd. Madero (a) was found to have a low MI proportion (Figure 4.4A).

Table 4.4. Grouping of *Ae. aegypti* populations by MIRs at 7 dpi.

Population	Non statistically different from
Apodaca	Coatzacoalcos
San Nicolas	Poza Rica, Minatitlan, Coatzacoalcos, Merida, Mazatan and Guerrero
Monterrey	Minatitlan, Coatzacoalcos
Cd. Madero	
Poza Rica	San Nicolas, Minatitlan, Coatzacoalcos, Merida, Mazatan and Guerrero

Minatitlan	San Nicolas, Monterrey, Poza Rica, Coatzacoalcos
Coatzacoalcos	Apodaca, San Nicolas, Monterrey, Poza Rica, Minatitlan
Merida	San Nicolas, Poza Rica, Mazatan and Guerrero
Mazatan	San Nicolas, Poza Rica, Merida and Guerrero
Guerrero	San Nicolas, Poza Rica, Merida and Mazatan

MIR, DI and SGI proportions variated more at 7 dpi. For San Nicolas, Monterrey and Cd. Madero, the SGIB was the main barrier limiting ZIKV transmission, while for the rest of the analyzed populations the SGEB was the most important (Table 4.5).

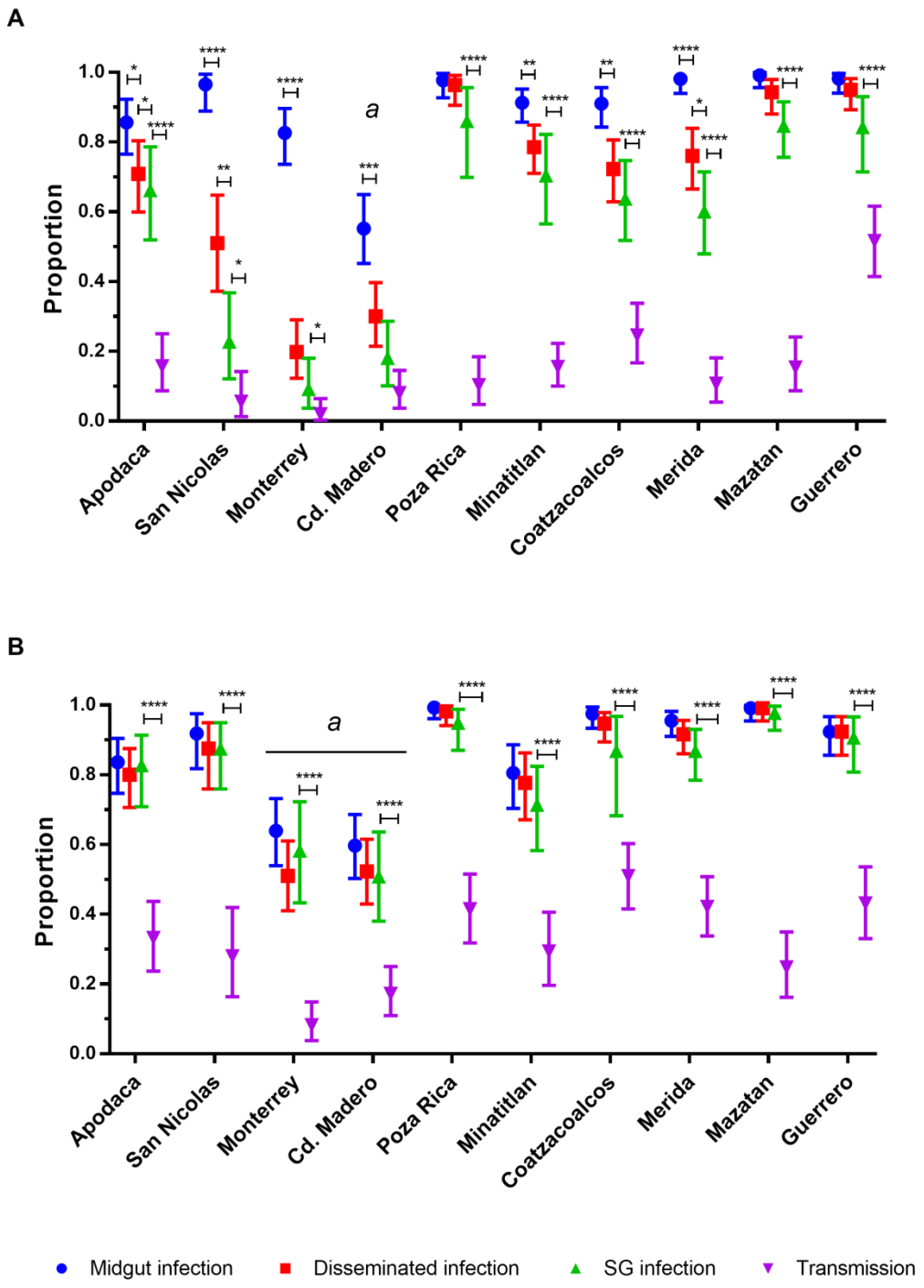

Figure 4.4. Proportion of ZIKV midgut infection, disseminated infection, salivary gland infection and transmission for *Ae. aegypti* populations by location. Midgut infection, disseminated infection, salivary gland infection and transmission proportions by location of the *Ae. aegypti* populations at 7 and 14 dpi (A and B, respectively). A North to South gradient is shown from left to right in the X-axis. Error bars represent 95% HDI confidence intervals. Data from at least 2 independent replicates. Statistical significance is depicted as **** for p <0.0001, *** p<0.001, **p<0.01 and * p<0.05 by two-tailed Fisher's exact test. A unique low MIR population was depicted with "a".

Table 4.5. Contribution of each of the barriers (MIB, MEB, SGIB and SGEB) to VC.

	Location	VC	MIB	MEB	SGIB	SGEB
Ae. aegypti 7 dpi	Apodaca	15%	8%	10%	21%	**61%**
	San Nicolas	4%	1%	21%	**50%**	29%
	Monterrey	1%	4%	33%	**63%**	0%
	Cd. Madero	7%	23%	24%	**66%**	0%
	Poza Rica	10%	1%	1%	6%	**93%**
	Minatitlan	15%	5%	8%	18%	**69%**
	Coatzacoalcos	24%	6%	16%	32%	**46%**
	Merida	10%	0%	11%	22%	**66%**
	Mazatan	15%	0%	3%	8%	**89%**
	Guerrero	52%	2%	5%	24%	**69%**
Ae. albopictus 7 dpi	San Nicolas	0%	**100%**	0%	0%	0%
	Coatzacoalcos	8%	0%	9%	29%	**61%**
	Huehuetan	0%	13%	**87%**	0%	0%
Ae. aegypti 14 dpi	Apodaca	33%	16%	4%	3%	**78%**
	San Nicolas	27%	5%	4%	8%	**82%**
	Monterrey	8%	17%	9%	20%	**54%**
	Cd. Madero	17%	29%	7%	**39%**	25%
	Poza Rica	41%	0%	1%	4%	**95%**
	Minatitlan	29%	17%	3%	26%	**54%**
	Coatzacoalcos	51%	3%	4%	17%	**76%**
	Merida	42%	5%	5%	15%	**76%**
	Mazatan	24%	0%	0%	1%	**99%**
	Guerrero	43%	9%	0%	11%	**81%**
Ae. albopictus 14 dpi	San Nicolas	2%	8%	35%	**57%**	0%
	Coatzacoalcos	27%	2%	7%	15%	**76%**
	Huehuetan	8%	6%	22%	**40%**	31%

The contribution of each of the barriers to the overall VC was calculated accordingly to equations 1-4. Major contributors to VC are shown in bold.

At 14 dpi, MI proportions determined the DI and SGI proportions. Predominantly high MI proportions were observed, most of the populations had statistically similar MI proportions and have been grouped together accordingly (Table 4.6).

Table 4.6. Grouping of *Ae. aegypti* populations by MIRs at 14 dpi.

Population	**Non statistically different from**
Apodaca	San Nicolas, Minatitlan, Guerrero

San Nicolas	Apodaca, Minatitlan, Coatzacoalcos, Merida and Guerrero
Monterrey	Cd. Madero
Cd. Madero	Monterrey
Poza Rica	Coatzacoalcos, Merida, Mazatan
Minatitlan	Apodaca, San Nicolas
Coatzacoalcos	San Nicolas, Poza Rica, Merida, Mazatan and Guerrero
Merida	San Nicolas, Poza Rica, Coatzacoalcos, Mazatan and Guerrero
Mazatan	Poza Rica, Coatzacoalcos and Merida
Guerrero	Apodaca, San Nicolas, Coatzacoalcos and Merida

The only exceptions were Cd. Madero and Monterrey (a), which had the lowest MI proportions (Figure 4.4B). In all 10 collections, TR proportions were significantly lower which indicated the involvement of a SGEB limiting ZIKV transmission. Based on the contribution of each barrier, we confirmed that the main barrier to ZIKV transmission in *Ae. aegypti* is the SGEB (Table 4.5). TR proportions or in other words VC ranged from 0.02 – 0.51 at 7dpi and 0.8- 0.51 at 14 dpi. In addition, except for Cd. Madero, TR and TE were not statistically different for *Ae. aegypti* populations at 7 and 14 dpi (Figure 4.5).

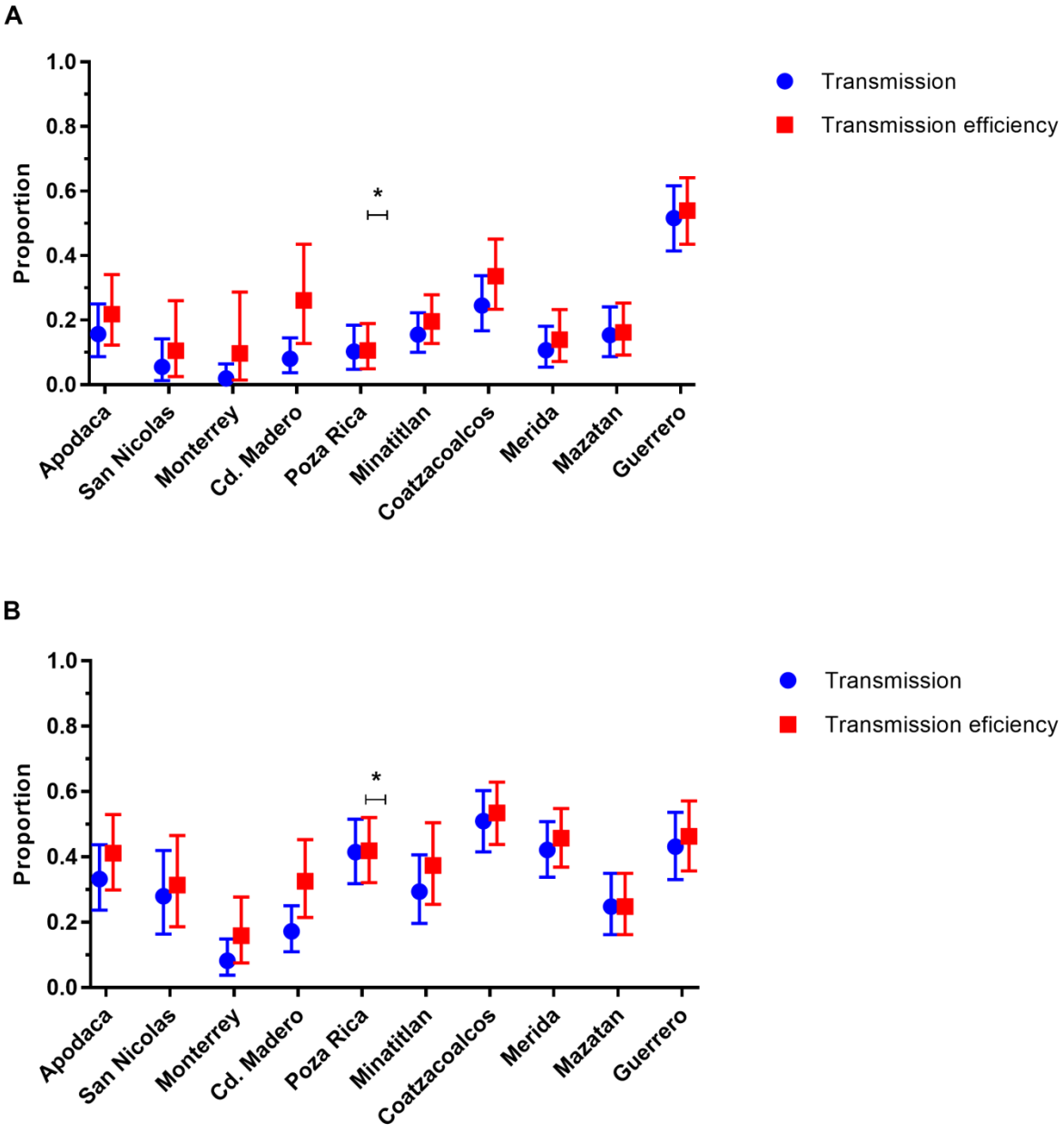

Figure 4.5. Comparison of ZIKV transmission and transmission efficiency proportions for the *Ae. aegypti* populations at 7 and 14 dpi. ZIKV transmission and transmission efficiency proportions for *Ae.aegypti* at 7 and 14 dpi (A and B), respectively. Error bars represent 95% HDI confidence intervals. Data from at least 2 independent replicates. * indicates $p<0.05$ by two-tailed Fisher's exact test.

For *Ae. albopictus* at 7 dpi, San Nicolas and Huehuetan had low MI and DI proportions which suggested that barriers at the midgut level (MIB and MEB) were responsible for the transmission

observed. The MIB and MEB were the main contributors influencing VC, respectively (Table 4.5). At 14 dpi, an increase in transmission was observed compared to 7 dpi. For San Nicolas and Huehuetan, the SGIB was the most important factor impacting ZIKV transmission at 14 dpi. However, for Coatzacoalcos, high MI resulted in low TR due to a SGEB at both 7 and 14 dpi (Figure 4.6 and Table 4.5). VC for *Ae. albopictus* ranged from 0 - 0.08 at 7 dpi and 0.02 - 0.26 at 14 dpi.

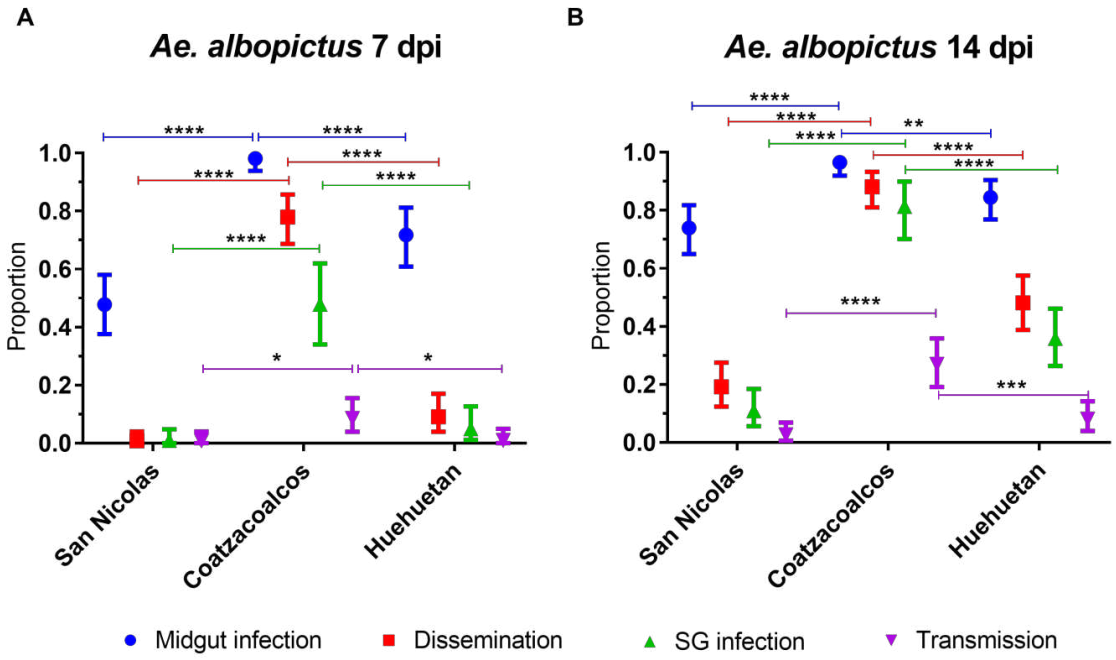

Figure 4.6. Proportion of ZIKV midgut infection, disseminated infection, salivary gland infection and transmission for *Ae. albopictus* populations by location. Midgut infection, disseminated infection, salivary gland infection and transmission proportions by location of the *Ae. albopictus* populations at 7 and 14 dpi (A and B, respectively). A North to South gradient is shown from left to right in the X-axis. Error bars represent 95% HDI confidence intervals. Data from at least 2 independent replicates. Statistical significance is depicted as **** for p <0.0001, *** p<0.001, **p<0.01 and * p<0.05 by two-tailed Fisher's exact test.

VC of co-occurring *Ae. aegypti* and *Ae. albopictus* populations

We evaluated the contribution of *Ae. aegypti* and *Ae. albopictus* to ZIKV transmission in areas where the species overlap. Both species were collected from breeding sites that were less than (<) 100 meters (m), < 3 kilometers (km), and < 20 km apart, and compared for differences in VC. At 7 and 14 dpi;

MI, DI, SGI and TR proportions were statistically different for *Ae. aegypti* and *Ae. albopictus* collected at < 3 and <20 km apart. Interestingly, for populations collected at closer proximity (<100 m apart), higher MI and TR were observed when compared with populations collected from 3 km and 20 km apart. At 14 dpi just the TR proportions were significantly different being higher in *Ae. aegypti* (Figure 4.7).

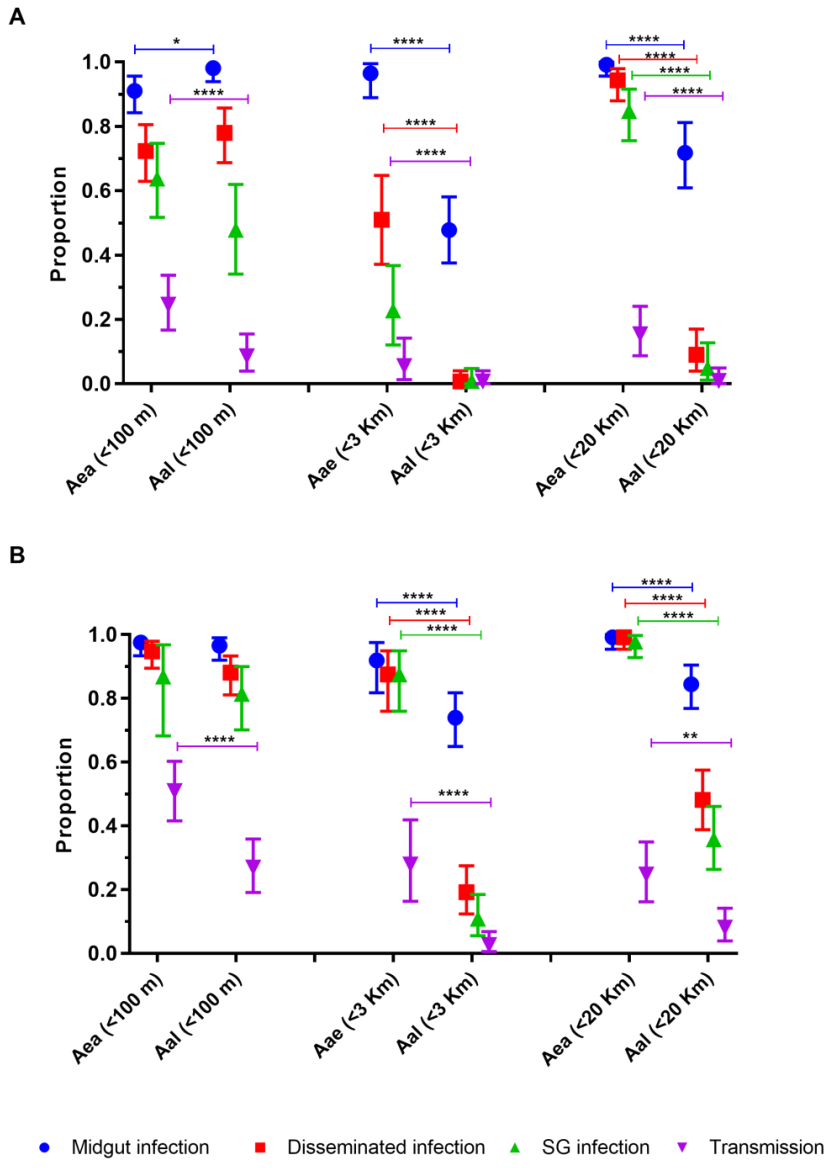

Figure 4.7. Comparison of three co-occurring *Ae. aegypti* and *Ae. albopictus* populations. Proportion of ZIKV midgut infection, disseminated infection, salivary gland infection and transmission for co-occurring *Ae.aegypti* (Aae) and *Ae. albopictus* (Aal) at 7 and 14 dpi (A and B), respectively. Error bars represent 95% HDI confidence intervals. Data from at least 2 independent replicates. Statistical significance is depicted as **** for p <0.0001, *** p<0.001, **p<0.01 and * p<0.05 by two-tailed Fisher's exact test.

Discussion

ZIKV has joined CHIKV [313] as a new arbovirus circulating in Latin America, a region already hyper-endemic for DENV [13]. The main transmission route is through the bite of an infectious Aedes mosquito [23]. Previous studies have reported VC for mosquitoes from the Americas [143, 144, 147, 148, 155, 161]. To date, two studies reported VC data for *Ae. aegypti* and/or *Ae. albopictus* for ZIKV from a disparate range of regions in the Americas [144, 161]. Even though it is useful to describe VC in risk areas, a more detailed geographical examination may help us to understand the mechanisms controlling VC in those populations. It is therefore important to determine how variable the VC is for ZIKV in Mexican mosquito populations. Thirteen recently colonized mosquito collections from 6 states throughout Mexico were analyzed at 7 and 14 dpi for VC, to have a better representation of the VC of mosquito populations in those areas now endemic for ZIKV. In some locations, *Ae. aegypti* and *Ae. albopictus* were found co-existing, while in others just *Ae. aegypti* was collected.

***Aedes aegypti* and *Ae. albopictus* are competent to transmit ZIKV**

Some studies have shown that both species are competent for ZIKV transmission [143-145, 148, 161]. In addition, ZIKV RNA has been detected in *Ae. aegypti* and *Ae. albopictus* from the field [18, 128, 311]. In this study *Ae. aegypti* and *Ae. albopictus* from Mexico were highly susceptible to ZIKV infection, both species had high MI with lower DI and SGI proportions. *Aedes aegypti* TR proportions were higher than the observed for *Ae. albopictus* but both species were competent for ZIKV transmission. Our observations were consistent with others that support *Ae. aegypti* as the principal vector for ZIKV because of higher TRs [161] and possibly shorter EIP [314] in addition to its cosmopolitan distribution and anthropophilic behavior [315].

Aedes aegypti ZIKV competence is determined by their region of origin

Previously, *Ae. aegypti* collections from Mexico were grouped into three regions based on genetic analysis [142]. Hence, we analyzed the vector competence of *Ae. aegypti* by their region of origin. We observed that populations from the Northeast had the lowest VC, increasing as southern regions were analyzed. Others have shown a limited ZIKV (Asian lineage) transmission by *Ae. aegypti* from the Rio Grande Valley area in Southern Texas [144] which is consistent with our observations for this region (Northeast of Mexico).

Interestingly, TR did not change after 7 dpi for the Pacific region, which may reflect a short EIP, which is important for its epidemiological implications affecting vectorial capacity [316]. High TR proportion at 7 dpi would potentially allow for sufficient capability as a ZIKV vector even in the face of shortened mosquito survival [158] enhancing the potential for ZIKV transmission in this region. In addition, the majority of the cases reported from Mexico belong to Southern states [36, 129]. However, those reports have to be interpreted with caution because of the limitations of the disease reporting systems [23, 36].

ZIKV transmission by *Ae. aegypti* is highly variable

The establishment of an infection in the midgut is one of the most important intrinsic factors that define the vector competence of the mosquito host [132]. Our data showed a high susceptibility of *Ae. aegypti* for ZIKV. We observed that *Ae. aegypti* DI and SGI depended on the MI. However, high variation in MIRs was observed at 7 dpi and 14 dpi. As previously reported for DENV-2 [140, 141], *Ae. aegypti* collections from Mexico also showed variability within and among populations for ZIKV. Collectively, populations from the Northeast showed low VC, but when analyzed by location (Apodaca through Poza Rica); we found variation in VC within this region. It has been shown that populations in proximity can become genetically distinct [135] which may affect genes that impact VC. Thus, interpreting VC for large areas may lead to wrong estimates of the impact VC has at a more local level.

Low VC in *Ae. albopictus* varies by location

In agreement with other studies using *Ae. albopictus* populations and ZIKV from the Asian lineage [147, 150, 154, 161] where the TR proportions were <0.25, the TR proportions from our populations ranged from 0.02 to 0.27. As observed for *Ae. aegypti*, *Ae. albopictus* from San Nicolas (Northern Mexico) had lower MI proportions when compared with other locations. The TR proportions for *Ae. albopictus* were low except for Coatzacoalcos, where *Ae. albopictus* was as competent as some of the *Ae. aegypti* populations from the North. It has been suggested that the existence of multiple locally-adapted vector populations could enhance the spread of arboviruses through space and time [317]. Thus, the relatively low VC for *Ae. albopictus* may be compensated by some characteristics of this species like the aggressive biting and blood feeding behaviors [105, 191, 318, 319] and abundance [320], which may also contribute to human transmission.

ZIKV transmission by *Ae. aegypti* and *Ae. albopictus* is limited by SG barriers

We also describe the contribution that each of the barriers has on the overall VC. We observed that for *Ae. aegypti* the SGIB and SGEB are limiting ZIKV at 7 dpi, while at 14 dpi the SGEB was the main barrier. For *Ae. albopictus* MIB, MEB and SGEB were limiting ZIKV at 7 dpi, which can be attributed to longer EIP in this species, as previously reported [314]. However, by 14 dpi SGIB and SGEB were limiting ZIKV.

In general, an arbovirus must penetrate the basal lamina of the salivary glands surrounding the acinar cells, where the virus has to replicate and subsequently be deposited into the apical cavities where mosquito saliva is stored prior to its delivery during feeding [166]. The molecular mechanisms involved in the SGEB are still unknown [136]. In addition, there is a limited knowledge on the antiviral response in the salivary glands [165]. However, the failure to exit from infected cells in the salivary glands may be one of the principal mechanisms limiting arbovirus transmission. Possible mechanisms may include, the presence of heparan sulfate proteoglycan (HSPG) which may act as a sponge retaining

virus [321] or due a defective secretory pathway [166]. For instance, no evidence has been found for salivary gland barriers for DENV [132]; however, the impact of SGEB has been described for bunyaviruses: Rift Valley fever virus (RVFV) [322] and La Crosse encephalitis virus (LACV) [323] and alphaviruses: Sindbis virus (SINV) [324] and chikungunya virus (CHIKV) [325]. The potential role of the SG barriers has been suggested for ZIKV [161, 326], and in our study we provide evidence of a strong SGEB limiting ZIKV transmission in Mexican *Ae. aegypti* populations. In addition, ZIKV is an RNA virus with high potential for mutation. However, no adaptation to American mosquitoes has been documented [143]. In addition, the SGEB may contribute to the selection of specific viral populations of ZIKV [182]. This finding may be fundamental for the development of new control methods for ZIKV in natural populations of mosquitoes. Mosquito control efforts have to be focused on both mosquito species in Mexico and in other Latin America countries, because, in addition to the barriers to transmission [135, 136], arbovirus transmission depends on the environmental conditions that influence geographical distribution, survival and abundance of the mosquito populations.

Proximity of collection sites of *Ae. aegypti* and *Ae. albopictus* determined their ZIKV competence

The potential role that *Ae. albopictus* may play in the spread of emerging and re-emerging diseases, especially in areas where both *Ae. aegypti* and *Ae. albopictus* coexist has been controversial. We report that when both species are found at closer proximity (<100 m), the TR proportion for the *Ae. albopictus* were higher than when they were collected from containers at 3 or 10 km apart, indicating a possible role for the microbiome [214] or possibly competition [224] having an effect on ZIKV VC, which has to be determined in future investigations. Overall, our data demonstrates how variable and dynamic VC is in *Ae. aegypti* and *Ae. albopictus* for ZIKV. In addition, we provided evidence on the main physiological barriers limiting ZIKV transmission in both species.

CHAPTER 5: CONCLUSIONS

My interest is in vector-borne diseases, especially those transmitted by mosquito vectors in Latin America. So, I was very fortunate to be a Fogarty training grant scholarship recipient so I could be trained and contribute to the control of Dengue virus (DENV) and other mosquito-borne viruses in my country. During the development of this dissertation, two arboviruses emerged in the western hemisphere. First, Chikungunya virus (CHIKV) emerged in 2013 which was followed by Zika virus (ZIKV) in 2015.

VC studies are an important way to get information about the impact that mosquito populations may have in the epidemiology of the arboviral diseases in areas where they co-occur. As recently exemplify by the abundant VC reports for ZIKV, where several mosquito species were challenged with ZIKV. Even when contrasting results from a couple of studies, the majority of the reports showed *Culex* mosquitoes are not competent vectors for ZIKV transmission. Also, far from the recognized vector *Aedes aegypti*, the potential transmission by local mosquito species upon viral introduction into template areas has been hypothesized after assessing their VC. In addition VC studies have provided evidence of the complex interactions between the arboviruses and their mosquito vectors. Importantly those reports may serve to alert the authorities and population for their protection through personal protective measures and government mediated initiatives like interventions for disrupting the transmission cycle (e.g. insecticide applications) or by funding research projects aiming to mitigate the burden of mosquito-borne diseases. It is fundamental to mitigate the burden that mosquito-borne diseases cause worldwide, especially for the groups that are more vulnerable to infection. This was observed following the recent ZIKV introduction to the Americas due the severe effects (microcephaly) associated with infection during pregnancy.

This dissertation includes studies based on vector competence of Mexican mosquitoes for DENV and ZIKV. DENV is a neglected tropical disease responsible for millions of cases worldwide and a public health problem throughout Latin America. The recently emerged ZIKV has caused millions of infections and has been associated with severe outcomes of disease as microcephaly and Guillain-Barré Syndrome.

If I could summarize the VC of Mexican mosquitoes for DENV-2 and ZIKV in one word, that will be dynamic. Previously it was shown that the Neovolcanic axis acts as a discrete barrier to gene flow for North and South populations which also differed in their VC phenotype for DENV-2. In this dissertation we found that those patterns have changed after 8 years. Concluding, that VC or other phenotypes like the insecticide resistance of the *Ae. aegypti* mosquitoes cannot be inferred from a one point in time study.

Subsequently, we profiled the microRNAs that were modulated in midguts infected (MEB-) with DENV-2 and also in midguts exposed to DENV-2. Some of the miRNAs that were modulated in infected or exposed midguts were reported previously and some are newly reported. Specifically, we included miRNAs modulated in *Ae. aegypti* midguts at 14 dpi , and also we suggest a number of miRNAs that may be implicated in the lack of MEB. Undoubtedly, the miRNA responses in the vector add a layer of complexity to the interaction between vector and pathogen. A future direction for this will be to validate their function.

Lastly, upon the emergence of ZIKV and its rapid spread in the Americas, initial reports showed low competence from American mosquitoes from geographically distant points; we assessed the VC of two *Aedes* species from Mexico. We found that *Ae. aegypti* and *Ae. albopictus* are competent vectors for ZIKV transmission. VC varied by species, as well as by region and geographic location, which demonstrates how variable is the VC phenotype. Variation in DENV-2 and Yellow fever virus (YFV) has been reported for *Ae. aegypti*, our results in addition to the growing number of ZIKV VC reports, point to variation as a share characteristic of VC for flaviviruses. Additionally, we observed that the salivary gland

escape barrier (SGEB) is the most important barrier to ZIKV transmission in *Ae. aegypti* populations. Further studies should focus in salivary glands and which mechanisms may be responsible of limiting ZIKV.

This dissertation represents a five-year effort. Hopefully, this work may contribute to the knowledge needed to mitigate mosquito-borne diseases.

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