

2011

Arbovirus phenotype alters transmission potential

Rebecca C. Christofferson

Louisiana State University and Agricultural and Mechanical College

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



Part of the [Veterinary Pathology and Pathobiology Commons](#)

Recommended Citation

Christofferson, Rebecca C., "Arbovirus phenotype alters transmission potential" (2011). *LSU Doctoral Dissertations*. 3885.

https://digitalcommons.lsu.edu/gradschool_dissertations/3885

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.

ARBOVIRUS PHENOTYPE ALTERS TRANSMISSION POTENTIAL

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 School of Veterinary Medicine
through the Department of Pathobiological Sciences

by
Rebecca C. Christofferson
B.S. Louisiana State University 2002
M.Ap.St. Louisiana State University 2005
August 2011

Acknowledgements

I would like to thank the following groups, without whom I would have been less productive and less entertained:

First and foremost: the Family. I cannot name you all because that would take a small eternity, but for everyone related or once-related to me, you have all helped shape me in some way; and for that I am grateful, especially my grandparents who lived/ are living long lives with grace and wisdom. To Mawmaw, the graveyard was always a fun place to play with you. That hasn't changed now that you reside there. To Gammy, I hope I can celebrate 91 years of independence with the same tenacity. To Pawpaw and Grandpa, who quietly shaped my childhood from the background. And to the myriad of cousins and aunts and uncles who showed me how to have a damn fine time.

To John, my ex-twin brother. If you hadn't have turned 30, we could still be twins. I am proud to be your sister and while this whole thing is about what I've done, you are a part of it, too. We aren't identical and we don't have ESP, but the sibling connection we share means that you are always at the front of my mind. It's probably the result of something you did to me *in utero*, but I can't prove anything. I love you, but don't tell anyone.

Thank you to my parents, John and Susan Carriere (Ph.D.). How do I even start? First of all, thanks for the whole life thing. That's worked out pretty well. (Thanks, Aunt Julia.) Y'all are the most supportive, wonderful parents anyone can have. Thanks for fussing at me when I started the "sing-song" voice so I'd quit (a thousand times, thanks!), and for teaching me how to pronounce "yellow." Thanks for threatening me with a cheese grater if I ever got a tattoo. Thanks for instilling in me the manners and respectfulness that have carried me through several phases of my life. Thanks for not getting too upset when I wrapped the carport post around the truck. Thanks for going to all my band concerts and piano recitals, even the ones that were probably painful. And thanks for

the love and support, the strength to take life in stride, the love of good music and art and the ability to tell the difference, the knowledge that I could at least try anything because you'd be there to pick me up when I fell, the love of coffee (very important here), the example of parenting, the advice and all the "I have to tell you something and you can't get mad at me" conversations, and the off the wall sense of humor. I love you 5&14. And lastly, thank you for trusting me to make my own choices, however grudgingly.

Speaking of, thanks to my wonderful husband, Mark A. Christofferson, II. I don't know what I'd do without you. You are the love of my life and- even though you have turned me into a heavy metal fan- I still look forward to the next several decades with you. You are the perfect husband, even when I say you aren't. And the fact that you are the perfect father makes me love you a hundred thousand times more. It helps that you have taken over all the housework and haven't thrown me out on the streets yet for indolence. Your only downfall is your horrible choice in lottery numbers.

And to my children: Evelyn Rose and the as yet unnamed Wookie. Evelyn, you are the light of my life. I cannot imagine how I lived without you or how the days passed so quickly, or what I did for entertainment. Your ability to make me laugh while I'm supposed to be stern will serve you well and turn my hair grey. Wookie, I look forward to meeting you in September and I can't wait to fall even more in love with you. But for now, please stop kicking my bladder.

Secondly: the Lab. To Dan "Dr. Dr." Chisenhall, for putting up with my gratuitous use of pronouns and my constant war with gravity. We may not always agree, but we somehow always laugh. (Sometimes at, not with.) To Jaime Lobo and Michael "Kracken" McCracken, for making me feel way smarter than I deserve, while not making me feel way older than I want to. To Ann-Marie Johnson, the best student worker ever: don't ever doubt you kept the ship afloat. And Ms. Julie Woody, thanks for the skeeters!

Thirdly, Dr. Christopher “Bossman” Mores. Thank you for pushing me as hard as I needed and then letting me have the freedom to push further. You have given me much more opportunity that I could have wished for and I am beyond grateful for that. You’ve also provided me with great entertainment, a renewed sense of fashion, and a healthy dose of cynicism. But probably most importantly, you are the greatest friend a student could ask for. I am privileged to have you as my mentor, and proud to call you friend. Oh, and you drove me to drink.

To Dean Kenneth Koonce, who pretty much told me to get a statistics degree and learn SAS. You were right; it is useful! Thank you for employing me right out of college, for putting up with too much “cute” sometimes, and for believing in me. Without the experiences I garnered under your employ, I don’t know where I’d be. Wherever, I wouldn’t know nearly as many one-hit-wonder songs. (“It’s so hard to be humble when you’re perfect in every way.....”)

To my committee members: Drs. Phil Elzer, Ivo Foppa, and Kevin Macaluso. You all contributed to my growth as a student, a researcher, and a person. (Metaphorically speaking. I’m blaming the weight gain on Evelyn and the Wookiee.) I am grateful to each of you for your time and efforts and for putting up, also, with my gratuitous use of pronouns.

To my friends, especially Danya Broussard, Leslie Carriere, Christie Landry, Claudia Leonardi, Ryan Machtmes, Andi Ocmond, and Lorna Ogden, etc. For listening to my rants and raves about what’s going on in the lab and pretending you are interested, and for giving me examples of what strong, intelligent people can do.

I am sure I am forgetting somebody, so whoever you are: thanks! This has been a fun trip.

Table of Contents

Acknowledgements.....	ii
List of Tables.....	viii
List of Figures.....	ix
Abstract.....	xi
Chapter 1: Introduction and Literature Review.....	1
Introduction.....	1
Arbovirus Transmission.....	2
Arboviral Fitness.....	6
West Nile Virus.....	14
Chikungunya Virus	18
Dengue Virus	21
Objectives and Rationale	24
References	27
Chapter 2: Factors Associated with Mosquito Pool Positivity and the Characterization of the West Nile Viruses Found within Louisiana during 2007....	40
Introduction	40
Materials and Methods.....	42
Samples.....	42
Mosquito Trap Sites and Field Collection of Mosquitoes.....	42
Virus Detection and Sequencing.....	42
Statistical Analysis.....	44
Phylogenetic Analysis.....	45
Results	45
Positive Pool Predictors.....	45
Phylogenetic Analysis.....	48
Discussion	48
References	53
Chapter 3: Estimating the Magnitude and Direction of Altered Arbovirus Transmission Due to Viral Phenotype	56
Introduction	56
Materials and Methods	60
Mosquitoes.....	60
Virus.....	60
Oral Exposure of Mosquitoes and Detection of Disseminated Infection.....	61
Effective Vector Competence (EVC) and Cumulative Vectorial Capacity (cVC) Model.....	62

Results	64
Statistical Test of Differences.....	68
Characterization of Fitness in the Vector.....	71
Discussion.....	72
References.....	76
Chapter 4: Viral Phenotype May Explain Chikungunya Expansion Phenomenon..	81
Introduction.....	81
Materials and Methods.....	84
Virus.....	84
Mosquitoes.....	85
Mosquito Exposure and Detection of Disseminated Infection.....	85
Data Analysis.....	85
Results.....	86
Reanalysis of Tsetsarkin et al. E226V Strain versus E226A in <i>Ae. aegypti</i>	86
Experimental Comparison of LR06 and SEA96.....	87
Discussion.....	87
References.....	91
Chapter 5: The Role of Interferon Responses in Dengue Transmission.....	94
Introduction.....	94
Materials and Methods	97
Mice.....	97
Virus.....	98
Virus Inoculation and Serum Collection.....	98
Virus Detection.....	98
Mosquito Exposure for Transmission to DKO Mice.....	99
Transmission from Viremic Mice to Mosquito.....	99
Cytokine Measurement and Statistical Analysis.....	100
Results.....	100
Successful Transmission in IRF3/7 DKO by Subcutaneous Needle Inoculation.....	100
Mosquito versus Needle Transmission.....	105
IRF3/7 ^{-/-} DKO Mouse Supports Transmission to Naïve Mosquitoes...	108
Discussion	108
References.....	114
Chapter 6: Discussion and Conclusions.....	117
Introduction.....	117
Summary of Results.....	118
Conclusions.....	125
References.....	127

Appendix 1: Permission to Reprint from BMC Virology Journal as per Creative Commons Attribution License.....	129
Appendix 2: Permission to Reprint from PLoS One as per Creative Commons Attribution License.....	130
Appendix 3: EVC and cVC SAS Code, Single Phase.....	131
Appendix 4: EVC and cVC SAS Code, Two Phase.....	142
Appendix 5: EVC and cVC Detailed Calculations.....	161
Vita.....	163

List of Tables

Table 1.1 Viral fitness affects transmission potential.....	9
Table 2.1 Species in positive mosquito pools.....	43
Table 2.2 Differences of least square means.....	49
Table 2.3 Genetic distances for 2007 samples.....	52
Table 2.4 Origin and classification of sequenced isolates.....	52
Table 3.1 Dissemination rates and samples sizes for 4 strains of dengue at 5, 7, and 9 days post exposure.....	63
Table 3.2 Primer and probe sequences for dengue serotypes 2 and 4.....	63
Table 3.3 Parameters of the vectorial capacity equation held constant.....	63
Table 3.4 Results from the cumulative formulation of vector competence and vectorial capacity method for 4 strains of dengue.....	69
Table 4.1 Primers and probe.....	89
Table 4.2 Dissemination rates.....	89
Table 4.3 Dissemination rates of experimentally exposed mosquitoes.....	89
Table 5.1 Summary of Transmission from Mosquito to IRF DKO mice.....	106

List of Figures

Figure 1.1 Flaviviruses.....	3
Figure 1.2 Alphaviruses.....	3
Figure 1.3 Arbovirus transmission cycles.....	7
Figure 1.4 West Nile virus maps reflect surveillance reports released by state and local health departments to CDC's ArboNET system for public distribution (2000).....	16
Figure 1.5 West Nile virus maps reflect surveillance reports released by state and local health departments to CDC's ArboNET system for public distribution (2002).....	17
Figure 1.6 Phylogram of the full-length CHIKV genomic sequence demonstrating the extreme genetic conservation seen during the course of the outbreak.....	20
Figure 1.7 Microevolution of DENV.....	25
Figure 2.1 Louisiana landcover.....	47
Figure 2.2 Positive pool probability over months by land cover.....	49
Figure 2.3 Maximum parsimony phylogenetic tree.....	50
Figure 3.1 Single versus cumulative VC values.....	65
Figure 3.2 Vector competence dynamics.....	67
Figure 3.3 Characterizing relative fitness.....	75
Figure 4.1 Displacement index characterizing relative fitness.....	90
Figure 5.1 Type I interferon production signals through IRF3 and IRF7.....	96
Figure 5.2 Mean viremia levels in dengue infections.....	101
Figure 5.3 IFN- γ response of infected and control mice.....	103
Figure 5.4 TNF- α response in dengue infected and control mice.....	104
Figure 5.5 IL-4 response in dengue infected and control mice.....	104
Figure 5.6 Viremia levels of mosquito vs. needle inoculated DKO mice.....	106
Figure 5.7 IFN- γ response in needle inoculated vs. mosquito inoculated infections.....	107

Figure 5.8 Mean viremia levels of mice (n-3) from which acquisition to mosquitoes was attempted.....	109
Figure 5.9 Illustration of the effective viremic period.....	111
Figure 6.1 A more complicated relationship.....	123

Abstract

Extrinsic and environmental factors are known to affect the transmission of arthropod-borne viruses (arboviruses), including variations in the arthropod vector populations. Differences among these factors have been associated with differential transmission and are sometimes used to control the spread of an arbovirus through a vertebrate population in an effort to prevent or disrupt an outbreak. However, diversity in intrinsic viral populations, such as genetic and phenotypic variability, is not often accounted for when considering alterations in transmission. Presented in this dissertation are four experimental studies that explore the contribution of viral intrinsic factors, especially phenotypic variability, to the transmission potential of arboviruses as judged by modeling parameters such as vectorial capacity (VC) and the basic reproductive number (R_0). The overall hypothesis of this research is that phenotypic differences of arboviruses alter the transmission potential of these arboviruses by conferring fitness advantages in either the vector or the vertebrate. Further, these phenotypic differences need not be large in magnitude to affect the relative transmission potential. To investigate this hypothesis, this research determined 1) whether intrinsic viral characteristics can lead to differential transmission in a given locale, 2) whether variability of viral fitness in the mosquito vector can lead to significant differential transmission potential, 3) how our newly formulated methods from our preceding aim could aid in the explanation of a currently puzzling phenomenon in the field of arbovirology, 4) whether phenotypic differences in the vertebrate host alters the potential for transmission, and 5) how

identified phenotypic differences in both the vector and vertebrate hosts could act synergistically or antagonistically to alter transmission potential of arboviruses. The research in this dissertation offers a more accurate tool for assessing transmission potential in the vector, provides a new model assessing transmission potential in the vertebrate, and provides several of the necessary steps towards a more appropriate calculation of R_0 . Our use of R_0 based on dynamic phenotypic differences provides a framework for a more dynamic formulation of transmission models, and provides an accessible framework for output validation and reporting to public health stakeholders.

Chapter 1

Introduction and Literature Review

Introduction

Arboviruses are **arthropod-borne viruses**, which require an amplification stage in an arthropod to complete the transmission cycles. Most arboviruses are transmitted by mosquitoes or ticks, though a few are transmitted by lice, black flies, midges or nest bugs (Kuno and Chang 2005). Most arboviruses are classified into three main families: Togaviridae, Bunyaviridae, Flaviviridae (Monath 1988; Weaver and Reisen 2010). Of those arboviruses of medical and veterinary importance, the disease manifestations include febrile illnesses, encephalitis and encephalomyelitis, and hemorrhagic diseases. Some of these same arboviruses cause no symptoms at all (Kuno and Chang 2005). Of specific interest are West Nile virus (WNV), dengue virus (DENV), and Chikungunya virus (CHIKV). WNV and DENV are members of the genus *Flavivirus*, a family of viruses, many of which are medically important (Figure 1.1). CHIKV is a member of the *Alphavirus* genus, family *Togaviridae*, which is a smaller group of viruses, but also have medical importance (Figure 1.2) (Schmaljohn and McClain 1996; Kuno and Chang 2005).

Flaviviruses have an approximately 11kb, positive-sense, single stranded RNA genome. The single open reading frame is co- and post-transcriptionally processed into three structural (Capsid, preMembrane, Envelope) and seven non-structural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Brinton 2002). Alphaviruses have a similar genome, with approximately 12kb, positive-sense, single stranded RNA, which encodes three structural proteins (C, E1, E2) and four non-structural proteins (NS1-4). Unlike flaviviruses, alphaviruses have a poly-A tail at the 3' end (Schmaljohn and McClain 1996; Tsetsarkin, Vanlandingham et al. 2007; Solignat, Gay et al. 2009).

The primary mechanism believed to mediate the success of arboviral infection establishment in the vertebrate host is the anti-viral, type I interferon response (Lobigs, Mullbacher et al. 2003; Couderc, Chretien et al. 2008; Tobler, Cameron et al. 2008; Munoz-Jordan 2010; Lazear, Pinto et al. 2011). Upon inoculation, arboviruses infect the cells that are localized to the skin tissue. DENV, WNV and CHIKV are suspected to have a tropism for dendritic cells upon initial infection (Palucka 2000; Wu, Grouard-Vogel et al. 2000; Kramer, Li et al. 2007; Kyle, Beatty et al. 2007). When the virus infiltrates a cell via endocytosis, Toll-like receptors (TLR) such as TLR3 or TLR7 recognize double stranded RNA and set off a cascade of signals resulting in the transcription of type I interferon (IFN- α/β). For example, through the activation of TLR-3, NF κ B and other transcription factors, particularly interferon regulatory factor (IRF) 3 and 7 lead to the production of interferon β . IFN β positively feeds back via the type I interferon receptor to activate the JAK/STAT pathway, involving IRF7 and IRF 9 transcription factors for IFN genes. These two cytokines, IFN α and β , are responsible for the establishment and maintenance of the anti-viral state in the immune response, a significant factor being the production of type II interferon (IFN- γ) (Katze, He et al. 2002; Haller, Kochs et al. 2006; Partidos, Weger et al. 2011). Other receptors in the cytoplasm, such as RIG-1 and MyD88 are also responsible for signaling the type I IFN pathway (Kawai and Akira 2006; Gilfoy and Mason 2007; Fredericksen, Keller et al. 2008). Downstream interferon-induced proteins, such as CXCL10 (IP10), have also been correlated to viral replication and ultimate seroconversion (Nightingale, Patkar et al. 2008; Tobler, Cameron et al. 2008).

Arbovirus Transmission

Arboviruses usually are maintained through a zoonotic transmission cycles where the arbovirus is passed from one or a few main vector species to one or a few main vertebrates

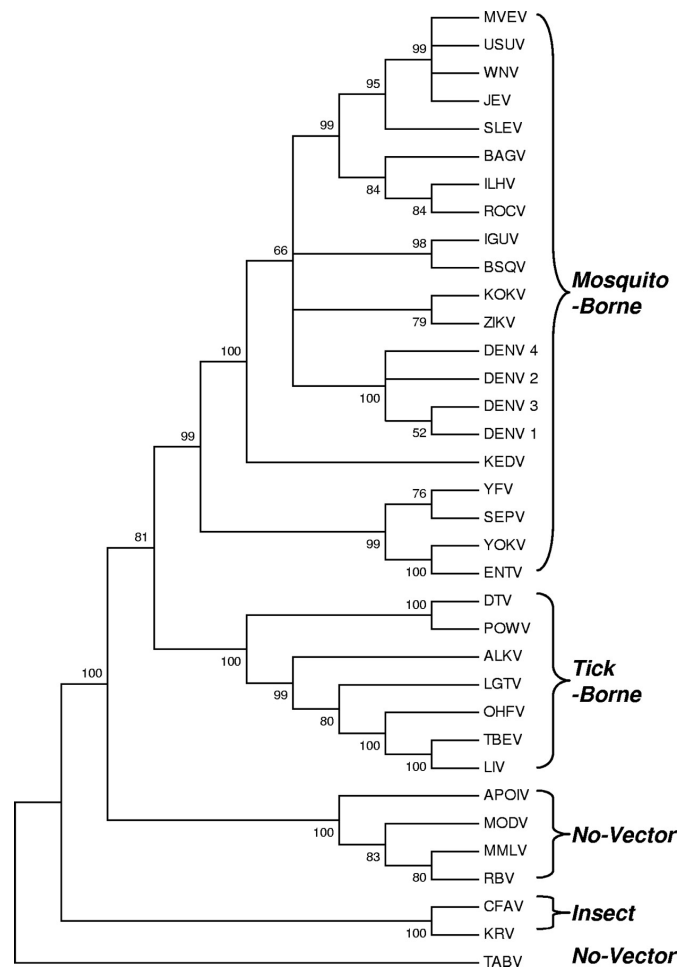


Figure 1.1: Flaviviruses. Phylogeny of Flaviviruses from Kuno, et al 2005 [2].

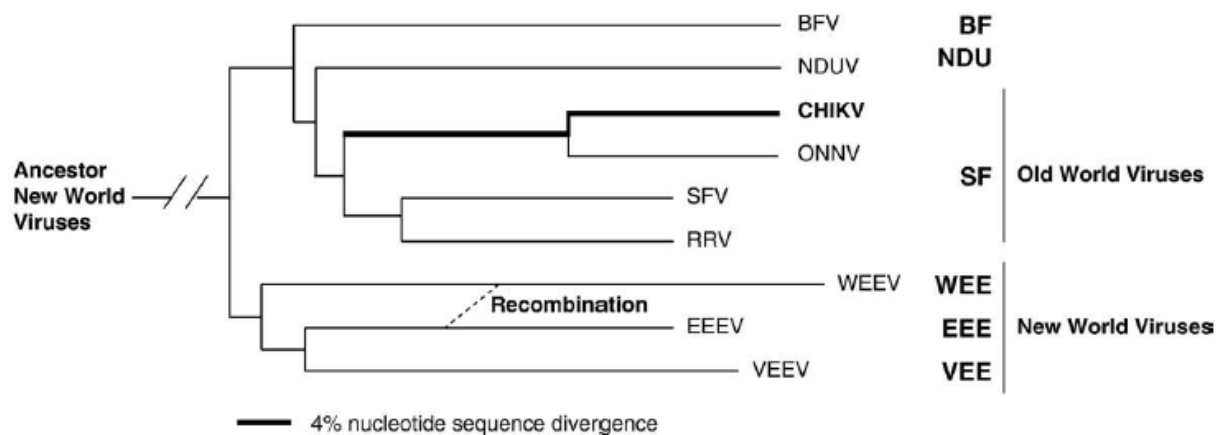


Figure 1.2: Alphaviruses. Simplified phylogeny of alphaviruses generated from E1 analysis from Solignat, et al 2009 (Solignat, Gay et al. 2009).

1. Viral characteristics, such as better replication in an unusual vertebrate/vector species (Weaver and Reisen 2010). A zoonotic disease then leaves the enzootic cycle and spills over into an unusual vertebrate population. This spill over can be due to many factors, such as, but not limited to:
 - a. The populations of the vertebrate that is the main preference of the vector (such as birds for ornithophilic mosquitoes) becomes scarce or
 - b. There is encroachment into the habitat of the vector, in which case infection of the unusual (ex-zootic) vertebrate host is incidental.
3. A bridge vector becomes infected and initiates the ex-zootic cycle by preferentially feeding on its specific host.

A classic example of the first factor is the adaptation of CHIKV virus to *Ae. albopictus* after a mutation caused a single amino acid change in the envelope protein, domain III region. This valine to alanine substitution made CHIKV much more fit in *Ae. albopictus* with dissemination rates being earlier and higher. The subsequent outbreak on La Reunion Island in 2006 was of epidemic proportions, and this strain of CHIKV quickly spread throughout the Indian subcontinent and SE Asia, displacing the pre-mutation strain (Tsetsarkin, Vanlandingham et al. 2007; Santhosh, Dash et al. 2009; Tsetsarkin, Chen et al. 2011). Another example is the similar mutation in WNV, in the envelope domain II, where an alanine to valine substitution increased the virus' fitness in *Culex* mosquitoes, again increasing dissemination rates as well as enabling earlier transmission (Moudy, Meola et al. 2007). Though first isolated in 2001, this strain was called WN02. After this mutation, the WN02 strain spread quickly across the western United States and has since completely displaced the originally invading NY99 strain (Jerzak, Bernard et al. 2005; Snapinn, Holmes et al. 2007; Kramer, Styer et al. 2008).

West Nile virus is an example of the second factor, where the usually ornithophilic but ubiquitous *Culex* mosquitoes take blood from viremic birds and then transmit WNV to people and/or horses, both of which are dead end hosts (Kramer, Li et al. 2007; Kutasi, Bakonyi et al. 2011; Sirbu, Ceianu et al. 2011). In addition, tick-borne Flavivirus infections (POWV, TBE, OMSKV) occur in humans who engage in activities such as hiking where they are in direct contact with the tick's habitat or directly from the small rodent reservoir (Dobler 2010; Weaver and Reisen 2010).

Yellow Fever (YFV) is an example of both the second and third factors. YFV is maintained by non-human primates in the forests of South America and Africa primarily by mosquitoes in the genres *Haemagogus* and *Sabethes* in the Americas, and non-urban *Aedes* (*Ae.*) spp. in Africa (1986; Huang 1986; Cardoso Jda, de Almeida et al. 2010). YFV has been diagnosed in people who have been exposed to the sylvatic transmission cycle of YFV either through recreational (hiking) or work-related habits (nomadic herdsman) (Gershman, Schroeder et al. 2009; Vasconcelos 2010; 2011). In addition, YFV sometimes escapes this sylvatic cycle and enters the urban cycle via *Ae. aegypti*, which is the bridge vector from sylvatic to urban Yellow Fever (Cardoso Jda, de Almeida et al. 2010; Vasconcelos 2010; 2011).

Vertical or transtadial transmission may play an important role in maintenance of many arboviruses, and specifically in tick-borne viruses (Baqar, Hayes et al. 1993; Lambrechts and Scott 2009; Ruzek, Yakimenko et al. 2010; Unlu, Mackay et al. 2010). However, vertical transmission of arboviruses, particularly WNV and DENV, has been only sporadically observed in natural mosquito populations (Gunther, Martinez-Munoz et al. 2007; Angel and Joshi 2008; Unlu, Mackay et al. 2010), and experimentally with SLE, Powassan viruses, and some bunyaviruses (Costero and Grayson 1996; Lambrechts and Scott 2009; Flores, Diaz et al. 2010). The relative importance of vertical transmission with mosquito-borne viruses, especially with regard to DENV and CHIKV transmission, is still considered low (Watts, Harrison et al. 1985;

Gunther, Martinez-Munoz et al. 2007; Hutamai, Suwonkerd et al. 2007; Vazeille, Mousson et al. 2009).

Dengue virus, however, has a different transmission cycle. A true anthroponosis, DENV does not have an enzootic cycle, but instead is maintained solely by the urban transmission cycle from *Ae. aegypti* mosquitoes and humans (Weaver and Reisen 2010). Some genetically distinct, sylvatic (forest associated) DENV has been detected, and may be maintained in Africa among non-human primates and associated mosquito species (Rico-Hesse, Harrison et al. 1997; de Thoisy, Lacoste et al. 2009). However, in endemic urban areas such as South America and Southeast Asia, sylvatic cycles are likely a spillover from the urban cycle. Additionally, in places where *Ae. aegypti* are absent, some transmission has been attributed to a secondary vector, *Ae. albopictus* (Gratz 2004; Moutailler, Barre et al. 2009; Webster, Farrar et al. 2009; Lambrechts, Scott et al. 2010; Vazeille, Mousson et al. 2010). Figures 1.3A and B contrast the anthroponotic cycle and zoonotic cycle of arboviruses.

Arboviral Fitness

Since no vaccine is available for many arboviruses, understanding and controlling the vector populations is often the best and only recourse for disease control (Weaver and Reisen 2010). However, vector control is problematic and if eradication is achieved, it is often transient (Teixeira, Costa Mda et al. 2009). Three important arboviruses have had significant changes in transmission due to fitness. Table 1.1 shows a summary of these changes and hypothesized effects on transmission.

When malaria was determined to be transmitted by mosquitoes, a new way of calculating the potential spread of the parasite in and from the vector population was formulated by Ronald Ross in a series of papers (Heesterbeek 2002). It wasn't until George McDonald, also working with malaria at the Ross Institute in London, that the term "basic reproduction rate" was coined.

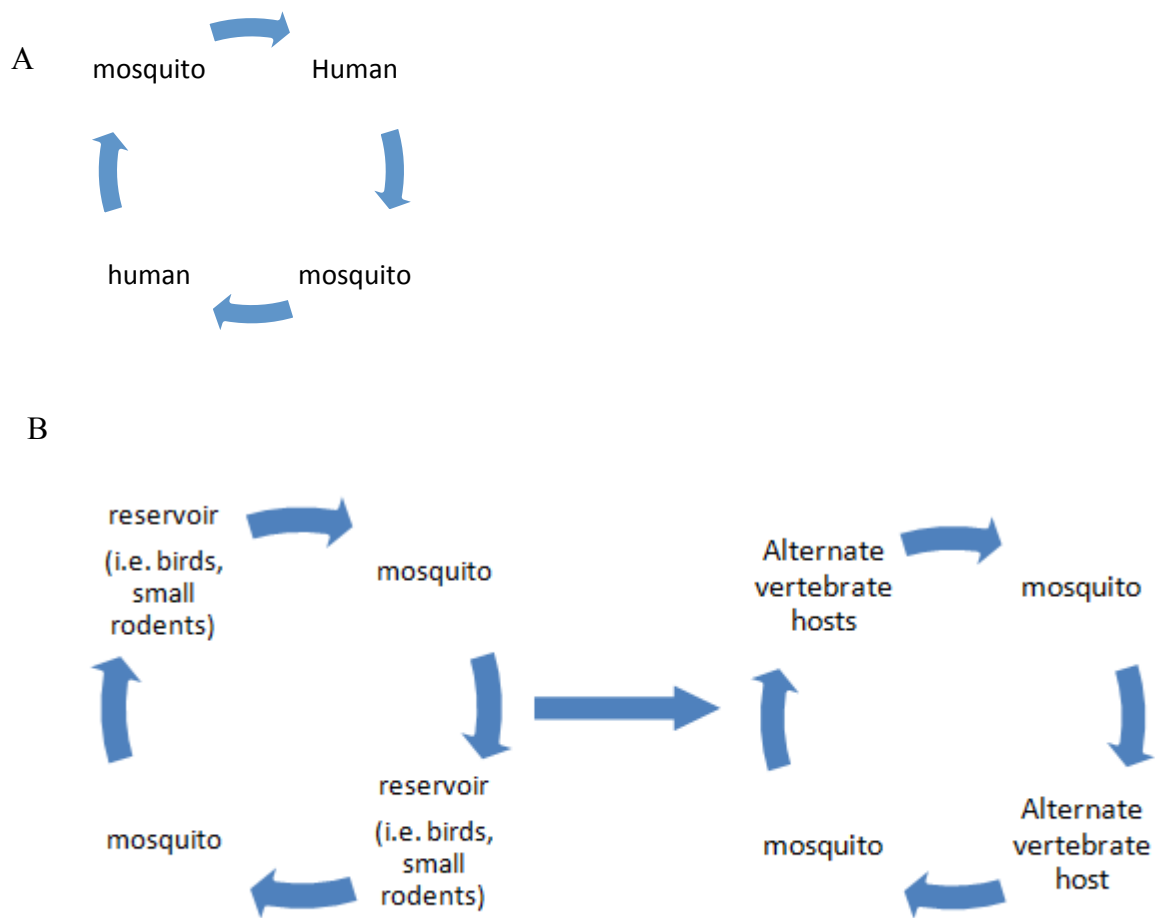


Figure 1.3: Arbovirus transmission cycles. A) shows the simplified anthroponotic transmission cycle of dengue virus. B) depicts the transmission cycle of such viruses as West Nile, Yellow Fever, and chikungunya.

He defined this as “The number of infections distributed in a community as the direct result of the presence in it of a single primary non-immune case.” Though he discusses the implications for other diseases, his paper was dedicated to the modeling of malaria (Macdonald 1952; Dye 1992; Heesterbeek 2002).

In 1964, Garrett-Jones isolated the entomological parameters from MacDonald’s equation and introduced the term “vectorial capacity,” though it excludes the parameter of vector competence discussed below (Garrett-Jones and Grab 1964; Dye 1986; Dye 1992). Vectorial capacity (V), is a complex parameter included for vector-borne diseases and is a measure of the transmission rate from a vector population to a susceptible host population. Itself a dynamic measure of transmission, V is defined as “the number [of infections] that a specific mosquito population can distribute per case per day (Garrett-Jones 1964; Dye 1986; Dye 1992; Smith and McKenzie 2004; Anderson and Rico-Hesse 2006).” The calculation of vectorial capacity is given by:

$$V = \frac{ma^2p^Nb}{-\ln(p)} \quad \text{Equation (Eq.) 1.1}$$

where **a** is the man biting rate and **m** is the mosquito density; these parameters are measures of contact between the vector and vertebrate hosts (Macdonald 1957). The probability of daily survival **p** is a measure of the mortality rate of the vector (Macdonald 1957). The extrinsic incubation period (EIP) **N** is the time, in days, it takes for a pathogen to infect the mosquito and disseminate to the salivary glands where it can be transmitted (Macdonald 1957). The original formula of vectorial capacity has been modified to include a transmission capability parameter, vector competence **b** (Dye 1986; Hardy 1988; Reisen 1989; Dye C 1995; Black and CG 1996). Vector competence is defined as the intrinsic ability of a vector to acquire a virus, become

Table 1.1: Viral fitness affects transmission potential. Role of viral fitness changes in transmission of three important arboviruses, West Nile Virus (WNV), chikungunya virus (CHIKV), and dengue virus (DENV).

Virus	Change	Affect on Fitness	Affect on Transmission
WNV	Amino acid change (A→V)	Faster and higher dissemination in vectors (Moudy, Meola et al. 2007)	Faster transmission through bird populations
CHIKV	Amino acid change (V→A)	Higher dissemination in alternate vector (Tsetsarkin, Vanlandingham et al. 2007)	Less competition for “new” vector = increased disease?
DENV	Genotype/strain differences	<ol style="list-style-type: none"> 1. Increased dissemination in mosquito population (see Ch. 3) 2. Longer/more intense viremia (see Ch. 5) 	<ol style="list-style-type: none"> 1. More transmission in vector population vs. “less fit” strains 2. More transmission to vector from vertebrates

infected with the virus, support sufficient replication that dissemination to the salivary glands is probable, and ultimately transmit the virus (Hardy 1988).

Several vector traits have been studied with regard to vector competence differences, such as mosquito species, mosquito strain within species, and mosquito size (Boromisa, Rai et al. 1987; Bennett, Olson et al. 2002; Vaidyanathan and Scott 2007; Alto, Reiskind et al. 2008). Vector competence for arboviruses particularly is impacted by extrinsic factors such as temperature differences during incubation, titer of virus offered during exposure, and larval competition (Hardy, Houk et al. 1983; Kramer, Hardy et al. 1983; Richards, Mores et al. 2007; Alto, Lounibos et al. 2008; Pesko, Westbrook et al. 2009). Estimates of vector competence can also be indicative of differences in vector susceptibility to arbovirus strains. Vector competence is estimated as the proportion of mosquitoes with a disseminated infection to the total number of exposed mosquitoes and can therefore be thought of as the dissemination rates within a vector population.

Comparisons of arbovirus strain-specific vector competence estimates have been used to support observed or hypothesized differences in transmission capability, and thus fitness (Anderson and Rico-Hesse 2006; Turell, Mores et al. 2006; Turell, Mores et al. 2006; Mores, Turell et al. 2007; van den Hurk, Hall-Mendelin et al. 2009). Typically, such comparisons are made at a single (optimal) time point during the extrinsic incubation period, and less commonly two or more time points might be used (Anderson and Rico-Hesse 2006; Moudy, Meola et al. 2007; Kilpatrick, Meola et al. 2008; Moutailler, Barre et al. 2009; van den Hurk, Hall-Mendelin et al. 2009). Using the appropriate extrinsic incubation period (EIP) is crucial when calculating vectorial capacity (Kramer and Ebel 2003; Anderson and Rico-Hesse 2006).

Many researchers have used vector competence and vectorial capacity to study the relative fitness and success within and between arboviruses either in single or multiple vector populations (Lucio-Zavaleta, Smith et al. 2001; Armstrong and Rico-Hesse 2003; Brault, Powers

et al. 2004; Myles, Pierro et al. 2004; Anderson and Rico-Hesse 2006; Tsetsarkin, Higgs et al. 2006; Gray, Caillaud et al. 2007; Kilpatrick, Meola et al. 2008; Brault 2009; Moutailler, Barre et al. 2009). In addition, vectorial capacity has been used to assess the effectiveness of control strategies either at the vector or viral levels (Busvine and Pal 1969; Rivero, Vezilier et al. 2010; Hancock, Sinkins et al. 2011). Vector competence has also been used as a phenotypic measure to explain the displacement of one viral strain relative to the other (Moudy, Meola et al. 2007; Tsetsarkin, Vanlandingham et al. 2007). Vector competence has also been used to assess the alteration of viral fitness in special circumstances. For example, to characterize the relative fitness between West Nile virus and St. Louis encephalitis virus in the context of superinfections, Pesko et al used dissemination rates (vector competence estimates) to infer that the virus that first infects *Culex quinquefasciatus* inhibits the second, but that some dissemination enhancement did occur in the case of mosquitoes primarily with St. Louis encephalitis and secondarily with West Nile virus (Pesko and Mores 2009).

While vectorial capacity can be used to assess viral fitness in vector populations, it is part of a larger measure to assess the overall transmission potential of an arbovirus in the entire transmission cycles from vector to vertebrate (Anderson and Rico-Hesse 2006). The basic reproductive number (R_0), has been long established as a measure of transmission, specifically speaking to the potential of a pathogen to become endemic, epidemic, or disappear from the population. If $R_0 < 1$, an outbreak is less likely to become an epidemic, and if $R_0 > 1$ an epidemic is likely to occur (Breban, Vardavas et al. 2007). R_0 speaks to the transmissibility of a pathogen as well as epidemic potential. With vector-borne diseases, vectorial capacity is an important component in R_0 . However, equally as important are the parameters that speak to the vertebrate involvement in the transmission cycle.

R0 has been calculated several different ways with the definition of parameters varying. The traditional equation used to calculate R0 for vector-borne diseases is given below (Garrett-Jones 1964).

$$R_0 = cV/r \quad \text{Eq. 1.2}$$

Recovery rate of the vertebrate ($1/r$)- which is sometimes written as the reciprocal (r), or days of infection - and infectivity to the vector (c) are vital to the transmission cycle. R0 has been used to judge or predict the probability and/or force of an epidemic due to pathogen introduction or evolution, and extrinsic factors such as climate change or seasonality (Wonham, Lewis et al. 2006; Massad, Ma et al. 2008; Rubel, Brugger et al. 2008; Brugger and Rubel 2009; Stoddard, Morrison et al. 2009; Pinho, Ferreira et al. 2010). In addition, it has been used to assess the effectiveness of control or intervention methods, such as educational programs or vector control strategies (Woolhouse, Dye et al. 1997; Dumont and Chiroleu 2010).

Arboviruses must be ingested by the arthropod vector via a viremic blood meal from the infected vertebrate host, then it must escape the midgut and disseminate to the salivary glands where it can then be transmitted to a naïve vertebrate host. The inherent ability of these arthropod vectors to support this biological life stage is called vector competence, and the time it takes to imbibe and then transmit the pathogen is the extrinsic incubation period. These biological factors within the vector are critical when studying disease transmission. Specifically, the replicative success of a particular virus within a particular vector will greatly inform any modeling efforts.

Replicative success is also a measure of viral fitness (Clarke, Duarte et al. 1993). In an epidemiological context, replication implies transmission from vertebrate host via arthropod vector to vertebrate host; therefore, the basic reproductive number (R0) has been proposed as a

metric for viral fitness (Anderson and May 1992; Lipsitch and Moxon 1997; Day 2001).

Selective pressure exists within these arbovirus systems in both the vector and vertebrate, leading to differential viral fitness, based on intrinsic viral characteristics (Cologna, Armstrong et al. 2005). The effects of this selective pressure can be identified by creation of fitness indices, which in turn will act as mathematical weights in multi-strain/serotype systems. Accounting for fitness and competition among strains will greatly increase the accuracy of any model, especially when the co-circulation of strains and serotypes translates to disease manifestation (Halstead 2007). Both kinetics and fitness must be accounted for in any accurate modeling system.

R_0 has been used as a measure of the transmission potential of arboviruses (Wonham, Lewis et al. 2006; Foppa and Spielman 2007; Rubel, Brugger et al. 2008). R_0 has also provided a basis of determining what characteristics affect transmission patterns of pathogens (Hartemink, Purse et al. 2009). Some other uses of R_0 include the determination of the relative importance of transmission routes (vertical vs. horizontal), the effects of ecological parameters such as temperature, humidity and climate change, and the effects of human movement on transmission (Foppa 2005; Brugger and Rubel 2009; Cosner, Beier et al. 2009; Gould and Higgs 2009; Jeger, Madden et al. 2009; Stoddard, Morrison et al. 2009; Reiter 2010).

Fitness of arboviruses has been hypothesized to be constrained by the requirement of two main hosts: vector and vertebrate. This “trade-off” hypothesis has been studied in several arboviruses both *in vivo* and *in vitro*. This idea was first proposed by Taylor and Marshall in 1975 using Ross River virus (Taylor and Marshall 1975; Taylor and Marshall 1975) and hypothesizes that extraordinary fitness in either the vector or the vertebrate is “traded” for adequate fitness in both. Since then, studies have been performed assessing the trade-off of fitness of arboviruses, and many of these studies have found that arboviruses that are increasingly adapted to a particular cell type (vector or vertebrate) become decreasingly efficient in that cell type or in that animal. Also, when alternating passages are used, the efficiency of a

viral strain can be “rescued” or maintained (Cologna, Armstrong et al. 2005; Greene, Wang et al. 2005; Ciota, Lovelace et al. 2007; Coffey, Vasilakis et al. 2008; Moutailler, Roche et al. 2011). Given this hypothesis, it was important that arboviral fitness and the affect on transmission be investigated in both the vector and vertebrate, and the potential importance of differential phenotypes in both hosts be accounted for.

West Nile Virus

WNV is an important zoonotic disease infecting avian species and occasionally causing neuroinvasive disease in mammals, most often horses and humans (Kramer, Li et al. 2007). First isolated from a patient in Uganda in the 1930s, WNV was not associated with epidemics until the 1950s (Smithburn, Hughes et al. 1940; May, Davis et al. 2011). In the 1990s, periodic epidemics began occurring in Europe, Israel, and North Africa, including human deaths (Reiter 2010). WNV is maintained primarily in a bird-mosquito transmission cycle, but occasionally an epizootic cycle involving humans and other vertebrates, most commonly horses, emerges (Wonham, Lewis et al. 2006; Kramer, Li et al. 2007; Kramer, Styer et al. 2008). The primary vectors of WNV belong to the genus *Culex* (*Cx.*), especially *Cx. pipiens*, *Cx. tarsalis*, and *Cx. quinquefasciatus* (Kramer, Li et al. 2007; Kilpatrick, Fonseca et al. 2010; Molaei, Cummings et al. 2010). However, other mosquito genera, including *Aedes*, have been shown to be competent for the virus and may act as bridge vectors (Moskvitina, Romanenko et al. 2008; CDC 2009; Larson, DeGroot et al. 2010).

In humans, most cases are mild or inapparent, requiring little to no medical intervention. As mentioned above, humans are dead end hosts and do not support viremia sufficient to infect mosquitoes. However, in the elderly or immunocompromised, the virus can invade the central nervous system and cause meningitis (WNM) or encephalitis (WNE). Most meningitis patients recover, though those with encephalitis have a bleaker prognosis. In an outbreak in Colorado,

18% of patients with WNE died and another 75% required increased care after discharge (Bode, Sejvar et al. 2006).

In equine species, a similar pattern of WNV infection is seen. Most cases are inapparent and the animals recover fully. However, in a small proportion of cases, neurological symptoms manifest and mortality can be as high as 57%. Several commercially licensed vaccines are available for use in horses, with indications for protection against and/or decrease in WNV viremia (Dauphin and Zientara 2007). In addition, some vaccines are available for use in bird species such as geese, in an effort to control transmission (Dauphin and Zientara 2007).

WNV is commonly separated into two distinct lineages, though some separate it into as many as five lineages. Some evidence associates lineage I more often with outbreaks of human and equine disease (Kramer, Li et al. 2007; Kramer, Styer et al. 2008; Weaver and Reisen 2010; Kauffman, Franke et al. 2011; May, Davis et al. 2011). However, others have shown that lineage II is just as capable of spilling over into epizootic cycles (Kutasi, Bakonyi et al. 2011; Papa, Bakonyi et al. 2011; Sirbu, Ceianu et al. 2011). Lineages III and IV are characterized by single isolates, and lineage 5 remains geographically confined to India (Kramer, Styer et al. 2008).

The WNV that first emerged in the United States in 1999 in New York City was of lineage I. This strain was most closely related to a 1998 isolate from Israel, with >99.8% identity (Lanciotti, Roehrig et al. 1999). This invading arbovirus caused significant morbidity and mortality in the resident bird population, especially in the American crow population (*Corvus spp.*), in addition to human cases of neuroinvasive disease (Lanciotti, Roehrig et al. 1999; Asnis, Conetta et al. 2000). There have been several studies linking clade changes or genetic diversity to phenotypic differences, some of which have been positively linked to epizootic transmission of the virus (Davis, Beasley et al. 2004; Ebel, Carricaburu et al. 2004; Bertolotti, Kitron et al. 2008). Further, in 2001, a mutation resulted in increased fitness in *Culex* mosquitoes and WNV quickly spread across the United States (Jerzak, Bernard et al. 2005; Snapinn, Holmes et al.

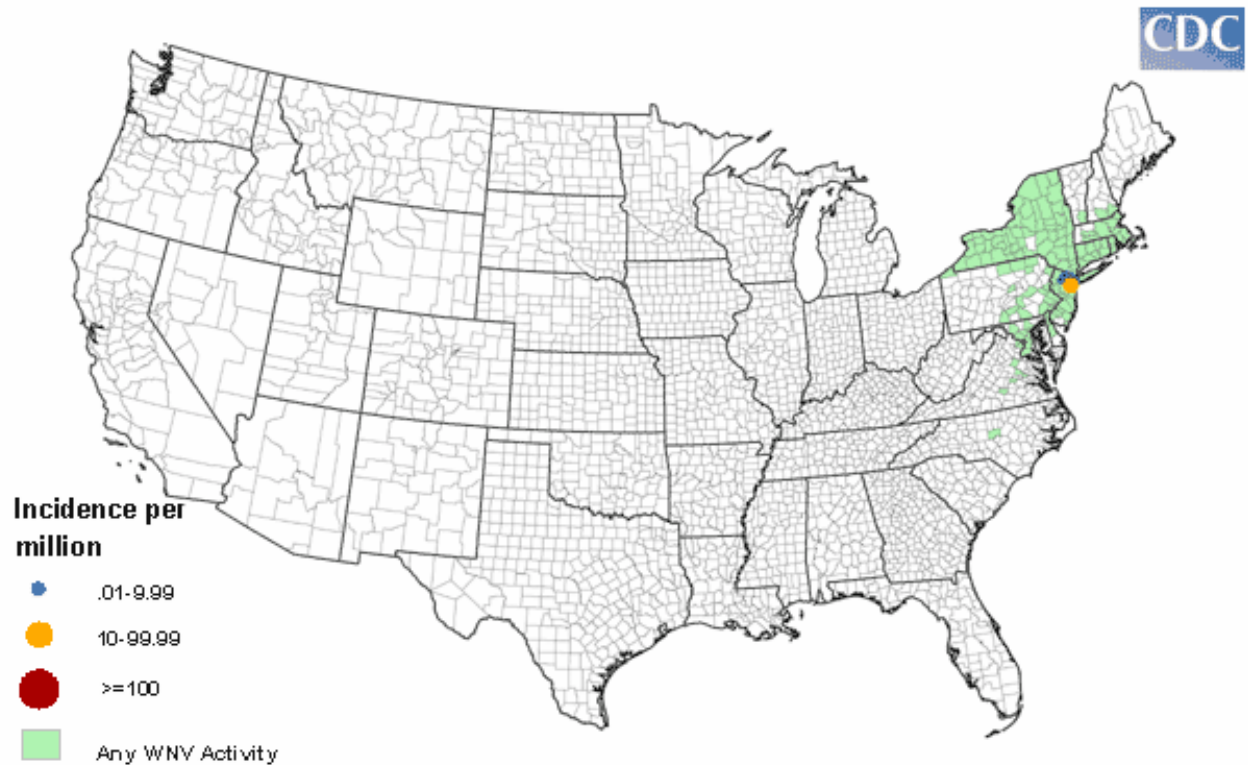


Figure 1.4: West Nile virus maps reflect surveillance reports released by state and local health departments to CDC's ArboNET system for public distribution (2000). Map shows the distribution of human neuroinvasive disease (encephalitis and/or meningitis) incidence occurring during 2000 with number of human cases shaded according to incidences ranging from .01 to 9.99, 10 to 99.99, greater than 100, and WNV activity (human, mosquito, veterinary, avian and sentinel data). (CDC)

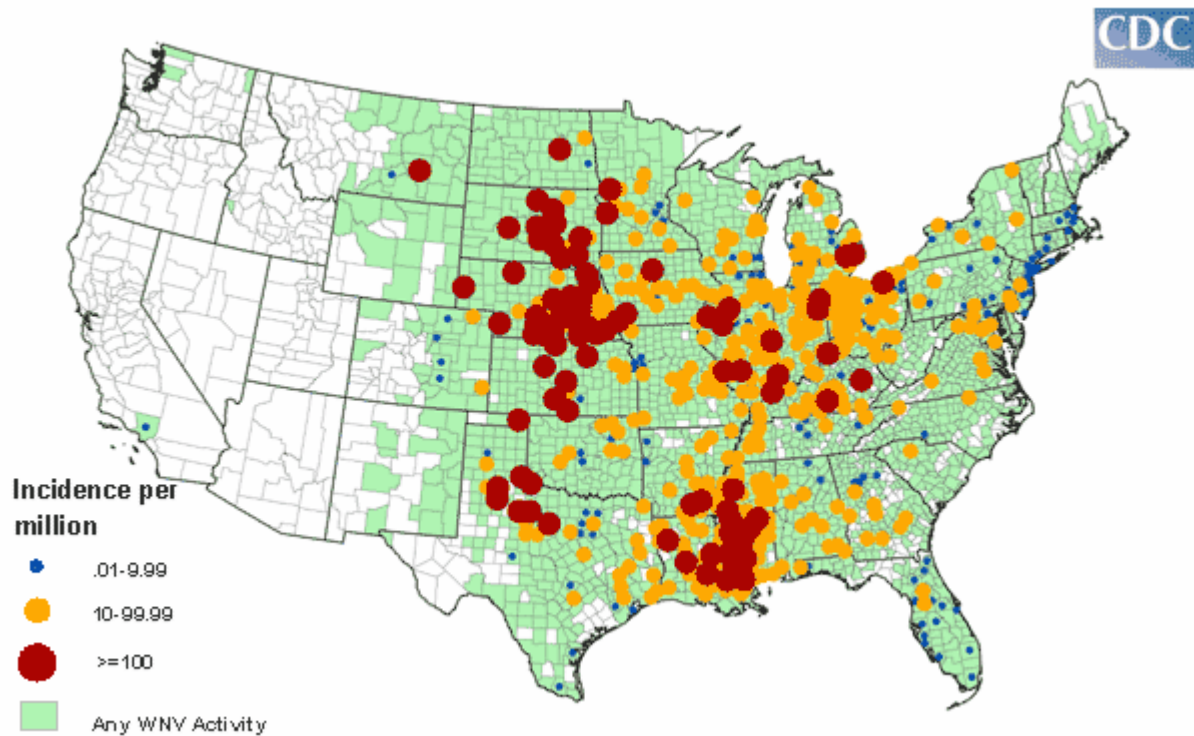


Figure 1.5: West Nile virus maps reflect surveillance reports released by state and local health departments to CDC's ArboNET system for public distribution (2002). Map shows the distribution of human neuroinvasive disease (encephalitis and/or meningitis) incidence occurring during 2002 with number of human cases shaded according to incidences ranging from .01 to 9.99, 10 to 99.99, greater than 100, and WNV activity (human, mosquito, veterinary, avian and sentinel data). (CDC)

2007; Kramer, Styer et al. 2008). The change in WNV activity in the United States from the year prior to this amino acid change (2000) to the year after (2002) are shown in figures 1.4 and 1.5, respectively.

Chikungunya Virus

Chikungunya (CHIKV) is a mosquito-borne Alphavirus (family Togaviridea) found in tropical areas such as Africa and Southeast Asia, as well as Italy. The virus was first isolated from a febrile patient in Tanzania in the early 1950s, and has since been responsible for periodic outbreaks of severe flu-like illnesses that can progress to severe arthralgia in some patients (Lumsden 1955; Robinson 1955; Mason and Haddow 1957). Because of its shared landscape ecology and symptoms with dengue, many CHIKV cases are likely misdiagnosed as dengue, so is probably more prevalent than thought (Chevillon, Briant et al. 2008; Santhosh, Dash et al. 2009).

CHIKV has historically been transmitted in its urban cycle by the mosquito *Ae. aegypti*, though *Ae. albopictus* was considered a fairly competent vector as well (Cavrini, Gaibani et al. 2009; Paupy, Ollomo et al. 2010). Likely the virus is maintained in a sylvatic cycle in Africa by non-human primates and their associated mosquitoes (*Ae. africanus*, *Ae. furcifer-taylori*, *Ae. dalzieli*, i.e.), as well as small rodents and possibly birds (Pialoux, Gauzere et al. 2007; Powers and Logue 2007; Thiboutot, Kannan et al. 2010). However, in Asian and India, the virus has persisted or re-emerged consistently in an urban, anthroponotic cycle (Figure 1.3A). This primarily involves the vector *Ae. aegypti* in Asia and in combination with *Ae. albopictus* in India, though the relative importance of interactions between these co-existing vectors has yet to be definitely assessed. Two distinct lineages of CHIKV have been identified: one comprised the West African isolates and the other of South/East African (sometimes referred to as Central/East

African) strains and strains isolated from Asia (Powers and Logue 2007; Kariuki Njenga, Nderitu et al. 2008). Interestingly, unlike WNV or DENV, very little localized genetic diversity is seen during an epidemic of CHIKV (Figure 1.6) (Powers and Logue 2007).

Beginning in 2004-2005, a significant outbreak of CHIKV began in Kenya and is believed to be the progenitor of the outbreaks in Comoros and La Reunion islands. Phylogenetic analysis of six strains from the 2004-2006 outbreaks (Comoros, Kenya, La Reunion) and several other earlier strains show that with high bootstrap probability, the isolates from 2004-2006 outbreaks formed a distinct clade with the amino acid changes resulting in little phylogenetic distance (Kariuki Njenga, Nderitu et al. 2008). This is especially interesting given that a single of these amino acid changes discussed below resulted in significant fitness differences, underscoring the importance of phenotypic analyses for transmission studies.

In 2006, an outbreak on La Reunion Island was particularly significant, resulting in hundreds of thousands of cases of CHIKV (Gerardin, Guernier et al. 2008; Staikowsky, Talarmin et al. 2009). The isolate from this island was noted to have an amino acid change at position 226 of the envelope (E1) protein caused an amino acid substitution, changing an alanine to a valine (Tsetsarkin, Vanlandingham et al. 2007; Kariuki Njenga, Nderitu et al. 2008). The presence of this amino acid change is not particularly phylogenetically relevant, since phylogenetic analysis firmly places this strain in a clade consisting of 16 other amino acid changes (Kariuki Njenga, Nderitu et al. 2008). Figure 1.6 from Kariuki Njenga, et al shows the extreme conservation of isolates from the 2004-2006 outbreak.

However, this mutation (E226V) has been shown to enhance the efficiency of viral replication within the mosquito *Ae. albopictus* (Tsetsarkin, Vanlandingham et al. 2007; Vazeille, Moutailler et al. 2007). There have been several reports that *Ae. aegypti* was absent from the island, and *Ae. albopictus* was determined to be the primary vector of that outbreak (Vazeille, Moutailler et al. 2007; Delatte, Dehecq et al. 2008; Delatte, Paupy et al. 2008). Thus, this fitness

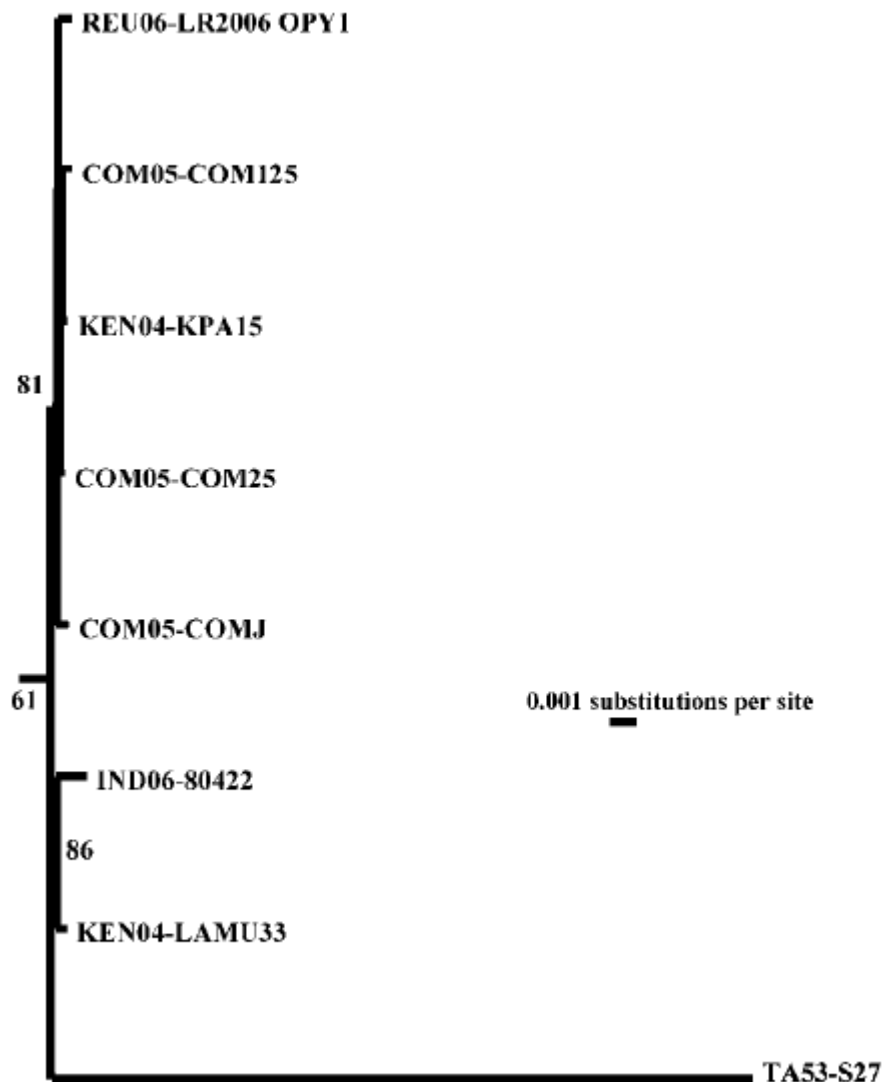


Figure 1.6: Phylogram of the full-length CHIKV genomic sequence demonstrating the extreme genetic conservation seen during the course of the outbreak. Complete nucleotide sequences of isolates from coastal Kenya, the Indian Ocean and India from 2004 to 2007 were compared using a neighbour-joining algorithm. The prototype strain (S27) was used to represent the historical Central/East African genotype. Bootstrap values are indicated at the nodes. Phylogram reproduced from (Kariuki Njenga, Nderitu et al. 2008).

advantage contributed significantly to the transmission of this CHIKV strain in this otherwise secondary vector species, resulting in considerable transmission and human cases. Additionally, CHIKV strains with this amino acid change have continued to spread throughout India and Southeast Asia and to Europe, where *Ae. aegypti* coexists with *Ae. albopictus*, and the potential role of this mosquito species has not been vigorously studied (Vazeille, Jeannin et al. 2008; Cavrini, Gaibani et al. 2009).

Dengue Virus

Dengue virus (DENV) is a significant international public health threat with the potential to become a health security issue as it continues to emerge throughout the tropics and reaches across national borders (2009). The virus has been responsible for episodic outbreaks since the first suspected epidemic in the 18th century (Rush 1789; Vasilakis and Weaver 2008). In recent years DENV has quickly become one of the leading causes of morbidity in tropical regions, and it is estimated that nearly two-fifths of the world is at risk for contracting DENV (2009).

The economic impact of DENV per family in highly endemic areas is often more than the average family's monthly income (Suaya, Shepard et al. 2009). The mean cost per case in international dollars when eight endemic countries were co-analyzed was \$514 per ambulatory patient and \$1,394 per hospitalized patient (Suaya, Shepard et al. 2009). In the Americas specifically, the median cost of ambulatory DENV cases is \$472 (USD) and hospitalized cases cost \$1,227 (Shepard, Coudeville et al. 2011). DENV infections are now routinely detected in the border region of Texas, in Hawaii, and in coastal/port cities such as Boston, New York, Miami, and the District of Colombia, with autochthonous transmission occurring in Key West and Miami, Florida in 2009 (2002; 2007; Brunkard, Robles Lopez et al. 2007; 2010).

The transmission of DENV is not fully explained by relative prevalence and case-reporting data alone. There are four antigenically related serotypes (D1, D2, D3 and D4) of DENV that cause acute febrile illness and less commonly lead to severe manifestations of the disease, DENV hemorrhagic fever (DHF) or DENV shock syndrome (DSS) (Armstrong and Rico-Hesse 2001; Ooi and Gubler 2009). The serotypes have overlapping spatial and temporal distributions in densely populated, endemic areas, where the incidence of infection and cases of severe disease have been steadily increasing since 1978 (Thu, Lowry et al. 2004; Zhang, Mammen et al. 2005; Balmaseda, Hammond et al. 2006; Suwandono, Kosasih et al. 2006; Jarman, Holmes et al. 2008; Kukreti, Chaudhary et al. 2008; 2009; Endy, Yoon et al. 2010). Additionally, since the late 1970s, DENV has become a yearlong public health concern, instead of a disease primarily of the rainy season (Nagao and Koelle 2008).

The problem of dengue arises from the complexity of the pathogenesis. As a person recovers from a primary infection, there is a transient immunity to all four serotypes, estimated to last from 3 to 4 months. However, after this period, protection only to the infecting serotype lasts (Endy, Nisalak et al. 2004). If the same individual were to contract a secondary infection (Bagny, Delatte et al. 2009) from a heterologous serotype, he/she is then at increased risk for contracting either DHF or DSS (Guzman, Kouri et al. 1991; Endy, Nisalak et al. 2004). Currently, the widely accepted hypothesis is that antibody to the primary infecting serotype enhance the infectivity of the secondary infecting serotype. This is called antibody dependent enhancement (ADE), but does not explain all cases of DHF and DSS. This concept was introduced by Halstead and O'Rourke in 1977, who showed that D2 virus entered and replicated in monocytes with greater efficiency when complexed with non-neutralizing antibody compared to virus without antibody. This study did not, however, identify the non-neutralizing antibodies as heterotypic; that is, as being primarily antibodies of a different serotype (Halstead and O'Rourke 1977). Studies in Cuba and Thailand offered evidence that heterotypic antibodies were

the driving force behind increased risk for DHF and DSS (Sangkawibha, Rojanasuphot et al. 1984; Burke, Nisalak et al. 1988; Thein, Aung et al. 1997; Rothman 2004). Further, there is evidence that the sequence of serotype infection ($D1 \rightarrow D2$ vs. $D2 \rightarrow D1$, i.e.) also affects the severity of disease, with a secondary infection with dengue serotype 2 increasing the likelihood of hemorrhagic manifestations (Guzman, Kouri et al. 2002; Alvarez, Rodriguez-Roche et al. 2006).

ADE has thus hindered the development of a vaccine as immunity inferred by a monovalent vaccine puts the population at risk for DHF or DSS. Recent vaccine efforts have focused on multi-valent formulations, though tetravalent vaccines have had problems with immunological equality. That is, a strong elicited immune response for one serotype precludes an effective response to another (Guy and Almond 2008; Webster, Farrar et al. 2009). Thus, efforts have focused on understanding and mitigating the transmission of dengue virus in endemic areas.

To that end, several studies have undertaken to understand the factors driving dengue transmission. As discussed above, external factors such as seasonality, temperature, vector populations, and human movement have been investigated. Intrinsic viral characteristics, such as genetic factors, have also been implicated in transmission differences. Outbreaks of the severe forms of DENV fever have been linked to changes in the dominant circulating serotype. For example, in Myanmar the largest outbreak in that region occurred in 2001. This outbreak was associated with the emergence of DENV 1 as the dominant serotype, displacing the other three. In that country, D1 completely displaced the other three serotypes for a definitive serotype switching event (Thu, Lowry et al. 2004). But also, within serotype changes have been implicated in changes in epidemic outbreaks or persistence. In Mexico, frequent lineage replacements were noted over the course of a 27 year epidemic. A total of 11 lineage changes were noted over this time course and little overlap among lineages was noted, though this had little impact on the co-circulation of the four serotypes (Carrillo-Valenzo, Danis-Lozano et al.

2010). Diversification of D1 in Colombia was suggested to be tied to atypical disease presentation (Mendez, Usme-Ciro et al. 2010). In Thailand, microevolution and the presence of genetic diversity in relative proximity was observed in a study involving 11 schools. The significant genetic variation within genotypes of D1, D2, and D3 in focused locations, such as the variation seen among these closely situated schools in Kamphaeng Phet, Thailand (Figure 1.7) was positively associated with differential disease outbreaks among students (Jarman, Holmes et al. 2008). This variability of strains speaks to the inter- and intra-serotype competition, which must surely be driven, at least in part, by viral fitness advantages.

Objectives and Rationale

The overall hypothesis of this research is as follows: Phenotypic differences of arboviruses alter the transmission potential of these arboviruses by conferring fitness advantages in either the vector or the vertebrate. Further, these phenotypic differences need not be of large magnitude to affect the relative transmission potential.

The specific aims addressed are:

1. To identify whether intrinsic viral characteristics can lead to differential transmission in a given locale. This was addressed in the first study (Chapter 2) where genetic diversity of West Nile virus could be associated with differential transmission of the virus. This study was the catalyst to the subsequent research involving phenotypic variation.
2. To assess viral fitness in the mosquito vector and the potential for differential fitness to lead to significant differential transmission potential. To address this aim, we first addressed the issue of mosquito mortality and the interaction with vector competence of arboviruses. This led to a novel formulation of vectorial capacity (cumulative vectorial capacity) and vector competence (effective vector competence). Further, we offer the first available statistical test of a vectorial capacity quantity. In addition, we determined that

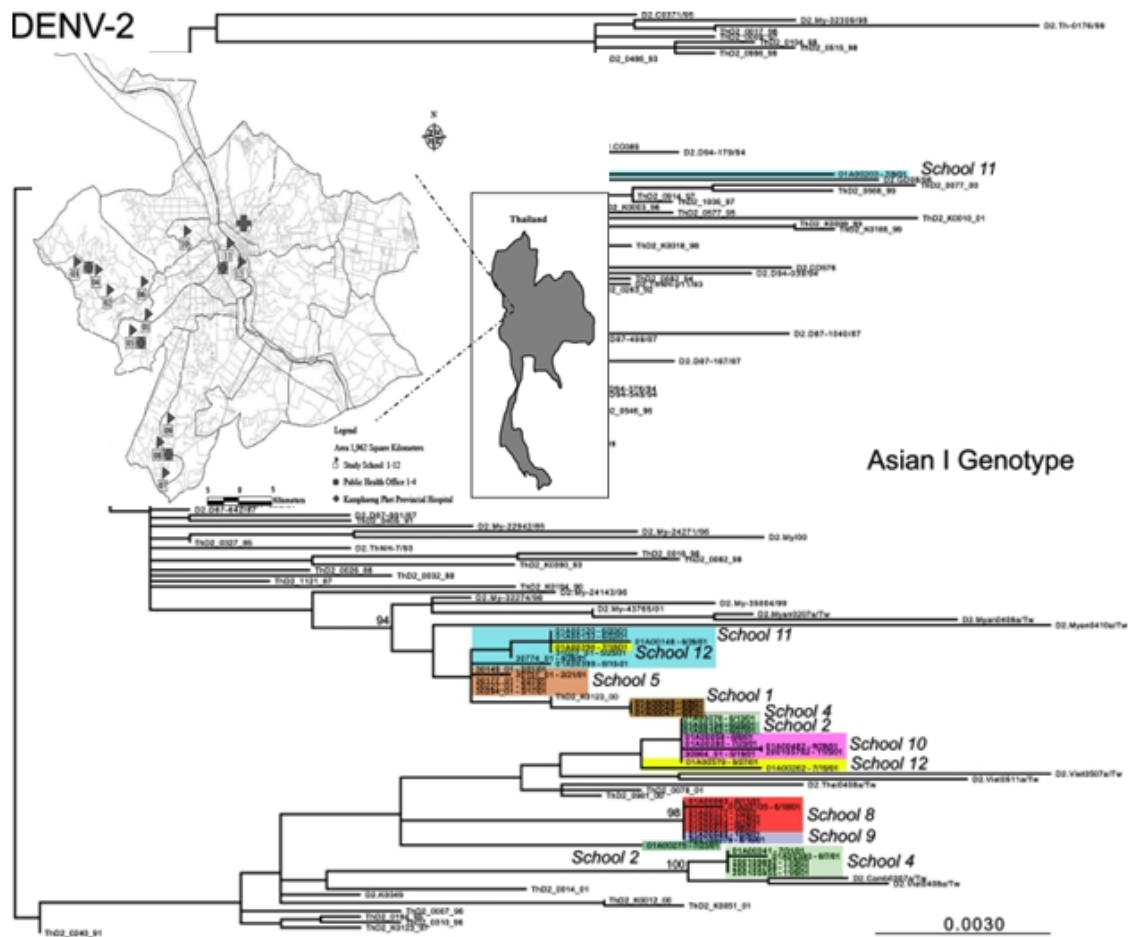


Figure 1.7: Microevolution of DENV. Genetic variation within D2 Asian I genotype in schools from a study in Thailand. In the same study, similar levels of diversity were seen within dengue serotypes 1 and 3. Adapted from (Jarman, Holmes et al. 2008).

even small phenotypic differences can have strong implications for the relative transmission potential of co-circulating arboviral strains.

3. To apply our newly formulated methods from Aim 2 to an ongoing question in the field of arbovirology to show that our methods could directly aid in the explanation of a still puzzling phenomenon. We show that our methodology can only detect significant differences in phenotype where traditional methods do not, applying our methods to already published data of chikungunya virus and the outbreak on La Reunion Island in 2006. We show that phenotypic changes among strains of CHIKV can offer explanation of the expansion of one strain over the previously dominant strain where genetic typing has thus far failed to do so.
4. To explore phenotypic differences in the vertebrate. Investigating the transmission potential of different dengue strains, we first developed a novel model for dengue transmission in a mouse which retains more of an immune response than other mouse models used in disease studies. This allows for more investigation of the immune responses that potentially mitigate infection establishment (transmission). In addition, we offer a potentially new avenue for fitness exploration in the innate immune response to different strains, specifically the type II interferon response. We also describe the first successful transmission of dengue virus from mosquitoes to mice, as well as acquisition from viremic mice to naïve mosquitoes.
5. To assess how these phenotypic differences in both hosts could alter transmission potential. We combined the data from Aims 3 and 4 into a single measure of transmission potential in the form of a modified R_0 . While this is not a final formulation, we determined even small differences in effective vector competence or the length and intensity of viremia in the vertebrate can significantly alter transmission potential of the arbovirus.

References

- (1986). Prevention and control of yellow fever in Africa. Geneva, World Health Organization.
- (2002). "Imported dengue--United States, 1999 and 2000." MMWR Morb Mortal Wkly Rep **51**(13): 281-283.
- (2007). "Dengue hemorrhagic fever--U.S.-Mexico border, 2005." MMWR Morb Mortal Wkly Rep **56**(31): 785-789.
- (2009). Dengue and dengue haemorrhagic fever, World Health Organisation.
- (2009). Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control. W. H. Organization. Geneva.
- (2010). "Locally acquired Dengue--Key West, Florida, 2009-2010." MMWR Morb Mortal Wkly Rep **59**(19): 577-581.
- (2011). "Yellow fever in Africa and Central and South America, 2008-2009." Wkly Epidemiol Rec **86**(4): 25-36.
- Alto, B. W., L. P. Lounibos, et al. (2008). "Larval competition alters susceptibility of adult *Aedes* mosquitoes to dengue infection." Proc Biol Sci **275**(1633): 463-471.
- Alto, B. W., M. H. Reiskind, et al. (2008). "Size alters susceptibility of vectors to dengue virus infection and dissemination." Am J Trop Med Hyg **79**(5): 688-695.
- Alvarez, M., R. Rodriguez-Roche, et al. (2006). "Dengue hemorrhagic Fever caused by sequential dengue 1-3 virus infections over a long time interval: Havana epidemic, 2001-2002." Am J Trop Med Hyg **75**(6): 1113-1117.
- Anderson, J. R. and R. Rico-Hesse (2006). "*Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus." Am J Trop Med Hyg **75**(5): 886-892.
- Anderson, R. M. and R. M. May (1992). Infectious diseases of humans. Oxford, UK, Oxford University Press.
- Angel, B. and V. Joshi (2008). "Distribution and seasonality of vertically transmitted dengue viruses in *Aedes* mosquitoes in arid and semi-arid areas of Rajasthan, India." J Vector Borne Dis **45**(1): 56-59.
- Armstrong, P. M. and R. Rico-Hesse (2001). "Differential susceptibility of *Aedes aegypti* to infection by the American and Southeast Asian genotypes of dengue type 2 virus." Vector Borne Zoonotic Dis **1**(2): 159-168.
- Armstrong, P. M. and R. Rico-Hesse (2003). "Efficiency of dengue serotype 2 virus strains to infect and disseminate in *Aedes aegypti*." Am J Trop Med Hyg **68**(5): 539-544.

- Asnis, D. S., R. Conetta, et al. (2000). "The West Nile Virus outbreak of 1999 in New York: the Flushing Hospital experience." Clin Infect Dis **30**(3): 413-418.
- Bagny, L., H. Delatte, et al. (2009). "Progressive decrease in *Aedes aegypti* distribution in Reunion Island since the 1900s." J Med Entomol **46**(6): 1541-1545.
- Balmaseda, A., S. N. Hammond, et al. (2006). "High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua." Trop Med Int Health **11**(6): 935-942.
- Baqar, S., C. G. Hayes, et al. (1993). "Vertical transmission of West Nile virus by *Culex* and *Aedes* species mosquitoes." Am J Trop Med Hyg **48**(6): 757-762.
- Bennett, K. E., K. E. Olson, et al. (2002). "Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States." Am J Trop Med Hyg **67**(1): 85-92.
- Bertolotti, L., U. D. Kitron, et al. (2008). "Fine-scale genetic variation and evolution of West Nile Virus in a transmission "hot spot" in suburban Chicago, USA." Virology **374**(2): 381-389.
- Black, W. I. and M. CG (1996). Population biology as a tool for studying vector-borne diseases. The biology of Disease Vectors. B. Beaty and W. Marquardt. Niwot, CO, University Press of Colorado. **I**: 393-416.
- Bode, A. V., J. J. Sejvar, et al. (2006). "West Nile virus disease: a descriptive study of 228 patients hospitalized in a 4-county region of Colorado in 2003." Clin Infect Dis **42**(9): 1234-1240.
- Boromisa, R. D., K. S. Rai, et al. (1987). "Variation in the vector competence of geographic strains of *Aedes albopictus* for dengue 1 virus." J Am Mosq Control Assoc **3**(3): 378-386.
- Brault, A. C. (2009). "Changing patterns of West Nile virus transmission: altered vector competence and host susceptibility." Vet Res **40**(2): 43.
- Brault, A. C., A. M. Powers, et al. (2004). "Venezuelan equine encephalitis emergence: enhanced vector infection from a single amino acid substitution in the envelope glycoprotein." Proc Natl Acad Sci U S A **101**(31): 11344-11349.
- Breban, R., R. Vardavas, et al. (2007). "Theory versus data: how to calculate R_0 ?" PLoS ONE **2**(3): e282.
- Brinton, M. A. (2002). "The molecular biology of West Nile Virus: a new invader of the western hemisphere." Annu Rev Microbiol **56**: 371-402.
- Brugger, K. and F. Rubel (2009). "Simulation of climate-change scenarios to explain Usutu-virus dynamics in Austria." Prev Vet Med **88**(1): 24-31.

- Brunkard, J. M., J. L. Robles Lopez, et al. (2007). "Dengue fever seroprevalence and risk factors, Texas-Mexico border, 2004." Emerg Infect Dis **13**(10): 1477-1483.
- Burke, D. S., A. Nisalak, et al. (1988). "A prospective study of dengue infections in Bangkok." Am J Trop Med Hyg **38**(1): 172-180.
- Busvine, J. R. and R. Pal (1969). "The impact of insecticide-resistance on control of vectors and vector-borne diseases." Bull World Health Organ **40**(5): 731-744.
- Cardoso Jda, C., M. A. de Almeida, et al. (2010). "Yellow fever virus in *Haemagogus leucocelaenus* and *Aedes serratus* mosquitoes, southern Brazil, 2008." Emerg Infect Dis **16**(12): 1918-1924.
- Carrillo-Valenzo, E., R. Danis-Lozano, et al. (2010). "Evolution of dengue virus in Mexico is characterized by frequent lineage replacement." Arch Virol **155**(9): 1401-1412.
- Cavrini, F., P. Gaibani, et al. (2009). "Chikungunya: an emerging and spreading arthropod-borne viral disease." J Infect Dev Ctries **3**(10): 744-752.
- CDC. "Final 2000 West Nile Virus Human Neuroinvasive Disease Incidence in the United States." from http://www.cdc.gov/ncidod/dvbid/westnile/Mapsincidence/surv&control00IncidMap_PrinterFriendly.htm.
- CDC "Final 2002 West Nile Virus Human Neuroinvasive Disease Incidence in the United States."
- CDC. (2009). "Mosquito species producing WNV positives by year." from <http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm>.
- Chevillon, C., L. Briant, et al. (2008). "The Chikungunya threat: an ecological and evolutionary perspective." Trends Microbiol **16**(2): 80-88.
- Ciota, A. T., A. O. Lovelace, et al. (2007). "Cell-specific adaptation of two flaviviruses following serial passage in mosquito cell culture." Virology **357**(2): 165-174.
- Clarke, D. K., E. A. Duarte, et al. (1993). "Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses." J Virol **67**(1): 222-228.
- Coffey, L. L., N. Vasilakis, et al. (2008). "Arbovirus evolution in vivo is constrained by host alternation." Proceedings of the National Academy of Sciences of the United States of America **105**(19): 6970-6975.
- Cologna, R., P. M. Armstrong, et al. (2005). "Selection for virulent dengue viruses occurs in humans and mosquitoes." Journal of virology **79**(2): 853-859.
- Cologna, R., P. M. Armstrong, et al. (2005). "Selection for virulent dengue viruses occurs in humans and mosquitoes." J Virol **79**(2): 853-859.

- Cosner, C., J. C. Beier, et al. (2009). "The effects of human movement on the persistence of vector-borne diseases." J Theor Biol **258**(4): 550-560.
- Costero, A. and M. A. Grayson (1996). "Experimental transmission of Powassan virus (Flaviviridae) by *Ixodes scapularis* ticks (Acari:Ixodidae)." Am J Trop Med Hyg **55**(5): 536-546.
- Couderc, T., F. Chretien, et al. (2008). "A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease." PLoS Pathog **4**(2): e29.
- Dauphin, G. and S. Zientara (2007). "West Nile virus: recent trends in diagnosis and vaccine development." Vaccine **25**(30): 5563-5576.
- Davis, C. T., D. W. Beasley, et al. (2004). "Emergence of attenuated West Nile virus variants in Texas, 2003." Virology **330**(1): 342-350.
- Day, T. (2001). "Parasite transmission modes and the evolution of virulence." Evolution **55**(12): 2389-2400.
- de Thoisy, B., V. Lacoste, et al. (2009). "Dengue infection in neotropical forest mammals." Vector Borne Zoonotic Dis **9**(2): 157-170.
- Delatte, H., J. S. Dehecq, et al. (2008). "Geographic distribution and developmental sites of *Aedes albopictus* (Diptera: Culicidae) during a Chikungunya epidemic event." Vector Borne Zoonotic Dis **8**(1): 25-34.
- Delatte, H., C. Paupy, et al. (2008). "[*Aedes albopictus*, vector of chikungunya and dengue viruses in Reunion Island: biology and control]." Parasite **15**(1): 3-13.
- Dobler, G. (2010). "Zoonotic tick-borne flaviviruses." Vet Microbiol **140**(3-4): 221-228.
- Dumont, Y. and F. Chiroleu (2010). "Vector control for the Chikungunya disease." Math Biosci Eng **7**(2): 313-345.
- Dye, C. (1986). "Vectorial capacity: must we measure all its components?" Parasitol Today **2**(8): 203-209.
- Dye, C. (1992). "The analysis of parasite transmission by bloodsucking insects." Annu Rev Entomol **37**: 1-19.
- Dye C, W. B. (1995). Non-Linearities in the Dynamics of Indirectly-Transmitted Infections (or, Does having a Vector make a Difference?). Ecology of Infectious Diseases in Natural Populations. D. A. Grenfell BT. Cambridge, UK, Cambridge University Press: 260-279.
- Ebel, G. D., J. Carricaburu, et al. (2004). "Genetic and phenotypic variation of West Nile virus in New York, 2000-2003." Am J Trop Med Hyg **71**(4): 493-500.

- Endy, T. P., A. Nisalak, et al. (2004). "Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand." J Infect Dis **189**(6): 990-1000.
- Endy, T. P., I. K. Yoon, et al. (2010). "Prospective cohort studies of dengue viral transmission and severity of disease." Curr Top Microbiol Immunol **338**: 1-13.
- Flores, F. S., L. A. Diaz, et al. (2010). "Vertical transmission of St. Louis encephalitis virus in *Culex quinquefasciatus* (Diptera: Culicidae) in Cordoba, Argentina." Vector Borne Zoonotic Dis **10**(10): 999-1002.
- Foppa, I. M. (2005). "The basic reproductive number of tick-borne encephalitis virus. An empirical approach." J Math Biol **51**(6): 616-628.
- Foppa, I. M. and A. Spielman (2007). "Does reservoir host mortality enhance transmission of West Nile virus?" Theor Biol Med Model **4**: 17.
- Fredericksen, B. L., B. C. Keller, et al. (2008). "Establishment and maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1." J Virol **82**(2): 609-616.
- Garrett-Jones, C. (1964). "Prognosis for Interruption of Malaria Transmission through Assessment of the Mosquito's Vectorial Capacity." Nature **204**: 1173-1175.
- Garrett-Jones, C. and B. Grab (1964). "The Assessment of Insecticidal Impact on the Malaria Mosquito's Vectorial Capacity, from Data on the Proportion of Parous Females." Bull World Health Organ **31**: 71-86.
- Gerardin, P., V. Guernier, et al. (2008). "Estimating Chikungunya prevalence in La Reunion Island outbreak by serosurveys: two methods for two critical times of the epidemic." BMC Infect Dis **8**: 99.
- Gershman, M., B. Schroeder, et al. (2009). Yellow Fever. Traveler's Health -mYellow Book. C. f. D. Control. Atlanta.
- Gilfoy, F. D. and P. W. Mason (2007). "West Nile virus-induced interferon production is mediated by the double-stranded RNA-dependent protein kinase PKR." J Virol **81**(20): 11148-11158.
- Gould, E. A. and S. Higgs (2009). "Impact of climate change and other factors on emerging arbovirus diseases." Trans R Soc Trop Med Hyg **103**(2): 109-121.
- Gratz, N. G. (2004). "Critical review of the vector status of *Aedes albopictus*." Med Vet Entomol **18**(3): 215-227.
- Gray, S. M., M. C. Caillaud, et al. (2007). "Transmission of two viruses that cause Barley Yellow Dwarf is controlled by different loci in the aphid, *Schizaphis graminum*." J Insect Sci **7**: 1-15.

- Greene, I. P., E. Wang, et al. (2005). "Effect of alternating passage on adaptation of sindbis virus to vertebrate and invertebrate cells." Journal of virology **79**(22): 14253-14260.
- Gunther, J., J. P. Martinez-Munoz, et al. (2007). "Evidence of vertical transmission of dengue virus in two endemic localities in the state of Oaxaca, Mexico." Intervirology **50**(5): 347-352.
- Guy, B. and J. W. Almond (2008). "Towards a dengue vaccine: progress to date and remaining challenges." Comp Immunol Microbiol Infect Dis **31**(2-3): 239-252.
- Guzman, M. G., G. Kouri, et al. (1991). "Sequential infection as risk factor for dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) during the 1981 dengue hemorrhagic Cuban epidemic." Mem Inst Oswaldo Cruz **86**(3): 367.
- Guzman, M. G., G. Kouri, et al. (2002). "Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks." Rev Panam Salud Publica **11**(4): 223-227.
- Haller, O., G. Kochs, et al. (2006). "The interferon response circuit: induction and suppression by pathogenic viruses." Virology **344**(1): 119-130.
- Halstead, S. B. (2007). "Dengue." Lancet **370**(9599): 1644-1652.
- Halstead, S. B. and E. J. O'Rourke (1977). "Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody." J Exp Med **146**(1): 201-217.
- Hancock, P. A., S. P. Sinkins, et al. (2011). "Strategies for introducing wolbachia to reduce transmission of mosquito-borne diseases." PLoS Negl Trop Dis **5**(4): e1024.
- Hardy, J. (1988). Arboviruses: Epidemiology and Ecology. Susceptibility and resistance of vector mosquitoes. M. TP. Boca Raton, CRC Press. **I**: 87-126.
- Hardy, J. L., E. J. Houk, et al. (1983). "Intrinsic factors affecting vector competence of mosquitoes for arboviruses." Annu Rev Entomol **28**: 229-262.
- Hartemink, N. A., B. V. Purse, et al. (2009). "Mapping the basic reproduction number (R) for vector-borne diseases: a case study on bluetongue virus." Epidemics **1**(3): 153-161.
- Heesterbeek, J. A. (2002). "A brief history of R0 and a recipe for its calculation." Acta Biotheor **50**(3): 189-204.
- Huang, Y. M. (1986). "*Aedes (Stegomyia) bromeliae* (Diptera: Culicidae), the yellow fever virus vector in East Africa." J Med Entomol **23**(2): 196-200.
- Hutamai, S., W. Suwonkerd, et al. (2007). "A survey of dengue viral infection in *Aedes aegypti* and *Aedes albopictus* from re-epidemic areas in the north of Thailand using nucleic acid sequence based amplification assay." Southeast Asian J Trop Med Public Health **38**(3): 448-454.

- Jarman, R. G., E. C. Holmes, et al. (2008). "Microevolution of Dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand." J Virol **82**(11): 5494-5500.
- Jeger, M. J., L. V. Madden, et al. (2009). "The effect of transmission route on plant virus epidemic development and disease control." J Theor Biol **258**(2): 198-207.
- Jerzak, G., K. A. Bernard, et al. (2005). "Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection." J Gen Virol **86**(Pt 8): 2175-2183.
- Kariuki Njenga, M., L. Nderitu, et al. (2008). "Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa." J Gen Virol **89**(Pt 11): 2754-2760.
- Katze, M. G., Y. He, et al. (2002). "Viruses and interferon: a fight for supremacy." Nat Rev Immunol **2**(9): 675-687.
- Kauffman, E. B., M. A. Franke, et al. (2011). "Detection of West Nile virus." Methods Mol Biol **665**: 383-413.
- Kawai, T. and S. Akira (2006). "Innate immune recognition of viral infection." Nat Immunol **7**(2): 131-137.
- Kilpatrick, A. M., D. M. Fonseca, et al. (2010). "Spatial and temporal variation in vector competence of *Culex pipiens* and *Cx. restuans* mosquitoes for West Nile virus." Am J Trop Med Hyg **83**(3): 607-613.
- Kilpatrick, A. M., M. A. Meola, et al. (2008). "Temperature, viral genetics, and the transmission of West Nile virus by *Culex pipiens* mosquitoes." PLoS Pathog **4**(6): e1000092.
- Kramer, L. D. and G. D. Ebel (2003). "Dynamics of flavivirus infection in mosquitoes." Adv Virus Res **60**: 187-232.
- Kramer, L. D., J. L. Hardy, et al. (1983). "Effect of temperature of extrinsic incubation on the vector competence of *Culex tarsalis* for western equine encephalomyelitis virus." Am J Trop Med Hyg **32**(5): 1130-1139.
- Kramer, L. D., J. Li, et al. (2007). "West Nile virus." Lancet Neurol **6**(2): 171-181.
- Kramer, L. D., L. M. Styer, et al. (2008). "A global perspective on the epidemiology of West Nile virus." Annu Rev Entomol **53**: 61-81.
- Kukreti, H., A. Chaudhary, et al. (2008). "Emergence of an independent lineage of dengue virus type 1 (DENV-1) and its co-circulation with predominant DENV-3 during the 2006 dengue fever outbreak in Delhi." Int J Infect Dis **12**(5): 542-549.
- Kuno, G. and G. J. Chang (2005). "Biological transmission of arboviruses: reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends." Clin Microbiol Rev **18**(4): 608-637.

- Kutasi, O., T. Bakonyi, et al. (2011). "Equine encephalomyelitis outbreak caused by a genetic lineage 2 west nile virus in hungary." J Vet Intern Med **25**(3): 586-591.
- Kyle, J. L., P. R. Beatty, et al. (2007). "Dengue virus infects macrophages and dendritic cells in a mouse model of infection." J Infect Dis **195**(12): 1808-1817.
- Lambrechts, L. and T. W. Scott (2009). "Mode of transmission and the evolution of arbovirus virulence in mosquito vectors." Proc Biol Sci **276**(1660): 1369-1378.
- Lambrechts, L., T. W. Scott, et al. (2010). "Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission." PLoS Negl Trop Dis **4**(5): e646.
- Lanciotti, R. S., J. T. Roehrig, et al. (1999). "Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States." Science **286**(5448): 2333-2337.
- Larson, S. R., J. P. DeGroot, et al. (2010). "Ecological niche modeling of potential West Nile virus vector mosquito species in Iowa." J Insect Sci **10**: 110.
- Lazear, H. M., A. K. Pinto, et al. (2011). "Interferon- β controls West Nile virus infection and pathogenesis in mice." J Virol.
- Lipsitch, M. and E. R. Moxon (1997). "Virulence and transmissibility of pathogens: what is the relationship?" Trends in Microbiology **5**(1): 31-37.
- Lobigs, M., A. Mullbacher, et al. (2003). "Role of type I and type II interferon responses in recovery from infection with an encephalitic flavivirus." J Gen Virol **84**(Pt 3): 567-572.
- Lucio-Zavaleta, E., D. M. Smith, et al. (2001). "Variation in Transmission Efficiency Among Barley yellow dwarf virus-RMV Isolates and Clones of the Normally Inefficient Aphid Vector, *Rhopalosiphum padi*." Phytopathology **91**(8): 792-796.
- Lumsden, W. H. (1955). "An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. II. General description and epidemiology." Trans R Soc Trop Med Hyg **49**(1): 33-57.
- Macdonald, G. (1952). "The analysis of equilibrium in malaria." Trop Dis Bull **49**(9): 813-829.
- Macdonald, G. (1957). The epidemiology and control of malaria. London, New York,, Oxford University Press.
- Mason, P. J. and A. J. Haddow (1957). "An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53; an additional note on Chikungunya virus isolations and serum antibodies." Trans R Soc Trop Med Hyg **51**(3): 238-240.
- Massad, E., S. Ma, et al. (2008). "The risk of chikungunya fever in a dengue-endemic area." J Travel Med **15**(3): 147-155.

- May, F. J., C. T. Davis, et al. (2011). "Phylogeography of West Nile virus: from the cradle of evolution in Africa to Eurasia, Australia, and the Americas." J Virol **85**(6): 2964-2974.
- Mendez, J. A., J. A. Usme-Ciro, et al. (2010). "Phylogenetic history demonstrates two different lineages of dengue type 1 virus in Colombia." Virol J **7**: 226.
- Molaei, G., R. F. Cummings, et al. (2010). "Vector-host interactions governing epidemiology of West Nile virus in Southern California." Am J Trop Med Hyg **83**(6): 1269-1282.
- Monath, T. P. (1988). The Arboviruses : epidemiology and ecology. Boca Raton, Fla., CRC Press.
- Mores, C. N., M. J. Turell, et al. (2007). "Experimental transmission of West Nile virus by *Culex nigripalpus* from Honduras." Vector Borne Zoonotic Dis **7**(2): 279-284.
- Moskvitina, N. S., V. N. Romanenko, et al. (2008). "[Detection of the West Nile Virus and its genetic typing in ixodid ticks (Parasitiformes: Ixodidae) in Tomsk City and its suburbs]." Parazitologiya **42**(3): 210-225.
- Moudy, R. M., M. A. Meola, et al. (2007). "A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes." Am J Trop Med Hyg **77**(2): 365-370.
- Moutailler, S., H. Barre, et al. (2009). "Recently introduced *Aedes albopictus* in Corsica is competent to Chikungunya virus and in a lesser extent to dengue virus." Trop Med Int Health **14**(9): 1105-1109.
- Moutailler, S., B. Roche, et al. (2011). "Host alternation is necessary to maintain the genome stability of rift valley Fever virus." PLoS neglected tropical diseases **5**(5): e1156.
- Munoz-Jordan, J. L. (2010). "Subversion of interferon by dengue virus." Curr Top Microbiol Immunol **338**: 35-44.
- Myles, K. M., D. J. Pierro, et al. (2004). "Comparison of the transmission potential of two genetically distinct Sindbis viruses after oral infection of *Aedes aegypti* (Diptera: Culicidae)." J Med Entomol **41**(1): 95-106.
- Nagao, Y. and K. Koelle (2008). "Decreases in dengue transmission may act to increase the incidence of dengue hemorrhagic fever." Proc Natl Acad Sci U S A **105**(6): 2238-2243.
- Nightingale, Z. D., C. Patkar, et al. (2008). "Viral replication and paracrine effects result in distinct, functional responses of dendritic cells following infection with dengue 2 virus." J Leukoc Biol **84**(4): 1028-1038.
- Ooi, E. E. and D. J. Gubler (2009). "Dengue in Southeast Asia: epidemiological characteristics and strategic challenges in disease prevention." Cad Saude Publica **25 Suppl 1**: S115-124.
- Palucka, A. K. (2000). "Dengue virus and dendritic cells." Nat Med **6**(7): 748-749.

- Papa, A., T. Bakonyi, et al. (2011). "Genetic characterization of west nile virus lineage 2, Greece, 2010." Emerg Infect Dis **17**(5): 920-922.
- Partidos, C. D., J. Weger, et al. (2011). "Probing the attenuation and protective efficacy of a candidate chikungunya virus vaccine in mice with compromised interferon (IFN) signaling." Vaccine **29**(16): 3067-3073.
- Paupy, C., B. Ollomo, et al. (2010). "Comparative role of *Aedes albopictus* and *Aedes aegypti* in the emergence of Dengue and Chikungunya in central Africa." Vector Borne Zoonotic Dis **10**(3): 259-266.
- Pesko, K. and C. N. Mores (2009). "Effect of sequential exposure on infection and dissemination rates for West Nile and St. Louis encephalitis viruses in *Culex quinquefasciatus*." Vector Borne Zoonotic Dis **9**(3): 281-286.
- Pesko, K., C. J. Westbrook, et al. (2009). "Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to chikungunya virus." J Med Entomol **46**(2): 395-399.
- Pialoux, G., B. A. Gauzere, et al. (2007). "Chikungunya, an epidemic arbovirosis." Lancet Infect Dis **7**(5): 319-327.
- Pinho, S. T., C. P. Ferreira, et al. (2010). "Modelling the dynamics of dengue real epidemics." Philos Transact A Math Phys Eng Sci **368**(1933): 5679-5693.
- Powers, A. M. and C. H. Logue (2007). "Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus." J Gen Virol **88**(Pt 9): 2363-2377.
- Reisen, W. (1989). "Estimation of vectorial capacity: relationship to disease transmission by malaria and arbovirus vectors." Bulletin of the Society of Vector Ecology **14**: 39-40.
- Reiter, P. (2010). "West Nile virus in Europe: understanding the present to gauge the future." Euro Surveill **15**(10): 19508.
- Richards, S. L., C. N. Mores, et al. (2007). "Impact of extrinsic incubation temperature and virus exposure on vector competence of *Culex pipiens quinquefasciatus* Say (Diptera: Culicidae) for West Nile virus." Vector Borne Zoonotic Dis **7**(4): 629-636.
- Rico-Hesse, R., L. M. Harrison, et al. (1997). "Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas." Virology **230**(2): 244-251.
- Rivero, A., J. Vezilier, et al. (2010). "Insecticide control of vector-borne diseases: when is insecticide resistance a problem?" PLoS Pathog **6**(8).
- Robinson, M. C. (1955). "An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. I. Clinical features." Trans R Soc Trop Med Hyg **49**(1): 28-32.
- Rothman, A. L. (2004). "Dengue: defining protective versus pathologic immunity." J Clin Invest **113**(7): 946-951.

- Rubel, F., K. Brugger, et al. (2008). "Explaining Usutu virus dynamics in Austria: model development and calibration." Prev Vet Med **85**(3-4): 166-186.
- Rush, B. (1789). Medical inquiries and observations. Philadelphia,, Printed and sold by Prichard & Hall.
- Ruzek, D., V. V. Yakimenko, et al. (2010). "Omsk haemorrhagic fever." Lancet **376**(9758): 2104-2113.
- Sangkawibha, N., S. Rojanasuphot, et al. (1984). "Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak." Am J Epidemiol **120**(5): 653-669.
- Santhosh, S. R., P. K. Dash, et al. (2009). "Appearance of E1: A226V mutant Chikungunya virus in Coastal Karnataka, India during 2008 outbreak." Virol J **6**: 172.
- Schmaljohn, A. L. and D. McClain (1996). "Alphaviruses (Togaviridae) and Flaviviruses (Flaviviridae)."
- Shepard, D. S., L. Coudeville, et al. (2011). "Economic impact of dengue illness in the americas." Am J Trop Med Hyg **84**(2): 200-207.
- Sirbu, A., C. S. Ceianu, et al. (2011). "Outbreak of West Nile virus infection in humans, Romania, July to October 2010." Euro Surveill **16**(2).
- Smith, D. L. and F. E. McKenzie (2004). "Statics and dynamics of malaria infection in *Anopheles* mosquitoes." Malar J **3**: 13.
- Smithburn, K. C., T. P. Hughes, et al. (1940). "A neurotropic virus isolated from the blood of a native of Uganda." American Journal of Tropical Medicine **20**: 471-492.
- Snapinn, K. W., E. C. Holmes, et al. (2007). "Declining growth rate of West Nile virus in North America." J Virol **81**(5): 2531-2534.
- Solignat, M., B. Gay, et al. (2009). "Replication cycle of chikungunya: a re-emerging arbovirus." Virology **393**(2): 183-197.
- Staikowsky, F., F. Talarmin, et al. (2009). "Prospective study of Chikungunya virus acute infection in the Island of La Reunion during the 2005-2006 outbreak." PLoS ONE **4**(10): e7603.
- Stoddard, S. T., A. C. Morrison, et al. (2009). "The role of human movement in the transmission of vector-borne pathogens." PLoS Negl Trop Dis **3**(7): e481.
- Suaya, J. A., D. S. Shepard, et al. (2009). "Cost of dengue cases in eight countries in the Americas and Asia: a prospective study." Am J Trop Med Hyg **80**(5): 846-855.

- Suwandono, A., H. Kosasih, et al. (2006). "Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004." Trans R Soc Trop Med Hyg **100**(9): 855-862.
- Taylor, W. P. and I. D. Marshall (1975). "Adaptation studies with Ross River virus: laboratory mice and cell cultures." J Gen Virol **28**(1): 59-72.
- Taylor, W. P. and I. D. Marshall (1975). "Adaptation studies with Ross River virus: retention of field level virulence." J Gen Virol **28**(1): 73-83.
- Teixeira, M. G., C. Costa Mda, et al. (2009). "Dengue: twenty-five years since reemergence in Brazil." Cad Saude Publica **25 Suppl 1**: S7-18.
- Thein, S., M. M. Aung, et al. (1997). "Risk factors in dengue shock syndrome." Am J Trop Med Hyg **56**(5): 566-572.
- Thiboutot, M. M., S. Kannan, et al. (2010). "Chikungunya: a potentially emerging epidemic?" PLoS Negl Trop Dis **4**(4): e623.
- Thu, H. M., K. Lowry, et al. (2004). "Myanmar dengue outbreak associated with displacement of serotypes 2, 3, and 4 by dengue 1." Emerg Infect Dis **10**(4): 593-597.
- Tobler, L. H., M. J. Cameron, et al. (2008). "Interferon and interferon-induced chemokine expression is associated with control of acute viremia in West Nile virus-infected blood donors." J Infect Dis **198**(7): 979-983.
- Tsetsarkin, K., S. Higgs, et al. (2006). "Infectious clones of Chikungunya virus (La Reunion isolate) for vector competence studies." Vector Borne Zoonotic Dis **6**(4): 325-337.
- Tsetsarkin, K. A., R. Chen, et al. (2011). "Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes." Proc Natl Acad Sci U S A **108**(19): 7872-7877.
- Tsetsarkin, K. A., D. L. Vanlandingham, et al. (2007). "A single mutation in chikungunya virus affects vector specificity and epidemic potential." PLoS Pathog **3**(12): e201.
- Turell, M. J., C. N. Mores, et al. (2006). "Laboratory transmission of Japanese encephalitis and West Nile viruses by molestus form of *Culex pipiens* (Diptera: Culicidae) collected in Uzbekistan in 2004." J Med Entomol **43**(2): 296-300.
- Turell, M. J., C. N. Mores, et al. (2006). "Laboratory transmission of Japanese encephalitis, West Nile, and Getah viruses by mosquitoes (Diptera: Culicidae) collected near Camp Greaves, Gyeonggi Province, Republic of Korea 2003." J Med Entomol **43**(5): 1076-1081.
- Unlu, I., A. J. Mackay, et al. (2010). "Evidence of vertical transmission of West Nile virus in field-collected mosquitoes." J Vector Ecol **35**(1): 95-99.
- Vaidyanathan, R. and T. W. Scott (2007). "Geographic variation in vector competence for West Nile virus in the *Culex pipiens* (Diptera: Culicidae) complex in California." Vector Borne Zoonotic Dis **7**(2): 193-198.

- van den Hurk, A. F., S. Hall-Mendelin, et al. (2009). "Vector Competence of Australian Mosquitoes for Chikungunya Virus." Vector Borne Zoonotic Dis.
- Vasconcelos, P. F. (2010). "Yellow fever in Brazil: thoughts and hypotheses on the emergence in previously free areas." Rev Saude Publica **44**(6): 1144-1149.
- Vasilakis, N. and S. C. Weaver (2008). "The history and evolution of human dengue emergence." Adv Virus Res **72**: 1-76.
- Vazeille, M., C. Jeannin, et al. (2008). "Chikungunya: a risk for Mediterranean countries?" Acta Trop **105**(2): 200-202.
- Vazeille, M., L. Mousson, et al. (2009). "Failure to demonstrate experimental vertical transmission of the epidemic strain of Chikungunya virus in *Aedes albopictus* from La Reunion Island, Indian Ocean." Mem Inst Oswaldo Cruz **104**(4): 632-635.
- Vazeille, M., L. Mousson, et al. (2010). "Orally co-Infected *Aedes albopictus* from La Reunion Island, Indian Ocean, can deliver both dengue and chikungunya infectious viral particles in their saliva." PLoS Negl Trop Dis **4**(6): e706.
- Vazeille, M., S. Moutailler, et al. (2007). "Two Chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*." PLoS ONE **2**(11): e1168.
- Watts, D. M., B. A. Harrison, et al. (1985). "Failure to detect natural transovarial transmission of dengue viruses by *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae)." J Med Entomol **22**(3): 261-265.
- Weaver, S. C. and W. K. Reisen (2010). "Present and future arboviral threats." Antiviral Res **85**(2): 328-345.
- Webster, D. P., J. Farrar, et al. (2009). "Progress towards a dengue vaccine." Lancet Infect Dis **9**(11): 678-687.
- Wonham, M. J., M. A. Lewis, et al. (2006). "Transmission assumptions generate conflicting predictions in host-vector disease models: a case study in West Nile virus." Ecol Lett **9**(6): 706-725.
- Woolhouse, M. E., C. Dye, et al. (1997). "Heterogeneities in the transmission of infectious agents: implications for the design of control programs." Proc Natl Acad Sci U S A **94**(1): 338-342.
- Wu, S. J., G. Grouard-Vogel, et al. (2000). "Human skin Langerhans cells are targets of dengue virus infection." Nat Med **6**(7): 816-820.
- Zhang, C., M. P. Mammen, Jr., et al. (2005). "Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence." J Virol **79**(24): 15123-15130.

Chapter 2

Factors Associated with Mosquito Pool Positivity and the Characterization of the West Nile Viruses Found within Louisiana During 2007¹

Introduction

West Nile Virus (WNV) is the most widely distributed arbovirus in the world, occurring on all continents save Antarctica (Kramer, Styer et al. 2008). Its lack of vector specificity compared to other arboviruses has allowed it to use a wide variety of mosquito species in its enzootic cycles (Lanciotti, Roehrig et al. 1999). WNV was introduced into the United States in 1999 and from its entry point of New York City it spread across the continental United States. Phylogenetic evidence traced this strain to a similar strain isolated in Israel in 1998 (Lanciotti, Roehrig et al. 1999). In 2001 a new WNV strain appeared. In 2002, this genotype became the dominant WN02 strain that was significantly associated with an increase in numbers of human morbidity and mortality cases in the US. In fact, the number of deaths from 1999-2001 were significantly less than the number of deaths in 2002 alone, though whether this association is due to direct virulence in humans or an indirect result of the virulence in birds and spread throughout the US remains unclear (Davis, Ebel et al. 2005; Moudy, Meola et al. 2007; Kramer, Styer et al. 2008).

WNV is a member of the genus *Flavivirus*, a group of positive sense RNA viruses. The genome is composed of a single open reading frame that produces ten viral proteins: three structural proteins (capsid C, membrane prM/M, envelope E) and seven non-structural proteins

¹ Christofferson RC, Roy AF, Mores CN: **Factors associated with mosquito pool positivity and the characterization of the West Nile viruses found within Louisiana during 2007.** *Virology journal* 2010, 7:139.

(NS1, NS2A, NS2B, NS3 NS4A, NS4B, and NS5) (Brinton 2002). The NS3 gene plays an important role in the replication of the virus, encoding a protein with four functions: a serine protease, a nucleoside triphosphatase, an RNA 5'triphosphatase, and a helicase (Brinton 2002). Phylogenetic analyses of WNV have most commonly utilized differences in the envelope protein, but the capsid, prM protein, and non-structural proteins have also been informative (Ebel, Dupuis et al. 2001; Lanciotti, Ebel et al. 2002; Beasley, Davis et al. 2003; Davis, Ebel et al. 2005; Jerzak, Bernard et al. 2005; Davis, Galbraith et al. 2007; Bertolotti, Kitron et al. 2008; Gray, Veras et al. 2010). Analyses done on complete genomes have given similar results to trees made from prM and envelope proteins (Davis, Ebel et al. 2005). To determine the genetic variability in Louisiana, the envelope coding and NS3 coding regions were analyzed. The NS3 gene has a high level of substitution and is phylogenetically informative (Gray, Veras et al. 2010) and mutations in the envelope region has been postulated to enable viruses subvert immune responses (Pfeffer, Foster et al. 2006). Analysis of these genes would illustrate the variation present in Louisiana as well as provide improved insight for our phylogenetic model.

Many phylogenetic studies have been geographically focused (Ebel, Dupuis et al. 2001; Andreadis, Anderson et al. 2004; Ebel, Carricaburu et al. 2004; Bertolotti, Kitron et al. 2008). Geographic Information Systems (GIS) based "region" classifications were successfully used to model WNV transmission risk in humans in northeast Ohio where local environmental features to model transmission (LaBeaud, Gorman et al. 2008). Employing a similar eco-regionalization method, we hypothesized that WNV pool positivity was correlated with regional environmental characteristics. Further, we postulated that the phylogenetic delineations would be associated with variations in regional environmental conditions.

Materials and Methods

Samples

According to the mosquito surveillance data, there are two primary species of mosquitoes that serve as possible WNV vectors to people in Louisiana: *Culex quinquefasciatus* and *Aedes albopictus*. There were twenty six species from eight genera captured and submitted for testing. Of these, fifteen species were found to be positive for WNV (Table 2.1).

Mosquito Trap Sites and Field Collection of Mosquitoes

Data was provided by Louisiana parish mosquito control departments. Each parish operates independently with its own trapping protocols and methods. Not all parishes actively sampled throughout the year due to considerations of the local mosquito activity levels. We therefore analyzed the months that all target parishes had in common (June-November), which captured the majority of the WNV transmission period (Richards, Mores et al. 2007).

Mosquitoes were sexed and the females were pooled according to genus and species; that is, one pool consisted of a single species. The pools were then homogenized and submitted to the Louisiana Animal Disease Diagnostic Laboratory (LADDL) at the Louisiana State University (LSU) School of Veterinary Medicine (SVM). LADDL is the state testing facility for mosquito pools for all parishes, so criteria for positive pools is the same across all parishes.

Virus Detection and Sequencing

Pools were obtained from the LADDL at the LSU SVM. Viral RNA was extracted from 140 µl of the supernatant from the mosquito pool homogenate using the QIAmp Viral RNA Extraction Kit following manufacturer's instructions (Qiagen, Valencia, CA). One microliter of the extracted viral RNA suspension was used as template for the reverse-transcriptase polymerase chain reaction (RT-PCR) using Superscript™ III RT-PCR kit (Invitrogen, Carlsbad, CA) with the

Table 2.1: Species in positive mosquito pools: Mosquito pools by species positive for WNV in 2007 from the four target parishes.

Species in Positive Mosquito Pools		
Genus	Species	Number of positive pools
<i>Aedes</i>	<i>albopictus</i>	12
	<i>triserriatus</i>	1
	<i>vexans</i>	5
<i>Anopheles</i>	<i>quadrимaculatus</i>	1
<i>Culex</i>	<i>coronator</i>	2
	<i>erraticus</i>	3
	<i>nigrapalpus</i>	1
	<i>quinquefasciatus</i>	129
	<i>restuans</i>	1
	<i>salinarus</i>	1
	<i>tarsalis</i>	1
<i>Mansonia</i>	<i>titillans</i>	2
<i>Psorophora</i>	<i>columbiae</i>	3
	<i>Ferox</i>	2
	<i>howardii</i>	1

previously described protocol (Chisenhall, Vitek et al. 2008). Upon confirmation of the presence of amplified viral DNA by gel electrophoresis, the remaining sample was cleaned using Qiagen PCR Cleanup kit following manufacturer's instructions. Cleaned DNA was then sent to the Gene Probes and Expression Systems Laboratories of the Division of Biotechnology and Molecular Medicine at the Louisiana State University School of Veterinary Medicine for sequencing. Sequencing was performed on a Beckman Coulter 8800 (Pasadena, CA) using the manufacturer's reagents and methods.

Statistical Analysis

SAS version 9.1.3 was used to code the data as a binary response, where a mosquito pool that was positive for was coded as '1' while the negative pools were coded as '0.' The probabilities reported are the probabilities of the event=1 (WNV positive). A confidence level of 95% was used for all tests; a stepwise selection process was invoked to cull out non-significant effects from the model.

PROC GLIMMIX with a binary distribution specified was used to construct the model and obtain the odds ratios, as well as to obtain differences in least squares means for the effects of parish and month. PROC GLIMMIX is a useful alternative to PROC LOGISTIC when wanting to obtain differences in least squares and/or modeling random effects, which is not easily done in PROC LOGISTIC. Predicted means and odds ratios are the same between the two procedures. Confidence intervals and tests for significance will vary when random effects are included the model in PROC GLIMMIX, but there were no random effects modelled here (Christofferson 2008).

Phylogenetic Analysis

The complete envelope (E) and non-structural protein 3 (NS3) genes were aligned separately for all successfully recovered isolates as well as 24 reference strains, which represented the Israel 1998 strain and isolates spanning the contiguous United States and one Mexican isolate (Ebel, Carricaburu et al. 2004). Alignments and the creation of the E-NS3 contigs were done using Vector NTI software and exported to GeneDoc for trimming. Alignments were imported into MEGA 4 and converted to MEGA format. Bootstrap analysis (n=1000) using maximum parsimony was performed and a tree produced. As the topologies of the E and NS3 phylogenies were the same, our tree represents a contiguous sequence of these two genes. The topology tree was collapsed with a node confidence of 70%. Genetic distances and means were also obtained using Mega 4 (Tamura, Dudley et al. 2007). Not all samples in the statistical model were available for the phylogenetic analysis.

Results

Positive Pool Predictors

The data used in the modelling study were from several parishes in Louisiana during 2007. There were 611 positives reported by the Louisiana Animal Disease Diagnostic Laboratory, 165 in our target parishes. Classification regions were constructed based on land cover data from the Louisiana GIS Digital Map, May 2007 (Figure 2.1). The land cover of a parish was determined by the majority rule. Ouachita and Caddo parishes are, for example, 50-75% forest lands; East Baton Rouge is a majority developed area comprised mostly of the urbanized capital of Baton Rouge (Harper 2006). Iberville Parish is classified as wetlands, defined as low-lying areas saturated with moisture.

Before building the WNV model, we tested several factors via multinomial regression to determine whether these were significant predictors of the mosquito species found, regardless of

WNV positivity. Interestingly, no factors (land cover, month) were significant for the 2007 data. Therefore, it was determined that no noteworthy correlation existed between these variables and thus no collinearity issues would arise by inclusion of all variables in the model.

The model originally included the effects of month, mosquito genus and species; but through a stepwise selection procedure, the variable species was not significant at the $\alpha=0.05$ level and thus eliminated from the model. This is likely due to the overwhelming number of *Culex quinquefasciatus*, which comprised over 61% of the total pools ($N_{\text{total}}=3246$) and 88% of the positive pools (Table 2.1). This is consistent with earlier studies where *Cx. quinquefasciatus* was found to be the most abundant and likely epizootic vector for the virus (Gleiser, Mackay et al. 2007; Mackay, Roy et al. 2008). In addition, all interactions were not significant and therefore removed from the model.

Locations of isolates were coded according to land cover. The model included land cover, genus, and month. Type of regional land cover was a significant effect ($p < 0.0001$) indicating that there is an ecological component driving WNV activity. Additionally, month was significant ($p < 0.0001$); and thus there is a temporal component that contributes to the probability of getting a positive mosquito pool. This trend can be seen in figure 2.2.

The pair wise differences in least squares means are given in table 2.2 with Tukey adjusted p-values showing where the significant differences lay. Forested lands are clearly more likely to have a positive mosquito pool as compared to developed areas and wetlands. Similarly, August appears to be the month where a positive mosquito pool is more likely. August and July are eight and six times more likely to see a positive mosquito pool than June, respectively. Interestingly, August and July are not significantly different from the months of September-November. All months are significantly different from June, which is the least likely month during the accepted transmission season to have a positive pool; the odds of a positive pool is

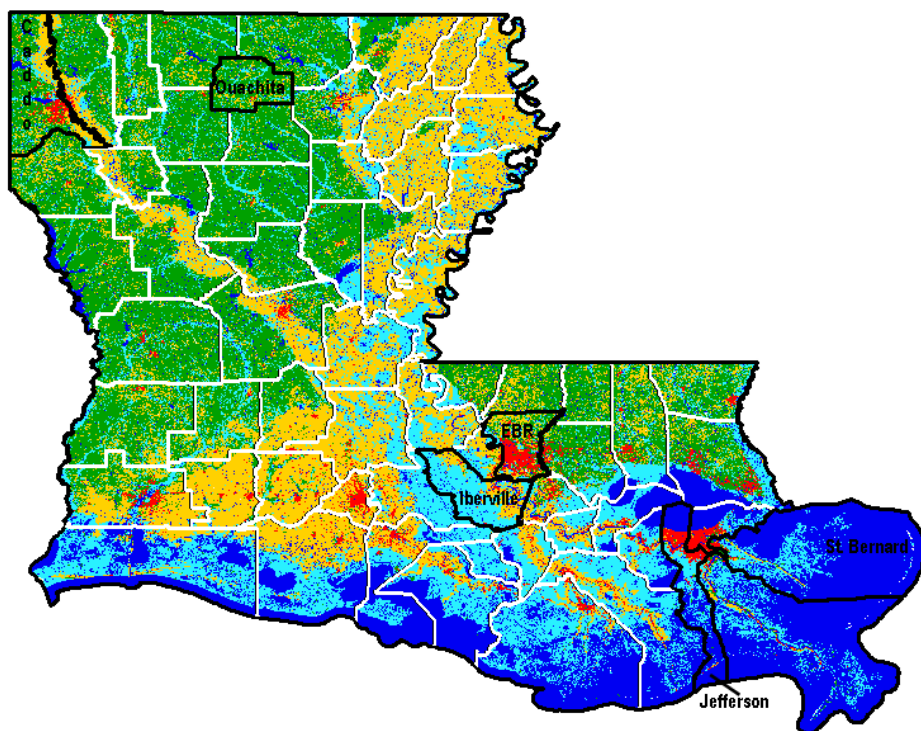


Figure 2.1: Louisiana landcover. Louisiana GIS Digital Map, May 2007 shows the land cover of Louisiana. Pertinent parishes are labelled with their parish names.

over seven times more likely in July than June. The overall trends of land cover and month are shown together in Figure 2.2.

Phylogenetic Analysis

The phylogenetic tree resulting from the contiguous segment comprised of the envelope and NS3 sequences is shown in figure 2.3. All samples are, unsurprisingly, of the 2002 lineage. However, there appears to be some diversity within both forested and wetland areas. Particularly, there is a possible small sub-group with samples 4893, 8441, and 3077. The samples from the wetlands show the possibility of a distinctive group; in particular, sample numbers 3766 and 3767. There is a clear delineation between forest and wetland samples based on two nucleotide substitutions in the NS3 gene: an adenine to guanine at positions 5760, and a cytosine to uracil at position 6324. Samples were grouped as follows: Israel 1998 as the root; the wetlands as a group; a small grouping of forest samples; a NY99 group; and the remaining were grouped together as representative of the North American 2002 clade. The between and within distances were computed according to the Jukes-Cantor model (table 2.3). Genetic diversity within Louisiana was modest when compared to isolates from a wide geographic range. The genetic distances within the eco-regions (forest vs. wetlands) in Louisiana were greater than the distances comparing Louisiana and those strains from outside of Louisiana. Table 2.4 identifies sample origins, classifications, and accession numbers.

Discussion

In some locations, WNV positivity in mosquito pools serves as a predictor for human cases (Andreadis, Anderson et al. 2004). Our findings that the month of collection and eco-region were significant predictors of positive pools suggest that ecological and temporal factors influence WNV activity and can assist the public health sector predict or prevent cases of human

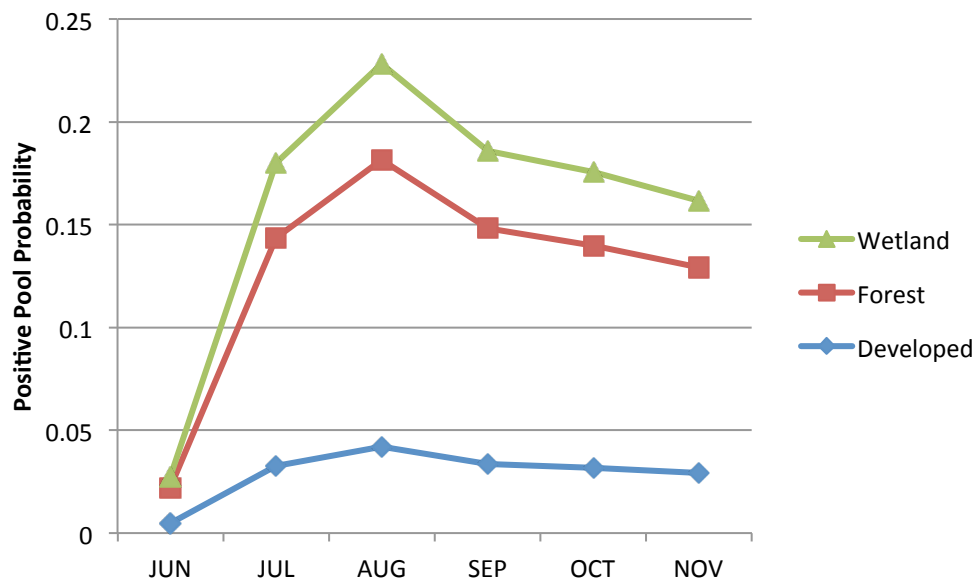


Figure 2.2: Positive pool probability over months by land cover. Predicted probability of a positive mosquito pool between June and November 2007 as predicted by land cover.

Table 2.2: Differences of least square means: The differences in the least square means reported as odds ratios with Tukey adjusted p-values show significant differences between types of land cover and month.

Differences of Least Squares Means			
Effects		Odds Ratio	Adjusted P-value
Developed	Forest	0.279	<.0001
Wetland	Forest	1.346	0.0076
July	June	6.65	0.0006
August	June	8.98	<.0001
September	June	7.09	0.0001
October	June	6.75	<.0001

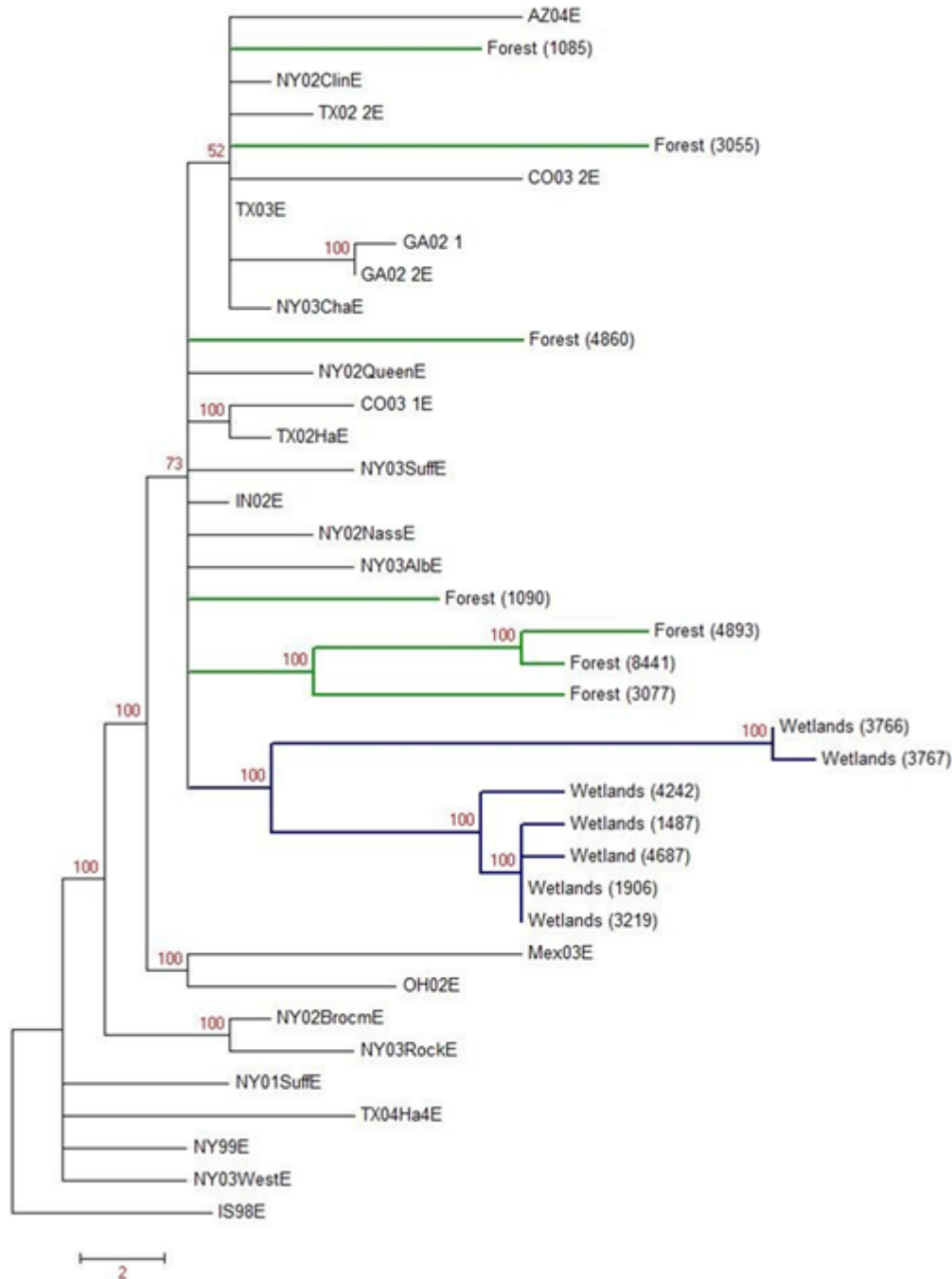


Figure 2.3: Maximum parsimony phylogenetic tree. Phylogenetic tree based on 1000 bootstraps of the maximum parsimony analysis of the WNV E and NS3 contiguous sequence. Branches indicating Louisiana isolates are coded by eco-region with isolate ID in parentheses. Other sequences included in this phylogeny include the following GenBank accession numbers: AF260967, DQ164201, DQ164195, DQ164191, DQ164190, DQ164189, DQ164203, DQ164204, DQ164199, AY712948, DQ164194, DQ164192, AY033389, DQ164196, DQ164197, DQ164193, DQ164205, AY963775, DQ164200, DQ164198, DQ164202, DQ164188, DQ164187, DQ164186.

WNV infection. Mosquito abatement programs are operated on a parish-wide basis, so any useful model would ideally work for the parish as a whole. Therefore, looking at the ecology of the parish as a whole- and even grouping parishes into ecological regions or types- will assist in determining a robust model as we did in our pool positivity model, can be used at the very least, on a parish level. Ideally, a model would serve the state as a whole, focusing on regional activity.

Phylogenetic analysis will also help us better understand how the changes in the genome may lead to a change in virulence. It is interesting that the statistical model predicts positive pools in forested areas while the phylogenetic study shows more diversity in the wetland areas. It is also interesting that there is more genetic diversity within Louisiana than without. That is, the diversity when comparing between the wetlands and forests within Louisiana is greater than the diversity when comparing the Louisiana isolates and those outside of the state. This suggests that WNV evolution is not as constrained in Louisiana as compared to other locations as has been suggested in other (Chisenhall and Mores 2009). Further, some component of eco-regions could have an important role in diversification. This could be due to a difference in bird populations found in each of these ecologies, or it could be a function of extrinsic, microhabitat conditions, such as temperature or humidity which could exert a selection pressure on the viruses.

The phylogenetic analysis shows that there has been no reversion in Louisiana from the 2002 lineage which replaced the originally introduced strain. Our statistical model serves as a basis for future testing, directing mosquito control efforts and surveillance programs. Though we believe our findings to be a significant start to a potentially long term project, it was not without its pitfalls. For example, there is a confounding factor of spatial clustering of the ecologies tested. However, if the spatial component was the only source of diversity, thus discounting the ecological diversity, we would not expect that the phylogenetic signature of the Southern Louisiana strains should be markedly different in topology than the Northern Louisiana strains. The fact that the wetland strains formed a distinct monophyletic group as compared to the forest

Table 2.3: Genetic distances for 2007 samples: Genetic distances of the 2007 samples from Mega 4.0 show more variability within Louisiana than when Louisiana is compared to the rest of North American isolates.

Genetic Distances for 2007 Samples			
	Within		Between
Louisiana	.29104		.4751
Outside LA	.56137		
Forest	.55029		.65608
Wetlands	.35147		

Table 2.4: Origin and classification of sequenced isolates: The isolates that were successfully sequenced are listed by land cover classification. The parish where each was collected is identified and accession numbers for E (envelope) and N (NS3) sequences are given as well.

Land Cover	ID Number	Parish	Accession Number (E/N)
Wetlands	3766	St. Bernard	HM538823/HM538811
	3767	St. Bernard	HM538824/HM538812
	3219	St. Bernard	HM538829/HM538807
	4242	Iberville	HM538825/HM538810
	1487	Jefferson	HM538827/HM538806
	4687	Jefferson	HM538826/HM538808
Forrest	1906	Jefferson	HM538828/HM538809
	3055	Caddo	HM538821/HM538813
	1085	Caddo	HM538817/HM538805
	4860	Ouachita	HM538822/HM538816
	4893	Ouachita	HM538818/HM538803
	8441	Ouachita	HM538819/HM538804
	3077	Ouachita	HM538820/HM538814

strains, which were characterized by a lack of phylogenetic structure, suggests the diversity seen within these regions is the result of some other, perhaps ecological, characteristic.

Additionally, the specificity of the land cover classification needs to be more precise. This land cover generalization to parish is a good start to determine if further investigation is garnered, and here we show that it is. Understanding how land cover and regional ecology effects mosquito pool positivity will help focus mosquito abatement efforts. This would especially help in areas where abatement programs are limited due to either funding or man power. Moreover, understanding how regional environments drive phylogenetic variation will lead to a greater understanding of the interactions between ecology and disease prevalence.

References

- Andreadis, T. G., J. F. Anderson, et al. (2004). "Epidemiology of West Nile virus in Connecticut: a five-year analysis of mosquito data 1999-2003." Vector Borne Zoonotic Dis **4**(4): 360-378.
- Beasley, D. W., C. T. Davis, et al. (2003). "Limited evolution of West Nile virus has occurred during its southwesterly spread in the United States." Virology **309**(2): 190-195.
- Bertolotti, L., U. D. Kitron, et al. (2008). "Fine-scale genetic variation and evolution of West Nile Virus in a transmission "hot spot" in suburban Chicago, USA." Virology **374**(2): 381-389.
- Brinton, M. A. (2002). "The molecular biology of West Nile Virus: a new invader of the western hemisphere." Annu Rev Microbiol **56**: 371-402.
- Chisenhall, D. M. and C. N. Mores (2009). "Diversification of West Nile virus in a subtropical region." Virol J **6**: 106.
- Chisenhall, D. M., C. J. Vitek, et al. (2008). "A method to increase efficiency in testing pooled field-collected mosquitoes." J Am Mosq Control Assoc **24**(2): 311-314.
- Christofferson, R. C. (2008). Old vs. New: A comparison of PROC LOGISTIC and PROC GLIMMIX. SAS Global Forum, San Antonio, TX.
- Davis, C. T., G. D. Ebel, et al. (2005). "Phylogenetic analysis of North American West Nile virus isolates, 2001-2004: evidence for the emergence of a dominant genotype." Virology **342**(2): 252-265.

- Davis, C. T., S. E. Galbraith, et al. (2007). "A combination of naturally occurring mutations in North American West Nile virus nonstructural protein genes and in the 3' untranslated region alters virus phenotype." J Virol **81**(11): 6111-6116.
- Ebel, G. D., J. Carricaburu, et al. (2004). "Genetic and phenotypic variation of West Nile virus in New York, 2000-2003." Am J Trop Med Hyg **71**(4): 493-500.
- Ebel, G. D., A. P. Dupuis, 2nd, et al. (2001). "Partial genetic characterization of West Nile virus strains, New York State, 2000." Emerg Infect Dis **7**(4): 650-653.
- Gleiser, R. M., A. J. Mackay, et al. (2007). "West Nile virus surveillance in East Baton Rouge Parish, Louisiana." J Am Mosq Control Assoc **23**(1): 29-36.
- Gray, R. R., N. M. Veras, et al. (2010). "Evolutionary characterization of the West Nile Virus complete genome." Mol Phylogenet Evol.
- Harper, R. A. (2006). Forest Inventory & Analysis Factsheet Louisiana 2005. USDA.
- Jerzak, G., K. A. Bernard, et al. (2005). "Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection." J Gen Virol **86**(Pt 8): 2175-2183.
- Kramer, L. D., L. M. Styer, et al. (2008). "A global perspective on the epidemiology of West Nile virus." Annu Rev Entomol **53**: 61-81.
- LaBeaud, A. D., A. M. Gorman, et al. (2008). "Rapid GIS-based profiling of West Nile virus transmission: defining environmental factors associated with an urban-suburban outbreak in Northeast Ohio, USA." Geospat Health **2**(2): 215-225.
- Lanciotti, R. S., G. D. Ebel, et al. (2002). "Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East." Virology **298**(1): 96-105.
- Lanciotti, R. S., J. T. Roehrig, et al. (1999). "Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States." Science **286**(5448): 2333-2337.
- Mackay, A. J., A. Roy, et al. (2008). "West Nile virus detection in mosquitoes in East Baton Rouge Parish, Louisiana, from November 2002 to October 2004." J Am Mosq Control Assoc **24**(1): 28-35.
- Moudy, R. M., M. A. Meola, et al. (2007). "A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes." Am J Trop Med Hyg **77**(2): 365-370.
- Pfeffer, M., J. E. Foster, et al. (2006). "Phylogenetic analysis of Buggy Creek virus: evidence for multiple clades in the Western Great Plains, United States of America." Appl Environ Microbiol **72**(11): 6886-6893.

- Richards, S. L., C. N. Mores, et al. (2007). "Impact of extrinsic incubation temperature and virus exposure on vector competence of *Culex pipiens quinquefasciatus* Say (Diptera: Culicidae) for West Nile virus." Vector Borne Zoonotic Dis **7**(4): 629-636.
- Tamura, K., J. Dudley, et al. (2007). "MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0." Mol Biol Evol **24**(8): 1596-1599.

Chapter 3

Estimating the Magnitude and Direction of Altered Arbovirus Transmission Due to Viral Phenotype¹

Introduction

The transmission potential of a vector borne disease has been used to predict risks of outbreaks, evaluate vector control strategies, and to compare strains of a pathogen (Garrett-Jones and Shidrawi 1969; Kramer and Ebel 2003; Anderson and Rico-Hesse 2006). An accurate measure of this potential is critical, and often estimated by vectorial capacity (VC) (Smith and McKenzie 2004). Vectorial capacity was originally devised by MacDonald in 1957 for malariologists, and represents “the number [of infections] that a specific mosquito population can distribute per case per day (Macdonald 1957; Garrett-Jones 1964; Dye 1992; Smith and McKenzie 2004).” The calculation of vectorial capacity is given by:

$$VC = \frac{ma^2p^Nb}{-\ln p} \quad \text{Equation (Eq.) 3.1}$$

where **a** is the man biting rate and **m** is the mosquito density; these parameters are measures of contact between the vector and vertebrate hosts (Macdonald 1957). The probability of daily survival **p** is a measure of the mortality rate of the vector (Macdonald 1957). The extrinsic incubation period (EIP) **N** is the time, in days, it takes for a pathogen to infect the mosquito and disseminate to the salivary glands where it can be transmitted (Macdonald 1957). The original formula of vectorial

¹Christofferson RC, Mores CN: **Estimating the magnitude and direction of altered arbovirus transmission due to viral phenotype**. *PLoS ONE* 2011, **6**:e16298.

capacity has been modified to include a transmission capability parameter, vector competence **b** (Dye 1986; Hardy 1988; Reisen 1989; Dye C 1995; Black and CG 1996). These last two parameters, EIP and **b**, capture intrinsic viral characteristics and have been used to evaluate differences in pathogen strains (Armstrong and Rico-Hesse 2001; Armstrong and Rico-Hesse 2003; Moudy, Meola et al. 2007; Tsetsarkin, Vanlandingham et al. 2007). Other researchers have recognized the importance of understanding the parameters in vectorial capacity as well as characterizing the interaction of important parameters, EIP, survival rate and vector competence, as a means for evaluating infection risks (Kramer and Ebel 2003; Cohuet, Harris et al. 2010).

Vector competence **b** is the ability of an arthropod to transmit an infectious agent following exposure to that agent (Hardy 1988). Several vector traits have been studied with regard to vector competence differences, such as mosquito species, mosquito strain within species and mosquito size (Boromisa, Rai et al. 1987; Bennett, Olson et al. 2002; Vaidyanathan and Scott 2007; Alto, Reiskind et al. 2008). Vector competence for arboviruses particularly is impacted by extrinsic factors such as temperature differences during incubation, titer of virus offered during exposure, and larval competition (Hardy, Houk et al. 1983; Kramer, Hardy et al. 1983; Richards, Mores et al. 2007; Alto, Lounibos et al. 2008; Pesko, Westbrook et al. 2009). Estimates of vector competence can also be indicative of differences in vector susceptibility to arbovirus strains. Indeed, several studies have shown that vector competence of dengue virus varies within a single serotype (Armstrong and Rico-Hesse 2001; Armstrong and Rico-Hesse 2003). Vector competence is estimated as the proportion of mosquitoes with a disseminated infection to the total number of exposed mosquitoes and can therefore be thought of as the dissemination rates within a vector population.

Comparisons of arbovirus strain-specific vector competence estimates have been used to support observed or hypothesized differences in transmission capability (Anderson and Rico-Hesse 2006; Turell, Mores et al. 2006; Turell, Mores et al. 2006; Mores, Turell et al. 2007; van den Hurk,

Hall-Mendelin et al. 2009). Typically, such comparisons are made at a single (optimal) time point during the extrinsic incubation period, and less commonly two or more time points might be used (Anderson and Rico-Hesse 2006; Moudy, Meola et al. 2007; Kilpatrick, Meola et al. 2008; Moutailler, Barre et al. 2009; van den Hurk, Hall-Mendelin et al. 2009). Using the appropriate extrinsic incubation period (EIP) is crucial when calculating vectorial capacity (Kramer and Ebel 2003; Anderson and Rico-Hesse 2006). However, the continuous interaction between EIP and vector competence has not been evaluated in terms of vectorial capacity estimates. Further, the importance of the mosquito survival rate is also critical given as it sets time constraints on the EIP. The importance of the interaction of mosquito lifespan, EIP and vector competence is recognized, but there has, until now, not existed a method to evaluate this interaction with more than rudimentary comparisons (Ghosh, Moreira et al. 2002; Kramer and Ebel 2003; Smith and McKenzie 2004; Anderson and Rico-Hesse 2006; Walker and Lynch 2007; Cohuet, Harris et al. 2010).

Instead of evaluating vectorial capacity and vector competence at discrete time points, as traditional use of vector competence data allows, vector competence can be modeled as a function of the rate of change over time in days, giving a value we term “effective vector competence (EVC).” This value is bounded by the survivability of the mosquito population, given that it includes p^N as a crucial evaluative parameter. This EVC can then be put back into the vectorial capacity equation as a parameter, resulting in a vectorial capacity curve as a function of time. Utilizing the EVC and vectorial capacity curve rather than single day or even day-by-day vector competence values accounts for the importance of the mosquito lifespan. When taken in concert, EIP (now a range of days), vector competence, and daily survival rate captures a more accurate picture of the natural transmission potential of a pathogen. Accordingly, we investigated the rate of change in vector competence, over a given interval, of dengue virus in *Aedes aegypti* mosquitoes, and the resulting EVC and vectorial capacity values, which resulted in a curvilinear function. The area under these

curves represents the average vector competence bounded by mosquito lifespan and the average cumulative transmission potential of the arboviruses within a population of mosquitoes for a given time interval, respectively. Four strains of dengue virus were evaluated in this manner to demonstrate the value of this model based on our experimental data. Further we use previously published data on both West Nile virus and chikungunya virus to prove the validity our model.

The use of vectorial capacity to statistically compare virus transmission differences has been limited due to the inability to efficiently test differences, and often the statistical comparisons are limited to the vector competence data (Rattanakul, Konishi et al. 1996; Reifenberg, Cuisance et al. 1997; Sithiprasasna, Linthicum et al. 2003; Anderson and Rico-Hesse 2006). Because vector competence is a parameter of proportion, each estimate of vectorial capacity contains within it an entire experiment aimed at estimating vector competence. Statistical methods that are readily available to researchers would require several replications of vectorial capacity estimates or artificial computer simulations. We used the calculated area under the curve for each virus strain in this study and calculated a corresponding variance estimate based on the inherent variance in the vector competence functions. We then used these estimates to test for differences in cumulative transmission potentials between strains of dengue virus based on our dynamic model.

The purpose of this modeling effort is to demonstrate that day-by-day comparisons of vector competence alone are not sufficient to offer consistent estimates of viral fitness. Additionally, the inclusion of the daily survival rate of the mosquito population makes the vector competence function much more relevant, as these bounds on EIP are important for fitness evaluation. Implicit in our calculation of a cumulative measure is the assertion that transmissibility at earlier time points is critical to characterizing epidemiologically relevant differences in viral strains, and that those differences are less apparent or lost when vectorial capacity is calculated with single day measures or even maximum measurements of vector competence at later times. Finally, because the single

day value of EIP in the field is impossible to pinpoint and unlikely to be meaningful (outside of point source introduction of virus), a range of time covering the transmission critical period will more accurately represent what is happening under natural conditions. In order to more accurately evaluate viral fitness, we offer a model of cumulative vectorial capacity and effective vector competence to show that 1) single day comparisons are inadequate and 2) even with the collection of day-by-day vector competence values, a cumulative evaluation is needed.

Materials and Methods

Mosquitoes

Aedes aegypti (Linnaeus) Rockefeller strain mosquitoes from the colony at Louisiana State University School of Veterinary Medicine were used in this experiment. Cartons containing approximately 100 individuals were kept in an environmental chamber at 28°C, 75-80% humidity, and subjected to a 16:8 light:dark regime. Mosquitoes were provided water after emergence until the time of blood-feeding; no mortality due to sugar starvation was observed. After blood-feeding, mosquitoes were supplied with fresh water and 10% sucrose solution for the remainder of the experiment.

Virus

Four strains of dengue from Southeast Asia were utilized in this experiment to demonstrate the hypothesized behavior of a multiple strain system. Three strains of serotype 2 (16803, 1232 and 16681) and one strain of serotype 4 (LN 634441) were propagated by inoculating a T-75 flask of confluent Vero cells with 100 µl of viral stock for 15 minutes (Alto, Lounibos et al. 2008). Ten mL of M199E medium with 10% newborn calf serum and 2% penicillin/ streptomycin/ fungizone was added. Flasks were incubated at 35°C with 5% CO₂ for 6-8 days when they were harvested for virus

at peak levels. Viral standard curves and concentrations were obtained via plaque assay as described previously before the beginning of the experiment and titers were verified throughout the experiment, including testing of blood meal titers, by qRT-PCR as previously described (Chisenhall and Mores 2009). We used the SuperScript III One-step qRT-PCR kit (Invitrogen, Carlsbad, CA) as per manufacturer's instructions.

Oral Exposure of Mosquitoes and Detection of Disseminated Infection

Mosquitoes were offered an infectious blood meal 3-5 days post emergence with an infectious titer of 10^6 pfu/ml for all strains. The blood meal consisted of bovine blood in Alsevier's anticoagulant (Hemostat, Dixon, CA) mixed 2:1 with a virus solution in a total volume of approximately 3 ml per carton, heated to 37°C and kept warm via the Hemotek device (Discovery Workshops, Arrington, Lancashire, UK). Mosquitoes were allowed to feed for 45 minutes before the blood meal was removed. Mosquitoes were then sorted and only fully engorged females were kept; all others were discarded. Engorged females were identified by the presence of red blood in the abdomen, visible with the naked eye and these mosquitoes were our exposed cohort. Mosquitoes were then sampled at days 5, 7, and 9 post exposure to test for dissemination status. Sample sizes at each day are given in table 3.1.

A disseminated infection where virus is present in legs and tissues other than the midgut, has been used to assess vector competence of dengue in *Aedes* mosquitoes, as well as other arboviruses in mosquito vectors. (Turell, Gargan et al. 1984; Armstrong and Rico-Hesse 2001; Moncayo, Fernandez et al. 2004; Alto, Reiskind et al. 2008). Mosquito legs were removed and analyzed separately for infection from the bodies. Legs and bodies were put into separate vials containing 900 µl of BA-1 diluent (Lanciotti, Kerst et al. 2000) and then homogenized at 20 Hz for 2 minutes using a TissueLyzer (Qiagen). RNA was extracted using the MagMax-96 kit (Ambion) on a King Fisher®

nucleic acid extraction instrument according to the manufacturer's instructions (Thermo Scientific). After extraction, the samples were tested for the presence of dengue viral RNA via qRT-PCR using the following protocol: RT step (1 cycle) 48°C for 2 minutes, 95°C for 2 minutes; amplification and data recording step (40 cycles) 95°C 15 seconds, 60°C 30 seconds. Primers were designed and obtained via Integrated DNA Technologies (Table 3.2) with 5' FAM fluorophore and 3' Black-Hole quencher for DEN-2 and 5' TAMRA fluorophore and 3' Iowa-Black quencher for DEN-4. These primers and probes do not cross react, and are specific to only the intended serotype of dengue. Vector competence was calculated as the number of disseminated infections divided by the total number of mosquitoes exposed, as described above. All analyses and modeling was performed in SAS 9.13 (Cary, NC). The SAS Code is given in Appendices 3 and 4 for the one and two-phase scenarios, respectively.

Effective Vector Competence (EVC) and Cumulative Vectorial Capacity (cVC) Model

Because we are interested in comparing viral characteristics only, we will hold **m**, as well as **a** (man biting rate) and **p** (probability of daily survival), constant. The values used in this effort are shown in table 3.3. The assumptions of our model are a naïve end-host population and no significant vertical transmission of the pathogen within the vector. While vertical transmission has been observed with dengue in *Aedes aegypti*, levels are very low and an impact on transmission has not been definitively proven (Gunther, Martinez-Munoz et al. 2007; Arunachalam, Tewari et al. 2008). Vector competence values used in this dengue vectorial capacity modeling effort are given in table 3.3.

Figure 3.1 shows traditional, single time point values of vectorial capacity calculated as in Eq. 3.1 and the comparison of our formulated cumulative vectorial capacity (cVC) described below.

Table 3.1: Dissemination rates and samples sizes for 4 strains of dengue at 5, 7, and 9 days post exposure.

Serotype	Strain	Origin	Dissemination Rates (n)		
			5 dpe	7dpe	9dpe
2	1232	Indonesia, human	0 (19)	0.35 (17)	0.44 (18)
2	16803	Thailand, human	0 (18)	0.06 (15)	0.58 (12)
2	16681	Thailand, human	0.045 (22)	0.28 (25)	0.35 (31)
4	LN 634441	Malaysia, human	0 (11)	0 (11)	0.18 (11)

Table 3.2: Primer and probe sequences for dengue serotypes 2 and 4. All sequences are 5' → 3'.

Serotype	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequence
DEN-2	CAGGTTATGGCACTGT CACGAT	CCATCTGCAGCAACACC ATCTC	CTCTCCGAGAACAGGCCTCGA CTTCA
DEN-4	TTGTCCTAATGATGCTG GTCG	TCCACCTGAGACTCCTTC CA	TTCCTACTCCTACGCATCGCA TTCCG

Table 3.3: Parameters of the vectorial capacity equation held constant. Parameter values of *Aedes aegypti* obtained from literature and held constant when calculating the Vectorial Capacity over a series of days, N, and corresponding vector competence values, b_N . *Denotes average of presented data.

Parameter	Value	Reference
Mosquito Density (m)	1.9	(Jeffery, Thi Yen et al. 2009)
Man Biting Rate (a)	3.125	(Yasuno and Tonn 1970)
Probability of Daily Survival (p)*	0.91	(Harrington, Buonaccorsi et al. 2001)

This figure highlights the importance of accounting for variation of vector competence over the course of time, as the rank of fitness as judged by vectorial capacity swaps from day to day.

Results

We devised a method that uses the rate of change in vector competence as part of the vectorial capacity equation. For each set of dissemination rates over a given interval meant to represent the EIP, a relationship defining b as a function of this interval was devised:

$$b_i(N) = \beta_1 N + \beta_0 \quad \text{Eq. 3.2}$$

where $b_i(N)$ is the function for strain i ; β_1 is the determined change in b per unit change in N (slope of the line); N is day post exposure, and β_0 is the y-intercept. This line represents the rate of change in dissemination rates over time and the variance inherent to this line will be used to construct variance estimates. The interval over which this line is constructed has a lower limit of time a and an upper limit of z . Using the rate of change function for each strain i , EVC is defined as:

$$\text{EVC} = \varphi = \int_a^z p^N (\beta_1 N + \beta_0) dN \quad \text{Eq. 3.3}$$

And cVC is now defined as:

$$cVC_i = \int_a^z \frac{ma^2 p^{N(\beta_0 N + \beta_1)}}{-\ln p} dN$$

As vector competence is a proportion, it is asymptotic at 0 and at 1 and the function of change is a sigmoidal (S) curve often analyzed via logistic regression. A sigmoidal curve is

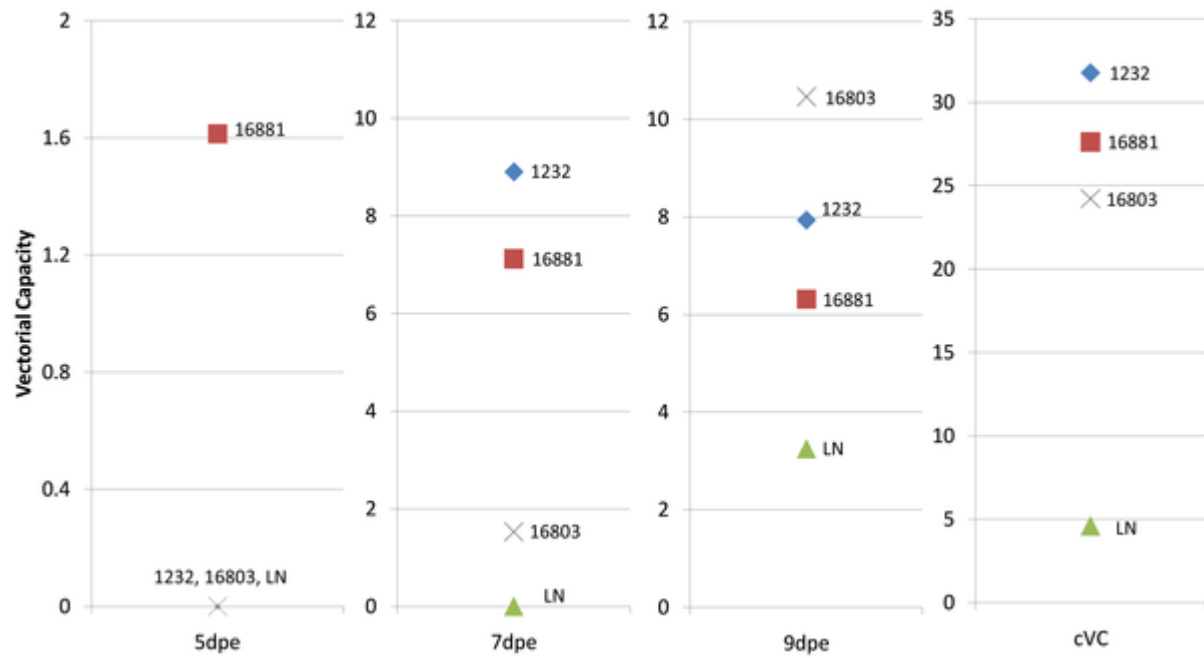


Figure 3.1: Single versus cumulative VC values: Vectorial capacity values for four strains of dengue at 5, 7, and 9 days post exposure (dpe) as well as the cumulative vectorial capacity (cVC) values. The relative fitness of the strains swaps places throughout all time points, highlighting the difficulty of choosing a single EIP value. The cVC values take into account all days (5, 7, 9) and the daily survival rate (.91) of the mosquito, offering a more accurate measure of viral fitness.

characterized by a plateau before (minimum =0) and after (maximum=M) a phase of exponential growth within the interval [a,t] where a is the beginning of the exponential growth phase and t is the end of the exponential phase (Figure 3.2). The interval (t,z] is the M-phase. At both the 0- and M-plateau phase of this curve, the rate of change of vector competence is either negligible or zero. During the 0-plateau phase, there is no mathematical contribution to the calculation of cVC, but during the M-plateau, the changes in cVC will largely be driven not by vector competence, but by the survival rate of the vector. If the interval of experimentation does not include the M-plateau phase, then vectorial capacity is calculated as above, using the linear function $b_i(N)$. If the M-phase is sufficient enough to contribute, then cVC is calculated by adding the areas under the curve of the exponential and M-phases (see Appendix 5 for more details).

As the dengue strains used in this study did not reach M-phase over the experimental time interval (days 5-9 post exposure), the cVC was calculated from the exponential phase only, as in Supplemental Definition 1 (Appendix 5). Corresponding variance estimates are calculated using the variation inherent in the linear function, $b_i(N)$, from the exponential growth phase of vector competence. One of the benefits of using linear regression equations to define cVC rather than directly incorporating the logistic function is the calculation of an accurate variance estimate, whereas logistic regression functions are based on maximum likelihood and thus variances often do not converge on true values of variance and confidence levels are approximations. Variance calculations are given in Appendix 5. The use of areas under the curve and corresponding variance estimates to test for differences has been established (Dill, Martin et al. 1978), but use in vector-borne disease transmission comparisons has not been explored. The results of this method, including EVC and cVC, for four dengue strains are given in table 3.4.

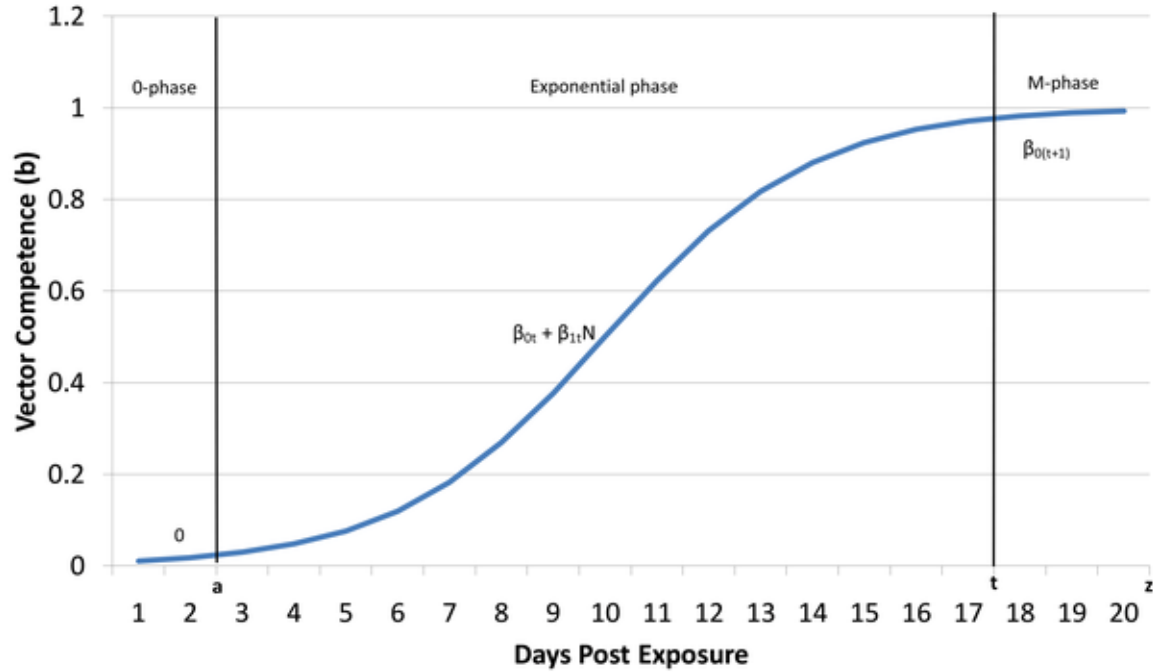


Figure 3.2 Vector competence dynamics: The complete lack of dissemination during the 0-phase will contribute 0 to the cumulative vectorial capacity calculation, while the functions in both the exponential growth and M- phases will be incorporated into the calculation of cVC. During the exponential growth phase, the linear function of vector competence growth will be incorporated in the cVC function, while in the M-phase, where dissemination rates have reached a plateau, only the y-intercept based on the value of the dissemination rate plateau value is necessary.

Statistical Test of Differences

We also provide a method for statistically testing the cVC. It is important to note that cVC itself is not a mean or a sum, but a fine scale sum of means. To demonstrate, we show here a simple sum, though in actuality we integrate over the interval to capture the continuous rates of change of vector competence. Let $E(VC_x)$ be the mean vectorial capacity at day X, then it follows that

$$E(VC5 + VC7 + VC9) = E(VC5) + E(VC7) + E(VC9) \quad \text{Eq. 3.5}$$

The importance of considering the cumulative transmission potential of a mosquito population is depicted in Figure 3.1 where the strain of DENV with the highest dissemination rate at N=9 (D2 16803) has the third highest cVC.

To test for differences between strains, confidence intervals about the mean differences in cVC estimates should be constructed based on an acceptable confidence level and the appropriate degrees of freedom (based on the sample size of the vector competence function). If the data are sufficiently normal, using critical values from the Student's t-distribution is acceptable. However, a robust alternative if data are not sufficiently normal is the construction of confidence intervals based on a t-like distribution of the differences. Area estimates and variances are obtained as above after bootstrap re-sampling with replacement for 1000 bootstrap iterations. The differences between these simulated area estimates and variances per strain are then used to develop a t-like distribution which given the number of bootstraps, is normally distributed. A t-like distribution has the properties of the Student's t-distribution, but the mean is shifted from 0 to a value dependent on the comparisons made. The distribution of the differences is constructed and the values of the 2.5 and 97.5 percentiles of this t-like distribution ($t_{.025}$ and $t_{.975}$, respectively) are obtained and used to construct a 95% confidence interval of the difference. For example, to compare strains 1 and 2, using the data

Table 3.4: Results from the cumulative formulation of vector competence and vectorial capacity method for 4 strains of dengue. Effective vector competence (ϕ) and cumulative vectorial capacity (cVC) estimates obtained from our model of integration and the associated variance estimates for each strain.*Indicates the 95% lower and upper confidence limits for the difference between each strain and D2 1232.

Strain	Lower 95% Confidence Limit of Difference*	cVC	Std. Err. (cVC) $\widehat{\text{Area}}$	Upper 95% Confidence Limit of Difference*	ϕ
1232	n/a	31.7616	6.66	n/a	.294
16803	-24.3807	24.2305	5.92	12.4718	.225
16681	-21.7204	27.6219	5.54	12.8051	.256
LN 634441	-42.1138	4.69539	3.00	-12.5657	.044

obtained experimentally and the t-like distribution from the bootstrap efforts, the upper and lower confidence limits are obtained by:

$$[(cVC_1 - cVC_2) - |t_{.025}| * \sqrt{Var_1 + Var_2}, (cVC_1 - cVC_2) + |t_{.975}| * \sqrt{Var_1 + Var_2}] \quad \text{Eq. 3.6}$$

The values cVC_1 , cVC_2 , Var_1 , and Var_2 are the values obtained from the original computations of cVC based on the experimental data and $b_i(N)$; the bootstrapping is to facilitate the t-like distribution and produce the values of $t_{.025}$ and $t_{.975}$ only. As in all interval tests of hypotheses, if the null value (i.e. a difference of 0) is contained within this interval, no significant difference exists between the compared strains. Results of pair wise testing for the cVC values of four strains of dengue are given in table 3.4.

By calculating the rate of change of vector competence and pairing this with the other parameters of the vectorial capacity equation, especially the survival function, a more accurate understanding of the comprehensive differences in the potential for transmission of arboviruses is obtained. For the sake of brevity, we present only the tested differences in strains with respect to an arbitrarily designated reference strain, D2 1232. We constructed 95% confidence intervals using cVC estimates and standard error estimates to test for significant differences between the reference strain and the other four strains. Only strain LN 634441 was significantly different from the reference strain (table 3.4). This demonstrates the ability of our model to distinguish between significant and non-significant differences. Using the definition of vectorial capacity and assuming a system of perfect transmission where every disseminated infection results in a successful transmission event, for every 10 cases of DENV transmitted from this population of mosquitoes that is attributable to strain 1232, one would expect only ≈ 2 to arise from strain LN 634441.

Characterization of Fitness in the Vector

To further characterize differences between dengue strains in the context of viral fitness, we devised a displacement index (DI). Viral fitness is a measure of the relative replicative abilities of a viral strain to a reference strain (Clarke, Duarte et al. 1993). Vector competence can be used as a measure of relative fitness, and thus so can vectorial capacity and cVC (Anderson and Rico-Hesse 2006). To isolate viral characteristics, and using the properties of integration, the “entomological” parameters can be moved to the outside of the integral (see Appendix 5).

This displacement index is then defined as:

$$DI = \frac{\frac{ma^2}{-\ln p} \varphi_i}{\frac{ma^2}{-\ln p} \varphi_{REF}} = \frac{cVC_i}{cVC_{REF}} \quad \text{Eq. 3.7}$$

When two strains (i and a reference strain REF) are co-circulating in a single population of mosquitoes, which is assumed to be homogeneous, the parameters considered to be intrinsic to the vector effectively cancel out. The DI is thus interpreted as a measure of the capability of a newly introduced strain to displace the established, dominant circulating strain:

$$DI = \frac{\varphi_i}{\varphi_{REF}} \quad \text{Eq. 3.8}$$

When the entomological parameters cancel out, the viral differences, measured as effective vector competence, are what determine whether one strain is capable of displacing the dominant circulating strain or serotype: the capability and speed of dissemination. Our model of EVC and cVC captures these parameters in a more thorough measurement, and as the DI utilizes cVC and/or EVC, it is likewise a more complete measure of viral fitness with regards to transmission. If entomological and vertebrate parameters of the vectorial capacity equation are held constant, when

$DI > 1$, there exists some intrinsic characteristic of that viral strain that will infer on the displacing strain a competitive advantage. In this way, the DI can be used to compare intrinsic viral fitness of viral strains within a vector population or can be used to determine the potential of a new virus to invade and hijack a vector population where an established arbovirus has sustained transmission.

Discussion

Expectation has been that arbovirus strains with lower vector competence at late EIPs will be at a competitive disadvantage and an arbovirus strain with the highest ultimate value of **b** should out-compete strains with lower dissemination rates at some fixed EIP. For example, the strain of West Nile virus originally introduced to North America (NY99) has since been displaced by another strain (WN02) which has a shorter extrinsic incubation period within its primary vectors, the *Culex* spp. mosquitoes (Moudy, Meola et al. 2007). Alone, the values of vector competence and EIPs are informative, however we detected highly variable times to initial and maximum dissemination and dissemination rates based on the strain of dengue virus. Taken at each time point, strain differences can be seen, but no clear pattern emerges for definitive conclusions. For example, if we were to compare the fastest start of dissemination, D2 16681 is the only strain with any dissemination at day 5. At day 7, however, this strain is out disseminated by D2 1232 which then falls behind D2 16803 at day 9 post exposure. The highest ultimate dissemination is seen in D2 16803 (58% at 9 dpe). These differences across time points show the difficulty in assessing fitness at discrete points. Further, though D2 16803 ultimately achieves the highest dissemination rate, because of the force of the survival function on vectorial capacity, this strain does not possess the highest cVC and therefore is not necessarily the most efficiently transmitted strain. In fact, D2 16803 did not have cVC above D2 1232 or D2 16681 which only had dissemination rates of 44% and 35% at day 9, respectively.

D4 LN 634441 lagged at all time points and was the only strain to be accurately assessed at each single time point, though this is attributable to the overall inefficiency of the strain.

While we use testing of legs to extrapolate transmission rates, we recognize that there has been no definitive evidence that proves this measure does not overestimate vector competence. There is no evidence to support the supposition that this overestimation would be differential across strains. The lack of an accessible transmission model for dengue has confounded such investigations, and this further highlights the importance of moving towards such a model.

As vector competence is a dynamic function of time, selection of an appropriate EIP (or range thereof) for testing is critical (Cohuet, Harris et al. 2010). However, it is also important to note that at later time points, survivorship of the mosquito cohort declines. The effect of the interaction of vector competence and declining survivorship has on estimates of vectorial capacity had not been rigorously explored. A strain that results in a smaller proportion of disseminated infections, but that invades much faster will infiltrate the mosquito population and perhaps render a portion of the vertebrate population immunologically unavailable to the strains with slower kinetics, as well as take advantage of a higher proportion of surviving vectors, a relationship not accurately reflected by simple vector competence comparisons. This demonstrates that there is a trade-off in vector competence and EIP, which this model accurately captures. This model should retain its accuracy and usefulness when comparing across vector populations and/or species by varying the other parameters according to the vector(s) of interest.

The argument that vectorial capacity values are most informative when used in a comparative way is not new (Dye 1992). A decisive, interpretable method for doing such a comparisons has until now, been unavailable. With this data, we demonstrate how the varying values of vector competence can be used to calculate the true magnitude of transmission potential and that these cumulative values are the basis of accurate tests of differences in these potentials.

In complex vector-borne disease transmission systems such as dengue, where multiple serotypes of an arbovirus co-circulate, understanding the relative kinetics of transmission of co-circulating strains and serotypes is a vital part of understanding the overall transmission. This is especially so in dengue endemic areas where serotype switching events have been linked to more severe disease outbreaks (Thu, Lowry et al. 2004). With the understanding that entomological parameters cancel out in our calculation of the DI, we formulated a comparison of relative fitness. In a theoretical system where D2 1232 is established as the dominant strain, and the other four dengue virus strains have been introduced and now co-circulate, none of the strains are capable of overtaking the system and displacing strain 1232. Conversely, if LN was the reference and 1232 the invader, 1232 would have the potential to displace LN with a ratio of infectious bites of 10:4. Excess secondary vertebrate cases of strain 1232 translate into enhanced transmission potential to naïve vectors, continuing the transmission cycle with more force than the other strains and thus perpetuating its dominance (Figure 3.3).

To further validate our model, we used data from Moudy et al. that investigated the differences in vector competence between the NY99 and WN02 strains of West Nile virus (Moudy, Meola et al. 2007). The displacement index of WN02 in relation to NY99 is 2.14, a value that is supported by the invasion of WN02 into the West Nile transmission system and its complete displacement of the established NY99 strain. A pathogen such as WNV which has multiple vector species, can add a new level of complexity to the model. In such cases, the entomological parameters would have a great impact on the transmission system. But like the cVC model in general, the DI can be used to make comparisons made across different populations or species of vectors.

Similarly, we demonstrate the use of the displacement index using data from two strains of chikungunya virus isolated from La Reunion Island during the 2005-2006 epidemic (Tsetsarkin, Vanlandingham et al. 2007). The mutation in the envelope changed an alanine to a valine and

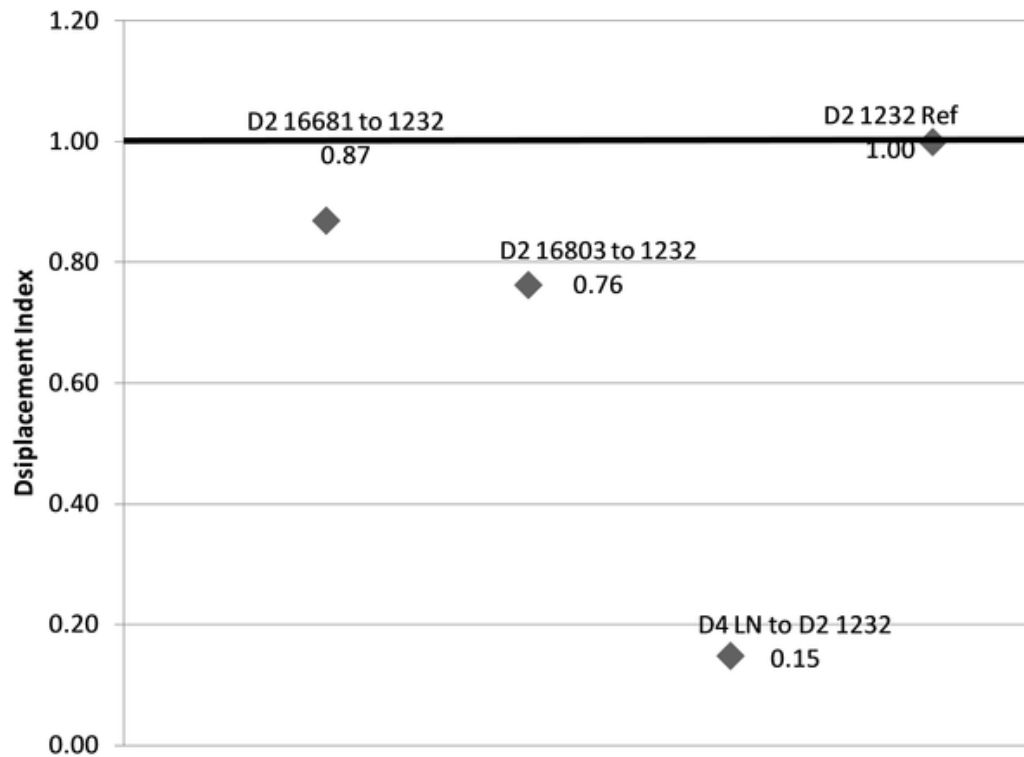


Figure 3.3: Characterizing relative fitness: The displacement index of three dengue strains (D4 LN 63441, D2 16681, D2 16803) compared to the reference strain (D2 1232) shows that no displacement should occur were these co-circulating strains.

increased the efficiency of the virus within the vector *Aedes albopictus* (Tsetsarkin, Vanlandingham et al. 2007). The displacement index of the viral strains (E226V:E226A) in *Ae. albopictus* was calculated to be 1.91, and further demonstrates the use of the displacement index as a measure of both viral fitness within a vector and a means of comparing transmission potential.

As these data indicate, our cVC methodology gives a more accurate measure of the magnitude of transmission potential, owing to the fact that it collapses several informative parameters into a single, standardized measure. In addition, it allows for direct statistical tests of differences in cumulative vectorial capacity where there has previously been none. The DI provides a scaled index by which viral fitness can be measured and compared, an assessment which further characterized the four dengue strains used. In addition, as the historical events highlight, the DI could indicate the potential emergence of new pathogen threats to public health, economy, and national security. The validation using West Nile and chikungunya data gives us confidence that this method will be a useful epidemiologic measure and future directions include investigations of field isolated dengue from endemic areas.

In summary, the cVC model along with the DI provides a conceptual and methodological basis by which virus fitness differences can be evaluated within an epidemiologically satisfying framework. This methodology will be additionally useful in retrospective characterizations of observed viral genetic and phenotypic differences detected during past epidemics, where attribution of the emergence event is of interest.

References

Alto, B. W., L. P. Lounibos, et al. (2008). "Larval competition alters susceptibility of adult *Aedes* mosquitoes to dengue infection." Proc Biol Sci **275**(1633): 463-471.

- Alto, B. W., M. H. Reiskind, et al. (2008). "Size alters susceptibility of vectors to dengue virus infection and dissemination." Am J Trop Med Hyg **79**(5): 688-695.
- Anderson, J. R. and R. Rico-Hesse (2006). "*Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus." Am J Trop Med Hyg **75**(5): 886-892.
- Armstrong, P. M. and R. Rico-Hesse (2001). "Differential susceptibility of *Aedes aegypti* to infection by the American and Southeast Asian genotypes of dengue type 2 virus." Vector Borne Zoonotic Dis **1**(2): 159-168.
- Armstrong, P. M. and R. Rico-Hesse (2003). "Efficiency of dengue serotype 2 virus strains to infect and disseminate in *Aedes aegypti*." Am J Trop Med Hyg **68**(5): 539-544.
- Arunachalam, N., S. C. Tewari, et al. (2008). "Natural vertical transmission of dengue viruses by *Aedes aegypti* in Chennai, Tamil Nadu, India." Indian J Med Res **127**(4): 395-397.
- Bennett, K. E., K. E. Olson, et al. (2002). "Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States." Am J Trop Med Hyg **67**(1): 85-92.
- Black, W. I. and M. CG (1996). Population biology as a tool for studying vector-borne diseases. The biology of Disease Vectors. B. Beaty and W. Marquardt. Niwot, CO, University Press of Colorado. **I**: 393-416.
- Boromisa, R. D., K. S. Rai, et al. (1987). "Variation in the vector competence of geographic strains of *Aedes albopictus* for dengue 1 virus." J Am Mosq Control Assoc **3**(3): 378-386.
- Chisenhall, D. M. and C. N. Mores (2009). "Diversification of West Nile virus in a subtropical region." Virology **6**: 106.
- Clarke, D. K., E. A. Duarte, et al. (1993). "Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses." J Virol **67**(1): 222-228.
- Cohuet, A., C. Harris, et al. (2010). "Evolutionary forces on Anopheles: what makes a malaria vector?" Trends Parasitol **26**(3): 130-136.
- Dill, G. M., F. A. Martin, et al. (1978). Computer Analysis of Sugarcane Data in Louisiana. Proceeding of the American Society of Sugarcane Technologists.
- Dye, C. (1986). "Vectorial capacity: must we measure all its components?" Parasitol Today **2**(8): 203-209.
- Dye, C. (1992). "The analysis of parasite transmission by bloodsucking insects." Annu Rev Entomol **37**: 1-19.

- Dye C, W. B. (1995). Non-Linearities in the Dynamics of Indirectly-Transmitted Infections (or, Does having a Vector make a Difference?). Ecology of Infectious Diseases in Natural Populations. D. A. Grenfell BT. Cambridge, UK, Cambridge University Press: 260-279.
- Garrett-Jones, C. (1964). "Prognosis for Interruption of Malaria Transmission through Assessment of the Mosquito's Vectorial Capacity." Nature **204**: 1173-1175.
- Garrett-Jones, C. and G. R. Shidrawi (1969). "Malaria vectorial capacity of a population of *Anopheles gambiae*: an exercise in epidemiological entomology." Bull World Health Organ **40**(4): 531-545.
- Ghosh, A. K., L. A. Moreira, et al. (2002). "Plasmodium-mosquito interactions, phage display libraries and transgenic mosquitoes impaired for malaria transmission." Insect Biochem Mol Biol **32**(10): 1325-1331.
- Gunther, J., J. P. Martinez-Munoz, et al. (2007). "Evidence of vertical transmission of dengue virus in two endemic localities in the state of Oaxaca, Mexico." Intervirology **50**(5): 347-352.
- Hardy, J. (1988). Arboviruses: Epidemiology and Ecology. Susceptibility and resistance of vector mosquitoes. M. TP. Boca Raton, CRC Press. **I**: 87-126.
- Hardy, J. L., E. J. Houk, et al. (1983). "Intrinsic factors affecting vector competence of mosquitoes for arboviruses." Annu Rev Entomol **28**: 229-262.
- Harrington, L. C., J. P. Buonaccorsi, et al. (2001). "Analysis of survival of young and old *Aedes aegypti* (Diptera: Culicidae) from Puerto Rico and Thailand." J Med Entomol **38**(4): 537-547.
- Jeffery, J. A., N. Thi Yen, et al. (2009). "Characterizing the *Aedes aegypti* population in a Vietnamese village in preparation for a Wolbachia-based mosquito control strategy to eliminate dengue." PLoS Negl Trop Dis **3**(11): e552.
- Kilpatrick, A. M., M. A. Meola, et al. (2008). "Temperature, viral genetics, and the transmission of West Nile virus by *Culex pipiens* mosquitoes." PLoS Pathog **4**(6): e1000092.
- Kramer, L. D. and G. D. Ebel (2003). "Dynamics of flavivirus infection in mosquitoes." Adv Virus Res **60**: 187-232.
- Kramer, L. D., J. L. Hardy, et al. (1983). "Effect of temperature of extrinsic incubation on the vector competence of *Culex tarsalis* for western equine encephalomyelitis virus." Am J Trop Med Hyg **32**(5): 1130-1139.
- Lanciotti, R. S., A. J. Kerst, et al. (2000). "Rapid detection of west nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay." J Clin Microbiol **38**(11): 4066-4071.

- Macdonald, G. (1957). The epidemiology and control of malaria. London, New York,, Oxford University Press.
- Moncayo, A. C., Z. Fernandez, et al. (2004). "Dengue emergence and adaptation to peridomestic mosquitoes." Emerg Infect Dis **10**(10): 1790-1796.
- Mores, C. N., M. J. Turell, et al. (2007). "Experimental transmission of West Nile virus by *Culex nigripalpus* from Honduras." Vector Borne Zoonotic Dis **7**(2): 279-284.
- Moudy, R. M., M. A. Meola, et al. (2007). "A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes." Am J Trop Med Hyg **77**(2): 365-370.
- Moutailler, S., H. Barre, et al. (2009). "Recently introduced *Aedes albopictus* in Corsica is competent to Chikungunya virus and in a lesser extent to dengue virus." Trop Med Int Health **14**(9): 1105-1109.
- Pesko, K., C. J. Westbrook, et al. (2009). "Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to chikungunya virus." J Med Entomol **46**(2): 395-399.
- Rattanarithikul, R., E. Konishi, et al. (1996). "Detection of *Plasmodium vivax* and *Plasmodium falciparum* circumsporozoite antigen in anopheline mosquitoes collected in southern Thailand." Am J Trop Med Hyg **54**(2): 114-121.
- Reifenberg, J. M., D. Cuisance, et al. (1997). "Comparison of the susceptibility of different Glossina species to simple and mixed infections with *Trypanosoma (Nannomonas) congolense* savannah and riverine forest types." Med Vet Entomol **11**(3): 246-252.
- Reisen, W. (1989). "Estimation of vectorial capacity: relationship to disease transmission by malaria and arbovirus vectors." Bulletin of the Society of Vector Ecology **14**: 39-40.
- Richards, S. L., C. N. Mores, et al. (2007). "Impact of extrinsic incubation temperature and virus exposure on vector competence of *Culex pipiens quinquefasciatus* Say (Diptera: Culicidae) for West Nile virus." Vector Borne Zoonotic Dis **7**(4): 629-636.
- Sithiprasasna, R., K. J. Linthicum, et al. (2003). "Some entomological observations on temporal and spatial distribution of malaria vectors in three villages in northwestern Thailand using a geographic information system." Southeast Asian J Trop Med Public Health **34**(3): 505-516.
- Smith, D. L. and F. E. McKenzie (2004). "Statics and dynamics of malaria infection in *Anopheles* mosquitoes." Malar J **3**: 13.
- Thu, H. M., K. Lowry, et al. (2004). "Myanmar dengue outbreak associated with displacement of serotypes 2, 3, and 4 by dengue 1." Emerg Infect Dis **10**(4): 593-597.

- Tsetsarkin, K. A., D. L. Vanlandingham, et al. (2007). "A single mutation in chikungunya virus affects vector specificity and epidemic potential." PLoS Pathog **3**(12): e201.
- Turell, M. J., T. P. Gargan, 2nd, et al. (1984). "Replication and dissemination of Rift Valley fever virus in *Culex pipiens*." Am J Trop Med Hyg **33**(1): 176-181.
- Turell, M. J., C. N. Mores, et al. (2006). "Laboratory transmission of Japanese encephalitis and West Nile viruses by molestus form of *Culex pipiens* (Diptera: Culicidae) collected in Uzbekistan in 2004." J Med Entomol **43**(2): 296-300.
- Turell, M. J., C. N. Mores, et al. (2006). "Laboratory transmission of Japanese encephalitis, West Nile, and Getah viruses by mosquitoes (Diptera: Culicidae) collected near Camp Greaves, Gyeonggi Province, Republic of Korea 2003." J Med Entomol **43**(5): 1076-1081.
- Vaidyanathan, R. and T. W. Scott (2007). "Geographic variation in vector competence for West Nile virus in the *Culex pipiens* (Diptera: Culicidae) complex in California." Vector Borne Zoonotic Dis **7**(2): 193-198.
- van den Hurk, A. F., S. Hall-Mendelin, et al. (2009). "Vector Competence of Australian Mosquitoes for Chikungunya Virus." Vector Borne Zoonotic Dis.
- Walker, K. and M. Lynch (2007). "Contributions of *Anopheles* larval control to malaria suppression in tropical Africa: review of achievements and potential." Med Vet Entomol **21**(1): 2-21.
- Yasuno, M. and R. J. Tonn (1970). "A study of biting habits of *Aedes aegypti* in Bangkok, Thailand." Bull World Health Organ **43**(2): 319-325.

Chapter 4

Viral Phenotype May Explain Chikungunya Expansion Phenomenon

Introduction

Chikungunya virus (CHIKV) is a mosquito-borne Alphavirus found in tropical areas such as Southeast Asia, India, and parts of Africa. This virus has been responsible for periodic outbreaks of severe flu-like illnesses and can lead to lifelong arthralgia in some patients (Powers and Logue 2007). CHIKV has historically been associated with transmission by the mosquito *Aedes (Ae.) aegypti*, though *Ae. albopictus* is a fairly competent vector as well (Moutailler, Barre et al. 2009; Pesko, Westbrook et al. 2009; Talbalaghi, Moutailler et al. 2010; van den Hurk, Hall-Mendelin et al. 2010). An outbreak in Kenya began in 2004 that is believed to be the progenitor of the outbreaks on the Comoros and La Reunion islands. Phylogenetic analysis of six strains from the 2004-2006 outbreaks (Comoros, Kenya, La Reunion) and several other earlier strains show that with high bootstrap probability, the isolates from 2004-2006 outbreaks formed a distinct clade with the amino acid changes resulting in little phylogenetic distance (Kariuki Njenga, Nderitu et al. 2008).

Despite this relative genetic stability, an increase in transmission was noted especially in 2006 when an outbreak on La Reunion Island resulted in over 100,000 cases of CHIKV (Gerardin, Guernier et al. 2008; Staikowsky, Talarmin et al. 2009). Since there had been several reports that the primary vector, *Ae. Aegypti*, was absent from the La Reunion Island, and *Ae. albopictus* was determined to be the primary vector of the outbreak there (Bagny, Delatte et al. 2009). Further investigation into the role of this alternate vector during this outbreak revealed an amino acid substitution at position 226 of the envelope (E1) protein, changing an alanine to a valine. This substitution (E226V) was shown to enhance the efficiency of viral replication within the mosquito

species *Ae. albopictus* (Vazeille, Moutailler et al. 2007; Delatte, Dehecq et al. 2008; Delatte, Paupy et al. 2008). The substitution was not associated with an increased fitness in vertebrate cell lines or baby mice, further attributing the epidemic to fitness in the vector (Bordi, Meschi et al. 2011; Tsetsarkin, Chen et al. 2011).

Curiously, this substitution reportedly did not cause a synonymous fitness advantage in *Ae. aegypti* and perhaps was even deleterious in the mosquito at days 7 post exposure (Tsetsarkin, Vanlandingham et al. 2007). This would suggest that the substitution resulted in an advantage only in *Ae. albopictus* and thus, the increase in transmission on La Reunion Island was due in large part to the absence of *Ae. aegypti* (Vazeille, Moutailler et al. 2007; Delatte, Dehecq et al. 2008; Delatte, Paupy et al. 2008). However, the E226V strain continued to spread throughout Africa, into Sri Lanka, the Indian subcontinent, and Southeast Asia over several years where such an absence of *Ae. aegypti* does not occur (Schuffenecker, Iteman et al. 2006; de Lamballerie, Leroy et al. 2008; Hapuarachchi, Bandara et al. 2010).

Even more intriguing is the recent study that showed that endemic strains, even with the Alanine→Valine substitution introduced, did not confer the same fitness advantage in native Thailand *Ae. albopictus* colonies as seen in the outbreak (E226V) vs. pre-outbreak (E226A) strains in experimental colonies (Tsetsarkin, Chen et al. 2011). The authors of this study also suggest that evolutionary forces have favored fitness advances in *Ae. aegypti* in Southeast Asia where *Ae. albopictus* has coexisted for at least 60 years. They also suggest that the A→V mutation accumulation takes over 15 transmission cycles, which would indicate that the fitness advantage in *Ae. albopictus* is not necessary for sustained transmission (Tsetsarkin, Chen et al. 2011). Therefore, the role of *Ae. aegypti* is not only still relevant, but vital to understanding the expansion of this mutation into Southeast Asia.

Given the magnitude of the displacement by the post-substitution genotype (E226V), there must be at least some fitness advantage of that genotype in *Ae. aegypti* mosquitoes compared to the previously endemic Southeast Asian CHIKV. The first step in determining whether CHIKV transmission can be solely attributed to *Ae. albopictus* in this region is to investigate the vector competence of the La Reunion 2006 outbreak strain (LR06) relative to the previously endemic Southeast Asian strains.

Most comparisons of arboviral fitness are done with vector competence measures. However, this measure alone is not sufficient to facilitate fine scale and field relevant comparisons. For example, a virus strain that achieves no dissemination in the mosquito vector population at a certain day post exposure will be determined to be less fit than a strain that has at least some dissemination at that time point. Days later, however, the first strain may have achieved higher dissemination than the second, and the resulting conclusions are completely opposite. Further, if dissemination rates are ultimately higher for one strain, but the time it takes to reach that proportion is so long that most of the mosquito population cannot survive, then the fitness advantage is not as apparent as it would seem. Thus, the effective vector competence (EVC) measure is a more accurate assessment of viral fitness. Furthermore, the use of the displacement index (DI) shows the directionality of any fitness advantage (Christofferson and Mores 2011).

We hypothesized that some significant phenotypic differences must exist between CHIKV strains with the E226V substitution and those without the substitution in order for the E226V genotype to have penetrated Southeast Asia where *Ae. aegypti* is still reportedly the primary vector. To test this, we reanalyzed the previously published data regarding the dissemination rates of the outbreak and pre-outbreak strains from La Reunion Island in *Ae. aegypti* mosquitoes, using our newly developed formulation of effective vector competence, a cumulative measure of viral fitness

(Tsetsarkin, Vanlandingham et al. 2007). Further, we experimentally compared the outbreak strain with a Southeast Asian (SVO 476-96) strain of CHIKV assumed to be more like the endemic strains found in that region. The goal was to determine whether the E226V genotype had a fitness advantage over viral types more similar to those formerly endemic to Southeast Asian that would facilitate the displacement in the region independent of its perceived fitness differences from the La Reunion Island-specific pre-outbreak conditions.

Materials and Methods

Virus

For our experimental infections of chikungunya, two strains were utilized. Viruses were the generous gift of Dr. Robert Tesh, University of Texas Medical Branch, Galveston and Dr. Ann Powers, CDC, Fort Collin, CO, respectively. The La Reunion 2006 strain used in the experimental study herein is an unadapted strain isolated originally from a patient on La Reunion Island in 2006 (LR06). The Southeast Asian strain is a human isolate from Northeast Thailand (Phayao) in 1996 (SEA96). Each strain was propagated in Vero cells, through a single passage. Viral standard curves and concentrations were obtained via plaque assay as described previously (Christofferson and Mores 2011). Titers were verified throughout the experiment by qRT-PCR using standard Taqman conditions with the SuperScript III One-step qRT-PCR kit as per manufacturer's instructions (Invitrogen). Primer and probe sequences for each strain are given in table 4.1 (Reiskind, Pesko et al. 2008). Strains utilized in Tsetsarkin, et al. are full length, infectious clones with GFP tags (Tsetsarkin, Vanlandingham et al. 2007). The outbreak strain (E226V) contains the substitution strain with the alanine→valine substitution and the pre-substitution strain (E226A) was isolated from La Reunion prior to the outbreak.

Mosquitoes

Ae. aegypti (Linnaeus) Rockefeller strain mosquitoes from the colony at Louisiana State University School of Veterinary Medicine were exposed to an artificial bloodmeal 3-5 days post emergence with approximately 10^6 pfu/.1 ml of either a SEA96 or LR06. Mosquitoes were kept at constant environmental conditions: 16:8 light:dark cycle, 28°C and 75-80% humidity.

Mosquito Exposure and Detection of Disseminated Infection

Feeding occurred as previously described (Christofferson and Mores 2011). Fully engorged females were placed into clean, one-quart cartons and then sampled at days 3, 5, and 7 days post exposure (dpe) to test for dissemination status. Approximately 15-17 mosquitoes per time point per strain were sampled. It has been previously shown that detectable arbovirus in the legs is indicative of a fully disseminated infection; thus the salivary glands are presumed to be infected as well (Turell, Gargan et al. 1984). Mosquito legs were removed and analyzed separately for infection from the bodies as previously described (Alto, Lounibos et al. 2008; Reiskind, Pesko et al. 2008; Turell, Dohm et al. 2008; Pesko, Westbrook et al. 2009; Christofferson and Mores 2011). RNA was extracted using the MagMax-96 kit (Ambion) on a King Fisher® nucleic acid extraction instrument according to the manufacturer's instructions (Thermo Scientific). After extraction, the samples were tested for the presence of chikungunya viral RNA via qRT-PCR using the following protocol on the Roche LightCycler480: RT step (1 cycle) 48°C for 2 minutes, 95°C for 5 minutes; amplification and data recording step (40 cycles) 95°C 15 seconds, 60°C 30 seconds.

Data Analysis

Vector competence was calculated as the number of disseminated infections divided by the total number of mosquitoes which fed to repletion (number exposed), cVC and DI values were

calculated as in (Christofferson and Mores 2011). All analyses and modeling was performed in SAS 9.13 (Cary, NC). EVC values are determined as part of the cumulative vectorial capacity (cVC) measurement. It is a cumulative measure of vector competence over a range of days, bounded and weighted by the mosquito lifespan. Briefly, define the rate of change of vector competence over days as $b_i(N)$:

$$b_i(N) = \beta_0 + \beta_1 N \quad \text{Equation (Eq.) 4.1}$$

where β_1 = rate of change of vector competence over time. EVC, represented by the Greek letter phi (ϕ) is then given by:

$$\phi = \int p^N b_i(N) dN \quad \text{Eq. 4.2}$$

where N is the extrinsic incubation period, here a range of days defined by the research; $b_i(N)$ is as above; and p = probability of daily survival. This is held constant so as to isolate the viral phenotypes and assess relative fitness (Christofferson and Mores 2011). The EVC value can be plugged into the cVC equation but EVC gives a measure of viral fitness while entomological and vertebrate density parameters are held constant and thus not included in EVC.

To determine fitness advantages between E226V and E226A, La Reunion data from Testsarkin, et al. was used and EVC obtained (Tsetsarkin, Vanlandingham et al. 2007). The same calculations were used for our experimental data.

Results

Reanalysis of Tsetsarkin, et al. E226V Strain vs. E226A in *Ae. aegypti*.

Dissemination rates of the two strains, E226V and E226A, were similar at each time point sampled but E226V had an initially higher dissemination rate as well as a steeper rate of change over

the first few time points. Dissemination rates, calculated EVC values, and the 95% lower and upper confidence limits for the test of difference are given in table 4.2 (Tsetsarkin, Vanlandingham et al. 2007). Though traditional day-by-day comparisons did not detect any statistical significance in the fitness of E226V in *Ae. aegypti* mosquitoes, the EVC estimate did. The difference of the two EVC calculations was significant at the $p=.05$ level. Using a t-test of the bootstrapped values, a significant difference between the two strains within *Ae. aegypti* mosquitoes was detected at the $p<.05$ level. Surprisingly, the results show that the substitution in E226V actually conferred a slight but statistically significant disadvantage in *Ae. aegypti*. Compared using the displacement index (DI), a measure of relative fitness. The E226A strain was 1.17 times as fit as the E226V strain when calculated based on the original Tsetsarkin 2007 data.

Experimental Comparison of LR06 and SE96

Dissemination rates and EVC for the SEA96 and LR06 strains are given in table 4.3. The LR06 strain had not only an earlier start of dissemination but ultimately reached three times the dissemination in *Ae. aegypti* compared to SEA96. Thus, the EVC calculation and bootstrapped test showed that LR06 had a statistically significant advantage in *Ae.aegypti* ($p<.05$). The overall advantage is again best reported as relative fitness. The DI value reported here is calculated as the ratio of fitness SEA96:LR06 and is .4. The reciprocal of this gives the level of fitness of LR06 compared to SEA96. Thus, LR06 is 2.5 times more fit than SEA96 in *Ae. aegypti*.

Discussion

Here we show that the La Reunion strains, E226V and E226A, differ significantly as judged by EVC in *Ae. aegypti*, while previous studies showed no significant difference. We concluded that the E226V mutation conferred a statistically significant disadvantage in *Ae. aegypti* compared to the

pre-outbreak La Reunion strain (E226A), based on reanalysis of those data (Tsetsarkin, Vanlandingham et al. 2007). The magnitude of this difference is further illustrated by the displacement index.

$$DI = \frac{cVC_i}{cVC_{REF}} \quad \text{Eq. 4.3}$$

With a DI of 1.17 indicating that the E226A strain is 1.17 times more fit than E226V, it would be expected that E226V would not displace the E226A strain in areas where *Ae. aegypti* would likely be the primary vector. (Figure 4.1) However, this has not been the case as this mutant has become the predominant strain detected (Schuffenecker, Itean et al. 2006; de Lamballerie, Leroy et al. 2008; Hapuarachchi, Bandara et al. 2010). Since this mutation nonetheless expanded into Southeast Asia where *Ae. aegypti* is still predominant, we hypothesized that some advantage must exist in *Ae. aegypti* outside of the focus of La Reunion Island. Thus, we investigated the relative fitness of the LR06 strain with a Southeast Asian strain of CHIKV that did not have the Ala→Val mutation. We found that the SEA96 strain without the substitution did have a substantial fitness disadvantage compared to the LR06 strain, with a DI of .4. Thus, there is a sufficient phenotypic advantage of this mutation in *Ae. aegypti* compared to the endemic, resident strain to allow for displacement of that endemic strain. Since the E226A/E226V data is a reanalysis and the SEA96 comparison is from our experimental efforts, direct comparisons of these two values should be made carefully as laboratory conditions and methods of virus detection varied. More investigation of the phenotypic relationship of this SEA96 strain to the pre-outbreak La Reunion strain is needed to fully correlate these results to the spread of the newly emerged strain across India and Southeast Asia. The expectation is that the pre-outbreak strain found on the island will be quite a bit more fit than the SEA96 strain.

Table 4.1: Primers and probe. The sequences for the primers and probe used to amplify CHIKV are given. All sequences are 5'→3'.

CHIKV E1	Forward Primer	Reverse Primer	Probe
	ACC CGG TAA GAG CGA TGA ACT	ACG CCG CAT CCG GTA TGT	CCG TAG GGA ACA TGC CCA TCT CCA

Table 4.2: Dissemination rates. Dissemination rates from Tsetsarkin, et al. and the calculated EVC rates with the 95% confidence limits for the test of difference between LR06 and Pre-LR strains.

Strain	P	Dissemination Rates in <i>Aedes aegypti</i> from						EVC	LCL of difference in EVC	UCL of difference in EVC
		1 dpe	3 dpe	5 dpe	7 dpe	9 dpe	12 dpe			
E226A	.91	0	0.15742	0.58279	0.89679	0.94816	1	2.92	-0.8364	-0.2729
E226V		0	0.39352	0.68547	0.74121	0.95084	0.945	2.49		

Southeast Asian strain vs. E226V in orally exposed Ae. aegypti.

Table 4.3: Dissemination rates of experimentally exposed mosquitoes. Dissemination rates of LR06 strain and the Southeast Asian strain in *Ae. aegypti* mosquitoes. The calculated EVC values and the 95% confidence limits for the test of difference between LR06 and 96 strains.

Strain	P	Dissemination Rates			EVC	LCL of difference in EVC	UCL of difference in EVC
		3 dpe	5 dpe	7 dpe			
LR06	.91	.0667	.4	.6875	0.95188	-0.58633	-0.075742
SEA96		0	.0625	.4375	0.38404		

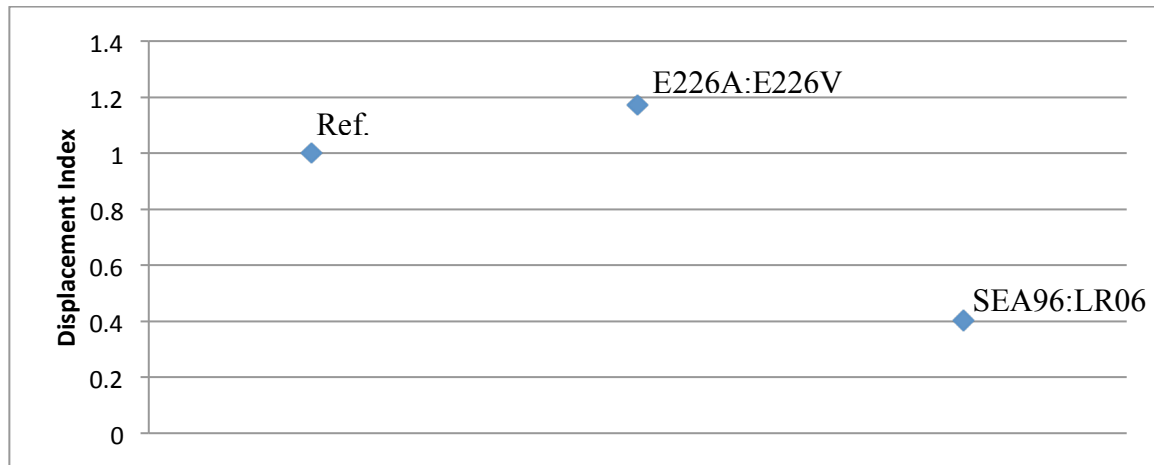


Figure 4.1: Displacement index characterizing relative fitness. The reference point is set a 1 as La Reunion 2006 compared to itself. The E226A:E226V point represents the relative EVC values of the pre-La Reunion strain to the mutant La Reunion strain. SEA96:LR06 shows the DI resulting from the experimental derivation of EVC of the Southeast Asian 1996 strain to the La Reunion 2006 strain.

We show that EVC values can be used to more accurately assess fitness as it can detect significant differences in fitness, likely due to the interaction with mosquito mortality rates. It is important to understand that EVC comparisons should be made in a single vector species with the assumption that either the viral strains compared, occur in the same population of vectors (i.e. holding daily survival constant is field relevant) or that differences in the probability of daily survival across vector populations are negligible.

Specifically, we report that the EVC more accurately captures fitness advantages conferred by the alanine to valine substitution at position 226 on the CHIKV E1 protein. We also use the DI to explore the relationship among phenotypes of CHIKV strains. Not only does the DI accurately direct the fitness advantage of one strain over the other, but it suggests that the phenotypic and phylogenetic relationships between the pre-substitution La Reunion strain (E226A) and the Southeast Asian strain are not sufficiently characterized to explain the spread of this mutation across the region, given the gap between the two in fitness. Both the EVC estimates and DI point towards the need for further investigation of the E226V strain in *Ae. aegypti* populations, as well as the need for further evaluation of the mixing of two equally as permissive vectors, *Ae. aegypti* and *Ae. albopictus*. This illustrates that the relative *in vivo*, within-vector phenotype can aide in further explaining an expansion phenomenon once genetic analysis has been exhausted.

References

- Alto, B. W., L. P. Lounibos, et al. (2008). "Larval competition alters susceptibility of adult *Aedes* mosquitoes to dengue infection." Proc Biol Sci **275**(1633): 463-471.
- Bagny, L., H. Delatte, et al. (2009). "Progressive decrease in *Aedes aegypti* distribution in Reunion Island since the 1900s." J Med Entomol **46**(6): 1541-1545.

- Bordi, L., S. Meschi, et al. (2011). "Chikungunya virus isolates with/without A226V mutation show different sensitivity to IFN- α , but similar replication kinetics in non human primate cells." New Microbiol **34**(1): 87-91.
- Christofferson, R. C. and C. N. Mores (2011). "Estimating the magnitude and direction of altered arbovirus transmission due to viral phenotype." PLoS ONE **6**(1): e16298.
- de Lamballerie, X., E. Leroy, et al. (2008). "Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come?" Virology **5**: 33.
- Delatte, H., J. S. Dehecq, et al. (2008). "Geographic distribution and developmental sites of *Aedes albopictus* (Diptera: Culicidae) during a Chikungunya epidemic event." Vector Borne Zoonotic Dis **8**(1): 25-34.
- Delatte, H., C. Paupy, et al. (2008). "[*Aedes albopictus*, vector of chikungunya and dengue viruses in Reunion Island: biology and control]." Parasite **15**(1): 3-13.
- Gerardin, P., V. Guernier, et al. (2008). "Estimating Chikungunya prevalence in La Reunion Island outbreak by serosurveys: two methods for two critical times of the epidemic." BMC Infect Dis **8**: 99.
- Hapuarachchi, H. C., K. B. Bandara, et al. (2010). "Re-emergence of Chikungunya virus in South-east Asia: virological evidence from Sri Lanka and Singapore." J Gen Virol **91**(Pt 4): 1067-1076.
- Kariuki Njenga, M., L. Nderitu, et al. (2008). "Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa." J Gen Virol **89**(Pt 11): 2754-2760.
- Moutailler, S., H. Barre, et al. (2009). "Recently introduced *Aedes albopictus* in Corsica is competent to Chikungunya virus and in a lesser extent to dengue virus." Trop Med Int Health **14**(9): 1105-1109.
- Pesko, K., C. J. Westbrook, et al. (2009). "Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to chikungunya virus." J Med Entomol **46**(2): 395-399.
- Powers, A. M. and C. H. Logue (2007). "Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus." J Gen Virol **88**(Pt 9): 2363-2377.
- Reiskind, M. H., K. Pesko, et al. (2008). "Susceptibility of Florida mosquitoes to infection with chikungunya virus." Am J Trop Med Hyg **78**(3): 422-425.
- Schuffenecker, I., I. Itean, et al. (2006). "Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak." PLoS Med **3**(7): e263.
- Staikowsky, F., F. Talarmin, et al. (2009). "Prospective study of Chikungunya virus acute infection in the Island of La Reunion during the 2005-2006 outbreak." PLoS ONE **4**(10): e7603.

- Talbalaghi, A., S. Moutailler, et al. (2010). "Are *Aedes albopictus* or other mosquito species from northern Italy competent to sustain new arboviral outbreaks?" Med Vet Entomol **24**(1): 83-87.
- Tsetsarkin, K. A., R. Chen, et al. (2011). "Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes." Proc Natl Acad Sci U S A **108**(19): 7872-7877.
- Tsetsarkin, K. A., D. L. Vanlandingham, et al. (2007). "A single mutation in chikungunya virus affects vector specificity and epidemic potential." PLoS Pathog **3**(12): e201.
- Turell, M. J., D. J. Dohm, et al. (2008). "Potential for North American mosquitoes to transmit Rift Valley fever virus." J Am Mosq Control Assoc **24**(4): 502-507.
- Turell, M. J., T. P. Gargan, 2nd, et al. (1984). "Replication and dissemination of Rift Valley fever virus in *Culex pipiens*." Am J Trop Med Hyg **33**(1): 176-181.
- van den Hurk, A. F., S. Hall-Mendelin, et al. (2010). "Vector competence of Australian mosquitoes for chikungunya virus." Vector Borne Zoonotic Dis **10**(5): 489-495.
- Vazeille, M., S. Moutailler, et al. (2007). "Two Chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*." PLoS ONE **2**(11): e1168.

Chapter 5

The Role of Interferon Responses in Dengue Transmission

Introduction

Dengue virus (DENV) is one of the most common arboviral infections, especially in tropical areas affecting millions of people annually (2009). There is presently no vaccine available for DENV, and control of the virus via mosquito abatement has not been successful in permanently eradicating the virus (Teixeira, Costa Mda et al. 2009). Understanding the transmission and factors that could potentially mitigate transmission of this virus are vital to controlling and understanding outbreaks. Modeling of DENV infections and transmission *in vivo* has been complicated by the difficulty of the virus to successfully establish infections in immunocompetent mice. Recently there have been successful studies involving several knockout mice, particularly the AG129 model, deficient in both type I and type II interferon receptors. These models have most often been used to model dengue disease and pathogenesis, especially the severe manifestations (Shresta, Kyle et al. 2004; Shresta, Kyle et al. 2004; Kyle, Beatty et al. 2007; Kyle, Balsitis et al. 2008; Yauch and Shresta 2008; Mota and Rico-Hesse 2009; Balsitis, Williams et al. 2010; Casseti, Durbin et al. 2010). Problems with these currently utilized mouse models include: 1) viremia levels are too transient or do not reach levels that adequately represent DENV infections in humans, 2) unnaturally high viral titers or lab adapted virus strains are necessary to establish infection and/or 3) routes of exposure do not resemble natural routes (i.e. intravenous or intracranial inoculations) (Johnson and Roehrig 1999; Chen, Lai et al. 2004; Shresta, Sharar et al. 2006; Chen, Hofman et al. 2007; Schul, Liu et al. 2007; Williams, Zompi et al. 2009).

In vitro studies have shown that dengue virus inhibits type I interferon production, indicating that this measure of immune response subversion is critical for dengue fitness and thus affects

transmission (Rodriguez-Madoz, Bernal-Rubio et al. 2010). Further, others have shown that downstream protein expression induced by type I interferon and the JAK/STAT pathway play important roles in dengue inhibition (Liu, Woda et al. 2009; Jiang, Weidner et al. 2010; Munoz-Jordan 2010). Much attention has been given to the JAK/STAT pathway, and it has been suggested that dengue subversion of the type I interferon response occurs only after this pathway has been initiated (Mazzon, Jones et al. 2009; Munoz-Jordan 2010). The NS5 and NS3 genes of the dengue virus has been shown to act as regulators of the JAK/STAT subversion, but these proteins have no direct affect on the type II interferon response (Mazzon, Jones et al. 2009).

In vivo studies have also shown the importance of the type I interferon and JAK/STAT pathways. Importantly, studies have established the crucial role of type I interferon in early clearance of dengue, though none have sought to explore infection establishment (Shresta, Sharar et al. 2005). Studies in the AG129 mouse and many important discoveries have been facilitated by the use of this mouse. However, with the additional deficiency in IFN-gamma, it is impossible to discern if the two interferon responses act in concert to inhibit infection establishment or if one or the other is responsible for establishment inhibition, though some have indicated both types I and II interferon are necessary for disease progression (Johnson and Roehrig 1999; Shresta, Kyle et al. 2004; Shresta, Sharar et al. 2005; Shresta, Sharar et al. 2006).

When a virus infiltrates a cell via endocytosis, the RIG-1 pathway is stimulated by the presence of single stranded RNA, and MDA5 and endosomal TLR3 recognize double stranded RNA. These pathways signal through the interferon regulatory factors (IRF) 3 and 7, which sets off a cascade of signals resulting in the transcription of type I interferon (IFN- α/β). Figure 5.1 demonstrates the importance of IRF3 and IRF7 in the type I interferon pathway. Briefly, through the activation of these pathways, NF κ B, IRF-3, and low-level, constitutively produced IRF-7 localize to the nucleuse to promote transcription of IFN- β . IFN- β binds the type I interferon receptor (IFNAR)

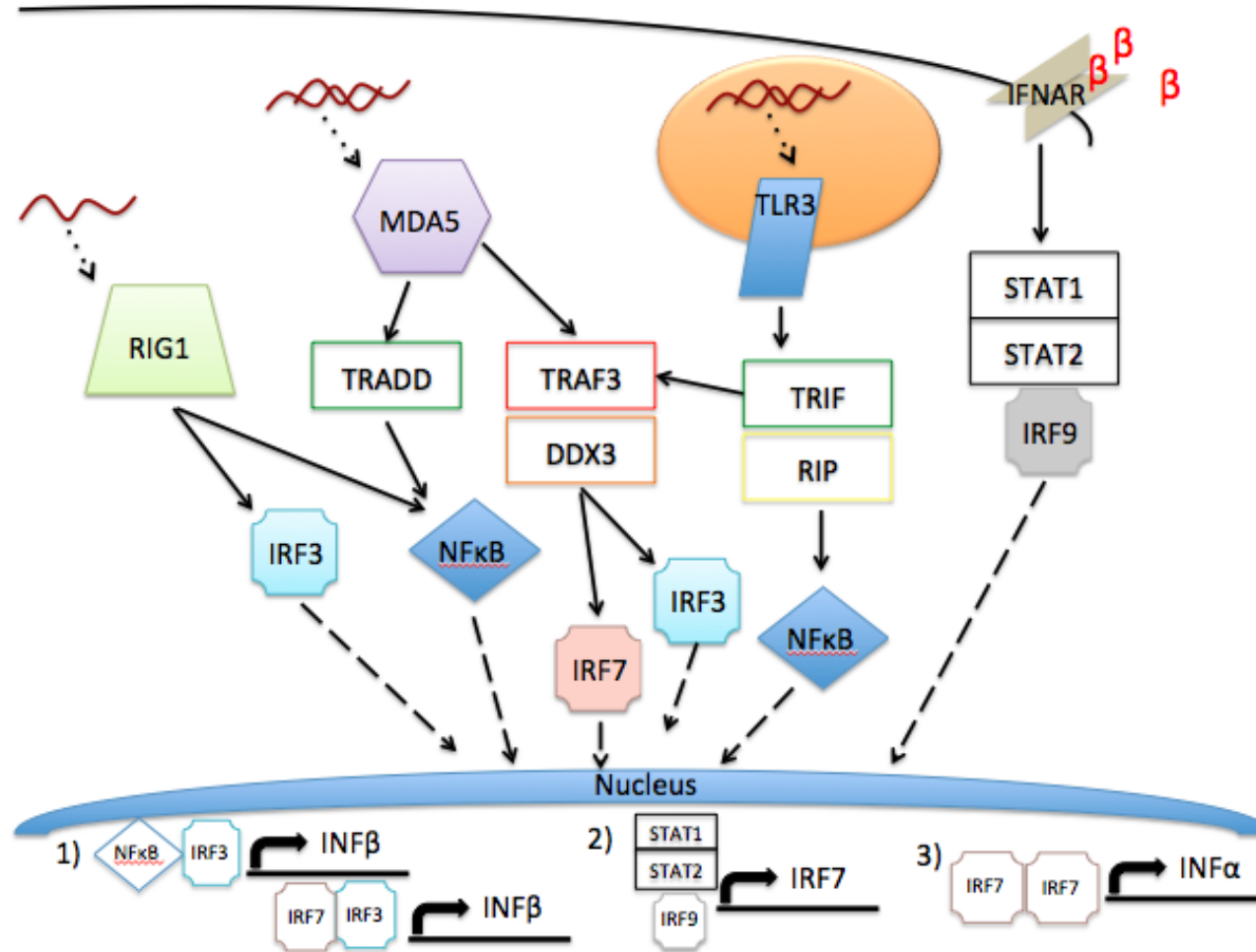


Figure 5.1: Type I interferon production signals through IRF 3 and IRF7. Single stranded RNA is recognized by RIG1 in the cytoplasm and double stranded RNA is recognized cytoplasmically by MDA5 and in the endosome by TLR3. The resulting signal cascades all utilize the interferon regulatory factors (IRF) 3 and 7 to first promote transcription of IFN- β , which positively feeds back via STAT1/STAT2/IRF9 to promote transcription of IRF7, which promotes transcription of IFN- α .

which leads to the localization of the STAT1/STAT2/IRF9 complex to the nucleus where IRF7 transcription is promoted. Upregulated production of IRF7 leads to increased localization of IRF7 dimers to the nucleus where the production of IFN- α is promoted. IFN α and β are responsible for the establishment and maintenance of the anti-viral state in the immune response (Katze, He et al. 2002; Haller, Kochs et al. 2006).

We hypothesized that type I interferon only, and not type II, was critical for the inhibition of dengue virus infection establishment (transmission). We tested this hypothesis using an IRF3/7 double knockout (DKO) strain of C57BL/6 mice. Given that mosquito saliva has been shown to alter infection kinetics in arbovirus infections, we further evaluated this hypothesis via a natural transmission route to determine whether the relative importance of types I and II interferon is changed by exposure route (Limesand, Higgs et al. 2003; Schneider and Higgs 2008; Thangamani, Higgs et al. 2010; Styer, Lim et al. 2011).

Materials and Methods

Mice

Mice were the generous gift of Drs. T. Taniguchi and M. Diamond. IRF 3/7 ^{-/- -/-} double knockout (DKO) mice are deficient in interferon regulatory factors 3 and 7, and thus have a significantly abrogated type I interferon. The type II interferon and all other immune responses are intact though blunted (Daffis, Suthar et al. 2009). Intact C57BL/6 mice were obtained from the Louisiana State University School of Veterinary Medicine Division of Laboratory Animal Medicine and were used as controls. All experiments met the approval and conditions of the LSU Institutional Animal Care and Use Committee (approved protocol # 09-077).

Virus

Three strains of dengue virus were used: two strains of dengue 2 (strain 1232 isolated from a patient in Indonesia, strain 16803 isolated from a patient in Thailand) and one strain of D4 (strain LN 634441 isolated from a patient in Malaysia). Virus was propagated as in (Alto, Lounibos et al. 2008) before inoculation into mice. Before the experiment, virus was titered via plaque assay and experimental titers were confirmed at 10^6 pfu/ml by qRT-PCR as in (Christofferson and Mores 2011). Since all titers were matched, no dilution was performed.

Virus Inoculation and Serum Collection

Mice were anesthetized with isoflurane and then injected subcutaneously with 100 μ l of supernatant from the virus inoculated cell culture. The total inoculum into each mouse was 10^5 pfu/mouse. Intact C57/Bl6 controls were inoculated with 10^5 pfu/mouse D2 16803 in the same manner as the other mice. Each treatment group consisted of 4-6 mice. Mice were then bled each day for five days via submandibular bleeding technique (Golde, Gollobin et al. 2005). Blood was allowed to clot for thirty minutes on bench top, and then centrifuged at 6 rcf for five minutes. Clarified serum was collected and placed into a clean tube for analysis. Though there were days when some mice did not bleed enough for a sample analysis, no less than three mice were analyzed on a given day.

Virus Detection

Viral RNA was extracted using the MagMax 95 kit (Ambion) or QIamp Viral RNA kit (Quiagen). Where necessary, serum samples were brought to volume with BA1 buffer and kits were run as per manufacturer's instructions. Detection of viral RNA was done using a One Step Taqman qRT-PCR assay (SuperScript III). Primers and probes are specific to a single serotype of dengue and

we have observed no cross reactivity among them (Christofferson and Mores 2011). In addition, all assays have been tested for their sensitivity and each has detected less than 1 pfu/ml.

Mosquito Exposure for Transmission to DKO Mice

Mosquitoes were offered an infectious blood meal 3-5 days post emergence with an infectious titer of 10^6 pfu/ml. The blood meal consisted of bovine blood in Alsevier's anticoagulant (Hemostat, Dixon, CA) mixed 2:1 with a virus solution in a total volume of approximately 3 ml per carton, heated to 37°C and kept warm via the Hemotek device (Discovery Workshops, Arrington, Lancashire, UK). Mosquitoes were allowed to feed for 45 minutes before the blood meal was removed. Each carton consisted of 100 mosquitoes and 7 cartons were fed for this study. Mosquitoes were then sorted and only fully engorged females were kept; all others were discarded.

Approximately 50% of mosquitoes were females, and there was approximately 100% successful feeding. Thus, approximately 350 engorged females were identified by the presence of red blood in the abdomen, visible with the naked eye. Seven mice were anesthetized with isoflurine and placed on top of each carton of dengue 2 (strain 1232) exposed mosquitoes. (Thus, each mouse was exposed to approximately 50 potentially infectious mosquitoes.) Mosquitoes were then tested for presence of virus in the legs as a proxy for disseminated infection. Testing was done via qRT-PCR on the Roche LightCycler 480 as previously described (Christofferson and Mores 2011).

Transmission from Viremic Mice to Mosquito

Mice were inoculated with 10^5 pfu/mouse D2 1232 via needle inoculation as above. (n=3) Every day post exposure, mice were anesthetized and placed on top of cartons of naïve mosquitoes approximately 3-4 days post emergence. Mosquitoes were allowed to feed for approximately 15-20 minutes and then the mice were removed. Engorged females were separated and placed in clean

cartons and kept at constant environmental conditions (16:8 hours light:dark, 28°C, 70-80% humidity). Whole mosquito bodies were tested for the presence of virus 3 days post exposure to determine infection (not dissemination) status.

Cytokine Measurement and Statistical Analysis

IFN- γ , TNF- α , IL-4 and IL-1 β were measured using the Millipore Milliplex MAP Mouse Cytokine/Chemokine kit as per manufacturer's instructions (Millipore, Billerica, MA). Viremia and cytokine levels were analyzed using a mixed linear model with repeated measures. All analyses were performed in SAS 9.13 (Carey, NC). Significance was detected at the $\alpha=.05$ level.

Results

Successful Transmission in IRF3/7 DKO by Subcutaneous Needle Inoculation

Transmission of virus via subcutaneous needle inoculation was successful in 100% of the DKO mice while no viremia was detected in the control mice challenged with D2 16803. Viremia levels were centered on the day of peak viremia (P0). Days before and after peak viremia were labeled relative to peak viremia: two days before peak day (P-2), the day before (P-1), peak day (P0), the day after peak (P1) and where relevant, two days after peak (P2). Mean viremia levels for each viremia day was calculated for each treatment group. Since no viremia was detected, the first day post-exposure was considered the day after peak viremia day.

There were significant differences in viremia intensity detected among all three strains of dengue. Mice infected with D2 1232 had the highest viremia at all time points followed by D2 16803 and lastly, D4 LN 634441. In addition, mice infected with D2 1232 had detectable viremia for four days while mice infected with either D2 16803 or D4 LN 634441 had only two days of viremia (Figure 5.2). The ability to achieve 100% infection using natural levels of virus inoculum from

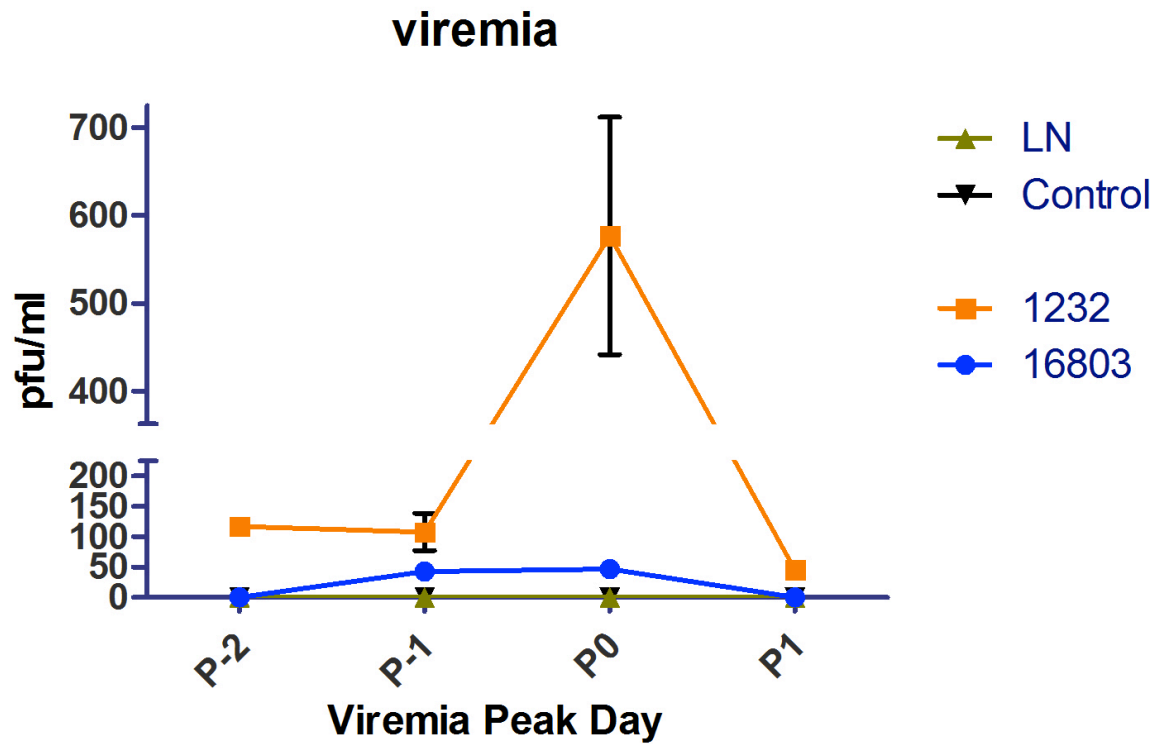


Figure 5.2: Mean viremia levels of dengue infections. Mean viremia levels are centered around viremia peak day (peak = P0) with the control mice beginning at P1 due to lack of viremia.

three unadapted dengue strains of two serotypes is an important step towards studying transmission of dengue, and is a first in dengue transmission research.

The type II interferon response to these dengue strains was investigated to determine whether any correlation existed between successful transmission. Across all dengue strains, INF- γ peaked on peak viremia day (P0), though no significant differences were observed between this and the day after viremia peak day across treatments. There was a statistical difference in INF- γ levels between D2 16803 and the other two strains, while D2 1232 and D4 634441 differed only at time points P-2 and P0. Since the controls do not have times points before P1, only that day was considered and it was statistically different from all three strains (Figure 5.3). Consistently, two days after peak INF- γ levels, viremia was either cleared (D2 16803, D4 LN) or drastically reduced (D2 1232).

The most robust INF- γ response was noted in mice infected with D2 16803. These levels peaked on P1 with levels over 200 times more than the controls on that day. There is a lack of direct correlation between relative viremia intensity and the INF- γ response. That is, the highest levels of viremia did not elicit the highest or the lowest INF- γ response and vice versa.

The TNF- α responses across dengue infected DKO mice did not differ except on day P1 where D2 16803 appeared to be significantly higher than the other groups. Again, only P1 data is available for the control mice, but it does not appear that controls differed from D2 1232 or D4 LN634441 infected mice (Figure 5.4). IL-1 β did not differ across any times points for any treatments and was largely undetectable.

Though the controls did not have detectable viremias or significant INF- γ or TNF- α responses, they did produce significantly higher levels of IL-4 at time points P1-P4 (Figure 5.5). Because of the sampling regime this could be due to true differences in the reaction of controls versus DKO mice, or it could be that the IL-4 response in the DKO mice was not detected since we did not sample those mice that many days post infection. Additionally, the variability among the

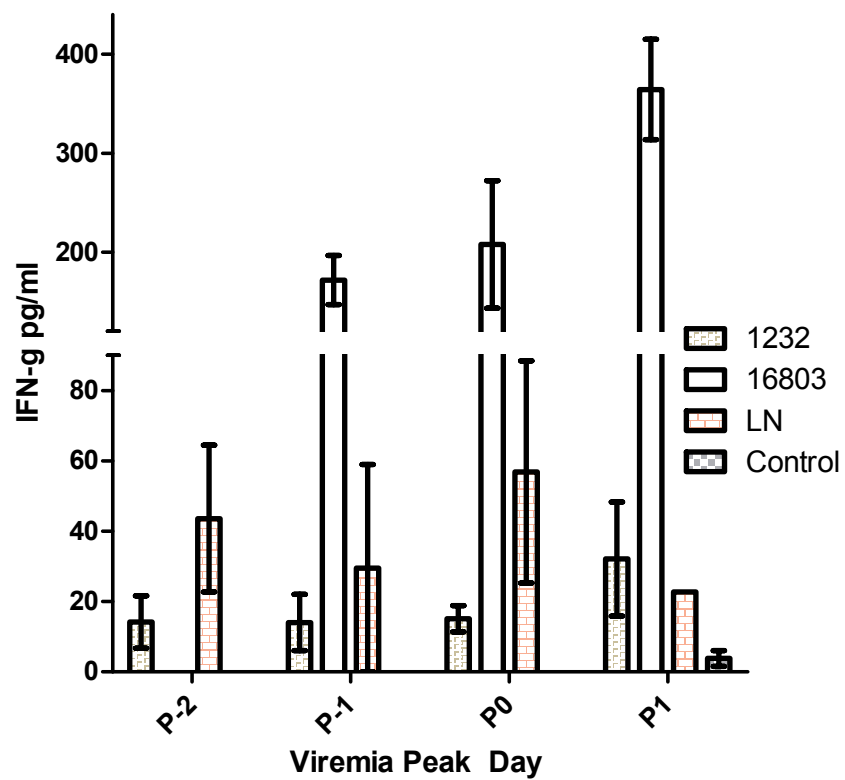


Figure 5.3: IFN- γ response of infected and control mice. D2 16803 infected mice elicited the highest levels of IFN- γ while the control mice had a slight, though significant response on P1.

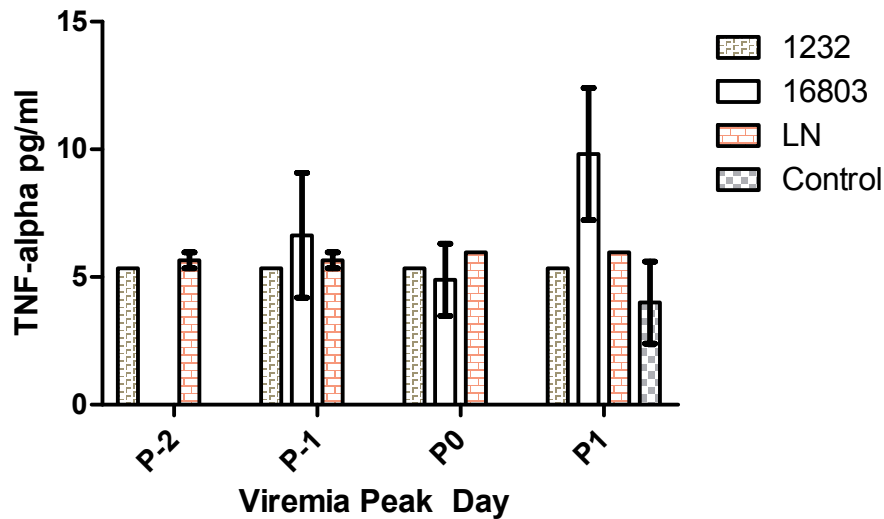


Figure 5.4: TNF- α response in dengue infected and control mice. The TNF- α response was not significantly different over most time points, but on P1 D2 16803 did differ from the controls and from the other two strains. Error bars cannot be seen for some of the time points because the standard errors were so small.

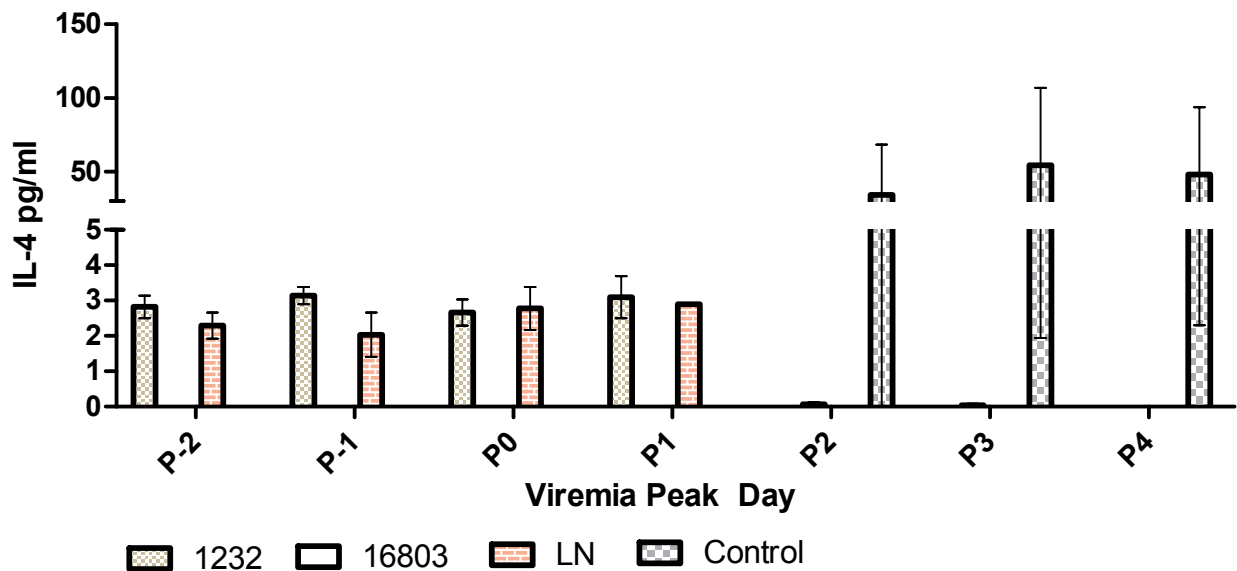


Figure 5.5: IL-4 responses in dengue infected and control mice. The IL-4 response of D2 1232 and D4 LN 634441 did not differ significantly, except for on P-1. D2 16803 did not elicit an IL-4 response during the sampled time points. The control mice had a comparatively higher IL-4 response at later time points, though this could be because of how we assigned time post peak viremia and did not sample out far enough in the DKO infected mice.

control mice constrain this analysis somewhat, though further investigation to specifically compare IL-4 to DKO would be interesting.

Mosquito versus Needle Transmission

To discern whether the roles of type I and type II interferon in dengue transmission is different when mice are exposed via infected mosquitoes, we inoculated seven mice by infected mosquito with D2 1232. Of the 7 mice exposed to infectious mosquitoes, 100% developed viremia. The day of peak viremia and the viremia titer on those days was not dependent on the number of infectious mosquitoes to which the mice were exposed ($p > .05$). The day of onset of viremia, however, was dependent on the number of infectious mosquitoes transmitting virus ($p < .05$). The average peak viremia was 7.14×10^2 pfu/ml (min. 3.0×10^1 pfu/ml, max. 2.15×10^3 pfu/ml). These results are summarized in Table 5.1.

Viremia levels in mosquito inoculated mice were statistically higher than those of the needle inoculated mice at all times except peak viremia (P0) (Figure 5.6). In addition, mosquito inoculated mice had detectable viremia for five days as compared to the needle inoculated mice, which had only four days detectable viremia. This also indicated that viremia peaked on average one day later post-exposure, though this was affected by the number of inoculating mosquitoes, as discussed above. As in the needle inoculated mice, IFN- γ production rose significantly on P1, and this day represented the only statistical difference between the groups in IFN- γ response (Figure 5.7). Between the needle and mosquito inoculated mice, there was no significant difference in either the IL-4 or the TNF- α response.

Table 5.1: Summary of transmission from mosquito to IRF DKO mice. *Indicates peak day for mouse 270 based on our obtained data, but no data was available for 4 dpi for this mouse.

Mouse Number	No. of Infectious Mosquitoes	Viremia				Peak Day
		1 dpi	2 dpi	3 dpi	4 dpi	
251	6	0	1.99	1.94e2	1.060e3	4
252	5	0	1.5e1	5.98e1	6.86e2	4
253	1	0	0	4.12	3.0e1	4
267	2	0	0	657	3.15e2	3
268	9	0	3.3	2.58e1	2.78e2	4
269	5	0	9.82	1.58e2	2.150e3	4
270	11	0	3.48	1.36e2	n/a	*3

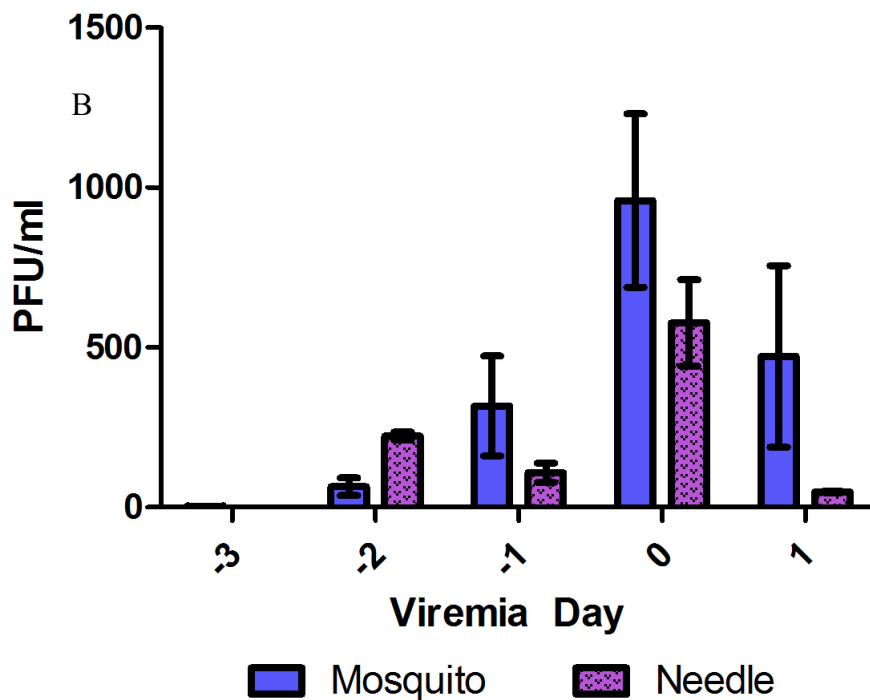


Figure 5.6: Viremia levels of mosquito vs. needle inoculated DKO mice. Viremia in mosquito inoculated mice was detectable for one day more than needle inoculated mice (5 days vs. 4 days, respectively). Viremia levels between the two treatment groups were statistically different at all times points except peak viremia day, P0.

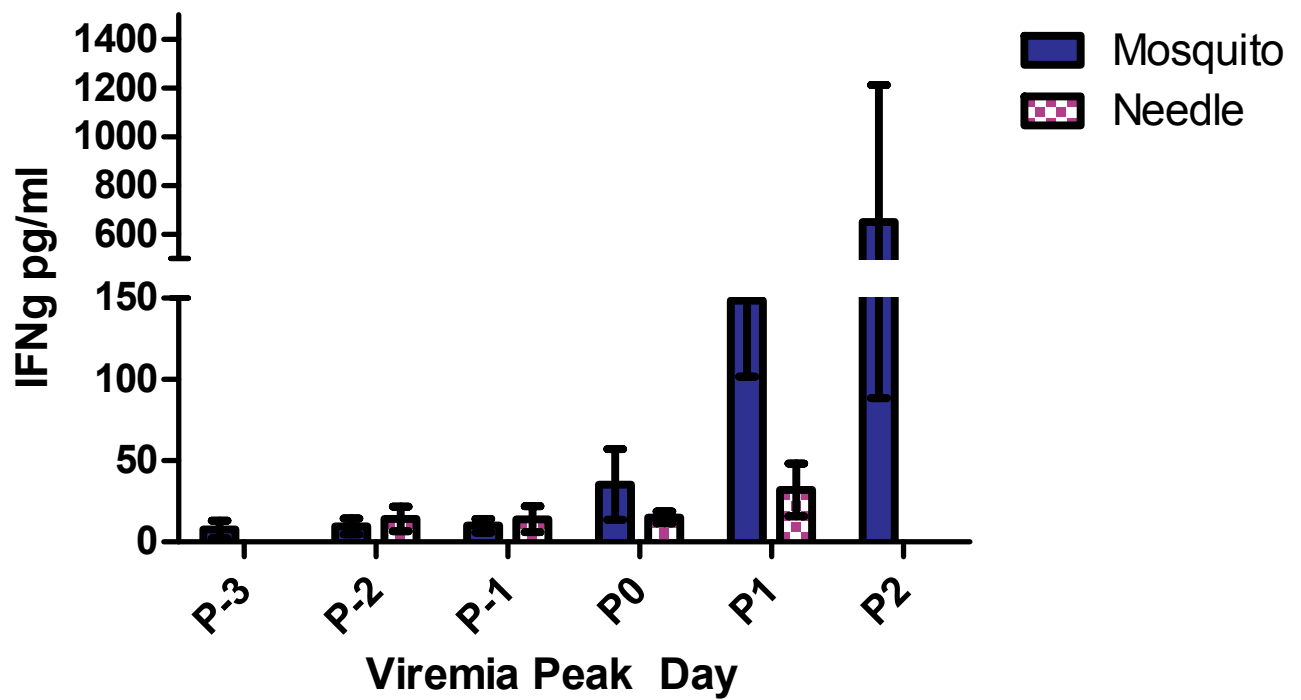


Figure 5.7: IFN- γ response in needle inoculated vs. mosquito inoculated infections. The IFN- γ response differed significantly on days P1 of infection and an additional day of IFN- γ was detected (P2) in mosquito inoculated mice.

IRF3/7^{-/-} DKO Mouse Supports Transmission to Naïve Mosquitoes

Viremic mice supported acquisition of virus by naïve mosquitoes on days P-1 and P0. Two of the three mice on day P-1 supported acquisition and on P0, again two mice supported acquisition, though only one mice supported acquisition on both days. Acquisition percentages were 7.14% (2 positive /28 total) and 11.1% (5/45) for days P-1 and P0, respectively. Results can be seen in Figure 5.8.

Discussion

We confirmed that this mouse is a successful and novel model of dengue transmission. It has the ability to support non-adapted dengue strains of at least 2 serotypes and can be used to test for differences in infection kinetics in a vertebrate host. In addition, it can be used to study the natural routes of transmission: infected mosquitoes. Importantly, we determined that the absence of the type I interferon is critical for establishment of dengue infection and thus a successful transmission event. Type II interferon, while necessary for viral clearance, is apparently not responsible for the inhibition of dengue infection establishment. In other words, given that transmission was 100% blocked in mice with an intact type I interferon response, and that when this response was abrogated, 100% infection was achieved. We conclude that type I interferon response is the critical response mitigating successful transmission of dengue virus. Also, given that in the control mice no viremia was detected and only a weak, limited IFN- γ response was elicited, and given that in the DKO mice the IFN- γ response was so temporally associated with cessation of viremia, we conclude that type II interferon response is necessary for and sufficient to clear dengue infection.

In addition, we determined that while mosquito salivary secretions have been known to alter the interferon responses, it does not change the relative importance of the systemic type I versus type II response. That is, type II still did not play a role in the initial establishment of infection while the

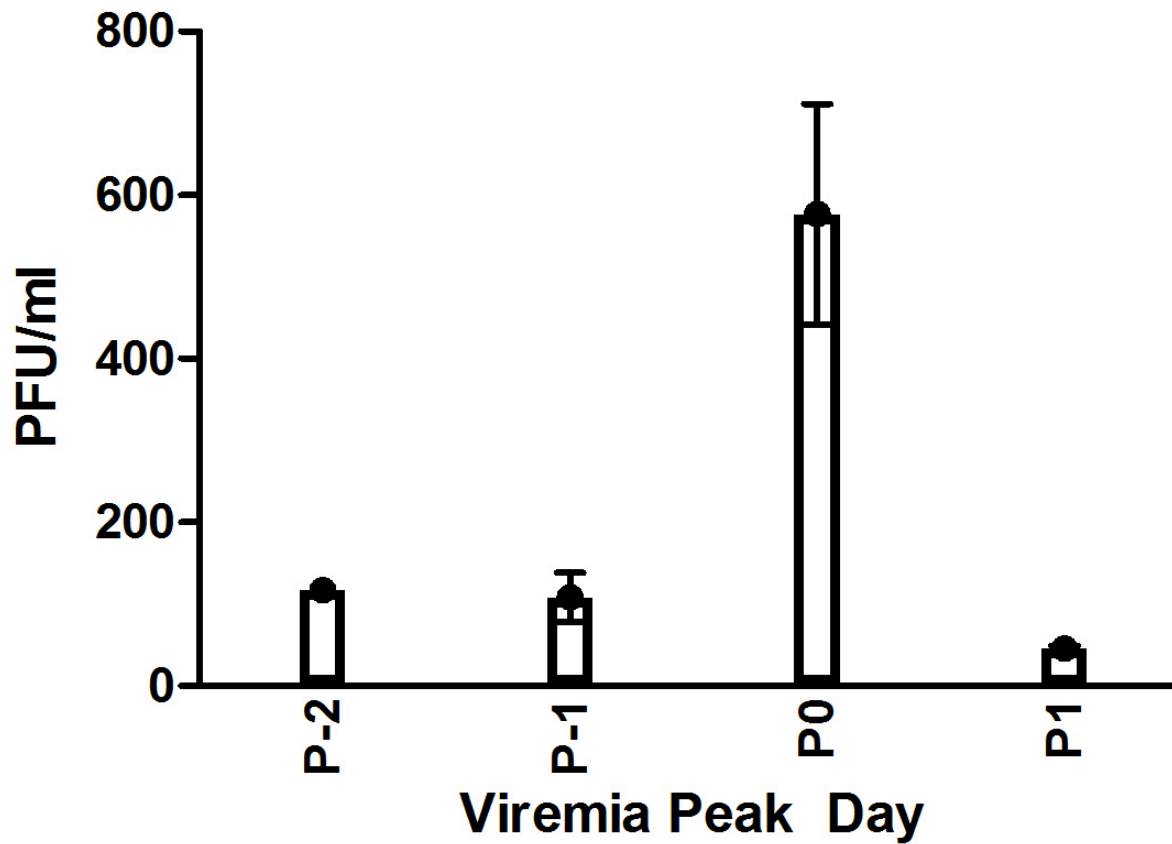


Figure 5.8: Mean viremia levels of mice (n=3) from which acquisition to mosquitoes was attempted. Mice were inoculated with 10^5 pfu/mouse of D2 1232. Each day post exposure, 25-50 mosquitoes were allowed to feed. Engorged females were kept and tested 3 days post feeding for the presence of virus in the body (i.e. infection, not dissemination). Acquisition of virus was achieved on days P-1 and P0 only at a rate of 7.14% and 11.1%, respectively.

absence of the type I response allowed for successful transmission via infectious mosquito. While some differences were noted in the IFN- γ response in mosquito versus needle inoculated mice (later peak of viremia and IFN- γ), the significance of these differences needs further study. In addition, the mechanistic origins of these differences, whether based on mosquito saliva or some intrinsic vertebrate or viral characteristic, requires investigation. An important limitation of this particular result is the measurement of systemic cytokine levels. Likely even more significant alteration of the immune response due to mosquito salivary secretions would be seen at the bite site, and not necessarily systemically. Therefore, it is important to continue to investigate the potential role of mosquito saliva in infection kinetics.

We also present, based on our findings, the concept of “effective viremic period” (EVP). That is, there exists a subset of the viremic period where transmission is unlikely. The EVP is the subset of the viremic period where transmission to a vector is probable. This is presented in Figure 5.9, where the acquisition rates from D2 1232 infected mice on days P-1 and P0 were 6% and 11%, respectively. The value of EVP would have a dramatic effect on calculations of the basic reproductive number (R_0), which includes a specific parameter encompassing the length of viremia (or, reciprocally, recovery rate). Possibly even more importantly would be the interaction among the parameters of EVP, intensity of viremia (c), and an acquisition parameter (q). Likely, the most critical parameter would be acquisition, as it measures the direction transmissibility resulting from the other two measures. Ultimately, it isn’t the absolute values of length and intensity of viremia, but whether a critical threshold is achieved which supports transmission to a naïve vector and how long viremia persists above that threshold.

In addition to allowing for transmission and infection establishment, this mouse model also has the ability to discern strain differences among dengue viruses. This makes it an ideal candidate

for viral fitness studies that could inform vaccine efforts. The three strains of dengue used here produced differential patterns of viremia and IFN- γ response. D2 1232 produced the highest viral

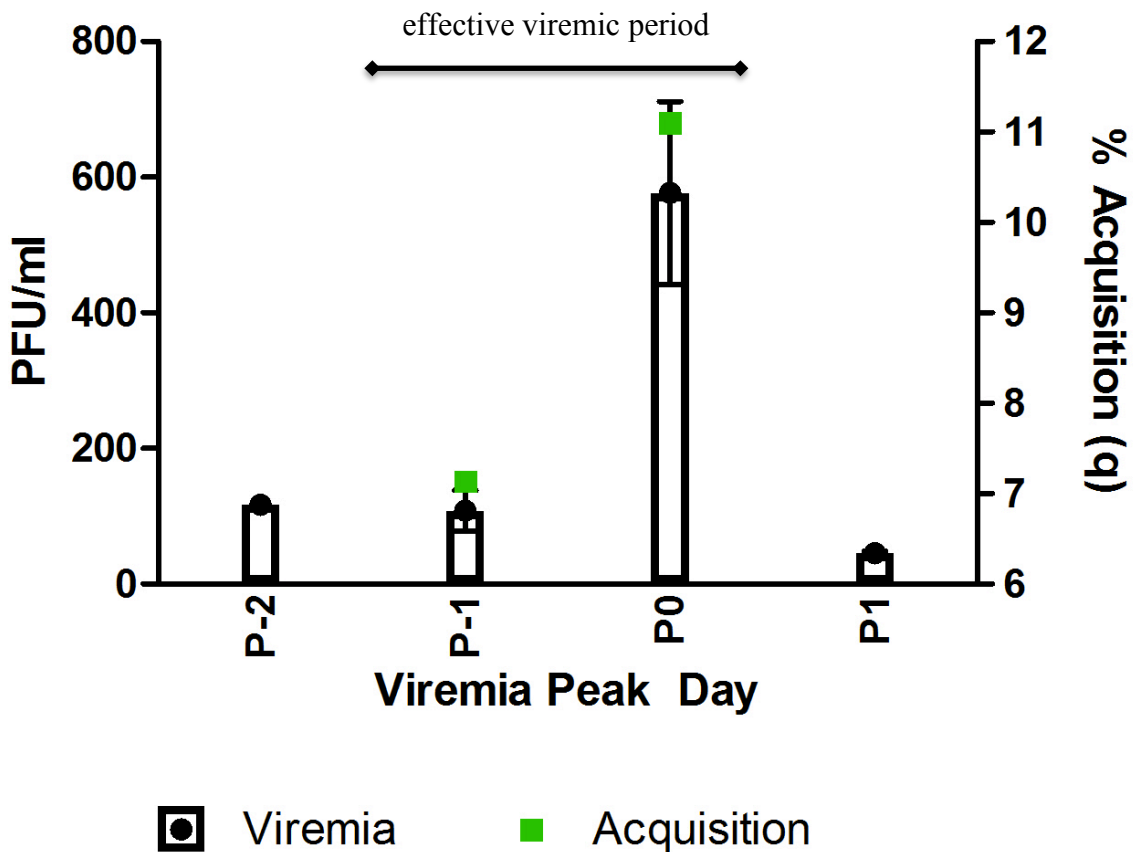


Figure 5.9: Illustration of the effective viremic period. Mean viremia levels for mice infected with D2 1232 (n=3) and the successful acquisition of virus by mosquitoes. In a D2 1232 infection, the actual viremic period lasts for four days. However, acquisition of virus from the mouse by a mosquito occurs only on two days as denoted by the green square. Thus, the effective viremic period is 2 days.

titer and longest viremic period suggesting it is the most fit of the three strains. This is especially interesting since this strain is also shown to be the most fit in the mosquito (Christofferson and Mores 2011). Infection with D4 LN 634441 resulted in the lowest viral titers in the serum. It is also interesting that the highest levels of INF- γ were not seen in the infection causing the highest viremia. This suggests that D2 1232 has some fitness advantage in that it does not elicit or somehow mitigates the INF- γ response, perhaps independently of the type I interferon pathways. As stated, the highest levels of INF- γ were detected with D2 16803 infection, and if the INF- γ response and viremia levels were monotypically correlated, the expectation would be that D4 LN 634441 would have the lowest INF- γ levels on peak days. This, however, is not the case, as D2 1232 has the lowest peak day IFN- γ levels. D2 16803 cumulatively elicited the highest levels of INF- γ , which was most likely the reason for the truncated viremic period.

There are, then, two possible interactions of viremia and INF- γ response. First, a viral strain like D2 1232 has evolved a fitness advantage to escape the INF- γ response which then allows for the higher viremia intensity and longer viremic period. Second, strains like D2 16803 and D4 LN 634441 do not have this fitness advantage. Therefore, the IFN- γ response occurs quickly, which is likely the reason for the truncated and lower viremia levels.

The fact that virus was no longer detectable following the peak of INF- γ reaffirms the importance of this response in the clearance of dengue virus infection (Chaturvedi, Elbishbishi et al. 1999; Green, Vaughn et al. 1999). This response mirrors the correlation between INF- γ response and viremia disappearance seen in children with dengue (Green, Vaughn et al. 1999; Chen, Yeh et al. 2001). This shows the ability to use this mouse model for inferences about dengue and its infection of humans. Further, this model highlights the role of the elicited INF- γ response in assessing viral fitness. In addition, the significant differences in the INF- γ response also indicates that even low

levels of viremia and the seemingly small differences in these low viremia levels may be biologically important.

Further fitness studies could also include differential responses to type I interferon. Since the IRF3/7 DKO mouse has an intact interferon receptor, IFN α/β can be exogenously introduced at different dosages in order to detect differential ability of DENV serotypes/strains to modulate and escape the antiviral immune response. Here we demonstrate the use of this DKO mouse model to detect differences in viral fitness in a vertebrate model among 3 DENV strains by comparing the infection establishment success and replicative abilities (viremia intensity). The importance of dengue viral fitness in understanding transmission dynamics is not only important in understanding dengue disease patterns, but in understanding the potential of different strains for use in vaccines.

Given that there is no vaccine, public health monitoring and a greater understanding of transmission dynamics leading to outbreak events is the first line of defense in the fight against DENV. The infection kinetics that mitigate the transmission of DENV from the vertebrate, namely the length and intensity of viremia, are crucial to understanding transmission. Part of the kinetic differences is the virus' ability to escape the antiviral immune response, specifically to modulate the type I interferon response. This ability cannot be tested in a receptor deficient mouse as no type I interferon response is possible. This mouse model offers the unique ability to use non-lab adapted strains to more accurately assess the true relative fitness of dengue strains. In addition, since this mouse retains the receptors for type I interferon, there remains the opportunity to assess the type I interferon responses elicited by dengue strains by exogenous introduction of type I interferon. Additionally, this mouse allows us to assess the immune response in a vertebrate model that is as immunologically intact as possible.

References

- (2009). Dengue and dengue haemorrhagic fever, World Health Organisation.
- Alto, B. W., L. P. Lounibos, et al. (2008). "Larval competition alters susceptibility of adult *Aedes* mosquitoes to dengue infection." Proc Biol Sci **275**(1633): 463-471.
- Balsitis, S. J., K. L. Williams, et al. (2010). "Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification." PLoS Pathog **6**(2): e1000790.
- Cassetti, M. C., A. Durbin, et al. (2010). "Report of an NIAID workshop on dengue animal models." Vaccine **28**(26): 4229-4234.
- Chaturvedi, U. C., E. A. Elbishbishi, et al. (1999). "Sequential production of cytokines by dengue virus-infected human peripheral blood leukocyte cultures." J Med Virol **59**(3): 335-340.
- Chen, H. C., F. M. Hofman, et al. (2007). "Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage." J Virol **81**(11): 5518-5526.
- Chen, H. C., S. Y. Lai, et al. (2004). "Lymphocyte activation and hepatic cellular infiltration in immunocompetent mice infected by dengue virus." J Med Virol **73**(3): 419-431.
- Chen, R. F., W. T. Yeh, et al. (2001). "A model of the real-time correlation of viral titers with immune reactions in antibody-dependent enhancement of dengue-2 infections." FEMS Immunol Med Microbiol **30**(1): 1-7.
- Christofferson, R. C. and C. N. Mores (2011). "Estimating the magnitude and direction of altered arbovirus transmission due to viral phenotype." PLoS ONE **6**(1): e16298.
- Daffis, S., M. S. Suthar, et al. (2009). "Induction of IFN-beta and the innate antiviral response in myeloid cells occurs through an IPS-1-dependent signal that does not require IRF-3 and IRF-7." PLoS pathogens **5**(10): e1000607.
- Golde, W. T., P. Gollobin, et al. (2005). "A rapid, simple, and humane method for submandibular bleeding of mice using a lancet." Lab Anim (NY) **34**(9): 39-43.
- Green, S., D. W. Vaughn, et al. (1999). "Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity." J Infect Dis **179**(4): 755-762.
- Haller, O., G. Kochs, et al. (2006). "The interferon response circuit: induction and suppression by pathogenic viruses." Virology **344**(1): 119-130.
- Jiang, D., J. M. Weidner, et al. (2010). "Identification of five interferon-induced cellular proteins that inhibit west nile virus and dengue virus infections." J Virol **84**(16): 8332-8341.

- Johnson, A. J. and J. T. Roehrig (1999). "New mouse model for dengue virus vaccine testing." J Virol **73**(1): 783-786.
- Katze, M. G., Y. He, et al. (2002). "Viruses and interferon: a fight for supremacy." Nat Rev Immunol **2**(9): 675-687.
- Kyle, J. L., S. J. Balsitis, et al. (2008). "Antibodies play a greater role than immune cells in heterologous protection against secondary dengue virus infection in a mouse model." Virology **380**(2): 296-303.
- Kyle, J. L., P. R. Beatty, et al. (2007). "Dengue virus infects macrophages and dendritic cells in a mouse model of infection." J Infect Dis **195**(12): 1808-1817.
- Limesand, K. H., S. Higgs, et al. (2003). "Effect of mosquito salivary gland treatment on vesicular stomatitis New Jersey virus replication and interferon alpha/beta expression in vitro." J Med Entomol **40**(2): 199-205.
- Liu, P., M. Woda, et al. (2009). "Dengue virus infection differentially regulates endothelial barrier function over time through type I interferon effects." J Infect Dis **200**(2): 191-201.
- Mazzon, M., M. Jones, et al. (2009). "Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation." J Infect Dis **200**(8): 1261-1270.
- Mota, J. and R. Rico-Hesse (2009). "Humanized mice show clinical signs of dengue fever according to infecting virus genotype." J Virol **83**(17): 8638-8645.
- Munoz-Jordan, J. L. (2010). "Subversion of interferon by dengue virus." Curr Top Microbiol Immunol **338**: 35-44.
- Rodriguez-Madoz, J. R., D. Bernal-Rubio, et al. (2010). "Dengue virus inhibits the production of type I interferon in primary human dendritic cells." J Virol **84**(9): 4845-4850.
- Schneider, B. S. and S. Higgs (2008). "The enhancement of arbovirus transmission and disease by mosquito saliva is associated with modulation of the host immune response." Trans R Soc Trop Med Hyg **102**(5): 400-408.
- Schul, W., W. Liu, et al. (2007). "A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs." J Infect Dis **195**(5): 665-674.
- Shresta, S., J. L. Kyle, et al. (2004). "Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice." Virology **319**(2): 262-273.
- Shresta, S., J. L. Kyle, et al. (2004). "Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical." J Virol **78**(6): 2701-2710.

- Shresta, S., K. L. Sharar, et al. (2006). "Murine model for dengue virus-induced lethal disease with increased vascular permeability." J Virol **80**(20): 10208-10217.
- Shresta, S., K. L. Sharar, et al. (2005). "Critical roles for both STAT1-dependent and STAT1-independent pathways in the control of primary dengue virus infection in mice." J Immunol **175**(6): 3946-3954.
- Styer, L. M., P. Y. Lim, et al. (2011). "Mosquito saliva causes enhancement of West Nile virus infection in mice." J Virol **85**(4): 1517-1527.
- Teixeira, M. G., C. Costa Mda, et al. (2009). "Dengue: twenty-five years since reemergence in Brazil." Cad Saude Publica **25 Suppl 1**: S7-18.
- Thangamani, S., S. Higgs, et al. (2010). "Host immune response to mosquito-transmitted chikungunya virus differs from that elicited by needle inoculated virus." PLoS ONE **5**(8): e12137.
- Williams, K. L., S. Zompi, et al. (2009). "A mouse model for studying dengue virus pathogenesis and immune response." Ann N Y Acad Sci **1171 Suppl 1**: E12-23.
- Yauch, L. E. and S. Shresta (2008). "Mouse models of dengue virus infection and disease." Antiviral Res **80**(2): 87-93.

Chapter 6

Discussion and Conclusions

Introduction

Much work has been done on genotypic studies of arboviruses, including West Nile virus (WNV), dengue (DENV), and chikungunya (CHIKV) (Lanciotti, Roehrig et al. 1999; Ebel, Carricaburu et al. 2004; Jerzak, Bernard et al. 2005; Schuffenecker, Iteaman et al. 2006; Bertolotti, Kitron et al. 2008; Jarman, Holmes et al. 2008; Kariuki Njenga, Nderitu et al. 2008; Santhosh, Dash et al. 2009; Carrillo-Valenzo, Danis-Lozano et al. 2010; Mendez, Usme-Ciro et al. 2010; Papa, Bakonyi et al. 2011). In the study described in Chapter 2, we showed that WNV genetic diversity was negatively correlated to transmission increases. Other researchers have shown that DENV diversity is positively associated with increases in transmission. Still, other studies have demonstrated that CHIKV has relative genetic stability even during intense transmission (Bertolotti, Kitron et al. 2008; Jarman, Holmes et al. 2008; Kariuki Njenga, Nderitu et al. 2008; Christofferson, Roy et al. 2010). These contradictory studies show that the association of genetic diversity and transmission is complex and unclear and suggests that rough measures of genetic diversity are insufficient to predict virus transmission differences. One possibility is that the phenotypes encoded by these genotypes are able to explain these differences and a more rigorous characterization of these traits could improve our forecasting of viral activity.

Extrinsic and environmental factors can affect phenotypic expression as seen in Chapter 2 where ecological niche also contributed to transmission differences and genetic diversity. Comparisons of viral fitness among different strains or species of arboviruses have been investigated, while the differences among cocirculating strains within a given focus and the effects on transmission of those strains have not been vigorously investigated. Presented in this dissertation

are four experimental studies that explore the contribution of differential phenotypes to the transmission potential of arboviruses as judged by modeling parameters such as vectorial capacity (VC) and the basic reproductive number (R_0). In this chapter we show the interrelatedness of these studies and the general conclusions. Additionally, future directions of this research are offered as well as continued utilization of the tools developed through this research.

Summary of Results

The study of West Nile virus in Louisiana (Chapter 2) prompted an interest in the potential role of strain differences in transmission potential. We noted that there was differential transmission among ecological settings, which was not totally surprising given the possible extrinsic factors affecting transmission (bird species richness, temperature, humidity, etc.). What was compelling were the differences in genetic diversity seen within ecologies, i.e. the wetland ecology predicted to have higher transmission (wetlands) had less diversity than did the forest ecologies.

Coincidentally, at the time we were contemplating the role of strain differences, Jarman, et al. published a paper detailing the transmission of dengue among eleven schools in a region of Thailand (Jarman, Holmes et al. 2008). They reported no clear correlation between genetic diversity changes in transmission of dengue fever (Jarman, Holmes et al. 2008). This lack of clear association between genetic diversity and transmission led to our hypothesis that the phenotypic differences, not just major phylogenetic characteristics are critical factors driving the transmission of arboviruses.

The studies in Chapters 3-5 of this dissertation address the parameters of the R_0 equation in order to achieve a dynamic, more accurate measure of the transmission potential, and thus epidemic potential of arboviruses. Chapters 3 and 4 address the vector parameter- vectorial capacity (V). Chapter 5 addresses the vertebrate parameters of intensity of viremia (c) and recovery rate ($1/r$) (or, reciprocally, length of viremia).

The equation for R_0 is given below:

$$R_0 = cV/r \quad \text{Eq. 6.1}$$

In Chapter 3, we undertook to assess how differences in phenotypes in the vector could alter transmission. We chose three virus strains from the Southeast Asian genotype of dengue type 2, as well as a Southeast Asian dengue type 4 to illustrate our theory. We found that strain differences can potentially affect the transmission potential. For example, as we show in Table 3.4 that while D2 1232 could potentially result in 32 secondary infectious bites, D2 16803 would result in only 28 secondary infectious. The significance of this difference is discussed in detail below. Of importance is the formulation of effective vector competence (EVC) and cumulative vectorial capacity (cVC), which provide more accurate assessments of viral fitness within mosquito vectors. In addition, we provide a statistical test of vectorial capacity (cumulative) where none existed before.

Further, the idea that phenotypic advantages *in vivo* could alter vectorial capacity in mosquito species led us to consider a potential two vector system (Chapter 4). Such a system is exemplified by CHIKV where in 2006, a genetic shift conferred a fitness advantage in a traditionally secondary vector, *Aedes (Ae.) albopictus*. However, *Ae. aegypti* could not be discounted as a vector of this new genotype. Using the novel EVC formulation we showed that on La Reunion Island, there was a significant fitness disadvantage in *Ae. aegypti*. However, the dynamics of this mutation on that island where *Ae. aegypti* is absent did not fully explain the spread of the mutant strain across India and into Southeast Asia where *Ae. aegypti* and *Ae. albopictus* coexist. Given that CHIKV has been relatively stable in these areas regardless of the presence of *Ae. albopictus*, it is unlikely that selection pressures for that vector alone could have driven the expansion of this mutant. This is further supported by the finding that the E226V mutation conferred no apparent fitness advantage in the vertebrate (Bordi, Meschi et al. 2011; Tsetsarkin, Chen et al. 2011). Therefore, using our EVC

method we experimentally investigated and confirmed that the E226V mutant strain had a significant advantage in *Ae. aegypti* relative to a likely resident CHIKV strain while there was a slight, but statistically significant disadvantage in *Ae. aegypti* as compared to the pre-mutation strain found on La Reunion island. Thus, some selection process that occurred in the presence of both vectors, and not just a scenario of *Ae. albopictus* (as on La Reunion Island) likely contributed to the expansion of this particular mutation. This illustrates that the relative *in vivo*, within vector phenotype can aide in explaining an expansion phenomenon where genetic analysis has been less informative.

Given these two chapters and the validation with known historical events (see Chapter 3, Discussion), we have reformulated the parameter V into cVC:

$$VC = \frac{ma^2p^Nb}{-\ln p} \quad \text{Equation (Eq.) 6.2}$$

becomes:

$$cVC = \frac{ma^2\varphi}{-\ln p} \quad \text{Eq. 6.3}$$

where:

$$\varphi = EVC = \int_a^z p^N (\beta_{1i}N + \beta_{0i})dN \quad \text{Eq. 6.4}$$

These results illustrated that even seemingly small phenotypic changes have the potential to significantly alter the relative transmission of cocirculating strains. Investigation of phenotypic differences in the vector is logically followed by a similar investigation in the vertebrate. Specifically, dengue virus usually exists in a hyperendemic situation where multiple serotypes and multiple strains within those serotypes cocirculate. Confounding studies of phenotypic differences as related to transmission was the lack of a transmission model for dengue in a small animal.

Therefore, our first undertaking was to identify and test a mouse model with the intention of studying not disease, but transmission of dengue virus. We chose the interferon regulatory factor (IRF) 3 and IRF 7 double knockout mouse strain since it is deficient in the type I interferon response (Daffis, Suthar et al. 2009). This murine model allows for strain comparisons in both viremia and immunological response, especially the IFN- γ response. The widely used AG129 mouse cannot support IFN- γ hypotheses as it is unable to mount an IFN- γ response (Shresta, Sharar et al. 2006; Yauch and Shresta 2008). In this study we showed a discordant relationship between viral fitness as measured by length and intensity of viremia and the immunological response. It was expected that the higher and/or longer viremia would elicit a stronger immune response, especially type II interferon because of the correlation seen between IFN- γ and disease state (Becquart, Wauquier et al. 2010; Tang, Kou et al. 2010). However, the highest and longest viremia did not cause the highest or longest IFN- γ response. In fact, the length of the IFN- γ response did not differ much among the three strains of dengue viruses. The more robust IFN- γ response was seen in those mice infected with D2 strain 16803 which had neither the highest nor lowest viremia. It is possible that this viral strain has an added advantage of being able to subvert the IFN- γ response, but the exact role of this and its relationship to viral fitness remains to be explored.

We are also the first to show that DENV transmission to and from a mosquito is possible in a small animal model. Within this model we also introduce the concept of effective viremic period (EVP), a subset of the traditional measure of viremic period used to calculate R_0 . By having a mouse model with the ability to support studies of transmission and acquisition by the natural vector route, and, using this EVP measure, the calculations of R_0 should be enhanced as assumptions of the acquisition success rate by mosquitoes can now be investigated.

The relationship of intensity and length of viremia with respect to mosquito-borne viruses would logically imply some function of acquisition. However, there is not a formulation of R_0 that

includes any such parameter. Such an acquisition parameter (q) would be defined as the proportion of mosquitoes, which become infected after feeding on a viremic animal. To illustrate how a final formulation of R_0 may look we determine the relationship among acquisition (q), intensity (c), and length of viremia (r).

In Chapter 3, we found that D2 strain 1232 has a cVC of ~ 32 . This is interpreted in the following manner: Over the course of 9 days (the total extrinsic incubation period measured), D2 1232, in a population of mosquitoes where the contact parameters of man biting rate and mosquito density remain constant, will result in 32 infectious bites to humans. In Chapter 5 we showed that in the mouse model, D2 1232 resulted in 4 days of detectable viremia. However, we further demonstrated that the EVP was, in fact only 2 days and that acquisition at viremia days P-1 and P0.

We postulate that a complex relationship exists among the differential phenotypes represented by these parameters. Namely, there exists a threshold of viremia above which acquisition is possible. This threshold intensity would identify a subset of the viremic period, defining the EVP. The intensity of viremia within this EVP would then correlate to some function of acquisition. This is illustrated in figure 6.1. For example, with D2 1232, the threshold of viremia intensity is defined by acquisition, and this threshold will then create a subset of the viremic period; the EVP where acquisition is probable. Likely, the parameters of intensity and length of viremia, as they are directly and positively correlated to acquisition, will be replaced by the acquisition parameter itself. Then, a function of acquisition over time, similar to the function devised in Chapter 3 for vector competence over time, could be developed.

In the case of D2 1232, the cumulative acquisition (q_c) is easily obtained by simple addition, since only two points of acquisition exist, and thus is calculated to be 17%. However, if the relationship were to become more complicated, a higher order function would be needed to define $q(c)$, and likely an integration of this function would be needed. Again, figure 6.1 shows this

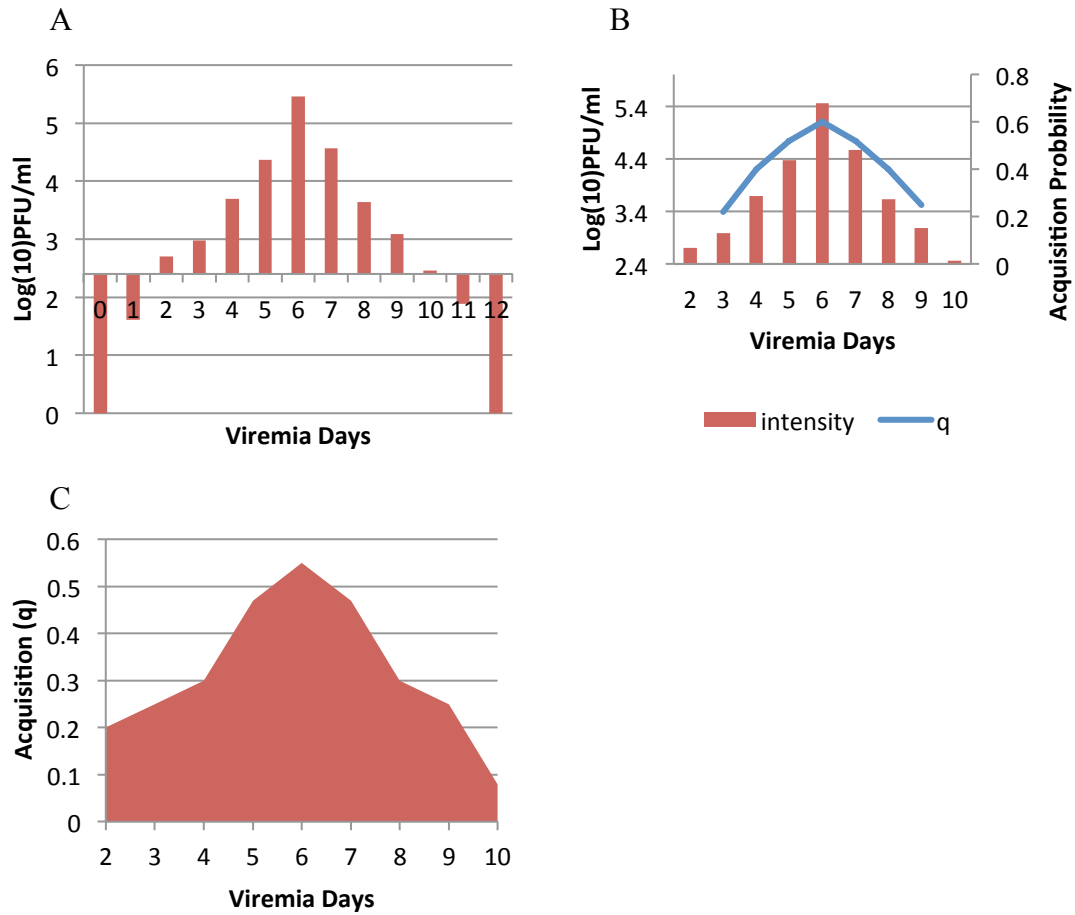


Figure 6.1: A more complicated relationship. A) A threshold is determined and then B) the EVP defined. The distribution of acquisition is also determined for the EVP based on the intensities. C) The area under the curve $q(\text{EVP})$ is a possible way to calculate the cumulative acquisition, and thus the cumulative transmission from an infected individual to the mosquito population.

possibility if viremia were to last 12 days, but only days 2-10 were high enough to support the probability of acquisition.

Using the formulations from these studies, R_0 could have the following form:

$$R_{0c} = q_c * EVC * \frac{ma^2}{-\ln p} \quad \text{Eq. 6.5}$$

Or, by combining the contact parameters and EVC into cumulative vectorial capacity:

$$R_{0c} = q_c * cVC \quad \text{Eq. 6.6}$$

This measure of cumulative R_0 would be interpreted over the period of days defined as the number of days of the mosquito extrinsic incubation period (the interval over which EVC was integrated), and the *total* number of viremic days. This is because, while no actual transmission is occurring during those days of the viremic period where acquisition is improbable, those days are equally as important in assessing the timing of potential outbreaks. This is especially so when comparing viral strains and assessing fitness as time to adequate viremia could be considered a measure of fitness itself.

To further simplify the interpretation, and since R_0 is traditionally defined as a measure of transmission per day, the measure of transmission per day can be derived by simply dividing R_{0c} by the total number of days in the transmission cycle. In the case of D2 1232, the extrinsic incubation period measured within the vector was 9 days. In the vertebrate there were a total of 4 viremic days, therefore the total transmission cycle could theoretically be calculated as $9+4 = 13$ days. Thus, using the contact parameters found in table 3.3,

$$R_0 = \frac{R_{0c}}{Tx\ Cycle\ (in\ days)} = \frac{.17*.294*107.89}{13} = \frac{5.39}{13} = .41 \quad \text{Eq. 6.7}$$

Given the definition of R_0 , since this estimate is less than 1, the risk of an outbreak is not probable. This is a conservative estimate of R_0 in that for this particular study, only the exponential phase of vector competence was utilized. Additionally, the viremia levels achieved by the mouse model are assumed to be proportional to, but not exact replicas of viremia levels in humans which can reach up to 10^7 pfu/ml. However, investigations into the proportionality of the mouse to human viremia can be made and the information garnered about the *relative* parameters of strains can still be used to model transmission differences.

Conclusions

Fitness of arboviruses has been hypothesized to be constrained by the requirement of two main hosts: vector and vertebrate. This idea was first proposed by Taylor and Marshall in 1975 using Ross River virus (Taylor and Marshall 1975; Taylor and Marshall 1975) and hypothesizes that extraordinary fitness in either the vector or the vertebrate is “traded” for adequate fitness in both. This “trade-off hypothesis” has been studied in several arboviruses both *in vivo* and *in vitro* (Cologna, Armstrong et al. 2005; Greene, Wang et al. 2005; Ciota, Lovelace et al. 2007; Coffey, Vasilakis et al. 2008; Moutailler, Roche et al. 2011). However, the measurements of fitness are often conservative and we show that even small changes can alter transmission potential.

We show that the contribution of infection kinetics in the vertebrate to the formulation of R_0 is one of a dilution effect. That is, acquisition is a proportion that will further subdivide the population of mosquitoes into those that will likely transmit and those that will not. It would therefore seem likely that even small fitness advantages in the vertebrate (that raise either q or

lengthen EVP) would have profound effects on the transmission potential of that viral strain. It might be that the trade-off hypothesis is a misrepresentation of the importance of small changes. That is, while big shifts in fitness in either host would compromise overall fitness in both hosts, big shifts are not necessary for advantages to appear.

An example of how these two fitness scenarios could potentially yield a new formulation of R_0 is proposed, but final validation will likely involve a more complex relationship and is beyond the scope of this dissertation. However, we do illustrate that the possibilities exist for phenotypic changes, even when relatively small, have the potential to affect transmission dynamics. This idea could have profound effects on the trade-off hypothesis as well as applied studies of transmission and public health policy. For example, whether an epidemic is being driven by strain fitness in the vector or in the vertebrate could decide whether mitigation of the outbreak could best be controlled by vector control strategies or by human behavior modifications.

In summary, this dissertation proposes that phenotypic variation is at least as important, if not more important, for assessing transmission potential of arboviruses than genotyping alone. Further, we offer a more accurate tool for assessing transmission potential in the vector and provide a new model assessing transmission potential in the vertebrate. While this dissertation does not propose a finalized, dynamic formulation of R_0 - the ultimate transmission estimate- it provides several of the necessary steps towards a more appropriate calculation. Furthermore, one of the major objectives of our studies is to provide not only these more accurate formulations, but to provide accessible parameters and calculations. In conclusion, our use of R_0 provides a framework for a more dynamic formulation of transmission models and provides such an accessible framework for output validation and reporting to public health stakeholders.


References

- Becquart, P., N. Wauquier, et al. (2010). "Acute dengue virus 2 infection in Gabonese patients is associated with an early innate immune response, including strong interferon alpha production." BMC infectious diseases **10**: 356.
- Bertolotti, L., U. D. Kitron, et al. (2008). "Fine-scale genetic variation and evolution of West Nile Virus in a transmission "hot spot" in suburban Chicago, USA." Virology **374**(2): 381-389.
- Bordi, L., S. Meschi, et al. (2011). "Chikungunya virus isolates with/without A226V mutation show different sensitivity to IFN- α , but similar replication kinetics in non human primate cells." The new microbiologica **34**(1): 87-91.
- Carrillo-Valenzo, E., R. Danis-Lozano, et al. (2010). "Evolution of dengue virus in Mexico is characterized by frequent lineage replacement." Arch Virol **155**(9): 1401-1412.
- Christofferson, R. C., A. F. Roy, et al. (2010). "Factors associated with mosquito pool positivity and the characterization of the West Nile viruses found within Louisiana during 2007." Virology journal **7**: 139.
- Ciota, A. T., A. O. Lovelace, et al. (2007). "Cell-specific adaptation of two flaviviruses following serial passage in mosquito cell culture." Virology **357**(2): 165-174.
- Coffey, L. L., N. Vasilakis, et al. (2008). "Arbovirus evolution in vivo is constrained by host alternation." Proceedings of the National Academy of Sciences of the United States of America **105**(19): 6970-6975.
- Cologna, R., P. M. Armstrong, et al. (2005). "Selection for virulent dengue viruses occurs in humans and mosquitoes." Journal of virology **79**(2): 853-859.
- Daffis, S., M. S. Suthar, et al. (2009). "Induction of IFN-beta and the innate antiviral response in myeloid cells occurs through an IPS-1-dependent signal that does not require IRF-3 and IRF-7." PLoS pathogens **5**(10): e1000607.
- Ebel, G. D., J. Carricaburu, et al. (2004). "Genetic and phenotypic variation of West Nile virus in New York, 2000-2003." Am J Trop Med Hyg **71**(4): 493-500.
- Greene, I. P., E. Wang, et al. (2005). "Effect of alternating passage on adaptation of sindbis virus to vertebrate and invertebrate cells." Journal of virology **79**(22): 14253-14260.
- Jarman, R. G., E. C. Holmes, et al. (2008). "Microevolution of Dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand." J Virol **82**(11): 5494-5500.
- Jerzak, G., K. A. Bernard, et al. (2005). "Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection." J Gen Virol **86**(Pt 8): 2175-2183.

- Kariuki Njenga, M., L. Nderitu, et al. (2008). "Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa." J Gen Virol **89**(Pt 11): 2754-2760.
- Lanciotti, R. S., J. T. Roehrig, et al. (1999). "Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States." Science **286**(5448): 2333-2337.
- Mendez, J. A., J. A. Usme-Ciro, et al. (2010). "Phylogenetic history demonstrates two different lineages of dengue type 1 virus in Colombia." Virol J **7**: 226.
- Moutailler, S., B. Roche, et al. (2011). "Host alternation is necessary to maintain the genome stability of rift valley Fever virus." PLoS neglected tropical diseases **5**(5): e1156.
- Papa, A., T. Bakonyi, et al. (2011). "Genetic characterization of west nile virus lineage 2, Greece, 2010." Emerg Infect Dis **17**(5): 920-922.
- Santhosh, S. R., P. K. Dash, et al. (2009). "Appearance of E1: A226V mutant Chikungunya virus in Coastal Karnataka, India during 2008 outbreak." Virol J **6**: 172.
- Schuffenecker, I., I. Iteman, et al. (2006). "Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak." PLoS Med **3**(7): e263.
- Shresta, S., K. L. Sharar, et al. (2006). "Murine model for dengue virus-induced lethal disease with increased vascular permeability." J Virol **80**(20): 10208-10217.
- Tang, Y., Z. Kou, et al. (2010). "Both viremia and cytokine levels associate with the lack of severe disease in secondary dengue 1 infection among adult Chinese patients." PLoS ONE **5**(12): e15631.
- Taylor, W. P. and I. D. Marshall (1975). "Adaptation studies with Ross River virus: laboratory mice and cell cultures." J Gen Virol **28**(1): 59-72.
- Taylor, W. P. and I. D. Marshall (1975). "Adaptation studies with Ross River virus: retention of field level virulence." J Gen Virol **28**(1): 73-83.
- Tsetsarkin, K. A., R. Chen, et al. (2011). "Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes." Proc Natl Acad Sci U S A **108**(19): 7872-7877.
- Yauch, L. E. and S. Shresta (2008). "Mouse models of dengue virus infection and disease." Antiviral Res **80**(2): 87-93.

Appendix 1

Permission to Reprint from BMC Virology Journal as per Creative Commons Attribution License



The Open Access Publisher

Log on/register
Feedback | Support | My details

home | journals A-Z | subject areas | advanced search | authors | reviewers | libraries | about | my BioMed Central

for authors
publishing services
for libraries
media information
press center
about us

What we do

- What is BioMed Central?
- About Membership
- Open Access Fund
- User comments
- Recent developments
- BioMed Central toolbar
- Online shop
- Jobs at BioMed Central

Our policies

- Peer-review policy
- Copyright
- Reprints and permissions
- Access to articles
- **Open access charter**
- Permanency of articles
- Duplicate publication
- Standards and affiliations

Who we are

- In-house team
- Board of Trustees

Promotion tools

- Advocacy home

Media

- Advertising information
- Forthcoming conferences

FAQ


- General FAQ
- Article-processing charge FAQ

Links

- Translated pages
- Links pages
- Link to us

Contact us

- Contact us

[Printer friendly version](#) 

BioMed Central Open Access Charter

Every peer-reviewed research article appearing in any journal published by BioMed Central is 'open access', meaning that:

1. The article is universally and freely accessible via the Internet, in an easily readable format and deposited immediately upon publication, without embargo, in an agreed format - current preference is XML with a declared DTD - in at least one widely and internationally recognized open access repository (such as PubMed Central).
2. The author(s) or copyright owner(s) irrevocably grant(s) to any third party, in advance and in perpetuity, the right to use, reproduce or disseminate the research article in its entirety or in part, in any format or medium, provided that no substantive errors are introduced in the process, proper attribution of authorship and correct citation details are given, and that the bibliographic details are not changed. If the article is reproduced or disseminated in part, this must be clearly and unequivocally indicated.


BioMed Central is committed permanently to maintaining this open access publishing policy, retrospectively and prospectively, in all eventualities, including any future changes in ownership.


BioMed Central has established an independent Board of Trustees. If and when a change of ownership should be considered, the Board of Trustees will be asked to judge and advise whether sufficient guarantees to continue a policy of unconditional open access for research articles are being offered and agreed by any prospective new owner. BioMed Central will not enter into a change of ownership agreement unless the Board of Trustees accepts these guarantees.

Any change in the composition of the Board of Trustees will be subject to approval by a majority of the existing members of the Board of Trustees.

The Board of Trustees consists of the following members:

- **Professor Brian Haynes** McMaster University (Canada)
- **Dr E Hyman** Harvard University (USA)
- **Professor Marc W Kirschner** Harvard Medical School (USA)
- **Professor Philippe Kourilsky** Singapore Immunology Network (Singapore)
- **Dr David G Nathan** Dana-Farber Cancer Institute (USA)
- **Sir Paul Nurse** Rockefeller University (USA)
- **Sir Richard Peto** University of Oxford (UK)
- **Dr Harold E Varmus** Memorial Sloan-Kettering Cancer Center (USA)
- **Professor Mitsuhiro Yanagida** Kyoto University (Japan)

Open access articles in BioMed Central's journals are identified by this logo: 

Search info pages


Appendix 2

Permission to Reprint from PLoS One as per Creative Commons Attribution License

Open-Access License

No Permission Required

The Public Library of Science (PLoS) applies the Creative Commons Attribution License (CCAL) to all works we publish (read the human-readable summary or the full license legal code). Under the CCAL, authors retain ownership of the copyright for their article, but authors allow anyone to download, reuse, reprint, modify, distribute, and/or copy articles in PLoS journals, so long as the original authors and source are cited. **No permission is required from the authors or the publishers.**



In most cases, appropriate attribution can be provided by simply citing the original article (e.g., Kaltenbach LS et al. (2007) Huntingtin Interacting Proteins Are Genetic Modifiers of Neurodegeneration. *PLoS Genet* 3(5): e82. doi:10.1371/journal.pgen.0030082). If the item you plan to reuse is not part of a published article (e.g., a featured issue image), then please indicate the originator of the work, and the volume, issue, and date of the journal in which the item appeared. For any reuse or redistribution of a work, you must also make clear the license terms under which the work was published.

This broad license was developed to facilitate open access to, and free use of, original works of all types. Applying this standard license to your own work will ensure your right to make your work freely and openly available. Learn more about open access. For queries about the license, please contact us.

All site content, except where otherwise noted, is licensed under a Creative Commons Attribution License.

Appendix 3

EVC and cVC SAS Code, Single Phase

```
/******EVC_cVC calculations Part
1******/
/****This part of the program simulates all bootstrap data and
calculates the linear function b(N)****/
/****The integration and EVC, cVC calculations are in Part2 ***/

libname chik "C:\Users\ODP\Desktop\SCHOOL\papers\ChikV";

data chikv;
set chik.exp_chik;
if strain="LR06" then n=15;
if strain="SEA" then n=16;
run;

data LR;
set chikv;
if strain="LR06" then output;
run;

data SEA;
set chikv;
if strain="SEA" then output;
run;

/*THIS MACRO IS FOR BOOTSTRAPPING WITH PROC SURVEYSELECT, SELECTION
WITH REPLACEMENT*/
/*Go me and my bad programming self*/
/*sx= sample size for timepoint=x*/
%macro samp_wr (data, s1, s2, s3, num);
ods listing close;
%do g=1 %to &num %by 1;
    proc surveyselect data=&data outseed method=pps_wr sampsize=(&s1,
&s2, &s3);
        size n;
        strata strain dpi;
        id strain dpi diss;
    run;
%put g;
%end;
ods listing;
%mend;
%samp_wr (LR, 15, 15, 15, 1001);
%samp_wr (SEA, 16, 16, 16, 1001);

/*COMBINE MACRO CREDIT:UCLA website-
http://www.ats.ucla.edu/stat/SAS/code/mac_set.htm*/
/*Modified by me*/
%macro combine (id, start, stop);
data big&id;
    set
    %do i = &start %to &stop %by 1;
        data&i
```



```

    %end;
;
run;
%mend;
%combine (LR, 1, 1001);
%combine (SEA, 1002, 2002);

ods listing;

/*Double checking the data to make sure the surveyselect worked
properly*/
/*InitialSeed is now the ID of each bootstrap*/
/* AN ID=1 INDICATES ORIGINAL DATA SET*/
data big; set bigLR bigSEA; run;
proc sort data=big; by strain dpi InitialSeed; run;
proc means data=big sum;
var NumberHits;
by strain dpi InitialSeed;
run;

data biglr; set biglr; rename InitialSeed=ID; run;
data bigsea; set bigsea; rename InitialSeed=ID; run;
data Lr; set Lr; ID=1; run;
data SeA; set SeA; ID=1; run;

/*Getting the calculated vector comp. values = total positive legs/
total tested */
/*THIS IS DONE ON THE ORIGINAL DATA AS WELL AS BOOTSTRAPPED DATA*/
%macro b (data);
proc sort data=&data; by dpi ID n; run;
ods listing close;
proc means data=&data;
by dpi ID n;
var diss;
output out=S&data sum=Sum;
run;
ods listing;

data S&data;
set S&data;
b=Sum/n;
run;
%mend;

%b (biglr);
%b (bigsea);
%b (Lr);
%b (SeA);

/*A NEW ID NEEDS TO BE ADDED TO THE BOOTSTRAPPED SAMPLES SO THAT THERE
IS A SET OF IDs NOT JUST PER DPI*/
data NID;
do i=2 to 1002 by 1;
put i;
output;
end;
run;

```

```

data NID3; set NID NID NID; run;

%macro ID (data, original, new_data);
data &data;
merge &data NID3;
drop ID;
run;

proc sort data=&data; by i; run;

data &data;
set &data;
rename i=ID;
run;

data &new_data;
set &data &original;
drop _TYPE_ _FREQ_;
run;
%mend;

%ID (sbiglr, slr, LR06);
%ID (sbigsea, ssea, SEA);

/*DOING REGRESSION TO GET THE LINEAR FUNCTION OF B(N)*/
/*THERE IS ONLY THE E-PHASE, SO ONLY 1 LINE IS NEEDED*/
%macro reg (data, id);
ods listing close;
proc sort data=&data; by ID; run;
proc reg data=&data outest=reg&id;
by ID;
ID=ID;
model b=dpi / cli;
run; quit;
%mend;
%reg (LR06, LR);
%reg (SEA, SEA);

data rLR06;
strain="LR06";
set Reglr;
keep ID _DEPVAR_ Intercept Dpi strain;
run;

data rSEA;
strain="SEA ";
set RegSea;
keep ID _DEPVAR_ Intercept Dpi strain;
run;

data chik.Reg;
set rLR06 rSEA;
run;

data chik.SEA;
strain="SEA ";
set SEA;

```

```

run;

data chik.LR06;
strain="LR06";
set LR06;
run;

/*****EVC_cVC calculations Part
2*****/
/****This part of the program performs the integration and EVC, cVC
calculations****/

libname chik "C:\Users\ODP\Desktop\SCHOOL\papers\ChikV";

data chik; set chik.LR06 chik.SEA; run;

data reg;
set chik.Reg;
if strain="LR06" then N=15;
else if strain="SEA" then N=16;
run;

proc sort data=reg; by strain ID dpi; run;

/****dataset all has the simulated data for 1000 bootstraps with
replacement + the original data****/
/****data has the following variables:      strain, ID (bootstrap ID),
dpi (day post exposure),
n (at each
dpi/strain), and b (dissemination rate)*****/
/****IMPORTANT to know which ID corresponds to the original experimental
data, as this will need to be extracted
from the large dataset
later*****/

/* COMPUTATION OF THE AREA UNDER THE CURVE:

Area (Ahat) = (m(a^2)/-ln p) * integral(lower to upper) {p^N * b(N)} dN
              = (m(a^2)/-ln p) * integral(lower to upper) {p^N *
(betal(N) + beta0)} dN
              = (m(a^2)/-ln p) * [ betal * integral(lower to upper) {p^N
* N} dN + beta0 integral(lower to upper) {p^N} dN]
              = (m(a^2)/-ln p) * c0*Beta0 + c1*betal
Where

Bmatrix (Bhat)= (b0 b1)' = [(X'X)^-1 * X' * p-hat]
p-hat = [p1 p2 p3]' where pi= proportion at time i
X=[1 t1
  1 t2
  1 ti] where ti are the time points of interest
(t1 would be lower limit, ti would be upper
limit of intergral)

c0 = integral(lower to upper) {p^N} dN = [1/(log p) ] *
[p^N_upper bound - p^N_lower bound]

```

```

c1      = integral(lower to upper) {p^N * N} dN = [1/(log p)^2 *
(p^N_upper * (N_upper* log p) - p^N_upper -

      p^N_lower * (N_lower * log p) + p^N_lower)]

*/
/*Using Trapezoidal Approximation for Area Under the Curve*/
/*Macro by Rebecca Christofferson*/
%macro auc (a, b, n, function, j);
data is;
cx= ((b-&a)/&n);
      do i = 0 to &n;
        put i; output;
      end;
run;

data x&j;
set is;
if i=0 then x=&a;
      else if i=&n then x=&b;
      else x=(&a + (i*cx));
run;

data fx&j;
set x&j;
if i=0 then fx= (&function/2);
      else if i=&n then fx= (&function/2);
      else fx= (&function);
run;

proc sort data=x&j; by i; run;
proc sort data=fx&j; by i; run;

data data&j;
set x&j fx&j;
by i;
run;

proc summary data=data&j;
var fx;
output out=area&j sum=auc;
run;

data area&j;
      cx= ((b-&a)/&n);
set area&j;
area=auc*cx;
run;

proc print data=area&j;
run;
%mend;

/*****SINCE ALL IN THE SAME MOSQUITO VECTOR, DO NOT NEED STRAIN
SPECIFIC QUANTITIES IN THIS PART*****/
/*****This is done on a per strain-ID basis*****/
%auc (3, 7, 150, (.91**x), c0);

```

```

%auc (3, 7, 150, ((.91**x)*x), c1);
/****These give quantities of the two matrices above****/

data c_matrix;
set areac0 areac1;
run;

/****Since I am holding constant m and a, arbitrarily assigned them a
value of 1****/
/**** The daily survival probably .91 is from:
Harrington LC, Buonaccorsi JP, Edman JD, Costero A,
Kittayapong P, et al. (2001)
Analysis of survival of young and old Aedes aegypti
(Diptera: Culicidae) from
Puerto Rico and Thailand. J Med Entomol 38: 537-547.
****/
data constants;
input m a p;
datalines;
1 1 .91
;
run;
data constants; set constants; const=m*(a**2)/ -(log(p)); run;

/*****Estimates of the Variance*****/
/* Variance (Ahat) = (constants^2) * (C0 C1)*Var(Bhat)*[(Co C1)']
(square root of this is the standard
error estimate)

where
Variance (Bhat) = [(X'X)^-1]*X'*[diag(var p-hat-i)]*X*(X'X)^-1

where
var (p-hat-i) = [ p1(1-p1)/n1      0      0
                  0      p2(1-p2)/n2      0
                  0      0      pi(1-pi)/ni]

*p has a binomial distribution; thus the binomial variances*
*****/
/*variance matrix calculations*/
proc sort data=chik; by strain ID dpi n; run;
data m; set chik;
var=b*(1-b)/N; *binomial variance computation where b=p;
run;

/*seperating each strain into its own dataset*/
%macro sep (dataset, set, var, condition);
data &dataset;
set &set;
if &var=&condition then output;
run;
%mend;

%sep (sLR06v, m, strain, "LR06");
%sep (sSEAv, m, strain, "SEA ");

```

```

%macro var (dataset, n, firstobs, label);
data s; set &dataset; run;
proc sort data=s; by ID dpi; run;
%do i=1 %to &n %by 1;
proc iml;
/*reading my variances calculated in the data step above*/
/*vp is my variance of p, where p= my b values (proportions)*/
/*variances calculated as binomial p(1-p)/n */
start var(varp1, varpsub, vp, x, xpx, vbhat, vb2, vb3, vb4);
    use s; read all into varp1 var{var}; create checkvp from varp1;
append from varp1;
    varpsub=varp1[1:3]; print varpsub;
/*****The varpsub matrix will need to be changed if a different
number of time points are used
    EXAMPLE: for 4 time points, varpsub=
varp1[1:4]*****/
    vp=diag(varpsub); print vp;
/*Rows 1-3 are for days 3, 5, 7 of ID=1, Rows 4-6 for days 3, 5, 7 for
ID=2, etc.*/
    create varp&label from vp; append from vp; print vp;
/*xpx is the x'x matrix*/
    x = {1 3, 1 5, 1 7};
/*****THIS MATRIX WILL NEED TO BE TAILORED TO SPECIFIC SAMPLING
TIME POINTS*****/
    xpx=x`*x;
    vbhat= inv(xpx)*x`;
    vb2=vbhat*vp;
    vb3=vb2*x;
    vb4=vb3*inv(xpx); print vb4;
    create vbhat&label from vb4; append from vb4;
finish;
run var(varp1, varpsub, vp, x, xpx, vbhat, vb2, vb3, vb4); quit;

data s;
set s (firstobs=&firstobs);
/****The firstobs takes the (firstobs-1) observations into the data
set****/
/****So if I want only the first three rows, firstobs=4****/
run;

    %if i=1 %then %do;
        data varps&label; set varp&label; run;
        data vbhats&label; set vbhat&label; run;
    %end;
    %else %if i ne 1 %then %do;
        proc datasets library=work nolist; append base=varps&label
data=varp&label; run; quit;
        proc datasets library=work nolist; append base=vbhats&label
data=vbhat&label force; run; quit;
    %end;
/*Varps: the variance matrices of Proportions concatenated on top each
other 3x3*/
/*VBHats: the vbhat matrices concatenated on top each other 2x2 */
/*****THE DIMENSIONS OF THIS WILL CHANGE BASED ON THE NUMBER OF TIME
POINTS, ETC*****/
%put i;

```

```

%end;
%mend;
%var (sLR06v, 1002, 4, LR06);
%var (sSEAv, 1002, 4, SEA);
/*
data chik.varpsLR06; set varpsLR06; run;
data chik.vbhatsLR06; set vbhatsLR06; run;
data chik.varpsSEA; set varpsSEA; run;
data chik.vbhatsSEA; set vbhatsSEA; run;

/*****
/*****AREA CALCS = EVC *****/
/*****/

data varpsLR06; set chik.varpsLR06; run;
data vbhatsLR06; set chik.vbhatsLR06; run;
data varpsSEA; set chik.varpsSEA; run;
data vbhatsSEA; set chik.vbhatsSEA; run;

/****This dataset contains my linear rate of change for each set of time
points (n=1001 bootstraps + original data)
Contains the following variables: strain, ID (of bootstrap/orig),
intercept (of the linear relationship),
                                dpi (slope of
the line, or rate of change)*****/

/****The way this macro works: It reads the first observations for a
set of data points then deletes those observations,
                                loops around to redo the macro for each ID (set of
data points).
There is no identifier, so it will be imperative to sort over ID
and know which set of obs in the output corresponds
                                to what ID
****/

/****Seperated Data Sets: LR06r and SEAr****/
%sep (LR06r, reg, strain, "LR06");
%sep (SEAr, reg, strain, "SEA ");

%macro area (b_data, vdata, n, firstobs, firstobsV, label, coef,
intercept);
/*****b_data is the data with the simulated linear relationship-
regression estimates*****/
/*****vdata is the data from the variance calculations above:
vbhats_label from above*****/
/*****n is the number of bootstraps plus original
data*****/
/*****firstobs, firstobsV, see
below*****/
/****coef is the name of the variable in b_data that is the slope of the
linear relationship**/
/*****intercept is the name of the variable in b_data corresponding to
the intercept*****/
/*****a is the day the integration
starts*****/

```

```

proc sort data=&b_data; by ID; run;
data b; set &b_data; drop strain ID; run;
data v; set &vdata; run;

%do i=1 %to &n %by 1;
proc iml;
start area (bs, c, bhat, bhatt, A, v, VAhat, vbhat);
    use b; read all into bs var{&coef &intercept}; print bs; *Column
one is int=B1, Column two is coef= B0;
    bhat=bs[2:1]; bhatt=bhat`; print bhat; *row one in bhat is
B0, row 2 is B1;
    create bhat&label from bhatt; append from bhatt;
    use c_matrix; read all into c var{area}; *row one is c0, row two
is C1; print c;
    A=c`*bhat;
    create area_est&label from A; append from A;

    /*use constants; read all into K var{const};
    Ahat=A*K; print Ahat;
    create area_est&label from Ahat; append from Ahat;*/

    use v; read all into v var{COL1 COL2};
    vbhat=v[1:2, 1:2]; *gives me the first two rows of the
matrix;
    print vbhat;
    VAhat=c`*vbhat*c; print VAhat;
    *K2=k*k;
    *VAhatc=VAhat*K2; *print VAhatc;
    create varA_&label from VAhat; append from VAhat;
finish area; run area(bs, c, bhat, bhatt, A, v, VAhat, vbhat); quit;

data b;
set b (firstobs=&firstobs);
run;
/*****points to the firstobs=n
observation*****/
/*****EXAMPLE: For b, we want the first row at each iteration, so
firstobs=2*****/

data v;
set v (firstobs=&firstobsV);
/**The firstobsV value is based on the vbhat matrix and is a 2x2 matrix
for 2 parameter line, so firstobsV=3**/
run;

%if i=1 %then %do;
    data areas_est&label; set area_est&label; run;
    data varAss_&label; set varA_&label; run;
%end;
%else %if i ne 1 %then %do;
    proc datasets library=work nolist; append
base=areas_est&label data=area_est&label force; run; quit;
    proc datasets library=work nolist; append base=varAs_&label
data=varA_&label force; run; quit;
%end;
%put i;

```



```

%end;
%mend;
ods listing close;
%area (LR06r, vbhatsLR06, 1002, 2, 3, LR06, dpi, intercept);
%area (SEAr, vbhatsSEA, 1002, 2, 3, SEA, dpi, intercept);

data chik.areas_estLR06; set areas_estLR06; run;
data chik.varAs_LR06; set varAs_LR06; run;
data chik.areas_estSEA; set areas_estSEA; run;
data chik.varAs_SEA; set varAs_SEA; run;

/*****Test*****/
data areas_estLR06; set chik.areas_estLR06; run;
data varsAs_LR06; set chik.varAs_LR06; run;
data areas_estSEA; set chik.areas_estSEA; run;
data varAs_SEA; set chik.varAs_SEA; run;

/*****To Test,
compute a t*-like statistics like so:

t*i=[Ahat(i,strainA) -
Ahat(i,ref_strain)]/sqrt[(se(i,straiA))^2 + (se(i,ref_strain))^2]
for i to J simulations (J=1001)

Then get the cdf for t*i and get upper 97.5 and lower 0.025 quantiles
of the cdf and then compute the confidence intervals
*****/

data id;
do id=1 to 1002 by 1;
output;
put id; end;
run;

data areas_estLR06; merge chik.areas_estLR06 id; rename AreaA=AreaLR06;
strain="LR06"; run;
data areas_estSEA; merge chik.areas_estSEA id; rename COL1=AreaSEA;
strain="SEA"; run;
data varAs_LR06; merge chik.varAs_LR06 id; rename VarA=VarLR06;
strain="LR06"; run;
data varAs_SEA; merge chik.varAs_SEA id; rename COL1=VarSEA;
strain="SEA"; run;

proc sort data=areas_estLR06; by ID; run;
proc sort data=VarAs_LR06; by ID; run;
proc sort data=areas_estSEA; by ID; run;
proc sort data=VarAs_SEA; by ID; run;

/****Merging the LR06 and SEA data*****/
%macro merge (newdata, data1, dataRef, by);
data &newdata;
update &data1 &dataRef; by &by; drop strain; run;
%mend;
%merge (comp, areas_estLR06, areas_estSEA, ID);
%merge (compv, VarAs_LR06, VarAs_SEA, ID);
%merge (compa, comp, compv, ID);

```

```

ods listing;
/****tstar = [(area1(obs)- area1(boot) - (Area2(obs)- area2(boot))] /
sqrt[(sel(boot)^2+se2(boot))^2]****/
%macro comp (data, REFid, COMPid, areaREF, areaComp);
data &data;
set &data;
diffREF = (&areaREF-Area&REFid); *extant strain;
diffComp= (&areaComp- Area&COMPid); *comparison strain;
diff = diffREF - diffComp;
denom= sqrt(Var&COMPid + Var&REFid);
tstar= (diff-0) / denom;
run;
proc gchart data=&data; vbar tstar; run;quit;
%mend;
%comp (compa, LR06, SEA, 0.9518782407, 0.3840366709);
/*Values obtained from distribution analysis of tstar distribution*/

/*****Percentiles*****/
/*    Using SAS INSIGHT to obtain: Solutions-->Analysis-->Interactive
Data Analysis
97.5 and 2.5 percentiles of T* will give us 95% confidence interval
Compa: 97.5 = 2.6850      2.5 = .1009
/*****/
/*****Building Confidence Intervals with the Original
Data*****/
/***This assumes the first set of data is the original, which it is
here*****/
data compO; set compa; if ID=1 then output; run;

%macro CI (datain, REFid, thi, tlow, COMPid);
data &datain;
set &datain;
diff= (Area&COMPid - Area&REFid);
sum=(Var&COMPid + Var&REFid);
se=sqrt(sum);
UCL= diff + (abs(&thi))*se;
LCL= diff - (abs(&tlow))*se;
run;
%mend;
%CI (compO, LR06, 2.6850, .1009, SEA);
proc print data=compO; run;

```

Appendix 4

EVC and cVC SAS Code, Two Phase

```
/******EVC_cVC calculations with 2-Phases Part 1******/
/*****This part of the program simulates all bootstrap data and
calculates the linear function b(N)*****/
/*****The integration and EVC, cVC calculations are in Part2 ****/

libname chik "C:\Users\ODP\Desktop\SCHOOL\papers\ChikV\Higgs";

data chikv;
set chik.chikvdatahiggsaegypti;
n=20;
rename b=diss;
run;

proc sort data=chikv; by strain dpi; run;

data LR;
set chikv;
if strain="A" then output;
run;

data pLR;
set chikv;
if strain="V" then output;
run;

/*THIS MACRO IS FOR BOOTSTRAPPING WITH PROC SURVEYSELECT, SELECTION
WITH REPLACEMENT*/
/*Go me and my bad programming self*/
/*sx= sample size for timepoint=x*/
%macro samp_wr (data, s1, s2, s3, s4, s5, s6, num);
ods listing close;
%do g=1 %to &num %by 1;
    proc surveyselect data=&data outseed method=pps_wr sampsize=(&s1,
&s2, &s3, &s4, &s5, &s6);
        size n;
        strata strain dpi;
        id strain dpi diss;
    run;
%put g;
%end;
ods listing;
%mend;
%samp_wr (LR, 20, 20, 20, 20, 20, 20, 1001);
%samp_wr (pLR, 20, 20, 20, 20, 20, 20, 1001);

/*COMBINE MACRO CREDIT:UCLA website-
http://www.ats.ucla.edu/stat/SAS/code/mac_set.htm*/
/*Modified by me*/
%macro combine (id, start, stop);
```

```

data big&iid;
  set
  %do i = &start %to &stop %by 1;
    data&i
  %end;
;
run;
%mend;
%combine (LR, 1, 1001);
%combine (pLR, 1002, 2002);

ods listing;

/*Double checking the data to make sure the surveyselect worked
properly*/
/*InitialSeed is now the ID of each bootstrap*/
/* AN ID=1 INDICATES ORIGINAL DATA SET*/
data big; set bigLR bigpLR; run;
proc sort data=big; by strain dpi InitialSeed; run;
proc means data=big sum;
var NumberHits;
by strain dpi InitialSeed;
run;

data biglr; set biglr; rename InitialSeed=ID; run;
data bigpLR; set bigpLR; rename InitialSeed=ID; run;
data Lr; set Lr; ID=1; run;
data pLR; set pLR; ID=1; run;

/*Getting the calculated vector comp. values = total positive legs/
total tested */
/*THIS IS DONE ON THE ORIGINAL DATA AS WELL AS BOOTSTRAPPED DATA*/
%macro b (data);
proc sort data=&data; by dpi ID n; run;
ods listing close;
proc means data=&data;
by dpi ID n;
var diss;
output out=S&data sum=Sum;
run;
ods listing;

data S&data;
set S&data;
b=Sum/n;
run;
%mend;

%b (biglr);
%b (bigpLR);
%b (Lr);
%b (pLR);

/*A NEW ID NEEDS TO BE ADDED TO THE BOOTSTRAPPED SAMPLES SO THAT THERE
IS A SET OF IDs NOT JUST PER DPI*/
data NID;
  do i=2 to 1002 by 1;

```

```

        put i;
        output;
    end;
run;
data NID3; set NID NID NID NID NID NID; run;

%macro ID (data, original, new_data);
data &data;
merge &data NID3;
drop ID;
run;

proc sort data=&data; by i; run;

data &data;
set &data;
rename i=ID;
run;

data &new_data;
set &data &original;
drop _TYPE_ _FREQ_;
run;
%mend;

%ID (sbiglr, slr, LR);
%ID (sbigpLR, spLR, pLR);

/*Need to separate out the E-phase from the M-phase*/
/*Looking at the data, the delineation is around day 8 post exposure*/
/*So first set will be days 1-7, second set will be days 9 and 12*/

data Elr; strain="LR "; set Lr; if dpi <8 then output; run;
data EpLR; strain="pLR"; set pLR; if dpi <8 then output; run;
data Mlr; strain="LR "; set Lr; if dpi >8 then output; run;
data MpLR; strain="pLR"; set pLR; if dpi >8 then output; run;

data chik.Elr; set Elr; run;
data chik.EpLR; set EpLR; run;
data chik.Mlr; set Mlr; run;
data chik.MpLR; set MpLR; run;

/*DOING REGRESSION TO GET THE LINEAR FUNCTION OF B(N)*/
/*THERE IS ONLY THE E-PHASE, SO ONLY 1 LINE IS NEEDED*/
%macro reg (data, id);
ods listing close;
proc sort data=&data; by ID; run;
proc reg data=&data outest=reg&id;
by ID;
ID=ID;
model b=dpi / cli;
run; quit;
%mend;

%reg (ELR, ELR);
%reg (MLR, MLR);
%reg (EpLR, EpLR);
%reg (MpLR, MpLR);

```

```

data ErLR;
strain="LR ";
set RegElr;
keep ID _DEPVAR_ Intercept Dpi strain;
run;

data ErpLR;
strain="pLR";
set RegEpLR;
keep ID _DEPVAR_ Intercept Dpi strain;
run;

data chik.Reg_E;
set ErLR ErpLR;
run;

data MrLR;
strain="LR ";
set RegMlr;
keep ID _DEPVAR_ Intercept Dpi strain;
run;

data MrpLR;
strain="pLR";
set RegMpLR;
keep ID _DEPVAR_ Intercept Dpi strain;
run;

data chik.Reg_M;
set MrLR MrpLR;
run;

/*****EVC_cVC calculations Part 2,
                                with 2 phases
                                E-
Phase*****/
/****This part of the program performs the integration and EVC, cVC
calculations****/

libname chik "C:\Users\ODP\Desktop\SCHOOL\papers\ChikV\Higgs";

data chik; set chik.ELR chik.EpLR; run;

data reg;
set chik.Reg_E;
N=20;
run;

proc sort data=reg; by strain ID dpi; run;

/**dataset all has the simulated data for 1000 bootstraps with
replacement + the original data***/
/**data has the following variables:      strain, ID (bootstrap ID),
dpi (day post exposure),
                                           n (at each
dpi/strain), and b (dissemination rate)*****/

```

```

/****IMPORTANT to know which ID corresponds to the original experimental
data, as this will need to be extracted
from the large dataset
later*****
*****/

/* COMPUTATION OF THE AREA UNDER THE CURVE:

Area (Ahat) = (m(a^2)/-ln p) * integral(lower to upper) {p^N * b(N)} dN
              = (m(a^2)/-ln p) * integral(lower to upper) {p^N *
(betal(N) + beta0)} dN
              = (m(a^2)/-ln p) * [ betal * integral(lower to upper) {p^N
* N} dN + beta0 integral(lower to upper) {p^N} dN]
              = (m(a^2)/-ln p) * c0*Beta0 + c1*betal
Where

      Bmatrix (Bhat)= (b0 b1)' = [(X'X)^-1 * X' * p-hat]
      p-hat = [p1 p2 p3]' where pi= proportion at time i
      X=[1 t1
          1 t2
          1 ti] where ti are the time points of interest
              (t1 would be lower limit, ti would be upper
limit of intergral)

      c0      = integral(lower to upper) {p^N} dN = [1/(log p) ] *
[p^N_upper bound - p^N_lower bound]
      c1      = integral(lower to upper) {p^N * N} dN = [1/(log p)^2 *
(p^N_upper * (N_upper* log p) - p^N_upper -
      p^N_lower * (N_lower * log p) + p^N_lower)]

*/
/*Using Trapezoidal Approximation for Area Under the Curve*/
/*Macro by Rebecca Christofferson*/
%macro auc (a, b, n, function, j);
data is;
cx= ((b-a)/n);
do i = 0 to n;
put i; output;
end;
run;

data x&j;
set is;
if i=0 then x=a;
else if i=n then x=b;
else x=(a + (i*cx));
run;

data fx&j;
set x&j;
if i=0 then fx= (function/2);
else if i=n then fx= (function/2);
else fx= (function);
run;

proc sort data=x&j; by i; run;

```

```

proc sort data=fx&j; by i; run;

data data&j;
set x&j fx&j;
by i;
run;

proc summary data=data&j;
var fx;
output out=area&j sum=auc;
run;

data area&j;
    cx= ((&b-&a)/&n);
set area&j;
area=auc*cx;
run;

proc print data=area&j;
run;
%mend;

/*****SINCE ALL IN THE SAME MOSQUITO VECTOR, DO NOT NEED STRAIN
SPECIFIC QUANTITIES IN THIS PART*****/
/*****This is done on a per strain-ID basis*****/
%auc (1, 7, 150, (.91**x), c0);
%auc (1, 7, 150, ((.91**x)*x), c1);
/****These give quantities of the two matrices above****/

data c_matrix;
set areac0 areac1;
run;

/****Since I am holding constant m and a, arbitrarily assigned them a
value of 1****/
/**** The daily survival probably .91 is from:
Harrington LC, Buonaccorsi JP, Edman JD, Costero A,
Kittayapong P, et al. (2001)
Analysis of survival of young and old Aedes aegypti
(Diptera: Culicidac) from
Puerto Rico and Thailand. J Med Entomol 38: 537-547.
****/
data constants;
input m a p;
datalines;
1 1 .91
;
run;
data constants; set constants; const=m*(a**2)/ -(log(p)); run;

/*****Estimates of the Variance*****/
/* Variance (Ahat) = (constants^2) * (C0 C1)*Var(Bhat)*[(Co C1)']
(square root of this is the standard
error estimate)

where
Variance (Bhat) = [(X'X)^-1]*X'*[diag(var p-hat-i)]*X*(X'X)^-1

```



```

where
var (p-hat-i) = [ p1(1-p1)/n1      0      0
                  0      p2(1-p2)/n2      0
                  0      0      0
pi(1-pi)/ni]

*p has a binomial distribution; thus the binomial variances*
*****
*****/
/*variance matrix calculations*/
proc sort data=chik; by strain ID dpi n; run;
data m; set chik;
var=b*(1-b)/N; *binomial variance computation where b=p;
run;

/*seperating each strain into its own dataset*/
%macro sep (dataset, set, var, condition);
data &dataset;
set &set;
if &var=&condition then output;
run;
%mend;

%sep (sLRv, m, strain, "LR ");
%sep (spLRv, m, strain, "pLR");

%macro var (dataset, n, firstobs, label);
data s; set &dataset; run;
proc sort data=s; by ID dpi; run;
%do i=1 %to &n %by 1;
proc iml;
/*reading my variances calculated in the data step above*/
/*vp is my variance of p, where p= my b values (proportions)*/
/*variances calculated as binomial p(1-p)/n */
start var(varp1, varpsub, vp, x, xpx, vbhat, vb2, vb3, vb4);
use s; read all into varp1 var{var}; create checkvp from varp1;
append from varp1;
varpsub=varp1[1:4]; print varpsub;
/*****The varpsub matrix will need to be changed if a different
number of time points are used
EXAMPLE: for 4 time points, varpsub=
varp1[1:4]*****/
vp=diag(varpsub); print vp;
/*Rows 1-4 are for days 1, 3, 5, 7 of ID=1, Rows 5-8 for days 1, 3, 5,
7 for ID=2, etc.*/
create varp&label from vp; append from vp; print vp;
/*xpx is the x'x matrix*/
x = {1 1, 1 3, 1 5, 1 7};
/*****THIS MATRIX WILL NEED TO BE TAILORED TO SPECIFIC SAMPLING
TIME POINTS*****/
xpx=x`*x;
vbhat= inv(xpx)*x`;
vb2=vbhat*vp;
vb3=vb2*x;
vb4=vb3*inv(xpx); print vb4;
create vbhat&label from vb4; append from vb4;

```

```

finish;
run var(varp1, varpsub, vp, x, xpx, vbhat, vb2, vb3, vb4); quit;

data s;
set s (firstobs=&firstobs);
/****The firstobs takes the (firstobs-1) observations into the data
set****/
/****So if I want only the first three rows, firstobs=4****/
/****so for 4 time points, firstobs=5****/
run;

    %if i=1 %then %do;
        data varps&label; set varp&label; run;
        data vbhats&label; set vbhat&label; run;
    %end;
    %else %if i ne 1 %then %do;
        proc datasets library=work nolist; append base=varps&label
data=varp&label; run; quit;
        proc datasets library=work nolist; append base=vbhats&label
data=vbhat&label force; run; quit;
    %end;
/*Varps: the variance matrices of Proportions concatenated on top each
other 3x3*/
/*VBHats: the vbhat matrices concatenated on top each other 2x2 */
/*****THE DIMENSIONS OF THIS WILL CHANGE BASED ON THE NUMBER OF TIME
POINTS, ETC*****/
%put i;
%end;
%mend;
%var (sLRv, 1002, 5, LR);
%var (splrv, 1002, 5, pLR);
/*
data chik.EvarpsLR; set varpsLR; run;
data chik.EvbhatsLR; set vbhatsLR; run;
data chik.EvarpspLR; set varpspLR; run;
data chik.EvbhatspLR; set vbhatspLR; run;

/*****
/*****AREA CALCS = EVC *****/
/*****

data varpsLR; set chik.EvarpsLR; run;
data vbhatsLR; set chik.EvbhatsLR; run;
data varpspLR; set chik.EvarpspLR; run;
data vbhatspLR; set chik.EvbhatspLR; run;

/****This dataset contains my linear rate of change for each set of time
points (n=1001 bootstraps + original data)
    Contains the following variables: strain, ID (of bootstrap/orig),
intercept (of the linear relationship),
                                dpi (slope of
the line, or rate of change)*****/

/****The way this macro works: It reads the first observations for a
set of data points then deletes those observations,
                                loops around to redo the macro for each ID (set of
data points).
```

```

        There is no identifier, so it will be imperative to sort over ID
        and know which set of obs in the output corresponds
            to what ID
    ***/

    /***Seperated Data Sets: LR06r and SEAr***/
    %sep (LRr, reg, strain, "LR");
    %sep (pLRr, reg, strain, "pLR ");

    %macro area (b_data, vdata, n, firstobs, firstobsV, label, coef,
    intercept);
    /*****b_data is the data with the simulated linear relationship-
    regression estimates*****/
    /*****vdata is the data from the variance calculations above:
    vbhats_label from above*****/
    /*****n is the number of bootstraps plus original
    data*****/
    /*****firstobs, firstobsV, see
    below*****/
    /****coef is the name of the variable in b_data that is the slope of the
    linear relationship**/
    /*****intercept is the name of the variable in b_data corresponding to
    the intercept*****/
    /*****a is the day the integration
    starts*****/
    proc sort data=&b_data; by ID; run;
    data b; set &b_data; drop strain ID; run;
    data v; set &vdata; run;

    %do i=1 %to &n %by 1;
    proc iml;
    start area (bs, c, bhat, bhatt, A, v, VAhat, vbhat);
        use b; read all into bs var{&coef &intercept}; print bs; *Column
        one is int=B1, Column two is coef= B0;
        bhat=bs[2:1]; bhatt=bhat`; print bhat; *row one in bhat is
        B0, row 2 is B1;
        create bhat&label from bhatt; append from bhatt;
        use c_matrix; read all into c var{area}; *row one is c0, row two
        is C1; print c;
        A=c`*bhat;
        create area_est&label from A; append from A;

        /*use constants; read all into K var{const};
        Ahat=A*K; print Ahat;
        create area_est&label from Ahat; append from Ahat;*/

        use v; read all into v var{COL1 COL2};
        vbhat=v[1:2, 1:2]; *gives me the first two rows of the
        matrix;

        print vbhat;
        VAhat=c`*vbhat*c; print VAhat;
        *K2=k*k;
        *VAhatc=VAhat*K2; *print VAhatc;
        create varA_&label from VAhat; append from VAhat;

```

```

finish area; run area(bs, c, bhat, bhatt, A, v, VAhat, vbhat); quit;

data b;
set b (firstobs=&firstobs);
run;
/*****points to the firstobs=n
observation*****/
/*****EXAMPLE: For b, we want the first row at each iteration, so
firstobs=2*****/

data v;
set v (firstobs=&firstobsV);
/**The firstobsV value is based on the vbhat matrix and is a 2x2 matrix
for 2 parameter line, so firstobsV=3**/
run;

%if i=1 %then %do;
    data areas_est&label; set area_est&label; run;
    data varAs_&label; set varA_&label; run;
%end;
%else %if i ne 1 %then %do;
    proc datasets library=work nolist; append
base=areas_est&label data=area_est&label force; run; quit;
    proc datasets library=work nolist; append base=varAs_&label
data=varA_&label force; run; quit;
%end;
%put i;
%end;
%mend;
ods listing close;
%area (LRr, vbhatsLR, 1002, 2, 3, LR, dpi, intercept);
%area (pLRr, vbhatspLR, 1002, 2, 3, pLR, dpi, intercept);

data chik.Eareas_estLR; set areas_estLR; run;
data chik.EvarAs_LR; set varAs_LR; run;
data chik.Eareas_estpLR; set areas_estpLR; run;
data chik.EvarAs_pLR; set varAs_pLR; run;

/*****Test*****/
data areas_estLR; set chik.Eareas_estLR; run;
data varsAs_LR; set chik.EvarAs_LR; run;
data areas_estpLR; set chik.Eareas_estpLR; run;
data varAs_pLR; set chik.EvarAs_pLR; run;

/*****To Test,
compute a t*-like statistics like so:

t*i=[Ahat(i, strainA) -
Ahat(i, ref_strain)]/sqrt[(se(i, strainA))^2 + (se(i, ref_strain))^2]
for i to J simulations (J=1001)

Then get the cdf for t*i and get upper 97.5 and lower 0.025 quantiles
of the cdf and then compute the confidence intervals
*****/

data id;
do id=1 to 1002 by 1;

```

```

output;
put id; end;
run;

data areas_estLR; merge chik.areas_estLR id; rename COL1=AreaLR;
strain="LR "; run;
data areas_estpLR; merge chik.areas_estpLR id; rename COL1=AreapLR;
strain="pLR"; run;
data varAs_LR; merge chik.varAs_LR id; rename COL1=VarLR; strain="LR
"; run;
data varAs_pLR; merge chik.varAs_pLR id; rename COL1=VarpLR;
strain="pLR"; run;

proc sort data=areas_estLR; by ID; run;
proc sort data=VarAs_LR; by ID; run;
proc sort data=areas_estpLR; by ID; run;
proc sort data=VarAs_pLR; by ID; run;

/****Merging the LR and pLR data*****/
%macro merge (newdata, data1, dataRef, by);
data &newdata;
update &data1 &dataRef; by &by; drop strain; run;
%mend;
%merge (compE, areas_estLR, areas_estpLR, ID);
%merge (compvE, VarAs_LR, VarAs_pLR, ID);
%merge (compaE, compE, compvE, ID);

ods listing;
/****tstar = [(area1(obs)- area1(boot) - (Area2(obs)- area2(boot))] /
sqrt[(se1(boot)^2+se2(boot))^2]****/
%macro comp (data, REFid, COMPid, areaREF, areaComp);*REF=LR;
data &data;
set &data;
diffREF = (&areaREF-Area&REFid); *extant strain;
diffComp= (&areaComp- Area&COMPid); *comparison strain;
diff = diffREF - diffComp;
denom= sqrt(Var&COMPid + Var&REFid);
tstar= (diff-0) / denom;
run;
proc gchart data=&data; vbar tstar; run;quit;
%mend;
%comp (compaE, LR, pLR, 1.4051738854, 1.8537182764);
/*Values obtained from distribution analysis of tstar distribution*/

/*****EVC_cVC calculations Part 2
With 2 phases
M-
Phase*****/
/****This part of the program performs the integration and EVC, cVC
calculations****/

libname chik "C:\Users\ODP\Desktop\SCHOOL\papers\ChikV\Higgs";

data chik; set chik.MLR chik.MpLR; run;

```

```

data reg;
set chik.Reg_M;
N=20;
run;

proc sort data=reg; by strain ID dpi; run;

/**dataset all has the simulated data for 1000 bootstraps with
replacement + the original data***/
/**data has the following variables:      strain, ID (bootstrap ID),
dpi (day post exposure),
n (at each
dpi/strain), and b (dissemination rate)*****/
/**IMPORTANT to know which ID corresponds to the original experimental
data, as this will need to be extracted
from the large dataset
later*****/
*****/

/* COMPUTATION OF THE AREA UNDER THE CURVE:

Area (Ahat) = (m(a^2)/-ln p) * integral(lower to upper) {p^N * b(N)} dN
              = (m(a^2)/-ln p) * integral(lower to upper) {p^N *
(beta1(N) + beta0)} dN
              = (m(a^2)/-ln p) * [ beta1 * integral(lower to upper) {p^N
* N} dN + beta0 integral(lower to upper) {p^N} dN]
              = (m(a^2)/-ln p) * c0*Beta0 + c1*beta1
Where

Bmatrix (Bhat)= (b0 b1)' = [(X'X)^-1 * X' * p-hat]
p-hat = [p1 p2 p3]' where pi= proportion at time i
X=[1 t1
  1 t2
  1 ti] where ti are the time points of interest
(t1 would be lower limit, ti would be upper
limit of intergral)

c0 = integral(lower to upper) {p^N} dN = [1/(log p) ] *
[p^N_upper bound - p^N_lower bound]
c1 = integral(lower to upper) {p^N * N} dN = [1/(log p)^2 *
(p^N_upper * (N_upper* log p) - p^N_upper -
p^N_lower * (N_lower * log p) + p^N_lower)]

*/
/*Using Trapezoidal Approximation for Area Under the Curve*/
/*Macro by Rebecca Christofferson*/
%macro auc (a, b, n, function, j);
data is;
cx= ((&b-&a)/&n);
do i = 0 to &n;
put i; output;
end;
run;

data x&j;
set is;

```

```

if i=0 then x=&a;
    else if i=&n then x=&b;
    else x=(&a + (i*cx));
run;

data fx&j;
set x&j;
if i=0 then fx= (&function/2);
    else if i=&n then fx= (&function/2);
    else fx= (&function);
run;

proc sort data=x&j; by i; run;
proc sort data=fx&j; by i; run;

data data&j;
set x&j fx&j;
by i;
run;

proc summary data=data&j;
var fx;
output out=area&j sum=auc;
run;

data area&j;
    cx= ((&b-&a)/&n);
set area&j;
area=auc*cx;
run;

proc print data=area&j;
run;
%mend;

/*****SINCE ALL IN THE SAME MOSQUITO VECTOR, DO NOT NEED STRAIN
SPECIFIC QUANTITIES IN THIS PART*****/
/*****This is done on a per strain-ID basis*****/
%auc (9, 12, 150, (.91**x), c0);
%auc (9, 12, 150, ((.91**x)*x), c1);
/****These give quantities of the two matrices above****/

data c_matrix;
set areac0 areac1;
run;

/****Since I am holding constant m and a, arbitrarily assigned them a
value of 1****/
/**** The daily survival probably .91 is from:
        Harrington LC, Buonaccorsi JP, Edman JD, Costero A,
Kittayapong P, et al. (2001)
        Analysis of survival of young and old Aedes aegypti
(Diptera: Culicidac) from
        Puerto Rico and Thailand. J Med Entomol 38: 537-547.
****/
data constants;
input m a p;

```

```

datalines;
1 1 .91
;
run;
data constants; set constants; const=m*(a**2)/ -(log(p)); run;

/*****Estimates of the Variance*****/
/* Variance (Ahat) = (constants^2) * (C0 C1)*Var(Bhat)*[(Co C1)']
(square root of this is the standard
error estimate)

where
Variance (Bhat) = [(X'X)^-1]*X'*[diag(var p-hat-i)]*X*(X'X)^-1

where
var (p-hat-i) = [ p1(1-p1)/n1      0      0
                  0      p2(1-p2)/n2      0
                  0      0      pi(1-pi)/ni]

*p has a binomial distribution; thus the binomial variances*
*****/
/*variance matrix calculations*/
proc sort data=chik; by strain ID dpi n; run;
data m; set chik;
var=b*(1-b)/N; *binomial variance computation where b=p;
run;

/*seperating each strain into its own dataset*/
%macro sep (dataset, set, var, condition);
data &dataset;
set &set;
if &var=&condition then output;
run;
%mend;

%sep (sLRv, m, strain, "LR ");
%sep (spLRv, m, strain, "pLR");

%macro var (dataset, n, firstobs, label);
data s; set &dataset; run;
proc sort data=s; by ID dpi; run;
%do i=1 %to &n %by 1;
proc iml;
/*reading my variances calculated in the data step above*/
/*vp is my variance of p, where p= my b values (proportions)*/
/*variances calculated as binomial p(1-p)/n */
start var(varp1, varpsub, vp, x, xpx, vbhat, vb2, vb3, vb4);
use s; read all into varp1 var{var}; create checkvp from varp1;
append from varp1;
varpsub=varp1[1:2]; print varpsub;
/*****The varpsub matrix will need to be changed if a different
number of time points are used
EXAMPLE: for 4 time points, varpsub=
varp1[1:4]*****/
vp=diag(varpsub); print vp;

```



```

/*Rows 1-4 are for days 1, 3, 5, 7 of ID=1, Rows 5-8 for days 1, 3, 5,
7 for ID=2, etc.*/
    create varp&label from vp; append from vp; print vp;
/*xpx is the x'x matrix*/
    x = {1 9, 1 12};
/*****THIS MATRIX WILL NEED TO BE TAILORED TO SPECIFIC SAMPLING
TIME POINTS*****/
    xpx=x`*x;
    vbhat= inv(xpx)*x`;
    vb2=vbhat*vp;
    vb3=vb2*x;
    vb4=vb3*inv(xpx); print vb4;
    create vbhat&label from vb4; append from vb4;
finish;
run var(varp1, varpsub, vp, x, xpx, vbhat, vb2, vb3, vb4); quit;

data s;
set s (firstobs=&firstobs);
/****The firstobs takes the (firstobs-1) observations into the data
set****/
/****So if I want only the first three rows, firstobs=4****/
/****so for 4 time points, firstobs=5****/
run;

    %if i=1 %then %do;
        data varps&label; set varp&label; run;
        data vbhats&label; set vbhat&label; run;
    %end;
    %else %if i ne 1 %then %do;
        proc datasets library=work nolist; append base=varps&label
data=varp&label; run; quit;
        proc datasets library=work nolist; append base=vbhats&label
data=vbhat&label force; run; quit;
    %end;
/*Varps: the variance matrices of Proportions concatenated on top each
other 3x3*/
/*VBHats: the vbhat matrices concatenated on top each other 2x2 */
/*****THE DIMENSIONS OF THIS WILL CHANGE BASED ON THE NUMBER OF TIME
POINTS, ETC*****/
%put i;
%end;
%mend;
%var (sLRv, 1002, 3, LR);
%var (splrv, 1002, 3, pLR);
/*
data chik.MvarpsLR; set varpsLR; run;
data chik.MvbhatsLR; set vbhatsLR; run;
data chik.MvarpspLR; set varpspLR; run;
data chik.MvbhatspLR; set vbhatspLR; run;

/*****
/*****AREA CALCS = EVC *****/
/*****
data varpsLR; set chik.varpsLR; run;
data vbhatsLR; set chik.vbhatsLR; run;
data varpspLR; set chik.varpspLR; run;

```

```

data vbhatspLR; set chik.vbhatspLR; run;

/****This dataset contains my linear rate of change for each set of time
points (n=1001 bootstraps + original data)
    Contains the following variables: strain, ID (of bootstrap/orig),
intercept (of the linear relationship),
                                dpi (slope of
the line, or rate of change)*****/

/****The way this macro works:  It reads the first observations for a
set of data points then deletes those observations,
                                loops around to redo the macro for each ID (set of
data points).
    There is no identifier, so it will be imperative to sort over ID
and know which set of obs in the output corresponds
                                to what ID
***/

/****Seperated Data Sets: LR06r and SEAr****/
%sep (LRr, reg, strain, "LR");
%sep (pLRr, reg, strain, "pLR ");

%macro area (b_data, vdata, n, firstobs, firstobsV, label, coef,
intercept);
/*****b_data is the data with the simulated linear relationship-
regression estimates*****/
/*****vdata is the data from the variance calculations above:
vbhats_label from above*****/
/*****n is the number of bootstraps plus original
data*****/
/*****firstobs, firstobsV, see
below*****/
/****coef is the name of the variable in b_data that is the slope of the
linear relationship**/
/*****intercept is the name of the variable in b_data corresponding to
the intercept*****/
/*****a is the day the integration
starts*****/
proc sort data=&b_data; by ID; run;
data b; set &b_data; drop strain ID; run;
data v; set &vdata; run;

%do i=1 %to &n %by 1;
proc iml;
start area (bs, c, bhat, bhatt, A, v, VAhat, vbhat);
    use b; read all into bs var{&coef &intercept}; print bs; *Column
one is int=B1, Column two is coef= B0;
    bhat=bs[2:1]; bhatt=bhat`; print bhat; *row one in bhat is
B0, row 2 is B1;
    create bhat&label from bhatt; append from bhatt;
    use c_matrix; read all into c var{area}; *row one is c0, row two
is C1; print c;
    A=c`*bhat;
    create area_est&label from A; append from A;

```

```

/*use constants; read all into K var{const};
  Ahat=A*K; print Ahat;
  create area_est&label from Ahat; append from Ahat;*/

use v; read all into v var{COL1 COL2};
  vbhat=v[1:2, 1:2]; *gives me the first two rows of the
matrix;

  print vbhat;
  VAhat=c`*vbhat*c; print VAhat;
  *K2=k*k;
  *VAhatc=VAhat*K2; *print VAhatc;
  create varA_&label from VAhat; append from VAhat;
finish area; run area(bs, c, bhat, bhatt, A, v, VAhat, vbhat); quit;

data b;
set b (firstobs=&firstobs);
run;
/*****points to the firstobs=n
observation*****/
/*****EXAMPLE: For b, we want the first row at each iteration, so
firstobs=2*****/

data v;
set v (firstobs=&firstobsV);
/*The firstobsV value is based on the vbhat matrix and is a 2x2 matrix
for 2 parameter line, so firstobsV=3*/
run;

%if i=1 %then %do;
  data areas_est&label; set area_est&label; run;
  data varAss_&label; set varA_&label; run;
%end;
%else %if i ne 1 %then %do;
  proc datasets library=work nolist; append
base=areas_est&label data=area_est&label force; run; quit;
  proc datasets library=work nolist; append base=varAs_&label
data=varA_&label force; run; quit;
%end;
%put i;
%end;
%mend;
ods listing close;
%area (LRr, vbhatsLR, 1002, 2, 3, LR, dpi, intercept);
%area (pLRr, vbhatspLR, 1002, 2, 3, pLR, dpi, intercept);

data chik.Mareas_estLR; set areas_estLR; run;
data chik.MvarAs_LR; set varAs_LR; run;
data chik.Mareas_estpLR; set areas_estpLR; run;
data chik.MvarAs_pLR; set varAs_pLR; run;

/*****Test*****/
data areas_estLR; set chik.Mareas_estLR; run;
data varsAs_LR; set chik.MvarAs_LR; run;
data areas_estpLR; set chik.Mareas_estpLR; run;
data varAs_pLR; set chik.MvarAs_pLR; run;

```

```

/*****To Test,
compute a t*-like statistics like so:

      t*i=[Ahat(i,strainA) -
Ahat(i,ref_strain)]/sqrt[(se(i,straia))^2 + (se(i,ref_strain))^2]
for i to J simulations (J=1001)

Then get the cdf for t*i and get upper 97.5 and lower 0.025 quantiles
of the cdf and then compute the confidence intervals
*****/

data id;
do id=1 to 1002 by 1;
output;
put id; end;
run;

data areas_estLR; merge chik.areas_estLR id; rename COL1=AreaLR;
strain="LR "; run;
data areas_estpLR; merge chik.areas_estpLR id; rename COL1=AreapLR;
strain="pLR"; run;
data varAs_LR; merge chik.varAs_LR id; rename COL1=VarLR; strain="LR
"; run;
data varAs_pLR; merge chik.varAs_pLR id; rename COL1=VarpLR;
strain="pLR"; run;

proc sort data=areas_estLR; by ID; run;
proc sort data=VarAs_LR; by ID; run;
proc sort data=areas_estpLR; by ID; run;
proc sort data=VarAs_pLR; by ID; run;

/****Merging the LR and pLR data*****/
%macro merge (newdata, data1, dataRef, by);
data &newdata;
update &data1 &dataRef; by &by; drop strain; run;
%mend;
%merge (compE, areas_estLR, areas_estpLR, ID);
%merge (compvE, VarAs_LR, VarAs_pLR, ID);
%merge (compaE, compE, compvE, ID);

ods listing;
/****tstar = [(area1(obs)- area1(boot) - (Area2(obs)- area2(boot))] /
sqrt[(se1(boot)^2+se2(boot))^2]***/
%macro comp (data, REFI, COMPID, areaREF, areaComp);*REF=LR;
data &data;
set &data;
diffREF = (&areaREF-Area&REFID); *extant strain;
diffComp= (&areaComp- Area&COMPID); *comparison strain;
diff = diffREF - diffComp;
denom= sqrt(Var&COMPID + Var&REFID);
tstar= (diff-0) / denom;
run;
proc gchart data=&data; vbar tstar; run;quit;
%mend;
%comp (compaE, LR, pLR, 1.0889, 1.06225);
/*Values obtained from distribution analysis of tstar distribution*/

```

```

/*****Percentiles*****/
/*      Using SAS INSIGHT to obtain: Solutions-->Analysis-->Interactive
Data Analysis
97.5 and 2.5 percentiles of T* will give us 95% confidence interval
Compa: 97.5 = 1.4017      2.5 = -1.2096
/*****/
/*****Building Confidence Intervals with the Original
Data*****/
/****This assumes the first set of data is the original, which it is
here*****/
data compO; set compaE; if ID=1 then output; run;

%macro CI (datain, REFid, thi, tlow, COMPid);
data &datain;
set &datain;
diff= (Area&COMPid - Area&REFid);
sum=(Var&COMPid + Var&REFid);
se=sqrt(sum);
UCL= diff + (abs(&thi))*se;
LCL= diff - (abs(&tlow))*se;
run;
%mend;

%CI (compO, LR, 1.4017, -1.2096, pLR);
proc print data=compO; run;

```

Appendix 5

EVC and cVC Detailed Calculations

For strain i , the cumulative vectorial capacity (cVC) is the sum of the components of the exponential and M-phases:

Exponential Growth Phase:

$$\frac{ma^2}{-\ln p} \int_x^y p^N (\beta_{1i} N + \beta_{0i}) dN = \frac{ma^2}{-\ln p} \int_x^y (p^N \beta_{1i} N + p^N \beta_{0i}) dN = \frac{ma^2}{-\ln p} \varphi$$

Where φ is the effective vector competence (EVC) parameter.

$$\begin{aligned} \varphi &= [\beta_{1i} \int_x^y p^N N dN + \beta_{0i} \int_x^y p^N dN] \\ &= [\beta_{1i} Z_0 + \beta_{0i} Z_1] \end{aligned}$$

$$\widehat{Area}_{Exponential\ Phase} = \frac{ma^2}{-\ln p} [\beta_{1i} Z_0 + \beta_{0i} Z_1]$$

S. Definition 1

M-phase:

$$\frac{ma^2}{-\ln p} \int_y^z p^N (\beta_{0i}) dN = \frac{ma^2}{-\ln p} \left[\int_y^z p^N dN \right]$$

$$\widehat{Area}_{M-Phase} = \frac{ma^2}{-\ln p} \left[\beta_{0i} \int_y^z p^N dN \right]$$

S. Definition 2

cVC then, is given by:

$$\widehat{Area}_{Exponential\ Phase} + \widehat{Area}_{M-Phase}$$

S. Eq. 1

To calculate variance estimates:

Let:

$$\widehat{\beta}_i = \begin{Bmatrix} \beta_{0i} \\ \beta_{1i} \end{Bmatrix} = (\mathbf{X}'\mathbf{X})^{-1} \mathbf{X}'\mathbf{b}$$

S. Definition 4

Where $\mathbf{X} = \begin{bmatrix} 1 & N_1 \\ 1 & N_2 \\ \vdots & \vdots \\ 1 & N_t \end{bmatrix}$ where N_i is the time value at sampled time point t

S. Definition 5

And $\hat{\mathbf{b}} = \begin{bmatrix} b_1 \\ b_2 \\ \vdots \\ b_n \end{bmatrix}$ where b is the observed proportion of disseminated infections at time t

S. Definition 6

Then for strain i at time points 1 to t :

$$Var(\hat{\boldsymbol{\beta}}_i) = (\mathbf{X}'\mathbf{X})^{-1}\mathbf{X}' \left[diag \left(\frac{p_{t1}(1 - b_{t1})}{n_{t1}}, \dots, \frac{b_{tn}(1 - b_{tn})}{n_{tn}} \right) \right] \mathbf{X}(\mathbf{X}'\mathbf{X})^{-1}$$

S. Definition 7

And the variance of the area estimate for strain i is:

$$Var(\widehat{Area}_i) = \left(\frac{ma^2}{-\ln p} \right)^2 (Z_0, Z_1) Var(\hat{\boldsymbol{\beta}}_i) \begin{pmatrix} Z_0 \\ Z_1 \end{pmatrix} \quad \text{S. Eq. 2}$$

For Variance (E-phase + M-phase)

a) Of EVC (without the inclusion of entomological constants)

$$= Var(\text{E-phase}) + Var(\text{M-phase})$$

b) Of cVC (with the inclusion of entomological constants)

$$= \left(\frac{ma^2}{-\ln p} \right)^2 Var(\text{E-phase}) + \left(\frac{ma^2}{-\ln p} \right)^2 Var(\text{M-phase})$$

Vita

Rebecca “Becky” Carriere Christofferson was born in Lafayette, Louisiana, and grew up with a slew of cousins around the Opelousas area. She has a twin brother, two loving and supportive parents, a remarkable husband, a beautiful daughter, and a second baby due in September 2011.

When she was six, her family moved to Houston for a very long 14-month period before coming to their senses and moving back to Louisiana. Settled in Baton Rouge, Becky eventually attended high school at St. Joseph’s Academy where she participated in the marching, symphonic and jazz bands as a trumpet player. This in addition to her first love, the piano, which she plays with her brother (drums) and father (bass), forming a jazz trio.

After high school, Becky attended Louisiana State University and was extremely proud to be accepted into the LSU Tiger marching band. It was while in college that she met her husband whom she married in 2002, just 2 months before graduating with her Bachelor of Science in Zoology.

After graduation, she continued to work for the LSU College of Agriculture, where she had been a student worker. She was hired full time as assistant to Dean Kenneth L. Koonce, and was incredibly lucky to learn a great deal about university workings as well as agriculture in the state. During that time, she worked on her Master of Applied Statistics part-time and graduated in December 2005. Two years later, she entered the doctoral program in the Department of Pathobiological Sciences at the LSU School of Veterinary Medicine where she was lucky enough to join the lab of Dr.

Christopher Mores. She is particularly interested in modeling different aspects of infectious disease transmission, especially arboviruses. She enjoys the creative and sometimes puzzling processes of grant writing, manuscript preparation and thinking of new avenues to explore. She also enjoys the quantitative aspects of data analysis and experimental design.

When she remembers what free time is, she enjoys reading, writing, music, and thinking of new and crazy ideas to research. Her most favorite pastime is, of course, playing with daughter Evelyn Rose.