DISSERTATION

EXPLORATIONS IN WEST NILE VIRUS ECOLOGY AND EVOLUTION

Submitted by

Alexandria D. Byas

Department of Microbiology, Immunology & Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2021

Doctoral Committee:

Advisor: Gregory D. Ebel

Richard Bowen
Rebekah Kading
Daniel Sloan

Copyright by Alexandria D. Byas 2021

All Rights Reserved

ABSTRACT

EXPLORATIONS IN WEST NILE VIRUS ECOLOGY AND EVOLUTION

West Nile virus (WNV) continues to be a major cause of human arboviral neuroinvasive disease. Susceptible non-human vertebrates are particularly diverse, ranging from commonly affected birds and horses to less commonly affected species such as alligators. The literature review in Chapter 1 summarizes the pathology caused by West Nile virus during natural infections of humans and non-human animals. While the most well-known findings in human infection involve the central nervous system, WNV can also cause significant lesions in the heart, kidneys and eyes. Time has also revealed chronic neurologic sequelae related to prior human WNV infection. Similarly, neurologic disease is a prominent manifestation of WNV infection in most non-human non-host animals. However, in some avian species, which serve as the vertebrate host for WNV maintenance in nature, severe systemic disease can occur, with neurologic, cardiac, intestinal and renal injury leading to death. The pathology seen in experimental animal models of WNV infection and knowledge gains on viral pathogenesis derived from these animal models are also briefly discussed. A gap in the current literature exists regarding the relationship between the neurotropic nature of WNV in vertebrates, virus propagation and transmission in nature. This and other knowledge gaps, and future directions for research into WNV pathology, are addressed.

In Chapter 2, experimental evolution work is described. For arboviruses, the vertebrate and invertebrate hosts in which they circulate shape viral evolution and can lead to the emergence of new genotypes. Previous work in mosquitoes and birds has identified species-specific effects on viral populations when species were assessed in isolation. We united mosquito and bird species to perform experimental evolution studies which paired *Culex (Cx.) pipiens* with American crows, *Cx. quinquefasciatus* with American crows and *Cx.*

quinquefasciatus with American robins. Crow and *Cx. pipiens* transmission cycles were the most successful and robin and *Cx. quinquefasciatus* transmission cycles were the least successful at reaching three complete rounds of bird-to-mosquito transmission. These findings suggest that crows may be more important to WNV maintenance in nature over robins. The greater success of crow cycles when paired with *Cx. pipiens* in comparison to crows paired with *Cx. quinquefasciatus* may also suggest fitness losses associated with *Cx. quinquefasciatus*. In multiple rounds of transmission, infection rates (WNV-positive mosquito midgut) and transmission-capability (WNV-positive mosquito saliva) decreased with each subsequent round of transmission, suggesting that pairings in isolation experience fitness losses. Competitive fitness assays of transmission cycles exhibited cyclical increases and decreases in fitness as virus moved through crows and mosquitoes, respectively. That the stronger competitive fitness tended to occur with samples from the avian host while virus from mosquitoes tended to have decreased fitness may be consistent with genetic restriction and strong purifying selection in birds and genetic expansion and weak purifying selection in mosquitoes. Sequencing is needed to assess whether differences in transmission cycle success and competitive fitness can be attributed to genetic changes.

In Chapter 3, the avian single cell viral environment is assessed. Error-prone replication of RNA viruses generates the viral diversity required for adaptation to rapidly changing environments. This is crucial for arboviruses whose viral populations exist as mutant swarms maintained between both mosquito and vertebrate hosts. By infecting cells and birds with barcoded WNV stock and sequencing single cells, we demonstrated that the richness and frequency of rare variants in crows far exceeded that found in robins. Moreover, those rare occurring variants were maintained by crows more than they were by robins. We further demonstrated that bird viremia functions as a determinant of multiplicity of infection in peripheral blood mononuclear cells (PBMCs), a significant site of viral replication. We found that increased viremia leads to increased polyinfections of individual PBMCs with maintenance of defective

genomes and less prevalent variants, specifically in crows, presumably through complementation. When two pairings of variably-fit viruses were used to co-infect American robins and American crows, we observed increases in replication for one of the less fit viruses when viremia was higher. The ability of the low fitness virus to better replicate at higher viremia is likely a result of polyinfections and complementation at the cellular level. Our findings suggest that weak purifying selection in highly susceptible crows is attributable to higher viremia, polyinfections and complementation while viral divergence and fewer variants rising to fixation in robins is a result of overall lower levels of viremia and fewer polyinfections.

In Chapter 4, the potential contributions of American alligators to natural WNV ecology are examined. West Nile virus (WNV) overwintering is poorly understood and likely multifactorial. Interest in alligators as a potential amplifying host arose when it was shown that they develop viremias theoretically sufficient to infect mosquitoes. We examined potential ways in which alligators may contribute to the natural ecology of WNV. We experimentally demonstrated that alligators are capable of WNV amplification with subsequent mosquito infection and transmission capability, that WNV-infected mosquitoes readily infect alligators and that water can serve as a source of infection for alligators but does not easily serve as in intermediate means for transmission between birds and alligators. These findings indicate potential mechanisms for maintenance of WNV outside of the primary bird-mosquito transmission cycle.

We performed a diverse array of experiments which utilize novel techniques and technologies to characterize the mechanisms of WNV evolution. We also identified a potential non-avian WNV amplifier host in alligators. This work represents a significant contribution to the West Nile virus literature by working with the unique species which contribute to virus propagation and assessing their effects on viral evolution and ecology.

ACKNOWLEDGEMENTS

This work could not have been completed without the enduring support of my family. JR, your patience and kindness over six years in Colorado and the past ten years have been what grounds me and what allows me to persist. Getting to come home to you and Zeva is the brightest part of my day, when things go well and when things go not so well. Thank you for everything.

To my parents: Mommy, thank you for pushing me to achieve academically and for staying on the phone with me late nights in lab. Daddy, thank you for teaching me that my voice and opinions deserve respect and to accept nothing less. To my brother, TJ, thanks for telling me, "it'll all pay off one day" while teasing me for staying in school forever.

To the Ebel Lab: I was lucky to be in a lab full of people who were there to do good work, help each other when they could and have fun doing it. Michael, I will always cherish our long dissection conversations. Dalit, we learned a lot during our shared bird work. Bekah, I wouldn't want to share a lab bench with anyone else. I'm eager to see where things take you after the PhD. Reyes, thank you for always being willing to help me when it came to library prep and sequencing. Nicole, thank you for teaching me cloning, for your help with my final sequencing work and for good laughs in the lab. Kendra, I enjoyed the short time we were able to share an office. Seph and James, thank you for all of your advice during my first years in the lab. Many thanks are also due to the undergraduate students who worked in the lab, particularly Lexi Robison and Cole Spangler.

Claudia: Thank you for pushing me to keep trying and to think creatively, even when it felt like my dissertation was going to disprove West Nile virus being transmitted by mosquitoes. Thank you for all the good feedback, advice, climbing and friendship.

Emily: Thank you for your contagious work ethic which inspired me to push through the last year of my PhD. Your guidance and encouragement made research during a pandemic possible. Equally important, thank you for being such a good example of how to make a difference outside of work.

Selene: Thank you for inviting me to share your office and for guiding me when I first joined the lab. You taught me everything I know about mosquitoes. Thank you for the best tamales and for making me feel like family.

To my residentmates, Lauren, Laura and Allison: Thank you for making my time in Colorado a fun one and for providing me with much needed work-life balance.

Anna: Thank you for honest conversation about science, music, politics, art, and everything else. You are a gift to the many communities you are a part of. Also, thank you so much for my awesome crow collage that will always serve as a souvenir of this time.

To my committee members: Rebekah and Dan, thank you for providing guidance during this experience. During our meetings, I was challenged to think critically and consider science in a way that was educational and constructive. I appreciate you both for contributing during various checkpoints and for your help in developing my project, my scientific skills and my confidence as a scientist.

Dick, I literally could not have done any of my work if it were not for your lab. Working with you all has been so much fun and it's been a pleasure to share a space with people who love science and truly enjoy what they do. I really enjoyed getting to work with other veterinarian scientists who are happy getting their hands dirty.

Bowen lab folks: Steph, Airn and Paul: Thank you all for being fun and keeping me safe during lots of time in the BSL3. You all were patient with me when I was new to the space and were ridiculously helpful when you didn't have to be.

Angela, I don't even know how to adequately thank you for all that you have done for me during my time here. You taught me everything I needed to know to work in the BSL3. You are patient and giving of your time, even when you are extremely busy. You guided me in ways I didn't know I needed guidance. I'm incredibly grateful for your mentorship and your friendship. Thank you for making the science fun.

Immense thanks go to the following collaborators: Colorado Parks & Wildlife and private landowners who let us trap birds; Aaron Brault of the CDC for help with numerous projects; LAR staff for animal care with particular thanks to Lon Kendall for guidance; Todd Felix of USDA APHIS for good conversation and assistance during field work; Heidi Runge for keeping me on track for 6 years.

Thank you Kristina Quynn and CSU Writes for writing spaces and guidance. Everything I wrote during the PhD from prelims to the last word of this dissertation was touched by CSU Writes and the community of writers that provided me with the motivation and the organization to achieve together what I could not have achieved alone.

Thank you to my mentors in the diagnostic lab including Gary Mason, Chad Frank, EJ Ehrhart, Paula Schaffer, Kristy Pabilonia, Christie Mayo, Amy MacNeill, Tawfik Aboellail and Pat Cole, who is no longer with us. The diagnostic lab was my home base, a place where I always felt certain of my footing. Thank you to CI group 1 for great conversations, particularly Danielle Adney, Kandi Mathiason and Christine Olver. Christine, I will always value our time together doing Feminist Frameworks and my time with Tango.

Many thanks are due to Ed Hoover and Sue VandeWoude for supporting the NIH T32 Program. That funding supported me during my PhD. When I was looking for a lab, I had a conversation with Sue about my goals and she suggested I go to talk to a few people including Greg. She predicted that I would join his lab and she was not wrong.

Lastly, for Greg: I never would have imagined that my pursuit of the PhD would lead me down the path that we have been on for the past five years. I didn't even know what I was looking for in a project, but I knew that I wanted to join a lab where people were valued and treated with respect. I found that in our lab and under your mentorship. Working with you on this project has taught me resilience and flexibility. In the face of failure, you show your students optimism. In the face of challenge, you show your students fortitude. During tough times, you reminded me to consider why we are doing this work. With enhanced conviction, I found even more pride and enjoyment in our work and the overall contributions of our lab to research. Thank you for everything.

If there is anyone I failed to acknowledge, please know that I am truly grateful.

DEDICATION

This work is dedicated to my grandparents:

Helen Byas (1929-2016)

Johnnie Mae Ferguson (1928-2019)

Robert Lee Byas, Sr. (1923-2010)

Hayward Lee Ferguson, Sr. (1923-2010)

Between the two of them, my grandmothers were a daily presence in my life until I left home for college. Granny, my maternal grandmother, had to quit school in the third grade to help raise her siblings. She always told me I had to stay in school. When I think about how far education has taken me in my life and when I think about the sacrifices that she made for her family, I recognize the privilege of education and it is with pride and solemnity that I celebrate this stage of my academic career. Grandma, my paternal grandmother, had a character that shaped who I am. She and my grandfather were organizers during the bus boycotts in the Macon, Georgia civil rights movement. She taught me to stand up for what is right and to be vocal in doing so. She showed me the joy in being an opinionated woman. My grandmothers passed away while I was at Colorado State University and so their memories in particular were on my mind during the time I did this work. All of my grandparents lived full lives dedicated to their families and their communities. I hope to achieve a fraction of their legacy.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS v

DEDICATION ix

Chapter 1. Literature Review: Comparative Pathology of West Nile Virus in Humans and Non-Human Animals 1

Chapter 2. Experimental West Nile Virus Evolution in Wild Birds and Mosquitoes Reveals Species-Specific Transmission Success 35

Chapter 3. Single Cell Analysis of WNV-Infected Birds Reveals Viremia-Influenced Alterations in Virus Evolution 56

Chapter 4. American Alligators are Capable of West Nile Virus Amplification, Mosquito Infection and Transmission 87

Concluding Remarks 106

Appendix 111

Chapter 1. Literature Review: Comparative Pathology of West Nile Virus in Humans and Non-Human Animals[1]

Introduction

Twenty years after its introduction, West Nile virus (WNV, *Flaviviridae: Flavivirus*) continues to be the leading cause of arboviral disease in the continental United States (US) (1). WNV was first isolated in 1937 from the blood of a woman in the West Nile district of Uganda (2). During the 1950s, the virus was isolated from birds, mosquitoes and people in Egypt (3,4). The virus continued to present primarily as recurrent epidemics of mild febrile disease in Africa, the Middle East and Europe (5). However, the disease phenotype was drastically different during outbreaks in the United States, Romania, Russia and Israel, which occurred in the late 1990s and early 2000s (6,7). In the United States, about 1 in 150 clinical patients developed neuroinvasive disease, characterized by meningitis, encephalitis, and/or poliomyelitis (8,9). Of those with neuroinvasive disease, 9% experienced mortality (10). The US outbreak represented the first known introduction of WNV to the Western hemisphere. Outbreaks during the late 1990s in Israel and eastern Europe were also characterized by higher rates of fatal neuroinvasive disease (11,12). Given that these outbreaks occurred in disparate locations, phylogenetic analysis was performed to assess the degree of relatedness between the virus isolates. The strain introduced into the United States (NY99) had greater than 99.8% nucleotide homology to virus isolated from the brain of a goose in Israel from 1998 and human Israeli cases in 1999 (13,14). Isolates from the US also held similar relatedness to isolates from Romania (13,15). It is surmised that these closely related viruses were able to cause disease in these distant locations as a result of globalization. Following its arrival in the United States, the

[1]This chapter includes the complete manuscript cited as “Byas, A.D.; Ebel, G.D. Comparative Pathology of West Nile Virus in Humans and Non-Human Animals. *Pathogens* **2020**, *9*, 48. https://doi.org/10.3390/pathogens9010048”. This article is reproduced with permission and only minimal modifications were made to meet formatting requirements.

virus showed remarkable adaptability to a new environment, quickly spread and is now endemic.

The Virus and Its Ecology

WNV is a member of the genus *Flavivirus* within the family *Flaviviridae*. The WNV genome is a positive-sense, single-stranded RNA molecule of approximately 11,000 nucleotides. Within host cells, viral RNA is translated and processed into 10 proteins: three structural (envelope, membrane and nucleocapsid) and seven nonstructural (NS) (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (13). The virus has two commonly accepted major genetic lineages and at least five additional proposed lineages (6,12,16–20,21). Lineage 1 is widely distributed and contains isolates from Africa, Asia, Australia, Europe, the Middle East, India and North America (21,22). Lineage 2 viruses have been isolated in Africa, Madagascar, the Middle East and Europe (23–26). Initially, lineage 1 viruses were considered to be more virulent than lineage 2 viruses; however, it has been shown that both lineages can cause neuroinvasive disease in humans and animals and lineage 2 has now displaced lineage 1 as the primary cause of WNV disease in Europe (23,27–31).

WNV is maintained in nature by ornithophilic *Culex* mosquitoes and wild birds, with the particular mosquito species varying by region in the United States and worldwide (reviewed extensively by Kramer et al. (32)). *Culex tarsalis* drive epidemic transmission in the western United States while *Culex pipiens* is the main vector in the eastern United States. *Culex quinquefasciatus* and *Culex nigripalpus* are important vectors for WNV in the southeastern United States (33,34). While many avian species have been infected with WNV, house sparrows and American robins have been identified as key to the virus' maintenance in nature (35–37). When the preferred bird hosts become less abundant during late summer and fall, ornithophilic mosquitoes shift their feeding to mammals leading to human epidemics (38).

In addition to horizontal circulation between birds and mosquitoes, WNV maintenance also has been described in the absence of a mosquito vector. There have been multiple reports

of WNV viral RNA detected in carcasses and feces in crow roosts during the winter when mosquitoes are unlikely contributors to transmission, although the transmission mechanism was not specifically elucidated (39,40). Similar occurrences of WNV disease during the winter have been seen in Bald Eagles, where feeding on the carcasses of infected Eared Grebes was considered the most likely source of infection (41). Experimentally, non-vector direct transmission also has been demonstrated in geese, with oral and cloacal shedding serving as the most likely sources of viral transmission (42). Chronic infection may also contribute to winter transmission as infectious virus has been isolated from house sparrows up to 43 days post inoculation (43). Overwintering of WNV may also occur by vertical transmission from adult female mosquitoes to their progeny, although this process is highly inefficient (44,45). In addition to the maintenance of WNV in nature via non-vector mechanisms, WNV disease has also been reported in humans potentially as a result of non-vector transmission including percutaneous exposure, transplacental transmission, breast milk consumption, blood transfusion and organ transplant (46–51).

Interestingly, despite the presence of numerous bird species and mosquitoes which should allow for its maintenance, WNV is not frequently seen in central and South America. There have been no major outbreaks and reports are rare in humans and horses (52–55). Possible causes may include cross-protection due to other circulating flaviviruses or a dilution effect on WNV due to high disease host diversity (56,57).

Much of the research surrounding West Nile virus focuses on (a) viral and ecological factors which affect viral transmission and (b) viral and immune factors which determine pathology and disease. A distinction must be made between the ability for transmission and pathology. While numerous species can be infected and experience pathology and disease, birds are well-established as the primary species which develop the high viral titers required to infect mosquitoes and contribute to virus perpetuation.

Experimental animal models have been used to examine the factors that affect WNV transmission. Some of that work has used wild birds, allowing for direct investigation of the most relevant virus ecology (58–60). Reports also demonstrate that additional species including Eastern cottontail rabbits, fox squirrels and alligators have the potential to infect mosquitoes (61–63), indicating that continued investigation into possible non-avian contributions to WNV maintenance is warranted. While WNV is primarily transmitted and maintained between birds and mosquitoes, it can infect and cause pathology and disease in a wide range of vertebrates. This plasticity is relatively unique amongst arboviruses and has been demonstrated in both natural and experimental infections. When it comes to the viral and immune factors leading to pathology, much has been examined in mouse models and is briefly described below. The pathology seen in natural and experimental infections will be discussed in detail in this review.

Natural WNV Disease

Humans

While approximately 80% of people infected with WNV are asymptomatic, the majority of symptomatic patients experience a mild febrile disease lasting up to a week, a syndrome known as West Nile fever (WNF) (64). In addition to fever, mild disease is characterized by headache, gastrointestinal problems, rash, myalgia, arthralgia and malaise. West Nile neuroinvasive disease (WNND) occurs in less than 1% of infected people and is manifested as multiple syndromes including West Nile meningitis (WNM), West Nile encephalitis (WNE) and West Nile poliomyelitis (WNP) (65). WNM describes inflammation which is primarily restricted to the meninges, the connective tissue coverings of the brain and spinal cord. WNM is typically associated with more favorable outcomes and is the most common manifestation of neuroinvasive disease in younger patients (66). WNE is a more invasive disease process, in which the brain parenchyma is infected and inflamed. This is more commonly seen in older adults and the immunosuppressed (10). Clinical signs associated with WNE range in severity and can include tremors, cerebellar ataxia, and general Parkinsonism (66). WNP is associated

with infection of the anterior horn cells (lower motor neurons) of the spinal cord, resulting in a polio-like flaccid paralysis, which at its most severe can cause quadriplegia and respiratory impairment (67–69). WNP is distinct from a rarer Guillain-Barre syndrome which has been reported in association with WNV infection (65). While these classifications are important, clinical presentation of WNV infection may present as any mixture of these syndromes.

Histological findings in West Nile neuroinvasive disease are nonspecific and typical of many viral encephalitides, and these are characterized by perivascular lymphocytic infiltrates, microglial nodules, neuronal loss, and neuronophagia (67,70–72). In severe cases, necrosis can be seen (73). Most commonly affected regions of the central nervous system (CNS) have extrapyramidal (movement-related) function and include the brainstem (medulla and pons), deep gray matter nuclei (substantia nigra of the basal ganglia and thalamus), and cerebellum with gray matter being the most severely affected (70,73–76). In the spinal cord, the anterior horns (ventral horns) and anterior spinal nerve roots are frequently involved and associated lower motor neuron loss results in muscle weakness (71,74,75,77,78). Clinically observed muscle weakness correlates histologically to neurogenic atrophy of the skeletal muscle (78).

While fever and neurological disease are the most well-known clinical manifestations of West Nile virus infection, there are less frequently observed non-neurological clinical findings. Ocular manifestations seen in WNV infection, specifically those associated with the optic nerve and the retina, can be considered an extension of the brain. In one report, 80% of patients with WNV neurologic disease have chorioretinal involvement, primarily multifocal chorioretinitis, although it is often asymptomatic and self-limiting (79,80). Additional WNV ocular manifestations include vitritis, optic neuritis and retinal hemorrhage (81–83). Renal failure has been documented in one study as present in 9% of hospitalized WNV patients and in another study, 21% of deceased WNV patients had renal failure (84,85). More commonly seen in other vertebrate species, myocarditis has been reported occasionally in humans (86–88). Other rare lesions include hepatitis, pancreatitis, orchitis and myositis (5,89–91).

There are also sequelae to acute disease which have been observed in both convalescent and fully recovered patients. Subsequent to WNF, WNM and WNE, there are estimates that 50% of patients are affected by neurocognitive or functional impairment (92,93). Neuropsychological sequelae include memory problems, headache, cognitive dysfunction, depression and fatigue (93–95). Motor skill abnormalities include tremors, fatigue, decreased strength and abnormal reflexes (93,94). On magnetic resonance imaging (MRI), WNV survivors had significant cortical thinning in the frontal and limbic cortices and regional atrophy in the cerebellum, brain stem, thalamus, putamen and globus pallidus (93). These neuropsychological and motor skills issues are interpreted to be the result of prolonged or permanent damage to the nervous system.

There is also some evidence for persistent infection of WNV (96–98). Sequelae from systemic infections have been observed with the kidneys being one of the most common extra-nervous system sites of disease. WNV is able to persist chronically in the kidneys up to seven years and previous infection lead to chronic kidney disease (97,99).

Birds

During the introduction of West Nile virus to the United States, mortality observed in the Bronx zoo and surrounding areas showed that avian species across multiple orders could be affected and included common crows, a fish crow, black-billed magpies, a black-crowned night heron, laughing gulls, a mallard duck, Himalayan Impeyan pheasants, a Blyth's tragopan, Chilean flamingos, guanay cormorants, bronze-winged ducks, a northern bald eagle and a snowy owl (100). Lesion variability observed between species is likely multifactorial and related to host factors and intrinsic viral factors which depend on virus strain (101). Specifically, levels of high viremia associated with being an amplifier host have been shown to correlate with mortality in some birds (102). Interestingly, high levels of mortality do not always correlate with histopathologic signs and this may be a measure of acute infection resulting in death and occurring in such rapid fashion that lesions do not develop histologically (101).

In contrast to humans and other affected species, most major organ systems have been shown to be affected in natural avian WNV infections. Neurological manifestations are indicative of viral encephalitis and are similar to findings seen in humans and other non-host vertebrate species. This encephalitis is characterized as a lymphoplasmacytic meningoencephalitis with an occasional heterophilic component, heterophils being the avian functional equivalent to the neutrophil. Histologically, there is perivascular cuffing, glial nodules and gliosis, neuronal necrosis and occasional hemorrhage. Frequently affected regions of the nervous system include the brain stem and, gray matter of the spinal cord, cerebellum and thalamus. In addition to the nervous system, myocarditis is a common lesion in birds (103–105). Inflammation and necrosis have also been reported in the gastrointestinal tract, kidney, spleen, liver, pancreas, lung, adrenal glands, thyroid, thymus, bursa, bone marrow and skeletal muscle (101). Lesions in naturally infected birds have been previously reviewed in detail by Gamino and Höfle (95). This chart has been modified to include the most recent literature as well as pathologic findings in birds experimentally infected with WNV (Table 1.1) (106–116). Ocular lesions are seen in raptors (red-tailed hawks, Cooper's hawks, bald eagles, golden eagles, goshawks) and owls (great horned owls, barred owls) and range from lymphoplasmacytic pectenitis and chorioretinal inflammation and scarring to generalized endophthalmitis (110,116–119). Vasculitis has occasionally been noted within multiple organs in a variety of affected avian species (103,120,121). While systemic infection is a hallmark of WNV infection in many species, adult domestic chickens and turkeys do not frequently experience significant disease (122,123). Age and a developed immune system likely contribute to this refractory nature of adult chickens as young chicks are susceptible and histologic lesions include myocardial necrosis, necrosis, nephritis and pneumonitis, and rare encephalitis (123).

Table 1.1. Distribution of West Nile virus (WNV)-infected bird lesions in natural and experimental infections. Modified from Gamino and Höfle (95).

Order: ACCI: Accipitriformes, ANSE: Anseriformes, CHAR: Charadriiformes, CICO: Ciconiiformes, FALC: Falconiformes, GALL: Galliformes, PASS: Passeriformes, PELE: Pelecaniformes, PHOE: Phoenicopteriformes, PSIT: Psittaciformes, and STRI: Strigiformes.

Family: ACCI: Accipitridae, ANAT: Anatidae, LARI: Laridae, ARDE: Ardeidae, FALC: Falconidae, PHAS: Phasianidae, CORV: Corvidae, LANI Laniidae, PASS: Passeridae, FRIN: Fringillidae, PHAL: Phalacrocoracidae, PHOE: Phoenicopteridae, PSIT: Psittacidae, STRO: Strigopidae, and STRI: Strigidae. ND: No described lesion. Tissues were collected at necropsy but no description of lesions (present or absent) is provided. NT: Tissue not tested. Tissue not analyzed in the necropsy. +: Lesion present. Lesion described by at least one author for the tissue. −: Lesion absent. Lesion stated as absent or not specifically described by any author for the tissue.

Table 1. Distribution of WNV-infected bird lesions in natural and experimental infections.

Order	**ACCI**	**ANSE**	**CHAR**	**CICO**	**FALC**	**GALL**	**PASS**				**PELE**	**PHOE**	**PSIT**		**STRI**
Family	**ACCI**	**ANAT**	**LARI**	**ARDE**	**FALC**	**PHAS**	**CORV**	**LANI**	**PASS**	**FRIN**	**PHAL**	**PHOE**	**PSIT**	**STRO**	**STRI**
Brain															
Perivascular cuffs	+	+	+	ND	+	+	+	+	+	-	-	+	+	+	+
Gliosis/glial nodules	+	+	+	ND	+	+	+	+	-	-	-	+	+	+	+
Meningeal inflammation	+	+	-	ND	+	-	+	-	-	-	+	-	-	-	+
Neuronal degeneration and necrosis	+	+	+	ND	+	+	+	-	-	-	+	-	-	+	+
Vasculitis	+	-	-	ND	-	-	+	-	+	-	-	-	-	-	-
Hemorrhage	+	-	-	ND	-	+	-	-	-	-	+	-	-	-	+
Spinal cord															
Inflammation	+	+	ND	ND	ND	ND	ND	NT	ND	NT	ND	ND	ND	NT	+
Neuronal degeneration and necrosis	-	+	ND	ND	ND	ND	ND	NT	ND	NT	ND	ND	ND	NT	+
Peripheral nervous system															
Inflammation	+	-	ND	ND	+	+	-	NT	NT	NT	ND	ND	ND	NT	+
Eye															
Retinitis	+	NT	NT	NT	NT	-	ND	NT	NT	NT	NT	NT	+	NT	+
Retinal necrosis	+	NT	NT	NT	NT	-	ND	NT	NT	NT	NT	NT	-	NT	-
Pectenitis	+	NT	NT	NT	NT	+	ND	NT	NT	NT	NT	NT	-	NT	-
Uveitis (inc. iris, choroid, ciliary body)	+	NT	NT	NT	+	+	ND	NT	NT	NT	NT	NT	-	NT	-
Heart															
Inflammation	+	+	+	+	+	+	+	+	-	-	+	+	+	NT	+
Myofiber necrosis	+	+	-	-	+	+	+	-	-	-	-	-	+	NT	+
Myofibril lysis and mineralization	+	+	+	+	+	+	+	-	-	-	+	+	+	NT	-
Vasculitis	-	-	-	-	+	-	+	-	-	-	-	-	-	NT	-
Hemorrhage	+	-	+	+	-	+	+	-	-	-	+	+	+	NT	-
Gastrointestinal tract															
Inflammation	+	-	ND	ND	-	+	+	-	ND	-	-	-	+	NT	+
Enterocyte necrosis	-	+	ND	ND	-	+	+	-	ND	-	-	-	-	NT	+
Crypt necrosis	-	-	ND	ND	-	-	+	+	ND	-	+	+	+	NT	+

Hemorrhage	-	+	ND	ND	-	-	+	-	ND	-	-	+	-	NT	-
Liver															
Inflammation	+	+	ND	ND	+	+	+	+	-	+	-	-	+	+	+
Hepatocyte necrosis	+	+	ND	ND	-	+	+	+	+	+	+	+	+	+	+
Vasculitis	-	-	ND	ND	+	-	-	-	-	-	-	-	-	-	-
Bile duct hyperplasia	+	-	ND	ND	-	-	-	-	-	-	-	+	-	-	-
Hemosiderosis	+	-	ND	ND	-	+	+	-	+	-	-	-	-	-	+
Hemorrhage	+	-	ND	ND	-	-	+	-	-	-	-	-	-	-	-
Kidney															
Inflammation (interstitial)	+	+	ND	+	+	+	+	ND	+	+	+	+	+	NT	+
Tubular necrosis	+	+	ND	-	-	+	+	ND	+	+	-	-	+	NT	+
Glomerular necrosis	-	-	ND	-	-	-	-	ND	-	-	-	-	+	NT	+
Vasculitis	-	-	ND	-	+	-	-	ND	-	-	-	-	-	NT	-
Hemorrhage	-	+	ND	-	-	-	-	ND	-	-	-	-	-	NT	-
Lung														NT	
Inflammation	+	-	ND	ND	-	+	+	-	+	-	ND	ND	-	NT	+
Necrosis	+	-	ND	ND	-	-	+	-	-	-	ND	ND	-	NT	+
Vasculitis	-	-	ND	ND	-	-	+	-	-	-	ND	ND	-	NT	-
Edema	+	-	ND	ND	+	-	-	+	+	-	ND	ND	-	NT	-
Spleen															
Lymphoid necrosis/apoptosis	+	+	ND	+	-	+	+	+	-	+	+	ND	+	NT	+
Lymphoid depletion	+	+	ND	-	+	+	-	-	-	-	-	ND	-	NT	+
Fibrin deposition	-	-	ND	+	-	+	-	+	-	-	+	ND	-	NT	+
Hemorrhage	-	+	ND	+	-	-	-	-	-	-	+	ND	-	NT	-
Hemosiderosis	+	+	ND	-	-	+	+	-	+	-	-	ND	-	NT	+
Vasculitis	-	-	ND	-	+	-	-	-	-	-	-	ND	-	NT	-
Other lymphoid organs															
Thymic lymphoid necrosis	NT	+	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	-	NT	+
Bursal epithelial atrophy-apoptosis	+	-	NT	NT	NT	ND	NT	NT	-	NT	NT	NT	ND	NT	+
Bursal lymphoid atrophy-apoptosis	+	+	NT	NT	NT	+	NT	NT	+	NT	NT	NT	ND	NT	+
Bone marrow necrosis	ND	-	NT	NT	NT	-	+	NT	NT	NT	NT	NT	ND	NT	ND
Endocrine system															
Pancreatic necrosis	-	+	ND	ND	-	+	-	+	ND	-	-	+	+	NT	+
Pancreatic inflammation	+	+	ND	ND	+	+	-	-	ND	-	+	+	+	NT	+
Adrenal gland necrosis	-	ND	ND	ND	NT	+	-	NT	NT	NT	ND	ND	+	NT	-
Adrenal gland inflammation	+	ND	ND	ND	NT	+	+	NT	NT	NT	ND	ND	+	NT	+
Thyroid gland necrosis	-	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	ND	NT	ND
Thyroid gland inflammation	+	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	ND	NT	ND
Skin															
Inflammation	ND	+	NT	NT	NT	-	NT	ND	NT	-	NT	NT	+	NT	-
Skeletal muscle															
Myofibril degeneration and necrosis	+	-	NT	NT	+	-	ND	NT	NT	-	NT	NT	+	NT	+
Inflammation	+	-	NT	NT	+	+	ND	NT	NT	-	NT	NT	+	NT	+
Fibrosis	+	-	NT	NT	-	-	ND	NT	NT	-	NT	NT	-	NT	+

Gonads															
Inflammation	-	-	ND	ND	-	+	NT	ND	NT	NT	ND	ND	-	NT	+
Necrosis	-	-	ND	ND	-	-	NT	ND	NT	NT	ND	ND	-	NT	+

Horses

Clinical signs of WNV infection in horses, aside from fever, are primarily related to nervous system infection and inflammation. Approximately 20% of infected horses develop clinical neurological signs (132). Mortality in unvaccinated horses is between 30 and 50%, inclusive of both natural death and elective euthanasia (55,133). Of equine survivors, remnant neurological signs are present in between 10–20% of horses (134). The most severe clinical signs in horses include limb ataxia, tetraparesis, paraparesis, recumbency, seizures and death (133,135). Additional signs include cranial nerve deficits, muscle fasciculations, hyperexcitability and behavioral changes (52,133,136,137).

In horses, histologically affected spinal cords are similar to those of humans with polioencephalomyelitis and are characterized by lymphocytes and fewer numbers of macrophages and neutrophils cuffing vessels, glial nodules, and occasional neuronophagia (138). Ventral and lateral horns of the spinal cord gray matter are most affected (136,138). In addition to the spinal cord, the gray matter of the midbrain and hindbrain are commonly affected (136). Perivascular hemorrhage is also seen in horses. The cerebral cortex seems to be least affected. Extraneural disease in horses includes sporadic renal hemorrhage, lymphoid atrophy and myocarditis (138).

Several commonalities unite the clinical presentation of WNV in the most affected vertebrates: birds, horses and humans. These include neurotropism characterized by primarily mononuclear inflammation, neuronal necrosis and gliosis which frequently affects gray matter and varies according to host and virus strain. In addition, renal and ocular tropism seems to be a conserved aspect of clinical disease in birds and humans.

Additional Affected Vertebrate Species

In addition to birds, humans and horses, WNV infects and causes disease in an extraordinary array of vertebrate species (see Figure 1.1).

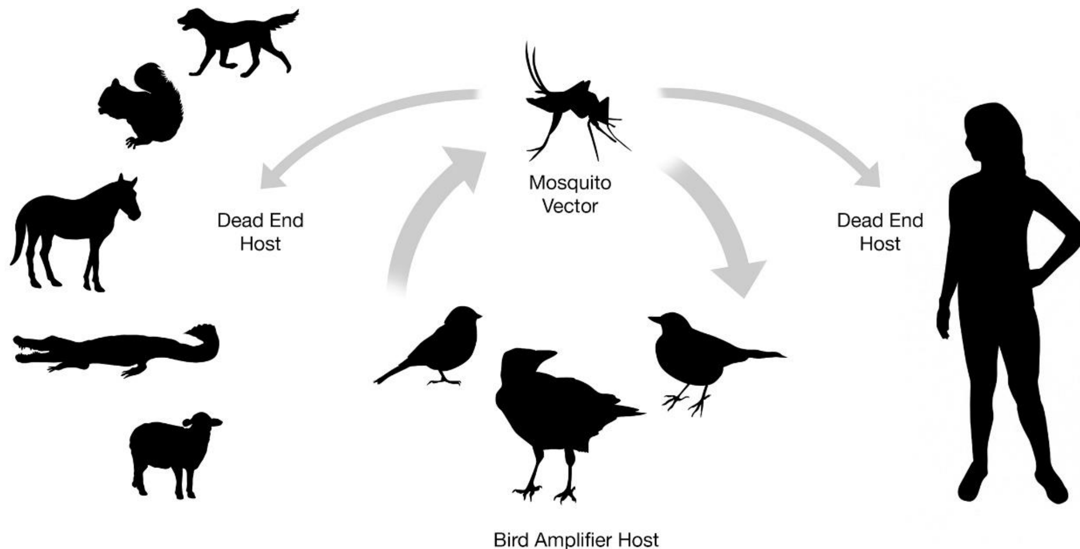


Figure 1.1. West Nile virus transmission cycle. West Nile virus circulates between Culex species mosquitoes and avian amplifying hosts. Humans and a wide array of vertebrate species can be affected as dead-end or non-amplifying hosts.

In many of these, clinical disease is solely neurological and thus similar to what is seen in humans and horses. This is observed in numerous single-animal case reports including those of an alpaca, harbor seal, reindeer, Barbary macaque, white-tailed deer and polar bear (139–144). Histologically, these animals had a nonsuppurative meningoencephalitis which frequently and preferentially affected the gray matter of the brainstem and spinal cord. Similarly, convulsions and ataxia in multiple WNV-infected sheep were seen in association with lymphoplasmacytic meningoencephalitis and myelitis characterized by perivascular cuffing and necrosis (139,145,146). Histopathology from a sheep which is representative of many species is shown in Figure 1.2.

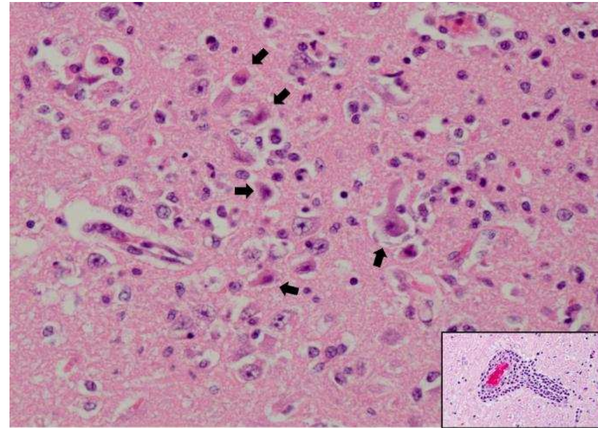


Figure 1.2. Hippocampus of a WNV-infected sheep with neuronal necrosis and necrotic debris (arrows) and abundant mixed inflammation admixed with remaining neurons. Hematoxylin and eosin staining at 400× magnification. Inset: Perivascular cuffing with lymphocytes and plasma cells. Hematoxylin and eosin staining at 200× magnification. Slide courtesy of Dr. Chad B. Frank, Colorado State University.

While the expected neurological disease is common in dead-end hosts, some species and individual animals have unique disease presentations. In addition to encephalitis, fox squirrels experience myocarditis, which has been mentioned as a common manifestation in birds. (147). In alligators infected with WNV, systemic disease is sometimes accompanied by inflammatory nodules in the skin composed of lymphocytes and macrophages (148–150). In a case report of a dog, polioencephalomyelitis and myocarditis were accompanied by vasculitis, pancreatitis and plasmacytic synovitis (inflammation of the articular synovial surface) (151). In an arctic wolf, renal vasculitis was a significant finding (152).

While WNV antigen distribution is frequently described in both natural infection in humans and animals and animal experimental infection, immunohistochemical antigen distribution and staining may indicate sites of replication but do not always correlate to pathology or organ dysfunction; therefore, extensive lists of IHC and PCR results will not be included in this review (76,153,154). Additionally, IHC does not always align with well-documented sites of virus replication (155). Antigen can be focal and sparse and is typically visible in only 50% of fatal WNV neuroinvasive disease cases, making this modality relatively insensitive (75).

Biomedical Models of WNV Infection

Mice

A vast body of knowledge regarding the pathogenesis of WNV encephalitis has resulted from experimental infections using animal models. Interestingly, wild-type mice are resistant to WNV infection, but all classical laboratory mice strains are susceptible to infections administered by the intracerebral and intraperitoneal routes (156). This susceptibility has been mapped to a mutation in the *OAS1b* gene, which is regulated by interferon and contributes to viral RNA degradation (157–159). Histopathology in the laboratory mouse is characterized by neuronal necrosis, lymphohistiocytic perivascular infiltrates, glial nodules, neuronal satellitosis and neuronophagia in the cerebral cortex, cerebellum, brainstem, hippocampus and spinal cord (154,160,161). Commonly used strains of mice include C57BL/6 mice, mice on a C57BL/6 background and C3H/HeN mice. Disease is not limited to the brain in mice. Thymic atrophy has been described in affected mice, and similarly, the thymus has been affected in ducklings and other birds (154,162). Gastrointestinal lesions in mice including dilation of the stomach and small intestine and villus blunting (shortening) have been seen in association with degeneration and necrosis of intestinal myenteric ganglia (155,163). This may serve as a model for gastrointestinal dysmotility seen in humans after flavivirus infection (164). Gastrointestinal disease is also frequently seen in birds.

An abundance of information about WNV pathogenesis has been yielded from mouse models of infection. After inoculation, initial viral replication is thought to occur in Langerhans dendritic cells of the skin (165). These infected Langerhans cells then migrate to draining lymph nodes where virus can be transported to the systemic circulation after return through the thoracic and lymphatic ducts (166,167). At that point a primary viremia allows for viral dissemination to the visceral organs for additional viral replication (168,169). Virus must then traverse the blood–brain barrier before replication in nervous tissue to cause encephalitis disease. The lesions of encephalitis and poliomyelitis seen in WNV infection cannot be

attributed solely to viral replication or the immune response, but rather are the result of both direct viral injury and immune-mediated pathogenesis. Specific work examines the immunologic mechanisms by which lymphocytes, the primary responding cell type, are recruited into the central nervous system where inflammatory mediators exacerbate viral-induced damage (161,170–175).

Hamsters

Golden hamsters are excellent models for WNV encephalitis because they mimic human disease in regards to length of viremia, muscle weakness, gastrointestinal signs, respiratory symptoms and clinical signs including tremors (156,176–178). Hamsters infected with WNV develop neurologic symptoms including tremors and hind limb paralysis associated with progressive pathology in the cerebellum and spinal cord (179). Histologically, this correlates with lymphoplasmacytic inflammation in those nervous system tissues. The significance of axonal transport as a contributor to entrance of WNV to the CNS has also been demonstrated in hamsters (180). Hamsters also have persistent renal infection, which mimics the chronic renal infection observed in humans (181). Overall, hamsters provide good models of disease, but mice are more easily genetically manipulated to assess specific alterations to the immune system.

Non-Human Primates

In addition to mice, some early experimental work examining WNV encephalitis was performed in non-human primates. Depending on the viral strain, intracerebral inoculation resulted in disease ranging from asymptomatic infection to febrile disease to overt encephalitis (182,183). Histopathology in acute WNV encephalitis includes severe perivascular and diffuse lymphoplasmacytic inflammation, neuronal degeneration and necrosis and glial nodules in the gray matter. Lesions were most frequent in the cerebellum, brainstem and anterior horns of the spinal cord. Chronic lesions also include loss of Purkinje cells in the cerebellum and spinal motor neurons. In contrast, more recent experimental infections examining antibody response to

WNV infection in immunocompetent rhesus macaques and marmosets resulted in low to absent viremia and minimal to absent infectious virus detection in the CNS (184,185). Persistent viral infection of the CNS, kidney and spleen were seen up to 167 days post infection in some non-human primates (183). Partially as a result of the associated financial costs and ethical implications, current use of non-human primates in WNV research is infrequent. However, they may be used in continued development of a human vaccine.

Additional Animal Species Used in WNV Biomedical Research

Multiple other species have been used in WNV biomedical research and contributed to varying aspects of the field. Rats were used in some of the earliest experimental work studying WNV and while older rats were resistant to fatal disease, newborn rats were susceptible (186,187). Histologically these infections were relatively mild and characterized by meningitis and mild inflammatory infiltrates and perivascular cuffs. The most severe lesions were seen in the hippocampus. In contemporary work, the use of rats is primarily related to toxicology studies which assess for the safety of WNV vaccines (188–190).

As the species most frequently and significantly affected by WNV infections aside from birds and humans, horses have been used in pathogenesis work and in efficacy and safety studies required for approval of WNV equine vaccines (191–196). There are currently four United States Department of Agriculture-licensed equine WNV vaccines available in the United States (197).

New Zealand White rabbits have been demonstrated to be appropriate models for non-lethal WNV infections as only weanling rabbits demonstrate severe lesions and clinical symptoms (198). Rabbits are also commonly used to generate antibodies against WNV which can be utilized in assays such as immunohistochemistry. Other species which have been examined as animal models for WNV infection include pigs, dog and cats (199,200). Experimental infection of snakes and bats failed to demonstrate significant viremia and disease

(201,202). Young chicks have occasionally been used as models for avian infection as wild birds can be difficult to work with and require trapping and special care (203–207).

Importantly, wild birds have been used for experimental WNV infections, not as a model for mammalian disease, but to assess pathogenesis and factors affecting viral evolution and transmission in the most relevant amplifying hosts (58,60,208–215). This use is crucial as cell culture and computer modelling are insufficient in their representation of complex living systems.

Summary and Future Directions

One of the more interesting features of the emergence of WNV is its somewhat unique ecological generalism. Whereas most flaviviruses productively infect a relatively restricted subset of animal species, WNV can infect an extraordinarily wide array of vertebrate taxa. This generalism has produced opportunities to learn about viral pathogenesis across taxa and increase knowledge regarding the extent to which pathogenic mechanisms may be conserved. For example, the most notable feature of WNV disease in humans is neurotropism resulting most frequently in encephalitis and, more uncommonly, meningitis and other neurologic syndromes. This set of clinical presentations is largely conserved in horses, birds and mice. The significance of this broad conservation is that human and avian neurological disease can be modeled with an uncommonly high degree of fidelity using mice, which has facilitated improved understanding of mechanisms of neuroinvasion and subsequent immune-mediated injury.

Other clinical syndromes are also present across broadly divergent vertebrate taxa. Renal tropism is a feature of WNV infection in birds that facilitated early surveillance efforts. Several human studies have demonstrated that renal failure may contribute to poor long-term outcomes among WNV survivors (99). These studies also documented the presence of WNV in urine sediment from individuals with a history of WNV (216). In some of these patients, WNV was detected up to nine years after acute infection. Similarly, avian kidneys have been found to persistently harbor WNV after natural and experimental infection. WNV, though typically considered a neurotropic virus, also has significant renal tropism that contributes to

pathogenesis in humans, and possibly avian hosts. The degree to which renal infection impacts the health of naturally infected hosts, including humans and birds remains to be fully described.

The detection of persistent infection in the kidneys of people and birds raises another important common feature of WNV pathogenesis that is conserved across several vertebrates and has been previously underappreciated. This is the extent to which WNV can persist within vertebrates despite the induction of a strong antibody and cell-mediated immune response. Monkeys, mice, birds and people have all been demonstrated to develop long-term persistent infections after acute WNV. This has also been noted in a human case of Russian spring-summer encephalitis (caused by tick-borne encephalitis virus) that resulted in progressive neurological disease approximately thirteen years after acute infection (217). Chronic lesions of prior viral and immune-mediated injury may manifest as the human populations previously affected by WNV continue to age. An important question that remains to be answered is whether persistent, chronic infection by WNV and other flaviviruses, years after acute infection, is an underrecognized aspect of viral pathogenesis.

References

1. Curren, E.J.; Lehman, J.; Kolsin, J.; Walker, W.L.; Martin, S.W.; Staples, J.E.; Hills, S.L.; Gould, C.V.; Rabe, I.B.; Fischer, M.; et al. West nile virus and other nationally notifiable arboviral diseases—United States, 2017. Morb. Mortal. Wkly. Rep. 2018, 67, 1137–1142.

2. Smithburn, K.C.; Hughes, T.P.; Burke, A.W.; Paul, J.H. A Neurotropic Virus Isolated from the Blood of a Native of Uganda. Am. J. Trop. Med. Hyg. 1940, 1, 471–492.

3. Melnick, J.L.; Paul, J.R.; Riordan, J.T.; Barnett, V.H.; Goldblum, N.; Zabin, E. Isolation from Human Sera in Egypt of a Virus Apparently Identical to West Nile Virus. Exp. Biol. Med. 1951, 77, 661–665.

4. Hurlbut, H.S.; Rizk, F.; Taylor, R.M.; Work, T.H. A study of the ecology of West Nile virus in Egypt. Am. J. Trop. Med. Hyg. 1956, 5, 579–620.

5. Perelman, A.; Stern, J. Acute pancreatitis in West Nile fever. Am. J. Trop. Med. Hyg. 1974, 23, 1150–1152.

6. Lvov, D.K.; Butenko, A.M.; Gromashevsky, V.L.; Larichev, V.P.; Gaidamovich, S.Y.; Vyshemirsky, O.I.; Zhukov, A.N.; Lazorenko, V.V.; Salko, V.N.; Kovtunov, A.I.; et al. Isolation of two strains of West Nile virus during an outbreak in southern Russia, 1999. Emerg. Infect. Dis. 2000, 6, 373–376.

7. Siegel-Itzkovich, J. Twelve die of West Nile virus in Israel. Br. Med. J. 2000, 321, 724.

8. Rossi, S.L.; Ross, T.M.; Evans, J.D. West Nile virus. Clin. Lab. Med. 2010, 30, 47–65.

9. Weaver, S.C.; Barrett, A.D.T. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat. Rev. Microbiol. 2004, 2, 789–801.

10. O'Leary, D.R.; Marfin, A.A.; Montgomery, S.P.; Kipp, A.M.; Lehman, J.A.; Biggerstaff, B.J.; Elko, V.L.; Collins, P.D.; Jones, J.E.; Campbell, G.L. The epidemic of West Nile virus in the United States, 2002. Vector-Borne Zoonotic Dis. 2004, 4, 61–70.

11. Outbreak of West Nile-like viral encephalitis—New York, 1999. Morb. Mortal. Wkly. Rep. 1999, 48, 845–849.

12. Hubálek, Z.; Halouzka, J. West Nile fever—A reemerging mosquito-borne viral disease in Europe. Emerg. Infect. Dis. 1999, 5, 643–650.

13. Lanciotti, R.S.; Roehrig, J.T.; Deubel, V.; Smith, J.; Parker, M.; Steele, K.; Crise, B.; Volpe, K.E.; Crabtree, M.B.; Scherret, J.H.; et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the Northeastern United States. Science 1999, 286, 2333–2337.

14. Giladi, M.; Metzkor-Cotter, E.; Martin, D.A.; Siegman-Igra, Y.; Korczyn, A.D.; Rosso, R.; Berger, S.A.; Campbell, G.L.; Lanciotti, R.S. West Nile Encephalitis in Israel, 1999: The New York Connection. Emerg. Infect. Dis. 2001, 7, 659–661.

15. Anderson, J.F.; Andreadis, T.G.; Vossbrinck, C.R.; Tirrell, S.; Wakem, E.M.; French, R.A.; Garmendia, A.E.; Van Kruiningen, H.J. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science 1999, 286, 2331–2333.

16. Bakonyi, T.; Hubálek, Z.; Rudolf, I.; Nowotny, N. Novel flavivirus or new lineage of West Nile virus, central Europe. Emerg. Infect. Dis. 2005, 11, 225.

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

18. Bondre, V.P.; Jadi, R.S.; Mishra, A.C.; Yergolkar, P.N.; Correspondence, V.A.A. West Nile virus isolates from India: Evidence for a distinct genetic lineage. J. Gen. Virol. 2007, 88, 875–884.

19. Vázquez, A.; Sánchez-Seco, M.; Ruiz, S.; Molero, F.; Hernandez, L.; Moreno, J.; Magallanes, A.; Tejedor, C.G.; Tenorio, A. Putative new lineage of West Nile virus, Spain. Emerg. Infect. Dis. 2010, 16, 549–552.

20. King, A.; Lefkowitz, E.; Adams, M.; Carstens, E. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier: New York, NY, USA, 2011; ISBN 978-0-12-384684-6.

21. Charrel, R.N.; Brault, A.C.; Gallian, P.; Lemasson, J.J.; Murgue, B.; Murri, S.; Pastorino, B.; Zeller, H.; De Chesse, R.; De Micco, P.; et al. Evolutionary relationship between Old World West Nile virus strains: Evidence for viral gene flow between Africa, the Middle East, and Europe. Virology 2003, 315, 381–388.

22. Lanciotti, R.S.; Ebel, G.D.; Deubel, V.; Kerst, A.J.; Murri, S.; Meyer, R.; Bowen, M.; McKinney, N.; Morrill, W.E.; Crabtree, M.B.; et al. Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. Virology 2002, 298, 96–105.

23. Bakonyi, T.; Ivanics, E.; Erdélyi, K.; Ursu, K.; Ferenczi, E.; Weissenböck, H.; Nowotny, N. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. Emerg. Infect. Dis. 2006, 12, 618–623.

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

25. Valiakos, G.; Touloudi, A.; Iacovakis, C.; Athanasiou, L.; Birtsas, P.; Spyrou, V.; Billinis, C. Molecular detection and phylogenetic analysis of West Nile virus lineage 2 in sedentary wild birds (Eurasian magpie), Greece, 2010. Euro Surveill. 2011, 16, 19862.

26. Shahhosseini, N.; Chinikar, S.; Moosa-Kazemi, S.H.; Sedaghat, M.M.; Kayedi, M.H.; Lühken, R.; Schmidt-Chanasit, J. West Nile Virus lineage-2 in Culex specimens from Iran. Trop. Med. Int. Heal. 2017, 22, 1343–1349.

27. Cotar, A.I.; Fălcuţă, E.; Dinu, S.; Necula, A.; Bîrluţiu, V.; Ceianu, C.S.; Prioteasa, F.L. West Nile virus lineage 2 in Romania, 2015-2016: Co-circulation and strain replacement. Parasites Vectors 2018, 11, 562.

28. Kolodziejek, J.; Seidel, B.; Jungbauer, C.; Dimmel, K.; Kolodziejek, M.; Rudolf, I.; Hubálek, Z.; Allerberger, F.; Nowotny, N. West Nile Virus Positive Blood Donation and Subsequent Entomological Investigation, Austria, 2014. PLoS ONE 2015, 10, e0126381.

29. Wodak, E.; Richter, S.; Bagó, Z.; Revilla-Fernández, S.; Weissenböck, H.; Nowotny, N.; Winter, P. 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. 2011, 149, 358–366.

30. Busquets, N.; Laranjo-González, M.; Soler, M.; Nicolás, O.; Rivas, R.; Talavera, S.; Villalba, R.; San Miguel, E.; Torner, N.; Aranda, C.; et al. Detection of West Nile virus lineage 2 in North-Eastern Spain (Catalonia). Transbound. Emerg. Dis. 2019, 66, 617–621.

31. Vilibic-Cavlek, T.; Savic, V.; Petrovic, T.; Toplak, I.; Barbic, L.; Petric, D.; Tabain, I.; Hrnjakovic-Cvjetkovic, I.; Bogdanic, M.; Klobucar, A.; et al. Emerging Trends in the Epidemiology of West Nile and Usutu Virus Infections in Southern Europe. Front. Vet. Sci. 2019, 6, 437.

32. Kramer, L.D.; Styer, L.M.; Ebel, G.D. A Global Perspective on the Epidemiology of West Nile Virus. Annu. Rev. Entomol. 2008, 53, 61–81.

33. Godsey, M.S.; King, R.J.; Burkhalter, K.; Delorey, M.; Colton, L.; Charnetzky, D.; Sutherland, G.; Ezenwa, V.O.; Wilson, L.A.; Coffey, M.; et al. Ecology of potential West Nile virus vectors in Southeastern Louisiana: Enzootic transmission in the relative absence of Culex quinquefasciatus. Am. J. Trop. Med. Hyg. 2013, 88, 986–996.

34. Bolling, B.G.; Moore, C.G.; Anderson, S.L.; Blair, C.D.; Beaty, B.J. Entomological Studies along the Colorado Front Range during a Period of Intense West Nile virus activity. J. Am. Mosq. Control Assoc. 2007, 23, 37–46.

35. Hamer, G.L.; Chaves, L.F.; Anderson, T.K.; Kitron, U.D.; Brawn, J.D.; Ruiz, M.O.; Loss, S.R.; Walker, E.D.; Goldberg, T.L. Fine-scale variation in vector host use and force of infection drive localized patterns of West Nile virus transmission. PLoS ONE 2011, 6, e23767.

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

37. Kilpatrick, A.M.; Daszak, P.; Jones, M.J.; Marra, P.P.; Kramer, L.D.; Marm Kilpatrick, A.; Daszak, P.; Jones, M.J.; Marra, P.P.; Kramer, L.D. Host heterogeneity dominates West Nile virus transmission. Proc. R. Soc. B Biol. Sci. 2006, 273, 2327–2333.

38. Kilpatrick, A.M.; Kramer, L.D.; Jones, M.J.; Marra, P.P.; Daszak, P. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. PLoS Biol. 2006, 4, 606–610.

39. 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. Crow deaths caused by West Nile virus during winter. Emerg. Infect. Dis. 2007, 13, 1912–1914.

40. Hinton, M.G.; Reisen, W.K.; Wheeler, S.S.; Townsend, A.K. West Nile Virus Activity in a Winter Roost of American Crows (Corvus brachyrhynchos): Is Bird-To-Bird Transmission Important in Persistence and Amplification? J. Med. Entomol. 2015, 52, 683–692.

41. 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. West Nile Virus Transmission in Winter: The 2013 Great Salt Lake Bald Eagle and Eared Grebes Mortality Event. PLoS Curr. 2014, 6, doi:10.1371/currents.outbreaks.b0f031fc8db2a827d9da0f30f0766871.

42. Banet-Noach, C.; Simanov, L.; Malkinson, M. Direct (non-vector) transmission of West Nile virus in geese. Avian Pathol. 2003, 32, 489–494.

43. Nemeth, N.; Young, G.; Ndaluka, C.; Bielefeldt-Ohmann, H.; Komar, N.; Bowen, R. Persistent West Nile virus infection in the house sparrow (Passer domesticus). Arch. Virol. 2009, 154, 783–789.

44. 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. West Nile virus in overwintering culex mosquitoes, New York City, 2000. Emerg. Infect. Dis. 2001, 7, 742–744.

45. Nelms, B.M.; Fechter-Leggett, E.; Carroll, B.D.; Macedo, P.; Kluh, S.; Reisen, W.K. Experimental and natural vertical transmission of West Nile virus by California Culex (Diptera: Culicidae) mosquitoes. J. Med. Entomol. 2013, 50, 371–378.

46. Laboratory-acquired West Nile virus infections—United States, 2002. Morb. Mortal. Wkly. Rep. 2002, 51, 1133–1135.

47. Centers for Disease Control and Prevention (CDC) West Nile virus infection among turkey breeder farm workers—Wisconsin, 2002. MMWR. Morb. Mortal. Wkly. Rep. 2003, 52, 1017–1019.

48. Centers for Disease Control and Prevention (CDC) Intrauterine West Nile virus infection—New York, 2002. Morb. Mortal. Wkly. Rep. 2002, 51, 1135–1136.

49. Hinckley, A.F.; O'Leary, D.R.; Hayes, E.B. Transmission of West Nile virus through human breast milk seems to be rare. Pediatrics 2007, 119, e666–e671.

50. Dodd, R.Y.; Foster, G.A.; Stramer, S.L. Keeping Blood Transfusion Safe From West Nile Virus: American Red Cross Experience, 2003 to 2012. Transfus. Med. Rev. 2015, 29, 153–161.

51. Anesi, J.A.; Silveira, F.P. AST Infectious Diseases Community of Practice Arenaviruses and West Nile Virus in solid organ transplant recipients: Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. Clin. Transplant. 2019, 33, e13576.

52. Morales, M.A.; Barrandeguy, M.; Fabbri, C.; Garcia, J.B.; Vissani, A.; Trono, K.; Gutierrez, G.; Pigretti, S.; Menchaca, H.; Garrido, N.; et al. West Nile virus isolation from equines in Argentina, 2006. Emerg. Infect. Dis. 2006, 12, 1559–1561.

53. Blitvich, B.J.; Fernandez-Salas, I.; Contreras-Cordero, J.F.; Marlenee, N.L.; Gonzalez-Rojas, J.I.; Komar, N.; Gubler, D.J.; Calisher, C.H.; Beaty, B.J. Serologic evidence of West Nile virus infection in horses, Coahuila State, Mexico. Emerg. Infect. Dis. 2003, 9, 853–856.

54. Komar, N.; Clark, G.G. West Nile virus activity in Latin America and the Caribbean. Rev. Panam. Salud Publica/Pan Am. J. Public Health 2006, 19, 112–117.

55. Silva, A.S.G.; Matos, A.C.D.; da Cunha, M.A.C.R.; Rehfeld, I.S.; Galinari, G.C.F.; Marcelino, S.A.C.; Saraiva, L.H.G.; Martins, N.R. da S.; Maranhão, R. de P.A.; Lobato, Z.I.P.; et al. West Nile virus associated with equid encephalitis in Brazil, 2018. Transbound. Emerg. Dis. 2019, 66, 445–453.

56. Yamshchikov, G.; Borisevich, V.; Kwok, C.W.; Nistler, R.; Kohlmeier, J.; Seregin, A.; Chaporgina, E.; Benedict, S.; Yamshchikov, V. The suitability of yellow fever and Japanese encephalitis vaccines for immunization against West Nile virus. Vaccine 2005, 23, 4785–4792.

57. Swaddle, J.P.; Calos, S.E. Increased avian diversity is associated with lower incidence of human West Nile infection: Observation of the dilution effect. PLoS ONE 2008, 3, e2488.

58. Grubaugh, N.D.; Smith, D.R.; Brackney, D.E.; Bosco-Lauth, A.M.; Fauver, J.R.; Campbell, C.L.; Felix, T.A.; Romo, H.; Duggal, N.K.; Dietrich, E.A.; et al. Experimental Evolution of an RNA Virus in Wild Birds: Evidence for Host-Dependent Impacts on Population Structure and Competitive Fitness. PLoS Pathog. 2015, 11, e1004874.

59. 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. A single positively selected West Nile viral mutation confers increased virogenesis in American crows. Nat. Genet. 2007, 39, 1162.

60. VanDalen, K.K.; Hall, J.S.; Clark, L.; McLean, R.G.; Smeraski, C. West Nile virus infection in American Robins: New insights on dose response. PLoS ONE 2013, 8, e68537.

61. Klenk, K.; Snow, J.; Morgan, K.; Bowen, R.; Stephens, M.; Foster, F.; Gordy, P.; Beckett, S.; Komar, N.; Gubler, D.; et al. Alligators as West Nile Virus Amplifiers. Emerg. Infect. Dis. 2004, 10, 2150–2155.

62. Platt, K.B.; Tucker, B.J.; Halbur, P.G.; Blitvich, B.J.; Fabiosa, F.G.; Mullin, K.; Parikh, G.R.; Kitikoon, P.; Bartholomay, L.C.; Rowley, W.A. Fox squirrels (Sciurus niger) develop West Nile virus viremias sufficient for infecting select mosquito species. Vector Borne Zoonotic Dis. 2008, 8, 225–233.

63. Tiawsirisup, S.; Platt, K.B.; Tucker, B.J.; Rowley, W.A. Eastern cottontail rabbits (Sylvilagus floridanus) develop West Nile virus viremias sufficient for infecting select mosquito species. Vector-Borne Zoonotic Dis. 2005, 5, 342–350.

64. Saxena, V.; Bolling, B.G.; Wang, T. Emerging Pathogens. In Clinics in Laboratory Medicine; Ismail, N., Snyder, J., William Pasculle, A., Eds.; Saunders: Philadelphia, USA, 2017.

65. Sejvar, J.J. Clinical manifestations and outcomes of West Nile virus infection. Viruses 2014, 6, 606–623.

66. Sejvar, J.J.; Haddad, M.B.; Tierney, B.C.; Campbell, G.L.; Marfin, A.A.; Van Gerpen, J.A.; Fleischauer, A.; Leis, A.A.; Stokic, D.S.; Petersen, L.R. Neurologic Manifestations and Outcome of West Nile Virus Infection. J. Am. Med. Assoc. 2003, 290, 511–515.

67. Agamanolis, D.P.; Leslie, M.J.; Caveny, E.A.; Guarner, J.; Shieh, W.J.; Zaki, S.R. Neuropathological findings in West Nile virus encephalitis: A case report. Ann. Neurol. 2003, 54, 547–551.

68. Doron, S.I.; Dashe, J.F.; Adelman, L.S.; Brown, W.F.; Werner, B.G.; Hadley, S. Histopathologically proven poliomyelitis with quadriplegia and loss of brainstem function due to West Nile virus infection. Clin. Infect. Dis. 2003, 37, e74–e77.

69. Fan, E.; Needham, D.M.; Brunton, J.; Kern, R.Z.; Stewart, T.E. West Nile virus infection in the intensive care unit: A case series and literature review. Can. Respir. J. 2004, 11, 354–358.

70. Kelley, T.W.; Prayson, R.A.; Ruiz, A.I.; Isada, C.M.; Gordon, S.M.; Kelley, MD, T.W.; Ruiz, MD, A.I.; Prayson, MD, R.A.; Gordon, MD, S.M.; Isada, MD, C.M. The Neuropathology of West Nile Virus Meningoencephalitis: A Report of Two Cases and Review of the Literature. Am. J. Clin. Pathol. 2003, 119, 749–753.

71. Bouffard, J.P.; Riudavets, M.A.; Holman, R.; Rushing, E.J. Neuropathology of the brain and spinal cord in human West Nile virus infection. Clin. Neuropathol. 2004, 23, 59–61.

72. Shieh, W.J.; Guarner, J.; Layton, M.; Fine, A.; Miller, J.; Nash, D.; Campbell, G.L.; Roehrig, J.T.; Gubler, D.J.; Zaki, S.R. The role of pathology in an investigation of an outbreak of West Nile encephalitis in New York, 1999. Emerg. Infect. Dis. 2000, 6, 370–372.

73. Schafernak, K.T.; Bigio, E.H. West Nile virus encephalomyelitis with polio-like paralysis & nigral degeneration. Can. J. Neurol. Sci. 2006, 33, 407–410.

74. Fratkin, J.D.; Leis, A.A.; Stokic, D.S.; Slavinski, S.A.; Geiss, R.W. Spinal Cord Neuropathology in Human West Nile Virus Infection. Arch. Pathol. Lab. Med. 2004, 128, 533–537.

75. Guarner, J.; Shieh, W.-J.; Hunter, S.; Paddock, C.D.; Morken, T.; Campbell, G.L.; Marfin, A.A.; Zaki, S.R. Clinicopathologic study and laboratory diagnosis of 23 cases with West Nile virus encephalomyelitis. Hum. Pathol. 2004, 35, 983–990.

76. Omalu, B.I.; Shakir, A.A.; Wang, G.; Lipkin, W.I.; Wiley, C.A. Fatal fulminant pan-meningo-polioencephalitis due to West Nile virus. Brain Pathol. 2003, 13, 465–472.

77. Leis, A.A.; Fratkin, J.; Stokic, D.S.; Harrington, T.; Webb, R.M.; Slavinski, S.A. West nile poliomyelitis. Lancet Infect. Dis. 2003, 3, 9–10.

78. Sampson, B.A.; Nields, H.; Armbrustmacher, V.; Asnis, D.S. Muscle weakness in West Nile encephalitis is due to destruction of motor neurons. Hum. Pathol. 2003, 34, 628–629.

79. Singh, S.; Kumar, A. Ocular manifestations of emerging flaviviruses and the blood-retinal barrier. Viruses 2018, 10, 530.

80. Khairallah, M.; Ben Yahia, S.; Ladjimi, A.; Zeghidi, H.; Ben Romdhane, F.; Besbes, L.; Zaouali, S.; Messaoud, R. Chorioretinal involvement in patients with West Nile virus infection. Ophthalmology 2004, 111, 2065–2070.

81. Garg, S.; Jampol, L.M. Systemic and intraocular manifestations of West Nile virus infection. Surv. Ophthalmol. 2005, 50, 3–13.

82. Bains, H.S.; Jampol, L.M.; Caughron, M.C.; Parnell, J.R. Vitritis and chorioretinitis in a patient with West Nile virus infection. Arch. Ophthalmol. 2003, 121, 205–207.

83. Hershberger, V.S.; Augsburger, J.J.; Hutchins, R.K.; Miller, S.A.; Horwitz, J.A.; Bergmann, M. Chorioretinal lesions in nonfatal cases of West Nile virus infection. Ophthalmology 2003, 110, 1732–1736.

84. Murray, K.O.; Baraniuk, S.; Resnick, M.; Arafat, R.; Kilborn, C.; Shallenberger, R.; York, T.L.; Martinez, D.; Malkoff, M.; Elgawley, N.; et al. Clinical investigation of hospitalized human cases of West Nile virus infection in Houston, Texas, 2002–2004. Vector Borne Zoonotic Dis. 2008, 8, 167–174.

85. Lindsey, N.P.; Sejvar, J.J.; Bode, A.V.; Pape, W.J.; Campbell, G.L. Delayed mortality in a cohort of persons hospitalized with West Nile virus disease in Colorado in 2003. Vector Borne Zoonotic Dis. 2012, 12, 230–235.

86. Albagali, C.; Chaimoff, R. A case of West Nile myocarditis. Harefuah 1959, 57, 274–276.

87. Kushawaha, A.; Jadonath, S.; Mobarakai, N. West nile virus myocarditis causing a fatal arrhythmia: A case report. Cases J. 2009, 2, 7147.

88. Pergam, S.A.; DeLong, C.E.; Echevarria, L.; Scully, G.; Goade, D.E. Myocarditis in West Nile Virus infection. Am. J. Trop. Med. Hyg. 2006, 75, 1232–1233.

89. Mathiot, C.C.; Gonzalez, J.P.; Georges, A.J. Current problems of arboviruses in central Africa. Bull. Soc. Pathol. Exot. Fil. 1988, 81, 396–401.

90. Smith, R.D.; Konoplev, S.; DeCourten-Myers, G.; Brown, T. West Nile virus encephalitis with myositis and orchitis. Hum. Pathol. 2004, 35, 254–258.

91. Sampson, B.A.; Ambrosi, C.; Charlot, A.; Reiber, K.; Veress, J.F.; Armbrustmacher, V. The pathology of human West Nile virus infection. Hum. Pathol. 2000, 31, 527–531.

92. Sejvar, J.J.; Curns, A.T.; Welburg, L.; Jones, J.F.; Lundgren, L.M.; Capuron, L.; Pape, J.; Reeves, W.C.; Campbel, G.L. Neurocognitive and functional outcomes in persons recovering from West Nile virus illness. J. Neuropsychol. 2008, 2, 477–499.

93. Murray, K.O.; Nolan, M.S.; Ronca, S.E.; Datta, S.; Govindarajan, K.; Narayana, P.A.; Salazar, L.; Woods, S.P.; Hasbun, R. The neurocognitive and Mri Outcomes of West nile Virus infection: Preliminary analysis Using an external control group Kristy. Front. Neurol. 2018, 9, 111.

94. Carson, P.J.; Konewko, P.; Wold, K.S.; Mariani, P.; Goli, S.; Bergloff, P.; Crosby, R.D. Long-Term Clinical and Neuropsychological Outcomes of West Nile Virus Infection. Clin. Infect. Dis. 2006, 43, 723–730.

95. Cook, R.L.; Xu, X.; Yablonsky, E.J.; Sakata, N.; Tripp, J.H.; Hess, R.; Piazza, P.; Rinaldo, C.R. Demographic and clinical factors associated with persistent symptoms after West Nile virus infection. Am. J. Trop. Med. Hyg. 2010, 83, 1133–1136.

96. Busch, M.P.; Kleinman, S.H.; Tobler, L.H.; Kamel, H.T.; Norris, P.J.; Walsh, I.; Matud, J.L.; Prince, H.E.; Lanciotti, R.S.; Wright, D.J.; et al. Virus and Antibody Dynamics in Acute West Nile Virus Infection. J. Infect. Dis. 2008, 198, 984–993.

97. Murray, K.; Walker, C.; Herrington, E.; Lewis, J.A.; McCormick, J.; Beasley, D.W.C.; Tesh, R.B.; Fisher-Hoch, S. Persistent Infection with West Nile Virus Years after Initial Infection. J. Infect. Dis. 2010, 201, 2–4.

98. Penn, R.G.; Guarner, J.; Sejvar, J.J.; Hartman, H.; McComb, R.D.; Nevins, D.L.; Bhatnagar, J.; Zaki, S.R. Persistent neuroinvasive West Nile virus infection in an immunocompromised patient. Clin. Infect. Dis. 2006, 42, 680–683.

99. Nolan, M.S.; Podoll, A.S.; Hause, A.M.; Akers, K.M.; Finkel, K.W.; Murray, K.O. Prevalence of chronic kidney disease and progression of disease over time among patients enrolled in the Houston West Nile virus cohort. PLoS ONE 2012, 7, e40374.

100. 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.; Larsen, T.; et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet. Pathol. 2000, 37, 208–224.

101. Gamino, V.; Höfle, U. Pathology and tissue tropism of natural West Nile virus infection in birds: A review. Vet. Res. 2013, 44, 39.

102. Komar, N.; Langevin, S.; Hinten, S.; Nemeth, N.; Edwards, E.; Hettler, D.; Davis, B.; Bowen, R.; Bunning, M. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg. Infect. Dis. 2003, 9, 311–322.

103. Nemeth, N.; Gould, D.; Bowen, R.; Komar, N. Natural and experimental West Nile virus infection in five raptor species. J. Wildl. Dis. 2006, 42, 1–13.

104. Palmieri, C.; Franca, M.; Uzal, F.; Anderson, M.; Barr, B.; Woods, L.; Moore, J.; Woolcock, P.; Shivaprasad, H.L. Pathology and Immunohistochemical Findings of West Nile Virus Infection in Psittaciformes. Vet. Pathol. 2011, 48, 975–984.

105. Eckstrand, C.D.; Woods, L.W.; Diab, S.S.; Crossley, B.M.; Giannitti, F. Diagnostic exercise: High mortality in a flock of chukar partridge chicks (Alectoris chukar) in California. Vet. Pathol. 2015, 52, 189–192.

106. Nemeth, N.M.; Bosco-Lauth, A.M.; Williams, L.M.; Bowen, R.A.; Brown, J.D. West Nile Virus Infection in Ruffed Grouse (Bonasa umbellus): Experimental Infection and Protective Effects of Vaccination. Vet. Pathol. 2017, 54, 901–911.

107. Bakonyi, T.; Gajdon, G.K.; Schwing, R.; Vogl, W.; Häbich, A.C.; Thaller, D.; Weissenböck, H.; Rudolf, I.; Hubálek, Z.; Nowotny, N. Chronic West Nile virus infection in kea (Nestor notabilis). Vet. Microbiol. 2016, 183, 135–139.

108. Gamino, V.; Escribano-Romero, E.; Gutiérrez-Guzmán, A.V.; Blázquez, A.B.; Saiz, J.C.; Höfle, U. Oculopathologic Findings in Flavivirus-Infected Gallinaceous Birds. Vet. Pathol. 2014, 51, 1113–1116.

109. Ziegler, U.; Angenvoort, J.; Fischer, D.; Fast, C.; Eiden, M.; Rodriguez, A.V.; Revilla-Fernández, S.; Nowotny, N.; de la Fuente, J.G.; Lierz, M.; et al. Pathogenesis of West Nile virus lineage 1 and 2 in experimentally infected large falcons. Vet. Microbiol. 2013, 161, 263–273.

110. Wünschmann, A.; Timurkaan, N.; Armien, A.G.; Bueno Padilla, I.; Glaser, A.; Redig, P.T. Clinical, pathological, and immunohistochemical findings in bald eagles (Haliaeetus leucocephalus) and golden eagles (Aquila chrysaetos) naturally infected with West Nile virus. J. Vet. Diagn. Investig. 2014, 26, 599–609.

111. Hofmeister, E.; Porter, R.E.; Christian Franson, J. Experimental susceptibility of Wood Ducks (Aix sponsa) for West Nile virus. J. Wildl. Dis. 2015, 51, 411–418.

112. Cox, S.L.; Campbell, G.D.; Nemeth, N.M. Outbreaks of West Nile virus in captive waterfowl in Ontario, Canada. Avian Pathol. 2015, 44, 135–141.

113. Gamino, V.; Escribano-Romero, E.; Blázquez, A.B.; Gutiérrez-Guzmán, A.V.; Martín-Acebes, M.; Saiz, J.C.; Höfle, U. Experimental North American West Nile Virus Infection in the Red-legged Partridge (Alectoris rufa). Vet. Pathol. 2016, 53, 585–593.

114. Hofmeister, E.K.; Lund, M.; Shearn Bochsler, V. West Nile Virus Infection in American Singer Canaries: An Experimental Model in a Highly Susceptible Avian Species. Vet. Pathol. 2018, 55, 531–538.

115. Hubálek, Z.; Kosina, M.; Rudolf, I.; Mendel, J.; Straková, P.; Tomešek, M. Mortality of Goshawks (Accipiter gentilis) due to west nile virus lineage 2. Vector-Borne Zoonotic Dis. 2018, 18, 624–627.

116. Wünschmann, A.; Armién, A.G.; Khatri, M.; Martinez, L.C.; Willette, M.; Glaser, A.; Alvarez, J.; Redig, P. Ocular Lesions in Red-Tailed Hawks (Buteo jamaicensis) With Naturally Acquired West Nile Disease. Vet. Pathol. 2017, 54, 277–287.

117. Lopes, H.; Redig, P.; Glaser, A.; Armien, A.; Wünschmann, A. Clinical findings, lesions, and viral antigen distribution in great gray owls (Strix nebulosa) and barred owls (Strix varia) with spontaneous West Nile virus infection. Avian Dis. 2007, 51, 140–145.

118. Wünschmann, A.; Shivers, J.; Bender, J.; Carroll, L.; Fuller, S.; Saggese, M.; van Wettere, A.; Redig, P. Pathologic and Immunohistochemical Findings in Goshawks (Accipiter gentilis) and Great Horned Owls (Bubo virginianus) Naturally Infected with West Nile Virus. Avian Dis. 2005, 49, 252–259.

119. Pauli, A.M.; Cruz-Martinez, L.A.; Ponder, J.B.; Redig, P.T.; Glaser, A.L.; Klauss, G.; Schoster, J.V.; Wünschmann, A. Ophthalmologic and oculopathologic findings in red-tailed

hawks and Cooper's hawks with naturally acquired West Nile virus infection. J. Am. Vet. Med. Assoc. 2007, 231, 1240–1248.

120. Nemeth, N.M.; Thomsen, B.V.; Spraker, T.R.; Benson, J.M.; Bosco-Lauth, A.M.; Oesterle, P.T.; Bright, J.M.; Muth, J.P.; Campbell, T.W.; Gidlewski, T.L.; et al. Clinical and Pathologic Responses of American Crows (Corvus brachyrhynchos) and Fish Crows (C ossifragus) to Experimental West Nile Virus Infection. Vet. Pathol. 2011, 48, 1061–1074.

121. Ernest, H.B.; Woods, L.W.; Hoar, B.R. Pathology associated with West Nile virus infections in the yellow-billed magpie (Pica nuttalli): A California endemic bird. J. Wildl. Dis. 2010, 46, 401–408.

122. Swayne, D.E.; Beck, J.R.; Zaki, S. Pathogenicity of West Nile Virus for Turkeys. Avian Dis. 2000, 44, 932.

123. Senne, D.A.; Pedersen, J.C.; Hutto, D.L.; Taylor, W.D.; Schmitt, B.J.; Panigrahy, B. Pathogenicity of West Nile Virus in Chickens. Avian Dis. 2000, 44, 642.

124. Rosenberg, K.V.; Dokter, A.M.; Blancher, P.J.; Sauer, J.R.; Smith, A.C.; Smith, P.A.; Stanton, J.C.; Panjabi, A.; Helft, L.; Parr, M.; et al. Decline of the North American avifauna. Science 2019, 366, 120–124.

125. George, T.L.; Harrigan, R.J.; Lamanna, J.A.; Desante, D.F.; Saracco, J.F.; Smith, T.B. Persistent impacts of West Nile virus on North American bird populations. Proc. Natl. Acad. Sci. USA 2015, 112, 14290–14294.

126. LaDeau, S.L.; Kilpatrick, A.M.; Marra, P.P. West Nile virus emergence and large-scale declines of North American bird populations. Nature 2007, 447, 710–713.

127. Wheeler, S.S.; Barker, C.M.; Fang, Y.; Armijos, M.V.; Carroll, B.D.; Husted, S.; Johnson, W.O.; Reisen, W.K. Differential Impact of West Nile Virus on California Birds. Condor 2009, 111, 1–20.

128. Kilpatrick, A.M.; Wheeler, S.S. Impact of West Nile Virus on Bird Populations: Limited Lasting Effects, Evidence for Recovery, and Gaps in Our Understanding of Impacts on Ecosystems. J. Med. Entomol. 2019, 56, 1491–1497.

129. Gibbs, S.E.J.; Wimberly, M.C.; Madden, M.; Masour, J.; Yabsley, M.J.; Stallknecht, D.E. Factors affecting the geographic distribution of West Nile virus in Georgia, USA: 2002–2004. Vector Borne Zoonotic Dis. 2006, 6, 73–82.

130. Crowder, D.W.; Dykstra, E.A.; Brauner, J.M.; Duffy, A.; Reed, C.; Martin, E.; Peterson, W.; Carrière, Y.; Dutilleul, P.; Owen, J.P. West nile virus prevalence across landscapes is mediated by local effects of agriculture on vector and host communities. PLoS ONE 2013, 8, e55006.

131. Bradley, C.A.; Gibbs, S.E.J.; Altizer, S. Urban land use predicts West Nile virus exposure in songbirds. Ecol. Appl. 2008, 18, 1083–1092.

132. Reisen, W.K.; Hahn, D.C. Comparison of immune responses of brown-headed cowbird and related blackbirds to West Nile and other mosquito-borne encephalitis viruses. J. Wildl. Dis. 2007, 43, 439–449.

133. Ward, M.P.; Schuermann, J.A.; Highfield, L.D.; Murray, K.O. Characteristics of an outbreak of West Nile virus encephalomyelitis in a previously uninfected population of horses. Vet. Microbiol. 2006, 118, 255–259.

134. Paré, J.; Moore, A. West Nile virus in horses-What do you need to know to diagnose the disease? Can. Vet. J. 2018, 59, 1119–1120.

135. Venter, M.; Pretorius, M.; Fuller, J.A.; Botha, E.; Rakgotho, M.; Stivaktas, V.; Weyer, C.; Romito, M.; Williams, J. West Nile virus lineage 2 in horses and other animals with neurologic disease, South Africa, 2008–2015. Emerg. Infect. Dis. 2017, 23, 2060–2064.

136. Porter, M.B.; Long, M.T.; Getman, L.M.; Giguère, S.; MacKay, R.J.; Lester, G.D.; Alleman, A.R.; Wamsley, H.L.; Franklin, R.P.; Jacks, S.; et al. West Nile virus encephalomyelitis in horses: 46 cases (2001). J. Am. Vet. Med. Assoc. 2003, 222, 1241–1247.

137. Delcambre, G.H.; Liu, J.; Streit, W.J.; Shaw, G.P.J.; Vallario, K.; Herrington, J.; Wenzlow, N.; Barr, K.L.; Long, M.T. Phenotypic characterisation of cell populations in the brains of horses experimentally infected with West Nile virus. Equine Vet. J. 2017, 49, 815–820.

138. Cantile, C.; Piero, F. Del; Di Guardo, G.; Arispici, M.; Del Piero, F.; Di Guardo, G.; Arispici, M. Pathologic and Immunohistochemical Findings in Naturally Occurring West Nile Virus Infection in Horses. Vet Pathol. 2001, 38, 414–421.

139. Yaeger, M.; Yoon, K.-J.; Schwartz, K.; Berkland, L. West Nile virus meningoencephalitis in a Suri alpaca and Suffolk ewe. J. Vet. Diagn. Investig. 2004, 16, 64–66.

140. Del Piero, F.; Stremme, D.W.; Habecker, P.L.; Cantile, C. West Nile Flavivirus Polioencephalomyelitis in a harbor seal (Phoca vitulina). Vet. Pathol. 2006, 43, 58–61.

141. Palmer, M.V.; Stoffregen, W.C.; Rogers, D.G.; Hamir, A.N.; Richt, J.A.; Pedersen, D.D.; Waters, W.R. West Nile virus infection in reindeer (Rangifer tarandus). J. Vet. Diagn. Investig. 2004, 16, 219–222.

142. Dutton, C.J.; Quinnell, M.; Lindsay, R.; DeLay, J.; Barker, I.K. Paraparesis in a polar bear (Ursus maritimus) associated with West Nile virus infection. J. Zoo Wildl. Med. 2009, 40, 568–571.

143. Miller, D.L.; Radi, Z.A.; Baldwin, C.; Ingram, D. Fatal West Nile virus infection in a white-tailed deer (Odocoileus virginianus). J. Wildl. Dis. 2005, 41, 246–249.

144. Ølberg, R.A.; Barker, I.K.; Crawshaw, G.J.; Bertelsen, M.F.; Drebot, M.A.; Andonova, M. West Nile virus encephalitis in a Barbary macaque (Macaca sylvanus). Emerg. Infect. Dis. 2004, 10, 712–714.

145. Tyler, J.W.; Turnquist, S.E.; David, A.T.; Kleiboeker, S.B.; Middleton, J.R. West Nile virus encephalomyelitis in a sheep. J. Vet. Intern. Med. 2003, 17, 242–244.

146. Rimoldi, G.; Mete, A.; Adaska, J.M.; Anderson, M.L.; Symmes, K.P.; Diab, S. West Nile Virus Infection in Sheep. Vet. Pathol. 2017, 54, 155–158.

147. Root, J.J.; Oesterle, P.T.; Nemeth, N.M.; Klenk, K.; Gould, D.H.; McLean, R.G.; Clark, L.; Hall, J.S. Experimental infection of fox squirrels (Sciurus niger) with West Nile virus. Am. J. Trop. Med. Hyg. 2006, 75, 697–701.

148. Nevarez, J.G.; Mitchell, M.A.; Morgan, T.; Roy, A.; Johnson, A. Association of West Nile Virus with Lymphohistiocytic Proliferative Cutaneous Lesions in American Alligators (Alligator mississippiensis) Detected by RT-PCR. J. Zoo Wildl. Med. 2008, 39, 562–567.

149. Jacobson, E.R.; Ginn, P.E.; Troutman, J.M.; Farina, L.; Stark, L.; Klenk, K.; Burkhalter, K.L.; Komar, N. West Nile virus infection in farmed American alligators (Alligator mississippiensis) in Florida. J. Wildl. Dis. 2005, 41, 96–106.

150. Miller, D.L.; Mauel, M.J.; Baldwin, C.; Burtle, G.; Ingram, D.; Hines, M.E.; Frazier, K.S. West Nile virus in farmed alligators. Emerg. Infect. Dis. 2003, 9, 794–799.

151. Cannon, A.B.; Luff, J.A.; Brault, A.C.; MacLachlan, N.J.; Case, J.B.; Green, E.N.G.; Sykes, J.E. Acute encephalitis, polyarthritis, and myocarditis associated with West Nile virus infection in a dog. J. Vet. Intern. Med. 2006, 20, 1219–1223.

152. Lanthier, I.; Hébert, M.; Tremblay, D.; Harel, J.; Dallaire, A.D.; Girard, C. Natural West Nile virus infection in a captive juvenile Arctic wolf (Canis lupus). J. Vet. Diagn. Investig. 2004, 16, 326–329.

153. Paddock, C.D.; Nicholson, W.L.; Bhatnagar, J.; Goldsmith, C.S.; Greer, P.W.; Hayes, E.B.; Risko, J.A.; Henderson, C.; Blackmore, C.G.; Lanciotti, R.S.; et al. Fatal Hemorrhagic Fever Caused by West Nile Virus in the United States. Clin. Infect. Dis. 2006, 42, 1527–1535.

154. Garcia-Tapia, D.; Hassett, D.E.; Mitchell, W.J.; Johnson, G.C.; Kleiboeker, S.B. West Nile virus encephalitis: Sequential histopathological and immunological events in a murine model of infection. J. Neurovirol. 2007, 13, 130–138.

155. Kimura, T.; Sasaki, M.; Okumura, M.; Kim, E.; Sawa, H. Flavivirus encephalitis: Pathological aspects of mouse and other animal models. Vet. Pathol. 2010, 47, 806–818.

156. Nalca, A.; Fellows, P.F.; Whitehouse, C.A. Vaccines and animal models for arboviral encephalitides. Antivir. Res. 2003, 60, 153–174.

157. Mashimo, T.; Lucas, M.; Simon-Chazottes, D.; Frenkiel, M.P.; Montagutelli, X.; Ceccaldi, P.E.; Deubel, V.; Guénet, J.L.; Desprès, P. A nonsense mutation in the gene encoding 2′-5′-oligoadenylate synthetase/L1 isoform is associated with West Nile virus susceptibility in laboratory mice. Proc. Natl. Acad. Sci. USA 2002, 99, 11311–11316.

158. Perelygin, A.A.; Scherbik, S.V.; Zhulin, I.B.; Stockman, B.M.; Li, Y.; Brinton, M.A. Positional cloning of the murine flavivirus resistance gene. Proc. Natl. Acad. Sci. USA 2002, 99, 9322–9327.

159. Scherbik, S.V.; Kluetzman, K.; Perelygin, A.A.; Brinton, M.A. Knock-in of the Oas1br allele into a flavivirus-induced disease susceptible mouse generates the resistant phenotype. Virology 2007, 368, 232–237.

160. Chambers, T.J.; Diamond, M.S. Pathogenesis of flavivirus encephalitis. Adv. Virus Res. 2003, 60, 273–342.

161. Shrestha, B.; Gottlieb, D.; Diamond, M.S. Infection and Injury of Neurons by West Nile Encephalitis Virus. J. Virol. 2003, 77, 13203–13213.

162. Himsworth, C.G.; Gurney, K.E.B.; Neimanis, A.S.; Wobeser, G.A.; Leighton, F.A. An outbreak of West Nile virus infection in captive lesser scaup (Aythya affinis) ducklings. Avian Dis. 2009, 53, 129–134.

163. Williams, J.H.; Mentoor, J.D.L.; Van Wilpe, E.; Venter, M. Comparative Pathology of Neurovirulent Lineage 1 (NY99/385) and Lineage 2 (SPU93/01) West Nile Virus Infections in BALBc Mice. Vet. Pathol. 2015, 52, 140–151.

164. White, J.P.; Xiong, S.; Malvin, N.P.; Khoury-Hanold, W.; Heuckeroth, R.O.; Stappenbeck, T.S.; Diamond, M.S. Intestinal Dysmotility Syndromes following Systemic Infection by Flaviviruses. Cell 2018, 175, 1198–1212.e12.

165. Johnston, L.J.; Halliday, G.M.; King, N.J. Phenotypic changes in Langerhans' cells after infection with arboviruses: A role in the immune response to epidermally acquired viral infection? J. Virol. 1996, 70, 4761–4766.

166. Byrne, S.N.; Halliday, G.M.; Johnston, L.J.; King, N.J.C. Interleukin-1β but not tumor necrosis factor is involved in West Nile virus-induced Langerhans cell migration from the skin in C57BL/6 mice. J. Investig. Dermatol. 2001, 117, 702–709.

167. Johnston, L.J.; Halliday, G.M.; King, N.J.C. Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus. J. Investig. Dermatol. 2000, 114, 560–568.

168. Diamond, M.S.; Shrestha, B.; Marri, A.; Mahan, D.; Engle, M. B Cells and Antibody Play Critical Roles in the Immediate Defense of Disseminated Infection by West Nile Encephalitis Virus. J. Virol. 2003, 77, 2578–2586.

169. Shrestha, B.; Wang, T.; Samuel, M.A.; Whitby, K.; Craft, J.; Fikrig, E.; Diamond, M.S. Gamma Interferon Plays a Crucial Early Antiviral Role in Protection against West Nile Virus Infection. J. Virol. 2006, 80, 5338–5348.

170. Shrestha, B.; Diamond, M.S. Role of CD8+ T Cells in Control of West Nile Virus Infection. J. Virol. 2004, 78, 8312–8321.

171. Shrestha, B.; Samuel, M.A.; Diamond, M.S. CD8+ T Cells Require Perforin To Clear West Nile Virus from Infected Neurons. J. Virol. 2006, 80, 119–129.

172. Wang, Y.; Lobigs, M.; Lee, E.; Mullbacher, A. CD8+ T Cells Mediate Recovery and Immunopathology in West Nile Virus Encephalitis. J. Virol. 2003, 77, 13323–13334.

173. Klein, R.S.; Lin, E.; Zhang, B.; Luster, A.D.; Tollett, J.; Samuel, M.A.; Engle, M.; Diamond, M.S. Neuronal CXCL10 Directs CD8+ T-Cell Recruitment and Control of West Nile Virus Encephalitis. J. Virol. 2005, 79, 11457–11466.

174. Glass, W.G.; Lim, J.K.; Cholera, R.; Pletnev, A.G.; Gao, J.L.; Murphy, P.M. Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. J. Exp. Med. 2005, 202, 1087–1098.

175. Shirato, K.; Kimura, T.; Mizutani, T.; Kariwa, H.; Takashima, I. Different chemokine expression in lethal and non-lethal murine West Nile virus infection. J. Med. Virol. 2004, 74, 507–513.

176. Xiao, S.Y.; Guzman, H.; Zhang, H.; Travassos da Rosa, A.P.; Tesh, R.B. West Nile virus infection in the golden hamster (Mesocricetus auratus): A model for West Nile encephalitis. Emerg. Infect. Dis. 2001, 7, 714–721.

177. Wang, T. Role of γδ T cells in West Nile virus-induced encephalitis: Friend or foe? J. Neuroimmunol. 2011, 240, 22–27.

178. Zukor, K.; Wang, H.; Hurst, B.L.; Siddharthan, V.; Van Wettere, A.; Pilowsky, P.M.; Morrey, J.D. Phrenic nerve deficits and neurological immunopathology associated with acute West Nile virus infection in mice and hamsters. J. Neurovirol. 2017, 23, 186–204.

179. Morrey, J.; Siddharthan, V.; Wang, H.; Hall, J.; Skirpstunas, R.; Olsen, A.; Nordstrom, J.; Koenig, S.; Johnson, S.; Diamond, M. West Nile virus-induced acute flaccid paralysis is prevented by monoclonal antibody treatment when administered after infection of spinal cord neurons. J. Neurovirol. 2008, 14, 152–163.

180. Samuel, M.A.; Wang, H.; Siddharthan, V.; Morrey, J.D.; Diamond, M.S. Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis. Proc. Natl. Acad. Sci. USA 2007, 104, 17140–17145.

181. Tesh, R.B.; Siirin, M.; Guzman, H.; Travassos da Rosa, A.P.A.; Wu, X.; Duan, T.; Lei, H.; Nunes, M.R.; Xiao, S. Persistent West Nile Virus Infection in the Golden Hamster: Studies on Its Mechanism and Possible Implications for Other Flavivirus Infections. J. Infect. Dis. 2005, 192, 287–295.

182. Manuelidis, E.E. Neuropathology of experimental West Nile virus infection in monkeys. J. Neuropathol. Exp. Neurol. 1956, 15, 448–460.

183. Pogodina, V.V.; Frolova, M.P.; Malenko, G.V.; Fokina, G.I.; Koreshkova, G.V.; Kiseleva, L.L.; Bochkova, N.G.; Ralph, N.M. Study on West Nile virus persistence in monkeys. Arch. Virol. 1983, 75, 71–86.

184. Ratterree, M.S.; Gutierrez, R.A.; Travassos da Rosa, A.P.A.; Dille, B.J.; Beasley, D.W.C.; Bohm, R.P.; Desai, S.M.; Didier, P.J.; Bikenmeyer, L.G.; Dawson, G.J.; et al. Experimental infection of rhesus macaques with West Nile virus: Level and duration of viremia and kinetics of the antibody response after infection. J. Infect. Dis. 2004, 189, 669–676.

185. Verstrepen, B.E.; Fagrouch, Z.; van Heteren, M.; Buitendijk, H.; Haaksma, T.; Beenhakker, N.; Palù, G.; Richner, J.M.; Diamond, M.S.; Bogers, W.M.; et al. Experimental infection of rhesus macaques and common marmosets with a European strain of West Nile virus. PLoS Negl. Trop. Dis. 2014, 8, e2797.

186. Eldadah, A.H. ; Nathanson, N. ; Sarsitis, R. Pathogenesis of West Nile virus encephalitis in mice and rats. I. Influence of age and species on mortality and infection. Am. J. Epidemiol. 1967, 86, 765–775.

187. Eldadah, A.H. ; Nathanson, N. Pathogenesis of West Nile virus encephalitis in mice and rats. II. Virus multiplication, evolution of immunofluorescence, and development of histological lesions in the brain. Am. J. Epidemiol. 1967, 86, 776–790.

188. Posadas-Herrera, G.; Inoue, S.; Fuke, I.; Muraki, Y.; Mapua, C.A.; Khan, A.H.; Parquet, M. del C.; Manabe, S.; Tanishita, O.; Ishikawa, T.; et al. Development and evaluation of a formalin-inactivated West Nile Virus vaccine (WN-VAX) for a human vaccine candidate. Vaccine 2010, 28, 7939–7946.

189. Bonafé, N.; Rininger, J.A.; Chubet, R.G.; Foellmer, H.G.; Fader, S.; Anderson, J.F.; Bushmich, S.L.; Anthony, K.; Ledizet, M.; Fikrig, E.; et al. A recombinant West Nile virus envelope protein vaccine candidate produced in Spodoptera frugiperda expresSF+ cells. Vaccine 2009, 27, 213–222.

190. Poore, E.A.; Slifka, D.K.; Raué, H.P.; Thomas, A.; Hammarlund, E.; Quintel, B.K.; Torrey, L.L.; Slifka, A.M.; Richner, J.M.; Dubois, M.E.; et al. Pre-clinical development of a hydrogen peroxide-inactivated West Nile virus vaccine. Vaccine 2017, 35, 283–292.

191. Seino, K.K.; Long, M.T.; Gibbs, E.P.J.; Bowen, R.A.; Beachboard, S.E.; Humphrey, P.P.; Dixon, M.A.; Bourgeois, M.A. Comparative efficacies of three commercially available vaccines against West Nile Virus (WNV) in a short-duration challenge trial involving an equine WNV encephalitis model. Clin. Vaccine Immunol. 2007, 14, 1465–1471.

192. Siger, L.; Bowen, R.; Karaca, K.; Murray, M.; Jagannatha, S.; Echols, B.; Nordgren, R.; Minke, J.M. Evaluation of the efficacy provided by a Recombinant Canarypox-Vectored Equine West Nile Virus vaccine against an experimental West Nile Virus intrathecal challenge in horses. Vet. Ther. 2006, 7, 249–256.

193. Chaintoutis, S.C.; Diakakis, N.; Papanastassopoulou, M.; Banos, G.; Dovas, C.I. Evaluation of cross-protection of a lineage 1 West Nile virus inactivated vaccine against natural infections from a virulent lineage 2 strain in horses, under field conditions. Clin. Vaccine Immunol. 2015, 22, 1040–1049.

194. Bunning, M.L.; Bowen, R.A.; Bruce Cropp, C.; Sullivan, K.G.; Davis, B.S.; Komar, N.; Godsey, M.S.; Baker, D.; Hettler, D.L.; Holmes, D.A.; et al. Experimental infection of horses with West Nile virus. Emerg. Infect. Dis. 2002, 8, 380–386.

195. Castillo-Olivares, J.; Mansfield, K.L.; Phipps, L.P.; Johnson, N.; Tearle, J.; Fooks, A.R. Antibody response in horses following experimental infection with West Nile Virus lineages 1 and 2. Transbound. Emerg. Dis. 2011, 58, 206–212.

196. Long, M.T.; Gibbs, E.P.J.; Mellencamp, M.W.; Zhang, S.; Barnett, D.C.; Seino, K.K.; Beachboard, S.E.; Humphrey, P.P. Safety of an attenuated West Nile virus vaccine, live Flavivirus chimera in horses. Equine Vet. J. 2007, 39, 486–490.

197.West Nile Virus|AAEP. Available online: https://aaep.org/guidelines/vaccination-guidelines/core-vaccination-guidelines/west-nile-virus (accessed on 6 December 2019).

198. Suen, W.W.; Uddin, M.J.; Wang, W.; Brown, V.; Adney, D.R.; Broad, N.; Prow, N.A.; Bowen, R.A.; Hall, R.A.; Bielefeldt-Ohmann, H. Experimental west nile virus infection in rabbits: An alternative model for studying induction of disease and virus control. Pathogens 2015, 4, 529–558.

199. Teehee, M.L.; Bunning, M.L.; Stevens, S.; Bowen, R.A. Experimental infection of pigs with West Nile virus. Brief Report. Arch. Virol. 2005, 150, 1249–1256.

200. Austgen, L.E.; Bowen, R.A.; Bunning, M.L.; Davis, B.S.; Mitchell, C.J.; Chang, G.J.J. Experimental Infection of Cats and Dogs with West Nile Virus. Emerg. Infect. Dis. 2004, 10, 82–86.

201.Steinman, A.; Banet-Noach, C.; Simanov, L.; Grinfeld, N.; Aizenberg, Z.; Levi, O.; Lahav, D.; Malkinson, M.; Perk, S.; Shpigel, N.Y. Experimental infection of common garter snakes (Thamnophis sirtalis) with West Nile virus. Vector Borne Zoonotic Dis. 2006, 6, 361–368.
202. Fagre, A.C.; Kading, R.C. Can bats serve as reservoirs for Arboviruses? Viruses 2019, 11, 215.

203. Dridi, M.; Van Den Berg, T.; Lecollinet, S.; Lambrecht, B. Evaluation of the pathogenicity of West Nile virus (WNV) lineage 2 strains in a SPF chicken model of infection: NS3-249Pro mutation is neither sufficient nor necessary for conferring virulence. Vet. Res. 2015, 46, 130.

204. Deardorff, E.R.; Fitzpatrick, K.A.; Jerzak, G.V.S.; Shi, P.Y.; Kramer, L.D.; Ebel, G.D. West nile virus experimental evolution in vivo and the trade-off hypothesis. PLoS Pathog. 2011, 7, e1002335.

205. Jerzak, G.V.S.; Brown, I.; Shi, P.-Y.; Kramer, L.D.; Ebel, G.D. Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching. Virology 2008, 374, 256–260.

206. Kading, R.C.; Biggerstaff, B.J.; Young, G.; Komar, N. Mosquitoes used to draw blood for arbovirus viremia determinations in small vertebrates. PLoS ONE 2014, 9, e99342.

207.Grubaugh, N.D.; Fauver, J.R.; Rückert, C.; Weger-Lucarelli, J.; Garcia-Luna, S.; Murrieta, R.A.; Gendernalik, A.; Smith, D.R.; Brackney, D.E.; Ebel, G.D. Mosquitoes Transmit Unique West Nile Virus Populations during Each Feeding Episode. Cell Rep. 2017, 19, 709–718.

208.Worwa, G.; Hutton, A.A.; Frey, M.; Duggal, N.K.; Brault, A.C.; Reisen, W.K. Increases in the competitive fitness of West Nile virus isolates after introduction into California. Virology 2018, 514, 170–181.

209.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.; Barrett, A.D.T.; et al. Evidence for Co-evolution of West Nile Virus and House Sparrows in North America. PLoS Negl. Trop. Dis. 2014, 8, e3262.

210.Langevin, S.A.; Brault, A.C.; Panella, N.A.; Bowen, R.A.; Komar, N. Variation in virulence of West Nile virus strains for house sparrows (Passer domesticus). Am. J. Trop. Med. Hyg. 2005, 72, 99–102.

211.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.; Romo, H.; et al. Host Competence and Helicase Activity Differences Exhibited by West Nile Viral Variants Expressing NS3-249 Amino Acid Polymorphisms. PLoS ONE 2014, 9, e100802.

212.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. West Nile Virus Temperature Sensitivity and Avian Virulence Are Modulated by NS1-2B Polymorphisms. PLoS Negl. Trop. Dis. 2016, 10, e0004938.

213.Melian, E.B.; Hall-Mendelin, S.; Du, F.; Owens, N.; Bosco-Lauth, A.M.; Nagasaki, T.; Rudd, S.; Brault, A.C.; Bowen, R.A.; Hall, R.A.; et al. Programmed ribosomal frameshift alters expression of west nile virus genes and facilitates virus replication in birds and mosquitoes. PLoS Pathog. 2014, 10, e1004447.

214.Maharaj, P.D.; Bosco-Lauth, A.M.; Langevin, S.A.; Anishchenko, M.; Bowen, R.A.; Reisen, W.K.; Brault, A.C. West Nile and St. Louis encephalitis viral genetic determinants of avian host competence. PLoS Negl. Trop. Dis. 2018, 12, e0006302.

215.Lim, S.M.; Brault, A.C.; van Amerongen, G.; Bosco-Lauth, A.M.; Romo, H.; Sewbalaksing, V.D.; Bowen, R.A.; Osterhaus, A.D.M.E.; Koraka, P.; Martina, B.E.E. Susceptibility of Carrion Crows to Experimental Infection with Lineage 1 and 2 West Nile Viruses. Emerg. Infect. Dis. 2015, 21, 1357–1365.

216.Murray, K.O.; Kolodziej, S.; Ronca, S.E.; Gorchakov, R.; Navarro, P.; Nolan, M.S.; Podoll, A.; Finkel, K.; Mandayam, S. Visualization of West Nile Virus in Urine Sediment using Electron Microscopy and Immunogold up to Nine Years Postinfection. Am. J. Trop. Med. Hyg. 2017, 97, 1913–1919.

217. Ogawa, M.; Okubo, H.; Tsuji, Y.; Yasui, N.; Someda, K. Chronic progressive encephalitis occurring 13 years after Russian spring-summer encephalitis. J. Neurol. Sci. 1973, 19, 363–373.

Chapter 2. Experimental West Nile Virus Evolution in Wild Birds and Mosquitoes Reveals Species-Specific Transmission Success

Introduction

RNA virus populations within hosts are highly diverse as the result of error-prone replication. These populations, also described as quasispecies, serve as the source of mutations which can arise over time and eventually dominate the circulating viral strains. Mutations may imbue increased pathogenicity, transmissibility and other characteristics associated with emergence. In West Nile virus (WNV), the dominant circulating genotype originally emerged as the result of increased transmission by *Culex* (*Cx.*) spp. mosquitoes attributable to an amino acid substitution in the viral envelope (1,2). Similarly, in chikungunya virus an envelope amino acid substitution altered vector specificity to increase infectivity in *Aedes albopictus* mosquitoes, allowing for transmission in new geographic locations (3). Despite emergence slightly more than a year ago, the global SARS-CoV-2 pandemic has brought awareness of mutations and their importance to non-scientists (4–6). These revelations highlight the significance of mutations and justify the interest in examining the conditions which lead to said mutations.

For arboviruses, those conditions include the vertebrate and invertebrate hosts in which these viruses circulate. In WNV, those are wild birds and *Culex* species mosquitoes. Since the initial incursion of WNV into the United States, American crows (*Corvus brachyrhynchos*) were shown to be highly susceptible as evidenced by high mortality associated with high levels of viremia (7,8). In contrast, American robins (*Turdus migratorius*) experience lower viremia and mortality is less common (7,9). Robins have previously been suggested as key to viral maintenance and human disease because their migratory dispersals influence *Cx. pipiens* mosquito feeding shifts leading to human infections (10). In addition to differences in avian

disease presentation, there are specific variations between species when it comes to the intrahost WNV populations. Examination of intrahost WNV populations demonstrated that genetic diversity is greater in mosquitoes than in birds as a consequence of relatively weak purifying selection in comparison to that of birds (11,12). This diversity may be driven by mosquito RNA interference (13). Sequencing revealed more unique mutations and defective genomes in crows in comparison to robins, indicative of greater virus mutational tolerance (14). These genetic alterations are associated with fitness impacts. In previous work, WNV passaged through robins was demonstrated to confer greater replicative fitness gains than when passaged through crows (14).

While the aforementioned work examined bird species or mosquitoes in isolation, some groups have previously performed challenging experimental evolution work utilizing mosquitoes and chicks together in multiple rounds of WNV transmission (12,15–17). Those specific experiments demonstrated initial viral diversification in mosquitoes followed by strong purifying selection in chicks with resulting restrictions in genetic diversity. In experiments where fitness of the transmission cycle products was assessed, fitness changes of WNV from alternating passage between chicks and mosquitoes were negligible (16). Similarly, experimental evolution of other viruses utilizing alternating cycles of vertebrates and invertebrates yielded products which were stable from a fitness standpoint (18–21). These findings are more variable when cell culture is used as a model for transmission (22–26).

For WNV, experimental evolution involving the relevant wild bird hosts in combination with mosquitoes had not been previously been reported. The use of wild birds is most relevant given that they serve as the natural amplifying host for WNV. We sought to examine transmission cycles utilizing *Cx. pipiens* and *Cx. quinquefasciatus* (*Cx. quinquefasciatus*) mosquitoe*s* with American crows and American robins. Pairings of *Cx. pipiens* with crows, *Cx. quinquefasciatus* with crows and robins with *Cx. quinquefasciatus* were assessed by their transmission success, mosquito tissue infection and competitive fitness. During a single round

of crow to mosquito transmission, we observed increasing mosquito infection (WNV-positive body) and transmission-capability (WNV-positive saliva) as a result of increasing viremia in crows. In multiple rounds of transmission, infection (WNV-positive midgut) and transmission-capability (WNV-positive saliva) decreased with each subsequent round of transmission. Crow and *Cx. pipiens* transmission cycles were the most successful while crow and *Cx. quinquefasciatus* transmission cycles were the second most successful. Robin and *Cx. quinquefasciatus* transmission cycles were least successful. Competitive fitness of transmission cycle products was similar regardless of the cell type utilized. Competitive fitness assays of transmission cycles using crows exhibited cyclical increases and decreases in fitness as virus moved through birds and mosquitoes, respectively. This pattern was not consistent in robin-*Cx. quinquefasciatus* cycles. Sequencing is needed to assess whether differences in transmission cycle success and competitive fitness can be attributed to genetic changes.

Materials and Methods

Artificial mosquito infections

To assess a baseline of mosquito infection and transmission rates at varying bloodmeal titers, we artificially infected mosquitoes with WNV spiked into a bloodmeal. Mosquito infections were performed under BSL3 conditions. Groups of ~50-60 four- to six-day-old lab colony *Cx. quinquefasciatus* and *Cx. pipiens* mosquitoes were exposed orally by artificial blood meal to WNV FtC-3699, a field isolate of WNV collected from a *Cx. tarsalis* mosquito pools in Fort Collins, CO in 2012 (GenBank: KR868734). The infectious bloodmeal contained a mixture of defibrinated calf blood and virus to yield titers of 10^3, 10^5, and 10^8 PFU/mL as confirmed by titration. Mosquito dissections and plaque assays of mosquito bodies and saliva to determine mosquito infections and transmission rates were performed as described below.

Animal care

Bird collection, housing and manipulation were performed according to methods approved by the Colorado State University Institutional Animal Care and Use Committee

(Protocols #15-5958 & 18-8080A), the United States Fish and Wildlife Service (Permit #MB68845B-0) and Colorado Parks and Wildlife (Permits 19TRb2106 & 20TRb2106) as previously described (7,14,27,28).

Single round mosquito infection by WNV-infected bird

To assess mosquito infection and transmission-capability when fed on a WNV-infected crow at 1 and 3 days post infection, we needle inoculated crows with 10,000 PFU of the FtC-3699 WNV in 100 µl of DMEM by subcutaneous needle inoculation and allowed cartons of ~50-60 four- to six-day-old *Cx. quinquefasciatus* and *Cx. pipiens* mosquitoes to feed on them. Birds were bled according to IACUC protocol on days of mosquito feeding and sera was separated and stored at -80°C. Bloodfed mosquitoes were separated and held for 14 days extrinsic incubation period for dissection. Bodies and saliva were collected as described below. Mosquito tissues were assayed by standard plaque assay protocol to determine whether they were WNV-positive or negative. Bird sera were titrated by standard plaque assay. Single round mosquito infection by WNV-infected robin is not included here due to the presence of antibody-positive birds as determined by PRNT.

Experimental WNV transmission summary

We performed experimental transmission of WNV through pairings of mosquitoes and birds (Figure 2.1).

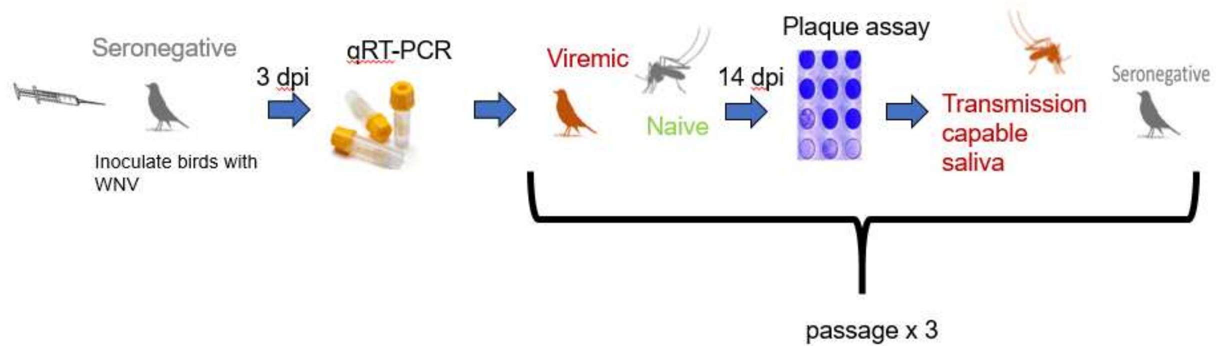


Figure 2.1. Transmission model. Birds were inoculated with West Nile virus. 3 days post inoculation, bird sera were tested for viral RNA by qRT-PCR. Viremic birds were fed on by naïve mosquitoes. After 14 days extrinsic incubation period, mosquitoes were dissected. Saliva was assessed for live virus by plaque assay. Transmission-capable saliva was used to inoculate the next seronegative bird. The bird-to-mosquito step was repeated for three passages of bird-to-mosquito transmission.

Mosquito species utilized included *Cx. quinquefasciatus* and *Cx. pipiens* mosquitoes. Birds were American crows and American robins. Mosquito-bird combinations evaluated include *Cx. quinquefasciatus* and American crow, *Cx. pipiens* and American crow, *Cx. quinquefasciatus* and American robin. Transmission from bird to mosquito was attempted with the goal of three sequential rounds of transmission in three replicates. Experiments were performed under BSL3 conditions. Birds were initially inoculated with WNV. Birds were bled daily throughout the infection and at day 3, RNA was extracted from serum and qRT-PCR was performed to ensure that they were viremic. Once viremia was confirmed, naïve mosquitoes were allowed to feed on birds and bloodfed female mosquitoes were sorted and held for 14 days. At 14 days post bird feed, mosquitoes were dissected and mosquito saliva was assessed for live virus by plaque assay. 100 µl of WNV-positive saliva was used to inoculate the next bird and the process was repeated.

WNV RNA-emia assessment

Blood was collected according to IACUC protocol and serum was separated. Serum RNA was extracted using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek) on the KingFisher Flex Magnetic Particle Processor (ThermoFisher Scientific). qRT-PCR was performed on a QuantStudio 3 (ThermoFisher) using previously published WNV primers and probes (29).

Mosquito use & dissections

Cx. quinquefasciatus and *Cx. pipiens* colonies maintained in the lab were used for this work. The colony was maintained with defibrinated calf blood and *ad libitum* sugar and water. After rearing to larvae, adult mosquitoes were maintained under 26-27°C, 70-80% relative humidity and 16:8 light:dark diurnal cycle. At the time of dissections, mosquitoes were cold anesthetized and legs and wings were removed. Saliva was collected by placing the mosquito proboscis in an immersion oil-containing capillary tube for 30 minutes. Saliva-containing oil was removed from the capillary tube by centrifugation at 15,000 *g* for 5 minutes at 4°C with collection in a 2 mL tube containing 100 µl mosquito diluent. When midguts were required, they were then dissected. Otherwise, the entire mosquito body was collected. All tissues were collected in a 2 mL tube containing 200 µl mosquito diluent. This mosquito diluent contains 1x PBS supplemented with 20% FBS, 50 µg/mL-1 penicillin/streptomycin, 50 µg/ml-1 gentamycin and 2.5 µg/ml-1 fungizone and a stainless steel bead. Tissues were homogenized at 25 cycles per second for 2 min and centrifuged at 15,000 *g* for 5 min at 4°C. Saliva and tissues were stored at -80 °C.

Plaque assays

Samples assessed for live virus included bird serum, mosquito bodies, midguts, legs/wings and saliva. Infectious virus in those samples was titrated using standard plaque assay protocol.

Competitive fitness assays

Mosquito legs/wings, mosquito saliva and avian sera were assessed in a competitive fitness assay against a WNV reference virus (WNVic-REF) which has previously been described (30,31). Competitions were performed on DF1 cells (chicken fibroblasts; ATCC® CRL-12203™) and CT cells (*Cx. tarsalis* embryonal cells; (32)) in duplicate when sufficient sample was present. Competitions were performed by mixing equal by PFU portions of the sample and reference virus. Cells plated in 24 well plates at 80% confluence were washed with PBS and the viral mixture was incubated on cells for 1 hour at 37°C temp for DF1 cells and 28°C temp for CT cells. After incubation, cells were washed with PBS and 2 mL of 2% FBS DMEM media and 2% FBS Schneider's media were added to each well of DF1 and CT cells, respectively. 50 µL of cell supernatant was collected at 2 days post-incubation. Viral RNA was extracted from cell supernatant and a 500 base pair region inclusive of the reference sequence was amplified by one-step PCR (Qiagen, primers available upon request) and submitted for Sanger sequencing. Sanger results were then analyzed using polySNP to determine the proportion of virus from transmission cycle products (avian sera, mosquito legs/wings and mosquito saliva) and proportion of WNVic-REF (30,31).

Results

High titer artificial bloodmeals increase mosquito infection rate and titer

We artificially infected *Cx. quinquefasciatus* and *Cx. pipiens* mosquitoes to establish baseline rates of mosquito infection and transmission capability at 14 days extrinsic incubation period after being administered varying titers of WNV-infected bloodmeal (Figure 2.2).

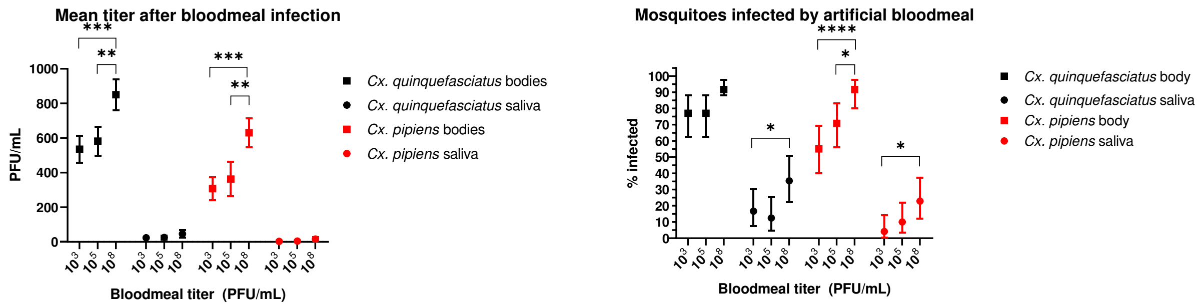


Figure 2.2. Bloodmeal West Nile virus infection of mosquitoes. Left: Artificial infections of *Cx. quinquefasciatus* and *Cx. pipiens* mosquitoes at varying bloodmeal titers (x-axis) were performed. At 14 days extrinsic incubation period, mosquito bodies and saliva were titrated for WNV (PFU/ml; y-axis). Bar=SEM. *=p<0.05, **=p<0.005, ***=p<0.0005, ****=p<0.0005. Right: Rates of infection (y-axis) for mosquito tissues at varying bloodmeal titers (x-axis) are shown. Bar=95% CI. Analyzed by Mann-Whitney Test.

Cx. quinquefasciatus and *pipiens* mosquitoes which received bloodmeals containing 10^8 PFU WNV had significantly greater mean infections (titer in body) than those which received bloodmeals containing 10^3 and 10^5 PFU WNV. There were no significant differences in the mean infection (titer in body) of mosquitoes which received blood meals containing 10^3 and 10^5 PFU WNV. There were no significant differences in the mean saliva titers of either mosquito species when comparing the initial bloodmeal titer; however, there was an upward trend in mean saliva titers as the bloodmeal titer increased. Generally, titers in *Cx. quinquefasciatus* bodies and saliva were higher than *Cx. pipiens* exposed to comparable virus titers in the blood meal. The percentage of WNV-positive bodies and saliva increased in *Cx. pipiens* with increasing bloodmeal titers. In *Cx. quinquefasciatus*, the percentage of WNV-positive bodies were similar among mosquitoes exposed to bloodmeals containing either 10^3 or 10^5 PFU bloodmeals and WNV-positive saliva were slightly lower in mosquitoes exposed to a 10^5 PFU bloodmeal compared to a 10^3 PFU bloodmeal. There were more WNV-positive bodies and saliva among *Cx. quinquefasciatus* fed 10^8 PFU blood meals than lower titer bloodmeals.

Mosquito infection and transmission-capability increase with increasing viremia in crows

We artificially infected American crows with WNV and fed *Cx. quinquefasciatus* and *Cx. pipiens* mosquitoes on them at 1 and 3 days post-infection. Mosquitoes were held for 14 days extrinsic incubation and bodies and saliva were collected. Plaque assays were performed to determine whether bodies and saliva were WNV-positive or WNV-negative (Figure 2.3).

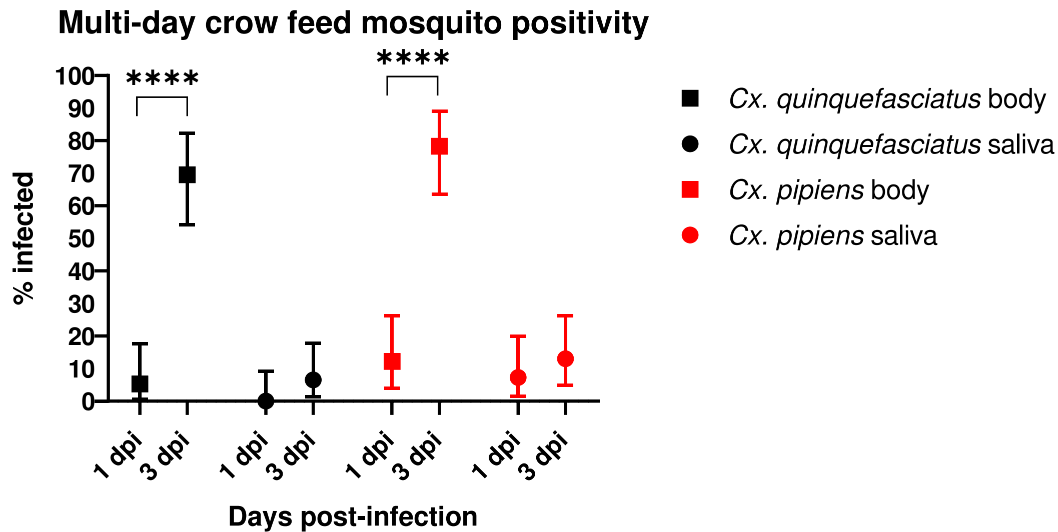


Figure 2.3. Mosquito infection and transmission-capability increased with increasing viremia in crows. *Cx. quinquefasciatus* and *Cx. pipiens* mosquitoes were fed on a WNV-infected crow at 1 and 3 dpi and held for 14 days extrinsic incubation period for dissection. Mosquito bodies and saliva were collected and assessed as WNV-positive or WNV-negative by plaque assay (y-axis). Viremia at 1 and 3 dpi were 10^3 and 10^6 PFU/mL. Bar= SEM, ****=p<0.0001. Analyzed by Mann-Whitney test.

On 1 day post infection (dpi), the crows fed on by *Cx. quinquefasciatus and Cx. pipiens* had a viremia of 1.6×10^3 and 1.2×10^3 PFU/mL, respectively. On 3 dpi, the crows fed on by *Cx. quinquefasciatus and Cx. pipiens* had viremia of 6×10^6 PFU/mL and 2×10^6 PFU/mL. From 1 dpi to 3 dpi, rates of WNV-positive *Cx. quinquefasciatus* and *Cx. pipiens* bodies increased significantly from 5.3% to 69.6% and 12.2% to 78.3%, respectively. Increases in rates of WNV-positive saliva for both mosquito species from 1 dpi to 3 dpi were less drastic. Generally, *Cx. pipiens* were slightly more likely to be infected than *Cx. quinquefasciatus* despite having slightly lower viremia as the infection source.

While identical amounts of WNV were not present in artificial bloodmeals (Figure 2.2) as in the viremia of crows when mosquitoes fed naturally (Figure 2.3), rough comparisons can be made. *Cx. quinquefasciatus* that fed on a crow with 10^6 PFU/mL WNV had slightly lower infection rates (WNV-positive body, 69.6%) than mosquitoes that fed on an artificial bloodmeal at 10^5 PFU/mL (77.1%). Similarly, *Cx. quinquefasciatus* saliva had lower WNV-positive rates (6.5%) when they fed on crows with 10^6 PFU/mL WNV than those that fed on an artificial bloodmeal at 10^5 PFU/mL (12.5%). *Cx. pipiens* that fed on a crow with 10^6 PFU/mL WNV had slightly higher infection rates (WNV-positive body, 78.3%) than *Cx. pipiens* that fed on an artificial bloodmeal at 10^5 PFU/mL (70.8%). Similarly, *Cx. pipiens* saliva had slightly higher WNV-positive rates (13.0%) when they fed on a crow with 10^6 PFU/mL WNV than *Cx. pipiens* that fed on an artificial bloodmeal at 10^5 PFU/mL WNV (10.0%). For both mosquito species, a higher percentage of bodies and saliva were WNV-positive when the infection source was a 10^3 PFU/mL artificial bloodmeal than a 10^3 PFU/mL crow viremia. In *Cx. quinquefasciatus*, 77.1% and 16.7% of bodies and saliva were WNV-positive when infected by 10^3 PFU/mL artificial bloodmeal in comparison to 5.3% and 0% of bodies and saliva being WNV-positive when infected by 10^3 PFU/mL crow viremia. In *Cx. pipiens*, 55.1% and 4.1% of bodies and saliva were WNV-positive when infected by 10^3 PFU/mL artificial bloodmeal in comparison to 12.2% and 7.3% of bodies and saliva being WNV-positive when infected by 10^3 PFU/mL crow viremia.

Experimental transmission cycles with bird-mosquito pairings are most successful when an American crow-Culex pipiens pairing is utilized

We performed experimental transmission of WNV through pairings of mosquitoes and birds (Figure 2.1). Combinations included *Cx. quinquefasciatus* and American crow, *Cx. pipiens* and American crow, and *Culex quinquefasciatus* and American robin. The goal of transmission cycles included initial needle-inoculation of birds with subsequent transmission to mosquitoes followed by two additional bird to mosquito cycles. The most successfully completed cycle

utilized crows and *Cx. pipiens*, as all three replicates reached completion of three rounds of bird to mosquito transmission (Figure 2.4).

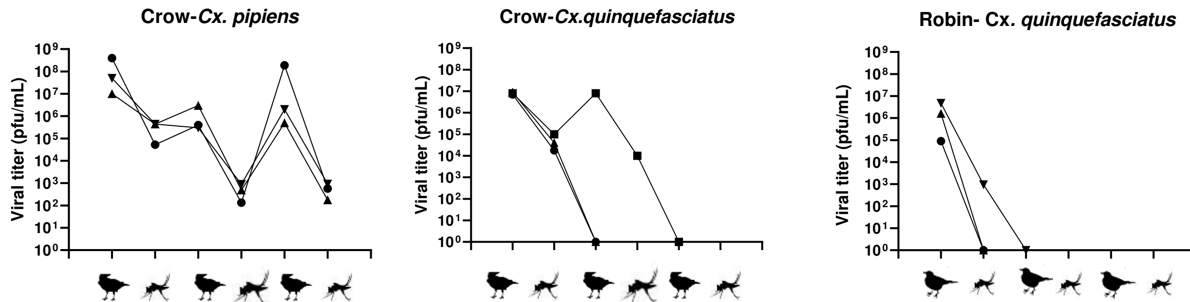


Figure 2.4. Experimental WNV transmission cycles result in variable transmission cycle success. The goal was three replicates in which three sequential rounds of bird to mosquito transmission were performed as detailed in Figure 4.1. The viral titer (y-axis) of avian sera and mosquito saliva at each transmission event are shown.

The crow and *Cx. quinquefasciatus* pairing was the next most successful pairing. Two replicates of this pairing terminated at the second round bird while the final replicate was terminated at the third round bird. The pairing utilizing robins and *Cx. quinquefasciatus* was the least successful with termination of two replicates at the first round mosquito and the third replicate terminating at the second round bird. Generally, the titer of sera and saliva utilized immediately before transmission cycle termination were comparable to sera and saliva titers which allowed for continuation of transmission cycles. Avian viremia resulting from mosquito saliva inoculation was capable of reaching magnitudes comparable to avian viremia initiated by needle inoculation, despite a lower inoculating titer.

Mosquito infection rates, dissemination and transmission-capability mirror the success of experimental transmission cycles

During bird-mosquito transmission cycles, mosquito legs/wings and saliva were collected at each dissection. The virus titer in mosquito tissues was determined by plaque assay (Figure 2.5).

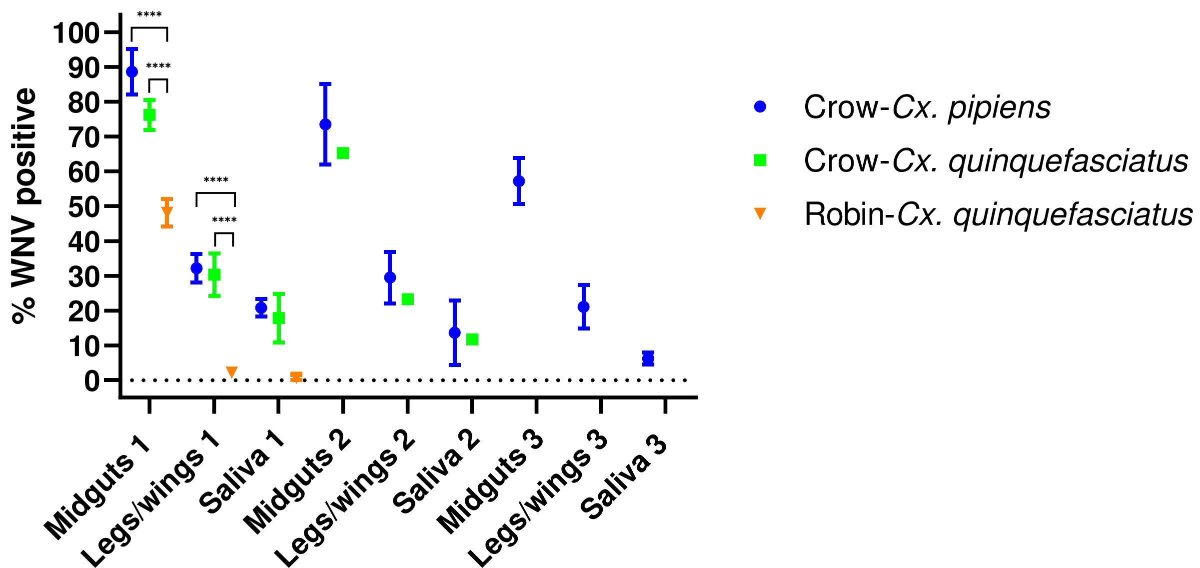


Figure 2.5. Mean transmission cycle mosquito infection rates. During each round of transmission, three replicates were attempted. The mean percentage of midguts, legs/wings and saliva which were WNV-positive by plaque assay are shown. Absent data points indicate prior termination of the cycle. Bar = SEM. Comparison by multiple t-tests. ****=p<0.00005.

The mean infection rate (WNV-positive midguts), dissemination rate (WNV-positive legs/wings) and transmission-capability (WNV-positive saliva) was always highest in the crow-*Cx. pipiens* cycles followed by crow-*Cx. quinquefasciatus* cycles. Interestingly, for all bird-mosquito pairings, the mean infection rate, dissemination rate and transmission-capability decreased with each subsequent round of transmission.

Sera and mosquito samples from transmission cycles exhibit variable competitive fitness in vitro

WNV-positive avian sera, mosquito legs/wings and mosquito saliva from each transmission event were assessed for fitness by competition assay in avian (DF1) and mosquito (CT) cell lines (Figure 2.6).

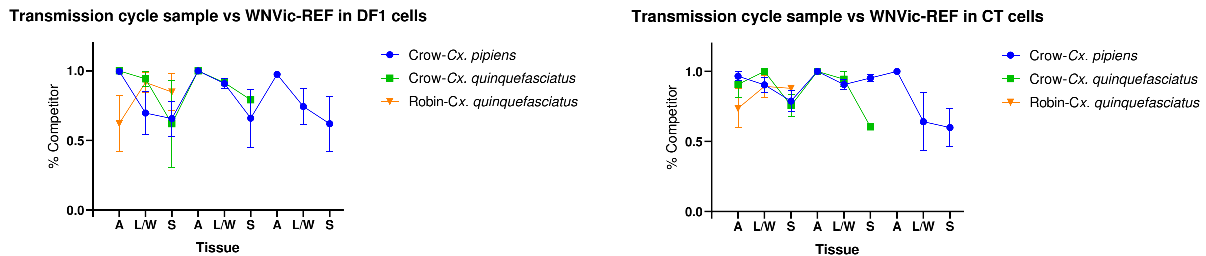


Figure 2.6. Transmission cycle sera and mosquito samples exhibit variable competitive fitness *in vitro.* WNV-positive avian sera (x-axis, A), mosquito legs/wings (x-axis, L/W) and saliva (x-axis,S) from each transmission event were mixed with equal Plaque Forming Units (PFU) of a reference virus (WNVic-REF) and incubated on DF1 (left) and CT (right) cells in duplicate. The proportion of the transmission cycle product present at 2 days post incubation is shown (y-axis). Bar=SEM.

Generally, each transmission cycle exhibited cyclical patterns in competitive fitness strength which were similar in both cell lines. For the crow-*Cx. pipiens* cycle samples, the virus population in the avian sera had the highest relative fitness compared to mosquito tissues. The mosquito tissues frequently had decreases in fitness from legs/wings to saliva. For the crow-*Cx. quinquefasciatus* cycle samples, aside from the first sera sample in CT cells, the mean relative fitness decreased from avian sera to mosquito legs/wings to saliva. For the robin-*Cx. quinquefasciatus* cycle samples, avian sera had lower relative fitness compared to mosquito tissues and mosquito legs/wings had greater relative fitness than saliva. Robin sera had lower relative fitness than crow sera. For all transmission cycles, none of the sera or mosquito samples were outcompeted by the reference virus.

Discussion

We performed challenging experimental evolution studies which paired different bird and mosquito species for sequential rounds of bird-to-mosquito transmission. Viremia is clearly a significant factor in WNV transmission as it determines virus availability to feeding mosquitoes. However, viremia is also associated with differences within the viral populations being

transmitted. For example, in crows which have high viremia, viral populations contain more defective genomes and are characterized by increased mutational tolerance (14). In robins, lower viremias are associated with fewer variants rising to fixation (14). These genetic changes have previously been associated with fitness alterations, with greater fitness gains observed in robin-passaged WNV than crow-passaged WNV (14). However, when crows were paired with mosquitoes in this work, transmission success suggests a crow fitness advantage.

Interestingly, a clear dose-dependent effect was not observed when we artificially infected *Cx. quinquefasciatus* and *Cx. pipiens* at bloodmeal titers of 10^3, 10^5, and 10^8 PFU/mL to establish a baseline of mosquito infection and transmission-capability without the influence of avian factors. At the highest administered bloodmeal titer, there were significantly greater mosquito body titers but differences between mosquito body titers were less distinct at the two lower bloodmeal titers. This was somewhat similar to a previous evaluation of dose-dependency for *Cx. quinquefasciatus* mosquitoes in which body titers and dissemination rates were not significantly different despite ingesting different amounts of WNV (33). In that particular study, infection rates *were* observed as a function of virus dose and that trend was present in *Cx. pipiens* mosquitoes in our work. These findings aligned with previously determination that mosquito artificial infections require a threshold viremia (34,35).

We incorporated the avian influence into our assessment of mosquito infection and transmission-capability by performing a single round crow infection and allowing *Cx. quinquefasciatus* and *Cx. pipiens* to feed on crows at both 1 and 3 days post-infection. For the artificial infections, infection rates of *Cx. quinquefasciatus* bodies and saliva were generally slightly higher than *Cx. pipiens.* In contrast, for the crow-to-mosquito infections we saw the opposite, with *Cx. pipiens* having slightly higher rates of infection and transmission capability than *Cx. quinquefasciatus* on both days of feeding. With a single round of infection and these differences being slight, we were curious whether greater differences in transmission cycle success would occur when exacerbated by multiple rounds of transmission. Our lab previously

observed high chick viremias leading to increasing mosquito mortality, but we did not see this in mosquitoes which fed on crows with extremely high levels of viremia, further highlighting the species-specific nature of vector-host interactions (15). While inter-species differences in infection and transmission capability rates were fairly similar, the intra-species infection rates and transmission capability with a blood meal containing 10^3 PFU/mL WNV were drastically different depending on whether the bloodmeal was imbibed from an artificial or avian source. The cause of this difference and its absence at higher titer bloodmeals is unclear; however, generation of defective genomes may be considered in the face of a rapidly growing crow viremia.

Outcomes of experimental WNV transmission cycles were quite different depending on the bird and mosquito pairings utilized and the only pairing which was capable of reaching completion of three sequential rounds of bird-to-mosquito transmission was that of crows and *Cx. pipiens.* The greater success of crow cycles when paired with *Cx. pipiens* in comparison to crows paired with *Cx. quinquefasciatus* may suggest relative fitness losses related to *Cx. quinquefasciatus.* This may be in part mediated by the slight observed increases in mosquito infection rates and transmission capability when *Cx. pipiens* were used instead of *Cx. quinquefasciatus* in a single round of infection, which was consistent with slightly higher *Cx. pipiens* tissue and saliva infection rates at each transmission step (Figure 2.5).

Transmission cycle success may also point to the significance of crows to WNV maintenance in nature over robins. When *Cx. quinquefasciatus* are the constant in experimental transmission cycles, the use of crows results in more successive transmission events in comparison to robins. Shifts in mosquito feeding habits from robins to humans were previously reported to be a major driver for human risk (36). Similar analyses regarding crows may be useful given their potential contributions in maintaining circulating WNV.

These experiments also highlight the difficulty of performing experimental evolution in a laboratory setting. The number of mosquitoes which were infected and then transmission-

capable decreased with each subsequent cycle, making transmission completion increasingly difficult. Cycles were terminated when no mosquitoes became transmission capable or when WNV-positive saliva did not infect the subsequent bird. Interestingly, when the initial bird viremia is ignored due to its initiation by a large needle-inoculum, most viremia and saliva titers within a transmission cycle pairing were similar across transmission events despite leading to subsequent transmission cycle continuation or termination. In nature, maintenance of WNV is likely multifactorial and additional factors which our model does not incorporate must be considered. These may include aspects such as the number of mosquitoes which feed at one time on birds, avian morbidity associated with WNV infection allowing for extended mosquito feeding, chronic avian infection, horizontal transmission between birds, vertical transmission in mosquitoes and potentially, non-avian viremic species (37–43). While this work may highlight specific species as more likely to contribute to natural WNV maintenance, it can also be speculated that this lack of transmission success with isolated species indicates that the diverse range of avian and mosquito species capable of transmitting WNV are essential to viral maintenance.

The virus population in avian sera, mosquito midguts, legs and wings and sera exhibited variable competitive fitness *in vitro* which was roughly cyclical in nature with peaks and valleys of relative fitness. That pattern necessitates consideration of the trade-off hypothesis, which proposes that viruses maintain replicative fitness in two divergent hosts by having reduced fitness in each individual host (44). In much of the work which has previously aimed to test the hypothesis, alternating host cycles yielded negligible or ambiguous effects on fitness (16,18–21). Likewise, our competitive fitness assays yielded similar outcomes for each transmission cycle, regardless of whether the cell type was avian or mosquito in origin. That the stronger competitive fitness tended to occur with samples from the avian host while virus from mosquitoes tended to have decreased fitness may be consistent with genetic restriction and strong purifying selection in birds and genetic expansion and weak purifying selection in

mosquitoes. These changes in the viral populations must be determined by viral genetic analyses.

Our work demonstrates the difficulty of modelling experimental evolution in a lab setting. While viremia obviously contributes to transmission cycle outcomes, success is also affected by the specific species of birds and mosquitoes utilized. In isolation, each of our pairings resulted in progressive decreases in mosquito infection and transmission-capability. This suggests that the natural fitness of WNV may require a diverse range of species for success. We anticipate that sequencing the samples utilized here will further elucidate how the specific species used in these studies effect variation in transmission success and competitive fitness.

References

1. Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD. Genetic and phenotypic variation of West Nile virus in New York, 2000-2003. Am J Trop Med Hyg [Internet]. 2004 Oct 1 [cited 2021 Feb 15];71(4):493–500.

2. Davis CT, Ebel GD, Lanciotti RS, Brault AC, Guzman H, Siirin M, et al. Phylogenetic analysis of North American West Nile virus isolates, 2001-2004: Evidence for the emergence of a dominant genotype. Virology [Internet]. 2005 Nov 25 [cited 2021 Feb 15];342(2):252–65. Available from: https://pubmed.ncbi.nlm.nih.gov/16137736/

3. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in Chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog [Internet]. 2007 Dec [cited 2021 Feb 15];3(12):1895–906. Available from: https://pubmed.ncbi.nlm.nih.gov/18069894/

4. Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, et al. Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. Cell [Internet]. 2020 Aug 20 [cited 2021 Feb 15];182(4):812-827.e19. Available from: https://pubmed.ncbi.nlm.nih.gov/32697968/

5. Grubaugh ND, Hanage WP, Rasmussen AL. Making Sense of Mutation: What D614G Means for the COVID-19 Pandemic Remains Unclear. Cell [Internet]. 2020 Aug 20 [cited 2021 Feb 15];182(4):794–5. Available from: https://doi.org/10.1016/j.cell.2020.06.040

6. Emerging SARS-CoV-2 Variants | CDC [Internet]. [cited 2021 Feb 15]. Available from: https://www.cdc.gov/coronavirus/2019-ncov/more/science-and-research/scientific-brief-emerging-variants.html

7. Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, et al. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis. 2003 Mar 1;9(3):311–22.

8. Bernard KA, Maffei JG, Jones SA, Kauffman EB, Ebel GD, Dupuis AP, et al. West Nile Virus Infection in Birds and Mosquitoes, New York State, 2000. Emerg Infect Dis [Internet]. 2001 Aug [cited 2021 Feb 15];7(4):679–85. Available from: https://pubmed.ncbi.nlm.nih.gov/11585532/

9. Duggal NK, Langwig KE, Ebel GD, Brault AC. On the Fly: Interactions between Birds, Mosquitoes, and Environment That Have Molded West Nile Virus Genomic Structure over Two Decades [Internet]. Vol. 56, Journal of Medical Entomology. Oxford University Press; 2019 [cited 2020 Oct 26]. p. 1467–74. Available from: https://pubmed.ncbi.nlm.nih.gov/31549720/

10. Kilpatrick AM, Daszak P, Jones MJ, Marra PP, Kramer LD, Marm Kilpatrick A, et al. Host heterogeneity dominates West Nile virus transmission. Proc R Soc B Biol Sci [Internet]. 2006 Sep 22 [cited 2019 Jul 30];273(1599):2327–33. Available from: https://royalsocietypublishing.org/doi/10.1098/rspb.2006.3575

11. Jerzak G, Bernard KA, Kramer LD, Ebel GD. Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection. J Gen Virol [Internet]. 2005 Aug [cited 2021 Feb 15];86(8):2175–83. Available from: https://pubmed.ncbi.nlm.nih.gov/16033965/

12. Jerzak GVS, Bernard K, Kramer LD, Shi PY, Ebel GD. The West Nile virus mutant spectrum is host-dependant and a determinant of mortality in mice. Virology [Internet]. 2007 Apr 10 [cited 2021 Jan 18];360(2):469–76. Available from: https://pubmed.ncbi.nlm.nih.gov/17134731/

13. Brackney DE, Beane JE, Ebel GD. RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification. PLoS Pathog [Internet]. 2009 Jul [cited 2021 Feb 15];5(7). Available from: https://pubmed.ncbi.nlm.nih.gov/19578437/

14. Grubaugh ND, Smith DR, Brackney DE, Bosco-Lauth AM, Fauver JR, Campbell CL, et al. Experimental Evolution of an RNA Virus in Wild Birds: Evidence for Host-Dependent Impacts on Population Structure and Competitive Fitness. PLoS Pathog. 2015;

15. Grubaugh ND, Fauver JR, Rückert C, Weger-Lucarelli J, Garcia-Luna S, Murrieta RA, et al. Mosquitoes Transmit Unique West Nile Virus Populations during Each Feeding Episode. Cell Rep [Internet]. 2017 [cited 2019 Nov 26];19(4):709–18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28445723

16. Deardorff ER, Fitzpatrick KA, Jerzak GVS, Shi PY, Kramer LD, Ebel GD. West nile virus experimental evolution in vivo and the trade-off hypothesis. PLoS Pathog [Internet]. 2011 Nov [cited 2019 Nov 26];7(11). Available from: https://pubmed.ncbi.nlm.nih.gov/22102808/

17. Jerzak GVS, Brown I, Shi P-Y, Kramer LD, Ebel GD. Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching. Virology [Internet]. 2008 May 10 [cited 2019 Nov 26];374(2):256–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18395240

18. Coffey LL, Vasilakis N, Brault AC, Powers AM, Tripet FR, Weaver SC. Arbovirus evolution in vivo is constrained by host alternation [Internet]. Vol. 105, Proceedings of the National Academy of Sciences of the United States of America. 2008 [cited 2021 Feb 15]. Available from: www.pnas.orgcgidoi10.1073pnas.0712130105

19. Riemersma KK, Jaeger AS, Crooks CM, Braun KM, Weger-Lucarelli J, Ebel GD, et al. Rapid evolution of enhanced Zika virus virulence during direct vertebrate transmission chains. J Virol [Internet]. 2021 Feb 3 [cited 2021 Feb 15]; Available from: http://jvi.asm.org/lookup/doi/10.1128/JVI.02218-20

20. Taylor WP, Marshall ID. Adaptation studies with Ross River virus: laboratory mice and cell cultures. J Gen Virol. 1975;28(1):59–72.

21. Taylor WP, Marshall ID. Adaptation studies with Ross River virus: retention of field level virulence. J Gen Virol [Internet]. 1975 [cited 2021 Feb 15];28(1):73–83. Available from: https://pubmed.ncbi.nlm.nih.gov/808589/

22. Klimstra WB, Ryman KD, Johnston RE. Adaptation of Sindbis Virus to BHK Cells Selects for Use of Heparan Sulfate as an Attachment Receptor. J Virol [Internet]. 1998 Sep 1 [cited 2021 Feb 15];72(9):7357–66. Available from: https://pubmed.ncbi.nlm.nih.gov/9696832/

23. Bernard KA, Klimstra WB, Johnston RE. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology [Internet]. 2000 Oct 10 [cited 2021 Feb 15];276(1):93–103. Available from: https://pubmed.ncbi.nlm.nih.gov/11021998/

24. Moutailler S, Roche B, Thiberge JM, Caro V, Rougeon F, Failloux AB. Host alternation is necessary to maintain the genome stability of Rift Valley fever virus. PLoS Negl Trop Dis [Internet]. 2011 May [cited 2021 Feb 15];5(5). Available from: https://pubmed.ncbi.nlm.nih.gov/21629727/

25. Cooper LA, Scott TW. Differential evolution of eastern equine encephalitis virus populations in response to host cell type. Genetics [Internet]. 2001 [cited 2021 Feb 15];157(4):1403–12. Available from: /pmc/articles/PMC1461603/?report=abstract

26. Weaver SC, Brault AC, Kang W, Holland JJ. Genetic and Fitness Changes Accompanying Adaptation of an Arbovirus to Vertebrate and Invertebrate Cells. J Virol [Internet]. 1999 May 1 [cited 2021 Feb 15];73(5):4316–26. Available from: https://pubmed.ncbi.nlm.nih.gov/10196330/

27. Brault AC, Langevin SA, Bowen RA, Panella NA, Biggerstaff BJ, Miller BR, et al. Differential virulence of West Nile strains for American Crows. Emerg Infect Dis [Internet]. 2004 [cited 2020 Oct 25];10(12):2161–8. Available from: https://pubmed.ncbi.nlm.nih.gov/15663854/

28. VanDalen KK, Hall JS, Clark L, McLean RG, Smeraski C. West Nile virus infection in American Robins: new insights on dose response. PLoS One [Internet]. 2013 [cited 2019 Nov 15];8(7):e68537. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23844218

29. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. 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 [Internet]. 2000 [cited 2020 Sep 10];38(11):4066–71. Available from: https://pubmed.ncbi.nlm.nih.gov/11060069/

30. Fitzpatrick KA, Deardorff ER, Pesko K, Brackney DE, Zhang B, Bedrick E, et al. Population variation of West Nile virus confers a host-specific fitness benefit in mosquitoes. Virology [Internet]. 2010 Aug [cited 2020 Oct 26];404(1):89–95. Available from: https://pubmed.ncbi.nlm.nih.gov/20552731/

31. Hall GS, Little DP. Relative quantitation of virus population size in mixed genotype infections using sequencing chromatograms. J Virol Methods. 2007 Dec 1;146(1–2):22–8.

32. Chao J, Ball GH. A COMPARISON OF AMINO ACID UTILIZATION BY CELL LINES OF CULEX TARSALIS AND OF CULEX PIPIENS. In: Invertebrate Tissue Culture. Elsevier; 1976. p. 263–6.

33. Richards SL, Mores CN, Lord CC, Tabachnick WJ. Impact of extrinsic incubation temperature and virus exposure on vector competence of Culex pipiens quinquefasciatus Say (Diptera: Culicidae) for West Nile virus. Vector-Borne Zoonotic Dis [Internet]. 2007 Dec 1 [cited 2021 Feb 22];7(4):629–36. Available from: https://pubmed.ncbi.nlm.nih.gov/18021028/

34. Sardelis MR, Turell MJ, Dohm DJ, O'Guinn ML. Vector competence of selected North American Culex and Coquillettidia mosquitoes for West Nile virus. Emerg Infect Dis [Internet]. 2001 [cited 2020 Sep 9];7(6):1018–22. Available from: /pmc/articles/PMC2631924/?report=abstract

35. Turell MJ, O'guinn M, Oliver J. POTENTIAL FOR NEW YORK MOSQUITOES TO TRANSMIT WEST NILE VIRUS. Vol. 62, Am. J. Trop. Med. Hyg. 2000.

36. Kilpatrick AM, Kramer LD, Jones MJ, Marra PP, Daszak P. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. PLoS Biol. 2006 Apr;4(4):606–10.

37. Dawson JR, Stone WB, Ebel GD, Young DS, Galinski DS, Pensabene JP, et al. Crow deaths caused by West Nile virus during winter. Emerg Infect Dis [Internet]. 2007 Dec [cited 2019 Nov 15];13(12):1912–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18258045

38. Hinton MG, Reisen WK, Wheeler SS, Townsend AK. West Nile Virus Activity in a Winter Roost of American Crows (Corvus brachyrhynchos): Is Bird-to-Bird Transmission Important in Persistence and Amplification? J Med Entomol [Internet]. 2015 Jul 1 [cited 2020 Sep 9];52(4):683–92. Available from: https://pubmed.ncbi.nlm.nih.gov/26335475/

39. Ip HS, Van Wettere AJ, McFarlane L, Shearn-Bochsler V, Dickson SL, Baker J, et al. West Nile Virus Transmission in Winter: The 2013 Great Salt Lake Bald Eagle and Eared Grebes Mortality Event. PLoS Curr [Internet]. 2014 [cited 2019 Nov 15];6. Available from: https://pubmed.ncbi.nlm.nih.gov/24761310/

40. Banet-Noach C, Simanov L, Malkinson M. Direct (non-vector) transmission of West Nile virus in geese. Avian Pathol [Internet]. 2003 Oct [cited 2019 Nov 15];32(5):489–94. Available from: https://pubmed.ncbi.nlm.nih.gov/14522704/

41. Nemeth N, Young G, Ndaluka C, Bielefeldt-Ohmann H, Komar N, Bowen R. Persistent West Nile virus infection in the house sparrow (Passer domesticus). Arch Virol [Internet]. 2009 May [cited 2019 Nov 15];154(5):783–9. Available from: https://pubmed.ncbi.nlm.nih.gov/19347246/

42. Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey MS, et al. West Nile Virus in Overwintering Culex Mosquitoes, New York City, 2000. Emerg Infect Dis [Internet]. 2001 Aug [cited 2020 Sep 10];7(4):742–4. Available from: https://pubmed.ncbi.nlm.nih.gov/11585542/

43. Nelms BM, Fechter-Leggett E, Carroll BD, Macedo P, Kluh S, Reisen WK. Experimental and natural vertical transmission of West Nile virus by California Culex (Diptera: Culicidae) mosquitoes. J Med Entomol [Internet]. 2013 Mar [cited 2019 Nov 15];50(2):371–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23540126

44. Ciota AT, Kramer LD. Insights into Arbovirus Evolution and Adaptation from Experimental Studies. Viruses [Internet]. 2010 Dec 2 [cited 2021 Feb 23];2(12):2594–617. Available from: http://www.mdpi.com/1999-4915/2/12/2594

Chapter 3. Single Cell Analysis of WNV-Infected Birds Reveals Viremia-Influenced Alterations in Virus Evolution[2]

Introduction

The genetic complexity of RNA viruses, including arthropod-borne viruses (arboviruses), within hosts is well described (1–9). This complexity contributes to virus fitness and pathogenesis, and their ability to adapt to rapidly changing environments (10–17). West Nile virus (WNV, Flaviviridae, flavivirus) population structure is shaped by the requirement for alternating replication in wild birds and mosquitoes (12,15,18–23). Birds exhibit distinct disease phenotypes during infection and species-specific impacts on WNV genetic diversity and fitness (12,15,16,24–26). In nature, highly susceptible American crows (*Corvus brachyrhynchos*) produce high viremia during acute infection that results in high mortality rates. Conversely, American robins (*Turdus migratorius*) produce lower viremias and are more resistant to severe disease (14,24). WNV that has replicated exclusively in robins bears the signature of strong purifying selection, with insertions, deletions and other lethal and nonsynonymous mutations rapidly removed by purifying selection (15). A subset of mutations arising during replication in robins, however, reaches relatively high population frequency, and robin-replicated virus is more fit than input virus. In contrast, WNV that has replicated exclusively in crows achieves high levels of population richness, carries abundant lethal mutations as a subset of its population, and experiences fitness losses and accumulation of defective genomes. Therefore, distinct avian species that serve as enzootic hosts for WNV in nature may have correspondingly distinct

[2] The work presented in this chapter is formatted for submission to *PLOS Pathogens* with Alex D.Byas as co-first author and is minimally modified for formatting. It was a highly collaborative project and is published here with the permission of the other co-first author, Dalit Talmi-Frank. Alex D. Byas' contributions include *in vitro* characterization of low fitness viruses, *in vivo* competitions using low fitness viruses and manuscript writing.

impacts on virus populations that shape their transmission and potential for emergence (11,15,27–31).

The evolutionary mechanisms that lead to these differences are not fully understood, but may derive from clear differences in viral load within crows compared to robins. WNV viremia within crows, and viral loads within their tissues, typically exceed those present in robins (11,15,24,32,33) . It may be, therefore, that the extent of polyinfection in crows vastly exceeds that which may occur within robins. If this is the case, the selective environment within crows could be quite different compared to that within robins: if polyinfection is more intensive and extensive within crows, the power of natural selection to remove deleterious variants from the population could be reduced due to more frequent complementation of defective or low-fitness genomes with those that are of high or average fitness. Conversely, higher fitness WNV genotypes may be less likely to reach high population frequency in crows compared to robins because their proteins and other functions contribute to the persistence of lower fitness genotypes. Notably, both of these phenomenae have been documented in *in vitro* studies (15,26,29,34,35) and are consistent with our prior observations of *in vivo* replication of WNV in a range of wild bird hosts. Nonetheless, whether polyinfection indeed occurs more frequently in crows compared to robins has not been addressed. Further, the impact of host viremia on the strength of selection has not been experimentally examined using ecologically relevant animals.

Accordingly, in this study we assessed the degree of polyinfection within individual cells in crows and robins, and *ex vivo*, compared to cultured avian fibroblast cells using a newly developed barcoded version of WNV. Specifically, we examined the dynamics of infection in PBMCs, key targets of WNV replication in mammals and birds, to explore the relationship between MOI-dependent polyinfection and complementation (36–41). Single cell analysis of avian PBMCs infected with barcoded WNV revealed more viral genotypes simultaneously infecting crow cells than robins. Rare viral genotypes were also more likely to be maintained during crow infections while they were eliminated in the robin. We also examined the effect of

viremia on relative fitness *in vivo*. To accomplish this, WNV variants of variable fitness were competed against a marked reference virus in robins and crows. One of the low fitness viruses was outcompeted by the reference virus as viremia increased (Low fitness WNV 2), while the other (Low fitness WNV 1) outcompeted the reference virus, regardless of the host infected. For one of our low fitness viruses, as crow and robin viremia increased the less fit virus was able to replicate to a greater proportion of the circulating virus while the more fit virus decreased in representation. The second low fitness virus decreased in representation during crow and robin infections when viremia was greater. Collectively, these results suggest that natural selection may be weakened within highly susceptible host species due to high viremias and MOI leading to frequent polyinfection of cells, increasing the likelihood of complementation. Our results also provide support for previous observations that document slower virus divergence in crows compared to robins, and point to the significance of American crows for maintaining virus genetic diversity under natural conditions.

Materials and Methods

Wild-type Infection of Ex-Vivo PBMCs and DF1 Cells

FtC-3699 is a wild type WNV collected from *Culex* spp. mosquito pools collected in Fort Collins, Colorado. PBMCs were separated from American crow and robin whole blood using a Histopaque-1077 (Sigma-Aldrich) gradient as previously described (37). PBMCs and chicken dermal fibroblasts (DF1) (ATCC® CRL-12203™) were infected with WNV strain FtC-3699 at MOIs of 0.1, 1 or 10, washed and supplemented with fresh RPMI medium containing 10% FBS as described (37). Supernatants were harvested at the designated time points and stored at -80ºC for plaque assays and RNA extraction.

Single Cell Sequencing of Barcoded WNV In Vivo PBMCs and In Vitro DF1 infections

Generation of a Molecularly Barcoded WNV (BC-WNV): A barcoded WNV was generated using a previously described WNV infectious clone (43,51). Briefly, a region was identified in the NS4b protein for the insertion of degenerate synonymous nucleotides at 11

consecutive third codon positions where any mutation would result in no alteration to the amino acid. PCR amplifications were performed with Q5 DNA polymerase (NEB, MA, USA) and assembly was performed using the HiFi DNA assembly master mix (NEB). The digested assembly reaction was amplified by rolling circle amplification using the Repli-g mini kit (Qiagen). The correct assembly was confirmed by assessing the banding pattern by restriction digestion and the sequence was confirmed by Sanger sequencing. Infectious RNA was generated by *in vitro* transcription using the ARCA 2X T7 master mix (NEB) with subsequent transfection in 293T cells (ATCC® CRL-3216™) using Lipofectamine 3000 (Thermo Fischer). Virus was harvested and aliquots were stored at -80 ºC.

Animals: Animal use was reviewed and approved by CSU Institutional Animal Care and Use Committee (15-5958; 18-8080A) according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Wild-caught American crows and American robins were housed in $20m^3$ rooms and provided water and a mixture of dry dog food (crows), moistened dry cat food (robins), berries (robins) and mealworms (robins) *ad libitum* in addition to various enrichment activities as described previously (11,15,24,33). Birds were tested for antibodies against WNV using a plaque-reduction neutralization test according to standard practices. Only serologically negative animals were used in infection experiments. Prior to infection, groups of 2-3 birds were moved to 0.5 to $1m^3$ cages within CSU biosafety level three (BSL3) facilities. After infection, birds were monitored several times daily for clinical symptoms. Jugular venipuncture was performed for blood collection. Birds were terminated at 5 days post-infection.

Tissue processing: After euthanasia, tissues (heart, lungs, spleen, liver, small intestines, large intestines, sciatic nerve and brain) were harvested and stored at -80°C. Tissues were weighed and added to DMEM to create a 10% weight: volume suspension. This suspension was homogenized, centrifuged and the supernatant was used for plaque assays and RNA

extractions. RNA extractions substituted lysis buffer containing proteinase K solution in PKD buffer (1:16) (Qiagen) for the Mag-Bind beads used in the manufacturer protocol.

Bird Infection: Birds were inoculated with BC-WNV by subcutaneous injection in the pectoral region with 10,000 plaque forming units (PFU) in 100 µL medium containing 1% FBS, MEM+ 2mM Glutamine+ 10% FBS + 1% Non-Essential Amino Acids (NEAA) Penicillin-streptomycin, and Sodium Bicarbonate pyruvate at a final concentration of 1.5-2.2g/L and Sodium pyruvate at a final concentration of 11,004 mg/L.

DF1 Infection: DF1 cells were infected with BC-WNV similarly to previous infection using FtC-3699 virus. DF1 cells were detached using TrypLE and trypsin was neutralized before centrifugation. After supernatant was aspirated, 4% PFA (EMS) in PBS was added to the cell pellet and cells were used for flow cytometry and sorting.

Blood Processing: PBMCs were separated from peak infection whole blood by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and utilized for flow cytometry and sorting, RNA extraction and library preparation. RNA was extracted from sera using the Mag-Bind® Viral DNA/RNA Kit (Omega Bio-tek) on a KingFisher Flex extraction robot (ThermoFisher Scientific) and viral RNA was quantified using qRT-PCR as previously described (52). Remaining sera were stored at -80°C for plaque assays.

PBMC and DF1 Preparation for Flow Cytometry: PBMC and DF1 cells were incubated on ice, pelleted and washed with a staining buffer containing 1xPBS, 1% RNAse-free BSA (Gemini) and 1:400 RNasin Plus (Promega) before storage at -80°C. Before flow cytometry, cells were washed with PBS, then permeabilized using 1X PBS, 0.1% Triton X100 (Sigma) 1% RNAse-free BSA (Gemini) and 1:400 of RNasin Plus (Promega). Cells were pelleted, washed, blocked with medium containing 2% FBS, incubated with an anti-WNV capsid antibody (GTX-131947; diluted 1:1000), and a subsequent AlexaFluor 647-labeled secondary antibody (diluted 1:1000).

Single-Cell Sorting and RNA Extraction: Cells were sorted through a 70 micron nozzle at the lowest speed possible using the BD FACSAria™ III sorter. We gated on and collected WNV-positive cells at different intensities. We used a modified protocol previously used to sequence individual fixed and stained single brain radial glial cells (53). Cells were sorted into 96-well plates containing lysis buffer with proteinase K solution in PKD buffer (1:16) (Qiagen) and were stored at -80ºC. Samples were incubated for 1 hour at 56ºC with the lid set to 66 ºC in a thermos block for reverse-crosslinking. Total RNA was extracted using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek, Norcross, GA) on the KingFisher Flex Magnetic Particle Processor (ThermoFisher Scientific, Waltham, MA), according to manufacturer's protocols, adjusted for small volumes.

Screening for Positive Cells: Only RNA derived from wells containing single WNV-positive cells, as determined by qPCR for the 18S housekeeping gene and WNV copies, were used for library preparation.

Library Preparation of Single-cell RNA: Previously described methods were modified to adapt the Primer ID approach to the Illumina MiSeq platform. Methods are provided below and development is provided in Supplementary Methods 1 (38, Zanini et al., 2019 for primer ID).

cDNA Generation and Purification: 5 µl of RNA was combined with 1 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 1 µl of cDNA primer (ID_cDNAWNV_7374_Rev) (10 µM) and 3 µl of nuclease-free water. The 10 µl reaction volume was incubated for 5 minutes at 65ºC and then placed on ice for 2 minutes. A reverse transcription reaction mixture containing 1µl of Superscript III RT enzyme, 1µl of RNaseOut, 2 µl dithiothreitol (DTT), 4 µl of 25 mM MgCl2, and 2 µl of 10x SSIII buffer was added to the previous reaction volume (20 µl total reaction volume) and incubated for 50 minutes at 50ºC, followed by 5 minutes at 85ºC. Reactions were chilled on ice, spun down and incubated for 20 minutes at 37 ºC after an addition of 1µl of RNaseH. cDNA was purified using Agencourt xp beads (Beckman Coulter) at 1X concentration with elution into 12µl of nuclease-free water.

PCR Amplification Step 1: Two steps were used for amplification of the target amplicon. 11 µl of cDNA was combined with 0.75 µl of 10 nM forward primer (R1_5'_WNV_for), 0.75 µl of 10 nM forward primer (5'_ID_Primer_Rev), and 12.5 µl of 2x KAPA HiFi HotStart mastermix (VWR). PCR conditions were 95ºC for 3 min, 98ºC for 20 s, 72ºC for 45 s, 72ºC for 1 min with 35 cycles. Samples were purified using Agencourt XP beads (Beckman Coulter) at 0.6X concentration and eluted in 20 µl of nuclease-free water.

PCR Amplification Step 2: The second round of PCR amplification served to add barcodes and adapters. 2 µl of purified PCR product from PCR amplification step 1 was combined with 9 µl of nuclease-free water, 0.75µl of 10 nM forward primer (illumina index i5), 0.75 µl of 10 nM forward primer (illumina index i7), 12.5 µl of 2x KAPA HiFi HotStart mastermix (VWR) and the same PCR conditions were followed as in PCR step 1, repeated for 10 cycles. Samples were purified using AMPure XP beads at 0.6x concentration with elution into 22 µl of nuclease-free water.

Samples were pooled at a volume of 5 µl each and concentrated using AMPure XP beads at 1.5x concentration. Pooled samples were quantified using Qubit and size distribution was verified by Tapestation. Additional size selection was performed using AMPure XP beads according to manufacturer's guidelines. Libraries were quantified using the NEB library quantification kit.

Library Pooling and Loading: Libraries were pooled by volume and concentration was normalized to 2nM. Libraries were denatured and a 15% PhiX control was spiked in. Samples were loaded at a 7pM concentration.

In Vitro and In Vivo Competitions of Variably Fit WNV Strains

Generation of low fitness-WNVic-REF virus mixtures: Low fitness WNV 1 and 2: Capsid-I111T and Envelope-V364M mutations were the highest nonsynonymous variants recovered from WNV-infected *Culex tarsalis* and *Aedes aegypti* mosquitoes, respectively and are identified as Low fitness WNV 1 and Low fitness WNV 2. These mutations demonstrated low competitive

fitness in cell culture and were engineered into a WNV infectious clone as previously described (44).

WNVic-REF: This genetically marked reference virus has a 5 nucleotide region of nonsynonymous mutations changing the parental sequence CTC TCA CGG to CTa agc aGG (NS5; genome position 8313-8317). This mutation allows for quantification of the virus' proportion in a mixture (28,55).

Low fitness viruses and WNVic-REF were amplified on Vero cells at 80% confluency at an MOI of 1. To confirm desired mutations and equal mixing of the low fitness viruses and WNVic-REF, a ~550 base pair region containing the reference region was amplified by one-step PCR (Qiagen, primers available upon request) and processed by Sanger sequencing. PolySNP analysis confirmed equal proportions of WNVic-REF and each competitor low fitness virus (28,55).

In vitro competition with low fitness-WNVic-REF virus mixtures: We used the low fitness-WNVic-REF virus mixtures to infect DF1 cells at MOIs of 0.1 and 1. Supernatant was collected daily until approximately 60-75% cytopathic effect was observed and aliquots were stored at -80 °C. Cell culture supernatant RNA was extracted and prepared for Sanger sequencing as previously described. The proportion of reference virus to the low fitness competitor was determined by polySNP.

In vivo competition with low fitness-WNVic-REF virus mixtures: Birds were inoculated with low-fitness-WNVic-REF virus mixtures as performed for BC-WNV infections. Whole blood was collected daily and serum was used for plaque assays and RNA extraction. Viral RNA was processed for Sanger sequencing with subsequent polySNP analysis.

Results

Ex vivo WNV Replication in PBMCs Demonstrates Host-Specific Accumulation of Non-Infectious Genomes

We assessed WNV replication in DF1 cells, *ex vivo* American robin and American crow PBMCs at MOI of 0.1, 1, and 10 (Figure 3.1).

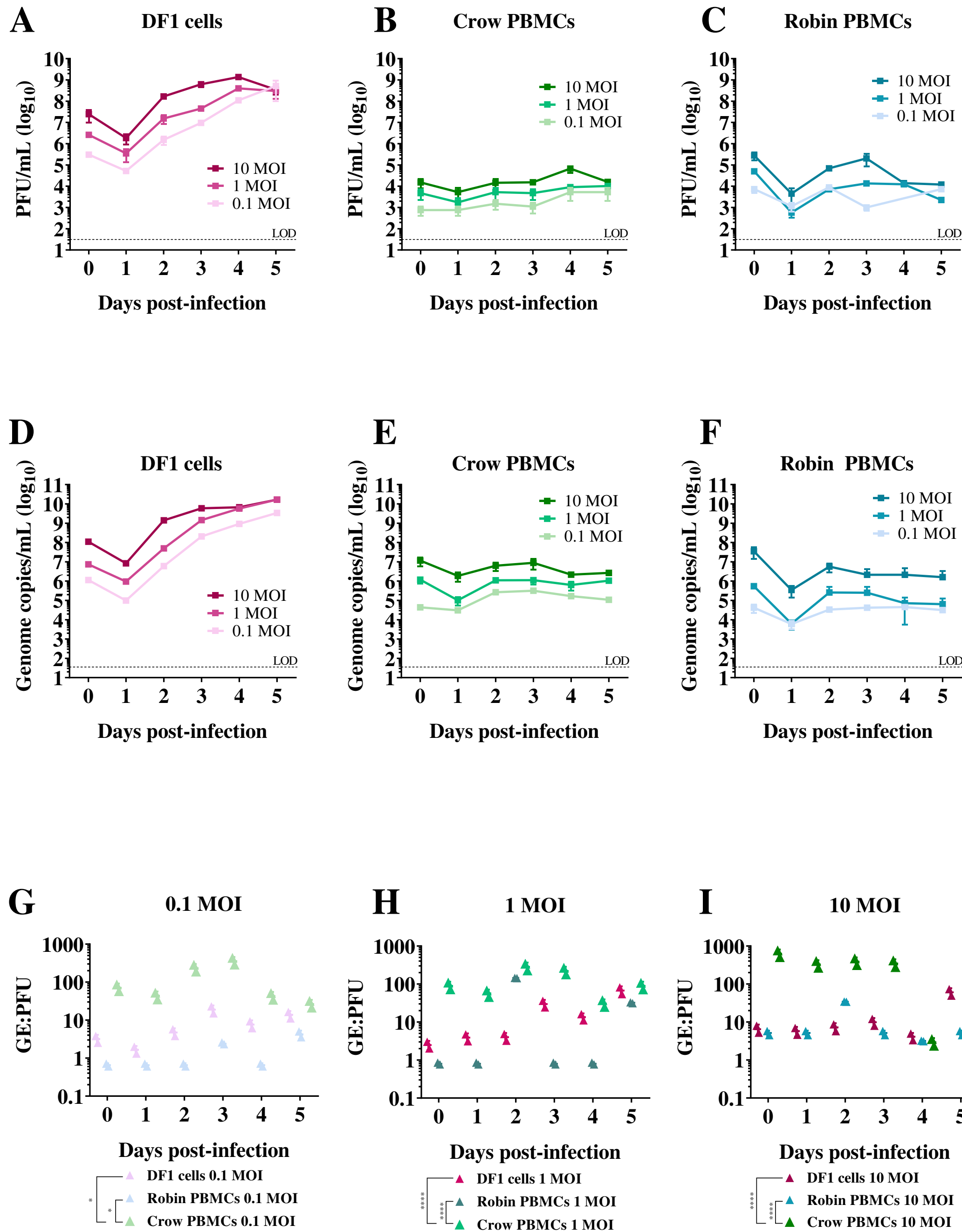


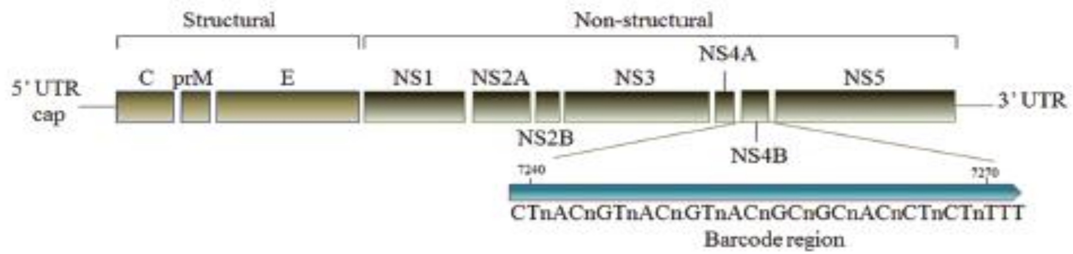
Figure 3.1. WNV replicates in DF1 Cells and *ex vivo* crow and robin PBMCs. Titers determined by plaque assay of DF1 cells (A), crow PBMCs (B) and robin PBMCs (C) infected with field strain WNV at MOI of 0.1, 1 and 10 (n=2 wells per cell type per MOI). Genome copies determined by qRT-PCR of DF1 cells (D), crow PBMCs (E) and robin PBMCs (F) (n=2 wells per cell type per MOI). WNV genome equivalents to plaque forming units (GE:PFU) at MOI of 0.1 (G) (*, DF1 vs crow PBMC, $P = 0.0215$; *, robin vs crow PBMC, $P = 0.0185$; 2-way ANOVA, Tukey's Multiple Comparison), 1 (H) (****, DF1 vs crow PBMC, $P < 0.0001$; ****, robin vs crow PBMC, $P < 0.0001$; 2-way ANOVA, Tukey's Multiple Comparison) and 10 (I) (****, DF1 vs crow PBMC, $P < 0.0001$; ****, robin vs crow PBMC, $P < 0.0001$; 2-way ANOVA, Tukey's Multiple Comparison) of DF1 cells, robin PBMCs and crow PBMCs.

Virus replication progressed in a cell-type dependent manner, with DF1 cells demonstrating higher titers than *ex vivo* cultured PBMCs; and robin PBMCs having higher peak titers than crow PBMCs (Figure 3.1A-C). Generally, a higher MOI inoculum led to higher titers in all cell types. qRT-PCR revealed a similar trend of higher initial MOI being associated with higher genome copies (Figure 3.1D-F). We calculated the specific infectivity (genome:PFU ratios) of WNV sampled across the 5-day infection (Figure 3.1G-I). Crow PBMCs had significantly higher specific infectivity across the three different MOI compared to DF1 cells and robin PBMCs, reflecting a higher fraction of non-infectious particles (2-way ANOVA, Tukey's Multiple Comparisons). Increases in MOI led to subtle increases in crow PBMC GE:PFU. At the lowest MOI, robin PBMCs had the lowest genome:PFU compared to DF1 cells and crow PBMCs, indicating that the majority of genomes are infectious. This distinction faded as the MOI increased to 1 and 10 and robin PBMC GE:PFU increased significantly. DF1 cells had intermediate specific infectivity compared to crow and robin PBMCs at 0.1 and 1 MOI and GE:PFU remained relatively stable regardless of MOI. Within each cell type, MOI-dependent GE:PFU differences were not observed (Mann-Whitney test).

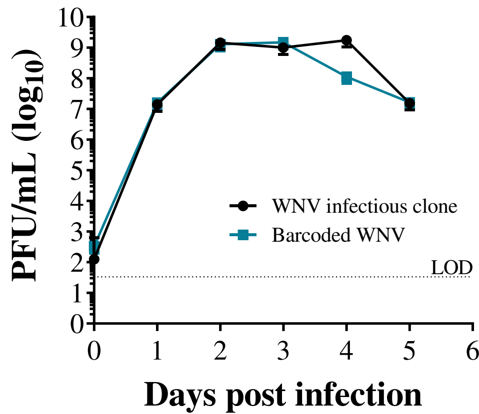
Establishing and Characterizing WNV Barcoded Virus (BC-WNV) Stock

To measure virus diversity within individual cells we generated a molecularly barcoded WNV (BC-WNV) as previously described for ZIKV (42,43). BC-WNV contains a segment in the NS4b region (7237-7269bp, Figure 3.2A) with 11 consecutive synonymous degenerate nucleotides at every third codon position.

A



B



C

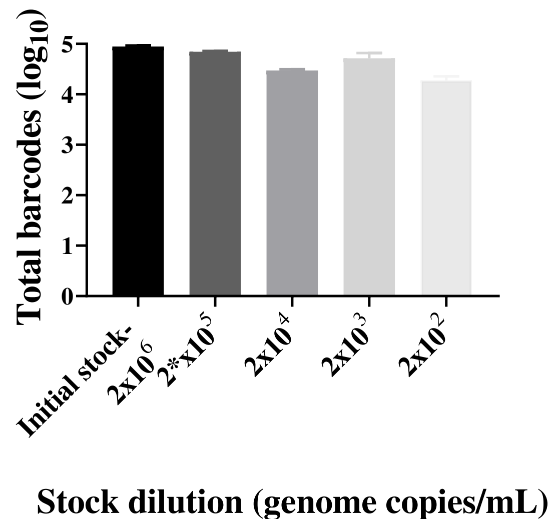


Figure 3.2. Establishing and Characterizing WNV Barcoded Virus (BC-WNV) Stock. (A) BC-WNV contains a segment in the NS4b region (7237-7269 bp) with 11 consecutive synonymous degenerate nucleotides at every third codon position. (B) Growth curve of WNV infectious clone backbone and BC-WNV in Vero cells (MOI = 0.1). (C) Total barcodes present in BC-WNV after dilution.

BC-WNV replicates similarly to unmodified WNV infectious clone in Vero cells (MOI = 0.1) (Figure 3.2B). Analysis of genetic diversity in the barcode region of the stock virus using unique molecular identifiers (UMIs) indicated that a total of 4,835 viral sequences were present per 50 µl of stock solution. These represented 2236 total unique barcodes. Three unique barcodes were detected more than 100 times in the stock and encompassed 14% of all viral sequences. Seven barcodes were detected 51-100 times (9%), 54 were detected 10-50 times (23%), 160

appeared 2-9 times (11%) and 2008 barcodes were detected 1 time and constituted 41% of the stock. We also assessed whether reductions in RNA copies impacted barcode composition. We diluted our stock 2x10^6 genome copies/mL to 2x10^2 genome copies/mL to confirm that barcode distribution was maintained regardless of concentration (Figure 3.2C).

Viral Barcode RNA Abundance Varies Between Cells and Across Different Wild Bird Hosts

We quantified barcodes in PBMCs from WNV-infected crows and robins after four days of *in vivo* replication and in DF1 cells inoculated at MOIs of 1 and 10 (Figure 3.3 & Supplementary Methods 1).

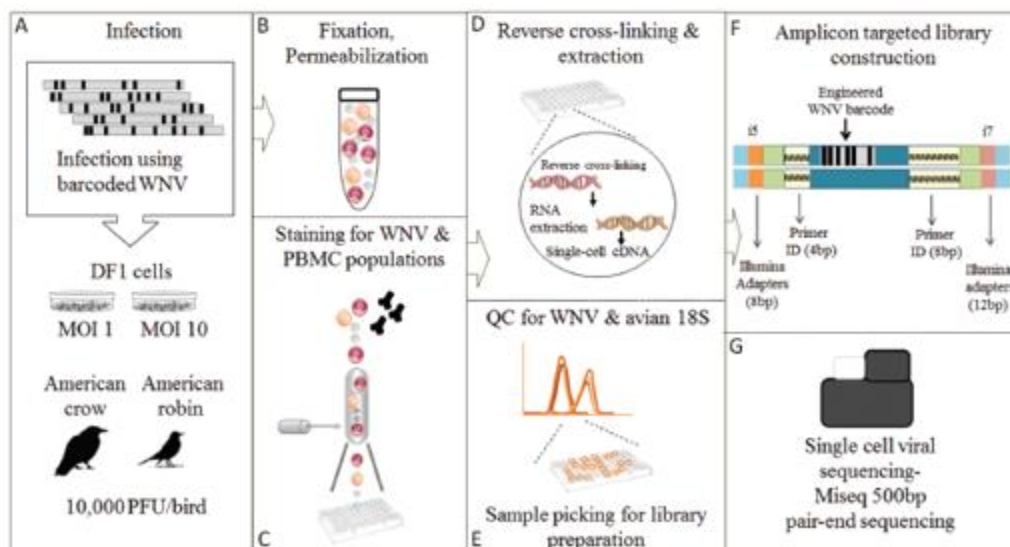


Figure 3.3 Barcode Quantification of WNV-infected Single DF1 cells and PBMCs by Flow Cytometry Sorting and Amplicon Targeted Library Construction. (A) DF1 cells at MOI 1 and 10 and American crows and robins at 10,000 PFU/bird were infected with BC-WNV. (B) PBMCs were collected from whole blood and DF1 cells were separated and fixed and permeabilized. (C) Cells were stained for WNV viral protein and cell type and sorted into a 96 well plate. (D) Cells were reverse cross-linked and viral RNA was extracted and reverse-transcribed into cDNA. (E) Individual cells were identified by PCR for WNV viral protein and avian 18S. (F) Libraries were constructed by adapting the Primer ID approach to the Illumina MiSeq platform (Supplementary Methods 1). (G) Sequencing was performed on an Illumina platform.

A total of 708 WNV-infected cells were sequenced including DF1 cells at MOI of 1 (n=94) and 10 (n=94), crow PBMCs (n=376) and robin PBCMs (n=144). Using qPCR with primer/probes to

detect the WNV E gene, we found that individual infected cells contained between 10^1-$10^{5.5}$ viral genomes/cell (Figure 3.4A).

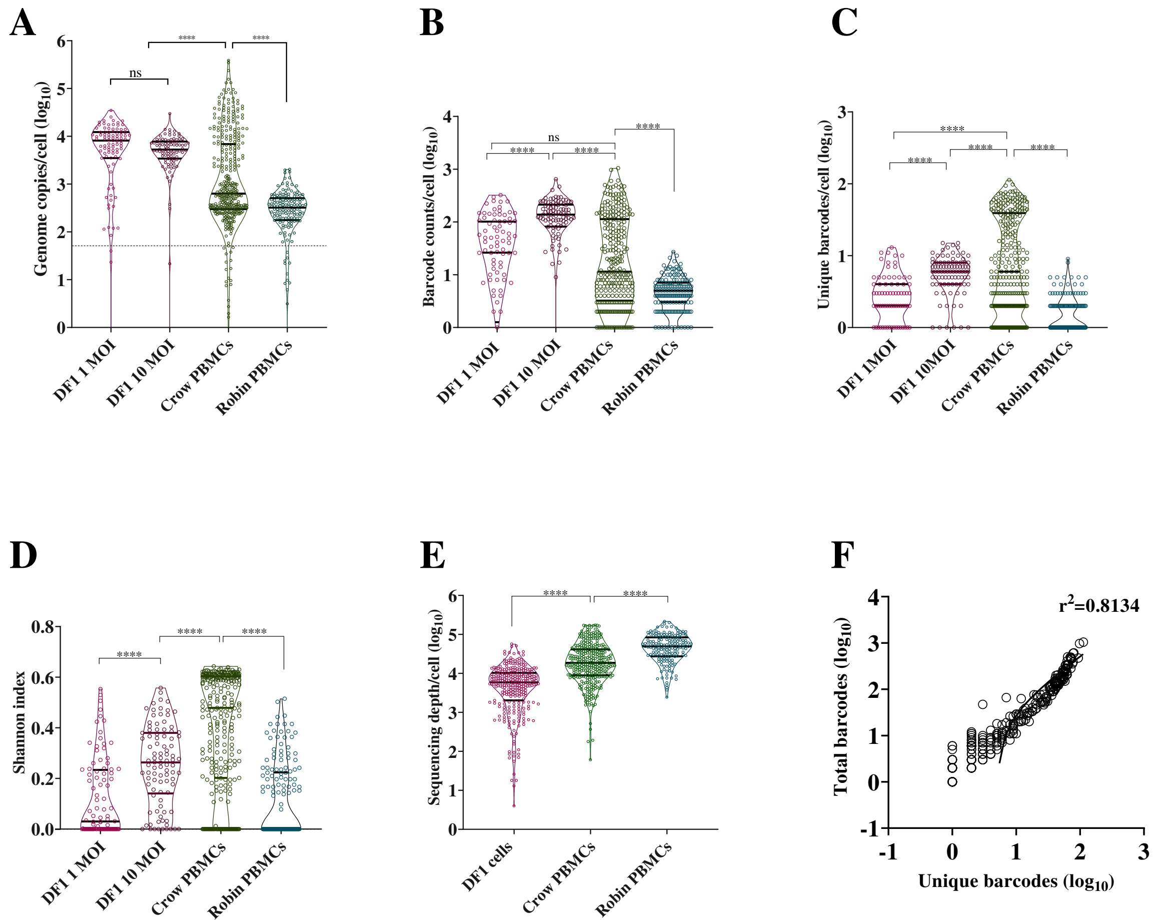


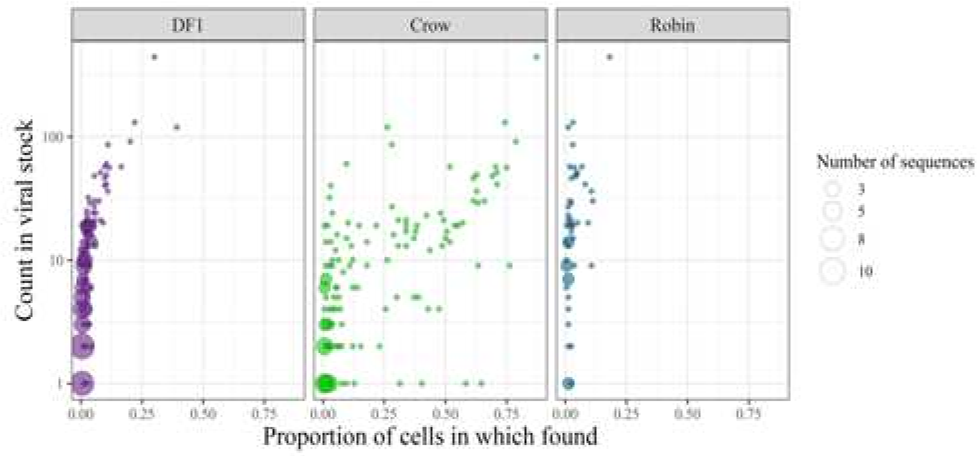
Figure 3.4. Viral Barcode RNA Abundance and Complexity Varies Between Cells and Across Wild Bird Hosts. (A) WNV Genome copies per cell ($\log_{10}$) in BC-WNV-infected DF1 cells at MOI 1 and 10 and crow and robin PBMCs as determined by qRT-PCR for E gene. (B) Barcode counts per cell ($\log_{10}$) in BC-WNV-infected DF1 cells at MOI 1 and 10 and crow and robin PBMCs as determined by sequencing of the NS4b barcode. (C) Unique barcodes per cell ($\log_{10}$) in BC-WNV-infected DF1 cells at MOI 1 and 10 and crow and robin PBMCs as determined by sequencing the NS4b barcode. (D) Shannon index (complexity) in DF1 cells at MOI 1 and 10 and crow and robin PBMCs. (E) Sequencing depth per cell ($\log_{10}$) in DF1 cells, crow and robin PBMCs. (Figures A-E, ****, P <0.0001) (F) Correlation of unique barcodes (log10) to total barcodes ($\log_{10}$) (r^2=0.8134).

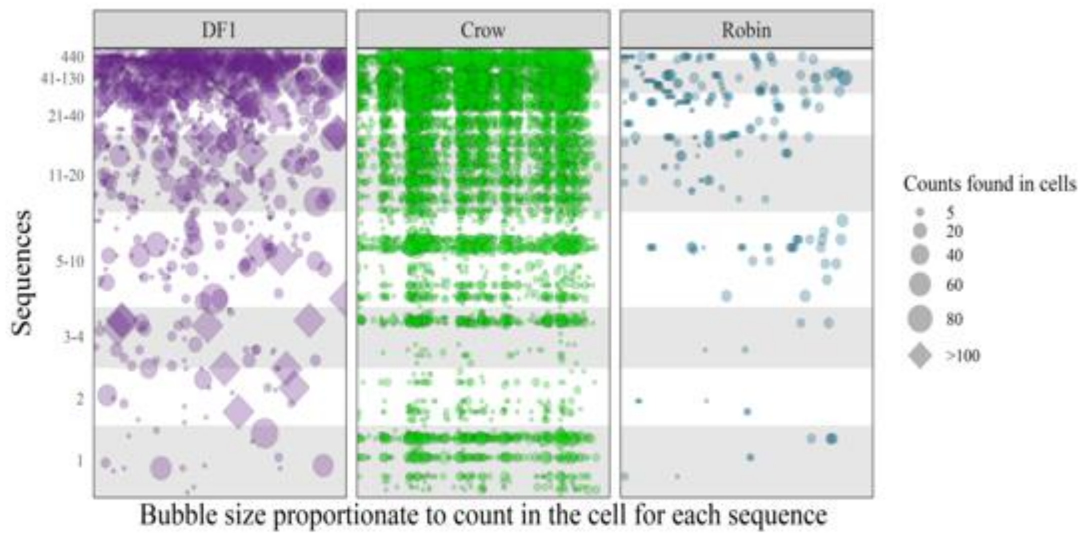
In crows, the maximum WNV genome capacity within a single cell was 388,094 with a mean of 11,265 RNA copies per cell, compared to a mean of 8530 or 5982 genomes in DF1 infected with MOI of 1 or 10, respectively, and 399 genomes per robin cell. There was a bimodal

distribution in crow cells, with 39% of cells containing an average of $10^{4.5}$ genomes (high subset) and 61% containing $10^{2.5}$ genomes (low subset). Robin PBMCs had significantly fewer WNV copies compared to crows and DF1 cells, which contained the highest mean genome copies per cell.

Single-cell sequencing supported our observations on viral load obtained using qRT-PCR (Figure 3.4B). Analysis of unique barcodes within cells revealed that crow PBMCs contained significantly higher barcode diversity (Figure 3.4C) and complexity (Shannon entropy) (Figure 3.4D) compared to both robin PBMCs and DF1 cells despite a slightly lower sequencing coverage depth compared to robins (Figure 3.4E). In all of our cells, total barcode count was significantly positively correlated with the number of unique barcodes (Figure 3.4F).

To determine the relationship between barcode frequency in the starting population and its likelihood of detection in single cells, we plotted the frequency of each unique barcode in the BC-WNV inoculum against its frequency of detection in single cells after replication (Figure 3.5A).


A
DF1
Crow
Robin
Count in viral stock
Proportion of cells in which found
Number of sequences
3
5
8
10


B
DF1
Crow
Robin
Sequences
440
41-130
21-40
11-20
5-10
3-4
2
1
Counts found in cells
5
20
40
60
80
>100
Bubble size proportionate to count in the cell for each sequence

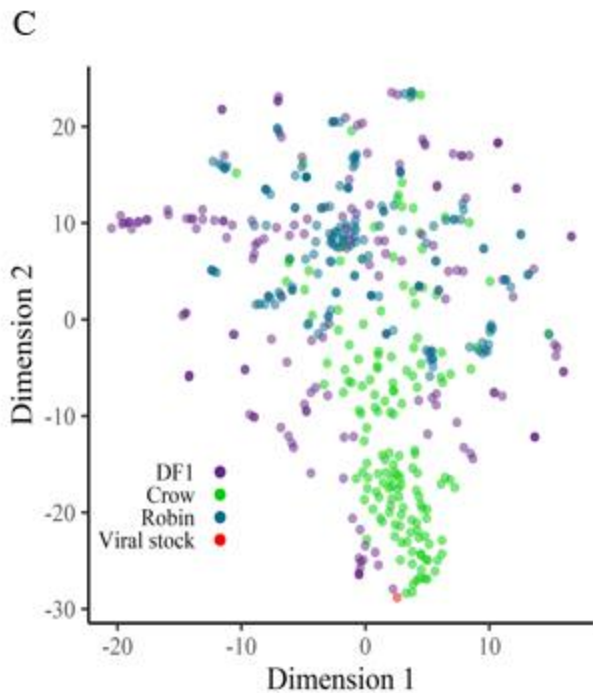


Figure 3.5. Barcode Prevalence Varies Between Cells and Across Wild Bird Hosts. (A) Frequency of each unique barcode in BC-WNV inoculum compared to detection in cells after replication. (B) Counts of unique barcode sequences in BC-WNV inoculum compared to counts found in cells after replication. (C) T-Distributed Stochastic Neighbor Embedding (t-SNE) analysis to plot the data in two-dimensional space.

Barcodes detected at lower frequencies in the input BC-WNV are almost never found in DF1 cells or robin PMBCs. In contrast, rare barcodes from the input frequently are detected in crow PBMCs, sometimes in greater than 50% of sequenced cells. In addition, more common barcodes from the input are almost always well maintained in crow PBMCs compared to robins (Figure 3.5B). Importantly, WNV populations within DF1 cells tend to become dominated by a single barcode sequence that rises to extremely high frequency, which almost never happens in crow or robin-derived cells. Finally, we used a t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis to plot the data in two-dimensional space (Figure 3.5C). Although we applied different perplexities to our data, cells did not cluster into distinct groups. Nevertheless, the crow cells consistently grouped closer to the stock sample than other cell types, supporting our

observations that barcode sequences, including those that are quite rare in the input stock virus population, are maintained during crow replication, but not in robins or DF1 cells.

Crows and robins promote replication of low-fitness WNV 1 at higher viremias

In divergent selective environments in crows and robins, the degree of viremia and PBMC polyinfection was a determinant of the ability of rare barcodes with no associated fitness differences to persist. To evaluate the significance of these findings to viral evolution, we needed to incorporate fitness implications. We hypothesized that variants with associated fitness losses would be able to better replicate systemically in higher viremia environments. Low fitness WNV variants were identified from previous experiments (44). We confirmed their low-fitness phenotype using replication studies and competition assays in DF-1 cells. All viruses exhibited similar growth kinetics in DF1 cells (Figure 3.6A).

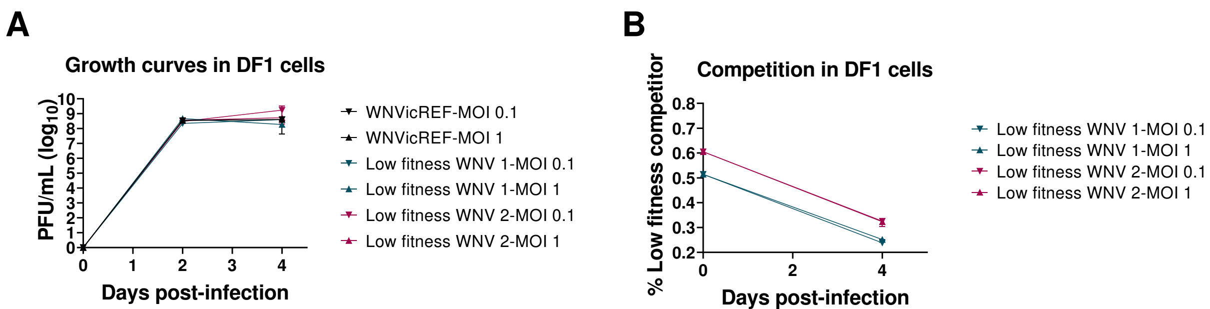


Figure 3.6. Characterization of low fitness WNVs *in vitro*. (A) Growth curves of Low fitness WNV 1 and 2 and WNVic-REF backbone in DF1 cells at MOIs of 0.1 and 1 with quantification by plaque assay (PFU/mL-$\log_{10}$; n=3 wells per virus per MOI; Bar=SEM). (B) Percent of low fitness competitor (y-axis) present during competition of low fitness viruses 1 and 2 against WNVic-REF at MOIs of 0.1 and 2 (PFU/mL in $\log_{10}$; n=3 wells per virus per MOI; Bar=SEM).

During the competition, DF1 cells were infected with a mixture of Low fitness WNV 1 or Low fitness WNV 2 and WNVic-REF at roughly equivalent genomic equivalents. By four days post-inoculation, Low fitness WNV 1 decreased by 26 and 27% at MOIs of 1 and 0.1, respectively and Low fitness WNV 2 decreased by 28% at both MOIs (Figure 3.6B).

We then infected crows and robins with our low fitness-WNVic-REF virus mixtures (Low fitness WNV 1-WNVic-REF or Low fitness WNV 2-WNVic-REF) and performed Sanger sequencing on their sera to assess the proportions of these two viruses at 2 and 4 days post inoculation. In crows for both infections and in robins for Low fitness WNV 1, day 4 post inoculation had greater mean viremia than at day 2 post inoculation (Figure 3.7A).

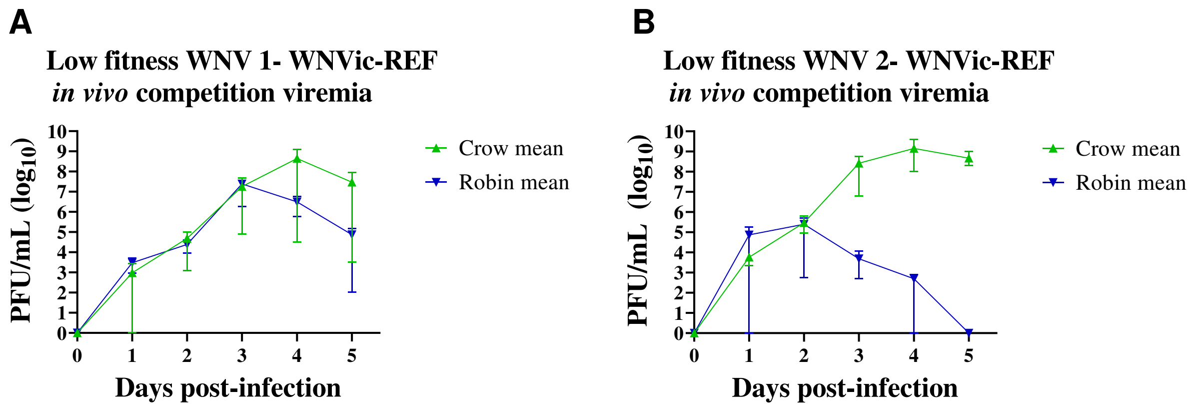


Figure 3.7. Avian Viremia During Low Fitness WNV-WNVic-REF Competitions. (A) Mean viremia (PFU/mL in $\log_{10}$) of American crows and American robins during Low Fitness WNV 1 and WNVic-REF competitions as determined by plaque assay (Bar = range). (B) Mean viremia (PFU/mL in $\log_{10}$) of American crows and American robins during Low Fitness WNV 2 and WNVic-REF competitions as determined by plaque assay (Bar = range).

In robins infected with Low fitness WNV 2, peak viremia was at 2 days post inoculation. At day 2, the mean robin viremia was 2.9×10^4 and 2.5×10^5 PFU/mL and the mean crow viremia was 4.8×10^4 and 3.0×10^5 PFU/mL for the Low fitness WNV 1-WNVic-REF and Low fitness WNV 2-WNVic-REF infections, respectively. Later at day 4, the mean robin viremia was 3.1×10^6 and 493 PFU/mL and the mean crow viremia was 4.42×10^8 and 1.4×10^9 PFU/mL for the Low fitness WNV 1-WNVic-REF and Low fitness WNV 2-WNVic-REF infections, respectively. At the day 4 time point when viremia was higher, the Low fitness WNV 1 was on average present at a higher proportion than it was at day 2 time points in both crows and robins (Figure 3.8A).

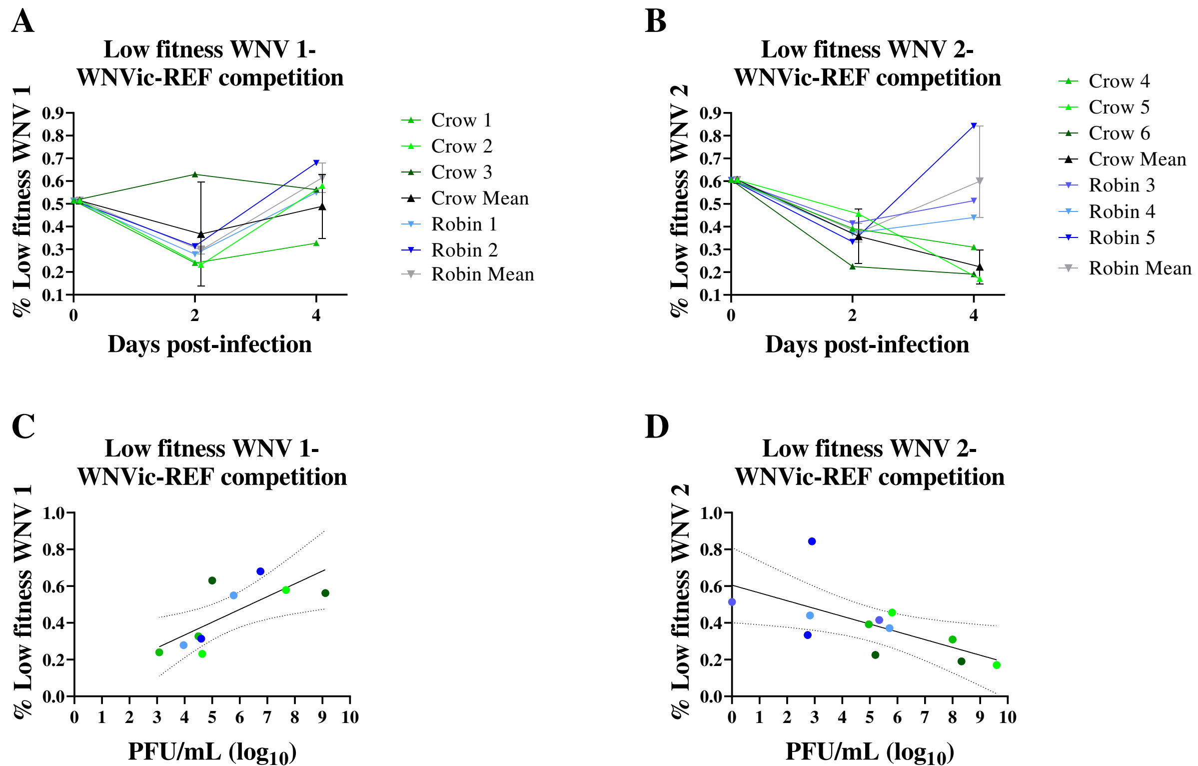


Figure 3.8. *In vivo* low fitness WNV vs WNVic-REF competition. (A) Percent of low fitness competitor (y-axis) present during competition of low fitness virus 1 against WNVic-REF in crows and robins (Bar=SEM). (B) Percent of low fitness competitor (y-axis) present during competition of low fitness virus 2 against WNVic-REF in crows and robins (Bar=SEM). (C) Comparison of percent of low fitness WNV 1 present during competition to viremia (PFU/mL in $\log_{10}$) with nonlinear regression (r^2=0.3902). (D) Comparison of percent of low fitness WNV 2 present during competition to viremia (PFU/mL in $\log_{10}$) with nonlinear regression (r^2=0.4131).

Of the birds infected with Low fitness WNV 1-WNVic-REF, 2/2 robins and 2/3 crows had increases in the low fitness competitor as viremia increased. For robins, Low fitness WNV 1 represented a mean 29.61% of viral RNA at day 2 and increased to 61.51% of viral RNA by day 4. In crows, Low fitness WNV 1 was on average 48.93% of viral RNA at day 2 and increased to 49.46% by day 4. Nonlinear regression of the relationship between viremia and the % Low fitness WNV 1 competitor yielded a curvilinear best fit line with a R-squared value of 0.5292 (Figure 3.8C).

Of the birds infected with Low fitness WNV 2-WNVic-REF, none of the robins or crows had increases in the low fitness competitor at higher viremia (Figure 3.8B). For robins, the Low fitness WNV 2 represented a mean 37.34% of viral RNA at day 2 when viremia was highest and increased to 59.95% of viral RNA by day 4 when viremia was lower. In crows, the Low fitness WNV 2 was on average 35.78% of viral RNA at day 2 and decreased to 22.34% by day 4 when viremia was higher. Nonlinear regression of the relationship between viremia and the % Low fitness WNV 2 competitor yielded a curvilinear best fit line with a R-squared value of 0.4131 (Figure 3.8D).

Discussion

Virus-host interactions clearly shape viral populations and evolution. We previously demonstrated that high WNV viremias in crows, coupled with error-prone virus replication, results in an extremely complex set of virus mutants that contains abundant nonviable virus genomes in addition to a wide array of low frequency virus variants (15). This contrasts with robins, in which lower viremia is associated with fewer genomes that contain lethal mutations and fewer overall variants. However, in robins, detected variants tended to rise to higher frequency. Importantly, PBMCs are a crucial site of WNV replication in birds. We hypothesized that high viremia in the crow leads to a high MOI of infection in PBMCs, resulting in polyinfection at the individual cell level. We expected this to facilitate the survival of rare and defective genomes via complementation in accordance with prior reports from studies conducted *in vitro* (34,45). Ultimately, the persistence of these low fitness genomes in natural populations would result in weakened selection. Similarly, we hypothesized that lower viremia in robins resulting in lower systemic MOI would lead to less PBMC polyinfection. Reduced polyinfection would then decrease the efficiency of complementation and reduce the survival of defective and rare genomes. To test these hypotheses, we assessed WNV replication in PBMCs, utilized a barcoded WNV to examine the replication of variably-represented genotypes at varying MOIs

and competed variably-fit viruses in *in vitro* and *in vivo* competitions to evaluate the effects of MOI on the survival of rare and less fit genotypes.

WNV replicated to modest titers in PBMCs *ex vivo*, consistent with previous reports of equine and avian PBMC infections (37,38). Crow PBMCs had the highest overall GE:PFU ratios during infection, indicating greater production of non-infectious particles. We also observed that higher MOIs in both robin and crow PBMC infections led to greater GE:PFU ratios. This is consistent with our previous *in vivo* studies that suggested high crow viremia may facilitate the persistence of defective genomes in the virus population (15). Defective genomes are commonly generated during lab and natural infections and have been demonstrated to survive multiple rounds of transmission suggesting that their production in birds can lead to maintenance in nature (29,46–48).

We next generated barcoded WNV for use in evaluating MOI *in vivo* in birds to explore the unique landscape of virus variants in single cells. While whole genome sequencing was performed on the BC-WNV stock and no undesired mutations were present for inoculation into PBMCs, we did not sequence the whole genome of virus extracted from PBMCs due to the expense required for sufficient coverage for over 700 individual cells. Therefore, it is possible that spontaneous mutations located outside the barcode could arise and confer fitness advantages or disadvantages during infection. We consider any rare undesired mutations in such a large data set largely inconsequential given that we observed no patterns of specific barcodes being preferentially replicated across the dataset.

This barcoded WNV virus, much like our previously published ZIKV barcoded virus (42) replicated similarly *in vitro* compared to wild-type WNV, indicating its suitability for *in vivo* studies. The barcoded WNV also exhibited *in vivo* phenotypes similar to wild-type as demonstrated by crow and robin viremia and organ viral loads (Supplementary Figure 1). When we performed NGS analysis of BC-WNV, we detected 2,236 barcodes, significantly fewer than the theoretical maximum. The cause of this is unclear; however, inefficient transfection of 293T

cells seems likely to have produced this result. Nevertheless, our data on BC-WNV replication *in vitro* and in birds, and our data on the complexity of the barcode region, indicated that it was a suitable tool to address the hypotheses we addressed in this work.

DF1 cells, crows and robins were infected with BC-WNV to determine the extent of polyinfection within cells of different origin. DF1 cells were infected at MOIs of 1 and 10 to compare *in vivo* findings to an established cell culture model of the avian-WNV interaction. DF1 cells contained up to 35,000 genome copies and had the highest mean genome copies per cell compared to avian PBMCs. This is not particularly surprising given that DF1 cell culture, while interferon-competent, is not affected by the influence of a full systemic innate immune response present during the *in vivo* infections performed to obtain PBMCs (40,49,50). WNV genome copies and barcode counts within DF1 cells did not differ significantly between MOI 1 and 10 suggesting that DF1 cells provide replication conditions that cause early saturation, and a difference might have been detected if shorter incubation periods were tested. Similarly, the generally higher titers in DF1 cells compared with PBMCs isolated from WNV-infected birds may be due to the absence of a complete innate immune response occurring in those cells *in vitro*. Analysis of barcode frequencies within DF1 cells demonstrated that rare input barcodes were typically lost during replication, and that each DF1 cell was frequently dominated by a single barcode at very high frequency, a trend not observed in crow or robin derived cells, indicating the limitations of DF1s as a model for WNV-avian interactions. We can only speculate that the preferable replication conditions and possibly shorter time to saturation would be able to cause such a rapid takeover of few single dominating variants (barcodes).

Analysis of viral loads within crow compared to robin PBMCs revealed striking differences in both genome copy numbers and total barcode counts. Crow PBMCs demonstrated the greatest range upper limit (individual cells reached the highest amounts) for genome copy numbers while robins contained the least, with up to a thousand-fold average difference. A single crow PBMC is capable of containing up to 388,000 genome copies while

robin PBMCs contained up to 2000 genome copies. This high viral load in crow compared to robin PBMC is consistent with viremia patterns in these animals and supports the role of PBMCs in generating host-specific viremia and mortality phenotypes in host animals. Moreover, these data indicate that clinically susceptible animals that develop strikingly high viremias contain high viral loads in circulating PBMCs.

Further, crows had higher numbers of unique barcodes within individual cells, indicating more frequent polyinfection compared to robins. When we compared changes in barcode frequency in the input inoculum to crow PBMCs, we observed previously rare barcodes rising in frequency, sometimes to greater than 50% of the population within an individual cell. In contrast, rare mutations in the initial stock tended not to be detected in robin PBMCs. We also observed more unique barcodes and higher levels of barcode diversity and complexity in crows compared to robin PBMCs. These data permit us to conclude that in crows, frequent polyinfection of PMBCs, and likely other cell types, facilitate the persistence of genetic diversity in these animals, including defective viral variants that may reduce the average fitness of the virus population. Moreover, hosts that experience high viremias may be key to maintaining virus genetic diversity at the population level and decrease the strength of purifying selection. As a consequence, high fitness variants may be prevented from rising in frequency within the population.

Conversely, infection of robins results in less frequent polyinfection and an overall reduction in population variation that may either result in fitness increases due to the action of purifying selection, or bottlenecks that lead to fitness declines over time. These somewhat divergent selective environments in crows and robins led us to hypothesize that WNV variants of reduced fitness would persist longer within crows compared to robins.

To test this, we measured the fitness of putatively low-fitness WNV strains identified and described previously in crows and robins(44). Our expectation was that their fitness would be higher in crows than robins due to weakened purifying selection imposed by high viremia and

consequently frequent polyinfection. We found that the ability of low fitness viruses to persist in the populations *in vivo* is related to viremia and less specific to the species of bird. This is further demonstrated at the second time point when mean viremia was similar between both species and there were similar trends in the representation of the low fitness competitor and reference virus. Given that the natural upper range limit of robin viremias overlaps with the lower range limit of crow viremias during these particular experiments, our results are perhaps unsurprising. This further supports previous assessments of species-specific viral diversity when WNV was passaged through crows and robins (15). Our *in vivo* competition of Low fitness WNV 2 against WNVic-Ref were different in that the low fitness variants were always outcompeted. The cause of this is unclear, but it seems likely that there is a threshold below which poorly fit variants cannot be 'rescued' by high viremia and polyinfection. The observed viremia curve during this particular infection peaked earlier and terminated faster than other experiments. While it is possible that viremia and MOI have less of an influence than hypothesized, it is also possible that there are specific phenotypic effects to the point mutation of this mutant which caused varying outcomes.

In summary, this study quantifies and characterizes genetic evolutionary processes within naturally infected relevant hosts at the single cell and systemic level. Our work with PBMCs demonstrated that individual cells, particularly in crows, can contain extremely high levels of virus and provide an environment for generation of defective genomes and genetic complementation. The novel use of single cell next-generation sequencing in conjunction with a barcoded WNV technique highlights the ability of single cells to contribute to viral population genetics in a species-specific manner. While we assessed variants of WNV, these sequencing methodologies could also be utilized to assess multi-infections between different viral etiologies and the associated competition for cellular replication resources. Our *in vivo* competition of a low fitness WNV and a high fitness reference virus demonstrated how viremia and MOI can potentially lead to multi-infections and complementation, thus linking our findings at the single

cell level to the circulating virus populations which go on to be transmitted to mosquitoes and maintained in nature.

References

1. Ehrbar DJ, Ngo KA, Campbell SR, Kramer LD, Ciota AT. High levels of local inter- and intra-host genetic variation of West Nile virus and evidence of fine-scale evolutionary pressures. Infect Genet Evol. 2017 Jul 1;51:219–26.

2. Romano CM, Lauck M, Salvador FS, Lima CR, Villas-Boas LS, Araújo ESA, et al. Inter- and Intra-Host Viral Diversity in a Large Seasonal DENV2 Outbreak. López-Galíndez C, editor. PLoS One [Internet]. 2013 Aug 2 [cited 2021 Jan 18];8(8):e70318. Available from: https://dx.plos.org/10.1371/journal.pone.0070318

3. Ketkar H, Herman D, Wang P. Genetic determinants of the re-emergence of arboviral diseases [Internet]. Vol. 11, Viruses. MDPI AG; 2019 [cited 2021 Jan 18]. Available from: /pmc/articles/PMC6410223/?report=abstract

4. Coffey LL, Vignuzzi M. Host Alternation of Chikungunya Virus Increases Fitness while Restricting Population Diversity and Adaptability to Novel Selective Pressures. J Virol [Internet]. 2011 Jan 15 [cited 2021 Jan 18];85(2):1025–35. Available from: http://jvi.asm.org/.http://jvi.asm.org/

5. Shriner D, Liu Y, Nickle DC, Mullins JI. EVOLUTION OF INTRAHOST HIV - 1 GENETIC DIVERSITY DURING CHRONIC INFECTION. Evolution (N Y) [Internet]. 2006 Jun 1 [cited 2021 Jan 18];60(6):1165–76. Available from: http://doi.wiley.com/10.1111/j.0014-3820.2006.tb01195.x

6. Azevedo SSD, Caetano DG, Côrtes FH, Teixeira SLM, Santos Silva K, Hoagland B, et al. Highly divergent patterns of genetic diversity and evolution in proviral quasispecies from HIV controllers. Retrovirology [Internet]. 2017 May 2 [cited 2021 Jan 18];14(1):29. Available from: http://retrovirology.biomedcentral.com/articles/10.1186/s12977-017-0354-5

7. Sobel Leonard A, McClain MT, Smith GJD, Wentworth DE, Halpin RA, Lin X, et al. Deep Sequencing of Influenza A Virus from a Human Challenge Study Reveals a Selective Bottleneck and Only Limited Intrahost Genetic Diversification. J Virol [Internet]. 2016 Dec 15 [cited 2021 Jan 18];90(24):11247–58. Available from: http://jvi.asm.org/

8. Lequime S, Fontaine A, Ar Gouilh M, Moltini-Conclois I, Lambrechts L. Genetic Drift, Purifying Selection and Vector Genotype Shape Dengue Virus Intra-host Genetic Diversity in Mosquitoes. Malik HS, editor. 2016 Jun [cited 2021 Jan 18];12(6):e1006111. Available from: https://dx.plos.org/10.1371/journal.pgen.1006111

9. Weaver SC, Forrester NL, Liu J, Vasilakis N. Population bottlenecks and founder effects: implications for mosquito-borne arboviral emergence. Nat Rev Microbiol [Internet]. 2021 Jan 11 [cited 2021 Jan 18];1. Available from: http://www.nature.com/articles/s41579-020-00482-8

10. Brault AC. Changing patterns of West Nile virus transmission: altered vector competence and host susceptibility. Vet Res [Internet]. 2009 Mar 1 [cited 2019 Jul 30];40(2):43. Available from: http://www.vetres.org/10.1051/vetres/2009026

11. Brault AC, Langevin SA, Bowen RA, Panella NA, Biggerstaff BJ, Miller BR, et al. Differential

virulence of West Nile strains for American Crows. Emerg Infect Dis [Internet]. 2004 [cited 2020 Oct 25];10(12):2161–8. Available from: https://pubmed.ncbi.nlm.nih.gov/15663854/

12. Deardorff ER, Fitzpatrick KA, Jerzak GVS, Shi PY, Kramer LD, Ebel GD. West nile virus experimental evolution in vivo and the trade-off hypothesis. PLoS Pathog [Internet]. 2011 Nov [cited 2019 Nov 26];7(11). Available from: https://pubmed.ncbi.nlm.nih.gov/22102808/

13. Duggal NK, Reisen WK, Fang Y, Newman RM, Yang X, Ebel GD, et al. Genotype-specific variation in West Nile virus dispersal in California. Virology [Internet]. 2015 Nov 1 [cited 2020 Oct 26];485:79–85. Available from: https://pubmed.ncbi.nlm.nih.gov/26210076/

14. Duggal NK, Langwig KE, Ebel GD, Brault AC. On the Fly: Interactions between Birds, Mosquitoes, and Environment That Have Molded West Nile Virus Genomic Structure over Two Decades [Internet]. Vol. 56, Journal of Medical Entomology. Oxford University Press; 2019 [cited 2020 Oct 26]. p. 1467–74. Available from: https://pubmed.ncbi.nlm.nih.gov/31549720/

15. Grubaugh ND, Smith DR, Brackney DE, Bosco-Lauth AM, Fauver JR, Campbell CL, et al. Experimental Evolution of an RNA Virus in Wild Birds: Evidence for Host-Dependent Impacts on Population Structure and Competitive Fitness. PLoS Pathog. 2015;

16. Jerzak GVS, Brown I, Shi P-Y, Kramer LD, Ebel GD. Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching. Virology [Internet]. 2008 May 10 [cited 2019 Nov 26];374(2):256–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18395240

17. Hadfield J, Brito AF, Swetnam DM, Vogels CBF, Tokarz RE, Andersen KG, et al. Twenty years of West Nile virus spread and evolution in the Americas visualized by Nextstrain. Vol. 15, PLoS Pathogens. Public Library of Science; 2019.

18. Grubaugh ND, Ebel GD. Dynamics of West Nile virus evolution in mosquito vectors. Vol. 21, Current Opinion in Virology. 2016.

19. Grubaugh ND, Fauver JR, Rückert C, Weger-Lucarelli J, Garcia-Luna S, Murrieta RA, et al. Mosquitoes Transmit Unique West Nile Virus Populations during Each Feeding Episode. Cell Rep [Internet]. 2017 [cited 2019 Nov 26];19(4):709–18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28445723

20. Ciota AT, Lovelace AO, Ngo KA, Le AN, Maffei JG, Franke MA, et al. Cell-specific adaptation of two flaviviruses following serial passage in mosquito cell culture. Virology [Internet]. 2007 Jan 20 [cited 2020 Oct 26];357(2):165–74. Available from: https://pubmed.ncbi.nlm.nih.gov/16963095/

21. Ciota AT, Ngo KA, Lovelace AO, Payne AF, Zhou Y, Shi PY, et al. Role of the mutant spectrum in adaptation and replication of West Nile virus. J Gen Virol [Internet]. 2007 Mar [cited 2020 Oct 26];88(3):865–74. Available from: https://pubmed.ncbi.nlm.nih.gov/17325359/

22. Ciota AT, Lovelace AO, Jones SA, Payne A, Kramer LD. Adaptation of two flaviviruses results in differences in genetic heterogeneity and virus adaptability. J Gen Virol [Internet]. 2007 Sep 1 [cited 2020 Oct 26];88(9):2398–406. Available from: https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.83061-0

23. Scroggs SLP, Grubaugh ND, Sena JA, Sundararajan A, Schilkey FD, Smith DR, et al. Endless Forms: Within-Host Variation in the Structure of the West Nile Virus RNA Genome during Serial Passage in Bird Hosts Downloaded from. 2019 [cited 2019 Jul 23]; Available from: https://msphere.asm.org/content/msph/4/3/e00291-19.full.pdf

24. Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, et al. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis. 2003 Mar 1;9(3):311–22.

25. Jerzak GVS, Bernard K, Kramer LD, Shi PY, Ebel GD. The West Nile virus mutant spectrum is host-dependant and a determinant of mortality in mice. Virology [Internet]. 2007 Apr 10 [cited 2021 Jan 18];360(2):469–76. Available from: https://pubmed.ncbi.nlm.nih.gov/17134731/

26. Pesko KN, Ebel GD. West Nile virus population genetics and evolution [Internet]. Vol. 12, Infection, Genetics and Evolution. Infect Genet Evol; 2012 [cited 2020 Oct 26]. p. 181–90. Available from: https://pubmed.ncbi.nlm.nih.gov/22226703/

27. Duggal NK, Bosco-Lauth A, Bowen RA, Wheeler SS, Reisen WK, Felix TA, et al. Evidence for Co-evolution of West Nile Virus and House Sparrows in North America. Tesh RB, editor. PLoS Negl Trop Dis [Internet]. 2014 Oct 30 [cited 2019 Mar 17];8(10):e3262. Available from: https://dx.plos.org/10.1371/journal.pntd.0003262

28. Fitzpatrick KA, Deardorff ER, Pesko K, Brackney DE, Zhang B, Bedrick E, et al. Population variation of West Nile virus confers a host-specific fitness benefit in mosquitoes. Virology [Internet]. 2010 Aug [cited 2020 Oct 26];404(1):89–95. Available from: https://pubmed.ncbi.nlm.nih.gov/20552731/

29. Pesko KN, Fitzpatrick KA, Ryan EM, Shi PY, Zhang B, Lennon NJ, et al. Internally deleted WNV genomes isolated from exotic birds in New Mexico: Function in cells, mosquitoes, and mice. Virology [Internet]. 2012 May 25 [cited 2020 Oct 26];427(1):10–7. Available from: https://pubmed.ncbi.nlm.nih.gov/22365325/

30. Brault AC, Huang CY-H, Langevin SA, Kinney RM, Bowen RA, Ramey WN, et al. A single positively selected West Nile viral mutation confers increased virogenesis in American crows.

31. Langevin SA, Brault AC, Panella NA, Bowen RA, Komar N. Variation in virulence of West Nile virus strains for house sparrows (Passer Domesticus). Am J Trop Med Hyg. 2005 Jan;72(1):99–102.

32. Nemeth NM, Thomsen B V, Spraker TR, Benson JM, Bosco-Lauth AM, Oesterle PT, et al. Clinical and Pathologic Responses of American Crows (Corvus brachyrhynchos) and Fish Crows (C ossifragus) to Experimental West Nile Virus Infection. Vet Pathol [Internet]. 2011 Nov [cited 2019 Nov 15];48(6):1061–74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21383115

33. VanDalen KK, Hall JS, Clark L, McLean RG, Smeraski C. West Nile virus infection in American Robins: new insights on dose response. PLoS One [Internet]. 2013 [cited 2019 Nov 15];8(7):e68537. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23844218

34. Froissart R, Wilke CO, Montville R, Remold SK, Chao L, Turner PE. Co-infection weakens

selection against epistatic mutations in RNA viruses. Genetics [Internet]. 2004 Sep [cited 2020 Oct 26];168(1):9–19. Available from: https://pubmed.ncbi.nlm.nih.gov/15454523/

35. Steinhauer DA, de la Torre JC, Meier E, Holland JJ. Extreme heterogeneity in populations of vesicular stomatitis virus. J Virol [Internet]. 1989 [cited 2020 Oct 26];63(5):2072–80. Available from: https://pubmed.ncbi.nlm.nih.gov/2539503/

36. Bai F, Kong K-F, Dai J, Qian F, Zhang L, Brown CR, et al. A Paradoxical Role for Neutrophils in the Pathogenesis of West Nile Virus. 2010 [cited 2019 Jan 18];202. Available from: https://academic.oup.com/jid/article-abstract/202/12/1804/2192117

37. Dietrich EA, Bowen RA, Brault AC. An ex vivo avian leukocyte culture model for West Nile virus infection. J Virol Methods. 2015;

38. Garcia-Tapia D, Loiacono CM, Kleiboeker SB. Replication of West Nile virus in equine peripheral blood mononuclear cells. Vet Immunol Immunopathol. 2006;

39. Rios M, Zhang MJ, Grinev A, Srinivasan K, Daniel S, Wood O, et al. Monocytes-macrophages are a potential target in human infection with West Nile virus through blood transfusion. Transfusion. 2006;

40. Suthar MS, Diamond MS, Gale M. West Nile virus infection and immunity. Nat Rev Microbiol [Internet]. 2013 Feb [cited 2019 Sep 18];11(2):115–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23321534

41. Uddin MJ, Suen WW, Bosco-Lauth A, Hartwig AE, Hall RA, Bowen RA, et al. Kinetics of the West Nile virus induced transcripts of selected cytokines and toll-like receptors in equine peripheral blood mononuclear cells. Vet Res [Internet]. 2016 [cited 2020 Oct 26];47(1). Available from: https://pubmed.ncbi.nlm.nih.gov/27267361/

42. Weger-Lucarelli J, Duggal NK, Bullard-Feibelman K, Veselinovic M, Romo H, Nguyen C, et al. Development and Characterization of Recombinant Virus Generated from a New World Zika Virus Infectious Clone. J Virol [Internet]. 2017 Jan 1 [cited 2020 Oct 26];91(1). Available from: https://pubmed.ncbi.nlm.nih.gov/27795432/

43. Weger-Lucarelli J, Garcia SM, Rückert C, Byas A, O'Connor SL, Aliota MT, et al. Using barcoded Zika virus to assess virus population structure in vitro and in Aedes aegypti mosquitoes. Virology [Internet]. 2018 Aug 1 [cited 2020 Oct 26];521:138–48. Available from: https://pubmed.ncbi.nlm.nih.gov/29935423/

44. Grubaugh ND, Weger-Lucarelli J, Murrieta RA, Fauver JR, Garcia-Luna SM, Prasad AN, et al. Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching. Cell Host Microbe [Internet]. 2016 Apr 13 [cited 2020 Oct 26];19(4):481–92. Available from: https://pubmed.ncbi.nlm.nih.gov/27049584/

45. García-Arriaza J, Manrubia SC, Toja M, Domingo E, Escarmís C. Evolutionary Transition toward Defective RNAs That Are Infectious by Complementation. J Virol [Internet]. 2004 Nov 1 [cited 2020 Oct 26];78(21):11678–85. Available from: https://pubmed.ncbi.nlm.nih.gov/15479809/

46. Li D, Lott WB, Lowry K, Jones A, Thu HM, Aaskov J. Defective interfering viral particles in acute dengue infections. PLoS One [Internet]. 2011 [cited 2020 Oct 26];6(4). Available from: https://pubmed.ncbi.nlm.nih.gov/21559384/

47. Aaskov J, Buzacott K, Thu HM, Lowry K, Holmes EC. Long-term transmission of defective RNA viruses in humans and Aedes mosquitoes. Science (80-) [Internet]. 2006 Jan 13 [cited 2020 Oct 26];311(5758):236–8. Available from: https://pubmed.ncbi.nlm.nih.gov/16410525/

48.Ke R, Aaskov J, Holmes EC, Lloyd-Smith JO. Phylodynamic Analysis of the Emergence and Epidemiological Impact of Transmissible Defective Dengue Viruses. PLoS Pathog [Internet]. 2013 Feb [cited 2020 Oct 26];9(2). Available from: https://pubmed.ncbi.nlm.nih.gov/23468631/

49. Samuel MA, Diamond MS. Pathogenesis of West Nile Virus Infection: a Balance between Virulence, Innate and Adaptive Immunity, and Viral Evasion. J Virol [Internet]. 2006 [cited 2019 Jan 18];80(19):9349–60. Available from: http://jvi.asm.org/

50. Ahlers LRH, Goodman AG. The immune responses of the animal hosts of West Nile virus: A comparison of insects, birds, and mammals [Internet]. Vol. 8, Frontiers in Cellular and Infection Microbiology. Frontiers Media S.A.; 2018 [cited 2021 Jan 19]. p. 96. Available from: /pmc/articles/PMC5891621/?report=abstract

51. Shi P-Y, Tilgner M, Lo MK, Kent KA, Bernard KA. Infectious cDNA Clone of the Epidemic West Nile Virus from New York City. J Virol [Internet]. 2002 Jun 15 [cited 2020 Oct 26];76(12):5847–56. Available from: http://jvi.asm.org/

52. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. 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 [Internet]. 2000 [cited 2020 Sep 10];38(11):4066–71. Available from: https://pubmed.ncbi.nlm.nih.gov/11060069/

53. Thomsen ER, Mich JK, Yao Z, Hodge RD, Doyle AM, Jang S, et al. Fixed single-cell transcriptomic characterization of human radial glial diversity. Nat Methods [Internet]. 2015 Dec 30 [cited 2020 Oct 26];13(1):87–93. Available from: https://pubmed.ncbi.nlm.nih.gov/26524239/

54. Zhou S, Jones C, Mieczkowski P, Swanstrom R. Primer ID Validates Template Sampling Depth and Greatly Reduces the Error Rate of Next-Generation Sequencing of HIV-1 Genomic RNA Populations. J Virol [Internet]. 2015 Aug 15 [cited 2020 Oct 26];89(16):8540–55. Available from: https://pubmed.ncbi.nlm.nih.gov/26041299/

55. Hall GS, Little DP. Relative quantitation of virus population size in mixed genotype infections using sequencing chromatograms. J Virol Methods. 2007 Dec 1;146(1–2):22–8.

Chapter 4. American Alligators are Capable of West Nile Virus Amplification, Mosquito Infection and Transmission[3]

Introduction

West Nile virus (Flavivirus, *Flaviviridae*, WNV) is naturally maintained in an enzootic cycle between mosquitoes and wild birds (1). 'Dead-end' hosts, such as humans and horses, are infected with WNV by mosquitoes but do not develop a viremia of sufficient magnitude to infect mosquitoes (1). Between 2001 and 2003, WNV outbreaks caused large-scale mortalities in farmed American alligators (*Alligator mississippiensis*) in the southeastern United States (2,3) and epizootics continue to be an issue for both alligators and multiple species of crocodiles (4,5). Clinical signs in primarily juvenile alligators, while uncommon, are neurologic or gastrointestinal in nature, with neurologic signs including unbalanced and uncoordinated swimming, ataxia, tremors and head tilt while gastrointestinal signs included anorexia and bloating (2,3,6). Histopathologic findings include multiorgan necrosis and inflammation (2,3). Lymphohistiocytic proliferative cutaneous lesions have also been observed in both alligators and crocodiles and arise 4-5 weeks after acute infections (5–7). These lesions, also known as "pix", grossly appear as transluminant 1-2 mm defects which are revealed during processing. The associated losses in hide quality cause significant economic impacts to farming operations. Through investigations of natural disease and experimental infections, potential causes of these outbreaks include horizontal transmission between alligators, consumption of WNV-infected meat and mosquito transmission(8–10).

[3] This chapter has been formatted for submission to *Virology* and has been minimally modified for formatting.

It is not fully understood how WNV overwinters or is maintained during seasonal absence of mosquitoes. Potential mechanisms include vertical transmission from mosquitoes to offspring, fecal-oral transmission in bird roosts, avian scavenging of WNV-infected carcasses, and chronic avian infections (11-18). Each of these potential modes of WNV overwintering have limitations or inefficiencies; therefore, we also sought to examine whether alligators may contribute to WNV maintenance in a mosquito-independent manner. Experimental infections of saltwater crocodiles (*Crocodylus porosus*) revealed the presence of WNV viral RNA in tank water, as well as oral and cloacal swabs; therefore, oral and cloacal shedding with mucosal exposure via water was interpreted as the mechanism of transmission to uninfected tankmates (7). In an experimental setting, needle-inoculated WNV-infected alligators also transmitted WNV to uninfected tankmates but the mechanism for this transmission was not explicitly demonstrated during this study (8). Accordingly, we assessed whether water can serve as a source of infection for alligators.

Similar to the mechanisms of horizontal transmission in alligators, oral and cloacal shedding of WNV has been demonstrated experimentally in birds and is a potential mechanism of WNV transmission in crow roosts when mosquitoes are inactive (12,13,19). Given that horizontal transmission is a potential means of mosquito-free WNV transmission in wild birds and alligators separately, we assessed the possibility of bidirectional horizontal transmission between alligators and wild birds (13,15). A feature that sets alligator WNV infections apart from dead-end hosts is the ability to develop viremia ranging between 10^5 and 10^6 plaque forming units per milliliter (PFU/mL) (3). That magnitude of viremia is sufficient for infection of *Culex* spp. mosquitoes (20,21). *Culex quinquefasciatus* is a significant vector for WNV transmission in the southeastern United States, the natural environment of American alligators, and testing of *Culex* mosquito bloodmeals demonstrated feeding on alligators (9,10). Accordingly, we sought to demonstrate transmission of WNV between alligators and mosquitoes.

To determine whether water serves as a potential source of WNV alligator infection, we inoculated tank water with WNV and confirmed that alligators developed a viremia. We subsequently assessed the possibility of bidirectional horizontal transmission between alligators and wild birds via water and did not observe infection in either species. This suggests that birds and alligators sharing water sources do not likely contribute to the natural maintenance of WNV. We also sought to examine the contributions of alligators to WNV maintenance in nature by mosquito. We experimentally demonstrated that WNV-infected mosquitoes transmit WNV to alligators, an unsurprising finding given the promiscuity of the virus and the mosquitoes that transmit it (1). Finally, we demonstrated that WNV-viremic American alligators were capable of infecting mosquitoes with subsequent ability for salivary transmission, indicating that they potentially amplify WNV in natural or farmed alligator environments.

Materials and Methods

Ethics statement

Wild birds were obtained and/or held under US Fish and Wildlife Service (#MB68845B-0) and Colorado Parks and Wildlife (# 19TRb2106, 19TR3387 & 20TRb2106) permits and with permissions from park supervisors and private landowners. No endangered or protected species were harmed during collections. Experiments involving animals were conducted in accordance with protocols approved by the Colorado State University Institutional Animal Care and Use Committee (Protocols #15-5958, 18-8080A &19-8763-A) and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Cells

Vero cells (ATCC CCL-81) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum, 50 µg/ml gentamycin, 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C with 5% CO_2.

Viruses

American isolates of WNV from a mosquito pool collected in Fort Collins CO (FtC-3699) and WNV strain NY99-4132 originally isolated from an American crow in New York 1999 were used for mosquito artificial infections and avian infections. FtC-3699 was used for bird and mosquito infections as these birds were being used for additional experiments in which FtC-3699 was the control. WNV NY99-4132 was used for alligators as it had previously been used in the lab and produced alligator infections which reached or exceeded viremia of ~10^5 PFU/mL. Viral titers were quantified using Vero cells and a standard plaque assay protocol (22). Viruses were propagated in Vero cells and stocks were diluted to 10,000 PFU/100 µl for inoculation. Stocks were stored at -80°C with 10% FBS and DMEM.

Mosquitoes

Culex quinquefasciatus (Sebring strain), *Culex tarsalis (*KR83*)* and *Culex pipiens* (Reeves strains) colonies maintained in the lab were used for artificial bloodfeed infections and feeding on alligators. For colony maintenance, mosquitoes were fed defibrinated calf blood and given sugar and water *ad libitum*. Larvae were reared and adults maintained under controlled conditions of temperature (26-27°C), relative humidity (70-80%) and light (16:8 light: dark diurnal cycle).

Alligator husbandry

Alligators were obtained from the Everglades Alligator Farm (Homestead, FL) under Colorado import permit 19HP3585. Alligators were fed gator chow pellets every other day (food volume ~5% of body weight). Alligators were housed in livestock tanks (either 1 or 2.37 m diameter) containing approximately 120 or 400 L of water, respectively, at 27°C and with adequate basking surfaces. Water was heated by aquarium heaters and aerated with aquarium water pumps. Function of heaters and water pumps were checked twice daily. Water was changed once a week. All animal infections were conducted under ABSL-3 containment.

Avian husbandry

American crows (*Corvus brachyrhynchos*) and American robins (*Turdus migratorius*) were housed in 0.5 to 1 m^3 cages in groups of up to 3 and fed *ad libitum* water and dry dog food (crows), moistened kitten food (robins), fruit (robins), dried mealworms (robins) and/or bird seed (sparrows). Birds and alligators were pre-screened for antibodies by PRNT before use.

Tankwater inoculation

To spike alligator tank water with WNV, 100 mL of freshly harvested viral stock was added immediately into the tanks prepared for housing alligators which contained 120 L of water. Virus was titrated in Vero cells to $1x10^{10}$ PFU/mL.

Vertebrate artificial inoculations

Birds and alligators were infected with 10,000 PFU of WNV in 100 of DMEM by subcutaneous and intracoelomic needle-inoculation, respectively.

Mosquito artificial bloodfeed infections

Mosquito infections were performed under BSL3 conditions. Groups of ~50-70 four- to six-day-old *Culex* mosquitoes of each species were orally exposed to blood containing WNV FtC-3699 virus using a water-jacketed glass feeder and hog-gut as a membrane. The infectious bloodmeal contained a 50:50 mixture of defibrinated calf blood and virus at a titer of $1.68x10^9$ PFU/mL as determined by back titration.

Mosquito feeding on alligators

Mosquito to alligator transmission: After artificial bloodfeed infection at 14 days extrinsic incubation period, mesh-covered cartons of 45 mosquitoes of each species were placed on the ventrum of manually restrained alligators for up to 15 minutes. After feeding on alligators, bloodfed mosquitoes were sorted into separate cartons.

Alligator to mosquito transmission: After needle inoculation of alligators at 8 days post infection, mesh-covered cartons of ~50-70 mosquitoes were placed on the

alligator's ventrum for up to 15 minutes. After feeding on alligators, bloodfed mosquitoes were sorted into separate cartons and held for 14 days for dissection.

Mosquito dissections and sample processing

Mosquitoes were cold-anesthetized. Legs and wings were removed. The mosquitoes' proboscis was placed into a capillary tube filled with immersion oil for 30 min to salivate. Bodies were placed into a 2 mL tube containing 200 µl mosquito diluent (1x PBS supplemented with 20% FBS, antibiotics/antifungals) and a stainless steel bead. The end of the capillary tube containing saliva was broken off into 100 µl mosquito diluent and centrifuged at 15,000 *g* for 10 min at 4°C. Mosquito bodies were homogenized at 25 cycles per second for 1 min and centrifuged at 15,000*g* for 10 min at 4°C. Samples were stored at -80°C.

Virus quantification

Samples assessed for live virus included alligator and bird serum, oral swabs of birds, cloacal swabs of birds and alligators and tank water. Infectious virus in the examined samples was titrated using standard plaque assays protocols as previously described (22). To measure viral RNA in the alligator serum and determine whether mosquitoes should be fed upon them, RNA was extracted from serum using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek) on the KingFisher Flex Magnetic Particle Processor (ThermoFisher Scientific) and qRT-PCR was performed using a QuantStudio 3 (ThermoFisher) and previously published WNV primers and probes (23).

Results

WNV-inoculated tank water serves as a source of infection for alligators

To assess whether WNV-inoculated tank water could serve as a source of infection for alligators, 100 mL of viral stock titrated to $1x10^{10}$ PFU/mL was mixed into the tank water. All three alligators developed viremia, which at day 6 ranged from $1.9x10^{3}$ to $3.7x10^{5}$ PFU/mL. Water was collected throughout this experiment (Figure 4.1).

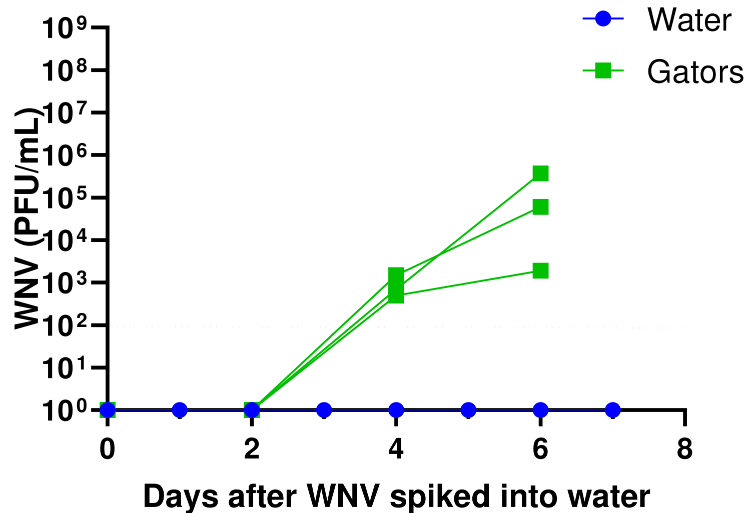
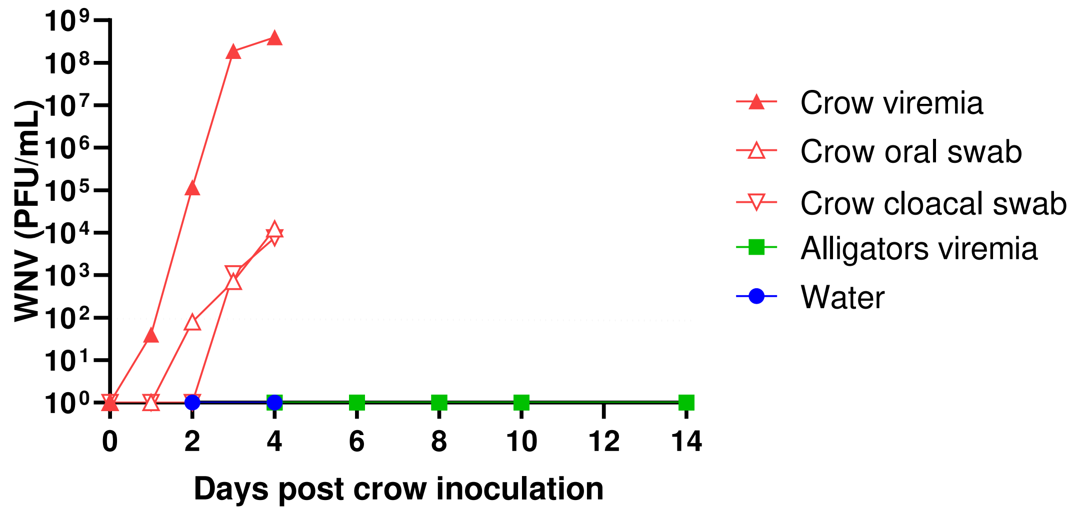


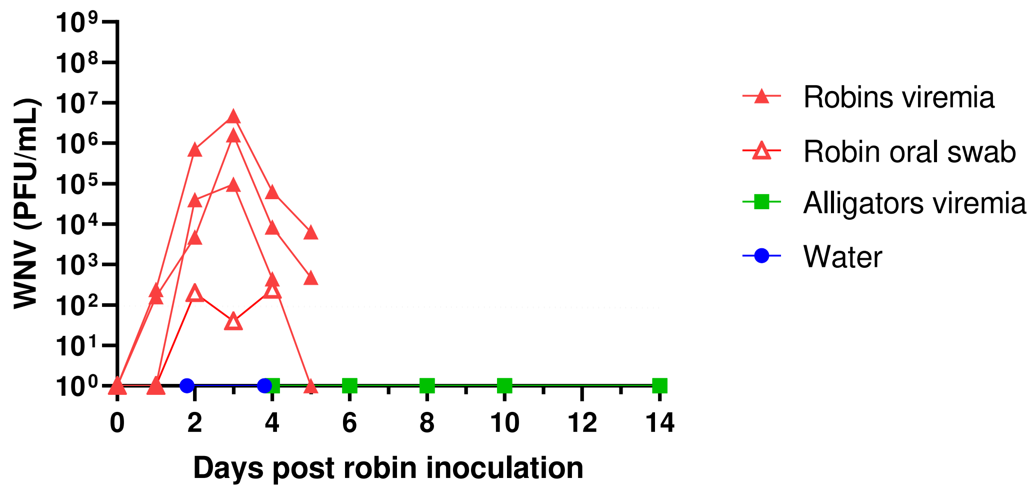
Figure 4.1. Water spiked with WNV led to alligator viremia even though virus was not detectable in water. Dotted line indicates limit of detection (10^2 PFU/ml).

Despite the large amount of virus added to the tank water and all three alligators becoming infected, virus was not detected by plaque assay or qRT-PCR, even at day 0.

Lack of transmission of WNV from birds to alligators via a shared water source

Because we demonstrated that alligators can become infected via virus in tank water, we sought to determine whether infected birds can shed sufficient virus into water to infect alligators. None of the experiments in which an American crow, American robins and house sparrows drank from and defecated into the water in which alligators were housed resulted in transmission to alligators (Figure 4.2).


WNV-infected crow housed with alligators
WNV (PFU/mL)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
2
4
6
8
10
12
14
Days post crow inoculation
Crow viremia
Crow oral swab
Crow cloacal swab
Alligators viremia
Water


WNV-infected robins housed with alligators
WNV (PFU/mL)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
2
4
6
8
10
12
14
Days post robin inoculation
Robins viremia
Robin oral swab
Alligators viremia
Water

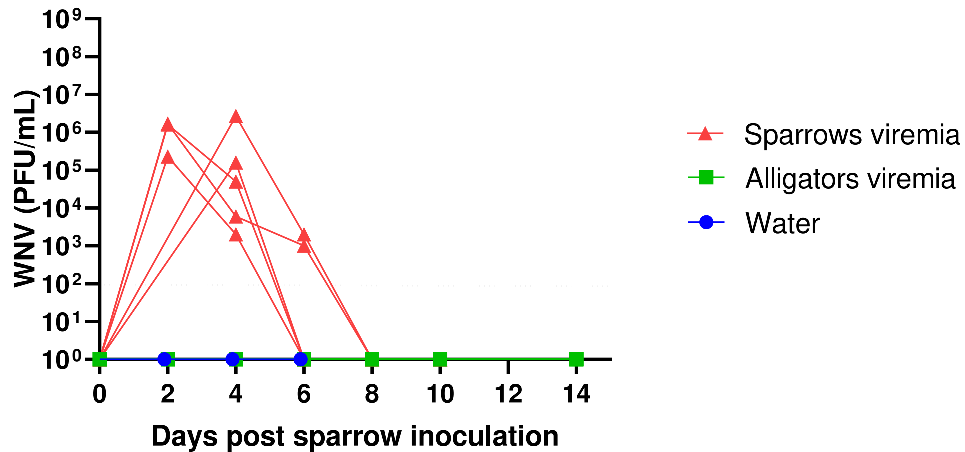


Figure 4.2. No birds transmitted WNV to alligators via a shared water source and virus was not detected in the water. Negative oral and cloacal swabs for robins and sparrows are not shown. Dotted line indicates limit of detection (10^2 PFU/ml).

In all experiments, needle-inoculated birds developed the expected levels of viremia, with crows developing highest titers and having detectable oral and cloacal shedding. WNV was rarely observed in oral swabs of robins. WNV was never detected in the water. Alligators also did not develop neutralizing antibodies after 14 days, further confirming lack of infection.

Lack of transmission of WNV from alligators to birds via a shared water source

While infected birds did not transmit WNV to alligators via the water, we sought to determine if WNV-infected gators could transmit to birds via a shared water source. Similarly, WNV-positive needle-inoculated alligators did not shed sufficient virus to quantify in the water. This did not occur despite viremia of greater than 10^5 PFU/mL and cloacal shedding up to 10^3 PFU/swab. House sparrows which drank from that water did not develop viremia (Figure 4.3).

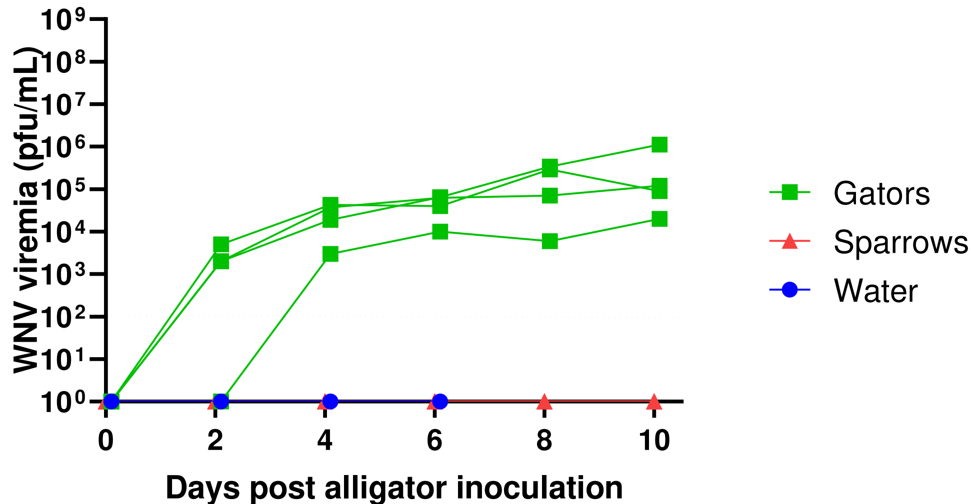


Figure 4.3. Viremic gators did not transmit WNV to HOSP via a shared water source. Dotted line indicates limit of detection (10^2 PFU/ml).

Mosquito transmission of WNV to alligators

Separate cartons of 45 WNV-exposed *Culex quinquefasciatus* (*Cx. quinquefasciatus*), *Culex tarsalis* (*Cx. tarsalis*) and *Culex pipiens* (*Cx. pipiens*) were allowed to feed on an individual juvenile alligator at 14 days extrinsic incubation period. Alligators were bled to evaluate viremia. *Cx. quinquefasciatus* exhibited the most successful feeding behavior with 82.2% (n=37) of mosquitoes engorged and bloodfed, as compared to 31.1% (n=14) bloodfed *Cx. pipiens* and 6.7% (n=3) bloodfed *Cx. tarsalis*. After feeding on alligators, mosquitoes were salivated and carcasses were collected. Of bloodfed mosquitoes, 100% (n=37), 100% (n=14) and 100% (n=3) of *Cx. quinquefasciatus*, *Cx. pipiens* and *Cx. tarsalis* were infected (WNV-positive mosquito body), respectively, at the time of feeding on alligators. Of bloodfed mosquitoes, 100% (n=37), 92.9% (n=13) and 100% (n=3) *Cx. quinquefasciatus*, *Cx. pipiens* and

Cx. tarsalis mosquitoes were transmission-capable (WNV-positive saliva) (Figure 4.4A) at the time of feeding on alligators.

Mosquito species	Total bloodfed on alligator	Total bodies positive (% of bloodfed)	Total saliva positive (% of bloodfed)
Cx. quinquefasciatus	37	37 (100%)	37 (100%)
Cx. pipiens	14	14 (100%)	13 (92.9%)
Cx. tarsalis	3	3 (100%)	3 (100%)

Figure 4.4A. Artificially-infected mosquitoes used to transmit WNV to alligators

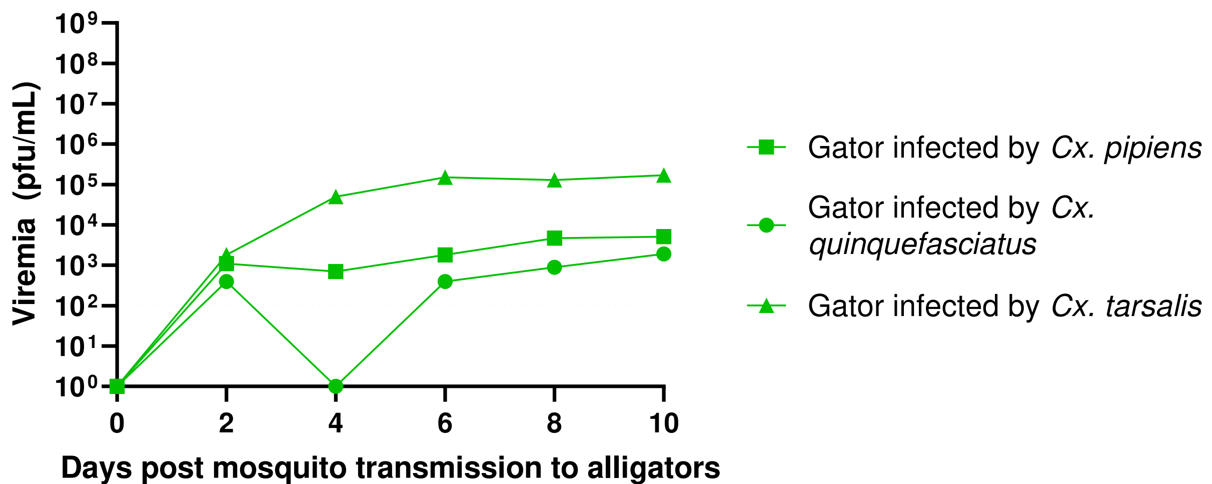


Figure 4.4B. Artificially infected mosquitoes of three mosquito species transmitted WNV to alligators and alligators developed viremia. Dotted line indicates limit of detection (10^2 PFU/ml).

All three alligators developed viremia (Figure 4.4B). Interestingly, the alligator fed on by the fewest number of mosquitoes (*Cx. tarsalis*) had high viremia throughout infection with peak titer reaching $1.7x10^5$ PFU/mL by 10 days after mosquito transmission. Alligators fed on by more *Cx. quinquefasciatus* and *Cx. pipiens* also developed viremia, reaching $1.9x10^3$ PFU/mL and $5.1x10^5$ PFU/mL, respectively.

Alligator transmission of WNV to mosquitoes with subsequent mosquito saliva transmission

To assess the potential of alligators as an amplifying host, alligators were needle-inoculated with WNV and cartons containing each of the three mosquito species were allowed to feed 8 days post-infection. Needle-inoculated alligators reached average higher levels of viremia, ranging from $1.3x10^5$ to $2.8x10^5$ PFU/mL (Figure 4.5A). Interestingly, this viremia was higher and more similar than those infected by mosquito transmission (Figure 4.4B), likely due to a higher and consistent inoculum. *Cx. quinquefasciatus* and *Cx. pipiens* exhibited similar feeding rates with 84.2% (n=48) and 82.3% (n=51) mosquitoes bloodfed (Figure 5A).

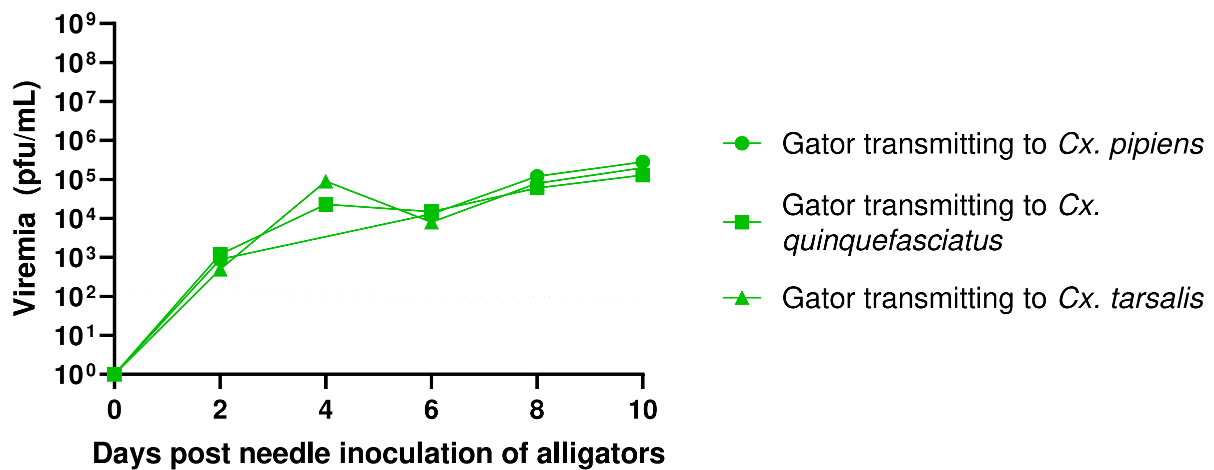


Figure 4.5A. Needle inoculated alligators developed viremia before we allowed naïve mosquitoes to feed on them. Dotted line indicates limit of detection (10^2 PFU/ml).

Mosquito species	Total bloodfed & alive at 14d post alligator feed	Total bodies positive (% of bloodfed)	Total saliva positive (% of bloodfed)
Cx. quinquefasciatus	48	4 (8.3%)	0 (0%)
Cx. pipiens	51	2 (13.7%)	2 (3.9%)
Cx. tarsalis	3	1 (33.3%)	1 (33.3%)

Figure 4.5B. Mosquitoes became infected and had WNV positive saliva after feeding on alligators.

Similar to the mosquito-to-alligator transmission attempts, very few *Cx. tarsalis* fed (5.8%; n=3). Of bloodfed mosquitoes, 8.3% (n=4), 13.7% (n=2) and 33.3% (n=1) of *Cx. quinquefasciatus*, *Cx. pipiens* and *Cx. tarsalis* became infected (WNV-positive mosquito body), respectively. Of bloodfed mosquitoes, 0% (n=0), 3.9% (n=2) and 33.3% (n=1) *Cx. quinquefasciatus*, *Cx. pipiens* and *Cx. tarsalis* mosquitoes became transmission-capable (WNV-positive saliva) (Figure 4.5B). Of infected mosquitoes (WNV-positive body), 0% (n=0), 100% (n=2) and 100% (n=1) *Cx. quinquefasciatus*, *Cx. pipiens* and *Cx. tarsalis* became transmission-capable (WNV-positive saliva). While feeding and infection rates are variable among mosquito species, both *Cx. tarsalis* and *Cx. pipiens* had virus-positive saliva, suggesting that they could transmit to a susceptible host.

Discussion

We experimentally demonstrated the ability of three species of *Culex* mosquitoes to transmit WNV to alligators. These findings are unsurprising given the multiple reports of WNV outbreaks in alligators and crocodiles, some of which were likely initiated by mosquito feeding. Interestingly, despite drastically different numbers of mosquitoes feeding, all three alligators developed viremia. Most impressive were *Culex tarsalis*, with three bloodfed mosquitoes causing the highest peak viremia. While *Culex quinquefasciatus* mosquitoes exhibited the most voracious feeding behavior and represent an important WNV vector in the southeastern US where most alligator farming occurs, this preference should be interpreted cautiously as we used mosquitoes which are significantly laboratory-adapted. Limited mosquito feeding may be related to the fact that mosquitoes were relatively old (29-30 days post emergence) by the time they fed on alligators and previously had a full bloodmeal, potentially decreasing willingness to refeed.

We inoculated alligators with WNV and then fed mosquitoes on them at peak viremia to assess the ability of alligators to function as amplifying hosts. At 14 days post-feeding, we collected mosquito bodies to assess infection and saliva to assess transmission capability.

Needle-inoculated alligator viremias were higher than mosquito-infected alligator viremias, measuring up to 10^5 PFU/mL, levels sufficient to infect mosquitoes. This is unsurprising as the needle inoculum was 10,000 PFU and the amount of virus provided when transmission occurs from mosquito saliva is much lower and thus it is interpreted as a dose-dependent difference. We observed low numbers of each mosquito species being infected (WNV-positive body) and occasional individuals in the *Cx. tarsalis* and Cx. *pipiens* groups going on to be transmission-capable (WNV-positive saliva). While the numbers of mosquitoes which fed on the WNV-viremic alligators were relatively low, the occurrence of low numbers of mosquitoes becoming transmission-capable indicates that alligators can serve as an amplifying host. Interestingly, even though few *Culex tarsalis* mosquitoes fed on the alligators, one of the three bloodfed mosquitoes became transmission capable. While results presented here may not necessarily provide statistically significant infection and transmission rates in mosquitoes which feed on alligators, they do provide proof of concept. Further investigation of ecologically-relevant species of *Culex* mosquitoes utilizing more mosquitoes to adequately assess vector competence specific to this amplifying host is needed.

We spiked tank water with WNV to assess whether water can function as a source of infection during horizontal transmission between alligators. All alligators became viremic, indicating that a high level of virus introduced into water can lead to alligator infection. Live virus and viral RNA were not detected in water at any time point during these infections, similar to previous alligator experiments. This may be the result of a sizeable tank of water diluting out virus to levels which were not quantifiable despite being sufficient for alligator infection. This may also suggest that alligators are highly susceptible to WNV, even when present in low quantities. In one report viral RNA was detected when filtered out of water during experimental crocodile transmission; thus, alternative means of water collection and sample preparation for extraction should be explored for future experiments (7). Artificial inoculation of water at extremely high titers ($1x10^{10}$ PFU/ml) is unlikely to be biologically relevant, but confirms

previous reports that environmental transmission of WNV can occur in the absence of a mosquito vector.

Given that horizontal transmission within tanks of alligators or crocodiles has been reported, we wanted to assess whether mosquito-free transmission between alligators and birds may serve as a means for WNV transmission in nature. For potential exposure from alligators to birds, we housed birds in cages partially submerged in alligator tank water, which served as the only source of drinking water. Our hypothesis was that avian oral and cloacal shedding into the tank water could infect alligators. For the reverse experiment, we hypothesized that alligator shedding into the tank water could potentially transmit to the birds as they drank that water. Transmission between birds and alligators via a shared water source did not result in WNV transmission in either direction. Oral and cloacal shedding were detected in the crow and one of three robins but live virus and viral RNA were not detected in the water in either experiment. Since we also did not detect WNV when we used a large amount of WNV to inoculate tank water and successfully infect alligators, it is likely that our means of detection was not sensitive. Therefore, during the transmission experiments between birds and alligators it is possible that virus was potentially there but not detectable. Nevertheless, given that the shared water source in our experiments is small relative to the amount of water in which alligators exist in nature or in farms, this mechanism is an unlikely contributor to natural WNV infection of alligators. However, transmission by mosquito and consumption of infected wild birds or infected feed remain plausible and reported sources for the initial infection.

Conclusion

We have demonstrated that American alligators are capable of serving as an amplifying host for WNV. Further investigations will be necessary to determine to what degree they actually contribute to maintenance of WNV in nature and whether this contributes to the overwintering of WNV. Our work also showed that mosquito-free transmission by shedding between birds and alligators is unlikely. This type of non-vectored transmission does not likely play a major role in

WNV maintenance and ecology, but also suggests that shedding birds are not a source of the outbreaks which occur in alligator farms. Those outbreaks are likely the result of a combination of mosquito to alligator transmission, horizontal transmission between alligators via the water and ingestion of WNV-contaminated feed as previously described. Previous evidence that consumption of WNV-infected carcasses leads to alligator infections still stands. Epizootics in alligator farms are likely to continue given that both the farms and natural swamplands of the region are good breeding grounds for mosquitoes. These swamplands are also prime habitat for avian amplifying hosts to infect the mosquitoes. Further work is necessary to determine how and whether these epizootics are related to natural WNV transmission cycles.

References

1. Byas AD, Ebel GD. Comparative Pathology of West Nile Virus in Humans and Non-Human Animals. Pathogens [Internet]. 2020 Jan 7 [cited 2020 Sep 10];9(1):48. Available from: https://www.mdpi.com/2076-0817/9/1/48

2. Miller DL, Mauel MJ, Baldwin C, Burtle G, Ingram D, Hines ME, et al. West Nile virus in farmed alligators. Emerg Infect Dis. 2003 Jul 1;9(7):794–9.

3. Jacobson ER, Ginn PE, Troutman JM, Farina L, Stark L, Klenk K, et al. West Nile virus infection in farmed American alligators (Alligator mississippiensis) in Florida. J Wildl Dis. 2005;41(1):96–106.

4. Simulundu E, Ndashe K, Chambaro HM, Squarre D, Reilly PM, Chitanga S, et al. West Nile Virus in Farmed Crocodiles, Zambia, 2019. Emerg Infect Dis J [Internet]. 2020 [cited 2020 Mar 17];26(4). Available from: https://wwwnc.cdc.gov/eid/article/26/4/19-0954_article

5. Isberg SR, Moran JL, De Araujo R, Elliott N, Davis SS, Melville L. First evidence of Kunjin strain of West Nile virus associated with saltwater crocodile (Crocodylus porosus) skin lesions. Aust Vet J. 2019 Oct 1;97(10):390–3.

6. Nevarez JG, Mitchell MA, Morgan T, Roy A, Johnson A. Association of West Nile Virus with Lymphohistiocytic Proliferative Cutaneous Lesions in American Alligators (Alligator mississippiensis) Detected by RT-PCR. J Zoo Wildl Med [Internet]. 2008 Dec 1 [cited 2019 Mar 25];39(4). Available from: http://www.ncbi.nlm.nih.gov/pubmed/19110697

7. Habarugira G, Moran J, Colmant AMG, Davis SS, O'Brien CA, Hall-Mendelin S, et al. Mosquito-Independent Transmission of West Nile virus in Farmed Saltwater Crocodiles (Crocodylus porosus). Viruses. 2020;12(2).

8. Klenk K, Snow J, Morgan K, Bowen R, Stephens M, Foster F, et al. Alligators as West Nile virus amplifiers. Emerg Infect Dis [Internet]. 2004 Dec [cited 2019 Jan 18];10(12):2150–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15663852

9. Unlu I, Kramer WL, Roy AF, Foil LD. Detection of West Nile Virus RNA in Mosquitoes and Identification of Mosquito Blood Meals Collected at Alligator Farms in Louisiana. J Med Entomol [Internet]. 2010 Jul 1 [cited 2020 Jan 7];47(4):625–33. Available from: http://openurl.ingenta.com/content/xref?genre=article&issn=0022-2585&volume=47&issue=4&spage=625

10. Rodrigues SCG, Maruniak JE. Blood meal identification from mosquitoes collected at a commercial alligator farm. J Am Mosq Control Assoc [Internet]. 2006 Sep [cited 2020 Sep 9];22(3):557–60. Available from: https://pubmed.ncbi.nlm.nih.gov/17067063/

11. WK R, Y F, HD L, VM M, J W, P O, et al. Overwintering of West Nile virus in Southern California. J Med Entomol [Internet]. 2006 [cited 2020 Sep 9];43(2). Available from: https://pubmed.ncbi.nlm.nih.gov/16619621/

12. Dawson JR, Stone WB, Ebel GD, Young DS, Galinski DS, Pensabene JP, et al. Crow deaths caused by West Nile virus during winter. Emerg Infect Dis [Internet]. 2007 Dec [cited 2019 Nov 15];13(12):1912–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18258045

13. Hinton MG, Reisen WK, Wheeler SS, Townsend AK. West Nile Virus Activity in a Winter Roost of American Crows (Corvus brachyrhynchos): Is Bird-To-Bird Transmission Important in Persistence and Amplification? J Med Entomol [Internet]. 2015 Jul [cited 2019 Nov 15];52(4):683–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26335475

14. Ip HS, Van Wettere AJ, McFarlane L, Shearn-Bochsler V, Dickson SL, Baker J, et al. West Nile Virus Transmission in Winter: The 2013 Great Salt Lake Bald Eagle and Eared Grebes Mortality Event. PLoS Curr [Internet]. 2014 [cited 2019 Nov 15];6. Available from: https://pubmed.ncbi.nlm.nih.gov/24761310/

15. Banet-Noach C, Simanov L, Malkinson M. Direct (non-vector) transmission of West Nile virus in geese. Avian Pathol [Internet]. 2003 Oct [cited 2019 Nov 15];32(5):489–94. Available from: https://pubmed.ncbi.nlm.nih.gov/14522704/

16. Nemeth N, Young G, Ndaluka C, Bielefeldt-Ohmann H, Komar N, Bowen R. Persistent West Nile virus infection in the house sparrow (Passer domesticus). Arch Virol [Internet]. 2009 May [cited 2019 Nov 15];154(5):783–9. Available from: https://pubmed.ncbi.nlm.nih.gov/19347246/

17. Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey MS, et al. West Nile virus in overwintering culex mosquitoes, New York City, 2000. Emerg Infect Dis. 2001;7(4):742–4.

18. Nelms BM, Fechter-Leggett E, Carroll BD, Macedo P, Kluh S, Reisen WK. Experimental and natural vertical transmission of West Nile virus by California Culex (Diptera: Culicidae) mosquitoes. J Med Entomol [Internet]. 2013 Mar [cited 2019 Nov 15];50(2):371–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23540126

19. Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, et al. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis. 2003 Mar 1;9(3):311–22.

20. Sardelis MR, Turell MJ, Dohm DJ, O'Guinn ML. Vector competence of selected North American Culex and Coquillettidia mosquitoes for West Nile virus. Emerg Infect Dis [Internet]. 2001 [cited 2020 Sep 9];7(6):1018–22. Available from: /pmc/articles/PMC2631924/?report=abstract

21. Turell MJ, O'guinn M, Oliver J. POTENTIAL FOR NEW YORK MOSQUITOES TO TRANSMIT WEST NILE VIRUS. Vol. 62, Am. J. Trop. Med. Hyg. 2000.

22. Bunning ML, Bowen RA, Bruce Cropp C, Sullivan KG, Davis BS, Komar N, et al. Experimental infection of horses with West Nile virus. Emerg Infect Dis. 2002;8(4):380–6.

23. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. 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 [Internet]. 2000 [cited 2020 Sep 10];38(11):4066–71. Available from: https://pubmed.ncbi.nlm.nih.gov/11060069/

Concluding Remarks

West Nile virus (WNV) continues to be an important cause of human arboviral neuroinvasive disease. Since the emergence of WNV, outbreaks of chikungunya virus and Zika virus, increased incidence of tick-borne encephalitis and continued widespread transmission of Dengue continue to highlight the importance of arbovirus research. Undoubtedly, undiscovered arboviruses will pose new threats to human and animal health. The two-host system provides a uniquely complex environment for viral maintenance and evolution. Understanding the two-host system of WNV from an evolutionary and ecological standpoint may grant us foresight for future arboviral emergence. To understand the way in which viral populations are shaped, this work requires the use of relevant avian hosts and mosquito vectors. It is also important to consider the potential contributions of unconventional species to the natural ecology of arboviruses, particularly when that virus is as promiscuous as WNV. Continued vigilance in the realm of arbovirology is necessary for the future of public health.

Experimental WNV Evolution

Conclusions

The hosts in which arboviruses circulate shape the virus' evolution, allowing for the emergence of mutations which have the potential to become established genotypes. Previous work examined the relevant wild birds and mosquitoes in isolation or mosquitoes in combination with chicks. Those experiments revealed that American crows exhibit weaker purifying selection while robins exhibit strong purifying selection. Examination of mosquito influences supported previous findings of viral diversification. We sought to pursue the logical, challenging next step: combining the relevant wild bird and mosquito species in cycles of transmission to assess effects of specific pairings on viral evolution. Cycles utilizing American crows and *Culex pipiens* were the most successful at transmission, completing three sequential rounds of bird-to-mosquito transmission in three replicates. This was followed in success by crow and *Culex*

quinquefasciatus cycles. Robin and *Culex quinquefasciatus* mosquitoes were the least successful. These findings suggest that the crow is likely important to viral maintenance in nature, potentially as a result of higher levels of viremia. It also suggests that *Culex quinquefasciatus* may provide a fitness disadvantage when paired with crows. Interestingly, all pairings exhibited a pattern of decreasing mosquito infection and transmission capability rates with each round of transmission. These losses may suggest that viral replication in a large range of mosquitoes and birds, as likely occurs in nature, is necessary for viral maintenance. Competitive fitness studies using avian sera, mosquito tissues and saliva from transmission events exhibited cyclical patterns of more relative fitness in avian sera and less relative fitness in mosquito tissues. Sequencing is need to determine whether that pattern correlates with previously observed genetic expansion in mosquitoes and restriction in birds.

Future Directions

Abundant opportunities for further exploration exist regarding the findings in this study of experimental evolution. Transmission studies using robins and *Cx. pipiens* would fill the final gap in the particular combinations we used. The use of *Culex tarsalis* in transmission cycles would also be prudent given its importance to the natural ecology of WNV. Given the progressive decreases in mosquito infection and transmission capability in all cycles that were restricted to a single species of bird and mosquito, it would be interesting to perform studies using intermixed species to see if that pattern persists. Additional investigations which explore ecological variables in nature may add to our understanding of why adequate titer saliva failed to infect birds and why adequate viremias did not infect mosquitoes. For example, multiple mosquitoes feeding on the same bird, extended periods of mosquito feeding on birds due to morbidity and increased probing may increase transmission success in nature. Sequencing of samples that failed to transmit will likely aide our understanding of whether the observed transmission patterns are attributable to genetic variation. Given the cyclical nature of

competitive fitness assay outcomes, sequencing the viral genomes from these transmission studies may also allow us to connect the viral genetic changes to relative fitness.

Single Cell Analysis of Avian WNV Infection

Conclusions

We assessed the avian single cell viral environment during WNV replication using a barcoded virus and observed high levels of intracellular virus which allow for generation of defective genomes and genetic complementation. The novel use of this single cell next-generation sequencing in conjunction with a barcoded WNV technique highlighted the importance of single cells to viral population genetics. We observed that high viremia in crows functions as a determinant of multiplicity of infection in peripheral blood mononuclear cells, leading to polyinfections. Those polyinfections allow for the maintenance of defective genomes and rare variants. The long term effect of this finding is that minority variants are allowed to persist with the future potential to emerge if provided appropriate environments. To assess whether this pattern is maintained in the circulating viremia and whether it would apply when rare variants are replaced by lower fitness variants, we performed *in vivo* competitions using low fitness viruses and reference virus. One low fitness WNV replicated better at higher viremias, consistent with our hypothesis. During the second low fitness WNV *in vivo* competition, we observed abbreviated viremia curves in robins and the low fitness virus failed to replicate better in crows and robins. These studies highlight the complexity of systemic infections in wild-caught birds.

Future Directions

Further investigation of the mechanisms of complementation at the cellular level may allow us to better understand how rare and low fitness variants are allowed to persist in viral populations. While we utilized PBMCs, the use of barcoded viruses to examine other sites of viral replication including avian lymphoid organs may be prudent. Utilizing barcoded viruses and *in vivo* competitions in mosquitoes would allow us to better understand the diversification and

bottlenecks which occur in mosquitoes at the single cell level. Additionally, these barcoded viruses which allowed us to examine evolutionary effects on variably-represented genotypes of WNV can be used to assess multi-infections of viral strains of any virus.

American Alligator WNV Amplification

Conclusions

Previous examination of natural and experimental infections of American alligators indicated relatively high titers for a "dead-end host" which were theoretically sufficient to infect mosquitoes. Alligators were also deemed capable of horizontal transmission. Our work which aimed how alligators contribute to the natural ecology of WNV demonstrated that transmission of WNV from water to alligators is possible, that transmission between birds and mosquitoes by a shared water source is unlikely, that mosquitoes readily infect alligators with WNV and that alligators are capable of infecting mosquitoes and that mosquitoes are subsequently capable of transmission.

Future Directions

Additional work is needed to determine the significance of these experimental findings to the natural maintenance of WNV. Information regarding the potential for chronic WNV infection in alligators could potentially point to a mechanism of WNV overwintering. Larger vector competence studies which included more mosquitoes and additional species which may serve as bridge vectors would allow for the better understanding of how alligators could infect naturally larger mosquito populations. Additional studies examining mosquitoes trapped in areas near alligator farms at the time of outbreaks as well as collection and filtration of nearby water sources may provide convincing evidence of the ways in which these outbreaks occur. Given the continued farming of alligators and WNV being a seasonal presence within the United States, further research examining the potential to vaccinate alligators may be useful from an economic standpoint. Finally, if sufficient evidence is demonstrated to indicate that alligators

play a routine role in the maintenance of WNV, it would be prudent to pursue studies which assess how replication within the species shapes WNV evolution.

Collectively, this work represents an advancement of experimental evolution work by modelling transmission in relevant species, demonstrates the significance of viremia and the single cell environment to WNV evolution and demonstrates the potential of alligators to contribute to natural WNV ecology.

Appendix

Supplementary Methods

Establishing an Amplicon Targeted Sequencing Approach

To overcome sequencing errors and PCR-mediated recombination that might introduce biases (Gorzer et al., 2010; Jabara et al., 2011; Keys et al., 2015; Meyerhans et al., 1990), we adjusted a library preparation strategy established by (Zhou et al., 2015). This strategy offers the inclusion of a stretch of degenerate nucleotides included into the cDNA synthesis primer that enables "tagging" of the input cDNA template before proceeding on to PCR amplification steps. This stretch of nucleotides served as an identifier sequence and was previously referred to as "Primer ID" (Zhou et al., 2015).

Since the focus of this study was to identify intra-host viral genetic diversity and we specifically aimed at sequencing single cells carrying a plethora of viral genomes, it was important to be able to identify low frequency genomes and to have as low sequencing bias as possible The inclusion of the Primer ID sequence allowed each original template copy to be tagged with an identifier sequence. Finding multiple identical Primer ID sequences during the analysis process of the sequencing products indicated that genomes tagged with the same Primer ID originated form a single original input template. These were subsequently collapsed to create a consensus sequence for each original template.

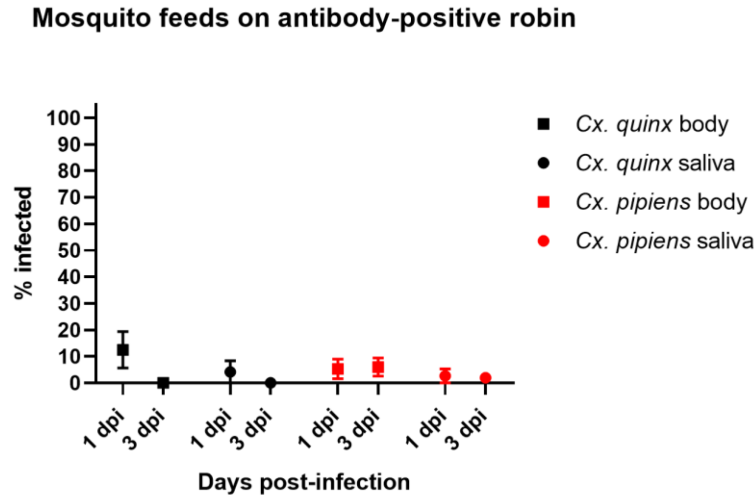
We adapted the Primer ID approach to the Illumina MiSeq platform and amplified a 335 bp long fragment within the WNV genome that included the barcoded region within the NS4b segment (Supplementary figure 2, table of Illumina barcodes used for this study, excel sheet table-Illumina barcodes used for sample multiplexing, (Zanini et al., 2018a). Library preparation is further detailed in the Experimental procedures section. Using the Primer ID method we were able to generate libraries from an input of as little as 50 viral genomes (Supplementary Methods Figure 1).

Supplementary Figures

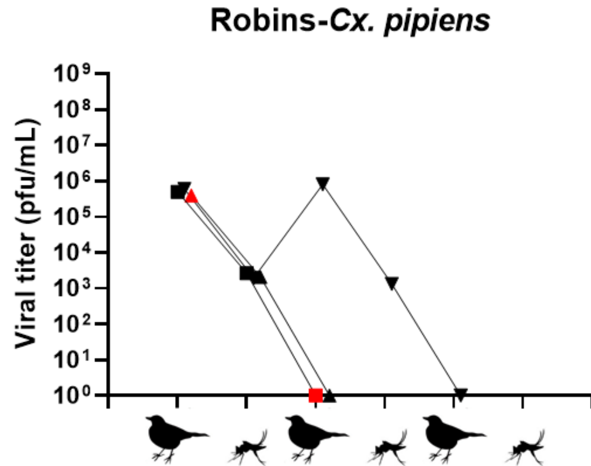
Supplementary Figure 1. Organ titers during infection with BC-WNV. (A) Titers of various organs in American crows during BC-WNV infection as determined by plaque assays (PFU/mL in $\log_{10}$). (B) Titers of various organs in American robins during BC-WNV infection as determined by plaque assays (PFU/mL in $\log_{10}$).

Supplementary Figure 2. (A) Table of illumina barcodes. (B) Table of primers for illumina sample multiplexing and primer ID. (C) Determination of limit of Primer ID detection.

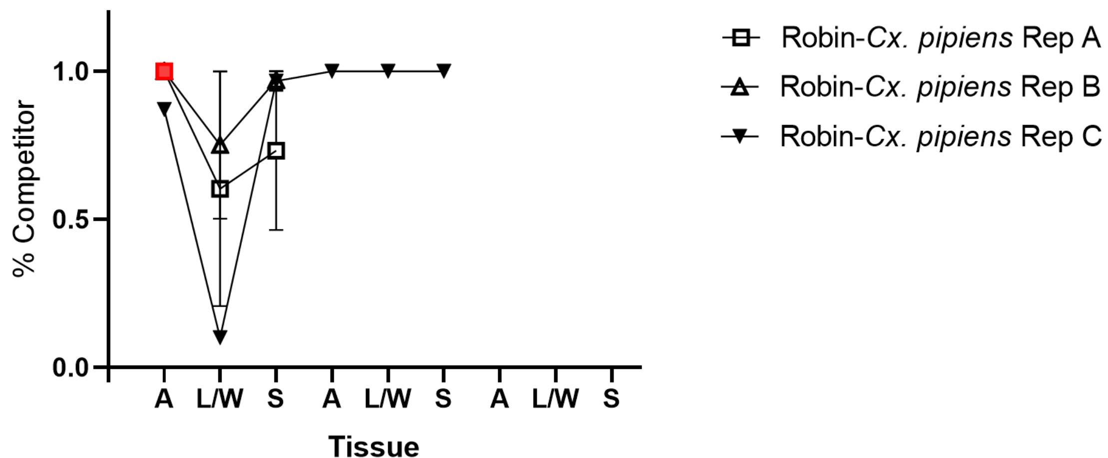
Figures included below are experiments associated with chapters 2 and 3 that were performed with antibody-positive robins.



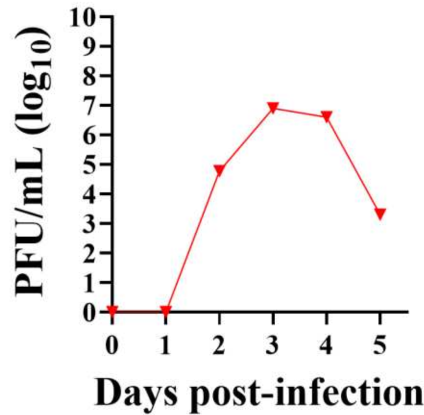
Supplementary Figure 3. Corresponds to Chapter 2, Figure 2.3. Mosquito infection and transmission-capability occur despite feeding on WNV antibody-positive robins. *Culex quinquefasciatus* and *Culex pipiens* mosquitoes were fed on a WNV-infected WNV antibody-positive robin at 1 dpi and 3 dpi (x-axis) and held for 14 days extrinsic incubation period for dissection. Mosquito bodies and saliva were collected and assessed as WNV-positive or WNV-negative by plaque assay (y-axis).



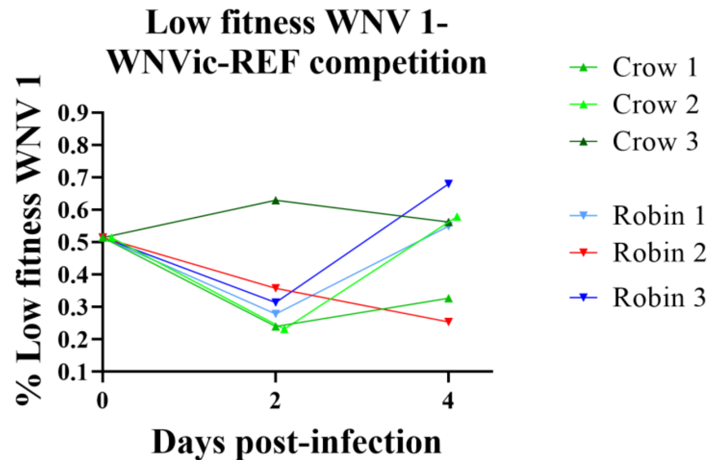
Supplementary Figure 4. Corresponds to Chapter 2, Figure 2.4. Experimental WNV transmission cycles proceed despite the presence of a WNV antibody-positive robin at initiation of one replicate. Mosquito transmission to another WNV antibody-positive bird fails to lead to viremia. The viral titer (y-axis) of avian sera and mosquito saliva at each transmission event are shown. WNV antibody-positive birds are denoted in red.



Supplementary Figure 5. Corresponds to Chapter 2, Figure 2.6. Transmission cycle sera and mosquito samples exhibit variable competitive fitness *in vitro.* WNV-positive avian sera (x-axis, A), mosquito legs/wings (x-axis, L/W) and saliva (x-axis,S) from each transmission event were mixed with equal Plaque Forming Units (PFU) of a reference virus (WNVic-REF) and incubated on DF1 cells in duplicate. The proportion of the transmission cycle product present at 2 days post incubation is shown (y-axis). WNV antibody-positive robin is denoted in red. Bar=SEM.



Supplementary Figure 6. Corresponds to Chapter 3, Figure 3.7A. Avian Viremia During Low Fitness WNV-WNVic-REF Competitions. Mean viremia (PFU/mL in log_{10}) of a WNV antibody-positive American robin during Low Fitness WNV 1 and WNVic-REF competitions as determined by plaque assay.



Supplementary Figure 7. Corresponds to Chapter 3, Figure 3.8. *In vivo* low fitness WNV vs WNVic-REF competition. Percent of low fitness competitor (y-axis) present during competition of low fitness virus 1 against WNVic-REF in crows and robins (Bar=SEM). WNV antibody-positive American robin 2 is denoted in red.