

Unravelling the factors behind differences in Sindbis virus load in *Aedes aegypti* after oral
infection

by

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B.S., Kwame Nkrumah University of Science and Technology, 2016
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AN ABSTRACT OF A DISSERTATION

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Abstract

Arbovirus transmission to their vertebrate hosts by mosquito vectors continues to linger on, causing major public health concerns. One of the reasons why mosquitoes that transmit arboviruses persist is due to failure of robust vector control strategies to limit or eradicate mosquitoes of public health importance. The shortfall of effective vector control strategies is due to insufficient understanding of what factors contribute to mosquito vector competence. The arbovirus load, or the amount of infectious virus in a mosquito, has been reported to affect vector competence. Related to this, after oral infection of inbred mosquitoes there is a high level of variation in virus load observed among individual mosquitoes. Unfortunately, there have been few studies conducted to understand what factors contribute to this titer variation. For this reason, Sindbis virus (SINV) was used as a model virus to better understand the factors contributing to variation in virus load among individual *Aedes aegypti* mosquitoes after oral infection. To achieve this goal, two specific aims were established. In Aim 1, the biological factors associated with variability in virus load in *Ae. aegypti* after SINV oral infection were investigated, while in Aim 2, the virus factors that contribute to variation in virus load after oral infection were examined. To address the first aim, highly inbred mosquitoes were reared and infected with SINV. The amount of virus load in the mosquitoes was determined and the results were analyzed to ascertain the biological factors contributing to titer variation. Results from this study showed that higher titer variation is observed after oral infection compared to intrathoracic infection, suggesting that the midgut is an important tissue barrier introducing variation in virus load. Additionally, decreased titer variation was observed with increased incubation period. This suggests that *Ae. aegypti* increasingly resist SINV infection over time following virus exposure. Biological factors such as mosquito age, mosquito strain, virus genotype, mosquito weight,

amount of blood mosquito ingested, time after adult mosquito emergence, and the batch of eggs used to produce adult mosquitoes did not affect titer variation. Furthermore, virus collected from high titer mosquitoes replicated better in cultured cells compared to virus from low titer mosquitoes, suggesting there may be genetic differences between these virus populations.

To address the second aim, the virus populations from individual low and high titer infected mosquitoes were sequenced, and the amount of sequence variation among the different populations was analyzed. Results from this study showed that there was a significant reduction in sequence variation when SINV moved from midguts to carcasses of low titer mosquitoes but not in high titer mosquitoes, suggesting that the midgut escape barrier is stronger in low titer mosquitoes compared to high titer mosquitoes. Also, carcasses of high titer mosquitoes had significantly higher population diversity and complexity than carcasses of low titer mosquitoes. Stronger purifying selection was also observed in the midguts and carcasses of low titer mosquitoes compared to high titer mosquitoes. Together these findings suggest that the virus populations present in low titer mosquitoes are less fit than those found in high titer mosquitoes, possibly due to genetic founder effect. Surprisingly, when the amount of SINV genomes was determined in the low and high titer mosquitoes it was found that the genome levels did not vary significantly, despite the large difference in infectious virus load. Additional analyses showed that high titer mosquitoes had significantly greater specific infectivity, or the ratio of infectious particles to viral genomes, than low titer mosquitoes. Low titer mosquitoes also had less viral protein translation and virion assembly than viruses from high titer mosquitoes. This could possibly contribute to the lower specific infectivity in low titer mosquitoes than high titer mosquitoes. Finally, immune suppression by antibiotic treatment caused an increase in specific infectivity, indicating that these differences were immune-related.

Altogether, this dissertation has contributed fundamental knowledge towards understanding the factors contributing to arbovirus titer in mosquitoes, which is driven by tissue barriers to infection and dissemination and immune-related differences in specific infectivity of SINV in the carcasses of mosquitoes.

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List of Abbreviations

Aedes.....	Ae
Basal lamina.....	BL
Culex.....	Cx
Chikungunya virus.....	CHIKV
Dengue virus	DENV
Eastern equine encephalitis virus.....	EEEV
Kilobase.....	kb
Midgut escape barrier.....	MEB
Midgut infection barrier.....	MIB
O'nyong'nyong virus.....	ONNV
Ribonucleic acid.....	RNA
Rift Valley fever virus.....	RVFV
Ross River virus.....	RRV
Semiliki Forest virus.....	SFV
Sindbis virus.....	SINV
West Nile virus.....	WNV
Western equine encephalitis virus.....	WEEV
World Health Organization.....	WHO
Usutu virus	USUV
Venezuelan equine encephalitis virus.....	VEEV
Yellow fever virus.....	YFV
Zika virus	ZIKV

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Dedication

I dedicate this work to my parents, Vivian and Godsman Hodoamede, and my spouse Patience Obeng, for their continued support throughout my PhD studies.

Chapter 1 - Introduction

1.1 Arboviruses

Viruses transmitted to humans and vertebrate animals by arthropod vectors, known as arboviruses, are transmitted predominately by the bite of infected mosquitoes, ticks, fleas, or gnats (WHO, 2017). Many arboviral diseases are persistent in tropical and sub-tropical geographical areas where their vectors mostly thrive, with a smaller number also present in temperate regions (WHO, 2017). Arboviral diseases of major public health concern include dengue fever and dengue hemorrhagic fever, chikungunya fever, and Zika fever (WHO, 2017).

Many arboviruses have a strong potential of emerging in new areas or re-emerging in pre-existing areas. The emergence and re-emergence of arboviruses has been attributed in many cases to climatic changes that affect the transmitting vector's life cycle (Pfeffer and Dobler, 2010). Arboviruses such as the West Nile virus (WNV), Usutu virus (USUV), or Rift Valley fever virus (RVFV) have emerged in new regions which were not known to them previously. West Nile virus has spread to the Americas, USUV to Central Europe, and the RVFV to the Arabian Peninsula (Pfeffer and Dobler, 2010). The introduction of arboviruses to new localities can be through travelers returning from countries located in the tropics with a high burden of arboviral diseases (Pfeffer and Dobler, 2010), and through the migration of infected birds from high to low arbovirus endemic areas (Rappole et al., 2000; Tsiodras et al., 2008). Viruses such as Zika virus (ZIKV), dengue virus (DENV), chikungunya virus (CHIKV), and yellow fever virus (YFV) have the potential of re-emerging to cause periodic or occasional epidemics in already existing regions (Stapleford et al., 2014; Wilder-Smith et al., 2017). Transmission of arboviruses in a community from person to person after they have emerged or re-emerged in an area is mostly through infected mosquitoes bites (Kraemer et al., 2015; Weaver and Lecuit, 2015). The

ability of mosquitoes to colonize new environments (Robert et al., 2016; Weaver, 2013) provides opportunities for arboviruses to spread to new geographical areas (Huang et al., 2019).

The most notable mosquito genera of medical importance are *Aedes* (Rückert et al., 2017), *Culex*, and *Anopheles*, the latter being vectors for malaria (Palmer, 2011). *Aedes spp* are especially problematic since they transmit ZIKV, DENV, Venezuelan equine encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV), Semliki Forest virus (SFV), YFV, Ross River virus (RRV), and CHIKV (Palmer, 2011; Rückert et al., 2017). O'nyong'nyong virus (ONNV) is transmitted by *An. gambiae* (Carissimo et al. 2015) while *Culex spp* transmit Sindbis virus (SINV) (Palmer, 2011).

The persistence of arbovirus transmission in endemic areas is due to a complex combination of factors including poor vector control (Achee et al., 2019), mosquito development of resistance to chemical insecticides (Dusfour et al., 2019), unavailability of specific antiviral drugs (Huang et al., 2017), lack of effective vaccines to manage most arbovirus diseases in the human host (Londono-Renteria et al., 2016), and lack of adequate funding (LaBeaud, 2008). Despite the problems associated with current vector control strategies, it is still the approach that has the most potential to drastically reduce the public health burden associated with arboviral disease by (1) reducing mosquito populations; (2) replacing susceptible mosquito populations with refractory mosquito populations or (3) eradicating mosquito populations of medical importance associated with arboviral diseases (Hemingway et al., 2006). Our quest to develop novel and robust vector control strategies can further be enhanced with an improved comprehensive understanding of what factor(s) fully contribute to vector competence in mosquitoes (Beerntsen et al., 2000). Both biotic and abiotic factors have been shown to contribute to mosquito vector competence. Abiotic factors that affect vector competence include

temperature and humidity while biotic factors that affect vector competence include mosquito microbiota, mosquito genotype, virus genotype, and mosquito innate immunity (Ciota and Kramer, 2013).

1.1.1 Major families of arboviruses

Arboviruses were initially classified based on serological data into three groups, namely Groups A, B, and C, in the mid-20th century. The great strides made in molecular biology have made it possible to now classify arboviruses based on their genomic sequences (Fauquet et al., 2005). Most arboviruses are classified under five different families, namely *Peribunyaviridae*, *Flaviviridae*, *Reoviridae*, *Rhabdoviridae*, and *Togaviridae* (Fauquet et al., 2005). The vast majority of arboviruses are RNA viruses. Arthropods that transmit arboviruses include mosquitoes, sandflies, midges, bugs, fleas, and ticks. Most individual arboviruses are transmitted by one family of arthropods. However, a few arboviruses can be transmitted by members of more than one arthropod family. For example, WNV can be transmitted by ticks and mosquitoes (Palmer, 2011; Lawrie et al., 2004). Most arboviruses are transmitted by mosquitoes, making these arthropods the principal targets in vector control strategies to reduce the public health burden associated with arboviral diseases. The mosquito species that transmit arboviruses are mainly in the family *Culicidae* (Palmer, 2011).

1.1.1.1 *Flaviviridae*

Arboviruses in the family *Flaviviridae* are in the genus *Orthoflavivirus* which includes YFV, DENV, Japanese encephalitis virus, WNV, St Louis encephalitis virus, Murray Valley equine encephalitis virus and UV, among others (Palmer, 2011). Species within two genera of mosquitoes, namely *Aedes spp* and *Culex spp*, and the tick *Ixodes spp* are responsible for transmitting most flaviviruses (Palmer, 2011).

Flavivirus virions are spherical with a relatively smooth surface and 50 nm in diameter. They have an electron-dense core which is 30 nm in diameter and is surrounded by a lipid membrane (Palmer, 2011). They contain genomes of positive-sense, single stranded (ss) RNA of 11 kb that encode both structural and non-structural proteins. The structural proteins are the capsid (C), envelope glycoprotein (E), and a membrane glycoprotein. The precursor membrane protein 18K (prM 18K) is found in immature virions (Palmer, 2011). The non-structural proteins of flaviviruses are NS3, NS4A, NS2A, NS2B, NS5B, NS5, and NS1. Flaviviruses of the most public health concern are DENV, YFV, WNV, ZIKV, and Japanese encephalitis virus (Palmer, 2011).

1.1.1.2 *Peribunyaviridae*

The family *Peribunyaviridae* includes seven genera, with arboviruses being found in the genus *Orthobunyavirus* (eg. La Crosse virus, California encephalitis virus, Oya virus, etc.) (Hughes et al. 2020). Bunyavirus particles are generally spherical or pleomorphic. They are approximately 80-120 nm in diameter (Palmer, 2011). They have surface glycoprotein projections which are approximately 10 nm. The glycoproteins are embedded in a lipid bilayer envelope. The lipid envelope is approximately 5 nm thick. The ss genomes of bunyaviruses are segmented into 3 portions designated as L (large), M (medium), and S (small) and contain negative or ambisense ssRNA. The total size of the genome is between 11 and 19 kb (Palmer, 2011). The L segment encodes the RNA polymerase, while the S segment encodes the nucleocapsid protein, and the M segment encodes the viral glycoproteins (Palmer, 2011). Bunyaviruses are transmitted by *Aedes spp*, *Culex spp*, and *Aedeomyia spp* (Palmer, 2011).

1.1.1.3 *Togaviridae*

Arboviruses in the family *Togaviridae* are within the genus *Alphavirus*, which includes members such as VEEV, SINV, EEEV, SFV, Mayaro virus, ONNV, RRV, WEEV, and CHIKV (Palmer, 2011). The *Aedes spp*, *Culex spp*, *Culiseta spp*, *Coquilettidia*, and *Mansonia spp* are examples of genera of mosquitoes that transmit alphaviruses, while ONNV is transmitted by *Anopheles spp* (Palmer, 2011).

The virions of alphaviruses are approximately 70 nm in diameter. They are spherical and have a lipid bilayer. The envelope contains heterodimeric spikes which consist of the glycoproteins E1 and E2. Alphaviruses have an enclosed nucleocapsid core which contains 240 capsid proteins and one copy of ~11.7 kb genomic ss positive-sense RNA (Palmer, 2011). In addition to structural proteins (glycoproteins E1 and E2, 6K, and capsid proteins), alphaviruses also express four non-structural proteins, namely nsP1, nsP2, nsP3, and nsP4. The structural proteins of alphaviruses are expressed via a subgenomic mRNA that is expressed from a promoter located between the nonstructural and structural ORFs. Some of the most notable viruses of public health importance in this family are CHIKV, VEEV, WEEV, SFV, and SINV (Palmer, 2011).

1.1.1.4 *Rhabdoviridae*

Viruses in the family *Rhabdoviridae* contain non-segmented, negative sense ss RNA. The genome encodes five structural proteins (Walker et al. 2015), consisting of nucleoprotein (N), matrix protein (M), glycoprotein (G), polymerase-associated phosphoprotein (P), and RNA dependent polymerase (L). The genome has an untranslated leader (*l*), and trailer (*t*) sequences. The genome size of rhabdoviruses is approximately 11 to 15 kb (Walker et al., 2015). Arboviruses in the *Rhabdoviridae* include vesicular stomatitis virus, Alagoas virus, Chandipura

virus, and Isfahan virus (Palmer 2011). Some genera of mosquitoes that transmit rhabdoviruses include *Coquillettidia spp*, *Culex spp*, and *Trichoprosopon spp* (Kuwata et al., 2011; Walker et al., 2015).

1.1.1.5 Vector competence and vectorial capacity for arboviruses

The ability of a mosquito to become infected with an arbovirus and transmit the arbovirus to a vertebrate host is termed as vector competence. Before an arbovirus can infect, disseminate, and transmitted by a mosquito, it needs to overcome certain barriers to infection and transmission (Franz et al., 2015). For example, the midgut infection barrier needs to be overcome to initiate a successful infection, while the midgut escape barrier needs to be overcome by the arbovirus for successful dissemination into the mosquito hemocytes, legs, abdomen, salivary glands, and other parts of the mosquito (Franz et al., 2015). After successful dissemination into other parts of the mosquito body, the arbovirus needs to overcome the salivary gland infection barrier to infect the salivary gland and salivary gland escape barrier to be transmitted in the saliva during blood feeding by the infected mosquito on a vertebrate host. Factors that affect vector competence are basal lamina permeability (which affects the midgut escape barrier), thickness of peritrophic matrix, digestive enzymes, immunity (for example RNAi) (Bartholomay and Michel, 2018; Kumar et al., 2018; Lee et al., 2019; Tikhe and Dimopoulos, 2021), microbial interactions (for example, the presence of *Wolbachia* reduces vector competence) (Cansado-Utrilla et al., 2021), salivary gland barriers (Franz et al. 2015), and the mosquito genetic background (Beerntsen et al., 2000).

Vectorial capacity is described as the number of potentially infectious bites by an arbovirus-infected mosquito on a vertebrate host. Vectorial capacity is affected by extrinsic factors and intrinsic factors (Brady et al., 2016). The vectorial capacity is influenced largely by

vector population density, vector blood feeding behavior, and vector longevity. In estimating vectorial capacity, four parameters are taken into account when using the classical formula, which are extrinsic incubation period of the pathogen, the ratio of mosquitoes to humans, mosquito survival per day, and human biting rate (Brady et al., 2016). Vectorial capacity accounts for the fact that only examining vector competence is not enough to estimate disease transmission if the frequency of contact with the infected host is low. Additionally, relatively short-lived vectors will reduce vectorial capacity even if a host is highly competent for the vector (Kramer and Ciota, 2015). Also, a poorly competent vector can cause an outbreak if there is frequent feeding, shorter extrinsic incubation period, and high vector population density. For example, the frequent blood feeding of the vector on human hosts and high mosquito population density was responsible for a YFV outbreak in Nigeria although the *Ae. aegypti* vector demonstrated poor competence for the virus (Miller et al., 1989).

1.1.1.6 Transmission of arboviruses

The transmission cycle of arboviruses starts when a female mosquito takes a blood meal from an infected person or other vertebrate during a blood meal. The ingested viruses infect mosquito midgut epithelial cells as their first point of interaction (Black et al., 2002). The virus interacts with host receptors in the process of initiating infection in the mosquito. For example, SINV binds to *Ae. aegypti* natural resistance-associated macrophage protein (NRAMP), laminin proteins, and L-sign receptors to initiate cell entry (Rose et al. 2011; Klimstra et al., 1998; Klimstra et al., 2003; Vancini et al., 2015). Upon entry into cells of the midgut, arboviruses uncoat and use the host machinery to translate viral proteins for assembly into viral particles (Black et al., 2002).

For a successful invasion, the virus needs to overcome physical barriers in the mosquito. These barriers are the midgut infection barrier, midgut escape barrier, salivary gland infection barrier, and salivary gland escape barrier (Black et al., 2002). The midgut infection barrier functions by (1) prevention of virus invasion of the epithelial midgut cells or (2) prevention of the replication of or spread of the virus to other cells after it has invaded the epithelial midgut cells (Black et al., 2002). The midgut escape barrier is made up of the midgut epithelium and the dense fibrous extracellular matrix called basal lamina (BL). The BL serves as a filter, allowing only molecules to pass through and preventing the passage of larger microorganisms. The matrix of the BL has pores smaller than that of most arboviruses. Differences in the BL structure and thickness may contribute to the differences in midgut escape rates (Black et al., 2002; Carpenter and Clem, 2023). After the arbovirus has escaped the MEB, it needs to disseminate through the mosquito body and to infect the salivary gland.

The salivary gland infection barrier prevents the virus from entry into the salivary gland tissue after it has escaped the midgut barriers, while the salivary gland escape barrier prevents the escape of the virus from the salivary gland into saliva after it has successfully infected the salivary gland tissue (Romoser et al., 2005). The successful evasion of these physical barriers by the viruses means it becomes available in the saliva to infect the next host during a subsequent blood meal. The duration of time needed for virus to infect, replicate, and subsequently be transmitted to the next vertebrate during a blood meal is termed as the extrinsic incubation period, while the intrinsic incubation period is defined as the time between arbovirus infection and the onset of arbovirus disease symptoms in a vertebrate host (Palmer, 2011; Chan and Johansson, 2012).

Some arboviruses can be transmitted from mother to offspring through eggs in a process termed vertical transmission (Romoser et al., 2005). For example, ZIKV can be transmitted by transovarian means (Li et al., 2017). Female mosquitoes can also sometimes be infected with certain arboviruses during mating with an infected male (Romoser et al., 2005).

Mechanical transmission occurs only in rare cases in arbovirus transmission. In this process, the virus attaches to the proboscis of a female mosquito which is inoculated in the vertebrate host during a blood meal without the requirement for replication in the mosquito vector (Palmer, 2011).

Certain arboviruses that infect humans can undergo sylvatic cycle transmission. This cycle involves transmission of arbovirus between non-human primates such as monkeys and mosquitoes in the forest. An example of an arbovirus that can undergo a sylvatic transmission cycle is YFV (Moreira-Soto et al., 2018). Additionally, in some cases vertebrates can be dead end hosts for arboviruses. This occurs when hosts do not develop high viral titers in their blood (known as viremia) after having been infected with an arbovirus. Well-known examples of dead end hosts are humans and horses infected with WNV. These hosts do not develop high viremia infections, hence mosquitoes do not get infected with WNV when they feed on an infected human or horse. In this case, mosquitoes can only be infected when they feed on infected birds that serve as amplifying hosts for WNV (Colpitts et al., 2012).

1.1.2 Sindbis virus (SINV)

1.1.2.1 SINV structure and genome

SINV virions are spherical with a diameter of 70nm. They have a $T=4$ icosahedral capsid which is made up of 240 monomers and a lipid membrane that is derived from the host cell with

80 trimeric spikes. Each spike consists of three heterodimers formed by the E1 and E2 glycoproteins (Strauss and Strauss, 1994). The viral genome is a positive sense, single-stranded RNA molecule with 11.7 Kb in length and contains two open reading frames. The first open reading frame codes for the four non-structural proteins, namely nsP1, nsP2, nsP3, and nsP4. The second open reading frame codes for five structural proteins which are the C (capsid), E3, E2, 6K, and E1 proteins (Strauss and Strauss, 1994). Expression of the structural proteins is via a subgenomic mRNA. Entry of SINV into cells is receptor-mediated via clathrin-coated vesicles. Replication of SINV is facilitated by specific membranous complexes (Spuul et al., 2011). Translation and nucleocapsid assembly take place in the cytosol while virion assembly is completed at the plasma membrane. Glycoprotein complexes and lipid membranes are acquired by the virus during virion assembly on the plasma membrane (Spuul et al., 2011).

1.1.2.2 Taxonomy of SINV

The International Committee on the Taxonomy of Viruses (ICTV) classifies 31 virus species in the genus *Alphavirus* (family *Togaviridae*). Genomic sequences used to construct a phylogenetic tree and the use of older serological cross-reactivity data clusters the alphaviruses in two main groups, which are the “Old World” and “New World” alphaviruses. The group name was assigned roughly due to the geographical location in which the viruses mainly occur (Fauquet et al., 2005). SINV is most closely related to western equine encephalitis virus (Weaver et al., 2012).

Phylogenetic analysis shows SINV can be classified into six genotypes that lie in north-south axes (Lundström and Pfeffer, 2010). The genotypes overlap with the routes of migrating bird hosts. European and African strains are grouped under genotype 1 while, the Australian,

Eastern, and Central Asian strains are grouped under genotypes 2-4 (Lundström and Pfeffer, 2010). The closely related Whataroa virus, first isolated from a town in New Zealand, falls under genotype 5. Genotype 6 strains are from southern and western Australia (Lundström and Pfeffer, 2010). Group 1 strains from sub-Saharan Africa and northern Europe share common ancestry (Lundström and Pfeffer, 2010).

1.1.2.3 History and epidemiology of SINV

SINV was first isolated from a *Culex* mosquito in the Sindbis district of Egypt in 1952 (reviewed in Adouchief et al., 2016). In nature, SINV is transmitted to humans by *Culex spp* and *Culiseta spp* mosquitoes, but SINV has also been isolated from several species of *Aedes* mosquitoes. In 1961, SINV was successfully isolated from the blood of patients from Uganda. Isolation of SINV from skin lesion biopsies from patients from South Africa was reported in 1963. Since then, there has been a series of SINV outbreaks reported in South Africa, Sweden, Finland, and Germany (Malherbe and Strickland-Cholmley, 1963, reviewed in Adouchief et al., 2016). SINV antibodies have been found in birds and children from Israel (reviewed in Adouchief et al., 2016). In 1965, antibodies produced in response to SINV infection were found in healthy individuals from Europe, with SINV infection occasionally causing an outbreak (reviewed in Adouchief et al., 2016). In 1974, a large SINV outbreak occurred in South Africa between January to February while an outbreak occurred in Sweden and Finland between August to September of the same year (reviewed in Adouchief et al., 2016). SINV infections have been diagnosed in patients periodically ever since. Between 1974 to 2002, epidemics occurred at an approximate 7-year intervals in Finland, with the largest outbreak occurring in 1995 with 1310 diagnosed patients (Brummer-Korvenkontio et al., 2002). In 2009, SINV was isolated from

mosquitoes in urban areas in southern Germany (Jöst et al., 2010) while the virus was isolated from crows in Benin in 2010 (Eiden et al., 2014).

1.1.2.4 SINV infects multiple host and tissue types

The primary vectors for SINV transmission are mosquitoes, while birds are the amplifying hosts (Hubálek, 2008). SINV also infects marsh frogs, rodents, and humans (reviewed in Adouchief et al., 2016). In adult mosquitoes, SINV infects and replicates in different tissues such as the midgut, hemolymph, eye, fat body, musculature, neurons, legs, and the salivary glands (Pierro et al., 2003).

SINV infects different species of mosquitoes. In northern Europe, SINV has been found in *Culex torrentium* and *Cx. pipiens*. In Sweden, SINV has been isolated from *Culiseta morsistans* and *Ae. cinereus* while SINV was isolated from *Ae. annulipes* in Finland, Norway, and western Russia (reviewed in Adouchief et al., 2016; Norder et al., 1996; Sane et al., 2012). In South Africa and Australia, SINV is isolated mostly from *Cx. unvittatus* and occasionally from *Cx. pipiens* and *Cx. theileri* (Storm et al., 2013).

Birds of the orders Passerines, Galliformes, and Anseriformes have been shown to be infected by SINV (Lundström et al., 1991). In Sweden, SINV infects passerines, thrushes (*Trudus philomelos*), fieldfares (*T. pilaris*) and redwings (*T. iliacus*) (Foy et al., 2004; Lundström et al., 1991) while SINV infects Galliformes, black grouse (*Tetrao tetrix*) and capercaillie (*Tetrao urogallus*) in Finland (Brummer-korvenkontio et al., 2002; Kurkela et al., 2008). SINV infection has been detected in carrion crows (*Corvus corone*) and magpies (*Pica pica*) in England (Medlock et al., 2007), and hooded crows (*Corvus cornix*) in Egypt (reviewed in Adouchief et al., 2016) and Germany (Eiden et al., 2014).

The ability of SINV to infect different mosquito species has allowed the use of multiple mosquito cell lines for studying SINV *in vitro*. For example, C6/36 and Aag-2 cell lines from *Ae. albopictus* and *Ae. aegypti*, respectively, can be used to study insect immune responses to SINV infection (Barletta et al., 2012; Brackney et al., 2010). The ability of SINV to infect a wide range of vertebrate cell types and cell lines such as Vero cells and BHK-21 cells from the kidneys of African green monkeys and baby hamsters, respectively, makes it possible to study mammalian host factors that affect SINV replication (Wu et al., 2020). Although *Ae. aegypti* does not serve as a natural vector for SINV, the ability of SINV to infect and replicate in different adult *Ae. aegypti* mosquito tissues makes it possible to study how this arbovirus infects (midgut infection), disseminates (leg infection), and is transmitted (salivary glands infection) in mosquitoes (Pierro et al., 2003). Virulent and avirulent SINV strains have been used extensively to study the pathogenesis of viral encephalitis in mice (Kulcsar et al., 2015).

1.2 *Aedes aegypti* as an arbovirus vector

1.2.3 *Aedes aegypti*

The yellow fever mosquito, *Ae. aegypti*, is thought to have originated in forests in Africa and has since spread to other tropical and subtropical regions of the world (Jansen and Beebe, 2010). They were introduced into urban environments through the activities of humans such as human settlements encroaching into forests and harvesting forest resources for economic gains. The spread of *Ae. aegypti* across urban areas in Africa was further facilitated by trading activities across the continent (Jansen and Beebe, 2010). *Ae. aegypti* were introduced to the New World through slave trade activities where merchant ships carried the mosquitoes from Africa to the

Americas and Europe. They were later introduced to the Asia continent and the Pacific region through the activities of the Second World War (Urdaneta-Marquez and Failloux, 2011).

The successful colonization of a new locality by *Ae. aegypti* is largely dependent on the climate conditions, availability of suitable vertebrate hosts, and stagnant water for breeding. *Ae. aegypti* breeds around human settlements in stagnant water in containers and choked gutters. Females feed on human blood for the development of eggs. They obtain blood meals by feeding on humans in the open or by moving indoors (Jansen and Beebe, 2010). The search for oviposition sites aids in the dispersal of the adult mosquito. The dispersal rate of *Ae. aegypti* is inversely proportional to the number of breeding sites available for oviposition (Edman et al., 1998). Dispersal of *Ae. aegypti* may also be facilitated by unintentional human transportation (Jansen and Beebe, 2010).

1.2.3.1 The life cycle of *Ae. aegypti*

The mosquito life cycle consists of four stages; the egg, larva, pupa, and the adult (Crans, 2004). A female *Ae. aegypti* lays approximately 100 to 200 eggs per batch which can withstand desiccation for approximately one year (Wong et al., 2011). The eggs are laid in stagnant water found in nature (such as tree holes or ground depressions) or human-made containers (including tires, water storage containers, or other types of containers) (Wong et al., 2011). A drop in oxygen level, infusion of bacteria, and a wide range of compounds are thought to stimulate the eggs for hatching. Most eggs hatch into the larval stage within 48 hours once stimulated. Harsh weather conditions in winter delays hatching of eggs until favorable weather condition returns (Crans, 2004). The larvae reside in the aquatic environment and move to the surface of the water, where they hang upside down and use a siphon to breathe. The larvae molt four times with the fourth molt resulting in the change to the pupa stage. Each time the larvae molt, they grow larger

(Crans, 2004). The pupa is a resting and non-feeding stage. The pupae are motile, making them able to respond to changes in environmental conditions. The pupae develop into adult mosquitoes within approximately 36 hours (Crans, 2004). The emergence of adult mosquitoes is followed by a short period of drying and hardening of body parts. The adult mosquito spreads its wings to dry properly before it commences flight. Mating and blood-feeding do not take place immediately, but after a few days (Crans, 2004).

Ae. aegypti males hatch first and become sexually mature to copulate after 24-48 hours, while the female becomes receptive for mating 30-60 hours after emergence. *Ae. aegypti* mate at dusk (Crans, 2004). Males are polygamous while females are monogamous. The female mates to receive sperm only once in her lifetime. The sperm received during the first mating is enough to fertilize all the eggs produced in the female's lifetime. Females store sperm in an organ called the spermatheca (Becker and Dahl, 1941; Crans, 2004).

Females require a diet with high protein content for ovary development, which is obtained from vertebrate blood through blood feeding (Harrington et al., 2009). After obtaining blood from its vertebrate host, extensive physiological changes occur which leads to oocyte maturation, and subsequently, oviposition (Harrington et al., 2009). The production of eggs in mosquitoes is a cyclic process termed the gonotrophic cycle. It starts with the uptake of a blood meal and ends with the laying of eggs (Becker and Dahl, 1941; Wong et al., 2011).

1.2.4 The infection process in mosquitoes involves bottlenecks

1.2.4.1 Structural composition of mosquito midgut

The midgut is made up of the anterior and posterior regions. The anterior region is used for sugar absorption. The posterior region has rough endoplasmic reticulum, and numerous mitochondria, and is the site of absorption of nutrients after intake of a blood meal. Drastic

changes occur in the midgut epithelium after blood acquisition. Some of the changes include nuclei condensation, mitochondria enlargement, and the formation of concentric whorls in the rough endoplasmic reticulum (Okuda et al., 2002; Kumar et al., 2018; Romoser et al., 2004). The midgut secretes peritrophic matrix, separates the ingested blood from the epithelium and act as a barrier to infection. Pathogens in the blood can enter epithelial cells through the microvilli before the immune response is initiated against the pathogens (Dong et al., 2009).

1.2.4.2 Midgut tissue barriers to infection

The main tissue barriers to midgut infection are the midgut infection barrier (MIB) and the midgut escape barrier (MEB) (Hardy et al., 1983; Smith et al., 2007). The MIB is the first tissue barrier of infection which is encountered by an arbovirus. The MIB uses two main processes to resist arboviruses from establishing a midgut infection. These processes are (1) preventing the arbovirus from invading the midgut epithelial cells and (2) the prevention of arboviral replication after the invasion of midgut cells, which blocks the spread of the arbovirus to other cells (Franz et al., 2006, 2014). Other mechanisms have been proposed as possible ways the MIB uses to prevent the establishment of arbovirus infection (Carpenter and Clem, 2023; Kato et al., 2008). These mechanisms may include (1) the diversion of blood and virus into the ventral diverticulum or crop; (2) filtration of the arbovirus by the peritrophic matrix (Hardy et al., 1983); (3) virion inactivation by midgut digestive enzymes; (4) cell charge interactions between the virus and midgut that do not result in binding; and (5) inappropriate receptors on the apical surfaces of midgut epithelial cells which prevent arbovirus binding to midgut epithelial cells. Although there is experimental evidence that supports mechanism (5), there is little scientific evidence supporting mechanisms (1) to (4) (Carpenter and Clem, 2023; Kato et al., 2008).

The midgut escape barrier (MEB) is the second barrier to arbovirus infection, which inhibits the escape of arboviruses from the midgut and thereby prevents the dissemination of infection to other tissues of the mosquito. For arboviruses to bypass the MEB and successfully disseminate to other parts of the body, the virus needs to overcome the midgut epithelium and the dense fibrous extracellular matrix underlying it, the basal lamina (BL). A hypothesized mechanism for arbovirus midgut escape is the passing of virions through large gaps in the midgut epithelium and basal lamina created after structure changes as a result of blood feeding. Also, arboviruses may be able to escape the midgut by infecting tracheal cells and using them as an escape route for dissemination throughout the mosquito (Franz et al., 2015). Furthermore, the MEB can be dose-dependent or dose-independent. An example of a dose-dependent MEB was demonstrated when midgut infection of *Cx. tarsalis* with low dose of WEEV led to the absence of WEEV transmission. The authors suggested that the reason for the absence of transmission after midgut infection was because the dose-dependent MEB may have modulated the replication of arbovirus in the MEB, hence preventing it from escaping (Franz et al., 2015). For dose-independent MEB, there is incompatibility between the virions and the tissue surfaces of the MEB, leading to the prevention of the virus binding and/or bypassing the MEB tissues. This leads to the prevention of dissemination of arboviruses in their natural mosquito hosts (Forrester et al., 2014). The BL is part of the midgut escape barrier and act as an obstacle to prevent escape of virions from the midgut. The BL is made of the proteinaceous matrix which is excreted by the epithelial cells (Yurchenco and O'Rear, 1994). The BL functions primarily by providing structural support to the epithelium and aiding in continuous renewal after injury. The BL is porous and serves as a filter. This filter allows only small molecules to pass through and not microorganisms, not even viruses (Grimstad and Walker, 1991). Thinner BL has been reported to

allow rapid dissemination of La Crosse virus from the midgut of *Ochlerotatus triseriatus* females (Grimstad and Walker, 1991; Thomas et al., 1993). On the contrary, differences in BL thickness were not found to be a determining factor in DENV dissemination in *Ae. albopictus* (Thomas et al., 1993). During blood feeding, mosquito guts expand several fold in diameter to be able to accommodate the influx of vertebrate blood. After the expansion, it returns to its pre-feeding state after a few days. During this expansion and contraction, tears may occur in the BL which may result in transient gaps. These transient gaps are mostly repaired after a few days. There is evidence suggesting that transient gaps in the BL may be exploited by some arbovirus to allow them to escape into the hemocoel or trachea cells (Cui et al., 2019; Dong et al., 2017).

1.2.4.3 Bottleneck effect caused by midgut barriers during arbovirus infection and its consequences for arbovirus genetic stability

The first bottleneck effect encountered by arboviruses during the process of establishing an infection in the mosquito is infection of the midgut. The midgut epithelium poses as a barrier to infection which results in only a few epithelial cells to be infected by the virus particles, allowing only a few viruses to successfully infect the midgut. To overcome the phenomenon where only a few virus particles infect a small number of midgut epithelial cells, high titer virus stocks are typically used for oral infection in laboratory experiments (Kenney et al., 2012). In a study by Brault et al., (2004), the use of high titer VEEV to infect *Culex taeniopus* resulted in most of the midgut cells becoming infected while the use of low titer VEEV resulted in few midgut cells becoming infected (Brault et al., 2004). Reducing the VEEV bloodmeal titer from 10^6 to 10^5 plaque forming units (PFU) ml^{-1} reduced the number of virions that infect and escape the midgut of *C. taeniopus* by 100-fold (Forrester et al., 2012).

The ability of arboviruses to overcome bottlenecks and avoid any fitness losses (where fitness is measured by the amount of infectious progeny) is vital for their continued circulation between their vertebrate hosts and invertebrate vectors. Overcoming major genetic drift and potentially deleterious mutations is important for arboviruses to be stable (Weaver et al., 1992). Genetic drift occurs when there is a random fluctuation in the frequency a particular allele in a population. During genetic drift, the fluctuation can cause an allele to become overwhelmingly frequent in a population or disappear from the population, particularly in small populations (Weaver et al., 1992). For arboviruses to be stable and circulate in nature, it has been proposed that they need to avoid Muller's Ratchet theory after encountering the midgut bottleneck. Muller's Ratchet predicts that viruses of smaller population size undergoing replication are doomed to accumulate ever-increasing deleterious mutations as a consequence of the magnified power of genetic drift and mutation that accompanies small population size (Howe and Denver, 2008). It is thought that arboviruses overcome Muller's Ratchet by restoring the mutant swarms quickly via low fidelity replication (Forrester et al., 2014; Lequime et al., 2016; Patterson et al., 2018).

1.3 Factors affecting vector competence

1.3.1 Vector genetics

1.3.1.1 *Ae. aegypti*-arbovirus interactions

The recent advancement of genetic and transcriptomic tools has made it possible to study host-pathogen interactions between arboviruses and mosquito vectors in much greater detail. Modern genetic tools have been used to identify important genes in medically important mosquitoes that can be manipulated for the development of vector control strategies. The use of

transcriptomic data has shown that the infection process is not limited to mosquito immune gene expression levels in response to arbovirus infection, but rather involves a complex response. Mosquito responses to arbovirus infection have been related to varied processes including metabolism, detoxification, apoptosis, DNA replication, and protein translation (Colpitts et al., 2011; Girard et al., 2010; Sanders et al., 2005; Tchankouo-Nguetchou et al., 2010). Genes involved in these cellular processes have been shown to play roles in arbovirus infection. Although genetic approaches have been used to study arbovirus interaction, limitations in these approaches have created controversial results in how some of these cellular processes affect arbovirus infection in mosquitoes. For example, there are contradictory results on the role of apoptosis on arbovirus in mosquitoes (Eng et al., 2016; Wang, et al., 2008; O'Neill et al., 2015). Furthermore, mosquito physiological responses may play a role in arbovirus infection (Huang et al., 2019). A study conducted by Troupin et al. showed that manipulating the expression of the mosquito ubiquitin protein Ub3881 in different tissues resulted in labelling DENV for degradation (Troupin et al., 2016). However, in the midgut of *Ae. aegypti*, the labeling of DENV with UB3881 helped the release of DENV particles from the midgut. The use of modern genomic and transcriptomic tools has shown that vector competence is dependent on diverse pathways in the mosquito vector (Huang et al., 2019).

The transcriptomic response of *Ae. aegypti* after infection with arboviruses has been studied extensively in order to understand the mechanisms of infection and transmission (Bonizzoni et al., 2012; Chauhan et al., 2012; Colpitts et al., 2011; Tchankouo-Nguetchou et al., 2012). These studies have identified numerous genes that are differentially expressed during arbovirus infection, with some of these genes having antiviral properties in *Ae. aegypti* (Severson and Behura, 2016; Sim et al., 2014). For example, a study conducted by Colpitts et al., (2011)

showed that infecting the Rockefeller strain of *Ae aegypti* with different flaviviruses, namely, WNV, DENV, and YFV, resulted in different gene expression profiles with functions of these differentially expressed genes being involved in metabolic processes, ion binding, transport, and peptidase activity. Another study conducted by Bonizzoni et al., reported that infecting *Ae. aegypti* Chetumal strain with DENV resulted in 132 significant differentially expressed genes in the midguts of infected mosquitoes compared to the control samples (Bonizzoni et al., 2012). A comparative midgut transcriptomic analysis of *Ae. aegypti* after CHIKV infection identified potential genes involved in midgut escape. In this study, Dong et al., (2017) orally infected *Ae. aegypti* with CHIKV by feeding them on a saline meal mixed with the virus. The control group were only fed with protein meal with no virus. Results from this study showed that there was significant upregulation of 33 genes functioning as trypsins, metalloproteinases, and serine-type endopeptidases in the midgut of mosquitoes at day 1 after saline or protein meal ingestion by the mosquitoes. One of these genes, named *Ae. aegypti* late trypsin (*AeLT*), was found to show a strong matrix metalloproteinase activity. The upregulated genes with trypsin, metalloproteinase, and serine-type endopeptidase functions may have potential involvement in facilitating midgut escape of CHIKV in *Ae aegypti* (Dong et al., 2017).

The different transcriptional responses of *Ae. aegypti* innate immune genes to three different viruses (DENV, WNV, and YFV) suggests unique vector responses to the arboviruses used (Colpitts et al., 2011). Examples include: the toll pathway in *Ae. aegypti* has been shown to control DENV infection (Xi et al., 2008), while Kaneko et al (2006) reported that distinct branches of the Imd pathway contribute to antiviral defense differently (Kaneko et al., 2006). For the Jak-Stat pathway, upregulation of the domeless receptor in this pathway was observed at 10 days after oral infection with DENV (Souza-Neto et al., 2009; Xi et al., 2008). Different

transcriptional expression of mosquito immune genes was observed when different inoculation routes, infection models and experimental conditions for the same virus were used (reviewed in Merklings and van Rij, 2013). Also, differential susceptibility of *Ae. aegypti* and *Ae. albopictus* to Mayaro virus correlated with differences in immune gene expression in the mosquito species (Diop et al., 2019). This suggests that there is a possibility of different expression of mosquito immune genes that results in different viral titers after oral infection. Since the significant variation in titer is specifically observed following oral infection, these critical differences could occur in the midgut, or alternatively, circumstances in the midgut could affect the systemic immune response of the mosquito, which is thought to be primarily associated with hemocytes (the circulating blood cells).

1.3.1.2 Heterozygosity measurement in mosquito vectors

The most common method for measuring genetic variation in a population is by measuring heterozygosity (observed and expected) and the proportion of nucleotide sites that are polymorphic (Nielsen et al., 2011). Conventionally, a large number of individual samples from each population is used to measure the level of heterozygosity. The availability of next-generation sequence technology has made it possible to generate large quantity and quality of sequence data making it possible to use fewer individuals in a population to measure the level of heterozygosity (Nielsen et al., 2011). In determining heterozygosity, multiple loci are selected and used for the analysis. For accurate measurement of heterozygosity, apparent diversity at a locus must not be due to sequencing errors or genotyping errors. Hence, there is the need for adequate sequencing coverage of both strands of diploid individuals (Nielsen et al., 2011). In measuring heterozygosity, the expected heterozygosity is estimated from allele frequency while

observed heterozygosity is estimated from the individual genotypes directly. Observed heterozygosity depends on the amount of genetic variation and the level of inbreeding in the population. Inbreeding increases homozygosity (Ritland, 1996). Inbreeding in a population is estimated by comparing observed heterozygosity to expected heterozygosity. A result of lower observed heterozygosity when compared to expected heterozygosity suggests that inbreeding is occurring in the population (Schmidt et al., 2021).

1.3.2 Virus genetics

1.3.2.1 SINV replication process in host cells

SINV replication begins with the attachment of the virus glycoproteins to host receptors. Several molecules have been implicated as receptors for SINV including laminin receptor, C-type lectins heparin sulfate, and L-SIGN, and Natural Resistance-Associated Macrophage Protein (NRAMP) (Zimmerman et al., 2023). The ability to use multiple receptors may help explain the broad host range of the virus. The attachment of the virus to these receptors initiates entry into the host cell through receptor-mediated endocytosis (Klimstra et al., 2004; Rose et al., 2011; Wang et al., 1992). The E2 transmembrane glycoprotein contains the receptor binding protein at the N-terminus for binding with host receptors while the E1 transmembrane glycoprotein is used to mediate fusion between the SINV envelope and the endosomal membrane of the host (Rose et al., 2011). The entry of SINV into the host cell is followed by translation of the viral genomic RNA in the cytoplasm to generate the P1234 nonstructural polyproteins (which are later processed into nsp1, nsp2, nsp3, and nsp4) (Xiong et al., 1989). Nsp2 has cysteine protease activity which mediates the processing of the polyprotein to produce nsp4. Nsp4 functions to generate the complete negative and positive strand RNA when it is still part of the

P1234 polyprotein (Kääriäinen and Ahola, 2002). Processing of P1234 in a sequentially regulated manner leads to a replication complex that synthesizes both positive and negative strand RNA. Complete proteolytic processing of the replication complexes results in individual nonstructural proteins nsP1, nsP2, nsP3, and nsP4. The function of nsP1 is the capping of newly generated genomic and subgenomic RNA transcripts. nsP2 contains nucleotriphosphatase, and has both proteolytic and helicase activities, making it function in regulation of RNA synthesis. nsP2 also alters host cellular processes such as shutting off host translation and transcription processes (Garmashova et al., 2006; Gorchakov et al., 2005; Perri et al., 2000). nsP3 may play a role in RNA synthesis. Additionally, nsP3 may have a possible role in modulating host cellular pathways as it has been reported to be associated with host factors (Cristea et al., 2006; Gorchakov et al., 2008). nsP4 is the RNA-dependent RNA polymerase (Rubach et al., 2009; Tomar et al., 2006).

The nonstructural proteins produced are involved in both negative and positive-strand RNA production. The positive-strand RNA produced comprises the full length genomic RNA as well as the subgenomic RNA, which is colinear with the 3'-end of the genome and contains the coding sequence for the SINV structural proteins (Kääriäinen and Ahola, 2002). The subgenomic RNA is 4,106 nucleotides long. It is capped and polyadenylated but does not serve as a template for minus-strand synthesis and is not packaged into virions (Strauss and Strauss, 1986). The subgenomic RNA is translated into a polyprotein which subsequently undergoes cotranslational and posttranslational cleavages to produce the virion structural proteins (Levis et al., 1990).

1.3.2.2 SINV and host factors interact during the virus replication process

SINV replication occurs in the cytoplasm of the infected cells and involves interactions with host proteins at both the early stage (6 hours post-infection) and late stage (12 hours post-infection) of replication (Westcott et al., 2022). These interactions may either enhance or repress the SINV replication process. The mammalian heterogeneous nuclear ribonucleoproteins (hnRNPs) have been shown to interact with SINV subgenomic RNA to regulate the expression of SINV structural proteins, promote particle infectivity, and enhance virus replication (Westcott et al., 2022). SINV nsPs have been reported to interact with several host factors during different stages of SINV replication processes. For example, the SINV nsP1 has been shown to interact with TMEM45B after SINV infection in 293A cells. TMEM45B is an interferon-stimulating protein with antiviral properties which the host cell uses to resist SINV infection (Yan et al., 2022). The interaction of TMEM45B and nsP1 leads to the destruction of the virus replication complex, the promotion of SINV RNA degradation, and the diminishing of SINV-induced cytopathic vacuoles. Infection of SINV in mammalian and invertebrate cells showed that nsP2 interacts with the host protein sorting nexin 5 (SNX5) which functions to enhance SINV replication (Schuchman et al., 2018).

In a study by Gorchakov et al., SINV was used to infect mammalian BHK-21 cells and mosquito C710 cells. The results from this study showed that SINV infection in BHK-21 led to the formation of two complexes: 1) nsP3-host endosome-like complexes and plasma membrane complexes, and 2) nsP3-host nuclei complexes (Gorchakov et al., 2008). The nsP3-host nuclei complexes could be co-isolated and solubilized only by treating with filament-destabilizing detergents. In both complexes formed, the GTPase activating protein (SH3 domain) binding protein 1 (G3BP1) and protein 3 (G3BP3), YBX1, and HSC70 host proteins were common. In

the mosquito-infected cells, Rasputin, an insect homolog of G3BP1 was found in nsP3-host membrane-associated complexes (Gorchakov et al., 2008). In addition, Frolova et al., reported that SINV nsP3 forms complexes with host factors, namely cytoskeleton proteins, chaperones, elongation factor 1A, some ribosomal proteins, and heterogeneous nuclear ribonucleoproteins (Frolova et al., 2006). These host factors may have roles in viral RNA synthesis (Gorchakov et al., 2008).

1.3.2.3 Genetic stability of SINV during replication

Arboviruses have been reported to undergo lower mutation rates when compared to other animal RNA viruses that replicate solely in vertebrates (Weaver et al., 1999). In SINV, the rate of nucleotide substitution is approximately 1×10^{-5} to 1×10^{-3} substitutions per nucleotide per cell infection (Strauss and Strauss, 1994) compared to poliovirus with approximately 1×10^{-4} to 1×10^{-2} substitutions per nucleotide per cell infection (Savolainen-Kopra and Blomqvist, 2010). It is thought that the alternation between infection of mosquitoes and vertebrates hosts during arbovirus replication helps constrain evolution in arboviruses, leading to more genetic stability compared to RNA viruses that replicate solely in vertebrates. In a study by Greene et al., SINV was passaged serially, either in one cell type or alternating between two different cell types, BHK (a vertebrate cell line) and C6/36 (a mosquito cell line). Results from this study showed that SINV accumulated less mutations and amino acid changes when passaged alternately between BHK-21 and C6/36 cells compared to when passaged in only BHK-21 or C6/36 cells (Greene et al., 2005).

In a study conducted by Kueberuwa et al., to understand the tissue specific attenuation of SINV without compromising the SINV genetic stability, microRNA (miR)-response elements

(MREs) corresponding to liver-specific miR122, muscle-specific miR133a and miR206, or hematopoietic-specific miR142-3p were inserted into the SINV genome with the goal of decreasing virus replication in the liver, muscle, and/or hematopoietic cells respectively (Kueberuwa et al., 2014). Unfortunately, SINV with the inserts mutated quickly *in vitro*, losing approximately 50% of the transgene sequences within an estimated 6.2 passages. To make the SINV containing the inserts more genetically stable, a shorter insert containing sequences of the MREs but not a full-length transgene was used. Complete genetic stability was observed after at least 10 passages. This suggests that SINV clinical properties can be improved through genetic modification (Kueberuwa et al., 2014). In a study conducted by Carpenter et al., midgut-specific miRNAs were inserted in SINV to study the effect of virus replication in midgut escape. Results from this study showed reduced infection but not the total elimination of disseminated infection. More importantly for this discussion, the SINV used for the study showed high genetic stability (Carpenter et al., 2021). In addition, insertion of a reaper gene in SINV to study the effect of apoptosis on virus infection in *Ae. aegypti* showed that apoptosis is used as an antiviral defense strategy to reduce virus infection in mosquitoes. Mutation in the reaper gene insert was low, suggesting there was genetic stability during SINV *in vivo* replication (Carpenter et al., 2022).

1.3.2.4 The use of SINV in scientific research due to low pathogenicity and fatality rate

SINV infection is associated with low pathogenicity and fatality rate in humans, which is one of the factors that make it a good model to study arboviruses. In Sweden, the ratio of subclinical to symptomatic SINV is estimated to be in a range of 20-40 to 1, while in Finland, it was 17 to 1 (Brummer-Korvenkontio et al., 2002; Lundström et al., 1991). After a SINV-infected mosquito bites an individual, it takes a median time of four days for symptoms to show although

the range of infection to symptoms is between 2 to 18 days (Kurkela et al., 2005; Sane et al., 2011). Acute phase symptoms usually take around two weeks to resolve (Sane et al., 2010). A report from Sane et al. showed that only 6% (20/337) of patients infected with SINV were hospitalized in Finland while Adouchief et al., reported that only 3% (2/65) were hospitalized in Sweden during a SINV outbreak (Adouchief et al., 2016; Sane et al., 2011). Viremia associated with SINV infection is low (10^3 genome copies/mL) as compared to other arboviruses such as CHIKV (10^9 - 10^{10} genome copies/mL), meaning that it is unlikely that humans serve as amplifying hosts. Furthermore, the pathogenicity associated with SINV disease is low compared to other arboviruses such as DENV, ZIKV, and CHIKV (reviewed in Adouchief et al., 2016; Appassakij et al., 2013; Sane et al., 2012). Specific IgM antibodies produced in response to SINV infection are detectable in patients within the first 7 to 14 days after infection (Kurkela et al., 2005; Vene et al., 1991) while IgG becomes detectable 11 days post-infection (Kurkela et al., 2005). SINV IgG antibodies may evoke a lifetime protection against new symptomatic infection (Kurkela et al., 2005).

Symptoms associated with SINV infection include rash, headaches, itching skin, fatigue, malaise, dizziness, nausea, and lymphadenopathy (reviewd in Adouchief et al., 2016; Kurkela et al., 2005). Skin lesions, mostly on the trunk and limbs, appear in approximately 92% to 100% of patients with symptomatic SINV infection (Sane et al., 2011; Turunen et al., 1998). In addition, joint symptoms appear in approximately 95% to 100% of patients with symptomatic SINV infection. For example, in a study conducted by Storm et al., five out of six patients with symptomatic SINV infection experienced joint pains. The most common joint pains associated with symptomatic SINV infection occur in the ankle (83% to 85%) followed by the wrists, knees, and fingers (56% to 72%) (Storm et al., 2014). Myalgia and arthralgia are also symptoms

that are commonly observed during symptomatic SINV infection. Laine and colleagues reported one possible case of meningitis in a symptomatic SINV infection while Adouchief et al., reported a case of recurring hemorrhagic skin lesions associated with symptomatic SINV infection. Overall, the evidence shows that severe complications of symptomatic SINV infection are rare (reviewed in Adouchief et al., 2016; Laine, 2002). Additionally, the pathogenicity of SINV is associated with capping of nonstructural proteins mRNA produced during SINV synthesis and replication. The absence of capped mRNAs during SINV synthesis and replication makes it more virulent (Lapointe et al., 2020). The use of SINV strains that produce capped mRNA in research make them safer for experimental use (Lapointe et al., 2020). Furthermore, *Ae. aegypti* mosquitoes resist SINV infection with an increase in the incubation period. The incubation period of fifteen days or more results in some SINV-infected *Ae. aegypti* mosquitoes being able to clear or drastically reduce SINV load. The low SINV load in *Ae. aegypti* mosquitoes later in infection may be associated with low or no virus transmission (Honório et al., 2020).

1.3.2.5 The use of SINV to study factors contributing to mosquito vector competence due to its ability to be easily engineered

Another advantage of using SINV as a model is the development of alphavirus transducing systems, which has made it possible to develop new expression vectors that can be used to study mosquito vector competence. SINV transducing system (STSs) have been used to express heterologous gene products transiently in mosquito cell culture and the larvae and adults of *Ae. aegypti* mosquitoes (Olson et al., 2000). The STSs are engineered to contain a second subgenomic promoter and a multiple cloning site (MCS). The insertion of a foreign gene into the MCS results in the transcription of a second subgenomic RNA containing the foreign insert

(Pierro et al., 2003). A gene of interest (GOI) with appropriate size of 1 kb or less can be inserted near the second promoter sequence for it to be transcribed in infected cells. Additionally, a GOI can be inserted as an in-frame fusion between two structural genes for a more stable expression compared to expression via the duplicated subgenomic promoter (Carpenter and Clem, 2022). The transcription of genes under control of a duplicated subgenomic promoter or as in-frame fusions between SINV structural proteins makes it possible to observe the biological effects of foreign gene products in either mosquito cell lines or larval and adult mosquitoes (Cheng et al., 2001; Olson et al., 1994). Engineering of SINV with a GOI can be delivered into mosquitoes orally by feeding mosquitoes with blood containing the genetically engineered SINV or the engineered virus can be injected in into adult mosquitoes, mosquito larvae, and mice. The GOI is expressed in the midgut and subsequently in other tissues (Blair et al., 2000).

Molecular engineering of SINV has also made it possible to develop new strains for the study of mosquito vector competence. For example, the characteristics of the TE/3'2J SINV strain did not make it conducive for use in studying vector competence since it does not readily infect midgut cells. The expression of genes using TE/3'2J in non-midgut tissues is mostly achieved by parenterally infecting mosquitoes through the thorax (Hahn et al., 1992; Olson et al., 1994). To address this problem, Olson and colleagues developed an MRE/3'2J construct that is made up of the structural coding region of the Malaysian MRE 16 SINV and the non-structural genes of the TE/3'2J virus. The structural coding region of the Malaysian MRE 16 SINV ensures the efficient midgut infection and dissemination of MRE/3'2J in *Ae. aegypti*. Additionally, TE/3'2J loses any inserted genes after three to four passages in cell culture (Hahn et al., 1992; Olson et al., 1994). The TE/3'2J duplicated subgenomic promoter is located in the 3' noncoding region of the virus RNA, immediately after the terminal nucleotides of structural genes. This

makes it less stable to carry and express heterologous genes. Due to this, the virus often deletes the inserted sequence after multiple cell passages (Olson et al., 2000; Seabaugh et al., 1998; Hahn et al., 1992). To increase insert stability, 5' dsMRE16ic was developed from the Malaysian MRE16 SINV strain. It has a T7 promoter at the 5' end to facilitate in vitro transcription of the viral RNA, and two subgenomic promoters located between the structural and non-structural genes. Heterologous genes can be inserted after the first subgenomic promoter to allow stable expression of the foreign gene product.

Genetic engineering of SINV has made it possible to study factors that contribute to mosquito vector competence. A study conducted by O'Neill et al., provided evidence on how apoptosis, an antiviral defense strategy in mosquitoes contributes to vector competence by using an engineered SINV. In this study, the pro-apoptotic reaper gene (Rpr) from *Drosophila* was inserted into a SINV infectious clone and named MRE/Rpr. The expression Rpr in mosquitoes resulted in an impaired infection phenotype. Additionally, strong selection against MRE/Rpr was observed in infected mosquitoes (O' Neill et al., 2015). This evidence showed that mosquitoes use apoptosis as a defense against arbovirus infection (O' Neill et al., 2015).

In all, the ability to develop new SINV strains and safely genetically engineer SINV to harbor and stably transcribe any GOI makes it easy for use in studying factors that contribute to mosquito vector competence.

1.3.2.6 Virus quasispecies

The low fidelity of viral polymerase introduces frequent errors during the replication process of RNA viruses. These errors introduce mutations, leading to the generation of a virus population consisting of multiple genotypes (Vignuzzi et al., 2006). High mutation rates are

dangerous for viruses as it has been reported that most of these mutations can produce nonviable individuals, but recent evidence shows the high mutational rate in RNA viruses can create swarms of variants containing potentially beneficial mutations, known as quasispecies. These quasispecies are more likely to overcome challenges encountered during infection and adapting to new environmental conditions (Vignuzzi et al., 2006). Additionally, quasispecies can help viruses to escape vaccines, resist antiviral effects, and increase pathogenicity. To measure the degree of quasispecies in a virus population, indices such as Shannon entropy (which measures complexity in the virus population) and nucleotide diversity (which measures diversity in the virus population) are used (Domingo et al., 2012).

1.3.3 Immunity

1.3.3.1 Molecular basis of the mosquito immune response to arbovirus infection

1.3.3.1.1 Recognition

For an immune response to be initiated against a pathogenic organism, there is the need for recognition of that pathogen by the immune system. The recognition of pathogens by the innate immune system is by the use of pattern recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs) of the pathogens (Kumar et al., 2018). PRRs are membrane bound or secreted proteins mostly found in the midgut and hemocoel. The majority of these proteins are secreted proteins having adhesive domains, which are used to interact with PAMPs to identify pathogens (Kumar et al., 2018; Prince et al., 2023). PRRs are comprised of different families of proteins. An example of a family of proteins functioning as PRRs is the thioester-containing proteins (TEPs) (Blandin et al., 2004; Cheng et al., 2011). One protein studied extensively in this family of proteins is TEP1. TEP1 is produced by hemocytes and

functions by recognizing pathogens for phagocytosis. The protein is secreted as an inactive single-chain peptide, and activated by proteolytic cleavage (Blandin et al., 2004). The activated protein is stabilized afterward by leucine-rich repeats complex proteins. The leucine-rich repeats complex proteins consist of the LRIM1 and APLC1 proteins. The TEP1 proteins can only function after stabilization. The TEP1 then binds to bacteria, fungi, or *Plasmodium* in the hemolymph or midgut leading to phagocytosis (Fraiture et al., 2009; Povelones et al., 2009). The functional importance of LRIM1 and APLC1 has been determined in the anti-*Plasmodium* response. Silencing LRIM1 and APLC1 in *An. gambiae* led to a weak immune response to *Plasmodium* infection (Fraiture et al., 2009). Different polymorphisms in the LRIM1 and APLC1 genes resulted in different degrees of susceptibility or resistance to *Plasmodium* infection (Fraiture et al., 2009). The role of TEPs in anti-flavivirus response in *Ae. aegypti* has been determined by Cheng and colleagues in 2011. The results of their study showed that overexpression of TEP1 led to a reduction in viral load while reduced expression levels of TEP3 resulted in high viral load. These results confirmed that TEP1 is involved in antiviral defense in *Ae. aegypti* (Cheng et al., 2011).

Another family of proteins that plays crucial roles in the recognition of PAMPs is the fibrinogen-related protein family (FREP). This family of proteins is the largest pattern-recognition protein family made up of 59 members. FREP proteins function in immune defense against fungi, bacteria, and parasite infections (Kumar et al., 2018; Wang et al., 2005). Gene knockdown techniques were used to demonstrate that FREP proteins play an important role in innate immunity (Hanington and Zhang, 2011; Kumar et al., 2018).

The C-type lectins are another family of proteins that function as PRR. They are both membrane-bound and/or soluble proteins. These groups of proteins have both positive and

negative regulation on the innate immune response. C-type lectin proteins are disulfide-linked heterodimers involved in killing *Escherichia coli* in a process independent of melanization. The knockdown of these proteins led to an increase in bacteria loads resulting in rapid mortality (Osta et al., 2004; Schnitger et al., 2009).

1.3.3.1.2 Immune signaling pathways in mosquitoes

Immune-signaling pathways help to initiate an immune response to infections and protect the mosquito from the harmful effects of pathogens. Induction of immune signaling pathways may result in the production of antimicrobial peptides (AMPs), which help in killing invading pathogens. Pathways that are also involved in AMP induction upon signaling are the Toll pathway, IMD pathway, and the Jak-Stat pathway. These pathways are triggered by the sensing of invading pathogens, and their signaling results in the production of effector molecules to either kill the pathogen or neutralize its harmful proteins (Kumar et al., 2018).

1.3.3.1.3 Toll pathway

The Toll pathway was first identified in *Drosophila melanogaster*. The identification of the Toll signaling cascade enhanced our understanding of the innate immune response in invertebrates and vertebrates (Kumar et al., 2018). The Toll pathway is induced by bacteria and fungi leading to the production of cytokines and/or AMPs to neutralize the effect of the pathogen (Lemaitre et al., 1996; Michel et al., 2001). To activate the pathway, proteolytically cleaved Spaetzle ligand binds to toll receptors to initiate a downstream signaling cascade leading to the induction of NF- κ B (Schneider et al., 1994). The downstream signaling cascade involves the toll receptors binding to MyD88, which leads to the recruitment of Tube, Pelle, and TRAF6 to activate Rel 1 (Wang et al., 2019). Genes involved in the Toll pathway are under the regulation of Rel 1. Rel 1 is also a transcription factor of NF- κ B and a homolog of the Dorsal gene in

Drosophila. The dorsal gene has been shown to be very important in the regulation of the *Drosophila* Toll pathway. *Ae. aegypti* has two isomers of Rel 1, which are Rel 1-A and Rel 1-B. The induction of Rel 1-A and Rel 1-B is initiated in mosquito larvae or female adults by septic injuries (Kumar et al., 2018). Rel 1 is crucial in activating the Toll pathway to initiate an antifungal immune response. In a study to determine the functional importance of Rel 1 in Toll pathway activation, two different transgenic mosquitoes were created. One of the transgenics was overexpressing Rel 1, while the other had Rel 1 knockdown (Bian et al., 2005). Results from this study showed that Rel 1-A homology domain led to the subsequent overexpression of Rel 1-B. Rel 1-A homology domain regulates the transcription activation of both Rel 1-A and Rel 1-B. The induction of Rel 1-A and Rel 1-B leads to Spaetzle 1A and Serpin-27A activation. The activation of Spaetzle 1A and Serpin-27A leads to downstream signaling resulting in immune defense against fungi (Bian et al., 2005). Transgenic *Ae. aegypti* with Rel 1 knockdown resulted in diminished activation of Spaetzle 1A and Serpin-27A and were highly susceptible to fungi infection (Bian et al., 2005). Proteolytic cleavage of Spaetzle has been shown in further studies to initiate the activation of the Toll pathway (Weber et al., 2003), while Serpin-27A regulates the melanization cascade (Gregorio et al., 2002). Cactus is a negative regulator of Rel 1, hence it downregulates induction of the Toll pathway when it is expressed (Bian et al., 2005). The silencing of Cactus using RNAi results in higher activation of the Toll pathway which subsequently leads to the expression of defensin to inhibit replication of the DENV (Xi et al., 2008).

1.3.3.1.4 Immune Deficiency (IMD) pathway

The activation of the IMD pathway begins with the recognition of bacteria, parasites, fungi, or viral PAMPs by the mosquito PRRs. The binding of the mosquito PRRs to

peptidoglycans initiates a downstream signaling cascade which leads to the activation of Rel 2. The downstream signaling cascade involves the peptidoglycan recognition protein-LC/LD, and LE receptors binding to Imd, which leads to the recruitment of FADD and Dredd to activate Rel 2 (Terradas et al., 2017). Rel 2 belongs to the same family of proteins as Rel 1 of the Toll pathway. The activation of Rel 2 results in the regulation of cecropin1 antimicrobial peptide. The Rel 2 gene undergoes alternative splicing to produce two isoforms namely Rel 2-F and Rel 2-S. The Rel 2-F is the longer protein while the Rel 2-S is the shorter protein (Meister et al., 2005). Both Rel 2-F and Rel 2-S have been shown to be involved in immune defense against Gram-negative and Gram-positive bacteria (Meister et al., 2005). Rel 2-F is involved in the defense against parasite infection in the mosquito (Meister et al., 2005). Just like the Cactus functioning as a negative regulator for Rel 1 in the Toll pathway, Caspar negatively regulates Rel 2 in the IMD pathway (Meister et al., 2005).

The PGRP-LC receptors activated the IMD pathway in a study which involved the infection of *An. gambiae* with *P. falciparum* (Garver et al., 2009; Meister et al., 2009). Another study that used Casper-silenced *Anopheles spp* mosquitoes resulted in over-activation of the IMD pathway, suggesting the importance of TEPI1, FBN9, LRRD7, APL2, and APL1A in *Plasmodium* defense (Dong et al., 2011; Mitri et al., 2009). The bacteria flora of mosquitoes has been shown to regulate the activation of the IMD pathway. A study conducted in *Ae. aegypti* infected with DENV shows interaction between mosquito microbiota, immune defense, and the virus resulted in an immune response inhibiting the replication of the virus (Ramirez et al., 2012). The authors further hypothesized that this interaction may not only apply in DENV infection but generally to other arboviruses as well (Ramirez et al., 2012).

1.3.3.1.5 JAK-STAT pathway

The Jak-STAT pathway is a very important antiviral immune pathway in mosquitoes. The components involved in the pathways are the Unpaired ligand peptide (Upd), transmembrane protein receptor (Dome), Janus kinase (JAK), and STAT proteins. The induction of the pathway is initiated by the binding of Upd to the Dome receptors. This results in the dimerization of the dome receptors leading to conformational changes (Agaisse and Perrimon, 2004). The Janus kinase associated with the receptor dimers is phosphorylated. The activated Janus kinase subsequently leads to receptor dimerization, leading to the production of binding pockets for STAT proteins to bind. The STAT proteins phosphorylate the JAK-Dome complex, resulting in activation and dimerization of the STAT proteins. The STAT proteins are translocated into the nucleus to induce the expression of target genes (Agaisse and Perrimon, 2004). In *Ae. aegypti*, the JAK-STAT pathway was shown to have antiviral activity against DENV. The silencing of the inhibitor of STAT showed reduced midgut susceptibility to DENV infection, while the silencing of the Dome receptors or Hop protein (which is a JAK homolog) led to increased midgut susceptibility to DENV infection (Jupatanakul et al., 2017).

1.3.3.1.6 RNA interference

Currently available evidence indicates that RNA interference (RNAi) is the major antiviral defense pathway in the mosquitoes. RNAi is a gene-silencing mechanism based on specific sequences. RNAi functions in maintaining homeostasis as well as in immune defense (Kumar et al., 2018). The small RNA molecules include small interfering RNAs (siRNAs), microRNAs (miRNAs), and the P element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) (Kumar et al., 2018).

In the RNAi pathway, small RNAs interact with the RNA-silencing complex (RISC) and Argonaute to elicit an immune response. The RISC complex interacts with Argonaute to serve as a guide for RNAi loaded on the RISC complex to cleave the targeted RNA sequence. Many viruses produce dsRNA during replication which serves as PAMPs for mosquito innate immunity to detect and initiate an immune response using its RNAi pathway. (Kumar et al., 2018).

Small interfering RNAs (siRNAs) are approximately 18-24 nucleotide sequences long and siRNAs derived from the viral genome typically accumulate in large amounts during a viral infection in mosquitoes. siRNAs are produced by the cleavage and processing of either strand of the double-stranded RNA (dsRNA). The guide strand which is derived after siRNA processing is always complementary to the targeted mRNA sequence to be degraded. The function of siRNA is dependent on its origin. siRNAs which are endogenously derived are involved in the regulation of cellular processes such as the formation of heterochromatin (Deshpande et al., 2005), and chromosomal segregation (Shao et al., 2015) by binding to endogenously encoded dsRNA. Exogenously derived siRNA is involved in antiviral immunity by targeting and degrading exogenous viral RNA (Bronkhorst and Van Rij, 2014). The siRNA pathway is initiated by Dicer2, R2D2, and Ago2. All mosquito groups have orthologs of these genes present in their genome (Campbell et al., 2008). The Dicer protein is very important in the biosynthesis of most small RNA molecules and is a member of the ribonuclease III family of proteins. Dicer proteins function by cleaving long dsRNA into small RNAs. Dicer has a phosphate-binding site for the identification of the phosphorylated 5' end of small RNAs (Tian et al., 2014). The Dicer protein also has an RNase domain which functions by cleaving long dsRNA strands into short dsRNA strands, and processing of viral dsRNAs or RNA intermediates to ultimately produce

viral siRNAs, during viral infection (Ding, 2010). The Argonaute (Ago) protein functions in the RNAi pathway by binding to the small RNAs generated by Dicer, which leads to the silencing of complementary mRNA sequence by either degradation or by translation inhibition. There are several homologs of the Ago family of proteins across vertebrates and invertebrates (Okamura et al., 2004). The primary role of Ago2 in insects is antiviral defense. Another important protein in the siRNA pathway is R2D2. The R2D2 protein has both a negative and positive role in small RNA biosynthesis. R2D2 functions by transferring the siRNA guide strand from Dicer-2 to Ago2. It also inhibits Dicer-2 from the processing of miRNA precursors (Cenik et al., 2011), and the sorting of endo-siRNA into Argonaute (Okamura et al., 2011). R2D2 promotes the passage of siRNA from Dicer to RISC (Liu et al., 2003).

1.3.3.1.7 Micro RNA (miRNA) pathway

miRNAs are noncoding RNAs involved in the regulation of gene expression by either preventing transcription, translation, or by degrading mRNA. They prevent the transcription of targeted genes by binding to sites mainly found in the 3' untranslated region (UTR) to induce mRNA deadenylation and decapping, although they can also be located in other parts of the target mRNA such as the 5' untranslated region (UTR), promoters, and coding sequences (Stroynowska-Czerwinska et al., 2014). Their size varies, ranging from 19 to 24 nucleotides in length (Abraham et al., 2005). The production of miRNA begins with RNA pol II expressing primary miRNA from either introns of protein-coding genes or exons of noncoding genes in the nucleus (Saj and Lai, 2011). Drosha in association with Pasha protein then cleaves the stem-loop structure in the primary miRNA to produce the precursor miRNA (Yeom et al., 2006). The precursor miRNA is later transported into the cytoplasm by Exportin-5. In the cytoplasm, the precursor protein is excised at its terminal loop by Dicer-1 together with Loquacious protein to

produce a miRNA duplex. The passenger strand in the miRNA duplex is degraded, while the guide miRNA is incorporated into RISC. The incorporated guide miRNA into RISC is now ready to carry its function by inhibiting transcription or translation and degrading mRNA (Yeom et al., 2006). Another protein, Ars2, is involved in the miRNA pathway. It functions by interacting with the microprocessor complex to stabilize the pri-miRNA to enhance target cleavage. This interaction involves Dicer-2 and Ars2 (Sabin et al., 2009).

miRNAs are likely important in antiviral immune defense in mosquitoes and the expression of some miRNAs has been shown to be regulated during viral infection. A study determined the antiviral defense functions of miRNA by sequencing small RNA in *Ae. aegypti* after ZIKV infection at 2, 7, and 14 days. Results from this showed the modulation of 17 host miRNAs after ZIKV infection at all the three time points. Most of these 17 miRNAs have been reported previously to be modulated during DENV and WNV infection. This suggests some miRNAs have antiviral effects against flavivirus infections (Saldana et al., 2017). Additionally, miR-252 has also been shown to inhibit the expression and function of the E2 protein of DENV in *Ae. albopictus* (Yan et al., 2014). The importance of Ars2 in the RNAi pathway was determined by silencing it in *Drosophila* cells. This made *Drosophila* very susceptible to viral infection, highlighting the importance of Ars2 in both siRNA and miRNA pathways (Sabin et al., 2009). miRNAs have been shown to have a higher rate of sequence variations hence targeting a repertoire of dsRNAs for degradation, while siRNA is involved in several different antiviral responses (Obbard et al., 2006).

1.3.3.1.8 PIWI-interacting RNA (piRNA) pathway

The piRNA pathway is best known for its protective role in germ cells during embryogenesis in *Drosophila*. The piRNA pathway protects germ cells from the harmful effect

of transposable elements in epigenetic programming (Ku and Lin, 2014; Shirayama et al., 2012). Mutations in piRNA pathway genes mostly results in infertility due to the increased activity of transposons. However, the piRNA pathway also has important roles in eliciting an immune response to viral infection (Miesen et al., 2015). The length of piRNAs range from 21-32 nucleotides (Ross et al., 2014).

There are eight different PIWI proteins, namely Piwi1-7 and Ago3. Piwi5 and Ago 3 are the core proteins of the viral piRNA ping-pong amplification loop. The other six proteins are involved in the transposon-derived piRNA biogenesis (Girardi et al., 2017). The PIWI proteins show a high level of divergence from one mosquito species to the other. For example, there are four homologs of Piwi4, three homologs of Piwi5, and two homologs or Armitage in *Aedes*, while in *Culex*, there are three homologs of Piwi4 and Piwi5 each and two homologs of Armitage. Only one homolog for these proteins exists in *Drosophila*. *An. gambiae* has the Spn-E proteins and one copy of Ago3 (Campbell et al., 2008). Studies carried in *Ae. aegypti* identified some variations in the components involved in the PIWI compared to *Drosophila*. For example, piRNA occurs in somatic and germ cells in *Ae. aegypti*, while it occurs in more the germ cells compared to the somatic cells in *Drosophila*. Also, the Aub gene is only found in *Aedes spp*, with its function performed in *Drosophila* by Piwi5. *Ae. aegypti* with PIWI knockdown showed increased susceptibility to SFV (Varjak et al., 2017). A study conducted by Walsh et al. to investigate the role of PIWI genes and piRNAs in *Culex spp* cells against arboviruses showed that the *Culex spp* PIWI is used for antiviral activity against arboviruses (Walsh et al., 2022). In this study, PIWI genes were silenced in *Culex quinquefasciatus* (Hsu) cells and *Culex tarsalis* cells (CT) and these cells were infected with La Crosse orthobunyavirus (LACV), USUV and Calbertado virus (CV). Silencing of PIWI genes in the insect cells only impacted LACV

replication and not USUV and CV. Sequencing and analyses of small RNA identified LACV-derived piRNAs, but not USUV-derived piRNAs and CT-derived piRNAs in the insect cells. This result suggests that *Culex spp* uses PIWI as an antiviral pathway against LACV infection (Walsh et al., 2022).

1.3.3.1.9 Complement proteins

The complement system initiates an immune response against a pathogen during the early phase of infection by directly mediating the elimination of pathogens (Shishido et al., 2012). Complement-like factors have been recently been identified in arthropods, and this shares a common ancestry with the complement-factors in other invertebrates and vertebrates (Blandin et al., 2004; Buresova et al., 2011; Cheng et al., 2011; Levashina et al., 2001). The thioester (TE)-containing proteins (TEPs) in mosquitoes have been shown to share high similarities with the Complement factor 3 (C3) in mammals (Dodds and Law, 1998). The TEPs families include alpha-2-macroglobulins (A2Ms), C3/C4/C5 complement factors, and insect TEPs (iTEP) (Blandin & Levashina, 2004). The macroglobulin complement-related factors (MCRs) are part of the iTEPs. A phylogenetic tree analysis shows MCRs forms an independent branch, which is separate from other subfamilies of iTEP. A study by Xiao et al., (2014) has shown that *Ae. aegypti* MCR (AaMCR) is effective in the defending the mosquito against flavivirus invasion. AaMCR has been shown to stimulate the production of AMPs in response to DENV infection (Xiao et al., 2014). Additionally, scavenger receptors (SR), which are cell-surface glycoproteins involved in pathogen recognition have been shown to defend *Ae. aegypti* against flavivirus infection (Xiao et al., 2014).

1.3.3.1.10 Antimicrobial peptides (AMPs)

Antimicrobial peptides are small peptides mostly produced in response to bacterial infection. They are produced in hemocytes, epithelial cells, and fat bodies, and are secreted into the hemolymph (Chalk et al., 1995; Kumar et al., 2018; Xiao et al., 2014). In *Ae. aegypti*, AMPs are categorized into 5 categories namely defensins, cecropins, gambicin, dipteracin,, and attacins (Chalk et al., 1995; Kumar et al., 2018; Xiao et al., 2014). A study conducted by Xiao and colleagues showed increased levels of attacin and dipteracin in response to DENV-2 infection in *Ae. aegypti* (Xiao et al., 2014). Additionally, a study conducted by Liu et al., (2020) showed that knockdown of ERK significantly enhanced DENV replication in *Ae. aegypti* whiles JNK or p38 did not significantly reduces DENV infection in the mosquitoes. The authors reported that the activation of Ras/ERK pathway in the midgut and carcass of *Ae. aegypti* through both genetical and pharmacological means resulted in decrease DENV titer in the infected mosquitoes. Additionally, a signaling crosstalk between Ras/ERK pathway and induction of defensin C, an AMP that inhibited DENV infection in *Ae. aegypti* was reported. This result reveals that Ras/ERK signaling pathway couples AMPs to mediate *Ae. aegypti* resistance against DENV infection (Liu et al., 2020).

1.3.3.1.11 Apoptosis

Apoptosis is a type of programmed cell death that is highly regulated in many organisms. Apoptosis is important in maintaining homeostasis by removing damaged and infected cells. Apoptosis is a major antiviral defense in many insects including mosquitoes (Clem, 2016). Studies have identified a family of related apoptotic genes in insects, including reaper, grim, hid, and sickle. These genes are highly regulated at the transcriptional level based on the developmental process of the cells. A related pro-apoptotic gene, Michelob-x (mx), first

identified in *An. gambiae*, has facilitated the study of the antiviral activity of apoptosis in other mosquito species including *Ae. aegypti* (Zhou et al., 2005; Liu & Clem, 2011).

Contradictory reports on the exact role apoptosis plays in mosquito response to viral infection have been published. Apoptosis has been shown to either increase virus infectivity and dissemination or reduce virus infectivity and dissemination. In one study by Wang et al., apoptosis was induced in *Ae. aegypti* by using dsRNA to knock down the apoptosis inhibitor gene *Aeiapl*, followed by oral infection with SINV. Silencing of *Aeiapl* resulted in induction of massive widespread apoptosis and increased SINV midgut infection and dissemination to other tissues (Wang et al., 2012). A possible explanation for this observed phenomenon is that apoptosis may have damaged physical barrier cells which enhanced SINV escape from the midgut to other parts of the body. Also, different studies using different viruses have reported different responses to apoptosis, mainly based on the virus's genetic background (Eng et al., 2016).

On the other hand, other several studies have shown that apoptosis can function as an antiviral defense. In a study by Vaidyanathan & Scott, the authors showed that the rate of apoptosis was highly correlated with WNV infection in the midguts of a refractory strain of *Cx. pipiens pipiens* (Vaidyanathan and Scott, 2006). Additionally, O'Neill et al. infected *Ae. aegypti* with SINV expressing the proapoptotic *reaper* gene and found out that this virus had replication defects in *Ae. aegypti* and reduced midgut infection and dissemination to other tissues (O'Neill et al., 2015). Also, Carpenter and Clem showed that expression of the pro-apoptotic reaper protein via insertion in the SINV structural open reading frame led to a more stable expression of the reaper protein, leading to increased apoptosis in *Ae. aegypti* and resulting in decreased midgut infection and dissemination compared to the virus constructed by O'Neill et al. (Carpenter and

Clem, 2022). A study conducted by Liu et al., (2013) showed that P53-mediated rapid induction of apoptosis in *Drosophila* after infection with either DNA or RNA viruses resulted in reduced virus infection in the *Drosophila*. The rapid apoptosis induction led to the prevention of viral gene expression and the overall prevention of infection (Liu et al., 2013).

1.3.3.1.12 Autophagy

Autophagy is a process well characterized in both invertebrates and vertebrates. Autophagy occurs in response to nutrient scarcity and/or pathogenic infection as it is generally considered as a pro-survival mechanism. Although apoptosis is considered as pro-death, some genes involved in the activation of apoptosis have been reported to have non-apoptotic effects, and an example of this is the activation of autophagy (Kuranaga, 2012). For example, *Aedronc*, *Casps7*, *Aep53* and several IAP orthologs (such as *IAP5*, *IAP9*, *AeBruce*) known to be involved in the apoptotic pathway have been shown to be transcriptionally activated during autophagy activation in *Ae. aegypti* (Bonizzoni et al., 2011; Eng et al., 2016; Puglise et al., 2016).

Our understanding of the role autophagy plays in the immune response to pathogenic infection was derived using viruses. Studies conducted using *Ae. aegypti* infected with DENV-2 saw an elevation of autophagy-related 5 (ATG5) gene expression in the midgut of susceptible mosquitoes, compared to refractory mosquitoes. An increase in the transcript levels of ATG5 coincided with increased expression of apoptosis-related genes (Eng et al., 2016). The silencing of autophagy-related genes resulted in elevated DENV-2 viral load. This was accompanied by a decreased accumulation of Atg8-phosphatidylethanolamine (PE). Atg8 functions to induce autophagy, and also serves as a progression marker for autophagy (Eng et al., 2016).

1.3.3.2 The role of midgut immune responses in the degree of susceptibility to arbovirus infection in mosquitoes

Immune responses in the midgut act as a barrier to prevent the establishment of virus infection. In *Ae. aegypti*, the midgut microbiota induces prostaglandin secretion, an immuno-active lipid that mediates immune responses. Prostaglandin production increases expression of the components of the Toll and IMD pathways, resulting in reduced susceptibility to DENV infection in the midgut (Barletta et al., 2020). In another example, *Ae aegypti* infected with *Wolbachia* had increased reactive oxygen species (ROS) levels when compared with *Ae. aegypti* not infected with *Wolbachia*. The higher levels of ROS induction in *Wolbachia*-infected *Ae. aegypti* was linked to Toll pathway activation which is essential in mediating the expression of antimicrobial peptides such as defensins and cecropins which inhibit the proliferation of DENV (Pan et al., 2012).

1.3.3.3 The role of midgut microbiota in determining susceptibility or refractoriness to arbovirus infection

The midgut of mosquitoes is colonized by an array of microbes forming the microbiota. The gut microbiota influences vital physiological processes of the mosquito related to susceptibility to pathogen infection (Guégan et al., 2018). The microbiota composition of the mosquito midgut shows high inter-individual variation among individual mosquitoes. The midgut microbiota composition of a mosquito can be affected by the species, ecological niche, geographical origin, source of food, and sex of the mosquito (Osei-Poku et al., 2012; Valiente Moro et al., 2013).

Signaling through immune pathways such as Toll, IMD, and JAK-STAT can also be initiated in response to natural microbiota in the gut. Many studies have shown that the mosquito natural microbiota boosts immune response and provides resistance against invading pathogens (Dong, et al., 2009; Ramirez et al., 2012). In *Ae. aegypti*, the midgut microbiota significantly affects the replication of DENV (Ramirez et al., 2014; Xi et al., 2008). Comparison of transcriptomic responses of antibiotic treated and non-antibiotic treated mosquitoes showed that antibiotic treated mosquitoes had lower expression levels of immune related genes compared to non-antibiotic treated mosquitoes. This result showed modifying the endogenous midgut microbiota by antibiotic treatment results in decreased immune response (Xi et al., 2008). Also, the DENV infection in the antibiotic treated mosquito midgut was two times higher than that of the midguts of non-antibiotic treated mosquitoes at 7 days post infection. This shows that depleting the midgut microbiota in *Ae. aegypti* using antibiotics rendered the mosquitoes more susceptible to DENV infection (Xi et al., 2008). In addition, the *Ae. aegypti* midgut microbiota can also activate basal levels of AMPs in response to virus infection (Ramirez et al., 2014).

In *Ae. aegypti* from the *Panama* strain, the bacteria *Chromobacterium* sp. has been reported to produce aminopeptidase which reduces DENV infection by degrading the virus envelope protein to prevent virus attachment and replication within host cells (Saraiva et al., 2018). Blood feeding in mosquitoes has been associated with an increase in bacteria number in the midgut which affects the mosquito's physiological processes such as the production of ROS and the peritrophic matrix. The ROS produced and the peritrophic matrix formed may affect vector competence (Oliveira et al., 2011). In a study by Charan et al. that assessed the midgut bacteria repertoire of DENV-susceptible and DENV-resistant *Ae. aegypti* strains, some bacteria genera were exclusively identified in either the susceptible strain or the resistant strain (Charan et

al., 2013). Bacteria such as *Pseudomonas rhodesiae*, *Enterobacter ludwigii*, and *Vagococcus salmoninarum* in *Ae. albopictus* midgut have been found to directly inhibit LACV infection, which suggests that these bacteria may produce antiviral molecules (Joyce et al., 2011). Contrary to most mosquito midgut microbiota preventing the establishment of pathogen infection, some midgut microbiota may enhance pathogenic infection in the arthropod host. For example, *Serratia odorifera* secrete P40, a polypeptide that interacts with prohibitin which leads to suppression of the *Ae. aegypti* immune system. This results in increased susceptibility of *Ae. aegypti* to DENV and CHIKV infection (Apte-Deshpande et al., 2014; Londono-Renteria et al., 2015). *S. marcescens* secretes a protein namely smEnhancin which functions by digesting mucins in the peritrophic matrix, making the mosquito more susceptible to arbovirus infection (Wu et al., 2019).

Another mechanism that microbes use to prevent viruses from establishing midgut infection is resource siphoning from the virus. *Wolbachia* sequester and deplete cholesterol and lipids in midgut cells to prevent DENV and CHIKV infection (Sinkins, 2013). This evidence shows that resource competition between midgut microbiota and arboviruses can contribute to mosquito pathogen resistance (Caragata et al., 2013).

1.3.3.4 Immune responses in hemolymph

Circulation of arboviruses in the hemolymph facilitates the invasion of mosquito tissues such as the fat body, neural system, muscles, and salivary glands (King and Hillyer, 2012). To limit invasion of these tissues after the arbovirus escapes from the midgut, the hemolymph possesses cellular and humoral immunity which is used as defense to limit the spread of invasion of these tissues (Xiao et al., 2014). An antiviral mechanism in the hemolymph used for immune

defense is the phenoloxidase (PO) cascade. The PO cascade is a humoral immune response leading to melanin formation after infection (Cerenius et al., 2008). A study conducted by Rodriguez-Andres et al. reported that a functional PO cascade in the hemolymph of *Ae. aegypti* is activated following SFV infection, suggesting PO may play a role in anti-viral response (Rodriguez-Andres et al., 2012).

The mosquito hemolymph also has thioester (TE)-containing proteins (TEPs) which has anti-viral properties. An *Ae. aegypti* TEP called macroglobulin complement-related factor (AaMCR) plays a role in protection against flavivirus infection (Cheng et al., 2011; Xiao et al., 2014). The AaMCR functions by recruiting a scavenger receptor AaSR-C to arrest DENV and initiate a cascade of reactions leading to the induction of AMPs against the virus during the early stages of viral infection (Xiao et al., 2014). Other immune cascades such as the Toll, IMD, and JAK–STAT pathways can also induce AMPs and/or other antiviral effectors for antiviral defense in the hemolymph (Dostert et al., 2005; Zamboni et al., 2005).

1.3.3.5 Antiviral immune responses in salivary glands

The saliva of mosquitoes, produced by the salivary glands, contains numerous anti-hemostatic and immunomodulatory molecules that aid in blood acquisition during blood feeding (Schneider and Higgs, 2008). Some of these immune molecules help to inhibit virus replication to a tolerable level (Calvo et al., 2009; Ribeiro et al., 2007; Sim et al., 2012). In a study conducted by Luplertlop et al., expression of the antibacterial peptide cecropin, which has anti-DENV properties, was strongly stimulated in salivary glands of DENV-infected mosquitoes compared to control mosquitoes (Luplertlop et al., 2011). Cecropin was shown to also have anti-CHIKV properties in the saliva of *Ae. aegypti* (Luplertlop et al., 2011). Additionally, IMD and

Toll immune signaling pathways have been reported to be upregulated in *Ae. aegypti* saliva following DENV infection, which suggests that these pathways may play a role in anti-viral defense in the saliva of mosquitoes. Furthermore, the complement-like factors AaMCR and AaSR-C are mildly upregulated in the salivary glands of DENV-infected mosquitoes compared to DENV-uninfected mosquitoes. This suggests that AaMCR and AaSR-C may have a role in viral recognition and induction of AMPs for antiviral defense (Xiao et al., 2014). Recent investigation has identified ankyrin repeat-containing protein (AnkP) and cystatin (Cyst) as anti-DENV factors in *Ae. aegypti* salivary glands (Sim et al., 2012). Together, all these mechanisms help to limit the propagation of arboviruses in the salivary glands (Cheng et al., 2016).

1.3.3.6 Tolerance and resistance as an immune defense strategy in mosquitoes to arbovirus infection

1.3.3.6.1 Mosquito resistance to arbovirus infection

Mosquitoes use two main strategies in response to arbovirus infection, either tolerance or resistance. While the resistance strategy prevents arbovirus transmission from mosquitoes to humans, the tolerance strategy does not (Lambrechts and Saleh, 2019). While the resistance strategy acts by preventing the virus from invading the cells of the mosquitoes or replicating after invasion (resulting in low levels or no virus in the mosquito), the tolerance immune strategy is characterized by high levels of virus replication without significant damage to the mosquito (Lambrechts and Saleh, 2019). Due to this, the identification of antiviral genes that are involved in resistance of arbovirus infection in mosquitoes is being used to develop transgenic mosquitoes as a vector control strategy to eliminate arbovirus infection and disease (Lambrechts and Saleh, 2019).

The RNAi pathway has been identified as the main antiviral defense used by mosquitoes to resist arbovirus infection (Kumar et al., 2018). Due to this, vector biologists are trying to harness the potential of the RNAi pathway and several other antiviral genes that block virus transmission into producing transgenic mosquitoes that will be resistant to arbovirus infection, hence leading to the eradication of arbovirus infections (Adelman et al., 2001; Blair et al., 2000; Olson et al., 1996). In addition, transposable elements (TE) such as MosI (Coates et al., 1998), piggyBAC (Kokoza et al., 2001), and Hermes (Jasinskiene et al., 1998; Pinkerton et al., 2000) can be used to provide a stable integration of exogenous DNA into mosquito genomes. This can be used to integrate and express antiviral effector genes in virus-susceptible *Ae. aegypti* mosquitoes. Using this approach, it may be possible to make DENV-susceptible mosquito refractory to DENV infection (Beaty, 2000).

Another genomic engineering tool that can be used to transgenic mosquitoes is CRISPR-Cas9. The CRISPR-Cas9 gene editing system can be used to introduce single or multiple mutation(s) leading to gain or loss of function of the targeted gene(s). The CRISPR-Cas9 system can also be used to insert gene segments in the genome of mosquitoes for the expression of the gene product of exogenous genes (Kistler et al., 2015). Additionally, current modification of the CRISPR-Cas9 gene editing system has led to the development of CRISPR activation (CRISPRa) system which is used for the transactivation of endogenous genes in mosquitoes. CRISPRa is made up of a catalytically-inactive Cas9 (dCas9) with a highly active tripartite activator termed as VP64-p65-Rta (VPR), and a synthetic guide RNA (gRNA). The gRNA is designed and synthesized to be complementary to the promoter of the targeted gene of interest. Using the CRISPRa system, Bui et al. were able to overexpress two developmental genes, namely *even-skipped (eve)* and *hedgehog (hh)*, in engineered *Ae aegypti* mosquitoes. This resulted in

observable morphological phenotypes in the transgenic mosquitoes (Bui et al., 2023).

Furthermore, this system was used to overexpress the *AaRel 1* gene product which is the positive transcriptional regulator of the Toll immune pathway. The overexpression of the Toll immune pathway in transgenic *Ae. aegypti* led to significant reduction of DENV2 titer in the mosquito. This result suggests that the CRISPR-Cas9 gene editing system can be used to produce transgenic mosquitoes that are refractory to arbovirus infection (Bui et al., 2023).

1.3.3.6.2 Mosquito tolerance to arbovirus infection

Tolerance in mosquitoes occurs when an arbovirus is able to establish a persistent infection in mosquitoes for the rest of the mosquito's life span. When a virus is able to establish tolerance in mosquitoes, active replication and high viral load typically do not lead to severe fitness defects, although some fitness cost occasionally occurs (Styer et al., 2007). Currently, there is little evidence of arbovirus virulence in mosquitoes after the establishment of tolerance (Ciota et al., 2011). For example, yellow fever virus has been reported to establish tolerance in mosquitoes but causes mortality in immunocompromised humans (Barrett and Higgs, 2007). The establishment of mosquito tolerance is important as it ensures high viral loads and mosquito survival which are necessary for arbovirus transmission to their vertebrate hosts.

During arbovirus infection, the immune response, physiological changes comprising of metabolic adaptation, tissue repair, and stress response are altered. However, the alteration of these processes does not result in pathology but rather improved health, a characteristic which defines tolerance (Troha et al., 2018). Recent evidence has shown that one of the reasons why arbovirus infections are pathogenic in their human vertebrate host and non-pathogenic in the invertebrate mosquito host is the antiviral RNAi response in mosquitoes, which is responsible for

mosquito defense against arbovirus infection (Myles et al., 2008). A study conducted by Myles et al. examined the role of RNAi in *Ae. aegypti* infected with SINV. In this study, *Ae. aegypti* were infected with recombinant alphaviruses expressing suppressors of RNA silencing. Results from this experiment showed that suppression of RNAi in infected *Ae. aegypti* led to increased virus replication and decreased mosquito survival. This result suggests that the exogenous siRNA pathway is important to maintain the survival of mosquitoes infected with arboviruses, maintain mosquito tolerance to arbovirus infection, and ensure the maintenance of arboviruses in nature (Myles et al., 2008).

1.3.4 Metabolism

1.3.4.1 Host metabolism important for the SINV replication process

The obligate intracellular nature of viruses makes them fully reliant on the host cell's metabolism to produce macromolecules required for virus replication. Viruses can activate or suppress the transcription of some host cell genes required for virus replication (Fontaine et al., 2015). One of the major processes a host cell uses to generate ATP for cellular processes is glycolysis. Glycolysis is an intracellular biochemical process that converts a glucose molecule into two molecules of pyruvate and the generation of two ATP molecules. The pyruvate generated during the glycolytic process has several fates and may include: 1) entering the tricarboxylic acid (TCA) cycle to produce NADH and FADH₂, reducing agents that donate electrons to the electron transport chain (ETC) leading to the generation of 34 molecules of ATP per glucose; and 2) conversion of pyruvate into lactate by the enzyme lactate dehydrogenase leading to the regeneration of NAD⁺ from NADH. Although no further ATP is produced in this process, NAD⁺ is used to drive the first biochemical step in glycolysis (DeBerardinis et al.,

2008). Some biochemical intermediates from the glycolytic process can be used in the process of cell growth and proliferation. For example, glucose-6-phosphate can be used in the pentose phosphate pathway to generate NADPH from NADP^+ . NADPH is used to reduce reactive oxygen species to maintain cellular redox balance and to protect the genome of the cell from mutations. Another glycolytic metabolite, namely 3-phosphoglycerate, is used in the synthetic process of different amino acids such as glycine, serine, and cysteine (Locasale, 2013). Recent evidence shows that glycolysis is important for multiple steps during SIN V replication. A study conducted by Findlay and Ulaeto reported that pre-treatment of Vero cells with different glycolysis inhibitors which targeted different steps or enzymes in the glycolysis pathway resulted in a significant reduction of SIN V replication, although it did not completely abolish it. This result suggests that glycolysis is needed for optimal SIN V replication (Findlay and Ulaeto, 2015).

Lipids are a major source of energy for cells and form part of the structural components of biological membranes. Several lipids play significant roles in signaling or functioning as secondary messengers. Lipids are mainly composed of cholesterol, fatty acids, phospholipids, sphingolipids, and triacylglycerides, and they all play important roles in cellular processes (Matsushita et al., 2021). Lipid-containing membranes such as cholesterol and sphingomyelin (SM) play vital roles in virus entry into cells, virus replication, and virus glycoprotein transport in cells (Pavoine and Pecker, 2009).

The phosphatidylinositol 3-kinase-Akt-TOR (PI3K/Akt/mTOR) pathway is a commonly activated signaling pathway in cells which functions to promote cellular metabolism, cell proliferation, survival, growth, and angiogenesis in response to extracellular signals (Beck et al., 2014). During infection, SIN V interacts with the host cell's PI3K/Akt/mTOR pathway to

manipulate cellular activities such as inhibition of apoptosis, promotion of virus replication, promotion of viral protein synthesis, and enhancement of host cell death. For example, the PI3K/Akt/mTOR pathway has been reported to enhance SINV replication by promoting cap-dependent SINV mRNA translation (Patel and Hardy, 2012).

1.3.4.2 SINV interferes with host cellular processes and metabolism pathways

Inhibition of host cell protein synthesis is a common phenomenon associated with alphavirus infection (Kääriäinen and Ahola, 2002). Some mechanisms alphaviruses use to inhibit host cell protein synthesis may include 1) reduction in the amount of intracellular ribonucleoside triphosphate caused by viral RNA synthesis, 2) alterations of the ionic environment of the cell which negatively affects the synthesis of host mRNAs more than viral mRNAs, and 3) competition between viral and host mRNAs for ribosomes and host cellular factors (Kääriäinen and Ahola, 2002).

Replication of SINV in vertebrate cells is characterized by cytopathic effect (CPE) within 48 hours after infection (Frolov and Schlesinger, 1994). SINV nsP2 and nsP3 proteins inhibit cellular transcription and translation, respectively, to induce CPE (Akhrymuk et al., 2018). Expression of SINV glycoproteins on the cell surface induces the rapid onset of CPE (Joe et al., 1998). Inhibition of the Na^+/K^+ ATPase, resulting in a decrease in Na^+/K^+ ATPase activity of SINV-infected host cell, may also contribute to CPE (Ulug et al., 1984). During replication, SINV affects fundamental processes of the host cell physiology (Gorchakov et al., 2004). SINV redirects cellular machinery and resources to synthesize structural proteins and the assembling of viral particles into a virion (Frolov and Schlesinger 1994; Gorchakov et al., 2004).

SINV infection of vertebrate hosts has been shown to interfere with the PI3K/Akt/mTOR pathway. A study conducted by Mohankumar et al., showed that infection of HEK cells with SINV leads to the drastic suppression of phosphorylated Akt (p-Akt) and phosphorylated-mTOR proteins (p-mTOR) when compared to cells infected with inactivated SINV or uninfected cells (Mohankumar et al., 2011). Reduction of p-mTOR in SINV-infected cells leads to reduced phosphorylated levels of S6 proteins (p-S6) when compared to cells infected with UV-treated SINV or uninfected cells. The p-S6 protein is a downstream effector of mTOR. In addition, SINV infection-induced phosphorylation of eIF2a and dephosphorylation of 4E-BP1 leads to translation shut-off in HEK cells. The reduction of p-Akt, p-mTOR, p-S6, and 4E-BP1 during the late stage of SINV infection of HEK cells suggests that SINV inhibits the Akt/mTOR pathway during viral infection to enhance RNA protein synthesis during replication (Mohankumar et al., 2011).

Additionally, SINV infection leads to drastic changes in the vertebrate host's mitochondrial metabolism. HEK cells infected with SINV decreased their oxygen consumption rate (OCR) and increased the extracellular acidification rate (ECAR) when compared to control cells which had no SINV infection. These results suggest that SINV infection leads to reduced respiration in vertebrate host cells (Rodriguez et al., 2022). In addition, SINV infection led to reduced ATP production in HEK cells compared to uninfected cells. The modulation of mitochondrial bioenergetics SINV infection is required to support active viral replication (Rodriguez et al., 2022; Silva da Costa et al., 2012).

1.4 Titer variation in arbovirus-infected mosquitoes

Large variation in viral titers between individual mosquitoes have been frequently observed after oral feeding with various arboviruses in mosquito populations that are highly inbred and raised in the same environmental conditions (Carpenter et al., 2021; Merwaiss et al., 2021; O'Neill et al., 2015). Prior to the work done in this dissertation, little was known about the factors that contribute to individual titer variation. In a landmark study, in order to understand the individual co-variation between DENV load and *Ae. aegypti* gene expression, Raquin and colleagues performed transcriptomic sequences on the midguts of individual field-caught *Ae. aegypti* infected with DENV. Results from this study were analyzed using an approach that accounted for individual co-variation between DENV load and mosquito gene expression. The method used was able to generate a prediction of the antagonist effect the selected mosquito genes have on DENV replication based on the correlation between the *Ae. aegypti* gene expression levels and DENV viral load. Using this new approach to compare DENV-infected midguts and uninfected controls, 39 candidate genes were detected which would have otherwise not have been detected using the conventional pairwise comparison. Using both the new and conventional pairwise comparison approaches, only four candidate genes were detected by both methods. This result suggests that the new approach used by the authors and the conventional pairwise comparison may be complementary. The authors demonstrated the validity of their new approach by the functional validation of an agonist gene which encodes a sterol regulatory element-binding protein (*SREBP*), which was identified using the authors' new correlation analysis and not by the conventional pairwise comparison. RNAi-mediated gene knockdown of *SREBP* in *Ae. aegypti* led to decreased midgut viral load. This suggests that *SERBP* may promote DENV midgut infection. The authors concluded that their new approach can be used to identify

agonist or antagonist mosquito host factors which account for inter-individual variation in arbovirus titer (Raquin et al., 2017). It is important to note that this study used mosquitoes that had been recently field-caught, not highly inbred mosquitoes.

Viral titer in mosquitoes after oral infection also depend on virus genotype (Ciota and Kramer, 2013). Different viral genotypes have been recorded to have different median titers after oral infection in mosquitoes (Anderson and Rico-Hesse, 2006). Variable viral titers are also observed when the same viral serotype is used to orally infect mosquitoes (Carpenter et al., 2021; Merwaiss et al., 2021; O'Neill et al., 2015; Raquin et al., 2017). In some cases, single nucleotide variations in viral populations that arise during viral replication may account for differences in viral titers in mosquitoes (Van Slyke et al., 2015). Viral mutant swarms are produced during replication, following the severe bottlenecks imposed on the initial viral populations (Van Slyke et al., 2015) by the mosquito midgut innate immune cells (Carissimo et al., 2015) and physical barriers (Black et al., 2002). The mutant swarms produced due to errors in viral replication may produce individual viruses with low fitness but make the viral population more adaptable to survive changing conditions and replicate more efficiently in the mosquito (Van Slyke et al., 2015). Mutations that result in fitness cost may result in low viral titers in the mosquito but some mutations that increase viral adaptability in the mosquito may result in high viral titers (Van Slyke et al., 2015).

The midgut serves as the first bottleneck to a successful arbovirus infection, hence overcoming it is imperative for the establishment of infection (Franz et al., 2015). Brackney et al., reported that the high mutational diversity of WNV in mosquitoes was driven by midgut RNA interference (RNAi) which led to virus diversification (Brackney et al., 2009). In addition, the different midgut transcriptional responses of *Ae. aegypti* innate immune cells to three

different viruses (DENV, WNV, and YFV) suggest unique vector responses to these arboviruses. The differences in transcriptional response results in differences in infection rate and virus titer in individual mosquitoes (Colpitts et al., 2011). Finally, different expression of mosquito immune genes is observed when different inoculation routes of infection such as intrathoracic versus oral infection with the same virus is used (Merkling and van Rij, 2013). Together, these studies suggest that there is a possibility that differential expression of mosquito immune genes in the midgut may result in different viral titers after oral infection.

As discussed above, the high error rates that introduce polymorphisms in virus genomes can affect midgut replication (Duffy 2018). For example, polymorphisms could affect the conserved pathogen-associated molecular patterns (PAMPs) in the virus that may not be recognized by pattern recognition receptors (PRRs) hence invading the mosquito innate immune defenses. There also may be mutations that make individual viruses either more or less efficient at replication. For example, the capsid protein of SFV antagonizes RNAi in mosquito cells to cause successful replication. Certain point mutations in SFV capsid protein showed defective viral replication in mosquito cells (Qian et al., 2020), hence the expression level of viral suppressors of RNAi may lead to differential replication of the viruses. Different DENV serotypes induce different immune responses in *Ae. aegypti* (Smartt et al., 2017) and this may be due to the slightly different genetic background of the virus. After a blood meal, the number of virus particles that successfully infect the midgut is thought to be very low (Forrester et al., 2012), suggesting that founder effect may explain the high degree of variation in virus titer observed after oral infection.

1.4.1 Specific infectivity of viruses

Not all individual virions or virus particles can infect cells, hence the need to distinguish between a virion or virus particle and a virus infectious unit (Sanjuán, 2018). More often than not, the virus titer obtained using methods like plaque assay or tissue culture infectious dose (TCID₅₀) assay can be tens to hundreds of thousand times lower than the amount of viral genomes or virus particles in a sample. The measure of what proportion of virus particles can cause an infection is termed as the virus specific infectivity, and it is calculated using the plaque forming units to particle ratio or TCID₅₀ to genome equivalent ratio (Klasse, 2015; Ojosnegros et al., 2011). The higher the specific infectivity value, the more virus particles in the sample can cause infection and vice versa. Many factors can potentially affect specific infectivity of viruses, including incomplete packaging of virions during the virus assembly stage, structural defects in the virion, lethal mutations, or incompatible interactions between the virus and the host factors that affect infectivity. Viruses normally have low specific infectivity in tissues that have infection barriers or other types of strong antiviral defenses (Li et al., 2011; Sanjuán 2018).

In this dissertation, studies were conducted to determine the underlying mechanisms responsible for the high degree of variation in the amount of infectious virus that is observed between individual *Ae. aegypti* mosquitoes following infection with SINV. The results of these studies are reported in Chapters 2 and 3.

1.5 References

- Abraham, E. G., Pinto, S. B., Ghosh, A., Vanlandingham, D. L., Budd, A., Higgs, S., ... & Michel, K. (2005). An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proceedings of the National Academy of Sciences*, 102(45), 16327-16332.
- Adelman, Z. N., Blair, C. D., Carlson, J. O., Beaty, B. J., & Olson, K. E. (2001). Sindbis virus-induced silencing of dengue viruses in mosquitoes. *Insect Molecular Biology*, 10(3), 265-273.
- Adouchief, S., Smura, T., Sane, J., Vapalahti, O., & Kurkela, S. (2016). Sindbis virus as a human pathogen—epidemiology, clinical picture and pathogenesis. *Reviews in Medical Virology*, 26(4), 221-241.
- Agaisse, H., & Perrimon, N. (2004). The roles of JAK/STAT signaling in *Drosophila* immune responses. *Immunological Reviews*, 198(1), 72-82.
- Akhrymuk, I., Frolov, I., & Frolova, E. I. (2018). Sindbis virus infection causes cell death by nsP2-induced transcriptional shutoff or by nsP3-dependent translational shutoff. *Journal of Virology*, 92(23), 10-1128.
- Anderson, J. R., & Rico-Hesse, R. (2006). *Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus. *The American Journal of Tropical Medicine and Hygiene*, 75(5), 886.
- Appassakij, H., Khuntikij, P., Kemapunmanus, M., Wutthanarungsan, R., & Silpapojakul, K. (2013). Viremic profiles in asymptomatic and symptomatic chikungunya fever: a blood transfusion threat?. *Transfusion*, 53(10pt2), 2567-2574.
- Apte-Deshpande, A. D., Paingankar, M. S., Gokhale, M. D., & Deobagkar, D. N. (2014). Serratia

- odorifera mediated enhancement in susceptibility of *Aedes aegypti* for chikungunya virus. *The Indian Journal of Medical Research*, 139(5), 762.
- Barletta, A. B. F., Alves e Silva, T. L., Talyuli, O. A., Luna-Gomes, T., Sim, S., Angleró-Rodríguez, Y., ... & Sorgine, M. H. F. (2020). Prostaglandins regulate humoral immune responses in *Aedes aegypti*. *PLoS Neglected Tropical Diseases*, 14(10), e0008706.
- Barletta, A. B. F., Silva, M. C. L. N., & Sorgine, M. H. F. (2012). Validation of *Aedes aegypti* Aag-2 cells as a model for insect immune studies. *Parasites & Vectors*, 5, 1-9.
- Barrett, A. D., & Higgs, S. (2007). Yellow fever: a disease that has yet to be conquered. *Annual Review Entomology*, 52, 209-229.
- Bartholomay, L. C., & Michel, K. (2018). Mosquito immunobiology: the intersection of vector health and vector competence. *Annual Review of Entomology*, 63, 145-167.
- Beaty, B. J. (2000). Genetic manipulation of vectors: a potential novel approach for control of vector-borne diseases. *Proceedings of the National Academy of Sciences*, 97(19), 10295-10297.
- Beck, J. T., Ismail, A., & Tolomeo, C. (2014). Targeting the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway: an emerging treatment strategy for squamous cell lung carcinoma. *Cancer Treatment Reviews*, 40(8), 980-989.
- Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C., & Kaiser, A. (2010). *Mosquitoes and their control*. Springer Science & Business Media.
- Beerntsen, B. T., James, A. A., & Christensen, B. M. (2000). Genetics of mosquito vector competence. *Microbiology and Molecular Biology Reviews*, 64(1), 115-137.
- Bian, G., Shin, S. W., Cheon, H. M., Kokoza, V., & Raikhel, A. S. (2005). Transgenic alteration of Toll immune pathway in the female mosquito *Aedes aegypti*. *Proceedings of the*

- National Academy of Sciences*, 102(38), 13568-13573..
- Black IV, W. C., Bennett, K. E., Gorrochótegui-Escalante, N., Barillas-Mury, C. V., Fernández-Salas, I., de Lourdes Muñoz, M., ... & Beaty, B. J. (2002). Flavivirus susceptibility in *Aedes aegypti*. *Archives of Medical Research*, 33(4), 379-388.
- Blair, C. D., Adelman, Z. N., & Olson, K. E. (2000). Molecular strategies for interrupting arthropod-borne virus transmission by mosquitoes. *Clinical microbiology reviews*, 13(4), 651-661.
- Blandin, S., Shiao, S. H., Moita, L. F., Janse, C. J., Waters, A. P., Kafatos, F. C., & Levashina, E. A. (2004). Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell*, 116(5), 661-670.
- Blandin, S., & Levashina, E. A. (2004). Thioester-containing proteins and insect immunity. *Molecular Immunology*, 40(12), 903-908.
- Boissière, A., Tchioffo, M. T., Bachar, D., Abate, L., Marie, A., Nsango, S. E., ... & Morlais, I. (2012). Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathogens*, 8(5), e1002742.
- Bonizzoni, M., Dunn, W. A., Campbell, C. L., Olson, K. E., Dimon, M. T., Marinotti, O., & James, A. A. (2011). RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, *Aedes aegypti*. *BMC Genomics*, 12(1), 1-13.
- Brackney, D. E., Scott, J. C., Sagawa, F., Woodward, J. E., Miller, N. A., Schilkey, F. D., ... & Ebel, G. D. (2010). C6/36 *Aedes albopictus* cells have a dysfunctional antiviral RNA interference response. *PLoS Neglected Tropical Diseases*, 4(10), e856.
- Brackney, D. E., Beane, J. E., & Ebel, G. D. (2009). RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification. *PLoS Pathogens*, 5(7), e1000502.

- Brady, O. J., Godfray, H. C. J., Tatem, A. J., Gething, P. W., Cohen, J. M., McKenzie, F. E., ... & Smith, D. L. (2016). Vectorial capacity and vector control: reconsidering sensitivity to parameters for malaria elimination. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 110(2), 107-117.
- Brault, A. C., Powers, A. M., Ortiz, D., Estrada-Franco, J. G., Navarro-Lopez, R., & Weaver, S. C. (2004). Venezuelan equine encephalitis emergence: enhanced vector infection from a single amino acid substitution in the envelope glycoprotein. *Proceedings of the National Academy of Sciences*, 101(31), 11344-11349.
- Bronkhorst, A. W., & van Rij, R. P. (2014). The long and short of antiviral defense: small RNA-based immunity in insects. *Current Opinion in Virology*, 7, 19-28.
- Brummer-Korvenkontio, M., Vapalahti, O., Kuusisto, P., Saikku, P., Manni, T., Koskela, P., ... & Vaheri, A. (2002). Epidemiology of Sindbis virus infections in Finland 1981–96: possible factors explaining a peculiar disease pattern. *Epidemiology & Infection*, 129(2), 335-345.
- Bui, M., Dalla Benetta, E., Dong, Y., Zhao, Y., Yang, T., Li, M., ... & Akbari, O. S. (2023). CRISPR mediated transactivation in the human disease vector *Aedes aegypti*. *PLoS Pathogens*, 19(1), e1010842.
- Buresova, V., Hajdusek, O., Franta, Z., Loosova, G., Grunclova, L., Levashina, E. A., & Kopáček, P. (2011). Functional genomics of tick thioester-containing proteins reveal the ancient origin of the complement system. *Journal of Innate Immunity*, 3(6), 623-630.
- Dusfour, I., Vontas, J., David, J. P., Weetman, D., Fonseca, D. M., Corbel, V., ... & Chandre, F. (2019). Management of insecticide resistance in the major *Aedes* vectors of arboviruses: Advances and challenges. *PLoS Neglected Tropical Diseases*, 13(10), e0007615.
- Calvo, E., Pham, V. M., Marinotti, O., Andersen, J. F., & Ribeiro, J. M. (2009). The salivary

- gland transcriptome of the neotropical malaria vector *Anopheles darlingi* reveals accelerated evolution of genes relevant to hematophagy. *BMC Genomics*, *10*, 1-27.
- Campbell, C. L., Black, W. C., Hess, A. M., & Foy, B. D. (2008). Comparative genomics of small RNA regulatory pathway components in vector mosquitoes. *BMC Genomics*, *9*, 1-16.
- Cansado-Utrilla, C., Zhao, S. Y., McCall, P. J., Coon, K. L., & Hughes, G. L. (2021). The microbiome and mosquito vectorial capacity: rich potential for discovery and translation *Microbiome*, *9*(1), 111.
- Caragata, E. P., Rancès, E., Hedges, L. M., Gofton, A. W., Johnson, K. N., O'Neill, S. L., & McGraw, E. A. (2013). Dietary cholesterol modulates pathogen blocking by *Wolbachia*. *PLoS Pathogens*, *9*(6), e1003459.
- Carissimo, G., Pondeville, E., McFarlane, M., Dietrich, I., Mitri, C., Bischoff, E., ... & Vernick, K. D. (2015). Antiviral immunity of *Anopheles gambiae* is highly compartmentalized, with distinct roles for RNA interference and gut microbiota. *Proceedings of the National Academy of Sciences*, *112*(2), E176-E185.
- Carpenter, A., Bryant, W. B., Santos, S. R., & Clem, R. J. (2021). Infection of *Aedes aegypti* mosquitoes with midgut-attenuated Sindbis virus reduces, but does not eliminate, disseminated infection. *Journal of Virology*, *95*(13), 10-1128.
- Carpenter, A., Santos, S. R., & Clem, R. J. (2022). Expressing the pro-apoptotic Reaper protein via insertion into the structural open reading frame of Sindbis virus reduces the ability to infect *Aedes aegypti* mosquitoes. *Viruses*, *14*(9), 2035.
- Carpenter, A., & Clem, R. J. (2023). Factors affecting arbovirus midgut escape in mosquitoes. *Pathogens*, *12*(2), 220.
- Cheng, G., Liu, Y., Wang, P., & Xiao, X. (2016). Mosquito defense strategies against viral

- infection. *Trends in Parasitology*, 32(3), 177-186.
- Cenik, E. S., Fukunaga, R., Lu, G., Dutcher, R., Wang, Y., Hall, T. M. T., & Zamore, P. D. (2011). Phosphate and R2D2 restrict the substrate specificity of Dicer-2, an ATP-driven ribonuclease. *Molecular cell*, 42(2), 172-184.
- Cerenius, L., Lee, B. L., & Söderhäll, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology*, 29(6), 263-271.
- Chalk, R., Albuquerque, C. M., Ham, P. J., & Townson, H. (1995). Full sequence and characterization of two insect defensins: immune peptides from the mosquito *Aedes aegypti*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 261(1361), 217-221.
- Charan, S. S., Pawar, K. D., Severson, D. W., Patole, M. S., & Shouche, Y. S. (2013). Comparative analysis of midgut bacterial communities of *Aedes aegypti* mosquito strains varying in vector competence to dengue virus. *Parasitology Research*, 112, 2627-2637.
- Chauhan, C., Behura, S. K., Debruyne, B., Lovin, D. D., Harker, B. W., Gomez-Machorro, C., ... & Severson, D. W. (2012). Comparative expression profiles of midgut genes in dengue virus refractory and susceptible *Aedes aegypti* across critical period for virus infection. *PloS One*, e47350.
- Cheng, G., Liu, L., Wang, P., Zhang, Y., Zhao, Y. O., Colpitts, T. M., ... & Fikrig, E. (2011). An in vivo transfection approach elucidates a role for *Aedes aegypti* thioester-containing proteins in flaviviral infection. *PloS One*, 6(7), e22786.
- Cheng, G., Liu, Y., Wang, P., & Xiao, X. (2016). Mosquito defense strategies against viral infection. *Trends in Parasitology*, 32(3), 177-186.
- Cheng, W. F., Hung, C. F., Chai, C. Y., Hsu, K. F., He, L., Rice, C. M., ... & Wu, T. C. (2001).

- Enhancement of Sindbis virus self-replicating RNA vaccine potency by linkage of Mycobacterium tuberculosis heat shock protein 70 gene to an antigen gene. *The Journal of Immunology*, 166(10), 6218-6226.
- Ciota, A. T., & Kramer, L. D. (2013). Vector-virus interactions and transmission dynamics of West Nile virus. *Viruses*, 5(12), 3021-3047.
- Ciota, A. T., Styer, L. M., Meola, M. A., & Kramer, L. D. (2011). The costs of infection and resistance as determinants of West Nile virus susceptibility in Culex mosquitoes. *BMC Ecology*, 11, 1-8.
- Clem, R. J. (2016). Arboviruses and apoptosis: the role of cell death in determining vector competence. *The Journal of General Virology*, 97(Pt 5), 1033.
- Coates, C. J., Jasinskiene, N., Miyashiro, L., & James, A. A. (1998). Mariner transposition and transformation of the yellow fever mosquito, Aedes aegypti. *Proceedings of the National Academy of Sciences*, 95(7), 3748-3751.
- Colpitts, T. M., Cox, J., Vanlandingham, D. L., Feitosa, F. M., Cheng, G., Kurscheid, S., ... & Fikrig, E. (2011). Alterations in the Aedes aegypti transcriptome during infection with West Nile, dengue and yellow fever viruses. *PLoS Pathogens*, 7(9), e1002189.
- Colpitts, T. M., Conway, M. J., Montgomery, R. R., & Fikrig, E. (2012). West Nile Virus: biology, transmission, and human infection. *Clinical Microbiology Reviews*, 25(4), 635-648.
- Crans, W. J. (2004). A classification system for mosquito life cycles: life cycle types for mosquitoes of the northeastern United States. *Journal of Vector Ecology*, 29, 1-10.
- Cristea, I. M., Carroll, J. W. N., Rout, M. P., Rice, C. M., Chait, B. T., & MacDonald, M. R. (2006). Tracking and elucidating alphavirus-host protein interactions. *Journal of Biological*

- Chemistry*, 281(40), 30269-30278.
- Cui, Y., Grant, D. G., Lin, J., Yu, X., & Franz, A. W. (2019). Zika virus dissemination from the midgut of *Aedes aegypti* is facilitated by bloodmeal-mediated structural modification of the midgut basal lamina. *Viruses*, 11(11), 1056.
- DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., & Thompson, C. B. (2008). The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell metabolism*, 7(1), 11-20.
- De Gregorio, E., Han, S. J., Lee, W. J., Baek, M. J., Osaki, T., Kawabata, S. I., ... & Brey, P. T. (2002). An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. *Developmental Cell*, 3(4), 581-592.
- Dennison, N. J., Jupatanakul, N., & Dimopoulos, G. (2014). The mosquito microbiota influences vector competence for human pathogens. *Current opinion in insect science*, 3, 6-13.
- Deshpande, G., Calhoun, G., & Schedl, P. (2005). *Drosophila argonaute-2* is required early in embryogenesis for the assembly of centric/centromeric heterochromatin, nuclear division, nuclear migration, and germ-cell formation. *Genes & Development*, 19(14), 1680-1685.
- Ding, S. W. (2010). RNA-based antiviral immunity. *Nature Reviews Immunology*, 10(9), 632-644.
- Diop, F., Alout, H., Diagne, C. T., Bengue, M., Baronti, C., Hamel, R., ... & Missé, D. (2019). Differential susceptibility and innate immune response of *Aedes aegypti* and *Aedes albopictus* to the haitian strain of the Mayaro virus. *Viruses*, 11(10), 924.
- Dodds, M. W., & Alex Law, S. K. (1998). The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and $\alpha 2$ -macroglobulin. *Immunological Reviews*, 166(1), 15-26.
- Domingo, E., Sheldon, J., & Perales, C. (2012). Viral quasispecies evolution. *Microbiology and*

Molecular Biology Reviews, 76(2), 159-216.

- Dong, S., Balaraman, V., Kantor, A. M., Lin, J., Grant, D. G., Held, N. L., & Franz, A. W. (2017). Chikungunya virus dissemination from the midgut of *Aedes aegypti* is associated with temporal basal lamina degradation during bloodmeal digestion. *PLoS Neglected Tropical Diseases*, 11(9), e0005976.
- Dong, Y., Das, S., Cirimotich, C., Souza-Neto, J. A., McLean, K. J., & Dimopoulos, G. (2011). Engineered anopheles immunity to Plasmodium infection. *PLoS Pathogens*, 7(12), e1002458.
- Dong, Y., Manfredini, F., & Dimopoulos, G. (2009). Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathogens*, 5(5), e1000423.
- Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., ... & Imler, J. L. (2005). The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nature Immunology*, 6(9), 946-953.
- Duffy, S. (2018). Why are RNA virus mutation rates so damn high?. *PLoS biology*, 16(8), e3000003.
- Dusfour, I., Vontas, J., David, J. P., Weetman, D., Fonseca, D. M., Corbel, V., ... & Chandre, F. (2019). Management of insecticide resistance in the major *Aedes* vectors of arboviruses: Advances and challenges. *PLoS Neglected Tropical Diseases*, 13(10), e0007615.
- Edman, J. D., Scott, T. W., Costero, A., Morrison, A. C., Harrington, L. C., & Clark, G. G. (1998). *Aedes aegypti* (Diptera: Culicidae) movement influenced by availability of oviposition sites. *Journal of Medical Entomology*, 35(4), 578-583.
- Eiden, M., Ziegler, U., Keller, M., Müller, K., Granzow, H., Jöst, H., ... & Groschup, M. H. (2014). Isolation of sindbis virus from a hooded crow in Germany. *Vector-Borne and*

Zoonotic Diseases, 14(3), 220-222.

- Eng, M. W., van Zuylen, M. N., & Severson, D. W. (2016). Apoptosis-related genes control autophagy and influence DENV-2 infection in the mosquito vector, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*, 76, 70-83.
- Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U., & Ball, L. A. (Eds.). (2005). *Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses*. Academic Press.
- Findlay, J. S., & Ulaeto, D. (2015). Semliki Forest virus and Sindbis virus, but not vaccinia virus, require glycolysis for optimal replication. *Journal of General Virology*, 96(9), 2693-2696.
- Fontaine, K. A., Sanchez, E. L., Camarda, R., & Lagunoff, M. (2015). Dengue virus induces and requires glycolysis for optimal replication. *Journal of Virology*, 89(4), 2358-2366.
- Forrester, N. L., Guerbois, M., Seymour, R. L., Spratt, H., & Weaver, S. C. (2012). Vector-borne transmission imposes a severe bottleneck on an RNA virus population. *PLoS Pathogens*, e1002897.
- Forrester, N. L., Coffey, L. L., & Weaver, S. C. (2014). Arboviral bottlenecks and challenges to maintaining diversity and fitness during mosquito transmission. *Viruses*, 6(10), 3991-4004.
- Fraiture, M., Baxter, R. H., Steinert, S., Chelliah, Y., Frolet, C., Quispe-Tintaya, W., ... & Levashina, E. A. (2009). Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of Plasmodium. *Cell Host & Microbe*, 5(3), 273-284.
- Franz, A. W., Sanchez-Vargas, I., Adelman, Z. N., Blair, C. D., Beaty, B. J., James, A. A., & Olson, K. E. (2006). Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proceedings of the National Academy of*

Sciences, 103(11), 4198-4203.

Franz, A. W., Sanchez-Vargas, I., Raban, R. R., Black IV, W. C., James, A. A., & Olson, K. E.

(2014). Fitness impact and stability of a transgene conferring resistance to dengue-2 virus following introgression into a genetically diverse *Aedes aegypti* strain. *PLoS Neglected Tropical Diseases*, 8(5), e2833.

Franz, A. W., Kantor, A. M., Passarelli, A. L., & Clem, R. J. (2015). Tissue barriers to arbovirus infection in mosquitoes. *Viruses*, 7(7), 3741-3767.

Frolov, I., & Schlesinger, S. (1994). Comparison of the effects of Sindbis virus and Sindbis virus replicons on host cell protein synthesis and cytopathogenicity in BHK cells. *Journal of Virology*, 68(3), 1721-1727.

Frolova, E., Gorchakov, R., Garmashova, N., Atasheva, S., Vergara, L. A., & Frolov, I. (2006). Formation of nsP3-specific protein complexes during Sindbis virus replication. *Journal of Virology*, 80(8), 4122-4134.

Garmashova, N., Gorchakov, R., Frolova, E., & Frolov, I. (2006). Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *Journal of Virology*, 80(12), 5686-5696.

Garver, L. S., Dong, Y., & Dimopoulos, G. (2009). Caspar controls resistance to *Plasmodium falciparum* in diverse anopheline species. *PLoS Pathogens*, 5(3), e1000335.

Girard, Y. A., Mayhew, G. F., Fuchs, J. F., Li, H., Schneider, B. S., McGee, C. E., ... & Bartholomay, L. C. (2010). Transcriptome changes in *Culex quinquefasciatus* (Diptera: Culicidae) salivary glands during West Nile virus infection. *Journal of Medical Entomology*, 47(3), 421-435.

Girardi, E., Miesen, P., Pennings, B., Frangeul, L., Saleh, M. C., & van Rij, R. P. (2017).

- Histone-derived piRNA biogenesis depends on the ping-pong partners Piwi5 and Ago3 in *Aedes aegypti*. *Nucleic Acids Research*, 45(8), 4881-4892.
- Gorchakov, R., Frolova, E., Williams, B. R., Rice, C. M., & Frolov, I. (2004). PKR-dependent and-independent mechanisms are involved in translational shutoff during Sindbis virus infection. *Journal of Virology*, 78(16), 8455-8467.
- Gorchakov, R., Frolova, E., & Frolov, I. (2005). Inhibition of transcription and translation in Sindbis virus-infected cells. *Journal of Virology*, 79(15), 9397-9409.
- Gorchakov, R., Garmashova, N., Frolova, E., & Frolov, I. (2008). Different types of nsP3-containing protein complexes in Sindbis virus-infected cells. *Journal of virology*, 82(20), 10088-10101.
- Greene, I. P., Wang, E., Deardorff, E. R., Milleron, R., Domingo, E., & Weaver, S. C. (2005). Effect of alternating passage on adaptation of sindbis virus to vertebrate and invertebrate cells. *Journal of Virology*, 79(22), 14253-14260.
- Grimstad, P. R., & Walker, E. D. (1991). *Aedes triseriatus* (Diptera: Culicidae) and La Crosse virus. IV. Nutritional deprivation of larvae affects the adult barriers to infection and transmission. *Journal of Medical Entomology*, 28(3), 378-386.
- Guégan, M., Zouache, K., Démichel, C., Minard, G., Tran Van, V., Potier, P., ... & Valiente Moro, C. (2018). The mosquito holobiont: fresh insight into mosquito-microbiota interactions. *Microbiome*, 6, 1-17.
- Malherbe, H., Strickland-Cholmley, M. & Jackson, A. L. (1963). Sindbis virus infection in man-report of a case with recovery of virus from skin lesions. *South African Medical Journal*, 37(21), 547-552.
- Hahn, C. S., Hahn, Y. S., Braciale, T. J., & Rice, C. M. (1992). Infectious Sindbis virus transient

- expression vectors for studying antigen processing and presentation. *Proceedings of the National Academy of Sciences*, 89(7), 2679-2683.
- Hanington, P. C., & Zhang, S. M. (2010). The primary role of fibrinogen-related proteins in invertebrates is defense, not coagulation. *Journal of Innate Immunity*, 3(1), 17-27.
- Hardy, J. L., Houk, E. J., Kramer, L. D., & Reeves, W. C. (1983). Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annual Review of Entomology*, 28(1), 229-262.
- Harrington, L. C., Edman, J. D., & Scott, T. W. (2001). Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood?. *Journal of Medical Entomology*, 38(3), 411-422.
- Honório, N. A., Câmara, D. C. P., Wiggins, K., Eastmond, B., & Alto, B. W. (2020). High-Throughput method for detection of Arbovirus infection of saliva in mosquitoes *Aedes aegypti* and *Ae. albopictus*. *Viruses*, 12(11), 1343.
- Howe, D. K., & Denver, D. R. (2008). Muller's Ratchet and compensatory mutation in *Caenorhabditis briggsae* mitochondrial genome evolution. *BMC Evolutionary Biology*, 8, 1-13.
- Huang, Y. J. S., Higgs, S., & Vanlandingham, D. L. (2017). Biological control strategies for mosquito vectors of arboviruses. *Insects*, 8(1), 21.
- Huang, Y. J. S., Higgs, S., & Vanlandingham, D. L. (2019). Arbovirus-mosquito vector-host interactions and the impact on transmission and disease pathogenesis of arboviruses. *Frontiers in Microbiology*, 10, 22.
- Hubálek, Z. (2008). Mosquito-borne viruses in Europe. *Parasitology research*, 103, 29-43.
- Jansen, C. C., & Beebe, N. W. (2010). The dengue vector *Aedes aegypti*: what comes

- next. *Microbes and Infection*, 12(4), 272-279.
- Hughes, H. R., Adkins, S., Alkhovskiy, S., Beer, M., Blair, C., Calisher, C. H., ... & ICTV Report Consortium. (2020). ICTV virus taxonomy profile: Peribunyaviridae. *Journal of General Virology*, 101(1), 1-2.
- Jasinskiene, N., Coates, C. J., Benedict, M. Q., Cornel, A. J., Rafferty, C. S., James, A. A., & Collins, F. H. (1998). Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes element from the housefly. *Proceedings of the National Academy of Sciences*, 95(7), 3743-3747.
- Joe, A. K., Foo, H. H., Kleeman, L., & Levine, B. (1998). The transmembrane domains of Sindbis virus envelope glycoproteins induce cell death. *Journal of Virology*, 72(5), 3935-3943.
- Jöst, H., Bialonski, A., Storch, V., Günther, S., Becker, N., & Schmidt-Chanasit, J. (2010). Isolation and phylogenetic analysis of Sindbis viruses from mosquitoes in Germany. *Journal of Clinical Microbiology*, 48(5), 1900-1903.
- Joyce, J. D., Nogueira, J. R., Bales, A. A., Pittman, K. E., & Anderson, J. R. (2011). Interactions between La Crosse virus and bacteria isolated from the digestive tract of *Aedes albopictus* (Diptera: Culicidae). *Journal of Medical Entomology*, 48(2), 389-394.
- Jupatanakul, N., Sim, S., Angleró-Rodríguez, Y. I., Souza-Neto, J., Das, S., Poti, K. E., ... & Dimopoulos, G. (2017). Engineered *Aedes aegypti* JAK/STAT pathway-mediated immunity to dengue virus. *PLoS Neglected Tropical Diseases*, 11(1), e0005187.
- Kääriäinen, L., & Ahola, T. (2002). Functions of alphavirus nonstructural proteins in RNA replication.
- Kaneko, T., Yano, T., Aggarwal, K., Lim, J. H., Ueda, K., Oshima, Y., ... & Silverman, N.

- (2006). PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. *Nature Immunology*, 7(7), 715-723.
- Kato, N., Mueller, C. R., Fuchs, J. F., McElroy, K., Wessely, V., Higgs, S., & Christensen, B. M. (2008). Evaluation of the function of a type I peritrophic matrix as a physical barrier for midgut epithelium invasion by mosquito-borne pathogens in *Aedes aegypti*. *Vector-borne and Zoonotic Diseases*, 8(5), 701-712.
- Kenney, J. L., Adams, A. P., Gorchakov, R., Leal, G., & Weaver, S. C. (2012). Genetic and anatomic determinants of enzootic Venezuelan equine encephalitis virus infection of *Culex (Melanoconion) taeniopus*. *PLoS Neglected Tropical Diseases*, 6(4), e1606.
- King, J. G., & Hillyer, J. F. (2012). Infection-induced interaction between the mosquito circulatory and immune systems. *PLoS Pathogens*, 8(11), e1003058.
- Kistler, K. E., Vosshall, L. B., & Matthews, B. J. (2015). Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Reports*, 11(1), 51-60.
- Klasse, P. J. (2015). Molecular determinants of the ratio of inert to infectious virus particles. *Progress in Molecular Biology and Translational Science*, 129, 285-326.
- Klimstra, W. B., Nangle, E. M., Smith, M. S., Yurochko, A. D., & Ryman, K. D. (2004). DC-SIGN and L-SIGN Can Act as Attachment Receptors for Alphaviruses and Distinguish between Mosquito Cell-and Mammalian Cell-Derived Viruses. *Journal of Virology*, 78(14), 7862.
- Kokoza, V., Ahmed, A., Wimmer, E. A., & Raikhel, A. S. (2001). Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the piggyBac transposable element vector pBac [3xP3-EGFP afm]. *Insect Biochemistry and Molecular Biology*, 31(12), 1137-1143.

- Kraemer, M. U., Sinka, M. E., Duda, K. A., Mylne, A. Q., Shearer, F. M., Barker, C. M., ... & Hay, S. I. (2015). The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. *Elife*, 4, e08347.
- Kramer, L. D., & Ciota, A. T. (2015). Dissecting vectorial capacity for mosquito-borne viruses. *Current Opinion in Virology*, 15, 112-118.
- Ku, H. Y., & Lin, H. (2014). PIWI proteins and their interactors in piRNA biogenesis, germline development and gene expression. *National Science Review*, 1(2), 205-218.
- Kueberuwa, G., Cawood, R., Tedcastle, A., & Seymour, L. W. (2014). Tissue-specific attenuation of oncolytic sindbis virus without compromised genetic stability. *Human Gene Therapy Methods*, 25(2), 154-165.
- Kulcsar, K. A., Baxter, V. K., Abraham, R., Nelson, A., & Griffin, D. E. (2015). Distinct immune responses in resistant and susceptible strains of mice during neurovirulent alphavirus encephalomyelitis. *Journal of Virology*, 89(16), 8280-8291.
- Kumar, A., Srivastava, P., Sirisena, P. D. N. N., Dubey, S. K., Kumar, R., Shrinet, J., & Sunil, S. (2018). Mosquito innate immunity. *Insects*, 9(3), 95.
- Kuranaga, E. (2012). Beyond apoptosis: caspase regulatory mechanisms and functions in vivo. *Genes to Cells*, 17(2), 83-97.
- Kurkela, S., Manni, T., Myllynen, J., Vaheri, A., & Vapalahti, O. (2005). Clinical and laboratory manifestations of Sindbis virus infection: prospective study, Finland, 2002–2003. *The Journal of Infectious Diseases*, 191(11), 1820-1829.
- Kurkela, S., Rätti, O., Huhtamo, E., Uzcátegui, N. Y., Nuorti, J. P., Laakkonen, J., ... & Vapalahti, O. (2008). Sindbis virus infection in resident birds, migratory birds, and humans, Finland. *Emerging Infectious Diseases*, 14(1), 41.

- LaBeaud, A. D. (2008). Why arboviruses can be neglected tropical diseases. *PLoS Neglected Tropical Diseases*, 2(6), e247.
- Laine, M., Luukkainen, R., Jalava, J., Ilonen, J., Kuusisto, P., & Toivanen, A. (2002). Prolonged arthritis associated with Sindbis-related (Pogosta) virus infection. *Rheumatology*, 41(7), 829-830.
- Lambrechts, L., & Saleh, M. C. (2019). Manipulating mosquito tolerance for arbovirus control. *Cell Host & Microbe*, 26(3), 309-313.
- LaPointe, A. T., Landers, V. D., Westcott, C. E., & Sokoloski, K. J. (2020). Production of noncapped genomic RNAs is critical to Sindbis virus disease and pathogenicity. *MBio*, 11(6), 10-1128.
- Lawrie, C. H., Uzcátegui, N. Y., Gould, E. A., & Nuttall, P. A. (2004). Ixodid and argasid tick species and West Nile virus. *Emerging Infectious Diseases*, 10(4), 653.
- Lee, W. S., Webster, J. A., Madzokere, E. T., Stephenson, E. B., & Herrero, L. J. (2019). Mosquito antiviral defense mechanisms: a delicate balance between innate immunity and persistent viral infection. *Parasites & Vectors*, 12, 1-12.
- Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., & Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette *spätzle*/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell*, 86(6), 973-983.
- Lequime, S., Fontaine, A., Ar Gouilh, M., Moltini-Conclois, I., & Lambrechts, L. (2016). Genetic drift, purifying selection and vector genotype shape dengue virus intra-host genetic diversity in mosquitoes. *PLoS Genetics*, 12(6), e1006111.
- Levashina, E. A., Moita, L. F., Blandin, S., Vriend, G., Lagueux, M., & Kafatos, F. C. (2001). Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout

- in cultured cells of the mosquito, *Anopheles gambiae*. *Cell*, 104(5), 709-718.
- Levis, R., Schlesinger, S., & Huang, H. V. (1990). Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. *Journal of Virology*, 64(4), 1726-1733.
- Li, D., Lott, W. B., Lowry, K., Jones, A., Thu, H. M., & Aaskov, J. (2011). Defective interfering viral particles in acute dengue infections. *PloS One*, 6(4), e19447.
- Liu, B., Behura, S. K., Clem, R. J., Schneemann, A., Becnel, J., Severson, D. W., & Zhou, L. (2013). P53-mediated rapid induction of apoptosis conveys resistance to viral infection in *Drosophila melanogaster*. *PLoS Pathogens*, 9(2), e1003137.
- Liu, Q., Rand, T. A., Kalidas, S., Du, F., Kim, H. E., Smith, D. P., & Wang, X. (2003). R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. *Science*, 301(5641), 1921-1925.
- Liu, W. Q., Chen, S. Q., Bai, H. Q., Wei, Q. M., Zhang, S. N., Chen, C., ... & Liang, S. H. (2020). The Ras/ERK signaling pathway couples antimicrobial peptides to mediate resistance to dengue virus in *Aedes* mosquitoes. *PLoS Neglected Tropical Diseases*, 14(8), e0008660.
- Locasale, J. W. (2013). Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nature Reviews Cancer*, 13(8), 572-583.
- Londono-Renteria, B., Troupin, A., Conway, M. J., Vesely, D., Ledizet, M., Roundy, C. M., ... & Colpitts, T. M. (2015). Dengue virus infection of *Aedes aegypti* requires a putative cysteine rich venom protein. *PLoS Pathogens*, 11(10), e1005202.
- Londono-Renteria, B., Troupin, A., & Colpitts, T. M. (2016). Arbovirosis and potential transmission blocking vaccines. *Parasites & Vectors*, 9(1), 1-11.
- Lundström, J. O., Vene, S., Espmark, Å., Engvall, M., & Niklasson, B. (1991). Geographical and

- temporal distribution of Ockelbo disease in Sweden. *Epidemiology & Infection*, 106(3), 567-574.
- Lundström, J. O., Vene, S., Espmark, Å., Engvall, M., & Niklasson, B. (1991). Geographical and temporal distribution of Ockelbo disease in Sweden. *Epidemiology & Infection*, 106(3), 567-574.
- Lundström, J. O., & Pfeffer, M. (2010). Phylogeographic structure and evolutionary history of Sindbis virus. *Vector-Borne and Zoonotic Diseases*, 10(9), 889-907.
- Luplertlop, N., Surasombatpattana, P., Patramool, S., Dumas, E., Wasinpiyamongkol, L., Saune, L., ... & Missé, D. (2011). Induction of a peptide with activity against a broad spectrum of pathogens in the *Aedes aegypti* salivary gland, following infection with dengue virus. *PLoS Pathogens*, 7(1), e1001252.
- Moreira-Soto, A., Torres, M. C., de Mendonça, M. L., Mares-Guia, M. A., dos Santos Rodrigues, C. D., Fabri, A. A., ... & de Filippis, A. B. (2018). Evidence for multiple sylvatic transmission cycles during the 2016–2017 yellow fever virus outbreak, Brazil. *Clinical Microbiology and Infection*, 24(9), 1019-e1.
- Matsushita, Y., Nakagawa, H., & Koike, K. (2021). Lipid metabolism in oncology: why it matters, how to research, and how to treat. *Cancers*, 13(3), 474.
- Medlock, J. M., Snow, K. R., & Leach, S. (2007). Possible ecology and epidemiology of medically important mosquito-borne arboviruses in Great Britain. *Epidemiology & Infection*, 135(3), 466-482.
- Meister, S., Kanzok, S. M., Zheng, X. L., Luna, C., Li, T. R., Hoa, N. T., ... & Zheng, L. (2005). Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*, 102(32),

11420-11425.

- Meister, S., Agianian, B., Turlure, F., Relógio, A., Morlais, I., Kafatos, F. C., & Christophides, G. K. (2009). *Anopheles gambiae* PGRPLC-mediated defense against bacteria modulates infections with malaria parasites. *Plos Pathogens*, 5(8), e1000542.
- Merkling, S. H., & van Rij, R. P. (2013). Beyond RNAi: antiviral defense strategies in *Drosophila* and mosquito. *Journal of insect physiology*, 59(2), 159-170.
- Merwaiss, F., Filomatori, C. V., Susuki, Y., Bardossy, E. S., Alvarez, D. E., & Saleh, M. C. (2021). Chikungunya virus replication rate determines the capacity of crossing tissue barriers in mosquitoes. *Journal of Virology*, 95(3), 10-1128.
- Michel, T., Reichhart, J. M., Hoffmann, J. A., & Royet, J. (2001). *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature*, 414(6865), 756-759.
- Miesen, P., Girardi, E., & van Rij, R. P. (2015). Distinct sets of PIWI proteins produce arbovirus and transposon-derived piRNAs in *Aedes aegypti* mosquito cells. *Nucleic Acids Research*, 43(13), 6545-6556.
- Miller, B. R., Monath, T. P., Tabachnick, W. J., & Ezike, V. I. (1989). Epidemic yellow fever caused by an incompetent mosquito vector. *Tropical Medical Parasitology*, 40(4), 396-399.
- Mitri, C., Jacques, J. C., Thiery, I., Riehle, M. M., Xu, J., Bischoff, E., ... & Bourgouin, C. (2009). Fine pathogen discrimination within the APL1 gene family protects *Anopheles gambiae* against human and rodent malaria species. *PLoS Pathogens*, 5(9), e1000576.
- Mohankumar, V., Dhanushkodi, N. R., & Raju, R. (2011). Sindbis virus replication, is insensitive to rapamycin and torin1, and suppresses Akt/mTOR pathway late during infection in HEK cells. *Biochemical and Biophysical Research Communications*, 406(2),

262-267.

- Myles, K. M., Wiley, M. R., Morazzani, E. M., & Adelman, Z. N. (2008). Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proceedings of the National Academy of Sciences*, 105(50), 19938-19943.
- Myles, K. M., Wiley, M. R., Morazzani, E. M., & Adelman, Z. N. (2008). Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proceedings of the National Academy of Sciences*, 105(50), 19938-19943.
- Nielsen, R., Paul, J. S., Albrechtsen, A., & Song, Y. S. (2011). Genotype and SNP calling from next-generation sequencing data. *Nature Reviews Genetics*, 12(6), 443-451.
- Norder, H., Lundström, J. O., Kozuch, O., & Magnus, L. O. (1996). Genetic relatedness of Sindbis virus strains from Europe, Middle East, and Africa. *Virology*, 222(2), 440-445.
- Obbard, D. J., Jiggins, F. M., Halligan, D. L., & Little, T. J. (2006). Natural selection drives extremely rapid evolution in antiviral RNAi genes. *Current Biology*, 16(6), 580-585.
- Ojosnegros, S., Garcia-Arriaza, J., Escarmis, C., Manrubia, S. C., Perales, C., Arias, A., ... & Domingo, E. (2011). Viral genome segmentation can result from a trade-off between genetic content and particle stability. *PLoS Genetics*, 7(3), e1001344.
- Okamura, K., Robine, N., Liu, Y., Liu, Q., & Lai, E. C. (2011). R2D2 Organizes Small Regulatory RNA Pathways in *Drosophila*. *Molecular and Cellular Biology*, 31(4), 884-896.
- Okamura, K., Ishizuka, A., Siomi, H., & Siomi, M. C. (2004). Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes & Development*, 18(14), 1655-1666.
- Oliveira, J. H. M., Gonçalves, R. L., Lara, F. A., Dias, F. A., Gandara, A. C. P., Menna-Barreto,

- R. F., ... & Oliveira, P. L. (2011). Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. *PLoS pathogens*, 7(3), e1001320.
- Olson, K. E., Higgs, S., Hahn, C. S., Rice, C. M., Carlson, J. O., & Beaty, B. J. (1994). The expression of chloramphenicol acetyltransferase in *Aedes albopictus* (C6/36) cells and *Aedes triseriatus* mosquitoes using a double subgenomic recombinant Sindbis virus. *Insect Biochemistry and Molecular Biology*, 24(1), 39-48.
- Olson, K. E., Higgs, S., Gaines, P. J., Powers, A. M., Davis, B. S., Kamrud, K. I., ... & Beaty, B. J. (1996). Genetically engineered resistance to dengue-2 virus transmission in mosquitoes. *Science*, 272(5263), 884-886.
- Olson, K. E., Myles, K. M., Seabaugh, R. C., Higgs, S., Carlson, J. O., & Beaty, B. J. (2000). Development of a Sindbis virus expression system that efficiently expresses green fluorescent protein in midguts of *Aedes aegypti* following per os infection. *Insect Molecular Biology*, 9(1), 57-65.
- Osei-Poku, J., Mbogo, C. M., Palmer, W. J., & Jiggins, F. M. (2012). Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. *Molecular Ecology*, 21(20), 5138-5150.
- Osta, M. A., Christophides, G. K., & Kafatos, F. C. (2004). Effects of mosquito genes on *Plasmodium* development. *Science*, 303(5666), 2030-2032.
- Palmer, S. R. (2011). Oxford textbook of zoonoses: biology, clinical practice, and public health control. 3-11.
- Pan, X., Zhou, G., Wu, J., Bian, G., Lu, P., Raikhel, A. S., & Xi, Z. (2012). Wolbachia induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue

- virus in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences*, 109(1), E23-E31.
- Patel, R. K., & Hardy, R. W. (2012). Role for the phosphatidylinositol 3-kinase-Akt-TOR pathway during sindbis virus replication in arthropods. *Journal of Virology*, 86(7), 3595-3604.
- Patterson, E. I., Khanipov, K., Rojas, M. M., Kautz, T. F., Rockx-Brouwer, D., Golovko, G., ... & Forrester, N. L. (2018). Mosquito bottlenecks alter viral mutant swarm in a tissue and time-dependent manner with contraction and expansion of variant positions and diversity. *Virus Evolution*, 4(1), vey001.
- Pavoine, C., & Pecker, F. (2009). Sphingomyelinases: their regulation and roles in cardiovascular pathophysiology. *Cardiovascular Research*, 82(2), 175-183.
- Perri, S., Driver, D. A., Gardner, J. P., Sherrill, S., Belli, B. A., Dubensky Jr, T. W., & Polo, J. M. (2000). Replicon vectors derived from Sindbis virus and Semliki forest virus that establish persistent replication in host cells. *Journal of Virology*, 74(20), 9802-9807.
- Pfeffer, M., & Dobler, G. (2010). Emergence of zoonotic arboviruses by animal trade and migration. *Parasites & Vectors*, 3, 1-15.
- Pierro, D. J., Myles, K. M., Foy, B. D., Beaty, B. J., & Olson, K. E. (2003). Development of an orally infectious Sindbis virus transducing system that efficiently disseminates and expresses green fluorescent protein in *Aedes aegypti*. *Insect Molecular Biology*, 12(2), 107-116.
- Povelones, M., Waterhouse, R. M., Kafatos, F. C., & Christophides, G. K. (2009). Leucine-rich repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites. *Science*, 324(5924), 258-261.

- Prince, B. C., Walsh, E., Torres, T. Z. B., & Rückert, C. (2023). Recognition of arboviruses by the mosquito immune system. *Biomolecules*, 13(7), 1159.
- Puglise, J. M., Estep, A. S., & Becnel, J. J. (2016). Expression profiles and RNAi silencing of inhibitor of apoptosis transcripts in *Aedes*, *Anopheles*, and *Culex* mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*, 53(2), 304-314.
- Qian, Q., Zhou, H., Shu, T., Mu, J., Fang, Y., Xu, J., ... & Zhou, X. (2020). The capsid protein of Semliki Forest virus antagonizes RNA interference in mammalian cells. *Journal of Virology*, 94(3), 10-1128.
- Ramirez, J. L., Souza-Neto, J., Torres Cosme, R., Rovira, J., Ortiz, A., Pascale, J. M., & Dimopoulos, G. (2012). Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS Neglected Tropical Diseases*, 6(3), e1561.
- Ramirez, J. L., Short, S. M., Bahia, A. C., Saraiva, R. G., Dong, Y., Kang, S., ... & Dimopoulos, G. (2014). *Chromobacterium* Csp_P reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and in vitro anti-pathogen activities. *PLoS Pathogens*, 10(10), e1004398.
- Rappole, J. H., Derrickson, S. R., & Hubalek, Z. (2000). Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerging Infectious Diseases*, 6(4), 319.
- Raquin, V., Merkling, S. H., Gausson, V., Moltini-Conclois, I., Frangeul, L., Varet, H., ... & Lambrechts, L. (2017). Individual co-variation between viral RNA load and gene expression reveals novel host factors during early dengue virus infection of the *Aedes aegypti* midgut. *PLoS Neglected Tropical Diseases*, 11(12), e0006152.
- Ribeiro, J. M., Arcà, B., Lombardo, F., Calvo, E., Phan, V. M., Chandra, P. K., & Wikel, S. K.

- (2007). An annotated catalogue of salivary gland transcripts in the adult female mosquito, *Aedes aegypti*. *BMC Genomics*, 8, 1-27.
- Ritland, K. (1996). Estimators for pairwise relatedness and individual inbreeding coefficients. *Genetics Research*, 67(2), 175-185.
- Robert, M. A., Christofferson, R. C., Silva, N. J., Vasquez, C., Mores, C. N., & Wearing, H. J. (2016). Modeling mosquito-borne disease spread in US urbanized areas: the case of dengue in Miami. *PloS One*, 11(8), e0161365.
- Rodriguez-Andres, J., Rani, S., Varjak, M., Chase-Topping, M. E., Beck, M. H., Ferguson, M. C., ... & Kohl, A. (2012). Phenoloxidase activity acts as a mosquito innate immune response against infection with Semliki Forest virus. *PLoS Pathogens*, 8(11), e1002977.
- Rodriguez, J. L., Costlow, J. L., Sheedy, M., Yoon, K. T., Gabaldón, A. M., & Steel, J. J. (2022). Sindbis Virus Replication reduces dependence on mitochondrial metabolism during infection. *Frontiers in Cellular and Infection Microbiology*, 12, 859814.
- Romoser, W. S., Turell, M. J., Lerdthusnee, K., Neira, M., Dohm, D., Ludwig, G., & Wasieloski, L. (2005). Pathogenesis of Rift Valley fever virus in mosquitoes—tracheal conduits & the basal lamina as an extra-cellular barrier. In *Infectious Diseases from Nature: Mechanisms of Viral Emergence and Persistence* (pp. 89-100). Springer Vienna.
- Rose, P. P., Hanna, S. L., Spiridigliozzi, A., Wannissorn, N., Beiting, D. P., Ross, S. R., ... & Cherry, S. (2011). Natural resistance-associated macrophage protein is a cellular receptor for sindbis virus in both insect and mammalian hosts. *Cell host & Microbe*, 10(2), 97-104.
- Ross, R. J., Weiner, M. M., & Lin, H. (2014). PIWI proteins and PIWI-interacting RNAs in the soma. *Nature*, 505(7483), 353-359.
- Rubach, J. K., Wasik, B. R., Rupp, J. C., Kuhn, R. J., Hardy, R. W., & Smith, J. L. (2009).

- Characterization of purified Sindbis virus nsP4 RNA-dependent RNA polymerase activity in vitro. *Virology*, 384(1), 201-208.
- Rückert, C., Weger-Lucarelli, J., Garcia-Luna, S. M., Young, M. C., Byas, A. D., Murrieta, R. A., ... & Ebel, G. D. (2017). Impact of simultaneous exposure to arboviruses on infection and transmission by *Aedes aegypti* mosquitoes. *Nature Communications*, 8(1), 15412.
- Sabin, L. R., Zhou, R., Gruber, J. J., Lukinova, N., Bambina, S., Berman, A., ... & Cherry, S. (2009). Ars2 regulates both miRNA-and siRNA-dependent silencing and suppresses RNA virus infection in *Drosophila*. *Cell*, 138(2), 340-351.
- Saj, A., & Lai, E. C. (2011). Control of microRNA biogenesis and transcription by cell signaling pathways. *Current Opinion in Genetics & Development*, 21(4), 504-510.
- Sanders, H. R., Foy, B. D., Evans, A. M., Ross, L. S., Beaty, B. J., Olson, K. E., & Gill, S. S. (2005). Sindbis virus induces transport processes and alters expression of innate immunity pathway genes in the midgut of the disease vector, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*, 35(11), 1293-1307.
- Sane, J., Guedes, S., Kurkela, S., Lyytikäinen, O., & Vapalahti, O. (2010). Epidemiological analysis of mosquito-borne Pogosta disease in Finland, 2009. *Eurosurveillance*, 15(2), 19462.
- Sane, J., Guedes, S., Ollgren, J., Kurkela, S., Klemets, P., Vapalahti, O., ... & Nuorti, J. P. (2011). Epidemic sindbis virus infection in Finland: a population-based case-control study of risk factors. *Journal of Infectious Diseases*, 204(3), 459-466.
- Sane, J., Kurkela, S., Lokki, M. L., Miettinen, A., Helve, T., Vaheri, A., & Vapalahti, O. (2012). Clinical Sindbis alphavirus infection is associated with HLA-DRB1* 01 allele and production of autoantibodies. *Clinical Infectious Diseases*, 55(3), 358-363.

- Sane, J., Kurkela, S., Levanov, L., Nikkari, S., Vaheri, A., & Vapalahti, O. (2012). Development and evaluation of a real-time RT-PCR assay for Sindbis virus detection. *Journal of Virological Methods*, 179(1), 185-188.
- Sanjuán, R. (2018). Collective properties of viral infectivity. *Current Opinion in Virology*, 33, 1-6.
- Saraiva, R. G., Fang, J., Kang, S., Angleró-Rodríguez, Y. I., Dong, Y., & Dimopoulos, G. (2018). Aminopeptidase secreted by *Chromobacterium* sp. Panama inhibits dengue virus infection by degrading the E protein. *PLoS Neglected Tropical Diseases*, 12(4), e0006443.
- Savolainen-Kopra, C., & Blomqvist, S. (2010). Mechanisms of genetic variation in polioviruses. *Reviews in Medical Virology*, 20(6), 358-371.
- Schmidt, T. L., Jasper, M. E., Weeks, A. R., & Hoffmann, A. A. (2021). Unbiased population heterozygosity estimates from genome-wide sequence data. *Methods in Ecology and Evolution*, 12(10), 1888-1898.
- Schneider, B. S., & Higgs, S. (2008). The enhancement of arbovirus transmission and disease by mosquito saliva is associated with modulation of the host immune response. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102(5), 400-408.
- Schneider, D. S., Jin, Y., Morisato, D., & Anderson, K. V. (1994). A processed form of the Spätzle protein defines dorsal-ventral polarity in the *Drosophila* embryo. *Development*, 120(5), 1243-1250.
- Schnitger, A. K., Yassine, H., Kafatos, F. C., & Osta, M. A. (2009). Two C-type lectins cooperate to defend *Anopheles gambiae* against Gram-negative bacteria. *Journal of Biological Chemistry*, 284(26), 17616-17624.
- Schuchman, R., Kilianski, A., Piper, A., Vancini, R., Ribeiro, J. M., Sprague, T. R., ... & Glaros,

- T. (2018). Comparative characterization of the Sindbis virus proteome from mammalian and invertebrate hosts identifies nsP2 as a component of the virion and sorting nexin 5 as a significant host factor for alphavirus replication. *Journal of Virology*, 92(14), 10-1128.
- Seabaugh, R. C., Olson, K. E., Higgs, S., Carlson, J. O., & Beaty, B. J. (1998). Development of a chimeric sindbis virus with Enhanced per OsInfection of *Aedes aegypti*. *Virology*, 243(1), 99-112.
- Severson, D. W., & Behura, S. K. (2016). Genome investigations of vector competence in *Aedes aegypti* to inform novel arbovirus disease control approaches. *Insects*, 7(4), 58.
- Shao, H., Huang, Y., Zhang, L., Yuan, K., Chu, Y., Dou, Z., ... & Yao, X. (2015). Spatiotemporal dynamics of Aurora B-PLK1-MCAK signaling axis orchestrates kinetochore bi-orientation and faithful chromosome segregation. *Scientific Reports*, 5(1), 12204.
- Shirayama, M., Seth, M., Lee, H. C., Gu, W., Ishidate, T., Conte, D., & Mello, C. C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell*, 150(1), 65-77.
- Shishido, S. N., Varahan, S., Yuan, K., Li, X., & Fleming, S. D. (2012). Humoral innate immune response and disease. *Clinical Immunology*, 144(2), 142-158.
- Silva da Costa, L., Pereira da Silva, A. P., Da Poian, A. T., & El-Bacha, T. (2012). Mitochondrial bioenergetic alterations in mouse neuroblastoma cells infected with Sindbis virus: implications to viral replication and neuronal death. *PloS One*, 7(4), e33871.
- Sim, S., Jupatanakul, N., & Dimopoulos, G. (2014). Mosquito immunity against arboviruses. *Viruses*, 6(11), 4479-4504.
- Sim, S., Ramirez, J. L., & Dimopoulos, G. (2012). Dengue virus infection of the *Aedes aegypti*

- salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior. *PLoS Pathogens*, 8(3), e1002631.
- Sinkins, S. P. (2013). Wolbachia and arbovirus inhibition in mosquitoes. *Future Microbiology*, 8(10), 1249-1256.
- Van Slyke, G. A., Arnold, J. J., Lugo, A. J., Griesemer, S. B., Moustafa, I. M., Kramer, L. D., ... & Ciota, A. T. (2015). Sequence-specific fidelity alterations associated with West Nile virus attenuation in mosquitoes. *PLoS pathogens*, 11(6), e1005009.
- Smartt, C. T., Shin, D., & Alto, B. W. (2017). Dengue serotype-specific immune response in *Aedes aegypti* and *Aedes albopictus*. *Memórias do Instituto Oswaldo Cruz*, 112, 829-837.
- Smith, D. R., Adams, A. P., Kenney, J. L., Wang, E., & Weaver, S. C. (2008). Venezuelan equine encephalitis virus in the mosquito vector *Aedes taeniorhynchus*: infection initiated by a small number of susceptible epithelial cells and a population bottleneck. *Virology*, 372(1), 176-186.
- Souza-Neto, J. A., Sim, S., & Dimopoulos, G. (2009). An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proceedings of the National Academy of Sciences*, 106(42), 17841-17846.
- Spuul, P., Balistreri, G., Hellström, K., Golubtsov, A. V., Jokitalo, E., & Ahola, T. (2011). Assembly of alphavirus replication complexes from RNA and protein components in a novel trans-replication system in mammalian cells. *Journal of Virology*, 85(10), 4739-4751.
- Stapleford, K. A., Coffey, L. L., Lay, S., Bordería, A. V., Duong, V., Isakov, O., ... & Vignuzzi, M. (2014). Emergence and transmission of arbovirus evolutionary intermediates with epidemic potential. *Cell Host & Microbe*, 15(6), 706-716.
- Storm, N., Weyer, J., Markotter, W., Nel, L. H., Paweska, J. T., Leman, P. A., & Kemp, A.

- (2013). Phylogeny of Sindbis virus isolates from South Africa. *Southern African Journal of Epidemiology and Infection*, 28(4), 207-214.
- Storm, N., Weyer, J., Markotter, W., Kemp, A., Leman, P. A., Dermaux-Msimang, V., ... & Paweska, J. T. (2014). Human cases of Sindbis fever in South Africa, 2006–2010. *Epidemiology & Infection*, 142(2), 234-238.
- Strauss, E. G., & Strauss, J. H. (1986). Structure and replication of the alphavirus genome. In *The Togaviridae and Flaviviridae* (pp. 35-90). Boston, MA: Springer New York.
- Strauss, J. H., & Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiological Reviews*, 58(3), 491-562.
- Stroynowska-Czerwinska, A., Fiszer, A., & Krzyzosiak, W. J. (2014). The panorama of miRNA-mediated mechanisms in mammalian cells. *Cellular and Molecular Life Sciences*, 71, 2253-2270.
- Styer, L. M., Meola, M. A., & Kramer, L. D. (2007). West Nile virus infection decreases fecundity of *Culex tarsalis* females. *Journal of Medical Entomology*, 44(6), 1074-1085.
- Tchankouo-Nguetcheu, S., Bourguet, E., Lenormand, P., Rousselle, J. C., Namane, A., & Choumet, V. (2012). Infection by chikungunya virus modulates the expression of several proteins in *Aedes aegypti* salivary glands. *Parasites & Vectors*, 5(1), 1-11.
- Tchankouo-Nguetcheu, S., Khun, H., Pincet, L., Roux, P., Bahut, M., Huerre, M., ... & Choumet, V. (2010). Differential protein modulation in midguts of *Aedes aegypti* infected with chikungunya and dengue 2 viruses. *PloS One*, 5(10), e13149.
- Terradas, G., Joubert, D. A., & McGraw, E. A. (2017). The RNAi pathway plays a small part in Wolbachia-mediated blocking of dengue virus in mosquito cells. *Scientific Reports*, 7(1), 43847.

- Thomas, R. E., Wu, W. K., Verleye, D., & Rai, K. S. (1993). Midgut basal lamina thickness and dengue-1 virus dissemination rates in laboratory strains of *Aedes albopictus* (Diptera: Culicidae). *Journal of Medical Entomology*, 30(2), 326-331.
- Tian, Y., Simanshu, D. K., Ma, J. B., Park, J. E., Heo, I., Kim, V. N., & Patel, D. J. (2014). A phosphate-binding pocket within the platform-PAZ-connector helix cassette of human Dicer. *Molecular Cell*, 53(4), 606-616.
- Tikhe, C. V., & Dimopoulos, G. (2021). Mosquito antiviral immune pathways. *Developmental & Comparative Immunology*, 116, 103964.
- Tomar, S., Hardy, R. W., Smith, J. L., & Kuhn, R. J. (2006). Catalytic core of alphavirus nonstructural protein nsP4 possesses terminal adenylyltransferase activity. *Journal of Virology*, 80(20), 9962-9969.
- Troha, K., Im, J. H., Revah, J., Lazzaro, B. P., & Buchon, N. (2018). Comparative transcriptomics reveals CrebA as a novel regulator of infection tolerance in *D. melanogaster*. *PLoS Pathogens*, 14(2), e1006847.
- Troupin, A., Londono-Renteria, B., Conway, M. J., Cloherty, E., Jameson, S., Higgs, S., ... & Colpitts, T. M. (2016). A novel mosquito ubiquitin targets viral envelope protein for degradation and reduces virion production during dengue virus infection. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1860(9), 1898-1909.
- Tsiodras, S., Kelesidis, T., Kelesidis, I., Bauchinger, U., & Falagas, M. E. (2008). Human infections associated with wild birds. *Journal of Infection*, 56(2), 83-98.
- Turunen, M., Kuusisto, P., Uggeldahl, P. E., & Toivanen, A. (1998). Pogosta disease: clinical observations during an outbreak in the province of North Karelia, Finland. *British journal of Rheumatology*, 37(11), 1177-1180.

- Ulug, E. T., Garry, R. F., Waite, M. R., & Bose Jr, H. R. (1984). Alterations in monovalent cation transport in Sindbis virus-infected chick cells. *Virology*, *132*(1), 118-130.
- Urdaneta-Marquez, L., & Failloux, A. B. (2011). Population genetic structure of *Aedes aegypti*, the principal vector of dengue viruses. *Infection, Genetics and Evolution*, *11*(2), 253-261.
- Vaidyanathan, R., & Scott, T. W. (2006). Apoptosis in mosquito midgut epithelia associated with West Nile virus infection. *Apoptosis*, *11*, 1643-1651.
- Valiente Moro, C., Tran, F. H., Nantenaina Raharimalala, F., Ravelonandro, P., & Mavingui, P. (2013). Diversity of culturable bacteria including *Pantoea* in wild mosquito *Aedes albopictus*. *BMC Microbiology*, *13*, 1-11.
- Varjak, M., Maringer, K., Watson, M., Sreenu, V. B., Fredericks, A. C., Pondeville, E., ... & Schnettler, E. (2017). *Aedes aegypti* Piwi4 is a noncanonical PIWI protein involved in antiviral responses. *MSphere*, *2*(3), 10-1128.
- Vignuzzi, M., Stone, J. K., Arnold, J. J., Cameron, C. E., & Andino, R. (2006). Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature*, *439*(7074), 344-348.
- Walker, P. J., Firth, C., Widen, S. G., Blasdel, K. R., Guzman, H., Wood, T. G., ... & Vasilakis, N. (2015). Evolution of genome size and complexity in the Rhabdoviridae. *PLoS Pathogens*, *11*(2), e1004664.
- Walsh, E., Torres, T. Z. B., & Rückert, C. (2022). *Culex* Mosquito Piwi4 Is Antiviral against Two Negative-Sense RNA Viruses. *Viruses*, *14*(12), 2758.
- Wang, H., Blair, C. D., Olson, K. E., & Clem, R. J. (2008). Effects of inducing or inhibiting apoptosis on Sindbis virus replication in mosquito cells. *The Journal of General Virology*, *89*(Pt 11), 2651.

- Wang, H., Gort, T., Boyle, D. L., & Clem, R. J. (2012). Effects of manipulating apoptosis on Sindbis virus infection of *Aedes aegypti* mosquitoes. *Journal of Virology*, 86(12), 6546-6554.
- Wang, J. M., Cheng, Y., Shi, Z. K., Li, X. F., Xing, L. S., Jiang, H., ... & Zou, Z. (2019). *Aedes aegypti* HPX8C modulates immune responses against viral infection. *PLoS Neglected Tropical Diseases*, 13(4), e0007287.
- Wang, K. S., Kuhn, R. J., Strauss, E. G., Ou, S. U. S. A. N., & Strauss, J. H. (1992). High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *Journal of Virology*, 66(8), 4992-5001.
- Wang, X., Zhao, Q., & Christensen, B. M. (2005). Identification and characterization of the fibrinogen-like domain of fibrinogen-related proteins in the mosquito, *Anopheles gambiae*, and the fruitfly, *Drosophila melanogaster*, genomes. *BMC Genomics*, 6, 1-15.
- Weaver, S. C., Rico-Hesse, R., & Scott, T. W. (1992). Genetic diversity and slow rates of evolution in New World alphaviruses. *Genetic Diversity of RNA Viruses*, 99-117.
- Weaver, S. C. (2013). Urbanization and geographic expansion of zoonotic arboviral diseases: mechanisms and potential strategies for prevention. *Trends in microbiology*, 21(8), 360-363.
- Weaver, S. C., Brault, A. C., Kang, W., & Holland, J. J. (1999). Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. *Journal of Virology*, 73(5), 4316-4326.
- Weaver, S. C., & Lecuit, M. (2015). Chikungunya virus and the global spread of a mosquito-borne disease. *New England Journal of Medicine*, 372(13), 1231-1239.
- Weaver, S. C., Winegar, R., Manger, I. D., & Forrester, N. L. (2012). Alphaviruses: population genetics and determinants of emergence. *Antiviral Research*, 94(3), 242-257.

- Weber, A. N., Tauszig-Delamasure, S., Hoffmann, J. A., Lelièvre, E., Gascan, H., Ray, K. P., ... & Gay, N. J. (2003). Binding of the Drosophila cytokine Spätzle to Toll is direct and establishes signaling. *Nature Immunology*, 4(8), 794-800.
- Westcott, C. E., Qazi, S., Maiocco, A. M., Mukhopadhyay, S., & Sokoloski, K. J. (2022). Binding of hnRNP I-vRNA Regulates Sindbis Virus Structural Protein Expression to Promote Particle Infectivity. *Viruses*, 14(7), 1423.
- Wilder-Smith, A., Gubler, D. J., Weaver, S. C., Monath, T. P., Heymann, D. L., & Scott, T. W. (2017). Epidemic arboviral diseases: priorities for research and public health. *The Lancet Infectious Diseases*, 17(3), e101-e106.
- Wong, J., Stoddard, S. T., Astete, H., Morrison, A. C., & Scott, T. W. (2011). Oviposition site selection by the dengue vector *Aedes aegypti* and its implications for dengue control. *PLoS Neglected Tropical Diseases*, 5(4), e1015.
- World Health Organization. (2017). Global vector control response 2017-2030. *Global vector control response 2017-2030*.
- Wu, P., Sun, P., Nie, K., Zhu, Y., Shi, M., Xiao, C., ... & Cheng, G. (2019). A gut commensal bacterium promotes mosquito permissiveness to arboviruses. *Cell Host & Microbe*, 25(1), 101-112.
- Wu, W., Simmons, C. A., Moffitt, J., Clem, R. J., & Passarelli, A. L. (2020). Effects of manipulating fibroblast growth factor expression on Sindbis virus replication in vitro and in *Aedes aegypti* mosquitoes. *Viruses*, 12(9), 943.
- Xi, Z., Ramirez, J. L., & Dimopoulos, G. (2008). The *Aedes aegypti* toll pathway controls dengue virus infection. *PLoS Pathogens*, 4(7), e1000098.
- Xiao, X., Liu, Y., Zhang, X., Wang, J., Li, Z., Pang, X., ... & Cheng, G. (2014). Complement-

- related proteins control the flavivirus infection of *Aedes aegypti* by inducing antimicrobial peptides. *PLoS Pathogens*, 10(4), e1004027.
- Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M., & Huang, H. V. (1989). Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science*, 243(4895), 1188-1191.
- Yan, F., Yang, W., Wang, X., & Gao, G. (2022). TMEM45B Interacts with Sindbis Virus Nsp1 and Nsp4 and Inhibits Viral Replication. *Journal of Virology*, 96(17), e00919-22.
- Yan, H., Zhou, Y., Liu, Y., Deng, Y., Puthiyakunnon, S., & Chen, X. (2014). MiR-252 of the Asian tiger mosquito *Aedes albopictus* regulates dengue virus replication by suppressing the expression of the dengue virus envelope protein. *Journal of Medical Virology*, 86(8), 1428-1436.
- Yeom, K. H., Lee, Y., Han, J., Suh, M. R., & Kim, V. N. (2006). Characterization of DGCR8/Pasha, the essential cofactor for Drosha in primary miRNA processing. *Nucleic Acids Research*, 34(16), 4622-4629.
- Yurchenco, P. D., & Julian, J. O. (1994). Basal lamina assembly. *Current Opinion in Cell Biology*, 6(5), 674-681.
- Zambon, R. A., Nandakumar, M., Vakharia, V. N., & Wu, L. P. (2005). The Toll pathway is important for an antiviral response in *Drosophila*. *Proceedings of the National Academy of Sciences*, 102(20), 7257-7262.
- Zhou, L., Jiang, G., Chan, G., Santos, C. P., Severson, D. W., & Xiao, L. (2005). Michelob_x is the missing inhibitor of apoptosis protein antagonist in mosquito genomes. *EMBO reports*, 6(8), 769-774.
- Zimmerman, O., Holmes, A. C., Kafai, N. M., Adams, L. J., & Diamond, M. S. (2023). Entry

receptors—the gateway to alphavirus infection. *The Journal of Clinical Investigation*, 133(2).

1.6 Chapter 1 figures

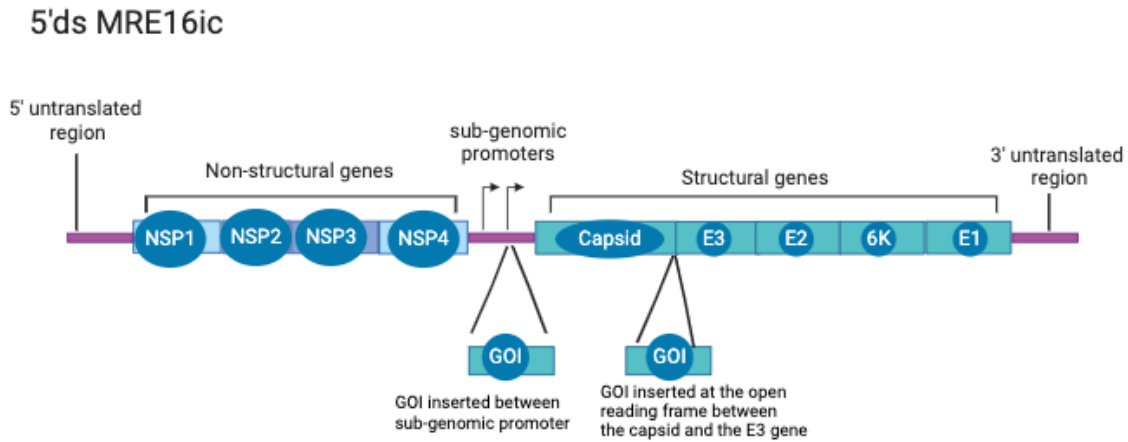


Figure 1.1. A schematic diagram of the genome of the SINV clone 5'dsMRE16ic.

A map of 5'dsMRE16ic showing the possible places where a gene of interest (GOI) can be inserted and expressed. In-frame insertion of a GOI within the structural open reading frame leads to a more stable gene insertion compared to insertion of GOI in between the two ORF regions (created in BioRender).

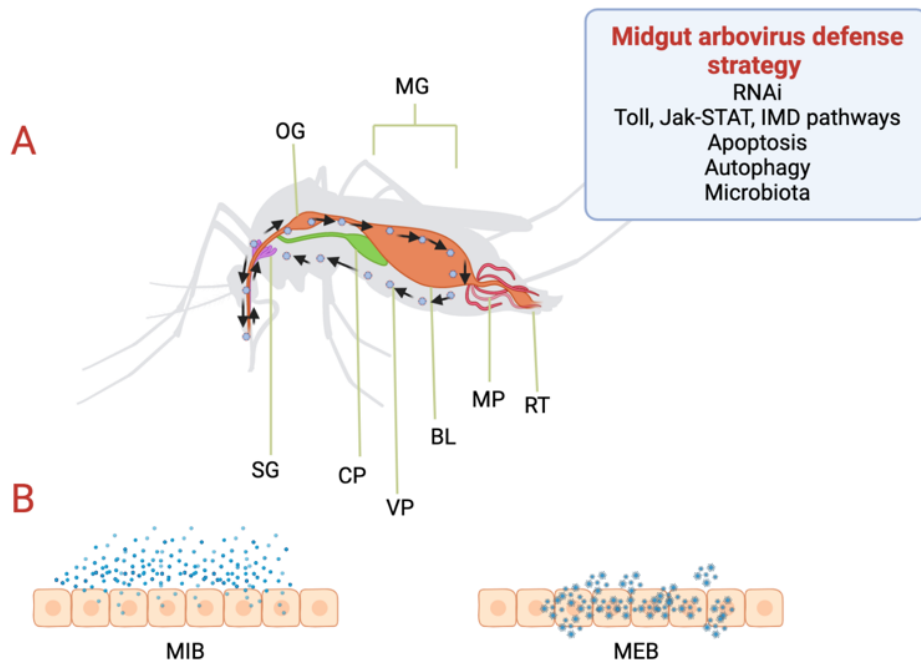


Figure 1.2. Diagram of infection route of arboviruses in mosquitoes.

Successful arbovirus infection of a mosquito vector requires crossing of the midgut tissue barrier before dissemination to other mosquito tissues including salivary glands. (A) Schematic representation of arbovirus route of infection in mosquito and the arbovirus defense strategy of the midgut. OG=Oesophagus; SG=Salivary glands; CP=Crop; VP=Virus particles; BL=Basal lamina; MP=Malpighian tubules; RT=Rectum; MG=Midgut. The arrow represents the route of arbovirus infection in mosquitoes. (B) Schematic representation of inhibition of infection by the midgut infection barrier (MIB) and prevention of escape of infection by the midgut escape barrier (MEB). Yellow squares represent midgut epithelial cells and blue spheres represent virus particles (created by BioRender).

Chapter 2 – Investigation of biological factors contributing to individual variation in viral titer after oral infection of *Aedes aegypti* mosquitoes by Sindbis virus

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Statistical analysis was performed by LA; all experiments were performed by PH; administration of project was performed by RJC.

2.1 Abstract

The mechanisms involved in determining arbovirus vector competence, or the ability of an arbovirus to infect and be transmitted by an arthropod vector, are still poorly understood. Vector competence can vary widely among different populations of a mosquito species. However, we and others have consistently observed a high degree of variation in viral titer (up to several logs) between individual infected mosquitoes, even those from highly inbred laboratory lines. Factors contributing to this variation in virus titer have been largely ignored in past studies. We investigated which biological factors can affect titer variation between individual mosquitoes of a laboratory strain of *Aedes aegypti*, the Orlando strain, after Sindbis virus infection. Greater titer variation was observed after oral versus intrathoracic infection, suggesting that the midgut barrier contributes to titer variation. Amongst the factors tested, only the length of the intrinsic incubation period affected the degree of titer variability, while virus strain, mosquito strain, mosquito weight, amount of blood ingested, and virus concentration in the blood meal had little effect. We also observed differences in culture adaptability and in the ability to orally infect mosquitoes between virus populations obtained from low and high titer mosquitoes, suggesting that founder effects may affect the virus titer in individual mosquitoes.

2.2 Introduction

Arboviral diseases account for approximately 17% of all infectious diseases globally and approximately 40,000 deaths each year, making it a great public health concern (WHO, 2017).

Some arboviruses of medical importance include chikungunya virus, dengue virus (DENV), Zika virus, West Nile virus, yellow fever virus, Sindbis virus (SINV), and many others (WHO, 2017).

SINV is classified as an emerging infectious virus that is mainly reported in northern Europe and South Africa (Lwande et al., 2015). In Sweden, disease caused by SINV infection in humans is called Ockelbo disease, which is characterized by rash, arthritis, and mild fever (Adouchief et al., 2016). Ockelbo disease is not fatal, and most patients recover within weeks or months, but arthralgia and myalgia can persist for years following infection, which suggests an inflammatory response or a persistent infection (Adouchief et al. 2016). The prevalence of infection for SINV is mostly low, resulting in short and sporadic epidemics (Lundström et al., 1991).

Arboviral diseases continue to persist due to the existence of the vector mosquitoes in endemic areas (Huang et al., 2019) and also due to the ability of these mosquitoes to spread to new environments to transmit the viruses (Robert et al., 2016; Weaver, 2013). A major factor contributing to the persistence of mosquitoes is a lack of robust vector control strategies (Achee et al., 2019). The inefficiency of current vector control strategies is due to a combination of factors including insufficient resources, a lack of technical know-how (Hemingway et al., 2006), and the development of mosquito resistance to chemical insecticides (Dusfour et al., 2019), especially in developing countries where the disease burden is highest (Hemingway et al. 2006). Also, the absence of effective antiviral drugs for the treatment of arboviral diseases (Huang et al.,

2017) and vaccines against most arboviruses contributes to the high morbidity and mortality caused by these viruses (Londono-Renteria et al., 2016).

A major limiting factor hindering the effort to develop new vector control strategies is the lack of a comprehensive understanding of what determines vector competence. Many studies have identified biological factors that contribute to differences in mosquito susceptibility to arbovirus infection such as mosquito age (Knecht et al., 2018), virus strain (Ciota et al., 2017), mosquito population or strain, virus concentration in host blood (Roundy et al., 2017), mosquito size (Nasci and Mitchell, 1994), and intrinsic incubation period (Weger-Lucarelli et al., 2016). However, an area of study that has been largely ignored is the extreme variation in the levels of viral replication that is observed amongst individual mosquitoes in a single experiment. We and many others have documented that a high degree of variation in viral titer exists between individual mosquitoes following oral infection, even between individual mosquitoes of inbred mosquito strains that have been raised in the same environmental conditions, and subsequently orally fed with the same virally infected blood (O'Neill et al., 2015). Large variation in virus titer between individual mosquitoes has been documented numerous times, but it is usually ignored by researchers and the causes of this variation are largely unaddressed. One study infected individual *Ae. aegypti* from field-caught populations with DENV and correlated differentially expressed genes with the levels of viral RNA (Raquin et al., 2017), resulting in the identification of 39 candidate genes that were proposed to affect DENV replication either positively or negatively. However, since these mosquitoes were from a wild population, they would be expected to have considerable genetic diversity, which likely contributed to the variation in titer.

In this study, we used SINV as a model arbovirus to investigate the contributions of several biological factors to variation in virus titer using the Orlando strain of *Ae. aegypti*, a

laboratory strain that was first established around 1940 and has been highly inbred since that time. Although we could not locate precise husbandry records, it appears that occasional outcrossing of Orlando with wild *Ae. aegypti* populations was performed up to 1992, but no outcrossing has occurred since at least 2003 and likely for longer (Kuno, 2010 and Alden Estep, personal communication). The biological factors we investigated included mosquito strain, virus strain, mosquito age and size, virus concentration in the blood meal, the amount of blood ingested, and intrinsic incubation period. To the best of our knowledge, this is the first study investigating which biological factors contribute to variation in virus titer between individual mosquitoes in a highly inbred mosquito line, where genetic variation between individuals is expected to be minimal.

2.3 Methods

2.3.1 Cell lines

BHK-21 cells were propagated in Dulbecco modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) at 37°C with 5% CO₂. C6/36 cells were maintained in Leibovitz's media (Gibco) plus 10% FBS at 27°C. Aag2 cells were maintained in Schneider's media (Gibco) plus 10% FBS at 27°C.

2.3.2 Mosquito rearing

Ae. aegypti mosquito strains Orlando (obtained in 2005 from James Becnel at the Agricultural Research Service, U.S. Department of Agriculture, Gainesville, FL) and Rex-D (obtained in 2018 from Alexander Franz, University of Missouri), were reared at 27°C, 80% humidity on a 12 h light/12 h dark cycle. *Ae. aegypti* eggs were hatched in defibrinated water and

brain heart infusion media (BHI) for 2 days to produce mosquito larvae. Mosquito larvae were separated into pans (each pan containing approximately 100 larvae) with a small amount of BHI and ground fish meal added. Larvae metamorphosized into pupae within 5-7 days. The pupae were transferred into pupa cups and transferred into mosquito cages. Adult mosquitoes emerged beginning at around 2 days after transfer. Adults were allowed to feed on raisins and water before and after blood feeding. All mosquito experiments were performed in an arthropod containment level 2 insectary at Kansas State University.

2.3.3 MRE-16 and TE RNA generation and cell transfection

The MRE-16 and TE infectious clones p5'dsMRE16ic and pTE5'2J, respectively, were obtained from Ken Olson (Colorado State University) and their construction has been described (Foy et al. 2004; Pierro et al. 2003). The p5'dsMRE16ic plasmid was linearized with *AscI* while the pTE5'2J plasmid was linearized with *XhoI*. Capped viral RNA was produced from the linearized plasmids using m7 G(5')ppp(5')G Cap Analog (Ambion) and MEGAscribe SP6 Transcription Kit (Thermo Fisher Scientific). A density of 5×10^5 cells BHK21 cells were plated in each well of 6-well plates in serum-free opti-MEM media (Gibco) and allowed to sit for 2 h. Six μ l of Lipofectamine 3000, 100 μ l opti-MEM, and 10 μ l of 13.5 ng/ μ l viral RNA were mixed and allowed to sit for 5 min. After incubation, 900 μ l of opti-MEM was then added to the tube containing the mixture and mixed with the seeded BHK-21 cells (Wang et al., 2008). The BHK-21 cells were incubated at 37°C for 3 days. At 3 days post-transfection, the medium was collected, aliquoted, and stored as P1 virus stock at -80 °C. The P1 virus stocks were passaged by adding 200 μ l of P1 virus to a T75 flask containing 90% confluent C6/36 cells, cultured in L-15 (Leibovitz) medium (Invitrogen) supplemented with 10% (vol/vol) FBS to obtain P2 viral

stocks. At 5 days post-infection (dpi), the P2 virus was harvested, aliquoted, and stored at -80°C . Viral titers were determined using TCID_{50} assays in BHK-21 cells, as described below. Virus stocks used in this study were thawed only once before use.

2.3.4 TCID_{50} assay

1×10^4 of BHK-21 cells were seeded in each well of 96-well tissue culture plates. The DMEM media was supplemented with $15 \mu\text{g/ml}$ of penicillin/streptomycin (Invitrogen). Frozen mosquito samples were thawed on ice and centrifuged at 4°C for 3 mins at 15,000 rpm to remove debris, while samples obtained from tissue culture cells were thawed on ice without centrifugation. Serial dilutions of each sample were added to 5 duplicate wells of BHK-21 cells. The plates were scored for cytopathic effects after 5 days, and the proportion of infected wells was used to calculate $\text{TCID}_{50}/\text{ml}$ values (O'Reilly et al., 1994). The $\text{TCID}_{50}/\text{ml}$ values were multiplied by 0.69 to obtain the PFU/ml values.

2.3.5 Oral infection with SINV

Newly emerged adult female mosquitoes were allowed to feed on raisins and water ad libitum. At 2 days post-emergence, raisins were removed but not water, and mosquitoes were starved for 1 day. For the mosquito age experiment, mosquitoes ages 3, 5, 7, 14, and 21 days post-emergence were used and they were starved a day before they turned the required age needed for the experiment. The mosquitoes were then separated into smaller containers. P2 virus stocks of 1.1×10^9 PFU/ml were mixed with defibrinated sheep blood in a ratio of 1:1 and fed to the mosquitoes for 1 h using a Hemotek membrane feeder (Hemotek Ltd.) covered with Parafilm. For the viral concentration experiment, the P2 virus stock was diluted with Leibovitz's medium

to a concentration of 1.0×10^7 , 1.0×10^8 , or 1.0×10^9 PFU/ml and used for the experiment. The mosquitoes were briefly cold-shocked at 4°C , and then fully engorged females were separated from the remaining mosquitoes. Fed females were subsequently maintained on raisins and water for 5 days post-blood meal (PBM) before dissecting. For the intrinsic incubation period experiment, mosquitoes were harvested at 5-, 10- and 15-days PBM. At the appropriate time points, mosquitoes were killed by freezing at -20°C , and the killed mosquitoes were placed in 200 μl DMEM plus 10% FBS and homogenized in 1.5-ml microcentrifuge tubes using disposable plastic pestles. The samples were frozen at -80°C until the titers were determined by TCID₅₀ assay.

2.3.6 Intrathoracic injection of SINV

Emerged adult female mosquitoes were fed on raisins and water until they were 3-, 7-, or 10-days old. These female mosquitoes were cold anesthetized and injected with one pulse of 69 nl of MRE-16 P2 virus stock, diluted with DMEM plus 10% FBS so that each mosquito received a dose of 10 PFU, using a Nanoject II injector (Drummond Scientific Company). After recovering, the mosquitoes were maintained on raisins and water for 5 days, after which they were harvested and tittered as described above.

2.3.7 Virus growth curves and mosquito reinfections

Virus that was obtained from mosquitoes that exhibited high titer (2.0×10^8 PFU/ml), medium titer (2.91×10^6 PFU/ml), or low titer (2.14×10^4 PFU/ml), or MRE-16 P2 stock virus were used to infect C6/36 and Aag2 cells at a multiplicity of infection (MOI) of 0.1 in a 6 well plate. Five individual mosquitoes from low titers, medium titers and high titers were pooled

together and used for the growth curve experiment. Two female mosquitoes were homogenized in 900 μ l of media Leibovitz's or Schneider's medium for serial dilution of medium and high titer viruses. After a 1 h absorption period, the cells were washed three times with PBS, and 2 ml of Leibovitz's or Schneider's medium containing 10% FBS was added into each well. At 0, 1, 2, 3, 4, and 5 days post-infection, 200 μ l of cell culture medium containing virus were collected and frozen at -80 °C until ready for TCID₅₀ assay.

2.3.8 Statistical analysis

GraphPad Prism 5 software was used for statistical analysis utilizing Mann Whitney U, Kruskal-Wallis, Fisher's Exact, and Pearson's chi-square tests. Dunn test was used for post-hoc multiple-comparison analysis for Kruskal-Wallis tests that showed significance difference. A p-value of <0.05 was considered significantly different. Since the data were not normally distributed, interquartile range (IQR) was used rather than standard deviation to measure variation within each set of data.

2.4 Results

2.4.1 Greater variation in virus titer is observed after oral infection than intrathoracic infection

The midgut is the first tissue to become infected when a mosquito takes a blood meal from an infected host and is thought to play a major role in determining vector competence due to the presence of both midgut infection and midgut escape barriers which must be overcome by the virus. However, mosquitoes can be artificially infected by injecting virus directly into the hemocoel, thus bypassing these midgut barriers. To determine the effect of midgut infection on

variation in titer, Orlando mosquitoes were either allowed to feed on a blood meal containing 1.1×10^9 PFU/ml or were injected with 10 PFU of passage 2 (P2) virus derived from an infectious clone of the MRE-16 strain of SINV, and then the virus titer in whole mosquitoes was determined by median tissue culture infectious dose (TCID₅₀) assay at 5 days after virus exposure. The results of this experiment revealed a significantly higher median titer in mosquitoes at this time point after oral infection compared to intrathoracic infection (p-value < 0.0001). More interestingly, there was greater variation in the individual titers of orally infected mosquitoes than injected mosquitoes. The median titer of orally infected mosquitoes was 2.0×10^7 PFU/ml with an interquartile range (IQR) of 4.61×10^7 , while the median titer for intrathoracic infection was 1.15×10^6 PFU/ml with an IQR of 1.89×10^6 . The overall titer range for oral infection was around 10^3 - 10^9 PFU/ml (100,000-fold) while that for intrathoracic infection was around 10^5 - 10^6 PFU/ml (100-fold) (Figure 2.1A-B). Similar levels of variation following oral infection were observed in two additional replicates using mosquitoes from different egg batches (Figure 2.8. (Supplementary)). Together these results suggest that infection of the midgut and the resulting bottlenecks associated with midgut infection and midgut escape can have a strong effect on the resulting overall titer of virus in individual mosquitoes, resulting in a high degree of variation in titer even in a highly inbred laboratory strain of mosquitoes.

2.4.2 Age of mosquito affects the median titer but not the variability or prevalence of infection after oral infection

One factor that is known to affect mosquito immune response is the age of the mosquito (Pigeault et al., 2015; Knecht et al., 2018). To examine if the age of mosquitoes has an effect on titer variability, mosquitoes of different ages (3, 5, 7, 14, and 21 days post-eclosion) were orally

infected with MRE-16 at a titer of 1.1×10^9 PFU/ml and then tittered at 5 days post-blood meal (PBM). The results from this experiment showed that mosquito age had a significant effect on median titer after oral infection (Figure 2.2A), although not in a consistent pattern. However, neither the amount of variation in titer nor the prevalence of infection were significantly affected by mosquito age (Figure 2.2B-C). This result shows that, although the age of mosquitoes can affect the median titer, it does not affect the amount of variation in viral titer in individual mosquitoes after oral infection.

We also examined whether the length of the mosquito developmental cycle had any effect on titer variation. We normally used female mosquitoes that emerged as adults at between 2 and 7 days after pupae were collected, but adults continue to emerge after this time. In this experiment, we compared adults that emerged on days 2, 4, 5, 6, or 7 after pupae collection. These mosquitoes were allowed to feed on blood containing MRE-16 at a concentration of 1.1×10^9 PFU/ml, and virus titers were determined at 5 days PBM. Our results indicated that the length of the developmental cycle did not affect median virus titer, infection prevalence, or titer variation (Figure 2.9. (Supplementary)).

2.4.3 Individual titer variation is also independent of mosquito age after intrathoracic infection

To determine whether mosquito age affects titer variation when the virus is delivered intrathoracically, we injected mosquitoes of ages 3, 7, and 10 days post-eclosion with 10 PFU of MRE-16 and measured virus titer at 5 days post-injection. All injected mosquitoes had detectable levels of virus, indicating no effect of age on infection prevalence. The results further indicated there were no significant differences in median titer or the amount of variation between

the different ages of mosquitoes after intrathoracic injection (Figure 2.2D-E). These results further support the conclusion that mosquito age does not affect variation in SINV titer between individual mosquitoes.

2.4.4 Mosquito strain and virus strain affect median titer but not individual variation, while virus strain also influences infection prevalence

Both mosquito strain and virus strain have been reported to affect vector competence (Ciota et al., 2017; Roundy et al., 2017). To determine whether a large amount of variation in virus titer between individual mosquitoes is observed in more than one mosquito line, MRE-16 SINV was used to orally infect mosquitoes from either the Orlando line or another laboratory *Ae. aegypti* line, Rex-D (Figure 2.3A-C). A significant difference in median virus titer was observed between the two mosquito lines. However, there was large variation observed in both lines, and infection prevalence was also similar.

To test whether virus strain can affect variation in titer, the MRE-16 strain was compared to another SINV strain, TE. The TE strain is more tissue culture-adapted than MRE-16 and is thus less efficient at causing oral infection in *Ae. aegypti* (Foy et al. 2004; Pierro et al. 2003). Orlando mosquitoes that had been fed MRE-16 or TE differed significantly in both median virus titer and infection prevalence (Figure 2.3D-F). However, both virus strains caused large variations in titer. These results indicate that large variation in SINV titer following oral infection occurs regardless of mosquito line or virus strain.

2.4.5 Both virus concentration and intrinsic incubation period affect median titer and infection prevalence, but only intrinsic incubation period affects titer variation

Virus concentration in the blood meal and intrinsic incubation period have both been reported to affect the susceptibility of mosquitoes to arbovirus infection (Roundy et al., 2017; Weger-Lucarelli et al., 2016). To assess the contribution of virus concentration and intrinsic incubation period to titer variation, Orlando mosquitoes were allowed to feed on blood meals containing MRE-16 titers of 1.5×10^7 , 1.0×10^8 , and 1.1×10^9 PFU/ml. Virus concentration significantly affected the median virus titer at 5 days PBM, with median titer correlating with increased SINV concentration (Figure 2.4A-B). Infection prevalence was also significantly affected by viral concentration, with prevalence being significantly lower at a concentration of 10^7 PFU/ml than at the higher concentrations (Figure 2.4C). However, there was a large amount of variation in titer regardless of the virus concentration in the blood meal (Figure 2.4A-B).

To examine the effect of intrinsic incubation period, Orlando mosquitoes were infected with MRE-16 at a titer of 1.1×10^9 PFU/ml and incubated for 5, 10, or 15 days. Results from the intrinsic incubation period experiment showed that intrinsic incubation period significantly affected the median viral titer, with the median titer dropping over time (Figure 2.4D-E). Interestingly, there was also less titer variation observed at the longest intrinsic incubation period than at the two shorter periods. Infection prevalence was also significantly reduced at 15 days compared to 5 or 10 days (Figure 2.4F). These results suggest that intrinsic incubation period, but not virus concentration, can affect the amount of variation in individual mosquito virus titer, with reduced titer variation observed after 15 days PBM.

2.4.6 No correlation between virus titer and either the weight of mosquito or the amount of blood ingested

Since mosquito size has been shown to affect arbovirus titer (Nasci and Mitchell, 1994), we considered the possibility that size variation between individual mosquitoes in a single experiment could be an underlying cause of variation in titer between individual mosquitoes. To test this, we measured mosquito size by weighing Orlando mosquitoes immediately after they had fed on blood containing 1.1×10^9 PFU/ml of MRE-16 and then weighing the same mosquitoes again 3 days later, after the blood meal was digested. In a separate experiment, comparing mosquito weight before blood feeding and at 3 days PBM indicated there was no significant weight gained or lost during this time period (Table 2.1 (Supplementary)), and thus the weight after 3 days was taken as the mosquito weight at the time of feeding. Results from this experiment showed that mosquito weight did not correlate with the virus titer (p -value = 0.961) (Figure 2.5A).

Another possible source of individual variation could be the amount of blood ingested (Nasci and Mitchell, 1994). In all experiments, we routinely select only mosquitoes that appear to have taken a full blood meal, as assessed by the amount of blood visible in the abdomen. However, to determine whether the amount of blood ingested contributes to variation in titer, the amount of blood ingested by the mosquitoes in Figure 2.5A was determined by the difference in weight immediately after blood feeding versus 3 days later. The amount of ingested blood ranged from 0.3 mg to 2.2 mg. However, there was not a significant correlation between the amount of blood ingested and the virus titer (p -value = 0.922) (Figure 2.5B). Together, these results indicate that the variation in virus titer that we routinely observe after oral infection is not due to either variation in mosquito size or the amount of ingested blood.

2.4.7 Virus from high titer mosquitoes replicates to a higher titer in cell culture than virus from low titer mosquitoes

Although the virus stocks used in this study were obtained from infectious cDNA clones and passaged only twice before use, any time a virus sample is prepared it will contain a swarm of viruses that differ from each other in their genome sequence. In addition, severe genetic bottlenecks occur during both midgut infection and midgut escape, raising the possibility that variation in titer could be due to founder effects caused by mosquitoes becoming infected with different sequence variants (Weaver et al., 2021). To begin to address this possibility, we collected virus from individual low titer (2.1×10^4), medium titer (2.9×10^6), and high titer (2.0×10^8) mosquitoes in order to determine if these virus populations differed in replication kinetics. We first used these virus populations to infect C6/36 and Aag2 cells, with a P2 stock of MRE-16 prepared by our normal procedure serving as a control. Results from the growth curves showed that viruses isolated from high titer mosquitoes replicated to a higher titer in tissue culture than the other viruses, while viruses collected from low titer mosquitoes replicated to a lower titer when compared to virus from medium titer mosquitoes or stock MRE-16 virus (Figure 2.6A & B).

Since the high titer virus stocks were diluted more than the medium and low titer stocks to reach the desired MOI for the growth curve experiment, it was possible that the results were influenced by dilution effect (ie, a potential inhibitor present in the mosquito homogenate). To rule this out, we repeated the growth curve by diluting the virus stocks in mosquito homogenate rather than DMEM. The results were not significantly affected by this change in procedure (Figure 2.10 (Supplementary)).

2.4.8 The virus obtained from high titer mosquitoes reinfects mosquitoes less efficiently than normally prepared virus stocks

To further examine possible differences in replication, viruses isolated from high titer mosquitoes were used to reinfect mosquitoes either after being passaged once in BHK-21 cells or without passage. If the high virus titer was due to founder effect in the first experiment, we would expect to see a skewing of virus titers towards higher values when the virus from high titer mosquitoes was used in a subsequent oral infection. The viral concentration for both high titer mosquitoes used for the re-infection experiment without passage in BHK-21 cells was 1.0×10^9 PFU/ml. The same concentration of normal stock virus was used to orally infect mosquitoes. The results from this experiment showed that non-passaged virus from high titer mosquitoes replicated to significantly lower median titer than stock virus (Figure 2.7A-B). The prevalence of infection was also significantly lower for the non-passaged high titer mosquitoes than for stock virus (99% versus 49%) (Figure 2.7C).

It has been previously observed that arboviruses obtained from mosquitoes are less infectious in mosquitoes than virus obtained from mammalian cells, possibly due to different glycosylation patterns for the envelope proteins (Bryant et al., 2007). We thus passaged virus from high titer mosquitoes one time in BHK-21 cells before feeding it to mosquitoes in a blood meal. The titer of the passaged virus was 2.0×10^6 PFU/ml, and so an equivalent concentration of stock virus was used as a control to orally infect mosquitoes and titer them at 5 days PBM. The results from this experiment showed that the virus obtained from high titer mosquitoes and passaged in BHK-21 cells resulted in a significantly higher median titer and infection prevalence than the stock MRE-16 virus when used at this relatively low concentration (Figure 2.7D-F), while mosquitoes infected with both virus stocks exhibited high variation in virus titer. This

result further supports the hypothesis that founder effects may play a role in causing the high degree of variation in virus titer that is observed when mosquitoes are orally infected.

2.5 Discussion

To begin to explore what biological factors contribute to variation in arbovirus titer between individual mosquitoes after oral infection, we used SINV as a model organism to study titer variation in a laboratory strain of *Ae. aegypti*. In doing so we learned several important things about the causes of individual variation. First, the route of infection contributes to variation in virus titer. We observed much greater variation in virus titers after oral infection than after intrathoracic infection, which is consistent with previous reports (Carpenter et al., 2021; O'Neill et al., 2015). This result suggests that a major source of individual variation involves midgut infection and/or midgut escape (Franz et al., 2015). In addition, the ability of SINV to successfully infect and escape the *Ae. aegypti* midgut is modulated by the midgut innate immune response (Lee et al., 2019). The differential expression of the innate immune genes such as those involved in RNA interference (RNAi) (Wang et al., 2006), Toll pathway, Jak/Stat pathway, and immune deficiency (IMD) pathway (Merkling and van Rij, 2013) can influence whether *Ae. aegypti* will be susceptible or refractory to viral infection (Barón et al., 2010). The gut microbiome also modulates midgut immune response by modulating the expression of antimicrobial peptides which can influence the degree to which arboviruses successfully infect and escape the midgut (Hegde et al., 2015; Johnson, 2015).

We also tested several other factors for their involvement in causing individual variation. Importantly, we showed that virus titer in *Ae. aegypti* did not correlate with either size of the mosquito or the amount of blood ingested. Reports from (Nasci and Mitchell, 1994) show that

although large *Ae. aegypti* mosquitoes were significantly more susceptible to Ross River virus than small mosquitoes when fed with viremias of 4.8, 5.7, 6.4, and 7.5 log₁₀ PFU/ml, the difference in susceptibility was less apparent at higher viremias. Since high viremia was used in this study, it may partially explain why there was no correlation between size of mosquito and viral titer. This result suggests that some other intrinsic factor is responsible for individual variation in virus titer, such as the strength of the midgut immune response or perhaps the number of midgut cells that are initially infected (Merkling and van Rij, 2013; Wang et al., 2006).

We considered the possibility whether the degree of individual variation that is observed is affected by other factors. Our results indicate that the amount of individual variation is not affected by mosquito age or by the concentration of virus in the blood meal. High levels of individual titer variation were observed when two different strains of SINV, MRE-16 and TE, were used to infect *Ae. aegypti*, and when MRE-16 was used to infect two different *Ae. aegypti* lines, Orlando and Rex-D. A sharp contrast between the median titer and prevalence of infection was observed for MRE-16 versus TE, which was expected since TE is better adapted to replicate in culture (Wang et al., 2008) than to infect mosquitoes (Foy et al., 2004; Pierro et al., 2003), but both viruses resulted in high titer variability. The intrinsic incubation period (the amount of time elapsed after exposure to virus) did affect the amount of individual variation in virus titer, with a longer intrinsic incubation resulting in decreased individual variation as well as decreased median titer and infection prevalence. This result suggests that the mosquito immune system, if given enough time, may be able to modulate the level of virus replication and bring down the levels of virus to a manageable level (Goic et al., 2016).

To begin to determine whether there were any differences between the virus populations that accumulated during infection of high, medium, and low titer mosquitoes, we examined the replication dynamics of these virus populations in mosquito cell lines. Results from this experiment showed that high titer mosquitoes replicated faster and to higher titer than our standard P2 virus stock, while low titer mosquitoes replicated slower and to lower titer than standard P2 virus. Medium titer replicated comparably with P2 virus. These results show that virus obtained from high titer mosquitoes is better able to replicate *in vitro* when compared to virus from low titer mosquitoes, suggesting that there may be genetic differences between these virus populations.

We also sought to examine whether the high degree of variation in titer values that were observed was because of changes in the viral genome. To test this hypothesis, refeeding experiments were performed using virus populations obtained from high titer mosquitoes that were either amplified in BHK-21 cells or not. Attempts to perform a refeeding experiment using low titer virus proved futile because we were not able to reinfect mosquitoes or rescue the virus from low titer mosquitoes in culture, presumably due to its low titer. However, results from feeding non-passaged high titer virus to mosquitoes resulted in similar titer variability compared to P2 stock virus but had lower median titer and lower prevalence of infection. Additionally, results from the refeeding experiment using high titer virus that was passaged once in BHK-21 cells showed similar variability compared to P2 virus but resulted in higher median titer and higher prevalence of infection. The lower median titer and lower prevalence of infection of P2 virus compared to other experiments in this study was likely due to the low concentration used in this experiment. It was interesting, however, that the virus derived from high titer mosquitoes that had been passaged once was better able to infect mosquitoes and resulted in a higher median

titer than P2 stock MRE-16. This may be attributable to genetic changes in the genome of the virus derived from high titer mosquitoes, which calls for future investigation to ascertain what these genetic changes are. Results from these experiments also showed that virus that came from high titer mosquitoes still caused highly variable virus titers when fed to additional mosquitoes, suggesting that there still is genetic variation in this virus population, and genetic bottlenecks could still result in founder effects (Clarke et al., 1993). The ability of virus derived from high titer mosquito to reinfect mosquitoes better after they are passaged in BHK-21 cells is consistent with previous reports that arboviruses need to go through the arthropod-mammalian-arthropod transmission cycle for effective infection (Coffey et al., 2008; Moutailler et al., 2011). This result is also consistent with literature indicating that arboviruses alternate between arthropod and vertebrate hosts to maintain their genetic stability to be able to replicate effectively in nature (Moutailler et al., 2011).

In summary, results from this study showed that a high degree of individual variation is observed when even a highly inbred laboratory strain of *Ae. aegypti* is orally infected with SINV. Of the several biological factors tested, only intrinsic incubation period affected this variation in SINV titer. The reduced titer variation that was observed due to longer intrinsic incubation period may be due to adaptation of SINV in *Ae. aegypti* (Hardy et al., 1983), or to action of the mosquito immune system in limiting virus replication. Variation in individual virus titer was significantly affected by the route of infection, leading us to conclude that the midgut appears to be an important tissue contributing to the variation in titer. Our findings also suggest that founder effects, due to the action of bottlenecks on the collection of virus variants present in a SINV population, may contribute to the variability in titer between individually infected mosquitoes. Deep sequencing of virus derived from high and low titer mosquitoes may help resolve this

question. However, consistent genetic changes may be difficult to detect between virus populations obtained from high and low titer mosquitoes since mutations continue to occur in the viral genome as the virus replicates after escaping from the midgut. It also remains possible that variation in the strength of the immune response between individual mosquitoes could play a role in the highly variable titer that is observed after oral infection. We are currently carrying out additional experiments to distinguish these possibilities.

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2.7 References

- Achee, N. L., Grieco, J. P., Vatandoost, H., Seixas, G., Pinto, J., Ching-NG, L., Martins, A. J., Juntarajumnong, W., Corbel, V., Gouagna, C., David, J.-P., Logan, J. G., Orsborne, J., Marois, E., Devine, G. J., & Vontas, J. (2019). Alternative strategies for mosquito-borne arbovirus control. *PloS Neglected Tropical Diseases*, 13(1), e0006822.
<https://doi.org/10.1016/j.pt.2006.05.003>.
- Adouchief, S., Smura, T., Sane, J., Vapalahti, O., & Kurkela, S. (2016). Sindbis virus as a human pathogen— epidemiology, clinical picture and pathogenesis. *Reviews in Medical Virology*, 26(4), 221-241. <https://doi.org/10.1002/rmv>
- Barón, O. L., Ursic-Bedoya, R. J., Lowenberger, C. A., & Ocampo, C. B. (2010). Differential gene expression from midguts of refractory and susceptible lines of the mosquito, *Aedes aegypti*, infected with Dengue-2 virus. *Journal of Insect Science*, 10(1), 1–23.
<https://doi.org/10.1673/031.010.4101>
- Bryant, J. E., Calvert, A. E., Mesesan, K., Crabtree, M. B., Volpe, K. E., Silengo, S., Kinney, R. M., Huang, C. Y. H., Miller, B. R., & Roehrig, J. T. (2007). Glycosylation of the dengue 2 virus E protein at N67 is critical for virus growth in vitro but not for growth in intrathoracically inoculated *Aedes aegypti* mosquitoes. *Virology*, 366(2), 415–423.
<https://doi.org/10.1016/j.virol.2007.05.007>
- Carpenter, A., Bryant, W. B., Santos, S. R., & Clem, R. J. (2021). Infection of *Aedes aegypti* mosquitoes with midgut-attenuated Sindbis virus reduces, but does not eliminate, disseminated infection. *Journal of Virology*, 95(13), 10-1128.
<https://doi.org/10.1128/jvi.00136-21>
- Ciota, A. T., Bialosuknia, S. M., Zink, S. D., Brecher, M., Ehrbar, D. J., Morrisette, M. N., &

- Kramer, L. D. (2017). Effects of Zika virus strain and *Aedes* mosquito species on vector competence. *Emerging Infectious Diseases*, 23(7), 1110–1117.
<https://doi.org/10.3201/eid2307.161633>
- Clarke, D. K., Duarte, E. A., Moya, A., Elena, S. F., Domingo, E., & Holland, J. (1993). Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses. *Journal of Virology*, 67(1), 222–228. <https://doi.org/10.1128/jvi.67.1.222-228.1993>
- Coffey, L. L., Vasilakis, N., Brault, A. C., Powers, A. M., Tripet, F. R., & Weaver, S. C. (2008). Arbovirus evolution in vivo is constrained by host alternation. *Proceedings of the National Academy of Sciences of the United States of America*, 105(19), 6970–6975.
<https://doi.org/10.1073/pnas.0712130105>
- Dusfour, I., Vontas, J., David, J. P., Weetman, D., Fonseca, D. M., Corbel, V., Raghavendra, K., Coulibaly, M. B., Martins, A. J., Kasai, S., & Chandre, F. (2019). Management of insecticide resistance in the major *Aedes* vectors of arboviruses: Advances and challenges. *PloS Neglected Tropical Diseases*, 13(10), 1–22.
<https://doi.org/10.1371/journal.pntd.0007615>
- Foy, B. D., Myles, K. M., Pierro, D. J., Sanchez-Vargas, I., Uhlirová, M., Jindra, M., Beaty, B. J., & Olson, K. E. (2004). Development of a new Sindbis virus transducing system and its characterization in three Culicine mosquitoes and two Lepidopteran species. *Insect Molecular Biology*, 13(1), 89–100. <https://doi.org/10.1111/j.1365-2583.2004.00464.x>
- Franz, A. W. E., Kantor, A. M., Passarelli, A. L., & Clem, R. J. (2015). Tissue barriers to arbovirus infection in mosquitoes. *Viruses*, 7(7), 3741–3767.
<https://doi.org/10.3390/v7072795>
- Goic, B., Stapleford, K. A., Frangeul, L., Doucet, A. J., Gausson, V., Blanc, H., Schemmel-Jofre,

- N., Cristofari, G., Lambrechts, L., Vignuzzi, M., & Saleh, M. C. (2016). Virus-derived DNA drives mosquito vector tolerance to arboviral infection. *Nature Communications*, 7, 1–10. <https://doi.org/10.1038/ncomms12410>
- Hardy, J. L., Houk, E. J., Kramer, L. D., & Reeves, W. C. (1983). Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annual Review of Entomology. Volume 28*, 229–262. <https://doi.org/10.1146/annurev.en.28.010183.001305>
- Hegde, S., Rasgon, J. L., & Hughes, G. L. (2015). The microbiome modulates arbovirus transmission in mosquitoes. *Current Opinion in Virology*, 15, 97–102. <https://doi.org/10.1016/j.coviro.2015.08.011>
- Hemingway, J., Beaty, B. J., Rowland, M., Scott, T. W., & Sharp, B. L. (2006). The Innovative Vector Control Consortium: improved control of mosquito-borne diseases. *Trends in Parasitology*, 22(7), 308–312. <https://doi.org/10.1016/j.pt.2006.05.003>
- Wang, H., Blair, C. D., Olson, K. E., & Clem, R. J. (2008). Effects of inducing or inhibiting apoptosis on Sindbis virus replication in mosquito cells. *The Journal of general virology*, 89(Pt 11), 2651. <https://doi.org/10.1099/vir.0.2008/005314-0>.
- Huang, Y. J. S., Higgs, S., & Vanlandingham, D. L. (2017). Biological control strategies for mosquito vectors of arboviruses. *Insects*, 8(1). <https://doi.org/10.3390/insects8010021>
- Huang, Y. J. S., Higgs, S., & Vanlandingham, D. L. (2019). Arbovirus-mosquito vector-host interactions and the impact on transmission and disease pathogenesis of arboviruses. *Frontiers in Microbiology*, 10(JAN), 1–14. <https://doi.org/10.3389/fmicb.2019.00022>
- Johnson, K. N. (2015). The impact of Wolbachia on virus infection in mosquitoes. *Viruses*, 7(11), 5705–5717. <https://doi.org/10.3390/v7112903>
- Knecht, H., Richards, S. L., Balanay, J. A. G., & White, A. V. (2018). Impact of mosquito age

- and insecticide exposure on susceptibility of *Aedes albopictus* (Diptera: Culicidae) to infection with Zika virus. *Pathogens*, 7(3), 1–9. <https://doi.org/10.3390/pathogens7030067>
- Kuno, G. (2010). Early history of laboratory breeding of *Aedes aegypti* (Diptera: Culicidae) focusing on the origins and use of selected strains. *Journal of Medical Entomology*, 47(6), 957–971. <https://doi.org/10.1603/ME10152>
- Lee, W. S., Webster, J. A., Madzokere, E. T., Stephenson, E. B., & Herrero, L. J. (2019). Mosquito antiviral defense mechanisms: A delicate balance between innate immunity and persistent viral infection. *Parasites and Vectors*, 12(1), 1–12. <https://doi.org/10.1186/s13071-019-3433-8>
- Londono-Renteria, B., Troupin, A., & Colpitts, T. M. (2016). Arbovirosis and potential transmission blocking vaccines. *Parasites and Vectors*, 9(1), 1–11. <https://doi.org/10.1186/s13071-016-1802-0>.
- Lundström, J. O., Vene, S., Espmark, Engvall, M., & Niklasson, B. (1991). Geographical and temporal distribution of Ockelbo disease in Sweden. *Epidemiology and Infection*, 106(3), 567–574. <https://doi.org/10.1017/S0950268800067637>
- Lwande, O. W., Obanda, V., Bucht, G., Mosomtai, G., Otieno, V., Ahlm, C., & Evander, M. (2015). Global emergence of alphaviruses known to cause arthralgia in humans. *Infection Ecology & Epidemiology*, 5. <https://doi.org/10.3402/iee.v5.29853>.
- Merkling, S. H., & van Rij, R. P. (2013). Beyond RNAi: Antiviral defense strategies in *Drosophila* and mosquito. *Journal of Insect Physiology*, 59(2), 159–170. <https://doi.org/10.1016/j.jinsphys.2012.07.004>
- Moutailler, S., Roche, B., Thiberge, J. M., Caro, V., Rougeon, F., & Failloux, A. B. (2011). Host alternation is necessary to maintain the genome stability of Rift Valley fever virus. *PloS*

- Neglected Tropical Diseases*, 5(5). <https://doi.org/10.1371/journal.pntd.0001156>
- Nasci, R. S., & Mitchell, C. J. (1994). Larval diet, adult size, and susceptibility of *Aedes aegypti* (Diptera, Culicidae) to infection with Ross River virus. *Journal of Medical Entomology*, 31(1), 123–126. <https://doi.org/10.1093/jmedent/31.1.123>
- O'Neill, K. O., Huang, N., Unis, D., & Clem, R. J. (2015). Rapid selection against arbovirus-induced apoptosis during infection of a mosquito vector. *Proceedings of the National Academy of Sciences of the United States of America*, 112(10), E1152–E1161. <https://doi.org/10.1073/pnas.1424469112>
- O'Reilly, D. R., Miller, L. K., & Luckow, V. A. (1994). *Baculovirus expression vectors: a laboratory manual*. Oxford University Press. [https://doi.org/10.1016/0092-8674\(93\)90288-2](https://doi.org/10.1016/0092-8674(93)90288-2)
- Pierro, D. J., Myles, K. M., Foy, B. D., Beaty, B. J., & Olson, K. E. (2003). Development of an orally infectious Sindbis virus transducing system that efficiently disseminates and expresses green fluorescent protein in *Aedes aegypti*. *Insect Molecular Biology*, 12(2), 107–116. <https://doi.org/10.1046/j.1365-2583.2003.00392.x>
- Pigeault, R., Nicot, A., Gandon, S., & Rivero, A. (2015). Mosquito age and avian malaria infection. *Malaria Journal*, 14(1), 1–11. <https://doi.org/10.1186/s12936-015-0912-z>
- Raquin, V., Merklings, S. H., Gausson, V., Moltini-Conclois, I., Frangeul, L., Varet, H., Dillies, M. A., Saleh, M. C., & Lambrechts, L. (2017). Individual co-variation between viral RNA load and gene expression reveals novel host factors during early dengue virus infection of the *Aedes aegypti* midgut. *PloS Neglected Tropical Diseases*, 11(12), 1–21. <https://doi.org/10.1371/journal.pntd.0006152>
- Robert, M. A., Christofferson, R. C., Silva, N. J. B., Vasquez, C., Mores, C. N., & Wearing, H. J.

- (2016). Modeling mosquito-borne disease spread in U.S. urbanized areas: The case of dengue in Miami. *PloS ONE*, 11(8), 1–28. <https://doi.org/10.1371/journal.pone.0161365>
- Roundy, C. M., Azar, S. R., Rossi, S. L., Huang, J. H., Leal, G., Yun, R., Fernandez-salas, I., Vitek, C. J., Paploski, I. a D., Kitron, U., Ribeiro, G. S., Hanley, K. a, Weaver, S. C., & Vasilakis, N. (2017). Mosquito competence for Zika virus transmission. *Emerging Infectious Diseases*, 23(4), 625–632. <https://doi.org/10.3201/eid2304.161484>
- Wang, X. H., Aliyari, R., Li, W. X., Li, H. W., Kim, K., Carthew, R., Atkinson, P., & Ding, S. W. (2006). RNA interference directs innate immunity against viruses in adult *Drosophila*. *Science*, 312(5772), 452–454. <https://doi.org/10.1126/science.1125694>
- Weaver, S. C. (2013). Urbanization and geographic expansion of zoonotic arboviral diseases: Mechanisms and potential strategies for prevention. *Trends in Microbiology*, 21(8), 360–363. <https://doi.org/10.1016/j.tim.2013.03.003>
- Weaver, S. C., Forrester, N. L., Liu, J., & Vasilakis, N. (2021). Population bottlenecks and founder effects: implications for mosquito-borne arboviral emergence. *Nature Reviews Microbiology*, 19(3), 184–195. <https://doi.org/10.1038/s41579-020-00482-8>
- Weger-Lucarelli, J., Rückert, C., Chotiwan, N., Nguyen, C., Garcia Luna, S. M., Fauver, J. R., Foy, B. D., Perera, R., Black, W. C., Kading, R. C., & Ebel, G. D. (2016). Vector competence of American mosquitoes for three strains of Zika virus. *PloS Neglected Tropical Diseases*, 10(10), 1–16. <https://doi.org/10.1371/journal.pntd.0005101>
- World Health Organization. (2017). *Global Vector Control Response 2017-2030*.

2.8 Chapter 2 figures and tables

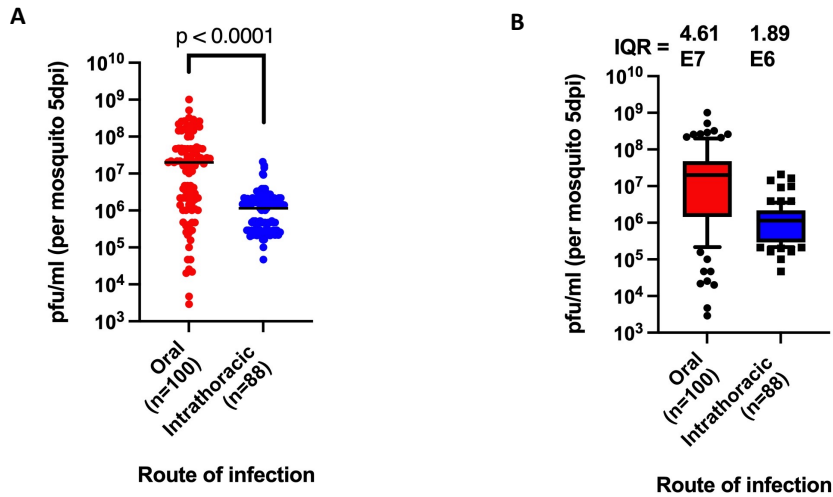


Figure 2.1. Oral and intrathoracic infection of *Ae. aegypti* mosquitoes.

(A) Titers of mosquitoes determined using TCID₅₀/mL assay after oral or intrathoracic infection at five days post virus exposure. (B) The same dataset as in (A) but displayed using box and whisker plots showing interquartile range (IQR) as a measure of variation. The sample size used of the oral the oral infection is 100, while the sample size for intrathoracic infection is 88. Mann Whitney test was used to analysis results for figure 2.1A with the p value showed top of the figure.

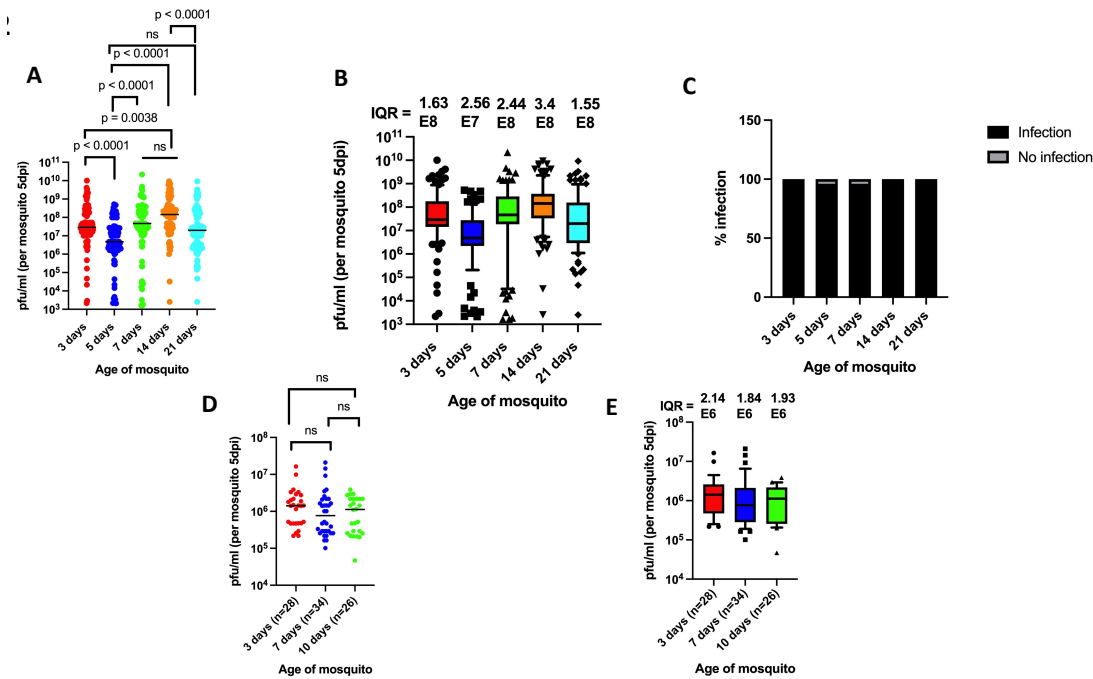


Figure 2.2. Age specific Oral and intrathoracic infection of *Ae. aegypti* adult mosquitoes (days post-emergence).

(A) Titers of mosquitoes of different ages after oral infection. (B) The use of IQR as a measure of variation in mosquitoes after oral infection. The dataset used in panel B is the same as in A. (C) Prevalence of infection of mosquitoes of different ages after oral infection. Sample size was 100. (D) Titers of mosquitoes of different ages after intrathoracic infection. (E) The use of IQR as a measure of variation in mosquitoes after intrathoracic infection. The dataset used in panel D is same as in E. Kruskal-Wallis test was used for statistical analysis in figure 2.2A and 2.2D while Dunn test was used for multiple comparison. Chi-square test was used for analysis in figure 2.2C.

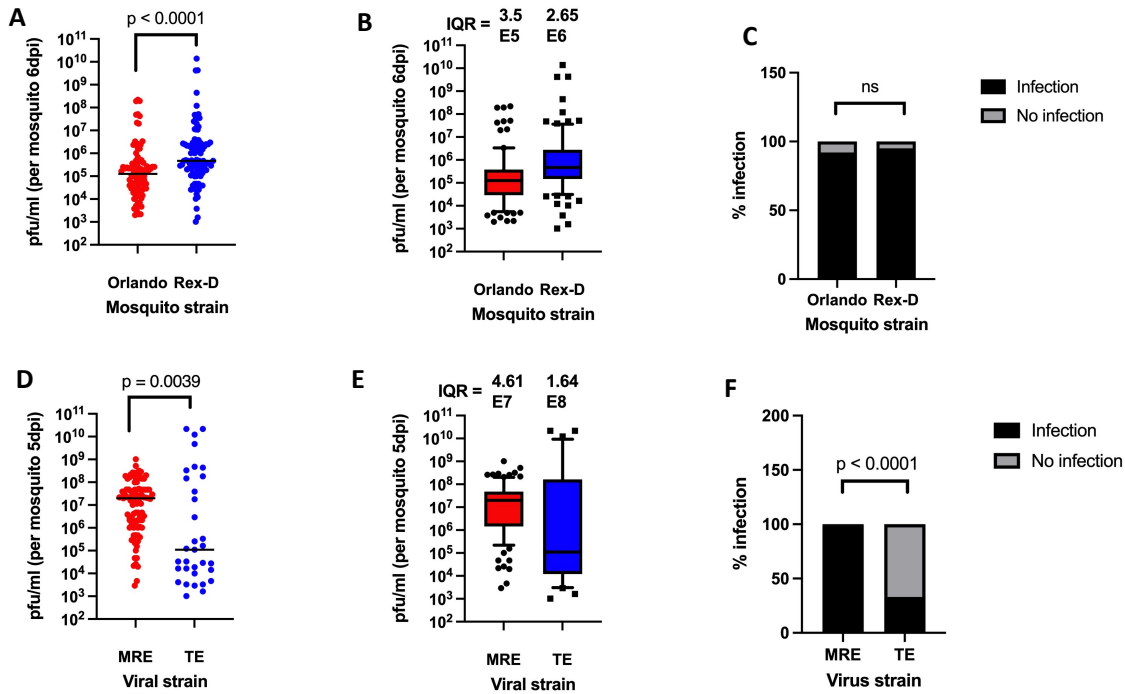


Figure 2.3: Mosquito vs. virus specific differences in Oral infection of *Ae. aegypti* mosquitoes

(A) Titers of Orlando and Rex-D mosquitoes that were orally infected with MRE-16. (B) The use of IQR as a measure of variation in mosquitoes of different strains. The dataset used in panel B is the same as in A. (C) Prevalence of infection of different mosquito strains after oral infection. (D) Titers of mosquitoes that were infected with MRE-16 or TE after oral infection. (E) The use of IQR as a measure of variation in mosquitoes using different viral strains after oral infection. The dataset used in panel E is the same as D. (F) Prevalence of infection of mosquito after oral infection with different viral strains. Sample size was 100 for MRE and 33 for TE. Statistical analysis was performed as described for Figure 2.1. Fisher exact test was used for statistical analysis in figure 2.3C and 2.3F.

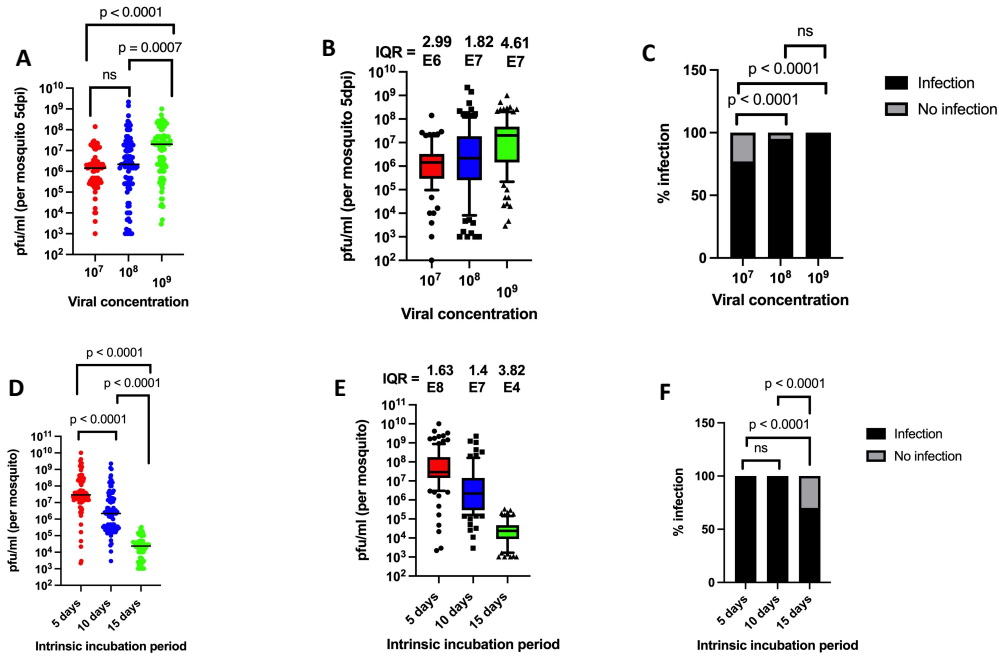


Figure 2.4: Oral infection of *Ae. aegypti* using different virus concentrations or intrinsic incubation periods.

(A) Titers of mosquitoes that were allowed to feed on blood containing different virus concentrations. (B) The use of IQR as a measure of variation in mosquitoes after oral infection with different virus concentrations. The dataset used in panel B is the same as in A. (C) Prevalence of infection of different mosquito strains after oral infection with different virus concentrations. Sample size was 100. (D) Titers of orally infected mosquitoes after different intrinsic incubation periods. (E) The use of IQR as a measure of variation in mosquitoes with different intrinsic incubation periods. The dataset used in panel E is the same as in D. (F) Prevalence of infection of mosquitoes with different intrinsic incubation periods. Sample size used was 100. Statistical analysis performed as described for figure 2.2.

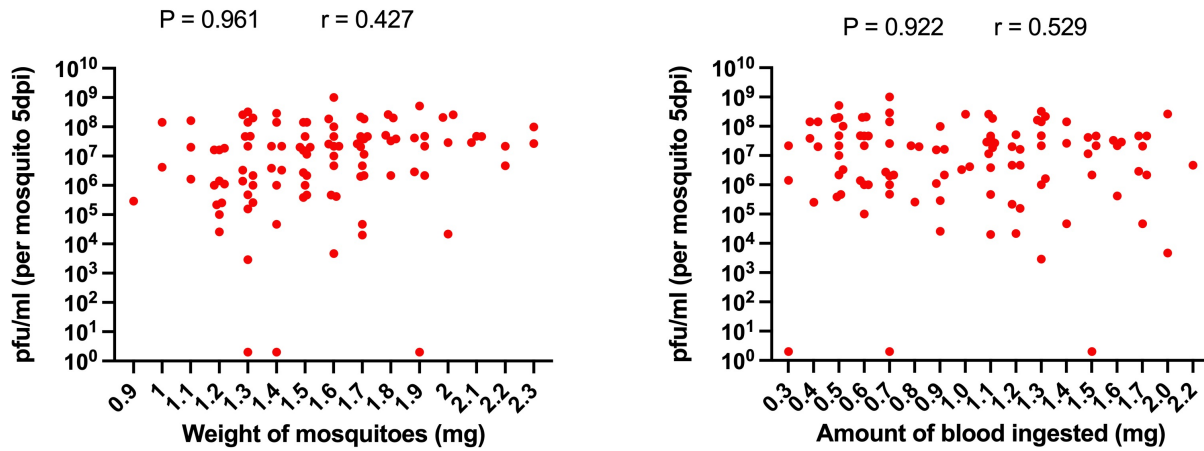


Figure 2.5: Correlation analysis to determine the correlation between amount of blood fed or mosquito weight and virus titer at 5 d PBM.

(A) Correlation analysis of amount of blood fed and virus titer (B) Correlation analysis of mosquito weight and virus titer. Sample size used was 100. Pearson's correlation chi square test was used for the statistical analysis.

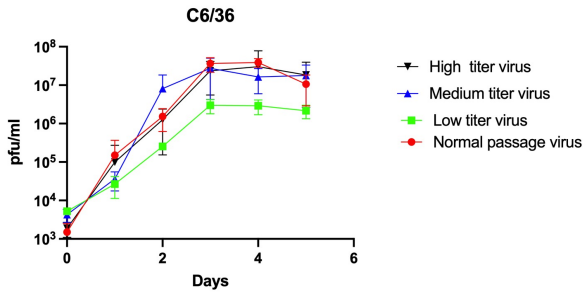
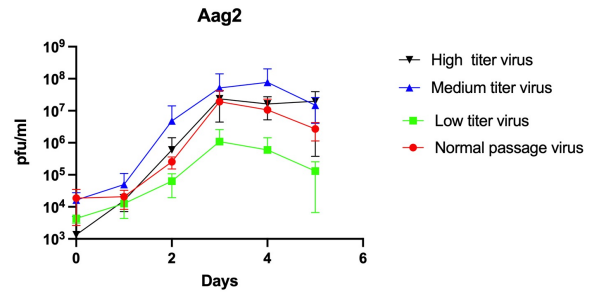
A**B**

Figure 2.6: Growth curves of viruses obtained from high, medium, and low titer mosquitoes.

Viruses from high, medium, and low titer mosquitoes normally passaged MRE-16 stock virus in (A) C6/36 cells and (B) Aag2 cells as determined by TCID₅₀ assay. Viruses from more than one mosquito was used in this experiment. The experiment was performed in two or three biological and technical replicates.

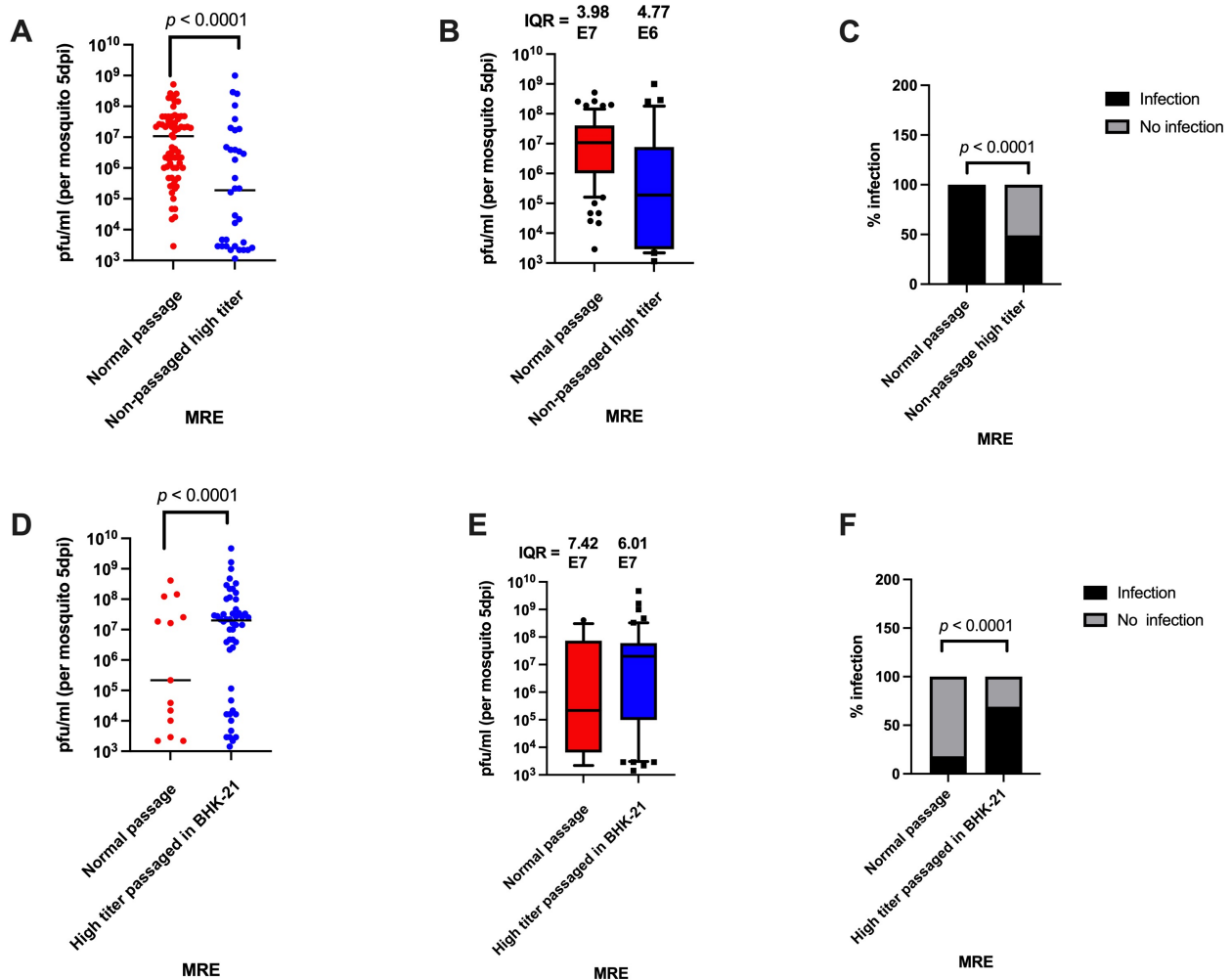


Figure 2.7: Refeeding experiment using viruses obtained from a single high titer mosquito, either passed once in BHK-21 cells or not passed.

This experiment was repeated two to three times (A) Titers of mosquitoes after oral infection with virus (2.0×10^9 PFU/ml) from high titer mosquitoes compared to stock MRE-16 virus. (B) The use of IQR as a measure of variation in mosquitoes after oral infection with virus from high titer mosquitoes or stock MRE-16 virus. The data used in panel B is the same as in A. (C) Prevalence of infection after oral infection with virus from high titer mosquitoes versus stock MRE-16 virus. (D) Titers of mosquitoes that were orally infected using virus from high titer mosquitoes passed once in BHK-21 cells versus MRE-16 stock virus (1×10^6 PFU/ml). (E) The

use of IQR as a measure of variation in mosquitoes after oral infection with virus from high titer mosquito passaged in BHK-21 cells. The data used in panel E is the same as in D. (F) Prevalence of oral infection after exposure to virus from high titer mosquitoes passaged once in BHK-21 cells versus stock MRE-16 virus. Sample size used was 72. Statistical analysis was performed as described for figure 2.3.

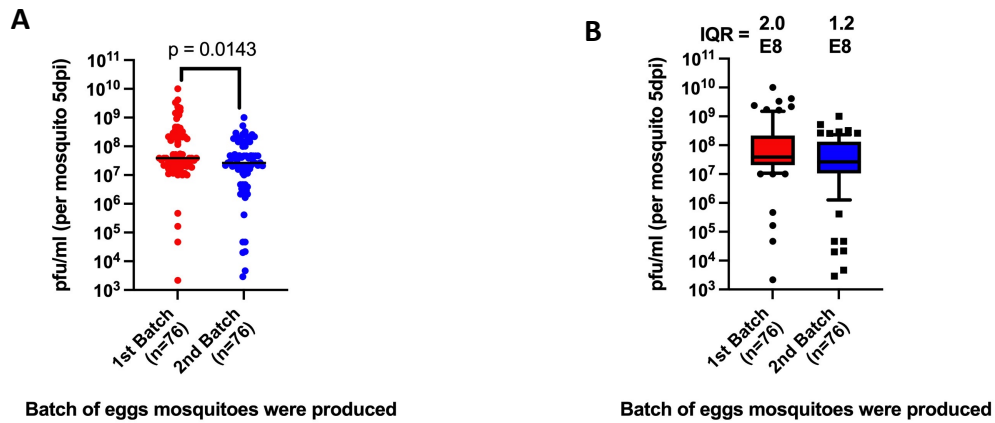


Figure 2.8 (Supplementary): Oral infection of different batches of mosquitoes.

(A) Titers of different batch after oral infection (B) The use of IQR as a measure of variation in different batch of mosquitoes after oral infection. Sample size used was 72. Statistical analysis figure 2.8 (supplementary) is as described in figure 2.1. The dataset used in B is the same as A.

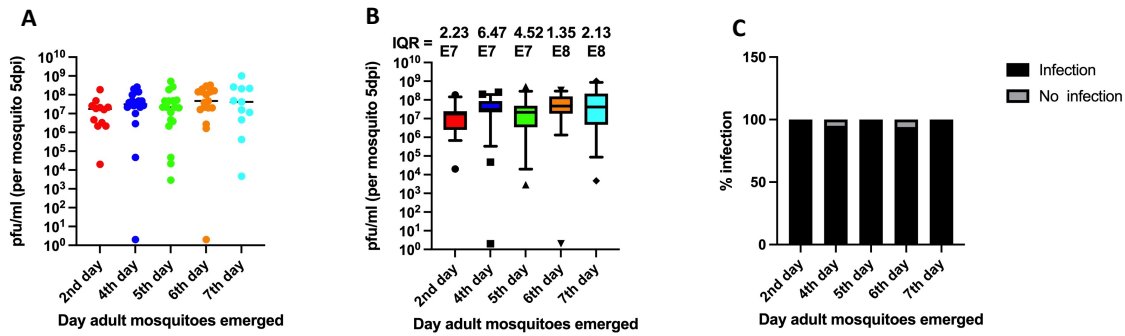


Figure 2.9 (Supplementary): Oral infection of day adult mosquito emergence.

(A) Titers of orally infected mosquitoes with different intrinsic incubation period (B) The use of IQR as a measure of variation in mosquitoes with different intrinsic incubation period. The dataset used in 6C is the same as 6D. (C) Prevalence of infection of adult mosquito emerging on different day. The dataset used in figure 2.9B (supplementary) is the same as figure 2.9A (supplementary). Statistical analysis used in figure 2.9 (supplementary) is as described in figure 2.2.

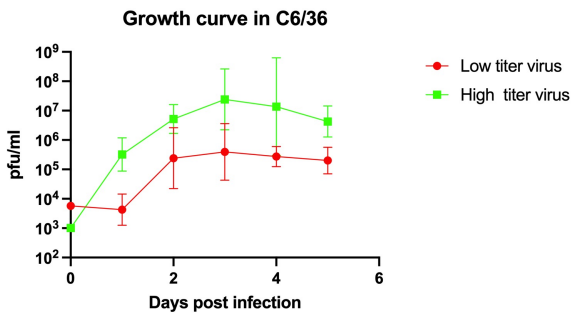
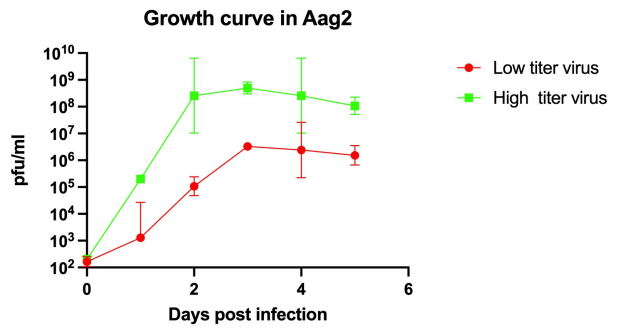
A**B**

Figure 2.10 (Supplementary): Growth curves of viruses obtained from low and high titer mosquitoes with viruses diluted in mosquito homogenate rather than DMEM.

In (A) C6/36 cells and (B) Aag2 cells as determined by TCID₅₀ assay. The bars indicate the means $\pm$ standard error of two independent experiments.

Table 2.1 (Supplementary). Weight of mosquitoes before blood meal and three days post blood digestion.

Mosquito ID #	Weight before blood fed (mg)	Weight 3 d PBM (mg)
U1	1.5	1.7
U2	1.8	2.1
U3	1.5	1.5
U4	1.6	1.8
U5	1.7	1.8
U6	1.5	1.6
U7	1.3	1.6
U8	1.9	2.0
U9	2.1	2.3
U10	1.3	1.4
U11	1.5	1.7
U12	1.5	1.7
U13	1.4	1.5

Chapter 3 - Extreme infectious titer variability in individual *Aedes aegypti* mosquitoes infected with Sindbis virus is associated with both differences in virus population structure and dramatic disparities in specific infectivity

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Sequence quality control and trimming and extraction and alignment of viral sequences were performed by GDE; Western blotting was performed by SM; all other experiments were performed by PH; administration of project was performed by RJC.

3.1 Abstract

Variability in how individuals respond to pathogens is a hallmark of infectious disease, yet the basis for individual variation in host response is often poorly understood. The titer of infectious virus among individual mosquitoes infected with arboviruses is frequently observed to vary by several orders of magnitude in a single experiment, even when the mosquitoes are highly inbred. To better understand the basis for this titer variation, we sequenced populations of Sindbis virus (SINV) obtained from individual infected *Aedes aegypti* mosquitoes that, despite being from a highly inbred laboratory colony, differed in their titers of infectious virus by approximately 10,000-fold. We observed genetic differences between these virus populations that indicated the virus present in the midguts of low titer mosquitoes was less fit than that of high titer mosquitoes, possibly due to founder effects that occurred during midgut infection. Furthermore, we found dramatic differences in the specific infectivity or SI (the ratio of infectious units/viral genome equivalents) between these virus populations, with the SI of low titer mosquitoes being up to 10,000-fold lower than that of high titer mosquitoes. Despite having similar amounts of viral genomes, low titer mosquitoes appeared to contain less viral particles, suggesting that viral genomes were packaged into virions less efficiently than in high titer mosquitoes. Finally, antibiotic treatment, which has been shown to suppress mosquito antiviral immunity, caused an increase in SI. Our results indicate that the extreme variation that is observed in SINV infectious titer between individual *Ae. aegypti* mosquitoes is due to both genetic differences between virus populations and to differences in the proportion of genomes that are packaged into infectious particles.

3.2 Introduction

Mosquito-transmitted arboviruses continue to take a substantial negative toll on public health worldwide, making them a significant global public health threat (Girard et al., 2020). A major factor contributing to the public health threat associated with arboviruses is due to their ability to emerge in new geographical locations or re-emerge at previously existing locations. Factors such as changes in mosquito anthropological behaviors, changing climatic conditions and the appearance of arbovirus genetic variants are some of the important contributing factors to arbovirus emergence and re-emergence (Liang et al., 2015).

The low fidelity of viral RNA-dependent RNA polymerases introduces frequent mutations into the genomes of RNA viruses during replication (Lauring and Andino 2010), and alphaviruses such as Sindbis virus (SINV) are no exception (Coffey et al., 2011; Rozen-Gagnon et al., 2014). The mutant swarms that result due to errors in viral replication produce many variants with low fitness, but at the same time these diverse genetic populations are important in making the virus more adaptable and able to replicate more efficiently in both the mosquito vector and the vertebrate host (Lauring and Andino, 2010). It is thought that the requirement for arboviruses to replicate in the diverse environments of the mosquito vector and vertebrate host, coupled with the multiple severe genetic bottlenecks that arboviruses experience in both vector and host, are major factors in maintaining their genetic stability over time (Coffey et al., 2013; Weaver et al., 2021; Weaver and Barrett, 2004).

For arboviruses to successfully establish infection in the mosquito, they need to traverse anatomical barriers including the midgut and salivary glands (Franz et al., 2015). These anatomical barriers, such as those associated with infecting and escaping the midgut and the salivary glands, exert strong genetic bottleneck effects on arboviruses (Fitzmeyer et al., 2023;

Forrester et al., 2012; Lequime et al., 2016; Patterson et al., 2018). Thus, during a single round of infection in a mosquito, individual sequence variants inevitably increase or decrease in frequency in a virus population due to these bottlenecks, which can be severe. For example, using low doses of marked clones of Venezuelan equine encephalitis virus (VEEV), it was shown that infection of *Culex taeniopus* results in two severe bottlenecks, each narrowing the virus population down to less than 10 virus particles that initiate midgut infection and escape the midgut, respectively (Forrester et al., 2012). The severe reduction in genetic diversity caused by these bottlenecks can cause stochastic founder effects, where the resulting population after a bottleneck differs genetically from the original population. However, genetic diversity is rapidly restored during subsequent rounds of virus replication because of high error rates (Patterson et al., 2018).

The titer of infectious virus in a mosquito is potentially of epidemiological importance because there is likely a positive correlation between virus titer in the mosquito and transmission risk. For example, in a study examining infection of *Aedes aegypti* by another alphavirus, chikungunya virus, it was found that for each 10-fold increase in viral titer in the mosquito legs there was a 49% increase in the probability of detecting virus in the saliva (Honório et al., 2020). Interestingly, numerous studies have documented that there is often extensive variation in arbovirus titer between individual mosquitoes after being given an infectious blood meal. This huge titer variation, which is often up to several orders of magnitude, is commonly observed in both field-caught mosquitoes and mosquitoes from highly inbred laboratory colonies (Carpenter et al., 2021; Hodoamedia et al., 2022; Merwaiss et al., 2021; Raquin et al., 2017). While this variation may not be so surprising in field-caught mosquitoes, which are relatively genetically diverse, the potential explanation for this extreme titer variation is less obvious when highly

inbred mosquitoes are infected. Despite the importance of mosquito titer in vector competence, until recently, there has been little attention paid to individual titer variation in arbovirus-infected mosquitoes (Hodoamede et al., 2022). One study published in 2017 examined transcriptomes in individual *Ae. aegypti* during infection with dengue virus (DENV) and identified several candidate genes whose expression levels correlated with virus RNA load, some of which affected DENV RNA levels when their expression was knocked down (Raquin et al., 2017). However, because the study was done using mosquitoes that had been recently field-caught, it was presumed by the authors that genetic differences between individuals was the primary cause of the variation in DENV RNA load that was observed (Raquin et al., 2017).

In a previous study, we began to investigate the causes of the extreme titer variation that exists between individual mosquitoes following SINV infection of highly inbred laboratory strains of *Ae. aegypti* (Hodoamede et al., 2022). Our work focused mainly on the Orlando strain, which has been in continuous culture since around 1940, with limited outcrossing up until 1992 (Kuno, 2010). Our Orlando colony has not been outcrossed since at least 2003. Despite this high degree of inbreeding, we routinely observe titer differences of several logs in a single experiment when using Orlando mosquitoes. This large amount of titer variation was not affected by individual mosquito size, the amount of blood ingested, the concentration of infectious virus fed to the mosquitoes, or the age of the mosquitoes when they were infected (Hodoamede et al., 2022). The only two factors that were identified as affecting titer variability were the route of infection, with less variation being observed following intrathoracic versus oral infection, and the incubation period after infection, with overall virus titer decreasing and becoming less variable over time (Hodoamede et al., 2022). The observation that virus titers are less variable when the midgut is bypassed by intrathoracic injection suggests that the midgut infection and escape

barriers and their associated genetic bottlenecks may play a role in causing individual variation. Interestingly, in the same study, we showed that SINV obtained from high titer mosquitoes replicated to higher titers in cultured cells compared to virus from low titer mosquitoes. This result further suggested that there may be genetic differences between the virus populations in low and high titer mosquitoes contributing to these phenotypic differences.

In this study, we sequenced SINV populations from individual high and low titer *Ae. aegypti* mosquitoes that had been infected as a single cohort, in order to investigate whether viral genetic factors contribute to virus titer variation after oral infection. Significant differences were observed between the virus populations obtained from low titer mosquitoes versus those from high titer mosquitoes, including stronger reductions in genetic complexity and diversity during dissemination from the midgut and evidence of stronger negative selection occurring in low titer mosquitoes. In addition, we also found that the specific infectivity (SI, or the ratio of infectious virus to viral genome equivalents) of these virus populations was up to 10,000-fold higher in high titer mosquitoes than in low titer mosquitoes, indicating that titer variability in this arbovirus-vector combination is primarily due to dramatic differences in SI.

3.3 Materials and methods

3.3.1 Cell lines

BHK-21 cells used were maintained in Dulbecco modified Eagle medium (DMEM, Gibco, Waltham MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Minneapolis, MN) at 37 °C with 5% CO₂. C6/36 cells were propagated in Leibovitz's L-15 medium (Gibco) plus 10% FBS at 27 °C. Aag2 cells were maintained at 27 °C in Schneider's medium (Gibco) plus 10% FBS.

3.3.2 Mosquito rearing

Ae. aegypti Orlando strain (maintained in our insectary since they were obtained in 2005 from James Becnel, Agricultural Research Service, U.S. Department of Agriculture, Gainesville, FL, USA) were reared at 27 °C, 80% humidity on a 12 h light/12 h dark cycle. *Ae. aegypti* eggs were hatched in water containing brain heart infusion media (BHI) for 2 days to produce mosquito larvae. Mosquito larvae were separated into pans (each pan containing approximately 100 larvae) with a small amount of BHI and ground fish meal added. Adult mosquitoes emerged beginning at around 2 days after pupation. Adults were fed on raisins and water before and after blood feeding. All mosquito experiments were conducted in an arthropod containment level 2 insectary at Kansas State University.

3.3.3 Preparation of virus stocks

The MRE-16 infectious clone p5'dsMRE16ic was obtained from Ken Olson (Colorado State University), and its construction was previously described (Foy et al., 2004; Pierro et al., 2003). The p5'dsMRE16ic plasmid was linearized with *Ascl*. Capped viral RNA was produced from the linearized plasmids using m⁷G(5')ppp(5')G Cap Analog (Ambion, Austin TX) and MEGAscribe SP6 Transcription Kit (Thermo Fisher, Waltham MA). A density of 5×10^5 cells BHK21 cells were plated in each well of 6-well plates in serum-free opti-MEM media (Gibco) and allowed to attach for 2 hours. Six µL of Lipofectamine 3000 (Thermo Fisher), 100 µL opti-MEM, and 10 µL of 13.5 ng/ul viral RNA were mixed and allowed to sit for 5 minutes. After incubation, 900 µL of opti-MEM was then added to the tube containing the mixture and mixed with the seeded BHK-21 cells (Wang et al., 2008). After incubation at 37 °C for 3 days, the medium was collected, aliquoted, and stored as P1 virus stock at -80 °C. The P1 virus stocks were passaged by adding

200 μ L of P1 virus to a T75 flask containing 90% confluent C6/36 cells and cultured in Leibovitz's L-15 medium supplemented with 10% (vol/vol) FBS to obtain P2 viral stocks. At 5 days post-infection (dpi), the P2 virus was harvested, aliquoted, and stored at -80°C . Viral titers were determined using median tissue culture infectious dose (TCID₅₀) assay in BHK-21 cells, as described below. Virus stocks were thawed only once before use.

3.3.4 Oral infection with SINV

Newly emerged adult female mosquitoes were fed on raisins and water ad libitum. At day 2 post-emergence, raisins were removed but not water, and mosquitoes were starved for 1 day. The mosquitoes were then separated into smaller containers. P2 virus stock was mixed with defibrinated sheep blood (Colorado Serum Company, Denver CO) in a ratio of 1:1 and mosquitoes were allowed to feed for 1 hour using a Hemotek membrane feeder (Hemotek Ltd., Blackburn, UK) covered with Parafilm. The mosquitoes were briefly cold-shocked at 4°C , and then fully engorged females were separated from the remaining mosquitoes. Fed females were subsequently maintained on raisins and water until 5 days post-blood meal (PBM) before dissecting. Mosquitoes were dissected in phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, 1 liter H₂O) and midguts were removed from the carcasses. Mosquito midguts and carcasses were placed in 200 μ L DMEM plus 10% FBS and homogenized in 1.5-mL microcentrifuge tubes using disposable plastic pestles. In cases where RNA extraction was performed, a volume of 180 μ L was placed into 500 μ L Trizol (ThermoFisher Scientific) solution. Both samples were frozen at -80°C until titration and RNA extraction.

3.3.5 TCID₅₀ assay

A total of 1×10^4 BHK-21 cells were seeded in each well of 96-well tissue culture plates. The DMEM media used in seeding the cells contained 10% FBS and was supplemented with Antibiotic-Antimycotic Solution (Caisson Labs). Frozen mosquito samples were thawed on ice and centrifuged at 4 °C for 3 minutes at $15,000 \times g$ to remove debris. Serial dilutions of each sample were added to 5 duplicate wells of BHK-21 cells. The plates were scored for cytopathic effects after 5 days, and the proportion of infected wells was used to calculate TCID₅₀/mL values (O'Reilly, et al., 1994).

3.3.6 TCID₅₀ assay in mosquito cells using immunofluorescence

Aag2 and C6/36 cells were plated in 96 well plates and incubated with 10-fold serial dilutions of virus stocks obtained from low or high titer mosquitoes (as previously determined using BHK cells). At 4 days post-infection, media was removed, 4% paraformaldehyde (ThermoFisher) was added to fix the cells and the plates were rocked for at least 2 hours at room temperature. The cells were then washed two times with 100 µL PBT (PBS + 0.05% Triton X-100). A volume of 50 µL anti-Sindbis E1 monoclonal antibody (mAb 30.11A, obtained from Stephen Higgs, Kansas State University) diluted 1:300 in PBT solution was added and rocked for 90 minutes at 25°C. The cells were then washed twice with PBT. A volume of 50 µL of anti-mouse IgG conjugated to Alexa488 (Life Technologies) diluted 1:300 in PBT was added and incubated for 60 minutes at 25 °C with rocking, after which the wells were washed two times with PBT. The presence or absence of antibody staining in each well was observed using a fluorescence microscope, from which TCID₅₀ values were determined.

3.3.7 SINV genome quantification by RT-qPCR

Total RNA from mosquito midguts and carcasses was extracted using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific) according to manufacturer instructions. Power SYBR™ Green RNA-C_T™ 1-Step Kit (Thermo Fisher Scientific) was used for the RT-PCR reaction according to the manufacturer instructions. The primers targeted the NSP3 region with sequences as follows: Forward primer 5'-AGAAGAGGCTTCAGGGCTGG-3' and Reverse primer 5'-TGACGAGGTCTTTGGCTGTG-3'. The cycling conditions for the reaction were reverse transcription step at 48°C for 30 minutes, enzyme activation step at 96°C for 10 minutes, followed by 40 cycles of denaturation at 96°C for 15 seconds and annealing/extension at 56°C for 60 seconds. A standard curve was generated using a 1:10 dilutions of the p5' dsMRE16ic plasmid. Cycling threshold (Ct) values were used to determine number of RNA copies in each sample. The RT-qPCR experiment for each sample was carried out in duplicate and the results were averaged.

3.3.8 Illumina sequencing

Six mosquito midgut and carcass samples each were selected for sequencing from low and high titer mosquitoes. RNA was extracted from the individual samples using PureLink™ RNA Mini Kit. The stranded mRNA-Seq was performed using the Illumina NovaSeq 6000 Sequencing System at the University of Kansas Medical Center – Genomics Core (Kansas City, KS). Quality control of the total RNA submissions was completed using the Agilent TapeStation 4200 using the RNA ScreenTape Assay kit (Agilent Technologies 5067-5576). Total RNA (228.06ng – 264.6ng) was used to initiate the library preparation protocol. The total RNA fraction was processed by oligo dT bead capture of mRNA, fragmentation of enriched mRNA, reverse

transcription into cDNA, end repair of cDNA, ligation with the appropriate Unique Dual Index (UDI) adaptors and strand selection and library amplification by PCR using the Universal Plus mRNA-seq with NuQuant library preparation kit (Tecan Genomics 0520-A01).

Library validation was performed using the D1000 ScreenTape Assay kit (Agilent Technologies 5067-5582) on the Agilent TapeStation 4200. Concentration of each library was determined with the NuQuant module of the library prep kit using a Qubit 4 Fluorometer (Thermo Fisher). Libraries were normalized to 4nM concentration and pooled. The multiplexed pool was quantitated, in triplicate, using the Roche Lightcycler96 with FastStart Essential DNA Green Master (Roche 06402712001) and KAPA Library Quant (Illumina) DNA Standards 1-6 (KAPA Biosystems KK4903). Using the qPCR results, the RNA-Seq library pool was adjusted to 1.9nM for multiplexed sequencing.

Pooled libraries were denatured with 0.2N NaOH (0.04N final concentration) and neutralized with 400mM Tris-HCl pH 8.0. A dilution of the pooled libraries to 380 pM was performed in the sample tube, on instrument, followed by onboard clonal clustering of the patterned flow cell using the NovaSeq 6000 S1 Reagent Kit v1.5 (200 cycle) (Illumina 20028318). A 2x101 cycle sequencing profile with dual index reads was completed using the following sequence profile: Read 1 – 101 cycles x Index Read 1 – 8 cycles x Index Read 2 – 8 cycles x Read 2 – 101 cycles. Following collection, sequence data was converted from bcl file format to fastq file format using bcl2fastq software and de-multiplexed into individual sequences for data distribution using a secure FTP site or Illumina BaseSpace for further downstream analysis.

3.3.9 Illumina sequence data analysis

NGS data was processed and analyzed essentially as described elsewhere (Grubaugh et al. 2016). Briefly, trimmed reads from fastq files were aligned to the reference 5'dsMRE16ic genome and duplicates removed prior to calling intrahost single nucleotide variants (iSNVs) using Vphaser2 (Yang et al. 2013). iSNVs with significant strand bias or those present in the population at less than five percent frequency were removed. Sequence data have been deposited in the Sequence Read Archive under BioProject ID PRJNA1000578.

3.3.10 Bottleneck size estimation

Estimation of the bottleneck size at the initial midgut site of infection was calculated by analyzing the change in frequency distribution of neutral markers between stock virus in bloodmeal (initial) and midgut infection (final) sample five days post infection. The single nucleotide variants (SNVs) that were presumed to be neutral were selected based on the following set of criteria: (i) change should occur at the third codon position, (ii) this change should be synonymous, and (iii) the SNVs should be found in virus in blood meal, low titer midgut and high titer midgut samples (Monsion et al., 2008). Assuming neutrality (i.e., in the absence natural selection), the idealized number of founding genomes (N) initiating midgut infection was determined using the formula

$$N = \frac{p(1 - p)}{Var(p') - Var(p)}$$

where p is the marker allele frequency of the stock virus in blood (initial population) and p' is the marker allele frequency of the infected midgut virus population (final population) (Monsion et al., 2008).

3.3.11 Calculation of mutation frequency

The mutation frequency was calculated for each ORF as the number of substitutions per nucleotide, averaged for the 6 mosquitoes of each sample type.

3.3.12 Natural selection assessment

The natural selection was assessed using the d_N/d_S ratio. The number of non-synonymous substitutions per non-synonymous site (d_N) and number of synonymous substitutions per synonymous site (d_S) were calculated using:

$$d_N = \frac{-3 \ln(1 - (4p_n)/3)}{4}$$

and

$$d_S = \frac{-3 \ln(1 - (4p_s)/3)}{4}$$

where p_n is the number of non-synonymous substitutions divided by the number of non-synonymous sites and p_s is the number of synonymous substitutions divided by the number of synonymous sites (Nei and Gojobori 1986). DnaSP software was used to calculate the synonymous and non-synonymous sites.

3.3.13 Virus complexity and diversity analysis

SINV population genetic complexity was estimated using normalized Shannon entropy (S_n) for each nucleotide site using the formula (Gregori et al. 2014):

$$S_n = - \frac{p \ln(p) + (1 - p) \ln(1 - p)}{\ln(4)}$$

where p is the minor allele frequency at the considered position, and $\ln(4)$ corresponds to maximum complexity (i.e., four possible nucleotides at each position). For individual

populations, Sn values range from 0 to 1 where 0 represents no complexity while 1 represents maximum complexity.

SINV population genetic diversity was estimated using nucleotide diversity at each nucleotide site using the formula (Cornman et al., 2013):

$$\pi = \left(\frac{D}{D-1}\right)(1 - (p^2 + (p-1)^2))$$

where D is the sequencing depth at the considered position and p is the SNV minor allele frequency. For individual SNVs, π values range from 0 to 1 where 0 represents no diversity while 1 represents maximum diversity.

3.3.14 Transmission electron microscopy

Low titer (3.2×10^3 - 4.0×10^4 TCID₅₀/ml), medium titer (1.5×10^5 - 3.0×10^6 TCID₅₀/ml, and high titer (1.9 - 4.5×10^8 TCID₅₀/ml) mosquitoes (3 of each) were individually homogenized in PBS and insoluble debris was removed by centrifugation in a microcentrifuge. The homogenates were fixed with 2% paraformaldehyde at room temperature for 2 hrs, negative stained with uranyl acetate, absorbed onto grids, and viewed by transmission electron microscopy. The entirety of each grid was scanned at low magnification to identify possible virions, followed up by examination at higher magnification to verify the presence of virions that were similar in size and shape to SINV.

3.3.15 Immunoblotting

Individual mosquitoes were homogenized in 200 μ L of PBS and centrifuged in a microcentrifuge at 12,000 RPM for 5 min to remove insoluble material. Following removal of 20 μ L of the lysate for TCID₅₀ assay, 80 μ L Laemmli SDS-Sample Buffer, 6X Reducing (Boston Bioproducts) was

added. The samples were then stored at 4°C until being analyzed. Samples were heated for 5 min at 95°C, and then loaded on a 12% Tris-Glycine gel (BioRad). Gels were transferred to nitrocellulose membrane using an iBlot2 (ThermoScientific) and blocked using non-protein blocking buffer (Li-Cor). Blots were incubated while rocking overnight at 4 °C in primary antibody (1:3000 anti-capsid, 1:3000 anti-glycoprotein, 1:1000 β -actin (SantaCruz)) and then washed and probed with IRDye800RD anti-rabbit for capsid and glycoprotein, and IRDye680RD anti-mouse for β -actin. Preparation of anti-capsid and anti-glycoprotein antisera was described previously (Ramsey et al., 2017). Images were obtained using a ChemiDoc system (Bio-Rad Laboratories) and bands were quantified using Gel Analyzer in the FIJI software package (Schindelin et al., 2012) with background correction.

3.3.16 Saliva collection and titration

Saliva was collected from 60 mosquitoes at 5 days PBM. Mosquitoes were starved of a sugar source for 24 h before saliva collection, then anesthetized by cold treatment, and their wings and legs were removed. The proboscis was placed in a pipette tip containing ~20 μ L FBS + 1 mM ATP. Mosquitoes were allowed to salivate for 60–90 min. After salivation the FBS was added to 100 μ L DMEM. Samples were vortexed, spun down, and then stored at -80°C. Meanwhile, the mosquito bodies were assayed by TCID₅₀, and the matching saliva samples from 13 high titer ($\geq 1 \times 10^8$ TCID₅₀/ml) and 12 low titer ($\leq 1 \times 10^4$ TCID₅₀/ml) mosquitoes were then assayed by TCID₅₀.

3.3.17 Antibiotic treatment

Pupae were randomly divided into 2 groups. The antibiotic treatment group was put in a pan with deionized H₂O containing an antibiotic cocktail consisting of 100 µg/mL each of penicillin and streptomycin, and 100 units/mL of gentamycin (Gibco). The control group was transferred to a separate pan without antibiotics. After eclosion, adults in the antibiotic treatment group were given access *ad libitum* to a 10% sucrose solution containing the same antibiotic cocktail as above, while control adults were maintained on 10% sucrose without antibiotics. At 2 days post-eclosion, the sucrose solutions were removed from both treatments and at 3 days post-eclosion, the antibiotic group was allowed to feed on a mixture of blood and virus containing the same antibiotic cocktail while the control group was allowed to feed on blood and virus alone. The mosquitoes were dissected at 5 days PBM and carcasses were stored in 200µl of DMEM at -80°C until being assayed.

3.3.18 Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. Fisher's exact, Mann-Whitney or Pearson's chi-square tests were used to determine statistically significant differences between treatments. A p-value of ≤ 0.05 was used as the threshold to indicate significant difference between treatments.

3.4 Results

3.4.1 SINV titer is highly variable and correlates with detection of virus in saliva

We and others have repeatedly shown that extreme variation in virus titer is observed between individual mosquitoes that are infected with various arboviruses (Carpenter et al., 2021; Dong et al., 2017; Hodoamede et al., 2022; Merwaiss et al., 2021; O'Neill et al., 2015), even when the mosquitoes are highly inbred. We first asked whether titer variation affects the likelihood of virus being detected in the saliva of infected mosquitoes, which is often used as a proxy to assess transmission potential. Saliva was collected at 5 days post-blood meal (PBM) from *Ae. aegypti* (Orlando) mosquitoes that had fed on blood containing passage 2 (P2) virus derived from p5'dsMRE16ic, an infectious clone of the MRE-16 strain of SINV. After determining the virus titers in the mosquito bodies by tissue culture median infectious dose (TCID₅₀) assay, the infectious titers in the saliva samples from 13 high titer ($\geq 10^8$ TCID₅₀/mL) and 12 low titer ($\leq 10^4$ TCID₅₀/mL) mosquitoes were determined. A significantly larger proportion of high titer mosquitoes had detectable virus in their saliva than low titer mosquitoes (Figure 3.1A). Furthermore, amongst those mosquitoes that had positive saliva titers, the median saliva titer was larger in high versus low titer mosquitoes (Figure 3.1B). Based on these results, it is likely that individual variation in carcass titer is an important factor in determining virus transmission dynamics.

We previously found that SINV collected from high titer mosquitoes replicated to a higher titer in cultured cells than virus from low titer mosquitoes (Hodoamede et al., 2022), suggesting there may be genetic differences between these virus populations. To further examine this, we performed Illumina sequencing on virus populations from high and low titer mosquitoes.

Mosquitoes were allowed to ingest blood containing 1.6×10^9 TCID₅₀/mL of P2 virus derived from p5'dsMRE16ic. At 5 days PBM, we dissected the mosquitoes into midguts and carcasses (defined as the rest of the mosquito besides the midgut) and determined the virus titers of the dissected mosquito carcasses using TCID₅₀ assay. Consistent with previous results, the individual carcass titers varied widely; the median carcass titer was 4.2×10^6 TCID₅₀/mL, but individual titers ranged from 1.5×10^9 to 3.7×10^3 (Figure 3.1C). From this group of mosquitoes, we selected six high titer ($\geq 1.5 \times 10^8$ TCID₅₀/mL) and six low titer ($\leq 6.8 \times 10^4$ TCID₅₀/mL) mosquitoes for Illumina sequencing of their individual midguts and carcasses, in order to compare the sequences of these individual virus populations (Figure 3.1D).

3.4.2 A significant reduction in numbers of sequence variants occurs following dissemination from midgut to carcass, but only in low titer mosquitoes

RNA isolated from the individual midgut and carcass samples of the 6 low and 6 high titer mosquitoes, as well as from a sample of the P2 virus stock that was fed to the mosquitoes, was used to prepare Illumina sequencing libraries, which were then subjected to sequencing. The resulting viral sequences were aligned and compared to the reference sequence of p5'dsMRE16ic. Viral genome sequence coverage in the mosquito tissue samples ranged from approximately 2,000X to 18,000X, with the exception of one low titer carcass sample which was around 40X, while genome coverage in the stock sample was around 25,000X (Figure 3.10. (Supplementary)). To reveal significant sequence changes, we focused on nucleotide variants that were $\geq 5\%$ in frequency within a sample. Overall, the amount of genetic diversity observed between samples was relatively low. Very few indels were observed, with a total of only 17 indels being $\geq 5\%$ in frequency in all samples combined, and those observed were small (≤ 3

nucleotides) (Figure 3.11. (Supplementary)). Nucleotide substitutions were distributed in what appeared to be a random pattern across the genome, although there were a few apparent “hot spots”, or sites that contained higher numbers of variants (Figure 3.2; sequence variants in stock virus are shown in Figure 3.12. (Supplementary)).

To examine whether there were differences in the degree of sequence variation amongst different viral ORFs, we first calculated the mutation frequencies (average mutations per nucleotide position) in order to account for differences in ORF length. The genome positions of ORFs and other genomic elements are provided in Table S1. Mutation frequencies were roughly similar in most of the 9 viral ORFs in midguts of low and high titer mosquitoes (Figure 3.3A), except that no mutations were identified at $\geq 5\%$ frequency in the NSP2 and 6K ORFs of high titer midguts. Greater variability was seen in carcasses, where three ORFs (NSP2, E3, and 6K) had no mutations at $\geq 5\%$, and mutations at $\geq 5\%$ were only seen in NSP3 and E2 of low titer carcasses. Mutation frequency varied somewhat in the 5' or 3' untranslated regions and the subgenomic promoter region, but the relatively small sizes of these regions and overall low number of mutations impacts the interpretation of these results.

Importantly, no single mutations were observed that correlated with virus titer. Mutations observed in the different samples were to a large extent unique, with a minority being shared between samples (Supplemental File 1 and Figure 3.13. (Supplementary)). There were also no clear patterns of differences in the proportions of mutations that were synonymous versus non-synonymous in the ORFs of viruses from low versus high titer mosquitoes (Figure 3.14. (Supplementary)).

Genetic bottlenecks exist for arboviruses at the levels of midgut infection and midgut escape. Consistent with a strong bottleneck occurring during midgut escape, we observed that the

numbers of sequence variants were reduced in the carcasses compared to the midguts of three of the six low titer mosquitoes, resulting in an overall significant reduction in the number of variants when low titer individuals were taken as a whole (Figure 3.4A-B). Interestingly, however, there was no significant difference in the number of sequence variants between the midguts and carcasses of the high titer mosquitoes (Figure 3.4C-D). This result suggests that there was more of a reduction in genetic complexity, or in other words a more stringent bottleneck, associated with virus dissemination in low titer mosquitoes than in high titer mosquitoes. However, there was no significant difference between low titer and high titer mosquitoes in terms of the numbers of variants that were observed in both midgut and carcass (conserved variants) or the numbers of variants seen only in carcass samples (emerged variants) (Figure 3.15. (Supplementary)). There was also no significant difference observed in bottleneck size when neutral markers were used to estimate the sizes of the bottlenecks associated with dissemination from midgut to carcass in low and high titer mosquitoes (Figure 3.16. (Supplementary)). Thus, although there was evidence of a more stringent genetic bottleneck in low titer than in high titer mosquitoes, bottleneck size did not appear to differ between these groups.

3.4.3 Differences in diversity and complexity between the virus populations in low titer and high titer mosquitoes

In order to gain a better understanding of the overall genetic differences between the various virus populations, we then compared the diversity (a measure of the degree of sequence variation) and complexity (a broader measure of variability, including richness, diversity and heterogeneity) of the virus populations in the cumulative midguts and carcasses of low and high

titer mosquitoes, as well as to those of the original stock virus that was used to infect the mosquitoes. This analysis showed that there was a significant reduction in both sequence diversity and complexity after dissemination from midgut to carcass in low titer mosquitoes, but not in high titer mosquitoes (Figure 3.5A-B). In addition, we examined the ratio of non-synonymous to synonymous substitutions (the d_N/d_S ratio) in the coding regions of the virus populations, in order to assess the strength of natural selection in these virus populations. A d_N/d_S ratio of less than 1 indicates that negative (purifying) selection is occurring in the population. This analysis revealed that purifying selection occurred in all the mosquito-derived virus populations, but the magnitude of the purifying selection was greater in both midguts and carcasses of low titer mosquitoes when compared to high titer mosquitoes (Figure 2.5C). Together, these analyses indicate that there was a stronger reduction in virus genetic diversity and complexity, accompanied by stronger purifying selection, during the process of virus dissemination from midgut to carcass in low titer versus high titer mosquitoes.

3.4.4 Low titer mosquitoes contain less viral particles and lower levels of capsid protein than high titer mosquitoes

Although we observed differences at the population genetic level between the virus populations in low and high titer mosquitoes, it seemed unlikely that these differences alone could cause the ~10,000-fold difference observed in infectious virus titers between low and high titer mosquitoes. To first rule out the possibility that titer was affected by the type of cells used to assay infectious virus, we compared TCID₅₀ values using mammalian BHK and mosquito Aag2 and C6/36 cells. Mosquito samples that had high or low titers, as measured in BHK cells, were re-titered in mosquito cells using an immunofluorescence-based TCID₅₀ assay. TCID₅₀ values

were highly consistent regardless of the cell line used (Figure 3.17. (Supplementary)), indicating that the type of cells used for virus titration does not affect the measurement of infectious virus.

We reasoned that the large differences in virus titer between individuals could be due to various reasons, including differences in the amounts of viral RNA (due to differences in synthesis or stability), differences in the amounts of viral proteins being translated from equivalent levels of viral RNA, differences in the proportion of viral RNA being packaged into particles, or the presence of large numbers of defective virus particles. To begin to decipher the basis for these large differences in titer, we orally infected another batch of mosquitoes and, after homogenization in PBS and titration by TCID₅₀, randomly selected three high titer, three medium titer, and three low titer mosquitoes in order to look for the presence of virus particles by transmission electron microscopy. A portion of the clarified lysate from the high, medium and low titer samples was negative stained and examined by TEM. Enveloped icosahedral virus particles consistent with the known size of SINV (~70 nm) were abundantly observed in homogenates from all three of the high titer mosquitoes we examined (Figure 3.18. (Supplementary) and Table 3.2 (Supplementary)). However, we rarely observed virus particles during examination of homogenates from 3 medium titer and 3 low titer mosquitoes (Table 3.2 (Supplementary)). Obviously, there must have been virus particles present in the low and medium titer samples, but they were apparently too dilute to be consistently observable by this technique, consistent with their lower TCID₅₀ values. Thus, based on this semi-quantitative method, it appeared that there were less viral particles in low and medium titer mosquitoes than in high titer mosquitoes. It should also be noted that that we did not observe any unenveloped nucleocapsid cores by TEM in any of the samples.

We next used immunoblotting to examine the levels of viral capsid protein in low and high titer mosquitoes. An additional batch of mosquitoes were orally infected and homogenized in PBS, and after a small portion of each homogenized sample was analyzed by TCID₅₀ assay, 7 high titer and 7 low titer mosquitoes were chosen for immunoblotting using antibodies against SINV capsid protein and glycoproteins E1 and E2. The results showed that, while there was variability in the levels of capsid protein between individuals, on average there was 1.6-fold less capsid protein in low titer compared to high titer mosquitoes (Figure 3.19. (Supplementary)). Although we were unable to detect E1 and E2 in this assay, this may have simply been a detection issue given the small amount of starting material in single mosquitoes, since in our experience the glycoproteins are more difficult to detect than capsid. Nevertheless, although levels of capsid protein would not necessarily need to be reduced in order to result in lower numbers of assembled virions, the reduced capsid protein levels in low titer mosquitoes is consistent with the lower numbers of virions observed by TEM.

3.4.5 Dramatic differences in specific infectivity (SI) between high titer and low titer mosquitoes

Since there appeared to be lower numbers of viral particles in low titer mosquitoes, it was important to examine the levels of viral RNA in the two groups, in order to distinguish between differences in viral genome replication versus differences in the proportion of genomes packaged into infectious particles. To examine this, we determined the SI (the ratio of infectious units to viral genomes) of virus populations from individual mosquitoes, initially from the same 6 low and 6 high titer mosquitoes that were used for sequencing. SINV genome equivalents were measured by RT-qPCR using primers that amplified a portion of the NSP3 region and were

compared to infectious units as measured by TCID₅₀ assay in the same mosquitoes. Interestingly, we found that genome equivalents did not vary to any great extent in 11 of the 12 mosquitoes, even though the infectious units were considerably different between the low and high titer mosquitoes (Figure 3.6A). This resulted in a large difference in SI (TCID₅₀/genome equivalents) between the two groups of mosquitoes (Figure 3.6B).

To determine whether this dramatic difference in SI was a general phenomenon and not restricted to this small group of mosquitoes, another batch of 50 mosquitoes were infected by blood feeding as before, and the SI of these individual mosquitoes was determined. Consistent with what was observed in the original 12 sequenced mosquitoes, we observed less variation in genome equivalents compared to infectious units amongst these mosquitoes (Figure 3.7A-B). Overall, the numbers of genome equivalents were greater than infectious units in almost all individuals (Figure 3.7B). Although this would be expected, in part since RT-PCR is more sensitive than TCID₅₀ assay, and also because all viruses produce more genomes than infectious particles, we noted that the difference between genome equivalents and virus titer in low titer individuals was strikingly large (Figure 3.7B). Genome equivalents also varied to some degree, but there was no significant overall correlation between genome equivalents and TCID₅₀ values (Figure 3.7C), with many individuals that had low titers of infectious virus containing high numbers of genomes. Notably, the top 10% of mosquitoes in terms of infectious titers had SI values ranging from 0.03 to 0.53, while the bottom 10% ranged from 5.6×10^{-7} to 9.1×10^{-4} (Figure 3.7D). These results, together with our TEM and immunoblot data, indicate that the large variation that is observed in individual mosquito titer is mainly driven by differences in the proportion of genomes that are packaged into infectious virus particles, rather than by differences in the levels of viral genomic RNA.

3.4.6 Carcass SI is significantly higher than midgut SI

Our sequence analysis indicated that there were significantly greater reductions in genetic diversity and complexity occurring during virus dissemination in low versus high titer mosquitoes. It was therefore of interest to compare the SI values in carcasses and midguts. To do this, another batch of 50 mosquitoes were orally infected as before. All mosquitoes had detectable virus titer in both midgut and carcass, indicating that variation in midgut titer does not affect the dissemination rate. When we compared midgut and carcass SI values in the individual mosquitoes, we again found that viral RNA levels did not correspond to infectious titer in either tissue (Figure 3.20. (Supplementary)). In addition, overall carcass SI was significantly higher than midgut SI (Figure 3.8A). Carcass SI values were greater than midgut SI in the large majority (88%) of individual mosquitoes (Figure 3.8B). Thus, there tended to be a higher ratio of non-infectious to infectious genomes in the midgut than in the carcass of most individuals. However, there was no significant correlation between midgut SI and carcass SI in individuals (Figure 3.8C), indicating that the magnitude of midgut SI does not appear to influence the SI of disseminated virus within an individual infected mosquito.

3.4.7 SI decreases with increased incubation period

Previously published results from our group showed that SINV titer variation decreases with time after an infectious blood meal (the incubation period); there was significantly decreased median titer as well as less titer variation at 15 days PBM than at earlier times (Hodoameda et al., 2022). To ask whether this reduction in titer variation with increased incubation period is also associated with differences in SI, we compared the SI of mosquitoes at 5 days and 15 days PBM. The results indicated that there was significantly lower SI at 15 days

PBM than at 5 days PBM (Figure 3.21. (Supplementary)). Thus, the overall reduction in virus titer over time that was previously observed also appears to be due to accumulation of unpackaged genomes.

3.4.8 Oral antibiotic treatment results in higher specific infectivity

It has been shown previously that feeding antibiotics to mosquitoes suppresses antiviral immunity due to the disruptive effects of antibiotics on the composition of the gut microbiome (Barletta et al. 2017; Ramirez et al. 2012; Xi, Ramirez, and Dimopoulos 2008). To test whether genome packaging efficiency is influenced by altering the immune response, we exposed pupae and adults to antibiotics in their water source prior to also providing antibiotics in an infectious blood meal. As expected based on previous reports, antibiotic treatment resulted in increases in both infection prevalence and median infectious titer compared to mosquitoes that were not exposed to antibiotics, likely due to the previously demonstrated immune suppressive effects of antibiotics (Figure 3.9A-B). However, antibiotic treatment did not significantly affect viral genome levels (Figure 3.9B). The resulting increase in median SI (Figure 3.9C) indicates that suppressing the immune response results in a higher proportion of viral genomes being packaged into infectious particles.

3.5 Discussion

Variation in how individual hosts respond to infections has been known to exist since antiquity; for example, some hosts survive while others succumb to infection, and symptom severity can vary widely between individuals. However, most of the past research on this topic has focused on genetic differences between hosts or differences in virulence genes between virus

strains. With the availability of deep sequencing technology, researchers have more recently begun to go deeper by comparing the viral quasispecies that are present in individual hosts. For example, the degree of intrahost variation of virus populations during SARS-CoV-2 and influenza infections, and the effect of bottlenecks associated with transmission between hosts, have recently been examined (Amato et al. 2022; Braun et al. 2021; McCrone et al. 2018; Tonkin-Hill et al. 2021). Interestingly, the findings with these other viruses are consistent with what we have observed, namely the presence of limited viral genome variation during acute infections in individual hosts, with strong negative selection and severe bottlenecks acting to limit the amount of diversity in these viruses as they spread through susceptible populations.

In this study, we explored whether there were differences between the virus populations in individual infected mosquitoes that could explain the tremendous amount of virus titer variation observed amongst highly inbred *Ae. aegypti* mosquitoes after oral infection with SINV. Our results revealed that there are both genetic differences at the population level and differences in the ratio of infectious units to genomes, or SI, between the virus populations found in low titer versus high titer mosquitoes. We did not find any evidence of specific candidate mutations that are associated with virus titer; instead, we found that the virus populations of low titer mosquitoes experienced significant reductions in genetic complexity and diversity during virus dissemination from the midgut, while those of high titer mosquitoes did not. There was also evidence of stronger purifying selection in both midguts and carcasses of low titer versus high titer mosquitoes. In addition, we observed dramatic differences in SI between low titer and high titer mosquitoes. Overall, our data indicate that there are both differences in the genetic composition and profound differences in SI between the virus populations found in low and high titer mosquitoes. At this point it is not clear whether these two types of observed differences are

causally related, but based on our results, the huge variation in SINV titer between individual *Ae. aegypti* mosquitoes is best explained by differences in SI, likely due to a component of the immune response that directly or indirectly affects packaging of viral genomes into virions. It should be noted that despite their being extensively inbred over the past ~80 years, the amount of genetic heterogeneity in Orlando mosquitoes is unknown, and it remains possible that there is genetic variation amongst individuals that affects their immune response.

The midgut, which is the initial site of infection, poses significant physical barriers to arbovirus infection (Franz et al., 2015). Genetic bottlenecks occur during the process of midgut infection as well as during escape of virus from the midgut to the hemocoel. The number of viruses that manage to pass through these bottlenecks is likely quite small (Fitzmeyer et al., 2023; Forrester et al., 2012). Thus, each genetic bottleneck has the potential to lead to founder effects, which can influence the eventual genetic structure of the virus population within an infected mosquito (Fitzmeyer et al., 2023; Lequime et al., 2016; Patterson et al., 2018). Our sequencing of viral populations indicated that there are higher numbers of genetic variants in the midguts than in the carcasses of both low and high titer mosquitoes, consistent with a midgut escape bottleneck. However, there were roughly similar numbers of genetic variants in the midguts of low and high titer mosquitoes, although some of the individual low titer midguts contained higher numbers. We did not observe a difference in the estimated bottleneck sizes between low and high titer mosquitoes, indicating that similar numbers of viruses escaped from the midgut in these individuals. Despite this, we found that low titer mosquitoes experienced a significant decrease in the number of variants during virus dissemination, while high titer mosquitoes did not. Measures of genetic complexity and diversity were also reduced following virus dissemination in low titer mosquitoes, but not in high titer individuals. In addition, there

was also evidence of stronger negative or purifying selection observed in low titer mosquitoes than in high titer mosquitoes. Finally, previously published data showed that virus obtained from high titer mosquitoes replicated better than virus from low titer mosquitoes in cell culture (Hodoamedia et al., 2022). All of these results are consistent with the conclusion that the virus populations in the midguts of low titer mosquitoes are overall less fit than those in the midguts of high titer mosquitoes. A likely source of this difference is genetic founder effect, occurring due to the midgut infection bottleneck. Despite the fact that the mosquitoes were all exposed to the same population of genetic variants present in the stock virus, stochastic effects caused by the midgut infection bottleneck may have led to some mosquitoes being infected with variants that were less fit than those infecting others.

To investigate this further, we examined the SI ratio (infectious units per genome equivalent) in orally infected mosquitoes. The results revealed that the huge variation in SINV infectious titer between individual mosquitoes is mainly due to differences in SI. The SI of SINV has been reported to vary somewhat depending on the cell line being infected, but to be in the range of 10^{-1} to 10^{-3} when infecting either BHK or C6/36 cells (Sokoloski et al., 2012). Thus, the SI values we observed in low titer mosquitoes, ranging from 10^{-3} to 10^{-6} , are exceptionally low compared to what has been previously reported from infection of cell lines.

The infectivity of SINV that is produced in vertebrate cells has been shown to be affected by encapsidation of host factors, resulting in two populations of SINV particles that differ in density as well as infectivity (Mackenzie-Liu et al., 2018; Sokoloski et al., 2013). However, SINV produced in mosquito cells was found to only produce the more infectious type of these particles (Sokoloski et al., 2013). Furthermore, we observed decreased numbers of viral particles in low titer mosquitoes, making differences in encapsidation of host factors an unlikely source of

the variation in infectivity observed in this study. Instead, it appears that viral RNA is not encapsidated efficiently in low titer mosquitoes, for reasons that are currently unclear. There are numerous possible explanations for why a large proportion of viral genomes are not being packaged in low titer mosquitoes. For example, viral RNA could be mislocalized in cells, preventing its encapsidation. Translation of viral RNA could be less efficient, resulting in lower amounts of viral proteins; indeed, our immunoblotting results indicated that there were lower levels of capsid protein on average in low titer mosquitoes, although the levels of capsid protein varied and did not entirely correlate with titer. Related to this, translation defects could be due to differences in RNA modifications such as capping or methylation, as in *Wolbachia*-colonized mosquitoes where it has been shown that decreased cytosine methylation in SINV RNA leads to reduced infectivity (Bhattacharya et al., 2020, 2022). It is also possible that one or more viral proteins are being modified somehow to make them less stable or less able to function, for example by altering their localization in cells. Unfortunately, we were not able to detect the viral glycoproteins E1 and E2 in individual mosquitoes in our immunoblots, but it is possible these proteins are mislocalized, unstable, or not functioning properly. However, it is notable that we did not observe unenveloped nucleocapsids by TEM, indicating the low production of infectious particles is not likely to be simply due to lack of envelopment of nucleocapsids. A significant clue as to what is affecting genome encapsidation is our observation that suppressing antiviral immunity using antibiotic treatment caused an increase in SI. Of course, additional work will be necessary to understand the precise underlying mechanisms causing inefficient genome packaging in low titer mosquitoes.

We further examined whether there were differences in SI between the virus populations in midguts and carcasses, and if SI changed over time in infected mosquitoes. Our results

indicated that the virus found in midgut at 5 days PBM had an overall lower SI than virus in carcass. However, there was no correlation between the midgut SI and carcass SI in individual mosquitoes, arguing against inefficient genome packaging being due to specific genetic differences in the viral populations. Furthermore, SI as well as median titer decreased over time, with SI being lower at 15 days PBM than at 5 days. These results further hint at the possibility that there may be a host response that can somehow affect the packaging efficiency of SINV genomic RNA, and that this response is more effective in some mosquitoes than in others, leading to extensive variation in the titer of infectious virus. In addition, this putative immune-related response appears to be more active in the midgut than in the carcass and appears to have a cumulative effect on virus infectivity over time, and/or is increasingly active as time goes on.

In conclusion, our findings indicate that the huge amount of variation in SINV infectious titer that is observed between individual infected *Ae. aegypti* mosquitoes within a single experiment is due to differences between the virus populations found in different individuals. These include genetic differences at the population level, which may be the result of founder effects that occur due to genetic bottlenecks associated with midgut infection and escape. In addition, we observed profound differences in the efficiency with which viral genomes were efficiently packaged into viral particles between mosquitoes, apparently due to some type of mosquito response that can affect the efficiency of packaging of SINV genomic RNA, such as by sequestering or modifying viral RNA, inhibiting translation of the viral structural proteins, or affecting their stability, localization, or function. Whether the genetic differences between virus populations and the differences in SI are causally related or only correlated remains to be determined. It will also be of interest to determine whether similar differences in virus populations and specific infectivity are observed in low and high titer infections of other vector

mosquitoes and in *Ae. aegypti* infected with other arboviruses, where high individual variability in virus titer has also been observed. Finally, our work highlights the importance of measuring titers of infectious virus when performing experiments in arbovirus-infected mosquitoes, since viral genome copy number may not always accurately reflect the amount of infectious virus present.

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3.7 References

- Amato, K. A., Haddock, L. A., Braun, K. M., Meliopoulos, V., Livingston, B., Honce, R., Schaack, G. A., Boehm, E., Higgins, C. A., Barry, G. L., Koelle, K., Schultz-Cherry, S., Friedrich, T. C., & Mehle, A. (2022). Influenza A virus undergoes compartmentalized replication in vivo dominated by stochastic bottlenecks. *Nature Communications*, *13*(1), 1–15. <https://doi.org/10.1038/s41467-022-31147-0>
- Barletta, A. B. F., Nascimento-Silva, M. C. L., Talyuli, O. A. C., Oliveira, J. H. M., Pereira, L. O. R., Oliveira, P. L., & Sorgine, M. H. F. (2017). Microbiota activates IMD pathway and limits Sindbis infection in *Aedes aegypti*. *Parasites and Vectors*, *10*(1), 1–9. <https://doi.org/10.1186/s13071-017-2040-9>
- Bhattacharya, T., Newton, I. L. G., & Hardy, R. W. (2020). Viral RNA is a target for Wolbachia-mediated pathogen blocking. *PLoS Pathogens*, *16*(6), 1–20. <https://doi.org/10.1371/journal.ppat.1008513>
- Bhattacharya, T., Yan, L., Crawford, J. M., Zaher, H., Newton, I. L. G., & Hardy, R. W. (2022). Differential viral RNA methylation contributes to pathogen blocking in Wolbachia-colonized arthropods. *PLoS Pathogens*, *18*(3), 1–25. <https://doi.org/10.1371/journal.ppat.1010393>
- Braun, K. M., Moreno, G. K., Wagner, C., Accola, M. A., Rehauer, W. M., Baker, D. A., Koelle, K., O'Connor, D. H., Bedford, T., Friedrich, T. C., & Moncla, L. H. (2021). Acute SARS-CoV-2 infections harbor limited within-host diversity and transmit via tight transmission bottlenecks. *PLoS Pathogens*, *17*(8), 1–26. <https://doi.org/10.1371/journal.ppat.1009849>
- Carpenter, A., Bryant, B., Santos, S. R., & Clem, R. J. (2021). Infection of *Aedes aegypti*

- mosquitoes with midgut-attenuated Sindbis virus reduces, but does not eliminate, disseminated infection. *Journal of Virology*, 95(13) <https://doi.org/10.1128/JVI.00136-21>
- Coffey, L. L., Beeharry, Y., Bordería, A. V., Blanc, H., & Vignuzzi, M. (2011). Arbovirus high fidelity variant loses fitness in mosquitoes and mice. *Proceedings of the National Academy of Sciences of the United States of America*, 108(38), 16038–16043. <https://doi.org/10.1073/pnas.1111650108>
- Coffey, L. L., Forrester, N., Tsetsarkin, K., Vasilakis, N., & Weaver, S. C. (2013). Factors shaping the adaptive landscape for arboviruses: implications for the emergence of disease. *Future microbiology*, 8(2), 155-176. <https://doi.org/10.2217/fmb.12.139>
- Cornman, R. S., Boncristiani, H., Dainat, B., Chen, Y., vanEngelsdorp, D., Weaver, D., & Evans, J. D. (2013). Population-genomic variation within RNA viruses of the Western honey bee, *Apis mellifera*, inferred from deep sequencing. *BMC Genomics*, 14(1). <https://doi.org/10.1186/1471-2164-14-154>
- Dong, S., Behura, S. K., & Franz, A. W. E. (2017). The midgut transcriptome of *Aedes aegypti* fed with saline or protein meals containing chikungunya virus reveals genes potentially involved in viral midgut escape. *BMC Genomics*, 18(1), 1–17. <https://doi.org/10.1186/s12864-017-3775-6>
- Fitzmeyer, E. A., Gallichotte, E. N., Weger-Lucarelli, J., Kapuscinski, M. L., Abdo, Z., Pyron, K., Young, M. C., & Ebel, G. D. (2023). Loss of West Nile virus genetic diversity during mosquito infection due to species-dependent population bottlenecks. *IScience*, 26(10), 107711. <https://doi.org/10.1016/j.isci.2023.107711>
- Forrester, N. L., Guerbois, M., Seymour, R. L., Spratt, H., & Weaver, S. C. (2012). Vector-Borne Transmission Imposes a Severe Bottleneck on an RNA Virus Population. *PLoS*

- Pathogens*, 8(9). <https://doi.org/10.1371/journal.ppat.1002897>
- Foy, B. D., Myles, K. M., Pierro, D. J., Sanchez-Vargas, I., Uhlirová, M., Jindra, M., Beaty, B. J., & Olson, K. E. (2004). Development of a new Sindbis virus transducing system and its characterization in three Culicine mosquitoes and two Lepidopteran species. *Insect Molecular Biology*, 13(1), 89–100. <https://doi.org/10.1111/j.1365-2583.2004.00464.x>
- Franz, A. W. E., Kantor, A. M., Passarelli, A. L., & Clem, R. J. (2015). Tissue barriers to arbovirus infection in mosquitoes. *Viruses*, 7(7), 3741–3767. <https://doi.org/10.3390/v7072795>
- Girard, M., Nelson, C. B., Picot, V., & Gubler, D. J. (2020). Arboviruses: A global public health threat. *Vaccine*, 38(24), 3989–3994. <https://doi.org/10.1016/j.vaccine.2020.04.011>
- Gregori, J., Salicrú, M., Domingo, E., Sanchez, A., Esteban, J. I., Rodríguez-Frías, F., & Quer, J. (2014). Inference with viral quasispecies diversity indices: Clonal and NGS approaches. *Bioinformatics*, 30(8), 1104–1111. <https://doi.org/10.1093/bioinformatics/btt768>
- Grubaugh, N. D., Weger-Lucarelli, J., Murrieta, R. A., Fauver, J. R., Garcia-Luna, S. M., Prasad, A. N., Black, W. C., & Ebel, G. D. (2016). Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching. *Cell Host and Microbe*, 19(4), 481–492. <https://doi.org/10.1016/j.chom.2016.03.002>
- Hodoameda, P., Addae, L., & Clem, R. J. (2022). Investigation of Biological Factors Contributing to Individual Variation in Viral Titer after Oral Infection of *Aedes aegypti* Mosquitoes by Sindbis Virus. *Viruses*, 14(1). <https://doi.org/10.3390/v14010131>
- Honório, N. A., Câmara, D. C. P., Wiggins, K., Eastmond, B., & Alto, B. W. (2020). High-throughput method for detection of arbovirus infection of saliva in mosquitoes *Aedes aegypti* and *Ae. albopictus*. *Viruses*, 12(11), 1–18. <https://doi.org/10.3390/v12111343>

- Kuno, G. (2010). Early history of laboratory breeding of *Aedes aegypti* (Diptera: Culicidae) focusing on the origins and use of selected strains. *Journal of Medical Entomology*, 47(6), 957–971. <https://doi.org/10.1603/ME10152>
- Lauring, A. S., & Andino, R. (2010). Quasispecies theory and the behavior of RNA viruses. *PLoS Pathogens*, 6(7), 1–8. <https://doi.org/10.1371/journal.ppat.1001005>
- Lequime, S., Fontaine, A., Ar Gouilh, M., Moltini-Conclois, I., & Lambrechts, L. (2016). Genetic Drift, Purifying Selection and Vector Genotype Shape Dengue Virus Intra-host Genetic Diversity in Mosquitoes. *PLoS Genetics*, 12(6), 1–24. <https://doi.org/10.1371/journal.pgen.1006111>
- Liang, G., Gao, X., & Gould, E. A. (2015). Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. *Emerging Microbes and Infections*, 4(3), 1–5. <https://doi.org/10.1038/emi.2015.18>
- Mackenzie-Liu, D., Sokoloski, K. J., Purdy, S., & Hardy, R. W. (2018). Encapsidated host factors in alphavirus particles influence midgut infection of *aedes aegypti*. *Viruses*, 10(5), 1–12. <https://doi.org/10.3390/v10050263>
- McCrone, J. T., Woods, R. J., Martin, E. T., Malosh, R. E., Monto, A. S., & Lauring, A. S. (2018). Stochastic processes constrain the within and between host evolution of influenza virus. *ELife*, 7, 1–19. <https://doi.org/10.7554/eLife.35962>
- Merwaiss, F., Filomatori, C. V., Susuki, Y., Bardossy, E. S., Alvarez, D. E., & Saleh, M.-C. (2021). Chikungunya Virus Replication Rate Determines the Capacity of Crossing Tissue Barriers in Mosquitoes. *Journal of Virology*, 95(3). <https://doi.org/10.1128/jvi.01956-20>
- Monsion, B., Froissart, R., Michalakakis, Y., & Blanc, S. (2008). Large bottleneck size in Cauliflower mosaic virus populations during host plant colonization. *PLoS Pathogens*,

4(10). <https://doi.org/10.1371/journal.ppat.1000174>

Nei, M., & Gojobori, T. (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Molecular Biology and Evolution*, 3(5), 418–426.

<https://doi.org/10.1093/oxfordjournals.molbev.a040410>

O'Neill, K., Huang, N., Unis, D., & Clem, R. J. (2015). Rapid selection against arbovirus-induced apoptosis during infection of a mosquito vector. *Proceedings of the National Academy of Sciences of the United States of America*, 112(10), E1152–E1161.

<https://doi.org/10.1073/pnas.1424469112>

O'Reilly, D. R., Miller, L. K., & Luckow, V. A. (1994). Baculovirus expression vectors: A laboratory manual. *Cell*, 74(1), 7–8. [https://doi.org/10.1016/0092-8674\(93\)90288-2](https://doi.org/10.1016/0092-8674(93)90288-2)

Patterson, E. I., Khanipov, K., Rojas, M. M., Kautz, T. F., Rockx-Brouwer, D., Golovko, G., Albayrak, L., Fofanov, Y., & Forrester, N. L. (2018). Mosquito bottlenecks alter viral mutant swarm in a tissue and time-dependent manner with contraction and expansion of variant positions and diversity. *Virus Evolution*, 4(1), 1–11.

<https://doi.org/10.1093/ve/vey001>

Pierro, D. J., Myles, K. M., Foy, B. D., Beaty, B. J., & Olson, K. E. (2003). Development of an orally infectious Sindbis virus transducing system that efficiently disseminates and expresses green fluorescent protein in *Aedes aegypti*. *Insect Molecular Biology*, 12(2), 107–116. <https://doi.org/10.1046/j.1365-2583.2003.00392.x>

Ramirez, J. L., Souza-Neto, J., Cosme, R. T., Rovira, J., Ortiz, A., Pascale, J. M., & Dimopoulos, G. (2012). Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS Neglected Tropical Diseases*, 6(3), 1–11. <https://doi.org/10.1371/journal.pntd.0001561>

- Ramsey, J., Renzi, E. C., Arnold, R. J., Trinidad, J. C., & Mukhopdhyay, S. (2017). Palmitoylation of Sindbis virus TF protein regulates its plasma membrane localization and subsequent incorporation into virions. *Journal of Virology*, *91*(3), 10-1128.
<https://doi.org/10.1128/jvi.02000-16>
- Raquin, V., Merkling, S. H., Gausson, V., Moltini-Conclois, I., Frangeul, L., Varet, H., Dillies, M. A., Saleh, M. C., & Lambrechts, L. (2017). Individual co-variation between viral RNA load and gene expression reveals novel host factors during early dengue virus infection of the *Aedes aegypti* midgut. *PLoS Neglected Tropical Diseases*, *11*(12), 1–21.
<https://doi.org/10.1371/journal.pntd.0006152>
- Rozen-Gagnon, K., Stapleford, K. A., Mongelli, V., Blanc, H., Failloux, A. B., Saleh, M. C., & Vignuzzi, M. (2014). Alphavirus Mutator Variants Present Host-Specific Defects and Attenuation in Mammalian and Insect Models. *PLoS Pathogens*, *10*(1).
<https://doi.org/10.1371/journal.ppat.1003877>
- Rupp, J. C., Sokoloski, K. J., Gebhart, N. N., & Hardy, R. W. (2015). Alphavirus RNA synthesis and non-structural protein functions. *Journal of General Virology*, *96*(9), 2483–2500.
<https://doi.org/10.1099/jgv.0.000249>
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682.
<https://doi.org/10.1038/nmeth.2019>
- Sokoloski, K. J., Hayes, C. A., Dunn, M. P., Balke, J. L., Hardy, R. W., & Mukhopadhyay, S. (2012). Sindbis virus infectivity improves during the course of infection in both mammalian

- and mosquito cells. *Virus Research*, 167(1), 26–33.
<https://doi.org/10.1016/j.virusres.2012.03.015>
- Sokoloski, K. J., Snyder, A. J., Liu, N. H., Hayes, C. A., Mukhopadhyay, S., & Hardy, R. W. (2013). Encapsidation of Host-Derived Factors Correlates with Enhanced Infectivity of Sindbis Virus. *Journal of Virology*, 87(22), 12216–12226.
<https://doi.org/10.1128/jvi.02437-13>
- Tonkin-Hill, G., Martincorena, I., Amato, R., Lawson, A. R., Gerstung, M., Johnston, I., Jackson, D. K., Park, N., Lensing, S. V., Quail, M. A., Gonsalves, S., Ariani, C., Spencer Chapman, M., Hamilton, W. L., Meredith, L. W., Hall, G., Jahun, A. S., Chaudhry, Y., Hosmillo, M., ... Kwiatkowski, D. (2021). Patterns of within-host genetic diversity in SARS-COV-2. *ELife*, 10, 1–25. <https://doi.org/10.7554/eLife.66857>
- Weaver, S. C., & Barrett, A. D. T. (2004). Transmission cycles, host range, evolution and emergence of arboviral disease. *Nature Reviews Microbiology*, 2(10), 789–801.
<https://doi.org/10.1038/nrmicro1006>
- Weaver, S. C., Forrester, N. L., Liu, J., & Vasilakis, N. (2021). Population bottlenecks and founder effects: implications for mosquito-borne arboviral emergence. *Nature Reviews Microbiology*, 19(3), 184–195. <https://doi.org/10.1038/s41579-020-00482-8>
- Xi, Z., Ramirez, J. L., & Dimopoulos, G. (2008). The *Aedes aegypti* toll pathway controls dengue virus infection. *PLoS Pathogens*, 4(7). <https://doi.org/10.1371/journal.ppat.1000098>
- Yang, X., Charlebois, P., Macalalad, A., Henn, M. R., & Zody, M. C. (2013). V-Phaser 2: Variant inference for viral populations. *BMC Genomics*, 14(1).
<https://doi.org/10.1186/1471-2164-14-674>

3.8 Chapter 3 figures and tables

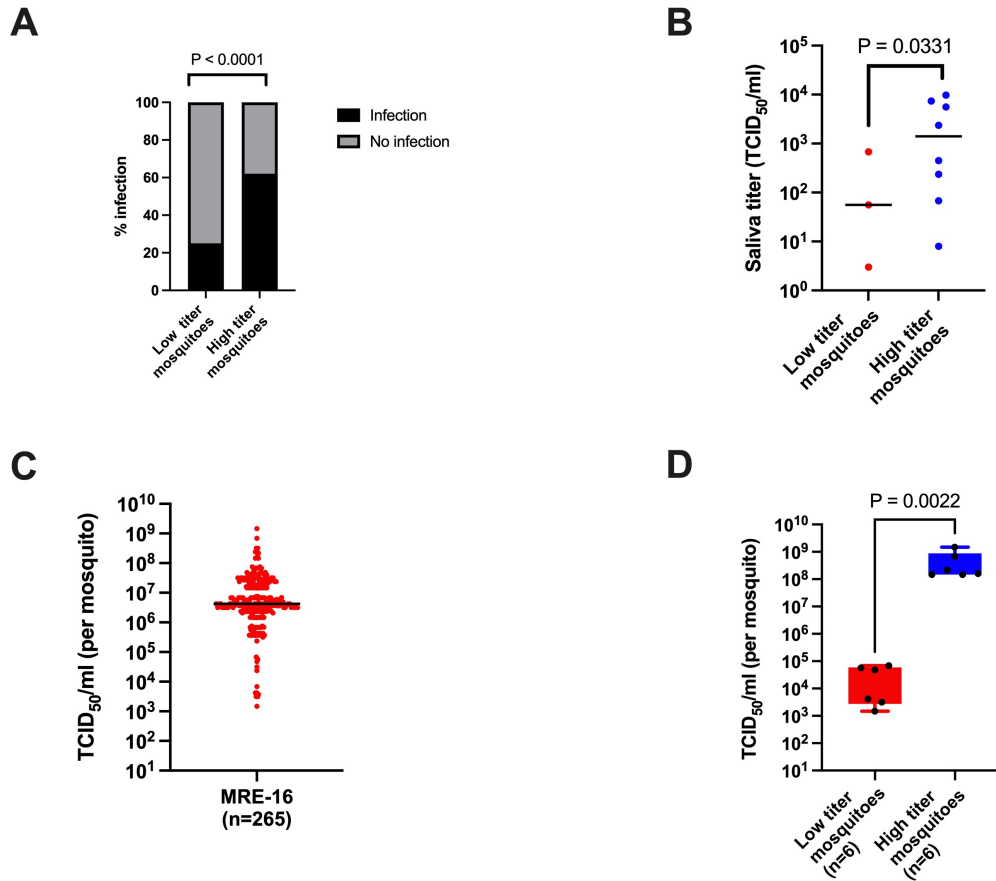


Figure 3.1. Oral infection of *Ae. aegypti* mosquitoes with SINV results in highly variable titers, and virus titer correlates with the presence of infectious virus in saliva.

(A) The proportions of virus-positive saliva samples collected from low titer and high titer mosquitoes. (B) Infectious titers of the virus-positive saliva samples in panel A. (C) Titers of mosquitoes collected at 5 days PBM (n=265). (D) The titers of the 12 low and high titer mosquitoes selected for sequencing. Fisher's exact test was used in panel A, while Mann-Whitney test was used in panels B and D.

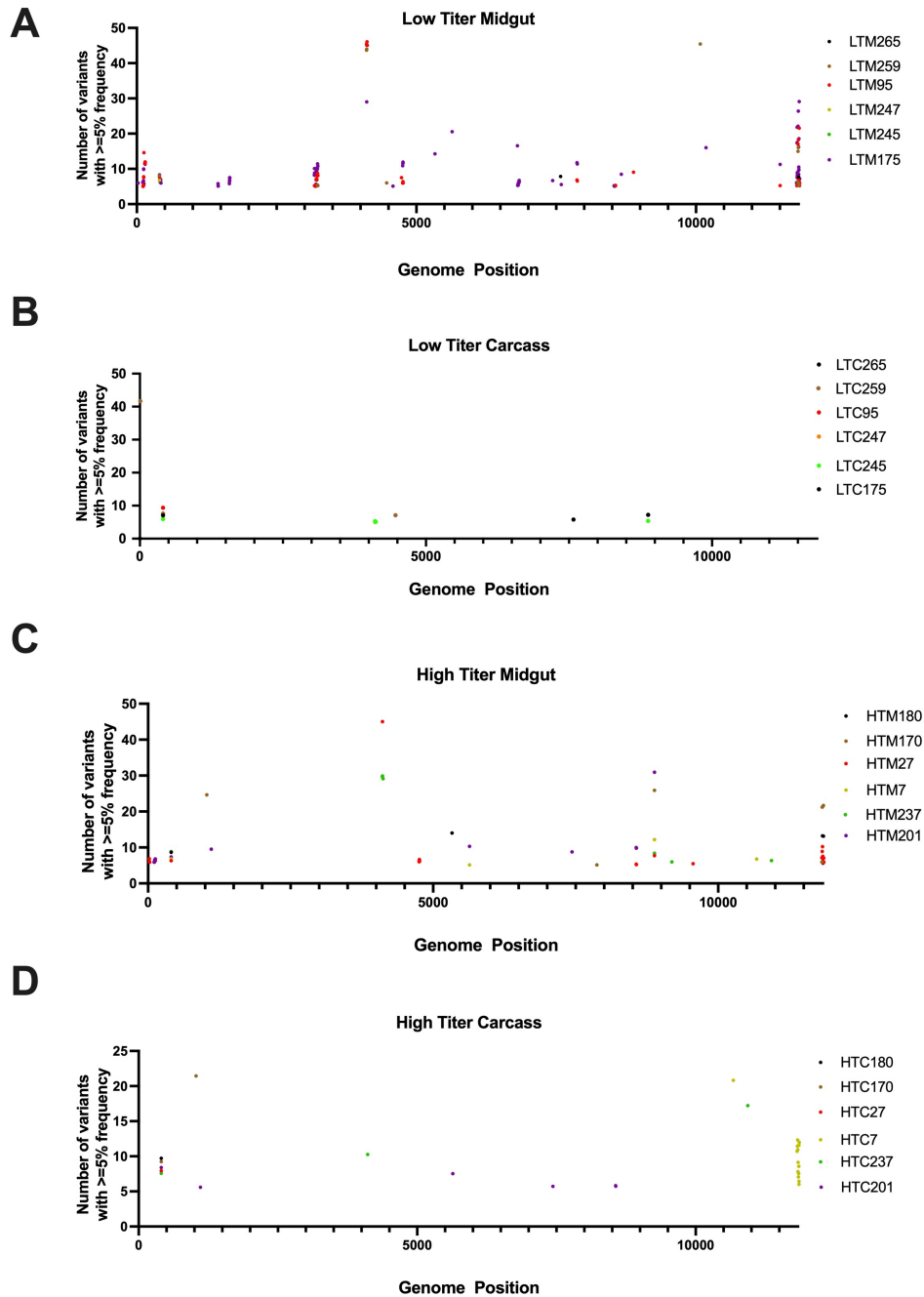


Figure 3.2. SINV variants with frequency $\geq 5\%$.

In (A) midgut and (B) carcass of low titer mosquitoes, and (C) midgut and (D) carcass of high titer mosquitoes. The individual mosquito sample identifiers are listed to the right of each panel. LTM, low titer midgut; LTC, low titer carcass; HTM, high titer midgut; HTC, high titer carcass.

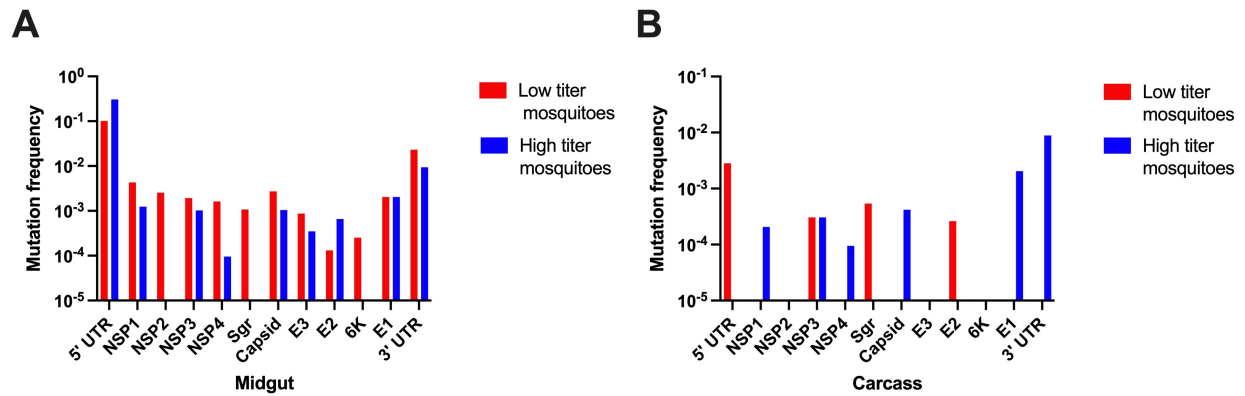


Figure 3.3. Distributions of nucleotide variants with frequency $\geq 5\%$ in the genomes of virus derived from (A) midguts and (B) carcasses.

Mutation frequency was calculated by dividing the number of variants within a given region by the length of the region. Non-structural proteins are represented by NSP1 to NSP4 while the structural proteins are represented by capsid, E3, E2, E, and 6K. Variants present in the 5' UTR, sub-genomic promoter (sgr), and 3' UTR regulatory elements are also shown.

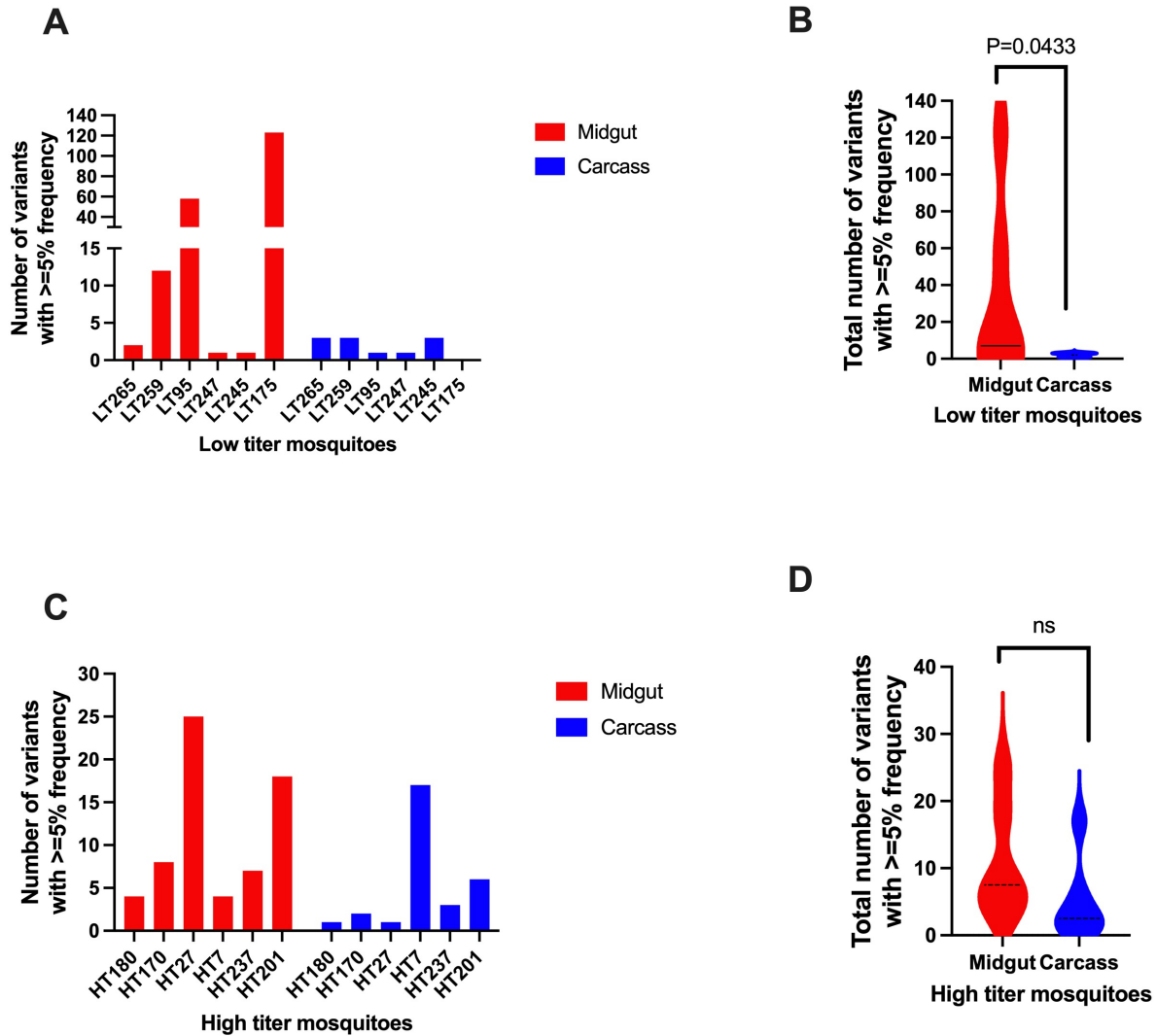


Figure 3.4. Reductions in the numbers of SINV variants following midgut escape in low and high titer mosquitoes.

The numbers of sequence variants in midguts and carcasses of individual low titer (A) and high titer (C) mosquitoes are shown, along with the total number of variants in midguts and carcasses of low titer (B) and high titer (D) mosquitoes. Mann-Whitney test was used for statistical analysis in panels B and D.

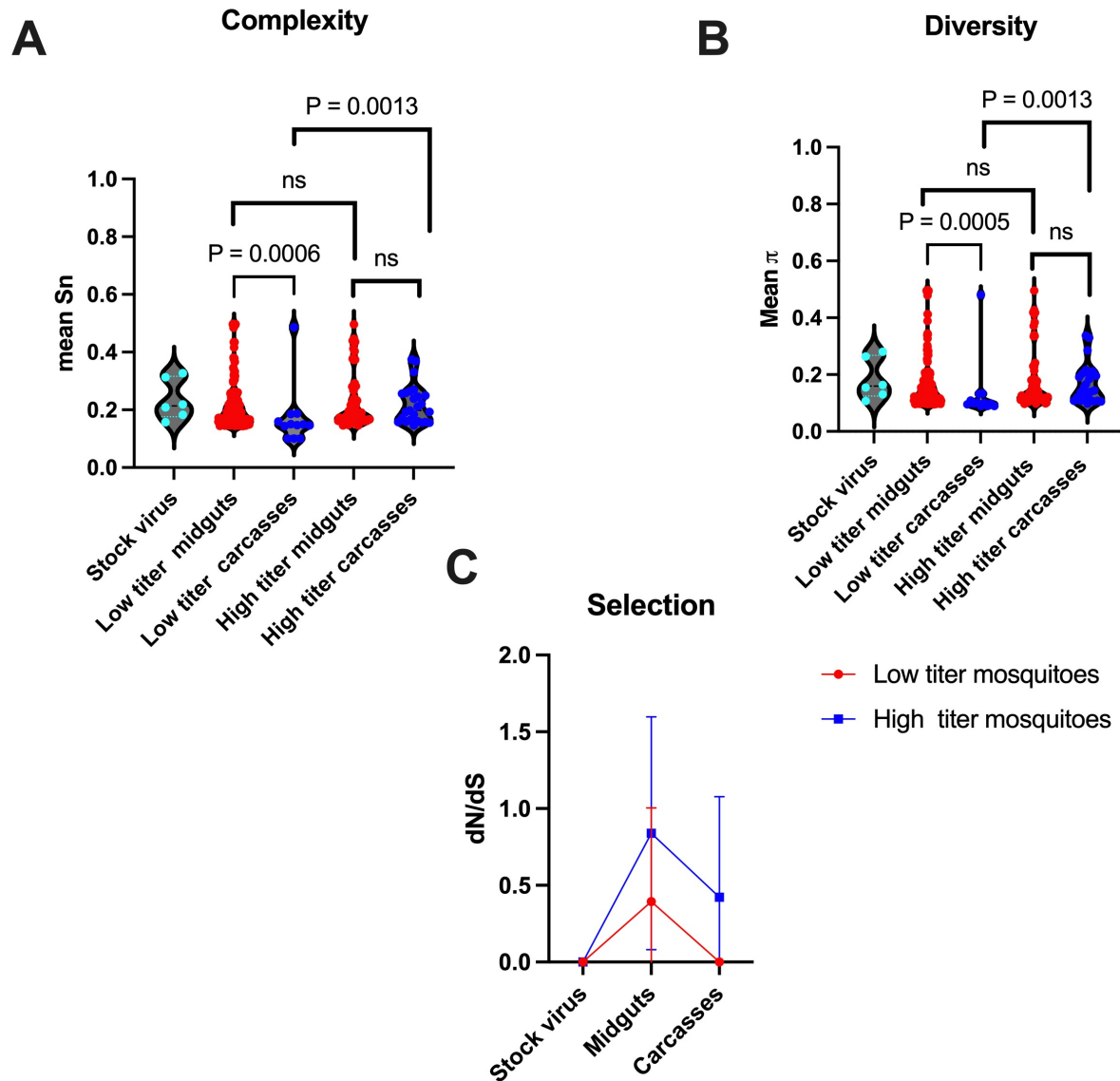


Figure 3.5. Genetic diversity, complexity, and purifying selection in low and high titer mosquitoes.

Both (A) diversity and (B) complexity was reduced in virus populations found in carcasses compared to midguts of low titer mosquitoes, but not of high titer mosquitoes. (C) Stronger purifying selection was observed in both midguts and carcasses of low titer mosquitoes than in high titer mosquitoes. Mann-Whitney test was used for statistical analysis in panels A and B. SINV variants with frequency $\geq 5\%$ were used in the analysis.

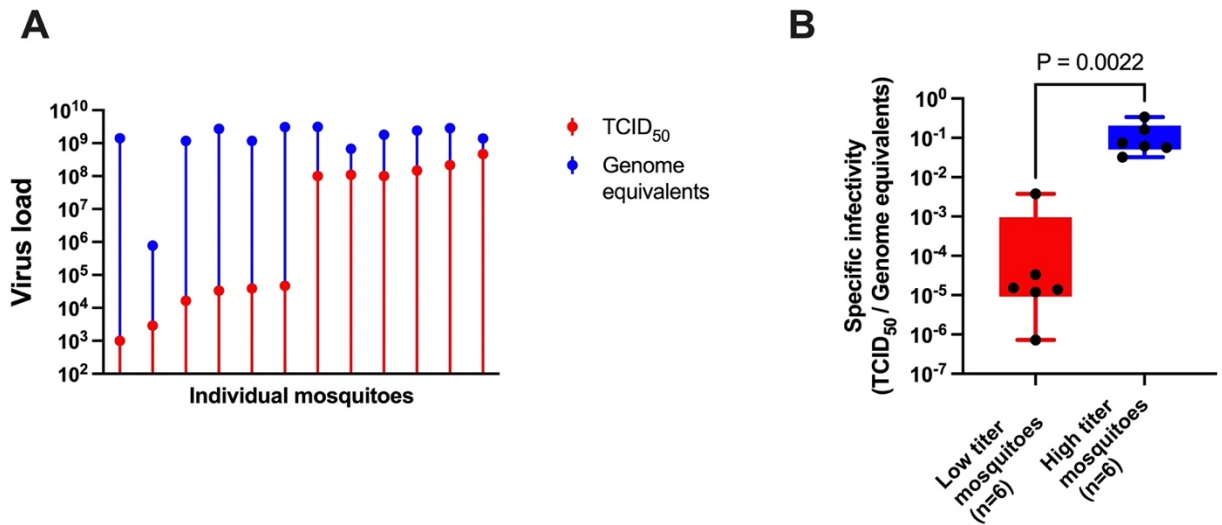


Figure 3.6. SINV genome equivalents do not correspond to virus titer in the carcasses of the 6 individual low titer and 6 high titer mosquitoes used for sequencing.

(A) Genome equivalents and virus titers in carcasses of the individual sequenced low titer and high titer mosquitoes. (B) Specific infectivity in carcasses of the individual low titer and high titer mosquitoes. Mann-Whitney test was used to analyze the results in (B).

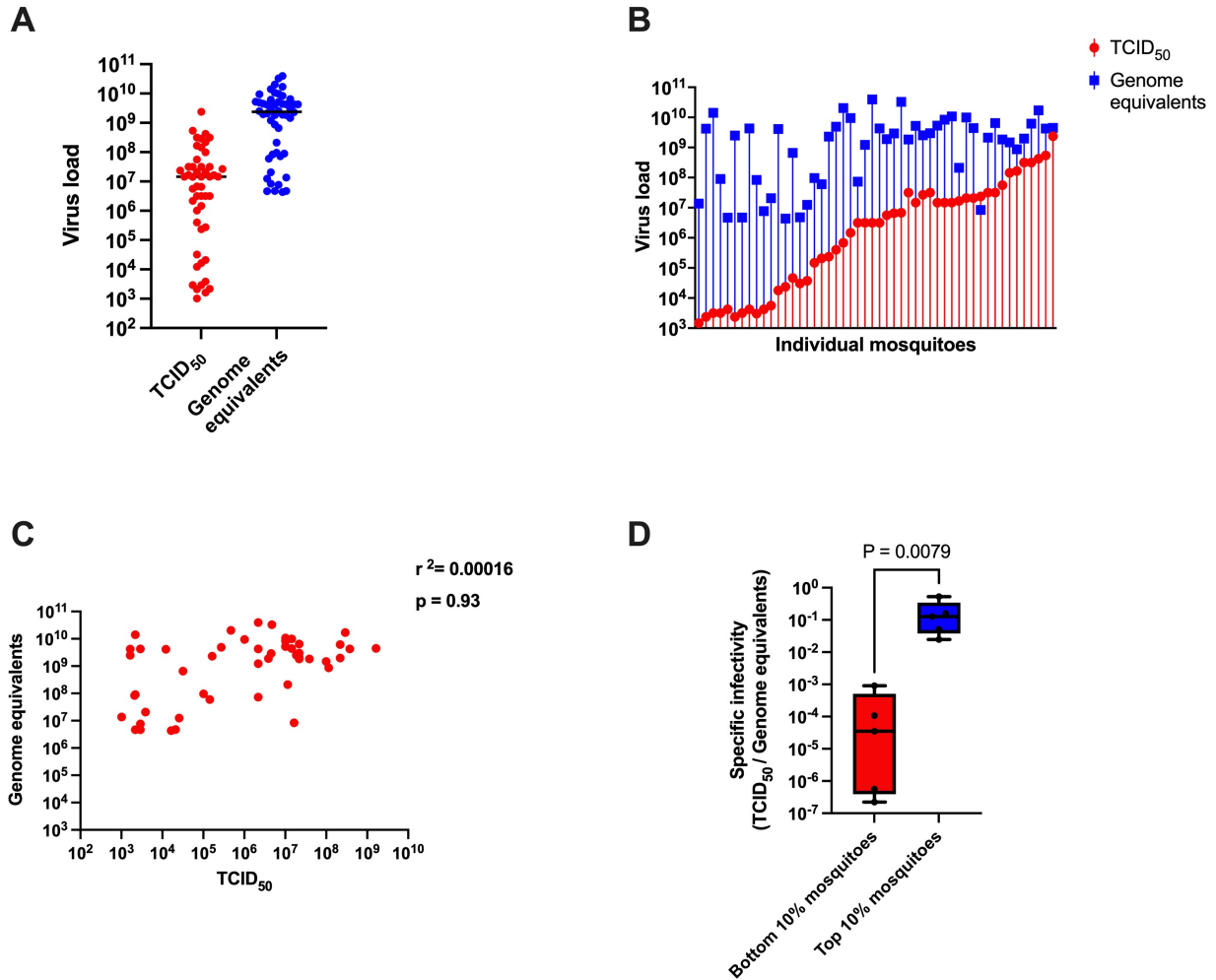


Figure 3.7. Dramatic differences are observed in SI between individual mosquitoes (n = 50).

(A) Genome equivalents and virus titers in individual mosquitoes. (B) Genome equivalents compared to their corresponding virus titers, arranged in ascending order of titer. (C) No significant correlation was observed between genome equivalents and virus titers. (D) SI values of the mosquitoes with the lowest 10% and highest 10% of titers. Pearson correlation test was used in panel C, while Mann Whitney test was used in D.

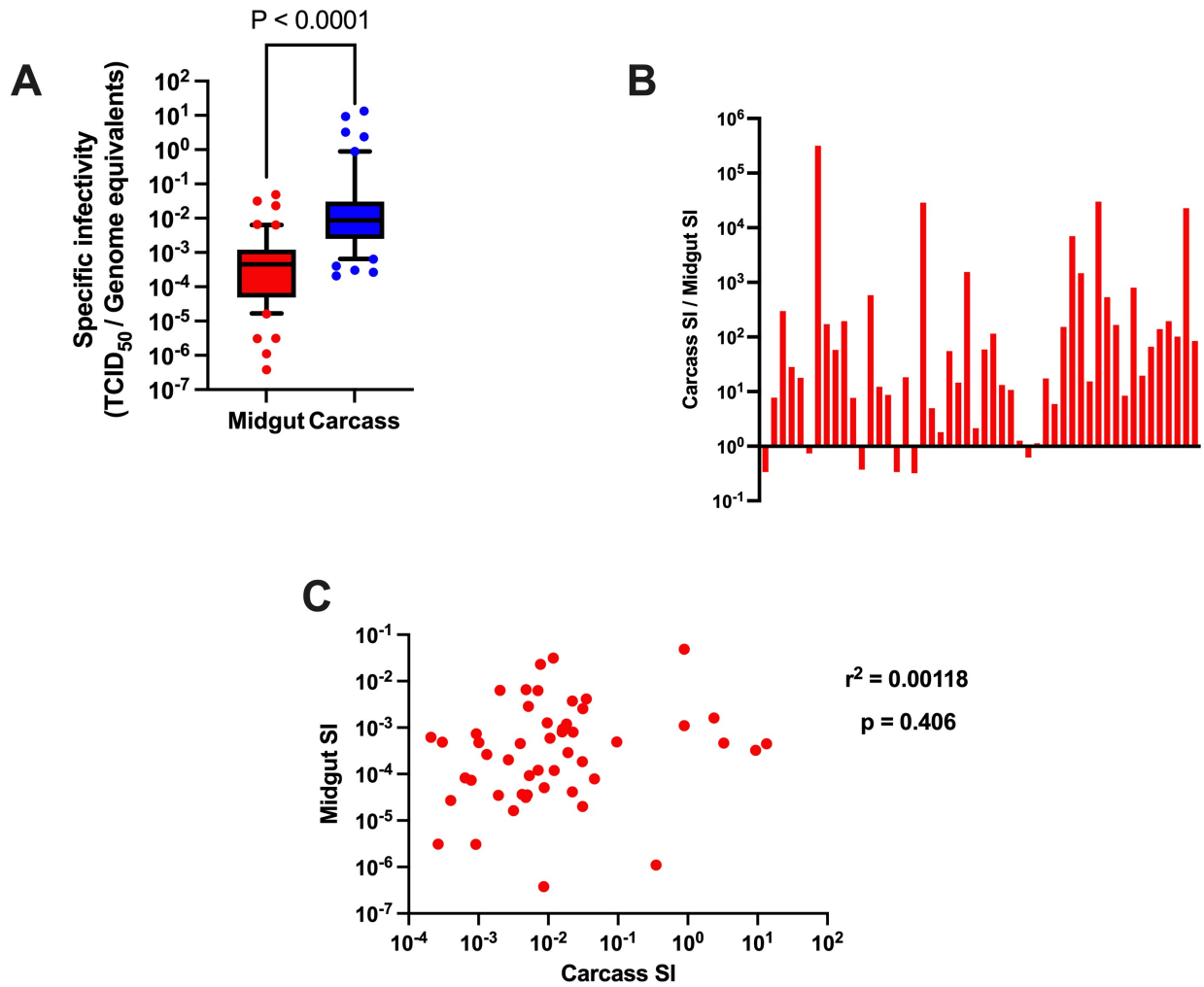


Figure 3.8. Midgut SI is lower than carcass SI in individual mosquitoes (n = 50).

(A) Box and whisker plot of midgut and carcass specific infectivity. (B) Ratio of carcass SI to midgut SI in individual mosquitoes, showing that carcass SI is higher than midgut SI in the majority of mosquitoes. (C) Pearson correlation analysis revealed a lack of significant correlation between midgut SI and carcass SI. Mann-Whitney test was used in panel A.

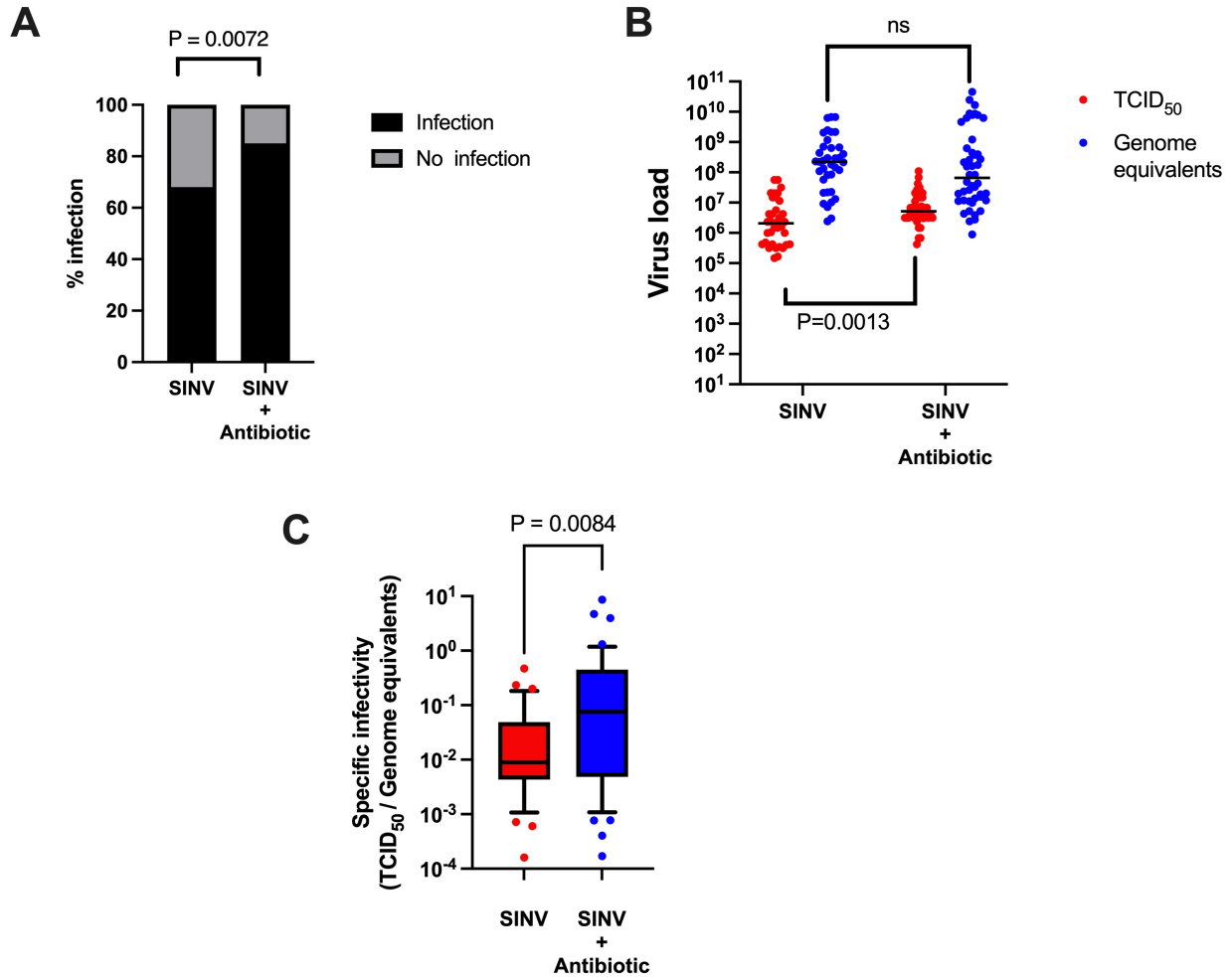


Figure 3.9. Oral treatment of mosquitoes with antibiotics results in higher titers of infectious virus but does not affect viral genome levels.

(A) The proportion of mosquitoes with detectable infectious virus is increased by antibiotic treatment (n=54 for antibiotic-treated and 56 for control). (B) Despite causing an increase in infectious titer, antibiotic treatment does not affect viral genome levels. (C) Specific infectivity is increased by antibiotic treatment. Fisher's exact test was used in panel A, Mann-Whitney test was used in panels B and C.

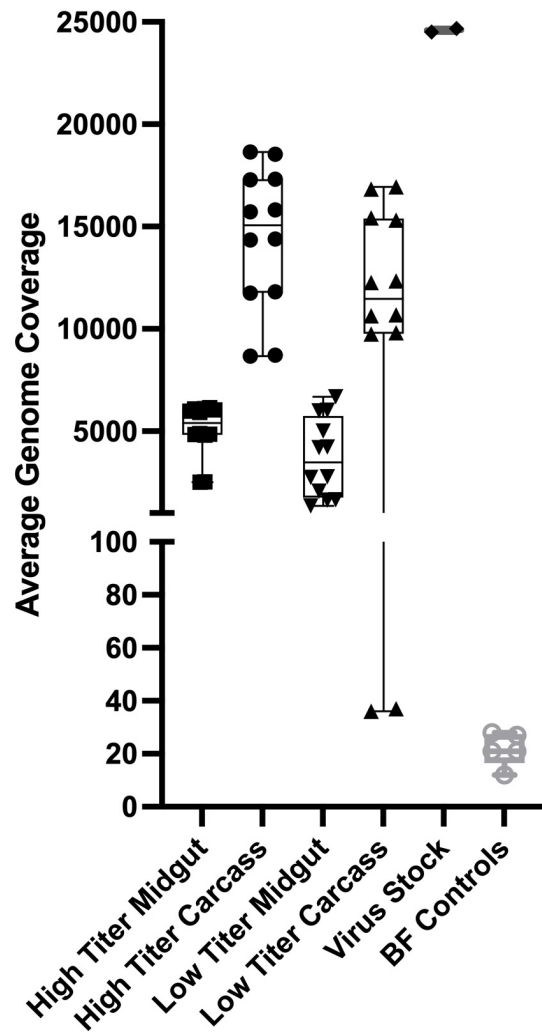


Figure 3.10. (Supplementary): Genome coverage of the SINV genome in samples sequenced in the study.

Each sequencing reaction was loaded in two lanes, resulting in two data points per sample. On average, carcass genome coverage was higher than midgut coverage. Blood fed (BF) controls were midguts and carcasses obtained from uninfected negative control mosquitoes that were given a blood meal lacking SINV.

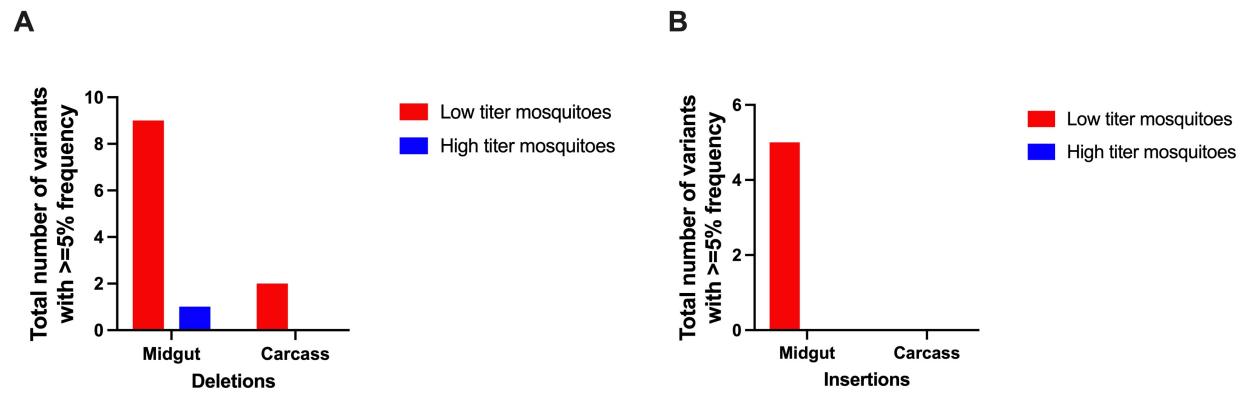


Figure 3.11. (Supplementary): Numbers of (A) deletion and (B) insertion variants present at $\geq 5\%$ frequency in midguts and carcasses of low and high titer mosquitoes.

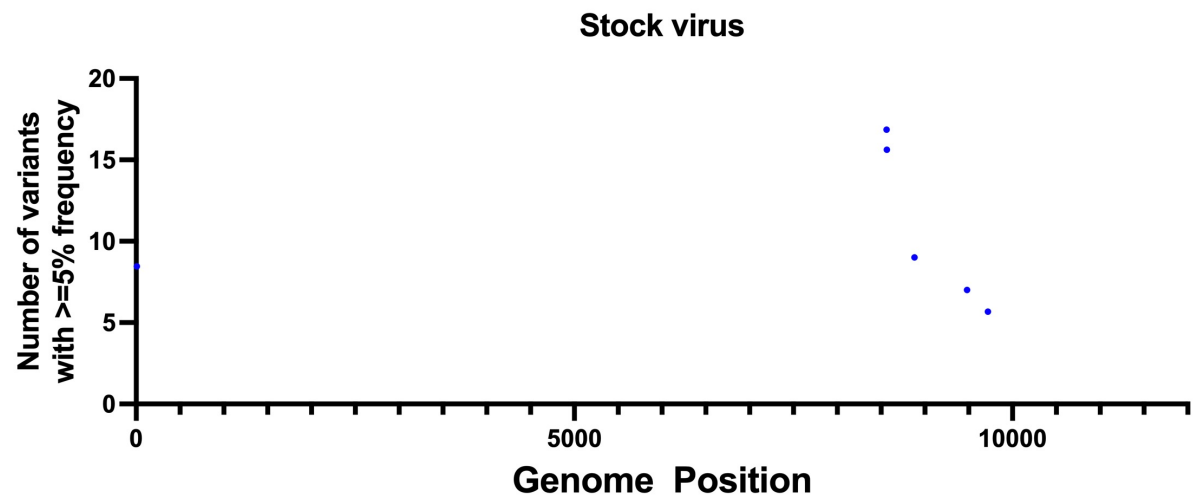


Figure 3.12. (Supplementary): Sequence variants present at $\geq 5\%$ frequency in P2 stock virus.

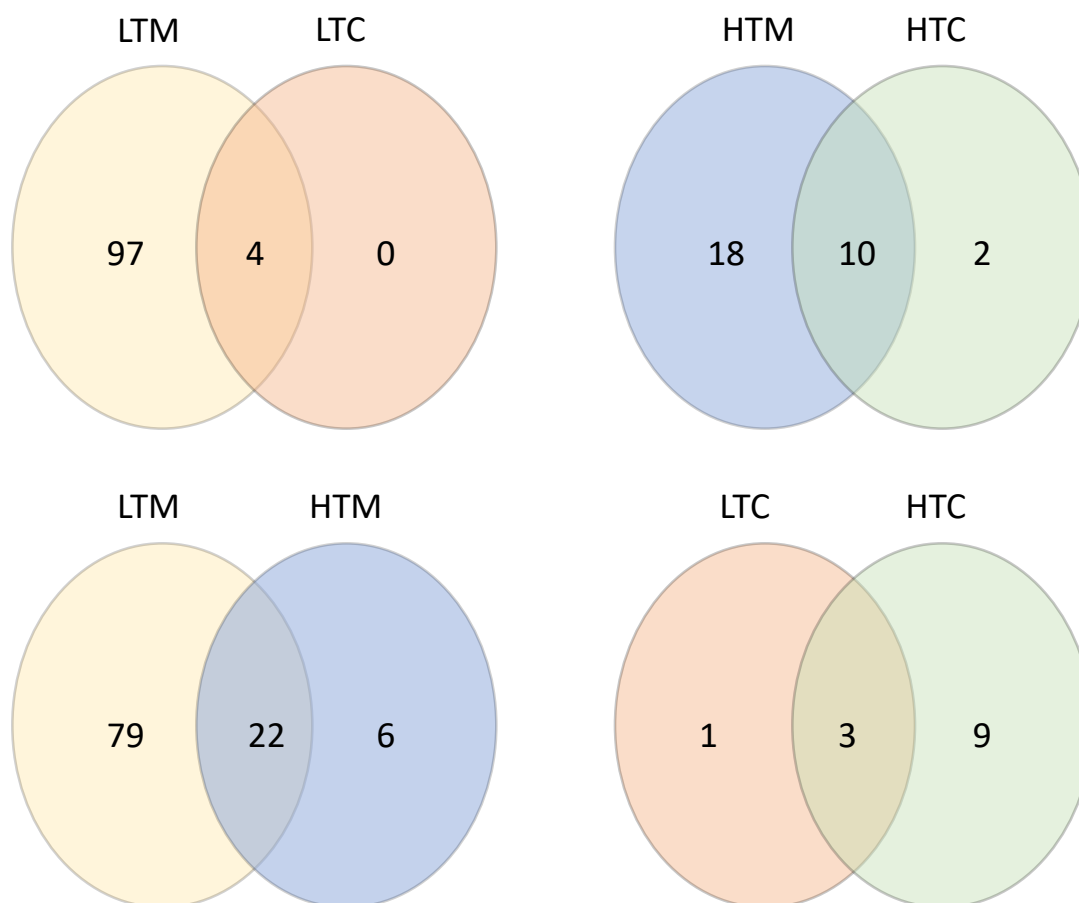


Figure 3.13. (Supplementary): Venn diagrams showing number of unique and shared open reading frame mutations between low titer mosquitoes and high titer mosquitoes.

LTM, low titer midgut; HTM, high titer midgut; LTC, low titer carcass; HTC, high titer carcass.

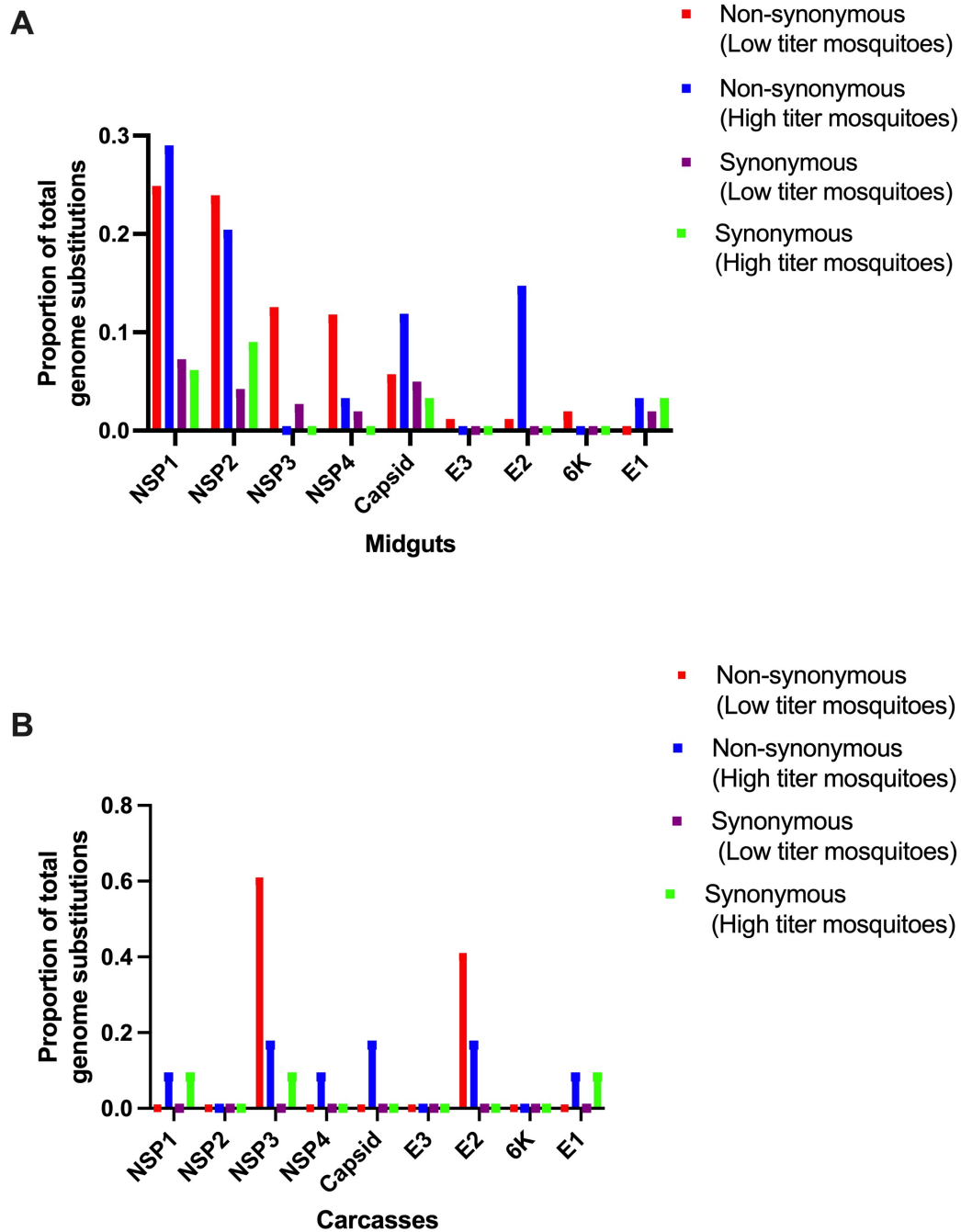


Figure 3.14. (Supplementary): Synonymous and non-synonymous nucleotide substitutions present in the ORF sequences of the virus populations.

From (A) midguts and (B) carcasses of low and high titer mosquitoes, shown as the proportion of total nucleotide substitutions in the viral genome.

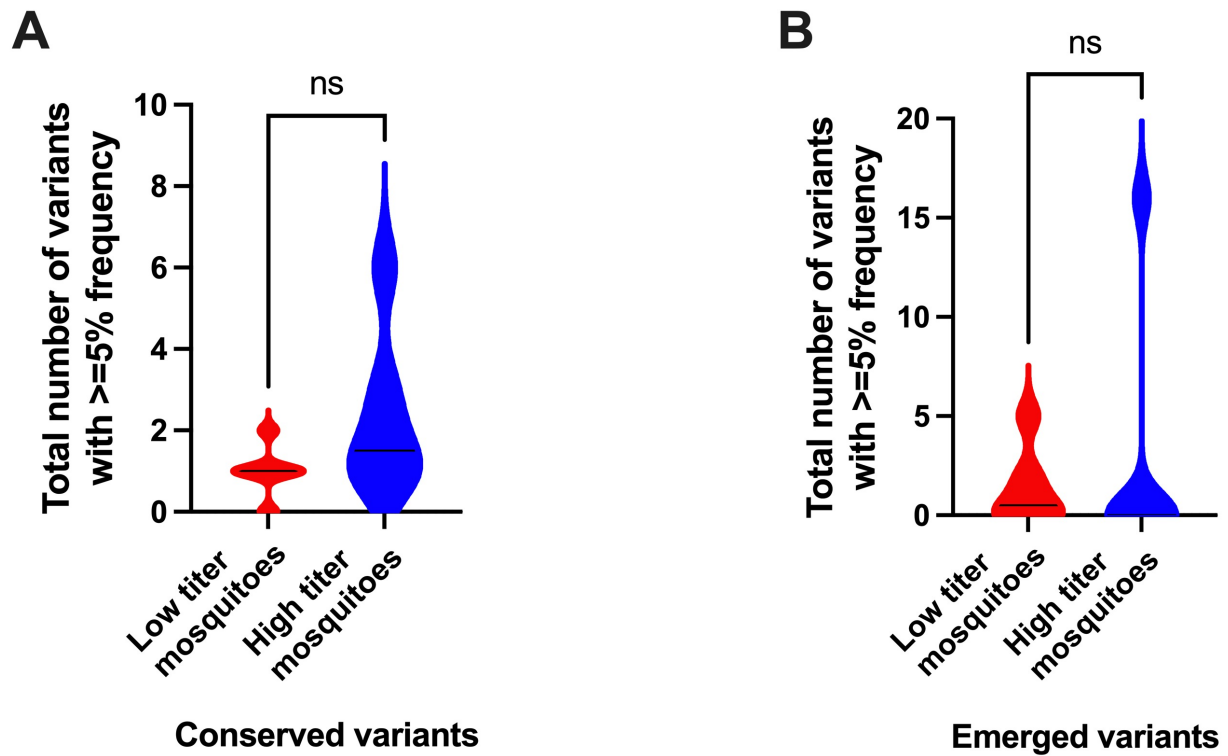


Figure 3.15. (Supplementary): Variants present at $\geq 5\%$ frequency.

That were (A) conserved and (B) emerged variants in low titer and high titer mosquitoes.

Conserved variants were those present in both midgut and carcass, while emerged variants were present in the carcass but absent in the midgut.

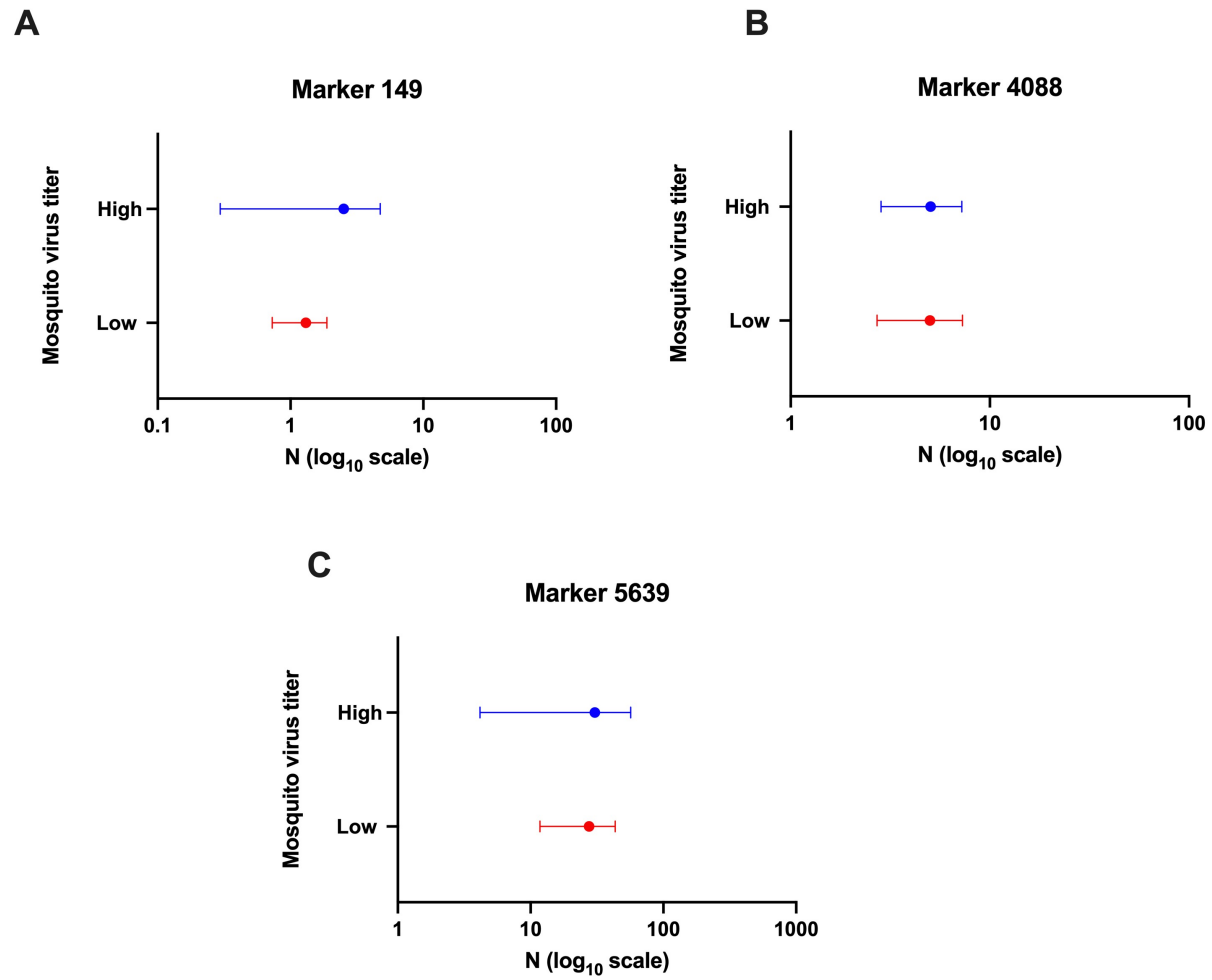


Figure 3.16. (Supplementary): Midgut bottleneck size estimation using nucleotide positions (A) 149, (B) 4088, and (C) 5639 in the viral genome.

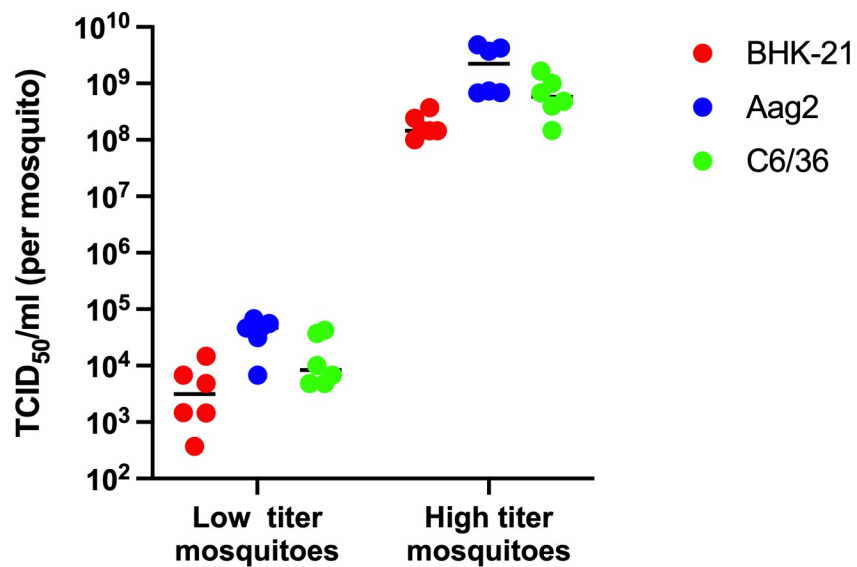
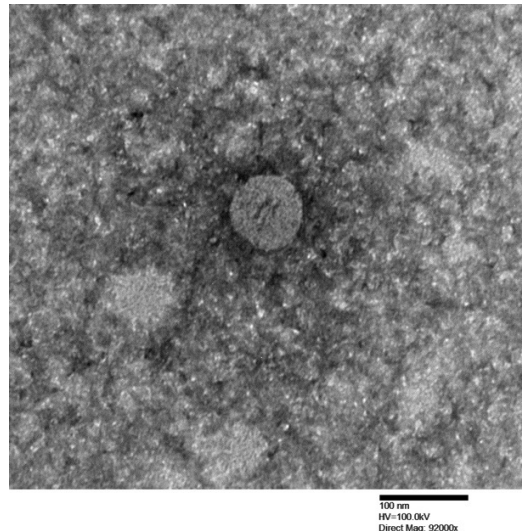
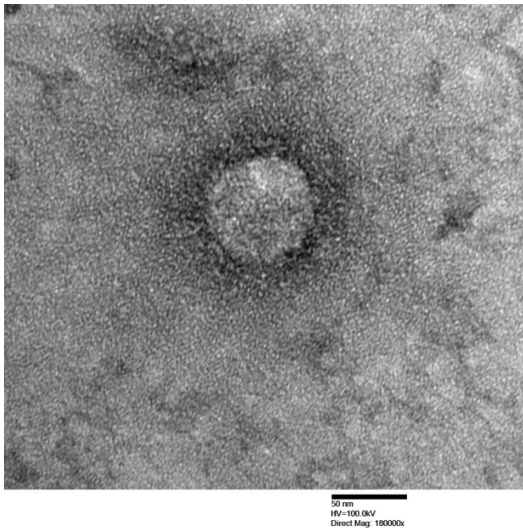


Figure 3.17. (Supplementary): Titration of infectious virus using mosquito C6/36 and Aag2 cells. Low titer and high titer stocks (6 of each) that had previously been titered by $TCID_{50}$ in BHK cells were re-titered using an immunofluorescence-based $TCID_{50}$ assay in C6/36 and Aag2 cells.

A



B

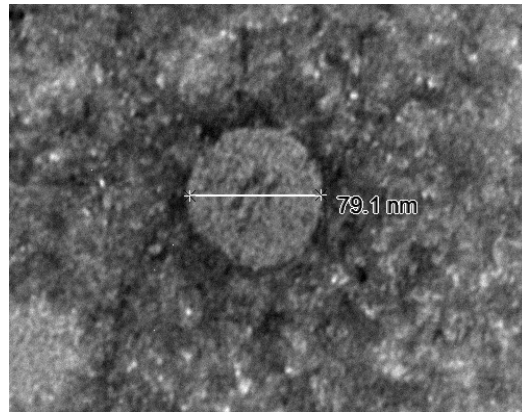
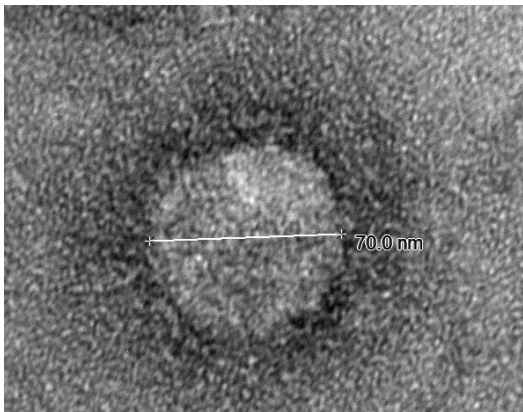


Figure 3.18. Example images of SINV-like virus particles in high titer mosquito homogenates obtained by negative staining and TEM.

(A) Two images that were obtained at different magnification (note scale bars below). (B) Estimated sizes are shown of the particles in (A), as measured using Image J software.

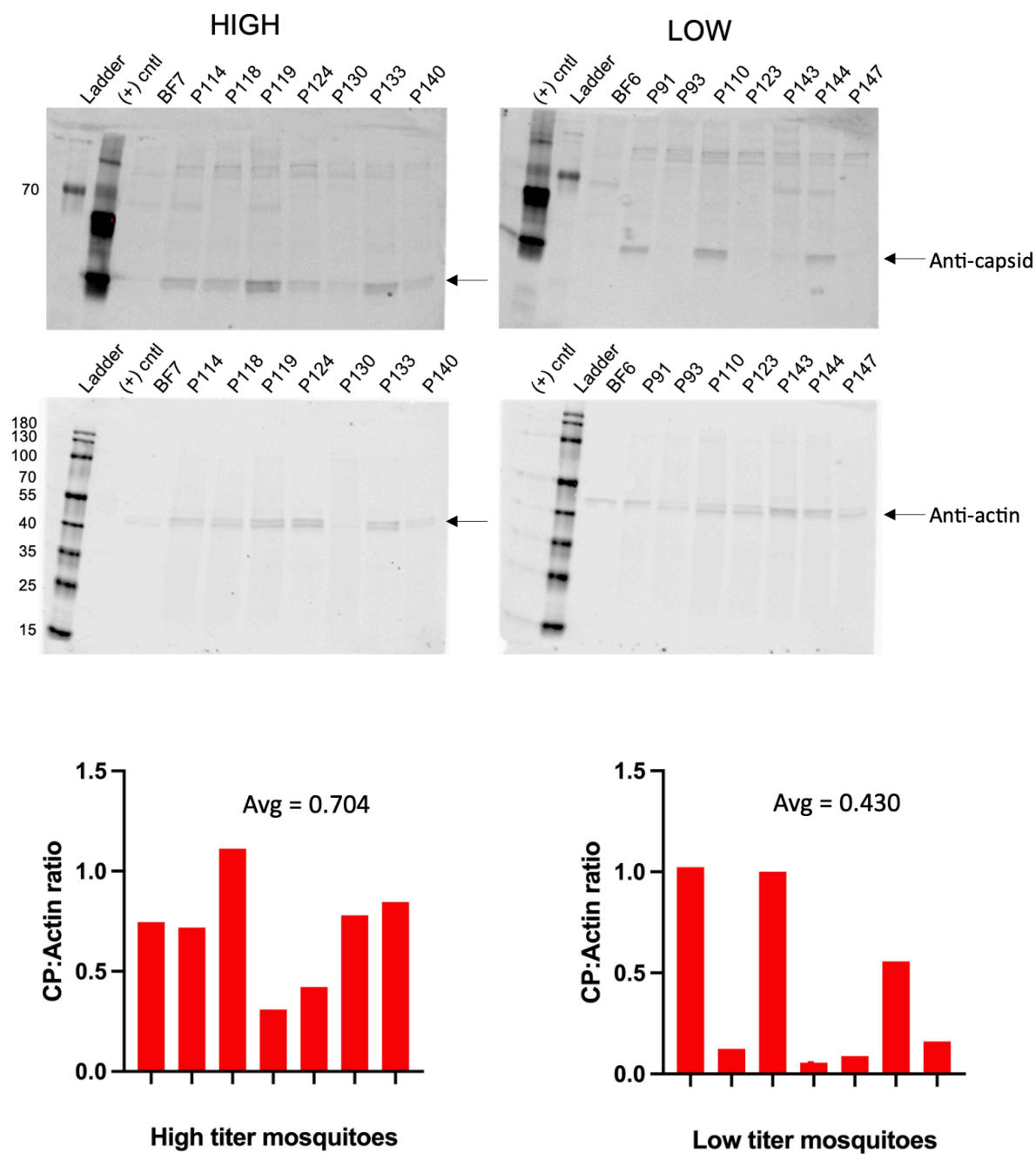


Figure 3.19. (Supplementary): Immunoblots of SINV capsid of low titer and high titer mosquitoes.

Immunoblots of lysates prepared from 7 high ($\geq 1.8 \times 10^8$ TCID₅₀/mL) and 7 low ($\leq 6.8 \times 10^4$ TCID₅₀/mL) titer mosquitoes probed with anti-capsid antibody or anti-actin antibody as a loading

control. The migration of size markers is indicated on the left. Arrows indicate migration of the proteins of interest. (+) cntl, positive control SINV stock; Ladder, mw size markers (in KDa); BF, blood-fed uninfected control mosquitoes. Individual mosquito samples are indicated by their number labels starting with P. The blots shown are representative of consistent results obtained by blotting the same lysates 4 times. Shown below are the results of quantification of the capsid bands in each sample after normalization to actin.

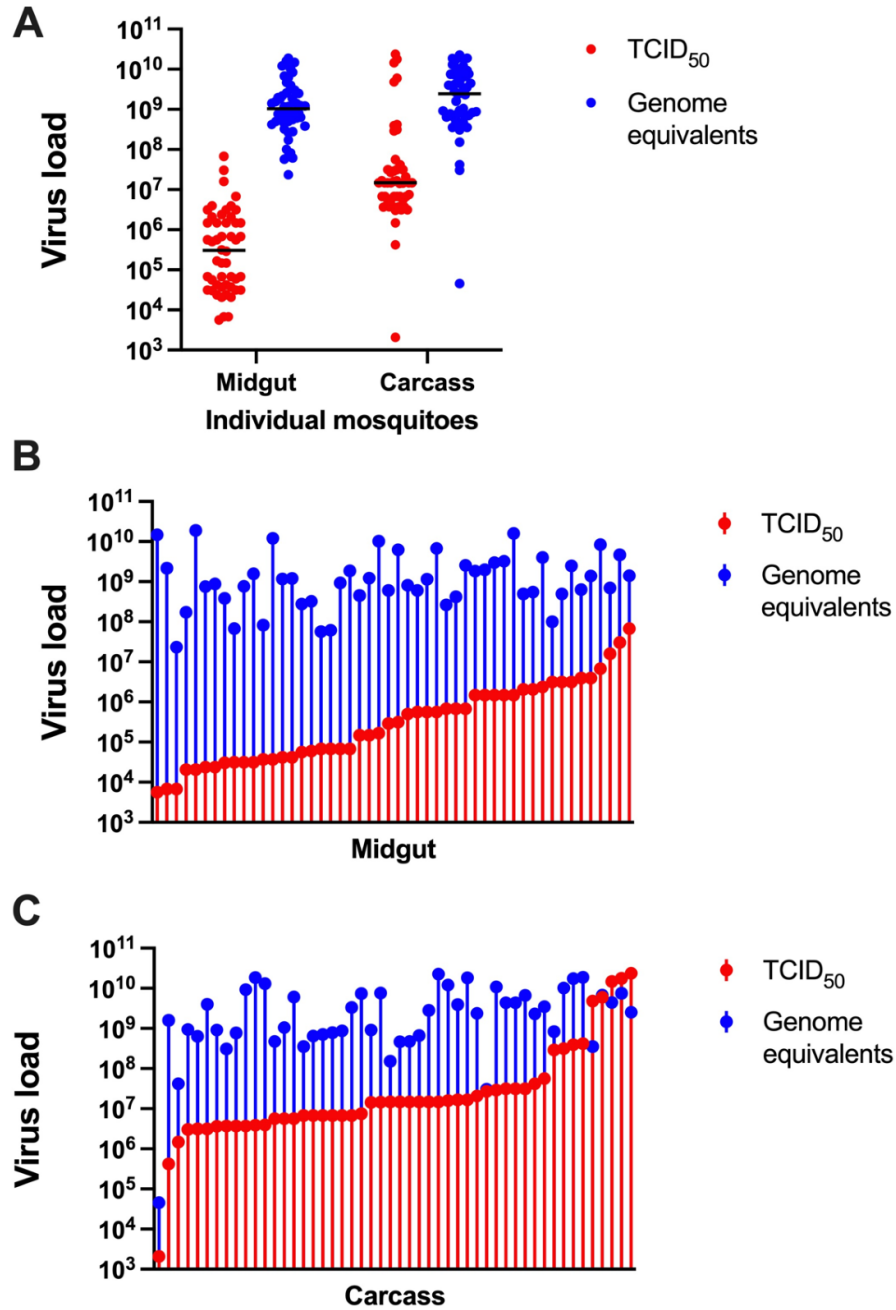


Figure 3.20. (Supplementary): Virus load in mosquito midguts versus carcasses (data corresponding to Figure 3.8).

(A) Overall virus titers and genome equivalents in midguts and carcasses (n = 50). (B) Virus load in midguts, arranged in ascending order of midgut titer. (C) Virus load in carcasses, arranged in ascending order of carcass titer.

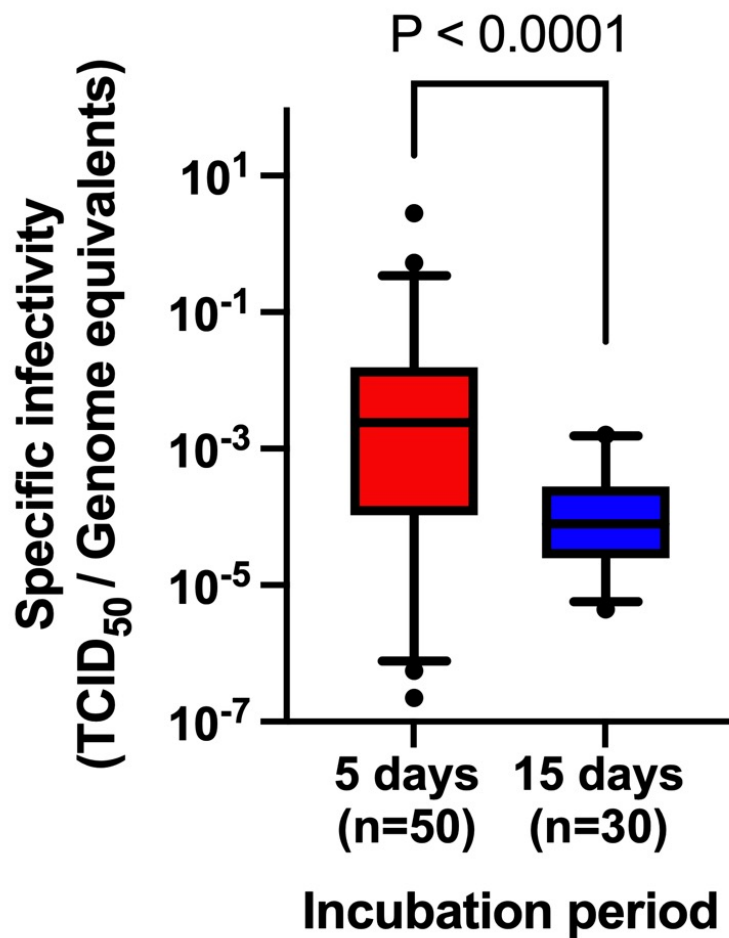


Figure 3.21. (Supplementary): Specific infectivity of mosquitoes at 5 days compared to 15 days PBM.

Mann-Whitney test was used for statistical analysis. n = 50 for 5 days and n = 30 for 15 days.

The data used for 5 days PBM was obtained from the experiment shown in Figure 3.7.

Table 3.1 (Supplementary). Genomic boundary positions of ORFs and other genome elements in the 5' dsMRE16ic genome.

Sequence	Start	End
5'UTR	1	59
NSP1	60	1679
NSP2	1680	4100
NSP3	4101	5735
NSP4	5736	7484
subgenomic promoter region	7485	7795
Capsid	7796	8592
E3	8593	8784
E2	8785	10055
6K	10056	10219
E1	10220	11539
3'UTR	11540	11858

Table 3.2 (Supplementary): Numbers of virus particles similar in size and shape to SINV that were observed in homogenates from high, medium and low titer mosquitoes.

Multiple fields of view were scanned for each sample, covering the entire TEM grid. Titer ranges were as follows: high titer, 1.9×10^8 - 4.5×10^8 TCID₅₀/mL; medium titer 1.5×10^5 - 3.0×10^6 TCID₅₀/mL; low titer 3.2×10^3 - 4.0×10^4 TCID₅₀/mL.

SAMPLE TYPE AND ID	NUMBER OF PARTICLES OBSERVED
HIGH TITER	
EL4	>10
EL7	>10
EL55	>10
MEDIUM TITER	
EL57	0
EL94	0
EL6	0
LOW TITER	
EL2	1
EL19	1
EL59	0

Table 3.3 (Supplementary): Mutations found in coding regions of the SINV genome in midguts and carcasses of low titer mosquitoes.

Midgut							
Nucleotide position	Variant	Reference	Original amino acid	Change in amino acid	Amino acid position	ORF	Synonymous or Non-synonymous
107	G	C	Phenylalanine	Leucine	16	NSP1	Non-synonymous
108	C	A	Isoleucine	Leucine	17	NSP1	Non-synonymous
111	G	A	Threonine	Alanine	18	NSP1	Non-synonymous
114	T	C	Glutamine	Stop/End	19	NSP1	Non-synonymous
116	G	A	Glutamine	Glutamine	19	NSP1	Synonymous
118	C	T	Leucine	Proline	20	NSP1	Non-synonymous
120	T	C	Glutamine	Stop/End	21	NSP1	Non-synonymous
122	C	G	Glutamine	Histidine	21	NSP1	Non-synonymous
122	T	G	Glutamine	Histidine	21	NSP1	Non-synonymous
123	G	A	Lysine	Glutamate	22	NSP1	Non-synonymous
126	C	A	Serine	Arginine	23	NSP1	Non-synonymous
128	A	T	Serine	Arginine	23	NSP1	Non-synonymous
143	C	G	Glutamine	Aspartic acid	28	NSP1	Non-synonymous
144	A	G	Valine	Isoleucine	29	NSP1	Non-synonymous
145	A	T	Valine	Glutamic acid	29	NSP1	Non-synonymous
149	C	A	Valine	Valine	30	NSP1	Synonymous
150	A	G	Alanine	Threonine	31	NSP1	Non-synonymous
420	T	C	Leucine	Phenylalanine	121	NSP1	Non-synonymous
422	T	C	Leucine	Leucine	121	NSP1	Synonymous
423	G	C	Arginine	Glycine	122	NSP1	Non-synonymous

427	G	C	Threonine	Arginine	123	NSP1	Non-synonymous
431	T	G	Valine	Valine	124	NSP1	Synonymous
1452	C	T	Phenylalanine	Leucine	465	NSP1	Non-synonymous
1454	T	C	Phenylalanine	Phenylalanine	465	NSP1	Non-synonymous
1455	A	C	Arginine	Arginine	466	NSP1	Synonymous
1456	C	G	Arginine	Proline	466	NSP1	Non-synonymous
1655	T	A	Glutamic acid	Aspartate	532	NSP1	Non-synonymous
1657	T	G	Glycine	Valine	533	NSP1	Non-synonymous
1659	G	C	Leucine	Valine	534	NSP1	Non-synonymous
1660	C	T	Leucine	Proline	534	NSP1	Non-synonymous
1661	G	A	Leucine	Leucine	534	NSP1	Synonymous
1662	G	C	Glutamine	Glutamate	535	NSP1	Synonymous
1663	C	A	Glutamine	Proline	535	NSP1	Non-synonymous
3174	C	G	Glycine	Arginine	1039	NSP2	Non-synonymous
3176	C	G	Glycine	Glycine	1039	NSP2	Synonymous
3180	A	C	Glutamine	Lysine	1041	NSP2	Non-synonymous
3181	G	A	Glutamine	Arginine	1041	NSP2	Non-synonymous
3184	T	G	Tryptophan	Leucine	1042	NSP2	Non-synonymous
3186	G	A	Serine	Glycine	1043	NSP2	Non-synonymous
3187	T	G	Serine	Isoleucine	1043	NSP2	Non-synonymous
3194	T	G	Leucine	Phenylalanine	1045	NSP2	Non-synonymous
3196	A	T	Phenylalanine	Tyrosine	1046	NSP2	Non-synonymous
3198	T	C	Proline	Serine	1047	NSP2	Non-synonymous
3200	C	A	Proline	Proline	1047	NSP2	Synonymous
3202	C	A	Glutamine	Proline	1048	NSP2	Non-synonymous

3206	A	C	Phenylalanine	Leucine	1049	NSP2	Non-synonymous
3208	T	C	Alanine	Valine	1050	NSP2	Non-synonymous
3211	T	A	Aspartate	Valine	1051	NSP2	Non-synonymous
3213	T	G	Aspartic acid	Tyrosine	1052	NSP2	Non-synonymous
3216	C	A	Lysine	Glutamine	1053	NSP2	Non-synonymous
3217	G	A	Lysine	Arginine	1053	NSP2	Non-synonymous
3219	A	C	Proline	Threonine	1054	NSP2	Non-synonymous
3220	G	C	Proline	Arginine	1054	NSP2	Non-synonymous
3223	T	A	Histidine	Leucine	1055	NSP2	Non-synonymous
3227	C	G	Serine	Serine	1056	NSP2	Synonymous
3229	G	C	Alanine	Glycine	1057	NSP2	Non-synonymous
4111	A	G	Tyrosine	Cysteine	1351	NSP3	Non-synonymous
4122	C	A	Arginine	Serine	1355	NSP3	Non-synonymous
4468	A	T	Valine	Aspartic acid	1470	NSP3	Non-synonymous
4581	C	T	Serine	Proline	1508	NSP3	Non-synonymous
4733	A	C	Alanine	Alanine	1558	NSP3	Synonymous
4754	A	G	Lysine	Lysine	1565	NSP3	Synonymous
4756	G	T	Valine	Glycine	1566	NSP3	Non-synonymous
4761	C	T	Phenylalanine	Leucine	1568	NSP3	Non-synonymous
4762	G	T	Phenylalanine	Cysteine	1568	NSP3	Non-synonymous
4763	A	T	Phenylalanine	Leucine	1568	NSP3	Non-synonymous
5332	T	A	Aspartic acid	Valine	1758	NSP3	Non-synonymous
5639	G	A	Glycine	Glycine	1860	NSP3	Synonymous
6806	C	T	Aspartic acid	Aspartic acid	2248	NSP4	Synonymous
6811	C	A	Aspartic acid	Alanine	2250	NSP4	Non-synonymous

6816	G	A	Isoleucine	Valine	2252	NSP4	Non-synonymous
6819	C	A	Isoleucine	Leucine	2253	NSP4	Non-synonymous
6821	G	C	Isoleucine	Methionine	2253	NSP4	Non-synonymous
6823	G	C	Alanine	Glycine	2253	NSP4	Non-synonymous
6826	G	A	Glutamate	Glycine	2255	NSP4	Non-synonymous
6828	A	C	Histidine	Asparagine	2256	NSP4	Non-synonymous
6829	T	A	Histidine	Leucine	2256	NSP4	Non-synonymous
6830	C	T	Histidine	Histidine	2256	NSP4	Synonymous
6831	G	T	Phenylalanine	Valine	2257	NSP4	Non-synonymous
6832	C	T	Phenylalanine	Serine	2257	NSP4	Non-synonymous
6833	A	T	Phenylalanine	Leucine	2257	NSP4	Non-synonymous
6834	A	C	Histidine	Asparagine	2258	NSP4	Non-synonymous
6838	C	A	Glutamine	Proline	2259	NSP4	Non-synonymous
6839	T	G	Glutamine	Histidine	2259	NSP4	Non-synonymous
7436	G	A	Isoleucine	Methionine	2458	NSP4	Non-synonymous
7873	A	G	Arginine	Arginine	26	Capsid	Synonymous
7877	A	C	Glutamine	Lysine	28	Capsid	Non-synonymous
8536	G	C	Valine	Valine	247	Capsid	Synonymous
8539	G	C	Threonine	Threonine	248	Capsid	Synonymous
8542	C	G	Tryptophan	Cysteine	249	Capsid	Non-synonymous
8545	A	T	Asparagine	Lysine	250	Capsid	Non-synonymous
8548	A	C	Serine	Arginine	251	Capsid	Non-synonymous
8551	A	G	Lysine	Lysine	252	Capsid	Synonymous
8556	C	A	Lysine	Threonine	254	Capsid	Non-synonymous
8557	C	G	Lysine	Asparagine	254	Capsid	Non-synonymous

8564	G	A	Lysine	Glutamine	257	Capsid	Non-synonymous
8664	T	C	Threonine	Isoleucine	290	E3	Non-synonymous
8883	G	A	Glutamine	Arginine	363	E2	Non-synonymous
10077	C	T	Threonine	Methionine	761	6K	Non-synonymous
10179	G	T	Phenylalanine	Serine	795	6K	Non-synonymous
11506	A	T	Alanine	Alanine	1237	E1	Synonymous
Carcass							
Nucleotide position	Variant	Reference	Original amino acid	Change in amino acid	Amino acid position	ORF	Synonymous or Non-synonymous
4111	G	A	Tyrosine	Cysteine	1351	NSP3	Non-synonymous
4122	A	C	Arginine	Serine	1355	NSP3	Non-synonymous
4468	A	T	Valine	Aspartic acid	1470	NSP3	Non-synonymous
8883	G	A	Glutamine	Arginine	363	E2	Non-synonymous

Table 3.4 (Supplementary): Mutations found in non-coding regions of the SINV genome in midguts and carcasses of low titer mosquitoes.

Midgut			
Nucleotide position	Variant	Reference	Genome element
22	A	T	5' UTR
7579	T	C	Subgenomic promoter
7579	A	C	Subgenomic promoter
11805	T	A	3' UTR
11807	T	C	3' UTR
11808	G	A	3' UTR
11809	T	A	3' UTR
11809	G	A	3' UTR
11810	G	T	3' UTR
11812	C	T	3' UTR
11812	G	T	3' UTR
11813	T	A	3' UTR
11813	G	A	3' UTR
11813	C	A	3' UTR
11814	C	T	3' UTR
11815	A	T	3' UTR
11815	C	T	3' UTR
11816	G	A	3' UTR
11816	T	A	3' UTR
11817	A	T	3' UTR
11819	A	T	3' UTR
11819	C	T	3' UTR
11821	A	C	3' UTR
11822	G	T	3' UTR
11823	A	T	3' UTR
11824	A	T	3' UTR
11827	A	T	3' UTR
11828	A	T	3' UTR
11828	C	T	3' UTR
11829	A	T	3' UTR
11830	A	T	3' UTR
11831	T	A	3' UTR
11832	G	T	3' UTR
11832	A	T	3' UTR

11832	C	T	3' UTR
11833	G	T	3' UTR
11834	A	C	3' UTR
11834	G	C	3' UTR
11834	T	C	3' UTR
11835	T	A	3' UTR
11836	T	C	3' UTR
11836	A	C	3' UTR
11837	T	A	3' UTR
11838	A	T	3' UTR
11839	T	A	3' UTR
11840	C	A	3' UTR
11840	T	A	3' UTR
11841	G	T	3' UTR
11841	A	T	3' UTR
11844	G	T	3' UTR
11844	A	T	3' UTR
11845	C	G	3' UTR
11845	T	G	3' UTR
11846	A	T	3' UTR
11852	T	A	3' UTR
11854	T	A	3' UTR
Carcass			
Nucleotide position	Variant	Reference	Genome element
22	A	T	5' UTR
7579	T	C	Subgenomic promoter

Table 3.5 (Supplementary): Mutations found in coding regions of the SINV genome in midguts and carcasses of high titer mosquitoes.

Midgut							
Nucleotide position	Variant	Reference	Original amino acid	Change in amino acid	Amino acid position	ORF	Synonymous or Non-synonymous
107	G	C	Phenylalanine	Leucine	16	NSP1	Non-synonymous
108	C	A	Isoleucine	Leucine	17	NSP1	Non-synonymous
111	G	A	Threonine	Alanine	18	NSP1	Non-synonymous
114	T	C	Glutamine	Stop/End	19	NSP1	Non-synonymous
116	G	A	Glutamine	Glutamine	19	NSP1	Synonymous
118	C	T	Leucine	Proline	20	NSP1	Non-synonymous
122	C	G	Glutamine	Histidine	21	NSP1	Non-synonymous
123	G	A	Lysine	Glutamine	22	NSP1	Non-synonymous
126	C	A	Serine	Glycine	23	NSP1	Non-synonymous
128	A	T	Serine	Serine	23	NSP1	Synonymous
1031	G	A	Glycine	Glycine	324	NSP1	Non-synonymous
1110	C	A	Isoleucine	Leucine	351	NSP1	Non-synonymous
4111	A	G	Tyrosine	Cysteine	1351	NSP3	Non-synonymous
4754	A	G	Lysine	Lysine	1565	NSP3	Synonymous
4756	G	T	Valine	Glycine	1566	NSP3	Non-synonymous
4761	C	T	Phenylalanine	Leucine	1568	NSP3	Non-synonymous
4762	G	T	Phenylalanine	Cysteine	1568	NSP3	Non-synonymous

4763	A	T	Phenylalanine	Leucine	1568	NSP3	Non-synonymous
5332	A	T	Aspartic acid	Valine	1758	NSP3	Non-synonymous
5639	G	A	Glycine	Glycine	1860	NSP3	Synonymous
7436	G	A	Isoleucine	Methionine	2458	NSP4	Non-synonymous
7873	A	G	Arginine	Arginine	26	Capsid	Synonymous
8564	G	A	Lysine	Glutamate	257	Capsid	Non-synonymous
8565	T	A	Lysine	Methionine	257	Capsid	Non-synonymous
8883	G	A	Glutamine	Arginine	363	E2	Non-synonymous
9561	C	T	Phenylalanine	Serine	589	E2	Non-synonymous
10676	A	C	Proline	Threonine	961	E1	Non-synonymous
10936	T	C	Glycine	Glycine	1047	E1	Synonymous
Carcass							
Nucleotide position	Variant	Reference	Original amino acid	Change in amino acid	Amino acid position	ORF	Synonymous or Non-synonymous
1031	G	A	Glycine	Glycine	324	NSP1	Synonymous
1110	C	A	Isoleucine	Leucine	351	NSP1	Non-synonymous
4111	G	A	Tyrosine	Cysteine	1351	NSP3	Non-synonymous
4122	A	C	Arginine	Serine	1355	NSP3	Non-synonymous
5639	G	A	Glycine	Glycine	1860	NSP3	Synonymous
7436	G	A	Isoleucine	Methionine	2458	NSP4	Non-synonymous

8564	G	A	Lysine	Glutamate	257	Capsid	Non-synonymous
8565	T	A	Lysine	Methionine	257	Capsid	Non-synonymous
8883	G	A	Glutamine	Arginine	363	E2	Non-synonymous
9188	T	G	Valine	Phenylalanine	465	E2	Non-synonymous
10676	A	C	Proline	Threonine	961	E1	Non-synonymous
10936	T	C	Glycine	Glycine	1047	E1	Synonymous

Table 3.6 (Supplementary): Mutations found in non-coding regions of the SINV genome in midguts and carcasses of high titer mosquitoes.

Midgut			
Nucleotide position	Variant	Reference	Genome element
22	A	T	5' UTR
26	G	A	5' UTR
27	C	A	5' UTR
11821	T	C	3' UTR
11821	A	C	3' UTR
11826	T	A	3' UTR
11831	T	A	3' UTR
11834	T	C	3' UTR
11835	T	A	3' UTR
11836	T	C	3' UTR
11836	A	C	3' UTR
11837	T	A	3' UTR
11838	A	T	3' UTR
11839	T	A	3' UTR
11845	T	G	3' UTR
11852	T	A	3' UTR
11854	T	A	3' UTR

Carcass			
Nucleotide position	Variant	Reference	Genome element
11821	T	C	3' UTR
11826	T	A	3' UTR
11831	T	A	3' UTR
11834	T	C	3' UTR
11837	T	A	3' UTR
11839	T	A	3' UTR
11844	A	T	3' UTR
11849	A	T	3' UTR
11851	T	A	3' UTR
11853	C	T	3' UTR
11854	T	A	3' UTR

Table 3.7 (Supplementary): Mutations found in coding regions of the SINV genome in stock virus.

Nucleotide position	Variant	Reference	Original amino acid	Change in amino acid	Amino acid position	ORF	Synonymous or Non-synonymous
8564	G	A	Lysine	Glutamine	257	Capsid	Non-synonymous
8565	T	A	Lysine	Methionine	257	Capsid	Non-synonymous
8883	G	A	Glutamine	Arginine	363	E2	Non-synonymous
9483	C	A	Glutamine	Proline	563	E2	Non-synonymous
9720	C	T	Isoleucine	Threonine	642	E2	Non-synonymous

Table 3.8 (Supplementary): Mutations found in non-coding regions of the SINV genome in stock virus.

Nucleotide position	Variant	Reference	Genome element
8	T	G	5' UTR

Chapter 4 – Conclusions and future directions

4.1 Conclusions

The overarching objective of the research presented in this dissertation was to unravel the factors behind variation in Sindbis virus (SINV) load in *Aedes aegypti* after oral infection. This was completed by (1) investigating the biological factors and (2) investigating the virus factors contributing to SINV titer variability in *Ae. aegypti* after oral infection. This final chapter provides a summary of the major findings towards addressing the objectives of this study, which provides important fundamental knowledge towards our understanding of mosquito vector competence. Additionally, this section provides an outlook on possible future studies.

1. Investigation of biological factors contributing to individual variation in viral titer after oral infection of *Ae. aegypti* mosquitoes by Sindbis virus.

The persistence of arboviruses, especially in the tropics where the disease burden is high, is largely due to a lack of easily implemented and effective vector control measures (Huang et al., 2019). The inability to develop robust control strategies for the management of arboviruses is hindered by our lack of comprehensive understanding of what factors contribute to vector competence. Although there are approximately 3,000 different mosquito species existing globally, only a few dozen of these mosquito species can transmit arboviruses. Also, there is a high degree of specificity between arboviruses and mosquitoes which determines which arboviruses a particular mosquito species can transmit (Viglietta et al., 2021).

Several studies have reported that individuals within a population of mosquitoes show variability in infection and transmission rates (Honório et al., 2020; Merwaiss et al., 2021; O'Neill et al., 2015). However, factors contributing to this variation in virus titer in mosquitoes

were largely unknown when these studies were initiated. Due to this, we first investigated which biological factors may contribute to variation in *Ae. aegypti* after SINV infection. Results from this study showed that biological factors such as mosquito strain, mosquito weight, amount of blood ingested, viral concentration, and viral strain did not contribute virus titer variability while only the route of infection and the incubation period contributed to virus titer variability (Hodoamedia et al., 2022). This suggests that *Ae. aegypti* reduces SINV titer or clears SINV infection with increased time. A possible explanation of the mechanism *Ae. aegypti* uses to resist SINV infection is by generating virus-derived small interfering RNAs (viRNAs) which the mosquito uses for antiviral defense against SINV infection (Myles et al., 2008). High virus titer variability was observed after oral infection compared to intrathoracic infection, suggesting that the midgut barrier may be an important tissue contributing to variation in virus titer (Franz et al., 2015). The midgut may impose a different degree of bottleneck to SINV infection and dissemination which is probably contributing to higher titer variation observed in oral infection compared to intrathoracic infection (Franz et al., 2015; Weaver et al., 2021). Furthermore, viruses from high titer mosquitoes re-infected mosquitoes better (with a significantly higher median titer and prevalence of infection) after having been passaged in BHK-21 cells (completing the mosquito-mammalian infection cycle) when compared to normally passaged SINV, suggesting that high titer mosquitoes may have a higher transmission potential compared to both normally passaged and/or viruses from low titer mosquitoes (Honório et al., 2020; Moutailler et al., 2011). Also, differences in culture adaptability between low and high titer mosquitoes and differences in infection prevalence and median virus titer of passaged or non-passage virus from high titer mosquitoes suggest that there might be genetic differences in the virus populations accounting for this observation (Novelo et al., 2023). Overall, results from this

chapter are the first to take a comprehensive look at the biological factors that affect titer variation in mosquitoes after infection, with the aim of adding to our knowledge of what factors can contribute to vector competence.

2. Extreme infectious titer variability in individual *Aedes aegypti* mosquitoes infected with Sindbis virus is associated with both differences in virus population structure and dramatic disparities in specific infectivity.

Individuals within a population of mosquitoes show variability in infection and transmission rates after oral infection with arboviruses (Hodoameda et al., 2022; Honório et al., 2020; Raquin et al., 2017). The positive correlation between mosquito viremia and virus transmission suggests that high titer mosquitoes are more likely to transmit arbovirus compared to low titer mosquitoes (Honório et al., 2020). Although a high level of variation in virus titer in mosquitoes is commonly observed after oral infection, there is limited knowledge on what virus factors contribute to this variation, especially in highly inbred mosquitoes (Hodoameda et al., 2022). For this reason, we investigated what virus factors may contribute to SINV titer variation in *Ae. aegypti* mosquitoes after oral infection. Results from this study showed high titer mosquitoes had significantly higher virus load in the saliva compared to low titer mosquitoes. Also, there was a significantly higher prevalence of virus load in the saliva of high titer mosquitoes compared to low titer mosquitoes suggesting that high titer mosquitoes are more vector competent than low titer mosquitoes, a result consistent with previous reports (Honório et al., 2020). Sequence analysis of the viral populations showed that SINV is relatively genetically stable compared to other RNA viruses, which was consistent to previous reports (Savolainen-Kopra and Blomqvist, 2010; Strauss and Strauss, 1994). Additionally, midguts in both low and

high titers had higher number of virus variants which were $\geq 5\%$ frequency when compared to the carcasses. A significant reduction in virus variants was recorded when they escaped from the midguts to the carcasses in low titers. However, there was no significant reduction in virus variants when they escaped from the midguts to the carcasses in high titers. This suggests that the viruses in low titer mosquitoes experienced increased genetic drift compared to high titer mosquitoes during midgut escape and dissemination (Patterson et al., 2018; Weaver et al., 2021). Additionally, the carcasses of high titer mosquitoes had significantly higher virus diversity and complexity in the virus population structure compared to carcasses of low titer mosquitoes. This suggests that the carcasses of high titer mosquitoes had a significant higher SINV quasi-species compared to the carcasses of low titer mosquitoes, a result consistent with results reported by Vignuzzi and colleagues as they also reported that quasi-species diversity determines the degree of pathogenesis (Vignuzzi et al., 2006). Furthermore, stronger purifying selection was observed in low titer mosquitoes compared to high titer mosquitoes, hence differences in purifying selection may have contributed to differences in SINV population diversity between low titer mosquitoes and high titer mosquitoes (Lequime et al., 2016).

To investigate further if other factors apart of differences in number of quasi-species are contributing to differences in SINV titer variability, we used transmission electron microscopy to semi-quantify the number of virions in low titer and high titer mosquitoes. Results from this experiment showed that high titer mosquitoes had more complete virions than low titer mosquitoes, suggesting that complete virion assembly occurs in high titer mosquitoes compared to low titer mosquitoes. Also, the amount of capsid protein measured in low titer mosquitoes was less than that measured in high titer mosquitoes, suggesting that translation of viral proteins was less efficient in low titer mosquitoes than high titer mosquitoes.

In order to understand if the less efficient translation of viral structural protein in low titer mosquitoes compared to high titer mosquitoes was due to differences in the amount of viral RNA, RT-qPCR was used to quantify viral RNA in low titer and high titer mosquitoes. Results from this experiment showed that the genome equivalent copies were similar between low titer and high titer mosquitoes. In addition, titer variation was not due to amount of genome equivalent copies, as there was no correlation between genome equivalent and infectivity, but significant differences in specific infectivity, or the ratio of infectious units to viral genomes, between low and high titer mosquitoes contributed to the observed variation between low titer and high titer mosquitoes. This suggest that virions in low titer mosquitoes are less fit compared to virions in high titer mosquitoes (Bhattacharya et al., 2022; Mackenzie-Liu et al., 2018; Sokoloski et al., 2012). Additionally, specific infectivity was found to be significantly higher in the carcasses than the midguts of the mosquitoes, suggesting that virions in the midguts are less infectious than virions in the carcasses. Furthermore, specific infectivity decreased with an increase in incubation period as mosquitoes infected with SINV and incubated for 15 days had significantly lower specific infectivity than mosquitoes incubated for 5 days. To understand the role immunity plays in virion assemblage, mosquitoes were treated with an antibiotic cocktail and specific infectivity was measured and compared to non-antibiotic treated control group. Results from this experiment showed that antibiotic treatment increased specific infectivity. Since antibiotic treatment is known to disrupt the midgut microbiota composition and decrease the mosquitoes' immune response to SINV infection (Barletta et al., 2017; Sim, et al., 2012), this provides evidence that mosquito immunity can affect the efficiency of virus assembly.

In all, this chapter is the first to show that SINV titer variation in *Ae. aegypti* is associated with virus population structure and specific infectivity. Altogether, Chapters 2 and 3 provide

important knowledge towards the understanding of what biological factors contributes to variation in SINV load in the *Ae. aegypti* after oral infection. We have shown that variation decreases with increase incubation period. The mechanism that *Ae. aegypti* uses to resist infection can now be investigated and this knowledge harnessed for the development of transgenic mosquitoes as novel vector control strategy. Additionally, since viruses from high titer mosquitoes are more likely to be transmitted than viruses from low titer mosquitoes, high titer mosquitoes may be of epidemiological importance for transmitting arboviruses than low titer mosquitoes. Also, specific infectivity contributes to differences in titer and differences in viral protein translation efficiency and complete virion assemblage may contribute to the differences in specific infectivity. Disruption of midgut microbiota with antibiotics affects mosquito immune response, which in turn affects specific infectivity. This shows that mosquito immune response is vital in determining vector competence.

4.2 Future directions

The results in this dissertation have revealed fundamental findings vital to help understand the factors contributing to arbovirus titer variation in *Ae. aegypti*, important information in the quest to understand vector competence. Although these results have improved our knowledge in this area, there is more work to be done in order to fully understand some of the results observed in this study. For this reason, further studies should be aimed at (1) investigating the mechanism behind the resistance of SINV infection by *Ae. aegypti* with increased incubation period and (2) investigating if epigenetic modification of SINV may contribute to the differences specific infectivity observed between low titer and high titer mosquitoes.

(1) Investigating the mechanism behind the resistance to SINV infection by *Ae. aegypti* with increased incubation period

An interesting observation reported in this study is the decrease in SINV titer variation with increase in incubation period. Additionally, approximately 30% of the *Ae. aegypti* mosquitoes may have cleared SINV infection at 15 days post infection as there was a lack of detection of SINV via TCID₅₀ assay (Hodoamede et al., 2022). These results show that *Ae. aegypti* uses the resistance approach as an immune response to SINV (Lambrechts and Saleh, 2019). The RNAi pathway is the primary antiviral defense used by mosquitoes to resist arbovirus infection, including SINV (Blair and Olson 2014, 2015; Franz et al., 2006; Keene et al., 2004). Previous reports by Myles et al., (2008) reported that virus-derived small interfering RNAs (viRNAs) in *Ae. aegypti* are used for antiviral defense against SINV infection. In this study, the authors reported that viRNAs that are 21 nt long are found in SINV-infected *Ae. aegypti*. The viRNAs were shown to be asymmetrically distributed over the length of the SINV genome (Myles et al., 2008). Suppressing production of viRNAs in *Ae. aegypti* infected with SINV-B2, a version of SINV expressing the B2 protein from Flock House virus (FHV) resulted in reduced survival and higher viral titers compared to *Ae. aegypti* infected with SINV with unsuppressed viRNAs. The B2 protein is a suppressor of RNA silencing that acts by binding to dsRNA to prevent the processing of dsRNA by dicer. Altogether, this result shows the importance of viRNAs in protecting *Ae. aegypti* against the pathogenic effect of SINV and survival of SINV infected mosquito, and the maintenance of the virus in nature (Myles et al., 2008). Hence, the role of RNAi and transcriptomic response in resisting SINV infection in *Ae. aegypti* with increased incubation period should be investigated. This future study should infect *Ae. aegypti* with SINV at 5 and 15-days post infection, the infected mosquitoes should be dissected into midguts and

carcasses, and RNAseq analysis should be performed on both midguts and carcasses, as well as sequencing of small RNAs. Differentially expressed genes between mosquitoes infected with SINV and incubated for 5 days and 15 days should be identified. The expression of candidate differentially expressed genes identified can be knocked down to validate the role of the differentially expressed genes in contributing to reduced SINV load in mosquitoes with increased incubation period. Additionally, the relative expression of genes in the RNAi pathway at 5 days and 15 days, as well as the amounts of virus-specific siRNAs should be determined to ascertain the role of RNAi in reducing SINV load in mosquitoes with increased incubation period.

- (2) Extreme infectious titer variability in individual *Aedes aegypti* mosquitoes infected with Sindbis virus is associated with both differences in virus population structure and dramatic disparities in specific infectivity.

SINV produces a mixture of infectious and non-infectious particles. In this study, the virus populations in high titer mosquitoes were found to have a significantly higher specific infectivity than viruses from low titer mosquitoes (Hodoamede et al., 2024). Epitranscriptomic RNA modification of viral RNA helps to regulate its stability and improve cellular and viral RNA functions (Ribeiro et al., 2023; Tsai and Cullen, 2020). Dang and colleagues reported that N-acetyltransferase 10 (NAT10) upregulation and ac4C modification of viral RNA could enhance SINV infectivity (Dang et al., 2024). Additionally, N6-methyladenosine modification of flavivirus viral RNA regulates infection (Gokhale et al., 2016). Also, alteration of m⁶A modification of specific host mRNA affects flavivirus infection (Gokhale et al., 2020). Altogether, these results suggest that epigenetic RNA modification of either host cellular transcripts and/or viral RNA may contribute to the differences in specific infectivity observed between low and high titer mosquitoes. Hence it will be imperative to investigate whether

acetylation, methylation, and/or glycosylation epigenetic modification of SINV RNA is different between viruses from low titer and high titer mosquitoes. For this future study, *Ae. aegypti* would be orally infected with SINV and at 5 days post infection, the infected mosquitoes would be dissected to separate them into midguts and carcasses. The viral RNA from midguts and carcasses will be sequenced (for example RNA methyl-seq, RNA acetyl-seq, etc), and results analyzed to determine if there is any epigenetic modification of SINV genome or not, and if this modification contributes to the differences in specific infectivity between low and high titer mosquitoes. For example, m⁶A is the most abundant modification of RNA in cells. The m⁶A modification in SINV can be detected using RNA immunoprecipitation sequencing (MeRIP-seq) which uses an m⁶A antibody that immunoprecipitate m⁶A sites after which next generation sequence is performed.

Furthermore, genes differentially expressed between low and high titer mosquitoes involved in epigenetic modification transcriptomic modification will be knocked down to validate their role in epigenetic modification transcriptomic modification of SINV genome and the effect this has on specific infectivity. Overall, results from this study will throw more light on the role of post-translational modification of SINV on specific infectivity in low titer and high titer mosquitoes.

Another study that is currently being conducted to understand factors contributing to SINV titer variation is elucidation of *Ae. aegypti* transcriptomic response to SINV infection. RNA-seq was performed on midguts and carcasses of (1) blood fed only mosquito samples, (2) low titer samples, and (3) high titer samples (the same RNAseq data that was used to obtain viral sequences in Chapter 3). Preliminary results from this study shows that more immune related genes, including genes involved in piRNA and miRNA pathways were upregulated in low titer

mosquitoes than high titer mosquitoes. Additionally, more genes involved in ATP hydrolysis were upregulated in in high titer mosquitoes than low titer mosquitoes. Also, more genes involved in methyltransferase activity were upregulated in low titer mosquitoes compared to high titer mosquitoes while more genes involved in acetyltransferase activity were upregulated in high titer mosquitoes compared to low titer mosquitoes. More genes involved in lipid metabolism/lipid transport activity were upregulated in high titer mosquitoes compared to low titer mosquitoes. Altogether, these results show that more ATP are hydrolyzed in the cells of high titer mosquitoes, making more energy available to SINV for replication. Also, more lipids are made available in the cells of high titer mosquitoes for SINV to use for replication. Stronger immune response in cells of low titer mosquitoes may limit the SINV replication and reduce infectivity. These experiments are currently being completed and although the results are not included in this dissertation, they will be published at a later date.

In conclusion, the results from this dissertation have shown that there is greater SINV titer variation in *Ae. aegypti* after oral infection compared to intrathoracic infection, and that variation in virus titer decreases with increased incubation period. Virus populations in the midguts of low titer mosquitoes overall were shown to be less fit than virus populations in the midguts of high titer mosquitoes, with a likely cause of this difference in fitness being due to founder effect occurring due to the midgut infection bottleneck. Finally, preliminary study of *Ae. aegypti* transcriptomic analysis of low titer mosquitoes and high titer mosquitoes shows that pro-viral genes are mostly upregulated in high titer mosquitoes and anti-viral genes are mostly upregulated in low titer mosquitoes. Overall, the results in this dissertation have provided imperative knowledge towards the understanding of factors contributing to differences in virus load in *Ae. aegypti* after oral infection with SINV.

4.3 References

- Barletta, A. B. F., Nascimento-Silva, M. C. L., Talyuli, O. A., Oliveira, J. H. M., Pereira, L. O. R., Oliveira, P. L., & Sorgine, M. H. F. (2017). Microbiota activates IMD pathway and limits Sindbis infection in *Aedes aegypti*. *Parasites & Vectors*, *10*(1), 1-9.
- Bhattacharya, T., Yan, L., Crawford, J. M., Zaher, H., Newton, I. L., & Hardy, R. W. (2022). Differential viral RNA methylation contributes to pathogen blocking in Wolbachia-colonized arthropods. *PLoS Pathogens*, *18*(3), e1010393.
- Blair, C. D., & Olson, K. E. (2014). Mosquito immune responses to arbovirus infections. *Current Opinion in Insect Science*, *3*, 22-29.
- Dang, Y., Li, J., Li, Y., Wang, Y., Zhao, Y., Zhao, N., ... & Ye, W. (2024). N-acetyltransferase 10 regulates alphavirus replication via N4-acetylcytidine (ac4C) modification of the lymphocyte antigen six family member E (LY6E) mRNA. *Journal of Virology*, *98*(1), e01350-23
- Franz, A. W., Sanchez-Vargas, I., Adelman, Z. N., Blair, C. D., Beaty, B. J., James, A. A., & Olson, K. E. (2006). Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proceedings of the National Academy of Sciences*, *103*(11), 4198-4203.
- Franz, A. W., Kantor, A. M., Passarelli, A. L., & Clem, R. J. (2015). Tissue barriers to arbovirus infection in mosquitoes. *Viruses*, *7*(7), 3741-3767.
- Gokhale, N. S., McIntyre, A. B., McFadden, M. J., Roder, A. E., Kennedy, E. M., Gandara, J. A., ... & Horner, S. M. (2016). N6-methyladenosine in flaviviridae viral RNA genomes regulates infection. *Cell Host & Microbe*, *20*(5), 654-665.
- Gokhale, N. S., McIntyre, A. B., Mattocks, M. D., Holley, C. L., Lazear, H. M., Mason, C. E., &

- Horner, S. M. (2020). Altered m6A modification of specific cellular transcripts affects Flaviviridae infection. *Molecular Cell*, 77(3), 542-555.
- Hodoameda, P., Addae, L., & Clem, R. J. (2022). Investigation of biological factors contributing to individual variation in viral titer after oral infection of *Aedes aegypti* mosquitoes by Sindbis virus. *Viruses*, 14(1), 131.
- Hodoameda, P., Ebel, G. D., Mukhopadhyay, S., & Clem, R. J. (2024). Extreme infectious titer variability in individual *Aedes aegypti* mosquitoes infected with Sindbis virus is associated with both differences in virus population structure and dramatic disparities in specific infectivity. *PLoS Pathogens*, 20(2), e1012047.
- Honório, N. A., Câmara, D. C. P., Wiggins, K., Eastmond, B., & Alto, B. W. (2020). High-Throughput method for detection of Arbovirus infection of saliva in mosquitoes *Aedes aegypti* and *Ae. albopictus*. *Viruses*, 12(11), 1343.
- Huang, Y. J. S., Higgs, S., & Vanlandingham, D. L. (2019). Arbovirus-mosquito vector-host interactions and the impact on transmission and disease pathogenesis of arboviruses. *Frontiers in Microbiology*, 10, 22.
- Keene, K. M., Foy, B. D., Sanchez-Vargas, I., Beaty, B. J., Blair, C. D., & Olson, K. E. (2004). RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*, 101(49), 17240-17245.
- Lambrechts, L., & Saleh, M. C. (2019). Manipulating mosquito tolerance for arbovirus control. *Cell Host & Microbe*, 26(3), 309-313.
- Lequime, S., Fontaine, A., Ar Gouilh, M., Moltini-Conclois, I., & Lambrechts, L. (2016). Genetic drift, purifying selection and vector genotype shape dengue virus intra-host genetic

- diversity in mosquitoes. *PLoS Genetics*, 12(6), e1006111.
- Mackenzie-Liu, D., Sokoloski, K. J., Purdy, S., & Hardy, R. W. (2018). Encapsidated host factors in alphavirus particles influence midgut infection of *Aedes aegypti*. *Viruses*, 10(5), 263.
- Merwaiss, F., Filomatori, C. V., Susuki, Y., Bardossy, E. S., Alvarez, D. E., & Saleh, M. C. (2021). Chikungunya virus replication rate determines the capacity of crossing tissue barriers in mosquitoes. *Journal of Virology*, 95(3), 10-1128.
- Moutailler, S., Roche, B., Thiberge, J. M., Caro, V., Rougeon, F., & Failloux, A. B. (2011). Host alternation is necessary to maintain the genome stability of rift valley fever virus. *PLoS Neglected Tropical Diseases*, 5(5), e1156.
- Myles, K. M., Wiley, M. R., Morazzani, E. M., & Adelman, Z. N. (2008). Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proceedings of the National Academy of Sciences*, 105(50), 19938-19943.
- O'Neill, K., Olson, B. J., Huang, N., Unis, D., & Clem, R. J. (2015). Rapid selection against arbovirus-induced apoptosis during infection of a mosquito vector. *Proceedings of the National Academy of Sciences*, 112(10), E1152-E1161.
- Novelo, M., Dutra, H. L., Metz, H. C., Jones, M. J., Sigle, L. T., Frentiu, F. D., ... & McGraw, E. A. (2023). Dengue and chikungunya virus loads in the mosquito *Aedes aegypti* are determined by distinct genetic architectures. *PLoS Pathogens*, 19(4), e1011307.
- Patterson, E. I., Khanipov, K., Rojas, M. M., Kautz, T. F., Rockx-Brouwer, D., Golovko, G., ... & Forrester, N. L. (2018). Mosquito bottlenecks alter viral mutant swarm in a tissue and time-dependent manner with contraction and expansion of variant positions and diversity. *Virus Evolution*, 4(1), vey001.

- Raquin, V., Merkling, S. H., Gausson, V., Moltini-Conclois, I., Frangeul, L., Varet, H., ... & Lambrechts, L. (2017). Individual co-variation between viral RNA load and gene expression reveals novel host factors during early dengue virus infection of the *Aedes aegypti* midgut. *PLoS Neglected Tropical Diseases*, *11*(12), e0006152.
- Ribeiro, D. R., Ribeiro, D., & Soares, A. R. (2023). The hidden RNA code: implications of the RNA epitranscriptome in the context of viral infections. *Frontiers in Genetics*, *14*, 1245683.
- Savolainen-Kopra, C., & Blomqvist, S. (2010). Mechanisms of genetic variation in polioviruses. *Reviews in Medical Virology*, *20*(6), 358-371.
- Sim, S., Ramirez, J. L., & Dimopoulos, G. (2012). Dengue virus infection of the *Aedes aegypti* salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior. *PLoS Pathogens*, *8*(3), e1002631.
- Sokoloski, K. J., Hayes, C. A., Dunn, M. P., Balke, J. L., Hardy, R. W., & Mukhopadhyay, S. (2012). Sindbis virus infectivity improves during the course of infection in both mammalian and mosquito cells. *Virus Research*, *167*(1), 26-33.
- Strauss, J. H., & Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiological Reviews*, *58*(3), 491-562.
- Tsai, K., & Cullen, B. R. (2020). Epigenetic and epitranscriptomic regulation of viral replication. *Nature Reviews Microbiology*, *18*(10), 559-570.
- Viglietta, M., Bellone, R., Blisnick, A. A., & Failloux, A. B. (2021). Vector specificity of arbovirus transmission. *Frontiers in Microbiology*, *12*, 773211.
- Vignuzzi, M., Stone, J. K., Arnold, J. J., Cameron, C. E., & Andino, R. (2006). Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature*, *439*(7074), 344-348.

Weaver, S. C., Forrester, N. L., Liu, J., & Vasilakis, N. (2021). Population bottlenecks and founder effects: implications for mosquito-borne arboviral emergence. *Nature Reviews Microbiology*, 19(3), 184-195.