Determinants of Fine-Scale Heterogeneity in Mosquito-Borne Virus Systems

Elizabeth Handly Mayton
Louisiana State University and Agricultural and Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Part of the Virology Commons

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_dissertations/5627

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
DETERMINANTS OF FINE-SCALE HETEROGENEITY IN MOSQUITO-BORNE VIRUS SYSTEMS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University
and Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Pathobiological Sciences

by

Elizabeth Handly Mayton
B.S., Louisiana State University, 2015
August 2021
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents, Bill and Cindy, for their never-ending support of my goals and aspirations. No matter the road bumps, trials, and teary-eyed phone calls, they have always been there to guide me. I would also like to thank my team at JM Modern Jiu Jitsu, for continuously pushing me to better myself. I’m greeted with a “what’s up, Doc?” every time I enter the gym, and I don’t think it will ever get old. To my fellow graduate students, past and present, this degree would have never happened without you. Whether it be technical support, or convincing me not to drop out and apply to Trader Joe’s, I would not have accomplished this without all of you. To my advisory committee, Dr. Kevin Macaluso, Dr. Jeremy Brown, Dr. John Hawke, and Dr. Lane Foil, thank you for your direction and guidance throughout the years. And last but certainly not least, my mentor Dr. Rebecca Christofferson. Thank you for convincing me to stay and pursue my passion, and setting the standard for research. Through laughs, tears, and the occasional naps under desks, you have helped me grow as a scientist and a person, and for that I am forever grateful.
# TABLE OF CONTENTS

Acknowledgements........................................................................................................ ii

Abstract.......................................................................................................................... v

Chapter 1. Introduction to Arboviral Systems ................................................................. 1
  1.1 Prevalence............................................................................................................... 1
  1.2 Mosquito-Borne Viruses....................................................................................... 2
  1.3 Mosquito Lifecycle............................................................................................... 4
  1.4 Vector Competence and Vectorial Capacity ....................................................... 5
  1.5 Vector Determinants............................................................................................. 9
  1.6 Viral Determinants ............................................................................................. 12
  1.7 Hypothesis and Rationale .................................................................................. 14
  1.8 Notes.................................................................................................................... 15

Chapter 2. Age-Structured Vectorial Capacity Reveals Timing, Not Magnitude of Within-Mosquito Dynamics is Critical for Arbovirus Fitness Assessment ....................... 21
  2.1 Introduction .......................................................................................................... 21
  2.2 Methods ............................................................................................................... 23
  2.3 Results .................................................................................................................. 31
  2.4 Discussion ............................................................................................................ 41
  2.5 Notes..................................................................................................................... 44

  3.1 Introduction .......................................................................................................... 51
  3.2 Methods ............................................................................................................... 53
  3.3 Results .................................................................................................................. 57
  3.4 Discussion ............................................................................................................. 64
  3.5 Notes..................................................................................................................... 69

Chapter 4. Phylogenetic Analysis of Existing Mayaro Virus Genome Sequences and Use in Vector Competence Studies ................................................................. 73
  4.1 Introduction .......................................................................................................... 73
  4.2 Methods ............................................................................................................... 75
  4.3 Results .................................................................................................................. 77
  4.4 Discussion ............................................................................................................ 84
  4.5 Notes..................................................................................................................... 86

Chapter 5. Conclusions and Future Directions .............................................................. 90
  5.1 Introduction .......................................................................................................... 90
  5.2 Summary of Results............................................................................................. 90
5.3 Conclusions and Future Directions ................................................................. 93
5.4 Notes .................................................................................................................. 95

Appendix 1. Permission to Reprint Chapter 2 from BMC ..................................... 97
Appendix 2. Permission to Reprint Chapter 3 from MDPI ................................... 98
Appendix 3. Supplementary Information from Chapter 2 .................................. 99
Appendix 4. Supplementary Information from Chapter 3 ................................. 106
Appendix 5. Vector Competence of South Texas Aedes aegypti for Mayaro Virus .... 109
Appendix 6. Chapter 3 Method Development Troubleshooting ....................... 111

Bibliography ............................................................................................................. 113
Vita .............................................................................................................................. 130
ABSTRACT

Arthropod-borne viruses (arboviruses) are the etiological agents of much morbidity and mortality, especially in low- and middle-income countries. Many of these viruses are spread and maintained by mosquitoes, particularly the urban mosquito Aedes aegypti. Zika virus (ZIKV) is responsible for one of the largest vector-borne disease outbreaks in the past decade, affecting millions in Central and South America including a wave of microcephaly among newborns. Mayaro virus (MAYV) is a mosquito-borne virus endemic to South America and is predicted to become an emergent public health threat. Describing the vector-virus transmission systems are critical for understanding the potential spread of these viruses. Traditionally, laboratory vector competence measures are used to evaluate the ability of a species of mosquito to take up and subsequently transmit an arbovirus by exposing mosquitoes to virus and terminally sampling for the presence of virus in the saliva or peripheral tissues at predetermined time points. However, traditional measures do not assess critical vector-virus interactions that will ultimately impact transmission potential, as these measures focus solely on rates of infectious mosquitoes. My overarching hypothesis is that there are undescribed sources of fine-scale heterogeneity within the vector-virus transmission system that will alter transmission potential. To test this hypothesis, I 1) investigated the impact of the age structure of the mosquito population on the transmission potential of ZIKV by Aedes aegypti, 2) developed a novel method for the quantification of observed heterogeneity among individual mosquitoes, and 3) characterized genotypic diversity among strains of MAYV and the potential impacts on vector competence measurements.
CHAPTER 1: INTRODUCTION TO ARBOVIRAL SYSTEMS

1.1 Prevalence

Arthropod borne viruses, or arboviruses, are responsible for a significant amount of morbidity worldwide, and have been a major public health threat for decades (WHO 2014). Arboviruses are transmitted by arthropods, referred to as vectors, which is why diseases caused by these viruses are called “vector-borne diseases”. Types of vectors responsible for carrying arboviruses include many species of ticks, biting midges, and mosquitoes. Some examples include Crimean Congo hemorrhagic fever virus (CCHFV), Zika virus (ZIKV), Mayaro virus (MAYV), and dengue virus (DENV). The most prevalent human disease-causing arboviruses are transmitted by mosquitoes (Jones et al. 2020). Newly detected mosquito-borne viruses are still making incursions into urban areas and are set to pose major public health threats.

*Aedes aegypti* and *Aedes albopictus* are capable of transmitting viruses such as DENV, CHIKV, ZIKV, and MAYV. Because these are primarily urban mosquitoes with a high probability of biting humans, they are often responsible for major outbreaks among human populations. With increased urbanization and global temperature changes, the habitat for these mosquitoes and the population at risk continues to expand (Figure 1) (Leta et al. 2018). Control of these viruses and potential outbreaks relies on preventative measures, such as surveillance and vector control. Both of these measures rely on the understanding of vector-virus systems.
Figure 1.1. Global habitat suitability for *Aedes aegypti* and *Aedes albopictus*. Yellow areas represent areas suitable for *Ae. aegypti*, blue areas represent areas suitable for *Ae. albopictus*, and red areas represent areas suitable for both species (Leta et al. 2018).

### 1.2 Mosquito-borne viruses

Species of mosquitoes carry Bunyaviruses, Alphaviruses, and Flaviviruses, however the viruses of interest in this dissertation work are Flaviviruses and Alphaviruses.

Flaviviruses are positive sense, single stranded, enveloped RNA viruses belonging to the family Flaviviridae. Examples of Flaviviruses of human health importance include yellow fever virus (YFV), ZIKV, and DENV. The viral genome of Flaviviruses consists of ten polyproteins: seven nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) and three structural (C, prM, E) (Valderrama, Diaz, and Lopez-Verges 2017). ZIKV is a Flavivirus of particular interest. ZIKV was originally isolated from a rhesus monkey in 1947 in Uganda (Musso and Gubler 2016). The primary vector for ZIKV is *Aedes aegypti*, an urban mosquito which frequently feeds upon humans, but *Aedes albopictus* has also been shown to transmit ZIKV (Azar and Weaver 2019). ZIKV commonly causes febrile illness; however, during the
2015 outbreak in Brazil, ZIKV was linked to fetal microcephaly and neurological symptoms (Krauer et al. 2017). Cases of ZIKV are consistently present in Southeast Asia and South America, but there have been cases of autochthonous (local) transmission in the southern United States and parts of Europe (Brady and Hay 2019; Grubaugh, Ladner, et al. 2017).

Alphaviruses are negative sense, single stranded, enveloped RNA viruses belonging to the family Togaviridae. Examples of mosquito-borne Alphaviruses include chikungunya virus (CHIKV) and Mayaro virus (MAYV). The Alphavirus genome consists of nine polyproteins: four nonstructural (NSP1-4) and five structural (C, E3, E2, 6k, E1) (Figueiredo and Figueiredo 2014). MAYV is an understudied arbovirus that has caused periodic outbreaks in South America (Mackay and Arden 2016; Blohm et al. 2019). MAYV causes febrile illness and arthralgia, which can persist for months to years. MAYV was originally isolated from the serum of a febrile human patient from Trinidad and Tobago in 1954. Outbreaks have been recorded in Trinidad and Tobago, Peru, Venezuela, and Brazil, and cases have recently been recorded in Haiti and French Guiana (Diagne et al. 2020). A primary urban vector has yet to be definitively established, however laboratory experiments indicate both Aedes aegypti and Aedes albopictus are capable of transmitting MAYV (Diop et al. 2019; Wiggins, Eastmond, and Alto 2018; Pereira et al. 2020).

Mosquito-borne viruses are maintained in a “cycle” of mosquito and vertebrate. Many mosquito-borne viruses are maintained outside of urban areas in undeveloped areas, such as rainforests; this is called the sylvatic cycle (Song et al. 2017). In the sylvatic cycle, viruses are transmitted by forest-dwelling mosquitoes to vertebrates, such as birds or monkeys. As
humans encroach on these areas, they can be fed upon by these sylvatic vectors, contract these viruses, and subsequently bring them back into urban areas. When the virus establishes itself in an urban cycle from a sylvatic cycle, it is referred to as a “spillover” event (Song et al. 2017) (Figure 1.2).

**Figure 1.2.** Sylvatic and urban cycles of mosquito-borne viruses. Mosquito-borne viruses circulate within a sylvatic cycle (left), which is maintained between forest-dwelling mosquitoes and non-human vertebrates, before spilling over into the urban cycle (right), which is maintained between urban mosquitoes and humans (Song et al. 2017).

### 1.3 Mosquito Lifecycle

Adult mosquitoes feed on nectar for daily sustenance. Female mosquitoes will feed on vertebrates only for egg production and laying (oviposition); therefore, male mosquitoes do not require a blood meal (Thais Chouin-Carneiro 2017). In the laboratory, adult mosquitoes are maintained using sugar water (10% sucrose solution) in place of nectar, and blood is provided by either an artificial system, a laboratory vertebrate (e.g., mice), or a human volunteer. Mosquitoes utilize a probiscis for feeding. When feeding on a vertebrate, the proboscis is inserted, and probes into the skin for a capillary. During this process, saliva is
deposited into the skin, which causes inflammatory responses from the vertebrate. Upon locating a blood source, the mosquito will ingest the blood. After blood feeding, females will then lay their eggs in an aquatic environment, often in shallow, stagnant water. Eggs will hatch into the water, as larval stages are entirely aquatic before adult emergence.

1.4 Vector Competence and Vectorial Capacity

1.4.1 Vector Competence

Vector competence is the physical ability of a vector to become infected by and eventually transmit a pathogen. A mosquito is exposed to an arbovirus in the process of the mosquito taking a blood meal from an infected vertebrate. Upon consuming an infectious blood meal, the virus will establish an infection in the midgut. From the midgut, the virus will disseminate into secondary tissues, and eventually the salivary glands, where the mosquito is capable of transmitting and considered infectious (Kramer and Ciota 2015). The time it takes for the virus to travel from the midgut to the salivary glands is called the extrinsic incubation period (EIP) (Thais Chouin-Carneiro 2017). Once the virus is present within the salivary glands, an infectious mosquito can transmit the virus by feeding on an uninfected vertebrate and depositing infectious saliva in the skin. Because saliva is deposited upon probing, a mosquito does not necessarily need to fully feed to infect a vertebrate (Styer, Kent, et al. 2007).
When a mosquito feeds on a viremic host (1), it takes the infected blood into the midgut, where the virus establishes an infection (2). The virus will then disseminate into secondary tissues (3) and establish an infection in the salivary glands (4). When a mosquito feeds again with infected salivary glands, infected saliva is deposited into a vertebrate host (5). The time it takes from midgut infection (2) to salivary gland infection (4) is referred to as the extrinsic incubation period (Thais Chouin-Carneiro 2017).

When a species of mosquito is capable of becoming infected by and subsequently transmitting a virus, it is considered to be a competent vector. Vector competence is often determined experimentally by exposing species of mosquitoes to virus and testing for infection and potential transmission. Testing for competent vectors is done by exposing a population of mosquitoes to virus either by an artificial feeding system, an infected vertebrate, or mechanical infection of the midgut (Souza-Neto, Powell, and Bonizzoni 2019). After exposure, groups of exposed mosquitoes are tested at set time points. Mosquitoes are dissected and abdomens are tested for infection, legs and/or wings for dissemination, and
salivary glands and/or saliva collections for transmission. Forced saliva collections are common, as they allow for direct, simple collection of saliva (Honorio et al. 2020). Saliva is forcibly collected by placing the proboscis of a mosquito into a tube (commonly a pipette tip or capillary tube) containing a medium (e.g., fetal bovine serum). Often the medium will contain adenosine triphosphate (ATP), which has been shown to trigger salivation (Galun, Avi-Dor, and Bar-Zeev 1963).

When determining a competent vector, the EIP and proportion of infectious mosquitoes is considered (Figure 1.3) (Thais Chouin-Carneiro 2017). When the EIP is short, the vector will become infectious faster, and in turn, there is a better chance of the population as a whole to become infectious (Christofferson et al. 2014). The proportion of infectious mosquitoes is a separate measure, however, as a short EIP does not always guarantee a 100% infectious population. More often than not, a population of exposed mosquitoes will not reach 100% infectious rates (Christofferson, Mores, and Wearing 2016). Additionally, EIPs can potentially be long, and the mosquito will not become infectious until later in its lifetime. While EIP and the proportion of infectious mosquitoes (vector competence) are separate measures, it is important that both are considered when determining a competent vector. If the sampling population of a set of mosquitoes reaches a high rate of infectiousness within its lifetime, it is considered a competent vector.

There exists a great deal of heterogeneity in vector competence both among and within species of vectors. This heterogeneity is due to a multitude of factors, including geographic location of the vector, viral strain, and viral dose. For example, the species Ae.
*aegypti* is considered to be a competent vector for ZIKV. However, depending on where the mosquitoes are isolated, they will have varying EIPs and vector competence measures (Azar and Weaver 2019).

1.4.2 Vectorial Capacity

Vector competence is a component of vectorial capacity. Vectorial capacity describes the potential of a group of vectors to successfully transmit a pathogen. Specifically, the vectorial capacity equation is the number of secondary vector infections resulting from the introduction of one infectious mosquito into an immunologically naïve population, meaning a population that has not been previously exposed to that pathogen (Kramer and Ciota 2015). Vectorial capacity is a function of the following: mosquito-to-human density (m), number of bites per mosquito per day (a²), vector competence as proportion of infectious mosquitoes (b), daily probability of survival (p), and the EIP in days (n) (Equation 1.1).

\[ VC = \frac{ma^2bp^n}{-\ln(p)} \]

*Equation 1.1. Vectorial capacity equation*

The vectorial capacity does indeed take into account many interactions that will drive transmission. Regardless, further investigation into the intrinsic and extrinsic interactions must be made, as this equation does not take into account variation among the included variables.

While the definitions of vector competence and vectorial capacity seem straightforward, assessment of vector competence remains a challenge (Christofferson et al. 2014). A mosquito may be physically capable of taking up and depositing virus into a
susceptible host, but many intrinsic and extrinsic factors will affect transmission success and subsequent outbreaks. These include environmental impacts, viral evolution, and mosquito life traits. In this body of work, aspects of mosquito life traits and viral evolution and their potential impacts are examined.

1.5 Vector determinants

1.5.1 Age

The lifespan of the mosquito will determine whether or not the mosquito will survive the EIP and be capable of transmitting before death; therefore, mortality studies and field age estimates are often performed (Bellan 2010). In the laboratory, mosquitoes are housed in controlled environments and daily observations are made in order to calculate mortality rates. Much like vector competence, mortality rates will vary depending on the species, in addition to environmental factors and the virus with which the vector is infected (Sylvestre, Gandini, and Maciel-de-Freitas 2013; Lambrechts and Scott 2009; Marinho et al. 2016).

Field estimates are performed to predict the survival of mosquitoes in a specific area. Traditionally, mark-release-capture experiments are used by marking mosquitoes with fluorescent stains, releasing marked mosquitoes into a specific area, and trapping mosquitoes within that area sometime later (Epopa et al. 2017). In addition, technological advancements have been made towards estimating mosquito age in the field using near-infrared spectroscopy, which measures the change in absorbance of light at different wavelengths from compounds within the mosquito (Lambert et al. 2018). There are many practical applications to determining age, including determining insecticide efficacy and mortality rate
values to be used in vectorial capacity calculations (Knecht et al. 2018; Brady et al. 2016).

When determining lifespan, it is also important to consider at what point during that lifespan will the mosquito become exposed to virus. Even when the EIP is short, if the mosquito is exposed too late in its lifetime, transmission will not occur (Bellan 2010).

1.5.2 Biting rates

Mosquito biting rates drive transmission as without bites, transmission cannot occur. In the vectorial capacity equation, the biting rate (a) is squared to indicate the two bites needed: one for initial mosquito infection and one for transmission to vertebrates. A variety of techniques are used to estimate biting rates, both in the field and the laboratory. In the field, traps and human landing catches (HLCs) are often used to measure human-vector contact. HLCs involve human volunteers waiting in an area with targeted mosquito populations, and mosquitos that approach the volunteers are observed for host-seeking behavior and collected (Djenontin et al. 2010; Gimnig et al. 2013). Traps often utilize an attractant to lure mosquitoes into collection containers, such as CO₂ and human odor (Barnard et al. 2011; Kweka and Mahande 2009). Traps will collect the mosquitoes themselves without the need for a human volunteer, making it safer than HLCs; however, HLCs are still considered the gold standard for estimating vector density (Tambwe et al. 2021).

Laboratory measures use vertebrates and artificial systems to observe mosquito biting behavior and are crucial for an in-depth understanding of this behavior. Mice are commonly used, and mosquitoes are observed biting the abdomen, tail, or ear. Artificially, blood and sugar have both been used in the form of blood droplets, artificial feeders, and sugar-soaked
Blood feeding by female mosquitoes is intended solely for oviposition; however, blood meals can have effects on both the mosquito and viral transmission (Ribeiro 2000; Sylvestre, Gandini, and Maciel-de-Freitas 2013). *Aedes* mosquitoes are unique in that they will feed multiple times within a gonotrophic cycle, or the time period between blood meal and oviposition (Scott, Clark, et al. 1993). This behavior is often not accounted for in vector competence studies, as mosquitoes are typically provided one infectious blood meal before being maintained on sugar solutions for the duration of the experiment (Azar and Weaver 2019). With this behavior comes increased transmission opportunities in each individual mosquito’s lifetime, as well as multiple blood meals. Two studies have shown a second blood meal within 2-3 days of the infectious blood meal has been shown to enhance viral dissemination and shorten the EIP (Armstrong et al. 2020; Shaw et al. 2020).
1.6 Viral determinants

1.6.1 Viral Deposition

Quantification of viral delivery from the mosquito continues to be a difficult task. Attempts of quantification have been made, often via forced salivation. However, because of the forcible nature of saliva collection, it is believed that this does not accurately represent the amount expectorated during feeding behavior (Smith et al. 2006). Vertebrates have been used to estimate viral deposition. In these experiments, mosquitoes are allowed to feed on a vertebrate, often a mouse. After being allowed to feed, tissue samples from the vertebrate are taken, and virus quantified (Styer, Kent, et al. 2007; Secundino et al. 2017). Other estimations have been used by having the mosquito feed on an artificial sugar source, such as a card, which is then tested for the presence of virus (Alto et al. 2017; Fourniol et al. 2021). In experiments with Culex and WNV, viral titer estimates were obtained by offering mosquitoes a single drop of blood which was collected and tested. Delivery of virus was observed with probing behavior alone (no blood meal), though on average probing delivered a lower titer of virus compared to blood feeding (Styer, Bernard, and Kramer 2006).

1.6.2 Viral genotype

Vector competence has been shown to vary not only among geographic location, but among viral strains as well. Flaviviruses and Alphaviruses are RNA viruses, which are prone to error due to frequent errors in replication and poor proofreading capabilities. This leads to high rates of mutation and subsequent evolution of the virus (Domingo et al. 1996). As viral evolution occurs and changes in the genome accumulate, viral strains will evolve to form
lineages, or groups with similar, yet distinct genotypic patterns. Differences in vector competence, transmissibility, and disease outcome can often be seen between lineages (Aubry et al. 2021; Tsetsarkin and Weaver 2011). For example, an amino acid change within the envelope polyprotein of CHIKV allowed Aedes albopictus to become a strongly competent vector, expanding its transmission potential to different areas and subsequently causing large outbreaks (Tsetsarkin et al. 2007).

For ZIKV, there are two distinct lineages: the African and Asian lineages. The African lineage was first recorded in 1947 from the Zika forest in Uganda and spread from Africa into Asia. The Asian lineage emerged in 1966 and was responsible for outbreaks in the Western Hemisphere (Hu et al. 2019). With the outbreaks in the Western Hemisphere, disease outcomes not previously seen were recorded, including neurological symptoms and congenital malformations in newborns. These novel disease outcomes are believed to have been caused by a mutation in the envelope protein (E), the portion of the virus that interacts and binds with host cells (Shan et al. 2020).

In MAYV, three distinct lineages have been identified: Genotype D, Genotype L, and Genotype N (Auguste et al. 2015). A Genotype D strain was first isolated in 1954 in Trinidad. One strain, collected in 2010 from Peru, has been classified as a Genotype N strain. Genotype L strains have been mostly restricted to Northern Brazil but have recently been isolated in Haiti (Blohm et al. 2019). Differences in disease presentation and vector competence have yet to be determined. Currently, there are only 72 full genome sequences of MAYV available, making it difficult to address these differences. Combined with a lack of
vector competence studies, very few individual strains have been used in these studies (Diagne et al. 2020).

1.7 Hypothesis and rationale

Arbovirus transmission relies heavily on the process of vector competence. Traditionally, this process is assumed to be relatively homogenous among mosquito populations, evidenced by common experimental designs. As suggested in previous research, trait variation exists at both the population and individual level in vector-virus systems; however, their consequences on transmission potential are largely unaccounted for. Traits such as vector competence, EIP, and biting are usually presented as averages or central tendencies. However, the data that make up these central tendency estimates demonstrate that there is a great deal of fine-scale heterogeneity in these processes stemming from both individual-level and population-level sources. The extent to which this fine-scale heterogeneity of vector competence, EIP, and biting affects transmission estimates at the population level remains unclear. Further, the tools to determine this have been lacking, as experimental set-ups favor traditional measures for central tendency which largely ignore fine-scale heterogeneity.

This body of work will address this gap in knowledge. The overarching hypothesis is: Fine-scale heterogeneity that exists within the mosquito-arbovirus systems has a significant impact on transmission potential. Specifically, fine-scale heterogeneity is defined as 1) mosquito age, 2) vector competence, 3) among and within population differences in biting, 4)
among and within individual differences in transmission, and 5) genetic diversity of viral
strains. This hypothesis is addressed by the following independent, but interconnected works:

1. “Age-structured vectorial capacity reveals timing, not magnitude of within-mosquito
dynamics is critical for arbovirus fitness assessment.” This study investigates the
impacts of the effects of mosquito age at the time of exposure, variability in vector
competence, and age-dependent biting habits on the transmission likelihood of Zika
virus by Ae. aegypti

2. “A Method for Repeated, Longitudinal Sampling of Individual Ae. aegypti for
Transmission Potential of Arboviruses.” This work describes the development of a
novel technique to longitudinally sample mosquitoes for vector competence
(ordinarily a terminal assay) and describes the variability in individual-level
transmission dynamics, as well as between two populations of Ae. aegypti.

3. “Phylogenetic analysis of existing Mayaro virus genome sequences and use in vector
competence studies”. This study describes the genotypic variability within known
Mayaro virus strains while investigating the presence of potential recombinants, and
the potential impact of this variability on vector competence of Ae. aegypti and Ae.
albopictus for Mayaro virus based on previously performed studies.

1.8 Notes

(WHO), W.H.O. A global brief on vector-borne disease. 2014; Available from:

Jones, R., et al., Arbovirus vectors of epidemiological concern in the Americas: A scoping
review of entomological studies on Zika, dengue and chikungunya virus vectors. PLoS


Knecht, H., et al., Impact of Mosquito Age and Insecticide Exposure on Susceptibility of Aedes albopictus (Diptera: Culicidae) to Infection with Zika Virus. Pathogens, 2018. 7(3).


2.1 Introduction

The transmission dynamics of arboviruses such as Zika virus (ZIKV) are evaluated over several characteristics, namely vector competence and the extrinsic incubation period (EIP). *Aedes aegypti* is the primary vector of ZIKV and several studies have evaluated its competence to transmit the virus (Costa-da-Silva et al. 2017; Roundy et al. 2017; Chouin-Carneiro et al. 2016; Musso and Gubler 2016). Vector competence is the ability of a mosquito to acquire and ultimately transmit a virus (Valderrama, Diaz, and Lopez-Verges 2017; Kramer and Ciota 2015). The time it takes for this process to occur is referred to as the extrinsic incubation period (EIP) (Christofferson, Mores, and Wearing 2016). Vector competence and EIP are interrelated measures of the proportion of vectors that become infectious given exposure and the time it takes for a vector to become infectious given exposure, respectively (Ohm et al. 2018; Kramer and Ciota 2015). Thus, EIP can be described as the temporality of vector competence and has been used to evaluate the relative fitness of arbovirus systems (Ohm et al. 2018; Bellan 2010; Kramer and Ciota 2015). In addition, EIP and vector competence are influenced by many parameters including vector species, mosquito-immune system, microbiota fauna, discrete populations within species, and environmental factors (Roundy et al. 2017; Chouin-Carneiro et al. 2016; Valderrama, Diaz, and Lopez-Verges 2017; Maciel-de-Freitas, Koella, and Lourenco-de-Oliveira 2011; Brady et al. 2016; Robert et al. 2016; Goidind et al. 2015; Hardy et al. 1983; Souza-Neto, Powell, and Bonizzoni 2019).

This chapter previously appeared as Mayton EH, Tramonte AR, Wearing HJ, and Christofferson RC. 2020. Age-structured vectorial capacity reveals timing, not magnitude of within-mosquito dynamics is critical for arbovirus fitness assessment. Parasites & Vectors 13, 310. It is reprinted by permission of BMC.
Indeed, changes in arbovirus fitness and thus transmission dynamics have been predicated on altered vector competence, especially as a critical component of vectorial capacity (Kramer and Ciota 2015; Lounibos and Kramer 2016; Macdonald 1957; Garrett-Jones 1964; Bagny et al. 2009; Tsetsarkin et al. 2007; Vazeille et al. 2007; Kilpatrick et al. 2008; Ciota and Kramer 2013; Moudy et al. 2007). The composite of vector competence and EIP into a single, dynamic measure allows for a more comprehensive understanding of this process (Valderrama, Diaz, and Lopez-Verges 2017; Dye 1992; Maciel-de-Freitas, Koella, and Lourenco-de-Oliveira 2011; Ruckert and Ebel 2018; Petersen et al. 2018). Not all mosquitoes that are exposed will be able to transmit (vector competence) and the time it takes for those mosquitoes that will transmit is not a constant (EIP), and so understanding this composite over several days post infection is critical (Kramer and Ciota 2015; Christofferson and Mores 2011).

Vectorial capacity (VC) was derived as a measure of transmission potential of a vector-borne pathogen by a competent vector, and incorporates both vector competence and EIP (Christofferson, Mores, and Wearing 2016; Dye 1992; Kramer and Ciota 2015; Macdonald 1957; Garrett-Jones 1964; Smith and McKenzie 2004). VC is the vector-centric component of the basic reproduction number ($R_0$), and VC represents the number of secondary cases resulting from the introduction of a single infectious human individual per infectious day of that human index case (Massad and Coutinho 2012; Dye 1986). VC is given by:

$$VC = \frac{ma^2bp^N}{-\ln(p)}$$  \hspace{1cm} (Equation 2.1)

where ‘m’ is the density of mosquitoes relative to humans, ‘a’ is the biting rate, ‘b’ is the vector competence of the mosquito for a particular virus, ‘N’ is the extrinsic incubation period, and ‘p’ is the daily probability of mosquito survival (Kramer and Ciota 2015). And $R_0$ is given by:
\[ R_0 = \frac{c}{r} \times VC \]  
(Equation 2.2)

where \( r \) is the human recovery rate, or the reciprocal of the average human infectious period, and \( c \) is the probability of transmission from the human to the vector given contact. \( R_0 \) is a threshold parameter, whereby an \( R_0 \) value \(< 1\) is not expected to sustain transmission and an \( R_0 \) value \( \geq 1\) is likely to result in an outbreak (Brady et al. 2016; Smith et al. 2012).

VC uses an average daily probability of survival to calculate the probability of a mosquito living through the EIP (\( p^N \)) and \( p^N/(-\ln(p)) \) represents the expected infectious days given \( N \) and \( p \) (Garrett-Jones and Shidrawi 1969). It is intuitive how mortality could impact transmission, but most studies address mortality based on a constant age at which the mosquito acquires the infection (Robert et al. 2016; Bellan 2010; Styer, Carey, et al. 2007; Maciel-de-Freitas, Koella, and Lourenco-de-Oliveira 2011; Novoseltsev et al. 2012). Mathematical modeling studies have addressed age-structure with respect to EIP or bite-structure, but no studies have empirically investigated all three processes simultaneously (Rock, Wood, and Keeling 2015; Bellan 2010). Thus, herein, we experimentally test the hypothesis that transmission dynamics of arboviruses are as or more affected by time as a function of age versus time as a function of days post-infection. We further develop an age-structured vectorial capacity equation (\( VC_{age} \)) to quantify these potential effects.

2.2 Methods

We first wanted to determine if age and/or prior bloodmeals affected the within-mosquito viral dynamics, as well as various life traits of the mosquito. To that end, we designated three treatment groups: YOUNG, OLDER, and S.OLDER. The YOUNG group was offered an infectious bloodmeal at approximately 5 days post-emergence (dpe). The OLDER group was offered a non-infectious bloodmeal at 5 dpe and then an infectious bloodmeal 1 week later (12 dpe). The S.OLDER group was not offered a prior, non-infectious bloodmeal, but a single infectious bloodmeal at approximately 12 dpe (to match
the OLDER timing). S refers to sugar, which was done in order to distinguish the absence of a bloodmeal at 5 dpe. All non-infectious bloodmeals are referred to as “mock” bloodmeals, as they contain non-infectious cell culture media in place of infectious media.

### 2.2.1 Virus and mosquitoes

ZIKV strain PRVABC59 (Asian lineage), originally isolated from a human patient in Puerto Rico in 2015, was provided by Dr. Barbara Johnson at the US Centers for Disease Control and Prevention (GenBank: KU501215). Viral stock was passaged three times in Vero cells before passage on Vero cells for mosquito exposure. Supernatant was collected 3 days post-inoculation and titer determined as previously described (Kawiecki et al. 2017). Titer of ZIKV was verified by qRT-PCR at approximately 8 x 10^7 plaque forming units (pfu)/mL, matched across all exposure experiments. Virus used for mosquito exposure was never frozen, as this has been shown to negatively affect vector competence when compared to freshly cultured virus (Ciota et al. 2017; Richards et al. 2007). Colony *Aedes aegypti* (Rockefeller) were provided by Dr. Daniel Swale of the LSU Entomology Department. To isolate the effect(s) of age, mosquitoes were maintained at constant conditions with 16:8 light/dark periods, approximately 80% humidity (monitored using a digital RH monitor), and at 28°C constant temperature. Mosquitoes were supplied with a 10% sucrose water solution after emergence via soaked cotton pledgets, which were replenished every 24 hours. Sucrose solution was removed 24 hours before experiments and was again provided after blood feeding/mock starvation.

### 2.2.2 Blood feeding and oral exposure of *Ae. aegypti*

Infectious and non-infectious bloodmeals were prepared with ZIKV-infected cell culture supernatant and non-infected cell culture supernatant, respectively. Whole bovine blood in Alsever’s solution from Hemostat Labs (Dixon, CA) was used in a 2:1 blood to supernatant ratio (Christofferson and Mores 2011). Mosquitoes were fed via the Hemotek
(Discovery Labs, UK) membrane feeding system for 45 minutes, after which mosquitoes were cold anesthetized and blood fed females were placed into clean cartons until further treatment. Because starvation and cold anesthetization could affect mortality rates, every treatment was subjected to the same schedule of each of these conditions. For example, all groups were starved 24 hours prior to days 5 and 12 post-emergence, regardless of whether that group would receive a bloodmeal. In addition, not only were blood fed mosquitoes cold anesthetized and moved to a new carton, but other groups that did not feed on that schedule were treated the same in order to mimic these conditions and control for these factors. The experimental design is depicted in Appendix 3, Figure S1.

2.2.3 Within-mosquito kinetics

In order to determine if the timing of the infectious bloodmeal affected the within-mosquito viral kinetics, mosquitoes were sampled at 5, 8, and 11 days post-infection (dpi) to test for infection and dissemination across all groups (sample sizes provided in Appendix 3, Table S1). Mosquito legs and bodies were put into separate tubes containing 900 µL BA-1 diluent media and BBs. Samples were then homogenized twice at 25 Hz for 3 minutes using a Qiagen Tissuelyzer. RNA was extracted (5X MagMax 96 Viral RNA Isolation Kit, Applied Biosystems) and tested for the presence of viral RNA via qRT-PCR (SuperScript III One-Step RT-PCR System, Invitrogen) as in (Tramonte and Christofferson 2019). Each treatment was repeated a total of three times and data was averaged over these replicates. Differences among the treatment groups for infection and dissemination rates were tested by a chi-square test for multiple proportions on 5, 8, and 11 dpi.

2.2.4 Transmission Assay

To directly assess transmission potential, 20 mosquitoes per treatment were force-salivated. Briefly, ZIKV-exposed mosquitoes were immobilized on ice before removing legs and wings. Mosquitoes were then placed on double-sided tape, and the proboscis of each
mosquito was placed into a pipette tip containing 35 μL FBS with 3 mmol/L ATP for 30 minutes, as previously described in (Tesla et al. 2018). Contents of the pipette tip were ejected into 100 μL BA-1 diluent and stored at -80 degrees Celsius before testing (see below).

Mosquitoes from the two OLDER groups were sampled at 5, 8, and 11 dpi, as well as 16 dpi to represent the end of the mortality study (28 dpe). In order to characterize the EIP across the mosquito’s lifetime, mosquitoes in group YOUNG were sampled at 5, 8, and 11 dpi, as well as additional days post infection in order to match the age at which the older groups were sampled. Thus, by adding 7 to the time points in the older groups, we additionally sampled the YOUNG group at 12, 15, and 18 dpi, as well as 23 dpi which corresponds to the oldest time point of 28 days old. Samples were tested for the presence of viral RNA in saliva using the techniques described above (Tramonte and Christofferson 2019; Tesla et al. 2018). Differences among the treatment groups’ transmission rates were tested by Chi-square test for multiple proportions at each sampling timepoint (Table 2.1).

2.2.5 Mortality Study

Mortality studies were performed for the same three treatments (YOUNG, OLDER, and S.OLDER). We added additional mock bloodmeal controls (that is, accounting for any infectious bloodmeal-associated alteration of mortality) where a mock bloodmeal was used in place of infectious bloodmeals. The three controls were: 1) a mock bloodmeal at 5 dpe (M.Y) to correspond to the YOUNG treatment, 2) a mock bloodmeal at 5 dpe, followed by another mock bloodmeal at 12 dpe (M.M) to correspond to the OLDER two bloodmeal treatment, and 3) a mock bloodmeal at 12 dpe (S.M) to correspond to the S.OLDER, one bloodmeal treatment. An additional negative control treatment was performed where the mosquitoes
were never blood fed (S). All treatments were cold anesthetized at 5 and 12 dpe, regardless of whether they were offered a bloodmeal so that all mosquitoes experienced the same treatment, and mosquito density per carton was kept relatively constant with an average of 47 mosquitoes/carton (range 36-58). Each mortality treatment was repeated a total of three times and data are averages of the three replicates.

Cartons were checked daily and, when present, dead mosquitoes were counted and removed up to 28 dpe (approximately 1 month), as this has been shown to be the upper limit of field survival of *Ae. aegypti* and is similar to the range used in Tesla, et al. (Tesla et al. 2018; Goindin et al. 2015). Only mosquitoes that took all offered bloodmeals were included in the mortality analyses.

To test for differences in mortality rates among treatments, Kaplan-Meier survival analyses were conducted and the average time to death (TTD) was estimated. Daily mortality estimates relative to age were then predicted using best fits in R (version 3.5.2) (Team 2018).

### 2.2.6 Determining age-structured willingness to probe

One of the most influential parameters for determining transmission dynamics of vector-borne pathogens is the biting rate (because it impacts vector-host transmission in both directions). Biting rate is sometimes parameterized as the reciprocal of the number of days between feeds (Paaijmans, Cator, and Thomas 2013). This assumes that the waiting time between bites is exponentially distributed, and we make this assumption for three biting rates: 0.5 (once every two days), 1 (once a day), and 2 (twice a day) (Christofferson, Mores, and Wearing 2014). However, we wanted to determine if the willingness of the mosquito to probe was affected by 1) timing of infectious bloodmeal and/or 2) age of the mosquito (Rock, Wood, and Keeling 2015). While mosquito biting is a function of many factors, it has been shown that heat cues are sufficient to initiate host seeking behaviors (Zermoglio et al. 2017). We use this to determine the willingness or probability of a mosquito probing, which can lead
to transmission (Styer, Kent, et al. 2007; Matsuoka et al. 2002; Dubrulle et al. 2009).

Ten to twelve mosquitoes per each ZIKV-infected treatment (YOUNG, OLDER, and S.OLDER) were placed in individual, clear plastic canisters (Bioquip) twenty-four hours before being provided a bloodmeal via membrane feeder using 1 mL discs with 800 μL of blood (Hemotek, Discovery Labs, UK). This was done at the same dpi schedule as the vector competence studies above. Willingness to bite was assessed using a two-tiered approach by a single observer to control for observation bias. First, mosquitoes were observed through the clear canister for their general position in the canister and second, the disc was removed to determine if they were on or near the mesh at the top of the canister. In all cases, these two methods of observation matched. That is, if a mosquito was observed to be at the bloodmeal prior to disc removal (looking through the canister), she did not move to the bottom of the canister upon disc removal.

This observation was done at 1, 20, and 45 minutes post placement of the disc and the disc was replaced between observation time points. Thus, a mosquito was assessed as “landed” and recorded as “1” if the female was at the top of the canister at any of the observation times. She was otherwise classified as “not landed” and coded as “0” if she was at the bottom of the canister for all three observation times. We then calculated the probability of biting, Z, as a function of age. Z(age) was determined by fitting the proportion of mosquitoes that landed or fed at least once a day using a self-starting non-linear least squares regression:

\[ Z(\text{age}) = s + (g - s)\exp(-\exp(h) \cdot (\text{age})) \]  

\text{(Equation 2.3)}

where \( s \) is the asymptote, \( g \) is the zero-response parameter, and \( h \) is the logarithmic rate constant. Comparison of probing differences among treatments was assessed both per day post infection as well as per mosquito age by Kruskal-Wallis test.
2.2.7 Age-structured vectorial capacity and $R_0$

We re-formulated the vectorial capacity equation to estimate VC as a function of the age at which the mosquito acquires an infectious bloodmeal, redefining the parameters with respect to age at the time of acquisition of infection. We define $Age_{acquisition}$ as the age at which a mosquito acquires an infection (the day she takes the infectious bloodmeal) and $Age_{transmission}$ as the age at which she subsequently transmits (Figure 2.1):

\[
Age_{transmission} = Age_{acquisition} + EIP \quad \text{(Equation 2.4)}
\]

Figure 2.1. Vector age at time of infection acquisition determines transmission opportunity. Uninfected mosquitoes (black, left) acquire an infection at $Age_{acquisition}$ (black vertical lines) and after a certain EIP (black dashed lines), a proportion will become infectious (red, right), but for a certain period of time (red horizontal lines) that is dependent on $Age_{acquisition}$.

In $VC_{age}$, $m$ is still the mosquito-to-human density and, for illustrative purposes, held constant here at 1. The parameters $z_{acquisition}$ and $z_{transmission}$ are the probability of a mosquito biting at age of acquisition and age at time of transmission, respectively. The traditional calculation of $p^N$ represents the probability of a mosquito living through a fixed number of days $N$, the EIP, and $p^N/(-\ln(p))$ represents the expected infectious days given $N$ and $p$. 
(Garrett-Jones and Shidrawi 1969). In the context of an age-dependent vectorial capacity framework, we can calculate a more precise probability of surviving based on the day the mosquito obtained an infection and the cumulative survival probability to $A_{age\_transmission}$ where the cumulative probability of living through the EIP given $A_{age\_acquisition}$ is given by:

$$ p(surviving\ to\ Age_{transmission}) = \prod_{j=1}^{A_{age\_transmission}} p_j $$  \hspace{1cm} (Equation 2.5)

Where $p_j$ is the probability of daily survival on day $j$ post emergence. We further estimate the infectious period ($L$) in an age-structured way by numerically deriving $L$:

$$ L = TTD - A_{age\_transmission} $$  \hspace{1cm} (Equation 2.6)

where TTD is the average time-to-death derived from the experimental mortality study.

Using the data from our experimental studies, we calculated $A_{age\_transmission}$ for two scenarios: $E_{min}$ and $E_{max}$ from our observed transmission data (Paaijmans et al. 2012), and calculated $VC_{age}$ as:

$$ VC_{age} = mb(z_{acquisition\ a})(z_{transmission\ a})\left(\prod_{j=0}^{A_{age\_transmission}} p_j\right)L $$  \hspace{1cm} (Equation 2.7)

$VC$ is a component of $R_0$, which also includes the average infectious period of the human and the probability of transmission from the human to the vector. For illustrative purposes, we calculated $R_0$ using both the traditional calculation of $VC$ (Eq. 1), as well as $VC_{age}$. We made the following assumptions: mosquito density is held constant at $m=1$; the average infectious period of the human is 5 days ($r^{-1}$) (Kucharski et al. 2016); and the average probability of transmission from human to vector is parameterized experimentally as the
average proportion of mosquitoes that develop a midgut infection given exposure (c). From these data, we can calculate the value of VC needed to push $R_0$ above this threshold. We use this value as a “threshold” to compare $VC_{age}$ across treatment groups and with the traditional VC measures.

All statistics and subsequent graphics were performed and generated using R version 3.5.2 (Team 2018). All packages used are provided in Supporting Text S1. All functions and function parameters used to fit the data and obtain age-dependent distributions of these parameters are given in Supplementary Table S2. Goodness of fit was assessed either through AIC (for non-linear models) or $R^2$ for linear models.

2.3 Results

2.3.1 Within-mosquito dynamics are more affected by time as a function of age versus days-post-infection:

We first wanted to determine if the age at which a mosquito is offered an infectious bloodmeal and acquires the ZIKV infection ($Age_{acquisition}$) impacted the within-vector kinetics of the mosquito. To do this, we looked at infection, dissemination, and transmission of the three treatments in the context of days post-infection. We found no significant difference in the infection ($p>0.05$) or dissemination rates ($p>0.05$) across all treatments (Appendix 3, Table S1). However, when direct age comparisons are made, the effect of $Age_{acquisition}$ on infection becomes obvious (Figure 2.2). The average rate of infection of mosquitoes given exposure was 78.2% across all treatments and days post infection (95% confidence interval: 74.3-82.0%).
Figure 2.2. Infection and dissemination rates in the context of mosquito age. Despite no significant effect of treatment on infection and dissemination rates when assessed over days post-infection, Ageacquisition has an obvious impact on the timing of these processes.

When we evaluated the proportion transmitting in terms of days post exposure (5, 8, and 11 only) none of the treatments were transmitting on days 5 or 8 dpi. On day 11, the transmission rates were 5%, 10%, and 15% in groups OLDER, S.OLDER, and YOUNG, respectively. These differences were not statistically significant using the Chi-square test for proportion (p>0.5) (Appendix 3, Table S1).

However, when we investigated mosquitoes from group YOUNG at time points that
age-matched OLDER and S.OLDER treatments (Table 2.1), the YOUNG group achieved a maximum transmission of 45% at 28 days old (23 dpi) versus only 15% for the S.OLDER group and 10% for the OLDER group at 28 days old (16 dpi).

**Table 2.1.** Transmission rates for each day post-infection (dpi) and corresponding mosquito age for each of the three treatment groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>dpi (day post-infection)</th>
<th>Age (day post-emergence)</th>
<th>% Transmission ($n = 20$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOUNG</td>
<td>5</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>28</td>
<td>45</td>
</tr>
<tr>
<td>OLDER</td>
<td>5</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>S.OLDER</td>
<td>5</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>28</td>
<td>15</td>
</tr>
</tbody>
</table>
We define the EIP\textsubscript{max} as the earliest EIP (days post-infection) where the maximum proportion of mosquitoes are transmitting, and we define the EIP\textsubscript{min} as the earliest EIP where the proportion of mosquitoes transmitting is minimal, but greater than 0. EIP values at different vector competence levels have been used to evaluate the vectorial capacity for malaria (Paaijmans et al. 2012). For the YOUNG group, EIP\textsubscript{max} was 23 dpi at 45%, and EIP\textsubscript{min} was 11 dpi at 15%. For the OLDER group, EIP\textsubscript{max} was 16 dpi at 10% and EIP\textsubscript{min} was 11 dpi at 5%. For the S.OLDER group, EIP\textsubscript{max} was 16 dpi at 15% and the EIP\textsubscript{min} was 11 dpi at 10%.

2.3.2 Mortality of \textit{Aedes aegypti} is modestly affected by timing of infectious bloodmeal

When each infectious treatment was compared to a matching non-infectious control group, the only significant difference in average time to death (TTD) was between the S.OLDER and S.M treatments, with an estimated difference in TTD of two days (Appendix 3, Table S3). Of interest, the non-blood fed sugar-only controls died significantly faster than any of the blood fed treatments with an average TTD of 19.6 days (Appendix 3, Table S3), which has been previously shown (Styer, Minnick, et al. 2007).

Pairwise comparisons of the ZIKV-exposed treatments determined that group YOUNG had a significantly longer average time to death (TTD) when compared to groups OLDER and S.OLDER, though this difference was modest (0.6 and 1.4 days, respectively) (Figure 2.3). The TTD for the YOUNG group was 25.9 days, 25.3 days for the OLDER group, and 24.5 days for the S.OLDER group. This corresponds to average daily survival probabilities of 0.962, 0.961, and 0.960 for the YOUNG, OLDER, and S.OLDER groups, respectively. Predicted daily survival rates were generated for the YOUNG group using a non-linear fit and a linear model was fit to the OLDER and S.OLDER groups. The parameters of these models and goodness of fit assessments are given in Appendix 3, Table S2, and the observed and predicted values are shown in Appendix 3, Figure S2 for all three
treatment groups. Additional comparisons were made among the treatment and control groups, detailed in Appendix 3, Supporting Text S2, Figure S3, and Table S3.

Figure 2.3. Survival curves of female *Ae. aegypti* by treatment. Each line represents the combined data from three replicates per treatment: YOUNG (gold line), OLDER (green line), and sugar-OLDER (S.OLDER, red line). Average time to death of treatment, YOUNG was significantly, but modestly, longer than treatments OLDER and S.OLDER

2.3.3 Age-dependence of willingness to probe

There was no significant difference in the willingness to probe based on treatment (YOUNG, OLDER, S.OLDER) at each day post infection via Kruskal-Wallis test, but there was a significant effect of age (p<0.05). We then were able to fit a daily probability of probing based on age (Figure 2.4).
Figure 2.4. Observed and predicted probabilities of daily biting. The observed daily biting frequencies (dots) from the laboratory experiments and the fitted daily predictions (green curve)

2.3.4 \( \text{VC}_{\text{age}} \) framework for assessing transmission as a function of age and effect on \( R_0 \)

To calculate \( \text{VC}_{\text{age}} \), we used treatment-group specific average TTD and predicted daily probabilities of survival (Appendix 3, Figure S4) and the overall daily prediction of willingness to bite in Figure 2.4. Since biting rate (the number of bites per day) is a field-derived parameter, we calculated \( \text{VC}_{\text{age}} \) over three biting rates – 0.5 (once every two days), 1 (once a day), and 2 (twice a day) (Christofferson and Mores 2011). For comparison, we calculated the traditional VC using the average life-dependent traits determined experimentally above, \( \text{EIP}_{\text{min}} \) and \( \text{EIP}_{\text{max}} \), and the biting rates referenced above. These VC values are given in Table 2, along with corresponding values for \( R_0 \). In the absence of published data regarding the probability of human to mosquito ZIKV transmission success, we used the average infection rate from our experimental data (78.2%) to parameterize
human to mosquito transmission, recognizing that this model system used a high dose of ZIKV titer, although such titers in individuals are not unheard of (Perkasa et al. 2016; Waggoner et al. 2016).

**Table 2.2** Traditional VC calculations for EIP\textsubscript{min} and EIP\textsubscript{max} for each treatment group (calculated according to Equation 2.1) and corresponding R\textsubscript{0} (according to Equation 2.2)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EIP</th>
<th>Biting rate</th>
<th>VC</th>
<th>R\textsubscript{0}</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOUNG</td>
<td>EIP\textsubscript{max}</td>
<td>2</td>
<td>19.06</td>
<td>74.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>4.77</td>
<td>18.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>1.19</td>
<td>4.65</td>
</tr>
<tr>
<td></td>
<td>EIP\textsubscript{min}</td>
<td>2</td>
<td>10.11</td>
<td>39.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2.53</td>
<td>9.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>0.632</td>
<td>2.47</td>
</tr>
<tr>
<td>OLDER</td>
<td>EIP\textsubscript{max}</td>
<td>2</td>
<td>4.03</td>
<td>15.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.01</td>
<td>3.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>0.25</td>
<td>0.978</td>
</tr>
<tr>
<td></td>
<td>EIP\textsubscript{min}</td>
<td>2</td>
<td>6.49</td>
<td>26.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.62</td>
<td>6.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>0.41</td>
<td>1.60</td>
</tr>
<tr>
<td>S.OLDER</td>
<td>EIP\textsubscript{max}</td>
<td>2</td>
<td>7.65</td>
<td>29.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.91</td>
<td>7.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>0.48</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>EIP\textsubscript{min}</td>
<td>2</td>
<td>6.25</td>
<td>24.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.56</td>
<td>6.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>0.39</td>
<td>1.52</td>
</tr>
</tbody>
</table>
2.3.5 YOUNG group

To achieve an $R_0$ of 1, the vectorial capacity needed to be at or above 0.256. When parameterized with $EIP_{\text{max}}$, the $VC_{\text{age}}$ at a biting rate of 0.5 (once every two days) did not achieve this minimal value, but at biting rates of once or twice per day, $VC_{\text{age}}$ was sufficient to push $R_0$ over the threshold of one (Figure 5). We next determined the window of opportunity, which encompasses the days post emergence when a mosquito acquires an infection and which results in $VC_{\text{age}}$ values where $R_0$ would be greater than one. The window of opportunity when we parameterized $VC_{\text{age}}$ with $EIP_{\text{max}}$ indicated that a virus must be acquired within the first 2 days post emergence when the bite rate was twice or once per day (Figure 2.5). Interestingly, when parameterized with $EIP_{\text{min}}$, all bite rates reached sufficient $VC_{\text{age}}$ values. The window of opportunity was 14 days post emergence for a bite rate of twice a day, 12 days post emergence for a bite rate of once a day, and 7 days post emergence for a bite rate of once every two days (Figure 2.5). Even at its highest, $VC_{\text{age}}$ indicates that traditional VC calculations are likely overestimates.
Figure 2.5 Vectorial capacity calculations using VC_{age}. VC_{age} values (y-axis) depend on the age at acquisition (x-axis). Each curve calculated based on pre-determined bite rates of 2 (yellow line), 1 (blue line), and 0.5 (black line) for (left) EIP_{max} of the YOUNG group, = 45% at 23 dpi, and (right) EIP_{min} of the YOUNG group = 15% at 11 dpi. The dotted line represents the lower limit of VC where below this threshold, R_0 is less than 1.
2.3.6 OLDER and S.OLDER groups

For both the OLDER and S.OLDER groups, the infectious bloodmeal occurred at 12 days post emergence, which was outside the window of opportunity for $\text{VC}_{\text{age}}$ in all but one scenario (Appendix 3, Figure S4). Only at a bite rate of twice daily did the OLDER group achieve the minimum $\text{VC}_{\text{age}}$ of 0.256 on or after $\text{Age}_{\text{acquisition}}$ of 12 days old, and only when parameterized with the $\text{EIP}_{\text{min}}$. The window of opportunity for this scenario was days 12-13 post emergence.

With these vector age-dependent traits, both EIP and vector competence would need to be altered significantly to achieve a $\text{VC}_{\text{age}} \geq 0.256$ at 12 days post emergence for other scenarios. For example, we consider a hypothetical $\text{EIP}_{\text{max}}$ of $\text{EIP}_{50}$ (the time it takes for 50% of exposed mosquitoes to transmit) and a hypothetical $\text{EIP}_{\text{min}}$ of $\text{EIP}_{10}$ to demonstrate how these group-specific mortality rates and the age-structure of willingness to bite interact to drive vectorial capacity above the threshold in the context of $\text{Age}_{\text{acquisition}}$ (Goindin et al. 2015; Carrington et al. 2013; Paaijmans et al. 2012). The S.OLDER group would need a maximum $\text{EIP}_{50}$ of 12 dpi or 11 dpi for biting rates of twice and once daily, respectively, and 8 dpi for a biting rate of once every two days. For the OLDER group, the maximum $\text{EIP}_{50}$ would need to be 13, 12, or 11 dpi for biting rates of twice or once daily, or once every two days, respectively. With the $\text{EIP}_{\text{min}}$, similar maximum EIPs were noted of 12 and 9 dpi for biting rates of twice or once daily, respectively. For both the OLDER and S.OLDER groups, with a hypothetical $\text{EIP}_{\text{min}}$ of 10%, $\text{VC}_{\text{age}}$ never got above the threshold needed to get $R_0$ at or above 1.
2.4 Discussion

Prediction of vector-borne disease spread remains difficult, as transmission of vector-borne disease is a dynamic, multifaceted system. This includes life traits of the mosquito, environmental factors, and vector-virus interactions (Lounibos and Kramer 2016; Alto et al. 2018; Ciota and Kramer 2013; Hardy et al. 1983; Muttis et al. 2018). Here we demonstrate through a combination of experimental and computational methods that mosquito age at the time of pathogen acquisition is a powerful driver of transmission potential due, in large part, to the age-dependence of daily mortality and biting habits. Further, these drivers lead to differences in the estimates of $R_0$.

Our study, which focused upon the age at the time of infectious bloodmeal, showed no significant impacts on the vector competence of colony Ae. aegypti for ZIKV. While a recent study did show there were significant effects of multiple bloodmeals on vector competence (Armstrong et al. 2019), we delivered the second bloodmeal at a much longer interval (7 days versus 3 dpi in (Armstrong et al. 2019)), which likely plays a role in this disparate finding and indicates that there may be age-dependence in this phenomenon as well. However, this hypothesis is outside the scope of the current study. In our mortality study, we did observe a modest difference in TTD. We also observed a very short TTD in mosquitoes only given sugar with no bloodmeal. We chose not to pursue these effects, as the differences in TTD were modest and the impacts of no bloodmeal have been previously observed.

The technology for determining the age-structure of natural mosquito populations in the field is currently still in development. For example, a study using near-infrared spectroscopy was able to predict the age of female Ae. aegypti +/- 2 days, indicating that determining the age-structure of a mosquito population is possible, and that such technology could be refined for field studies (Sikulu-Lord et al. 2016; Lambert et al. 2018). Further, mid-infrared spectroscopy had varying, but some promising results in determining the age-
structure of *Anopheles* mosquitoes (Gonzalez Jimenez et al. 2019; Lambert et al. 2018). As these technologies are pursued and refined, there will be a need for ways to understand and quantify age-dependent interactions among vector competence, EIP, mosquito lifespan, and biting behavior (Lounibos and Kramer 2016; Johnson et al. 2020).

The methodologies in this study highlight the need to understand the quantitative interaction between vector competence, mosquito mortality, and age at time of infection acquisition. Thus, we anticipate that this methodology could be used to explore other important modifiers of vector competence and mosquito mortality and the interaction of the two, such as innate mosquito immunity response to infection, adult and larval microbiome, as well as other extrinsic factors known to affect these traits (Maciel-de-Freitas, Koella, and Lourenco-de-Oliveira 2011; Valderrama, Diaz, and Lopez-Verges 2017; Robert et al. 2016; Goindin et al. 2015; Chouin-Carneiro et al. 2016; Roundy et al. 2017; Brady et al. 2016; Hardy et al. 1983; Souza-Neto, Powell, and Bonizzoni 2019). Several recent studies have focused on environmental factors such as temperature, and found that temperature not only affects vector competence of many arboviruses, especially in *Aedes aegypti*, but also several life traits of the mosquito (Tesla et al. 2018; Alto et al. 2018; Muttis et al. 2018). This means that transmission is ultimately impacted by the interactions of all of these temporally dependent processes, and future studies should consider age-structure when assessing these impacts. Studies have also shown the impact of vector species, in particular the difference between field-derived and lab strains (Chouin-Carneiro et al. 2016; Roundy et al. 2017).

Here, we use a lab strain *Ae. aegypti*, which has been shown to vary in its competence when compared to a field-derived strain. Future use of VC_{age} could highlight these differences, as well as differences among various arbovirus-arthropod systems.

Our results demonstrate the importance of age-structure when evaluating the fitness of a mosquito-virus system and indicate that R_{0} may be overestimated when it is not considered.
This framework further shows that how within-mosquito arbovirus fitness is measured – often by comparing proportions of transmitting mosquitoes at arbitrary time points – is not sufficient. Here, when the Age\textsubscript{acquisition} was advanced, the difference in hypothetical EIPs necessary for the system to succeed was not very different. For example, in the OLDER and S.OLDER groups, there was only one day difference between the EIP\textsubscript{min} and EIP\textsubscript{max} needed for success at biting rates of 2 and 1, though these quantities represented a difference of 40% in the proportion of mosquitoes transmitting. The same trend was demonstrated in the YOUNG group where EIP\textsubscript{min} resulted in longer windows of opportunity compared to EIP\textsubscript{max}. Additionally, when the minimum time to transmission is shorter, a larger portion of the mosquito population contributes to transmission, because VC\textsubscript{age} implicates older mosquitoes in this scenario given no significant reduction in mortality due to extrinsic factors (Onyango et al. 2020). Thus, VC\textsubscript{age} reveals that the temporality of the within-mosquito arbovirus dynamics is more impactful than the ultimate magnitude of this widely used fitness measure. More investigations into the earlier and minimal transmission rates – such as at earlier time points that we did not consider – may be warranted to quantify fully the contribution of lower but faster dissemination profiles in arbovirus systems.

A study by Althouse et. al. also found that the temporality of transmission from non-human primates was sometimes more impactful than the magnitude of the viremia leading to transmission to the mosquito (Althouse and Hanley 2015). They proposed a “tortoise-and-the-hare” (TatH) model to describe this relationship between arboviral viremia profiles in non-human primates and the predicted transmission success to vectors, showing that the strategy of “slow and steady” viremia – lower levels for longer periods – resulted in higher predicted transmission success of arboviruses (Althouse and Hanley 2015). This same TatH model recently described macro-transmission dynamics in Colombia, where it was...
demonstrated that slow burn-in epidemics actually resulted in cumulatively more cases and higher \( R_0 \) values than in initially explosive outbreaks (Pena-Garcia and Christofferson 2019). Vector competence is a continuous process over time, and as such, \( \text{EIP}_{\text{min}} \) and \( \text{EIP}_{\text{max}} \) are not independent measures. Thus, vector competence profiles with higher transmission rates at earlier times (“hare strategy”) will always be more fit than lower proportions at the same EIP. However, this study demonstrates that in systems where the maximum measured vector competence is low, but the time to minimum transmission is short (“tortoise strategy”) (Goddard et al. 2002), there is still a good chance the system will succeed. \( V\text{C}_{\text{age}} \) also suggests that how arbovirus phenotypes are compared and ranked, and perhaps even how the field identifies ‘highly’ or ‘negligibly’ competent vectors, may need adjustment in the context of mosquito age.

2.5 Notes:


Goindin, D., C. Delannay, C. Ramdini, J. Gustave, and F. Fouque. 2015. 'Parity and longevity of Aedes aegypti according to temperatures in controlled conditions and consequences on dengue transmission risks', PLoS One, 10: e0135489.


Massad, E., and F. A. Coutinho. 2012. 'Vectorial capacity, basic reproduction number, force of infection and all that: formal notation to complete and adjust their classical concepts and equations', Mem Inst Oswaldo Cruz, 107: 564-7.


3.1 Introduction

Vector-borne viruses remain a major cause of morbidity in low- and middle-income countries and have been making incursions into more temperate regions recently (Pietsch et al. 2020; Aubry et al. 2020; Vermeulen et al. 2020). Because there is no treatment available for many of these viruses, determining the factors that promote arboviral transmission, emergence, and expansion is critical for predicting and controlling the impact on human and animal health. Dengue virus (DENV), chikungunya virus (CHIKV), and Zika virus (ZIKV) are transmitted by Aedes aegypti, an urban-dwelling mosquito widespread throughout tropical and subtropical areas (Brady et al. 2013; Kraemer et al. 2019; Messina et al. 2014; Kraemer et al. 2015; Lounibos and Kramer 2016). The viruses this vector species transmits are responsible for large outbreaks affecting millions of people every year (Valderrama, Diaz, and Lopez-Verges 2017; Brathwaite Dick et al. 2012; Marano et al. 2017; Braack et al. 2018; "Dengue/DHF update (12): Americas” 2020). Complete understanding of the transmission systems of these arboviruses provides insight into the spread of the virus, especially when parameterizing prediction models that may be used in decision-making (Campos et al. 2020; Ciota and Kramer 2013).

Vector competence is the intrinsic susceptibility of a vector species to infection with and subsequent transmission of a pathogen (Valderrama, Diaz, and Lopez-Verges 2017; Kramer and Ciota 2015; Ciota and Kramer 2013; Christofferson and Mores 2011).

In a study on which Dr. William Black is the senior author, the importance of vector competence is explained as follows: “Understanding the relative vector competence of mosquitoes at the species, population, and individual levels is critical to the study of vector biology and the success of future vector-borne disease control programs” (Richardson et al. 2006).

Measures of vector competence have evolved since first being included in the vectorial capacity equation in the late 20th century (Garrett-Jones 1964; Kramer and Ciota 2015). Vector competence has been determined by calculating either the proportion of vectors that are infected, that have a disseminated infection in the legs, or have detectable viral particles in forcibly collected saliva (Kramer and Ciota 2015; Smith et al. 2005). Usually, these measures are done at discrete, systematic time points, which may or may not accurately capture the process of vector competence (Christofferson, Mores, and Wearing 2016; Bellan 2010). For example, recent studies have demonstrated that discrete, terminal sample strategies do not capture the impact of individual heterogeneity on transmission efficiency (Mayton et al. 2020; Christofferson et al. 2014; Christofferson, Mores, and Wearing 2014; Armstrong et al. 2020) and that there are a multitude of factors that govern the ultimate success of transmission of an arbovirus, including mosquito behavior and within-virus kinetics (Lounibos and Kramer 2016, Kramer and Ciota 2015, Hardy et al. 1983).

While traditional ways of measuring vector competence are essential in determining successful vector–virus systems, the impact of these other factors, along with the impact of individual mosquito heterogeneity, must be investigated in order to further describe the transmitting population (Cator et al. 2020). Here, we present a method of measuring transmission potential that longitudinally samples the same individual mosquitoes, capturing biting behavior and transmission capability over time, as well as heterogeneity in viral output
from single mosquitoes. We use a model system of Rockefeller colony mosquitoes, field-derived mosquitoes, and ZIKV to demonstrate the method and describe the output data.

3.2 Methods

3.2.1 Cell Culture and Virus

ZIKV strain PRVABC59 (Asian lineage), which was isolated from human serum in Puerto Rico in 2015, was provided by Dr. Barbara Johnson at the US Centers for Disease Control and Prevention. Prior to use, the viral stock was passaged four times in Vero cells. On the fourth passage, cells were inoculated onto Vero cells at a multiplicity of infection (MOI) of 1. Supernatant was collected at 4 days post inoculation (dpi) and titer was determined by a neutral red plaque assay and qRT-PCR as previously described (Kawiecki et al. 2017). Virus was passaged onto Vero cells before being exposed to mosquitoes, as frozen virus has been shown to negatively affect mosquito susceptibility (Weger-Lucarelli et al. 2016; Ciota et al. 2017). Supernatant was collected at 4 dpi and titer was determined using qRT-PCR before being used the same day for exposure. Titers were matched across all experiments as \( \sim 5 \times 10^7 \) pfu/mL as previously described (Mayton et al. 2020; Kawiecki et al. 2017).

3.2.2 Viral Quantification and Testing

RNA extraction was performed using the 5× MagMax96 viral nucleic acid isolation kit (Applied Biosystems, Foster City, CA, USA) and the KingFisher Flex (Thermo Fisher). Viral RNA was detected and quantified by qRT-PCR, using the SuperScript III Platinum Taq kit (Invitrogen) and the Roche LightCycler 96 as previously described (Mayton et al. 2020; Faye et al. 2008). A standard curve was run on all plates, with the lowest detectable dilution being our limit of detection (LoD). Any samples between our LoD Cq value and a Cq of 40 were
inoculated onto Vero cells for confirmation of replicating virus. A neutral red plaque assay was used to titer our viral stock and indicated samples.

3.2.3 Mosquito Exposure and Maintenance

Lab-strain *Ae. aegypti* (Rockefeller) and fifth generation (F5) field-derived *Ae. aegypti* collected from southern Texas were used in this experiment. The Rockefeller strain was provided by Dr. Daniel Swale of the Louisiana State University Entomology Department, Baton Rouge, LA (Mayton et al. 2020). For field-derived *Ae. aegypti*, eggs were collected using oviposition traps in 7 cities across the Texas/Mexico border. Field collected eggs (F0) were hatched in a 1 g/L aerated nutrient broth mixture and reared to adult emergence in larval rearing pans stored at 23.9 degrees Celsius with 1:1 liver powder as needed. Once pupated, adults were moved to an environmental chamber kept at 24.6 °C and 70% relative humidity and a 16:8 light/dark cycle. One week after emergence, female mosquitoes were fed defibrinated cow blood using a Hemotek artificial feeding system (Hemotek, Blackburn, UK) and allowed to oviposit on oviposition papers in cages. These F1 generation eggs were provided to our lab and were reared in the same manner in the laboratory for four more generations. Generation five (F5) were used for experimental purposes here.

At 3–5 days post-emergence, mosquitoes were starved of sugar solution for 24 h before being exposed for 45 min to a ZIKV bloodmeal containing 2 mL whole bovine blood in Alsevers (Hemostat Labs, Dixon, CA, USA) and 1 mL viral supernatant using the Hemotek artificial feeding system with a 3 mL reservoir. Mosquitoes were then cold anesthetized and engorged females were sorted into new cartons. Cotton soaked with 10% sugar solution was provided for all mosquitoes *ad libitum*. Mosquitoes were housed at 28 °C, 16:8 light/dark schedule, and 80% relative humidity (Tramonte and Christofferson 2019). Wet oviposition paper was provided in each canister and carton and was rehydrated once per day.
3.2.4 Traditional Vector Competence Assay

ZIKV-exposed mosquitoes were sampled \((n = 15–20)\) at corresponding time points—10, 14, and 18 days post exposure (dpe) for Rockefeller with an additional time point of 24 dpe from the individual cohort. Similarly, traditional vector competence included 10, 15, and 24 dpe for field-derived mosquitoes. These mosquitoes were not offered additional bloodmeal between exposure and terminal sampling. Infectious rates were determined by the presence of ZIKV in the saliva. Mosquitoes were cold anesthetized and placed on a cold pan before removing the legs and wings. Saliva was then collected via forced salivation by placing the proboscis into a micropipette tube containing 35 μL of fetal bovine serum (FBS) with 3 mmol/L ATP for 30 min as previously described (Tesla et al. 2018). Tip contents were then ejected into 100 μL of BA-1 (1% bovine serum albumin in M199X) media. RNA was extracted and qRT-PCR was performed on all samples as described above. In order to confirm the presence of replicating virus in the saliva, 50 μL of sample was inoculated onto 6-well plates of confluent Vero cells. Plates were rocked for 30 min at room temperature before 1.5 mL of M199X + 10% FBS, 2% antibiotic-antimycotic was added. Plates were observed for the presence of cytopathic effect (CPE) and supernatant was collected at 3- and 7-dpi and tested for the presence of viral RNA via qRT-PCR to confirm positive growth and thus the presence of infectious virus.

3.2.5 Limit of Detection

We compared the limits of viral recovery and detection using a known amount of virus and Hemotek reservoirs without having been offered to a mosquito. First, 180 μL of blood was spiked with 10 μL of the ZIKV viral stock described above at varying titers \((10^4–10^{-1} \text{ pfu/100 μL})\) and placed into Hemotek reservoirs. The Hemotek reservoirs were placed on the feeding system, which heats the reservoirs to 37 °C, for 45 min in order to mimic the conditions during blood offering to mosquitoes. Serum was collected and viral concentration
determined via qRT-PCR. To determine agreement between qRT-PCR results and infectious viral particles, the same serum collections were plaqued using a neutral red assay.

3.2.6 Longitudinal Sampling

Twenty-four hours before the start of the experiment, individual mosquitoes were placed into a clear, plastic canister (Figure 3.1). For our proof-of-principal trial with Rockefeller colony mosquitoes, one group of twelve mosquitoes was used every other day. We expanded this with the field-derived mosquitoes by assaying two staggered cohorts of individuals, one group observed/tested on even days, the other group on odd days. The tops of the canisters were removed and replaced with black fiberglass screen to avoid tearing the parafilm covering the Hemotek reservoir. Starting at 9 dpe (field-derived Group 1) or 10 dpe (Rockefeller/field-derived Group 2), mosquitoes were each provided an individual bloodmeal using a 0.3 mL reservoir containing 180 μL of bovine blood in Alsevers (see above). Blood was provided for 45 min at 37 °C. During the 45 min, behavior was observed by looking through the canister and/or lifting the reservoir and looking through the top of the canister to observe probing behavior at 1, 20, and 45 min as in (Mayton et al. 2020). Mosquitoes were classified as down (no probing or red abdomen observed), probed (exhibited probing behavior, no red abdomen observed), or fed (red abdomen observed).

After 45 min, blood was removed from the reservoirs by piercing the parafilm with a pipette tip, removing the blood with the pipette, and placing into individual microcentrifuge tubes. Tubes were centrifuged for 6 min at 4000 rpm at 4 °C to separate the serum. Serum was removed and placed in a new tube for further testing. This was done until 24 dpe, determined by previously observed vector competence studies and average time to death [23,33,34]. Collected serum was tested for the presence of viral RNA via qRT-PCR. In order to confirm the presence of replicating virus in the serum, 25 μL of sample was inoculated onto 12-well plates of confluent Vero cells. Plates were rocked for 30 min at room
temperature before 1.5 mL of M199X + 10% FBS, 2% antibiotic-antimycotic was added. Plates were observed for the presence of CPE and supernatant was collected at 3- and 7-dpi and tested for the presence of viral RNA via qRT-PCR to confirm positive growth and thus the presence of infectious virus. On the final day of the experiment, mosquitoes that survived were force salivated and processed as described for traditional vector competence.

We wanted to determine whether this method could be used to test whether the proportion of probing events vs. feeding events resulting in transmission was significantly different. To do this, we used a chi-square test of proportion (function prop.test, R version 3.5.3), with a confidence level of 95%. As a means of determining general reproducibility (lack of variation between replicates), we compared the proportions of overall biting events and transmission events within the two field-derived groups using the chi-square test of proportion as above.

![Experimental setup. Individual mosquitoes were housed in plastic canisters covered with fiberglass screen (left) and offered blood using the Hemotek artificial feeding system with the 300 μL reservoir and a custom stand (right).](image)

**Figure 3.1.** Experimental setup. Individual mosquitoes were housed in plastic canisters covered with fiberglass screen (left) and offered blood using the Hemotek artificial feeding system with the 300 μL reservoir and a custom stand (right).

### 3.3 Results

#### 3.3.1 Model System Development

##### 3.3.1.1 *In Vitro* Limit of Detection

To determine the limit of detection of our method, we first measured the recoverability of virus from sera using a controlled scenario. When we compare the viral concentration from
recovered sera via qRT-PCR and paired plaque assay, there was complete agreement at dilutions $10^{0-2}$. At higher viral concentrations, quantification of the plaque assay was hampered by too many plaques to count. We did determine that our limit of detection was 1 pfu/100 μL. However, our qRT-PCR assay was more sensitive and detected down to 0.1 viral RNA copies/100 μL (Appendix 4, Table S1). Based on these results, we determined our method to be sensitive and moved forward with the experiment using the qRT-PCR to determine viral concentration and delineate between pfu/volume versus RNA equivalents/volume.

### 3.3.1.2 Vector Competence by Traditional Measures

Rockefeller mosquitoes were terminally tested for the presence of ZIKV in the saliva at 10, 14, 18, and 24 dpe. Forced saliva results revealed 0% of mosquitoes transmitted at 10 dpe, 26.7% transmitted at 14 dpe, 46.7% transmitted at 18 dpe, and 87.5% at 24 dpe. All positive qRT-PCR samples were confirmed infectious by observation of viral growth in vitro. Titers of forced saliva and in vitro collections from 24 dpe are reported here (Appendix 4, Table S2). This indicates moderate to high vector competence as per traditional vector competence, which is consistent with previous studies (Mayton et al. 2020; Weger-Lucarelli et al. 2016).

### 3.3.1.3 Individual, Longitudinal Vector Competence Method

A novel method was developed to assess vector competence, extrinsic incubation period (EIP), and biting habits at the individual mosquito level. Biting behavior was observed when bloodmeals were offered every other day starting at 10 dpe and ending at 24 dpe. Biting behaviors were recorded as either blood fed or probed. Over the course of the study period, 11/12 Rockefeller mosquitoes bit (either blood fed or probed) and all 11 bit more than once (either blood fed or probed) (Figure 3.2).
Despite robust biting habits, only four individuals from the Rockefeller colony successfully transmitted (ID# 1, 5, 7, and 10), and of those all four transmitted more than once with a total number of nine transmission events (Figure 3.3a). From this method, we are able to discern time to first transmission, which was 14 dpe (Mosquito #5). In addition, it was possible to observe and characterize repeated transmission from the same mosquito specimen. Mosquito #1 had the most transmission events, with three starting at 18 dpe (Figure 3.3a).

The role of different biting behaviors and the subsequent transmission was observed. There was a total of three transmission events with probing and seven associated with blood feeding from Rockefeller mosquitoes (Figure 3.3a). We calculated the proportion of probing and feeding events that resulted in transmission as 13.6% and 15%, respectively. There was not a significant difference between transmission proportion relative to type of behavior ($p > 0.05$). The range of recovered viral quantities from serum collections was 0.2 viral RNA copies/100 μL (below in vitro limit of detection), and 1.9 pfu/100 μL to 290 pfu/100 μL.
(within in vitro limit of detection). Interestingly, we observed variability in output from the same mosquito over different transmission events (Figure 3.3b) (Appendix 4, Table S3). Both the lowest (probing) and highest (blood feeding) recovered virus quantity was from the same mosquito (ID# 5) (Figure 3.3a).

**Figure 3.3.** Viral titers expectorated with each successful transmission event by Rockefeller individuals. Only mosquitoes which had successful transmission over time are shown. A successful transmission event is defined by a positive serum sample via qRT-PCR and growth on Vero cells. (a) Each shaded square represents a transmission event, with white squares representing no detectable virus. Other squares are scored from lowest viral titer (light blue) to highest viral titer (navy). (b) Viral titers present in serum were compared for blood fed (F) vs. probed (P). Viral titers range from lowest detectable titer (light blue) to highest detectable titer (navy). Asterisks (*) indicate where recovered quantity is below the limit of detection from our sensitivity analysis and reflect qRT-PCR values of genome equivalents (viral RNA copies/100 μL) rather than pfu/100 μL.

Next, we demonstrated the differences in traditional vector competence measures to findings from our longitudinal sampling methodology (Figure 3.4). There was no transmission at 10 dpe in either method (Figure 3.4). The proportion of transmission events observed from the longitudinal sampling method was calculated two different ways: (1) as the proportion of mosquitoes that successfully transmitted over the total number of living mosquitoes per sampling day and (2) the number of mosquitoes that successfully transmitted over the total number of mosquitoes that bit per sampling day. Overall, lower proportions of transmission events (calculated either way) were observed compared to the proportion
infectious mosquitoes measured by traditional vector competence (Figure 3.4). In the longitudinal sampling method, we found that the proportion of mosquitoes that transmitted out of biting mosquitoes was higher than the proportion that transmitted out of total mosquitoes, indicating that the denominator (and thus transmitting proportion) is sensitive to inclusion of biting behavior.

![Figure 3.4. Rockefeller transmission profile. Traditional vector competence was calculated as the proportion of mosquitoes with positive forced saliva samples divided by the number sampled (black line). From the longitudinal sampling method, the total number of transmitting mosquitoes over the total number of living mosquitoes was calculated per sampling day (yellow line). Lastly, the total number of transmitting mosquitoes over the total number of mosquitoes that bit was calculated per sampling day (blue line).](image)

3.3.2 Application of Method to Field-Derived Mosquitoes

Field-derived mosquitoes were terminally tested for the presence of ZIKV in the saliva at 5, 10, 15, and 24 dpe. Results revealed 0% of mosquitoes had viral detection at 5 and 10 dpe, 5% had detection at 15 dpe, and 62.5% had detection at 24 dpe. Again, all positive qRT-PCR samples were tested for infectious virus by observation of viral growth in vitro (Table...
S2). Biting behavior was observed when bloodmeals were offered every other day starting at 9 dpe (field-derived Group 1) or 10 dpe (field-derived Group 2) and ending at 24 dpe to get complete coverage of all days of the study of field-derived mosquitoes. When compared, biting frequencies between Group 1 and Group 2 of the field-derived mosquitoes were not significantly different; therefore, field-derived groups were combined and will be described as one population of 30. Over the course of the study period, 25/30 mosquitoes bit, and 20/25 bit more than once (Figure 3.5). When compared via chi-square test of proportions, Rockefeller mosquitoes exhibited a significantly higher proportion of biting behavior (72.1%) than field-derived mosquitoes (42.1%) ($p$-value < 0.05).

![Diagram](image1.png)

**Figure 3.5.** Biting behavior of field-derived individuals over the study period. Each row represents one mosquito across days post exposure. Each shaded square represents a potential transmission event, with mosquitoes being classified as no biting (grey), probing (light blue), and blood fed (dark blue). White squares indicate no opportunity (dead). Mosquito IDs 1–14 represent Group 1 (left), while IDs 15–30 represent Group 2 (right).

Six field-derived individuals successfully transmitted (ID# 12, 15, 24, 25, 28, 30), but there were only seven transmission events (Figure 3.6a). Time to first transmission occurred at 18 dpe, and only one mosquito had more than one successful transmission event (Figure 3.6a). Of the seven successful transmission events, five resulted from feeding behavior and two events resulted from probing behavior. Similar to the Rockefeller colony, blood feeding
behavior yielded a higher maximum titer compared to probing behavior (Figure 3.6b). When compared, the proportion of transmission events by feeding (71.4%) was significantly higher than events by probing (28.6%) ($p < 0.05$). Mosquito #12 was the only mosquito to successfully transmit more than once. Again, titers were variable among transmission events.

Field-derived mosquitoes expectorated viral quantities ranging from 0.2 viral RNA copies (below in vitro limit of detection), and 1.5 to 28.3 pfu/100 μL (Appendix 4, Table S3).

Figure 3.7 shows the differences in traditional vector competence measures and the longitudinal sampling method for field-derived mosquitoes. Again, the traditional measure reached higher transmission rates compared to the longitudinal sampling measures (Figure 3.7).

![Figure 3.6](image)

**Figure 3.6.** Viral titers expectorated with each successful transmission event by field-derived individuals. Only mosquitoes which had successful transmission over time are shown. A successful transmission event is defined by both a positive serum sample via qRT-PCR and growth on Vero cells. (a) Each shaded square represents a transmission event, with white squares representing no detectable virus. Other squares are scored from lowest viral titer (light blue) to highest viral titer (navy). (b) Viral titers present in serum were compared for blood fed (F) vs. probed (P). Viral titers range from lowest detectable titer (light blue) to highest detectable titer (navy). Asterisks (*) indicate where recovered quantity is below the limit of detection from our sensitivity analysis and reflect qRT-PCR values of genome equivalents (viral RNA copies/100 μL) rather than pfu/100 μL.
Figure 3.7. Field-derived transmission profile. Traditional vector competence was calculated as the proportion of mosquitoes with positive forced saliva divided by the number sampled (black line). The total number of transmitting mosquitoes over the total number of living mosquitoes was calculated per sampling day (yellow line). Lastly, the total number of transmitting mosquitoes over the total number of mosquitoes that exhibited biting behavior was calculated per sampling day (blue line).

3.4 Discussion

* Ae. aegypti * are unique in that they take multiple bloodmeals during a gonotrophic cycle (Scott, Clark, et al. 1993; Cebrian-Camison, Martinez-de la Puente, and Figuerola 2020). Being an urban mosquito, they are often present in or near households, making it likely to bite humans more than once (Scott, Chow, et al. 1993; Stoddard et al. 2013). Traditional vector competence tells us the subset of mosquitoes that are capable of transmitting arboviruses. However, actual transmission is a function of several other conditions. Here, we have developed a method which can account for the interaction of some of the vector traits that define these conditions and the subset of mosquitoes that do the transmitting; namely,
biting behavior, vector competence, and EIP at one time. Due to the many factors affecting vector competence, such as geographic location, viral strain, and mosquito population/species, it is imperative that we continue exploring the heterogeneity of transmission potential both at the population and individual levels (Aubry et al. 2020, Christofferson and Mores 2011, Weger-Lucarelli et al. 2016, Ciota et al. 2017). Further, modification of this method could target time points of the same mosquito to get more traditional measures (% at discreet time points) to determine the distribution of EIP in a population after exposure.

Importantly, this method successfully reveals the heterogeneity of transmission potential among individuals. The relationship between biting and viral output were observed. Overall, titers of recovered serum ranged from $10^2$–$10^{-1}$, which is consistent with a previous study of ZIKV in *Ae. aegypti* (Aubry et al. 2020). Our data suggests that those that fed tended to have higher viral titers recovered from the serum than those that probed, which was previously observed in *Culex* spp. (Styer, Bernard, and Kramer 2006). Congruency with these two studies suggests our method will be a useful tool for assessing vector competence and testing hypotheses regarding viral transmission at the individual-mosquito level. For example, we observed one mosquito having both the highest and the lowest viral titer output, associated with a bite and a probing event, respectively. Further, we observed that one mosquito had an “empty” feeding event between transmission events, while other transmitters had consistent transmission. Further, average viral titer of serum collections differed between the two colonies, suggesting differences of viral output at both the individual and population level. Differences in biting frequency between the Texas and Rockefeller mosquitoes were noted, with biting proportions significantly lower for field-derived Texas mosquitoes compared to the Rockefeller colony. This is not surprising, as lab colonies are likely adapted to lab conditions, which is why we chose to validate the method in field-derived mosquitoes (Ross,
Endersby-Harshman, and Hoffmann 2019). This longitudinal method is thorough enough to detect these differences and thus allows for further hypothesis testing regarding the mechanisms behind this phenomenon and other heterogeneity observed. Other methods have also investigated vector competence in longitudinal ways, highlighting the importance of this research (Hol, Lambrechts, and Prakash 2020; Kumar et al. 2020). Of course, with an artificial system, there is a lack of biological cues associated with feeding (Zermoglio et al. 2017; Dekker and Carde 2011; Healy et al. 2002). Although our method uses an artificial system, this makes it both accessible and cost effective while longitudinally sampling individuals for virus transmission in the context of mosquito behaviors.

Traditional vector competence is a cumulative measure, which is monotonically increasing and often described by a logistic function (Christofferson et al. 2014; Aubry et al. 2021). In contrast, our results are highly variable, indicating the process of transmission is likely heterogeneous at the population and individual levels. For example, when we further consider cumulative transmission events as the proportion of transmission events over cumulative biting events, these were not significantly different between Rockefeller and field-derived (14.5% vs. 16.7%, respectively, \( p > 0.05 \)), despite field-derived mosquitoes having a lower overall biting frequency. This suggests that continued study is needed to elucidate the functional relationship between population bite frequency and transmission intensity.

The discrepancy between traditional vector competence and the results from the longitudinal sampling method could be due to several factors. First, our mosquitoes were offered multiple bloodmeals, which has been shown to increase vector competence for some arboviruses (Armstrong et al. 2020). However, previous work from our laboratory showed contrasting results (Mayton et al. 2020). Second, our method takes into account mosquito behavior, which traditional vector competence measures cannot. Third, forced salivation
assays by definition, compels salivation and all but guarantees virus recovery. The traditional assay does not account for the myriad of micro-processes that occur during mosquito contact with human hosts, including variability in saliva deposition, behaviors, and the possibility of inherent heterogeneity among mosquitoes. We hypothesize that this individual, longitudinal method provides a means to test how these and other factors define the successful contacts that result in transmission. Importantly, we successfully observed these differences, and were able to isolate virus from these biting events.

Traditional vector competence remains a crucial part of identifying vectors with the potential to support viral spread, and is an important step towards investigating intricate interactions between the vector, virus, and environment occurring at all stages of vector competence (Azar and Weaver 2019). The novel method proposed herein can also be performed at limited, discrete time points, similar to traditional vector competence sampling methods, which would be more directly comparable to vector competence data output. However, our method—unlike cohort sampling—allows for observation of individual heterogeneity of metrics such as the extrinsic incubation period, viral titer output, and associated biting behavior of the mosquito. Further, longitudinal measurement of individual feeding opportunities, for example, can be used to determine not only post-exposure dynamics (transmission), but interrogate the role of pre-exposure behaviors that affect the infection and dissemination dynamics within cohorts (e.g., number of bloodmeals).

Ultimately, this method can be used to ask nuanced questions about effectors of vector competence and transmission, such as the role of length of time probing, vector–virus interactions, and the role of environmental factors (Alto et al. 2018; Christofferson and Mores 2016; Muttis et al. 2018).

Mosquito populations in arbovirus-endemic areas can be subset according to exposure (Figure 8). First, only a subset of the total mosquito population will become exposed to
infectious individuals (“Exposed”). Second, a subset of these mosquitoes will become infected, meaning the infection will remain sequestered in the midgut (“Infected”) (Danet et al. 2019; Kramer and Ciota 2015). Third, some of these infected mosquitoes will develop disseminated infections—which is what is measured by traditional vector competence assays that use periphery tissues such as legs, wings, or heads to detect the presence of virus (Ciota and Kramer 2013, Weger-Lucarelli et al. 2016, Ciota et al. 2017). Forced saliva, while thought to be a better indication of truly infectious individuals, still only identifies that subset of mosquitoes that are infectious because the data inherently describes those mosquitoes that can transmit. Longitudinal sampling in the manner described herein includes the characteristics of individual mosquito behavior, which further funnels the population of mosquitoes into those that do transmit (Figure 3.8). Further, our method identifies repeat transmitters, which is impossible with terminal assays, and with this we can begin to investigate the concept of super-spreaders and the multitude of individual and heterogeneous vector–virus interactions that drive transmission.

Figure 3.8. Schematic representing the process of vector competence. As susceptible mosquitoes progress through the stages of vector competence, the population funnels down into the transmitting population, the small group that our method aims to further describe.
3.5 Notes


*Dengue/DHF update (12): Americas,* in ProMED-mail. 2020.


Kumar, S., et al., 2020.


CHAPTER 4: PHYLOGENETIC ANALYSIS OF EXISTING MAYARO VIRUS GENOME SEQUENCES AND USE IN VECTOR COMPETENCE STUDIES

4.1 Introduction.

Mayaro virus (MAYV) is a mosquito-borne Alphavirus belonging to the Togaviridae family. MAYV was first isolated from human serum in 1954 in Trinidad and Tobago, and is known to cause febrile illness characterized by persistent myalgia. Because it presents in a similar manner to a closely related, co-circulating Alphavirus, Chikungunya virus (CHIKV), it is likely that it is misdiagnosed (Acosta-Ampudia et al. 2018). Autochthonous transmission of MAYV has been detected in multiple countries in South America, including Peru, Brazil, and Venezuela, as well as in Mexico (Mackay and Arden 2016; Diagne et al. 2020). In 2014, cases of MAYV emerged in Haiti, indicating introduction into a naïve population (Blohm et al. 2019). Most recently, in 2020, a cluster of cases were detected in urban areas of French Guiana (WHO 2020). In the sylvatic cycle, MAYV is known to be transmitted by *Haemogogus spp.*, though a primary urban vector has yet to be identified (Mota et al. 2015; Diop et al. 2019). *Aedes aegypti* and *Aedes albopictus* are urban-dwelling mosquitoes that could be responsible for transmission, as they are capable of transmitting many other mosquito-borne viruses, such as CHIKV (Vega-Rua et al. 2014; Figueiredo and Figueiredo 2014; Souza-Neto, Powell, and Bonizzoni 2019).

Genetic variability and emergence of new lineages of arboviruses has been associated with changes in vector competence, which has in some cases led to significant expansion events such as in West Nile virus (WNV) (Moudy et al. 2007; Ebel et al. 2004) and CHIKV.
(Vega-Rua et al. 2014). While a primary urban vector has yet to be identified, there are still very few vector competence studies performed with MAYV, and of these studies, there is little diversity among the strains used (Dieme, Ciot, and Kramer 2020; Diagne et al. 2020). Therefore, there is a general gap in knowledge regarding the interplay between genotype and within-mosquito phenotype. However, there is also a general lack of data regarding MAYV genetic diversity in general, indicating that a first step in determining the role of this interplay in MAYV transmission is to characterize MAYV genetic diversity. Currently, a total of 72 full genome sequences of MAYV are publicly available in GenBank. To date, no phylogenetic analyses have been published that contain all 72 sequences.

Three lineages have been described for MAYV: Genotype D, which makes up the majority of available sequenced strains; Genotype N, which consists of a single strain from Peru; and Genotype L, which has been mostly isolated to Northern Brazil. There are two known D/L Genotype recombinants: a strain isolated from Haiti in 2015, and a strain isolated from Brazil in 2014, both of which were identified in 2017 (Mavian et al. 2017). While only these two recombinants were detected at the time, additional strains have been identified and sequenced and it is possible that more recombinants are circulating. To begin further characterizing the genetic diversity of MAYV, we chose to conduct a number of phylogenetic analyses addressing potential recombination among available MAYV strains. In addition, we reviewed available vector competence data using Ae. aegypti and Ae. albopictus in an attempt to identify potential variation in vector competence and extrinsic incubation period (EIP) based on viral lineage.
4.2 Methods

4.2.1 Phylogenetic analyses

All sequences were collected from GenBank. Alignment of sequences was performed using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) online (Edgar 2004). A best-fit substitution model (GTR+F+I+G4) was chosen based on the Bayesian information criterion (BIC) score using ModelFinder in IQ-TREE v2.1.3, and maximum likelihood (ML) tree construction performed using IQ-TREE v2.1.3 with Ultra-Fast bootstrap (Minh et al. 2020; Hoang et al. 2018; Kalyaanamoorthy et al. 2017). This was run three times to ensure the same tree was chosen each time. The resulting ML tree was visualized using FigTree v1.4.4 (Rambaut 2018). PHIPack was used to perform a Pairwise Homoplasy Index (PHI) test of recombination (Bruen, Philippe, and Bryant 2006). IQ-TREE was used to perform an approximate unbiased (AU) test on clade constraints (Genotype D and Genotype L clades) of the three detected recombinants (Shimodaira 2002). Recombination Detection Program 4 (RDP4) was used to test for the presence of recombinants and potential breakpoints (Martin et al. 2015).

4.2.2 Vector competence review

In PubMed, “Mayaro virus” and “Aedes” were searched. Papers with titles mentioning MAYV were opened, and abstracts read. Abstracts containing “Mayaro virus” and “Aedes” were read in their entirety. Those that orally exposed mosquitoes and tested for transmission via salivary gland extraction or forced saliva were included. Studies using intrathoracic inoculation were not included, as this method will shorten the EIP (Chan et al. 2020). Studies that did not state the strain of MAYV used were not included. In addition, to augment
existing data, a combination of Texas field-caught *Ae. aegypti* and MAYV strain TRVL 4675 were assayed for vector competence in the Christofferson laboratory (Appendix 5).

As we show above in Chapter 2 (Mayton et al. 2020), time to first infectious bite, or the minimum EIP (EIP\textsubscript{MIN}), and the time to maximum proportion of infectious mosquitoes, or the maximum EIP (EIP\textsubscript{MAX}) are important measurements for determining the window of opportunity for transmission. Using the data provided in our literature review, we calculated the average EIP\textsubscript{MIN} and EIP\textsubscript{MAX} for each lineage used. This was done for EIP\textsubscript{MIN} by averaging the earliest days of observed infectious saliva for each mosquito-virus combination. The average EIP\textsubscript{MAX} was determined by averaging the sampling days with the highest proportion of infectious mosquitoes. Corresponding average transmission rates were determined by averaging transmission rates at that time point. Transmission rates were determined by the number of positive saliva/salivary gland samples over the total number of mosquitoes sampled.

In order to observe and compare the overall transmission potential for the genotypes/mosquito species, the reproductive number (R\textsubscript{0}) was calculated using a modified vectorial capacity equation developed in Chapter 2 (VC\textsubscript{Age}) (Mayton et al. 2020). To calculate the basic reproductive number (R\textsubscript{0}) from a modified VC\textsubscript{Age}, we parameterized the MAYV infectious period as 7 days (Torres et al. 2004) and held constant the probability of transmission to the vector as 1. Further, to determine maximum transmission potential for each EIP-lineage-mosquito combination, we held constant the biting rate at 2 (Mayton et al. 2020). The probability of daily survival of *Ae. aegypti* was taken from (Mayton et al. 2020),
while the probability of daily survival of *Ae. albopictus* was taken from (Muhammad et al. 2020). The modified VC<sub>Age</sub> does not include the probability of biting, as that is not available for *Ae. albopictus*, and our goal was to observe relative values of R<sub>0</sub> and windows of opportunity for the combinations.

### 4.3 Results

#### 4.3.1 Support for recombination

The program RDP4 was used to identify potential recombination signals, as well as parental strains and breakpoints within the genome (Table 1) (Martin et al. 2015). RDP4 utilizes multiple methods of detection, including Rdp, BOOTSCAN, GENECONV, and SiScan, which utilize phylogenetic methods of detection, as well as Maxchi, 3seq, and Chimaera, which utilize nucleotide substitution methods to test for and characterize recombination events (Posada 2002; Gibbs, Armstrong, and Gibbs 2000; Boni, Posada, and Feldman 2007; Martin et al. 2015; Martin et al. 2005; Graham, McNeney, and Seillier-Moiseiwitsch 2005; Sawyer 1989). In addition to the previously identified recombinants, a third recombinant (Haiti-1/2014) was detected as well, although the program warned that this new recombination event may be due to evolutionary events other than recombination. Strains BeH473130 and FPI_1738 were suggested as the major and minor parents for all three recombinant strains. All detection methods were significant when testing for recombination signal from all three recombinants (p<0.05).
Table 4.1. Results of RDP4 analysis of sequence alignment. The detected recombinant strain, major and minor parents, and breakpoint locations within the recombinant’s sequence are listed below.

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>Major Parent</th>
<th>Minor Parent</th>
<th>Breakpoint Locations</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR/SJRP/LPV01/2015</td>
<td>BeH473130</td>
<td>FPI_1738</td>
<td>140; 10926</td>
</tr>
<tr>
<td>Haiti-1/2015</td>
<td>BeH473130</td>
<td>FPI_1738</td>
<td>169; 10957</td>
</tr>
<tr>
<td>Haiti-1/2014</td>
<td>BeH473130</td>
<td>FPI_1738</td>
<td>40; 11233</td>
</tr>
</tbody>
</table>

The PHI test was utilized to further test for the presence of recombinants. This test simply tests for the presence of recombination and does not attempt to characterize recombinants (Bruen, Philippe, and Bryant 2006). An initial PHI test of all sequences yielded a p-value of $p<5.86\times10^{-31}$, indicating recombination signals. When the known recombinants, Haiti-1/2015 and BR/SJRP/LPV01/2015, were removed from the alignment, PHI test returned a p-value of $3.8\times10^{-6}$, indicating additional recombinants were present among the remaining strains. After removing the suggested recombinant Haiti-1/2014, PHI test gave a p-value of 0.997, indicating no other additional recombinants. This further supports Haiti-1/2014 as a recombinant strain.

In the subsequent ML tree, all three recombinants fell into the Genotype L clade and are grouped together (Figure 4.1). As the recombinants are suggested as Genotype D and L recombinants, tree constraints and topology tests were used to determine their most supported placement, which should coincide with the placement of the major parent (Genotype L). To do this, each recombinant was constrained to either the Genotype D or L clade for tree
building. An AU-test was used to test for support of resulting topologies. An AU-test p-value less than 0.05 indicates low support for the proposed tree. Tests for all three recombinants supported that their position in the Genotype L clade, as constraint of each sequence to the D Genotype clade was rejected by AU-test (Table 4.2). This further supports BeH473130 as a major parent.

Figure 4.1. Maximum likelihood tree of 72 MAYV strains. Maximum likelihood (ML) tree built using IQ-TREE. Genotypes L, D, and N are noted. Potential recombinant strains are highlighted.
Table 4.2. Clade constraint and approximate unbiased (AU) test p-value. P-values < 0.05 indicate rejection of a tree with that constraint and is indicated by a negative (-). Positive (+) indicates the support of a tree with that constraint. AU test was performed for trees with no constraint, constraint to the Genotype L clade, and constraint to the Genotype D clade.

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>Constraint</th>
<th>AU p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haiti-1/2015</td>
<td>None</td>
<td>0.443 (+)</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>0.557 (+)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>1.67e-132 (-)</td>
</tr>
<tr>
<td>BR/SJRP/LPV01/2015</td>
<td>None</td>
<td>0.432 (+)</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>0.568 (+)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>1.01e-5 (-)</td>
</tr>
<tr>
<td>Haiti-1/2014</td>
<td>None</td>
<td>0.44 (+)</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>0.56 (+)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>5.64e-51 (-)</td>
</tr>
</tbody>
</table>

4.3.2 Vector Competence Results and Review

A total of six vector competence studies that fit our search criteria have been performed for *Ae. aegypti*, including the study performed here (Table 4.3). Of the six studies presented, three used the TRVL4675 strain. Only one study (Brustolin et. al.) used both a Genotype D strain and a Genotype L strain. The laboratory colony used in this study was not competent for either strain. Interestingly, Diop et. al. used one of the suspected recombinants (Haiti-1/2015), and the laboratory colony used reached a transmission rate of 40%. The average EIP$_{MIN}$ for Genotype D and L strains were 8 dpe and 5 dpe, with average corresponding transmission rates (vector competence) of 39% and 11.7%, respectively. The average EIP$_{MAX}$ for Genotype D and L strains were 13.3 dpe and 10.5 dpe, with 55.8% and 21.7% average vector competence, respectively.

A total of four vector competence studies that fit our search criteria have been performed for *Ae. albopictus* (Table 4.3). Three of the studies used the TRVL4675 strain, and
the fourth study used the suspected recombinant strain Haiti-1/2015. All of the different mosquito colonies used in these studies were competent for MAYV, with all colonies reaching a transmission rate greater than 50%. The average EIP$_{MIN}$ and EIP$_{MAX}$ for Genotype D and L strains were 5.7 dpe and 12.3 dpe, with corresponding transmission rates of 28.7% and 66.6%, respectively. Only one Genotype L strain was used. The EIP$_{MIN}$ and EIP$_{MAX}$ for this strain was 3 dpe and 14 dpe, with corresponding transmission rates of 40% and 75%, respectively. Laboratory colony *Ae. albopictus* reached a higher transmission rate than laboratory colony *Ae. aegypti* when the Haiti-1/2015 strain was used, with maximum rates of 75% vs. 40%.

**Table 4.3. Summary of previous vector competence studies.** Results of previous studies that fit our search criteria are listed, as well as the results of vector competence studies performed for this study. Mosquito species and location of collection, laboratory colony (Col) or field-caught/generation (FC), days post-exposure (dpe), transmission rate (positive saliva/total saliva samples), MAYV strain used and respective lineage, sample size, and citation are included.

<table>
<thead>
<tr>
<th>Mosquitoes</th>
<th>Colony</th>
<th>dpe</th>
<th>Tx Rate (%)</th>
<th>Strain</th>
<th>n</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ae. aegypti</em> (Brazil)</td>
<td>Col</td>
<td>7</td>
<td>42.5</td>
<td>TRVL4675 (D)</td>
<td>40</td>
<td>Pereira et. al. 2020 (Pereira et al. 2020)</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Brazil)</td>
<td>Col</td>
<td>14</td>
<td>72.5</td>
<td>TRVL4675 (D)</td>
<td>40</td>
<td>Pereira et. al. 2020</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (French Polynesia)</td>
<td>Col</td>
<td>3</td>
<td>20</td>
<td>Haiti-1/2015 (L)</td>
<td>20</td>
<td>Diop et. al. 2019 (Diop et al. 2019)</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (French Polynesia)</td>
<td>Col</td>
<td>5</td>
<td>30</td>
<td>Haiti-1/2015 (L)</td>
<td>20</td>
<td>Diop et. al. 2019</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (French Polynesia)</td>
<td>Col</td>
<td>7</td>
<td>35</td>
<td>Haiti-1/2015 (L)</td>
<td>20</td>
<td>Diop et. al. 2019</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (French Polynesia)</td>
<td>Col</td>
<td>14</td>
<td>40</td>
<td>Haiti-1/2015 (L)</td>
<td>20</td>
<td>Diop et. al. 2019</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Florida)</td>
<td>FC (F2)</td>
<td>3</td>
<td>3.89</td>
<td>TRVL4675 (D)</td>
<td>52</td>
<td>Wiggins et. al. 2018 (Wiggins et al. 2018)</td>
</tr>
</tbody>
</table>

(Table Cont’d)
<table>
<thead>
<tr>
<th>Mosquitoes</th>
<th>Colony</th>
<th>dpe</th>
<th>Tx Rate (%)</th>
<th>Strain</th>
<th>n</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ae. aegypti</em> (Florida)</td>
<td>FC (F2)</td>
<td>6</td>
<td>9.02</td>
<td>TRVL4675 (D)</td>
<td>102</td>
<td>Wiggins et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Florida)</td>
<td>FC (F2)</td>
<td>9</td>
<td>4.82</td>
<td>TRVL4675 (D)</td>
<td>101</td>
<td>Wiggins et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Florida)</td>
<td>FC (F2)</td>
<td>12</td>
<td>24.25</td>
<td>TRVL4675 (D)</td>
<td>102</td>
<td>Wiggins et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Peru)</td>
<td>FC (F1)</td>
<td>14</td>
<td>70.6</td>
<td>Iquitos (D) 1997</td>
<td>17</td>
<td>Long et. al. 2011 (Long et al. 2011)</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Rockefeller)</td>
<td>Col</td>
<td>7</td>
<td>3.4</td>
<td>BeAr505411 (L)</td>
<td>29</td>
<td>Brustolin et. al. 2018 (Brustolin et al. 2018)</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Rockefeller)</td>
<td>Col</td>
<td>14</td>
<td>0</td>
<td>BeAr505411 (L)</td>
<td>29</td>
<td>Brustolin et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Rockefeller)</td>
<td>Col</td>
<td>7</td>
<td>0</td>
<td>BeAn343102 (D)</td>
<td>28</td>
<td>Brustolin et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Rockefeller)</td>
<td>Col</td>
<td>14</td>
<td>0</td>
<td>BeAn343102 (D)</td>
<td>29</td>
<td>Brustolin et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Texas)</td>
<td>FC (F4)</td>
<td>6</td>
<td>0</td>
<td>TRVL4675 (D)</td>
<td>20</td>
<td>Mayton, 2021 (unpublished)</td>
</tr>
<tr>
<td><em>Ae. aegypti</em> (Texas)</td>
<td>FC (F4)</td>
<td>10</td>
<td>0</td>
<td>TRVL4675 (D)</td>
<td>20</td>
<td>Mayton, 2021 (unpublished)</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (Brazil)</td>
<td>Col</td>
<td>7</td>
<td>43.3</td>
<td>TRVL4675 (D)</td>
<td>30</td>
<td>Pereira et. al. 2020</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (Brazil)</td>
<td>Col</td>
<td>14</td>
<td>80</td>
<td>TRVL4675 (D)</td>
<td>30</td>
<td>Pereira et. al. 2020</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (La Reunion)</td>
<td>Col</td>
<td>3</td>
<td>40</td>
<td>Haiti-1/2015 (L)</td>
<td>20</td>
<td>Diop et. al. 2019</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (La Reunion)</td>
<td>Col</td>
<td>5</td>
<td>40</td>
<td>Haiti-1/2015 (L)</td>
<td>20</td>
<td>Diop et. al. 2019</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (La Reunion)</td>
<td>Col</td>
<td>7</td>
<td>55</td>
<td>Haiti-1/2015 (L)</td>
<td>20</td>
<td>Diop et. al. 2019</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (La Reunion)</td>
<td>Col</td>
<td>14</td>
<td>75</td>
<td>Haiti-1/2015 (L)</td>
<td>20</td>
<td>Diop et. al. 2019</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (New York)</td>
<td>FC (F4)</td>
<td>7</td>
<td>35.5</td>
<td>TRVL4675 (D)</td>
<td>90</td>
<td>Dieme et. al. 2020 (Dieme, Ciota, and Kramer 2020)</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (New York)</td>
<td>FC (F4)</td>
<td>14</td>
<td>64.4</td>
<td>TRVL4675 (D)</td>
<td>90</td>
<td>Dieme et. al. 2020 (Dieme, Ciota, and Kramer 2020)</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (Florida)</td>
<td>FC (F2)</td>
<td>3</td>
<td>7.2</td>
<td>TRVL4675 (D)</td>
<td>14</td>
<td>Wiggins et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (Florida)</td>
<td>FC (F2)</td>
<td>6</td>
<td>0</td>
<td>TRVL4675 (D)</td>
<td>29</td>
<td>Wiggins et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (Florida)</td>
<td>FC (F2)</td>
<td>9</td>
<td>55.34</td>
<td>TRVL4675 (D)</td>
<td>29</td>
<td>Wiggins et. al. 2018</td>
</tr>
<tr>
<td><em>Ae. albopictus</em> (Florida)</td>
<td>FC (F2)</td>
<td>12</td>
<td>26.42</td>
<td>TRVL4675 (D)</td>
<td>34</td>
<td>Wiggins et. al. 2018</td>
</tr>
</tbody>
</table>
Results from $R_0$ calculations using VC$_{Age}$ revealed that all combinations achieved $R_0$ values greater than 1, indicating the potential for outbreak from each of the lineages in both mosquito species (Figure 2). In *Ae. aegypti*, Genotype D outperformed Genotype L at both the EIP$_{MIN}$ and the EIP$_{MAX}$ with both higher magnitude $R_0$ and longer windows of opportunity. In *Ae. albopictus*, Genotypes D and L were more homogenous, with Genotype D at EIP$_{MIN}$ having the lowest $R_0$ and shortest window of opportunity. However, overall, all lineages performed better in *Ae. albopictus* compared to *Ae. aegypti*, indicating that regardless of lineage (D vs. L), *Ae. albopictus* is a more likely outbreak vector than *Ae. aegypti*.

![Figure 4.2. Calculations of reproductive numbers based on VC$_{Age}$ equation for *Ae. aegypti* and *Ae. albopictus*. The reproductive number ($R_0$) is represented on the y-axis, and the age at time of virus acquisition by the mosquito is represented on the x-axis (measured by days). The horizontal black dotted line indicates a $R_0 = 1$, indicating outbreak potential. Colored lines represent calculations based on the average EIP$_{MAX}$ (dashed lines) and EIP$_{MIN}$ (solid lines) for each genotype used in *Ae. aegypti* (left) and *Ae. albopictus* (right) vector competence studies. Genotype L strains are represented by green (EIP$_{MIN}$) and orange (Legend cont’d)
(EIP_{\text{MAX}}) lines, while Genotype D strains are represented by yellow (EIP_{\text{MIN}}) and blue (EIP_{\text{MAX}}) lines.

4.4 Discussion

Complex interactions between the vector and virus drive viral transmission. Evolution of the viral genome can potentially lead to phenotypic effects, such as altered vector competence or EIPs (Aubry et al. 2021; Ebel et al. 2004; Moudy et al. 2007; Armstrong and Rico-Hesse 2003). Specifically, changes in the viral genome have led to expansion and devastating outbreaks, as seen with CHIKV (Tsetsarkin et al. 2007). Viral recombination is another means whereby genetic differences can arise to alter viral spread and transmission potential, even with the selective pressure arboviruses experience due to host adaptation (Ciota and Kramer 2010). While recombinants are rare, they have previously occurred in Alphaviruses. Notably, Eastern equine encephalitis virus (EEEV) and Sinbis-like virus (SINV) recombined to create Western equine encephalitis virus (WEEV) (Hahn et al. 1988; Weaver et al. 1997).

Importantly, recombination does not require two species of viruses, as within-virus recombination is possible (Simon-Loriere and Holmes 2011).

Here, we demonstrated further support for the MAYV recombinants identified in 2017, as well as identified a possible new recombinant. All three recombinant strains were inferred to have the same major and minor parental strains, though these were not the parental strains previously suggested (Mavian et al. 2017). The parental strains suggested by our analysis are MAYV sequences that were not included in the 2017 study, indicating that our analysis and the inclusion of all 72 genomes has identified novel origins of these recombinants. RDP4
warned that one of the newly identified could have resulted from evolutionary processes other than recombination. This could include genetic drift or an accumulation of mutations within the genome (Coffey et al. 2013). Further analyses did support this strain as a recombinant, so we addressed it as such.

Two of the three recombinants were isolated in Haiti within the same year, with both belonging to Genotype L. Additional isolates from Haiti within the same timeframe belonged to Genotype D, indicating the possibility of multiple introductions into the country (Mavian et al. 2017; Diagne et al. 2020). Whether or not Genotype L or Genotype D is more transmissible in that region remains to be seen. Selective pressures could be driving viral evolution, leading to increased transmission among humans or adaptation to new vectors (Ciota et al. 2013; Ebel et al. 2004). Results from phenotypic studies reviewed here suggest both *Ae. albopictus* and *Ae. aegypti* are capable of transmission of MAYV (Dieme, Ciota, and Kramer 2020; Brustolin et al. 2018; Diop et al. 2019; Wiggins, Eastmond, and Alto 2018; Pereira et al. 2020; Long et al. 2011). However, Texas and Rockefeller *Ae. aegypti* colony mosquitoes were not competent for the Trinidad 1954 strain. Overall, *Ae. albopictus* reached higher proportions of infectious mosquitoes than *Ae. aegypti*. While *Ae. aegypti* bites primarily humans, *Ae. albopictus* have more variable feeding behaviors, though in urban areas this is not always the case (Ponlawat and Harrington 2005). However, the age dependent $R_0$ calculation indicated that *Ae. albopictus* is more likely to be an outbreak vector, though this will be dependent on the human-biting rate. Variability was also seen between
Genotype D and Genotype L, warranting further investigation into the impacts of genotype and within-vector phenotype.

The number of available sequences for MAYV is sparse relative to other systems. For example, a ZIKV GenBank search (“zika virus complete genome”) returns 494 complete genome sequences, and a CHIKV search (“chikungunya virus complete genome”) returns 781 complete genome sequences. The lack of MAYV sequences will continue to hinder investigations of the interactions among mosquito and viral strain pairings. Indeed, a majority of studies reviewed here focused on one MAYV strain isolated in 1954, and this can lead to bias in data and transmission estimates. Considering MAYV has recently emerged in new areas and a primary urban vector has yet to be established, it is imperative that vector competence studies be performed with more current attention to the interaction of genotypes and within-mosquito phenotypes. Further study of both genetic variability and its role in vector competence will improve monitoring and surveillance for MAYV, which could lessen the impacts of future outbreaks.

4.5 Notes


Mota, M.T., et al., Complete Genome Sequence of Mayaro Virus Imported from the Amazon Basin to Sao Paulo State, Brazil. Genome Announc, 2015. 3(6).


Rambaut, A. FigTree v1.4.4. 2018; Available from: http://tree.bio.ed.ac.uk/software/figtree/.


CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Introduction

When evaluating transmission potential of viruses among vector-virus systems, vector competence studies are considered the gold standard. Vector competence methods use terminal, discrete time points to determine the rate of infectious mosquitoes; however, the nature of these studies do not describe the detailed process of transmission from the mosquito, nor do they capture all of the interactions among vector-virus systems.

Presented in this dissertation are three studies which describe the individual and population level heterogeneities within mosquito-virus systems and the potential impacts on vector competence and subsequent viral transmission. Chapters 2 and 3 have been published in peer-reviewed academic journals (Mayton et al. 2020; Mayton et al. 2021).

5.2 Summary of results

In Chapter 2, I conducted an investigation of the potential impacts of the age-structure of the mosquito population on transmission potential of ZIKV by Aedes aegypti. During traditional vector competence studies, proportions of infectious mosquitoes and the average extrinsic incubation period (EIP) (time taken for a mosquito to become infectious) of the study population are evaluated. However, mosquito age is largely ignored, which can affect vector competence primarily through its interplay with mosquito mortality and the EIP. Ae. aegypti mosquitoes were exposed to ZIKV at either 5 days post-emergence or 12 days post-emergence, and EIPs, willingness to bite over time, and mosquito mortality were compared based upon age at time of exposure. Based on observed changes in daily probability of survival and willingness to bite over time, the vectorial capacity equation was restructured to
account for age-based impacts (VC_{Age}). Results demonstrated the importance of timing of infection rather than high proportions of mosquitoes on transmission potential, as a low proportion of mosquitoes that became infectious earlier in their lifetime were more likely to cause an outbreak when estimated by VC_{Age}. These results could change how we view highly vs. poorly competent vectors by highlighting window of transmission opportunity rather than high proportion of infectious mosquito populations. Estimated bite frequency had a clear impact on transmission potential as well, as mosquitoes who are capable of biting more during their infectious period will drive transmission, providing the rationale for Chapter 3.

Though the results from Chapter 2 were impactful, I recognized that the way that vector competence and EIP are measured is still dependent on arbitrarily chosen timepoint and forced salivation. That is, though there was some additional consideration of the natural system of mosquitoes, the method of vector competence is still relatively artificial and could be refined. Therefore, in Chapter 3, I set forth developing a method capable of observing biting behavior, EIP, and viral deposition together at the individual level over time. A common practice involves forcibly collecting saliva and testing for the presence of virus, indicating an infectious vector. These methods, however, do not address known transmission factors such as biting and time-based effects on vector-virus interactions (Sylvestre, Gandini, and Maciel-de-Freitas 2013; Scott, Chow, et al. 1993; Bellan 2010), nor do they address the transmission potential of vectors at the individual level. Previous studies have highlighted the existing heterogeneities that exist among vector-virus transmission systems, but there lacks a method to address these heterogeneities collectively over time. I successfully developed a
method using *Ae. aegypti* and ZIKV that allows for the observation of biting behavior, transmission events, and viral output at the individual level over time, allowing me to follow up the initial age observations made in Chapter 2. Notably, our results demonstrated heterogeneity among biting behavior, viral output based on such behavior, and transmission success. In future investigations, this method can be used with various vector-virus combinations, as well as in tandem with environmental impacts.

Based on previous literature that described the impact of viral genotype on vector competence (Armstrong and Rico-Hesse 2003; Ebel et al. 2004; Moudy et al. 2007; Zouache et al. 2014), I explored the genetic diversity within available Mayaro virus strains and compared to vector competence studies performed with *Ae. aegypti* and *Ae. albopictus* to observe evidence of potential genotypic impacts on vector competence. One particular study has presented evidence of potential recombinants between the Genotype L lineage and the Genotype D lineage (Mavian et al. 2017). Since that study in 2017, additional full genome sequences have been made available. Using phylogenetic analyses, results supported the existence of the previously discovered recombinants. Additionally, evidence of a third recombinant is present. A review of *Aedes*-MAYV vector competence studies revealed one of these recombinants has been used in traditional studies. From the review, the average EIP_{MIN} and EIP_{MAX} for each species-genotype combination was calculated. Using a modified version of VCAge, R_0 was calculated using the average EIP and vector competence values. Interestingly, *Ae. albopictus* not only reached higher average transmission rates than *Ae. aegypti*, but were also more likely to reach outbreak potential (R_0 > 1). Additionally, variability was observed
between the L Genotype and D Genotype strains. This work highlights the need for investigation into MAYV-vector systems and provides a basis for moving forward.

5.3 Conclusions and future directions

The work performed in this dissertation can be used to further improve vector competence determinations and supplement future protocol design. I successfully observed and brought attention to the heterogeneities that exist among vector-virus systems, as well as designed quantitative and observational methods for examining the aforementioned heterogeneities.

The results of Chapter 2 raise important concerns regarding vector competence and how vectors are classified as competent for transmission. Within this study, the impact of multiple blood meals was investigated, and was found to have no significant impact on EIP. This contradicts another study using a ZIKV-Ae. aegypti system which observed changes in EIP based upon number of blood meals (Armstrong et al. 2020). This could be due to the length of time between the second blood meal – 7 days in my study vs 2-3 days in the aforementioned work. I believe it necessary to explore the impact of blood meal timing in the future to assess why our results differ. Regardless, my work has successfully shown and, importantly, quantified the importance of mosquito age in the process of arbovirus transmission. I propose that the restructured vectorial capacity equation be used in future vector competence studies, as this will allow for a more in-depth description of transmission success.

The method developed in Chapter 3 is versatile and can be employed in a multitude of different vector-virus systems, as well as interactions between these systems and the
environment. Many studies have highlighted the impact of temperature on vector competence and transmission potential (Christofferson and Mores 2016; Mordecai et al. 2017; Alto et al. 2018; Muttis et al. 2018). The method developed here can be used in a controlled environment to investigate those impacts. Further improvement of the method can assist in quantifying viral output from the vector. There has since been technology developed to track and record mosquito probing behavior, which can be used in tandem with this method to relate probing and/or feeding times to viral output.

The developed method was the primary basis of a proposal written for the National Research Council for a project aimed at quantifying and standardizing viral delivery for eventual use in infection models. It is crucial that mosquitoes be used as a method of delivery for infection in human infection models, as there are noted differences in pathogenesis when delivered via mosquito vs needle inoculation. With my developed method, viral delivery from individual mosquitoes can be standardized, and the model can be inoculated with desired amounts of virus with a minimal number of mosquitoes.

Results from Chapter 4 highlight the need for further investigation into MAYV vector competence and the impacts of viral genotypes. Variability between viral lineages and vector combinations was shown using \( VC_{Age} \), however the lack of vector competence studies and lack of diversity in MAYV strains used in these studies warrants further attention. Phylogenetic analyses proved to be an important tool when evaluating changes in viral genotype, which can in turn point to potential phenotypic changes. The possibility of recombination shown by my and other analyses should continue to be explored as more
sequences become available, as it is possible recombination events could have phenotypic effects. The results from this chapter provide a rationale for comparative investigations into phenotypic differences among MAYV lineages, particularly vector competence.

The work presented here is not intended to downplay traditional vector competence experiments. Rather, the results are meant to build upon these practices. Determining the success of vector-virus pairings remains imperative, and methods needed will vary based on the goals of the study. Indeed, Chapter 4 highlights the need for traditional practices, as more data will aid in determining potential vectors responsible for spread of an emerging pathogen.

While traditional methods provide a look into the infectious population of mosquitoes, Chapters 2 and 3 provide ways of examining the smaller, transmitting population of mosquitoes, and the complex heterogeneities that define this population.

5.4 Notes


Age-structured vectorial capacity reveals timing, not magnitude of within-mosquito dynamics is critical for arbovirus fitness assessment

Author: E. Hardly Mayton et al
Publication: Parasites & Vectors
Publisher: Springer Nature
Date: Jun 15, 2020
Copyright © 2020. The Author(s)

Creative Commons
This is an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
You are not required to obtain permission to reuse this article.
CC0 applies for supplementary material related to this article and attribution is not required.
APPENDIX 2: PERMISSION TO REPRINT CHAPTER 3 FROM MDPI

MDPI Open Access Information and Policy

All articles published by MDPI are made immediately available worldwide under an open access license. This means:

- everyone has free and unlimited access to the full-text of all articles published in MDPI journals;
- everyone is free to re-use the published material if proper accreditation/citation of the original publication is given;
- open access publication is supported by the authors’ institutes or research funding agencies by payment of a comparatively low Article Processing Charge (APC) for accepted articles.

Permissions

No special permission is required to reuse all or part of article published by MDPI, including figures and tables. For articles published under an open access Creative Common CC BY license, any part of the article may be reused without permission provided that the original article is clearly cited. Reuse of an article does not imply endorsement by the authors or MDPI.

A Method for Repeated, Longitudinal Sampling of Individual Aedes aegypti for Transmission Potential of Arboviruses

by E. Handly Mayton 1, Heather M. Hernandez 2, Christopher J. Vitek 2 and Rebecca C. Christofferson 1,3, * 1

1 Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA
2 Center for Vector-Borne Diseases, The University of Texas Rio Grande Valley, Edinburg, TX 78539, USA
3 Center for Computation and Technology, Louisiana State University, Baton Rouge, LA 70803, USA

* Author to whom correspondence should be addressed.

Academic Editor: Corey L. Campbell

Insects 2021, 12(4), 292; https://doi.org/10.3390/insects12040292

Received: 1 March 2021 / Revised: 24 March 2021 / Accepted: 25 March 2021 / Published: 27 March 2021
Supplementary Figure S1. Illustration of main treatment design for vector competence experimentation. Three treatments were applied to assess our hypothesis. Treatment YOUNG received an infectious bloodmeal at 5 days old; Treatment OLDER received a mock (blood and non-infectious supernatant) bloodmeal at 5 days old, followed by an infectious bloodmeal at 12 days old; and Treatment S.OLDER (Sugar.OLDER) received an infectious bloodmeal at 12 dpe (no bloodmeal at 5 dpe).
**Supplementary Table S1.** Infection and dissemination rates for each day post-infection (dpi) and corresponding mosquito age for each of the three ZIKV treatments. Percent infection was determined by the proportion of infected abdomens over total exposed; and percent dissemination was determined as the proportion of infected legs over total exposed. Three replicates were performed for each treatment, but proportions and sample sizes (n) are combined from all three replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>dpi (Age)</th>
<th>% Infected (n)</th>
<th>% Disseminated (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOUNG</td>
<td>5 (10)</td>
<td>77.45 (52)</td>
<td>38.53 (52)</td>
</tr>
<tr>
<td></td>
<td>8 (13)</td>
<td>81.90 (50)</td>
<td>48.25 (50)</td>
</tr>
<tr>
<td></td>
<td>11 (16)</td>
<td>83.38 (52)</td>
<td>69.93 (52)</td>
</tr>
<tr>
<td>OLDER</td>
<td>5 (17)</td>
<td>74.67 (45)</td>
<td>4.00 (45)</td>
</tr>
<tr>
<td></td>
<td>8 (20)</td>
<td>69.40 (32)</td>
<td>37.50 (32)</td>
</tr>
<tr>
<td></td>
<td>11 (23)</td>
<td>87.88 (44)</td>
<td>66.67 (44)</td>
</tr>
<tr>
<td>S.OLDER</td>
<td>5 (17)</td>
<td>78.27 (41)</td>
<td>9.80 (41)</td>
</tr>
<tr>
<td></td>
<td>8 (20)</td>
<td>80.89 (50)</td>
<td>35.30 (50)</td>
</tr>
<tr>
<td></td>
<td>11 (23)</td>
<td>74.09 (73)</td>
<td>51.94 (73)</td>
</tr>
</tbody>
</table>

**Supplementary Table S2.** Modeled fits of parameters, the type of model, and the parameter values. For each parameter where predictions were made from experimental data, the type of model (and R self-starting function when applicable) and parameter estimates for each fit are given below. Goodness of fit was assessed either through AIC (for non-linear models) or R² for linear models.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Model (R self-start function)</th>
<th>Parameter estimates</th>
<th>Goodness of fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily probability of survival for YOUNG group</td>
<td>Asymptotic regression (SSasymp)</td>
<td>s = 0.796, r = 1.13, c = -2.56</td>
<td>AIC = -43.8</td>
</tr>
<tr>
<td>Daily probability of survival for OLDER group</td>
<td>Linear model</td>
<td>slope = -0.016, intercept = 1.13</td>
<td>Adj. R² = .88</td>
</tr>
<tr>
<td>Daily probability of survival for S.OLDER group</td>
<td>Linear model</td>
<td>slope = -0.012, intercept = 1.04</td>
<td>Adj. R² = .95</td>
</tr>
<tr>
<td>Probability of daily biting</td>
<td>Asymptotic regression (SSasymp)</td>
<td>s = 0.996, r = 0.995, c = -1.665</td>
<td>AIC = -0.71</td>
</tr>
</tbody>
</table>
Supplementary Figure S2. Observed and predicted daily probabilities of survival for the three treatment groups. Observed daily survival rates (dots) and the predicted daily survival rates (green curve) for YOUNG group (a), OLDER group (b), and S.OLDER group (c).
Supporting Text S1: R packages

The R packages used in this study are as follows: ggrepplot2, ggthemes, gridExtra, doBy, MASS, stats, nlme, nls2, ggfortify, survival, ggpubr, ggplotify, lattice, vcd, reshape.

Supporting Text S2: Mortality of Aedes aegypti with respect to bloodmeals

The supporting controls for each of the treatment groups were as follows:

1. For YOUNG group: a mock bloodmeal at 5 days post emergence (dpe) followed by sugar sustenance (“M.S”)
2. For OLDER group: a mock bloodmeal at 5 dpe followed by a second mock bloodmeal at 12 dpe (“M.M”)
3. For S.OLDER group: a mock bloodmeal at 12 dpe only (“S.M”)
4. A sugar-only control (“S”) that received no bloodmeals.

Of interest, the non-blood fed sugar-only controls (group S) died significantly faster than any of the other treatments with an average TTD of 19.6 days (Figure S3). To determine if there was a generalized effect of exposure on mortality, we compared all groups with a ZIKV exposure to those without, excepting the group that received no bloodmeal at all which was removed from the analysis. There was no significant difference between ZIKV-exposed mosquitoes (groups YOUNG, OLDER, and S.OLDER) and the non-exposed groups (p>0.05).
Supplementary Figure S3: Mortality curves for all treatments and associated controls. The sugar-only group had significantly faster mortality compared to the other groups which received at least one bloodmeal.
Supplementary Table S3. Mean time to death and sample size for ZIKV-infection treatments and unexposed controls used in the mortality study. Exposed groups were the three treatments exposed to ZIKV: YOUNG -- ZIKV at 5 days post emergence (dpe); OLDER – mock bloodmeal at 5 dpe and ZIKV bloodmeal at 12 dpe; S.OLDER – only a ZIKV bloodmeal at 12 dpe. Unexposed groups were not exposed to ZIKV but matched for bloodmeal uptake: M.S – mock bloodmeal at 5 dpe; M.M – mock bloodmeals at 5 and 12 dpe; S.M – mock bloodmeal at 12 dpe; and S – no bloodmeal, sugar only. Mean times to death and total sample sizes per treatment are given below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Time to death</th>
<th>Sample size (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed</td>
<td>YOUNG</td>
<td>25.9</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>OLDER</td>
<td>25.3</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>S.OLDER</td>
<td>24.5</td>
<td>137</td>
</tr>
<tr>
<td>Unexposed</td>
<td>M.S</td>
<td>25.5</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>M.M</td>
<td>25.8</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>S.M</td>
<td>26.5</td>
<td>132</td>
</tr>
<tr>
<td>Sugar Only</td>
<td>S</td>
<td>19.6</td>
<td>158</td>
</tr>
</tbody>
</table>
Supplemental Figure S4. VCage values for the OLDER and S.OLDER groups indicates that Ageacquisition of 12 days post emergence was too late in all but one case to result in $R_0 > 1$ in this model system. The dotted line is where VCage = 0.256 (where applicable).
Supplemental Table 1. Limit of detection for novel assay for qRT-PCR and neutral red plaque assay. Bloodmeals were spiked with a known titer of virus and loaded into a Hemotek reservoir before incubating on an artificial feeding system for 45 minutes. Blood was collected from the reservoir and serum was titered using qRT-PCR. Additionally, serum was plaqued using neutral red and Vero cells. Five replicates were performed. Plaque counts are listed, with excessive plaques being classified as TTC (too many to count). Titers obtained via qRT-PCR are listed below.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Plaque Assay</th>
<th>Dilution (pfu/100 μL)</th>
<th>Rep 1</th>
<th>Rep 2</th>
<th>Rep 3</th>
<th>Rep 4</th>
<th>Rep 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁴</td>
<td>TTC</td>
<td>TTC</td>
<td>TTC</td>
<td>TTC</td>
<td>TTC</td>
<td>TTC</td>
</tr>
<tr>
<td></td>
<td>10³</td>
<td>TTC</td>
<td>TTC</td>
<td>TTC</td>
<td>TTC</td>
<td>TTC</td>
<td>TTC</td>
</tr>
<tr>
<td></td>
<td>10²</td>
<td>59</td>
<td>15</td>
<td>14</td>
<td>17</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10¹</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁰</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁻¹</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assay</th>
<th>qRT-PCR</th>
<th>Dilution (pfu/100 μL)</th>
<th>Rep 1</th>
<th>Rep 2</th>
<th>Rep 3</th>
<th>Rep 4</th>
<th>Rep 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁴</td>
<td>4.2 x 10⁴</td>
<td>9.9 x 10⁴</td>
<td>1.2 x 10⁴</td>
<td>1.7 x 10⁴</td>
<td>2.1 x 10⁴</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10³</td>
<td>5.8 x 10³</td>
<td>4.5 x 10³</td>
<td>2.4 x 10³</td>
<td>1.0 x 10³</td>
<td>3.2 x 10³</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10²</td>
<td>7.7 x 10²</td>
<td>1.2 x 10³</td>
<td>1.2 x 10³</td>
<td>9.1 x 10²</td>
<td>7.1 x 10²</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10¹</td>
<td>8.6 x 10¹</td>
<td>1.4 x 10²</td>
<td>2.8 x 10¹</td>
<td>5.7 x 10¹</td>
<td>3.1 x 10¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁰</td>
<td>1.1 x 10¹</td>
<td>4.4 x 10⁰</td>
<td>3.9 x 10⁰</td>
<td>1.0 x 10¹</td>
<td>9.5 x 10⁰</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁻¹</td>
<td>8.6 x 10⁻¹</td>
<td>1.1 x 10⁰</td>
<td>0</td>
<td>1.9 x 10⁰</td>
<td>3.8 x 10⁻¹</td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Table 2. Titers of individual forced saliva at 24 days post-exposure.
Surviving mosquitoes at the end of the model system experiment were force salivated. Positive forced saliva samples were determined via qRT-PCR. Positive individuals and their respective titers are listed below. To confirm the presence of replicating virus, 50 μL of remaining saliva was inoculated onto 6 well plates of confluent Vero cells. At 3 and 7 days post-inoculation, supernatant was collected and tested for viral replication via qRT-PCR for the positive growth.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Mosquito ID</th>
<th>Titer of saliva (Viral RNA Copies/100 μL)</th>
<th>Replication in cell culture (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rockefeller</td>
<td>1</td>
<td>7.7</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.9</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>45</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6.8</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.4</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>240</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3.0</td>
<td>Y</td>
</tr>
<tr>
<td>Field-derived</td>
<td>1</td>
<td>1.7</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.1</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5.1</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.2</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>3.5</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>70</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.8</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>9.4</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>13</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.8</td>
<td>Y</td>
</tr>
</tbody>
</table>
Supplemental Table 3. Titers of collected serum and collections from Vero inoculation. 
Serum collected from successful transmission events were titered via qRT-PCR. Colony, individual mosquito ID, and days post mosquito exposure at time of transmission event are listed below. For confirmation of replicating virus, 25 μL of remaining serum was inoculated onto 12 well plates of confluent Vero cells. At 3 and 7 days post-inoculation, supernatant was collected and tested for viral replication via qRT-PCR for the positive growth.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Colony ID</th>
<th>Days post-exposure</th>
<th>Titer of serum (Viral RNA Copies/100 μL)</th>
<th>Replication in cell culture (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rockefeller</td>
<td>5</td>
<td>14</td>
<td>290</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>16</td>
<td>0.2</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>16</td>
<td>3.3</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>18</td>
<td>3.9</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>18</td>
<td>35</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>18</td>
<td>1.9</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>20</td>
<td>7.8</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>22</td>
<td>5.4</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>24</td>
<td>1.6</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>24</td>
<td>0.6</td>
<td>Y</td>
</tr>
<tr>
<td>Field-derived</td>
<td>15</td>
<td>18</td>
<td>4.6</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>18</td>
<td>0.2</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>18</td>
<td>1.5</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>19</td>
<td>0.7</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>20</td>
<td>2.3</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>21</td>
<td>0.3</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>22</td>
<td>4.9</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>23</td>
<td>3.1</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>24</td>
<td>28</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>24</td>
<td>4.8</td>
<td>Y</td>
</tr>
</tbody>
</table>
APPENDIX 5: VECTOR COMPETENCE OF SOUTH TEXAS Aedes Aegypti FOR MAYARO VIRUS

Introduction:
To augment existing data, a basic vector competence assay was conducted to determine the dynamics of the TRVL4675-Ae. aegypti system. Basic MAYV characterization was also performed prior to experiments.

Methods:
A strain of MAYV collected from a human in Trinidad and Tobago in 1954 (TRVL4675) was used for vector competence studies received from Dr. Christopher Mores. Viral stock used was titered using a neutral red plaque assay before use as previously described (Kawiecki et al. 2017). Virus was passaged onto a confluent flask of Vero cells two days before being used for mosquito exposure, and supernatant was titered via qRT-PCR before use. All mosquitoes were exposed to ~1 x 10^6 plaque forming units (pfu)/mL. If necessary, viral supernatant was diluted in M199X media to obtain desired titer.

Field-caught Ae. aegypti collected from sites in southern Texas were provided by Christopher Vitek. F3 eggs were hatched and maintained at 37 degrees Celsius at 80% relative humidity with a 16:8 light:dark cycle as previously described (Mayton et al. 2020). Cartons of 3-5 day old mosquitoes were exposed using the Hemotek artificial feeding system. Reservoirs containing 2 mL of whole bovine blood in Alsevers and 1 mL of viral supernatant were provided for 45 minutes. After 45 minutes, mosquitoes were cold anesthetized and blood fed mosquitoes were sorted into new cartons. Groups of mosquitoes (n=20) were sampled at 6 and 10 days post-exposure via forced salivation as previously described (Mayton et al. 2020). RNA was extracted from saliva samples using the MagMax96 total nucleic isolating kit and a KingFisher extraction robot. Extractions were tested for the presence of MAYV RNA via qRT-PCR. Primer and probe sequences used were taken from (Wiggins, Eastmond, and Alto 2018).

Results:
Saliva samples from field-caught Texas Ae. aegypti were negative at both 6 and 10 days post-exposure time points via qRT-PCR. Since forced salivation was negative, no further testing was done.

Conclusion:
Despite South Texas experiencing local transmission of Aedes-driven arboviruses in the past (Thomas et al. 2016; Martin et al. 2019), mosquitoes derived from eggs collected from Brownsville, TX were not competent for MAYV. Other Ae. aegypti populations were shown to be competent for MAYV (Diop et al. 2019; Long et al. 2011; Wiggins, Eastmond, and Alto 2018; Pereira et al. 2020), reconfirming the fact that mosquito populations of the same
species but from disparate geographic locations can differ in their competence for the same virus (Vega-Rua et al. 2014; Calvez et al. 2018; Severini et al. 2018).
APPENDIX 6: CHAPTER 3 METHOD DEVELOPMENT
TROUBLESHOOTING

Introduction:
Several attempts were made towards measuring individual mosquito transmission over time before successfully developing the method presented in Chapter 3. These attempts and, of course, problems, are presented here. This may be helpful in the future when looking towards similar method developments.

Method Development:
First and foremost, I wanted to ensure the mosquitoes would bite and probe into what was provided. The following attempts were made before successfully developing the method used in Chapter 3:

1. Attempt 1: feeding into cell culture media for direct inoculation onto cells
   - Vero cells plated in 35 mm cell culture dishes
   - After 24 hours, media (2% M199X) was replaced, and dish covered with parafilm
   - Dish flipped and provided to individual mosquitoes (parafilm side down)
   - After allowing mosquitoes opportunity to feed for 45 minutes, dishes were flipped, and any expectorated virus would be directly inoculated onto cells
   - Cells would then be observed for CPE
   - PROBLEM: mosquitoes would not feed on media

2. Attempt 2: feeding into bovine serum
   - Same method as attempt 1, but using bovine serum
   - Mosquitoes were also intrathoracically inoculated to increase chances of infection
   - Mosquitoes did feed, but at a very low rate
   - High rates of mortality from IT inoculation
   - PROBLEM: high mortality, low feeding rates

3. Attempt 3: feeding into 3 mL Hemotek reservoirs
   - Hemotek used to keep blood warm, which could contribute to biting
   - 900 μL of blood used in a 3 mL Hemotek reservoir
   - Wire mesh cut off of canisters and replaced with fiberglass screen to prevent tearing of the parafilm
   - Mosquitoes fed well
   - Blood collected after feeding, serum separated for further testing
   - Successfully isolated virus from blood, but only for a few samples
   - PROBLEM: blood volume too high, making it difficult to isolate virus

4. Attempt 4: feeding into 300 μL Hemotek reservoirs
   - Both blood and serum attempted, using 180 μL
   - Mosquitoes fed well with blood, not with serum
• *This attempt was successful using blood, employed in Chapter 3*

Next, I set forth quantifying virus collected. This was done throughout each attempt. qRT-PCR was successful, however quantifying via *in vitro* methods served as a challenge due to contamination in Vero cells. The following are attempts at addressing the contamination as well as changes that were implemented as a result of those attempts.

1. **Attempt 1: syringe filter**
   - Serum collected and drawn up into a syringe
   - 0.22 μm filter attached and used to filter serum
   - **PROBLEM:** major loss of serum

2. **Attempt 2: *in vitro* methods**
   - Antibiotic/antimycotic concentrations in cell culture media were increased from 2% to 4%
   - I also attempted to wash Vero monolayers. To do this, serum was inoculated onto the monolayer. After incubating for 30 minutes to allow any virus present to bind to the cells, the monolayer was washed with 1X PBS in an attempt to remove contaminants.
   - **PROBLEM:** not effective in reducing contamination.

3. **Attempt 3: mosquito hatching**
   - Contamination suspected to be yeast. Up until this point, mosquito eggs had been hatched using dry yeast to create a low oxygen, nitrogen rich environment to trigger hatching.
   - Vacuum method employed for hatching rather than yeast to reduce contamination
   - **Contamination reduced, able to detect virus in collections**
   - We continued to use this vacuum hatching method in the lab for all mosquito experiments
   - **PROBLEM:** contamination reduced, but still present.

**Conclusions:**
I successfully developed a biting and viral collection method, which is presented in Chapter 3. While I was not able to completely eliminate contamination in Vero cells, I was still able to detect replicating virus from collections, which was used to confirm qRT-PCR results.
BIBLIOGRAPHY

(WHO), World Health Organization. 2014. 'A global brief on vector-borne disease'.

(WHO), World Health Organization. 2020. 'Mayaro virus disease - French Guiana'.


Christofferson, R. C., C. N. Mores, and H. J. Wearing. 2014. 'Characterizing the likelihood of dengue emergence and detection in naive populations', *Parasit Vectors*, 7: 282.


population size, survival and dispersal of male mosquitoes of the Anopheles gambiae complex in Bana, a west African humid savannah village', *Parasit Vectors*, 10: 376.


Mackay, I. M., and K. E. Arden. 2016. 'Mayaro virus: a forest virus primed for a trip to the city?', Microbes Infect, 18: 724-34.


Massad, E., and F. A. Coutinho. 2012. 'Vectorial capacity, basic reproduction number, force of infection and all that: formal notation to complete and adjust their classical concepts and equations', *Mem Inst Oswaldo Cruz*, 107: 564-7.


Rambaut, Andrew. 2018. 'FigTree v1.4.4'. [http://tree.bio.ed.ac.uk/software/figtree/](http://tree.bio.ed.ac.uk/software/figtree/).


VITA

E. Handly Mayton was born and raised in Greenville, Mississippi. She is the daughter of Bill Mayton and Cindy Fava. Handly graduated with a Bachelor of Science in Biological Sciences from Louisiana State University in 2015. During her senior year, she worked as an undergraduate assistant at LSU School of Veterinary Medicine, where she discovered her interest in mosquito-borne viruses in the Mores Lab. After graduating in 2015, she joined the laboratory of Rebecca C. Christofferson as a research technician. She then decided to pursue a career in research and joined the Christofferson Lab as a PhD student in 2016, where she began her research into vector-virus interactions. Handly will graduate with a Doctor of Philosophy in Veterinary and Biomedical Sciences in August 2021. She has accepted a post-doctoral position at the Walter Reed Army Institute of Research as part of the National Research Council Research Associateship Program.