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Vector Competence of *Aedes aegypti* for Zika Virus and Effects of Colonization

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Vector Competence of *Aedes aegypti* for Zika Virus and Effects of Colonization

by

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Vector Competence of *Aedes aegypti* for Zika Virus and Effects of Colonization

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Abstract: The following dissertation aims to determine how vector colonization of influences the vector competence of *Aedes aegypti* for Zika virus (ZIKV) as well as the microbiome as a correlating factor. *Ae. aegypti* is the vector of multiple arthropod-borne viruses including dengue, yellow fever, and Zika virus, making it one of the most globally significant disease vectors and is studied in laboratories world-wide with significant research focus on vector competence studies. Many of these studies, however, utilize strains of *Ae. aegypti* that have been colonized in insectaries for laboratory use and may not reflect the phenotype of wild mosquitoes. While studies have shown differences lab adaptation of mosquitoes resulting in an altered phenotype compared to field mosquitoes, a comprehensive study examining the process of adaptation and effects on vector competence has not been conducted. **I hypothesize that the colonization of *Ae. aegypti* results in an increase in vector competence for ZIKV, correlated with a change in microbiome diversity and composition.** First, the vector competence of multiple species of mosquitoes (*Ae. aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus*) was determined for ZIKV, using various strains of both virus and each vector species. A field-collected population of *Ae. aegypti* was then colonized and experimentally examined for vector competence for ZIKV and microbiome over the course of ten generations. I found that the vector competence of this population did increase over the course of the study and that this change occurred abruptly after multiple generations, resulting in two distinct groups of low and high competence. I then identified a number of bacteria that exhibited different levels of abundance between the low and high competence groups, many of which remain uncharacterized in the mosquito microbiome. Further studies to elucidate the role of these bacteria in determining vector competence as well as the development methods to minimize the effects of colonization could lead to better standardization across vector competence studies and increased relevance to field mosquitoes. These findings are incorporated into the existing literature with recommendations on the design of vector competence studies.

Dedication

This work is dedicated to my grandmother, Barbara Poole, who was always incredibly supportive of my pursuits in academia and beyond; she encouraged me to work hard and enjoy the journey. Her persistent curiosity encouraged me to never stop asking questions.

Acknowledgements

This work would not have been possible without the support of excellent lab mates and great mentors. My mentor, Scott Weaver, provided invaluable guidance but always allowed me the freedom and resources to explore additional questions and pursue numerous opportunities within and beyond my dissertation work. This support enabled me to develop a skill set and network that will continue to carry me as I move forward in my scientific career.

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Additionally, I would like to thank so many of my mentors and teachers, current and former. I have been so fortunate in my path through academia to have excellent mentors along every step of the way. Their patience and encouragement are the reason I have worked to make it this far. I hope to emulate their passion for the field of global health and infectious disease as well as their dedication to process of teaching and learning. Thank you.

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CHAPTER 1: INTRODUCTION

ZIKA VIRUS

Virology

Zika virus (ZIKV) is a positive-sense single stranded RNA (+ssRNA) virus that belongs to the genus *Flavivirus* (1-3). ZIKV's genome, roughly 11 kilobases, is organized as a single open reading frame (ORF) (3-7) (Figure 1.1B). This ORF results in a single polypeptide, which is then processed by a number of proteases (3-8). The genes and resulting proteins are: Envelope (E), Membrane (M), Capsid (C), non-structural 1 (NS1), NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Figure 1.1A,B) (3-8). An untranslated region can be found at both the 5' and 3' ends of the ORF, including a cap at the 5' end which initiates translation of the genome (3-8).

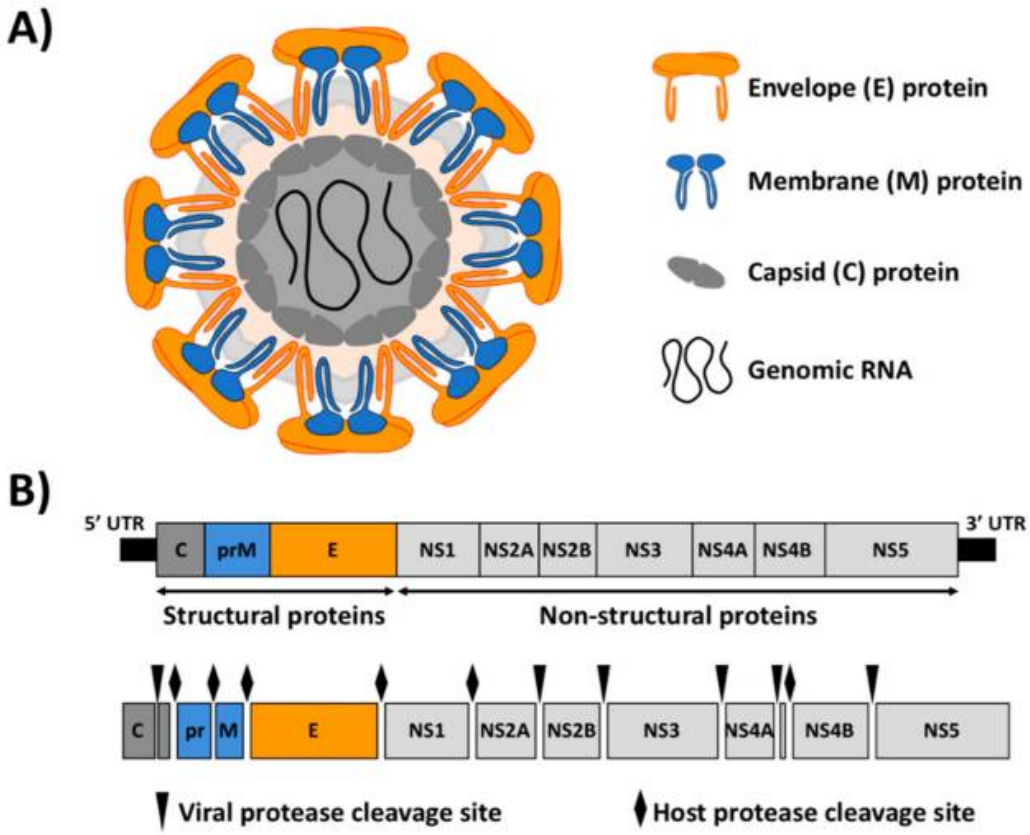

Figure 1.1 Zika Virus viral structure and genome organization

A) ZIKV's virion is structured with the viral RNA encapsulated by viral capsid protein (C), organized within a lipid bilayer derived from the host cell along with viral membrane protein (M) and the external envelop protein (E).

B) ZIKV's genome is a single open reading frame (ORF) flanked by untranslated regions (UTR) on both the 5' and 3' ends. The structural proteins are coded for by the corresponding genes: C, prM (whose product is cleaved into the M protein), and E. The non-structural genes are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, whose proteins eventually form the viral replication complex. Following translation into protein products, the resulting single polypeptide is cleaved at a number of sites by viral and host proteases.

Ávila-Pérez G, Nogales A, Martín V, Almazán F, Martínez-Sobrido L. Reverse Genetic Approaches for the Generation of Recombinant Zika Virus. *Viruses* 2018;10(11):597. Published 2018 Oct 31. doi:10.3390/v10110597

Upon entry into a host cell and acidification of the endosome, the viral RNA is directly translated by host ribosomes (9-11). The resulting polypeptide is cleaved by both host and viral proteases, resulting in the viral structural proteins and non-structural proteins (NSPs) (Figure 1.1 B, Figure 1.2) (9-11). The non-structural proteins form the replication complex, which forms negative strands followed by new strands of positive-sense viral RNA for packaging (9-11). The viral structural proteins are assembled into immature virions, and bud around the viral RNA from the host endoplasmic reticulum. These immature virions undergo additional processing, namely the cleavage of the viral prM protein by furin, a host protease (9-11). The mature ZIKV virion is comprised of viral RNA encapsulated in the C protein within a lipid bilayer derived from host cells and viral E protein (9-11) (Figure 1.2).

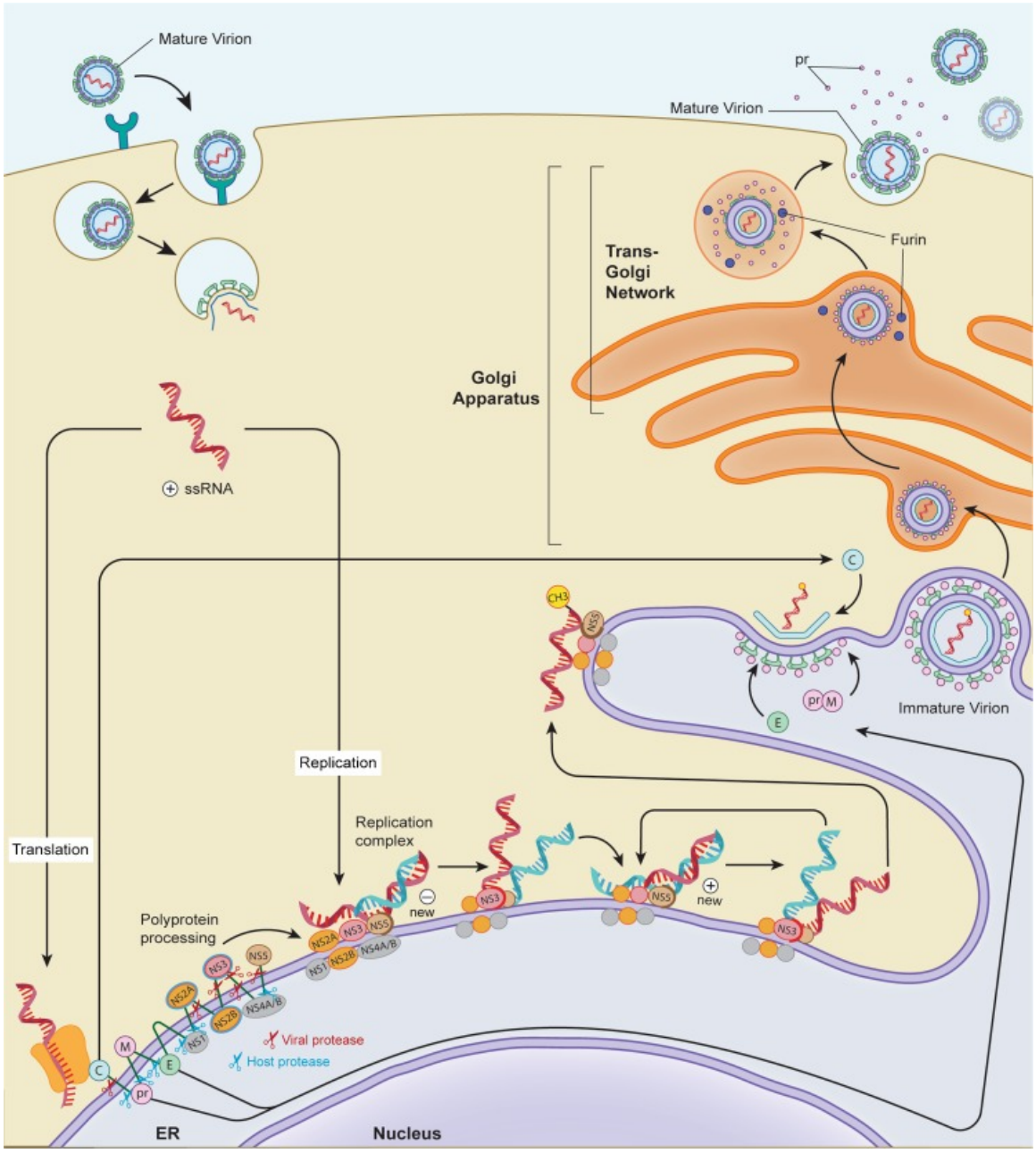

Figure 1.2 Zika Virus Replication Cycle

Following viral binding, endocytosis, and acidification of the endosome, viral RNA is released into the host cell. Translation of the +ssRNA occurs at the host ribosome, resulting in a single polyprotein that is then cleaved by viral and host proteases. NSPs form the replication complex for viral genome replication. Structural protein C forms the capsid around the genomes, further packaged into host lipid bilayer assembled with E and prM proteins. Immature viruses before maturation by host furin, cleaving prM into mature M protein. Following maturation, virions bud into the endoplasmic reticulum and are then exocytosed from the host cell.

Abram RPM, Solis J, Nath A, 2017. Therapeutic Approaches for Zika Virus Infection of the Nervous System. *Neurotherapeutics 14*(4):1027-1048. doi: 10.1007/s13311-017-0575-2.

Discovery and Early Findings

ZIKV was first detected in 1947 during yellow fever virus (YFV) surveillance in the serum of a sentinel rhesus macaque in the Ziika forest of Uganda (12,13). Months later, during additional YFV surveillance, sylvatic mosquitoes were collected and pooled for viral detection. A series of experiments involving inoculating animals with homogenized sylvatic mosquito pools found a filterable agent that caused illness in mice that failed to be neutralized by convalescent serum containing antibodies from YFV and dengue virus (DENV) (12,13). Following this initial characterization in animals, serosurveys were conducted throughout the region, including Ziika, Bwamba, Kampala, and western Nile region. These serosurveys found that evidence of ZIKV infection in about 6% of individuals studied, suggesting the first possibility of human infection (12). The first detected human infections were believed to have occurred in 1954 in Nigeria. During a jaundice outbreak, two patients exhibited an increase in ZIKV neutralizing titers and a viral isolate was able to be isolated from a third patient, but this was later determined to be Spondweni virus (13). Nearly a decade later in Entebbe, a patient with similar clinical presentation had serological evidence of ZIKV infection (14).

In parts of Asia, ZIKV has likely also been circulating at least since the 1950s, as supported by positive serosurveys in Malaya, India, and Borneo (15-18). The first detection of ZIKV in a non-sylvatic mosquito occurred in Malaysia in 1966 when the virus was isolated from a pool of *Aedes aegypti*, a species that is highly anthropophilic and anthropophagic (19). Surprisingly given this isolation from such an urban vector, there was no indication of human infection until multiple patients in Indonesia showed serological

evidence of ZIKV infection (20). Furthermore, there were no findings of ZIKV circulation for decades afterwards (Figure 1.3) (21).

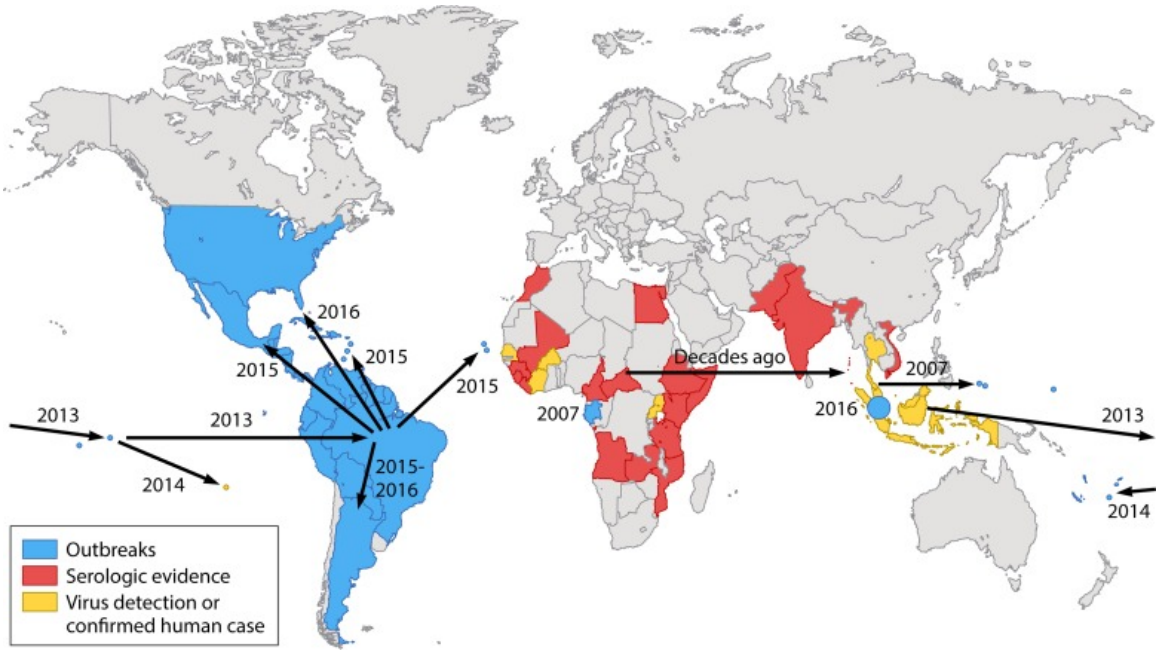

Figure 1.3 Recent Zika Virus Spread

Following historic spread in parts of Africa, India, and Southeast Asia as determined by serologic evidence, a series of outbreaks in the South Pacific began in 2007. This modern circulation of ZIKV eventually led to an introduction in Brazil in 2013 where the virus spread through many countries in the Americas.

Weaver SC, 2017. Emergence of Epidemic Zika Virus Transmission and Congenital Zika Syndrome: Are Recently Evolved Traits to Blame? *mBio* 8(1): e02063-16; DOI: 10.1128/mBio.02063-16

Recent Zika Virus Activity

In 2007, ZIKV caused an outbreak on a scale previously unseen for this virus (Figure 1.3) (16, 22-26). On the island of Yap of the Federated States of Micronesia, there were 59 suspected cases, of which 49 were confirmed. Follow-up serosurveys and modeling of the outbreak found that there were likely over 5000 cases in the total population of 6800, indicating that over 70% of persons living on the island had been infected (24). Also in 2007, ZIKV caused an outbreak in Gabon (15-16) Six years later,

ZIKV caused an outbreak in French Polynesia, which was linked to a 2010 outbreak in Cambodia based on sequencing (16, 27-28).

Within a decade, ZIKV was detected in human cases in 2015 in Brazil, although retrospective studies indicate that ZIKV was introduced as early as 2013 and was not detected due to clinical similarity to DENV and other arboviruses endemic in the region. This outbreak was detected partially due to and rapidly drew international concern due to association with a spike in birth defects, the most notable of which was microcephaly (16,21,25-26). ZIKV rapidly spread throughout major urban regions in Brazil and into surrounding countries, followed by a spike in microcephaly in many regions (Figure 1.3) (29). In 2016, the ZIKV outbreak in the Americas was declared a "public health emergency of international concern" by the World Health Organization (30). Additional surveillance revealed that microcephaly was just one of the possible birth defects, which were termed congenital Zika syndrome (31).

In response to the introduction and rapid spread of Zika virus, Brazil amplified vector control efforts. In addition to traditional use of insecticide and larvicide, regions in Brazil also employed novel control techniques. A long-term, widespread trapping program was used in Recife (32), a mosquito-disseminated pyriproxyfen was tested in Amazonian Brazil (33), and field studies of transgenic sterile male mosquitoes were conducted in Bahia (34). Each of these techniques exhibited local success in the reduction of *Aedes aegypti*.

TRANSMISSION CYCLES AND VECTOR-VIRUS INTERACTIONS

Urban and Sylvatic Transmission Cycles

Similar to other arboviruses, ZIKV maintains an enzootic transmission cycle in forests between non-human primates (NHPs) and sylvatic mosquitoes. For ZIKV, this

transmission occurs between African green monkeys and *Aedes furcifer* and *Aedes africanus* (16). Spillover from this cycle occurs in areas where humans come into contact with these sylvatic vectors. This includes not only areas where humans live on the edge of sylvatic habitats but also cases where individuals may regularly work in forested areas, such as logging (16).

Once a human has been infected with the virus through exposure to a sylvatic cycle, there is potential to introduce the cycle into an urban transmission cycle. During this cycle, the virus circulates between humans and urban and/or peri-urban vector species, such as *Ae. aegypti* and *Ae. albopictus* (16). In order for this urban cycle to be maintained, humans must develop a high enough viremia to infect a susceptible vector during blood feeding and the mosquito species present must be competence vectors or the virus.

Vector Competence vs Vectorial Capacity

When examining vector-virus interactions and the role played in arboviral outbreaks, two important terms to define and differentiate are vector competence and the broader vectorial capacity. While it is only one aspect of vectorial capacity, vector competence can be studied in a laboratory setting and therefore has a much larger representation in the literature. Vector competence is defined as the innate ability of a mosquito to transmit a given agent (32, 36). More specifically, vector competence describes the susceptibility of mosquito to infection with an infectious agent and then the subsequent transmission of the agent. Vectorial capacity, on the other hand, is defined as the daily rate of new infections arising from current infections and incorporates a number of factors related to both vectors and humans (32-35).

In order to closely examine vector competence of arboviruses, it is necessary to understand interactions that take place within the mosquito during infection, dissemination, and transmission. When a female mosquito takes a blood meal from an individual with high enough viremia, the virus ingested first makes its way to the mosquito midgut (Figure

1.4, step 1) (36). Here the virus faces the first barriers to infection. In the midgut, the virus must overcome effects of the mosquito microbiome, digestive enzymes, and mosquito immune response as well as physical barriers (36). Following ingestion of a blood meal, the mosquito's midgut cells excrete a peritrophic matrix, which envelops the blood meal and blocks viral access to the mosquito's midgut epithelium cells (36). The virus must attach to and infect the midgut epithelial cells prior to the production of this matrix. This hurdle to infection is called the midgut infection barrier (MIB) (36) (Figure 1.4 step 2). If the virus is able to infect the midgut epithelium, it must next replicate within these cells and be released from the basolateral end of the cells. Here it faces additional pressure from the mosquito immune response and the tissue barrier of the basal lamina (36). This stage is called the midgut escape barrier (MEB) (36) (Figure 1.4 step 3).

If a virus is able to overcome both the MIB and the MEB, it enters the mosquito hemolymph, resulting in what is referred to as a disseminated infection. From here, the virus is able to infect tissues throughout the mosquito, including the legs, wings, ovaries, and, importantly, the salivary glands (Figure 1.4 step 4). The last major barrier for the virus is the salivary gland infection barrier (SGIB) (Figure 1.4 step 5). The basal lamina of the salivary glands may be the major tissue barrier of this stage (36). Once the salivary glands have been infected, the virus can replicate within the salivary glands and be shed in the mosquito saliva, which is injected into a host during blood feeding. Each of these major barriers presents a significant bottleneck to the virus population which results in a significant reduction in the diversity of viral population present in the mosquito following each stage of infection (37,38). The amount of time it takes a virus to complete this process from acquisition of virus from viremic blood meal to shedding virus in saliva is called the extrinsic incubation period (EIP), one of the main factors of interest in determining vector competence and one of the most important for vectorial capacity.

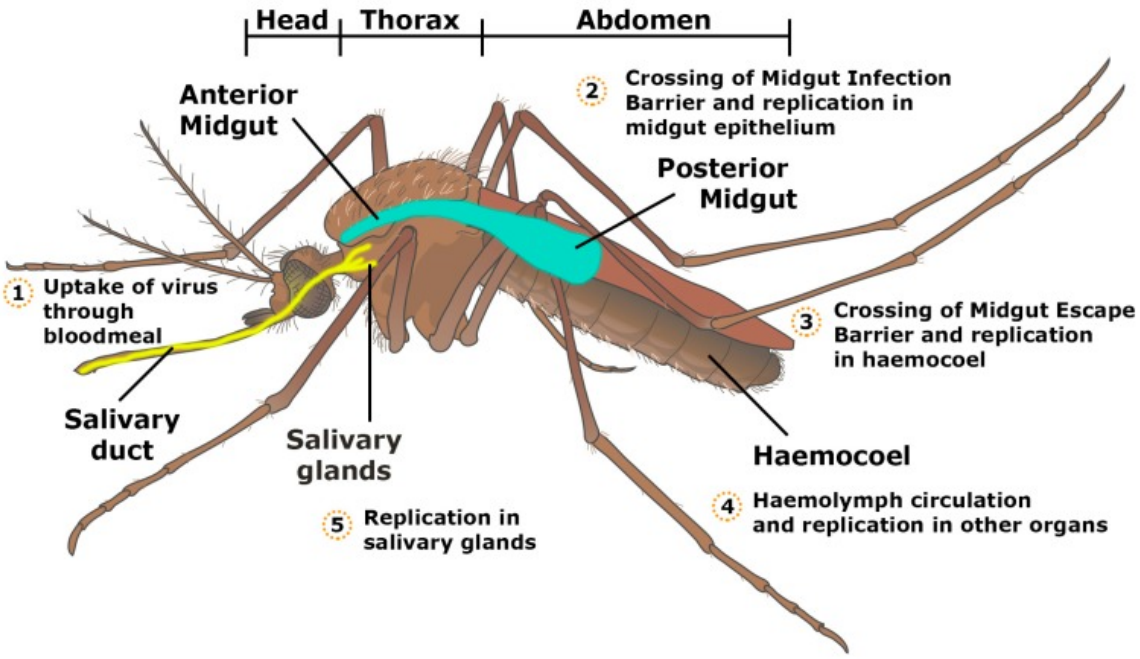

Figure 1.4 Viral Infection of and Replication within a Mosquito

Following uptake of the virus in a viremic blood meal (1), the virus enters the midgut of the mosquito where it faces the midgut infection barrier (2). After crossing the midgut escape barrier (3), the virus enters the hemolymph where it can invade and replicate in additional organs throughout the mosquito (4). Lastly, the virus enters the salivary glands and replicates (5) before being shed in the saliva during blood feeding.

Lim EXY, Lee WS, Madzokere ET, Herrero LJ. Mosquitoes as Suitable Vectors for Alphaviruses. *Viruses*. 2018;10(2):84. Published 2018 Feb 14. doi:10.3390/v10020084

Extrinsic factors also influence vector-virus interaction. Most notably, ambient temperature has been shown to affect infection, dissemination, and transmission. One study focused on ZIKV in *Ae. aegypti* found that the virus had an optimal temperature for infection and exhibited decreased efficiency at temperatures above or below the optimal range (39). This trend was also observed for dissemination and transmission, though the optimal temperature range was narrower (39).

While each of the components of vector competence can be studied experimentally and determined in a laboratory, vectorial capacity is a much more complicated concept. Specifically defined as the daily rate of new infections arising from current infections and

incorporates a number of factors related to both vectors and humans, vectorial capacity is described by the equation:

$$ma^2bp^n/-log_e p$$

where *m*= the number of female mosquitoes relative to human hosts, *a*= the daily rate of blood feeding, *b*= the transmission rate of exposed mosquitoes (the variable most closely related to vector competence), *p*= the survival rate of the mosquito in days, and *n*= the number of days between ingesting an infectious blood meal and shedding of virus in saliva (EIP, also related to vector competence) (32,42-43). Vectorial capacity combines factors of arbovirus transmission that can be determined in a laboratory setting, namely vector competence, with other factors that directly impact the spread of viruses during an outbreak. By examining both vector-virus and vector-human interactions, vectorial capacity gives a more complete picture than described by vector competence alone.

The important differences between vector competence and vectorial capacity are most evident when more than one vector species is capable of transmitting a given virus. In the example of chikungunya virus, *Ae. aegypti* was the primary vector while *Ae. albopictus* was a secondary vector (38). This was in part driven by the differences in feeding patterns between these two mosquitoes. The highly anthropophilic *Ae. aegypti* feeds primarily on human blood meals, making it much more likely to ingest an infectious blood meal and subsequently more likely to transmit the virus to human hosts following the EIP. The less anthropophilic *Ae. albopictus* is more catholic in its feeding, taking blood meals from more varied hosts, and is therefore less likely to become infected and transmit virus (38). However, *Ae. albopictus* occupies a different ecologic and global niche, more likely to be found in peri-urban areas than *Ae. aegypti* and more likely to be found in temperate climates, partially due to the ability of *Ae. albopictus* to overwinter as eggs (44) (38). For these reasons, *Ae. albopictus* could possibly spread the virus into new regions, were it to become a more efficient vector. This is ultimately what was observed with chikungunya virus when a series of adaptive mutations occurring in the Indian Ocean

Lineage significantly increased transmission efficiency in *Ae. albopictus*, which subsequently became the primary vector in outbreaks in regions where *Ae. aegypti* was less prevalent (38). This example shows the importance of the relative effects of each factor in the vectorial capacity equation. Prior to the *Ae. albopictus* adaptive mutations, the relatively low human exposure (variable *a*) resulted in low enough vectorial capacity that *Ae. albopictus* was not an important vector of chikungunya virus. However, the adaptive mutations increased variables *b* and *n*, which increased the overall vectorial capacity even though other factors remained low.

KNOWN EFFECTS OF COLONIZATION

Vector colonization, the long-term rearing of mosquitoes over multiple generations in insectary and laboratory settings, is a necessary component of vector research. Many labs that study mosquitoes simply do not have regular access to field collections due to their geographic location away from tropical and subtropical regions. Even for labs in regions with the vector species of interest, mosquitoes often need to be mass-reared in numbers that would be difficult to collect from the field. Additionally, it would be challenging to control field-collected mosquitoes for age and other factors. However, the insectary setting is very different from the natural setting of mosquitoes, lacking many of the natural pressures that mosquitoes face during survival in nature as well as much of the diversity of bacteria they are exposed to throughout the course of their lives. Insectary colonies are maintained in controlled conditions to maximize survival, reproduction, and ease of use. In some cases, mosquito colonies are maintained for decades, such as the Rockefeller strain of *Ae. aegypti,* which was originally collected from Cuba in the 1930s (45). This Rockefeller strain is widely used due to its ease of use and availability, but how it differs from field populations has been of interest for decades (46). The specific effects that long-term colonization has on the vectors and on experimental outcomes of interest (i.e. vector competence, microbiome, behavior) is not well-established, but studies have

made it clear that mosquitoes quickly adapt to these laboratory settings and this adaptation may have consequences for experimental studies.

Mosquito Development and Reproduction

In their natural environment, immature mosquitoes may have to deal with aquatic predators, interspecies competition (47), and limited nutritional resources (48). In a laboratory setting, however, larvae and pupae are often given food *ad libitum* and do not have to deal with significant competition or predation. Studies have found that this alters how quickly mosquitoes develop during immature stages (49). Dense adult mosquito populations combined with mosquitoes in a given colony being closely age-matched has also been shown to affect sexual maturation and breeding (50,51).

In a large study (49), *Ross et al*. collected *Ae*. *aegypti* mosquitoes from Townsville, Australia and started multiple colonies under varying conditions such as consensus size, level of inbreeding (including use of isofemale lines), and nutritional status. These conditions were run in replicate and compared during early colonization (F4) and nine generations later (F13). In examining larval development time, *Ross et al*. found that results depended on colony condition. Smaller colonies (<100 individuals as compared to 400 individuals in large study colonies) exhibited developmental delays only under low nutrition settings as compared to both the large colonies and the ancestral (F4) colony. Under high nutritional settings, developmental rate were mixed, even between replicate colonies. However, the large colony populations consistently developed faster than the ancestral colony, suggesting adaptation to laboratory settings (49).

Ross et al. also examined other aspects of these mosquito colonies (49), such as survival to adulthood, fecundity, egg hatch rate, and mating competitiveness. In each of these factors, the isofemale lines and smaller mosquito colonies exhibited significant changes from the ancestral colony, resulting in lower survival to adulthood, lower egg

hatch rate, and decreased mating competitiveness. For the larger colony populations, however, there were no statistically significant differences found in these factors (49)

In nature, male mosquitoes typically require around between 12 and 40 hours to reach sexual maturity, depending on the species, which involves a 180 degree rotation of the genitalia before successful mating can be achieved (50). However, a study comparing *Anopheles arabiensis* field-collected as pupae to a colony maintained for over 100 generations found an accelerated male development rate in the colonized population. At 11 hours post-emergence, 42% of colonized males had already completed genital rotation, which further increased to 96% at 17 hours post-emergence during which time none of the wild-collected males had reached sexual maturity (50).

While reproductive traits such as sperm length and size of testes and male accessory gland have not been significantly correlated with mating success in mosquitoes (51), they can be used as indicators to track changes to reproductive phenotype that occur during colonization. In comparing these traits between F1 progeny of field-collected *An. gambiae* versus a colony maintained for over 25 years, it was found that colonized males exhibited shorter sperm length, larger testes, and smaller accessory gland, suggesting laboratory adaptation and potential reproductive cost (51).

Mosquito Microbiome

The mosquito microbiome is comprised of the bacterial communities that occupy the midgut and other organs (52). Mosquitoes likely acquire their microbiota (the specific bacteria that comprise the microbiome) from a number of exposures including larval water (53), blood meals (54), and other sources. Portions of the microbiome have also been shown to be transferred vertically, during egg laying, as well as horizontally (52). While much research focuses on the gut microbiome, recent research has shed light on the microbiome of other organs in the mosquito, especially the salivary glands (55). In the wild, immature mosquitoes develop in bacteria-rich aquatic habitats and female adult

mosquitoes acquire blood meals from varied hosts. However, in an insectary setting, standardization of rearing protocols leads to mosquitoes being exposed to a different and likely less diverse set of bacteria from larval rearing through blood feeding as adults.

One study (56) examined the microbiome of three species of mosquitoes, comparing laboratory-reared mosquitoes to field-collected mosquitoes from two different trap types. It was found that *Ae. aegypti* from the lab had a significantly lower diversity of their microbiome when compared to field mosquitoes from either trap type. However, no difference was observed within *Ae. albopictus* and *Culex quinquefasciatus* actually showed the opposite, with field-collected mosquitoes exhibiting lower diversity than the laboratory-reared group. This study also found that the laboratory-reared mosquitoes exhibited a distinct microbiome community structure when compared to their field counterparts across all three species. This observation was particularly evident with *Ae. aegypti* (56).

In a study examining the conservation of the midgut microbiome during *An. gambiae* colonization, it was found that after 10 generations, microbiome diversity was decreased and only 38% of the bacterial genera from the field-collected population had been maintained (57). Another study by *Dickson et al.* (58) compared the microbiome of six *Ae. aegypti* colonies with different geographic origins and varying levels of colonization. The mosquito colonies examined in this study originated from field collections in Australia (F10), Cambodia (F7), French Guiana (F4), Gabon (F10), Guadeloupe (F5), and Uganda (F3). The study found that the microbiomes of these mosquitoes were remarkably similar, with no significant differences detected in the richness, diversity, or composition of the microbiome between any groups (58). This suggests not only that the microbiome of colonized mosquitoes is largely determined by the insectary in which they are reared, but also that this shift to an insectary microbiome occurs relatively quickly. The mosquito microbiome is not an isolated element, but has been shown to influence other factors of interest. The microbiome plays significant roles

in the development (59), immune response (60), vector competence (61), among other factors.

Genetic Diversity

One important indication of divergence of a colonized population from its field-collected origin is genetic diversity. While a decrease in genetic diversity may be minimized over the course of colonization by using a large initial population, the genetic effects of using small starting populations, including isofemale lines, and colony inbreeding have been observed. One study (62) that examined the published sequences of over a dozen *Ae. aegypti* colonies and then compared them to field counterparts across different parts of the world found that the lab strains were less genetically diverse. Also of interest, *Gloria-Soria et al.* found that a widely used laboratory strain of *Ae. aegypti*, the Liverpool strain, was so divergent from mosquitoes collected from the reported strain origin, that they suggest the strain was possibly contaminated (62). A study comparing *An. arabiensis* colonized for 13 generations to mosquitoes collected form their site of origin found that the colonized group had significantly lower total number of alleles, hypothesizing that some differences observed are due to a small initial starting population (63).

Vector Competence

Vector competence has been shown to be affected by a number of factors, including microbiome (64) and modulation of immune genes (65, 66). As both microbiome and vector genes have been shown to be affected by vector colonization, it follows that colonization would consequently have an impact on vector competence. However, as vector competence is impacted by many underlying factors, determining causality and predicting direction of change may be difficult.

A study examining vector competence for strains of dengue virus in two low-generation populations of *Ae. aegypti* (Peru, F2 and Texas, F3) and comparing them to the highly colonized Rockefeller strain found that while the low-colonized mosquitoes exhibited different levels of disseminated virus when infected with different strains, the Rockefeller mosquitoes exhibited no variation among strains (67). However, in comparing vector competence of these low-colonized mosquitoes to Rockefeller, there was no clear correlation with level of colonization (67). When a colony of *Ae. aegypti* from Vero Beach, Florida was followed for 16 generations and examined for yellow fever virus vector competence, it was found that levels of disseminated infections decreased with colonization (68).

In studies of non-*Aedes* species, when the vector competence of *Culex pipiens* for Rift Valley fever virus was examined across 16 generations of colonization, the susceptibility to infection increased over the course of colonization, but the rate of virus transmission decreased (69). In another study, field-collected *An. stephensi* were compared to *An. stephensi* that had been colonized for over 65 generations (71). When fed with *Plasmodium vivax* from patient blood, the field-collected mosquitoes and colonized mosquitoes exhibited the same level of susceptibility to infection with the oocyst stage (71). However, when the level of sporozoite loads were compared, the wild mosquitoes exhibited increased levels of sporozoites compared to the colonized group (71).

The described colonization studies provide evidence that colonization does significantly affect the phenotype of mosquitoes as compared to natural populations. Insectary conditions and protocols for rearing and maintenance influence vector development, microbiome, genetic diversity, and vector competence. These studies, however, examine colonization as an endpoint, comparing late-generation mosquito strains to earlier generations or field populations. Understanding the process of colonization, how quickly vector changes occur and correlations between the changing factors would greatly

increase our ability to mitigate these factors, increasing reproducibility as well as relevance of lab populations to natural populations.

Chapter 2: Methods and Materials

CELL CULTURE

Vero cells (African green monkey kidney, CCL-81) were purchased from American Type Culture Collection (ATCC). Vero cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% Penicillin-Streptomycin (Pen-Strep). Vero cells were incubated at 37˚ C with humidity and 5% CO_2. C6/36 (*Aedes albopictus*) cells were maintained in a medium with Eagle's Minimum Essential Medium (EMEM) and L-15 medium in a 1:1 ratio supplemented with 10% heat-inactivated FBS, 1% nonessential amino acids, 5% tryptose phosphate broth, 0.5% sodium bicarbonate, and 1% Pen-Strep. C6/36 cells were incubated at 28˚ C with humidity and 5% CO_2.

VIRUSES

Viruses used for all described studies were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). Lyophilized viruses were reconstituted in DMEM supplemented with 3% FBS. Once reconstituted, viruses were put on nearly confluent Vero or C6/36 cells to grow viral stocks. These cells were then observed daily during viral growth for signs of cytopathic effect (CPE). Once proper CPE was reached, cell supernatant was collected and cell debris was removed by centrifugation (4000 RPM for 15 min). Additional heat-inactivated FBS was then added to clarified until total FBS volume reached 30%. Virus stocks were aliquoted into 1mL single-use amounts and stored at -80˚ C. Viruses used as full length infectious clones (FLIC) were obtained as lyophilized stocks following preparation as previously described (71). Viruses used throughout the described studies are listed and described in Table 2.1.

Table 2.1: Zika Virus Strains Used Throughout These Studies

Strain	Genbank Accession	Species of Origin	Location Isolated	Year Isolated	Passage History*	Experiment(s) Used in
DakAR 41525	KU955591.1	*Ae. africanus*	Senegal	1984	AP61, C6/36 (2), Vero (3)	*Ae. aegypti* *Ae. albopictus* *Culex quinquefasciatus* (Chapter 3)
FSS 130125 (isolate)	KU955593.1	Human	Cambodia	2010	Vero, C6/36 (2), Vero (3)	*Ae. aegypti* *Ae. albopictus* *Culex quinquefasciatus* (Chapter 3)
MEX 1-7	KX247632.1	*Ae. aegypti*	Chiapas State, Mexico	2015	Vero (4), C6/36, Vero (3)	*Ae. aegypti* *Ae. albopictus* *Culex quinquefasciatus* (Chapter 3)
MEX 1-44	KX856011.1	*Ae. aegypti*	Chiapas State, Mexico	2015	Vero (7)	*Culex quinquefasciatus* (Chapter 3)
PB81	KU365780	Human	Brazil	2015	Vero, C6/36, Vero	*Ae. albopictus* *Culex quinquefasciatus* (Chapter 3)
PRVABC 59	KX377337.1	Human	Puerto Rico	2015	Vero (5)	*Ae. albopictus* *Culex quinquefasciatus* (Chapter 3)
PRVABC 59 FLIC	KX377337.1	Human	Puerto Rico	2015	Vero-P1, post electroporation	*Ae. aegypti* (Chapter 4)

MOSQUITO MAINTENANCE

Mosquito populations used in the described studies, listed in table 2.2, were kept in an incubator with a temperature of 27±1˚C, relative humidity of 80±10%, and light:dark cycle of 16:8. Mosquitoes were sex-sorted 3 days post-eclosion and only female mosquitoes were used for studies, including microbiome sequencing. After sorting mosquitoes were housed within .5L cardboard containers with mesh tops in the incubators for the remainder of studies. Immediately following sorting, mosquitoes were given access to water soaked cotton balls, which were removed approximately 12 hours prior to blood feeding to increase feeding efficiency. Following feeding and sorting for engorgement, mosquitoes were given access to 10% filtered sucrose soaked into cotton balls *ad libitum* for the remainder of the studies (72,73).

Table 2.2: Mosquito genus, species, and populations utilized

Mosquito Population	Location	Generation	Experiment(s) Used in
Aedes aegypti	Salvador, Brazil	F2	Chapter 3
Aedes aegypti	Dominican Republic	F6	Chapter 3
Aedes aegypti	Rio Grande Valley, Texas, USA	F4	Chapter 3
Aedes albopictus	Rio Grande Valley, Texas, USA	F5	Chapter 3
Aedes albopictus	Houston, Texas, USA	F2	Chapter 3
Aedes albopictus	Salvador, Brazil	F3	Chapter 3
Aedes taeniorhynchus	Galveston, Texas, USA	Long term colony	Chapter 3
Culex quinquefasciatus	Galveston, Texas, USA	Long term colony	Chapter 3
Culex quinquefasciatus	Houston, Texas, USA	F2	Chapter 3
Aedes aegypti	Weslaco, Texas, USA	F0-F10	Chapter 4
Aedes aegypti	Rockefeller strain (Cuba)	Long term colony	Chapter 4

MOSQUITO PROCESSING AND SCREENING

To determine whether ZIKV-exposed mosquitoes were productively infected, and whether the infections had disseminated and where being shed into saliva, cohorts of mosquitoes from each population were selected for analysis (either 2, 3, 4, 7, 10, 14, 17 or 21 days post-oral exposure). To facilitate collection of saliva, mosquitoes were anesthetized on ice, and legs were removed and placed into microfuge tubes containing a sterilized steel ball bearing and 500µL of mosquito collection media (MCM) (DMEM, 2% FBS, 1% Pen-Strep, and 2.5µg/mL of amphotericin B). Following removal of legs, mosquitoes were restrained on a glass slide with mineral oil, and their proboscises were inserted into a 10µL micropipette tip containing 8µL of FBS. Following a 30-minute period in which the mosquitoes were allowed to salivate, the FBS/saliva were ejected into a microfuge tube containing 100µL of MCM. The carcasses of the mosquitoes were placed into independent tubes with 500µL of MCM matched to the corresponding legs/saliva tube. In some experiments, salivary glands and midguts were utilized in lieu of saliva and carcasses, and in some experiments only bodies and legs were collected. Bodies, legs, midguts, and salivary glands were processed by trituration for 5 minutes at 26Hz in

TissueLyser II (Qiagen), and all samples (including saliva) were clarified in a centrifuge at 200 x g for 5 minutes.

VIRAL ASSAYS

To determine titers of virus present in stocks, mouse/NHP sera, or bloodmeals, samples underwent 10-fold serial dilution series in dilution media (DMEM, 2% FBS, 1% Pen-Strep) in 96 well culture plates. Following dilution, 100µL of dilutions were added to nearly confluent (80-95%) monolayers of Vero cells on either 12 or 24 well tissue culture plates. Viral dilutions were allowed to adsorb for one hour in a humidified 37˚C incubator with 5% CO_2, at which point plates were overlayed using a solution of DMEM containing 3% FBS, 1% Pen-Strep, 1.25µL/mL amphotericin B, and 0.8% (weight/vol) methylcellulose. Overlayed plates were incubated for 3-5 days (dependent on viral strain) in a humidified 37˚C incubator with 5% CO_2.

To determine the presence or absence of virus in homogenized mosquito samples or saliva, either 50 or 100µL of clarified supernatant were added to nearly confluent (80-95%) monolayers of Vero cells on either 96 or 24 well tissue culture plates respectively. Inocula were adsorbed for one hour in a humidified 37˚C incubator with 5% CO_2, at which point plates were overlayed using a solution of DMEM containing 3% FBS, 1% Pen-Strep, 1.25µL/mL amphotericin B (for 96 well plates) or DMEM containing 3% FBS, 1% Pen-Strep, 1.25µL/mL amphotericin B and 0.8% (weight/vol) methylcellulose (for 24 well plates). 96 well plates were incubated for 3 days, while 24 well plates were incubated for 3-5 days (strain dependent) in a humidified 37˚C incubator with 5% CO_2.

Following incubation, liquid or semisolid overlays were removed and each well was washed twice with phosphate buffered saline (PBS), and fixed for a minimum of 30 minutes in an ice-cold solution of methanol:acetone (1:1, vol/vol). Following fixation, organic fixative was removed and plates were air-dried. Following complete air drying, plates were washed with PBS and then blocked with 3% FBS in PBS, followed by an

overnight incubation with mouse hyperimmune serum against-ZIKV strain MR-766 (1:2,000 in blocking solution) (WRCEVA, UTMB). Plates were then washed with PBS followed by incubation with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, MD) diluted1:2,000 in blocking solution. Plates were washed with PBS, after which an aminoethylcarbazole solution (Enzo Diagnostics, Farmingdale, NY) prepared according to manufacturer's protocol, was added and plates were incubated in the dark. Development was halted by washing in tap water and plates were allowed to air dry at room temperature before scoring.

ANIMALS USE

Animal experiments described in chapter 3 were performed in full compliance with the guidelines established by the Animal Welfare Act for the housing and care of laboratory animals and conducted as laid out in University of Texas Medical Branch Institutional Animal Care and Use Committee (UTMB-IACUC) approved protocols. Mice (*Mus musculus*, A129 (IFN α/β receptor null, IFNAR) were approved for use on IACUC protocol #0209068B.

Chapter 3: Vector Competence of *Aedes aegypti*, *Aedes albopictus*, and other Potential Vectors for Zika Virus*

RATIONALE

Following its initial discovery and characterization, Zika virus (ZIKV) was found in a series of serosurveys to have spread through parts of Africa and Southeast Asia (12,13,16,78-80). ZIKV had caused less than two dozen reported human cases in the 50 years subsequent to 1954 and most of these were mild febrile illnesses (16). With the exception of being isolated from a pool of *Aedes aegypti* in Malaysia (19), ZIKV had not been associated with urban transmission and appeared to be largely caused by sylvatic transmission and spillover events (16).

After remaining unseen for many years, ZIKV began causing outbreaks in the South Pacific, beginning with the outbreak on Yap Island in 2007 and rapidly spreading to other islands in the region including French Polynesia, New Caledonia, Easter Island, and the Cook Islands (16,22,23,25,81). ZIKV would next emerge in Brazil in 2013 (70-86), though it remained undetected in the region until 2015 (81), most probably due to the high levels of endemic dengue virus (DENV) circulation as well as the introduction of chikungunya virus (CHIKV) around the same time (86). ZIKV rapidly spread through the Americas, causing outbreaks and increases of associated birth defects in many countries throughout the region (26,87). Of particular note during this American outbreak was the emergent association with urban settings and transmission by urban vectors, specifically *Ae. aegypti* (21).

* Portions of this chapter are verbatim comprised of previously published work:
Roundy CM, Azar SR, Rossi SL, et al. Variation in Aedes aegypti Mosquito Competence for Zika Virus Transmission. *Emerg Infect Dis*. 2017; 23(4): 625–632. doi:10.3201/eid2304.161484
Azar SR, Roundy CM, Rossi SL, et al. Differential Vector Competency of *Aedes albopictus* Populations from the Americas for Zika Virus. *Am J Trop Med Hyg*. 2017; 97(2): 330–339. doi:10.4269/ajtmh.16-0969
Hart CE, Roundy CM, Azar SR, et al. Zika Virus Vector Competency of Mosquitoes, Gulf Coast, United States. *Emerg Infect Dis*. 2017; 23(3): 559–560. doi:10.3201/eid2303.161636
Roundy CM, Azar SR, Brault AC, et al. Lack of evidence for Zika virus transmission by Culex mosquitoes. *Emerg Microbes Infect*. 2017; 6(10): e90. Published 2017 Oct 18. doi:10.1038/emi.2017.85

This association led to three leading hypotheses by arbovirus researchers: 1) ZIKV was simply introduced into a large, naïve population living in proximity to competent vectors, leading to large scale outbreaks; 2) ZIKV had adapted for increased level or duration of human viremia, causing an increase in potential mosquito infectivity; 3) ZIKV had adapted for increased fitness in urban vectors, such as *Ae. aegypti*, increasing transmission rates in areas with high human density (21). The third of these hypotheses was suggested by a similar adaptation that took place in CHIKV; mutations that occurred in the Indian Ocean Lineage of the virus caused an increase in fitness in *Ae. albopictus* mosquitoes and aided the further spread of CHIKV (76-86).

In order to test the hypothesis that the American strain of ZIKV had adapted for increased fitness in and transmission by urban vectors, a series of vectors competence studies was run. In the first set of experiments, two viral strains from before the American outbreak, DakAr 41525 (African lineage, Senegal, 1984) and FSS13025 (Asian lineage, Cambodia 2010), were compared to an American outbreak isolate, MEX1-7 (American lineage, Mexico, 2015). Each of these strains was used to infect three groups of *Ae. aegypti* collected from Salvador, Brazil; Rio Grande Valley, Texas; and the Dominican Republic.

Following experiments with *Aedes aegypti*, similar experiments were conducted with *Ae. albopictus*, another important urban vector. *Aedes albopictus* was of particular interest due to its ability to occupy a wider array of ecological niches and climates as compared to *Ae. aegypti* (80). Even though *Ae. albopictus* may not have as many anthropophilic behaviors as *Ae. aegypti*, its wider geographic range, including portions of the Northeast US (80), caused specific concern if it were found to be a highly competent vector of ZIKV. These experiments largely mirrored those conducted in *Ae. aegypti* with a few changes to note. The artificial blood meal system utilized with *Ae. aegypti* was replaced by the use of infectious murine blood meals, as this was found to increase vector competence (91). Additionally, some different strains of mosquito and virus were utilized,

based on availability. This was especially true of ZIKV strains as additional isolates became available throughout the ongoing outbreak.

As the outbreak progressed and as publications showed a relatively low level of vector competence for *Ae. aegypti* (92), some became concerned that additional vectors, namely *Culex quinquefasciatus*, may be playing a cryptic role in viral transmission (89,90). This concern was of unique interest because *Cx. quinquefasciatus* occupies a much broader geographical and ecological niche than either *Ae. aegypti* and *Ae. albopictus*. If *Cx. quinquefasciatus* were in fact a significant vector of ZIKV, significantly greater regions of the Americas would be at risk, particularly those outside of urban centers.

CHAPTER METHODS

Viruses

The viruses used throughout these studies were low passage isolates, as described in Chapter 2 and listed in Table 2-1.

Mosquitoes

The mosquitoes used throughout these studies were low colonized populations, as described in Chapter 2 and listed in Table 2-2.

Animal Use

Four-week-old Type I interferon receptor-knockout ($IFNAR^{-/-}$, A129) mice were infected via IP injection with 10^5 FFU of ZIKV strain FSS13025 in a volume of 100µL. Inocula were diluted in sterile phosphate-buffered saline (PBS). Based on previous work, this infection can be expected to demonstrate viremias ranging from 10^4-10^7 FFU/mL on days 1-3 days pot-infection (DPI) with peak viremia anticipated at 2 DPI. A single animal per DPI was selected, anesthetized with 100mg/kg Ketamine, and placed atop the mesh lid of a 0.5L cardboard cup containing starved *Ae. aegypti*. Mosquitoes were allowed to feed

for 30 min, then cold-anesthetized, and fully engorged specimens were incubated as described in Chapter 2. After blood feeding, mice were euthanized and exsanguinated for viremia quantification by FFA.

Artificial Blood Meals

Artificial blood meals containing ZIKV were prepared at $\approx 4 \times 10^4$, 4×10^5, or 4×10^6 FFU/mL. Blood meals comprised 1% (wt./vol) sucrose, 7.5% fetal bovine serum (FBS), 12.5% washed human erythrocytes (University of Texas Medical Branch blood bank), 900 μM adenosine triphosphate, and viral dilutions in DMEM containing 2% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. After 1 h of feeding, mosquitoes were cold-anesthetized, and engorged females were extrinsically incubated. Infections were conducted in 4 separated experiments, with 1 of the 3 mosquito strains studied at a time, followed by the murine blood meal experiments.

Viral Sample Screening

CPE-positive saliva samples were titrated using the "Viral Assay" methodology described in Chapter 2 on 24-well plates of Vero cells. Means and SDs were calculated for all positive samples. Samples that were positive during initial screening but below the limit of detection (10 FFU) for the titration assay were given a value of limit of detection–1 for statistical analysis.

Statistical Analysis

For mosquitoes fed on artificial blood meals, the effect of mosquito strain, virus strain, and dpi, as well as interactions among these, on percentage of bodies infected was analyzed by using a nominal logistic regression, with separate analyses for each blood meal titer (≈ 4, 5, or 6 $\log_{10}$ FFU/mL). Because of the large number of comparisons, the threshold for significance (α) was set to an arbitrary but conservative value of 0.005. Next, the effects

of mosquito strain, virus strain, and dpi on disseminated infections, measured as the percentage of infected bodies that produced infected legs, were analyzed by using a nominal logistic regression, with separate analyses for each blood meal titer. Similarly, the effects of mosquito strain, virus strain, and dpi on transmission, measured as the percentage of mosquitoes with disseminated infection that secreted virus in the saliva, were analyzed by using a nominal logistic regression, with separate analyses for each blood meal titer. For both disseminated infection and saliva infection, interactions among each of the 3 independent variables were not fully explored, because some combinations were not included (i.e., some mosquito strain × virus strain × dpi combinations did not yield infected bodies). Virus titer in the saliva was not subject to statistical analysis because of small sample sizes. The effects of feeding mode (mouse versus artificial blood meal), virus titer, and dpi on the percentage of infections, disseminated infection, and transmission in Salvador *Ae. aegypti* mosquitoes fed on ZIKV virus strain FSS were analyzed by nominal logistic regression.

AEDES AEGYPTI RESULTS

We detected no statistically significant interactions among mosquito strain, virus strain, and dpi in any analysis of infection, dissemination, or transmission after mosquitoes fed on an artificial blood meal. Frequently, dpi significantly affected infection, dissemination, and transmission as expected based on the need for replication and dissemination in the mosquito, so these data are not presented in detail here.

When *Ae. aegypti* mosquitoes were fed on artificial blood meals at doses of 5 or 6 $\log_{10}$ FFU/mL of ZIKV, DAK AR 41525 (Figure 3.1, panels B, C; Figure 3.2, panels B, C; Figure 3.3, panels B, C) produced a significantly higher percentage of infection than did the same titers of strain FSS and MEX1–7 (Figure 3.1, panels B, C; Figure 3.2, panels B, C; Figure 3.3, panels B, C) across all 3 strains of *Ae. aegypti* ($p<0.001$ at 5 $\log_{10}$ FFU/mL, $p<0.002$ at 6 $\log_{10}$ FFU/mL). In addition, at the 2 higher doses, disseminated DAK AR

41525 infections produced a higher percentage of infectious saliva ($p<0.004$ at 5 $\log_{10}$ FFU/mL, $p<0.0001$ at 6 $\log_{10}$ FFU/mL). DAK AR 41525, however, did not result in a higher percentage of infections resulting in dissemination to the legs (a proxy for the hemocoel). At an artificial blood meal concentration of ≈4 $\log_{10}$ FFU/mL, we found no significant difference among the 3 ZIKV strains in infection, dissemination, or transmission. For all artificial blood meal concentrations, FSS 13025 and MEX 1–7 produced similar infection, dissemination, and transmission percentages in each mosquito population.

Fig. 1

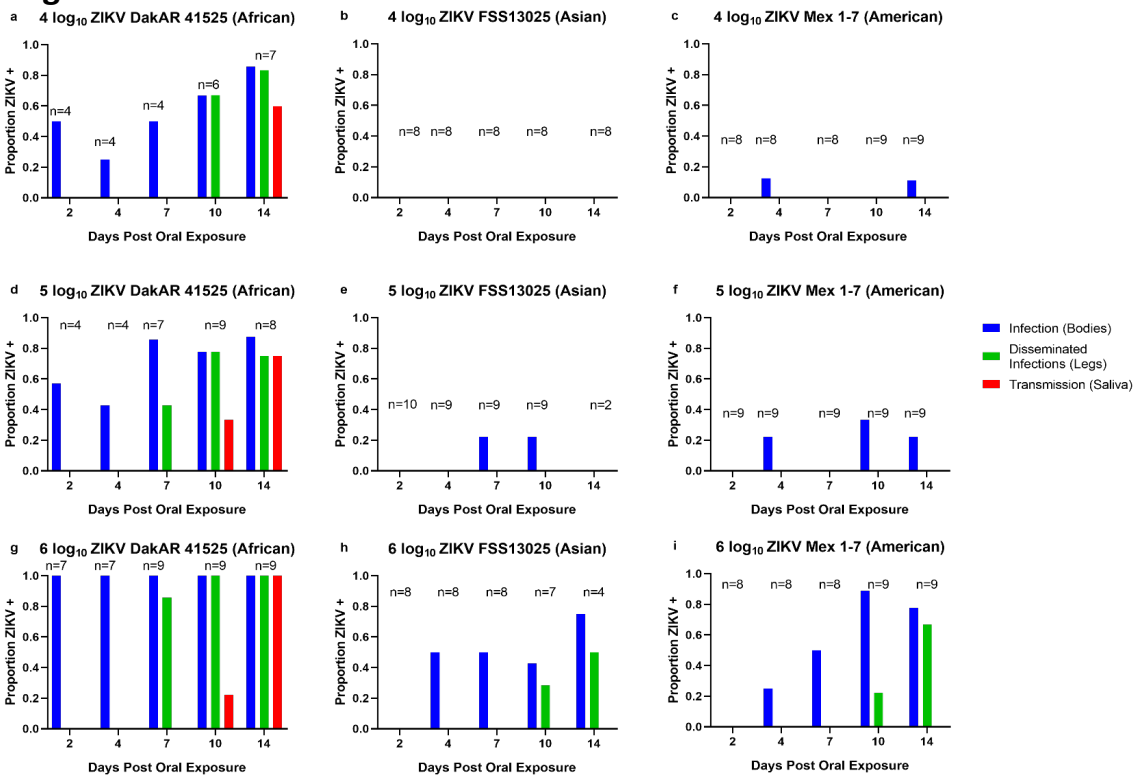

Figure 3.1 Vector Competence of *Aedes aegypti* (Salvador, Brazil F2) for Zika viruses

Infection, disseminated infection, and transmission of 3 Zika virus strains by *Aedes aegypti* mosquitoes from Salvador, Brazil, after artificial blood meals with a concentration of 4 $\log_{10}$ (A-C), 5 $\log_{10}$ (D-F), or 6 $\log_{10}$ (G-I) focus-forming units/mL.

Fig. 2

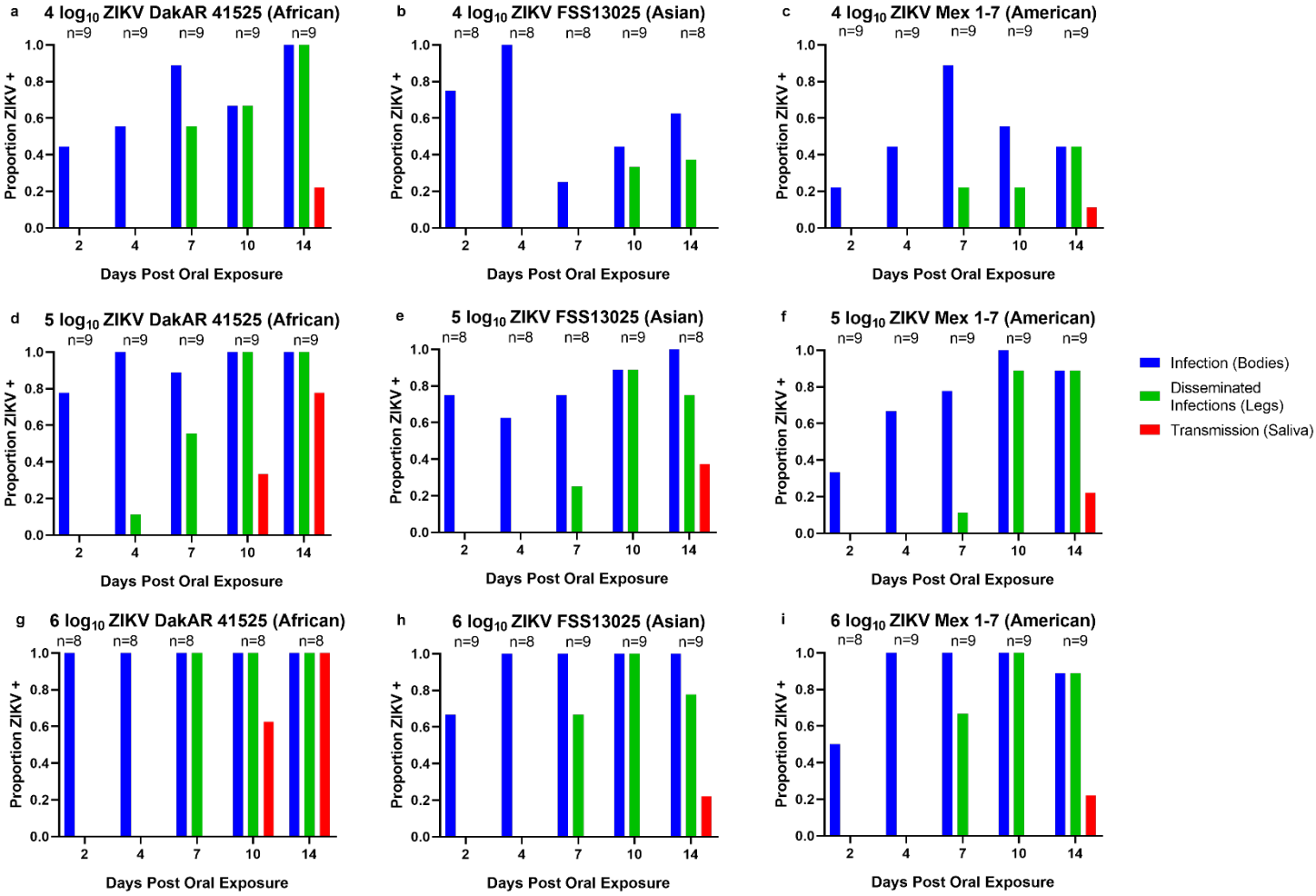

Figure 3.2 Vector Competence of *Aedes aegypti* (Salvador, Brazil F6) for Zika viruses

Infection, disseminated infection, and transmission of 3 Zika virus strains by *Aedes aegypti* mosquitoes from the Dominican Republic after artificial blood meals with a concentration of 4 $\log_{10}$ (A-C), 5 $\log_{10}$ (D-F), or 6 $\log_{10}$ (G-I) focus-forming units/mL.

Fig. 3

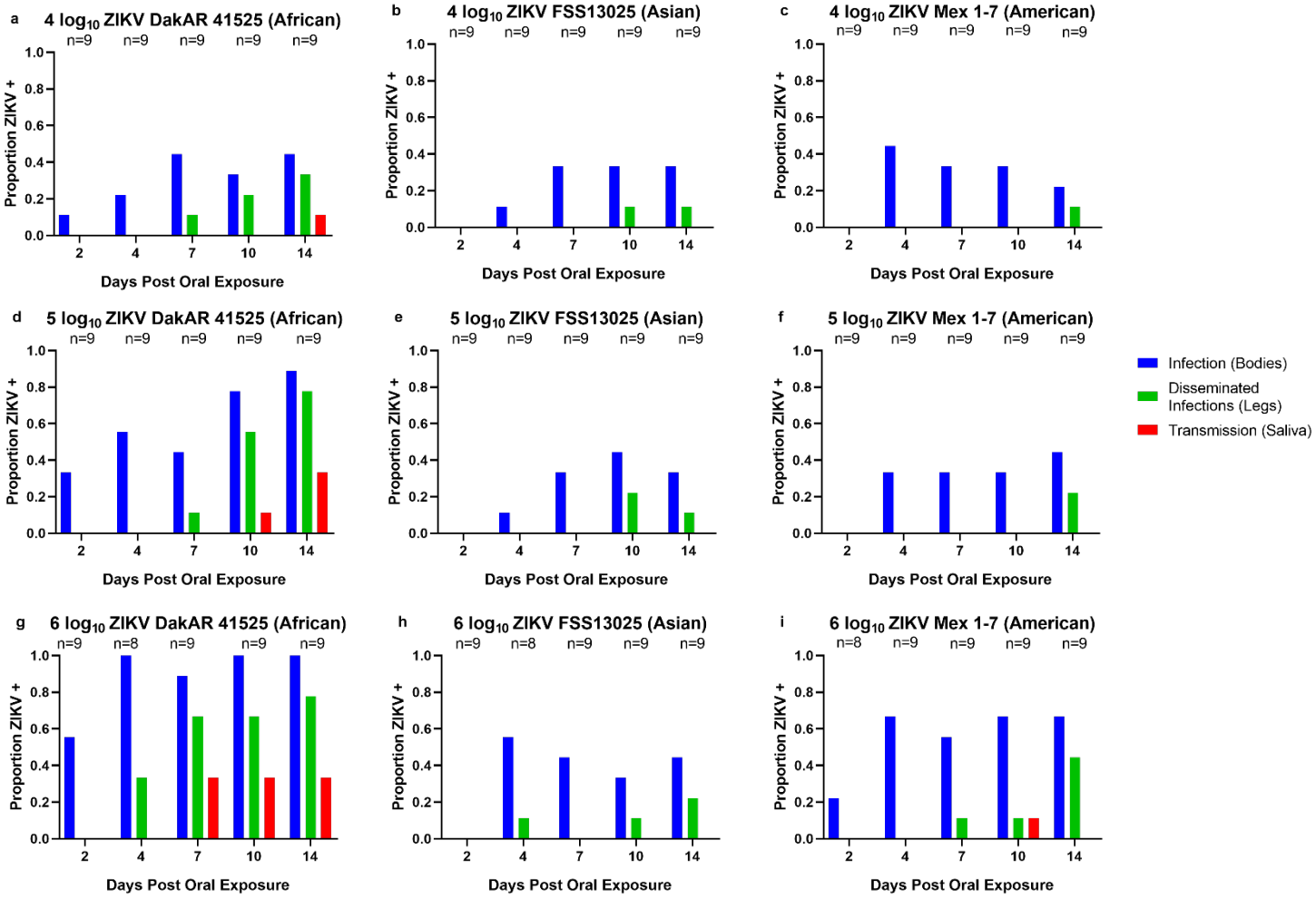

Figure 3.3 Vector Competence of *Aedes aegypti* (Rio Grande Valley F4) for Zika viruses

Infection, disseminated infection, and transmission of 3 Zika virus strains by *Aedes aegypti* mosquitoes from the Dominican Republic after artificial blood meals with a concentration of 4 $\log_{10}$ (A-C), 5 $\log_{10}$ (D-F), or 6 $\log_{10}$ (G-I) focus-forming units/mL.

When *Ae. aegypti* mosquitoes were fed on artificial blood meals at doses of ≈4, 5, or 6 $\log_{10}$ FFU/mL of Zika virus, a significantly greater percentage of mosquitoes from the DR (Figure 3.2) became infected than from the RGV (Figure 3.3) and Salvador populations (Figure 3.1) ($p<0.001$ at 4 and 5 $\log_{10}$ FFU/mL, $p<0.002$ at 6 $\log_{10}$ FFU/mL). At doses of 5 and 6 $\log_{10}$ FFU/mL, a greater percentage of *Ae. aegypti* mosquitoes from the DR with

disseminated infections had infectious saliva ($p<0.004$ at 5 $\log_{10}$ FFU/mL, $p<0.0001$ at 6 $\log_{10}$ FFU/mL). *Ae. aegypti* mosquitoes from the DR, however, did not have significantly higher percentages of infections that disseminated. For all artificial blood meal doses and Zika virus strains, *Ae. aegypti* from Salvador and the RGV had similar infection, dissemination, and transmission percentages.

Because virus titers and sampling days for mosquitoes fed on mice (4, 6, and 7 $\log_{10}$ FFU/mL sampled 7 and 14 days post-feeding) and artificial blood meals (4, 5, and 6 $\log_{10}$ FFU/mL sampled 2, 4, 7, 10, and 14 dpi) did not completely overlap, we first compared mosquito infection only for blood meal titers (≈4 and 6 $\log_{10}$ FFU/mL) and dpi (7, 14 dpi) that coincided between the 2 feeding methods Figure 3.1, panels A, C; Figure 3.4). A nominal logistic regression using these data (N = 81) showed no significant interactions among the independent variables; virus titer (χ^2 24.3, df = 1, $p<0.0001$) and feeding method (χ^2 9.7, df = 1, $p<0.0019$) significantly affected the likelihood of infection, whereas dpi did not (χ^2 0.33, df = 1, $p = 0.56$). Using this same dataset, we found that virus titer, feeding method, and dpi all significantly affected dissemination from infected bodies to legs (N = 50, $p<0.0001$ for all 3 variables). Because only 8 mosquitoes in this group produced infected saliva, we did not attempt analysis on this small sample. However, it was striking that only mosquitoes fed on mice produced infected saliva. An analysis using all data from Salvador mosquitoes fed on Zika virus strain FSS 13025 in artificial blood meals and mice revealed a significant effect of all 3 independent variables on infection ($p<0.0001$ for all comparisons), with infection being greater at higher blood meal titers and later time points after infection and from blood meals acquired from mice.

Fig. 4

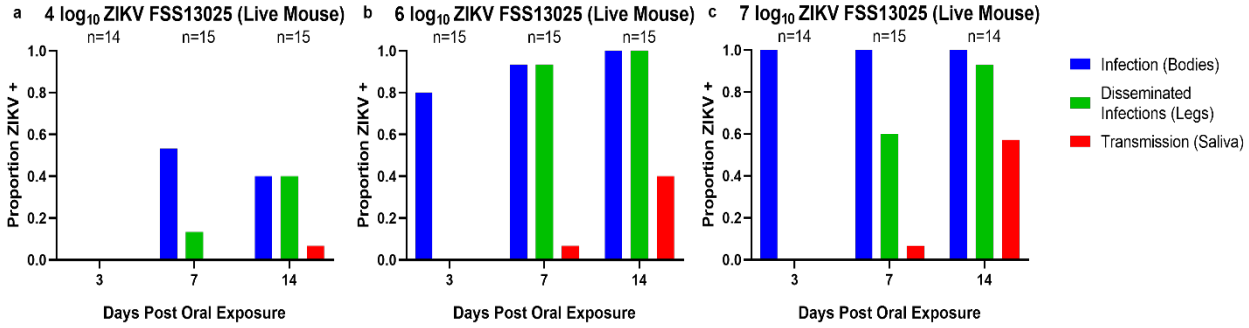

Figure 3.4 Vector Competence of *Aedes aegypti* (Salvador, Brazil F2) for ZIKV FSS13025 via viremic bloodmeal

Infection, disseminated infection, and transmission of the Zika virus strain FSS13025 by *Aedes aegypti* mosquitoes from Salvador, Brazil, after blood meals from infected A129 mice with viremic titers of 4 $\log_{10}$, 6 $\log_{10}$, or 7 $\log_{10}$ focus-forming units/mL.

Ae. aegypti mosquitoes from Salvador exhibited a minimum EIP of 10 days after artificial infection with Zika virus strain DAK AR 41525 at 5 and 6 $\log_{10}$ FFU/mL and 14 days after infection with FSS 13025 or MEX1–7 strains at 6 $\log_{10}$ FFU/mL and DAK AR 41525 at 4 $\log_{10}$ FFU/mL. *Aedes aegypti* mosquitoes from the DR exhibited an EIP of 10 days after artificial infection with Zika virus strain DAK AR 41525 at 5 and 6 $\log_{10}$ FFU/mL and 14 days after infection with FSS 13025 at 5 or 6 $\log_{10}$ FFU/mL, MEX1–7 strains at all 3 doses, and DAK AR 41525 at 4 $\log_{10}$ FFU/mL. *Ae. aegypti* mosquitoes from the RGV did not effectively transmit FSS 13025 or MEX1–7 at any titer (only 1 positive MEX1–7 saliva sample on 10 dpi) but showed an EIP of 7 days with strain DAK AR 41525 at 6 $\log_{10}$ FFU/mL, 10 days at 5 $\log_{10}$ FFU/mL, and 14 days at 4 $\log_{10}$ FFU/mL. Mosquitoes infected through murine blood meals showed an EIP of 7 days after a 6 or 7 $\log_{10}$ FFU/mL blood meal, and 14 days after a 4 $\log_{10}$ blood meal.

AEDES ALBOPICTUS RESULTS

Comparison of ZIKV Strains

RIO GRANDE AE. ALBOPICTUS

With respect to our analyses, it should be noted that ideally, the virus strains tested in all three populations of mosquitoes would be geographically matched, so as to facilitate easier comparisons. However, on availability, we replaced DakAR 41525 and FSS13025 with modern epidemic strains PRVABC 59 and PB 81 due to their increased public health relevance. Therefore, because virus and mosquito strains were not completely blocked, we could not compare all virus strains among all mosquito strains. Thus we first compared the impact of virus strain, viremia titer in the mouse, and day of extrinsic incubation, as well as interactions among all three factors, on infection of Rio Grande mosquitoes, which fed on MEX 1-7-, FSS 133205-, and DakAR 41525-infected mice. Substantially higher viremia titers were generated in mice infected by the DakAR 41525 strain compared with the other two (Figure 3.5). We found no significant interactions among the three factors, and a significant effect only of virus strain (df = 2, $\chi^2 = 7.07$, $P = 0.029$), with DakAR 41525 and MEX 1-7 infecting a significantly higher proportion of mosquitoes than FSS 13025. This effect was driven primarily by the greater infectivity of DakAR 41525 and MEX 1-7 at the lower viremia titers. We then compared the effect of the three factors on ZIKV dissemination in infected mosquitoes. This analysis revealed a significant interaction between viral strain and days of extrinsic incubation (df = 2, $\chi^2 = 10.5$, $P = 0.005$), but no significant interactions among the remaining factors. We therefore conducted a simple effects test of the impact of viral strain and viremia titer for each day of extrinsic incubation (3, 7, 14) individually. There were very few disseminated infections on day 3, precluding comparison, but on days 7 and 14 there were significant effects of both virus strain and viremia titer ($P \leq 0.001$ for all comparisons). Virus dissemination increased with increasing viremia titer, and at a comparable viremia titer, the DakAR 41525 strain of ZIKV (1.6×10^6 FFU/mL) disseminated more efficiently than the FSS 13025 (3.5×10^6 FFU/mL) or MEX 1-7 (1.0×10^6 FFU/mL) strains. Finally, we compared the efficiency of dissemination

by different ZIKV strains following different viremia titers and days of extrinsic incubation. This analysis detected a significant interaction between viremia titer and days of extrinsic incubation (df = 2, $\chi^2 = 12.8$, $P = 0.002$). Thus we conducted a simple effects test of the impact of virus strain and titer at days 7 and 14 of extrinsic incubation (no dissemination was detected at day 3 of extrinsic incubation). This analysis detected no significant differences at day 7 but a significant interaction of virus titer and strain on day 14, with virus secretion into the saliva increasing with viremia titer, and with the DakAR 41525 strain of ZIKV generating a higher percentage of infectious saliva than the other two strains. Overall, these results indicated that the DakAR 41525 disseminated more efficiently and was shed into the saliva more frequently in Rio Grande Valley *Ae. albopictus* compared with the MEX 1-7 and FSS 13025 ZIKV strains.

Fig. 5

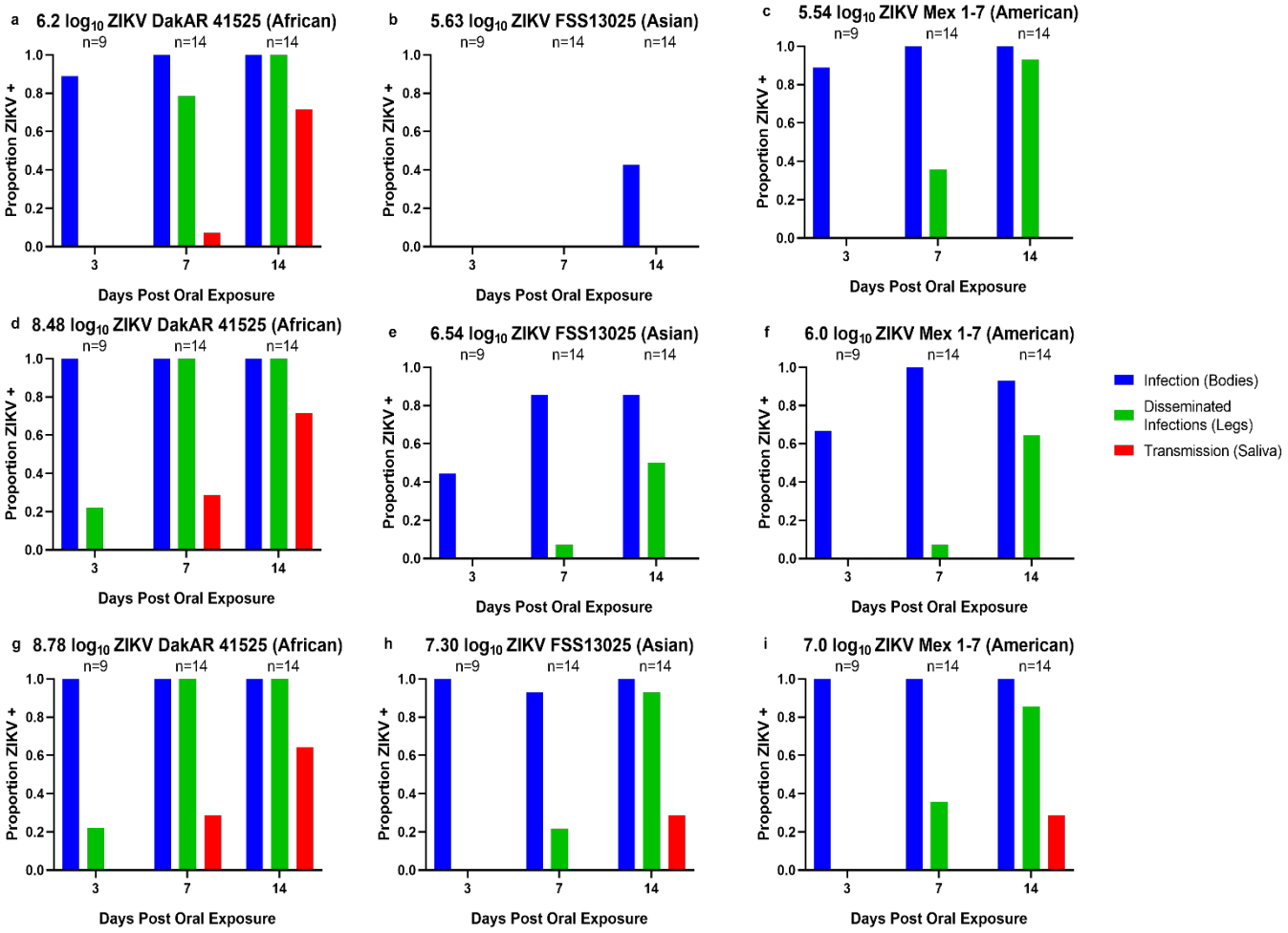

Figure 3.5 Vector Competence of *Ae. albopictus* (Rio Grande Valley, TX, F5) for ZIKV

Infection, disseminated infection, and potential transmission of three Zika virus strains by *Aedes albopictus* from the Rio Grande Valley, TX, following bloodmeal from viremic A129 mice infected with (A, D, G) DakAR 41525 (Senegal, 1984), (B, E, H) FSS13025 (Cambodia, 2010) or (C, F, I) MEX 1-7 (Mexico, 2015) and assays on day 3 (N = nine mosquitoes per virus), day 7 (N = 14), and day 14 (N = 14).

HOUSTON AE. ALBOPICTUS

A similar analysis to the one described earlier detected no significant interactions or main effects of virus strain, virus titer, or days of extrinsic incubation on infection of Houston mosquitos exposed to PB 81, MEX 1-7, and PRVABC 59 ZIKV strains. This analysis detected no significant interactions or main effects of virus strain, virus titer, or

days of extrinsic incubation on infection. Analysis of dissemination in infected bodies revealed a three-way interaction among the independent variables. A simple effects test of virus strain at viremia titer = 7.0 ± 0.2 $\log_{10}$ FFU/mL and day 14 of extrinsic incubation showed no differences among the three ZIKV strains. There were no interactions among the three factors on secretion of virus into saliva and only virus strain influenced this outcome, with PRVABC 59 ZIKV shed into saliva more efficiently than the other two strains (df = 2, χ^2 = 17.2, P = 0.0002), which failed to produce detectable virus in saliva. Overall, ZIKV strain PRVABC 59 was more efficiently shed into the saliva of Houston *Ae. albopictus* compared with PB 81 and MEX 1-7 (Figure 3.6).

Fig. 6

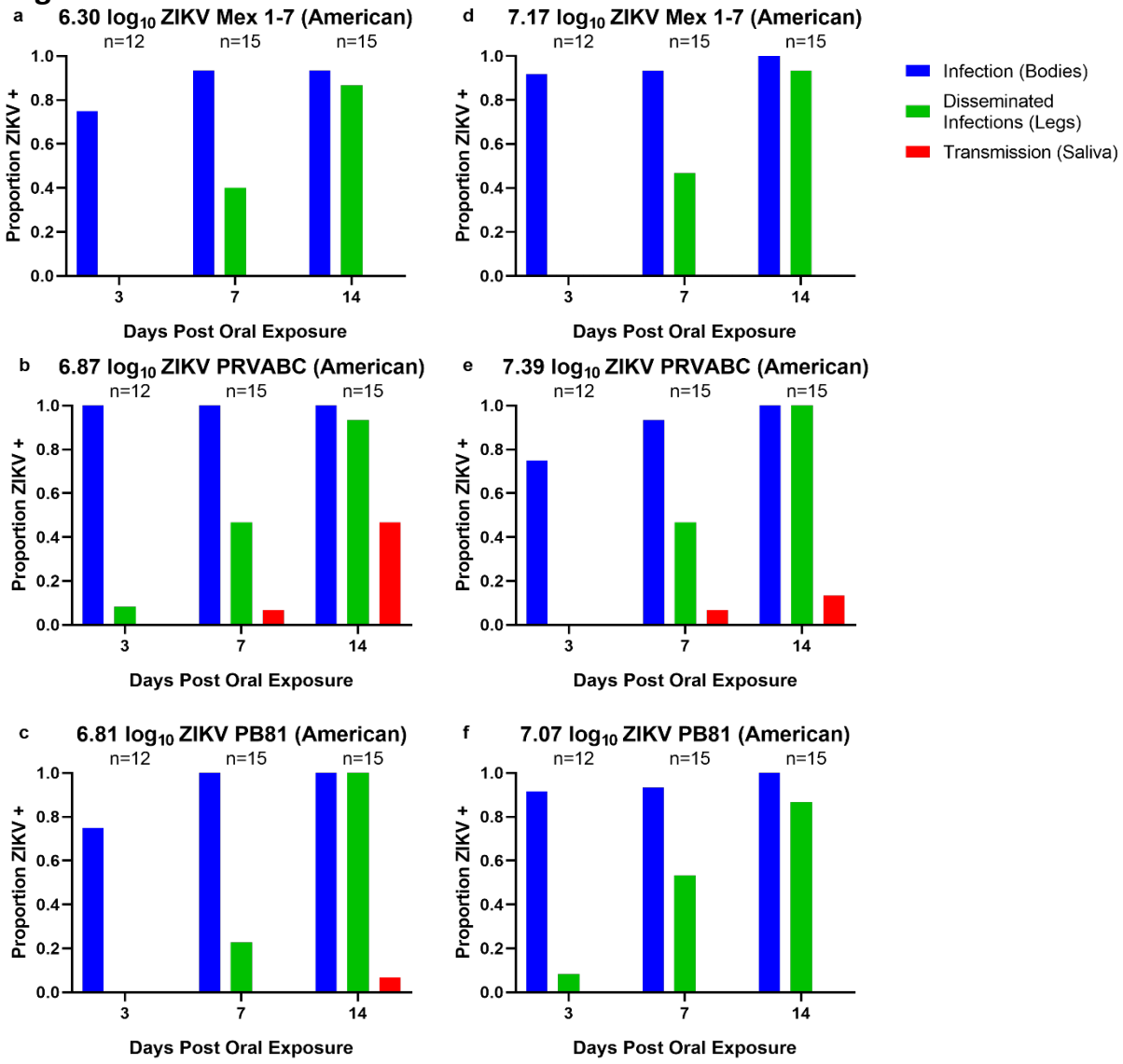

Figure 3.6 Vector Competence of *Ae. albopictus* (Houston, TX, F2) for ZIKV

Infection, disseminated infection, and potential transmission of three Zika virus strains by *Aedes albopictus* from the Houston, TX, following bloodmeal from viremic A129 mice infected with (A, D) MEX 1-7 (Mexico, 2015), (B, E) PRVABC59 (Puerto Rico, 2015) or (C, F) PB81 (Brazil, 2015) and assays on day 3 (N = 12 mosquitoes per virus), day 7 (N = 15), and day 14 (N = 15).

Only days of extrinsic incubation significantly influenced infection or dissemination of PB 81 and MEX 1-7 in Salvador mosquitoes ($P < 0.003$ for both comparisons). Neither virus strain was detected in mosquito saliva (Figure 3).

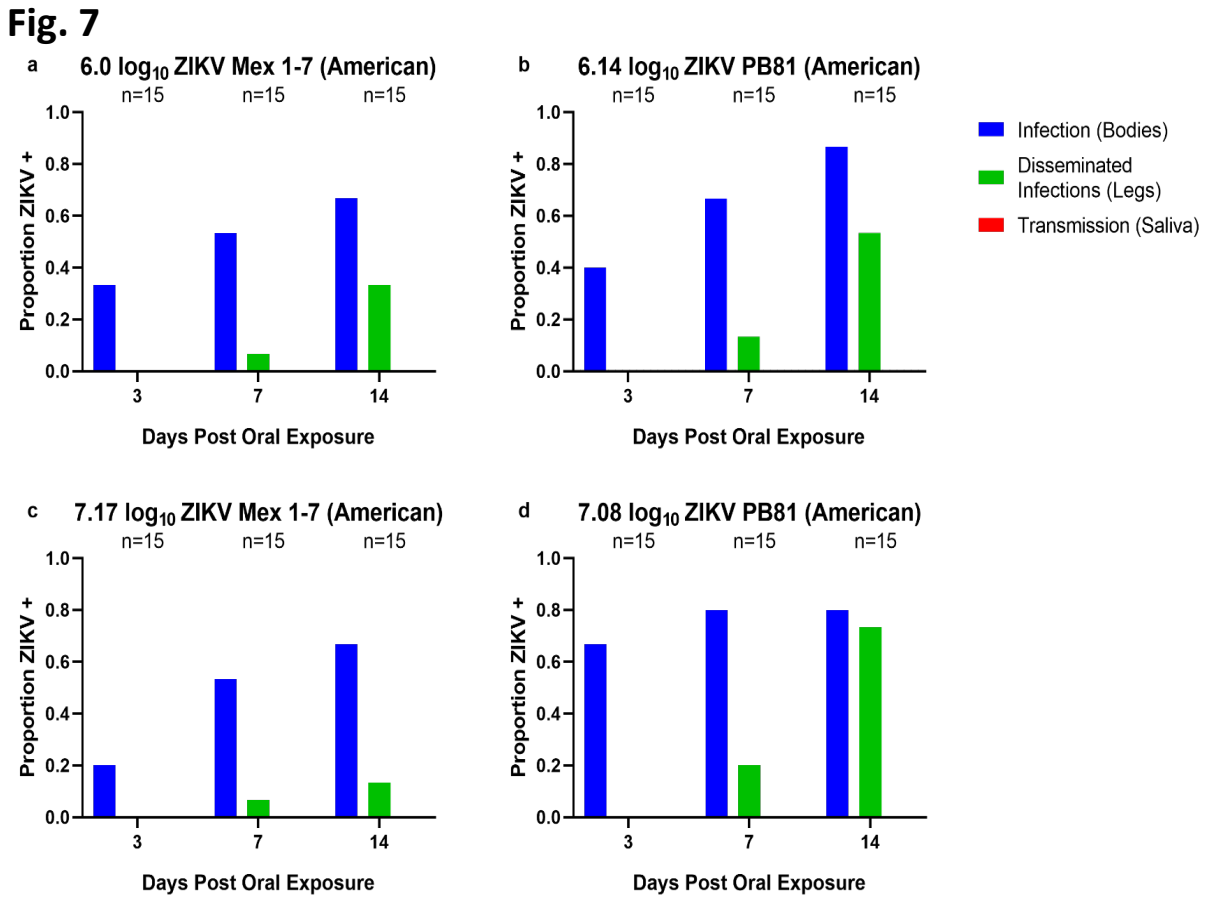

Figure 3.7 Vector Competence of *Ae. albopictus* (Salvador, Brazil, F3) for ZIKV

Infection, disseminated infection, and potential transmission of three Zika virus strains by *Ae. albopictus* from Salvador, Brazil, following bloodmeal from viremic A129 mice infected with (A, C) MEX 1-7 (Mexico, 2015), or (B, D) PB81 (Brazil, 2015) and assays on day 3 ($N = 15$ mosquitoes per virus), day 7 ($N = 15$), and day 14 ($N = 15$).

Comparison of Mosquito Strains

MEX 1-7 ZIKV

Using the same data set that we used to compare virus strains, we next compared the effect of mosquito strain, virus titer, and days of extrinsic incubation on infection by MEX 1-7, the only ZIKV strain fed to all three *Ae. albopictus* populations. We detected a significant interaction between virus titer and mosquito strain ($df = 2, \chi^2 = 6.48, P = 0.039$). We therefore proceeded to conduct a simple effects test of the impact of mosquito strain and days of extrinsic incubation with a viremia titer of 7.0 ± 0.2 $\log_{10}$ FFU/mL. This comparison revealed no significant interaction between the two factors and a significant effect only of mosquito strain ($df = 2, \chi^2 = 43.7, P < 0.0001$), with Houston and Rio Grande mosquitoes showing significantly higher susceptibility than Salvador mosquitoes. For dissemination from infected mosquitoes, we detected no significant interactions among factors, with a significant impact of both mosquito strain ($df = 2, \chi^2 = 16.7, P < 0.0002$) and days of extrinsic incubation ($df = 1, \chi^2 = 59.6, P < 0.0001$). As expected, dissemination increased as extrinsic incubation increased. Additionally, dissemination was significantly lower in the Salvador strain of *Ae. albopictus* than in the two U.S. strains. MEX 1-7 produced detectable virus in the saliva of only the Rio Grande mosquitoes. Overall, only *Ae. albopictus* from the Rio Grande Valley proved competent for transmission potential of MEX 1-7. However, *Ae. albopictus* from Houston proved more susceptible to disseminated infections of ZIKV strain MEX 1-7 when compared with mosquitoes from Salvador, Brazil.

PB 81 ZIKV

We then compared the infectivity of ZIKV strain PB 81 in the two mosquito strains tested: Houston and Salvador. We detected a significant interaction between mosquito

strain and days of extrinsic incubation ($df = 2, \chi^2 = 4.8, P = 0.03$), so we conducted a simple effects test of the impact of mosquito strain and virus titer for each day of extrinsic incubation, individually. This analysis revealed a significant effect of mosquito strain on days 7 and 14 ($P < 0.03$ for both comparisons), with Houston mosquitoes showing significantly greater susceptibility than Salvador mosquitoes. Dissemination among infected mosquitoes was shaped by a significant three-way interaction among mosquito strain, virus titer, and days of extrinsic incubation, with the general pattern that dissemination increased with virus titer and days of extrinsic incubation, but dissemination was generally higher in Houston mosquitoes. Virus was detected in the saliva of only the Houston mosquitoes. Overall, *Ae. albopictus* from Houston were more competent for transmission of ZIKV strain PB 81 compared with the Salvador, Brazil, population.

SALIVA TITERS

Because *Ae. albopictus* from the Rio Grande Valley and Houston were fed on different sets of ZIKV strains, it was not possible to compare the two mosquito populations. Instead, we compared the saliva titers of three viruses, DakAR 41525, FSS 13025, and MEX 1-7, in Rio Grande mosquitoes that were fed on viremia titers of 8.5, 7.3, and 7.0 $\log_{10}$ FFU/mL, respectively, and sampled on day 14 of extrinsic incubation. All three viruses reached median saliva titers of 1.0 $\log_{10}$ FFU per collection sample and mean titers of 2.1, 1.5, and 1.6 $\log_{10}$ FFU per collection sample in saliva, respectively, which did not differ significantly (Wilcoxon, $df = 2, N = 18, P = 0.78$). We then compared the saliva titers in Rio Grande mosquitoes fed on the DakAR 41525 strain of ZIKV at three different viremia titers (6.2, 8.5, 8.8 $\log_{10}$ FFU/mL, respectively) and sampled at day 14 of extrinsic incubation; these titers (3.3, 3.7, and 3.6 $\log_{10}$ FFU per collection sample) also did not differ significantly (Wilcoxon test, $df = 2, N = 29, P = 0.74$). Titers generally increased between

days 7 and 14 of extrinsic incubation, but the large number of negative day 7 samples precluded analysis of titer as a continuous variable.

50% Oral Infectious Dose

The 50% oral infectious dose (OID_{50}) values were interpolated utilizing the method of Reed and Muench when infection encompassed 50% at the doses used in the study. For a majority of *Ae. albopictus* populations fed on ZIKV strains the OID_{50} could not be calculated due to infection rates exceeding 50%, even at the lowest tested viremia titers (e.g., DakAR 41525). The FSS 13025 strain demonstrated an OID_{50} of 6.7 log_{10} FFU/mL for assay on day 7 of extrinsic incubation which decreased to 5.9 log_{10} FFU/mL by day 14 of extrinsic incubation in Rio Grande population of *Ae. albopictus*. PB 81 exhibited an OID_{50} of 6.8 log_{10} FFU/mL for assay on day 3 of extrinsic incubation in Salvador *Ae. albopictus*.

Culex quinquefasciatus and *Aedes taeniorhynchus* Results

Additional vector populations, two *Cx. quinquefasciatus* and one *Ae. taeniorhynchus*, from the Houston area were orally exposed to multiple strains of ZIKV including three American strains (MEX 1-7, MEX 1-44, and PRVABC59), one Asian strain (FSS13025), and one African strain (DakAR41525) via artificial bloodmeal or viremic A129 mice (Table 3.1). Samples were taken up to 17 days post-feed and were found to be altogether negative. No matter the combination of mosquito strain, viral strain, bloodmeal type, or bloodmeal titer, no sample showed positive infection (Table 3.1).

Table 3.1: Experimental Parameters for *Cx. quinquefasciatus* vector competence

Virus strain	Mosquito species/strain	Blood meal	Dose, log_{10} FFU/mL	No./time point	Days tested after feeding
MEX 1–44 (Mexico 2015)	*Culex quinquefasciatus* (colonized)	Artificial	6	20	10, 17

	Aedes taeniorhynchus (colonized)	Artificial	6	20	10, 17
DAK AR 41525 (Senegal 1985)	*Cx. quinquefasciatus* (colonized)	Artificial	4, 5, 6	20	3, 7, 14
FSS 13025 (Cambodia 2010)	*Cx. quinquefasciatus* (colonized)	Artificial	4, 5, 6	20	3, 7, 14
	Cx. quinquefasciatus (Houston F2)	Murine	4, 6, 7	5	3, 7, 14
MEX 1–7 (Mexico 2015)	*Cx. quinquefasciatus* (colonized)	Artificial	4, 5, 6	20	3, 7, 14
	Cx. quinquefasciatus (Houston F2)	Murine	6	26	14
PRABC59 (Puerto Rico 2015)	*Cx. quinquefasciatus* (Houston F2)	Murine	7	21	14

DISCUSSION

Aedes aegypti

Because no vaccine or therapeutic drugs are available, ZIKV prevention depends on controlling the mosquito vector or avoiding bites. Although some previous studies (92) showed relatively low ZIKV competence in *Ae. aegypti* mosquitoes, raising questions about the role of other potential vectors, others have shown this species to be highly

competent (89,90). We demonstrated that *Ae. aegypti* mosquito competency as a vector for ZIKV in the Americas varies greatly and depends on mosquito origin, ZIKV strain, and type of blood meal used. Recent studies demonstrated that preexisting DENV antibodies in ZIKV–endemic areas might enhance ZIKV infection in vitro (95) while others show evidence of protection (96) One in vivo study demonstrated that monoclonal antibodies to DENV envelope neutralize Zika virus in vitro and protect immunocompromised mice from lethal infection (97). The role of preexisting immunity to heterologous viruses remains unclear (98); thus, even a moderately competent vector, such as *Ae. aegypti* mosquitoes, might be able transmit efficiently because of its highly anthropophilic behavior and ready access to homes without screening or air conditioning in much of Latin America and the Caribbean.

In agreement with previous studies (92), we demonstrated significant variation in competency for ZIKV transmission among *Ae. aegypti* mosquito populations from 3 different parts of the Americas. After artificial blood meals, strains FSS 13025 and MEX1–7 were refractory to transmission in all populations; we detected only 1 positive saliva sample after large oral doses. In contrast, mosquitoes from the DR were susceptible to and able to transmit all 3 ZIKV strains. A similar difference in DENV competency has been noted in comparisons of *Ae. aegypti* mosquito populations from different geographic locations (93). This variation could be due to genetic differences among mosquitoes or differences in microbiome, virome, or immune activation. Understanding differences in competency and underlying mechanisms could help guide new strategies to control this vector.

In addition to differences in competency among *Ae. aegypti* mosquito populations, we showed a significant difference in infectivity among Zika virus strains. DAK AR 41525 was the only strain capable of disseminating and being transmitted by all mosquito strains. Furthermore, in mosquitoes from the DR, which were susceptible to all 3 ZIKV strains, DAK AR 41525 disseminated the most rapidly and resulted in the greatest proportion of

infectious saliva. This finding is surprising given that African ZIKV strains have never been associated with outbreaks involving *Ae. aegypti* mosquitoes.

Another contribution of our findings is the higher infectivity from murine blood meals than from artificial meals. Artificial blood meals are known to be less infectious than natural meals, at least in part because of the lack of coagulation and concentration of the virus adjacent to the mid-gut epithelium (97,103). Also, in the case of DENV and St. Louis encephalitis virus, frozen stocks are less infectious for *Ae. aegypti* mosquitoes than freshly harvested, cell culture–derived virus (104). The FSS 13025 strain of Zika virus infected only 75% of Salvador *Ae. aegypti* mosquitoes at 6 $\log_{10}$ FFU/mL by 14 dpi from an artificial blood meal, with 67% of these infections disseminating, and 0% involving the saliva. In contrast, 14 dpi after feeding on an infected mouse with a 6 $\log_{10}$ FFU/mL viremia, 100% infection occurred; 92% of these were disseminated, and 61% of disseminated infections reached the saliva. With titers as low as 4 $\log_{10}$ FFU/mL in murine blood meals, 40% of mosquitoes became infected, of which 100% were disseminated and had ZIKV detected in saliva. This dramatic difference in competency after artificial versus viremic blood meals undoubtedly contributed to the underestimation of *Ae. aegypti* mosquitoes as a ZIKV vector in previous studies (92).

An important determinant of vectorial capacity is the EIP, that is, the time before a virus can be found in the saliva of a mosquito after an infectious blood meal. A short EIP facilitates rapid spread, whereas a long EIP gives a larger window for mosquito death, including by human intervention. The 7-day minimum EIP we estimated after a murine blood meal, and 7–10 days after an artificial blood meal, are comparable to those of other flaviviruses in mosquitoes incubated at similar temperatures.

Another major factor in vector transmission is the amount of virus inoculated in the saliva, which can affect pathogenesis (105); this value is critical for determining realistic animal model doses. We found saliva titers of up to 4 $\log_{10}$ FFU per collection, with the following mean ±SD $\log_{10}$ FFU/collection for each mosquito–virus strain combination:

Salvador mosquitoes, DAK AR 41525: 2.49 ±2.93; DR mosquitoes, DAK AR 41525: 2.72 ±3.26; DR mosquitoes, MEX1–7: 2.30 ±2.35; RGV mosquitoes, DAK AR: 2.20 ±1.96; Salvador mosquitoes, FSS 13025 infected through a murine blood meal: 2.77 ±3.00. Because of the dearth of positive saliva samples, no statistically significant differences were found for these means. These infectious saliva titers are based only on a small number of positive samples after artificial blood meals. Some studies have found that in vitro salivation overestimates the amount of an arbovirus inoculated in vivo (106); others have found the inverse (67). Additional studies are needed to precisely determine the amount of virus transmitted by a ZIKV–infected *Ae. aegypti* mosquito.

Ideally, in investigations of viral adaption to vectors, virus and mosquito origins should be matched. Mosquitoes colonies matching the locations of the ZIKV strains reported here were unavailable. However, vector-adaptive mutations in arboviruses are unlikely to remain geographically isolated because they spread more efficiently (107,88). Therefore, adaptive evolution was investigated on the basis of available mosquitoes with minimal colonization histories, from sites at risk for Zika virus transmission or with reported autochthonous transmission. Surprisingly, despite the use of minimally colonized mosquitoes, most susceptible population of *Ae. aegypti* from the DR had the longest history of 6 generations. Previous studies demonstrated altered DENV-2 susceptibility for *Ae. aegypti* colonized for >4 generations (107).

Although human Zika virus viremia is not well characterized, a Micronesia study found viral RNA concentrations of 900–729,000 RNA copies/mL (94). Recent case studies have estimated ranges of 1.47–2 $\log_{10}$ PFU/mL (95), 0.49–3.39 $\log_{10}$ FFU/mL [49], 2.20–2.75 $\log_{10}$ PFU/mL, and 1.88–2.80 $\log_{10}$ PFU/mL (110). This wide range might reflect the sampling of most patients after peak viremia has passed, which complicates selecting realistic doses for mosquito competency studies. As our artificial blood meal titers were higher than those described during human infection, these results may overestimate the role of these vectors.

Aedes albopictus

These results demonstrate the vector competence of *Ae. albopictus* from various geographic locations in the Americas for multiple strains of ZIKV, an important component of establishing risk and designing control strategies. To determine the role that *Ae. albopictus* may play in outbreak settings (vectorial capacity), additional factors such as range, longevity and feeding behaviors, especially as compared with the domestic and highly anthropophilic *Ae. aegypti* must be considered. *Aedes albopictus* is widespread throughout many temperate regions of the United States where *Ae. aegypti* is not typically found, such as the upper Midwest and the northeast (111-113). Anthropophilic and endophilic feeding behaviors of *Ae. aegypti*, however, make the species more apt at transmitting human arboviruses when compared with the more opportunistic and exophilic blood feeding behavior of *Ae. albopictus* (111,112). Additionally, the tendency of *Ae. aegypti* to take multiple blood meals per gonotrophic cycle means these mosquitoes are more likely to become infected and also more likely to transmit to multiple people once infected compared with *Ae. albopictus* mosquitoes (112,113). ZIKV has been detected in both species during outbreaks, *Ae. albopictu*s in Gabon in 2007 (115) and *Ae. aegypti* in Mexico in 2015 (109) and Brazil in 2016 (101), indicating that each species may play a role in ZIKV epidemics.

As demonstrated in previous studies with both *Ae. aegypti* and *Ae. albopictus*, we found that vector competence of *Ae. albopictus* varies with the geographic origin of both vector and virus strain. To more accurately reflect natural infection, we used viremic A129 mice, previously shown to be more infectious for mosquitoes than artificial bloodmeals. For example, when *Ae. aegypti* from Salvador, Brazil, were orally infected with 6 $\log_{10}$ FFU/mL of ZIKV (FSS 13025) by artificial bloodmeal, by day 14 of extrinsic incubation, 75% of mosquitoes were infected, with 67% of those infections disseminating, but never with virus detected in the saliva. When the same population of mosquitoes was

infected via a viremic A129 mouse circulating 6 $\log_{10}$ FFU/mL, on day 14 of extrinsic incubation all tested mosquitoes were infected, with 92% of infections disseminating and 61% of the disseminated infections reaching the saliva (80). This pronounced difference has been observed with other arboviruses such as western equine encephalitis virus, and is at least partly explained by clotting of blood ingested from an animal resulting in greater viral concentration directly adjacent to the mosquito midgut epithelium (102). Recently, the reduction of infectivity as a result of freeze-thaws was demonstrated for ZIKV infection of *Ae. aegypti* (102). All of these studies support the use of viremic animals for accurately assessing vector competence.

Strikingly, our findings with *Ae. albopictus* from the Rio Grande Valley of Texas corroborate field findings in Gabon during the 2007 outbreak (115). When Rio Grande Valley mosquitoes were exposed to the African lineage ZIKV strain DakAR 41525 at 6 and 8 $\log_{10}$ FFU/mL, they were uniformly infected and developed disseminated infections, such that by day 14 of extrinsic incubation, 60% of mosquitoes had shed virus into their saliva (Figure 1C). Phylogenetically, the Gabon 2007 strain clusters closely with the 1984 Senegal ZIKV strain (DakAR 41525) based on envelope and NS3 genes (113). Additionally, recent studies (116) as well as our own data (80) have also demonstrated efficient transmission of African lineage ZIKV strains by American *Ae. aegypti*. In sum, these data suggest that the African lineage of ZIKV is well adapted for urban transmission by both *Ae. aegypti* and *Ae. albopictus*.

A limitation of our understanding of vector competence for ZIKV is the dearth of data for infectious human viremia profiles, with titers ranging from 0.49 to 3.39 $\log_{10}$ infectious particles/mL or 900 to 729, 000 RNA copies/mL (94,109,117), orders of magnitude below the viremia titers to which mosquitoes in our analyses were exposed, and well below our estimated OID_{50} of both FSS 13025 and PB 81 in *Ae. albopictus* from the Rio Grande and Salvador, Brazil, respectively. Another limitation is that previous studies found that colonization of *Ae. aegypti* and *Ae. albopictus* alters their competence for

DENV (107,115). However, our use of low-generation colonies should have minimized these potential artifacts. Also, ideally our mosquito and virus strains would have been matched, and all possible combinations would have been tested. This was not possible because of logistical constraints and the acquisition of virus and mosquito strains after the study was initiated. For example, on availability, we replaced DakAR 41525 and FSS13025 with recent epidemic strains PRVABC 59 and PB 81 due to their increased public health relevance.

Our data strongly demonstrated that, while all tested populations of *Ae. albopictus* proved susceptible to midgut and disseminated infections at varying efficiencies, Houston and Salvador mosquitoes were relatively incapable of transmitting ZIKV. In order for an arbovirus to be transmitted by a competent vector, it must 1) be ingested via a bloodmeal from an infected host; 2) infect epithelial cells of the mosquito midgut; 3) disseminate from the midgut into the hemocoel and infect further tissues; 4) infect the salivary glands; and 5) be shed into acinar cavities for inoculation into a new host upon subsequent feedings (38,119). The relative inability of Houston and Salvador *Ae. albopictus* to transmit ZIKV strains, despite the presence of disseminated virus in hemocoel, suggests the possibility of a salivary gland infection or salivary egress barrier [66]. In the case of the former, ZIKV may be failing to infect secondary amplification tissues such as the fat bodies, hemocytes, nerve, or muscle tissues following midgut escape, preventing sufficient replication to efficiently infect the salivary glands. Anatomic analyses utilizing ZIKV reporter systems (59) in transmission-competent versus transmission-incompetent *Ae. albopictus* populations are needed to address these hypotheses.

The explosive spread of ZIKV throughout tropical and subtropical regions of the Americas has raised concerns that mosquitoes other than *Ae. aegypti* may be transmitting ZIKV [68]. In our study, *Ae. albopictus* from Salvador, Brazil, orally exposed to two American strains of ZIKV (MEX 1-7 and PB 81) at high titers (6 or 7 $\log_{10}$ FFU/mL) shed no virus into saliva, even by day 14 of extrinsic incubation. Although we tested only one

Brazilian mosquito population, the lack of transmission competence in the population tested with high viremia titers, coupled with a lack of field data from Mexico and Brazil reporting ZIKV positive *Ae. albopictus* pools, calls a significant role for this species into question, especially when taken together with previous reports on the competency of *Ae. albopictus* from Jurjuba, Rio de Janeiro. Of the seven populations of mosquitoes (all of which were populations from the Americas, five *Ae. aegypti* populations and two *Ae. albopictus* populations) tested in that study, the Jurjuba *Ae. albopictus* proved to be the least susceptible, although the transmission potential of this population was not tested (116).

A critical component of viral pathogenesis is the infectious dose, or the saliva titer for arboviruses (104). We found a range of saliva titers, with a maximum titer of 3.72 $\log_{10}$ FFU/collection of DakAR 41525 in a Rio Grande Valley mosquito sample. Analyses in other arboviruses have reported a large variation in saliva titers dependent on the virus and vector in question. For example, when the *alphavirus* Venezuelan equine encephalitis virus (VEEV) was used to infect *Ae. albopictus* and *Ae. taeniorhynchus* (Wiedemann) and saliva was collected in vitro, the resultant titers ranged from 0.2 to 1.1 $\log_{10}$ and 0.2 to 3.2 $\log_{10}$ plaque forming units (PFU) per collection, respectively (121). Further analyses comparing in vitro saliva collection of VEEV to in vivo inoculation revealed that artificial salivation (mean 74 PFU) overestimates the in vivo inoculum by nearly 10-fold (mean 11 PFU) (104). Similar analyses of in vivo saliva deposition of WNV from four different species of mosquitoes (*Culex tarsalis* Coquillet, *Culex pipiens* Linnaeus, *Aedes japonicus* [Theobald], and *Aedes triseriatus* [Say]) into a murine host yielded a range of saliva titers (3.4–6.1 $\log_{10}$ PFU) (105), whereas a separate analysis of WNV utilizing saliva collected from *Cx. tarsalis in vitro* yielded a titer of 1.41 $\log_{10}$ PFU (122). Further complicating in vitro collection of mosquito saliva is the finding that mosquitoes demonstrate host-seeking behavior until imbibing 2.5–3.5 μL of blood (120). In vitro saliva collections (our own as well as previous reports (80,81) comprise minimal volumes (≤

potential for mosquitoes to imbibe a significant percentage of expectorated saliva). To account for potential volume loss, we report our results as FFU per collection, as opposed to FFU/mL. In summary, disparate methodologies of saliva collection from different mosquito populations infected with different viruses limit what conclusions can be drawn from salivary viral titers and underscore the necessity for standardization of methodologies.

Like other vector competence studies (92), our data suggest significant variation as a function of mosquito origin and viral strain. Laboratory competency studies have produced disparate findings, with *Ae. albopictus* populations being shown to be both poor and relatively competent vectors (124,125). These disparities underscore the need for further studies in both the laboratory and the field to determine the potential role that *Ae. albopictus* may play in future ZIKV outbreaks, especially in temperate climates where *Ae. aegypti* cannot survive cold winters. Variables among this and other vector competence studies reported in the literature, such as colonization, mosquito microbiome composition, and genetics differences, should be further explored to determine their impact on ZIKV transmission.

Culex quinquefasciatus

While almost all published results support *Ae. aegypti* and potentially *Ae. albopictus* as urban vectors, some articles (126-129) suggest that *Cx. quinquefasciatus* may serve as a ZIKV vector. Accurately identifying the vector of a pathogen enables public health agencies to implement appropriate control strategies and inform citizens of proper prevention measures (130). Additionally, establishing the vector for an emerging pathogen paves the way for researchers to advance our understanding of virus–vector interactions and pursue novel methods of control. In contrast, erroneously incriminating a vector could lead to misdirected use of limited government funds, diversion of research efforts and misinforming the public through misdirected media and educational programs.

Traditional criteria for arthropod vector incrimination include: (i) demonstration of feeding or other effective contact with pathogen's host; (ii) association in time and space of the vector and the pathogen-infected host; (iii) repeated demonstration of natural infection of the vector and (iv) experimental transmission of the pathogen by the vector (130).

For ZIKV transmission in the Americas, criterion 3 has been met only for *Ae. aegypti*, with detection of naturally infected mosquitoes with titers compatible with transmission competence in Mexico (109) and Brazil (115). *Ae. albopictus* has also been shown to be capable of laboratory transmission (81,124,131-133). Although no field infections have been reported for *Ae. albopictus* in the Americas, they were detected during a 2007 Gabon outbreak (114). In locations where *Ae. aegypti* has been found infected at high rates in the Americas, testing of *Cx. quinquefasciatus*, typically the most common urban tropical mosquito, was unsuccessful aside from three pools collected in Recife, Brazil described by Guedes *et al.* (90). However, the ZIKV RNA levels measured in these Recife pools, reflected in high Ct values (37.6–38.15) representing <10 infectious units in typical RT-qPCR assays, are incompatible with transmission-competent mosquitoes, which typically have viral titers several orders of magnitude higher (81,131-133). Even naturally infected mosquitoes without viral dissemination to the salivary glands typically have higher titers (133) than reported by some of the *Culex* transmission studies (90). Thus, the wild-caught *Culex* mosquito pools were likely contained trace amounts of residual viremic blood in their guts or legs or other dislodged appendages from other infected mosquitoes of different species, or were false-positives.

Many other studies have found no transmission competence (131,132,134,135), even after examining several combinations of geographic strains of mosquito as well as ZIKV, along with different methods of oral exposure. These include other studies from Brazil with colonies established in 2016 and another study from China (132). Even after intrathoracic inoculation, generally the most permissive route for arbovirus infection of

mosquitoes, *Culex* mosquitoes were found to be refractory to disseminated infection (117,136). Although some of these studies found infection of the midgut without dissemination to the saliva, the majority found no indication of any infection after oral exposure. Many of these ZIKV-refractory populations tested are highly competent for West Nile and St. Louis encephalitis flaviviruses, so the specific ZIKV block in these populations would need to be restricted to most but not all *Cx. quinquefasciatus* populations to explain the results of the *Culex* transmission studies. In addition, *Cx. quinquefasciatus* from Recife challenged in another study with the same BRPE243/2015 ZIKV strain used by one study (126) as well as with two other ZIKV strains, were consistently refractory to oral infection (137).

The discrepancy between the negative results from so many published studies and the questionable findings of a select few studies should engender caution in interpretation and conclusions reported by media and public health authorities unless they are verified by more robust results including detection of genuinely transmission-competent mosquitoes in nature. Until further data are collected and other groups can replicate the *Culex*-positive transmission findings, it is important that public education and interventions remain focused on the conclusion supported by the vast majority of studies: *Ae. aegypti* is the only mosquito species for which we have strong evidence of ZIKV transmission in the Americas.

Chapter 4: The Effect of Colonization on *Aedes aegypti* Vector Competence and Microbiome

RATIONALE

As the primary vector of a number of arboviruses, *Aedes aegypti* is of significant concern to global health and emerging infectious diseases (32). Studying the vector competence, the susceptibility of a mosquito to infection and subsequent transmission ability of a given agent, is one of the most important tools available to researchers as it can help identify the mosquito species potentially responsible for transmission and give insight into vector-virus interactions (32,37). These studies inform major vector control responses and can lead to novel vector intervention strategies. Studies that come to unsupported conclusions may lead to misappropriation of funds and research effort, and undue public concern.

While vector competence is only one component of vectorial capacity, which gives a more complete picture of the interaction between vector, virus, and human populations (as described in chapter 1), vector competence can be readily studied in laboratory settings. This, combined with the ease of manipulation of *Ae. aegypti* compared to other vector species, leads to a large number of publications examining vector competence (32). This was especially evident following the ZIKV outbreak in the Americas (32). With such interest and research effort focused on examining vector competence, it is evidently important that the mosquito populations being used for this work resemble mosquitoes in the wild as closely as possible. However, for the functions of mass-rearing, ease of use, and other experimental purposes, mosquitoes are reared under controlled insectary conditions for multiple generations before being used in experiments.

During these generations of rearing in the insectary, mosquitoes can quickly become adapted to laboratory settings. As discussed in chapter one, the mating (50,53),

development (49), genes (62-63), microbiome (56-58), and vector competence (68-70) have all been shown to change as mosquitoes spend generations in an insectary setting. Furthermore, one large scale study found that observed changes were not consistent across replicate populations, suggesting a strong founder effect (49), a reduction in genetic diversity in a population due to starting with a small number of individuals. This makes predicting and accounting for these changes even more difficult.

Many of the existing studies regarding the colonization of mosquitoes only examine single timepoints of colonized populations to either field populations or populations from earlier in colonization (49,56,58-63,67-70). While this does provide some valuable data regarding overall effects of colonization, it does not allow for examining how quickly the process of adaptation to laboratory conditions occurs. Additionally, when examining multiple factors, single timepoints make it more difficult to establish cause-effect relationships that may happen temporally over multiple generations. This is particularly important when considering vector competence, which is impacted by a number of underlying factors (37).

A significant factor of recent focus is the mosquito microbiome (64). The microbiome is capable of directly interacting with viruses within the mosquito as well as interacting with the mosquito immune system, activating pathways which are then primed to additionally respond to viruses (64). As both the microbiome (56-58) and vector competence (68-70) have been shown to be altered following colonization, understanding changes to these factors may help elucidate interactions that lead to the colonized phenotype.

In order to test the hypothesis that vector colonization would result in a decrease in microbiome diversity and an overall increase in vector competence, we observed the colonization process of field-collected mosquitoes for ten generations. *Aedes aegypti* were collected from Rio Grande Valley, Texas and reared under standard insectary conditions for ten generations. Each generation, beginning with the field-collected mosquitoes, vector

competence was determined for ZIKV. The microbiome of mosquitoes was also characterized for several of the generations to examine any trends in changes as well as any correlation with changes in vector competence. The microbiome sequencing was done prior to any exposure to blood meal or ZIKV in order to examine the mosquitoes' baseline populations.

CHAPTER METHODS

Viruses

The virus used throughout these studies was derived from the PRVABC59 full length infectious clone, as described in Chapter 2 and listed in Table 2-1. A single, large-scale viral stock was prepared at the start of the study and the same stock was used throughout all vector competence experiments for subsequent mosquito generations. The stock was checked by focus forming assay, as described in Chapter 2, every 6 months to ensure that viral titer had not significantly dropped.

Mosquitoes

The mosquitoes used throughout these studies were a colony started from field-collected adults (F0 throughout this Chapter), as described in Chapter 2 and listed in Table 2-2. Adult mosquitoes were collected using BG Sentinel traps baited with dry ice in Weslaco, Texas in June 2018. *Aedes aegypti* mosquitoes were identified and sorted based on morphology. For microbiome sequencing of the F0 generation, 20 females were randomly selected once all *Ae. aegypti* from traps had been sorted; 114 females were used for the competence study of the F0 generation. Remaining females (>200 individuals) and all males (>200 individuals) were maintained as a colony to minimize founder effect.

Artificial Bloodmeals

Artificial blood meals containing ZIKV were prepared at ~1×10^6 FFU/mL. Blood meals comprised 1% (wt./vol) sucrose, 7.5% fetal bovine serum (FBS), 12.5% washed

sheep erythrocytes (Colorado Serum Company), 900 μM adenosine triphosphate, and viral dilutions in DMEM containing 2% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. After 1 hour of feeding, mosquitoes were cold-anesthetized, and engorged females were extrinsically incubated as described in Chapter 2.

Microbiome Sequencing

High-throughput sequencing of the bacterial 16S ribosomal RNA gene was performed using genomic DNA isolated from each sample. Sequencing libraries for each isolate were generated using universal 16S rRNA V3-V4 region primers in accordance with Illumina 16S rRNA metagenomic sequencing library protocols (71). The samples were barcoded for multiplexing using Nextera XT Index Kit v2. Sequencing was performed on an Illumina MiSeq instrument using a MiSeq Reagent Kit v2 (500-cycles).

To identify the presence of known bacteria, sequences were analyzed using the CLC Genomics Workbench 12.0.3 Microbial Genomics Module. Reads containing nucleotides below the quality threshold of 0.05 (using the modified Richard Mott algorithm) and those with two or more unknown nucleotides or sequencing adapters were trimmed out. Reference based OTU picking was performed using the SILVA SSU v132 97% database (72). Sequences present in more than one copy but not clustered to the database were placed into de novo OTUs (97% similarity) and aligned against the reference database with 80% similarity threshold to assign the "closest" taxonomical name where possible. Chimeras were removed from the dataset if the absolute crossover cost was three using a k-mer size of six. Alpha diversity was measured using Shannon entropy and total number of genera, rarefaction sampling without replacement, and with 100 replicates at each point. Plots show the rarefication at 2106 reads. Statistical

comparisons were performed using Kruskal-Wallis and Dunn's multiple comparisons tests. Beta diversity was calculated using the Bray-Curtis diversity measure (OTU level. Differentially abundant bacteria (genus level) were identified using analysis of composition of microbiomes (ANCOM) (73) with a significance level of $P < 0.05$, while values quantifying fold change were obtained using the log2 fold change formula (74).

RESULTS

Vector Competence

The infection, dissemination, and saliva infection status of mosquitoes was determined at 10 days after oral exposure to ZIKV. Generations F0 through F6 showed no statistically significant differences in rates of infection, dissemination, or transmission potential. Generations F7 through F10 showed increased infection, dissemination, and transmission as compared to generations F0-F6. There were no statistically significant differences in generations F7-F10 in rates of infection, dissemination, or transmission potential. The Rockefeller strain mosquitoes exhibited higher rates of infection, dissemination, and transmission than any generation F0-F10; this difference was especially pronounced in rates of transmission (Figure 4.1).

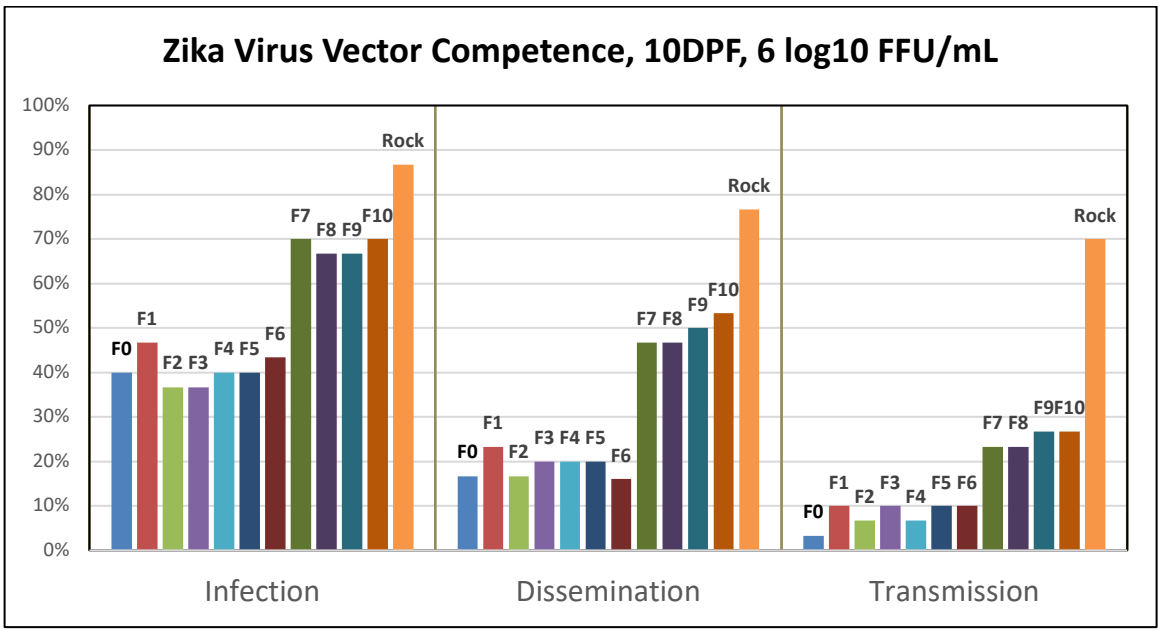

Figure 4.1 Vector Competence of 10 Generations of *Ae. aegypti* for ZIKV

Infection, disseminated infection, and potential transmission of three Zika virus strains by a colony of *Aedes aegypti* originating from Weslaco, Texas, 10 days post-feeding (DPF) artificial blood meal containing 6 $\log_{10}$ focus forming units (FFU) per mL of ZIKV PRVABC59 FLIC.

Microbiome Alpha Diversity

The effect of colonization on microbiome diversity was examined using alpha diversity analysis by comparing the total number of genera and the Shannon diversity of generations F0-F5, F7, F10 of the Weslaco strain. Shannon diversity accounts for both abundance and evenness of bacteria species present in a population. Rockefeller strain mosquitoes were used as a heavily colonized comparison group. Sequencing and analysis was biased towards early generations in order to observe changes to microbiome diversity that may have occurred within a few generations in the colonization process. The total number of genera was determined for each of these generations (Figure 4.2) and no significant trend was found. Differences existed between F5 and F0, F1, F7, and

Rockefeller. F5 had a higher number of genera than each of these groups, except F1 (Figure 4.2). The Shannon Entropy was calculated (Figure 4.3) and the only significant difference was found between F1 and F5, with F1 exhibiting higher Shannon entropy than F5 (Figure 4.3).

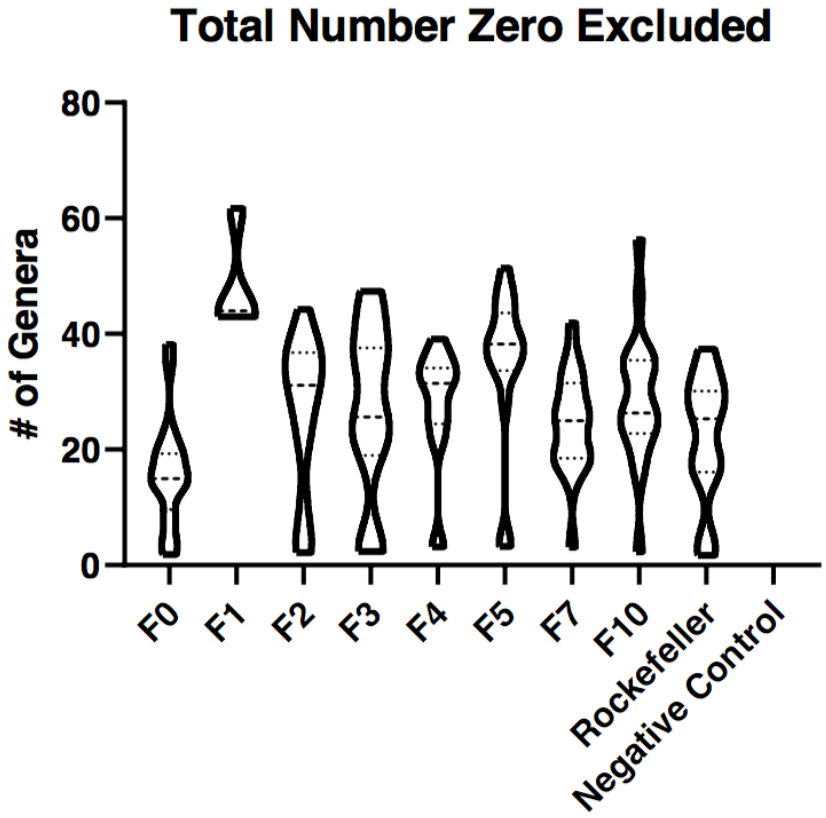

Figure 4.2 Total Number of Genera Detected in Microbiome of Multiple Generations of *Ae. aegypti*

The number of genera in generations F0, F1, F2, F3, F4, F5, F7, and F10 of the Weslaco strain and the Rockefeller strain of *Aedes aegypti*. Dashed lines represent the mean and dotted lines the interquartile range.

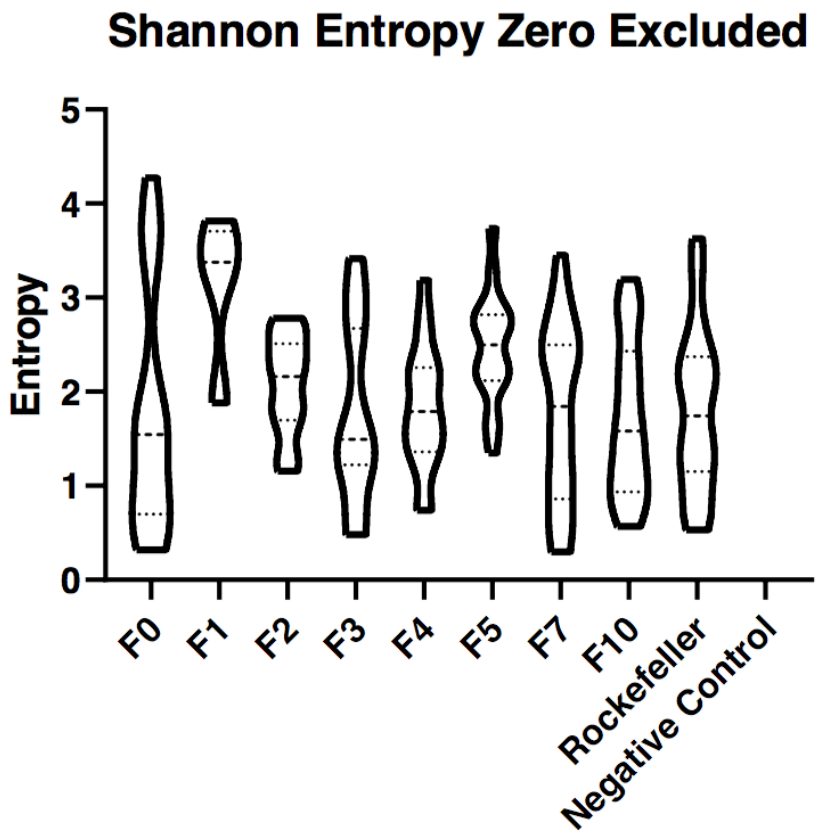

Figure 4.3 Shannon Diversity in Microbiome of Multiple Generations of *Ae. aegypti*

Shannon diversities for generations F0, F1, F2, F3, F4, F5, F7, and F10 of the Weslaco strain as well as the Rockefeller strain of *Aedes aegypti*. Dashed lines represent the mean and dotted lines the interquartile range.

Microbiome Beta Diversity

The effect of colonization on bacterial structure was determined using beta diversity analysis by comparing composition of microbiomes of individual mosquitoes from generations F0, F1, F2, F3, F4, F5, F7, and F10 of the Weslaco strain. The Rockefeller strain was used as a heavily colonized group for comparison. Sequencing and analysis was biased towards early generations in order to observe changes to microbiome structure that may have occurred within a few generations in the colonization process. Generation groups exhibited clustering patterns of microbiome, with some generations clustering more tightly than others (Figure 4.4). This clustering is particularly evident with the F0 generation

which shows little clustering and the Rockefeller strain which clusters tightly (Figure 4.4). The F0 generation and the Rockefeller also exhibit very different structures, as exhibited by the distance between these groups in the principal coordinates analysis (Figure 4.4).

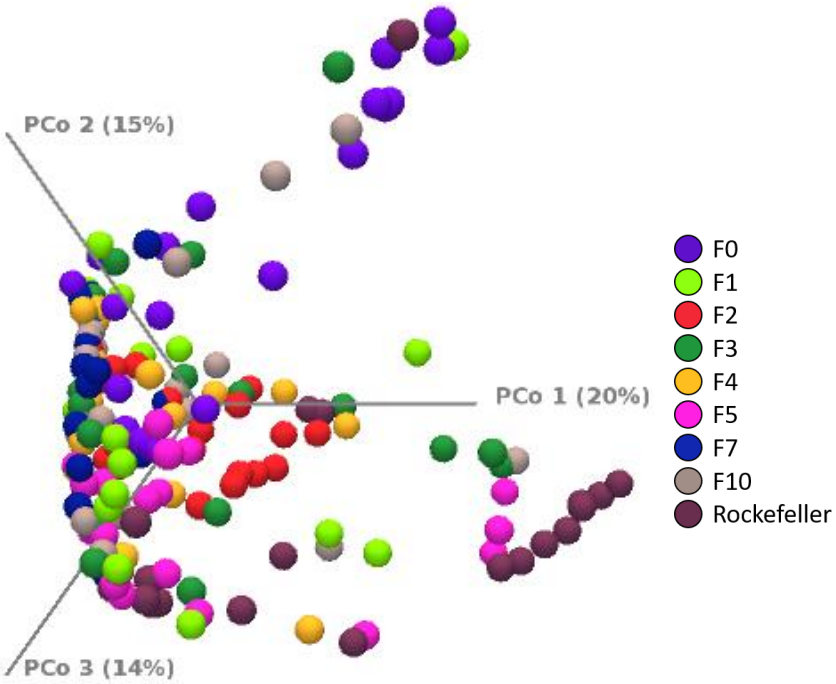

Figure 4.4 Principal Coordinates Analysis of Multiple Generations of *Ae. aegypti*

Principle Coordinates Analysis at the operational taxonomic level (OTU) level in order to compare identified OTU similarities between generations. Each point represents the microbiome of an individual mosquito.

Analysis of Composition of Microbes (ANCOM)

The effect of colonization on microbiome composition as well as association between competence level and certain OTUs was determined by comparing abundance of identified OTUs among mosquito groups (F0-F5 as low competence; F7 and F10 as high competence). Twenty-seven OTUs were identified with statistically different abundances

between low competence (F0-F5) and high competence (F7, F10) generation groups (Figure 4.5). Six of these OTUs were at a higher abundance in the low competence group and the remaining 21 showed a higher abundance in the high competence group. The OTUs and corresponding competence group are listed in Table 4.1.

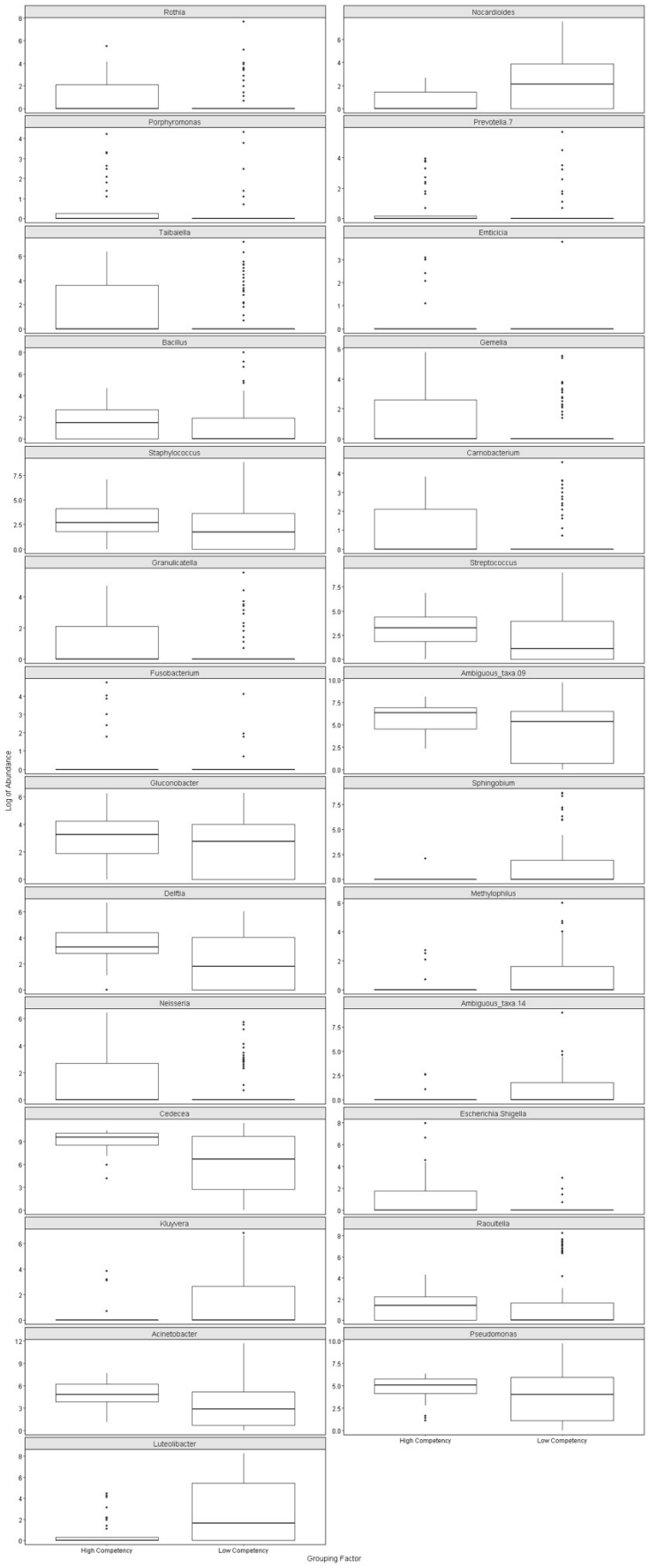
Rothia
Nocardioides
Porphyromonas
Prevotella.7
Taibaiella
Emticicia
Bacillus
Gemella
Staphylococcus
Carnobacterium
Granulicatella
Streptococcus
Fusobacterium
Ambiguous_taxa.09
Gluconobacter
Sphingobium
Delftia
Methylophilus
Neisseria
Ambiguous_taxa.14
Cedecea
Escherichia.Shigella
Kluyvera
Raoultella
Acinetobacter
Pseudomonas
Luteolibacter
Log of Abundance
High Competency
Low Competency
Grouping Factor

Figure 4.5 Analysis of Composition of Microbes Comparing High Vector Competence and Low Vector Competence Generation Groups

ANCOM of bacteria that exhibited significantly different abundances between high competence (F7, F10) (left) and low competence (F0-F5) (right) generation groups.

Table 4.1: Summary of ANCOM Results

OTU	Competence Group with Higher Abundance
Rothia	High Competence
Porphyromonas	High Competence
Taibaiella	High Competence
Bacillus	High Competence
Staphylococcus	High Competence
Granulicatella	High Competence
Fusobacterium	High Competence
Gluconobacter	High Competence
Delftia	High Competence
Neisseria	High Competence
Cedecea	High Competence
Kluyvera	Low Competence
Acinetobacter	High Competence
Luteolibacter	Low Competence
Nocardioides	Low Competence
Prevotella	High Competence
Emticicia	High Competence
Gemelia	High Competence
Camobacter	High Competence
Streptococcus	High Competence
Ambiguous taxa 09	High Competence
Sphingobium	Low Competence
Methylophilus	Low Competence
Ambiguous taxa 14	Low Competence
Escherichia Shigella	High Competence

Raotella	High Competence
Pseudomonas	High Competence

DISCUSSION

Vector Competence

This study is the first report tracking colonization of *Ae. aegypti* over the course of multiple generations and examining correlations with vector competence. While other studies (49,56,58-63,67-70) have identified changes when comparing low colonized to high colonized populations, they do not provide insight into how rapidly these changes occur and minimize correlations that can be drawn to other factors. Contrary to the hypothesis that changes to vector competence would occur slowly over the course of colonization, our data show an abrupt increase in vector competence after the first 6 generations of insectary-reared mosquitoes showed little or no changes from the field-collected population (Figure 4.1). Following this abrupt increase in vector competence, levels of infection, dissemination, and transmission were stable through the end of the study at F10 (Figure 4.1). This suggests a change occurred to an underlying factor between F6 and F7 that was then maintained through F10. As there was no change to rearing protocol between these generations and the colony experienced no bottleneck or stochastic founder effect, shown to rapidly alter mosquito populations (49) is unlikely, though not impossible. One explanation for the abrupt change in vector competence is an increase or decrease in certain bacterial populations, which may contribute to vector competence (64). To examine this possibility, microbiome analysis was conducted on generations grouped into low vector competence (F0-F6) and high vector competence (F7-F10) rather than analyzed as individual generations, discussed below.

Importantly, even the most colonized F10 generation in this study exhibited lower vector competence than the Rockefeller strain. With each stage of vector competence (infection, dissemination, transmission), the contrast between the Rockefeller strain and

Weslaco strain became more evident (Figure 4.1), suggesting the Rockefeller strain has particularly low midgut barriers and salivary gland barriers compared to the other mosquitoes examined. This heavily colonized strain is still used throughout many labs and my results provide further evidence that it is a strikingly poor model for vector competence studies, unless the aim of a study is to examine a virus in the most permissive vector environment.

Microbiome

Previous studies (56) have established that laboratory-reared mosquitoes have distinct microbiome structures as compared to their field counterparts, but this is the first study to examine how quickly this change occurs and its possible role in changes to vector competence that also occur during colonization. While changes to alpha diversity, as measured by both total number of genera and by Shannon diversity, continued to occur throughout the first 5 colonized generations (Figures 4.2, 4.3), the F7 and F10 generations were comparable to one another. This suggests that the microbiome may be more prone to changes during early colonization and then stabilizes into an insectary-reared phenotype. This is further supported by the alpha diversity of the Rockefeller strain, which is comparable to both F7 and F10.

The beta diversity shows clustering patterns for each generation, but very few trends in terms of how tightly clusters occur over the course of colonization, or any major separation of most microbiomes (Figure 4.4). The F0 group is the least tightly clustered generation, which is to be expected as they were field collected and were not controlled for any factors other than species and sex. All insectary reared mosquitoes were controlled for age, larval water, larval feeding, and other rearing conditions and had no exposure to sugar or blood meals prior to collection, which likely results in a more similar microbiome. The Rockefeller strain was tightly clustered and was the most divergent group.

The differential abundance results give insight into specific OTUs associated with increased colonization and their possible role in vector competence. Of the 21 OTUs identified with a difference in abundance between high and low vector competence, only 6 of them exhibited an increased abundance in the low competence group (Figure 4.5, Table 4.1). Less than half (11 of 27) of these OTUs have been previously studied in the context of the mosquito microbiome. Those that appeared in the literature are listed in Table 4.2 along with the vector species in which they were identified and corresponding reference. While some OTUs had only been identified in one or very few prior publications, others, such as *Staphylococcus* and *Pseudomonas* were found throughout much of the literature on mosquito microbiomes. However, almost none of the listed publications identify the role these bacteria play in vector competence. One notable exception is *Kalappa et al*. (138) which examined the role of the midgut microbiome of *An. stephensi* vector competence in *Plasmodium berghei* infection. Rather than examining the role of specific bacteria, this study found that treating the mosquito microbiome with antibiotics to clear much of the microbiome resulted in higher levels of infection as compared to infection with the native microbiome (138).

Table 4.2: Previous Literature on OTUs of Interest

OTU	Previous Description In Mosquito Microbiome
Bacillus	*Anopheles stephensi* (138), *Aedes albopictus* (139,140), *Ae. aegypti* (139,140), *Culex spp.* (141), *An. culicifacis* (142), *An. stephensi* (143), *An. maculipennis* (143)
Staphylococcus	*An. culcifacies* (142), *An. stephensi* (144), Cx. quinquefasciatus (145), *An. darlingi* larval water (146), *An. crucians* (147), *An. punctipennis* (147), *An. quadrimaculatus,*(147), *Ae. spp.* (147), *An. spp.* (147), *Cx spp.* (147), *Cs.spp*. (147), *Ps. spp.* (147)
Gluconobacter	*An. crucians* (147), *An. punctipennis* (147), *An. quadrimaculatus,*(147), *Ae.*

	spp. (147), *An. spp.* (147), *Cx spp.* (147), *Cs.spp*. (147), *Ps. spp.* (147)
Delftia	*An. culicifacies* (142), *Ae. albopictus* (148)
Cedecea	*Ae. aegypti* (149), *An. gambiae* (150)
Kluyvera	*Ae. aegypti* (151)
Acinetobacter	*An. stephensi* (138), *Ae. aegypti* (140, 152), *An. stephensi* (143), *An. maculipennis* (143), *An. culicifacies* (142), *Cx. pipiens* (153), *Coquillettidia perturbans* (153), *An. spp.* (154)
Prevotella	*An. stephensi* (138)
Streptococcus	*An. culicifacies* (155), *Cx. pipiens* (156,157)
Escherichia Shigella	*An. darlingi* larval water (146), *Cx. spp.* (141), *Cx. quinquefasciatus* (170)
Pseudomonas	*An. spp.* (154), *An. darlingi* larval water (146), *Ae. aegypti* (146, 152, 147), *An. culcifacies* (142, 155), *An. stephensi* (143, 138, 148), *An. maculipennis* (143), *An. albimanus* (146), *Ae. koreicus* (147), *An. gambiae* (149)

Without previous studies on any of the OTUs associated with higher or lower vector competence, it becomes difficult to test the hypothesis that observed changes in vector competence were due to an increase or decrease in certain OTUs. Additional studies examining each OTU and possibly multiple as a community structure would give significant insight specific roles played by the microbiome.

As a whole, these studies exemplify some of the rapid changes that can occur during early colonization of *Ae. aegypti* and identify possible microbiome mechanisms for the changes observed in vector competence. Unexpectedly, the change in vector competence occurred rapidly and resulted in two competence phenotypes: low (F0-F6) and high (F7-F10). In this population, vector competence significantly increased abruptly after 6 generations of insectary rearing with later generations exhibiting increased rates of infection, dissemination, and transmission for ZIKV which remained elevated through

generation F10. However, even the generation that exhibited increased vector competence were more refractory than the Rockefeller strain. While there was not a significant decrease in microbiome diversity as hypothesized, a number of bacteria exhibited significant differences in abundance between the low and high competence groups, interesting targets for further experiments to examine specific interactions and effects on vector competence.

Chapter 5: Conclusions and Future Directions

SUMMARY STATEMENT

Aedes aegypti is the vector of multiple arboviruses, including yellow fever virus, dengue virus, and Zika virus (ZIKV) (35). Highly anthropophilic and anthropophagic behavior (37) paired with competence for these viruses (35) makes *Ae. aegypti* one of the most important pathogen vectors worldwide. Until 2003, ZIKV had only caused 14 reported cases (87). Following a series of outbreaks in the South Pacific, ZIKV emerged into the Americas in 2013, later detected in Brazil in 2015 and declared a public health emergency of international concern when it was linked to microcephaly and other birth defects (30). ZIKV rapidly spread through the Americas due to large, naïve human populations in dense urban centers with high populations of *Ae. aegypti* (21). This outbreak rapidly brought international global attention to both the virus and its vector, resulting in a large number of publications focused on the vector competence of *Ae. aegypti* for ZIKV (35). While other mosquito species were also examined, much of the research was focused on *Ae. aegypti* due to its relevance as a vector of human arboviruses, driven partially by its anthropophilic behavior, as described in Chapter 1. My research described above aimed to:

1. Determine the vector competence of *Ae. aegypti* as well as *Ae. albopictus* and other suspected vectors for ZIKV, including differences in competence associated with strain of virus and strain of vector species
2. Examine changes that occur during early colonization of *Ae. aegypti*, including vector competence for ZIKV and the microbiome as a possible mechanism to changes observed.

The hypothesis driving this research was: colonization increases the vector competence of *Aedes aegypti* for Zika virus and is associated with changes to the microbiome. To this end, these studies support the following main conclusions:

1. Both *Ae. aegypti* and *Ae. albopictus* are competent vectors of ZIKV. Their vector competence, as described by their ability to become infected and transmit virus, varied by strain of virus and strain of mosquito. *Culex quinquefasciatus* is not a competent vector species for ZIKV, though this conclusion is at odds with a small number of other studies (90). These other studies, however, used very highly colonized mosquitoes and/or unique strains of virus that are unavailable to other labs for confirmatory testing.
2. All competent vectors were the most susceptible to African strain DakAR 41525, with higher levels of infection, dissemination, and transmission compared to Asian and American strains, which does not support the hypothesis that ZIKV had mutated for increased transmission by urban vector species since leaving Africa in the distant past.
3. During early colonization, there was a significant increase in vector competence of *Ae. aegypti* for ZIKV. Rather than occurring gradually, however, the competence remained similar to that of field-collected mosquitoes for 6 insectary-reared generations before suddenly and significantly increasing, and remaining increased through the 10th generation. The vector competence of all study generations remained significantly below that of the highly colonized Rockefeller strain.

The microbiome of *Ae. aegypti* underwent significant changes during early colonization, including changes to diversity and composition. For the first 5 colonized generations, diversity continued to fluctuate but F7 and F10 were comparable, which were comparable to the diversity of the Rockefeller strain. In comparing the bacterial composition of early low competence generations and later high competence generations, a total of 27 bacterial OTUs was identified with different abundance between the two group.

My studies are the first to examine the process of colonization and its effects on both vector competence and microbiome. While other studies have addressed effects of colonization, they have examined colonization as an endpoint rather than a process (49,47,58-63,67-71). Knowing how quickly these changes occur as well as correlations between changes allows researchers to begin identifying causative relationships, as well as identify ways to delay or even reverse effects of colonization.

Previous studies (49,57) examining vector colonization have examined ways to minimize certain effects of colonization. In a study examining changes to the microbiome, *Akorli et al*. (57) found that *An. gambiae* insectary-reared in field-collected water rather than tap water helped conserve more of the microbiome from earlier generations. By generation F10, mosquitoes reared in field water conserved 50% of the F5 bacterial genera compared to 38% in mosquitoes reared in tap water, though both groups experiences a decrease in diversity (57). Another study into the effects of inbreeding on *Ae. aegypti* (49) found that the fitness, as measured by development time and survival, decreased over 20 generations of insectary inbreeding, but fitness was improved by a single generation of outbreeding by introducing mosquitoes from an earlier generation (49). While neither of these studies examined vector competence, they still exemplify simple methods that can be used to minimize the progression to an artificial, colonized phenotype. Additional studies and the development of alternative methodologies should be pursued to develop insectary protocols that result in mosquito populations that better reflect vectors in their field setting.

Two important considerations for vector competence studies are reproducibility and relevance to natural vector populations. Utilizing a vector strain that has been heavily adapted to insectary rearing, such as Rockefeller *Ae. aegypti*, may maximize both ease of manipulation and result reproducibility as it produces a consistently colonized phenotype, through this has not been well-studied. This, however, is at odds with using field-collected mosquitoes, which are as relevant as possible to natural vectors but would be very difficult to replicate as each field collection would result in a different set of individual mosquitoes

with varied microbiome, genetics, and other traits. To that end, it is important to note that the findings described here regarding insectary colonization may be specific to the mosquito population and insectary utilized throughout the study. While there is strong evidence that colonization changes multiple aspects of vector biology (46, 49-44, 47-63, 67-71), the direction and extent of those changes likely depend on a number of factors such as bacteria present in the insectary, traits of the mosquitoes used to initiate the colony, and maintenance size of the colony. It has been observed that even replicate colonies can acquire different traits during the process of colonization (49). To best maximize relevance to natural vectors while not sacrificing reproducibility, arbovirus researchers need to incorporate strategies to minimize effects of colonization. These may include collection of field mosquitoes when possible, outbred (field-collected) crossing into insectary-reared colonies, the use of field-collected larval water for rearing, and regular characterization of colony traits such as gene expression and microbiome. The characterization of colonies would also allow results such as vector competence to be examined in a more complete context and could lead to identifying correlation across experimental outcomes.

FUTURE DIRECTIONS

The studies presented here focus on changes that occur during early colonization of *Ae. aegypti*, namely changes to vector competence for ZIKV, and the microbiome. An important factor missing from these analyses is changes to gene expression. These data could provide a link between changes observed in microbiome and vector competence via immune pathways, or could identify other pathways that provide additional correlations. The RNA for these studies has been extracted from each generation and will be analyzed prior to publication.

To more fully explore the consequences of colonization with the aim of identifying protocols to minimize these effects on experimental outcomes, additional long-term studies

could be run using multiple vector populations and insectary protocols. Overall, these studies would be similar to those described in Chapter 4 with regular determination of vector competence and microbiome, along with gene expression and other factors. However, beginning with more than one vector population as well as replicate colonies from a single starting population would explore the relative influences of the founding population, stochasticity, and insectary setting. If possible, running the study across two insectaries would further dissect these influences. Strategies to minimize colonization effects, including outbreeding and larval rearing in field-collected water, would be utilized alongside control groups to examine their effects and their relative ability to aid reproducibility across vector populations. Lastly, these studies would run for multiple years in order to determine at what point the vector populations would approach a heavily colonized phenotype, as exemplified by the Rockefeller strain, and for how long the strategies to mitigate colonization are effective. While these proposed experiments represent an extensive undertaking, they would provide a wealth of valuable data to arbovirus researchers and allow for better reproducibility of experiments. Additionally, as vector competence studies provide only a part of estimating vectorial capacity, increasing the relevance of the experimental vector populations to wild populations maximizes the applicability of results.

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