DISSERTATION

MOLECULAR ANALYSIS OF THE GENETIC DETERMINANTS THAT CONTRIBUTE TO VIRULENCE IN LINEAGE 2 WEST NILE VIRUS

Submitted by

Hannah Elizabeth Romo

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: Greg Ebel
Co-Advisor: Aaron Brault

Carol Blair
Richard Bowen
Kate Huyvaert

ABSTRACT

MOLECULAR ANALYSIS OF THE GENETIC DETERMINANTS THAT CONTRIBUTE TO VIRULENCE IN LINEAGE 2 WEST NILE VIRUS

The ability of arboviruses to impart significant global disease burdens is related to the corresponding capacity of arboviruses to emerge in naïve environments or re-emerge in endemic environments. The introduction of West Nile virus (WNV) into North America was marked by rapid spread across the continent, high rates of neuroinvasive disease in humans and horses, and subsequent displacement by newer evolved genotypes. In the last 12 years, an underrepresented lineage of WNV, lineage 2 (L2) has similarly emerged from sub-Saharan Africa into areas of Europe and Russia, causing widespread neurological disease and recurrent enzootic transmission. Given the potential for further geographic spread of L2 WNV and to understand mechanisms that drive emergence events for WNV, I sought to characterize L2 WNV in a comprehensive and comparative manner by investigating potential molecular mechanisms of pathogenesis in mosquitoes, birds, and mice (as models for human disease). A more thorough understanding of the mechanisms that dictate rapid dispersal and endemic maintenance of arboviruses will improve our ability to predict emergence events, increase the effectiveness of surveillance mechanisms, and develop effective intervention strategies.

Within lineage 1 (L1) WNV, the role of the NS3-249P amino acid in modulating severe virogenesis in American Crows (AMCRs) has been well established and is predicted to be involved in facilitating the emergent capacity of L1 WNV. The evolution of a proline at the same NS3-249 locus in L2 WNV was initially observed during the first L2 WNV associated outbreak in Europe. However, no bird mortality was observed during the NS3-249P associated L2 WNV outbreak, and the extent of L2 WNV pathogenesis in birds is unclear. In this aim, I examined

the viremia titers and mortality profiles of North American AMCRs and house sparrows following infection with African and European L2 WNV strains with and without amino acid mutations at the NS3-249 locus. Our results demonstrate that L2 WNV strains can elicit severe virogenic and fatal outcomes in AMCRs and HOSPs. Additionally, I found that the NS3-249 locus is modulating AMCR viremia titer outcomes, similar to what has been previously observed for the NS3-249 locus in L1 WNV strains. I also demonstrated the 3' UTR of NS10 reduces viremia titers of AMCRs at later time points.

The vast majority of our understanding regarding the vector competence of *Culex* mosquitoes for WNV originates from studies performed with L1 WNV strains, and as such, little information is available regarding the competency of *Culex* mosquitoes for L2 WNV. To remediate this, I assessed the vector competence phenotypes of two different North American *Culex* mosquito species for multiple L2 WNV strains. Our results demonstrate that *Culex pipiens and Culex quinquefasciatus* mosquitoes can effectively transmit L2 WNV. I also identified a L2 strain harboring an NS3-249P mutation (NS10) that limited infection to the midgut of *Culex pipiens* mosquitoes. The competence of North American *Culex* mosquitoes to transmit L2 WNV taken together with the ability of AMCRs and HOSPs to serve as reservoir hosts for L2 WNV demonstrates the capacity for L2 WNV transmission in the Western Hemisphere.

Previous studies generated in this dissertation demonstrated that high viral titers in AMCRs were modulated by the NS3-249P mutation in the NS10 L2 WNV strain and that this same strain also generated lower infection rates in *Culex pipiens* compared to other L2 WNV strains, suggesting that the NS3-249 locus might be involved in concurrently modulating vector competence in *Culex pipiens* and viral titers in AMCRs. To conclusively determine the role the NS3-249P mutation in facilitating emergence of L2 WNV, I examined the phenotype of NS3-249P and NS3-249H L2 WNV mutations in a transmission cycle inclusive manner. Specifically, I found that the NS3-249P mutation was directly involved in decreasing fitness in *Culex pipiens*. Furthermore, I found that following infection in mice, the NS3-249 residue did not modulate

neuroinvasive disease phenotypes and suggests that the emergence of L2 WNV in Greece was potentially facilitated by increases in force of transmission related to the occurrence of the NS3-249P mutation, rather than the emergence of a more neuroinvasive L2 genotype.

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisors Drs. Aaron Brault and Greg Ebel, who have both encouraged and supported me throughout my time as a student. Dr. Brault has been an excellent advisor and mentor and I am very grateful for the support and patience he has offered me. I owe a special thanks to Dr. Ebel for his continuously helpful insights. I am also very grateful to the other members of my committee, Drs. Carol Blair, Dick Bowen, and Kate Huyvaert for their advice and contributions to my project.

I owe a huge thanks to the many people at the CDC who have assisted with my research and offered their friendship in the process. I would like to acknowledge Jason Velez for his helpful advice regarding cell culture, his extreme kindness and his willingness to always be able to culture cells for countless plaque assays. I am especially grateful to Andrea Sherman who guided me through the nuances of insectary work, maintained our colonies and provided me with adult mosquitoes for my vector competence studies. I am very appreciative to the postdoctoral fellows that have rotated through Aaron's lab (Bethany Bolling, Joan Kenney, Angela Bosco-Lauth, Elizabeth Dietrich, and Nisha Duggal) for their supportive guidance and friendship. Their keen insight and helpful suggestions have played a large role in shaping the outcome of this dissertation.

I am especially grateful to the folks at CSU and USDA who facilitated trapping and maintenance of birds used in this study. The generosity and expertise of Dick Bowen, Angela Bosco-Lauth and others at the Animal Disease laboratory enabled a large part of my research. I especially want to thank Todd Felix for his guidance and expertise and Nathan Grubaugh and Joseph Fauver for their assistance in trapping and maintaining our AMCR colony.

Finally, I would like to thank my family and friends for always offering their support and encouragement. I am especially thankful to my husband for keeping me grounded and always being my biggest supporter.

DEDICATION

I dedicate this to Justin Tanner

TABLE OF CONTENTS

ABSTRACT .....……..……..ii

ACKNOWLEDGEMENTS …….……...…...v

CHAPTER 1: LITERATURE REVIEW …….……...……1

CHAPTER 2: AVIAN HOST COMPETENCE OF LINEAGE 2 WEST NILE VIRUSES AND PHENOTYPIC EFFECT OF POLYMORPHISMS AT THE NS3-249 LOCUS…….……..…34

CHAPTER 3: VECTOR COMPETENCE OF *CULEX PIPIENS* AND *CULEX QUINQUEFASCIATUS* MOSQUITOS FOR AFRICAN AND EUROPEAN L2 WNV STRAINS ……...……61

CHAPTER 4: THE NS3-H249P SUBSTITUTION MODULATES DISPARATE PHENOTYPES IN AMCRS AND *CULEX PIPIENS*, EVIDENCE FOR HOST-SPECIFIC ADAPTIVE EVOLUTION……..79

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS…….……..……...…….....97

REFERENCES...……..…….……101

CHAPTER ONE: LITERATURE REVIEW

Introduction

Arboviruses are viral pathogens that are maintained primarily within zoonotic transmission cycles between hematophagic arthropods and reservoir hosts. Humans serve as either direct amplification hosts or incidental hosts in which serious and often fatal disease can be evident in the absence of sufficient viremia titers to serve for the infection of arthropod vectors. The ability of arboviruses to emerge as agents of disease or to be transmitted in new locations or between novel vectors and hosts has been exemplified by the recent appearance of West Nile virus (WNV) and Zika virus (ZIKV) in the Americas. Selective pressures that modulate arboviral emergence and relate to the capacity for host range expansion can lead to novel human virulence patterns. As such, gaining a more thorough understanding of arboviral emergence mechanisms will be necessary in order to advance our ability to predict emergence events and to develop effective infection mediation mitigation strategies.

Flaviviridae

The *Flavivirus*, *Pestivirus*, *Hepacivirus,* and *Pegivirus* genera compose the viral family *Flaviviridae*. Viruses within the *Flaviviridae* family are distributed worldwide and are a significant cause of human and animal disease. The family name '*flavivirus*' is derived from the Latin word "flavus*"* (meaning yellow) that was used to describe the jaundice associated with yellow fever virus (YFV) disease. *Pestivirus* is derived from the Latin word "*pestis"* meaning plague and includes viruses that often infect livestock such as bovine viral diarrhea virus, border disease virus and classical swine fever(Schweizer & Peterhans, 2014). The *Hepacivirus* genus, derived from the Greek word "hepatos" meaning liver, contains hepatitis C virus (seven genotypes), GB virus, and canine hepacivirus. The *Pegivirus* group is a tentatively proposed inclusion and contains GB virus (GBV-A, GBV-D, GBV-D)(Karabatsos, 1985).

There are four major ecological groups within the *Flavivirus* genus: tick-borne flaviviruses, insect specific flaviviruses, mosquito-borne flaviviruses, no known vector flaviviruses, and non-classified flaviviruses. Currently, 73 viruses comprise the *Flavivirus* genus and include dengue virus (DENV), Japanese encephalitis virus (JEV), Saint Louis encephalitis virus (SLEV), WNV, YFV, and ZIKV. Flaviviruses have an established capacity for emergence as demonstrated by the arrival of WNV and more recently, ZIKV in the Americas and together, significantly impact the global disease burden.

Approximately 300 million cases of DENV infection are estimated to occur annually(Bhatt *et al.*, 2013). Sylvatic DENV occurs in non-human primates and is transmitted by arboreal *Aedes* mosquitoes while urban DENV transmission occurs between humans and the urban *Aedes aegypti* mosquito (Vasilakis *et al.*, 2011). Dengue virus disease in humans ranges from febrile illness to severe dengue hemorrhagic fever and shock syndrome. There are four DENV serotypes and infection with one serotype produces immunity that is fully protective against that serotype, but increases the risk for developing severe hemorrhagic fever following exposure to a second serotype(Halstead, 2007). Several live attenuated vaccines are in the later stages of clinical trials, but no vaccines or therapies are currently effective for human use, so treatment for DENV disease remains largely supportive (Khetarpal & Khanna, 2016).

WNV is the most widely dispersed encephalitic flavivirus and exists in a transmission cycle between birds and mosquitoes (May *et al.*, 2011). WNV was first isolated from the blood of a febrile woman in 1937 in Uganda and has since been isolated in every continent except Antarctica (Smithburn *et al.*, 1940; Chancey *et al.*, 2015). Infection with WNV can result in febrile illness, but can also cause severe or fatal encephalitis in less than 1% of infected individuals(Mostashari *et al.*, 2001). No therapies or vaccines are approved for WNV and so the primary method of reducing human exposure is through vector control.

Similar to DENV, YFV cycles between humans and the *Aedes aegypti* mosquito in an urban transmission cycle or a jungle cycle between non-human primates and *Aedes africanus*

and other mosquitoes (Monath, 2001). A cycle intermediate to the jungle and urban cycles is maintained by tree-hole breeding mosquitoes that transmit YFV to both humans and non-human primates (Barrett & Monath, 2003). Infection in humans produces a viremia (levels of virus in the serum) that lasts up to 5 days during which they are infectious to mosquitoes (Barrett & Monath, 2003). In most cases, YFV disease in humans produces an asymptomatic or mild disease. However, in approximately 30% of individuals, a more severe and potentially fatal form of disease characterized by fever, jaundice, and hemorrhaging can occur(Huang *et al.*, 2014). A recent resurgence of YFV occurred in Angola in 2015 (Grobbelaar *et al.*, 2016, pp. 2015–2016). The outbreak spread to the Democratic Republic of the Congo the following year and prompted the initiation of a large vaccination campaign(Burki, 2016). A single dose of the attenuated YFV vaccine provides lifelong protection for most individuals and is considered the safest and most effective vaccine created (Barrett & Higgs, 2007).

ZIKV has recently emerged in the Americas and is cause for considerable worldwide concern due to rapid dispersal across the Americas and the association of microcephaly in infants(Shan *et al.*, 2016). Originally isolated in the Zika forest in Uganda in 1947, ZIKV is transmitted by *Aedes* spp. mosquitoes, with humans and non-human primates serving as amplification hosts (Dick *et al.*, 1952; Wikan & Smith, 2016). Infection can produce a mild febrile disease in humans, but on rare occasions can also lead to an autoimmune disorder known as Guillain–Barré syndrome(Parra *et al.*, 2016). Guillain–Barré syndrome is an autoimmune disorder that targets the peripheral nervous system and a rise in the number of cases has been reported in areas with recent history of ZIKV transmission(Parra *et al.*, 2016). More notably, ZIKV can also be transmitted from mother to fetus, causing miscarriage or birth deformities(Ellington *et al.*, 2016; Mlakar *et al.*, 2016; Schuler-Faccini, 2016). Currently, no vaccines or therapies are approved for ZIKV so vector control remains the principal means of reducing infection(Weaver *et al.*, 2016).

The severe pathogenic capacity and global mobility of flaviviruses such as YFV, WNV, DENV and ZIKV characterize a continuing source of disease occurrences and emphasize the need for virus specific therapies and vaccines(Barrett, 2016; Weaver *et al.*, 2016). Understanding the factors associated with flavivirus emergence is critical for developing effective control strategies.

West Nile Virus

WNV is a mosquito-borne flavivirus belonging to the genus *Flavivirus* and groups to the JEV serological complex (Calisher *et al.*, 1989). The JEV serological complex grouping resulted from a seminal study by Calisher et al., in which the cross-neutralization status of sixty-six flaviviruses was assessed. The study led to the classification of eight serocomplexes within the family *Flaviviridae*, with viruses such as JEV, WNV, SLEV, Murray Valley encephalitis, Kokobera, Alfuy, Stratford, Usutu, Kunjin, and Koutango demonstrating serological cross-reactivity and thereby grouping to the JEV serocomplex(Calisher *et al.*, 1989).

WNV virion are approximately 50 nm in diameter and contain a single-stranded positive-sense RNA genome, approximately 11 kb in length encoding 10 proteins (Fig. 1a). Virions are enveloped by envelope (E) and membrane (M) glycoproteins embedded in an underlying host derived lipid bilayer. The nucleocapsid is made up of a single positive sense RNA genome complexed with C protein. WNV gains cellular entry by binding of the E protein into host cell receptors and triggering receptor-mediated endocytosis (Fig. 1b)(Chu & Ng, 2004a). Host cell proteins that function as receptors for WNV have not been well characterized, however several cell surface proteins such as αVβ3 integrin and DC-SIGN (dendritic-cell-specific ICAM-grabbing non-integrin) are believed to directly or indirectly act as receptors for WNV infection(Chu & Ng, 2004b; Davis *et al.*, 2006). A decrease in pH within the trafficking endosome results in a conformation change in the E protein resulting in the fusion of viral and endosomal membranes and subsequent release of the viral genome into the cytoplasm(Modis *et al.*, 2004). Cap-

dependent translation is mediated by a 2'-O methylated guanosine cap at the 5' end of the viral genome. The genome encodes a single open reading frame (ORF) from which a single polyprotein is translated. This polyprotein is post-transnationally processed to yield 10 viral proteins. The three structural proteins, capsid (C), prM (pre-membrane), and envelope (E) are encoded on the 5' end of the genome and the seven non-structural proteins (NS1, NS2A, NSB, NS3, NS4A, NS4B, and NS5) are encoded by the 3' portion of the polyprotein (Fig. 1a) (Mukhopadhyay *et al.*, 2005).

The viral C, prM, and E proteins are necessary for virion formation and assembly. The C protein is a highly basic protein that encapsidates the RNA genome in a nucleocapsid structure and is potentially involved in other non-structural functions(Beatch *et al.*, 2005; Hunt *et al.*, 2007; Urbanowski & Hobman, 2013). The prM protein is a glycoprotein precursor for M that prevents E from undergoing premature rearrangement and fusion as immature virions traverse through the secretory pathway. The E glycoprotein is the major protein on the surface of the virions and mediates host cell receptor binding and membrane fusion. The seven non-structural proteins NS1 – NS5 facilitate replication and synthesis of the negative and positive RNA intermediates. The function of NS1 is unclear, but it is believed that NS1 induces cellular humoral responses and is important for RNA replication(Falgout *et al.*, 1990; Mackenzie *et al.*, 1996; Timofeev *et al.*, 1998). NS2A is a hydrophobic, potentially membrane spanning protein possibly involved in virion assembly(Kümmerer & Rice, 2002). NS2B interacts as a cofactor to NS3 to facilitate serine protease activity(Arias *et al.*, 1993) The NS3 protein is a large, highly conserved protein that contains a helicase domain in the C terminal region and a protease domain on the N-terminal region. The helicase domain encodes a helicase and an RNA-stimulated nucleoside triphosphatase (NTPase)(Gorbalenya *et al.*, 1989; Wengler & Wengler, 1991, 1993). NS3 has also been shown to bind the terminal stem loop structure of the 3' UTR in conjunction with NS5(Chen *et al.*, 1997). The functions of NS4A and NS4B are unknown, however it its speculated that NS4B may be involved in RNA replication(Lindenbach & Rice, 1999). NS5

contains a methyltransferase domain in the N terminal region and RNA-dependent RNA polymerase in the C terminal portion(Kamer & Argos, 1984; Koonin, 1991).

Clinical Disease Outcomes

To initiate WNV infection, a mosquito that is transmitting WNV must attempt to take a blood meal from a naïve individual. Salivary secretions are injected at the site of infection as the mosquito probes for target blood vessels. Infectious virions from the saliva are deposited at the probe site and infect keratinocytes and/or resident Langerhans cells(Lim *et al.*, 2011). Cells infected with WNV drain to the lymph nodes, whereby infection becomes disseminated(Johnston *et al.*, 2000). WNV disease in humans is generally characterized by an approximate 75% asymptomatic to 25% symptomatic disease ratio, with less than 1% of patients developing West Nile neuroinvasive disease (WNND) (Fig. 2a)(Petersen LR *et al.*, 2013). WNND is distinguished by the presence of meningitis, encephalitis, acute flaccid paralysis, or a combination of all three (Fig. 2b). The incubation period following WNV infection ranges from 2-14 days and clinical symptoms can persist from 3 to 5 days. Symptomatic WNV disease is characterized by a period of flu-like symptoms and self-limiting febrile illness(Sejvar, 2014). The risk for developing WNND significantly increases with populations over the age of 65 and immunosuppressed individuals(Carson *et al.*, 2012).Pre-existing chronic disease conditions such as cancer, diabetes, renal disease, and hypertension also increase the risk of developing severe WNND(Cho & Diamond, 2012). WNND induced meningitis is characterized by inflammation of the meninges only, a membrane that cloaks the brain and spinal cord. Symptoms include headaches, fever as well as rigidity and stiffness in the neck (Beckham & Tyler, 2009). Encephalitis, or generalized inflammation of the brain, is distinguished from meningitis by the presence of injury in the brain parenchyma, focal neurological symptoms and evidence of inflammation.

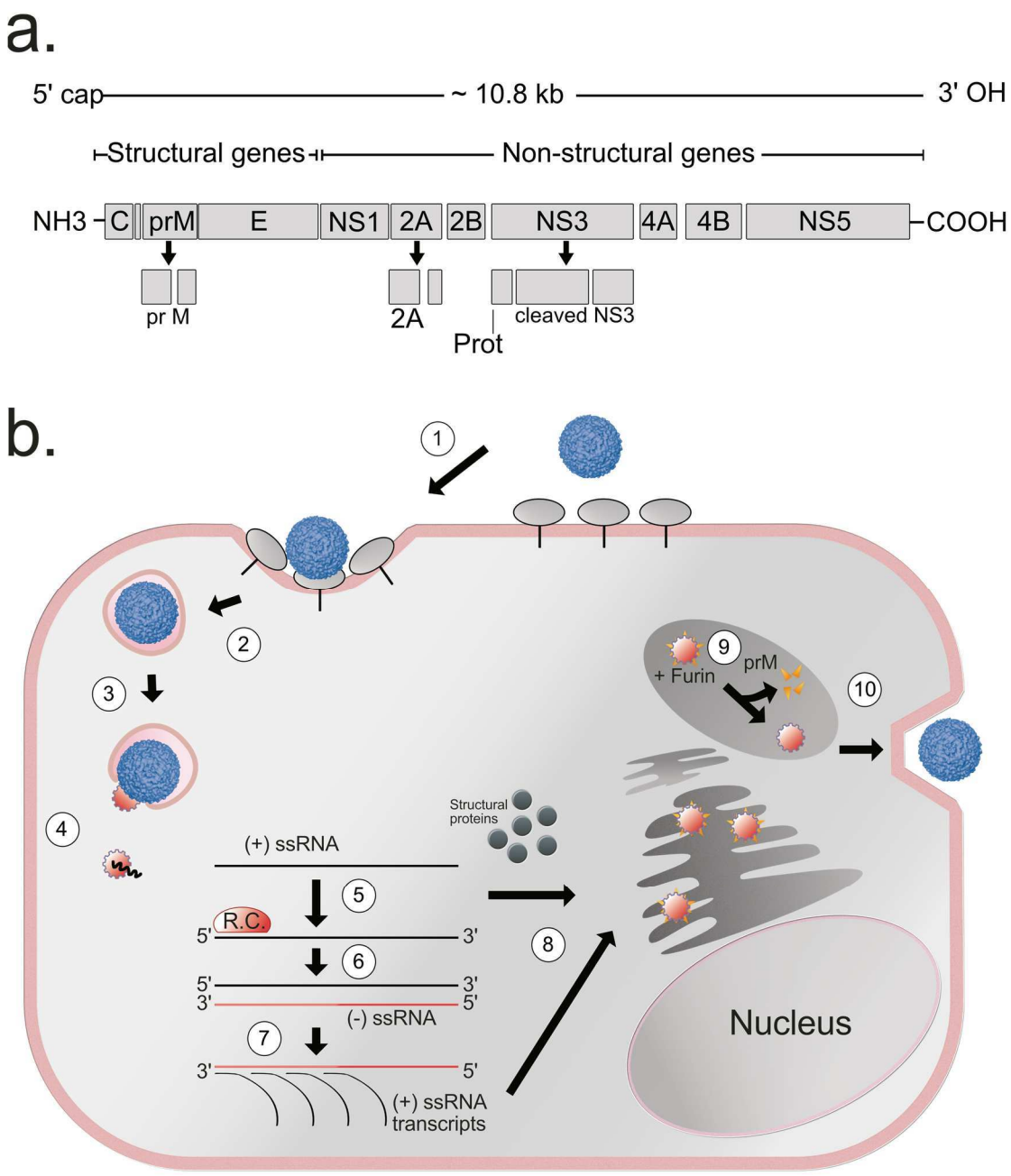

Figure 1. Genomic structure and replication cycle of WNV.

a) Schematic of WNV genome, polyprotein and polyprotein cleavage products. b) Replication of WNV in a cell is depicted: 1) Recognition of virion with cellular receptor 2) Receptor mediated endocytosis 3) Fusion of endosomal membrane triggered by drop in pH 4) Uncoating of the virion 5) + sense ssRNA translation initiated and polyprotein is processed 6) Replication complex is formed and negative-sense ssRNA is synthesized 7) Full length positive-sense ssRNA is formed 8) Capsid surrounds genomic RNA on rough endoplasmic reticulum (ER) 9) To prevent premature rearrangement of the immature virion to the fusogenic form, furin mediates cleavage of prM to M in the late secretory pathway 10) Mature virions exit the cell via exocytosis (adapted from (Suthar *et al.*, 2013).

Focal neurological symptoms of encephalitis are characterized by general weakness, ataxia, and movement disorders. WNND associated acute flaccid paralysis can present symptomatically as acute limb weakness or co-present with either meningitis or encephalitis(Marciniak *et al.*, 2004).

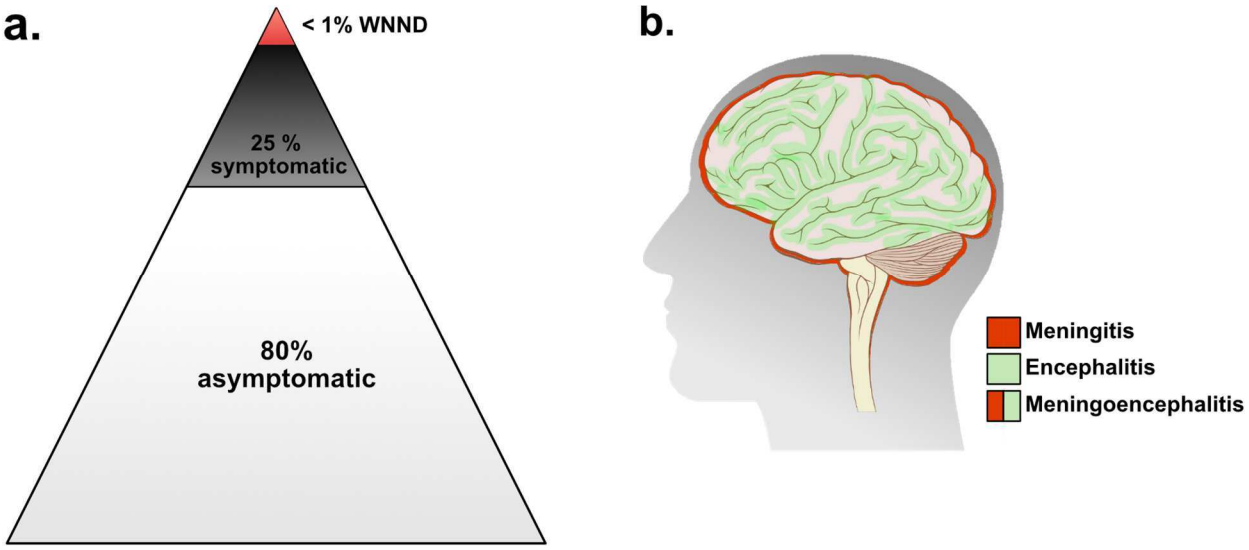

Figure 2. Potential disease outcomes in humans following WNV infection.

a) A pyramid is used to depict the various outcomes of WNV infection in humans. Less than 1% of all infected individuals develop WNND b) Variants of WNND include development of meningitis (inflammation of the meninges), encephalitis (inflammation of the brain), or a combination of both (meningoencephalitis). In some cases, some WNND patients may also develop acute flaccid paralysis (not pictured) alone or in combination with the WNND presented in Fig 2.b.

WNV transmission cycle

WNV exists in transmission cycles between mosquitoes and birds (Fig. 3). Birds, mainly in the order Passeriformes, that generate viremia titers sufficient to infect mosquitoes serve as reservoir hosts for WNV. Passerines, also known as perching birds, are the largest order of

birds worldwide and their respective contribution to the WNV transmission cycle varies by species(Mayr, 1946). For example, a multitude of experimental infections of different Passerines species with WNV (NY99) have demonstrated that some species such as American Crows (*Corvus brachyrhynchos*; AMCRs), and blue Jays (*Cyanocitta cristata)* are highly competent reservoir species, while mourning Doves (*Zenaida macroura*) and European Starlings (*Sturnus vulgaris*) demonstrate only moderate host competence for WNV(Komar *et al.*, 2003). *Culex* mosquitoes drive the transmission cycle between avian hosts due to their avian host feeding preference(Farajollahi *et al.*, 2011). Overwintering has been demonstrated in multiple *Culex* species and may also be an important mechanism of WNV persistence(Reisen *et al.*, 2006a). WNV infection in humans generates a transient viremic response that is not sufficiently high for the infection of mosquitoes(Zou *et al.*, 2010). Consequently, humans and most mammals function as incidental, dead-end hosts in the WNV transmission cycle(Zou *et al.*, 2010). Routes to WNV infection that are alternative to the mosquito-bird-mosquito transmission cycle are rare, but have been demonstrated. WNV infections in birds during periods of non-observable mosquito activity is believed to be facilitated by bird-to-bird transmission (Dawson *et al.*, 2007). Potential bird-to-bird transmission can occur through the fecal-oral route or through ingestion of WNV positive bird carcass by birds of prey(Ip *et al.*, 2014; Wheeler *et al.*, 2014). Some rabbits, rodents, and alligators have been shown to develop WNV viremia titers sufficient to infect mosquitoes following experimental exposure to WNV and could potentially be involved in WNV maintenance and transmission (Klenk et al., 2004; Padgett et al., 2007; Platt et al., 2007; Tiawsirisup et al., 2005). WNV has been isolated from many different species of hard (*Ixodidae*) and soft (*Argasidae*) ticks but their contribution to the WNV transmission cycle remains unclear as evidence of laboratory transmission has been inconclusive (Hutcheson et al., 2005; Reisen et al., 2007). Blood transfusion and organ transplants can be sources of WNV infection to recipient patients when donor sources are not pre-screened for WNV (Iwamoto et al., 2003; Stramer et al., 2005).

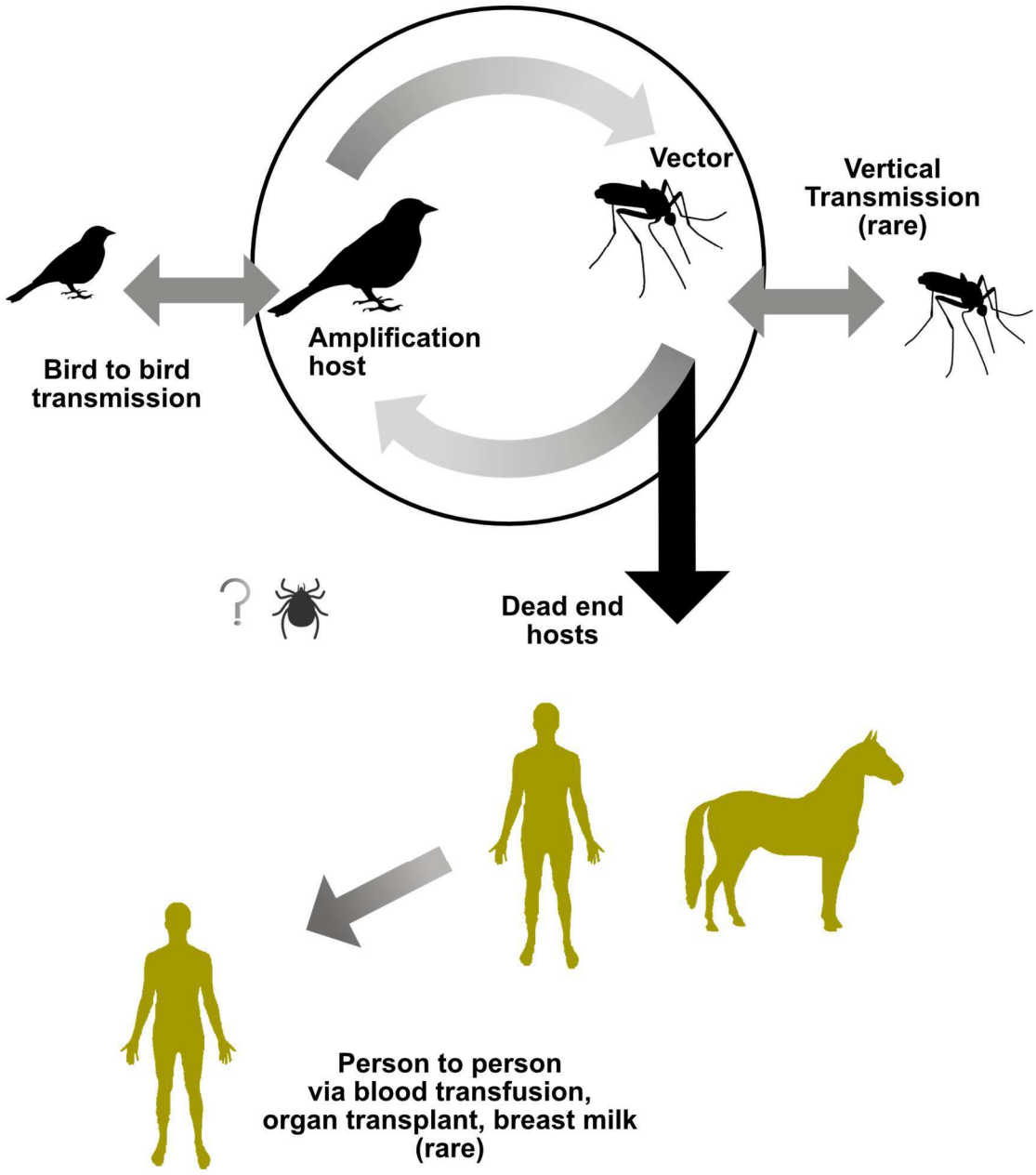

Figure 3. WNV Transmission Cycle.

The main WNV transmission cycle is between *Culex* mosquitoes and passerine birds. In addition to infection by mosquito-borne transmission, the carcasses of birds that are WNV positive can be a source of oral exposure to WNV for scavenger avian species. Vertical transmission can be an important mechanism of WNV persistence. Spill-over from the enzootic cycle between mosquitoes and birds results in incidental infection of humans, horses and other mammals. Although rare, person-to-person transmission though blood transfusion and organ transplantation has also been demonstrated (Iwamoto et al., 2003; Stramer et al., 2005). WNV has been isolated from field caught ticks, but the laboratory experiments regarding WNV transmission remain inconclusive (Hutcheson et al., 2005; W. K. Reisen et al., 2007).

Phylogenetic lineages of West Nile virus

Hemagglutination inhibition assays and monoclonal antibody-binding assays were used to geographically classify WNV isolates into African, Indian, and Europe/Middle East groupings(Hammam *et al.*, 1965; Price & O'Leary, 1967). Nucleotide sequencing and phylogenetic comparison of 47 full-length WNV genome sequences determined that WNV groups to two main lineages based on genetic relatedness(Lanciotti *et al.*, 2002). Lineage 1 (L1) viruses have been identified on all continents except Antarctica and lineage 2 (L2) strains are detected in sub-Saharan Africa, Madagascar, and more recently Russia and Europe(Lanciotti *et al.*, 2002; Ciccozzi *et al.*, 2013). Additionally phylogenetic studies based only on the genetic relatedness of the E protein gene propose the inclusion of an additional 4 lineages(Bakonyi *et al.*, 2005; Lvov *et al.*, 2004; Pachler *et al.*, 2014; Umrigar & Pavri, 1977).

Phylogenetic analysis further subdivides L1 into three monophyletic clades (clade 1a, clade 1b, clade 1c)(Lanciotti *et al.*, 2002). Clade 1a is distributed across Europe, the Middle East, Asia, and the Americas. The emergence of clade 1a in the Americas was discovered following the co-occurrence of a cluster of human encephalitis cases and bird deaths in New York City, NY in 1999(Nash *et al.*, 2001). Genome sequence analyses of isolates collected from dead birds, mosquitoes and human clinical samples identified WNV as the causative agent of infection. The isolations in New York, NY in 1999 marked the first identification of WNV in North America(Briese *et al.*, 1999; Jia *et al.*, 1999). By 2004, the introduced genotype had spread to most parts of the continental United States(Petersen LR *et al.*, 2013). The original genotype was subsequently displaced by a second genotype (WN02) that differed by one non-synonymous change (U1442C in the E gene) and two synonymous changes (C2466U in the E gene, C9352U in the NS5 gene)(Davis *et al.*, 2005). Studies have demonstrated that the WN02 genotype is transmitted with a shorter extrinsic incubation period (EIP) and more efficiently than the original genotype (NY99) in *Culex* mosquitoes, suggesting that viral evolution can impact the force of transmission for WNV(Moudy *et al.*, 2007; Kilpatrick *et al.*, 2008). Clades 1b (Kunjin virus) and

1c have been identified in Australia and India, respectively (Bondre *et al.*, 2007; May *et al.*, 2011).

L2 WNV was previously only associated with sporadic outbreaks in sub-Saharan Africa and Madagascar. However, in 2004 a L2 isolate was made from an encephalitic goshawk in Hungary and L2 WNV is now circulating in some parts of Europe and Russia. The abundance of epidemiological data concerning WNV disease in humans associated with L1 viral infection led to the assumption that L2 WNVs were only mildly pathogenic and transmission was strictly confined to the sub-Saharan region of Africa. However, the enzootic emergence of L2 in Europe, the association of neurovirulent disease in humans and neurovirulence studies in mice and hamsters have demonstrated that L2 WNV are capable of geographic expansion and can elicit severe pathology(Botha *et al.*, 2008).

The third WNV lineage [lineage 3 (L3)] comprises 2 isolates from the Czech Republic(Bakonyi *et al.*, 2005; Hubálek *et al.*, 1998). The L3 Rabensburg virus (RabV) strain was isolated in 1997 following injection of homogenate from a pool of *Culex pipiens* mosquitoes into the brains of suckling mice(Hubálek *et al.*, 1998). Characterizations in mice have demonstrated RabV to be less pathogenic than similarly tested Eg-101 (L1 clade a)(Bakonyi *et al.*, 2005). Lineage 4 is represented by a single isolate collected from a tick in 1988 in the Caucuses and 4 isolates from ticks and frogs collected in 2002 and 2004 (Lvov *et al.*, 2004; May *et al.*, 2011). Lineage 5 (L5) WNV stains are characterized by strains isolated in India in 1955 through 1982(Bondre *et al.*, 2007). Phylogenetic studies have demonstrated that L1 and L5 have been co-circulating in India for the past 27 years. Pathogenic studies in mice demonstrated that L5 WNV strains are also slightly less pathogenic than similarly tested L1 strains(Umrigar & Pavri, 1977; Bondre *et al.*, 2007).

Phylogenic analysis of additional isolates using complete genome sequences as well as defined parameters for lineage additions are needed to validate inclusion of these proposed lineages.

Lineage 2 Emergence in Europe

WNV was first isolated from a febrile woman in Uganda in 1937 by Smithburn, *et al*(Smithburn *et al.*, 1940). Genome sequencing later determined that this isolate phylogenetically grouped with L2 WNV strains, indicating that L2 WNVs have been endemic in Africa since discovery(Lanciotti *et al.*, 2002). L2 WNV have actively circulated in sub-Saharan Africa and have been repeatedly associated with sporadic human and equine outbreaks (Jupp, 2001).

L2 WNV was first detected in Europe when the first L2 isolate (HUN04) was made from an encephalitic goshawk in southern Hungary in 2004(Bakonyi *et al.*, 2006). In the same year, a L2 WNV was isolated from a febrile patient in Russia and this case marked the first time WNV L2 was associated with human infection outside of Africa(Platonov *et al.*, 2011). A second isolation of L2 WNV was made in Hungary in 2005(Bakonyi *et al.*, 2006). The 2004 and 2005 L2 WNV isolates were nearly genetically identical, suggesting that the virus had overwintered in Hungary the previous year(Bakonyi *et al.*, 2006). In 2008, an isolate nearly genetically identical to HUN04 was made in Austria from wild birds, suggesting that enzootic transmission of L2 WNV had occurred outside Hungary(Wodak *et al.*, 2011). The emergence of L2 WNV out of Africa occurred once prior to 2004, when an isolate was made from a passerine bird (*Sylvia nisoria*) in Cyprus in 1968. The Cyprus isolate (CYP68) was passaged twice in suckling mice brains before being sequenced. Subsequent genomic and *in vivo* analysis revealed that CYP68 lacks the glycosylation site in L1 and is also highly attenuated in mice(McMullen *et al.*, 2012).Correspondingly, this isolate was not associated with outbreak events or human infections in Cyprus(Beasley *et al.*, 2002).

Phylogeographic studies have determined that two separate introductions of L2 WNV were made in Central Europe and Russia in 1999 and 2000, respectively. The central European clade comprises isolates from Hungary, Italy, Austria, and Greece and is associated with recurrent epidemics in Italy and Greece. Isolates from Russia, Romania, and Italy make up the

L2 WNV Russia clade. The first human L2 isolate made in Russia in 2004 grouped to the L2 WNV Russian clade, indicating that both of the introduced genotypes are pathogenic to humans (Ciccozzi et al., 2013). It is believed that L2 WNV was introduced into central Europe by migratory birds that had overwintered in central Africa where L2 is endemic(Bakonyi *et al.*, 2006).

It is believed that migratory birds mediated two separate introductions of L2 WNV into Europe from Africa, as the first isolates collected in Europe were most genetically similar to L2 isolates from South Africa(Ciccozzi *et al.*, 2013, p. 2). Additionally, the sporadic nature of WNV epidemics in Europe has been partially explained by the likelihood that migratory birds can simultaneously serve as introductory hosts and amplification hosts, thereby facilitating significant increases in enzootic transmission when large populations of migratory birds potentially infected with WNV return to spend the summer in Europe(Hubalek & Halouzka, 1999).

In 2010, the first outbreak of WNV was observed in Greece(Papa *et al.*, 2011a). Sequence analysis of an isolate derived from a pool of *Culex pipiens* mosquitoes collected during the outbreak identified the circulating WNV as an L2 virus(Papa *et al.*, 2011a). This isolate (NS10) exhibited closest identity (99.6%) to the first L2 WNV isolate collected in Hungary in 2004(Papa *et al.*, 2011a). L2 WNV outbreaks in Greece were recurrent through 2014 and all Greek isolates phylogenetically grouped within the Hungarian L2 clade(Papa *et al.*, 2011a). Interestingly, L2 Greek WNV isolates were found to all have a non-synonymous mutation at the NS3-249 locus. All previously sequenced L2 WNV genomes from Africa and Europe have been found to encode a histidine at this locus(Papa *et al.*, 2011a). In L1 backbones, the NS3-249 locus was the only site found to be under strong positive selection. Additionally, studies showed that this site strongly modulates viremia titers and mortality phenotypes in AMCRs, a corvid species highly susceptible to WNV.(Brault *et al.*, 2007) The effect of the histidine to proline

mutation in L2 WNV genetic backbones was unknown when the dissertation studies described in the preceding chapters were initiated.

In 2011, evidence of L2 WNV circulation was reported in Italy, where L1 WNV is endemic(Bagnarelli *et al.*, 2011; Hernández-Triana *et al.*, 2014). Since 2011, L1 and L2 WNV have been isolated from mosquitoes during the same transmission season, indicating the capacity for the co-circulation of two WNV lineages in Italy(Barzon *et al.*, 2013). A single L2 WNV isolate containing a NS3-H249P mutation was isolated from the pool of *Culex pipiens* in North Eastern Italy, but this isolate was not associated with reported cases of WNV disease in humans or horses nor was this mutation observed in L2 isolates collected in subsequent years(Capelli *et al.*, 2013).

Sporadic events of L2 WNV transmission have been noted in surrounding European countries and in Russia. For example, small L2 mediated WNV outbreak occurred in 2010 in Romania (57 reported cases) and in Serbia (52 cases)(Popović *et al.*, 2013; Sirbu *et al.*, 2011). Additionally, two L2 isolations were made from humans in 2004 in Russia and L2 mediated outbreaks occurred in the same area in 2007 and 2010(Platonov *et al.*, 2011). Outbreaks of L1 WNV have been reported in Russia, indicating the potential for L1 and L2 co-circulating(Platonov *et al.*, 2008).

The isolation of L2 WNV from 6 other countries and their corresponding genetic relatedness to either the Hungary (2004) or Russian (2004) strains indicate that L2 WNV are being spread in central and southern Europe and suggest the potential for further geographic dissemination.

L2 WNV Pathogenesis

In Africa, outbreaks and individual sporadic L2 WNV disease cases have been observed in humans and horses. Large outbreaks have been rare, but tend to correlate with increased rainfall or seasonal temperatures(Venter & Swanepoel, 2010). Cases of fatality in horses

following neurological disease in horses and WNV induced hepatic disease in humans have also been observed in South Africa(Venter *et al.*, 2009). Since the emergence of L2 WNV in Hungary in 2004, L2 WNV infections in humans and horses have been detected across Russia, Austria, Greece, and Italy(Hernández-Triana *et al.*, 2014). In Greece, the 2010 outbreak resulted in 262 cases of human disease. Of these cases, 197 were reported as WNND and 33 of these patients died, generating a case fatality rate of 17%. A L2 mediated in Romania in 2010 reported 57 cases of human disease with a case fatality rate of 8.8%(Sirbu *et al.*, 2011). The case fatality rate of L1 in the United States is 10% (reported cases from 1999 through 2008) and is similar to those reported for L2 in Europe(Lindsey *et al.*, 2010, pp. 1999–2008). Clinical symptoms in patients with WNND included encephalitis (85%) or meningitis (12%) and less than 5% of cases reported acute flaccid paralysis. Underlying medical conditions such as hypertension, diabetes and immunosuppression were found in 74% of patients with WNND(Danis *et al.*, 2011). The case fatality rate following WNND varied from 10%-16% in Greece in subsequent epidemic years(Papa, 2013). In Italy and Russia, where L1 and L2 co-circulate, limited epidemiological information is available regarding L2 disease, but it is known that L2 WNV infections have resulted in disease in humans as numerous L2 isolates have been made from patients with WNND(Hernández-Triana *et al.*, 2014).

A study by Beasley et al., 2002 demonstrated that L2 WNV strains (isolated in Africa and Cyprus) are equally pathogenic as L1 WNV strains in mouse and Syrian hamster models. In this study, mice or hamsters were inoculated intraperitoneally or intracranially with 12 L1 WNV strains (from Africa, United states, Australia, India) or 7 L2 WNV strains (from Africa, Madagascar, and Cyprus) and corresponding LD_{50} values were calculated(Beasley *et al.*, 2002)(94). Results demonstrated that strains with varying pathogenicity were distributed across both lineages. For example, an approximate 6-fold difference was observed between the LD_{50} values calculated for L1 USA99 [0.5 plaque forming units (PFU)] and L2 SA58 (3.2 PFU).

Similarly, LD_{50} values in excess of 10,00 PFU were observed in WNV strains from either lineage, suggesting that pathogenic variants are interlineage (present in either L1 or L2 WNV strains)(Beasley *et al.*, 2002).

WNV mosquito vectors

The WNV transmission cycle progresses when a female mosquito imbibes a blood meal from an infected host to ensure egg development. A female mosquito may go through several gonotrophic cycles in her lifetime and may also feed on multiple hosts within each cycle, thereby increasing the potential for human- WNV infectious mosquito contact(Muturi *et al.*, 2008; Scott *et al.*, 1993). An amplification host must generate viremia titers [greater than 4.7 $\log_{10}$ (PFU/sera)] sufficient to infect mosquito vectors(Komar, 2003). Mosquito species and genetic variations within a species' population dictate how susceptible a mosquito is to infection such that higher viremia titers in birds are needed to infect less susceptible vectors(Reisen *et al.*, 2005).

Global distribution of WNV transmission vectors

Field isolations of WNV have been made in as many as 63 different mosquito species stratified across 8 different genera (Hubalek & Halouzka, 1999). Despite this, species within the *Culex* genus are regarded as the main vectors of WNV due to their relative field abundance, host feeding preferences and high vector competence (Fig. 4)(Andreadis, 2012; Turell *et al.*, 2005). Members of the *Culex (Culex)* complex are currently taxonomically defined by the Catalog of the Mosquitoes of the World and include such mosquito species as *Culex pipiens, quinquefasciatus, tarsalis,* and *restuans*(Knight, 1978). Some *Culex* species are geographically limited while others such as *Culex pipiens* and *Culex quinquefasciatus* are disseminated worldwide in either temperate or tropical regions, respectively (Fig. 4)(Smith & Fonseca, 2004). The widespread distribution and competence for transmitting make *Culex pipiens* and *Culex*

quinquefasciatus important transmission vectors worldwide(Turell, 2012). Close human association coupled with the widespread distribution in either the northern or southern hemisphere earn *Culex pipiens* and *Culex quinquefasciatus* mosquitoes the common names of the northern house mosquitoes or the southern house mosquitoes(Farajollahi *et al.*, 2011).

In the United States, the most important WNV vector in the Midwest and northeast is *Culex pipiens*, while *Culex quinquefasciatus* and *Culex tarsalis* predominate in the south and northwest, respectively (Andreadis, 2012). In Europe, mosquitoes within the *Culex pipiens* spp. group function as the primary enzootic and epizootic transmission vectors(Farajollahi *et al.*, 2011).

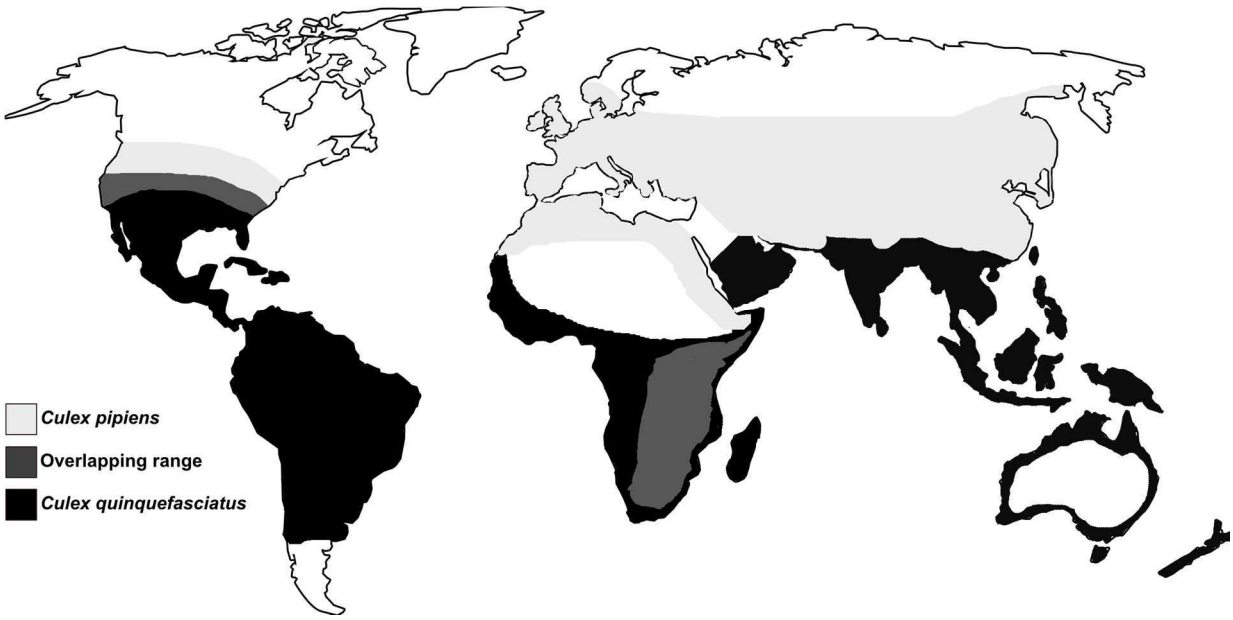

Figure 4. Global distribution of *Culex pipiens* and *Culex quinquefasciatus.*

The distribution of *Culex pipiens and Culex quinquefasciatus* is displayed by color. The range of *Culex pipiens* is depicted by light gray. Overlapping ranges of *Culex pipiens* and *Culex quinquefasciatus* are depicted by dark gray and the range of *Culex quinquefasciatus* is depicted in black (adapted from Smith and Fonseca, 2004)

In Greece, *Culex pipiens* and *Culex modestus* were identified as the most abundantly trapped *Culex* mosquito vectors during periods of WNV transmission(Papa *et al.*, 2013). In Italy, *Culex pipiens* and *Culex impudicus* were the most commonly trapped *Culex* species(Romi *et al.*, 2004). The primary vector for WNV in Africa is *Culex univittatus* (Jupp & McIntosh, 1970). However, the abundance and number of recent field isolations of WNV in *Culex quinquefasciatus* and *Culex pipiens* in Africa have demonstrated that these species may also play a role in transmission and maintenance of WNV (Motayo *et al.*, 2016; Mutebi *et al.*, 2012; Muturi *et al.*, 2008). In Asia, the three most common mosquito vectors for WNV are contained in the *Culex vishnui* group, which include *Culex tritaeniorhynchus* Giles, *Culex pseudovishnui* Colless, and *Culex vishnui* Theobald(Hasegawa *et al.*, 2008; Sirivanakarn, 1975).

Vector competence

Vector competence describes the ability of a mosquito to become infected and to amplify and subsequently transmit the agent after a period of time(Hardy *et al.*, 1983). When a mosquito imbibes an infectious blood meal, four barriers have been described with which an arbovirus must contend to be transmitted. The time period that the virus takes to traverse these barriers following oral exposure is known as EIP(Franz *et al.*, 2015).

Infection in a mosquito is initiated in the apical side of midgut epithelial cells following the ingestion of an infectious blood meal. Following infection, WNV replicates in the midgut epithelial cells and spreads to other midgut epithelial cells. Barriers to either of these processes constitute a midgut infection barrier (MIB). Virions must then be released basolaterally and traverse through the basal lamina into the hemocoel and infect other organs and cell types such as hemocytes, nerve cells, and fat cells within the body cavity of the mosquito. A midgut escape barrier (MEB) describes interference with any of these steps. Failure to initiate infection in the salivary glands constitutes a salivary gland infection barrier (SGIB). Replication of virus occurs

within tissues of the salivary gland and virions along with other salivary proteins are released by the mosquito when probing. Failure to release virions in the salivary gland tissues following amplification demonstrates the presence of a salivary gland escape barrier (SGEB)(Franz *et al.*, 2015). The mosquito deposits virus and other salivary components in the host during probing. The amount of WNV that is deposited during probing ranges from $10^{1.2}$ to $10^{5.9}$ PFU(Styer *et al.*, 2006, 2007). Studies by Reisen et al., 2007 have also demonstrated that uninfected mosquitoes can potentially become infected after imbibing near an area where concurrent probing of infected female mosquitoes was occurring.

Determinants of vector competence of *Culex* spp. for WNV can be modulated by differences in viral genetics, temperature, geography, time, and mosquito species. Following the initial identification of WNV in New York in 1999, WNV was rapidly dispersed across the rest of the country and by 2004, had spread to most parts of the contiguous United States. The introduced genotype was subsequently displaced by a second genotype (WN02) that differed by a single amino acid change in the envelope protein (E-V159A) and two synonymous mutations (Davis *et al.*, 2005; McMullen *et al.*, 2011). In *Culex* mosquitoes, this genotype demonstrated a shorter extrinsic incubation period and higher peak viremia titers in house sparrows compared to the original introduced genotype (Moudy *et al.*, 2007; Kilpatrick *et al.*, 2008; Duggal *et al.*, 2014). This same WN02 strain also demonstrated a shorter extrinsic incubation period (EIP) compared to east coast genotype WNV strains in mosquitoes when incubated at increased temperature, but overall, both strains transmitted earlier with increasing temperatures(Kilpatrick *et al.*, 2008; Reisen *et al.*, 2006b). Temperature has also been demonstrated to decrease time to transmission in newly emergent L2 WNV European isolates and it has been postulated that a localized increased in seasonal temperatures helped facilitate the epidemic spread of L2 in Europe(Fros *et al.*, 2015). Spatio-temporal dynamics can also alter vector competence profiles. For example, in California, the consistency of vector competence varied dramatically for the same mosquito species that were either geographically or temporally separated(Kilpatrick *et al.*,

2010). Studies assert that vector competence is also under genetic control within the same mosquito species. For example, in the United States, the degree of genetic relatedness and vector competence differed more for *Culex pipiens* from distant locations than mosquitoes collected from closely related sites, suggesting that vector competence is genetically influenced by the mosquito(Kilpatrick *et al.*, 2010). Hybridization between *Culex* spp. can occur in areas where separate ranges overlap, leading to altered vector competence phenotypes or changes in host seeking patterns(Kothera *et al.*, 2009). For example, Ciota et al., 2013 demonstrated that altered vector competence phenotypes and enhanced transmission was observed for laboratory hybrids between North American *Culex pipiens*, *Culex quinquefasciatus,* and *Culex molestus* compared to parental mosquito strains. Taken together these studies demonstrate that transmission of WNV in mosquitoes is a dynamic and complex process.

Feeding patterns of WNV transmission vectors

The preferential and mixed host feeding patterns of specific *Culex* spp. drive the WNV transmission cycle and incidental host infection rate. For example, *Culex pipiens* mosquitoes are known as ornithophilic bridge vectors. Specifically, they are distinguished by a strong host feeding preference for avian species, but also feed on humans, thus “bridging” WNV infection between birds and humans(Apperson *et al.*, 2002). Both *Culex quinquefasciatus* and *Culex tarsalis* also exhibit a strong feeding preference for birds but feed on humans and other mammals more frequently than *Culex pipiens*(Molaei *et al.*, 2007; Rizzoli *et al.*, 2015). In a study by Kent et al., the *Culex tarsalis* mosquito demonstrated a clear preference for avian derived blood meals in early summer but increasingly sought mammalian host blood meals as the WNV transmission season progressed(Kent *et al.*, 2009). This same species also demonstrated preferences for species-specific avian blood meals that seemed to alter in early season (robins) compared to late season (sparrows)(Kent *et al.*, 2009). In Europe, the principle vector for WNV transmission is *Culex pipiens*. Similar to studies in North America, blood meal analysis studies

for *Culex pipiens* in Italy have demonstrated a clear preference for birds over mammals. The same study demonstrated that the avian preference can also be species specific(Rizzoli *et al.*, 2015). Other *Culex* species such *Culex stigmatasoma, Culex nigripalpus,* and *Culex restuans* are also preferential bird feeders and contribute to enzootic maintenance of WNV(Turell *et al.*, 2005).

Overwintering mechanisms

Overwintering mechanisms maintain WNV in the environment during seasonal cycles unfavorable to mosquito activity and/or facilitate reintroduction of WNV. These overwintering mechanisms are important because they ensure that the virus is maintained until spring or summer months when enzootic transmission can resume. Overwintering mosquitoes can enter a true diapause (dormancy), temperature-dependent quiescence, or enter a state that results in reduced mosquito activity(Bugbee & Forte, 2004; Faraji & Gaugler, 2015; Nasci *et al.*, 2001; Nelms *et al.*, 2013; Robich & Denlinger, 2005). In nature, both WNV RNA and infectious WNV have been detected in pools of overwintering *Culex* spp. mosquitoes, suggesting that when a female mosquito becomes active in more environmentally favorable conditions, it can immediately initiate WNV transmission (Farajollahi *et al.*, 2005; Nasci *et al.*, 2001). Vertical transmission occurs when an infected female passes WNV to her offspring. Vertical transmission of WNV in *Culex* spp. and *Aedes* spp. has been demonstrated in experimental studies and in nature, yet the overall efficiency of vertical transmission is relatively low(Anderson & Main, 2006; Baqar *et al.*, 1993; Reisen *et al.*, 2006c).

WNV reservoir hosts

WNV enzootic transmission occurs between *Culex* mosquitoes and passerine birds. In order to function as a competent reservoir host for WNV, a bird must amplify a level of viremia that surpasses the infectivity threshold for mosquito oral infectivity. Birds that survive WNV

infection develop lifelong neutralizing antibodies and cease to contribute to the WNV transmission cycle. Birds in the order *Passeriformes* such as house sparrows (*Passer domesticus*), American Robins (*Turdus migratorius*), and house finches (*Carpodacus mexicanus*) are competent amplification hosts but rarely fatally succumb to mortality(Komar *et al.*, 2003).

A reservoir competence (RC) index value derived from the viremia titers and mortality profiles of multiple species of birds experimentally exposed to WNV was developed by Komar et al., in 2003. The RC index values can be used to describe the potential number of infectious mosquitoes that result from a WNV infected avian host(Komar *et al.*, 2003). In this study, Komar estimated the RC index values for specific avian hosts based on the generation of infectious viremia titers greater than $10^{5.0}$ PFU/mL (therefore infectious to *Culex pipiens* and *Culex quinquefasciatus* mosquitoes), mean daily viremia titer, and susceptibility. Birds from 25 different species representing 10 orders were experimentally exposed to WNV by WNV-infectious mosquito bites. The resulting RC index values for each bird species were derived from resulting mean daily viremia titers and mortalities. High RC index values were observed for highly susceptible avian species such as blue jays (*Cyanocitta cristata*) (RC=2.25), AMCRs (RC=1.62), and HOSPs (RC=1.59). Avian species such as the Japanese quail (*Coturnix japonica*), ring-necked pheasant (*Phasianus colchicus*), and the European starling (*Sturnus vulgaris*), which generated low viremia titers, similarly demonstrated low RC index values, indicating these avian species either contribute little to WNV transmission or are completely incompetent hosts (don't infect imbibing mosquitoes). Additionally, the RC index value was utilized in a study by Duggal et al., 2014 to show that an increase in the host competence of HOSPs from North America occurred for newer evolved strains of WNV(Duggal *et al.*, 2014)(131). In this study, North American HOSPs were experimentally exposed to the three North American genotypes of WNV (East coast, WN02, and SW03), viremias titers and

mortalities were measured through 7 days after infection and the RC index values were calculated. Results demonstrated that the RC index values increased over time for newer evolved WNV compared to older WNV genotypes, whereas the RC index value decreased for the originally introduced east coast genotypes, thereby demonstrating that opposing selective pressures are concurrently acting on viral and avian host(Duggal *et al.*, 2014, p.).

Virulence determinants of avian pathogenicity

WNV disease in birds can vary by species. Some species do not exhibit clinical symptoms or mortality, whereas others are highly susceptible to WNV. Pathological examination of fatal cases demonstrated that WNV disease can produce brain hemorrhage, splenomegaly, meningoencephalitis, and myocarditis but the most common cause of death is widespread organ failure(Steele *et al.*, 2000). As many as 198 documented species of birds in North America have succumbed to fatal WNV disease(Komar, 2003). Corvids are particularly susceptible to mortality following WNV infection. For example, in 1999 when WNV was first identified in New York, 295 fatal cases of WNV infection were confirmed in birds and 262 (89%) of these birds were identified as AMCRs. The mortality rates of AMCRs were so high that crow deaths were proposed as a sentinel surveillance system for tracking the dispersal of WNV across the United States(Eidson *et al.*, 2001). For some species, high levels of mortality following WNV infection substantially and persistently impacted population magnitudes. When WNV was first identified in New York in 1999, an upsurge of avian mortality was observed and the mortality continued as WNV dispersed across the United States. In a study using a continental-wide data set, the impact of WNV was estimated on bird populations in North America, George et al., 2015 found that a negative population impact on 23 of the 49 species evaluated (47%) was correlated with the arrival of WNV in North American, and that the negative impact was greatest among certain passerines (New World sparrows, finches, and vireos). When L2 WNV was first identified in Hungary, isolates were made from encephalitic

goshawks that succumbed to lethal WNV disease, indicating that the introduced L2 genotype was highly pathogenic(Bakonyi *et al.*, 2006). However, the first outbreak of L2 WNV in Greece was not accompanied by massive bird die-offs, similar to what was observed New York in 1999 and no large events of bird mortality were noted during subsequent L2 epidemics(Papa, 2012). The contrasting epidemiological scenario for birds between Europe and North America raises questions concerning avian host adaptation. For example, the strain responsible for the first L2 WNV outbreak in Greece (NS10) harbored a mutation that associates with significantly elevated viremia titers and mortality in AMCRs. However, no massive bird mortality in any species of corvid was observed during this outbreak. The earliest evidence of WNV in Europe dates back to 1960 and suggests the avian reservoirs have been exposed to WNV much longer than similar amplification species in North America(Hubalek & Halouzka, 1999). One study suggests that opposing selective pressures can rapidly develop in avian hosts following WNV exposure over time. However Lim et al., 2014 showed that fatal pathogenesis can occur in European jackdaws (*Corvus monedula*) following L1 and L2 WNV infection(Lim *et al.*, 2014)(152). A study examining the susceptibility of wild-caught HOSPs to WNV strains from North America found the competence of HOSPs decreased over time for the North American founding WNV strain(Duggal *et al.*, 2014). However, the competency of HOSPs collected in 2012-2013 was shown to have increased over time when exposed to WNV isolates made over successive years in North America, indicating that a net increase in host competency was occurring in North American HOSPs(Duggal *et al.*, 2014). HOSPs are Old World species that were introduced to the United States in the late 19th century and have since become invasive throughout the Americas(Robbins, 1973). In North America, it is well established that HOSPs are important amplifying reservoir hosts for WNV due to large abundance and high exposure rates(Langevin *et al.*, 2005). The ability of HOSPs to serve as competent reservoirs for circulating L2 European viruses and the potential for co-evolution of HOSPs to newer L2 WNV strain has not been tested.

In the years preceding the introduction of WNV to New York, considerable WNV induced avian mortality was observed in Israel during several L1 WNV outbreaks. Prior to the avian mortality in Israel, fatal infections in birds were not extensively observed(Brault, 2009; Malkinson & Banet, 2002). The absence of avian mortality stemming from WNV enzootic transmission of L2 WNV in Europe could be related to a lack of surveillance, decreased host susceptibility or viral mediated virulence changes(Brault *et al.*, 2004). Experimental studies have demonstrated that viral genetics can affect virulence and mortality phenotypes in specific avian species. For example, a Kunjin (KUN) and a Kenyan (KEN) isolate both demonstrated low viremia titers in AMCRs.

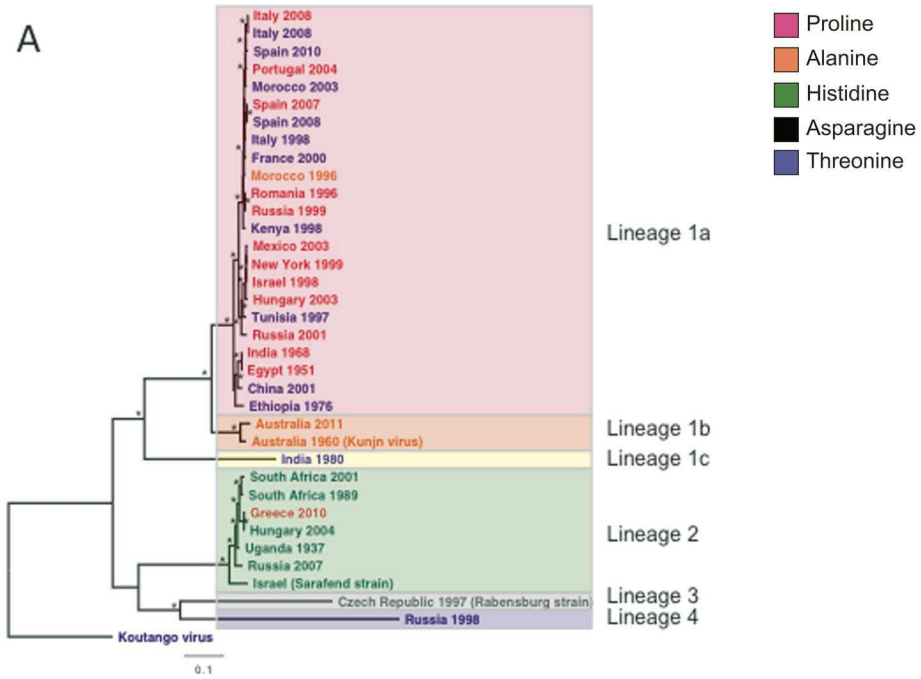

Figure 5. Phylogenetic analysis of WNV and amino acid relatedness at the NS3-249 locus.

Phylogenetic analyses of WNV lineages 1-4. Isolates are color coded to match amino acid identity at NS3-249 locus. a) Full genomic analysis of 36 strains of WNV. Koutango virus is used an outgroup (Langevin *et al.*, 2014).

In contrast, the isolate associated with the first introduction of WNV into North America (NY99) and associated with widespread AMCR mortality, similarly elicited drastically elevated viremia

titers and mortality in AMCRs, demonstrating that viral genetics can influence avian pathogenicity(Brault *et al.*, 2004, 2007). Studies later demonstrated (described in the next section) that the varying degree of virulence between KEN and NY99 was due to a threonine to proline mutation at the NS3-249 locus.

WNV virulence modulated by the NS3-249 locus in AMCRs

Positive selection analysis as determined by the measure of the ratio of non-synonymous (dN) and synonymous (dS) mutations in the coding regions of the L1 WNV genome was performed and the NS3-249 locus was the only site that was found to be under positive selection (ratio of dN/dS greater than 1 for the NS3-249 site)(Brault *et al.*, 2007). Phylogenetic analysis of L1 through L4 determined that considerable heterogeneity at the NS3-249 locus is present between and within lineages (Fig. 5)(Brault *et al.*, 2007). For example, amino acids threonine, proline and alanine (only one strain) are found at NS3-249 locus in L1 WNVs. However, the threonine to proline substitution at the NS3-249 site occurs more frequently in recently evolved L1 WNV isolates and this substitution event has occurred on at least 3 independent occasions. Notably each substitution event directly precedes epidemic occurrences (Egypt, 1950; Romania, and Russia, 1996; Israel, 1997–98)(Langevin *et al.*, 2014). For L2 WNVs, 6 of 7 the isolates in the phylogeny have a histidine at this site; however all L2 WNV isolates made from Greece exhibit a histidine to proline substitution at the NS3-249 locus. The L3 isolate from the Czech republic contains an NS3-249Asn and L4 consists of NS3-249Thr(Brault *et al.*, 2007). Previous studies have established that a threonine to proline substitution at the NS3-249 locus results in significantly elevated viremia titers and mortality in AMCRs(Brault *et al.*, 2007). The L1 WNV KEN parental isolate contains an NS3-249Thr and the parental NY99 harbors an NS3-249Pro identity. An alternating proline or threonine substitution was engineered into the KEN or NY99 strains, respectively, and compared to the virulence phenotypes of the parental strains. Results demonstrate that the proline elicited severe viremia

titers compared to the threonine in either L1 backbone. Alternatively, both the KEN (NS3-249Thr) and the NY99 NS3-Pro249Thr demonstrated low viremia titers and mortalities in AMCRs(Brault *et al.*, 2007).

Five (Ala, Thr His, Pro, and Asn) amino acid identities occupy NS3-249 locus across lineages 1-4. To assess the effect of additional polymorphisms at the NS3-249 locus on phenotypic variability, variable NS3-249 point mutations found in nature (Ala, Thr His, Pro, and Asn) were engineered into the NY99 (L1 WNV) backbones and tested in AMCRs(Langevin *et al.*, 2014). Similar to what was observed in previous studies, the threonine NS3-249 variant elicited the greatest reduction in pathogenicity compared to the parental genotype containing NS3-249Pro. The alanine, histidine, and asparagine NS3-249 substitutions all elicited moderate viremia titers. To assess whether the NS3-249 locus could modulate neuroinvasiveness, the same viruses tested in AMCRs were assessed in a murine model. No appreciable differences in the LD_{50} were observed in mice inoculated with the viruses by the intraperitoneal route.

The NS3 gene has two primary domains; a helicase domain and protease domain. The function of the NS3 helicase domain, which contains the NS3-249 locus, is to relax duplexed RNA products and catalyze the corresponding function by hydrolyzing ATP. Since the NS3-249 locus sits within the helicase domain of the NS3, the helicase and ATPase activities were assessed using the same polymorphic L1 isolates. Changes in the activities of the NS3 helicase and ATPase were generated by changing the identity of the NS3-249 amino acid. However, increases or decreases in either ATPase or helicase activities did not correlate to similar increases or decreases in viremia titers observed in AMCRs when experimentally exposed to the same NS3-249 mutants, indicating the host-specific interactions were occurring in conjunction with the NS3-249 locus to modulate AMCR-specific virulence phenotypes(Langevin *et al.*, 2014).

Questions regarding the mechanism of the histidine to proline NS3-249 mutation in newly emergent Greek L2 WNV remain unanswered. Specifically, does the proline mutation in

L2 WNVs confer a significant increase in avian host competence or virulence similar to what has been observed in L1 WNV and if so, is the effect consistent among newly emergent L2 WNV from Europe compared to older, African L2 strains. Furthermore, do additional polymorphisms (NS3-249 Ala, Thr, Asn), exhibit a potential to alter virulence phenotypes in AMCRs Lastly, does the NS3-His249Pro mutation affect the neuroinvasiveness of newly emergent European L2 isolates compared to African isolates in mice.

Globally, flaviviruses have demonstrated a capacity for rapid dispersal to new geographic areas with the potential to elicit severe pathogenic effects in humans. WNV is one of the most widely distributed flaviviruses, with a demonstrated capacity to cause neurological disease and potentially fatal outcomes in humans, horses, and birds. In 2004, lineage 2 (L2) WNV emerged in Hungary and mediated the first L2-associated outbreaks across central and southern Europe(Bakonyi *et al.*, 2006). The purpose of this dissertation is to understand the genetic factors related to the emergence of lineage 2 WNVs and maintenance in Europe by (1) assessing the phenotypic effects of polymorphisms at the NS3-249 site in disparate L2 WNV backbones for modulation of avian host competence, (2) characterizing the fitness and transmission potential of L2 WNV viruses in mosquito vectors, and (3) determining the phenotypic effect of the NS3-His249Pro mutation in L2 WNV in reservoir, vector and incidental hosts. Understanding the genetic determinants involved in affecting host range adaptation and virulent outcomes will further the development of transmission control and infection prevention strategies.

SUMMARY AND SPECIFIC AIMS

Summary

WNV is a flavivirus that exists in a bird-mosquito transmission cycle. WNV infection in humans can result in severe and potentially fatal neurological disease. The rapid spread of L1 WNV across North America and more recently, the emergence of L2 WNV in Europe highlight the adaptive capacity of WNV to establish enzootic transmission in new environmental niches. Little is known about the genetic determinants that dictate the capacity of WNV to adapt to new host ranges and elicit novel pathogenic phenotypes.

A histidine to proline substitution at the NS3-249 locus was observed for the first time during a L2 WNV outbreak in Greece in 2010. Polymorphisms at the NS3-249 locus in L1 WNV have been associated with variable virulence phenotypes in AMCRs, a passerine species believed to be an important amplification host for WNV. Selection analyses have also demonstrated that NS3-249 is the only site in the NS3 gene to be under strong selective pressure. The goal of this dissertation has been to phenotypically delineate unique genetic factors associated with the emergence and establishment of L2 WNV in south and central Europe and to examine the corresponding role of the NS3-H249P substitution in enzootic transmission.

Specific aim 1) Assess the phenotypic effect of polymorphisms at the NS3-249 locus in different L2 WNV backbones for modulation of avian host competence.

Passerine species highly susceptible to WNV infection are critical to maintenance and amplification of WNV. AMCRs and other corvids are an important reservoir species. The virogenesis potential of WNV with the NS3-H249P substitution and other polymorphic substitutions in disparate L2 WNV isolates have been addressed in highly competent reservoir

hosts (Chapter 2). Additionally, chimeric viruses were generated to test the potential of additional genetic elements to enhance or alter pathogenic outcomes. *Studies performed in Chapter 2 have been designed to test the hypothesis that genetic factors such as the variable amino acid identity at the NS3-249 locus and other epistatic elements in L2 WNV can modulate increased virogenesis and pathogenic outcomes in important amplification avian hosts.*

Specific aim 2) Characterize the vector competence and transmission potential of *Culex* mosquitoes for WNV L2 viruses in enzootic and epizootic mosquito vectors.

Mosquito vectors that display high vector competence for WNV and correspondingly demonstrate the ability to bridge transmission between humans and birds are necessary for maintaining enzootic and epidemic transmission of WNV. Arboviruses have repeatedly demonstrated a capacity to modulate the infection phenotypes of mosquitoes and establish new geographic transmission ranges. To examine whether genetic changes in newly emergent European L2 WNV lead to altered vector competence phenotypes compared to older African L2 isolates, the vector competence of two *Culex* mosquito species were assessed for various geographically and temporally disparate L2 WNV isolates. *Experiments in Chapter 3 were designed to specifically address the hypothesis that variable efficiencies in vector competence will be elicited by viral genetic changes in L2 WNVs.*

Specific aim 3) Assess the comprehensive effect of the NS3-His249Pro mutation in L2 WNV backbones on the mosquito-bird transmission cycle and incidental disease.

Previous studies have demonstrated that polymorphism in the NS3-249 locus can modulate levels of L1 WNV amplification in AMCRs, potentially also altering levels of enzootic transmission. It is unknown whether the genetic changes in newly evolved L2 WNV are associated with increased force of transmission between vectors and avian hosts or if increases

in human disease rates are due to the emergence of more virulent phenotypes. *Experiments described in Chapter 4 have been designed to specifically address the hypothesis that the genetic changes at the NS3-249 locus within L2 WNVs dictate alternating and inversely correlated changes in vector and reservoir host and that these changes do not modulate pathogenic phenotypes in incidental hosts.*

CHAPTER 2: AVIAN HOST COMPETENCE OF LINEAGE 2 WEST NILE VIRUSES AND PHENOTYPIC EFFECT OF POLYMORPHISMS AT THE NS3-249 LOCUS

INTRODUCTION

In 2010, a WNV outbreak occurred in Greece and represented the first documented occurrence of WNV clinical disease that was observed in that area(Danis *et al.*, 2011). Several human cases of encephalitis were reported in Nea Santa (Kilkis prefecture), Greece and $C0_2$ baited traps were used to collect adult mosquitoes in that area(Papa *et al.*, 2011a). A WNV isolate was made from pools of *Culex pipiens* mosquitoes and full genome sequencing determined that this strain phylogenetically grouped with other L2 strains, sharing the closest genetic identify to a L2 strain that had been isolated from an encephalitic goshawk in Hungary in 2004 and also contained a mutation (His to Pro) at the NS3-249 locus(Papa *et al.*, 2011b a, a b). WNV outbreaks in Greece were recurrent through 2013 (2010-2014) and all were L2 WNV mediated, indicating that the L2 strain had become established in that region(eCDC, 2014).

The L2 WNV strain (NS10) that was connected with the first outbreak of L2 WNV in Greece was also the first L2 WNV strain to be identified with an NS3-H249P mutation(Papa *et al.*, 2011a). The identification of an NS3-249P in L2 represents the fourth known emergence of a proline at this locus. All previously sequenced L2 WNV isolates, including those initially isolated in Hungary in 2004 and 2005, associated with an NS3-249H identity(Bakonyi *et al.*, 2006; Ciccozzi *et al.*, 2013, p. 2). Whether the NS3-H249P mutation is directly related to increased enzootic transmission via increases in avian host competence and whether polymorphisms at the NS3-249 locus similarly modulate virulence phenotypes in L2 WNV is unknown.

Within L1 WNV, selection analysis studies have shown that a single amino acid (249) within the NS3 gene is under strong selective pressure(Brault *et al.*, 2007). Phenotypic studies have demonstrated that a threonine to proline NS3-249 substitution elicits a significant increase

in viremia titers and mortality in American Crows (AMCRs; *Corvus brachyrhynchos*), an avian host highly susceptible to L1 WNV (NY99) infection(Brault *et al.*, 2007). The emergence of the NS3-T249P substitution has preceded large WNV outbreak events on three separate occasions, indicating the potential association between this positively selected locus and outbreaks of human disease(Langevin *et al.*, 2014). Amino acid heterogeneity at the NS3-249 locus is evident among the different WNV lineages, with five naturally occurring amino acid identities [alanine (L1), asparagine (L3), threonine (L1, L4), proline (L1, L2), and histidine (L2)] having been identified. Previous studies have demonstrated that variable growth and virulence phenotypes in AMCRs are dependent upon specific amino acid residues present at the NS3-249 locus in a common NY99 L1 WNV genetic backbone. For example, the NS3-249T mutation in a L1 WNV strain (NY99) severely restricts peripheral replication and mortality with a 3.5 $\log_{10}$ (PFU/ml sera) mean peak viremia titer and 12.5% mortality, whereas the proline elicits the greatest increase in virogenesis with an mean peak titer of 9.5 $\log_{10}$ (PFU/ml sera) and 100% mortality. Histidine, alanine or asparagine NS3-249 variants elicit virogenesis intermediate to that of NS3-249P or NS3-249T in AMCRs(Langevin *et al.*, 2014).

The NS3-249 locus is located in the helicase domain of NS3 and adjacent to the NS3 ATP binding domain. To examine whether functional differences in the helicase or ATPase activities could be affected by amino acid differences at the NS3-249 locus, the efficiency of ATP hydrolysis (ATPase activity) and capacity to unwind short double-stranded RNA (helicase activity) was examined. Results demonstrated that while differences in helicase and ATPase actives related to NS3-249 mutations occurred, these changes do not correlate with virulence phenotypes in AMCRs(Langevin *et al.*, 2014).

House sparrows (HOSPs) are an Old World passerine species that display moderate host competence for WNV (L1)(Komar *et al.*, 2003). The relative abundance of HOSPs and associated frequency of WNV infection have established HOSPs as important amplifying hosts(Godsey *et al.*, 2005; Komar *et al.*, 2003). Unlike what has been observed in AMCRs, the

same NS3-249P in L1 WNV (NY99) did not elicit drastic changes in virogenesis compared to other NS3-249 variants in HOSPs(Langevin *et al.*, 2014).

AMCRs are highly competent avian hosts for WNV and will be used as a model for assessing pathogenic differences between African and European L2 WNV strains. House sparrows are native to Europe and prevalent in Africa and North America and will be used to model relative competence in a moderate avian susceptibility species. Additionally, polymorphisms at the NS3-249 locus were tested in 2 L2 WNV for the potential to modulate virulence phenotypes. Finally, multivariate genetic elements between a L2 WNV strain isolated in South Africa in 1989 (SA89) and the NS10 WNV strain were phenotypically examined by generating chimeras and testing in AMCRs. Taken together these results demonstrate that avian specific adaptive evolutionary changes are occurring in L2 WNV and these changes have the potential to influence WNV emergence and alter enzootic transmission.

MATERIALS AND METHODS

Parental L2 WNV viruses and cells

Four parental L2 WNV strains were utilized in this study. The L2 WNV Hungarian 2004 (HUN04) strain and the Ugandan 2009 (UG2274) are isolates while the South African 1989 (SA89) and Nea Santa, 2010 (NS10) strains are derived from a two-plasmid infectious complementary DNA (cDNA) clone system (described in detail below). The four L2 WNV strains, corresponding accession numbers and amino acid differences used in this study are listed in Table 2.1. The geographic association of each of the 4 L2 WNV isolates is depicted in Fig. 2.1. The SA89 and UG2274 strains originated from Africa but were not associated with epidemics of human disease(Brault *et al.*, 2007). The HUN04 was isolated from a moribund goshawk (*Accipiter gentilis*) (Bakonyi et al., 2006) following a fatal case of WNV encephalitis in Hungary in 2004 and the NS10 isolate was made from a pool of *Culex pipiens*

Table 2.1 L2 WNV strains SA89, UG2274, HUN04, and NS10.

The origin, year of isolation, source, accession of each strain in amino acid differences are depicted.

Virus strain	Origin	Year	Source	Genbank accession	Amnio acid differences																					
					E	NS1			NS2A			NS2B		NS3		NS4B						NS5				
					88	35	44	69	1	98	181	88	119	11	249	14	23	32	49	79	113	25	190	197	374	643
African isolates																										
SA89 IC	South Africa	1989	Human	EU068667.1	P	H	K	E	Y	K	V	M	V	K	H	S	A	S	T	T	V	T	K	I	H	R
UG2274	Uganda	2009	*Culex neavei*	NA	P	Y	K	E	Y	R	I	M	V	K	H	S	A	S	T	A	V	T	K	T	Y	G
European isolates																										
HUN04	Hungary	2004	*Accipiter gentilis*	DQ116961	S	Y	R	G	H	R	V	I	V	R	H	S	T	N	T	A	V	A	R	T	Y	G
NS10 IC	Greece	2010	*Culex pipiens*	HQ537483.1	P	Y	R	E	Y	R	V	I	I	R	P	G	T	N	A	A	M	T	R	T	Y	G

mosquitoes in Nea Santa, Greece in 2010 (Papa et al., 2011). Low passage isolates, HUN04 and UG2274 were grown on African green monkey kidney cells (Vero cells; ATCC no. CCL-81). Infection was performed at a multiplicity of infection (MOI) of 0.1 and cell culture medium was harvested following evidence of cytopathic effects (CPE). The collected supernatant was centrifuged at 1400 rpm for 10 minutes to remove cellular debris and aliquots were frozen at -80^{0}C. Plaque assays on Vero cells were used to determine viral titers(Brault *et al.*, 2004).

Construction of SA89 and NS10 infectious cDNA clones

The SA89 and NS10 ICs were generated using a two plasmid cloning system. The generation of a two plasmid IC for L1 WNV constructs has been previously described(Kinney *et al.*, 2006). For the generation of an infectious cDNA clone of the SA89 virus (SA89-IC), the first plasmid was designed to contain the genetic elements of SA89 (accession number EU068667) corresponding to the 5' UTR and structural proteins (capsid, pre-membrane, envelope) and the second plasmid contains all 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), the 3' UTR, and an engineered terminal XbaI site for plasmid linearization at the 3' terminus of the genomic cDNA. The NS10 infectious cDNA clone (NS10-IC) was generated by the introduction of 17 non-synonymous point mutations in the coding region, and 7 nucleotide changes in the 3' UTR into the WNV SA89-IC backbone via site directed mutagenesis. The amino acid and 3' UTR genetic differences between SA89 and NS10 are noted in Table 1.2.

Construction of SA89 NS3-249 mutants, NS10 NS3-249 mutants, and SA89 / NS10 chimeras

For the generation of the NS3-249 mutants, introductions of non-synonymous amino acid point mutations for alanine (CAC to GCC), asparagine (CAC to AAC), threonine (CAC to ACC), and proline (CAC to CCC) for SA89 and alanine (CCC to GCC), asparagine (CCC to AAC), threonine (CCC to ACC) and histidine (CCC to CAC) for NS10 into the second plasmid of

the IC system were generated by site directed mutagenesis. Four chimeras between SA89 and NS10 NS3-P249H (NS1-NS3, NS4B, NS5, and 3' UTR) were generated by restriction enzyme mediated cloning techniques. Genetic regions of NS10 were cut at restriction enzyme sites common to NS10 and SA89 and ligated into the corresponding sites in the second plasmid of SA89. Sanger sequencing was performed to confirm genetic identity of all plasmids. Table 2.2 lists the amino acid and 3' UTR differences between SA89 and NS10 as well as the NS3-249 mutants and 4 chimeras (NS1-NS3, NS4B, NS5, and 3' UTR) derived from the SA89 and NS10 infectious clones.

Recovery of virus from infectious clones

For *in vitro* ligation, plasmids were digested with Nsi I and Not I, treated with calf intestinal alkaline phosphatase (CIP; New England Biolabs). The smaller, excised Nsi I and Not I fragments were removed by eluting larger fragments in a second PCR purification spin column. The cut cDNAs were ligated, linearized with Not I, and treated with proteinase K (Invitrogen) to removed excess enzyme. The resulting cDNA products were extracted in a third PCR purification spin column.

A T7 RNA polymerase (AmpliScribe T7 RNA Transcription kit; Epicentre) was used to transcribe positive sense RNA from the linearized cDNA products for 2 hours at 37^0C. Reactions were composed of 6 mM m7G- (59)ppp- (59)A cap analog (New England Biolabs), 20% of the manufacturer recommended concentration of ATP, and 0.5–2 mg of *in vitro* ligated DNA. Following the reaction, the product was treated with DNAase to remove traces of *in vitro* ligated cDNA template and separated on a PCR purification spin column. RNA pellets were precipitated with 70% ethanol with sodium acetate and resuspended in RNAse-free water. For transfections, one T-150 flask of BHK cells were grown to 70-80% confluency (containing 10^7 cells) and electroporated (two pulses) with genomic RNA using a ECM 630 electroporator (BTX Harvard Apparatus) set at 800 V, 25uF and 25 Ω.

Table 2.2. Summary of NS3-249 mutants and chimeras derived from SA89 and NS10
Amino acid or 3' UTR differences of NS3-249 mutants and chimeras (NS1-NS3, NS4B, NS5, and 3' UTR) compared to the parental strain (SA89 or NS10) are bolded and shaded in grey.

Infectious clone	Amino acid																		3' UTR							Tested species (corresponding *n*)	
	NS1		NS2A	NS2B		NS3		NS4B						NS5					10467	10469	10497	10512	10614	10681	10702		
	35	44	98	88	119	11	249	14	23	32	49	79	113	190	197	374	643									HOSP	AMCRs
SA89	H	K	K	M	V	K	H	S	A	S	T	T	V	K	I	H	R		t	t	c	a	c	a	t	7	6
SA89 NS3-His249Pro	H	K	K	M	V	K	**P**	S	A	S	T	T	V	K	I	H	R		t	t	c	a	c	a	t	-	6
SA89 NS3-His249Ala	H	K	K	M	V	K	**A**	S	A	S	T	T	V	K	I	H	R		t	t	c	a	c	a	t	-	6
SA89 NS3-His249Asn	H	K	K	M	V	K	**N**	S	A	S	T	T	V	K	I	H	R		t	t	c	a	c	a	t	-	6
SA89 NS3-His249Thr	H	K	K	M	V	K	**T**	S	A	S	T	T	V	K	I	H	R		t	t	c	a	c	a	t	-	6
NS10	Y	R	R	I	I	R	P	G	T	N	A	A	M	R	T	Y	G		a	c	t	t	t	g	c	7	5
NS10 NS3-Pro249His	Y	R	R	I	I	R	**H**	G	T	N	A	A	M	R	T	Y	G		a	c	t	t	t	g	c	-	5
NS10 NS3-Pro249Ala	Y	R	R	I	I	R	**A**	G	T	N	A	A	M	R	T	Y	G		a	c	t	t	t	g	c	-	5
NS10 NS3-Pro249Asn	Y	R	R	I	I	R	**N**	G	T	N	A	A	M	R	T	Y	G		a	c	t	t	t	g	c	-	5
NS10 NS3-Pro249Thr	Y	R	R	I	I	R	**T**	G	T	N	A	A	M	R	T	Y	G		a	c	t	t	t	g	c	-	5
Chimeras																											
NS1-NS3	**Y**	**R**	**R**	**I**	**I**	**R**	**H**	S	A	S	T	T	V	K	I	H	R		t	t	c	a	c	a	t	-	5
NS4B	H	K	K	M	V	K	H	**G**	**T**	**N**	**A**	**A**	**M**	K	I	H	R		t	t	c	a	c	a	t	-	5
NS5	H	K	K	M	V	K	H	S	A	S	T	T	V	**R**	**T**	**Y**	**G**		t	t	c	a	c	a	t	-	5
3' UTR	H	K	K	M	V	K	H	S	A	S	T	T	V	K	I	H	R		**a**	**c**	**t**	**t**	**t**	**g**	**c**	-	5

After transfection cells were transferred to T-75 cm^2 flasks following electroporation. Culture supernatant was harvested when cytopathic effects CPE were observed. The harvested medium was centrifuged at 1400 rpm for 10 minutes to remove cellular debris, aliquoted and stored at -80^0C. Titers of frozen virus stocks were determined by plaque assay as previously described(Brault *et al.*, 2004). RNA was extracted with a viral RNA extraction kit (QIAamp Viral RNA Mini Kit, Qiagen) and complete genomic sanger sequencing was used to confirm genetic identity of all viruses.

Avian studies

American Crows (*Corvus brachyrhynchos*; AMCR) were collected under a US Fish and Wildlife Services and Colorado Parks and Wildlife permit (MB91672A-0). The Institutional Animal Care and Use Committee at the Colorado State University granted approval for an avian studies protocol (12-3871A). After hatch-year wild AMCRs were netted using pressurized cannon nets at two baited sites in Bellvue, Colorado, with the permission of Morning Fresh Dairy (40° 38' 51''N, 105° 11'15'' W) and the managers of the Colorado State Fisheries Unit (40° 37' 35'' N, 105° 10' 32'' W). Both sites were baited daily with dog food and discarded meat products for one month. Nets were launched following the arrival of large numbers of AMCRs to the bait site. Birds were extracted from the net and placed in temporary large plastic holding cages while being transported to a permanent holding facility. Captured birds were affixed with a unique identification band to their leg and bled by jugular venipuncture. Serum from individual birds was tested using a 90% plaque reduction neutralization test to determine the presence of WNV neutralizing antibody as previously described (Langevin *et al.*, 2005). Prior to inoculation with WNV, birds were housed at the Colorado State University Animal Disease Laboratory and fed an *ad libitum* mixture of dry dog and cat food. For WNV inoculations, groups of 5 (NS10 IC, NS10 NS3-249 mutants, NS1-NS3, NS4B, NS5, and 3' UTR chimeras) or 6 (SA89 IC, HUN04, UG2274, SA89 NS3-249 mutants) birds were subcutaneously inoculated by needle on the

breast region with a 100 µl inocula containing 1,500 PFU of WNV diluted in phosphate buffered saline solution (PBS). HOSPs were collected using Japanese mist nets in Mead, Colorado. Birds were banded and bled as described above to test for prior exposure to WNV by $PRNT_{90}$ assay. Birds were housed at the Colorado State University Animal Disease Laboratory and fed dry multi-seed feed *ad libitum*. Groups of 7 HOSPs were inoculated with 100 µl containing 1,500 PFU of the parental SA89 and NS10-ICs diluted in PBS as described for the AMCR inoculations. Following inoculation, birds were checked twice daily for signs of clinical disease through 7 days post infection (dpi). Birds displaying clinical signs of ataxia, lack of coordination or having difficulty feeding or accessing water were euthanized by phenobarbital overdose administered through the jugular vein. After 7 dpi, birds were monitored daily through 14 dpi. Daily bleeds for AMCRs and HOSPs were performed through day 7 for each bird by jugular venipuncture using a syringe with a 26-gauge needle for AMCRs and 29-gauge needle for HOSPs with blood volumes collected of 0.2 and 0.1mL respectively. Collected blood was diluted 1:10 in medium (DMEM, 10% fetal bovine serum 100 U/mL penicillin, 100 mg/mL Streptomycin) and spun for 10 minutes at 3,500 rpm following a 30-minute coagulation period. Viral titrations of sera were performed using a Vero cell plaque assay as previously described (Brault *et al.*, 2004). All surviving birds were euthanized at 14 dpi by intravenous phenobarbital overdose.

Reservoir competence index

The RC index was calculated for AMCRs exposed to SA89 and the NS1-NS3, NS4B, NS5, and 3' UTR chimeras. The viremia infection threshold for mosquitoes was set at 4.7 $\log_{10}$ (PFU/ml) as previously determined by Komar et al., 2003(Komar *et al.*, 2003). RC values were calculated as the product of susceptibility, the relative duration of infectiousness (number of days with viremia titer above infection threshold), and mean daily infectiousness (mean of viremia titers above infection threshold). As all AMCRs were susceptible to WNV infection, a value of 1.0 was used for susceptibility. The average RC value for each group is displayed

(Table 2.3). RC index values of 1.0 indicate that for one day, 100% of mosquitoes feeding on a specific WNV infected host would potentially become infected. The RC index values were originally developed to compare host competences of various avian species exposed to the same WNV strain. In this study, RC index values will be used to evaluate host competences of AMCRs exposed to different viral constructs as previous results have demonstrated that different WNV strains can impact the ability of an avian host to serve as a competent amplification host for WNV(Langevin *et al.*, 2014).

3'-UTR structural characterization

The Mfold web server (http://unafold.rna.albany.edu) was used to computationally predict the RNA secondary structure of the 3' UTR for SA89 and NS10 viruses. The maximum distance between paired bases was set to 80 and folding temperature was fixed at 37°C as previously described(Gritsun *et al.*, 2014). The model that was generated with the lowest minimum free energy was selected. Outputs were displayed as structure outlines with flat exterior loop types.

Statistical analysis

Mean peak viremia titers (highest daily mean) for parental L2 WNV strains (SA89, NS10 UG227, HUN04), the SA89 NS3-249X and NS10 NS3-249X mutant viruses, and each of the four corresponding SA89 / NS10 NS3-P249H chimeric viruses (NS1-NS3, NS4B, NS5, 3' UTR) in experimentally infected AMCRs were compared by analyses of variance (ANOVA). An ANOVA analysis was also used to compare mean peak viremia titers between HOSPs exposed to SA89 or NS10. Mean comparisons were performed using Tukey's HSD adjustment for multiple comparisons. RC values were calculated as previously described and statistical differences between the calculated RC values for each group were performed using a t-test.

RESULTS

Phenotype of lineage 2 WNV in AMCRs

The virogenesis of geographically and genetically distinct African and European L2 WNV strains (Fig. 2.1) was evaluated in the highly susceptible AMCR model. Mean peak viremia titers (highest daily means) observed in inoculated AMCRs ranged from 7.5 to 10.1 $\log_{10}$ (PFU/ml sera) for the four L2 WNV viruses assessed (Fig. 2.2a, 2.2b). The highest peak viremia titers and 100% mortality were exhibited in AMCRs infected with the NS10 and HUN04 strains. Contrastingly, the SA89 virus elicited a mean peak viremia titer that was 400-fold lower than HUN04 ($p<0.05$) and demonstrated only 33% mortality. The UG2274 strain demonstrated a mortality that was seemingly intermediate to the mortality of SA89 and NS10 or HUN04 L2 WNV strains, but differences were not statistically significant.

Effects of polymorphisms at the NS3-249 locus in AMCRs

A wide range of viremia titers and mortality profiles were exhibited by AMCRs infected with viruses with the different amino acids (alanine, asparagine, threonine, proline, and histidine) at the NS3-249 locus in both the SA89 or NS10 backbones (Fig. 2.3, 2.4).

For SA89, each alternative NS3-249 polymorphism elicited either an increase (proline) or decrease (alanine, asparagine, threonine) in mean peak viremia titers (highest daily average) and mortality compared to the parental SA89 strain (Fig. 2.3a, 2.3b). Specifically, the introduction of a threonine at the NS3-H249 locus in the SA89 backbone resulted in a peak viremia titer that was 100,000 fold lower ($p< 0.0001$) compared to the parental strain. Onset of detectable viremia titers was also delayed by 48 hours and a peak viremia titer of 2.4 $\log_{10}$ (PFU/ml sera) was not observed till 6 dpi. Correspondingly, no mortality was observed for AMCRs within SA89 NS3-H249T AMCR inoculated group.

Figure 2. 1. Map depicting the geographic location of L2 WNV isolates.

The distribution of L2 WNVs assessed in this study is depicted. The asterisk denotes the only L2 WNV strain (of the four depicted) that harbors an NS3-249P mutation. The circled numbers reference the viral strains as follows: 1) SA89 2) UG2274 3) HUN04 4) NS10.

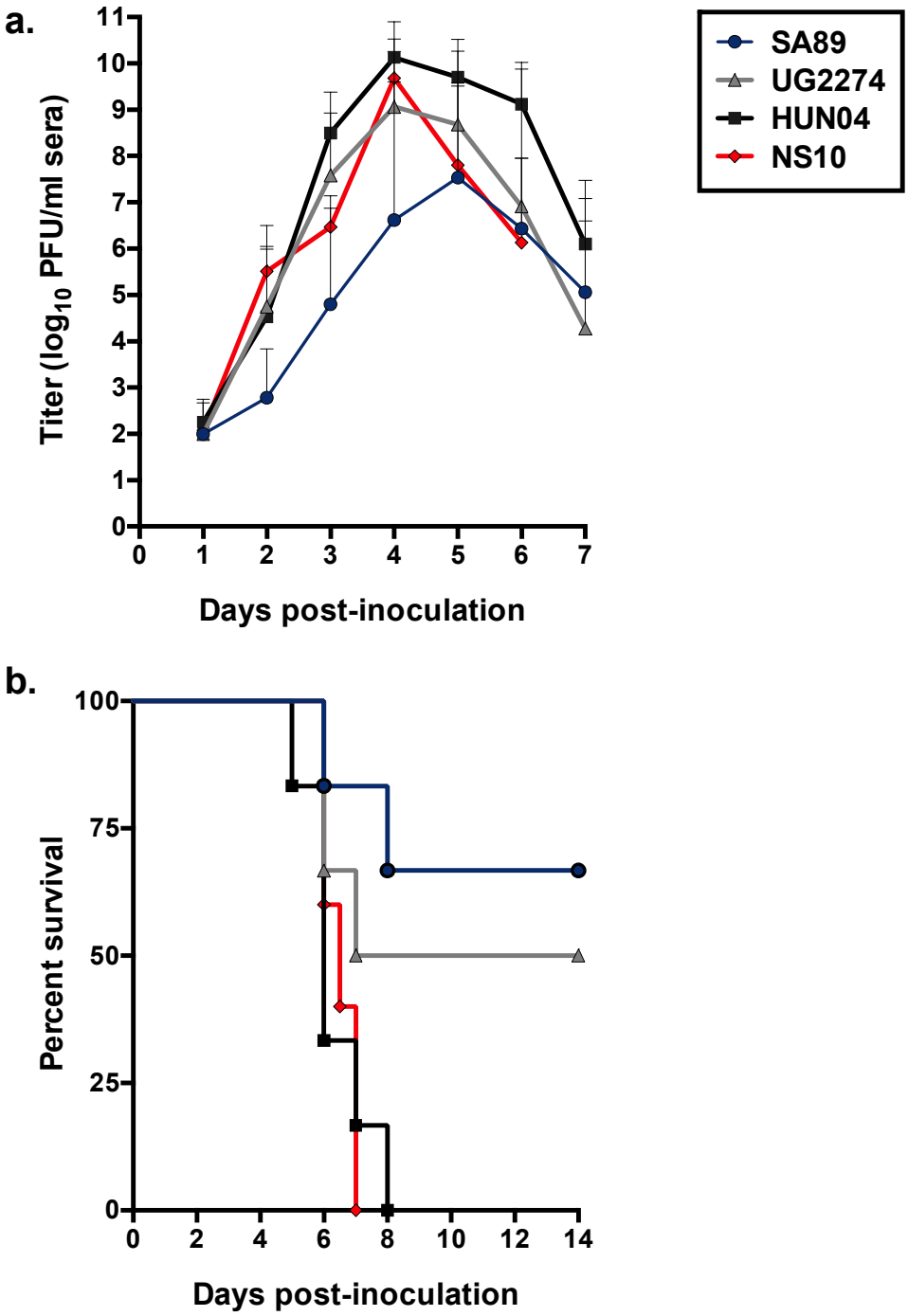

Figure 2.2 Viremia titers and mortality profiles of AMCRs inoculated with SA89, UG2274, HUN04, and NS10.

a) AMCRs were inoculated with 1,500 PFU of SA89, UG2274, HUN04, and NS10 and bled daily to assess viremia titers. Mean daily viremia titers were calculated through 7 dpi with error bars representing standard deviations. b) Surviorship of AMCRs through 14 dpi is depicted.

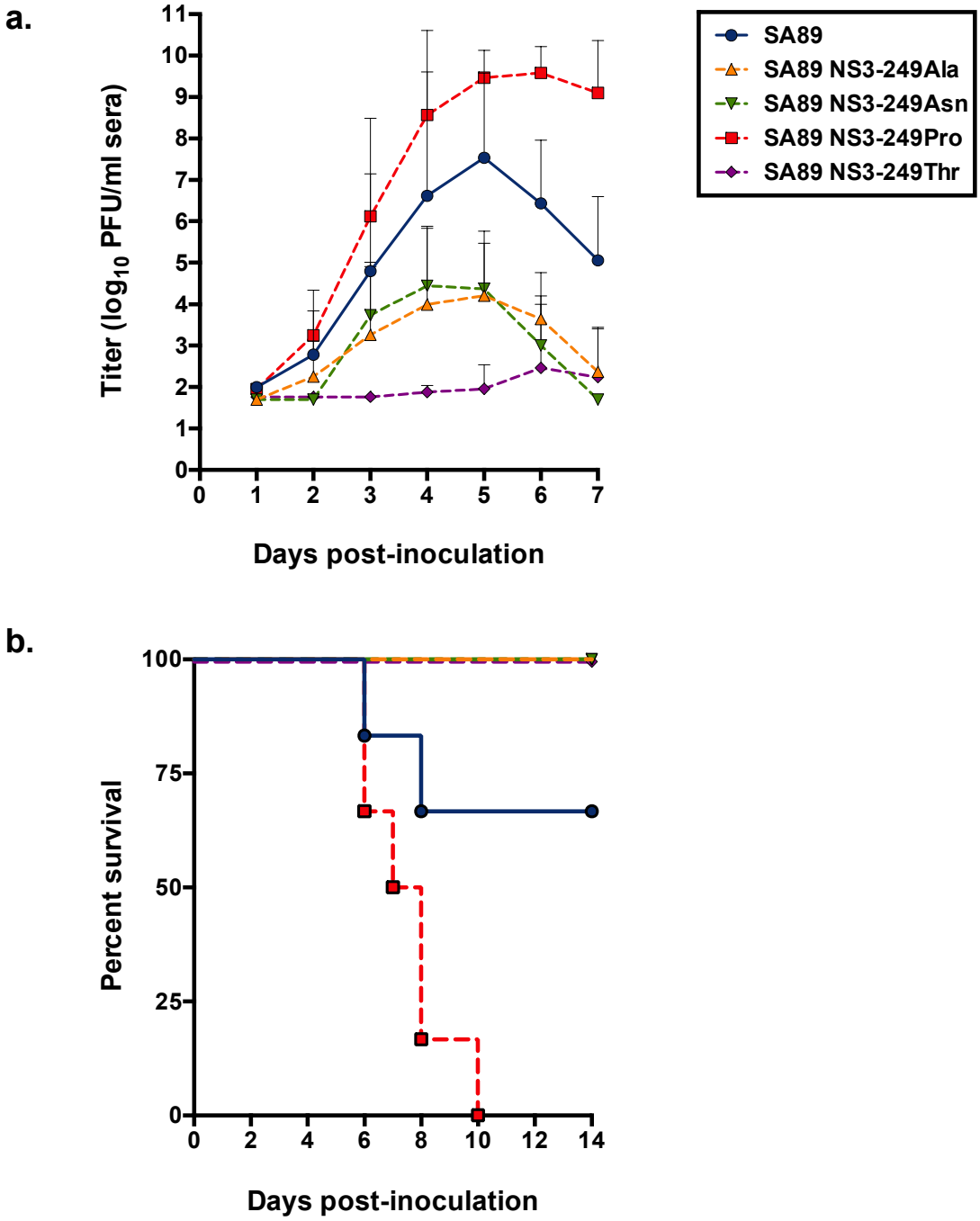

Figure 2.3 Viremia titers and mortality profiles of AMCRs inoculated with SA89 and NS3-249 (alanine, asparagine, proline, and threonine) mutants.

AMCRs were inoculated with 1,500 PFU of SA89 and corresponding NS3-249 mutants. a) AMCRs were bled through 7 dpi, mean daily viremia titers were calculated and standard deviations are similarly displayed. b) Surviorship of AMCRs inoculated with corresponding L2 WNV strains through 14 dpi is depicted. The parental strain is depicted in solid lines, while dashed lines represent NS3-249 mutants.

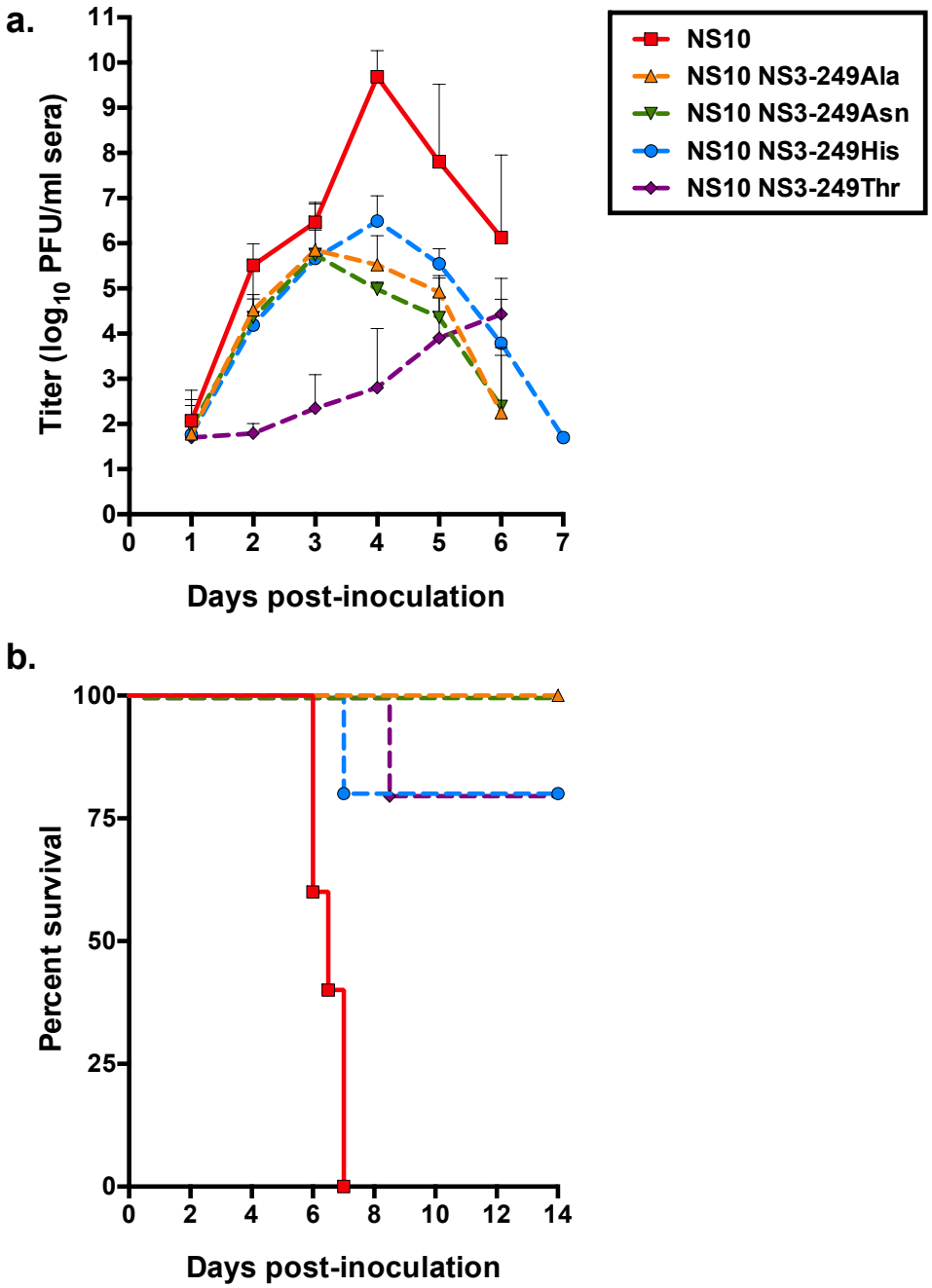

Figure 2.4. Viremia titers and mortality profiles of AMCRs inoculated with NS10 and NS3-249 (alanine, asparagine, histidine, and threonine) mutants.

AMCRs were inoculated with 1,500 PFU of NS10 and corresponding NS3-249 mutants. a) AMCRs were bled through 7 dpi, mean daily viremia titers were calculated and standard deviations are similarly displayed. b) Surviorship of AMCRs inoculated with corresponding L2 WNV strains through 14 dpi is depicted. The parental strain is depicted in solid lines, while dashed lines represent NS3-249 mutants.

Recombinant SA89 WNVs containing the NS3-H249A and NS3-H249N mutations at the NS3-249 locus both elicited a 1,000-fold lower ($p=0.0003$, $p< 0.0001$) mean peak viremia titer compared to SA89, with no mortality being observed in either group. The mean peak viremia titer (highest daily mean viremia titer) for the SA89 NS3-H249P strain demonstrated a 100-fold higher mean daily peak viremia titer compared SA89 (not statistically significant), and mortality rates were observed to be 100% versus 33%, respectively ($p=0.0269$). Despite this, a 1,000 ($p=0.0006$) or 10,000-fold ($p=0.0001$) higher mean daily viremia titer was observed for the SA89 NS3-H249P recombinant virus on 6 and 7 dpi, respectively compared to the parental strain at the same time points. AMCRs inoculated with the SA89 NS3-H249P virus were uniformly susceptible with 100% mortality being observed. Of the four NS3-249 mutants, differences in mortality were only statistically significant between the parental SA89 and the SA89 NS3-H249P mutant ($p=0.02$).

AMCRs inoculated with each of the NS10 NS3-249 (alanine, asparagine, histidine, and threonine) mutants demonstrated significantly lower viremia titers (highest daily mean viremia titer) and mortality rates compared to NS10 (Fig. 2.4a 2.4b). The most significant reduction in viremia titers was exhibited by the NS10 NS3-P249T virus for which a 100,000-fold ($p< 0.0001$) reduction in mean daily peak viremia titer was observed. Furthermore, the NS10 NS3-P249T mutant peak viremia titer, in addition to being significantly diminished, was delayed by 48 hours, being observed on 6 dpi. Only 20% mortality was observed for the NS10 NS3-P249T inoculated crows as compared to 100% mortality for NS10. The histidine mutant, NS10 NS3-P249H, demonstrated the smallest reduction in peak viremia titers compared to NS10 with a mean peak daily viremia titer of 6.5 $\log_{10}$ PFU/ml sera ($p=0.0003$) on 4 dpi. The NS10 NS3-P249H strain resulted in a 1,000-fold reduction ($p=0.0003$) of mean daily peak viremia titers compared to NS10. Similar to NS10 NS3-P249T, the NS10 NS3-P249H virus only demonstrated 20% mortality. Both the NS10 NS3-P249N and NS10 NS3-P249A mutants exhibited a 7,000-fold lower ($p< 0.0001$) mean peak daily viremia titer compared to NS10 and demonstrated 100%

survivorship. Differences in mortality were statistically significant between the parental NS10 and each of the NS10 NS3-P249 mutants (p=0.002).

Characterization of the NS1-NS3, NS4B, NS5, and 3' UTR chimeras in AMCRs

Significant differences in viremia titers at later time points (6 and 7 dpi) were observed for AMCRs inoculated with SA89 compared to AMCRs inoculated with the SA89 NS3-H249P strain. However, the same differences in viremia titers at later time points was not observed for NS10 IC compared to NS10 NS3-P249H strain, suggesting that alternate genetic elements within SA89 could be potentiating a higher viremia titer phenotype at later time points in AMCRs.

Comparative analysis of the NS10 and SA89 genomic sequences revealed a total of 17 amino acids differences (Table 2.2, Fig. 2.5a), located within the protein coding regions for six non-structural WNV proteins (NS1, NS2A, NS2B, NS3, NS4B, NS5), and 7 nucleotide differences in the non-coding 3'UTR. To incriminate other genetic elements dictating growth and/or virulence determinants between the two genetic backbones, independent of the extreme virogenesis in AMCRs elicited by the NS3-249P identity, four chimeric WNV viruses between the SA89 IC and NS10 NS3-P249H IC were generated (Fig 2.5a, Table 2.2) and tested in AMCRs (Fig. 2.6a, 2.6b).

The parental SA89 IC and the four chimeras (NS1-NS3, NS4B, NS5 and 3' UTR) produced mean peak viremia titers (highest daily average) and mortalities in AMCRs that were indistinguishable between all groups (all $p>0.05$) (Fig 2.6a, 2.6b). However, at the later time points of 5, 6, and 7 dpi, the parental SA89 had higher mean viremia titers compared to 3' UTR ($p=0.001$ for 5 dpi, $p<0.001$ for 6 dpi; $p= 0.007$ for 7 dpi). Specifically, a 1,000-fold (5, 7 dpi) and 10,000-fold (6 dpi) lower mean viremia titer (day matched means) was observed for the 3' UTR chimeric virus compared to SA89. The viremia titers of the chimeric NS5 and NS1-NS3 strains were statistically indistinguishable from those of the SA89 parental strain at all time points.

a

Virus strain	Amnio acid differences																								
	NS1		NS2A	NS2B		NS3		NS4B						NS5				3' UTR							
	35	44	98	88	119	11	249	14	23	32	49	79	113	190	197	374	643	10467	10469	10497	10512	10614	10681	10702
SA89	H	K	K	M	V	K	H	S	A	S	T	T	V	K	I	H	R	t	t	c	a	c	a	t
NS10	Y	R	R	I	I	R	P	G	T	N	A	A	M	R	T	Y	G	a	c	t	t	t	g	c

b

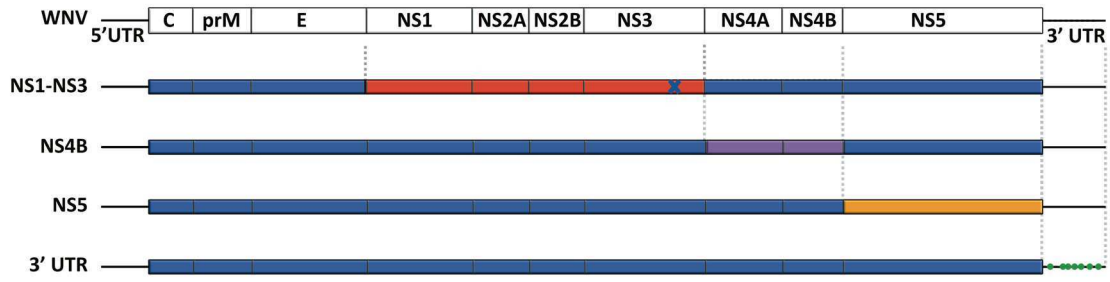

Figure 2.5 Chimerization schematic for SA89 and NS10 NS3-P249H

a) Amino acid and 3'UTR nucleotide differences between SA89 and NS10. The NS3-249 amino acid is depicted in light grey for the SA89 or NS10 strains. No nucleotide or amino acid differences were identified in the 5'UTR and structural proteins. b) Chimeras were constructed between SA89 and NS10 NS3-P249H ICs to target genetic regions in the NS10 backbone that were modulating avian virogenesis separate from the extreme pathogenicity generated by the NS3-249P mutation. A chimerization scheme for the chimeras generated between the SA89 and NS10 NS3-H249P ICs is depicted. The NS3-P249H mutation in the NS1-NS3 chimera is noted. Chimeras in panel b are color matched to the amino acid differences or nucleotide differences in panel a such that NS1-NS3=red, NS4B=purple, NS5=yellow, and 3"UTR=green.

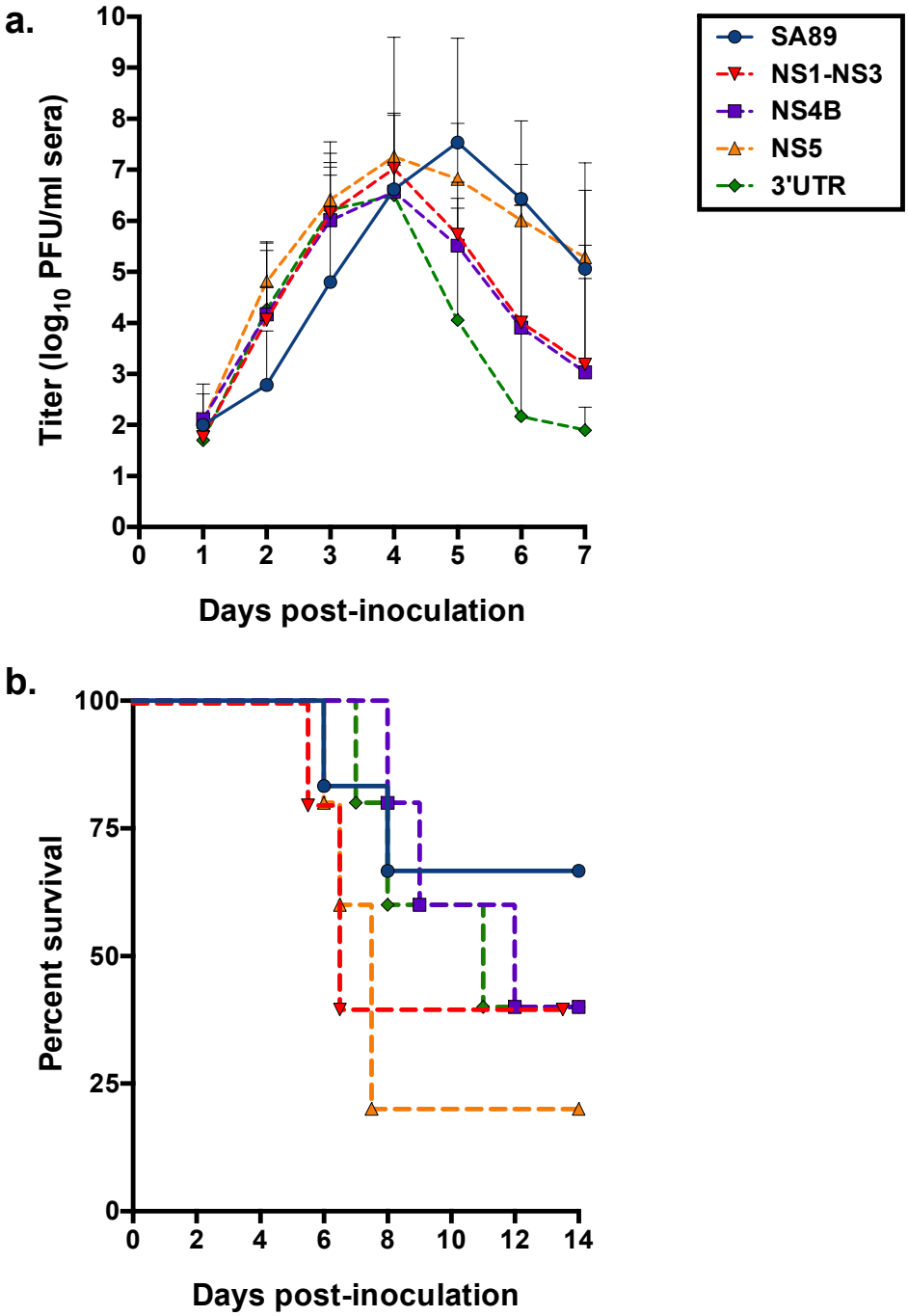

Figure 2.6 Viremia titers and mortality profiles of AMCRs inoculated with SA89 and NS10 NS3-H249H chimeric viruses: NS1-NS3, NS4B, NS5, and 3' UTR

AMCRs were inoculated with 1,500 PFU of SA89 and corresponding chimeras (NS1-NS3, NS4B, NS5, and 3' UTR). a) AMCRs were bled through 7 dpi, mean daily viral titers were calculated and standard deviations are similarly displayed. b) Surviorship of AMCRs inoculated with the corresponding L2 WNV strains through 14 dpi is depicted. Parental strain is depicted in solid lines, while dashed lines represent chimeras.

The NS4B chimera generated 100-fold decreased viremia titers compared to the SA89 IC in inoculated AMCRs, but only at 6 dpi (p=0.03).

Reservoir competence

The RC index represents a value that can be used to describe the relevance of a particular avian species for WNV enzootic transmission(Komar *et al.*, 2003). To determine if RC indices could be used to quantify the impact of the prolonged viremia titers of AMCRS inoculated with the SA89 strain compared to the 3' UTR and other chimeric viruses (NS1-NS3, NS4B, NS5), RC values were calculated for each of the chimeras based on the empirical data generated from AMCR inoculations and compared against the RC index values of the parental SA89 strain (Table 2.3). No statistical differences in calculated RC values were observed between any of the viruses.

Table 2.3. Calculated RC index values for SA89, and the chimeras NS1-NS3, NS4B, NS5, and 3' UTR.
RC index values are calculated as a product of the susceptibility, mean infectiousness, and duration of viremic days [(identified as the days where viremia titers were greater than 4.7 log10 (PFU/ml sera)]

Virus	Susceptibility	Mean infectiousness	Duration of viremic days	RC index
SA89	1.00	0.34	3.33	1.13
NS1-NS3	1.00	0.24	3.60	0.86
NS4B	1.00	0.20	3.40	0.67
NS5	1.00	0.29	4.40	1.26
3' UTR	1.00	0.25	2.40	0.59

3' UTR characterization

Our previous results demonstrated that differences in mean daily viremia titers at later time points (5-7 dpi) were observed in AMCRs between the SA89 strain and the 3' UTR

chimeric virus, indicating that the 3' UTR of NS10 could modulate later viremia titers in AMCRs. The 3' UTR of flaviviruses mediates negative strand RNA synthesis, facilitates formation of sfRNA, enables packaging of virions and interacts with host and WNV viral proteins(Markoff, 2003). To determine if structural disparities are exhibited between the 3' UTR of SA89 and NS10, secondary RNA structures were computationally generated using the Mfold web server and structure differences were examined.

The 3' UTR Mfold generated structures of either the SA89 or NS10 L2 WNV strains contained the highly conserved sequence regions (CS) 1, CS2, CS3 as well as the 3' terminal long-stem loop (3' SL) pentanucleotide sequence as previously described for WNV by Markoff et al., 2003 (Fig. 2.7a, 2.7b)(Markoff, 2003)(164). However, within NS10, the CS1 sequence (Fig 2.7b) was partially contained within stem loop 2 (SL2), and the highly conserved 3' terminal SL contained only two structural bulges compared to the three structural bulges observed for SA89 (Fig 2.7a). The CS1 sequence of the 3' UTR derived from the SA89 L2 WNV strain was not associated with any secondary RNA structure. Additionally, the NS10 3' UTR had 2 fewer stem loop (SL) structures and 1 more dumbbell (DB) than SA89 (Fig. 2.7b compared to Fig. 2.7a). The SA89 3' UTR had three DB structures and 9 SL structures, while the NS10 3' UTR contained 4 DB and only 7 SL structures.

Phenotype of select L2 WNVs in House sparrows

To evaluate the capacity of HOSPs to serve as competent amplification hosts for L2 WNV, the two L2 WNV viruses that displayed the most divergent phenotypes in AMCRs, SA89 and NS10, were chosen for inoculation of HOSPs (Fig. 2.8a, 2.8b). Mean peak daily viremia titers of 7.8 and 6.4 $\log_{10}$ (PFU/ml sera) with either 71% or 14% mortality were elicited by SA89 or NS10 respectively. A high degree of inter-bird titer variability was observed in HOSPs that rendered the SA89 and NS10 strains statistically indistinguishable. Despite this, both viruses generated mean peak viremia titers that were in excess of 10^5 (PFU/ml).

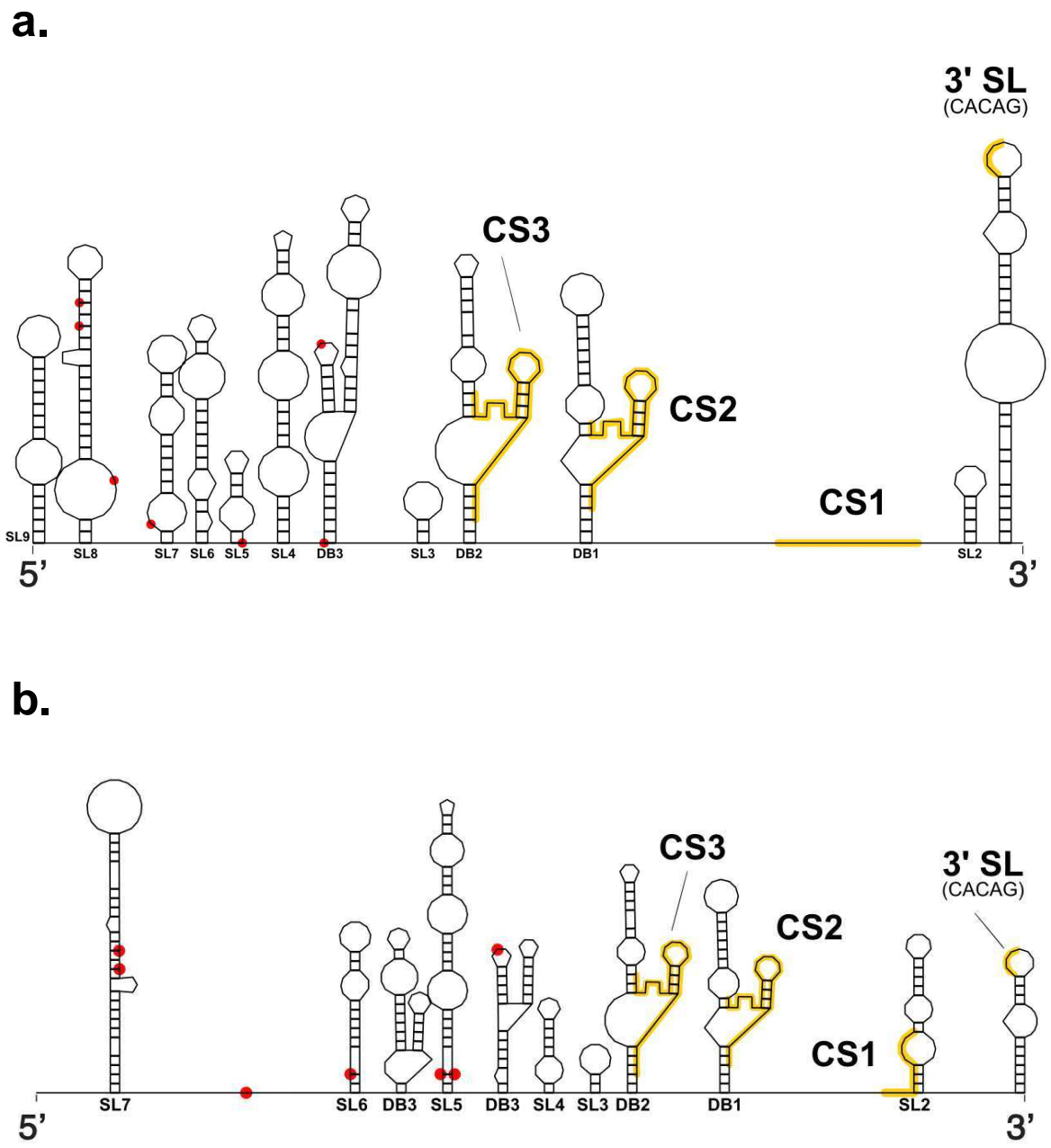

Figure 2.7 Mfold generated 3' UTR secondary RNA structures.

The outlined predicted RNA structures of a) SA89 and b) NS10 are depicted. Conserved regions 1-3 (CS1, CS2, CS3) and the pentanucleotide sequence (CACAG) within the 3' SL are highlighted in yellow. Red circles denote the position of the nucleotide differences between SA89 and NS10. Three DB structures and 9 SL structures were determined for SA89, while NS10 contained 4 DB and 7 SL structures.

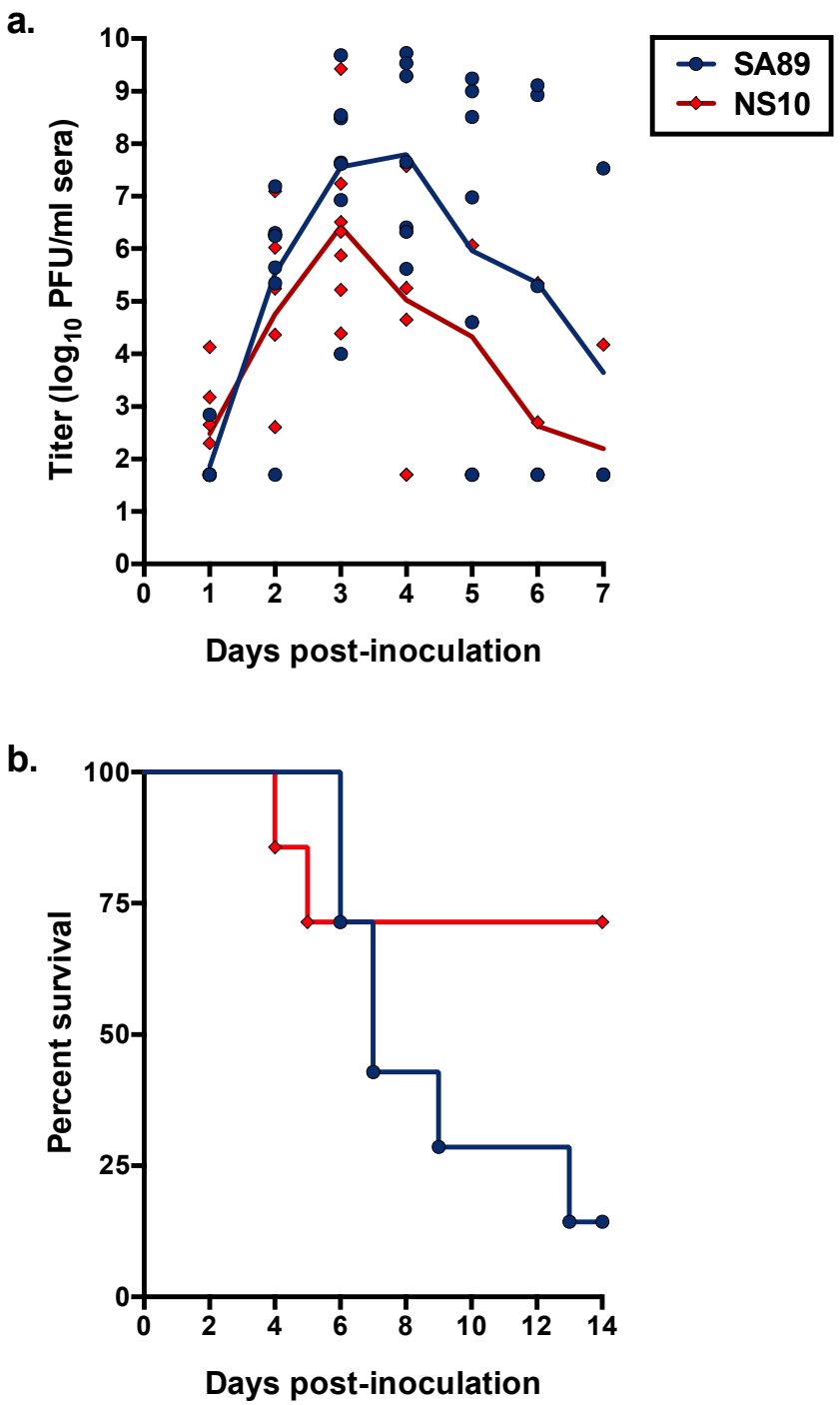

Figure 2.8 Viremia titers and mortality profiles of HOSPs inoculated with the SA89 IC and the NS10 IC.

HOSPs were inoculated with 1,500 PFU of SA89 IC and NS10 IC. a) HOSPs were bled through 7 dpi and mean daily viremia titers were calculated (solid red or blue lines represent means at each time point). Each dot represents the viral titers for each bird at that time point. b) Surviorship of HOSPs infected with L2 WNV strains through 14 dpi is depicted.

DISCUSSION

Although surveillance studies in Africa have identified multiple avian species with WNV neutralizing antibodies, avian mortality during WNV outbreaks in Africa has not been extensively characterized(Jupp, 2001). An absence of bird mortality was also noted during the initial 2010 L2 WNV outbreak in Greece. However, Lim et al., 2014, demonstrated the susceptibility of European jackdaws (*Corvus monedula*), an Old World corvid species, to pathogenic and lethal infection with L1 and L2 WNV strains, indicating that European avian species are susceptible to fatal WNV infection. Additionally, Work et al., 1955, demonstrated mortality for African HOSPs and hooded crows (*Corvus corone sardonius*) following experimental infection with a WNV strain isolated from Egypt (unknown lineage). In our model, both African and European L2 WNV isolates generated lethal outcomes in AMCRs and HOSPs. Studies have demonstrated that HOSPs from North America have become less susceptible to WNV disease over time(Duggal *et al.*, 2014). It is likely that pathogenic L2 WNV strains actively circulate in Africa, but resident birds are no longer susceptible to fatal infection(Jupp, 2001). Alternately, it is also likely that surveillance efforts during WNV transmission periods failed to identify bird deaths. However, WNV has been documented in Europe (by evidence of neutralizing antibodies in humans) since the late 1960s(Hubalek & Halouzka, 1999). Additionally, exposure of HOSPs from Hawaii (where WNV is not transmitted) to WNV infection produces 100% mortality and mean peak viremia titers of 9.24 $\log_{10}$ (PFU/ml sera) and contrasts with the attenuated levels of viremia titers and mortality exhibited by HOSPs in the continental United States in response to infection with the same WNV strain, thereby providing evidence of host adaptive responses(Duggal *et al.*, 2014; Hofmeister *et al.*, 2015). Our studies demonstrate that L2 WNV strains originating from either Africa or Europe have the potential to elicit both fatal and viremic responses in birds sufficient to sustain enzootic transmission and highlight the susceptibility of a North American corvid species to severe pathogenesis by L2 WNV. It is likely that avian deaths are still

occurring in Europe in response to WNV infection, but that the levels are low enough that bird mortality events go undetected.

The emergence of the NS3-249P mutation was initially identified in L2 during the first L2 mediated WNV outbreak in Greece, but was not associated with European L2 strains isolated in prior years. In our study, the NS3-249 proline mutants elicited higher virogenesis in AMCRs compared to the same strains harboring an NS3-249H identity suggesting that increases in enzootic transmission in Greece in 2010 were potentially facilitated by increased viremia titers elicited by the NS3-249P L2 WNV variant in susceptible avian species.

In Italy, the NS3-249P variant has only been isolated once, despite the multiple occurrences of L2 WNV mediated outbreaks and contrasts to the repeated association of the NS3-239P variant and L2 Greece outbreaks(Capelli *et al.*, 2013). However, the epidemiological scenario of WNV in Italy contrasts with that of Greece; evidence of WNV epizootic transmission (L1) dates back to 1998, whereas Greece only experienced its first outbreak of WNV in 2010(Autorino *et al.*, 2002; Danis *et al.*, 2011). The first WNV strain isolated in 1998 in Italy demonstrated a NS3-249T identity (L1 WNV) but this genotype is no longer detected and instead has been replaced by a second L1 WNV genotype(Barzon *et al.*, 2009). Strains within the second genotype are associated with an NS3-249P identity(Barzon *et al.*, 2009). Additionally, outbreaks of WNV seem to be smaller compared to those observed in Greece, with the largest WNV outbreak having occurred in 2010 with just 25 cases of reported neuroinvasive diseases(Barzon *et al.*, 2013). Smaller outbreaks could reflect a decrease of non-naïve WNV populations as a result of prior WNV exposure or could reflect decreased enzootic transmission potentially mediated by the absence of increased virogenesis elicited by the NS3-249P mutation within circulating L2 WNV strains. Additionally, the prior emergence and ongoing circulation of the NS3-249P mutation in L1 WNV might preclude the maintenance of an NS3-249P emergence in an alternate lineage. This might be used to explain the prior absence of NS3-

249P emergence events in areas where L1, harboring the NS3-249P mutation, and L2 (NS3-249H) actively co-circulate.

Previous studies by Langevin et al., 2014 demonstrated that phenotypic variations related to polymorphisms at the NS3-249 locus have been observed in a L1 WNV genetic backbone. Similarly, I found that interchanging NS3-249 polymorphisms modulate severe differences in AMCR virogenesis in alternate L2 WNV strains, suggesting that the NS3-249 locus modulates viremia titers and mortality phenotypes in AMCRs in a similar manner across WNV lineages. The most significant phenotypic differences in either L2 WNV strains were elicited by either proline or threonine NS3-249 variants. The emergence of the NS3-T249P substitution has preceded large WNV outbreak on four separate occasions. Taken together, these results suggest a possible role of the NS3-249 locus for an avian selective capacity for influencing L2 WNV emergence. Negative selective influences that have constrained prior emergence of the NS3-249P in L2 WNV need to be further investigated.

Genetic elements independent of the NS3-249 locus involved in modulating avian disease have been documented for L1 WNV strains. Amino acid polymorphisms within the NS1-NS2B genes have been found to modulate AMCR pathogenicity in an independent and additive manner between a Kenyan WNV isolate and the NY99 North American founding isolate(Dietrich *et al.*, 2016). Additionally, several amino acid mutations within the structural proteins, envelope and pre-membrane of a WNV strain isolated in Mexico, attenuated virulence phenotypes in AMCRs, HOSPs and house finches(Langevin *et al.*, 2011). In our study, the SA89 strain generated AMCR viremia titers that would be predicted to be infectious for mosquitoes (greater than 10^5 PFU/ml) 2 days longer than an alternative L2 WNV containing the same NS3-249H identity. Protracted viremia titers in birds greater than 10^5 PFU/ml increase infectious exposure events to imbibing mosquitoes and potentially would lead to greater enzootic transmission. Additionally, the chimeric virus incorporating elements from the 3' UTR of NS10 into the SA89 backbone (3' UTR), was found to significantly reduce peripheral viral titers below the 10^5

PFU/ml threshold three days sooner compared to SA89 in AMCRs. Despite this, no differences in the calculated RC index values were observed between any of the chimeras (NS1-NS3, NS4B, NS5, and 3' UTR) and SA89. The attenuating 3' UTR region of NS10 contrasts with the highly pathogenic effector substitution NS3-249P substitution in that same strain. The NS10 3' UTR secondary structure lacks a prominent bulge in the 3' SL and harbors fewer stem loop structures than the SA89 3' UTR structure. Previous studies have demonstrated the relative importance of the bulge structures, rather than specific nucleotide sequences in the 3' SL, as enhancers of cell-specific WNV replication(Yu & Markoff, 2005). Additionally, specific sequences and bulge structures in the 3' SL have also been shown to disrupt WNV and DENV replication(Tilgner & Shi, 2004; Zeng *et al.*, 1998). The 3' SL of JEV has been shown to directly bind with WNV nonstructural proteins NS5 and NS3 and it is thought that these interactions facilitate formation of the replication complex and may be involved in minus-strand RNA synthesis(Chen *et al.*, 1997). Stem loop structures within the 3' UTRs of rubella virus can also directly interact with host cell proteins and potentially modulate viral replication (Nakhasi *et al.*, 1990). Structural differences between the 3' UTR of SA89 and NS10, in conjunction with the phenotypic observation that the 3' UTR of NS10 restricts viremia titer production in AMCRs at later time points, indicates that the 3' UTR might be involved in modulating viral replication either directly or indirectly by impacting viral production through increased cell death in avian hosts. However, the direct impact of the altered 3' UTR structures between NS10 and SA89 and their respective role in potentially altering replication phenotypes in AMCRs needs to be further examined.

CHAPTER 3: VECTOR COMPETENCE OF *CULEX PIPIENS* AND *CULEX QUINQUEFASCIATUS* MOSQUITOES FOR AFRICAN AND EUROPEAN L2 WNV STRAINS

INTRODUCTION

Enzootic transmission of lineage 2 (L2) WNVs, previously limited to circulation in sub-Saharan Africa, emerged as a disease threat in 2004 in Europe with subsequent sporadic human disease cases in Western Europe and larger epidemics observed in Russia (2004) and Greece (2010-2014).

L2 WNV was first observed in Europe in 2004 when an isolate was made from an encephalitic goshawk in Hungary(Bakonyi *et al.*, 2006). The Hungarian L2 isolate (HUN04) demonstrated the closest genetic identity to other L2 isolates from southern and central Africa. Other L2 isolates from birds were subsequently made in Hungary (2005) and Austria (2008) and demonstrated a high degree of sequence conservation with the original HUN04 strain, suggesting that the introduced genotype virus was subsequently circulating in an enzootic transmission cycle in Western Europe. In 2010, a L2 mediated outbreak of WNV was observed in Greece and represented the first documented L2 associated WNV outbreak of human disease in Europe(Papa *et al.*, 2011a). Sequence analysis of an isolate (NS10) made from a pool of *Culex pipiens* mosquitoes collected during the outbreak in Nea Santa, Greece, demonstrated closest sequence identity to the HUN04. Notably, an NS3-249Pro substitution, previously associated with increased viremia titers and mortality in corvids in L1 WNVs(Brault *et al.*, 2007; Langevin *et al.*, 2014) and previously unidentified in L2 viruses, was also described for the NS10 isolate. All previously sequenced L2 WNV strains identified with a histidine at the NS3-249 position (Langevin et al., 2014).

Consecutive L2 mediated outbreaks were observed in Greece from 2011 to 2014 and small L2 mediated WNV outbreaks were later described for Italy beginning in 2014(Magurano *et al.*, 2012). Sporadic L2 WNV human disease cases in Russia in 2004 and in Serbia in 2012

were also observed, indicating that L2 WNV was circulating in central Europe and Russia. The potential for further geographic spread of L2 WNV beyond central Europe and Russia likely depends on the availability of competent transmission vectors for L2 WNV strains. Limited information regarding the vector competence of *Culex* species for L2 WNV is available. Given the recent demonstrated capacity of L2 WNV to emerge and become endemic in new environmental habitats, inquiry regarding the capacity of North American mosquitoes to transmit L2 WNV is necessary to address the potential of L2 WNV maintenance in the Western Hemisphere.

Species within the genus *Culex* are the main transmission vectors of WNV worldwide(Farajollahi *et al.*, 2011). The importance of individual species within the *Culex* complex for enzootic maintenance and transmission of L2 WNV geographically varies(Fonseca *et al.*, 2004). In Europe, WNV has been isolated from 8 species of mosquitoes, but WNV transmission is primarily facilitated by ornithophilic *Culex pipiens and Culex modestus* mosquitoes. Studies in southwest Europe have identified both human and avian blood meals in *Culex pipiens*, indicating that European *Culex pipiens* mosquitoes can serve as bridge vectors due to their ability to readily host switch(Farajollahi *et al.*, 2011; Hamer *et al.*, 2008).

Culex univittatus is the main enzootic transmission vector for WNV in Africa, where both L2 and L1 WNV co-circulate(Hubalek & Halouzka, 1999). However, ornithophilic *Culex quinquefasciatus* and *Culex pipiens* have also been identified as important mosquito vectors for WNV in some parts of Africa(Jupp, 2001; Motayo *et al.*, 2016; Mutebi *et al.*, 2012; Muturi *et al.*, 2008).

Different mosquitoes within the *Culex* complex have demonstrated varying capacity to serve as competent transmission vectors for WNV in the laboratory. For example, in a study by Balenghien et al., 2008 European *Culex modestus* and *Culex pipiens* mosquitoes were per orally exposed to WNV (L1) and heads and salvia expectorants were assayed for WNV as evidence of disseminated infections and capacity to transmit WNV, respectively. Results

demonstrated that higher rates of dissemination (evidence of WNV in the legs or wings of the mosquito) and transmission were observed for *Culex modestus (*89.2% dissemination, 54.5% transmission) compared to *Culex pipiens (*38.5%, dissemination 15.8% transmission), and indicate that *Culex modestus* is a far more efficient transmission vector than *Culex pipiens*(Balenghien *et al.*, 2008). European *Aedes albopictus* mosquitoes have demonstrated transmission competence by evidence of WNV in saliva samples following experimental per oral exposure to WNV, but host-feeding preferences make these mosquitoes unlikely to contribute to the enzootic transmission cycle(Fortuna *et al.*, 2015).

Vector competence phenotypes can also be modulated by changes in viral genetics. In the United States, a genotype (WN02) completely displaced the introduced strain and differed only by a single amino acid(Davis *et al.*, 2005; McMullen *et al.*, 2011). Decreased EIP and more effective transmission of WN02 by *Culex pipiens* and *Culex tarsalis* likely facilitated higher levels of enzootic transmissions and enabled displacement of the originally introduced genotype(Moudy *et al.*, 2007). The comparative assessment of the vector competence for newly evolved L2 strains and older L2 WNV isolates as well as a relative evaluation of the vector competence between geographically distinct L2 strains has not been performed.

To assess if diverse transmission phenotypes are elicited by genetically distinct L2 WNVs, as well as to assess the competence of North American mosquitoes for L2 NWV transmission, the vector competence of North American *Culex pipiens* and *Culex quinquefasciatus* mosquitoes for two European L2 isolates and two African L2 isolates was examined. I found that key differences in rates of infection, dissemination (by evidence of WNV in mosquito legs) and transmission are genetically influenced, indicating that viral genetics can potentially alter enzootic transmission of L2 WNV. This study also determined that two species of North American *Culex* mosquitoes can effectively transmit L2 WNV, thus widening the potential range of L2 WNV transmission.

MATERIAL AND METHODS

Virus stocks

The L2 WNV strains HUN04, SA89, NS10, and UG2274 used in this study have been previously described in Chapter two. Virus stocks were prepared by inoculating C6/36 mosquito cells (*Aedes albopictus*) at an MOI of 0.1. Supernatant of infected cells was collected at 4 days post infection, centrifuged at 1400 rpm for 10 minutes to remove cellular debris, and frozen in aliquots at -80^{0}C. Titer of stocks was determined by plaque assay on African green monkey kidney cells (Vero cells; ATCC no. CCL-81) as previously described(Brault *et al.*, 2004).

Mosquitoes

Culex pipiens (L.) and *Culex quinquefasciatus* (Say) mosquitoes were derived from laboratory colonies. The *Culex pipiens* colony originated in 2010 from egg rafts collected in Chicago, Illinois, and the *Culex quinquefasciatus* colony was established in 1988 from Sebring County, Florida. Colonized mosquitoes were maintained on goose blood (*Culex pipiens*) or calf blood (*Culex quinquefasciatus*) for egg laying and provided 10% sucrose *ad libitum*. Larvae were reared and adults were maintained at ~70-80% humidity at 28°C with a 16:8 (light dark diurnal cycle) photoperiod.

Laboratory vector competence assessment of *Culex pipiens* and *Culex quinquefasciatus*

Three- to 5-day-old adult female *Culex pipiens* mosquitos or four- to seven- day old *Culex quinquefasciatus* were deprived of sucrose 24 hours before feeding and water 6-8 hours before feeding. Frozen viral stocks were thawed to room temperature and added to defibrinated calf blood (Colorado Serum Company, Denver, CO) with 0.1mM ATP to a final WNV titer of 10^7 or 10^8 PFU/ml. Mosquitoes were offered infectious blood meals for 0.5-1 hour (s) by use of a Hemotek membrane feeding apparatus (Discovery Workshops, Accrington, UK). An aliquot of the infectious blood meal was reserved and stored at -80°C for later back-titration of viral titers

by plaque assay. Fully engorged females were sorted under cold anesthesia, placed in 1-pint cartons, provided with 10% sucrose *ad libitum* and held at 27°C at a 16: L:D photoperiod with 70-80 % humidity through 14 days post exposure (dpe). Mosquitoes from each group were assessed at incubation periods of 5, 7, 9 and 14 dpe to evaluate potential differences in the extrinsic incubation period (EIP). Mosquitoes were anesthetized following incantation by exposure to triethylamine (Flynap; Carolina Biological Supply Company, Burlington, NC), their legs removed and placed into 500 µl mosquito diluent [DMEM containing 10% FBS, 5% penicillin/streptomycin (10 µg/ml), and 1% Fungizone (2.5 µg/ml)]. Saliva samples were collected by placing each mosquito proboscis into a capillary tube charged with Type B immersion oil (Cargille Labs, Cedar Grove, NJ). After a 30 minute period of expectoration, the contents of capillary tubes were collected and expelled into 200 µl mosquito diluent by centrifugation at 5,000 rpm for 10 minutes and stored at -80°C until assessed for the presence of virus by plaque assay. Following salivation, mosquito bodies were added to 1 ml mosquito diluent and stored at -80°C. Mosquito bodies and legs were homogenized in a mixer mill with the addition of 2 copper-coated steel shot BBs (4.5mm diameter, 0.177" caliber) (Qiagen). Each body or leg sample was triturated for 2 minutes in a 2 ml round bottom polypropylene tube in either 1 ml (body) or 0.5 ml (leg) mosquito diluent media and clarified by centrifugation at 10,000 rpm for 10 minutes. To expel the saliva samples from capillary tubes, saliva samples were centrifuged at 10,000 rpm for 10 minutes. Mosquito bodies, legs and saliva samples were assayed for infectious virus by non-dilution plaque assay. Briefly, undiluted mosquito homogenate (bodies or leg) or saliva suspension was allowed to adsorb on Vero cells (ATCC no. CCL-81) for 1 hour at 37^0C and an agarose (0.8%) nutrient overlay was added. After 2 days post inoculation (dpi), a second overlay containing 0.005% neutral red was added and plaques or evidence of cytopathic effects (CPE) was recorded on 3 dpi. Rates of infection (proportion of positive bodies from tested mosquitoes), dissemination (proportions of positive legs from

positive bodies) and transmission (proportion of positive salvia samples from positive bodies) between virus groups were calculated.

Statistics

Logistics regression analysis was used to determine differences in proportions of WNV positive bodies (infection rates), WNV positive legs (dissemination rates) or WNV positive saliva samples (transmission rates) between SA89, NS10, UG2274 and HUN04 across 4 time points (5, 7, 9 and 14 dpe) for *Culex pipiens* and *Culex quinquefasciatus* mosquitoes. A generalized linear model assuming a binomial distribution was fit for each proportion for *Culex pipiens*. Each model had utilized time and virus as main effects and together as an interaction effect. The models were used to estimate odds ratios (OR) and 95% confidence intervals (CI), which were adjusted for multiplicity. All 95% CI that do not contain 1.0 imply there is a statistically significant difference between two viruses. No model was fit for infection rates across all time points and for both the leg and saliva data at time point 5 dpe for *Culex* quinquefasciatus due to the lack of variation. Due to the small variation among the *Culex quinquefasciatus* exposed to 7 $\log_{10}$ (PFU/ml) data, difference in proportions and corresponding 99% CI between viruses were computed for each infection rates only at each time point. No model was fit for dissemination or transmission rates across all time points and for both the leg and saliva data *for Culex quinquefasciatus* [7 $\log_{10}$ (PFU/ml)] due to the lack of observable values.

RESULTS

Vector competence of *Culex pipiens*

Two African L2 strains and two European L2 strains were used to examine the vector competence of *Culex pipiens* (Chicago) at 5, 7, 9, and 14 days post oral exposure (dpe) (Fig. 3.1). Vector competence proportions calculated with the denominators as either the number of WNV positive bodies or the number of WNV exposed mosquitoes is summarized in Table 3.1.

Infection (number of positive bodies by number of WNV exposed mosquitoes), dissemination (number of positive legs by number of WNV positive bodies), and transmission rates (number of positive saliva by number of WNV positive bodies) are displayed in Fig 3.1 and analyzed statistically in Table 3.2.

A lower proportion of mosquitoes were infected after per oral exposure to NS10 compared to mosquitoes that were exposed to SA89, HUN04 and UG2274 at all time points except for HUN04 compared to NS10 at 7 dpe and SA89 compared to NS10 at 14 dpe (Fig. 3.1a). Specifically, the L2 WNV strains SA89, UG2274 and HUN04 were between 6-23 times more likely (OR range displayed in Table 3.2) to infect mosquitoes following per oral exposure than NS10 at most time points. There are no significant differences between dissemination ortransmission for the L2 WNV (Fig. 3.1b, 3.1c). Mosquitoes were able to transmit all viruses at 5 dpe and continued to transmit through 14 dpe (Fig. 3.1c). Odds ratios and corresponding 95% CI are presented in Table 3.2.

Vector competence of *Culex quinquefasciatus*

To evaluate the vector competence of an alternate *Culex* mosquito species important for WNV enzootic transmission in North America, the vector competence of *Culex quinquefasciatus* for L2 WNV was similarly assessed (Fig. 3.2a, 3.2b, 3.2c). Vector competence proportions calculated with the denominators as either the number of WNV positive bodies or the number of WNV exposed mosquitoes is summarized in Table 3.4. Infection (number of positive bodies by number of WNV exposed mosquitoes), dissemination (number of positive legs by number of WNV positive bodies), and transmission rates (number of positive saliva by number of WNV positive bodies) are displayed in Fig 3.2 and analyzed statistically in Table 3.3.

Infection rates were at or near 100% for all mosquitoes per orally exposed to the four L2 WNV isolates and differences could not be statistically compared due to the lack of variation

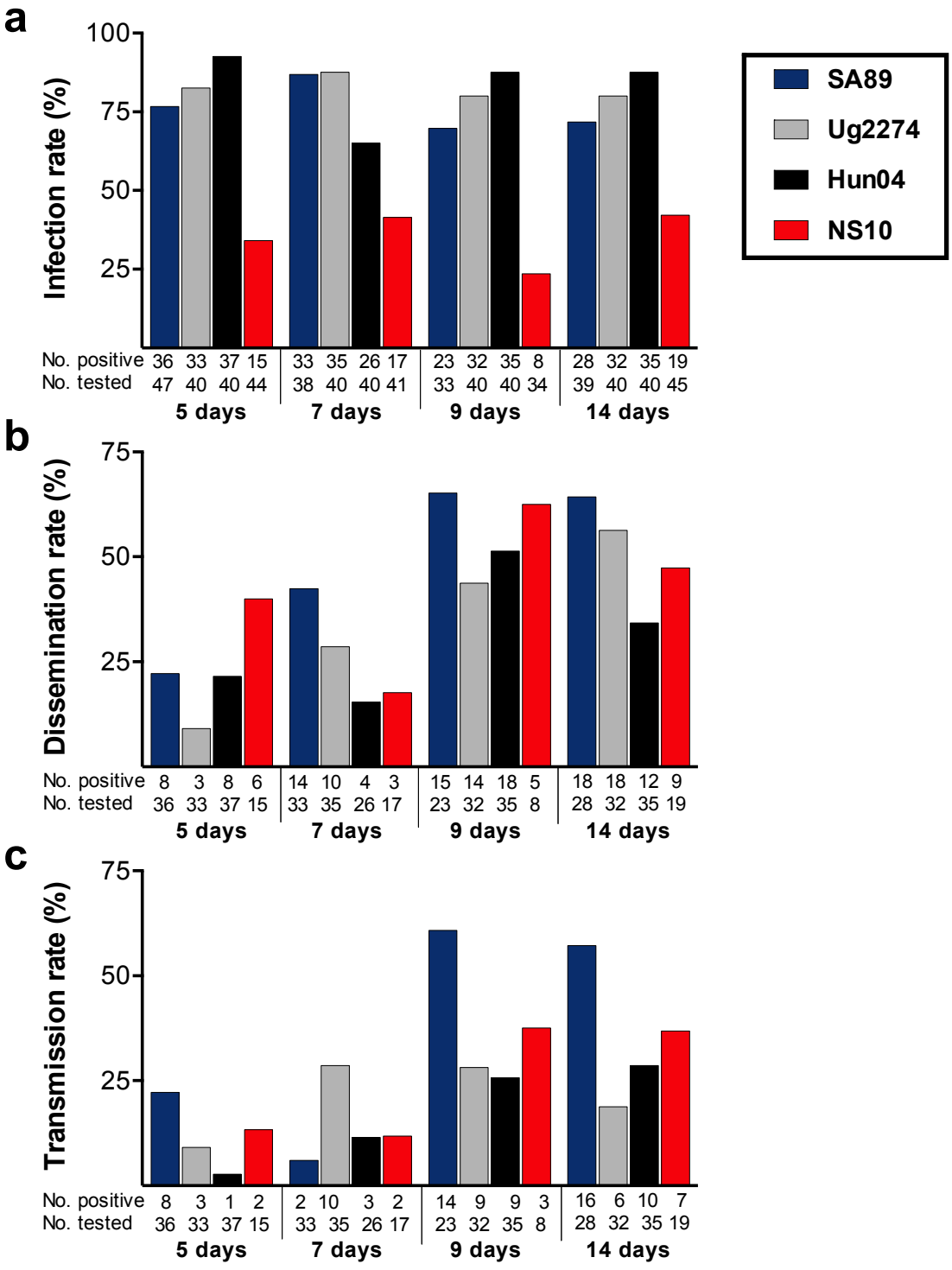

Figure 3.1 Vector competence of *Culex pipiens* for L2 WNV

Mosquitoes were per orally exposed to 8 $\log_{10}$ PFU/ml of SA89, Ugan09, HUN04 or NS10. a) Infection rate is number of WNV positive bodies over WNV exposed mosquitoes. b) Dissemination rate is number of WNV positive legs over positive bodies. c) Transmission rate is number of WNV positive saliva over positive bodies.

Table 3.1. Summary of vector competence of *Culex pipiens*.
Proportions are calculated two different ways: a) proportions calculated with number of WNV exposed mosquitoes as denominator. b) Proportions calculated with number of WNV exposed mosquitoes as denominator for infection. Denominator for Dissemination and Transmission is number of WNV positive bodies.

			5			7			9			14		
Mosquito group	**Proportions calculations**	**L2 strains**	**Infct.**	**Dissm.**	**Trans.**	**Infct.**	**Dissm.**	**Trans.**	**Infct.**	**Dissm.**	**Trans.**	**Infct.**	**Dissm.**	**Trans.**
Culex pipiens		SA89	76.5% (36/47)	17 % (8/47)	17% (8/47)	86.8% (33/38)	36.8% (14/38)	5.2% (2/38)	69.7% (23/33)	45.5% (15/33)	42.4% (14/33)	71.8% (28/39)	46.2%(18/39)	41% (16/39)
8 $\log_{10}$ (PFU/ml) blood meal titer	Infection, dissemination, transmission: (denominator is WNV exposed mosquitoes)	UG2274	92.5% (37/40)	20% (8/40)	2.5% (1/40)	65% (26/40)	10% (4/40)	7.5% (3/40)	87.5% (35/40)	45% (18/40)	22.5% (9/40)	87.5% (35/40)	30% (12/40)	15% (6/40)
		HUN04	82.5% (33/40)	7.5% (3/40)	7.5% (3/40)	87.5% (35/40)	25% (10/40)	25% (10/40)	80% (32/40)	35% (14/40)	22.5% (9/40)	80% (32/40)	45% (18/40)	25% (10/40)
		NS10	34.1% (15/44)	13.6% (6/44)	4.5% (2/44)	41.5% (17/41)	7.3% (3/41)	4.8% (2/41)	23.5% (8/34)	14.7% (5/34)	8.8% (3/34)	42.2% (19/45)	20% (9/45)	15.5% (7/45)
	Infection: (denominator is WNV exposed mosquitoes) Dissemination and transmission: (denominator is WNV positive bodies)	SA89	76.5% (36/47)	22.3% (8/36)	22.2% (8/36)	86.8% (33/38)	42.4% (14/33)	6.1% (2/33)	69.7% (23/33)	65.2% (15/23)	60.9% (14/23)	71.8% (28/39)	64.3% (18/28)	57.1% (16/28)
		UG2274	92.5% (37/40)	21.6% (8/37)	2.7% (1/37)	65% (26/40)	15.4% (4/26)	11.5% (3/26)	87.5% (35/40)	51.4% (18/35)	25.7% (9/35)	87.5% (35/40)	34.3% (12/35)	18.8% (6/32)
		HUN04	82.5% (33/40)	9.1% (3/33)	9.1% (3/33)	87.5% (35/40)	28.6% (10/35)	28.6% (10/35)	80% (32/40)	43.8% (13/32)	28.1% (9/32)	80% (32/40)	56.3% (18/32)	28.6% (10/35)
		NS10	34.1% (15/44)	40% (6/15)	13.3% (2/15)	41.5% (17/41)	17.6% (3/17)	11/7% (2/17)	23.5% (8/34)	62.5% (5/8)	37.5% (3/8)	42.2% (19/45)	47.4% (9/19)	36.8% (7/19)

Table 3.2 Odds ratios and 95% CI for *Culex pipiens* [8 log10 (PFU/ml)]
OR with 95% CI not containing 1.0 are bolded, indicating strength of significance

Time	Virus Comparison	Infection		Dissmienation		Transmission	
		OR	95% CI	OR	95% CI	OR	95% CI
5	Hun04 - SA89	3.77	(0.46, 30.74)	1.22	(0.23, 6.54)	0.13	(0, 3.34)
	Hun04 - Ug2274	2.62	(0.29, 23.97)	3.08	(0.35, 27.28)	0.32	(0.01, 11.17)
	Hun04 - NS10	**23.84**	**(3.04,187.12)**	1.58	(0.26, 9.51)	0.54	(0.01, 23.34)
	SA89 - Ug2274	0.69	(0.13, 3.57)	2.53	(0.29, 22.12)	2.53	(0.29, 22.04)
	SA89 - NS10	**6.33**	**(1.53, 26.23)**	1.3	(0.22, 7.69)	4.31	(0.36, 51.79)
	Ug2274 - NS10	**9.11**	**(1.86, 44.62)**	0.51	(0.05, 4.9)	1.7	(0.1, 29.36)
7	Hun04 - SA89	0.28	(0.05, 1.65)	0.19	(0.03, 1.27)	0.23	(0.03, 1.92)
	Hun04 - Ug2274	0.27	(0.05, 1.55)	0.33	(0.05, 2.33)	0.38	(0.04, 3.49)
	Hun04 - NS10	2.62	(0.65, 10.54)	1.41	(0.12, 15.85)	1.58	(0.09, 27.34)
	SA89 - Ug2274	0.94	(0.12, 7.36)	1.75	(0.39, 7.89)	1.68	(0.31, 9.06)
	SA89 - NS10	**9.32**	**(1.63, 53.31)**	7.39	(0.92, 59.47)	6.96	(0.59, 81.72)
	Ug2274 - NS10	**9.88**	**(1.74, 56.28)**	4.22	(0.5, 35.52)	4.14	(0.33, 51.99)
9	Hun04 - SA89	3.04	(0.48, 19.35)	0.98	(0.23, 4.12)	0.39	(0.08, 1.89)
	Hun04 - Ug2274	1.75	(0.27, 11.48)	1.52	(0.38, 6.12)	1	(0.2, 5.06)
	Hun04 - NS10	**22.75**	**(3.41,151.92)**	4.75	(0.82, 27.5)	3	(0.35, 26.02)
	SA89 - Ug2274	0.57	(0.11, 3.02)	1.55	(0.36, 6.67)	2.54	(0.53, 12.15)
	SA89 - NS10	**7.48**	**(1.39, 40.11)**	4.83	(0.79, 29.58)	7.61	(0.91, 63.36)
	Ug2274 - NS10	**13**	**(2.34, 72.22)**	3.12	(0.53, 18.52)	3	(0.35, 26.02)
14	Hun04 - SA89	2.75	(0.45, 16.76)	0.56	(0.14, 2.31)	0.48	(0.11, 2.11)
	Hun04 - Ug2274	1.75	(0.27, 11.48)	0.59	(0.14, 2.4)	1.89	(0.33, 10.74)
	Hun04 - NS10	**9.58**	**(1.72, 53.21)**	1.93	(0.42, 8.85)	1.81	(0.34, 9.57)
	SA89 - Ug2274	0.64	(0.13, 3.19)	1.05	(0.27, 4.13)	3.94	(0.75, 20.82)
	SA89 - NS10	3.48	(0.85, 14.34)	3.43	(0.77, 15.25)	3.78	(0.77, 18.49)
	Ug2274 - NS10	**5.47**	**(1.21, 24.73)**	3.27	(0.74, 14.46)	0.96	(0.15, 5.97)

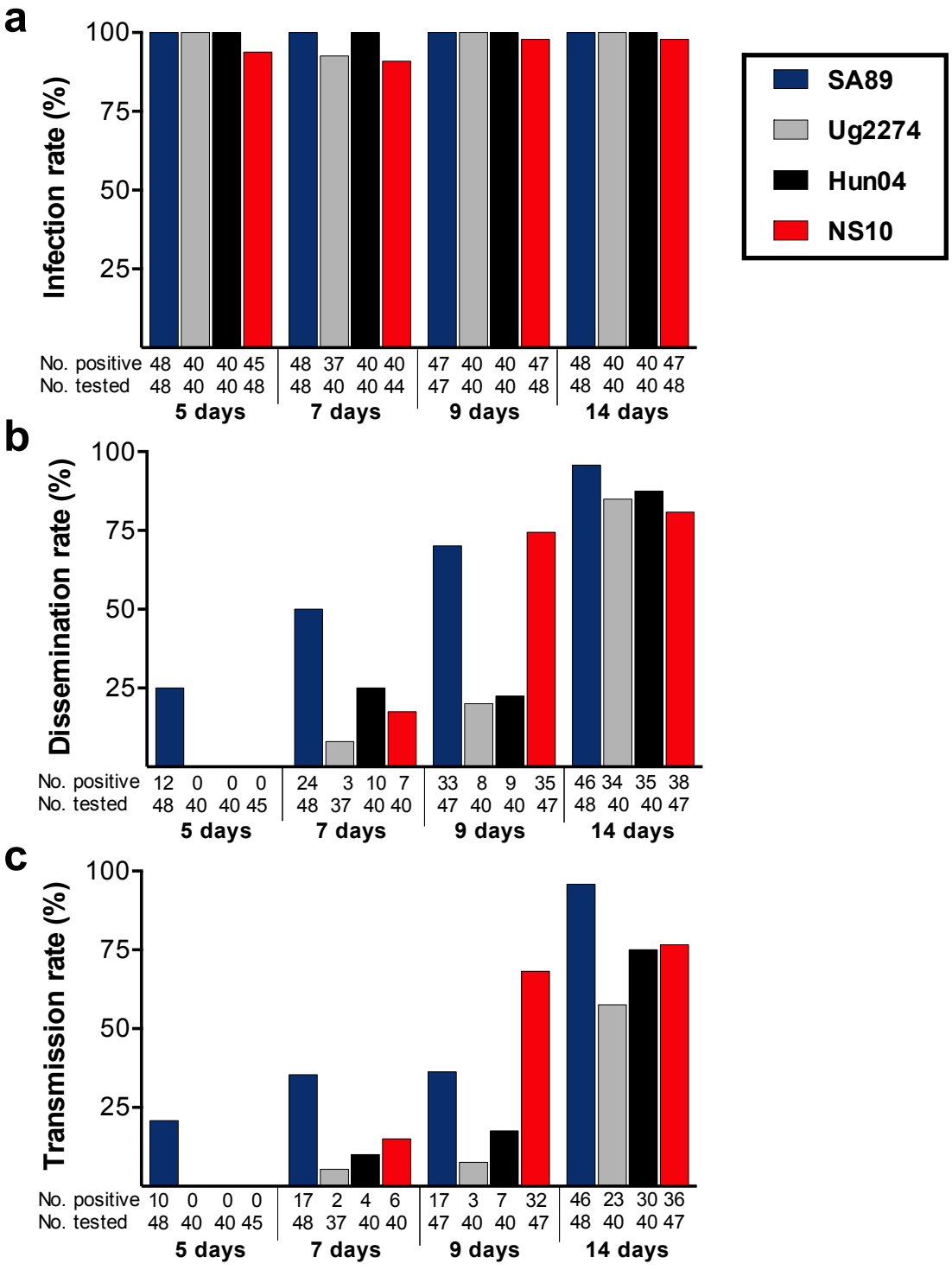

Figure 3.2 Vector competence of *Culex quinquefasciatus* for L2 WNV

Mosquitoes were per orally exposed to 8 $\log_{10}$ PFU/ml of SA89, Ugan09, HUN04 or NS10. a) Infection rate is number of WNV positive bodies over WNV exposed mosquitoes. b) Dissemination rate is number of WNV positive legs over positive bodies. c) Transmission rate is number of WNV positive saliva over positive bodies.

Table 3.3 Odds ratios and 95% CI for *Culex quinquefasciatus* [8 log10 (PFU/ml)]
OR with 95% CI not containing 1.0 are bolded, indicating strength of significance

Time	Virus Comparison	Dissmienation		Transmission	
		OR	95% CI	OR	95% CI
5	Hun04 - SA89	-	-	-	-
	Hun04 - Ug2274	-	-	-	-
	Hun04 - NS10	-	-	-	-
	SA89 - Ug2274	-	-	-	-
	SA89 - NS10	-	-	-	-
	Ug2274 - NS10	-	-	-	-
7	Hun04 - SA89	0.33	(0.08 , 1.31)	0.2	(0.03 , 1.20)
	Hun04 - Ug2274	4.11	(0.52 , 32.39)	2.11	(0.15 , 29.18)
	Hun04 - NS10	1.76	(0.35 , 8.88)	0.7	(0.09 , 5.25)
	SA89 - Ug2274	**12.33**	**(1.74 , 87.28)**	10.42	(1.04 , 104.05)
	SA89 - NS10	**5.29**	**(1.21 , 23.18)**	3.47	(0.73 , 16.54)
	Ug2274 - NS10	0.43	(0.05 , 3.64)	0.33	(0.03 , 4.00)
9	Hun04 - SA89	0.12	(0.03 , 0.53)	0.37	(0.08 , 1.69)
	Hun04 - Ug2274	1.16	(0.23 , 5.8)	2.62	(0.31 , 22.22)
	Hun04 - NS10	0.11	(0.02 , 0.47)	0.11	(0.02 , 0.48)
	SA89 - Ug2274	**9.43**	**(2.12 , 41.92)**	6.99	(0.97 , 50.13)
	SA89 - NS10	0.88	(0.23 , 3.33)	0.28	(0.08 , 1.00)
	Ug2274 - NS10	0.09	(0.02 , 0.42)	0.04	(0.01 , 0.29)
14	Hun04 - SA89	0.3	(0.02 , 3.88)	0.13	(0.01 , 1.40)
	Hun04 - Ug2274	1.24	(0.18 , 8.38)	2.22	(0.54 , 9.19)
	Hun04 - NS10	1.62	(0.27 , 9.54)	1	(0.24 , 4.25)
	SA89 - Ug2274	4.06	(0.34 , 48.88)	**17**	**(1.68 , 171.83)**
	SA89 - NS10	5.31	(0.49 , 57.55)	7.67	(0.75 , 78.76)
	Ug2274 - NS10	1.31	(0.24 , 7.12)	0.45	(0.12 , 1.74)

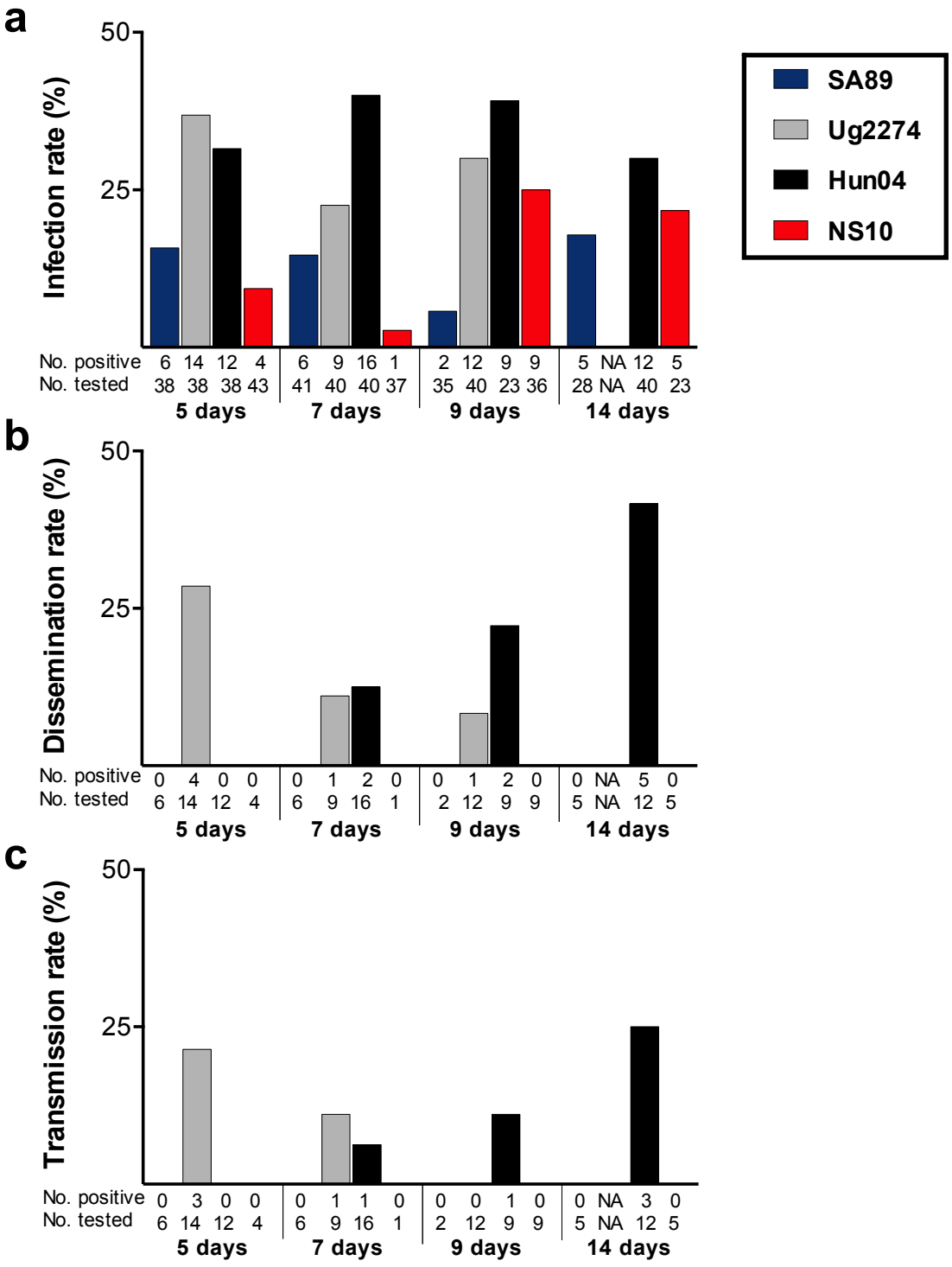

Figure 3.3 Vector competence of *Culex quinquefasciatus* for L2 WNV

Mosquitoes were per orally exposed to 7 $\log_{10}$ (PFU/ml) of SA89, Ugan09, HUN04 or NS10. a) Infection rate is number of WNV positive bodies over WNV exposed mosquitoes. b) Dissemination rate is number of WNV positive legs over positive bodies. c) Transmission rate is number of WNV positive saliva over positive bodies.

Table 3.4. Summary of vector competence of *Culex quinquefasciatus*.
Proportions are calculated two different ways: a) proportions calculated with number of WNV exposed mosquitoes as denominator. b) Proportions calculated with number of WNV exposed mosquitoes as denominator for infection. Denominator for Dissemination and Transmission is number of WNV positive bodies.

			5			7			9			14		
Mosquito group	**Proportions calculations**	**L2 strains**	**Infct.**	**Dissm.**	**Trans.**	**Infct.**	**Dissm.**	**Trans.**	**Infct.**	**Dissm.**	**Trans.**	**Infct.**	**Dissm.**	**Trans.**
Culex quinquefasciatus 8 $\log_{10}$ (PFU/ml) blood meal titer	Infection, dissemination, transmission: (denominator is WNV exposed mosquitoes)	SA89	100% (48/48)	25% (12/48)	20.8% (10/48)	100% (48/48)	50% (24/48)	35.4% (17/48)	100% (47/47)	70.2% (33/47)	36.1% (17/47)	100% (48/48)	95.8% (46/48)	95.8% (46/48)
		UG2274	100% (40/40)	0% (0/40)	0% (0/40)	100% (40/40)	25% (10/40)	10% (4/40)	100% (40/40)	22.5% (9/40)	17.5% (7/40)	100% (40/40)	87.5% (35/40)	75% (30/40)
		HUN04	100% (40/40)	0% (0/40)	0% (0/40)	92.5% (37/40)	7.5% (3/40)	5% (2/40)	100% (40/40)	20% (8/40)	7.5% (3/40)	100% (40/40)	85% (34/40)	57.5% (23/40)
		NS10	93.8% (45/48)	0% (0/48)	0% (0/48)	90.9% (40/44)	15.9% (7/44)	13.6% (6/44)	97.9% (47/48)	72.9% (35/48)	66.7% (32/48)	97.9% (47/48)	79.1% (38/48)	75% (36/48)
	Infection: (denominator is WNV exposed mosquitoes) Dissemination and transmission: (denominator is WNV positive bodies)	SA89	100% (48/48)	25% (12/48)	20.8% (10/48)	100% (48/48)	50% (24/48)	35.4% (17/48)	100% (47/47)	70.2% (33/47)	36.1% (17/47)	100% (48/48)	95.8% (46/48)	95.8% (46/48)
		UG2274	100% (40/40)	0% (0/40)	0% (0/40)	100% (40/40)	25% (10/40)	10% (4/40)	100% (40/40)	22.5% (9/40)	17.5% (7/40)	100% (40/40)	87.5% (35/40)	75% (30/40)
		HUN04	100% (40/40)	0% (0/40)	0% (0/40)	92.5% (37/40)	8.1% (3/37)	5.4% (2/37)	100% (40/40)	20% (8/40)	7.5% (3/40)	100% (40/40)	85% (34/40)	57.5% (23/40)
		NS10	93.8% (45/48)	0% (0/45)	0% (0/45)	90.9% (40/44)	17.5% (7/40)	15% (6/40)	97.9% (47/48)	74.5% (35/47)	68.1% (32/47)	97.9% (47/48)	80.9% (38/47)	76.6% (36/47)
Culex quinquefasciatus 7 $\log_{10}$ (PFU/ml) blood meal titer	Infection, dissemination, transmission: (denominator is WNV exposed mosquitoes)	SA89	15.8% (6/38)	0% (0/38)	0% (0/38)	14.6% (6/41)	0% (0/41)	0% (0/41)	5.7% (2/35)	0% (0/35)	0% (0/35)	17.9% (5/28)	0% (0/28)	0% (0/28)
		UG2274	31.6% (12/38)	0% (0/38)	0% (0/38)	40% (16/40)	5% (2/40)	2.5% (1/40)	39.1% (9/23)	8.7% (2/23)	4.3% (1/23)	30% (12/40)	12.5% (5/40)	7.5% (3/40)
		HUN04	36.8% (14/38)	10.5% (4/38)	7.9% (3/38)	22.5% (9/40)	2.5% (1/40)	2.5% (1/40)	30% (12/40)	2.5% (1/40)	0% (0/40)	NA	NA	NA
		NS10	9.3% (4/43)	0% (0/43)	0% (0/43)	2.7% (1/37)	0% (0/37)	0% (0/37)	25% (9/36)	0% (0/36)	0% (0/36	21.7% (5/23)	0% (0/23)	0% (0/23)
	Infection: (denominator is WNV exposed mosquitoes) Dissemination and transmission: (denominator is WNV positive bodies)	SA89	15.8% (6/38)	0% (0/6)	0% (0/6)	14.6% (6/41)	0% (0/6)	0% (0/6)	5.7% (2/35)	0% (0/2)	0% (0/2)	17.9% (5/28)	0% (0/5)	0% (0/5)
		UG2274	316.% (12/38)	0% (0/12)	0% (0/12)	40% (16/40)	12.5% (2/16)	6.25% (1/16)	39.1% (9/23)	8.3% (1/12)	11.1% (1/9)	30% (12/40)	41.7% (5/12)	25% (3/12)
		HUN04	36.8% (14/38)	28.56% (4/14)	21.4% (3/14)	22.5% (9/40)	11.1% (1/9)	11.1% (1/9)	30% (12/40)	22.2% (2/9)	0% (0/12)	NA	NA	NA
		NS10	9.3% (4/43)	0% (0/4)	0% (0/4)	2.7% (1/37)	0% (0/1)	0% (0/1)	25% (9/36)	0% (0/9)	0% (0/9)	21.7% (5/23)	0% (0/5)	0% (0/5)

between the groups (Fig. 3.2a). SA89 was the only L2 WNV strain that demonstrated evidence of dissemination (25%) and transmission (20%) on 5 dpe (Fig 3.2b, 3.2c). However, dissemination and transmission was evident for all strains by 7 dpe. The likelihood for dissemination infections for SA89 was 12.33 (95% CI: 1.74, 87.28) and 5.29 times greater (95% CI: 1.21, 23.18) than UG2274 and NS10, respectively. At 9 dpe, SA89 was 9.43 (95% CI: 2.12, 41.92) times more likely to show evidence of dissemination infections than UG2274. Likewise, SA89 was 10.4 (95% CI: 1.04, 104.05) more likely to transmit WNV at 7 dpe and 17 times more likely (95% CI: 1.68, 171.83) at 14 dpe than UG2274. Odds ratios and corresponding 95% CI for L2 WNV exposed *Culex quinquefasciatus* mosquitoes are presented in Table 3.3.

Previous results demonstrated that the four L2 WNV strains exhibited equal infection rates at all time points. To assess if differences in infection could be observed for *Culex quinquefasciatus* mosquitoes exposed to a lower viral dose in a blood meal, age-matched mosquitoes were per orally exposed to 7 $\log_{10}$ (PFU/ml) and vector competence similarly assessed (Fig. 3.3a, 3.3b, 3.3c). Vector competence proportions calculated with the denominators as either the number of WNV positive bodies or the number of WNV exposed mosquitoes is summarized in Table 3.4. Infection (number of positive bodies by number of WNV exposed mosquitoes), dissemination (number of positive legs by number of WNV positive bodies), and transmission rates (number of positive saliva by number of WNV positive bodies) are displayed in Fig32.3.

Expectedly, lower infection rates were observed for each of the 4 L2 WNV strains. The HUN04 L2 WNV isolate infected the greatest proportion of mosquitoes at 7, 9 and 14 dpe but was only determined to be significantly greater compared to UG2274 at 5 dpe (27% larger proportion, 99% CI 0.031, 0.521). Dissemination and transmission were sporadically observed for some viruses, yet statistical significance could not be addressed due to the lack of observable positive values.

DISCUSSION

A previous study comparing the vector competence of European and North American *Culex pipiens* for NS10 found North American mosquitoes to be poorly competent for L2 WNV transmission(Fros *et al.*, 2015). However, in the study described here, I have demonstrated North American *Culex pipiens* and *Culex quinquefasciatus* mosquitoes to efficiently transmit multiple L2 WNV isolates and that the likelihood and efficiency of transmission continues to increase over time for all viruses. Comparable infection and dissemination proportions have been observed for *Culex quinquefasciatus* (Florida) mosquitoes similarly exposed to L1 WNV isolates(Richards *et al.*, 2014). However, a higher rate of infection (91%) was observed for *Culex quinquefasciatus* (Sebring) per orally exposed to 7 $\log_{10}$ (PFU/ml) L1 WNV at similar conditions to those in this study. Variation in the response of different mosquito vectors to WNV infection is expected and taken together these results demonstrate that L2 WNV transmission can be supported by North American *Culex* species, thereby expanding the potential range of L2 WNV enzootic transmission.

Previous studies have demonstrated that increases in temperature decrease the time to transmission for *Culex pipiens*, *Culex tarsalis*, and *Culex univittatus* mosquitoes exposed to L1 WNV ((CORNEL *et al.*, 1993; Dohm *et al.*, 2002; Reisen *et al.*, 2006b). The WN02 L1 WNV strain is believed to have displaced the originally introduced genotype (NY99) in North America due, in part, to a transmission advantage over NY99 in the presence of increased transmission(Kilpatrick *et al.*, 2008). The WN02 strain differed from the NY99 strain by the presence of one non-synonymous change (U1442C in the E gene) and two synonymous changes (C2466U in the E gene, C9352U in the NS5 gene)(Davis *et al.*, 2005). Variation at these sites is present in the genetic backbone of SA89 (1442T, 2446T) NS10 (1442U, 2446U), UG2274 (1442T, 2446T), and HUN04 (1442U, 2446U). Curiously, the nucleotide identity at these sites is matched between geographically associated L2 WNV strains. The exact role of nucleotide variations between geographically dissimilar L2 WNV strains in unknown. However,

localized increases in temperature were noted in Greece in 2011, 2012 and 2013 and it is believed that warmer temperatures may have facilitated increases in enzootic transmission for NS10(Fros *et al.*, 2015).

In *Culex quinquefasciatus* mosquitoes, the SA89 strain demonstrated the greatest dissemination and transmission efficiencies. The presence of *Culex quinquefasciatus* mosquitoes in South Africa(Jupp, 2001), where SA89 was isolated, could reflect a strong geographic association between the virus and a primary mosquito vector and could explain the high vector competence demonstrated for this species for the SA89 strain.

Curiously, in this experimental setup, the NS10 was the only L2 WNV that demonstrated a strong reduction in infection in *Culex pipiens* mosquitoes, the principle enzootic vector for WNV in Europe. NS10 was the first sequenced L2 strain that demonstrated an NS3-H249P substitution and similarly the only L2 isolate to be associated with a large WNV outbreak. Variable amino acid identities (Ala, Asn, His, Thr, Pro) have occupied the NS3-249 locus across lineages 1-4 and this site has also been demonstrated to modulate avian specific phenotypes, with the NS3-249Pro substitution eliciting the greatest virogenesis in AMCRs (Langevin et al., 2014). Studies from Chapter 2, aim 1 demonstrate the same pathogenic NS3-249P related associations in AMCRs for NS10. A previous study demonstrated that a reduced proportion of infected bodies were observed for North American *Culex pipiens* compared to *Culex pipiens* from the Netherlands per orally exposed to NS10(Fros *et al.*, 2015), perhaps indicating that this altered infection phenotype observed for NS10 might be specific to North American *Culex pipiens*. The impaired ability of NS10 to efficiently infect *Culex pipiens* mosquitoes and the correlation of the emergence of the NS3-H249P substitution in the same strain needs to be further examined. The fact that this site strongly modulates virogenesis in AMCRs strengthens the rationale for direct evaluation of the NS3-249 locus in contrasting vertebrate and invertebrate host systems for the potential role in supporting the trade-off hypothesis or other fitness model assessments.

Together, these studies demonstrate that disparate L2 isolates have the potential to elicit distinct transmission phenotypes, indicating that viral genetic changes are occurring for L2 WNV. Furthermore, I established that *Culex pipiens* and *Culex quinquefasciatus* mosquitoes from North America could serve as competent transmission vectors of L2 WNV. These results further highlight the need to maintain proper surveillance and vector control efforts to control and limit further geographic distribution of L2 WNV.

CHAPTER 4: THE NS3-H249P SUBSTITUTION MODULATES DISPARATE PHENOTYPES IN AMCRs AND *CULEX PIPIENS*, EVIDENCE FOR HOST-SPECIFIC ADAPTIVE EVOLUTION

INTRODUCTION

The L2 WNV Greek outbreak in 2010 was the first instance in which WNV resulted in an outbreak of WNV neurologic disease in Greece(Danis *et al.*, 2011). Retrospective serosurveys in Greece identified a 1% seroprevalence rate in humans for neutralizing antibodies against WNV, indicating that a low level of enzootic WNV transmission was taking place prior to the 2010 outbreak. The upsurge in the reported number of human WNV cases during the 2010 outbreak could be due an increase in pathogenicity by the newly introduced L2 genotype and potentially related to emergence of the NS3-H249P mutation(Papa *et al.*, 2011b). However, previous studies have determined the NS3-T249P substitution was sufficient in a L1 WNV backbone to significantly increase viral titers and subsequent mortality in crows , but the same substitution was not found to alter neuroinvasiveness in mice. The effect of the histidine to proline mutation in a L2 WNV backbone on virulence phenotypes in mice has not been directly assessed. Alternatively, the increase of WNV human cases in 2010 could be the result of increased force of transmission between vector and host and a subsequent rise in the frequency of spillover events.

The competency of North American *Culex pipiens* and *Culex quinquefasciatus* mosquitoes for infection, dissemination and transmission of African and European L2 WNV isolates was previously examined (aim 2, Chapter 3). A significantly lower proportion of *Culex pipiens* mosquitoes were found to become infected following *per oral* exposure to NS10 than to the African SA89 L2 WNV strain. The unique presence of the NS3-H249P substitution in the NS10 strain and the finding that all other L2 isolates (HUN04, SA89, UG2274), with a histidine at the NS3-249 locus, demonstrated higher oral infection rates, indicate the potential that this

locus could serve to modulate vector competence in addition to viral growth and virulence in avian hosts.

For this study, I sought to comprehensively examine how the NS3-H249P substitution is directly involved in transmissibility of L2 WNV. Specifically, I assessed the vector competence of *Culex pipiens* mosquitoes for the L2 NS3-H249P mutation in SA89 and NS10 WNVs and compared the resulting data to the peak viremia titer of AMCRs for these same strains. I also assessed whether the newly emergent L2 WNVs (NS10) and the accompanying NS3-H249P mutation substitution could affect neuroinvasive phenotypes in a murine model compared to an older, NS3-249H harboring L2 WNV (SA89).

MATERIALS AND METHODS

Viruses and cells

Low passage infectious cDNA clone-derived virus stocks were used for the vector competence studies and the lethal dose LD_{50} determinations. High titered virus stocks used for the vector competence studies were generated by infecting C6/36 (*Aedes albopictus*) cells at an MOI of 0.1. Stocks for the lethal dose LD_{50} determination in mice and *in vitro* growth curves were prepared on African green monkey kidney cells (Vero cells; ATCC no. CCL-81). After infection, supernatant was collected at 4 dpi and centrifuged at 1400 rpm for 10 minutes for remove cellular debris. Aliquots were prepared and frozen at -80^0C. Vero cell plaque assays, as previously described in Chapter 2, were used to determine titers of stocks (Brault et al., 2004).

Peak viremia titer profiles in AMCRs

Peak viremia titers for AMCRs previously exposed (aim 1, chapter 2) to SA89-IC, SA89 NS3-H249P, NS10-IC and NS10-P249H were determined again in this study to directly contrast with the phenotype of the same strains tested in birds and mice.

In vitro replication

Triplicate Vero and C6/36 cell cultures were used to assess the growth capacity of SA89-IC, SA89 NS3-H249P, NS10-IC or NS10 NS3-P249H. The NY99 genotype was included as a reference strain. All viruses were inoculated in triplicate onto confluent monolayers of the aforementioned cells at an MOI of 0.1. Inocula were absorbed on Vero cells at 37^0C or C6/36 cells at 27^0C for one hour. After absorption, medium was removed and cells washed three times with PBS. Following the cell washes, 3 ml of fresh cell culture medium (DMEM, 10% FBS and 5% Pen/Strep) was added to each well. To assess the quantity of residual virus present from the initial inocula, a 50 µl aliquot was taken immediately following addition of fresh medium [0 hours post inoculation (hpi)] and diluted 1:10 in cell culture medium. At 24 hour intervals, an additional 50 µl of supernatant was removed, diluted 1:10 in medium and frozen at -80^0C for later titration. Viral titers were quantified by plaque assay on Vero cells.

Mosquitoes

The mosquitoes used in this study were derived from laboratory maintained colonies. The *Culex pipiens* (Chicago) mosquitoes were originally collected as egg rafts in Chicago, Illinois in 2010. The colony was maintained on goose blood for egg laying (Colorado Serum Company, Denver, CO). Colonies were provided with 10% sucrose *ad libitum* and maintained in a 70% relative humidity chamber at 28°C. Mosquitoes were also maintained on a 16:8 hr light: dark cycle.

Vector competence studies

For vector competence studies, 3-5 day old female *Culex pipiens* mosquitoes were deprived of sucrose and water 24 hours or 8 hours prior to feeding, respectively. Previously frozen, titrated virus stocks were thawed and diluted with defibrinated goose blood (Colorado Serum Company, Denver, CO) to a final titer of 8.0 $\log_{10}$ PFU/ml. A portion of the blood meal

was stored at -80°C for confirmation of viral titer by plaque assay. A Hemotek membrane feeding apparatus (Discovery Workshops, Accrington, UK) was used to present infectious blood meals for 0.5-1 hour to mosquitoes. Fully engorged females were sorted by cold anesthesia and held in 1-pint cartons. Females were provided with 10% sucrose *ad libitum*, held at 27°C with a 16:8 hr L:D photoperiod and kept at 70-80% humidity through 14 days post feeding (dpf). At 14 dpf, mosquitoes were anesthetized by triethylamine (Flynap; Carolina Biological Supply Company, Burlington, NC) exposure. Two legs were removed from each mosquito and placed into 500-µl mosquito diluent [DMEM with 10% FBS, 5% penicillin/streptomycin (10 µg/ml) and 1% Fungizone (2.5 µg/ml)]. Capillary tubes pre-charged with Type B immersion oil (Cargille Labs, Cedar Grove, NJ) were used to salivate mosquitoes for 30 minutes. The contents of capillary tubes were collected in 200-µl mosquito diluent. Following salivation, mosquito bodies were placed in 1 ml mosquito diluent. The body, leg and saliva samples were stored at -80°C. Mosquito bodies and legs were homogenized in a mixer mill with the addition of 2 copper-coated steel shot BBs (4.5mm diameter, 0.177" caliber) (Qiagen) and clarified by centrifugation at 10,000 rpm for 10 minutes. Each body or leg sample was triturated for 2 minutes in a 2 ml round bottom polypropylene tube. A neat (undiluted) sample was added to a well of a 6-well plate and plaque assay performed as previously described for the assaying of individual bodies, legs and saliva samples for identification of the presence of infectious virus. Briefly, body or leg mosquito homogenate supernatants and saliva suspensions were adsorbed on Vero cells for 1 hour at 37^0C and an 0.8% agarose nutrient overlay was added. After 2 days, a second 0.8% nutrient overlay with 0.005% neutral red was added. Evidence of plaques was recorded the following day. Infection rates were determined as the proportion of positive bodies from tested mosquitoes (Fig. 4.3a), dissemination (Fig 4.3b) was calculated as the proportion of positive legs from positive bodies and transmission (Fig 4.3c) was determined as the proportion of positive salvia sample from positive bodies. The total transmission rate was calculated as proportion of mosquitoes with a positive saliva samples as a function of the number of fully

engorged females (Fig 4.3d). Proportions were calculated two different ways: a) proportions calculated with number of WNV exposed mosquitoes as denominator. b) Proportions calculated with number of WNV exposed mosquitoes as denominator for infection. The denominator for dissemination and transmission was calculated using the number of WNV positive bodies. A summary of the proportions is outlined in Table 4.1.

LD_{50} determination

Outbred, three week old CD-1/ICR mice (Charles River Laboratories) were allowed to acclimate to housing conditions for one week before experimental manipulations were performed. For LD_{50} determinations, CD-1/ICR mice were intraperitoneally inoculated with serial 10-fold dilutions of virus in 0.1 mL inoculum containing PBS with a PBS sham inoculum serving as an inoculation control. Doses of 0.1 PFU, 1.0, 10 and 100 PFU of SA89, SA89 NS3-H249P, NS10 or NS10 NS3-P249H were inoculated intraperitoneally in mice. Five mice were used per dose such that 20 mice total were tested for each virus. Five mice were used for PBS-only control group. Viral inocula were back titrated the same day (without freeze-thaw) to confirm doses. Mice were observed for clinical signs of morbidity and any mouse that demonstrated severe signs of disease was euthanized by isoflurane anesthesia followed by cervical dislocation. At 28dpi, surviving mice were isoflurane anesthetized, bled by cardiac puncture and euthanized as described above. The 50% lethal dose endpoint (LD_{50}) was determined using a probit analysis. The use of mice in this study was approved by the Division of Vector-borne Diseases, Centers for Disease Control and Prevention Institutional Animal Care and Use Committee (protocol #12-3871A**)**. All manipulations were performed in biosafety level 3 facilities.

Statistics

For studies assessing the vector competence of *Culex pipiens* mosquitoes for L2 WNV strains, a chi-squared analysis was used to compare proportions of infection, dissemination,

transmission or total transmission between SA89-IC and SA89 NS3-H24P or NS10-IC and NS10 NS3-P249H. An analysis of variance (ANOVA) was used to compare *in vitro* replication studies. Multiple comparisons were made using Tukey's HSD adjustment. A probit analysis was used to calculate LD_{50} values and differences between the calculated values were statistically analyzed by nonlinear regression (a curve comparison). The GraphPad Prism 6.0 (GraphPad San Diego, CA) software program was used to perform all statistically calculations.

RESULTS

Growth of L2 WNV in cell culture.

To assess the relative *in vitro* growth characteristics of the recombinant NS3-249 mutant and parental viruses, viral titers in supernatants of Vero and C6/36 cells were determined following inoculation with SA89-IC, SA89 NS3-H249P, NS10-IC, and NS10 NS3-P249H clone-derived viruses. Titers from these samples were compared to NY99 genotype (L1 WNV) as a reference (Fig. 4.1). No differences in growth curves were observed between any L2 viruses at any time point in the mammalian (Vero) cell line. Additionally, no differences were observed between L2 viruses and the reference L1 strain, indicating that viral growth between L1 and L2 was indistinguishable in mammalian cells. In mosquito (*Aedes albopictus*, C6/36) cells, there were no consistent differences between any of the L2 WNV strains or the reference L1 WNV and all viruses reached similar peak titers by 4 and 5 dpi. Together, these results indicate there are no consistent *in vitro* growth differences between SA89, NS10 and the corresponding NS3-249 mutants (SA89 NS3-H249P and NS10 NS3-P249H) in mammalian (Vero) or mosquito (C6/36) cells.

SA89, NS10, and derived NS3-249 mutants in AMCRs

In order to evaluate the effect of the L2 WNV histidine to proline substitution on avian amplification as a marker for enzootic transmission competence, the peak viremia titers of

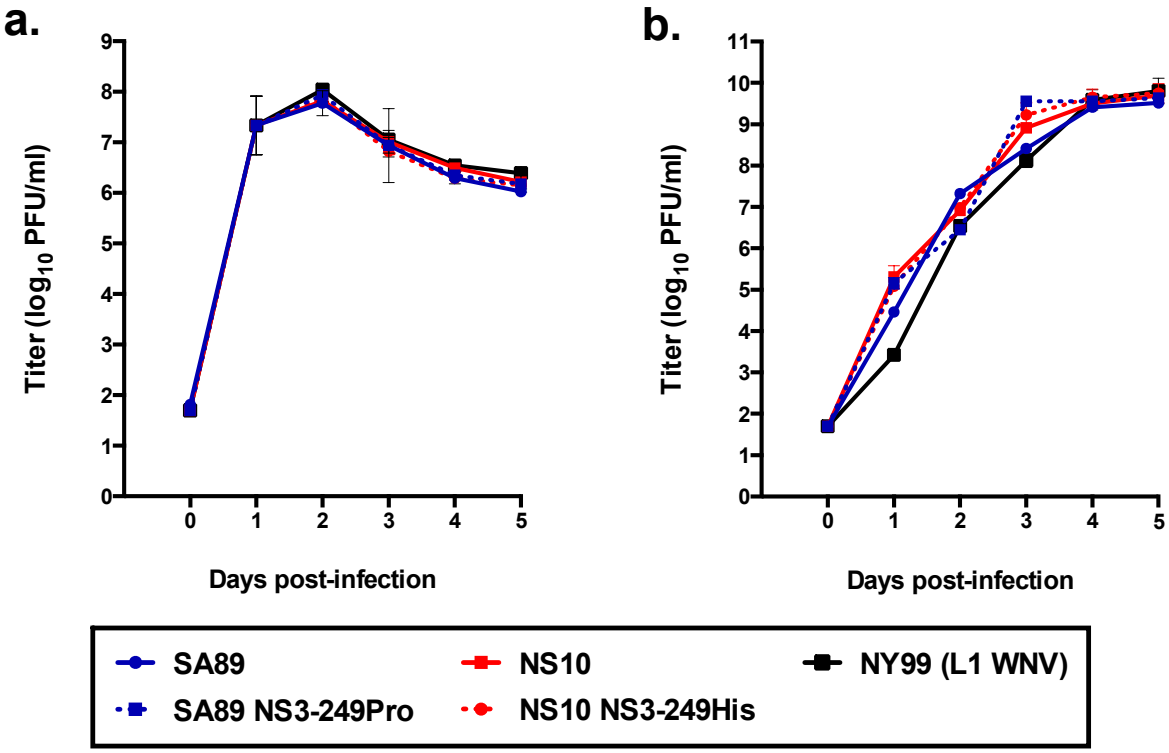

Figure 4.1. Replication efficiency of L2 isolates compared to reference L1 strain.

Monolayers of a) Vero cells at 37°C or b) C6/36 cells at 27°C were inoculated with recombinant WNV L2 viruses or with the NY99 L1 parental WNV strain at an MOI of 0.1. Samples of supernatant were taken through 5 days post infection and titers determined by plaque assay on Vero cells.

AMCRs experimentally inoculated with SA89, SA89 NS3-H249P, NS10 or NS10-H249P (previously performed in aim 1, chapter 2) were directly compared (Fig. 4.2). For SA89 and SA89 NS3-H249P, viremia titers peaked on 5 dpi and dpi 6, respectively.

The mean peak viremia titer for SA89 NS3-H249P was 9.4 ± 0.6 $\log_{10}$ (PFU/ml sera) and seemingly higher compared to parental SA89 [7.5 ± 2.1 $\log_{10}$ (PFU/ml sera)], but these differences were not statistically significant (p=0.07). However, increases in viremia titers for SA89 NS3-H249P compared SA89 were observed, but only at later time points (1,000 fold at 6 dpi with p=0.0006; 10,000 fold at 7 dpi with p=0.0001) (not shown in Fig. 4.2 but demonstrated in Fig. 2.6). The mean peak viremia titers in AMCRs inoculated with the NS10-IC and NS10 NS3-P249H clone-derived viruses were observed on 5 dpi, with a significantly lower viremia titer demonstrated by the NS10 NS3-P249H virus (6.5 ± 0.5 $\log_{10}$ PFU/ml sera) compared to parental NS10 (9.7 ± 0.6 $\log_{10}$ PFU/ml sera) virus. These results indicate that, in AMCRs, the presence of a proline at the NS3-249 locus results in higher viral growth regardless of the L2 WNV backbone I assayed.

Vector competence

Previous results have also demonstrated that the histidine to proline mutation at the NS3-249 locus in NS10 and SA89 backbones enhances viremia titer levels and increases virulence in AMCRs. To determine whether the NS3-249 locus similarly modulates vector competence phenotypes in mosquitoes, the effect of the histidine to proline substitution at the NS3-249 locus in L2 WNV on vector competence was assessed in *Culex pipiens* mosquitoes (Fig. 4.3 a-d). Vector competence proportions calculated with the denominators as either the number of WNV positive bodies or the number of WNV exposed mosquitoes is summarized in Table 4.1. Infection (number of positive bodies by number of WNV exposed mosquitoes), dissemination

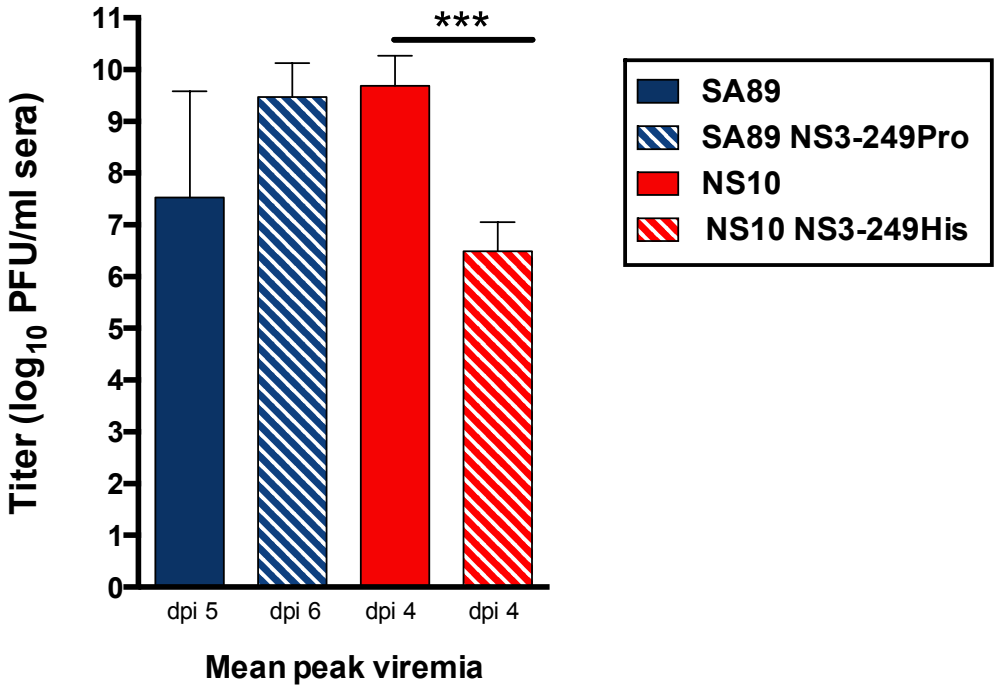

Figure 4.2. Mean peak viremia titers of NS3-249 point mutants in AMCRs.

The peak viremia titers of individual AMCRs inoculated with SA89 and derived NS3-H249P point mutant (n=6) or NS10-IC and NS10 NS3-P249H (n=5) clone-derived viruses. The days on which the mean peak viremia titers occurred for each virus are noted. Peak titers of parental isolates were compared with corresponding NS3-249 point mutants using an ANOVA test.

(number of positive legs by number of WNV positive bodies), transmission rates (number of positive saliva by number of WNV positive bodies), and transmission exposure rates (number of positive saliva by number of WNV exposed mosquitoes) in are displayed in Fig 4.3. A significantly higher proportion of *Culex pipiens* mosquitoes were infected by the NS10 NS3-249H mutant (89%) compared to the NS10 IC clone-derived virus (20%), indicating that the NS3-249 amino acid could modulate infection of the midgut epithelium ($p< 0.0001$) (Fig. 4.3a). In contrast, at 14 dpi, no statistically observed difference in the infection rates for SA89 and SA89 NS3-H249P were observed ($p=0.5995$) (Fig. 4.3a). No difference in the dissemination or transmission rates was observed for NS10 compared to NS10 NS3-P249H ($p=0.6568$ and $p=0.1017$, respectively) (Fig. 4.3b 4.3c). However, a lower dissemination rate was observed for SA89 NS3-H249P (35%) compared to SA89 (80%) ($p< 0.0001$) (Fig. 4.3b). Similarly, a lower transmission rate was elicited by SA89 NS3-H249P (17%) compared to SA89 (62%) ($p=0.0005$) (Fig. 4.3c).

To examine the theoretical effect of the NS3-H249P on transmission, I compared the number of mosquitoes that were able to transmit WNV to the number of mosquitoes that received an artificial infectious blood meal (Fig. 4.3d). I found that in cases where a proline was present at the NS3-249 locus a significant reduction in transmissibility was observed, The SA89 infected mosquitoes demonstrated 57% transmission rate compared to only 15% transmission by SA89 NS3-H249P ($p= 0.0004$). Similarly, I found that a transmission rate of 15% for NS10 was increased to 39% by the presence of the NS3-P249H mutation ($p=0.0095$), indicating that regardless of the L2 backbone, the NS3-H249P mutation significantly impacts mosquito transmission fitness. Vector competence studies with these four L2 clone-derived viruses have indicated that infection, dissemination, and transmission rates of *Culex pipiens* could be altered by the identity of the amino acid at the NS3-249 locus and that the genetic backbone could alter the effect of the polymorphism on mosquito competence phenotype.

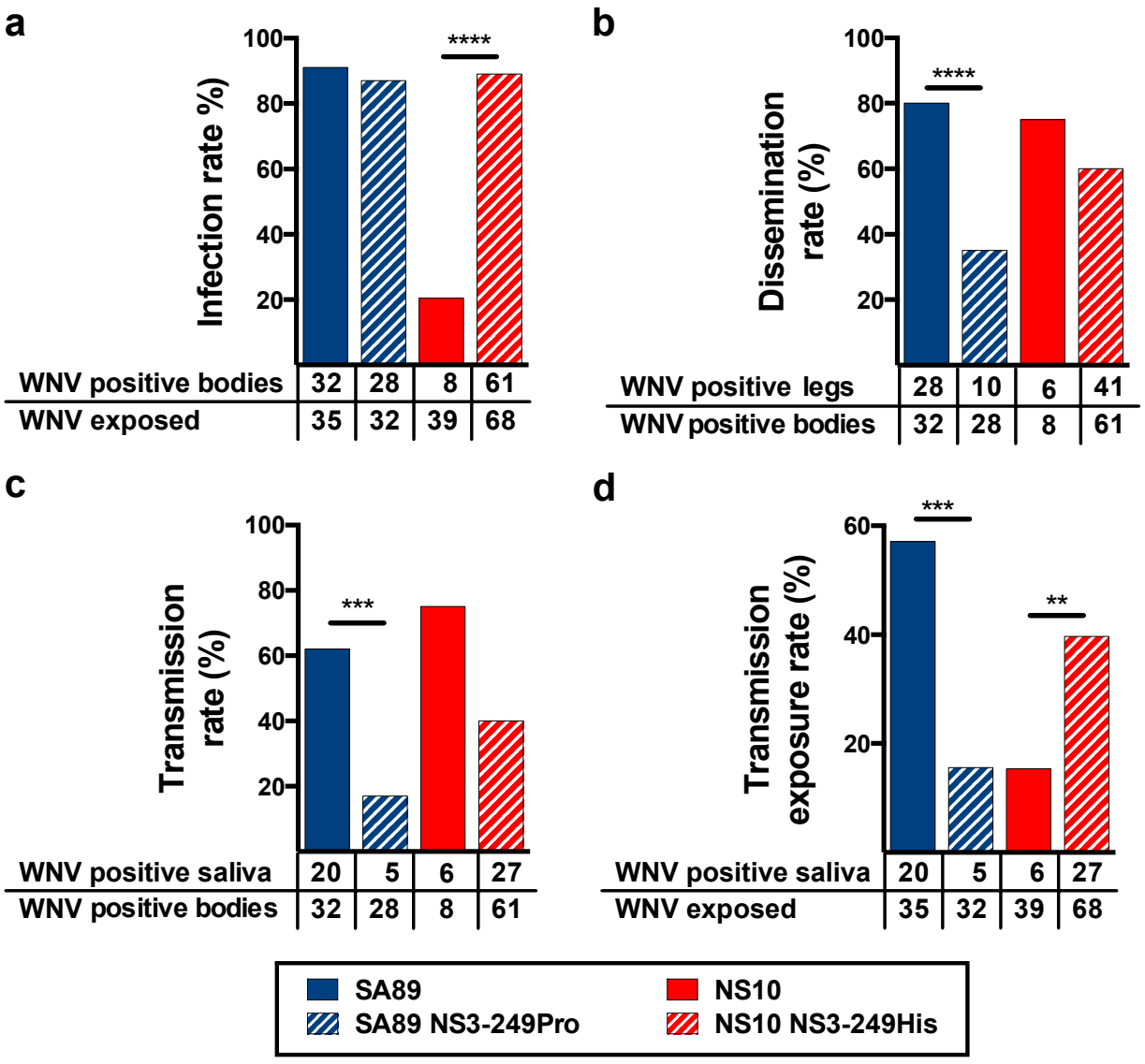

Figure 4.3. Vector competence of *Culex pipiens* for NS3-249 mutants

Culex pipiens were per orally exposed to 8 $\log_{10}$ PFU/ml of virus and infection, dissemination and transmission assessed at 14 dpi. a) Infection rates as determined by the proportion of exposed mosquitoes with virus positive bodies b) Dissemination rates as determined by the proportion of mosquitoes with positive bodies with virus positive legs c) Transmission rates as determined by the proportion of mosquitoes with positive bodies with virus-positive expectorants d) Transmission exposure rates were determined as the proportion of mosquitoes exposed to virus that had virus positive expectorants. Statistical significance calculated using Chi-squared test.

Table 4.1. Summary of vector competence of *Culex pipiens* for SA89, SA89 NS3-H249P, NS10, and NS10 NS3-P249H.

Proportions are calculated two different ways: a) proportions calculated with number of WNV exposed mosquitoes as denominator. b) Proportions calculated with number of WNV exposed mosquitoes as denominator for infection. Denominator for Dissemination and Transmission is number of WNV positive bodies.

L2 WNV strains	**Infection**	**Dissemination**	**Transmission**
	a) Proportions calculated with number of WNV exposed mosquitoes as denominator		
SA89	91.4% (32/35)	80% (28/35)	57.1% (20/35)
SA89 NS3-H249	87.5% (28/32)	31.25% (10/32)	15.6% (5/32)
NS10	20.5% (8/39)	15.4% (6/39)	15.3% (6/39)
NS10 NS3-P249	89.7% (61/68)	60.2% (41/68)	39.7% (27/68)

L2 WNV strains	**Infection**	**Dissemination**	**Transmission**
	b) Proportions calculated with number of WNV exposed mosquitoes as denominator for infection. Denominator for Dissemination and Transmission is number of WNV positive bodies.		
SA89	91.4% (32/35)	87.5% (28/32)	62.5% (20/32)
SA89 NS3-H249	87.5% (28/32)	35.7% (10/28)	17.9% (5/28)
NS10	20.5% (8/39)	75% (6/8)	75% (6/8)
NS10 NS3-P249	89.7% (61/68)	67.% (41/61)	44.3% (27/61)

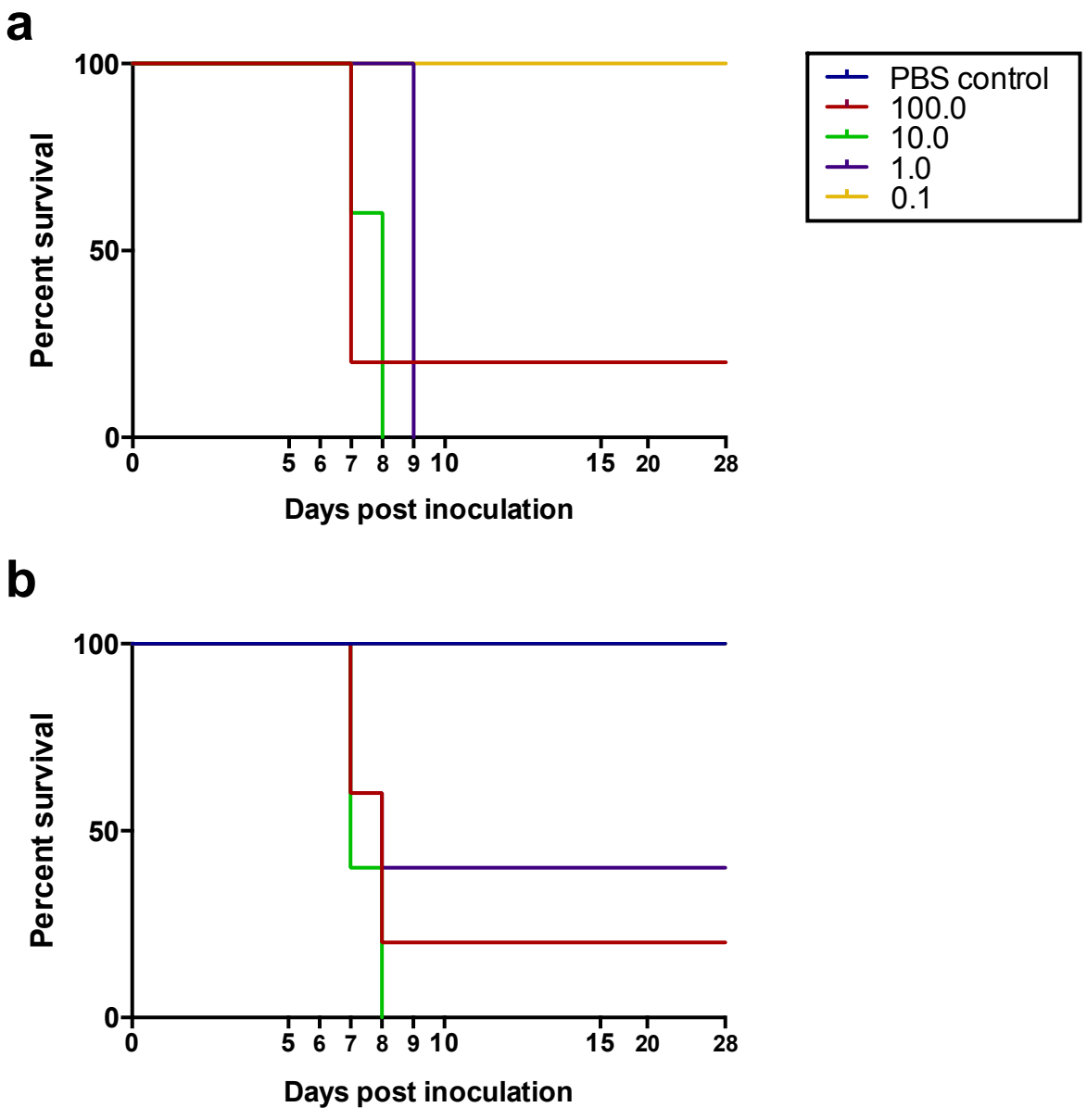

Figure 4.4. Dose response curves of SA89 and SA89 NS3-H249P

Dose response curves of CD-1 mice IP inoculated with input doses ranging from 0.1 PFU to 100 PFU of either a) SA89-IC or b) SA89 NS3-H249P clone-derived viruses. Mice were monitored daily for signs of morbidity and all surviving mice were euthanized at 28 dpi.

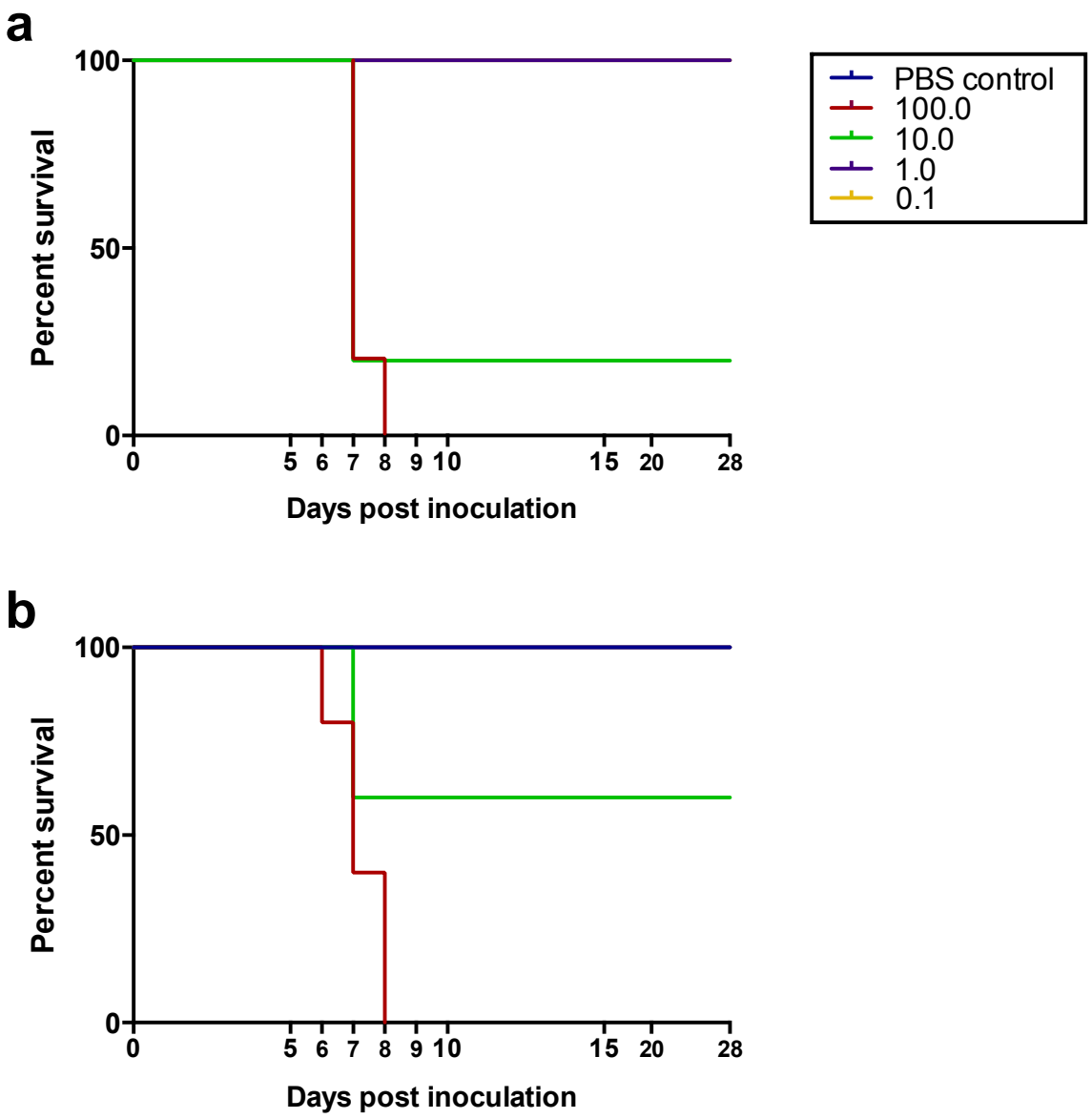

Figure 4.5. Dose response curves of NS10 and NS10 NS3-P249H

Dose response curves of CD-1 mice IP inoculated with input doses ranging from 0.1 PFU to 100 PFU of either a) NS10-IC or b) NS10 NS3-P249H clone-derived viruses. Mice were monitored daily for signs of morbidity and all surviving mice were euthanized at 28 dpi.

Neuroinvasive phenotype of NS3-249 mutant

To determine if the NS3-H249P mutation affects virulence in mice, 3-4 week-old CD-1/ICR mice were intraperitoneally challenged with serial 10 fold dilutions of NS10 and the derived NS3-249 mutants (Fig. 4.5). As a comparison, SA89 and the SA89 NS3-H249P mutant were similarly examined (Fig. 4.4). Mortality was observed within each virus group by 7-9 dpi. The LD_{50} values for SA89-IC, SA89 NS3-H249P, NS10-IC, and NS10 NS3-P249H clone-derived viruses were calculated at 0.38, 0.87, 2.49, and 1.31 PFU, respectively. No significant differences were observed the LD_{50} values between the four L2 viruses.

DISCUSSION

Phylogenetic analysis and murine studies together have demonstrated that more recent L1 and/or L2 isolates have been correlated with increased neuroinvasive pathogenicity in mice, suggesting that WNV has become more virulent in humans over time (McMullen *et al.*, 2012). However, the low viremia titers produced in humans following WNV infection preclude the contribution of humans to the transmission cycle, so the potential selection of more neuroinvasive WNV phenotypes in humans is likely a bystander result stemming from selection between vector and reservoir host. The first case of L2 WNV mediated neurological disease in humans in Europe coincided with emergence of a L2 variant with a NS3-H249P substitution (Papa *et al.*, 2011a). The neuroinvasive potential (as determined by comparisons between LD_{50} values) did not differ between the older African (SA89) and newer evolved European (NS10) strains. Additionally, I demonstrate that the histidine to proline NS3-249 mutation seemingly does not affect neuroinvasive potentials as assessed by peripheral inoculation in outbred mice. Together this demonstrates that the high rates of WNV encephalitis in humans observed during the 2010-2014 Greek outbreaks are very likely a result of high human exposure rates driven by increased infection of mosquitoes resulting from a high force of enzootic transmission rather than bystander increases in neuroinvasiveness modulated by newer evolved L2 genotypes or the evolution of the NS3-249P mutation.

When transmission proportions were calculated to examine the effect on total transmission (i.e. denominator is total number of virus exposed mosquitoes), a significant reduction in the proportion of mosquitoes with positive WNV saliva was observed for mosquitoes per orally exposed to NS10 and SA89 NS3-H249P, demonstrating that a proline at the NS3-249 locus severely impacts total levels of transmission albeit due to different barriers for which the mutations are restricted. The effect of significantly reduced transmission in *Culex pipiens* inversely correlates with the increased virogenesis elicited by the same NS3-H249P mutation in AMCRs. Other genetic elements within SA89 and NS10 are likely driving the differential vector competence phenotypes elicited by the NS3-249 locus in *Culex pipiens* and it is likely that these differences in combination with altered virogenesis profiles in AMCRs hosts either restrict or enhance the emergence potential of these mutations. For example, the SA89 and SA89 NS3-H249P mutant demonstrated robust infection rates, but the overall transmission of SA89 NS3-H249P was severely constrained by a restriction in its capacity to disseminate. Whatever increase in enzootic amplification that was achieved by the higher viremia titers in AMCRs elicited by the theoretical emergence of SA89 NS3-H249P variant would be negatively modulated by the severely reduced transmission in *Culex pipiens*, thereby restricting emergence of Pro mutations derived from that genetic backbone. Alternatively, the higher viremia titers elicited by the NS3-249P substitution could offset the negative effect this mutation was found to have on midgut infectivity of NS10. Under this scenario, the net effect on the overall transmissibility of mosquitoes could be offset. Taken together, these findings indicate that the NS3-249 locus modulates components of transmission that dictate fitness effects directly impacting enzootic maintenance.

Arboviruses must continually contend with the disparate selective environments present within vertebrate and invertebrate host systems to maintain enzootic transmission. It has been postulated that WNV evolution modulated by key vector and avian species may drive the emergence of selective mutations, such as those cause that mortality in AMCRs or facilitate

more efficient transmission by *Culex pipiens*(Brault *et al.*, 2007; Coffey & Reisen, 2016; Kilpatrick *et al.*, 2008; Moudy *et al.*, 2007). The co-evolution of attenuating (*Culex pipiens*) and pathogenic determinants (AMCRs) within NS10 may reflect the inherent necessity of arboviruses to host switch between mosquito invertebrate and vertebrate systems in a trade-off dependent manner, such that positive selection leads to advantages in one host but are unfavorable in a secondary host. For example, a single point mutation in NS4B in Dengue virus inversely modulates replication phenotypes in mammalian and mosquito hosts systems and demonstrates *in vitro* support of the trade-off hypothesis (Hanley *et al.*, 2003, p. 4). A study by Deardorff et al, demonstrated a mosquito adapted (serial passaged) WNV elicited slight fitness gains in *Culex pipiens* mosquitoes, but led to significant fitness losses in chickens(Deardorff *et al.*, 2011). This evidence was contrasted by a similar experiment in the same study where fitness gains were observed across *Culex pipiens* and chickens following infection with a chicken adapted (serially passaged in chickens) WNV strain(Deardorff *et al.*, 2011). Our results demonstrate that the NS3-249P mutation in a context dependent manner (NS10) increases fitness gain in AMCRs while concurrently reducing vector competence in *Culex pipiens* mosquitoes and represents naturally occurring evidence in support of the trade-hypothesis for WNV transmission between *Culex pipiens* and AMCRs.

However, one key limitation associated with our experimental design is the evaluation of African and European L2 WNV strains in North American vectors and amplification hosts. Significant phenotypic variation in the response of disparate mosquito vectors and amplification hosts to WNV infection has been well documented. It is possible that more geographically relevant transmission vectors or amplification hosts do not reflect the results demonstrated here following exposure to L2 WNV. However, the data provided herein demonstrate *in vivo* evidence in support of the trade-off hypothesis and provide a framework for these studies to be addressed in more closely matched vertebrate and invertebrate host systems. Taken together, these results establish in this experimental system that the role of the NS3-249P substitution in

enzootic transmission is not linked to increases in incidental host pathogenesis. Rather, adaptive evolutionary changes at the NS3-249 locus are occurring in L2 WNV, thereby concurrently modulating the viremic responses of key amplification hosts and primary enzootic vectors.

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

SUMMARY

For this dissertation, an integrated approach was utilized for characterizing an understudied yet emerging lineage of WNV with a special emphasis on the direct impact of evolution at the NS3-249 locus on host competence. Specifically, the phenotype of African and European L2 strains with and without polymorphic NS3-249 mutations was examined in 1) mice serving as a surrogate for human disease, 2) in AMCRs and HOSPs as models of extreme and moderately susceptible avian hosts, and 3) multiple geographically relevant *Culex* transmission vectors.

Avian pathogenesis of L2 WNV

The pathogenesis of L2 WNV strains from African and Europe has been confirmed in our AMCR and HOSP models and demonstrates the potential of North American avian species to support L2 WNV enzootic transmission. Additionally, I demonstrated that the NS3-249 locus modulates virulence phenotypes in L2 WNV and that the severe virogenesis accompanying strains containing variable NS3-249P identities might serve as a mechanism for increased force of transmission and facilitate emergence of L2 WNV into new geographic territories.

Vector competence of L2 WNV in North American mosquitoes

Little information was available regarding the competency of *Culex* mosquitoes to transmit L2 WNV. In this study I have demonstrated a range of vector competence phenotypes for L2 WNV in multiple *Culex* species. I showed that *Culex pipiens and Culex quinquefasciatus* mosquitoes can transmit L2 WNV with varying efficiencies but that these differences are species and WNV strain dependent. This study also identified that a L2 WNV strain, NS10, demonstrated reduced infection rates in *Culex pipiens*, a key transmission vector of WNV in

Europe and raised questions regarding the effect of the NS3-249P contained within NS10 on vector competence phenotypes.

Impact of the NS3-249P evolution on L2 WNV transmissibility

In this last aim, I interrogate the role of the NS3-249P mutation on the three main facets of WNV the transmission cycle; human pathogenesis, reservoir amplification and vector competence. I found that while murine neuroinvasive phenotypes were not impacted by NS3-249P variations, a striking inverse phenotype was controlled by the NS3-249P mutation in *Culex pipiens* mosquitoes and AMCRs. I found the high viremia titers elicited by NS3-249P mutations are offset by significant decreases in transmissibility and indicates that the NS3-249 locus is directly modulating transmission and fitness phenotypes and subsequently impacting enzootic maintenance.

FUTURE DIRECTIONS

L2 WNV was isolated in Madagascar from parrots in 1978 and was recognized as the most abundant arbovirus in that area through 1990(Boyer *et al.*, 2014). Recently an upsurge of L2 WNV mediated disease amongst various domestic avian species in Madagascar has been detected(Maquart *et al.*, 2016). Circulating isolates most closely match other L2 WNV collected from the same area in 1980, indicating that the current epizootics are not a result of recent L2 WNV introductions. Only the E gene of recent L2 WNV isolates from Madagascar has been sequenced, making it impossible to identify from available sequence information whether the NS3-249 locus is altered from the normal histidine variant. However, the resurgence of L2 WNV in Madagascar and correspondingly genetic relatedness to other Madagascar L2 WNV strains likely indicates that L2 WNV can persist at levels undetectable through passive surveillance for decades. This suggests that despite the nonappearance of L2 WNV mediated outbreaks in Greece (via the absence of clinical WNV cases) in 2015 and 2016, L2 WNV is still an important

arboviral threat. Preservation of L2 WNV through periods of non-evident enzootic transmission exemplifies the need to limit further dissemination and restrict endemic establishment of L2 WNV. Surveillance is a key component of identifying arboviral threats. However, traditional methods of detection for WNV may not capture emergence events for specific lineages. In Larimer County, CO surveillance of field caught mosquitoes for WNV is carried out using traditional qRT-PCR methodologies(Lanciotti *et al.*, 2000). Primer sequences (sequence courtesy of Reyes Murrieta and Greg Ebel) were computationally tested against representative strains from L1 and L2 for evidence of binding (Primer test, Geneious 9.1.6). Primers bound L1 WNV strains only, indicating that traditional WNV surveillance methods would be unable to capture evidence of L2 WNV transmission and highlight the need for a more comprehensive approach for arbovirus surveillance.

The role of alternate polymorphisms at the NS3-249 locus in WNV needs to be further examined in mosquitoes and contrasted with data already generated for the same NS3-249 polymorphic mutations in AMCRs. For example, the results herein demonstrate that reduced infection rates in *Culex pipiens* mosquitoes are potentially offset by increases in viremia titers in AMCRs for the L2 WNV prototype strain NS10. Previous studies by Langevin and Brault et al., demonstrated that NS3-249 mutations (Ala, Asp, Thr) that naturally circulate elicit reduced virogenesis in AMCRs(Brault *et al.*, 2007; Langevin *et al.*, 2014). Studies should be performed to assess whether increases in vector competence offset decreases in AMCR virogenesis elicited by the same NS3-249 mutations such that despite reduced amplification in reservoir hosts, transmission for these strains still occurs. These studies might shed light on emergence mechanisms of NS3-249P in regions where NS3-249 WNV harboring strains currently circulate.

It is curious that European L2 WNV mediated outbreaks have occurred only recently given the potential role of migratory birds in long distance WNV dispersal and the existence of ancient avian migratory pathways between Africa and Europe that could potentially facilitate repeated L2 WNV introductions. The seemingly restricted emergence of L2 WNV out of Africa

may be related to specific maintenance of L2 WNV rather than predicated on introduction events. For example, L2 WNV was detected in Cyprus in 1968, but not associated with outbreaks or identified surrounding areas (Beasley et al., 2002). Our studies demonsrate that viral genetic changes within L2 WNV have occurred and are potentially modulating increases in enzootic transmission. Increased force of transmission may increase the likelihood that introduced WNV genotypes would become endemic and be transmitted in subsequent transmission seasons. It will be interesting to see if L2 WNV continues to spread across Europe and corresponding occurrence of new viral genetic changes might facilitate further geographic dissemination and sustain enzootic transmission.

REFERENCES

Anderson, J. F. & Main, A. J. (2006). Importance of Vertical and Horizontal Transmission of West Nile Virus by Culex pipiens in the Northeastern United States. *J Infect Dis* 194, 1577–1579.

Andreadis, T. G. (2012). The Contribution of *Culex pipiens* Complex Mosquitoes to Transmission and Persistence of West Nile Virus in North America. *J Am Mosq Control Assoc* 28, 137–151.

Apperson, C. S., Harrison, B. A., Unnasch, T. R., Hassan, H. K., Irby, W. S., Savage, H. M., Aspen, S. E., Watson, D. W., Rueda, L. M. & other authors. (2002). Host-feeding habits of *Culex* and other mosquitoes (Diptera: Culicidae) in the Borough of Queens in New York City, with characters and techniques for identification of *Culex* mosquitoes. *J Med Entomol* 39, 777–785.

Arias, C. F., Preugschat, F. & Strauss, J. H. (1993). Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. *Virology* 193, 888–899.

Autorino, G. L., Battisti, A., Deubel, V., Ferrari, G., Forletta, R., Giovannini, A., Lelli, R., Murri, S. & Scicluna, M. T. (2002). West Nile virus Epidemic in Horses, Tuscany Region, Italy. *Emerg Infect Dis* 8, 1372–1378.

Bagnarelli, P., Marinelli, K., Trotta, D., Monachetti, A., Tavio, M., Del Gobbo, R., Capobianchi, M., Menzo, S., Nicoletti, L. & other authors. (2011). Human case of autochthonous West Nile virus lineage 2 infection in Italy, September 2011. *Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull* 16.

Bakonyi, T., Hubálek, Z., Rudolf, I. & Nowotny, N. (2005). Novel Flavivirus or New Lineage of West Nile Virus, Central Europe. *Emerg Infect Dis* 11, 225–231.

Bakonyi, T., Ivanics, E., Erdelyi, K., Ursu, K., Ferenczi, E., Weissenbock, H. & Nowotny, N. (2006). Lineage 1 and 2 Strains of Encephalitic West Nile Virus, Central Europe. *Emerg Infect Dis* 12, 618–623.

Balenghien, T., Vazeille, M., Grandadam, M., Schaffner, F., Zeller, H., Reiter, P., Sabatier, P., Fouque, F. & Bicout, D. J. (2008). Vector competence of some French *Culex* and *Aedes* mosquitoes for West Nile virus. *Vector Borne Zoonotic Dis Larchmt N* 8, 589–595.

Baqar, S., Hayes, C. G., Murphy, J. R. & Watts, D. M. (1993). Vertical transmission of West Nile virus by *Culex* and *Aedes* species mosquitoes. *Am J Trop Med Hyg* 48, 757–762.

Barrett, A. D. T. & Higgs, S. (2007). Yellow fever: a disease that has yet to be conquered. *Annu Rev Entomol* 52, 209–229.

Barrett, A. D. T. & Monath, T. P. (2003). Epidemiology and ecology of yellow fever virus. In *Adv Virus Res*, The Flaviviruses: Detection, Diagnosis, and Vaccine Development, pp. 291–315. Edited by T. J. C. and T. P. Monath. Academic Press.

Barrett, A. D. T. (2016). Yellow Fever in Angola and Beyond — The Problem of Vaccine Supply and Demand. *N Engl J Med* 0, null.

Barzon, L., Franchin, E., Squarzon, L., Lavezzo, E., Toppo, S., Martello, T., Bressan, S., Pagni, S., Cattai, M. & other authors. (2009). Genome sequence analysis of the first human West Nile virus isolated in Italy in 2009. *Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull* 14.

Barzon, L., Pacenti, M., Franchin, E., Squarzon, L., Lavezzo, E., Cattai, M., Cusinato, R. & Palù, G. (2013). The Complex Epidemiological Scenario of West Nile Virus in Italy. *Int J Environ Res Public Health* 10, 4669–4689.

Beasley, D. W. C., Li, L., Suderman, M. T. & Barrett, A. D. T. (2002). Mouse Neuroinvasive Phenotype of West Nile Virus Strains Varies Depending upon Virus Genotype. *Virology* 296, 17–23.

Beatch, M. D., Everitt, J. C., Law, L. J. & Hobman, T. C. (2005). Interactions between Rubella Virus Capsid and Host Protein p32 Are Important for Virus Replication. *J Virol* 79, 10807–10820.

Beckham, J. D. & Tyler, K. L. (2009). Clinical Manifestations of Neurological Disease. In *West Nile Enceph Virus Infect*, Emerging Infectious Diseases of the 21st Century, pp. 69–95. Springer New York.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G. & other authors. (2013). The global distribution and burden of dengue. *Nature* 496, 504–507.

Bondre, V. P., Jadi, R. S., Mishra, A. C., Yergolkar, P. N. & Arankalle, V. A. (2007). West Nile virus isolates from India: evidence for a distinct genetic lineage. *J Gen Virol* 88, 875–884.

Botha, E. M., Markotter, W., Wolfaardt, M., Paweska, J. T., Swanepoel, R., Palacios, G., Nel, L. H. & Venter, M. (2008). Genetic Determinants of Virulence in Pathogenic Lineage 2 West Nile Virus Strains. *Emerg Infect Dis* 14, 222–230.

Boyer, S., Tantely, M. L., Randriamaherijaona, S., Andrianaivolambo, L. & Cardinal, E. (2014). Mosquitoes sampling strategy for studying West Nile Virus Vectors in Madagascar: Abundance, Distribution and Methods of Catching in High Risk Areas. *Arch Inst Pasteur Madagascar* 71, 1–4.

Brault, A. C. (2009). Changing patterns of West Nile virus transmission: altered vector competence and host susceptibility. *Vet Res* 40, 43.

Brault, A. C., Langevin, S. A., Bowen, R. A., Panella, N. A., Biggerstaff, B. J., Miller, B. R. & Komar, N. (2004). Differential Virulence of West Nile Strains for American Crows. *Emerg Infect Dis* 10, 2161–2168.

Brault, A. C., Huang, C. Y.-H., Langevin, S. A., Kinney, R. M., Bowen, R. A., Ramey, W. N., Panella, N. A., Holmes, E. C., Powers, A. M. & Miller, B. R. (2007). A single positively selected West Nile viral mutation confers increased virogenesis in American crows. *Nat Genet* 39, 1162–

1166.

Briese, T., Jia, X. Y., Huang, C., Grady, L. J. & Lipkin, W. I. (1999). Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. *Lancet Lond Engl* 354, 1261–1262.

Bugbee, L. M. & Forte, L. R. (2004). The discovery of West Nile virus in overwintering *Culex pipiens* (Diptera: Culicidae) mosquitoes in Lehigh County, Pennsylvania. *J Am Mosq Control Assoc* 20, 326–327.

Burki, T. (2016). Yellow fever in Africa: a disaster waiting to happen. *Lancet Infect Dis* 16, 896–897.

Calisher, C. H., Karabatsos, N., Dalrymple, J. M., Shope, R. E., Porterfield, J. S., Westaway, E. G. & Brandt, W. E. (1989). Antigenic Relationships between Flaviviruses as Determined by Cross-neutralization Tests with Polyclonal Antisera. *J Gen Virol* 70, 37–43.

Capelli, G., Ravagnan, S., Montarsi, F., Ciocchetta, S., Cazzin, S., Bonfanti, L., Gennaro, A. D., Portanti, O., Mulatti, P. & other authors. (2013). Further evidence of lineage 2 West Nile Virus in Culex pipiens of North-Eastern Italy. *Vet Ital* 49.

Carson, P. J., Borchardt, S. M., Custer, B., Prince, H. E., Dunn-Williams, J., Winkelman, V., Tobler, L., Biggerstaff, B. J., Lanciotti, R. & other authors. (2012). Neuroinvasive Disease and West Nile Virus Infection, North Dakota, USA. *Emerg Infect Dis* 18.

Chancey, C., Grinev, A., Volkova, E. & Rios, M. (2015). The Global Ecology and Epidemiology of West Nile Virus. *BioMed Res Int* 2015.

Chen, C. J., Kuo, M. D., Chien, L. J., Hsu, S. L., Wang, Y. M. & Lin, J. H. (1997). RNA-protein interactions: involvement of NS3, NS5, and 3' noncoding regions of Japanese encephalitis virus genomic RNA. *J Virol* 71, 3466–3473.

Cho, H. & Diamond, M. S. (2012). Immune Responses to West Nile Virus Infection in the Central Nervous System. *Viruses* 4, 3812–3830.

Chu, J. J. H. & Ng, M. L. (2004a). Infectious Entry of West Nile Virus Occurs through a Clathrin-Mediated Endocytic Pathway. *J Virol* 78, 10543–10555.

Chu, J. J. & Ng, M.-L. (2004b). Interaction of West Nile Virus with αvβ3 Integrin Mediates Virus Entry into Cells. *J Biol Chem* 279, 54533–54541.

Ciccozzi, M., Peletto, S., Cella, E., Giovanetti, M., Lai, A., Gabanelli, E., Acutis, P. L., Modesto, P., Rezza, G. & other authors. (2013). Epidemiological history and phylogeography of West Nile virus lineage 2. *Infect Genet Evol* 17.

Coffey, L. L. & Reisen, W. K. (2016). West Nile Virus Fitness Costs in Different Mosquito Species. *Trends Microbiol* 24, 429–430.

CORNEL, A. J., JUPP, P. G. & BLACKBURN, N. K. (1993). Environmental Temperature on the

Vector Competence of *Culex univittatus (*Diptera: Culicidae) for West Nile Virus. *J Med Entomol* 30, 449–456.

Danis, K., Papa, A., Theocharopoulos, G., Dougas, G., Athanasiou, M., Detsis, M., Baka, A., Lytras, T., Mellou, K. & other authors. (2011). Outbreak of West Nile Virus Infection in Greece, 2010. *Emerg Infect Dis* 17, 1868–1872.

Davis, C. T., Ebel, G. D., Lanciotti, R. S., Brault, A. C., Guzman, H., Siirin, M., Lambert, A., Parsons, R. E., Beasley, D. W. C. & other authors. (2005). Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: Evidence for the emergence of a dominant genotype. *Virology* 342, 252–265.

Davis, C. W., Nguyen, H.-Y., Hanna, S. L., Sánchez, M. D., Doms, R. W. & Pierson, T. C. (2006). West Nile Virus Discriminates between DC-SIGN and DC-SIGNR for Cellular Attachment and Infection. *J Virol* 80, 1290–1301.

Dawson, J. R., Stone, W. B., Ebel, G. D., Young, D. S., Galinski, D. S., Pensabene, J. P., Franke, M. A., Eidson, M. & Kramer, L. D. (2007). Crow Deaths Caused by West Nile Virus during Winter. *Emerg Infect Dis* 13, 1912–1914.

Deardorff, E. R., Fitzpatrick, K. A., Jerzak, G. V. S., Shi, P.-Y., Kramer, L. D. & Ebel, G. D. (2011). West Nile Virus Experimental Evolution in vivo and the Trade-off Hypothesis. *PLOS Pathog* 7, e1002335.

Dick, G. W. A., Kitchen, S. F. & Haddow, A. J. (1952). Zika virus. I. Isolations and serological specificity. *Trans R Soc Trop Med Hyg* 46, 509–520.

Dietrich, E. A., Langevin, S. A., Huang, C. Y.-H., Maharaj, P. D., Delorey, M. J., Bowen, R. A., Kinney, R. M. & Brault, A. C. (2016). West Nile Virus Temperature Sensitivity and Avian Virulence Are Modulated by NS1-2B Polymorphisms. *PLoS Negl Trop Dis* 10.

Dohm, D. J., O'Guinn, M. L. & Turell, M. J. (2002). Effect of environmental temperature on the ability of Culex pipiens (Diptera: Culicidae) to transmit West Nile virus. *J Med Entomol* 39, 221–225.

Duggal, N. K., Bosco-Lauth, A., Bowen, R. A., Wheeler, S. S., Reisen, W. K., Felix, T. A., Mann, B. R., Romo, H., Swetnam, D. M. & other authors. (2014). Evidence for Co-evolution of West Nile Virus and House Sparrows in North America. *PLoS Negl Trop Dis* 8.

eCDC. (2014). Danis.

Eidson, M., Komar, N., Sorhage, F., Nelson, R., Talbot, T., Mostashari, F., McLean, R. & West Nile Virus Avian Mortality Surveillance Group. (2001). Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. *Emerg Infect Dis* 7, 615–620.

Ellington, S. R., Devine, O., Bertolli, J., Quiñones, A. M., Shapiro-Mendoza, C. K., Perez-Padilla, J., Rivera-Garcia, B., Simeone, R. M., Jamieson, D. J. & other authors. (2016). Estimating the Number of Pregnant Women Infected With Zika Virus and Expected Infants With

Microcephaly Following the Zika Virus Outbreak in Puerto Rico, 2016. *JAMA Pediatr* 170, 940–945.

Falgout, B., Bray, M., Schlesinger, J. J. & Lai, C. J. (1990). Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. *J Virol* 64, 4356–4363.

Faraji, A. & Gaugler, R. (2015). Experimental host preference of diapause and non-diapause induced Culex pipiens pipiens (Diptera: Culicidae). *Parasit Vectors* 8, 389.

Farajollahi, A., Crans, W. J., Bryant, P., Wolf, B., Burkhalter, K. L., Godsey, M. S., Aspen, S. E. & Nasci, R. S. (2005). Detection of West Nile viral RNA from an overwintering pool of Culex pipens pipiens (Diptera: Culicidae) in New Jersey, 2003. *J Med Entomol* 42, 490–494.

Farajollahi, A., Fonseca, D. M., Kramer, L. D. & Kilpatrick, A. M. (2011). 'Bird biting' mosquitoes and human disease: a review of the role of *Culex pipiens* complex mosquitoes in epidemiology. *Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis* 11, 1577–1585.

Fonseca, D. M., Keyghobadi, N., Malcolm, C. A., Mehmet, C., Schaffner, F., Mogi, M., Fleischer, R. C. & Wilkerson, R. C. (2004). Emerging Vectors in the Culex pipiens Complex. *Science* 303, 1535–1538.

Fortuna, C., Remoli, M. E., Severini, F., Di Luca, M., Toma, L., Fois, F., Bucci, P., Boccolini, D., Romi, R. & Ciufolini, M. G. (2015). Evaluation of vector competence for West Nile virus in Italian *Stegomyia albopicta* (=*Aedes albopictus*) mosquitoes. *Med Vet Entomol* 29, 430–433.

Franz, A. W. E., Kantor, A. M., Passarelli, A. L. & Clem, R. J. (2015). Tissue Barriers to Arbovirus Infection in Mosquitoes. *Viruses* 7, 3741–3767.

Fros, J. J., Geertsema, C., Vogels, C. B., Roosjen, P. P., Failloux, A.-B., Vlak, J. M., Koenraadt, C. J., Takken, W. & Pijlman, G. P. (2015). West Nile Virus: High Transmission Rate in North-Western European Mosquitoes Indicates Its Epidemic Potential and Warrants Increased Surveillance. *PLoS Negl Trop Dis* 9, e0003956.

Godsey, M. S., Blackmore, M. S., Panella, N. A., Burkhalter, K., Gottfried, K., Halsey, L. A., Rutledge, R., Langevin, S. A., Gates, R. & other authors. (2005). West Nile Virus Epizootiology in the Southeastern United States, 2001. *Vector-Borne Zoonotic Dis* 5, 82–89.

Gorbalenya, A. E., Donchenko, A. P., Koonin, E. V. & Blinov, V. M. (1989). N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. *Nucleic Acids Res* 17, 3889–3897.

Gritsun, D. J., Jones, I. M., Gould, E. A. & Gritsun, T. S. (2014). Molecular Archaeology of Flaviviridae Untranslated Regions: Duplicated RNA Structures in the Replication Enhancer of Flaviviruses and Pestiviruses Emerged via Convergent Evolution. *PLOS ONE* 9, e92056.

Grobbelaar, A. A., Weyer, J., Moolla, N., Jansen van Vuren, P., Moises, F. & Paweska, J. T. (2016). Resurgence of Yellow Fever in Angola, 2015–2016. *Emerg Infect Dis* 22, 1854–1855.

Halstead, S. B. (2007). Dengue. *Lancet Lond Engl* 370, 1644–1652.

Hamer, G. L., Kitron, U. D., Brawn, J. D., Loss, S. R., Ruiz, M. O., Goldberg, T. L. & Walker, E. D. (2008). Culex pipiens (Diptera: Culicidae): A Bridge Vector of West Nile Virus to Humans. *J Med Entomol* 45, 125–128.

Hammam, H. M., Clarke, D. H. & Price, W. H. (1965). Antigenic Variation of West Nile Virus in Relation to Geography,. *Am J Epidemiol* 82, 40–55.

Hanley, K. A., Manlucu, L. R., Gilmore, L. E., Blaney, J. E., Hanson, C. T., Murphy, B. R. & Whitehead, S. S. (2003). A trade-off in replication in mosquito versus mammalian systems conferred by a point mutation in the NS4B protein of dengue virus type 4. *Virology* 312, 222–232.

Hardy, J. L., Houk, E. J., Kramer, L. D. & Reeves, W. C. (1983). Intrinsic Factors Affecting Vector Competence of Mosquitoes for Arboviruses. *Annu Rev Entomol* 28, 229–262.

Hasegawa, M., Tuno, N., Yen, N. T., Nam, V. S. & Takagi, M. (2008). Influence of the Distribution of Host Species on Adult Abundance of Japanese Encephalitis Vectors—Culex vishnui Subgroup and Culex gelidus—in a Rice-Cultivating Village in Northern Vietnam. *Am J Trop Med Hyg* 78, 159–168.

Hernández-Triana, L. M., Jeffries, C. L., Mansfield, K. L., Carnell, G., Fooks, A. R. & Johnson, N. (2014). Emergence of West Nile Virus Lineage 2 in Europe: A Review on the Introduction and Spread of a Mosquito-Borne Disease. *Front Public Health* 2.

Hofmeister, E. K., Dusek, R. J. & Brand, C. J. (2015). Surveillance Potential of Non-Native Hawaiian Birds for Detection of West Nile Virus. *Am J Trop Med Hyg* 93, 701–708.

Huang, Y.-J. S., Higgs, S., Horne, K. M. & Vanlandingham, D. L. (2014). Flavivirus-Mosquito Interactions. *Viruses* 6, 4703–4730.

Hubalek, Z. & Halouzka, J. (1999). West Nile fever--a reemerging mosquito-borne viral disease in Europe. *Emerg Infect Dis* 5, 643–650.

Hubálek, Z., Halouzka, J., Juricová, Z. & Sebesta, O. (1998). First isolation of mosquito-borne West Nile virus in the Czech Republic. *Acta Virol* 42, 119–120.

Hunt, T. A., Urbanowski, M. D., Kakani, K., Law, L.-M. J., Brinton, M. A. & Hobman, T. C. (2007). Interactions between the West Nile virus capsid protein and the host cell-encoded phosphatase inhibitor, I2PP2A. *Cell Microbiol* 9, 2756–2766.

Hutcheson, H. J., Gorham, C. H., Machain-Williams, C., Loroño-Pino, M. A., James, A. M., Marlenee, N. L., Winn, B., Beaty, B. J. & Blair, C. D. (2005). Experimental transmission of West Nile virus (Flaviviridae: Flavivirus) by *Carios capensis* ticks from North America. *Vector Borne Zoonotic Dis Larchmt N* 5, 293–295.

Ip, H. S., Van Wettere, A. J., McFarlane, L., Shearn-Bochsler, V., Dickson, S. L., Baker, J., Hatch, G., Cavender, K., Long, R. & Bodenstein, B. (2014). West Nile Virus Transmission in

Winter: The 2013 Great Salt Lake Bald Eagle and Eared Grebes Mortality Event. *PLoS Curr* 6.

Iwamoto, M., Jernigan, D. B., Guasch, A., Trepka, M. J., Blackmore, C. G., Hellinger, W. C., Pham, S. M., Zaki, S., Lanciotti, R. S. & other authors. (2003). Transmission of West Nile virus from an organ donor to four transplant recipients. *N Engl J Med* 348, 2196–2203.

Jia, X. Y., Briese, T., Jordan, I., Rambaut, A., Chi, H. C., Mackenzie, J. S., Hall, R. A., Scherret, J. & Lipkin, W. I. (1999). Genetic analysis of West Nile New York 1999 encephalitis virus. *Lancet Lond Engl* 354, 1971–1972.

Johnston, L. J., Halliday, G. M. & King, N. J. (2000). Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus. *J Invest Dermatol* 114, 560–568.

Jupp, P. G. & McIntosh, B. M. (1970). Quantitative experiments on the vector capability of Culex (Culex) univittatus Theobald with West Nile and Sindbis viruses. *J Med Entomol* 7, 371–373.

Jupp, P. G. (2001). The Ecology of West Nile Virus in South Africa and the Occurrence of Outbreaks in Humans. *Ann N Y Acad Sci* 951, 143–152.

Kamer, G. & Argos, P. (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Res* 12, 7269–7282.

Karabatsos, N. (1985). *International catalogue of arboviruses, including certain other viruses of vertebrates*, 3rd ed. San Antonio, Texas: American Society of Tropical Medicine and Hygiene for The Subcommittee on Information Exchange of the American Committee on Arthropod-borne Viruses.

Kent, R., Juliusson, L., Weissmann, M., Evans, S. & Komar, N. (2009). Seasonal Blood-Feeding Behavior of Culex tarsalis (Diptera: Culicidae) in Weld County, Colorado, 2007. *J Med Entomol* 46, 380–390.

Khetarpal, N. & Khanna, I. (2016). Dengue Fever: Causes, Complications, and Vaccine Strategies. *J Immunol Res* 2016.

Kilpatrick, A. M., Meola, M. A., Moudy, R. M. & Kramer, L. D. (2008). Temperature, Viral Genetics, and the Transmission of West Nile Virus by *Culex pipiens* Mosquitoes. *PLoS Pathog* 4, e1000092.

Kilpatrick, A. M., Fonseca, D. M., Ebel, G. D., Reddy, M. R. & Kramer, L. D. (2010). Spatial and temporal variation in vector competence of Culex pipiens and Cx. restuans mosquitoes for West Nile virus. *Am J Trop Med Hyg* 83, 607–613.

Kinney, R. M., Huang, C. Y.-H., Whiteman, M. C., Bowen, R. A., Langevin, S. A., Miller, B. R. & Brault, A. C. (2006). Avian virulence and thermostable replication of the North American strain of West Nile virus. *J Gen Virol* 87, 3611–3622.

Klenk, K., Snow, J., Morgan, K., Bowen, R., Stephens, M., Foster, F., Gordy, P., Beckett, S., Komar, N. & other authors. (2004). Alligators as West Nile Virus Amplifiers. *Emerg Infect Dis* 10, 2150–2155.

Knight. (1978). WRBU: NORTHCOM Medically Important Arthropods.

Komar, N. (2003). West Nile virus: epidemiology and ecology in North America. *Adv Virus Res* 61, 185–234.

Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D., Davis, B., Bowen, R. & Bunning, M. (2003). Experimental Infection of North American Birds with the New York 1999 Strain of West Nile Virus. *Emerg Infect Dis* 9, 311–322.

Koonin, E. V. (1991). The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *J Gen Virol* 72 (Pt 9), 2197–2206.

Kothera, L., Zimmerman, E. M., Richards, C. M. & Savage, H. M. (2009). Microsatellite characterization of subspecies and their hybrids in Culex pipiens complex (Diptera: Culicidae) mosquitoes along a north-south transect in the central United States. *J Med Entomol* 46, 236–248.

Kümmerer, B. M. & Rice, C. M. (2002). Mutations in the Yellow Fever Virus Nonstructural Protein NS2A Selectively Block Production of Infectious Particles. *J Virol* 76, 4773–4784.

Lanciotti, R. S., Kerst, A. J., Nasci, R. S., Godsey, M. S., Mitchell, C. J., Savage, H. M., Komar, N., Panella, N. A., Allen, B. C. & other authors. (2000). Rapid Detection of West Nile Virus from Human Clinical Specimens, Field-Collected Mosquitoes, and Avian Samples by a TaqMan Reverse Transcriptase-PCR Assay. *J Clin Microbiol* 38, 4066–4071.

Lanciotti, R. S., Ebel, G. D., Deubel, V., Kerst, A. J., Murri, S., Meyer, R., Bowen, M., McKinney, N., Morrill, W. E. & other authors. (2002). Complete Genome Sequences and Phylogenetic Analysis of West Nile Virus Strains Isolated from the United States, Europe, and the Middle East. *Virology* 298, 96–105.

Langevin, S. A., Brault, A. C., Panella, N. A., Bowen, R. A. & Komar, N. (2005). Variation in Virulence of West Nile Virus Strains for House Sparrows (passer Domesticus). *Am J Trop Med Hyg* 72, 99–102.

Langevin, S. A., Bowen, R. A., Ramey, W. N., Sanders, T. A., Maharaj, P. D., Fang, Y., Cornelius, J., Barker, C. M., Reisen, W. K. & other authors. (2011). Envelope and pre-membrane protein structural amino acid mutations mediate diminished avian growth and virulence of a Mexican West Nile virus isolate. *J Gen Virol* 92, 2810–2820.

Langevin, S. A., Bowen, R. A., Reisen, W. K., Andrade, C. C., Ramey, W. N., Maharaj, P. D., Anishchenko, M., Kenney, J. L., Duggal, N. K. & other authors. (2014). Host Competence and Helicase Activity Differences Exhibited by West Nile Viral Variants Expressing NS3-249 Amino Acid Polymorphisms. *PLoS ONE* 9, e100802.

Lim, P.-Y., Behr, M. J., Chadwick, C. M., Shi, P.-Y. & Bernard, K. A. (2011). Keratinocytes Are Cell Targets of West Nile Virus In Vivo□. *J Virol* 85, 5197–5201.

Lim, S. M., Brault, A. C., van Amerongen, G., Sewbalaksing, V. D., Osterhaus, A. D. M. E., Martina, B. E. E. & Koraka, P. (2014). Susceptibility of European Jackdaws (Corvus monedula)

to Experimental Infection with Lineage 1 and 2 West Nile Viruses. *J Gen Virol*.

Lindenbach, B. D. & Rice, C. M. (1999). Genetic Interaction of Flavivirus Nonstructural Proteins NS1 and NS4A as a Determinant of Replicase Function. *J Virol* 73, 4611–4621.

Lindsey, N. P., Staples, J. E., Lehman, J. A., Fischer, M. & Centers for Disease Control and Prevention (CDC). (2010). Surveillance for human West Nile virus disease - United States, 1999-2008. *Morb Mortal Wkly Rep Surveill Summ Wash DC 2002* 59, 1–17.

Lvov, D. K., Butenko, A. M., Gromashevsky, V. L., Kovtunov, A. I., Prilipov, A. G., Kinney, R., Aristova, V. A., Dzharkenov, A. F., Samokhvalov, E. I. & other authors. (2004). West Nile virus and other zoonotic viruses in Russia: examples of emerging-reemerging situations. *Arch Virol Suppl* 85–96.

Mackenzie, J. M., Jones, M. K. & Young, P. R. (1996). Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. *Virology* 220, 232–240.

Magurano, F., Remoli, M. E., Baggieri, M., Fortuna, C., Marchi, A., Fiorentini, C., Bucci, P., Benedetti, E., Ciufolini, M. G. & other authors. (2012). Circulation of West Nile virus lineage 1 and 2 during an outbreak in Italy. *Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis* 18, E545-547.

Malkinson, M. & Banet, C. (2002). The Role of Birds in the Ecology of West Nile Virus in Europe and Africa. In *Jpn Enceph West Nile Viruses*, Current Topics in Microbiology and Immunology, pp. 309–322. Edited by P. D. J. S. Mackenzie, P. D. A. D. T. Barrett & D. V. Deubel. Springer Berlin Heidelberg.

Maquart, M., Boyer, S., Rakotoharinome, V. M., Ravaomanana, J., Tantely, M. L., Heraud, J.-M. & Cardinale, E. (2016). High Prevalence of West Nile Virus in Domestic Birds and Detection in 2 New Mosquito Species in Madagascar. *PLoS ONE* 11.

Marciniak, C., Sorosky, S. & Hynes, C. (2004). Acute flaccid paralysis associated with West Nile virus: motor and functional improvement in 4 patients. *Arch Phys Med Rehabil* 85, 1933–1938.

Markoff, L. (2003). 5'- and 3'-noncoding regions in flavivirus RNA. *Adv Virus Res* 59, 177–228.

May, F. J., Davis, C. T., Tesh, R. B. & Barrett, A. D. T. (2011). Phylogeography of West Nile Virus: from the Cradle of Evolution in Africa to Eurasia, Australia, and the Americas. *J Virol* 85, 2964–2974.

Mayr, E. (1946). The Number of Species of Birds. *The Auk* 63, 64–69.

McMullen, A. R., Albayrak, H., May, F. J., Davis, C. T., Beasley, D. W. C. & Barrett, A. D. T. (2012). Molecular evolution of lineage 2 West Nile virus. *J Gen Virol* 94, 318–325.

McMullen, A. R., May, F. J., Li, L., Guzman, H., Bueno, R., Dennett, J. A., Tesh, R. B. & Barrett, A. D. T. (2011). Evolution of New Genotype of West Nile Virus in North America. *Emerg Infect Dis* 17, 785–793.

Mlakar, J., Korva, M., Tul, N., Popović, M., Poljšak-Prijatelj, M., Mraz, J., Kolenc, M., Resman Rus, K., Vesnaver Vipotnik, T. & other authors. (2016). Zika Virus Associated with Microcephaly. *N Engl J Med* 374, 951–958.

Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature* 427, 313–319.

Molaei, G., Andreadis, T. G., Armstrong, P. M., Bueno, R., Dennett, J. A., Real, S. V., Sargent, C., Bala, A., Randle, Y. & other authors. (2007). Host Feeding Pattern of Culex quinquefasciatus (Diptera: Culicidae) and Its Role in Transmission of West Nile Virus in Harris County, Texas. *Am J Trop Med Hyg* 77, 73–81.

Monath, T. P. (2001). Yellow fever: an update. *Lancet Infect Dis* 1, 11–20.

Mostashari, F., Bunning, M. L., Kitsutani, P. T., Singer, D. A., Nash, D., Cooper, M. J., Katz, N., Liljebjelke, K. A., Biggerstaff, B. J. & other authors. (2001). Epidemic West Nile encephalitis, New York, 1999: results of a household-based seroepidemiological survey. *Lancet Lond Engl* 358, 261–264.

Motayo, B. O., Onoja, B. A., Faneye, A. O. & Adeniji, J. A. (2016). Seasonal abundance and molecular identification of West Nile virus vectors, Culex pipens and Culex quinquefasciatus (diptera: culicidae) in Abeokuta, South-West, Nigeria. *Afr Health Sci* 16, 135–140.

Moudy, R. M., Meola, M. A., Morin, L.-L. L., Ebel, G. D. & Kramer, L. D. (2007). A Newly Emergent Genotype of West Nile Virus Is Transmitted Earlier and More Efficiently by *Culex* Mosquitoes. *Am J Trop Med Hyg* 77, 365–370.

Mukhopadhyay, S., Kuhn, R. J. & Rossmann, M. G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol* 3, 13–22.

Mutebi, J.-P., Crabtree, M. B., Kading, R. C., Powers, A. M., Lutwama, J. J. & Miller, B. R. (2012). Mosquitoes of Western Uganda. *J Med Entomol* 49, 1289–1306.

Muturi, E. J., Muriu, S., Shililu, J., Mwangangi, J. M., Jacob, B. G., Mbogo, C., Githure, J. & Novak, R. J. (2008). Blood-feeding patterns of Culex quinquefasciatus and other culicines and implications for disease transmission in Mwea rice scheme, Kenya. *Parasitol Res* 102, 1329.

Nakhasi, H. L., Rouault, T. A., Haile, D. J., Liu, T. Y. & Klausner, R. D. (1990). Specific high-affinity binding of host cell proteins to the 3’ region of rubella virus RNA. *New Biol* 2, 255–264.

Nasci, R. S., Savage, H. M., White, D. J., Miller, J. R., Cropp, B. C., Godsey, M. S., Kerst, A. J., Bennett, P., Gottfried, K. & Lanciotti, R. S. (2001). West Nile Virus in Overwintering *Culex* Mosquitoes, New York City, 2000. *Emerg Infect Dis* 7, 742–744.

Nash, D., Mostashari, F., Fine, A., Miller, J., O’Leary, D., Murray, K., Huang, A., Rosenberg, A., Greenberg, A. & other authors. (2001). The outbreak of West Nile virus infection in the New York City area in 1999. *N Engl J Med* 344, 1807–1814.

Nelms, B. M., Macedo, P. A., Kothera, L., Savage, H. M. & Reisen, W. K. (2013). Overwintering

Biology of *Culex* (Diptera: Culicidae) Mosquitoes in the Sacramento Valley of California. *J Med Entomol* 50, 773–790.

Pachler, K., Lebl, K., Berer, D., Rudolf, I., Hubalek, Z. & Nowotny, N. (2014). Putative New West Nile Virus Lineage in *Uranotaenia unguiculata* Mosquitoes, Austria, 2013. *Emerg Infect Dis* 20, 2119–2122.

Padgett, K. A., REISEN, W. K., KAHL-PURCELL, N., FANG, Y., CAHOON-YOUNG, B., CARNEY, R., ANDERSON, N., ZUCCA, L., WOODS, L. & other authors. (2007). West Nile Virus Infection In Tree Squirrels (*Rodentia: Sciuridae*) In California, 2004–2005. *Am J Trop Med Hyg* 76, 810–813.

Papa, A., Xanthopoulou, K., Gewehr, S. & Mourelatos, S. (2011a). Detection of West Nile virus lineage 2 in mosquitoes during a human outbreak in Greece. *Clin Microbiol Infect* 17, 1176–1180.

Papa, A. (2012). West Nile virus infections in Greece: an update. *Expert Rev Anti Infect Ther* 10, 743–750.

Papa, A. (2013). West Nile virus infections in humans—Focus on Greece. *J Clin Virol*.

Papa, A., Bakonyi, T., Xanthopoulou, K., Vázquez, A., Tenorio, A. & Nowotny, N. (2011b). Genetic Characterization of West Nile Virus Lineage 2, Greece, 2010. *Emerg Infect Dis* 17, 920–922.

Papa, A., Xanthopoulou, K., Tsioka, A., Kalaitzopoulou, S. & Mourelatos, S. (2013). West Nile virus in mosquitoes in Greece. *Parasitol Res* 112, 1551–1555.

Parra, B., Lizarazo, J., Jiménez-Arango, J. A., Zea-Vera, A. F., González-Manrique, G., Vargas, J., Angarita, J. A., Zuñiga, G., Lopez-Gonzalez, R. & other authors. (2016). Guillain–Barré Syndrome Associated with Zika Virus Infection in Colombia. *N Engl J Med* 375, 1513–1523.

Petersen LR, Brault AC & Nasci RS. (2013). West nile virus: Review of the literature. *JAMA* 310, 308–315.

Platonov, A. E., Karan', L. S., Shopenskaia, T. A., Fedorova, M. V., Koliasnikova, N. M., Rusakova, N. M., Shishkina, L. V., Arshba, T. E., Zhuravlev, V. I. & other authors. (2011). [Genotyping of West Nile fever virus strains circulating in southern Russia as an epidemiological investigation method: principles and results]. *Zh Mikrobiol Epidemiol Immunobiol* 29–37.

Platonov, A. E., Fedorova, M. V., Karan, L. S., Shopenskaya, T. A., Platonova, O. V. & Zhuravlev, V. I. (2008). Epidemiology of West Nile infection in Volgograd, Russia, in relation to climate change and mosquito (Diptera: Culicidae) bionomics. *Parasitol Res* 103, 45–53.

Platt, K. B., Tucker, B. J., Halbur, P. G., Tiawsirisup, S., Blitvich, B. J., Fabiosa, F. G., Bartholomay, L. C. & Rowley, W. A. (2007). West Nile Virus Viremia in Eastern Chipmunks (*Tamias striatus*) Sufficient for Infecting Different Mosquitoes. *Emerg Infect Dis* 13, 831–837.

Popović, N., Milošević, B., Urošević, A., Poluga, J., Lavadinović, L., Nedelijković, J., Jevtović, D.

& Dulović, O. (2013). Outbreak of West Nile virus infection among humans in Serbia, August to October 2012. *Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull* 18.

Price, W. H. & O'Leary, W. (1967). Geographic variation in the antigenic character of West Nile virus. *Am J Epidemiol* 85, 84–86.

Reisen, W. K., Fang, Y. & Martinez, V. M. (2005). Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. *J Med Entomol* 42, 367–375.

Reisen, W. K., Brault, A. C., Martinez, V. M., Fang, Y., Simmons, K., Garcia, S., Omi-Olsen, E. & Lane, R. S. (2007). Ability of transstadially infected *Ixodes pacificus* (Acari: Ixodidae) to transmit West Nile virus to song sparrows or western fence lizards. *J Med Entomol* 44, 320–327.

Reisen, W. K., Fang, Y., Lothrop, H. D., Martinez, V. M., Wilson, J., O'Connor, P., Carney, R., Cahoon-Young, B., Shafii, M. & Brault, A. C. (2006a). Overwintering of West Nile Virus in Southern California. *J Med Entomol* 43, 344–355.

Reisen, W. K., Fang, Y. & Martinez, V. M. (2006b). Effects of Temperature on the Transmission of West Nile Virus by *Culex tarsalis* (Diptera: Culicidae). *J Med Entomol* 43, 309–317.

Reisen, W. K., Fang, Y., Lothrop, H. D., Martinez, V. M., Wilson, J., O'Connor, P., Carney, R., Cahoon-Young, B., Shafii, M. & Brault, A. C. (2006c). Overwintering of West Nile Virus in Southern California. *J Med Entomol* 43, 344–355.

Richards, S. L., Anderson, S. L. & Lord, C. C. (2014). Vector competence of *Culex pipiens quinquefasciatus* (Diptera: Culicidae) for West Nile virus isolates from Florida. *Trop Med Int Health* 19, 610–617.

Rizzoli, A., Bolzoni, L., Chadwick, E. A., Capelli, G., Montarsi, F., Grisenti, M., de la Puente, J. M., Muñoz, J., Figuerola, J. & other authors. (2015). Understanding West Nile virus ecology in Europe: Culex pipiens host feeding preference in a hotspot of virus emergence. *Parasit Vectors* 8.

Robbins, C. S. (1973). Introduction, Spread, and Present Abundance of the House Sparrow in North America. *Ornithol Monogr* 3–9.

Robich, R. M. & Denlinger, D. L. (2005). Diapause in the mosquito *Culex pipiens* evokes a metabolic switch from blood feeding to sugar gluttony. *Proc Natl Acad Sci U S A* 102, 15912–15917.

Romi, R., Pontuale, G., CIufolini, M. G., Fiorentini, G., Marchi, A., Nicoletti, L., Cocchi, M. & Tamburro, A. (2004). Potential vectors of West Nile Virus following an equine disease outbreak in Italy. *Med Vet Entomol* 18, 14–19.

Schuler-Faccini, L. (2016). Possible Association Between Zika Virus Infection and Microcephaly — Brazil, 2015. *MMWR Morb Mortal Wkly Rep* 65.

Schweizer, M. & Peterhans, E. (2014). Pestiviruses. *Annu Rev Anim Biosci* 2, 141–163.

Scott, T. W., Chow, E., Strickman, D., Kittayapong, P., Wirtz, R. A., Lorenz, L. H. & Edman, J. D. (1993). Blood-Feeding Patterns of Aedes aegypti (Diptera: Culicidae) Collected in a Rural Thai Village. *J Med Entomol* 30, 922–927.

Sejvar, J. J. (2014). Clinical Manifestations and Outcomes of West Nile Virus Infection. *Viruses* 6, 606–623.

Shan, C., Xie, X., Barrett, A. D. T., Garcia-Blanco, M. A., Tesh, R. B., Vasconcelos, P. F. da C., Vasilakis, N., Weaver, S. C. & Shi, P.-Y. (2016). Zika Virus: Diagnosis, Therapeutics, and Vaccine. *ACS Infect Dis* 2, 170–172.

Sirbu, A., Ceianu, C. S., Panculescu-Gatej, R. I., Vazquez, A., Tenorio, A., Rebreanu, R., Niedrig, M., Nicolescu, G. & Pistol, A. (2011). Outbreak of West Nile virus infection in humans, Romania, July to October 2010. *Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull* 16.

Sirivanakarn, S. (1975). The Systematics of Culex vishnui Complex in Southeast Asia with the Diagnosis of Three Common Species (Diptera: Culicidae). *Mosq Syst* 19.

Smith, J. L. & Fonseca, D. M. (2004). Rapid Assays for Identification of Members of the Culex (culex) Pipiens Complex, Their Hybrids, and Other Sibling Species (diptera: Culicidae). *Am J Trop Med Hyg* 70, 339–345.

Smithburn, K. C., Hughes, T. P., Burke, A. W. & Paul, J. H. (1940). A Neurotropic Virus Isolated from the Blood of a Native of Uganda. *Am J Trop Med Hyg* s1-20, 471–492.

Steele, K. E., Linn, M. J., Schoepp, R. J., Komar, N., Geisbert, T. W., Manduca, R. M., Calle, P. P., Raphael, B. L., Clippinger, T. L. & other authors. (2000). Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. *Vet Pathol* 37, 208–224.

Stramer, S. L., Fang, C. T., Foster, G. A., Wagner, A. G., Brodsky, J. P. & Dodd, R. Y. (2005). West Nile virus among blood donors in the United States, 2003 and 2004. *N Engl J Med* 353, 451–459.

Styer, L. M., Bernard, K. A. & Kramer, L. D. (2006). Enhanced Early West Nile Virus Infection in Young Chickens Infected by Mosquito Bite: Effect of Viral Dose. *Am J Trop Med Hyg* 75, 337–345.

Styer, L. M., Kent, K. A., Albright, R. G., Bennett, C. J., Kramer, L. D. & Bernard, K. A. (2007). Mosquitoes Inoculate High Doses of West Nile Virus as They Probe and Feed on Live Hosts. *PLOS Pathog* 3, e132.

Suthar, M. S., Diamond, M. S. & Gale Jr, M. (2013). West Nile virus infection and immunity. *Nat Rev Microbiol* 11, 115–128.

Tiawsirisup, S., Platt, K. B., Tucker, B. J. & Rowley, W. A. (2005). Eastern cottontail rabbits

(Sylvilagus floridanus) develop West Nile virus viremias sufficient for infecting select mosquito species. *Vector Borne Zoonotic Dis Larchmt N* 5, 342–350.

Tilgner, M. & Shi, P.-Y. (2004). Structure and Function of the 3′ Terminal Six Nucleotides of the West Nile Virus Genome in Viral Replication. *J Virol* 78, 8159–8171.

Timofeev, A. V., Ozherelkov, S. V., Pronin, A. V., Deeva, A. V., Karganova, G. G., Elbert, L. B. & Stephenson, J. R. (1998). Immunological basis for protection in a murine model of tick-borne encephalitis by a recombinant adenovirus carrying the gene encoding the NS1 non-structural protein. *J Gen Virol* 79, 689–695.

Turell, M. J. (2012). Members of the *Culex pipiens* Complex as Vectors of Viruses. *J Am Mosq Control Assoc* 28, 123–126.

Turell, M. J., Dohm, D. J., Sardelis, M. R., O'guinn, M. L., Andreadis, T. G. & Blow, J. A. (2005). An Update on the Potential of North American Mosquitoes (Diptera: Culicidae) to Transmit West Nile Virus. *J Med Entomol* 42, 57–62.

Umrigar, M. D. & Pavri, K. M. (1977). Comparative serological studies on Indian strains of West Nile virus isolated from different sources. *Indian J Med Res* 65, 603–612.

Urbanowski, M. D. & Hobman, T. C. (2013). The West Nile Virus Capsid Protein Blocks Apoptosis through a Phosphatidylinositol 3-Kinase-Dependent Mechanism. *J Virol* 87, 872–881.

Vasilakis, N., Cardosa, J., Hanley, K. A., Holmes, E. C. & Weaver, S. C. (2011). Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. *Nat Rev Microbiol* 9, 532–541.

Venter, M. & Swanepoel, R. (2010). West Nile Virus Lineage 2 as a Cause of Zoonotic Neurological Disease in Humans and Horses in Southern Africa. *Vector-Borne Zoonotic Dis* 10, 659–664.

Venter, M., Human, S., Zaayman, D., Gerdes, G. H., Williams, J., Steyl, J., Leman, P. A., Paweska, J. T., Setzkorn, H. & other authors. (2009). Lineage 2 West Nile Virus as Cause of Fatal Neurologic Disease in Horses, South Africa. *Emerg Infect Dis* 15, 877–884.

Weaver, S. C., Costa, F., Garcia-Blanco, M. A., Ko, A. I., Ribeiro, G. S., Saade, G., Shi, P.-Y. & Vasilakis, N. (2016). Zika virus: History, emergence, biology, and prospects for control. *Antiviral Res* 130, 69–80.

Wengler, G. & Wengler, G. (1991). The carboxy-terminal part of the NS 3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. *Virology* 184, 707–715.

Wengler, G. & Wengler, G. (1993). The NS 3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. *Virology* 197, 265–273.

Wheeler, S. S., Woods, L. W., Boyce, W. M., Eckstrand, C. D., Langevin, S. A., Reisen, W. K. & Townsend, A. K. (2014). West Nile virus and non-West Nile virus mortality and coinfection of

American crows (Corvus brachyrhynchos) in California. *Avian Dis* 58, 255–261.

Wikan, N. & Smith, D. R. (2016). Zika virus: history of a newly emerging arbovirus. *Lancet Infect Dis* 16, e119-126.

Wodak, E., Richter, S., Bagó, Z., Revilla-Fernández, S., Weissenböck, H., Nowotny, N. & Winter, P. (2011). Detection and molecular analysis of West Nile virus infections in birds of prey in the eastern part of Austria in 2008 and 2009. *Vet Microbiol* 149, 358–366.

Yu, L. & Markoff, L. (2005). The Topology of Bulges in the Long Stem of the Flavivirus 3′ Stem-Loop Is a Major Determinant of RNA Replication Competence. *J Virol* 79, 2309–2324.

Zeng, L., Falgout, B. & Markoff, L. (1998). Identification of Specific Nucleotide Sequences within the Conserved 3′-SL in the Dengue Type 2 Virus Genome Required for Replication. *J Virol* 72, 7510–7522.

Zou, S., Foster, G. A., Dodd, R. Y., Petersen, L. R. & Stramer, S. L. (2010). West Nile fever characteristics among viremic persons identified through blood donor screening. *J Infect Dis* 202, 1354–1361.