DISSERTATION

INVESTIGATING THE POTENTIAL ROLE OF WILDLIFE AS RESERVOIRS OF CHIKUNGUNYA VIRUS:

EXPERIMENTAL INFECTIONS AND FIELD STUDIES

Submitted by

Emily Lauren Blizzard

Department of Microbiology, Immunology, and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2017

Doctoral Committee:

Advisor: Richard A. Bowen

Kristy Pabilonia
Tony Schountz
Matthew Johnston

ABSTRACT

INVESTIGATING THE POTENTIAL ROLE OF WILDLIFE AS RESERVOIRS

OF CHIKUNGUNYA VIRUS:

EXPERIMENTAL INFECTIONS AND FIELD STUDIES

Chikungunya virus (CHIKV) is a mosquito-borne human pathogen endemic to Africa and Asia and the etiologic agent of chikungunya fever (CHIKF), a severe debilitating and often chronic arthralgic disease. The recent introduction of CHIKV into the Western Hemisphere has led to an increased initiative to investigate the role that mammals other than non-human primates might play during CHIKV sylvatic transmission cycles. The focus of the studies presented in this dissertation was to investigate the potential of several common rodent species to serve as reservoir and/or amplifying hosts for CHIKV during outbreaks in the Americas.

Nine rodent species were subcutaneously inoculated with one of two strains of chikungunya virus during initial experimental infections. Of these, 7 out of 9 species became infected with CHIKV. Groundhogs (*Marmota monax)* were the mostly likely candidate to serve as a reservoir host in North America based on magnitude of viremia. All groundhogs included in this study developed significant viremias ranging from 4.0E2 to 1.6E6 and lasting 2-4 days post infection. The viral loads observed were sufficient to infect *Ae.* spp mosquitoes indicating that these animals are capable of serving as reservoir hosts. Additionally, groundhogs undergo periods of hibernation and further research is need to determine if these animals are capable of overwintering CHIKV in the U.S.

Based on the findings from the CHIKV experimental infections, Cotton rats (*Sigmodon hispidus*), were evaluated as a laboratory animal model to study CHIKV pathogenesis. Thirteen of seventeen cotton rats developed a low-titer viremia, but no clinical or post-mortem pathological findings were observed. Mosquitoes fed on viremic cotton rats failed to become infected, suggesting that this species is not likely to play a role in CHIKV transmission cycles.

Field studies investigating the likelihood of three mammals and eight reptilian and amphibian species were conducted in select regions of Cambodia and the Grand Cayman islands. Individuals from nine of eleven species examined had low levels of detectable CHIKV antibodies, suggesting that they may have been infected with CHIKV. The results of these studies provide some insight into the potential role of wildlife, mainly rodents, in CHIKV transmission cycles in the Americas.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Richard Bowen for providing me the opportunity to work in his laboratory and for his patience as a mentor and advisor. Secondly, I would like to thank Dr. Tom Deliberto, Dr. Dale Nolte, and Dr. Thomas Gidlewski from the USDA for facilitating this project and providing support and guidance. I would also like to thank my graduate committee members – Dr. Kristy Pabilonia, Dr. Tony Schountz, and Dr. Matthew Johnston for their assistance and expertise over the past few years. Numerous people have come in and out of the Bowen lab during my career, and I would like to thank all of them, especially Airn Tolnay Hartwig, for their assistance, guidance, and support. A big thank you to Paul Gordy for his help with constructing animal housing, providing technical assistance, and for always listening.

Lastly, I would like to thank my family and friends for their endless love, support, encouragement, and guidance during my graduate career. Most importantly I would like to thank my fiancé (Tanya Miller) and my dog children for sticking with me throughout this very long journey and helping me to stay grounded. We made it!!!

TABLE OF CONTENTS

Abstract ii

Acknowledgments iv

List of Tables v

List of Figures ix

Chapter 1: Introduction and Literature Review 1

1.1: Introduction 1

1.2: Alphaviruses: Background 2

1.2.1: Alphavirus Genome Structure 2

1.2.2: Alphavirus Replication Cycle 4

1.2.3: Non-structural Proteins 5

1.2.4: Structural Proteins 6

1.3: Epidemiology of Chikungunya Virus Infections 8

1.4: Chikungunya Virus Genotypes 10

1.5: Chikungunya Virus Vectors and Transmission Cycles 11

1.6: Mosquito Vector-Virus Biology 13

1.7: Clinical Disease and Pathogenesis of Chikungunya Fever 14

1.8: Immune Responses to Chikungunya Virus Infection 16

1.8.1: Innate Immune Response to Chikungunya Virus Infection 17

1.8.2: Adaptive Immune Response to Chikungunya Virus Infection 20

1.9: Animal Models of Chikungunya Virus (CHIKV) 23

1.9.1:Non-human Primate Models 23

1.9.2: Rodent Models 25

1.10: Evidence for Primates and Rodents As Reservoir Hosts 26

1.11: Rationale for the Current Study 28

References 30

Chapter 2: Experimental Infection of North American Rodents with Chikungunya Virus 40

2.1: Introduction 40

2.2: Materials and Methods 41

2.2.1: Animal Collection and Husbandry 41

2.2.2: Cell Culture 44

2.2.3: Virus Strains 44

2.2.4: Experimental Infection and Sample Collection 44

2.2.5: Necropsy and Tissue Processing 45

2.2.6: Virus Isolation 45

2.2.7: Serology 46

2.3: Results 46

2.3.1: Clinical Responses 46

2.3.2 Viremia, Tissue Virus Burdens, and Serologic Responses 46

2.4: Discussion 51

References 58

Chapter 3: Experimental Infection of Cotton Rats With Chikungunya Virus 62

3.1: Introduction 62

3.2: Materials and Methods 64

3.2.1: Cotton Rats 64

3.2.2: Mosquitoes 64

3.2.3: Viruses 64

3.3: Experimental Design 65

3.3.1: Cotton Rat to Mosquito Transmission 65

3.3.2: Virus Isolation 66

3.3.3: Serology 66

3.3.4: Immunohistochemistry 67

3.4: Results 68

3.4.1: Responses of Cotton Rats to Infection with Chikungunya Virus 68

3.4.2: Tissue Virus Burdens and Immunohistochemistry 72

3.4.3: Transmission of Virus to Mosquitoes 72

3.5: Discussion 73

References 77

Chapter 4: Chikungunya Antibodies Detected In Wildlife Species In Endemic And Epidemic Chikungunya Regions 81

4.1: Introduction 81

4.2: Materials and Methods 82

4.2.1: Animals 82

4.2.2: Viruses 83

4.2.3: Serology 83

4.3: Results 84

4.3.1: Cambodia 85

4.3.2: Grand Caymans 86

4.4: Discussion 87

Reference 90

Chapter 5: Conclusions 102

LIST OF TABLES

Table 2.1: study..... 48

Table 2.2: study..... 49

Table 2.3: study..... 54

Table 2.4: study..... 55

Table 2.5: study..... 56

Table 3.1: study..... 80

Table 3.2: study..... 81

Table 3.3: study..... 82

Table 4.1: study..... 96

LIST OF FIGURES

Figure 1.1: CHIKV genome map 3

Figure 1.2: Organization of CHIKV genome and replication products 8

Figure 1.3: Alphavirus replication cycle 9

Figure 1.4: Representation of immune response against CHIKV infection 26

Figure 2.1: Groundhog body temperatures 57

Figure 3.1: Cotton rat body temperatures 78

Figure 3.2: Cotton rat left hock measurements 78

Figure 3.3: Cotton rat right hock measurements 79

Figure 3.4: Cotton rat weights 79

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Understanding the ecology of emerging and re-emerging arboviruses is critical in predicting, preventing, and mitigating a large number of human and veterinary diseases. One such emerging mosquito-borne disease is chikungunya virus (CHIKV). CHIKV is the etiologic agent of chikungunya fever (CHIKF), a severe debilitating and often chronic arthralgic disease. This virus was originally isolated in Africa in 1952 (Robinson, 1955) and has long been recognized in both Africa and Asia. The largest CHIKV documented epidemic began in May of 2004 along coastal Kenya and Lamu Island and spread from Africa to the Indian Ocean islands, into India, and parts of Europe (Sergon et al., 2007). In India, more than 1.5 million people became infected and CHIKV subsequently spread to parts of Europe through viremic travelers (Sergon, 2007; Njenga et al., 2008). In 2013, an autochthonous transmission cycle involving CHIKV and *Aedes* sp. mosquitoes was established on the island of St. Martin and quickly spread throughout the Caribbean and Americas. Since its introduction, CHIKV has been documented in 45 countries throughout the Americas and has infected more than 1.8 million people (Pan American Health Organization, August 2016). The emergence of CHIKV in the New World highlights the global health threat from CHIKV and other emerging alphaviruses. Despite these large outbreaks, numerous questions remain about the natural history of this pathogen. For instance, the sylvatic cycle of CHIKV and the role of non-primate vertebrates during transmission cycles remain largely unknown. Serological evidence suggests that forest-dwelling primates and humans are the only reservoirs for CHIKV (Powers and Logue, 2007; Diallo et al., 1999; Jupp et al., 1990). However, experimental infections suggest that various rodent species may potentially serve as competent CHIKV hosts (McIntosh 1961; Bosco-Lauth et al., 2015). Chapter two of this dissertation describes studies that investigated the potential role of wildlife during CHIKV epidemics by experimentally infecting rodents from eight species that are either native or invasive within North America. In chapter three, I describe investigation of the pathogenesis of CHIKV

infection in a cotton rat model (*Sigmodon hispidus*), and in chapter four I report on a sero-surveillance project with various wildlife species in Cambodia and the Grand Cayman islands designed to determine whether CHIKV circulated at the human-wildlife interface in endemic and recent epidemic regions. The final chapter is a summary of my findings and suggestions on future research.

1.2 Alphaviruses: Background

The *Alphavirus* genus, family *Togaviridae*, are spherical enveloped viruses transmitted by arthropod vectors. Viruses within this genus are globally distributed and are composed of many geographic variants and strains (Kuhn, 2007; Strauss and Strauss, 1994). Typically, all alphaviruses are maintained in sylvatic transmission cycles involving specific mosquito vectors and susceptible wildlife hosts, independent of humans. Occasional spillover events from sylvatic cycles to domestic animals and humans often cause intermittent emerging and re-emerging epidemics. Alphaviruses are classified as Old or New World viruses based on the location of their discovery and occurrence (Calisher et al., 1988; Schwartz and Albert, 2010). New World alphaviruses, found in the Americas, are primarily associated with potentially fatal encephalitic disease. These encephalitic alphaviruses include Venezuelan, Eastern, and Western equine encephalitis viruses, each of which can cause debilitating febrile disease and severe encephalitis in equids and humans. Old World alphaviruses are endemic to Africa, Europe, and Australia, and are generally associated with arthritic or rheumatic disease in humans. Arthritogenic alphaviruses include Ross River, Barmah Forest, O'nyong-nyong, chikungunya, and Sindbis group viruses. Disease manifestations are usually dominated by chronic and debilitating polyarthralgia and/or polyarthritis.

1.2.1 Alphavirus Genome Structure

Alphaviruses are enveloped viruses with a positive-sense, single-stranded RNA genome consisting of two open reading frames (ORFs) flanked by two non-coding regions containing structures important in RNA replication (**Figure 1.1**). The ORF located at the 5' end of the genome encodes four nonstructural proteins: nsP1, nsP2, nsP3, and nsP4. These nonstructural proteins are essential for viral replication and processing within the host cell. The second ORF is located at the 3' end of the genome

and encodes five structural proteins: capsid, E1 and E2 envelope glycoproteins, and E3 and 6K proteins. These structural proteins are translated from a 26S subgenomic RNA.

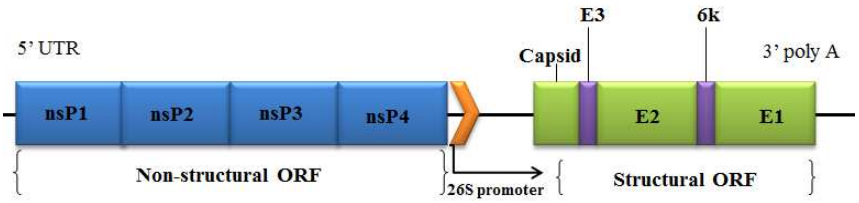

Figure 1.1. CHIKV genome map. Adapted from Weaver et al., 2012.

Mature alphavirus virions are spherical with a diameter of 60 – 70 nm. The genomes range approximately 10 – 12 kb in length with a 5' methyl-guanosine cap and a polyadenylated tail, and is encapsulated by the capsid protein to form the nucelocapsid. The E1 and E2 glycoproteins form heterodimers, on the surface of the cell membrane, and upon budding out from the cell, these heterodimers form the spikes on the surface of the virion (Zhang et al., 2002).

1.2.2 Alphavirus Replication Cycle

The replication cycle of alphaviruses has been reviewed recently (Leung, 2011; Weaver et al., 2015). Entry of alphaviruses into susceptible host cells is mediated by pH-dependent receptor mediated endocytosis into clathrin-coated vesicles (**Figure 1.3**) (Sourisseau et al., 2007). During the translocation of the viral genome from the clathrin-coated vesicles to the low pH endosome, a conformational change occurs in the E1-E2 heterodimer that exposes the E1 fusion domain (Wahlberg et al., 1989). Exposure of the E1 fusion domain and subsequent binding with the endosomal membrane leads to release of the 49S RNA genome (encapsulated in nucleocapsid) into the cytoplasm (Powers et al., 2001). The 49S RNA genome is then translated to yield non-structural proteins that aid in viral replication. In some alphaviruses two polyproteins P123 and P1234 are produced (**Figure 1.2**). The resulting polyprotein is then cleaved by viral proteases contained in the nsP2, resulting in the creating of nsP1, nsP2, nsP3, and nsP4. RNA replication then proceeds through the synthesis of minus –strand RNA, which is used as a template for the synthesis of positive-strand RNA and the 26S subgenomic plus-strand RNA. The plus

stranded RNA is produced at a constant rate throughout the replication cycle, although the minus strand is undetectable during late stage infections (Chevillon et al., 2008). Throughout this process host-cell transcription is shut down by the nsP2 protein, which also inhibits host cell production of IFNα/β (Fros et al., 2010).

Synthesis of the 26S mRNAs is initiated near the 5' end and continues to the end of the approximately 3,700 nucleotide open reading frame. During the translation of the 26S ORF, five structural proteins are generated including C, E3, E2, 6K, and E1. The C protein is the first to be translated and autocatalitically cleaved from the nascent precursor polyprotein. Through this translation a signal sequence is created and is bound by a cellular signal recognition particle (SRP). This momentarily blocks the translation of the 26S mRNA. The SRP also targets the ribosome bound nascent polyprotein to the rough endoplasmic reticulum where the ribosome binds to a protein translocation complex, triggering the SRP to be released and the signal sequence to be inserted into the translocation complex. The precursor for the E2 and E3 proteins, p62, has now been created and translation can begin again. The p62 protein passes into the lumen of the endoplasmic reticulum through the translocation complex. After the completion of p62 translation, the protein is cleaved from the nascent polypeptide by a signal peptidase. This exposes the nascent polypeptide 6K, which is ribosome-bound. The 6K protein serves as the signal sequence for the E1 protein. The E1 protein is translated as it passes through the translocation complex until it meets with a stop-transfer sequence and leaves two amino acid extensions protruding out into the cytoplasm. Next, the 6K protein is cleaved from E1by the signal peptidase. Once the p62 and E1 proteins are inserted into the endoplasmic reticulum membrane, heterodimers are formed and transported to the Golgi body. Later, during virion maturation, these heterodimers will be incorporated into the virus envelope. During transportation of the p62, E1 heterodimers to the plasma membrane, p62 is cleaved via a furin-like protease and produces E2 and E3 proteins. Depending on the particular virus the E3 protein may remain outside the plasma membrane or may be incorporated along with the E2/E1 proteins onto the virion heterodimers. Concurrently with the formation of the envelope proteins, the synthesized 49S

genomic RNAs are encapsulated within capsids of C protein. Finally, the assemblage of a mature virion begins at the plasma membrane where the envelope proteins interact with the RNA containing capsids. Interactions between the E2 protein and the C termini causes the plasma membrane to engulf the RNA containing capsids and fully enveloped virions bud off from the cell.

1.2.3 Non-structural proteins

nsP1 – Is required for the synthesis of the (-) RNA strands and acts as a meythltransferase and guanyltransferase in the formation of the 5' methyl-guanosine cap (Hahn et al., 1989; Sawicki et al., 1981; Wang et al., 1991; Durbin and Stollar, 1985; Mi and Stollar, 1990; Mi and Stollar 1991). NsP1 also regulates the activity of the nsP2 protease as demonstrated by de Groot et al. when in the presence of nsP1, Sinbus virus reduced the cleavage of nsP2 and nsP3 by regulating the nsP2 protein (De Groot et al., 1990).

nsP2 – The nsP2 protein is the largest non-structural protein and performs multiple enzymatic activities including acting a helicase, a triphosphatase, and a protease (Rupp et al., 2015; Breakwell et al., 2007). Additionally nsP2 suppresses type 1 interferon responses in infected cells (Breakwell et al., 2007). RNA-RNA duplexes are unwound during replication and transcription by the nsP2 N-terminal domain and RNA 5'-triphosphatase which function as a RNA helicase (Russo et al., 2006; Vasiljeva et al., 2000; Gomez de Cedron et al., 1999). The C-terminal of nsP2 protein is involved in the proteolytic processing of the CHIKV nonstructural polyproteins. It is also involved in the regulation of 26S subgenomic RNA synthesis, downregulating (-) RNA synthesis during late stage CHIKV infections, RNA 5'-triphosphatase activity, and directing nsP2 for nuclear transport (Suopanki et al., 1998; Sawicki et al., 2006; Sawicki and Saeicki, 1993; Peranen et al., 1990; Vasiljeva et al., 2000).

nsP3 – The role of nsP3 protein in CHIKV replication is not well understood. It is thought to play a role in 26S subgenomic and (-) RNA synthesis and affect the cleavage of the nsP2 proteinase (Hahn et al., 1989; LaStarza et al., 1994; Lemm and Rice, 1993; Shirako and Strauss, 1994; Wang et al., 1994).

nsP4 – The nsP4 protein serves as a RNA polymerase and is associated with the viral replicative complex (Strauss and Strauss, 1994).

1.2.4 Structural Proteins

Capsid – The capsid protein forms a nucelocapsid core structure containing the genomic RNA beneath the viral membrane. It acts as a viral autoprotease to recognize and assemble the genomic RNA into an ordered protein shell (Warrier et al., 2008). The capsid protein also plays a key role in virion formation and budding. It contains a hydrophobic pocket that interacts with the cytoplasmic tail of the E2 protein (Skoging et al., 1996). Additionally, in 2007 the capsid protein of New World alphaviruses was found to regulate host cell antiviral mechanisms (Aguilar et al., 2007; Garmashova et al., 2007; Garmashova et al., 2006).

6K – The 6k protein is ~58-61 amino acids in length and is incorporated into small amounts into the mature virions. The function of this protein remains unclear although it is thought that it may help control lipid bilayer deformability by regulating interactions between the lipids that interact with the transmembrane domains of the E1-E2 heterodimers (Cadd et al., 1997).

E2 – The E2 protein is a type I transmembrane protein that consists of an N-terminal hydrophilic region, a membrane-spanning region, and a cytoplasmic endodomain. Its' main function is to interact with host cell specific surface receptors which aid in virion endocytosis.

E1 – The E1 protein is responsible for triggering viral fusion and host cell receptors during viral entry. This protein is composed of three globular domains: DI, DII, and DIII. The DI domain is an eight strained β sheet (Kielian and Rey 20006). The DII domain is composed of "finger-like" extensions with a fusion loop at the tip of the molecule. Mutations in these fusion peptides influence the entry and exit of alphaviruses into susceptible cells (Chatterjee et al., 2000; Lu et al., 1999; Vashishtha et al., 1998). The DIII domain is connected to the C terminus of the DI domain by a ~ twelve amino acid polyprotein (Kielian and Rey, 2006). The E1 protein region interacts with the membrane region of the E2 protein and plays a vital role in virus fusion and assembly (Sjoberg and Garoff, 2003).

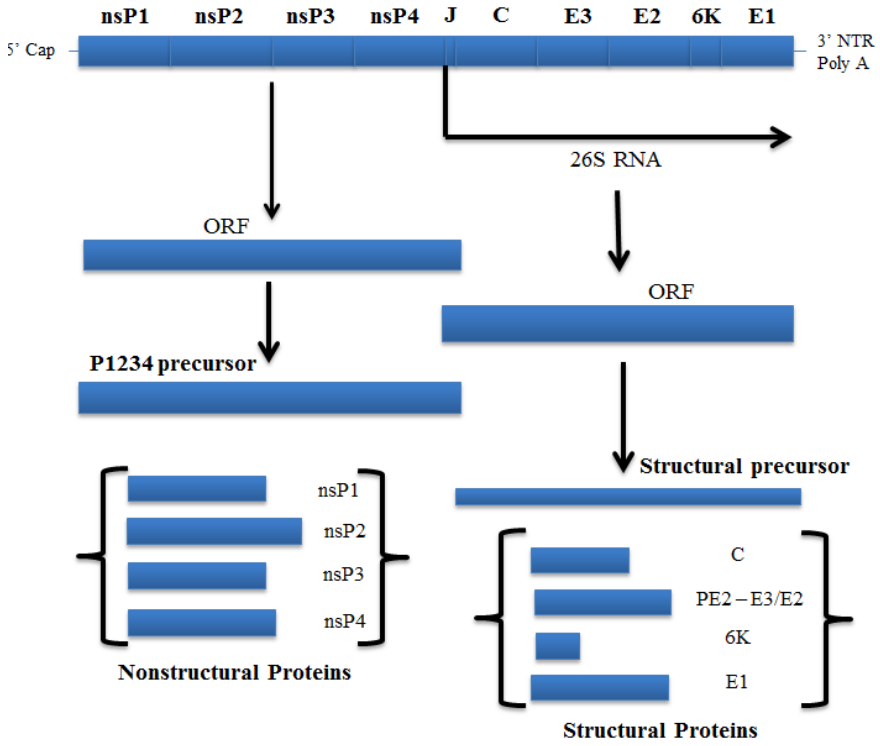

Figure 1.2. Organization of CHIKV genome and replication products. Adapted from Thiberville et al., 2013.

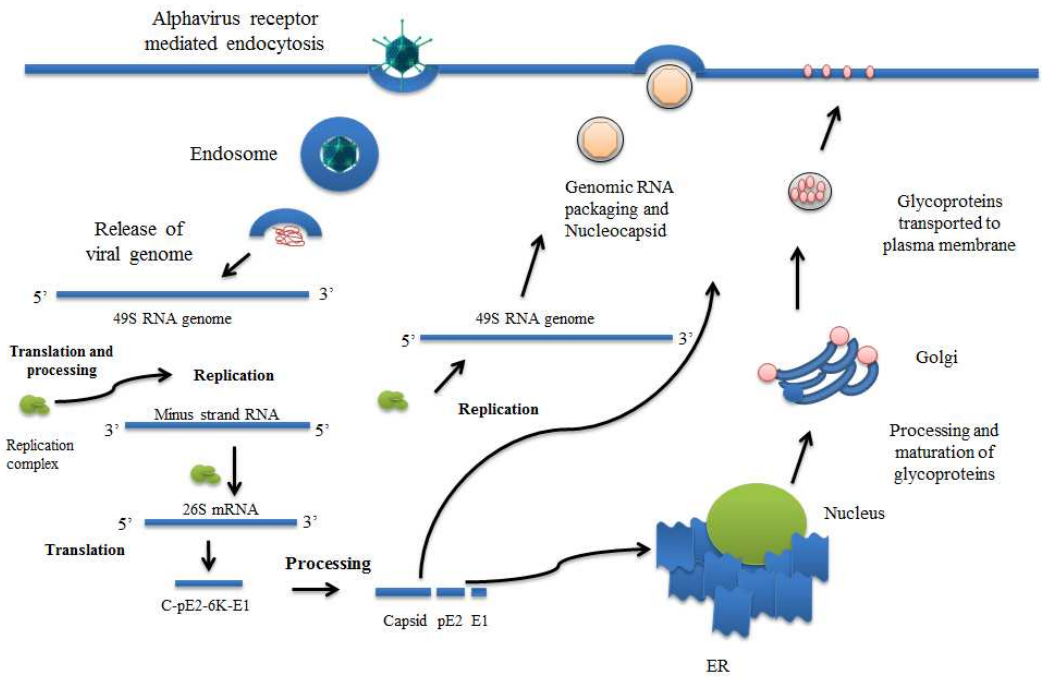

Figure 1.3. Alphavirus replication cycle. Adapted from Thiberville et al., 2013.

1.3 Epidemiology of Chikungunya Virus Infections

The first recorded occurrence of CHIKF was speculated to have occurred as far back as 1779 during an epidemic in Batavia, Dutch East Indies (present day Jakarta, Indonesia), where the outbreak was mistaken as an outbreak of dengue fever (Carey, 1971). A Dutch physician, Dr. David Bylon, recorded a *Knokkel-koorts* (a Dutch word meaning knuckle or joint) febrile epidemic characterized by acute fever, rash, joint pain, and chronic arthralgia. Similar outbreaks followed in 1823 and 1870 in Zanzibar; India in 1824-25, 1871, 1902, 1923, and 1963-64; 1827-28 in the southeastern U.S. and the Caribbean (Carey, 1971). During the 1828 epidemic in New Orleans, physicians described 99% of the population as having severe and persistent arthralgia weeks after recovering from an acute disease (Carey, 1971). Since yellow fever and dengue viruses were known to have been disseminated aboard sailing ships during this period, and have similar transmission cycles, it is conceivable that CHIKV may have similarly been introduced into the New World (Weaver and Lecuit, 2015).

Retrospective analysis of physicians' records indicate that outbreaks of CHIKF have frequently been misdiagnosed as dengue fever over the centuries. The first isolation of CHIKV from patient serum and characterized case of CHIKF was documented in Tanganyika (Tanzania), East Africa during a dengue-like outbreak in 1952-53. The virus and associated disease were called "chikungunya" meaning "that which contorts or bend up" by the local Makonde people and refers to the stooped posture of infected patients experiencing severe joint pain (Robinson, 1955). One of the largest outbreaks of CHIKF occurred in India during 1963, where approximately 400,000 human infections were documented in Chennai alone (Arankalle et al., 2007). Although there were no additional epidemics documented for 30 years, CHIKV re-emerged widely from 2004 onwards. In late 2004, the Lamu province and city of Mombasa, Kenya, experienced the beginning of one of the largest documented CHIKF epidemics ever recorded. This epidemic quickly spread in 2005 to the neighboring Comoros islands, Seychelles, Mauritius, Madagascar, and La Reunion Island (Chretien et al., 2007; Njenga et al., 2008). Prevalence rates of CHIKF were estimated to be 75% in Kenya (Lamu), 63% in the Comoros, and 26% in Mayotte (2006) (Pialoux, 2007). On Reunion Island, approximately 87,780 people out of a population of 266,000 were diagnosed with CHIKF and 254 people died (Powers et al., 2007). Subsequent epidemics arose in many areas around the Indian Ocean including India, where millions of people were infected from 2005-2007. Each year hundreds of travelers return to the Americas from CHIKV-endemic regions infected with CHIKV and have the potential of introducing the virus into a naïve geographic region. In December of 2013, CHIKV was isolated for the first time in the Western hemisphere on the island of Saint Martin (Fischer et al., 2014). The CHIKV epidemic in the New World spread rapidly throughout the Americas and as of May 11, 2016 more than 1.7 million cases had been documented in 45 countries throughout Central, South, and North America, and the Caribbean. To date, CHIKV had been documented in 25 countries in Africa, 20 countries in Asia, 45 countries in the Americas, 2 countries in Europe, and 10 countries in Oceania and the Pacific islands (Pan America Health Organization, August 2016).

1.4 Chikungunya Virus Genotypes

Phylogenetic analysis of CHIKV strains isolated between the 1950s and 1990s led to the recognition of three distinct phylogroups: (i) East/Central/South African (ECSA), (ii) West African, and (iii) Asian genotypes (Powers et al., 2000; Pastorino et al., 2004; Schuffenecker et al., 2006; Volk et al., 2010). Based on analysis of the E1 protein or full genome sequences, these three phylogroups are between 95.2 to 99.8% conserved, despite genomic variations linked to geographical origin and evolutionary rates (Chevillon et al., 2008; Schuffenecker et al., 2006). Analysis of CHIKV strains emerging throughout the Indian Ocean region since 2000 revealed limited sequence variation from the ESCA strains isolated in the 1950s (Schuffenecker et al., 2006; Yergolkar et al., 2006). Additionally, the West African phylogroup appears to be ancestral to the ECSA cluster. the ECSA cluster appears to have diverged from West African ancestors and the Asian and Indian Ocean genotypes have evolved more recently and independently from the ECSA variants (Powers et al., 2000; Schuffenecker et al., 2006). Therefore, CHIKV conceivably emerged in West Africa, proceeded to colonize other geographical regions of Africa, and then emerging again from these separate African regions after acquiring phenotypic mutations and spread to Asia and into the Indian Ocean regions.

At a viral genetic level, CHIKV, like most alphaviruses, involves a host-dependent balance between regulating mutation rates and adapting to evolutionary constraints. In the course of each replication cycle CHIKV has an average mutation frequency of $\sim 10^{-4}$ and for each new CHIKV virus replicated, the resulting progeny will possess at least one mutation (Coffey et al., 2014). These mutations may result in the formation of geographic variants, which may or may not enhance virus fidelity, fitness, or adaptability. Although most mutants are deleterious and removed by negative selection, some result in positive adaptive advantages that lead to increased expression in some populations of vectors or hosts. An important advantage for having multiple mutation events during viral replication is that it allows the viral population to quickly adapt when negative or restrictive pressures are placed upon certain genotypes, viral vectors, and/or hosts.

1.5 Chikungunya Virus Vectors and Transmission Cycles

CHIKV is mosquito-borne and transmitted in forested and urban areas by *Aedes* species mosquitoes. The capacity of such mosquitoes to serve as a vector of CHIKV is influenced by several external and internal factors including ambient temperature, abundance and availability of susceptible hosts, vector population densities, predation, vector survival, efficiency of viral replication, and pathogen genotype (Zouache et al., 2014; Coffey et al., 2014). The availability of vector and/or host species may support variations among genotypes and may explain the utilization of two distinct transmission cycles – the sylvatic and urban cycle.

Chikungunya virus is endemic to sub-Saharan Africa, Southeast Asia, and India. As a zoonotic virus, CHIKV, has historically been maintained in sylvatic cycles with occasional spillover events leading to local epidemics of human disease. In Africa, CHIKV circulates in an enzootic cycle between forest-dwelling *Aedes spp.* mosquitoes (*Aedes furcifer, Aedes taylori, Aedes africanus, Aedes luteocephalus*) and is maintained in non-human primates and other vertebrate reservoirs (rodents and bat spp.) (Diallo et al. 2012; Jupp and McIntosh, 1990; Diallo et al., 1999). In Senegal, enzootic strains of CHIKV have been isolated from diverse species of mosquito including*: Aedes (Diceromyia) furcifer, Ae. (Diceromyia) taylori, Ae. (Stegomyia) luteocephalus, Ae. (Stegomyia) africanus* and *Ae. (Stegomyia) neoafricanus* (Diallo et al., 1999). Sporadic spillover of enzootic CHIKV into urban inter-human transmission cycles is amplified by the involvement of anthropophilic mosquito species such as: *Ae. (Stegomyia) aegepti* and *Ae. (Stegomyia) albopictus* (Coffey et al., 2014). *Ae. aegypti*, the yellow fever mosquito, is endemic to Africa and was first described in 1862 (Christophers, 1960). The behavior and ecology of *Ae. aegypti* make it an ideal vector during epidemic cycles due to its anthropophilic nature. Moreover, adult females often take several blood meals during a single gonotrophic cycle and artificial containers are preferred larval sites (Gubler, 2002). *Ae. aegypti* has two distinct phenotypes in Africa: a dark sylvatic form found in forested habitats (*Ae. aegypti formosus*) and a pale, domestic form that is widespread in urban environments (*Ae. aegypti aegypti*) (McClelland 1974; Tabachnick et al., 1979; Failloux et al., 2002). It

is believed that negative selective pressures forced the forest-dwelling *Ae. aegypti formosus* to adapt to breeding in artificial water storage containers in urban environments and led to the emergence of the *Ae. aegypti aegypti* phenotype (Powell et al., 2013). These domesticated *Ae. aegypti aegypti* mosquitoes then colonized most of the tropical and subtropical regions of the globe, exploiting the slave trade from the 15th to 19th centuries into the New World, Asia in the 18th and 19th centuries, and World War II troop movements to the Pacific islands (Coffey et al., 2014).

In Asia, CHIKV is maintained in an urban transmission cycle vectored by the mosquito *Ae. aegypti* and *Aedes albopictus.* (Tsetsarkin et al., 2007). *Ae. albopictus*, the Asian tiger mosquito, was discovered in 1894 in India and is endemic to Southeast Asia (Skuse, 1894) . *Ae. albopictus* mosquitoes have successfully colonized all five continents throughout both temperate and tropical regions (Lounibos, 2002). *Ae. albopictus* mosquitoes are both zoophilic and anthropophilic, and are active throughout the day. Although these mosquitoes do not have a particular ecological niche, distinct temperate and tropical populations have arisen. The temperate populations were originally introduced to Houston, Texas from Japan and then from the U.S. to 20 European countries (Medlock et al., 2012; Hawley et al., 1987). The Asian tiger mosquito has successfully spread throughout temperate zones mainly due to its ability to thrive in arid and cold conditions, undergo periods of adult diapause, and overwinter by laying desiccation-resistant eggs. Tropical populations serve as vectors for over 26 arboviruses (Paupy et al., 2010). Climate change will likely significantly contribute to changes in distribution and abundance of these vector populations and consequently to changes in pathogen transmission rates. In areas affected by climate change, increasing temperatures may allow vector populations to survive at higher latitudes and altitudes, alter vector seasonality, and increase population sizes. Prior to the 2004 CHIKV epidemic on La Reunion island, *Ae. albopictus* was not a primary vector for CHIKV transmission (Tsetsarkin et al., 2009; Carey et al., 1969) and *Ae. aegypti* mosquitoes were uncommon on the island during this epidemic. However, a mutation at position 226 in the chikungunya E1 glycoprotein from an alanine to a valine resulted in enhanced infectivity of the more prevalent *A. albopictus* population during subsequent

transmission cycles. This A226V mutation greatly enhanced the efficiency of dissemination of CHIKV from the midgut to the secondary organs of these mosquitoes (Tsetsarkin et al., 2007; Vazeille et al., 2007; Coffey et al., 2014). The A226V mutation appears to be limited to ECSA strains of CHIKV, and the negative epistatic interactions between the E1 glycoprotein and an E1-98 theronine found in Asian strains seems to hinder similar mutations in Asian strains (Tsetsarkin et al., 2011). Spontaneous duplications and accumulations of mutations in the 3'-UTR of the CHIKV genome are responsible for variations in viral fitness within arbovirus vectors and evolutionary limitations among CHIKV viral strains (Tsetsarkin et al., 2014).

1.6 Mosquito Vector-Virus Biology

Alphaviruses, including CHIKV, multiply rapidly in a variety of cell cultures including both mosquito cell lines (C6/36, Ae, A20) and mammalian cell lines (Vero, BHK21, HEK-213T, MRC5, BGM, HeLa) (Thiberville et al., 2013; Strauss and Strauss, 1994). Generally these viruses persist in infected insect cells; however, in mammalian cells they cause acute cytocidal effects infections. Common cytocidal effects observed in permissive cells include rounding of infected cells, fusion of adjacent cells to form polykaryocytes, and the presence of cytoplasmic inclusions. The replication of alphaviruses in mammalian cells causes apoptosis. Alphaviruses replicate to peak titers in mosquito cells within the first 48 hours of infection, followed by a decline in virus production.

Most arthropod-borne viruses cause little or no pathology in their invertebrate vectors (Chen et al., 2015) and mechanisms responsible for allowing persistent infections of alphaviruses in invertebrates are not well understood. Mosquitoes become infected by ingesting an arbovirus-contaminated blood meal. Once the arbovirus has reached the midgut, it replicates in the midgut epithelial cells, and escapes to the hemolymph through the tracheal system. Hemolymph circulation allows the virus to spread to the fat body, salivary glands, muscles, and neural tissues. Once a vector is infected with an arbovirus, the infection persists in its tissues for the length of its life. This persistent viral infection does not affect the behavior or life span of the mosquito vector, and appears to have few fitness costs (Kuno et al., 2005).

The extrinsic incubation period is the interval between a vector acquiring an infectious agent and the time it takes for the agent to replicate and be transmissible to a susceptible host. This period for a vector is determined by the arbovirus, mosquito species, and a number of external factors including especially ambient temperature and humidity. Geographic variations in vector populations result in varying infection, dissemination, and transmission rates among *Aedes spp.* mosquitoes (Tsetsarkin et al., 2006; Pesko et al., 2009).

The main route of CHIKV infection is through horizontal transmission from a vector to a susceptible host species. CHIKV is introduced into the host's skin capillaries during probing and feeding attempts by infected female *Aedes spp.* mosquito, and subsequently disseminates throughout the lymphatic system. Mosquito vectors typically become infected after feeding upon viremic hosts with CHIKV viral titers ranging from 10^3-10^5 PFU/mL (Pesko et al., 2009; Tsetsarkin et al., 2006). CHIKV viral titers in humans have been reported as high as $10^{5.5}$ plaque-forming units (PFU) per mL, and similar viremia titers have been detected in non-human primates (Chen et al., 2010; McIntosh et al., 1964). Direct human-to-human transmission of CHIKV has not been documented. However, limited evidence suggests that mother-to-child transmission may occur when CHIKV is acquired during late gestation periods (Couderc et al. 2009).

1.7 Clinical Disease and Pathogenesis of Chikungunya Fever

CHIKV infection begins when a human host is fed on by a CHIKV infected *Aedes* species mosquito (Reiter et al., 2006). Following feeding, CHIKV particles are thought to circulate through subcutaneous capillaries of the skin and begin replicating in the fibroblasts of the dermis (Reiter et al., 2006). *In vitro* studies have demonstrated that lymphocytes, dendritic cells, and natural killer cells are refractory to CHIKV infection (Sourisseau et al., 2007; Solignat et al., 2009). Within two to four days following infection, viral particles are transported to the circulatory system and disseminate to infect cells in other target organs, such as the liver, spleen, joints, and muscles (Weaver and Lecuit, 2015). Less frequently, CHIKV infects target cells in the liver, eye, kidneys, and the central nervous system (Van

Duijl-Richter et al., 2015). Three to five days following infection, patients develop a viral load of approximately 10^7 copies of RNA per milliliter serum, a concentration that may persist for three to ten days (Thiberville et al., 2013). This correlates with an increase in type I interferons and signals that the innate immune system is attempting to control the spread of CHIKV replication. In most cases the innate and adaptive immune responses, mainly CD4 T cells and CHIKV-specific antibodies, will eradicate CHIKV replication in approximately 7 days.

The acute stage of infection is characterized by a sudden onset of fever (on average 104◦F) and, in some cases, patients experience a relapse one to two days following an afebrile period lasting four to ten days this phenomenon is known as "saddleback fever" (Hawman et al., 2013; Carey, 1971). Patients may develop severe polyarthralgia in the wrists, ankles, knees, elbows, shoulders, and spine (Suhrbier et al., 2012). These polyarthralgia events are characteristically symmetrical and bilateral and patients may experience extreme tenderness, swelling, and may remain incapacitated for several weeks or months (Powers and Logue, 2007). Other commonly described symptoms include retro-orbital pain, lumbar back pain, chills, weakness, headache, myalgia, photophobia, and petechial or maculopapular rash (Brighton et al., 1983; Calisher, 1988; McGill, 1995). Maculopapular rashes primarily appear on the trunk, face, and extremities two to five days post-infection and may last up to 10 days. Most acute symptoms resolve within seven to ten days of onset (Ryman et al., 2000; Suhrbier et al., 2012). Roughly 5-15% of patients experience a "silent" or an asymptomatic infection without overt disease symptoms (Gerardin et al., 2008).

While most of the acute symptoms of CHIKF resolve in one to two weeks, a variable, sometimes large fraction of patients experience chronic arthritic symptoms that may last from several months to years (Sissoko et al., 2009; Das et al., 2010; Chaaitanya et al., 2011). A high percentage of these patients tend to be elderly, have high viral loads, or have previously diagnosed, underlying medical conditions (Sissoko et al., 2009; Das et al., 2010). These chronic symptoms may include: arthralgia, myalgia, asthenia, polyarthritis, severe debilitating arthritis, joint stiffness, bursitis, and tenosynovitis (Thiberville

et al., 2013). Even though most CHIKV infections are not life threatening, recent severe life-threatening symptoms have been described in the Americas (2013-2016) and in the Indian Ocean epidemics (2006-2008). Severe symptoms included encephalitis, meningoencephalitis, Guillain-Barre syndrome, heart failure and myocarditis, pneumonia and nephritis (Das et al., 2010; Crosby et al., 2016; Lemant et al., 2008; Leburn et al., 2009). Although these severe symptoms occur in less than 1% of persons infected, 280 fatalities have been documented, mainly in the elderly or in patients with underlying health conditions (CDC, August 2016).

In rare cases, vertical transmission of CHIKV from mother-to-child has been documented, specifically in 2005 during the La Reunion Island outbreak (Gerardin et al., 2008). A study conducted at the Groupe Hospitalier Sud-Reunion during this outbreak found that mother-to-child transmission is rare with only 2.5% of exposed neonates becoming infected (Couderc et al., 2009). Of the 2.5% exposed, only 10% were exposed during pregnancy. Neonates infected with CHIKV all appeared asymptomatic at birth with the onset of disease occurring approximately four days post-partum (Couderc et al., 2009). Thrombocytopenia occurred in 89% of neonates, with patients exhibiting pain, prostration, and fever. Encephalopathy occurred in 50% of cases with magnetic resonance imaging (MIR) showing abnormalities in the white mater of the brain (Couderc et al., 2009).

1.8 Immune Responses to Chikungunya Virus Infection

CHIKV infections in humans are rarely fatal. In most cases, the immune system of a healthy adult is capable resolving the infection within one to two weeks (Suhrbier et al., 2012). Elderly and neonatal patients constitute the majority of CHIKV infections, perhaps due to their waning and immature, respectively, immune systems. These individuals exhibit severe and often chronic CHIKF disease manifestations and have higher fatality rate than other patient cohorts (Gerardin et al., 2013; Jaffar-Bandjee et al., 2010). The pathogenesis of chronic polyarthralgic disease induced by CHIKV infection remains largely unknown, although it has been proposed that an influx of inflammatory mediators in joints results in excessive inflammation and tissue damage that leads to arthritic manifestations (Suhriber

and Mahalingam, 2009). The use of non-human primates, neonatal mice, and/or interferon (IFN) –α/β knockout mice have provided insights into the innate immune system's response during CHIKV infection similar to those conditions observed in chronic patients.

1.8.1 Innate Immune Response to Chikungunya Virus Infection

Current understanding of CHIKV–stimulated immune responses, suggests that viral dissemination and arthritic manifestation are mediated by the host's innate immune responses. Alphaviruses are typically strong inducers of type I IFN (Farber and Glasgow, 1972; Glasgow et al., 1971; Grieder and Vogel, 1999). CHIKV is typically eliminated from patients before IgG is produced and before T_H cells and cytokines become activated (Kam et al., 2009). During acute CHIKV infections, high levels of cytokines and chemokines are produced including: IFN-α/β, interleukin (IL)-1β, IL-5, IL-6, IL-7, IL-2R, IL-12, IL-10, IL-15, IL-18, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma-induced protein 10 (CXCL10), monokine induced by gamma interferon (MIG), and monocyte chemotactic protein-1 (MCP-1) (Teo et al., 2012; Chow et al., 2011; Ng et al., 2009; Hoarau et al., 2010; Chirathaworn et al., 2010; Chaaitanya et al., 2011; Kelvin et al., 2011). In vertebrate hosts, the production of type I IFNs, mainly IFN-α and IFN-β, restrict and/or inhibit CHIKV replication and spread. INF-α/β are primarily produced by leukocytes and fibroblasts (Schwartz and Albert, 2010). Type I IFN production is regulated by pathogen recognition receptors (PRRs) that detect pathogen associated molecular patterns (PAMPS) (Akira, 2004; Stetson et al., 2006). There are two types of PRRs: 1) toll-like receptors (TLRs) located on membranes and endosomes and 2) retinoic acid-inducible gene I-like receptors (Takeuchi and Akira, 2007). Upon recognition of the PAMP molecules, PRRs stimulate a cascade of events that ultimately result in the hallmark of the innate immune response, production of a type I IFN (Teng et al., 2011).

While it was previously suggested that CHIKV directly activated hematopoietic cells, in vitro studies have demonstrated that CHIKV induces the production of IFNs when fibroblasts become infected (Dalod et al., 2003; Schilte et al., 2010). Furthermore, upregulation of type I IFN expression has been

documented in acute-phase infections of non-human primates, mouse models, and in human patient cohorts (Wauquier et al., 2011; Labadie et al., 2010; Gardner et al., 2010; Chow et al., 2011; Ng et al., 2009; Hoarau et al., 2010; Rudd et al., 2012; Messaoudi et al., 2013; Palha et al., 2013). Infection of mouse strains deficient in IFN –α/β receptors (IFNAR) or IFN response factors 3/7 resulted in very high mortality rates within days of inoculation (Rudd et al., 2012; Gardner et al., 2012; Couderc et al., 2008).

Immunological studies utilizing animal models demonstrated that up-regulation of type I IFN is rapid but transient during acute CHIKV infection (Gardner et al., 2010; Labadie et al., 2010). IFN-α/βR$^{-/-}$ knockout mice exhibit severe CHIKV disease correlated with an increase in viral titers. Heterozygous mice with at least one functioning copy of the IFN- α/β receptor exhibit mild CHIKV disease (Courdec et al., 2008). Follow up studies indicated that IFN- α/β production is stimulated in fibroblasts, but not in hematopoietic dendritic cells nor in peripheral blood mononuclear cells (Schilte et al., 2010). Other knockout mouse models, mainly Cardiff and Myd88, have indicated that RIG-like receptors (RLR), in combination with TLR, are responsible for the recognition and mediation IFN- α/β production in fibroblasts (Schilte et al., 2010; Teo et al., 2012). Furthermore, ISG-15, an early IFN-stimulated gene, is critical in protecting neonatal mice from CHIKV-induced mortality in knockout models (Werneke et al., 2011). Collectively, these results indicate that IFN- α/β may protect vital organs such as the brain, heart, liver, and kidneys from severe CHIKV disease by limiting viral dissemination.

Dendritic cells (DC), natural killer cells (NK), and monocytes play a key role during the innate immune response and have been associated with the immunopathogenesis of many viral diseases (Pastorino et al., 2005; Coleman et al., 2009; Grieder et al., 1996; Rehermann et al., 2009; Her et al., 2010). Monocytes and macrophages seem to be ideal viral targets as they are located, largely, in the circulatory system and peripheral tissues. The role of monocytes and macrophages during the acute phase of CHIKF has been investigated in a variety of animal models (Gardner et al., 2010; Hoarau et al., 2010; Labadie et al., 2010; Morrison et al., 2011; Ozden et al., 2007). Monocytes are derived from hematopoietic stem cells in the bone marrow and migrate from the blood stream throughout the tissues of

the body. In these tissues, monocytes will differentiate into resident macrophages or dendritic cells. Macrophages may be classified as classically activated macrophages (M1) or alternatively activated macrophages (M2) (Mosser and Edwards, 2008; Martinez et al., 2009). M1 macrophages promote a T_H1 immune response, inhibit cell proliferation, and promote inflammation (Mosser and Edwards, 2008). M2 macrophages promote a T_H2 immune response, promote cell proliferation, and tissue repair (Mosser and Edwards, 2008). Immunohistochemistry of muscle and synovial tissues obtained from human patients, non-human primates, and rodent models during acute CHIKF suggest that viral replication in joint tissues leads to prolific infiltrations of monocyte, macrophage, and natural killer cells (Ozden et al., 2007; Hoarau et al., 2010). *In vitro* experimental studies demonstrated that monocytes derived from PBMCs could be infected by CHIKV and actively maintain infections (Her et al., 2010). Additionally, CHIKV has been shown to infect synovial macrophages (Hoarau et al., 2010). Microarray analysis of mice inoculated via foot pad with CHIKV demonstrated that, like rheumatoid arthritis, CHIKV infection leads to increased expresion of genes associated in both diseases with macrophage activation and recruitment (Nakaya et al., 2012). Similarly, B-cell deficient knockout mice (μMT) inoculated with CHIKV via a footpad challenge, developed a persistent viremia lasting over a year (Lum et al., 2013; Poo et al., 2014). CHIKV has also been shown to persist in macaque spleen macrophages two months post infection (Labadie et al., 2010; Messaoudi et al., 2013). Likewise, CHIKV has been isolated from human synovial fluid macrophages in CHIKF patients 18 months post clinical disease presentation (Soden et al., 2000). Together, these data suggest that monocytes/macrophages serve as vehicles for viral dissemination and mediate inflammation in synovial tissues and perhaps promote viral persistence.

The role of NK cells during CHIKV infections remains unclear. However, activated NKs have been found in CHIKF patients' synovial fluid (Hoarau et al., 2010). NK cells provide anti-viral protection by eliminated infected cells via cytolytic mediators (perforin and granzymes) and IFN-γ (Bryceson et al., 2009; Lanier et al., 2008). During the acute phase of CHIKV infection, NKs express receptors for HLA-C1 alleles and undergo clonal expansion (Petitdemange et al., 2011). In CHIKV

infected mice, NK cells and macrophages have been detected at elevated levels along with the up-regulation of IL-12 (Gardner et al., 2012). This suggests that NK cells may play a prominent role in viral control and should be explored further.

1.8.2 Adaptive Immune Response to Chikungunya Virus Infection

Following the onset of CHIKF clinical disease, seroconversion takes place within the first week of infection. CHIKV IgM antibodies are detectable subsequent to disease onset and reach peak titers 2-3 dpi. These antibodies may persist for several weeks to months (Kam et al., 2012; Zim et al., 2013; Nitatpattan et al., 2014; Panning et al., 2009). The persistence of IgM CHIKV antibodies for months following infection has been observed in chronic CHIKF patients and may play a role in CHIKV persistence (**Figure 1.4**) (Petitdemange et al., 2015). The detection CHIKV antibodies in patient serum by ELISA allows for more rapid detection of CHIKF than traditional RT-PCR (Kam et al., 2009). However, in regions were other alphaviruses are present, cross-reactivity has been observed (Kam et al., 2009). Similarly, CHIKV neutralizing immunoglobulin G (IgG) antibodies appear ten to thirteen following viral clearance from the blood and may persist for several years at high titers (Kam et al., 2009). Together both IgM and IgG CHIKV antibodies provide an early measure of protection by binding to cell receptors and preventing viral entry, cause the agglutination of CHIKV virions, and triggering phagocytosis by neutrophils and macrophages (Kam et al., 2009). *In vitro* studies using CHIKF human patient serum, have shown that long-lasting CHIKV antibody responses are triggered by the presence of the N-terminal region of the E2 CHIKV glycoprotein (Kam et al., 2012). The predominated IgG neutralizing antibody in humans is the IgG_3 subtype (Kam et al., 2012). In several CHIKF cohorts a correlation exists between the presence of the IgG3 subtype and the absence of chronic disease (Kishishita et al., 2015). Patients producing IgG_3, do not develop fever and joint pain, and are resistant to secondary CHIKV infections by other genotypes (Kishishita et al., 2015).

Recently, Toll-like receptor 3 has been shown to detect CHIKV virions and trigger a humoral immune response (Her et al., 2014). The specific roles of B cells and the cytokines they produce during

CHIKV infection remain unclear. While most of the current CHIKV animal models focus on investigating innate immunity, these models are unsuitable for investigating the adaptive immune responses to CHIKV infection (Zeigler et al., 2008; Couderc et al., 2008; Gardner et al., 2010; Morrison et al., 2011). Similarly, our current understanding of T-cell involvement during CHIKV infection need to be more intensively explored. T cell receptors, in collaboration with T cells, are the main mediators of the adaptive immune system. T cell receptors are further separated into two groups depending on the co-receptors expressed, either CD4+ or CD8+. Generally, CD4+ T cells recognize viral antigen peptides presented by the major histocompatibility complex I and help mediate antiviral activity by aiding B cells in generating immunoglobulins. CD8+ T cells produce anti-viral cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor (TNF). During CHIKV infection both human patients and animal models generate a robust CHIKV-specific CD4+ and CD8+ T cell immune response (Messaoudi et al., 2013; Wauquier et al., 2011; Hoarau et al., 2010; Gardner et al., 2010). Studies have demonstrated that the frequencies and absolute values of T cells are significantly lower in CHIKF patients as compared to healthy donors (Peititdemange et al., 2015). However, T cell abundance returned to normal levels in these patients after a two-month period (Petitdemange et al., 2011). Of the remaining peripheral T cells, a significant portion of these expressed CD95, and activation of these cells, in addition to the overstimulation of a T cell immune response, is thought to lead to early lymphopenia in some patients (Hoarau et al., 2010; Wauquier et al., 2011). Clinical patient cohort and animal experimental studies have demonstrated that CHIKV infection leads to a robust CHIKV-specific CD4+ and CD8+ T cell response (Hoaurau et al., 2010; Messaoudi et al., 2013; Gardner et al., 2010; Wauquier et al., 2011). Clinical patient studies have shown that CD8+ T cells are activated 2-3 days following infection and switch from CD8+ to CD4+ T-cells during the later stages of acute infection (Wauqueir et al., 2011). Studies using immunocompromised mouse models demonstrated that during chronic CHIKV infections, IFN-γ T-cell responses were driven by CD8+ T cells (Hoarau et al., 2013). Additionally, CD8+ and CD4+ T-cells were shown to infiltrate inflamed joints in several of these mouse models (Morrison et al., 2011).

Collectively these and similar studies suggest that CD8+ T-cells do not play a major role in mediating CHIKV viral clearance. It remains unclear whether CD8+ T-cells play a role in clearance of viral CHIKV RNA from persistent joint macrophages in chronic patients. While almost all CHIKF patients develop CHIKV-specific T-cell immune responses, only 20% of these patients maintain memory T-cells after one to two years (Hoarau et al., 2013).

These data suggest that an efficient and robust T and B cell response may be crucial in CHIKV viral clearance.

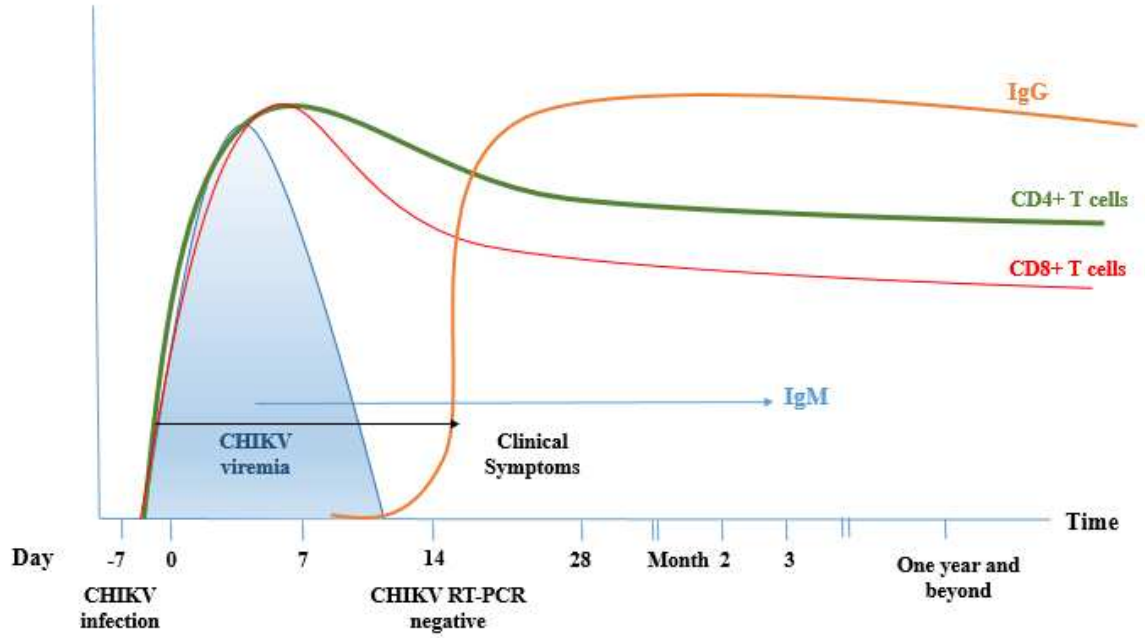

Figure 1.4. Representation of immune response against CHIKV infection. Adapted from Kam et al., 2009.

1.9 Animal Models of Chikungunya Virus (CHIKV)

Identifying animal models that adequately recapitulate the signs and symptoms of CHIKF seen in humans is essential for understanding CHIKV host-virus interactions and disease pathogenesis. Ideally, clinical research studies involving CHIKV-infected patients would aid in developing vaccines and treatment for CHIKF. However, while several clinical studies examining CHIKV-infected patients have provided accurate demonstrations of viral pathogenesis, this research is limited by ethical concerns.

Additionally, the study of virus tropism in these patients is restricted due to the invasive procedures required to obtain tissue and organ biopsies. To overcome these limitations, animal models have been exploited to give a better illustration of the mechanisms involved during CHIKV pathogenesis and disease development in an *in vivo* setting.

1.9.1 Non-human Primate Models

The first non-human primate studies with CHIKV were performed in 1953 and resulted in the demonstration that rhesus macaques (*Macaca mulata*) were susceptible to CHIKV infection and were capable of developing CHIKV neutralizing antibodies (Ross, 1956). Rhesus macaques have also been used to examine immune responses triggered by CHIKV infection and in the development of CHIKV vaccines and therapeutics (Broeckel et al., 2015). Rhesus macaques inoculated with CHIKV develop high levels of circulating virus ($10^4 – 10^6$ pfu/mL) two to four days post-infection that up to four to five days (Binn et al., 1967). Other non-human primate species including *Cercopithecus* monkeys, baboons, and bonnet monkeys (*Macaca radiate*) have been shown to become viremic following CHIKV inoculation (McIntosh et al., 1964; Paul and Singh, 1968). More recently, cynomolgus macaques (*Macaca fascicularis*), have been evaluated and found to develop high viral loads, and exhibit similar clinical and pathological features to those described for human CHIKF patients (Labadie et al., 2010). Importantly, studies using both rhesus and cynomolgus macaques demonstrated long-term persistence of CHIKV in secondary lymphoid organs and joints, a feature that may aid in investigating similar chronic arthralgia that accompanies CHIKF disease in human patients (Labadie et al., 2010; Messaoudi et al., 2010).

Human and experimentally infected non-human primates experience similar acute stage responses to CHIKV infection. During the first week after infection with CHIKV, cynomolgus macaques developed peak viremia of approximately 10^8 pfu/mL (Labadie et al., 2010). Cynomolgus macaques exhibited a high fever one to two following infection that persisted for two to seven days (Chen et al. 2010; Labadie et al., 2010; Messauodi et al., 2013). The most severe CHIKV symptoms were seen in macaques

administered the highest dose of CHIKV (10^9 pfu), which included joint swelling of the wrists and ankles, fever, and rash (Labadie et al., 2010). Animals that received moderate doses (10^2-10^6 pfu) developed low-titer viremia with fever and rash, while those receiving only 10 pfu failed to devleop clinical signs of disease (Labadie et al., 2010). Corresponding to human infections, cynomolgus macaques infected with an intermediate dose ($10^2 – 10^6$ pfu) of CHIKV underwent viral dissemination to multiple tissues, including liver, joint, muscle, skin, brain, spinal cord, and lymph nodes (Labadie et al., 2010). Histological abnormalities were detected in CHIKV infected cynomolgus macaques throughout the liver, spleen, and lymph nodes (Chen et al., 2010). In the red pulp of the spleen and the cortex of lymph nodes mononuclear cell infiltrates were observed, and in the liver, increased levels of hepatocyte death due to apoptosis was observed (Labadie et al., 2010). While it is unclear whether CHIKV chronic joint pain is caused by the persistence of CHIKV replication or due to disruptions of inflammatory equilibrium caused by CHIKV replication, there is evidence that CHIKV persists in both humans and non-human primates. Labadie documented the first evidence of CHIKV persistence in cynomolgus macaques by recovering infectious virus from spleen, liver, and muscle as late as 44 dpi (Labadie et al., 2010).

Non-human primate models were originally developed due to the limited availability of clinical human specimens, their involvement in sylvatic disease transmission cycles, and notably their ability to replicate CHIKV-induced pathology. Due to the close evolutionary relationship between non-human primates and humans, scientists are able to better understand aspects of the innate and adaptive immune system during CHIKV infection. Despite these advantages, primate models suffer from several disadvantages including difficulty handling and obtaining samples, ethical controversies, and high costs associated with purchasing and housing. To overcome these limitations, inbred mouse models of different genetic backgrounds and ages are commonly used to study CHIKV disease pathogenesis and evaluate potential antiviral therapies.

1.9.2 Rodent Models

Development and testing of CHIKV therapeutics and vaccines are dependent upon identifying animal models that accurately mimic CHIKF in humans. Historically, mouse models have been extensively used to study arbovirus pathogenicity and viral transmission cycles (Thiberville et al., 2013). Several key factors have plagued use of rodent models of CHIKV infection, including high variability in results depending on genetic background, age, and site of viral inoculation (Srivastava et al., 2008; Chan et al., 2015). The C57BL/6 mouse strain is the most common genetic background for developing a wide range of genetically modified mutant strains used to understand CHIKV infection and immunity.

CHIKV studies involving C57BL/6 mice infected by intradermal (i.d.) inoculation demonstrated muscle weakness that eventually resulted in paralysis six dpi (Couderc et al., 2008). Similarly, nine day-old C57BL/6 mice inoculated with CHIKV exhibited a 50% mortality rate, while six day old mice died by twelve dpi (Couderc et al., 2008). Low levels of CHIKV were detected in blood, spleen, liver, and brain, whereas higher levels of virus were detected in skin, joints, and muscles (Couderc et al., 2008). A second investigation using nine-day-old C57NL6/J mice inoculated subcutaneously (SC) with CHIKV reported a 60% mortality from ten dpi, with viremia detected as early as one dpi and as late as ten dpi, accompanied by detection of virus in muscle, liver, lung, and brain (Werneke et al., 2011). Morrison and colleagues observed that fourteen day old C57BL/6 mice exhibited CHIKV-induced disease manifestations three weeks post infection (Morrison et al., 2011). Viral RNA was detected in musculoskeletal tissues three weeks post infection and histologic analysis showed severe necrotizing myositis, chronic tenosynovitis, inflammatory cell arthritis, and multifocal vasculitis (Morrison et al., 2011). Likewise, two to three day old outbred CD-1 and ICR mice exhibited dragging of the hind limbs, lethargy, and weight loss starting seven dpi (Ziegler et al., 2008). Dhanwani et al. found that two to three day-old Balb/c mice inoculated with CHIKV exhibited locomotor abnormalities such as partial paralysis of hind limbs associated with detectable levels of several inflammatory mediators in muscle tissues (Dhanwani et al., 2014). Discrepancies between these studies highlight the importance of age and immune competence in CHIKV

pathogenesis. Since adult wild-type mice are resistant to CHIKV infection via SC and ID inoculation routes, subsequent studies investigated alternative routes of inoculation. By shifting the site of inoculation from intradermal i.d. and subcutaneous s.c. to either the ventral footpad, intranasal, or intracranial inoculation, adult mice demonstrated significant differences in disease pathogenesis (Gardner et al., 2010). Adult six week old C57BL6/J mice inoculated in the foot pad experienced an infection localized to the foot, joint, synovial membranes, tendon, lymph nodes, spleen, and muscle starting at six dpi (Gardner et al., 2010). Similarly other studies found that intranasal inoculation induced encephalopathy in mice. Not surprisingly, intranasal and intracranial inoculation resulted in high mortality and neurotropic disease (Powers et al., 2000; Ryman et al., 2008; Wang et al. 2008). These latter models involving neurotropic disease are unsuitable for viral pathogenesis studies and vaccine testing as they do not accurately replicate CHIKF as seen in humans.

1.10 Evidence for Primates and Rodents as Reservoir Hosts

Wild birds and mammals serve as essential reservoirs for arthropod-borne virus disease cycles. While some of these pathogens occasionally spill over into human and domestic animal populations, these incidental hosts are usually not essential to the survival of the pathogen. Wildlife species, including rodents and primates, are of particular importance in zoonotic cycles because they serve as reservoirs for many arboviruses (Meerburg et al., 2009; Davis et al., 2005; Jackson et al., 2015).

In Africa, serological evidence suggests that non-human primates, rodents, and birds may maintain CHIKV during inter-epidemic periods (Cornet et al., 1968; McIntosh1961; Vourch et al., 2014; Bres et al., 1969; Cornet et al., 1979; Osterrieth et al., 1960). Wild non-human primates are predominately thought to serve as virus reservoirs during inter-epidemic periods (Osterrieth et al., 1960). CHIKV neutralizing antibodies have been detected in chimpanzees in the Democratic Republic of Congo, vervet monkeys (*Cerocpithecus aethiops*), and chacma baboons (*Papio ursinus*) in southern Africa (Osterrieth et al., 1960; McIntosh and Jupp, 1970; McIntosh et al., 1977; McIntosh et al., 1964). In Nigeria, CHIKV antibodies have been detected in 10% of Patas monkeys (*Erythrocebus patus*), 7% of

Tantalus monkeys (*Cercopithecus aethiops tantalus*), and 20% of Mona monkeys (*Cercopithecus mona*) (Boorman and Draper, 1968). McCrae found evidence of CHIKV circulating in zoophilic mosquito species (*Ae. africanus*) and non-human primates, specifically red-tailed monkeys (*Cercopithecus ascanisua*) in Uganda (McCrae et al., 1971). Antibodies to CHIKV have also detected in other wild non-human primate species including baboons, chimpanzees, gorillas, and vervet monkeys imported to the Unites States from Africa for scientific research purposes (Harrison et al., 1967). In Asia, although there is no known sylvatic transmission cycle, CHIKV antibodies have been detected in non-human primates in Thailand, Malaysia, and the Philippines (Halstead et al., 1966; Marchette et al., 1978; Inoue et al., 2003; Sam et al., 2015).

Previous epidemiological studies have assumed that CHIKV vertebrate reservoirs were limited to non-human primates. However, other reservoir/amplifying hosts, such as rodents, may maintain CHIKV in a secondary sylvatic cycle in the absence of human hosts. The order Rodentia represents the largest group of mammals with more than 2,000 species in 30 families and offers a large number of potential reservoirs for emerging zoonotic arboviruses. In Senegal, CHIKV has been isolated from various vertebrate species, including vervet monkeys (*Cercopithecus aethiops*), Senegal galago (*Galago senegalensis*), a palm squirrel (*Xerus erythropus*), baboons (*Papio papio*), a golden sparrow (*Auripasser luteus*), and bats (*Scotophilus spp.*) (Bres et al., 1969; Cornet et al., 1979). Experimental infections of wild African rodent species in the genera *Mastomys*, *Arvicanthis*, and *Aethomys* with CHIKV were found to be capable of developing a detectable viremia (McIntosh, 1961). While species within the *Arcicanthis* and *Aethomys* genera developed low viral loads of CHIKV, the *Mystromys* species produced a high viremia followed by the development of CHIKV neutralizing antibodies (McIntosh, 1961). Some studies have also detected low viremia levels following experimental infections of rodent species including Syrian Golden hamsters and bats (Bedekar and Pavri, 1969; Bosco-Lauth et al., 2015; Bosco-Lauth et al., 2016). Diallo isolated several strains of CHIKV in Senegal from thirteen mosquitoes including some zoophilic mosquito species (Diallo et al., 1999). It also appears that some domestic animals such as

cattle, pigs, dogs, rabbits, sheep, goats, horses, and several species of birds do not develop a detectable viremia but are capable of producing CHIKV neutralizing antibodies (Karabatos, 1985; Halstead et al., 1966; Bosco-Lauth et al., 2015). These finding suggest that a variety of wildlife host species may play a role in CHIKV sylvatic transmission cycles and may involve rodents and other zoophilic mosquito species (Diallo et al., 1999; Bosco-Lauth et al., 2015).

1.11 Rationale for the Current Study

The overall aim of the research described in the next three chapters of this dissertation was to study chikungunya infection in wildlife species that cover a range of diverse taxonomic families that are common throughout the Americas. Several aspects of chikungunya pathogenesis, virulence, and transmission have been evaluated in non-human primate and inbred mouse models but very few studies have been performed using species that are more genetically diverse and may be involved in natural sylvatic transmission cycles. Similarly, the potential role wildlife species play in sylvatic transmission cycles both in endemic and epidemic regions remains unclear. Decades of CHIKV research have focused on establishing a CHIKF model to replicate human infections and have identified non-human primates as the best animal model. In addition to replicating CHIKF disease pathology similar to those observed in humans, non-human primates have also been identified as the main wildlife species responsible for CHIKV replication during sylvatic transmission cycles. While several studies have identified CHIKV neutralizing antibodies in additional wildlife species, these potential reservoirs have largely been ignored. Here I sought to evaluate the potential of a diverse set of rodent species in order to evaluate their potential role as reservoirs/amplifying hosts for CHIKV. This research is novel and significant as it examines CHIKV disease dynamics in wild, genetically diverse individuals that potentially may play a role in sylvatic transmission cycles as CHIKV spreads throughout the Americas

REFERENCES

Aguilar PV, Weaver SC, Basler CF. 2007. Capsid protein of eastern equine encephalitis virus inhibits host cell gene expression. *J Virol 81*: 3866-3876.

Akira S. 2004, February. Toll receptor families: structure and function. In *Seminars in Immunology* (Vol. 16, No. 1, pp. 1-2). Academic Press.

Arankalle VA, Shrivastava S, Cherian S, Gunjikar RS, Walimbe AM, Jadhav SM, ... Mishra AC 2007. Genetic divergence of Chikungunya viruses in India (1963–2006) with special reference to the 2005–2006 explosive epidemic. *J Gen Virol 88:* 1967-1976.

Bedekar SD, Pavri KM. 1969. Studies with chikungunya virus. Part I. Susceptibility of birds and small mammals. *Indian J Med Res 57*: 1181-92.

Binn LN, Harrison VR, Randall R. 1967. Patterns of viremia and antibody observed in rhesus monkeys inoculated with chikungunya and other serologically related group A arboviruses. *Am J Trop Med Hyg* 16: 782-5.

Boorman JPT, Draper CC. 1968. Isolations of arboviruses in the Lagos area of Nigeria, and a survey of antibodies to them in man and animals. *Transact Royal Soc Trop Med Hyg 62*: 269-277.

Bosco-Lauth AM, Han S, Hartwig A, Bowen RA. 2015. Development of a hamster model for chikungunya virus infection and pathogenesis. *PloS One 10*: e0130150.

Bosco-Lauth AM, Nemeth NM, Kohler, DJ, Bowen, RA. 2016. Viremia in North American Mammals and Birds After Experimental Infection with Chikungunya Viruses. *The Am J Trop Med Hyg 94*: 504-506.

Breakwell L, Dosenovic P, Hedestam GBK, D'Amato M, Liljeström P, Fazakerley J, McInerney GM. 2007. Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. *J Virol 81*: 8677-8684.

Bres P, Hery G. 1969. Kinetics of Production of Haemagglutination Inhibiting and Complement Fixing Antibodies in Mice Immunized with Chikungunya, Onyong-nyong and Sinbus Viruses. 3. Effect of interval between inoculations and Use of an Adjuvant. *Revue Immunol 33*: 147.

Brighton SW, Prozesky OW, De La Harpe AL. 1983. Chikungunya virus infection. A retrospective study of 107 cases. *South Af Med J 63*: 313-315.

Broeckel R, Haese N, Messaoudi I, Streblow DN. 2015. Nonhuman Primate Models of Chikungunya Virus Infection and Disease (CHIKV NHP Model). *Pathogens 4*: 662-681.

Bryceson YT, Long EO. 2009. Line of attack NK cell specificity and integration of signals. *Cur Opin Immunol* 20: 344–352.

Cadd TL, Skoging U, Liljeström P. 1997. Budding of enveloped viruses from the plasma membrane. *Bioessays 19*: 993-1000.

Calisher CH, Karabatsos N. 1988. Arbovirus serogroups: definition and geographic distribution. In: *The Arboviruses: Epidemiology and Ecology* 1: 19-57.

Carey DE. 1971. Chikungunya and dengue: a case of mistaken identity? *J History Med Allied Sci 26*: 243-262.

Carey DE, Myers RM, DeRanitz CM, Jadhav M, Reuben R. 1969. The 1964 chikungunya epidemic at Vellore, South India, including observations on concurrent dengue. *Transact Royal Soc Trop Med Hyg 63*: 434-445.

Chaaitanya IK, Muruganandam N, Sundaram SG, Kawalekar O, Sugunan AP, Manimunda SP, Ghosal SR, Muthumani K, Vijayachari P. 2011. Role of proinflammatory cytokines and chemokines in chronic arthropathy in CHIKV infection. *Viral Immunol* 24: 265–271.

Chatterjee PK, Vashishtha M, Kielian M. 2000. Biochemical consequences of a mutation that controls the cholesterol dependence of Semliki Forest virus fusion. *J Virol 74*: 1623-1631.

Chan YH, Lum FM, Ng LFP. 2015. Limitations of current in vivo mouse models for the study of chikungunya virus pathogenesis. *Med Sci 3*: 64-77.

Chen LH, Wilson ME. 2010. Dengue and chikungunya infections in travelers. *Curr Opin Infect Dis 23*: 438-444.

Chen XG, Jiang X, Gu J, Xu M, Wu Y, Deng Y, ... Armbruster P. 2015. Genome sequence of the Asian Tiger mosquito, *Aedes albopictus*, reveals insights into its biology, genetics, and evolution. *Proc Natl Acad Sci 112*: e5907-e5915.

Chevillon C, Briant L, Renaud F, Devaux C. 2008. The chikungunya threat: An ecological and evolutionary perspective. *Trends in Microbiol 16*: 80–88.

Chirathaworn C, Rianthavorn P, Wuttirattanakowit N, Poovorawan Y. 2010. Serum IL-18 and IL-18BP levels in patients with Chikungunya virus infection. *Viral Immunol* 23: 113–117.

Chow A, Her Z, Ong EK, Chen JM, Dimatatac F, Kwek DJ, Barkham T, Yang H, Renia L, Leo YS, Ng LF. 2011. Persistent arthralgia induced by Chikungunya virus infection is associated with interleukin-6 and granulocyte macrophage colony-stimulating factor. *J of Infect Dis* 203: 149–157.

Chretien JP, Anyamba A, Bedno SA, Breiman RF, Sang R, Sergon K, ... Linthicum KJ. 2007. Drought-associated chikungunya emergence along coastal East Africa. *Am J Trop Med Hyg 76*: 405-407.

Christophers S. 1960. *Aedes aegypti* (L.) the yellow fever mosquito: its life history, bionomics and structure. *Rickard.*

Centers for Disease Control and Prevention. 2015. "Clinical Evaluation & Disease." Accessed August 12, 2016. https://www.cdc.gov/chikungunya/hc/clinicalevaluation.html.

Coffey LL, Failloux AB, Weaver SC. 2014. Chikungunya virus–vector interactions. *Viruses 6*: 4628-4663.

Coleman KD, Wright JA, Ghosh M, Wira CR, Fahey JV. 2009. Estradiol modulation of hepatocyte growth factor by stromal fibroblasts in the female reproductive tract. *Fertil Steril 92*: 1107-1109.

Cornet M, Robin Y, Adam C, Valade M, Calvo MA. 1979. Transmission expérimentale comparée du virus amaril et du virus Zika chez *Aedes aegypti*. *L Cahiers ORSTOM série Entomologie médicale et Parasitologie*, 47-53.

Cornet M, Robin Y, Hannoun C, Corniou B, Bres P, Causse G. 1968. An epidemic of yellow fever in Senegal in 1965. Epidemiological studies. *Bull World Health Organ* 39: 845—858.

Couderc T, Chrétien F, Schilte C, Disson O, Brigitte M, Guivel-Benhassine F, ... Desprès P. 2008. A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. *PLoS Pathog 4*: e29.

Couderc T, Khandoudi N, Grandadam M, Visse C, Gangneux N, Bagot S, ... Lecuit M. 2009. Prophylaxis and therapy for Chikungunya virus infection. *J Infect Dis 200*: 516-523.

Crosby L, Perreau C, Madeux B, Cossic J, Armand C, Herrmann-Storke C, ... Thiéry G. 2016. Severe manifestations of chikungunya virus in critically ill patients during the 2013–2014 Caribbean outbreak. *Int J Infect Dis 48*: 78-80.

Dalod M, Hamilton T, Salomon R, Salazar-Mather TP, Henry SC, Hamilton JD, Biron CA, 2003. Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta. *J Exp Med* 197: 885–898.

Das T, Jaffar-Bandjee MC, Hoarau JJ, Trotot PK, Denizot M, Lee-Pat-Yuen G, ... Alessandri JL. 2010. Chikungunya fever: CNS infection and pathologies of a re-emerging arbovirus. *Prog Neurobiol 91*: 121-129.

Davis CT, Ebel GD, Lanciotti RS, Brault AC, Guzman H, Siirin M, ... Elizondo-Quiroga D. 2005. Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: evidence for the emergence of a dominant genotype. *Virology 342*: 252-265.

De Groot RJ, Hardy WR, Shirako Y, Strauss JH. 1990. Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing *in vivo*. *The EMBO J 9*: 2631.

Dhanwani R, Khan M, Lomash V, Rao PVL, Ly H, Parida M. 2014. Characterization of Chikungunya virus induced host response in a mouse model of viral myositis. *PloS One 9*: e92813.

Diallo M, Thonnon J, Traore-Lamizana M, Fontenille D. 1999. Vectors of Chikungunya virus in Senegal: current data and transmission cycles. *Am J Trop Med Hyg 60*: 281-286.

Diallo D, Sall AA, Buenemann M, Chen R, Faye O, Diagne CT, ... Weaver SC. 2012. Landscape ecology of sylvatic chikungunya virus and mosquito vectors in southeastern Senegal. *PLoS Negl Trop Dis 6*: e1649.

Durbin RK, Stollar V. 1985. Sindbis virus mutants able to replicate in methionine-deprived *Aedes albopictus* cells. *Virology 144*: 529-533.

Failloux AB, Vazeille M, Rodhain F. 2002. Geographic genetic variation in populations of the dengue virus vector *Aedes aegypti*. *J Mol Evol 55*: 653-663.

Farber PA, Glasgow LA. 1972. Effect of Corynebacterium acnes on interferon production in mice. *Infect Immun 6*: 272-276.

Fischer M, Staples JE. 2014. Notes from the field: chikungunya virus spreads in the Americas—Caribbean and South America, 2013–2014. *MMWR Morb Mortal Wkly Rep 63*: 500-1.

Fros JJ, Liu WJ, Prow NA, Geertsema C, Ligtenberg M, Vanlandingham DL, ... Pijlman GP. 2010. Chikungunya virus nonstructural protein 2 inhibits type I/II interferon-stimulated JAK-STAT signaling. *J Virol 84*: 10877-10887.

Gardner CL, Burke CW, Higgs ST, Klimstra WB, Ryman K.D. 2012. Interferon-alpha/beta deficiency greatly exacerbates arthritogenic disease in mice infected with wild-type chikungunya virus but not with the cell culture-adapted live-attenuated 181/25 vaccine candidate. *Virology 425*: 103-112.

Gardner J, Anraku I, Le TT, Larcher T, Major L, Roques P, Schroder WA, Higgs S, Suhrbier A. 2010. Chikungunya virus arthritis in adult wild-type mice. *J Virol 84*: 8021-8032.

Garmashova N, Gorchakov R, Frolova E, Frolov I. 2006. Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J Virol 80*: 5686-5696.

Garmashova N, Gorchakov R, Volkova E, Paessler S, Frolova E, Frolov I. 2007. The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J Virol 81*: 2472-2484.

Gérardin P, Guernier V, Perrau J, Fianu A, Le Roux K, Grivard P, ... Favier F. 2008. Estimating Chikungunya prevalence in La Reunion Island outbreak by serosurveys: two methods for two critical times of the epidemic. *BMC Infectious Diseases 8*: 1.

Gérardin P, Fianu A, Michault A, Mussard C, Boussaïd K, Rollot O, ... Gaüzère BA. 2013. Predictors of Chikungunya rheumatism: a prognostic survey ancillary to the TELECHIK cohort study. *Arthritis Res Ther 15*: 1.

Gérardin P, Barau G, Michault A, Bintner M, Randrianaivo H, Choker G, ... Le Roux K. 2008. Multidisciplinary prospective study of mother-to-child chikungunya virus infections on the island of La Reunion. *PLoS Med 5*: e60.

Glasgow LA, Odugbemi T, Dwyer P, Ritterson AL. 1971. *Eperythrozoon coccoides* I. Effect on the interferon response in mice. *Infect Immun 4*: 425-430.

Gomez de Cedrón M, Ehsani N, Mikkola ML, García JA, Kääriäinen L. 1999. RNA helicase activity of Semliki Forest virus replicase protein NSP2. *FEBS letters 448*: 19-22.

Grieder FB, Nguyen HT. 1996. Virulent and attenuated mutant Venezuelan equine encephalitis virus show marked differences in replication in infection in murine macrophages. *Microb Pathog 21*: 85-95.

Grieder FB, Vogel SN. 1999. Role of interferon and interferon regulatory factors in early protection against Venezuelan equine encephalitis virus infection. *Virology 257*: 106-118.

Gubler DJ. 2002. Epidemic Dengue/Dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends in Microbiol 10*: 100-103.

Hahn YS, Grakoui A, Rice CM, Strauss EG, Strauss JH. 1989. Mapping of RNA-temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4. *J Virol 63*: 1194-1202.

Halstead SB. 1966. Mosquito-borne haemorrhagic fevers of South and South-East Asia. *Bull Wor Heal Org 35*: 3.

Harrison VR, Binn LN, Randall R. 1967. Comparative Immunogenicities of Chikungunya vaccines prepared in avian and mammalian tissues. *Am J Trop Med Hyg 16*: 786-91.

Hawman DW, Stoermer KA, Montgomery SA, Pal P, Oko L, Diamond MS, Morrison TE. 2013. Chronic joint disease caused by persistent Chikungunya virus infection is controlled by the adaptive immune response. *J Virol 87*: 13878-13888.

Hawley WA, Reiter P, Copeland RS, Pumpuni CB, Craig GB. 1987. *Aedes albopictus* in North America: probable introduction in used tires from northern Asia. *Science, 236*:1114-1116.

Her Z, Malleret B, Chan M, Ong EK, Wong SC, Kwek DJ, ... Ng LF. 2010. Active infection of human blood monocytes by Chikungunya virus triggers an innate immune response. *The J Immunol 184*: 5903-5913.

Her Z, Teng TS, Tan JJ, Teo TH, Kam YW, Lum FM, … Lulla V. 2014. Loss of TLR3 aggravates CHIKV replication and pathology due to an altered virus-specific neutralizing antibody response. *EMBO Molecular Medicine*: e201404459.

Hoarau JJ, Bandjee MCJ, Trotot PK, Das T, Li-Pat-Yuen G, Dassa B, ... Tallet F. 2010. Persistent chronic inflammation and infection by Chikungunya arthritogenic alphavirus in spite of a robust host immune response. *J Immuno 184*: 5914-5927.

Inoue S, Morita K, Matias RR, Tuplano JV, Resuello RRG, Candelario JR, Cruz DJM, Mapua CA, Hasebe F, Igarashi A, Natividad FF. 2003. Distribution of three arbovirus antibodies among monkeys (Macaca fascicularis) in the Philippines. *J Med Primat 32*: 89-94.

Jackson JA. 2015. Immunology in wild nonmodel rodents: an ecological context for studies of health and disease. *Parasite Immunol 37*: 220-232.

Jaffar-Bandjee MC, Ramful D, Gauzere BA, Hoarau JJ, Krejbich-Trotot P, Robin S, ... Gasque P. 2010. Emergence and clinical insights into the pathology of Chikungunya virus infection. *Expert Rev Anti Infect Ther 8*: 987-996.

Jupp PG, McIntosh BM. 1990. *Aedes furcifer* and other mosquitoes as vectors of chikungunya virus at Mica, northeastern Transvaal, South Africa. *J Am Mosq Control Assoc 6*: 415-420.

Kam YW, Lum FM, Teo TH, Lee WW, Simarmata D, Harjanto S, ... Lin RT. 2012. Early neutralizing IgG response to Chikungunya virus in infected patients targets a dominant linear epitope on the E2 glycoprotein. *EMBO Mol Med 4*: 330-343.

Kam YW, Ong EK, Rénia L, Tong JC, Ng LF. 2009. Immuno-biology of Chikungunya and implications for disease intervention. *Microbes Infect* 11: 1186-1196.

Karabastos N, ed. International Catalogue of Arboviruses, Including Certain Other Viruses of Vertebrates. San Antonio, TX: American Society of Tropical Medicine and Hygiene, 1985, updated in 2001.

Kelvin AA, Banner D, Silvi G, Moro ML, Spataro N, Gaibani P, ... Bermejo-Martin JF. 2011. Inflammatory cytokine expression is associated with chikungunya virus resolution and symptom severity. *PLoS Neg Trop Dis 5*: e1279.

Kielian M, Rey FA. 2006. Virus membrane-fusion proteins: more than one way to make a hairpin. *Nature Rev Microbiol 4*: 67-76.

Kishishita N, Sasayama M, Takeda N, Sa-ngasang A, Anuegoonpipat A, Anantapreecha S. 2015. Neutralization Activity of Patient Sera Collected during the 2008-2009 Chikungunya Outbreak in Thailand. *J Clin Microb 53*: 184-190.

Kuhn RJ. 2007. Togaviridae: the viruses and their replication. *Fields Virology 1*: 1001-1022.

Kuno G, Chang GJJ. 2005. Biological transmission of arboviruses: reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends. *Clin Microbiol Rev 18*: 608-637.

Labadie K, Larcher T, Joubert C, Mannioui A, Delache B, Brochard P, ... de Lamballerie X. 2010. Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages. *J Clin Invest 120*: 894-906.

Lanier LL. 2008. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol* 8: 259–268.
LaStarza MW, Lemm JA, Rice CM. 1994. Genetic analysis of the nsP3 region of Sindbis virus: evidence for roles in minus-strand and subgenomic RNA synthesis. *J Virol 68*: 5781-5791.
Lebrun G, Chadda K, Reboux AH, Martinet O, Gauzere BA. 2009. Guillain-Barré syndrome after chikungunya infection. *Emerg Infect Dis 15*: 495-497.
Lemant J, Boisson V, Winer A, Thibault L, André H, Tixier F, ... Besnard M. 2008. Serious acute chikungunya virus infection requiring intensive care during the Reunion Island outbreak in 2005–2006. *Crit Care Med 36*: 2536-2541.
Lemm JA, Rice CM. 1993. Assembly of functional Sindbis virus RNA replication complexes: requirement for coexpression of P123 and P34. *J Virol 67*: 1905-1915.
Leung JYS, Ng MML, Chu JJH. 2011. Replication of alphaviruses: a review on the entry process of alphaviruses into cells. *Adv Virol 2011*.
Lounibos LP. 2002. Invasions by insect vectors of human disease. *Annu Rev Entomol 47*: 233-266.
Lu YE, Cassese T, Kielian M. 1999. The cholesterol requirement for sindbis virus entry and exit and characterization of a spike protein region involved in cholesterol dependence. *J Virol 73*: 4272-4278.
Lum FM, Teo TH, Lee WW, Kam YW, Rénia L, Ng LF. 2013. An essential role of antibodies in the control of Chikungunya virus infection. *J Immunol 190*: 6295-6302.
Martinez FO, Helming L, Gordon S. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol 27*: 451-483.
Marchette NJ, Rudnick A, Garcia R, Mac Vean S. 1978. Alphaviruses in Peninsular Malaysia. I. Virus isolations and animal serology. *Southeast Asian J Trop Med Public Health* 9: 317- 29.
McClelland GAH. 1974. A worldwide survey of variation in scale pattern of the abdominal tergum of *Aedes aegypti* (L.)(Diptera: Culicidae). *Trans R Entomol Soc Lond 126*: 239-259.
McCrae AWR, Henderson BE, Kirya BG, Sempala SDK. 1971. Chikungunya virus in the Entebbe area of Uganda: isolations and epidemiology. *Trans R Soc Trop Med Hyg 65*: 152-168.
McGill PE. 1995. Viral infections: α-viral arthropathy. *Bailliere's clinical rheumatology 9*: 145-150.
McIntosh BM. 1961. Susceptibility of some African wild rodents to infection with various arthropod-borne viruses. *Trans R Soc Trop Med Hyg* 55: 63–68.
McIntosh BM, Paterson HE, McGillivray G, De Sousa J. 1964. Further Studies on the Chikungunya Outbreak in Southern Rhodesia in 1962: I.—Mosquitoes, Wild Primates and Birds in Relation to the Epidemic. *Ann Trop Med Parasitol 58*: 45-51.
McIntosh BM, Jupp PG. 1970. Attempts to transmit chikungunya virus with six species of mosquito. *J Med Entomol 7*: 615-618.
McIntosh BM, Jupp PG, Dos Santos I. 1977. Rural Epidemic of Chikungunya in South Africa with Involvement of Ae s (Diceromyia) furcifer (Edwards). *S Afr J 73:* 267.
Medlock JM, Hansford KM, Schaffner F, Versteirt V, Hendrickx G, Zeller H, Bortel WV. 2012. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. *Vector-borne Zoonotic Dis 12*: 435-447.
Meerburg BG, Kijlstra A. 2009. Changing climate—changing pathogens: *Toxoplasma gondii* in North-Western Europe. *Parasitol Res 105*: 17-24.
Messaoudi I, Vomaske J, Totonchy T, Kreklywich CN, Haberthur K, Springgay L, ... Streblow, DN. 2013. Chikungunya virus infection results in higher and persistent viral replication in aged rhesus macaques due to defects in anti-viral immunity. *PLoS Negl Trop Dis 7*: e2343.
Mi S, Stollar V. 1991. Expression of Sindbis virus nsPl methyltransferase activity in *Escherichia coli*. *Virology* 184:423- 427.
Mi S, Stollar V. 1990. Both amino acid changes in nsP1 of Sindbis virusLM21 contribute to and are required for efficient expression of the mutant phenotype. *Virology 178*: 429-434.
Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol 8*: 958-969.

Morrison TE, Oko L, Montgomery SA, Whitmore AC, Lotstein AR, Gunn BM, ... Heise MT. 2011. A Mouse Model of Chikungunya Virus–Induced Musculoskeletal Inflammatory Disease: Evidence of Arthritis, Tenosynovitis, Myositis, and Persistence. *Am J Pathol 178*: 32-40.

Nakaya HI, Gardner J, Poo YS, Major L, Pulendran B, Suhrbier A. 2012. Gene profiling of Chikungunya virus arthritis in a mouse model reveals significant overlap with rheumatoid arthritis. *Arthritis Rheum 64*: 3553-3563.

Nitatpattana N, Kanjanopas K, Yoksan S, Satimai W, Vongba N, Langdatsuwan S, Nakgoi K, Ratchakum S, Wauquier N, Souris M, Auewarakul P. 2014. Long-term persistence of Chikungunya virus neutralizing antibodies in human populations of North Eastern Thailand. *Virol J 11*: 1.

Ng LF, Chow A, Sun YJ, Kwek DJ, Lim PL, Dimatatac F, ... Kourilsky P. 2009. IL-1β, IL-6, and RANTES as biomarkers of Chikungunya severity. *PloS One 4*: e4261.

Njenga MK, Nderitu L, Ledermann JP, Ndirangu A, Logue CH, Kelly CHL, Sang R, Sergon K, Breiman R, Powers AM. 2008. Tracking epidemic chikungunya virus into the Indian Ocean from East Africa. *J Gen Virol 89*: 2754-2760.

Osterrieth P, Blanes-Ridaura G. 1960. Research on the Chikungunya virus in the Belgian Congo. I. Isolation of the virus in upper Uele *Annales de la Societe belge de medecine tropicale (1920)*, *40*: 199-203.

Ozden S, Huerre M, Riviere JP, Coffey LL, Afonso PV, Mouly V, ... Jaffar MC. 2007. Human muscle satellite cells as targets of Chikungunya virus infection. *PloS One 2*: e527.

Palha N, Guivel-Benhassine F, Briolat V, Lutfalla G, Sourisseau M, Ellett F, ... Levraud JP. 2013. Real-time whole-body visualization of Chikungunya virus infection and host interferon response in zebrafish. *PLoS Path 9*: e1003619.

Pan American Health Organization. Chikungunya. *World Health Organization*, 12 Aug. 2016. Web.

Panning M, Hess M, Fischer W, Grywna K, Pfeffer M, Drosten C. 2009. Performance of the RealStar chikungunya virus real-time reverse transcription-PCR kit. *J Clin Microbiol* 47: 3014–6.

Pastorino B, Bessaud M, Grandadam M, Murri S, Tolou HJ, Peyrefitte CN. 2005. Development of a TaqMan® RT-PCR assay without RNA extraction step for the detection and quantification of African Chikungunya viruses. *J Virol Methods 124*: 65-71.

Pastorino B, Muyembe-Tamfum JJ, Bessaud M, Tock F, Tolou H, Durand JP, Peyrefitte CN. 2004. Epidemic resurgence of Chikungunya virus in Democratic Republic of the Congo: identification of a new central African strain. *J Med Virol 74*: 277-282.

Paul SD, Singh KRP. 1968. Experimental infection of *Macaca radiata* with Chikungunya virus and transmission of virus by mosquitoes. *Indian J Med Res 56*: 802-811.

Paupy C, Ollomo B, Kamgang B, Moutailler S, Rousset D, Demanou M, ... Simard F. 2010. Comparative role of *Aedes albopictus* and *Aedes aegypti* in the emergence of Dengue and Chikungunya in central Africa. *Vector-Borne Zoonotic Dis 10*: 259-266.

Peränen J, Rikkonen M, Liljeström P, Kääriäinen L. 1990. Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. *J Virol 64*: 1888-1896.

Pesko K, Westbrook CJ, Mores CN, Lounibos LP, Reiskind MH. 2009. Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to chikungunya virus. *J Med Entomol 46*: 395-399.

Petitdemange C, Becquart P, Wauquier N, Béziat V, Debré P, Leroy EM, Vieillard V. 2011. Unconventional repertoire profile is imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. *PLoS Path 7*: e1002268.

Petitdemange C, Wauquier N, Vieillard V. 2015. Control of immunopathology during chikungunya virus infection. *J Allergy Clin Immunol 135*: 846-855.

Pialoux G, Gaüzère BA, Jauréguiberry S, Strobel M. 2007. Chikungunya, an epidemic arbovirosis. *Lancet Infect Dis 7*: 319-327.

Poo YS, Rudd PA, Gardner J, Wilson JA, Larcher T, Colle MA, ... Bielefeldt-Ohmann H. 2014. Multiple immune factors are involved in controlling acute and chronic chikungunya virus infection. *PLoS Neg Trop Dis 8*: e3354.

Powell JR, Tabachnick WJ. 2013. History of domestication and spread of *Aedes aegypti*-A Review. *Memorias do Instituto Oswaldo Cruz 108*: 11-17.

Powers AM, Brault AC, Tesh RB, Weaver SC. 2000. Reemergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. *J Gen Virol* 81: 471–479.

Powers AM, Brault AC, Shirako Y, Strauss EG, Kang W, Strauss JH, Weaver SC. 2001. Evolutionary relationships and systematics of the alphaviruses. *J Virol* 75: 10118-31.

Powers AM, Logue CH. 2007. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J Genl Virol 88*: 2363-2377.

Rehermann B, Nascimbeni M. 2005. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 5: 215–29.

Reiter P, Fontenille D, Paupy C. 2006. *Aedes albopict*us as an epidemic vector of chikungunya virus: another emerging problem? *Lancet Infect Dis 6*: 463-464.

Robinson MC, 1955. An epidemic of virus disease in Southern Province, Tanganyika territory, in 1952–1953. *Trans R Soc Trop Med Hyg 49*: 28-32.

Ross RW. 1956. The Newala epidemic: III. The virus: isolation, pathogenic properties and relationship to the epidemic. *J Hyg 54*: 177-191.

Rudd PA, Wilson J, Gardner J, Larcher T, Babarit C, Le TT, ... Akira S. 2012. Interferon response factors 3 and 7 protect against Chikungunya virus hemorrhagic fever and shock. *J Virol 86*: 9888-9898.

Rupp JC, Sokoloski KJ, Gebhart NN, Hardy RW. 2015. Alphavirus RNA synthesis and non-structural protein functions. *J Gen Virol 96*: 2483-2500.

Russo AT, White MA, Watowich SJ. 2006. The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. *Structure 14*(9), 1449-1458.

Ryman KD, Klimstra WB, Nguyen KB, Biron CA, Johnston RE. 2000. Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. *J Virol 74*: 3366-3378.

Sam IC, Kümmerer BM, Chan YF, Roques P, Drosten C, AbuBakar S. 2015. Updates on chikungunya epidemiology, clinical disease, and diagnostics. *Vector-Borne and Zoonotic Dis 15*: 223-230.

Sawicki DL, Sawicki SG, Keränen S, Kääriäinen L. 1981. Specific Sindbis virus-coded function for minus-strand RNA synthesis. *J Virol 39*: 348-358.

Sawicki DL, Sawicki SG. 1993. A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis. *J Virol 67*: 3605-3610.

Sawicki DL, Perri S, Polo JM, Sawicki SG. 2006. Role for nsP2 proteins in the cessation of alphavirus minus-strand synthesis by host cells. *J Virol 80*: 360-371.

Schilte C, Couderc T, Chretien F, Sourisseau M, Gangneux N, Guivel-Benhassine F, ... Arenzana-Seisdedos F. 2010. Type I IFN controls chikungunya virus via its action on nonhematopoietic cells. *J Exp Med 207*: 429-442.

Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, ... Biscornet L. 2006. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med 3*: e263.

Schwartz O, Albert ML. 2010. Biology and pathogenesis of chikungunya virus. *Nature Rev Microbiol 8*: 491-500.

Sergon K, Yahaya AA, Brown J, Bedja SA, Mlindasse M. 2007. Seroprevalence of Chikungunya virus infection on Grande Comore Island, Union of the Comoros, 2005. *Am J Trop Med Hyg* 76: 1189-1193.

Shirako Y, Strauss JH. 1994. Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *J Virol 68*: 1874-1885.

Sissoko D, Malvy D, Ezzedine K, Renault P, Moscetti F, Ledrans M, Pierre V. 2009. Post-epidemic Chikungunya disease on Reunion Island: course of rheumatic manifestations and associated factors over a 15-month period. *PLoS Neg Trop Dis 3*: e389.

Sjöberg M, Garoff H. 2003. Interactions between the transmembrane segments of the alphavirus E1 and E2 proteins play a role in virus budding and fusion. *J Virol 77*: 3441-3450.

Skoging U, Vihinen M, Nilsson L, Liljeström P. 1996. Aromatic interactions define the binding of the alphavirus spike to its nucleocapsid. *Structure 4*: 519-529.

Skuse E, FAA. 1894. The banded mosquito of Bengal. *Indian Mus Notes* 30, 20.

Soden M, Vasudevan H, Roberts B, Coelen R, Hamlin G, Vasudevan S, La Brooy J. 2000. Detection of viral ribonucleic acid and histologic analysis of inflamed synovium in Ross River virus infection. *Arthrit Rheum 43*: 365.

Solignat M, Gay B, Higgs S, Briant L, Devaux C. 2009. Replication cycle of chikungunya: a re-emerging arbovirus. *Virology* 393: 183-197.

Sourisseau M, Schilte C, Casartelli N, Trouillet C, Guivel-Benhassine F, Rudnicka D, ... Frenkiel, M. P. 2007. Characterization of reemerging chikungunya virus. *PLoS Path 3*: e89.

Strauss JH, Strauss EG. 1994. The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev 58*: 491-562.

Srivastava U, Nelson M, Su YC, Mahalingam S. 2008. Mechanisms of Chikungunya virus disease informed by Ross River virus research. *Future Virol* 3: 509-11.

Stetson DB, Medzhitov R. 2006. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 24: 93-103.

Suhrbier A, Jaffar-Bandjee MC, Gasque P. 2012. Arthritogenic alphaviruses—an overview. *Nat Rev Rheumatol 8*: 420-429.

Suhrbier A, Mahalingam S. 2009. The Immunobiology of viral arthritides. *Pharmacol Ther* 124: 301-8.

Suopanki J, Sawicki DL, Sawicki SG. 1998. Regulation of alphavirus 26S mRNA transcription by replicase component nsP2. *J Gen Virol 79*: 309-319.

Tabachnick WJ, Powell JR. 1979. A world-wide survey of genetic variation in the yellow fever mosquito, *Aedes aegypti*. *Genet Res 34*: 215-229.

Takeuchi O, Akira S. 2007. Signaling pathways activated by microorganisms. *Curr Opin Cell Biol 19*: 185-191.

Teng TS, Kam YW, Tan JJ, Ng LF. 2011. Host response to Chikungunya virus and perspectives for immune-based therapies. *Future Virol 6*: 975-984.

Teo TH, Lum FM, Lee WW, Ng LF. 2012. Mouse models for Chikungunya virus: deciphering immune mechanisms responsible for disease and pathology. *Immunol Res 53*: 136-147.

Thiberville SD, Moyen N, Dupuis-Maguiraga L, Nougairede A, Gould EA, Roques P, de Lamballerie X. 2013. Chikungunya fever: epidemiology, clinical syndrome, pathogenesis and therapy. *Antiviral Res 99*: 345-370.

Tsetsarkin KA, Chen R, Leal G, Forrester N, Higgs S, Huang J, Weaver SC. 2011. Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes. *Proc Natl Acad Sci 108*: 7872-7877.

Tsetsarkin KA, Chen R, Yun R, Rossi SL, Plante KS, Guerbois M, ... Huang J. 2014. Multi-peaked adaptive landscape for chikungunya virus evolution predicts continued fitness optimization in *Aedes albopictus* mosquitoes. *Nat Commun 5*.

Tsetsarkin K, Higgs S, McGee CE, Lamballerie XD, Charrel RN, Vanlandingham DL. 2006. Infectious clones of Chikungunya virus (La Reunion isolate) for vector competence studies. *Vector-Borne Zoonotic Dis 6*: 325-337.

Tsetsarkin KA, McGee CE, Volk SM, Vanlandingham DL, Weaver SC, Higgs S. 2009. Epistatic roles of E2 glycoprotein mutations in adaption of chikungunya virus to *Aedes albopictus* and *Ae. aegypti* mosquitoes. *PLoS One 4*: e6835.

Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. 2007. A single mutation in chikungunya virus affects vector specificity and epidemic potential. *PLoS Path 3*: e201.
Van Duijl-Richter MK, Hoornweg TE, Rodenhuis-Zybert IA, Smit JM. 2015. Early Events in Chikungunya Virus Infection—From Virus Cell Binding to Membrane Fusion. *Viruses 7*: 3647-3674.
Vashishtha M, Phalen T, Marquardt MT, Ryu JS, Ng AC, Kielian M. 1998. A single point mutation controls the cholesterol dependence of Semliki Forest virus entry and exit. *J Cell Bio 140*: 91-99.
Vasiljeva L, Merits A, Auvinen P, Kääriäinen L. 2000. Identification of a Novel Function of the Alphavirus Capping Apparatus RNA 5′-Triphosphatase Activity of nsp2. *J Biol Chem 275*: 17281-17287.
Vazeille M, Moutailler S, Coudrier D, Rousseaux C, Khun H, Huerre M, ... Despres P. 2007. Two Chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. *PloS One 2*: e1168.
Volk SM, Chen R, Tsetsarkin KA, Adams AP, Garcia TI, Sall AA, ... Maharaj PD. 2010. Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. *J Virol 84*: 6497-6504.
Vourc'h G, Halos L, Desvars A, Boué F, Pascal M, Lecollinet S, Zientara S, Duval T, Nzonza A, Brémont M. 2014. Chikungunya antibodies detected in non-human primates and rats in three Indian Ocean islands after the 2006 ChikV outbreak. *Vet Res 45*: 1.
Wahlberg JM, Boere WA, Garoff H. 1989. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. *J Virol 63*: 4991-4997.
Wang YF, Sawicki SG, Sawicki DL. 1994. Alphavirus nsP3 functions to form replication complexes transcribing negative-strand RNA. *J Virol 68*: 6466-6475.
Wang YF, Sawicki SG, Sawicki DL. 1991. Sindbis virus nsP1 functions in negative-strand RNA synthesis. *J Virol 65*: 985-988.
Wang E, Volkova E, Adams AP, Forrester N, Xiao SY, Frolov I, Weaver SC. 2008. Chimeric alphavirus vaccine candidates for chikungunya. *Vaccine 26*: 5030-5039.
Warrier R, Linger BR, Golden BL, Kuhn RJ. 2008. Role of sindbis virus capsid protein region II in nucleocapsid core assembly and encapsidation of genomic RNA. *J Virol 82*: 4461-4470.
Wauquier N, Becquart P, Nkoghe D, Padilla C, Ndjoyi-Mbiguino A, Leroy EM. 2011. The acute phase of Chikungunya virus infection in humans is associated with strong innate immunity and T CD8 cell activation. *J Infect Dis 204*: 115-123.
Weaver SC, Osorio JE, Livengood JA, Chen R, Stinchcomb DT. 2012. Chikungunya virus and prospects for a vaccine. *Expert review of vaccines 11*:1087-1101.
Weaver SC, Lecuit M. 2015. Chikungunya virus and the global spread of a mosquito-borne disease. *New England J Med 372*: 1231-1239.
Werneke SW, Schilte C, Rohatgi A, Monte KJ, Michault A, Arenzana-Seisdedos F, ... Lenschow DJ. 2011. ISG15 is critical in the control of Chikungunya virus infection independent of UbE1L mediated conjugation. *PLoS Path 7*: e1002322.
Yeo LS, Chu JJH. 2013. Recent developments and challenges in mouse models of Chikungunya virus infection. *Future Virol 8*: 423-426.
Yergolkar PN, Tandale BV, Arankalle VA, Sathe PS, Sudeep AB, Gandhe SS, ... Mishra AC. 2006. Chikungunya outbreaks caused by African genotype, India. *Emerg Infect Dis 12*: 1580.
Zhang W, Mukhopadhyay S, Pletnev SV, Baker TS, Kuhn RJ, Rossmann MG. 2002. Placement of the structural proteins in Sindbis virus. *J Virol 76*: 11645-11658.
Ziegler SA, Lu L, da Rosa APT, Xiao SY, Tesh RB. 2008. An animal model for studying the pathogenesis of chikungunya virus infection. *Amer J Trop Med Hyg 79*: 133-139.

Zim MM, Sam IC, Omar SS, Chan YF, AbuBakar S, Kamarulzaman A. 2013. Chikungunya infection in Malaysia: comparison with dengue infection in adults and predictors of persistent arthralgia. *J Clin Virol 56*: 141-145.

Zouache K, Fontaine A, Vega-Rua A, Mousson L, Thiberge JM, Lourenco-De-Oliveira R, ... Failloux AB. 2014. Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential. *Proc R Soc Lond B: Biol Sci 281*: 20141078.

CHAPTER TWO

EXPERIMENTAL INFECTION OF NORTH AMERICAN RODENTS WITH CHIKUNGUNYA VIRUSES

2.1 Introduction

The emergence and re-emergence of infectious diseases is largely driven by environmental, ecological, and socio-economic drivers (Jones et al., 2008; Johnson et al. 2015; Wiethoelter et al., 2015). The emergence and subsequent spread of these pathogens have a significant global impact on economies and human health. Chikungunya virus (CHIKV) is an emerging arthropod-borne alphavirus (*Togaviridae*) endemic to Africa, Southeast Asia, and several islands in the Indian Ocean. In the past decade, global travel, the presence of both native and invasive competent vector(s), and increased human contact has facilitated the emergence of CHIKV into the Western hemisphere. As a result, widespread epidemics of CHIKV infection have been reported in 45 countries affecting 1.7 million people throughout the Americas and Caribbean islands (Pan America, August 2016). Viral pathogens, especially RNA viruses like CHIKV, pose the highest probability of global pandemics due to their high rate of nucleotide substitution and lack of mutation correction machinery (Taylor et al., 2001; Woolhouse et al., 2005; Burke et al., 1998; Cleaveland et al., 2001; Jones et al., 2008). These mutations enhance the ability of these viruses to adapt to new vector(s) and host species, and managing emerging infectious diseases requires a clear understanding of both sylvatic and urban transmission cycles.

Chikungunya virus was originally discovered during a dengue-like outbreak in present day Tanzania by Dr. Marion Robinson (Robinson, 1955). It is believed that CHIKV originated in forest dwelling non-human primates in Africa and is maintained in a sylvatic cycle involving *Aedes spp.* mosquitoes. Presumably, spillover of CHIKV from sylvatic transmission to urban human-mosquito-human transmission cycles occurred, associated with land-use changes, deforestation, human encroachment, and agricultural farming encouraged greater wildlife-human interactions (Jones et al., 2008; Perry et al., 2013; Bengis et al., 2002; Wiethoelter et al., 2015). In Asia, CHIKV is primarily

transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes in urban cycles, although wild non-human primates in Malaysia have been seropositive for CHIKV (Inoue, et al., 2003; Marchette et al., 1980). The spread of CHIKV into the Americas highlights the ease with which arboviruses are transported via infected global travelers to new naïve geographic locations and become endemic in a relatively short time frame. Like most RNA viruses, CHIKV has the ability to mutate and adapt to utilize novel vectors and vertebrate host species.

The objective of this study was to identify potential reservoir or amplifying hosts among several taxa of wild rodent located throughout the Americas using the approach of experimental infection. Studies evaluating and identifying reservoir host species for CHIKV will help advance our understanding of CHIKF disease tropism and host disease tolerance.

2.2 Materials and Methods

2.2.1 Animal Collection and Husbandry

Animals were chosen for CHIKV experimental infection trials based on their abundance, availability, and ability to represent broader rodent taxonomic groups. Rodents of nine species from five families were collected (N=4 to 20 individuals per species) for this study. The geographic distribution of these animals is presented in **Table 2.1** and details on their acquisition in **Table 2.2**.

Table 2.1. Overview of rodent species examined including taxonomic descriptions and geographic distribution.

Species	Family	Genus	Species	Distribution of Genus
Nutria	Myocastoridae	*Myocastor*	*Coypus*	Introduced into U.S. during the late 19th century for fur farming; native to subtropical and temperate South America.
Norway rat	Muridae	*Rattus*	*norvegicus*	Most likely originating on the plains of Asia, China, and Mongolia and now exist worldwide.
Fox squirrel	Sciuridae	*Sciurus*	*Niger*	Occur sympatrically throughout the eastern and central U.S., west to the Dakotas, Colorado, and Texas.
Deer mice	Cricetidae	*Peromyscus*	*maniculatus*	Alaska, Quebec, New Brunswick, central Mexico, and east to Georgia.
Brushy-tailed woodrat	Cricetidae	*Neotoma*	*Cinerea*	British Columbia, Alberta, to northern Arizona and New Mexico, and the Dakotas.
Cotton rat	Cricetidae	*Sigmodon*	*Hispidus*	Southeastern U.S. and west to Utah, and into South America.
Meadow voles	Cricetidae	*Microtus*	*Pennsylvanicus*	North to Canada, west to Alaska, and to South Carolina. Subset occurs in central Colorado to northwest New Mexico.
Prairie voles	Cricetidae	*Microtus*	*orchrogaster*	Central Canada to northern New Mexico, and West Virginia.
Groundhog	Sciuridae	*Marmota*	*monax*	Canada to Kansas and across south to parts of Alabama.

Table 2.2 Source of rodents experimentally infected with CHIKV.

Common Name	**Scientific Name**	**Source**
Cotton rats	*Sigmodon hispidus*	Colony, Miami University
Prairie vole	*Microtus orchrogaster*	Colony, Texas Tech University
Meadow vole	*Microtus pennsylvanicus*	Live trapped, Colorado
Deer mice	*Peromyscus maniculatus*	Colony, Colorado State University
Nutria	*Myocastor coypus*	Live trapped, Louisiana
Brushy-tailed wood rat	*Neotoma cinerea*	Live trapped, Colorado
Norway rat	*Rattus norvegicus*	Live trapped, Colorado
Groundhogs	*Marmota monax*	Live trapped, New York
Fox squirrels	*Sciurus niger*	Live trapped, Colorado

Animals were given an individual identification number and bled upon arrival, and then caged or allowed free-range in rooms with clean water and food *ad libitum*. Diet varied by species and consisted of alfalfa pellets, commercial laboratory chow, seed mix (sunflower, cracked corn, peanuts, millet, milo,), carrots, apples, potatoes, yams, and celery root. Aquatic rodents were provided with pools of fresh water. Arboreal rodent species were given wooden perches to climb. Rodents were acclimated to captivity for at least 1 week before inoculation. Animals ranged in age from weaned offspring to adults. This work was approved by the Animal Care and Use Committee at Colorado State University (approval 14-5258A) and conducted in strict accordance with the NIH Guide for Care and Use of Laboratory Animals.

2.2.2 Cell Culture

Vero cells were maintained in accordance to commonly used cell culture techniques (Bosco-Lauth et al., 2015). Cells were grown at 37 °C with 5% carbon dioxide in Dulbecco's minimum essential medium (DMEM) supplemented with 4% fetal bovine serum, 0.225% sodium bicarbonate, and antibiotics (Bosco-Lauth et al., 2015).

2.2.3 Virus Strains

Two strains of CHIKV were provided by Ann Powers at the Centers for Disease Control and Prevention, Fort Collins, CO. The SAH2123 (SAH) strain was isolated from a human patient in South Africa in 1976 and had been passaged three times in Vero cells. The COM2005 (COM) virus was isolated from mosquito pools collected during the Comoros Island outbreak in 2005 and had been passaged twice in suckling mice and seven times in Vero cells. Both of these strains are members of the East-Central-South African clade of CHIKV.

2.2.4 Experimental Infection and Sample Collection

Rodents were separated into rooms and/or cages depending upon the size of the species and inoculating strain of virus. Virus inoculation was performed by subcutaneous injection (0.1 mL) on the right hock and the inoculum dose was between $10^4 – 10^5$ plaque-forming units (PFU). Following inoculation, all animals were monitored daily for clinical signs of disease and bled (0.05 to 0.5 mL), depending on species, via tail-bleed, vena cava, saphenous vein, or submandibular daily for up to 7 days post inoculation (DPI), and at 14 and 21 DPI. The total amount of blood taken did not exceed 10% blood volume. Norway rats and voles were bled and euthanized serially (groups of 2 or 3 animals) on 1-5 and 21 DPI. After sampling, blood samples for smaller rodent species (deer mice, meadow voles, and prairie voles) were placed in BA-1 medium (M199-Hank's salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100U/mL penicillin, 100 ug/mL streptomycin, 2.5 ug/mL amphotericin B in 0.05 M Tris, pH 7.6). Blood was centrifuged for 3 minutes at 12,000 x g for serum separation. All samples were stored at -80°C until virus isolation and antibody testing. Nutria and groundhogs were sedated with ketamine-

xylazine for bleeding, and groundhogs had subcutaneously-implant transponders (LifeChips, Detron, NSW, Australia) that were used to measure body temperature at each sampling point.

2.2.5 Necropsy and Tissue Processing

Animals were euthanized by intravenous or intraperitoneal over dose of pentobarbital on 21 DPI or serially on days (2, 3, 4, 7, 10 DPI) (Norway rats, meadow voles, and prairie voles) depending upon the number of animals available. Animals were necropsied within two hours of euthanasia and the following tissues were collected: heart, liver, lung, spleen, kidney, quadriceps muscle, forelimb, hind limbs, small intestine, and brain. Tissues were weighed (100 ug) and added to 1 ml of BA-1 medium containing 20% fetal bovine serum. One copper BB was then added to each tube of tissue and homogenized using a mixer mill (Retsch GmbH, Haan, Germany) at 25 cycles/second for 5 minutes). Supernatant was then separated from the tissue pellet by centrifugation (12,000 x g for 3 minutes). Samples were then stored at -80°C until processing. Tissue samples collected from animals with low or absent viremia titers were not tested and tissues collected after 21 DPI were not tested.

2.2.6 Virus Isolation

Vero cell plaque assays were used to determine viremia in animal serum and back titrate virus inocula as described (Robinson, 1955; Lumsden, 1955; Soumahoro et al., 2009; Ng and Haparachchi, 2010; Her et al., 2009; Schuffenecker et al., 2006; Bosco-Lauth et al., 2015). Briefly, Vero cells were grown in 6-well plates in Dulbecco's Modified Eagle Medium (DMEM) containing 7.5% bovine calf serum and antibiotics. For virus titration, serum samples were serially diluted in 10-fold increments beginning with a 1:10 serum in BA-1 dilution and 0.1 mL of each dilution inoculated per well. Inoculated cells were incubated at 37°C for one hour prior to addition of a 2 mL overlay per well of Minimum Essential Media (MEM) and agarose supplemented with 4% fetal bovine serum, sodium bicarbonate, and antibiotics. Plates were then incubated for 24 hours at 37°C, after which a second overlay containing 0.004% neutral red dye was added. After 24 and 48 hours plates plaques were counted. The detection limit for this assay was 100 PFU/mL.

2.2.7 Serology

Pre and post inoculation sera from all CHIKV-challenged animals were tested for anti-CHIKV neutralizing antibodies by plaque reduction neutralization testing (PRNT) on Vero cells in 6 well plates as described by Beaty et al, 1995. Serum samples were heat-inactivated at 56°C for 30 minutes and serial two-fold dilutions from 5 to 160 were prepared in 96-well plates in BA-1 medium. An equal amount of CHIKV SAH2123, diluted to approximately 100 pfu/mL, and the afore mentioned serial dilutions were incubated for 1 hour at 37°C. Samples were then inoculated at 100 uL per well onto 6 well plates of Vero cells and incubated at 37°C for one hour. The wells then received a first and second overlay as described above for plaque assay, and plaques were counted one day after the second overlay. Antibody-free control wells received virus diluted in BA1. Antibody titers were calculated as a reciprocal of the highest serum dilution with ≥ 80% reduction ($PRNT_{80}$) of viral plaques compared to control wells. Sera with ≥80% neutralization of CHIKV were considered positive.

2.3 Results

2.3.1 Clinical Responses

Throughout the course of the studies, none of the animals displayed clinical signs of disease attributed to CHIKV infection.

2.3.2 Viremia, Tissue Virus Burdens, and Serologic Responses

Detectable viremia was not observed for nutria, brushy-tailed woodrats, and Norway rats. Variable portions of other species developed viremia (as calculated by PA in sera), with the highest proportion of these occurring in cotton rats (75%) and deer mice (42%). Species for which at least one animal developed detectable viremia by 5 DPI included fox squirrels, deer mice, cotton rats, meadow voles, prairie voles, and groundhogs (**Table 2.3**). In many cases, viremia was observed on a single post-inoculation day. For example, between the two groups of 12 fox squirrels inoculated with two strains of CHIKV SAH and COM, only one adult male squirrel inoculated with SAH was viremic (500 pfu/mL) on

day 1 post inoculation. Although there were too few viremic animals for statistical testing, it did not appear that animal sex was a significant factor in development of viremia.

Table 2.3. Summary of viremia titers and antibody responses in rodents experimentally inoculated with chikungunya viruses.

Species	Sex Ratio	Virus strain	Number tested	Number viremic (range of peak viremia*)	Number PRNT positive at 21 DPI	$PRNT_{80}$ antibody titer range
Nutria	2 F	SAH	2	0/2	50 (1/2)	10
	1 F, 1 M	COM	2	0/2	50 (1/2)	10
Norway rat	10 F, 10 M	SAH	20	0 /20	60 (3/5)	40
Fox squirrel	2 F, 4 M	SAH	6	1/6 (5E2)	83 (5/6)	10 – 40
	3 F, 3 M	COM	6	0/6	0 (0/6)	< 10
Deer mice	3 F, 3 M	SAH	6	3/6 (2.7E3-5E3)	0 (0/6)	≥10-20
	3 F, 3 M	COM	6	3/6 (1.1E3-3.1E3)	33 (2/6)	≥20-40
Brushy-tailed woodrat	2 F, 1 M	SAH	3	0/3	33 (1/3)	10
	3 F	COM	3	0/3	33 (1/3)	≥20
Cotton rat	3 F, 3 M	SAH	6	5/6 (2E2-7.7E5)	66 (4/6)	10 – 40
	3 F, 3 M	COM	6	4/6 (1.3E3-5.7E5)	33 (2/6)	≥20
Meadow vole	4 F, 3 M	SAH	7	2/3 (4E2-6.3E4)	0 (0/1)	< 10
Prairie vole	11 F, 12 M	SAH	23	3/4 (2.5E3-2.6E7)	0 (0/5)	< 10
Groundhog	4 M	SAH	4	4/4 (4E2-1.6E6)	100 (4/4)	≥320

DPI = days postinoculation; PRNT = plaque reduction neutralization tests.

* Peak viremia titer in $\log_{10}$ plaque-forming units/mL serum.

As expected, none of the animals manifest viremia on the day of inoculation (0 DPI). With the exception of one groundhog, the duration of viremia was one to two days, and viremia was not detected after 4 DPI, as depicted in **Table 2.4**.

Table 2.4. Viremia titers over time after virus inoculation in animals that developed detectable viremia.

Animal	**Virus Strain**	**1 DPI***	**2 DPI**	**3 DPI**	**4 DPI**	**6 DPI**
Meadow Vole 1	SAH	2E5	-	-	-	-
Meadow Vole 2	SAH	6.3E4	-	-	-	-
Meadow Vole 6	SAH	-	-	4E2	-	-
Prairie Vole 1	SAH	1.2E4	-	-	-	-
Prairie Vole 2	SAH	1.7E5	-	-	-	-
Prairie Vole 3	SAH	6.1E5	-	-	-	-
Prairie Vole 4	SAH	2.6E7	-	-	-	-
Prairie Vole 8	SAH	-	-	2.5E3	-	-
Fox Squirrel 2	SAH	5E2	-	-	-	-
Deer Mouse 2	SAH	<100	<100	<100	4.4E3	<100
Deer Mouse 5	SAH	<100	5.0E3	<100	<100	<100
Deer Mouse 6	SAH	<100	2.7E3	<100	<100	<100
Deer Mouse 1	COM	<100	6.0E2	<100	<100	<100
Deer Mouse 4	COM	<100	<100	<100	3.1E3	1.1E3
Cotton Rat 1	SAH	<100	2E2	<100	<100	<100
Cotton Rat 2	SAH	<100	3.7E5	<100	<100	<100
Cotton Rat 3	SAH	<100	5.3E4	<100	<100	<100
Cotton Rat 4	SAH	<100	7.7E5	<100	<100	<100
Cotton Rat 5	SAH	<100	1.8E3	<100	<100	<100
Cotton Rat 3	COM	<100	5.7E5	<100	<100	<100
Cotton Rat 4	COM	<100	3.5E4	<100	<100	<100
Cotton Rat 5	COM	<100	1.3E3	<100	<100	<100
Cotton Rat 6	COM	<100	1.3E5	<100	<100	<100
Groundhog 8579	SAH	<10	2.1E5	1.3E5	4.0E2	<10
Groundhog 8641	SAH	6.8E4	1.1E3	<10	<10	<10
Groundhog 8660	SAH	1.6E6	2.4E5	1.2E4	2.3E3	<10
Groundhog 8665	SAH	1.7E4	1.9E3	<10	<10	<10

* Data indicate PFU/mL serum.

Groups of meadow vials, prairie voles, and Norway rats were euthanized at daily intervals following virus inoculation and tissues (skin from the inoculate site, heart, liver, lung, spleen, kidney, quadriceps muscle, small intestine, and brain) were homogenized and titrated by plaque assay. Comparison of tissue titers of virus versus viremia in the same animal indicated that significant virus replication took place in skin, muscle, and liver (**Table 2.5**). None of the tissues from Norway rats contained detectable infectious virus.

Table 2.5. Virus titers in tissue homogenates shown from voles. Organs not listed here did not have detectable virus titers.

Animal	Animal number	Organ	DPI	Tissue virus (PFU/gram)	Viremia (PFU/mL)
Meadow Vole	1	Skin	1	9E4	2E5
	1	Spleen	1	3E2	2E5
	2	Skin	1	2E5	6.3E4
	4	Skin	2	3E2	<100
	6	Muscle	3	3.9E7	4E2
Prairie Vole	1	Muscle	1	2E4	1.2E4
	1	Liver	1	1E4	1.2E4
	1	Skin	1	4E4	1.2E4
	2	Skin	1	8.2E6	1.7E5
	2	Liver	1	1E4	1.7E5
	3	Kidney	1	7E4	6.1E5
	3	Lung	1	4E4	6.1E5
	3	Skin	1	7E3	6.1E5
	6	Skin	2	1.1E6	<100
	6	Spleen	2	2.4E4	<100
	6	Liver	2	7E4	<100
	6	Muscle	2	1E2	<100
	7	Muscle	2	1.1E5	<100
	7	Skin	2	2E5	<100

Among the animals tested, groundhogs had the highest titer and, in two animals, longest viremia. As with other animals evaluated however, they failed to display clinical signs of disease and fever associated with infection was not obvious (**Figure 2.1**).

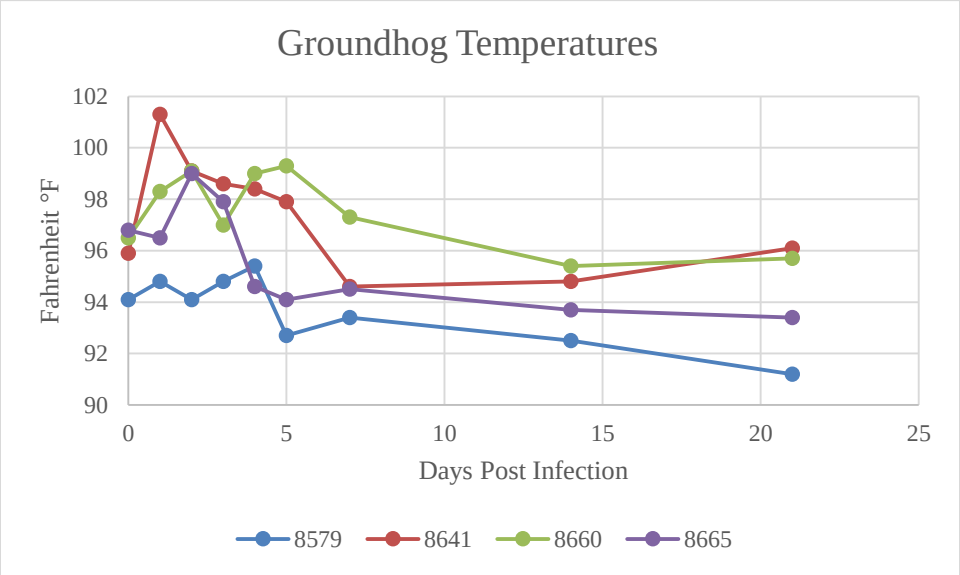

Figure 2.1. Groundhog body temperatures from 0 to 21 days post infection.

2.4 Discussion

This study providence evidence for CHIKV replication and development of viremia in several species of rodents during experimental infections, including cotton rats, deer mice, prairie voles, meadow voles, fox squirrels, and groundhogs. Identifying rodent species susceptible to CHIKV infection is crucial in investigating the role rodents may play in maintaining CHIKV in both endemic and novel geographic sylvatic cycles and in exploiting new animal models that accurately recapitulate human CHIKF pathogenesis.

The results of this study show that rodents from 6 out of 9 species tested became infected and developed viremia following subcutaneous inoculation with CHIKV strains COM and/or SAH). However, CHIKV infection in these animals did not cause overt signs of disease. Among the 6 species examined, groundhogs developed the highest CHIKV viremia titer (up to 10^6 pfu) lasting from 2-4 days post infection. The CHIKV viral titers observed in these adult groundhogs should be sufficient to infect

Ae. albopictus mosquitoes (Pesko et al., 2009), indicating that groundhogs may be capable of serving as reservoir hosts. Groundhogs may also be capable of overwintering CHIKV if these animals are infected prior to or during hibernation.

Some endothermic mammals, such as groundhogs, enter periods of dormancy (hibernation) throughout winter months when food supplies are scare and energy demands increase (Bouma et al., 2010). The exact mechanisms leading to hibernation are unclear; however, it has been suggested that several factors are linked to its' induction including: a lack of food, alteration in circadian rhythms, and increased lipid metabolism. Hibernation consist of euthermic periods (arousal lasting < 24 hours) and periods of hibernation (lasting several days to week) when individuals' body temperatures are typically around 10°C and have been recorded as low as -2°C (Schmaljoh and Hjelle, 1997; Storey, 1997; Carey et al., 2003). Interestingly, the repetitive cycles of cooling and rewarming do not cause any gross histological damage in hibernating animals; although apoptosis and necrosis has been observed in non-hibernating animals experiencing similar extreme temperatures (Arendt et al., 2003; Fleck and Carey 2005; Sandovici et al., 2004). One adaptation that may help hibernating animals to avoid tissue damage is the suppression of the immune system (Bouma et al., 2013). During hibernation the functions of the innate and adaptive immune systems of mammals' are drastically reduced (Bouma et al., 2010). As the body temperature of hibernating animals decreases, the innate immune system undergoes a reduction in circulating neutrophils and monocytes (Bouma et al., 2010; Frerichs et al., 1994; Reznik et al., 1975; Spurrier and Dawe, 1973). This limits humoral immune responses, which could lead to overwintering of viruses such as CHIKV that would normally be cleared rapidly during warmer seasons. The adaptive immune response in hibernating animals is also limited by a reduction in lymphocyte production before and during hibernation and a diminished ability to produce cytokines such as TNF and IFN. While research investigating the immune functions of hibernating mammals is scare, experimental inoculations of Golden-mantled ground squirrels with Lipopolysaccharides (LPS), a main component of gram negative bacterial cellular walls, did not induce febrile illness during torpor (Prendegast et al., 2002).

Additionally, peritoneal macrophages from artic ground squirrels maintained 37°C demonstrated that TNF production significantly decreased prior to and during hibernation, and returned to normal levels during the summer (emergence from hibernation; Novoselova et al., 2000). However, if Golden-mantled ground squirrels were injected with the cytokine PGE1, a signaling molecule that induces fever, these individuals developed a fever and immediately emerged from hibernation (Prendegast et al., 2002). Thus, it is thought that the lack of response to LPS injection was due to a lack of macrophages and an inability to produce cytokines in hibernating animals (Prendegast et al., 2002). Other evidence for a reduction in immune functions during hibernation can be seen in skin allograph rejections following emergence from hibernation. Skin allographs transplanted into hibernating 13-lined ground squirrels were not rejected until the animals fully emerged from hibernation (Shivatcheva, 1988). This evidence suggests that the adaptive cellular immune response was inhibited during hibernation in these animals (Shivatcheva, 1988). Furthermore, most pathogens do not replicate well at low temperatures; however one notable exception is White Nose Syndrome in bats (Bouma et al., 2010). While scientists do not completely understand the reasons for immune suppression in hibernating mammal, it probably aids in energy conservation and prevents cellular damage to vital organs. This evidence suggests that groundhogs infected with CHIKV prior to or during hibernation would be able to maintain an uninhibited CHIKV viral infection since the production of type I IFNs, mainly IFN-α and IFN-β, restrict and/or inhibit CHIKV replication and spread would be suppressed. Additionally, monocytes/macrophages appear to serve as vehicles for viral dissemination and mediate inflammation in synovial tissues during CHIKV infection. With the production of monocytes/macrophages uninhibited it is plausible that infected groundhogs would exhibited increased levels of viral replication in vital organs such as the brain, heart, liver, kidneys and peripheral tissues.

While laboratory infections, such as those reported here, do not completely replicate natural disease transmission cycles due to their highly controlled and often unnatural inoculation routes; environmental studies investigating arbovirus prevalence in natural systems provides evidence for small

bird and mammal involvement in arbovirus sylvatic transmission cycles. Once infected, these species develop viral titers capable of infecting competent vector species during blood feeding. The majority of these arboviruses do not cause overt signs of disease following infection but instead, use these hosts as reservoirs or incidental hosts (Williams and Barker, 2001). The role of vertebrate hosts in the epidemiology of many arboviruses remains unclear and further research is needed. Here I will discuss several arboviruses that have been found to circulate, to some degree, in small rodent hosts in order to demonstrate that it is possible that CHIKV is circulating at some level in wild rodent populations in endemic and epidemic areas.

Saint Louis encephalitis virus (SLEV) is an arthropod-borne flavivirus endemic to North America (Williams and Barker, 2001). Although birds primarily serve as the amplifying host for SLEV throughout North America, wild mammals may play a role in secondary transmission cycles. In the Southeastern United States, SLEV and antibodies have been detected in raccoons (*Procyon lotor*), Virginia opossums (*Didelphis virginiana*), and cotton rats (*Sigmodon hispidus*) (McLean and Bowen, 1980). Additionally, SLEV antibodies were detected in cotton mice (*Peromyscus gossypinus*) and armadillos (*Dasypus novemcinctus*) in Florida (Bigler and Hoff 1975; Day et al., 1996). In Ohio, SLEV antibodies have been isolated from big brown bats (*Eptesicus fuscus*) and little brown bats (*Myotis lucifugus*). During experimental SLEV infections, bats have been shown to overwinter the virus through hibernation and develop viremias following arousal (Herbold et al., 1983). On the Pacific coast of the U.S., antibodies have been detected in black-tailed jackrabbits (*Lepus californicus*), snowshoe hares *(Lepus americanus*), Nuttall's cottontails (*Sylvilagus nuttallii)*, Fresno and Heerman's kangaroo rats (*Dipodoms nitrtoides*) and (*Dipodomys heermanni*), southern grasshopper mice (*Onychomys torridus*), deer mice (*Peromyscus maniculatus*), harvest mice (*Reithrodontomys megalotis*), San Joachin antelope ground squirrels (*Ammospermophilus nelson*), California bellied marmots (*Marmota flaviventris*), wood rats (*Neotoma fuscipes*), pocket gophers (*Thomoys talpoides*), house mice (*Mus musculus*), black rats (*Rattus rattus*), and Norway rats (*Rattus norvegicus*) (Hardy et al., 1974; McLean and Bowen, 1980). In South and

Central America SLEV has been isolated from a vesper mouse (*Calomys musculinus*), house mouse, grass mouse (*Akodon arviculoides*), rice rat (*Orzomys nigripes*), three-toed sloth (*Bradypus variegation*), spiny rat (*Proechimys semispinosus*), and neotropical fruit bat (*Artibeus jamaicensis*) (Hardy et al., 1974; McLean and Bowen 1980; Trainer and Hanson 1969; Trainer and Hoff 1971; Hoff et al., 1970, 1973; Seymor et al., 1983; De Rodaniche and Galindo, 1961; Ubico and McLean 1995).
Experimental infections of wild mammals with SLEV by peripheral routes has not been shown to cause clinical signs of disease however, species known to naturally become infected have consistently produced viremia and antibodies (Herbold et al., 1983; Kokernot et al., 1969). SLEV experimental infection studies in Mexican free-tailed bats, cotton rats, and black-tailed jackrabbits have demonstrated variable results in which some, but not all animals develop viremias or antibodies (Sulkin et al., 1963; McLean and Bowen 1980).

Snowshoe hare virus (SSHV) is a variant of La Crosse virus and is a member of the *Bunyaviridae* family. It occurs from Alaska to Canada and into New York State (Artsob 1983; Srihongse et al., 1984; Calisher et al., 1986). SSHV is a mosquito-borne virus vectored by *Culiseta* and at least 10 *Aedes spp.* of mosquitoes (Newhouse et al., 1967; Iversen et al., 1969; McLean et al., 1970). The primary vertebrate host for SSHV are snowshoe hares. However, SSHV has been isolated from northern red-backed voles (*Clethrionomys rutilus*) and collared lemmings (*Dicrostonyx rubicans*) (Ritter and Feltz, 1974). Antibodies have also been detected in Arctic ground squirrels (*Spermophilus parryii*), Golden-mantled squirrels (*Spermophilus lateralis*), Yellow-bellied marmots, Yellow-pine chipmunks (*Eutamias amoenus*), black bears (*Ursus americanus*), bighorn sheep (*Ovis canadensis*), red fox (*Vulpes vulpes*), Norway rat, and common vole (*Microtus arvalis*) (Ritter and Feltz, 1974; McLean et al., 1971; Zarnke and Yuill, 1981).

Western equine encephalitis virus (WEEV) is a member of the Alphavirus genus in the Togaviridae family. The primary vector of WEEV is *Culex tarsalis*, although *Aedes melanimon* is also a vector (Hardy et al., 1976; Hardy et al., 1974). Although birds are thought to be the primary vertebrate

hosts for WEEV wild rodents may play a role in some areas (Holden et al., 1973). *Culex tarsalis* primarily feeds on birds however, in some regions this species has been documented to feed on black-tailed jackrabbits (Nelson et al., 1976). WEEV has also been isolated sporadically from western gray squirrels (*Sciurus griseus*), California ground squirrels, San Joachin antelope ground squirrels, house mice, Virginia opossum, Richardson's ground squirrel (*Spermopilus richardsonii*), and vole (*Microtus spp.*) (Karabatsos, 1985; Hardy et al., 1974; Smart et al. 1972). The role these mammals play in WEEV remains unclear, however some have been shown to harbor the virus until spring (Hardy et al., 1974). This may allow WEEV to overwinter in temperate zones.

Since its discovery in 1952, CHIKV received minimal attention by the scientific community until it caused large epidemic outbreaks. For instance, the role of non-human vertebrates during sylvatic transmission cycles remains largely unknown. In Africa and Asia, serological evidence suggests that non-human primates, rodents, and birds may maintain CHIKV during interepidemic periods (Halstead et al. 1966; Marchette et al., 1980, Apandi et al., 2010; Sam et al., 2015; Wolfe et al., 2001; Vourch et al., 2014; McIntosh et al., 1961; Inoue et al., 2003; MacKenzie et al., 2013; Diallo et al., 1999; Kading et al., 2013). Following the circulation of CHIKV throughout susceptible primate populations, it may take several years for a naïve subset of individuals to either be born or migrate from non-endemic areas. During these periods it is plausible that rodents and other wildlife species may maintain CHIKV in a secondary sylvatic cycle in endemic and non-endemic areas; while human and non-human primate populations remain immunologically protected and/or in areas with low populations of humans and primate hosts (Diallo et al., 1999). It is also possible that wildlife reservoirs, including rodents, may serve as amplifying hosts for this virus while not displaying overt physiological signs of disease. The extent of involvement of these alternative reservoir hosts during CHIKV transmission cycles remains unknown. The results of the studies reported here show that some rodents are capable of developing CHIKV viremia and, among the species examined, several developed a viremia of sufficient magnitude to possibly infect susceptible vector hosts. Further serological surveillance and experimental infection studies with various

rodent species are crucial in elucidating ecological and epidemiological drivers of CHIKV outbreaks. Understanding the natural ecology of CHIKV in both emerging and endemic zoonotic transmission cycles is critical in predicting, preventing, and mitigating human and veterinary disease.

REFERENCES

Apandi Y, Izmawati SK, Amal NM, Faudzi Y, Mansor W, ... Zainah S. 2010. Identification of Chikungunya virus strains circulating in Kelantan, Malaysia in 2009. *Southeast Asian J Trop Med Public Health 41*: 1374.

Arendt T, Stieler J, Strijkstra AM, Hut RA, Rudiger J, … Hartig W. 2003. Reversible paired helical filament-like phosphorylation of tau is an adaptive process associated with neuronal plasticity in hibernating animals. *J Neurosci* 23: 6972-6981.

Artsob H, Spence L, Th'ng C, Lampotang V, Johnson D, … Watt I. 1986. Arbovirus infections in several Ontario mammals. *Canadian J Vet Res* 50: 42-46.

Beaty BJ, Calisher CH, Shope RE. 1995. Diagnostic procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette E.H., Lennette D.A, Lennette E.T. eds *Arboviruses*. Washington, D.C.: American Public Health Association 189-212.

Bengis RG, Kock RA, Fischer J. 2002. Infectious animal diseases: the wildlife/livestock interface. *Rev Sci Tech OIE 21*: 53-66.

Bigler WJ, Hoff GF. 1975. Arbovirus surveillance in Florida: Wild vertebrate studies, 1965-1974. *J Wild Dis* 11: 348-356.

Bouma HR, Carey HV, Kroese FGM. 2010. Hibernation: the immune system at rest? *J Leukoc Biol* 88: 619-624.

Bouma HR, Henning RH, Kroese FGM, Carey HV. 2013. Hibernation is associated with depression of T-cell independent humoral immune responses in the 13-lined ground squirrel. *Dev Comp Immunol* 39: 154-160.

Bosco-Lauth AM, Nemeth NM, Kohler DJ, Bowen RA. 2016. Viremia in North American Mammals and Birds After Experimental Infection with Chikungunya Viruses. *Am J Trop Med Hyg 94*: 504-506.

Bosco-Lauth AM, Han S, Hartwig A, Bowen RA. 2015. Development of a hamster model for chikungunya virus infection and pathogenesis. *PloS One 10*: e0130150.

Burke DS. in Pathology of Emerging Infections (eds Nelson, A. M. & Horsburgh, C. R.)1-12 (American Society for Microbiology, Washington DC, 1998).

Calisher CH, Francy DB, Smith GC, Muth DJ, Lazuick JS, Karabatsos N, … McLean RG. 1986. Distribution of Bunyamwera serogroup viruses in North America, 1956-1984. *Am J Trop Med* 35: 429-443.

Carey HV, Andrews MT, Martin SL. 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol Rev* 83: 1153-1181.

Cleaveland S, Laurenson MK, Taylor LH. 2011. Diseases of humans and their domestic mammals: Pathogen characteristics, host range and the risk of emergence. *Phil Trans R Soc Lond B* 356: 991-999.

Day JF, Stark LM, Zhang JT, Ramsey AM, Scott TW. 1996. Antibodies to arthropod-borne encephalitis viruses in small mammals from southern Florida. J Trop Med 32: 431-436.

De Rodaniche E, Galindo P. 1961. St Louis encephalitis in Panama: III, Investigation of local mammals and birds as possible reservoir hosts. *Am J Trop Med* 10: 390-392.

Diallo M, Thonnon J, Traore-Lamizana M, Fontenille D. 1999. Vectors of Chikungunya virus in Senegal: current data and transmission cycles. *Am J Trop Med Hyg 60*: 281-286.

Fleck CC, Carey HV. 2005. Modulation of apoptotic pathways in intestinal mucosa during hibernation. *Am J Physiol Regul Integr Comp Physiol* 289: 586-595.

Frerichs KU, Kennedy C, Sokoloff L, Hallenbeck JM. 1994. Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia." *J Cereb Blood Flow Metab* 14: 193-205.

Halstead SB. 1966. Mosquito-borne haemorrhagic fevers of South and South-East Asia. *Bull World Health Organ 35*: 3.

Hanley KA, Nelson JT, Schirtzinger EE, Whitehead SS, Hanson CT. 2008. Superior infectivity for mosquito vectors contributes to competitive displacement among strains of dengue virus. *BMC ecology 8*: 1.

Hardy JL, Reeves WC, Scrivani RP, Roberts Dr. 1974. Wild mammals as hosts of group A and group B arboviruses in Kern County, California. *Am J Trop Med* 23: 1165-1177.

Hardy JL, Reeves WC, Sjogren RD. 1976. Variations in the susceptibility of field and laboratory populations of *Culex tarsalis* to experimental infection with western equine encephalomyelitis virus. *Am J Epi* 103:498-505.

Her Z, Kam YW, Lin RTP, Ng LFP. 2009. Chikungunya: a bending reality. *Microb Infect* 11: 1165–1176.

Herbold JR, Heuschele WP, Parsons MA. 1983. Reservoir of St. Louis encephalitis virus in Ohio bats. *Am J Vet Res* 44: 1889-1893.

Hoff GL, Yuill TM, Iversen JO, Hanson RP. 1970. Selected microbial agents in snowshoe hares and other vertebrates of Alberta. *J Wild Dis* 6: 472-478.

Hoff GL, Issel GJ, Trainer DO, Richard SH. 1973. Arbovirus serology in North Dakota mule and white-tailed deer. *J Wild Dis* 9: 291-295.

Holden P, Hayes RO, Mitchell CJ, Francy DB, Lazuick JS, Hughes TB. 1973. House sparrows, *Passer domesticus* (L.) as hosts of arboviruses in Hale County, Texas: I. Field studies 1965-1969. *Am J Trop Med* 22: 244-253.

Inoue S, Morita K, Matias RR, Tuplano JV, Resuello RRG, Candelario JR, ... Natividad FF. 2003. Distribution of three arbovirus antibodies among monkeys (*Macaca fascicularis*) in the Philippines. *J Med Primatol 32*: 89-94.

Iversen JO, Hanson RP, Papadopoulus O, Morris CV, de Foliart GR. 1969. Isolation of viruses of California encephalitis virus group from boreal *Aedes* mosquitoes. *Am J Trop Med* 18: 735-742.

Johnson PT, De Roode JC, Fenton A. 2015. Why infectious disease research needs community ecology. *Science*, *349*: 1259504.

Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P. 2008. Global trends in emerging infectious diseases. *Nature 451*: 990-993.

Jupp PG, McIntosh BM, Dos Santos I, De Moor P. 1981. Laboratory vector studies on six mosquito and one tick species with chikungunya virus. *Trans R Soc Trop Med Hyg 75*: 15-19.

Kading RC, Borland EM, Cranfield M, Powers AM. 2013. Prevalence of antibodies to alphaviruses and flaviviruses in free-ranging game animals and nonhuman primates in the greater Congo basin. *J Wildl Dis 49*: 587-599.

Karabatsos, N. 1985. International catalog of arboviruses including certain other viruses of vertebrates. American Society of Tropical Medicine and Hygiene. *San Antonio, Texas, USA*.

Kokernot RH, Radivojevic B, Anderson RJ. 1969. Susceptibility of wild and domesticated animals four arboviruses. *Am J Vet Res* 30: 2197-2203.

Lumsden WHR. 1955. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952–1953: II. General Description and Epidemiology. *Trans R Soc Trop Med Hyg* 49: 33–57.

Mackenzie JS, Gubler DJ, Petersen LR. 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nature Med 10*: S98-S109.

Mackenzie JS, Jeggo M. 2013. Reservoirs and vectors of emerging viruses. *Curr Opin Virol 3*: 170-179.

Marchette NJ, Rudnick A, Garcia R. 1980. Alphaviruses in Peninsular Malaysia: II. Serological evidence of human infection. *Southeast Asian J Trop Med Public Health 11*: 14-23.

McLean DM, Bergman SKA, Goddard EJ, Graham EA, Purvin-Good KW. 1971. North-south distribution of arbovirus reservoirs in British Columbia, 1970. *Canadian J Pub Health* 62: 120-124.

McLean RG, Bowen GS. 1980. Vertebrate hosts. In *St. Louis encephalitis*, ed. TP Monath. Washington, DC: American Public Health Association pp. 381-450.

McIntosh BM. 1961. Susceptibility of some African wild rodents to infection with various arthropod-borne viruses. *Trans R Soc Trop Med Hyg* 55: 63–68.

Nelson RL, Temples CH, Reeves WC, Milby MM. 1976. Relation of mosquito density to bird: Mammal feeding ratios of *Culex tarsalis* in stable traps. *Am J Trop Med* 25: 644:654.

Newhouse VF, Burgdorfer JA, Corwin D. 1967. Field and laboratory studies on the hosts and vrctors of the snowshow hare strain of California virus. *Mosq New* 31: 401-408.

Ng LC, Hapuarachchi HC. 2010. Tracing the path of chikungunya virus- Evolution and adaptation. *Infect Gene Evol* 10: 876–885.

Novoselova EG, Kolaeva SG, Makar VR, Agaphonova TA. 2000.Production of tumor necrosis factor in cells of hibernating ground squirrels *Citellus undulates* during annual cycle. *Life Scu* 67: 1073-1080.

Pan American Health Organization. "Chikungunya." World Health Organization, 12 Aug. 2016. Web. 08 July 2016.

Perry BD, Grace D, Sones K. 2013. Current drivers and future directions of global livestock disease dynamics. *Proc Nat Acad Sci 110*: 20871-20877.

Pesko K, Westbrook CJ, Mores CN, Lounibos LP, Reiskind MH. 2009. Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to chikungunya virus. *J Med Entomol 46*: 395-399.

Prendergast BJ, Freeman DA, Zucker I, Nelson RJ. 2002. Periodic arousal from hibernation is necessary for initiation of immune responses in ground squirrels. *Am J Physiol Regul Integr Comp Physiol* 282: 1054-1062.

Reznik F, Renik-Schuller H, Emminger A, Mohr U. 1975. Comparative studies of blood from hibernating and nonhibernating European hamsters (*Cricetus cricetus* L). *Lab Anim Sci* 25: 210-215.

Ritter DG, Feltz ET. 1974. On the natural occurrence of California encephalitis virus and other arboviruses in Alaska. *Canadian J Mico* 20: 1359-1366.

Robinson MC. 1955. An epidemic of virus disease in Southern Province, Tanganyika territory, in 1952–1953. *Trans R Soc Trop Med Hyg 49*: 28-32.

Sam IC, Kümmerer BM, Chan YF, Roques P, Drosten C, AbuBakar S. 2015. Updates on chikungunya epidemiology, clinical disease, and diagnostics. *Vector-Borne Zoonotic Dis 15*: 223-230.

Sandovici M, Henning RH, Hut RA, Strijkstra AM, … Deelman LE. 2004. Differential regulation of glomerular and interstitial endothelial nitric oxide synthase expression in the kidney of hibernating ground squirrel. *Nitric Oxide* 11: 194-200.

Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, ... Biscornet, L. 2006. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med*, *3*: e263.

Seymour C, Peralta PH, Montgomery GG. 1983. Serologic evidence of natural togavirus infections in Panamanian sloths and other vertebrates. *Am J Trop Med* 32: 854-861.

Shivatcheva TM. 1988. Survival of skin allografts in European ground squirrels, *Spermophillus citellus* L., during hibernation. *Folia Biol* (Krakow) 36: 213-221.

Schmaljohn C, Hjelle B. 1997. Hantaviruses: A Global Disease Problem. *Emer Infect Dis* 3:95.

Smart KL, Elbel RE, Woo RFL, Kern GT, Crane GL, … Hill DW. 1972. California and western encephalitis viruses from Bonneville Basin Utah, in 1965. *Mosq News* 32: 382-390.

Soumahoro MK, Gerardin P, Boelle PY, Perrau J, Fianu A, Pouchot J. 2009. Impact of chikungunya virus infection on health status and quality of life: A retrospective cohort study. *Plos One* 4: e7800. doi: 10.1371/journal.pone.0007800. pmid:19911058.

Spurrier WA, Dawe AR. 1973. Several blood and circulatory changes in the hibernation of the 13-lined ground squirrel. *Citellus tridecemlineatus. Comp Biochem Physiol A: Comp Physiol* 44: 267-282.

Srihongse S, Grayson MA, Diebel R. 1984. California serogroup viruses in New York State: The role of subtypes in human infections. *Am J Trop Med* 33: 1218-1227.

Storey KB. 1997. Metabolic regulation in mammalian hibernation: enzyme and protein adaptations. *Comp Biochem Physiol Part A: Physiol* 118: 1115-1124.

Sulkin SE, Allen R, Sims R. 1963. Studies of arthropod-borne virus infections in Chiroptera: I. Susceptibility of insectivorous species to experimental infection with Japanese B and St. Louis encephalitis viruses. *Am J Trop Med* 12: 800-814.
Taylor LH, Latham SM, Woolhouse MEJ. 2001. Risk factors for human disease emergence. *Phil. Trans. R. Soc. Lond. B* 356: 983-989.
Trainer DO, Hanson RP. 1969. Serologic evidence of arbovirus infections in wild ruminants. *Am J Epi* 90: 354-358.
Trainer DO, Hoff GL. 1971. Sereologic evidence of arbovirus infections activity in a moose population in Alberta. J Wild Dis 7: 118-119.
Tsetsarkin KA, Chen R, Weaver SC. 2016. Interspecies transmission and chikungunya virus emergence. *Curr Opin Virol 16*: 143-150.
Vourc'h G, Halos L, Desvars A, Boué F, Pascal M, Lecollinet S, ... Brémont M. 2014. Chikungunya antibodies detected in non-human primates and rats in three Indian Ocean islands after the 2006 ChikV outbreak. *Vet Res 45*: 1.
Ubico SR, McLean RG. 1995. Serologic survey of neotropical bats in Guatemala for virus antibodies. *J Wild Dis* 31: 1-9.
Weaver SC, Reisen WK. 2010. Present and future arboviral threats. *Antiviral Res 85*: 328-345.
Weaver SC, Osorio JE, Livengood JA, Chen R, Stinchcomb DT. 2012. Chikungunya virus and prospects for a vaccine. *Expert Rev Vaccines 11*: 1087-1101.
Wiethoelter AK, Beltrán-Alcrudo D, Kock R, Mor SM. 2015. Global trends in infectious diseases at the wildlife–livestock interface. *Proc Nat Acad Sci 112*: 9662-9667.
Williams ES, Barker IK. 2001. Infectious Diseases of Wild Mammals. Ames: Iowa State Press.
Wolfe ND, Kilbourn AM, Karesh WB, Rahman HA, Bosi EJ, Cropp BC, ... Gubler DJ. 2001. Sylvatic transmission of arboviruses among Bornean orangutans. *Am J Trop Med Hyg 64*: 310-316.
Woolhouse MEJ, Gowtage-Sequeria S. 2005. Host range and emerging and reemerging pathogens. *Emerg Infect Dis* 11: 1982-1847.
Zarnke RL, Yuill TM. 1981. Powassan virus infection in snowshoe hares (*Lepus americanus*). *J Wildl Dis* 17: 303-310.

CHAPTER THREE

EXPERIMENTAL INFECTION OF COTTON RATS WITH CHIKUNGUNYA VIRUS

3.1 Introduction

Chikungunya virus (CHIKV) is an arthropod-borne *Alphavirus* of animal origin that has recently emerged in the Americas. Since its discovery in Tanzania in 1953, CHIKV has caused numerous periodic outbreaks through Africa, Asia, and the Indian Ocean region; although historical evidence describes probable CHIKV epidemics in the Americas occurring early as the nineteenth century (Carey, 1971; Dickson, 1839; Dickson, 1859). Currently, two of the three geographically isolated lineages of CHIKV, East-Central-South African and Asian, have been documented circulating in the Western Hemisphere (Tsetsarkin et al., 2016; Lanciotti et al., 2016). CHIKF pathogenesis, in humans, is characterized by acute onset of fever, nausea, headache, rash, myalgia and acute/chronic polyarthritis (Yeo and Chu, 2013; Her et al., 2010; Hapuarachchi et al., 2010). The virus is primarily transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes (De Lamballerie et al., 2008; Powers and Logue, 2007). In 2013, CHIKV was isolated for the first time in the Western Hemisphere and has since spread to over 45 countries and territories throughout the Americas and Caribbean (Pan America, August 2016). Both *Aedes spp.* vectors are common throughout the Americas with *Ae. aegypti* mosquitoes being more prevalent in neotropical regions and *Ae. albopictus* occupying both tropical and northern temperate countries (Higa et al., 2011; Benedict et al., 2007; Fernandez-Salas et al., 2015). As expected, CHIKV has followed a similar pattern of expansion in the Americas as seen with dengue epidemics and, as of 2015, Zika virus outbreaks. Since these arboviruses share mosquito vectors and ecological conditions, it is likely that once established, periodic epidemics may occur cyclically as seen in other endemic regions.

As an arbovirus, CHIKV has traditionally been maintained in enzootic transmission cycles between competent mosquito vectors and susceptible vertebrate hosts, with occasional spillover into human populations (Powers et al., 2000; Weaver, 2006). In Africa, the virus is thought to be maintained in a sylvatic cycle between *Aedes spp.* mosquitoes and primarily non-human primates. Serological

evidence suggests that vertebrate species such as rodents, birds, and reptiles may play a secondary role in CHIKV transmission cycles (Vourch et al., 2014; Osterrieth et al., 1960; McIntosh, 1970; McIntosh et al., 1977; McIntosh et al., 1964). Studies conducted in the 1970s allowed isolation of CHIKV in Senegal from three species of non-human primates, one palm squirrel (*Xerus erythropus*), bats (*Scotophilus spp.*), and a golden sparrow (*Auripaser luteus*) (Bres et al., 1969; Cornet et al., 1979). Furthermore some studies have found antibodies to CHIKV in rodents, birds, reptiles in addition to domestic livestock including horses, cattle, pigs, and cattle (Bosco-Lauth et al., 2015; Vourch et al., 2014; Cornet et al., 1968; McIntosh et al., 1964). Experimental infections of wild African rodent species in the genera *Mastomys*, *Arvicanthis*, and *Aethomys* with CHIKV have also demonstrated these species are capable of developing viremia (McIntosh, 1961). While animals within the *Arcicanthis* and *Aethomys* genera demonstrated low levels of viremia, individuals in the *Mystromys* produced high titer viremia followed by the development of neutralizing antibodies (McIntosh, 1961). Other experimental studies have also shown that hamsters, cotton rats, and bats are competent hosts for CHIKV viral replication (Bedekar and Pavri, 1969; Bosco-Lauth et al., 2015). Diallo isolated multiple strains of CHIKV from thirteen mosquito species in Africa, including many that have never been experimentally examined for their vector competence with CHIKV (Diallo et al., 1999). Repeated viral isolation of CHIKV from these species suggests that other zoophilic non-*Aedes* mosquitoes may play a role in CHIKV transmission cycles (Diallo et al., 1999). These findings highlight the need to investigate the possibility that there may be other competent hosts and or vectors involved in CHIKV transmission cycles in novel geographic areas. The purpose of this study was to evaluate the course of infection of CHIKV in cotton rats (*Sigmodon hispidus*) and to attempt isolation of CHIKV from mosquitoes that had fed on viremic cotton rats and enhance our understanding of the potential role of rodents as reservoir hosts for this pathogen.

3.2 Materials and Methods

3.2.1 Cotton Rats

Eighteen female 6-8 week cotton rats (*Sigmodon hispidus*) were obtained from Charles River Laboratories and housed individually in the animal biosafety level (ABSL) 3 facility at Colorado State University. Animals were provided a commercial rodent chow and water *ad libitum*, and acclimated to the laboratory settings for 3-4 weeks prior to infection. This work was approved by the Animal Care and Use Committee at Colorado State University, Fort Collins, Colorado, USA (approval 14-5258A) and conducted in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals).

3.2.2 Mosquitoes

Aedes aegypti (Rex) were obtained from the Centers for Disease Control laboratory raised colonies and received into the BSL-3 facility as egg rafts. Egg rafts were placed in 6"x12" Tupperware pans with tap water and placed in an incubator with an 8:16 hours dark: light cycle at 75% relative humidity and 27°C. Larvae were hatched and fed a mixture of crushed Tetramin fish food twice a week and pupae were picked daily as needed. Pupae were placed in small ramekins water and kept in cardboard cartons until hatched. Adult mosquitoes were kept in separate cardboard containers with approximately 100 mosquitoes per carton and maintained on sugar cubes and water with a 12:12 hour light: dark cycle at 75% relative humidity and 27°C. Mosquitoes were maintained for one to three days before being fed on cotton rats.

3.2.3 Viruses

The SAH2123 (SAH) strain of CHIKV was provided by Ann Powers at the Centers for Disease Control and Prevention, Fort Collins, CO. This isolate was derived from a human isolate in 1976 and had been passage three times in Vero cells. This CHIKV strain is classified as a member of the East-Central-South African lineage.

3.3 Experimental Design

For this study, 17 cotton rats were acclimated for 6 weeks prior to infection, during which time they were weighed, implanted with IPTT300 temperature transponder tags (BioMedic Data Systems, Inc., Seaford, DE), and joint measurements were taken of the left and right hock joints using microcalipers (measuring 1/100th inch increments). The SAH strain of CHIKV was diluted in sterile PBS to prepare an inoculum at final titer of 2 x 10^6 pfu/mL and animals were infected by subcutaneous injection of 0.1 mL in the right lateral thigh region. Following inoculation, rats were weighed daily, temperatures were recorded, and joint measurements were taken of both the left and right hock. Blood was collected by puncture of the submandibular vein followed by centrifugation. Animals were bled on days 0, 2, 3, 4, 7, 14, and 21 days post infection. On days 2, 4, 7, 14, and 21, groups of 3 cotton rats were euthanized and necropsied. Serum and samples of organs (brain, heart, lung, liver, spleen, kidney, small intestine, skin, and muscle) were frozen for virus titration or fixed by immersion in 10% neutral-buffered formalin. Tissues were then transferred into 70% ethanol solution after 48 to 72 hours before being sent to a commercial laboratory for embedding, sectioning and staining. Sera from terminal blood samples were frozen undiluted and saved for serological assay.

3.3.1 Cotton Rat to Mosquito Transmission

Two days following inoculation of CHIKV, cotton rats were lightly anesthetized with ketamine-xylazine and a small region of their abdominal region was shaved to allow mosquitoes to feed. Cardboard cartons containing approximately 100 adult (male and female) *Ae. aegypti* mosquitoes were placed membrane side up and shaved cotton rats were draped over the top. Mosquitoes were allowed to feed for 20-30 minutes. Immediately following feeding, engorged mosquitoes were placed in an incubator and maintained on a 12:12 hour light: dark cycle at 75% relative humidity and 27°C. After ten days of extrinsic incubation mosquitoes were frozen for approximately 10-15 minutes and then were sorted while being chilled on a cold plate.

3.3.2 Virus Isolation

Frozen mosquitoes were thawed briefly before being individually added to 0.5 mL bovine albumin media (BA-1: MEM medium, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 ug/mL streptomycin, 2.5 ug/mL amphotericin B in 0.05 M Tris, pH 7.6) with a single copper BB in a conical cryovial. Samples were then triturated using a mixer mill at 25 cycles/second for 3 minutes at room temperature and then centrifuged for 2-4 minutes at 1,130 x g (1,200 rpm). Following centrifugation, 150 uL of each sample were added to a 96 well plate. Serial 10-fold dilutions were then prepared in BA-1 medium with 100 uL samples from each dilution being plated onto wells in 6 well plates containing a monolayer of 90-100% confluent Vero cells, as described (Westaway et al., 1966).

Vero cells were grown in 6-well plates in Dulbecco's Modified Eagle medium (DMEM) containing 7.5% newborn calf serum and antibiotics until a monolayer of cells formed. A 100 uL aliquot of sample serial dilutions was added to each of the wells and incubated for 1 hour at 37°C. Following the one hour incubation, a primary overall of 2 mL minimum essential media (MEM) and 0.8% agarose supplemented with 2% fetal bovine serum, sodium bicarbonate, and antibiotics per well. Plates were incubated for 24 hours at 37°C at which time a second 2 mL overlay containing 0.004% neutral red was added. Plaques were counted at 24 and 48 hours following the addition of the secondary overlay. Virus was back-titrated from the mosquito blood meals using the same protocol as described above.

3.3.3 Serology

Plaque reduction neutralization tests (PRNT) were used to detect CHIKV neutralizing antibodies in serum samples as described in (Lindsey et al., 1976). Briefly, Vero cells were grown in 6 well plates to a 90-100% confluent monolayer, as described above. Serum samples were heat-inactivated at 56°C for 30 minutes and serially diluted in BA-1 with antibiotics from 1:10 to 1:320 in 96 well plates. Diluted serum samples were then combined with an equal volume of a suspension of CHIKV SAH to yield approximately 100 PFU/0.1 mL. Samples were then inoculated onto 6 well plates, incubated for 1 hour at

37°C, and first and second overlays were added as described above. Antibody titers were expressed as a reciprocal of the highest serum dilution with ≥ 80% reduction ($PRNT_{80}$) compared to control wells. Sera with ≥80% neutralization of CHIKV were considered positive.

3.3.4 Imunohistochemistry

Cotton rat tissues (brain, liver, spleen, lung, kidney, heart, small intestine, and skin and muscle from site of infection) were collected immediately following euthanasia and placed into 10% neutral-buffered formalin. Tissues were then transferred into 70% ethanol solution after 48 hours, sections of heart, lung, small intestine, spleen, and a whole leg were placed in cassettes, and submitted to a commercial laboratory for embedding. Sections (5 um) were cut on a microtome and placed onto charged slides. Slides were deparaffinized and subjected to antigen retrieval by heating to 95°C in Target Retrieval (DAKO) for 25 min followed by a 20 min cooling-down period at room temperature (rt). The sections were then subjected to the following blocking steps at rt incubation: (i) peroxidase-block (DAKO) for 10 min, (ii) 0.15M glycine in PBS for 15 min, (iii) DAKO antibody-diluent solution for 30 min, with a brief rinse in TRIS-buffered saline with 0.1% Tween-20 (TBST) between each. The sections were then incubated for 2 hours at room temperature with a monoclonal antibody (5.5G9) specific to the capsid protein of CHIKV (Goh, et al., 2015), followed by 10 min of repeated TBST-washes. Antibody binding was visualized using anti-mouse-IgG Envision kit (DAKO) according to the manufacturer's protocol. Slides were then counterstained with Meyer's hematoxylin, mounted with Glycergel (DAKO) and examined under a microscope.

3.4 Results

3.4.1 Responses of Cotton Rats to Infection with Chikungunya Virus

Clinical signs of disease were not observed in any of the infected animals. Cotton rat baseline body temperature ranged from 99.8 to 102.5 °F (mean of 100.6 °F) and none of the animals developed discernible fever following virus inoculation (**Figure 3.1**). Similarly, daily measurements of both right and left hock widths failed to detect significant swelling associated with infection (**Figure 3.2 and Figure**

3.3). A number of the animals lost a considerable amount of body weight over the course of the study, despite lack of other overt clinical signs, and this was attributed to the stress of frequent handling (**Figure 3.4**).

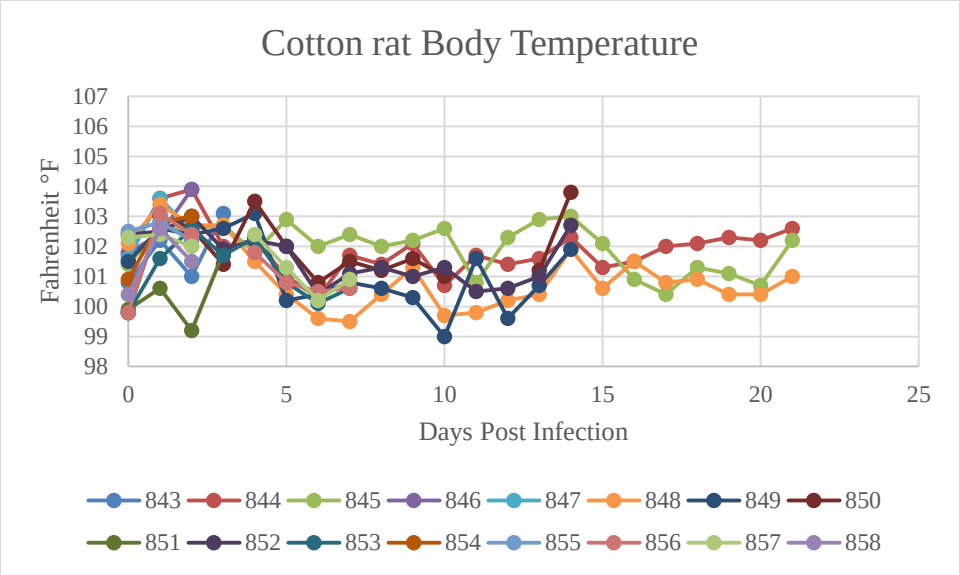

Figure 3.1. Cotton rat body temperatures over the course of the study.

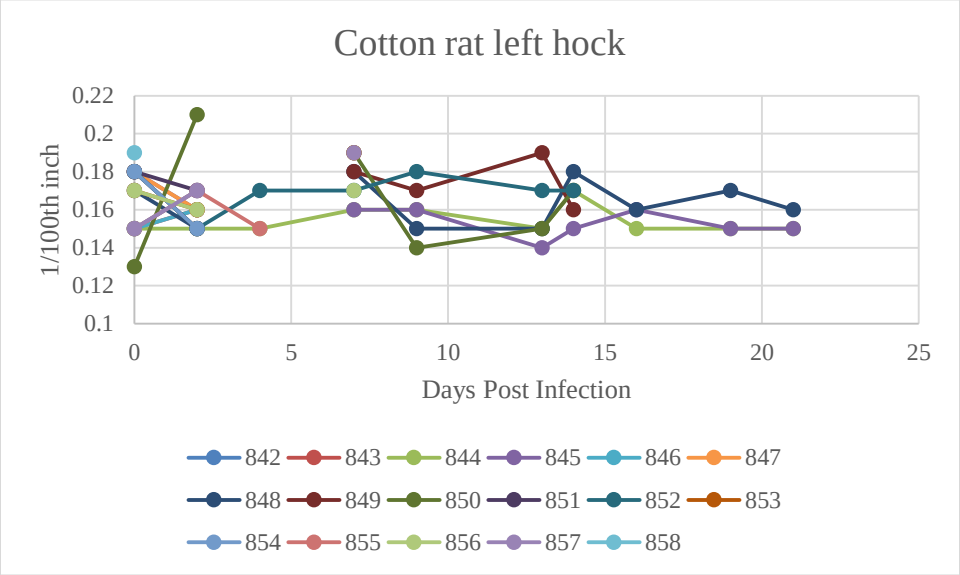

Figure 3.2. Cotton rat left hock measurements in 1/100th inch increments over the course of the study.

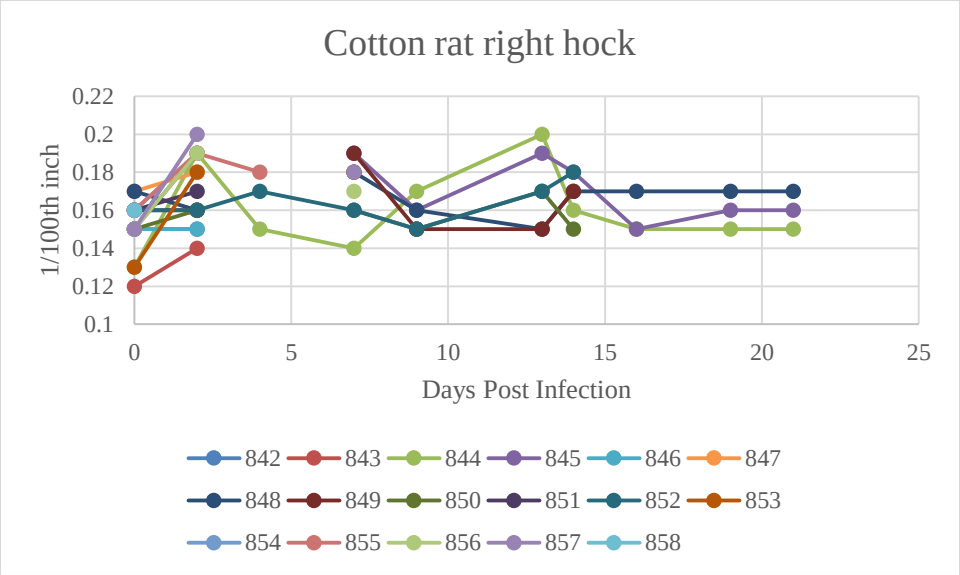

Figure 3.3. Cotton rat right hock measurements in 1/100th inch increments over the course of the study.

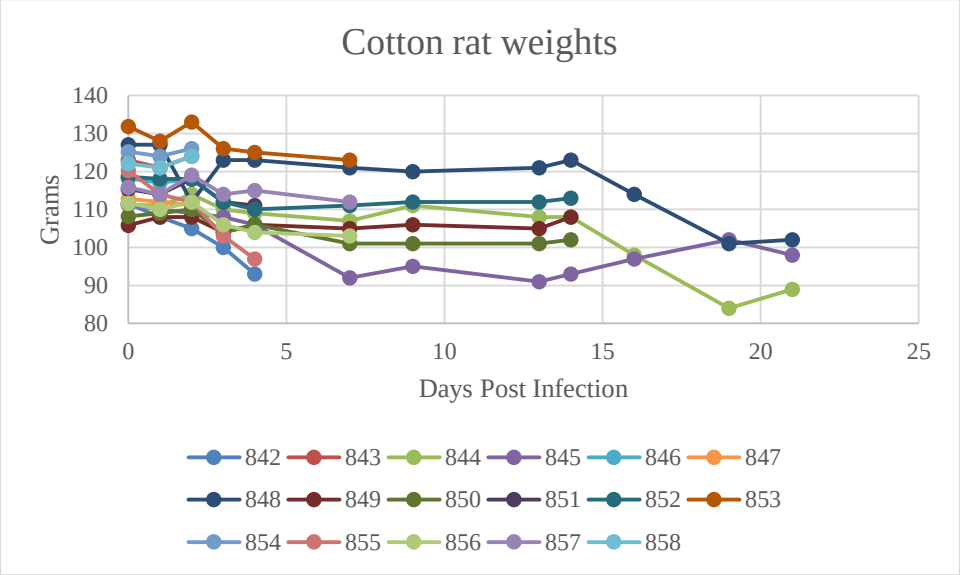

Figure 3.4. Cotton rat weight changes over course of the study.

None of the 17 cotton rats used in this study were viremic prior to virus inoculation, and 13 of 17 animals developed detectable viremia after inoculation. Viremia was detected only at 2 DPI and ranged from 2 x 10^2 to 6 x 10^4 PFU/mL (**Table 3.1**).

All 17 animals were screened for negative (<10) for neutralizing antibodies to CHIKV prior to infection. Sera collected on 7 DPI, 14 DPI, and 21 DPI were tested for anti-CHIKV antibodies and eight of nine had neutralization titers of at least 10 with titers ranging from 10 to ≥80 (**Table 3.1**).

Table 3.1. Virus titers in serum and serologic responses of cotton rats infected with chikungunya virus.

	Viremia virus titer (PFU/mL)			Antibody Titers ($PRNT_{80}$)		
Animal	**2 DPI**	**3 DPI**	**4 DPI**	**7 DPI**	**14 DPI**	**21 DPI**
842	2E2	<100	<100			
843	6.3E4					
844	5E2	<100	<100	NT	NT	20
845	<100	<100	<100	NT	NT	40
846	4.5E4					
847	1.1E4					
848	1.1E3	<100	<100	NT	NT	20
849	2.2E3	<100	<100	NT	≥40	
850	<100	<100	<100	NT	≥80	
851	7E2	<100	<100			
852	3.9E3	<100	<100	NT	≥40	
853	6.0E2	<100	<100	<10		
854	1.5E3					
855	2.6E3	<100	<100			
856	<100	<100	<100	≥40		
857	<100	<100	<100	≥40		
858	5.0E4					

NT = not tested

3.4.2 Tissue Virus Burdens and Immunohistochemistry

A battery of tissues was collected from each of the cotton rats euthanized on days 2 to 4 post-inoculation and homogenates of those tissues tested by plaque assay for titer of infectious virus. Virus was isolated only from tissues collected at day 2 post-inoculation, and was present in liver, spleen, and lung in titers substantially greater than the corresponding titer in serum (**Table 3.2**).

Table 3.2. Organ burdens of virus in cotton rats inoculated with chikungunya virus.

Animal	ID number	Organ	DPI	Tissue virus (PFU/gram)	Viremia (PFU/mL)
Cotton Rat	843	Liver	2	5.2E5	6.3E4
Cotton Rat	843	Spleen	2	1.4E5	6.3E4
Cotton Rat	846	Skin	2	2.0E2	4.5E4
Cotton Rat	846	Liver	2	2.7E5	4.5E4
Cotton Rat	846	Spleen	2	1.6E5	4.5E4
Cotton Rat	847	Lung	2	3.8E5	1.1E4
Cotton Rat	854	Lung	2	1.7E5	1.5E3
Cotton Rat	854	Liver	2	2.0E6	1.5E3
Cotton Rat	858	Liver	2	9.0E4	5.0E4

Tissues from four cotton rats that had the highest viremia on day 2 (843, 848, 853, 855) that had been euthanized 1, 2, 3, and 4 days after virus inoculation were immunohistochemically processed to detect CHIKV antigens. Virus antigen was not detected in any of the tissues examined (liver, brain, lung, heart, muscle, and small GI tract).

3.4.3 Transmission of Virus to Mosquitoes

Each of the 17 cotton rats was exposed to feeding by 20 mosquitoes and the number than were visibly engorged ranged from 2 to 13 (**Table 3.3**). A total of 137 engorged mosquitoes survived the 10-day extrinsic incubation period and were tested for CHIKV infection by plaque assay. Thirteen of the 17 rats fed upon were viremic at the time of feeding, with titers that ranged from 2×10^2 to 6×10^4 PFU/mL. None of the tested mosquitoes were virus positive, indicating that transmission did not occur.

Table 3.3. Infection of *Aedes aegypti* mosquitoes fed on viremic cotton rats.

Animal	Viremia titer on day of feeding (PFU/mL)	Number of engorged mosquitoes
842	2E2	5/20
843	6.3E4	4/20
844	5.0E2	13/20
845	<100	8/20
846	4.5E4	14/20
847	1.1E4	11/20
848	1.1E3	12/20
849	2.2E3	10/20
850	<100	1/20
851	7E2	17/20
852	3.9E3	8/20
853	6E2	3/20
854	1.5E3	9/20
855	2.6E3	2/20
856	<100	13/20
857	<100	2/20
858	5E4	5/20

3.5 Discussion

In general, experimental investigation of infectious diseases are primarily influenced by heterogeneities among the host populations involved (Jackson, 2015). Genetic differences within these host populations influence disease severity and transmission rates (Loyd-Smith et al., 2005). Most often, rodent models are used to investigating immunological dynamics during infectious disease studies given their ecological importance and propensity to serve as reservoirs during arbovirus transmission cycles (Meerburg et al., 2009; Davis et al., 2005). Worldwide there are over 1,500 species of rodents within the family *Rodentia*. Modern immunology studies involving laboratory derived rodent populations provide skewed and/or restricted comparison data between laboratory and natural reservoir dynamics. The lack of allelic variation within these models make them unrepresentative of natural rodent populations and can

muddle infectious disease dynamic investigations; however the utilization of laboratory models to make inferences about natural systems is still vital to infectious disease research.

In this study I utilized cotton rats (*Sigmodon hispidus*) to investigate CHIKF disease dynamics in a rodent host. In the wild, cotton rat populations are ubiquitous throughout South, Central and southern North America and live in habitats that should be conducive to transmission of mosquito-borne pathogens, including CHIKV. Notably, while the animals used in this study were from a commercial vendor and were more than likely to have a limited genetic background, the results of this study, and my previous pilot study, indicate that cotton rats are capable of becoming infected with CHIKV and may have more diverse immunological infection dynamic in natural systems. Of the 17 cotton rats inoculated, 13 developed detectable viral loads on day two, the only day viremia was detected. Viremia titers on day two ranged from 2×10^2 to 6.3×10^4 PFU/mL; four of the 13 viremic animals had titers greater than 10^4 PFU/mL. While the peak in CHIKV viremia is short, other infectious disease rodent systems (i.e. hantavirus) have also demonstrated short periods of viremia. Additionally, in human CHIKF 5-15% of patients are asymptomatic, indicating that subclinical CHIKV infections with unspecific or mild symptoms are often undiagnosed (Gerardin et al., 2008).

Reservoir hosts for pathogens that normally cause disease in humans and other animal hosts typically do not show severe or clinical signs of infection due to the virus being cytopathic in the non-natural host but not in reservoirs, differences in viral tropism between non-natural hosts and reservoir hosts, differences in interactions between the virus and host resistance mechanisms in non-natural hosts, and reservoir hosts, interactions between the virus and other microbes or pathogens within the non-natural hosts and reservoir hosts, reservoir host response may control viral replication more efficiently, and/or reservoir hosts may tolerate higher viral loads than non-natural hosts (Mandl et al., 2015). As seen in Chapter 2, there is a lack of knowledge regarding arbovirus viral infection dynamics in reservoir rodent hosts and the systems that influence disease replication/resistance mechanisms.

A few examples of viruses that use wildlife as reservoir hosts include influenza A in wild waterfowl, and Hendra and Nipah viruses in fruit bats (Mandl et al., 2015). Wild water fowl (*Anas* spp.) infected with influenza A virus exhibited transient infections (Jourdain et al., 2010). This may be an example of the reservoir hosts (the waterfowl) being previously exposed to another strain of influenza A and being only partially protected from infection with co-circulating viral strains (Mandl et al., 2015). Pterpoid fruit bat species also exhibited transient Hendra and Nipah virus infections (Halpin et al., 2011; Middleton et al., 2007). Seroprevalence of these viruses in fruit bat species have been documented as high as 60% although prevalence rates remain low at 1% (Breed et al., 2013; Rahman et al., 2013). Since these bat species are seasonal synchronous breeders it is difficult to determine if these infections are seasonal increases due to juvenile bats losing maternal immunity or changes in bat immunity that result in reinfections or reactivation of low-level infections (Mandl et al., 2015; Epstein et al., 2013; Sohayati et al., 2011). In some cases differences in viral tropism may influence viral-host cell interaction. The sooty mangabey, a host for SIV, may be more resistant to SIV infection due to a lack of a key SIC receptor, CCR5, on host T cells (Paiardini et al., 2011). Similarly, African green money CD4+ memory T cells down regulate expression during SIV infection making them resistant to infection (Beaumier et al., 2009).

Several examples of zoonotic viruses replicating to high viral levels in their reservoirs have been described. In African primates species, SIV establishes chronic high viral load infections with limited immune control (Pandrea and Apetrei, 2010; Silvestri et al., 2003). Hantavirus infections of rodent species result in acute viral loads and chronic infections (Easterbrook and Klein, 2008; Miedema et al., 2013; Pandrea and Apetrei, 2010; Mandl et al., 2015; Schountz and Prescott, 2014). The exact host mechanisms of how rodents switch from an initial immune activation to a dampened response during hantavirus infection still remains unclear. It is thought that specific viral proteins interact with rodent immune pathways to limit proinflammatory responses and prevent viral clearance. Within hantavirus rodent reservoirs there is a high level of variation in prevalence rates among species (Essbauer and Krautkramer, 2015). For example, prevalence rates of Puumala virus (PUUV) in rodents varies

significantly depending on time, region, and viral strain (Essbauer et al., 2006; Mertens et al., 2011; Augot et al., 2008). Several hantavirus have been shown to be capable of infecting a variety of rodent species in addition to host switching including Andes virus, Dobrava virus, Puumala virus, and Tula virus (Gonzalez et al., 2002; Sibold et al., 2001; Dekonenko et al., 2003; Plyusnin et al., 1994). As you can tell from these host-virus interactions extensive within-host and between-host diversity exists through multiple virus transmission cycles in both non-native hosts and reservoir species.

Given the recent emergence of both known and novel viruses worldwide (Zika, CHIK, Heartland, and MERS viruses) a better understanding of natural disease transmission cycles, reservoir hosts, and mechanisms for reservoir hosts to tolerate viral infections is critical. An ideal solution to this dilemma would be to identify other wildlife species (rodents, reptiles, amphibians) involved in CHIKV sylvatic transmission cycles, other than non-human primates. In this study we investigated the potential role of rodents in the *Sigmodon* genus that has representatives in North, Central, and South America. Cotton rats in general, are used extensively to investigate human infectious diseases including herpes simplex virus, poliovirus, measles virus, and respiratory syncytial virus (Ward et al., 2001; Green, 2013; Niewiesk and Prince et al., 2002). While many commonly utilized laboratory rat or mouse species are refractory to human pathogen infection, cotton rats have been shown to be uniquely susceptible (Niewiesk and Prince, 2002). Due to the increased use of cotton rats in scientific research, cell lines, antibodies, cytokine genes, and reagents have been created for use in these studies (Niewiesk and Prince, 2002). In this study, we demonstrated that the majority of animals 12/18 (67%) infected with CHIKV developed a detectable viremia. The primary mosquito vectors for CHIKV are *Aedes aegypti* and *Aedes albopictus* (Pialoux et al., 2007). In order to become infected with CHIKV, competent host vectors (e.g. *Aedes spp.)* must feed on viremic vertebrate hosts containing $10^{3.36}$ to $10^{5.0}$ pfu/mL of blood (Pesko et al., 2009). Although several of the animals infected in this study did not produce viremia within this threshold, our study indicates that other rodent species may be useful in investigating CHIKV pathogenesis.

REFERENCES

Bedekar SD, Pavri KM. 1969. Studies with chikungunya virus. Part I. Susceptibility of birds and small mammals. *Indian J Med Res 57*: 1181-92.

Benedict MQ, Levine RS, Hawley WA, Lounibos LP. 2007. Spread of the tiger: global risk of invasion by the mosquito Aedes albopictus. *Vector-borne Zoonotic Dis 7*: 76-85.

Bosco-Lauth AM, Han S, Hartwig A, Bowen RA. 2015. Development of a hamster model for chikungunya virus infection and pathogenesis. *PloS One, 10*: p.e0130150.

Breaumier CM, Harris LD, Goldstein S, Klatt NR, Whitted S, McGinty J, … Brenchley JM. 2009. CD4 downregulation by memory CD4+ T cells in vivo renders African green monkey resistant to profressive SIVagm infection. *Nat Med* 15: 879-885.

Breed AC, Meers J, Sendow I, Bossart KN, Barr JA,…Field HE. 2013. The distribution of henipaviruses in Southeast Asia and Australasia: is Wallace's line a barrier to Nipah virus? *PLoS One e61316.*

Bres P, Hery G. 1969. Kinetics of Production of Haemagglutination Inhibiting and Complement Fixing Antibodies in Mice Immunized with Chikungunya, Onyong-nyong and Sinbus Viruses. 3. Effect of interval between inoculations and Use of an Adjuvant. *Revue Immunol 33*: 147.

Carey DE. 1971. Chikungunya and dengue: a case of mistaken identity? *J History Med Allied Sci 26*: 243-262.

Cornet M, Robin Y, Adam C, Valade M, Calvo MA. 1979. Transmission expérimentale comparée du virus amaril et du virus Zika chez *Aedes aegypti*. *L Cahiers ORSTOM série Entomologie médicale et Parasitologie*, 47-53.

Cornet M, Robin Y, Hannoun C, Corniou B, Bres P, Causse G. 1968. An epidemic of yellow fever in Senegal in 1965. Epidemiological studies. *Bull World Health Organ* 39: 845—858.

Couderc T, Chrétien F, Schilte C, Disson O, Brigitte M, Guivel-Benhassine F, ... Desprès P. 2008. A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. *PLoS Pathog 4*: e29.

Davis S, Valvet E, Leris H. 2005. Fluctuating rodent populations and risk to humans from rodent-borne zoonoses. *Vector Borne Zoonotic Dis* 5: 305-314.

De Lamballerie X, Leroy E, Charrel RN, Ttsetsarkin K, Higgs S, Gould EA. 2008. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? *Virology J 5*: 1.

Dekonenko A, Yakimenko V, Ivanov A, Morozov V, Nikitin P. 2003. Genetic similarity of Puumala viruses found in Finland and western Siberia and of the mitochondrial DNA of their rodent hosts suggests a common evolutionary origin. *Infect Genet Evol* 3: 245-257.

Diallo M, Thonnon J, Traore-Lamizana M, Fontenille D. 1999. Vectors of Chikungunya virus in Senegal: current data and transmission cycles. *Am J Trop Med Hyg 60*: 281-286.

Dickson SH. 1839. On dengue: its history, pathology and treatment. *Philadelphia: Haswell, Barrington and Haswell* 3: 5–23.

Dickson SH. 1859 Dengue. Elements of medicine: a compendious view of pathology and therapeutics; or the history and treatment of diseases. *Philadelphia: Blanchard and Lea* 744–49.

Easterbrook JD, Klein SL. 2008. Immunologial Mechanisms Mediating Hantavirus Persistence in Rodent Reservoirs. *PLoS One* 4 e1000172.

Epstein JH, Baker ML, Zambrana-Torrelio C, Middleton D, Barr JA, … Crameri G. 2013. Duration of Maternal Antibodies against Canine Distemper Virus and Hendra Virus in Pteropid Bats. *PLoS ONE* 8 e67584.

Essbauer S, Schmidt J, Conraths FJ, Friedrich R, Koch J, Hautmann W,… Pfeffer M. 2006. A new Puumala hantavirus subtype in rodents associated with an outbreak of Nephropathia epidemica in South-East Germany in 2004. *Epidemiol Infect* 134:1333– 1344.

Essbauer SS, Krautkramer. "Hantaviruses – Infections, Epidemiology and Hosts." In *Zoonoses – Infections Affecting Humand and Animals,* 749-780. New York, NY: Springer, 2015.

Fernández-Salas I, Danis-Lozano R, Casas-Martínez M, Ulloa A, Bond JG, Marina CF, Lopez-Ordóñez T, Elizondo-Quiroga A, Torres-Monzón JA, Díaz-González EE. 2015. Historical inability to control *Aedes aegypti* as a main contributor of fast dispersal of chikungunya outbreaks in Latin America. *Antiviral Res 124*: 30-42.

Gardner J, Anraku I, Le TT, Larcher T, Major L, Roques P, Schroder WA, Higgs S, Suhrbier A. 2010. Chikungunya virus arthritis in adult wild-type mice. *J Virol 84*: 8021-8032.

Gerardin P, Guernier V, Perrau J, Fianu A, Le Roux,…Favier F. 2008. Estimating Chikungunya prevalence in La Reunion Island outbreak by serosurveys: Two methods for two critical times of the epidemic. *BMC Infectious Dis* 8: 99.

Green MG, Huey D, Niewiesk S. 2013. The cotton rat (*Sigmodon hispidus*) as an animal model for respiratory tract infections with human pathogens. *Lab Animal* 42:170.

Goh LY, Hobson-Peters J, Prow NA, Gardner J, Bielefeldt-Ohmann H, Suhrbier A, Hall R.A. 2015. Monoclonal antibodies specific for the capsid protein of chikungunya virus suitable for multiple applications. *Journal of General Virology* 96: 507-512.

Gonzalez DVM, Edelstein A, Miguel S, Martinez V, Cortez J, Cacace ML, … Padula P. 2002. Andes virus associated with hantavirus pulmonary syndrome in northern Argentina and determination of he precise site of infection. *Am J Trop Med Hyg* 66: 713-720.

Haplin K, Hyatt AG, Fogarty R, Middleton D, Bingham J, … Daszak P. 2011. Pteropid bats are confirmed as the reservoir hosts of henipaviruses: a comprehensive experimental study of virus transmission. *Am J Trop Med Hyg* 85: 946-951.

Hapuarachchi HC, Bandara KBAT, Sumanadasa SDM, Hapugoda MD, Lai YL, Lee KS, Tan LK, Lin RT, Ng LF, Bucht G, Abeyewickreme W. 2010. Re-emergence of Chikungunya virus in South-east Asia: virological evidence from Sri Lanka and Singapore. *J Gen Virol 91*: 1067-1076.

Her Z, Malleret B, Chan M, Ong EK, Wong SC, Kwek DJ, ... Ng LF. 2010. Active infection of human blood monocytes by Chikungunya virus triggers an innate immune response. *The J Immunol 184*: 5903-5913.

Higa Y, Yen NT, Kawada H, Son TH, Hoa NT, Takagi M. 2010. Geographic distribution of *Aedes aegypti* and *Aedes albopictus* collected from used tires in Vietnam. *J Am Mosquito Cont Ass 26*:1-9.

Hiramatsu H, Nishikomori R, Heike T, Ito M, Kobayashi K, Katamura K, Nakahata T. 2003. Complete reconstitution of human lymphocytes from cord blood CD34+ cells using the NOD/SCID/γcnull mice model. *Blood* 102: 873-880.

Jackson JA. 2015. Immunology in wild nonmodel rodents: an ecological context for studies of health and disease. *Parasite Immunol* 37:220–32.

Jourdain E, Funnarsson G, Wahlgren J, Latorre-Margalef N, Brojer C, … Olsen B. 2010. Influenza virus in a natural host, the mallard: experimental infection data. *PLoS ONE* 5 e8935.

Lanciotti RS, Lambert AJ, Holodniy M, Saavedra S, Signor LDCC. 2016. Phylogeny of Zika virus in western hemisphere, 2015. *Emerg Infect Dis 22*: 933.

Lindsey HS, Calisher CH, Mathews JH. 1976. Serum dilution neutralization test for California group virus identification and serology. *J Clin Microbiol* 4: 503–51.

Loyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM. 2005. Superspreading and the effect of individual variation on disease emergence. Nature 5: 305-314.

Mandl J, Ahmed R, Barreiro LB, Daszak P, Epstein H, … Feinberg MB. 2015. Reservoir Host Immune Responses to Emerging Zoonotic Viruses. *Cell* 160: 20-33.

McIntosh BM, Paterson HE, McGillivray G, De Sousa J. 1964. Further Studies on the Chikungunya Outbreak in Southern Rhodesia in 1962: I.—Mosquitoes, Wild Primates and Birds in Relation to the Epidemic. *Ann Trop Med Parasitol 58*: 45-51.

McIntosh BM, Jupp PG. 1970. Attempts to transmit chikungunya virus with six species of mosquito. *J Med Entomol 7*: 615-618.

McIntosh BM, Jupp PG, Dos Santos I. 1977. Rural Epidemic of Chikungunya in South Africa with Involvement of *Aedes* (Diceromyia) *furcifer* (Edwards). *S Afr J 73:* 267.

McIntosh BM, Worth CB, Kokernot RH. 1961. Isolation of semliki forest virus from *Aedes* (Aedimorphus) *argenteopunctatus* (theobald) collected in Portuguese East Africa. *Trans R Soc Trop Med Hyg 55*: 192-198.

Meerburg BG, Singleton GR, Kijlstra A. 2009. Rodent-borne diseases and their risks for public health. *Crit Rev Micro* 35: 221-270.

Middleton DJ, Morrisy CJ, van der Heide BM, Russell GM, Braun MA, Westbury HA,… Daniels PW. 2007. Experimental Nipah virus infection in pteropid bats (*Pteropus poliocephalus*). *J Comp Pathol* 136: 266-272.

Miedema F, Hazenberg MD, Tesselaar K, van Baarle D, de Boer RJ, Borghans JA. 2013. Immune activation and collateral damage in AIDS pathogenesis. *Front Immunol* 4:298.

Morrison TE, Oko L, Montgomery SA, Whitmore AC, Lotstein AR, Gunn BM, ... Heise MT. 2011. A Mouse Model of Chikungunya Virus–Induced Musculoskeletal Inflammatory Disease: Evidence of Arthritis, Tenosynovitis, Myositis, and Persistence. *Am J Pathol 178*: 32-40.

Niewiesk S, Prince G. 2002. Diversifying animal models: the use of hispid cotton rats (*Sigmodon hispidus*) in infectious diseases. *Lab Animals* 36: 357-372.

Osterrieth P, Blanes-Ridaura G. 1960. Research on the Chikungunya virus in the Belgian Congo. I. Isolation of the virus in upper Uele *Annales de la Societe belge de medecine tropicale (1920), 40,* 199-203.

Paiardini M, Cervasi B, Reyes-Aviles E, Micci L, Ortiz AM, Chahroudi A, … Francella N. 2011. Low levels of SIV infection in sooty mangabey central memory CD4+ T cells are associated with limited CCR5 expression. *Nat Med 17: 830-836.*

Pan American Health Organization. Chikungunya. *World Health Organization*, 12 Aug. 2016. Web. July 2016.

Pandrea I, Apetrei C. 2010. Where the wild things are: pathogenesis of SIV infection in African nonhuman primate hosts. *Curr HIV/AIDS Rep* 7:28–36.

Pesko K, Westbrook CJ, Mores CN, Lounibos LP, Reiskind MH. 2009. Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to chikungunya virus. *J Med Entomol 46*: 395-399.

Pialoux G, Gaüzère BA, Jauréguiberry S, Strobel M. 2007. Chikungunya, an epidemic arbovirosis. *Lancet Infect Dis 7*: 319-327.

Plyusin A, Vapalahti O, Lankinen H, Lehvaslaiho H, Apekina N, Myasnikov Y,…Vahier A. 1994. Tula virus: a newly detected hantavirus carried by European common voles. *J Virol* 68: 7833-7839.

Powers AM, Brault AC, Tesh RB, Weaver SC. 2000. Reemergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. *J Gen Virol* 81: 471–479.

Powers AM, Logue CH. 2007. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J Genl Virol 88*: 2363-2377.

Rahman SA, Hassan L, Epstein JH, Mamat ZC, Yatim AM. 2013. Risk Factors for Nipah virus infection among pteropid bats, Peninsular Malaysia. *Emerg Infect Dis* 19: 51–60.

Schountz T, Prescott J. 2014. Hantavirus immunology of rodent reservoirs: current status and future directions. *Viruses* 6:1317–1335.

Sibold C, Ulrich R, Labuda M, Lundkvist A, Martens H, Schutt M,…Druger DH. 2001. Dobrava hantavirus causes hemorrhagic fever with renal syndrome in central Europe and is carried by two different Apodemus mice species. *J Med Virol* 63: 158-167.

Silvestri G, Sodora DL, Koup RA, Paiardini M, O'Neil SP, McClure HM,…Feinberg MB. 2003. Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. *Immunity* 18:441–452.

Sohayati AR, Hassan L, Sharifah SH, Lazarus K, Zaini CM, Epstein JH. 2011. Evidence for Nipah virus recrudescence and serological patterns of captive *Pteropus vampyrus*. *Epidemiol Infect* 139:1570–1579.

Tsetsarkin KA, Chen R, Weaver SC. 2016. Interspecies transmission and chikungunya virus emergence. *Curr Opin Virol 16*: 143-150.

Vourc'h G, Halos L, Desvars A, Boué F, Pascal M, Lecollinet S, Zientara S, Duval T, Nzonza A, Brémont M. 2014. Chikungunya antibodies detected in non-human primates and rats in three Indian Ocean islands after the 2006 ChikV outbreak. *Vet Res 45*: 1.

Ward LE. 2001. Handling the cotton rat for research. *Lab Animal Europe* 1: 31-6.

Weaver SC. 2006. Evolutionary influences in arboviral disease. *Curr Top Microbiol Immunol* 299: 285-314.

Westaway EG. 1966. Assesment and application of a cell line from pig kidney for plaque assay and neutralization tests with twelve group B arboviruses. *Am J Epidemiol* 84: 439-456.

World Health Organization, 1999. Removing obstacles to healthy development: report on infectious diseases Vancouver.

Yeo LS, Chu JJH. 2013. Recent developments and challenges in mouse models of Chikungunya virus infection. *Future Virol 8*: 423-426.

Ziegler SA, Lu L, da Rosa APT, Xiao SY, Tesh RB. 2008. An animal model for studying the pathogenesis of chikungunya virus infection. *Amer J Trop Med Hyg 79*: 133-139.

CHAPTER FOUR

CHIKUNGUNYA ANTIBODIES IN WILDLIFE SPECIES FROM ENDEMIC AND EPIDEMIC REGIONS

4.1 Introduction

Chikungunya virus (CHIKV) is a single stranded negative sense RNA virus that causes acute febrile illness and debilitating arthritis. CHIKV has been responsible for causing numerous outbreaks in Africa, Southeast Asia, the Indian subcontinent, and recently the Americas. Historically, CHIKV outbreak have been limited to Africa and Southeast Asia but globalization and ease of overseas travel have introduced this arthropod borne virus into the New World. In December of 2013, CHIKV infections were first documented on the Caribbean island of St. Martin (Leparc-Goffart et al., 2014). CHIKV has since been introduced to over 45 countries, territories, and provinces throughout the Caribbean, Central, South and North America (Pan America, July 2016).

As an arbovirus, CHIKV is primarily transmitted by *Aedes aegypti* and *Aedes albopicuts* mosquitoes (Weaver, 2006). Like most arboviruses, CHIKV circulates in both sylvatic transmission cycles (involving wildlife species mainly non-human primates) and urban cycles (human to mosquito to human transmission) (Powers et al., 2007). In Africa, CHIKV is maintained in sylvatic transmission cycles involving *Aedes spp.* mosquitoes and arboreal non-human primate *spp*. While the virus is thought to be maintained in non-human primate populations, other vertebrate reservoirs may be involved in secondary sylvatic transmission cycles when non-human primate populations have developed immunity to CHIIV infection (Diallo et al., 2012; Jupp et al., 1990; Diallo et al., 1999; McIntosh et al., 1961). Although sylvatic transmission has not been documented in Asia, CHIKV has been isolated from non-human primate species and CHIKV-specific antibodies have been found in non-human primate serum (Apandi et al., 2009; Inoue et al., 2003; Marchette et al., 1978). This evidence suggests a possible zoonotic sylvatic transmission cycle does exist in Asia however, greater investigation is needed.

Previous experimental infections of big brown bats (*Eptesicus fuscus*), cotton rats (*Sigmodon hispidus*), deer mice (*Peromyscus maniculatus*), green iguanas (*Iguana iguana*), leopard frogs (*Rana sphenocephala*), and Texas toads (*Anaxyrus speciosus*) with CHIKV in our lab suggested that rodent, reptile, and amphibian species may be involved in CHIKV sylvatic transmission cycles (Bosco-Lauth et al., 2015; Blizzard et al., unpublished; Hartwig et al. unpublished). Individuals from each of these species became viremic following CHIKV infection and amphibian and reptile species remained viremic for 6-10 days post infection (Hartwig et al., unpublished). These findings suggest that rodents, bats, reptile, and amphibian species may play a role in CHIKV sylvatic transmission cycles. In this study we sought to determine if CHIKV antibodies were present in wildlife species in areas with historical CHIKV outbreaks, Cambodia, and during an on-going epidemic in the Americas, Grand Cayman islands.

4.2 Materials and Methods

4.2.1 Animals

The diversity of wildlife species collected during November of 2012 from five provinces of Cambodia including Kandal, Kompong Chhnang, Kampong Thom, Siem Reap, and Preah Vihear (**Table 4.1**). Animal species were collected by villagers from the surrounding areas and brought to wildlife traders in each community village. Species collected include ricefield rats (*Rattus argentiventer*), Rugulose bullfrogs (*Hoplobatrachus rugulosus*), lesser horseshoe bats (*Rhinolophus hipposideros*), lesser asian house bats (*Scotophilus kuhlii*), Flower's Long-headed lizards (*Pseudocalotes floweri*), tokay geckos (*Gekko gecko*), Asian black-spined toad (Bufo melanastictus), rainbow snake (*Enhydris enhydris*), Indo-chinese water snake (*Enhydris subtaniata*), glossy marsh snake (*Gerarda prevostiana*), and Bocourt's water snake (*Enhydris bocourti*). After collecting blood samples, animals were returned alive to the wildlife brokers to be sold for human consumption and/or to sell to local crocodile farmers. Blood samples were collected by tail bleeding (snake and lizard species) and/or heart (rats, bats, frog, and toad species).

Blood samples were collected during June 2015 from green iguanas on the Grand Cayman islands by Dr. Matthew Johnston as part of an invasive green iguana research program. In all 10 juvenile males, 6 juvenile females, 5 subadult males, 11 subadult females, 13 adult females, and 3 adult males were sampled. Blood samples were collected by tail bleeding.

Serum samples from Cambodia and Grand Cayman were shipped back to our laboratories for testing, under the appropriate APHIS and CITES permits. This work was approved by the Animal Care and Use Committee at Colorado State University, Fort Collins, Colorado, USA (approval 14-5258A) and conducted in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals).

4.2.2 Viruses

One strain of CHIKV was graciously provided by Ann Powers at the Centers for Disease Control and Prevention, Fort Collins, CO. The SAH2123 (SAH) virus was isolated from a human patient during the Comoros Island outbreak in 2005 and had been passaged twice in suckling mice and seven times in cultured Vero cells. This strain is classified as a member of the East-Central-South African clade of CHIKV.

4.2.2 Serology

Antibody serological screening was performed using plaque reduction neutralization assays (PRNT) as previously described (Lindsey et al., 1976). Briefly, Vero cells were grown in 6 well plates until a confluent monolayer was obtained. Serum samples were diluted to 1:320 in 96 well plates with BA-1 containing antibiotics. CHIKV SAH virus was diluted to yield approximately 100 plaques/well for the control titration and 75 ul of virus was added to 75 ul of sample and incubated at 37 °C for one hour. Samples were then inoculated onto 6 well Vero plates and allowed to incubate for one hour at 37 °C. First and second overlays were added as described previously and plaques were counted 24 and 48 hours following the second overlay application. Virus titers were depicted as the reciprocal of the highest dilution of serum resulting in 80% neutralization.

4.3 Results

4.3.1 Cambodia

Of the 466 animals sampled throughout Cambodia a limited number of animals from all five regions had antibodies at low levels (≥10-40). In Kandal 3/39 (8%) of ricefield rats were positive for CHIKV antibody. Of the species sampled in Kompong Chhang, 11/64 (17%) lesser horseshoe bats, 2/4 (50%) Flower's long-headed lizards, and 9/33 (27%) lesser Asian house bats were positive for CHIKV antibodies. In Preah Vihear, 14/37 (38%) Rugulose bullfrogs and 8/59 (4%) water snake *spp.* had detectable antibody. Within the Kampong Thom province, 2/6 (33%) tokay geckos, 5/71(7%) water snake *spp.*, and 2/20 (10%) Rugulose bullfrogs had detectable antibodies. And finally in Siem Reap, 18/48 (37%) of Rugulose bullfrogs and 1 of 51 (2%) water snake *spp.* samples had detectable antibodies.

Table 4.1. Wildlife species sampled from five regions throughout Cambodia. (Percentages of animals positive for CHIKV antibodies by PRNT).

Species	Location	Number tested	Number PRNT positive %(number/total)	$PRNT_{80}$ antibody titer range
Ricefield Rats	Kandal	39	8 (3/39)	40
Rainbow Snake Indo-Chinese Water Snake		5	0	-
Rugulose Bullfrogs		14	0	-
Lesser Horseshoe Bats	Kompong Chhnang	64	17 (11/64)	≥10-≥20
Asian black-spined toad		2	0	-
Flower's Long-headed Lizard		4	50 (2/4)	≥10-≥20
Lesser Asian House Bat		33	27 (9/33)	≥10-≥20
Tokay Gecko		6	3 (2/6)	≥10-≥20
Rainbow Snake Indo-Chinese Water Snake Glossy Marsh Snake Bocourt's Water Snake	Kampong Thom	71	7 (5/71)	≥10-≥20
Asian black-spined toad		10	0	≥10-≥20
Flower's Long-headed Lizard		3	0	≥10-≥20
Rugulose Bullfrogs		20	10 (2/20)	≥10-≥20
Rugulose Bullfrogs	Siem Reap	48	38 (18/48)	≥10-≥20
Rainbow Snake Indo-Chinese Water Snake Glossy Marsh Snake Bocourt's Water Snake		51	2 (1/51)	≥10-≥20
Rugulose Bullfrogs	Preah Vihear	37	38 (14/37)	≥10-≥20
Rainbow Snake Indo-Chinese Water Snake Glossy Marsh Snake Bocourt's Water Snake		59	4 (8/59)	≥10-≥20

4.3.2 Grand Caymans Island

Of the 48 green iguanas (*Iguana iguana*) sampled only one juvenile male animal had a low level of antibody present at time of sampling (40).

4.4 Discussion

Our investigation detected the presence of low titer neutralizing antibodies to CHIKV in several wild vertebrates from five provinces throughout Cambodia and on the Grand Cayman islands in the Caribbean. CHIKV was first detected in Cambodia in 1961 and has continued to co-circulate at low levels with dengue virus. In 2011, a CHIKV outbreak began in the Northwest region of Battambang province (May) and subsequently spread to Siem Reap (June/July), Kampong Thom (July), Preah Vihear (August/December), Kampong Cham (October), and Kandal (December) (Duong et al., 2012). Although this outbreak was short in duration and had a limited number of identified cases (approximately 24) it indicated that CHIKV was still circulating at some level throughout the country. While we identified the presence of CHIKV antibodies at low titer in all of the provinces investigated it remains unclear the role these species play in CHIKV transmission.

As of June of 2015, the Grand Cayman islands were recovering from a CHIKV epidemic that had spread throughout the Americas. On the Grand Cayman islands there are no native or invasive non-human primate species to maintain an endemic CHIKV sylvatic transmission cycle. In the absence of NHPs it would be interesting if, similar to the results seen in experimental trials, reptiles and or amphibian species would be capable of serving as amplifying and/or reservoir hosts. Although we did detect the presence of CHIKV neutralizing antibodies in 1/48 invasive green iguana species, the role these animals play in CHIKV remains unclear.

Previous studies have also detected the presence of CHIKV antibodies at low levels in other terrestrial vertebrate species during CHIKV outbreaks. In Senegal, CHIKV has been isolated from several vertebrate species including: including vervet monkeys (*Cercopithecus aethiops*), Senegal galago (*Galago senegalensis*), a palm squirrel (*Xerus erythropus*), baboons (*Papio papio*), a golden sparrow

(*Auripasser luteus*), and bats (*Scotophilus spp.*) (Bres et al., 1969; Cornet et al., 1979). In Malaysia Marchette et al. detected low levels of circulating CHIKV antibodies in *Rattus sabanus* (Marchette et al., 1978). Experimental infections of wild rodent species in the genera *Mastomys*, *Arvicanthis*, and *Aethomys*, *Mesocricetus*, bat *spp.*, and reptile *spp.* indicate that these species are capable of developing low levels of viremia and develop low level antibody responses (McIntosh, 1961; Bedekar and Pavri, 1969; Bosco-Lauth et al., 2015). One study conducted on La Reunion island 2005-2006, one of the largest CHIKV outbreaks documented indicated that NHP species, including lemurs, and ship rats had detectable levels of CHIKV antibodies. It also appears that some domestic animals such as cattle, pigs, dogs, rabbits, sheep, goats, horses, and several species of birds do not develop a detectable viremia but are capable of producing CHIKV neutralizing antibodies (Karabatos, 1985; Halstead et al., 1966; Bosco-Lauth et al., 2015). These finding suggest that a variety of wildlife host species may play a role in CHIKV sylvatic transmission cycles and may involve rodents and other zoophilic mosquito species (Diallo et al., 1999; Bosco-Lauth et al., 2015). Similar to other studies, these findings should be considered with caution. At present, the amount of cross reaction between CHIKV and antibodies to other circulating alphaviruses in these animals remains unclear. Additionally, if these animals have developed neutralizing antibodies to CHIKV does this indicate they developed a detectable infection? Could these animals serve as reservoir hosts for CHIKV? Further investigation is needed to determine if these species can develop detectable CHIKV viremias and infect *Aedes spp*. mosquitoes; how long viremias last; and the extent of alphavirus antibody cross reaction.

In conclusion, our findings indicate that species other than non-human primates are being exposed to CHIKV, almost certainly as a result of being fed upon by infected mosquitoes. The presence of antibodies in no way indicates competence of that host for transmission of virus back to mosquitoes or that those animals play a role in the ecology of the virus infection. Nonetheless, considering the dogma that CHIKV persists only in primates, these findings justify expanded efforts to identify alternative vertebrate hosts for this virus.

REFERENCES

Apandi Y, Lau, Izmawati SK, Amal NM, Faudzi Y, Mansor W, ... Zainah S. 2010. Identification of Chikungunya virus strains circulating in Kelantan, Malaysia in 2009. *Southeast Asian J Trop Med Public Health 41*: 1374.

Bedekar SD, Pavri KM. 1969. Studies with chikungunya virus. Part I. Susceptibility of birds and small mammals. *Indian J Med Res 57*: 1181-92.

Bosco-Lauth AM, Han S, Hartwig A, Bowen RA. 2015. Development of a hamster model for chikungunya virus infection and pathogenesis. *PloS One*, *10*: p.e0130150.

Bres P, Hery G. 1969. Kinetics of Production of Haemagglutination Inhibiting and Complement Fixing Antibodies in Mice Immunized with Chikungunya, Onyong-nyong and Sinbus Viruses. 3. Effect of interval between inoculations and Use of an Adjuvant. *Revue Immunol 33*: 147.

Cornet M, Robin Y, Adam C, Valade M, Calvo MA. 1979. Transmission expérimentale comparée du virus amaril et du virus Zika chez *Aedes aegypti*. *L Cahiers ORSTOM série Entomologie médicale et Parasitologie*, 47-53.

Diallo M, Thonnon J, Traore-Lamizana M, Fontenille D. 1999. Vectors of Chikungunya virus in Senegal: current data and transmission cycles. *Am J Trop Med Hyg 60*: 281-286.

Diallo D, Sall AA, Buenemann M, Chen R, Faye O, Diagne CT, ... Weaver SC. 2012. Landscape ecology of sylvatic chikungunya virus and mosquito vectors in southeastern Senegal. *PLoS Negl Trop Dis 6*: e1649.

Duong V, Andries AC, Ngan C, Sok T, Richner B, Asgari-Jirhandeh N, Bjorge S, Huy R, Ly S, Laurent D, Hok, B. 2012. Reemergence of Chikungunya virus in Cambodia. *Emerg Infect Dis* 18: 2066-9.

Halstead SB. 1966. Mosquito-borne haemorrhagic fevers of South and South-East Asia. *Bull Wor Heal Org 35*: 3.

Hartwig AT, Bosco-Lauth A, Bowen RA. Unpublished. Experimental infection of North American Reptile species.

Inoue S, Morita K, Matias RR, Tuplano JV, Resuello RRG, Candelario JR, Cruz DJM, Mapua CA, Hasebe F, Igarashi A, Natividad FF. 2003. Distribution of three arbovirus antibodies among monkeys (Macaca fascicularis) in the Philippines. *J Med Primat 32*: 89-94.

Jupp PG, McIntosh BM. 1990. Aedes furcifer and other mosquitoes as vectors of chikungunya virus at Mica, northeastern Transvaal, South Africa. *J Am Mosq Control Assoc* 6:415-420.

Karabastos N, ed. International Catalogue of Arboviruses, Including Certain Other Viruses of Vertebrates. San Antonio, TX: American Society of Tropical Medicine and Hygiene, 1985, updated in 2001.

Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, De Lamballerie X. 2014. Chikungunya in the Americas. *The Lancet* 383: 514.

Marchette NJ, Rudnick A, Garcia R, Mac Vean S. 1978. Alphaviruses in Peninsular Malaysia. I. Virus isolations and animal serology. *Southeast Asian J Trop Med Public Health* 9: 317- 29.

McIntosh BM, Worth CB, Kokernot RH. 1961. Isolation of semliki forest virus from *Aedes* (Aedimorphus) *argenteopunctatus* (theobald) collected in Portuguese East Africa. *Trans R Soc Trop Med Hyg 55*: 192-198.

Pan American Health Organization. Chikungunya. *World Health Organization*, 12 Aug. 2016. Web. July 2016.

Powers AM, Logue CH. 2007. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J Genl Virol 88*: 2363-2377.

Weaver SC. 2006. Evolutionary influences in arboviral disease. *Curr Top Microbiol Immunol* 299: 285-314.

CHAPTER FIVE

CONCLUSIONS

Chikungunya virus (CHIKV) is an arthropod-borne alphavirus endemic to Africa and Asia. The experiments in this dissertation investigated the potential for selected rodent species in North America to serve as reservoir and/or amplifying hosts during CHIKV epidemics. The conclusions from these investigations suggest that some species are capable of developing a CHIKV viremia that may be capable of infecting *Aedes spp.* vectors and could contribute to CHIKV transmission.

Nine rodent species were investigated for their potential to develop CHIKV viremia: nutria, brushy-tailed woodrats, fox squirrels, cotton rats, meadow voles, prairie voles, Norway rats, deer mice, and groundhogs. Six of these species, fox squirrels, cotton rats, meadow voles, prairie voles, deer mice, and groundhogs developed viremias capable of being transmitted to *Aedes spp.* mosquitoes. Four of nine species additionally developed CHIKV antibodies which may aid in detecting CHIKV outbreaks before human cases are detected. These results confirm that there are competent vertebrate hosts for CHIKV in the Americas, although their importance as such during sylvatic transmission cycles remains unknown. Additional studies are needed to evaluate the role of rodent species during CHIKV sylvatic transmission cycles in both emerging and historically-endemic CHIKV areas.

Experimental infection of cotton rats by subcutaneous CHIKV inoculation resulted in asymptomatic viremic infection of thirteen of seventeen cotton rats. Immunohistochemistry of select tissues did not detect viral replication on 2, 3, and 4 dpi. However, CHIKV virus was detected in homogenized liver, spleen, and lung tissues on 2 dpi. Viremia was short-lived and virus was cleared within four days of infection. These results indicate that cotton rats develop chikungunya viremia of potentially sufficient magnitude to serve as competent hosts.

Field study investigations conducted in CHIKV endemic Cambodia and a recent outbreak in the Grand Cayman Islands demonstrated that wildlife species have some exposure to CHIKV. Antibody titers were detected in rats, bats, frogs, snakes, and green iguana species. In wildlife, CHIKV immunity

could also be attributed to cross protection from prior alphavirus infections. These results tentatively suggest that CHIKV may infect these species however, the extent of their role in CHIKV sylvatic transmission cycles in historically endemic and newly emerging CHIKV regions remains unknown.

In retrospect, had I discovered earlier that groundhogs were competent CHIKV hosts I would have performed additional experimental studies investigating the effects of hibernation on virus titers and potential viral resurgence during the emergence from hibernation. Additional hibernating rodents and reptiles should be examined for their capacity to overwinter CHIKV in both endemic and naïve geographic regions. Additionally, it would be interesting to further examine immunological differences/alterations during hibernation and the effects these differences have on CHIKV pathogenesis and replication. Future directions for CHIKV research should include a more extensive investigation of reservoir competence among rodent taxa (including genetic diversity among individuals in populations, differences based on diet, gut flora diversity, and previous exposure to pathogens), investigating additional reptile hosts (both endemic and naïve geographic regions), and a better understanding of immune responses to CHIKV in rodent hosts in both hibernating and non-hibernating species. Moreover, the examination of potential wildlife hosts for CHIKV, both reptile and mammalian, through sero-surveillance would help in predicting and managing the impact of future CHIKV emergence.