DISSERTATION

# STUDIES OF AEDES AEGYPTI IMMUNE PATHWAYS IN RESPONSE TO DENGUE VIRUS INFECTION: EVALUATION OF GENETICALLY MODIFIED MOSQUITO FITNESS, IMMUNE PATHWAY EXPRESSION AND NATURAL GENETIC VARIATION

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2016

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ABSTRACT

STUDIES OF AEDES AEGYPTI IMMUNE PATHWAYS IN RESPONSE TO DENGUE VIRUS INFECTION: EVALUATION OF GENETICALLY MODIFIED MOSQUITO FITNESS, IMMUNE PATHWAY EXPRESSION AND NATURAL GENETIC VARIATION

Mosquito-borne diseases, such as dengue, are global health priorities, since they affect hundreds of millions of people per year. Vector control is one of the most successful tools for preventing mosquito-borne disease transmission and characterization of anti-viral mechanisms has led to the development of novel vector control strategies. One of the main mechanisms of mosquito antiviral defense is the small interfering RNA (siRNA) pathway, which has been shown to influence Aedes aegypti dengue viral infection. The RNA interference (RNAi) response of this pathway has been utilized to create transgenic Ae. aegypti lines, which are refractory to dengue virus type-2 (DENV2) infection. Additionally, genetic studies can also provide insights into function and natural variation of anti-viral pathways, potentially leading to the development of new approaches to vector control.

The recent advancements in transgenic technologies are increasing the potential of genetically-modified vectors for disease management. In this project an RNAi based genetically-modified mosquito, Carb109, was evaluated for fitness after a backcrossing to a genetically diverse laboratory strain (GDLS). This method improved the mosquito fitness and transgene stability over previous non-backcrossed strains, making it more feasible for use in genetic vector control programs. However, positional effects of the transgene made the homozygote less fit and stable regardless of introgression into a GDLS.

The next two aims explored the genetics of siRNA genes in a gene expression and a population genetic study. Gene expression of multiple potential anti-viral immunity genes in

Ae. aegypti strains artificially selected for differences in midgut infection rates showed some evidence for the involvement of dicer2 (Dcr2), a siRNA gene in DENV2 midgut escape in these strains. Gene expression also varied naturally diurnally and over the lifetime of the mosquito, which demonstrated the importance of keeping consistent sample collection schedules for gene expression studies. Lastly, four non-synonymous substitutions in the Dcr2 gene were evaluated for association with susceptibility to DENV2 Jamaica 1409 infection, but no association was found.

ACKNOWLEDGEMENTS

I would like to thank my parents William and Judith Raban, my grandfather William Phillips and my fiancé Jonathan Grava for always supporting me in my academic efforts. I would also like to thank my friends and colleagues at Colorado State University. Their friendship and support were vital to this work. I would like to also thank my laboratory colleagues especially Monica Heersink, Cynthia Meredith, Dr. Alexander Franz and Dr. Irma Sanchez-Vargas for their assistance with this work. I also would like to thank my committee, especially Dr. Ken Olson and Dr. Bill Black, for making me a better scientist and for their support and effort towards completion of this degree. I would also like to thank Dr. Carol Blair and Dr. Boris Kondratieff for their assistance improving my dissertation.

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# CHAPTER I: LITERATURE REVIEW

## Dengue past and present

Arthropod-borne disease research began in the late 1800s when Fredrick Kilbourne and Theobald Smith determined the Babesia transmission cycle between cattle and ticks (Assadian and Stanek, 2002). These discoveries were followed by Patrick Manson and Ronald Ross, who first demonstrated transmission of filariasis and malaria by blood-feeding arthropods. This discovery elucidated the filariasis and malaria transmission cycles and ultimately led to the reward of a Nobel Prize to Ross. The later work of Josiah Nott, Carlos Finlay and Walter Reed led to the discovery of the yellow fever transmission cycle. Since this discovery, there are over 500 known arthropod-borne viruses with more being discovered every year (CDC, 2010).

Dengue virus (DENV) is an arthropod-borne virus (arbovirus) that affects more humans than any other arbovirus today. The earliest descriptions of dengue like disease are found in medical encyclopedias from the Chinese Chin dynasty (265-420 AD) ("Etymologia of dengue," 2006). Modern discovery of the transmission cycle of DENV started in the early 1900s with Graham (Graham, 1903) and Bancroft (Bancroft, 1906) who first determined that DENV was mosquito-borne and identified the principal vector, respectively. Soon after, Ashburn and Craig isolated the etiological agent and by the 1930s many aspects of DENV transmission had been defined (Ashburn and Craig, 1907). A few of the subsequent findings concerned the identification of secondary vectors (Simmons et al., 1930b) (Snijders et al., 1931). During this time researchers also determined the extrinsic incubation period, (Schule, 1928; Simmons et al., 1930a) seasonality and duration of Aedes aegypti infection, (Blanc and Caminopetros, 1929) human DENV symptomology (Sabin and Schlesinger, 1945) and the intrinsic incubation period (Lumley, 1943).

Current studies indicate that dengue has been circulating globally for hundreds of years (Twiddy et al., 2003), but in recent years the frequency and severity of this disease has increased (Gubler, 1998). Current records suggest that there are over 50 million and as many as 400 million cases of dengue each year (Bhatt et al., 2013; WHO, 2009a), with an increase in the more severe manifestations of the disease, dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (WHO, 2009b). The Centers for Disease Control (CDC) defines dengue fever as a self-limiting disease lasting approximately 7 to 10 days. Dengue fever is the most common manifestation of the disease, but occasionally it may progress into DHF, or DSS. These more serious forms of the disease are characterized by vascular permeability, plasma leakage, and shock (WHO, 1997). In dengue endemic countries, the WHO estimated the disability-associated life years (DALYS) associated with dengue ranged from 400 to over 500, and the economic burden exceeds hundreds of millions of dollars (WHO, 2009b). With the increase in the number and severity of dengue cases, and with no current treatment or vaccine for the disease, these numbers are predicted to increase drastically in the next decade. Currently, vector control is still the principal approach for controlling DENV.

Even though DENV has been known to the modern world for over 100 years, it has been increasing in incidence. There are many possible explanations for the global increase in the incidence of dengue fever (DF), DSS and DHF. The increased severity of the disease associated with DSS and DHF has been attributed to the introduction of new serotypes into naïve populations, co-circulation of multiple DENV serotypes (Halstead, 1988), global spread of the more virulent southeast Asian genotypes (Armstrong and Rico-Hesse, 2001; Rico-Hesse et al., 1997) regional changes in the primary vector (Vazeille et al., 2003), and the global expansion of the primary vector, Aedes aegypti (Gubler, 1998). The increased incidence is also attributable to

human population expansion and urbanization, the lack of effective mosquito control in most underdeveloped countries, insecticide resistance, increased global travel, and the lack of public health infrastructure in most developing countries (Carabali et al., 2015; Gubler, 1995, 1998; Gubler and Clark, 1996). These factors coupled with the lack of an effective vaccine make dengue one of the most important arthropod-borne infectious diseases in the world today.

## Dengue molecular virology

Dengue virus (DENV) is a single stranded positive-sense RNA virus in the family Flaviviridae, genus Flavivirus. There are four distinct DENV serotypes. The flaviviruses contain two other genera, Pestivirus and Hepacivirus. Within the genus, Flavivirus, most of the viruses are arthropod-borne and they are transmitted by either mosquitoes or ticks. Medically important mosquito-borne viruses in this genus include West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), as well as the type species yellow fever virus (YFV).

Dengue viruses are small (40-60nm) enveloped viruses with an approximately 11 kb RNA genome. The genome has a type 1 5' $m^7$G5'ppp5' cap with no 3' poly A tail (Chambers et al., 1990; Wengler, 1981). During the replication cycle it is hypothesized virus cell entry occurs by receptor mediated endocytosis (Acosta et al., 2009, 2008; Hung et al., 1999; Mosso et al., 2008) and by clathrin mediated endocytosis (Acosta et al., 2011, 2008; Kuadkitkan et al., 2010; Mosso et al., 2008), but there are exceptions to this dogma that vary by virus serotype and host cell type (Acosta et al., 2009, 2008; Bielefeldt-Ohmann et al., 2001) . After entry, the virus is encapsulated in an endosome eand the fusion of viral and endosomal membranes and leads to the release of the viral genomic RNA into the cytoplasm. The genomic RNA is then translated as a single large polyprotein which is cleaved co- and posttranslationally into mature proteins. The genome has a single open reading frame that contains 3 structural, capsid (C), premembrane (prM), envelope(E) and 7 nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and

NS5 (Henchal and Putnak, 1990). After translation, viral RNA synthesis occurs in viral replication complexes that are composed of vesicles derived from cellular membranes (den Boon and Ahlquist, 2010; Paul and Bartenschlager, 2013). First, negative strand RNA is generated using non-structural proteins translated from the genomic RNA, which serves as template for positive strand genomic RNA. The newly made genomic RNA associates with the C protein, and forms a complex that buds into the endoplasmic reticulum (ER) at which point it acquires its envelope consisting of ER originated lipid bilayer and E and prM proteins. After entering the trans-Golgi, the prM protein is cleaved (Stadler et al., 1997) resulting in the creation of the M protein, conformational changes in the E protein (Allison et al., 2003) and the creation of a mature viral particle.

The structural proteins are associated with the core and envelope of the dengue virion. The C protein is one of the main components of the viral core along with the viral genomic RNA. The C protein is involved in virion assembly and can be found in the cytoplasm of dengue infected cells (Wang et al., 2002). The viral envelope consists of a host derived lipid membrane and the prM and E proteins. The prM protein prevents E protein associated fusion, and when it is cleaved forms the M protein (Allison et al., 1995; Guirakhoo et al., 1992; Heinz et al., 1994). The E protein is associated with cell attachment and fusion (Heinz, 1986) and along with the M protein, is the most abundant protein on the viral envelope (Allison et al., 1995).

The non-structural proteins aid in viral replication. The function of NS1 is not well established but it may have a role in RNA replication (Lindenbach and Rice, 1999; Mackenzie et al., 1996; Westaway et al., 1997). NS2A is hypothesized to recruit RNA templates in membrane associated replication complexes and has been found to bind to NS3, NS5 and the 3'UTR (Chambers et al., 1989; Mackenzie et al., 1998, 1996). NS3 with NS2B as a cofactor, function as

the viral serine protease, which cleaves the viral polypeptide at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions, as well as processing NS2A, NS3, NS4A, and C (Westaway et al., 1997). NS3 also has NTPase and helicase activity important in viral RNA replication (Cui et al., 1998; Kapoor et al., 1995) and may be involved in induction of apoptosis (Prikhod et al., 2002). NS4B co-localization with NS3 has been demonstrated to improve NS3 helicase activity (Umareddy et al., 2006). NS4A, and NS4B localized to the site of RNA replication, but overall little is known about their function (Lindenbach and Rice, 1999; Zou et al., 2015b). However, the co-localization of NS3 and NS4B is required for efficient viral replication (Zou et al., 2015a). The NS5 protein is the viral RNA-directed RNA polymerase (RDRP), and also has methyltransferase activity required for the methylation of the 5' cap. The 5' and 3' untranslated regions (UTR) have conserved sequences that initiate negative strand synthesis (Olsthoorn and Bol, 2001; Wengler and Castle, 1986; Wengler, 1981; Zeng et al., 1998) and the 3' UTR is involved in translational efficiency (Alvarez et al., 2005; Wei et al., 2009). Secondary structure in the coding regions also directs the translational start sites and influences replication efficiency (Clyde and Harris, 2006).

Dengue epidemiology

There are four serotypes of DENV (1-4) that have a tropical and sub-tropical distribution. Dengue viruses occur in a sylvatic cycle between nonhuman primates and mosquito species, such as Ae. furcifer, Ae. taylori, Ae. luteocephalus in Africa(Diallo et al., 2003) and Haemagogus leucocelaenus in the Americas(de Figueiredo et al., 2010) and an epidemic or urban cycle which occurs between humans and Ae. aegypti and to a lesser extent Ae. albopictus. The epidemic cycle is thought to have arisen from the sylvatic cycle within the last 1,000 to 2,000 years (Rico-Hesse, 2003). The timing of these transmission cycles is influenced by the intrinsic and extrinsic incubation periods. The intrinsic incubation period of dengue is defined as the time required for a

vertebrate host to develop disease symptoms after an initial infectious bite. DENV intrinsic incubation period is 3 to 14 days with a duration of infectivity to mosquitoes ranging from 2 to 10 days (Gubler et al., 1981). Laboratory experiments have also demonstrated the possibility of non-viremic transmission of other flaviviruses like WNV, which happens by infected mosquitoes feeding in proximity of a non-infected mosquito resulting in the transmission of the virus from an infected to non-infected mosquito (Higgs et al., 2005; Reisen and Fang, 2007). Non-viremic transmission may therefore be a mechanism to greatly enhance a transmission cycle due to its ability to bypass the intrinsic incubation period in the vertebrate host.

The extrinsic incubation period is defined as the time from initial ingestion of the virus by the vector to the time when it can transmit to a vertebrate host, and is typically 7 to 14 days for DENV with variations due to other factors, especially temperature (Chan and Johansson, 2012; Watts et al., 1987). During the dengue transmission cycle, a mosquito may become infected with DENV after imbibing a blood meal from a viremic primate. Before transmission to another primate, the virus has to attach to the mosquito midgut, replicate within the midgut, escape the midgut, disseminate to the mosquito salivary glands, and then be transmitted to a primate in a subsequent blood feed (Woodring and Higgs, 1996). The speed of these processes dictates the extrinsic incubation period of the virus and is influenced by environmental factors, such as temperature (Watts et al., 1987) and vector and virus genetics.

Dengue vectors and vector competence

The main vector of DENV in urban cycles is Ae. aegpyti with Ae. albopictus as a secondary vector. Known colloquially as the yellow fever mosquito, and the Asian tiger mosquito respectively, they have tropical and sub-tropical distributions. However, due to its ability to over winter Ae. albopictus is also found in temperate regions. Both are container inhabiting, because their eggs are oviposited in containers where the larvae develop. Aedes

aegypti subspecies aegypti is commonly distributed in man-made artificial containers, which contributes to their domestic and peridomestic distribution (Moore et al., 1978). In contrast Aedes aegypti subspecies formosus is mostly found in natural containers such as tree holes, husks or coconuts and other fruits and nuts, or rock pools, and adults largely feed outdoors in sylvatic habitats (Lounibos, 1981; Powell and Tabachnick, 2013). Aedes aegypti formosus also has a lower DENV vector competence than its sibling species. Aedes albopictus is also common in urban environments as it also inhabits natural containers leading to a more diverse oviposition and host feeding preferences (Clements, 1999; Gratz, 2004; Kramer and Ebel, 2003) as compared to Ae. aegpyti. There are many factors that contribute to the transmission of a virus from a vector to a vertebrate host. A few examples are vector density in the population, host feeding frequency, vector longevity, length of the extrinsic incubation period, and vector competence. These factors are all part of the Ross- Macdonald vectorial capacity model (Macdonald, 1957) modified by Garret-Jones (Garret-Jones, 1964). To further the complexity of arboviral transmission, these factors themselves are also influenced by outside environmental factors that affect the vector competence of the host (Adelman et al., 2013; Alto et al., 2008, 2005; Anderson et al., 2010; Bates and Roca-Garcia, 1946; Chamberlain and Sudia, 1955; Dye, 1992; Epstein, 2001; Grimstad and Haramis, 1984; Grimstad and Walker, 1991; Gubler et al., 2001; Pant and Yasuno, 1973; Platt et al., 1997; Reeves et al., 1994; Schneider et al., 2007; Smith, 1987; Takahashi, 1976; Watts et al., 1987). Viral factors can also influence the vector competence of a mosquito (Anderson and Rico-Hesse, 2006; Armstrong and Rico-Hesse, 2001; Bosio et al., 2000; Miller and Mitchell, 1986; Myles et al., 2004). The complexity of arboviral transmission is further demonstrated by the fact that there are over 3,200 recognized species of mosquito, and only a small fraction of these are known to vector a pathogen.

The vector competence of a mosquito is defined as its permissiveness to infection (Severson and Black, 2005) or its ability to be infected with a virus that results in viral replication and transmission to a susceptible host (Kramer and Ebel, 2003). As mentioned previously, when a mosquito imbibes an infected blood meal there are many steps before the virus can be transmitted. If any of these events do not occur, there is a barrier to transmission and the vector is incompetent or refractory to infection with the virus. On the other hand, if the virus passes all of these barriers, then it can be transmitted to a vertebrate host, and the vector is considered to be a competent vector. The most common barriers to arboviral infection are midgut infection barriers (MIB) (Chamberlain and Sudia, 1961; Cooper et al., 2007; Paulson et al., 1989), midgut escape barriers (MEB) (Hardy et al., 1983; Kramer et al., 1981) and salivary gland infection and escape barrier (Grimstad et al., 1985; Kramer et al., 1981; Paulson et al., 1989; Romoser et al., 2005). In a permissive vector, DENV-2 infection results in an initial infection of the posterior mesenteronal midgut epithelial cells (Hardy and Reeves, 1990; Whitfield et al., 1973). Then the arbovirus replicates in the midgut, spreads to and replicates in the fat body and other cells or tissues and finally spreads to the salivary glands (Franz et al., 2015; Hardy et al., 1983; Kuberski, 1979; Parikh et al., 2009; Romoser et al., 2005; Woodring and Higgs, 1996). In A. albopictus a MEB was found to be the common barrier to disseminated DENV-1 infections (Boromisa et al., 1987). Midgut infection barriers and MEB both appear to be common in Ae. aegypti flavivirus infection (Bennett et al., 2005; Bosio et al., 2000; Gomez-Machorro et al., 2004). Receptors, miRNA, and innate immune variations in the midgut have been described as influencing these barriers during DENV infection (Black et al., 2002; Carvalho-Leandro et al., 2012; Mercado-Curiel et al., 2008; Zhou et al., 2014), but in some cases

viral dissemination was dependent on viral strain, rather than midgut variations (Dickson et al., 2014; Khoo et al., 2013b).

Even among competent vectors within the same species, there is variation in MIB and MEB among individuals for DENV (Bennett et al., 2002; Boromisa et al., 1987; Gubler and Rosen, 1976; Gubler et al., 1979; Lozano-Fuentes et al., 2009; Rosen et al., 1985; Tabachnick et al., 1985; Tran et al., 1999; Vazeille-Falcoz et al., 1999) which is due to genetic factors in both the virus and the vector. Different strains of DENV within the same serotype (Armstrong and Rico-Hesse, 2001; Bennett et al., 2005, 2002; Dickson et al., 2014; Rico-Hesse et al., 1997; Rosen et al., 1985) and among serotypes (Bennett et al., 2005) vary in their infectivity to both vertebrate and invertebrate hosts. Previous studies showed that vector competence was a function of both the viral and mosquito genotypes (Lambrechts et al., 2009). This work resolved earlier studies that attempted to determine the genetic variation associated with DENV vector competence. In Ae. albopictus, genetic variation was hypothesized to be additive (Gubler et al., 1976) and in Ae. aegypti the refractory genotype was dominant to the susceptible genotype (Gubler et al., 1979). Previous work hypothesized that susceptibility phenotypes in A.aegypti were controlled by a small number of loci based on their stability over time (Miller and Mitchell, 1991) . More recently, a study determined that approximately 40% of the variation in Ae. aegypti dengue susceptibility was attributed to genetic factors, with the remaining 60% of the variation being attributed to “environmental” or uncontrolled factors (Bosio et al., 1998). There were two quantitative trait loci (QTL) associated with MIB to DENV infection in Ae. aegypti and one weak association with MEB (Bosio et al., 2000). These loci were located on different chromosomes and the effects of these three loci were additive and independent. Additionally studies tested these loci for anti-viral association (Bernhardt et al., 2012; Gorrochotegui-

Escalante et al., 2005). These studies showed that MIB and MEB were not independent and this quantitative trait had multiple genes influencing DENV susceptibility (Bennett et al., 2002). Trypsin was not found to be associated with vector competence of Ae. aegypti for DENV2 (Gorrochotegui-Escalante et al., 2005), nor was an association found with multiple anti-viral genes in the small interfering RNA (siRNA) pathway: Argonaute 2 (Ago2), Dicer 2 (Dcr2), R2D2 and the apoptosis pathway related gene inhibitor of apoptosis 2 (IAP2). Thus, while these studies have shown that the virus and vector both play a role in vector competence of Ae. aegypti for DENV, no finite gene or set of genes has been attributed to vector competence. Instead, the large genetic component to vector competence appears to be controlled by multiple loci with both dominant and additive effects.

## Mosquito immunity

### Midgut proteases

Adult female mosquitoes have a type 1 peritrophic membrane (PM), which is produced in response to a blood meal. The peritrophic membranse does not begin formation for 8 hours and is not fully formed for 20-24 hours post blood meal (Houk, 1977), but this can occur at different rates in different mosquito species (Ponnudurai et al., 1988). Arboviruses are presumed to bind and infect the midgut epithelium before formation of the PM (Hardy et al., 1983), but there is some indication that the digestive biology of the midgut cells may influence viral proliferation. The mosquito midgut is composed primarily of columnar epithelial cells, which absorb nutrients and produce enzymes for nutrient digestion (Clements, 1992). The proteases secreted by these cells may degrade envelope viral proteins (Tellam et al., 1999) and thereby aid in viral infection of La Crosse virus (LACV) in Ae. triseriatus and DENV2 in Ae. aegpyti (Ludwig et al., 1991, 1989; Molina-Cruz et al., 2005). Likewise, abundant trypsin, an abundant midgut protease was found to be associated with Ae. aegypti DENV2 vector competence suggesting a role for midgut

digestion in viral immunity (Gorrochotegui-Escalante et al., 2005). Other studies have also demonstrated that late phase midgut trypsin activity may decrease DENV2 infection in Ae. aegypti (Brackney et al., 2008).

### Insect innate immunity

For mosquitoes, as for other insects, there is little evidence for an adaptive immune response (Dong et al., 2006; Watson et al., 2005). Instead, mosquitoes have an innate immune response comprised of cellular and humoral responses (Blair and Olson, 2014; Sim et al., 2014). There is evidence that all of the known innate immune pathways may play some role in the anti-viral response to viruses in insects (Kingslover et al., 2013).

### Insect cellular and humoral immune responses

There is evidence that the cellular immune response plays a role in insect viral infections (Lavine and Beckage, 1995; Luo and Pang, 2006; Washburn et al., 1996). However, there is little evidence for the involvement of the cellular immune response to arboviral infection other than observational reports of midgut sloughing during alphavirus infection of Culex spp. (Weaver et al., 1992, 1988). The cellular immune response generally involves phagocytosis and encapsulation followed by melanization. In Ae.aegypti there are three main hemocyte types: prohemocytes, which are precursors of all hemocytes; granulocytes, which are adhesive and phagocytic; oenocytoids, which exhibit phenoloxidase (PO) activity (Castillo et al., 2006). There is evidence for hemocyte involvement in Plasmodium infection of some Anopheles via PO activity (Meister et al., 2004) or nitric oxide activity (Herrera-Ortíz et al., 2004; Kumar et al., 2004; Luckhart et al., 1998). Hemocytes produce effector molecules against Plasmodium (Lavine and Strand, 2002; Whitten et al., 2006) and thus have involvement in the humoral immune response. Hemocytes may also play a role in JAK/STAT signaling in the fat body (Agaisse et al., 2003) and the Toll humoral pathway may be involved in hemocyte proliferation

(Qiu et al., 1998). These studies indicate that the insect cellular and humoral immune response are not exclusive of each other.

The three main humoral insect immunity pathways are the Toll, immunodeficiency (IMD), and the Janus kinase signal transducer and activator of transcription (JAK/STAT) (Fig. 1.1). These pathways produce effector molecules that target pathogens. The Toll pathway has traditionally been associated with immune defense against gram-positive bacteria (Filipe et al., 2005; Rutschmann et al., 2002) and fungi (Gottar et al., 2006; Lemaitre et al., 1996). Recently, the Toll pathway has been found to be associated with anti-viral immunity in Drosophila (Ferreira et al., 2014; Zambon et al., 2005) and mosquitoes (Luplertlop et al., 2011; Xi et al., 2008). As reviewed in Hoffman (2003), in Drosophila this pathway is activated by spaetzle protein binding to the Toll receptor (Hoffmann, 2003; Trinchieri and Sher, 2007). Spaetzle activation results from a proteolytic cascade that becomes activated by the pathogen (Jang et al., 2006). Then the myeloid differentiation primary response 88 (myD88) and tube adaptor proteins are activated, which in turn activate pelle. Pelle has kinase activity that results in the phosphorylation of cactus, which is an inhibitory protein of nuclear factor- kappa b (NF-$_{kB}$) transcription factors dorsal related immunity factor (dif, larvae) and dorsal (adult) (Filipe et al., 2005; Gottar et al., 2006). Mosquitoes have a dorsal orthologue, isoforms Relish 1A and 1B (Rel1-A and Rel1-B) (Shin et al., 2005) and Relish orthologue Rel2, isoforms, long, short, and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor ($I_K$B-type) (Shin et al., 2002). This interaction causes cactus to be degraded and releases NF-$_{kB}$ homologs, dif/dorsal, which are transcription factors for anti-pathogen effectors.

The IMD pathway also activates a NF -$_{kB}$ -like Relish, but this pathway is associated with the immunity against gram-negative bacteria (Hoffmann and Reichhart, 2002; Kaneko and

Silverman, 2005; Kaneko et al., 2005, 2004) and recently with viral immunity (Avadhanula et al., 2009; Lemaitre et al., 1995). As reviewed in the literature a pathogen activates the peptidoglycan recognition protein LC, PGRP-LC, receptor, which leads to the activation of caspase-8 like death-related ced-3/Nedd2-like (DREDD) and Fas-associated protein with death domain (FADD), and a mitogen activated protein (MAP) kinase, TGF-beta activating kinase 1 (TAK1) (Hoffmann and Reichhart, 2002). TAK1 activates a signalsome and DREDD and FADD cleave the inhibitory domain of a transcription factor called Relish. This cleavage leads to the production of anti-pathogen effectors. Sometimes the IMD pathway is associated with the JNK (c-Jun N-terminal kinase pathway) (Delaney et al., 2006; Silverman et al., 2003), which is involved in apoptosis, (Moreno et al., 2002) melanization, (Bidla et al., 2007) defense of bacterial infection (Brandt et al., 2004; D. S. Schneider et al., 2007) and with the immune response of C6/36 A. albopictus cells to WNV infection (Mizutani et al., 2003). Recently, the IMD and JNK have also been implicated in D. melanogaster immunity to Nora virus (Cordes et al., 2014). The IMD pathway has also been shown to play a role in Sindbis virus (SINV) (Huang et al., 2013) and cricket paralysis virus (CrPV) (Costa et al., 2009) immunity in in D. melanogaster. The IMD pathway has also been shown to be upregulated in the salivary glands of Ae. aegypti during DENV infection (Luplertlop et al., 2011). Overall though, there is currently little evidence for a large role of this pathway in arboviral infection in mosqutioes.

The JAK/STAT pathway is associated with anti-viral defense in Drosophila (Agaisse and Perrimon, 2004; Dostert et al., 2005) , during dengue infection of Ae. aegypti (Souza-Neto et al., 2009; Xi et al., 2008) and WNV infection of Culex cell lines (Franz et al., 2014). This pathway is associated with hemocytes and the fat body (Agaisse et al., 2003; Dostert et al., 2005). As described in previous reviews, the JAK/STAT pathway in Drosophila involves binding of the

cytokine ligand unpaired (UPD) to the DOME receptor, domeless (Agaisse and Perrimon, 2004). This causes DOME to by phosphorylated by hopscotch (HOP), a receptor associated JAK tyrosine kinase. This causes the recruitment of STAT, a cytoplasmic transcription factor that translocates into the nucleus and activates anti-pathogen effectors. Recent studies in Drosophila indicate that the JAK/STAT pathway is anti-viral (Cordes et al., 2014; Kemp et al., 2013), but it is specific to certain viruses, while other viruses are unaffected by alterations in this pathway (Kemp et al., 2013). However, for the most part the JASK/STAT pathway is broad against non-arboviral pathogens and Drosophila specific. There are only a few studies that show a relationship between this pathway and arboviral infection in mosquitoes or mosquito cell lines (Franz et al., 2014; Souza-Neto et al., 2009; Xi et al., 2008).

Apoptosis and immunity

The apoptosis pathway is known to be associated with anti-viral defense in many organisms. This pathway involves an activation of caspases that result in the death of a cell (Salvesen and Duckett, 2002). This pathway can be activated by cellular (Han et al., 2000; Kumar et al., 2004) and humoral immunity (Horng and Medzhitov, 2001; Sanders et al., 2005). In response, many viruses possess apoptosis inhibitors or, in some cases, hijack the pathway to aid in viral proliferation (Best, 2008). There is some evidence that apoptosis pathways are associated with anti-viral immunity in mosquitoes. Apoptosis-like phenomena have been seen in the salivary glands and midguts of Culiseta melanura infected with eastern equine encephalitis virus (EEEV) (Scott and Lorenz, 1998), Culex pipiens infected with West Nile virus (WNV) (Girard et al., 2007, 2005; Vaidyanathan and Scott, 2006), Ae. albopictus infected with SINV (Bowers et al., 2003), and Ae. aegypti infected with Semliki Forest virus (SFV) (Mims et al., 1966). In other studies, changes in apoptotic gene expression during DEV infection of Ae. aegypti suggested a potential role for this pathway (Xi et al., 2008). The Toll pathway, which

has been implicated in anti-viral immunity in mosquitoes (Xi et al., 2008), has also been found to be associated with the apoptosis pathway in Drosophila (Horng and Medzhitov, 2001) and Ae. aegypti (Sanders et al., 2005). Bryant et al. (2008) determined that there is an expansion of anti-viral apoptosis related genes in Ae. aegypti as compared to Drosophila, which was hypothesized to be an immune mechanism developed to combat the increased pathogen exposure in blood feeding insects (Bryant et al., 2008). More recently alterations in the apoptosis pathway in A. aegpyti led to changes in the SINV replication and dissemination (Wang et al., 2012) and similar results were seen with flock house virus (FHV) replication in Drosophila (Liu et al., 2013).

## Small RNAs and anti-viral immunity

The small RNA pathways have been shown to be vital to antiviral defense in insects. RNA interference (RNAi) post-transcriptionally silences viral genes in response to the viral dsRNA. Many RNA viruses like DENV are single stranded, but they have double stranded replicative intermediates (Chambers et al., 1990) and secondary structures, that are thought to be the targets for RNAi (Sanchez-Vargas et al., 2004). These replicative intermediates are associated with the cellular derived membrane bound vesicles (Khromykh et al., 2000; Mackenzie et al., 1998) and are composed of numerous nascent positive sense RNA bound to an antisense complementary strand (Vaughan et al., 2002). There are 3 main classes of small RNAs that have been found to be associated with modulation of virus infection, the small interfering RNA (siRNA), microRNA (miRNA) and P-element-induced wimpy testis (PIWI)-associated RNA (piRNA).

### miRNA pathway and anti-viral immunity

The miRNA pathway is activated by small stem loop dsRNAs. These dsRNAs called long primary RNA (pri-miRNA) can be cellularly derived in the nucleus or may have viral

origins. This pri-miRNA is cleaved by an RNase, Drosha, to form pre-miRNA before being translocated out of the nucleus by an exportin. Once in the cytoplasm, another RNase, Dicer1 (Dcr1), further cleaves and processes the pre-miRNA to a mature miRNA, which is a 17-24 base pair (bp) partially double stranded RNA (dsRNA). One strand of the miRNA, the guide strand, is then loaded into the RNA induced silencing complex (RISC) for use in targeting mRNA for translational suppression. The RISC contains an RNase, Argonaute 1 (Ago1), that facilitates RNA cleavage and degradation thus reducing protein translation of the transcript (Höck and Meister, 2008).

There is evidence that anti-viral immunity is associated with the miRNA pathway (Asgari, 2015). Drosophila X virus (DXV) immunity in D. melanogaster may be attributed to the miRNA pathway (Zambon et al., 2005). Keene et al. (2004) demonstrated that the miRNA pathway may be important to o'nyong nyong virus (ONNV) infection in A. gambiae by knocking down Dcr1, which increased ONNV titer (Keene et al., 2004). QTL mapping studies by Bernhardt et al (2012) showed no association between, Ago1, Dcr1 and R3D1, and DENV2 susceptibility in Ae. aegypti. However, this study was inconclusive because a chromosomal inversion blocked recombination (Bernhardt et al., 2012). Viruses may also produce small viral RNAs to regulate their own replication processes. In a recent study, one DENV derived small RNA may have regulated the replication of NS1 (Hussain and Asgari, 2014); however, the DENV derived small RNA was likely expressed at levels too low to influence NS1 expression to the degree seen in the study (Skalsky et al., 2014). Therefore, there is some evidence that the miRNA pathway may play a role in the anti-viral response to viral infection, but the potential role of miRNA in the self-regulation of viral replication is still undetermined.

siRNA pathway and anti-viral immunity

The siRNA pathway is also associated with silencing of selfish genetic elements (endo-siRNA), but appears to also play a large role in anti-viral immunity (exo-siRNA) (Blair and Olson, 2014; Blair, 2011; Gammon and Mello, 2015). As depicted in figure 1.2, during viral infection, Dicer 2 (Dcr2) cuts viral dsRNA into 21-23 nt RNAs, which are loaded into the RISC (Du and Zamore, 2005; Zamore et al., 2000). The ssRNA produced and use by the RISC as a guide to target complementary viral RNA sequences. The RISC is composed of Dcr2, R2D2, a double stranded RNA binding protein (dsRBP), and Argonaute 2 (Ago2), which cleaves the viral region targeted by the ssRNA. The viral sequence targeted is then degraded if there is perfect base pairing; if not then there is often translation silencing. Translational silencing involves RISC complex or complementary target sequence binding to a transcript to block its translation, but not resulting in the immediate degradation of the transcript.

The siRNA pathway has been determined to be primarily anti-viral as opposed to the miRNA and piRNA pathway whose primary purposes is developmental, regulatory or for protection against selfish elements. However, there is evidence that the piRNA pathway is also antiviral (Schnettler et al., 2013; Vodovar et al., 2012), but the siRNA pathway is the primary antiviral pathway amongst the small RNA pathways. Firstly, in D. melanogaster, there is an increased rate of evolution in the siRNA pathway compared to the miRNA pathway, presumably a result of positive selection due to counterdefense to viral evolution (Obbard et al., 2006). Also, during viral infection, there is often an accumulation of virus-derived siRNAs (viRNAs), mostly siRNA pathway associated, and viruses often encode suppressors of RNAi. Both of these observations suggest the importance of the RNAi pathway in anti-viral defense (Ding and Voinnet, 2007; Li and Ding, 2006; Wu et al., 2010). In D. melanogaster, the siRNA pathway has been associated with anti-viral defense against FHV (Galiana-Arnoux et al., 2006; C. Kemp

et al., 2013; Li et al., 2002; Wang et al., 2006), Drosophila C virus (DCV) (Galiana-Arnoux et al., 2006; Kemp et al., 2013; van Rij et al., 2006), Drosophila X virus (DXV) (Kemp et al., 2013), cricket paralysis virus (CrPV) (Kemp et al., 2013; Wang et al., 2006), Sindbis virus (SINV) (Galiana-Arnoux et al., 2006; Kemp et al., 2013), WNV (Chotkowski et al., 2008), and vesticular stomatitis virus (VSV) (Kemp et al., 2013; Zambon et al., 2006).

In mosquitoes, there is evidence that the siRNA pathway is involved in anti-viral defense against arboviruses. Anopheles gambiae had increased ONNV loads after silencing the Ago2 in the siRNA pathway (Keene et al., 2004). The siRNA pathway is involved in regulation of SINV in Ae. aegypti. Sindbis viral load was increased after dsRNA knock downs of Tudor staphylococcal nuclease (TSN), a member of the RNAi RISC (Campbell et al., 2008), in Dcr2 defective mosquito strains (Khoo et al., 2010) and in the presence of the RNAi inhibitor, Flock House Virus-B2 (FHV-B2) (Cirimotich et al., 2009). Accumulation of viral associated small RNAs in other studies indicate that the mosquito siRNA pathway targets SINV (Campbell et al., 2008; Khoo et al., 2010).

Studies have also shown that during DENV infection, virus-derived interfering RNAs (viRNAs) correspond with an increase in viral titer and decrease in extrinsic incubation time when components of the RNAi pathway were knocked down (Sanchez-Vargas et al., 2009). Knocking down the expression of members of the RNAi pathway caused an increase in viral titer and dissemination in Ae. aegypti engineered to have an RNAi based resistance to DENV2 (Franz et al., 2006). Additionally, Dcr2 knock down studies indicate that Rift Valley fever virus (RVFV) infection is altered by the RNAi pathway in mosquito cells (Léger et al., 2013). A recent study also demonstrated that Dcr2 genotype influenced Ae. aegypti susceptibility to DENV infection (Lambrechts et al., 2013). Additionally, gene expression studies have shown a small

but significant increase in Dc2 and R2D2 expression in response to DENV infection in Ae. aegypti (Bonizzoni et al., 2012a).

The accumulation of the virus derived siRNAs (viRNAs) in multiple studies also suggests that the siRNA pathway is involved in the anti-viral response. In a Drosophila model, Dcr2 was required for the formation of viRNAs, while R2D2 was not vital to viRNA production (Wang et al., 2006). In another study, there were viRNAs detected in DXV infection in Drosophila, but this accumulation of viRNAs was not associated with Dcr2, and therefore it was hypothesized that either the miRNA or piRNA pathway were involved in this process (Zambon et al., 2006). Therefore, there is an abundance of evidence to support the involvement of the siRNA pathway in the anti-viral immune response to arboviral infection.

piRNA pathway and anti-viral immunity

The piRNA pathway involves PIWI associated proteins that are in the Ago protein family. The three known Drosophila PIWI proteins are Piwi, Aub, and Ago3. The piRNAs are either maternally derived, meaning they are deposited in the maturing oocyte from the maternal nurse cells, or they result from a transposon created piRNA cluster (Senti and Brennecke, 2010). In Drosophila, these piRNAs are associated with a piwi/Aub or Ago3 complex that cleaves and processes the target, which inactivates the target and creates another piRNA that can associate with another Piwi-associated complex (Senti and Brennecke, 2010). This process continues creating a loop of gene silencing referred to as the ping-pong model (Aravin et al., 2007). This pathway is typically associated with the regulation of selfish elements, like transposons, and early development, but has also been found to inhibit viral replication (e.g. DXV infection in Drosophila) (Zambon et al., 2006). However, Ago3 and PIWI were found to be the only two piRNA pathway proteins in Ae. aegypti associated with anti-viral activity (Miesen et al., 2015).

Furthermore, Ago3 dsRNA knock down experiments demonstrated that An. gambiae was more susceptible to ONNV infection with an impaired piRNA pathway (Keene et al., 2004). More recent studies have demonstrated that the piRNA pathway plays a direct role in the DENV antiviral response in Ae. aegypti (Hess et al., 2011), SINV infection in mosquito cell lines (Vodovar et al., 2012), CHIKV infection in Ae. aegypti and Ae. albopictus (Morazzani et al., 2012) and in SFV infection in mosquito cell lines (Schnettler et al., 2013; Siu et al., 2011). Multiple studies on the anti-viral response in mosquitoes and mosquito cell lines have also demonstrated the accumulation of piRNA associated small RNAs, given further proof for the role of this pathway in mosquito anti-viral immunity (Léger et al., 2013; Morazzani et al., 2012; Vodovar et al., 2012).

## Small RNA pathway components

The small RNA pathways are composed of many proteins that interact with dsRNA in order to regulate their expression. As discussed previously, there is evidence that these pathways are associated with DENV2 susceptibility in Ae. aegypti and therefore the molecular biology of these proteins and interactions between these proteins are important in understanding their role in anti-viral immunity.

### Dicers

Dicers are a RNAse III enzymes, that recognize and cleave dsRNA into 19-27 nucleotide small interfering RNA (siRNA) or micro RNAs (miRNA) (Hammond, 2005). They contain DexD/H-box RNA helicase domains and a Piwi/Argonaute/Zwille (PAZ) domain, as well as a double stranded RNA binding domains (dsRBD) and two RNase III cleavage domains. The PAZ domain binds to the 3’ overhang of the siRNA and miRNA, and one RNase III domain binds each RNA strand (Hammond, 2005; Yan et al., 2003). The dsRBD binds dsRNA between the RNase and PAZ binding sites, whereas the function of the helicase domain is related to signal transmission (Kemp and Imler, 2009).

Dicer 2 is the initiator of the siRNA pathway by recognizing and cleaving dsRNA (Bass, 2000; Tabara et al., 2002) while dicer 1 (Dcr1) recognizes and cleaves pre-miRNA in the miRNA pathway (Kim, 2005; Lee et al., 2004) . There is also some indication that Dcr1 and Dcr2 play a role in RISC assembly (Pham et al., 2004; Schwarz et al., 2003; Tomari et al., 2004). The cleavage of dsRNA by Dcr2 is aided by ATP (Liu et al., 2003; Nishikura, 2001). Dcr2 then loads the siRNA into Argonaute 2 (Ago2) and remains associated with the complex (Bernstein et al., 2001).

RNA induced silencing complex (RISC)

The RISC is a group of proteins that facilitate the loading, targeting and cleavage of dsRNA via the siRNA and miRNA pathways (Kobayashi and Tomari, 2015). The currently known members of the RISC are the argonautes (Ago1 or Ago2), R2D2, fragile X mental retardation protein (Fmr-1) and vasa intronic gene (VIG), but it is hypothesized that there are still many unknown components to the RISC (Schwarz et al., 2004). In the case of the siRNA pathway, target pairing is exact, which results in the mRNA cleavage as the mechanism of gene silencing (Zeng and Cullen, 2003). In the miRNA pathway, there is only a small part of the miRNA called the seed sequence that is required for target pairing. This incomplete match pairing tends to cause the blockage of translation initiation, which is the primary mechanism of silencing in this pathway (Iwasaki et al., 2009; Pillai, 2005; Zeng and Cullen, 2003), but there is some indication that like the siRNA pathway, the miRNA pathway can have a role in mRNA degradation as well (Bagga et al., 2005).

R2D2 and R3D1(Loquacious/Loq)

R2D2 and R3D1/Loq are associated with the loading of either the siRNA, or the miRNA, respectively into the RISC. They are both dsRNA binding proteins (dsRBP) that bind the

siRNA or miRNA and thereby play some role in determining the guide strand incorporated into the RISC (Preall et al., 2006).

### The Argonautes (Ago) and PIWI proteins

Argonaute proteins contain PAZ and PIWI domains. The PIWI domain attaches to the 5' end of the strand used to target complementary dsRNA, known as the guide strand, and the PAZ domain binds the 3' end of this same strand (Lingel and Izaurralde, 2004; Lingel et al., 2003; Ma et al., 2004; Yan et al., 2003). The PIWI domain has RNase H endonuclease activity that provides the slicer activity for the cleavage of the target dsRNA.

Argonaute proteins provide the slicing activity in the RISC, Ago2 being associated with the siRNA associated RISC, and Ago1 being associated with the miRNA associated RISC. However, Ago1 and Ago2 are not exclusive to their respective pathways. In Drosophila, Ago1 interacted with both miRNAs and siRNAs (Caudy et al., 2003; Förstemann et al., 2007; Okamura et al., 2004), but if miRNAs were not associated with Ago1 and siRNAs were not associated with Ago2 the miRNA or siRNA pathways had reduced or no function (Förstemann et al., 2007). Additionally, argonaute 3 (Ago3) associates with the transposon derived piRNAs to target and cleave complementary RNA (Angelica and Fong, 2008). Aubergine (Aub) and PIWI also contain a PIWI cleave domain and they associate with piRNAs, but they contain anti-sense transcript that target RNA (Ku and Lin, 2014).

### Other RISC components

Other proteins have been found to be associated with the RISC. Fmr1, the Drosophila homolog of Fragile x, VIG, a protein from the intron of the Vasa gene,(Caudy et al., 2002), Dmp68, an RNA helicase, (Ishizuka et al., 2002) and Tudor-SN, potentially involved in product degradation, have all been found to associated with the Drosophila RISC (Hammond, 2005). Fmr1 is a negative translational regulator that binds dsRNA (Wan et al., 2000). While not

required for the RISC, Fmr1 has been shown to associate with Ago2, Dcr2, Dmp68, Ago1 and miRNAs in vivo resulting in the increased efficiency and stability of the RISC (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004) VIG also has an RNA binding domain (Caudy et al., 2002). Dmp68 unwinds short dsRNA and appears to be required for RNAi in D. melanogaster. It either mediates unwinding of the siRNA duplex to create an active RISC, or is involved in later RNA recognition (Ishizuka et al., 2002).

VSRs

The anti-viral RNA response to viral infection probably led viruses to evolve suppressors of RNAi (VSRs) (Obbard et al., 2009). Insect viruses such as FHV, DCV, and CrPV had all been found to encode proteins that are suppressors of RNAi (Nayak et al., 2010; Wang et al., 2006). Another study showed that dsRNA knock downs of FHV-B2, and DCV-1A in transgenic D. melanogaster expressing FHV-B2, and FHV-1A genes had increased titers of DCV (Berry et al., 2009). In mosquitoes, studies demonstrated that expression of FHV-B2 reduced the number of viRNAs and increased viral titers during SINV and ONNV infection (Cirimotich et al., 2009; Myles et al., 2008).

Multiple mechanisms of viral counterdefenses to RNAi immunity have been seen in many studies. Viruses have been shown to prevent Dcr processing by binding of VSRs to dsRNA (Chao et al., 2005), or by binding the small RNA to prevent loading in the RISC (Lakatos et al., 2006), or by directing or interfering with the interaction with proteins in the RNAi pathway (Bortolamiol et al., 2007; Chapman et al., 2014; Moon et al., 2012; E. Schnettler et al., 2013; Zhang et al., 2006). The production of subgenomic flavivirus RNAs (sfRNAs) during flavivirus infection has been suggested as playing a role in viral counter defenses (Chapman et al., 2014). The structure of the sfRNA blocks the cellular endonuclease Xrn1 from

cleaving viral RNA. DCV 1A has been shown to bind dsRNA to prevent cleavage by Dcr2 (Nayak et al., 2010) and FHV B2 binds dsRNA to prevent Dcr2 processing (Chao et al., 2005; Galiana-Arnoux et al., 2006; Lu et al., 2005). Additionally, flavivirus proteins have been shown to inhibit autophagy and apoptosis, which would reduce the spread of viral infection (Roy et al., 2014).

## Anti-viral immunity in mosquitoes: the big picture

As it has been suggested throughout this review, many anti-viral immune response pathways are involved in immunity to viral infection in insects. After studying FHV, and DCV infection in D. melanogaster, Galiana-Arnoux et al. (2006) proposed that the immune system in D. melanogaster is driven by the RNAi pathway, and various cytokine signaling pathways (Galiana-Arnoux et al., 2006). More specifically this study suggested that the RNAi pathway limits viral replication in cells while the cytokine signaling pathways signals the creation of antiviral effectors to prevent spread of the infection to uninfected cells. During viral infection, the cellular pathway can trigger the JAK/STAT pathway in nearby cells, can produce anti-viral molecules and can trigger apoptosis. Apoptosis pathway inhibitor, IAP2, is necessary for signaling in the IMD and JNK pathways of D. melanogaster (Gesellchen et al., 2005). In D. melanogaster, the anti-viral response is regulated by the siRNA (Zambon et al., 2006), JAK/STAT (Dostert et al., 2005) and the Toll pathways (Zambon et al., 2005).

Arboviral immunity in mosquitoes, such as Ae. aegypti, is likely governed by different mechanisms than the Drosophila response to insect viruses. Firstly, mosquito arboviral infection rates are low and there is little impact of arboviral infection on mosquito survivorship or fecundity; therefore, there is little selection pressure to develop a strong response to arboviral infection (Blair and Olson, 2014). On the other hand, the Drosophila viruses, such as DCV,

typically have large impacts on host fitness (Gravot et al., 2000), which could potentially result in stronger, broader immune response. Additionally, insect viruses tend to have VSR to combat these strong immune responses, but VSRs have not been identified for arboviruses. Mosquito viruses that are not arboviral have also been recently found to encode VSRs (Van Cleef et al., 2014) indicating that the mosquito immune response to arboviruses may be reduced. The strongest evidence for siRNA pathway involvement in anti-arboviral immunity are from studies where the siRNA pathway is impaired artificially prior to an infected blood meal (Keene et al., 2004; Khoo et al., 2010; Khoo et al., 2013a; Sánchez-Vargas et al., 2009), whereas studies that assess natural changes in siRNA pathway gene expression in response to infection demonstrate only small changes in expression (Bonizzoni et al., 2012b). In addition, blood feeding in the absence of virus induces the expression of immune genes (Bonizzoni et al., 2011), thereby indicating siRNA genes may be upregulated during oral infection for purposes other than regulation of arboviral infection. The siRNA and piRNA pathways have other functions that may be driving their expression in mosquitoes, but the large increase in arboviral infection and dissemination rates in mosquitoes with a reduced siRNA response is strong evidence for the role of this pathway in mosquito arboviral immunity.

The Toll and c-Jun kinase and JAK/STAT pathways were also found to be activated in Ae. aegypti during dengue infection (Sanders et al., 2005; Xi et al., 2008). The Toll pathway has also been linked to autophagy, a cell degradation mechanism, during viral infection (Moy et al., 2014). However, these pathways are typically associated with anti-microbial immunity and the anti-viral mechanisms of these pathways are not well understood. Additionally, in comparison to the siRNA and piRNA pathways there is less evidence for the involvement of the

Toll and c-Jun kinase and JAK/STAT pathways in the regulation of arboviral infection in mosquitoes.

## Anti-viral immunity and vector control

The evolving understanding of insect immunity has begun to complement new genetic control technologies. The two current strategies employed for genetic vector control involve genetic modification of naturally occurring effector genes or the introduction of an engineered immunity gene. In both cases, the expression of these genes is enhanced by specialized promoters, typically inducible upon uptake of the pathogen and typically tissue specific. Early genetic control experiments used virus expressed antibodies that blocked sporozoite infection of salivary glands of An. gambiae (de Lara Capurro et al., 2000). Genetically modified A. gambiae have also been created to express effector genes that also blocked sporozoite infection (Ito et al., 2002; L. a Moreira et al., 2002). Since this time, there have been dozens of anti-microbial peptides used to modify the immune response of A. gambiae to Plasmodium spp. infection (Carter and Hurd, 2010). Modifying the expression of the naturally occurring Cecropin A and Defensin A genes have also been used to modify the immune response of Ae. aegypti to bacterial infection (Shin, 2003). Other groups also engineered Ae. aegypti or An. gambiae to have modified expression of anti-microbial effectors (Kokoza et al., 2000). Another study created a RNA interference based refractory gene that used the siRNA pathway to target the prM gene of DENV2 (Franz et al., 2006). More recently, an apoptosis pathway has been modified to suppress DENV infection in Ae. aegypti (Carter et al., 2014) and certainly as the genetic manipulation technologies improve, new methods for developing refractory vectors will be established in the future.

Recently, a release of insects with dominant lethality (RIDL) strain of Ae. aegypti (Alphey and Andreasen, 2002; Phuc et al., 2007; Thomas et al., 2000) was shown to suppress

wild populations of Ae. aegypti (Harris et al., 2012). These conditional, dominant, sex-specific and lethal systems have provided a useful tool for the suppression of mosquito populations. Modified traditional sterile insect technique (SIT) methods are also being considered for mosquito population suppression (Bouyer and Lefrançois, 2014) . After population suppression, a vector population replacement strategy could then be used to replace the wild-type population with one refractory to pathogen, thus reducing the possibility for pathogen transmission. Currently, the population replacement strategy is in its infancy, with only proof of concept experimentation thus far, but with the advancement of transgenic technologies, a population reduction strategy may be an important part of an integrated vector management program (Robert et al., 2014).

Genetically modified mosquitoes for vector control

One of the major obstacles to the utilization of transgenic mosquitoes for vector control is the lack of a strategy to drive these modified genes into a natural population. These drive mechanisms are needed to compensate for the small numbers of transgenics in the population compared to non-transgenics, and they will also have to compensate for any fitness reduction in the transgenic vector that may prevent transgene maintenance in the population. The currently proposed gene drive mechanism for vector control are underdominance, killer-rescue systems, the paratransgenic Wolbachia system, homing endonucleases, the Medea systems and CRISPR/Cas9 systems (Alphey et al., 2013; Gantz et al., 2015; Sinkins and Gould, 2006). With underdominance, transgenic mosquitoes are released that contain toxin genes associated with the gene of interest and an unlinked antidote gene which will protect the mosquito from the effects of the toxin gene (Akbari et al., 2013; Davis et al., 2001; Magori and Gould, 2006). Killer rescue approaches use a lethal transgene and an unlinked repressor gene (Gould et al., 2008). These systems are self-limiting and easily allow vector control programs to limit the geographic

dispersal of the transgene. In one example of this strategy, Semele (Marshall et al., 2011), males are engineered to express a toxin that kills or renders females infertile that do not express the antidote to the toxin. This toxin and repressor system can be linked to an anti-pathogen gene, and thus only females with this transgene will be able to reproduce if mated with the transgenic male. This system unfortunately requires a very large initial population of transgenic vectors.

In the Wolbachia system, an endosymbiont bacterium of mosquitoes, the Wolbachia bacteria will be genetically transformed with the gene of interest and will be driven into the population with its natural drive mechanism (Curtis, 2006; Hancock et al., 2011). Basically, females have to be infected with the same Wolbachia spp. as their male mating partner, otherwise the progeny are unviable. Likewise, the progeny of any mating with a Wolbachia infected female will also be infected with Wolbachia. So, Wolbachia infected females have an advantage over non-infected females, and the Wolbachia is able to quickly drive itself through the population (Rasgon, 2008). Recent studies have shown that Wolbachia may reduce DENV infection rates in Ae. aegypti (Frentiu et al., 2014), which is an additional benefit to this approach. The main drawback to the system is the difficulty of transforming the Wolbachia to express genes of interest.

Homing endonucleases (HEGs) have also been proposed as genetic drive systems for population replacement genetic vector control strategies (Burt, 2003). HEGs induce breaks in dsDNA that results in HEG being copied into the broken chromosome during repair, thus resulting in the integration of additional copies of the HEG associated genes into the genome (Goddard et al., 2001). A recent study showed that the HEG, I-SceI, could be successfully driven into an A. gambiae laboratory cage population and provided proof of principal for a population replacement strategy for vector control (Windbichler et al., 2011).

Lastly, the Medea system is based on the Medea element, which is a naturally occurring selfish genetic element, that results in the death of all offspring without the element (Beeman et al., 1992; Wade and Beeman, 1994). These elements have been artificially constructed in the lab and essentially consists of a maternally expressed toxin and a zygotically expressed antidote that results in the death of offspring that do not contain the antidote (Beeman and Friesen, 1999) or the converse where the toxin is zygotically expressed and the antidote maternally (Marshall and Hay, 2011). An anti-pathogen transgene can then be linked with this system and driven into a population. Essentially, all of these drive systems have shown potential, but have not been evaluated extensively, so they will require more evaluation before their utility in a vector replacement control strategy is understood.

The two current approaches to mosquito control are strategies that reduce or eliminate the abundance of mosquito populations and those which replace the wild mosquito populations with individuals that are engineered to be refractory to pathogen transmission. In recent years the former approach has been successful with lethal dominant strategies (Alphey and Andreasen, 2002; Phuc et al., 2007; Thomas et al., 2000). The latter technique involves the insertion of an anti-pathogen effector gene into the germline of a mosquito via a linked transposon and injection of a corresponding transposase. This gene is under the control of an engineered tissue specific promoter and carries a marker gene for identification. Fitness effects associated with this transformation can result from the transgene product(s), the location of the insertion site, or from inbreeding in the parent line. There are multiple studies of the fitness of transgenic Ae. aegypti and many of these studies have found a fitness cost associated with transgenesis. These fitness problems have been attributed to the transgene marker, but in other mosquitoes, such as An.

stephensi fitness costs due to transgensis varied (Catteruccia et al., 2003; C. Li et al., 2008), sometimes even being neutral or advantageous (Amenya et al., 2010; Moreira et al., 2004).

Current research goals

The following chapters examine the vector competence of Ae. aegypti for DENV2 with emphasis on the RNAi pathway. Chapter 2 explores the fitness and vector competence of transgenic mosquitoes created to be refractory to DENV2 infection by utilizing the RNAi response to target the virus. These studies give insight into the possibility of using transgenic mosquitoes that use an RNAi based resistance to DENV2 in a vector control program for DENV transmission. Chapter 3 examines the utility of integrating an RNAi based transgene engineered for resistance to DENV2 into a genetically diverse laboratory strain (GDLS) to improve the fitness of the transgenic mosquito. Chapters 2 and 3 provide preliminary evidence that a population replacement strategy using an RNAi based transgenic may be possible if the effects of the integration site are evaluated and the transgene is introgessed into a GDLS population.

Chapter 4 examines the expression of immunity related genes associated with vector competence of Ae. aegypti for DENV. This study uses two different Ae. aegypti strains, D2MEB, selected for a midgut escape barrier to DENV2, and D2S3, selected for a limited midgut escape barrier to DENV2. These strains are used to examine whether anti-viral gene expression is associated with MEBs. This chapter also examines the expression of the siRNA pathway genes over the lifetime of the mosquito and also examines the change in the expression of the genes throughout the day. Chapter 4 attempts to determine whether transcriptional changes in gene expression are associated with viral infection and midgut escape barriers. Finally, Chapter 5 examines the association of DENV2 susceptibility in Ae. aegypti with four SNPs in the siRNA pathway gene, Dcr2, within 12 Senegalese populations. This population study attempts to link a single nucleotide polymorphism (SNP) to DENV susceptibility. So,

basically both Chapters 4 and 5 study the relationship between the vector competence of Ae. aegypti and ant-viral immune pathways from a transcriptional and genetic level.

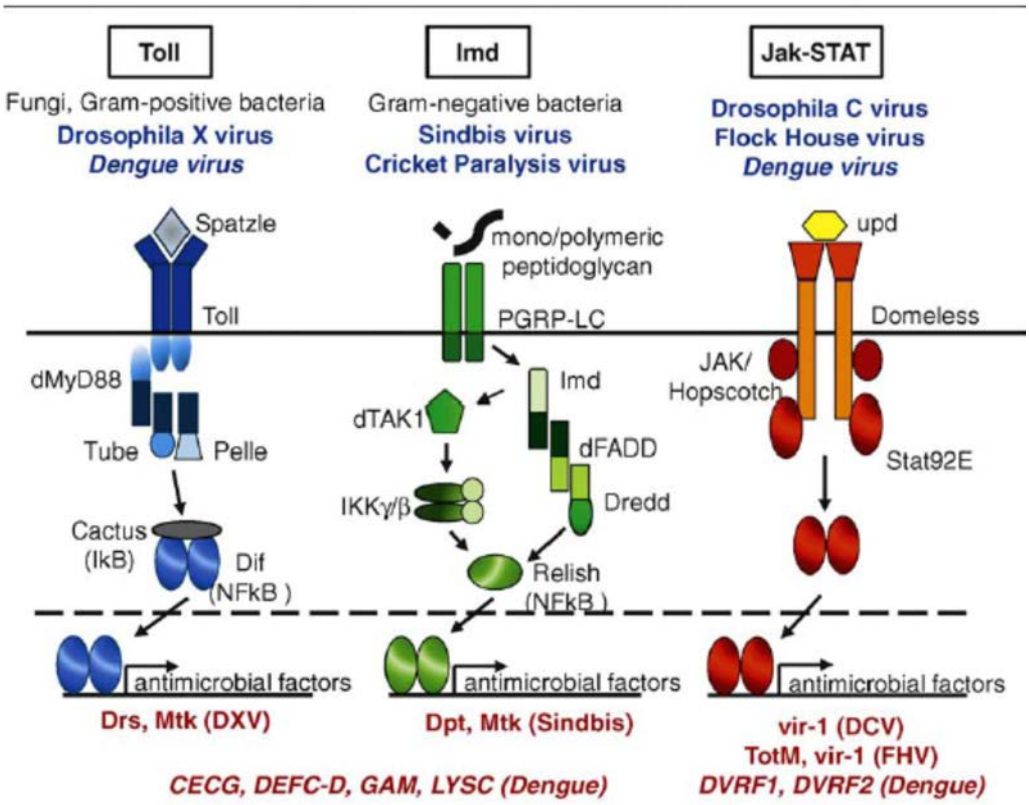



Figure 1.1: Humoral immune pathways in response to virus infections. Adapted from Sabin et al., (2010). Left- The Toll pathway is primarily involved fungi and gram-positive bacteria immunity, but has also been shown to be involved in viral and arboviral immunity. Spatzle becomes activated in the presence of a pathogen and triggers a proteolytic cascade resulting in the formation of antimicrobial factors, drs, mtk, CECG, DEFC-D, GAM, LYSC. Middle- The Imd pathway is associated with gram-negative bacteria immunity, but has also been shown to be involved in viral and antiviral immunity. The Imd pathway is a caspae-8 like pathway that leads the production of antimicrobials dpt, mtk, CECG, DEFC-D, GAM, LYSC. Right- The JAK/STAT pathway is more commonly associated with anti-viral immunity in insects. This pathway produces multiple anti-viral effector molecules including TotM, vir-1, DVRF1 and DVRF2. Abbreivations: CECG- cecropin G; DCV- Drosophila C virus; DEFC-D- defensin D; dFADD- Fas-associated protein with death domain; Dif- dorsal related immunity factor; dmyD88- myeloid differentiation primary response 88; Dpt- diptericin; Dredd- death-related ced-3/Nedd2-like; Drs- drosomycin; DVRF- dengue virus restriction factor; FHV- flock house virus; GAM- gambicin; DXV- Drosophila X virus; IkB- inhibitor of kappa B; IKKγ/β- inhibitor of kappa gamma kinase beta; Imd- immunodeficiency; JAK- janus kinase; Jak-STAT- Janus kinase-signal transducers and activators of transcription; LYSC- lysozyme C; Mtk- metchnikowin; NFkB- nuclear factor- kappa B; PGRP-LC- peptidoglycan recognition protein LC receptor; Stat92E- signal-transducer and activator of transcription protein at 92E; TAK1- TGF-beta activating kinase 1; TotM- Turandot M; upd- unpaired; vir-1- virus-induced RNA 1

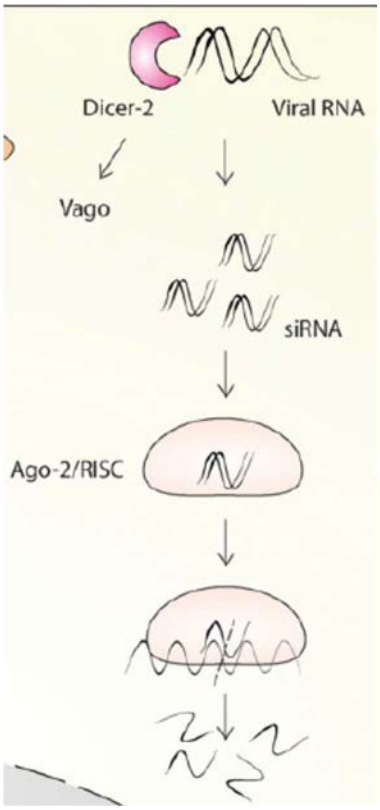



Figure 1.2: siRNA pathway response to virus infections. Adapted from Merkling and van Rij (2013). In the cytoplasm, dicer-2 recognizes and cleaves the viral derived double-stranded RNA(dsRNA) into small interfering RNA (siRNA). The siRNA is then incorporated in an Argonaute-2 (Ago-2) and RNA-induced silencing complex (RISC), which identify and cleave the complementary viral RNA sequences.

# CHAPTER II: FITNESS IMPACT AND STABILITY OF A TRANSGENE CONFERRING RESISTANCE TO DENGUE-2 VIRUS FOLLOWING INTROGRSSION INTO A GENETICALLY DIVERSE AEDES AEGYPTI STRAIN

## Introduction

This chapter includes work published by Franz et al., (2014). With increasing advancements in transgenic technologies, and the increasing need for novel vector control strategies, the use of genetically-modified mosquitoes for vector control is closer to becoming a reality. However, gene replacement in the field requires stable, multi-generational effector gene expression that is functional across diverse genetic backgrounds. The effector genes developed for these purposes should have minimal fitness costs relative to wild-type mosquitoes (Beaty, 2000; James, 2005; Lambrechts et al., 2008; Scott et al., 2002). There have been multiple studies that demonstrate evidence of a fitness load associated with transgenes (Catteruccia et al., 2003; Nic Irvin et al., 2004; Moreira et al., 2004) and these fitness loads could prevent the spread of these transgenes into wild populations. However, these studies were conducted in inbred, homozygous lines. Therefore, the deleterious recessive alleles in these homozygous inbred lines may mask transgene associated fitness effects. A genetically diverse background is therefore imperative to assess transgene loads of genetically modified mosquitoes (Scott et al., 2002).

In previous studies, to complement a vector population replacement disease control strategy, a transgenic Aedes aegypti was created with a midgut infection barrier (MIB) to dengue virus serotype 2 (DENV2) infection (Franz et al., 2006). The transgene in this line utilized the intrinsic anti-viral properties of the RNA interference (RNAi) innate immune pathway, which aids in the regulation of viral infection in mosquitoes and many other organisms (Blair, 2011). This Carb77 transgenic line was engineered to express an internal repeat (IR) effector RNA in

midgut tissues after initiation by a blood meal inducible carboxypeptidase A promoter (CPA) (Franz et al., 2006). The effector RNA created a dsRNA to the DENV2 prM gene. This strategy initiated an early DENV2-specific RNAi response in midgut epithelial cells preventing midgut infection. However, Carb77 mosquitoes eventually lost their DENV2 refractory phenotye, even though the transgene sequence was intact in later Carb77 generations (Franz et al., 2009). The IR RNA was no longer detected by northern blot analysis, so it was hypothesized that IR-RNA expression loss may be due to chromatin/heterochromatin rearrangements which silenced the transgene (Franz et al., 2009). To further evaluate the genetic and phenotypic stability of this transgene, this study engineered new transgenic Ae. aegypti lines with the same transgene as the Carb77 line. The resulting Carb109M, was highly refractory to DENV2 infection and was used in the current study to evaluate the effect of genetic background on transgene stability. This current strain has now been stably expressing the transgene for over 40 generations.

Carb109M was subjected to five generations of backcrossing into a genetically-diverse laboratory strain (GDLS) derived from field collections of Ae. aegypti from southern Mexico (de Valdez et al., 2011; Deus et al., 2012). The enhanced green fluorescence protein (EGFP) eye marker associated with the transgene was selected in each of the five backcross generations to produce six backcross 5 (BC5) lines (Carb109M/GDLS.BC5). To increase the frequency of the transgene prior to family selection, the BC5 lines underwent five generations of selection for the EGFP eye marker. In the end, the Carb109M/GDLS.BC5.HZ maintained the DENV2 refractory phenotype for multiple generations. These results demonstrate the importance of outcrossing transgenes into recently-colonized, genetically diverse strains to more accurately assess transgene-associated fitness loads and prior to releasing genetically modified strains into cage or wild populations.

## Materials and Methods

### Transgene design and establishment of transgenic families

Plasmid DNA construction and germ-line transformation are as described in previous studies (Franz et al., 2006). In brief, Ae. aegypti Higgs white eye (HWE), a Rexville D strain variant, pre-blastoderm embryos were microinjected with a mariner MosI nonautonomous Class II transposable element (TE) helper plasmid and a pMos-Carb/Mnp+/i/Mnp-/svA donor plasmid (Franz et al., 2014).

We obtained a total number of 201 G0 larvae, 191 developed into adults. Male survivors were individually mated to 20 HWE females and female survivors were separated into groups of 5 and mated to one HWE male. After the initial mating, to minimize the number of families the female founders were pooled into groups of from 28-32 females and the male founders were pooled into groups of three families. The overall number of families was reduced to 40 'super-families', three of which were from female founders. EGFP eye expression was found in 11 families, Carb1M, Carb1F, Carb22M, Carb96M, Carb96F, Carb109M, Carb109F, Carb175M, Carb175F, Carb194M, and Carb203F by use of a fluorescence microscope (Olympus SZ12, Melville, NY) with an EGFP-specific filter. These lines were backcrossed to the HWE parent, and the G1 progeny of these seven lines were tested for susceptibility to DENV2 Jamaica 1409. Carb109M, Carb109F, Carb175M and Carb175F were resistant to DENV2 infection. Carb109M resulted from 3 males that were then intercrossed to 20 HWE females each resulting in 2 male and 2 female EGFP positive progeny. The 2 males were then combined to make the male Carb109 line (Carb109M) and the 2 females were combined to make the female Carb109 line (Carb109F). These two lines were then out crossed to the HWE line and maintained separately in colonies throughout the study.

Mosquito colony maintenance

All stages of mosquitoes were maintained at 28 ºC ± 2 ºC, 80% ± 5% relative humidity and 14:10 hour light dark photoperiod. Larvae were raised at a density of ~100 mosquitoes per L and were fed ~0.35g of 50:50 flake fish food (Tetramin, Melle, Germany) and rodent diet (Harlan Labs) every other day until pupation. At pupation they were placed into 2.5L cartons and were provided sugar and water upon eclosion. Five to eight days post emergence the colonies were given a citrated sheep blood meal (Colorado Serum Co., Boulder, CO).

Detection of transgene integration and characterization of integration site

Total DNA was extracted using the Puregene kit (Qiagen, CA) from three females per sample following the manufacturer's protocol of the Puregene kit. DNA pellets were resuspended in 50 µl hydration solution overnight at room temperature. Approximately 5 µg of total DNA were digested with PstI in a 30 µl reaction mix (25 µl DNA and water, 3 µl buffer, 2 µl (=40 U) PstI) for 4 h at 37ºC. Before electrophoresis on a 0.8% agarose gel, the entire reaction mix was further diluted with water to a volume of 50 µl. The samples were boiled with loading dye for 5 min and then quickly chilled on ice. The DNA transfer to a positively charged nylon membrane (Brightstar, Applied Biosystems, CA) was carried out according to standard procedures. Blots were hybridized with a $^{32}$P-dCTP (3,000 Ci/mmol) labeled probe corresponding to the 354 bp mariner Mos1 left arm generated with the DECAprime II Random Primed Labeling kit (Applied Biosystems). Hybridizations were conducted at 48°C overnight.

Genome Walking was carried out using the Clontech Genome Walker Universal Kit (Takara Bio Company, Mountain View, CA) and Advantage2 polymerase mix (Franz et al., 2009; Khoo et al., 2010). The mariner left and right arm primer are listed in previous studies (Franz et al., 2009). Carb109 transgene integration into genomic DNA of supercontig 1.470 was confirmed by PCR using primers 10318 FWD: 5'-ctcacacggcattacatgaaatatgttagtatttaatc-3',

maRight REV: 5’-gagcagcgcttcgattcttacgaaagtgtg-3’ and maLeft FWD: 5’caattatgacgctcaattcgcgccaaac3’, 10318 REV: 5’--aacagtagcttgtatgcttaggcatactaattgag-3’.

Detection of transgene expression by northern blot analysis

RNA extractions and northern blot analyses were conducted as described in previous studies (Adelman et al., 2002; Franz et al., 2006). Approximately 10-15 µg of total RNA from the midguts of blood fed and non-blood fed females of the Carb109 and the Carb52 lines were blotted onto a positively charges nylon membrane (Ambion) after separation on an 1.2% agarose gel. Carb52 was used as a negative control. The Carb52 line also expressed EGFP from the CPA promoter (Franz et al., 2011). Labelled probes from the prM region of the transgene were generated as previously described and probes were hybridized using the same procedure (Franz et al., 2006).

Oral virus challenge

To prepare virus for the DENV infectious blood meal, high passage DENV2 Jamaica 1409 virus at a multiplicity of infection (MOI) of 0.01 was used to infect C6/36 cells in L-15 medium supplemented with 3% heat inactivated fetal bovine serum, 1% streptomycin and 1% L-glutamine for 12 days at 28º C. The media was replaced 5 to 6 days after infection. For the chikungunya virus (CHIKV) infectious blood feeds, monolayers of Vero cells were infected with CHIKV 37997 at a MOI of 0.01 and incubated for 36 hours at 37ºC. The infectious blood meal was prepared for oral feeding as described previously (Bosio et al., 1998). A sample of each blood meal was taken and frozen at -80ºC for later quantification by plaque assay by the methods described below.

Infectious virus titration by plaque assay

Individual mosquitoes were triturated in 1.0 ml of L15 medium (Franz et al., 2006). Twenty-four well plates of LLC-MK2 cells were grown to confluency and infected with 10-fold

serial dilutions of virus for 1 hour and then overlaid with an agar nutrient mixture. Plates were held at 37ºC for 10 days before they were stained with 5mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution and incubated overnight. Plaques were counted and titers were calculated in pfu/ml.

## Evaluation of transgene fitness by introgression into a GDLS

### Introgression mating and transgene frequency

A subset of the eye marker positive mosquitoes from both Carb109 lines (G5) were reciprocally backcrossed (BC1) with a GDLS (Valdez et al., 2010) followed by 4 reciprocal backcrosses (BC2-BC5) to the GDLS. The GDLS was created by mixing equal numbers of larvae from 10 separately-maintained Ae. aegypti populations recently colonized from Chiapas, Mexico (de Valdez et al., 2011). An equal number of larvae from each of the 10 strains were placed into 1 liter of tap water, maintained as described above and mated to one another to create the GDLS. The virgin individuals from the transgenic strains and the GDLS were reciprocally mated and then blood fed. The subsequent offspring were screened for EGFP and virgin EGFP positive individuals were then reciprocally crossed to virgin GDLS individuals. This was repeated for a total of 5 backcrosses (GDLS.BC5). At each cross, the percentage of transgenic mosquitoes was calculated from ~150 3$^{rd}$ instar larvae screened with an EGFP-specific filter on a fluorescence microscope (Olympus SZ12, Melville, NY). Larvae lacking the EGFP eye marker were culled.

Five generations of backcrossing should generate mosquitoes in which 31 of every 32 alleles (97%) unlinked to the transgene are expected to have originated from the GDLS strain. Proportions of EGFP-expressing larvae in GDLS.BC1 through BC5 were analyzed by estimating the 95% highest density interval (HDI) with WinBUGS (Lunn et al., 2000) and the Credible

Intervals for Proportions script (McCarthy, 2007). An additional backcross (BC6) was generated for some analyses described in the results section.

Evaluation of transgene frequencies in HTL/BC1 and HTL/BC5 populations with no selection

Two lines named HTL1 and HTL2 were selected for transgene frequency studies, because they appeared to be the most fit and had consistent DENV2 refractory phenotype. Sixty lines were created with 4 different experimental conditions. The first group of 15 lines was a HTL1/GDLS.BC1 heterozygote and GDLS mating resulted in an initial HTL1 allele frequency of 0.25. The offspring were allowed to randomly mate through five generations (F1–F5) with no selection. The EGFP positive frequency was expected to be 0.4375, based on Hardy-Weinberg expectations ($0.25^2$ transgene homozygotes + 2*0.25*(1–0.25) transgene heterozygotes). The second set of 15 lines was generated by intercrossing HTL1/GDLS.BC1 heterozygotes so that the initial frequency of the HTL1 allele was 0.5. The expected frequency of EGFP-expressing larvae in F1–F5 was 0.75 ($0.5^2$ transgene homozygotes +2*0.5*(1–0.5) transgene heterozygotes).

The third and fourth sets of 15 lines were the same as for the first and second sets except that HTL2/GDLS.BC1 were used. These 60 lines were maintained without selection of EGFP-expressing larvae for five generations and the frequency of EGFP larvae was estimated in ~150 larvae from each of the 15 lines in each generation. This same process was repeated for the BC5 offspring. The relative fitness loads of the transgene in homozygotes, heterozygotes and fitness of wild-type homozygotes were estimated by identifying the fitness coefficients in Fisher's Selection Model (Fisher, 1950) using the observed proportion of larvae expressing EGFP (pEGFPt) in each of the six generations. Fisher's model is:

$$p_{t+1} = \frac{p_t(p_t w_{AA} + (1-p_t)w_{Aa})}{\left(p_{t2} w_{AA} + 2p_t(1-p_t)w_{Aa} + (1-p_t)^2 w_{aa}\right)}$$

where: $p_t$ = transgene frequency in generation t, $w_{AA}$ = fitness of transgene homozygotes, $w_{Aa}$ = fitness of transgene heterozygotes and $w_{aa}$ = fitness of wild-type homozygotes.

A FORTRAN program was written that generated a three-dimensional matrix containing all combinations of $W_{AA}$, $W_{Aa}$ and $W_{aa}$ each incremented by 0.01 from 0.0–1.0. The matrix therefore contained 100 x 100 x100 = $10^6$ combinations. Fisher's model was run for five generations starting with a $p_0$ (starting allele frequency) of either 0.5 or 0.25 for each combination. $p_t$ were transformed into proportion predicted EGFP expressing larvae in each generation t ($pEGFP_t$) by:

$$pEGFP_t = p_{t2} + 2p_t(1-p_t)$$

All six values of $pEGFP_t$ were compared with observed proportions oEGFPt for t =0….5 generations as:

$$diff = \sum_{t=0}^{5} |oEGFP_t - pEGFP_t|$$

The FORTRAN program identified the smallest diff and reported the associated $w_{AA}$, $w_{Aa}$ and $w_{aa}$ values. Statistical comparisons among groups, generations and backcrosses were based on calculating Bayesian 95% highest density intervals (95% HDI) using WinBUGS (Lunn et al., 2000) and the estimation of mean and variance script (McCarthy, 2007). Proportions with non-overlapping 95% HDI were considered statistically different.

Relative fitness of HTL1/GDLS.BC5 and HTL2/GDLS.BC5 mosquitoes

Three crosses between HTL1/GDLS.BC5 heterozygotes (200 individuals/cross; F1, F2, F3) and three crosses between HTL2/GDLS.BC5 heterozygotes (200 individuals/cross; M.1,

M.2, M.3) were performed in six separate $1ft^3$ cages. One-week-old females received non-infectious bloodmeals. All larvae from each cross were screened for EGFP expression and all wild-type larvae were culled. EGFP-expressing F1 larvae were reared to adults. Following random mating, females received blood meals and their F2 progeny were again screened for EGFP expression and wild-type larvae were culled. This procedure was followed for three more generations (F3–F5) to increase the frequency of the HTL1 or HTL2 transgene in the population while minimizing inbreeding. Results were again compared to values expected under Fisher's Model. Only $w_{AA}$ and $w_{Aa}$ were estimated because $w_{aa} = 0$ since all wild-type larvae were discarded during the selection process. Frequencies of EGFP expressing larvae were recorded for five generations for each of the six lines. Mean observed-to-expected proportions were compared by estimating the 95% HDI with WinBUGS and the estimating proportions script.

Family-based selection to generate homozygous line from HTL/GDLS.BC5

At the end of five generations of selection and assuming $W_{AA}=W_{Aa} = 1$, Fisher's model simplifies to:

$$p_{t+1} = 1/(2-p_t)$$

and predicts that 98.22% of larvae were expected to express EGFP, and the transgene frequency was expected to be 0.83. Another 25 generations of selection would be required before nearly all larvae (99.9%) could be expected to express EGFP. Therefore, a family based selection scheme was used to generate HTL homozygous (HZ) families from the six lines (HTL1/GDLS.BC5 F.1, F.2, F.3 and HTL2/GDLS.BC5 M.1, M.2, M.3), 30 families were established each consisting of three F5 females placed in a cage with one male. Siblings from each of the thirty families were screened for EGFP expression and families with all siblings expressing EGFP were reared to adults and intercrossed. These offspring were reared to adults, intercrossed, bloodfed, eggs

collected and hatched. These offspring were again screened for EGFP expression. Families, in which all siblings expressed EGFP were combined and maintained as homozygous (HZ) lines for further experiments. Ultimately this process yielded one or more HTL/GDLS.BC5.HZ lines which can be tested for vector competence to DENV2-Jamaica1409.

## Results

### Transgenic mosquito families and selection of resistant strains by DENV2 challenge

Seven pools of injected Ae. aegypti had EGFP positive individuals. After outcrossing to a HWE line, 11 transgenic lines were generated and at generation 3 (G3), the Carb1M, Carb1F, Carb22M, Carb96M, Carb96F, Carb109M, Carb109F, Carb175M, Carb175F, Carb194M, and Carb203F mosquito families were orally challenged with a $1.5 \times 10^6$–$1.6 \times 10^7$ pfu/mL of DENV2-Jamaica1409 infected blood meal to determine their susceptibility to this virus by plaque assay (Fig. 2.1A and B). The Carb109M and Carb109F lines and the Carb175F lines were initially highly resistant to DENV2 infection (Carb175F, $p<0.004$; Carb109M and Carb109F, $p<0.0001$). DENV2 prevalence in lines Carb194M and Carb203F was also significantly lower than prevalence in HWE and Carb52 controls (Carb194M, $p<0.01$; Carb203F, $p<0.02$), but these lines were lost in subsequent generations (Fig. 2.1A). The Carb109F, Carb109M and Carb175F lines were then retained and tested for susceptibility to DENV2 infection in generation 5 (G5) (Fig. 2.1A), but by this generation only the Carb109 lines retained a high resistance to infection (Fig. 2.1B). Therefore, the Carb175F line was omitted from the introgression studies and the Carb109F and Carb109M were maintained as separate lines throughout this study and were selected as HTL1 and HTL2 lines for the introgression studies.

### Resistance to DENV3, CHIKV

Anti-viral resistance in the 3 initial high refractory strains remained specific to the target dengue serotype, DENV2. Neither the Carb109F, Carb109M nor the Carb175F line was

resistant to an alternate dengue serotype, DENV3 6889, nor were they resistant to the alphavirus, QR-MX/97 CHIKV (Fig. 2.2A and 2.2B). At 7 dpi, HWE mosquitoes had mean DENV3 6889/QR-MX/97 titers of 1300 pfu/mosquito and Carb109M had mean virus titers of 750 pfu/mosquito (Fig. 2.2A). At 7 dpi, DENV3 prevalence was similar between HWE, Carb109M, Carb175F, and the Carb52 control. However, at 14 days dpi, DENV3 prevalence was significantly higher for HWE (80%) than for Carb109M or Carb175F (55%) (Fisher's exact test, $p = 0.0307$). Infecting the same mosquito lines with CHIKV 37997 showed no statistical differences in prevalence (90–100%). There is a >60 % sequence identity in the 568 nt target region between the genome of DENV3 6889/QR-MX/97 and that of DENV2-Jamaica1409, so the reduction in 14 dpi DENV3 titers may be due to the high sequence identity.

Molecular analysis of transgene integration and expression

At G3, genome walking determined that the Carb109M line had two integration sites, while the Carb175F line had only one integration site (Fig. 2.3A and 2.3B). The transgene integration patterns of Carb109M and Carb109F were identical by Southern blot analysis and maintained the same pattern after introgression of the transgene into the GDLS genetic background (Fig. 2.3B). One integration site of the Carb109M line was identified by genome walking to be in the 3' UTR of AAEL010318 (VectorBase supercontig 1.470; contig 18391; nt position 98,722-98,869 on chromosome 3) (Fig. 2.3C). The second integration site is in a repetitive (>50 copies) sequence motif and included a 920 bp portion of the pMos1 plasmid backbone extending from the left arm of the TE (data not shown). The physical integration pattern of the second integration event extending from the right arm of the TE was not determinable by genome walking. Genome walking was not performed on the Carb109F line.

Northern blot analysis detected Mnp+/i/Mnp- RNA in Carb109 G3 females 20 hours post blood meal, but not in non-blood fed Carb109 mosquitoes, or Carb109 mosquitoes 48 hours post

blood meal (Fig. 2.4A). The Carb109 line had higher expression of the Mnp+/i/Mnp- RNA than the Carb175 line (Fig. 2.4B), which lost the refractory phenotype by G5.

Resistance to DENV2 after introgression into GDLS

The DENV2 refractory phenotype was maintained after introgression of the Carb109 transgene into a diverse genetic background. The GDL is highly susceptible to DENV2-Jamaica1409 at 7 and 14 dpi (Fig. 2.5). Following five consecutive backcrosses to GDLS, Carb109F/GDLS.BC5 and Carb109M/GDLS.BC5 mosquitoes remained highly refractory to DENV2 infection at both time points, similar to line Carb109M, although Carb109F/GDLS.BC5 tended to be more susceptible to the virus at 14 dpi than Carb109M/GDLS.BC5 (Fig. 2.5). However, there was no significant difference between the DENV2 titers of Carb109F/GDLS/BC5 and Carb109M/GDLS.BC5 mosquitoes. HWE, GDLS and BC5 Neg (negative for the EGFP marker) mosquitoes showed a significantly higher prevalence of DENV2 (59.6–67.5%) than Carb109M, Carb109F, Carb109M/GDLS.BC5, and Carb109F/GDLS.BC5 mosquitoes (1.8–7.4%) at 7 and 14 dpi (Fig. 2.5). Both Carb109M/GDLS.BC5 and Carb109F/GDLS.BC5 mosquitoes also retained expression of the transgene 20 hours post infection after 6 backcrosses to the GDLS (Fig. 2.4C).

In the end two homozygous lines derived from the Carb109F/GDLS.BC5 and one homozygous line derived from the Carb109F/GDLS.BC5 line were refractory to challenge with $5.2x10^6$ pfu/ml of DENV2 (data not shown). The HWE positive control strain had a 48.0% infection rate at 7dpi (n=25) and 53.8% infection rate at 12dpi (n=39), while at 7 dpi the BC6 strains the Carb109M had a 0.0% infection rate (n=25) and the Carb109F line had an 8.0% infection rate (n=25). At BC6 both lines had a 0.0% infection at 12 dpi in (n=27-29).

Fitness of the Carb109F and Carb109M transgenes during selection

At each back cross, the proportion of EGFP positive larvae was recorded and compared to 0.5, the number of expected EGFP positive individuals (Fig. 2.6). Selection was applied to each of three lines of Carb109F/GDLS.BC5 and Carb109M/GDLS.BC5 heterozygotes. In each generation wild-type larvae were culled and mosquitoes were allowed to inter-mate. This was repeated over four consecutive generations (Fig. 2.7). Observed values were compared with values predicted from the simplified Fisher's model, $p_{t+1} = 1/(2 - p_t)$. The Carb77 line had reduced EGFP expression during the $F_2$-$F_4$ larval stage. This was evaluated from the 100 EGFP larvae in each generation that seemed to fall into two phenotypic classes. All EGFP larvae had fluorescent green anal papillae, but some also had GFP fluorescent slits behind the eyes. When these samples were grouped by phenotype and then were genotyped by melting curve PCR (mcPCR) all 12 larvae with GFP only in the anal papillae were Carb77 / + heterozygotes while 10 of the 12 with EGFP behind the eyes were Carb77 homozygotes. Thus it could be that the EGFP phenotype was differentially expressed in the multitude of different genetic backgrounds in the GDLS. However, this phenomenon was not seen in the Carb109 lines.

Replicate F.2 reached Fisher model predictions for generation 2 but then remained lower than predictions from generations 3 through 5. Replicate F.3 reached model predictions for generation 4 but was lower than predicted in generation 5. The 95% HDI surrounding proportions of EGFP-expressing larvae in generation 5 for all three F replicates did not cover the expected 0.9822 allele frequency. Replicate M.1 started below the expected 0.75 value but exceeded predictions in generation 2 and overlapped predictions in generations 3–5 (Fig. 2.8). Replicate M.2 fell below predictions for generation 2 and 3 but reached predictions in generations 4 and 5. Only replicate M.3 tracked model predictions in all generations. The 95% HDI surrounding proportions of EGFP expressing larvae in generation 5 for all three M

replicates contained the expected 0.9822 allele frequency. Among the three F replicated lines the fitness coefficients of transgene homozygotes ($W_{AA}$) ranged from 0.32–0.51, a dramatic improvement over the BC5 values of $W_{AA}$ = 0.01 (Table 2). Fitness of transgene heterozygotes ($W_{Aa}$ =0.68–1.00) on the other hand overlapped the BC5 values (WAa = 0.95). Among the three M replicates the fitness coefficients of transgene homozygotes ($W_{AA}$) ranged from 0.72–0.77, far exceeding the BC5 values of $W_{AA}$ = 0.01 (Table 2). However, the fitness of M replicate transgene heterozygotes ($W_{Aa}$ =0.75–0.86) was lower than the BC5 values ($W_{Aa}$= 0.94–1.00). Family-based selection was conducted in 30 families in each of replicates M.1–M.3 and F.1–F.3. However, we breed only one homozygous family (Carb109M/GDLS.BC5.HZ) from Replicate M.3.

The proportion of EGFP larvae did not significantly vary from the expected frequency of 0.50 during backcross introgression into the GDLS (Fig. 2.6A and 2B). The 95% HDI for the proportion of larvae expressing EGFP overlapped among all of the five backcross generations of both Carb109F/GDLS and Carb109M/GDLS. The frequency of EGFP larvae in populations that were selected for the EGFP marker at each generation deviated significantly from Fisher's selection model for the Carb77 line (Fig. 2.7A) but were as expected for the Carb109M line, and were as expected in the Carb109F by G5 (Fig. 2.7B). At intercrosses F2 to F4 the Carb109F line had lower frequencies of the EGFP marker than expected by Fisher's selection model ($\chi 2>14.24$, df=1, $p<0.001$) by $\chi 2$ test and the same was seen for the Carb77 line ($\chi 2>*$, df=1, $p<0.001$).

Transgene fitness without selection

The frequencies of EGFP-expressing larvae were compared among Carb109F/GDLS.BC1, Carb109M/GDLS.BC1, Carb109F/GDLS.BC5 and Carb109M/GDLS.BC5 at starting frequencies of 0.25 and 0.5. Frequencies were measured over five generations without selection for EGFP (Fig. 2.8A, 2.8B). In all BC1 lines, the proportion of

larvae expressing EGFP declined rapidly and reached zero by the fifth generation. This occurred whether $p_0$ was 0.5 (Fig. 2.8A) or 0.25 (Fig. 2.8B). Estimated fitness coefficients among these four BC1 variants (Carb109F/GDLS.BC1, $p_0$=0.25, Carb109M/GDLS.BC1, $p_0$=0.25, Carb109F/GDLS.BC1, p0=0.5, Carb109M/GDLS.BC1, $p_0$=0.5) were $W^{AA}$ =0.01 in all four experiments while $W_{Aa}$ varied from 0.11–0.21 and $W_{aa}$ varied between 0.94–1.00, respectively.

These results suggest that either the transgene has a dominant fitness load or that one or more deleterious alleles were linked to the transgene insertion site. In contrast, the proportions of EGFP expressing larvae in Carb109M/GDLS.BC5 and Carb109F/ GDLS.BC5 when initiated at $p_0$=0.5 were 0.4881 and 0.4782 in F5. However, the 95% HDI surrounding these frequencies did not contain the predicted 0.75 allele frequency. Again the predicted fitness coefficient for transgene homozygotes was $W_{AA}$ =0.01 in all four experiments while $W_{Aa}$ varied from 0.94–1.00, which actually exceeded $W_{aa}$ (0.65–0.93) in the four BC5 experiments. The proportions of EGFP expressing larvae in Carb109F/GDLS.BC5 and Carb109M/GDLS.BC5 initiated at $p_0$=0.25 (Fig. 2.8B) were 0.4538 and 0.2365 in F5. However, only the 95% HDI surrounding frequencies in Carb109F/GDLS.BC5 (BC5F in Fig. 2.8) contained the predicted 0.4375 frequency. The fact that the predicted fitness coefficient for transgene homozygotes was very low ($W_{AA}$=0.01), whereas fitness coefficients for transgene heterozygotes ($W_{Aa}$=0.94–0.95) and those for wildtypes ($W_{aa}$=0.81–0.93) were significantly higher supports the interpretation that the wild-type allele had a dominant positive effect on fitness. Thus, five generations of backcrossing did not change the fitness of transgene but greatly improved the fitness of transgene heterozygotes.

Discussion

Previous attempts to evaluate the fitness of antiviral effector RNA transgenic Ae. aegypti engineered for resistance to dengue virus type 2 in a GDLS backbone (Franz et al., 2006) was

unsuccessful due to loss of transgene function (Franz et al., 2009). In this study we developed and characterized new transgenic Ae. aegypti lines using similar methods to the previous studies. We then identified additional lines highly refractory to DENV2 and used them for introgression studies. Carb175 showed initial resistance to DENV2, but quickly lost this resistance (Fig. 2.1A). This was faster than the loss of the Carb77 DENV2 refractory phenotype, but currently, the homozygous Carb109 line created from this study has retained its refractory phenotype through 33+ generations. Additionally, the Carb109 transgenes were stable after 5 to 6 backcrosses into a genetically diverse population.

The prolonged success of the Carb109 line can be attributed to multiple differences in the strain, despite their derivation from the same construct. The Carb109 line had an additional integration site as compared to the Carb77 line (Franz et al., 2006) and the Carb175 line from this study. In a transgenic A. stephensi study, two copies of a transgene had a lower fitness cost in comparison to a one copy (Li et al., 2008). Also, the location of the integration likely varied between strains leading to positional effects. Positional effects could be a possible explanation for the higher expression of the transgene in the Carb109 line compared to the Carb175 line (Fig. 2.4B). Even within the Carb109 line, the Carb109M line was slightly more refractory than the Carb109F line, even though they resulted from the same transformation event (Fig. 2.5A and 2.5B).

The original line, Carb77, had lower than expected EGFP frequencies (Fig. 2.7A). In this study, the Carb109 strains, especially the Carb109M line, maintained expected EGFP frequencies in selection studies. The fitness differences between strains with the same construct, but different integration sites suggest fitness effects associated with the location of the integration opposed to transgene or reporter expression. This phenomenon was also seen in other

studies where the integration site was suggested as the cause of differences in the fitness in lines with the same construct (Li et al., 2008). In this study there were also differences in the fitness of mosquitoes with the same construct and the same integration site. The Carb109F had lower EGFP frequencies than the Carb109M line (Fig. 2.7B). Differences in the fitness of transgenic lines from the same construct have also been found in other studies sometimes resulting in a higher fitness than seen in the parent strain (Amenya et al., 2010).

Introgression of the transgene into a GDLS strain improved the fitness of the transgene. Most notably, the fitness of the transgene heterozygotes increased from $W_{Aa}$ =0.11–0.21 after one backcross to $W_{Aa}$ = 0.94–1.00 after five generations of backcrossing. However, the fitness was not improved in transgene homozygotes ($W_{AA}$ = 0.01-0.01) after five generations of backcrossing. The four generations of selection also improved transgene homozygous fitness from $W_{AA}$ = 0.01 to 0.32–0.51 in the female strain and from $W_{AA}$ = 0.01 to 0.72–0.77 in the male strains. These results suggest that a deleterious recessive allele is linked with the transgene, but has become unlinked with recombination. Additionally, the resulting single homozygous line, despite initiating the family based homozygous selection with 180 families, also indicates that there is a deleterious recessive allele linked with the transgene. This study emphasizes the importance of creating multiple transgenic lines and introgression of these lines into a genetic background to maintain genetic diversity and improve strain fitness. This strategy should improve the outcomes of “reduce and replace” genetic vector control strategies (Okamoto et al., 2013; Robert et al., 2013). Moreover, genetic drive systems for population replacement strategies still need further improvement, thus improving strain fitness through backcrossing to genetically diverse strains can reduce the rate of transgene elimination in the field, thereby

reducing the number of individuals needed for release and reducing the need for strong genetic drive systems.

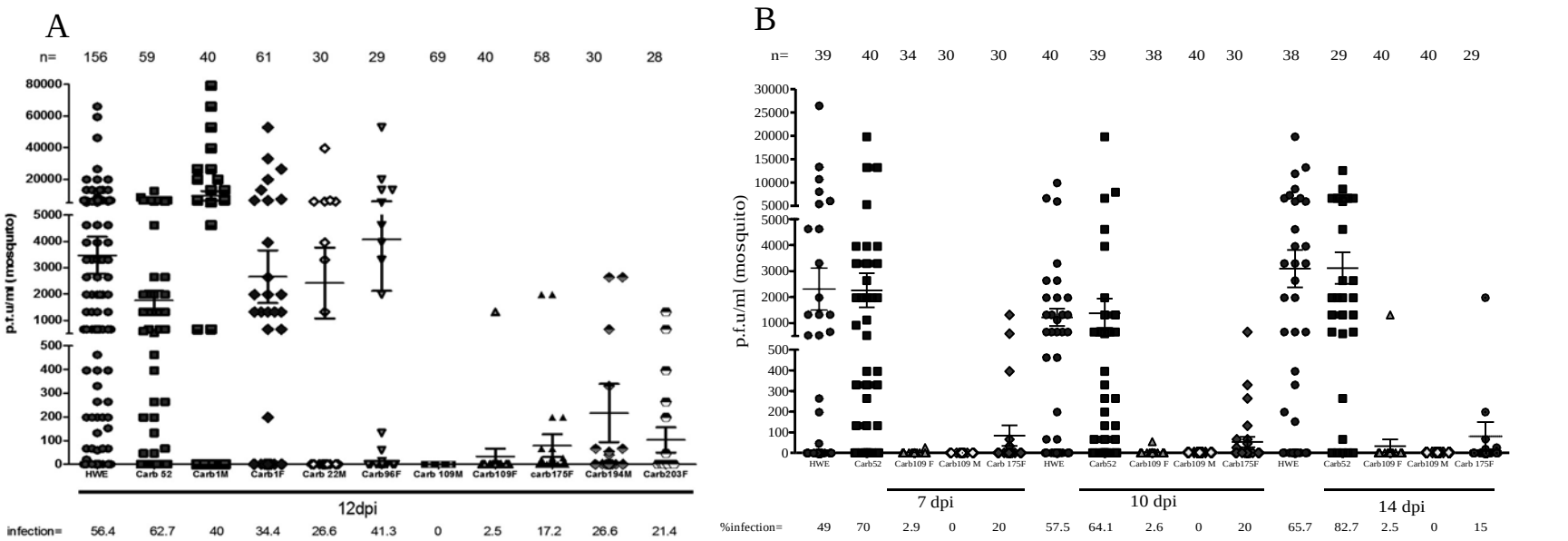



Figure 2.1: Titer and infection rate of the early EGFP expressing Carb lines A) G3 B) G5 infected with DENV2 Jamaica 1409. A) depicts 1 replicate of individual whole mosquito titers in plaque forming units (pfu) per mosquito of multiple lines at 12 days post infection (dpi). The groups are the Higgs white eye (HWE), parent strain of the Carb lines, the Carb52 line, the Carb1male line and female lines, the Carb22 male line, the Carb 96 female, the Carb109 male and female lines, the Carb175 female line, the Carb194 male line, the Carb203 female line. B) depicts 1 replicate of individual whole mosquito titers in plaque forming units (pfu) per mosquito infected with 1.6 X $10^7$ pfu/ml DENV2 Jamaica 1409 at 7, 10 and 14 dpi. The groups are the HWE line, the Carb52 line, the Carb109 female and male lines, and the Carb175 male line. The numbers following the strain name indicate whether the line is male, M, or female, F, derived. The horizontal lines indicate the mean titer of each group and the bars indicate standard error. Above the graph, the number of individuals titrated in each group, n, is indicated. Below the graph shows the percentage of mosquitoes infected in each group.

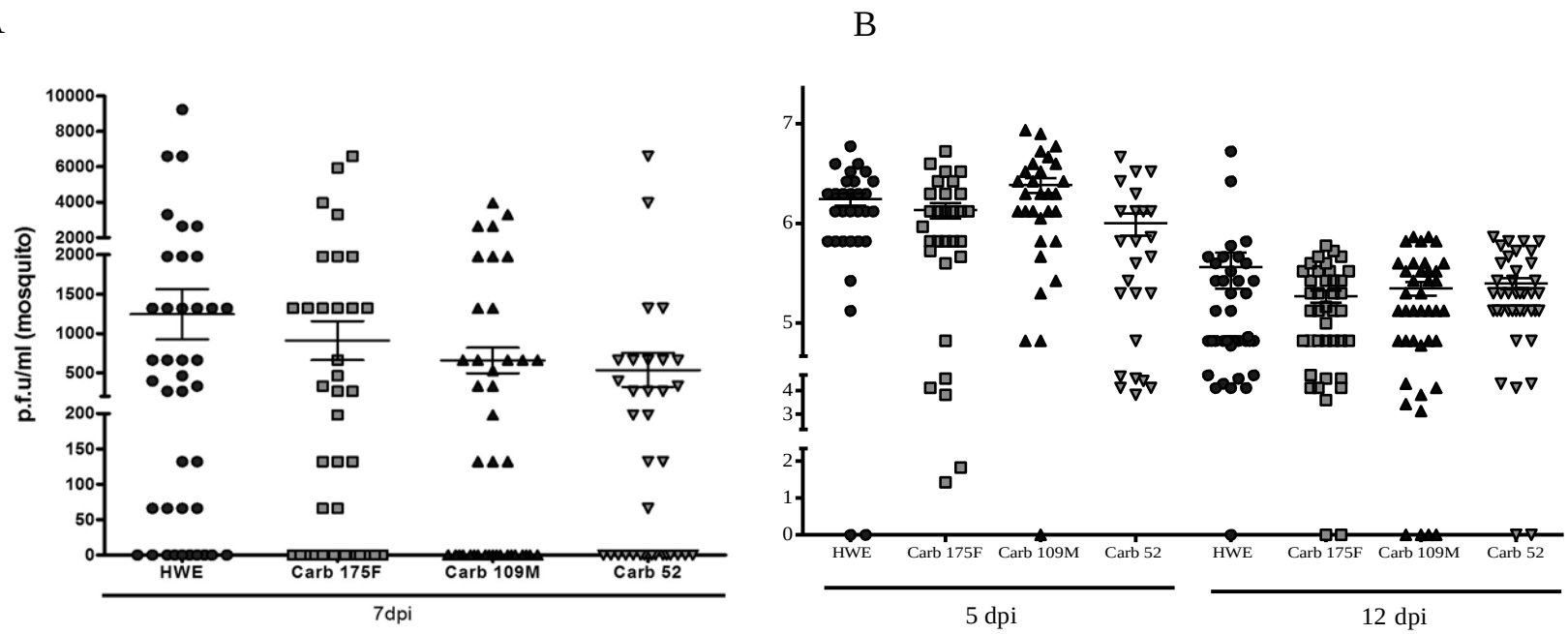



Figure 2.2: Titers and infection rate of Carb lines (G3) infected with A) DENV3 Mex BC177 and B) CHIKV 37997. Both graphs depict individual whole mosquito titers in plaque forming units (pfu) per mosquito infected with either A) $2.3x10^8$ pfu/ml of DENV3 Mex BC177 or B) $8.1x10^7$ pfu/ml of CHIKV 37997. Mosquitoes were titrated in graph A 7dpi and in graph B 5dpi and 12dpi. The groups shown above are HWE line, Higgs white eye, parent strain of the Carb lines, the Carb175 female line, the Carb109 male, the Carb52 line. The numbers following the strain name indicate whether the line is male, M, or female, F, derived. The horizontal lines indicate the mean titer of each group and the bars indicate standard error. Below the graph shows the days post infection (dpi). n= 35-40 for all groups.

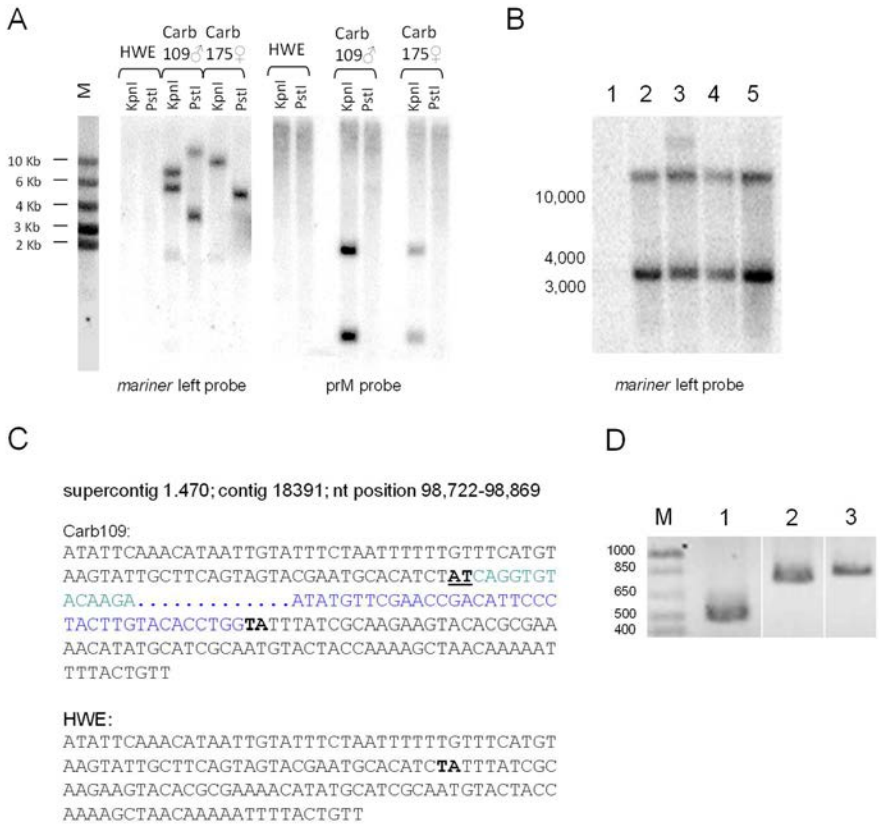



Figure 2.3: Transgene integration characterization of Carb109 mosquitoes. (A). Southern blot analysis to detect transgene integration sites among pMos-Carb/Mnp/i/Mnp/svA transformed Ae. aegypti. Total DNA was extracted from five $G_4$ females per sample and digested with KpnI or PstI. Blots were hybridized overnight at 48ºC with random-primed $^{32}$P-dCTP-labeled DNA probes corresponding to the left arm of the mariner Mos1 TE or the prM encoding region of the IR effector. (B). Southern blot analysis to detect transgene integration sites among Carb109 lines in $G_9$ mosquitoes and Carb109 x GDLS backcrosses. Total mosquito DNA was extracted from three females and digested with PstI. Blots were hybridized overnight at 48ºC with a random-primed $^{32}$P-dCTP-labeled DNA probe corresponding to the left arm of the mariner Mos1 TE. Lane 1: HWE; lane2: Carb109F G9 line; lane 3: Carb109M G9; lane 4: GDLS BC6 X Carb109F line; lane 5: GDLS BC6 X Carb109M line. (C). Physical mapping of a transgene integration site in Carb109 mosquitoes. In bold: mariner Mos1 TE target sequence motif; in bold and underlined: duplication of the TE target sequence as a consequence of mariner Mos1 integration. (D) PCR assay and gel electrophoresis to confirm integration site of the TE in Carb109 mosquitoes. M=size marker; lane1: Carb109, amplicon of mariner Mos1 left arm and flanking sequence of supercontig 1.470 (primers maLeft FWD/10318 REV); lane2: Carb109, amplicon of mariner Mos1 right arm and flanking sequence of supercontig 1.470 (primers 10318 FWD/maRight REV); lane3: HWE, amplicon of the same region of supercontig 1.470 (primers 10318 FWD/10318 REV).

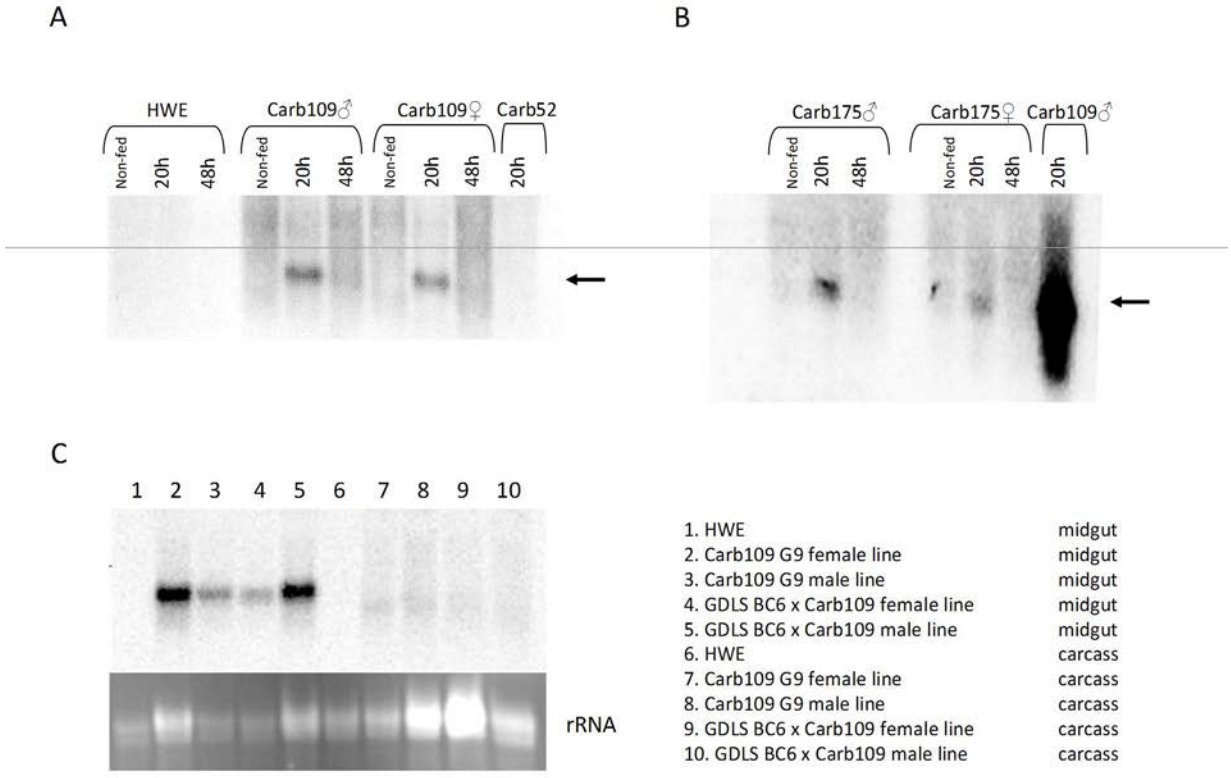



Figure 2.4: Characterization of transgene expression. (A to C) detection of transcripts derived from the DENV2 targeting IR effector among midguts of bloodfed Carb109 females. (A) Northern blot to detect the DENV2 prM derived IR RNA in midguts of bloodfed Carb109 females from the male and female derived lines, respectively (arrow). HWE and Carb52 mosquitoes were used as controls. (B) The same effector RNA was only weakly detectable in midguts of bloodfed Carb175 females of male and female derived lines, respectively (arrow). Blots were overexposed for 72 h using Carb109 RNA as control. (C) Detection of IR effector RNA at 20 h post-bloodmeal in midguts of Carb109 females that had been backcrossed for six generations with the GDLS strain. The ethidium-bromide stained gel is shown as loading control. Total RNA was extracted from pools of 20 midguts. Blots were hybridized with a $^{32}$P-dCTP labeled random primed DNA probe corresponding to the prM encoding cDNA of DENV2. Hybridization temperatures ranged between 45 and 48$^{o}$C.

A

B

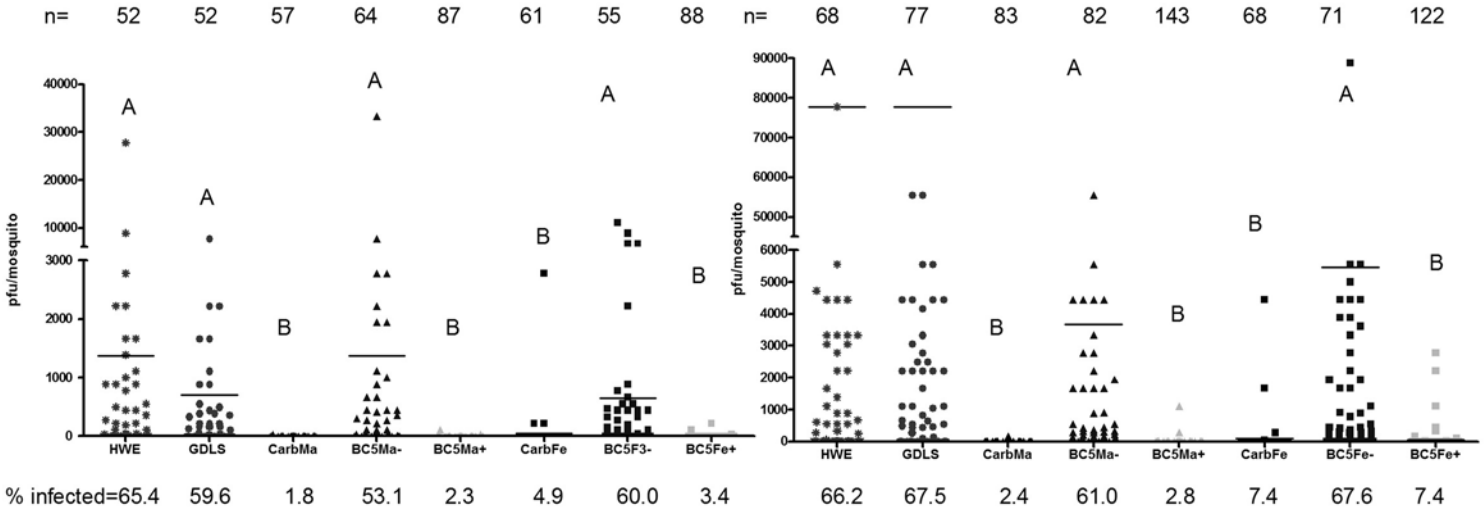



Figure 2.5: Titer by plaque titration of whole mosquitoes 7 (A) and 14 (B) days post infection (dpi) with DENV2 Jamaica 1409. The graph depicts 3 replicates of individual titers in plaque forming units (pfu) per mosquito of the HWE line, Higgs white eye, parent strain of the Carb109 line, the GDLS line, genetically diverse lab strain, the CarbFe line, Carb109 female line, the CarbMa line, the Carb109 male line, the MaBC5- line, the introgressed Carb109 male line negative for the eye marker at the 5th BC, the FeBC5- line, the introgessed Carb109 female line negative for the eye marker at the 5th BC, the MaBC5+, the introgressed Carb109 male line positive for the eye marker at the 5th BC, and the FeBC5+, the introgressed Carb109 female line positive for the eye marker at the 5th BC. The numbers following the strain name indicate the number of dpi the samples were collected, 7 or 14 days. The horizontal lines indicate the mean titer of each group. Groups found to differ significantly by titer ($H=520$, $df=14$, $p < 0.001$) by a Kruskall-Wallis and a Dunn's multiple comparison post hoc test ($p <0.001$) are designated with a different letter (A or B) above each group. A Pearson's chi-squared test was also performed between the groups designated by the Dunn's test which showed that the proportion of zeros between the groups A and B were statistically significant ($\chi 2= 306.8$, $df=1$, $p<0.0001$). The number above the graph depicts the number of individuals titrated in each strain, N, and the number below the graph indicates the percentage infected.

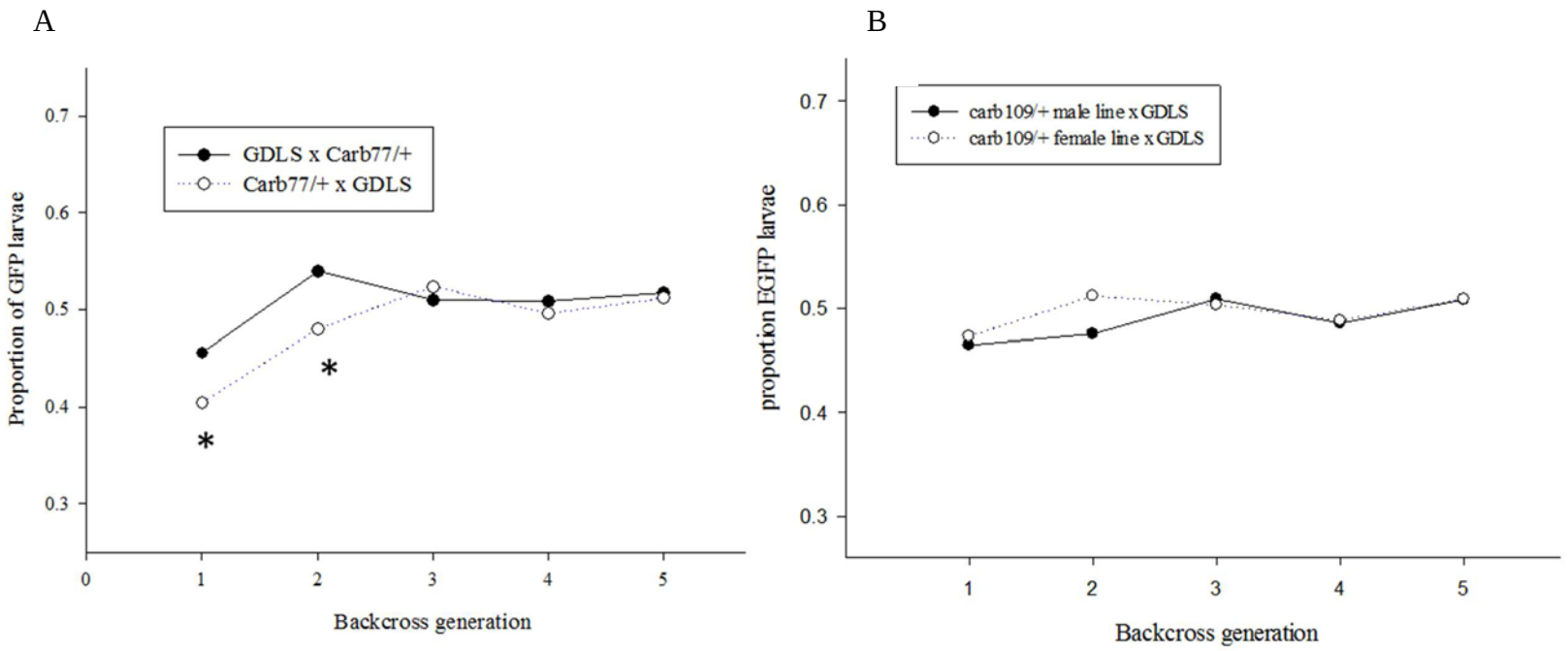



Figure 2.6: Transgene stability in the Carb77 (A) and Carb109 (B) lines after 5 backcrosses to the GDLS. The graphs show the frequency of EFGP positive larvae after 5 backcrosses to a GDLS with selection over 5 generations. (A) Shows the proportion of EGFP larvae in the Carb77 backcrosses. (B) Shows the proportion of EGFP larvae in the Carb109 backcrosses. * indicates significantly different from the expected 0.50 frequency (Fisher's exact test, $p \leq 0.001$).

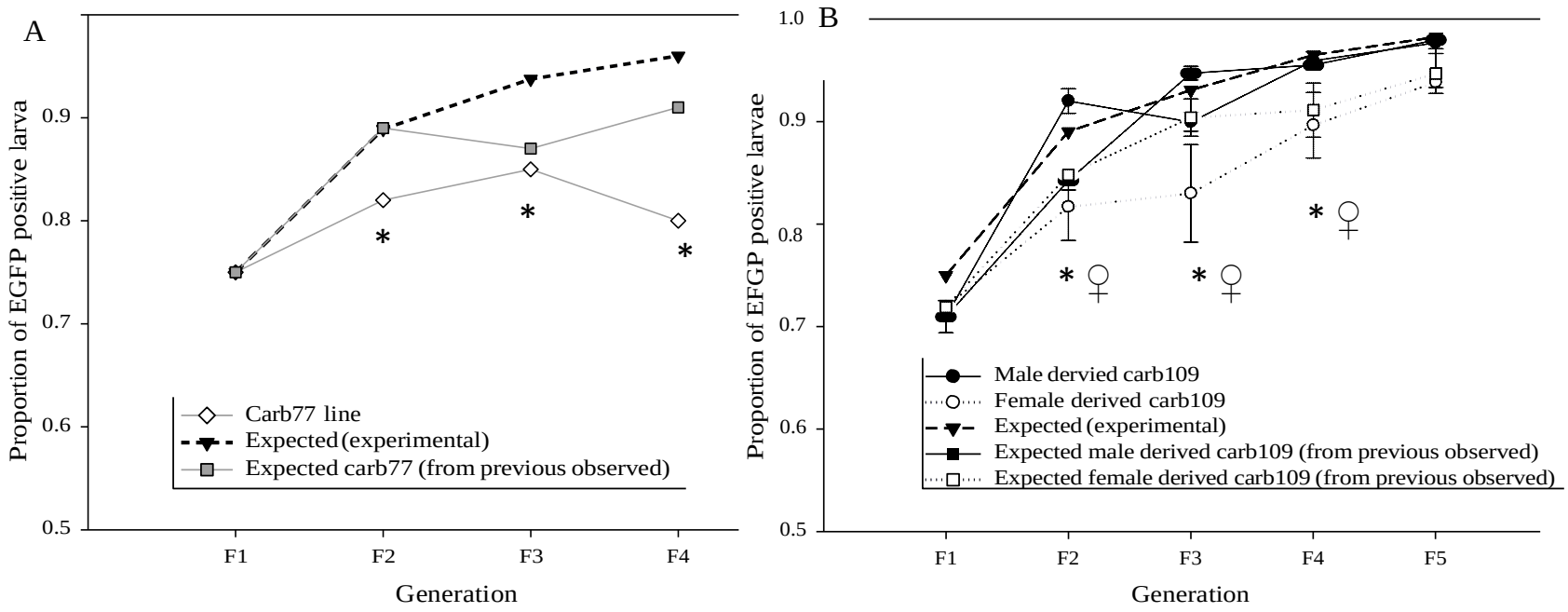



Figure 2.7: Transgene stability in the Carb77 (A) and Carb109 (B) lines after 4 to 5 intercrosses. The graphs show the frequency of EFGP positive larvae after 5 backcrosses to a GDLS with selection over 5 generations. (A) Shows the frequency of EGFP larvae in the Carb77 versus the expected frequencies and the frequencies expected based on the previous generation of EGFP larvae. (B) Shows the frequency of EGFP larvae in the Carb109 male and female derived line versus the expected frequencies and the frequencies expected based on the previous generation of EGFP positive larvae. * indicates significantly different from expected frequencies ($\chi 2$ goodness-of fit, $p < 0.001$). A ♀ or ♂ following the * indicates differences in the female or male only at the time point.

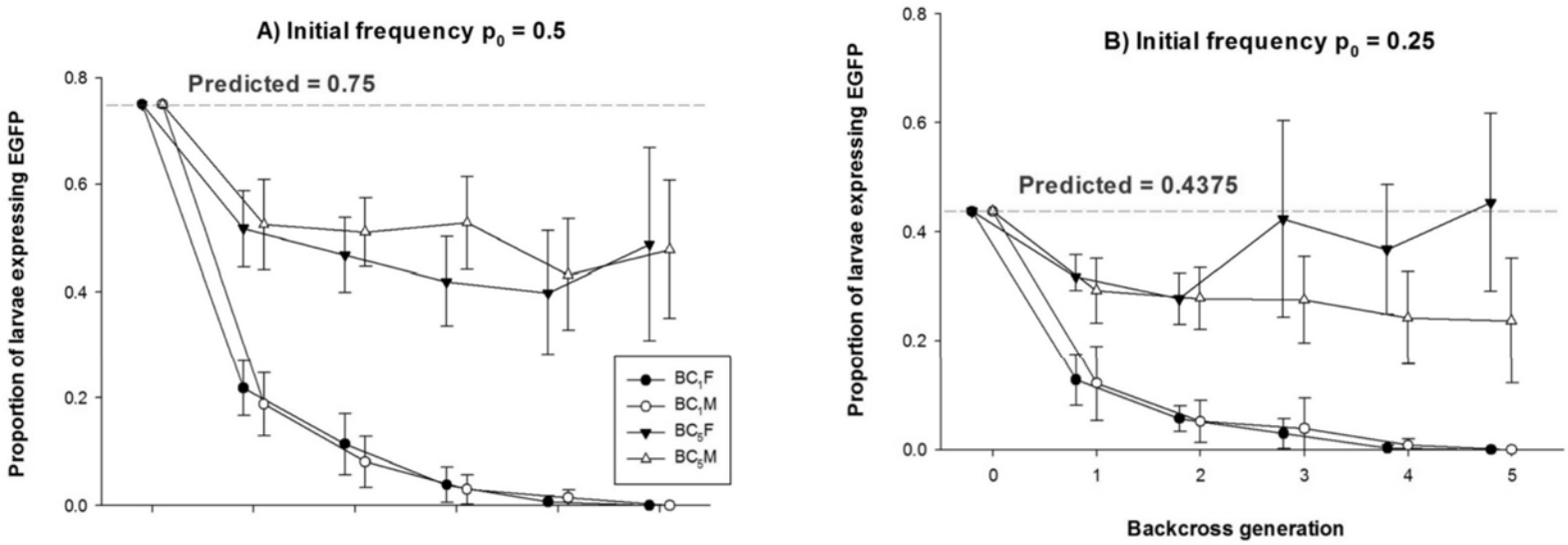



Figure 2.8: Transgenic allele frequencies among Carb109/GDLS backcrossed mosquitoes over five generations (F1–F5) in absence of selection for the transgenic phenotype. Initial frequencies (p0) of the Carb109 transgene were either (A) 0.5 (transgenic heterozygote x transgenic heterozygote) for Carb109F/GDLS.BC1, Carb109M/GDLS.BC1, Carb109F/GDLS.BC5, and Carb109M/GDLS.BC5 or (B) 0.25 (transgenic heterozygote x GDLS) for Carb109F/GDLS.BC1, Carb109M/GDLS.BC1, Carb109F/GDLS.BC5, and Carb109M/GDLS.BC5. Fifteen lines were established for each of the eight experiments. Proportions of EGFP-expressing offspring were estimated by examining 150 larvae from each of the 15 lines over five successive generations (F1–F5) of inter-breeding without selection for the transgenic phenotype. Bars around mean proportions represent the 95% Highest Density Intervals (95% HDI). Proportions showing non-overlapping 95% HDI are significantly different.

# CHAPTER III: LIFE HISTORY CHARACTERISTICS OF A TRANSGENIC AEDES AEGYPTI STRAIN REFRACTORY TO DENGUE VIRUS 2 INFECTION AFTER INTROGRESSION INTO A GENETICALLY DIVERSE LAB STRAIN

## Introduction

Dengue viruses (DENV) are estimated to infect almost 400 million people annually making dengue the most important human arboviral disease (Bhatt et al., 2013). Difficulty in development of a dengue vaccine and the absence of a nonhuman reservoir host in the urban dengue cycle makes vector control the most effective current strategy to reduce DENV transmission. Historically, vector control for the main DENV vector, Aedes aegypti, has involved environmental modification, such as removal of containers used as oviposition sites, biological control through introduction of a predatory species, or insecticides to reduce the vector population size, but these strategies are plagued by ethical issues and have transient effectiveness. Most control programs now implement an integrated approach to vector control and genetic vector control has become an option in an integrated vector control program. The first field releases of genetically modified Ae. aegypti used irradiated, sterilized males, but these were unsuccessful due to fitness reduction associated with the methods of sterilization, lack planning, (Curtis et al., 1976; McDonald et al., 1977; Petersen et al., 1977) or were proof of concept experiments (Rai et al., 1973). Recent genetic population reduction strategies using lethal dominant technology have been more successful (Harris et al., 2012), but this strategy would be most successful in concert with other vector control methods (Curtis and Graves, 1988; Whitten and Foster, 1975).

Another genetic control strategy to complement vector suppression strategies involves replacement of a vector population with a population that is engineered to be refractory to a

pathogen (Curtis and Graves, 1988). A previously developed a RNA interference based transgenic Ae. aegypti resistant to DENV2, but after $G_{13}$ the resistance phenotype was lost and there were reductions in the laboratory population fitness of the transgenics (Franz et al., 2009, 2014; A. Franz et al., 2006). Some of the fitness associated with backcrossing this strain into a GDLS were described in Chapter 2 and in previous studies (Franz et al., 2014). In these studies, introgression of the transgene into a GDLS background led to improvement of the fitness of transgene heterozygotes. This chapter further evaluated the fitness costs associated with using an RNAi based transgenic Ae. aegypti engineered with resistance to DENV2 infection as a method for DENV control. This study focuses on the creation of the transgenic A. aegpyti strains refractory to DENV2 and the fitness of these new transgenic strains. Transgenic strains that maintained stability and function over time, as assessed in Chapter 2, were used in fitness experiments to determine the mosquito fitness after introgression of the transgenes into a genetically diverse lab population (GDLS). Additionally, in this chapter I further characterized the fitness of the homozygous transgenic lines, which were found to gain little benefit from introgression as described in Chapter 2. These experiments support the possibility of a population replacement strategy as part of DENV control program and demonstrate the importance of outcrossing transgenic lines to improve fitness.

## Materials and Methods

### Transgene Design

Plasmid DNA construction and germ-line transformation were described previously (A. Franz et al., 2006). In brief, 1505 Ae. aegypti Higgs white eye (HWE), a Rexville D strain variant, preblastoderm embryos were microinjected with a mariner MosI nonautonomous Class II transposable element (TE) helper plasmid and a pMos-Carb/Mnp+/i/Mnp-/svA donor plasmid.

Mosquito colony maintenance

All stages of mosquitoes were maintained at 28 ºC ± 2 ºC, 80% ± 5% relative humidity and 14:10 hour light dark photoperiod. Unless otherwise stated, larvae were reared at a density of ~100 mosquitoes per L and were fed ~0.35g of 50:50 flake fish food (Tetramin, Melle, Germany) and rodent diet (Harlan Labs) every other day until pupation after which they were placed into 2.5L cartons. After emergence adults were provided sugar and water and were given a citrated sheep blood meal (Colorado Serum Co., Boulder, CO) 5 to 8 days later.

Immunofluorescence assay (IFA)

Indirect IFAs were conducted as described earlier (Kuberski, 1979) using the 3H5 DENV2 E specific antibody on midguts stored in a 4% paraformaldehyde PBS solution, or head squashes fixed on a glass slide with 100% acetone. The IFAs were conducted to characterize the midgut infection phenotype of each strain.

Vector competence of BC5 and phenotypic recovery by transient silencing of Dcr2

Total RNA was extracted from adult HWE strain Ae. aegypti with the RNeasy kit (Qiagen, Valencia, CA) and cDNA was generated by RT-PCR using the Superscript III RT kit (Invitrogen, Carlsbad, CA) with oligo (dT) primers. Then PCR amplification and gel purification were performed on a ~500bp region of the RNA binding domain of Dcr2 (Sanchez-Vargas et al., 2009) and on a ~500bp region of the βgal gene of an Escherichia coli cDNA clone (Keene et al., 2004) both with incorporated 5' T7 promoter sequences. The products were then in vitro transcribed, purified, and the quality and quantity of the dsRNA was checked (Keene et al., 2004). Approximately 200, four to six day old females that were positive for the EGFP eye marker by fluorescence microscopy of both the original (G7) and backcrossed ($5^{th}$ BC) carb109 lines were intrathoracically injected with Dcr2 dsRNA, or subjected to one of the 3 controls; βgal dsRNA injection, PBS injection, or no injection. The dsRNA was diluted to 6µg/µl in

phosphate buffer solution (PBS) resulting in 892 ng dsRNA per injection using the Nanoject II injection system (Drummond Scientific, Broomall, PA). Reduction in Dcr2 mRNA was calculated from 5 Carb109M and 5 Carb109F non-injected and Dcr2 injected females by qPCR using the Quanifast SYBR green kit (Qiagen, Valencia, CA), using the primers described in previous studies (Khoo et al., 2010).

Approximately 36 hours post-injection the mosquitoes were given an infectious blood meal containing 8.6 X $10^6$ pfu/ml DENV2. As described in previous studies, high passage DENV2 Jamaica 1409 at an MOI of 0.01 was used to infect C6/36 cells in L-15 media supplemented with 3% heat inactivated fetal bovine serum, 1% streptomycin and 1% L-glutamine for 12 days at 28º C (Bosio et al., 1998). The medium was replaced 5 to 6 days after infection. Non-injected HWE mosquitoes were also included as a positive control. Mosquitoes were collected 14 days post infection (dpi) and virus titers were assessed by plaque assay.

## Evaluation of transgene fitness by introgression into a GDLS

### Introgression mating

At G5 a subset of the eye marker positive mosquitoes from both Carb109 lines (G5) were reciprocally intercrossed (BC1) with the genetically diverse lab strain (GDLS) followed by 4 reciprocal backcrosses (BC2-BC5) to the GDLS. Fitness traits were calculated separately for each reciprocal cross of each BC unless specified, but for creation of the next generation the reciprocal BCs were combined at each generation.

### Fitness of backcrossed Carb109 lines

Adult fecundity at first oviposition, hatch rate, egg viability, ratio of transgenic offspring, sex ratio, transgenic sex ratio, adult survival, larval development, larval survival and larval energy reserves were the fitness traits examined in this study. To determine fecundity, hatch rate and egg viability, 40 to 80 blood fed females from each cross were caged individually in 1.25L cardboard cartons (Huhtamaki, Fulton, NY) with organdy lids. At BC1 and BC4, forty

individually caged blood fed GDLS females were also used as a control. These two GDLS controls were combined and used as a control for all BCs.

Each cage was provided with a sugar source and an oviposition site. After 4 days post blood meal the egg liner was removed and the eggs were counted to determine the fecundity of each female at first oviposition. Then the eggs were dried for at least 4 days then hatched in ~1L of water. Egg liners were left in the pans for approximately 12 hours. The number of larvae during the first hatch was recorded and the larvae were raised to adults. To determine the viability of the unhatched eggs, the egg liners were removed from the water, dried for another 2 days and then hatched a second time under the same conditions. After 48 hours all larvae present were counted. Any remaining unhatched eggs were counted and examined for viability using the Trpis method (Trpis, 1970). Transgene fitness was evaluated by determining offspring sex ratio and the transgenic sex ratio. For each strain, approximately 60 larvae were randomly selected at each backcross and all of the resulting pupae were sexed and separated by EGFP marker expression. The percent transgenic larvae was determined by the presence of the EFGP eye marker under a fluorescence microscopy. The differences between GDLS and the Carb109M and Carb109F hatch rates were calculated by Kruskall-Wallis analysis on the ranked data followed by a Dunn's test (SigmaPlot, Systat Software, San Jose, CA). Fecundity differences between groups were evaluated by a 2-way ANOVA with strain and backcross as main effects (SigmaPlot, Systat Software, San Jose, CA).

To compare adult survival rates, the reciprocal crosses of both Carb109 BC lines were screened for the EGFP eye marker, and then combined to make two lines, one Carb109M and one Carb109F. The GDLS line was used as a control. At BC1-4, each line was separated into two groups of 8 cages containing either 20 blood fed or 20 non-fed 4 day old females. All

females were also provided sugar ad libitum. The insectary conditions, blood feeding protocol and general mosquito maintenance were as described in the colony maintenance section. However, the blood fed group was fed on day 4 and day 11 post emergence and an oviposition site was not provided to prevent oviposition associated mortality. Every day mosquito survival was recorded until all mosquitoes had died or until the mosquitoes were 31 days post emergence. For each strain and each backcross, adult survival curves were created and compared by a log rank test (SigmaPlot, Systat Software, San Jose, CA).

To compare larval development rates and estimate larval energy reserves the reciprocal crosses of both Carb109 BC lines were screened for the EGFP eye marker, and then combined to make two lines, one Carb109M line and one Carb109F line. Larval development was compared between 8 pans for each Carb109 BC and the GDLS at two pan densities 200 larvae/L (high) and 50 larvae/L (low). On days 5 through 7 post hatch the survival and developmental stage of each individual was calculated by head capsule width (Lardeux and Tetuanui, 1995). On days 6 and 7 post hatch, the number of emerged mosquitoes were counted, and the percentage of emerged mosquitoes was probit transformed and analyzed in a general linear model (GLM) with strain, backcross, and density level as main factors (SigmaPlot, Systat Software, San Jose, CA). During the first two days of pupation, each individual was removed and discarded, but for BCs 1, 3 and 5 on the third day 100 females were randomly selected from each group and split into 5 cages of 20 individuals. These females were starved and provided with only water to estimate the energy reserves acquired during the larval stage. Each day the number of dead females were counted and their survival curves were compared by a Log Rank test.

Creation of homozygous Carb109/GDLS lines

At intercross 2, 30 lines from each cross were created by intercrossing 3 EGFP positive females with 1 EGFP positive male. The offspring from each cross were screened and those

groups with 100% EGFP expression were retained and put into EGFP positive groups of 5 females and 1 male. The offspring from this cross were screened for the EGFP eye marker, all lines with 100% positive offspring were retained. This process was repeated twice to verify homozygosity and to obtain large enough numbers for colonization. To further confirm homozygosity, both lines were backcrossed to the HWE line and the offspring were screened for EGFP expression.

### Fitness and vector competence of the homozygous, GDLS introgressed Carb109 lines

The number of eggs per female, hatch rate and sex ratio were calculated as described above from 30 individual females from each homozygous strain. The GDLS and 20 individuals from the early generation Carb109M line were used as a control. DENV2 Jamaica 1409 oral infections were conducted by the methods described previously on the three homozygous Carb109 strains to determine their DENV2 vector competence.

## Results

### Characterization of Carb109M midgut DENV2 infection by IFA

The proportion of DENV2 infected midguts was reduced in the Carb109M line as compared to the HWE line (Fig. 3.1) 4 days post infection (dpi). Additionally, the intensity of infection was also lower 4 dpi in the Carb109M line versus the HWE line, by indication of larger infection foci in the HWE line (Fig. 3.1). By 10dpi 74.3% of the Carb109M line had no DENV2 infection in the midgut (Fig. 3.1, D-B). In the remaining 25.7% Carb109M individuals with a midgut infection at 10 dpi, none had an infection disseminated to the head, while 94.0% of individuals in the HWE line with a midgut infection at 10 dpi had a disseminated infection. Most of the infected midguts at 10 dpi had a limited infection in the anterior midgut with just a few small foci of infection (Fig. 3.1, D-C). Only 5.7% of Carb109M midguts had an infection in

the posterior midgut. Furthermore, they had much smaller infection foci as compared to the HWE positive mosquitoes (Fig. 3.1, D-A and D-D) and had no disseminated infection.

### Partial susceptible phenotype recovery from dsRNA Dcr2 knock down

Carb109M and Carb109F G9 mosquitoes showed a recovery of their DENV2 susceptible phenotype after injection with dsRNA to Dcr2. The Carb109M strain had a 33.33% disseminated infection rate (n=24) and the Carb109F had a 37.50% disseminated infection rate (n=24) 12 dpi after injection with dsRNA to Dcr2 as compared to the non-injected, PBS injected and βgal injected control groups (n=15), which all had a 0.00% disseminated infection rate. The positive control, HWE strain (n=15), showed a 66.67% dissemination rate. The average dsRNA knock down of Dcr2 was 37.0 fold in the Carb109F and 28.4 fold in Carb109M 36 hours post injection by qPCR (data not shown).

## Fitness of the GDLS introgressed Carb109 lines

### Sex ratios of Carb109M, Carb109F introgressed and GDLS lines

The overall sex ratio for each line was slightly skewed towards males, however, it was not a large deviation from a 1:1 ratio (Fig. 3.2). Once the mosquitoes were separated by their EGFP expression the differences in sex ratio were skewed more towards males in the first two backcrosses (Fig. 3.2). Mosquitoes skewed towards males are fairly common in the wild and in colony, and this ratio, while skewed is within the range of other studies (Hapairai et al., 2014; Hickey and Craig, 1966; Owusu-Daaku et al., 1997; Vinogradova, 2011). This sex ratio could be skewed for a variety reasons including linkage of the transgene to the sex loci or sex linked meiotic drive (Hickey and Craig, 1966; Owusu-Daaku et al., 1997). However, there was no significant difference in Carb109 sex ratios compared to the GDLS in all mosquitoes ($p>0.05$) or for transgenic mosquitoes only ($p>0.05$).

Hatch rate

Figure 3 shows that there is initially a lower hatch rate in the Carb109 lines compared to the GDLS line, but by BC3 there is no difference in hatch rate in any of the groups. Comparison of the hatch rate after the first hatch (Fig. 3.3A), and the total hatch rate (Fig. 3.3B), shows that there was no difference in hatch overall between the GDLS and Carb109 lines indicating a delayed hatch in the Carb109 lines before BC3. Egg viability was not statistically different from GDLS ($H=0.76$, $df=8$, $p>0.05$) at any time point for any line.

Fecundity

The fecundity at first oviposition was also lower for the first two backcrosses, but was recovered by BC3 (Fig. 3.4). There were also significant differences between strains, but these differences were attributed to the differences in the earlier backcrosses as there were no significant differences after applying a Tukey post hoc test to the strain main effects (Fig. 3.4).

High and low density larval development and energy reserves

Larval development time was longer for the Carb109 lines raised at high densities, but not for those at low densities (Table 3.1). At the lowest larval density, the mean percentage of larvae that emerged at day 7 did differ from the GDLS at backcross 5, but this is due to the high emergence rates in the Carb109 lines (Table 3.1). Therefore, the Carb109 lines did not develop significantly slower than the GDLS at any backcross in the low density pans. There is a significant difference between Carb109M and the GDLS larvae at high densities (Table 3.1).

To assess the energy reserves acquired by the larvae, Carb109F and Carb109M adult females raised in the high and low density larval conditions were given no food resources, only water, after emergence. Carb109M and Carb109F larvae raised at high densities had significantly lower survival as adults compared to the GDLS at BC1 and BC3, but the mean time to death did not vary by even 1 day at any backcross (Fig. 3.5). Females raised at a low density had no significant differences in adult survival between any group in any backcross (Fig. 3.6).

Adult survival with sugar only and sugar and blood resources

Survival of adult females fed only sugar was lower in the Carb109M line through BC4 and through BC3 in the Carb109F line compared to the GDLS line (Fig. 3.7), but there was no difference in survival during any cross for mosquitoes fed sugar and provided with weekly blood meals (Fig. 3.8). Differences in mean time to death was never greater than 4 days between any group in the sugar only or weekly blood feeding groups, even when significantly different (Figs. 3.7 and 3.8).

Fitness of homozygous strains

The mean number of eggs per female was significantly lower in all of the Carb109 homozygous strains compared to the GDLS and the early intercross Carb109M homozygous strain (Figs. 3.9A, 3.9D). The large difference in the number of eggs laid per female resulted from a large number of females in the Carb109M homozygous groups not laying any eggs. After the 20% to 30% of females that did not lay any eggs were omitted, the mean number of eggs laid was still significantly different from the GDLS ($H=73.49$, $df=4$, $p<0.001$, Dunn's multiple comparison $p<0.05$), but not from the early intercross Carb109M homozygous strain. The hatch rate was also significantly lower in the Carb109 homozygous strains compared to the GDLS and the early intercross Carb109M homozygous strain (Figs. 3.9B, 3.9D). There was no significant difference between the sex ratios of the GDLS, the early intercross Carb109M, or the Carb109 homozygous strains (Fig. 3.9C, 3.9D).

Discussion

The Carb77 line from Franz et al., (2006) demonstrated fitness deficits compared to non-transgenic strains, which has been commonly found in many recent studies that examine the fitness of transgenic mosquitoes (Catteruccia et al., 2003; N Irvin et al., 2004; Junitsu Ito et al., 2002; Koenraadt et al., 2010; Marrelli et al., 2006; Massonnet-Bruneel et al., 2013; L. a Moreira

et al., 2002; L. Moreira et al., 2002; Moreira et al., 2004). Recent studies that showed no fitness reduction associated with transgenic mosquitoes, only compared the transgenic fitness to a highly laboratory adapted parent strain (Amenya et al., 2010; Massonnet-Bruneel et al., 2013; McArthur et al., 2014). The focus of these studies was centered on the transgene itself with negative effects associated with the transgene insertion site, the transgene product (Marrelli et al., 2006); inbreeding effects also play a large role in the fitness reduction (Koenraadt et al., 2010). These inbreeding effects are associated with the highly interbred parent strain and the inbreeding associated with the creation of a homozygous strain. In this study, the parent strain was the HWE line, which has been colonized for 20+ years, and maintains a recessive white eye phenotype utilized for ease of screening for the EGFP marker. Backcrossing the Carb109 lines to a GDLS increased the mean number of eggs laid, reduced hatch delay, increased the survival of sugar fed females, and increased the competitiveness of the Carb109 line in small GDLS laboratory populations. A similar backcrossing strategy was used previously before a small cage release study of RIDL transgenic mosquitoes, and these also remained competitive in a GDLS population (Valdez et al., 2010). Rapid creation of a homozygous strain requires inbreeding. Previous studies have shown that there is a large inbreeding depression associated with homozygosity (Catteruccia et al., 2003; Nic Irvin et al., 2004; Junitsu Ito et al., 2002; L. Moreira et al., 2002). This inbreeding depression is caused by allele similarity between closely related individuals resulting in an increase in deleterious recessive alleles. The early intercross of the Carb109M also showed a reduced fitness compared to the GDLS, but this was not significant. These results suggest that in this case, inbreeding may have a large influence on the fitness reduction in this line. Additionally, since the transgene is located in the 3' UTR of a coding region there may be no large fitness detriment when heterozygous, because expression of

the non-transgenic allele may be sufficient for a normal phenotype. In contrast, once the mosquito is homozygous it has fitness deficits. However, previous work on this line indicated that the transgene is likely linked to a recessive mutation and thus in the homozygous strain the recessive allele becomes fixed (Franz et al., 2014).

Additionally, not all fitness parameters were improved with backcrossing to the GDLS, mainly larval survival when raised at a high density. Aedes aegypti is highly influenced by density dependent in the larval stage. High density larval conditions have been shown to adversely affect RIDL transgenic Ae. aegypti (Bargielowski et al., 2011). High food competition, similar to high density conditions, increased mortality in transgenic A. aegpyti compared to wild and inbred larvae in other studies (Koenraadt et al., 2010). Similar results were also seen in this study; the high density lines had reduced fitness compared to those raised in low density conditions. The increased high density mortality of the Carb109 strain may potentially detrimental in the field as conditions are more often sub-optimal. This study did not examine larval competition between the GDLS and Carb109 lines, but another study examined larval competition between transgenic and wild type lines suggested that there also may be further mortality associated with larval competition (Koenraadt et al., 2010). But, in general, introgression of the Carb109 transgene into a GDLS genetic background improved the overall fitness of the mosquito.

In this study, introgression of a transgene into a population of interest has been shown to increase the fitness of a transgenic mosquito. Applications of this strategy in a field release program may increase the probability for programmatic success. Yet, while we used the GDLS to attempt to simulate a wild population, this strain is still laboratory adapted, and this may pose an obstacle for utilization in the field. Additionally, previous backcrossing experiments with the

Carb77 strain did not result in the same success, and thus this strategy may only be effective on a line to line basis. These experiments were only conducted with a GDLS created from one region in Mexico. The wide range of genetic variability in A. aegpyti would likely require introgression into genetic backgrounds specific to that region, which is time consuming and possibly not practical on a large scale. With these considerations, this study still demonstrates the utility of introgression of a transgene into the population of interest prior to evaluating the fitness potential of a transgenic mosquito.

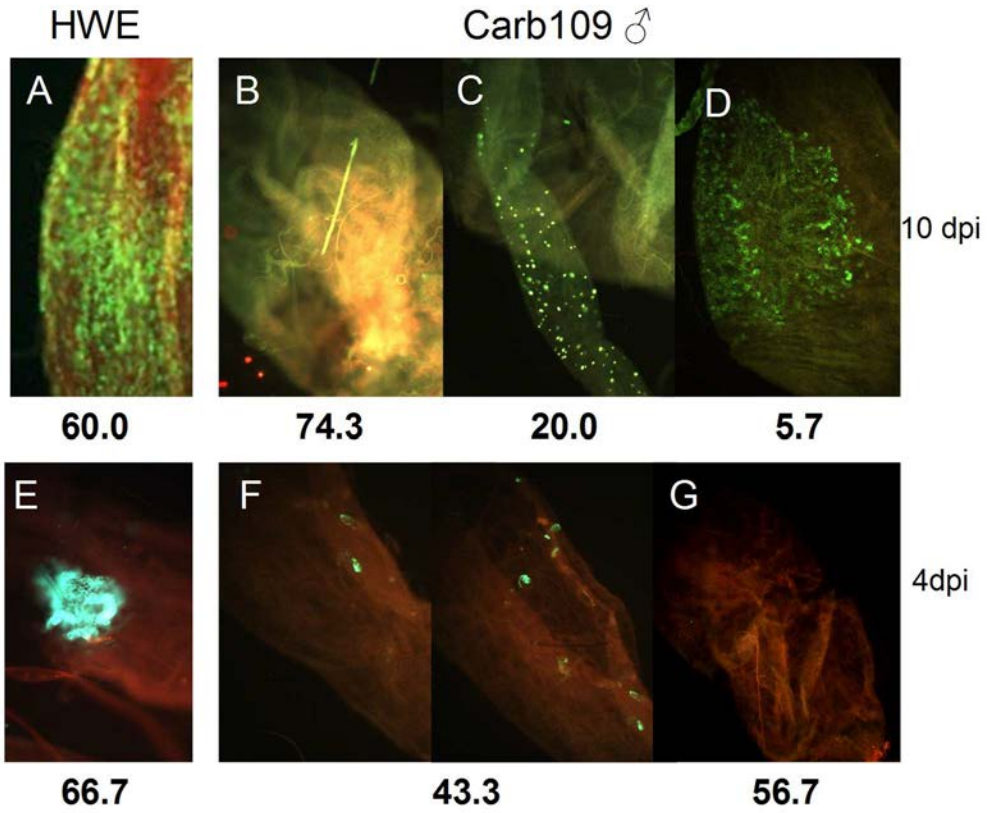



Figure 3.1: Detection of DENV2 by IFA in the HWE and Carb109M lines 4 and 10 dpi with DENV2 Jamaica 1409. (B-D) shows the midgut infection of the Carb109M male line 10dpi. Below each picture shows the percentage of midguts (n=34) with (B) no infection, (C) infection of the anterior midgut, (D) foci of midgut infection. At 4 dpi the Carb109M had either small foci of infection (F) or no infection (G) with the percentage in each group shown below (n=34). The positive controls HWE 10dpi (A) and HWE 4dpi (E) with the percentage positive shown below.

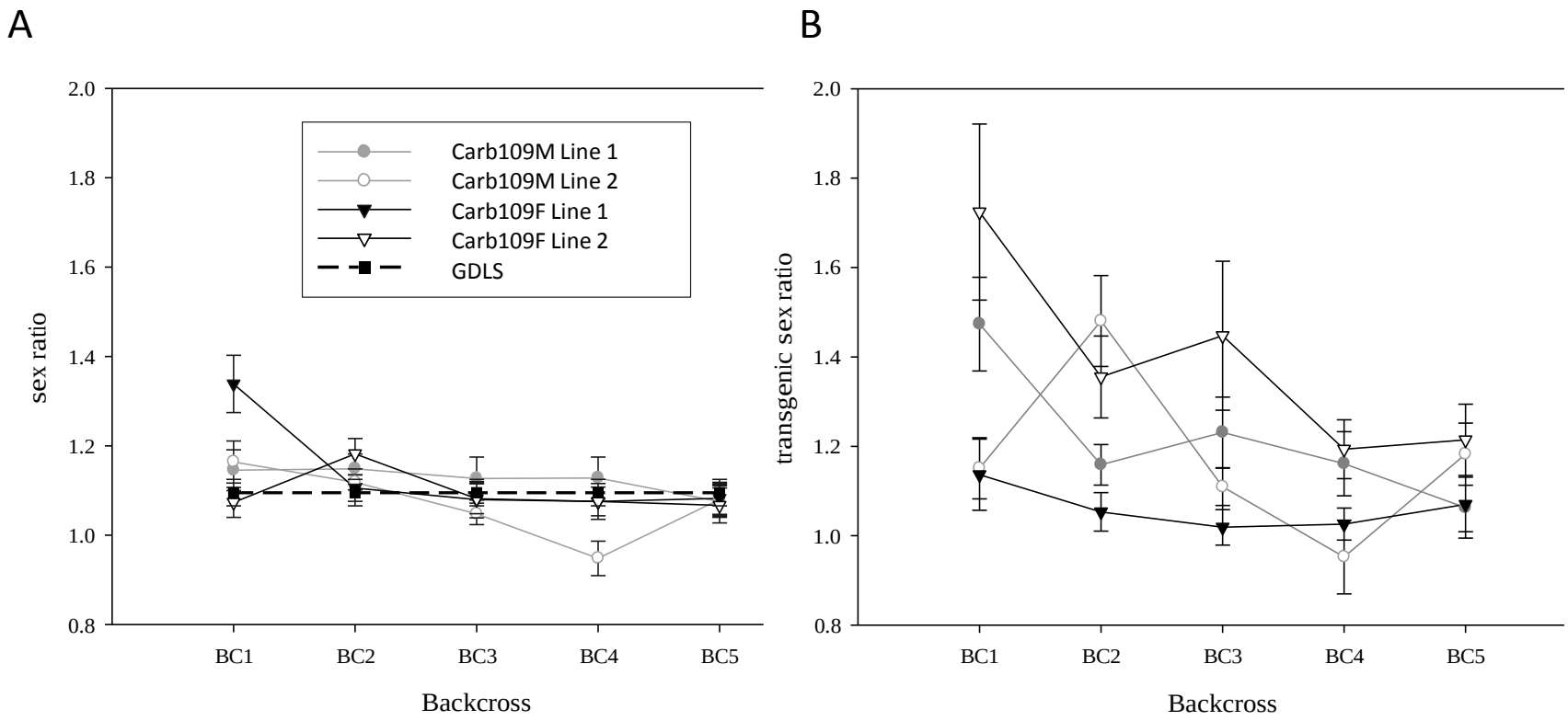
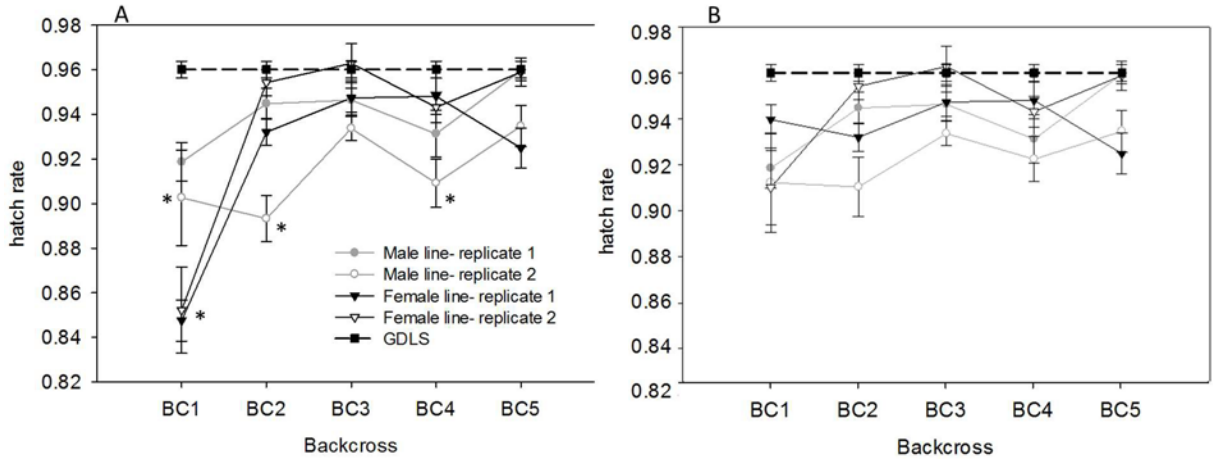


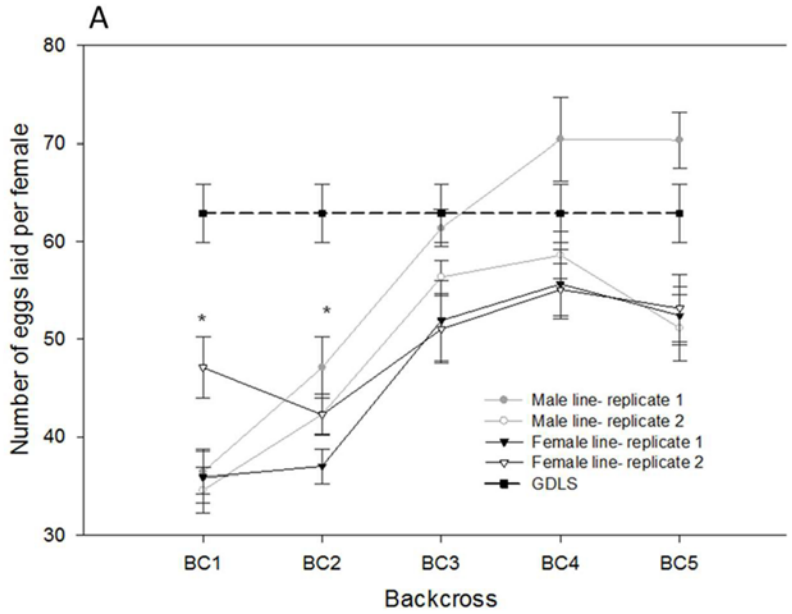

Figure 3.2: Sex ratio and and transgenic sex ratio over the 5 backcrosses to the GDLS. (A) shows the mean sex ratio EFGP positive mosquitoes over 5 BCs. The Carb109M lines (gray circles) and the Carb109♀ lines (black triangles) is the observed EGFP frequency the first replicate (filled shapes) or the second replicate (open shapes). The sex ratio is calculated as the number of males/number of females. Therefore, sex ratio >1 is skewed towards males. n= approx. 30 to 60 offspring from 60-80 individual females. The GDLS control (black square) is represented by the dotted black line. The bars indicate standard error.




C

| Backcross | Strain | H, p | Difference of Ranks | q |
|---|---|---|---|---|
| 1 | Carb109F | 220.71, p<0.001 | 357.52 | 6.81* |
| | Carb109M | | 311.55 | 5.95* |
| 2 | Carb109F | | 280.28 | 5.76* |
| | Carb109M | | 221.33 | 4.52* |
| 3 | Carb109F | | 140.83 | 2.59 |
| | Carb109M | | 15.23 | 0.31 |
| 4 | Carb109F | | 71.95 | 1.33 |
| | Carb109M | | 32.67 | 0.603 |
| 5 | Carb109F | | 110.07 | 2.01 |
| | Carb109M | | 3.89 | 0.07 |

Figure 3.3: Hatch rate of eggs over 5 backcrosses to the GDLS. (A) shows the mean hatch rate EFGP positive mosquitoes over 5 BCs for the first hatch only. (B) shows the mean hatch rate EFGP positive mosquitoes over 5 BCs for the combination of the first and second hatch . The Carb109M lines (gray circles) and the Carb109♀ lines (black triangles) are the observed EGFP frequency in the first replicate (filled shapes) or the second replicate (open shapes). The GDLS control (black square) is represented by the dotted black line. The bars indicate standard error. (C) shows the results of the Kruskall-Wallis analysis of the ranked percentage hatched eggs. The H statistic was derived for all group comparisons, and the difference of ranks and q statistic were calculated through a post hoc Dunn's test. * indicates significant differences ($p < 0.05$) between the experimental group and GDLS control.




B

| Source of Variation | DF | SS | MS | F | p |
|---|---|---|---|---|---|
| Strain | 2 | 9531.88 | 4765.94 | 14.65 | <0.001 |
| Backcross | 4 | 69217.36 | 17304.34 | 53.19 | <0.001 |
| Residual | 933 | 303547.18 | 325.35 | | |
| Total | 939 | 390910.18 | 416.31 | | |

| | p values | | | |
|---|---|---|---|---|
| Backcross | BC1 | BC2 | BC3 | BC4 |
| BC1 | - | 0.17 | <0.001 | <0.001 |
| BC2 | 0.17 | - | <0.001 | <0.001 |
| BC3 | <0.001 | <0.001 | - | 0.45 |
| BC4 | <0.001 | <0.001 | 0.45 | - |
| BC5 | <0.001 | <0.001 | 0.79 | 0.94 |

Figure 3.4: Fecundity of the Carb109 lines during 5 backcrosses to the GDLS. (A) each data point is the mean number of eggs laid per female from n=60-80 individual females. Females that did not lay eggs were omitted from the calculations. The gray lines with circles indicate replicates of the crosses of the Carb109 male line. The solid black lines with triangles indicate replicated of the crosses of the Carb109 female line. The GDLS control (dashed line with black squares) is represented by the dotted black line. The bars depict standard error. (B) the upper table shows the statistical results of a 2-way ANOVA with strain and back cross as main effects. Analysis was conducted on ranked data meet to the assumptions of the model. The lower table shows the p-value table from a Tukey post hoc test to identify the differences in the main effect of back cross. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value.

Table 3.1: Statistical results from larval development experiments. A) Mean percentage of larvae that emerged at day 7 post hatch. SE indicates standard error of the 8 replicates. B) The results of a 3-way ANOVA with strain, back cross and larval density as the 3 main effects. The data was probit transformed to meet the normality and equal variances assumptions of the model. Significant main effects and interactions were then compared by a Tukey test. The p-values for the significant interactions in the low density (lower left) and the high density groups (lower right) are shown. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value.

A

| Strain | BC | Density | Mean percentage emerged at day 7 | SE |
|---|---|---|---|---|
| Carb109M | 1 | Low | 38.26 | 2.41 |
| Carb109M | 3 | Low | 43.88 | 2.56 |
| Carb109M | 5 | Low | 57.41 | 3.81 |
| Carb109F | 1 | Low | 29.23 | 1.87 |
| Carb109F | 3 | Low | 27.55 | 2.00 |
| Carb109F | 5 | Low | 51.38 | 2.85 |
| GDLS | 1 | Low | 33.75 | 2.74 |
| GDLS | 3 | Low | 25.99 | 1.99 |
| GDLS | 5 | Low | 39.68 | 1.92 |
| Carb109M | 1 | High | 10.86 | 1.30 |
| Carb109M | 3 | High | 11.57 | 1.06 |
| Carb109M | 5 | High | 13.01 | 1.34 |
| Carb109F | 1 | High | 6.08 | 0.53 |
| Carb109F | 3 | High | 9.01 | 0.97 |
| Carb109F | 5 | High | 12.62 | 0.87 |
| GDLS | 1 | High | 7.35 | 0.82 |
| GDLS | 3 | High | 10.00 | 0.68 |
| GDLS | 5 | High | 11.17 | 0.91 |

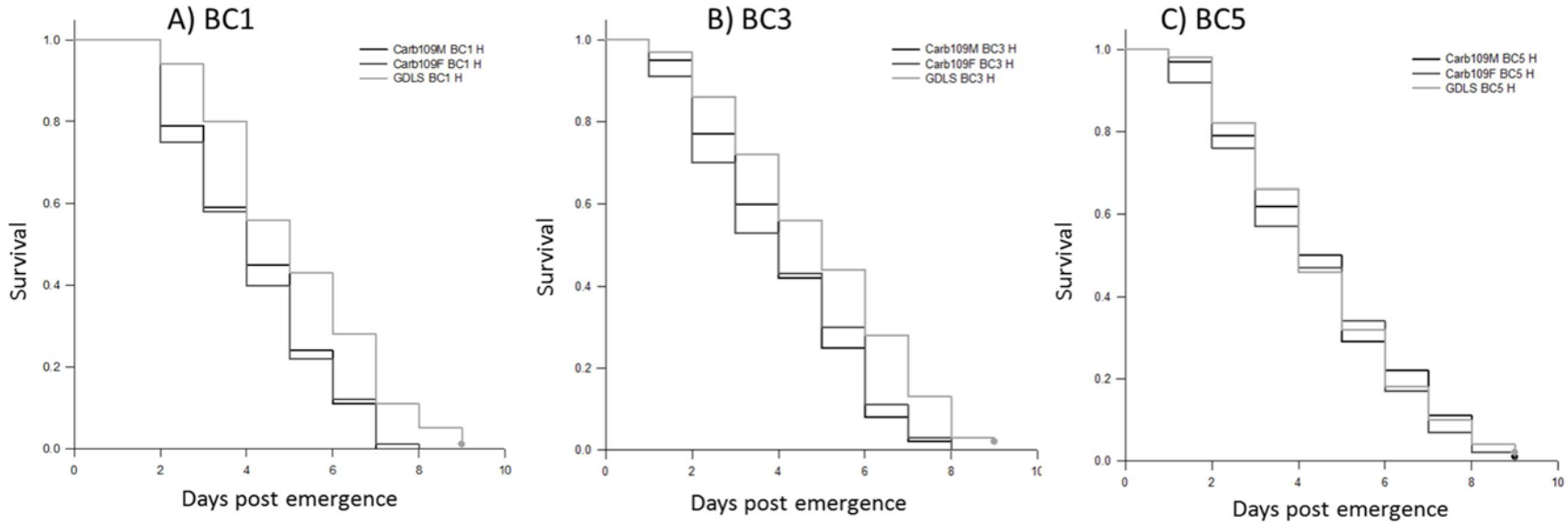
B

| Source of Variation | DF | SS | MS | F | p |
|---|---|---|---|---|---|
| Strain | 2 | 1.73 | 0.86 | 28.27 | <0.001 |
| BC | 2 | 3.12 | 1.56 | 51.01 | <0.001 |
| Density | 1 | 35.39 | 35.39 | 1157.35 | <0.001 |
| Strain x BC | 4 | 0.43 | 0.11 | 3.52 | 0.009 |
| Strain x Density | 2 | 0.33 | 0.16 | 5.36 | 0.006 |
| BC x Density | 2 | 0.73 | 0.37 | 11.93 | <0.001 |
| Strain x BC x Density | 4 | 0.34 | 0.09 | 2.79 | 0.029 |
| Residual | 126 | 3.85 | 0.03 | | |
| Total | 143 | 45.92 | 0.32 | | |

| Low Density Simple Main Effects | | | |
|---|---|---|---|
| Backcross | Factor | Carb109M | Carb109F |
| BC3 | Carb109M | - | <0.001 |
| | Carb109F | <0.001 | - |
| | GDLS | <0.001 | 0.85 |
| BC5 | Carb109M | - | 0.16 |
| | Carb109F | 0.16 | - |
| | GDLS | <0.001 | 0.002 |

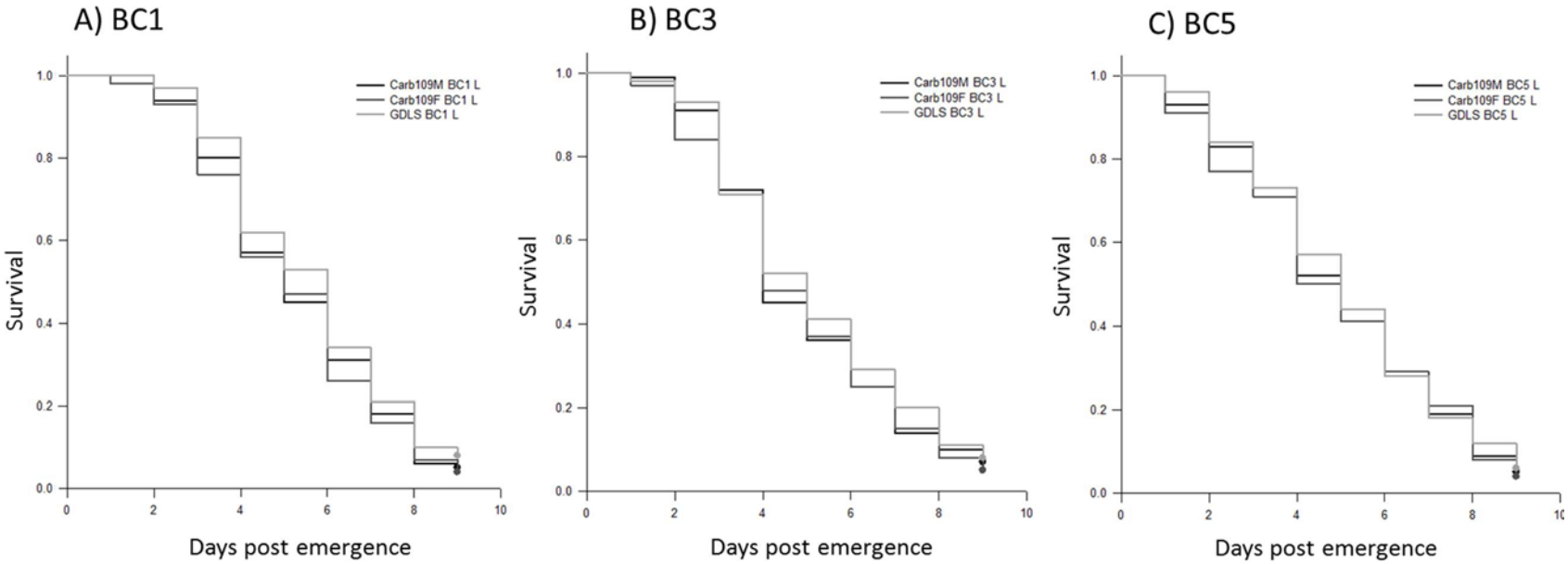
| High Density Simple Main Effects | | |
|---|---|---|
| Factor | Carb109M | Carb109F |
| Carb109M | - | 0.006 |
| Carb109F | 0.006 | - |
| GDLS | 0.03 | 0.85 |

| Backcross | Factor | BC1 | BC3 |
|---|---|---|---|
| Carb109M | BC1 | - | 0.21 |
| | BC3 | 0.21 | - |
| | BC5 | <0.001 | <0.001 |
| Carb109F | BC1 | - | 0.82 |
| | BC3 | 0.82 | - |
| | BC5 | <0.001 | <0.001 |
| GDLS | BC1 | - | 0.03 |
| | BC3 | 0.03 | - |
| | BC5 | 0.15 | <0.001 |



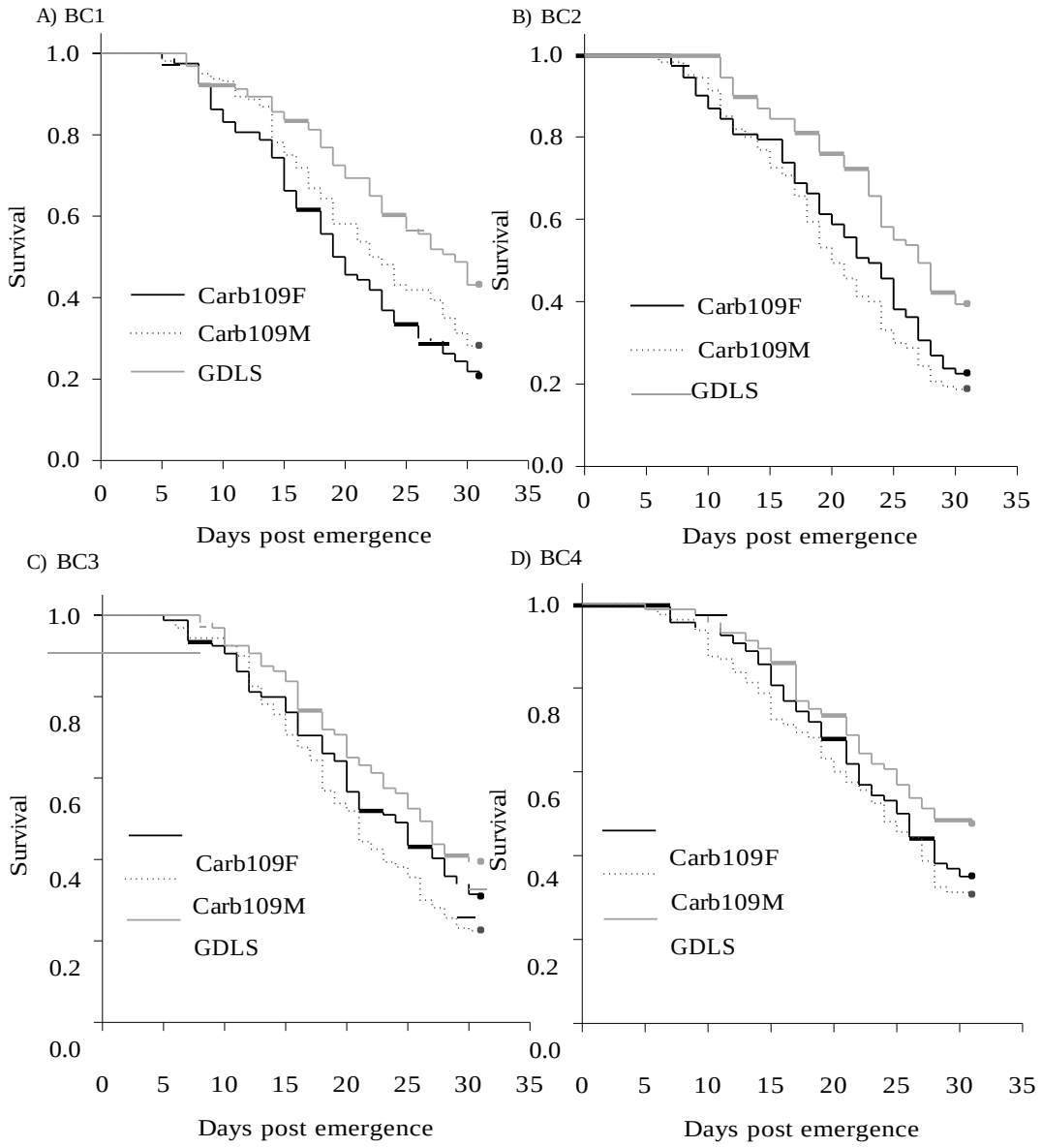

| | BC1 | | | BC3 | | | BC5 | | |
|---|---|---|---|---|---|---|---|---|---|
| Strain | Mean | 95% lower | 95% upper | Mean | 95% lower | 95% upper | Mean | 95% lower | 95% upper |
| Carb109F | 4.10** | 3.75 | 4.42 | 4.01* | 3.62 | 4.40 | 4.50 | 4.12 | 4.96 |
| Carb109M | 4.18** | 3.86 | 4.51 | 4.09** | 3.74 | 4.43 | 4.32 | 3.90 | 4.74 |
| GDLS | 5.20 | 4.80 | 5.54 | 4.99 | 4.58 | 5.40 | 4.56 | 4.16 | 4.96 |

Figure 3.5: Survival of Carb109 adults raised at high larval densities. This figures shows the survival curves and mean survival of adult female Carb109F (dark gray), Carb109M (black) and GDLS raised as larvae at a density of 200 larvae/1L. Females were given no sugar or blood as adults. The top survival curves are for A) backcross (BC) 1, B) BC3 and C) BC5. The lower table depicts the mean survival time (days) and 95% confidence intervals. These values were calculated using Kaplan-Meier survival curves. ** indicates significant differences between the experimental group and the GDLS control with a $p < 0.001$ and an * indicates significant differences with $p < 0.05$ as determined by a Log Rank test and Holm-Sidak multiple comparison test.



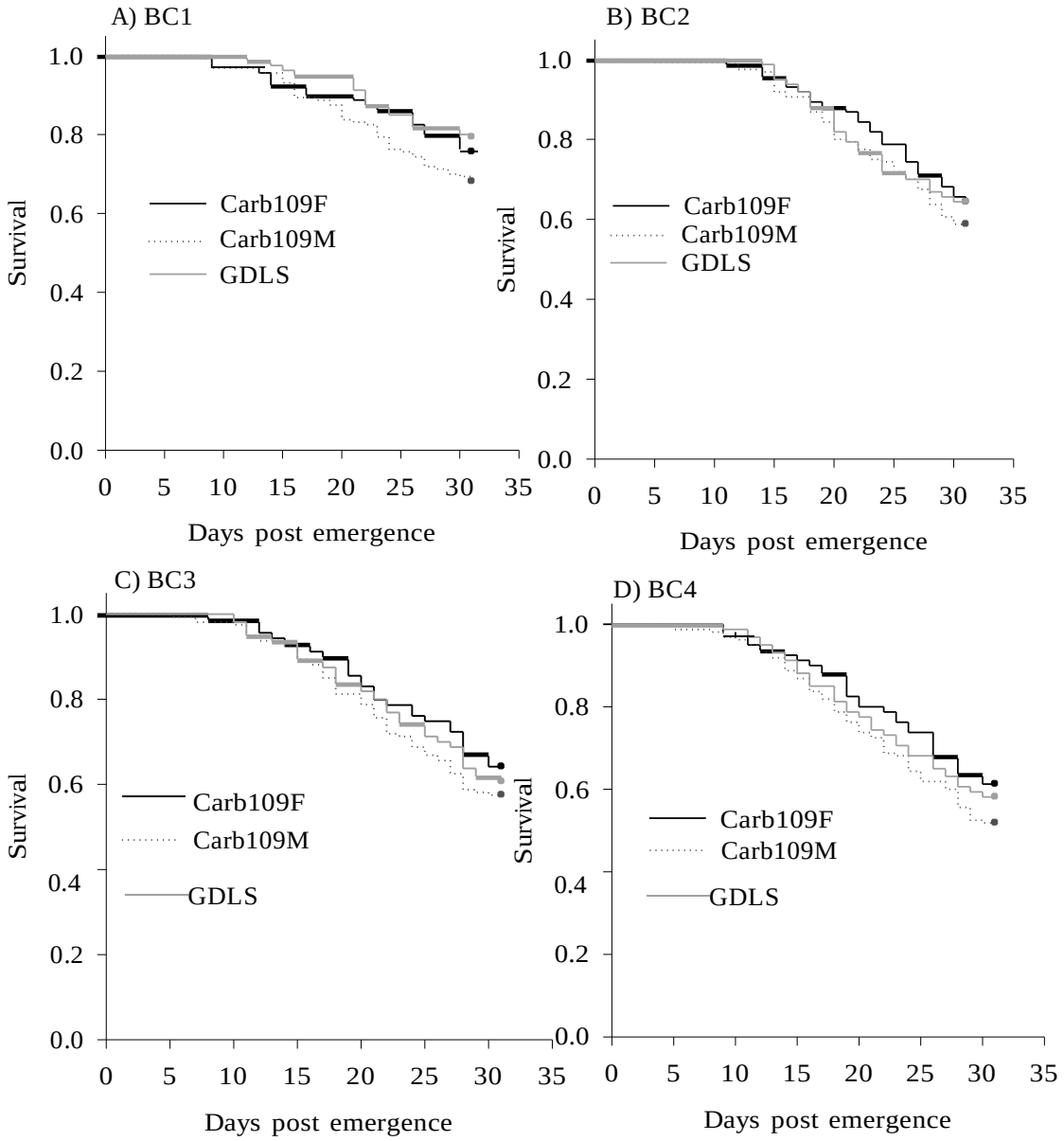

| BC1 | | | BC3 | | | BC5 | | |
|---|---|---|---|---|---|---|---|---|
| Mean | 95% lower | 95% upper | Mean | 95% lower | 95% upper | Mean | 95% lower | 95% upper |
| 5.19 | 4.79 | 5.59 | 4.85 | 4.42 | 5.23 | 4.88 | 4.40 | 5.36 |
| 5.31 | 4.92 | 5.70 | 4.92 | 4.51 | 5.33 | 4.97 | 4.51 | 5.43 |
| 5.62 | 5.23 | 6.01 | 5.15 | 4.72 | 5.59 | 5.12 | 4.67 | 5.58 |

Figure 3.6: Survival of Carb109 adults raised at low larval densities. This figures shows the survival curves and mean survival of adult female Carb109F (dark gray), Carb109M (black) and GDLS raised as larvae at a density of 50 larvae/1L. Females were given no sugar or blood as adults. The top survival curves are for A) backcross (BC) 1, B) BC3 and C) BC5. The lower table depicts the mean survival time (days) and 95% confidence intervals. These values were calculated using Kaplan-Meier survival curves. There were no significant differences between any survival curve by a Log Rank test ($p > 0.05$).



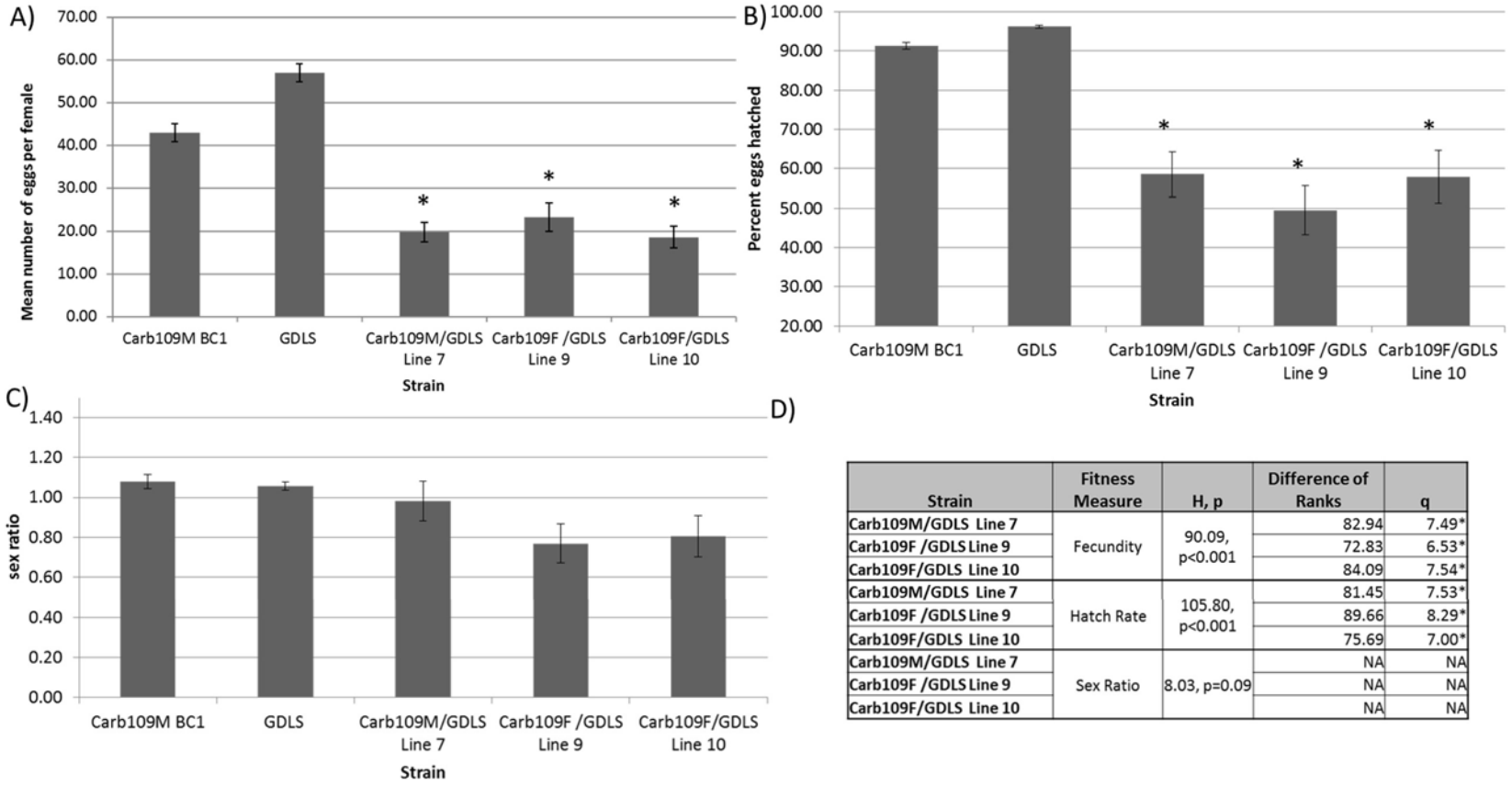

| | BC1 | | | BC2 | | | BC3 | | | BC4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | Mean | 95% lower | 95% upper | Mean | 95% lower | 95% upper | Mean | 95% lower | 95% upper | Mean | 95% lower | 95% upper |
| Carb109F | 20.33**# | 19.06 | 21.59 | 21.80** | 20.59 | 23.01 | 22.30 | 21.02 | 23.58 | 23.51 | 22.34 | 24.68 |
| Carb109M | 22.25*# | 21.03 | 23.47 | 20.73** | 18.29 | 21.71 | 20.92** | 19.72 | 22.12 | 22.43* | 21.17 | 23.68 |
| GDLS | 24.58 | 23.38 | 25.78 | 24.91 | 23.86 | 25.97 | 23.94 | 22.79 | 25.09 | 24.88 | 23.76 | 26.01 |

Figure 3.7: Survival of sugar fed adult female Carb109F and the Carb109M. The figures show survival curves for sugar fed adult females. The graphs depict survival at A) BC1, B) BC2, C) BC3 and D) BC4. The mean survival time (days) and 95% confidence intervals were calculated using Kaplan-Meier survival curves. The significant differences between curves were calculated by a Log Rank test. Curves statistically different were evaluated by a Holm-Sidak multiple comparison test. Significantly different groups from the GDLS are indicated by * ($p < 0.05$) and ** ($p < 0.001$).




| Strain | BC1 | | | BC2 | | | BC3 | | | BC4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | 95% Lower | 95% Upper | Mean | 95% Lower | 95% Upper | Mean | 95% Lower | 95% Upper | Mean | 95% Lower | 95% Upper |
| Carb109F | 28.50 | 27.61 | 29.39 | 27.95 | 27.12 | 28.78 | 27.40 | 26.45 | 28.36 | 26.95 | 25.95 | 27.95 |
| Carb109M | 27.64 | 26.70 | 28.58 | 27.11 | 26.19 | 28.03 | 26.26 | 25.21 | 27.32 | 25.55 | 24.43 | 26.67 |
| GDLS | 29.04 | 28.34 | 29.75 | 27.48 | 26.62 | 28.33 | 26.91 | 25.91 | 27.90 | 26.29 | 25.25 | 27.34 |

Figure 3.8: Survival of blood fed adult female Carb109F and the Carb109M. The figures show survival curves for blood fed adult females. The graphs depict survival at A) BC1, B) BC2, C) BC3 and D) BC4. The mean survival time (days) and 95% confidence intervals were calculated using Kaplan-Meier survival curves. The significant differences between curves were calculated by a Log Rank test. Curves statistically different were evaluated by a Holm-Sidak multiple comparison test. Significantly different groups from the GDLS are indicated by * ($p < 0.05$) and ** ($p < 0.001$).




| Strain | Fitness Measure | H, p | Difference of Ranks | q |
|---|---|---|---|---|
| Carb109M/GDLS Line 7 | Fecundity | 90.09, p<0.001 | 82.94 | 7.49* |
| Carb109F /GDLS Line 9 | | | 72.83 | 6.53* |
| Carb109F/GDLS Line 10 | | | 84.09 | 7.54* |
| Carb109M/GDLS Line 7 | Hatch Rate | 105.80, p<0.001 | 81.45 | 7.53* |
| Carb109F /GDLS Line 9 | | | 89.66 | 8.29* |
| Carb109F/GDLS Line 10 | | | 75.69 | 7.00* |
| Carb109M/GDLS Line 7 | Sex Ratio | 8.03, p=0.09 | NA | NA |
| Carb109F /GDLS Line 9 | | | NA | NA |
| Carb109F/GDLS Line 10 | | | NA | NA |

Figure 3.9: Fitness of the Carb109 homozygous introgressed lines compared to GDLS and early introgression lines.These graphs show (A) the mean number of eggs laid per female, (B) proportion of eggs hatched at first hatch, (C) the sex ratio. D) is statistics table for the Kruskall-Wallis and Dunn's post hoc tests. The Carb109 BC1 is an early backcross cross of the male homozygous line, and the Carb109M/GDLS Line 7 and Carb109F Lines 9 and 10 are homozygous lines. Bars indicate SE. * indicates groups significantly different by a Kruskall-Wallis test and a Dunn's post hoc test.

# CHAPTER IV: EXPRESSION OF ANTI-VIRAL GENES IN AEDES AEGYPTI ARTIFICIALLY SELECTED FOR DENGUE VIRUS SEROTYPE 2 MIDGUT ESCAPE BARRIERS OR HIGH DISSEMINATION RATES

## Introduction

Understanding the mosquito's anti-viral response to arboviral infection is key to developing refractory mosquitoes for genetic control programs. The mosquito anti-viral response involves the small RNA pathways, siRNA, miRNA, piRNA and innate immune signaling cascades. The siRNA pathway identifies dsRNA viral intermediates, and prevents viral replication and dissemination by binding and cutting viral genomes and transcripts. In mosquito disease vectors there have been many studies that indicate a role of these pathway in the anti-arboviral response (Blair and Olson, 2014; Brackney et al., 2010; Keene et al., 2004; Khoo et al., 2013a; Sánchez-Vargas et al., 2009; Scott et al., 2010). While the siRNA pathway appears to be the predominant small RNA pathway in anti-arboviral immunity, another small RNA pathway, the microRNA (miRNA) has also been implicated in the regulation of ONNV in A. gambiae (Keene et al., 2004). However, more research is needed to understand how these pathways regulate arboviral infection. Additionally, there are multiple potential anti-viral pathways that regulate the permissiveness for arboviral infection of mosquitoes. The apoptosis gene IAP2 is necessary for signaling in the IMD and JNK pathways of D. melanogaster (Gesellchen et al., 2005) and the apoptosis pathway has recently been demonstrated to prevent SINV infection and dissemination in Ae. aegypti (Wang et al., 2012). In this study, two Ae. aegypti strains, artificially selected for low and high permissiveness for DENV2 were used to assess differences in gene expression of siRNA pathway genes, Ago2, Dcr2, R2D2, the miRNA pathway gene, R3D1, and the apoptosis pathway gene, IAP2.

This chapter explores the involvement of anti-viral gene expression in the dissemination of viral infection. The main barriers to arborviral infection are midgut infection barriers (MIB), midgut escape barriers (MEB) or salivary gland infection barriers (SIB). While all of these barriers have been demonstrated in nature, the most common DENV infection barrier is the MEB. Therefore, the expression of anti-viral genes in the midgut may determine whether an arbovirus can be replicated and disseminated in a vector. To study the role of these pathways in arboviral dissemination in the midgut, two Ae. aegypti strains were selected, D2S3 and D2MEB, which have a similar genetic background, but have been selected to have high or low viral dissemination rates. The D2S3 strain was selected for a high ( >80%) DENV2 dissemination rate and the D2MEB strains was selected for a low (<30%) DENV2 dissemination rate, but high (>80%) midgut infection rate (Bennett et al., 2005). Therefore, the D2S3 is considered to have low MIB and MEB, while the D2MEB strain has a high MEB. These strains were examined for differences in the expression of small RNA pathway genes and apoptosis genes in DENV2 infected mosquitoes. Since, the D2MEB Ae. aegypti strain has a MEB, and the D2S3 Ae. aegypti does not, differences in gene expression in the midgut between these two strains may suggest a role of the gene in viral midgut escape.

Additionally, small RNA pathway genes also have other functions that may influence their expression. The siRNA pathway also has a role in immunity to insect viruses . Additionally, the small RNA pathways have been shown to be involved with development and biochemical processing in other insects. Multiple studies have demonstrated that siRNAs are not only derived from exogenous RNA sources for anti-viral immunity, but endo-siRNAs are also involved in the silencing of genomic transposable elements (Lee et al., 2006) and influence tissue development (Ghildiyal et al., 2008) and embryogenesis (Lucchetta et al., 2009). These studies

demonstrate that the same components required for arboviral immunity are also used for these other functions. Therefore, I hypothesize that the small RNA pathway genes naturally fluctuate in expression over the mosquito lifetime and diurnally.

## Materials and Methods

### Mosquito colony maintenance

All stages of mosquitoes were maintained at 28 ºC ± 2 ºC, 80% ± 5% relative humidity and 14:10 hour light dark photoperiod. Larvae were raised at a density of ~100 mosquitoes per L and were fed ~0.35g of 50:50 flake fish food (Tetramin, Melle, Germany) and rodent diet (Harlan Labs) every other day until pupation, after which they were placed into 2.5L cartons. Post emergence adults were provided 10% (w/v) sugar water.

### Vector competence and phenotypic recovery by silencing of Dcr2 and Ago2

To evaluate the role of Dcr2 and Ago2 in the susceptibility to DENV2 infection of D2MEB and D2S3, D2S3 and D2MEB females were injected with dsRNA to Dcr2 or Ago2, created as described in Chapter 2 of this dissertation, approximately 28 to 36 hours prior to an infectious blood meal. For the infectious blood meal, high passage DENV2 Jamaica 1409 at an MOI of 0.01 was used to infect C6/36 cells in L-15 medium supplemented with 3% heat inactivated fetal bovine serum, 1% streptomycin and 1% L-glutamine for 12 days at 28º C. The medium was replaced 5 to 6 days after infection. The infectious blood meal was prepared for oral blood feed as previously described (Bosio et al., 1998). An infectious blood meal containing 1.1 x$10^7$ pfu/ml DENV2, as determined by plaque assay, was administered in an artificial feeding system, when the female mosquitoes were 4-6 days post emergence. At 12 days post infection females were collected and evaluated for head infection/dissemination rates by indirect IFA as described in previous studies (Kuberski, 1979). This experiment was conducted twice which resulted in a total sample size of between 30 and 48 individuals per group. DENV2

antigen was detected using the 3H5 DENV2 E protein-specific antibody on individual heads fixed to glass slides with 100% acetone (Kuberski, 1979).

Total RNA extracted from adult female D2S3 and D2MEB strain Ae. aegypti with the RNeasy kit (Qiagen, Valencia, CA). cDNA was created from the mRNA using the Superscript III RT kit (Invitrogen, Carlsbad, CA) using oligo(dT) primers. A ~500bp region of the RNA binding domain of Dcr2 (Sanchez-Vargas et al., 2009), a ~500bp region of the PIWI domain in Ago2 (Sanchez-Vargas et al., 2009) and a ~500bp region of the βgal gene of an Escherichia coli cDNA clone (Keene et al., 2004) were transcribed produce the dsRNA. The primers used in amplification had a 5' T7 sequence, which were then used in vitro transcribe the dsRNA. The products were purified, and the quality and quantity of the dsRNA was evaluated as previously described (Keene et al., 2004). The dsRNA was diluted to 6µg/µl in phosphate buffer saline (PBS) resulting in 892 ng dsRNA per injection using the Nanoject II injection system (Drummond Scientific, Broomall, PA). Reduction in Dcr2 and Ago2 mRNA expression was determined by Qrt-PCR using the Quanifast SYBR green kit (Qiagen, Valencia, CA), with the primers described in previous studies (Khoo et al., 2010). Ago2 and Dcr2 expression were compared to all controls using a $\chi^2$ test.

DENV infection associated expression studies

Female D2S3 and D2MEB Ae. aegypti were given either an infectious or non-infectious blood meal at day 5 post emergence. The non-infectious blood meal was prepared with the same medium as the infectious except the cells were not infected. A sample of the blood meal was taken and frozen at -80ºC for later quantification by plaque assay. At 2 hours on days 2, 5, 7, and 10 post infection, between 13:30 and 15:00 hours, 20 D2MEB and 20 D2S3 females were dissected to separate the midgut and carcass. The samples were stored at -80°C in Trizol for RNA extraction.

Lifetime expression studies

Additional D2S3 and D2MEB females from the same cohort were collected as 4$^{th}$ instar and pupae and on days 1, 3, 5, 7, 10, 12, 15 post emergence between 13:30 and 15:00. At each time point, 20 D2MEB and 20 D2S3 females were dissected to separate the midgut and carcass. The 4$^{th}$ instar larvae and pupae were not dissected and were evaluated as whole samples. On day 5 female mosquitoes were collected and a portion of the mosquitoes were given a blood meal with no virus. Thus, for days 5, 7, 10, 12, and 15 two groups of samples were taken at each time point, 1) blood fed on day 5 and 2) never blood fed. The sample taken on day 5 was collected 2 to 3 hours after the blood meal. All samples were stored at -80°C in Trizol for RNA extraction.

Diurnal expression studies

Subsets of the females fed a non-infectious blood meal in the previous studies were set aside to examine the variation in gene expression over the day. On day 2 post blood meal, 20 D2S3 and 20 D2MEB females were collected at 4 hour intervals from 8 am to 8 pm. The midgut and carcass were dissected from each other and stored at -80°C in Trizol for RNA extraction.

RNA extraction and preparation

RNA was extracted from the samples by the Trizol method (Invitrogen, Carlsbad, CA, USA) per the manufacturer's protocol and was DNase I digested (Fermentas, Pittsburgh, PA, USA) per the manufacturer's protocol. RNA quality and quantity were calculated by NanoDrop 2000 (ThermoScientific, Pittsburgh, PA, USA) and the samples were diluted into multiple aliquots at a concentration of 0.25 ng per µl for use in qPCR analysis. Based on the quantity and quality of the RNA extracted and the sample size limitations of the qRT-PCR plate only 12-14 individuals of the 20 collected for each sampling point were analyzed for each time point.

Quantitative RT-PCR (qRT-PCR) analysis

For each sample, Ct values were determined from a standard curve developed from serially diluted standards. Standards were created from total RNA extracted from ~20 HWE strain Ae. aegypti mosquitoes using an RNeasy kit (Qiagen, Valencia, CA). Then cDNA was synthesized using the Superscript III RT kit (Invitrogen, Carlsbad, CA) using oligo(dT) primers. PCR amplification was performed using a iQ™ SYBR® Green Supermix (BIORAD, USA) kit per the manufacturer's instructions using the primers in Table 1A. The reaction products were separated on a 1.0% agarose gel and the QIAQuick gel extraction kit was used to isolate fragments of the appropriate size. Clones were constructed using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions and the T7 MAXI script invitro transcription kit (Ambion) was used to transcribe RNA. Each sample was DNAse treated and purified using the MEGAclear kit (Ambion). RNA was quantified on the NanoDrop system and 10-fold serial dilutions were aliquoted into single use tubes prior to storage at -80°C.

A 0.25 ng aliquot of each sample was run on the iQ5 Real-Time PCR Detection System (BioRad, Hercules, CA, USA) in duplicate using the Quantifast qPCR kit (Qiagen, Valencia, CA, USA) per the manufacturer's protocol using the primers and corresponding annealing temperatures in Table 1B. The RT reaction was conducted for 10 min at 50°C followed by a PCR reaction (5min at 95°C and 35 cycles of 10 s at 95°C and 30 s at annealing temperatures in table 1B). The Dcr2 primers used were described elsewhere (C. Khoo et al., 2010). Primers were designed in Primer3 (Untergasser et al., 2012), optimized and melting curves were performed to eliminate primer dimers and non-target amplification. All runs were within the 90-105% efficiency range and the data was normalized to rsp7 as described previously (Boisson et al., 2006). The standard deviation between technical replicates was typically <0.250 indicating that the analysis can detect >2 fold differences 95% of the time.

mRNA copy numbers of each target were analyzed to evaluate the hypotheses that target gene expression a) is higher in infected versus non-infected individuals, b) is higher in the midgut than the carcass in infected versus non-infected individuals, c) is higher in the D2MEB infected versus D2S3 infected individuals, d) varied over the lifetime and e) varied diurnally.

For each gene, the number of transcripts were compared at each time point in analysis of variance (ANOVA) to determine whether the source of variation in the number of transcripts per gene was associated with the main effects of strain (D2MEB versus D2S3), tissue (midgut versus carcass) and when applicable infection status (infected versus non-infected), time of day the collection occurred (8am, 12pm, etc.) or blood feeding status (yes versus no). The 4$^{th}$ instar larva and pupae transcript data were analyzed by strain and time by ANOVA, since whole carcasses were analyzed. When necessary, the data were log or square root transformed to comply with the normality and equal variances assumptions of ANOVA. Interactions between factors were examined in a single, double and triple interaction model to include all possible interactions and Tukey multiple comparison tests were used to compare all significant associations.

## Results

### Ago2 gene expression during DENV infection

Ago2 gene expression was not altered by viral infection, but appeared to be induced in mosquito midguts within 5 days of blood feeding, and generally expressed at a higher level in the D2MEB mosquito. At 2 hours post blood meal, and 2 days post blood meal there were significantly more Ago2 transcripts in the midgut of mosquitoes in both the D2S3 and the D2MEB strains (Table 4.2). The D2MEB strain had significantly higher overall Ago2 expression than the D2S3 strain. In fact, the effect size (r) is largest for the strain main effect at 2 days post blood meal, which indicates a large difference in Ago2 expression between strains

(Table 4.2). However, mosquitoes that had an infectious blood meal did not have more Ago2 transcripts at any time point demonstrating that the differences in expression between strains was not associated with viral infection (Table 4.2). None of the two way or three way interactions were significant between the independent variables: tissue, strain, infection status (Table 4.2). At 5 days post blood meal, the midguts of both strains had significantly higher numbers of Ago2 transcripts, but no other comparisons or interaction was significant at this time point (Table 4.2). On day 7 post infection, none of the comparisons or interactions were significant.

From these results it appeared that the Ago2 transcript increases after blood feeding in both the D2S3 and D2MEB strain, but this increase was slightly higher in the D2MEB strain (Fig. 4.3). By day 5, however, the strain differences were no longer significant, so the lower number of Ago2 transcripts in the D2S3 strain only occurred during the first two days following a blood meal. Even though there was a highly significant difference between strains and tissues at the early time points, these differences were small, <2 fold (Table 4.3), so the biological significance is questionable. Based on the standard deviation of the technical replicates, the assay had a 95% chance to detect >2 fold difference in transcript levels (Quellhorst et al., 2014).

Ago2 expression over the mosquito life span

The expression of Ago2 varies over the lifetime of the mosquito in the absence of viral infection and blood feeding (Fig. 4.4). These differences varied depending on strain and tissue over time. For the early life stages, 4$^{th}$ instar larvae and pupae, there were no significant differences in the number of Ago2 transcripts between life stage or mosquito strain. (data not shown $F(0.76,2)$, $p>0.05$). But on days 3 and 5 post emergence, there were significantly more Ago2 transcripts in the D2MEB strain than the D2S3 (Table 4.4). There were no significant differences between strains on days 1, 7, 12 and 15 post emergence ($p> 0.5$). Interestingly, the main effects interaction of tissue and strain on day 5 demonstrated that there was a larger number

of transcripts in the midgut of the D2MEB strain (Table 4.4). The fold differences of Ago2 gene expression in the midgut versus the carcass were high at many time points, especially in D2MEB mosquitoes (Table 4.5). This difference was not associated with blood feeding, or viral infection. Additionally, on day 10 post emergence the midguts of both groups of mosquitoes had significantly more Ago2 transcripts than the carcass (Table 4.6). These results indicate that there is a natural variation in Ago2 expression between strains that changes over the lifetime of the mosquito.

On day 5 the mosquitoes were either offered a blood meal or were not fed. On day 1 post blood meal, there were significantly more Ago2 transcripts in D2MEB mosquitoes than the D2S3 mosquito (Table 4.7). These differences had disappeared by day 2 post blood meal, when there were no longer significant differences in the number of Ago2 transcripts between any independent variable. However, on day 10 post emergence both strains had significantly more Ago2 transcripts in their midgut versus their carcasses. Again by day 12 and 15 post emergence, these differences were gone. It should be noted, however, that while these differences are statistically significant, the fold change in expression is small for each interaction.

Ago 2 diurnal expression

Ago 2 expression varied diurnally within and between mosquito strains, and this difference was influenced by infection status (Fig. 4.5). There was a significant difference in the main effects of strain, and infection status for Ago2 (Table 4.8) and there was almost a significant difference in the main effect of time ($p=0.056$). The D2MEB strain had significantly higher numbers of transcripts than the D2S3 strain ($q= 4.350$, $p= 0.002$). The two earliest time points, 8:00 and 12:00, had a higher number of transcripts than the later time points, 16:00 and 20:00 (Table 4.8). Additionally, in this analysis infection status was found to be significantly different, although this difference was less than 2 fold. No other interactions were significant.

Dcr2 expression during DENV infection

Factors influencing the expression of Dcr2 were complicated, with many significant main effects influencing gene expression. However, while the main effect of infection status was not significant or very high for Ago2, there were significant differences in the midgut expression of Dcr2 between infected and non-infected mosquitoes. At 2 hours and 2 days post blood meal, there were significant main effects of tissue, strain, infection status and the interaction of infection status and tissue and infection status and strain in Dcr2 gene expression (Table 4.9). At 2 hours post blood meal, there was also a significant 3-way interaction between main effects. A subsequent Tukey test suggests that the reason for this significant 3-way interaction was that the midgut of an infected D2MEB mosquito had significantly more transcripts. This was a greater than 2-fold difference, which is likely biologically significant (Table 4.3). Additionally, at 2 hours strain was the main effect with the highest effect size (r=0.86), which indicates that at this earlier time point, there is a large difference in expression between the two strains. Tukey post-hoc tests to evaluated the main effect interaction of tissue and strain at 2 hours (r=0.65) indicated that the non-infected D2MEB strain also had more transcripts in its midgut compared to the carcass, which indicates that the increase in Dcr2 was blood meal associated (Table 4.9). In fact, both strains had higher expression levels in their midgut than their carcasses at all time points (Table 4.8). The number of transcripts, however, was significantly different and about 3 fold higher in the midgut of a D2MEB individual receiving an infectious blood meal compared to the midgut of the D2MEB strain that received a non-infectious blood meal (Tables 4.3 and 4.7). There were also significantly more Dcr2 transcripts in the carcass of D2MEB at two hours when compared to the carcass of D2S3 in both groups given either an infectious or non-infectious blood meal (Fig. 4.3). These results suggest that the D2MEB strain has a greater increase in Dcr2 expression in response to blood feeding, and infection than the D2S3 strain.

On day 2 post blood meal, the 3-way interaction between main effects was no longer significant, but the significant 2 way interactions (infection status x tissue and infection status x strain) were significant (Table 4.9). The main effects of tissue, strain and the interaction of tissue and strain were still significant at this time point. The significant two- way interaction was attributed to significantly more Dcr2 transcripts in the D2MEB strain that received an infectious blood meal than the D2MEB strain given the uninfected blood meal, but this difference was not seen in the D2S3 strain (Table 4.9). The D2MEB mosquitoes given a DENV2 infected blood meal had a higher number of transcripts than D2S3 mosquitoes given a DENV2 infected blood meal, but there was no difference between the strains if they were given a non-infected blood meal. Both strains had a higher number of Dcr2 transcripts in their midguts versus their carcasses (Table 4.9). Therefore, it appears that only the D2MEB has an increased number of Dcr2 transcripts when given an infectious blood meal.

On days 5 and 7 post blood meal, the main effect of infection status no longer significantly affected the number of Dcr2 transcripts, but tissue and strain and their interaction had a significant effect (Table 4.9). Both strains had more Dcr2 transcripts in their midgut compared to their carcass, and there were more transcripts in the D2MEB strain overall than the D2S3 strain. There were also more Dcr2 transcripts in the midgut of the D2MEB strain than the D2S3 strain. However, on day 7 post blood meal, the difference in the number of Dcr2 transcripts in mosquitoes given an infectious blood meal and infection status and strain only just barely missed the significance cut off (Table 4.9). This difference appears to be due to the increased number of Dcr2 transcripts in the D2MEB strain.

Dcr2 expression over the mosquito life span

Similar to Ago2 expression, Dcr2 expression varies over the life time of the D2MEB and D2S3 strains in the absence of blood feeding or arboviral infection (Fig. 4.4). On day 1 post

emergence, the main effect of tissue, and the two-way interaction of tissue and strain were significantly different (Table 4.10). On day 3 post emergence, all main effects, two-way interactions and the three way interactions were significant. On day 5, tissue, stain and the tissue and strain interaction were significant main effects (Table 4.10).

On day 5 the mosquitoes were either blood fed or not. On day 7, there were significantly more Dcr2 transcripts in the midguts of D2MEB mosquitoes, however, this difference was also seen in the D2S3 mosquitoes. The number of transcripts was not influenced by blood feeding status. Within blood fed D2MEB there were more Dcr2 transcripts in the midgut versus the carcass of the mosquito. There were no significant differences in any effect between the number of Dcr2 transcripts in 4$^{th}$ instars and pupae.

Dcr2 diurnal expression

Dcr2 expression varied diurnally within and between mosquito strains, and this difference was influenced by infection status (Fig.4.5). There is a significant difference in the main effects of strain and time for Dcr2 (Table 4.8). The D2MEB strain had significantly higher numbers of transcripts than the D2S3 strain. The 12:00 and 16:00 had a higher number of transcripts than the other time points (Table 4.8). No other interactions were significant.

IAP2 expression during DENV infection

In the D2S3 and D2MEB strains there was no difference in IAP2 expression between individuals receiving an infectious versus non-infectious blood meal. There were no significant associations at any time point between the independent variables and the number of IAP2 transcripts (Table 4.2).

R2D2 expression during DENV infection

There was no difference in R2D2 expression between infected and non-infected mosquitoes of either strain. There were no significant associations at any time point between the independent variables and the number of R2D2 transcripts (Table 4.2).

R3D1 expression during DENV infection

On day 1 post blood meal, strain significantly affected the number of R3D1 transcripts (Table 4.2). A Tukey test demonstrated that these differences are attributed to a higher number of transcripts in the D2MEB strain versus the D2S3 strain. All other associations between transcript number and the independent variable were not significant at any other time point.

Discussion

The Ago2 and Dcr2 dsRNA knock down experiments in the D2MEB strain resulted in a partial recovery of the susceptible phenotype as demonstrated by the increase in disseminated DENV infections. These results indicate that the siRNA pathway has a role in DENV midgut escape in Ae. aegypti.

In previous studies, DENV infection reduced the expression of many innate immunity genes (Bonizzoni et al., 2012b), but other studies show this to be strain specific, with some strains exhibiting increased expression after blood feeding (Bonizzoni et al., 2012a). In this study, DENV2 infection did not significantly change the expression of any gene except for Dcr2, which was higher at the two earliest time points, but only in the D2MEB strain. However, this study also demonstrated that at early time points D2MEB mosquitoes have higher Dcr2 gene expression after blood feeding regardless of infection status, which is similar to other studies (Bonizzoni et al., 2012a).

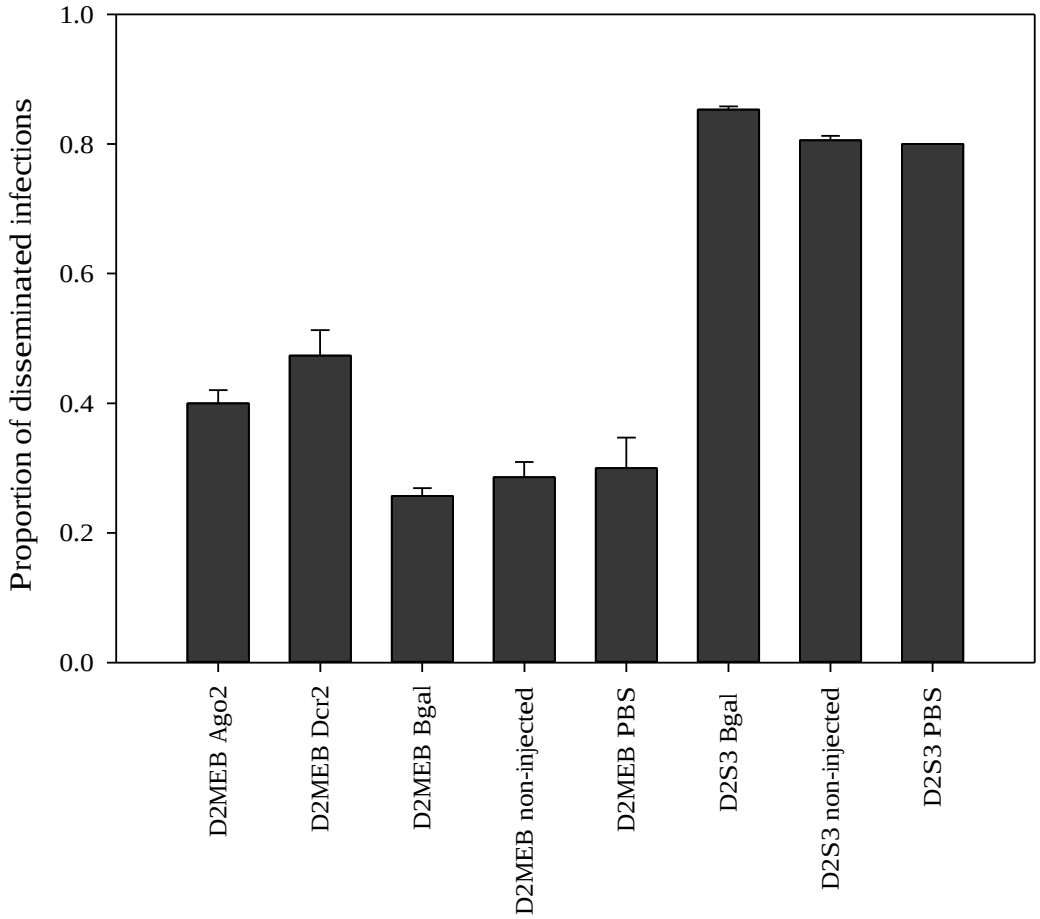
While infection status was not significantly associated with Ago2 expression, the D2MEB strain had a higher Ago2 expression level than D2S3 and both strains had higher Ago2 expression

in the midgut compared to the carcass. The increase in the midgut expression of Ago2 was highest in the D2MEB (~5 fold) at day 3 post blood meal, but remained at least ~2 fold higher in the midgut after day 1. The higher expression levels in the midgut may indicate that the differences between the midgut and carcass may be blood meal induced rather than virus induced. The earliest time points post blood meal (2 hours and 2 days) also had the highest number of Ago2 transcripts in both strains, regardless of infection status, which gives further evidence that Ago2 expression may increase from blood feeding. Other studies conducted on 3 different laboratory strains of Ae. aegypti also showed an increase in Ago2 expression after blood feeding (Bonizzoni et al., 2012a), so the increase in Ago2 expression in response to blood feeding may be common between strains.

R2D2, R3D1 and IAP2 expression did not significantly differ in this study by infection status, blood feeding, tissue or strain. Other studies have shown that R2D2 expression changed in some Ae. aegypti strains in response to blood feeding, while other strains did not change (Bonizzoni et al., 2012a). In this same study, R3D1 gene expression increased in all blood fed strains, but our study 50% of the time points showed a decrease in R3D1 expression in response to infection. These results are similar to other studies which showed a decrease in expression of some innate immune gene in response to DENV infection (Bonizzoni et al., 2012b).

The diurnal and lifetime studies also demonstrated diurnal and age-related variation in gene expression. This variation is not surprising since these genes are not just modulating arbovirus infections, but they defend against other pathogens and regulate mosquito physiology. Additionally, with the minimal changes in gene expression (<2 fold) typically seen in many expression studies, the collection time and mosquito age could entirely mask significant differences. Many studies use 3-5 day old mosquitoes, but in this study Ago2 and Dcr2

expression were shown to vary between strains and tissues over the lifetime of the mosquito. Typically, the differences in expression between time points were not large. However, in this study the differences between the variation of Dcr2 expression in 5 and 7 day old mosquitoes was large enough to likely impede analysis of differences associated with strain and tissue. More notably, during diurnal studies of Ago2 expression, which shown not to be significantly affected by infection, infection status was a significant main effect. Therefore, if another collection time had been chosen for the large infection study, then infection may have significantly influenced Ago2 expression. In this study, the choice of collection time likely did not alter the overall conclusions, since the differences in gene expression between the groups were often small and thus their biological significance would still be questionable. The differences in diurnal and lifetime Ago2 and Dcr2 expression in this study may be more pronounced than others, since many gene expression studies pool mosquitoes and this study was conducted with individuals. Another study conducted by Ptitsyn et al., (2011) demonstrated that Dcr2 expression levels in field collected Ae. aegypti significantly changed over 72-104 hours post emergence, while the Ago2 expression levels changed over time, but these changes were not significant (Fig. 4.5). This study does demonstrate the importance of collection protocols and study design in gene expression studies.




| Strain and injection | Ago 2 | | Dcr 2 | |
|---|---|---|---|---|
| | □2 statistic | p value | □2 statistic | p value |
| D2S3 βgal | 57.96 | <0.001 | 42.65 | <0.001 |
| D2S3 not injected | 34.76 | <0.001 | 24.02 | <0.001 |
| D2S3 PBS | 34.04 | <0.001 | 23.46 | <0.001 |
| D2MEB βgal | 4.96 | 0.03 | 13.00 | <0.001 |
| D2MEB not injected | 3.14 | 0.08 | 9.54 | <0.001 |
| D2MEB PBS | 4.00 | 0.05 | 11.30 | <0.001 |

Figure 4.1: siRNA gene knockdown of Dcr2 and Ago2. D2MEB females were injected with dsRNA against the Ago2 and the Dcr2 transcript. The D2S3 mosquito was used as a positive control. βgal and PBS injections were controls for the dsRNA and carriers. The non-injected control was used to control for a potential immune response to the injection. Disseminated infections were determined by head squash IFA. The experiment was replicated twice. $\chi^2$ statistics were done to assess the differences in the number of disseminated infections per group.

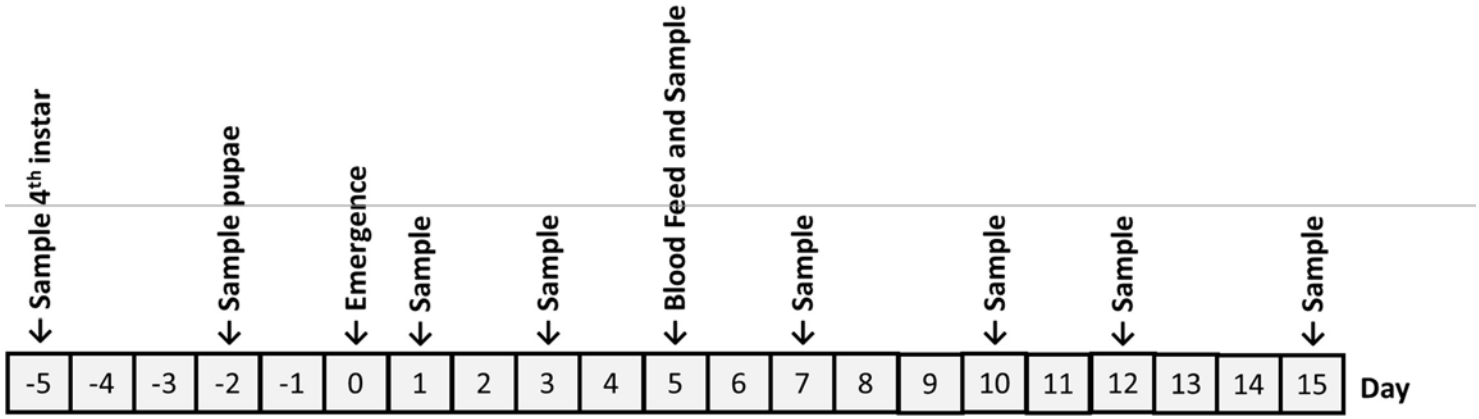



Figure 4.2: Sampling design for infection and lifetime studies. The figure shows the sampling and blood feeding regime for the infection and life span studies.

Table 4.1A: cDNA cloning primers

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | Annealing temp. | Product size (bp) |
|---|---|---|---|---|
| Ago2 | GAACTGTGCAGTATTCCGCC | GGACACCCTGCTTTAGGGAC | 55°C | 430 |
| Dcr2 | TGACCCCAGCAAATACGGTC | TGACCCCAGCAAATACGGTC | 55°C | 526 |
| IAP2 | GAACTGAAGCGCGAAGAACA | ATTCGCGAGTCCAACGAACT | 55°C | 317 |
| R2D2 | GGACCGACATCGGTTCTTCG | TTACACCGATGGCGTAAGGG | 55°C | 424 |
| R3D1 | ACCAGCGTTTTAAATGCATACG | GCTGAAAACCTAGACAAAACTGT | 52°C | 573 |

Table 4.1B: qPCR primers for genes of interest

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | Annealing temp. | Product size (bp) |
|---|---|---|---|---|
| Ago2 | TAGGAAGTCGGCCACCGATA | TTTCCGACGCTCACTCCAAA | 54°C | 114 |
| Dcr2 | CTCACTGCAGTTAAGGGCCA | ACCGTTGCTAGAGGTGGTTG | 54°C | 134 |
| IAP2 | AGGTCAAATGTGCCTGGTGT | TTCCGATGGTGGGATCCAGA | 54°C | 150 |
| R2D2 | ATTGCTCTGGACGAAACGCT | CCAGATCGCTGTTGTCTCGT | 55°C | 100 |
| R3D1 | ACCCGAAGTTGAACGGAACCT | TAATTGCCAAAACGGAGGGC | 54°C | 113 |

Table 4.2: ANOVA table of Ago2 expression between infected and non-infected D2S3 and D2MEB mosquitoes. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value, r- effect size.

| Source of Variation | Time | df | SS | MS | F | p | r |
|---|---|---|---|---|---|---|---|
| Tissue | 2 hours | 1 | 0.559 | 0.559 | 16.73 | <0.001 | 0.40 |
| Strain | 2 hours | 1 | 0.203 | 0.203 | 6.07 | 0.016 | 0.25 |
| Infection | 2 hours | 1 | 0.005 | 0.005 | 0.15 | 0.697 | 0.04 |
| Tissue x Strain | 2 hours | 1 | 0.059 | 0.059 | 1.77 | 0.187 | 0.14 |
| Tissue x Infection | 2 hours | 1 | 0.002 | 0.002 | 0.07 | 0.79 | 0.03 |
| Strain x Infection | 2 hours | 1 | 0.003 | 0.003 | 0.08 | 0.773 | 0.03 |
| Tissue x Strain x Infection | 2 hours | 1 | 0.002 | 0.002 | 0.06 | 0.814 | 0.03 |
| Residual | 2 hours | 88 | 2.939 | 0.033 | | | |
| Total | 2 hours | 95 | 3.771 | 0.040 | | | |
| Tissue | Day 2 | 1 | 0.267 | 0.267 | 8.77 | 0.004 | 0.30 |
| Strain | Day 2 | 1 | 0.511 | 0.511 | 16.81 | <0.001 | 0.40 |
| Infection | Day 2 | 1 | 0.098 | 0.098 | 3.23 | 0.076 | 0.19 |
| Tissue x Strain | Day 2 | 1 | 0.099 | 0.099 | 3.25 | 0.075 | 0.19 |
| Tissue x Infection | Day 2 | 1 | 0.009 | 0.009 | 0.30 | 0.584 | 0.06 |
| Strain x Infection | Day 2 | 1 | 0.006 | 0.006 | 0.21 | 0.651 | 0.05 |
| Tissue x Strain x Infection | Day 2 | 1 | 0.001 | 0.001 | 0.04 | 0.844 | 0.02 |
| Residual | Day 2 | 88 | 2.676 | 0.030 | | | |
| Total | Day 2 | 95 | 3.668 | 0.039 | | | |
| Tissue | Day 5 | 1 | 0.000 | 0.00 | 0.0141 | 0.906 | 0.01 |
| Strain | Day 5 | 1 | 0.209 | 0.21 | 6.327 | 0.014 | 0.26 |
| Infection | Day 5 | 1 | 0.039 | 0.04 | 1.182 | 0.28 | 0.12 |
| Tissue x Strain | Day 5 | 1 | 0.099 | 0.10 | 3.016 | 0.086 | 0.18 |
| Tissue x Infection | Day 5 | 1 | 0.008 | 0.01 | 0.233 | 0.63 | 0.05 |
| Strain x Infection | Day 5 | 1 | 0.003 | 0.00 | 0.0986 | 0.754 | 0.03 |
| Tissue x Strain x Infection | Day 5 | 1 | 0.001 | 0.00 | 0.0396 | 0.843 | 0.02 |
| Residual | Day 5 | 88 | 2.900 | 0.03 | | | |
| Total | Day 5 | 95 | 3.260 | 0.03 | | | |
| Tissue | Day 7 | 1 | 0.002 | 0.00 | 0.0544 | 0.816 | 0.02 |
| Strain | Day 7 | 1 | 0.011 | 0.01 | 0.336 | 0.563 | 0.06 |
| Infection | Day 7 | 1 | 0.018 | 0.02 | 0.583 | 0.447 | 0.08 |
| Tissue x Strain | Day 7 | 1 | 0.018 | 0.02 | 0.58 | 0.448 | 0.08 |
| Tissue x Infection | Day 7 | 1 | 0.004 | 0.00 | 0.127 | 0.723 | 0.04 |
| Strain x Infection | Day 7 | 1 | 0.001 | 0.00 | 0.03 | 0.863 | 0.02 |
| Tissue x Strain x Infection | Day 7 | 1 | 0.006 | 0.01 | 0.196 | 0.659 | 0.05 |
| Residual | Day 7 | 88 | 2.753 | 0.03 | | | |
| Total | Day 7 | 95 | 2.812 | 0.03 | | | |

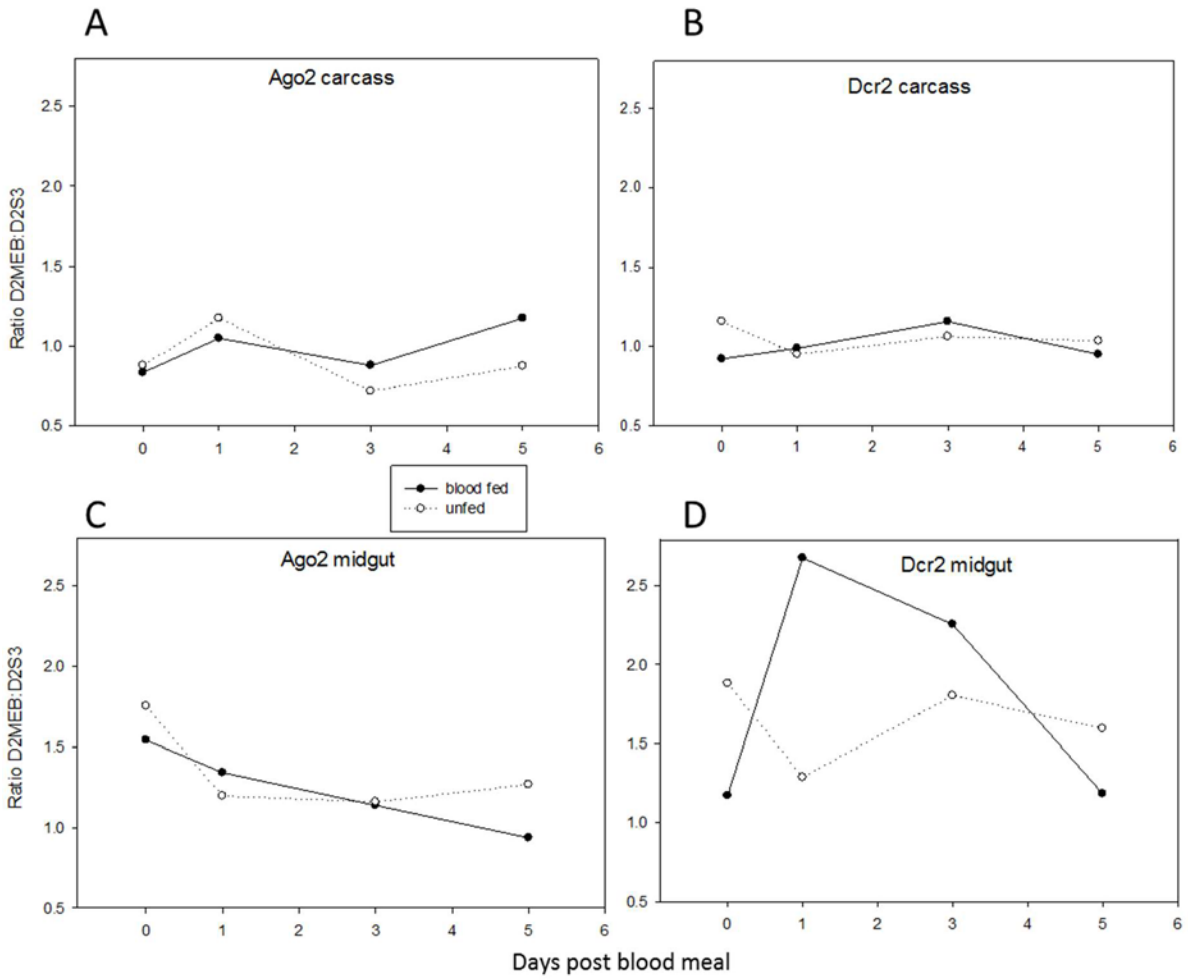



Figure 4.3: Ratio of transcripts in blood fed or unfed D2MEB versus D2S3 mosquitoes. The ratio of D2MEB:D2S3 carcass expression of Ago2 (A) and Dcr2 (B) in blood fed (black line, black circle) versus unfed (dotted line, open circle) are shown at the top. The ratio of D2MEB:D2S3 midgut expression of Ago2 (C) and Dcr2 (D) in blood fed (black line, black circle) versus unfed (dotted line, open circle) are shown at the top. The x-axis is the time (days) post blood meal (pbm). The zero time point is 2 hours pbm.

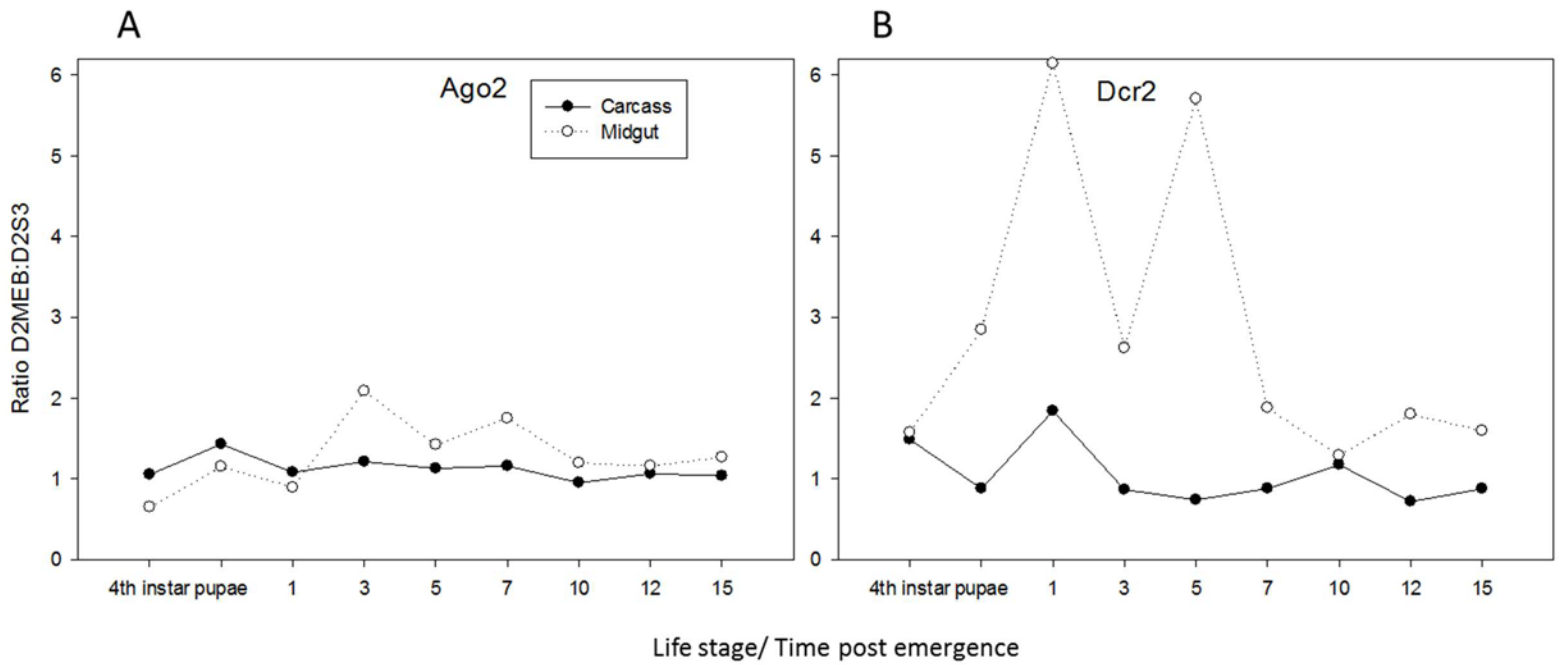



Figure 4.4: Ratio of transcripts in the carcass and midgut over the mosquito lifetime of D2MEB versus D2S3 mosquitoes. The ratio of D2MEB:D2S3 expression of Ago2 (A) and Dcr2 (B) in the carcass (black line, black circle) versus the midgut (dotted line, open circle) are shown. The x-axis is the life stage ($4^{th}$ instar larvae or pupae) or the day post emergence.

Table 4.3: Ratio of expression of potential immunity genes in DENV infected versus non-infected between D2MEB and D2S3 Ae. aegypti strains. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value, r- effect size.

| Mosquito Strain | Tissue | Day Post Blood Meal | Genes | | | | |
|---|---|---|---|---|---|---|---|
| | | | Ago2 | Dcr2 | IAP2 | R2D2 | R3D1 |
| D2MEB | Carcass | 1 | 1.05 | 1.24 | 1.03 | 1.05 | 1.04 |
| D2MEB | Carcass | 2 | 1.06 | 1.56 | 1.12 | 1.05 | 0.97 |
| D2MEB | Carcass | 5 | 0.93 | 1.25 | 0.93 | 0.96 | 0.96 |
| D2MEB | Carcass | 7 | 1.01 | 1.51 | 1.26 | 0.96 | 1.01 |
| D2S3 | Carcass | 1 | 1.03 | 0.96 | 0.94 | 1.14 | 0.96 |
| D2S3 | Carcass | 2 | 1.03 | 0.96 | 0.85 | 1.08 | 0.99 |
| D2S3 | Carcass | 5 | 1.01 | 1.10 | 1.10 | 0.97 | 1.08 |
| D2S3 | Carcass | 7 | 0.91 | 1.01 | 1.06 | 0.92 | 1.06 |
| D2MEB | Midgut | 1 | 0.95 | 2.49 | 1.04 | 1.19 | 1.03 |
| D2MEB | Midgut | 2 | 1.16 | 2.95 | 1.22 | 0.96 | 1.01 |
| D2MEB | Midgut | 5 | 1.07 | 1.12 | 1.03 | 1.01 | 0.96 |
| D2MEB | Midgut | 7 | 0.98 | 1.46 | 1.07 | 1.09 | 1.09 |
| D2S3 | Midgut | 1 | 1.00 | 1.02 | 0.95 | 1.12 | 0.97 |
| D2S3 | Midgut | 2 | 1.05 | 1.01 | 0.93 | 1.09 | 0.99 |
| D2S3 | Midgut | 5 | 1.02 | 0.97 | 1.13 | 1.00 | 0.92 |
| D2S3 | Midgut | 7 | 1.03 | 1.09 | 1.19 | 1.10 | 1.05 |

Table 4.4: ANOVA table of Ago2 expression over the lifetime of D2S3 and D2MEB mosquitoes. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value, r- effect size.

| Source of Variation | Time | df | SS | MS | F | p | r |
|---|---|---|---|---|---|---|---|
| Tissue | Day 1 | 1 | 0.002 | 0.002 | 0.04 | 0.85 | 0.03 |
| Strain | Day 1 | 1 | 0.000 | 0.000 | 0.00 | 0.97 | 0.01 |
| Tissue x Strain | Day 1 | 1 | 0.020 | 0.020 | 0.36 | 0.55 | 0.09 |
| Residual | Day 1 | 44 | 2.375 | 0.054 | | | |
| Total | Day 1 | 47 | 2.396 | 0.051 | | | |
| Tissue | Day 3 | 1 | 0.009 | 0.009 | 0.20 | 0.66 | 0.07 |
| Strain | Day 3 | 1 | 0.568 | 0.568 | 11.76 | 0.00 | 0.46 |
| Tissue x Strain | Day 3 | 1 | 0.048 | 0.048 | 0.98 | 0.33 | 0.15 |
| Residual | Day 3 | 44 | 2.126 | 0.048 | | | |
| Total | Day 3 | 47 | 2.750 | 0.059 | | | |
| Tissue | Day 5 | 1 | 0.102 | 0.102 | 1.38 | 0.25 | 0.17 |
| Strain | Day 5 | 1 | 0.537 | 0.537 | 7.29 | 0.01 | 0.38 |
| Tissue x Strain | Day 5 | 1 | 0.666 | 0.666 | 9.04 | 0.00 | 0.41 |
| Residual | Day 5 | 44 | 3.241 | 0.074 | | | |
| Total | Day 5 | 47 | 4.546 | 0.097 | | | |
| Tissue | Day 7 | 1 | 0.030 | 0.030 | 0.31 | 0.58 | 0.08 |
| Strain | Day 7 | 1 | 0.266 | 0.266 | 2.78 | 0.10 | 0.24 |
| Tissue x Strain | Day 7 | 1 | 0.124 | 0.124 | 1.29 | 0.26 | 0.17 |
| Residual | Day 7 | 44 | 4.216 | 0.096 | | | |
| Total | Day 7 | 47 | 4.636 | 0.099 | | | |
| Tissue | Day 10 | 1 | 0.366 | 0.366 | 4.47 | 0.04 | 0.30 |
| Strain | Day 10 | 1 | 0.040 | 0.040 | 0.49 | 0.49 | 0.10 |
| Tissue x Strain | Day 10 | 1 | 0.021 | 0.021 | 0.25 | 0.62 | 0.08 |
| Residual | Day 10 | 44 | 3.608 | 0.082 | | | |
| Total | Day 10 | 47 | 4.035 | 0.086 | | | |
| Tissue | Day 12 | 1 | 0.033 | 0.033 | 0.29 | 0.60 | 0.08 |
| Strain | Day 12 | 1 | 0.008 | 0.008 | 0.07 | 0.79 | 0.04 |
| Tissue x Strain | Day 12 | 1 | 0.004 | 0.004 | 0.03 | 0.86 | 0.03 |
| Residual | Day 12 | 44 | 5.023 | 0.114 | | | |
| Total | Day 12 | 47 | 5.067 | 0.108 | | | |
| Tissue | Day 15 | 1 | 0.079 | 0.079 | 0.45 | 0.51 | 0.10 |
| Strain | Day 15 | 1 | 0.109 | 0.109 | 0.62 | 0.43 | 0.12 |
| Tissue x Strain | Day 15 | 1 | 0.010 | 0.010 | 0.06 | 0.81 | 0.04 |
| Residual | Day 15 | 44 | 7.666 | 0.174 | | | |
| Total | Day 15 | 47 | 7.863 | 0.167 | | | |

Table 4.5: Ratio of siRNA gene expression in midguts versus carcasses in D2MEB and D2S3 Ae. aegypti strains.

| Strain | Gene | Day Post Emergence | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 3 | Day 5 | Day 7 | Day 10 | Day 12 | Day 15 |
| D2MEB | Ago2 | 0.95 | 4.82 | 2.23 | 3.51 | 1.90 | 3.57 | 2.82 |
| D2S3 | Ago2 | 0.71 | 1.22 | 1.27 | 0.82 | 0.48 | 1.72 | 1.30 |
| D2MEB | Dcr2 | 4.82 | 1.24 | 1.75 | 1.42 | 1.04 | 1.10 | 0.90 |
| D2S3 | Dcr2 | 0.71 | 0.37 | 0.81 | 0.58 | 1.00 | 0.62 | 1.40 |

Table 4.6: ANOVA table of Ago2 expression in non-infectious blood fed and blood fed D2S3 and D2MEB mosquitoes. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value, r- effect size.

| Source of Variation | Day Post Blood Meal | DF | SS | MS | F | p | r |
|---|---|---|---|---|---|---|---|
| Tissue | Day 1 | 1 | 1098.92 | 1098.92 | 2.76 | 0.10 | 0.17 |
| Strain | Day 1 | 1 | 3655.643 | 3655.643 | 9.17 | 0.00 | 0.31 |
| Blood fed | Day 1 | 1 | 223.811 | 223.811 | 0.56 | 0.46 | 0.08 |
| Tissue x Strain | Day 1 | 1 | 1093.981 | 1093.981 | 2.74 | 0.10 | 0.17 |
| Tissue x Blood fed | Day 1 | 1 | 58.213 | 58.213 | 0.15 | 0.70 | 0.04 |
| Strain x Blood fed | Day 1 | 1 | 9.373 | 9.373 | 0.02 | 0.88 | 0.02 |
| Tissue x Strain x Blood fed | Day 1 | 1 | 46.434 | 46.434 | 0.12 | 0.73 | 0.04 |
| Residual | Day 1 | 88 | 35098.15 | 398.843 | | | |
| Total | Day 1 | 95 | 41284.52 | 434.574 | | | |
| Tissue | Day 2 | 1 | 579.595 | 579.595 | 1.62 | 0.21 | 0.13 |
| Strain | Day 2 | 1 | 800.384 | 800.384 | 2.23 | 0.14 | 0.16 |
| Blood fed | Day 2 | 1 | 408.362 | 408.362 | 1.14 | 0.29 | 0.11 |
| Tissue x Strain | Day 2 | 1 | 419.467 | 419.467 | 1.17 | 0.28 | 0.11 |
| Tissue x Blood fed | Day 2 | 1 | 973.535 | 973.535 | 2.71 | 0.10 | 0.17 |
| Strain x Blood fed | Day 2 | 1 | 110.308 | 110.308 | 0.31 | 0.58 | 0.06 |
| Tissue x Strain x Blood fed | Day 2 | 1 | 3.248 | 3.248 | 0.01 | 0.92 | 0.01 |
| Residual | Day 2 | 88 | 31575.54 | 358.813 | | | |
| Total | Day 2 | 95 | 34870.44 | 367.057 | | | |
| Tissue | Day 5 | 1 | 17.608 | 17.608 | 0.04 | 0.84 | 0.02 |
| Strain | Day 5 | 1 | 112.247 | 112.247 | 0.28 | 0.60 | 0.06 |
| Blood fed | Day 5 | 1 | 4.808 | 4.808 | 0.01 | 0.91 | 0.01 |
| Tissue x Strain | Day 5 | 1 | 152.76 | 152.76 | 0.38 | 0.54 | 0.07 |
| Tissue x Blood fed | Day 5 | 1 | 9.047 | 9.047 | 0.02 | 0.88 | 0.02 |
| Strain x Blood fed | Day 5 | 1 | 33.662 | 33.662 | 0.08 | 0.77 | 0.03 |
| Tissue x Strain x Blood fed | Day 5 | 1 | 31.552 | 31.552 | 0.08 | 0.78 | 0.03 |
| Residual | Day 5 | 88 | 35658.47 | 405.21 | | | |
| Total | Day 5 | 95 | 36020.16 | 379.16 | | | |
| Tissue | Day 10 | 1 | 727.063 | 727.063 | 1.74 | 0.19 | 0.14 |
| Strain | Day 10 | 1 | 136.546 | 136.546 | 0.33 | 0.57 | 0.06 |
| Blood fed | Day 10 | 1 | 2.313 | 2.313 | 0.01 | 0.94 | 0.01 |
| Tissue x Strain | Day 10 | 1 | 14.101 | 14.101 | 0.03 | 0.86 | 0.02 |
| Tissue x Blood fed | Day 10 | 1 | 142.622 | 142.622 | 0.34 | 0.56 | 0.06 |
| Strain x Blood fed | Day 10 | 1 | 365.431 | 365.431 | 0.87 | 0.35 | 0.10 |
| Tissue x Strain x Blood fed | Day 10 | 1 | 75.163 | 75.163 | 0.18 | 0.67 | 0.05 |
| Residual | Day 10 | 88 | 36844.81 | 418.691 | | | |
| Total | Day 10 | 95 | 38308.05 | 403.243 | | | |

Table 4.7: Ratio of siRNA gene expression in blood fed versus non-blood fed D2MEB and D2S3 Ae. aegypti strains

| Strain | Gene | Days post blood meal | | | |
|---|---|---|---|---|---|
| | | 2 hours | Day 3 | Day 5 | Day 7 |
| D2MEB | Ago2 | 1.04 | 0.83 | 0.95 | 0.89 |
| D2S3 | Ago2 | 1.19 | 0.74 | 0.97 | 1.20 |
| D2MEB | Dcr2 | 0.91 | 2.71 | 1.35 | 1.16 |
| D2S3 | Dcr2 | 1.46 | 1.13 | 1.08 | 1.56 |

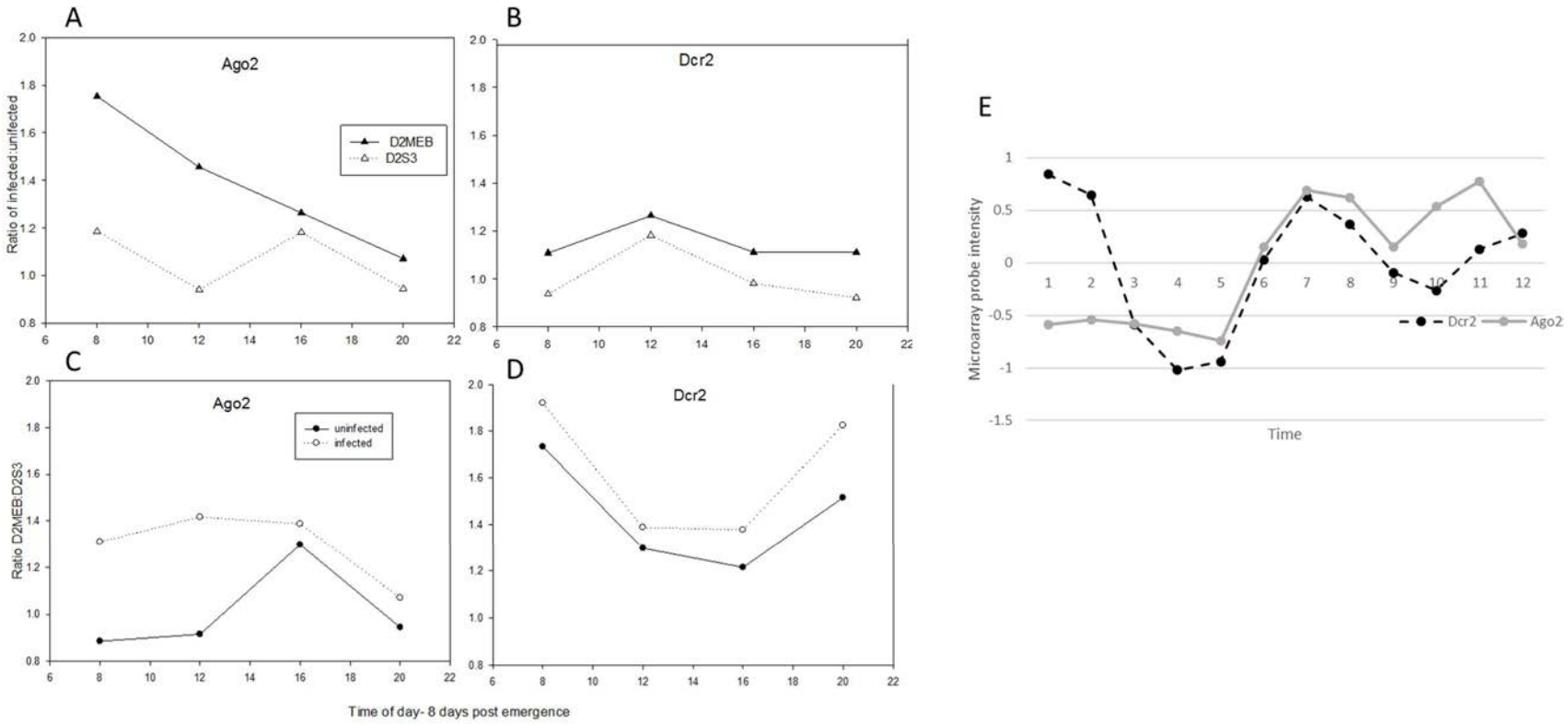



Figure 4.5: Diurnal changes in gene expression level of Ago2 and Dcr2 in D2MEB and D2S3 mosquitoes. The ratio of the number of Ago2 (A) and Dcr2 (B) transcripts in infected versus uninfected D2MEB (black line, black triangle) and D2S3 (dotted line, open triangle) are shown in the top figures. The ratio of the number of Ago2 (C) and Dcr2 (D) transcripts in D2MEB versus D2S3 in uninfected (black line, black circle) and infected (dotted line, open circle) are shown in the bottom figures. The time points are 8- 8:00, 12- 12:00, 16- 16:00 and 20- 20:00 on day 8 post emergence. E) shows the variation in Dcr2 (dashed line, black circle) and Ago2 (gray line, gray circle) over 12 time points 72-104 hours post emergence from the supplementary data from Ptitsyn et al., (2011).

Table 4.8: ANOVA table of the diurnal siRNA gene expression in non-infectious blood fed and infectious blood fed D2S3 and D2MEB mosquitoes. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value, r- effect size.

| Gene | Source of Variation | df | SS | MS | F | p | r |
|---|---|---|---|---|---|---|---|
| Ago2 | Strain | 1 | 0.19 | 0.19 | 3.14 | 0.078 | 0.13 |
| | Infection | 1 | 0.42 | 0.42 | 6.94 | 0.009 | 0.19 |
| | Time | 3 | 0.46 | 0.16 | 2.56 | 0.056 | 0.12 |
| | Strain x Infection | 1 | 0.17 | 0.17 | 2.82 | 0.095 | 0.12 |
| | Strain x Time | 3 | 0.04 | 0.01 | 0.20 | 0.898 | 0.03 |
| | Infection x Time | 3 | 0.29 | 0.10 | 1.58 | 0.197 | 0.09 |
| | Strain x Infection x Time | 3 | 0.14 | 0.05 | 0.78 | 0.508 | 0.06 |
| | Residual | 184 | 11.12 | 0.06 | | | |
| | Total | 199 | 12.84 | 0.06 | | | |
| Dcr2 | Strain | 1 | 16221.05 | 16221.05 | 35.30 | <0.001 | 0.40 |
| | Infection | 1 | 643.25 | 643.25 | 1.40 | 0.238 | 0.09 |
| | Time | 3 | 6416.53 | 2138.84 | 4.65 | 0.004 | 0.16 |
| | Strain x Infection | 1 | 620.59 | 620.59 | 1.35 | 0.247 | 0.09 |
| | Strain x Time | 3 | 2862.34 | 954.11 | 2.08 | 0.105 | 0.11 |
| | Infection x Time | 3 | 394.67 | 131.56 | 0.29 | 0.835 | 0.04 |
| | Strain x Infection x Time | 3 | 85.88 | 28.63 | 0.06 | 0.980 | 0.02 |
| | Residual | 184 | 84562.70 | 459.58 | | | |
| | Total | 199 | 111632.69 | 560.97 | | | |

Table 4.9: ANOVA table of Dcr2 expression in infected and non-infected D2S3 and D2MEB mosquitoes. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value, r- effect size.

| Source of Variation | Time | df | SS | MS | F | p | r |
|---|---|---|---|---|---|---|---|
| Tissue | 2 hours | 1 | 3.754 | 3.754 | 95.41 | <0.001 | 0.72 |
| Strain | 2 hours | 1 | 9.508 | 9.508 | 241.65 | <0.001 | 0.86 |
| Infection | 2 hours | 1 | 0.428 | 0.428 | 10.87 | 0.001 | 0.33 |
| Tissue x Strain | 2 hours | 1 | 2.526 | 2.526 | 64.20 | <0.001 | 0.65 |
| Tissue x Infection | 2 hours | 1 | 0.177 | 0.177 | 4.50 | 0.037 | 0.22 |
| Strain x Infection | 2 hours | 1 | 0.432 | 0.432 | 10.97 | 0.001 | 0.33 |
| Tissue x Strain x Infection | 2 hours | 1 | 0.184 | 0.184 | 4.67 | 0.033 | 0.22 |
| Residual | 2 hours | 88 | 3.463 | 0.039 | | | |
| Total | 2 hours | 95 | 20.471 | 0.215 | | | |
| Tissue | Day 2 | 1 | 1.919 | 1.919 | 36.72 | <0.001 | 0.54 |
| Strain | Day 2 | 1 | 0.618 | 0.618 | 11.83 | <0.001 | 0.34 |
| Infection | Day 2 | 1 | 0.932 | 0.932 | 17.83 | <0.001 | 0.41 |
| Tissue x Strain | Day 2 | 1 | 0.369 | 0.369 | 7.06 | 0.009 | 0.27 |
| Tissue x Infection | Day 2 | 1 | 0.135 | 0.135 | 2.59 | 0.111 | 0.17 |
| Strain x Infection | Day 2 | 1 | 0.592 | 0.592 | 11.34 | 0.001 | 0.34 |
| Tissue x Strain x Infection | Day 2 | 1 | 0.071 | 0.071 | 1.37 | 0.246 | 0.12 |
| Residual | Day 2 | 88 | 4.598 | 0.052 | | | |
| Total | Day 2 | 95 | 9.235 | 0.097 | | | |
| Tissue | Day 5 | 1 | 1.253 | 1.253 | 29.29 | <0.001 | 0.50 |
| Strain | Day 5 | 1 | 0.742 | 0.742 | 17.35 | <0.001 | 0.41 |
| Infection | Day 5 | 1 | 0.077 | 0.077 | 1.81 | 0.182 | 0.14 |
| Tissue x Strain | Day 5 | 1 | 1.699 | 1.699 | 39.71 | <0.001 | 0.56 |
| Tissue x Infection | Day 5 | 1 | 0.023 | 0.023 | 0.55 | 0.462 | 0.08 |
| Strain x Infection | Day 5 | 1 | 0.002 | 0.002 | 0.04 | 0.852 | 0.02 |
| Tissue x Strain x Infection | Day 5 | 1 | 0.005 | 0.005 | 0.12 | 0.726 | 0.04 |
| Residual | Day 5 | 88 | 3.766 | 0.043 | | | |
| Total | Day 5 | 95 | 7.568 | 0.080 | | | |
| Tissue | Day 7 | 1 | 1.232 | 1.232 | 34.13 | <0.001 | 0.53 |
| Strain | Day 7 | 1 | 0.959 | 0.959 | 26.56 | <0.001 | 0.48 |
| Infection | Day 7 | 1 | 0.141 | 0.141 | 3.91 | 0.051 | 0.21 |
| Tissue x Strain | Day 7 | 1 | 0.976 | 0.976 | 27.04 | <0.001 | 0.48 |
| Tissue x Infection | Day 7 | 1 | 0.001 | 0.001 | 0.02 | 0.884 | 0.02 |
| Strain x Infection | Day 7 | 1 | 0.141 | 0.141 | 3.89 | 0.052 | 0.21 |
| Tissue x Strain x Infection | Day 7 | 1 | 0.002 | 0.002 | 0.04 | 0.838 | 0.02 |
| Residual | Day 7 | 88 | 3.177 | 0.036 | | | |
| Total | Day 7 | 95 | 6.629 | 0.070 | | | |

Table 10: ANOVA table of Dcr2 expression over the lifetime of D2S3 and D2MEB mosquitoes. Abbreviations are as follows, BC- backcross, DF- degrees of freedom, SS- sum of squares, MS- mean sum of squares, F- F statistic, p- p-value, r- effect size.

| Source of Variation | Time | df | SS | MS | F | p | r |
|---|---|---|---|---|---|---|---|
| Tissue | Day 1 | 1 | 1.246 | 1.246 | 17.824 | <0.001 | 0.54 |
| Strain | Day 1 | 1 | 2.179 | 2.179 | 31.157 | <0.001 | 0.64 |
| Tissue x Strain | Day 1 | 1 | 0.89 | 0.89 | 12.734 | <0.001 | 0.47 |
| Residual | Day 1 | 44 | 3.077 | 0.0699 | | | |
| Total | Day 1 | 47 | 7.392 | 0.157 | | | |
| Tissue | Day 3 | 1 | 0.102 | 0.102 | 1.378 | 0.25 | 0.17 |
| Strain | Day 3 | 1 | 0.537 | 0.537 | 7.293 | 0.01 | 0.38 |
| Tissue x Strain | Day 3 | 1 | 0.666 | 0.666 | 9.044 | 0.00 | 0.41 |
| Residual | Day 3 | 44 | 3.241 | 0.0737 | | | |
| Total | Day 3 | 47 | 4.546 | 0.0967 | | | |
| Tissue | Day 5 | 1 | 1.15 | 1.15 | 24.851 | <0.001 | 0.60 |
| Strain | Day 5 | 1 | 2.944 | 2.944 | 63.593 | <0.001 | 0.77 |
| Tissue x Strain | Day 5 | 1 | 0.673 | 0.673 | 14.548 | <0.001 | 0.50 |
| Residual | Day 5 | 44 | 2.037 | 0.0463 | | | |
| Total | Day 5 | 47 | 6.805 | 0.145 | | | |
| Tissue | Day 7 | 1 | 0.183 | 0.183 | 1.303 | 0.26 | 0.17 |
| Strain | Day 7 | 1 | 0.198 | 0.198 | 1.413 | 0.24 | 0.18 |
| Tissue x Strain | Day 7 | 1 | 0.593 | 0.593 | 4.219 | 0.05 | 0.30 |
| Residual | Day 7 | 44 | 6.181 | 0.14 | | | |
| Total | Day 7 | 47 | 7.156 | 0.152 | | | |
| Tissue | Day 10 | 1 | 0.0495 | 0.0495 | 0.384 | 0.54 | 0.09 |
| Strain | Day 10 | 1 | 0.0662 | 0.0662 | 0.514 | 0.48 | 0.11 |
| Tissue x Strain | Day 10 | 1 | 0.0214 | 0.0214 | 0.166 | 0.69 | 0.06 |
| Residual | Day 10 | 44 | 5.671 | 0.129 | | | |
| Total | Day 10 | 47 | 5.808 | 0.124 | | | |
| Tissue | Day 12 | 1 | 0.37 | 0.37 | 2.845 | 0.10 | 0.25 |
| Strain | Day 12 | 1 | 0.00559 | 0.00559 | 0.043 | 0.84 | 0.03 |
| Tissue x Strain | Day 12 | 1 | 0.659 | 0.659 | 5.073 | 0.03 | 0.32 |
| Residual | Day 12 | 44 | 5.72 | 0.13 | | | |
| Total | Day 12 | 47 | 6.754 | 0.144 | | | |
| Tissue | Day 15 | 1 | 0.000403 | 0.000403 | 0.00266 | 0.96 | 0.01 |
| Strain | Day 15 | 1 | 0.41 | 0.41 | 2.708 | 0.11 | 0.24 |
| Tissue x Strain | Day 15 | 1 | 0.17 | 0.17 | 1.124 | 0.30 | 0.16 |
| Residual | Day 15 | 44 | 6.662 | 0.151 | | | |
| Total | Day 15 | 47 | 7.243 | 0.154 | | | |

# CHAPTER V: GENETIC VARIATION AND DENGUE SUSCEPTIBILITY ASSOCIATED WITH FOUR DCR2 SINGLE NUCLEOTIDE POLYMORPHISMS IN NATURAL AEDES AEGYPTI POPULATIONS FROM SENEGAL

## Introduction

As outlined in Chapter 1, the siRNA pathway has an influence on anti-viral immunity and arboviral infection in mosquitoes. In Chapters 2 and 3, the siRNA pathway was utilized to create genetically modified Ae. aegypti refractory to DENV2. Chapter 4 demonstrates that the siRNA pathway influences midgut infection barriers in artificially selected Ae. aegypti. Therefore, in this chapter the natural variation in Senegalese populations of Ae. aegypti are evaluated for genetic variability based on their susceptibility to DENV2. Bernhardt et al. (2012) determined that there were multiple highly variable loci in many of the Aedes aegypti RNAi genes. Therefore, twelve sites from a wide geographic range in Senegal, Africa were evaluated for their DENV susceptibility phenotype. These collection sites were chosen from previous work that demonstrated large differences in the susceptibility of Ae. aegypti to DENV infection at these locations (Sylla et al., 2009). The four single nucleotide polymorphisms (SNPs) were selected because they were described by Bernhardt et al., (2012) as being alternatively fixed in mosquitoes from northwest and southeastern Senegal. In general populations from the southeast are refractory to DENV-2 infection while populations from the northwest are susceptible to DENV-2 infection (Table 5.1). Therefore, in populations with a higher prevalence of northwest alleles should have lower MIB and MEB and greater SUSC. Alternatively, populations in which alleles from the southeast are more prevalent should have greater MIB and MEB and lower SUSC.

The chosen loci in the Dcr2 gene were nonsynonymous single nucleotide polymorphisms (SNPs). The first SNP, 2336, is located in a domain of unknown function/non-functional domain 133 base pairs (bp) upstream of the Dcr2 Piwi/Argonaute/Zwille (PAZ) domain (Fig. 5.1A). The PAZ domain is highly conserved and is the primary binding site for the siRNA and miRNA 3' overhang (Hammond, 2005; Yan et al., 2003). The second SNP, 2521, is located 52bp into the PAZ domain (Fig. 5.1A). The variability at both of these sites results in a change in the amino acid side chain charge. The other two SNPs, 4474 and 4540 are located within the Dcr2 RiboC domain (Fig. 5.1B). This domain is an RNAse III domain, which functions as RNA binding and cleavage site (Tahbaz et al., 2004) and is also involved in Ago binding (Sasaki and Shimizu, 2007).

## Materials and methods

### Sample collection and colony establishment

Aedes aegypti samples were taken from 12 locations in Senegal, Africa (Fig. 5.2). Mosquitoes were collected as larvae from water storage containers and tree holes from urban and rural areas. The individuals were raised to adult hood, blood fed, and the eggs were shipped to the Arthropod-borne and Infectious Disease Lab (Fort Collins, CO, USA) to establish colonies. A separate colony was created from each of the 12 sampling locations.

Egg papers were hatched in sterile water containing brewer's yeast and were maintained at 28°C and 80.0% humidity. Larvae were raised as described in previous chapters and were blood fed on human volunteers approximately twice a week. All experiments were conducted on $F_3$ individuals since there were too few individuals to conduct the study in earlier generations.

### Mosquito infection

Four to 6 days post-emergence $F_3$ mosquitoes were given a blood meal consisting of 1:1 defibrinated cow blood and DENV2 infected cell culture medium. The blood meal was made by

infecting C6/36 cells with high passage DENV2 Jamaica 1409 virus at an MOI of 0.01. The C6/36 cells maintained in L-15 medium supplemented with 3% heat inactivated fetal bovine serum, 1% streptomycin and 1% L-glutamine. The cells were held at 28 ºC for 12 days and the medium was replaced 5 to 6 days post infection. The infectious blood meal was prepared and administered to mosquitoes from each location as described by Bosio et al. (1998) and Sánchez-Vargas et al. (2009). At 14 days post infection, individuals were collected and dissected to obtain a thorax for single nucleotide polymorphism (SNP) analysis. The head and abdomen were retained for immunofluorescent assays (IFAs). Heads were fixed on a glass slide with 100.0% acetone and the midgut was dissected from each abdomen and stored in a 4.0% paraformaldehyde PBS solution.

IFAs

Indirect IFAs were conducted as described in previous studies (Kuberski, 1979) using the 3H5 DENV2 E protein specific antibody. All midguts were examined for presence of DENV2 antigen by fluorescence microscopy. Individuals with positive midguts were then examined for the presence of antigen in the head by fluorescence microscopy. Individuals with no detectable antigen in the midgut were labeled as having a midgut infection barrier (MIB). Individuals with detectable antigen in their midgut, but not in their head were labeled as having a midgut escape barrier (MEB). Individuals with detectable antigen in their midgut and head were labeled as being susceptible (SUSC) to infection.

DNA extraction

The thorax of each individual was homogenized in 30µl of Pat Roman's buffer (0.1M NaCl, 0.2M sucrose, 0.1M tris buffer, 0.05 M EDTA and 0.5% SDS) (Black and DuTeau, 1997). The samples were then incubated for 30 minutes at 65°C before adding 7 µl of 8M potassium acetate. The samples were then chilled on ice for 35 min. and then microcentrifuged at

maximum speed for 15 minutes. The supernatant was removed and transferred to a new tube with 100 µl of ~100.0% ethanol. Contents of the tube were then mixed and left at room temperature for 5 minutes. The tube was centrifuged again at maximum speed for 15 minutes. The ethanol was removed and the pellet resuspended in 75% ethanol and centrifuged at maximum speed for 5 minutes. The previous step was repeated once more with 100% ethanol before vacuum drying in a centrifuge for approximately 20 minutes. The sample was then resuspended in 50 µl TE buffer, aliquoted and stored at -80°C.

Multiple displacement amplification

The amount of recoverable DNA in each thorax was small, so multiple displacement amplification (MDA) was conducted using the GE Healthcare Templiphi Kit (Pittsburgh, PA, USA). The amplification was conducted by adding 1µl DNA to 5µl sample buffer. This reaction was kept on ice and 5µl of reaction buffer and 0.2 µl Φ29 DNA enzyme were added to the tube. The reaction was incubated at 30°C in a heated lid thermal cycler for 18 hours. Then the samples were incubated at 65°C for 10 minutes and then held at 4°C. Then each sample was diluted into 80µl sterile water and aliquoted into 4 tubes. A few samples from each group of MDAs were run on a 0.8% agarose gel to confirm and visualized to confirm amplification. All samples were stored at -20°C.

Melting curve PCR

Samples were analyzed using an allele specific SYBR green melting curve PCR reaction (iQ™ SYBR® Green Supermix (BIORAD, USA)) using the manufacturer's protocol. The primers used in the assay are listed in Table 5.2. The annealing temperature for all reactions was 57 °C. The primers were designed for 4 SNPs, located at variable sites in the Dcr2 gene. Primer's were diluted to 500 pmol/µl. For each loci, forward primers were designed for each allele, and one reverse primer was designed for both alleles. One forward primer at each loci

was given a long G-C extension to allow for defined differences in melting temperatures (Table 5.1). The genotype at each loci was determined by examining the melting curve profiles of each sample.

### Data analysis

The melting curve data were sorted into groups based on their viral dissemination. Mosquitoes with an IFA positive head were classified as dengue susceptible (SUSC). Mosquitoes with an IFA negative and an IFA positive midgut were classified as having a midgut escape barrier (MEB) and individuals that had an IFA negative midgut and an IFA negative head were classified as having a midgut infection barrier (MIB). The sorted melting curve data were converted to allele frequency tables using the CONVERT program (Purdue University, v.1.31). ARLEQUIN (Berne University, ver. 3.5.1.2) was also used to conduct analysis of molecular variance (AMOVA) for differences in pairwise loci within the SUSC, MEB, MIB groups. Linkage disequilibrium (LD) analysis was performed in ARELQUIN (Berne University, ver. 3.5.1.2) for all pairwise loci comparisons by location within the SUSC, MEB and MIB groups. Genotype-phenotype associations were compared by a $\chi^2$ test. Location clustering of genotypes was conducted by correspondence analysis (CA) using PAST software (Oyvind Hammer, v.3.1) (Hammer et al., 2001).

## Results

### Location genotype clustering and genotype by phenotype associations

At locus 2336, the GG genotype was predominant at most geographic locations except for the central, inland locations Sokone and Fatick which had more of the CC and CG genotypes, respectively (Fig. 5.3). I hypothesized that at locus 2336, there would be a greater abundance of DENV MIB and MEB individuals associated with the CC genotype followed by the GC genotype and the GG phenotype would be the most susceptible, but there were no significant

genotype by phenotype associations (Table 5.3). At locus 2521, there was more genotype diversity, but there was a less clear geographic clustering (Fig. 5.3). More urban locations, such as Ouakam, Thies and Bambey had an abundance of AA genotype, but the AC and CC genotypes were in abundance throughout the geographic sampling range. For this locus, we hypothesized that the refractory individuals would have a higher abundance of the AA genotype followed by the AC and the CC phenotype would be the most SUSC. However, there was no significant genotype by phenotype association to support this hypothesis (Table 5.4). At locus 4474, we hypothesized that there would be more MIB and MEB individuals that were the GG genotype followed by the GT. The TT genotype was supposed to have the most SUSC individuals, but the genotype by phenotype associations were not significant (Table 5.5). There were more GT genotypes seen in the central and southern locations of Sokone, Fatick, Sedhiou and Gossas. The only central locations that were not predominately the GT genotype were Joal, which is on the coast and Bignona, which is the southern-most collection site. At locus 4540, only Ouakam, the most urban and western most collection location, was predominately the AA genotype (Fig. 5.3). Pire and Gossas, the furthest north and furthest north-east sites were predominantly AC and the rest of the locations were predominantly CC. I hypothesized that the CC genotypes followed by the AC genotype would have the most MEB individuals, while the AA would be associated with SUSC, but no significant associations were found (Table 5.6).

Discussion

There was no association with genotype and phenotype between any of the 4 SNPs in Dcr2 and DENV2 susceptibility. However, there is a great deal of genetic diversity at these SNP loci among collection sites. The genotype-phenotype associations were not significant for any of the loci, which may be due to the selection of DENV2 Jamaica 1409 over regionally circulating DENV strains (Dickson et al., 2014). Locus 4540, however, had the most significant

associations between genotypes and the MIB and SUSC phenotypes at multiple locations. This locus is in the RiboC domain of the Dcr2 gene, which is a RNAse III domain (Tahbaz et al., 2004). The RiboC domain is highly conserved in vertebrates, but often is more variable in non-vertebrates (Sasaki and Shimizu, 2007) and locus 4540 was also the most variable locus in this study. Therefore, this study demonstrated variability by collection location in the Dcr2 gene of Ae. aegypti in Senegal, but this variability was not associated with DENV2 Jamaica 1409 susceptibility.

Table 5.1: Locus descriptions from previous studies. Locus descriptions from the Bernhardt et al., (2012) study. Abbreviations: SNP- single nucleotide polymorphism; Gly- glycine; Arg- arginine; Asn- asparagine; His- histidine; Tyr- tyrosine; Asp- aspartic acid;

| Locus | SNP | Amino Acid | Genotype distribution |
|---|---|---|---|
| 2236 | GGN | Gly | greater prevalence in northwest Senegal |
| | CGN | Arg | greater prevalence in southeast Senegal |
| 2521 | CAR | Asn | greater prevalence in northwest Senegal |
| | AAR | His | greater prevalence in southeast Senegal |
| 4474 | TAY | Tyr | greater prevalence in northwest Senegal |
| | GAY | Asp | greater prevalence in southeast Senegal |
| 4550 | AAY | Asn | greater prevalence in northwest Senegal |
| | CAY | His | greater prevalence in southeast Senegal |

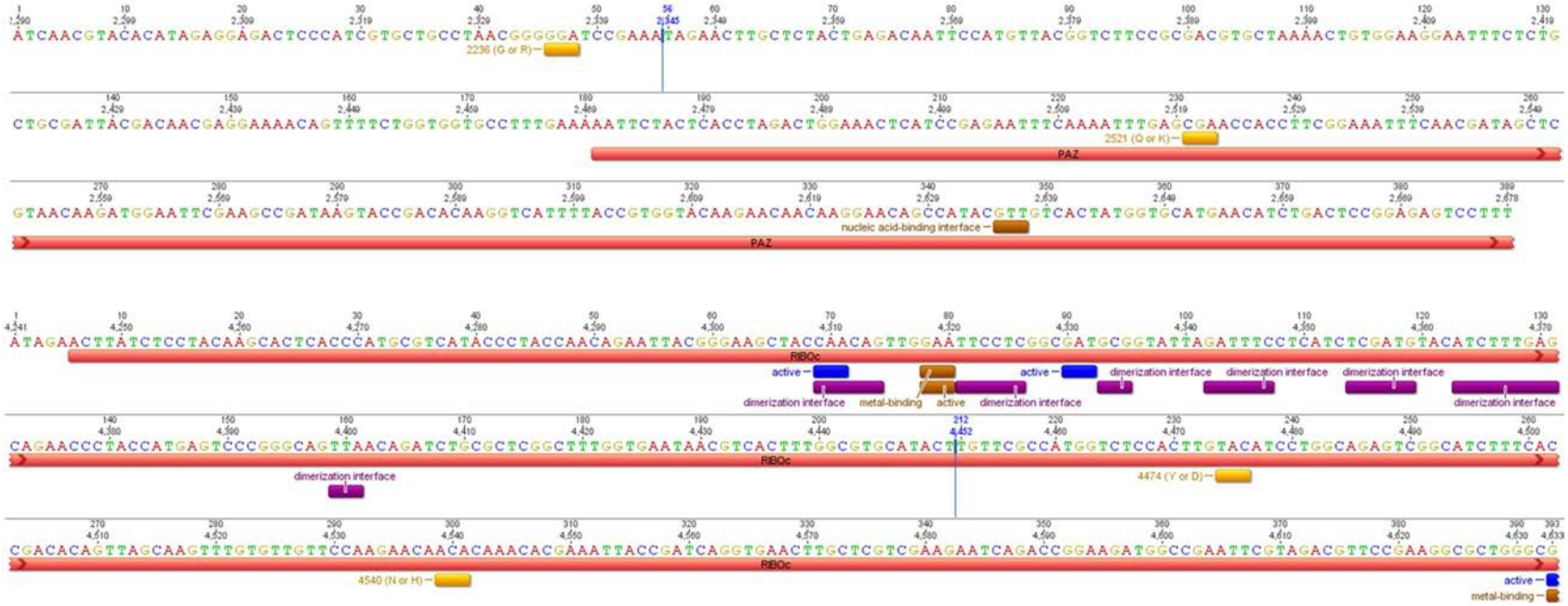



Figure 5.1: Location of SNPs. A) the location of loci 2236 and 2521 (yellow) upstream and within the Dcr2 PAZ domain, respectively. A 5' region of the PAZ domain is shown in red and one nucleic acid binding site is also shown (brown). B) the location of loci 4474 and 4540 (yellow) in the Dcr2 RIBOc domain. A 5' region of the RIBOc domain (red) and multiple active sites (blue) dimerization interfaces (purple) and metal binding sites (brown) are also shown.


Figure 5.2: Map of the 12 collection sites in Senegal, Africa. The white squares indicate the location of the 12 collection sites where Ae. aegypti mosquitoes were collected prior to colonization and analysis.

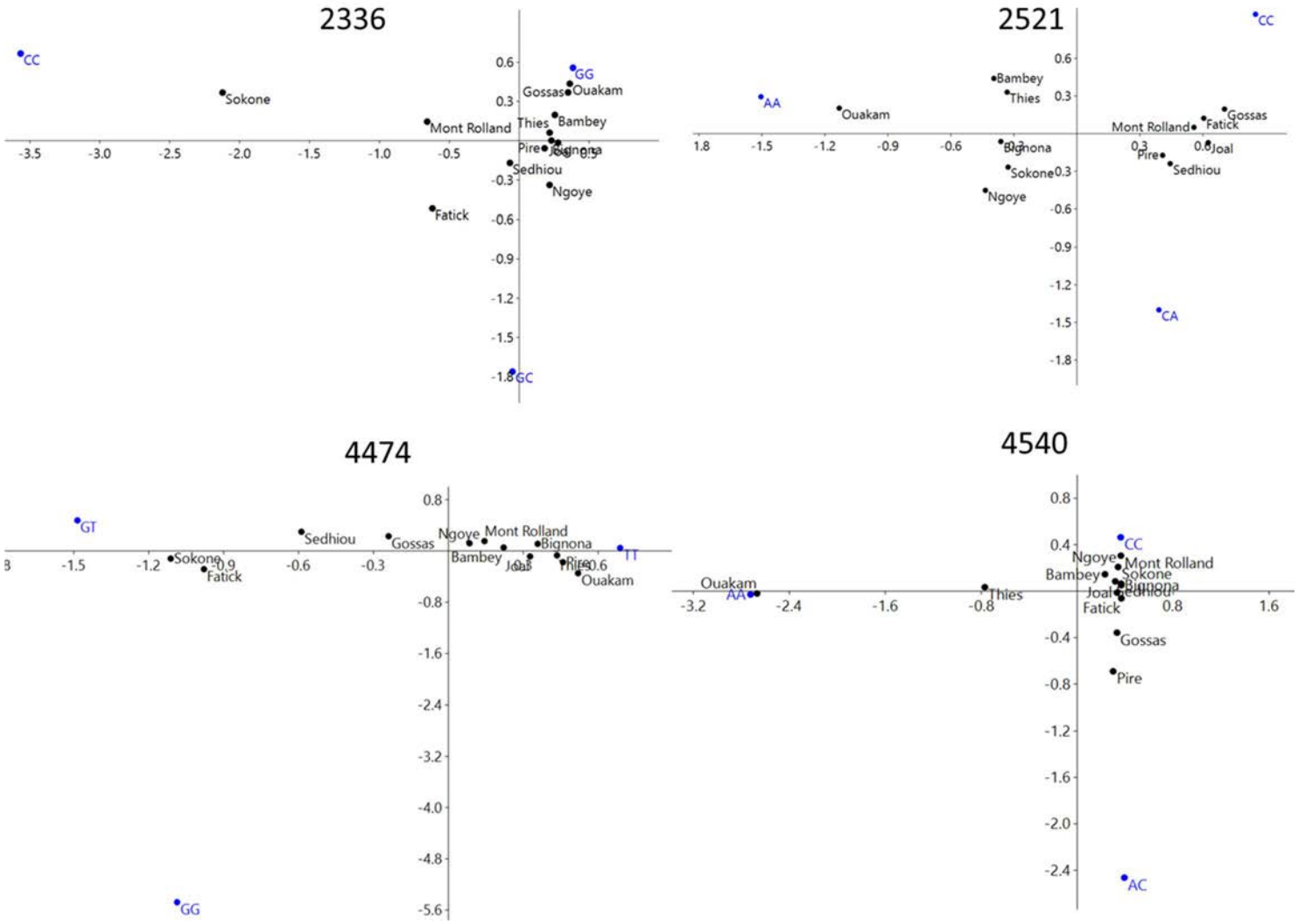



Figure 5.3: Correspondence analysis of geographic genotype clustering. The 4 loci were analyzed for geographic clustering by CA. Graphs of the genotypes at locus 2336 (top left), 2521 (top right), 4474 (bottom left) and 4540 (bottom right) for each collection location.

Table 5.2: Primers used for melting curve PCR. Primers are named by gene, locus (2236, 2521, 4474, 4540), primer direction (F-forward, r-reverse) and allele number (1 or 2).

| Name | Sequence |
|---|---|
| Dicer2 2336F1 | GCGGGCAGGGCGGCGGGGGCGGGGCCTTGGGACTAATCAACGTACACAGAG |
| Dicer2 2336F2 | GCGGGCTTGGGACTAATCAACGTACACAAAA |
| Dicer2 2336r | AGAGCAAGTTCTATTTCGGA |
| Dicer2 2521F1 | GCGCGGCGGGCAGGGCGGCGGGGGCGGGGCCAAAAATTCTACTCACCTAGACTCGA |
| Dicer2 2521F2 | GCGGGCAAAAATTCTACTCACCTAGACTTGG |
| Dicer2 2521r | GAAGGTGGTTCGCTCAAA |
| Dicer2 4474F1 | GCGGGCAGGGCGGCGGGGGCGGGGCCCTTGTTCGCCATGGTCTCCACTAGT |
| Dicer2 4474F2 | GCGGGCCTTGTTCGCCATGGTCTCCACTGGG |
| Dicer2 4474r | GTGTCGGTGAAAGATGCC |
| Dicer2 4540F1 | GCGGGCAGGGCGGCGGGGGCGGGGCCAAGTTTGTGTTGGTCCAAGAACCAC |
| Dicer2 4540F2 | GCGGGCAAGTTTGTGTTGGTCCAAGAACTAA |
| Dicer2 4540r | CGGCCATCTTCCGGTCTG |

Table 3: Genotype-phenotype associations at locus 2336. The genotype-phenotype associations were compared for the DENV susceptibility phenotypes at each of the 12 collection sites. p-values highlighted in gray are significantly different by $\chi^2$ test. n- sample size; $\chi^2$ stat- the chi-squared statistic; df- degrees of freedom; p-value- calculated probability

| Location | Locus | Phenotype | n | $\chi$2 stat | df | p-value |
|---|---|---|---|---|---|---|
| Bambey | 2336 | MIB | 64 | 0.94 | 2 | 0.63 |
| | | MEB | 36 | 0.61 | 2 | 0.74 |
| | | SUSC | 64 | 1.68 | 2 | 0.43 |
| Bignona | | MIB | 79 | 1.56 | 2 | 0.46 |
| | | MEB | 51 | 1.06 | 2 | 0.59 |
| | | SUSC | 79 | 1.32 | 2 | 0.52 |
| Fatick | | MIB | 74 | 3.48 | 2 | 0.18 |
| | | MEB | 45 | 0.99 | 2 | 0.61 |
| | | SUSC | 74 | 4.11 | 2 | 0.13 |
| Gossas | | MIB | 86 | 0.46 | 1 | 0.50 |
| | | MEB | 60 | 0.13 | 1 | 0.71 |
| | | SUSC | 86 | 0.35 | 2 | 0.84 |
| Joal | | MIB | 89 | 0.82 | 2 | 0.66 |
| | | MEB | 57 | 0.00 | 2 | 1.00 |
| | | SUSC | 89 | 0.45 | 1 | 0.50 |
| Mont Rolland | | MIB | 37 | 2.04 | 2 | 0.36 |
| | | MEB | 23 | 5.38 | 2 | 0.07 |
| | | SUSC | 37 | 7.34 | 2 | 0.03 |
| Ngoye | | MIB | 132 | 1.65 | 1 | 0.20 |
| | | MEB | 80 | 1.36 | 2 | 0.51 |
| | | SUSC | 131 | 0.05 | 1 | 0.82 |
| Pire | | MIB | 45 | 2.98 | 2 | 0.23 |
| | | MEB | 26 | 2.86 | 2 | 0.24 |
| | | SUSC | 45 | 2.42 | 2 | 0.30 |
| Ouakum | | MIB | 57 | 0.13 | 1 | 0.72 |
| | | MEB | 43 | 0.10 | 1 | 0.76 |
| | | SUSC | 57 | 0.19 | 1 | 0.66 |
| Sedhiou | | MIB | 63 | 0.43 | 2 | 0.80 |
| | | MEB | 45 | 2.23 | 2 | 0.33 |
| | | SUSC | 63 | 0.49 | 2 | 0.78 |
| Sokone | | MIB | 45 | 3.84 | 2 | 0.15 |
| | | MEB | 16 | 2.93 | 2 | 0.23 |
| | | SUSC | 45 | 3.50 | 2 | 0.17 |
| Theis | | MIB | 107 | 1.64 | 2 | 0.44 |
| | | MEB | 62 | 1.02 | 2 | 0.60 |
| | | SUSC | 107 | 1.47 | 2 | 0.48 |

Table 4: Genotype-phenotype associations at locus 2521. The genotype-phenotype associations were compared for the DENV susceptibility phenotypes at each of the 12 collection sites. p-values highlighted in gray are significantly different by $\chi^2$ test. n- sample size; $\chi^2$ stat- the chi-squared statistic; df- degrees of freedom; p-value- calculated probability

| Location | Locus | Phenotype | n | $\chi^2$ stat | df | p-value |
|---|---|---|---|---|---|---|
| Bambey | 2521 | MIB | 68 | 5.28 | 2 | 0.07 |
| | | MEB | 51 | 1.45 | 2 | 0.48 |
| | | SUSC | 67 | 2.03 | 2 | 0.36 |
| Bignona | | MIB | 79 | 1.77 | 2 | 0.41 |
| | | MEB | 51 | 1.45 | 2 | 0.48 |
| | | SUSC | 79 | 0.25 | 2 | 0.88 |
| Fatick | | MIB | 79 | 1.02 | 2 | 0.60 |
| | | MEB | 46 | 3.31 | 2 | 0.19 |
| | | SUSC | 79 | 2.52 | 2 | 0.28 |
| Gossas | | MIB | 84 | 0.03 | 1 | 0.87 |
| | | MEB | 58 | 0.00 | 1 | 0.95 |
| | | SUSC | 84 | 0.00 | 1 | 0.95 |
| Joal | | MIB | 88 | 2.37 | 2 | 0.31 |
| | | MEB | 56 | 0.93 | 2 | 0.63 |
| | | SUSC | 88 | 0.45 | 1 | 0.50 |
| Mont Rolland | | MIB | 38 | 0.19 | 2 | 0.91 |
| | | MEB | 23 | 3.59 | 2 | 0.17 |
| | | SUSC | 38 | 3.44 | 2 | 0.18 |
| Ngoye | | MIB | 132 | 11.56 | 2 | 0.00 |
| | | MEB | 79 | 1.09 | 2 | 0.58 |
| | | SUSC | 132 | 4.98 | 2 | 0.08 |
| Pire | | MIB | 40 | 3.64 | 2 | 0.16 |
| | | MEB | 22 | 5.45 | 2 | 0.07 |
| | | SUSC | 40 | 10.37 | 2 | 0.01 |
| Ouakum | | MIB | 57 | 8.22 | 2 | 0.02 |
| | | MEB | 43 | 4.39 | 2 | 0.11 |
| | | SUSC | 57 | 4.82 | 2 | 0.09 |
| Sedhiou | | MIB | 64 | 3.61 | 2 | 0.16 |
| | | MEB | 45 | 1.04 | 2 | 0.59 |
| | | SUSC | 63 | 0.38 | 1 | 0.54 |
| Sokone | | MIB | 45 | 3.28 | 2 | 0.19 |
| | | MEB | 16 | 2.81 | 2 | 0.25 |
| | | SUSC | 45 | 1.60 | 2 | 0.45 |
| Theis | | MIB | 107 | 7.32 | 2 | 0.03 |
| | | MEB | 62 | 1.07 | 2 | 0.59 |
| | | SUSC | 107 | 2.59 | 2 | 0.27 |

Table 5: Genotype-phenotype associations at locus 4474. The genotype-phenotype associations were compared for the DENV susceptibility phenotypes at each of the 12 collection sites. p-values highlighted in gray are significantly different by $\chi^2$ test. n- sample size; $\chi^2$ stat- the chi-squared statistic; df- degrees of freedom; p-value- calculated probability

| Location | Locus | Phenotype | n | $\chi^2$ stat | df | p-value |
|---|---|---|---|---|---|---|
| Bambey | 4474 | MIB | 69 | 0.91 | 2 | 0.63 |
| | | MEB | 41 | 2.27 | 2 | 0.32 |
| | | SUSC | 69 | 5.89 | 2 | 0.05 |
| Bignona | | MIB | 79 | 2.18 | 1 | 0.14 |
| | | MEB | 51 | 0.24 | 1 | 0.63 |
| | | SUSC | 79 | 1.34 | 1 | 0.25 |
| Fatick | | MIB | 80 | 0.71 | 2 | 0.70 |
| | | MEB | 47 | 4.74 | 2 | 0.09 |
| | | SUSC | 80 | 4.40 | 2 | 0.11 |
| Gossas | | MIB | 75 | 0.01 | 1 | 0.92 |
| | | MEB | 53 | 2.42 | 1 | 0.12 |
| | | SUSC | 75 | 2.35 | 1 | 0.13 |
| Joal | | MIB | 87 | 1.23 | 2 | 0.54 |
| | | MEB | 55 | 0.57 | 2 | 0.75 |
| | | SUSC | 87 | 0.12 | 2 | 0.94 |
| Mont Rolland | | MIB | 36 | 0.34 | 1 | 0.56 |
| | | MEB | 21 | 0.18 | 1 | 0.68 |
| | | SUSC | 36 | 0.44 | 1 | 0.50 |
| Ngoye | | MIB | 129 | 2.37 | 2 | 0.31 |
| | | MEB | 77 | 6.33 | 2 | 0.04 |
| | | SUSC | 129 | 2.04 | 2 | 0.36 |
| Pire | | MIB | 44 | 4.29 | 2 | 0.12 |
| | | MEB | 25 | 5.77 | 2 | 0.06 |
| | | SUSC | 44 | 13.45 | 2 | 0.00 |
| Ouakum | | MIB | 55 | 1.92 | 2 | 0.38 |
| | | MEB | 42 | 0.74 | 2 | 0.69 |
| | | SUSC | 55 | 1.92 | 2 | 0.38 |
| Sedhiou | | MIB | 63 | 0.42 | 1 | 0.52 |
| | | MEB | 44 | 0.06 | 1 | 0.80 |
| | | SUSC | 63 | 0.38 | 1 | 0.54 |
| Sokone | | MIB | 45 | 4.70 | 2 | 0.10 |
| | | MEB | 16 | 0.37 | 1 | 0.54 |
| | | SUSC | 45 | 1.60 | 2 | 0.45 |
| Theis | | MIB | 107 | 4.49 | 2 | 0.11 |
| | | MEB | 62 | 4.07 | 2 | 0.13 |
| | | SUSC | 107 | 2.55 | 2 | 0.28 |

Table 6: Genotype-phenotype associations at locus 4540. The genotype-phenotype associations were compared for the DENV susceptibility phenotypes at each of the 12 collection sites. p-values highlighted in gray are significantly different by $\chi^2$ test. n- sample size; $\chi^2$ stat- the chi-squared statistic; df- degrees of freedom; p-value- calculated probability

| Location | Locus | Phenotype | n | $\chi$2 stat | df | p-value |
|---|---|---|---|---|---|---|
| Bambey | 4540 | MIB | 69 | 2.85 | 2 | 0.24 |
| | | MEB | 41 | 1.86 | 2 | 0.40 |
| | | SUSC | 68 | 0.18 | 2 | 0.91 |
| Bignona | | MIB | 78 | 0.00 | 1 | 0.97 |
| | | MEB | 50 | 0.77 | 1 | 0.38 |
| | | SUSC | 78 | 0.58 | 1 | 0.45 |
| Fatick | | MIB | 78 | 0.57 | 1 | 0.45 |
| | | MEB | 46 | 1.63 | 1 | 0.20 |
| | | SUSC | 78 | 1.83 | 1 | 0.18 |
| Gossas | | MIB | 79 | 0.47 | 2 | 0.79 |
| | | MEB | 55 | 5.10 | 2 | 0.08 |
| | | SUSC | 79 | 6.29 | 2 | 0.04 |
| Joal | | MIB | 87 | 0.60 | 2 | 0.74 |
| | | MEB | 55 | 0.47 | 2 | 0.79 |
| | | SUSC | 87 | 1.30 | 2 | 0.52 |
| Mont Rolland | | MIB | 37 | 3.10 | 1 | 0.08 |
| | | MEB | 22 | | | |
| | | SUSC | 37 | 0.68 | 1 | 0.41 |
| Ngoye | | MIB | 128 | 6.29 | 2 | 0.04 |
| | | MEB | 76 | 0.46 | 1 | 0.50 |
| | | SUSC | 128 | 3.92 | 2 | 0.14 |
| Pire | | MIB | 41 | 0.74 | 2 | 0.69 |
| | | MEB | 24 | 1.63 | 2 | 0.44 |
| | | SUSC | 41 | 2.64 | 2 | 0.27 |
| Ouakum | | MIB | 57 | 0.33 | 1 | 0.56 |
| | | MEB | 43 | 1.57 | 1 | 0.21 |
| | | SUSC | 57 | 0.85 | 1 | 0.36 |
| Sedhiou | | MIB | 63 | 6.03 | 2 | 0.05 |
| | | MEB | 44 | 1.97 | 1 | 0.16 |
| | | SUSC | 63 | 0.98 | 2 | 0.61 |
| Sokone | | MIB | 44 | 2.76 | 1 | 0.10 |
| | | MEB | 16 | 4.75 | 1 | 0.03 |
| | | SUSC | 44 | 7.80 | 1 | 0.01 |
| Theis | | MIB | 0 | 6.42 | 2 | 0.04 |
| | | MEB | 0 | 0.02 | 2 | 0.99 |
| | | SUSC | 0 | 15.73 | 2 | 0.00 |

# CHAPTER VI: SUMMARY AND CONCLUSIONS

In Chapter 2, a transgenic mosquito engineered to utilize the RNAi pathway to prevent DENV2 infection, Carb109 was backcrossed to a GDLS. Backcrossing to a GDLS improved the fitness of the transgenic mosquito making it more feasible for use in genetic vector control programs. These results improved upon a previous transgenic strain that was engineered with the same construct, Carb77. The original Carb77 strain lost its DENV refractory phenotype and as this chapter showed had low fitness over time. Creating a new line, Carb109, and backcrossing it to the GDLS improved the transgene stability and fitness of the new line compared to its predecessor.

Chapter 3 focused on the fitness of transgenic vectors. Backcrossing the Carb109 transgenic lines to a GDLS improved many life history traits that would improve the ability of the transgenic line to be driven into natural populations. Hatch rate, fecundity, larval energy reserves and adult survival on a sugar diet were all improved by backcrossing the Carb109 to a GDLS. Once the lines were made homozygous on the other hand, their fecundity and hatch rates decreased significantly, even significantly below the rates seen in the early backcrosses. Therefore, the homozygote, regardless of introgression into the GDLS, had too high of a genetic load or reduced fitness to be maintained for many generations. The integration site of the transgene along with positional effects likely contributed to the loss of most of the homozygous lines in subsequent generations.

In Chapter 4, gene expression of multiple potential anti-viral immunity genes was examined in two Ae. aegypti strains artificially selected for a high MEB, D2MEB, and high susceptibility, D2S3. This work showed that Ago2 and Dcr2 expression is higher in the D2MEB strain versus the D2S3 strain and higher expression of both of these genes in the midgut versus

the carcass. Only Dcr2 had higher expression levels in infected versus non-infected, but this difference was only in the D2MEB strain, which implicates a role of Dcr2 and possibly the siRNA pathway in the MEB characteristic of this strain. These studies provided further evidence of the role of siRNA pathway genes in the midgut escape in these strains. Additionally, the expression of these genes varied naturally over the course of a day and over the lifetime of the mosquito. Thus, this chapter also showed the importance of keeping consistent collection times when conducting gene expression studies.

Chapter 5 evaluated 4 non-synonymous substitutions in the Dcr2 gene for association with susceptibility to DENV2 Jamaica 1409 infection. These experiments showed that there was high diversity at these site; however, there was not an association between DENV susceptibility and Dcr2 genotype at these loci.

These studies further evaluated the role of the siRNA pathway in mosquito anti-viral immunity. In the first two chapters, the siRNA pathway was utilized to create mosquito refractory to DENV2 infection. Ultimately, these studies showed that the Carb109 strain is not suitable for field release due to its low homozygote fitness, but these studies showed that outbreeding transgenic strains to a genetically diverse mosquito population can better prepare the mosquito for successful field release. Additionally, these studies showed that the transgene did not cause fitness deficits in the Carb109 mosquito, but the inbred parent strain caused the lower fitness of this strain. This study also suggests the need for improved transgenic technologies to allow simplify transformation and screening genetically diverse parent strains trains, instead of highly inbred strains with reduced fitness and increased genetic loads. Therefore, in the future, novel transgenic lines can be created and improved using the knowledge gained from this work.

The fourth chapter gave further evidence of the role of the siRNA pathway in regulating viral infection in the mosquito midgut. There are many other studies that have shown differences in immune gene expression in response to mosquito infection, but this study was the first to compare immune gene expression in mosquito strains with strong differences in their MEB phenotypes. Additionally, this is the first study characterize in-depth the natural expression of Ago2 and Dcr2 over time. The natural variation of Dcr2 and Ago2 seen in this study may be indicative of the role of these genes in the regulation of other processes in the mosquitoes. Thus, this work is a first step in identifying the other roles of these genes in the mosquito.

The fifth chapter aimed to find an association between the siRNA pathway and DENV susceptibility in wild populations. The Dcr2 gene is highly variable at the 4 loci evaluated in this study and the loci evaluated in other studies. There are likely other factors driving the diversity in this gene, but conducting a similar study with a locally derived, low passage virus would likely have a better chance of success. Therefore, even though the results of these studies were not significant further evaluation of these sites with different virus strains is needed to conclude whether there is an association between the genotype at these loci and the DENV susceptibility phenotype.

# REFERENCES


Acosta, E.G., Castilla, V., Damonte, E.B., 2011. Infectious dengue-1 virus entry into mosquito C6/36 cells. Virus Res. 1–7. doi:10.1016/j.virusres.2011.06.008

Acosta, E.G., Castilla, V., Damonte, E.B., 2009. Alternative infectious entry pathways for dengue virus serotypes into mammalian cells. Cell. Microbiol. 11, 1533–49. doi:10.1111/j.1462-5822.2009.01345.x

Acosta, E.G., Talarico, L.B., Damonte, E.B., 2008. Cell entry of dengue virus. Future Virol. 3, 471–470. doi:10.2217/17460794.3.5.471

Adelman, Z.N., Anderson, M. a E., Wiley, M.R., Murreddu, M.G., Samuel, G.H., Morazzani, E.M., Myles, K.M., 2013. Cooler temperatures destabilize RNA interference and increase susceptibility of disease vector mosquitoes to viral infection. PLoS Negl. Trop. Dis. 7. doi:10.1371/journal.pntd.0002239

Adelman, Z.N., Sanchez-Vargas, I., Travanty, E.A., Carlson, J.O., Beaty, B.J., Blair, C.D., Olson, K.E., 2002. RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. J. Virol. 76, 12925–12933.

Agaisse, H., Perrimon, N., 2004. The roles of JAK/STAT signaling in Drosophila immune responses. Immunol. Rev. 198, 72–82.

Agaisse, H., Petersen, U.M., Boutros, M., Mathey-Prevot, B., Perrimon, N., 2003. Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. Dev. Cell 5, 441–50.

Akbari, O.S., Matzen, K.D., Marshall, J.M., Huang, H., Ward, C.M., Hay, B. a., 2013. A synthetic gene drive system for local, reversible modification and suppression of insect

populations. Curr. Biol. 23, 671–677. doi:10.1016/j.cub.2013.02.059

Allison, S.L., Schalich, J., Stiasny, K., Mandl, C.W., Kunz, C., Heinz, F.X., 1995. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. J. Virol. 69, 695–700.

Allison, S.L., Tao, Y.J., Riordain, G.O., Mandl, C.W., Harrison, S.C., Heinz, F.X., Al, A.E.T., 2003. Two distinct size classes of immature and mature subviral particles from tick-borne encephalitis virus. J. Virol. 77, 11357–11366. doi:10.1128/JVI.77.21.11357

Alphey, L., Andreasen, M., 2002. Dominant lethality and insect population control. Mol. Biochem. Parasitol. 121, 173–178. doi:10.1016/S0166-6851(02)00040-3

Alphey, L., McKemey, A., Nimmo, D., Neira Oviedo, M., Lacroix, R., Matzen, K., Beech, C., 2013. Genetic control of Aedes mosquitoes. Pathog. Glob. Health 107, 170–179. doi:10.1179/2047773213Y.0000000095

Alto, B.W., Lounibos, L.P., Higgs, S., Juliano, S. a, 2005. Larval competition differentially affects arbovirus infection in Aedes mosquitoes. Ecology 86, 3279–3288.

Alto, B.W., Lounibos, L.P., Mores, C.N., Reiskind, M.H., 2008. Larval competition alters susceptibility of adult Aedes mosquitoes to dengue infection. Proc. R. Soc. B Biol. Sci. 275, 463–71. doi:10.1098/rspb.2007.1497

Alvarez, D.E., De Lella Ezcurra, A.L., Fucito, S., Gamarnik, A. V, 2005. Role of RNA structures present at the 3'UTR of dengue virus on translation, RNA synthesis, and viral replication. Virology 339, 200–12. doi:10.1016/j.virol.2005.06.009

Amenya, D.A., Bonizzoni, M., Isaacs, A.T., Jasinskiene, N., Chen, H., Marinotti, O., Yan, G., James, A.A., 2010. Comparative fitness assessment of Anopheles stephensi transgenic lines receptive to site-specific integration. Insect Mol. Biol. 19, 263–269. doi:10.1111/j.1365-

2583.2009.00986.x

Anderson, J.R., Rico-Hesse, R., 2006. Aedes aegypti vectorial capacity is determined by the infecting genotype of dengue virus. Am. J. Trop. Med. Hyg. 75, 886.

Anderson, S.L., Richards, S.L., Tabachnick, W.J., Smartt, C.T., 2010. Effects of West Nile virus dose and extrinsic incubation temperature on temporal progression of vector competence in Culex pipiens quinquefasciatus. J. Am. Mosq. Control Assoc. 26, 103–7.

Angelica, M.D., Fong, Y., 2008. Argonautes confront new small RNAs. Curr Opin Chem Biol. 141, 520–529. doi:10.1016/j.surg.2006.10.010.Use

Aravin, A. A, Hannon, G.J., Brennecke, J., 2007. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science. 318, 761–4.doi:10.1126/science.1146484

Armstrong, P.M., Rico-Hesse, R., 2001. Differential susceptibility of Aedes aegypti to infection by the American and southeast Asian genotypes of dengue type 2 virus. Vector borne zoonotic Dis. 1, 159–168. doi:10.1016/j.bbi.2008.05.010

Asgari, S., 2015. Regulatory role of cellular and viral microRNAs in insect–virus interactions. Curr. Opin. Insect Sci. 8, 104–110. doi:10.1016/j.cois.2014.12.008

Ashburn, P., Craig, C., 1907. Experimental investigations regarding the etiology of dengue fever. J. Infect. Dis. 4, 440.

Assadian, O., Stanek, G., 2002. Theobald Smith-the discoverer of ticks as vectors of disease. Wien. Klin. Wochenschr. 114, 479–81.

Avadhanula, V., Weasner, B., Hardy, G., 2009. A novel system for the launch of alphavirus RNA synthesis reveals a role for the Imd pathway in arthropod antiviral response. PLoS Pathog. 5, e1000582. doi:10.1371/journal.ppat.1000582

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., Pasquinelli, A.E., 2005.

Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 122, 553–63. doi:10.1016/j.cell.2005.07.031

Bancroft, T., 1906. On the etiology of dengue fever. Australas. Med. Gaz. 25, 17–18.

Bargielowski, I., Nimmo, D., Alphey, L., Koella, J.C., 2011. Comparison of life history characteristics of the genetically modified OX513A line and a wild type strain of Aedes aegypti. PLoS One 6, 1–7. doi:10.1371/journal.pone.0020699

Bass, B.L., 2000. Double-stranded RNA as a template for gene silencing. Cell 101, 235–8.

Bates, M., Roca-Garcia, M., 1946. The development of the virus of yellow fever in Haemagogus mosquitoes. Am. J. Trop. Med. Hyg. 26, 585–605.

Beaty, B.J., 2000. Genetic manipulation of vectors: A potential novel approach for control of vector-borne diseases. Proc. Natl. Acad. Sci. U. S. A. 97, 10295–10297.

Beeman, R., Friesen, K., 1999. Properties and natural occurrence of maternal-effect selfish genes (’Medea' factors) in the red flour beetle, Tribolium castaneum. Heredity. 82, 529–534.

Beeman, R.W., Friesen, K.S., Denell, R.E., 1992. Maternal-effect selfish genes in flour beetles. Science. 256, 89–92.

Bennett, K., Beaty, B., Black, W.C., 2005. Selection of D2S3, an Aedes aegypti (Diptera: Culicidae) strain with high oral susceptibility to dengue 2 virus and D2MEB, a strain with a midgut barrier to dengue 2. J. Med. Entomol. 42, 110–119. doi:10.1603/0022-2585(2005)042

Bennett, K.E., Olson, K.E., Muñoz, M.D.L., Fernandez-Salas, I., Farfan-Ale, J. a, Higgs, S., Black, W.C., Beaty, B.J., 2002. Variation in vector competence for dengue 2 virus among 24 collections of Aedes aegypti from Mexico and the United States. Am. J. Trop. Med. Hyg. 67, 85–92.

Bernhardt, S. a, Simmons, M.P., Olson, K.E., Beaty, B.J., Blair, C.D., Black, W.C., 2012. Rapid intraspecific evolution of miRNA and siRNA genes in the mosquito Aedes aegypti. PLoS One 7, e44198. doi:10.1371/journal.pone.0044198

Bernstein, E., Caudy, a a, Hammond, S.M., Hannon, G.J., 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409, 363–6. doi:10.1038/35053110

Berry, B., Deddouche, S., Kirschner, D., Imler, J.-L., Antoniewski, C., 2009. Viral suppressors of RNA silencing hinder exogenous and endogenous small RNA pathways in Drosophila. PLoS One 4, e5866. doi:10.1371/journal.pone.0005866

Best, S.M., 2008. Viral subversion of apoptotic enzymes: escape from death row. Annu. Rev. Microbiol. 62, 171–92. doi:10.1146/annurev.micro.62.081307.163009

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Myers, M.F., George, D.B., Jaenisch, T., William, G.R., 2013. The global distribution and burden of dengue 496, 504–507. doi:10.1038/nature12060.The

Bidla, G., Dushay, M.S., Theopold, U., 2007. Crystal cell rupture after injury in Drosophila requires the JNK pathway, small GTPases and the TNF homolog Eiger. J. Cell Sci. 120, 1209–15. doi:10.1242/jcs.03420

Bielefeldt-Ohmann, H., Meyer, M., Fitzpatrick, D.R., Mackenzie, J.S., 2001. Dengue virus binding to human leukocyte cell lines: receptor usage differs between cell types and virus strains. Virus Res. 73, 81–9.

Black, W.C., Bennett, K.E., Gorrochótegui-escalante, N., Barillas-Mury, C. V, Fernández-salas, I., de Lourdes Muñoz, M., Farfán-alé, J. a, Olson, K.E., Beaty, B.J., 2002. Flavivirus

susceptibility in Aedes aegypti. Arch. Med. Res. 33, 379–88.

Black, W.C., DuTeau, N.M., 1997. RAPD-PCR and SSCP analysis for insect population genetic studies., in: Crammpton J, Beard, C., Louis, C. (Eds.), The Molecular Biology of Insect Disease Vectors: A Methods Manual. Chapman and Hall, New York, pp. 361–373.

Blair, C.D., 2011. Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission. Future Microbiol. 6, 265–77. doi:10.2217/fmb.11.11

Blair, C.D., Olson, K.E., 2014. Mosquito immune responses to arbovirus infections. Curr. Opin. Insect Sci. 3, 22–29. doi:10.1016/j.cois.2014.07.005.Mosquito

Blanc, G., Caminopetros, J., 1929. Duration of conservation of the virus of dengue in Stegomyas. Influence of the cold season on the infecting power. Comptes Rendus Hebdomardaires des Seances l'Academie des Sci. 188, 1273–1275.

Boisson, B., Jacques, J.C., Choumet, V., Martin, E., Xu, J., Vernick, K., Bourgouin, C., 2006. Gene silencing in mosquito salivary glands by RNAi. FEBS Lett. 580, 1988–1992. doi:10.1016/j.febslet.2006.02.069

Bonizzoni, M., Dunn, W.A., Campbell, C.L., Olson, K.E., Dimon, M.T., Marinotti, O., James, A.A., 2011. RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, Aedes aegypti. BMC Genomics 12, 82. doi:10.1186/1471-2164-12-82

Bonizzoni, M., Dunn, W.A., Campbell, C.L., Olson, K.E., Marinotti, O., James, A. a, 2012a. Strain variation in the transcriptome of the dengue fever vector, Aedes aegypti. G3 (Bethesda). 2, 103–14. doi:10.1534/g3.111.001107

Bonizzoni, M., Dunn, W.A., Campbell, C.L., Olson, K.E., Marinotti, O., James, A. a., 2012b. Complex modulation of the Aedes aegypti transcriptome in response to dengue virus

infection. PLoS One 7, e50512. doi:10.1371/journal.pone.0050512

Boromisa, R., Rai, K., Grimstad, P., 1987. Variation in the vector competence of geographic strains of Aedes albopictus for dengue 1 virus. J. Am. Mosq. Control Assoc. 3, 378.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., Ziegler-Graff, V., 2007. The Polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Curr. Biol. 17, 1615–21. doi:10.1016/j.cub.2007.07.061

Bosio, C.F., Beaty, B.J., Black, W.C., 1998. Quantitative genetics of vector competence for Dengue-2 virus in Aedes aegypti. Am. J. Trop. Med. Hyg. 59, 965–970.

Bosio, C.F.F., Fulton, R.E.E., Salasek, M.L.L., Beaty, B.J.J., Black, W.C.C., 2000. Quantitative trait loci that control vector competence for dengue-2 virus in the mosquito Aedes aegypti. Genetics 156, 687-698.

Bouyer, J., Lefrançois, T., 2014. Boosting the sterile insect technique to control mosquitoes. Trends Parasitol. 30, 271–273. doi:10.1016/j.pt.2014.04.002

Bowers, D.F., Coleman, C.G., Brown, D.T., 2003. Sindbis virus-associated pathology in Aedes albopictus (Diptera: Culicidae). J. Med. Entomol. 40, 698–705. doi:10.1603/0022-2585-40.5.698

Brackney, D.E., Foy, B.D., Olson, K.E., 2008. The effects of midgut serine proteases on dengue virus type 2 infectivity of Aedes aegypti. Am. J. Trop. Med. Hyg. 79, 267–274.

Brackney, D.E., Scott, J.C., Sagawa, F., Woodward, J.E., Miller, N.A., Schilkey, F.D., Mudge, J., Wilusz, J., Olson, K.E., Blair, C.D., Ebel, G.D., 2010. C6/36 Aedes albopictus cells have a dysfunctional antiviral RNA interference response. PLoS Negl. Trop. Dis. 4, e856. doi:10.1371/journal.pntd.0000856

Brandt, S.M., Dionne, M.S., Khush, R.S., Pham, L.N., Vigdal, T.J., Schneider, D.S., 2004.

Secreted bacterial effectors and host-produced Eiger/TNF drive death in a Salmonella-infected fruit fly. PLoS Biol. 2, e418. doi:10.1371/journal.pbio.0020418

Bryant, B., Blair, C., Olson, K., 2008. Annotation and expression profiling of apoptosis-related genes in the yellow fever mosquito, Aedes aegypti. Insect Biochem. Mol. Biol. 38, 331–345.

Burt, A., 2003. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc. R. Soc. Biol. Sci. 270, 921–928. doi:10.1098/rspb.2002.2319

Campbell, C.L., Keene, K.M., Brackney, D.E., Olson, K.E., Blair, C.D., Wilusz, J., Foy, B.D., 2008. Aedes aegypti uses RNA interference in defense against Sindbis virus infection. BMC Microbiol. 8, 47. doi:10.1186/1471-2180-8-47

Carabali, M., Hernandez, L.M., Arauz, M.J., Villar, L.A., Ridde, V., 2015. Why are people with dengue dying? A scoping review of determinants for dengue mortality. BMC Infect. Dis. 15, 301. doi:10.1186/s12879-015-1058-x

Carter, J.R., Keith, J.H., Fraser, T.S., Dawson, J.L., Kucharski, C. a, Horne, K.M., Higgs, S., Fraser, M.J., 2014. Effective suppression of dengue virus using a novel group-I intron that induces apoptotic cell death upon infection through conditional expression of the Bax C-terminal domain. Virol. J. 11, 111. doi:10.1186/1743-422X-11-111

Carter, V., Hurd, H., 2010. Choosing anti-Plasmodium molecules for genetically modifying mosquitoes: focus on peptides. Trends Parasitol. 26, 582–590.

Carvalho-Leandro, D., Ayres, C.F.J., Guedes, D.R.D., Suesdek, L., Melo-Santos, M.A. V, Oliveira, C.F., Cordeiro, M.T., Regis, L.N., Marques, E.T., Gil, L.H., Magalhaes, T., 2012. Immune transcript variations among Aedes aegypti populations with distinct susceptibility to dengue virus serotype 2. Acta Trop. 124, 113–119. doi:10.1016/j.actatropica.2012.07.006

Castillo, J., Robertson, A., Strand, M., 2006. Characterization of hemocytes from the mosquitoes

Anopheles gambiae and Aedes aegypti. Insect Biochem. Mol. Biol. 36, 891–903. doi:10.1016/j.ibmb.2006.08.010.Characterization

Catteruccia, F., Godfray, H.C.J., Crisanti, A., 2003. Impact of genetic manipulation on the fitness of Anopheles stephensi mosquitoes. Science. 299, 1225–1227. doi:10.1126/science.1081453

Caudy, A. a, Myers, M., Hannon, G.J., Hammond, S.M., 2002. Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev. 16, 2491–6. doi:10.1101/gad.1025202

Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M.P., Tops, B.B.J., Silva, J.M., Myers, M.M., Hannon, G.J., Plasterk, R.H.A., 2003. A micrococcal nuclease homologue in RNAi effector complexes. Nature 425, 411–414. doi:10.1038/nature01956

CDC, 2010. International catalog of arboviruses including certain other viruses of vertebrates.

Chamberlain, R.W., Sudia, W., 1961. Mechanism of transmission of viruses by mosquitoes. Annu. Rev. Entomol. 6, 371–390. doi:10.1146/annurev.en.06.010161.002103

Chamberlain, R.W., Sudia, W.D., 1955. The effects of temperature upon the extrinsic incubation of eastern equine encephalitis in mosquitoes. Am. J. Hyg. 62, 295–305.

Chambers, T.J., Hahn, C.S., Galler, R., M., R.C., 1990. Flavivirus genome organization, expression and replication. Annu. Rev. Microbiol. 44, 649–688.

Chambers, T.J., Mccourt, D.W., Rice, C.M., 1989. Yellow fever virus proteins NS2A, NS2B, and NS4B: identification and partial N-terminal amino acid sequence analysis. Virology 169, 100–109.

Chan, M., Johansson, M. a., 2012. The incubation periods of dengue viruses. PLoS One 7, 1–7. doi:10.1371/journal.pone.0050972

Chao, J. a, Lee, J.H., Chapados, B.R., Debler, E.W., Schneemann, A., Williamson, J.R., 2005.

Dual modes of RNA-silencing suppression by flock house virus protein B2. Nat. Struct. Mol. Biol. 12, 952–957. doi:10.1038/nsmb1005

Chapman, E.G., Costantino, D. a, Rabe, J.L., Moon, S.L., Wilusz, J., Nix, J.C., Kieft, J.S., 2014. The structural basis of pathogenic subgenomic flavivirus RNA (sfRNA) production. Science 344, 307–310. doi:10.1126/science.1250897

Chotkowski, H.L., Ciota, A.T., Jia, Y., Puig-Basagoiti, F., Kramer, L.D., Shi, P.-Y., Glaser, R.L., 2008. West Nile virus infection of Drosophila melanogaster induces a protective RNAi response. Virology 377, 197–206. doi:10.1016/j.virol.2008.04.021

Cirimotich, C.M., Scott, J.C., Phillips, A.T., Geiss, B.J., Olson, K.E., 2009. Suppression of RNA interference increases alphavirus replication and virus-associated mortality in Aedes aegypti mosquitoes. BMC Microbiol. 9, 49.

Clements, A.N., 1999. The biology of mosquitoes. Volume 2: sensory reception and behaviour. CABI Publishing, NY

Clements, A.N., 1992. The biology of mosquitoes Volume 1: development nutrition and reproduction. CABI Publishing, NY

Clyde, K., Harris, E., 2006. RNA secondary structure in the coding region of dengue virus type 2 directs translation start codon selection and is required for viral replication RNA secondary structure in the coding region of dengue virus. J. Virol. 80, 2170–2182. doi:10.1128/JVI.80.5.2170

Cooper, D., Thi, E., Chamberlain, C., 2007. Aedes Dronc: a novel ecdysone-inducible caspase in the yellow fever mosquito, Aedes aegypti. Insect Mol. Biol. 16, 563–572.

Cordes, E.J., Licking-Murray, K.D., Carlson, K.A., 2014. Differential gene expression related to Nora virus infection of Drosophila melanogaster. Virus Res. 175, 95–100.

doi:10.1016/j.virusres.2013.03.021.Differential

Costa, A., Jan, E., Sarnow, P., Schneider, D., 2009. The IMD pathway is involved in antiviral immune responses in Drosophila. PLoS One 4, e7436. doi:10.1371/journal.pone.0007436

Cui, T., Sugrue, R.J., Xu, Q., Lee, a K., Chan, Y.C., Fu, J., 1998. Recombinant dengue virus type 1 NS3 protein exhibits specific viral RNA binding and NTPase activity regulated by the NS5 protein. Virology 246, 409–417. doi:10.1006/viro.1998.9213

Curtis, C.F., 2006. Models to investigate some issues regarding the feasibility of driving refractoriness genes into mosquito vector populations, in: Knols, B.G.J., Louis, C. (Eds.), Bridging Laboratory and Field Research for Genetic Control of Disease Vectors. pp. 199–202.

Curtis, C.F., Graves, P., 1988. Methods for replacement of malaria vector populations. J. Trop. Med. Hyg. 91, 43–48.

Curtis, C.F., Grover, K.K., Suguna, S.G., Uppal, D.K., Dietz, K., Agarwal, H. V, Kazmi, S.J., 1976. Comparative field cage tests of the population suppressing efficiency of three genetic control systems for Aedes aegypti. Heredity. 36, 11–29.

Davis, S., Bax, N., Grewe, P., 2001. Engineered underdominance allows efficient and economical introgression of traits into pest populations. J. Theor. Biol. 212, 83–98. doi:10.1006/jtbi.2001.2357

de Figueiredo, M.L.G., de C Gomes, A., Amarilla, A. a, de S Leandro, A., de S Orrico, A., de Araujo, R.F., do S M Castro, J., Durigon, E.L., Aquino, V.H., Figueiredo, L.T.M., 2010. Mosquitoes infected with dengue viruses in Brazil. Virol. J. 7, 152. doi:10.1186/1743-422X-7-152

de Lara Capurro, M., Coleman, J., Beerntsen, B.T., Myles, K.M., Olson, K.E., Rocha, E., Krettli,

A.U., James, A.A., 2000. Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in Plasmodium gallinaceum-infected Aedes aegypti. Am. J. Trop. Med. Hyg. 62, 427–33.

de Valdez, M., Nimmo, D., Betz, J., Wise de Valdez, M.R., Nimmo, D., Betz, J., Gong, H.-F., James, A. a, Alphey, L., Black, W.C., 2011. Genetic elimination of dengue vector mosquitoes. Proc. Natl. Acad. Sci. U. S. A. 108, 4772–4775. doi:10.1073/pnas.1019295108

Delaney, J.R., Stöven, S., Uvell, H., Anderson, K. V, Engström, Y., Mlodzik, M., 2006. Cooperative control of Drosophila immune responses by the JNK and NF-kappaB signaling pathways. EMBO J. 25, 3068–3077. doi:10.1038/sj.emboj.7601182

den Boon, J.A. Den, Ahlquist, P., 2010. Organelle-like membrane compartmentalization of positive-strand RNA virus replication factories. Annu. Rev. Microbiol. 64, 241–256. doi:10.1146/annurev.micro.112408.134012

Deus, A.K.M., Butters, M.P., Black, W.C., Foy, B.D., 2012. The effect of ivermectin in seven strains of Aedes aegypti (Diptera : Culicidae) including a genetically diverse laboratory strain and three permethrin resistant strains. J Med Entomol. 49, 356-363.

Diallo, M., Ba, Y., Sall, A.A., Diop, O.M., Ndione, J.A., Mondo, M., Girault, L., Mathiot, C., 2003. Amplification of the sylvatic cycle of dengue virus type 2, Senegal, 1999-2000: entomologic findings and epidemiologic considerations. Emerg. Infect. Dis. 9, 362–367.

Dickson, L.B., Sanchez-Vargas, I., Sylla, M., Fleming, K., Black, W.C., 2014. Vector competence in West African Aedes aegypti is flavivirus species and genotype dependent. PLoS Negl. Trop. Dis. 8, e3153. doi:10.1371/journal.pntd.0003153

Ding, S.-W., Voinnet, O., 2007. Antiviral immunity directed by small RNAs. Cell 130, 413–26. doi:10.1016/j.cell.2007.07.039

Dong, Y., Taylor, H.E., Dimopoulos, G., 2006. AgDscam , a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system. PLoS Biol. 4, e229. doi:10.1371/journal.pbio.0040229

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J.A., Imler, J.-L., 2005. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of Drosophila. Nat. Immunol. 6, 946–953. doi:10.1038/ni1237

Du, T., Zamore, P.D., 2005. microPrimer: the biogenesis and function of microRNA. Development 132, 4645–4652. doi:10.1242/dev.02070

Dye, C., 1992. The analysis of parasite transmission by bloodsucking insects. Annu. Rev. Entomol. 37, 1–19. doi:10.1146/annurev.ento.37.1.1

Epstein, P.R., 2001. West Nile virus and the climate. J. urban Heal. 78, 367–71. doi:10.1093/jurban/78.2.367

Etymologia of dengue, 2006. . Emerg. Infect. Dis. 12, 893.

Ferreira, lvaro G., Naylor, H., Esteves, S.S., Pais, I.S., Martins, N.E., Teixeira, L., 2014. The Toll-dorsal pathway is required for resistance to viral oral infection in Drosophila. PLoS Pathog. 10, e1004507. doi:10.1371/journal.ppat.1004507

Filipe, S.R., Tomasz, A., Ligoxygakis, P., 2005. Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway. EMBO Rep. 6, 327–33. doi:10.1038/sj.embor.7400371

Fisher, R. a, 1950. Gene frequencies in a cline determined by selection and diffusion. Biometrics 6, 353–361. doi:10.2307/3001780

Förstemann, K., Horwich, M.D., Wee, L., Tomari, Y., Zamore, P.D., 2007. Drosophila microRNAs are sorted into functionally distinct argonaute complexes after production by

dicer-1. Cell 130, 287–297. doi:10.1016/j.cell.2007.05.056

Franz, Sanchez-Vargas, I., Adelman, Z.N.Z., Blair, C.D., Beaty, B.J., James, A.A., Olson, K.E., 2006. Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified Aedes aegypti. Proc. Natl. Acad. Sci. U. S. A. 103, 4198–4203. doi:10.1073/pnas.0600479103

Franz, a W.E., Sanchez-Vargas, I., Piper, J., Smith, M.R., Khoo, C.C.H., James, a a, Olson, K.E., 2009. Stability and loss of a virus resistance phenotype over time in transgenic mosquitoes harbouring an antiviral effector gene. Insect Mol. Biol. 18, 661–672. doi:10.1111/j.1365-2583.2009.00908.x

Franz, A., Kantor, A., Passarelli, A., Clem, R., 2015. Tissue barriers to arbovirus infection in mosquitoes. Viruses 7, 3741–3767. doi:10.3390/v7072795

Franz, A., Sanchez-Vargas, I., Adelman, Z., 2006. Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified Aedes aegypti. Proc. Natl. Acad. Sci. U. S. A. 103, 4198–4203.

Franz, A.W.E., Jasinskiene, N., Sanchez-vargas, I., Isaacs, A.T., Smith, M.R., Khoo, C.C.H., Heersink, M.S., James, A. a, Ken, E., 2011. Comparison of transgene expression in Aedes aegypti generated by mariner Mos1 transposition and phiC31 site-directed recombination. Insect Mol. Biol. 20, 587–598. doi:10.1111/j.1365-2583.2011.01089.x.Comparison

Franz, A.W.E., Sanchez-Vargas, I., Raban, R.R., Black IV, W.C., James, A. a., Olson, K.E., 2014. Fitness impact and stability of a transgene conferring resistance to dengue-2 virus following introgression into a genetically diverse Aedes aegypti strain. PLoS Negl. Trop. Dis. 8, e2833. doi:10.1371/journal.pntd.0002833

Frentiu, F.D., Zakir, T., Walker, T., Popovici, J., Pyke, A.T., van den Hurk, A., McGraw, E.A.,

O'Neill, S.L., 2014. Limited dengue virus replication in field-collected Aedes aegypti mosquitoes infected with Wolbachia. PLoS Negl. Trop. Dis. 8, e2688. doi:10.1371/journal.pntd.0002688

Galiana-Arnoux, D., Dostert, C., Schneemann, A., Hoffmann, J. a, Imler, J.-L., 2006. Essential function in vivo for dicer-2 in host defense against RNA viruses in Drosophila. Nat. Immunol. 7, 590–597. doi:10.1038/ni1335

Gammon, D.B., Mello, C.C., 2015. RNA interference-mediated antiviral defense in insects. Curr. Opin. Insect Sci. 8, 111–120. doi:10.1016/j.cois.2015.01.006

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M., Bier, E., James, A. a., 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. Proc. Natl. Acad. Sci. E6736-E6743. doi:10.1073/pnas.1521077112

Garret-Jones, C., 1964. The human blood index of malaria vectors in relation to epidemiological assessment. Bull. World Health Organ. 30, 241–261.

Gesellchen, V., Kuttenkeuler, D., Steckel, M., Pelte, N., Boutros, M., 2005. An RNA interference screen identifies inhibitor of apoptosis protein 2 as a regulator of innate immune signalling in Drosophila. EMBO Rep. 6, 979–984. doi:10.1038/sj.embor.7400530

Ghildiyal, M., Seitz, H., Horwich, M.D., Li, C., Du, T., Lee, S., Xu, J., Kittler, E.L.W., Zapp, M.L., Weng, Z., Zamore, P.D., Phillip, D., 2008. Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells. Science. 320, 1077–1081. doi:10.1126/science.1157396

Girard, Y.A., Popov, V., Wen, J., Han, V., Higgs, S., 2005. Ultrastructural study of West Nile virus pathogenesis in Culex pipiens quinquefasciatus (Diptera: Culicidae). J. Med. Entomol.

42, 429–44.

Girard, Y.A., Schneider, B.S., McGee, C.E., Wen, J., Han, V.C., Popov, V., Mason, P.W., Higgs, S., 2007. Salivary gland morphology and virus transmission during long-term cytopathologic West Nile virus infection in Culex mosquitoes. Am. J. Trop. Med. Hyg. 76, 118–28.

Goddard, M.R., Greig, D., Burt, A., 2001. Outcrossed sex allows a selfish gene to invade yeast populations. Proc. R. Soc. B Biol. Sci. 268, 2537–2542. doi:10.1098/rspb.2001.1830

Gomez-Machorro, C., Bennett, K.E., del Lourdes Munoz, M., Black, W.C., 2004. Quantitative trait loci affecting dengue midgut infection barriers in an advanced intercross line of Aedes aegypti. Insect Mol. Biol. 13, 637–648. doi:10.1111/j.0962-1075.2004.00522.x

Gorrochotegui-Escalante, N., Lozano-Fuentes, S., Bennett, K.E., Molina-Cruz, A., Beaty, B.J., Blackiv, W.C., 2005. Association mapping of segregating sites in the early trypsin gene and susceptibility to dengue-2 virus in the mosquito Aedes aegypti. Insect Biochem. Mol. Biol. 35, 771–788. doi:10.1016/j.ibmb.2005.02.015

Gottar, M., Gobert, V., Matskevich, A.A., Reichhart, J.-M., Wang, C., Butt, T.M., Belvin, M., Hoffmann, J.A., Ferrandon, D., 2006. Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. Cell 127, 1425-1437. doi:10.1016/j.cell.2006.10.046

Gould, F., Huang, Y., Legros, M., Lloyd, A.L., 2008. A killer-rescue system for self-limiting gene drive of anti-pathogen constructs. Proc. Biol. Sci. 275, 2823–2829. doi:10.1098/rspb.2008.0846

Graham, H., 1903. The dengue: a study of its pathology and mode of propagation. J. Trop. Med. 6, 209–214.

Gratz, N.G., 2004. Critical review of the vector status of Aedes albopictus. Med. Vet. Entomol. 18, 215–27. doi:10.1111/j.0269-283X.2004.00513.x

Gravot, E., Thomas-Orillard, M., Jeune, B., 2000. Virulence variability of the Drosophila C virus and effects of the microparasite on demographic parameters of the host (Drosophila melanogaster). J. Invertebr. Pathol. 75, 144–151. doi:10.1006/jipa.1999.4913

Grimstad, P.R., Haramis, L.D., 1984. Aedes triseriatus (Diptera: Culicidae) and La Crosse virus. III. Enhanced oral transmission by nutrition-deprived mosquitoes. J. Med. Entomol. 21, 249–56.

Grimstad, P.R., Paulson, S.L., Craig, G.B., 1985. Vector competence of Aedes hendersoni (Diptera: Culicidae) for La Crosse virus and evidence of a salivary-gland escape barrier. J. Med. Entomol. 22, 447–53.

Grimstad, P.R., Walker, E.D., 1991. Aedes triseriatus (Diptera: Culicidae) and La Crosse virus. IV. Nutritional deprivation of larvae affects the adult barriers to infection and transmission. J. Med. Entomol. 28, 378–86.

Gubler, D., 1995. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg. Infect. Dis. 1, 55–57. doi:10.3201/eid0102.950204

Gubler, D.J., 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. 11, 480–496.

Gubler, D.J., Clark, G.G., 1996. Community involvement in the control of Aedes aegypti. Acta Trop. 61, 169–179.

Gubler, D.J., Nalim, S., Tan, R., Saipan, H., Sulianti Saroso, J., 1979. Variation in susceptibility to oral infection with dengue viruses among geographic strains of Aedes aegypti. Am. J. Trop. Med. Hyg. 28, 1045–1052.

Gubler, D.J., Reiter, P., Ebi, K.L., Yap, W., Nasci, R., Patz, J. a, 2001. Climate variability and

change in the United States: potential impacts on vector- and rodent-borne diseases. Environ. Health Perspect. 109 Suppl, 223–233.

Gubler, D.J., Rosen, A., Rosen, L., 1976. Variation among geographic strains of Aedes albopictus in susceptibility to infection with dengue viruses. Am. J. Trop. Med. Hyg. 25, 318–325.

Gubler, D.J., Rosen, L., 1976. Variation among geographic strains of Aedes albopictus in susceptibility to infection with dengue viruses. Am. J. Trop. Med. Hyg. 25, 318–325.

Gubler, D.J., Suharyono, W., Tan, R., Abidin, M., Sie, A., 1981. Viraemia in patients with naturally acquired dengue infection. Bull. World Health Organ. 59, 623–630.

Guirakhoo, F., Bolin, R. a, Roehrig, J.T., 1992. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. Virology 191, 921–931.

Halstead, S.B., 1988. Pathogenesis of dengue: challenges to molecular biology. Science (80-. ). 239, 476–481.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. Paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 9–18. doi:10.1016/j.bcp.2008.05.025

Hammond, S.M., 2005. Dicing and slicing: the core machinery of the RNA interference pathway. FEBS Lett. 579, 5822–5829. doi:10.1016/j.febslet.2005.08.079

Han, Y.S., Thompson, J., Kafatos, F.C., Barillas-Mury, C., 2000. Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes. EMBO J. 19, 6030–6040. doi:10.1093/emboj/19.22.6030

Hancock, P. a, Sinkins, S.P., Godfray, H.C.J., 2011. Population dynamic models of the spread of Wolbachia. Am. Nat. 177, 323–333. doi:10.1086/658121

Hapairai, L.K., Marie, J., Sinkins, S.P., Bossin, H.C., 2014. Effect of temperature and larval density on Aedes polynesiensis (Diptera: Culicidae) laboratory rearing productivity and male characteristics. Acta Trop. 132, S108–S115. doi:10.1016/j.actatropica.2013.11.024

Hardy, J.L., Houk, E.J., Kramer, L.D., Reeves, W.C., 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annu. Rev. Entomol. 28, 229–262. doi:10.1146/annurev.en.28.010183.001305

Hardy, J.L., Reeves, W., 1990. Experimental studies of infection in vectors, in: Epidemiology and Control of Mosquito-Borne Arboviruses in California. Californian Mosquito Vector Control Association, Sacramento, pp. 145–253.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S. a, Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I., Collado, A., Stevenson, J., Scaife, S., Dafa'alla, T., Fu, G., Phillips, C., Miles, A., Raduan, N., Kelly, N., Beech, C., Donnelly, C. a, Petrie, W.D., Alphey, L., 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nat. Biotechnol. 30, 828–830. doi:10.1038/nbt.2350

Heinz, F.X., 1986. Epitope mapping of flavivirus glycoproteins. Adv. Virus Res. 31, 103–168.

Heinz, F.X., Stiasny, K., Puschener-Auer, G., Holzmann, H., Allison, S.L., Mandl, C.W., Kunz, C., 1994. Structual changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. Virology 198, 109–117.

Henchal, E. a, Putnak, J.R., 1990. The dengue viruses. Clin. Microbiol. Rev. 3, 376–96. Herrera-Ortíz, A., Lanz-Mendoza, H., Martínez-Barnetche, J., Hernández-Martínez, S., Villarreal-Treviño, C., Aguilar-Marcelino, L., Rodríguez, M.H., 2004. Plasmodium berghei ookinetes induce nitric oxide production in Anopheles pseudopunctipennis midguts cultured in vitro. Insect Biochem. Mol. Biol. 34, 893–901. doi:10.1016/j.ibmb.2004.05.007

Hess, A.M., Prasad, A.N., Ptitsyn, A., Ebel, G.D., Olson, K.E., Barbacioru, C., Monighetti, C., Campbell, C.L., 2011. Small RNA profiling of dengue virus-mosquito interactions implicates the PIWI RNA pathway in anti-viral defense. BMC Microbiol. 11, 45. doi:10.1186/1471-2180-11-45

Hickey, W.A., Craig, G.B., 1966. Genetic distortion of sex ratio in a mosquito, Aedes aegypti. Genetics 53, 1177–1196.

Higgs, S., Schneider, B.S., Vanlandingham, D.L., Klingler, K. a, Gould, E. a, 2005. Nonviremic transmission of West Nile virus. Proc. Natl. Acad. Sci. U. S. A. 102, 8871–8874. doi:10.1073/pnas.0503835102

Höck, J., Meister, G., 2008. The Argonaute protein family. Genome Biol. 9, e120. doi:10.1186/gb-2008-9-2-210

Hoffmann, J. a, 2003. The immune response of Drosophila. Nature 426, 33–38. doi:10.1038/nature02021

Hoffmann, J. a, Reichhart, J.-M., 2002. Drosophila innate immunity: an evolutionary perspective. Nat. Immunol. 3, 121–126. doi:10.1038/ni0202-121

Horng, T., Medzhitov, R., 2001. Drosophila MyD88 is an adapter in the Toll signaling pathway. Proc. Natl. Acad. Sci. U. S. A. 98, 12654–12658. doi:10.1073/pnas.231471798

Houk, E.J., 1977. Midgut ultrastructure of Culex tarsalis (Diptera: Culcidae) before and after a bloodmeal. Tissue Cell 9, 103–118.

Huang, Z., Kingsolver, M.B., Avadhanula, V., Hardy, R.W., 2013. An antiviral role for antimicrobial peptides during the arthropod response to alphavirus replication. J. Virol. 87, 4272–4280. doi:10.1128/JVI.03360-12

Hung, S.L., Lee, P.L., Chen, H.W., Chen, L.K., Kao, C.L., King, C.C., 1999. Analysis of the

steps involved in dengue virus entry into host cells. Virology 257, 156–167. doi:10.1006/viro.1999.9633

Hussain, M., Asgari, S., 2014. MicroRNA-like viral small RNA from dengue virus 2 autoregulates its replication in mosquito cells. Proc. Natl. Acad. Sci. U. S. A. 111, 2746–27451. doi:10.1073/pnas.1320123111

Irvin, N., Hoddle, M.S.M., O'Brochta, D. A, Carey, B., Atkinson, P.W., 2004. Assessing fitness costs for transgenic Aedes aegypti expressing the GFP marker and transposase genes. Proc. Natl. Acad. Sci. U. S. A. 101, 891–896. doi:10.1073/pnas.0305511101

Ishizuka, A., Siomi, M.C., Siomi, H., 2002. A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev. 16, 2497–508. doi:10.1101/gad.1022002

Ito, J., Ghosh, A., Moreira, L., 2002. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. Nature 417, 452–455.

Ito, J., Ghosh, A., Moreira, L.L.A., Wimmer, E.A., Jacobs-Lorena, M., 2002. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. Nature 417, 452–455. doi:10.1038/417452a

Iwasaki, S., Kawamata, T., Tomari, Y., 2009. Drosophila argonaute1 and argonaute2 employ distinct mechanisms for translational repression. Mol. Cell 34, 58–67. doi:10.1016/j.molcel.2009.02.010

James, A. a, 2005. Gene drive systems in mosquitoes: rules of the road. Trends Parasitol. 21, 64–7. doi:10.1016/j.pt.2004.11.004

Jang, I.-H., Chosa, N., Kim, S.-H., Nam, H.-J., Lemaitre, B., Ochiai, M., Kambris, Z., Brun, S., Hashimoto, C., Ashida, M., Brey, P.T., Lee, W.-J., 2006. A Spätzle-processing enzyme

required for toll signaling activation in Drosophila innate immunity. Dev. Cell 10, 45–55. doi:10.1016/j.devcel.2005.11.013

Jin, P., Zarnescu, D.C., Ceman, S., Nakamoto, M., Mowrey, J., Jongens, T. a, Nelson, D.L., Moses, K., Warren, S.T., 2004. Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. Nat. Neurosci. 7, 113–117. doi:10.1038/nn1174

Kaneko, T., Goldman, W.E., Mellroth, P., Steiner, H., Fukase, K., Kusumoto, S., Harley, W., Fox, A., Golenbock, D., Silverman, N., 2004. Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. Immunity 20, 637–49.

Kaneko, T., Golenbock, D., Silverman, N., 2005. Peptidoglycan recognition by the Drosophila Imd pathway. J. Endotoxin Res. 11, 383–389. doi:10.1179/096805105X76823

Kaneko, T., Silverman, N., 2005. Bacterial recognition and signalling by the Drosophila IMD pathway. Cell. Microbiol. 7, 461–469. doi:10.1111/j.1462-5822.2005.00504.x

Kapoor, M., Zhang, L., Ramachandra, M., Kusukawa, J., Ebner, K., Padmanabhan, R., 1995. Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. J. Biol. Chem. 270, 19100–19106.

Keene, K., Foy, B., Sanchez-Vargas, I., 2004. RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of Anopheles gambiae. Proc. Natl. Acad. Sci. U. S. A. 101, 17240–17245.

Kemp, Mueller, S., Goto, A., Barbier, V., Paro, S., Bonnay, F., Dostert, C., Troxler, L., Hetru, C., Meignin, C., Pfeffer, S., Hoffmann, J. a, Imler, J.-L., 2013. Broad RNA interference-

mediated antiviral immunity and virus-specific inducible responses in Drosophila. J. Immunol. 190, 650–658. doi:10.4049/jimmunol.1102486

Kemp, C., Imler, J.-L., 2009. Antiviral immunity in Drosophila. Curr. Opin. Immunol. 21, 3–9. doi:10.1016/j.coi.2009.01.007

Kemp, C., Mueller, S., Goto, A., Barbier, V., Paro, S., Bonnay, F., Dostert, C., Troxler, L., Hetru, C., Meignin, C., Pfeffer, S., Hoffmann, J.A., Imler, J.L., 2013. Broad RNA interference-mediated antiviral immunity and virus-specific inducible responses in Drosophila. J Immunol 190, 650–658. doi:10.4049/jimmunol.1102486

Khoo, Doty, J.B., Heersink, M.S., Olson, K.E., Franz, A., 2013a. Transgene-meidated suppression of the RNA interference pathway in Aedes aegypti interferes with gene silencing and enhances Sindbis virus and dengue virus type 2 replication. Insect Mol. Biol. 29, 997–1003. doi:10.1016/j.biotechadv.2011.08.021.Secreted

Khoo, Doty, J.B., Held, N.L., Olson, K.E., Franz, A.W.E., 2013b. Isolation of midgut escape mutants of two American genotype dengue 2 viruses from Aedes aegypti. Virol. J. 10, 257. doi:10.1186/1743-422X-10-257

Khoo, Piper, J., Sanchez-Vargas, I., Olson, K.E., Franz, A.W.E., 2010. The RNA interference pathway affects midgut infection- and escape barriers for Sindbis virus in Aedes aegypti. BMC Microbiol. 10, 130.

Khromykh, a a, Sedlak, P.L., Westaway, E.G., 2000. Cis- and trans-acting elements in flavivirus RNA replication. J. Virol. 74, 3253–3263.

Kim, V.N., 2005. MicroRNA biogenesis: coordinated cropping and dicing. Nat. Rev. Mol. cell Biol. 6, 376–385. doi:10.1038/nrm1644

Kingslover, M., Huang, Z., Hardy, R., 2013. Insect antiviral innate immunity: pathways,

effectors, and connections. J. Mol. Biol. 425, 4921–4936. doi:10.1016/j.jmb.2013.10.006.Insect

Kobayashi, H., Tomari, Y., 2015. RISC assembly: Coordination between small RNAs and Argonaute proteins. Biochim. Biophys. Acta. doi:10.1016/j.bbagrm.2015.08.007

Koenraadt, C.J.C., Kormaksson, M., Harrington, L.C.L., 2010. Effects of inbreeding and genetic modification on Aedes aegypti larval competition and adult energy reserves. Parasit. Vectors 3, 92. doi:10.1186/1756-3305-3-92

Kokoza, V., Ahmed, A., Cho, W., 2000. Engineering blood meal-activated systemic immunity in the yellow fever mosquito, Aedes aegypti. Proc. Natl. Acad. Sci. U. S. A. 97, 9144–9149.

Kramer, L.D., Ebel, G.D., 2003. Dynamics of flavivirus infection in mosquitoes. Adv. Virus Res. 60, 187–232.

Kramer, L.D., Hardy, J.L., Presser, S.B., Houk, E.J., 1981. Dissemination barriers for western equine encephalomyelitis virus in Culex tarsalis infected after ingestion of low viral doses. Am. J. Trop. Med. Hyg. 30, 190–197.

Ku, H.-Y., Lin, H., 2014. PIWI proteins and their interactors in piRNA biogenesis, germline development and gene expression. Natl. Sci. Rev. 1, 205–218. doi:10.1093/nsr/nwu014

Kuadkitkan, A., Wikan, N., Fongsaran, C., Smith, D.R., 2010. Identification and characterization of prohibitin as a receptor protein mediating DENV-2 entry into insect cells. Virology 406, 149–161. doi:10.1016/j.virol.2010.07.015

Kuberski, T., 1979. Fluorescent antibody studies on the development of dengue-2 virus in Aedes albopictus (Diptera: Culicidae). J. Med. Entomol. 16, 343–349.

Kumar, S., Gupta, L., Han, Y.S., Barillas-Mury, C., 2004. Inducible peroxidases mediate nitration of Anopheles midgut cells undergoing apoptosis in response to Plasmodium

invasion. J. Biol. Chem. 279, 53475–53482. doi:10.1074/jbc.M409905200

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y.-P., Dolja, V. V, Calvino, L.F., López-Moya, J.J., Burgyán, J., 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J. 25, 2768–27680. doi:10.1038/sj.emboj.7601164

Lambrechts, L., Chevillon, C., Albright, R.G., Thaisomboonsuk, B., Richardson, J.H., Jarman, R.G., Scott, T.W., 2009. Genetic specificity and potential for local adaptation between dengue viruses and mosquito vectors. BMC Evol. Biol. 9, 160.

Lambrechts, L., Koella, J.C., Boëte, C., 2008. Can transgenic mosquitoes afford the fitness cost? Trends Parasitol. 24, 4–7.

Lambrechts, L., Quillery, E., Noël, V., Richardson, J.H., Jarman, R.G., Scott, Thomas, W., Chevillon, C., 2013. Specificity of resistance to dengue virus isolates is associated with genotypes of the mosquito antiviral gene dicer-2. Proc. R. Soc. B; Biol. Sci. 280, 20122437. doi:10.1098/rspb.2012.2437

Lardeux, F., Tetuanui, A., 1995. Larval growth of Aedes polynesiensis and Aedes aegypti (Diptera: Culicidae). Mosq. Syst. 27, 118–124.

Lavine, M., Beckage, N.E., 1995. Polydnaviruses: potent mediators of host insect immune dysfunction. Parasitol. Today 11, 368–378. doi:10.1016/0169-4758(95)80005-0

Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32, 1295–1309.

Lee, R.C., Hammell, C.M., Ambros, V., 2006. Interacting endogenous and exogenous RNAi pathways in Caenorhabditis elegans. RNA 12, 589–597. doi:10.1261/rna.2231506.sequences

Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., Carthew, R.W., 2004. Distinct roles for Drosophila dicer-1 and dicer-2 in the siRNA/miRNA silencing pathways. Cell 117, 69–81.

Léger, P., Lara, E., Jagla, B., Sismeiro, O., Mansuroglu, Z., Coppée, J.Y., Bonnefoy, E., Bouloy, M., 2013. Dicer-2- and Piwi-mediated RNA interference in Rift Valley fever virus-infected mosquito cells. J. Virol. 87, 1631–1648. doi:10.1128/JVI.02795-12

Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J.M., Hoffmann, J. a, 1995. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. Proc. Natl. Acad. Sci. U. S. A. 92, 9465–9469.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86, 973–983.

Li, C., Marrelli, M., Yan, G., 2008. Fitness of transgenic Anopheles stephensi mosquitoes expressing the SM1 peptide under the control of a vitellogenin promoter. J. Hered. 99, 275–282.

Li, C.C., Marrelli, M.M.T., Yan, G.G., Jacobs-Lorena, M., 2008. Fitness of transgenic Anopheles stephensi mosquitoes expressing the SM1 peptide under the control of a vitellogenin promoter. J. Hered. 99, 275–282. doi:10.1093/jhered/esn004

Li, F., Ding, S.-W., 2006. Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. Annu. Rev. Microbiol. 60, 503–531. doi:10.1146/annurev.micro.60.080805.142205

Li, H., Li, W.X., Ding, S.W., 2002. Induction and suppression of RNA silencing by an animal

virus. Science (80-. ). 296, 1319–1321. doi:10.1126/science.1070948

Lindenbach, B.D., Rice, C.M., 1999. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. J. Virol. 73, 4611–4621.

Lingel, A., Izaurralde, E., 2004. RNAi: finding the elusive endonuclease. RNA 10, 1675–1679. doi:10.1261/rna.7175704

Lingel, A., Simon, B., Izaurralde, E., Sattler, M., 2003. Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. Nature 426, 465–469. doi:10.1038/nature02123

Liu, B., Behura, S.K., Clem, R.J., Schneemann, A., Becnel, J., Severson, D.W., Zhou, L., 2013. P53-mediated rapid induction of apoptosis conveys resistance to viral infection in Drosophila melanogaster. PLoS Pathog. 9, e1003137. doi:10.1371/journal.ppat.1003137

Liu, Q., Rand, T. a, Kalidas, S., Du, F., Kim, H.-E., Smith, D.P., Wang, X., 2003. R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. Science. 301, 1921–1925. doi:10.1126/science.1088710

Lounibos, L.P., 1981. Habitat segregation among african treehole mosquitos. Ecol. Entomol. 6, 129–154. doi:10.1111/j.1365-2311.1981.tb00601.x

Lozano-Fuentes, S., Fernandez-Salas, I., de Lourdes Munoz, M., Garcia-Rejon, J., Olson, K.E., Beaty, B.J., Black, W.C., 2009. The neovolcanic axis is a barrier to gene flow among Aedes aegypti populations in Mexico that differ in vector competence for Dengue 2 virus. PLoS Negl. Trop. Dis. 3, e468. doi:10.1371/journal.pntd.0000468

Lu, R., Maduro, M., Li, F., Li, H.W., Broitman-Maduro, G., Li, W.X., Ding, S.W., 2005. Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature 436, 1040–1043. doi:10.1016/j.bbi.2008.05.010

Lucchetta, E.M., Carthew, R.W., Ismagilov, R.F., 2009. The endo-siRNA Pathway is essential

for robust development of the Drosophila embryo. PLoS One 4, e7576. doi:10.1371/journal.pone.0007576

Luckhart, S., Vodovotz, Y., Cui, L., Rosenberg, R., 1998. The mosquito Anopheles stephensi limits malaria parasite development with inducible synthesis of nitric oxide. Proc. Natl. Acad. Sci. U. S. A. 95, 5700–5705.

Ludwig, G. V, Christensen, B.M., Yuill, T.M., Schultz, K.T., 1989. Enzyme processing of La Crosse virus glycoprotein G1: a bunyavirus-vector infection model. Virology 171, 108–113.

Ludwig, G. V, Israel, B.A., Christensen, B.M., Yuill, T.M., Schultz, K.T., 1991. Role of La Crosse virus glycoproteins in attachment of virus to host cells. Virology 181, 564–571.

Lumley, G., 1943. Dengue. Part 1., Number 3. ed. Australasian Med. Pub. Co., Glebe, New South Wales.

Lunn, D.J., Thomas, A., Best, N., Spiegelhalter, D., 2000. WinBUGS – A Bayesian modelling framework: Concepts, structure, and extensibility. Stat. Comput. 10, 325–337. doi:10.1023/A:1008929526011

Luo, K., Pang, Y., 2006. Spodoptera litura multicapsid nucleopolyhedrovirus inhibits Microplitis bicoloratus polydnavirus-induced host granulocytes apoptosis. J. Insect Physiol. 52, 795–806.doi:10.1016/j.jinsphys.2006.04.007

Luo, K.J., Pang, Y., 2006. Disruption effect of Microplitis bicoloratus polydnavirus EGF-like protein, MbCRP, on actin cytoskeleton in lepidopteran insect hemocytes. Acta Biochim. Biophys. 38, 577–85.

Luplertlop, N., Surasombatpattana, P., Patramool, S., 2011. Induction of a peptide with activity against a broad spectrum of pathogens in the Aedes aegypti salivary gland, following infection with dengue virus. PLoS Pathog. 7, e1001252. doi:10.1371/journal.ppat.1001252

Ma, J.-B., Ye, K., Patel, D.J., 2004. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature 429, 318–322. doi:10.1038/nature02519

Macdonald, G., 1957. The epidemiology and control of malaria. London, New York: Oxford University Press. 201 p.

Mackenzie, J.M., Jones, M.K., Young, P.R., 1996. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology 220, 232–240. doi:10.1006/viro.1996.0307

Mackenzie, J.M., Khromykh, A.A., Jones, M.K., Westaway, E.G., 1998. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology 245, 203–215. doi:10.1006/viro.1998.9156

Magori, K., Gould, F., 2006. Genetically engineered underdominance for manipulation of pest populations: a deterministic model. Genetics 172, 2613–2620. doi:10.1534/genetics.105.051789

Marrelli, M., Moreira, C., Kelly, D., 2006. Mosquito transgenesis: what is the fitness cost? Trends Parasitol. 22, 197–202.

Marshall, J.M., Hay, B.A., 2011. Inverse Medea as a novel gene drive system for local population replacement: a theoretical analysis. J. Hered. 102, 336–341. doi:10.1093/jhered/esr019

Marshall, J.M., Pittman, G.W., Buchman, A.B., Hay, B.A., 2011. Semele: a killer-male, rescue-female system for suppression and replacement of insect disease vector populations. Genetics 187, 535–551. doi:10.1534/genetics.110.124479

Massonnet-Bruneel, B., Corre-Catelin, N., Lacroix, R., Lees, R.S., Hoang, K.P., Nimmo, D., Alphey, L., Reiter, P., 2013. Fitness of transgenic mosquito Aedes aegypti males carrying a

dominant lethal genetic system. PLoS One 8, e62711. doi:10.1371/journal.pone.0062711

McArthur, C.C., Meredith, J.M., Eggleston, P., 2014. Transgenic Anopheles gambiae expressing an antimalarial peptide suffer no significant fitness cost. PLoS One 9, e88625. doi:10.1371/journal.pone.0088625

McCarthy, M.A., 2007. Bayesian Methods for Ecology. Cambridge University Press.

McDonald, P.T., Hausermann, W., Lorimer, N., 1977. Sterility introduced by release of genetically altered males to a domestic population of Aedes aegypti at the Kenya coast. Am. J. Trop. Med. Hyg. 26, 553–561.

Meister, S., Koutsos, a C., Christophides, G.K., 2004. The Plasmodium parasite-a "new" challenge for insect innate immunity. Int. J. Parasitol. 34, 1473–1482. doi:10.1016/j.ijpara.2004.10.004

Mercado-Curiel, R.F., Black, W.C., Muñoz, M.D.L., 2008. A dengue receptor as possible genetic marker of vector competence in Aedes aegypti. BMC Microbiol. 8, 118. doi:10.1186/1471-2180-8-118

Merkling, S.H., van Rij, R.P., 2013. Beyond RNAi: Antiviral defense strategies in Drosophila and mosquito. J. Insect Physiol. 59, 159–170. doi:10.1016/j.jinsphys.2012.07.004

Miesen, P., Girardi, E., van Rij, R.P., 2015. Distinct sets of PIWI proteins produce arbovirus and transposon-derived piRNAs in Aedes aegypti mosquito cells. Nucleic Acids Res. 43, 6545–6556. doi:10.1093/nar/gkv590

Miller, B.R., Mitchell, C.J., 1991. Genetic selection of a flavivirus-refractory strain of the yellow fever mosquito Aedes aegypti. Am. J. Trop. Med. Hyg. 45, 399–407.

Miller, B.R., Mitchell, C.J., 1986. Passage of yellow fever virus: its effect on infection and transmission rates in Aedes aegypti. Am. J. Trop. Med. Hyg. 35, 1302–1309.

Mims, C.A., Day, M.F., Marshall, I.D., 1966. Cytopathic effect of Semliki Forest virus in the mosquito Aedes aegypti. Am. J. Trop. Med. Hyg. 15, 775–784.

Mizutani, T., Kobayashi, M., Eshita, Y., Shirato, K., Kimura, T., Ako, Y., Miyoshi, H., Takasaki, T., Kurane, I., Kariwa, H., Umemura, T., Takashima, I., 2003. Involvement of the JNK-like protein of the Aedes albopictus mosquito cell line, C6/36, in phagocytosis, endocytosis and infection of West Nile virus. Insect Mol. Biol. 12, 491–499.

Molina-Cruz, A., Gupta, L., Richardson, J., Bennett, K., Black, W., Barillas-Mury, C., 2005. Effect of mosquito midgut trypsin activity on dengue-2 virus infection and dissemination in Aedes aegypti. Am. J. Trop. Med. Hyg. 72, 631–637.

Moon, S.L., Anderson, J.R., Kumagai, Y., Wilusz, C.J., Akira, S., Khromykh, a. a., Wilusz, J., 2012. A noncoding RNA produced by arthropod-borne flaviviruses inhibits the cellular exoribonuclease XRN1 and alters host mRNA stability. Rna 18, 2029–2040. doi:10.1261/rna.034330.112

Moore, C.G., Cline, B.L., Ruiz-Tiben, E., Lee, D., Romney-Joseph, H., Rivera-Correa, E., 1978. Aedes aegypti in Puerto Rico: environmental determinants of larval abundance and relation to dengue virus transmission. Am. J. Trop. Med. Hyg. 27, 1225–1231.

Morazzani, E.M., Wiley, M.R., Murreddu, M.G., Adelman, Z.N., Myles, K.M., 2012. Production of virus-derived ping-pong-dependent piRNA-like small RNAs in the mosquito soma. PLoS Pathog. 8, e1002470. doi:10.1371/journal.ppat.1002470

Moreira, L. a, Ito, J., Ghosh, A., Devenport, M., Zieler, H., Abraham, E.G., Crisanti, A., Nolan, T., Catteruccia, F., Jacobs-Lorena, M., 2002. Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. J. Biol. Chem. 277, 40839–40843. doi:10.1074/jbc.M206647200

Moreira, L., Ghosh, A., Abraham, E., 2002. Genetic transformation of mosquitoes: a quest for malaria control. Int. J. Parasitol. 32, 1599–1605.

Moreira, L.L.A., Wang, J., Collins, F.F.H., Jacobs-Lorena, M., 2004. Fitness of anopheline mosquitoes expressing transgenes that inhibit Plasmodium development. Genetics 166, 1337–1341.

Moreno, E., Yan, M., Basler, K., 2002. Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily. Curr. Biol. 12, 1263–1268.

Mosso, C., Galván-Mendoza, I.J., Ludert, J.E., del Angel, R.M., 2008. Endocytic pathway followed by dengue virus to infect the mosquito cell line C6/36 HT. Virology 378, 193–199. doi:10.1016/j.virol.2008.05.012

Moy, R.H., Gold, B., Molleston, J.M., Schad, V., Salzano, M., Yagi, Y., Fitzgerald, K.A., Ben, Z., Soldan, S.S., Cherry, S., 2014. Antiviral autophagy restricts Rift Valley Fever virus infection and is conserved from flies to mammals. Immunity 40, 51–65. doi:10.1016/j.immuni.2013.10.020.Antiviral

Myles, K.M., Wiley, M.R., Morazzani, E.M., Adelman, Z.N., 2008. Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. Proc. Natl. Acad. Sci. U. S. A. 105, 19938–19943. doi:10.1073/pnas.0803408105

Myles, K.M.K., Pierro, D.J.D., Olson, K.E., 2004. Comparison of the transmission potential of two genetically distinct Sindbis viruses after oral infection of Aedes aegypti (Diptera : Culicidae). J. Med. Entomol. 41, 95–106.

Nayak, A., Berry, B., Tassetto, M., Kunitomi, M., Acevedo, A., Deng, C., Krutchinsky, A., Gross, J., Antoniewski, C., Andino, R., 2010. Cricket paralysis virus antagonizes Argonaute

2 to modulate antiviral defense in Drosophila. Nat. Struct. Mol. Biol. 17, 547–554. doi:10.1038/nsmb.1810

Nishikura, K., 2001. A short primer on RNAi: RNA-directed RNA polymerase acts as a key catalyst. Cell 107, 415–418.

Obbard, D.J., Gordon, K.H.J., Buck, A.H., Jiggins, F.M., 2009. The evolution of RNAi as a defence against viruses and transposable elements. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 364, 99–115. doi:10.1098/rstb.2008.0168

Obbard, D.J., Jiggins, F.M., Halligan, D.L., Little, T.J., 2006. Natural selection drives extremely rapid evolution in antiviral RNAi genes. Curr. Biol. 16, 580–585. doi:10.1016/j.cub.2006.01.065

Okamoto, K.W., Robert, M.A., Lloyd, A.L., Gould, F., 2013. A reduce and replace strategy for suppressing vector-borne diseases : insights from a stochastic, spatial model. PLoS One 8, e81860. doi:10.1371/journal.pone.0081860

Okamura, K., Ishizuka, A., Siomi, H., Siomi, M.C., 2004. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. 18, 1655–1666. doi:10.1101/gad.1210204

Olsthoorn, R., Bol, J., 2001. Sequence comparison and secondary structure analysis of the 39 noncoding region of flavivirus genomes reveals multiple pseudoknots. RNA 7, 1370–1377.

Owusu-Daaku, K.O., Wood, R.J., Butler, R.D., 1997. Selected lines of Aedes aegypti with persistently distorted sex ratios. Heredity. 79, 388–393.

Pant, C.P., Yasuno, M., 1973. Field studies on the gonotrophic cycle of Aedes aegypti in Bangkok, Thailand. J. Med. Entomol. 10, 219–223.

Parikh, G.R., Oliver, J.D., Bartholomay, L.C., 2009. A haemocyte tropism for an arbovirus. J.

Gen. Virol. 90, 292–296. doi:10.1099/vir.0.005116-0

Paul, D., Bartenschlager, R., 2013. Architecture and biogenesis of plus-strans virus replication factories. World J. Virol. 2, 32–48. doi:10.5501/wjv.v2.i2.32

Paulson, S.L., Grimstad, P.R., Craig, G.B., 1989. Midgut and salivary gland barriers to La Crosse virus dissemination in mosquitoes of the Aedes triseriatus group. Med. Vet. Entomol. 3, 113–123.

Petersen, J.L., Lounibos, L.P., Lorimer, N., 1977. Field trials of double translocation heterozygote males for genetic control of Aedes aegypti (L.) (Diptera: Culicidae). Bull. Entomol. Res. 67, 313-324. doi:10.1017/S0007485300011135

Pham, J.W., Pellino, J.L., Lee, Y.S., Carthew, R.W., Sontheimer, E.J., 2004. A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. Cell 117, 83–94.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C. a, Coleman, P.G., White-Cooper, H., Alphey, L., 2007. Late-acting dominant lethal genetic systems and mosquito control. BMC Biol. 5, 11. doi:10.1186/1741-7007-5-11

Pillai, R.S., 2005. MicroRNA function: multiple mechanisms for a tiny RNA? RNA 11, 1753–1761. doi:10.1261/rna.2248605

Platt, K.B., Linthicum, K.J., Myint, K.S., Innis, B.L., Lerdthusnee, K., Vaughn, D.W., 1997. Impact of dengue virus infection on feeding behavior of Aedes aegypti. Am. J. Trop. Med. Hyg. 57, 119–125.

Ponnudurai, T., Billingsley, P.F., Rudin, W., 1988. Differential infectivity of Plasmodium for mosquitoes. Parasitol. Today 4, 319–21.

Powell, J.R., Tabachnick, W.J., 2013. History of domestication and spread of Aedes aegypti - A Review. Mem. Inst. Oswaldo Cruz 108, 11–17. doi:10.1590/0074-0276130395

Preall, J.B., He, Z., Gorra, J.M., Sontheimer, E.J., 2006. Short interfering RNA strand selection is independent of dsRNA processing polarity during RNAi in Drosophila. Curr. Biol. 16, 530–535. doi:10.1016/j.cub.2006.01.061

Prikhod, G.G., Prikhod, E.A., Pletnev, A.G., Cohen, J.I., 2002. Langat flavivirus protease NS3 binds caspase-8 and induces apoptosis. Apoptosis 76, 5701–5710. doi:10.1128/JVI.76.11.5701

Ptitsyn, A. a, Reyes-Solis, G., Saavedra-Rodriguez, K., Betz, J., Suchman, E.L., Carlson, J.O., Black, W.C., 2011. Rhythms and synchronization patterns in gene expression in the Aedes aegypti mosquito. BMC Genomics 12, 153. doi:10.1186/1471-2164-12-153

Qiu, P., Pan, P.C., Govind, S., 1998. A role for the Drosophila Toll/Cactus pathway in larval hematopoiesis. Development 125, 1909–20.

Quellhorst, G., Prabhakar, S., Han, Y., Yang, J., 2014. PCR array : A simple and quantitative method for gene expression profiling. Bioscience 12, pgs 1-12.

Rai, K.S., Grover, K.K., Suguna, S.G., 1973. Genetic manipulation of Aedes aegypti: incorporation and maintenance of a genetic marker and a chromosomal translocation in natural populations. Bull. World Health Organ. 48, 49–56.

Rasgon, J.L., 2008. Using predictive models to optimize Wolbachia-based strategies for vector-borne disease control. Adv. Exp. Med. Biol. 627, 114–125. doi:10.1007/978-0-387-78225-6_10

Reeves, W.C., Hardy, J.L., Reisen, W.K., Milby, M.M., 1994. Potential effect of global warming on mosquito-borne arboviruses. J. Med. Entomol. 31, 323–332.

Reisen, W., Fang, Y., 2007. Is nonviremic transmission of West Nile virus by Culex mosquitoes (Diptera: Culicidae) nonviremic? J. Med. Entomol. 44, 299–302. doi:10.1603/0022-2585(2007)44

Rico-Hesse, R., 2003. Microevolution and virulence of dengue viruses. Adv. Virus Res. 59, 315–341.

Rico-Hesse, R., Harrison, L.M., Salas, R.A., Tovar, D., Nisalak, A., Ramos, C., Boshell, J., de Mesa, M.T., Nogueira, R.M., da Rosa, a T., 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230, 244–251.

Robert, M.A., Okamoto, K., Lloyd, A.L., Gould, F., 2013. A reduce and replace strategy for suppressing vector-borne diseases: insights from a deterministic model. PLoS One 8, e73233. doi:10.1371/journal.pone.0073233

Robert, M.A., Okamoto, K.W., Gould, F., Lloyd, A.L., 2014. Antipathogen genes and the replacement of disease-vectoring mosquito populations : a model-based evaluation. Evol. Appl. 7, 1238–1251. doi:10.1111/eva.12219

Romoser, W.S., Turell, M.J., Lerdthusnee, K., Neira, M., Dohm, D., Ludwig, G., Wasieloski, L., 2005. Pathogenesis of Rift Valley fever virus in mosquitoes--tracheal conduits & the basal lamina as an extra-cellular barrier. Arch. Virol. 89–100.

Rosen, L., Roseboom, L.E., Gubler, D.J., Lien, J.C., Chaniotis, B.N., 1985. Comparative susceptibility of mosquito species and strains to oral and parenteral infection with dengue and Japanese encephalitis viruses. Am. J. Trop. Med. Hyg. 34, 603–615.

Roy, S., Sadigh, B., Datan, E., Lockshin, R. A., Zakeri, Z., 2014. Regulation of cell survival and death during Flavivirus infections. World J. Biol. Chem. 5, 93–105. doi:10.4331/wjbc.v5.i2.93

Rutschmann, S., Kilinc, A., Ferrandon, D., 2002. Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in Drosophila. J. Immunol. 168, 1542–6.

Sabin, A., Schlesinger, R., 1945. Production of immunity to dengue with virus modified by propagation in mice. Science. 101, 640–642.

Sabin, L.R., Hanna, S.L., Cherry, S., 2010. Innate antiviral immunity in Drosophila. Curr. Opin. Immunol. 22, 4–9. doi:10.1016/j.coi.2010.01.007

Salvesen, G.S., Duckett, C.S., 2002. IAP proteins: blocking the road to death's door. Nat. Rev. Mol. cell Biol. 3, 401–410. doi:10.1038/nrm830

Sanchez-Vargas, I., Scott, J.C., Poole-Smith, B.K., Franz, A.W.E., Barbosa-Solomieu, V., Wilusz, J., Olson, K.E., Blair, C.D, 2009. Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway. PLoS Pathog. 5, e1000299. doi:10.1371/journal.ppat.1000299

Sanchez-Vargas, I., Travanty, E., Keene, K., 2004. RNA interference, arthropod-borne viruses, and mosquitoes. Virus Res. 102, 65–74.

Sanders, H.R., Foy, B.D., Evans, A.M., Ross, L.S., Beaty, B.J., Olson, K.E., Gill, S.S., 2005. Sindbis virus induces transport processes and alters expression of innate immunity pathway genes in the midgut of the disease vector, Aedes aegypti. Insect Biochem. Mol. Biol. 35, 1293–307. doi:10.1016/j.ibmb.2005.07.006

Sasaki, T., Shimizu, N., 2007. Evolutionary conservation of a unique amino acid sequence in human DICER protein essential for binding to Argonaute family proteins. Gene 396, 312–320. doi:10.1016/j.gene.2007.04.001

Schneider, D.S., Ayres, J.S., Brandt, S.M., Costa, A., Dionne, M.S., Gordon, M.D., Mabery, E.M., Moule, M.G., Pham, L.N., Shirasu-Hiza, M.M., 2007. Drosophila eiger mutants are

sensitive to extracellular pathogens. PLoS Pathog. 3, e41. doi:10.1371/journal.ppat.0030041

Schneider, J.R., Mori, A., Romero-Severson, J., Chadee, D.D., Severson, D.W., 2007. Investigations of dengue-2 susceptibility and body size among Aedes aegypti populations. Med. Vet. Entomol. 21, 370–376. doi:10.1111/j.1365-2915.2007.00699.x

Schnettler, E., Donald, C.L., Human, S., Watson, M., Siu, R.W.C., McFarlane, M., Fazakerley, J.K., Kohl, A., Fragkoudis, R., 2013. Knockdown of piRNA pathway proteins results in enhanced semliki forest virus production in mosquito cells. J. Gen. Virol. 94, 1680–1689. doi:10.1099/vir.0.053850-0

Schnettler, E., Leung, J.Y., Sterken, M.G., Metz, S.W., Geertsema, C., Goldbach, R.W., Vlak, J.M., Kohl, A., Khromykh, A. A., Pijlman, G.P., 2013. Non-coding flavivirus RNA displays RNAi suppressor activity in insect and mammalian cells. J. of Virol. 86, 13486–13500. doi:10.1128/JVI.01104-12

Schule, P.A., 1928. Dengue fever: transmission by Aedes aegypti. Am. J. Trop. Med. Hyg. 8, 203-213.

Schwarz, D.S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., Zamore, P.D., 2003. Asymmetry in the assembly of the RNAi enzyme complex. Cell 115, 199–208.

Schwarz, D.S., Tomari, Y., Zamore, P.D., 2004. The RNA-induced silencing complex is a Mg2+-dependent endonuclease. Curr. Biol. 14, 787–791. doi:10.1016/j.cub.2004.03.008

Scott, J.C., Brackney, D.E., Campbell, C.L., Bondu-Hawkins, V., Hjelle, B., Ebel, G.D., Olson, K.E., Blair, C.D., 2010. Comparison of dengue virus type 2-specific small RNAs from RNA interference-competent and -incompetent mosquito cells. PLoS Negl. Trop. Dis. 4, e848. doi:10.1371/journal.pntd.0000848

Scott, T.W., Lorenz, L.H., 1998. Reduction of Culiseta melanura fitness by eastern equine

encephalomyelitis virus. Am. J. Trop. Med. Hyg. 59, 341–346.

Scott, T.W., Takken, W., Knols, B.G.J., Boëte, C., 2002. The ecology of genetically modified mosquitoes. Science 298, 117–119. doi:10.1126/science.298.5591.117

Senti, K.-A., Brennecke, J., 2010. The piRNA pathway: a fly's perspective on the guardian of the genome. Trends Genet. 26, 499–509. doi:10.1016/j.tig.2010.08.007

Severson, D., Black, W., 2005. Genome evolution in mosquitoes, in: Marquardt, W. (Ed.), Biology of Disease Vectors. Elsevier, pp. 449–463.

Shin, S., Kokoza, V., Bian, G., 2005. REL1, a homologue of Drosophila dorsal, regulates Toll antifungal immune pathway in the female mosquito Aedes aegypti. J. Biol. Chem. 280, 16499–16507.

Shin, S.W., 2003. Transgenesis and reverse genetics of mosquito innate immunity. J. Exp. Biol. 206, 3835–3843. doi:10.1242/jeb.00640

Shin, S.W., Kokoza, V., Ahmed, A., Raikhel, A.S., 2002. Characterization of three alternatively spliced isoforms of the Rel/NF-kappa B transcription factor Relish from the mosquito Aedes aegypti. Proc. Natl. Acad. Sci. U. S. A. 99, 9978–83. doi:10.1073/pnas.162345999

Silverman, N., Zhou, R., Erlich, R.L., Hunter, M., Bernstein, E., Schneider, D., Maniatis, T., 2003. Immune activation of NF-kappaB and JNK requires Drosophila TAK1. J. Biol. Chem. 278, 48928–48934. doi:10.1074/jbc.M304802200

Sim, S., Jupatanakul, N., Dimopoulos, G., 2014. Mosquito immunity against arboviruses. Viruses 6, 4479–4504. doi:10.3390/v6114479

Simmons, J.S., John, J.H. St., Reynolds, F.H.K., 1930a. Dengue fever transmitted by Aedes albopictus, Skuse. Am. J. Trop. Med. Hyg. 1, 17–21.

Simmons, J.S., St. John, J., Reynolds, F.H.K., 1930b. Transmission of dengue fever by Aedes

albopictus Skuse. Philipp. J. Sci. 41, 215–229.

Sinkins, S.P., Gould, F., 2006. Gene drive systems for insect disease vectors. Nat. Rev. Genet. 7, 427–435. doi:10.1038/nrg1870

Siu, R.W.C., Fragkoudis, R., Simmonds, P., Donald, C.L., Chase-Topping, M.E., Barry, G., Attarzadeh-yazdi, G., Rodriguez-Andres, J., Nash, A.A., Merits, A., Fazakerley, J.K., Kohl, A., Rodriguez-, J., Nash, A.A., Merits, A., Fazakerley, J.K., Kohl, A., 2011. Antiviral RNA interference responses induced by Semliki Forest virus infection of mosquito cells: characterization, origin, and frequency-dependent functions of virus-derived small interfering RNAs. J. Virol. 85, 2907–2917. doi:10.1128/JVI.02052-10

Skalsky, R.L., Olson, K.E., Blair, C.D., Garcia-Blanco, M. a, Cullen, B.R., 2014. A "microRNA-like" small RNA expressed by dengue virus? Proc. Natl. Acad. Sci. U. S. A. 111, E2359. doi:10.1073/pnas.1406854111

Smith, C.E., 1987. Factors influencing the transmission of western equine encephalomyelitis virus between its vertebrate maintenance hosts and from them to humans. Am. J. Trop. Med. Hyg. 37, 33S–39S.

Snijders, E.P., Schuffner, W.A.P., Dinger, E.J., 1931. On the transmission of dengue in Sumatra. Am. J. Trop. Med. Hyg. 11, 171–197.

Souza-Neto, J. a, Sim, S., Dimopoulos, G., 2009. An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. Proc. Natl. Acad. Sci. U. S. A. 106, 17841–17846. doi:10.1073/pnas.0905006106

Stadler, K., Allison, S.L., Schalich, J., Heinz, F.X., 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J. Virol. 71, 8475–84781.

Sylla, M., Bosio, C., Urdaneta-Marquez, L., Ndiaye, M., Black, W.C., 2009. Gene flow,

subspecies composition, and dengue virus-2 susceptibility among Aedes aegypti collections in Senegal. PLoS Negl. Trop. Dis. 3, e408. doi:10.1371/journal.pntd.0000408

Tabachnick, W.J., Wallis, G.P., Aitken, T.H., Miller, B.R., Amato, G.D., Lorenz, L., Powell, J.R., Beaty, B.J., 1985. Oral infection of Aedes aegypti with yellow fever virus: geographic variation and genetic considerations. Am. J. Trop. Med. Hyg. 34, 1219–12124.

Tabara, H., Yigit, E., Siomi, H., Mello, C.C., 2002. The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. Cell 109, 861–871.

Tahbaz, N., Kolb, F.A., Zhang, H., Jaronczyk, K., Filipowicz, W., Hobman, T.C., 2004. Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. EMBO Rep 5, 189–194. doi:10.1038/sj.embor.7400070

Takahashi, M., 1976. The effects of environmental and physiological conditions of Culex tritaeniorhynchus on the pattern of transmission of Japanese encephalitis virus. J. Med. Entomol. 13, 275–284.

Tellam, R.L., Wijffels, G., Willadsen, P., 1999. Peritrophic matrix proteins. Insect Biochem. Mol. Biol. 29, 87–101.

Thomas, D.D., Donnelly, C. a, Wood, R.J., Alphey, L.S., 2000. Insect population control using a dominant, repressible, lethal genetic system. Science 287, 2474–2476. doi:10.1126/science.287.5462.2474

Tomari, Y., Matranga, C., Haley, B., Martinez, N., Zamore, P.D., 2004. A protein sensor for siRNA asymmetry. Science. 306, 1377–1380. doi:10.1126/science.1102755

Tran, K.T., Vazeille-Falcoz, M., Mousson, L., Tran, H.H., Rodhain, F., Ngugen, T.H., Failloux, A.B., 1999. Aedes aegypti in Ho Chi Minh City (Viet Nam): susceptibility to dengue 2 virus

and genetic differentiation. Trans. R. Soc. Trop. Med. Hyg. 93, 581–586.

Trinchieri, G., Sher, A., 2007. Cooperation of Toll-like receptor signals in innate immune defence. Nat. Rev. Immunol. 7, 179–190. doi:10.1038/nri2038

Trpis, M., 1970. A new bleaching and decalcifying method for general use in zoology. Can. J. Zool. 892–893.

Twiddy, S.S., Holmes, E.C., Rambaut, A., 2003. Inferring the rate and time-scale of dengue virus evolution. Mol. Biol. Evol. 20, 122–129.

Umareddy, I., Chao, A., Sampath, A., Gu, F., Vasudevan, S.G., 2006. Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. J. Gen. Virol. 87, 2605–2614. doi:10.1099/vir.0.81844-0

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3-new capabilities and interfaces. Nucleic Acids Res. 40, 1–12. doi:10.1093/nar/gks596

Vaidyanathan, R., Scott, T.W., 2006. Apoptosis in mosquito midgut epithelia associated with West Nile virus infection. Apoptosis 11, 1643–1651. doi:10.1007/s10495-006-8783-y

Valdez, M.R.W. De, Suchman, E.L., Carlson, J.O., Black, W.C., 2010. A large scale laboratory cage trial of Aedes densonucleosis virus (AeDNV). J. Med. Entomol. 47, 392–399. doi:10.1603/ME09157

Van Cleef, K.W.R., Van Mierlo, J.T., Miesen, P., Overheul, G.J., Fros, J.J., Schuster, S., Marklewitz, M., Pijlman, G.P., Junglen, S., Van Rij, R.P., 2014. Mosquito and Drosophila entomobirnaviruses suppress dsRNA- and siRNA-induced RNAi. Nucleic Acids Res. 42, 8732–8744. doi:10.1093/nar/gku528

van Rij, R.P., Saleh, M.-C., Berry, B., Foo, C., Houk, A., Antoniewski, C., Andino, R., 2006.

The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev. 20, 2985–2995. doi:10.1101/gad.1482006

Vaughan, G., Olivera, H., Santos-Argumedo, L., Landa, A., Briseno, B., Escobar-Gutiérrez, A., 2002. Dengue virus replicative intermediate RNA detection by reverse transcription-PCR. Clin. Diagn. Lab. Immunol. 9, 198-200. doi:10.1128/CDLI.9.1.198

Vazeille, M., Rosen, L., Mousson, L., Failloux, A.-B., 2003. Low oral receptivity for dengue type 2 viruses of Aedes albopictus from Southeast Asia compared with that of Aedes aegypti. Am. J. Trop. Med. Hyg. 68, 203–208.

Vazeille-Falcoz, M., Mousson, L., Rodhain, F., Chungue, E., Failloux, a B., 1999. Variation in oral susceptibility to dengue type 2 virus of populations of Aedes aegypti from the islands of Tahiti and Moorea, French Polynesia. Am. J. Trop. Med. Hyg. 60, 292–299.

Vinogradova, E.B., 2011. The sex structure of the larval populations of the urban mosquito Culex pipiens pipiens f. molestus Forskal (Diptera, Culicidae) in St. Petersburg. Entomol. Rev. 91, 729–734. doi:10.1134/S0013873811060054

Vodovar, N., Bronkhorst, A.W., van Cleef, K.W.R., Miesen, P., Blanc, H., van Rij, R.P., Saleh, M.C., 2012. Arbovirus-derived piRNAs exhibit a ping-pong signature in mosquito cells. PLoS One 7, e30861. doi:10.1371/journal.pone.0030861

Wade, M.J., Beeman, R.W., 1994. The population dynamics of maternal-effect selfish genes. Genetics 138, 1309–1314.

Wan, L., Dockendorff, T.C., Jongens, T.A., Dreyfuss, G., 2000. Characterization of dFMR1, a Drosophila melanogaster homolog of the fragile X mental retardation protein. Mol. Cell. Biol. 20, 8536.

Wang, H., Gort, T., Boyle, D.L., Clem, R.J., 2012. Effects of manipulating apoptosis on sindbis

virus infection of Aedes aegypti mosquitoes. J. Virol. 86, 6546–6554. doi:10.1128/JVI.00125-12

Wang, S.-H., Syu, W.-J., Huang, K.-J., Lei, H.-Y., Yao, C.-W., King, C.-C., Hu, S.-T., 2002. Intracellular localization and determination of a nuclear localization signal of the core protein of dengue virus. J. Gen. Virol. 83, 3093–3102.

Wang, X.H., Aliyari, R., Li, W.X., Li, H.W., Kim, K., Carthew, R., Atkinson, P., Ding, S.W., 2006. RNA interference directs innate immunity against viruses in adult Drosophila. Science. 312, 452-454.

Washburn, J.O., Kirkpatrick, B.A., Volkman, L.E., 1996. Insect protection against viruses. Nature 383, 767.

Watson, F.L., Pu, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M., Rebel, V.I., Schmucker, D., 2005. Extensive diversity of Ig-superfamily proteins in the immune system of insects Science 309, 1874–1878.

Watts, D.M., Burke, D.S., Harrison, B. A., Whitmire, R.E., Nisalak, A., 1987. Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. Am. J. Trop. Med. Hyg. 36, 143–152.

Weaver, S.C., Lorenz, L.H., Scott, T.W., 1992. Pathologic changes in the midgut of Culex tarsalis following infection with Western equine encephalomyelitis virus. Am. J. Trop. Med. Hyg. 47, 691–701.

Weaver, S.C., Scott, T.W., Lorenz, L.H., Lerdthusnee, K., Romoser, W.S., 1988. Togavirus-associated pathologic changes in the midgut of a natural mosquito vector. J. Virol. 62, 2083–2090.

Wei, Y., Qin, C., Jiang, T., Li, X., Zhao, H., Liu, Z., Deng, Y., Liu, R., Chen, S., Yu, M., Qin, E.,

2009. Translational regulation by the 3’ untranslated region of the dengue type 2 virus genome. Am. J. Trop. Med. Hyg. 81, 817–824. doi:10.4269/ajtmh.2009.08-0595

Wengler, G., 1981. Terminal sequences of the genome and replicative-from RNA of the flavivirus West Nile virus: absence of poly(A) and possible role in RNA replication. Virology 113, 544–555.

Wengler, G., Castle, E., 1986. Analysis of structural properties which possibly are characteristic for the 3’-terminal sequence of the genome RNA of flaviviruses. J. Gen. Virol. 67 ( Pt 6), 1183–1188.

Westaway, E.G., Mackenzie, J.M., Kenney, M.T., Jones, M.K., Khromykh, a a, 1997. Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J. Virol. 71, 6650–6661.

Whitfield, S.G., Murphy, F. A., Sudia, W.D., 1973. St. Louis encephalitis virus: an ultrastructural study of infection in a mosquito vector. Virology 56, 70–87.

Whitten, M., Foster, G., 1975. Genetic methods of pest control. Annu. Rev. Entomol. 461–476.

Whitten, M.M.A., Shiao, S.H., Levashina, E. a, 2006. Mosquito midguts and malaria: cell biology, compartmentalization and immunology. Parasite Immunol. 28, 121–130. doi:10.1111/j.1365-3024.2006.00804.x

WHO, 2009a. Dengue Fact Sheet.

WHO, 2009b. Dengue: Guidelines for diagnosis, treatment, prevention and control. WHO Press, World Health Organization, Geneva, Switzerland.

WHO, 1997. Clinical Diagnosis, in: Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control. Geneva, Switzerland, pp. 397–397. doi:10.1111/j.1444-

0938.2007.00167.x

Windbichler, N., Menichelli, M., Papathanos, P.-A.A., Thyme, S.B., Li, H., Ulge, U.Y., Hovde, B.T., Baker, D., Monnat, R.J., Burt, A., Crisanti, A., 2011. A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. Nature 473, 212–215. doi:10.1038/nature09937

Woodring JL, Higgs S, B.B., 1996. Natural cycles of vector borne pathogens., in: Marquardt WC, B.B. (Ed.), Biology of Disease Vectors. University Press of Colorado, Boulder, CO, pp. 51–72.

Wu, Q., Wang, X., Ding, S.-W., 2010. Viral suppressors of RNA-based viral immunity: host targets. Cell Host Microbe 8, 12–15. doi:10.1016/j.chom.2010.06.009

Xi, Z., Ramirez, J., Dimopoulos, G., 2008. The Aedes aegypti Toll pathway controls dengue virus infection. PLoS Pathog. 4, e1000098. doi:10.1371/ journal.ppat.1000098

Yan, K.S., Yan, S., Farooq, A., Han, A., Zeng, L., Zhou, M.-M., 2003. Structure and conserved RNA binding of the PAZ domain. Nature 426, 468–474. doi:10.1038/nature02129

Zambon, R.A., Nandakumar, M., Vakharia, V.N., Wu, L.P., 2005. The Toll pathway is important for an antiviral response in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 102, 7257–7262. doi:10.1073/pnas.0409181102

Zambon, R.A., Vakharia, V.N., Wu, L.P., 2006. RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. Cell. Microbiol. 8, 880–889. doi:10.1111/j.1462-5822.2006.00688.x

Zamore, P.D., Tuschl, T., Sharp, P.A., Bartel, D.P., 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101, 25–33. doi:10.1016/S0092-8674(00)80620-0

Zeng, L., Falgout, B., Markoff, L., 1998. Identification of specific nucleotide sequences within the conserved 3'-SL in the dengue type 2 virus genome required for replication. J. Virol. 72, 7510–7522.

Zeng, Y., Cullen, B.R., 2003. Sequence requirements for micro RNA processing and function in human cells. RNA 9, 112–123.

Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J., Chua, N.H., 2006. Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. Genes Dev. 20, 3255. doi:10.1101/gad.1495506.hang

Zhou, Y., Liu, Y., Yan, H., Li, Y., Zhang, H., Xu, J., Puthiyakunnon, S., Chen, X., 2014. miR-281, an abundant midgut-specific miRNA of the vector mosquito Aedes albopictus enhances dengue virus replication. Parasit. Vectors 7, 488. doi:10.1186/s13071-014-0488-4

Zou, J., Lee, L.T., Wang, Q.Y., Xie, X., Lu, S., Yau, Y.H., Yuan, Z., Geifman Shochat, S., Kang, C., Lescar, J., Shi, P.-Y., 2015a. Mapping the interactions between the NS4B and NS3 proteins of dengue virus. J. Virol. 89, 3471-3483. doi:10.1128/JVI.03454-14

Zou, J., Xie, X., Wang, Q.-Y., Dong, H., Lee, M.Y., Kang, C., Yuan, Z., Shi, P.-Y., 2015b. Characterization of dengue virus NS4A and NS4B protein interaction. J. Virol. 89, 3455–3470. doi:10.1128/JVI.03453-14