DISSERTATION

ASSESSMENT OF MOSQUITO AND ANIMAL MODEL FACTORS IN AEDES-BORNE ARBOVIRUS TRANSMISSION AND DISEASE

Submitted by

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ABSTRACT

ASSESSMENT OF MOSQUITO AND ANIMAL MODEL FACTORS IN AEDES-BORNE ARBOVIRUS TRANSMISSION AND DISEASE

Aedes aegypti mosquitoes are the main vector for Dengue viruses (DENV 1-4), chikungunya virus (CHIKV) and Zika virus (ZIKV). The reemergence of DENV and yellow fever virus (YFV) along with the emergence of arthropod-borne viruses (arboviruses) in non-native regions, as seen with CHIKV and ZIKV, have resulted in outbreaks and pandemics that result in millions of human cases of disease each year causing significant morbidity and mortality. The aforementioned arboviruses differ in their genetic relatedness and structure (DENV, YFV and ZIKV are all separate virus species in the family *Flaviviridae,* while CHIKV is a virus species in the family *Togaviridae*) and the diseases they cause. However, all are transmitted in the saliva of infected *Aedes aegypti* mosquitoes, and key aspects of this *Aedes*-borne arbovirus transmission remain understudied, including the composition of transmitted virus, dose, and mosquito and mammalian host factors that can influence virus transmission and subsequent disease. In this dissertation, I attempt to elucidate some of these virus and host factors that are thought to influence the successful transmission of Zika and chikungunya viruses to vertebrate hosts and subsequent host disease.

Zika virus (ZIKV) is primarily transmitted to humans through the bite of an infected mosquito but it also can be transmitted sexually. Zika disease usually results in fever, headache, rash and arthralgia, but complications of Guillain-Barré syndrome and birth defects in infants born to mothers infected during pregnancy can occur. Interestingly, there is an unexplained

female bias that occurs with ZIKV disease whereby the attack rate and incidence are significantly higher in females compared to males. One hypothesis for this bias is that different disease outcomes occur depending on infection by different ZIKV transmission routes.

Different immunocompetent animal models, other than non-human primates, were tested for their susceptibility to ZIKV infection by different transmission routes, and their ability to transmit the virus onward. While mice have been extensively used for modeling ZIKV infection, there are no ZIKV-susceptible immunocompetent mouse models that have been used to investigate sexual and mosquito bite transmission. Here, the multimammate mouse (*Mastomy natalensis*), the New Zealand white rabbit (*Oryctolagus cuniculus),* the Hartley strain Guinea pig (*Cavia porcellus*), and the Jamaican fruit bat (*Artibeus jamaicensis*) were explored as potential animal models for sexual and mosquito bite transmission of ZIKV. These animals were chosen for their association with flavivirus transmission and potential to support viral replication. It was found that the multimammate mouse and the New Zealand White (NZW) rabbit are not susceptible ZIKV infection. Sexually mature male Hartley guinea pigs were inoculated subcutaneously using a needle, and by mosquito bite, but found to be not susceptible to ZIKV infection. Lastly, the Jamaican fruit bat was found to be a poor model for transmission and disease as low loads of viral RNA were rarely detected in their tissues.

To investigate the effects of mosquito bite on ZIKV disease between biological sex, we used a mouse model of ZIKV infection (immunodeficient A129 mice). Early infection kinetics in male mice were found to be dependent on infection with ZIKV that is derived from mosquito salivary glands. However, rapid infection of most female tissues was negatively influenced by pre-exposure to mosquito saliva. It was also found that this mouse model is refractory to ZIKV transmission by the sexual route, despite robust infections of tissues in the male reproductive

tract. These data lead to a better understanding of how differences in biological sex and mosquito bite can influence ZIKV infection dynamics in this mouse strain and should be an impetus for examining similar differences in other ZIKV infection models, and in humans.

Estimating the titer of virus transmitted from infectious mosquitoes to a host is critical for a better understanding of the effects of mosquito virus transmission on disease outcome. Different experimental procedures and methods were tested to estimate the titer of virus transmitted by *Ae. aegypti* mosquitoes after being infected with either ZIKV or CHIKV. It was found that immersion oil is a more efficient media for collecting mosquito saliva containing virus in the capillary tubes used with the forced salivation (FS) technique. With this technique, it was shown that FS virus titers were similar between mosquitoes that never received a blood meal compared to those that were blood fed immediately prior. When immunocompromised mice were fed on by Zika virus-infected mosquitoes, they always became infected, even when no infectious virus was detected in their saliva. However, ZIKV and CHIKV was never detected in the blood remaining in artificial feeders after these were fed on by infected mosquitoes. To address this discrepancy, the virus titer in ingested bloodmeals of individual mosquitoes was compared to virus in their saliva from FS. These experiments revealed there to be higher virus titers in the dissected bloodmeals compared to those detected in the same mosquitoes' saliva. This demonstrates how mosquitoes re-ingest much of their saliva during artificial blood feeding and highlight a large increase in virus transmission during blood feeding. Lastly, these experiments showed ~100-10,000 times more viral RNA is transmitted in mosquito saliva than infectious virus.

A hypothesis to address the difference between viral RNA and infectious virus in saliva is the preferential secretion of packaged defective viral genomesgenomes (DVG). Both CHIKV

and ZIKV are RNA viruses dependent on RNA-dependent RNA polymerases (RdRp) for replication. RdRp generate relatively high error rates during replication of the viral RNA and can lead to insertions and deletions in the genetic material. In certain replication conditions, large regions of the virus genome can be deleted resulting in DVGs. DVGs are able to be packaged into virus-like particles and can then be released from the cell. Both alphaviruses and flaviviruses have been documented to produce DVGs, however, there has been little research on naturally-transmitted, packaged arboviral DVGs transmitted in host biofluids (mainly mosquito saliva, but also semen that is involved in the sexual transmission of ZIKV). Here, DVGs were characterized from cell cultures infected with CHIKV and ZIKV, as well as from infected mosquito saliva and other tissue samples. It was found that CHIKV readily produced large quantities of DVGs *in vitro*, especially when passaged at a high multiplicity of infection, as well as in mosquito saliva, but this was not observed with ZIKV. Sequencing analysis of CHIKV cell culture samples showed a high abundance of DVG deletions over the genomic regions encoding the structural genes. Additionally, several DVG variants were detected in individual mosquito samples. Interestingly, variation of DVGs decreased though dissemination to the saliva in the mosquito, while abundance increased. In comparison, there were little to no DVGs detected in ZIKV-infected mosquitoes. Understanding of DVGs naturally produced in biofluids during arbovirus infections may further our understanding arbovirus transmission, infection and disease.

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CHAPTER 1: OVERVIEW OF THE LITERATURE

1.1 Aedes Mosquitoes

Aedes is a genus of mosquitoes found in tropical and subtropical zones and have more recently become an invasive species outside of tropical regions[1]. The *Aedes* genus contains over 700 species. Species of medical importance include, but are not limited to *Aedes aegypti, Aedes albopictus, Aedes atlanticus, Aedes atropalpus, Aedes cinereus*, and *Aedes melanimon*[1]. This review will focus on *Aedes aegypti* as it is one of the primary vectors for many arthropod-borne viruses (arboviruses). These include yellow fever (YFV), dengue viruses 1-4 (DENV), chikungunya virus (CHIKV), Zika virus (ZIKV), and more recently Mayaro virus (MAYV)[2]. *Aedes aegypti* mosquitoes are recognized by white markings on the legs and a distinctive lyre mark on the surface of the thorax. Although it was first identified in 1700s, the domesticated form of *Ae. aegypti* is estimated to have originated approximately 600 years ago in Africa[3]. The domestication of this species is thought to have occurred by the use of human-generated water containers supplanting water-filled tree holes as places for this species to lay its eggs, and in turn allowing the mosquito larvae to develop close proximity to humans. *Ae. aegypti* were brought to the New World during the slave trade from West Africa in the 1600s and quickly became established enough to sustain yellow fever epidemics throughout central America[3]. As ships returned to the Old World, reports of yellow fever began to emerge in the 1700s in the Americas, and by the 1900s *Ae. aegypti* were established throughout the Mediterranean, Asia, Australia, and the Pacific.

During the 1950s and 1970s, approximately twenty countries in the Americas eradicated *Ae. aegypti* through the use of dichloro-diphenyl-trichloroethane (DDT)[4]. *Ae. aegypti* were

found to be high susceptible to DDT, however the continual use of DDT led to the rise of resistance against the insecticide. This resistance, along with growing human populations and degradation of sanitary condition in cities, tourism, and trade in old automobiles tires coupled with the abandonment of mosquito control[5] led to a reinfestation of *Ae. aegypti*. With *Ae. aegypti* mosquitoes established in hundreds of countries throughout the world and their capability of transmitting dangerous arboviruses, understanding their behaviors and interaction with humans is critical to addressing arboviral disease control and prevention.

1.1.1 Aedes life cycle

It takes 7-10 days for a mosquito to hatch from an egg and develop into an adult; it progresses, progressing through four life stages (i.e., embryo, larva, pupa, adult). Female mosquitoes store spermatozoa that are used to fertilize the oocytes as they ovulate. After a bloodmeal, a female mosquito lays 50-500 eggs on moist surfaces— usually on the inner walls of a water container above the waterline[6]. Embryos begin to develop once the eggs are laid and will hatch as larvae within two days if submerged in water. The eggs are resistant to desiccation, and the embryos are able to survive up to eight months and warmer winters[6]. Once the embryos hatch into larva, they begin to develop and go through four instar stages. Larvae emerge from the egg fully adapted for living in water. They are able to use atmospheric oxygen for respiration and feed off of water borne particles[6]. Larvae molt four times as they pass into each instar stage. At the 4th instar the larvae develop the most and then transition into pupae. Pupae are mobile aquatic organisms preferring to settle in dark sections of water. During the pupal stage, the development of adult organs starts (i.e., fat body development). The final stage from pupa to adult takes one to two days depending on environmental conditions[6]. Adults emerge within a few hours and 2-3

days post emergence; adult reproductive organs are developed and males are able to fertilize females.

1.1.2 Aedes aegypti feeding behaviors

Ae. aegypti originated in Sub-Saharan Africa from the zoophilic ancestral species *Ae. aegypti formosus*. After expansion into forest-adjacent villages and adaptation to human-generated water containers, *Ae. aegypti* became anthropophilic, preferring to blood feed on humans and therefore are more often found within human dwellings and living within close proximately to humans. Studies within the U.S. have found that 53% to 95% of *Ae. aegypti* blood meals are from humans while domestic animals make up the rest of the rest[3,7]. Similar studies in regions of Africa and Asia have found that hosts of *Ae. aegypti* include human, monkeys, ox, goat, and other domestic animals[8]. *Ae. aegypti* take multiple blood meals in a gonotrophic cycle. Additionally, interrupted feeding by a host results in a multiple partial meal[7] leading to feeding on multiple humans within a short time frame. After blood feeding, *Ae. aegypti* rest indoors and are most frequently found in bedrooms, bathrooms, and kitchens on vertical surfaces. These behaviors highlight the interaction between humans and *Ae. aegypti*.

1.1.3 Aedes aegypti distribution

The distribution of *Ae. aegypti* is driven by climate change, leading to new suitable climates, and urbanization. As *Ae. aegypti* shifted from zoophily to anthropophily and expanded their habitat into urban communities, their distribution became more widespread. Human population growth and international trade then led to global spread. *Ae. aegypti* mosquitoes are now established in Europe, the Middle East, throughout Africa, southern Russia, Georgia, Azerbaijan, Portugal, the Atlantic archipelago, the Canaries, the Azores, South and Central America, the south-eastern United States, southeast Asia, the Pacific and India Islands, and northern

Australia[9–11]. Of importance, the geographical range of *Ae. aegypti* has dramatically changed over the past sixty years[12]. Most of this change is due to climate change. *Ae. aegypti* are unable to survive at low temperatures, but as temperatures increase worldwide, *Ae. aegypti* are able to expand their habitat and become established in new areas. Within the U.S., the range of *Ae. aegypti* has increased from seven states (southern California, Arizona, Texas, Louisiana, Georgia, Florida, Alabama and Mississippi) to twenty-four states[12,13]. Prediction models show that by 2050, *Ae. aegypti* could expand throughout the eastern U.S. and Canada along with new regions of Europe and Russia[12,14,15]. The regional expansion of arboviruses like *Ae. aegypti* suggests that we can expect to see outbreaks of these arboviruses in native populations.

1.1.4 Aedes-borne arboviruses and their impact on human health

Ae. aegypti are the main vector for DENV, CHIKV, ZIKV, and more recently MAYV[16]. Reemergence of DENV and YFV along with emergence of arboviruses in non-native regions, as seen with CHIKV and ZIKV, have resulted in pandemics leading to thousands of cases and significant morbidity and mortality each year[17].

In 2019, there were over four million case of infection with DENV, ZIKV and CHIKV worldwide, with 3.9 million due to DENV alone[18,19]. During 2015-2016, there were more than one million cases of infection with ZIKV and since 2013, there have been over three million cases of infection with CHIKV. Symptoms of infection with *Aedes*-borne arboviruses vary between virus species and can lead to long-term effects. Many infections are asymptomatic while some result in symptomatic or clinically mild disease with low fever. However, long term effects are seen with DENV, ZIKV and CHIKV infections. DENV infections can lead to alopecia, joint pain, and muscle pain. ZIKV infections have been associated with complications including Guillain-Barré syndrome and birth defects in newborns of mothers infected during pregnancy[20].

CHIKV infection often leads to persistent and chronic joint pain that can last years after infection. The impact of the disease burden of these viruses can be measured by mortality and by disability-adjusted life years (DALYs). CHIKV caused at least 500,000 DALYs in 2005 due to the long term chronic conditions as a result of infection[21] and outbreaks of dengue disease in 2015 resulted in 1.14 million DALYs[22]. Additionally, the global financial cost of these arboviruses can reach billions of dollars a year. For example, the dengue outbreak of 2013 had a global impact of $9 billon[23].

The expansion of these arboviruses is due to the previously highlighted mosquito behaviors and distributions, however other factors including inefficient vector and disease control greatly influence the emergence and reemergence of arboviruses.

1.1.5 Problems with control: diagnostics, treatment, vaccines and vector control

Diagnostic testing is established for most *Aedes*-borne arboviruses, however each method has challenges. Serological detection is often used to determine infection and past exposure. Enzyme-linked immunosorbent assays (ELISAs) are used to detect antibodies and can target immunoglobulin M (IgM) or immunoglobulin G (IgG)[24]. These assays demonstrated high false-positive rates, which is more confounded by vaccination[25]. Further complications result from cross-reactivity between the four dengue serotypes and ZIKV. In addition to ELISAs, plaque reduction-neutralization tests (PRNTs) can be used to quantify neutralizing antibodies but require a long turnaround time[24]. Molecular diagnostics include qRT-PCR and metagenomic sequencing (mNGS). qRT-PCR is used to determine infection by detecting viral genomes in biofluids including urine, saliva, and blood[24].

Many arboviruses lack vaccines and proper treatment options. There are vaccines against YFV and Japanese encephalitis virus. However, there is only one currently-approved vaccine for

DENV, and none for ZIKV and CHIKV[26]. Dengvaxia is a vaccine for DENV, approved in at least 10 countries including the United States, and two other vaccines are in Phase III evaluation[26]. There are several ZIKV and CHIKV vaccine candidates in clinical development and phase 2 trials[26]. Unfortunately, treatment for arboviruses is also limited as there are very few medications or therapies proven to clear infection. Most often, supportive care is the only treatment for arbovirus infection.

There are numerous methods for vector control. The most common method is the use of insecticides to reduce populations of adults, including *Aedes* spp. Mosquitoes often build resistance to insecticides and over the past fifty years, different approaches have been implemented to help decrease the rate of resistance such as using different classes of insecticides in combination or during altering years. In addition, other methods including the use of *Wolbachia* endosymbionts, the sterile insect technique (SIT) and Release of Insects carrying a Dominate Lethal (RIDL) technologies are used for vector control[27]. *Wolbachia* is a genus of intracellular bacteria naturally found in many insects and tends to be localized to the reproductive system. Although results are dependent on species and strain, two different outcomes generally occur when *Aedes* species are infected with *Wolbachia* species/strains that are designed to ultimately control *Aedes*-borne arboviruses, a) the induction of cytoplasmic incompatibility (CI) in the mosquitoes that reduces subsequent progeny, or b) significantly reduced transmission of some viral pathogens[28]. SIT and RIDL technologies use irradiation to cause insect sterility or genetic engineering to introduce a dominate lethal gene, respectively[29]. When the males are released, these modified insects will mate with wild-type female insects and the progeny will die from the genetic load, leading to a decrease in the population[29]. Both these technologies require repeated release of insects intowild populations.

Ae. aegypti mosquitoes are established in hundreds of countries throughout the world. Due to their anthropophilic behavior and close association with human habitats, *Ae. aegypti* are the primary vector for multiple human pathogens. Climate change is predicted to increase the distribution of *Ae. aegypti* and therefore potentially introduce pathogens into native populations. With limited vaccines and treatment, *Aedes*-borne arbovirus infections result in long-term complications and high medical costs each year. Vector control remains the primary way to limit *Ae. aegypti* mosquitoes and their associated pathogens. Better understanding of transmission and vector-virus interactions will result in new strategies for disease prevention.

1.2 Classification of Aedes-borne arboviruses

There are four *Aedes*-borne arboviruses that cause human epidemics: YFV, DENV, CHIKV and ZIKV[30]. These viruses have emerged in both hemispheres. Other *Aedes*-borne arboviruses have emerged in specific regions of the world. These include, Spondweni virus (SPOV), O'nyong nyong virus (ONNV), and MAYV[31]. These viruses are grouped into three virus families with the majority of viruses belonging to the *Flaviviridae* and *Togaviridae* and RVFV being part of the *Phenuiviridae* family[32].

Many *Aedes*-borne arboviruses are maintained in a sylvatic transmission cycle between nonhuman primates (NHPs) and mosquitoes in natural forest habitats. Arboreal mosquitoes that dwell in the forest canopy maintain viral infection by interacting with NHPs and transmitting viruses from viremic to naïve hosts[33]. Spillover events occur when people encroach on forest habitats and are fed upon by infected mosquitoes. That infected person enters back into the urban or peri-urban environment, they are bitten by peri-/urban mosquitoes that then spread infection within that population[33]. This has been shown repeatedly with DENV where infection in NHPs

lead to outbreaks in human populations. However, DENV, CHIKV and ZIKV have become fully adapted to urban cycles and no longer require a sylvatic cycle to be maintained[34]. As they are maintained in these urban cycles, these RNA viruses adapt to both vertebrate and invertebrate host systems and are under selective pressure. Rapid replication rates of these viruses lead to high levels of mutation caused by the error-prone polymerase[35]. Such factors allow for these RNA viruses to adapt to new environments and may encourage new variants to arise.

1.2.1 Flavivirus

The genus *Flavivirus,* within the family *Flaviviridae,* is comprised of over 50 viruses including many medically important human and veterinary pathogens. Many of these pathogens are horizontally transmitted between hematophagous arthropods and a vertebrate host. Approximately 50% of flaviviruses are mosquito-borne and approximately 28% are tick-borne. The remaining flaviviruses have no known arthropod vector and are transmitted between rodents and bats or are insect-specific[32]. Tick-borne flaviviruses consist of 12 species, and mosquito-borne flaviviruses consist of 27 species. Mosquito-borne flaviviruses can generally be grouped by the disease manifestations they can cause; hemorrhagic-type disease is caused by infection with viruses such as YFV and DENV. Infection with these two viruses, and also ZIKV, can also result in arthralgias. Encephalitis and meningitis are the most severe disease type that result in infections with WNV and JEV[36]. Mosquito-borne flaviviruses are primary transmitted by *Aedes* or *Culex* mosquito species. Medically important *Culex*-borne flaviviruses include JEV, WNV, and St. Louis encephalitis virus (SLEV) and medically important *Aedes*-borne flaviviruses include YFV, DENV, and ZIKV[36].

Structure

Flaviviruses are small icosahedral viruses that consist of a host-derived lipid membrane surrounded by a surface envelope proteins. Flaviviruses have a positive sense, ssRNA genome that is ~11kb and consists of a single long ORF that encodes all structural and nonstructural proteins. The ORF genome is flanked by non-coding RNAs (NCRs) at the 5'- and 3'- terminal ends[37]. These NCRs contain RNA sequence motifs that are important for RNA translation, replication and packaging. Components of the NCRs, including secondary structure, are conserved between genera; however there can be large variations in sequence composition, length, and localization of certain elements[37]. The 5'- end is a type 1 cap (m^7GppAmp) where the A is followed by highly conserved G nucleotides. The 3'- end of most flaviviruses lacks a terminal poly (A) tail and instead has the dinucleotide CU[38]. There are three structural proteins: capsid (C), envelope protein (E) and either the pre-membrane protein (prM) in immature virions or the M in mature. The E protein is important for virus binding and undergoes acid pH-dependent fusion with the host cell upon infection[39]. It has three distinct domains and varies between flaviviruses[39]. Two virion forms have been identified: mature and immature. Mature virions express two membrane-associated proteins, E and M, while intracellular immature virions contain the precursor prM, which is cleaved into M during maturation[40].

The seven nonstructural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These genes all serve important functions, however, not all gene functions are fully understood. The most well studied include NS1, NS2B, NS3 and NS5NS5[41]. NS1 has multiple forms. The cell association form aids in RNA replication and the secreted form regulates complement activation. In addition, NS1 has been documented to play a role in neuroinvasinveness[41]. NS1 also forms a complex with NS2B that is involved in processing polyproteins[41]. NS3 contains a RNA helicase domain and a RNA triphosphatase needed for RNA replication and formation of

the 5' terminal cap. NS5 acts as the RNA dependent RNA polymerase (RdRP) that is critical for replication during infection[41].

1.2.3 Replication

The first step in replication is cell entry. This occurs when the E glycoprotein binds to cell surface receptors in a process referred to as "receptor-mediated endocytosis"[39]. This results in the invagination of the virion into an endosome. The host receptor used by flaviviruses are specific for each virus. DENV has been shown to bind with several cell membrane receptors including lectins and claudin-1[42]. *In vivo* mouse studies with JEV show that heat shock protein 70 may be the receptor. DC-SIGN, TIM1 and TAM receptors are used by ZIKV for entry and to date there has been no identified receptor for YFV[42].

The low pH of the endosome causes a conformational change in the E glycoprotein resulting in fusion of the viral membrane with the endosomal membrane and the release of the nucleocapsid into the cell cytosol[39]. From here, the genomic RNA travels to the endoplasmic reticulum (ER) where direct translation of the positive sense RNA can occur. The end of the RNA binds to translation initiation factors to form a complex that attaches to the ribosome to start translation of the whole viral genome[43]. Capsid proteins are held on the ER, while the E and prM proteins are found on the lumen side[43]. The E and prM proteins are activated by the host peptidase enzyme. In the cytoplasm, the viral proteases activate all the other proteins that aggregate to form the replication complex (RC). The viral RNA is then synthesized in multiple steps. The ends of the RNA bind to form a positive sense RNA circle that is processed by the RC to form a double helix with the negative strand RNA. This negative strand is then used as a template to copy more mRNA to be translated into proteins. E proteins aggregate in the lumen while the capsid proteins aggregate in the cytoplasm[39]. Genomic RNA binds to the capsid protein

and is packaged into new virus particles as it buds off from the ER[37]. In the immature virus, the prM covers the outer facing portions of the envelope proteins to prevent premature fusion with the cell membrane. The virus then unbinds, buds off and travels through Golgi apparatus towards the cell surface. Before reaching the surface, the prM is processed and mature virus buds from the cell[37]. Sometimes, partially mature/immature forms are released from infected cells. The amount of immature virus released varies between flaviviruses. Immature virus has been shown to be non-infectious but plays a role in host immune recognition[40].

1.2.4 ZIKV

ZIKV is an *Aedes*-borne flavivirus that was first isolated in the Zika forest in 1947 from a febrile sentinel rhesus monkey and then later from a pool of *Ae. africanus* mosquitoes[44]. With only a few human infections occurring before the 2000s, ZIKV epidemics were underreported due to misdiagnoses. However, an outbreak in 2007 on Yap Island occurred resulting in >5,000 infections[44]. Following this, an epidemic in 2013 of ZIKV was reported in French Polynesia with more than 30,000 cases and some neurological complications. ZIKV was introduced into Brazil in 2013-2014 from the Pacific Islands, but disease was not recognized in a human population until Nov. 2015. Over 1 million cases of ZIKV disease occurred during this pandemic. Moreover, there were hundreds of cases of spontaneous abortions reported and thousands of cases of microcephaly, ocular malformations and other birth defects[14]. Though not well studied, the virus has been associated with some unique epidemiological patterns and pathologies, including higher attack rates in women compared to men, and urogenital pathology that is linked to the sexual transmission of the virus.

ZIKV is unique among flaviviruses in its ability to be efficiently transmitted sexually, as well as by the bites of *Aedes* spp. mosquitoes. The true impact of sexual transmission on the

epidemiology of ZIKV is unclear. However, mathematical models have shown that sexual transmission contributed to 4.8% of transmission, and has greater impact in places where mosquito transmission is not present[46]. Sexual transmission has been reported from 13 countries without simultaneous mosquito-borne transmission[47]. Data from 2011 to 2016 found 27 published cases of ZIKV that were known to be transmitted by sex. Ninety-two percent of these cases were caused by male-to-female transmission from symptomatic and asymptomatic infected individuals[48]. It is clear that both genders can transmit the virus sexually, although shedding is seen in the male genital tract for more prolonged periods of time[48]. Semen samples from 2016 to 2017 found that ZIKV RNA can be detected in semen 281 days after symptom onset, but decreases substantially during the first three months[49]. Infectious ZIKV was found less often in semen, and only within the first 1 month after infection[49].

A common finding of ZIKV outbreaks is that a higher percentage of cases are females. The Puerto Rico outbreak from Nov. 2015 to Oct. 2016 resulted in a significantly higher disease incidence among women (936 per 100,000) than men (576 per 100,000)[50]. Interestingly the 2007 Yap Island outbreak also resulted in a higher attack rate for females relative to males (17.9 to 11.4 per 1,000)[51,52], even though more males had IgM antibodies against ZIKV (75% to 68%)[52], suggesting males were exposed more often but had less disease. Additionally, of the total, 65% of patients that had travel-associated ZIKV infections in the continental U.S. between 2015-2016 were female[53].

The ZIKV genome is 10.9 kb and consists of a single open reading frame with terminal 5'- and 3'- untranslated regions. The 5'-end of the genome has a type 1 cap, m7GppAmp. The genome encodes for three structural proteins and seven nonstructural proteins. There are two major lineages, African and Asian. Certain variations created by a high mutation rate have been

identified in ZIKV strains which contribute to the phenotypic difference in virulence and vector competence[54]. ZIKV evolution and adaption is influenced by the dual host (mosquito and mammalian) system; not only does ZIKV have to evade two immune systems, but it also goes through microenvironments as it disseminates within each system. This influences viral diversity and the defective viral genomes (DVGs) of ZIKV. Although there is evidence of other DVGs in flaviviruses, there is little investigation into ZIKV DVGs and the effects of them in transmission[55]. A recent study showed ZIKV RNA isolates from multiple organs in a susceptible mouse resulted in different ZIKV diversity. The production of DVGs were organ-specific, suggesting that dissemination through tissues leads to selective pressure and bottlenecks that drive viral variants and impact disease and spread[56]. Furthermore, data in the study showed that infection in the liver generated high viral diversity yet low RNA and infectious viral titers, suggesting that diversity could influence viral replication[56].

1.2.5 Alphaviruses

At least 27 alphaviruses have been recognized that cause disease in humans and animals including rodents, birds and horses. Alphaviruses are arthropod-borne with mosquitoes being the main vectors and avian hosts contributing to the global distribution for many species[57]. *Aedes* and *Culex* mosquitoes are the main vectors for CHIKV and are important in the transmission of Sindbis virus (SINV), equine encephalitis virus (EEEV), Semliki Forest virus (SFV), Venezuelan equine encephalitis virus (VEEV), CHIKV, MAYV, and RRV.

1.2.6 Structure

Alphaviruses are small, enveloped viruses that contain a single stranded, positive-sense RNA genome. The genome is approximately 11.8kb in length and encodes for two open reading frames (ORFs). The virion envelope is a host derived lipid bilayer that is embedded with E1 and

E2 proteins[32]. Alphaviruses have a 5' type 0 cap (N7mGppp) and a poly(A) tail at the 3' end. These factors aid in the ability for alphaviruses to evade the host immune system as it appears as host mRNA[58]. Additionally, there are untranslated regions (UTRs) that flank the ORFs. The 3' UTR of alphaviruses has been shown to help in virus replication and play a role in determining the virus host range.[59] The 5' ORF encodes for four nonstructural proteins that are critical to genome replication, RNA capping, polyprotein cleavage and viral replication[58]. These proteins are nsP1, nsP2, nsP3 and nsP4. NsP1 is important in negative strand RNA synthesis and RNA capping while nsP2 contains the helicase and proteinase that cleave the nonstructural polyprotein[60]. The role of nsP3 is the least known but has been shown to have a role in RNA synthesis. Lastly, nsP4 codes for the RdRP. The subgenomic mRNA generates a single polypeptide that codes for the capsid, E3, E2 6K and E1[60]. These proteins are cleaved during and after translation to form the functional structural proteins. E3, E2 6K and E1 are the envelope glycoproteins[60].

1.2.7 Replication

Envelope proteins bind to host receptors and the virion is transported into the cell by endocytosis of clathrin-coated vesicles[61]. There is little information known on the exact interaction, but it is hypothesized that E2 binds to the host receptor. Attachment factors including heparan sulfate proteoglycan, C- type lectins and phosphatidylserine have all be documented to aid in SINV, SFV and CHIKV attachment[62]. Mxra8 is reported as a receptor for CHIKV, RRV, MAYV, and ONNV. Additionally, prohibitin 1 is documented as a receptor of CHIKV[62]. In the case of SINV, it was shown that binding to dendric cells was SIGN receptor dependent and with CD-SIGN and L-SIGN (C-type lectins) acting as attachment factors[63], while the natural resistance-associated macrophage proteins are credited as the receptor[64]. After attachment the

virion enters the cell through endocytosis. Within the endosome, the pH level is reduced. This leads to a conformational rearrangement of the envelope protein in which E1 protein fuses with the endosomal membrane[61]. This allows the genomic RNA to be released in the cytoplasm, where replication can occur. Early in infection the non-structural proteins are cleaved between the nsP3 and the nsP4[65]. The non-structural polypeptide, containing nsP1-3, and nsP4 form an initial replication complex, including the RdRP, that is used to synthesize negative-strand RNA[57]. Additional cleavage of the nsP1-3 into nsP1 and nsP2-3 only occurs when there is a high concentration of polyprotein[57]. Once all the non-structural proteins are individually produced, negative synthesis switches to synthesis of the positive strand and sub genomic structural RNA. During translation of the structural RNA, the C protein is cleaved and aggregates with the genomic RNA by recognition of the packaging signal in the 5' half of the genome. This guarantees that only full-length RNA is packed[57]. These glycoproteins are transported to the Golgi network and cleaved by host proteases to generate mature E2 and E3 proteins. E2 interacts with the C protein and upon release the virion acquires a membrane bilayer[57].

1.2.8 CHIKV

CHIKV is an alphavirus transmitted by *Aedes* species mosquitoes. Infections with CHIKV result in arthralgia and myalgia that can persist for long periods of time after infection is cleared[16]. CHIKV is in 40 countries worldwide but was first reported in Tanzania in 1952 with other epidemics across Africa and Asia from 1960-1990[66]. In 2004, more than 500,000 cases were reported in an outbreak in Kenya and in 2005, 1.4 million people were affected in India[66]. Other small outbreaks were seen during the 2000s. Interestingly, a new variant, E1-226V, emerged in 2009 that allowed the virus to better adapt to *Ae. albopictus* resulting in epidemics in new regions[66]. CHIKV emerged in the Americas in 2013 and 1.1 million case were reported

within that year[67]. Since then, CHIKV has spread to 45 countries and territories in the Americas and has caused 3 million cases.

The CHIKV genome is 11.8kb long and encodes two polyproteins and contains two open reading frames. The first one encodes for four non-structural proteins, nsp1, nsp2 nsp3 and nsp4 and the second for the five structural proteins, capsid E3, E2, 6k and E1 [68]. The 3'-UTR is the longest among alphaviruses and is diverse across different lineages. It is hypothesized the insertion and deletion within the 3'-UTR led to this diversification and influenced the phenotypic difference between lineages[69]. The chikungunya genome contains a non-structural polyprotein that codes for nsP1, nsP2, nsP3, and RdRp nsP4. The RdRp is only expressed when there is suppression of translation termination, which happens about 10% of the time and regulates the expression of RdRp. During later stages of infection, subgenomic mRNA is transcribed. Its translation results in the production of the capsid (C), envelope (E), and 6k proteins. Interestingly, a frameshift occurs about 10% of time at the 6k sequence resulting in the production of TF protein. CHIKV translation is 5' cap dependent. The advantage to this system is efficient replication by regulating protein production as needed.

There are three phylogenetically different groups of CHIKV: Asian genotype, the Western African genotype, and the East, Central and Southern African (ECSA) genotype[70]. In 2005, the ECSA genotype virus was responsible for epidemics in Kenya, southeastern Africa and India where over 1 million cases were reported[71]. The E1 mutation that is present in many ESCA strains is accountable for these outbreaks and leads to efficient replication in *Ae. albopictus* mosquitoes[72]. Additionally, ESCA lineage CHIKV was isolated from an outbreak in St. Martin in 2013 that subsequently lead to the epidemic in the Western Hemisphere.

CHIKV RNA evolution is influenced by recombination and high mutation rate. The

highly error-prone RdRP generates many mutants and produces quasispecies. Quasispecies contain mutated genomes but also genomes with large deletions (DVGs) that are unable to replicate without the co-infection of a full-length virus. The production of CHIKV DVGs and defective interfering particles (DIPs; DVGs that interfere with the replication and production of wild-type virus) has well been documented in cell culture. DVGs were first described in influenza virus and have since been observed in many viral families when viruses have been passed at high multiplicity of infection (MOI). Multiple passaging at a high MOI drives the production of DVGs as it allows for co-infection of cells with whole length virus and DVGs, where DVGs take advantage of the proteins encoded by the full-length virus and hijack their replication machinery to produce more copies of themselves relative to full length virus because their genomes are shorter and thus replicate faster[55]. CHIKV DVGs have been generated in cell culture with both mammalian and mosquito cells and were shown to inhibit CHIKV infection in vitro as well dissemination in *Ae. aegypti* [73]. The production of DVGs during a natural CHIKV infection within a host (mammalian or invertebrate) is unknown, however it is likely that DVGs are generated during the course of infections, as seen with other alphaviruses, and these may influence transmission and disease outcome[74].

1.3. Mosquito vector-virus interaction

1.3.1 Mosquito immunity

There are many mosquito driven factors that influence the emergence of arboviruses. Cellular and molecular interactions between the virus and mosquito biomolecules drive virus diversification include those with the mosquito immune system, and virus interactions with tissues encountered as the virus disseminates to other tissues. Mosquitoes possess an innate immune system, but unlike a mammalian host, cannot mount an adaptive immune response. The

main antiviral mechanism in mosquitoes are the RNA interference (RNAi) pathways[75]. RNAi controls viral infections by cleaving long double-stranded RNA into small RNAs that can then be used to target and prevent viral replication. There are three type of small RNAs, small interfering RNA (siRNA), micro RNAs (miRNAs), and PIWI-interacting RNAs (piRNAs)[75]. The siRNA pathway is the major player in the antiviral response, with transcription, cleavage and processing of siRNA occurring primary in the cytoplasm[76]. Many viruses, including those discussed above produce dsRNA intermediate forms during replication. The siRNA pathway is initiated as the Dicer-2-R2D2 complex binds dsRNA[76]. Here the RNase II host enzyme, Dicer-2, cuts the dsRNA into siRNAs of 21-23 nt. These siRNAs activate the RNAi machinery by binding the RNA-induced silencing complex (RISC). The ssRNA serves as a guide strand to detect and degrade viral RNA by the host endonuclease Argonaute-2[76]. The role of siRNA is well documented in DENV, SINV and other arboviruses. Silencing of the siRNA pathway molecule results in increased virus replication and rates of dissemination in mosquitoes infected with DENV[77], SINV[78,79], and ZIKV[80,81].

miRNAsM are ~22 nt non-coding RNAs that are involved in post-transcriptional regulation of target genes. MiRNA pathways are similar to siRNA as they are both activated by dsRNA which is cleaved and bound to the RISC to sever as a guide-strand to detect and degrade viral ssRNA[75]. The main differences between siRNA and miRNA are the cellular location and effector proteins. miRNA) and processed into precursor RNA (pre-RNA) by the protein Drosha in the nucleus[82]. The pre-miRNA is then exported into the cytoplasm to be matured by Dicer1 and loaded into Ago-1 to generate the RISC. Due to a lack of miRNAs that are produced during infection, it is thought that miRNA does not have an antiviral role in mosquitoes. It is hypothesized that this is because RNA virus replication in the cytoplasm prevents access to

Drosha for processing in the nucleus[82]. However, miRNAs have been shown to affect modulating host genes that can control viral infection. During CHIKV infection of *Ae. albopictus*, expression of miRNAs are altered in the saliva[83]. It was also found that 17 miRNA in *Ae. aegypti* are differentially expressed during ZIKV infection[84]. Similar results have been document for DENV, WNV, an ONNV[75].

Until recently the role of the piRNA pathway in mosquito's antiviral response has been fairly unknown. The piRNA pathway produces 24-30 nt piRNAs in a dicer independent manner[75]. piRNA has been studied in *Drosophila* however homologues in *Ae. aegypti* and *Ae. albopictus* have been predicted to have the same function[85]. There are three Piwi proteins, the p-element induce wimpy testes (Piwi), Aubergine (Aub) and Argonaute 3 (Ago3) form the piRNA induced silencing complex (piRISC)[75]. ssRNA precursors in the cytoplasm are processed by the protein Zucchini into primary piRNAs that are then loaded onto Piwi and Aub proteins[75]. Piwi-associated proteins are translocated to the nucleus where they further used to silence the transcription of target sequences. Aub-associated piRNAs result in products that are bound to Ago3 and are mature into secondary piRNAs, and enter the "ping-pong" cycle for amplification[75]. piRNAs have been found to be expressed in mosquitoes after viral infections; although, the exact role of piwi RNA in mosquito antiviral immunity may have a great effect on viral diversity[85].

Other innate immune pathways in mosquitoes include JAK-STAT and Toll pathways. These pathways are initiated by the binding of a cytokine receptor. This leads to the activation of a protein kinase that in turn activates transcription factors. Innate immune gene are then expressed, including antimicrobial peptides (AMPs) and reactive oxygen species (ROS). These pathways have been shown to have a role in arbovirus infection in *Ae. aegypti*. During ZIKV

infection, AMPs are induced along with activation of the JAK-STAT pathway[86]. Activation of JAK-STAT and Toll during DENV infection has been shown to reduce infection of the midgut and fat bodies[87]. Interesting CHIKV nsP2 inhibits interferon signaling by inactivation of the JAK-STAT pathway[88], which aids in infection and dissemination[89]. Additionally, SFV infection downregulates the transcription of JAK-STAT[90]. Together this shows that different virus families have developed common mechanisms to interfere with the mosquito immune system.

Along with innate immune pathways, mosquitoes have cellular immune mechanisms that aid in antiviral activities. These defenses include phagocytosis, modulation and encapsulation of pathogens by hemocytes. Hemocytes are cells that circulate in the hemolymph of mosquitoes and have been shown to be infected by viruses, including DENV, SINV and WNV. In response to SFV infection, hemocytes produce a phenoloxidase cascade which catalyzes the formation of melanin around pathogens to prevent the spread of infection[91]. Additional hemocytes perform phagocytosis when activated by pathogens[75]. Lastly, fat body-mediated antiviral responses play a role in mosquito immunity. The fat-body organ in mosquitoes is similar to the liver in mammals in that it regulates metabolism and growth. It's role in anti-viral immunity is unclear, but it has been hypothesized to aid in inhibition of fatty acid synthase which decreases infectious virus[40].

1.3.2 Replication and dissemination in mosquitoes

After uptake of a viremic bloodmeal, the virus infects and replicates in the epithelial cells of the midgut[92]. The midgut consists of a single layer of epithelial cells that touches the bloodmeal, has a basal lamina (BL) on the hemocoelic side and these are wrapped with crisscrossing muscle fibers. The midgut is composed of two regions - the anterior midgut and the posterior midgut which hold and digests the bloodmeal. The two regions have distinctive characteristics, as the anterior region cells have noticeable microvilli, a smooth ER and a well-

developed basal labyrinth[93]. The posterior region's cells have a prominent rough ER and more mitochondria[93]. Once an infection is established in the midgut, the virus escapes from the midgut and replicates in the hemocoelic tissues. Virus circulate through the hemolymph allowing for translocation to other organs, including the salivary glands (SG)[92]. Mosquitoes have a pair of salivary glands that each consist of three lobes connected by the main salivary duct. These lobes are divided into two lateral lobes and median lobe. Each lobe has a smaller duct that is surrounded by epithelial cell and basal lamina (BL) layer. The lateral lobes are divided into proximal and distal regions[94]. Virus infects the acinar cells of the SG to replicate. To be transmitted during another bloodmeal, the virus also must escape the salivary glands and be excreted in the saliva.

It has been said that action of taking a bloodmeal for a mosquito is equal to 'humans drinking a 12 gallon smoothie containing 25 pounds of hamburger'. The blood components influence the immune and physiological state of the midgut, which affect the susceptibility of the epithelial cells to viral infection[95]. Shortly after bloodmeal acquisition, arboviruses must infect the gut epithelial cells. This step can be specific for certain mosquito-virus species/strain pairs and so is often referred to as the midgut infection barrier (MIB). This process is limited by mosquito gut immunity, host blood factors and the microbiota of the midgut[92,96]. RNAi, Toll and JAK-STAT pathways all play a role in limiting viral infection in the midgut. Physical barriers to the MIB include the lack of cell surface receptors on epithelial cells and the peritrophic matrix (PM)[97]. The PM is a semi-permeable layer that is secreted into the lumen in response to a blood meal. It surrounds the bloodmeal within the midgut to prevent tissue damage and protect it from infection. During infection, viruses infect the epithelial cells before the PM is expressed (4-12 hours post bloodmeal). Virions sit close to the epithelial cell layer and enter the epithelial cells

through the microvilli[97]. Interestingly, virus-mosquito species combinations have specific patterns of infection in the midgut. Experimental studies with DENV2 show that infection is only in the posterior midgut while JEV infected the entire midgut[98,99]. Additionally, EEEV infects 20-30% of midgut cells[100], while VEEV and WNV infect less than 5% of midgut cells[101,102]. These studies illustrate how infection is dependent on virus-vector pairs, infection and escape barriers.

Once in the midgut epithelium, replication occurs and virions pass through the BL by overcoming the midgut escape barrier (MEB) and enter the hemolymph. Interestingly, a recent study showed that successive non-infectious bloodmeals decrease the extrinsic incubation period (EIP), or the time from virus acquisition to the mosquito becoming infectious, by enhancing virus escape from the midgut[103]. A second bloodmeal leads to increased permeability of the BL layer and allows for virus to escape more readily. Cells circulating in the hemocoel become infected and transport virions to secondary organs, such as the fat body and nerve tissue[93]. Additionally, it is thought that viruses use the hemocoel as vehicle to disseminate from the midgut to SG. Experimental infections with SINV show that hemocytes aid in amplification of virus outside of SG[104]. Studies with sevral arboviruses all show that the distal lateral lobes of the SG become infected and thus it has been hypothesized that these lobes contain receptors that aid in virus endocytosis[93,105]. After replication virus travels into the apical cavities of the SG acinar cells to eventually be transmitted in the saliva that is expelled into the host during blood feeding.

Viral replication and dissemination play at critical role in vector competence, which is the ability of an arthropod to transmit a virus. Mosquito vector competence is influenced by genetic factors, such as those stated above (e.g. replication, dissemination, immune response etc.), and environmental factors[106]. Environmental factors include finding a bloodmeal source, virus acquisition during feeding, and environmental conditions. Vector competence is a quantitative

biological measurement and represents the proportion of mosquitoes in a population that are able to transmit a given virus[107].

1.3.3 Vectorial Capacity

Mosquito vectorial capacity is a measure of the ability of a mosquito species to successfully transmit a pathogen, and include vector competence as well as all of the life traits of the vector that influence its success as a vector. Mathematically, vectorial capacity is defined by 5 factors: 1) vector density with respect to host, 2) daily probability of the bitten 3) vector competence, 4) probability of survival, and 5) the extrinsic incubation period (EIP)[107]. Vector density and probability of daily survival are often the target of vector control methods and are affected by climate change and globalization. The daily probability of a host being fed on is affected by differences in human behavior and living conditions. Vector competence is affected by many factors intrinsic to the vector's biology, including microbiome, virus adaptation, and genetics[108]. EIP is the amount of time that a virus takes to be transmitted after taken up in a mosquito bloodmeal, and is affected by biology as well as by climate and virus evolution. Many of these factors are specific to individual viruses and mosquito pairs.

Viral diversity drives changes in vectorial capacity as new variants may have decreased EIPs or increased vector competence. For example, the production of novel viral RNA sequences during replication in the mosquito can be beneficial for the virus because dsRNA will not match target sequences and won't be targeted for degradation by siRNA[109]. The production of new variants results in a virus population with a large number of variant genomes (quasispecies) that drive changes in vector competence and host expansion[110]. As arboviruses infect and replicate in

multiple hosts, they encounter bottlenecks at various stages of infection. These bottlenecks lead to the reduction of quasispecies and have an important impact on virus evolution[111].

1.3.4 Transmission by mosquitoes

Mosquito saliva contains many proteins with vasodilatory, antihemostatic, anti-inflammatory, and immune-modulating effects[112]. During a blood feeding event, these proteins are secreted into the host along with any virus the mosquito may be transmitting. Many studies have shown that mosquito saliva facilitates viral transmission and contributes to subsequent disease. A number of studies in mice have demonstrated this for WNV[113,114], DENV[115], SFV[116] and CHIKV[117]. The broad hypothesis from mosquito bite enhancement studies is that viral infection is facilitated by saliva components that modulate immune pathways by several mechanisms. These include 1) immune cell activation which leads to blood capillary leakage and thus retention of virus in extravascular tissues[112], 2) immune cell activation which enables the virus to hijack the immune cell to facilitate its spread[118], 3) disruption of endothelial cell barriers which leads to virus dissemination and enhanced cell migration[118], 4) suppression of the innate immune response, and 5) subversion of the host adaptive immune response[119].

A mosquito transmits only ~5 nL of saliva as measured by forced salivation[120]; however, the quantity of infectious virus found to be secreted in the saliva of individual mosquitoes can vary by 5 orders of magnitude[106,121] and this is not always correlated with the quantity of saliva secreted[120]. In models of interhost transmission, stochastic virus population bottlenecks and expansions greatly influence the number of virions being transmitted between hosts[122–124]. Furthermore, observations with ZIKV, DENV, CHIKV and WNV[120,124] have shown a large disparity between infectious virus and genome copies in individual mosquito saliva samples. Experiments with EEEV[125,126], WNV[127,128], VEEV[129], have showed a large range, from

undetectable to 5 $\log_{10}$[125], of infectious virus transmitted . These results show that there is variation in the amount of virus transmitted in saliva, that may be dependent on the virus-vector pairing, Notably, the disparity between infectious virus and genome copies in the saliva of individual mosquitoes has been observed throughout all vector studies. Current studies have implicated a role of defective viral genomes (DVGs) as an explanation for this pattern, and may even play a role in viral persistence in the invertebrate host[130,131].

DVGs have been described in most RNA viruses and have been associated with antiviral immunity during infection[55]. There are different types of DVGs that can be generated by mutations, frame shifts, deletions, and copy-backs. DVGs are usually distinguished by large deletions in the genome; however, many DVGs have small alterations resulting from mutation or frame shift[74]. A point mutation in particular regions, such as the replicase, yield genomes that cannot replicate but have proper structural proteins. Internal deletions can occur during viral replication where serval essential genes are removed but the 5' and 3' ends are retained[74]. Alphaviruses also retain the packing signals in DVGs[132]. Multiple deletions across the genome result in different DVG variants within samples obtained from infections. Copy-back and snap back DVGs are rearranged genomes in which a sequence is duplicated in the reverse complement to create stem-like structures[74]. These are generated when the RdRP detaches from the template and reattaches to the nascent strand, copying back the end of the genome. Interestingly, it has been hypothesized that the generation of DVGs is not completely spontaneous but may be encoded in viral genomes. This has been observed with vesicular stomatitis virus, respiratory syncytial virus and influenza virus, where hotspots for generation of copy back DVGs have been identified[133–135].

DVGs have been more well characterized in alphaviruses in comparison to flaviviruses. This may be due to the fidelity of the RdRp. Alphavirus have a low-fidelity RdRp in comparison, and infection with Sindbis virus shows that low-fidelity RdRP enhances production of DVGs[136]. DVGs have been identified during natural DENV infection, however, the role and impact they have on disease outcome is unknown[137]. Additionally, WNV DVGs were isolated from dead birds in New Mexico, though the DVGs were not linked to disease[138]. It was found that mice inoculated with high levels of SFV DVGs in additional to standard virus were protect from disease[139]. Recently, the role of DVGs has expanded into antiviral immunity in mosquitoes and persistent infection. *Drosophila* infected with Sindbis virus DIPs and full length virus survived longer than those without DIPs[140]. Recently, similar results were shown with CHIKV, where DVGs limited infection in *Ae. aegypti* mosquitoes[73]. Together, these studies demonstrate that the function of DVGs in natural infection is not yet understood, but they may play more important role arbovirus transmission and disease dynamics.

CHAPTER 2: IN SEARCH OF AN IMMUNOCOMPETENT SMALL-ANIMAL MODEL FOR THE STUDY OF SEXUAL TRANSMISSION OF ZIKA VIRUS

2.1 Introduction

ZIKV is a positive-stranded RNA virus in family *Flaviviridae*. ZIKV is primarily transmitted to humans through the bite of an infected mosquito. Transmission also occurs perinatally, through sexual activity, and blood transfusion[141–145]. The 2015-2016 ZIKV pandemic in the Americas resulted in over 1 million suspected cases, with hundreds of spontaneous abortions reported and thousands of infants born with microcephaly, ocular malformations and other birth defects[146–148]. Following the epidemic, many groups sought to characterize animal models for ZIKV infection as a means of characterizing viral pathogenesis and the species' immune response for future pre-clinical studies.

Non-human primates (NHPs) and mice are the most widely used animal models for ZIKV infection[149–151]. There are advantages and disadvantages to both models. NHPs are naturally susceptible to ZIKV infection and are similar to humans anatomically and physiologically, such as developmentally and *in utero,* including comparable gestational periods[152]. However, they are also costly to maintain, have restrictions on group size, and are surrounded by ethical considerations[153]. Mice are small, have a fast-reproductive rate, and are easy to genetically manipulate. However, immunocompetent mice are not naturally susceptible to ZIKV infection[153].

There are several large immunocompetent animal models for ZIKV—including goats, sheep, water buffalos, lions, and NHPs[154]. The NHP models are the most favorable for ZIKV owing to anatomic and physiologic similarity between humans and NHPs. Challenge studies in rhesus (*Macaca mulatta*), pigtail (*Macaca nemestrina*), and cynomolgus macaques (*Macaca*

fascicularis) have shown that viremia lasts for weeks even in the absence of clinical symptoms[155,156]. This holds true for other NHPs such as owl monkeys (*Aotus* sp.*)*, squirrel monkeys (*Saimiri* sp.), and the marmoset (*Callithrix jacchus*)[157,158]. The use of NHP models has provided guidance and information on the safety and efficacy of vaccine and drug treatments. However, high costs, space constraints, and ethical issues surrounding NHP research limits the numbers of animals that can be used in studies and reduces the statistical power[159]. Therefore, NHPs models have challenges if to be used to study how transmission route effect disease outcome.

Mice have been extensively used for modeling ZIKV infection, as reviewed in Bradley et al[160]. However, very few ZIKV-susceptible immunocompetent mouse models have been established since efforts began in 2015, and none of these studies investigated sexual transmission[161,162]. All non-NHP studies establishing sexual transmission of ZIKV have relied on genetically modified knockdown mice lacking a fully intact IFN 1 response[163,164]. Strains of mice successfully established for investigation of vaccines and other therapeutics include *Ifngr1* knockout, *Stat2* knockout, *Irf3/Irf5* double knockout *Irf3/Irf5/Irf7* triple knockout[150,165–167]. ZIKV is able to evade human type I interferon (IFN) response due to species-specific evasion mechanisms[153]. However, the IFN response in mice is able to interfere with viral replication and prevent infection[168]. While these models are helpful in assessing transmission routes, a major limitation is their inability to provide information about the immune response mounted in the face of infection.

To investigate potential small animal models with intact innate immune systems for studying the sexual transmission of ZIKV, we experimentally inoculated the New Zealand white (NZW) rabbit (*Oryctolagus cuniculus*), Natal multimammate mouse (*Mastomys natalensis*),

Hartley guinea pig (*Cavia porcellus*), and the Jamaican fruit bats (*Artibeus jamaicensis*). These four animal models were of particular interest owing to previous studies characterizing their susceptibility to ZIKV (Hartley guinea pigs[169–173], Jamaican fruit bat) or susceptibility to other flaviviruses (NZW rabbit[174] and multimammate mouse[175]). Further, these animal models are outbred, which more closely mimic the human population in their genetic heterogeneity and allow for analysis of diverse responses to vaccines and other therapeutics[176,177].

Hartley guinea pigs have been used for a model of ZIKV infection and are susceptible to ZIKV when infected subcutaneously and intranasally[169,170,172]. Advantages to using guinea pigs as an animal model are their small size and high reproductive rate, facilitating the use of larger sample sizes. The reproductive physiology of the guinea pig is also similar to that of humans, making them optimal for translational animal models[178]. Recent studies have examined the effects of ZIKV on fetal development when females are infected during pregnancy, showing that pregnant dams are susceptible to infection, resulting in abnormal pregancies[171,173]. However, all studies with Hartley guinea pigs have investigated susceptibility of very young animals or animals that may otherwise be immunocompromised (for instance, due to pregnancy). The susceptibility of sexually mature adult guinea pigs has not yet been assessed, nor has the potential for guinea pigs to transmit ZIKV sexually.

Challenge of New Zealand white rabbits with ZIKV has not yet been reported, though they have been established as animal models for West Nile virus and Murray Valley encephalitis virus, two other mosquito-borne flaviviruses. When inoculated, NZW rabbits demonstrate a resistant phenotype similar to that appreciated in horses and humans[174]. Also of interest, cottontail rabbits (*Sylvivagus* spp.) inoculated with Asian-lineage ZIKV (PRVABC59) were

shown to seroconvert 28 days post-infection, though none demonstrated viremia[179]. To date, the susceptibility of NZW rabbits to ZIKV has not been described.

Jamaican fruit bats are one of the most abundant bats throughout the Caribbean, Mexico and Central America, and closely related *Artibeus* species and subspecies are similarly abundant throughout South America. Furthermore, these fruit-eating bats are abundant in known ZIKV urban and peri-urban hotspots because of the presence of many fruit trees in human population centers[180,181]. Male Jamaican fruit bats subcutaneously inoculated with Asian-lineage ZIKV become infected, demonstrating viral RNA in some tissues and mounting an antibody response[181]. Histopathology and immunohistochemistry showed evidence of virus replication in brain tissue, testes, lungs and salivary glands[181]. Older studies in the 1960's demonstrated susceptibility of Angolan fruit bats (*Lissonycteris angolensis*), straw-colored fruit bats (*Eidolon helvum*), Egyptian fruit bats (*Rousettus aegyptiacus*), and new world little brown bats (*Myotis lucifugus*) to African-lineage ZIKV (strain MR766)[182–184]. Additionally, serologic investigations indicate the exposure of several wild bat populations to ZIKV, or a closely related flavivirus[185].

Lastly, the multimammate mouse is known to be a host for several viruses, including arenaviruses and flaviviruses[186,187]. Usutu virus, a *Culex*-associated mosquito-borne flavivirus, was isolated from three multimammate mice in Senegal, warranting further investigation into this rodent's role in sylvatic flavivirus transmission[175]. Additionally, the closely related *Mastomys coucha* are used for pre-clinical models in papillomavirus research[188]. To date there are no published studies with these animals examining their potential as a viral reservoir for medical important pathogens.

ZIKV is primarily transmitted by mosquito bite. A number of studies have demonstrated that mosquito transmission, as compared to needle inoculation, of West Nile Virus (WNV)[113,114],

dengue viruses (DENV)[115], Semliki Forest virus (SFV)[116] and chikungunya virus (CHIKV)[117] affect infection outcome. A study in ZIKV-infected NHPs resulted in delayed viremia when the animals were infected by mosquito bite, as well as differences in tissue tropism from individuals who were subcutaneously inoculated[189]. These results suggest that inoculation by infected mosquito bite alters replication kinetics and pathogenesis, and thus, investigating the effect of mosquito saliva is an important area of study when establishing an animal model.

Despite the devastating impacts of ZIKV and its rapid global spread in 2015-2016, to date no immunocompetent small animal model exists allowing for the study of sexual transmission dynamics and associated pathology. This study was undertaken to identify potential candidates for such a model to characterize mechanisms underpinning the sexual transmission of ZIKV.

2.2 Results

2.21 Multimammate mice (Mastomys natalensis) are not susceptible to ZIKV

A total of 15 multimammate mice, both male and female, were inoculated with Asian lineage ZIKV (PRVABC59) and an African strain of ZIKV (DAR41525) (Table 2.1 and 2.2). Additionally, two A129 mice (previously confirmed to be susceptible[190]) were infected in parallel as positive controls and were inoculated with ZIKV PRVABC59 only (Table 2.2). All animals were inoculated with $2.6x10^6$ PFU of respective the ZIKV strains. All mice were euthanized five days post-inoculation, and all tissues (saliva, blood, brain, heart, lungs, liver, kidney, bladder, testis, seminal vesicles and ovary) were negative by qRT-PCR. All tissues from positive control A129 mice were positive for ZIKV viral RNA loads (10^8- 10^9 PFU equivalents/gram) (Figure 2.1).

Table 2. 1 Summary of all animals inoculations and sample collections

Animal	n	Sex	Inoculation route	Animals per Inoculum Group				Euthanasia timepoint	Samples collected from each animal		
				ZIKV 41525	ZIKV PRVABC59	ZIKV M766	Sham-inoculated		Ante-mortem samples**	Non-reproductive organs***	Reproductive organs
Mastomys natalensis	6	F	SC	2	3	0	1	5dpi	Blood, Saliva	Brain, heart, lungs, liver, kidney, bladder	Ovary
	6	M		2	3		1				Testes, Seminal vesicles
New Zealand white rabbit	2	F	Ivag	0	2	0	0	Not euthanized	Blood, Saliva, Vaginal swab, Urine		N/A
	6	M	SC	0	6		2	7dpi, 28dpi*	Blood, Saliva, Semen Urine		Testes, Seminal vesicles
Hartley guinea pig	8	M	SC	0	3	0	1	7dpi	Blood, Saliva, Urine		Testes, Cowper's gland
			MB		3		1				
Jamaican fruit bat	9	F	SC	2	5	0	2	2dpi, 3dpi, 5dpi, 28dpi, 35dpi and 48dpi	Blood, Saliva, Urine		Ovary
	6	M		1	4		1				Testes

*for each timepoint, 2 inoculated rabbits and 1 sham-inoculated rabbit where euthanized
**ante-mortem samples were collected every two days
*** no organs were collected from female rabbits
Ivag= intravaginal, SC = subcutaneous, MB = mosquito bite. Sham-inoculated animals (with 100 µL of PBS) were negative controls. N/A= non-applicable
ZIKV PRVABC59= ZIKV strain PRVABC59 (ZIKV-PR; GenBank:KU501215), ZIKV 41525= ZIKV strain DAK 41525 (GenBank:KU955591.1), ZIKV M766= ZIKV strain MR766 (GenBank:LC002520),

Table 2. 2 Multimammate mice (*Mastomys natalensis*) inoculations

Animal ID	Species	Sex	Virus inoculated
1	*M. natalensis*	Male	Zika 41525
2	*M. natalensis*	Male	Zika 41525
3	*M. natalensis*	Male	Zika PRVACB59
4	*M. natalensis*	Male	Zika PRVACB59
5	*M. natalensis*	Male	Zika PRVACB59
6	*M. natalensis*	Male	Mock
7	*Mus musculus* (A129 strain)	Male	Zika PRVACB59
8	*Mus musculus* (A129 strain)	Male	Mock
9	*M. natalensis*	Female	Zika 41525
10	*M. natalensis*	Female	Zika 41525
11	*M. natalensis*	Female	Zika PRVACB59
12	*M. natalensis*	Female	Zika PRVACB59
13	*M. natalensis*	Female	Zika PRVACB59
14	*M. natalensis*	Female	Mock
15	*Mus musculus* (A129 strain)	Female	Zika PRVACB59
16	*Mus musculus* (A129 strain)	Female	Mock

All animals were subcutaneously inoculated with $2.6x10^6$ PFU in 100 µL of virus or 100 µL of PBS (Mock) and euthanized at 5 d.p.i. *Mus musculus* (A129 strain) were used as positive control.
ZIKV PRVABC59= ZIKV strain PRVABC59 (ZIKV-PR; GenBank:KU501215), ZIKV 41525= ZIKV strain DAK 41525 (GenBank:KU955591.1)

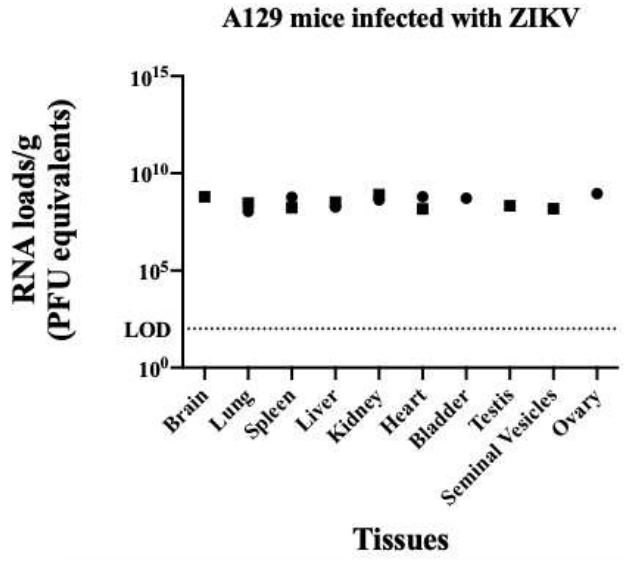


Figure 2. 1 ZIKV RNA levels of infected *Mus musculus* (A129 strain) positive controls sampled at 5 dpi; square= male, n=1 and circle= female, n=1. LOD= limit of detection.

2.2.2 New Zealand white rabbits are not susceptible to ZIKV

As our preliminary investigations sought to characterize male-to-female sexual transmission using the Asian linage, the susceptibility of male rabbits to ZIKC PRVABC59 was carried out first. Six male rabbits were subcutaneously inoculated with $2.6x10^6$ PFU of ZIKV PRVABC59 (Table 2.3). No significant changes in body weight or temperature were observed during the course of the study (Figure 2.2). Additionally, all tissues (brain, heart, lungs, liver, kidney, bladder, testis, and seminal vesicles) and fluid samples (saliva, blood, and semen) were negative by qRT-PCR. After male rabbits were euthanized, two female rabbits were intravaginally inoculated with $2.6x10^6$ PFU of ZIKV PRVABC59 (Table 2.3). Blood, saliva, and vaginal swabs samples from females were negative by qRT-PCR. Serum collected from the 3 males euthanized at 28 d.p.i. did not neutralize ZIKV, indicating lack of seroconversion (Figure 2.3).

Table 2. 3 Study design for the inoculation of New Zealand white rabbits with ZIKV

Animal ID	ZIKV Strain	Sex	Euthanized d.p.i
Rabbit 1	Zika PRVACB59	Male	7
Rabbit 2	Zika PRVACB59	Male	7
Rabbit 3	Mock	Male	7
Rabbit 4	Zika PRVACB59	Male	28
Rabbit 5	Zika PRVACB59	Male	28
Rabbit 6	Mock	Male	28
Rabbit 7	Zika PRVACB59	Female	N/A
Rabbit 8	Zika PRVACB59	Female	N/A

All animals were subcutaneously inoculated with $2.6x10^6$ PFU in 100 µL of virus or 100 µL of PBS (Mock). Females were not euthanized after 28 days; saliva, urine and vaginal swabs were negative by q-RT-PCR (N/A= non-applicable). ZIKV PRVABC59= ZIKV strain PRVABC59 (ZIKV-PR; GenBank:KU501215)

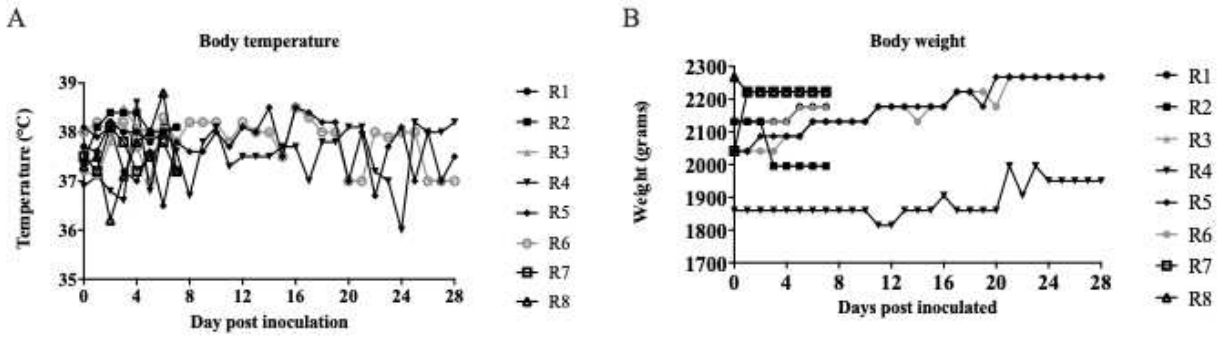


Figure 2. 2 NZW rabbit body temperature and weight collected every two days. A) body temperature and B) body weight. Black = inoculated with ZIKV, Grey=Mock inoculated (negative)

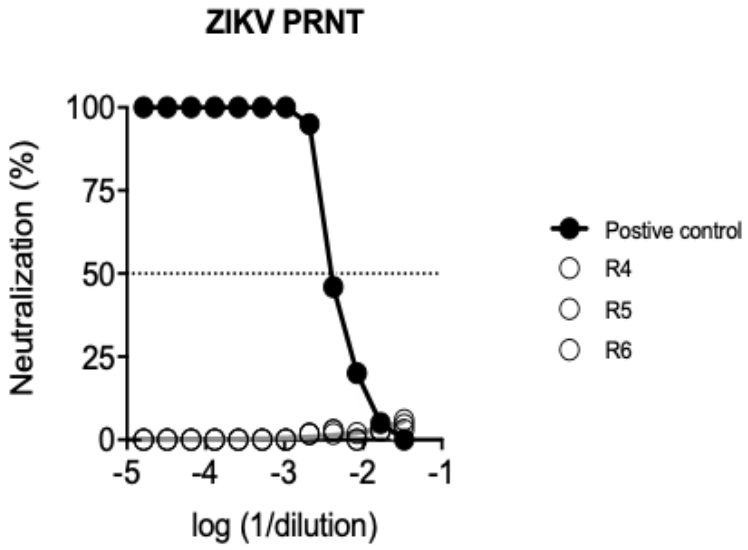


Figure 2. 3 ZIKV PRNTS of rabbit serum from 28 d.p.i. Positive control is serum from human previously infected with ZIKV.

2.2.3 Mature Hartley guinea pigs are not models for sexual transmission for ZIKV or susceptible to ZIKV by mosquito bite.

Males were once again predominantly used to evaluate the use of these animals as a model of sexual transmission of ZIKV. Males were inoculated either subcutaneously or by infectious mosquito bite to evaluate whether mosquito saliva would potentiate infection, as multiple studies have demonstrated them to be susceptible to ZIKV infection (Table 2.1 and 2.4). Following infection by mosquito bite, mosquito bodies (pooled by guinea pig) were all positive for ZIKV RNA via qRT-PCR (Figure 2.4). However, all tissues (brain, heart, lungs, liver, kidney, bladder, and testis) and fluid samples (saliva and blood) collected from guinea pigs either infected subcutaneously or by infectious mosquito bite were negative by qRT-PCR. Body weight and temperature showed no significant changes during the course of the study, additionally no clinical signs (fatigue, weight loss, hunched posture, scruffy fur or labored breathing) where observed (Figure 2.5).

Table 2. 4 Inoculations of ZIKV into adult male guinea pigs (GP).

Animal ID	ZIKV strain	Inoculation Route	Sex
Guinea Pig 1	Zika PRVACB59	SC	Male
Guinea Pig 2	Zika PRVACB59	SC	Male
Guinea Pig 3	Zika PRVACB59	SC	Male
Guinea Pig 4	Mock	SC	Male
Guinea Pig 5	Zika PRVACB59	MB	Male
Guinea Pig 6	Zika PRVACB59	MB	Male
Guinea Pig 7	Zika PRVACB59	MB	Male
Guinea Pig 8	Mock	MB	Male

All animals were euthanized at 7 d.p.i. SC = subcutaneous, MB = mosquito bite. SC inoculated animals were subcutaneously inoculated with $2.6x10^6$ PFU in 100 µL of virus. Mock animals were negative controls and inoculated with 100 µL of PBS or bit by non-infectious mosquitoes.

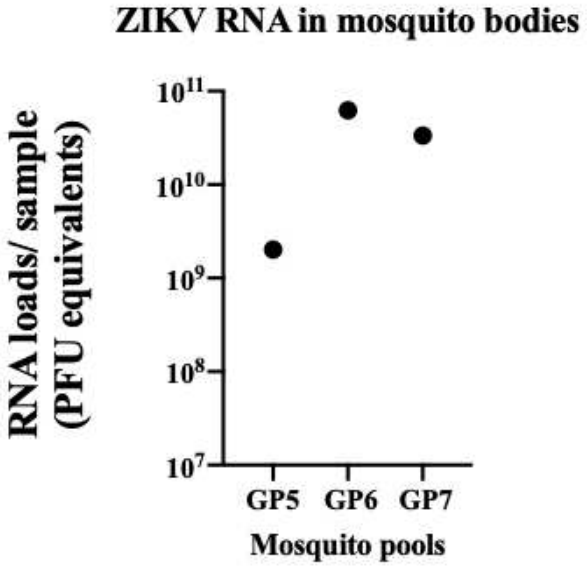


Figure 2. 4 ZIKV RNA levels in pools of mosquito bodies that fed on guinea pigs

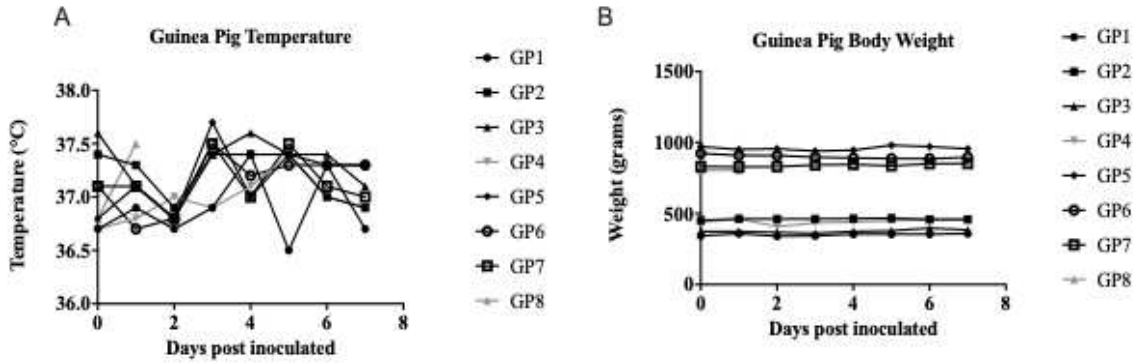


Figure 2. 5 Guinea pig body temperature and weight collected every two days. A) body temperature and B) body weight. Black = inoculated with ZIKV, Grey=Mock

2.2.4 Artibeus jamaicensis are not good models for sexual transmission for ZIKV

Male Jamaican fruit bats (*Artibeus jamaicensis*) have previously been shown to support ZIKV PRVABC59 replication[181]. We evaluated the infection potential of two additional ZIKV strains (DAR41525 and MR766) and explored the use of Jamaican fruit bats as a sexual transmission model by evaluating infection in both males and females after s.c. inoculation (Table 5). Samples from bats euthanized late during after the course of infection, including urine, saliva, blood, and tissues (brain, heart, lungs, liver, kidney, bladder and reproductive track-testis, seminal vesicles and ovary) were negative by qRT-PCR, using an assay targeting the NS5 gene. However, using a set of primers target 3' sfRNA and traditional PCR, viral cDNA was detected in some samples (Table 6). Fluid and tissue samples from bats euthanized earlier on during the course of infection (2, 3, and 5 dpi) were also negative by qRT-PCR with primers targeting the NS5 gene and when targeting the 3'sfRNA with traditional PCR. With only low and infrequent levels of detectable RNA in select tissue AJs are not a good model for sexual transmission as robust and sustained infection are needed.

Table 2. 5 Jamaican fruit bat inoculations with ZIKV

Animal ID	Sex	ZIKV strain	Euthanized d.p.i
AJ_001	Female	MR766	35
AJ_002	Female	MR766	28
AJ_003	Male	MR766	35
AJ_004	Female	PRVABC59	35
AJ_005	Male	PRVABC59	35
AJ_006	Female	PRVABC59	28
AJ_007	Female	41525	48
AJ_008	Male	41525	48
AJ_009	Female	41525	48
AJ_010	Male	PRVABC59	2
AJ_011	Female	PRVABC59	2
AJ_012	Female	PRVABC59	3
AJ_013	Male	PRVABC59	3
AJ_014	Male	PRVABC59	5
AJ_015	Female	PRVABC59	5

All animals were subcutaneously inoculated with $2.6x10^6$ PFU in 100 µL. ZIKV PRVABC59= ZIKV strain PRVABC59 (ZIKV-PR; GenBank:KU501215), ZIKV 41525= ZIKV strain DAK 41525 (GenBank:KU955591.1), ZIKV MR766= ZIKV strain MR766 (GenBank:LC002520)

Table 2. 6 Results of PCR and Sanger sequencing for bats held at 28dpi and 48dpi. PCR target was 3' sfRNA

Tissue	Aj002 (MR766, 28 dpi)	Aj006 (PRVABC59, 28 dpi)	Aj009 (41525, 48 dpi)
Brain	No	Yes	Yes
Heart	Yes	No	Yes
Lung	Yes	No	Yes
Liver	No	No	No
Spleen	Yes	No	No
Kidney	Yes	No	Yes
Bladder	Yes	No	Yes
Ovary	No	No	Yes
Uterus	Yes	Yes	No
Blood	Yes	Yes	No

2.3 Discussion

There are many murine models for the study of ZIKV, however most are immunodeficient or immunosuppressed mice that lack an intact IFN pathway. NHP models have been successfully used but the restrictions on sample size limits statistical power. Further, NHP's are more difficult to obtain for experimental studies due to their current prioritization for pre-clinical SARS-CoV-2 research. Much was learned about ZIKV pathogenesis following the Westward expansion of Asian lineage ZIKV, though many questions remain – questions whose answers may lay important groundwork for the study of other emerging viruses capable of sexual transmission. Establishment of an immunocompetent small animal model for the sexual transmission of ZIKV would help illustrate a more comprehensive portrait of ZIKV transmission and pathogenesis, informing future drug development studies and risk mitigation strategies.

We examined the multimammate mouse, New Zealand white rabbit, Hartley guinea pig, and Jamaican fruit bat as immunocompetent small animal models for ZIKV infection with the goal of developing one into a model for studying ZIKV sexual transmission. In these studies, it was found that the multimammate mouse and NZW rabbit are not susceptible ZIKV infection. Our data also show that sexually mature Hartley guinea pigs were also not susceptible to ZIKV infection, contrary to other studies[169,170,172]. Additionally, ZIKV infection of guinea pigs was not established even when inoculation by infectious mosquito bite. Because our preliminary goal of this study was to establish a ZIKV sexual transmission model, we focused on male infections because up to 90% of sexually transmitted ZIKV cases are due to male-to-female transmission[191]. Here the susceptibility of only male animals was determined with the guinea pigs and to a lesser extent rabbits, this led to a male bias in these studies. Additionally, the Jamaican fruit bat is susceptible to ZIKV but is not a good model for sexual transmission and

disease due to low viremia, lack of viral RNA in sexual reproductive organs, and the lack of clinical symptoms.

Artibeus species and subspecies are abundant throughout South America and reside in known ZIKV urban and suburban hotspots[180,181]. A recent study challenging male Jamaican fruit bats with ZIKV strain PRVABC569 demonstrated the animals were acutely infected and mounted an antibody response to ZIKV[181]. Our data support this, as we found limited viral RNA in tissues at late phases of infection. Jamaican fruit bats are a relatively new animal model for the study of viral infection[181,192,193]. and much is yet to be learned surrounding their immune response, reproduction, mating behavior, and normal blood chemistry values[194]. As this animal model becomes more well-characterized, its value for the study of ZIKV and other medically important viruses will increase exponentially for enhanced understanding of viral ecology and innate immunity. More importantly, bats may play a critical role in arbovirus ecology and transmission as zoonotic virus reservoirs[195]. Interestingly, bats and hematophagous arthropods fill many of the same ecological niches, and mosquito species that transmit arboviruses have been known to take blood meals from bats[196]. The role as bats as reservoir hosts for ZIKV should be further explored, and the use of Jamaican fruit bats as a model system to investigate the potential for sylvatic transmission of mosquito-borne viruses is high priority.

Although the results of our study indicate that the multimammate mouse was not susceptible to ZIKV, it plays an important role in viral ecology and its permissiveness to other viruses should be further explored in controlled settings. The multimammate mouse is natively found in West Africa and is the main reservoir of Lassa virus (LASV). The prevalence of LASV in multimammate mouse can be 8%-30% in the wild, and can transmit to human by direct or indirect exposure to infected rodent fluids[197]. Other viruses have been isolated from natural

multimammate mouse populations including alphaviruses, bunyaviruses and flaviviruses[198]. We were interested in the susceptibility of the multimammate mouse to ZIKV not only for its use as an animal model, but in characterizing potential sylvatic reservoirs of ZIKV owing to the geographic overlap of the multimammate mouse and ZIKV risk (as characterized by suitable *Ae. aegypti* habitat), in addition to the fact that mosquitoes known to transmit ZIKV (genus *Aedes*) are known to feed on rodents.[199–201]

Previous studies inoculating Hartley guinea pigs via subcutaneous and intranasal inoculation resulted in low levels of viremia and effects on fetal development. However, guinea pigs used in these studies were either very young (under 5 weeks)[169–172] or pregnant[173]. One study used 6-month-old non-pregnant females, and observed low levels of RNAemia (10^3-10^4 RNA copies), but these animals were also subcutaneously inoculated with higher titers (10^7 or 10^8 PFU) than we used and that are unlikely to be transmitted by mosquito bite[173]. Younger animal have a less robust immune response[202] and pregnancy leads to an immunosuppressed state[203], making individuals more prone to viral infection. Therefore, it is interesting that the sexually mature guinea pigs used in our study (inoculated subcutaneously with $2.6x10^6$ PFU) did not become infected with ZIKV at detectable levels, and that infection by mosquito bite did not potentiate infection, as in other studies demonstrating altered replication kinetics by this route[189]. Although guinea pigs may not be a good model for sexual transmission, they may be useful to address question surrounding fetal development during infection.

Other small animal models including hamsters, humanized STAT2 mice, and more recently the treeshrew, have been used in ZIKV infection studies[204–206]. Overall, these models, with the exception of the treeshrew, are insufficient models for sexual ZIKV transmission. Syrian golden hamsters developed neutralizing antibodies after inoculation with ZIKV, but no

viremia was detected[204]. In addition, a STAT2 humanized mouse was created to make a more fully immunocompetent animal model[205]. Although this model does allow for ZIKV replication and has been used to look at drug candidates and effects on pregnancy, it is a transgenic mouse and still lacks a fully intact immune system comparable to humans. However, the tree shrew proved to be susceptible to ZIKV with high viremia and viral RNA secreted in saliva. They also developed typical dermatological manifestation[206]. The treeshew seems to be a promising animal model for ZIKV pathogenesis and future efforts should investigate its susceptibility via different transmission routes, including via coitus and infectious mosquito bite.

Interestingly, different ZIKV strains result in variable pathogenicity, both in clinical cases and animal models. Previous studies demonstrated that the African strain of ZIKV causes more severe infection, including *in utero*[207,208]. *In vivo* studies in which mice were infected with different strains of ZIKV also demonstrate variation in tissues tropism[209], neuropathology[210] and innate immune response[211]. Comparative studies with multiple strains of ZIKV are critical for defining genetic variation that may contribute to differences in immune response, pathology, and forward transmission potential. In this study the strain of ZIKV did not make a difference in susceptibly to of this animal species.

While our investigation did not result in the establishment of a small animal model for the sexual transmission of ZIKV as we had hoped, the findings are still valuable as we have demonstrated three outbred animal species that are not permissive to infection, one of which was confirmed susceptible at more immunocompromised life stages (the Hartley guinea pig). These models should continue to be used for sexual transmission studies to more fully characterize at which point in infection the risk of transmission is highest. These data would help guide future

animal model work for sexually transmitted viruses, as well as inform risk mitigation for ZIKV-infected individuals.

2.4 Materials and Methods

2.4.1Virus and cells

African Green Monkey kidney cells (Vero; ATCC #CCL-81) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, and incubated at 37°C in 5% CO_2. ZIKV strain PRVABC59 (ZIKV-PR; GenBank:KU501215) was originally isolated from a human traveler to Puerto Rico in 2015 and passaged three times on Vero cells prior to obtaining it from Aaron Brault (CDC, Ft. Collins, CO). ZIKV strain DAK 41525 (GenBank:KU955591.1) was passaged twice on Vero cells, and was obtained from Rushika Perera (Colorado State University, Ft. Collins, CO). ZIKV strain MR766 (GenBank:LC002520), passaged twice on Vero cells, was obtained from Greg Ebel (Colorado State University, Ft. Collins, CO). ZIKV strain MR766 (GenBank:LC002520), passaged twice on Vero cells, was obtained from Greg Ebel (Colorado State University, Ft. Collins, CO).

2.4.2 Ethics statement and animals

Use of all animals was approved by the Colorado State University Institutional Animal Care and Use Committee (protocol 15-6677AA). All procedures were done in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

NZW rabbit and the Hartley guinea pigs were obtained from Charles River. The multimammate mice (*Mastomys natalensis*) were obtained from Heinz Feldmann (Chief, Laboratory of Virology NIH, NIAID). Jamaican fruit bats (*Artibeus jamaicensis*) were obtained from our colony at CSU. These bats are reared and maintained using SOPs under CSU's Laboratory Animal Resources

A129 mice were also obtained from the Colorado State University breeding colony. As the first published study characterizing experimental infection of multimammate mice with BSL-3 viruses, it is worth noting that these animals are particularly fractious and most be handled with care. To this end, these animals were never handled unless fully anesthetized.

2.4.3 ZIKV subcutaneously inoculation into animals

Four A129 mice and 12 multimammate mice, 8-12 weeks old, were anesthetized in a holding chamber with 1-3% isoflurane to effect with an oxygen flow rate of 1.5 L/min. Once the animal was anesthetized, it was removed from the chamber and $2.6x10^6$ PFU (100 µL) ZIKV or PBS (100 µL) was administered subcutaneously between the scapulae with a sterile hypodermic 34-gauge needle in a biosafety cabinet. Sexually mature 6-month-old male rabbits were restrained by one researcher while the other used a sterile hypodermic 34-gauge needle to subcutaneously inoculate $2.6x10^6$ PFU (100 µL) of virus or PBS (100 µL) between the scapulae. Four, sexually mature male guinea pigs 8-12 weeks of age were inoculated subcutaneously with $2.6x10^6$ PFU (100 µL) of ZIKV PRVABC59 or PBS (100 µL). Animals were restrained by one researcher while the other used a sterile hypodermic 34-gauge needle to subcutaneously inoculate $2.6x10^6$ PFU (100 µL) of virus between the scapulae. As these animals were previously shown to be susceptible, another four male guinea pigs were inoculated by infectious mosquito bite (one was fed on by non-infectious mosquitoes) to evaluate if mosquito saliva would potentiate infection, as shown in other arbovirology transmission studies (see below for methods). All animals were individually housed. Bats were inoculated in two experiments were performed. The first group composed of nine bats that were subcutaneously inoculated with $7.5x10^5$ PFU of ZIKV PRVABC59, MR766 or DAR41525 and held for 28, 35 of 48 d.p.i. A second set of six

bats were s.c. inoculated with $7.5x10^5$ PFU with ZIKV PRVABC59 and euthanized at early time points, 2, 3- and 5-days post inoculation

2.4.4 Mosquito infections of guinea pigs

To infect guinea pig by mosquito bite, *Ae. aegypti* strain Poza Rica mosquitoes were fed an infectious blood meal and held for 14-17 days to ensure dissemination of virus to the salivary glands. Infectious blood meals were prepared with 1mL fresh virus contained in the cell culture supernatant of infected Vero cells and 1 mL of defibrinated calf blood. Back titration of the bloodmeals ranged between $1x10^6$ - $5x10^6$ PFU/mL. Mosquitoes were sorted post-feeding and 10-20 blood fed mosquitoes were placed in cartons with an organdy cover and provided water and sugar source. To allow the mosquitoes to feed on the guinea pigs, each guinea pig was anesthetized using 100mg/kg ketamine combined with 10mg/kg xylazine and placed on the organdy cover of one carton for ~20 minutes. After allowing the mosquitoes to feed on the guinea pigs, blood fed mosquitoes were immediately knocked down, their saliva was collected by the forced salivation method described previously[212], and their bodies homogenized in media for later testing. ZIKV infections of mosquito bodies were determined by plaque assay and qRT-PCR. Samples were titrated by Vero cell plaque assay, with a tragacanth gum overlay and staining at day 5 post-cell culture inoculation.

2.4.5 Intravaginal inoculation of female rabbits

For intravaginal inoculation, two female rabbits, 6-month-old, were restrained in a seated position by one researcher. Another researcher used a blunt 200µl pipette tip to gently inoculate

$2.25x10^4$ PFU (100µL) ZIKV of virus into the vagina. Each rabbit was held in this seated position for 3 minutes to help facilitate absorption.

2.4.6 Sample collection: Urine, rectal swab, oral swabs, semen, and blood

A129 mice and multimammate mice: 20-50 µL of blood was collected every two days from all mice by a small nick in the lateral tail vain. A129 mice were restrained in a mouse restrainer during bleeding and due to fractious nature of multimammate mouse, only blood and saliva samples were taken when the mice were anesthetized no other ante-mortem samples were collected

NZW Rabbits: Urine, rectal swabs, oral swabs, and blood were collected every two days. Urine was collected as produced during handling, animals were manipulated over plastic wrap, and if urine was released it was pipetted off the plastic wrap and placed into 1.7 mL tube and put directly onto dry ice. Rectal swabs and oral swabs were taken while animals were restrained by one researcher. Blood was collected from a venipuncture performed on the marginal ear vein. Semen was collected from male rabbits every two days. Semen was collected from rabbits only using an artificial vagina made from 2 inches of PVC pipe. PVC was lined with a plastic tube seal on the outside of one end. The plastic liner was filled with hot water and the other side was sealed. In one end, a 15ml conical tube was placed the other end was lubricated. A male rabbit was placed into the cage with the female and once interest was expressed by the male, the researcher placed the artificial vagina between the male and female. The rabbit's penis was guided into the artificial vagina and semen was collected upon ejaculation.

Guinea pigs: Urine, rectal swabs, oral swabs, and blood were collected every two days. Urine was collected as produced during handling. Animals were manipulated over plastic wrap, and if urine was released it was pipetted off the plastic wrap and placed into 1.7 mL tube and put

directly onto dry ice. Rectal swabs and oral swabs were taken while animals were restrained by one researcher. Blood was collected by cranial vena cava venipuncture.

Bats: Urine, rectal swabs, oral swabs, and blood was collected every two days. Urine was collected as produced during handling, animals were manipulated over plastic wrap, and if urine was released it was pipetted off the plastic wrap and placed into 1.7 mL tube and put directly onto dry ice. Bats were anesthetized in a holding chamber with 1-3% isoflurane to effect with an oxygen flow rate of 1.5 L/min. Once the animal was anesthetized, it was removed from the chamber rectal swabs, oral swabs, and blood was collected. 20-50 µL of blood was collected from a small nick in the wing.

2.4.7 Body weight and temperature

Body weight was taken daily for rabbits and guinea pigs. Animals were placed on a scale and weight was recorded. Rectal temperatures for rabbits and guinea pigs were taken with a lubricated standard thermometer.

2.4.8 Euthanasia, blood collection and necropsy

A129 mice and multimammate mice were euthanized by cervical dislocation following inhalation anesthesia using isoflurane. Blood was collected by cardiac puncture with a 34-gauge sterile needle inserted into the apex of the heart. Bats were anesthetized with isoflurane and appropriate depth of anesthesia was confirmed prior to thoracotomy and cardiocentesis with a 34-gauge needle. The NZW rabbits and guinea pigs were euthanized by overdose of ketamine and xylene consistent with Institutional Animal Care and Use Committee recommendations. For all animals, pieces of each tissue were removed and placed in a pre-weighed tube with 500ul of DMEM media and kept at 80°C for RNA extraction. Tissue for each animal included saliva,

blood, and tissues including brain, heart, lungs, liver, kidney, bladder and reproductive track (ovary, testis, and seminal vesicles for rodents).

2.4.9 RNA extractions

Tubes containing pieces of tissue were re-weighed, homogenized for 1 minutes at 24 c/s, and spun for 5 minutes at 14000 x g. RNA was extracted from all samples using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek) on the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific). RNA was eluted in 30 µL nuclease-free water.

2.4.10 qRT-PCR

Promega GoTaq Probe 1-Step RT-qPCR System Time was used on RNA extracted from blood and tissues to quantify ZIKV RNA according to manufacturers' instructions. Primers targeting the NS5 gene were used (ZIKV 1086 (CCGCTGCCCAACACAAG) and ZIKV 1162c (CCACTAACGTTCTTTTGCAGACAT)). The probe used was ZIKV 1107-FAM (AGCCTACCTTGACAAGCAGTCAGACACTCAA)[52]. An addition primer set targeting the 3' UTR of multiple strains of ZIKV (F: TTCCCCACCCTTYAATCTGG and R: TGGTCTTTCCCAGCGTCAAT) was used.

2.4.11 qRT-PCR Standards Generation

Standards for ZIKV were generated by establishment of PFU equivalence. RNA was extracted from stock virus with a known viral titer and was diluted to achieve serial 10-fold PFU equivalence dilutions. The standard curve detection of 10^3-10^8 ZIKV PFU equivalence/reaction and had a primer efficiency of 88.62% with an R^2 value of 0.971, a slope of -3.629, and y-intercept = 47.270

2.4.12 Plaque reduction neutralization test (PRNTs)

PRNTS were performed on serum that was heat-inactivated by incubating at 56°C for 30 minutes. Samples were serially diluted (ten-fold) into DMEM media and then mixed with 125µl of ZIKV virus. Serum and virus were incubated at 37°C for one hour and then plated onto confluent Vero cells and incubated for one more hour at 37°C. Tragacanth gum overlay was added, and cells were stained at 5 days post-inoculation.

CHAPTER 3: THE INFLUENCE OF BIOLOGICAL SEX AS A DETERMINANT OF ZIKA VIRUS INFECTION AND DISEASE IN SUSCEPTIBLE MICE

3.1 Introduction

ZIKV is a positive-strand RNA virus in the *Flaviviridae* family that is primarily transmitted to humans through the bite of an infected mosquito. The 2015-16 ZIKV pandemic in the Americas resulted in over one-million suspected cases, with hundreds of spontaneous abortions and thousands of microcephaly cases, ocular malformations, and other birth defect[146] cases reported. Interestingly, ZIKV is the only known flavivirus that has been definitively proven to be sexually transmitted between humans[213,214]. This feature likely underlies some unique epidemiological patterns of ZIKV infection and disease, including higher risk of sexual partners being ZIKV seropositive in affected households, higher attack rates in women compared to men, and urogenital pathology in infected men[215,216]. It follows that biological sex may influence some of these unique infection and disease patterns. Female ZIKV disease bias was first observed during the pre-pandemic 2007 Yap Island outbreak where a higher disease attack rate was observed in females compared to males (17.9 vs. 11.4 per 1000) despite evidence that males had higher exposure by assessing anti-ZIKV IgM prevalence (75% in males vs. 68% in females) [51]. Similar patterns were repeatedly observed across endemic regions afflicted by the pandemic. For example, the 2015 Puerto Rico outbreak resulted in higher ZIKV disease incidence among women (936 per 100,000) than men (576 per 100,000)[217]. This disease bias may partly relate to male-to-female ZIKV sexual transmission, but this is unlikely to be the only explanation given that ZIKV is more often a mosquito-transmitted virus[218]. Inherent physiological differences (e.g., differential cytokine expression or hormone influences) may also allow ZIKV to spread more easily or cause more severe disease in females compared to males.

Experimental infection of animal models with ZIKV is necessary to help understand ZIKV infection and pathology in infected humans, including the disease bias observed between males and females, and to identify details of sexual versus mosquito transmission and how these routes of infection may influence disease bias among the biological sexes. Non-human primates (NHPs) are considered the model that best recapitulates human pathology. NHPs have been studied to examine the effect of transmission route on ZIKV disease. It was found that there is a delay in peak viremia when NHPs were infected by mosquito bite compared to subcutaneous inoculation[189]. Two other studies have used both male and female NHPs. However, the low sample size prevents any conclusion to be drawn on how biological sex may affect disease outcome[219,220]. The use of NHPs in research is expensive, ethically controversial, and is complicated for ZIKV studies because sexually maturity in many NHPs is not reached for years[221–224]. Several groups have instead successfully used immunodeficient mouse models to address critical questions of ZIKV pathogenesis and disease outcomes. Highly-immunodeficient gene knock-out mice that cannot express all interferon receptors (AG129 strain; IFN α/ß/γ receptor$^{-/-}$) have been used to elucidate many ZIKV infection processes, including: 1) how males sexually transmit ZIKV to females, 2) how exposure to ZIKV by subcutaneous (s.c.) inoculation, intravaginal (ivag) inoculation, or sexual transmission alters viral titers, and 3) the impact of viral strain on transmission and disease outcome[225–227]. The less-immunodeficient A129 gene knock-out mouse strain which cannot express α- and ß-interferon receptors (IFN α/ß R$^{-/-}$), has been studied to understand: 1) how viral lineages differ in disease progression, and 2) whether mosquitoes can transmit ZIKV to mice[166,228–230]. Other immunodeficient mice, including stat2$^{-/-}$, C57BL/6 *Ifnar1*$^{-/-}$, and C57BL/6 *Rag1*$^{-/-}$ strains, have also been tested as models for ZIKV infections [150,167,205,231,232]. Each of these studies in mice elucidate our understanding of ZIKV

infections, yet we are not aware of studies that have examined the differences in ZIKV infection and pathology relative to biological sex other than by examination of ZIKV sexual transmission. Here, we use the A129 mouse model to begin to address the role of biological sex as a factor that affects ZIKV pathogenesis.

3.2 Results

3.2.1 ZIKV infections by mosquito bite

We first examined differences in ZIKV pathogenesis between male and female mice when infected by mosquito bite. ZIKV transmission from mosquito bites, estimated to be a mean inoculum of 9.76 $x10^2$ ZIKV PFU (see Methods section and Table S1) ultimately resulted in all mice developing detectable viremia via qRT-PCR by 4 days post inoculation (d.p.i.) (Table S2). However, at the earliest time point (2 d.p.i.), significant differences were observed in the proportion of males that had detectable viral RNA in brain tissue (males = 77% [7/9]) compared to females = 22% [2/9]; p-value = 0.018; Table 3.1). Immunohistochemical examination of the infected brain tissue of qRT-PCR positive males and females at the earliest timepoint showed marginally more antigen-positive neurons in the brains of the infected males (mean of 940 ± 50.86; positive cells per 10 random fields examined per 5 mice) compared to the brains of the infected females (mean of 750 ± 66.56; positive cells per 10 random fields examined per 2 mice) (Figure 3.2). Regarding the reproductive tract tissues at the earliest time point, viral antigen was most prevalent in spermatogonia of male seminiferous tubules and in females was limited to uterine endometrial cells (Figure 3.2). At the later time points, neither the proportions of infected mice nor the number of tissues per mouse differed (Table S2). Similarly, the tissue viral RNA loads were generally the same between males and females at these later time points, with the exception of the mean viral RNA loads in blood at 7 d.p.i., which was $1x10^{7.3}$ viral genome

copies/mL in females and $1x10^{8.4}$ viral genome copies/mL in males (p-value = 0.0256, un-paired T-test; $P \geq 0.05$) (Figure 3.1c and Supplemental figure 3.1). Mice held for 28 d.p.i. were used to monitor for any progression of disease, differences in survival, and for seroconversion. All 12 mice survived the infection and remained free of clinical signs of ZIKV disease (e.g., lethargy, ataxia, and paralysis). Viral RNA load present in the tissues of these mice were generally reduced compared to earlier time points, but no differences in the tissue viral RNA loads were detected between males and females (Supplemental Figure 3.1).

Table 3. 1 Proportions of female and male tissues positive for ZIKV by qRT-PCR from mosquito bite at 2 days post-inoculation.

Tissue	**Female**	**Male**	**p-value**
Brain	2/9	7/9	0.018
Heart	4/9	5/9	NS
Lung	4/9	6/9	NS
Liver	4/9	7/9	NS
Spleen	6/9	7/9	NS
Kidney	4/9	6/9	NS
Bladder	5/9	3/9	NS
Blood	8/9	6/9	NS
Testis	N/A	5/9	N/A
Epididymis	N/A	7/9	N/A
Seminal Vesicle	N/A	5/9	N/A
Ovary	4/9	N/A	N/A
Vagina	5/9	N/A	N/A

NS= non-significant (p-value > 0.05); Fisher Exact test

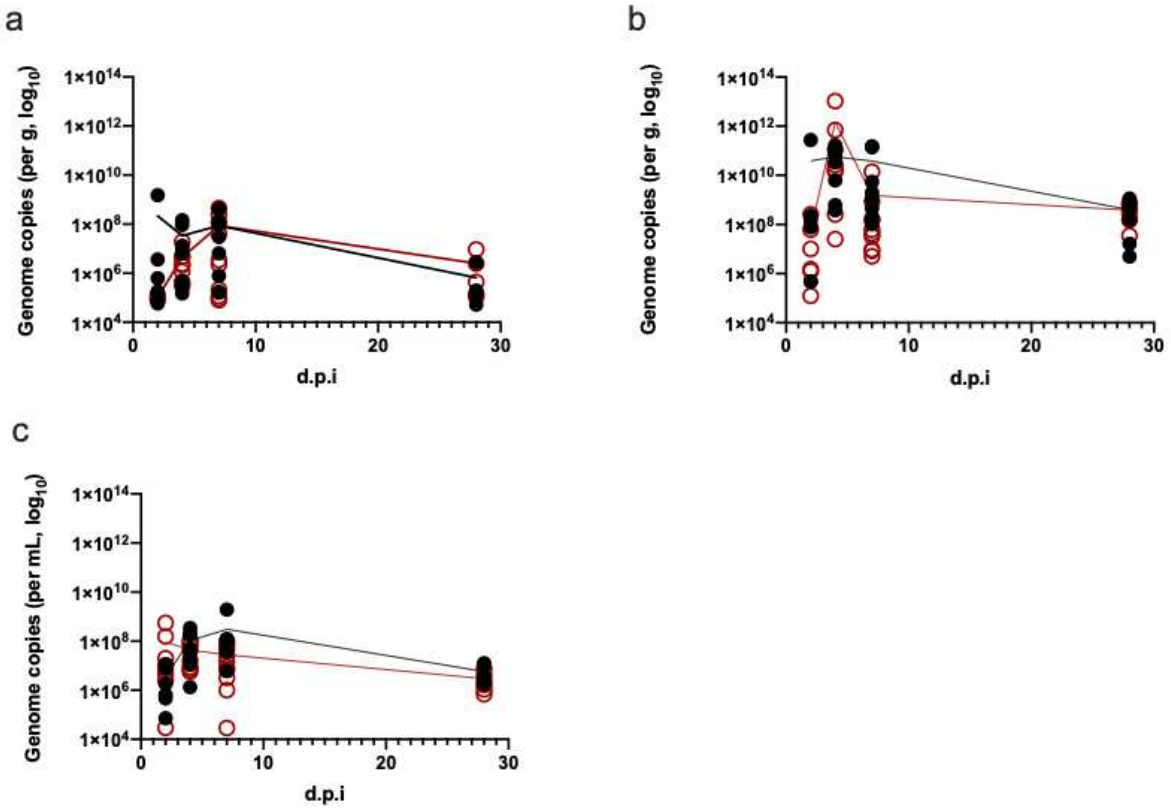


Figure 3. 1 ZIKV RNA genome copies detected by qRT-PCR in positive samples of a) brains, b) spleens, and c) blood of mice inoculated by mosquito bite. Black closed circles= males, red open circles= females. Limit of detection is 10 genome copies. Lines connect at mean with individual variation showed (un-paired T-test; $P \geq 0.05$).

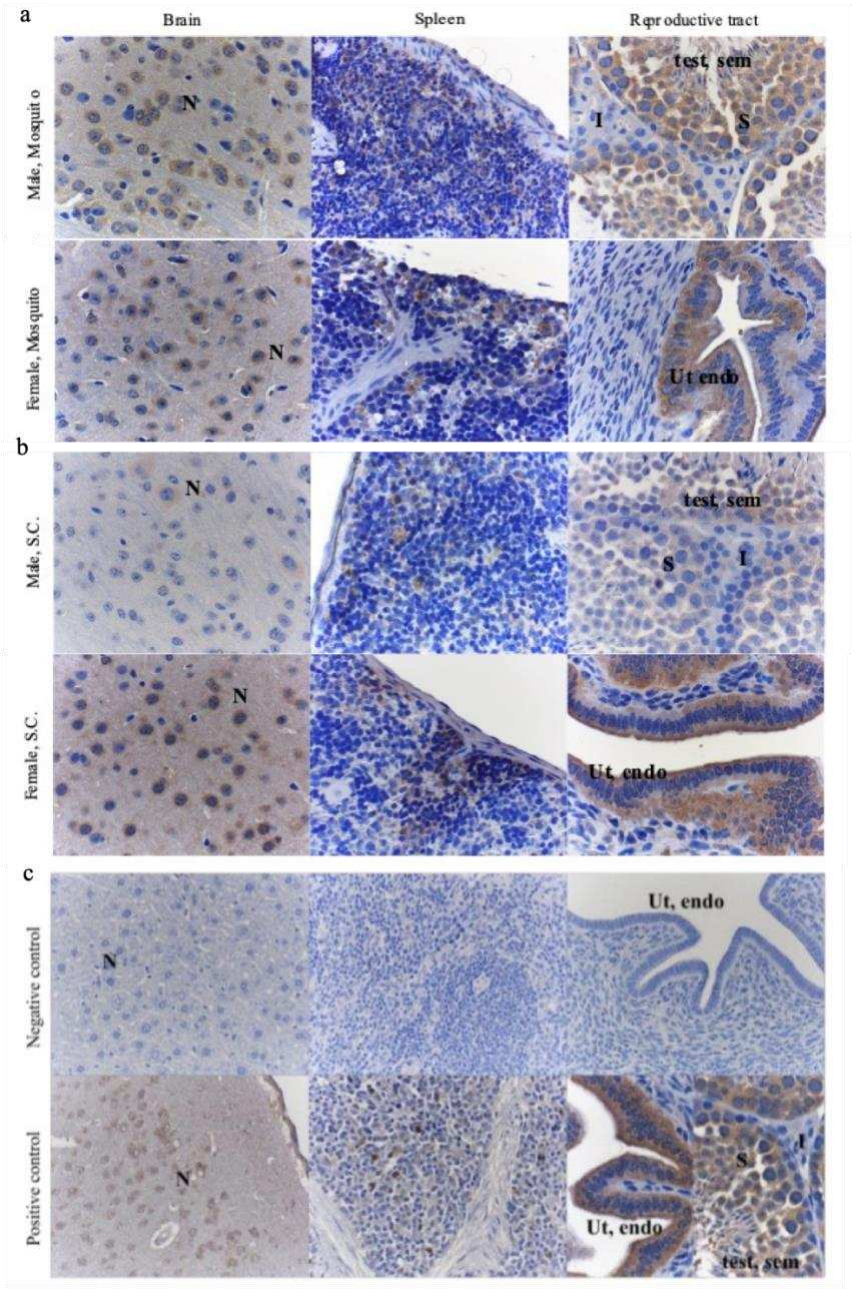


Figure 3. 2 Immunohistochemical (IHC) comparison of Zika virus infection in the brain, spleen, and reproductive tracts of male and female mice inoculated by mosquito bite and by subcutaneous injection 2 days post inoculation. A) IHC labelling shows Zika virus infection in the brain, spleen and the reproductive tracts of males and females mosquito bite B) IHC labelling shows Zika virus infection in the brain, spleen, and the reproductive tracts of males and females s.c. injection. C) IHC staining negative (non-infected mouse) and positive control (s.c. inoculated mouse from previous study). I, Interstitial cells; N, neuron; test semi, testis and seminiferous tubule; f, female; S, spermatogonia; m, male; Ut endo, uterus endometrium; S.C., subcutaneous inoculation; mosq, mosquito inoculation.

3.2.2 ZIKV infection by subcutaneous needle inoculation

To determine if the infection differences observed between males and females at the early time point was due to mosquito bite delivery of virus, we exposed a second set of mice to ZIKV by subcutaneous (s.c.) needle inoculation using a virus titer estimated to be roughly equivalent to that introduced by those from our mosquito bite experiments (1000 PFU). Again, ZIKV RNA was detectable in all tissues via qRT-PCR by 4 days post inoculation (d.p.i.) (Table S2). However, the results at the earliest 2 d.p.i. time point were markedly different in males. While the proportions of female tissues containing viral RNA did not noticeably change in comparison to the mosquito bite infection experiments, most male tissues did not contain detectable viral RNA except for the blood and spleen. Similarly, viral RNA was rarely detected in the reproductive tract tissues of s.c.-inoculated males at 2 d.p.i. (Table 3.2). Overall, we observed significantly lower proportions of detectable infections in 6 of 8 common tissues among both males and females, especially in the brain tested between s.c.-inoculated males compared to s.c.-inoculated females at the earliest time point (Table 3.2, Figure 3.3a). However, when examining histological sections of viral RNA-positive spleens between the s.c.-inoculated males and females, there were similar numbers of ZIKV antigen positive cells between the two groups (mean of 505.2 ± 22.64 for males and 505.2 ± 22.64; positive cells per 10 random fields examined per 5 mice of each sex) (Figure 3.2). Similar to the mosquito bite experiments, no differences in biological sex were observed in s.c.-inoculated mice at the later time points post-infection (Figure 3.3 and Supplemental figure 3.1). No mice displayed clinical signs of ZIKV disease, all survived to 28 days post-infection, and there was no significant difference in

neutralizing antibody production at this latest time point between males and females regardless of the infection route (Figure 3.4).

Table 3. 2 Proportions of female and male tissues positive for ZIKV by qRT-PCR from subcutaneous inoculation (s.c.) at 2 days post inoculation.

Tissue	Female	Male	p-value
Brain	6/10	0/10	0.0108
Heart	9/10	0/10	0.001
Lung	8/10	0/10	0.0007
Liver	1/10	0/10	NS
Spleen	10/10	5/10	0.0325
Kidney	7/10	0/10	0.0031
Bladder	4/10	0/10	NS
Blood	10/10	4/10	0.0325
Testis	N/A	1/10	N/A
Epididymis	N/A	0/10	N/A
Seminal Vesicle	N/A	0/10	N/A
Ovary	8/10	N/A	N/A
Vagina	4/10	N/A	N/A

NS= non-significant (p-value > 0.05); Fisher Exact test

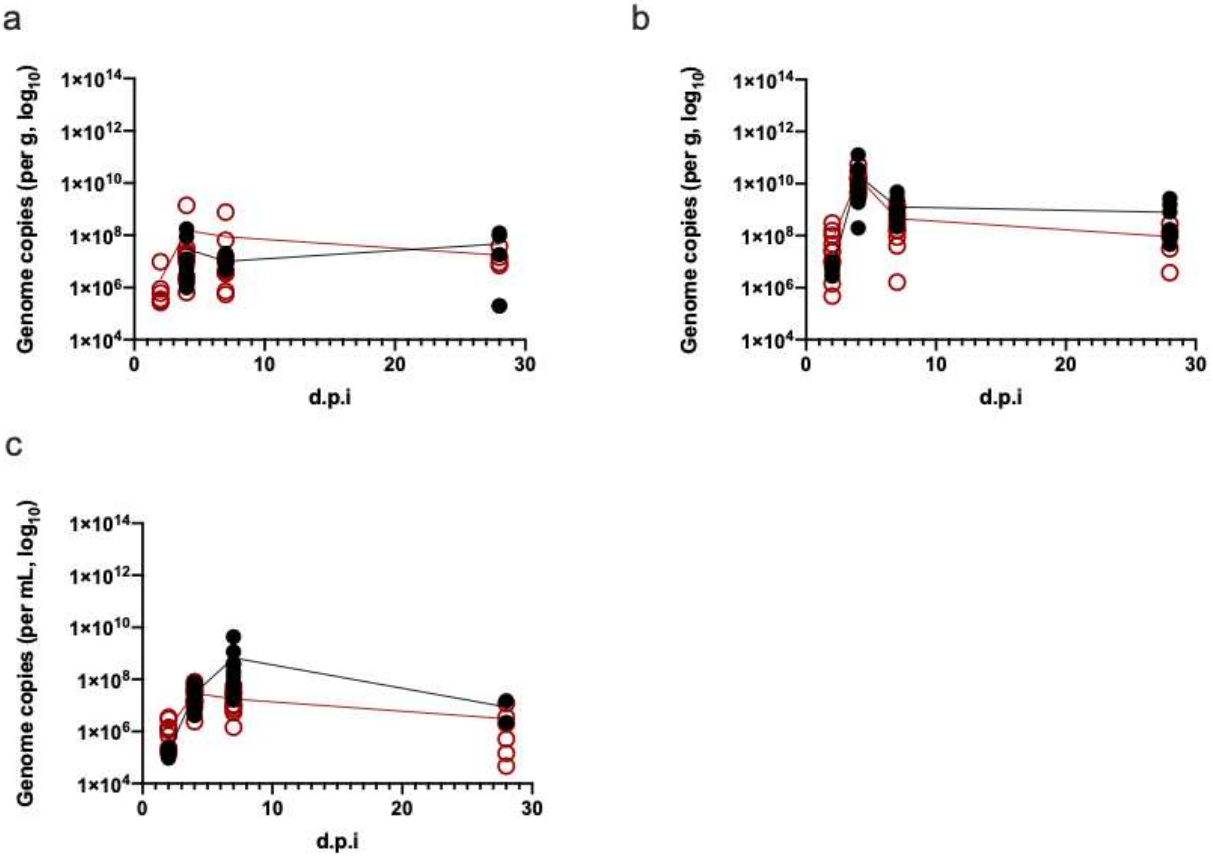


Figure 3. 3 ZIKV RNA genome copies detected by qRT-PCR in positive sample of a) brains b) spleens and c) blood of mice inoculated by s.c. injections. Black closed circles= males, red open circles= females. Limit of detection is 10 genome copies**.** Lines connect at mean and individual variation showed. No significant different between means of sex at different time points as determined by un-paired T-test; $P \geq 0.05$.

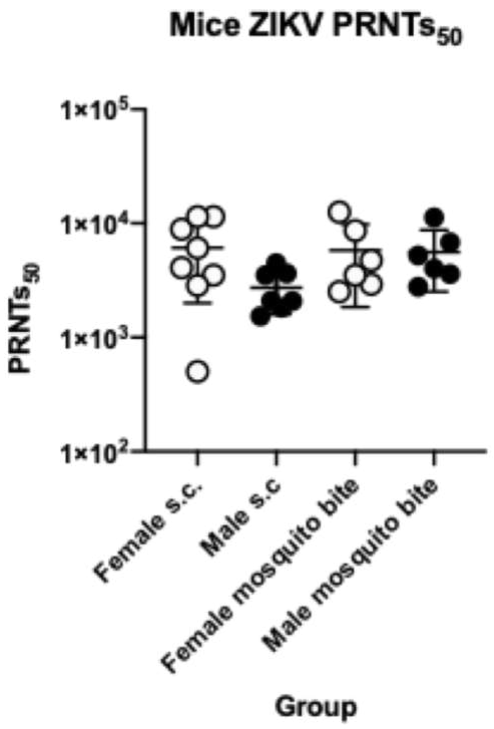


Figure 3. 4 Plaque reduction neutralization tests (PRNTs) from male and female mice infection by subcutaneous inoculation (s.c.) or mosquito bite(s) 28 d.p.i. Means (horizontal bars) were not significantly different (one-way ANOVA and post-hoc Tukey test; $P \geq 0.05$).

3.2.3 ZIKV infection by subcutaneous needle inoculation after uninfected mosquito bites

As shown above, the speed of initial (early time point) ZIKV infections reversed in certain tissues of males compared to females when the virus was delivered by mosquito bites or subcutaneous needle inoculation. We hypothesized that either mosquito saliva or mosquito saliva-derived ZIKV was potentially affecting initial virus infection dynamics most noticeably in males. To test the hypothesis, we examined tissues in both sexes at 2 d.p.i. only following an experiment whereby we first blood fed uninfected mosquitoes on mice of each biological sex, and then immediately infected these mice by subcutaneous needle inoculation at the bite site using the same dose of virus from the same aliquots as the needle inoculation experiments above. This experiment resulted in similar infection rates of the blood and the blood-laden spleen as the

prior two experiments, but mostly absent or few detectable infections of other tissues. Overall, no differences were observed in this 2 d.p.i. examination of ZIKV infection in any common tissues between the male and female mice (Table 3.3).

Table 3. 3 Proportions of female and male tissues positive for ZIKV by qRT-PCR from mosquito bite follow by s.c inoculation 2 days post-inoculation.

Tissue	Female	Male	p-value
Brain	0/9	0/6	NS
Heart	0/9	0/6	NS
Lung	0/9	0/6	NS
Liver	0/9	0/6	NS
Spleen	9/9	4/6	NS
Kidney	0/9	0/6	NS
Bladder	1/9	0/6	NS
Blood	8/9	3/6	NS
Testis	N/A	0/6	N/A
Epididymis	N/A	0/6	N/A
Seminal Vesicle	N/A	0/6	N/A
Ovary	0/9	N/A	N/A
Vagina	3/9	N/A	N/A

NS= non-significant (p-value > 0.05); Fisher Exact test

3.2.4 ZIKV transmission by the sexual route

We examined male-to-female sexual transmission by two methods. Initially, we examined semen collected by vaginal lavage from 3 female mice mated to s.c.-inoculated males (n = 5) or males infected by mosquito bites (n = 10) that were held to 7-21 d.p.i. prior to mating. All of these infected males, regardless of infection route, developed detectable viral RNA in blood (represented above in Figures 3.1 and 3.3). However, ZIKV was infrequently detected in their semen. Twenty-six semen samples were collected from females mated to the 10 s.c.-inoculated males, but only one sample was positive by plaque assay and qRT-PCR ($9x10^2$ PFU and $2.9x10^5$ genome copies, respectively). Similarly, 16 semen samples were collected from females mated to the 5-mosquito bite-infected males, but only one was positive by plaque assay and qRT-PCR ($6.5x10^2$ PFU and $2.9x10^5$ genome copies, respectively). An additional experiment was

performed to determine if female mice were susceptible to ZIKV infection by intravaginal inoculation with $2.6x10^4$ PFU ZIKV, but none of the females (0/10) became infected after 7 days post-inoculation.

Despite the low proportion of males with detectable levels of ZIKV in their semen, we attempted to infect female mice by mating. Females were mated only with s.c.-inoculated males because the viral RNA load in the male reproductive tissues did not differ significantly at later time points depending on infection route. In these experiments, ZIKV could not be detected in any of the tissues of the mated females, regardless of whether the female was observed to have a copulation plug (definitively mated; n= 7), or if a copulation plug was never observed after 7 days of co-habitation (n = 3). Importantly, all mated males tested positive for viral RNA by qRT-PCR in their blood and tissues.

3.3 Discussion

Here we describe the role of biological sex on ZIKV infection and disease in the context of several different infection routes and using an immunodeficient mouse model. Both sexes were equally susceptible to ZIKV infection by mosquito bite and by subcutaneous inoculation with a needle and virus ultimately disseminated to all tissues tested, but neither sex exhibited overt clinical signs of disease, and all animals survived to 28 d.p.i. as well as developed anti-ZIKV neutralizing antibodies. However, the rate of initial tissue/organ infections differed by biological sex and the differences changed depending on infection route. While the natural mosquito-bite route of infection was relatively efficient overall across both sexes, at the earliest time point tested viral RNA was detected in the brains of few female mice but in the brains of most mosquito-bitten males. When a similar dose was given to both sexes by s.c.-inoculation, the

observation was reversed and broadened to most tissues, whereby early time point ZIKV infections were undetectable in most male tissues but prevalent in nearly all female tissues. Overall, these data suggest that the virus delivered to mice by mosquito bite facilitates ZIKV infections of nervous system tissue more easily in males compared to females and that taking away the mosquito component of transmission slowed ZIKV infections of nearly all male tissues, but was not a factor in the initial infection rates of female tissues. From these results, a third experiment that decoupled the mosquito saliva and virus inoculations was performed to test the competing hypotheses that mosquito saliva or mosquito-derived ZIKV affects initial virus-infection dynamics. Here we found delayed tissue infections of both the male and female groups, except for the female vagina. Because the pre-infection treatment with uninfected mosquito bites (which would deposit mosquito saliva at the injection site) did not facilitate rapid tissue infections of either sex, the results suggest that early infection kinetics in male mice is more dependent on being infected with ZIKV that is derived from mosquito salivary glands, but that rapid infection of most female tissues was negatively influenced by pre-exposure to mosquito saliva.

A number of studies in mice have demonstrated that mosquito-borne viruses, including West Nile Virus (WNV)[113,114], dengue viruses (DENV)[115], Semliki Forest virus (SFV)[116] and chikungunya virus (CHIKV)[117] are enhanced by mosquito bite. On the other hand, a study in ZIKV-infected NHPs demonstrated delays in viremia when the animals were infected by mosquito bite, however there was no comparison between sexes[189]. The broad hypothesis from the mosquito bite enhancement studies is that viral infection is facilitated by saliva components that modulate immune pathways by several mechanisms. These include 1) immune cell activation which leads to blood capillary leakage and thus retention of virus in extravascular

tissues[112], 2) immune cell activation which leads to endocytosis of virus but the virus subsequently hijacks the immune cell to facilitate its spread[118], 3) disruption of endothelial cell barriers which leads to virus dissemination and enhanced cell migration[118], 4) suppression of the innate immune response, and 5) subversion of the host adaptive immune response[119]. Our data suggest a similar enhancement of ZIKV infections when it is transmitted by mosquitoes, but only in our male mice and it seemingly was more connected to mosquito salivary gland derived virus compared to mosquito saliva. The early ZIKV infection differences of tissues we observed between the biological sexes may be due to a number of sex specific immune interactions between either the virus composition or mosquito saliva. Flavivirus samples recovered from mosquito saliva are known to be comprised of far more viral genomes (presumably defective virus particles) than infectious particles (seen in Supplemental Table 1) compared to virus derived from cell culture[233], and the post-translational modifications of virus structural proteins are also likely to differ. Alternatively, mosquito salivary factors may be more or less effective in affecting anti-viral immunity in males relative to females. The X chromosome of mammals contains the largest number of immune-related genes and the process of X chromosome inactivation aids in equalizing gene expression from this chromosome in males and females[234]. However, ~23% of X-linked genes have been shown to escape X inactivation, many of which are immune-related genes and regulatory elements[235,236]. In humans, this is thought to contribute to female's increased resistance to microbial diseases relative to males, but also their higher preponderance of autoimmune diseases. Further research should be done to decipher the potential interactions of ZIKV and mosquito salivary factors with immune factors in females compared to males.

One potential bias in our experiments is that the ZIKV dose administered to mice by s.c.-inoculation could only be estimated to match what was likely delivered by the mosquito bites through comparisons to what mosquitoes transmitted by the forced salivation technique after blood feeding. We also could not account for the possible delivery of differential doses of virus to each mouse by a few probing but non-blood fed mosquitoes, nor the likelihood that a wider range of titers were delivered by the mosquitoes due to the uncertainties of the numbers that would blood feed on any one mouse. Indeed, viral RNA loads in tissues harvested from mice infected by mosquito bite were of a wider range compared to viral RNA loads in tissues harvested from mice inoculated by a needle.

Because sexual transmission has been evidenced in humans and other mouse models, we also evaluated this mouse model for its ability to recapitulate sexual transmission. Viral RNA loads in tested male sex organs were high after 4 d.p.i., similar to those of other tissues, but virus was rarely detected in the semen of infected males. Furthermore, females failed to develop detectable ZIKV infections when they were directly inoculated intravaginally or mated to ZIKV-infected males. This was unexpected, because the more immunocompromised AG129 mouse model can efficiently be infected intravaginal and sexually[225]. The difference between these mouse models is the presence of the IFN-γ receptor in the A129 mice we used. This may suggest a role for IFN-γ in mouse ZIKV transmission via male semen, as well as in anti-ZIKV vaginal immunity in females. Vaginal immunity is partly hormone-dependent as changes in hormone levels influence the production of cytokines, chemokines, α/ß defensin and TLR expression[237,238]. It has also been shown that IFN-γ expression is hormonally-dependent and aids in control of ZIKV infection[239]. Our results should prompt further studies of the role of IFN-γ in ZIKV infections of both male and female sex organs.

Overall, this study demonstrates how biological sex can influence ZIKV infection dynamics in this immunocompromised mouse model and the potential role of mosquito derived virus or mosquito saliva in this phenomenon. Consideration of these differences should be incorporated into future ZIKV experimental infections in mouse and other animal models, and prompt collection and comparisons of similar data from natural ZIKV infections of humans.

3.4 Materials and Methods

3.4.1 Virus and cells

African Green Monkey kidney cells (Vero; ATCC #CCL-81) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, and incubated at 37°C in 5% CO_2. ZIKV strain PRVABC59 (ZIKV-PR; GenBank:KU501215), originally isolated from a human traveler to Puerto Rico in 2015 with three rounds of amplification on Vero cells, was obtained from Aaron Brault (CDC, Ft. Collins, CO).

3.4.2 Mice

Sexually mature (8-12 weeks) A129 mice were obtained from breeding colony maintained at Colorado State University. Use of mice was approved by the Colorado State University Institutional Animal Care and Use Committee (protocol 15-6677AA). All procedures were done in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

3.4.3 Mosquito infections

To infect mice by mosquito bite, *Ae. aegypti* strain Poza Rica mosquitoes were fed an infectious blood meal and held for 14-17 days to ensure dissemination of virus to the salivary glands.

Infectious blood meals were prepared with 1 mL fresh virus contained in the cell-culture supernatant of infected Vero E6 cells and 1 mL of defibrinated calf blood. Back titration of the bloodmeals ranged between $1x10^6$ - $5x10^6$ PFU/mL. Mosquitoes were sorted post blood feeding and 10-20 blood fed mosquitoes were place in cartons with an organdy cover and given water and sugar source. To allow the mosquitoes to feed on the mice, each mouse was anesthetized using 100mg/kg ketamine+ 10mg/kg xylazine and placed on the organdy cover of one carton for ~20 minutes. After 20 minutes, blood fed mosquitoes were immediately knocked down, their saliva was collected by the forced salivation method described previously[212], and their bodies homogenized in media for later testing. ZIKV infections of mosquito bodies and saliva were determined by plaque assay and q-RT-PCR. Samples were titrated by Vero cell plaque assay, with a tragacanth gum overlay and staining at day 5 post-cell culture inoculation.

3.4.4 Normalizing ZIKV infections from needle inoculations and mosquito bite.

To normalize needle inoculations with mosquito bite inoculations, we immediately salivated all engorged mosquitoes post mouse feeding and determined the quantity of viral RNA in the saliva. The number of mosquitoes that were found engorged after blood feeding ranged from 1-10 per mouse (Supplemental Table 1), and we observed that most mosquitoes which probed also engorged, while most non-engorged mosquitoes did not probe. The mean viral titer from each saliva pool was $9.76\ x10^2$ PFU (Supplemental Table 1); we therefore needle-inoculated each mouse with $1.00x10^3$ PFU.

3.4.5 ZIKV inoculation into mice

Mice were anesthetized in a holding chamber with 1% to 3% isoflurane to effect with an oxygen flow rate of 1.5 L/min. Once the animal was anesthetized, it was removed from the chamber and

$1x10^3$ PFU of virus in a volume of 100 µL was administered subcutaneously (s.c.) in the region of the back between the scapulae with a sterile hypodermic 34-gauge needle in a biosafety cabinet.

3.4.6 Intravaginal inoculation of female mice

Female mice were intravaginally-inoculated with $2.25x10^4$ PFU of ZIKV. Mice were restrained with their ventral side up with one hand and with the tail held down exposing the vaginal opening. Using a blunt 20 µL pipette tip, 15µL of virus was gently inoculated into the vagina. Each mouse was held in this position for 3 minutes.

3.4.7 Sexual Transmission and semen collection

Methods for sexual transmission and semen collection were adopted from Duggal et al[225], and we were trained on these techniques by a member from the same laboratory (E.M. McDonald). Infected male mice were mated to three to four female mice starting on the evening of day 7 post infection. In preparation, bedding from male cages was placed in female cages 3 day before mating to simulate the estrous cycle. Each evening, females were placed into male cages and each subsequent day, females were removed from male cages and checked for a copulatory plug. Females with a plug were placed into a new cage and held for 7 days. Females without a plug were left in the cage with the male to allow for mating, and this process continued for up to two weeks. The same procedure was used for semen collection, however, females with a plug were euthanized and semen was collected from the vagina and uterine horns. This was done immediately after euthanasia by first dissecting the reproductive track (uterus and uterine horns), subsequently, the tips of the uterine horns were cut and then washed with 150µL of sterile PBS while placed over collection tube.

3.4.8 Euthanasia, blood collection and necropsy

Mice were deeply anesthetized with isoflurane followed by cervical dislocation. Cardiac blood was collected with a 34-gauge sterile needle inserted into the apex of the heart. Pieces of each tissue were removed and placed in a pre-weighed tube with 500 µL of DMEM media and kept at -80°C for RNA extraction. The rest of the tissues were placed in 10% neutral buffered formalin for histology at a 1:10 weight to volume ratio.

3.4.9 Plaque reduction neutralization test (PRNTs)

PRNTs were performed on serum that was heat inactivated by incubating at 56°C for 30 minutes. Samples were serially diluted and then mixed with 125 µL of ZIKV. Serum and virus were incubated at 37°C for one hour and then placed on to cells and incubated for one more hour at 37°C. Tragacanth gum overlay was added and cells were stained at day 5 post-cell culture inoculation.

3.4.10 RNA extractions

Tubes containing pieces of tissue were re-weighed, homogenized and spun for 5 minutes at 14000 x g. RNA was extracted from all samples using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek) on the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific). RNA was eluted in 30 µL nuclease-free water.

3.4.11 q-RT-PCR

Progmeg GoTaq Probe 1-Step RT-qPCR System Time was used on RNA extracted from blood and tissues to quantify ZIKV RNA according to manufacturers' instructions. Primers used were

ZIKV 1086 (CCGCTGCCCAACACAAG) and ZIKV 1162c (CCACTAACGTTCTTTTGCAGACAT). The probe used was ZIKV 1107-FAM (AGCCTACCTTGACAAGCAGTCAGACACTCAA)[52]. Approximately 100 ng of RNA was added to each reaction. Standards were generated using a forward primer containing a T7 promoter and a non-modified reverse primer to amplify ZIKV stain PRVABC59 viral RNA. The amplicon was used as template to generate RNA transcripts using the T7 Megascript Kit according to the manufacturer's instructions (ThermoFisher Scientific). RNA was quantified on a Qubit Fluorometer (ThermoFisher Scientific) and diluted to achieve serial 10-fold genome equivalent (GE) dilutions. The standard curve detection of 10^4-10^7 ZIKV GE/reaction had a primer efficiency of 88.62% with an R^2 value of 0.971, a slope of -3.629, and y-intercept = 47.270.

3.4.12 Histopathology and Immunohistochemistry

Mouse tissues immediately fixed in 10% neutral buffered formalin were submitted to the Veterinary Diagnostic Laboratory at Colorado State University (Fort Collins, CO) for standard paraffin embedding, sectioning and staining with hematoxylin and eosin, as well as immunohistochemistry (IHC). Tissues were dehydrated by using a graded ethanol series, embedded in dimethylbenzene-paraffin before sectioning 5 µM thick samples onto charged glass slides. For IHC, sections were dewaxed using xylene and rehydrated through graded ethanol and then boiled in antigen retrieval solution. The cooled sections were blocked with 3% H_2O_2 followed by goat serum (Abcam). Antibody for IHC was a polyclonal rabbit antibody that targets the pre-M and E proteins of ZIKV and was provided by CDC-Division of Vector-Borne Diseases and diluted in TBS to final concentrations of 1:750. Sections were incubated in primary antibody at 4°C overnight then incubated at room temperature for 1 hour with HRP-goat anti-rabbit

secondary (Vector Laboratories, Burlingame, CA). Finally, DAB and substrate (Vector Laboratories, Burlingame, CA) was applied and sections were washed with TBS prior to cover slipping for imaging. All slides and controls (see supplementary figure) were simultaneously stained and were blindly read by a diplomat of the American College of Veterinary Pathologists (F. Magunda). The mean number of IHC labelled cells per 10 representative 600X fields in similar regions of each tissue type were determined.

3.4.13 Statistical Analyses

Results were expressed as mean values (where line connect or horizontal lines) with individual variation. The statistical details are noted in the figures and/or in the corresponding figure legends. Statistical significance was primarily determined using either Fisher's exact test, unpaired Student's t-test or a one-way Analysis of Variance (ANOVA) with a Tukey's multiple-comparison in the GraphPad Prism (GraphPad Software, La Jolla California USA).

CHAPTER 4: CHARACTERIZING AND QUANTIFYING ARBOVIRUS TRANSMISSION BY AEDES AEGYPTI USING FORCED SALIVATION AND ANALYSIS OF BLOODMEALS

4.1 Introduction

Estimating the titer of arboviruses transmitted by mosquitoes during blood feeding on a host is critical to understand arbovirus transmission, especially by accurately simulating these natural infections in laboratory studies. There are several documented methods for collecting saliva and/or determining the efficiency and titer of virus transmitted from mosquito saliva to a host: (1) forced salivation (FS) of infectious mosquitoes into media contained in a capillary tube and testing the captured saliva for virus, (2) detecting virus in host tissues immediately after infectious mosquitoes take a blood meal or by later examining host infection or seroconversion rates, and (3) detecting virus in the remaining blood from artificial feeders fed upon by infectious mosquitoes[128,129,212,240–242]. It is also possible to detect arboviral nucleic acids transmitted into sugar solutions when wild or laboratory mosquitoes sugar feed on special collection devices. However, this does not estimate virus transmitted to a host and tends to only be a qualitative measure of transmission potential because the number of sugar-feeding mosquitoes and the frequency at which they sugar-feed is often unknown[243]. The three former quantitative methods each have challenges and their success is dependent on mosquito species and virus. Therefore, it is essential to evaluate the effectiveness of these methods on different mosquitoes and virus combinations to determine the best laboratory practices for predicting viral transmission.

Forced salivation is often used as it can determine virus expectorated from a single mosquito and is not dependent on the mosquito blood feeding. Within this method, different media have been used within the capillary tube, the most common being either microscope

immersion oil[233] or fetal bovine serum (FBS)[244]. FBS was thought to be a better medium to use because it may aid in viral stabilization and preservation, however, placing the mosquito proboscis into FBS is more difficult due to the hydrophobic properties of the mosquito cuticle[129,245]. In this sense, immersion oil is an easier choice to work with. Results with *Ae. albopictus* infected with Venezuelan equine encephalitis virus (VEEV) showed that there was no difference between virus titers when saliva was collected in either immersion oil or FBS using the FS technique[129].

In addition to forced salivation, vertebrate hosts or artificial feeders can be used to estimate the amount of virus being transmitted from an infectious mosquito bite. However, mosquito feeding behaviors are inconsistent, especially in high-containment (BSL-3) laboratory settings because of rapid air exchange and personal protective equipment that limit body heat and odor cues, so there is no way to ensure any one mosquito, or recalcitrant species or strains, will take a blood meal[246]. A study with Eastern equine encephalitis virus (EEEV)-infected *Ae. aegypti* used mouse intracerebral 50% lethal doses to show that the amount of virus transmitted varied from being undetectable to 1.0×10^5 [126]. An additional study with EEEV showed mosquitoes transmitted ~1.0×10^3 PFU as measured by FS collections with immersion oil[127]. Comparing these two results suggested that the quantities of EEEV transmitted during blood feeding and FS collection were approximately equal. However, other studies with different virus-vector pairings have given disparate results. For example, a study that quantified the amount of West Nile virus (WNV) transmitted by *Culex tarsalis* after blood feeding determined that virus transmitted was approximately 600-fold higher than virus transmitted during the FS technique[127], but another study showed similar virus titers transmitted from *Culex pipiens quinquefasciatus* by blood feeding and forced salivation[128]. Taken together, these results show that there is variation in the

amount of virus transmitted in saliva that may be dependent on the virus-vector pairing, and that detection methods vary widely in their accuracy and precision.

Here, we have attempted to quantify virus titers transmitted from *Ae. aegypti* mosquitoes infected with Zika virus (ZIKV) and chikungunya virus (CHIKV). Our efforts examined variations on the FS technique and compared it to virus transmission during blood feeding on animals and artificial feeders, and also to re-ingested virus recovered from bloodmeals dissected out of the mosquitoes (Figure 4.1). The results from this study add critical information to understanding the transmission of *Ae. aegypti*-borne arboviruses, which are responsible for frequent human disease epidemics across the tropical and sub-tropical areas of the world.

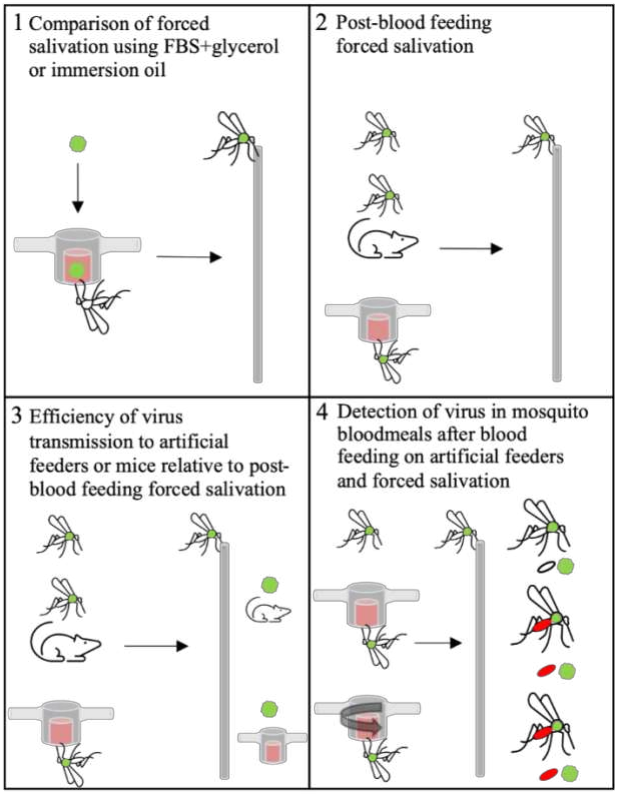


Figure 4. 1 Graphical picture of experiments completed in this study. Green ball represents Zika virus (ZIKV) or chikungunya virus (CHIKV). Green thorax mosquitoes = mosquitoes infected by infectious bloodmeal as shown in panel 1.

4.2 Results

4.2.1 Comparison of Virus Detection from Saliva Collected in FBS + Glycerol or Immersion Oil

When ZIKV- and CHIKV-infected *Ae. aegypti* were subjected to FS using two different media, there was a significant difference in the proportion of positive saliva samples from mosquitoes that salivated into oil compared to FBS + glycerol regardless of virus (Table 4.1). Overall, infectious ZIKV was detected in 31% (30/96) of saliva samples collected in oil, compared to 18% (18/100) of samples collected in FBS + glycerol ($p \leq 0.05$). The same pattern was seen with detection of infectious CHIKV, with 38% (38/100) of positive saliva samples collected in oil compared to 14% (14/100) from FBS + glycerol ($p \leq 0.05$). When testing for viral RNA in the same samples, the same pattern was observed, with oil resulting in more positive samples than the FBS + glycerol media for both viruses. However, viral RNA was detected in more samples overall than infectious virus for both viruses. Despite the increased virus prevalence in saliva samples collected using oil, positive samples from both FS media did not significantly differ in the quantity of infectious virus or viral RNA for either virus (Figure 4.2).

Table 4. 1 Proportions of saliva collections from individual mosquitoes that were ZIKV- or CHIKV-positive using either qRT-PCR or plaque assays when force salivated using oil or FBS-glycerol as the collection media.

Virus	**Collection media**	**qRT-PCR**	**P-value**	**Plaque assay**	**P-value**
ZIKV	FBS+glycerol	42% (42/100)	*0.0170	18% (18/100)	*0.0455
	Oil	65% (62/96)		31% (30/96)	
CHIKV	FBS+glycerol	47% (46/98)	*≤0.0001	14% (14/98)	*0.0002
	Oil	82% (82/100)		38% (38/100)	

Data are the sum of two biological replicates. P-value from Fisher's exact text; * indicates P ≤ 0.05.

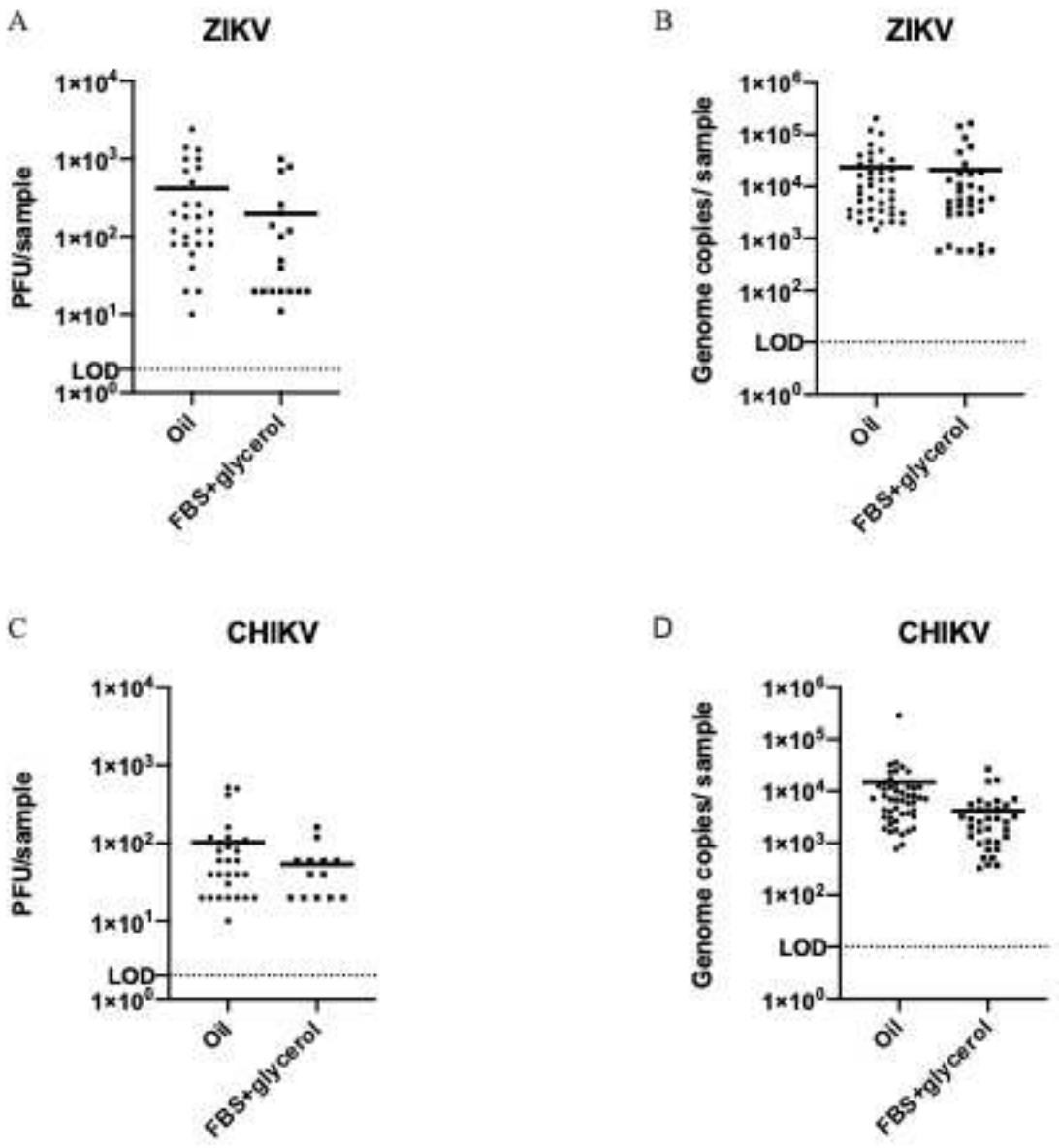


Figure 4. 2 Quantification of virus and viral RNA from virus-positive forced salivations using oil or FBS-glycerol as the collection media; titer and genome copies of virus-positive saliva samples from individual mosquitoes infected with ZIKV (A and B) or CHIKV (C and D). LOD= limit of detection. Titer LOD = 2 PFU; genome copies LOD = 10. The means of virus titers or genome copies from saliva collected by either method (horizontal bars), regardless of virus, were not significantly different (un-paired T-test; $P \geq 0.05$).

4.2.2 Detection of ZIKV or CHIKV from Mosquito Saliva Post-Blood Feeding

Comparisons of virus titers transmitted by ZIKV- and CHIKV-infected *Ae. aegypti* through FS were made post-blood feeding on a mouse or an artificial feeder, in groupings of 1–10 mosquitoes, relative to those that never blood fed (see Figure 4.1 panel 2 for experimental outline). We strived to group increasing numbers (from 1 to 10) of mosquitoes' saliva after they blood fed (artificial feeder or mouse) to compare titers from these groups, and made similar groupings from the non-blood feds for equal comparisons (Tables 4.2 and 4.3). There was a small increase in the proportions of groups positive for infectious ZIKV from mosquitoes salivated post-blood feeding (either on an artificial feeder or a mouse) relative to non-blood feds (≥50% vs. 40%, respectively). However, this was not observed when the same samples were tested for viral RNA, and similarly no differences were observed between the proportions of mosquito groups transmitting infectious CHIKV. There were also no significant differences in the quantities of infectious virus or viral RNA expectorated from mosquitoes among the treatment groups (Figure 4.3). Overall, the quantity of transmitted viral RNA was between 10 and 10,000 times greater than infectious virus in all treatment groups, and there was no correlation between the quantity of virus or viral RNA in the treatment groups and the number of mosquitoes salivated in each group (ZIKV, $p = 0.692$; ZIKV RNA, $p = 0.121$; CHIKV, $p = 0.576$; CHIKV RNA $p = 0.706$, determined by Spearman's rank-order correlation)

Table 4. 2 Detection of ZIKV from forced salivation samples of groups of infected mosquitoes that underwent different blood feeding treatments.

Blood feeding treatments	**Group ID**	**Number of mosquitoes that blood fed**	**Titer (PFU/sample)**	**Genome copies/sample**
None	1	1	0	$4.0x10^4$
None	2	1	0	$2.1x10^5$
None	3	2	$1.0 x10^2$	$4.7x10^3$
None	4	2	0	$8.1x10^3$
None	5	3	0	$1.1x10^5$
None	6	3	$4.0 x10^1$	$6.3x10^5$
None	7	5	$2.0 x10^2$	$3.1x10^5$
None	8	5	0	$2.6x10^6$
None	9	7	$1.8 x10^2$	$2.3x10^5$
None	10	7	0	$1.5x10^5$
None	11	10	0	$2.2x10^5$
None	12	10	0	$7.3x10^5$
None	13	7	$5.0 x10^2$	$7.3 x10^5$
None	14	10	0	$1.1 x10^5$
None	15	10	$6.0 x10^1$	$3.2 x10^5$
Mouse	1	1	$2.8 x10^2$	$1.3 x10^7$
Mouse	2	1	0	$9.8 x10^3$
Mouse	3	2	$4.0 x10^2$	$2.0 x10^5$
Mouse	4	2	0	$2.0 x10^5$
Mouse	5	3	$2.6 x10^2$	$6.4 x10^5$
Mouse	6	3	$2.0 x10^1$	$1.7 x10^4$
Mouse	7	5	$3.0 x10^2$	$3.6 x10^5$
Mouse	8	5	0	$1.1 x10^5$
Mouse	9	7	0	$1.7x10^5$
Mouse	10	7	$2.0 x10^1$	$3.1x10^6$
Mouse	11	7	$2.0 x10^2$	$1.1x10^6$
Artificial feeder	1	1	$3.0 x10^2$	$6.4x10^4$
Artificial feeder	2	1	$6.0 x10^2$	$1.6x10^6$
Artificial feeder	3	3	$6.0 x10^1$	$6.4 x10^5$
Artificial feeder	4	2	0	$6.3x10^3$
Artificial feeder	5	1	0	$1.1x10^4$
Artificial feeder	7	1	$4.0 x10^1$	$2.2x10^4$
Artificial feeder	10	3	$6.0 x10^1$	$4.2x10^5$

Table 4. 3 Detection of CHIKV from forced salivation samples of groups of infected mosquitoes that underwent different blood feeding treatments

Blood feeding treatments	**Group ID**	**Number Blood Feed Mosquitoes**	**Titer (PFU/mL)**	**Genome copies**
None	1	1	0	0
None	2	1	1.0×10^1	2.4×10^3
None	3	2	6.2×10^1	6.7×10^3
None	4	2	8	1.7×10^3
None	5	3	6	1.8×10^3
None	6	3	3.4×10^2	6.2×10^4
None	7	5	9.0×10^1	1.9×10^4
None	8	5	1.0×10^3	1.3×10^6
None	9	7	3.2×10^1	1.3×10^5
None	10	7	8.8×10^1	6.9×10^4
None	11	4	2.01×10^2	1.1×10^5
Mouse	1	1	0	8.7×10^2
Mouse	2	1	3.0×10^1	9.5×10^2
Mouse	3	2	1.0×10^2	1.4×10^4
Mouse	4	2	1.1×10^3	3.4×10^5
Mouse	5	3	1.4×10^2	0
Mouse	6	3	4.3×10^3	7.5×10^5
Mouse	7	5	2.6×10^2	1.6×10^5
Mouse	8	5	2.6×10^2	1.3×10^5
Mouse	9	7	1.1×10^2	8.0×10^4
Mouse	10	7	2.6×10^3	1.7×10^6
Mouse	11	6	9.2×10^1	2.2×10^4
Artificial feeder	1	1	0	0
Artificial feeder	2	1	4	2.2×10^4
Artificial feeder	3	2	0	5.3×10^2
Artificial feeder	4	2	1.4×10^2	1.1×10^4
Artificial feeder	5	3	1.0×10^1	2.4×10^3
Artificial feeder	6	3	2.3×10^2	7.4×10^4
Artificial feeder	7	5	1.2×10^3	7.4×10^5
Artificial feeder	8	5	5.2×10^3	1.4×10^6
Artificial feeder	9	7	1.7×10^3	1.1×10^6
Artificial feeder	10	7	3.9×10^2	5.2×10^5
Artificial feeder	11	5	4.4×10^1	2.4×10^4

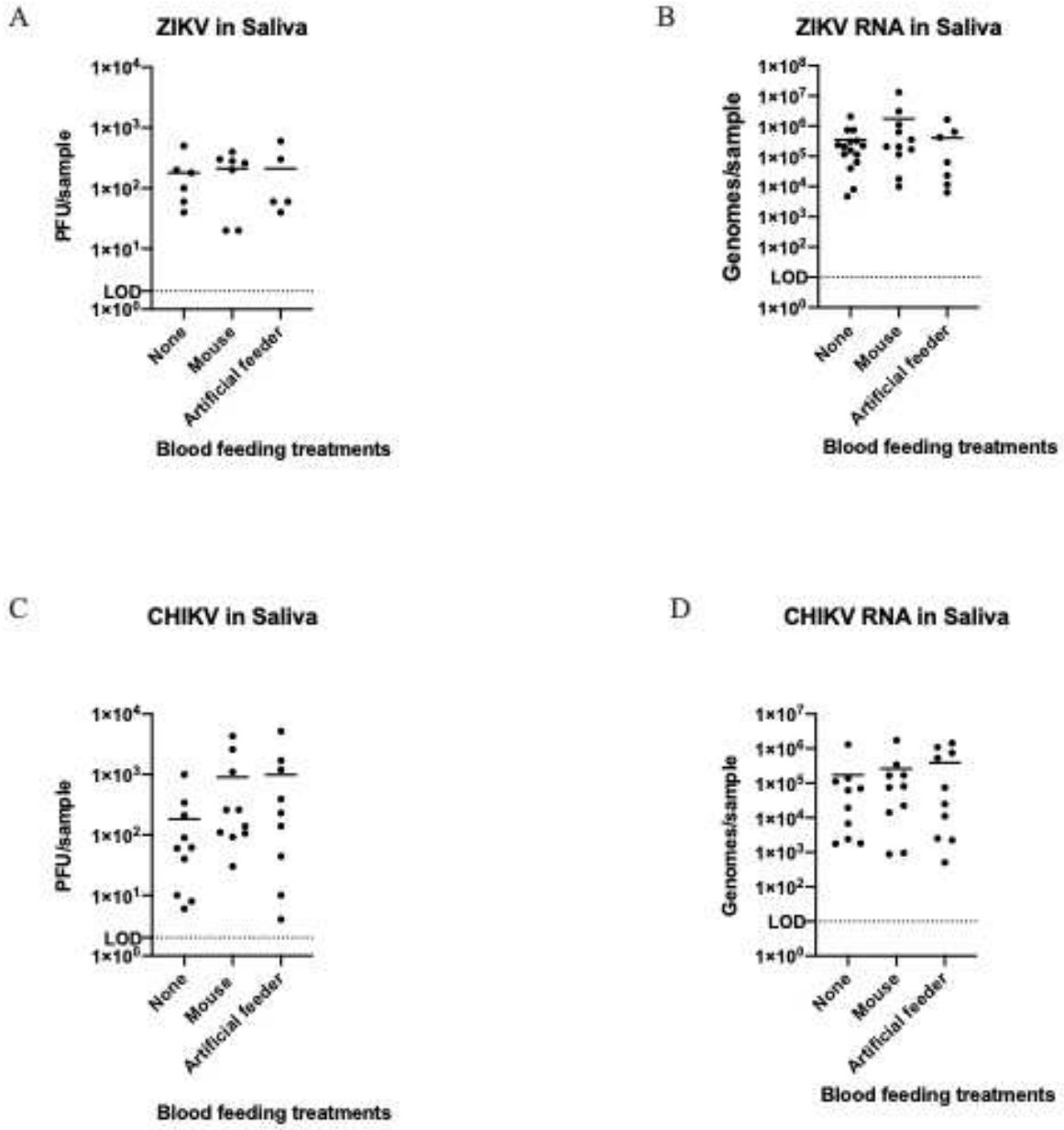


Figure 4. 3 Quantification of ZIKV and CHIKV from groups of virus-positive saliva-samples collected from forced salivation samples of mosquitoes that underwent different blood feeding treatments. Titer and genome copies of saliva samples from positive pools of mosquitoes infected with ZIKV (A and B; displayed in Table 2) or CHIKV (C and D; displayed in Table 3). LOD = limit of detection. Titer LOD = 2 PFU; genome copies LOD = 10. Means of virus titers or genome copies from saliva collected by either method (horizontal bars) were not significantly different (one-way ANOVA and post-hoc Tukey test; $P \geq 0.05$)

4.2.3. Efficiency of ZIKV or CHIKV Transmission to Artificial Feeders or Mice Relative to Post-Blood Feeding Forced Salivation

Overall, 53 immunocompromised mice were fed upon by groups of ZIKV-infected mosquitoes (between 1–4 individual mosquitoes) and all became infected with ZIKV. Table 4.4 shows the results from eight of these experiments when FS of the mosquitoes' post-blood feeding was unsuccessful in detecting infectious virus. Notably, some mice became infected even after being bitten by only one or two mosquitoes from which infectious virus could not be detected and that transmitted low levels of viral RNA (7.2×10^2–6.8×10^4 genome copies) via FS (Table 4.4). In contrast, infectious virus was never detected, and viral RNA rarely detected, from artificial feeders fed upon by groups of ZIKV- and CHIKV-infected mosquitoes, even though infectious virus and viral RNA was detected in their saliva via FS (Tables 4.5 and 4.6). These data highlighted the sensitivity of immunocompromised mice for detecting transmission of potentially low titers of ZIKV from *Ae. aegypti*, but also highlighted the surprising insensitivity of artificial feeder blood for detecting transmitted virus (both ZIKV and CHIKV) from *Ae. aegypti*. Considering that quantification of viral RNAs from FS of both groups of blood fed mosquitoes was roughly equal, the combined data suggested that some factor was inhibiting our ability to detect arbovirus transmission by *Ae. aegypti* from the blood in the artificial feeders. We hypothesized that this inhibitory factor was mosquito re-ingestion of their saliva, and thus the virus they expectorated, during blood feeding on artificial feeders.

Table 4. 4 Detection of ZIKV from forced salivations after infected mosquitoes blood fed on immunocompromised mice.

Mouse ID	Number of mosquitoes that blood fed	Pooled saliva-Titer (PFU)	Pooled saliva-Genome copies	Mouse became infected as determine by viral RNA detection in tissue/blood?
080	4	0	$2.8x10^5$	Yes
086	2	0	$2.0x10^4$	Yes
095	1	0	$7.2x10^2$	Yes
109	2	0	$2.0x10^4$	Yes
165	3	0	$1.3X10^3$	Yes
166	1	0	$5.0X10^3$	Yes
169	2	0	$6.8X10^4$	Yes
174	3	0	$3.3X10^3$	Yes

Pooled saliva titers were determined by plaque assay, limit of detection (LOD) = 2 PFU. Pooled saliva genome copies were determined by qRT-PCR, LOD = 10 genome copies. In total, 53 mice became infected after being bitten by ZIKV-infected mosquitoes. Eight of these mice (shown above) became infected even though the mosquitoes that bit them had undetectable titers of virus in their saliva as measured by FS post-blood feeding.

Table 4. 5 Detection of ZIKV from forced salivations and from the blood remaining in the artificial feeders after infected mosquitoes blood fed on them.

Group Number	Number of mosquitoes that blood fed	Pooled saliva-Titer (PFU)	Pooled saliva-Genome copies	Remaining blood in the artificial feeder-Titer (PFU)	Remaining blood in the artificial feeder- Genome copies
1	2	$8.0x10^1$	$1.1x10^5$	0	$2.1x10^3$
2	6	$3.0x10^1$	$1.6x10^4$	0	$7.0x10^2$
3	4	$1.8x10^1$	$9.5x10^3$	0	0
4	5	$1.2x10^2$	$2.2x10^5$	0	$3.5x10^3$
5	9	$4.0x10^1$	$1.9x10^5$	0	0
6	2	$5.0x10^1$	$4.5x10^5$	0	0
7	7	$2.3x10^2$	$1.9x10^5$	0	0
8	5	$1.0x10^2$	$1.2x10^6$	0	0
9	3	$2.3x10^2$	$9.0x10^4$	0	$1.7x10^3$

Pooled saliva titers determined by plaque assay, limit of detection (LOD) = 2 PFU. Pooled saliva genome copies determined by qRT-PCR LOD = 10 genome copies.

Table 4. 6 Detection of CHIKV from forced salivations and from the blood remaining in the artificial feeders after infected mosquitoes blood fed on them.

Group Number	Number of mosquitoes that blood fed	Pooled saliva-Titer (PFU)	Pooled saliva-Genome copies	Remaining blood in the artificial feeder-Titer (PFU)	Remaining blood in the artificial feeder- Genome copies
1	2	$2.1x10^2$	$1.1x10^5$	0	0
2	11	$3.7x10^3$	$1.8x10^6$	0	0
3	10	$2.0x10^2$	$9.5x10^4$	0	0
4	7	$3.3x10^2$	$5.0x10^4$	0	$2.8x10^3$
5	6	$2.1x10^2$	$1.0x10^5$	0	0

Pooled saliva titers determined by plaque assay, limit of detection (LOD) = 2 PFU. Pooled saliva genome copies determined by qRT-PCR LOD = 10 genome copies.

4.2.4 Detection of ZIKV or CHIKV in Mosquito Bloodmeals

To test our hypothesis of re-ingested virus during blood feeding, we dissected and tested the bloodmeals of ZIKV- and CHIKV-infected mosquitoes after they fed on artificial feeders containing uninfected blood and then underwent forced salivation. As the process of dissecting out the blood meal results in some contamination of virus into the dissecting media from the hemolymph and midgut tissue of these infected mosquitoes, mock blood meal dissections were performed as treatment controls on groups of mosquitoes that did not get a second bloodmeal. Approximately 100-fold more infectious ZIKV or ZIKV RNA, as well as infectious CHIKV or CHIKV RNA, was detected in the bloodmeals from individual mosquitoes relative to the treatment controls (Tables S3 and S4). These results were consistent between groups that fed on artificial feeders that were manipulated so that the blood in them was mixed during the blood feed, compared to those that were not. Importantly, however, a similar increase was not observed in the virus/viral RNA detected in the saliva from these same treatment groups, nor from the bodies of mosquitoes in these same treatment groups (Tables S3 and S4). Overall, the log transformed mean differences of virus quantified between the bloodmeal and saliva collected

from ZIKV-infected mosquitoes given blood feeding treatments were significantly different from the control treatment (none = 2.214 PFU/5.059 genome copies (gc), artificial feeder = 3.656 PFU/7.064 gc, artificial feeder + mixing = 3.699 PFU/6.350 gc) (Figure 4.4 A,B; one-way ANOVA and post hoc Tukey test; $p \leq 0.05$). A similar difference was seen with CHIKV-infected mosquitoes (none = 2.067 PFU/5.195 genome copies (gc), artificial feeder = 3.443 PFU/6.832 gc, artificial feeder + mixing = 3.739 PFU/6.659 gc) (Figure 4.4 C,D; one-way ANOVA and post hoc Tukey test; $p \leq 0.05$).

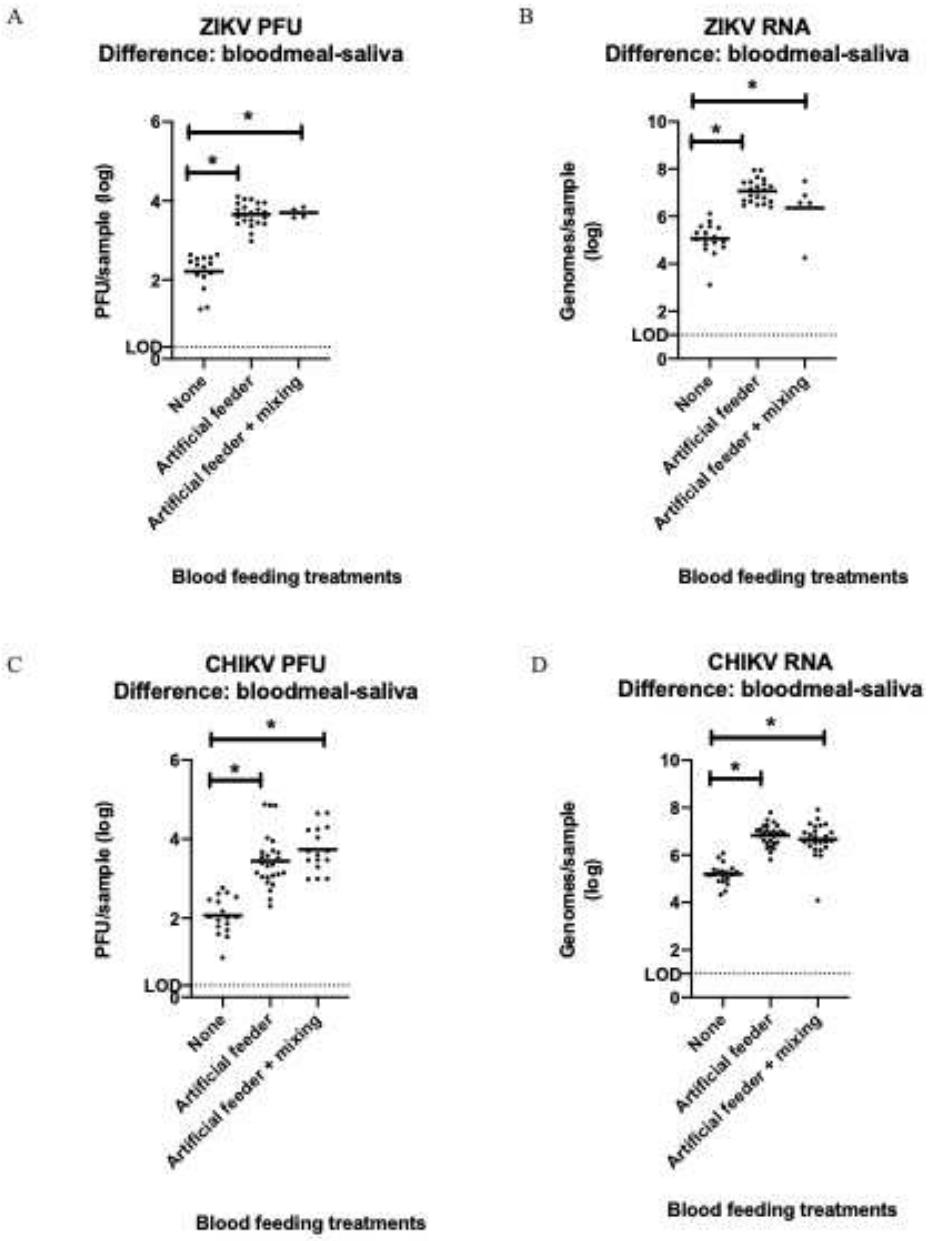


Figure 4. 4 Log transformed difference between ZIKV and CHIKV detected in the bloodmeals and saliva of individual infected mosquitoes after they were given different blood feeding treatments. A) Infectious ZIKV mean difference: None = 2.21, Artificial feeder = 3.65, Artificial feeder + mixing = 3.69. B) ZIKV genome copies mean difference: None = 5.05, Artificial feeder = 7.06, Artificial feeder + mixing = 6.35. C) Infectious CHIKV mean difference: None = 2.06, Artificial feeder = 3.41, Artificial feeder + mixing = 3.73. D) CHIKV genome copies mean difference: None = 5.19, Artificial feeder = 6.82, Artificial feeder + mixing = 6.65. Graphs show both PFUs determined by plaque assay and RNA determined by qRT-PCR. Titer LOD= 2 PFU; genome copy LOD = 10 genomes. Means are horizontal bars (one-way ANOVA and post-hoc Tukey test; *$P \leq 0.05$).

4.3 Discussion

We used different experimental procedures and methods to estimate the titer of virus transmitted by *Ae. aegypti* mosquitoes after being infected with either ZIKV or CHIKV. Different medias have been previously evaluated for FS, including immersion oil and a mixture of 1:1 FBS + glycerol, as well as blood and other medias[129,233,242,244,247–250]. Results from these studies showed that the effectiveness of the media depends on mosquito and virus species, but no other study compared these methods with *Ae. aegypti* infected with CHIKV or ZIKV. We found no evidence that FBS + glycerol aided in viral stabilization and preservation as was previously suggested[129,245] and no difference in titers from ZIKV- or CHIKV-positive samples, however, infectious saliva collected using oil resulted in significantly more positive samples from individual mosquitoes. The use of oil with FS is also easier to perform because the mosquitoes are drawn into the capillary tube via the similar hydrophobic properties of the mosquito's cuticle and the oil, which draws saliva out of the salivary glands into the oil. Additionally, proof of successful saliva capture can be observed, and its quantity estimated, because the hydrophobic oil and aqueous saliva do not mix[120]. In the same paper, Sanchez-Vargas et al. estimated that individual *Ae. aegypti* expectorated a mean of 6.8 nL using oil-based FS and observed no correlation of CHIKV titers with the saliva volume expectorated. Another potential benefit of oil-based FS is that the mosquitoes are unable to re-ingest their own saliva. It has been previously shown that anopheline mosquitoes will re-ingest many of the *Plasmodium* sporozoites they deposit in the host when blood feeding[251]. It follows that saliva re-ingestion could also influence the virus detection success in FS.

FS has been used as the standard method to determine transmission of mosquito-borne arboviruses[129,212,233,241], but we are not aware of any examination of forced salivation on

mosquitoes that blood fed immediately prior. Given the large variances in virus transmission by any one mosquito (Tables 2 and 3 and the supplemental tables), we wanted to know if virus transmission estimates using FS were different if performed immediately after they imbibed a second blood meal relative to estimates using FS from sibling mosquitoes never given a second blood meal. If there were no differences, one would be able to estimate the amount of virus transmitted in laboratory experiments, or even natural experiments in the field, using FS immediately after one or more mosquitoes took a blood meal on a host. For example, one could capture indoor resting blood fed mosquitoes from the walls of a house, perform FS on them immediately post-capture, and reliably estimate the titer that they may have just transmitted to the people in the house whom they bit. We used groups of mosquitoes (1–10 mosquitoes/group) that were given three different blood feeding treatments (none, blood fed on a mouse, blood fed on an artificial feeder) and demonstrated that infectious virus and viral RNA titers determined from FS were not different between the treatments. Furthermore, the ratios of infectious virus to viral RNA quantified were not different between the treatment groups. Each treatment group showed ~100–10,000 times more viral RNA than infectious virus as has been reported in many other studies using FS on unfed mosquitoes alone[233,252]

To compare our post-blood feeding FS data with the quantity of virus transmitted during the blood feed, we analyzed outcomes of ZIKV-infected mosquitoes that blood fed on immunocompromised mice, and virus transmitted to artificial feeders after ZIKV- or CHIKV-infected mosquitoes fed on them. Mice became infected after mosquito feeding even when no infectious virus was detected in the mosquito saliva post feeding. Our limit of detection is 2 PFU and 10 genomes copies; however, we never detected anything under 10 PFU. Based on these results, we can assume these mice can become infected with less than 10 PFU transmitted by

mosquitoes blood feeding on them. When examining the blood remaining in the artificial feeders, however, infectious virus was never recovered, and viral RNA was only recovered in four out of nine ZIKV groups and one out of nine CHIKV groups. This observation could be explained in at least two non-exclusive hypotheses. It may be that live virus was quickly inactivated and viral nucleic acid sequences were destroyed by proteases and nucleases in the artificial blood meal, making their detection difficult by plaque assay and qRT-PCR, respectively. However, this seems unlikely given that we rarely record a drop in virus titer of the original blood meal used to first infect the mosquitoes when it is 'back-titered' after sitting in the artificial feeder for ~30 min during the blood feed. Another possibility is that virus expelled with the saliva into the artificial blood meal may be immediately re-ingested through the suction force needed to bring blood into the food canal.

To address the latter hypothesis, we dissected out bloodmeals from the infected mosquito midguts after they were given different blood feeding treatments and then were processed with FS. For one of the blood feeding treatments, blood was pipetted up and down in the blood feeder during the time of feeding to determine if blood mixing might counteract the re-ingestion of a mosquito's own expectorated virus during blood feeding. Compared to the control treatment (mock dissection of bloodmeals from empty midguts), significantly more virus and viral RNA was recovered from the bloodmeals of the two blood feeding treatment groups, and the mixing of the blood in the artificial feeder did not influence this. This indicated that mosquitoes re-ingest much of their expectorated virus while feeding on the artificial feeder and that poor detection of virus in the remaining blood from artificial feeding is likely due to re-ingestion. As each mosquito dissected of its bloodmeal was also processed via FS, we could determine the difference of virus titers between the bloodmeal and saliva to estimate the quantity of virus

transmitted during blood feeding. The estimate was consistent between *Ae. aegypti* transmitting either ZIKV or CHIKV and between quantities of infectious virus or viral RNA detected; between 50–100 times more virus is secreted during blood feeding than is detected in FS performed immediately after blood feeding, suggesting a large increase in virus transmission during blood feeding. One limitation of these data is that they are estimates of virus transmission by *Ae. aegypti* derived from blood feeding on artificial feeders, which may not accurately reflect what occurs during blood feeding on live hosts, including transmission during probing but not blood feeding [253]. However, blood feeding on live hosts results in diverse outcomes. Mosquitoes will capillary feed by either fully cannulating capillaries, or just pierce the capillary at a right angle with the tip of the labrum, or sometimes might only nick a capillary and perform 'pool feeding' on the blood that pools into the interstitial space of the dermis[254]. Each of these methods are likely to result in differing quantities of saliva/virus deposited as well as being re-ingested back into the blood meal. As such, artificial feeders may be a more consistent blood source for this estimation. In natural blood feeding experiments, Secundino et al. determined that the ZIKV cDNA ranged from 2.0×10^{2}–2.1×10^{10} when the mouse ear tissue was immediately removed and homogenized after being fed on by ZIKV-infected *Ae. aegypti* mosquitoes [230]. In our study, this quantity is more comparable to the RNA loads in bloodmeals rather than saliva, indicating that mosquitoes re-ingest much of their saliva during natural blood feeding.

Studies using different combination of viruses and mosquito species have evaluated the use of vertebrate hosts or artificial feeders to estimate the amount of virus being transmitted from an infectious mosquito and found varying results[125–128]. Our data allow for estimation of the amount of ZIKV or CHIKV from an infectious *Ae. aegypti* mosquito by performing FS on it immediately post-blooding, quantifying infectious virus or viral RNA and then multiplying the

titer determined by ~50–100. Quantifying infectious virus ensures measurement of true infectious units, but it is clearly of low sensitivity and so simultaneously quantifying viral RNA will give the best estimates of transmission dose. More experiments will be necessary to determine if the increase in virus transmission during blood feeding relative to FS we observed is because of (a) more saliva being released by *Ae. aegypti* or (b) more virus being released from the salivary glands, or both. Similarly, results from this study should be replicated with other arbovirus vectors to determine if they are consistent across mosquito species. Overall, the methods developed here can be used as a better way to estimate the titer of arboviruses transmitted by blood feeding *Ae. aegypti* and may be valuable for similar estimations with other mosquitoes.

4.4 Materials and Methods

4.4.1 Virus and Cells

African Green Monkey kidney cells (Vero; ATCC #CCL-81) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (DMEM; Gibco Thermo Fisher, FBS; Hyclone, Logan, UT, USA), 2 mM L-glutamine (Gibco Thermo Fisher), 1.5 g/L sodium bicarbonate (Gibco Thermo Fisher), 100 U/mL penicillin (Gibco Thermo Fisher) and incubated at 37 °C in 5% CO_2. ZIKV strain PRVABC59 (ZIKV-PR; GenBank:KU501215), originally isolated from a human traveler to Puerto Rico in 2015 with three rounds of amplification on Vero cells, was obtained from Dr. Aaron Brault (CDC, Ft. Collins, CO, USA). CHIIKV strain LR2006_OPY1 (GenBank: KT449801.1) was obtained from the University Texas Medical Branch and isolated from outbreak in Reunion Island in 2006 with three rounds of amplification on Vero cells.

4.4.2 Mosquito Infections

Ae. aegypti Poza Rica strain mosquitoes were fed an infectious artificial blood meal containing either CHIKV or ZIKV and held for 10–14 days before all subsequent experiments to ensure dissemination of virus to the salivary glands. Infectious bloodmeals were prepared with 1 mL fresh virus contained in the cell-culture supernatant of infected Vero cells and 1 mL of defibrinated calf blood. Back-titering of the bloodmeals ranged between 1×10^6–5×10^6 PFU/mL. Mosquitoes were sorted post blood feeding and were placed in cartons (Huhtamaki, paper food container 64oz) with an organdy cover and given water and a sugar source.

4.4.3 Mice Infection

A129 mice (interferon alpha/beta receptor -/-) 8–12 weeks old were obtained from breeding colony maintained at Colorado State University. Use of mice was approved by the Colorado State University Institutional Animal Care and Use Committee (protocol 15-6677 AA). All procedures were done in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. To infect mice by mosquito bite, *Ae. aegypti* Poza Rica strain mosquitoes were fed an infectious blood meal and held for 14–17 days. Mosquitoes were sorted post blood feeding and 10–20 blood-fed mosquitoes were place in cartons with an organdy cover and given water and a sugar source. To allow the mosquitos to feed on the mice, each mouse was anesthetized using 100 mg/kg ketamine/10 mg/kg xylazine (ketamine: Zetamine from VetOne, xylazine: XylaMed from VET ONE) and placed on the organdy cover of one carton for ~20 min.

4.4.4 Mosquito Sample Collections

Mosquitoes were immediately cold-anesthetized post-blood feeding and their saliva was collected by the FS method described previously[212], briefly their legs and wings were removed and their proboscis was placed into a capillary tube containing either mineral oil or FBS + glycerol at a ratio of 1:1. After 20–30 min, mosquitoes were pulled off the capillary tube and the capillary tube contents were centrifuged into 150 µL of 2x DMEM and held at −80 °C. The bodies were place in a separate tube held at −80 °C to be homogenized in media for later testing. Infections of mosquito bodies and saliva were determined by plaque assay and qRT-PCR. Samples were titrated by Vero cell plaque assay, with a tragacanth gum overlay and staining at day 5 post-cell culture inoculation for ZIKV and day 2 post-cell culture inoculation for CHIKV.

4.4.5 Bloodmeal Dissections

Bloodmeal dissections were done immediately after individual mosquitoes underwent FS. Mosquitoes were dissected on the sides of glass wells partially filled with 200 µL of DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin. The midguts were dissected out and spilt open so that the blood meal contents could spill out into the media, and the torn midgut swished into media to extract the whole blood meal. Mock blood meal dissections were performed on non-blood fed mosquitoes exactly the same way but there was no blood meal that could spill out into the media. The media (plus blood meal) was then collected and placed into a tube for later testing, and the body plus torn midgut were placed into another tube and frozen at −80 °C to be homogenized in media for later testing.

4.4.6 RNA Extractions and qRT-PCR

Tubes containing mosquito bodies were homogenized and both saliva and bodies where centrifuged for 5 min at 14,000× *g*. Bloodmeals were collected in 150 µL of DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin. RNA was extracted from all samples using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek) on the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific). RNA was eluted in 30 µL nuclease-free water. Progmeg GoTaq Probe 1-Step RT-qPCR System Kits were used on RNA extracted from saliva and bodies to quantify CHIKV and ZIKV RNA according to manufacturers' instructions. Standard cycling condition were followed, one cycle at 45 °C for 15 min, one cycle at 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers used for CHIKV were Forward (5′-CTTTGAAGTTTCCTTTCGGTGG-3′) and Reverse (5′-ACFFAAFFRAAACTGGTATGG-3′) and Probe-FAM (5′-TCTGCAGCGTCTTTATCCACGGG-3′). Primers used were ZIKV 1086 (5′-CCGCTGCCCAACACAAG-3′) and ZIKV 1162c (5′-CCACTAACGTTCTTTTGCAGACAT-3′). The probe used was ZIKV 1107-FAM (5′-AGCCTACCTTGACAAGCAGTCAGACACTCAA-3′)[52]. Approximately 100 ng of RNA was added to each reaction. Standards were generated for each virus using a full-length viral RNA. RNA was quantified on a Qubit Fluorometer (ThermoFisher Scientific) and diluted to achieve serial 10-fold genome equivalent (GE) dilutions. The standard curve detection of 104–107 GE/reaction had a primer efficiency of 88.62% to 102% with an R^2 value of 0.971 to 0.997, a slope of −3629 to −3269, and y-intercept = 37.966 to 47.270.

4.4.7 Statistical Analyses

Results in figures were expressed as mean values (horizontal bars) with individual values showing the variance. The statistical details are noted in the figures and/or in the corresponding figure legends. Statistical significance was primarily determined using either Fisher's exact test, unpaired Student's *t*-test or a one-way analysis of variance (ANOVA) with a Tukey's multiple-comparison in GraphPad Prism. Correlation was determined by Spearman's rank-order correlation in the GraphPad Prism (GraphPad Software, La Jolla CA,USA).

CHAPTER 5: THE ROLE AND CHARACTERIZATION OF DEFECTIVE INTERFERING PARTICLES IN THE TRANSMISSION OF *AEDES*-BORNE ARBOVIRUSES

5.1 Introduction

Aedes species mosquitoes transmit several arboviruses that cause disease outbreaks that result in widespread morbidity and thousands of deaths each year [31]. These viruses include chikungunya virus (CHIKV), dengue virus (DENV), Zika virus (ZIKV) and yellow fever virus[16]. CHIKV and ZIKV represent two *Aedes*-borne arboviruses from different viral families. CHIKV (genus:*Alphavirus*; family:*Togaviridae*) has a single-stranded, positive sense RNA genome and was seen for the first time in the Americas in 2013 [255]. The CHIKV genome is organized with the nonstructural genes encoded on the 5' end and the structural genes encoded on the 3' end. The nonstructural genes are the first to be translated, while the structural genes are translated later from a subgenomic RNA that derives from the 3' end[256]. ZIKV (genus:*Flavivirus*; family:*Flaviviridae*) also has a single-stranded, positive sense RNA genome but its genome is organized with the structural genes on the 5' end and the non-structural genes on the 3' end, and these are all simultaneously translated as a single polypeptide [256].

Both CHIKV and ZIKV are maintained in a mosquito-vertebrate transmission cycle, thus they have evolved to replicate in both host systems [107]. These RNA viruses are dependent on RNA-dependent RNA polymerases (RdRp) for replication[257]. RdRp often produce single nucleotide mutations due to a lack of proof-reading functions and so generate relatively high error rates during replication of the viral RNA [258]. The mechanisms of RNA recombination can help to rescue wild type genomes when deleterious mutations occur, which can lead to advantageous viral variants [259]. However, recombination can also result in insertions and deletions in the genetic material. Deletions usually occur when the RdRp hits a break point, falls

off and then continues replication at a different point in the genome [260]. The two genome parts then recombine, but potentially large amounts of genetic material can be missing. This process results in the production of a defective viral genome (DVG). DVGs can range from deletions of a few nucleotides to loss of complete genes or more, leaving only genomic termini that contain replication promoters [260].

Defective viral genomes (DVGs) are able to be packaged into virus-like particles, sometimes due to the retention of a packaging signal, and can then be released from the cell [261,262]. Importantly, packaged DVGs must co-infect with at least one virus with a complete genome in order to replicate as they lack their own replication machinery [260]. These DVGs can then affect the production of virus from the co-infected cell because their smaller genomes are replicated faster, leading to a reduction in infectious virus produced from the cell [74]. When packaged DVGs interfere with standard viral replication in co-infection assays they are referred to as defective interfering particle (DIPs) [260]. Both alphaviruses and flaviviruses have been documented to produce packaged DVGs when serial passaged in cell culture at a high multiplicity of infection (MOI) [130,136,263,264]. In addition, it has been shown that these DVGs affect the propagation of standard virus by decreasing the amount of infectious virus produced in each serial passage and are indeed DIPs[265–267].

Until recently, very little has been known about the role of DVGs outside of *in vitro* studies. Mice infected with Semliki Forest virus (SFV), influenza A virus, and murine respirovirus were all protected from disease to some degree when inoculated with DIPs along with standard virus [139,268–271]. More recently, DVGs have also been identified in fluids and tissue from human and animals during natural infections with dengue virus, influenza A virus, hepatitis C and A viruses and West Nile virus [137,138,272–274]. Interestingly, one study found a

correlation between DVGs in clinical samples from children infected with Respiratory Syncytial Virus (RSV) and strong innate immune responses [275]. Lastly, a recent study showed ZIKV RNA isolates from multiple organs in a susceptible mouse resulted in different ZIKV genome diversity [56]. Taken to together these studies demonstrate that DIPs are produced during infection in vertebrate hosts and that they may affect the course of disease.

There has been little to no research on packaged arboviral DVGs or DIPs transmitted in host biofluids (mainly mosquito saliva, but also semen that is involved in the sexual transmission of ZIKV). From experimental studies, we and others have documented large disparities between titers of infectious virus and virus genome copies detected in the same mosquito saliva samples from mosquitoes infected with CHIKV, DENV-2, ZIKV and West Nile virus (WNV) [233,252]. Interestingly, the same disparity is seen in detections of ZIKV from semen of infected humans and mice [49,226]. These disparities could be due to a preferential secretion of packaged DVGs into mosquito saliva and the semen of infected male vertebrates, but it is unclear what effect these may have on subsequent infections or disease. In addition, very few studies have characterized or examined the effect of DIPs in nonvertebrate hosts. *Drosophila melanogaster* that were infected with samples of Sindbis virus that had a high DIP content were found to survive longer [140]. In addition, mosquitoes infected with samples of WNV that had a high DIP content show reduced infection phenotypes [138]. More recently, infection with packaged CHIKV DIPs were shown to inhibit CHIKV infection *in vitro* as well dissemination in *Ae. aegypti* [73].

Understanding of DVGs/DIPs naturally produced in biofluids during arbovirus infections may further our understanding arbovirus transmission, infection and disease. The aim of this chapter is to lay the foundation for evaluation the role of natural *Aedes*-borne arbovirus packaged DVGs/DIPs during mosquito infection, dissemination and transmission by characterizing

packaged DVGs/DIPs of CHIKV and ZIKV in biofluids involved in the transmission of these viruses, relative to those produced in other infected mosquito and vertebrate tissues and in cell culture.

5.2 Results

5.2.1 Generation and detection of CHIKV and ZIKV DVGs in cell culture

After each of five serial passages at 0.01 MOI, the quantity of CHIKV in the cell culture supernatant gradually increased approximately by 1 log10 PFU/mL, but when serially-passaged at 10 MOI over the same number of passages, there was over a log decrease in infectious virus detected in the supernatant (Figure 5.1A). In comparison, the quantity of infectious virus in the supernatant over time did not change with serially-passaged ZIKV, regardless of the infecting MOI, and regardless of being passaged 4 more times (Figure 5.1B). Deep sequencing analysis of CHIKV RNA derived from the supernatant virus of the first (p1) and last (p5) serial passages identified a variety of DVGs, with the majority of deletions detected in the region of the structural genes (Figure 5.2). CHIKV DVG deletions from serially passaging at 0.01 MOI were heterogeneous but mostly similar between p1 and p5, the normalized abundance was less than < 1% relative to median total coverage, and the quantity of DVGs did not noticeably change between the passages (Figure 5.3). In contrast, DVG deletions from serially passaging at 10 MOI were consistently larger and became more uniform between p1 and p5 (Figure 5.2). Furthermore, there was ~30% increase in the relative abundance of these CHIKV DVGs in p5 compared to p1 (Figures 5.2 & 5.3), whereby most consisted of a normalized abundance between 1% and 10% relative to median total coverage. Additional molecular analyses using q-RT-PCR targeting conserved and DVG deletion regions of the genome (Table 5.1; Figure 5.4) supported these sequencing results. When comparing the mean genome copies detected from supernatant CHIKV

generated over five serial passages with 0.01 MOI, there was little variation as determined using primer sets designed to detect the conserved region (mean genome copies targeting nsP1= $3.8x10^{10}$) compared to the DVG deletion genome region (mean genome copies targeting CE2 = $1.98x10^{10}$) (P = 0.03) (Figure 5.5A). However, for the same comparison over five serial passages with 10 MOI, there was a significant difference in mean genome copies determined using primer sets designed to detect the conserved region (mean genome copies targeting nsP1 = $1.6x10^{9}$) compared to the DVG deletion genome region (mean genome copies targeting CE2 = $1.3x10^{8}$) (P = 0.0006) (Figure 5.5B). Similar analyses of supernatant ZIKV at passage nine at either MOI did not detect changes in mean genome copies using any of the primer sets against conserved or predicted DVG deletion regions (3' and E, MOI 0.01: P = 0.2, MOI 10: P = 0.8; 5' and E, MOI 0.01: P = 0.1, MOI 10: P =0 .4) (Figure 5.5C and 5.5D).

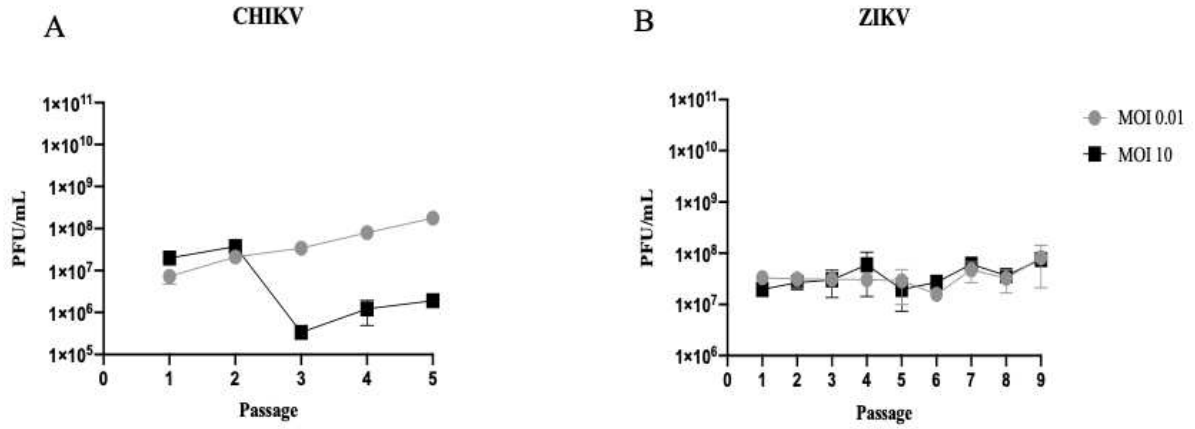


Figure 5. 1 Analysis of CHIKV and ZIKV grown *in vitro* from two independent replicates A) CHIKV; five passages at a MOI of 0.01 or 10. B) ZIKV; nine passages at a MOI of 0.01 or 10. Mean and standard deviations shown. Unpaired T-tests were used to determine significance differences of titers at each passage between MOI 10 and MOI 0.01. CHIKV; significant difference at each passage (p), p1-p5 $P \geq 0.01$. ZIKV; significance difference at p1 and p6 P = 0.02

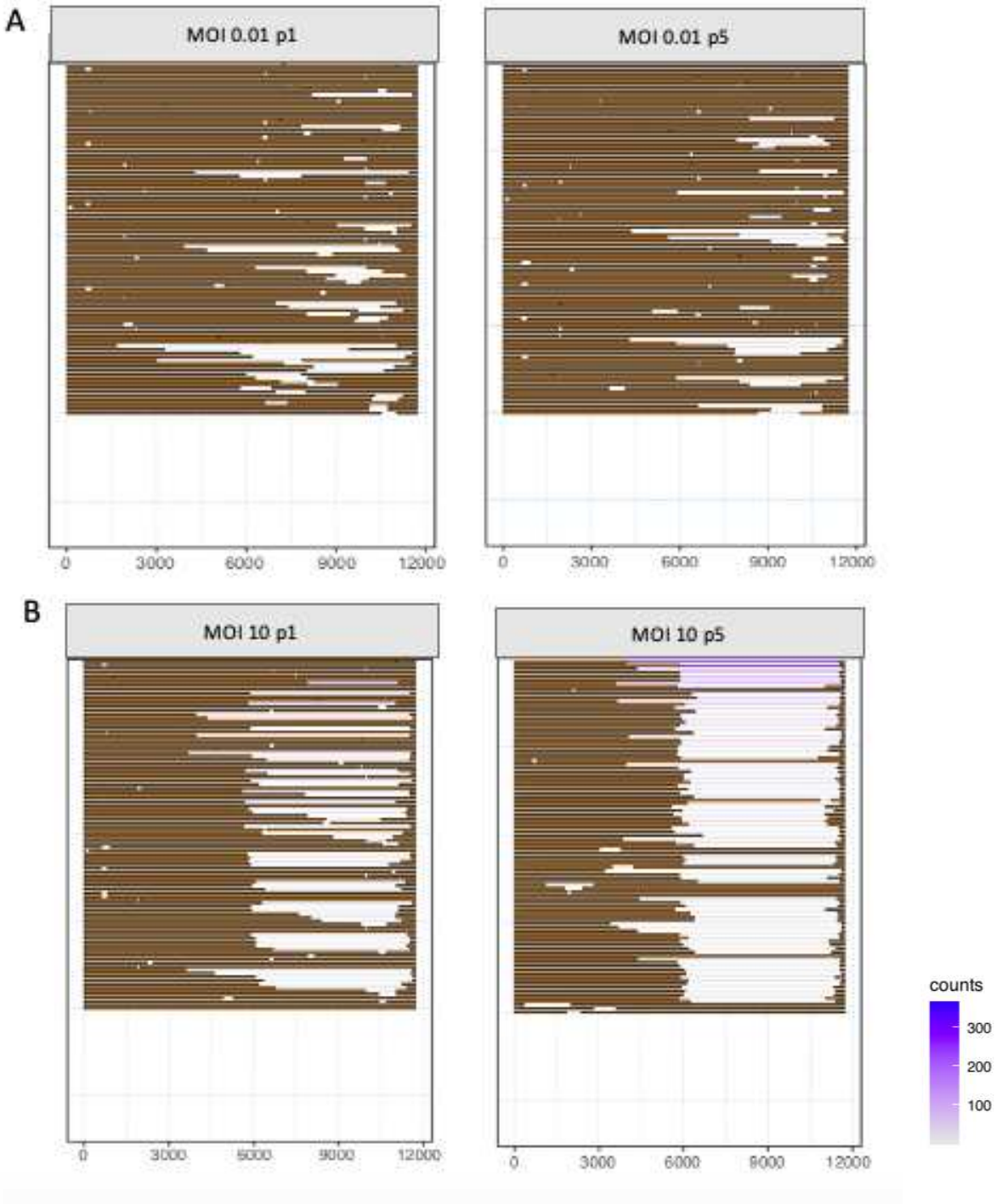


Figure 5. 2 Deep sequencing analyses of DVG variants produced from *in vitro* CHIKV infections through five passages. A) MOI of 0.01 at passages 1 (p1) or 5 (p5). B) MOI of 10 at passages 1 (p1) or 5 (p5). X-axes denoted nucleotide positions across the genome. Each line represents a DVG variant with the white space representing deletions. The counts of each variant are represented by range of blue added to the deletion space as observed in the legend.

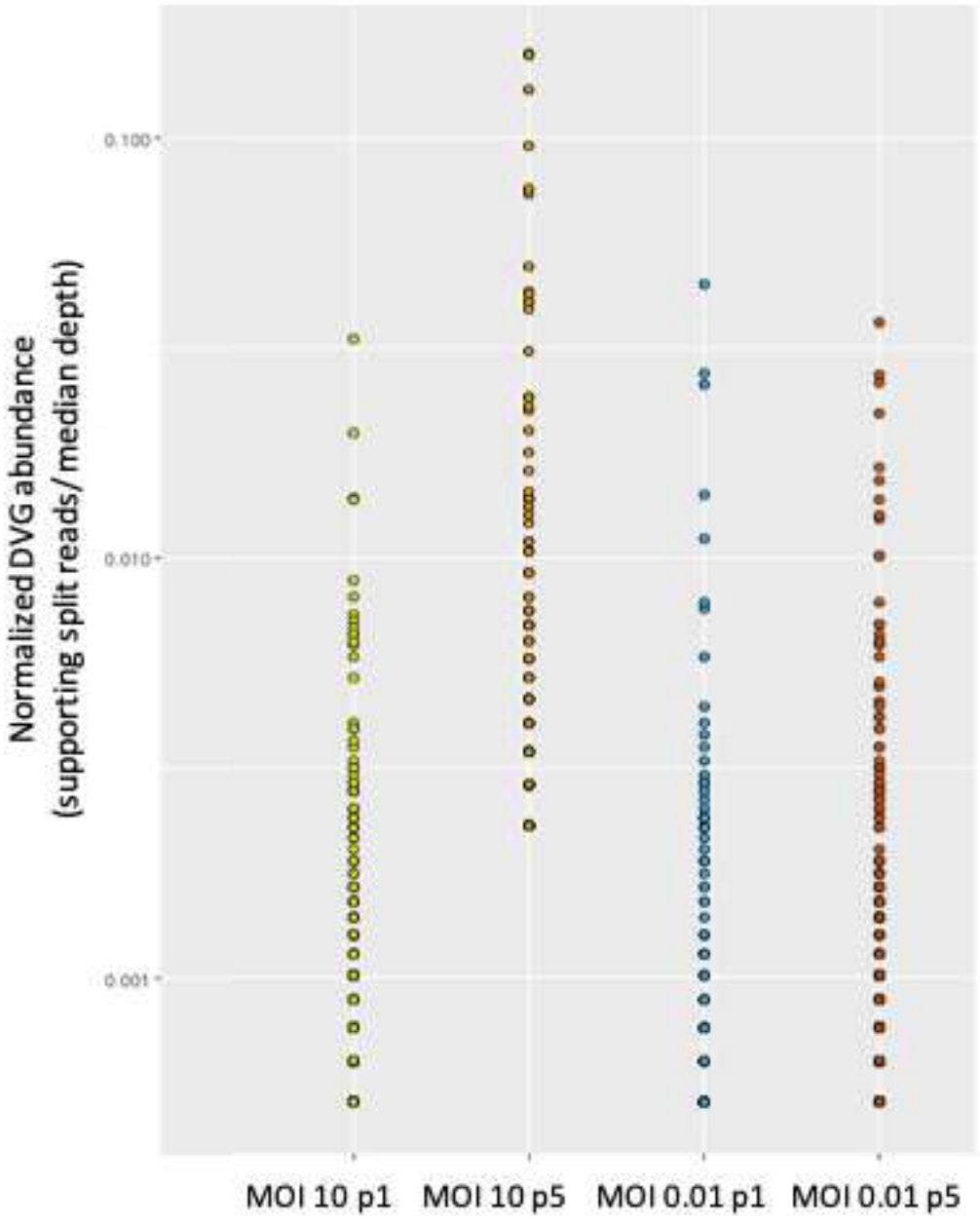


Figure 5. 3 Normalized abundance of CHIKV DVG variants relative to all reads from sequencing. Y-axis displays proportions. MOI either 10 or 0.01, Passage either 1st (p1), or 5th (p5). Each dot= DVG variants with 5x coverage (5 read count cutoff)

Table 5. 1 **q-RT-PCR primers and probes designed to target predicted DVG deletions and conserved regions of the CHIKV and ZIKV genomes**.

Virus	Target genomic region	Predicted DVG deletion or conserved regions	Forward Primer	Reverse Primer	Probe - FAM
CHIKV	nsP1	conserved	**5'-**CCCATGTTTG AGGTGGAACC-**3'**	**5'-**ACTGCCGAT ATCCAGGATGG-**3'**	**5'-** AGGCAGGTCACA CCGAATGACCA-**3'**
	nsP2	conserved	**5'-**ACGGAAGGTA AACTGGTATGG-**3'**	**5'-**CTTTGAAGTT TCCTTTCGGTGG-**3'**	**5'-** TCTGCAGCGTC TTTATCCACGGG-**3'**
	Capsid/ E2 (CE2)	DVG	**5'-**CATACTTAGCT CACTGTCCCG-**3'**	**5'-**GCTGTCATCC GTCTTTATTCCG-**3'**	**5'-** CGTCCCGTCTGT CGCTTCATTTCT-**3'**
ZIKV	5'	conserved	**5'-**GTTGTTGATCT GTGTGAA-**3'**	**5'-**TGACCAGAAA CTCTCGTTTC-**3'**	**5'**GCGACAGTTCG AGTTTGAAGCGA AAGC-**3'**
	3'	conserved	**5'-**AAGAGGGACT AGTGGTTAGAGG-**3'**	**5'-**CTCATGGAGT CTCTGGTCTTTC-**3'**	**5'-** CCCCGGAAAAC GCAAAACAGCAT-**3'**
	Envelope (E)	DVG	**5'-**TCCACGACATT CCATTACCTTG-**3'**	**5'-**ATGTGCGTCC TTGAACTCTAC-**3'**	**5'-** TTCCGGTGTCTG CCCCAGC-**3'**

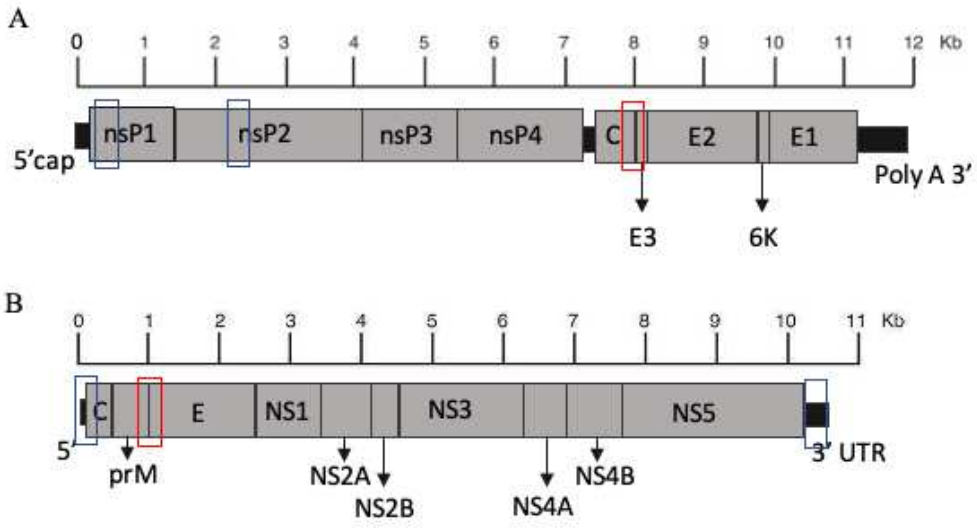


Figure 5. 4 q-RT-PCR primers and probes designed to target predicted DVG deletion and conserved regions of the A) CHIKV genome and B) ZIKV genome. Blue boxes = conserved region, red boxes = DVG deletion region (observed from CHIKV) in Fig. 2 and predicted from ZIKV

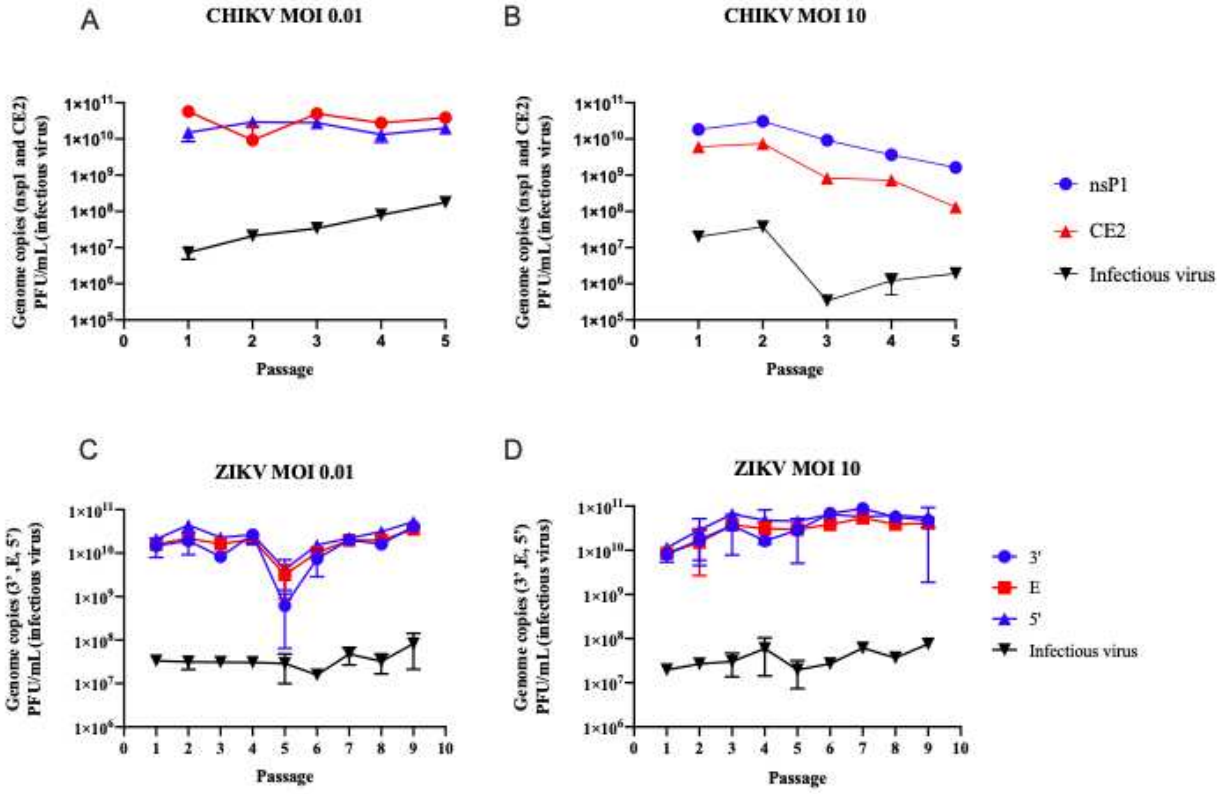


Figure 5. 5 Analysis of CHIKV and ZIKV from supernatants collected after serial-passages *in vitro* from two independent replicates. CHIKV was passaged 5 times at a MOI of (A) 0.01 or (B) 10, and CHIKV genome copies (gc) were determined using q-RT-PCR targeting a region of the nsP1 gene that is conserved during passaging or targeted the CE2 gene region that is deleted in most DVGs developed during passaging. ZIKV was passaged 9 times at a MOI of (A) 0.01 or (B) 10, and ZIKV gc were determined using q-RT-PCR targeting regions of the 5' and 3' genome that are conserved during passaging or targeted the E region that is predicted to be deleted in most DVGs developed during passaging. Infectious virus (black line) in all panels is shown for comparison. Multiple un-paired t-tests compared gc at each passage. At an MOI of 0.01, CHIKV gc between nsp1 and CE2 were only significantly different at passage 1 ($P \geq 0.05$), and at an MOI 10, CHIKV gc between nsp1 and CE2 were significantly different at every passage ($P \geq 0.03$). At an MOI 0.01, ZIKV gc between 3' and E were only significantly different at passage 3 ($P \geq 0.03$), and only significantly different at passage 2 and 9 ($P \geq 0.01$) between 5' and E. At an MOI 10, ZIKV gc were only significantly different at passage 6 and 7 ($P \geq 0.01$) between 3' and E, and not significantly different at any passage between 5' and E.

5.2.2 Detection and characterization of naturally occurring CHIKV and ZIKV DVGs in infected mosquitoes

Virus-infected mosquitoes were examined using many of the same methods developed with samples of virus passaged in cell culture above, with a focus on CHIKV-infected mosquitoes due the higher potential of DVG production with this virus. We first analyzed CHIKV-infected mosquito bodies and saliva from replicate pools of 15 individuals using plaque assays and qRT-PCR using primers targeting the previously identified conserved genomic region (Table 5.1; nsP2). As has been shown in previous chapters and in other virus-vector systems [252,276], the abundance of viral RNA detected in both bodies and saliva of these samples is significantly greater than the abundance of infectious virus detected by plaque assays on the same samples ($P = 0.006$). However, the ratio of viral RNA to infectious virus is much larger in mosquito saliva, suggesting a preferential secretion of packaged DVGs into this biofluid (Figure 5.6). Sequence analysis was performed on two of the mosquito groups. CHIKV reads were successfully detected in both the bodies and saliva samples from group 2 and revealed five distinct DVG variants, one in the bodies but four in the saliva (Figure 5.7). The four variants detected in group 1 bodies were all similar as they were missing most of the genome other than the 5' and 3' termini. Although few, the relative abundance of these DVG variants in both the bodies and saliva of mosquitoes was relatively high (Figure 5.8) as it compared most favorably to the relative abundance of most DVG variants observed in cell culture supernatant when CHIKV was passaged 5 times at the highest MOI (Figure 5.3). DVG variant detection was less successful in tissues of individual mosquitoes due to the low number of CHIKV reads detected in any sample (Figure 5.9). Overall, the depth of sequencing reads across most nucleotide positions in the virus genome in these samples was approximately between 5,000-10,000X, but this noticeably dropped off at the 3' terminus. The sequencing depth of reads that detected DVG breakpoints

was usually between 1-100X. DVG reads were successfully detected from input samples and were relatively homogenous in their distribution across the virus genome but most DVGs in the input were of low relative abundance (Supplemental figure 1). In contrast, DVG reads in the mosquito midgut samples were more often skewed to the 5' half of the CHIKV genome, there were many more assembled DVGs detected, and their relative abundances spanned the low and high range (Figure 5.9). CHIKV DVG reads detected in mosquito legs (representing disseminated virus) more resembled that of the input virus, being spread across the genome and most of the assembled DVGs being of low abundance. Only one full set of CHIKV DVGs were detected across all tissue compartments of one mosquito (Figure 5.9A), but the pattern of DVG reads reflected the others and it was notable that one assembled DVG variant occurred at a relatively high abundance (Figure 5.10). Individual ZIKV-infected mosquitoes were also dissected, and their tissues sequenced for comparison. However, the depth of ZIKV sequencing reads across the ZIKV genome was more variable overall in these samples, and very few reads of DVG breakpoints were detected (Figure 5.11). Similarly, the few DVGs that could be assembled were of low abundance (Figure 5.12).

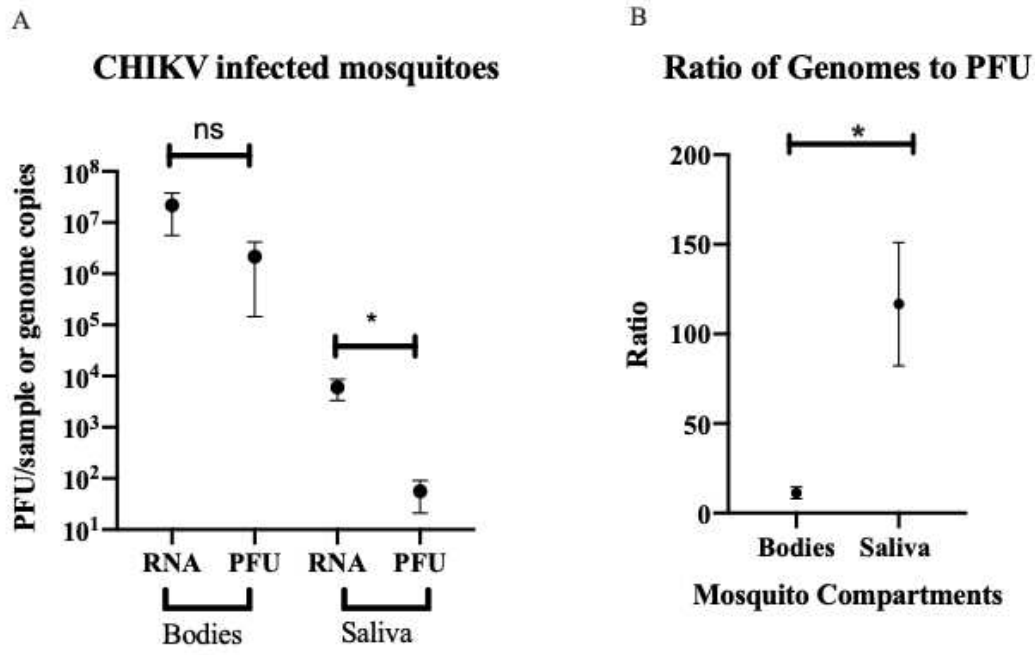


Figure 5. 6 Molecular analysis of CHIKV in three groups of infected mosquitoes (n=15/group). RNA was measured in genome copies by q-RT-PCR targeting conserved regions (nsP2). PFU determined by plaque assay. A) comparison of RNA and PFU in bodies and saliva samples. B) ratio of genome to PFU in the paired samples. Mean and standard deviations shown. Significant difference of the groups was as determined by paired T-tests; * = $p \leq 0.05$

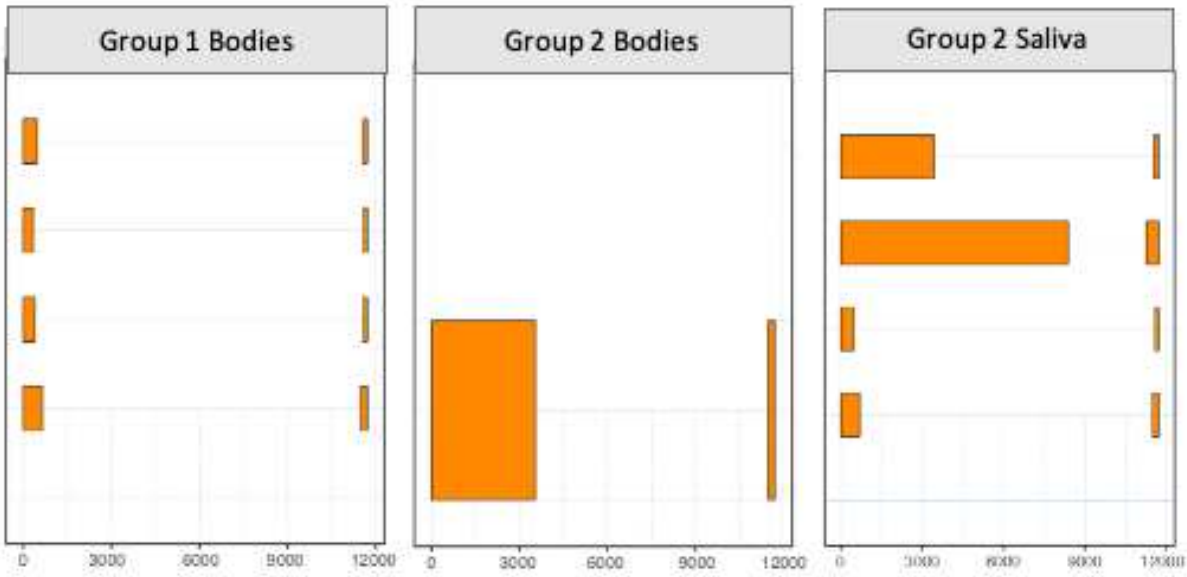


Figure 5. 7 DVG variants in the bodies and saliva of CHIKV-infected mosquitoes from two groups (n=15/group). X-axes denote nucleotide positions across the genome. Each orange line represents a DVG variant with the white space representing deletions. Group 1 saliva not shown because no DVGs were detected.

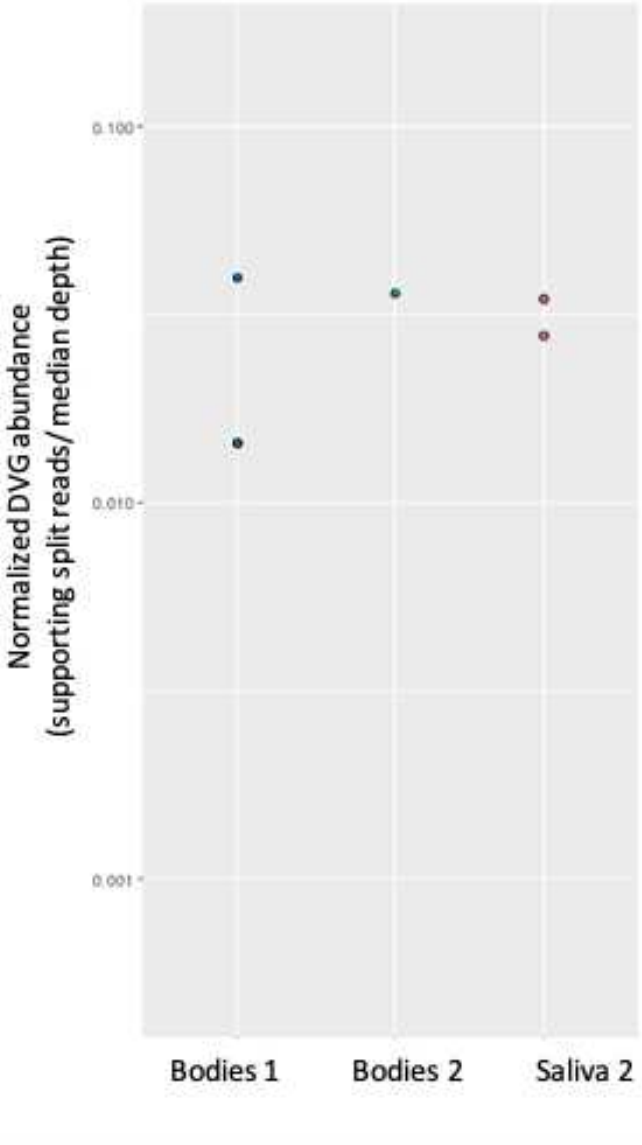
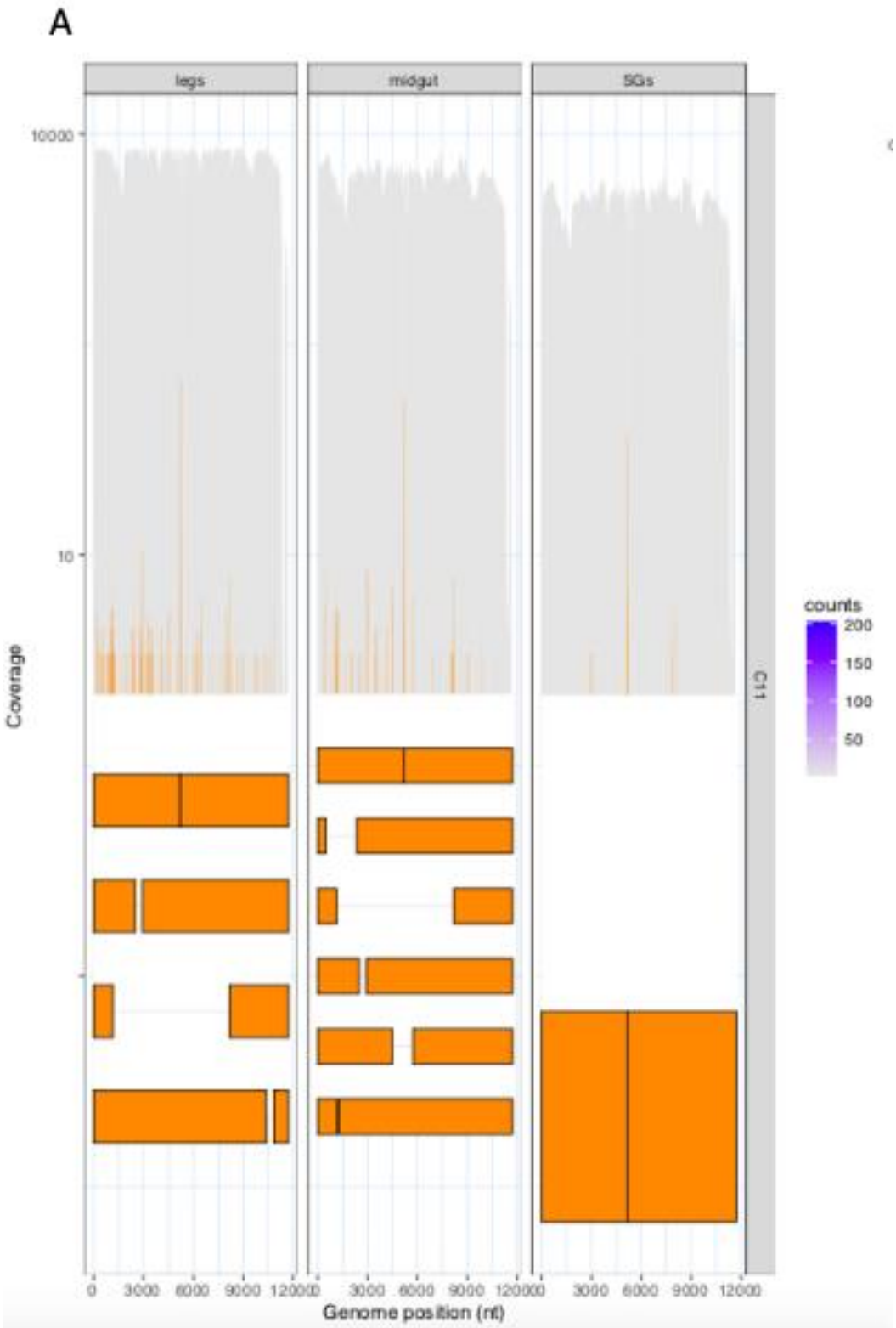
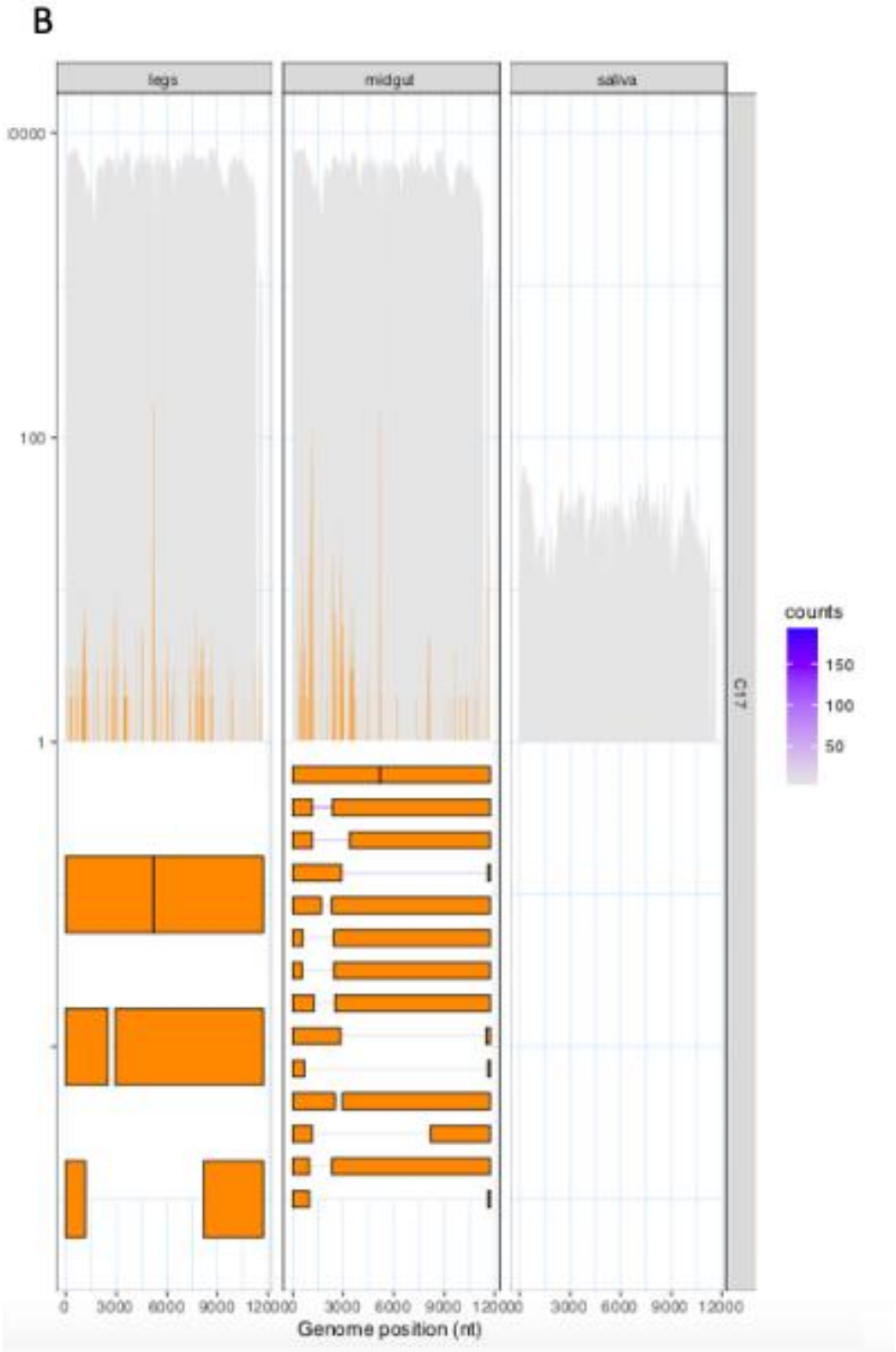
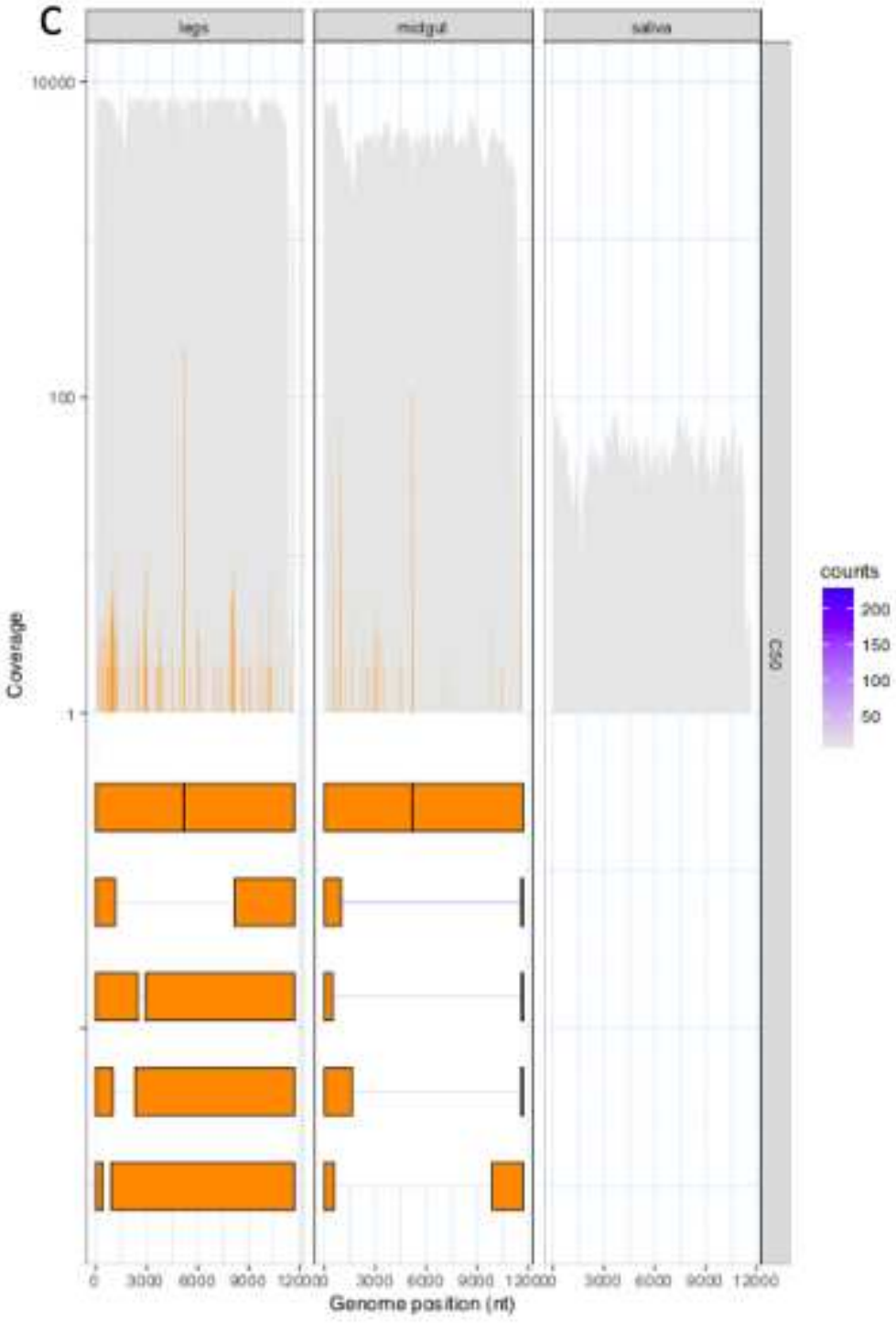


Figure 5. 8 Normalized abundance of CHIKV DVG variants relative to all reads from mosquito sequencing. Y-axis displays proportions. Each dot= DVG variants with 5x coverage (5 read count cutoff)


A
legs
midgut
SGs
10000
10
Coverage
counts
200
150
100
50
C11
0
3000
6000
9000
12000
Genome position (nt)


B
legs
midgut
saliva
10000
100
1
counts
150
100
50
Ctr7
0
3000
6000
9000
12000
Genome position (nt)


C
legs
midgut
saliva
10000
100
1
Coverage
CDS
counts
200
150
100
50
0
3000
6000
9000
12000
Genome position (nt)

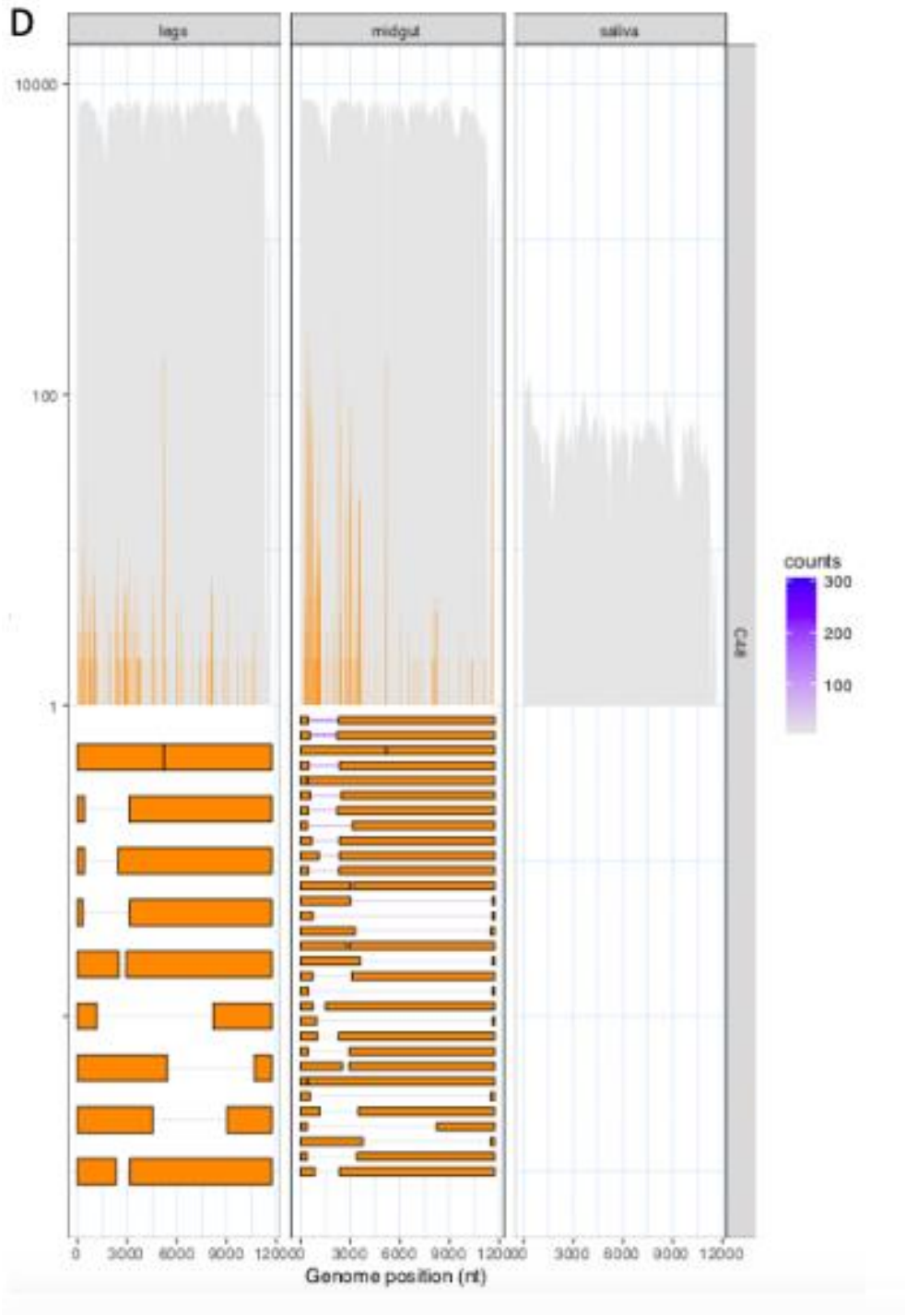


Figure 5. 9 Deep sequencing analysis of DVG variants in individual CHIKV infected mosquitoes (A-D). Y-axis is nt across the genome; 0-12,000nt. Each vertical gray line in the top half of each panel represents depth of cover over the genome. Orange vertical lines represent deletions. Each horizontal orange bar in the bottom half of each panel represents a DVG variant with the white space representing deletions these The counts of each variant are represented by range of blue added to the deletion space as observed in the legend. DVG variants have 5x coverage (5 read count cutoff).

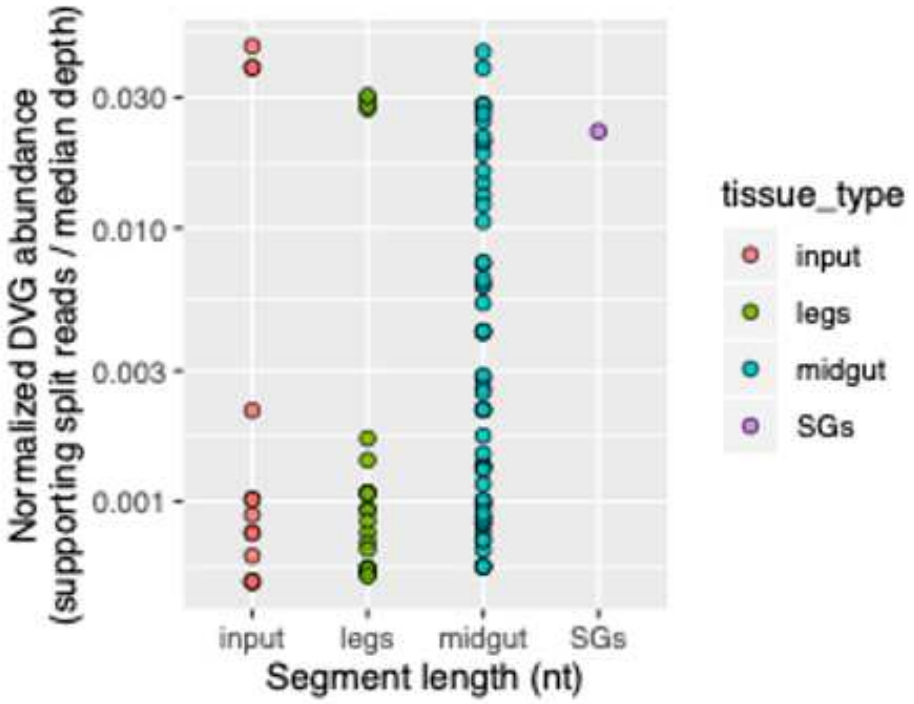
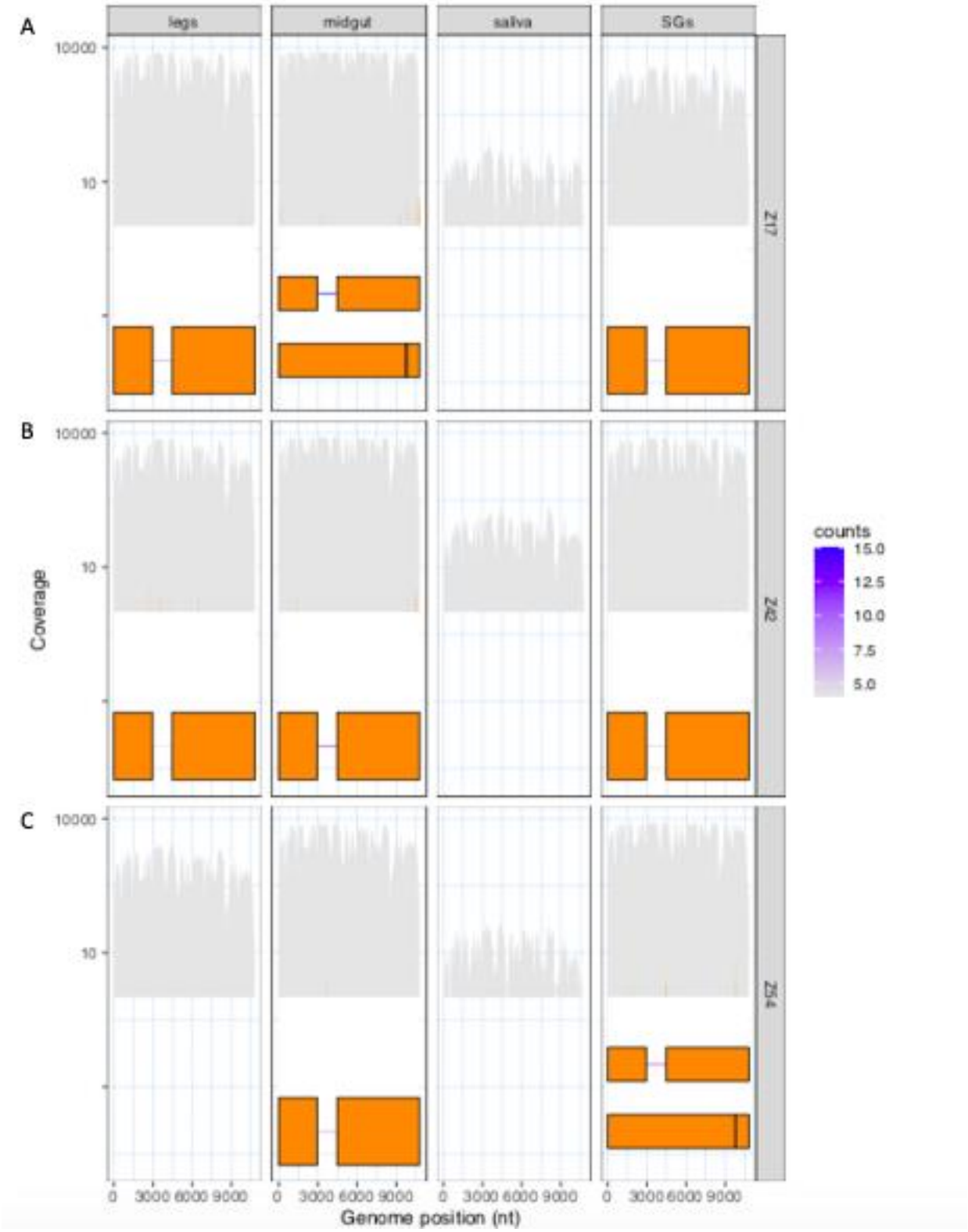


Figure 5. 10 Normalized abundance of CHIKV DVG variants in tissues from all samples relative to all reads. Y-axis displays proportions. Each dot = one DVG variant. Each dot= DVG variants with 5x coverage (5 read count cutoff).


A
B
C
legs
midgut
saliva
SGs
Z17
Z42
Z54
10000
10
Coverage
counts
15.0
12.5
10.0
7.5
5.0
0
3000
6000
9000
Genome position (nt)

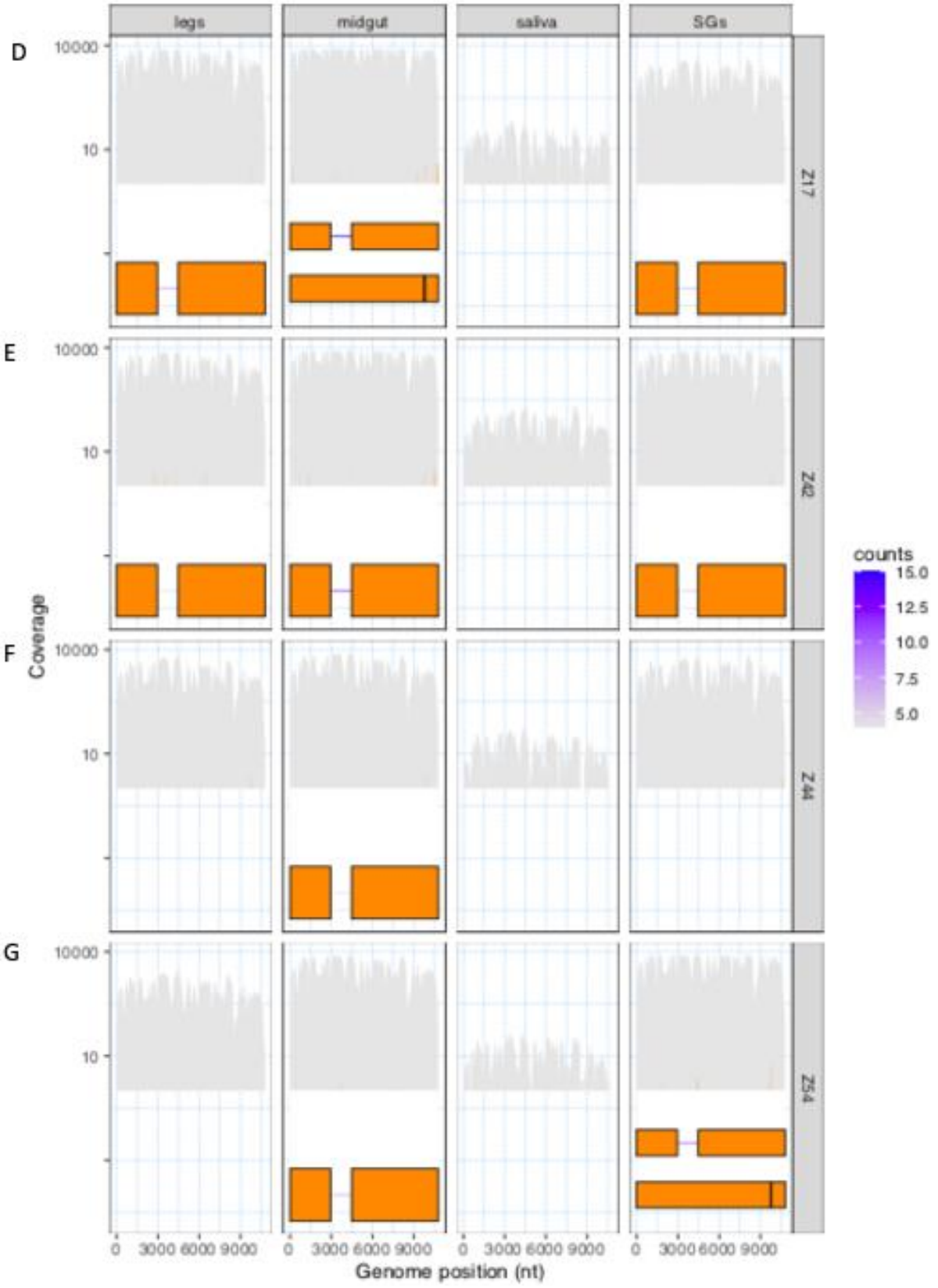

legs
midgut
saliva
SGs
D
E
F
G
Z17
Z42
Z44
Z54
10000
10
Coverage
counts
15.0
12.5
10.0
7.5
5.0
0
3000
6000
9000
Genome position (nt)

Figure 5. 11 Deep sequencing analysis of DVG variants in individual ZIKV infected mosquitoes (A-G). Y-axis is nt across the genome; 0-12,000nt. Each vertical gray line in the top half of each panel represents depth of cover over the genome. Orange vertical lines represent deletions. Each horizontal orange bar in the bottom half of each panel represents a DVG variant with the white space representing deletions. The counts of each variant are represented by range of blue added to the deletion space as observed in the legend. DVG variants have 5x coverage (5 read count cutoff).

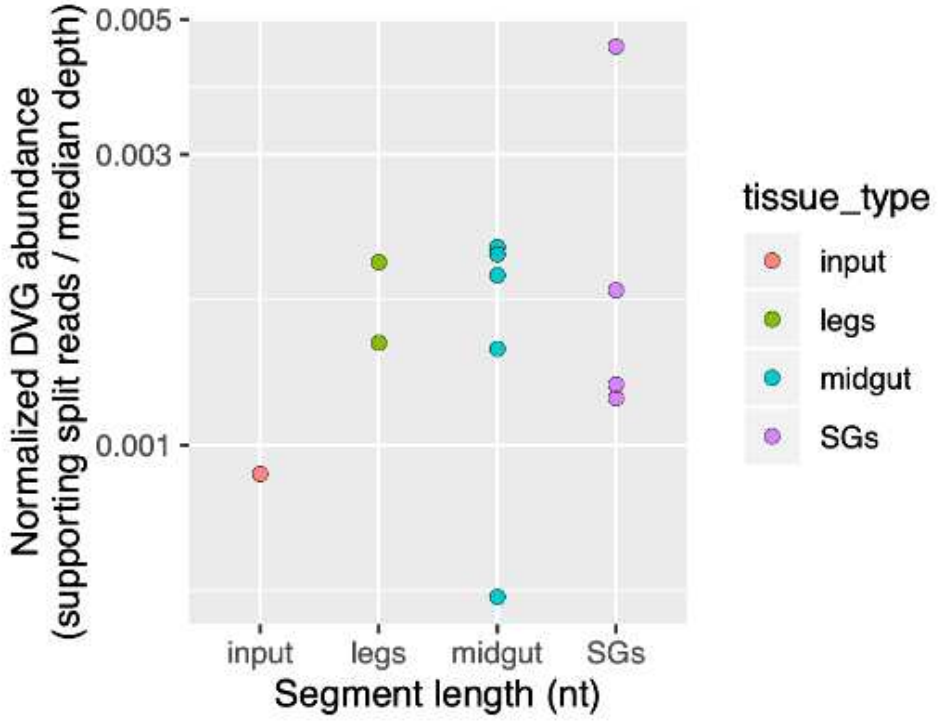


Figure 5. 12 Normalized abundance o ZIKVDVG variants in tissues from all samples relative to all reads. Y-axis displays proportions. Each dot+ DVG variants wit 5x coverage (5 read count cutoff).

5.2.3 Supplementary detection of DVGs in CHIKV- and ZIKV-infected mosquitoes compared to input and further investigation of ZIKV DVGs in other biofluids

To address the deficiencies in the preliminary sequencing experiments presented above, a larger and more deliberate experiment was designed (Figure 5.13). Three groups of 20 mosquitoes each, either infected with CHIKV or ZIKV, were held for 14 days post infection and samples of

the following tissues/biofluids were pooled for sequencing analysis: midgut, legs, salivary glands and saliva. These samples along with input virus will be analyzed by high throughput sequencing and molecular assays as described above. In addition to these samples, ZIKV-infected male mouse tissues (including brain, liver, epididymis, seminal vesicles and testes), along with ZIKV-positive semen collected from some of these mice, will be similarly evaluated. We hypothesize that the experiment will reveal that DVG diversity will be stochastic as virus disseminates through mosquito and mouse tissues, undergoing contraction and expansion, and that that DVG variants ultimately found in the transmissible biofluids (mosquito saliva and mouse semen) will be few but very abundant. The analyses to be completed on these samples will provide insight on how selective pressure and bottlenecks drive DVG variants and may impact transmission and disease.

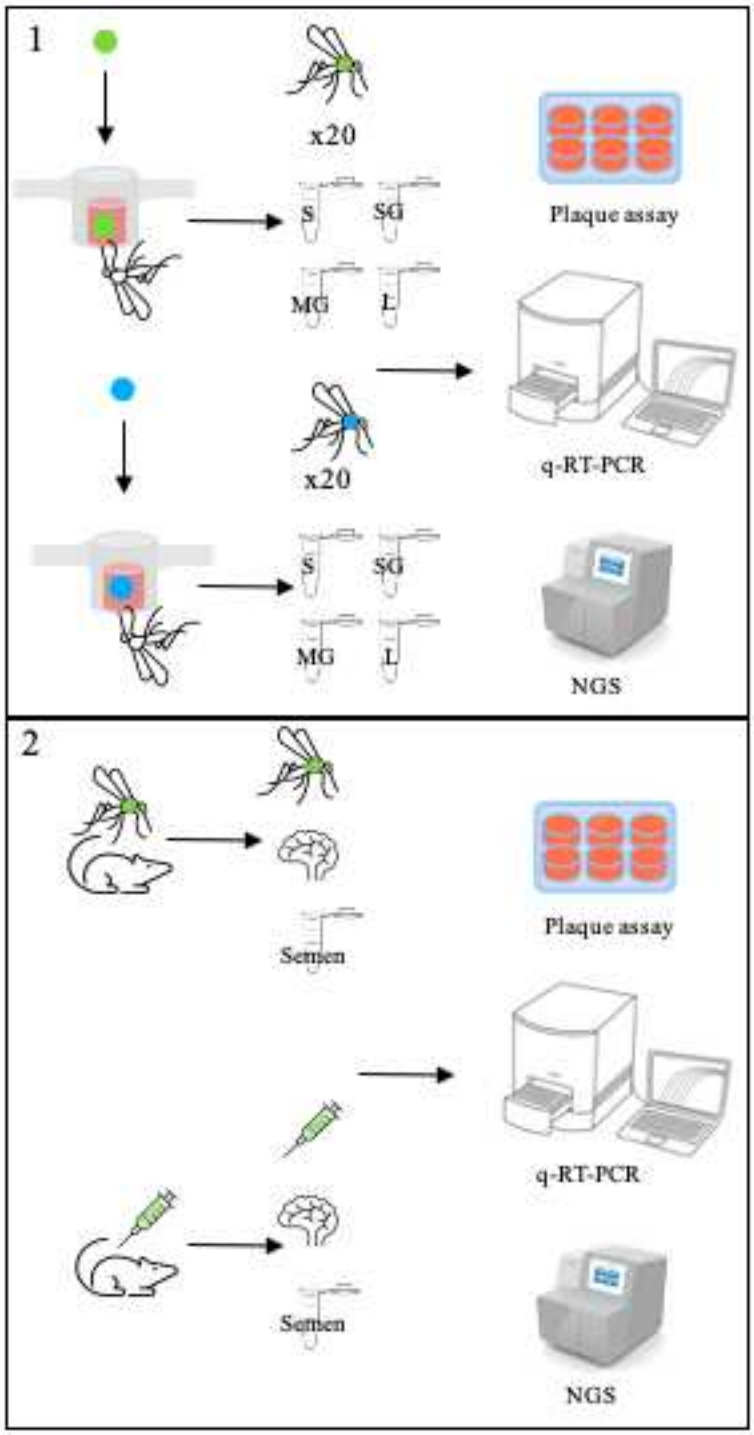


Figure 5. 13 Graphical image of experimental layout. S = saliva, SG = Salivary glands, MG = midgut and L = legs. Panel 1, supplement detection of DVGs in CHIKV (blue) and ZIKV (green)- infected mosquitoes compared to input. Panel 2, supplement detection of ZIKV DVGs in other biofluids.

5.3 Discussion

The impact of DVGs/DIPs on viral transmission and disease outcome is relatively unexplored. Much of what is known comes from *in vitro* studies that initiate the generation of DVGs at a high MOI [260,263,266,277]. Some studies have looked into the natural occurrences of DVGs/DIPs during clinical infection and have found DVGs in tissues and serum of both humans and animals; however, the presence of DVGs has not been linked to outcome or disease severity [137,138,262,267,271,274,275]. Functionally, DVGs have been shown to reduce infectious virus in *in vitro* and *in vivo* studies with mice [139,268–270,275]. Additionally, in nonvertebrate hosts, DVGs have been documented to aid in viral persistence and limit dissemination [73,264,277,278]. Characterizing DVGs in infectious biofluids (mosquito saliva and semen involved in sexually transmission of ZIKV) would help to illustrate a more comprehensive portrait of the function and impact DVGs have on viral disease, transmission, and spread.

We examined and characterized DVGs during *in vitro* infections with CHIKV and ZIKV. In these studies, it was found that CHIKV, an alphavirus, generates a large quantity of DVGs after only three passages at a high MOI. Others studies have shown similar results [279]. However, this was not observed with ZIKV, which after nine passages at a high MOI showed no change in viral titer. This in part may be due to the fidelity of the RdRp as alphaviruses have a lower fidelity RdRp in comparison to flaviviruses[136]. Infection with Sindbis virus demonstrated that its low-fidelity RdRP enhanced production of DVGs[136], as it led to more break points across the genome resulting in recombination events and deletions. Sequencing analysis of CHIKV cell culture samples showed a high abundance of deletions over the subgenomic RNA in passage five at an MOI of 10. Two studies, one by Levi *et al* and the other by Langsjoen *et al*, support these results, showing that *in vivo* passaging results in deletion across the second half of the genome

[73,279]. The former study also looked at DVG formation in other cell lines and found variation in the location of the deletions, demonstrating that cellular environments are factors in the generation and maintenance of DVGs [73].

Previous studies, along with our own observation have found a large disparity between titers of infectious virus and virus genome copies detected in the same mosquito saliva samples from infected mosquitoes[233,252]. Results from this study support this as it was found that the ratio of RNA to infectious virus was 10:1 in bodies compared to 100:1 in saliva. Although the depth of coverage in the sequence analysis was limited, we detected several DVG variants in both saliva and bodies. Interestingly, the abundance of variants in saliva were greater than that in bodies. This trend was similarly seen in one CHIKV infected individual mosquito sample as variation of DVGs decreased though dissemination in the mosquito while abundance of one DVG in the saliva was high. This data is in direct comparison with sequencing analysis of individual ZIKV infected mosquitoes. There were little to no DVGs detected in ZIKV samples. Notably, a study that looked into ZIKV DVGs in organs from infected mice found that DVGs are influenced by organ microenvironments [56]. These results, together with our findings, indicate that vector and mammalian cells/tissues may differ in their DVG production.

To the best of our knowledge, no study has looked at natural production of arbovirus DVGs in the vector, although inoculation with artificial DVGs resulted in limited infection and dissemination in invertebrates [73,264,278]. Experiments with animal models have used inoculums with high initial level of DVGs, but as seen in models of interhost transmission, mosquito bottlenecks restrict the number of virions being transmitted between hosts [122]. It seems unlikely that there would be co-infection of a host cell by a DVG and standard virion unless the infectious biofluids contained a high DVG-to-standard-virus-particle ratio [260]. Secondary planned

experiments highlighted in figure 13 will address this hypothesis. Samples including saliva, salivary glands, midguts, and legs from groups of twenty mosquitoes will provide sufficient amount of viral RNA to be analyzed by NGS. We predict to see a high abundance of DVGs in saliva. Moreover, tissues from ZIKV infected male mice along with corresponding infectious semen samples will be analyzed. The results from these experiments will begin to address how DVGs are produced through infection and transmission from both the mammalian and vector hosts.

5.4 Materials and methods

5.4.1 Virus and Cells

African Green Monkey kidney cells (Vero; ATCC #CCL-81) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, and incubated at 37°C in 5% CO_2. ZIKV strain PRVABC59 (ZIKV-PR; GenBank:KU501215), originally isolated from a human traveler to Puerto Rico in 2015 with three rounds of amplification on Vero cells, was obtained from Dr. Aaron Brault (CDC, Ft. Collins, CO). CHIIKV strain LR2006_OPY1 (GenBank: KT449801.1) was obtained from the University Texas Medical Branch and isolated from outbreak in Reunion Island in 2006 with three rounds of implications of Vero cells.

5.4.2 Cell culture serial passaging

CHIKV (passaged five times) and ZIKV (passaged nine times) were passaged on VEROs. Cell were infected at a MOI of 0.01 or 10 in duplicated. CHIKV supernatant was collect 48 h.p.i and was spun at x10,000g for 5 minutes. ZIKV supernatant was collected 72 h.p.i. and was spun at

x10,000g for 5 minutes. Each passage was titer by plaque assay before next passage. Samples were stored at -80°C. Samples were titrated by Vero cell plaque assay, with a tragacanth gum overlay and staining at day 5 post-cell culture inoculation for ZIKV and 2 days post cell culture inoculation for CHIKV.

5.4.3 Mosquito infections

Ae. aegypti strain Poza Rica mosquitoes were fed an infectious blood containing either CHIKV or ZIKV and held for 10-14 days to ensure dissemination of virus to the salivary glands. Infectious blood meals were prepared with 1mL fresh virus contained in the cell-culture supernatant of infected Vero cells and 1 mL of defibrinated calf blood. Back-titering of the bloodmeals ranged between $1x10^6$ - $5x10^6$ PFU/ml. Mosquitoes were sorted post blood feeding and were place in cartons with an organdy cover and given water and a sugar source.

5.4.4 Mice infection by mosquito bite

To infect mice by mosquito bite, *Ae. aegypti* strain Poza Rica mosquitoes were fed an infectious blood meal and held for 14-17 days to ensure dissemination of virus to the salivary glands. Infectious blood meals were prepared with 1 mL fresh virus contained in the cell-culture supernatant of infected Vero E6 cells and 1 mL of defibrinated calf blood. Back titration of the bloodmeals ranged between $1x10^6$ - $5x10^6$ PFU/mL. Mosquitoes were sorted post blood feeding and 10-20 blood fed mosquitoes were place in cartons with an organdy cover and given water and sugar source. To allow the mosquitoes to feed on the mice, each mouse was anesthetized using 100mg/kg ketamine+ 10mg/kg xylazine and placed on the organdy cover of one carton for ~20 minutes. After 20 minutes, blood fed mosquitoes were immediately knocked down, their

saliva was collected by the forced salivation method described previously[43], and their bodies homogenized in media for later testing. ZIKV infections of mosquito bodies and saliva were determined by plaque assay and q-RT-PCR. Samples were titrated by Vero cell plaque assay, with a tragacanth gum overlay and staining at day 5 post-cell culture inoculation.

5.4.5 ZIKV inoculation into mice

Mice were anesthetized in a holding chamber with 1% to 3% isoflurane to effect with an oxygen flow rate of 1.5 L/min. Once the animal was anesthetized, it was removed from the chamber and $1x10^3$ PFU of virus in a volume of 100 µL was administered subcutaneously (s.c.) in the region of the back between the scapulae with a sterile hypodermic 34-gauge needle in a biosafety cabinet

5.4.5 Mosquito sample collections

Mosquitoes were immediately cold-anesthetized post-blood feeding and their saliva was collected by the forced salivation method described previously[280], briefly their legs and wings were removed and their proboscis was placed into a capillary tube containing immersion oil. After 20-30 minutes mosquitoes were pull off the capillary tube and the capillary tube contents centrifuged into media held at 4°C. The bodies were place in a separate tube held at 4°C to be homogenized in media for later testing. Infections of mosquito bodies and saliva were determined by plaque assay and q-RT-PCR. For secondary experiments, legs were kept and pooled in 500 µL of DMEM, after forced salivation of individual mosquito, saliva samples were pooled into 500 µL of DMEM. Salivary glands and midgut were then dissected out and pooled into 500 µL of DMEM. Salivary glands, midguts and legs were processed as describe below.

5.4.6 Semen collection

Semen collection were adopted from Duggal et al[16], and we were trained on these techniques by a member from the same laboratory (E.M. McDonald). Infected male mice were mated to three to four female mice starting on the evening of day 7 post infection. In preparation, bedding from male cages was placed in female cages 3 day before mating to simulate the estrous cycle. Each evening, females were placed into male cages and each subsequent day, females were removed from male cages and checked for a copulatory plug. Females with a plug were euthanized and semen was collected from the vagina and uterine horns. This was done immediately after euthanasia by first dissecting the reproductive track (uterus and uterine horns), subsequently, the tips of the uterine horns were cut and then washed with 150µL of sterile PBS while placed over collection tube.

5.4.7 Euthanasia, blood collection and necropsy

Mice were deeply anesthetized with isoflurane followed by cervical dislocation. Cardiac blood was collected with a 34-gauge sterile needle inserted into the apex of the heart. Pieces of each tissue were removed and placed in a pre-weighed tube with 500 µL of DMEM media and kept at -80°C for RNA extraction. The rest of the tissues were placed in 10% neutral buffered formalin for histology at a 1:10 weight to volume ratio.

5.4.8 RNA extractions and q-RT-PCR

Tubes containing mosquito bodies where homogenized and both saliva and bodies where centrifuged for 5 minutes at 14000 x g. RNA was extracted from all samples using the Mag-Bind

Viral DNA/RNA 96 kit (Omega Bio-Tek) on the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific). RNA was eluted in 50 µL nuclease-free water. Progmeg GoTaq Probe 1-Step RT-qPCR System Time was used on RNA extracted from saliva and bodies to quantify CHIKV and ZIKV RNA according to manufacturers' instructions. Primers used for CHIKV and ZIKV are in table 1 Approximately 100 ng of RNA was added to each reaction. Standards were generated for each virus RNA extracted from viral stocks. RNA was quantified on a Qubit Fluorometer (ThermoFisher Scientific) and diluted to achieve serial 10-fold genome equivalent (GE) dilutions. The standard curve detection of 10^4-10^7 GE/reaction.

5.4.9 Short-read Illumina Sequencing and analysis

The KAPA RNA HyperPrep was used following manufacturer's protocol for generating library with ½ volume reactions. Resulting libraries were quantified using a Qubit 3.0 fluorometer (Invitrogen) and their size determined using a 2200 TapeStation (Agilent). Libraries were pooled in equimolar concentrations and sequenced on Illumina NextSeq (NextSeq 500/550 Mid Output v2 kit, 300 cycles, pair end). Data underwent quality filtering[281] by importing FastQ flies, adapter trimming[282], and collapse of non-unique read pairs using CD-HIT[283]. Remaining, quality reads were be aligned to the ZIKV Puerto Rican (ZIKV-PR; GenBank:KU501215),and CHIKV LR2006_OPY1 (GenBank: KT449801.1) genome using Bowtie2[284]. Aligned reads were analyzed with DI-tector to identify deletions, insertions, copy-back and snap-back genomes[285]. Briefly DI-tector assigns each read with a first and last segment and mapping them so that the first and last segment flank the ends of DI genome junctions. Parameters here were left at default settings of a minimum segment length of 15 ("--Min_Segment"). Additionally, the "-n" or –Nb_Reads" parameters was set to 5 which only shows DI genomes with count reads superior to

5. Next both read segments are compared and clustering by the nature of their junctions: copyback or deletion/insertion. These DI genomes are then counted, sorted by type and exported. Generation of figures was done in with R script in R Studio (RStudio Team, version 3.6.3) *http://www.rstudio.com/*) stringr (‘1.4.9’) dplyr (‘1.0.2’) ggplot2 (‘3.3.2’), gridExtra (‘2.3’) and cowplot (‘1.1.0’) packages are required.

5.4.10 Statistical Analyses

Results in figures were expressed as mean values (horizontal bars) and standard deviation. The statistical details are noted in the figures and/or in the corresponding figure legends. Statistical significance was primarily determined using unpaired or paired t-test in the GraphPad Prism (GraphPad Software, La Jolla California USA).

CHAPTER 6: SUMMARY AND FUTURE CONSIDERATIONS

Mosquito and animal model factors were evaluated regarding their effects on *Aedes*-borne arbovirus disease outcome and transmission. The first goal was to establish an immunocompetent small animal model to study ZIKV transmission. Although no new animal model was discovered, new information was gained from this study. It was found that the multimammate mouse and NZW rabbits are not susceptible to ZIKV infection as determined by a lack of viral RNA in tissues collected. Additionally, there was no detectable antibodies in rabbits at 28-day post infections. This leads to the conclusion that these species do not support viral replication. Most likely, ZIKV is unable to enter into cells from a lack of receptor binding[286], or able to enter cells but is quickly eliminated by innate and adaptive immune mechanisms[287]. Additionally, there may be inherent cellular factors that do not allow or actively limit virus replication[288]. To further investigate these hypotheses, immune signaling cascades such as those driven by as IFN- β, RIG-I, and CXCL10 could be targeted and evaluated for changes in regulation[289]. Interestingly, sexually mature male Hartley guinea pigs were inoculated subcutaneously, and by mosquito bite, but found to be not susceptible to ZIKV infection by these routes. As both young and pregnant Guinea pigs have successfully been used in ZIKV studies[169–173], this suggests that only guinea pigs in underdeveloped immune or immunosuppressed states to be susceptible to ZIKV. The Jamaican fruit bat has been previously shown to be susceptible to ZIKV[181] infection. However, infrequent and low detection of ZIKV RNA in tissues make this model inefficient. There is still a need for establishing a small immunocompetent animal of ZIKV, especially to understand infection and disease when infected by differing transmission routes.

There is an unexplained female bias occurring within ZIKV disease whereby the attack rate and incidence are significantly higher in females compared to males [51,215–217]. To this end, many questions around ZIKV disease remain including: (1) whether infection by different transmission routes affects disease presentation or severity and (2) whether transmission leads to different ZIKV disease symptoms, prevalence, and/or incidence in females compared to males. In an attempt to answer some of these questions, we used an immunodeficient mouse model (A129 mice). Females and males were infected by subcutaneous inoculation with a needle and by infected mosquito bite. Early in infection, the proportion of infected brains was higher in males than females when infected by mosquito bite. This result highlights how biological sex and infection route can influence ZIKV infection. What remains unknown is the cause of this phenomenon. Differences in disease outcome between biological sex has been observed with other viral infections[290]. One area to further explore is what differences there are between female and male immune response leading to these differences. Interestingly, there is an over-expression of the X-linked immune genes in female mammals relative to males, including in humans and mice that is due to incomplete X chromosome inactivation. This is thought to contribute to an increased female resistance to microbial diseases relative to males[234–236]. Here, we could explore the expression of three X-linked immune genes: (1) Toll-like receptor 7, (2) CD40 ligand, and (3) C-X-C chemokine receptor 3[291,292]. These genes are known to escape X-chromosome inactivation and have also been previously implicated in flavivirus pathogenesis[293,294]. Another question to further explore is whether mosquito saliva perpetuates these biological sex differences. Because vector saliva is immunomodulatory[295], it may be that differential interactions with male and female immune molecules leads to differential disease outcomes

between the sexes. This could be investigated by studying mosquito salivary and immune factor interactions in females compared to males.

This set of experiments also demonstrates the limitations of mosquito infection studies. It was a challenge to match the inoculation titer of a mosquito bite to subcutaneous inoculation. The dose of ZIKV delivered by mosquito bite was estimated by forced salivation technique after blood feeding. Forced salivation techniques were also evaluated to accurately quantify virus titers transmitted from *Ae. aegypti* mosquitoes infected with ZIKV and CHIKV. Here, we demonstrated that forced salivation immediately after blood feeding reflected the same titers achieved from forced salivation without blood feeding, and that this method could be used to estimate the titer of virus inoculated during a bloodmeal. Moreover, we found that there is ~50-100 times higher virus titers in the dissected bloodmeals compared to the titer in saliva. This demonstrates how mosquitoes reingest much of their saliva during artificial blood feeding and highlights a large increase in virus transmission during *Ae. aegypti* blood feeding. Both forced salivation and the dissected bloodmeals of artificially blood-fed mosquitoes revealed that the quantity of viral RNA expectorated by mosquitoes was 2-5 logs more than the quantity of infectious virus. What remains to be explored is the quantity of virus secreted during probing prior to uptake of blood as probing has been documented to release virus[127]. Additionally, results from this study should be replicated with other arbovirus-vector combinations to determine whether they are consistent across mosquito species.

Our experiments along with other similar studies[252,276] have shown mosquito saliva to contain ~100-10,000 times more viral RNA than infectious virus. A hypothesis to address the difference between viral RNA and infectious virus is the preferential secretion of packaged defective viral genome (DVG) into mosquito saliva. We examined the presence of ZIKV and

CHIKV DVGs in mosquito saliva. Molecular and sequencing analyses detected several DVG variants in individual mosquito samples infected with ZIKV or CHIKV. Notably, variations of CHIKV DVGs decreased as the virus disseminated to the saliva in the mosquito, while abundance in the saliva increased relative to the other mosquito tissues. There are many more questions that remain regarding the role of DVGs in arbovirus transmission. Our next steps are to better characterize the variations of DVGs through dissemination within mosquitoes and to explore the expression of ZIKV DVGs in other biofluids. This study will provide insight on how selective pressure and bottlenecks drive DVG variants and may impact transmission and disease. The latter point is an important factor to be investigated. It has been shown that DGVs protect from disease when inoculated alongside infectious virus[139,268,269]**.** Using animal models, the effects of DVG inoculum on infection could be further explored**.** If this hypothesis is proven correct and there is a large quantity of arbovirus DVGs in biofluids (i.e., saliva and semen), we can explore how this may lead to differences in disease between the biological sexes.

Different animal species were evaluated as models for ZIKV infection with the goal to characterize how biological sex and transmission route affect disease outcome. We moved forward with an immunocompromised mouse model and demonstrated how biological sex can influence ZIKV infection dynamics. This may be due to sex-specific interactions with mosquito-derived virus or saliva. To accurately estimate the quantity of virus transmitted during mosquito bite, we evaluated forced salivation techniques, developed new methods that can be used as a better way to estimate the titer of arboviruses transmitted by *Ae. aegypti.* Finally, we explored the role of DVGs to account for disparity of the viral RNA loads and infectious virus in saliva. This work explores mosquito and animal model factors and their effects on *Aedes*-borne

arbovirus transmission and disease. This work has led to novel information of how biological sex and mosquito saliva can play an important role in viral transmission.

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APPENDIX A: SUPPLEMENTAL MATERIAL

Chapter 3 Supplement Material

Table S 1 Estimated ZIKV inoculum delivered from mosquitoes that blood fed on mice.

MOUSE_ID#	Sex	Infection route and day (D) mouse tested	# mosquitoes blood fed	Estimated inoculum titer (PFU)	Estimated inoculum titer/ mosquito (PFU)	Estimated inoculum genome copies
MM_MOUSE_165	M	mosquito bite D2	3	0	0	$1.3X10^3$
MM_MOUSE_166	M	mosquito bite D2	1	0	0	$5.0X10^3$
MM_MOUSE_168	M	mosquito bite D2	1	$1.2X10^2$	$1.2X10^2$	$6.6X10^4$
MM_MOUSE_169	M	mosquito bite D2	2	0	0	$6.8X10^4$
MM_MOUSE_170	M	mosquito bite D2	7	$1.8X10^2$	$2.5X10^1$	$5.0X10^4$
MM_MOUSE_171	M	mosquito bite D2	7	$1.5X10^3$	$2.14X10^2$	$4.8X10^5$
MM_MOUSE_172	M	mosquito bite D2	6	$2X10^2$	$3.3X10^1$	$8.7X10^4$
MM_MOUSE_173	M	mosquito bite D2	3	0	0	$3.3X10^3$
MM_MOUSE_174	M	mosquito bite D2	4	$1.2X10^2$	$3.0X10^1$	$1.4X10^4$
MM_MOUSE_175	F	mosquito bite D2	3	$1.8X10^2$	$6.0X10^1$	$5.9X10^4$
MM_MOUSE_176	F	mosquito bite D2	3	0	0	$5.7X10^4$
MM_MOUSE_177	F	mosquito bite D2	4	$3.8X10^2$	$9.5X10^2$	$2.5X10^5$
MM_MOUSE_178	F	mosquito bite D2	9	$3.0X10^2$	$3.3X10^1$	$3.5X10^5$
MM_MOUSE_179	F	mosquito bite D2	4	$1.0X10^2$	$2.5X10^1$	$2.4X10^4$
MM_MOUSE_180	F	mosquito bite D2	6	$1.2X10^2$	$2.0X10^1$	$5.1X10^5$
MM_MOUSE_181	F	mosquito bite D2	4	$1.2X10^2$	$3.0X10^1$	$3.3X10^5$
MM_MOUSE_182	F	mosquito bite D2	2	0	0	$6.9X10^3$
MM_MOUSE_183	F	mosquito bite D2	2	0	0	$2.0X10^4$
MM_MOUSE_079	M	mosquito bite D4	3	$8.0x10^1$	$2.6x10^1$	$4.8x10^4$
MM_MOUSE_080	M	mosquito bite D4	4	0	0	$2.8x10^5$
MM_MOUSE_081	M	mosquito bite D4	5	$1.4x10^3$	$2.8x10^2$	$2.9x10^5$
MM_MOUSE_083	M	mosquito bite D4	3	$2.0\ x10^2$	$6.6x10^1$	$1.3\ x10^5$
MM_MOUSE_085	M	mosquito bite D4	1	$4.0x10^2$	$4.0x10^2$	$1.19x10^5$
MM_MOUSE_087	M	mosquito bite D4	10	$2.4x10^3$	$2.4x10^2$	$1.1x10^6$
MM_MOUSE_091	M	mosquito bite D4	2	$3.6x10^2$	$1.8x10^2$	5.4x103
MM_MOUSE_094	M	mosquito bite D4	6	$2.6\ x10^2$	$4.3x10^2$	$3.6x10^4$
MM_MOUSE_099	F	mosquito bite D4	2	$1.4x10^2$	$7.0x10^1$	$1.5x10^4$
MM_MOUSE_100	F	mosquito bite D4	4	$2.4x10^2$	$6.0x10^1$	$2.02x10^5$
MM_MOUSE_103	F	mosquito bite D4	8	$1.4x10^3$	$1.75x10^2$	$5.1x10^5$
MM_MOUSE_105	F	mosquito bite D4	5	$1.6x10^2$	$3.2x10^1$	$1.7x10^6$
MM_MOUSE_106	F	mosquito bite D4	2	$6.0x10^3$	$3.0x10^2$	$7.0x10^4$
MM_MOUSE_113	F	mosquito bite D4	1	$2.0x10^2$	$2.0x10^2$	$1.5x10^4$
MM_MOUSE_114	F	mosquito bite D4	3	N/A	N/A	$8.3x10^4$
MM_MOUSE_118	F	mosquito bite D4	9	$1.3x10^3$	$1.44x10^2$	$8.3x10^3$
MM_MOUSE_082	M	mosquito bite D7	2	$2.0x10^1$	$1.0x10^1$	$1.2\ x10^4$
MM_MOUSE_084	M	mosquito bite D7	2	$7.0\ x10^3$	$3.5x10^3$	$5.1\ x10^4$
MM_MOUSE_086	M	mosquito bite D7	2	0	0	$2.0x10^4$
MM_MOUSE_088	M	mosquito bite D7	3	$6.0\ x10^2$	$2.0x10^2$	$6.1x10^4$
MM_MOUSE_092	M	mosquito bite D7	4	$3.2x10^2$	$8.0x10^1$	$2.1x10^5$
MM_MOUSE_093	M	mosquito bite D7	6	$1.2x10^3$	$2.0x10^2$	$2.8x10^5$
MM_MOUSE_095	M	mosquito bite D7	1	0	0	$7.2x10^2$
MM_MOUSE_096	M	mosquito bite D7	9	$3.1x10^4$	$3.44x10^2$	$5.5x10^6$
MM_MOUSE_101	F	mosquito bite D7	5	$3.6x10^2$	$7.2x10^1$	$7.5x10^4$
MM_MOUSE_104	F	mosquito bite D7	2	$1.6x10^3$	$8.0x10^2$	$7.37x10^4$
MM_MOUSE_102	F	mosquito bite D7	1	$1.2x10^2$	$1.2x10^2$	$2.8x10^4$
MM_MOUSE_108	F	mosquito bite D7	6	$3.8x10^2$	$6.3x10^1$	$1.6x10^5$
MM_MOUSE_109	F	mosquito bite D7	2	0	0	$2.0x10^4$
MM_MOUSE_110	F	mosquito bite D7	4	$3.0x10^2$	$7.5x10^1$	$1.8x10^5$

MM_MOUSE_111	F	mosquito bite D7	5	$6.0x10^2$	$1.2x10^2$	$4.2x10^4$
MM_MOUSE_115	F	mosquito bite D7	4	$3.2x10^2$	$8.0x10^2$	$5.7x10^5$
MM_MOUSE_116	F	mosquito bite D7	3	$8.0x10^2$	$2.66x10^2$	$1.5x10^4$
MM_MOUSE_117	F	mosquito bite D7	4	$9.0x10^2$	$2.25x10^2$	$1.9x10^5$
MM_MOUSE_220	M	mosquito bite D28	2	N/A	N/A	$1.3\ x10^5$
MM_MOUSE_221	M	mosquito bite D28	5	N/A	N/A	$5.4\ x10^4$
MM_MOUSE_222	M	mosquito bite D28	4	N/A	N/A	$2.1\ x10^5$
MM_MOUSE_223	M	mosquito bite D28	6	N/A	N/A	$3.8\ x10^4$
MM_MOUSE_224	M	mosquito bite D28	8	N/A	N/A	$8.9\ x10^5$
MM_MOUSE_225	M	mosquito bite D28	9	N/A	N/A	$3.1\ x10^5$
MM_MOUSE_214	F	mosquito bite D28	6	N/A	N/A	$2.2\ x10^5$
MM_MOUSE_215	F	mosquito bite D28	2	N/A	N/A	$1.6\ x10^5$
MM_MOUSE_216	F	mosquito bite D28	7	N/A	N/A	$8.8\ x10^5$
MM_MOUSE_217	F	mosquito bite D28	4	N/A	N/A	$1.4x10^5$
MM_MOUSE_218	F	mosquito bite D28	5	N/A	N/A	$6.9\ x10^5$
MM_MOUSE_219	F	mosquito bite D28	5	N/A	N/A	$8.7\ x10^5$

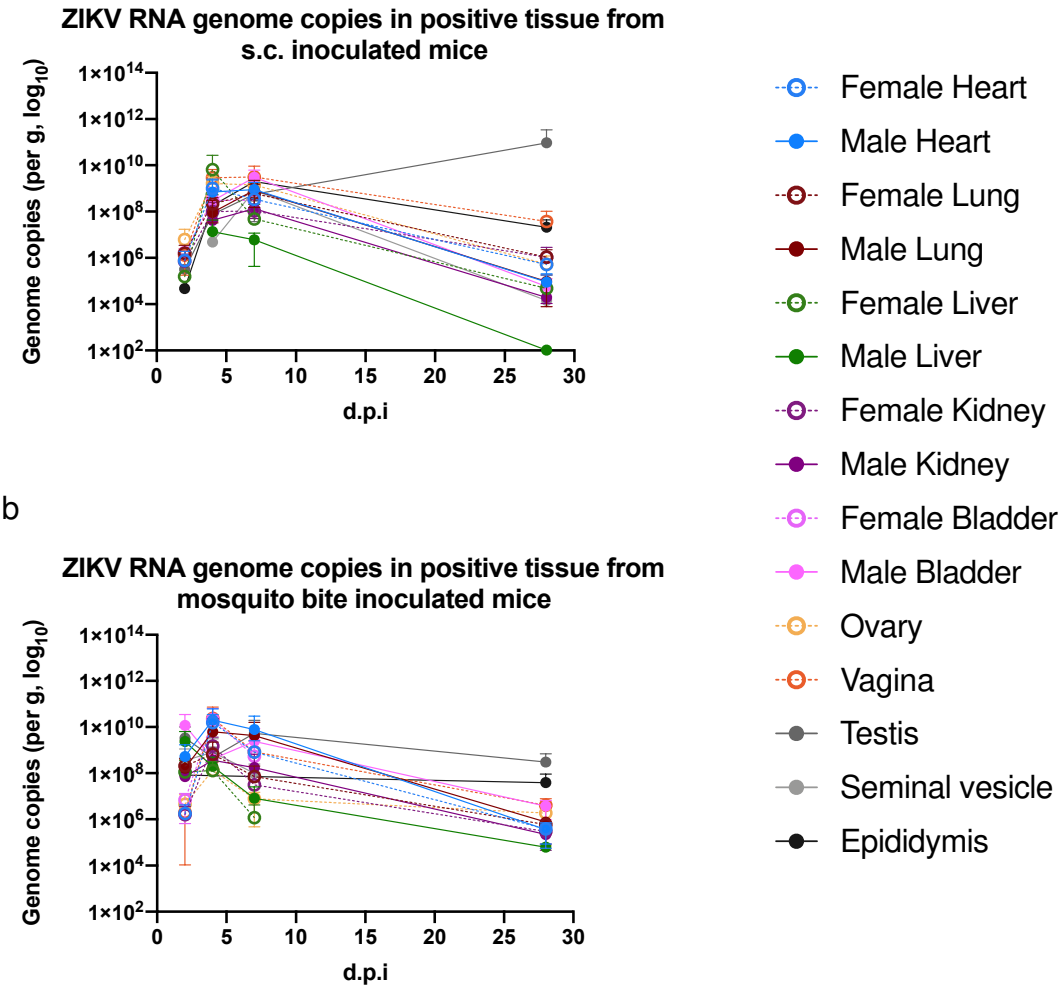
Individual engorged mosquitoes were force salivated after blood feeding. Plaque assay and qRT-PCR was then performed on the saliva samples and inocula.

Table S 2 Sample size and proportions of ZIKV RNA positive tissue

Inoculation route	d.p.i	Sex	N	Proportion of positive tissue determined by qRT-PCR												
				Blood	Brian	Heart	Lung	Liver	Spleen	Kidn.	Bladd.	Testis	Epid.	Sem. Ves.	Ovary	Vagina
Mosquito bite(s)	2	F	9	8/9	2/9	4/9	4/9	4/9	6/9	4/9	5/9	N/A	N/A	N/A	4/9	5/9
		M	9	6/9	7/9	5/9	6/9	7/9	7/9	6/9	3/9	5/9	7/9	5/9	N/A	N/A
	4	F	8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	N/A	N/A	N/A	8/8	8/8
		M	8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	N/A	8/8	N/A	N/A
	7	F	10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	N/A	N/A	N/A	10/10	10/10
		M	8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	N/A	8/8	N/A	N/A
	28	F	6	6/6	5/6	6/6	3/6	0/6	6/6	4/6	0/6	N/A	N/A	N/A	4/6	6/6
		M	6	6/6	5/6	6/6	6/6	1/6	6/6	6/6	6/6	6/6	0/6	5/6	N/A	N/A
Subcutaneous inoculation (s.c.)	2	F	10	10/10	6/10	9/10	8/10	1/10	10/10	7/10	4/10	N/A	N/A	N/A	8/10	4/10
		M	10	4/10	0/10	0/10	0/10	0/10	5/10	0/10	0/10	1/10	0/10	0/10	N/A	N/A
	4	F	10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	N/A	N/A	N/A	10/10	10/10
		M	10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	N/A	10/10	N/A	N/A
	7	F	11	11/11	11/11	11/11	11/11	11/11	11/11	11/11	11/11	N/A	N/A	N/A	11/11	11/11
		M	10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	N/A	10/10	N/A	N/A
	28	F	6	6/6	4/6	4/6	4/6	1/6	5/6	3/6	0/0	N/A	N/A	N/A	5/6	4/6
		M	7	2/7	5/7	7/7	6/7	1/7	7/7	6/7	2/7	7/7	1/7	6/7	N/A	N/A

Proportion of positive tissue determined by qRT-PCR. Kidn.= Kidney, Bladd.= Bladder. Epid.= Epididymis, Sem. Ves.= Seminal Vesicles,

a



b

Figure S 1 ZIKV RNA in tissues detected by qRT-PCR in a) s.c. inoculated mice b) mosquito bite inoculated mice. Limit of detection is10 genome copies. Mean and SEM are shown.

Table S 3 Quantification of virus from ZIKV-infected mosquitoes given different blood feeding treatments, then force salivated and dissected of their bloodmeals.

Treatment	Mosquito number	Bodies		Saliva		Bloodmeal		Difference: bloodmeal-saliva	
None		Titer	Genome Copy	Titer	Genome Copy	Titer	Genome Copy	Titer	Genome Copy
	1	1.60E+05	8.20E+08	4.00E+00	6.70E+03	1.40E+02	2.10E+05	1.36E+02	2.03E+05
	2	1.30E+05	3.50E+08	2.00E+00	9.90E+03	1.50E+02	1.30E+06	1.48E+02	1.29E+06
	3	4.00E+04	6.00E+08	0.00E+00	9.30E+03	4.30E+02	3.80E+05	4.30E+02	3.71E+05
	4	5.00E+04	2.70E+08	0.00E+00	0.00E+00	2.90E+02	3.30E+05	2.90E+02	3.30E+05
	5	5.00E+04	8.00E+08	2.00E+00	6.20E+03	2.00E+01	4.80E+04	1.80E+01	4.18E+04
	6	1.30E+05	4.30E+08	0.00E+00	1.10E+04	2.00E+01	8.00E+04	2.00E+01	6.90E+04
	7	1.30E+05	2.00E+08	0.00E+00	5.80E+03	2.40E+02	3.30E+04	2.40E+02	2.72E+04
	8	6.00E+02	1.20E+08	0.00E+00	1.40E+04	1.20E+02	6.30E+05	1.20E+02	6.16E+05
	9	0.00E+00	1.50E+03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
	10	0.00E+00	4.50E+08	2.00E+00	0.00E+00	4.40E+02	3.80E+05	4.38E+02	3.80E+05
	11	6.00E+04	6.20E+04	0.00E+00	0.00E+00	0.00E+00	1.30E+03	0.00E+00	1.30E+03
	12	1.90E+05	3.10E+08	1.20E+01	1.70E+04	3.70E+02	1.30E+05	3.58E+02	1.13E+05
	13	1.50E+05	5.70E+08	0.00E+00	2.50E+04	6.00E+01	7.70E+04	6.00E+01	5.20E+04
	14	0.00E+00	3.70E+08	0.00E+00	8.30E+03	3.40E+02	1.40E+05	3.40E+02	1.32E+05
	15	1.30E+05	2.81E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
	16	1.70E+05	7.20E+08	0.00E+00	0.00E+00	2.60E+02	7.90E+04	2.60E+02	7.90E+04
	17	1.30E+05	6.00E+08	0.00E+00	0.00E+00	2.10E+02	9.00E+04	2.10E+02	9.00E+04
	18	0.00E+00	3.90E+08	0.00E+00	0.00E+00	3.60E+02	2.00E+05	3.60E+02	2.00E+05
	19	9.00E+04	4.00E+08	0.00E+00	0.00E+00	0.00E+00	1.20E+05	0.00E+00	1.20E+05
	20	2.90E+05	5.60E+08	0.00E+00	0.00E+00	3.00E+01	5.40E+05	3.00E+01	5.40E+05
	Mean	9.50E+04	3.98E+08	1.10E+00	5.66E+03	1.74E+02	2.38E+05	1.73E+02	2.33E+05
	Log transform of mean							2.2142563	5.05911457
Artificial feeder	1	1.30E+05	5.40E+08	5.00E+01	1.90E+04	5.00E+03	3.30E+06	4.95E+03	3.28E+06
	2	6.00E+04	3.40E+08	1.10E+02	1.20E+05	5.30E+03	2.60E+06	5.19E+03	2.48E+06
	3	2.10E+05	8.20E+08	0	0	9.00E+03	6.40E+06	9.00E+03	6.40E+06
	4	2.80E+02	2.40E+08	0	5.10E+04	9.00E+03	4.30E+06	9.00E+03	4.25E+06
	5	2.10E+05	1.30E+08	1.70E+02	9.30E+04	7.00E+03	2.80E+07	6.83E+03	2.79E+07
	6	1.00E+04	1.80E+08	6	9.50E+04	0	3.00E+06	-6.00E+00	2.91E+06
	7	2.00E+05	5.90E+08	4.80E+01	5.90E+04	4.00E+03	2.30E+07	3.95E+03	2.29E+07
	8	6.00E+04	4.10E+08	2	6.60E+03	1.10E+04	8.60E+07	1.10E+04	8.60E+07
	9	1.50E+05	3.20E+08	1.00E+02	9.30E+04	6.00E+03	1.10E+07	5.90E+03	1.09E+07
	10	1.40E+05	3.90E+08	5.10E+01	1.00E+04	1.00E+03	2.70E+07	9.49E+02	2.70E+07
	11	1.00E+04	1.20E+09	1.50E+02	3.10E+04	1.10E+04	1.30E+07	1.09E+04	1.30E+07
	12	2.70E+05	5.70E+08	1.90E+02	2.90E+05	3.00E+03	7.90E+06	2.81E+03	7.61E+06
	13	1.10E+05	3.00E+08	1.00E+02	6.50E+04	3.30E+03	3.50E+04	3.20E+03	-3.00E+04
	14	2.50E+05	1.30E+09	3.30E+01	3.30E+04	3.80E+03	9.00E+07	3.77E+03	9.00E+07
	15	2.00E+04	5.10E+08	6.00E+01	1.50E+04	1.50E+03	4.70E+06	1.44E+03	4.69E+06
	16	3.10E+05	1.00E+09	1.20E+02	3.60E+04	4.70E+03	1.40E+07	4.58E+03	1.40E+07
	17	4.80E+05	4.30E+08	9.00E+01	3.70E+04	2.70E+03	3.10E+06	2.61E+03	3.06E+06
	18	4.10E+05	7.20E+08	2.10E+01	3.50E+04	3.20E+03	4.30E+06	3.18E+03	4.27E+06
	19	0	4.20E+08	1.00E+02	1.50E+05	2.40E+03	6.20E+06	2.30E+03	6.05E+06
	20	1.60E+05	6.20E+08	2.10E+01	1.90E+03	2.60E+03	1.80E+07	2.58E+03	1.80E+07
	21	9.00E+04	3.40E+08	1.50E+02	3.40E+04	1.30E+04	1.80E+07	1.29E+04	1.80E+07
	22	8.00E+04	8.80E+09	2.10E+01	3.00E+04	6.00E+03	3.70E+07	5.98E+03	3.70E+07
	23	1.80E+05	3.20E+08	3.00E+02	1.80E+05	9.00E+03	4.40E+07	8.70E+03	4.38E+07
	Mean	1.42E+05	8.20E+08	7.58E+01	5.94E+04	4.94E+03	1.82E+07	4.86E+03	1.81E+07
	Log transform of mean							3.65639081	7.06358567

Artificial feeder + mixing	1	9.00E+04	5.40E+08	6	1.00E+04	6.00E+03	3.10E+07	5.99E+03	3.10E+07
	2	0	1.80E+04	0	0	0	1.80E+04	0.00E+00	1.80E+04
	3	9.00E+04	2.30E+08	2.60E+02	3.50E+05	4.00E+03	4.00E+06	3.74E+03	3.65E+06
	4	1.60E+05	5.00E+08	3.00E+01	3.60E+03	7.00E+03	3.70E+06	6.97E+03	3.70E+06
	5	8.00E+04	3.50E+08	0	0	4.00E+03	7.50E+06	4.00E+03	7.50E+06
	Mean	8.40E+04	3.24E+08	5.92E+01	7.27E+04	4.20E+03	9.24E+06	4.14E+03	9.17E+06
	Log transform of mean							3.69897028	6.35032543

Table S 4 Quantification of virus from CHIKV-infected mosquitoes given different blood feeding treatments, then force salivated and dissected of their bloodmeals.

Treatment	**Mosquito number**	**Bodies**		**Saliva**		**Bloodmeals**		**Difference: bloodmeal-saliva**	
None		Titer	Genome Copy	Titer	Genome Copy	Titer	Genome Copy	Titer	Genome Copy
	1	2.80E+05	3.20E+08	6.60E+01	1.50E+04	1.00E+02	2.20E+05	3.40E+01	2.05E+05
	2	4.10E+04	6.90E+07	1.00E+01	9.10E+03	1.00E+01	8.10E+05	0.00E+00	8.01E+05
	3	1.00E+05	8.30E+07	3.20E+01	2.70E+03	2.00E+01	2.70E+05	-1.20E+01	2.67E+05
	4	2.50E+05	2.80E+08	0	0	2.90E+02	2.10E+05	2.90E+02	2.10E+05
	5	3.00E+05	2.10E+08	1.80E+01	1.60E+03	8.00E+01	5.40E+05	6.20E+01	5.38E+05
	6	4.80E+04	1.10E+08	2	0	1.10E+02	1.80E+05	1.08E+02	1.80E+05
	7	3.90E+04	7.60E+07	8	1.10E+03	8.00E+01	2.30E+04	7.20E+01	2.19E+04
	8	4.80E+04	1.10E+08	2	0	1.50E+02	1.50E+05	1.48E+02	1.50E+05
	9	2.70E+05	1.40E+08	0	0	3.40E+02	1.90E+05	3.40E+02	1.90E+05
	10	8.00E+04	1.90E+08	2	0	4.20E+02	1.30E+05	4.18E+02	1.30E+05
	11	2.50E+05	1.40E+08	6	0	4.50E+02	1.00E+05	4.44E+02	1.00E+05
	12	5.20E+04	8.90E+07	0	0	4.00E+01	3.00E+04	4.00E+01	3.00E+04
	13	1.50E+05	1.40E+08	4	0	5.90E+02	1.20E+06	5.86E+02	1.20E+06
	14	1.90E+05	1.50E+08	0	0	2.60E+02	7.80E+04	2.60E+02	7.80E+04
	15	4.60E+04	6.50E+07	0	0	1.10E+02	6.00E+04	1.10E+02	6.00E+04
	16	8.00E+04	1.10E+08	0	0	5.00E+01	7.90E+04	5.00E+01	7.90E+04
	17	5.50E+05	4.20E+08	1.80E+02	2.90E+04	2.70E+02	2.80E+05	9.00E+01	2.51E+05
	18	2.10E+06	1.00E+04	3.80E+01	0	1.40E+02	0	1.02E+02	0.00E+00
	19	4.80E+04	1.70E+08	0	2.00E+04	1.00E+01	1.70E+05	1.00E+01	1.50E+05
	20	0	5.30E+07	0	0	0	1.40E+05	0.00E+00	1.40E+05
	Mean	2.46E+05	1.46E+08	1.84E+01	3.93E+03	1.76E+02	2.43E+05	1.58E+02	2.39E+05
	Log transform of mean							2.06679183	5.19506467
Artificial feeder	1	1.60E+04	9.90E+04	0	0	2.00E+02	6.50E+05	2.00E+02	6.50E+05
	2	1.10E+05	4.20E+07	0	0	4.70E+03	3.20E+06	4.70E+03	3.20E+06
	3	2.50E+05	8.00E+07	0	0	1.40E+03	2.50E+07	1.40E+03	2.50E+07
	4	9.00E+04	1.40E+08	0	1.70E+04	1.20E+03	3.40E+06	1.20E+03	3.38E+06
	5	1.00E+05	1.50E+08	0	2.30E+04	7.00E+02	4.10E+06	7.00E+02	4.08E+06
	6	1.20E+05	1.20E+08	0	0	1.30E+03	3.10E+06	1.30E+03	3.10E+06
	7	3.50E+05	2.80E+08	2.80E+01	2.20E+04	4.50E+03	8.70E+06	4.47E+03	8.68E+06
	8	1.30E+05	3.10E+08	0	0	3.80E+03	1.80E+07	3.80E+03	1.80E+07
	9	4.30E+05	2.20E+08	2	2.10E+03	9.00E+03	1.00E+07	9.00E+03	1.00E+07
	10	2.30E+05	4.40E+08	1.00E+01	2.00E+04	7.50E+04	1.70E+07	7.50E+04	1.70E+07
	11	1.30E+05	1.00E+08	2.20E+01	5.60E+02	1.10E+03	8.30E+06	1.08E+03	8.30E+06
	12	3.30E+05	9.00E+07	0	0	2.50E+03	1.90E+06	2.50E+03	1.90E+06
	13	1.00E+06	4.40E+08	0	0	7.00E+04	2.20E+06	7.00E+04	2.20E+06
	14	3.90E+05	7.70E+08	3.00E+02	3.30E+05	2.40E+03	1.80E+07	2.10E+03	1.77E+07
	15	1.70E+05	1.90E+08	4	0	3.30E+03	6.40E+06	3.30E+03	6.40E+06
	16	1.30E+05	2.30E+08	6	1.00E+03	3.00E+02	1.10E+07	2.94E+02	1.10E+07
	17	3.40E+05	1.60E+08	4	1.80E+04	5.10E+03	2.00E+06	5.10E+03	1.98E+06
	18	2.70E+06	3.10E+08	5.00E+01	2.90E+04	7.20E+04	8.30E+06	7.20E+04	8.27E+06
	19	2.00E+05	1.10E+08	0	0	1.40E+03	6.20E+07	1.40E+03	6.20E+07
	20	1.40E+05	1.50E+08	1.20E+01	2.40E+03	2.30E+03	1.20E+07	2.29E+03	1.20E+07
	21	1.60E+05	1.50E+08	1.20E+01	1.60E+04	3.50E+03	3.00E+07	3.49E+03	3.00E+07
	22	1.40E+05	2.20E+08	0	0	1.10E+03	1.00E+07	1.10E+03	1.00E+07
	23	4.00E+04	1.90E+08	2	1.80E+03	8.00E+02	7.80E+06	7.98E+02	7.80E+06
	24	9.00E+04	1.20E+08	0	6.40E+02	5.00E+02	4.20E+06	5.00E+02	4.20E+06
	25	0	1.30E+08	0	0	0	1.30E+06	0.00E+00	1.30E+06
	Mean	3.11E+05	2.06E+08	1.81E+01	1.93E+04	1.07E+04	1.11E+07	1.07E+04	1.11E+07

	Log transform of mean							3.41899626	6.82390797
Artificial feeder + mixing	1	1.90E+05	2.00E+08	1.40E+01	4.40E+03	4.00E+03	3.40E+06	3.99E+03	3.40E+06
	2	1.10E+05	1.20E+08	1.40E+01	3.70E+04	5.00E+03	6.00E+06	4.99E+03	5.96E+06
	3	7.50E+05	5.60E+08	4.60E+01	6.60E+04	11000	4.00E+06	1.10E+04	3.93E+06
	4	1.10E+05	1.00E+08	8	6.20E+03	2.00E+03	8.90E+06	1.99E+03	8.89E+06
	5	1.10E+05	9.40E+07	1.60E+01	5.50E+03	3.00E+03	2.20E+06	2.98E+03	2.19E+06
	6	9.00E+04	1.70E+08	6	5.80E+03	1.00E+03	2.40E+06	9.94E+02	2.39E+06
	7	1.00E+05	2.90E+08	1.10E+02	5.70E+04	0	5.90E+06	-1.10E+02	5.84E+06
	8	1.40E+05	2.50E+08	2.80E+01	2.00E+04	0	1.70E+06	-2.80E+01	1.68E+06
	9	9.00E+04	2.40E+08	4.40E+01	3.90E+04	3.00E+03	3.50E+06	2.96E+03	3.46E+06
	10	1.50E+05	1.90E+08	0	0	0	1.60E+06	0.00E+00	1.60E+06
	11	1.00E+05	1.40E+08	1.00E+01	2.20E+03	1.00E+03	8.80E+06	9.90E+02	8.80E+06
	12	2.70E+05	1.80E+08	4.20E+01	6.90E+03	1.80E+04	1.60E+07	1.80E+04	1.60E+07
	13	1.30E+06	1.30E+09	8	7.70E+03	4.60E+04	9.90E+06	4.60E+04	9.89E+06
	14	2.80E+05	3.30E+08	1.60E+01	3.40E+03	0	3.70E+06	-1.60E+01	3.70E+06
	15	2.00E+04	3.40E+06	4	4.50E+03	0	1.70E+04	-4.00E+00	1.25E+04
	16	1.70E+05	1.80E+08	1.40E+01	1.60E+04	0	4.20E+06	-1.40E+01	4.18E+06
	17	1.50E+05	2.20E+08	3.80E+01	1.30E+04	1.00E+03	3.60E+06	9.62E+02	3.59E+06
	18	3.10E+05	1.40E+08	1.20E+01	2.60E+03	2.00E+04	3.40E+07	2.00E+04	3.40E+07
	19	9.00E+04	1.80E+08	1.20E+01	3.80E+04	0	1.00E+06	-1.20E+01	9.62E+05
	20	9.00E+04	7.50E+08	4.00E+01	9.60E+04	0	1.10E+06	-4.00E+01	1.00E+06
	21	1.60E+05	2.80E+08	5.40E+01	3.60E+04	3.00E+03	7.20E+06	2.95E+03	7.16E+06
	22	7.20E+05	5.70E+08	2.00E+02	7.50E+04	4.50E+04	8.40E+07	4.48E+04	8.39E+07
	23	6.00E+04	1.00E+08	2.60E+01	1.30E+04	0	2.00E+07	-2.60E+01	2.00E+07
	24	2.50E+05	3.60E+08	2.60E+01	1.50E+04	1.70E+04	1.80E+07	1.70E+04	1.80E+07
	25	6.00E+04	1.30E+08	8	1.00E+03	5.00E+03	2.10E+07	4.99E+03	2.10E+07
	Mean	2.35E+05	2.83E+08	3.18E+01	2.28E+04	7.40E+03	1.09E+07	7.37E+03	1.09E+07
	Log transform of mean							3.73907067	6.65855854

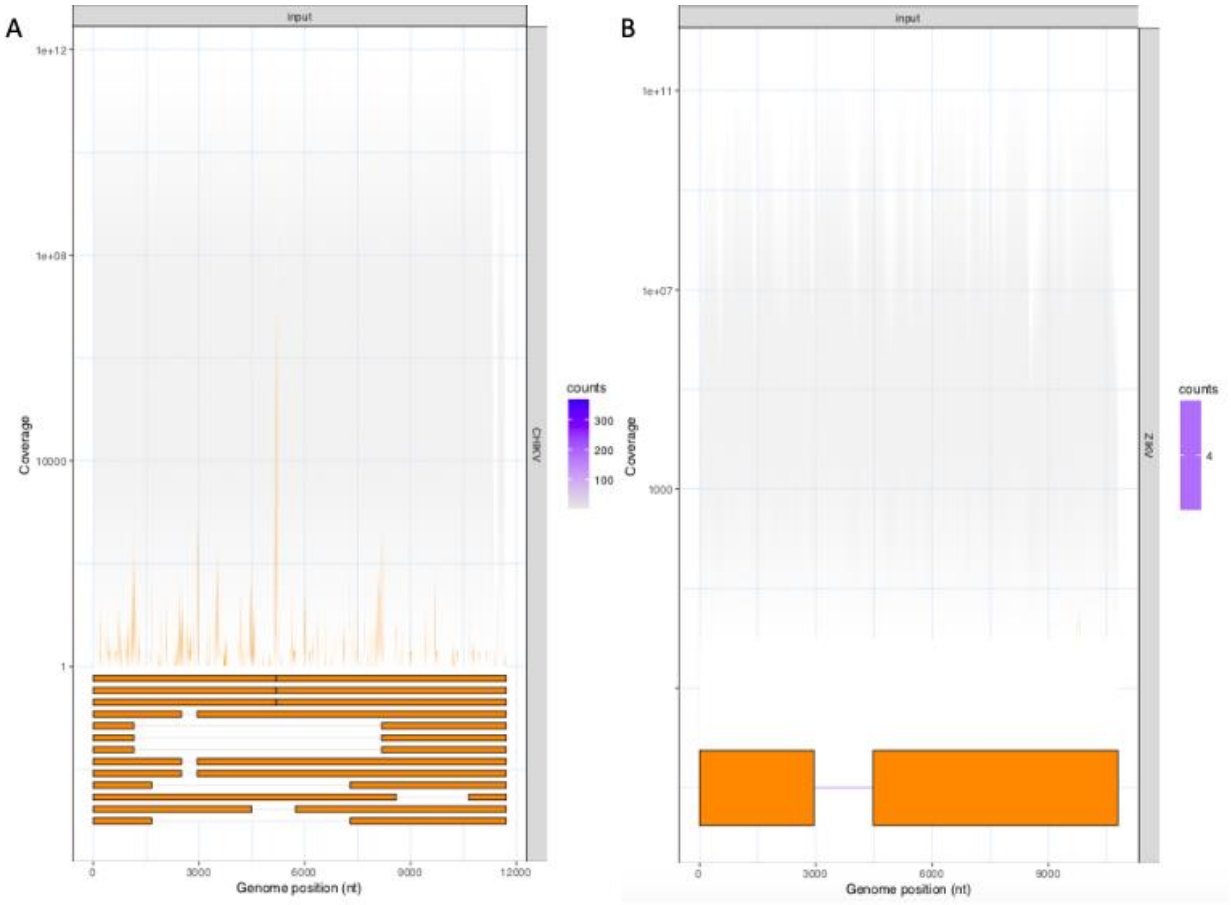


Figure S 2 Deep sequencing analysis of DVG variants in the input (frozen virus stock aliquots) of CHIKV (A) and ZIKV (B) in each experiment. Y-axis is nt across the genome; 0-12,000nt. Each vertical gray line in the top half of each panel represents depth of cover over the genome. Orange vertical lines represent deletions. Each horizontal orange bar in the bottom half of each panel represents a DVG variant with the white space representing deletions, these DVG variants have 5x coverage (5 read count cutoff).