The Dissertation Committee for Nicholas Andrew Bergren Certifies that this is the approved version of the following dissertation:

EVOLUTIONARY INFLUCENCES ON THE REDUCTION IN ENZOOTIC CIRUCLATION AND HUMAN INCIDENCE OF WESTERN EQUINE ENCEPHALITIS

Committee:

Scott C. Weaver, M.S., Ph.D. Supervisor

Robert B. Tesh, M.D.

Judith F. Aronson, M.D.

Dennis A. Bente, D.V.M. Ph.D.

William K. Reisen, Ph.D.

_____

Dean, Graduate School

EVOLUTIONARY INFLUCENCES ON THE REDUCTION IN ENZOOTIC CIRUCLATION AND HUMAN INCIDENCE OF WESTERN EQUINE ENCEPHALITIS

by

Nicholas Andrew Bergren, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

July 2016

Dedication

This dissertation is dedicated to

my Wife, Rebekkah Bergren,

my Mother, Denise Pool,

my Grandmothers, Barbara Bowman and Bette Joy

and my Great Grandmother Jane Russell.

Without their unwavering support, love, encouragement, and influence

this endeavor would have been impossible

S.D.G.

Acknowledgements

This dissertation would not have been possible if it weren't for the help and support of a great multitude of people. First and most importantly, I would thank my mentor, Scott Weaver, for his patience, kindness, willingness to teach, and expertly executing the tasks required of a successful PI (1. fundraising, 2) throwing parties to keep the masses at bay, and 3) making sure the masses don't get in trouble with the Feds!). Special thanks goes to Shannan Rossi who has spent much time and energy teaching, mentoring, helping with experiments and being a great friend. Robert Seymour has been a great friend, always quick with a Monty Python reference or a good conversation; he also provided much help in analyzing the histopathology. Thanks to the patient and kind efforts of Rodion Gorchakov, I learned that cloning is equal parts art and science. Jonathan Auguste and Naomi Forrester helped teach me the magic methods of phylogenetics. Jonathan also was very kind in including me on side projects that would allow me to practice and hone my skills in phylogenetics. I am thankful for the example of Konstantin Tsetsarkin, who showed me how to ask questions. Special thanks to Nik Vasilakis for teaching me the techniques necessary to work with mosquitoes, and demanding a high level of competency. Furthermore, Nik kindly included me on several projects that expanded my interest in the virus-mosquito interaction; he also introduced me to top-shelf gin, to which I am very thankful. I am very thankful for the efforts of Grace Leal and Ruimei (Mei) Yun who keep the lab running smoothly and are always willing to help. Thanks to Jing Huang I always had mosquitoes ready to work with and she helped significantly with dissecting an overwhelming amount of mosquitoes. All of the other members of the Weaver Legion helped generate a spirit of comradery and unity that made the Weaver Lab a joyful place to work for the last six years. I thank Rick Pyles and Aaron Miller in the Assay Development Service Division for their efforts in conducting the pyrosequencing work. I also want to thank my committee members

Robert Tesh, Judy Aronson, Dennis Bente, and William Reisen all of whom were always supportive and willing to share their knowledge.

Many people outside of UTMB also helped make this dissertation come to fruition. Lauren Wilkerson with the Houston Mosquito Control Division of Harris County Public Health Services helped catch house sparrows and teach me how to handle and manipulate them. Aaron Brault with the Centers for Disease Control and Prevention provided the Imperial181 clone which was critical for the completion of the dissertation. Richard Bowen, of Colorado State University, out of the kindness of his heart and interest in science agreed to compete some intensive experiments involving house sparrows. In short, this dissertation would not have been possible without the efforts of any of these kind people.

EVOLUTIONARY INFLUCENCES ON THE REDUCTION IN ENZOOTIC CIRUCLATION AND HUMAN INCIDENCE OF WESTERN EQUINE ENCEPHALITIS

Publication No._____

Nicholas Andrew Bergren, Ph.D.

The University of Texas Medical Branch, 2016

Supervisor: Scott C. Weaver

Understanding the evolutionary and ecological circumstances in which arboviruses emerge into naïve geographical areas is critical for the development of targeted maintenance and prevention strategies. In order to develop a complete understanding of the ways in which viruses emerge, the factors surrounding a reduction in virus activity, or submergence, must also be studied. Western equine encephalitis virus (WEEV) provides a unique case study on how submergence can be understood by observing evolutionary and ecological factors. WEEV caused several epizootic events in the early 20th century that account for the death of thousands humans and equids. Later in the century, the number of reported cases dropped with the last human case occurring in 1998. However, WEEV has still been detected in mosquito, albeit at reduced levels. My studies identified six nonsynonymous mutations that were phylogenetically significant for the evolution of WEEV through the 20th century. I also found that these mutations have a phenotypic effect on WEEV's enzootic hosts. Using competitive fitness assays, contemporary mutations have a competitive advantage in *Culex tarsalis* and possibly house sparrows. Additionally, these mutations have no effect on virulence in the Syrian

golden hamster. Given this data I propose that the mutations WEEV has accumulated by positive selection only enhance this ability to transmit enzootically. I also hypothesize the mutations that confer mammalian virulence were purified out of the population by negative selection due to a reduction in selective pressure on those residues. Overall, the evolution of WEEV over the 20th century trends away from disease in mammals and toward its enzootic cycle. A number of factors could account for this ranging from the vaccination and drastic reduction of the US equine population, the use of screens on windows and doors, and/or changes in agricultural practices. The submergence of WEEV was likely precipitated by an ecological shift critical for the virus' maintenance of the mutations that confer virulence in mammals and subsequently compensated by increasing its adaptability to its enzootic hosts. However, these compensatory mutations may be an example of too little too late as evidenced by the subsequent decline in population size.

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List of Abbreviations

A	adenosine
C	cytosine
CEF	chick embryonic fibroblast
CF	complement fixation
CHIKV	chikungunya virus
CNS	central nervous system
CSU	Colorado State University
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
EEEV	Eastern equine encephalitis virus
EIP	extrinsic incubation period
ESS	estimated sample size
FBS	fetal bovine serum
FEL	fixed-effect likelihood
FUBAR	fast unbiased Bayesian approximation
G	guanine
HI	hemagglutination-inhibition
HP	heparan sulfate
hpi	hours post infection
HPD	highest posterior density
HOSP	house sparrow
ICA	infectious center assay
IFEL	internal fixed-effect likelihood
IMP181	Imperial181
IN	intranasal
MCC	maximum clade credibility
MCMC	Markov chain Monte Carlo
MOI	multiplicity of infection
MTD	mean time to death
mRNA	messenger RNA
NHP	non-human primate
nsP	nonstructural protein
ORF	open reading frame
PDB	protein database
Penn-Strep	Penicillin & Streptomycin
PFU	plaque forming unit
polyA	polyadenylated
RNA	ribonucleic acid
SC	subcutaneous
SINV	Sindbis virus
SLAC	single likelihood ancestor counting
T	thymine

TB	Terrific Broth
TE	Tris-EDTA
U	uracil
UCEX	uncorrelated exponential clock
UCLN	uncorrelated log-normal clock
UPGMA	unweighted pair group method with arithmetic
UTMB	University of Texas Medical Branch
UTR	untranslated region
VEEV	Venezuelan equine encephalitis virus
WCSP	white-crowned sparrow
WEE	Western equine encephalomyelitis/encephalitis
WEEV	Western equine encephalitis virus

Chapter 1: Introduction

EVOLUTION AND THE PHYLOGENETIC APPROACH

Since Darwin's 1859 publication of *On the Origin of Species by Natural Selection* where he uses his observation of finches on the Galapagos Islands to describe his theory of Decent with Modification (Darwin, 1859), scientists have been attempting to create models that accurately reflect the evolutionary path of different subsets of life on Earth. Before the introduction of sequencing technology this process was almost exclusively accomplished by the comparison of various morphological traits among similar organisms. However, with the ability to sequence an organism's genes the discipline of phylogenetics, estimating the evolutionary history of an organism based on sequence information, has become a general practice among scientists. However, many pitfalls exist in the development of these phylogenies and if the theory and methodologies are not properly understood then errors in approach and conclusions are close at hand.

Principles of Viral Evolution

GENETIC INFORMATION

The genome is the entity of organisms that carries its genetic information. In most organisms this is stored as deoxyribonucleic acid (DNA), however some viruses have a genome that consists of ribonucleic acid (RNA). The structure of DNA is that of a double helix where two polynucleotide strands run antiparallel with each other. The backbone consists of a series of deoxyriboses with phosphodiester groups between each sugar moiety covalently linking the 5' and 3' carbons. On each sugar, covalently linked to the 1' carbon is a base. Bases consist of adenine (A), guanine (G) (purines), thymine (T), or cytosine (C) (pyrimidines). The two main differences between DNA and RNA are their sugar moieties, deoxyriboses in DNA and ribose in RNA, and T in DNA being replaced

with uracil (U). Bases pair together via hydrogen bonding (A bonds with T/U, resulting in two hydrogen bonds; C bonds with G, resulting in three hydrogen bonds). This base pairing is the basis for the antiparallel nature of double stranded nucleic acids.

In order to use the information stored in nucleic acids RNA is encoded into protein (for organisms with DNA genomes, the DNA is first transcribed to RNA). Note that RNA has many other functions including transferring amino acids to their proper location in the ribosome/RNA complex (tRNA), participating in the functions of the ribosome (rRNA), regulating gene expression (siRNA), etc., however, here focus is given to RNA acting as a means of providing the information necessary to synthesize proteins (mRNA). A universal genetic code exists for all organisms, with few exceptions, and is the biological language that allows specific amino acid sequences to be constructed. Every three bases consists of a codon which codes for a particular amino acid, while there are 64 (4^3) different codon possibilities only 20 amino acids and 3 stop codons are possible, given the inherent redundancy of this code, it is said to be degenerate.

One of an organism's necessary functions is the replication of its genomic material in order to grow and produce progeny. Through a number of different processes: environmental or errors in the polymerase non-complementary nucleotides can be incorporated into the genome. While the genetic code has allowed for some variation most mutations will cause a change in amino acid. For example, 60% of all mutation occurring in the third base position of a codon will not change the amino acid residue while only 4% of mutations at the first or second codon position will result in no change in amino acid (Vandamme, 2009). Mutations that result in no change in amino acid are referred to as synonymous mutations and mutations that change an amino acid are called non-synonymous mutations. In addition to non-complementary bases being able to be incorporated into the nucleic acid sequence, insertions or deletions (indels) of nucleotides are another mechanism of introducing mutation into an organism's genome. Indels can be particularly deleterious to an organism when the number of bases being inserted or

deleted is not a multiple of three. This type of mutation alters the reading frame of the sequence (hence the term frame-shift mutation) and usually causes the accumulation of stop codons resulting in a truncated protein. However, some viruses do make use of this phenomenon to make other proteins accessible. Other factors are known to influence the genetic variation of an organism, however, as this dissertation is focused on the viral evolution, they are outside the scope of discussion.

EVOLUTIONARY PRINCIPLES

Alterations that are passed on in the genome of an organism are referred to as polymorphisms. Generally polymorphisms can either be maintained in the population while coexisting with the original variant, become fixed in the population by displacing the original variant, or be lost due to some type of disadvantage or stochastic change. The rate at which these polymorphisms are fixed in the population is called the fixation rate. Fixation rate is primarily used when comparing two different populations or species. However, when comparing mutations among individuals within a population that will eventually become fixed the term is changed to substitution rate.

As polymorphisms arise in a population the selective pressures act on the polymorphism in a manner dependent on the properties of the polymorphism. If the polymorphism, here referred to as an allele, increases the fitness of the organism, as compared to individuals with the original allele then it will be subject to positive selection. That is, the new allele will be selected for due to its beneficial properties. If the new allele imparts a negative impact on the organism then it will be purified out of the population because the deleterious impact of the new allele will lower the fitness of the organism hence the allele will not be passed on to successive generations. These types of selection are collectively referred to natural selection and represent a deterministic model of evolution (Vandamme, 2009). Conversely, stochastic evolution refers to probabilistic mechanisms in play when random fluctuations determine allele frequency. This model is

commonly interchanged with the term genetic drift, however even though these two concepts are inextricably linked, genetic drift specifically refers to the random selection on alleles based on the random selection of individuals that will contribute to the next generation (Vandamme, 2009). The stochastic model can only provide probability of allele frequencies in the next generation. Note that this model still takes into account reproductive fitness of the allele variants. Under this model mutations can arise in organisms that have no impact on fitness hence their substitution rate is solely attributed to stochastic events within the population.

One major factor that affects the rate at which mutations get fixed in the population through stochastic processes is the effective population size. This is defined as the size of an idealized population that is randomly mating and that has the same gene frequency changes as the population being studies (the "census" population) (Vandamme, 2009). Note that the effective population size can be significantly different from the total population size if a significant proportion of the total population is not producing offspring (e.g. interbreeding, population subdivision, selection on linked mutations). As implied above the rate of evolution is heavily influenced by the effective population size and when the effective population size varies from generation to generation the rate of evolution can be significantly affected. For example, if the effective population size is greatly diminished and thus the effective population becomes more homogeneous a genetic bottleneck is said to have occurred (Vandamme, 2009). This situation is particularly problematic for asexual organisms because as their effective population size is decreased a phenomenon called Muller's Ratchet can have a larger effect. Muller's Ratchet refers to the phenomenon where fitness declines as a result of the accumulation of deleterious mutations and the populations' inability to purify them out of the population due to its reduced size (i.e. all individuals have the deleterious mutations resulting in the loss of mutation-free individuals) (Page and Holmes, 1998).

Evolution, as it occurs in nature, is never entirely deterministic or stochastic, but a mixture of the two with the effective population size and selective factors both influencing the evolutionary trajectory of the organism. While genetic mutations always arise due to random events, they can sometimes result in some sort of fitness advantage. For these mutations positive selection will push the mutation toward fixation in the population. Mutations that are deleterious will be purified from the population due to the reduction in fitness, which is negative selection. Sometimes mutations under negative selection can be fixed in the population by genetic drift if they are not significantly deleterious, though more generation times are needed for fixation to occur.

Generating Molecular Phylogenies

Sequence Data and Alignments

The first question one must ask when conducing a phylogenetic analysis is: Are the sequences being analyzed homologous? That is developing a phylogeny operates under the assumption that the sequence information being studies all derived from a common ancestor. If the answer is yes then the next step is to assess whether or not the sequences are similar enough to determine the relationship between the sequences. DNA and RNA are both composed of four bases. Therefore, if gaps are not allowed, on average 25% of two random sequences would be identical. When gaps are allowed this similarity between two random sequences increases to 50% (Vandamme, 2009). Thus phylogenetic analyses should be conducted on sequences that have 60% or greater sequence similarity. Sometimes investigators will study genes with regions of hyper-variability. Generally these sequences are excluded from the analyses and only the conserved sequence fragments are studied.

Arguably the most critical aspect of developing a phylogeny is the alignment. The establishment of evolutionary relationships between two different sequences is the ultimate goal of phylogenetics. In order to accomplish the goal of establishing

evolutionary relationships, homologous sites within the gene must be compared to one another. This is accomplished by aligning homologous sites of sequence information into columns, commonly referred to the alignment. For closely related organisms alignments can be easy, but as organisms diverge a proper alignment becomes more difficult due to increasing sequence dissimilarity (Vandamme, 2009). While alignments can be constructed by eye, software packages do exist that automate the process though fine-tuning of the alignment is required after ran through a software package.

PHYLOGENETIC TREES

Phylogenetic trees are the graphical representations scientists use to describe the relatedness of different organisms. The terminology used to reflect specific parts of the tree (i.e. root, branch, node) reflect the fact that these diagrams are similar in structure to actual trees. There are a number of different ways to develop a tree and the resulting branching pattern is collectively referred to as a tree topology. This section will cover the basics of the different algorithms scientists can use to develop phylogenetic trees and the benefits and drawbacks to each method.

UPGMA

The oldest and simplest method of developing phylogenetic trees is the unweighted pair group method with arithmetic (UPGMA) method. Initially, clusters are developed by grouping taxa with the smallest pairwise distance. Resulting clusters are then considered one taxa and this process is repeated until all taxa are clustered (Vandamme, 2009). One draw back to the UPGMA tree is that it assumes the evolutionary rate between all taxa are identical. This assumption frequently results in a tree that does not reflect what occurred in nature.

Neighbor-joining

The Neighbor-joining method develops first by assuming a star-like tree that has no internal branches. The next steps involve introducing internal branches followed by

calculating the length of the tree. The resulting tree that is shortest is considered the most likely tree (Page and Holmes, 1998). Neighbor-joining trees differ from UPGMA in that it does not attempt to cluster the most closely related taxa, but minimizes the internal branch length. This method represents a significant improvement on the UPGMA method, however this method is still prone to error due to its inability to explore the theoretical "tree-space" (Vandamme, 2009).

Maximum Parsimony

The maximum parsimony method determines the tree topology by finding the smallest number of substitutions between sequences (Page and Holmes, 1998). This algorithm searches a number of different tree topologies and determines a parsimony length for each. Parsimony length is the sum of the character changes required to generate the tree topology being analyzed. Of the trees analyzed the tree that has the lowest parsimony length is determined to be the maximum parsimony tree (Vandamme, 2009). One drawback to maximum parsimony is its sensitivity to long-branch attraction. That is, maximum parsimony assumes a common character is always inherited from a common ancestor, which results in underestimations of actual divergence between more distantly related taxa.

Maximum Likelihood

Maximum likelihood and maximum parsimony are similar in that different tree topologies are examined and relative support is determined by summing all sequence positions. Maximum likelihood searches for a tree that maximizes the likelihood of a tree topology while taking into account a model for nucleotide substitution (Vandamme, 2009). The main drawback to this method is that it tends to be computationally intensive, though this is becoming less of an issue as computational capacity increases.

Bayesian

Bayesian methods are conceptually different from the other methods discussed because they do not search for the single best tree. Like maximum likelihood, Bayesian methods utilize the concept of likelihood, but rather than targeting the most likely tree it targets a probability distribution of trees (Vandamme, 2009). This method requires scientists to specify specific prior beliefs about the data set, termed prior distribution, on the model. The algorithm then returns a confidence interval on the priors. Some common priors include substitution models and branch lengths (ex. time). Usually, the specific tree topology is left uniform meaning no priors are defined concerning it. Confidence is reported as posterior probabilities, which are obtained by searching the tree space using a sampling technique called Markov chain Monte Carlo (MCMC). Briefly, MCMC works by setting a set of random parameters and proposes a new set of parameters; likelihood ratio and prior ratio are then calculated and the new parameters are either accepted or rejected. MCMC lends to a large amount of noise in the initial part of the analysis and is usually discarded by removing the first 10% as "burn-in". Recently, Bayesian methods have become widely used because of their ability to estimate divergence times accurately, while using optimal data sets and measure the relative genetic diversity in a population (Drummond et al., 2012).

ALPHAVIRUS OVERVIEW

Taxonomy and Classification

Alphaviruses are enveloped single-stranded positive-sense RNA viruses. They are members of the family *Togaviridae* genus *Alphavirus* (Virus Taxonomy, 2012). The genus consists of 31 distinct species (Virus Taxonomy, 2012), each one grouping into one of eight distinct antigenic complexes (Calisher and Karabatsos, 1988; Powers et al., 2001; Travassos da Rosa et al., 2001). Two viruses, Salmonid alphavirus (Villoing et al., 2000) and Southern elephant seal virus (La Linn et al., 2001) remain to be classified into

a complex; however, they will probably eventually form one or more new complexes. This relatedness was initially determined by immunologic tests (e.g. hemagglutination-inhibition (HI), complement fixation (CF), cytotoxic T-cell lysis of infected cells) (Calisher, 1980; Linn et al., 1998; Mullbacher et al., 1979). Subtyping of viruses within each complex can be accomplished by using similar immunological methods taking into account the direction of the assay (e.g. virus X against antibody Y and virus Y against antibody X) (discussed in detail on page 16) (Calisher, 1994).

Evolution and Phylogeny

The phylogenies of alphaviruses derived from nucleotide sequence information generally recapitulate their relationships derived from antigenic tests (Calisher and Karabatsos, 1988; Powers et al., 2001) and provide a powerful tool for understanding current, past, and possible future evolutionary relationships between viruses (Podsiadlo and Polz-Dacewicz, 2013). Geographically, alphaviruses are broadly categorized into one of two groups: Old World and New World (Figure 1.1). This bifurcation has led to questions concerning the geographic and evolutionary origins of alphaviruses. Moreover, questions concerning the geographic origin of alphaviruses are further compounded due to issues of recombination, differing phylogenetic patterns when trees are constructed based on different parts of the genome, and New World viruses residing within Old World clades. This generated a variety of theories that address the geographic origin and evolutionary history of alphaviruses.

Before the North and South American lineages of eastern equine encephalitis virus (EEEV) diverged, a recombination event took place between an EEEV-like ancestor and a Sindbis virus (SINV)-like ancestor (Hahn et al., 1988). This event created the clade within the western equine encephalitis complex that comprises Fort Morgan, Buggy Creek, and Western equine encephalitis virus (WEEV). The timing of the recombination event is thought to have occurred approximately 1300 and 1900 years ago (Weaver et al.,

1997); however, this date should be taken with a certain amount of skepticism as the phylogenetic methodologies applied to achieve that date can be inaccurate when relatively recent isolates are used to assess ancient lineages because purifying selection within the population can obscure the findings (Wertheim and Kosakovsky Pond, 2011). In order for the recombination event to have occurred, a single cell would have to have been co-infected with both viruses simultaneously, which would imply some geographic overlap between the ancestors of Old and New World alphaviruses.

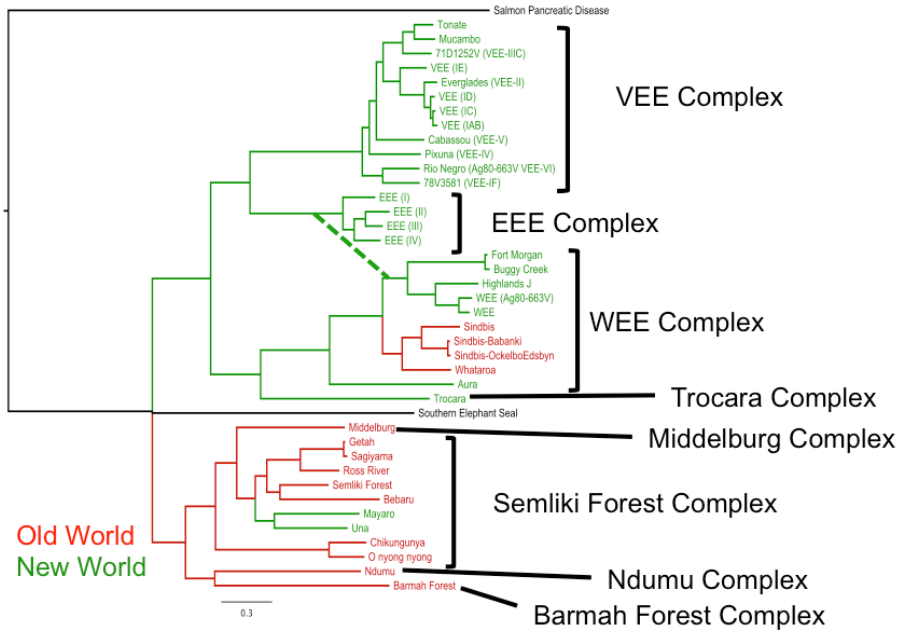

Figure 1.1 Alphavirus phylogenetic tree generated using Bayesian methods.
The tree was generated in MrBayes using Markov Chain Monte Carlo (MCMC) methods and midpoint rooted using full genomes except regions of nsP3 and capsid where the amount of divergence between different species has rendered them incapable of distinguished as related, that is they appear random. Representatives from all alphavirus species are included. The dashed line represents the recombination event between the EEEV-like and SINV-like ancestors. Roman numerals indicate major subtypes within a species. The scale of 0.3 indicates 30% nucleotide sequence divergence and all nodes have a posterior probability greater than or equal to 0.9. (Figure courtesy of Naomi Forrester, UTMB)

Phylogenies of alphaviruses can be dissimilar depending upon the genome region used to generate the tree. Trees constructed using the envelope glycoproteins are unlike trees using the nonstructural genes (the reason being discussed in detail below). For example, when the E1 sequences are used SINV groups with the Western complex viruses and the Eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV) (both New World complexes) and Semliki Forest and other Old World complexes diverge independently from each other (Figure 1.2a). Contrast that with phylogenies are generated on the basis of the nonstructural and capsid genes, where SINV and Aura virus appear to reside in the same clade as viruses in the Semliki Forest complex (Figure 1.2b) (Gould et al., 2010). Although this phenomenon is primarily due to the recombination event between SINV-like and EEEV-like ancestors it illustrates that when constructing phylogenies across alphavirus species investigators must have a detailed understanding of previous findings regarding the evolution of alphaviruses. In other words, a clear hypothesis and a logical method to test that hypothesis should be formulated before studies are conducted.

Evidence of geographic boundaries being surmounted exists independent of the genes the analysis is based on. This is evident with the New World viruses Mayaro and Una always clustering within the Semliki Forest complex, which removes the possibility of convergent evolution (Figures 1.1 & 1.2) (Gould et al., 2010). This is a clear example of some type of transoceanic crossing of the virus without the confounding variable of recombination. The argument of Mayaro and Una viruses surmounting large geographical boundaries is supported by the fact that symptoms of these viruses are similar to Old World viruses: fever, arthralgia, rash, etc. (Causey et al., 1961; Halsey et al., 2013; Pinheiro and LeDuc, 1988; Powers et al., 2001).

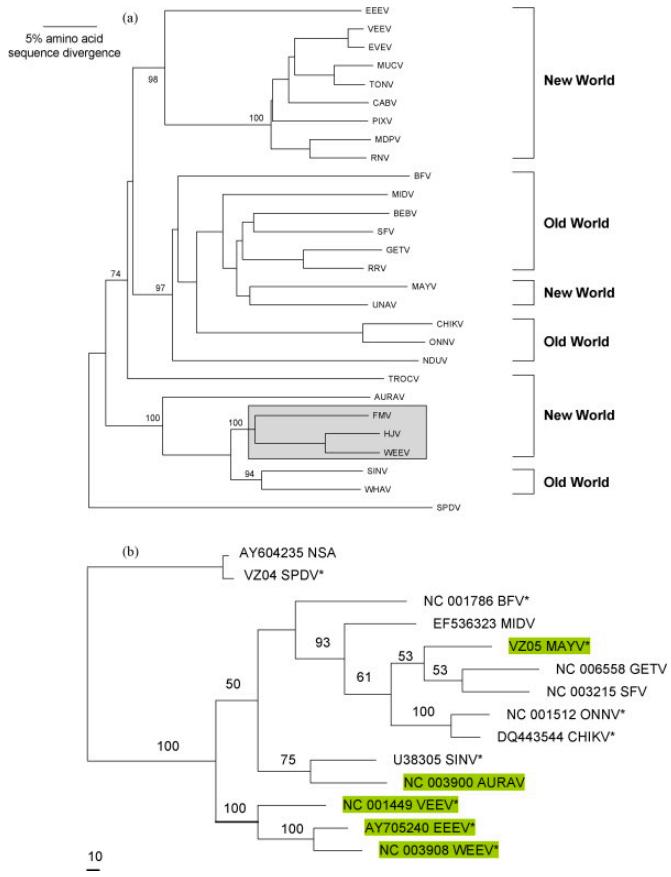

Figure 1.2 Phylogenetic analysis of alphaviruses (a) E1 and (b) nsP4 genes.
(a) Midpoint rooted Neighbor Joining tree based on E1 sequences. Bootstrap values generated using 1000 resamplings. Gray box indicates recombinant viruses. (b) Midpoint rooted parsimony tree based on nsP4 sequences. Bootstrap values generated using 1000 resamplings. Highlighted viruses indicate New World viruses. Reprinted from, Antiviral Research, 87, Gould, E.A. *et al.* Understanding the alphaviruses: Recent research on important emerging pathogens and progress towards their control, 111-124, (2010), with permission from Elsevier.

The question stands: from where did alphaviruses originate? Theories describing alphavirus origin occurring in the New World and Old World have been described (Forrester et al., 2012; Hahn et al., 1988; Powers et al., 2001). If alphaviruses originated in the New World then an ancestral SINV had to be present either by evolutionary origin or reintroduction so that the recombination event between the SINV-like and EEEV-like ancestors could occur. If the SINV ancestor originated in the New World then it must also have been transported to the Old World by some means. Furthermore, an ancestral

virus to the Middelburg, Semliki Forest, Ndumu, and Barmah Forest complexes must have been introduced to the Old World (Gould et al., 2010). After that introduction to the Old World the ancestor to Una and Mayaro viruses was transported back to the New World (Lavergne et al., 2006; Powers et al., 2001). One benefit to this theory is that it allows for the origin of alphaviruses occurring where they are most diverse, the neotropics (Weaver et al., 1997). A limitation of this theory is that it requires at least three independent introductions of different ancestors to the Old World. Conversely, alphaviruses could have originated in the Old World. Many alphaviruses viruses share ecological landscapes in the Old World and the plausibility of a mixed infection resulting in a recombination event that gave rise to the recombinant WEE complex clade could theoretically occur (Lavergne et al., 2006; Powers et al., 2001). However, there is no evidence of any encephalitic alphaviruses in the Old World. Furthermore, if the alphaviruses originated in the Old World and the recombination event occurred in the New World the issue of crossing the ocean becomes a bigger issue. Not only would two separate introductions have to have occurred (the common ancestor to the Venezuelan, Eastern, Western, and Trocara complexes and the common ancestor to Mayaro and Una virus), but also a SINV-like ancestor had to be introduced back to the Old World. Implicit in both theories is that these viruses, before the Age of Discovery (pre-1400s) (Roberts, 1993), were able to surmount several transoceanic crossings. Migratory birds have been proposed to account for this phenomenon (Weaver et al., 1997). However, the question stands: why isn't this phenomenon occurring today?

The most recent theory describing the geographic and evolutionary origins of alphaviruses hypothesizes that the mosquito-borne alphaviruses originated from a marine environment where lice are the primary vector (Forrester et al., 2012). This theory describes a pattern of mosquito-borne alphavirus emergence where initial emergence from marine to terrestrial vertebrate hosts occurred in the Pacific; however, this emergence could have occurred in any ocean due to the large geographic range

alphaviruses inhabit today. Once established in terrestrial vertebrates the virus then moved both to the Old and New World relatively simultaneously (Figure 1.3). One drawback is that this situation would require several reintroduction events to account for the recombinant clade and the presence of Mayaro and Una viruses in the New World (Forrester et al., 2012). This theory is supported by a Bayesian phylogeny based on full genome sequencing and incorporation of salmon pancreatic disease virus (SPDV) (Forrester et al., 2012). Furthermore, various mosquito-borne alphaviruses are capable of replicating in fish cells and at lower temperatures which provides support to the theory on the marine origin of alphaviruses (Peleg and Pecht, 1978; Wolf and Mann, 1980). Additional inclusion of the Southern elephant seal virus (isolated from the seal louse *Lepidophthirus macrorhini*) also highlights the ability of alphaviruses to inhabit a marine ecosystem. While the theory of marine emergence is most compelling, more work must be done in order to fully understand how and where alphaviruses evolved over past millennia. Indeed, with the viruses available, researchers are probably seeing only the "tip of the iceberg" and more concerted efforts toward discovering new marine and terrestrial alphaviruses would allow for a more definitive theory on alphavirus origins to be developed. One effective way to accomplish this would be to conduct a robust sampling of marine and aquatic environments for alphaviruses that may share similar ecological niches as the Salmon pancreatic disease virus or Southern elephant seal viruses.

Recently arboviruses, viruses transmitted by arthropods, have been emerging, spreading, and causing epizootic events at unprecedented rates (Leparc-Goffart et al., 2014; Musso et al., 2015). Several examples exist where a detailed understanding of the evolutionary relationships have allowed for a mechanistic understanding of disease spread and emergence (Brault et al., 2004; Brault et al., 2002; Greene et al., 2005b; Tsetsarkin et al., 2014; Tsetsarkin and Weaver, 2011; Tsetsarkin et al., 2009; Tsetsarkin et al., 2007). Understanding the evolutionary relationships of alphaviruses, and

arboviruses in general, has direct consequences on efforts to control, treat, and mitigate these diseases and should not be neglected (Beaty, 2005; Wilder-Smith et al., 2010).

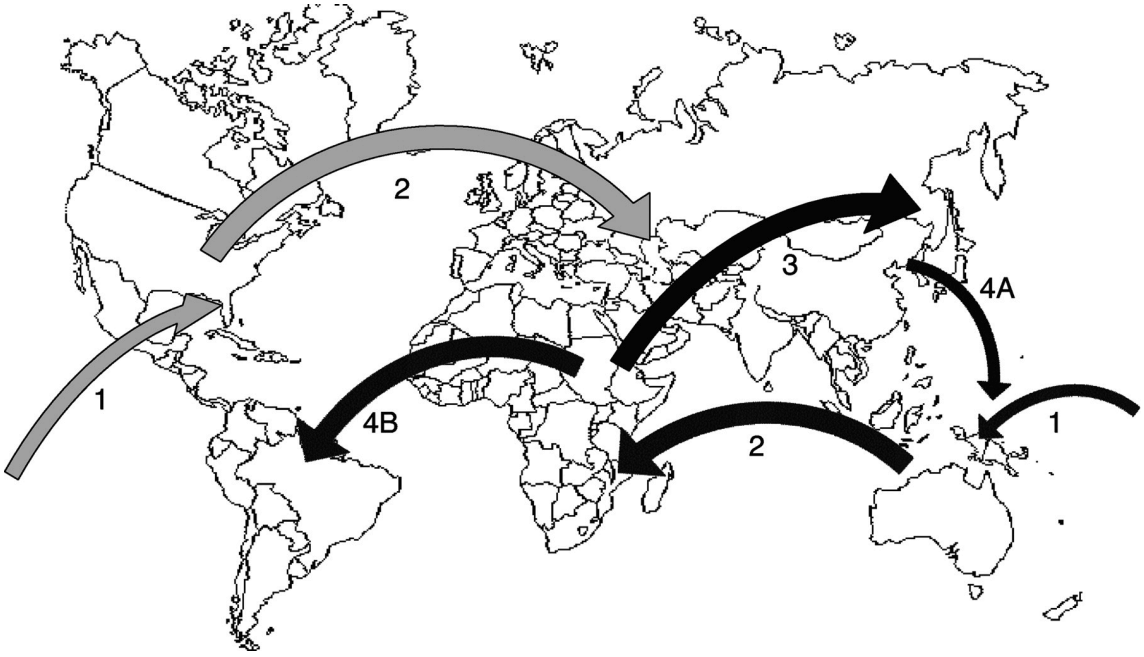

Figure 1.3 World map representing the theoretical emergence of alphaviruses from the Pacific Ocean.
New World alphaviruses (gray arrows): 1) introduction to the New World from the Pacific, 2) introduction of New World alphaviruses to the Old World. Old World alphaviruses (black arrows): 1) introduction to the Old World (e.g. Australasia) from the Pacific, 2) introduction from Australasia to Southern Africa, 3) spread from Southern Africa to Eurasia, 4A) introduction of Ross River virus to Australia from Asia, and 4B) introduction of Mayaro and Una viruses to South America. Reprinted from, Journal of Virology, 86, Forrester, N.L. *et al.* Genome-Scale Phylogeny of the Alphavirus Genus Suggests a Marine Origin, 2729-2738, (2012), with permission from the American Society for Microbiology.

Virion Structure and Genetic Organization

The structural components of an alphavirus virion consist of a nucleocapsid, envelope, and glycoproteins (Figure 1.4). The alphavirus nucleocapsid is icosahedral where the triangulation number equals 4 and functions to contain the genome (Choi et al., 1991; Strauss and Strauss, 1994; Tong et al., 1993). The entire nucleocapsid consists of 240 copies (determined and varied through a variety of methods) of the 30 kDa capsid

protein and has a fenestrated structure which leaves the genomic RNA susceptible to RNase degradation (Choi et al., 1991; Coombs et al., 1984; Fuller and Argos, 1987; Strauss and Strauss, 1994; Tong et al., 1993). The diameter of the nucleocapsid varies slightly based on the methodology used to measure it: using X-ray scattering of intact virus particles it measures 41 nm (Stubbs et al., 1991), X-ray diffraction of nucleocapsid crystals reports a diameter of 40.4 nm (Harrison et al., 1992), 34-38 nm as measured by transmission electron microscopy of isolated virus particles (Coombs et al., 1984), and cryoEM studies report a diameter of 30-46 nm (Sherman and Weaver, 2010). The virion envelope is a lipid bilayer that closely resembles the composition of the host from which it came (Strauss and Strauss, 1994). SINV's envelope is 4.8 nm wide and has a radius of 23.2 nm; interestingly, because the bilayer is curved, the outer layer is 40% larger than that of the inner (Strauss and Strauss, 1994). Alphaviruses also have two main glycoproteins, E1 and E2 of approximately 50 kDa each. These glycoproteins pass thorough the lipid bilayer via alpha-helices and E2 interacts with capsid (Mukhopadhyay et al., 2006). Both E1 and E2 are glycosylated, but glycosylation patterns are not completely conserved among alphaviruses (Strauss and Strauss, 1994) and can differ depending upon the host from which the virion came from (e.g. vertebrate or arthropod) which may enhance infectivity and facilitate transition to the reciprocal host (Shabman et al., 2007). E1 and E2 associate together to form a heterodimer, three of which interact to form a trimeric spike present on the surface of the virion (Figure 1.4) (Rice and Strauss, 1982; Ziemiecki and Garoff, 1978). The virion contains an equimolar amount of both E1 and E2 proteins as compared to capsid and the trimeric spikes also have an icosahedral symmetry where the triangulation number equals 4 (von Bonsdorff and Harrison, 1975). A hydrophobic fusion loop in E1 facilitates membrane fusion, though E2 plays an important role in regulating that function (Mukhopadhyay et al., 2006; Roussel et al.; White and Helenius, 1980). E2's primary function is receptor binding (Dubuisson and

Rice, 1993; Strauss et al., 1991). The roles E1 and E2 play in attachment and entry and assembly will be described in detail below.

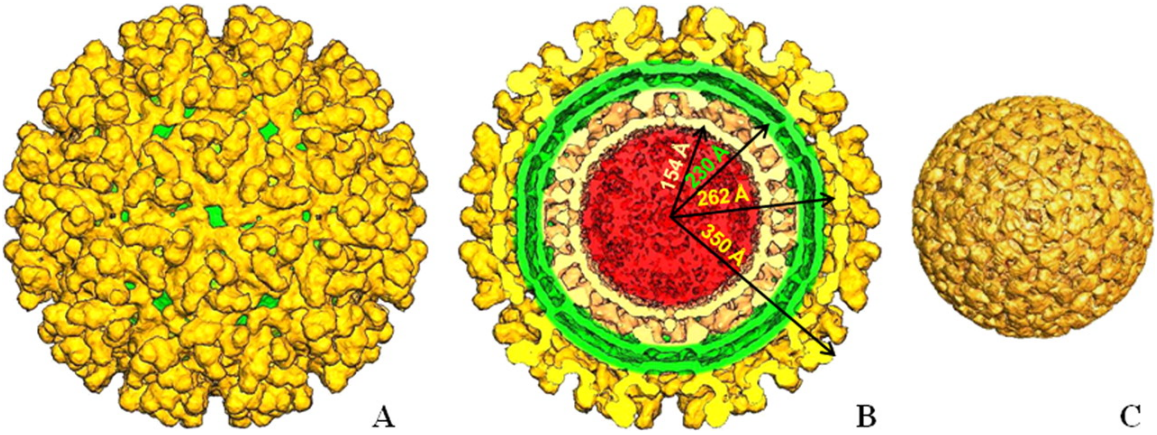

Figure 1.4 Diagram of alphavirus structural elements.
3D rendering of WEEV using cryoelectron microscopy. A) Depicts of complete virion, B) shows half of the complete virion detailing internal structure, and C) represents the nucleocapsid without other structural components. The yellow spikes, visible in both panels A and B represent the glycoprotein trimers. E2 can be seen passing through the lipid bilayer (green) and interacting with the nucleocapsid (beige). The area in red, visible in panel B, indicates the space occupied within the virion by the RNA genome. Reprinted from, Journal of Virology, 84, Sherman, M.B. and Weaver S.C. Structure of the Recombinant Alphavirus Western Equine Encephalitis Virus Revealed by Cryoelectron Microscopy, 9775-9782, (2010), with permission from the American Society for Microbiology.

The alphavirus genome consists of a single positive-sense single-stranded RNA molecule of approximately 11.5 kilobases (kb) (Strauss et al., 1984). The genome mimics a messenger RNA (mRNA) molecule complete with a 5' cap and 3' polyadenylated (polyA) tail (Figure 1.5) (Strauss and Strauss, 1994). The two major regions of the alphavirus genome are the nonstructural and structural ORFs. The nonstructural genes are translated directly from the genomic RNA and consist of approximately the first two-thirds of the genome. Generally, the nonstructural proteins facilitate the genome synthesis. The structural genes consist of approximately the last third of the genome and are under the control of a subgenomic promoter. Recently, the transframe (TF) protein has been discovered and characterized as component of the structural ORF; not much is

known to date, but TF may play a role in virus particle release and virulence (Snyder et al., 2013). Translation occurs from the subgenomic RNA, which also has a 5' cap and a polyA tail. Between the nonstructural and structural cassettes is a small non-coding intergenic region (Strauss and Strauss, 1994). Flanking the open reading frames of the genome are the 5' and 3' untranslated regions (UTR)s (Strauss and Strauss, 1994) The UTRs play many different roles for example the 5' UTR in VEEV plays an important role in the induction of interferon-α/β which has a direct effect on virulence (White et al., 2001), the 3' UTR in SINV represses deadenylation of the poly A tail (Garneau et al., 2008), the 3' UTR may even play a role in chikungunya virus' evolutionary adaptation to mosquitoes (Chen et al., 2013). The mechanism by which the UTRs are able to exert these effects is mostly through their interaction with host factors as a result of RNA secondary structure.

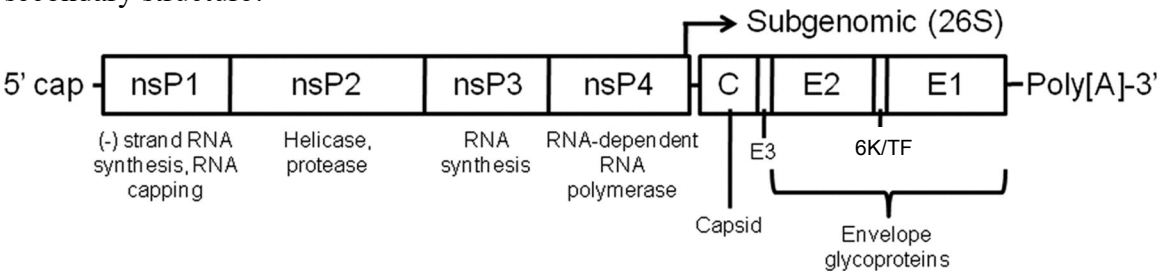

Figure 1.5 Alphavirus genomic organization.
Diagram depicts the 5' cap and UTR, nonstructural genes (with the major functions noted), subgenomic promoter, intergenic region, structural genes, 3' UTR, and polyadenylated tail. Reprinted from, Journal of Virology, 84, Sherman, M.B. and Weaver S.C. Structure of the Recombinant Alphavirus Western Equine Encephalitis Virus Revealed by Cryoelectron Microscopy, 9775-9782, (2010), with permission from the American Society for Microbiology.

Replication

Attachment and Entry of Host Cells

The specific host receptors or host factors alphaviruses use to attach to host cells are not completely understood (Kielian et al., 2010). However a number of molecules have been proposed: DC- and L-SIGN (Klimstra et al., 2003), heparan sulfate (HP)

(Klimstra et al., 1998), α1β1 integrin (La Linn et al., 2005), high-affinity laminin, class I major histocompatibility antigen, and other proteins identified by monoclonal antibodies (Kielian, 1995; Kielian et al., 2010). Binding of the virus to the host cell is mediated primarily by E2 (Kielian et al., 2010), which is clearly seen in HS binding of tissue culture-adapted alphaviruses due to mutations accumulated in E2 (Heil et al., 2001; Klimstra et al., 1998). However, the viruses adapted for HS affinity show attenuation in *in vivo* models (Bernard et al., 2000; Byrnes and Griffin, 2000), therefore the biological role of HS, as a means of virus attachment, is not fully understood (Wang et al., 2007a).

Alphaviruses enter cells primarily via receptor-mediated endocytosis. Alternatively, direct fusion of the virus into the cell has been proposed as the entrance mechanism of alphaviruses (Kononchik et al., 2011; Wang et al., 2007b). Clathrin-mediated endocytic uptake followed by pH-dependent fusion was first identified with Semliki Forest virus (Helenius et al., 1980; Marsh and Helenius, 1980) and has been repeatedly verified as a primary entry mechanism for alphaviruses (Kielian et al., 2010). As endosomes mature ATP-dependent proton pumps acidify the compartment (Mellman et al., 1986). This acidification causes the association between E1 and E2 to destabilize (Wahlberg et al., 1989; Wahlberg and Garoff, 1992). After dissociation, the hydrophobic E1 fusion loop is exposed and inserted into the host membrane (Li et al., 2010). Next, and critical for membrane fusion, is formation of the E1 post-fusion homotrimer (Li et al., 2010; Wahlberg and Garoff, 1992). The force needed to provide membrane fusion is accomplished through the effort of several trimers via conformation changes with E1 in a pH-dependent fashion (Kielian et al., 2010).

TRANSCRIPTION AND TRANSLATION

Translation of the nonstructural proteins occurs directly from the genomic RNA once it is exposed to the cytosol after exit from the endocytic pathway. For most alphaviruses, the 5' cap mediates translation of the nonstructural proteins resulting in the

production of polyproteins P123 (including nsP1, nsP2, and nsP3) and nsP1234 (including nsP1, nsP2, nsP3, and nsP4) via a leaky UGA termination codon prior to nsP4, thus the two polyproteins are able to be produced (Li and Rice, 1989; Strauss et al., 1988; Strauss et al., 1983). If an alphavirus lacks this stop codon then only nsP1234 will be produced (Myles et al., 2006; Takkinen, 1986). The polyproteins are subsequently cleaved to produce the individual nonstructural proteins via nsP2 proteinase activity (Hardy and Strauss, 1989), however the polyproteins themselves and several intermediates do have critical biological functions independent from the function of the individual nonstructural protein (Rupp et al., 2015). The resulting protein products, together with host factors, recruit genomic RNA to cytoplasmic membranes and form replication complexes, where subsequent transcription of minus-strand and positive-strand genomic RNA occurs (Grimley et al., 1968; Kujala et al., 2001). Additionally, a subgenomic promoter on the minus-strand facilitates transcription of the subgenomic RNA from which the structural proteins are translated as a polyproteins and subsequently cleaved to their individual peptides (Clegg and Kennedy, 1975; Rice and Strauss, 1981). The subgenomic RNA is 5' capped and polyadenylated and made in molar excess from that of the genomic RNA (Glanville et al., 1976). This allows for many more viral proteins, necessary for virion production, to be generated. Furthermore, ablating subgenomic transcription is an effective means for viral attenuation (Plante et al., 2011).

ASSEMBLY AND RELEASE

The alphavirus structural polyprotein is processed into the individual structural genes both co- and post-translationally (Schlesinger and Schlesinger, 1986). Capsid is autocatalytically cleaved from the structural polyprotein through a *cis* acting serine proteinase (Strauss and Strauss, 1990). The capsid protein then forms core like particles when in the presence of nucleic acid (Tellinghuisen and Kuhn, 2000). This formation is

due to a sequence element that causes a nucleation event that leads to the formation of core particles (Linger et al., 2004; Weiss et al., 1994; Weiss et al., 1989).

The resulting structural polyprotein, without capsid, is then initially inserted into the endoplasmic reticulum via an N-terminal signal sequence (Garoff et al., 1990). Then the envelope glycoproteins undergo post-translational modification in the form of glycosylating all N-linked glycosylation sites after they are inserted into the membrane (Sefton, 1977). Glycosylation is extremely important for proper protein folding and stability. Additionally, glycosylation facilitates prevention of aggregate protein formation. Furthermore, the type of glycosylation that occurs can vary depending upon the infected host and the infected cell's state of growth (Hsieh and Robbins, 1984; Knight et al., 2009). For example, alphaviruses have very different glycosylation profiles depending on whether they arose from insect or vertebrate cells which can affect a number of attributes of the virus including heparin binding, infectivity and growth kinetics (Knight et al., 2009). As a result of further post-translational modifications (e.g. glycosylation and palmitoylation), conserved sequence elements and signal sequences in the envelope glycoproteins form a specific configuration within the membrane (Gaedigk-Nitschko and Schlesinger, 1991; Ivanova and Schlesinger, 1993; Liljeström and Garoff, 1991). Further processing and maturation of the envelope glycoproteins are mediated by furin and signalase cleavage (Gaedigk-Nitschko and Schlesinger, 1990). Throughout this process the envelope glycoproteins are transported from the endoplasmic reticulum, through the Golgi apparatus, and deposited on the plasma membrane (Jose et al., 2009).

Assembled capsid particles then interact with processed E2 on the plasma membrane (Liu and Brown, 1993; Zhao and Garoff, 1992). E1 is present during this interaction but has no specific role in budding (Kail et al., 1991; Metsikkö and Garoff, 1990). The interaction of capsid with E2 provides the free energy necessary for capsid to transverse the plasma membrane and as it translocates picks up an envelope complete with a complement of glycoprotein spikes (Garoff and Simons, 1974; Jose et al., 2009).

WESTERN EQUINE ENCEPHALITIS VIRUS

Western equine encephalitis virus resides in the family *Togaviridae* genus *Alphavirus* (Virus Taxonomy, 2012). The virus is one of several New World alphaviruses that is vectored by mosquitoes and causes severe disease in horses, and is the etiologic agent of western equine encephalomyelitis or encephalitis (WEE) (Kelser, 1933; Meyer et al., 1931). WEEV is endemic in both North and South America however, individual lineages and ecological niches exist for North and South American subtypes (Reisen and Monath, 1988; Weaver et al., 1997).

Discovery

During an October night in the San Joaquin Valley of California Karl F. Meyer found himself hiding behind a bush with a syringe full of strychnine, a very sharp knife, and his tobacco pipe. While crouched behind the bush Dr. Meyer contemplated the series of events that brought him to this point (Sabin, 1980).

During the summer of 1930 a number of cases of equine botulism was reported in the San Joaquin Valley of California. Due to Dr. Meyer's earlier work on the disease he was immediately interested. However, he had significant reservations because there was not enough moisture in the feed for botulism to grow during the summer time. As a result, Dr. Meyer sent his colleague, Dr. Geirger, to observe the diseased horses and report on any findings. What Dr. Geiger found were horses exhibiting signs of encephalitis (e.g. circling, paralysis, etc.). Additionally, Dr. Geirger found lesions present in the brains of horses that succumbed to the disease. This convinced Dr. Meyer that the horses were dying of encephalitis and not botulism. However, in spite of these efforts and additional efforts made by Dr. Meyer, an etiologic agent (presumed to be a virus) was not able to be isolated from animals that succumbed to the illness.

This result convinced Dr. Meyer that if an isolation of the agent were to be successful, it would have to be isolated from a horse just starting to show the signs of

disease. This conclusion brought Dr. Meyer to his uncomfortable position of crouching behind a bush at night. He found a horse that was beginning to show symptoms; however the owner phoned Dr. Meyer and said, "I won't sell the horse, and if you ever do anything to the horse, I shoot you." Needless to say Dr. Meyer walked down to the farm with a $20 bill in his pocket. Before he left for the farm he was warned by his colleagues not to attempt to reason with the farmer, to which he quipped, "I'm not going to talk to him. I'm going to talk to his wife." When he arrived at the farm he told the farmer's wife, "Look here, this horse is going to die anyhow, and when it's dead you haven't anything. It just goes to the rendering plant and you get a couple of dollars. On the other hand, you see, you could contribute to the knowledge of what this is and perhaps to its prevention." She replied, "Well, my husband is just irate about this." To which Dr. Meyer said, "Yes, I can readily understand, but look here, suppose I trust you, and give you $20 and the next morning you will find in the backyard the horse without a head?" "How will you do this?" she asked. "Look here, about nine o'clock at night when it is dark, I'll be over here behind some bushes, where I can see the window of your house. When your husband is sound asleep you lift up the shade."

After waiting for a while Dr. Meyer noticed light was coming from the house. The shade was up. He rushed to the stables where the horse was and within ten minutes the head was off. He threw it over the fence, wrapped it in burlap, tossed it in his truck, and speed off into the night. He later said concerning the night in question, "We drove back, and I tell you, naturally, I was fantastically excited." [This story is chronicled in the memoir to Dr. Meyer published by the National Academy of Sciences (Sabin, 1980).]

The findings of that night's events were documented in the journal *Science*, without the exciting story (Meyer et al., 1931). As he initially thought the causative agent was determined to be a virus which was elucidated by intraocular injection of filtered spinal fluid taken from horse he obtained from the kind wife of the farmer (Meyer et al., 1931). While the virus was initially discovered in 1930, epizootic activity is thought to

have occurred prior to 1930. Documented evidence exists that the virus was possibly causing equine illness as early as 1912 in Kansas and Nebraska (Udall, 1913). Any evidence for virus circulation prior to 1912 is lacking. Ultimately, knowledge of prior circulation of WEEV is unknown, however, it is likely the virus did circulate and cause disease long before the early 20th century.

WEEV Classification

WEEV is one of seven species that reside in the western complex of the genus *Alphavirus* (Figure 1.1) (Calisher and Karabatsos, 1988; Forrester et al., 2012; Powers et al., 2001). Before viruses were classified on a species-level basis, due to advancements in the field of sequence analysis, the relationship between arboviruses were defined on the basis of antigenic similarity as determined by several serological techniques including HI, complement fixation (CF), and neutralization tests (Clarke and Casals, 1958; Karabatsos et al., 1963). Using these serological tests viruses are considered different if both heterologous versus homologous anti-immune sera from the two viruses in question, i.e. both directions, causes a fourfold or greater difference in cross-reactivity (Clarke and Casals, 1958). If a fourfold difference in cross-reactivity is achieved in only one direction the distinction was considered a subtype. Originally, there was thought to be a significant amount of antigenic variation between WEEV strains from the western and eastern United States (Hayes and Wallis, 1977; Karabatsos et al., 1963). However, upon closer inspection the "eastern isolates" of WEEV were determined to be the distinct, albeit related, virus, Highlands J (Calisher et al., 1988). WEEV also circulates in South America, most notably in Argentina (Calisher and Karabatsos, 1988; Calisher et al., 1985; Mitchell et al., 1985). Antigenic studies show that WEEV isolates from South America constitute a virus subtype due to directional neutralization of South American isolates against North American serum (Calisher et al., 1988; Calisher et al., 1985).

Evolution

WEEV IS A RECOMBINANT VIRUS

Before the advent of nucleic acid sequencing WEEV always remained a bit of a puzzle in regards to its serological relationships. WEEV demonstrates greater cross-reactivity with the Old World viruses than it does with the New World viruses (with Sindbis virus (SINV) being WEEV most closely related virus as determined by serology) (Calisher, 1980; Calisher et al., 1988). The conundrum lies in the fact that WEEV causes encephalitis, an attribute of the New World viruses, and WEEV's geographical range resides in the New World (Reisen and Monath, 1988). Additionally, alphavirus evolution, in most cases, demonstrates some linear descent from a common ancestor (Strauss and Strauss, 1986). This apparent incongruity was resolved when sequence analysis revealed that WEEV is a recombinant virus between EEEV and SINV (Hahn et al., 1988). By cloning the 3'-terminal 4,288 nucleotides, which contains the structural polyprotein ORF and the 3'-terminal end of nsP4, comparisons between WEEV, EEEV, SINV, and VEEV were made. Comparing the percent sequence identity among each gene between viruses produces a general pattern of what genes arose from which ancestor. Once the genes flanking the recombination event were determined, the specific location of the recombination event was determined by inspection of the sequence alignment. The study concluded that the nonstructural proteins, most of capsid, and 80 nucleotides in the 3'UTR arose from an EEEV-like ancestor and the envelope glycoproteins arose from a SINV-like ancestor. Further experiments using phylogenetic methods indicated that this recombination event occurred between 1,300 to 1,900 years ago (Weaver et al., 1997); however, this information should be taken with skepticism, as these methodologies are highly dependent on the samples used, quality of the sequence data, and mathematical assumptions that may not accurately reflect reality.

PREVIOUS EVOLUTIONARY FINDINGS

In addition to hypothesizing the date of recombination for western complex viruses, Weaver *et al*. (1997) also explored evolutionary patterns within the Western complex. Two monophyletic linages were found to circulate in North America, designated Group A and Group B (Figure 1.11). Another monophyletic group comprised isolates from Argentina though one Argentinian isolate fell in Group B. Group A contained highly virulent viruses from North America in addition to isolates from Russia and Brazil. Group B contained viruses found to be presently circulating and recent samplings of epizootic and endemic WEEV, this finding lead to the conclusion that Group A WEEV was extinct. WEEV has also been found circulating in China, and phylogenetic analysis groups the isolate with the Russian and a Texas isolate (TBT-235) that Weaver *et al*. (1997) determined to be Group A viruses, which implies that the Chinese isolate is a Group A virus. However, a recent phylogenetic study of WEEV utilizing complete genomes (described in Chapter II) found the TBT-235 isolate was a Group B3 isolate (Bergren et al., 2014). With the TBT-235 firmly in the Group B3 sublineage the phylogenetic lineage of the Chinese and Russian WEEV isolates are brought into question. A study investigating the phylogenetic relationship of all WEEV strains, and not just North American isolates, would help determine the lineage of several non-North American WEEV isolates.

Disease Manifestations, Treatment, and Prevention

HUMANS

WEEV infection can result in a broad spectrum of disease outcomes from subclinical, febrile symptoms, to full-blown encephalitis (Reisen and Monath, 1988). The ratio of inapparent infection to disease symptoms is dependent on age and is skewed strikingly toward infants: 1:1 in infants less than 1 year-old, 58:1 in children between 1

and 4 years-old, and 1150:1 in people greater than 14 years-old (Reeves and Hammon, 1958). Specifically, signs and symptoms manifest suddenly and include fever, chills, headache, nausea, and vomiting (Reisen and Monath, 1988). After initial onset of illness neurological signs and symptoms can manifest within a few days. Neurological signs and symptoms include lethargy, drowsiness, neck stiffness, photophobia, vertigo, and mental status changes (Kokernot et al., 1953). Infants are particularly prone to irritability, convulsions, upper motor neuron deficits, and tremors, though these signs can appear in older patients as well (Medovy, 1943). Case fatality generally ranges between 3 and 4%; however, during severe epidemics case fatality can increase as high as 15% (McGowan et al., 1973; Reisen and Monath, 1988). Due to the relative ease of WEEV aerosolization, more severe outcomes are observed in laboratory acquired infections (Hanson et al., 1967). Presence of neurological sequelae is often seen in patients recovering from neurological complications associated with the disease and is also skewed toward younger individuals (Earnest et al., 1971; Herzon et al., 1957).

When diagnosing WEEV infection, signs and symptoms are generally non-specific. Cerebrospinal fluid shows elevated leukocyte counts ($\geq 500 mm^3$), a slightly elevated protein concentration, and normal glucose levels (Beaty et al., 1995; Reisen and Monath, 1988). As with most non-specific viral infections the early stage of infection is characterized by a predominance of polymorphonuclear cells, which, later in infection, are replaced by mononuclear cells (Reisen and Monath, 1988). Accurate diagnosis is dependent upon testing serum of CSF for virus-specific neutralizing or IgM antibodies (Beaty et al., 1995). In fatal cases diagnosis can be confirmed by virus isolation, nucleic acid amplification, and histopathology with immunohistochemistry (CDC, 2016a). There is currently no vaccine available to protect humans against WEEV, though a formalin-inactivated vaccine was classified as an investigational new drug and was available to laboratory workers. This vaccine is not available today.

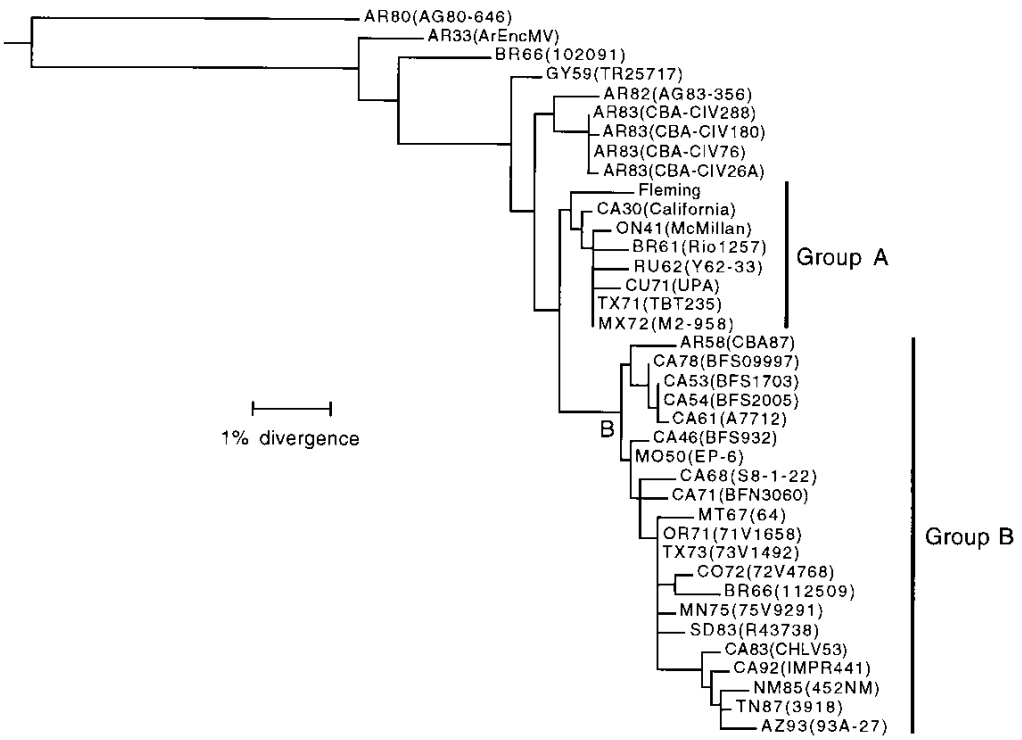

Figure 1.6 Phylogenetic tree derived from E1-3'-untranslated sequences of WEEV. Tree was rooted by using an outgroup consisting of the homologous alphavirus E1 sequence. Node B indicates thy hypothetical ancestor of Group B. Reprinted from, Journal of Virology, 71, Weaver, S.C. *et al.* Recombinational history and molecular evolution of Western equine encephalomyelitis complex alphaviruses, 613-623, (1997), with permission from the American Society for Microbiology.

EQUIDS

The case fatality rate for equids ranges from 10 to 50%. Disease usually manifests in equids after a 1 to 3 week incubation period. Initially, febrile signs will manifest early in the disease progression including fever, anorexia, restlessness, irritability, decreased movement, and ataxia (Reisen and Monath, 1988). Subsequent neurological involvement can manifest as head pressing, stupor, head drooping, blindness, flaccid lips, involuntary movements, inability to stand, partially closed eyelids, convulsions, and paralysis. Terminally the animal can exhibit involuntary eye movement, paddling, and coma (Monath and Trent, 1981).

As with humans, accurate diagnosis is dependent upon testing serum or CSF for virus-specific antibodies (Monath and Trent, 1981). However, this is done mostly to rule out other infections that are treatable. A formalin-inactivated vaccine against WEEV is available for purchase and is routinely used to immunize horses against infection. Unfortunately the vaccine has low immunogenicity and requires annual boosters, though coverage for affected areas is very good (Minke et al., 2004).

Ecology of WEEV

Transmission Cycle

The primary summer transmission cycle of WEEV in North America is well understood. The primary vector is the *Culex (Cx.) tarsalis* mosquito and amplification hosts are usually passeriform birds (Chamberlain, 1962; Hayes, 1981; Hess and Hayes, 1967; Reeves and Hammon, 1958). Horses and humans are usually considered dead-end hosts; however, there may be some evidence that horses do develop viremias high enough to infect naïve mosquitoes (Byrne et al., 1964; Giltner and Shahan, 1936; Sponseller et al., 1966). Even though low proportions of mosquitoes would become infected from viremic horses, the increased number of horses in America in the early 20th century could potentially compensate for the low proportions. Other vertebrates and mosquitoes can play a role in the transmission cycle (Reisen and Monath, 1988) and will be described in detail below.

Vectors

With the discovery of the disease agent in 1930 (Meyer et al., 1931), efforts to elucidate the means by which it was transmitted were immediately undertaken. The idea that WEEV was transmitted by mosquitoes was introduced in a study that showed after subcutaneous inoculation 10% of horses developed encephalitis and the ability to detect virus in the blood three days post intracerebral inoculation (Meyer, 1932). Experimental evidence of the vector competence of *Aedes* (*Ae.*) *aegypti* was then established (Giltner

and Shahan, 1936; Kelser, 1933; Merrill and Tenbroeck, 1935). Further studies established horses were able to become infected via the bite of artificially infected *Aedes* mosquitoes (Madsen and Knowlton, 1935).

Table 1.1 Summary of field-collected arthropods tested for WEEV and virus isolations made in Kern County, 1943-1952.

Reprinted from, University of California Publications in Public Health, Volume IV, Reeves, W.C. and Hammon, W. McD. Epidemiology of the arthropod-borne viral encephalitides in Kern County, California 1943-1952 (1962) with permission from the University of California.

Species	Number tested	Number of virus isolations
Mosquitoes		
Cx tarsalis.	92,635	140
Cx. quinquefasciatus	23,765	1
Cx. peus	3,391	3
Culiseta inornata	249	0
Culex thriambus	125	0
Ae. melanimon	13,890	2
Ae. nigromaculis	14,220	0
Ae. vexans	433	0
Anopheles spp.	983	0
Mites		
Ornithonyssus sylviarum *Dermanyssus americanus*	135,334	10
Ticks		
Argas persicus	4,352	0
Haemaphysalis leporis-palustris	333	0
Ornithodorus turicata	69	0
Miscellaneous		
Fleas (rodent)	2,972	0
Anoplura	86	0
Mallophaga	196	0
Protocalliphora larvae	1,480	0
Oeciatus vicarious	864	0
Total	295,377	156

Table 1.2 List of mosquito species found naturally infected with WEE virus in North America.

Reprinted from, The Arboviruses: Epidemiology and Ecology, Vol. V, Ch. 50, Reisen, W.K. and Monath, T.P. Western equine encephalomyelitis, 89-137, (1988), with permission from CRC Press. Data that could not be independently verified were omitted.

Mosquito Species	Locality of Infection	Reference
Aedes		
campestris	Saskatchewan	(McLintock et al., 1970)
dorsalis	Saskatchewan	(McLintock et al., 1970)
	Utah	(Smart et al., 1972)
	Arizona, New Mexico	(Hayes et al., 1976)
flavescens	Saskatchewan	(McLintock et al., 1970)
melanimon	California	(Reeves and Hammon, 1962) (Emmons et al., 1982)
nigromaculis	California	(Reeves and Hammon, 1962)
pionips	Manitoba	(Sekla et al., 1980)
sollicitans	Texas	(Sudia et al., 1975)
spencerii	Saskatchewan	(McLintock et al., 1970)
sticticus	Manitoba	(Sekla et al., 1980)
taeniorhynchus	Texas	(Sudia et al., 1975)
thelcter	Texas	(Sudia et al., 1975)
trivittatus	Iowa	(Rowley et al., 1973)
vexans	Saskatchewan	(McLintock et al., 1970)
	Manitoba	(Sekla et al., 1980)
	New Mexico	(Hayes et al., 1976)
	Texas	(Hill et al., 1971)
	Minnesota	(Burroughs and Burroughs, 1954)
Anopheles		
earlei	Manitoba	(Sekla et al., 1980)
freeborni	Washington	(Hammon et al., 1945)
punctipennis	Iowa	(Rowley et al., 1973)
Culex		
erraticus	New Mexico	(Hayes et al., 1976)
erythrothorax	California	(Emmons et al., 1974)
peus	California	(Reeves and Hammon, 1962) (Meyers et al., 1960) (Emmons et al., 1974)
pipiens	Washington	(Hammon et al., 1945)
	California	(Reeves and Hammon, 1962) (Meyers et al., 1960) (Emmons et al., 1974)
quinquefasciatus	Texas	(Hill et al., 1971)
	California	(Reeves and Hammon, 1962) (Emmons et al., 1982)
tarsalis	Western U.S.	(Hammon et al., 1945) (Burroughs and Burroughs, 1954) (Meyers et al., 1960) (Reeves and Hammon, 1962) (Rowley et al., 1973) (Hayes et al., 1967) (Emmons et al., 1974) (Sudia et al., 1975) (Hayes et al., 1976)
	Central Canada	(McLintock et al., 1970; Sekla et al., 1980)
	Mexico	(Sudia et al., 1975)
Coquilletidia perturbans	Washington	(Hammon et al., 1945)
	Central Canada	(McLintock et al., 1970; Sekla et al., 1980)
Psorophora		
confinnis/columbiae	Arizona, Texas, New Mexico	(Hayes et al., 1976)
	California	(Emmons et al., 1974)
discolor	Texas	(Sudia et al., 1975)
signipennis	Arizona, New Mexico	(Hayes et al., 1976)

Cx. tarsalis was determined to be an important vector in the natural transmission of WEEV as a result of several studies conducted in the Yakima Valley, Washington. These studies were able to repeatedly recover WEEV from *Cx. tarsalis* mosquitoes, while virus was not found in other hematophagous arthropods (Hammon et al., 1942; Hammon et al., 1941; Hammon et al., 1943; Reeves, 1943). These findings were then experimentally verified showing that *Cx. tarsalis* is capable of becoming infected and transmitting the virus to guinea pigs and chickens after feeding on a suspension of WEEV (Hammon and Reeves, 1943; Reeves, 1943). Shortly thereafter, similar studies were conducted Kern County, California (Reeves and Hammon, 1962). These studies further confirmed that the primary vector for WEEV in Kern County was *Cx. tarsalis* (Table 1.1). *Cx. tarsalis* acting as the main vector for WEEV has been indicated time and again giving certainty to the fact that it is the main vector for WEEV in North America (Emmons et al., 1974; Green et al., 1980; Hayes et al., 1976; Hayes et al., 1967; Meyers et al., 1960). Interestingly, a number of isolates were made from mites (Table 1.1), but these were found to have recently feed on the blood of a viremic host and their role as a vector was ruled out (Reeves and Hammon, 1962; Reeves et al., 1955).

WEEV has been found in a number of different mosquito species other than *Cx. tarsalis* (Table 1.2). However, the significance, or lack thereof has not been determined in all mosquitoes WEEV has been isolated from. The mammalophilic mosquitoes *Ae. melanimon* , *Ae. dorsalis*, and *Culiseta inornata* have all been shown to be susceptible to infection and are capable of transmitting virus to susceptible hosts (Hardy, 1987; Hardy et al., 1979; Kramer et al., 1998; Reeves and Hammon, 1962). *Ae. melanimon* was implicated as a potential vector between 1969 and 1974 where near identical levels of WEEV was found in both *Cx. tarsalis* and *Ae. melanimon* during the same months (Hardy, 1987; Reisen, 1984). Unfortunately, this finding was unable to be followed up due to reduced levels of enzootic activity. However, during the summer transmission season of 1983 virus was isolated in Kern County from *Ae. melanimon* six weeks after it

was initially identified in *Cx. tarsalis* (Hardy, 1987). These findings clearly show *Ae. melanimon* participating in the summer transmission cycle of WEEV in California. Transmission in *Ae. melanimon* could be a recent phenomenon because 13,890 *Ae. melanimon* were tested between 1943 and 1952 yielding only 2 WEEV isolates (Reeves and Hammon, 1962), although previous experimental infections of *Ae. melanimon* confirmed its ability to become infected and transmit virus (Reeves, 1943). The involvement of *Ae. melanimon* (and other mammalophilic mosquitoes such as *Ae. dorsalis*) could account for the perpetuation of the virus cycles involving mammals during the late summer (discussed in detail in the *Hosts* section) (Smart et al., 1972). Additionally, while *Cx. tarsalis* are catholic feeders they do have a preference for birds over mammals (75% to 25%), while *Ae. melanimon* strongly prefer mammals over birds (99%) (Hardy, 1987). In Iowa WEEV was isolated from *Ae. trivittatus*. *Cx. tarsalis* were refractory to infection from the *Ae. trivattatus* WEEV isolate, but *Ae. trivattatus* was not (Green et al., 1980). Interestingly other ornithophilic mosquitoes (*Cx. quinquefasciatus, Cx. pipiens,* and *Cx. peus*) were all shown to be relatively refractory to infection and/or unable to transmit the virus efficiently (Hammon and Reeves, 1943; Hardy et al., 1979).

A number of intrinsic factors can contribute to making a potential vector refractory to infection. Vector competence is determined by the virus' ability to infect and escape both the midgut and salivary gland epithelium. The ability of the virus to successfully surmount all barriers is also dependent on genetic factors within the mosquito and the virus (Green et al., 1980; Kramer et al., 1981; Mossel et al., 2013).

Hosts

Certain criteria must be met in order for a vertebrate species to act as an efficient reservoir host, the species must: 1) be abundant in the area, 2) show no apparent or mild signs of infection, 3) have a high titer viremia that is not short-lived after inoculation with a small amount of virus, 4) not passively transfer protective antibodies to offspring,

thereby suppressing potential viremia in juveniles, and 5) be a favored host for the mosquito vector (Hammon et al., 1943; Reeves and Hammon, 1962). The listed qualities of a reservoir host represent an ideal scenario; actual reservoir hosts in nature could deviate from these criteria in a virus dependent manner. Initially, chickens were thought to be the reservoir host for WEEV because they met most the criteria mentioned above, except criterion 3 (Reeves and Hammon, 1962). In order to empirically determine if chickens are an important reservoir host for WEEV, DDT was applied to the walls of chicken coups; if the WEEV-positive serum of young chickens in the DDT group was significantly reduced from that of non-treated young chickens then the conclusion that chickens were an important reservoir for WEEV could be made. Upon analysis the serum for young chickens showed no significant difference in infection rates between DDT-treated and non-treated areas. This result meant that either: 1) wild birds were heavily infected and mosquitoes were being infected from them, 2) *Cx. tarsalis* has a large flight range and mosquitoes that were infected outside the study area they were flying in, 3) some other hematophagous arthropod was serving as a vector, 4) an internal parasite in the chickens, possibly a helminth, was participating as a carrier, or 5) direct bird-to-bird transmission was occurring (Reeves and Hammon, 1962). As a result a vast serological survey of wild birds was conducted along with a series of controlled experiments designed to rule out the possible explanations for the failed DDT experiment listed above. These studies resulted in conclusive evidence that wild birds are infected with WEEV on a regular basis. Specifically, house sparrows (*Passer domesticus*) (HOSP), house finches (*Haemorhous mexicanus*), red-winged blackbirds (*Agelaius phoniceus*), mourning doves (*Zenaida macroura*), and Bullock's orioles (*Icterus bullockii*) were frequently shown to have positive antibody titers against WEEV (Reeves and Hammon, 1962). Follow-up laboratory studies have shown that the HOSP, house finch, tricolored blackbird (*Agelaius tricolor*), white-crowned sparrow (*Zonotrichia leucophrys*) (WCSP), domestic chicken (*Gallus gallus domesticus*) and California Valley and bobwhite quail (*Callipepla*

californica and *Colinus virginianus*) were capable of developing titers high enough to infect *Cx. tarsalis* (Hammon, 1951; Hammon and Reeves, 1945; Howitt, 1940; LaMotte et al., 1967; Watts and Williams, 1972). However, experiments conducted on galliform birds showed high viremias with chicks but adult galliforms did not produce high viremias, which indicates that galliforms are most likely not major actors in the WEEV transmission cycle (Reisen et al., 2003; Reisen et al., 2006). Prior exposure to WEEV renders birds inefficient hosts due to seroconversion; interestingly, a robust immune response is mounted regardless of strain virulence (Reisen et al., 2003; Reisen et al., 2000). Together the serology and laboratory infections indicate that passerine birds are the major reservoir for WEEV with HOSPs playing a major role. Additionally, nestling HOSPs generate high titer viremias for a duration of 3 to 5 days (the significance of this will be discussed in *Annual Patterns of Emergence*) (Holden et al., 1973).

While passerine birds are the main reservoirs for WEEV during the months in which the virus is active, some mammals can also play a role in the transmission cycle. Significant proportions of the black-tailed jackrabbit (*Lepus californicus*), California ground squirrel (*Otospermophilus beecheyi*), and western grey squirrel (*Sciurus griseus*) tested for WEEV antibodies between 1943 and 1973 returned positive results, however, only the black-tailed jackrabbit appears to tolerate infection well, that is a significant portion of infected black-tailed jackrabbits did not become moribund upon infection (Bowers et al., 1966; Hardy, 1987; Hardy et al., 1977; Hardy et al., 1974b; Reeves and Hammon, 1962). Additionally, the San Joaquin antelope squirrel (*Ammospermophilus nelsoni*), Heermann's kangaroo rat (*Dipodomys heermanni*), Fresno kangaroo rat (*Dipodomys nitratoides*), and deer mouse (*Peromyscus maniculatus*) all develop viremias high enough to infect a susceptible mosquito, though none tolerate infection well except the San Joaquin antelope squirrel and deer mouse (Hardy et al., 1974a). Though unlike the black-tailed jackrabbit, California ground squirrel, and western grey squirrel, both species seldom exhibit seropositivity in serological surveys (Hardy et al., 1974b). Due to

the ability of the black-tailed jackrabbit to tolerate infection and develop a significant viremia it is probably the predominant participant in a mammalian transmission cycle with *Ae. melanimon, which* is initiated by spillover from infected *Cx. tarsalis* feeding on a naïve black-tailed jackrabbit and subsequently infecting *Ae. melanimon* or other susceptible *Aedes* spp. mosquitoes. The jackrabbit-*Aedes* spp. cycle has been proposed to explain the persistence of WEEV in arid environments where *Cx. tarsalis* is not abundant (Hardy, 1987; Reisen and Monath, 1988).

Horses are generally thought of as dead-end hosts and their inability to transmit virus has been well established (Bowen, 2007; Reeves and Hammon, 1962; Reisen and Monath, 1988). However, some studies have reported equids capable of developing low-level viremias sufficient to transmit the virus to feeding susceptible mosquitoes (Byrne et al., 1964; Giltner and Shahan, 1936; Sponseller et al., 1966). However, an initial wave of horse infections followed by human infections was not seen on a consistent basis when no vaccine was available for horses; which is seen with other arboviruses where a domestic animal serves as an amplifying host. Additionally, mosquito species that regularly feed on horses (other than *Cx. tarsalis*) are rarely seen infected with WEEV (Reeves and Hammon, 1962).

Annual Patterns of Emergence

WEEV typically follows an annual pattern of emergence during the spring, summer maintenance, decline during the fall, and quiescence in the winter months (Reisen and Monath, 1988). Nestling house finches, sparrows and other nestling passerines amplify the virus during the spring (Cockburn et al., 1957; Hayes et al., 1967; Reeves et al., 1958b; Reeves and Hammon, 1962). The means by which the virus enters this amplification cycle remains elusive; however, studies in Texas and Colorado indicate that nestling passerines become infected before *Cx. tarsalis,* thus implicating some unknown sylvatic vector (Cockburn et al., 1957; Hayes et al., 1967), though, initial

amplification in nestling passerines as a means of emergence is not found in all regions (Reeves and Hammon, 1962). Furthermore, in the northern latitudes WEEV has been found actively circulating in snowshoe hares and ground squirrels before *Cx. tarsalis* terminates diapause indicating a sylvatic transmission cycle precedes the springtime amplification, possibly vectored by an *Aedes* spp. mosquito or *Culiseta inornata* (Burton et al., 1966b; Iversen et al., 1971; Leung et al., 1975; McLintock et al., 1970; Yuill and Hanson, 1964).

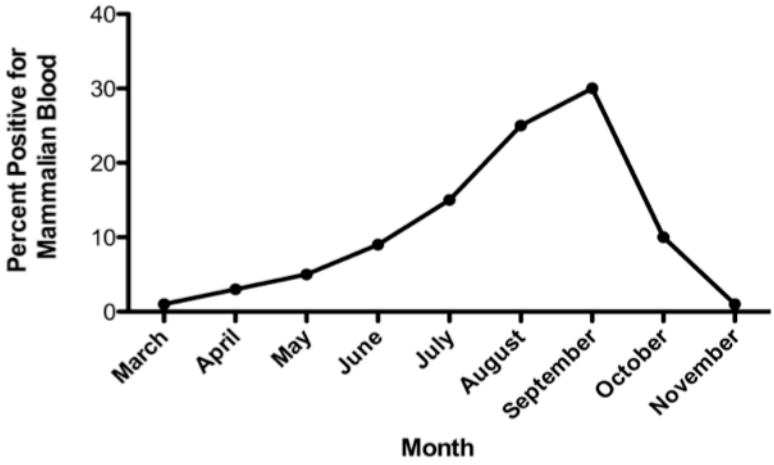

Figure 1.7 *Cx. tarsalis* shift in host feeding pattern
Annual shift of *Cx. tarsalis* blood feeding patterns toward mammals. Data from Kern County, California. Reformatted from, the *American Journal of Tropical Medicine and Hygiene*, 14, Tempelis, C.H. *et al.* A three-year study of the feeding habits of *Culex tarsalis* in Kern County, California, 170-177, (1965) with permission from the American Journal of Tropical Medicine and Hygiene.

As spring progresses to summer transmission includes a greater proportion of adult passerines. Additionally domestic galliforms (chickens) and columbiforms (doves) can play a role in virus maintenance and amplification, though their ability to amplify virus is dependent on their age which makes them poor hosts (Hayes et al., 1967; Reeves and Hammon, 1962). Infections of equines and humans are typically seen during the summers in which virus transmission exceeds some minimum threshold. Other ornithophilic mosquitoes may also be important, though their importance to virus

maintenance is limited due to them being poor vectors (discussed above). Interestingly, as the year persists the feeding pattern of *Cx. tarsalis* becomes increasingly catholic (Figure 1.7). While this shift does result in the infection of many dead-end hosts, it does allow for the initiation of the *Ae. melanimon*-jackrabbit (or more generally *Aedes* spp.-mammal cycle). A graphic of the summer transmission cycle for WEEV is depicted in Figure 1.8.

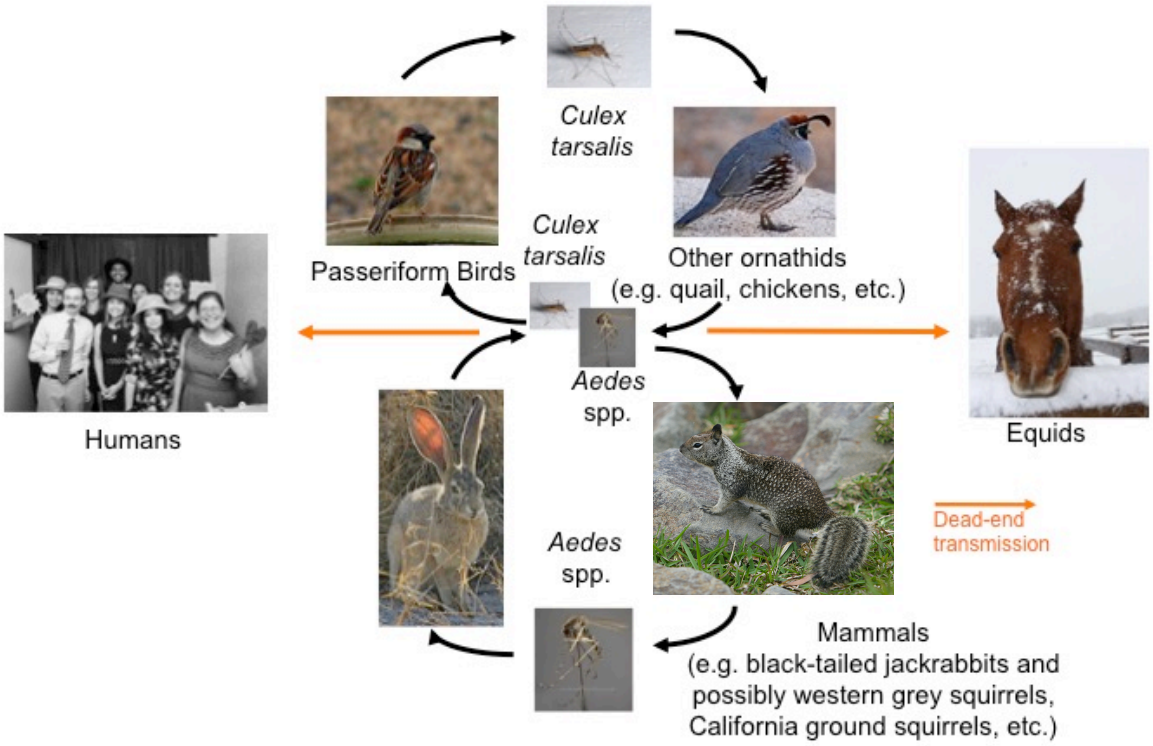

Figure 1.8 Summer transmission cycle for WEEV.
Once the cycle between *Cx. tarsalis* and passeriformes is established as a result of springtime amplification in nestling passeriformes, virus can then spill over in to other avian species, mammals and *Aedes* spp. mosquitoes. Typically involvement of mammals and *Aedes* spp. mosquitoes does not occur until late summer.

Coinciding with the onset of autumn, the number of infections begins to subside (Reisen and Monath, 1988). The presence of WEEV in the *Cx. tarsalis* population also begins to decline, although some mammalian transmission cycles may persist into the fall (Hammon et al., 1945; McLintock et al., 1970; Sekla et al., 1980). Ultimately few infections occur after October (McGowan et al., 1973; Reeves and Hammon, 1962).

Possible Overwintering Mechanisms

Vectors

The mechanism by which WEEV overwinters remains cryptic. Significant work has been undertaken to determine potential overwintering mechanisms; however no firm conclusions could be made (Fulhorst et al., 1994; Gebhardt and Hill, 1960; Gebhardt et al., 1964; Hess and Hayes, 1967; Reeves, 1961, 1974; Thomas and Eklund, 1960). Of the variety of hypotheses explored they can all fall into one of three categories: 1) maintenance within the vector, 2) chronic infection in vertebrates, or 3) annual extinction and reintroduction (Reisen and Monath, 1988).

Perhaps the most natural place to look for WEEV's overwintering mechanism(s) is the vectors. Indeed, *Cx. tarsalis*, experimentally infected with St. Louis encephalitis virus (a flavivirus that circulates in the same geographic region as WEEV), can enter quiescence and remain transmissible to an avian host for up to 8 months (Bellamy et al., 1968). This concept was also experimentally shown with WEEV in *Cx. tarsalis* lending to the concept that WEEV could overwinter in *Cx. tarsalis* (Bellamy et al., 1967). The specific biology of *Cx. tarsalis'* overwintering behavior varies based on geographical and environmental factors (Nelms et al., 2013; Nelson, 1971). Additionally, the late summer/early fall and late winter/early spring transmission dynamics of WEEV are tied to *Cx. tarsalis'* overwintering behavior and, thus specific transmission dynamics and even some differences in transmission cycle will vary by geographic region (some examples are described above). *Cx. tarsalis* that reside in northern, more temperate latitudes enter diapause in the late summer/early fall; importantly, the great majority of females that enter diapause are non-blood-fed, which implies that WEEV would have difficulty overwintering by this route (Bellamy and Reeves, 1963; Kliewer et al., 1969; Mitchell, 1981; Nelson, 1966). In intermediate latitudes, *Cx. tarsalis* females either undergo diapause or quiescence (Reisen et al., 1986a, b, c), though few females remain

reproductively active (Bellamy and Reeves, 1963). Alternatively, reproductive activity in *Cx. tarsalis* can persist through winter in southern, subtropical latitudes, though at reduced levels (Nelson, 1971; Reisen et al., 1995a; Reisen et al., 1995b). The continued reproductive activity through the winter supports the possibility that WEEV could persist in a transmission cycle throughout the winter, at reduced levels; however, the overwhelming lack of corroborating field evidence makes this unlikely. Several studies attempted to find virus circulating in the winter months between summers of virus activity in the Imperial Valley of California and found no circulating virus ruling this possibility out (Emmons et al., 1974; Emmons et al., 1973; Reisen et al., 1992). One study conducted in Boulder County, Colorado did find WEEV in *Cx. tarsalis* on December 30, 1953 (Mitchell, 1981). Proceeding further south *Cx. tarsalis* appears to be a winter species in the Rio Grande Valley and could easily transmit WEEV during the winter months (Eads, 1965).

Another possible overwintering solution for WEEV is maintenance in other mosquito vectors. One candidate is *Culiseta (Cs.) inornata*. During the winter a portion of *Cs. inornata* females keep their gonotrophic cycle intact (Dow et al., 1976) and are active during the late summer/early spring (McLintock et al., 1970; Washino et al., 1962). However, attempts to isolate virus during the winter and early spring have been unsuccessful; additionally, *Cs. inornata* does not feed on birds (Dow et al., 1976; McLintock et al., 1970; Reisen et al., 1990; Reisen et al., 1989; Sekla et al., 1980; Washino et al., 1962).

Early attempts were made to detect WEEV virus transmitted vertically in *Cx. tarsalis*. Theses studies used a variety of methods ranging from controlled laboratory experiments (Chamberlain and Sudia, 1957; Henderson and Burst, 1977; Thomas, 1963), testing adult males in nature (Reeves and Hammon, 1962), or testing adults from field-collected immature mosquito forms (Hammon and Reeves, 1947). As a result *Cx. tarsalis* has not demonstrated an ability to vertically transmit WEEV. Three isolations of WEEV

were made from *Ae. dorsalis* males and females collected as larvae collected in August 1991 and 1992 (Fulhorst et al., 1994). These isolations purportedly represent examples of vertical transmission of WEEV. However, the significance of these data has been brought into question due to sequencing information (Weaver, unpublished, 1997) and an inability to repeat the results (Kramer et al., 1998; Reisen et al., 1996). Similar evidence has never been found in *Cx. tarsali*s apart from a single isolation in Colorado (Mitchell, 1981). Little evidence has been documented of a mammal-*Aedes* transmission cycle occurring early in the spring. Additionally, *Ae. dorsalis* failed to be incriminated in in the ecology of WEEV in in the Coachella Valley of California during the years of 1994 thru 1996 (Reisen et al., 1998); though this could have been due to reduced activity of the virus. Interestingly, activity of WEEV has been documented in Canada in snowshoe hares and ground squirrels before *Cx. tarsalis* terminates diapause, which does indicate that some sylvatic cycle could precede springtime amplification between *Cx. tarsalis* and nestling passeriformes (Burton et al., 1966b; Iversen et al., 1971; McLintock et al., 1970; Yuill and Hanson, 1964). One drawback to this theory is the inability to demonstrate vertical transmission in nature by *Ae. melanimon,* which is arguably a more important vector in the late summertime mammal-mosquito transmission cycle (Reeves et al., 1990).

Hosts

Chronic infection in vertebrates is another possible strategy WEEV could utilize to over winter. Since WEEV is initially amplified by nestling passerines and the summer cycle is primarily perpetuated by infection of juvenile and adult passerines, it stands to reason that passerines, or other avian species, could potentially develop a chronic infection and maintain a viremia that is sufficient to infect a susceptible vector for an extended period of time. An experiment designed to explore this hypothesis found 8 of 224 experimentally infected birds had virus in various tissues 198 to 234 days post-

infection, 2 of which had viremia (Reeves et al., 1958a). Though the specific mechanism by which these viruses were unable to escape the immune response remains unclear. However, a follow-up study was unable to cause infection in *Cx. tarsalis* by feeding on birds one or more months post-infection (Reeves, 1974). Overall, the maintenance of the virus in passerines during the winter months seems unlikely.

Since passerines are unlikely candidates for virus maintenance during the winter, our attention is turned to other vertebrate species. Reptiles and amphibians do not usually demonstrate seropositivity in surveillance studies (Spalatin et al., 1964), however experimental infections indicate that WEEV infections do not always produce antibody levels high enough to be detected in the assays used (Thomas et al., 1980) and *Cx. tarsalis* does occasionally feed on reptiles (Hayes et al., 1973; Henderson and Senior, 1961; Tempelis et al., 1965; Tempelis and Washino, 1967). However, a recent dissertation found little evidence for *Cx. tarsalis* feeding regularly on exothermic animals (Thiemann and Reisen, 2012; Thiemann et al., 2011). Moreover, WEEV has been isolated from garter snakes (*Thamnophis* spp.) and leopard frogs (*Rana pipiens*) in the early summer (Burton et al., 1966a; Gebhardt et al., 1964). Also, viremias sufficient to infect *Cx. tarsalis* develop in garter snakes (Thomas and Eklund, 1960; Thomas and Eklund, 1962) and desert tortoises (Bowen, 1977) when experimentally infected with WEEV. Importantly, when garter snakes and desert tortoises are inoculated with virus and hibernation is subsequently induced, viremias drop to near-undetectable levels after the temperature is lowered to below 10°C. However, when the reptiles are brought out of hibernation the viremia returns to levels sufficient to infect *Cx. tarsalis* (Bowen, 1977; Thomas and Eklund, 1960; Thomas and Eklund, 1962). While the reptile overwintering hypothesis may be appealing, the rarity with which *Cx. tarsalis* feeds on reptiles makes the possibility less likely (Reeves, 1974; Reisen and Monath, 1988).

The last obvious option for WEEV maintenance in a host during the winter months is in mammals. As described above mammals typically become involved in the

transmission cycle during the late summer (Bowers et al., 1966). However, infection in some species does result in a fatal outcome, which makes for a poor virus reservoir (Hardy et al., 1974a; Kissling, 1958; Lennette et al., 1955). Independent transmission cycles do exist involving only mammals and their appropriate vector. In terms of overwintering, it has been hypothesized that physiological alterations associated with hibernation could result in maintenance of the virus through the winter months by delaying the viremia until spring (Karstad, 1963; Reisen and Monath, 1988). Isolations of WEEV have been made in the winter months from the common house mouse (*Mus musculus*) and the San Joaquin antelope squirrel (*Ammospermophilus nelsoni*) (Hardy et al., 1974b). Additionally, virus has been isolated from snowshoe hares and ground squirrels before *Cx. tarsalis* terminates diapause (Burton et al., 1966b; Iversen et al., 1971; McLintock et al., 1970; Yuill and Hanson, 1964).

Reintroduction

Evidence of extinction and reintroduction events have been demonstrated with several arboviruses including: West Nile, Japanese encephalitis, St. Louis encephalitis, Venezuelan equine encephalitis, Ross River, and eastern equine encephalitis viruses (Chen et al., 1990; Dusek et al., 2009; Kramer et al., 1997; Lindsay et al., 1993; Reisen et al., 2010; Rico-Hesse et al., 1995; Sammels et al., 1995; Seidowski et al., 2010; Weaver et al., 1991). Naturally, this evidence would lend investigators to ask if WEEV follows a pattern of extinction and reintroduction that accounts for its maintenance through the winter months and years of periodic inactivity. When a portion of the E2 gene from 55 strains of WEEV from various regions in California was analyzed phylogenetically results indicated that WEEV strains are maintained in distinct geographic foci and reintroduction events are quite rare, though they may occur infrequently reintroductions are certainly not a mechanism that would allow for a viable overwintering strategy (Kramer and Fallah, 1999).

Summary

The specific overwintering mechanism(s) WEEV uses is not understood despite decades of study. While some support exists for vertical transmission in *Ae. dorsalis* (Fulhorst et al., 1994), the significance of this finding has been brought into question as a result of sequencing data (Weaver, unpublished, 1997) and an inability to recapitulate the findings (Kramer et al., 1998; Reisen et al., 1996). Also, one would expect to isolate more virus from mammalian species early in the spring if this were the main overwintering mechanism. Reptiles could also play a role in the maintenance of WEEV over the winter months (Bowen, 1977; Thomas and Eklund, 1960; Thomas and Eklund, 1962), but the attack-rate of *Cx. tarsalis* against reptiles reduces the likelihood of this being the main mechanism for WEEV overwintering (Reeves, 1974; Reisen and Monath, 1988); more supporting data is needed before this mechanism is conclusively demonstrated. Ultimately, the mechanism(s) for WEEV maintenance through the winter months are most likely multi-faceted and dependent on the specific ecological parameters in the immediate geographic area.

ENVIRONMENTAL FACTORS

Ambient Temperature

Once a mosquito ingests an infectious blood meal the time between ingestion and the ability to transmit the virus is referred to as the extrinsic incubation period (EIP) (Hardy, 1988). EIP is a critical factor in the ecology of arboviruses because it governs the amount of time a vector must survive before it can transmit the virus. Conventional understanding of EIP indicates that temperature has an inverse relationship with EIP; in other words, when temperature increases the EIP decreases due to increased rates of viral replication and metabolic activity within the mosquito. The effect is established with several arboviruses including yellow fever virus (Whitman, 1937), Rift Valley fever virus (Turell et al., 1985), and EEEV (Chamberlain and Sudia, 1955). However, when infected

Cx. tarsalis females are incubated at a higher temperature (32°C) the infection rate decreases (Hardy et al., 1983; Kramer et al., 1983). In a later study, an increase in temperature was found to decrease transmission rates after peaking between days 7 and 10, though infection rates were not significantly altered by incubation temperature (Reisen et al., 1993). Field studies have also shown amount of virus required to infect 50% of mosquitoes increases during the summer months in California (Hardy et al., 1990; Reisen et al., 1996).

Weather

The transmission of WEEV is significantly affected by weather patterns due to their influence on habitats for mosquito larvae, which directly affects the abundance of adult female mosquitoes (Wegbreit and Reisen, 2000). A study of the relationships between weather and mosquito abundance in Kern County California showed the abundance of *Cx. tarsalis* was directly and quantitatively related to rainfall, snow depth and water content, and runoff in the Kern River. This study also found that WEEV returned to Kern County, after a prolonged absence of greater than ten years, only after relatively wet years (Wegbreit and Reisen, 2000).

With the world undergoing climatic warming and California and the southwest United States experiencing a record-breaking drought, knowing how WEEV is reacting to these changing conditions is critical for planning prevention strategies and understanding how WEEV's epidemic potential could change. The reliance WEEV demonstrates on both weather and lower ambient temperatures for transmission and a more efficient EIP, respectively, demonstrates that WEEV would disappear from southern regions for more temperate climates where the temperature and water abundance are more hospitable for *Cx. tarsalis* larvae (Reeves et al., 1994).

Epidemic and Epizootic Incidence in North America

WEEV has caused numerous documented epizootic events during the 20th century. The epizootic that prompted the discovery of the agent occurred in 1930 where approximately 6,000 horses were infected with a 50% case fatality rate (Meyer et al., 1931). Subsequently severe outbreaks appeared to progress toward the east from California during the years of 1931 thru 1934, reaching the grasslands of Canada in 1935 (Reisen and Monath, 1988). Further, several severe outbreaks occurred during the mid 1930s and throughout the 1940s (Buss and Howitt, 1941). More equids than humans exhibit WEEV disease (due to the increased amount of exposure to *Cx. tarsalis* and equids exhibiting more severe symptoms than humans) during epidemics though the specific intensity of each epidemic can vary widely. For example, during 1937 and 1938 more than 300,000 horses were infected with WEEV (Davison, 1942). An especially severe epidemic that occurred in 1941 resulted in 3,336 human deaths in the United States (mostly in the Dakotas, Minnesota, and Nebraska) and Canada (Manitoba and Saskatchewan) (Davison, 1942; Eklund, 1946).

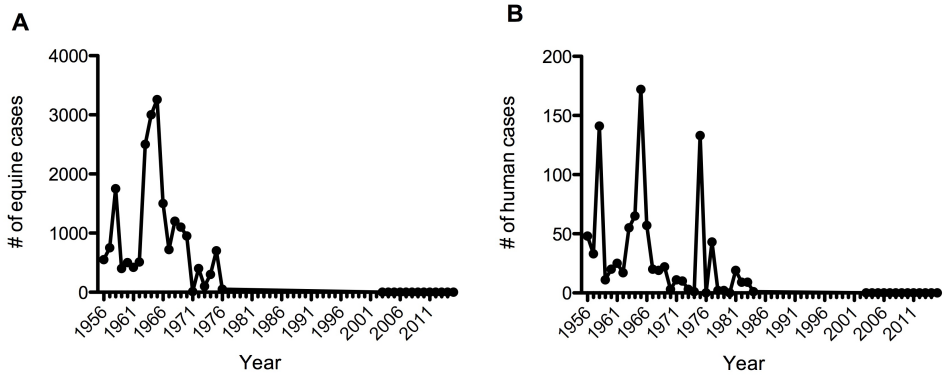

Figure 1.9 Reported case incidence of WEE each year in A) horses and B) humans from 1955-2015.
Data obtained from CDC and USDA reports and Reisen and Monath (1988). A) Epizootiological data from USDA/APHIS between 1977 and 2002 not available. One case was reported in 2003 and 2004 however, diagnostic testing not conclusive (USDA APHIS, personal communication, 2016) B) Epidemiological data from CDC between 1986-2002 taken down from CDC site and unavailable. Last human case reported in 1998.

After the 1930s and 40s the number of human and equine cases began to decline. This decline resulted in the zero case incidence seen today (Figure 1.9). Several severe outbreaks did occur. One well-studied epidemic that occurred in 1952 in the Central Valley of California resulted in 375 cases of WEEV-induced human encephalitis, 9 of which resulted in death. Other epidemics occurred in 1958, 1965, and 1975 (Hollister et al., 1953; Potter et al., 1977). Interestingly, some of the most severe epizootic events occurred in Midwestern North America and southern Manitoba, as in 1941. Several epidemics occurred in the late 19th century in the same region with the 1975 epidemic causing 53 human and 420 equine cases (Eadie and Friesen, 1982). WEE continued to occur sporadically throughout the 1980s and 90s with the last reported human case in North America occurring in 1998. One case of fatal WEE did arise in Uruguay during April of 2009 in an otherwise healthy 14-year-old boy (Delfraro et al., 2011). The infecting virus showed significant phylogenetic relationship with the WEEV/Imperial181 isolate that is generally thought to be avirulent; however, more intensive phylogenetic techniques would have been useful to verify this relationship.

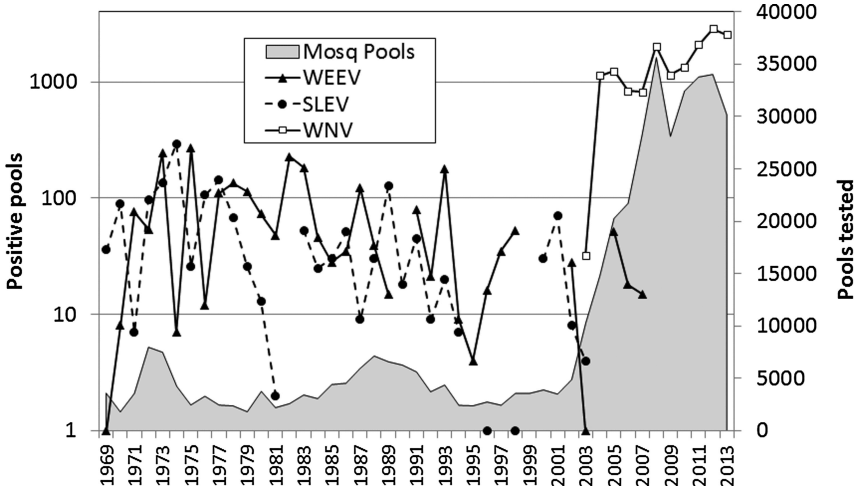

Figure 1.10 Number of mosquito pools positive for WEEV and tested for arboviruses in California, 1969-2013.
Reformatted from, Vector-Borne and Zoonotic Diseases, 16(4), Reisen, W. *et al.* Surveys for antibodies against mosquitobourne encephalitis viruses in California birds, 1996-2013, (2016) With permission from Vector-Bourne and Zoonotic Diseases.

WEEV is still found circulating in its enzootic cycle, as evidenced by positive mosquito pools, albeit at reduced levels (Figure 1.10) (Anderson et al., 2015; CDC, 2016b; Reisen, 1984). The last instances of identifying WEEV in its enzootic cycle occurred in Harris County, Texas with one seropositive house sparrow (*Passer domesticus*) (HOSP) in the summer of 2015 (Wilkerson, 2016) and a mosquito positive pool found in 2013 in Clark County, Nevada (CDC, 2016b). The specific reason WEEV has stopped causing significant amounts of human disease is something of a mystery, though reduction in incidence does correlate with a drastic decline in the US horse population (Figure 1.11) and increased vaccination coverage of horses (Reisen and Monath, 1988). To add to the confusion while the number of human cases decreased, the population living in endemic areas have increased and human antibody prevalence has decreased (Reeves and Milby, 1979; Reisen and Monath, 1988).

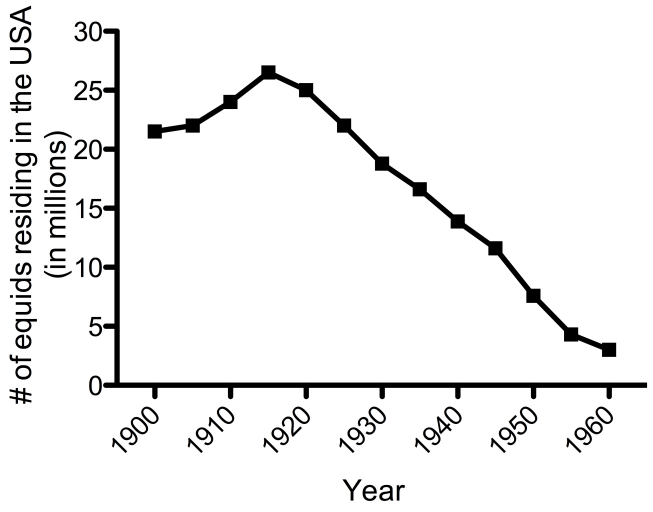

Figure 1.11 United States horse population 1900-1960 in millions.
Data reprinted with permission from (Kilby, 2007)

Previous Attempts at Understanding WEEV's Reduction in Human Incidence

Several attempts have been made to explain why the human incidence of WEE has decreased over the nineteenth century. These studies investigated a number of

different hypotheses using a variety of methodologies. The following sections constitute a review of these studies and their relevant findings.

INFECTIVITY VARIATION AND GENETIC DIVERSITY AMONG STRAINS OF WEEV

(Nagata et al., 2006)

BALB/c mice were used to assess variations in virulence among eight strains of WEEV. All strains analyzed in this study were of epizootic origin with isolation dates ranging from 1930 to 1984. When BALB/c mice were inoculated with $5.0x10^3$ plaque forming units (PFU) via the intranasal (IN) route 100% mortality was achieved, though, mean time to death (MTD) varied among strains (up to 7 days difference between strains). A simple cluster diagram was then constructed to determine any correlation between phylogenetic ancestry and virulence. The cluster diagram recapitulated the results found in Weaver *et al*. (1997) and indicated that the recent isolates (Weaver's Group B) had a longer MTD than the isolates made earlier in the 19th century (Weaver's Group A). The investigators then categorized these short and long MTD groups into two different "pathotypes": low virulence and high virulence. While this study indicated that there may be some change affecting WEEV's virulence in mammals, no definitive connection was made that would account for WEEV's reduction in human incidence.

LIMITED INTERDECADAL VARIATION IN MOSQUITO AND AVIAN HOST COMPETENCE FOR WEEV

(Reisen et al., 2008)

In order to determine if differences in the virus' enzootic host and vector competence could account for WEEV's reduction in human incidence, six isolates, each representing a decade from the 1950s to the 2000s were assessed in HOSPs, WCSPs, and *Cx. tarsalis*. For the sparrows virus was inoculated subcutaneously (SC) at approximately 10^3 PFU. The WCSPs showed no difference among the different viruses. The HOSPs showed similar results as the WCSPs, though the isolate representing the 2000's,

designated Imperial181, was unable to achieve a viremia greater than 10^4 PFU/ml while the other HOSPs all achieved viremias greater than 10^7 PFU/ml. Vector competence was measured by determining the WEEV infectious dose 50 (ID_{50}) required to infect *Cx. tarsalis* females. Groups of *Cx. tarsalis* were allowed to feed on artificial blood meals with varying amounts of virus ranging from 10^2 to 10^7 PFU/ml. Vector competence was then determined by detection of viral RNA from expectorated saliva samples. No significant differences were determined among the strains tested (BFS1703, from the 1950s; and IMP181, from the 2000s). Generally this study indicates that the date of virus isolation fails to account for any differences in WEEV's ability to replicate in its enzootic hosts or be transmitted by its enzootic vector. Though there was a difference in the HOSP viremia from the representative 2000s isolate (Imperial181), this could indicate that WEEV's ability to generate a robust viremia in HOSPs is dependent on certain aspects of the virus. Also, this study took representative samples from each decade; these isolates could fall in the same or different lineages, which may complicate the results.

WEE SUBMERGENCE: LACK OF EVIDENCE FOR A DECLINE IN VIRULENCE

(Forrester et al., 2008)

To investigate differences in strain virulence among enzootic and epizootic strains of WEEV, ten isolates, collected between the 1940s and 1990s, were chosen to determine any correlation between time of isolation and virulence in 6-8 week-old NIH Swiss-Webster mice. Briefly, mice were inoculated with 10^3 PFU virus SC, viremia and weight were monitored following infection. Results are suggestive that a decrease in virulence may have occurred over time, but are by no means definitive.

VIRULENCE VARIATION AMONG ISOLATES OF WEEV IN AN OUTBRED MOUSE MODEL

(Logue et al., 2009)

To better understand viral determinants of virulence six WEEV isolates underwent full genome sequencing. Sequence divergence was greatest between McMillan

(a Group A isolate collected in 1941) and Imperial181 (a Group B isolate collected in 2005) isolates with 2.7% amino acid sequence divergence. Specific amino acid differences between the two isolates were then noted. McMillan and Imperial181 were then assessed for virulence in 6-8 week-old CD1 mice. 10^3 PFU virus were administered SC. The differences obtained between these two isolates were drastic; McMillan achieved significantly higher brain titers than Imperial181 and 100% mortality where Imperial181 cause no mortality. These observed differences in virulence appeared to highlight differences intrinsic to each virus as a factor of time of isolation; however, due to differences between the isolates not controlled for, such as passage history, the conclusions drawn cannot solely be attributed to intrinsic differences between the viruses that arose in nature.

VARIATION IN MAMMALIAN, AVIAN, AND MOSQUITO CELLS FAILS TO EXPLAIN TEMPORAL CHANGES IN ENZOOTIC AND EPIDEMIC ACTIVITY IN CALIFORNIA

(Zhang et al., 2011)

As a follow-up to Reisen *et al*. (2008) this study assessed the *in vitro* replication kinetics and cell death in a variety of relevant cell cultures (C6/36, duck embryonic fibroblasts, and Vero) of the viruses investigated previously. No significant differences between interdecadal isolates were detected.

MOLECULAR DETERMINANTS OF MOUSE NEUROVIRULENCE AND MOSQUTO INFECTION FOR WESTERN EQUINE ENCEPHALITIS VIRUS

(Mossel et al., 2013)

In a follow up study to Logue *et al*. (2009) mutations identified in the previous study were tested for specific phenotypes in the CD1 murine disease model. The investigators found that when they swapped the glutamine at position 214 of the E2 glycoprotein of McMillan with the arginine present at the same position on Imperial181 mouse neurovirulence was ablated. Interestingly, the reciprocal mutation did not confer mouse neurovirulence to Imperial181. However, the 214 mutation and a glutamic acid

(Imperial181)-to-Lysine (McMillan) mutation at position 181 of E2 both confer reductions in mosquito infectivity on the Imperial181 backbone. These data account for inverse relationship between mouse neurovirulence and mosquito infectivity seen in the McMillan and Imperial181 isolates; however, the evolutionary significance of these mutations was not assessed. As a result, these virulence mutations could potentially have been artifacts of repeated mouse brain passages or other passage methodologies.

CONCLUSIONS

Accounting for WEEV's decline in human incidence has been extremely difficult for investigators to determine. Part of the difficulty lies in the lack of low passage isolates from the early 20th century. Additionally, many hypotheses proposed that account for the reduction of WEEV incidence in humans and reduced levels of enzootic activity tend to be challenging to test experimentally. Some of these hypotheses include a switch from ditch to piped irrigation, decreased exposure to infectious vectors (thanks to screen doors/windows and air conditioning), influences of global warming on the virus ecology, and a challenge to the dogma that horses are dead-end hosts, and thus vaccination and population decline resulted in the reduction of an important amplification host. Unfortunately, investigators cannot control for fifty years of human activity. However, studying the evolution of WEEV using a large data set would allow investigators to control variables such as passage history more effectively, possibly allowing other hypotheses to be tested more effectively. Studying the evolution of WEEV would also allow me to identify important evolutionary events in WEEV's evolutionary history that may have altered some phenotype of the virus. Thus my goal for my dissertation was to determine if the evolution of WEEV could account for the reduction in human incidence and enzootic circulation observed in the second half of the 20th century.

Chapter II: Evolutionary Analysis of Western Equine Encephalitis Virus*

INTRODUCTION

Western equine encephalitis virus (WEEV) is a mosquito-borne arbovirus and the causative agent of western equine encephalitis (WEE). Infections of humans and horses can be fatal, and survivors often suffer permanent neurological sequelae (CDC, 1995; Whitley and Gnann, 2002). WEEV belongs to the genus *Alphavirus* in the family *Togaviridae* and has a positive-sense, single-stranded RNA genome approximately 11.5kb in length, including two open reading frames (ORFs) flanked by 5'- and 3'-untranslated regions (UTRs) (Netolitzky et al., 2000; Strauss and Strauss, 1994). One unusual feature of WEEV is that it is the descendant of an ancient recombination event between Sindbis virus (SINV)-like and eastern equine encephalitis virus (EEEV)-like ancestors (Hahn et al., 1988; Weaver et al., 1997).

WEEV is found in North and South America. In North America, it circulates enzootically among passerine birds and is transmitted by its primary mosquito vector, *Culex (Culex) tarsalis* (Reeves and Hammon, 1958). Mammals can participate in a secondary cycle (Bowers et al., 1966; Hardy et al., 1977; Hardy et al., 1974b). Both humans and horses are thought to be dead-end hosts (Reisen and Monath, 1988), although some equids, such as burros and ponies, develop low to moderate levels of viremia (slightly under 10^4 PFU/ml) (Byrne et al., 1964; Sponseller et al., 1966), which could allow these hosts to contribute to epizootic amplification.

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* The data in this chapter were previously published in the Journal of Virology and the article is copyright © American Society for Microbiology. The content is reprinted with permission from the American Society for Microbiology. The article citation is Bergren, N.A., Auguste, A.J., Forrester, N.L., Negi, S.S., Braun, W.A. and Weaver, S.C., 2014. Western equine encephalitis virus: evolutionary analysis of a declining alphavirus based on complete genome sequences. Journal of Virology 88, 9260-9267.

In the 1930s through the 1950s, WEEV produced widespread outbreaks encompassing western North America, extending north into Saskatchewan, Canada (Reisen and Monath, 1988). Western states were affected by several outbreaks during the 1930s and, by 1937, the epidemic/epizootic reached the eastern side of the Canadian Rockies (Artsob and Spence, 1979; Cameron, 1942). Sporadic outbreaks continued to occur throughout the early 20th century in the western and Midwestern United States. However, the incidence of WEE has drastically decreased over the past four decades. The 1970s saw 209 human cases; 87 were reported in the 1980s, only 4 cases in the 1990s, and no cases have been reported in the United States or Canada since 1998 (CDC, 2010).

In order to explain these epidemiological data, several studies investigated possible reasons WEE incidence has decreased so rapidly (Forrester et al., 2008; Logue et al., 2009; Mossel et al., 2013; Nagata et al., 2006; Reisen et al., 2008; Zhang et al., 2011). While some suggested a reduction in mammalian virulence, interpretations were confounded by the viral strains used (e.g. different viral lineages, various passage histories, etc.)

Only two detailed phylogenetic studies of WEEV have been conducted (Kramer and Fallah, 1999; Weaver et al., 1997). By sequencing partial E1 envelope glycoprotein and nsP4 genes Weaver *et al*. (1997) identified two monophyletic lineages and proposed that one had become extinct. Kramer and Fallah (1999) sequenced the E2 envelope glycoprotein gene of a large collection of WEEV isolates from California and observed the maintenance over time of local enzootic lineages. However, both studies were limited by the short sequence fragments employed and the phylogenetic methods available at the time.

To accurately assess the evolutionary history of WEEV and identify population changes and mutations that might be related to the historic decline in WEEV incidence, we conducted a robust phylogenetic analysis using complete WEEV genomic sequences representing a diverse temporal and geographic distribution, with a focus on low-passage-

number virus trains. Also, a three-dimensional (3D) homology model of the E1 and E2 proteins was generated with mutations of interest mapped on the model.

MATERIALS AND METHODS

Virus strain selection, propagation, and isolation of RNA

Thirty-three WEEV strains were chosen based on varied locations and years of collection, with a focus on low-passage-number histories (Table 2.1). Viruses were propagated on C6/36 cells (Forrester et al., 2008) and precipitated with polyethylene glycol (Vasilakis et al., 2013). RNA was extracted using TRIzol LS (Invitrogen, Carlsbad, CA) as per the manufacture's instructions.

RT-PCR, PCR amplification, and sequencing

cDNA was prepared using SuperScript III (Invitrogen) per the manufacturer's instructions. Overlapping PCR amplicons coving the WEEV genome were generated using WEEV-specific primers (see primer sequences in Appendix 1) and Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA). PCR amplicons were purified from agarose gels using a gel extraction kit (Qiagen, Netherlands), and direct sequencing of amplicons was performed using WEEV-specific internal primers and BigDye terminator v3.1 (ABI, Foster City, CA) and a 3500 Genetic Analyzer (ABI). Sequences were assembled using Sequencher v5.0.1 (Gene Codes Corporation, Ann Arbor, MI).

Sequence analysis

The ORFs from 27 genomic WEEV sequences were aligned with all genomic sequences from the GenBank library using Seaview v4.1 (Glatier et al., 1996). MacVector v.11.0.2 (MacVector Inc., Cary, NC) was used to determine percent

nucleotide and amino acid identity for the complete concatenated ORFs and for each gene. Comparisons of all strains were made against Imperial181.

Table 2.1 Collection of WEEV sequences used for analysis

Strain	Location	Date (day-mo-yr)	Host	Passage History	Accession no.
AG80646	Chaco Province, Argentina	1980	*Culex ocossa*	v (2), sm (1)	GQ287646
California	San Joaquin Valley, CA	1930	Horse	gp (?), sm (27), C6 (1)	KJ554965
McMillan	Ontario Province, Canada	1941	Human	mp (2), sm (2), v (2), C6 (1)	GQ287640
BFS932	Bakersfield, CA	1946	*Culex tarsalis*	sm (1), v (1)	KJ554966
EP-6	Missouri	1950	Mosquito	ce (1), C6 (1)	KJ554967
BFS1703	Bakersfield, CA	1953	*Culex tarsalis*	sm (1), C6 (1)	KJ554968
BFS2005	Bakersfield, CA	1954	*Culex tarsalis*	de (1)	GQ287644
E1416	Kern County, CA	25-Jan-1961	*Zonotrichia leucophrys*	bhk (4), C6 (1)	KJ554969
Montana64	Montana	1967	Horse	de (1), C6 (1)	GQ287643
S8-122	Butte County, CA	2-Aug-1968	*Sclurus griseus*	sm (1), C6 (1)	KJ554970
BFS3060	Butte County, CA	19-Jul-1971	*Culex tarsalis*	ce (1), sm (1), C6 (1)	KJ554972
71V1658	Oregon	12-Aug-1971	Horse	v (2), smb (1)	GQ287645
TBT-235	Texas	1971	*Gopherus berland*	wc (1), de (1), sm (1), bhk (1), C6 (1)	KJ554971
75V9291	Wilkin City, MN	26-Jul-1975	*Culex tarsalis*	v (2), C6 (1)	KJ554973
BFS09997	Kern County, CA	30-Jun-1978	*Culex tarsalis*	v (1), C6 (1)	KJ554974
CHLV53	Riverside County, CA	19-Jul-1983	*Culex tarsalis*	v (1), C6 (1)	KJ554976
KERN5547	Kern County, CA	1983	*Culex tarsalis*	v (1), C6 (1)	KJ554975
85452NM	New Mexico	1985	*Culex tarsalis*	sm (2), C6 (1)	GQ287647
PV0208A	Lubbock County, TX	1990	Mosquito	v (1), or sm (1), C6 (1)	KJ554977
IMPR441	Imperial County, CA	21-Jul-1992	*Culex tarsalis*	sm (1), C6 (1)	KJ554978
CO921356	Larimer County, CO	30-Jul-1992	*Culex tarsalis*	v (1), C6 (1)	KJ554979
93A38	Tacna, AZ	8-Jun-1992	Mosquito	v (1), C6 (1)	KJ554980
93A27	Parker, AZ	9-Jun-1992	Mosquito	v (1)	KJ554981
93A30	Phoenix, AZ	10-Jun-1993	Mosquito	v (1), C6 (1)	KJ554982
93A79	Yuma, AZ	13-Jul-1993	Mosquito	v (1), C6 (1)	KJ554983
CNTR34	Contra Costa County, CA	1993	*Culex tarsalis*	v (1), C6 (1)	KJ554984
LAKE43	Lake County, CA	1994	*Culex tarsalis*	v (2), C6 (1)	KJ554985
PV72102	El Paso County, TX	1997	Mosquito	v (1) or sm (1), C6 (1)	KJ554986
PV012357A	El Paso County, TX	2001	Mosquito	v (1) or sm (1), C6 (1)	KJ554987
R02PV002957B	El Paso County, TX	2002	Mosquito	v (1) or sm (1), C6 (1)	KJ554988
R02PV001807A	El Paso County, TX	2002	Mosquito	v (1) or sm (1), C6 (1)	KJ554989
R05PV003422B	El Paso County, TX	2005	Mosquito	v (1) or sm (1), C6 (1)	KJ554990
R0PV00384A	El Paso County, TX	2005	Mosquito	v (1) or sm (1), C6 (1)	KJ554991
Imperial181	Imperial County, CA	2005	*Cx. tarsalis*	v (2)	GQ287641

[a]Passage numbers are in parentheses. Abbreviations: gp, guinea pig; mp, mouse; sm, suckling mouse; smb, suckling mouse brain; v, Vero cells; bhk, baby hamster kidney cells; wc, wet chicks; de, duck embryonic fibroblasts; cd, chick embryonic fibroblast; C6, C6/36; p, passage in unknown medium; ?, unknown passage number.

Phylogenetic methods

A coalescent phylogenetic analysis of the WEEV sequences was preformed using BEAST v.1.7.5 (Drummond et al., 2012). The analysis was run once for 50 million steps,

sampling every 10,000 steps and discarding the first 10% as burn-in; 1st, 2nd, and 3rd codon positions were analyzed independently. A Bayesian skyline analysis was conducted under the strict clock, uncorrelated log-normal clock (UCLN), and uncorrelated exponential clock (UCEX) models, and convergence was assessed by examining the stationary ln-likelihood and effective sample size (ESS, >200) parameters in Tracer v1.4 (http://tree.bio.ed.ac.uk/sofware/tracer/). To determine the best-fit substitution and clock models, path-sampling and stepping-stone analyses were used (Baele et al., 2012; Baele et al., 2013). A maximum clade credibility (MCC) tree, node heights (hypothesized dates of divergence events), evolutionary rates, and a Bayesian skyline plot were then generated. The BEAST output tree file was analyzed using Tree Annotator (included in the BEASTv.1.7.5 software package), discarding the first 10% as burn-in, and visualized in FigTree v.1.3.1. To verify its accuracy, a Bayesian phylogeny was inferred in MrBayes (http://mrbayes.sourceforge.net/) using the general time-reversible (GTR+I+Γ_4) model, which was determined to be optimal using Modeltest (Posada and Crandall, 1998). The analysis was performed for 1 million steps, with sampling every 1,000 steps and discarding the first 10% as burn-in.

Nonsynonymous synapomorphic mutations of interest

To identify mutations that define major WEEV lineages, each amino acid was manually traced on the inferred MCC tree using MacClade v4.08 (http://macclade.org/macclade.html). To assess potential selective pressures accompanying WEEV evolution, WEestimated the number and locations of nonsynonymous and synonymous nucleotide substitutions per site and determined if the sites were positively or negatively selected using the Data Monkey server (Pond and Frost, 2005a). The d_N/d_S ratio reflects the predominance of synonymous mutations, which generally reflect neutral change, versus nonsynonymous mutations that more often reflect phenotypic alterations. Codon-based selection analyses use d_N/d_S to estimate the overall

impact of selection on specific codons, which, when pared with nucleotide substitution models and viewed in a phylogenetic framework, can identify selected mutations across lineages (Pond et al., 2009). The overall d_N/d_S ratio and selection pressure were determined by single likelihood ancestor counting (SLAC) and fast unbiased Bayesian approximation (FUBAR) methods (Pond and Frost, 2005b; Pond et al., 2009). Positive and negative selection events at each codon also were inferred using internal fixed-effect likelihood (IFEL), FEL, and FUBAR methods, and appropriate statistical tests were run on these tests as part of the Data Monkey Server package (Murrell et al., 2013; Pond and Frost, 2005b; Pond et al., 2006).

Molecular modeling of WEEV E1 and E2 envelope proteins

Sequences of the E1 and E2 proteins from the BFS932 and Imperial181 WEEV strains were submitted to fold recognition servers (Söding et al., 2005) for homologous sequence alignment using crystal structures of SINV (protein data base (PDB) entry 3MUU) and chikungunya virus (CHIKV) (PDB entry 3N40) proteins as templates. Because domain 2 is missing from the SINV E2 structure, we used the SINV E1 protein to generate 3D model structures of the BFS932 and Imperial181 proteins, while the CHIKV E2 proteins was used to generate 3D models of E2. MPACK (Mumenthaler and Braun, 1995; Sanner et al., 1989) was used to build homology model structures, which were energy minimized using the Fantom program (Schaumann et al., 1990). Finally, trimeric model structures of BFS932 and Imperial181 were obtained by fitting their E1 and E2 proteins into a trimeric structure of SINV (http://pymol.org).

RESULTS

Percent nucleotide and amino acid identities indicated that WEEV has maintained a highly conserved genome since 1930 (Table 2.2). The percent identities for the genome and individual genes were greater than 95%. Some genes, including E2, contained higher

nucleotide than amino acid identities, and E2 had the greatest variation in both amino acids and nucleotides. Conversely, nsP1 was the most highly conserved gene.

Table 2.2 Nucleotide and amino acid divergence of complete WEEV genome ORFs and individual genes compared to the Imperial181 strain

Reported as percent nucleotide (amino acid) divergence from Imperial181 strain.

	California	McMillan	BFS932	EP6	BFS1703	BFS2005	E1416	Montana64	S8122	TBT235	BFN3060
Genome	97.3 (98.0)	97.3 (98.1)	98.0 (98.6)	97.9 (98.6)	97.8 (98.4)	97.7 (98.4)	97.8 (98.4)	98.4 (98.8)	98.2 (98.8)	98.6 (99.0)	98.2 (98.7)
nsP1	98.7 (99.4)	98.7 (99.4)	99.2 (99.8)	99.1 (99.8)	98.9 (99.2)	99 (99.4)	98.9 (99.2)	99.2 (99.6)	99.2 (99.8)	99.3 (99.6)	99.2 (99.8)
nsP2	97.1 (98.9)	97.2 (98.9)	97.8 (99.1)	97.8 (99.1)	97.6 (99.1)	97.6 (99.0)	97.6 (99.1)	98.3 (99.1)	98.1 (99.0)	98.6 (99.2)	98.1 (99.0)
nsP3	97.0 (97.6)	97.0 (97.6)	97.5 (98.1)	97.6 (98.1)	97.2 (97.6)	97.2 (97.6)	97.2 (97.6)	98.0 (98.5)	97.9 (98.3)	97.8 (98.5)	97.9 (98.1)
nsP4	97.5 (98.4)	97.5 (98.4)	98.1 (99.0)	98.1 (99.2)	97.7 (98.7)	97.7 (98.5)	97.7 (98.7)	98.7 (99.2)	98.4 (99.3)	99.1 (99.5)	98.4 (99.3)
Capsid	97.0 (97.3)	97.0 (97.3)	97.9 (98.1)	97.7 (97.7)	97.8 (98.1)	97.7 (98.1)	97.8 (98.1)	98.5 (98.5)	98.3 (98.5)	99.1 (99.2)	98.1 (98.2)
E3	97.2 (96.7)	97.8 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)
E2	96.3 (95.1)	96.3 (95.6)	97.4 (97.0)	97.2 (97.2)	97.4 (97.0)	97.4 (97.0)	97.4 (97.0)	97.7 (97.4)	97.7 (97.4)	98.1 (97.7)	97.7 (97.4)
6K	97.3 (100)	97.3 (100)	97.3 (98.0)	97.3 (98.0)	97.3 (98.0)	97.3 (98.0)	97.3 (98.0)	98.7 (98.0)	98 (100)	98.0 (98.0)	98.0 (100)
E1	97.0 (98.6)	97.2 (98.9)	97.9 (99.1)	98 (98.9)	97.7 (98.9)	97.7 (98.9)	97.7 (98.9)	98.2 (99.3)	98 (99.3)	98.3 (99.1)	97.8 (98.6)

	71V1658	75V9291	BFS09997	KERN5547	CHLV53	85452NM	PV02808A	IMPR441	CO921356	93A38	93A27
Genome	98.2 (98.7)	98.2 (98.8)	97.7 (98.4)	98.9 (99.2)	98.8 (99.1)	99.2 (99.4)	97.7 (98.7)	99.0 (99.2)	97.7 (98.6)	97.7 (98.6)	99.0 (99.2)
nsP1	99.5 (99.8)	99.2 (99.8)	98.9 (99.2)	99.4 (99.8)	99.4 (99.8)	99.7 (99.8)	99.1 (99.8)	99.2 (99.6)	99.1 (99.8)	99.1 (99.8)	99.4 (99.6)
nsP2	98.2 (99.0)	98.2 (99.1)	97.6 (99.1)	98.7 (99.2)	98.6 (99.1)	99.2 (99.6)	97.3 (99.0)	99.0 (99.5)	97.3 (99.0)	97.4 (98.9)	99.0 (99.6)
nsP3	97.7 (98.1)	97.6 (98.1)	97.2 (97.6)	98.6 (98.7)	98.6 (98.9)	99.0 (99.1)	97.0 (97.9)	98.8 (98.3)	96.9 (97.6)	97.1 (97.7)	98.8 (98.5)
nsP4	98.5 (99.2)	98.5 (99.0)	97.7 (98.7)	99.2 (99.7)	99.1 (99.5)	99.3 (99.5)	98.2 (99.3)	99.2 (99.5)	98.2 (99.3)	98.0 (99.2)	99.0 (99.3)
Capsid	97.9 (98.1)	97.8 (98.5)	97.8 (98.1)	99.4 (99.6)	99.1 (99.6)	99.5 (100)	97.7 (98.1)	99.0 (100)	97.4 (98.1)	97.7 (98.1)	99.1 (100)
E3	97.2 (96.7)	97.8 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)	98.3 (98.3)	96.7 (98.23)	98.3 (98.3)	96.7 (98.3)	96.7 (98.3)	98.3 (98.3)
E2	97.4 (97.0)	97.7 (97.7)	97.2 (96.5)	98.6 (98.6)	98.3 (98.4)	98.9 (98.8)	97.3 (97.2)	98.8 (98.8)	97.4 (97.2)	97.4 (97.2)	98.5 (98.8)
6K	98.0 (98.0)	98.0 (98.0)	97.3 (98.0)	98 (96.0)	98 (96.0)	98.7 (98.0)	97.3 (96.0)	98.7 (98.0)	97.3 (96.0)	97.3 (96.0)	98.7 (98.0)
E1	98.1 (99.3)	98.2 (99.3)	97.7 (98.9)	98.6 (99.5)	98.8 (99.5)	99.1 (99.5)	97.7 (99.3)	98.9 (99.5)	97.8 (99.3)	97.7 (99.3)	98.9 (99.3)

	93A30	93A79	CNTR34	Lake43	PV71202	PV012357A	R02PV002957B	R02PV001807A	R02PV003422B	R0PV003814A
Genome	99.5 (99.7)	99.0 (99.4)	97.7 (98.6)	97.7 (98.6)	99.5 (99.7)	99.6 (99.7)	99.6 (99.8)	99.6 (99.7)	99.9 (100)	99.9 (99.9)
nsP1	99.8 (99.8)	99.6 (99.8)	99.0 (99.8)	99.1 (99.8)	99.6 (99.8)	99.7 (99.8)	99.9 (100)	99.7 (99.8)	99.9 (100)	99.7 (99.8)
nsP2	99.6 (99.9)	98.9 (99.6)	97.4 (99.0)	97.4 (98.9)	99.5 (99.9)	99.6 (99.7)	99.5 (99.7)	99.6 (99.7)	99.9 (100)	99.9 (100)
nsP3	99.4 (99.6)	98.8 (99.1)	97.0 (97.7)	96.9 (97.6)	99.4 (99.6)	99.4 (99.4)	99.2 (99.4)	99.4 (99.2)	99.8 (100)	99.7 (99.8)
nsP4	99.5	99.0	98.0	98.1	99.6	99.8	99.7	99.8	99.9	99.9

	(99.6)	(99.7)	(99.3)	(99.3)	(99.8)	(99.8)	(99.8)	(99.8)	(100)	(100)
	99.6	99.2	97.7	97.7	99.7	99.9	99.9	99.9	99.9	99.9
Capsid	(100)	(99.6)	(98.1)	(98.1)	(100)	(100)	(100)	(100)	(100)	(100)
	98.3	98.3	96.7	96.7	98.3	99.4	99.4	99.4	100	100
E3	(98.3)	(98.3)	(98.3)	(98.3)	(98.3)	(100)	(100)	(100)	(100)	(100)
	99.4	98.7	97.3	97.4	99.4	99.5	99.4	99.5	99.8	99.8
E2	(99.3)	(98.8)	(97.2)	(97.2)	(99.3)	(99.5)	(99.5)	(99.5)	(100)	(100)
	99.3	98.7	97.3	97.3	99.3	100	100	100	100	100
6K	(100)	(98.0)	(96.0)	(96.0)	(100)	(100)	(100)	(100)	(100)	(100)
	99.2	99.0	97.7	97.7	99.3	99.5	99.5	99.5	99.9	100
E1	(99.5)	(99.5)	(99.3)	(99.3)	(99.8)	(99.8)	(99.8)	(99.8)	(100)	(100)

Stepping-stone and path-sampling analyses indicated GTR+I+Γ_4 as the best-fit nucleotide substitution model along with the UCEX model. The inferred Bayesian MCC phylogeny showed the presence of four main WEEV lineages (Figure 2.1). The California and McMillan isolates were not monophyletic, as in previous analyses (Weaver et al., 1997), due to the absence of a more divergent WEEV strain as an outgroup. However, Group A was confirmed as monophyletic using an Markov chain Monte Carlo (MCMC) analysis with the South American isolate AG80-646 as an outgroup.

Estimated dates of lineage divergence were obtained for Groups B1 to B3. Group A sequences were difficult to resolve, and the divergence of Group A from Group B1 could not be reliably estimated. Based on the phylogenetic data and the history of WEE outbreaks, this divergence probably occurred in the mid-1930s to early 1940s. Group B2 diverged from Group B1 in approximately 1944, with 95% highest posterior density (HPD) values of 1942 to 1946. Finally, group B3 diverged around 1967 (95% HPD = 1965-1970). The overall rate of WEEV evolution was estimated at 2.8×10^{-4} substitutions/site/year (95% HPD = $3.4 \times 10^{-}4$ to 2.2×10^{-4}), with rates for individual lineages in a narrow range of 8.0×10^{-4} to 3.0×10^{-4} substitutions/site/year.

A Bayesian skyline plot showed a slight increase in the WEEV estimated population size between 1940 and 1965 during the year of the last major outbreaks (Figure 2.3) (Reisen and Monath, 1988). This increase was followed by a plateau and then by a decline beginning around 1990, corresponding to the establishment of Group B3 that contains all currently circulating strains.

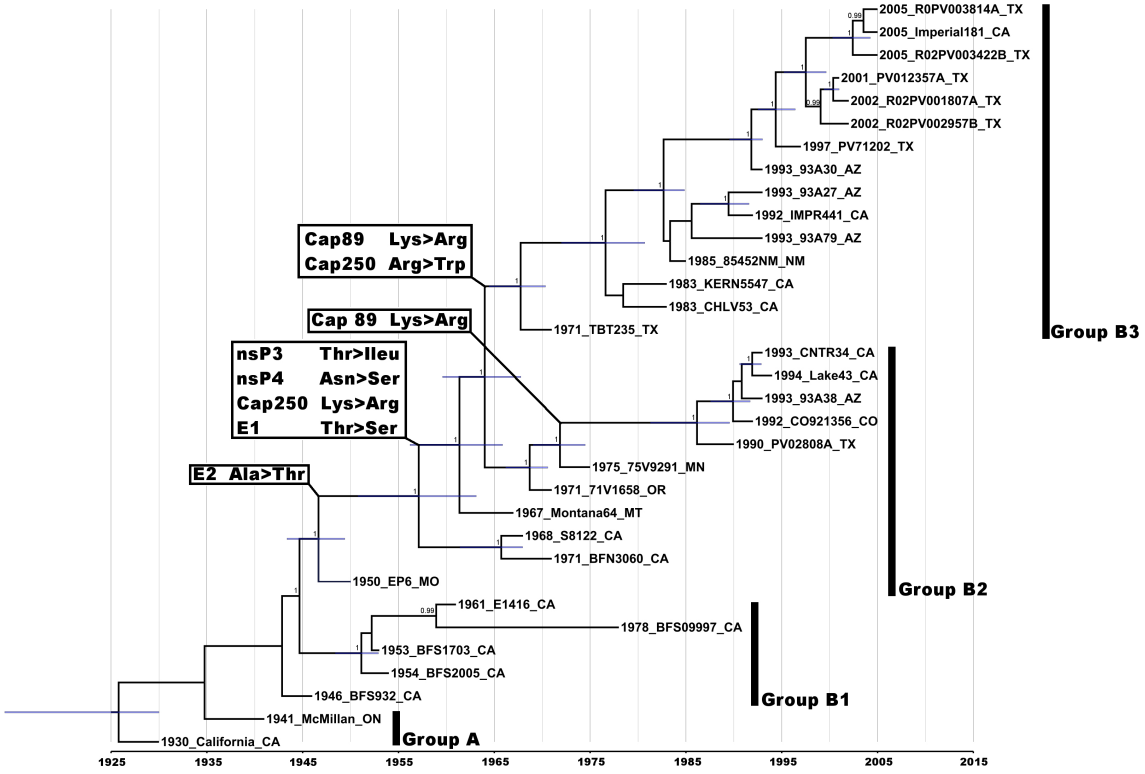

Figure 2.1 Maximum clade credibility tree based on 33 WEEV complete genomes. Numbers at nodes indicate posterior probabilities of ≥0.9. Bars at nodes indicate 95% confidence intervals of divergence dates, and the *x*-axis represents time in years. The four distinct lineages, Groups A, B1 to B3, are indicated. Nonsynonymous synapomorphic mutations are indicated on the tree based on their identified nodes of occurrence. Taxon/tip labels include year of isolation, strain name, and state where the virus was isolation.

Upon manual analysis of the MCC tree and alignment file using MacClade v4.08, six nonsynonymous synapomorphic mutations of interest were found that delineated the clades resolved on the MCC tree (Figure 2.1 and Table 2.3). Selection analysis showed that the WEEV genome has evolved mainly under purifying selection (d_N/d_S ratio of 0.145). The IFEL analysis detected only one positively selected site verses 39 negatively selected sites at $P \leq 0.1$. The positively selected site, encoding a Val-to-Ile substitution involved part of Group B3 (strain 93A30 and more recent trains) as well as strains S8122, BFN3060, California, and McMillan (Figure 2.1). When the mutations manually traced were analyzed, IFEL analysis suggested positive selection; however, P values were > 0.1

(Table 2.3). Positive selection on these sites also was suggested by both FEL and FUBAR analyses (although *P* values were still > 0.1).

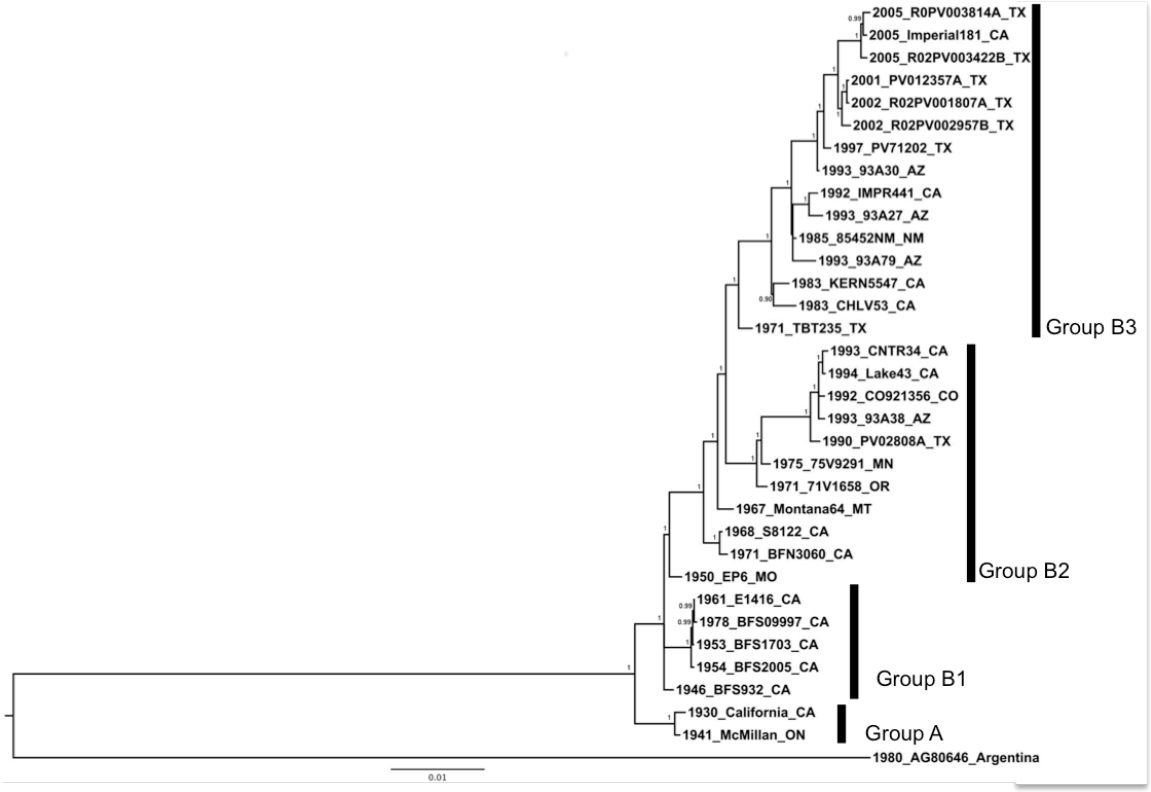

Figure 2.2. Markov chain Monte Carlo tree based on 33 WEEV complete genomes with AG80-646 as an outgroup.
Numbers at nodes indicate posterior probabilities of ≥0.9. The four distinct lineages, Groups A, B1 to B3, are indicated. Nonsynonymous synapomorphic mutations are indicated on the tree based on their identified nodes of occurrence. Taxon/tip labels include year of isolation, strain name, and state where the virus was isolation.

3D models for both the BFS932 and Imperial181 E1 proteins indicated three domains: 1 and 2 had an interlinking beta sheet structure with a long hydrophobic fusion loop at one end of domain 2, while domain 3 shared high structural similarity with the immunoglobulin domain.

A complete list of amino acid positions that differed between BFS932 and Imperial181 was obtained for the E1 and E2 proteins. To visualize these amino acids, a trimeric structure of the BFS932 E1-E2 heterodimer was constructed (Figure 2.4). The BFS932 E1 protein had 11 differences compared to all other WEEV strains (Figure 2.4 A

to C, yellow), while there were only 4 differences compared to Imperial181 (Figure 2.4 D to F, yellow). There were 25 differences in the BFS932 E2 protein compared to all WEEV strains (Figure 2.4 A to C, red) and 13 differences compared to Imperial181 (Figure 3 D to F, red). Most of the E2 substitutions were located at the E2-E2 interface. Nonsynonymous, synapomorphic mutations of interest were mapped on the D3 structure and are indicated in magenta (Thr->Ser at position 374 in E1) and green (Ala->Thr at position 23 in E2) (Figure 2.4)

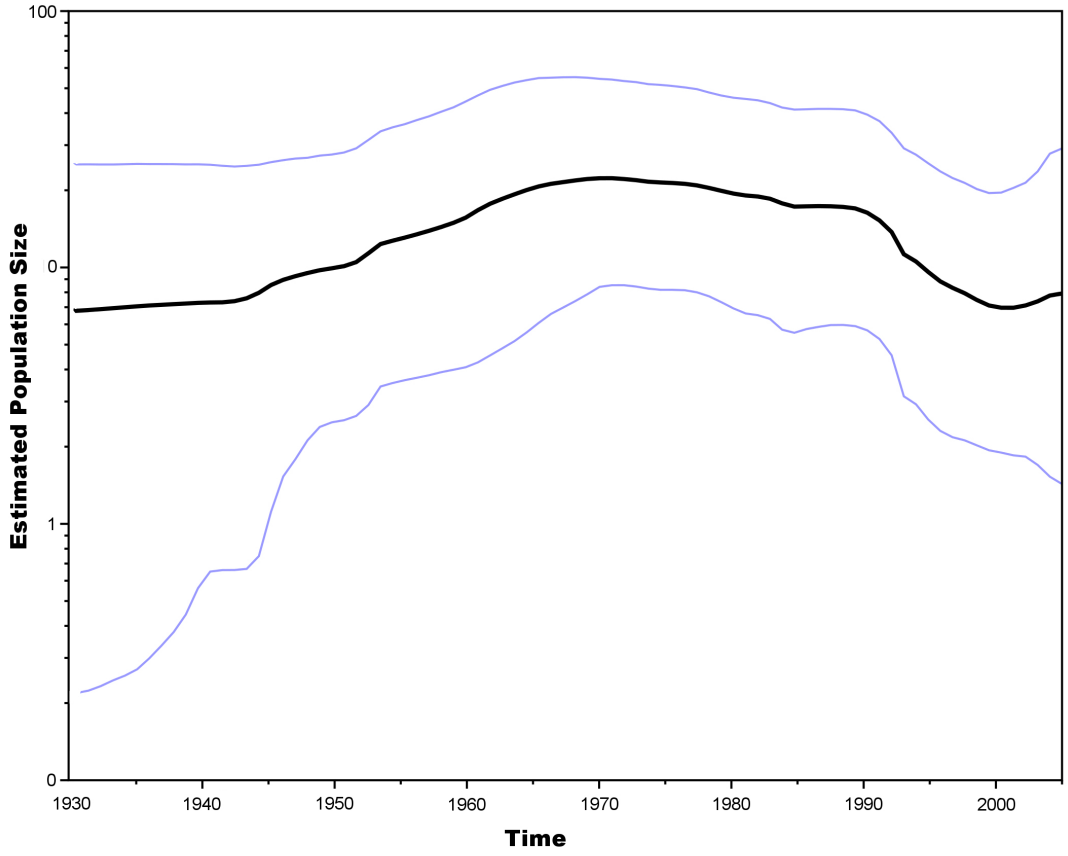

Figure 2.3 Bayesian skyline plot of WEEV strains in North America between 1930 and 2005.

the centerline represents the estimate for mean estimated population size (or mean genetic variation) in the population through time. The upper and lower blue lines represent the 95% HPD.

DISCUSSION

This report consists of the first detailed phylogenetic and evolutionary analysis of WEEV using complete genomic sequences. The presence of two major North American lineages was confirmed as previously described (Weaver et al., 1997) and further delineated several sub-groups (B1 thru 3) within the Group B lineage. Purifying selection was also identified as the major influence on WEEV evolution in North America, as described previously for several other arboviruses (Auguste et al., 2010; Volk et al., 2010). Additionally, several mutations that define the divergence of Groups A and B1 thru B3 were recognized. No phylogenetically related differences in glycosylation sites, cysteine residues, and UTR folding patterns were recognized.

Table 2.3 Mutations that contribute to the definition of WEEV lineages

Gene	Change in Amino Acid	Amino Acid Position	Mutation	Nucleotide Position (from beginning of nonstructural protein ORF)	Codon Position	IFEL Analysis	
						Selection Type	p-value
nsP3	Thr > Ileu	152	C > T	4436	2	+	0.11
nsP4	Asn > Ser	602	A > G	7382	2	+	0.23
Capsid	Lys > Arg	89	A > G	7714	2	+	0.16
Capsid	Lys > Trp	250	A > T	8196	1	+	0.28
			A > G	8197	2		
E2	Ala > Thr	23	G > A	8472	1	+	0.38
E1	Thr > Ser	374	A > T	10959	1	+	0.28

In North America, WEEV was first isolated in 1930 from a fatal case of equine encephalitis (Meyer et al., 1931). This Californian strain fell into the Group A lineage along with the 1941 McMillan isolate. This relationship suggests WEEV's early epidemic spread to the eastern side of the Canadian Rockies from California. We hypothesize that Group A became extinct in the 1940s and was displaced by Group B1, which then became predominant. Strains from ancestral Groups A and B1 generally are more virulent, in murine models, than more contemporary strains from Groups B2 and B3 (Forrester et al., 2008; Logue et al., 2009; Nagata et al., 2006).

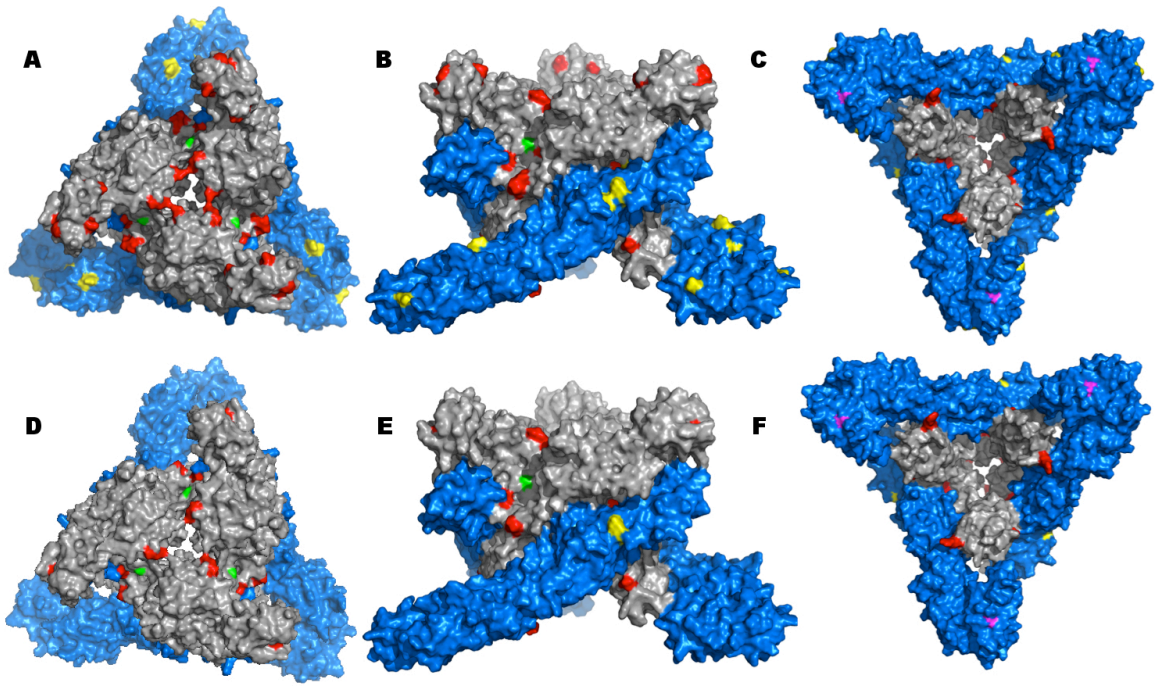

Figure 2.4 Predicted E1-E2 WEEV trimers.
(A thru C) Mapping of all mutations on WEEV/BFS932 surface compared to all WEEV strains. (A) Top view; (B) side view; (C) bottom view. (D thru F) Mapping of all mutations on the WEEV/BFS932 surface compared to WEEV/Imperial181. (D) Top view; (E) side view; (F) bottom view. All mutations in the E1 protein are shown in yellow, and those from the E2 proteins are shown in red. Amin acids 374 in E1 (magenta) and 23 in E2 (green) are also indicated.

In the late 1940s, Groups B2 displaced Group B1, which probably went extinct. Subsequently, in the late 1960s, the Group B3 lineage emerged from Group B2, eventually replacing it. The consistency of the tightly grouped HPDs throughout the MCC tree supports the reliability of these temporal estimates (Figure 2.1).

When all three Group B sublineages were circulating, the Bayesian skyline analysis indicated a concurrent increase in WEEV estimated population size between 1965 and the late 1980s. However, after the late 1980s a reduction in estimated population size occurred when Group B3 viruses became predominant in North America. The interpretations require caution due to the 95% HPD values for 1930 to 1950 being relatively broad, which can be a result of sampling bias. There is more confidence in the estimates between the years 1950 and 1995, when the HPD range was tighter (Figure

2.3). However, when the estimated time to most recent common ancestor (tMRCA) and Skyline analyses are both considered in concert, the evidence for these population size interpretations is compelling. Interestingly, the pattern seen on the skyline analysis is similar to that of the annual influenza A virus cycle, although not as pronounced and over a much longer period of time (Rambaut et al., 2008). This could be an effect of purifying selection on WEEV, trimming the tree to the Group B3 lineage.

The selection analyses indicated that many nucleotide sites within the WEEV genome are under purifying selection. When population sizes are reduced, stochastic drift could result in the accumulation of deleterious mutations, fitness declines, and lineage extinction events that could explain not only the lineage replacements we observed but also the decline in WEEV genetic diversity. However, manual inspection of our WEEV alignment revealed several mutations that may represent positively selected codons. These mutations were, for the most part, just under the threshold of significance as determined by IFEL analysis ($P > 0.1$) (Table 2.3). Both FEL and FUBAR flagged these same mutations at P values that narrowly missed the threshold of significance, suggesting that they have important phenotypes. Furthermore, current codon-based analyses sometimes lack the sensitivity to detect positive selection. For example, analysis preformed on CHIKV sequences failed to identify all mosquito vector adaptive mutations shown by experimental studies (Tsetsarkin et al., 2014; Tsetsarkin and Weaver, 2011; Tsetsarkin et al., 2009; Tsetsarkin et al., 2007; Volk et al., 2010).

The homology models suggested that the E2-E2 and E2-E1 interfaces, locations far removed from receptor binding or potential antibody binding sites, are important sites of WEEV evolution. Mutations at these interfaces (Figure 2.4), including the substitution at E2 position 23 (Table 2.3), may stabilize the E2-E2 trimer spikes and further prevent the release of genomic RNA during fusion.

The recent epidemiology of WEE, with no reported human cases in North America since 1998, the dearth of WEEV detected in mosquito surveillance since 2008

(CDC, 1995, 2010), and the pattern of lineage displacement observed in our phylogeny (Figure 2.1 and 2.2), with one lineage becoming predominant along with a decline in genetic diversity in Group B3, raises a key question: what factor(s) caused the apparent reduction in WEEV circulation and resulting spillover of disease in humans? We hypothesize that a significant disturbance in WEEV circulation occurred roughly between 1945 and 1965. This event affected WEEV evolution in one of two ways: 1) changes in selective pressures altered the trajectory of WEEV evolution during the late 20th century, or 2) a reduction in WEEV populations and/or diversity caused genetic drift and a decline in WEEV fitness, possibly coupled with reduced mammalian virulence. The key synapomorphic mutations we delineated, including those that may be subject to positive selection, deserve reverse genetic analyses to test these hypotheses by assessing the phenotypic properties.

In summary, using comprehensive phylogenetic analyses, we confirmed the major Group A and B lineages described previously (Weaver et al., 1997) and determined the further divergence of Group B into 3 sublineages, two of which probably went extinct. we also delineated several mutations that define Groups A and B1 thru B3, which may have been positively selected. However, overall, WEEV's evolution has been dominated by purifying selection. WEEV has undergone a reduction in genetic diversity coincident with the circulation of only the Group B3 lineage since the 1970s, suggesting that drift reduced its fitness, levels of circulation, and possibly its virulence in mammals. These data, as well as the apparent submergence of WEEV as an and important arbovirus affecting both humans and equids, provides a unique opportunity to study a phenomenon that, compared to studies of arboviral emergence, may be equally instructive regarding their maintenance, evolution, and the ability to predict future trends.

Chapter III: Construction and *in vitro* Characterization of Infectious Clones

INTRODUCTION

Development of genetic systems where the sequence of a virus can be specifically altered has been a major cornerstone in modern virology. By altering the sequence of organisms researchers are able to determine the function of specific residues and larger sequence elements, create new assays and methodologies for studying these organisms, and develop new treatments and prevention strategies for various diseases. For example, by exploiting the replication strategy of alphaviruses live-attenuated vaccine candidates can be generated and are both safe and efficacious (Atasheva et al., 2009; Kim et al., 2011; Pandya et al., 2012; Plante et al., 2011; Wang et al., 2007a). These systems have also been used to determine structures critical for the replication of alphaviruses (Frolov et al., 2001; Gorchakov et al., 2004; Hardy, 2006; Niesters and Strauss, 1990). Additionally, these systems allow researchers to assess epizootic and enzootic phenotypes between less divergent alphavirus strains in vertebrate and mosquito models (Brault et al., 2002; Greene et al., 2005a; Kuhn et al., 1996; Powers et al., 2000).

Generating a genetic system for alphaviruses is relatively easy due to their genomes being positive sense and relatively short (approximately 11.5kb). The first step in developing a genetic system for alphaviruses (termed reverse-genetic system for alphaviruses due to their RNA genome) is reverse transcribing their genomic RNA and cloning it into a DNA plasmid. Directly adjacent to the virus genome on the 5' end is an RNA transcription promoter that facilitates *in vitro* transcription of the DNA to RNA. The plasmid also contains a linearization site and antibiotic resistance gene, both 3' of the virus genome, to facilitate the generation of single genome RNA copies and plasmid DNA in bacterial culture, respectively. Once the plasmid DNA has been transcribed to

RNA, the RNA can then be transfected into cells through a variety of methods. The transfected cells are then allowed to incubate and generate virus a genomic sequence identical to the plasmid DNA. Virus is then harvested and characterized in a variety of ways that answer pertinent research questions.

Through studying the comprehensive phylogeny of WEEV complete genomes (discussed in Chapter II), we were able to identify several mutations that may have participated in WEEV's reduction in enzootic circulation and human incidence (Table 2.3). In order to assess whether these mutations impart a phenotype that could potentially contribute to WEEV's reduction in enzootic and human incidence, infectious clones containing each mutation and various combinations thereof were generated on the WEEV/Imperial181 (IMP181) backbone. IMP181 was isolated from a pool of *Cx. tarsalis* mosquitoes in Imperial County, California in 2005 (Table 2.1). This virus has been used as the prototypical contemporary isolate of WEEV in the majority of recent studies (Logue et al., 2009; Mossel et al., 2013; Reisen et al., 2008; Zhang et al., 2011) and will serve a similar role for the studies described below. Additionally the WEEV isolate BFS932, isolated from Bakersfield California in 1942 from a pool of mosquitoes and has a relatively low passage history (Table 2.1), was used as a virulent wild-type control in the replication curve experiments and several experiments throughout the project due to its low passage history, high virulence and isolation date (Forrester et al., 2012). Interestingly, while the isolate is highly neurovirulent (see Chapter V) it does not possess the murine neurovirulent adaptation mutation R214Q in E2 described in Mossel et al. (2013), which may have arisen due to passage history or the species from which the virus was isolated; for these reasons BFS932 serves as an appropriate WEEV strain to use as an ancestral control.

In order to understand the effects of the identified mutations on viral fitness *in vitro* multi-step replication curves were conducted in Vero, 3T3, and C7-10 cells. Additionally, competition assays were conducted in Vero and C7-10 cells. Replication

curves are a common tool used to assess the replicative fitness (e.g. the ability to infect a cell and generate infectious virions) of a virus *in vitro* (Dulbecco and Vogt, 1954; Weaver et al., 1999; Zhang et al., 2011). Competitive fitness assays are a sensitive assay that reports on the competitive advantage of one virus over another in the context of the system they are tested in (Holland et al., 1991; Tsetsarkin and Weaver, 2011; Weaver et al., 1999). Briefly, two viruses are mixed at equal ratios and allowed to infect a chosen cell type. After infection and replication, virus is harvested and the ratio of viruses is determined. The virus that replicates with greater efficiency will occupy a greater proportion of the overall virus population. Both multi-step and competitive fitness assays were conducted *in vitro* before moving into more complicated systems in order to strategize efforts in *in vivo* systems.

Cell types used throughout this study include Vero, 3T3, and C7-10 cells. Vero cells, isolated from the kidney of an African green monkey (*Cercopithecus aethiops*), are an extremely common cell line used in modern virology (Ammerman et al., 2008). While they are extremely useful by virtue of their ease of handling and the ubiquity with which they are used (which more easily allows for comparison among different studies), Vero cells are incapable of producing alpha- or beta-interferon (Desmyter et al., 1968), thus Vero cells may not be able to discriminate the fitness of a mutation if it alters the virus' relationship with the interferon pathway and/or the effects thereof. Because Vero cells do not mount an interferon response, NIH-3T3 (3T3) cells were also used. 3T3 cells are mouse embryo fibroblasts (Todaro and Green, 1963) and are commonly used in virological research (Frolov et al., 2012). In addition to the two vertebrate cell lines, C7-10 cells, derived from *Aedes albopictus*, were used to determine if any differences between mutations could be determined in a cell line relatively close to its vector.

MATERIALS AND METHODS

Cloning Point Mutations

The IMP181 cDNA infectious clone was obtained from Aaron Brault at the Centers for Disease Control and Prevention (CDC). PCR-mediated mutagenesis was used to introduce each mutation individually (Horton, 1995), generating six clones each with one mutation. Additionally, in order to perform competition assays (described bellow) an IMP181 clone with the *XbaI* site in nsP2 silently ablated was developed. A graphical representation the process of site directed mutagenesis is depicted in Figure 3.1.

PCR AMPLIFICATION, RESTRICTION, AND LIGATION

Antiparallel WEEV-specific primers with the mutation of interest present in the primer sequence were used in conjunction with flanking WEEV-specific forward and reverse primers to amplify the two primary PCR fragments. Primary fragments were then concatenated via a secondary PCR reaction with the flanking WEEV-specific forward and reverse primers. All PCR amplifications were conducted using NEB's Phusion Polymerase (Ipswich, MA) as per manufacturers instructions. Proper PCR amplification and restriction digests were all verified by size exclusion gel electrophoresis using 1% agarose gel on 1X TAE buffer with 0.5 mg/ml ethidium bromide (all gels prepared in this manner unless otherwise noted). After band size was verified, amplified cDNA (cloned DNA) was excised out of the agarose gel and purified using Qiagen's (Hilden, Germany) Gel Extraction kit in accordance with the manufacturers instructions. The secondary fragments and IMP181 infectious clone were then cut using unique sequence specific restriction enzymes from NEB. Ligation reactions between the restricted fusion fragment and the restricted infectious clone were conducted using Agilent Genomics' Ligation Kit (Santa Clara, CA). Table 3.1 details the mutagenesis primers, flanking primers and restriction enzymes used for each clone.

Cloning 6X and 3X-1/2 Point Mutants

In order to more efficiently determine if any of the key discovered mutations have a phenotypic effect in relevant systems, plasmids with all or half of the discovered nonsynonymous synapomorphic mutations were developed. These clones were designed in-house and constructed by Epoch Life Sciences (Missouri City, TX). WEEV/IMP181-6X contains all mutations listed in Table 2.3. The half mutant clones, were divided into two clones based on their change in amino acid residues, changes that were from one type to the same type (e.g. polar to polar) were included in one clone and changes that were from one type to another (e.g. polar to non-polar) were included in the other. The resulting clones were named WEEV/IMP181-nsP3-Cap250-E1 and WEEV/IMP181-nsP4-Cap89-E2, with each gene listed representing the mutation that occurs in the gene. Once the clones were received from Epoch Life Sciences, stocks were made as described below and sequences were verified by Sanger sequencing. Table 3.2 outlines the name of each clone generated both in-house and by Epoch Life Sciences and the mutations they contain.

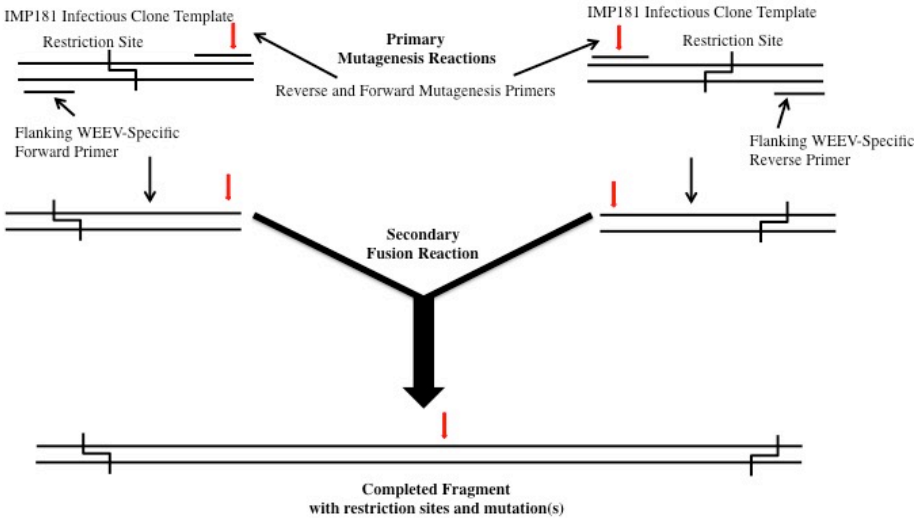

Figure 3.1 Diagram detailing the steps required for PCR-mediated mutagenesis. Figure details both primary and secondary PCR reactions. Required restriction digest of completed fragment and infectious clone and subsequent ligation of the restated fragment and infectious clone not shown.

Rescuing Virus from Plasmid DNA

TRANSFORMING TOP10 E. COLI *CELLS WITH PLASMID*

Ligated cDNA plasmids were then used to transform One Shot Top10 *E. coli* from Invitrogen (Carlsbad, CA). Briefly, vials of One Shot Top10 cells were thawed on ice. Afterwards, 5µl of ligation mix was pipetted directly into individual vials. Samples were allowed to incubate on ice for 30 minutes followed by a 30 second heat shock at 42°C. Samples were placed back on ice and 250µl S.O.C. media was added to each sample aseptically. Samples were allowed to incubate at 37°C with 225 rpm in a shaking incubator. After 1 hour samples were spread on LB plates with ampicillin (100µg/ml unless otherwise noted) and allowed to incubate overnight at 37°C.

Table 3.1 List of primers and restriction enzymes used to clone point mutations into IMP181 via PCR mediated mutagenesis.

IMP181 Mutation	Mutagenesis Primers*†	Flanking Primers	Restriction Enzymes
XbaI Ablation (nsP2-*XbaI*)	GAAGGCTCT**G**GAGCCAGT	WEEV-2089F CTGGTAGATCCACCATTTCAC	*NdeI*
		WEEV-4213R GTCCTACAGCATGTATGATGAG	*BssHII*
nsP3 Ileu152Thr	ACCAGGATAA**C**CGAGGCCATTC	WEEV-3288F GTATGGGCTTAATAGAGAGGTAG	*BssHII*
		WEEV-5553R CAACTCCGCTTCTGAGATC	*BlpI*
nsP4 Ser602Asn	CATAAGAGGGA**G**CCCAATCAC	WEEV-6296F CATGCAATGATGAGTACTG	*HindIII*
		WEEV-8010R GTTTGTCGCTGGTGTACTGCAG	*BbvCI*
Capsid Arg89Lys	CAAGCCAAGAA**A**GACGAAAC	WEEV-6893F GTATGTTCCTTAACGCTGTTTG	*StuI*
		WEEV-9704R TGTTTCAGCGTTGGTTGG	*PspOMI*
Capsid Trp250Lys	GATCC**TT**AACGGTCACC	WEEV-6893F	*StuI*
		WEEV-9704R	*PspOMI*
E1 Ser374Thr	GGCAAGAAG**A**CCACCTGCAATG	WEEV-9337F GAGCAGACGCAACAGCAG	*PspOMI*
		WEEV-11484R GAAATTTTAAAAACAAAATAAAAACAAAAG	*AvaI*
E2 Thr374Ala	GTATTGCAGACACTCA**G**CGC	WEEV-6893F	*StuI*
		WEEV-9704R	*PsPOMI*

*Mutated bases are indicated in bold.
†Reverse mutagenesis primer is antiparallel to the forward mutagenesis primer (shown).

Approximately 10 random colonies that successfully grew overnight were then selected and used to inoculate 10ml Terrific Broth (TB) (Corning Life Sciences, Tewksbury, MA) with ampicillin. Liquid cultures were then allowed to incubate overnight at 37°C with 225 rpm in a shaking incubator. After incubation, 7ml were taken for isolation of plasmid DNA using Qiagen's QIAprep Spin Miniprep Kit as per manufactures instructions. Upon isolation of plasmid DNA, a PCR that ran over the

region cloned was performed using NEB's Phusion polymerase and WEEV specific primers to grossly determine success of the cloning procedure.

LARGE SCALE PREPARATIONS OF PLASMID cDNA

DNA plasmids for all mutants were prepared in the same manner. Fluted 1000ml Erlenmeyer flasks with 250ml TB with ampicillin, were inoculated using the remaining 3ml liquid culture leftover from the miniprep, and allowed to incubate overnight at 37°C with 225 rpm in a shaking incubator. After incubation, bacteria were pelleted by centrifugation at 4070 rcf at 4°C for 30 minutes. After the supernatant was discarded the pellet was resuspended in 8ml of BF1 buffer (0.025M Tris-HCl, 0.1M NaCl, 0.01M EDTA pH 7.5). Samples were then transferred to 50ml conical tubes with 16 ml BF2 buffer (0.1M NaOH, 1% SDS) and mixed until homogeneous. Samples were then incubated on ice for 10 minutes, after which 12ml BF3 buffer (3M Potassium Acetate) was added, samples were mixed and placed on ice for 15 minutes. Cell debris and protein were pelleted via centrifugation at 4070 rcf at 4°C for 10 minutes. Supernatant was decanted through a 100μm cell strainer (Falcon, Tewksbury, MA) into a new 50ml conical tube. Isopropanol (Sigma-Aldrich, St Louis, MO) was then added to the 45ml mark of the conical tube. Solution was mixed thoroughly and placed at -20°C for 30 minutes to precipitate the nucleic acids. The nucleic acids were then pelleted via centrifugation at 4070 rcf for 10 minutes. The supernatant was discarded and the pellet was dissolved in 2ml of Tris-EDTA (TE). Two milliliters 5M LiCl was added to precipitate the RNA, samples were mixed and placed on ice for 10 minutes. RNA was pelleted via centrifugation at 4070 rcf for 10 minutes. Remaining supernatant was transferred to 15 ml conical tubes with 8ml 100% ethanol. Samples were mixed thoroughly and placed at -20°C for 1 hour to precipitate the DNA. DNA was pelleted via centrifugation at 4070 rcf for 10 minutes. After, the supernatant was discarded and the pellet was dissolved in 1ml TE. In a separate 15ml conical tube 4.8g CsCl, 40μl ethidium

bromide (10mg/ml), plasmid solution, and additional TE was added for a total weight of 9.1g. Dissolved solutions were then transferred to OptiSeal™ tubes for NVT-90 rotors (Beckman Coulter, Brea, CA) and appropriately balanced. Tubes were centrifuged overnight in an ultracentrifuge at 292,438 rcf. After ultracentrifugation, the plasmid band was extracted by piercing the side of the tube with a 14-gauge syringe (only approximately 0.5ml was taken). Plasmid was transferred to a 15 ml conical tube with 1 ml TE and 3.5 ml 100% ethanol. Solution was mixed and stored at -20°C for 1 hour. The resulting precipitated plasmid was then pelleted via centrifugation at 4070 rcf for 10 minutes. Supernatant was discarded and pellet was dissolved in 0.4 ml TE and transferred to a microcentrifuge tube. Concentration of the solution was measured by OD260. After concentration was recorded, 400µl Phenol-Chloroform (Ambion, Austin, TX) was added and mixed thoroughly. Aqueous and organic phases were separated via centrifugation at 16,873 rcf for 5 minutes. The aqueous phase was then transferred to a new tube with 70µl 5M NaCl and 850µl 100% ethanol. After mixing, the solution was placed at -20°C for 15 minutes. Precipitated DNA was then pelleted via centrifugation at 16,873 rcf for 5 minutes. Supernatant was then discarded and TE was added to a total concentration of 1mg/µl. Resulting stock solutions were stored at -80°C. All stock plasmids underwent full genome sequencing via Sanger methods (described in Chapter II) to verify the absence of any undesired mutations or errors in the sequence that could have arisen by virtue of the cloning methods.

Table 3.2 List of all infectious clones generated and the mutations they contain.

Infectious Clone	Mutation(s) Present in Each Infectious Clone*					
IMP181-nsP2-*XbaI*	nsP2 Leu481Leu					
IMP181-6X	nsP3 Thr152Ileu	nsP4 Asn602Ser	Capsid Lys89Arg	Capsid Lys250Trp	E2 Ala23Thr	E1 Thr374Ser
IMP181-nsP3-Cap250-E1	nsP3 Thr152Ileu	Capsid Lys250Trp	E1 Thr374Ser			
IMP181-nsP4-Cap89-E2	nsP4 Asn602Ser	Capsid Lys89Arg	E2 Ala23Thr			
IMP181-nsP3	nsP3 Thr152Ileu					
IMP181-nsP4	nsP4 Asn602Ser					
IMP181-Cap89	Capsid Lys89Arg					
IMP181-Cap250	Capsid Lys250Trp					
IMP181-E1	E1 Thr374Ser					
IMP181-E2	E2 Ala23Thr					

*Gene | Ancestral Mutation | Position | Contemporary Mutation

IN VITRO *TRANSCRIPTION*

In order to generate infectious RNA from a DNA plasmid 10µl of plasmid DNA was linearized with NotI (NEB) and phenol/chloroform purified prior to *in vitro* transcription. RNA transcripts of virus genome were generated using the T7 mMessage mMachine® Kit (Ambion, Austin, TX). Briefly, 5µl of linearized DNA was mixed with 10µl 2X NTP-CAP, 2µl 10X Reaction Buffer, 2µl Enzyme Mix, and 1µl nuclease-free water. Preps were incubated at 37°C for 2 hours. RNA was purified by adding 30µl nuclease-free water and 30µl LiCl Precipitation Solution, mixing, and placing at -20°C for 1 hour. RNA was pelleted by centrifugation at 4070 rcf at 4°C for 15 minutes. Supernatant was discarded and samples were washed with 70% ethanol and centrifuged again. Pellet was dissolved in 11ul nuclease-free water and concentration was determined by spectrometry. Samples were stored at -80°C until electroporation (no more than 24 hours).

ELECTROPORATION

Electroporation was performed in Vero cells (2 T-150s (Corning Life Science) @ ~85% confluence for each sample) (ATCC CCL-81, American Type Culture Collection, Manassas, VA) maintained in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, Carlsbad, CA), 10% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) 100X Non-Essential Amino Acids (Sigma-Aldrich), 1% (vol/vol) Sodium Pyruvate (100mM) (Gibco) and 1% (vol/vol) Penn-Strep (Penicillin & Streptomycin) (5,000 U/ml) (Gibco) and washed three times in 1X Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco). Prior to DPBS washing, the total number of cells was determined. After washing, cells were suspended in 700µl DPBS and mixed with the RNA product from the *in vitro* transcription and immediately electroporated to minimize degradation of the RNA. Electroporation was performed on a BTX ECM 830 (BTX, Holliston, MA) set to: 250V, 10ms per pulse, 3 pulses, with a 1s interval between pulses. After electroporation samples

were allowed to sit at room temperature for 10 minutes, after which the suspension of electroporated cells was transferred to 10ml DMEM supplemented as described above. Cell suspension was then transferred to a T-75 flask (Corning Life Science) and supplemented DMEM was added to total 20ml. Flasks were incubated at 37°C with 5% CO_2 in a humidified incubator for 48-72 hours. After incubation infectious media was harvested and clarified by centrifugation at 4070 rcf for 10 minutes. One-milliliter aliquots of clarified infectious media were then made in 2ml screw-cap tubes and stored at -80°C.

INFECTIOUS CENTER ASSAY

In order to determine the efficiency of the electroporation and detect potential cloning/mutagenesis errors an infectious center assay (ICA) was preformed. Vero cells at 90% confluence were prepared on 6-well plates and serial dilutions of the electroporated cells from -1 to -6 were prepared and transferred to the 6-well plate. After 1 hour incubation an overlay consisting of 0.05% agarose and supplemented media (2% agarose on H_2O diluted with supplemented DMEM to 0.05%) was applied to the 6-well plate. Assays were incubated at 37°C with 5% CO_2 in a humidified incubator for 48 hours. After incubation 10% neutral buffered formalin (Sigma-Aldrich) was applied to each well in order to fix the plates. After fixing formalin was removed along with the agarose plug. Plates were developed with crystal-violet stain (0.5% crystal-violet (Fisher Scientific, Waltham, MA), 25% (vol/vol) methanol (Sigma-Aldrich)) and plaques were counted to determine efficiency.

PLAQUE ASSAY

Stock virus concentration was determined by plaque assay on Vero cells. Briefly samples were serially 10-fold diluted from -1 to -6 and an appropriate amount of each dilution was inoculated onto each well (100µl for 12-well and 200µl for 6-well plates).

Cells were then allowed to incubate at 37°C with 5% CO_2 in a humidified incubator for one hour. After incubation a 0.05% agarose DMEM overlay was applied to each well. Samples were allowed to incubate for 48 hours. After the 48 hour incubation, plates were fixed with 10% neutral buffered formalin. After fixation, formalin was removed along with the agarose plug. Plates were then stained with crystal-violet to visualize plaques, which were counted for calculation of viral titer.

Replication Curves

Multi-step replication curves were conducted in 6-well plates in triplicate in order to examine potential changes in replication kinetics of the different mutants in Vero, 3T3 (ATCC, Manassas, VA), and C7-10 (courtesy of Ilya Frolov) cells. Cells were maintained with DMEM supplemented with 10% FBS (5% for Veros), 1% Penn-Strep, 1% 100X Non-Essential Amino Acids, and 1% 100mM Sodium Pyruvate. C7-10 media was also supplemented with 1% Tryptose Phosphate Broth (Sigma-Aldrich). Each virus was diluted to a multiplicity of infection (MOI) of 0.1 and back titrated to verify proper MOI. Media in the plates was aspirated and virus inoculum was added (0.200ml). Plates were allowed to incubate at 37°C and 5% CO_2 in a humidified incubator for 1 hour with frequent rocking. After incubation inoculum was removed and cells were washed three times with DPBS and 2ml supplemented DMEM was added to each well. Supernatant samples were taken at 0, 3, 6, 12, 24, 36, and 48 hours post infection (hpi) and plaque assayed on Vero cells to determine replication and infection kinetics.

***in vitro* Competition Assays**

Competitive fitness assays were conducted in Vero and C7-10 cells. Mutant viruses were competed against the ablated restriction site mutant (WEEV/IMP181-nsP2-*XbaI*) acting as a surrogate wild-type virus. Mixtures of viruses were prepared to an MOI of 0.01 at a 1:1 ratio calculated based on plaque assay titration. Infection of cells was

conducted as described above for replication curves. After 48 hpi RNA was extracted from cell culture using Trizol (Invitrogen) as per manufactures instructions. Following RNA extraction a 200bp fragment with the *XbaI* site in nsP2 directly in the center was generated using SuperScript III reverse transcriptase (Invitrogen) and NEB's Phusion Polymerase with WEEV specific primers. Generation of correct amplicons was verified on a 1% agarose gel and extracted using Qiagen's Gel Extraction Kit. Approximately 500ng of purified DNA product was digested with *XbaI* for 2 hours at 37°C. Digested product was then ran on a 1% agarose gel. The ratio of mutant (100bp) to WEEV/IMP181-nsP2-*XbaI* (200bp) was then estimated by quantifying band densitometry using myImageAnalysis™ (ThermoFisher Scientific, Waltham, MA).

RESULTS

Electroporation Titers and Efficiencies

All clones listed in Table 3.2 were successfully electroporated and were capable of producing viable virus with no undesired mutations (as determined by Sanger sequencing of the full genome). Table 3.3 lists each virus' electroporation efficiency and titer. Some viruses had to be passed once in Vero cells to generate a high titer (also listed in Table 3.3), as before genomes were sequenced after passage to insure proper sequence. IMP181-nsP3-Cap250-E1 and IMP181-nsP4-Cap89-E2 did not require additional passage in Vero cells, as the electroporation titer was sufficient to conduct the required experiments.

Replication Curves

The initial means of measuring fitness between mutated viruses consisted of multi-step replication curves in Vero, 3T3, and C7-10 cells. Infectious titers at sequential time-points are presented in Figure 3.2. A two-way ANOVA with a Bonferroni post-test comparing mutated viruses to IMP181 was conducted on all samples. In all three cell-

types, statistical analysis indicated that there were significant differences between the individual curves. Significant differences between the mutated viruses and IMP181 at specific time-points are recorded in Table 3.4. IMP181-nsP3-Cap250-E1 and IMP181-nsP4-Cap89-E2 were not included in these assays due to their construction and incorporation to experiments occurring at a later date. Also, 3T3 cells were carried out an additional 24 hours due to significant amount of intact monolayer at 48 hpi.

Table 3.3 Electroporation efficiencies and titers of infectious clones

Infectious Clone	Electroporation Efficiency (cells infected/μg RNA)	Post-Electroporation Titer (PFU/ml)	Vero Passage Titer (PFU/ml)
IMP181	$4.6x10^5$	$1.5x10^5$	$1.2x10^7$
IMP181-nsP2-*XbaI*	$2.6x10^6$	$3.0x10^5$	$2.0x10^7$
IMP181-6X	$7.4x10^5$	$4.0x10^4$	$6.8x10^6$
IMP181-nsP3-Cap250-E1	$1.0x10^6$	$9.5x10^6$	N/A
IMP181-nsP4-Cap89-E2	$5.8x10^5$	$1.2x10^7$	N/A
IMP181-nsP3	$6.0x10^5$	$8.5x10^4$	$1.8x10^7$
IMP181-nsP4	$1.5x10^6$	$3.0x10^5$	$1.3x10^7$
IMP181-Cap89	$7.3x10^4$	$1.7x10^4$	$2.6x10^7$
IMP181-Cap250	$2.1x10^5$	$1.2x10^5$	$8.8x10^7$
IMP181-E1	$2.0x10^6$	$4.3x10^4$	$3.3x10^7$
IMP181-E2	$1.1x10^6$	$3.5x10^5$	$1.3x10^7$

Taking into account the Bonferroni post-test data, viruses at terminal, or near terminal time-points showed significance more often than earlier time-points. Interestingly, mutated viruses in 3T3 cell culture typically grew to lower titers as compared to IMP181; while in C7-10 cell culture, mutated viruses generally replicated more efficiently than IMP181. Moreover, mutated viruses, except for IMP181-Cap89, replicated to titers similar to BFS932 in C7-10 cells. IMP181 and BFS932 replicated to titers higher than most mutated virus (though IMP181-nsP3 and IMP181-nsP4 do replicate to titers similar to IMP181 and BFS932) in 3T3 cells. Vero cells did not recapitulate the results seen in the 3T3 nor C7-10 cells with IMP181 residing approximately in the median of the final titers measured. IMP181-nsP3, IMP181-Cap250, IMP181-E2, IMP181-E1, and BFS932 all indicated significance at terminal or near-terminal time-points in all cell types (Table 3.4). 3T3 cells had more significant hits than either Vero cells or C7-10 cells. IMP181-nsP2-*XbaI* showed significance in both C7-10

and 3T3 cells, indicating that it may not accurately mimic wild-type virus; however, Vero cells showed no significant difference (Table 3.4).

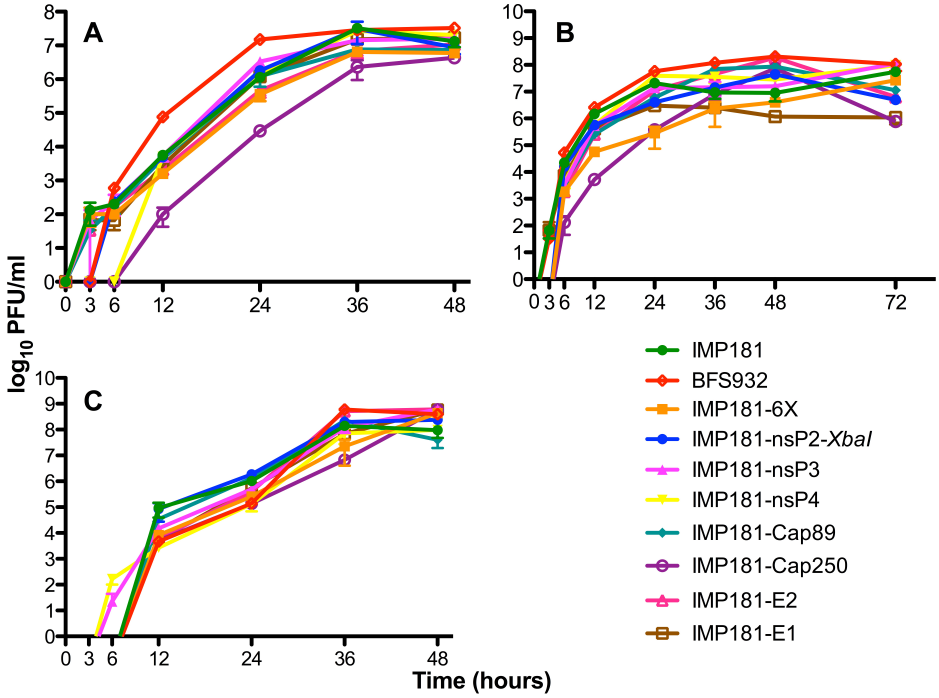

Figure 3.2 Multi-step replication curves of viruses with phylogenetically conserved mutations.

Replication curves conducted in A) Vero, B) 3T3, and C) C7-10 cells. Cells were infected at a MOI of 0.1. Error bars show standard deviations of mean titers. Proper inoculum titers were verified by plaque titration in all samples and were all within two fold of the desired amount of virus for an MOI of 0.1.

Table 3.4 Significant differences between IMP181 & mutated viruses at specific time-points as determined by Bonferroni post-tests.

Vero			3T3			C7-10		
Virus	Time (hours)	P-value	Virus	Time (hours)	P-Value	Virus	Time (hours)	P-value
IMP181-6X	36	P<0.001	IMP181-nsP3	72	P<0.001	IMP181-6X	36	P<0.05
IMP181-nsP3	36	P<0.001	IMP181-nsP4	72	P<0.01	IMP181-6X	48	P<0.001
IMP181-Cap89	36	P<0.001	IMP181-Cap89	36	P<0.001	IMP181-nsP3	48	P<0.001
IMP181-Cap250	36	P<0.001	IMP181-Cap89	48	P<0.001	IMP181-Cap250	36	P<0.01
IMP181-E2	36	P<0.001	IMP181-Cap89	72	P<0.001	IMP181-Cap250	48	P<0.001
IMP181-E1	36	P<0.001	IMP181-Cap250	48	P<0.001	IMP181-E2	36	P<0.001
BFS932	24	P<0.01	IMP181-Cap250	72	P<0.001	IMP181-E2	48	P<0.001
BFS932	48	P<0.001	IMP181-E2	36	P<0.05	IMP181-E1	48	P<0.001
			IMP181-E2	48	P<0.001	IMP181-nsP2-*XbaI*	48	P<0.001
			IMP181-E2	72	P<0.001	BFS932	36	P<0.001
			IMP181-E1	72	P<0.001	BFS932	48	P<0.001
			IMP181-nsP2-*XbaI*	48	P<0.05			
			IMP181-nsP2-*XbaI*	72	P<0.001			
			BFS932	24	P<0.05			
			BFS932	36	P<0.001			
			BFS932	48	P<0.001			
			BFS932	72	P<0.001			

in vitro Competition Assays

Relative fitness was further assessed using *in vitro* competition assays. Competition assays provide a more sensitive means of determining the relative fitness of one virus to another, which makes this method particularly useful for studies investigating single or few polymorphisms. Competitive fitness assays were run with WEEV181-6X and WEEV181-nsP2-*XbaI* acting as a surrogate for wild-type virus and were conducted in triplicate in Vero and C7-10 cells (Figure 3.3).

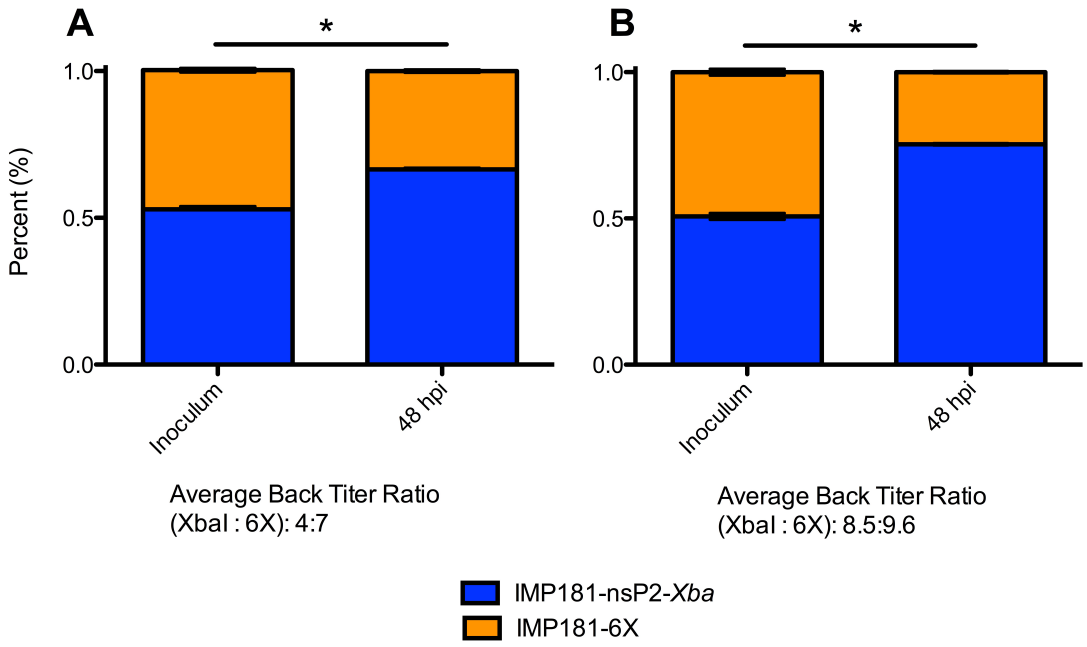

Figure 3.3 IMP181-6X v. IMP181-nsP2-*XbaI* *in vitro* competition assay reported as percent of total population.
Competition assays conducted in A) Vero cells and B) C7-10 cells. Cells infected with equal proportions of virus, as determined by plaque assay titer. Inocula before mixing were back titrated. After mixing the ratio between the two viruses was determined (indicated in the figure). All samples were run in triplicate and error bars indicate standard deviation. *Indicates $P<0.001$ as determined by two-way ANOVA.

Examination of *in vitro* competition assays indicated that IMP181-nsP2-*XbaI* out competed IMP181-6X in both Vero and C7-10 cells after 48 hpi. Additionally there was no statistical difference in a competition between IMP181-nsP2-*XbaI* and IMP181

indicating that IMP181-nsP2-*XbaI* can be used as a surrogate wild-type virus in these experiments (data not shown). This indicates that IMP181-nsP2-*XbaI* (the surrogate wild-type) has a competitive advantage over IMP181-6X in both Vero cells and C7-10 cells, with the effect being more pronounced in C7-10 cells. This shows that *in vitro* mutations that WEEV has acquired and conserved over the 20th century confer a competitive advantage in the models tested.

DISCUSSION

In this study, I was able to develop infectious clones and rescue viruses individually containing all phylogenetically conserved mutations and combinations thereof. I also ran initial characterizations of these viruses using multi-step replication curves and *in vitro* competition assays in a variety of cell types. This involved generating six point mutation clones on the IMP181 backbone through site-directed mutagenesis by fusion PCR methodologies. Due to time constraints the IMP181-6X, IMP181-nsP3-Cap250-E1 and IMP181-nsP4-Cap89-E2 clones were constructed courtesy of Epoch Life Sciences. All infectious clones were successfully electroporated and proper sequence was verified via Sanger sequencing. All cloned viruses except IMP181-nsP3-Cap250-E1 and IMP181-nsP4-Cap89-E2 developed low titers and subsequently had to be passed in Vero cells once to achieve higher titers (sequences were verified with Sanger sequencing). In thinking about the disparity in titer between clones that developed low and high titers, one viable explanation I can come up with is an increase in my technical proficiency of the electroporation protocol; the two clones that developed high titers were conducted more recently than the others (on the order to two years). However, all electroporation efficiencies were in the same range of 10^5-10^6 cells/μg RNA, indicating that the optimization of the electroporation protocol may not have been the main component in the increased titers. In addition to optimizing several aspects of the protocol (mostly focusing on minimizing cell loss during washing and electroporation), the clones that

achieved higher titers were harvested 72 hours post-electroporation versus 48 hours post-electroporation, which was the case with all other electroporated clones. The reason for extending the incubation time for 24 hours was due to the monolayer not being completely obliterated. The increased incubation time is most likely the main factor in achieving the higher titers. However, in-spite of the differences between post electroporation titers all viruses were able to achieve high titers after passage in Vero cells. Additionally, no clone mutations reverted and no additional mutations arose during electroporation or passage, thus passaged viruses were used as stocks for all subsequent experiments.

Significant time was put into the generation of a WEEV/BFS932 infectious clone. Through many attempts and different methodologies a viable clone was unable to be constructed and rescued. After considerable effort done in-house, the cloning was also outsourced to Epoch Life Sciences, who after six months failed to achieve a viable clone as well. In light of these efforts, the quest to generate a viable WEEV/BFS932 infectious clone was abandoned; though, the isolate was still used in subsequent experiments as an ancestral control.

The initial assessment I made to compare potential phenotypic effects of these mutants was multi-step replication curves. Usually an MOI of 0.1 to 0.01 is used when conducing multi-step replication curves. This low MOI results in the infection of only a small fraction of the total cells; the specific proportions of cells receiving no virus particles, one virus particle, and multiple virus particles is determined by a Poisson distribution (Flint et al., 2008). Having only a fraction of cells initially infected allows both the replication and subsequent infection of cells by progeny virus to be assessed. Multi-step replication curves are contrasted from one-step replication curves in that a much higher MOI is used, usually around 10, to ensure infection of the vast majority of cells, thus the kinetics of a single replication cycle can be assessed (Dulbecco and Vogt, 1954; Ellis and Delbrück, 1939). Replication in Vero cells resulted in the least amount of

significant variation between mutated viruses and IMP181. This result is possibly due to the permissive nature of the cell line. Additionally, C7-10 cells showed similar results as the Vero cells with a few additional significant differences, though C7-10 cells do retain their anti-viral RNAi response (Walker et al., 2014). 3T3 cells revealed the most significant differences between mutated viruses and IMP181. This could possibly be because 3T3 cells are able to mount an interferon response to viral infection, unlike Vero cells, and the mutations were, in some way, compromising the viruses' ability to replicate in the system. Although, significantly different at 72 hpi both IMP181 and BFS932 were able to replicate to high titers, indicating that the point mutations may not be exhibiting an ancestral phenotype, but may be lacking some compensatory mutations which impart some epistatic effect. Moreover, this idea is supported by the fact that IMP181-6X replicated to titers statistically similar to IMP181. Giving further support to this theory, a large majority of the discovered mutations arose at the same time or within a very short timeframe of one another (Figure 2.1).

Competitive fitness assays conducted *in vitro* revealed a trend of the surrogate wild-type virus IMP181-nsP2-*XbaI* to outcompete IMP181-6X. This trend, shown in both Vero and C7-10 cells, indicates that mutations accumulated overtime (present in IMP181-nsP2-*XbaI*) confer a competitive advantage over ancestral mutations cloned into the identical backbone *in vitro*. An alteration in experimental design of the *in vitro* competitive assay experiments would have benefited from the inclusion of a control group competing IMP181-nsP2-*XbaI* and BFS932. This would have shown that the contemporary viruses outcompete ancestral viruses *in vivo* due to, at least in part, the phylogenetically conserved mutations tested in IMP181-6X. In spite of this limitation, the results obtained are still compelling.

In addition to testing Vero, 3T3, and C7-10 cells, chick embryonic fibroblasts (CEF) (Charles River, Wilmington, MA) were used for competitive fitness assays and multi-step replication curves; however, due to the timing of the experiments (i.e. studies

in HOSPs were already being conducted) the samples taken were not analyzed. This decision was mostly due to the fact that experiments were already being conducted in HOSPs and 21-day old chickens and additional *in vitro* tests would be redundant and limited in their ability to address the questions I was attempting to answer.

All viruses were successfully rescued and high titers were achieved. Additionally, the replication curves showed that the viruses replicate efficiently, although some differences did exist between point mutations and IMP181. This effect is most pronounced in 3T3 cells were a majority of the point mutations were significantly lower than IMP181. Interestingly, IMP181-6X replicated to titers statistically indistinguishable between itself and IMP181, indicating that there may be some epistatic effect seen when all mutations are present. This is not unsurprising because of the timing in which all the mutations arose in the population. Additionally, the competitive fitness assays conducted *in vitro* indicate that contemporary mutations may confer a competitive advantage over ancestral mutations. Taken together, the replication curves and *in vitro* competition assays provide compelling evidence to study the effects of these mutations in more biologically relevant models.

Chapter IV: Characterization of Ancestral Mutations in Enzootic Vector and Reservoir Species

INTRODUCTION

Western equine encephalitis virus (WEEV), a member of the genus *Alphavirus*, family *Togaviridae*, was an important human and veterinary pathogen in the early to mid 20th century (Reisen and Monath, 1988). The WEEV genome consists of a single-stranded, positive-sense RNA, approximately 11.5kb in length, with a 5' cap and polyadenylated tail. The genome codes for four nonstructural genes (nsP1-4) and a subgenomic RNA codes for the structural proteins: capsid, E1-3 and 6K/TF (Strauss and Strauss, 1994). WEEV resides in both North and South America and is a member of the western serocomplex. Generally WEEV can be divided into two lineages: North American and South American. Two primary genetic lineages (Groups A and B) comprise the North American lineage, with Group B having three sublineages (Groups B1-B3) (Bergren et al., 2014; Weaver et al., 1997). Of the North American lineage, all Group A strains were isolated between approximately 1930 and 1941, Group B1 strains between 1946 and 1961, Group B2 strains between 1950 and 1993, and Group B3 strains from 1971 to 2005 (Bergren et al., 2014).

WEEV is an arbovirus, and as such it is maintained in nature by circulating between mosquito vectors and vertebrate hosts. The ecology of WEEV in North America has been well studied and characterized; the annual transmission cycle can be divided on the basis of season with the virus amplifying in the spring, maintaining during the summer, declining in the fall, and quiescent during the winter (Reisen and Monath, 1988). During the spring virus is amplified in nestling HOSPs and other nestling passerines via *Culex tarsalis* (*Cx. tarsalis*) mosquitoes (Cockburn et al., 1957; Hayes et al., 1967; Reeves et al., 1958b; Reeves and Hammon, 1962). The virus is then maintained

in passerines throughout the summer (Reeves and Hammon, 1962). During years of high enzootic activity and *Cx. tarsalis* affinity for mammalian hosts increasing during the summer months (Tempelis et al., 1965), WEEV can spillover into mammalian species such as black-tailed jackrabbit (*Lepus californicus*), western grey squirrels (*Scirus griseus*), California ground squirrels (*Otospermophilus beecheyi*) and other mammals initiating an independent mammal/*Aedes* spp. cycle (Bowers et al., 1966; Hardy, 1987; Hardy et al., 1977; Hardy et al., 1974a). The onset of fall initiates an annual reduction in virus circulation, with virus falling below detectable levels by October or November (McGowan et al., 1973; Reeves and Hammon, 1962). Winter is a time when relatively no virus can be found, however, the means by which WEEV overwinters, while having been studied extensively, remains elusive. Some overwintering theories include maintenance in *Cx. tarsalis* (though the biology of their diapause makes this unlikely) (Bellamy and Reeves, 1963; Kliewer et al., 1969; Mitchell, 1981), maintenance in *Culiseta inornata* (Dow et al., 1976), vertical transmission in *Aedes dorsalis* (Fulhorst et al., 1994), maintenance in its reservoir species or another bird (Reeves et al., 1958a; Reeves, 1974), maintenance in a mammal (Bowers et al., 1966; Hardy et al., 1974a; Kissling, 1958; Lennette et al., 1955), or maintenance in a reptile (Bowen, 1977; Thomas and Eklund, 1960; Thomas and Eklund, 1962). Whatever the means by which WEEV overwinters, it likely varies between different ecological niches and could look quite different in varying geographic localities. The ecology of WEEV in South America is markedly different and includes *Aedes albifasciatus* as a potential enzootic vector with various birds and mammals acting as reservoir hosts (Aviles et al., 1992; Calisher et al., 1985; Mitchell et al., 1985; Mitchell et al., 1987).

WEEV is the etiologic agent of western equine encephalitis or encephalomyelitis (WEE) (Meyer et al., 1931). Disease occurs in both humans and horses, both of which are thought of as dead-end hosts (i.e. viremia levels do not reach a level sufficient to infect a naïve biting mosquito) (Reeves and Hammon, 1962; Reisen and Monath, 1988).

However, some studies have shown some equine species, such as ponies and burros, are able to develop viremias capable of infecting a naïve biting mosquito (Byrne et al., 1964; Giltner and Shahan, 1936; Sponseller et al., 1966). Disease can range from a mild febrile illness to full-blown encephalitis leading to coma or death (Reisen and Monath, 1988). Specific neurological signs for humans include: lethargy, drowsiness, neck stiffness, photophobia, vertigo, and mental status changes (Kokernot et al., 1953). For horses, neurological signs and symptoms include head pressing, stupor, head drooping, blindness, flaccid lips, involuntary movements, an inability to stand, partially closed eyelids, convulsions, and paralysis (Reisen and Monath, 1988). The case fatality rate in humans can range from 3% to 15% depending on the epizootic event, though disease is skewed toward infants, young children, and the elderly (McGowan et al., 1973; Medovy, 1943; Reisen and Monath, 1988). The case fatality rate in horses ranges from 10% to 50% (Reisen and Monath, 1988). Diagnosis is accomplished primarily by serology. Though, in fatal cases further confirmation can be obtained by isolating virus from infectious tissue (Beaty et al., 1995; CDC, 2016a; Reisen and Monath, 1988). No vaccine is available for human use, though a formalin-inactivated vaccine has been available for equids since the 1940s and is administered annually to this day (Minke et al., 2004).

Over the 20th century WEEV's human case incidence in the United States and Canada has declined precipitously with the last case presenting in 1998 (CDC, 2010). Additionally, the rates of finding virus positive mosquito pools and seropositive birds have declined with periodic identification. The last instances of identifying WEEV in its enzootic cycle occurred in Harris County, Texas with one seropositive house sparrow (*Passer domesticus*) (HOSP) in the summer of 2015 (Wilkerson, 2016) and a mosquito positive pool found in 2013 in Clark County, Nevada (CDC, 2016b). Despite the drastic reduction in human incidence and apparent enzootic activity, the WEEV genome is highly conserved with only 3.7% nucleotide sequence divergence between the most disparate North American isolates (Bergren et al., 2014).

Previously, we identified six nonsynonymous synapomorphic mutations that were important in delineating the internal branching patterns of a phylogenetic tree constucted using 33 complete WEEV genomes (Bergren et al., 2014). These six nonsynonymous synapomorphic mutations all indicate the potential for them to be positivly selected, and thus may play an important role in WEEV's reduction in human incidence and enzootic activty. In oder to elucidate any effects on enzootic transmission they may have we studied the effect of these mutations by conducing competion assays in two enzootic reservoir models, HOSPs and 21-day old chickens, and the enzootic vecor, *Cx. tarsalis*.

MATERIALS AND METHODS

Cell culture

Vero cells (ATCC CCL-81, American Type Culture Collection, Manassas, VA) were maintained in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, Carlsbad, CA), 5% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) 100X Non-Essential Amino Acids (Sigma-Aldrich), 1% (vol/vol) Sodium Pyruvate (100mM) (Gibco) and 1% (vol/vol) Penn-Strep (Penicillin & Streptomycin) (5,000 U/ml) (Gibco). Cells were incubated at 37°C with 5% CO_2 in a humidified incubator.

Viral infectious clone plasmids

The WEEV/Imperial181 (IMP181) infectious clone was provided by Aaron Brault at the Centers of Disease Control and Prevention (CDC). Epoch Life Sciences (Missouri City, TX) generated infectious clones containing all or half of the mutations previously identified. Briefly, WEEV/IMP181-6X contains all mutations listed in Table 2.3. The half mutant clones, were divided into two clones based on their change in amino acid residues or potential importance based on the location the residue occupies within the protein. The resulting clones were named WEEV/IMP181-nsP3-Cap250-E1 and

WEEV/IMP181-nsP4-Cap89-E2, with each gene listed in the name representing the mutation occurring in the corresponding gene listed on Table 2.3.

Plasmid purification and *in vitro* transcription

Stocks of infectious clones were made via transforming chemically competent Top10 *E. coli* (Invitrogen, Carlsbad, CA) and subsequent large-scale preparation and purification on CsCl gradients (Sambrook et al., 2006). Proper sequences of stock infectious clones were verified using BigDye Terminator v3.1 Cycle Sequencing kit on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). Prior to *in vitro* transcription, plasmids were linearized using *NotI*. The mMessage mMachine T7 Transcription Kit (Ambion, Austin, TX) was used to transcribe the linearized DNA plasmid to RNA as per manufacturers instructions. RNA was stored at -80°C for no more than 24 hours prior to electroporation.

Virus stocks

Stocks of WEEV/BFS932 (BFS932) were generated by harvesting a plaque of virus and subsequently using the plaque-pick to infect Vero cells and generate a stock of virus (Lidbury et al., 2011). Electroporation of *in vitro* transcribed RNAs was used to generate stocks of all other wild-type and mutant WEEV strains (Gorchakov et al., 2012). Proper sequence was verified using BigDye Terminator v3.1 Cycle Sequencing kit on a 3500 Genetic Analyzer (Applied Biosystems) for both virus stocks generated by plaque-harvesting and electroporation. Stock titers were determined by plaque assay as previously described (Beaty et al., 1989).

Competition assays with IMP181-nsP2-*XbaI*

RELATIVE DENSITOMETRY ANALYSIS

In order to distinguish wild-type virus from mutated virus a wild-type virus with the restriction site *XbaI* in nsP2 was ablated using synonymous mutations, the mutant was designated IMP181-nsP2-*XbaI* (specifics regarding the generation of this virus are described in Chapter III). Briefly, viruses were mixed at a ratio of 1:1 as determined by PFU titer. The virus mixture was then introduced into a desired system. After an appropriate incubation time RNA was extracted using Trizol (Invitrogen) as per manufacturers instructions. Following RNA extraction a 200bp fragment with the *XbaI* site in nsP2 directly in the center was generated using SuperScript III reverse-transcriptase (Invitrogen) and NEB's Phusion Polymerase with WEEV specific primers. Generation of correct amplicons was verified on a 1% agarose gel and extracted using Qiagen's (Hilden, Germany) Gel Extraction Kit. Approximately 500ng of purified DNA product was digested with *XbaI* for 2 hours at 37°C. Digested product was run on a 1% agarose gel. The ratio of mutant virus (100bp) to WEEV/IMP181-nsP2-*XbaI* (200bp) was then estimated by quantifying band densitometry using myImageAnalysis™ (ThermoFisher Scientific, Waltham, MA).

CULEX TARSALIS

Mixtures of viruses were diluted to ~7 $\log_{10}$ PFU/ml mixed at a 1:1 ratio based on PFU. Assays competing IMP181-6X v. IMP181-nsP2-*XbaI* and IMP181 v. IMP181-nsP2-*XbaI* were conducted. Proper titers were verified by conducing plaque assays on each diluted virus before they were mixed. Virus mixtures were subsequently mixed at a ratio of 1:1 with artificial blood meals consisting of 33% (vol/vol) PBS-washed chicken blood (Colorado Serum Company, Denver, CO), 33% (vol/vol) DMEM (Gibco), 20% (vol/vol) FBS, 1% (weight/vol) sucrose (Fisher Scientific, Waltham, MA), 5 mmol ATP (Sigma-Aldrich, St. Louis, MO). Artificial infectious blood meals were transferred to artificial membrane feeders, warmed to 37°C (Discovery Workshops, Accrigton, UK), an

aliquot was stored at -80°C for densitometry analysis. Loaded membrane feeders were placed on cartons containing 100 *Cx. tarsalis* females and allowed to feed for 2-3 hours. Following feeding non-engorged mosquitoes were discarded and engorged mosquitoes were maintained for 14 days in a humidified incubator with a diurnal light/dark cycle with 10% sucrose available *ad libitum*. After incubation, heads were harvested from surviving mosquitoes and stored in a 2ml Eppendorf Safe Lock tube (Hamburg, Germany) with 250µl DMEM with 25 mg/ml Amphotericin B and a steal BB. Heads were homogenized at 26 pulses/sec for 5 minutes on a TissueLyser II (Qiagen). Homogenates were centrifuged at 16,873 rcf for 5 minutes and supernatant collected for RNA extraction and analyzed as described above.

HOUSE SPARROWS (HOSPS)

Through collaboration with Harris County Mosquito Control, a division of the Harris County Public Health Department, HOSPs were caught using mist nets and transferred to the ABSL3 at UTMB. Previous exposure to WEEV was determined in HOSPs via hemagglutination-inhibition assays (HI); only HOSPs negative for WEEV were used in the described studies. IMP181-6X was competed against IMP181-nsP2-*XbaI*. Additionally, an assay competing IMP181 v. IMP181-nsP2-*XbaI* and IMP181-nsP2-*XbaI* were conducted as controls in order to ensure IMP181-nsP2-*XbaI* was behaving as wild-type IMP181 and was not reverting, respectively. Mixtures of viruses were diluted to 4 $\log_{10}$ PFU/ml mixed at a 1:1 ratio based on plaque forming units (PFU) and 100µl was used to inoculate each HOSP subcutaneously (SC). Proper titers were verified by conducing plaque assays on each diluted virus before they were mixed and an aliquot of the mixed virus inoculum was stored at -80°C and analyzed as a comparator. Approximately, 100µl of blood was collected via jugular bleeds each day post infection. Serum was collected from blood via centrifugation at 16,873 rcf for 5 minutes, and stored at -80°C until analysis. Analysis of the ratio between wild-type and mutant virus is

described above. These HOSP experiments were conducted at UTMB under Dr. Weaver's approved IACUC protocol 0209068B.

Competition assays with wild-type IMP181

PYROSEQUENCING ANALYSIS[†]

In order to have a better understanding of the phenotypic effect of the mutations elucidated phylogenetically without the confounding factor of having a mutated wild-type virus, competition assays between mutant virus and wild-type IMP181 were conducted in various models with the appropriate controls. Since ratios of wild-type to mutated virus can not be determined by restriction digest and agarose band densitometry the ratio of wild-type to mutated virus was determined by pyrosequencing.

Prior to pyrosequencing analysis virus positive samples were determined by CPE assay (cytopathic effect). Briefly, all mosquito samples were homogenized at 26 pulses/sec for 5 minutes. Resulting homogenates were clarified via centrifugation at 16,873 rcf for 5 minutes. 96-well plates with Vero cell monolayers were infected with 50µl of the supernatant and allowed to incubate at 37°C and 5% CO_2 in a humidified incubator for 1 hour. After one hour 150µl DMEM supplemented as described above was added. Cultures were then incubated in the same conditions for 48 hours. After 48 hours, cultures were fixed with 10% neutral-buffered formalin for 30 minutes and stained with crystal violet.

Sample Preparation and Nucleic Acid Extraction

Aliquots of mosquito lysates and serum samples were prepared in Roche (Basel, Switzerland) external lysis buffer IVD (200µl) and deposited into individual wells of 96 deep-well processing plates (Roche Applied Science). Nucleic acids were subsequently extracted in a high-throughput fashion using a Magna Pure 96 instrument employing

[†] All sample handling, nucleic acid extraction and qPCR were performed in the Assay Development Service Division of the Galveston National Laboratory.

large-volume Cellular RNA extraction kits (Roche) according to the manufacturer's protocol for fresh/frozen biological samples. After extraction, a portion of the RNA was immediately converted to cDNA and the remaining material archived at -80°C.

Reverse Transcription

cDNA was synthesized from extracted RNA using an iScript™ synthesis kit (Bio-Rad, Hercules, CA). 40µl reactions were assembled in 96 well PCR plates (ThermoFisher Scientific) containing 8µl iScript reaction mix, 2µl reverse transcriptase and 30µl of extracted RNA. RT was completed using a Bio-Rad C1000™ thermocycler using the following protocol: 1) 1.5 min, 25°C, 2) 42°C, 30 min, 3) 85°C, 5 min, 4) indefinite hold at 4°C. Generated cDNA was analyzed immediately then stored at -20°C.

Pyrosequencing

WEEV pyrosequencing was performed using PCR-based pyrosequencing. Primers were designed using Pyromark Assay Design 2.0 software for SNP analysis. Initial PCR was carried out as follows: 12.5µl of iQ supermix™ (Bio-Rad, Hercules, CA) was mixed within a 25µl PCR reaction containing 200nM of both forward and reverse primers (Table 3.1), 3µl cDNA and nuclease-free water. Thermocycling was completed using a Bio-Rad C1000™ thermocycler using the following protocol: 1) 3.0 min, 95°C, 2) 95°C, 30s, 3) 60°C, 30s, 4) 72°C, 30s repeat 50x, 5) 72°C, 2 min, 6) indefinite hold at 4°C. Generated biotinlyated PCR products were pyrosequenced using PyroMark Gold reagents on a PyroMark Q96 ID platform according to the manufacturers' instructions (Qiagen). Sequencing primers were diluted to a final concentration 0.3µM in combination with SNP mode setting were used to perform pyrosequencing. Two distinct pyrosequencing assays were developed so all viruses used in the experiments could be analyzed; details regarding the primer used, sequences analyzed, and different viruses used in each assay are listed on Table 4.1.

Initial validation of the PCR assay was performed using a WEEV infectious clone plasmids that had been sequenced-confirmed using conventional Sanger-based dideoxynucleotide methodology. Preparation of PCR amplicons for pyrosequencing was performed using a Qiagen Pyromark Q96 vacuum workstation. Pyrosequencing was run in SNP mode on the Pyromark ID using Pyromark Gold reagents.

Table 4.1 Primers and viral sequence analyzed in pyrosequencing assay.

Pyrosequencing Assay	nsP3 Based	nsP4 Based
Primers (Forward/Reverse)	GCCGATGTCACCATATATTGCTT	Biotin-CGTTAGCCGAAAGCGTTAAGAACT
	Biotin-CATCCAGTATTTCGACGCTTTCTT	TTTAGGTCAGCCGTAGAGGGTGAT
Sequencing Primers	AATGGGAGACCAGGA	CGTAGAGGGTGATTGG
Sequence Analyzed	TAAYCGAGGCCATTCACCGCAAAGAA	GNTCCCTCTTATGCTCTTGAAGTTCTT
Viruses used in Analysis	IMP181-6X	IMP181-nsP4-Cap89-E2
	IMP181-nsP3-Cap250-E1	
	BFS932	

CULEX TARSALIS

Competition assays in the Bakersfield colony of *Culex tarsalis* (Bellamy and Kardos, 1958) were conducted in triplicate. Experimental design was the same as described above, except that five mosquitos were harvested on day 3 and 5 post infection and ten mosquitoes were harvested on day 10. Salivary glands, midguts, legs and wings, and bodies were all dissected and stored in 2ml Eppendorf Safe Lock tubes (Hamburg, Germany) with 250µl DMEM with 25 mg/ml Amphotericin B and a steel BB. Samples were stored at -80°C.

21-DAY OLD CHICKENS

The 21-day old chicken model has been shown to develop elevated viremias without causing mortality and is a potential model for replication of WEEV in an avian host (Fraizer et al., 1985). Rhode Island Red chicken eggs were obtained from Stromberg's (Pine River, MN) and incubated in a RCOM 50 Digital Incubator (Stromberg's). Chickens were reared to 21-days old upon which they were infected SC

with 3 $\log_{10}$ PFU of viruses mixed at a ratio of 1:1 based on PFU. Proper titers were verified by conducing plaque assays on each diluted virus before they were mixed and an aliquot of the mixed virus inoculum was stored at -80°C and analyzed as a comparator. Chickens were then bled via brachial vein bleeds alternately on days 1 thru 3-post infection. Four days post infection chickens were euthanized and terminally bled. Serum was collected from blood via centrifugation at 16,873 rcf for 5 minutes and stored at -80°C until analysis. Pyrosequencing analysis was conducted as described above. All chicken work was conducted under approved IACUC protocol 0209068B at UTMB.

HOSPs

HOSPs were caught using mist nets in Larimer Co., CO and transferred to the ABSL3 at Colorado State University (CSU). A lack of previous exposure to WEEV was determined by HI. Only naïve HOSPs were used in the described studies. Mixtures of viruses were diluted to ~4 $\log_{10}$ PFU/ml and mixed at a 1:1 ratio based on PFU. HOSPs were infected with 100µl diluted virus mixture SC. Titers of viruses before mixing were taken to verify proper concentrations. Also, an aliquot of the inoculum was reserved for testing the initial ratio of viruses. HOSPs were then bled days 1 thru 4-post infection via jugular bleeds. Serum was collected from blood via centrifugation at 16,873 rcf for 5 minutes, and stored at -80°C until analysis. Pyrosequencing analysis was conducted as described above. The described HOSP work was conducted under Dr. Bowen's approved IACUC protocol 16-6420A at CSU.

RESULTS

Competition assays with IMP181-nsP2-*XbaI*

CULEX TARSALIS

In order to assess the effect of the elucidated nonsynonymous synapomorphic mutations in WEEV's enzootic vector, adult female *Cx. tarsalis* mosquitoes were exposed to mixtures containing equal proportions of either IMP181 v. IMP181-nsP2-*XbaI* as a control or IMP181-nsP2-*XbaI* v. IMP181-6X. Proper dilutions were verified by plaque assay in each virus before they were mixed. After feeding engorged mosquitoes were separated and maintained for 14 days. Upon day 14-post blood meal, heads were harvested to assess the ratio of disseminated virus within each mosquito. Analysis was accomplished by comparing the relative intensity of two bands of DNA on an agarose gel using myImageAnalysis™. Size discrimination was achieved via IMP181-nsP2-*XbaI* having its *XbaI* site in nsP2 ablated.

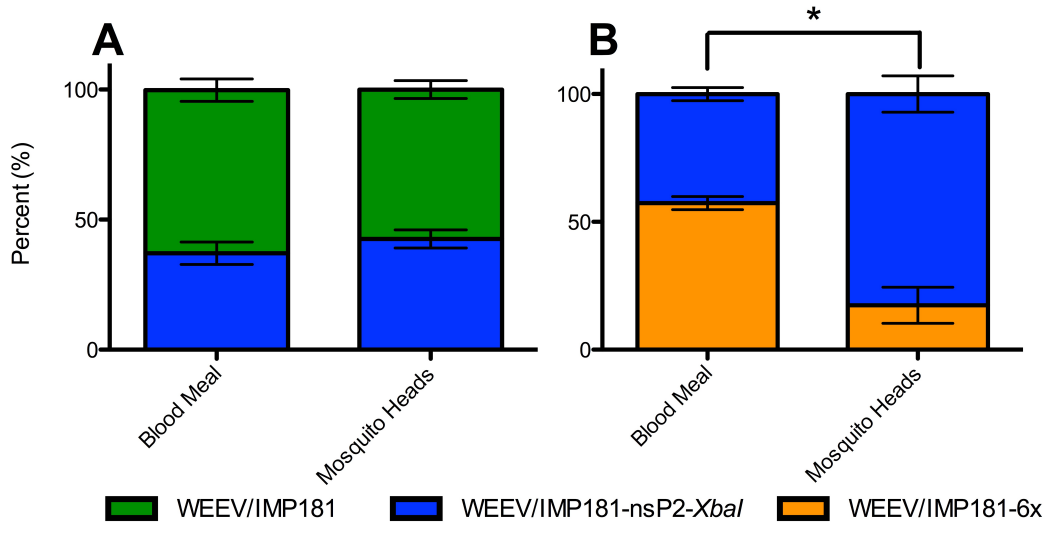

Figure 4.1 Competition assays in *Cx. tarsalis* using the IMP181-nsP2-*XbaI* construct. A) Control experiment competing IMP181 v. IMP181-nsP2-*XbaI* (n=26) to ensure IMP181-nsP2-*XbaI* is an appropriate surrogate for wild-type. B) Experiment competing IMP181-6X v. IMP181-nsP2-*XbaI* (n=34). Error bars indicate standard error. *Indicates significance between groups as determined by an unpaired t-test.

The assay competing IMP181 against IMP181-nsP2-*XbaI* showed no significant difference between the virus ratio in the artificial blood meal and disseminated virus taken on day 14 post blood meal, indicating that the use of IMP181-nsP2-*XbaI* is an appropriate surrogate for wild-type (Figure 4.1 A). When IMP181-nsP2-*XbaI* was competed against IMP181-6X, IMP181-nsP2-*XbaI* demonstrated a clear competitive advantage over IMP181-6X in its ability to disseminate throughout the mosquito (Figure 4.1 B).

HOSP

Assessing the effect of the elucidated mutations on viral fitness in a natural enzootic host is critical as an increase or decrease in fitness could substantially affect the ecology of the virus in nature. However, before attempting the competition assays, I first wanted to practice handling, infecting and bleeding HOSPs in order to obtain proficiency. A total of six HOSPs were caught and transported to the ABSL3. HOSPs then received 10^3 PFU of virus SC, monitored and bleed on days 1 and 2 post infection for viremia. My initial plan was to measure viremia on days 3 and 4; however, due to issues with housing and bleeding, I was only able to collect blood on days 1 and 2. After infection all HOSPs developed viremia, though some did not exhibit viremia until day 2 (Figure 4.2). Additionally, most viremias lasted at least 2 days, however one was short lived, only detecting virus on day 1 post infection.

After gaining competence with the HOSP, competition assays were conducted assessing the effect of the elucidated nonsynonymous synapomorphic mutations on viral fitness in the HOSP. Briefly, HOSPs were infected with 10^3 PFU mixtures of virus at a ratio of 1:1 as determined by PFU. Proper dilutions were verified by plaque assay and an aliquot of the final inoculum was taken to verify proper inoculum ratio. HOSPs were bled on days 1 and 2 post infection and the ratio of virus mixtures were determined by myImageAnalysis™ as described above.

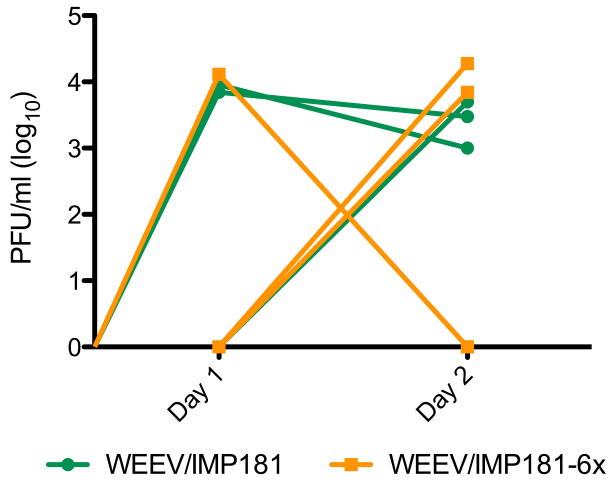

Figure 4.2 Viremia in HOSPs infected with 10^3 PFU mutated and wild-type WEEV. Each point represents one subject. Lines connect data points between the same subjects (n=3 HOSPs per group).

Groups of six HOSPs were either infected with mixtures of IMP181-nsP2-*XbaI* and IMP181-6X, IMP181-nsP2-*XbaI* and IMP181, or IMP181-nsP2-*XbaI* alone. For the HOSPs infected with IMP181-nsP2-*XbaI* and IMP1816X, IMP181-nsP2-*XbaI* displayed a significant advantage over IMP181-6X on day 2 post infection with the trend beginning on day 1 post infection (Figure 4.3 A). This indicates that the nonsynonymous synapomorphic mutations present in recent lineages display an advantage in its replicative fitness in the HOSP. Furthermore, I verified that the mutations ablating the restriction site in IMP181-nsP2-*XbaI* do not revert in the HOSP (Figure 4.3 C). Unfortunately, when IMP181 was competed against IMP181-nsP2-*XbaI*, IMP181-nsP2-*XbaI* showed a clear advantage over IMP181 (significant on days 1 and 2), even though the inoculum was ~9:1 IMP181:IMP181-nsP2-*XbaI*. This finding indicates that IMP181-nsP2-*XbaI* is not an appropriate surrogate for wild-type WEEV competition assays in HOSP competition assays.

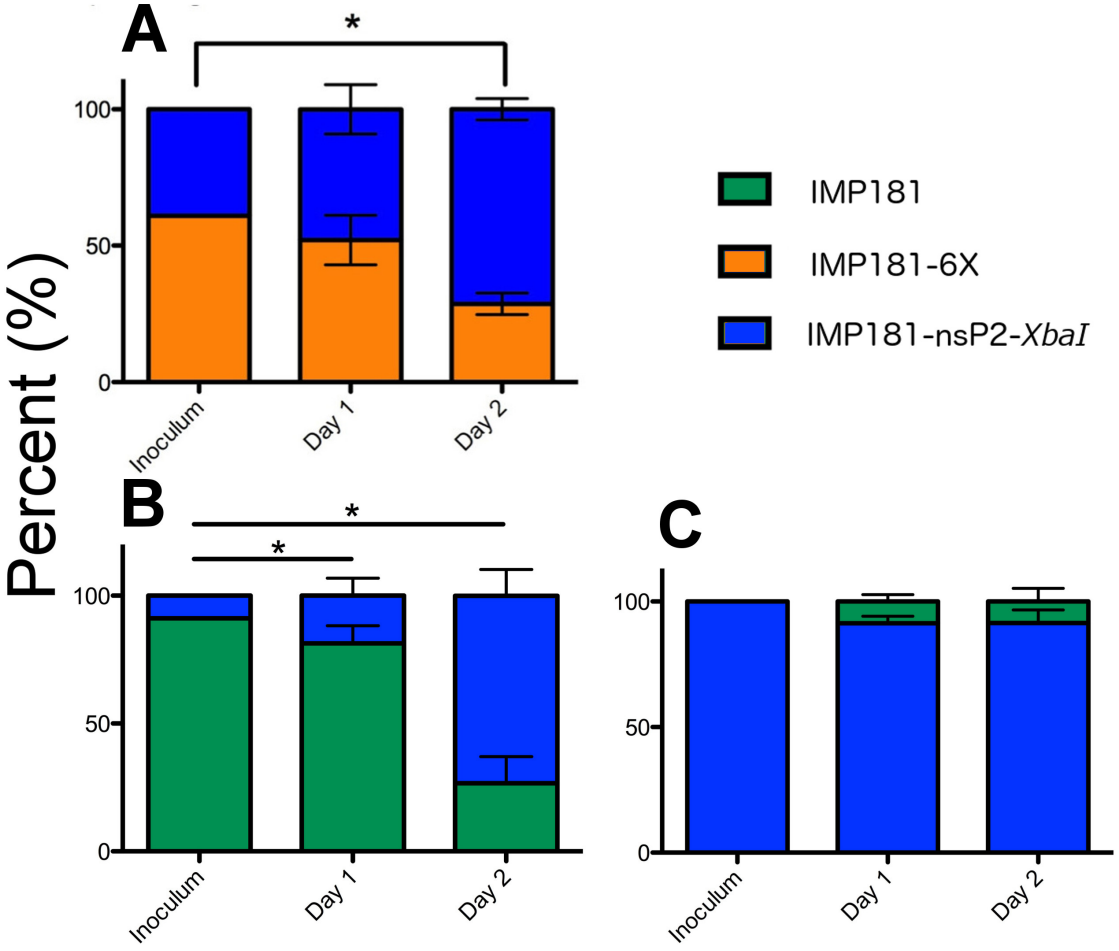

Figure 4.3 Competition assays in HOSPs using the IMP181-nsP2-*XbaI* construct. A) Assay competing IMP181-6X against IMP181-nsP2-*XbaI* (n=6), B) Control competing IMP181 against IMP181-nsP2-*XbaI* (n=6), C) Control ensuring no reversion of IMP181-nsP2-*XbaI* in the HOSP model (n=6). Error bars show standard error. *Indicates statistical significance as determined by a one-way ANOVA with a Tukey post-test.

Competition assays with wild-type IMP181

Due to the ability of IMP181-nsP2-*XbaI* to outcompete IMP181 in the HOSP, I decided to conduct the experiments testing the competitive advantage of the nonsynonymous synapomorphic mutations using IMP181 as the wild-type virus that the mutant viruses will be competed against. The analysis was conducted by pyrosequencing as described above.

CULEX TARSALIS

Female *Cx. tarsalis* mosquitoes were allowed to feed on artificial blood meals containing mixtures, each conducted in triplicate, of IMP181 and BFS932, to control for potential epistatic interactions, IMP181 and IMP181-6X, IMP181 and IMP181-nsP3-Cap250-E1, and IMP181 and IMP181-nsP4-Cap89-E2. The clones with three mutations were used to try to further understand which mutations are exerting a phenotypic effect. All artificial blood meals had a viral titer within 2-fold of the desired titer. After feeding engorged mosquitoes were separated and maintained for 10 days. On days 3 and 5 post blood meal 5 mosquitoes and on day 10 post blood meal 10 mosquitoes were taken from each replicate and salivary glands, midgut, legs and wings, and body were dissected for analysis. Prior to pyrosequencing analysis CPE assays were conducted to ensure only infectious samples were sequenced (Figure 4.4). On day 3 post-blood meal the majority of midguts tested were infected. Additionally, presence of virus in the legs and wings, which is an indicator of disseminated infection, was between 10% and 40%. Bodies and salivary glands were between 0% and 20% infected. On day 5 the majority of midguts were still infected with the rates in other tissues increasing as compared to day 3. Interestingly, the IMP181 v. BFS932 group appeared to infect tissues at a faster rate, though this could not be validated statistically. By day 10 all midguts were infected and at least 90% of all other tissues tested positive for virus.

Salivary glands from day 10 post blood meal, which were positive for virus, were submitted for pyrosequencing. When competed against IMP181-6X and IMP181-nsP3-Cap250-E1, IMP181 outcompeted both (Figure 4.5 A&B). This finding is further validated by IMP181 outcompeting BFS932 (Figure 4.5 D), though not by the large margin seen in the competition assays with IMP181-6X and IMP181-nsP3-Cap250-E1. When IMP181 was competed against IMP181-nsP4-Cap89-E2 no competitive advantage could be determined between them (Figure 4.5 C).

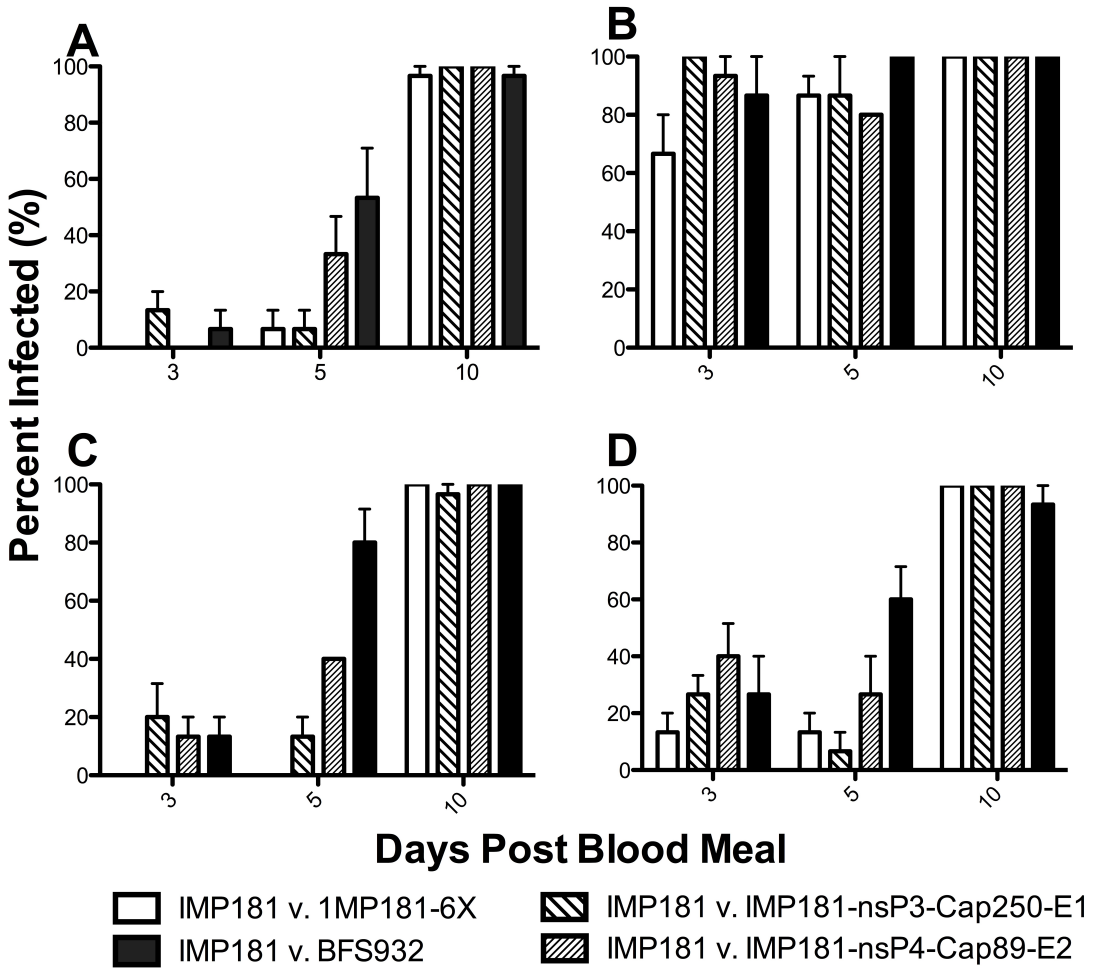

Figure 4.4 Mosquito infection rates for competition assays.
WEEV infection rates in the A) salivary glands, B) midgut, C) bodies, and D) legs and wings of mosquitoes used in competition assays. Day 3 n=5 per replicate, day 5 n=5 per replicate, day 10 n=10 per replicate. Error bars indicate standard error. No samples were significantly different from one another on each day as determined by a one-way ANOVA with Tukey post-tests.

21-DAY OLD CHICKENS

When infected with WEEV, 21-day-old chickens develop a high short-lived viremia (Fraizer et al., 1985). Chickens were infected with virus mixtures containing 3 $\log_{10}$ PFU. All viruses were back titrated and were found to be within 2.1-fold of the desired inoculum dose. Serum samples from days 1 and 2-post infection were submitted

for pyrosequencing. When IMP181 was competed against both IMP181-6X and IMP181-nsP3-Cap250-E1, IMP181 outcompeted both viruses on days 1 and 2-post infection by a significant margin (Figure 4.6 A&B). In the assay competing IMP181 against IMP181-nsP4-Cap89-E2 significance between the two viruses was seen on day 2-post infection, as compared to the inoculum (Figure 4.6 C). Additionally, days 1 and 2-post infection were significantly different from each other. The results in this assay are difficult to interpret as the inoculum dose was skewed toward IMP181-nsP4-Cap89-E2. When IMP181 and BFS932 were competed against each other no significant difference could be determined between any of the time-points taken. Moreover, the outcome of the assay (IMP181 or BFS932 winning) varied drastically with 100% IMP181 present in some subjects or 100% BFS932 present in others, which accounts for the large error bars (Figure 4.6 D).

HOSP

HOSPs are the natural enzootic reservoir for WEEV and develop a high-titer, short-lived viremia upon infection (Reisen et al., 2008). HOSPs were infected with virus mixtures containing 3 $\log_{10}$ PFU of virus mixtures. Individual virus dilutions were back titrated and were within 2-fold of the desired titer. After mixing, aliquots of the inoculum were taken and analyzed as a comparator. Serum samples from day 1 and 2-post infection were analyzed by pyrosequencing. When IMP181-6X and IMP181-nsP3-Cap250-E1 were competed against IMP181, IMP181 outcompeted both on days 1 and 2-post infection as compared to the inoculum by significant margins (Figure 4.7 A&B). No significance could be determined when IMP181 was competed against IMP181-nsP4-Cap89-E2 (Figure 4.7 C), though a small trend can be seen in favor of IMP181-nsP4-Cap89-E2. Additionally, the assay competing BFS932 and IMP181 did not have a clear winner and no general trends can be seen, indicating that IMP181 and BFS932 are equally adapted to the HOSP (Figure 4.7 D).

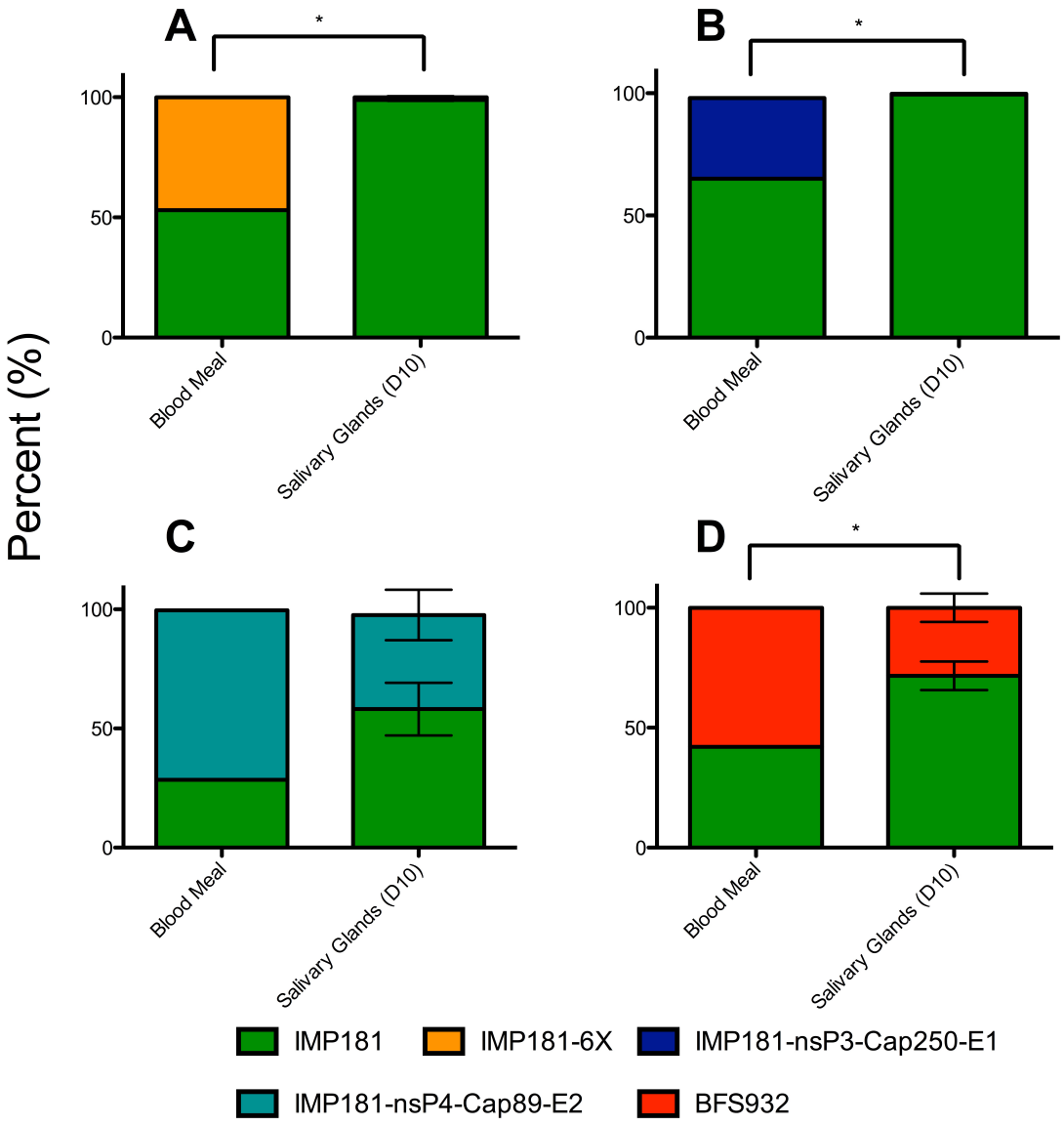

Figure 4.5 Competition assays in *Cx. tarsalis* showing the ratios of virus in the blood meal and salivary gland on day 10 post blood meal.

Mean and standard error of replicates (n=5 per replicate) in assays competing A) IMP181 v. IMP181-6X, B) IMP181 v. IMP181-nsP3-Cap250-E1, C) IMP181 v. IMP181-nsP4-Cap89-E2, and D) IMP181 v. BFS932. *Indicates significance between the blood meal and salivary glands on day 10 post blood meal ingestion as determined by an unpaired t-test.

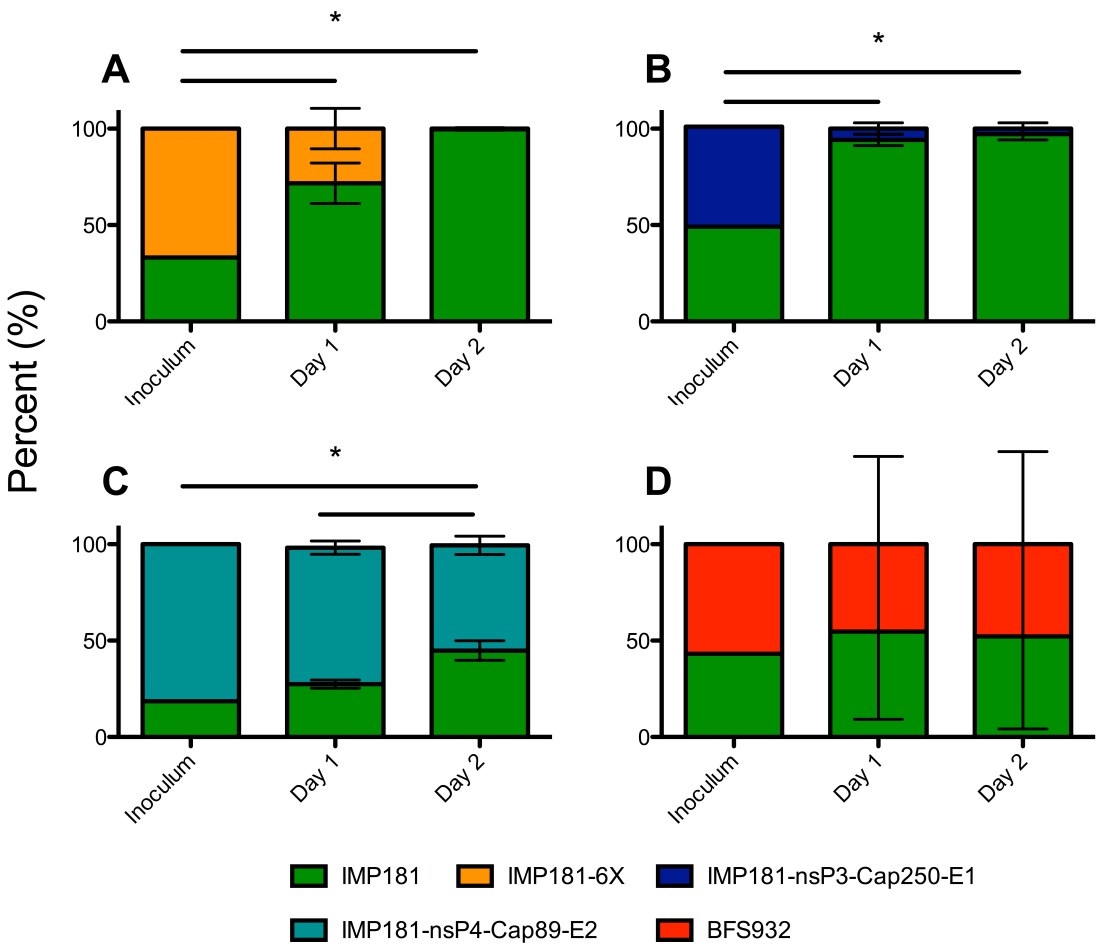

Figure 4.6 Competition assays in 21-day-old chickens showing the ratios of virus in the inoculum and serum days 1 and 2 post infection.
Mean and standard error in assays competing A) IMP181 v. IMP181-6X, B) IMP181 v. IMP181-nsP3-Cap250-E1, C) IMP181 v. IMP181-nsP4-Cap89-E2, and D) IMP181 v. BFS932 (n=4 per group). *Indicates significance between time-points as determined by a one-way ANOVA with a Tukey post-test.

DISCUSSION

Understanding the effect different mutations can have on the fitness of an arbovirus in relationship to its ecological niche is an important factor when determining reasons for reductions or increases in population size and its potential to emerge into new areas or niches. In this series of experiments, relative fitness of the nonsynonymous synapomorphic mutations important in WEEV's evolutionary history were tested in both

WEEV's enzootic vector and reservoir host. I found that the mutations that WEEV accumulated over the 20th century, are important in distinguishing between the Group B sublineages, and do have a competitive advantage in *Cx. tarsalis*. Competition assays were also conducted in 21-day-old chickens and HOSPs; however the effect of the mutations is inconclusive.

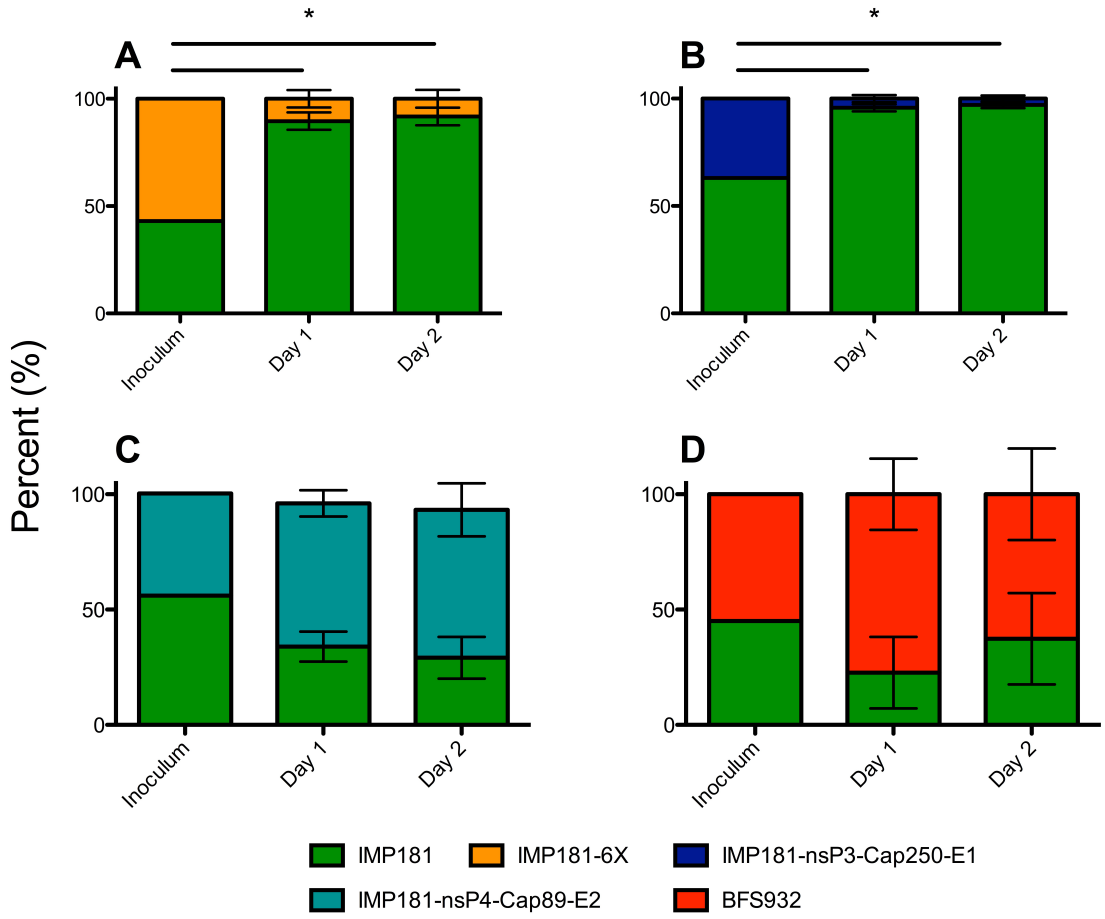

Figure 4.7 Competition assays HOSPs showing the ratios of virus in the inoculum and serum days 1 and 2 post infection.
Mean and standard error in assays competing A) IMP181 v. IMP181-6X, B) IMP181 v. IMP181-nsP3-Cap250-E1, C) IMP181 v. IMP181-nsP4-Cap89-E2, and D) IMP181 v. BFS932 (n=7 per group). *Indicates significance between time-points as determined by a one-way ANOVA with a Tukey post-test.

Initially, experiments were conducted using the surrogate wild-type clone, IMP181-nsP2-*XbaI*, so samples could be compared via restriction digest followed by

measurement of band intensity on an agarose gel. Utilizing this method experiments were conducted in *Cx. tarsalis* and HOSPs. In each case the nonsynonymous synapomorphic mutations WEEV has acquired during the 20th century (present in IMP181-nsP2-*XbaI*) out competed the corresponding ancestral mutations, present in IMP181-6X. These finding indicate that the mutations WEEV has accumulated over time demonstrate a competitive advantage in its enzootic hosts and vectors. Although, the control experiment competing IMP181-nsP2-*XbaI* against IMP181 to ensure no artificial advantage on the part of IMP181-nsP2-*XbaI*, showed no statistical difference between the two viruses when conducted in *Cx. tarsalis*, a significant difference could be found when the two viruses were competed in the HOSP model, with IMP181-nsP2-*XbaI* winning the competition. Unfortunately, this throws the findings discovered in these experiments into question and a method of conducting these experiments without the use of mutating the wild-type virus was sought out. The specific reason(s) for IMP181-nsP2-*XbaI* outcompeting IMP181 remain unknown however, the only plausible explanation I surmise is that while only silent mutations were made, the mutations could possibly have altered secondary structure of the RNA in such a way as to increase its replicative fitness. A study attempting to elucidate the reason IMP181-nsP2-*XbaI* demonstrated a competitive advantage over IMP181 could be conducted and may yield interesting results due to the low probability that the two silent mutations introduced would confer an increase in fitness. Additionally, one limitation of these experiments was the lack of a competition between IMP181, or its surrogate IMP181-nsP2-*XbaI*, and BFS932. This assay would have helped control for potential epistatic interactions and determine if the effects seen in the experimental group were biologically relevant.

In order to reliably determine which virus is winning in the competition assay without using the surrogate wild-type (IMP181-nsP2-*XbaI*), pyrosequencing was used to analyze the competition assays thereby eliminating the need of a surrogate wild-type. A similar study in *Cx. tarsalis* was conducted differing in that midguts, salivary glands,

bodies, and legs & wings were harvested on days 3, 5, and 10 post infection. CPE assays showed that at least 90% of mosquitoes developed a disseminated infection, which resulted in infection of the salivary glands. No significant differences in the rate or proportion of mosquitoes infected between groups could be determined. When salivary glands were analyzed by pyrosequencing, IMP181 outcompeted all other viruses except IMP181-nsP4-Cap89-E2. Thus, the mutations that confer a shift in amino acid residue phenotype confer a greater competitive advantage over the mutations that do not. This conclusion is further verified by IMP181 outcompeting BFS932 in the salivary glands of *Cx. tarsalis* by a significant margin, though the margin is not quite as drastic as seen in the IMP181-6X and IMP181-nsP3-Cap250-E1 groups. Interestingly, the mutations that didn't confer drastic phenotype changes to the amino acid residues (IMP181-nsP4-Cap89-E2) did not display a competitive advantage or disadvantage, indicating that these mutations do not confer significant changes to the virus' replicative fitness in the salivary gland of *Cx. tarsalis* mosquitoes. The colony of *Cx. tarsalis* used for these experiments were collected in 1953 (Bellamy and Kardos, 1958). Data exists that as mosquito colonies increase in generation their susceptibility to infection also increases (Hardy et al., 1976). This phenomenon likely affects the colony of *Cx. tarsalis* used in these experiments. However, the likelihood of the factors affecting susceptibility to infection also affecting the outcome of an internally controlled experiment like the competition assay is extremely low. If these experiments were to be conducted in a newly established colony of *Cx. tarsalis*, I hypothesize that the results would be recapitulated, potentially with greater differences due to less fit viruses being unable to surmount specific infection barriers. In short, the competition assays indicate that the nonsynonymous synapomorphic mutations WEEV has acquired over the 20th century and are important for distinguishing between the Group B sublineages have a competitive advantage in the salivary glands of *Cx. tarsalis* mosquitoes.

To assess the effects of these conserved nonsynonymous synapomorphic mutations in enzootic hosts, competition assays were conducted in 21-day-old chickens and wild-caught HOSPs. Birds received 3 $\log_{10}$ PFU of virus mixtures and were bled on days 1 thru 4-post infection. Pyrosequencing analysis was conducted on sera from days 1 and 2-post infection, because the viremias peaked on those days in HOSPs and 21-day-old chickens (Fraizer et al., 1985; Reisen et al., 2008). In HOSPs, analysis indicated that IMP181 outcompeted both IMP181-6X and IMP181-nsP3-Cap250-E1 by significant margins on days 1 and 2 post infection. However, no significant differences could be determined when IMP181 was competed against IMP181-nsP4-Cap89-E2 and BFS932. The lack of significance in the assay between IMP181 and BFS932 brought the significant results achieved in the assays containing IMP181-6X and IMP181-nsP3-Cap250-E1 into question with regard to their biological significance. BFS932 likely has epistatic residues that play a compensatory role in relation to the nonsynonymous synapomorphic mutations being tested so that the virus can replicate efficiently in the HOSP. Likely as the contemporary nonsynonymous synapomorphic mutations were being introduced to the WEEV population these epistatic residues were probably purified out of the population, which is supported by the overwhelming amount of negative selection on the majority of the WEEV genome (Bergren et al., 2014). The 21-day-old chickens generally recapitulated the results found in the HOSPs, except the assay competing IMP181-nsP4-Cap89-E2 was found to have significant differences between the inoculum dose and day 2 and day 1 and day 2 (Figure 4.6 C). However, given the greater relevance of the HOSP model and the inoculum dose being far from a 1:1 mixture (approximately 9:41) the relevance of this finding is questionable.

These experiments sought to demonstrate the significance of the nonsynonymous synapomorphic mutations that are important in defining the evolutionary lineage of WEEV in its enzootic hosts. I accomplished this by conducting competition assay in several relevant systems that model enzootic hosts and vectors. As a result of these

experiments, I was able to determine that the discovered phylogenetically significant mutations WEEV has acquired during the 20th century demonstrate a clear competitive advantage over the ancestral mutations in the salivary gland of *Cx. tarsalis*. Furthermore, I was able to specifically implicate that the nsP3-T152I, Capsid-K250W, and E1-T374S mutations play a critical role in this effect. Conversely, nsP4-N602S, Capsid-K89R, and E2-A23T were not shown to provide a significant competitive advantage in either direction. This finding is not surprising as the nsP4-N602S and Capsid-K89R mutations do not confer a significant property change of the amino acid residue. Furthermore, while E2-A23T does provide a change from nonpolar to an uncharged-polar residue the placement of the mutation on the E2 structure (Figure 2.4 C&F), buried in the base of E2, makes the relative importance of the mutation less likely. Interestingly, the competition assays conducted in HOSPs and 21-day-old chickens revealed that IMP181 was unable to outcompete BFS932, indicating that ancestral virus was not significantly different, in terms of fitness in the avian species tested, with the contemporary IMP181 isolate. However, I did find that IMP181 was able to outcompete both IMP181-6X and IMP181-nsP3-Cap250-E1. I hypothesize that this effect is due to the presence of epistatic mutations in BFS932 that allows it to replicate efficiently in avian species and have subsequently been purified out of the population. In short, through these studies I was able to determine that the evolutionary history of WEEV throughout the 20th century has been characterized by a selection toward its enzootic cycle, specifically transmission by *Cx. tarsalis*.

Chapter V: Characterization of Ancestral Mutations in the Golden Syrian Hamster Virulence Model

INTRODUCTION

Western equine encephalitis virus (WEEV) is one of several arboviruses in North America that cause a disease in humans characterized by encephalitis (Reisen and Monath, 1988). The agent was discovered in 1930 in the brain of an infected horse (Meyer et al., 1931). Maintenance of the virus is mediated by a transmission cycle where *Culex tarsalis* is the main vector and house sparrows (HOSPs) and other passerines are the primary reservoir hosts (Hammon et al., 1945; Reeves and Hammon, 1958). The case fatality rate can reach 15% in humans and 50% in equids depending upon the severity of the epidemic (McGowan et al., 1973; Reisen and Monath, 1988). WEEV resides in the western complex in the genus *Alphavirus,* family *Togaviridae* (Calisher and Karabatsos, 1988; Calisher et al., 1988). Two main lineages distinguish North American from South American WEEV. Within the North American lineage two lineages (Group A and Group B) are present, with Group B having three sublineages (Groups B1-3) (Bergren et al., 2014; Weaver et al., 1997).

WEEV can naturally infect a wide range of vertebrates (including species from classes Amphibia, Reptilia, Aves, and Mammalia) in addition to its incidental, dead-end, hosts: humans and horses. Disease that results from infection primarily occurs in mammals with fatal outcomes consistent with involvement of the central nervous system (CNS). Specifically, black-tailed jackrabbits (*Lepus californicus*), California ground squirrels (*Otospermophilus beecheyi*), western grey squirrels (*Sciurus griseus*), San Joaquin antelope squirrel (*Ammospermophilus nelsoni*), Heermann's kangaroo rats (*Dipodomys heermanni*), Fresno kangaroo rats (*Dipodomys nitratoides*), and deer mice (*Peromyscus maniculatus*) all have been shown to develop viremias high enough to

participate in the transmission cycle. However, only the San Joaquin antelope squirrel, black-tailed jackrabbit, and deer mouse tolerate infection well (Bowers et al., 1966; Hardy, 1987; Hardy et al., 1977; Hardy et al., 1974a; Hardy et al., 1974b; Reeves and Hammon, 1962). Infection is generally inapparent in amphibians, reptiles, and birds (Holden et al., 1973; Reisen et al., 2003; Reisen et al., 2008; Thomas and Eklund, 1960; Thomas and Eklund, 1962). Inapparent infection in these vertebrates has led to several overwintering hypotheses (discussed in detail in Chapter I).

Disease can progress from a mild-febrile illness to the development of severe encephalitis. In cases of severe encephalitis coma or death can follow. Additionally, life-long neurological sequelae are commonly associated with patients that recover from severe disease (Palmer and Finley, 1956). Pathologic examination of cases that have a fatal outcome show unusual cystic degeneration in the brain (Noran, 1944). Blood vessels within the brain typically show severe endothelial proliferation and mural thickening sometimes accompanied with vascular calcification. Mononuclear cells are usually observed near and around vessels (Noran and Baker, 1945). Additionally, parenchymal changes occur throughout the brain that consist of focal areas of demyelination (Noran and Baker, 1945; Noran, 1944). Ventricles are usually dilated with the area consisting of closely packed cysts of various size (Noran and Baker, 1945). Due to the unique presentation, physicians in the 1940s felt confident diagnosing WEE based on pathologic alterations in the brain without the addition of any virological studies to confirm.

Since WEEV has the potential to cause severe illness in both humans and horses and its ability to be used as an agent of bioterrorism, several animal models for disease pathogenesis have been characterized including several types of mice, hamsters, and non-human primates (NHPs).

The murine model for WEEV infection has been widely used in studying the pathogenesis of the virus. However, little consistency has been achieved due to multiple mouse strains and infection methods being used. The most frequently used murine model

for WEEV infection is the 3-4-week-old NIH Swiss-Webster mouse (Aguilar, 1970; Atasheva et al., 2009; Bianchi et al., 1993; Forrester et al., 2008; Liu et al., 1970; Monath et al., 1978). These mice are thought to reasonably mimic human illness when infected subcutaneously (SC). NIH Swiss-Webster mice show infectious virus in the muscle and blood 2 and 3 days post infection (dpi). As the infection progresses virus can be found in the heart, lung and spleen (6 dpi) and finally in the brain (4 to 12 dpi) (Liu et al., 1970). As expected, intracranial inoculation (IC) of NIH Swiss-Webster mice results in rapid proliferation of virus in nervous tissue in the first 56 hours followed by infection of the heart, lungs, and spleen (Liu et al., 1970). Mice that do not exhibit neurological signs of the disease still may become moribund due to necrosis observed in the myocardium; whether or not this is a manifestation that can be found in human disease is unknown (Monath et al., 1978). The virus is extremely lethal when administered via IC or intranasal (IN) routes in both adult and newborn NIH Swiss-Webster mice (Atasheva et al., 2009; Bianchi et al., 1993; Liu et al., 1970). 6-8-week-old CD1 mice are also used as an outbred mouse model (Logue et al., 2009). SC inoculation in CD1 mice reveal similar disease to that seen in NIH Swiss mice except the viremia is transient and not useful as a marker of virulence; CD1 mice exposed via aerosol show similar results with NIH Swiss-Webster mice infected via IC and IN routes (Logue et al., 2009). Lastly, inbred BALB/c mice have also been utilized (Barabé et al., 2007; Nagata et al., 2005; Nagata et al., 2006; Wu et al., 2007) because they exhibit high mortality when inoculated by the IN route. Time to morbidity seems to be a distinguishing metric in determining WEEV virulence in BALB/c mice (Nagata et al., 2006).

WEEV introduced via the aerosol route was confined to the brain and other neural tissues in cynomolgus macaques (Reed et al., 2005). No virus was found in the blood or throat swabs. The infection resulted in the death of 33% (3 of 9) macaques, and appeared to mimic the disease and mortality observed in aerosol-exposed laboratory personnel, which has a rate of morbidity at 40% (2 of 5) (Hanson et al., 1967).

The hamster model was first used as an efficacy model for WEEV vaccines in 1969 (Cole and McKinney, 1969). Later in 1972, a study showed hamsters develop encephalitis independent of infection route with a virulent WEEV strain (Zlotnik et al., 1972). In addition to encephalitis, general pathologic lesions were observed in all infected organs. Viral load was detected in brain, spleen, liver, and serum 1-5 dpi (Julander et al., 2007). Hamsters infected IP are extremely susceptible to disease (Julander et al., 2007). Hamsters show signs of encephalitis and upon histological analysis showed lesions in the brain. The hamster model is well suited to observe severe WEEV infection due to morbidity as a result of neurological complications and peripheral organ involvement. Unfortunately the hamster model does not recapitulate the ventricular involvement observed in humans (Noran and Baker, 1945; Noran, 1944); however, no small animal model recapitulates those disease manifestations. Given the susceptibility of the hamster to disease caused by WEEV and the reports indicating neurologic and peripheral organ involvement, I chose to use the Syrian golden hamster model for the subsequent virulence assays.

In a previous study, we identified six nonsynonymous synapomorphic mutations that were critical in delineating diverging sublineages within the Group B lineage of WEEV (Bergren et al., 2014). Analysis of the selective pressures affecting the WEEV population in the 20th century indicates that these six nonsynonymous synapomorphic mutations may be positivley selcted. This potential for positive selection increases the likelihood that these mutations would confer some phenotype to the virus. In order to detrmine if these mutations led to a phenotype that affects mammalian virulence, we ran virulence assays in Syrian golden hamsters with clones containing the mutations of interest.

MATERIALS AND METHODS

Animal Studies

Female Golden Syrian hamsters 5-6 weeks of age were purchased from Charles River (Wilmington, MA). All work with hamsters was conducted under approved IACUC protocol 0209068B.

HAMSTER MODEL VALIDATION

Cohorts (n=6) were infected SC in the back with 10^4 PFU virus. Proper inoculum doses were verified by back titration. Body weights were measured each day with blood harvested retro-orbitaly (RO) every other day in each subject. On day 3 and 4 post infection two hamsters and one hamster, respectively, from each group were perfused with PBS as previously described (Plante et al., 2015). Blood and major organs were harvested as a result of the perfusion. Of the major organs: half were placed in 10% neutral buffered formalin for histopathological analysis and the remaining half (contralateral or same organ) were placed in DMEM containing 5% FBS for measurement of viral load by plaque assay.

ASSESSMENT OF NONSYNONYMOUS SYNAPOMORPHIC MUTATIONS EFFECT ON VIRULENCE

Cohorts (n=16 per virus infection; n=6 for MOCK) were infected SC in the back with 10^4 PFU virus. Proper inoculum doses were verified by back titration. Body weights were measured each day with blood harvested alternatively every other day in each subject. On days 2 thru 5 three hamsters from each group (one from MOCK) were perfused with PBS as previously described (Plante et al., 2015). Briefly, subjects were perfused through the left atrium with ~80 ml of PBS at a flow rate of 2 ml/min, blood left the circulatory system through an incision in the right atrium, an aliquot of which was collected. Major organs were harvested and half of each organ, or the contralateral organ (quadriceps muscle was taken for analysis of skeletal muscle), was placed in 10% neutral

buffered formalin and the remaining half in supplemented DMEM for measurement of viral load by plaque assay.

Histopathological Analysis

Fixation was conducted as described previously (Plante et al., 2011). Tissues were allowed to fix in 10% neutral buffered formalin (RICCA, Arlington, TX) for a 72 hour minimum. Tissues were then embedded in paraffin wax with 5μm sections taken. Samples were rehydrated using an ethanol gradient and stained with hematoxylin and eosin.

RESULTS

Hamster Model Validation

The hamster model was a common model for WEE in the 1970s, though one contemporary study did use the model (Cole and McKinney, 1969; Julander et al., 2007; Zlotnik et al., 1972). The data show that the hamster model is well suited to mimic WEE disease; however, due to the infrequency with which it is used I wanted to verify that the model worked as previously reported. I tested the virulence of three separate WEEV strains in the hamster model, BFS932, R02PV001807A (R02), and IMP181. BFS932 is a low passage virulent isolate of WEEV that was collected from a mosquito pool in 1942 in Bakersfield, CA (Table 2.1) (Forrester et al., 2008). R02 was isolated in El Paso, TX in 2002 from a pool of mosquitoes. The strain falls within the Group B3 sublineage but in a separate terminal clade than IMP181 (Figure 2.1). Since IMP181 is commonly used as the prototypical contemporary isolate, I included R02 to verify that the assumption about IMP181 is valid. Female hamsters were 5-6 weeks old upon infection with 10^4 PFU. Back titrations were conducted on the inoculum for each virus and all were within 2-fold of the desired dose. Weights and survival were monitored each day and viremia was measured every other day for each subject (Figure 5.1A-C). Additionally, two and one

subjects were perfused on days 3 and 4, respectively, in order to assess the viral load in their major organs; one BFS932 subject was moribund on day 5 post infection and was perfused for analysis (Figure 5.1D-I).

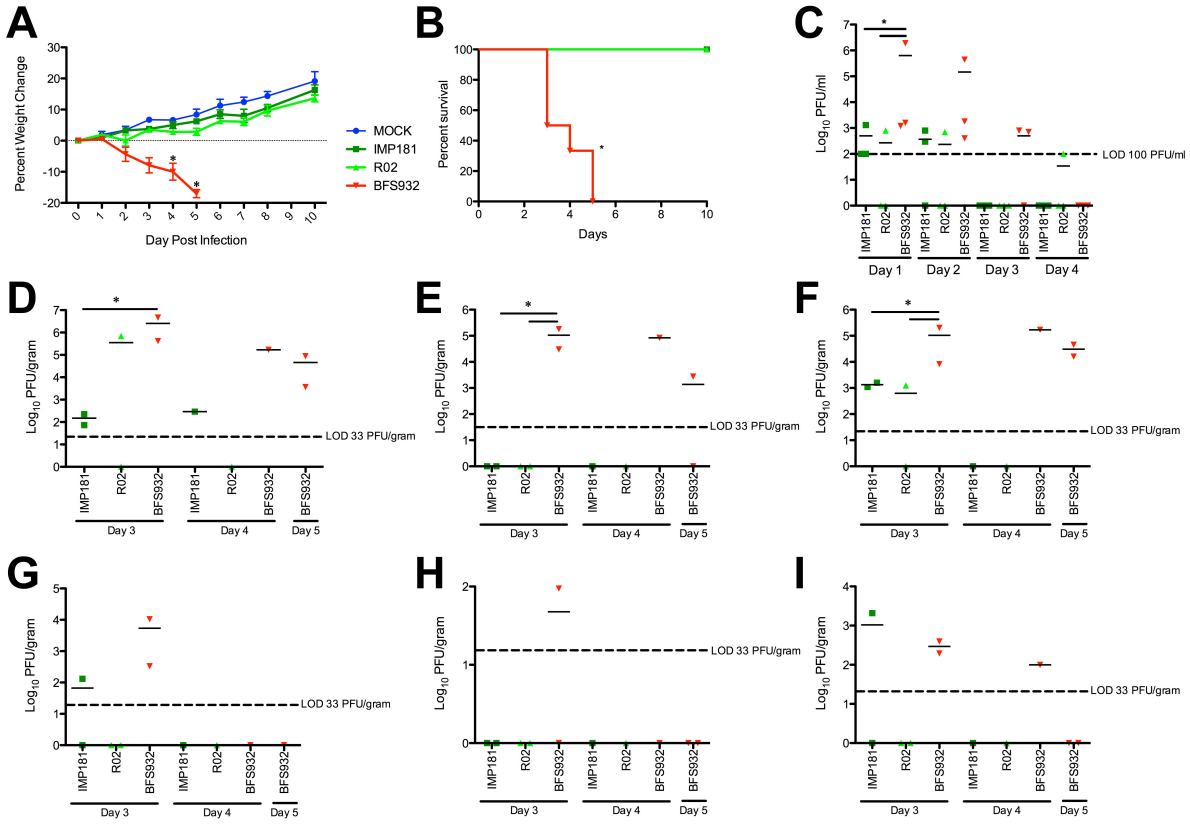

Figure 5.1 Weight, survival, and viral burden in 5-6 week old Syrian golden hamsters following infection with a virulent and avirulent strains of WEEV.

Panels show A) weight, B) survival, C) viremia; and viral titers in the D) brain, E) muscle, F) heart, G) lung, H) liver, and I) kidney. *Indicate statistical significance as determined by A) two-way ANOVA with a Bonferroni post-test, B) Log-Rank, C-I) one-way ANOVA with Tukey post-tests.

IMP181 and R02 groups both tolerated infection well with no significant differences in weight when compared to MOCK and 100% survival. BFS932 caused marked disease leading to significant weight loss on days 4 and 5 post infection as compared to MOCK and 100% morbidity by day 5 (Figure 5.1 A-B). BFS932 also had

significantly higher viremias on day 1 post infection between both IMP181 and R02 (Figure 5.1 C). Viral load in the brain on day 3-post infection was also significantly higher in BFS932 than IMP181; although, significance could not be achieved between BFS932 and R02 (Figure 5.1 D). However, there was no histopathological similarity between BFS932 and R02, with BFS932 causing classic signs of encephalitis including hemorrhage, perivascular cuffing, and infiltration of mononuclear cells in the brain parenchyma (marked accordingly on Figure 5.2). Conversely brains from the R02 group were indistinguishable from IMP181 and MOCK groups, which showed no signs of disease (Figure 5.2). Muscle titers were also significantly higher in BFS932 as compared to IMP181 and R02 on day 3-post infection (Figure 5.1 E). This finding was corroborated by the histopathology showing mild myositis in the BFS932 group (indicated by an arrow) and no pathology in MOCK, IMP181, and R02 groups (Figure 5.2). Additionally, heart titers were significantly higher in BFS932 as compared to IMP181 and R02 groups on day 3-post infection (Figure 5.1 F). The hearts of all groups were reported as normal when analyzed by histopathology (data not shown). Viral loads in the lung, liver and kidney all were statically indistinguishable from one another (Figure 5.1 G-I). Interestingly, sites of mild focal necrosis and possible edema were found in BFS932 livers (indicated by arrows on Figure 5.2).

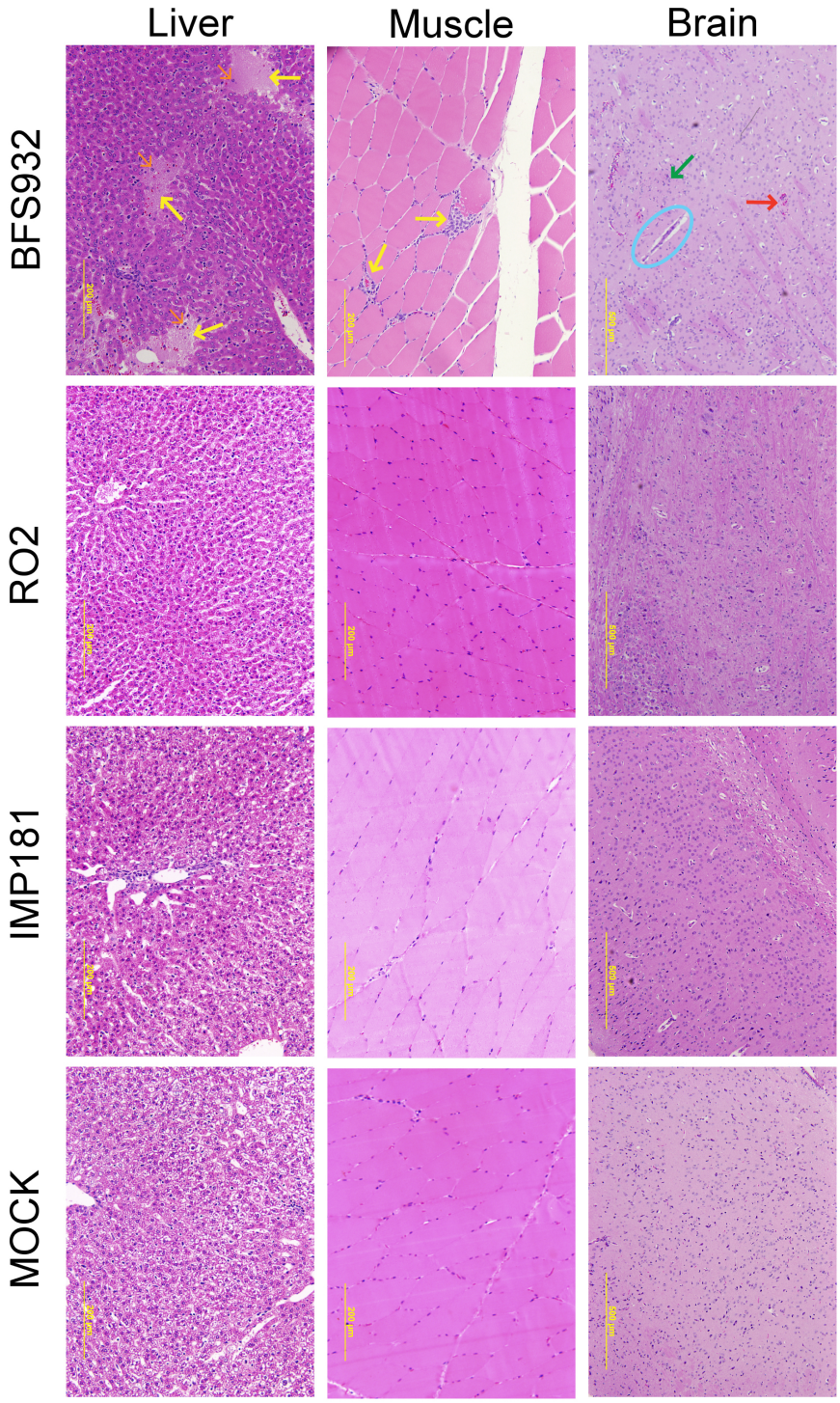

Figure 5.2 Histology for validation of 5-6 week old Syrian golden hamsters as a WEEV pathogenesis model.

Brain images at 10X; perivascular cuffing, hemorrhage, and mononuclear infiltration marked with blue circles, red arrows, and green arrows, respectively. Muscle and liver images taken at 20X; myositis and focal locations of necrosis marked with yellow arrows in the muscle and liver, respectively. Orange arrows indicate free-floating nuclei in the liver.

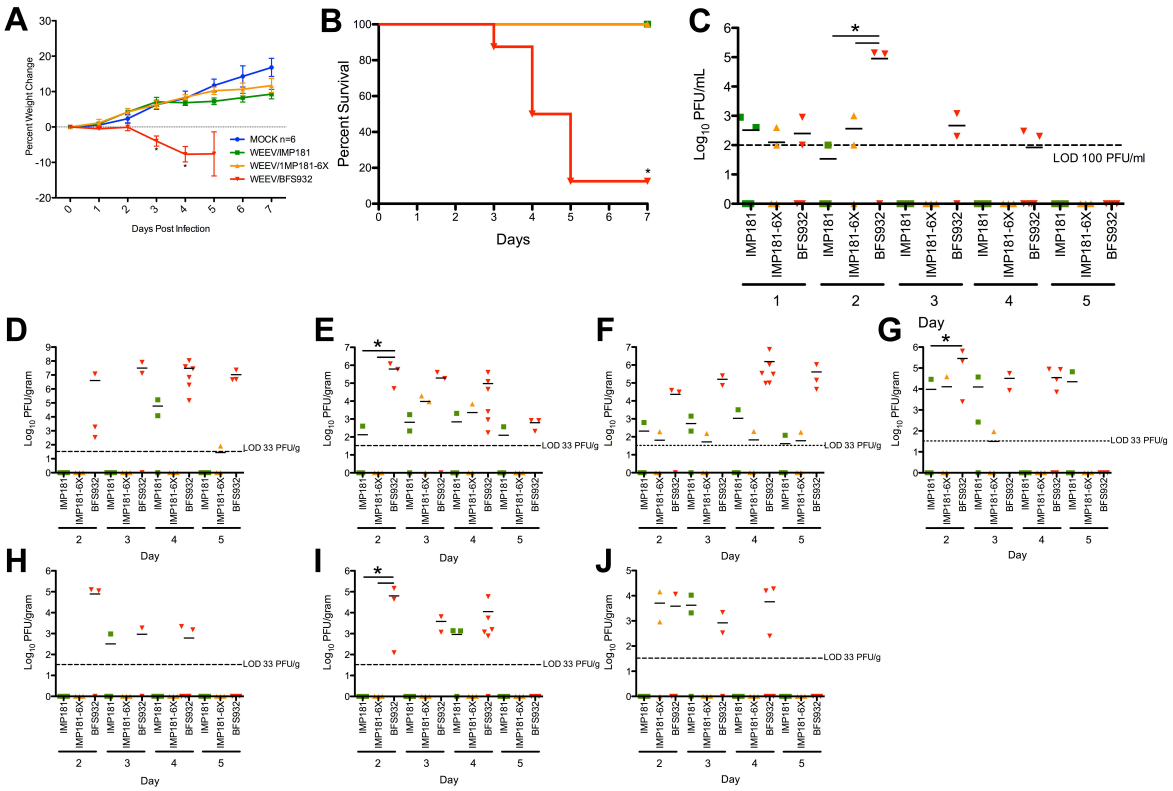

Figure 5.3 Weight, survival, and viral burden in 5-6 week old Syrian golden hamsters following infection of WEEV with cloned ancestral nonsynonymous synapomorphic mutations and appropriate controls.
Panels show A) weight, B) survival, C) viremia; and viral titers in the D) brain, E) heart, F) muscle, G) lung, H) liver, I) kidney, and J) spleen. *Indicate statistical significance as determined by A) two-way ANOVA with a Bonferroni post-test, B) Log-Rank, C-J) one-way ANOVA with Tukey post-tests.

Assessment of Nonsynonymous Synapomorphic Mutations' Effect on Virulence

Having validated the Syrian golden hamster as a viable model for assessing the relative virulence of WEEV strains, I sought to elucidate any changes in virulence the nonsynonymous synapomorphic ancestral mutations would have on the IMP181 backbone. In order to accomplish this I compared the relative virulence of two separate WEEV strains along with the IMP181-6X construct. The rationale for using BFS932 was to be able to determine if IMP181-6X was producing an infection more similar to BFS932 than its parental clone, IMP181. Briefly, female hamsters, 5-6 weeks old, were

infected with 10^4 PFU. Back titrations were conducted on the inoculum for each virus and all were within 1.8-fold of the desired dose. Weights and survival were monitored each day and viremia was measured every other day for each subject (Figure 5.1 A-C). Additionally, three subjects were perfused with DPBS on days 2, 3, 4, and 5 in order to assess the viral load in their major organs (Figure 5.1 D-J).

Subjects receiving IMP181 tolerated infection well with no significant difference as compared to MOCK at all time-points and having 100% survival. For the most part, IMP181-6X followed the same pattern of virulence as IMP181, also showing no significant difference in weight as compared to MOCK at all time-points and having 100% survival. Given these data, the nonsynonymous synapomorphic mutations important in delineating diverging sublineages within the Group B lineage of WEEV do not appear to be major determinants of mammalian virulence. All but one BFS932 subject became moribund and succumbed to infection (Figure 5.3 A and B). BFS932 also had significantly higher viremias than both IMP181 and IMP181-6X on day 2 post infection (Figure 2.3 C). Interestingly, viral burden in the brain showed no significant difference between the groups (Figure 5.3 D). However, upon histopathological analysis of the brain tissue no lesions could be found in IMP181 or IMP181-6X, whereas signs of severe encephalitis were present in BFS932 including perivascular cuffing, mononuclear infiltrate, and hemorrhage (Figure 5.4). Titers in the heart were found to be significantly higher in BFS932 as compared to IMP181 and IMP181-6X (Figure 5.3 E). When hearts were observed for histology mild myocarditis could be found in all groups (Figure 5.5). Muscle titers showed no significant differences between groups (Figure 5.3 F). Analysis of the muscle by histopathology revealed mild myositis in BFS932 subjects. Interestingly, severe myositis was found in IMP181-6X subjects while IMP181 was indistinguishable from MOCK (Figure 5.4). Lung titers were only significantly different between BFS932 and IMP181 (Figure 5.3 G). IMP181 and IMP181-6X showed no signs of pathology in the lung, whereas BFS932 showed significant amounts of hemorrhage

into the alveolar spaces (Figure 5.4). Significantly higher titers were found in the kidneys of BFS932 subjects (Figure 5.3 I). No difference in viral burden could be determined in the liver or spleen (Figure 5.3 H and J), however, significant amounts of necrosis were observed in BFS932 livers (Figure 5.4).

DISCUSSION

Through these experiments, I verified that the Syrian golden hamster is an appropriate animal model for testing virulence among different WEEV strains. Additionally, using the Syrian golden hamster, I compared the disease progression and pathogenesis between a contemporary isolate containing the ancestral nonsynonymous synapomorphic mutations critical for delineating WEEV's evolutionary history to both the parental virus (IMPP181-6X) against the parental contemporary clone, IMP181, and an ancestral virulent strain, BFS932. Survival, weight change, viremia, viral burden in the organs, and histopathology were all used as metrics to assess potential differences in virulence between the viruses used. I also sacrificed subjects in such a way as to investigate the progression of the disease. In short, these studies were conducted in such a way as to determine any potential obvious differences in pathogenesis the discovered nonsynonymous synapomorphic mutations may have on virulence and general pathogenesis in the Syrian golden hamster.

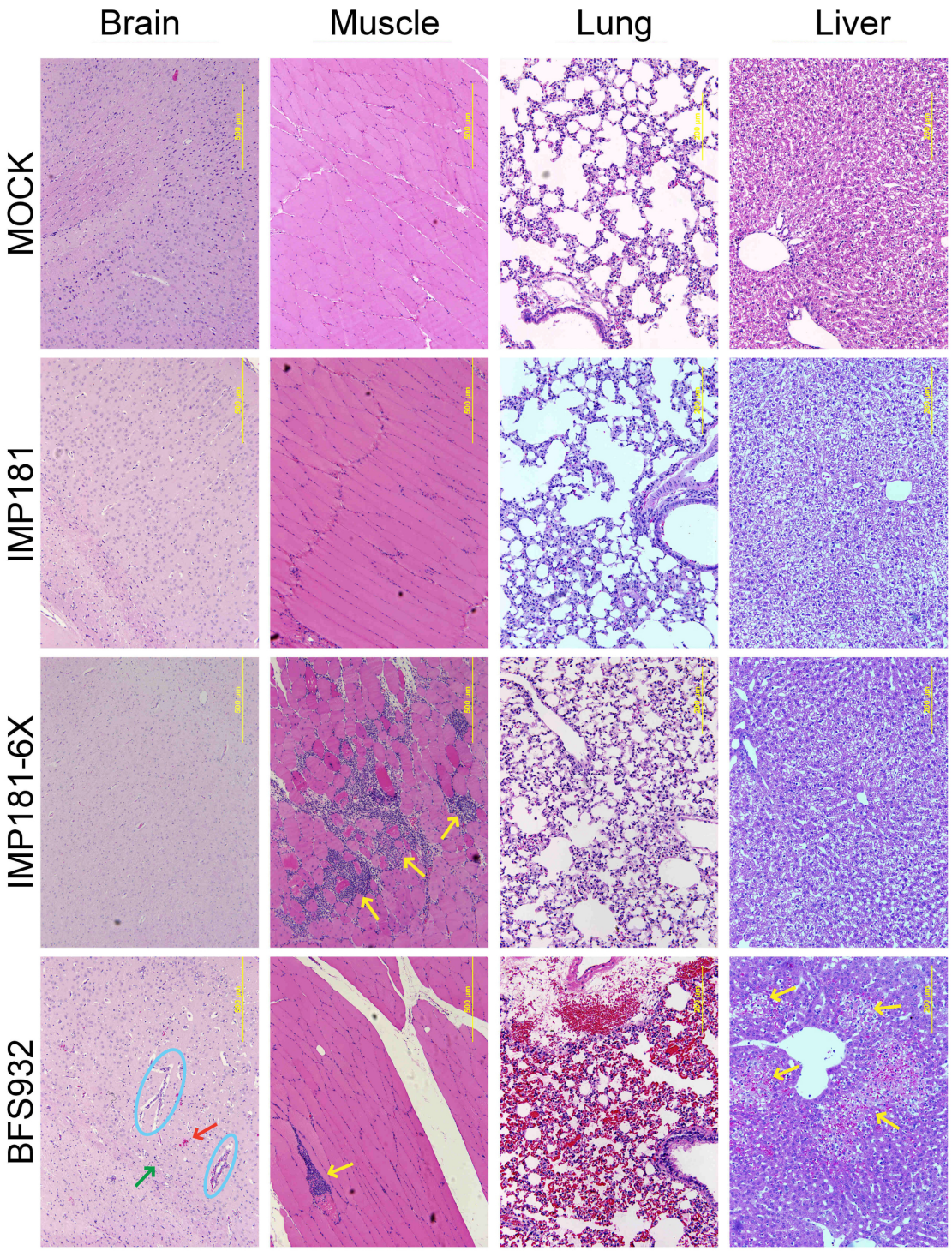

Figure 5.4 Differences in histopathology during peak disease of 5-6 week old Syrian golden hamsters.

Brain and muscle images at 10X; perivascular cuffing, hemorrhage, and mononuclear infiltration marked with blue circles, red arrows, and green arrows, respectively. Yellow arrows on muscle slides indicate myositis. Liver and Lung images taken at 20X; yellow arrows on liver slides indicate foci of necrosis. All BFS932 images are from day 4 post infection. IMP181-6X, IMP181, and MOCK were taken at day 5 post infection.

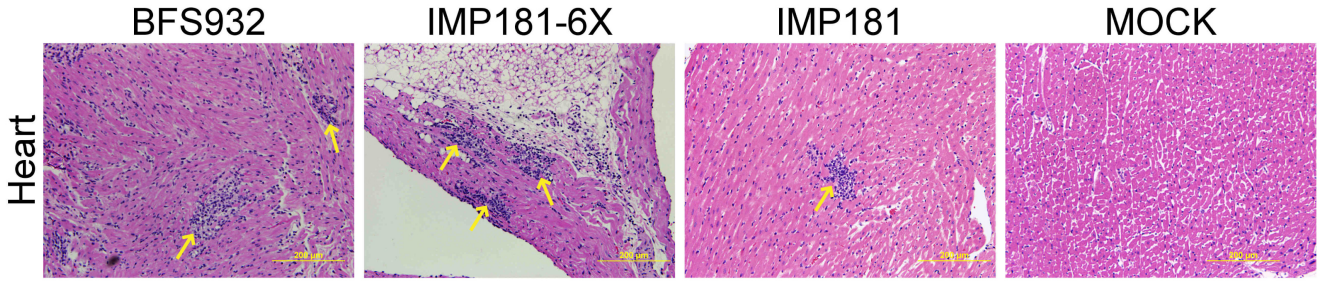

Figure 5.5 Similar histopathology during peak disease of 5-6 week old Syrian golden hamsters.
Heart images taken at 20X. Yellow arrows indicate foci of myocarditis. BFS932 image is from day 4 post infection. IMP181-6X, IMP181, and MOCK images were taken at day 5 post infection.

During the validation study, marked differences were discovered between the virulence and pathogenesis of IMP181 and BFS932. Weight and survival data indicated that IMP181 was not statistically different from MOCK with 100% survival and no decrease in weight following infection. Conversely, BFS932 showed noticeable weight loss with significant differences from MOCK on days 4 and 5-post infection. Additionally, all subjects succumbed to illness by day 5-post infection. Both IMP181 and BFS92 developed a short-lived viremia with BFS932 developing significantly higher titers on only day 1 post infection. These three metrics alone indicate that BFS932 is significantly more virulent than IMP181 and the Syrian golden hamster model is appropriate for distinguishing between the two. Furthermore, when viral burden in the organs was determined significant differences between IMP181 and BFS932 was found in the brain, muscle, and heart on day 3-post infection. This finding was further validated upon examination of histological analysis of the brain and muscle. Marked signs of encephalitis were present in the brains of BFS932 subjects with no lesions detectable in IMP181 subjects. Mild myositis was found in the skeletal muscle of BFS932 subjects; conversely, no myositis was found in the skeletal muscle of IMP181 subjects. Interestingly, while viral burden indicated no significant difference in the liver in any

groups, mild signs of focal necrosis was discovered in the livers of the BFS932 group. The pathology described in the BFS932 subjects was consistent with the pathology previously described in studies using the Syrian golden hamster as an animal model for WEE (Cole and McKinney, 1969; Julander et al., 2007; Zlotnik et al., 1972). The only deviations were a lack of viral burden in the liver and an absence of myocarditis upon examination of the cardiac muscle. Even though higher amounts of viral burden wasn't discovered in the liver the presence of focal necrosis indicates that the liver was involved. Moreover, the absence of myocarditis does not indicate that the heart muscle wasn't involved, as viral burden in the heart were noticeably high. Not obtaining the full spectrum of pathology previously reported in the literature could be due limitations in the study design. Only two hamsters on day 3 and 1 on day 4-post infection were analyzed for viral loads and pathology in the organs. The lack of subjects on day 2 and 5-post infection and the limited number of subjects analyzed could have participated in not observing the full spectrum of disease previously reported. Additionally, the virulent strain, BFS932, has not been previously used in the Syrian golden hamster model and may have a slightly different pathogenesis as compared to the strains previously reported in the model. However, in spite of these limitations the Syrian golden hamster model indicated its ability to discriminate between virulent and avirulent strains of WEEV primarily through neurological involvement.

The IMP181 strain has been used in numerous studies as the prototypical contemporary isolate of WEEV (Logue et al., 2009; Mossel et al., 2013; Nagata et al., 2006; Reisen et al., 2008). In order to verify this assumption I included R02; R02 is a Group B3 strain however, it falls in a terminal clade distinct from IMP181. Additionally, it was isolated from a different geographical location (El Paso, TX) than IMP181. RO2 behaved similarly to IMP181 by nearly every metric. The only point of deviation was that BFS932 had significantly higher titers than IMP181 in the brain on day 3, but not R02. Furthermore, R02 did not demonstrate significance between itself and IMP181 at that

time-point. However, upon examination of the brain histology, no lesions could be found in R02 subjects. The lack of significant difference between BFS932 and R02 in the brain on day 3-post infection could simply be a result of the low sample size. Indeed, while one R02 subject reported nearly 10^6 PFU/g the other subject reported no titer at all. In spite of this small discrepancy, comparing IMP181 to R02 in the Syrian golden hamster model validated the use of IMP181 as an appropriate prototype for contemporary WEEV isolates.

Having validated the Syrian golden hamster as an appropriate animal model for comparing relative virulence among WEEV strains, I then tested the ancestral nonsynonymous synapomorphic mutations on the IMP181 backbone effect on virulence. IMP181 and BFS932 were used as avirulent and virulent controls to compare IMP181-6X against. Disease within IMP181 and BFS932 groups were recapitulated as in the experiment assessing the viability of the Syrian golden hamster model. Generally, IMP181-6X tracked with IMP181 indicating the ancestral nonsynonymous synapomorphic mutations have no effect on virulence. Subjects in the BFS932 group succumbed to illness, except for one. However, statistical significance between BFS932 and the other groups was achieved as determined by a log-rank test. Additionally, on days 3 and 4 post infection significant differences in weight as compared to MOCK were evident, whereas IMP181 and IMP181-6X were indistinguishable from MOCK in terms of survival and weight change. All groups developed viremia, though BFS932 developed a more pronounced viremia (significant from both IMP181 and IMP181-6X on day 2-post infection) and a longer lasting viremia (detectable out to day 4-post infection, whereas viremia in IMP181 and IMP181-6X were detectable only out to day 2-post infection). Interestingly, significantly different titers were not achieved in the brain, however upon visual inspection of the data while all viruses were present in the brain at one time or another, BFS932 seems more pronounced. Moreover, the histology supports this conclusion as the only signs of encephalitis occurred in the BFS932 group. Viral load

in the heart between BFS932 and IMP181 and IMP181-6-X were significantly different on day 2-post infection. Interestingly, all groups showed signs of mild myocarditis. Additionally, significant differences in viral burden could not be determined in the skeletal muscle, though BFS932 seems to develop higher titers than the other groups. Upon histological analysis, mild myositis was found in BFS932 subjects and, interestingly, moderate myositis was discovered in IMP181-6X subjects with no myositis being found in IMP181. This is the only instance where IMP181-6X displayed a pathology more similar to that of BFS932. Oddly, the pathology seemed more pronounced in IMP181-6X than BFS932 subjects. Viral burden in the lung was significantly different between BFS932 and IMP181, but not between BFS932 and IMP181-6X. Upon investigation of the histology significant amounts of hemorrhage was found in the lungs of BFS932 subjects, though no pathology was found in the lungs of IMP181-6X subjects. Significant differences in viral burden were found in IMP181 and IMP181-6X as compared to BFS92 in the kidneys on day 2-post infection, though no corroborating pathology was found. Viral burden in all other organs were indistinguishable between groups. Although, viral burden in the liver wasn't significantly different (one BFS932 subject didn't develop any viral burden in the organ, resulting in a non-significant result), moderate foci of necrosis was discovered in the livers of BFS932 subjects with no pathology appearing in the other groups.

In summary, IMP181-6X displayed no propensity to revert to a virulent phenotype in the Syrian golden hamster. Interestingly, moderate myositis was discovered in the skeletal muscle and comparably high titers were found in the lung on day 2-post infection. While the lung titers can be explained due to relatively small sample sizes on each day (n=3), the presence of moderate myositis is more difficult. Independent of virus strain, skeletal muscle tissue seems to be ubiquitously infected by WEEV in the Syrian golden hamster model. While virus was present in the muscle of all groups only BFS932 and IMP181-6X displayed signs of myositis, compared to IMP181 showing no signs of

myositis. This could potentially represent a change in the pathogenesis of WEEV as a result of the ancestral nonsynonymous synapomorphic mutations included in IMP181-6X. However, while this may represent a small change in pathology, the overall outcome remains unchanged. All IMP181-6X subjects survive infection and show no signs of illness, indicating that the ancestral nonsynonymous synapomorphic mutations included in IMP181-6X do not play a role in increasing WEEV's virulence in mammals.

Chapter VI: Conclusions and Future Directions

Western equine encephalitis virus (WEEV) was the causative agent in many outbreaks of human and equine encephalitis in western North America throughout the early- to mid-20th century. However, the mid- to late-20th century was characterized by a marked reduction in WEEV activity with the last human case in North America occurring in 1998. This is based on reduced levels on enzootic activity found in states that still actively survey for WEEV including California, Texas, and New Mexico. While these states are currently and have experienced drought over the past few years, I think this reduction in enzootic activity is valid given the reductions started to occur before these states underwent drought conditions, though these droughts could have easily exacerbated the reduction. The overall objective of my research was to use phylogenetic and experimental approaches to better understand associated genetic changes WEEV has undergone that could account for its reduction in enzootic circulation and human incidence. In order to address this objective, my studies were designed to assess which mutations may play an important role in defining the evolutionary lineages of North American WEEV in the 20th century; once I elucidated the mutations that were critical in defining the internal branching pattern in WEEV's phylogeny, I then assessed their phenotype in a number of different relevant systems including the enzootic vector and reservoir hosts and a mammalian disease model.

Evolutionary History of WEEV During the 20th Century

In order to investigate the evolutionary history and population structure of North American WEEV during the 20th century, I compiled a diverse library of WEEV isolates and used their complete genomes to conduct a Bayesian coalescent analysis and other phylogenetic methods. I was able to identify the two major North American lineages, Group A and B, as previously described (Weaver et al., 1997). Also I further defined the

Group B lineage into three distinct sublineages (Groups B1-3). When analyzing the population of WEEV, I found purifying selection to be the predominant form of selection on the WEEV genome throughout the 20th century. Even though purifying selection was the main selective pressure on WEEV, as with most arboviruses, several mutations that define the divergence of Groups A thru B3 were recognized as potential sites for positive selection. In addition to the phylogenetic studies I attempted to determine any phylogenetically relevant differences between the viruses based on glycosylation patterns, cysteine residues, and UTR folding patterns, of which all were found to be uniformly conserved.

WEEV was implicated as the etiologic agent of several severe outbreaks of encephalitis in humans and horses in the 1930s. The virus spread northeast during the 1930s and early 1940s affecting Western and Midwestern states and Canada. Viruses attributed to this epidemic spread fall into the Group A lineage as evidenced by the California strain (1930, CA) and McMillan (1941, Ontario) isolates both residing in the Group A lineage. However, shortly after that initial spread, I determined that Group B1 emerged and displaced Group A, which ultimately became extinct in the 1940s. Anecdotally, I found that the vast majority of WEEV isolates that have a mammalian virulent phenotype are from Group A or B1 in comparison to isolates from Group B2 and B3 (Forrester et al., 2008; Logue et al., 2009; Nagata et al., 2006). Shortly after the divergence of Group B1 from Group A, Group B2 emerged from Group B1. I hypothesize that shortly after the emergence of Group B2, Group B1 became extinct. After Group B2 became predominate, Group B3 emerged from Group B2 during the 1960s. The consistent tight grouping of the highest posterior densities (HPDs) throughout the phylogeny supports this timeline.

Bayesian skyline analysis indicated an increased population size between 1965 and 1980, which coincided during the co-circulation of all Group B sublineages. This was followed by a reduction in population size as the Group B3 sublineage became the only

circulating lineage of WEEV in North America. The predominance of the Group B3 sublineage is most likely an example of the effects of purifying selection on the WEEV population, which resulted in a trimming of WEEV evolution toward Group B3.

I further found purifying selection to be the predominant form of selection on the WEEV genome. Taken in concert with the decreased genetic diversity observed in the Bayesian coalescent analysis this finding is not surprising. If the size of a population is reduced, it becomes more susceptible to stochastic drift, which can cause several of the phenomena observed such as lineage extinction events mediated by the phenomenon known as Muller's Ratchet. In spite of these results, when inspected manually, I found several mutations that could represent positive selection. Furthermore, IFEL, FEL, and FUBAR analyses all indicated these mutations were under positive selection, though the statistical significance could not be verified. Moreover, these codon-based analyses have been found to lack sensitivity when detecting sites of positive selection as indicated by the previous studies (Tsetsarkin et al., 2014; Tsetsarkin and Weaver, 2011; Tsetsarkin et al., 2009; Tsetsarkin et al., 2007; Volk et al., 2010).

Given WEEV's recent epidemiology, the reduction in detection of WEEV in mosquitoes, and the predominance of the Group B3 sublineage the question arises: what environmental/ecological factor(s) precipitated this evolutionary course for WEEV? I hypothesize that a significant disturbance affected WEEV circulation sometime between 1945 and 1965, affecting the evolution of WEEV in one of two ways: 1) changes in selective pressures resulted in the above detailed evolutionary trajectory of WEEV during the 20th century, or 2) the reduction in WEEV population and diversity caused WEEV to drift genetically which precipitated a reduction in fitness.

Using a Bayesian coalescent framework in concert with phylogenetic methods detailing the selective pressures on the WEEV genome, I was able to verify the two major North American lineages of WEEV (Group A and B) and further delineate Group B into three sublineages, of which Group B3 is the only currently circulating WEEV lineage in

North America. While I discovered that purifying selection is the predominant form of selective pressure on the WEEV population, I also determined that several nonsynonymous synapomorphic mutations could have been positively selected. After these findings, my focus shifted to testing this hypothesis experimentally by determining if these mutations play a role in the fitness of WEEV, and subsequently its evolution or if the WEEV genome has simply been undergoing genetic drift during the 20th century.

ROLE OF NONSYNONYMOUS SYNAPOMORPHIC MUTATIONS OF INTEREST

In accordance with the data obtained in the phylogenetic analyses, I sought to apply an experimental approach to determine if the elucidated nonsynonymous synapomorphic mutations were fixed in the WEEV population by positive selection. In order to accomplish this I conducted a variety of fitness competition assays using *in vitro* and *in vivo* models. I also tested the effect of these mutations have on virulence in a mammalian disease model. Through the course of these experiments I found evidence of positive selection for the mutations in WEEV's enzootic transmission cycle; I also found that these mutations have no effect on virulence in the model tested.

I initially conducted multi-step replication curves to assess potential differences between the various mutations and the wild-type parental clone derived from a recent WEEV isolate. Unfortunately the resulting data were a bit confusing. In spite of the messy nature of the data several conclusions could be drawn: 1) Vero cells showed the least amount of movement between mutated and wild-type viruses, possibly due to the permissiveness of the cell line; 2) C7-10 cells generally recapitulated the results obtained with Vero cells; and 3) 3T3 cells showed a large amount of variation between the mutated and wild-type viruses, possibly due to the presence of an intact interferon response in 3T3 cells. However, the results obtained from the 3T3 cells is difficult to decipher because IMP181 (original clone), BFS932, and IMP181-6X all replicated to titers statistically indistinguishable from each other, while the single mutants replicated to

titers significantly lower than IMP181, implicating potential epistatic interactions for maximal affect.

To further understand any potential effects of these mutations *in vitro*, competitive fitness assays were conducted in both Vero and C7-10 cells. These competitive fitness assays revealed a trend for the wild-type virus (in this case IMP181-nsP2-*XbaI*) to outcompete the virus with the ancestral nonsynonymous synapomorphic mutations (IMP181-6X). However, these studies were limited because a control assay competing IMP181-nsP2-*XbaI* against BFS932 was not run. Having this assay would have allowed me to make a more definitive statement on the relative fitness of the nonsynonymous synapomorphic mutations that define the distinctions in the Group B sublineages because potential epistatic effects could have been ruled out.

Having obtained compelling data from the *in vitro* competition assays, I then conducted competition assays in relevant model systems for WEEV's enzootic transmission cycle. My initial experiments, using the IMP181-nsP2-*XbaI* clone as a surrogate wild-type virus, indicated nonsynonymous synapomorphic mutations demonstrate a competitive advantage in both *Cx. tarsalis* and HOSPs. However, the control assay competing IMP181 with IMP181-nsP2-*XbaI* indicated that IMP181-nsP2-*XbaI* conferred a significant competitive advantage in the HOSP, while not in *Cx. tarsalis*. Unfortunately, this result threw my initial findings into question because if IMP181-nsP2-*XbaI* is more fit than wild-type, then it cannot reliably be used in the competition assays.

In order to determine if the competitive advantage of the surrogate wild-type virus was real, I conducted competition assays directly between IMP181 and the mutated viruses, without the use of the genetic marker, in *Cx. tarsalis*, HOSPs, and 21-day-old chickens. Due the sequences of the viruses being exactly identical apart from the points at which they were mutated I was unable to conduct restriction digest densitometry readings or a differential qRT-PCR to determine the relative ratios. Instead, the samples were

analyzed by pyrosequencing over one of the mutated nucleotides, thus the relative ratio of virus could be determined. I did not conduct a more powerful deep sequencing method (such as MiSeq, Illumina, San Diego, CA) because the increased sensitivity was not required for the assays being conducted. In addition to altering the analysis methodology, I altered the design of the *Cx. tarsalis* experiment where I harvested the midgut, salivary glands, body, and legs & wings on days 3, 5, and 10-post blood meal. The intention was to determine the infection kinetics within the mosquito and if the viruses had differing fitness levels dependent on the infected organ. Unfortunately, cost was restrictive in the analysis, so only day 10-post blood meal salivary glands were analyzed for virus ratio. Additionally, no differences in infection kinetics were found; however, if differences were found the specific source from which those differences were arising would have been difficult to determine because each mosquito group was infected with a mixture of two viruses. When analyzed by pyrosequencing a clear competitive advantage of the nonsynonymous synapomorphic mutations that define WEEV's Group B sublineage can be seen as indicated by significant results in the groups competing IMP181 against IMP181-6X, IMP181-nsP2-Cap250-E1 and BFS932. Interestingly, this advantage is only seen in the mutations that provide a significant change to the chemistry of their amino acid residue or reside in a region of the envelope glycoproteins that may have biological significance. This result indicates that these nonsynonymous synapomorphic mutations are likely the result of positive selection in *Cx. tarsalis* mosquitoes.

The competition assays were also repeated in HOSPs and 21-day-old chickens. In HOSPs, IMP181 outcompeted both IMP181-6X and IMP181-nsP3-Cap250-E1 by significant margins on day 1 and 2-post infection. However, no significant difference in virus ratios could be determined in the assay competing IMP181 and BFS932. These results were recapitulated in the experiments with 21-day-old chickens. The only difference between the HOSP and 21-day-old chicken experiments was that IMP181 was found to have a significant advantage over IMP181-nsP4-Cap89-E2 between the

inoculum dose and day 2-post infection and between days 1 and 2-post infection in the 21-day-old chicken. Though I have some doubt regarding the biological relevance of this finding as the original inoculum dose was approximately 9:41, far from the desired 1:1 ratio. Due to the lack of significant results in the assays competing IMP181 and BFS932, the significant results achieved in the assays containing IMP181-6X and IMP181-nsP3-Cap250-E1 were brought into question. Most likely, BFS932 has other, possibly epistatic residues, which compensate somehow for the effect of the nsP3-Cap250-E1 substitutions avian fitness, but were eliminated from the population by drift or purifying selection.

Lastly, I sought to determine if the nonsynonymous synapomorphic mutations played any role in the reduction in mammalian virulence observed in WEEV during the 20th century. In order to determine if the ancestral nonsynonymous synapomorphic mutations conferred increased virulence I infected Syrian golden hamsters with IMP181-6X, IMP181, and BFS932. IMP181 and BFS932 were used as avirulent and virulent controls, respectively, to compare against the effects of IMP181-6X. I found that IMP181-6X replicated the disease progress of IMP181. Specifically, no significant differences between IMP181 and IMP181-6X could be determined in weight, survival , viremia, or viral load in any organ. Conversely, BFS932 caused severe disease resulting in morbidity in all subjects, except one. Generally, viremia, viral load in the organs, and the presence of pathological lesions were significantly higher in BFS932 hamsters compared to the others, though all subjects were found to have mild myocarditis as a result of infection. In short, since all IMP181-6X subjects survived and showed no significant signs of disease I conclude that the nonsynonymous synapomorphic mutations do not play a role in altering the virulence of WEEV.

FINAL CONCLUSIONS

Taken together, the results of my dissertation provide a compelling story of how WEEV has evolved through the 20th century in North America. Phylogenetic analysis

demonstrated that WEEV is following a pattern of emergence and purification in relation to its circulating lineages. This pattern ultimately resulted in the emergence of Group B3 and a subsequent reduction in population size. While a majority of the WEEV genome is undergoing negative selection, purifying out deleterious mutations as they arise in the population, I was able to determine that at least one of three residues were under positive selection. Furthermore, I determined that these residues were important in defining the various evolutionary lineages of WEEV. These studies provided a theoretical base supporting my hypothesis that these mutations caused altered phenotypes in either WEEV's enzootic transmission cycle or virulence in mammals. Competitive fitness assays in *Cx. tarsalis*, HOSPs, and 21-day-old chickens and virulence assays in Syrian golden hamsters were the basis for determining if the nonsynonymous synapomorphic mutations had any significant phenotypic effect. Through these experiments I found evidence that the non-conservative nonsynonymous synapomorphic mutations that WEEV acquired during the 20th century and that are critical for defining the Group B sublineage and mediate a competitive advantage in *Cx. tarsalis* mosquitoes. In avian species I found the mutations do not have a significant advantage over the ancestral residues when in a virus genome that presumably contains compensatory epistatic mutations. Furthermore, the positively selected substitutions involve changes in the chemical property of amino acid residue and/or reside in an protein domains known to be functionally important (in this case stabilizing the E1-E1 interface). Additionally, I found that these mutations have no effect on virulence in the Syrian golden hamster.

Initially I developed two hypotheses that could theoretically account for the submergence of WEEV over the 20th century; they included: 1) genetic drift and bottleneck events were predominating in the WEEV population leading to a Muller's Ratchet effect; or 2) positive selection toward its enzootic cycle despite the predominating purifying selection, population decline, and reduced enzootic activity. In order to test these hypotheses I wanted to define the phenotype of the nonsynonymous

mutations that were fixed in the WEEV population during the 20th century I found during my initial phylogenetic analysis (Bergren et al., 2014).

After the initial phylogenetic experiments, the tree topology and patterns in population I obtained led me to believe that WEEV would likely be succumbing to a Muller's Ratchet phenomenon (i.e. hypothesis 1). If the mutations conferred no change in fitness or virulence or were deleterious then I could conclude that WEEV was undergoing genetic drift and, if the mutations were deleterious, that drift was leading to a Muller's Ratchet phenomenon, due to the lowered population size of WEEV. This finding would explain the lowered human case incidence and the reduced levels of enzootic activity, and would be experimentally supported if I saw changes in mammalian virulence or fitness in enzootic hosts, respectively.

Conversely, I thought that these mutations might also represent evidence of positive selection toward WEEV's enzootic cycle. If the second hypothesis was supported, I would expect the mutations to have a beneficial advantage in enzootic hosts with no affect on virulence. This hypothesis operates under the assumption that mammals (possibly equids) once participated to a greater extent in the transmission of WEEV and their removal from the transmission cycle caused beneficial residues to mammalian transmission to be purified out by either negative selection or eliminated stochastically by genetic drift. The underlying ecological factors that could account for this reduction in mammalian participation could be one or a combination of two things. First, over the 20th century the population of *Cx. tarsalis* has diminished, therefore because of this diminished vector population WEEV is unable to reach the enzootic threshold required to spill over into a mammalian transmission cycle and therefore the residues adapted to that cycle were purified out. Alternatively, if equids participated in the transmission of WEEV prior to their vaccination, beginning in the 1940s, this removal from the transmission cycle would result in the loss of residues that conferred fitness in that cycle, especially if they were even slightly deleterious in the *Cx. tarsalis* – passerine cycle.

Taken together, the results presented herein support the hypotheses that WEEV, immediately preceding a population decline, has undergone positive selection for circulation in the enzootic cycle. Thus the hypothesis that the evolution of WEEV over the 20th century has been characterized solely by genetic drift, resulting in a Muller's Ratchet phenomenon is refuted. Previous phylogenetic analysis demonstrated that WEEV is following a pattern of emergence and purification in relation to its lineages (Bergren et al., 2014). This pattern ultimately resulted in the emergence of Group B3 and a subsequent reduction in population size. While a majority of the WEEV genome is undergoing negative selection, purifying out deleterious mutations as they arise in the population, six residues were identified as possibly being under positive selection (Bergren et al., 2014). Using the phylogenetic studies as a theoretical base that informed the experiments described in this manuscript, I found evidence that the three non-conservative nonsynonymous synapomorphic mutations that have become fixed in the WEEV population during the 20th century demonstrate evidence for positive selection in *Cx. tarsalis*. In avian species I found the mutations do not have a significant advantage over an ancestral virus strain, possibly due to compensatory epistatic mutations. Additionally, none of the fixed nonsynonymous synapomorphic mutations has an effect on mammalian virulence. Evidence of positive selection is difficult to square with the previous phylogenetic findings because of WEEV's declining population and the reduced levels of enzootic activity. Additionally, because WEEV is only being collected in states where there is active surveillance (Texas and California) a sampling bias could have been incorporated into the data set if WEEV strains in other parts of North America have a different evolutionary lineage. However, it is important to note that the mutations that were positively selected for arose during a period of elevated population size (Bergren et al., 2014). Thus I can conclude that during this time of elevated population levels these residues were positively selected for in *Cx. tarsalis* and possibly by avian hosts. Moreover, this finding fits with the second hypothesis that these mutations, while having

an affect in the enzootic transmission of WEEV will show no affect in mammalian virulence. Ultimately, while these data are suggestive of past increased levels of mammalian participation in the WEEV transmission cycle no definitive conclusions can be made. One potential area of future study would be to determine which mutations affect mammalian virulence and determine if they were purified out of the population by genetic drift or negative selection. Interestingly, three conservative amino acid changes were found fixed in the WEEV population showed no affect in any of the experiments conducted, indicating that these mutations were most likely fixed in the population due to genetic drift. This finding is not surprising given the reduction in the WEEV population and stepladder type tree that describes WEEV's evolutionary history over the 20th century (Bergren et al., 2014). I hypothesize that a significant disturbance in WEEV circulation occurred between 1945 and 1965. The results presented herein suggest that WEEV attempted to compensate for this disturbance by selecting residues that allow it to transmit more efficiently in its enzootic cycle. However, this disturbance may have altered the ecology of the virus such that previously recorded levels of enzootic transmission are no longer possible, hence the declining population size and stepladder type tree (Bergren et al., 2014).

This research provides a unique look at the evolution of WEEV over the 20th century. I have demonstrated that, even while attempting to compensate for an ecological disturbance, some shifts in virus ecology are so significant that the virus is unable to establish a similar ecological niche. In the case of WEEV, despite positive selection toward its enzootic vector, enzootic circulation of WEEV remains so reduced that it is not able to amplify to a point where consistent levels of transmission are observed. The specific ecological circumstances that precipitated this evolutionary trajectory for WEEV remain cryptic, but may involve decreased populations of *Cx. tarsalis* (Reisen, 2012). Additionally, the population reduction in North America and the vaccination of equids may have also participated in the evolutionary trajectory if equids did participate as

amplifying hosts for WEEV (Kilby, 2007). Experiments attempting to test these hypotheses, for the most part, are logistically challenging and/or cost restrictive. Thus, the precise nature of the disturbance may never be known. Even so this research provides unique insight into the nature of an arbovirus on the verge of potential extinction.

Notwithstanding the information and insight into the evolution of WEEV during the 20th century, there are limitations inherent in the phylogenetic and experimental studies that ought to be considered in the analysis of the results. 1) While the strains used for the phylogenetic study were the optimal choices for the type of study conducted, any time when virus isolates from the early 20th century are used the question of passage history and bias come up. There is always a chance that an ancestral mutation observed only appears to be ancestral because it is selected for by a specific passage method, such as suckling mouse brain. Confidence can be derived from these studies because, as much as was possible, only low passage isolates were used and none of the mutations were found to affect virulence, which is the phenotype most likely affected by past passaging methodologies. Additionally, bias can significantly affect the outcome of a phylogenetic tree. In order to address bias, I attempted to use virus isolates from a number of geographic locations and time points (while still controlling for passage history). 2) The experiments are limited in that the effects of these mutations are only tested in one direction, which is on the IMP181 background. The results would be much more compelling if reciprocal results were obtained by cloning the nonsynonymous mutations into an ancestral isolate of WEEV (such as BFS932). Indeed, I went to great lengths to construct a BFS932 infectious clone. However, after considerable effort on my and a company's part plans for a BFS932 infectious clone were abandoned. Regardless, the studies presented herein would have greatly benefited from reciprocal experiments. 3) The *Cx. tarsalis* colony used in the mosquito experiments is approximately 40 years old. Even though colonies can become more susceptible to infection as their generation increases, I have confidence that the findings in the experiments are valid due to the fact

that competition assays are internally controlled. However, it may have been informative to conduct the experiments in colony of *Cx. tarsalis* with fewer generations. In fact, since a younger colony would be less susceptible to infection, I hypothesize that the observed differences would be amplified.

Despite the previously mentioned limitations, my dissertation provides a unique look at the evolution of WEEV over the 20th century. I have shown that the majority of the WEEV genome is undergoing purifying selection; however, up to three sites in nsP3, Capsid, and E1 have been positively selected as evidenced by their increased fitness in *Cx. tarsalis*. Interestingly, these findings coincide with an observed reduction in enzootic circulation of WEEV, indicating that the mutations positively selected for may not have been able to overcome the population decline and resulting genetic drift, which was precipitated by some unknown ecological event. Additionally, it appears that residues associated with virulence in mammals were purified out of the population by drift or negative selection. The ecological disturbance that precipitated this evolutionary trajectory still remains cryptic, with hypothesis ranging from, horses participating in the transmission cycle, possibly on a small scale, and their vaccination took them out of the transmission cycle, changes in agricultural practices, or changes in human behavior (decreased exposure to mosquitoes via screen doors/windows and air conditioning). Experiments attempting to test these hypothesize, for the most part, are either not possible or cost restrictive. Thus, the precise nature of the disturbance may never be known. Not only does this work contribute to a comprehensive body of research on WEEV, but it also provides insight into a comprehensive understanding of the mechanisms that control viral emergence and submergence.

POTENTIAL FUTURE DIRECTIONS

While the experiments detailed in this dissertation demonstrate compelling evidence that WEEV is evolving in such a way that favors its enzootic cycle, additional

experiments that would assist in shoring up some of the limitations in experimental design would be helpful. Additionally, the future study of WEEV could be expanded into other directions, using this research as a starting point. As stated previously, conducing the experiments using an ancestral virulent strain of WEEV with the reciprocal nonsynonymous synapomorphic mutations would make the findings presented herein more compelling. Another avenue of research one could take is to explore potential areas of positive selection at the node tips on the phylogenetic tree. For example, IFEL, FEL, and FUBAR analysis flagged a mutation occurring in the 6K gene as being positively selected for. These mutations may shed light on how WEEV is continuing to evolve and attempt to adapt to its environment. Furthermore, I think additional field surveillance for WEEV would assist in filling many gaps currently present in regards to the present state of WEEV in North America. Moreover, it would behoove researchers to test a variety of ecological niches in addition to *Cx. tarsalis* and HOSPs. One specific question researchers could ask is if WEEV is transmitting more often between *Ae. melanimon* and black-tailed jackrabbits or some as yet unidentified cycle. In terms of attempting to determine the source of the ecological disturbance that WEEV underwent, replicating the results obtained in previous studies that show horses obtaining a viremia would be beneficial. However, even if positive results were achieved the results would have to be questioned due to the lack of WEEV observed in other mosquito species that commonly feed on horses. In short, WEEV provides a unique glimpse into arbovirus evolution and many different research tracks can be taken to answer important questions regarding the mechanisms surrounding emergence and submergence. Perhaps WEEV shouldn't be as neglected by the scientific community as it currently is.

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Vita

NAME: **Nicholas Andrew Bergren**

Nicholas Bergren was born in Portland, Oregon on July 14, 1987 to Denise Pool and Andrew Bergren. Nicholas grew up in Clackamas, Oregon and later in Sunnyvale, Texas. He graduated from Dr. John D. Horn High School in 2005. Thinking he wanted to be a military pilot, Nicholas attended Embry-Riddle Aeronautical University in Daytona Beach, Florida for his freshmen year of college. After deciding a career in aviation wasn't for him, Nicholas transferred to LeTourneau University in Longview, Texas to pursue a degree in biology. Subsequently he graduated cum laude in 2010 with a Bachelor's of Science in Biology. During his time at LeTourneau Nicholas participated in an internship with the North Texas University Health Science Center's School of Public Health where he conducted research on West Nile virus evolution. Desiring a career in higher education with a focus on arbovirology, Nicholas enrolled in the GSBS at UTMB where he has pursued his Ph.D. in Experimental Pathology. Through his doctoral work at UTMB, he has developed the skills necessary to have a successful career in academia and an appreciation for the scientific endeavor. His dissertation has matured and expanded his views of infectious disease, specifically the importance of a changing ecological landscape and the impacts that has on arbovirus emergence.

PRESENT POSITION:

Graduate Assistant
The University of Texas Medical Branch
Department of Pathology
4.138 Keiller Building
301 University Boulevard
Galveston, Texas 77555-0609

BIOGRAPHICAL:

Date of Birth: July 14, 1987
Born: Portland, Oregon
Citizenship: United States of America
Home Address: 320 Ferry Road Apt 1206
Galveston TX 77550
Phone: (409) 747-2440
Cell: (469) 826-8140
Fax: (409) 747-2429
E-mail: nabergre@utmb.edu
nicholasbergren@gmail.com

EDUCATION:

Aug 2010—July 2016	*Ph.D. Experimental Pathology* Department of Pathology University of Texas Medical Branch Mentor: Scott Weaver, Ph.D. Admitted to Candidacy: May 2013 GPA: 3.767
Aug 2006—May 2010	*Bachelor of Science in Biology, Cum Laude* LeTourneau University Advisor: Dr. Andree Elliott GPA: 3.55

PROFESSIONAL WORK HISTORY:

Aug 2010—Present	*Graduate Assistant* Department of Pathology University of Texas Medical Branch
Aug 2009—May 2010	*Lead Lab Assistant, General Biology I and II* Department of Biology LeTourneau University
Aug 2008—May 2009	*Lab Assistant, Integrated Vertebrate Anatomy and Physiology I and II* Department of Biology LeTourneau University
Aug 2007—May 2009	*Lab Assistant, General Biology I and II* Department of Biology LeTourneau University

CERTIFICATIONS:

July 2015	*Arthropod Containment Level 3* Independent Access
Feb 2014	*Arthropod Containment Level 2* Independent Access
May 2012	*Animal Biosafety Level 3* Independent Access
Dec 2011	*Animal Biosafety Level 2* Independent Access
Dec 2011	*Biosafety Level 3* Independent Access
Aug 2011	*Department of Justice Clearance* Class B and C Select Agents

RESEARCH ACTIVITIES:

AREA OF RESEARCH:

Understanding and preventing the emergence of arboviral diseases in humans and domestic animals is of vital importance to human health and the economies of nations. My primary area of research interest is geared toward understanding environmental, pathogen, host and vector factors that play a role in the emergence, maintenance, virulence, and pathogenesis of arboviral diseases. These problems can be addressed in a number of various ways including field studies, systematic approaches, reverse genetic techniques, transmission studies, and pathological/immunological characterizations.

GRANT SUPPORT:

Current
FY2015-2016
2T32AI060549-11-National Institutes of Health
"Effect of evolution on western equine encephalitis evolution on enzootic hosts & human disease"
PI: Dr. Weaver: $32,400 + tuition and fees/year. Two years.

Past
FY2013-2014
5T32AI007526-13-National Institutes of Health
"Evolution of western equine encephalitis virus accounts for virulence reduction"
PI: Dr. Barrett; $32,400 + tuition and fees/year. Two years.

DOCTORAL RESEARCH:

Department of Pathology, The University of Texas Medical Branch
Mentor: Dr. Scott Weaver
Field of Study: Potential evolutionary influences on the reduction in enzootic circulation and human incidence of western equine encephalitis.

UNDERGRADUATE RESEARCH:

Department of Biology, LeTourneau University
Advisor: Dr. Andree Elliott
Field of Study: Applied Microbiology
Produce biodiesel product from genetically altered *E. coli.*

School of Public Health, University of North Texas Health Science Center
Advisor: Dr. Joon-Hak Lee
Field of Study: Viral Phylogenetics and Epidemiology
Isolate and sequence three genes that are attributed to virulence in West Nile virus.

Department of Biomedical Engineering, LeTourneau University
Advisor: Karen Rispin, M.S.
Field of Study: Computer Model Verification
Assist biomedical engineers in verifying the current computational model in order to develop a scientific basis for either ACL surgery or knee therapy.

TEACHING RESPONSIBILITIES

TEACHING RESPONSIBILITIES AT UTMB
Courses:
Graduate School of Biomedical Sciences

Spring 2015	Small Group Facilitator, Molecular Biology and Genetics

Students/Mentees:

PhD Students

Jan 2015—April 2015 Preceptor to Miguel Saldana
Jan 2012—April 2015 Preceptor to Josef Lopez

High School Students

June 2015—July 2015 Preceptor to Kevin Le
Aug 2013—June 2015 Bench Tutorials Mentor to Ellie Cherryhomes

TEACHING RESPONSIBILITIES AT LETOURNEAU UNIVERSITY
Courses:

School of Arts and Sciences

Aug 2007—Dec 2007 Tutorial Instructor, General Biology I

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

August 2013—Present	American Society for Virology
March 2012—Present	American Society of Tropical Medicine and Hygiene
Feb 2011—Present	American Society of Microbiology

HONORS AND AWARDS:

2014	*Robert Shope, Ph.D. Endowed Scholarship* University of Texas Medical Branch
2013	*Edith and Robert Zinn Presidential Scholarship* University of Texas Medical Branch
2013	*ACAV Kelly Labell Travel Award* American Society of Tropical Medicine and Hygiene
2010	*Outstanding Lab Assistant* LeTourneau University

ADDITIONAL INFORMATION

JOURNAL REVIEWER FOR:
Jan 2015 – Present Journal of Virology
Feb 2016 – Present Parasites & Vectors

PUBLICATIONS

ARTICLES IN PEER-REVIEWED JOURNALS:

1. Plante, K.; Rossi, S.L.; **Bergren, N.A.**; Seymour, R.L.; Weaver, S.C. (2015) Extended preclinical safety, efficacy and stability testing of a live-attenuated Chikungunya vaccine candidate. PLoS: Neglected Tropical Diseases. 9(9): e0004007. doi: 10.1371/journal.pntd.0004007.
2. Rossi, S.L.; Russell-Lodrigue, K.E.; Killeen, S.Z.; Wang, E.; Leal, G.; **Bergren, N.A.**; Vinet-Oliphant, H.; Weaver, S.C.; Roy, C.J. (2015) IRES-containing VEEV vaccine protects cynomolgus macaques from IE Venezuelan equine encephalitis virus aerosol challenge. PLoS: Neglected Tropical Diseases. 9(5): e0003797. doi: 10.137/journal.pntd.003797.
3. August, A.J.; Lemey, P., **Bergren, N.A.**; Giambalvo, D.; Moncada, M.; Moron, D.; Hernandez, R.; Navarro, J.; Weaver, S.C. (2015) Enzootic transmission of Yellow fever virus, Venezuela. Emerging Infectious Diseases. 21(6): 94-97.
4. **Bergren, N.A.**; Auguste, A.J.; Forrester, N.L.; Negi, S.S.; Braun, W.A.; Weaver, S.C. (2014) Western equine encephalitis virus: evolutionary analysis of a declining alphavirus based on complete genome sequences. Journal of Virology. 88(16).
5. Seymour, R.L.; Rossi, S.L.; **Bergren, N.A.**; Weaver, S.C. (2012) The role of innate versus adaptive immune responses in a mouse model of o'nyong-nyong virus infection. American Journal of Tropical Medicine and Hygiene. 88(6): 1170-1179.

CONFERENCE PRESENTATIONS

POSTER PRESENTATIONS

1. **Bergren, N.A.;** Auguste, A.J.; Forrester, N.L.; Negi, S.S.; Braun, W.A. Weaver, S.C. (2015) Potential evolutionary influences on the reduction in enzootic circulation and human incidence of Western equine encephalitis. Poster Presentation at the 2015 McLaughlin Colloquium on Infection and Immunity, Galveston, TX, April 2015.
2. **Bergren, N.A.;** Auguste, A.J.; Forrester, N.L.; Negi, S.S.; Braun, W.A. Weaver, S.C. (2014) Western equine encephalitis virus: evolutionary analysis of a declining alphavirus based on complete genome sequences. Poster Presentation at the 2014 McLaughlin Colloquium on Infection and Immunity, Galveston, TX, April 2014.
3. **Bergren, N.A.;** Weaver, S.C. (2013) Mutations present in western equine encephalitis virus possibly account for reduction in virulence. Poster Presentation at the 62nd annual meeting of the American Society of Tropical Medicine and Hygiene, Washington DC, November 2013.
4. **Bergren, N.A.;** Weaver, S.C. (2013) Mutations present in western equine encephalitis virus possibly account for reduction in virulence. Poster Presentation at the 18th International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology, Gainesville, FL, August 2013.

5. **Bergren, N.A.**; Gorchakov, R.V.; Rossi, S.L.; Plante, K.S.; Weaver, S.C. (2012) Development for an IRES-based Vaccine for Western Equine Encephalitis Virus. Poster Presentation at the 61st annual meeting of the American Society of Tropical Medicine and Hygiene, Atlanta, GA, November 2012.
6. Seymour, R.L.; Rossi, S.L.; **Bergren, N.A.**; Weaver, S.C. (2012) The role of innate versus adaptive immune responses in a mouse model of o'nyong-nyong virus infection. Poster Presentation at the 61st annual meeting of the American Society of Tropical Medicine and Hygiene, November 2012.
7. Rossi, S.L.; Guerbios, M.; Gorchakov R.; Plante, K.; **Bergren, N.A.**; Forrester, N.A.; Weaver, S.C. Venezuelan Equine Encephalitis Virus Subtypes ID and IE Containing IRES Sequences are Potential Vaccine Candidates. Poster Presentation at the 61st annual meeting of the American Society of Tropical Medicine and Hygiene, November 2012.
8. **Bergren, N.A.**; Gorchakov, R.V.; Rossi, S.L.; Plante, K.S.; Weaver, S.C. (2012) Development for an IRES-based Vaccine for Western Equine Encephalitis Virus. Poster Presentation at the Western Regional Center for Excellence Annual Meeting, Dallas, TX, October 2012.
9. **Bergren, N.A.**, Finch, K.E., Lee, J.H. (2009) Genetic analysis of West Nile Virus from North Central Texas. 2009 Annual Society for Advancing Hispanics/Chicanos & Native Americans in Science Conf, Dallas, TX, Nov, 2009.
10. **Bergren, N.A.**, Terrell, K.R., Rispin, K.L. and Gonzalez, R.V. (2009) Using orthopedic suturing techniques for muscles attachment in load application apparatus for cadaveric knee. 26th Annual Houston Conference on Biomedical Engineering Research, Houston, TX, Mar, 2009.

Permanent address: Current: 320 Ferry Road Apt 1206, Galveston TX 77550

As of Aug 9: 2960 W. Stuart #D106, Fort Collins CO 80526

This dissertation was typed by Nicholas Bergren.