ARBOVIRUS INFECTION DYNAMICS IN THE MOSQUITO Aedes aegypti

A Dissertation in
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by
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ABSTRACT

Arboviral diseases account for more than 17% of all infectious diseases globally, among the most prevalent are dengue fever and chikungunya, with over half of world’s population at risk of infection. Although mortality is generally low, the morbidity caused by these pathogens is associated with a substantial socioeconomic burden. Occasionally, infection with dengue virus (DENV) can lead to death due to dengue shock syndrome in all ages and chikungunya virus (CHIKV) infection can be severe for infants, with nearly half of infected newborns experiencing encephalopathy and multiple organ dysfunction. Both DENV and CHIKV are RNA viruses transmitted through the bite of the mosquito *Aedes aegypti*. Arboviral infection in the mosquito is a complex process, as the dynamics of infection can be influenced by the genetic variation of the virus altering the infection rates and transmission potential in mosquitoes. Competition between strains or serotypes can also affect viral population dynamics within the vector, thus modulating the transmission potential. This happens in nature when mosquitoes take multiple blood meals from several different hosts, each infected with a different or multiple virus. How different viruses interact and compete inside the mosquito during the stepwise process of infection or how the vector immune response changes in response to these different viruses is an area of vital research.

Due to the lack of an effective vaccine and antiviral drugs, vector control is the most efficient strategy in reducing arbovirus transmission. Therefore, understanding the basic processes shaping vector-virus interactions is necessary for both implementation of more effective control strategies and the development of genetic modifications (GMO) and
biocontrol in mosquitoes to limit transmission. Current example of such technologies includes clustered regularly interspaced short palindromic repeats (CRISPR) and the use of the endosymbiont Wolbachia. The three major aims of my dissertation are to investigate the 1) infection dynamics of DENV in the mosquito *Ae. aegypti*; 2) competitive interactions between co-infecting DENV serotypes in both wild type and *Wolbachia* – infected mosquitoes and 3) genetic basis of *Ae. aegypti* immune response to DENV and CHIKV infection.

In **chapter 1** I reviewed the history and role of *Ae. aegypti* as a disease vector and the importance of my research by studying the process of infection with different arboviruses inside the vector. **In chapter 2** I examined the infection dynamics of DENV in the mosquito *Ae. aegypti*, specifically, the effect of infectious dose and serotype on the growth kinetics of virus in the tissues define the stepwise process of infection in the mosquito body. In **chapter 3** I studied the competitive interactions between co-infecting DENV serotypes both in wild type mosquitoes and in *Wolbachia*-infected *Ae. aegypti*. **Chapter 4** examines the comparative genetic basis of *Ae. aegypti* response to DENV and CHIKV infection using a family-breeding design. Finally, **chapter 5** synthesizes the findings of the dissertation, including future directions, as well as discussing unanswered questions in this research area.
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“Try not. Do or do not, there is no try” – Master Yoda.
Chapter 1
Introduction

1.1 *Aedes aegypti*

The yellow fever mosquito, *Ae. aegypti*, is one of the most feared and important dipterans around the world (McGregor & Connelly, 2020). The reason behind its notoriety is because no other mosquito species has had such a dramatic impact on human history (Jeffrey R. Powell & Tabachnick, 2013). Often called, “the most dangerous animal in the planet”, *Ae. aegypti* alone is responsible for the transmission of at least half a dozen medically important pathogens to humans (Jeffrey R. Powell, 2016), with the earliest recorded mosquito-borne virus outbreak of yellow fever occurring in the XV century (Staples & Monath, 2008). Several of the pathogens transmitted by *Ae. aegypti* are responsible for substantial global socioeconomic losses. Chiefly among them are: yellow fever (YFV), Zika (ZIKV), chikungunya (CHIKV) and dengue (DENV) viruses (Leta et al., 2018). Globally, DENV is the most important vector-borne viral disease, with estimates of 3.6 billion people living in areas of high transmission risk, and up to $20 billion in economic losses linked to DENV infection. Additionally, newly emerging pathogens like CHIKV have caused several major epidemics in the past 20 years, and although mortality remains low, CHIKV infection can be severe in the young and cause ongoing arthritis like complications. Congenital infection rates are upwards of 50% and nearly half of infected
newborns will experience central nervous system disease (encephalopathy) (Morrison, 2014).

The *Ae. aegypti* life cycle occurs in four defined stages: 1) female mosquitoes lay eggs on wet walls of water containers above the waterline, preferably in artificial containers around human habitation, 2) larvae hatch once the water rises enough to cover the eggs, 3) pupae develop until the adult mosquito is ready to emerge and, 4) after emergence, male mosquitoes feed on nectar and females feed almost exclusively on human blood to produce eggs (Christophers, 1960). Because of this, the natural history of *Ae. aegypti* is deeply intertwined with human history (McNeill, 2010). Native and once confined to the African continent, where ancestral populations exhibit sylvatic cycles, *Ae. aegypti* territorial expansion around the globe coincides with the transatlantic slave trade by European colonialists (Lounibos, 1981; Tabachnick, 1991). “Domestication” of the mosquito is thought to have occurred in two ways; 1) with the transition from nomadic to sedentary lifestyles, humans began living in villages in close proximity and storing water year around, creating an ideal niche for the mosquito to exploit, and 2) during slave or trade voyages, where ships had optimal breeding conditions such as crowding and long-term water storages (Jeffrey R. Powell & Tabachnick, 2013; Wilke et al., 2020). Regardless of the true domestication origin of *Ae. aegypti*, its current preference for both human habitat and blood, made this species and efficient disease vector (J. R. Powell, 2018; Scott A Ritchie, 2014).

Since the first confirmed outbreak of YFV in Yucatán, México, in 1614 (Staples & Monath, 2008) and the first time *Ae. aegypti* was identified as an arboviral vector in Cuba
in 1906 (Bancroft, 1906; Reed & Carroll, 1901), there has been a search for mosquito abatement methods, eventually leading to the current concept of vector control. From an epidemiological point of view, vector control methods can only be effective if they are contrasted with a mosquito efficacy in transmitting a pathogen (Roiz et al., 2018; David L. Smith et al., 2012). This efficacy has been described as the vectorial capacity (VC), a mathematical link between the mosquito’s natural history and pathogen transmission (Macdonald, 1957). VC defines, within a vector population, the pathogen transmission potential by using mosquito metrics such as: daily survival rate, biting rate and the extrinsic incubation period (EIP) (Macdonald, 1957; David L. Smith et al., 2012; D. L. Smith et al., 2014; Souza-Neto, Powell, & Bonizzoni, 2019). The EIP has been defined as the time it takes from pathogen acquisition to transmission by a vector, to a susceptible vertebrate host, and it represents one of the most influential parameters in modeling mosquito-borne pathogen transmission (Ohm et al., 2018; Claudia Rückert & Ebel, 2018; Tjaden, Thomas, Fischer, & Beierkuhnlein, 2013). Several factors can influence the length of the EIP, such as temperature, vector microbiome, initial pathogen dose, and both the mosquito and viral genotype (Armstrong & Rico-Hesse, 2003; Carrington & Simmons, 2014; Fontaine et al., 2018; A. Gloria-Soria, Armstrong, Powell, & Turner, 2017; Gould & Higgs, 2009; Kauffman & Kramer, 2017; L. Lambrechts et al., 2009; Le Flohic, Porphyre, Barbazan, & Gonzalez, 2013). The interactions between any of these factors in shaping the transmission dynamics of a pathogen represents vital research area. Specifically, the vector - pathogen interactions are the main focus of this dissertation, using Ae. aegypti and DENV and CHIKV viruses.
1.2 Dengue and chikungunya

Dengue fever is one of the most prevalent arboviral diseases globally (Murray, Quam, & Wilder-Smith, 2013), with estimates of more than 390 million infections per year (Bhatt et al., 2013) and over half of the world’s population at risk (Brady et al., 2012). Although mortality is generally low, the morbidity caused by dengue fever is associated with a substantial socioeconomic burden (Duane J. Gubler, 2011). Dengue fever is caused by four different serotypes of virus (DENV 1–4), belonging to the *Flaviviridae* family, (Deen et al., 2006) that are 65–70% similar at the DNA sequence level across their ~11-kb genomes (Perera & Kuhn, 2008), while also exhibiting a high-average within-serotype diversity of ~3% at the amino acid level (Azhar et al., 2015).

Compared to DENV, CHIKV was relatively unknown until it started disseminating across the globe in 2004, causing large outbreaks in Latin America, Asia and Africa (Petersen & Powers, 2016). Chikungunya fever is caused by a single CHIKV serotype that belongs to the *Alphavirus* genus and it has an ~11 kb single stranded positive-sense RNA genome (Tanabe et al., 2018). The more substantial impact of the disease, is in long term morbidity (Silva & Dermody, 2017). While adults tend to experience a syndrome involving rash, fever and arthritis, many go on to have long-term complications and up to 50% of infected newborns will develop encephalopathy. Additionally, recent studies have determined that 48% of infected individuals develop chronic arthritis within 20 months of infection (Yactayo, Staples, Millot, Cibrelus, & Ramon-Pardo, 2016).
1.3 Vector-virus dynamics

Arboviral infection in the mosquito is a complex process (Lequime, Fontaine, Ar Gouilh, Moltini-Conclois, & Lambrechts, 2016). Viruses must infect several tissues, including the midgut and salivary glands, in a stepwise fashion (Franz, Kantor, Passarelli, & Clem, 2015). Following ingestion, the virus enters the midgut epithelial cells, where it replicates. Subsequently, it disseminates and infects secondary tissues, ultimately reaching the salivary glands (Khoo, Doty, Held, Olson, & Franz, 2013). The midgut is thought to represent a key barrier in the infection process, preventing many mosquitoes from reaching a disseminated infection stage (Cox, Brown, & Rico-Hesse, 2011). Once the mosquito is infected with a virus, the infection can persist in multiple tissues and the vector has the potential of constantly spreading the pathogen throughout its life (Miller, Mitchell, & Ballinger, 1989). A key determinant in the kinetics of infection is the antiviral immunity of the mosquito, that ultimately affects the EIP and the mosquito VC. Several innate immune pathways have been reported to control or modulate viral infection inside the mosquito in both systemic and tissue specific ways. Some of the main pathways are, RNAi, Toll, Janus Kinase - signal transduction and activators of transcription (JAK-STAT), the Immune deficiency factor (IMD) and antimicrobial peptides (AMP’s) (Bartholomay et al., 2010; Blair & Olson, 2015; Cheng, Liu, Wang, & Xiao, 2016; R. Zhang et al., 2017).

The kinetics of infection are equally influenced by genetic variation in the virus. For DENV, comparisons between strains within single serotypes, have revealed variation in both infection rates and EIP in mosquitoes (Anderson & Rico-Hesse, 2006; Armstrong & Rico-Hesse, 2003), potentially due to differences in viral replication rates (Cox et al.,
In humans, genetic variation within and between serotypes also determines relative viral fitness (replication rate) (Louis Lambrechts et al., 2012; Ty Hang et al., 2010), with particular strains linked to more severe disease outcome. The intra-host/vector diversity of DENV can also play a role in transmission, such as variants with a replicative advantage can spread more rapidly overall, eventually displacing those lower replication rates. (Guzman & Harris, 2015). For example, an uncharacteristically large outbreak of dengue in Cairns, Australia in 2008/2009 was attributed to the very short EIP of the DENV-3 strain in the mosquito (S. A. Ritchie et al., 2013). DENV-2 strains from the American and South East Asian genotypes differ in their EIP lengths, with the South East Asian genotypes having shorter EIPs. This shorter EIP was thought, in part, to explain the displacement of the American DENV strains in South America by the Asian lineage (Anderson & Rico-Hesse, 2006). Surprisingly, little is known about DENV kinetics in the mosquito and how the virus interacts with individual tissues during infection. Given that the virus moves in a stepwise fashion, selective pressures in initial tissues might have cumulative effects on the viral kinetics in downstream tissues (Hall, Bento, & Ebert, 2017).

Competition between strains or serotypes can also affect viral population dynamics within the vector thus modulating the transmission potential (Louis Lambrechts et al., 2012; Vogels et al., 2019). This happens in nature when mosquitoes take multiple blood meals from several different hosts, each infected with a different or multiple DENV serotypes (Shrivastava et al., 2018). In controlled laboratory experiments, using field derived mosquito populations, there are no differences in dissemination and transmission rates between DENV-1 and DENV-4 mono-infections in Ae. aegypti, but during co-
infection, DENV-4 has a much higher dissemination rate, leading to the exclusive presence of DENV-4 in the saliva (Vazeille, Gaborit, Mousson, Girod, & Failloux, 2016). Furthermore, differential replication between DENV-2 and DENV-3 have been shown, with DENV-2 exhibiting a much higher replication efficiency in both *in vitro* and *in vivo* during co-infection (Quintero-Gil, Ospina, Osorio-Benitez, & Martinez-Gutierrez, 2014). Additionally, the impact of co-infection with different families of arboviruses in vector competence has just been recently studied. For example, mosquitoes exposed to double or triple CHIKV, ZIKV and DENV viruses were capable of transmitting all pathogens concurrently, without noticeable changes to mosquito infection and dissemination rates (C. Rückert et al., 2017). Co-infection studies may shed light on the outcome of competitive processes in field mosquitoes, even if rare, but also importantly allow for direct comparisons of the transmissibility between viruses.

Compared to other viruses, CHIKV is much less studied. This is in part due to its very recent re-emergence (Wahid, Ali, Rafique, & Idrees, 2017), but also the requirement that all in vitro and in vivo CHIKV research be carried out under BSL-3 conditions. To date, there are only a handful of studies in the literature that examine the basis of the mosquito’s antiviral response to CHIKV infection (Chowdhury et al., 2020; McFarlane et al., 2014; Zhao, Alto, Jiang, Yu, & Zhang, 2019). Interestingly, the traditional mosquito innate immune pathways (Toll, JAK/STAT, RNAi) play little or no role in limiting CHIKV infection (McFarlane et al., 2014; Shrinet, Srivastava, & Sunil, 2017). Not surprisingly then, the transcriptional response of mosquitoes infected with CHIKV and DENV also share little overlap (Shrinet et al., 2017). Additionally, there have been several recent
discoveries of novel non-canonical antiviral genes in *Drosophila* (Kounatidis et al., 2017; L. Zhang et al., 2020) and in *Ae. aegypti* (Sigle & McGraw, 2019). The antiviral action of each of these genes was revealed only by examining population level genetic variation in the antiviral response. Quantitative genetic studies have also revealed differences in the transmission potential between arboviruses (Sanchez-Vargas, Olson, & Black, 2021) and helped understand the genetic basis of *Wolbachia* mediated pathogen blocking in in *Ae. aegypti* (Terradas, Allen, Chenoweth, & McGraw, 2017).

### 1.4 Vector control

One of the most important outcomes of researching mosquito-pathogen interactions is the development and optimization of vector control methods. *Ae. aegypti* natural history has allowed this species to encroach and establish in new geographic areas (McGregor & Connelly, 2020). Classic vector control methods, used to this day, include the removal of breeding containers and areas of stagnant water (D. J. Gubler & Clark, 1996; Jones, Ant, Cameron, & Logan, 2021; Lobo, Achee, Greico, & Collins, 2018). However, because of the mosquito’s preference for small and often cryptic breeding grounds, complete removal its challenging, even for the most prepare abatement programs particularly in areas of high annual rainfall (Cheong, 1967; McGregor & Connelly, 2020). Additionally, *Ae. aegypti* has been shown to breed in septic tanks, sewage plants, cesspits and stormwater drains, making the task of monitoring mosquito populations even more cumbersome (Arana-Guardia et al., 2014; Babu, Panicker, & Das, 1983; Barrera et al., 2008). Insecticide-based approaches offer the next line of suppression against mosquito populations. This method can target both larvae and adult mosquitoes and it has been responsible for the eradication
of diseases like typhus, malaria, yellow fever and even dengue fever in many countries (Achee et al., 2015; Keatinge, 1949; Rogan & Chen, 2005). The two main problems with the use of insecticides are that 1) they can cause widespread negative health effects in non-targets, such as humans, animals or beneficial insects and 2) target insects can develop resistance, driving the use of higher insecticides concentrations and increasing the negative effects (Aktar, Sengupta, & Chowdhury, 2009; Calvo-Agudo et al., 2019; Rivero, Vézilier, Weill, Read, & Gandon, 2010). For *Ae. aegypti*, many studies have shown low efficiency of insecticides in wild populations (Maciel-de-Freitas et al., 2014; Marcombe et al., 2011; Moyes et al., 2017; Pleresub et al., 2016) particularly due to point mutations in the voltage sodium channel, the main target of several classes of insecticides (Brito et al., 2013; Moyes et al., 2017).

Novel biotechnological vector control strategies that could work in tandem with the classic methods are currently being developed (Barrera et al., 2008; Jones et al., 2021; Roiz et al., 2018; Wilson et al., 2020). Some of the new approaches for reducing arthropod borne diseases include the use of gene editing technologies like RNA interference (RNAi) and CRISPR to induce pathogen resistant phenotypes or to kill mosquitoes at early developmental stages (Chaverra-Rodriguez et al., 2018; Macias, Ohm, & Rasgon, 2017). Other strategies are the use of sterile insect technique (SIT) involving mass rearing of the targeted mosquito, followed by sterilization and release into the wild to suppress the population reproductive potential (Alphey et al., 2010; Kandul et al., 2019; Zheng et al., 2019). Similarly, the release of insects carrying a dominant lethal (RIDL) uses dominant lethal transgenes that are suppress during mass laboratory rearing, but once the mosquitoes
are release in the wild their offspring will die due to the lack of lethal transgene suppression in the field (Lin & Wang, 2015; Phuc et al., 2007; Watkinson-Powell & Alphey, 2017). Finally, the bacteria *Wolbachia pipiens* is currently being trialed and used as a vector control method (John H. Werren, Baldo, & Clark, 2008; P.-S. Yen & Failloux, 2020). *Wolbachia* is an intracellular Gram-negative bacterium that is maternally transmitted and naturally occurs in more than 60% of insect species but not in *Ae. aegypti* (Andrea Gloria-Soria, Chiodo, & Powell, 2018; Hertig & Wolbach, 1924; Hilgenboecker, Hammerstein, Schlattmann, Telschow, & Werren, 2008; J. H. Werren, 1997). *Wolbachia*'s ability to manipulate the host reproductive biology has been shown to cause a several phenotypes in its host, including sex ratio distortions such as male-killing, feminization, parthenogenesis and cytoplasmic incompatibility (CI). In the case of CI, *Wolbachia*-infected males mate with uninfected females and prevent them from producing viable offspring, whereas all other possible crosses are successful. Because *Wolbachia* is vertically-inherited, infected females have a reproductive advantage by default, and the infection tends to spread in populations (Asgharian, Chang, Mazzoglio, & Negri, 2014; McGraw & O'Neill, 1999; Weeks & Breeuwer, 2001; J. H. Yen & Barr, 1971; Zabalou et al., 2004). After Wolbachia was introduced into *Ae. aegypti*, where it formed stably inherited infections, it was also shown to limit the replication of several pathogens during coinfection in the mosquito including; ZIKV, YFV, CHIKV and DENV (Hoffmann et al., 2014; Hurk et al., 2012; Moreira et al.; Walker et al., 2011). Subsequently, *Wolbachia* has been tested in large scale field release programs in numerous locations around the globe, where the bacterium has spread to near fixation and lead to overall reductions in human disease incidence,
measuring up to 77% for dengue virus and 60% for chikungunya virus (Durovni et al., 2019; Indriani et al., 2020; Pinto et al., 2021).

Regardless of the vector control strategy, the effectiveness of these methods can be shaped by vector-pathogen interactions. Therefore, any insights into vector x virus interactions will inform the development and successful implementation of control practices. My research will provide a better understanding of virus-vector dynamics, which will assist in developing intervention points for genetic modification. My findings will help expand our model of vectorial capacity by adding components such as dose and serotype, and our understanding of DENV epidemiology, such as the effect of serotype competition in both transmission and Wolbachia mediated pathogen blocking. Additionally, my work will reveal mosquito genes involved with mosquito immunity to CHIKV, that is less well studied than DENV, offering new potential targets for control.
1.5 References


Asgharian, H., Chang, P. L., Mazzoglio, P. J., & Negri, I. (2014). *Wolbachia* is not all about sex: male-feminizing *Wolbachia* alters the leafhopper *Zyginidia pullula* transcriptome in a mainly sex-independent manner. *Frontiers in microbiology*, 5, 430-430. Do i:1 0.3 389 /fmi cb.2 014.00430


Chapter 2

Intra-host growth kinetics of dengue virus in the mosquito *Aedes aegypti*

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2.1 Abstract

Dengue virus (DENV) transmission by mosquitoes is a time-dependent process that begins with the consumption of an infectious blood-meal. DENV infection then proceeds stepwise through the mosquito from the midgut to the carcass, and ultimately to the salivary glands, where it is secreted into saliva and then transmitted anew on a subsequent bite. We examined viral kinetics in tissues of the *Ae. aegypti* mosquito over a finely graded time course, and as per previous studies, found that initial viral dose and serotype strain diversity control infectivity. We also found that a threshold level of virus is required to establish body-wide infections and that replication kinetics in the early and intermediate tissues do not predict those of the salivary glands. Our findings have implications for mosquito GMO design, modeling the contribution of transmission to vector competence and the role of mosquito kinetics in the overall DENV epidemiological landscape.
2.2 Author summary

DENV infection in the mosquito is a complex and dynamic process. Following ingestion of an infected blood meal, DENV enters the mosquito midgut epithelial cells, where it replicates. Subsequently, the virus disseminates and infects other tissues, including hemocytes, fat body and reproductive organs, ultimately reaching the salivary glands. The kinetics of infection are influenced by genetic variation in the virus. Comparisons between strains within single serotypes, have revealed variation in infection rates in mosquitoes. To explore the role of infectious dose, serotype and tissue in viral infection kinetics we sampled DENV loads in populations of infected mosquitoes over numerous, sequential time-points. We reveal that the kinetics of DENV infection in the midgut, carcass and salivary glands of the mosquito *Ae. aegypti* are strikingly different among the strains selected for this study, and that these differences are also driven by the initial infectious dose.

**Keywords:** mosquito; *Aedes aegypti*; DENV; infection dynamics; within-vector infection; EIP, intra-host; viral kinetics; dengue virus.
2.3 Introduction

Dengue is the most prevalent arboviral disease globally (Murray, Quam, & Wilder-Smith, 2013), with estimates of more than 390 million infections per year (Bhatt et al., 2013) and over half of the world’s population at risk (Brady et al., 2012). Although mortality is generally low, the morbidity caused by dengue disease is associated with a substantial socioeconomic burden (Duane J. Gubler, 2011). Dengue virus (DENV), transmitted to humans through the bite of the *Ae. aegypti* mosquito (Simmons, Farrar, van Vinh Chau, & Wills, 2012), is spreading in large part due to the expanding geographic range of the vector (Powell & Tabachnick, 2013). Previously confined to Africa, *Ae. aegypti* is now present in tropical and temperate regions worldwide, and its spread is assisted by climate change, globalization and ineffective vector control programs (Colón-González, Fezzi, Lake, & Hunter, 2013).

DENV infection in the mosquito is a complex and dynamic process (Lequime, Fontaine, Ar Gouilh, Moltini-Conclois, & Lambrechts, 2016). The virus must circumvent multiple tissue barriers, including the midgut and salivary glands, and infect a range of intermediate tissues in a stepwise fashion (Franz, Kantor, Passarelli, & Clem, 2015). Following ingestion, DENV enters the midgut epithelial cells, where it replicates. Subsequently, the virus disseminates and infects secondary tissues, including hemocytes, fat body and reproductive tissue, ultimately reaching the salivary glands (Khoo, Doty, Held, Olson, & Franz, 2013). The midgut is thought to represent the primary barrier to the process of infection (Franz et al., 2015), capable of preventing many mosquitoes from reaching the stage of disseminated infections (Cox, Brown, & Rico-Hesse, 2011). The rate
of this progression dictates the extrinsic incubation period (EIP), or the delay before a mosquito can infect another human on a subsequent bite (Black Iv et al., 2002). The EIP plays an important role in shaping transmission rates (Macdonald, 1957), with longer time windows reducing the number of opportunities for pathogen transmission over a mosquito’s lifetime.

The kinetics of infection are equally influenced by genetic variation in the virus. Dengue fever is caused by four different serotypes of virus (DENV 1–4) (Deen et al., 2006) that are 65–70% similar at the DNA sequence level across their ~11-kb genomes (Perera & Kuhn, 2008), while also exhibiting a high-average within-serotype diversity of ~3% at the amino acid level (Azhar et al., 2015). Comparisons between strains within single serotypes, have revealed variation in both infection rates and EIP in mosquitoes (Anderson & Rico-Hesse, 2006; Armstrong & Rico-Hesse, 2003), most likely due to differences in viral replication rates (Cox et al., 2011). In humans, genetic variation within and between serotypes also determines relative viral fitness (replication rate), epidemic potential and virulence (Lambrechts et al., 2012; Ty Hang et al., 2010), with particular strains linked to more severe clinical manifestations. The intra-host/vector diversity of DENV can also play a role in transmission, such as variants with a replicative advantage can spread more rapidly overall, eventually displacing those with lower fitness (Guzman & Harris, 2015). For example, an uncharacteristically large outbreak of dengue in Cairns, Australia in 2008/2009 was attributed to the very short EIP of the DENV-3 strain in the mosquito (Ritchie et al., 2013).
Surprisingly, little is known about DENV kinetics in the mosquito and how the virus interacts with individual tissues during infection. Mosquitoes have evolved both systemic and tissue specific antiviral mechanisms to limit viral replication (Cheng, Liu, Wang, & Xiao, 2016); thus, viral kinetics may differ between tissues. Given that the virus moves in a stepwise fashion, selective pressures in an initial tissue might therefore have cascading effects on the viral kinetics in downstream tissues (Hall, Bento, & Ebert, 2017). Additionally, different tissue types may offer a diversity of cell types and cellular niches that may vary in their capacity to support DENV replication. For example, the midgut epithelium is a dynamic niche, with cells being shed frequently (Zieler, Garon, Fischer, & Shahabuddin, 2000), whereas other cell types in intermediate tissues may be more stable sites of virus production.

A better understanding of intra vector kinetics will assist with developing optimal intervention points for genetic modification, improve our model of vectorial capacity (Macdonald, 1957) by adding components of dose and serotype, etc. (Christofferson & Mores, 2011) and, expand our understanding of DENV epidemiology, and vector-virus interactions such as peaks in viral load and latency periods in the host. Herein we compared midgut, carcass and salivary gland loads for 4 strains of DENV representing each of the 4 serotypes, fed at either high or low infectious doses, daily over a period of 3 weeks. In so doing, we were able to assess how the factors of dose and serotype strain diversity define susceptibility, EIP and transmissibility but also how tissue specific differences and the inter relationships between tissue kinetics drive transmissibility.
2.4 Methods

2.4.1 *Ae. aegypti* rearing and virus preparation

Approximately 8000 eggs of an *Ae. aegypti* inbred line collected in Townsville, Australia 13 generations previously were vacuum hatched and divided into individual 30 × 40 × 8-cm plastic trays containing 1 liter of reverse osmosis (RO) autoclaved water. Larvae (~150) were placed in individual trays containing 3 liters of RO autoclaved water, supplemented with common fish food (Tetramin®, Melle, Germany). Pupae were collected and placed into breeding cages, containing approximately 450 mosquitoes each. Adults were provided 10% sucrose *ad libitum*. All mosquitoes were reared in a controlled environment at 26°C, 75% relative humidity and a 12-hr light/dark cycle.

The DENV serotypes/strains used for this experiment are listed in Table 2.1. The virus was propagated in cell culture, as described previously (Frentiu, Robinson, Young, McGraw, & O'Neill, 2010). *Ae. albopictus* C6/36 cells were grown at 26°C in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1× Glutamax (Invitrogen) and HEPES buffer. Cells were first allowed to form monolayers of around 60–80% confluence in T175 flasks (Sigma Aldrich, St. Louis, MO), and then inoculated with DENV and maintained in RPMI medium supplemented with 2% FBS. After 7 days post-inoculation, live virus was harvested, titrated via absolute quantification PCR and adjusted to two final viral loads of 10^5 and 10^8 DENV copies per ml. Single-use aliquots were stored at −80°C for subsequent plaque assay titration.
2.4.2 Mosquito infections

Prior to infection, female mosquitoes were sorted and placed in 1-liter plastic cups with a density of ~150 individuals. Sucrose was removed from the mosquitoes 24 hrs, prior to oral infection. Double-chamber glass feeders were covered with pig intestine previously immersed in a 10% sucrose solution. Water heated to 37°C was circulated in the outer chamber of the feeders, and a 1:1 mix of defibrinated sheep blood and the previously titrated DENV virus was placed inside the feeder. The mosquitoes were split into 2 feeding groups, each allowed to feed for ~2 hrs. One group was fed with a DENV infectious blood meal concentration of $10^8$ and the other with $10^5$ DENV copies/ml. After 24 hrs, blood fed mosquitoes were identified by visual inspection and separated into cups of 10 individuals.

2.4.3 Mosquito dissection and RNA extraction

Blood fed *Ae. aegypti* mosquitoes were collected daily, 10 per feeding group, over 20 days. Mosquitoes were anesthetized by chilling and dissected on a chill plate in a drop of sterile phosphate buffer saline (PBS). Midgut, salivary glands and carcass tissues were

<table>
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<th>Serotype</th>
<th>Strain</th>
<th>Passage</th>
<th>Accession number</th>
<th>Place of origin</th>
<th>Collection date</th>
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<td>DENV-2</td>
<td>ET-300</td>
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<td>EF440433.1</td>
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<td>2000</td>
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<tr>
<td>DENV-3</td>
<td>Cairns/08/09</td>
<td>9</td>
<td>JN406515.1</td>
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<td>DENV-4</td>
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<td>None</td>
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NA: not available
placed in separated 1.5-ml microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) containing 200 µl of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and 2-mm glass beads. Samples were homogenized and frozen at −80°C. RNA extraction was performed using the TRIzol RNA extraction protocol according to the manufacturer’s instructions, and RNA was eluted in 25 µl of nucleic acid-free water. Samples were DNase treated (Life Technologies, Carlsbad, CA, USA) according to the manufacturer instructions (Amuzu, Simmons, & McGraw, 2015). Total RNA was determined with a NanoDrop™ lite spectrophotometer (Thermo Scientific, Waltham, MA).

2.4.4 DENV absolute quantification via RT-qPCR

Quantitative real-time PCR (RT-qPCR) reactions for DENV detections were performed with 4× TaqMan® fast virus 1-step master mix (Roche Applied Science, Switzerland), PCR-grade water, 10 µM of DENV primers and probe and 2.5 µl of RNA in a final volume of 10 µl. Reactions were run in microseal 96 microplates (Life Technologies Life, Technologies, Carlsbad, CA) covered with optically clear film. LightCycler 480 (Roche Applied Science, Switzerland) thermal cycling conditions were 50°C for 5 min for reverse transcription, 95°C for 10 s for RT inactivation/denaturation followed by 50 amplification cycles of 95°C for 3 s, 60°C 30 s and 72°C for 1 s.

Standard curves were generated from triplicate samples on each plate spanning the range of 10 to 10⁸ copies/reaction of DENV fragment copies. DENV standards were constructed as described elsewhere (Ye et al., 2015), and they contained the 3’UTR of the DENV genome. The limit of detection was set at 10 copies in this experiment; water was
used as a negative control and standards were run in triplicates. The concentration of DENV genome copies in each sample was extrapolated from the standard curve as DENV copies per nanogram of total RNA.

The primer sequences used for the detection of DENV, as previously used (Ye et al., 2014), were F: 5′-AAGGACTAGAGGTTAGAGGAGACCC-3′, R: 5′CGTTCTG TG CCTGGAATGATG-3′ and P: 5′-HEX- AACAG CATATTGA CGCTGGAGAG ACC AGA-BHQ1-3′.

2.4.5 Survival assay

A second set of Townsville mosquitoes were reared in the same conditions as above. Mosquitoes were blood fed with the two viral concentrations as above (10⁸ and 10⁵ DENV copies/ml). A total of 80 fed mosquitoes per concentration were separated into clear 200-ml cups, each containing 10 individuals. They were monitored daily to assess death until all had expired. Neither DENV serotypes nor infectious doses had an effect on survival compared to a blood-only fed control (Appendix A).

2.4.6 Data analysis

Data analysis was carried out in R v 3.6.0 (http://www.r-project.org/). For statistical analysis of the infection frequency, quasibinomial GLM was used to correct for overdispersion of data, and Tukey for contrasts was used for post hoc comparisons. For the kinetics of DENV virus, first we fit a 3-parameter logistic DRC model to DENV loads in each tissue, dose and serotype combination, which modeled the upper asymptote
or the maximum DENV load, the slope or growth rate at the midpoint, and the midpoint or infection age which is halfway between the lower and upper asymptotes (i.e. ED50). We then estimated separate trends for each treatment combination. From the candidate models, the best fitting model for each treatment combination was selected using Akaike’s information criterion (AIC). For MG and CA tissues the best fitting model was the 3-parameter logistic model, while for the SG tissue we identified a linear relationship between DPI and DENV load. DRC parameters and pairwise comparisons were estimated with the DRC package (Ritz, Baty, Streibig, & Gerhard, 2016). The best model was selected based on the AIC (yielding the lowest) values. In general, for the salivary glands, the best fit was a linear model; thus, they were subsequently analyzed in this fashion. For the average DENV load at DPI 20, significant differences were based on Tukey post hoc comparison following ANOVAs. All DENV loads were reported on a log scale given the value range.

2.5 Results

We orally challenged inbred wild type *Ae. aegypti* mosquitoes with two infectious doses (10^8 and 10^5 DENV copies/ml), representative of plasma viremia ranges in humans (Duong et al., 2015), of the 4 DENV serotypes. In each vector competence experiment, mosquitoes were blood fed with a strain representing each of the 4 DENV serotypes at the 2 infectious doses. Ten individuals were then collected daily for 20 days, starting from the first day post-infection (DPI), to measure DENV infection status and infection kinetics in 3 key tissues - midgut (MG), carcass (CA) and salivary glands (SG) - per mosquito.
2.5.1 The proportion of DENV-infected mosquitoes is affected by both infectious dose and DPI

Infection status was determined by analyzing the proportion of individuals positive for DENV in each tissue at each DPI, based on the presence of a positive qRT-PCR. Infection prevalence (Figure 2.1) in the MG, CA and SG in all the serotypes was significantly influenced by the infectious dose and DPI (Table 2.2). In only one case was there a significant interaction between these 2 factors (Table 2.2, DENV-3 SG, GLM, F=5.23, p=0.02), indicating that, in general, dose and age of infection act independently to shape infection status.

Mosquito populations fed with higher doses had higher infection rates across tissues and strains (Figure 2.1). The DENV-1 and -2 strains exhibited a higher infection rate in all tissues and at both infectious doses compared to the DENV-3 and -4 strains. DENV-4 exhibited especially low infection rates in all tissues at the low infectious dose. The significance of DPI demonstrates that infection status is heavily time-dependent. For the DENV-1 and -2 strains, in the high infectious dose, infection rates were constantly high throughout 20 DPI in contrast to the low infectious dose, where there is noticeable difference between the infection rates between early and late DPI. For the DENV-3 strain, both infectious doses peaked in infection rate at ~12 days in all tissues. In the DENV-4 strain, infection levels are weaker than the other strains, which may contribute to the greater variation in the effect of time across tissues and doses.
2.5.2 EIP is affected by the serotype strain and initial infectious dose

If we take SG detection of viral load as a proxy for presence of virus in the saliva, the susceptibility data (Figure 2.1) can also be used to shed light on EIP. EIP can be measured in multiple ways, including the day of first arrival of virus in the tissue/saliva or the point at which 50% of the population has infected tissue/saliva. For all strains and infectious doses, viral load can be detected in the SG already by day 2. In many cases, especially for low doses, viral load appears in the SG and then detection is lost for several days before returning and becoming stable (Figure 2.1). If we calculate an EIP$_{50}$ for populations where infection continues for more than two days, we see that infectious dose and serotype strain diversity are predictors; for the strains representing DENV 1-4 at low-dose, they are 5, 6, 9 and 15 days, respectively, while for the high-dose they are 1, 3, 7 and 7 days.
Figure 2.1: Susceptibility of DENV strains by DPI, tissue and infectious dose. The percentage of DENV infectious mosquitoes calculated daily out of 10 individuals. Low infectious dose in red and high infectious dose in blue. Stars represent EIP50 at the salivary gland for both infectious doses. Statistical analysis is presented in Table 2.3.
Table 2.2. Susceptibility by Strain, Tissue, Dose and DPI

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*P<0.05, **P<0.01, ***P<0.001. ns. not significant.
2.5.3 Intra-host DENV kinetics are influenced by initial infectious dose and strain

To quantify the kinetics of DENV infection, we assessed whether the cumulative change in DENV load in each tissue followed a trend that was either a sigmoidal or linear over time. Thus, we first fit a 3-parameter logistic model to DENV loads in each tissue, dose and serotype combination, which modeled the upper asymptote or the maximum DENV load, the slope or growth rate at the midpoint, and the midpoint or infection age which is halfway between the lower and upper asymptotes (i.e., ED50). We then estimated separate trends for each treatment combination. From the candidate models, the best fitting model for each treatment combination was selected using Akaike’s information criterion (Appendix A). For MG and CA tissues the best fitting model was the 3-parameter logistic model, while for the SG tissue we identified a linear relationship between DPI and DENV load. We used the slope and maximum DENV load parameters to compare the behavior of the different serotypes at different infectious doses and across tissues and times post-infection (Figure 2.2).

2.5.4 Some treatments do not show substantial DENV replication and others exhibit subpopulation structure of DENV load in infected mosquitoes

For a number of treatments DENV loads did not rise to very high levels (Figure 2.2). For example, in the low-dose categories neither the DENV-3 nor -4 strains reached a load higher than log 10^3 in any tissue as measured by population mean. In these cases, although we were able to fit a dose response curve (DRC) to the data, the parameters of max load and growth rate are not informative (Appendix A). Therefore, the subsequent
discussion of growth parameter estimates focuses only on those treatments with substantial DENV loads and replication (Tables 2.3 and 2.4). For the remaining treatments we aimed to fit a single DRC to the different treatment combinations (infectious dose, tissue and strain). In several treatments, however, we identified potential subpopulation structure, with a proportion of the mosquitoes exhibiting high loads and the rest exhibiting very low loads. Those treatments were the low infectious dose in the MG and CA for strains of serotype DENV-1 and -2 and the high infectious dose in the MG and CA for strains of serotypes DENV-3 and -4 (Figure 2.2). After noticing subpopulation structure in our data, we applied Hartigan’s dip test for all datasets. For any with a significant result, we then used a bimodality coefficient test to partition the data. We found that using a coefficient of bimodality of >0.75 effectively split our datasets into two clear subpopulations (Appendix A) using the following criteria: (1) for the y-axis, we selected the lowest load in the histogram located between the two highest peaks (Appendix A); and (2) for the x-axis, we identified the DPI where the two populations began to diverge.
**Figure 2.2:** DENV kinetics by strain, tissue and infectious dose. For the midgut and carcass, lines represent the fitted parametric growth curves for DENV loads. Growth curves were fitted on logarithmic transformed data. For the salivary glands, lines represent a linear regression for DENV load. Linear regressions were fitted on logarithmic transformed data. For all tissues and serotypes, points represent individual mosquito values (n=10); in red, low infectious dose ($10^5$ DENV copies/ml); in blue, high infectious dose ($10^8$ DENV copies/ml). Points for the subpopulations have been “jittered” to avoid overlap. Dashed lines represent subpopulations.
Table 2.3 DRC parameters for successful infections by tissue, infectious dose and strain

<table>
<thead>
<tr>
<th>Infectious dose</th>
<th>Serotype</th>
<th>Tissue</th>
<th>Parameter</th>
<th>Parameter estimate</th>
<th>Standard error</th>
<th>p-value</th>
<th>Sig.</th>
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</thead>
<tbody>
<tr>
<td>HIGH</td>
<td>DENV-3</td>
<td>MIDGUT</td>
<td>Growth rate</td>
<td>0.66</td>
<td>0.07</td>
<td>&lt;0.001</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>DENV-1</td>
<td>MIDGUT</td>
<td>Growth rate</td>
<td>0.64</td>
<td>0.21</td>
<td>0.003</td>
<td>**</td>
</tr>
<tr>
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<td>DENV-4</td>
<td>MIDGUT</td>
<td>Growth rate</td>
<td>0.59</td>
<td>0.11</td>
<td>&lt;0.001</td>
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</tr>
<tr>
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<td>DENV-2</td>
<td>MIDGUT</td>
<td>Growth rate</td>
<td>0.31</td>
<td>0.04</td>
<td>&lt;0.001</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>DENV-4</td>
<td>CARCASS</td>
<td>Max DENV load</td>
<td>1.36</td>
<td>0.48</td>
<td>0.005</td>
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<td>DENV-3</td>
<td>CARCASS</td>
<td>Max DENV load</td>
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<td>0.1</td>
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<td>DENV-2</td>
<td>CARCASS</td>
<td>Max DENV load</td>
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<td>DENV-1</td>
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<tr>
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<td>DENV-4</td>
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<td>ED50</td>
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<td>DENV-1</td>
<td>MIDGUT</td>
<td>ED50</td>
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<td>***</td>
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<td>MIDGUT</td>
<td>ED50</td>
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<td>CARCASS</td>
<td>ED50</td>
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<td>MIDGUT</td>
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</tr>
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*P<0.05, **P<0.01, ***P<0.001. p-value indicates if parameter estimate is different than zero.
Standard error of parameter estimates. Treatments ranked by parameter estimate.
Table 2.4 DRC parameter contrasts for successful infections between strains, infectious dose and subpopulations

<table>
<thead>
<tr>
<th>Infectious dose</th>
<th>Serotype contrast</th>
<th>Tissue</th>
<th>Parameter estimate</th>
<th>Standard error</th>
<th>p-value</th>
<th>Sig.</th>
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<td>ns</td>
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<td></td>
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<td>0.001</td>
<td>**</td>
</tr>
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<td>ns</td>
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<tr>
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<td>DENV-1 vs DENV 2</td>
<td>MIDGUT</td>
<td>Growth rate</td>
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<td>0.07</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Max DENV load</td>
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<td>&lt;0.001</td>
<td>***</td>
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<tr>
<td></td>
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<td>CARCASS</td>
<td>Growth rate</td>
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<tr>
<td></td>
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<tr>
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<td>CARCASS</td>
<td>Growth rate</td>
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<tr>
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<td>DENV-1 vs DENV-4</td>
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<td>0.08</td>
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<tr>
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<td>Max DENV load</td>
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<td>0.01</td>
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<tr>
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<td>0.01</td>
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<td></td>
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<td>0.01</td>
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<td>0.05</td>
<td>ns</td>
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<td>0.01</td>
<td>**</td>
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<tr>
<td></td>
<td></td>
<td>CARCASS</td>
<td>Growth rate</td>
<td>0.37</td>
<td>0.04</td>
<td>**</td>
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<tr>
<td></td>
<td></td>
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<td>**</td>
</tr>
<tr>
<td>HIGH</td>
<td>DENV-2 vs DENV-4</td>
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<td>Growth rate</td>
<td>0.30</td>
<td>&lt;0.001</td>
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</tr>
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<td></td>
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<td>Max DENV load</td>
<td>0.30</td>
<td>&lt;0.001</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CARCASS</td>
<td>Growth rate</td>
<td>0.30</td>
<td>&lt;0.001</td>
<td>***</td>
</tr>
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<td>Max DENV load</td>
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<tr>
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<td>Growth rate</td>
<td>0.32</td>
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<tr>
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<tr>
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<td></td>
<td>CARCASS</td>
<td>Growth rate</td>
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<td>&lt;0.001</td>
<td>***</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001; ns, not significant.
2.5.5 DENV load is always higher in the midgut than other tissues for high infectious dose

In all serotypes, maximum DENV loads were consistently higher in the MG than the CA. Because we fit linear models to the SG data, we examined the average DENV load at the last DPI (20 days) in lieu of a maximum load (Appendix A). For all serotypes and high infectious doses, the SG load at this point was lower than the CA loads. This strong pattern suggests a “trickledown” effect for DENV load across the sequentially infected tissues (Figure 2.3). In contrast, low infectious doses resulted in low DENV loads in all tissues and for all strains, and DENV-3 and -4 could not be evaluated due to their lack of successful infection and failure of the selected model to run. Additionally, we ran generalized linearized models on DENV load in the MG and CA (for high dose only) as a predictor for the SG DENV load. We found no significant predictive ability regardless of the serotype and tissue (Appendix A).
2.5.6 Tissue-specific DENV kinetics vary by strain in the high infectious dose

In the MG, the maximum loads of DENV-2 (12.2 log c/ng) and -3 (11.5 log c/ng) are similarly high and greater than the strains representing serotypes 1 (9.11 log c/ng) and 4 (9.74 log c/ng) (Table 2.3). In the CA, the DENV-1 (8.99 log c/ng) and -2 (9.53 log c/ng) strains have the highest loads and are both significantly different from DENV-3 (8.85 log c/ng) and -4 (6.44 log c/ng) strains (Table 2.4). In the SG, the average final load (DPI 20) for the DENV-2 strain is not different from the DENV-1 and -3 strains but is greater than the DENV-4 (Appendix A). Although we have examined only one representative strain from each serotype, these patterns suggest that DENV-2 may be better at replicating in
mosquitoes and that the other serotypes do not show consistent patterns of dominance over one another across tissues. For growth rate, no one strain appeared to dominate over the others across multiple tissues (Tables 2.4 and 2.5). For example, the DENV-3 strain has the highest growth rate (change in viral copies/day) in the MG (0.66 log DENV copies per DPI), whereas DENV-2 strain has the lowest (0.31 log DENV copies per DPI). In the CA, DENV-4 has the highest growth rate (1.36 log DENV copies per DPI), whereas DENV-1 has the lowest (0.21 log DENV copies per DPI). Interestingly, in the SG all strains have distinctly different growth rates, ranked in the following order: DENV-1, 2, 3, and 4 (Table 2.5). This suggests that the growth rates in early process/upstream tissues are not predictive of rates in the final tissue controlling transmissibility.

2.5.7 The DENV-2 strain has a higher maximum DENV load than the DENV-1 strain in the low infectious dose

For the low infectious dose, we only considered mosquitoes infected with either DENV-1 or -2 strains, as the remaining strains did not exhibit “successful” infections meaning that even though infection rate was high, the actual DENV load was substantially low. Here, we saw that the DENV-2 strain had a significantly higher max DENV load in the MG and a higher average final load in the SG than the DENV-1 strain. For growth rate, there were no differences between the DENV-1 and DENV-2 strains in the MG or CA, but in SG, the DENV-1 strain was significantly higher (Table 2.6).
Table 2.5 Linear regression model for SG

<table>
<thead>
<tr>
<th>Infectious dose</th>
<th>Serotype Term</th>
<th>Slope</th>
<th>Standard error</th>
<th>F</th>
<th>p-value</th>
<th>Sig</th>
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</thead>
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<td>DENV-3 DPI</td>
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<td>-1.81</td>
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<td>DENV-4 DPI</td>
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<td>2.31</td>
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</tr>
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<td>0.04</td>
<td>2.87</td>
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*P<0.05, **P<0.01, ***P<0.001. Tukey for contrasts. ns, not significant.

Table 2.6 Serotype contrasts for growth rate in SG

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<tr>
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<th>Serotype contrast</th>
<th>Standard error</th>
<th>df</th>
<th>p-value</th>
<th>Sig</th>
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<td>DENV-1 – DENV-3</td>
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<td>DENV-1 – DENV-4</td>
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<td>DENV-2 – DENV-3</td>
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</tr>
<tr>
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<td>DENV-2 – DENV-4</td>
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<td>DENV-3 – DENV-4</td>
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*P<0.05, **P<0.01, ***P<0.001. Tukey for contrasts. ns, not significant.
2.6 Discussion

To explore the role of infectious dose, serotype and tissue in viral infection kinetics we sampled DENV loads in populations of infected mosquitoes over numerous, sequential time-points of the mosquito’s lifespan relevant to transmission in the field. We reveal that the kinetics of DENV infection in the midgut, carcass and salivary glands of the mosquito *Ae. aegypti* are strikingly different among the strains selected for this study, and that these differences are also driven by the initial infectious dose. Specifically, we showed that (1) initial infectious dose dictates infection frequency, with the DENV-1 and -2 strains analyzed here infecting a greater proportion of mosquitoes than the DENV-3 and -4 strains; (2) for DENV-3 and -4 strains only, the high infectious dose led to strong infection, with lower doses producing lower viral replication; (3) viral growth rates in particular tissues did not predict the total DENV load in that same tissue, with some strains having higher growth rate but lower total DENV load; (4) in the salivary glands all strains have independent growth rates not predicted by growth rates in previous tissues; and (5) for every tissue and infectious dose, the DENV-2 strain reached a higher viral load according to both the average final load at 20 DPI and max DENV load.

The initial infectious dose has been shown to influence the likelihood of a mosquito becoming infected following an infectious blood meal and whether there is ultimately dissemination to the salivary glands after a blood meal (D. J. Gubler, Nalim, Tan, Saipan, & Sulianti Saroso, 1979; Rosen, Roseboom, Gubler, Lien, & Chaniotis, 1985). The amount of virus in human blood required to infect ~50% of a mosquito population varies between serotypes, with DENV-1 and -2 requiring ~10-fold less than DENV-3 and -4. This same
rank order is observed here. However, strain variation within each serotype is substantial (Fontaine et al., 2018), so further comparisons across multiple strains within each serotype are clearly needed to draw any conclusions about whether the strains we have selected are predictive of the entire serotype.

We were also able to identify a potential threshold of virus needed in the blood meal to initiate successful infections and assure likely transmissibility via the salivary glands. A similar threshold effect has been observed in *Drosophila melanogaster* populations challenged with systemic bacterial infections (Duneau et al. 2017). The study identified two possible outcomes of infection; (1) where hosts died with high bacterial burden or (2) where hosts survived with chronic infection and low pathogen load. Bacterial load and the time it took the host to establish an immune response were found to determine the trajectory of infection. Similarly, we saw either high infection (leading to transmissibility) or low/loss of infection. Interestingly, for our study, the threshold effect was driven by the initial infectious dose and by serotype. DENV-1 and -2 strains required less virus to establish an infection, whereas, DENV-3 and -4 strains required substantially more. The failure to initiate a successful infection could be due to poor replication in the midgut or the midgut preventing dissemination (Cox et al., 2011; Khoo et al., 2013; Taracena et al., 2018). This pattern is not unique to the midgut, however, with low and inefficient replication also in disseminated tissues, such as the carcass. The data may simply be the result of low population sizes of virus in combination with the large number of vacant cellular niches. During infection DENV selectively annexes and manipulates host metabolism and machinery to increase viral translation and replication (Chotiwan et al.,
2018; Choy, Sessions, Gubler, & Ooi, 2015; Reid et al., 2018), while also enhancing replication of mosquito cells to increase niche availability in the mosquito (Helt & Harris, 2005). The more virions that infect a given cell, the more efficient this process becomes and, thus, more viruses are produced. Initial viral load combined with inherent replicative differences between strains is likely to produce the pattern we see in our data, that the DENV-1 and 2 strains require a lower dose to produce successful infections.

Interestingly, higher viral growth rates in tissues did not necessarily lead to higher DENV loads. For example, for the high infectious dose of DENV-1, the midgut had the highest max DENV load compared to the carcass, however its growth rate was smaller. This relationship was seen for the other treatments as well. It is possible that the migration of viruses between tissues via the tracheal system or hemolymph (Forrester, Guerbois, Seymour, Spratt, & Weaver, 2012) or directly from nearby tissues is impacting the total viral counts. In support of tracheal or hemolymph travel is the initial spike in infection rate in salivary glands followed by a reduction to zero and then a second front of infection several days later. It is possible that the latter wave represents the larger migration of virus from bodily tissues that then establishes permanent infection. Given how the circulatory system of the mosquito works, viral particles could be carried from tissues that are not in immediate proximity to the salivary glands. This process could directly affect growth rate in upstream tissues. The salivary glands also represent a special case of the entry and exit balance, with virus being excreted into the saliva and hence lost from tissue measures. Under a more natural setting additional factors would likely affect kinetics including, exposure to non-infectious blood meals prior to consumption of an infectious (Zhu et al.,
consumption of sequential dengue infectious meals (Muturi, Buckner, & Bara, 2017) or interactions between DENV and other viruses present in blood meals or the mosquito body (Le Coupanec et al., 2017).

The disconnect between kinetics in intermediate tissues and those of the salivary glands suggests that there are either stochastic processes driving kinetics in early tissues that do not predict how many viruses make it to the salivary glands, or that factors affecting replication rate in the salivary glands are somewhat independent (Cheng et al., 2016). For example, previous research has demonstrated that the strength and efficacy of immunity varies across tissues (Cheng et al., 2016) and in response to different serotypes (Smartt, Shin, & Alto, 2017). It is also possible that DENV is modulating the vector immune response in a strain- or tissue-dependent manner (Sim & Dimopoulos, 2010). Differences between the salivary glands and other tissues may also be due to the number of cellular niches, and/or unique replication rates in those niches. For example, in contrast to the midgut epithelium, DENV tropism in the salivary glands appears to be heterogenous within the tissue; this might be due to the distribution of cellular receptors (Cao-Lormeau, 2009), with a possible higher concentration of viral entry receptors in the lateral distal lobes of the tissue (Salazar, Richardson, Sánchez-Vargas, Olson, & Beaty, 2007). In future studies, it would be useful to quantify the production rate of virus using negative strand strain-specific PCR methods (Raquin & Lambrechts, 2017) in the midgut and in the salivary glands relative to the availability of cellular niches.

The differences in viral kinetics between the DENV strains analyzed here, particularly in the salivary glands, might explain disparities in transmission potential in the
field. A range of studies have linked different genotypes to overall dominance in terms of circulation in human populations both at local and global scales (Dumre et al., 2017; Liebman et al., 2012; Sasmono et al., 2018; Shrivastava et al., 2018). Additionally, DENV variants with a replicative advantage in both the host and vector can spread more rapidly, eventually displacing those with lower fitness (Guzman & Harris, 2015). Our findings indicate that 2 of the 4 DENV strains studied here show greater capacity to replicate in the mosquito. In some cases, these differences may have consequences for public health, as some specific variants of DENV have been linked to more severe clinical manifestations than others (Balmaseda et al., 2006; Halsey et al., 2012; Nisalak et al., 2000; OhAinle et al., 2011). These findings also have implications for the mosquito’s contribution to the DENV epidemiological landscape, with the understanding that aspects of human immunity, including cross-reactivity and non-specific serotype responses also shape the nature of circulating variants (Reich et al., 2013). Lastly, our work provides better insight into ideal design of vector competence experiments and the selection of gene candidates for the engineering of virus refractory mosquitoes. Specifically, our findings support a greater focus on salivary gland or saliva-based measures of infection and genes that either completely reduce viral loads in the midgut or that control viral replication in the endpoint tissue, salivary glands.

2.7 Acknowledgments

The authors would like to thank Emily Kerton, for her help with insect rearing and sample preparation.
2.8 References


Chapter 3

The effects of DENV serotype competition and co-infection on viral kinetics
in Wolbachia-infected and uninfected Aedes aegypti mosquitoes


3.1 Abstract

Background: The Ae. aegypti mosquito is responsible for the transmission of several medically important arthropod-borne viruses, including multiple serotypes of dengue virus (DENV-1, -2, -3, and -4). Competition within the mosquito between DENV serotypes can affect viral infection dynamics, modulating the transmission potential of the pathogen. Vector control remains the main method for limiting dengue fever. The insect endosymbiont Wolbachia pipientis is currently being trialed in field releases globally as a means of biological control because it reduces virus replication inside the mosquito. It is not clear how co-infection between DENV serotypes in the same mosquito might alter the pathogen-blocking phenotype elicited by Wolbachia in Ae. aegypti.

Methods: Five- to 7-day-old female Ae. aegypti from two lines, namely, with (wMel) and without Wolbachia infection (WT), were fed virus-laden blood through an artificial membrane with either a mix of DENV-2 and -3 or the same DENV serotypes
singly. Mosquitoes were subsequently incubated inside environmental chambers and collected on the following days post-infection: 3, 4, 5, 7, 8, 9, 11, 12, and 13. Midgut, carcass, and salivary glands were collected from each mosquito at each timepoint and individually analyzed to determine the percentage of DENV infection and viral RNA load via RT-qPCR.

**Results:** We saw that for WT mosquitoes DENV-3 grew to higher viral infection rates across multiple tissues when co-infected with DENV-2 than when it was in a mono-infection. Additionally, we saw a strong pathogen-blocking phenotype in wMel mosquitoes independent of co-infection status.

**Conclusion:** In this study, we demonstrated that the wMel mosquito line is capable of blocking DENV serotype co-infection in a systemic way across the mosquito body. Moreover, we showed that for WT mosquitoes, serotype co-infection can affect infection frequency in a tissue- and time-specific manner and that both viruses have the potential of being transmitted simultaneously. Our findings suggest that the long-term efficacy of *Wolbachia* pathogen blocking is not compromised by arthropod-borne virus co-infection.

**Key words:** dengue; co-infection, *Aedes aegypti*; serotype; *Wolbachia*; infection dynamics
3.2 Introduction

Dengue viruses (DENVs) are medically important arthropod-borne viruses (arboviruses) responsible for up to 300 million cases of dengue fever a year, and it can be caused by any of the four related but antigenically distinct DENV serotypes (DENV-1 to -4) (Murray, Quam, & Wilder-Smith, 2013). In regions with endemic transmission of all four serotypes of DENV, varying predominance of certain serotypes has been observed between seasons (Vasilakis & Weaver, 2008). Differences in the DENV infection kinetics and transmission potential are influenced by the genetic diversity of the four different DENV serotypes. The overall genome sequence-level differences between serotypes are estimated at 20-30% (Holmes & Twiddy, 2003; Rico-Hesse, 2003).

Substantial evidence indicates that variation in the DENV genome of serotypes and strains can have epidemiological significance by altering the extrinsic incubation period (EIP) (Fontaine et al., 2018; Ko, Salem, Chang, & Chao, 2020; L. Lambrechts et al., 2009), defined as the time it takes for the pathogen to be transmitted by the vector (Macdonald, 1957), and therefore has a powerful effect on the scale and speed of epidemics. DENV-2 strains from the American and Southeast Asian genotypes differ in their EIP lengths, with the Southeast Asian genotypes having shorter EIPs. This shorter EIP was thought, in part, to explain the displacement of the American DENV strains in South America by the Asian lineage (Anderson & Rico-Hesse, 2006). Additionally, different DENV serotypes exhibit various degrees of infectivity across the same mosquito populations (C. V. F. Carrington, Foster, Pybus, Bennett, & Holmes, 2005; Ekwudu et al., 2020; Gubler, Nalim, Tan, Saipan, & Sulianti Saroso, 1979; Novelo et al., 2019; Weaver & Vasilakis, 2009). Moreover, oral
susceptibility to DENV-1 was shown to be up to four times higher than that of DENV-3 in *Ae. aegypti* from Senegal, with DENV-1 having higher infection and dissemination rates (Gaye et al., 2014). Finally, systematic analyses of DENV replication kinetics of all four DENV serotypes found significant differences in the infection rate and EIP between serotypes (Ekwudu et al., 2020; Novelo et al., 2019).

Competition between DENV strains and serotypes can also affect viral population dynamics within the vector, thus modulating the transmission potential (Louis Lambrechts et al., 2012; Vogels et al., 2019). This happens in nature when mosquitoes take multiple blood meals from several different hosts that are each infected with a different or multiple DENV serotypes (Shrivastava et al., 2018). In controlled laboratory experiments, using field-derived mosquito populations, there were no differences in dissemination and transmission rates between DENV-1 and DENV-4 mono-infections in *Ae. aegypti*, but during co-infection, DENV-4 had a much higher dissemination rate, leading to the exclusive presence of DENV-4 in the saliva for this particular experimental mosquito population (M. Vazeille, Gaborit, Mousson, Girod, & Failloux, 2016). Furthermore, differential replication between DENV-2 and DENV-3 has been shown, with DENV-2 exhibiting a much higher replication efficiency both *in vitro* and *in vivo* during co-infection (Quintero-Gil, Ospina, Osorio-Benitez, & Martinez-Gutierrez, 2014). Additionally, the effect of co-infection with different families of arboviruses on vector competence has just been recently studied. For example, mosquitoes exposed to double or triple infections with chikungunya virus (CHIKV; *Togaviridae*), Zika virus (ZIKV; *Flaviviridae*), and DENV (*Flaviviridae*) were capable of transmitting all pathogens concurrently, without noticeable
changes to mosquito infection and dissemination rates (Rückert et al., 2017). Co-infection studies may shed light on the outcome of competitive processes in field mosquitoes, even if rare, but also importantly allow for direct comparisons of the transmissibility between viruses.

Given the ease of rearing *Ae. aegypti* in the laboratory, vector competence experiments are an important tool with which to study the effect and interaction between DENV and mosquito genotypes in the transmission potential of the virus. However, one of the main issues with artificial vector competence experiments is that there is too much heterogeneity between experiments that results from environmental variation and its interaction with genetic variation in both the mosquitoes and viruses (Souza-Neto, Powell, & Bonizzoni, 2019). Individual vector competence experiments using single DENV serotypes or strains often give varying results in both infection and transmission rates, making pairwise comparisons difficult to interpret. Moreover, limited data are available on co-infections with different serotypes, with some experiments suggesting competitive disadvantage or superinfection interference between DENV serotypes (Muturi, Buckner, & Bara, 2017). Additionally, it is not clear how these viral dynamics and interactions may be altered in the presence of *Wolbachia* infection, which is currently being trialed in global releases as a means of reducing virus transmission to humans (Dorigatti, McCormack, Nedjati-Gilani, & Ferguson, 2018) and that is known to reduce viral replication in serotype-specific ways (Bell, Katzelnick, & Bedford, 2019; L. B. Carrington et al., 2018; Ferguson et al., 2015). To the best of our knowledge, only one study has looked at the effects of
arboviral co-infection in *Wolbachia*-infected mosquitoes using ZIKV and DENV (Dutra et al., 2016).

To assess the effect of DENV serotype co-infection on transmissibility and any corresponding interactions with *Wolbachia* infection, we used two interdependent approaches. First, we challenged two *Ae. aegypti* mosquito lines that were either *Wolbachia* infected (wMel) or *Wolbachia* uninfected (WT) in both mono- and co-infection vector competence experiments with DENV-2 and DENV-3. We collected midgut, carcass, and salivary glands at nine timepoints post-infection. We then used the infection rate and viral RNA load data to assess the effects of competing serotypes and evaluate their performance in individual mosquitoes and between wMel and WT lines. Second, we determined whether serotype co-infection altered viral infection dynamics relative to the mono-infected state by comparing viral RNA load and infection rate between the two vector competence experiments.

### 3.3 Methods

**3.3.1 Mosquito lines and rearing**

The mosquito lines used for this work have been described previously (Terradas, Allen, Chenoweth, & McGraw, 2017; Walker et al., 2011). The wMel line was collected from the *Wolbachia* release zone in Cairns, Australia, as part of the Eliminate Dengue Program, whereas the WT line, naturally free from *Wolbachia*, was collected outside the *Wolbachia* release zone. Both lines were identified morphologically and with genetic markers as well as screened for the presence/absence of pathogens before being used in
our study (31). WT and wMel larvae were fed Tetramin® fish food (Melle, Germany), and adults were maintained on 10% sucrose. All mosquitoes were reared in a controlled environment at 26°C, 75% relative humidity, and a 12-hr light/dark cycle.

3.3.2 Virus culture and titration

The DENV serotypes/strains used for this experiment are listed in Table 3.1. The virus was propagated in cell culture, as described previously (Frentiu, Robinson, Young, McGraw, & O'Neill, 2010). Briefly, *Ae. albopictus* C6/36 cells were grown at 26°C in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1× Glutamax (Invitrogen), and HEPES buffer. Cells were first allowed to form monolayers of around 60–80% confluence in T175 flasks (Sigma Aldrich, St. Louis, MO), and then, they were inoculated with DENV and maintained in RPMI medium supplemented with 2% FBS. After 7 days post-inoculation, live virus was harvested, titrated via absolute quantification PCR (qPCR) and plaque forming unit assay (as per below), and adjusted to a final concentration of ~4 × 10^5 plaque-forming units (PFU)/ml for both serotypes (Table 3.2) prior to mixing with blood. Live virus was used for all vector competence experiments.

Prior to the above steps, we isolated the viruses at different timepoints from C6/36 cells and assessed their viral RNA loads by qPCR and plaque assay to select the most appropriate day to harvest virus for the vector competence experiments. These pilot experiments also revealed the relationship between viral RNA load estimates by qPCR and live virus estimates by plaque assay. In general, we saw that higher viral RNA loads
correlated with higher plaque assay titers (Appendix B) with viral RNA loads ranging from ~10- to 2000-fold higher than live virus titers, as expected (Choy, Ellis, Ellis, & Gubler, 2013; Richardson, Molina-Cruz, Salazar, & Black, 2006). The average ratio of viral RNA copies relative to PFU/ml was larger for DENV-3 (1300-fold) than for DENV-2 (270-fold), indicating that RT-qPCR was more sensitive for DENV-3. This effect was consistent across the two tested collection timepoints.

### Table 3.1 Dengue serotypes and strains use

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strain</th>
<th>Passage</th>
<th>Accession number</th>
<th>Place of origin</th>
<th>Collection date</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-2</td>
<td>ET-300</td>
<td>11</td>
<td>EF440433.1</td>
<td>East Timor</td>
<td>2000</td>
</tr>
<tr>
<td>DENV-3</td>
<td>Cairns 2008</td>
<td>9</td>
<td>JN406515.1</td>
<td>Australia</td>
<td>2008</td>
</tr>
</tbody>
</table>

### Table 3.2. Viral titers

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Undiluted RT-qPCR titer of infected supernatant (copies/ml)</th>
<th>Final plaque assay titer for the blood meal (PFU/ml)</th>
<th>Fold change difference between RNA copies/mL and PFU/mL</th>
<th>Average ratio of viral RNA copies/ml relative to PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-2</td>
<td>$4 \times 10^7$</td>
<td>$\sim 3 \times 10^5$</td>
<td>146</td>
<td>277.1</td>
</tr>
<tr>
<td>DENV-3</td>
<td>$8 \times 10^8$</td>
<td>$\sim 4 \times 10^5$</td>
<td>2046</td>
<td>1382.1</td>
</tr>
</tbody>
</table>

### 3.3.3 Mosquito infections

The methods for mosquito oral infections have been described previously (Novelo et al., 2019; Terradas et al., 2017). Briefly, prior to oral DENV infections, 6- to 7-day-old adult female mosquitoes were sorted and starved for 24 hrs. For mono-infections, a 1:1 mix of
virus culture and defibrinated sheep blood was prepared. For co-infections, 1 ml of each DENV serotype was combined, and from that blend, 1 ml was combined with 1 ml of defibrinated sheep blood. Glass feeders with double chambers were covered with pig intestine, and water heated to 37°C was circulated in the outer chamber of the feeders. The mosquitoes were allowed to feed for ~2 hrs. Immediately after blood feeding, mosquitoes were knocked down and sorted on ice. Unfed females were discarded. The remaining mosquitoes were returned to 70-ml plastic cups and maintained on 10% sucrose. At days post-infection (DPI) 3, 4, 5, 7, 8, 9, 11, 12, and 13, mosquitoes were anesthetized and dissected in sterile phosphate-buffered saline (PBS). We collected midgut, carcass, and salivary glands from individual mosquitoes. For our vector competence experiments, the mosquito carcass was the collection of tissues that remained after dissecting the midgut and salivary glands. We used the carcass as a proxy for viral dissemination from the mosquito midgut. All tissue collections were conducted on live mosquitoes. Individual tissues were collected in 1.5-ml microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) containing 200 µl of Trizol reagent (Invitrogen) and 2-mm glass beads. Samples were homogenized and frozen at −80°C until RNA extraction.

3.3.4 DENV absolute quantification via RT-qPCR

The RT-qPCR mixture contained 2.5 µl of LC480 master mix (Roche); 4.25 µl of PCR-grade water; 0.25 µl of 10 µM forward (F) primer, reverse (R) primer, and probe (P) specific to each DENV serotype; and 2 µl of RNA. Reactions were run in the LightCycler 480 instrument (Roche), and the thermal cycling conditions were 95°C for 3 minutes, 30 cycles of 95°C for 30 seconds, 50°C for 1 min, and 68°C for 1 min, finalizing with 68°C
for 5 min. Standard curves were generated by triplicate on each plate by analyzing 100 to \(10^7\) copies/reaction of DENV fragment copies with a limit of detection of set at 100 copies. DENV genome copies were extrapolated from the standard curve as DENV copies per tissue. The standards for both serotypes were generated using a ~100-bp conserved region of the NS5 protein of the DENV genome. The detection threshold for both DENV-2 and DENV-3 was set at ~35 Ct. The primers and probes used for the detection of specific DENV serotypes were as follows:

DENV-2-ET300: F primer (primer 9873681; TCCATACACGCCACACATGAG), R primer (primer 98736818; GGGATTTCCCTCCCATGATTC) and Probe-FAM (probe 98084286; 56-fam/AGGTTTGGATTCGAGAAAACCCATGG/3BHQ_1).

DENV-3-Cairns08/09: F primer (primer 98644632; TTTCTG CTCCCAC CAC TTTC), R primer (primer 98451787; CCATCCYGCTCCCTGAGA) and Probe-LC640 (probe9845178; 5LtC640N/AAGAA AGTTGGT AGTCCCC TGCAGACC TCA/3IAb R QSp).

3.3.5 Statistical analysis

All statistical analysis used R v3.6.0 (http://www.r-project.org/). For the analysis of the infection frequency, a one-way ANOVA was fitted, and Tukey for contrasts was used for *post hoc* comparisons. Viral RNA load analysis was carried out using Kruskal-Wallis by rank test for the non-parametric data. All DENV RNA loads were reported on a log10 scale given the value range.
3.4 Results

In this study, we challenged both WT and wMel Ae. aegypti mosquitoes with two serotypes in the co- and mono-infected states. Specifically, we investigated (1) the relative infection dynamics of two DENV serotypes inside the mosquito by competing them directly and (2) how individual serotypes behave when they are in a co-infection relative to mono-infection. Mosquitoes were blood fed with DENV-2 and DENV-3, both separately and together. In the case of mono-infections, a 1:1 mix of each DENV serotype and blood was used. For co-infections, the two serotypes were first combined, and from that blend, a 1:1 mix of blood and the two serotypes was used. Overall, the viral titers for both experiments were equivalent. We used RT-qPCR with serotype-specific TaqMan probes to quantify DENV RNA load in midgut, carcass, and salivary gland tissues to assess dissemination and infectiousness at nine timepoints post-infection.

3.4.1 DENV serotype competition in co-infection

By competing the serotypes in same mosquitoes, we were able to powerfully compare their performance, controlling some of the substantial variation that can occur across vector competence experiments. Infection rates in the WT mosquito line indicate that DENV-3 was a better competitor than DENV-2 but that the magnitude of this difference changed depending on the tissue and DPI. In the co-infection experiments, we classified the infection rates as uninfected (no viral RNA load detectable for either serotype), only DENV-2 infected (DENV-2/alone), only DENV-3 infected (DENV-3/alone), or co-infected (both serotypes detected). We saw significant variation in the
percentage of infected WT mosquitoes between serotypes (df=1, F=22.2, P < 0.0001). In the midgut, most mosquitoes were co-infected at all DPIs (infection rate: 25% to 100%). Mosquitoes were DENV-3/alone at 6 timepoints with an infection rate between 10 and 50% and DENV-2/alone at DPI 4 and 9 with an infection rate of ~15% (Figure 3.1, midgut).

In the carcass, most WT mosquitoes were either co-infected or DENV-3/alone, but we saw no DENV-2/alone mosquitoes at any timepoint (Figure 3.1, carcass). For the salivary glands, most WT mosquitoes were similarly either co-infected or DENV-3/alone, and there was only one timepoint at which we observed DENV-2/alone mosquitoes, with an infection frequency of ~10% at DPI 3 (Figure 3.1, salivary glands). For wMel mosquitoes, no pairwise comparison between serotypes was possible, as there were too few infected mosquitoes to perform statistical analysis, due to the action of Wolbachia-mediated pathogen blocking. Overall, our results indicate that DENV-3 is better at replicating and disseminating in mosquitoes, regardless of tissue or timepoint, than DENV-2.
We also compared viral RNA loads between serotypes across tissues and DPI. Overall, we saw no significant differences between DENV-2 and DENV-3 (Figure 3.2, Chi square = 1.66, P = 0.19, df = 1). The viral RNA load for both serotypes in the WT mosquitoes ranged from ~10^3 to ~10^8 in the midgut, ~10^2 to ~10^7 in the carcass, and ~10^2 to ~10^6 log10 DENV copies per tissue in the salivary glands. In contrast, the viral RNA load in the wMel mosquitoes was diminished and only observed in 10 timepoints across all
tissues, ranging from ~10^2 to ~10^5 in the midgut, ~10^2 to ~10^8 in the carcass, and ~10^2 log10 DENV copies per tissue in the salivary glands. Additionally, in the wMel mosquitoes, viral RNA loads from both serotypes were observed at only one timepoint (Figure 3.1, wMel, carcass, DPI 5).

![Figure 3.2. DENV load in co-infection by DPI, tissue, and mosquito line.](image)

**Figure 3.2. DENV load in co-infection by DPI, tissue, and mosquito line.** *Ae. aegypti* were orally challenged with DENV-2 (orange circles) and DENV-3 (navy diamonds) simultaneously, and both viruses were fed to mosquitoes at ~3 × 10^5 DENV genome copies/ml. Mosquito tissues (midgut, carcass, and salivary glands) were collected at nine days post-infection, and DENV RNA load was determined via RT-qPCR using serotype-specific probes. Mosquitoes with undetectable viral RNA load are not represented in this graph. Black bars represent treatment medians. Each symbol represents a single mosquito sample. Total number of mosquitoes screened per day was n=7 for the wild type line and n=30 for wMel.

Viral RNA loads from both serotypes were expressed as DENV-2/DENV-3 ratio (Figure 3.3). Although for all tissues there was a general trend of co-infected samples
having higher levels of DENV-3 than DENV-2, they were not significantly different from one another. For the wMel mosquitoes, only DPI 5 had sufficient data with which to calculate a ratio due to the action of Wolbachia pathogen blocking; in this case, DENV-3 appeared higher than DENV-2, but with so few data points, statistical comparisons were not possible.

Figure 3.3. Co-infection serotype ratio by DPI and tissue. Graphs depict the log10 DENV-2/DENV-3 ratio for all wild type (grey) and wMel (red) samples found to be positive for infection with both viruses via RT-qPCR for each tissue. Ratios greater than zero indicate that DENV-2 levels were higher than DENV-3 levels for that sample. Color lines represent mean DENV-2/DENV-3 ratio and the standard error estimate of the mean ratio.
3.4.2 Co-infection alters infection dynamics of DENV serotypes in wild type mosquitoes

After examining the competition dynamics between DENV-2 and DENV-3, we then sought to determine whether or not co-infection altered viral infection dynamics relative to the mono-infected state. For the wMel mosquitoes, no pairwise comparisons between mono- and co-infection for each serotype were possible due to the action of *Wolbachia*-mediated viral blocking. In the WT line, we saw higher infection rates when DENV-3 was in co-infection relative to mono-infection (Figure 3.4) but that the magnitude of this difference varied by DPI and tissue (df=1, F=7.5, P < 0.005). In the salivary glands, infection was only observed at four timepoints in the mono-infected state, whereas it was present in eight out of the nine timepoints in the co-infected mosquitoes (Figure 3.4). For viral RNA load, we saw no significant difference between DENV-3 mono- and DENV-3 co-infection (Figure 3.5, Chi square = 1.54, P = 0.21, df =1). For DENV-2, there were no differences in either infection frequency (Figure 3.4, df= 1, F=0.97, P=0.33) or viral RNA load (Figure 3.5, Chi square = 0.01, P = 0.91, df =1) between mono- and co-infection, indicating that co-infection did not alter dynamics.

3.4.3 Tissue specific differences in viral dynamics

Overall, for DENV-2, the infection rates were highest in the midgut and declined as the virus moved into the carcass and salivary glands (Figure 3.4). DENV-3, in contrast, demonstrated the highest infection rates in the midgut, followed by the salivary glands (Figure 3.4). Viral RNA loads for the two serotypes were highest in the midgut and
decreased in the carcass and then the salivary glands (Figure 3.5, DENV-2; Chi square = 57.48, P < 0.001, df = 2; DENV-3; Chi square = 57.48, P < 0.001, df = 2). The carcass was the tissue most likely to have either serotype present despite the action of wMel-mediated pathogen blocking. Dissemination rates of each of the two serotypes in the presence of wMel were similar across all three tissues (Figure 3.1).

Figure 3.4. WT line susceptibility to DENV in co- and mono-infections by DPI, tissue, and serotype. *Ae. aegypti* mosquitoes were orally challenged with DENV-2 and DENV-3 in both mono- and co-infection experiments. Each bar represents the proportion of mosquitoes positive for either serotype in the mono- (grey) and co-infection (blue) experiments for each day post-infection. Mosquito tissues (midgut, carcass, and salivary glands) were collected at nine days post-infection, and DENV RNA load was determined via RT-qPCR using serotype-specific probes. Sample size n=7 per day.
3.5 Discussion

We sought to explore the role of DENV serotype co-infection and competition in both Wolbachia-mediated blocking and within-vector infection dynamics. Particularly, we examined DENV RNA loads and infection rates in DENV-2/DENV-3 co-infected Ae. aegypti midguts, carcasses, and salivary glands of two mosquito lines with (wMel) and without Wolbachia infection (WT). We identified that in WT mosquitoes, there was a
competitive advantage for DENV-3 when co-infected with DENV-2 across multiple tissues compared to a mono-infection. Additionally, we saw a strong pathogen-blocking phenotype in wMel mosquitoes independent of co-infection status, tissue, and DPI.

Our data showed that for the WT mosquitoes, DENV serotype co-infection altered the infection frequency of each serotype in a tissue-specific manner, with DENV-3 having a competitive advantage over DENV-2. This advantage was clearer at the dissemination stage of infection once the virus reached the hemocoel from the midgut and ultimately at the transmission level when the virus arrived at the salivary glands. Moreover, the DENV-3 competitive advantage was confirmed when we compared each serotype in co-infected mosquitoes vs mono-infected mosquitoes, and we identified that DENV-3 produced higher infection rates in all tissues when mosquitoes were co-infected. Conversely, DENV-2 infection dynamics did not change significantly when mosquitoes were co-infected compared to mono-infected. Only one other study has looked at the infection dynamics of co-infection with DENV-2 and -3 both in vitro and in vivo (Quintero-Gil et al., 2014); contrary to our findings, they showed an increase in replication efficiency of 1000-fold for DENV-2. These contradictory results may be due to the use of different DENV and mosquito genotypes.

When the replication capacities of the two DENV serotypes were assessed in WT mosquitoes, no significant differences in viral RNA load between DENV-2 and DENV-3 in either mono- and co-infection were found. Differences in viral replication rates without affecting the viral RNA load in each tissue have been previously reported (Novelo et al., 2019), in which DENV serotypes that had high growth rates did not necessarily achieve
high viral RNA loads or high infection frequencies in experimental mosquito populations. The disconnect between viral RNA load and infection frequency for each virus suggests that stochastic processes are potentially taking place or and genotype-by-genotype interactions are affecting the virus infection dynamics. For example, previous research has shown that the strength of the mosquito immune response can be tissue and serotype dependent (Cheng, Liu, Wang, & Xiao, 2016; Smartt, Shin, & Alto, 2017; Taracena et al., 2018) and could lead to scenarios in which mosquitoes are more susceptible to dengue infection with a particular serotype (i.e., higher infection frequency) but can also mount a relatively strong immune response in particular tissues (i.e., low viral RNA load).

Arboviral co-infection is not limited to DENV serotypes; although most reported cases are with DENV, co-circulation of DENV with CHIKV and/or ZIKV is increasing around the globe (Bisanzio et al., 2018; Rodriguez-Morales, Villamil-Gómez, & Franco-Paredes, 2016). This co-circulation represents a major challenge for many national and international public health organizations, particularly because there is little information about the potential clinical and biological consequences of these interactions. Individual case reports of arboviral co-infection in humans suggest enhanced disease severity. Co-infection with ZIKV and CHIKV has been associated with severe meningoencephalitis in a male patient, and co-infection with DENV and CHIKV was linked to severe metrorrhagia in a female patient (Brito, Azevedo, Cordeiro, Marques, & Franca, 2017; Schilling, Emmerich, Günther, & Schmidt-Chanasit, 2009). Increased disease severity may occur when both viruses interfere with different parts of the same immune pathways. For example, interferon signaling is a major part of the human antiviral response, and it is
mediated by the signal transducer and activator of transcription one and two (STAT-1 and STAT-2) (Murira & Lamarre, 2016). DENV has been shown to block STAT-1, and CHIKV can potentially interfere with STAT-2 (Hollidge, Weiss, & Soldan, 2011), therefore blocking the activation cascade of interferon and potentially increasing disease severity.

The wMel mosquitoes challenged in DENV serotype co-infection were far less susceptible than the WT line, indicating that the pathogen-blocking phenotype caused by Wolbachia infection is not affected by concomitant DENV serotypes. Additionally, the effect of Wolbachia blocking was seen in all three tissues and was stable across nine timepoints, from 3 to 13 DPI, encompassing days of the mosquito’s lifespan relevant to viral transmission in the field. Pathogen blocking by Wolbachia-infected mosquitoes challenged with co-infecting arboviruses has only been shown once before (Caragata et al., 2019). Co-infection was performed using DENV/ZIKV challenges but not with multiple DENV serotypes at the same time, and it was limited to three timepoints and only one mosquito tissue. Although co-circulation of novel emerging arboviruses like ZIKV or CHIKV coupled with DENV has been reported (Carrillo-Hernández, Ruiz-Saenz, Villamizar, Gómez-Rangel, & Martínez-Gutierrez, 2018; dos Santos S. Marinho et al., 2020; Villamil-Gómez et al., 2016), most countries where DENV is endemic have reported co-circulation of all 4 DENV serotypes, resulting in hyperendemicity for the virus (Bastos Mde et al., 2012; Villabona-Arenas et al., 2014). Specifically, for DENV-2 and DENV-3 infections, one study showed that from 303 human serum samples, up to 21% were infected with both viruses (Senaratne, Murugananthan, Sirisena, Carr, & Noordeen, 2020). This
phenomenon has been linked to an increased frequency of severe dengue cases and an overall increase of virulence (Guzman & Harris, 2015).

Another effect of co-transmission can be epidemiological, where co-infection occurring from a single biting event can significantly increase disease burden. Mathematical modeling using in silico data in Ae. aegypti has shown that co-transmission events can potentially lead to an increased number of cases for both viruses (Vogels et al., 2019). An additional unanswered question in co-infection in Ae. aegypti is how sequential viral infections might affect pathogen transmission dynamics, a scenario that can also occur in nature (Nuckols et al., 2015; Marie Vazeille, Mousson, Martin, & Failloux, 2010). Whether or not viruses can potentially interact after sequential acquisition by the mosquito has yet to be determined. Last, in our co-infection experiments, we used half as much of each virus but the same overall viral RNA load as mono-infections; however, no statistically significant differences were observed in viral RNA load between mono-infection and co-infection for either serotype. This still begs the question of the potential outcome of infecting the mosquitoes with twice the amount of each serotype and twice the overall amount of virus in co-infection.

3.6 Conclusion

Here, we present the first examination of DENV serotype co-infection and its effect on Wolbachia-mediated pathogen blocking. We demonstrated that the wMel mosquito strain is capable of blocking DENV serotype co-infection in a systemic way across the mosquito body. Moreover, we showed that for WT mosquitoes, serotype co-infection can
affect infection frequency in a tissue- and time-specific manner and that both viruses have the potential of being transmitted simultaneously.

3.7 Acknowledgements

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3.8 References


Vazeille, M., Mousson, L., Martin, E., & Failloux, A.-B. (2010). Orally co-infected Aedes albopictus from la Reunion island, Indian ocean, can deliver both dengue and chikungunya infectious viral particles in their saliva. *PLoS Neglected Tropical Diseases*, 4(6), e706. doi:10.1371/journal.pntd.0000706


Chapter 4

Family level variation in the mosquito *Aedes aegypti* susceptibility and immune response to dengue and chikungunya viral infection.

4.1 Abstract

*Aedes aegypti* is the main vector of the arboviruses dengue (DENV) and chikungunya (CHIKV). Viral susceptibility and transmissibility in mosquito populations depends on the GxG interactions between the virus and the vector. Both genotypes can also have a great impact in the evolutionary potential of the vector:virus association. Here, we used a modified full-sibling design to estimate the contribution of mosquito genetic variation to the susceptibility and immune response to DENV and CHIKV. We demonstrated substantial genetic variation in the susceptibility to both viruses. Heritabilities were significant, but much higher for DENV susceptibility than to CHIKV, at 40 and 18%, respectively. The differences are largely driven by greater within family variation for CHIKV. Interestingly, we showed lack of genetic correlation between DENV and CHIKV susceptibility between siblings of the same families. These data suggest that the mosquito is much better at controlling DENV infection, and that the mosquito responds to the viruses with two very different mechanisms. These disparities may result from different coevolutionary histories, with CHIKV only recently emerging and acting as a selective agent in mosquito populations. Last, in we tested for correlations between viral
susceptibility and the expression levels of several immune genes, identifying two genes that may underpin the insect’s response to CHIKV (TEP3 and CECH).

4.2 Introduction

Arboviral diseases account for more than 17% of all infectious diseases globally (Kading, Brault, & Beckham, 2020), among the most prevalent are dengue and Chikungunya, with over half of the world’s population at risk and no effective vaccines or treatments (Wilder-Smith et al., 2017). Although mortality is generally low, the morbidity caused by these pathogens is associated with a substantial socioeconomic burden. In some cases, infection with any of the four dengue virus serotypes (DENV-1, 2, 3, 4) can lead to dengue shock syndrome in all ages (Rajapakse, 2011), and chikungunya virus (CHIKV) infection can be severe for infants, with nearly half of infected newborns experiencing encephalopathy and multiple organ dysfunction (Mehta et al., 2018). Both DENV and CHIKV are single stranded RNA viruses, belonging to the Flaviridae and Togaviridae viral families respectively, and are transmitted primarily through the bite of the mosquito *Ae. aegypti* (Huang, Higgs, Horne, & Vanlandingham, 2014; Lim, Lee, Madzokere, & Herrero, 2018). Unlike DENV, CHIKV is less well studied, largely as it was a relatively unknown pathogen causing small outbreaks around the Indian ocean until recently (Rougeron et al., 2015). In 2013 the virus was first reported in the Americas, and by 2018 there have been more than 3 million cases in 45 countries (Yactayo, Staples, Millot, Cibrelus, & Ramon-Pardo, 2016; Zeller, Van Bortel, & Sudre, 2016).
Arboviral infection in the mosquito is a complex and dynamic process, as the kinetics of infection can be influenced by the genetic variation of both the mosquito and the virus (Novelo et al., 2019). Natural *Ae. aegypti* populations have been shown to vary in their susceptibility to multiple arboviruses, including DENV and CHIKV (Gaye et al., 2014; Gokhale, Paingankar, Sudeep, & Parashar, 2015; Gubler, Nalim, Tan, Saipan, & Sulianti Saroso, 1979; Reiskind, Pesko, Westbrook, & Mores, 2008; Y. H. Ye et al., 2014), however, studies have shown that diverse mosquito populations from the Americas, India and the Pacific region have, are highly susceptible to CHIKV infection (Alto et al., 2017; Chen et al., 2015; Gokhale et al., 2015; Tesh, Gubler, & Rosen, 1976; Vega-Rúa, Zouache, Girod, Failloux, & Lourenço-de-Oliveira, 2014). Transmission efficiency in *Ae. aegypti* populations from America have been shown to be up to 83% for multiple CHIKV genotypes (Vega-Rúa et al., 2014). Moreover, CHIKV infectious particles have been detected in *Ae. aegypti* salivary glands as early as 2 days post infection (DPI), indicating that the dissemination of the virus within the mosquito is rapid and concomitant with infection of the salivary glands (Dubrulle, Mousson, Moutailler, Vazeille, & Failloux, 2009). Comparatively, infectious DENV particles have been detected in the mosquito salivary glands 4 DPI (Salazar, Richardson, Sánchez-Vargas, Olson, & Beaty, 2007). Overall, the differences in susceptibility between CHIKV and other arboviruses, such as DENV, have been linked to viral replication inside the mosquito, where genetic variation and specific pathogen infection mechanisms can have an important role in determining susceptibility and transmission.
Upon ingestion of a viral infected blood meal, the molecular interactions between the mosquito cells and the pathogen starts with the viral particle attachment to cellular receptors, facilitating viral endocytosis or activating signaling pathways to gain intracellular access (Alonso-Palomares, Moreno-García, Lanz-Mendoza, & Salazar, 2018). For DENV, the specific identity of mosquito cellular receptors remains elusive, although several putative receptors have been suggested (Cruz-Oliveira et al., 2015; Smith, 2012). To date, only one DENV cell expressed receptor has been characterized, *Prohibitin*, a ubiquitously expressed conserved protein shown to interact with DENV-2 (Kuadkitkan, Wikan, Fongsaran, & Smith, 2010). CHIKV cellular internalization is thought to be mediated through clathrin-mediated endocytosis (CME), a key process in vesicular trafficking (Dong, Balaraman, et al., 2017; Hoornweg et al., 2016; Lee et al., 2013). Upon entry, both viruses are uncoated through intracellular specific conditions, such as pH, and replicate in the cell cytoplasm (Rodriguez, Muñoz, Segura, Rangel, & Bello, 2019). Cellular tropism for both DENV and CHIKV appears widespread to multiple mosquito organs, including midgut, fat body tissue, nervous, tracheal and reproductive systems and salivary glands (Kantor, Grant, Balaraman, White, & Franz, 2018; Matusali et al., 2019; Salazar et al., 2007; Vega-Rúa, Schmitt, Bonne, Krijnse Locker, & Failloux, 2015; Wong, Chan, Sam, Sulaiman, & Vythilingam, 2016; Zhang et al., 2010; Ziegler et al., 2011). Interestingly, variation in the replication rates between viruses have been reported inside *Ae. aegypti*. Specifically, one study measuring viral RNA copies of each virus (Le Coupanec et al., 2017), showed that in the mosquito midguts, DENV had a lower copy number than CHIKV at 3 DPI, similarly, in the salivary glands, CHIKV was detected as early as 4 DPI whereas DENV was detected no earlier than 10 DPI. Moreover, co-infection
with DENV and CHIKV has shown to increased overall mosquito infection rates up to 100% for both viruses, while also enhancing CHIKV and DENV viral replication in the midgut and salivary glands, compared to each virus alone (Le Coupangec et al., 2017).

Viral infection triggers cellular and humoral immune responses inside the mosquito. The core pathways of the insect immune response are the Janus Kinase-Signal Transducer Activator of transcription (JAK/STAT), Toll, Immune Deficiency (IMD) and RNA interference (RNAi) (Alonso-Palomares et al., 2018). Viral components also interact with pattern recognition receptors, triggering processes, such as melanization and the production of anti-microbial peptides (AMP), including acting, defensin and cecropin among others (Agaisse & Perrimon, 2004; Antonova, Alvarez, Kim, Kokoza, & Raikhel, 2009; Sim & Dimopoulos, 2010). Additionally, complement related proteins in the mosquito hemolymph, such as thioester containing proteins (TEP), can recognize arboviruses and trigger further AMP production (Mukherjee, Das, Begum, Mal, & Ray, 2019). Interestingly, some of the traditional innate immune pathways (Toll, JAK/STAT, IMD) that have been shown to contribute to the reduction of DENV replication both in vitro and in vivo, play little role in limiting CHIKV infection. Specifically, exogenous activation of the JAK-STAT pathway has been shown to modulate DENV infection, but resulted in no enhanced resistance to CHIKV (Jupatanakul et al., 2017). Moreover, CHIKV infection has also been shown to significantly repress the Toll pathway stimulation, and neither the IMD pathway has been found to mediate viral activities (McFarlane et al., 2014). Of the major insect immune pathways known to control infection of arboviruses such as DENV, only the RNAi pathway has been shown to play a vital role in limiting
CHIKV replication. Knockdown of Argonaute2 (AGO2), an RNAi effector molecule, resulted in significant increase of viral RNA replication and titers (McFarlane et al., 2014).

Transcriptomic profiles of DENV and CHIKV infected mosquitoes have shown to be distinct, with little overlap between responsive genes (Shrinet, Srivastava, & Sunil, 2017). Expression profiles of CHIKV-infected mosquitoes have revealed upregulation of immune genes encoding AMPs, such as defensin and cecropin (CECH), and TEP (Dong, Behura, & Franz, 2017). Both TEP and CECH have been shown to be highly upregulated upon CHIKV infection up 10 DPI. In the same study, TEP3 expression was shown to be highly induced with up to 16-fold greater expression than other genes (Zhao, Alto, Jiang, Yu, & Zhang, 2019). Another study in malaria infected Anopheles gambie mosquitoes, revealed that TEP molecules can function as positive immune regulators, reducing pathogen intensity (Clayton, Dong, & Dimopoulos, 2014). Further reports have shown increased expression of the CECH gene upon pathogen infection (Antonova et al., 2009; Kokoza et al., 2010; Muturi, Blackshear, & Montgomery, 2012), including with Sindbis virus (SINV) infection, a member of the Togaviridae family (Kim & Muturi, 2013). Overall, the current data indicates that these genes might be important for CHIKV infection response inside the vector.

There have been several recent discoveries of novel non-canonical antiviral genes in the fly (Magwire et al., 2012; Piontkivska et al., 2016) and in Ae. aegypti (Ford et al., 2019) revealed by examining population level genetic variation in the antiviral response. Studying the natural genetic variation in Ae. aegypti could lead to insights in the basis of the vector’s own natural immune response to CHIKV and DENV, and may offer a means
to identify candidate antiviral genes to target with emerging insect genetic tools, such as CRISPR-cas9, (Terradas, Allen, Chenoweth, & McGraw, 2017; Terradas & McGraw, 2019). Here, we used a modified full-sib breeding design to assess the family level variation in CHIKV and DENV susceptibility, using a U.S. population of *Ae. aegypti*. By examining the susceptibility trait for both viruses in the same population, we can show the contribution of the genetic variation to the viral load variance. We then used the families showing extreme phenotypes in viral load to screen candidate genes relate to infection susceptibility. The set of candidate genes selected, were either common responders to viral infection of both CHIKV and DENV, or were shown to have a specific effect only in CHIKV-infection based on the literature.

4.3. Methods

4.3.1 Mosquito line and stock practices

An $F_3$ *Ae. aegypti* mosquito line originating from Monterrey, Mexico, was reared in the laboratory for 3 generations to expand and adapt the colony to laboratory feeding regimes. Mosquitoes were reared under standard conditions; $26^\circ$C, 65% relative humidity and a 12 h light/dark cycle. Larvae were maintained on fish food (Tetramin, Tetra). Adults were fed with 10% sucrose solution *ad libitum*. Mosquitoes were fed human blood (BioIVT, Hicksville, NY, USA) for egg collection using a Hemotek system (Hemotek).
4.3.2. Breeding design

A modified full – sib breeding design (William G. Hill & Mackay, 2004; Terradas et al., 2017; Yixin H. Ye et al., 2016) was carried out on the mosquitoes in combination with DENV and CHIKV infection. After expanding the mosquito line over 3 generations, F$_6$ eggs were hatched in synchrony and reared at low density (~150 larvae per 3 ltr of RO water). After pupation, males and females were separated and transferred to 30 x 30 x 30 cm cages at a density of ~250 individuals per cage. Six to seven- day old virgin adult females were blood-fed and then 250 virgin males were then added for mating. A total of 600 families were created by placing 600 blood-fed and mated females in small individual housings containing moist filter paper. Egg papers were collected and dried 3-4 days later, for short term storage. We selected female lineages that had produced a minimum of 60 eggs. For each family, eggs were hatched separately and after pupation, females were separated and split into two cups with a minimum of 8 individuals per cup. Mosquitoes were maintained on sucrose until vector competence experiments.

4.3.3. Virus culture

All experiments were carried out using the CHIKV strain 20235-St Martin 2013 (BEI Resources), and the DENV-2 strain 429557-Mex 2005 (BEI Resources), both from Latin American outbreaks. Virus was cultured in C6/36 cells as previously reported (Frentiu, Robinson, Young, McGraw, & O'Neill, 2010; Novelo et al., 2019; Terradas et al., 2017; Yixin H. Ye et al., 2016). Briefly, C6/36 Ae albopictus cells were grown in RPMI 1640 media (Life Technologies, Carlsband, CA, USA) and supplemented with 10% heat-
inactivated fetal bovine serum (FBS, Life Technologies), 1% Glutamax (Life Technologies) and 25 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown to a confluency of 80% and then independently infected with DENV-2 and CHIKV. Infected culture flasks were incubated at 27 °C. For DENV-2, after 7 days post-infection, supernatant was harvested at a titer of 1.0 x 10^5 focus forming units per ml (FFU/ml). For CHKV, supernatant was harvested at 2 days post infection at a titer of 8.7 x 10^5 and adjusted to a final concentration of 1.0 x 10^5 FFA/ml. Both viruses were store at -80°C in 1 ml single use aliquots for vector competence experiments.

4.3.4. Mosquito infections

Methods for oral mosquito infections have been fully described previously (Amuzu, Simmons, & McGraw, 2015; Novelo et al., 2019; Terradas et al., 2017). Prior to infections, mosquitoes were starved for 24 hrs. Half of the 6–7-day old females of each family were challenged with DENV-2 (1st cup) and the other half (2nd cup) with CHIKV at equal viral titers using a 1:1 mix of the frozen titrated aliquots and human blood. Mosquitoes were allowed to feed for 30 minutes and shortly after they were anesthetized with CO_2 and unfed individuals were removed and discarded. All CHIKV work was carried out at the Penn State Eva J. Pell ABSL-3 laboratory.

4.3.5. Viral quantification

After 7 days post infection, whole mosquitoes were collected, homogenized and stored in trizol (Invitrogen, Carlsbad, CA, USA) reagent at -80 °C for downstream molecular analysis. RNA was extracted according to the manufacturer’s protocol. RNA
was eluted in 25 ul of DNA/RNA free water and DNase treated (Life Technologies, Carlsbad, CA, USA). DENV and CHIKV was quantified using 4x TaqMan fast virus 1-step master mix (Applied biosystems, Foster city, CA, USA) in individual 10 ul reactions containing virus specific primers and probes (Table 4.1). The RT-qPCR reactions were run in a LightCycler 480 instrument (Roche Applied Science, Switzerland). The thermal cycling conditions were 50°C for 5 min for reverse transcription, 95°C for 10s for RT inactivation/denaturation followed by 50 amplification cycles of 95°C for 3s, 60°C for 30s and 72°C for 1s. Standard curves for both DENV and CHIKV were generated as described elsewhere (Rückert et al., 2017; Yixin H. Ye et al., 2016) and contained a ~100 bp fragment of the 3'UTR of the DENV genome and a ~140 bp fragment of the CHIKV genome, respectively. The standard curve spanned from $10^7$ to 10 copies/reaction with a limit of detection of 100 copies for both viruses. The viral load in each sample was extrapolated from the standard curve as copies per mosquito.

4.3.6. Selection of immune genes for expression analysis

Both viral load and infection intensity have been shown to be modulated by the mosquito immune response (Blair & Olson, 2014). To assess the potential role of specific immune genes in viral infection, we used families with extreme viral loads to test the for associations with gene expression. The null genetic correlation between DENV and CHIKV infected mosquitoes suggests a genetic decoupling in the mechanisms that drive the susceptibility to either virus in mosquitoes. Therefore, we aimed to test immune genes that represent mosquito immune pathways that have 1) shown to be of great importance in modulating infection for both viruses, and 2) that have might be important in the immune
response against CHIKV. We examined whether virus type and viral load group altered the expression level of several immune related genes. We compared the expression levels of four genes associated with viral infection in *Ae. aegypti*. For the first group, we focused in two genes, *AGO2* and *DCR2*, that are part of the mosquito RNA RNAi pathway which have been shown to modulate both CHIKV and DENV. For the second group, we tested the antimicrobial peptide (AMP), cecropin (*CECH*), a TOLL pathway-regulated immune effector (Mukherjee et al., 2019; Ramirez & Dimopoulos, 2010; Xi et al., 2008) and the complement - related immune protein *TEP3* (Blandin & Levashina, 2004; Mukherjee et al., 2019) which have been shown to be upregulate in CHIKV infection but not DENV.

### 4.3.7. Immune gene expression

Retrotranscription of RNA to cDNA and gene expression analysis was carried out on a LightCycler 480 instrument (Roche) using the Script One-step SYBR Green qRT-PCR (Quantabio, Beverly, MA, USA) according to the manufacturer’s protocol. All CT values were normalized to the housekeeping *Ae. aegypti* gene *RpS17*. Gene expression ratios were obtained using the ∆∆Ct method (Livak & Schmittgen, 2001). PCR amplification cycled 45 times at 95° C for 3 sec and 60° C for 30 sec, and the final cycle was followed by a melting curve analysis. All primers for candidate genes and viral quantification are listed in table 4.1.
We tested for genetic variation and broad-sense heritability ($H^2$) for DENV and CHIKV loads across our family design. We estimated the parameters using a modified full-sib breeding design and the random effect linear model previously described (Terradas et al., 2017; Yixin H. Ye et al., 2016):

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

$z_{ij}$ is the trait value for the $j$th female from the $i$th family, $f_i$ is the random effect of the $i$th family and $\varepsilon_{ij}$ is the unexplained error. To test if the genetic variation was significant, the family term was fitted as a random effect and the model (1) was compared to a reduce model without the family term. Loglikelihoods of both models were compared and twice the difference was tested against a chi-squared distribution with a single degree
of freedom. Broad sense heritability was estimated as twice the family variance component \( (\sigma_{\text{family}}) \) divided by the total phenotypic variance \( (\sigma_{\text{family}} + \sigma_{\text{error}}) \), (Saxton, 2004). All models were constructed in SAS studio version 3.8 (SAS Institute, Cary, NC, USA).

Genetic correlations between DENV and CHIKV infected siblings was estimated using a bivariate version of model (1) and fitting unrestrictive covariance correlations at the family level (\text{TYPE=}UNR option) suing SAS Proc MIXED command. Significance of the genetic correlation was tested by loglikelihood ratio tests.

4.4 Results

4.4.1. Family level variation in the heritability of DENV and CHIKV viral loads

To test for differences in the heritability across DENV and CHIKV viral loads we assayed a total of 800 DENV and CHIKV fed individual mosquitoes from 50 families in a modified full-sib design. Overall, we obtained 37 families for DENV and 38 for CHIKV that had enough infected mosquitoes for further analysis. Infected mosquitoes were tested for both viruses at day 7 post infection. After RNA extraction, we quantified DENV and CHIKV via RT-qPCR in 3-5 individuals per family. The broad sense heritability (\( H^2 \)) of DENV load was estimated to be 0.40 (Figure 4.1A. LRT: \( X^2=24.8, P=>0.001 \)) and for CHIKV load, \( H^2 \) was 0.18 (Figure 4.1B. LRT: \( X^2= 5.9, P=0.015 \)) across all the families. Both heritability values were significantly greater than zero, indicating a role for multi-genetic interactions in determining the viral load trait across the mosquito families for DENV and CHIKV. The range of viral load was extremely variable across the families,
spanning from hundreds to millions of copies; $10^2$-$10^7$ per body for DENV and $10^2$-$10^8$ per body for CHIKV.

**Figure 4.1. Ranked families by viral load.** A) DENV load ranked families and B) CHIKV load ranked families. Each point represents a single infected mosquito. Box plots show mean viral load ± SEM of each distinct *Ae. aegypti* family. Whole mosquito viral load was log10 transformed. N=3-5 individuals per family.
4.4.2. Genetic correlation between DENV and CHIKV across mosquito families

We found no correlation between DENV and CHIKV loads between siblings of the same family based on 37 families (Figure 4.2. Pearson’s $r = 0.0018, p = 0.99$). Furthermore, the correlation was not significantly greater than zero (LRT: $X^2 > 0.001, P = 1.00$). The decoupling between susceptibility traits for siblings indicates a lack of shared control mechanisms for the two viruses.

![Figure 4.2. Scatter plot showing the relationship between mean DENV and CHIKV loads from individual families. Gray area represents the 95% confidence interval of the regression line. N = 37 mosquito families.](image)

4.4.3 Phenotypic extremes of viral loads for both DENV and CHIKV infection in the mosquito families

We selected the five most extreme mosquito families with respect to mean viral load for both viruses. We then tested for differences between the viral loads between these groups and found a statistically significant difference between our family extremes for both DENV (Figure 4.3A, $w=256, df=1, p<0.0001$) and CHIKV (Figure 4.3B, $w=393, df=1,$
We then used these families to study the comparative expression of key immunity genes relevant to the mosquito response to each virus as determined by the literature.

Figure 4.3. Whole mosquito viral loads per family representing the extremes of the distribution. A) DENV extreme families and B) CHIKV extreme families. Each point represents a single infected mosquito. Whole mosquito viral load was log10 transformed. Boxplots show family mean and SEM. N=3-5 individuals per family. * 0.05 < p < 0.01, ***0.001 < 0.0001, **** p < 0.0001.
4.4.4. Immune genes relative expression

We evaluate the relative expression of two genes known to be involved in viral control of both CHIKV and DENV, *DCR2* (AAEL006794) and *AGO2* (AAEL017251), part of the RNAi pathway. For DENV infected mosquitoes, there was a significant difference in relative *AGO2* expression between family groups, with higher expression of the gene in the High viral load group (Figure 4.4A, \(w=236, \text{df}=1, p=0.001\)). The average gene expression fold change of was 1.49 between High/Low viral load groups. For *DCR2* expression, there was no significant difference between extreme family groups (Fig 4.4C, \(w=116, \text{df}=1, p=0.48\)) with a average fold change in gene expression of 1.22 between High/Low viral load groups. For CHIKV infected mosquitoes, we saw no significant difference in relative *AGO2* gene expression between extreme family groups (Fig 4.4B, \(w=288, \text{df}=1, p=0.08\)). The average gene expression fold change of High/Low viral load groups was 1.39. However, there was a significant difference in *DCR2* expression between family groups were the High viral load group had higher expression (Fig 4.4D, \(w=352, \text{df}=1, p<0.001\)) with an average gene expression fold change of 2.98 over the Low viral load group.

The second set of genes we evaluate are two effector molecules that have been shown to be important in the overall control of CHIKV but not DENV infection in *Ae. aegypti*, *CECH* (AAEL017211) and *TEP3* (AAEL008607). For DENV infected mosquitoes we identify a significant difference between groups (Fig 4.5A, \(w=67, \text{df}=1, p=0.01\)), with a higher expression of the *TEP3* in the Low viral load group. The average gene expression fold change between High/Low viral load groups was 0.17. However, we
saw no significant difference in *CECH* gene expression between the High and Low viral load groups (Fig 4.5C, w=115, df=1, p=0.31) with an average gene expression fold change of 0.83. For CHIKV infected mosquitoes we saw a significant difference in both *TEP3* (Fig 4.5B, w=342, df=1, p=<0.0001) and *CECH* (Fig 4.5D, w=394, df=1, p=<0.0001) and, with higher gene expression in the High CHIKV group. The average gene expression fold change between High/Low viral load groups was 3.84 for *TEP3* and 2.98 for *CECH*.

Additionally, we looked at the relationship between gene expression and viral load regardless of the family groups (i.e., High and Low) for both DENV and CHIKV infected mosquitoes. This was done to take into account the variation in gene expression in families that might not correspond to their viral load groups. For DENV infected mosquitoes the only significant interaction was for *AGO2* expression, that was positively correlated to viral load (Appendix C, Spearman’s $r = 0.63$, $p=.05$). For CHIKV infected mosquitoes all genes were positively correlated to viral load (Appendix C). In general, the correlation trends corresponded to the viral load family groupings previously observed.
Figure 4.4. AGO2 and DCR2 expression relative to the housekeeping gene RPS17 in family groups classified as High and Low Viral loads. A) AGO2 expression in DENV infected families; B) AGO2 expression in CHIKV infected families and C) DCR2 expression in DENV infected families; D) DCR2 expression in CHIKV infected families. Boxplots show family mean with SEM. N=3-5 individuals per family. * 0.05 < p < 0.01, ***0.001 < 0.0001, **** p < 0.0001. The average fold change of High viral load/Low viral load groups is A) 1.49, B) 1.36, C) 1.22, D) 2.98.
Figure 4.5. TEP3 and CECH expression relative to the housekeeping gene RPS17 in family groups classified as High and Low Viral loads. A) TEP3 expression in DENV infected families; B) TEP3 expression in CHIKV infected families and C) CECH expression in DENV infected families; D) CECH expression in CHIKV infected families. Boxplots show family mean with SEM. N=3-5 individuals per family. * 0.05 < p < 0.01, ***0.001 < 0.0001, **** p < 0.0001. The average fold change of High viral load /Low viral load groups is A) 0.17, B) 3.84, C) 0.83, D) 2.98.
4.5. Discussion

We performed a modified full-sib breeding design to study the contribution of the mosquito’s genetic variation, while limiting environmental variation, to susceptibility to each virus. By exploring susceptibility to the two viruses in sisters of the same family, we were also able to measure the genetic correlation between the individual susceptibilities. We used the distribution of susceptibilities to select families representing the phenotypic extremes for each virus. In these extreme families we tested for differences in expression for key genes previously shown to represent the mosquito immune response to each virus. Our experiments demonstrate the role of multi-gene interactions in determining the viral load trait across mosquito families for both DENV and CHIKV. Additionally, the lack of genetic correlation between DENV and CHIKV viral loads across siblings, demonstrated independence of the genetic response to these two viruses. Finally, our gene expression analysis on the extreme families showed that, although some viral control mechanisms, such as the RNAi pathway, play a universal role in modulating viral replication, the mosquitoes’ response to infection appears markedly different depending on the infecting virus.

The heritability values of the viral susceptibility for both DENV and CHIKV were significant but fell within different ranges. For DENV, the $H^2$ was 40%, this is in accordance with previous studies examining susceptibility in *Ae. aegypti* using multiple tissues as proxy. Both Bosio *et al.* (Bosio, Beaty, & Black, 1998) and Ye *et al.* (Yixin H. Ye et al., 2016) reported heritabilities of ~40% in the midgut, head and saliva of DENV infected mosquitoes. This suggests, that for DENV infection susceptibility, the mosquito
genotype differences contribute to vector competence almost equally as the viral genotype or environmental effects do. Heritability of CHIKV susceptibility was estimated at 18%, almost half that estimated for siblings in the same family. The lower heritability for CHIKV suggests that environmental or stochastic factors as well as the viral genotype have a greater role than the mosquito’s own genetic factors in determining the susceptibility to infection (Charmantier & Garant, 2005; William G. Hill, Goddard, & Visscher, 2008; William G. Hill & Mackay, 2004). CHIKV infected mosquitoes also exhibited a wider range in viral loads than DENV and reached higher viral loads than DENV infected mosquitoes. Overall, the wider range of viral loads in CHIKV is a key contributor to the overall low heritability for this trait. The genetic correlation between siblings for the DENV and CHIKV susceptibility trait was not significantly different than zero. This shows independence between the genes that control the susceptibility trait between viruses. Additionally, a value less than one indicates the presence of genotype x environment interactions (William G. Hill & Mackay, 2004). In general, the primary source of genetic correlation is the pleiotropy of genes, though linkage disequilibrium can be a contributing factor (Sgrò & Hoffmann, 2004; Slatkin, 2008). Our results could suggest that genes controlling this trait are present in different loci among the mosquito population or that they lack pleiotropic effect, meaning that different genes control susceptibility to infection depending on the virus (W. G. Hill, 2013).

Phylogenetic studies on Ae. aegypti and DENV have revealed a coevolutionary process between the virus and the co-indigenous mosquito populations (Alonso-Palomares et al., 2018; Barón, Ursic-Bedoya, Lowenberger, & Ocampo, 2010; Sim & Dimopoulos, 2010) which has in turn shape the susceptibility to the virus. Since CHIKV is a re-emerging
disease, the lack of the coevolutionary process with naïve, such as American, *Ae. aegypti* populations, could be primary source of the differences between the mechanisms that control infection inside the vector (Alonso-Palomares et al., 2018). The global spread of CHIKV and DENV was preceded by the global expansion of *Ae. aegypti* and *Ae. albopictus*. While the expansion of *Ae. aegypti* started over 400 years ago, the geographic expansion of *Ae. albopictus*, the secondary vector of DENV and CHIKV started since the 1960’s, previously being limited to Southeast Asia (Louis Lambrechts, Scott, & Gubler, 2010). Among the evolutionary forces that drive the evolution of arboviruses, vector interactions may play an important role in selecting viral genotypes with low fitness costs and high transmissibility rates (L. Lambrechts et al., 2009). CHIKV emergence in the Indian Ocean was linked to a single mutation that enhanced transmission by *Ae. albopictus* over *Ae. aegypti* (Tsetsarkin, Vanlandingham, McGee, & Higgs, 2007). It has been hypothesized that the emergence of arboviruses is partially caused by adaptations to native vector populations. Invasive viral genotypes, that outcompete local genotypes tend to have enhanced viral replication in the vector (Chevillon & Failloux, 2003).

Using the natural genetic variation in a mosquito population, we were able to select family extremes with respect to DENV and CHIKV viral loads. Using these families, we then quantify the expression levels of four genes associated with the mosquito immunity after viral infection with DENV and CHIKV. We tested two genes involve in the immune response for both viruses and two immune genes that have been recently shown to be of importance upon CHIKV infection. We decided for this this approach since very little is known about *Ae. aegypti* immune response to CHIKV compared to DENV. Additionally,
the two genes of importance for CHIKV that we chose, have been shown to be upregulated
in transcriptomic studies of CHIKV infected mosquitoes (Zhao et al., 2019).

The first set of genes, \textit{AGO2} and \textit{DCR2}, are part of the RNAi-induced silencing
complex (RISC) (Pratt & MacRae, 2009) and been shown to play a vital role in limiting
CHIKV and DENV replication (Franz et al., 2006; Liu, Swevers, Kolliopoulou, &
Smagghe, 2019; McFarlane et al., 2014; Mukherjee et al., 2019). Interestingly, the
expression pattern of both genes was different depending on the infecting virus. \textit{AGO2}
expression was positively correlated to higher viral loads in DENV infected families but
not so for CHIKV. In contrast, \textit{DCR2} expression was positively correlated with load in
CHIKV infected families, but no correlation was seen for DENV loads. Induction of RISC
starts with \textit{DCR2} recognition and cleavage of exogenous viral double stranded RNA
dsRNA) into small interfering RNAs (siRNAs). The siRNAs are loaded into RISC and
cleaved by \textit{AGO2} leading to further viral degradation (Blair, 2011). Arbovirus infection
studies in \textit{Ae. aegypti} have indicate that, given the ability of viruses to replicate despite
RNAi response, that arboviruses must possess a mechanism of immune evasion or
suppression (Blair & Olson, 2014). Studies with Flock House Virus (FHV) infected
\textit{Drosophila} have shown that some viral genome siRNAs are inefficiently loaded into RISC,
potentially reducing the efficacy of RNAi downstream of \textit{DCR2} and allowing the virus to
maintain replication (Flynt, Liu, Martin, & Lai, 2009). For DENV-2, it has been shown
that its genome contains an overrepresentation of \textit{DCR2} target sites suggesting a decoy
mechanism for RNAi evasion (Sánchez-Vargas et al., 2009). These hotspots have also been
shown to be a poor target for \textit{AGO2} cleavage potentially due to secondary intra-strand
structures (Siu et al., 2011). Additionally, RNAi targeting has been shown to produce viral
genotype diversification, leading to the proliferation of escape mutants within the same viral population that are not easily recognized by the effectors in this pathway (Brackney, Beane, & Ebel, 2009). Whether either virus genotype actively promotes these evasion strategies, that result in differential RISC viral modulation, remains unknown.

The second set of genes, *CECH* and *TEP3*, are a TOLL pathway-regulated (Mukherjee et al., 2019; Ramirez & Dimopoulos, 2010; Xi, Ramirez, & Dimopoulos, 2008) antimicrobial peptide (AMP), and a complement-related immune protein (Blandin & Levashina, 2004; Mukherjee et al., 2019), respectively. The expression pattern for both genes was also different depending on the virus. *TEP3* expression was negatively correlated with DENV viral load while it was positively correlated to CHIKV viral load. No expression pattern was seen for *CECH* in DENV infected families but it was positively correlated to CHIKV viral load. The expression pattern of both genes in is in line with other studies suggesting their role in CHIKV immune modulation (Zhao et al., 2019). Interestingly, the inverse correlation between *TEP3* expression and CHIKV and DENV viral loads suggest a differential interaction with the viruses. For CHIKV, the *TEP3* expression pattern suggest that the immune response mediated through the immune protein is reactive to the pathogen while for DENV, it suggests viral suppression. The mechanisms by which each virus might affect the expression of these genes remains unknown.

Our experimental design has some caveats that may limit the scope of its overall interpretation. Immune effector expression can vary depending on the time post infection and its possible that the expression levels of specific genes might peak immediately after blood feeding and were missed from our sampling regime (Erler, Popp, & Lattorff, 2011; Sim & Dimopoulos, 2010). Given the scale of the family-based design we could only
sample one single time point. The experimental conditions differ from past transcriptomic and gene expression studies used to identify our gene candidates. The expression results shown here do not exclude genes that might be relevant in different tissues and timepoints. Finally, the immune response of *Ae. aegypti* is complex and dynamic and several questions regarding the mosquitoes’ immunity remain outstanding, since most of what is known about the general immunity of arthropods has been elucidated in the *Drosophila* model system (Wang et al., 2015). Specifically, the precise molecular details of *Ae. aegypti* antiviral immune systems remains undescribed (Cheng, Liu, Wang, & Xiao, 2016).

From an evolutionary perspective, the differences in quantitative genetics shown here indicate that the selective pressures that lead to DENV infected mosquitoes having a higher heritability and more homogeneous viral loads are stronger than those for CHIKV, suggesting that the selection for higher or lower viral loads within a mosquito population would occur faster for DENV than CHIKV infection (William G. Hill & Mackay, 2004). This would have implication in our ability to predict the strength and impact of new outbreaks and the overall infection dynamics between *Ae. aegypti* and CHIKV. These changes could also explain CHIKV re-emergence and explosion in the American continent in naïve *Aedes* populations. Additionally, the results shown here suggest that the infection dynamics and some of the mechanisms that control viral susceptibility in mosquito populations are markedly different between DENV and CHIKV. From an epidemiological perspective, if the genes that control viral susceptibility to multiple viruses are different and are located at different loci in the mosquito genome, this could introduce a challenge to vector control methods aiming to target and modify universal loci that could be used against multiple arbovirus. In this case, more individualized gene editing approaches would
be necessary depending on the predominant virus present (Scott & Zhang, 2017). Our gene expression results also provide with some candidate genes that are not traditionally associated with antiviral activity, that should be further examined as potential targets to make refractory mosquito populations through gene editing techniques.
4.6. References


Chapter 5

Synthesis and General Discussion

*Aedes aegypti* remains as one of the deadliest animals in the world (McGregor & Connelly, 2020). Because of this, the development of vector control methods remains as the primary tool to fight the spread of vector borne diseases. A key part of development of optimal and effective vector control strategies depends on a deep understanding of the vector-virus interactions that take place when a mosquito takes pathogen-laden blood meal. The viral infection process inside the mosquito is a complex and dynamic process that is poorly understood. (Guzman & Harris, 2015). Additionally, the strength and efficacy of the mosquito immunity varies across tissues and in response to different serotypes and viruses. These vector-virus interactions can ultimately shape the transmission potential of multiple arboviruses (Bennett et al., 2002; Gubler, Nalim, Tan, Saipan, & Sulianti Saroso, 1979).

**Chapter 2** focused on comparing tissue-specific mosquito responses to DENV infection using four DENV serotypes. **Chapter 3** further expanded this work by examining the effect of DENV serotype co-infection in tissue-specific responses to infection, for both a *Wolbachia* infected and uninfected mosquito lines. Finally, in **Chapter 4**, we explored the mosquito infection dynamics from a population perspective and between different
arboviral families. We tested the role of the *Ae. aegypti* own genetic variation in determining susceptibility to DENV and CHIKV.

Differences between individual infections of viruses of the same family, in our case, of DENV serotypes, had markedly different dynamics in our vector competence assays in Chapter 2, DENV-1 and 2 outperform DENV-3 and 4, reaching higher viral and infection rates. This can potentially explain the disparities in the transmission potential in the field between the different DENV serotypes, since DENV variants with replicative advantages can move more rapidly in susceptible vector or host population. If individual infections of DENV serotypes have different dynamics inside the mosquito, how do they behave when they are co-infecting the vector? moreover, how can this scenario affect the development and deployment of vector control methods? In Chapter 3 we try to answered these questions by analyzing the co-infection process of DENV-2 and DENV-3 inside a *Wolbachia*-infected *Ae. aegypti* mosquito line. We saw that DENV co-infection has little effect in *Wolbachia’s* ability to block pathogen replication and that *Wolbachia’s* blocking phenotype was systemic across the mosquito body. From a vector control point of view, this seems promising, since it suggests that the long-term efficacy of *Wolbachia* won’t be compromised by DENV-coinfections. In *Wolbachia*-free mosquitoes, DENV co-infection had a significant effect in the dynamics of each DENV serotype. While in Chapter 2, DENV-2 ha greater capacity to replicate in the mosquito than DENV-3, when in co-infection, DENV-3 had a competitive advantage over DENV-2, suggesting either enhancement of DENV-3 or competitive inhibition of DENV-2, although the mechanisms by which this occur remain unknown. These chapters support the notions that the infection
process inside the vector, that ultimately affects transmissibility, is variable and dynamic and can be depended on the infecting virus.

If the infection dynamics of DENV differ across (chapter 2) and between serotypes (chapter 3) at an individual level, how do the differences in the infectious process translate to a populations level? And how much the mosquito population’s own genetic variation can impact this process, when infection is caused by an arbovirus from a different viral family? In chapter 4, we demonstrate not only that the heritability to the susceptibility to DENV and CHIKV is different within a population but that some of the immune mechanisms that control it are different. We showed that indeed, the mosquitoes immunity responds differently to each virus and that although some viral control mechanisms, such as the RNAi pathway, play a universal role in modulating viral replication, the mosquitos’ response to infection appears markedly different depending on the infecting virus.

While these experiments shed light on the G x G interaction on the susceptibility and viral transmission potential, I was only able to test individual viral and mosquito strains, thus extrapolating the results for other genotypes is not without limitations. Further expedients could use our framework with a diverse arrange of mosquito and viral genotypes incorporating different variables such as more initial infectious doses, different modes of co-infection and different immune genes (Souza-Neto, Powell, & Bonizzoni, 2019). Presently, my findings from chapter 2 have been further expanded not only by testing more DENV strains per serotype but also doing with Ae. albopictus, in the work done by Ekwudu et al. (2020). Additionally, the tissue specific infection dynamics and the presence of a viral subpopulation structure caused by a dose-dependent effect was reported in
CHIKV infected mosquitoes by Merwaiss et al. (2021). Similarly, chapter’s 3 results are in agreement with the work done by Caragata et al. (2019) showing that co-infection does not adversely affect pathogen blocking caused by Wolbachia, currently being trialed worldwide.

The selection for viral genotypes that have the potential for rapid and explosive spread is actively driven by within vector selection (Chevillon & Failloux, 2003). Single adaptive mutations within the viral genotype have been linked to the re-emergence and spread of viruses like CHIKV. Viruses with such selective advantages can displace local and more mild viral genotypes of the same or different viral families (Tsetsarkin, Vanlandingham, McGee, & Higgs, 2007). Outcompeted genotypes tend to be milder clinical manifestations and slow disease transmission within mosquito populations. How much does the viral populations reflect the genetic structure of their vectors? Worldwide, Ae. aegypti populations consists of connected islands of genetically different populations (Apostol, Black, Reiter, & Miller, 1996). The vector competence within a population is, up to some degree, determine by the G x G interactions, therefore viral genotype structure is a result from adaptations to the vector (Lambrechts et al., 2009). The transmission potential of a virus is therefore dependent on the vector-virus compatibility, i.e., the combination of initial viral load, host and pathogen genotypes.

Through my work, I demonstrate the importance of the viral genetic diversity, initial viral dose and tissues - specific response in dictating infectivity. We showed that a threshold level of virus is required to establish body-wide infection and that this threshold might be different between DENV serotypes. We also showed that the pathogen blocking
effect in *Wolbachia*-infected mosquitoes is not affected by DENV serotype co-infection. Finally, we showcase the contribution of mosquito’s genetic variation in the differential susceptibility to infection between DENV and CHIKV. Taken together, my dissertation addresses different areas of the complex vector-virus interactions and their effect on pathogen transmissibility. While many unanswered questions remained, my research provides valuable insight into the infection dynamics, immunity and feasibility of vector control strategies.
5.1 References


## Appendices

### Appendix A: Supplementary materials from Chapter 2

**Appendix A:** DRC estimates for unsuccessful infections by tissue, infectious dose and DENV strain

<table>
<thead>
<tr>
<th>Infectious dose</th>
<th>Serotype</th>
<th>Tissue</th>
<th>Subpopulation threshold</th>
<th>Parameter</th>
<th>Estimate</th>
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<td>MIDGUT</td>
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<td>Max DENV load</td>
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<td>0.14</td>
<td>&lt;0.001  ***</td>
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<td></td>
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<td>Max DENV load</td>
<td>ED50 Growth rate</td>
<td>Max DENV load</td>
<td>ED50</td>
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<td>2.96 0.28 &lt;0.001 ***</td>
<td>4.56 1.09 &lt;0.001 ***</td>
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<td>7.25 2.17 0.001 **</td>
<td></td>
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*NA, not available due to poor fitting to the log logistic model. *NTA no threshold applied. *P<0.05, **P<0.01, ***P<0.001. SE of the estimate, p-value indicates if estimate is different than zero.
Appendix A: Multimodal distribution analysis

<table>
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<th>Treatments</th>
<th>Hartigan’s dip test</th>
<th>Bimodality coefficient</th>
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</tr>
<tr>
<td>DENV-1 MG LOW</td>
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<td>0.86</td>
</tr>
<tr>
<td>DENV-2 CA LOW</td>
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<td>DENV-2 MG LOW</td>
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<tr>
<td>DENV-3 MG HIGH</td>
<td>&lt;2.2e-16</td>
<td>0.75</td>
</tr>
<tr>
<td>DENV-4 CA HIGH</td>
<td>5.18E-06</td>
<td>0.89</td>
</tr>
<tr>
<td>DENV-4 MG HIGH</td>
<td>&lt;2.2e-16</td>
<td>0.93</td>
</tr>
<tr>
<td>DENV-1 CA HIGH</td>
<td>7.83E-06</td>
<td>0.67</td>
</tr>
<tr>
<td>DENV-1 MG HIGH</td>
<td>0.5921</td>
<td>0.63</td>
</tr>
<tr>
<td>DENV-2 CA HIGH</td>
<td>0.0001161</td>
<td>0.75</td>
</tr>
<tr>
<td>DENV-2 MG HIGH</td>
<td>0.9904</td>
<td>0.63</td>
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</tbody>
</table>

If the p value is >0.05, it suggests bimodality.

Appendix A: Survival curve statistical analysis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Observed</th>
<th>Expected</th>
<th>(O-E)^2/E</th>
<th>(O-E)^2/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80</td>
<td>80</td>
<td>88.5</td>
<td>0.82</td>
<td>1.06</td>
</tr>
<tr>
<td>DENV-1 High</td>
<td>80</td>
<td>80</td>
<td>98.7</td>
<td>3.52</td>
<td>4.93</td>
</tr>
<tr>
<td>DENV-1 Low</td>
<td>80</td>
<td>80</td>
<td>75.2</td>
<td>0.31</td>
<td>0.39</td>
</tr>
<tr>
<td>DENV-2 High</td>
<td>80</td>
<td>80</td>
<td>64.7</td>
<td>3.62</td>
<td>4.44</td>
</tr>
<tr>
<td>DENV-2 Low</td>
<td>80</td>
<td>80</td>
<td>86.4</td>
<td>0.47</td>
<td>0.60</td>
</tr>
<tr>
<td>DENV-3 High</td>
<td>80</td>
<td>80</td>
<td>81.1</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>DENV-3 Low</td>
<td>80</td>
<td>80</td>
<td>82.1</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>DENV-4 High</td>
<td>80</td>
<td>80</td>
<td>68.7</td>
<td>1.85</td>
<td>2.29</td>
</tr>
<tr>
<td>DENV-4 Low</td>
<td>80</td>
<td>80</td>
<td>74.3</td>
<td>0.43</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Chisq = 12.9 on 8 degrees of freedom, p= 0.01.
**Appendix A: AIC values for the DRC model**

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Dose</th>
<th>Tissue</th>
<th>Subpopulation threshold</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-1</td>
<td>High</td>
<td>MG</td>
<td>NA</td>
<td>967</td>
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<tr>
<td>DENV-1</td>
<td>Low</td>
<td>MG</td>
<td>Successful</td>
<td>318</td>
</tr>
<tr>
<td>DENV-1</td>
<td>Low</td>
<td>MG</td>
<td>Unsuccessful</td>
<td>341</td>
</tr>
<tr>
<td>DENV-2</td>
<td>High</td>
<td>MG</td>
<td>NA</td>
<td>775</td>
</tr>
<tr>
<td>DENV-2</td>
<td>Low</td>
<td>MG</td>
<td>Successful</td>
<td>386</td>
</tr>
<tr>
<td>DENV-2</td>
<td>Low</td>
<td>MG</td>
<td>Unsuccessful</td>
<td>360</td>
</tr>
<tr>
<td>DENV-3</td>
<td>High</td>
<td>MG</td>
<td>Successful</td>
<td>392</td>
</tr>
<tr>
<td>DENV-3</td>
<td>High</td>
<td>MG</td>
<td>Unsuccessful</td>
<td>306</td>
</tr>
<tr>
<td>DENV-3</td>
<td>Low</td>
<td>MG</td>
<td>NA</td>
<td>263</td>
</tr>
<tr>
<td>DENV-4</td>
<td>High</td>
<td>MG</td>
<td>Successful</td>
<td>605</td>
</tr>
<tr>
<td>DENV-4</td>
<td>High</td>
<td>MG</td>
<td>Unsuccessful</td>
<td>228</td>
</tr>
<tr>
<td>DENV-4</td>
<td>Low</td>
<td>MG</td>
<td>NA</td>
<td>282</td>
</tr>
<tr>
<td>DENV-1</td>
<td>High</td>
<td>CA</td>
<td>NA</td>
<td>786</td>
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<td>CA</td>
<td>Successful</td>
<td>181</td>
</tr>
<tr>
<td>DENV-1</td>
<td>Low</td>
<td>CA</td>
<td>Unsuccessful</td>
<td>300</td>
</tr>
<tr>
<td>DENV-2</td>
<td>High</td>
<td>CA</td>
<td>NA</td>
<td>621</td>
</tr>
<tr>
<td>DENV-2</td>
<td>Low</td>
<td>CA</td>
<td>Successful</td>
<td>199</td>
</tr>
<tr>
<td>DENV-2</td>
<td>Low</td>
<td>CA</td>
<td>Unsuccessful</td>
<td>340</td>
</tr>
<tr>
<td>DENV-3</td>
<td>High</td>
<td>CA</td>
<td>Successful</td>
<td>340</td>
</tr>
<tr>
<td>DENV-3</td>
<td>High</td>
<td>CA</td>
<td>Unsuccessful</td>
<td>301</td>
</tr>
<tr>
<td>DENV-3</td>
<td>Low</td>
<td>CA</td>
<td>NA</td>
<td>160</td>
</tr>
<tr>
<td>DENV-4</td>
<td>High</td>
<td>CA</td>
<td>Successful</td>
<td>362</td>
</tr>
<tr>
<td>DENV-4</td>
<td>High</td>
<td>MG</td>
<td>Unsuccessful</td>
<td>163</td>
</tr>
<tr>
<td>DENV-4</td>
<td>Low</td>
<td>MG</td>
<td>NA</td>
<td>189</td>
</tr>
</tbody>
</table>

**Appendix A: Results of the GLM fitted to salivary glands DENV load**

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>SE</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1.06</td>
<td>2.36</td>
<td>0.44</td>
<td>0.66</td>
</tr>
<tr>
<td>DENGUE-2</td>
<td>41.94</td>
<td>75.43</td>
<td>0.55</td>
<td>0.58</td>
</tr>
<tr>
<td>DENGUE-3</td>
<td>0.14</td>
<td>2.41</td>
<td>0.05</td>
<td>0.95</td>
</tr>
<tr>
<td>DENGUE-4</td>
<td>-29.65</td>
<td>20.55</td>
<td>-1.44</td>
<td>0.16</td>
</tr>
<tr>
<td>Carcass load</td>
<td>0.13</td>
<td>0.29</td>
<td>0.44</td>
<td>0.66</td>
</tr>
<tr>
<td>Midgut load</td>
<td>0.05</td>
<td>0.24</td>
<td>0.21</td>
<td>0.83</td>
</tr>
<tr>
<td>DENGUE-2:Carcass load</td>
<td>-4.69</td>
<td>8.34</td>
<td>-0.56</td>
<td>0.58</td>
</tr>
<tr>
<td>DENGUE-3:Carcass load</td>
<td>-0.03</td>
<td>0.30</td>
<td>-0.13</td>
<td>0.89</td>
</tr>
<tr>
<td>DENGUE-4:Carcass load</td>
<td>6.15</td>
<td>4.24</td>
<td>1.44</td>
<td>0.16</td>
</tr>
<tr>
<td>DENGUE-2:Midgut load</td>
<td>-3.50</td>
<td>6.34</td>
<td>-0.55</td>
<td>0.58</td>
</tr>
<tr>
<td>DENGUE-3:Midgut load</td>
<td>-0.01</td>
<td>0.24</td>
<td>-0.07</td>
<td>0.94</td>
</tr>
<tr>
<td>DENGUE-4:Midgut load</td>
<td>3.02</td>
<td>2.09</td>
<td>1.44</td>
<td>0.16</td>
</tr>
<tr>
<td>Carcass load:Midgut load</td>
<td>-0.01</td>
<td>0.03</td>
<td>-0.33</td>
<td>0.73</td>
</tr>
<tr>
<td>DENGUE-2:Carcass load:Midgut load</td>
<td>0.39</td>
<td>0.70</td>
<td>0.56</td>
<td>0.58</td>
</tr>
<tr>
<td>DENGUE-3:Carcass load:Midgut load</td>
<td>0.008</td>
<td>0.03</td>
<td>0.27</td>
<td>0.78</td>
</tr>
<tr>
<td>DENGUE-4:Carcass load:Midgut load</td>
<td>-0.62</td>
<td>0.42</td>
<td>-1.45</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Appendix A: Density plots. Dotted line indicates data split point

DENV-1 MG LOW

DENV-2 MG LOW

DENV-1 CA LOW

DENV-2 CA LOW

DENV-3 MG HIGH

DENV-4 MG HIGH
Appendix A: Average salivary gland DENV load at the last DPI (20 days).

Average DENV load at DPI 20 for all 4 DENV serotypes at 2 infectious doses (High: $1 \times 10^8$, low: $1 \times 10^5$ DENV copies/ml). All box plots show median and interquartile ranges (n = 10 per treatment). Significant differences are based on Tukey post hoc comparison following ANOVAs on log-transformed data. Only significant differences are shown. *p<0.05.
Appendix A: Survival curves for all treatments

Survival curve analysis of control and the 4 DENV serotypes at 2 infectious doses (High: $1 \times 10^8$, low: $1 \times 10^5$ DENV copies/ml). There was no significant difference between treatments ($\chi^2 = 12.9$, df = 8, p = 0.1). Dotted outside lines represent 95% confidence intervals. Each line at the top represents individual treatments.
Appendix B: Supplementary materials from Chapter 3

Appendix B: Supplementary figure 1. Relationship between PFU and viral RNA copies for both DENV-2 and DENV-3.

Relationship between PFU and viral RNA copies for both DENV-2 and DENV-3. Viruses were harvested from C6/36 cells at DPI 5 and 7 and on the day of the experiment (DPI7-Exp). A) PFU/ml for both DENV-2 and DENV-3 for DPI5 (red), DPI7 (blue), and on the day of the experiment DPI7 (black). B) Log10 viral RNA copies/ml for both DENV-2 and DENV-3 for DPI5 (red), DPI7 (blue), and on the day of the experiment DPI7 (black). All assays were done using live virus.
Appendix C: Supplementary materials from Chapter 4

**Appendix C:** Supplementary figure 1. Relationship between DENV viral load and gene expression levels.

Relationship between DENV viral load and gene expression levels in extreme DENV load mosquito families. DENV viral load was correlated with expression levels of A) AGO2 (Spearman's $r = 0.63$, $p = 0.05$), B) DCR2 (Spearman's $r = 0.0022$, $p = 0.95$), C) TEP3 (Spearman's $r = -0.46$, $p = 0.18$) and D) CECH (Spearman's $r = -0.35$, $p = 0.32$). Each point represents a mosquito family. N=10 families.
Appendix C: Supplementary figure 3. Relationship between DENV viral load and gene expression levels.

Relationship between CHIKV viral load and gene expression levels in extreme CHIKV load mosquito families. CHIKV viral load was correlated with expression levels of A) AGO2 (Spearman's $r = 0.7$, $p = 0.016$), B) DCR2 (Spearman's $r = 0.64$, $p = 0.035$), C) TEP3 (Spearman's $r = 0.76$, $p = 0.0061$) and D) CECH (Spearman's $r = 0.62$, $p = 0.043$). Each point represents a mosquito family. N=10 families.
VITA

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Education

2018 – 2021 PhD in Entomology
Penn State University, University Park, PA, USA
2014 – 2016 Master of Science in Molecular Medicine (Distinction)
University of Sheffield, United Kingdom
2009 – 2014 Bachelor of Science in Biology
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- Dean's Postgraduate Research Scholarship and Deans International Postgraduate Research Scholarship - Monash University (AUS).
- National Council of Science and Technology Scholarship - CONACyT (MEX)

Teaching experience

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- Teaching assistant: ENT216 Plagues Through the Ages (Spring 2020)