

DISSERTATION

DYNAMICS OF WEST NILE VIRUS EVOLUTION DURING INFECTION OF WILD
BIRDS, MOSQUITOES, AND THE HUMAN BRAIN: UNRAVELING THE
COMPELEXITIES OF SELECTION, DRIFT, AND FITNESS

Submitted by

Nathan D. Grubaugh

Department of Microbiology, Immunology, and Pathology

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Colorado State University

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Doctoral Committee:

Advisor: Gregory D. Ebel

William C. Black IV

Brian D. Foy

Aaron C. Brault

Daniel B. Sloan

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ABSTRACT

DYNAMICS OF WEST NILE VIRUS EVOLUTION DURING INFECTION OF WILD BIRDS, MOSQUITOES, AND THE HUMAN BRAIN: UNRAVELING THE COMPLEXITIES OF SELECTION, DRIFT, AND FITNESS

Over the last half century diseases caused by RNA viruses have emerged with increasing frequency. Emerging viral diseases have profound public health and economic consequences as highlighted by the recent epidemics of Ebolavirus in West Africa, MERS coronavirus in the Middle East, and avian influenza A(H7N9) virus in China. Furthermore, the recent emergence of several arthropod-borne viruses (arboviruses) in the Americas are of significant concern. West Nile virus (WNV) was introduced to the United States in 1999 and is now the leading cause of viral encephalitis in North America. Chikungunya virus (CHIKV) caused more than 1.7 million human infections in the Western Hemisphere since its introduction in 2013. Zika virus (ZIKV) was first detected in Brazil in 2015 and is associated with thousands of severe birth defects.

RNA virus emergence can in large part be attributed to their rapid rates of evolution. Low fidelity of viral RNA polymerases (10^{-6} to 10^{-4} substitutions per nucleotide copied), coupled with rapid replication rates leads to the formation of large and genetically complex intrahost populations. Intrahost diversity provide viruses with the ability to quickly adapt to shifting fitness landscapes, either as a product of infecting new hosts or host environments. These intrahost variants can also collectively contribute to the phenotype of the population, influencing viral fitness and disease. Moreover, the dynamic nature of viral populations provides a “moving target” for antiviral defenses and severely limits our ability to develop new drugs and vaccines.

Arbovirus transmission imposes unique evolutionary pressures due to the requirement to constantly replicate in disparate hosts. Several studies using WNV have assessed how different host types impact arbovirus population structure, revealing that viral populations are more diverse in mosquitoes compared to birds. In mosquitoes, purifying selection is weak and virus diversification is driven by the action of RNA interference, which creates an intracellular milieu that favors rare genotypes. In contrast, purifying selection in birds is strong and the innate antiviral response is suspected to be dominated by type I interferon. This cycling of genetic diversification in mosquitoes and selective constraint in birds leads to slower rates of evolution compared to many single-host viruses. Despite these constraints, adaptations to local mosquitoes facilitated invasions of WNV and CHIKV. The mechanisms for arbovirus adaptation, however, are incompletely understood.

Within hosts, genetically and phenotypically complex viral populations are formed by genetic drift and natural selection. Defining these processes in different hosts can help to predict future emergence, inform treatment paradigms, and enhance control efforts. Accordingly, we allowed WNV to replicate in wild-caught American crows, house sparrows and American robins to assess how natural selection shapes RNA virus populations in ecologically relevant hosts that differ in susceptibility to virus-induced mortality. After five sequential passages in each bird species, we examined the phenotype and population diversity of WNV through fitness competition assays and next-generation sequencing (NGS). We demonstrate that fitness gains occur in a species-specific manner, with the greatest replicative fitness gains in robin-passaged WNV and the least in WNV passaged in crows. Sequencing data revealed that intrahost WNV populations were strongly influenced by purifying selection and that the overall complexity of the viral populations was similar among passaged hosts. However, the selective pressures that

control WNV populations seem to be dependent on the bird species. Specifically, crow-passaged WNV populations contained the most unique mutations and defective genomes, but the lowest average mutation frequency. Therefore, our data suggest that WNV replication in the most disease-susceptible bird species is positively associated with virus mutational tolerance, likely via complementation, and negatively associated with the strength of selection.

Different bird species clearly differ in their impacts on WNV population structure, but the role of distinct mosquito vector species on viral population genetics has not been addressed. We sought to determine whether important enzootic (*Culex tarsalis*, *Cx. quinquefasciatus*, and *Cx. pipiens*) and bridge vectors (*Aedes aegypti*) of WNV have differential impacts on viral mutational diversity and relative fitness. Using NGS, we report high genetic diversity during WNV infection of mosquitoes, with species dependent impacts on rates of WNV evolution ($\sim 2\times$ greater divergence within *Cx. quinquefasciatus*). Within mosquitoes, WNV that escaped known anatomical barriers to transmission also underwent stochastic reductions in genetic diversity that was subsequently recovered during intratissue population expansions. Cycles of genetic drift and weak purifying selection within a single mosquito infection resulted in accumulation of deleterious mutations in the virus population (i.e. mutational load). Consequently, the expectorated (i.e. transmitted) WNV had lower relative fitness in avian cells compared to input virus. These findings demonstrate that the adaptive potential associated with mosquito transmission carries a significant fitness cost in vertebrates and that this fitness cost arises during a single systemic infection in a wide array of mosquitoes.

The structure of WNV populations transmitted to humans from mosquitoes likely influences whether infection progresses into severe, acute encephalitis. However, WNV replication and population structures within specific human brain regions have not been studied

and could reveal important insights into the virus-host interactions that occur during acute encephalitis. We describe a fatal case of WNV encephalitis in which we analyzed tissue obtained from specific brain regions at autopsy using NGS and immunohistochemistry. Despite similar levels of WNV replication between the cortical and subcortical regions, injury was only observed in the subcortical grey matter brain regions. In addition, expression of specific interferon-stimulated genes and WNV amino acid variation was higher in injured tissues. Analysis of WNV populations revealed no evidence for bottlenecks between tissues, indicating that the viral populations could move relatively freely among the regions studied and viral genetic diversity is more likely shaped by natural selection than genetic drift. Therefore, this observational, patient-based data suggests that neuronal injury and the strength of viral selection pressure may be associated with the level of the innate immune response; however, confirmation is needed with additional human samples and in animal models.

Taken together, these results reveal important insights into the deterministic and stochastic forces that shape WNV populations during infection of different hosts and tissues. In general, birds maintain fitness through natural selection and mosquitoes randomly shuffle the variant repertoire, decreasing relative fitness. Therefore, arboviruses cycle between levels of relative fitness as they cycle between hosts. Moreover, we can now make predictions about the evolutionary rates, fitness outcomes, and adaptive potential from specific transmission cycles. For example, WNV may evolve more rapidly while maintaining higher relative fitness from a *Cx. quinquefasciatus*-robin cycle than a *Cx. pipiens*-crow cycle, which will increase adaptive potential and facilitate emergence.

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Chapter 1: Literature Review

Historical perspective

Arthropod-borne viruses (arboviruses) have been significant causes of morbidity and mortality over a long history of human interaction. Clinical descriptions similar to dengue virus (DENV) infections were reported as early as the 3rd Century in China [1]. After centuries of silence, similar reports surfaced from the French West Indies and Panama in the 1600s [1]. By the 1700s and 1800s, DENV had gone global, perhaps aided by commercial sailing ships [2]. Likewise, yellow fever virus (YFV) and its mosquito vector, *Aedes aegypti*, probably emerged in the Americas via the slave trade, and became one of the most important tropical diseases of the 15th to 20th centuries [3,4].

The role of arthropods for the transmission of these pathogens would be overlooked for thousands of years. Early reports of DENV-like disease were described as “water poison”, and even the mosquito-borne parasitic disease known as malaria literally translates to “bad air”. Not until landmark discoveries by Carlos Finlay (1881 [5]), Sir Ronald Ross (1897 [6]), and Walter Reed (1901 [7]) was it known that mosquitoes could be vectors of pathogens, and even more important, that these diseases could be combated with vigilant mosquito control programs. Armed with insecticides, a world war against mosquitoes began. These programs had many early successes, however, the rapid emergence of insecticide resistance – and the push to reduce their use due to environmental concerns – greatly reduced their effectiveness [5,8].

Today, greater than a third of the global population is at risk of DENV infection [9-11], and the number of human cases are predicted to continuously rise [12]. Other arboviruses, such as West Nile virus (WNV), chikungunya virus (CHIKV), and Zika virus (ZIKV), have emerged

from previously restricted foci to cause disease outbreaks around the world [13-15]. Even YFV has resurged despite an efficacious human vaccine [16]. Thus, the unfortunate reality is that arboviruses and humans will likely have a long future together.

Diversity of arboviruses

Arboviruses must replicate in arthropod vectors and vertebrate hosts to maintain biological transmission, however, they are ubiquitous in nature and can be found worldwide. To achieve this feat, arboviruses utilize several diverse hematophagous (blood feeding) arthropods, including mosquitoes (order Diptera: family Culicidae [17]), biting midges (Diptera: Ceratopogonidae [18]), sand flies (Diptera: Psychodidae [19]), and ticks (Ixodida: Ixodidae and Argasidae [20]), as competent vectors. Likewise, arboviruses can cause substantial viremia to sustain transmission in a wide variety of vertebrates, primarily mammals and birds, but also reptiles and possibly amphibians [21-23]. This convergent evolution and adaptive radiation to perpetuate in several ecological niches means that arboviruses are themselves numerous and taxonomically diverse. In fact, there are > 500 suspected and confirmed arbovirus species, most of which have RNA genomes. These include the flaviviruses (family *Flaviviridae*: genus *Flavivirus*), alphaviruses (*Togaviridae*: *Alphavirus*), bunyaviruses (*Bunyaviridae*: *Orthobunyavirus*, *Nairovirus*, and *Phlebovirus*), orbiviruses (*Reoviridae*: *Orbivirus*), vesiculoviruses (*Rhabdoviridae*: *Vesiculovirus*), and thogotoviruses (*Orthomyxoviridae*: *Thogotovirus*). African swine fever virus (*Asfarviridae*: *Asfarvirus*) is the only known representative with a DNA genome [24-27]. Despite their diversity, the vast majority of the medically important arboviruses belong to the flavivirus, alphavirus, and bunyavirus groups.

The flaviviruses include some of the world's most important and pervasive arboviruses. Their RNA genomes are single stranded, positive sense, and contain a single open reading frame (ORF) (discussed in detail below) [26]. The genus can be divided into viruses that are transmitted between vertebrate hosts by 1) mosquitoes or 2) ticks, 3) that infect vertebrates with no known vector, and 4) that infect mosquitoes but cannot replicate in vertebrates. The mosquito-borne group can be further subdivided into viruses primarily vectored by *Aedes* spp. (e.g. DENV and ZIKV) or *Culex* spp. (e.g. WNV and Japanese encephalitis virus [JEV]). The tick-borne viruses can be also subdivided by their host-vector pairings: 1) mammals and ixodid (hard) ticks and 2) seabirds and argasid (soft) ticks [28,29]. Mammalian tick-borne flaviviruses comprise some of the most pathogenic arboviruses known, sometimes causing encephalitis (e.g. Tick-borne encephalitis and Powassan viruses [30]) or hemorrhagic fever (e.g. biosafety level 4 Omsk hemorrhagic fever and Kyasanur Forest disease viruses). The last two flavivirus groups can only replicate in vertebrate cells (e.g. Rio Bravo virus in bats [31]) or arthropod cells (e.g. *Culex* flavivirus [32]) and are not considered arboviruses.

The alphaviruses also have single stranded, positive sense RNA genomes, but unlike the flaviviruses, they contain two ORFs (one for each set of non-structural and structural polyproteins) [26]. Most of the ~30 species are enzootic mosquito-borne viruses that use rodents and birds as reservoir hosts [33]. However, notable exceptions include CHIKV which has a mosquito-primate cycle, the mosquito-specific Eilat virus [34], the potentially louse-borne Southern elephant seal virus [35], and the non-vectored salmon pancreas disease virus [36]. The mosquito-borne alphaviruses can be subdivided by geography: New World (i.e. the Americas) and Old World (i.e. Africa, Europe, and Asia). The New World alphaviruses (e.g. eastern, western, and Venezuelan equine encephalitis viruses [VEEV]), as their names indicate, can cause

severe encephalitic disease in mammals and have the potential for use as biological weapons [33,37]. The Old World alphaviruses (e.g. CHIKV, Sindbis virus [SINV], and o'nyong-nyong virus [ONNV]), on the other hand, are typically associated with nonfatal but debilitating rheumatic disease, commonly polyarthralgia and/or polyarthritis [38].

The third major group of arboviruses, the bunyaviruses, have a genomic architecture quite different from the flaviviruses and alphaviruses. They have tripartite genomes consisting of large, medium, and small negative sense and single-stranded RNA segments [26]. These viruses are incredibly diverse, not only fueled by their high mutation rates, but they can also rapidly diverge by genome reassortment in dually infected hosts (i.e. genetic shift) [39-41]. Many of the nearly 300 distinct bunyaviruses are pathogens of humans and livestock [42,43]; therefore a more complete understanding of the ecology and evolution of these understudied viruses is of critical need. The orthobunyaviruses (e.g. La Crosse virus) occur almost world-wide and have been isolated from a variety of vertebrates (e.g. humans, cattle, rodents, marsupials, and bats) and arthropods (e.g. mosquitoes and midges) [44,45]. Phleboviruses are named after their association with phlebotomine sand flies; however they are not exclusively limited to these vectors [46]. For example, the most notorious member, Rift Valley fever virus, is vectored by several species of mosquitoes but has never been isolated from sand flies. Moreover, it was recently discovered that phleboviruses causing severe disease in humans can also be transmitted by ticks (e.g. Heartland virus [47]). Nairoviruses, on the other hand, are predominantly tick-borne, and appeared to have coevolved with their hard and soft tick vectors [48].

The scarcity of DNA viruses suggests that the highly error prone RNA virus replication [49-51] is likely required for the arthropod-borne lifestyle (i.e. host-switching). However, the arbovirus families utilize a variety of replication strategies and genome architectures, and is

exemplified by the RNA genomes of the orbiviruses (double-stranded and segmented), vesiculoviruses (negative sense, single-stranded, and monopartite), and thogotoviruses (negative sense, single stranded, and segmented) in addition to what was described above. This indicates that arboviruses, along with hematophagy in arthropods [52], likely arose independently several times (i.e. convergent evolution) [53]. This also indicates that arboviruses from different groups will follow divergent evolutionary trajectories set forth by their specific environments.

Global emergence of arboviruses

Today there are many viruses that were once considered as endemic pathogens of Africa that can now be found all around the world. The emergences of WNV and CHIKV are of particular interest due to the speed at which they traveled the globe and the explosive outbreaks they caused. They also happened in an era with advanced molecular diagnostics, allowing their introductions to be chronicled like none other before [13,15]. WNV was originally isolated in 1937 from a febrile native of the West Nile district of Uganda [54], and subsequently was associated with sporadic outbreaks throughout Africa, Eurasia, and Australia [13]. Outbreaks were generally mild (few cases of neurological disease) and small in scale during this period. Then things started to change in the 1990s when frequent WNV epidemics occurred in Romania, Europe, and the Mediterranean Basin [55]. These outbreaks were striking for three reasons: 1) they were often large, 2) they were associated with neurological symptoms and high case-fatality rates (~10% in Romania [56]), and 3) they occurred in temperate regions. The increasing trend of the disease burden caused by WNV continued into North America after it was first detected in the New York City area in 1999 [57]. WNV demonstrated a remarkable ability to act as an ecological generalist and utilized many different native mosquito vector and avian host species.

This allowed WNV to rapidly spread throughout North America and into South America in 5 to 10 years [13,58,59].

The history of CHIKV follows a similar narrative. The virus was first isolated in what is now called Tanzania in 1953 [60], then was associated with intermittent outbreaks of arthralgia throughout sub-Saharan Africa and Asia in the 1950s and 1960s [15]. In 2004, a drought helped to fuel an outbreak on the Kenyan coast [61]. From there CHIKV spilled over onto several Indian Ocean islands and to India, sparking epidemics that infected millions of people [15]. Infected travelers from the outbreak regions returned home with the virus [62-64] and in some cases initiated autochthonous transmission in temperate regions [65]. Travelers from the 2006-2009 CHIKV epidemics did not initiate outbreaks in the Americas despite the opportunities [64], however its introduction was inevitable. In December 2013, local transmission of CHIKV was confirmed on the islands of St. Martin and Martinique. A month later autochthonous cases were reported from several Caribbean islands, then in Central America, northern South America, and Florida within a year [66-69]. According to the Pan American Health Organization, the suspected CHIKV case count in the Americas now exceeds 1.7 million from 45 countries or territories [70]. These examples demonstrate that when the stage is set for arbovirus emergence they can do so at a rapid pace and have significant consequences. But what sets the stage?

Arbovirus transmission is primarily constrained by the availability of competent vectors and hosts. Nevertheless, arboviruses can emerge from tightly restricted enzootic foci by several processes including the natural movement of vectors and hosts, human activities, and virus adaptive potential. Patterns of arbovirus dispersal vary considerably depending on the movements of their hosts. Mosquitoes can travel several kilometers over consecutive days [71,72], not only helping to locally disperse viruses within a season, but potentially across larger

expanses of land over the course of several years [73]. A wide taxonomic range of vertebrates serve as hosts, and therefore their impacts on daily, seasonal, and perennial viral dispersal also vary substantially. Birds, however, are the most common arbovirus host. This coincides with the tendency for many mosquito species to prefer avian bloodmeals [74,75], likely stemming from their long-shared evolutionary history [76,77]. In addition to being competent amplifying hosts for many arboviruses, birds also greatly aid in arbovirus radiation and global emergence. For example, it is hypothesized that alphaviruses originated in the Americas and were distributed multiple times to the Old World by migratory birds then evolved independently after thousands of years of isolation [78-81]. Additionally, dense groups of migratory birds, such as thrushes, gulls, and storks, are potential sources of transcontinental introductions of WNV [56,82-85].

Anthropogenic processes of globalization, urbanization, and industrialization also facilitate arbovirus emergence [59]. First, humans broke down biogeographical barriers and connected the world through the ever increasing amount of global travel. The story of *Ae. aegypti* highlights the dangers of globalization [86]. *Ae. aegypti* is believed to have originated in North Africa and spread throughout Africa via the trans-Saharan trade network. Then the mosquitoes boarded ships and colonized much of the tropical and subtropical world during the fifteenth to seventeenth centuries. Once *Ae. aegypti* became established in the Americas, the stage was set for the introduction of several important *Ae. aegypti*-borne viruses. First came YFV and DENV via the trans-Atlantic commerce and slave trade [87,88]. An effective vaccine developed in the 1930s (17D) successfully eliminated YFV in all but the unvaccinated regions of the Americas and Africa [89,90], but DENV persisted to alarming levels [88,91]. The DENV burden was exacerbated by several other anthropogenic processes such as the increased dispersion of *Ae. aegypti*, introduction of new serotypes, and human population expansion.

Furthermore, humans took all of the critical pieces required for sylvatic transmission (competent vectors, mosquito breeding sites, and competent primate amplifying hosts [i.e. humans]) and conveniently placed them in dense urban centers to create new endemic and epidemic cycles. These same factors also enabled the recent emergences of CHIKV and ZIKV in the Americas [14,15] and even helped to perpetuate arboviruses that do not use humans as amplifying hosts. For example, both agriculture and urbanization can increase the risk of human WNV incidence by providing suitable habitats for important avian hosts and mosquito vectors [59,92]. Finally, the unintended consequences of industrialization, namely greenhouse gas emissions, are continuing to induce climate change at an unprecedented rate. Rainfall, temperature, and other climate variables can directly impact many facets of arbovirus transmission, including mosquito abundance, behavior, and vector competence [61,93-96]. These are just a few examples of how humans are altering arbovirus ecology. Since these activities are unlikely to cease, it is unlikely that human participation in arbovirus emergence will also cease.

Dispersion and habit changes offer the opportunities for viral emergence, but their ability to undergo rapid evolution allows them to adapt and thrive in novel environments. A central feature of RNA virus biology is they have high mutation rates and form genetically complex populations [49-51]. This provides the viruses with opportunities for rapid selection, and therefore adaptation [97,98]. WNV and CHIKV are well documented examples of RNA virus evolution leading to successful integration into new environments. The unfortunate events following WNV introduction into North America also provided scientists with the perfect laboratory to follow the adaptive steps a virus takes to survive in a foreign environment. Repeated analysis of WNV sequences revealed a few key events. First, there appeared to be a single point of introduction into the New York City area in 1999 demonstrated by the extreme

genetic homogeneity during the first two years of transmission [57,99,100]. Then a new subtype of WNV (WN02) with single nucleotide substitution conferring a conservative amino acid change in the envelope protein (A159V) was detected [101]. The new WN02 strain had a shorter extrinsic incubation period (EIP) in mosquitoes compared to the original subtype (NY99) [93,102,103], which increased vectorial capacity (the basic reproductive rate of vector borne pathogens [104]) and likely led to the displacement of NY99 [102,105]. Finally, replication within North American birds imposed selective pressures for increased viral replication and pathogenesis (possibly by overcoming the host's antiviral response) [106-111], which again increased transmission potential. In the end, WNV managed to become endemic with only a few important adaptive changes.

The 2006-2009 Indian Ocean CHIKV epidemic was vectored by *Ae. albopictus* [15,65], a highly invasive species that recently colonized much of the world, including several temperate regions [112]. Previous CHIKV epidemics, however, were driven by the urban *Ae. aegypti* mosquitoes, and *Ae. albopictus* was not implicated as a major vector. The older Asian CHIKV lineage is significantly less infective in *Ae. albopictus* than *Ae. aegypti*, making *Ae. albopictus* a less efficient vector [113]. In addition, the Asian CHIKV lineage is genetically constrained in its ability to adapt to *Ae. albopictus* [114]. So what changed? The emerging CHIKV strains during the Indian Ocean epidemic came from Africa and were able to acquire multi-step *Ae. albopictus*-adaptive mutations that were unavailable to the Asian strains [113-116]. The envelope glycoprotein 1 (E1) A226V mutation was the first step and provided a 50-100 fold fitness increase [113], followed by additional fitness increases by the secondary E2-L210Q mutation [115,116]. These mutations allowed CHIKV to better utilize *Ae. albopictus* as a vector and fueled an epidemic involving millions of people.

An interesting aspect of the CHIKV story is that the adaptive mutations were also created *de novo* experimentally [117], and several other second-step mutations are hypothesized to further enhance CHIKV fitness in *Ae. albopictus* [115]. These studies force us to ask the question, “What can be predicted about virus evolution and emergence?”. Specific predictions about viral emergence, such as when and where, are likely to be difficult, but forecasting how viruses will adapt to new environments should be feasible as long as genetic variation is driven by natural selection [118-120]. While emergence may never be predicted with any certainty, moving experimental evolution towards a predictive science is a challenge worth pursuing.

Unique aspects of arbovirus evolution

Seminal studies conducted from the late 1970s to the mid-1990s discovered that the formation of genetically complex viral populations presented a challenge to the traditional paradigms of population genetics: evolution was rapid, population sizes were near-infinite, and selection could act at the level of population [49-51,121-123]. The quasispecies theory, a mathematical formulation for the rapid evolution and self-organization of RNA-like molecules [124,125], was adopted to help describe the complex evolutionary dynamics of RNA viruses [126-128]. Currently a viral quasispecies is defined as a population of related but non-identical virus genomes (i.e. mutant swarms or mutant clouds) under continuous processes of mutation, inter-variant competition, and selection for the fittest groups of variants in a given landscape [129,130]. Thus variants within a quasispecies can collectively contribute to the phenotype of the population, including viral fitness and host disease, through cooperative interactions [131-134]. These important theories and experiments provided the framework for understanding the molecular mechanisms underlying viral genetic diversity and fitness during host infection.

Multihost pathogens present another complex challenge [135]. Multihost viruses, like arboviruses, are no different than single host RNA viruses in that they form large intrahost populations of related but non-identical viral genomes [117,136-139]; however, their evolutionary trajectories are uniquely different because they must constantly replicate in different hosts. Moreover, compared to some other multihost viruses, like ebolaviruses that occasionally “jump” between rodents, bats, and humans (all mammals), arboviruses must cycle between hosts of different phyla (Arthropoda and Chordata) to persist. Therefore arbovirus populations possess qualities unlike most other classes of viruses. Releasing arboviruses from their dual host cycles removes genetic constraint and facilitates host-specific adaptation, much like single host viruses [140-144]. Yet natural transmission does not favor host specialization, rather they are predicted to be selected for as generalists [145,146]. So why do arboviruses exist in dual host cycles? What are the costs? While many resources have been dedicated to these questions, the answers are still not entirely clear.

It seems that a major consequence of replication in disparate hosts is that it leads to slower rates of evolution than their single host counterparts [147-150]. A hypothesis is that adaptive mutations in mosquitoes can be deleterious in the vertebrate hosts (and vice versa), thus leading to fitness trade-offs and less positive selection [151,152]. However, this hypothesis is controversial because experimental evolution studies provided conflicting results. Some studies reject [140,153-156], partially reject [141,157], and support [142,144] that alternating replication cycles lead to overall fitness declines in both hosts. These differing results may represent the complex nature of virus-host interactions and the vast differences among the experimental models. For example, evolution of flaviviruses in *Culex*-bird cycles [141,156] may not be similar to alphaviruses in *Aedes*-rodent cycles [142]. Most of these studies were also performed *in vitro*

[140,144,153-155,157] and these idealized conditions may less accurately reflect evolution in nature than *in vivo* experiments [141,142,156]. The general sentiment from these studies is that there are fitness trade-offs in vertebrates but not in mosquitoes.

The relative stasis of arbovirus evolution may not be due to fitness tradeoffs *per se*, but perhaps by incongruent fitness landscapes that constrain genetic diversity [143]. Replication in mosquitoes may select for variants to better recognize specific receptor molecules and evade the arthropod antiviral response, however, many of these variants may start in a fitness landscape valley when transmitted to birds and will likely be removed by mass selection [98]. The overall effect of shifting landscapes may not change the fitness of the viral population, but would be predicted to massively purge genetic diversity. *In vivo* experiments and analysis of natural populations reinforce the idea that the arbovirus lifecycle limits genetic diversity [137,158-160]. In fact, three studies that reject the fitness trade-off hypothesis still demonstrate that there was less acquired genetic diversity after host alternation than after sequentially passaging in one or both hosts individually (i.e. lowered genetic diversity did not directly affect the measured fitness) [140,153,154]. Again, these studies disagree as to which host contributes the most to genetic diversity. Experimental and natural infections with WNV [131,137,161] or St. Louis encephalitis virus (SLEV [156]) demonstrate that viral populations are more diverse in *Culex* mosquitoes than birds. Meanwhile, infections with DENV reveal that the levels of genetic diversity are similar, if not a bit higher in humans than *Aedes* mosquitoes [158-160]. *In vitro* studies with DENV and two alphaviruses also state that genetic diversity is higher in mammalian than mosquito cells [137,156,161]. However, all three of those studies were conducted using *Ae. albopictus* C6/36 cells which lack a functional RNA interference (RNAi) response [162,163] that drives viral diversification [164,165] and therefore the results should be interpreted with caution.

Nonetheless, there may be host- and virus-dependent processes that greatly contribute to viral evolution [157,166].

Arbovirus evolution is best described to date in the WNV-*Culex*-bird system. There is substantial data demonstrating that WNV genetic diversity is higher in mosquitoes than birds, and that WNV genetic diversity in mosquitoes is generated by strong diversifying selection and maintained by weak purifying selection [131,137,161,164,165,167,168]. Diversifying selection is driven by the primary innate antiviral response in mosquitoes, RNAi, where viral RNA is targeted for degradation by sequence complementarity to a small template RNA loaded into the RNA-induced silencing complex (RISC) [169,170]. Mutated viruses are less susceptible to sequence complementation, and thus silencing (degradation), than are un-mutated viruses, creating an intracellular milieu where rare viral haplotypes are favored and have a competitive advantage [164,165,171]. The continued measurement of high (> 1) d_N/d_S ratios from intra-mosquito WNV populations is highly suggestive of weak purifying selection [131,137,161]. This could be directly related to the RNAi response, where selection happens at the nucleotide level and neither synonymous nor nonsynonymous mutations are favored. Complementation of multiple viral genomes and proteins within mosquito cells may also decrease purifying selection as they allow for the persistence of low fitness and deleterious mutations [121,167,172]. However, cellular multiplicities of infection (MOI) and complementation have yet to be directly tested for their direct impacts on selection within mosquitoes.

The mosquito replicative environment allows WNV to explore a broader sequence space and facilitates rapid adaptation during shifting fitness landscapes, as predicted by some of the fundamental theories of natural selection [97,98]. The fitness landscape of birds, as one would expect, is vastly different from that of mosquitoes. For one, WNV replication in birds does not

allow for the accumulation of nonsynonymous mutations, likely because the primary innate antiviral response, the type I interferon (IFN) pathway [173], acts primarily upon the amino acids (i.e. phenotype). The WNV master sequence is hypothesized to already sit at a high fitness peak, encoding for key properties leading to resistance to IFN, replicative fitness, and virulence [174]. Therefore any amino acid substitution could alter the infection phenotype [131], decrease fitness, and be rapidly removed by strong purifying (removal of deleterious variants) or mass selection (outcompeted by more fit variants) [131,137,161]. These processes in birds are predicted to constantly revert mutations accumulated in mosquitoes back towards the master sequence, and argue for evolutionary stasis. However, Jerzak et al. showed that alternating WNV replication between mosquitoes and birds over the course of 20 passages led to similar nucleotide diversity as WNV replicating in mosquitoes alone [161], suggesting that genetic constraint may be due to infection of birds and not necessarily by alternating hosts (i.e. genetic diversity is rapidly reintroduced by mosquitoes).

To date, arbovirus evolution is primarily defined by its generalities and is far from a predictive science. The notion that arboviruses have slow rates of evolution is widely accepted but paradoxically contrasts other aspects of RNA virus biology: generation of genetically diverse populations and rapid adaptation. More detailed descriptions of the minority variant dynamics during arbovirus transmission are needed to further understand how arboviruses maintain complex intrahost populations and rapidly adapt to novel conditions in the context of minor long-term change to their master sequence. Specifically, studies are urgently needed to understand how replication within different cells, tissues, and species can alter arbovirus diversity and fitness. These data will also help to define the innate mechanisms that drive arbovirus evolution and the ecological conditions that alter its path. Advancing the knowledge base of the WNV-

Culex-bird system, along with spillover into humans, is a direct route towards achieving these goals. The features of the WNV life cycle that impact both stochastic (random genetic drift) and deterministic (selection) processes of evolution and the knowledge gaps are discussed in detail.

West Nile virus as a model to study arbovirus evolution

WNV is an excellent model to study arbovirus evolution and emergence. First and foremost, WNV is an important human pathogen that caused the largest outbreaks of viral neuroinvasive disease ever reported in the Western Hemisphere (in 2003 and 2012) [175]. Second, WNV is an ecological generalist and infects a wide-range of bird and mosquito species that can easily be maintained in a laboratory setting [176-178]. Conversely, to replicate the transmission cycle of DENV and CHIKV, difficult primate or often inappropriate rodent animal models are required [179,180]. Finally, the WNV system is well defined, from its ecology [181] and emergence [13] to its replication [182] and pathology [173]. These traits enable experimental manipulation of the WNV replication environment to track infections with diverse outcomes and to better understand how arboviruses respond to novel and dynamic conditions. In this way, the microhabitat-specific aspects of arbovirus transmission, such as different avian and mosquito species, can be examined for their influence on arbovirus evolution. From there the finer details of selective pressures from different cell types and immune response that could alter WNV population structure can start to be uncovered.

Ecology

WNV is found in tropical and temperate latitudes and on every continent except Antarctica, making it perhaps the most widely distributed arbovirus in the world [13,181]. It is

maintained in an enzootic cycle primarily by ornithophilic (bird “loving”) *Culex* mosquitoes and passerine birds. Given its global distribution, several species of *Culex* mosquitoes and birds have been implicated as competent vectors and hosts. The possible WNV vectors include *Cx. univittatus* and *Cx. neavei* in Africa [183-185], *Cx. molestus* and *Cx. perexiguus* in Europe [186], *Cx. annulirostris* in Australia [187], *Cx. bitaeniorhynchus*, *Cx. vishnui*, *Cx. pseudovishnui*, and *Cx. tritaeniorhynchus* in Asia and the Middle East [188-190], *Cx. tarsalis*, *Cx. restuans*, *Cx. nigripalpus*, and *Cx. salinarius* in North America [177,178,191-194], and members of the *Cx. pipiens* complex (e.g. *Cx. pipiens pipiens* and *Cx. p. quinquefasciatus*) around the world [177,178,185,191,192,195-197]. In addition, several species of *Aedes* and *Culiseta* mosquitoes serve as secondary vectors or act as bridge vectors to dead-end mammalian hosts, including humans [178,191,194,197-199]. The role of different mosquito species in WNV transmission is largely determined by microhabitat conditions, such as availability of breeding sites and avian hosts [200-205], and macrohabitat conditions, such as climate [95,206-208].

WNV can amplify to sufficient titers in many different bird species, predominately in the order Passeriformes, to sustain mosquito transmission ($\sim 10^4$ plaque forming units [PFU]/mL) [176,209-214]. In the Americas, these include important amplifying hosts such as corvids (e.g. American crows [*Corvus brachyrhynchos*], fish crows [*C. ossifragus*], and blue jays [*Cyanocitta cristata*]), thrushes (e.g. American robins [*Turdus migratorius*] and Swainson's thrushes [*Catharus ustulatus*]), and sparrows (e.g. house sparrows [*Passer domesticus*]), as well as other diverse bird taxa such as Columbiformes (e.g. doves), Galliformes (e.g. grouse), and Anseriformes (e.g. mallards) [214]. In addition, young birds are generally more susceptible to arbovirus infection [215-218], therefore chicks and juveniles born to refractory adults may still be competent hosts and/or succumb to disease [219,220]. For example, adult domestic chickens

develop very low WNV viremia levels [185], but days-old chicks produce enough virus to sustain transmission and are often used as laboratory models for bird infection [131,141,161,221].

Again, the avian species involved in local enzootic WNV cycles are also dependent upon several ecological factors that influence species distribution and diversity, such as urbanization (which decreases avian diversity) [222]. Perhaps even more essential are the various blood feeding preferences of local *Culex* mosquitoes [74,75,223-226]. For example, in the highly urbanized environments in Chicago, IL and Washington, DC, *Cx. pipiens* preferentially choose to take blood meals from American robins (highly competent WNV hosts [176]) despite their relatively low abundance [74,226]. Meanwhile in a more suburban/rural environment in Weld County, CO, *Cx. tarsalis* often choose Eurasian collared-doves and mourning doves (moderately competent WNV hosts [176,227]) over robins [75]. Adding to the complexity is that mosquito blood feeding shifts with seasonal patterns [228], as exemplified by the continent-wide decreasing preferences for robin bloodmeals from early to late summer [74,75,225,229]. The shift appears to be related to the robin roosting period [230]. Nestling birds have minimal feather coverage and weak defensive behaviors making them easy and preferred targets for mosquito bloodmeals [231-233]. This concentration of highly competent hosts greatly amplifies the number of WNV-infected mosquitoes which then seek out new sources of bloodmeals after the robins disperse in late summer [230], often leading them to humans [74,75,229].

The dynamic and often synergistic ecological conditions of local WNV transmission cycles can have significant consequences on transmission intensity and can help to fuel WNV epidemics [59,229]. George Macdonald's expansion of the basic reproductive rate for vector-borne pathogens, called vectorial capacity (VC), is a tool often used to model these conditions

[104]. His formula is founded on a few basic intrinsic and extrinsic principles of vector-borne pathogen transmission:

$$VC = \frac{ma^2bp^n}{-\log_e p}$$

where m is the density of mosquitoes, a is the biting rate, b is the vector competence, p is the mosquito survival rate, and n is the EIP. Local and seasonal conditions, such temperature and rainfall, can directly influence several of these variables [203,207]. For example, increased temperatures can increase *Culex* population sizes (m) [234], shorten the length of the gonotrophic cycle (i.e. time between bloodmeals, and therefore increase a) [235], increase viral dissemination rates (b) [236], and shorten the EIP (n) [93]. Slight alterations to VC can have significant effects on the outcome of local WNV transmission, and likely virus evolution.

These dynamic variables underscore the heterogeneity of WNV transmission and suggest that the fitness landscapes must be constantly changing. It is not known, however, how much the landscapes change. How different are the replication environments between different species of *Culex* vectors, or between *Culex* and *Aedes*? Brackney et al. demonstrated that WNV populations change temporally in mosquitoes [167]; therefore, environmental factors that influence the biting rates (e.g. gonotrophic cycles and availability of bloodmeals) and EIP could also alter the transmitted WNV populations. WNV is also likely to evolve differently in bird species with different levels of susceptibility to infection and disease. Even intrinsic and extrinsic temperatures can have a significant impact on virus diversification. Flamand estimated that the frequency of spontaneous vesiculovirus mutation doubles when the temperature is raised from just 39 to 39.8 °C [237]. These data indicate that WNV mutation rates could increase when their avian hosts develop a fever, or even more profoundly increase when the mosquito environmental

temperatures increase. In all, little is known about the ecology of WNV evolution and how effectively WNV can adapt given the constraints of the arbovirus lifecycle.

Cellular infection and the replication cycle

The ~50 nm diameter enveloped WN virions attach to a wide range of cell types, possibly by interacting with several different or conserved host surface proteins (including DC-SIGNR expressed on dendritic cells [238]). They enter the cells via receptor-mediated endocytosis of clathrin-coated pits, the endosome acidifies, and the viral envelope (E) proteins fuse to the endosomal membrane [239-241]. The inner surface of the virus becomes exposed to the cytoplasmic environment, so the capsid (C) proteins associate with the viral genome to protect from host nucleases and RNA sensors while the genome prepares for replication and translation [242,243]. The capped positive sense genome serves as the viral mRNA (despite the lack of a poly A tail) and therefore viral proteins can be immediately translated. The single WNV ORF is composed of three structural (C, pre-membrane [prM], and E) and seven nonstructural protein coding regions (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [26]. The polyprotein is co- and post-translationally cleaved into mature proteins by the viral serine protease complex (NS2B-NS3) and cellular proteases [244].

The genome also serves as the template for complementary minus strand synthesis (template for replication), but the RNA must cyclize first. The 5' and 3' termini untranslated regions (UTRs) form conserved secondary structures that are used for long distance *cis*-acting RNA-RNA interactions [245-248]. This cyclization is critical for viral replication and mutations that alter the RNA structures and base pairing can be highly deleterious [249]. All of the NS proteins are involved in the formation of viral replication complexes on the endoplasmic

reticulum (ER). The membrane-spanning proteins, NS2A, NS2B, NS4A, and NS4B, cause invaginations in the ER and anchor the complex [250-253]. NS1 co-localizes with the replication complex on the luminal side of the ER and is required to initiate RNA synthesis [182]. NS3 and NS5 are located on the cytoplasmic side of the ER and accept the minus strand viral RNA as the template for replication. NS5 replicates the RNA (RNA-dependent RNA polymerase [RdRp] [254]) and adds a 5' cap ($m^7GpppAmp$ methyltransferase [255]). NS3 dephosphorylates the 5' end of the nascent RNA prior to capping (5'triphosphatase [256]) and splits the bonds (RNA-stimulated nucleoside triphosphatase [257]) and unwinds the double-stranded RNA (ARPAse/helicase [258]). During the exponential phases of replication, multiple positive strand RNAs are simultaneously copied from a single template, exit into the cytoplasmic side, then associate with the structural viral proteins anchored to the ER [259-261]. The structural proteins bud into the lumen, transport through the Golgi network to the plasma membrane to be released by exocytosis [182]. Mature virions are typically released starting at 8-10 hours post infection and peaks at 24 hours.

The replication process, though efficient, is far from error proof. Eukaryotic cells can correct mismatched bases during DNA replication to stabilize their cellular genomes [262]. RNA viruses, on the other hand, encode for a RdRp that lacks proofreading activity [49]. The intrinsic error rates of viral RNA polymerases are 10^{-6} to 10^{-4} per nucleotide [263,264]. WNV is estimated to mutate at a rate of ~1 to 3 substitutions per round of genome replication [137,265]; therefore with a genome size of ~11,000 nucleotides it is theoretically possible that all single nucleotide substitutions could be generated within 10^4 rounds of replication. There is evidence suggesting that these mutation rates are selected for optimal viral fitness [266-270]. Increasing replication fidelity (i.e. decreasing mutation rates of the RdRp) restricts genetic diversity and limits

phenotypic plasticity. Decreasing replication fidelity continuously lowers population fitness through the irreversible accumulation of lethal mutations, a process analogous to a ratchet [122,271,272].

Separate cycles of viral replication and translation means that newly synthesized RNA genomes can be replicated by or form virions with proteins with different genetic backgrounds. For example, a WNV RNA genome is translated and cleaved into functional structural and NS proteins. The negative strand copy of the genome is copied with several errors, one of which would alter the coding sequence of the E protein and reduce its ability to infect new cells. Yet at this stage, the genome can still associate with the non-mutated structural proteins to create an infectious virion. The lethality of the genome, and selection to remove it, is not applied until the genome is translated in new cells, produces new virions with the mutated E protein, and then tries to infect a third set of susceptible cells.

These epistatic interactions of intra-cellular viral genomes and proteins (i.e. complementation) can significantly impact the viral population structure and are highly influenced by the number of viruses infecting a cell (i.e. density-dependent selection) [121,273]. When the MOI is high, complementation of viral genomes allows for the maintenance of specific mutations as part of a viral population [121,273-275]. Even defective viruses can be maintained when they co-infect cells with functional viruses [172,276,277]. Conversely, complementation can also help mutants reach adaptive landscapes where they have a fitness advantage and target organs where they can significantly alter the infection phenotype [132]. When the MOI is low, and therefore complementation is minimal, the frequency of any one virus in the population is roughly equal to its fitness, and the relative fitness of the entire population is high [121]. Moreover, a single high fitness virus can rapidly become dominant and homogenize the

population. However, a recent study by Combe et al. complicates the density-dependence theory [278]. They demonstrate that multiple viral genomes can be transmitted within a single infectious unit, implying that complementation can even arise when the MOI is very low, helping to maintain genetic diversity. The influence of MOI, genetic diversity, and fitness needs to be further investigated for arboviruses that infect many different hosts and cell types. Specifically, does complementation work the same in mosquito midgut epithelial cells as they do in avian dendritic cells? Is there a correlation between complementation and transmission fitness, and would it work to select for specific levels of viremia in birds? Intracellular WNV evolution is certainly a critical component of long-term viral success.

Mosquito infection and the exo-siRNA pathway

WNV must pass through several anatomical barriers within mosquitoes for transmission to occur [279]. The first and most important barrier to viral infection is the mosquito mesenteron (midgut). This section of the alimentary canal is composed of a single layer of epithelial cells encircled by a multilayer extracellular matrix called the basal lamina [279,280]. In some cases, viruses may be able to bypass this barrier through “leaky” midguts [281,282]. However, in most competent species, infection is initiated at the posterior portion of the midgut where contents of the bloodmeal are absorbed. Eventually the virus must pass through the basal lamina of the midgut and infect hemocytes (invertebrate immune cells [283]), fat bodies, neurons, and muscle tissue throughout the mosquito hemocoel (i.e. a disseminated infection) [279,284]. It is hypothesized that infection of these cells is a part of an important amplification step to overcome the next major barrier, infection of the salivary glands [284,285].

Mosquito salivary glands are lobed structures that consist of a single layer of cells surrounding the salivary duct [280,286]. The distal regions of the lateral lobes are the first to become infected followed by the spread to all of the salivary gland cells [284,287,288]. After replication in the cytoplasm, mature virions are transported by intracellular vacuoles and/or are directly released through the plasma membrane into an extracellular acinus (a holding place for saliva proteins). The contents of the acinus, including the salivary proteins and virus, are emptied and expectorated during mosquito probing and feeding. The acinus is refilled soon after so that the process can continue with the next feeding attempt [289,290]. Styer et al. estimates that *Culex* expectorate 10^4 - 10^6 PFU of WNV during bloodfeeding, about 10-100× more than *Aedes* [291].

The ability of an arbovirus population to overcome these anatomical barriers is the foundation of vector competence and a key component of vectorial capacity. However, overcoming these barriers may have significant costs to the viral population. While Brackney et al. showed that WNV genetic diversity is maintained throughout *Cx. quinquefasciatus* infection, many other studies document the existence of population bottlenecks within the barriers that can lead to fitness declines [160,292-296]. Studies using virus-like particles to track binding and internalization of WNV and VEEV in *Cx. quinquefasciatus* and *Ae. taeniorhynchus*, respectively, demonstrate that midgut infection is established by only a few cells and then spreads from these foci [293,294]. The extent of the bottleneck upon midgut infection is dependent upon the diversity and amount of virus in the bloodmeal that infects the few susceptible cells [292,295,296]. At higher doses, more viruses infect the midgut which helps to maintain the integrity of the founding population despite the few number of susceptible cells [295]. Therefore, the midgut bottleneck may be less severe when *Culex* mosquitoes feed upon

American crows that can produce WNV viremia levels $>10^{10}$ PFU/ml of serum compared to mourning doves that produce a maximum of $\sim 10^6$ PFU/ml. At low oral doses of VEEV fed to *Cx. taeniopus* ($\sim 10^5$ PFU/ml), Forrester et al. estimated that as few as two virions seeded the midgut infection and as few as one disseminated into the hemoceol [295]. Given the potential for severe bottlenecks, it makes sense statistically that higher frequency haplotypes are more likely to survive [292]. However, defective virus genomes present at low frequencies are known to persist through anatomical barriers [167] and multiple rounds of transmission [172]. These data suggest that the bottleneck sizes are dynamic and contingent on many factors including mosquito species and tissue, virus dose and diversity, and perhaps ecological factors, such as temperature.

Seminal work by Ernst Mayr (founder principle [297]) and H.J. Muller (Muller's ratchet [271]) predicts that small populations formed by random sampling at bottlenecks (i.e. non-selective) are prone to accumulating deleterious mutations and severe fitness declines [122,272]. If this is true, then how is virus replication in mosquitoes supported? For one, the severity of bottlenecks at mosquito anatomical barriers may not be as extreme as previously reported. Perhaps using genetically marked viral clones and PCR assays [292,295] are not comprehensive enough to calculate the true bottleneck sizes because they cannot account for locally acquired *de novo* haplotypes. In addition, the demonstration that multiple genomes can be transmitted within a single infectious unit lends support that some genetic diversity can be maintained even under extreme genetic bottlenecks [278]. The consequences of bottlenecks may also be offset by rapid regeneration of genetic diversity and compensatory mutations. However, it appears that these factors do not remove all of the deleterious alleles [298]. Deardorf et al. showed that passaging WNV within mosquitoes led to fitness decreases in birds but not in mosquitoes [141], so perhaps the mosquito environment is very tolerant of mutations that are deleterious in birds. High d_N/d_S

ratios from intra-mosquito WNV populations support this statement [131,137,161], but the virus and mosquito factors that govern this tolerance are unknown.

The most widely used measure of virus-mosquito interactions is vector competence, which is determined by a highly complex relationship of biotic and abiotic factors [285,299]. As discussed above, external conditions such as temperature certainly can alter viral replication and vector competence [93,236]. Genetics of the vector and the virus [93,102,300], previous viral infections (i.e. superinfection exclusion) [301-303], and the mosquito microbiome [304] also impact susceptibility to infection. As demonstrated with *Anopheles* and hemoparasite *Plasmodium* [305], the mosquito innate immune response is likely a critical component of controlling systemic WNV infection [169]. RNAi, specifically the exogenous small interfering RNA (exo-siRNA) pathway, is the primary antiviral immune response in arthropods [169,306]. Studies using various arthropod-virus pairings, including *Drosophila*-SINV [307], *An. gambiae*-ONNV [308], *Ae. aegypti*-SINV [309], and *Ae. aegypti*-DENV [310], demonstrate that the exo-siRNA pathway helps to limit arthropod mortality and viral replication. However, the extent to which it contributes to vector competence is not yet known.

The exo-siRNA pathway is expected to play a major role in the generation of viral genetic diversity [164,165]. This “virus-induced gene silencing” [311] pathway is activated when viral dsRNAs that naturally occur during replication and as secondary structures are recognized by Dicer-2. This RNase III enzyme cleaves the RNA into 19-23 base pair fragments (predominately 21 base pairs) which are loaded into the RISC. One strand of the cleaved RNA is discarded and the remaining strand, termed the guide strand, remains to recognize new target viral RNA by sequence complementarity. Finally, Argonaute-2, which has endonuclease activity, “slices” the target RNA and “silences” the virus [169]. The exo-siRNA pathway is sensitive to

mismatches between the guide strand and the target viral RNA [312], therefore RNAi can drive diversification by selecting for point mutations that differ from the guide strands [164,313,314]. It is also hypothesized that RNAi, which is less prominent as an antiviral defense in vertebrates (a debated subject [315]), is at least partially responsible for the greater WNV genetic diversity in mosquitoes than birds [131,137,161]. In fact, genetic diversity itself likely provides a fitness benefit in mosquitoes [171], and the decreased fitness associated with high fidelity WNV in mosquitoes may be related to its relative inability to generate RNAi escape mutants [269].

There are still many questions regarding intra-mosquito WNV evolution. For a start, there is little known about the temporal and spatial dynamics. Brackney et al. found that genetic diversity decreases overtime, but this may have been confounded by the use of an unnaturally diverse input population [167]. There is also evidence that genetic diversity is different between mosquito tissues (including saliva) [117,158,167], but it is not yet known if this is solely due to bottlenecks or if other areas of mosquito-virus interactions (e.g. RNAi) play a significant role. Second, vector competence is highly variable between mosquito populations and environmental conditions [93,178,191-193,236]. The high genetic diversity produced within mosquitoes likely means that even minor alterations to vector competence can significantly impact viral population structure. However, a correlation between vector competence and viral genetic diversity has not been directly investigated. Comparing intrahost evolution, fitness of transmitted populations, and RNAi responses to WNV between different mosquito species and strains will start to address some of the major knowledge gaps of arbovirus evolution. Moreover, there seems to be two opposing forces within mosquitoes: RNAi and bottlenecks which act to diversify and homogenize populations, respectively. Yet both forces share a commonality in that they act upon the viral genome and not the protein, which does not always optimize viral fitness. Perhaps

arboviruses may be successful *despite* their requirements to replicate within mosquitoes and selection in birds may remove enough of the deleterious mutations to sustain future transmission. Or it might just be a numbers game when at any given foci literally thousands of infected mosquitoes may be seeking hosts to continue the transmission cycle. Likely a more detailed understanding of vector competence, WNV evolution within mosquitoes, and the fitness costs associated with mosquito replication is needed to fully grasp how arbovirus transmission is maintained despite the many reasons why it should not.

Avian susceptibility and infection

WNV primarily causes disease in birds that has devastated North American populations since its introduction in 1999 [316-319]. LaDeau et al. demonstrated that American crows, a highly susceptible species [176,320,321], experienced a 45% population decline within 5 years after the arrival of WNV [316]. More recently, George et al. found that 47% of 49 bird species studied in the United States were negatively affected by the introduction of WNV [319]. Some species, such as the field sparrow (*Spizella pusilla*), experienced sharp population declines that quickly recovered. However, 25% of the species, including the purple finch (*Caprodacus purpureus*) experienced population declines that have not yet recovered. Susceptibility to infection and mortality, which are influenced by the bird's age [215-220] and immune response [214,320], are obvious factors that affect avian populations. There are also several ecological factors, including climate [322], microhabitat conditions [323], and human land use [59,200,324], that are more likely to put birds at risk to infection and/or alter their overall health. Taken together, these factors suggest that there is a complex and somewhat unknown network of

interactions that determines the fate of a WNV-exposed bird population, and ultimately, virus transmission.

WNV clinical disease is primarily caused by virus dissemination into the major organs: the lungs, heart, liver, kidneys, spleen, and the central nervous system (CNS). The clinical symptoms are often non-specific and include fever, ataxia, and anorexia. Some WNV-susceptible birds experience rapid systemic infection, multiple organ failure, and death within a few days, while acute disease is infrequent in others [176,214,320,321]. Alternatively, chronic infections are known to occur when viral RNA persists for months in various tissues and may contribute to virus overwintering [325-327]. The underlying reasons for differences in clinical outcome among bird species are not clear, but are likely dependent upon the viral load, inflammatory response, and production of neutralizing antibodies. While there is a great deal of knowledge about WNV pathogenesis and the host immune response in mammals (reviewed in the next section), unfortunately very little is known about birds. Birds that clear infection often experience a rise in antibody titers (primarily IgM) around 5-7 days post infection and peak in about one month (IgY, avian equivalent of IgG) [214,320,328,329]. The critical links between the innate and adaptive immune responses [330] have not been studied in the WNV-bird system, therefore there is little understanding of how certain bird species can clear infection. Limiting the advancement of research are the general lack of commercially available reagents for ecologically relevant hosts (such as antibodies to IFN components), difficulties in obtaining birds (often need to be caught), and special animal biosafety level 3 facilities for WNV-infected birds.

WNV evolution within both naturally infected wild birds [137] and experimentally infected young chickens [131,161] is dominated by purifying selection. Since WNV tends to cause acute infection and transmission to mosquitoes occurs before the development of a

neutralizing antibody response, the selective pressures are likely a reflection of the early innate immune response. In addition, there is some evidence that wild birds paradoxically select for particular WNV variants. Pesko et al. detected large in frame deletions within some of the WNV genomes recovered from dead lorikeets [331]. These variants acted as defective interfering particles and reduced infection in Vero cell culture and mosquitoes, but did not decrease pathogenesis in mice. Moreover, Brault et al. reported a single T249P amino acid substitution in the NS3 helicase coding region that was positively selected by and significantly increased virulence in American crows [106]. While these examples represent important findings, they do not provide enough data to understand the role of particular birds as drivers of viral evolution and emergence. Does the selection strength vary between hosts? Is it correlated with disease or viral replication? And how does it impact viral fitness in birds and mosquitoes?

Filling in some of these knowledge gaps about WNV-bird interactions will significantly increase our ability to understand virus evolution. Viruses and hosts are under continuous pressures from one another to increase their own defenses, an evolutionary conflict to maintain fitness described by the ‘Red Queen’ hypothesis (i.e. race to stay in the same place) [332]. Furthermore, host immunity modulates viral adaptation [333]. Maximum viral adaptation occurs at the crossing point of the strength of selection and viral abundance, which often occurs with a moderate immune response. This is the point where the virus-host conflict is the most intense. Deviations from this response will decrease the net viral adaptation rate. At the extremes when there is either no effective host response (no selection) or overwhelming immune pressure (all virus is cleared), there is no adaptation. Unlike HIV, where the intrahost dynamics and adaptation rates are well characterized [334,335], strong predictions about WNV evolution cannot be made

without further investigation into virus-bird interactions. Moreover, we cannot predict in which bird species the evolutionary conflict is the most intense.

WNV evolution within birds is expected to directly impact disease in humans. When WNV spills-over into humans and other mammals, it can replicate, attenuate host antiviral responses, and cause pathogenesis [173], but the selective pressures from the mammalian immune system cannot drive WNV evolution because they are dead-end hosts. Even so, outbreaks of WNV encephalitis in Europe in 2008 [336] and in Australia in 2011 [337] are examples of human and equine disease caused by WNV strains previously described with low neuroinvasiveness. Therefore, these virulence changes most likely evolved during the natural transmission cycle and could be the result of bird-specific WNV adaptations coopted for enhanced cell entry, replication, and/or immune evasion in mammals. When these assumptions are applied to the evidence that WNV is becoming more virulent in birds by co-evolving with North American house sparrows [107], it suggests that WNV may also be becoming more virulent in humans. Investigation of ecologically relevant avian hosts infected with WNV may help us understand the mechanisms that drive virulence in humans and perhaps even predict future change. Of course, this assumes that the viral mechanisms of pathogenicity are similar in avian and mammalian hosts, which may not be true and also requires further examination.

Mammalian infection, type-I interferon, and neuroinvasive disease

Experimental WNV infection in mice and hamsters provided key information about viral dissemination and the host innate immune response. Following subcutaneous transmission of WNV from a mosquito, early phase WNV replication is thought to primarily occur in fibroblasts, keratinocytes, and Langerhans cells [173,338]. Infected Langerhans cells, or bone marrow-

derived epidermal dendritic cells, migrate from the skin to the draining lymph nodes [339]. WNV replication in the lymph nodes seeds the establishment of primary viremia and visceral-organ dissemination. Secondary WNV replication occurs in cells such as dendritic cells and macrophages of the peripheral tissues [173,340,341]. In humans, most WNV infections are asymptomatic (70-80%), about a quarter develop acute disease without neurologic involvement, and very few (<1%) progress to severe West Nile neuroinvasive disease (WNND) [56,342,343]. WNV is both neuroinvasive and neurotropic, but the very low proportion of individuals that develop WNND suggests that host restriction is robust.

The first and primary line of host defense against WNV replication is the innate immune response, dominated by type I IFN (α/β) [344]. Cells recognize WNV infection by sensing double- and single-stranded viral RNA using pattern recognition receptors. Engagement of these receptors leads to the production of chemokines and cytokines, including, IFN- α and $-\beta$. The secreted type I IFNs activate a signal transduction pathway in a paracrine and autocrine manner to induce hundreds of antiviral effector IFN-stimulated genes (ISGs) [345,346]. These ISGs can either directly or indirectly impede WNV replication. Important ISGs include RSAD2 (aka viperin, restricts early WNV replication and promotes IFN induction [347]), OAS1 (binds to dsRNA to activate an endoribonuclease [348]), IFIT1 (binds to translation factors and sequesters 5'-triphosphate RNA [349]), IRF-1 (induces IFN and other ISGs, aids CD8⁺ T cell expansion [350]), and IFI-27 (aka ISG12, sensitizes cells to apoptosis [351,352]). Additionally, several studies have demonstrated that there are cell- and tissue-specific antiviral responses to WNV infection due to distinct transcriptional programs [346,352-354]. This dynamic IFN-induced antiviral state is often powerful enough to prevent viral dissemination and clear infection.

To counteract, WNV has evolved functions to evade and antagonize the IFN response and the antiviral actions of ISGs. WNV suppresses early replication and hides its RNA in replication complexes to avoid detection and the antiviral actions of ISGs [355,356]. The viral encoded 2'-O methyltransferase caps the 5' end of the viral genome to evade host restriction by IFIT proteins [357,358]. Several WNV NS proteins can also directly antagonize the host response. NS1 can disrupt IFN and ISG induction by altering toll-like receptor 3 activation [359]. Both NS4B and NS5 (and maybe other NS proteins) can obstruct ISG expression by activating a pathway to degrade the IFN receptor (i.e. IFNAR1) and blocking the phosphorylation of several signal transduction components (i.e. JAK1, Tyk2, STAT1 and STAT2) [360-363]. If the virus can successfully counteract the host defenses, or if the host is immunocompromised, then the virus has a chance to reach the CNS.

The blood-brain-barrier (BBB) is formed by tight junctions between the microvasculature endothelial cells at the interface of the CNS. It is not entirely clear how WNV overcomes this formidable physical barrier [364]. One option is the hematogenous route where viruses enter by directly infecting the endothelial cells [365] or infecting immune cells that can pass freely into the CNS (i.e. a "Trojan horse") [366]. Data also suggest that the proinflammatory response to WNV infection facilitates neuroinvasion by compromising the integrity of the BBB [367,368]; however, subsequent studies found that inflammatory cytokines, including IFN α/β , act to tighten the junctions [369,370]. Alternatively, or in parallel, WNV may enter the CNS by the transneural route via peripheral neurons [371,372] or olfactory nerves [364]. Once in the CNS, WNV spread is thought to be limited by the relative susceptibilities of different neurons to infection and IFN protection [352,373]. The subcortical region, particularly the thalamus, medulla, pons, midbrain, basal ganglia, and the anterior horn of the spinal cord are especially vulnerable to neuronal loss

and injury, while the cortical neurons are left unharmed [374]. WNND can develop into meningitis, encephalitis, or poliomyelitis [375]. Meningitis, the most common (~40% of WNND), is also the least severe (<1% case fatality rate). Approximately 20% of the cases that develop encephalitis succumb to infection with symptoms of encephalitis including altered mental status, weakness, and movement disorders. Poliomyelitis, the most severe form of the disease, is often accompanied by abrupt and asymmetric weakness or paralysis and a case fatality rate of 10-50%. Unfortunately, the symptoms of WNND commonly persist as neurologic sequelae in survivors.

Despite the wealth of knowledge about WNV-host interactions, the intrahost dynamics of WNV evolution are vastly understudied. This is a significant shortcoming because several studies demonstrated that the viral population structure can directly impact viral fitness and disease [131-134]. Since viral populations can be formed in virtually endless possibilities, experimentation is needed to define the important structures. Mouse models that were developed to dissect the role of specific IFN pathway components during WNV infection, such as *Rsad2*^{-/-} and [347] and *Ifnar*^{-/-} [352] knockout mice, can be used to uncover their role in intrahost WNV evolution. Temporal sampling of various tissues can reveal how the dynamic host responses and potential bottlenecks shape viral populations during systemic infection [376]. Together, the pressures within a host may create distinct viral subpopulations with specific properties that can aid in viral dissemination. Some of these factors may help highly virulent strains of WNV reach the brain and cause severe disease. Taken further, understanding the selective pressures imposed by components of the host IFN response and knowing why certain variants make it to the brain may aid the development of novel antiviral treatments.

Chapter 2: Experimental evolution of an RNA virus in wild birds: evidence for host-dependent impacts on population structure and competitive fitness

Introduction

RNA viruses pose some of the most complex, persistent and challenging problems facing public health and medicine. The ongoing outbreaks of avian influenza A(H7N9) virus (*Orthomyxoviridae*) in China [377], Ebola virus (*Filoviridae*) in West Africa [378], and chikungunya virus (CHIKV, *Togaviridae*, *Alphavirus*) and West Nile virus (WNV, *Flaviviridae*, *Flavivirus*) in the Americas [13,15] highlight the health and societal impacts imposed by RNA virus-induced diseases. Several factors contribute to the emergence of these agents and the continued burdens they impose on human health. Among these is their ability to undergo rapid evolution in new and/or changing environments. Well documented examples of RNA virus evolution leading to increased virus transmission include WNV and CHIKV. In both cases, small, conservative amino acid substitutions (residues with similar physiochemical properties) to the viral envelope proteins resulted in more efficient transmission by mosquito vectors [102,113]. Adaptive changes to RNA virus genomes first arise as minority components within a genetically complex population of related but non-identical virus variants. The genetic diversity present in naturally occurring RNA virus populations has been clearly shown through a large and expanding body of observational and experimental studies to be critical to their biology. For example, several studies have demonstrated that the diversity of an intrahost viral population, rather than the fitness of individual variants, correlates with pathogenesis, disease progression and therapeutic outcome [132,379,380]. Moreover RNA viruses have the capacity for rapid

evolutionary change because within infected hosts, all single nucleotide mutations may be generated.

This has been particularly clear in the case of WNV, an arthropod-borne virus (arbovirus) that persists in nature in enzootic cycles between ornithophilic mosquitoes (mainly *Culex* spp.) and birds. After its initial identification in the New York City area in 1999, WNV spread throughout the continental United States, producing the largest outbreaks of flaviviral encephalitis ever recorded in North America. The explosive spread of the virus was accompanied by the displacement of the introduced genotype by a derived strain that is more efficiently transmitted by local *Culex* mosquitoes [105]. Studies of intrahost population dynamics of WNV demonstrated that genetic diversity is greater in mosquitoes than in birds [131]. The selective basis for the host-specific patterns of WNV genetic diversity is that the strong purifying selection that predominates in birds is relaxed in mosquitoes [131,137]. In addition, the RNA interference-based antiviral response in mosquitoes creates an environment where negative frequency-dependent selection may drive rare variants to higher population frequency [164]. Moreover, WNV maintains both adaptive plasticity and high fitness by alternating between hosts that impose different selective forces on the virus population [141].

Nonetheless, important gaps remain in our understanding of how error-prone replication interacts with selective and stochastic reductions in viral genetic diversity under natural conditions. This is particularly the case for arboviruses, which tend to cause acute infection in vertebrates, with transmission occurring before the development of a neutralizing antibody response. Therefore, well-described mechanisms of immune selection such as those that occur during chronic hepatitis C and human immunodeficiency virus infections are comparatively weak during acute arbovirus infection of vertebrates. Thus, the ways that ecologically relevant,

natural hosts can influence arbovirus genetic diversity remain poorly understood. WNV in particular provides an excellent experimental system to study the influences of natural vertebrate hosts on viral evolution. The virus infects a large number of wild bird species [177] with a wide-range of infection outcomes [176]. In addition, several studies have provided evidence that particular WNV variants may arise through adaptation to birds [106,331].

Therefore, we sought to determine whether different wild bird species may have distinct impacts on WNV population structure. Specifically, we allowed WNV to replicate in wild-caught American crows (*Corvus brachyrhynchos*), house sparrows (*Passer domesticus*), and American robins (*Turdus migratorius*), bypassing the mosquito portion of the arbovirus cycle in order to focus on the impact of different vertebrate environments on virus populations during acute infection. Virus was passaged in individuals of each species five times in order to amplify host-specific patterns of selection that may remain cryptic after a single passage. Bird species were selected on the basis of ecological relevance and resistance to WNV-induced mortality. American crows experience high viremia and mortality following inoculation with WNV [321] and can directly transmit virus to roost mates without mosquito involvement [381]; house sparrows experience high viremia and intermediate mortality [382] and are frequently involved in WNV perpetuation [383]; and American robins experience intermediate viremia but very low mortality [328] and can be drivers for human WNV risk [229]. Virus populations were characterized using next generation sequencing (NGS) and through *in vivo* fitness competition studies in birds and mosquitoes. Our findings demonstrate that relevant vertebrate hosts with varying levels of disease susceptibility differentially shape WNV population structure with direct impacts on fitness during host shifts.

Materials and methods

Ethics statement

Wild birds were collected from under US Fish and Wildlife Service (#MB91672A-0) and Colorado Parks and Wildlife (#13TRb2106) permits and with permissions from landowners. No endangered or protected species were caught or harmed during the study. Experiments involving animals were conducted in accordance with protocols approved by the Colorado State University (CSU) Institutional Animal Care and Use Committee (#12-3694A) and the recommendations set forth in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Serial passage of WNV in wild-caught birds

A WNV infectious clone (WNVic) was previously constructed from an American crow kidney isolate collected during the 2000 outbreak in New York City [99,384]. The WNVic contains a naturally selected proline at amino acid site 249 in nonstructural protein 3 (NS3) allowing it to replicate to high titers in wild birds [106,109]. Wild birds were collected in Northern Colorado from 2013 to 2014 using mist nets (house sparrows and American robins) and cannon nets (American crows). All birds were bled prior to inoculation and serum was tested by plaque reduction neutralization test to confirm that all birds used for subsequent studies were WNV seronegative. The virus strain used to initiate the passage series was derived from a WNVic as previously described [384]. Virus was harvested from the supernatant of BHK cells transfected with linearized plasmid, stored at -80°C and used without further passage. Viruses were administered to birds by subcutaneous inoculation to the breast region with 1,000 WNV plaque forming units (PFU)/100 µl, a dose similar to mosquito transmission [291], in inoculation

medium (endotoxin and cation-free phosphate buffered saline with 1% FBS). Birds were bled from the jugular vein at the time of peak viremia on 3 days post-infection (dpi). Serum was titered by standard plaque assay on African green monkey kidney cells (Vero, ATCC CCL-81) and stored at -80°C until used for subsequent passage or sequencing as described below. The first passage series utilized seven birds for each wild-caught species and the three birds with the median viral titers were used to start three independent replicate lineages, each including three naïve birds (i.e. replicates 'a', 'b', and 'c'). From each group of three birds, the serum with the median viral titer was used to continue passaging to another cohort until five serial passages were completed. The WNVic derived virus was also passaged once in three young chickens for 3 dpi and two individual *Cx. quinquefasciatus* mosquitoes for 14 dpi to compare viral populations from commonly used laboratory vertebrate host and invertebrate vector models, respectively.

Wild-caught birds were housed in 0.5 to 1m³ cages in groups of 3-4 with space for limited flight (sparrows and robins) and fed *ad libitum* water and a mixture of dry dog food (crows and robins), raisins (robins), earth worms (robins) and/or bird seed (sparrows) as described previously [321,328,382,385]. Chickens (two-days old) were hatched from specific pathogen free eggs (Charles River Specific Pathogen Free Avian Services, Franklin, CT) and maintained as described previously [131]. *Cx. quinquefasciatus* mosquitoes were reared from a long laboratory-established colony. Mosquitoes were maintained at 26-27°C and 70-80% relative humidity with a 16:8 L:D photoperiod. Water and 10% sucrose was provided *ad libitum*. Adult mosquitoes used for experiments were 4-7 days post-emergence. All animal infections were conducted within the Colorado State University ABSL-3.

Phenotypic assessment

The infection phenotype of each WNV lineage after five passages (p5) in wild-caught birds was compared to the unpassaged (p0) WNV in the same bird species as virus passage, young chickens (two-days old), and *Cx. quinquefasciatus* mosquitoes (4-7 days post emergence). Viremia and survival was measured from birds were inoculated with 1,000 PFU of p5 or p0 WNV (n = 4-5 birds/virus) for up to 6 dpi. As defined here, competitive fitness compares the replication of a competitor virus (i.e. serial passaged p5 WNV) and a standard WNV reference (WNV-REF) during infection of the same host. Competitive fitness is quantified by the proportion of competitor to WNV-REF genotypes using sequence chromatograms (i.e. quantitative sequencing) [386]. The WNV-REF was created using site-directed mutagenesis [248]. Five sequential synonymous changes were made to nucleotide positions 8313-8317 in the NS5 region of the genome, changing the parental sequence CTC TCA CGG to CTa agc aGG. The non-coding changes to WNV-REF did not affect the replication kinetics and infectivity compared to the WNVic [171], making WNV-REF a useful standard to measure the fitness changes of WNVic after serial passage in birds [141,171].

Birds were co-inoculated with 1000 PFU of equally mixed WNV-REF and p5 competitor virus (n = 4-5 birds/competition) and serum was collected 3 dpi as described above. *Cx. quinquefasciatus* mosquitoes were intrathoracically (IT) inoculated with 10 PFU/69 nl of equally mixed WNV-REF and p5 competitor virus (n = 40-60 mosquitoes/competition) using a Nanoject II (Drummond Scientific Company, Boomall, PA). Injected mosquitoes were held in quart-sized cardboard containers with water and 10% sucrose provided *ad libitum*. After 14 dpi, the mosquitoes were anesthetized with triethylamine and saliva was collected in capillary tubes for 30 minutes as previously described [387]. Individual whole mosquito bodies were homogenized

in 100 µl of cell culture medium (Eagle's minimum essential medium, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, 50µg/ml gentamicin, 1x L-glutamine and 125ng/ml fungizone) and a ball bearing using a Mixer Mill MM300 (Qiagen, Valencia, CA) for 30 s at 24 cycles/s. The homogenates were clarified by centrifugation for 5 min at 10,000 × g. Additionally, 6 log₁₀ PFU/ml of crow p5 viruses mixed equally with WNV-REF was offered to mosquitoes in an infectious bloodmeal (n = 30-60 mosquitoes each). Mosquito midguts, legs/wings and saliva were collected from individual mosquitoes at 14 dpi and homogenized. Total RNA was isolated from 50 µl of bird serum, mosquito tissue homogenates and mosquito saliva using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek, Norcross, GA) on the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA), according to manufacturer's protocols, and RNA eluted in 50 µl of nuclease-free water.

Sequencing and data analysis

Viral RNA was extracted from 50 µl of bird serum or mosquito body homogenate as described above with the addition of 1 µl of the RNase inhibitor SUPERase-In (Ambion, Austin, TX) and 0.5 µl of linear polyacrylamide (Ambion) added to the RNA followed by Turbo DNase treatment (Ambion). Total RNA was amplified using the NuGEN Ovation RNA-Seq System V2 (San Carlos, CA) and cDNA amplicons were sheared using the Covaris S2 Focused-ultrasonicator (Covaris, Woburn, MA) according to the manufacturer's recommendations. Sequencing libraries were prepared from 22-100 ng of sheared cDNA using NuGEN's Ovation Ultralow Library Kit according to the manufacturer's recommendations. Agencourt RNAClean XP beads (Beckman Coulter Genomics, Pasadena, CA) were used for all purification steps. WNV genome equivalents (GE)/ml concentrations were quantified following 1) RNA extraction,

2) DNase treatment and 3) RNA-Seq cDNA synthesis steps by qRT-PCR using the iScript One-step RT-PCR Kit for probes (Bio-Rad Laboratories Inc., Hercules, CA) and a previously described probe and primer set [388]. Briefly, 25 µl reactions were amplified using the CFX96 Real-Time instrument (Bio-Rad Laboratories Inc.), standard amplification conditions and WNV RNA standards prepared as previously described [167]. Finished libraries were analyzed for correct size distribution using the Agilent Bioanalyzer High Sensitivity DNA chips (Agilent, Santa Clara, CA). Deep sequencing was performed using the Illumina HiSeq 2000 platform at Beckman Coulter Genomics (Danvers, MA) and 20-30 uniquely barcoded libraries were multiplexed per lane.

Fastq files containing 100nt paired-end read data were demultiplexed using CASAVA and custom scripts that impose high stringency (0 mismatches) in the barcode region of each read. The sequence of the input WNV strain was determined from three independent biological sequencing replicates of the input virus using the Trinity assembler [389]. 100 nt paired-end reads were then aligned to this “input” sequence using MOSAIK [390]. Duplicate reads were removed using the MarkDuplicates tool within Picard to limit the influence of PCR artifacts and multiply sequenced clusters on variant calling with Vphaser2 [391]. Variants with significant strand bias were removed to reduce the potential for false-positives [392]. Variants called using Vphaser2 were used for subsequent data analysis unless otherwise specified. Analysis was limited to the protein coding sequences; and intrahost single nucleotide variants (iSNVs) and intrahost length variants (iLVs, includes both insertions and deletions) were analyzed separately.

Hamming distances from the p0 “input” virus were calculated for each population by dividing the total number of polymorphisms by the average coding sequencing coverage. Mean

viral population complexity was calculated by the normalized Shannon entropy (S_N) at each site using the following equation [393]:

$$S_N = - \sum p_i (\ln p_i) + (1 - p_i) (\ln (1 - p_i)) / \ln N,$$

where p is the frequency of the iSNV at site i and N is the coverage at that site. At a single nucleotide position, a S_N score of 0 indicates a single nucleotide was present (i.e. no polymorphism) while a score of 1 represents maximum complexity (i.e. equal numbers of alternate nucleotides). The S_N at all protein coding sequence nucleotides loci were averaged to estimate the viral population complexity.

High frequency iSNVs were subjected to an additional analysis to reduce the possibility that conclusions drawn from the complete dataset were dependent on extremely rare variants. To establish a threshold for “high frequency” iSNVs, all of the Vphaser2 accepted variants detected in this study ($n = 6052$) were $\log_{10}$ transformed, increased by 3.75 (to make all of the values positive) and fit to a gamma distribution, where $\alpha = \mu^2/s^2$ and $\beta = E[\mu]/s^2$, using R (data did not fit a beta distribution). An iSNV frequency >0.02 was determined to be in the upper 5% of the gamma distribution and was used to define high frequency SNVs detected through WNV passage in birds ($n = 341$ individual SNVs). The sequencing reads from p0, p1 and p5 were aligned to the WNV genome using *mpileup* from the VarScan2 software package [394] and haplotypes were reconstructed using QuasiRecomb 1.2 [395] with the flags ‘-r 97-10395’, to reconstruct haplotypes from the entire coding sequence with respect to reference genome numbering, ‘-K 1-10’, to use a bigger interval of generators and ‘-noRecomb’, to disable the recombination process because it was not expected from the viral population and to reduce the runtime. To increase haplotype specificity, the flag ‘-conservative’ was employed and analysis was restricted to haplotypes containing high frequency SNVs (i.e. >0.02).

The proportion of mutations in each population that were nonsynonymous (pN) and the ratios of nonsynonymous to synonymous variants per site (d_N/d_S) were used to test for intrahost selection [147]. DnaSP (version 5) [396] was used to determine the number of nonsynonymous and synonymous sites to calculate d_N/d_S using the Nei-Gojorori method [397] with the following modifications for NGS data. N_d and S_d (i.e. the numbers of detected nonsynonymous and synonymous mutations, respectively) were calculated for each viral population by the sum of individual nonsynonymous and synonymous VPhaser2 accepted iSNV frequencies and the passage consensus sequence was used to determine the number of nonsynonymous and synonymous sites. The number of nonsynonymous (7843.67) and synonymous (2455.33) sites in the ancestral p0 consensus sequence were used to determine that pN prior to selection is ~ 0.76 . In addition, 50 most frequent haplotypes reconstructed from p1 and p5 from each bird species were analyzed using the Fu and Li's F [398] and Fay and Wu's H [399] statistical tests of neutrality in DnaSP with a window length of 100, a step size of 25 and the p0 consensus sequence as an outgroup to infer the ancestral nucleotide state.

The standardized variance in iSNV frequencies (F_{ST}) was used to estimate the extent of interhost genetic divergence using a scale between 0 and 1, and the extent of F_{ST} change between populations represents the degree of genetic divergence. Specifically, in-house FORTAN scripts were used to calculate F_{ST} using equations 1, 2 and 4 by Fumagalli et al. [400]. Intrahost SNV frequencies determined by *mpileup* and *readcounts* from the VarScan2 software package [394] were used to estimate the per site heterozygosity in biological replicates compared to the total population (e.g. all biological replicates within passage) at a single passage (i.e. intra-passage) and the per site heterozygosity between passage replicates (i.e. inter-passage).

For estimation of the probability of resampling for the iLV data, we used the phyper command in R (www.R-project.org). We calculated that a total of 51,490 single nucleotide iLVs were possible by multiplying the length of the coding sequence (10,299 nt) by the 5 different kinds of iLVs that could occur at each site (one deletion and four different nt insertions). We then used phyper to obtain the probability of sampling overlap of 400 iLVs out of 600 sampled (reflecting a reasonable approximation of our observed data for crows) given that 51,490 iLVs are possible. Simulation studies were conducted in R by randomly sampling 600 individuals, with replacement, from a set of 51,490 and comparing the sets. T-tests, Kruskal Wallis tests, and correlation statistics were obtained using R and GraphPad Prism (La Jolla, CA).

Results

Virus passage and phenotypic assessment

The WNV used in these studies was derived from an infectious clone of the NY99 genotype and is described in detail elsewhere [384]. Clone-derived WNV was passaged five times in wild-caught American crows, house sparrows and American robins. To avoid systematically selecting high- or low-replicating strains and population bottlenecks during passage, and since titers are highly variable in wild-caught birds, the sera from the individuals with the intermediate viral load were passed into the next cohort at a standard dose of 1000 PFU. Virus titer was variable but did not change significantly or consistently during the course of passage (**Figure 2.1A**). Further, five passages in wild birds did not alter viremia production or mortality in crows and sparrows (**Figure 2.2A-B**). WNV replication and fitness after passage was assessed using young chickens and *Culex quinquefasciatus* mosquitoes to directly compare the viral populations in hosts not used for passaging and to remove the variability of wild-caught

birds (e.g. age and infection history) (Fig 1B-1C). Passaged virus (p5) was similar to the WNVic (p0) in peak viremia production in chickens (i.e. at 2 and 3 dpi) (**Figure 2.1B**).

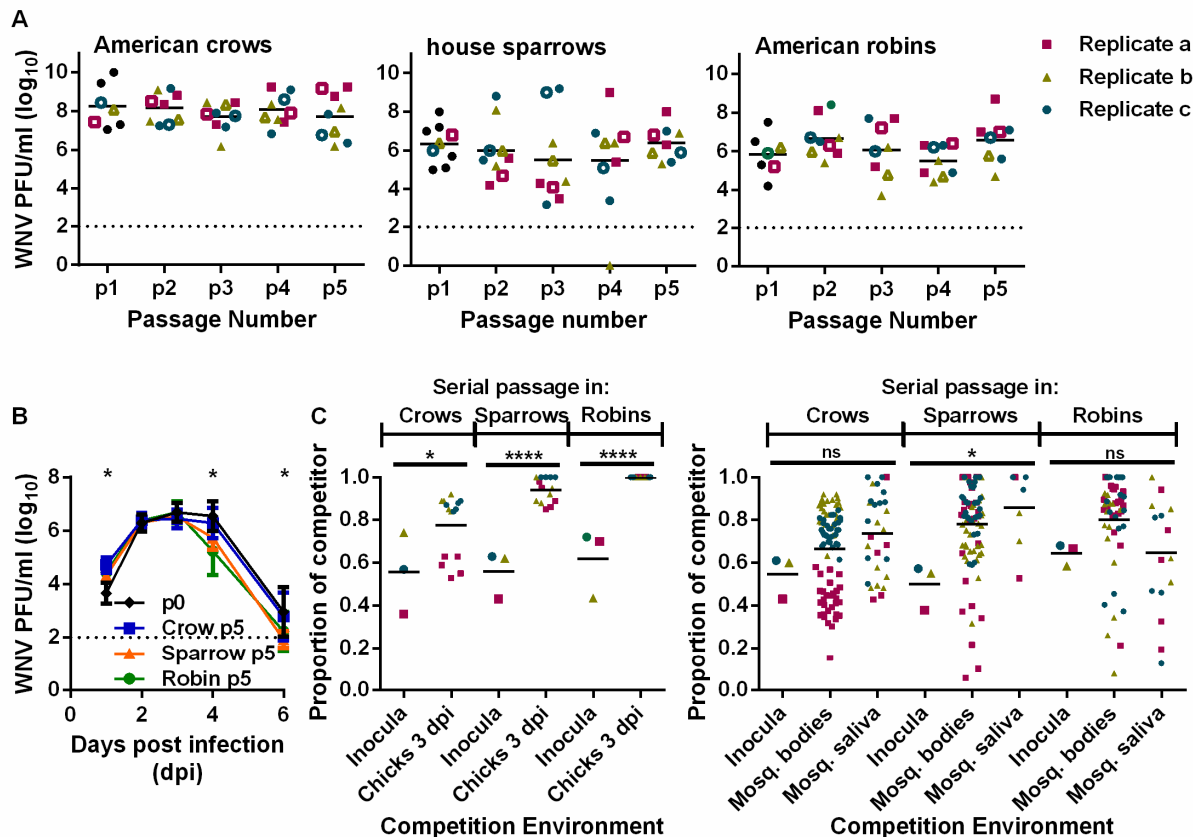


Figure 2.1. Passage of WNV in birds results in competitive fitness increases while viremia remains unchanged. (A) WNV titers during passage. Open symbols represent samples with median viremias that were used for subsequent passage. (B) Viremia production after sequential passage, measured in young chickens (mean $\pm$ SD, $n = 12-15$ chickens each, data from passage replicates combined, *, $P < 0.01$, two-way ANOVA with Tukey's correction). Dashed lines indicate the assay detection limits. (C) Competitive replicative fitness in young chickens (left; *, $P = 0.0339$; ****, $P < 0.0001$, unpaired t-test) and mosquitoes (right; ns, not significant; *, $P < 0.05$, unpaired t-test for both bodies and saliva). Passage replicates are colored as in (A) and horizontal lines represent the mean proportion of bird-passed WNV. Phenotypic assessment of wild bird passaged virus in its passaged host and in orally infected mosquitoes are in **Figure 2.2**.

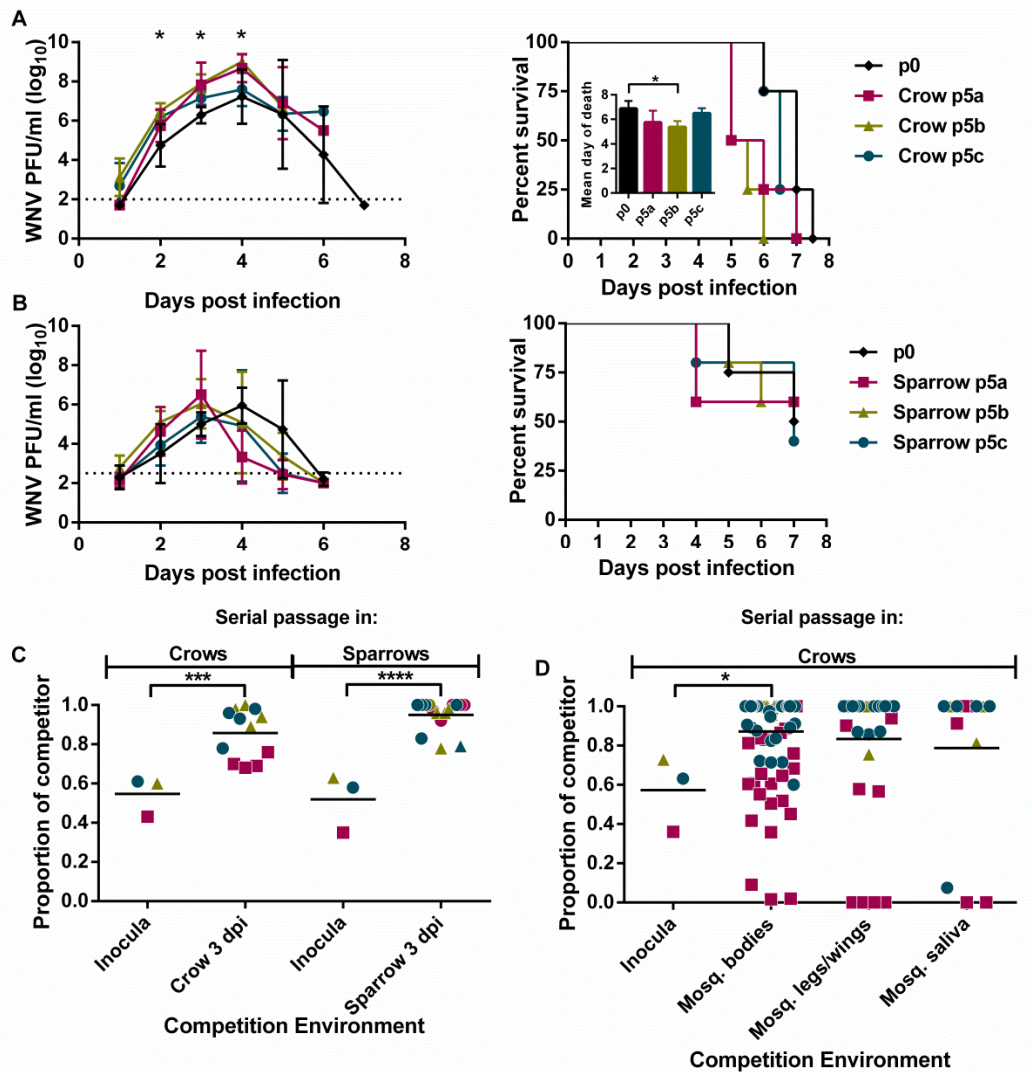


Figure 2.2. Phenotypic analysis in wild-caught birds and bloodfed mosquitoes. (A) Wild-caught crows infected with crow-passaged WNV ($n = 4$ each) were assessed daily for viremia production (represented as the mean $\pm$ SD of WNV plaque forming units [pfu]/ml of serum) and survival were compared by two-way ANOVAs with Tukey's corrections for multiple comparisons. Left: *, crow-passage 5 replicate "b" (p5b) vs p0 at 2, 3 and 4 days post infection (dpi), $P = 0.0206$, 0.0382 and 0.0185 , respectively. Right inset: *, p5b vs p0, $P = 0.0309$. However, this significance is likely due to lower than expected WNVic p0 virus titers ($100\times$ lower at 3 dpi than the first passage of WNVic shown in Figure 2.1). Crow passages p5a and p5c did not lead to significant differences in viremia and survival. Due to decreasing sample sizes caused by mortality, analysis was limited to 1-4 dpi. The dashed lines indicate the assay detection limits. (B) Wild-caught sparrows infected with sparrow-passaged WNV were assessed as described in (A) and led to no significant differences in viremia and survival compared to p0 viruses ($n = 4$ each). (C) WNV competitive fitness was measured by the change of mean proportion of the competitor (i.e. p5 WNV) from the inocula compared to after 3 days post infection (dpi) in crows and sparrows by unpaired t -tests (crows, $P = 0.0017$; sparrows $P < 0.0001$). The biological replicates are shown as magenta squares (replicate 'a'), yellow triangles

(replicate 'b') and teal circles (replicate 'c'). **(D)** Crow p5 WNV competitive fitness in *Culex quinquefasciatus* mosquitoes 14 dpi by oral inoculation was determined as described in **(C)** (bodies, $P = 0.0258$; legs/wings and saliva, not significant).

Fitness assays were used to directly compare passaged viruses to a standard reference WNV in head-to-head competition. These assays can detect subtle fitness differences that are inapparent in comparative studies. Competitive fitness of all wild-bird p5 WNV was significantly enhanced in chickens. Crow-passaged virus had the smallest fitness gains and robin-passaged virus the largest (**Figure 2.1C**). Fitness studies conducted in wild birds produced the same results as those in chickens (**Figure 2.2C**). Competitive fitness was slightly increased in mosquitoes, but no bird-specific differences were noted (**Figure 2.1C**, **Figure 2.2D**).

Patterns of intrahost mutational diversity

At each passage virus was examined by NGS to determine whether the consensus sequence changed during passage and to characterize the diversity of intrahost viral populations (**Figure 2.3**). WNV genome coverage was variable across the genome and between samples (**Figure 2.3A**), and positively correlated with viral population size (**Figure 2.3C**). The lower relative WNV genome coverage from robin sera can in part be explained by smaller intrahost viral population sizes and smaller virus to host RNA ratios. Approximately 68%, 29% and 7% of NGS reads aligned to the WNV genome from crow, sparrow and robin sera, respectively. Comparatively, 20% and 0.5% of the NGS reads aligned to the WNV genome from chicken sera and mosquito bodies, respectively.

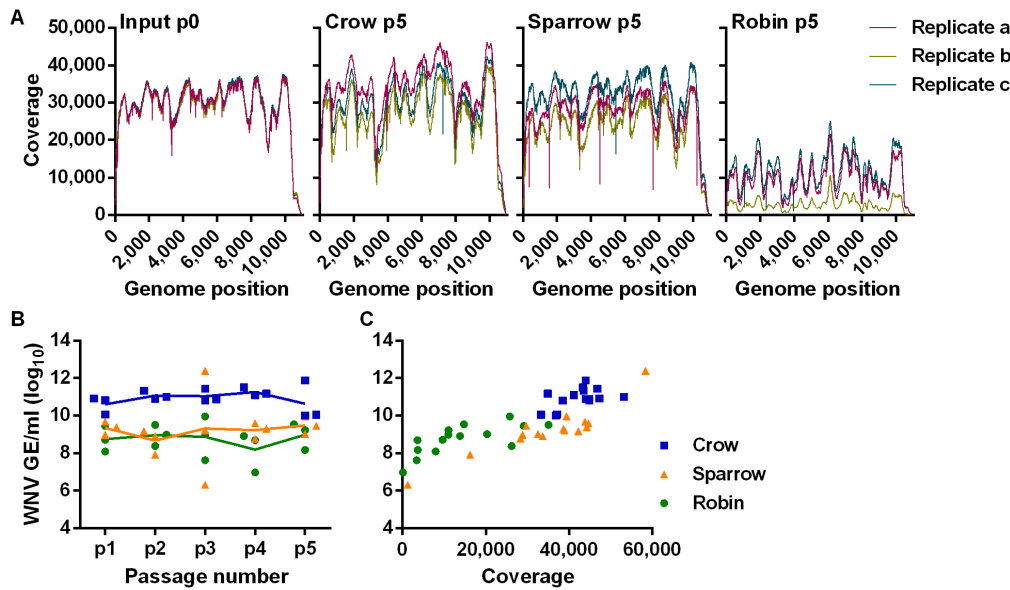


Figure 2.3. Sequencing coverage of the virus genome is correlated with intrahost virus population sizes. (A) WNV sequencing coverage plotted by genome position for the input virus used to initiate passaging (sequenced as technical replicates, p0a-p0c) and after five passages in wild-caught crows, sparrow and robin viruses (sequenced as biological replicates, p5a-p5c). (B) Intrahost virus population sizes measured by genome equivalents (GE)/ml of bird serum after each sequential passage. (C) Correlations of sequencing coverage of the WNV genome to the intra-host virus population sizes from each bird species using individuals were made by the Pearson's correlation coefficient (crows, $r = 0.5249$, $P = 0.0445$; sparrows, $r = 0.9145$, $P = <0.0001$; robins $r = 0.7041$, $P = 0.0034$).

Three nucleotide mutations that led to consensus amino acid substitutions were detected though passaging in birds, but none became fixed (i.e. frequency = 1) in the population. In contrast, three consensus amino acid substitutions were detected after a single mosquito passage.

We estimated intrahost variation from NGS data to determine whether WNV population diversity was bird species-dependent. The mean number of unique iSNVs in each virus population was relatively constant between passages, but differences were apparent among bird species (**Figure 2.4A**). WNV populations passaged in crows five times (p5) had significantly more unique iSNVs than WNV passaged in sparrows and robins. In addition, the frequency of individual iSNVs increased during passage in a species-dependent manner: The mean iSNV

frequency after p5 in robins was significantly higher than after p5 in crows or sparrows (**Figure 2.4B**). Despite these differences, the viral populations had similar S_N , Hamming distances (i.e. SNVs per coding sequence) and amino acid substitutions per coding sequence after p5 in different species (**Figure 2.4C**).

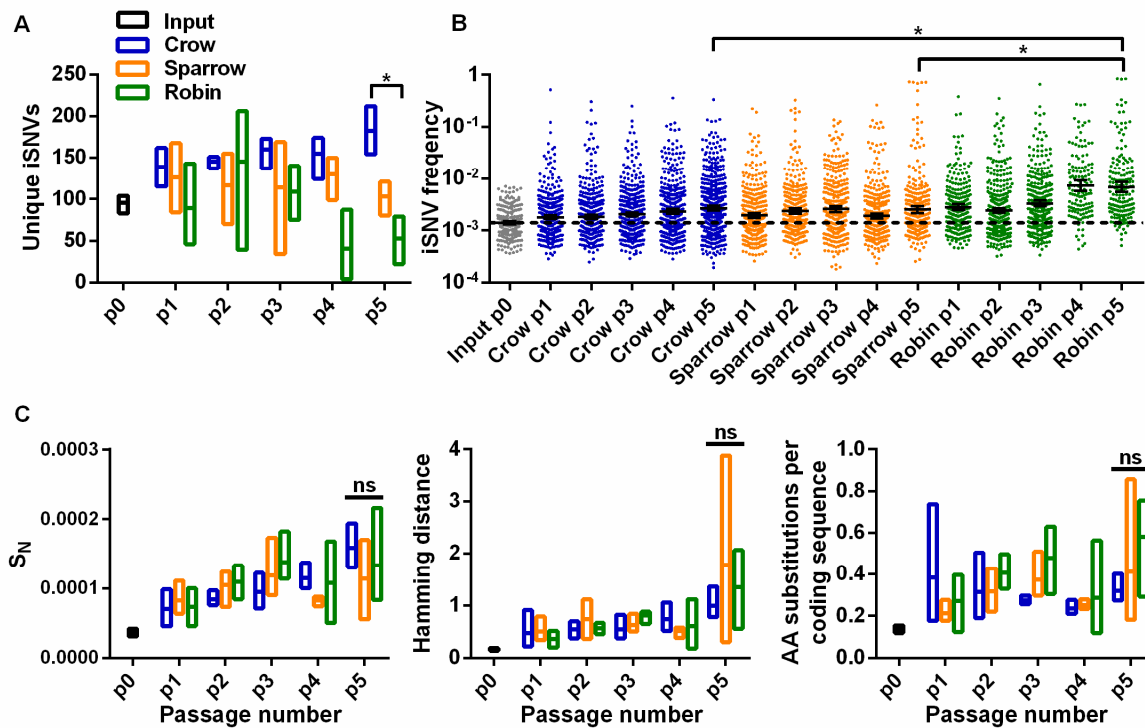


Figure 2.4. Disparate adaptive routes in birds lead to similar overall intrahost population complexity and diversity. The number (A, mean $\pm$ range) and frequency (B, geometric mean $\pm$ 95% CI) of unique intrahost single nucleotide variants (iSNVs,) from the WNV coding sequence during passage in wild-caught crows, sparrows or robins (*, $P < 0.05$, Kruskal-Wallis test with Dunn's correction). (C) Mean ($\pm$ range) normalized Shannon entropy (S_N , measure of population complexity) (left), Hamming distance from the p0 consensus sequence (SNVs per coding sequence) (middle) and the number of amino acid (AA) substitutions per coding sequence (right) (ns, not significant).

We examined the ratio of viral GE to PFUs and intrahost single nucleotide length variants (iLVs, including both insertions and deletions) to assess defective viral genomes in WNV populations during passage. Crow-passaged WNV had the highest GE:PFU ratio (**Figure 2.5A**) and the most unique iLVs (**Figure 2.5B**). In addition, a greater proportion of the iLVs in crows

were found in subsequent passages compared to sparrows and robins (**Figure 2.5C**). The number of iLVs per coding sequence was positively correlated with the titer of infectious virus (**Figure 2.5D**). We then evaluated the possibility that greater levels of iLV carry through in crows, which can only occur via complementation (**Figure 2.5C**), were due to sampling artifacts. To do this, we used a hypergeometric test implemented in R that indicated that selecting 400 common iLVs in two samples of 600 from the total pool of available single-nucleotide iLVs ($n=51,490$) was 0. Simulation studies confirmed that it is extremely unlikely that random sampling produced the observed data.

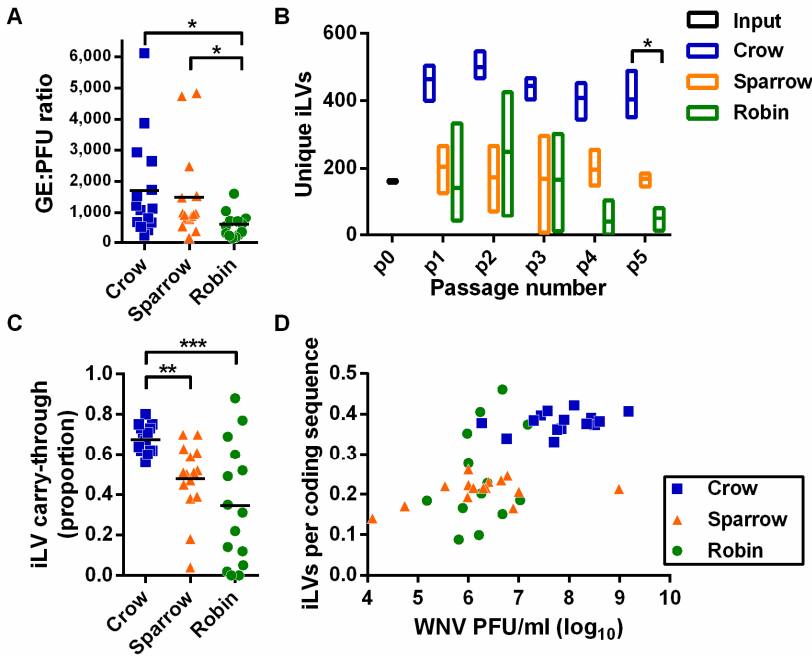


Figure 2.5. Intra-host virus population density contributes to the accumulation of deleterious mutations. (A) WNV genome equivalent to plaque-forming unit (GE:PFU) ratio from all bird passages ($n = 15$ per species; *, $P < 0.05$, Kruskal-Wallis test with Dunn's correction). (B) Unique intra-host length variants (iLVs, i.e. single nucleotide insertions and deletions, mean $\pm$ range) from the WNV coding sequence (*, crow p5 vs robin p5, $P = 0.0219$, Kruskal-Wallis test with Dunn's correction) and (C) proportion of unique iLVs detected in the subsequent replicate series passages (e.g. carry-through from p1a to p2a) calculated after each passage (**, $P = 0.0084$; ***, $P = 0.0005$, Kruskal-Wallis test with Dunn's correction). (D) Correlation of virus population sizes (PFU/ml) to the number of iLVs per coding sequence from each individual (Pearson $r = 0.6150$, $P < 0.001$).

Intrahost selective pressures

Evidence for natural selection was assessed in WNV populations using intrahost neutrality tests. The pN and d_N/d_S ratios were the highest in the input p0 WNV population and decreased significantly during passage in each bird species (**Table 2.1**). Separate analysis of d_N and d_S shows that d_N did not significantly increase during passage while d_S increased significantly at p5 in all bird species, a hallmark of purifying selection. The Fu and Li's F and Fay and Wu's H statistics were obtained from reconstructed haplotypes. The F statistic at p1 and p5 was consistently negative, indicating that the haplotypes contained excessive amounts of rare SNVs, again indicative of purifying selection (**Table 2.1**). The H statistic measures an excess of high compared to intermediate frequency SNVs. The insignificant H values suggest that the deviations from neutrality were due to natural selection rather than selective sweeps (**Table 2.1**). Analysis of reconstructed haplotypes that arose during passage and high frequency iSNVs (i.e. frequency > 0.02) was conducted to minimize the impact of differences in sequencing coverage and to assess positive selection. 0.02 was selected as a cutoff for "high frequency" mutations because it includes the top 5% of a gamma distribution of all VPhaser2-accepted iSNVs. The proportion of iSNVs that were high frequency after p5 was the greatest within robin-passaged WNV populations (16.5%) compared to sparrows (4.9%) and crows (4.8%) (**Figure 2.6A**).

Reconstructed haplotypes from high frequency iSNVs were then used to assess the selective pressures that lead to haplotype replacement during passage (**Figure 2.6B**). The ancestral p0 virus population was composed of a single dominant haplotype that remained dominant after a single passage in all bird species. After p5, the ancestral haplotype remained dominant in crows, but not in sparrows and robins. Furthermore, high frequency iSNVs from crows contributed significantly fewer amino acid substitutions per coding sequence compared to

robins after p5 (**Figure 2.6C**). Examination of d_N/d_S , amino acid diversity and high frequency nonsynonymous iSNVs across the WNV genome demonstrated that, in general, selection was the strongest in the structural protein coding regions (**Figure 2.6D-E**). Specifically, passage in robins imposed significant selective pressures on the envelope (E) protein coding region that heavily targeted ectodomains (ED) I and II. The apparent selection of the nonstructural protein 4B (NS4B) from sparrow passaging is the result of a single high frequency nonsynonymous iSNV. Individual high frequency iSNVs fluctuated in frequency through passaging and all nonsynonymous high frequency iSNVs were unique to its passage lineage (i.e. no “signature mutations” were detected that served as markers for replication in any particular bird species).

Table 2.1. Intrahost tests of neutrality.

Passage	pN	d_N/d_S	d_N	d_S	Fu and Li's F	Fay and Wu's H
p0	0.84	1.57	2×10^{-5}	1×10^{-5}	NA	NA
Crow p1	0.80	1.22	5×10^{-5}	4×10^{-5}	-0.62029	0.36792
Crow p5	0.35*	0.18*	4×10^{-5}	$3 \times 10^{-4*}$	-4.17419**	0.77866
Sparrow p1	0.45	0.25	3×10^{-5}	1×10^{-4}	-1.83693	0.4298
Sparrow p5	0.33*	0.23*	6×10^{-5}	$6 \times 10^{-4*}$	-1.16389	1.87529
Robin p1	0.73	0.84	4×10^{-5}	4×10^{-5}	-0.83631	0.22204
Robin p5	0.46*	0.25*	9×10^{-5}	$4 \times 10^{-4*}$	-1.21878	1.85143

*, $P < 0.05$, compared to p0 by the Kruskal-Wallis test with Dunn's correction for multiple comparisons.

**, $P < 0.02$, critical values compared by two tailed tests in DnaSP.

NA, not applicable because there was only one dominant haplotype.

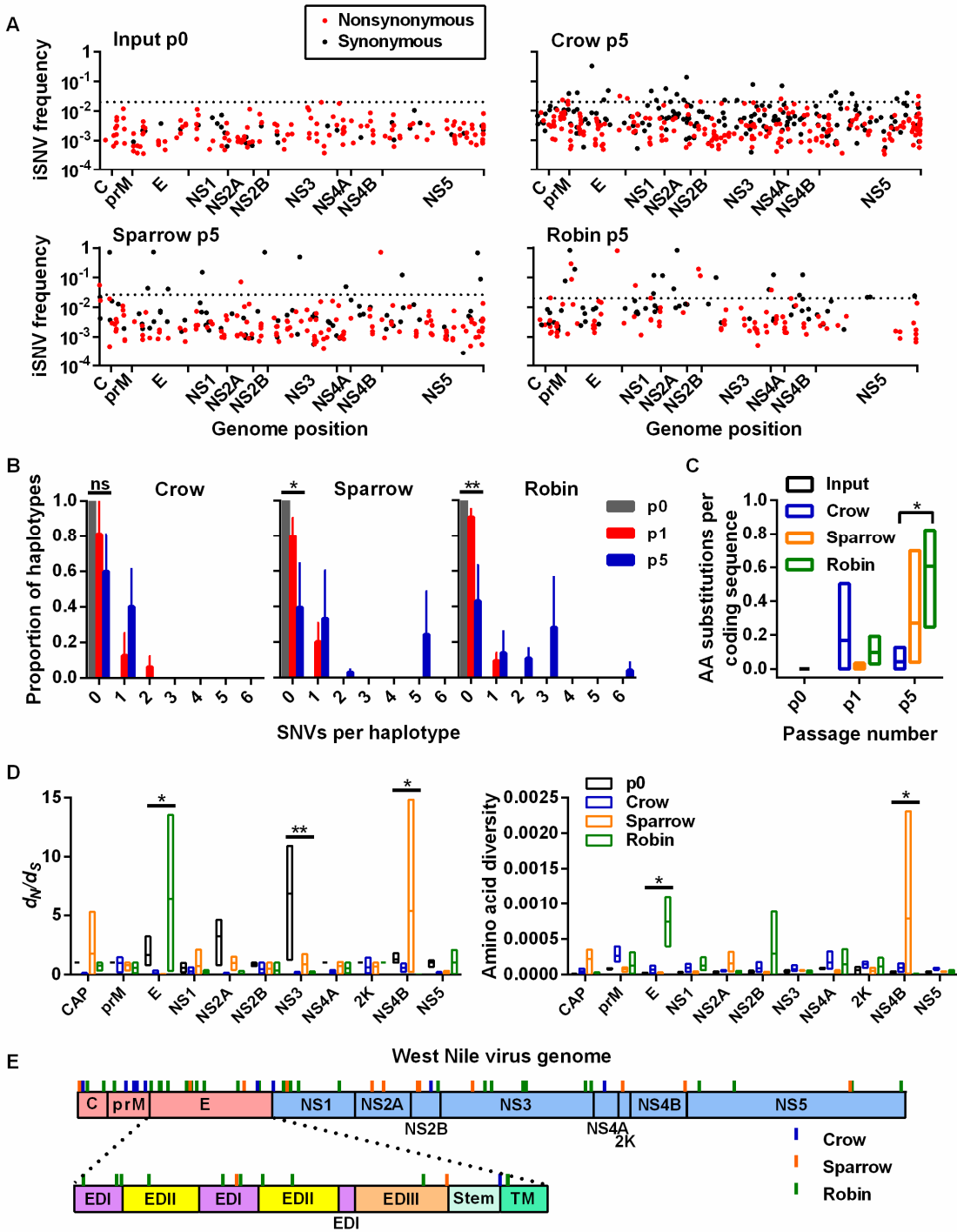


Figure 2.6. High-frequency iSNVs contribute to haplotype displacement in a bird-species dependent manner. (A) iSNVs from input virus (p0) and after passage 5 (p5, all replicates combined) plotted according to genome position. Red and black dots represent synonymous and nonsynonymous iSNVs, respectively. Dotted line represents division between high and low frequency iSNVs (0.02). (B) Haplotypes were reconstructed from high frequency iSNVs represented by the number of SNVs per haplotype (i.e. Hamming distance from the p0 haplotype, $\pm$ SEM) (ns, not significant; *, $P = 0.0250$; **, $P = 0.0036$, Kruskal-Wallis test). (C)

Mean ($\pm$ range) number of amino acid (AA) substitutions per coding sequence from high frequency iSNVs at p5 in each bird species (*, crow p5 vs robin p5, $P = 0.0429$, Kruskal-Wallis test with Dunn's correction). **(D)** Mean ($\pm$ range) ratios of nonsynonymous to synonymous variants per site (d_N/d_S) (left) and amino acid diversity (right) from p0 and p5 for each WNV protein coding region. Left: * E protein, $P = 0.0284$; **, nonstructural protein 2A (NS2A), $P = 0.0064$; *, NS4B, $P = 0.0175$. d_N/d_S was set at 1 for replicates without synonymous single nucleotide variants (SNVs) and 0 without nonsynonymous SNVs in the coding region. Right: *, E, $P = 0.0284$; *, NS4B, $P = 0.0328$, Kruskal-Wallis test. **(E)** High frequency nonsynonymous iSNVs from all bird passages were plotted according to their position in the WNV genome.

Interhost genetic divergence

F_{ST} was then estimated from the coding sequence to determine the degree of genetic divergence among replicates within a passage and between passages (**Figure 2.7**). Viral populations from robins were more divergent compared to those from crows and sparrows. F_{ST} from WNV passaged once in young chickens was similar to wild-caught birds, but WNV passaged once in mosquitoes was much more divergent. These results are supported by analysis of haplotypes (**Figure 2.8**). The p0 haplotype was still dominant in chicken p1 populations with a small minority of haplotypes containing single iSNVs, similar to wild birds (**Figure 2.6B**). In mosquitoes the ancestral haplotype became a minority after a single passage.

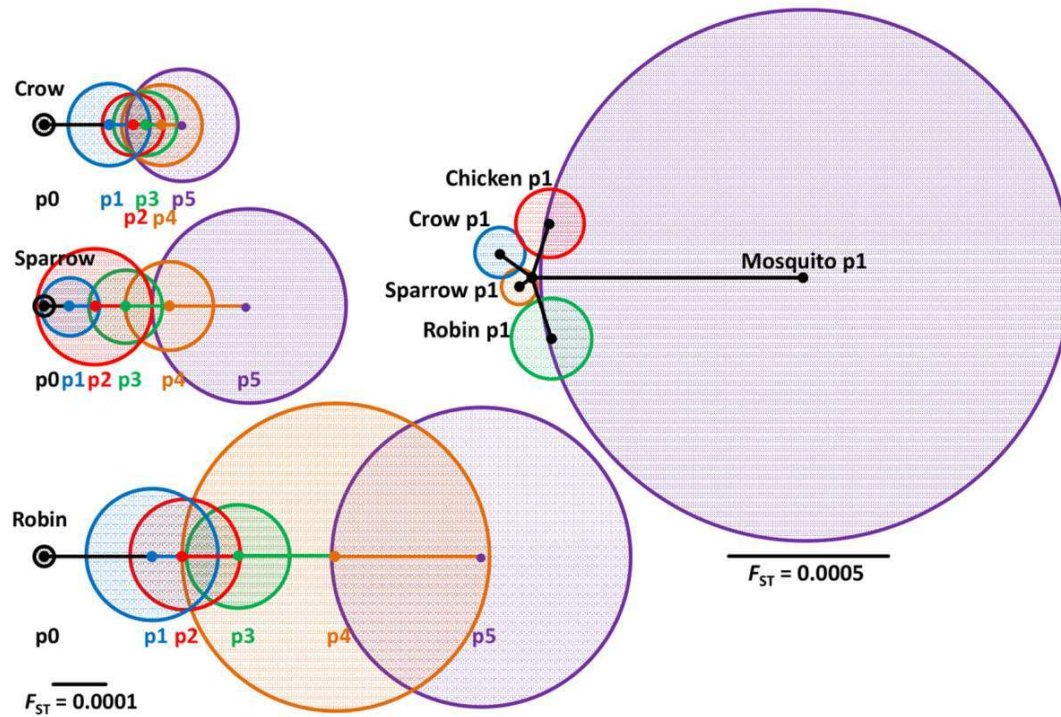


Figure 2.7. Differential interhost divergence of viral populations between individuals, sequential passage and host species. Circle diameters represent divergence (F_{ST}) between individuals within a passage. Lines connecting circle centers represent between-passage divergence and were measured using combined replicates. F_{ST} from replicate means at p5 among crows (2×10^{-4}), sparrows (4×10^{-4}) and robins (6×10^{-4}) were significantly different ($P = 0.0500$). F_{ST} was similar after a single passage in wild birds and chickens ($\sim 2 \times 10^{-4}$) and significantly different from F_{ST} after a single mosquito passage (2×10^{-3} , $P = 0.0174$, Kruskal-Wallis test).

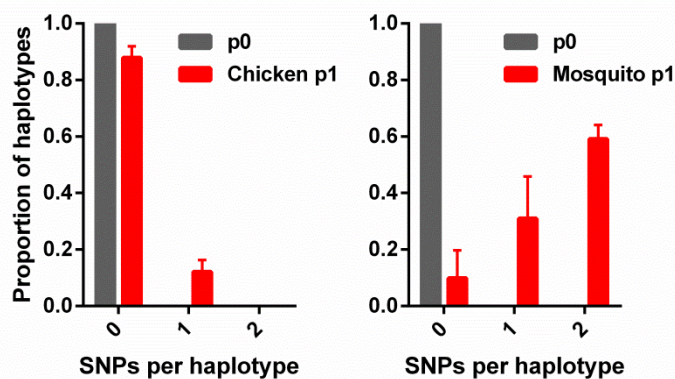


Figure 2.8. Haplotype reconstruction of viral populations passaged in chickens and mosquitoes. Haplotypes were reconstructed from the high frequency iSNVs (i.e. > 0.02) from input WNV and after one passage in young chickens and *Culex quinquefasciatus* mosquitoes and are represented by the number of single nucleotide variants (SNVs) per haplotype (Hamming distance from the p0 haplotype).

Discussion

Virus passage and phenotypic assessment

We examined WNV genetic diversity during the course of passage in birds that experience varying mortality due to WNV infection to assess how different hosts influence virus population structure and fitness. Passage in each host was accomplished in three concurrent biological replicates in order to control for the impact of individual wild-caught birds that may vary in several ways that could impact virus replication. Titers during passage were highly variable between individuals. However, mean titers did not significantly change during the course of passage, indicating that replication competence was retained and that overt increases in competitive fitness were not selected through our passage strategy.

Wild-bird passaged virus was similar to unpassaged WNV in viremia production. Only when more sensitive *in vivo* competitive fitness assays (i.e. comparative replication of the passaged and reference WNV in the same host) were conducted were changes apparent. Note that our definition of fitness here is restricted to the specific competition environment (within the bird or mosquito) and does not consider the larger ecological fitness required for maintenance in a complex arbovirus transmission cycle. Passage in all birds resulted in significant competitive fitness gains during replication in chickens. Interestingly, the fitness gains were smallest after WNV was passaged in the host that experiences the most mortality (crows), and largest in the most disease-resistant avian host (robins). Fitness gains were far less clear when virus competition was measured in mosquitoes. A limitation to our mosquito studies is that competition was conducted via intrathoracic inoculation, which bypasses the midgut, a major physiological barrier in mosquitoes. Intrathoracic inoculation was used because the volume of blood available and the virus titers would have likely made oral infection highly inefficient.

Importantly, our results on WNV replication and fitness are supported by previous observations [141] indicating that high fitness is maintained through purifying selection in vertebrates, and that no tradeoff occurs when the virus is re-introduced into mosquitoes. Moreover, replicative fitness increases occur during passage in ecologically relevant wild birds, and these gains occur in a species-specific manner.

Patterns of intrahost mutational diversity and selective pressures

To investigate the viral genetic and population determinants of the observed fitness gains, we characterized WNV at each passage using NGS. Our data suggests that although the overall complexity of the virus population was similar among different bird species, its composition, and the selective pressures that produced it appear to be bird species-dependent. Interestingly, WNV replication in the most disease-susceptible bird species seems to be positively associated with the number of unique iSNVs (i.e. mutational tolerance) and negatively associated with iSNV frequency (i.e. strength of selection). This observation requires further investigation using additional resistant and susceptible birds, but may provide important insights into which bird species are most likely to drive virus evolution toward fitness gains. Our data thus far suggests that more disease resistant birds such as robins would be most likely to fill this role as long as they produce sufficiently high titers to infect mosquitoes.

In this study we used various neutrality tests to determine whether intrahost WNV populations from each bird species were evolving non-randomly through purifying selection. While these tests all measure slightly different aspects of genetic diversity, all clearly demonstrate purifying selection in birds. This result confirms previous studies of WNV passaged in young chickens [131], and indicates that our approaches to sequencing and analysis, although

they differ significantly from those reported previously, produce results consistent with other methods.

Our studies also provide some evidence for positive selection during bird infection. We found that WNV passage in robins resulted in more amino acid substitutions that reach high frequency compared to crows. In addition, the ancestral haplotype tended to be displaced by novel mutants that arose during passage in sparrows and robins. These data suggest that positive selection within hosts is stronger in less susceptible bird species [107].

Examination of patterns of variation across the WNV genome provides additional evidence for differences in host selective environment. We found, consistent with previous reports on dengue virus populations [139], the highest variant frequencies in ectodomains I and II of the E coding sequence of WNV passaged in robins. The mechanisms that lead to the emergence of these variants are not currently clear. Although the E protein contains most neutralizing epitopes, the earliest neutralizing antibody responses observed in birds generally occur at around 5 to 7 days post infection [214,328]. Other mechanisms that could impact selection on the E protein include resistance to the early antiviral states induced by type I interferon [174,373] and alternate methods for virus entry and uncoating of the viral RNA [182]; though these mechanisms need further investigation, especially in birds. Our results suggest that in relatively resistant hosts, novel variants may rise to high frequency within the context of purifying selection. The notion that positive selection occurs in robins is further supported by our data showing that virus diverged most during replication in them. It is, however, balanced by a lack of evidence of a selective sweep, i.e. a rapid reduction in genetic diversity as a novel variant becomes very prominent in the population. Clearly further studies are needed to confirm whether and how positive selection contributes to WNV population structure in birds.

Defective genomes

Compared to other RNA viruses, arboviruses have low long-term rates of amino acid substitution [150]. This is at least partially due to the fact that most mutations are deleterious because of evolutionary constraints on arbovirus genomes [152]. We provide evidence that accumulation of deleterious mutations, or defective viral genomes, is unequal between hosts; WNV populations replicating in wild-caught crows accumulate the most defective genomes, and WNV replicating in robins accumulate the least. Defective genomes are often found during laboratory and natural virus infections [331,401] and can persist through multiple rounds of transmission [172,277]. Using both bioassays (i.e. GE:PFU) and sequencing data (i.e. iLVs per coding sequence), we found that the accumulation of WNV defective genomes during infection was positively correlated with viral load. This apparent density-dependent selection of deleterious mutations likely occurs via functional complementation, which becomes more efficient as effective multiplicity of infection (MOI, i.e. intrahost viral load) increases [274,275]. In addition, high MOI environments tend to tolerate neutral mutations that can become deleterious in a new environment [402]. Taken together, these studies provide a framework to understand how WNV replication in high-viremic crows leads to a broader network of potentially deleterious mutations and limited selection for adaptive amino acid substitutions, especially when compared to WNV replication in robins. The rather modest fitness gains experienced by crow-passaged WNV support this observation.

Conclusions

The results presented here shed light on the selective forces that shape WNV populations in nature. We demonstrate that selective pressures that control WNV populations seem to occur

in a species-specific manner (**Figure 2.9**). All three bird species evaluated have been suggested to be significant drivers of WNV outbreaks, with robins receiving particular attention due to findings indicating that this species is more frequently fed upon by mosquito vectors [229]. During intrahost WNV replication, our studies suggest that disease-susceptibility is positively associated with mutational tolerance and negatively associated with the strength of selection. This means that robins also may better maintain high fitness in WNV populations than do birds that are more susceptible to disease. While it is tempting to speculate that robins are significant generators of WNV genetic diversity, we also confirm herein that mosquitoes are much more efficient in generating mutational diversity in the WNV system. Moreover, these data suggest that intrahost virus evolutionary dynamics are associated with host resistance to disease in several ways and provide an important insight towards the genetic and ecological factors that influence RNA virus emergence.

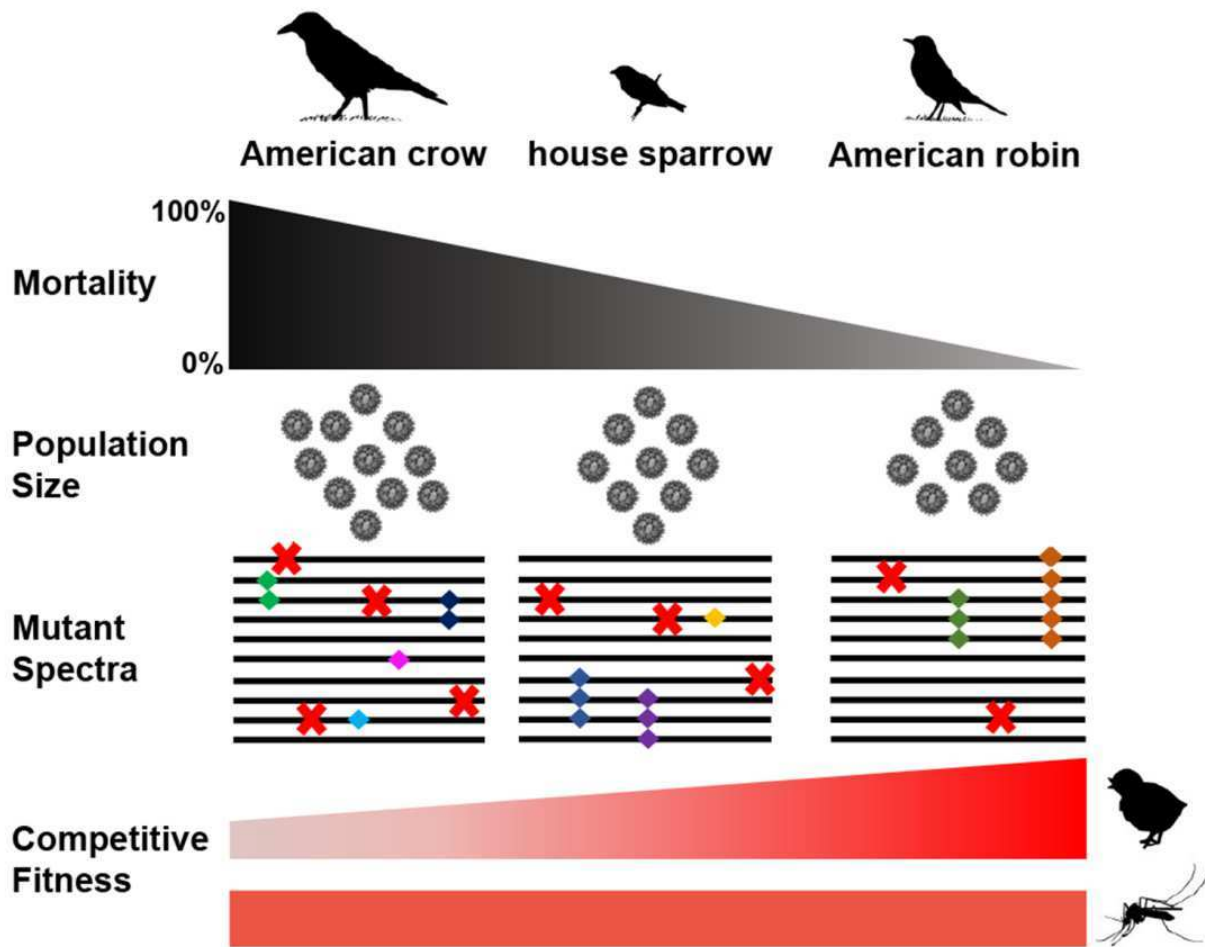


Figure 2.9. Species-specific composition of WNV populations and competitive fitness. Host mortality and intrahost WNV population sizes are associated with WNV population structure and competitive fitness. The WNV populations from all bird species contain ~1 mutation per genome. However in the crow environment, WNV populations are more tolerant of unique and deleterious mutations (e.g. insertions and deletions), but few mutations rise to high frequency. In the most disease-resistant bird species, robins, the WNV populations are under stronger selection pressures. Robin-associated WNV populations are less tolerant of unique and deleterious mutations, and more mutations reach high frequency. The selective environment of more disease-resistant birds was also positively associated with competitive fitness in young chickens, but not in mosquitoes. Population size: each “virus” represents a $\log_{10}$ of GE/ml. Mutant spectra: “X” represents deleterious mutations, “diamonds” represent neutral or advantageous mutations, and diamonds of the same color represents the same mutation.

Chapter 3: Genetic drift during systemic arbovirus infection of mosquito vectors leads to decreased relative fitness during host switching

Introduction

The emergence of arthropod-borne RNA viruses (arboviruses) is an ongoing problem that imposes significant health and economic burdens on communities worldwide. West Nile (WNV), chikungunya (CHIKV), dengue (DENV), and Zika viruses are all in various states of emergence at local or global scales [13-15]. The mechanisms underlying arbovirus emergence are complex and include, for example, altered land use and increased global travel. In addition, RNA viruses have an inherent ability to rapidly mutate and thus generate opportunities for adaptation in novel environments through an error-prone polymerase [50]. WNV is an excellent example of an introduced RNA virus that adapted to a new environment (i.e. new genotype linked to a shorter extrinsic incubation period in local mosquitoes), promoting its spread throughout the Americas [103]. Several studies have assessed how different host types impact WNV population structure, and have shown that WNV populations are more diverse in mosquitoes compared to birds [131,137]. In mosquitoes, purifying selection is weak and virus diversification is driven by the action of RNA interference (RNAi), which creates an intracellular milieu that favors rare genotypes [164,165]. In contrast, purifying selection in birds is strong and the innate antiviral responses are dominated by type I interferon [161]. Thus, in the WNV system, mosquitoes and birds have distinct impacts on virus population biology: Mosquitoes allow for increased adaptive plasticity, while birds maintain high fitness through purifying selection [141].

A wide array of studies has suggested that arboviruses can adapt to microhabitat-specific conditions. CHIKV is capable of adapting to transmission by *Aedes albopictus* during a single

round of infection [117]. WNV evolutionary dynamics have been shown to vary in response to environmental conditions [201,202]. Indeed, different avian hosts of WNV have distinct impacts on virus population structure and fitness (Chapter 2). The impacts of different mosquito species on WNV population biology and fitness, however, have not been directly addressed. This is a critical shortcoming in the field because throughout its distribution, WNV is maintained in its enzootic cycle by several *Culex* species, including *Culex tarsalis*, *Cx. pipiens quinquefasciatus*, and *Cx. pipiens pipiens* [13]. In addition, mosquitoes of several divergent genera have been found infected and/or demonstrated to be competent vectors [177,178]. These include *Aedes* mosquitoes that may act as a “bridge” between enzootic cycles and mammals, including humans [198]. Although it seems clear that infection of mosquitoes leads to genetically complex virus populations [117,160,161], the impact of any particular mosquito species on WNV population biology has not been determined. In addition, the full range of selective forces acting on WNV during systemic infection of mosquitoes is poorly understood, and important inconsistencies persist in the literature. For example, whereas some studies have documented the existence of population bottlenecks during arbovirus transmission by mosquitoes [292,293,295], others have not [167,296]. The genetic implications of high mutational diversity coupled with population bottlenecks during mosquito transmission have not been fully elaborated, and the impacts of different vector species on virus population diversity and fitness are not known.

Therefore, we determined the extent to which mosquito vectors of WNV differ in their propensity to drive virus diversification and impact fitness. In particular, using next-generation sequencing (NGS) we characterized virus populations within distinct tissue compartments including midguts, hemolymph, salivary glands and expectorated saliva of *Cx. tarsalis*, *Cx. quinquefasciatus*, *Cx. pipiens*, and *Ae. aegypti* during a single mosquito infection. Taken

together, our results 1) demonstrate that mosquito species have differential impacts on virus evolution, 2) illustrate sequential reductions and expansions in virus population size that occur during the spread of virus from one mosquito compartment to another, and 3) confirm the importance of purifying selection during vertebrate infection in maintaining WNV fitness. Ironically, our results document a profound loss in relative fitness imposed by arthropod transmission of an arbovirus.

Materials and methods

Mosquito infections

The WNV infectious clone (WNVic) of the NY99 strain collected during the 2000 outbreak in New York City was produced as described [384]. Laboratory colonies of *Cx. quinquefasciatus*, *Cx. pipiens*, *Cx. tarsalis*, and *Ae. aegypti* mosquitoes were reared, fed bloodmeals containing $\sim 2 \times 10^8$ plaque forming units (PFU)/mL of WNVic, dissected, and processed as described in Chapter 2. Mosquito midguts, legs, salivary glands, and saliva were collected after 14 days extrinsic incubation for NGS and midguts, legs, and bodies (without midguts and legs) were collected after 3-7 and 10 days extrinsic incubation for viral growth curves.

Production of mutant viruses

The WNVic (NY99 strain) was used to generate mutations that were recovered from mosquito saliva. Mutations were engineered using mutagenic primers and fragments containing overlapping sequences were cloned using AQUA cloning [403] and subsequently transformed into NEB Stable competent cells (NEB). Resulting bacterial colonies were grown overnight in

LB media and plasmid was purified using the Zippy Plasmid Miniprep kit (Zymo Research). The presence of desired mutations was confirmed with Sanger sequencing. Following sequence validation, the plasmid was amplified using rolling circle amplification with the TempliPhi Amplification Kit (GE Healthcare) and then linearized using XbaI (NEB). The linearized construct was then column purified with the NucleoSpin PCR clean-up kit (Macherey-Nagel). Capped infectious RNA was then produced using the HiScribe T7 ARCA mRNA kit (NEB) for 4 hours at 37 °C. RNA was subsequently quantified using the Qubit HS RNA kit (Invitrogen). 10 µg of RNA was then used for electroporation in 1×10^7 BHK-21 cells using a BTX ECM 630 electroporator with settings 450 V, 1200 Ω, and 150 µF using two pulses. After electroporation, cells were seeded in T25 flasks and placed in a 37 °C incubator. Supernatant was harvested after 3-5 days of incubation, clarified by centrifugation, and aliquots were made and stored at -80 °C. Virus concentrations were determined via plaque assay in Vero cells (PFU) and qRT-PCR (genome equivalents [GE]) (**Table 3.1**).

Table 3.1. WNV clones made with the highest frequency nonsynonymous mutation detected in each saliva sample compared to the input (WNVic) and reference (WNV-REF) viruses.

Species	Rep.	Genome position	nt change	Coding region	aa change ^a	iSNV freq	<u>Virus rescued in BHK cells^b</u>		
							GE/ml	PFU/ml	GE:PFU ^c
<i>Cx. tarsalis</i>	A	1066	AT	Envelope	M34L	0.86	6.1×10 ¹¹	9.0×10 ⁷	6,769
	B	6107	TC	NS3	I499T	0.07	6.8×10 ¹¹	5.8×10 ⁸	1,174
	C	6257	AG	NS3	Y549C	0.05	1.9×10 ¹¹	5.3×10 ⁷	3,514
<i>Cx. quinquefasciatus</i>	A	434	TC	Capsid	V113A	0.69	6.4×10 ¹¹	2.3×10 ⁸	2,742
	B	428	TC	Capsid	I111T	1.00	4.4×10 ¹¹	1.0×10 ⁸	4,319
	C	1703	AT	Envelope	H246L	0.04	1.3×10 ¹¹	1.4×10 ⁷	9,614
<i>Ae. aegypti</i>	A ^d	10104	GT	NS5	W808C	0.03	2.9×10 ⁹	N.D. ^e	N.D.
	B	2763	GT	NS1	K98N	0.30	6.0×10 ¹¹	1.5×10 ⁸	4,059
	C	2056	GA	Envelope	V364M	0.03	5.3×10 ¹¹	2.2×10 ⁷	24,472
WNV-REF		8313-8317 ^f		NS5			6.0×10 ¹¹	1.7×10 ⁸	3,660
WNVic ^g							7.7×10 ¹¹	2.3×10 ⁸	3,420

^a Amino acid (aa) substitution position based on coding region position.

^b Clones with point mutations were constructed as described [403], amplified by rolling circle amplification, linearized, capped, and rescued in BHK-21 cells. WNV genome equivalents (GE) and plaque forming units (PFU) were determined by qRT-PCR and standard plaque assays, respectively.

^c The GE:PFU ratio is an inverse proxy for viral infectivity. For example, a viral stock with a ratio of 100 contains a higher proportion of infectious particles than a viral stock with a ratio of 1000.

^d The highest frequency mutation in this sample encoded for a stop codon in the NS5 region (frequency = 0.04) and was not engineered into the WNVic due the assumption that the mutation is lethal.

^e The recovered virus replicated in BHK-21 cells as determined by qRT-PCR but failed to form plaques in Vero cells. A GE:PFU ratio of 10,000 was used to estimate the viral titer for replicative fitness studies.

^f Five sequential synonymous changes were made to nucleotide positions 8313-8317 in the NS5 region of the WNV-REF genome, changing the parental sequence CTC TCA CGG to CTa agc aGG.

^g Parental virus to all point mutation clones and WNV-REF.

nt, nucleotide; aa, amino acid; iSNV, intrahost single nucleotide variant; GE, genome equivalents; PFU, plaque forming units.

Phenotypic assessment

Replication fitness of the reconstructed WNV mutants was compared to the WNVic during infection of chicken fibroblast DF-1 cells (ATCC no. CRL-12203) and *Ae. albopictus* clone C6/36 cells (ATCC no. CRL-1660) at a multiplicity of cellular infection (MOI) of 0.01 for 1-6 days post infection. RNA was extracted from 50 µl of cell culture supernatant, homogenized mosquito tissues, and saliva collected in diluent using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek) on the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific) and the RNA was eluted in 50 µl of nuclease-free water. Viral GE was determined by qRT-PCR of the WNV envelope coding region using the following forward primer (5'-TCA GCG ATC TCT CCA CCA AAG-3'), reverse primer (5'-GGG TCA GCA CGT TTG TCA TTG-3'), and probe (5'-TGC CCG ACC ATG GGA GAA GCT C-3') sequences [388] and was standardized by the total sample volume. Viral PFUs were determined by standard plaque assay in Vero cells. Competitive fitness of saliva WNV populations and the reconstructed WNV mutants (competitors) was determined by directly comparing their replication to a reference WNV during co-infection in DF-1 cells [171]. Reference and competitors were equally mixed and added to DF-1 cells in 24-well plates at MOIs of ~ 0.0008 for the recovered saliva populations and 0.01 for the reconstructed WNV mutants. Supernatants were collected at 2, 4, and 6 days post infection and RNA was extracted as described above. Quantitative Sanger sequencing was used to determine the proportion of competitor (saliva and mutants) to reference WNV genotypes [386].

Library preparations and viral population analysis

Total RNA from mosquito tissues and saliva after 14 days extrinsic incubation were amplified and prepared for NGS as described in Chapter 2. 100 nt paired-end reads were generated using the Illumina HiSeq 2500 platform at Beckman Coulter Genomics. All NGS data can be accessed from the NCBI BioProject PRJNA311123.

Demultiplexed reads were aligned to the WNVic sequence using MOSAIK [390] and duplicate reads were removed using the MarkDuplicates tool within Picard to limit the influence of PCR artifacts. Intrahost single nucleotide variants (iSNVs) and intrahost length variants (iLVs, includes both insertions and deletions) were called using VPhaser2 [391] and variants with significant strand bias were removed. Analysis was limited to the protein coding sequences and was done separately for iSNVs and iLVs. Richness was calculated by the sum of the iSNV sites detected in each population and was normalized by the number of WNV reads (i.e. sites per million WNV reads). Genetic distance was calculated by the sum of the iSNV and amino acid substitution frequencies from each population and reported as the iSNVs and amino acid variants per coding sequence, respectively. Shannon entropy (S) was calculated for each intrahost population (i) using the iSNV frequency (p) at each nucleotide position (s):

$$S_{i,s} = -p_s(\ln p_s) + (1 - p_s) \times \ln(1 - p_s) \quad (1)$$

The mean S from all sites s is used to estimate the mutant spectra complexity.

The standardized variance in iSNV frequencies (F_{ST}) was used to estimate genetic divergence between two viral populations as described [400]:

$$a_s = \frac{4n_i(p_{i,s} - p_s)^2 + 4n_j(p_{j,s} - p_s)^2 - b_s}{2(2n_i n_j / (n_i + n_j))} \quad (2)$$

and

$$b_s = \frac{n_i(2p_{i,s}(1 - p_{i,s}) + n_j(2p_{j,s}(1 - p_{j,s}))}{n_i + n_j - 1} \quad (3)$$

where $p_{i,s}$, $p_{j,s}$, and p_s are the frequencies of the input WNV consensus nucleotide at site s from populations i , j , and combined, respectively. Only VPhaser2-called iSNVs were used, all other sites $p = 1$. The number of individuals sampled, n , was set to the average WNV coverage depth (12,599 nt) to normalize for sequencing variations. The estimate of F_{ST} for the protein coding *locus* of m sites (10,299 nt) is:

$$F_{ST}^{(locus)} = \frac{\sum_{s=1}^m a_s}{\sum_{s=1}^m (a_s + b_s)} \quad (4)$$

High frequency (HF) variants were calculated by $\log_{10}$ transforming the variant frequency, increasing the values to make them all positive (iSNVs increased by 3.68 and iLVs increased by 4.3), and each data set was fitted to a gamma distribution (iSNVs $n = 5265$ and iLVs $n = 6828$). iSNV frequencies > 0.034 ($n = 259$) and iLV frequencies > 0.007 ($n = 346$) were determined to be in the upper 5% of their respective gamma distributions. Haplotypes were reconstructed using QuasiRecomb 1.2 [395] using parameters described in Chapter 2 and manually edited to only include HF iSNVs. The thirty most common predicted haplotypes from each population were used estimate how the viral demographics were changing within each host by calculating Tajima's D [404] and Harpending's raggedness index [405,406] using DnaSP (v5) [396]. Haplotype phylogenies were constructed using the Bayesian Markov chain Monte Carlo method in BEAUti and BEAST (v1.8) [407] with a HKY substitution and gamma site heterogeneity model and a lognormal relaxed molecular clock.

The number of founder genomes (i.e. effective population size [N_e]) initiating infection in the mosquito midguts was determined by the genetic variance within and between populations using F_{ST} [408]:

$$N_e = \frac{1 - F_{ST}}{F'_{ST} - F_{ST}} \quad (5)$$

where F_{ST} is the genetic variance between the input populations (bloodmeal) and F'_{ST} is the genetic variance between the midgut populations. F_{ST} was calculated from all replicate combinations for species (i.e. replicate A vs B, A vs C, and B vs C) using equations 2-4. To minimize the influence of selection on variant frequencies, only third codon synonymous iSNVs not predicted to co-occur on haplotypes with nonsynonymous mutations (remove genetic hitchhiking) were used in F_{ST} calculations.

Intrahost selection was estimated by the ratio of nonsynonymous (d_N) to synonymous (d_S) SNVs per site (d_N/d_S) using the Jukes-Cantor formula [409]:

$$d_N = \frac{-3 \times \ln(1 - ((4p_n)/3))}{4} \quad (6)$$

and

$$d_S = \frac{-3 \times \ln(1 - ((4p_s)/3))}{4} \quad (7)$$

where p_n equals Nd (sum of the synonymous iSNV and iLV frequencies accepted by VPhaser2) divided by the number of nonsynonymous sites and p_s equals Sd (sum of the synonymous iSNVs) divided by the number of synonymous sites. DnaSP was used to determine the number of nonsynonymous (7843.67) and synonymous (2455.33) sites from the ancestral input WNV consensus sequence using the Nei-Gojorori method [397]. d_N/d_S values >1 for divergent lineages are the hallmark of positive selection, but the power of d_N/d_S to detect positive selection within hosts is very low considering that the majority of the nonsynonymous mutations are under

strong purifying selection [410]. Therefore we only used the d_N/d_S ratio to cautiously estimate the strength of purifying selection. Statistical comparisons were performed using GraphPad Prism (version 6.04) for Windows.

Results

Vector competence and anatomical barriers to virus transmission

Arboviruses must overcome anatomical barriers within mosquitoes for transmission to occur (**Figure 3.1A**). Three enzootic vectors, *Cx. tarsalis*, *Cx. quinquefasciatus*, and *Cx. pipiens*, and one representative of *Aedes* mosquitoes acting as a potential bridge vector, *Ae. aegypti*, were exposed to WNVic derived from the NY99 genotype to evaluate vector competence and obtain samples for analysis. Vector competence was determined by examining the percent of midguts, legs (containing hemolymph), salivary glands, and saliva infected with WNV. After 14 days extrinsic incubation, 75% of *Cx. tarsalis*, 38% of *Cx. quinquefasciatus* and *Ae. aegypti*, and 28% of *Cx. pipiens* had WNV RNA in their saliva (**Figure 3.1B**). These differences in overall vector competence were related to differences in the strengths of barriers to infection and escape from the midguts and salivary glands of tested mosquitoes (**Figure 3.1B**). Furthermore, the mosquitoes with the highest viral GE in their salivary glands were more likely to have virus detected in their saliva (**Figure 3.1C** and **Figure 3.2**, $p < 0.05$ Mann-Whitney test). However, viral GE in the midguts and saliva were not significantly different among mosquito species (Figure 1C, $p > 0.05$ by Kruskal-Wallis-Dunn's corrections) despite species-specific differences in susceptibility to oral infection and transmission, respectively. Moreover, the rates of WNV replication in the midguts are not directly correlated with the midgut infection and escape

barriers (**Figure 3.1D**). Importantly, these data demonstrate a context-dependent relationship between the strength of any given anatomical barrier and viral load.

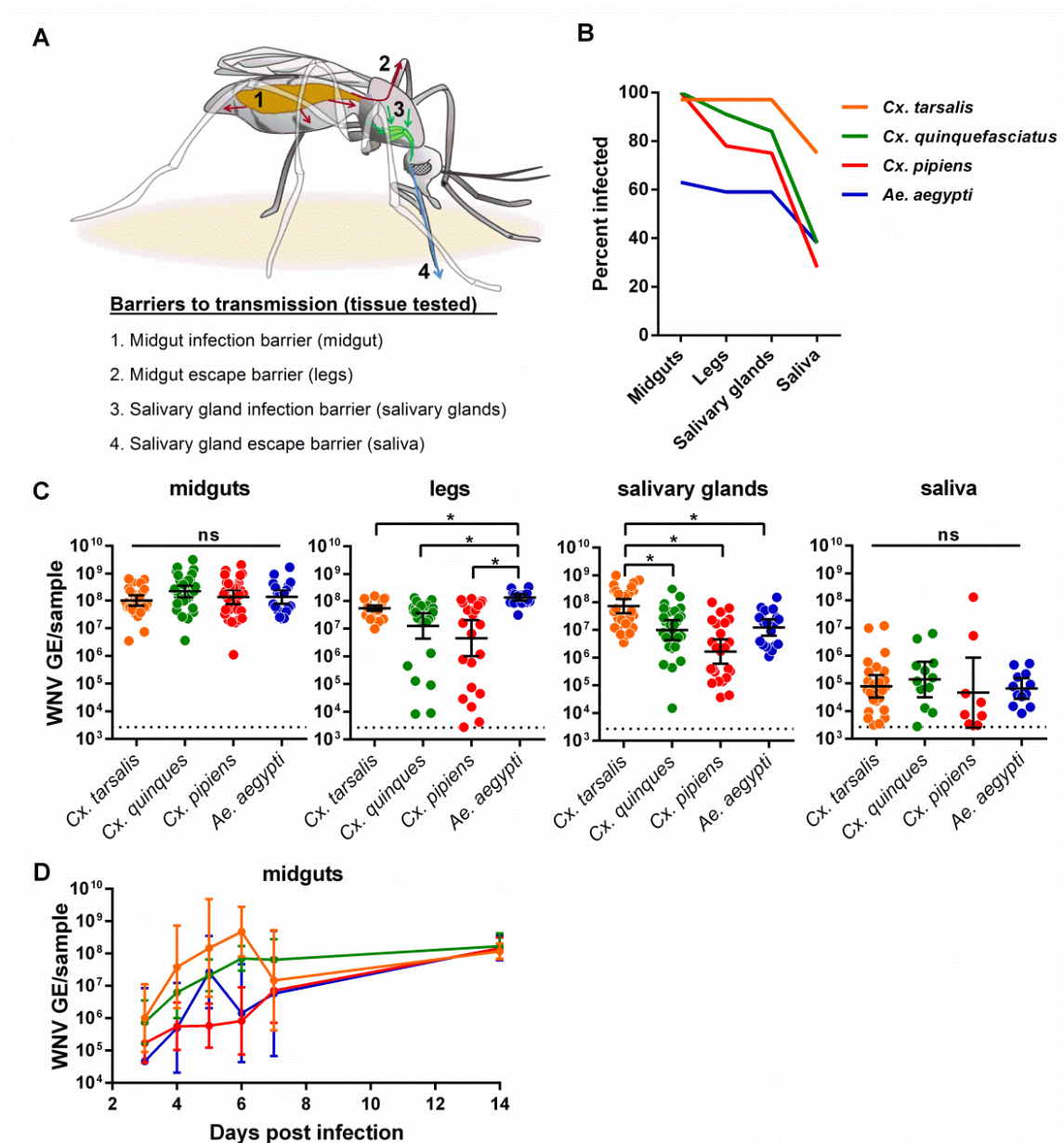


Figure 3.1. Vector competence of mosquitoes and characterization of specimens used in this study. (A) Overview of the anatomical barriers to virus transmission. Infected tissues indicate that WNV could overcome the barrier (e.g. infected legs indicate there was not a midgut escape barrier in that mosquito). (B) Percent of tissues and saliva with WNV RNA determined by qRT-PCR ($n = 32$ mosquitoes for each species) at 14 days post exposure. (C) WNV GE per tissue or saliva sample from only the WNV-infected tissues determined by qRT-PCR (*, $p < 0.05$; ns, not significant). (D) WNV replication rates determined by collecting midguts at 3-7 and 14 days post infection ($n = 16$).

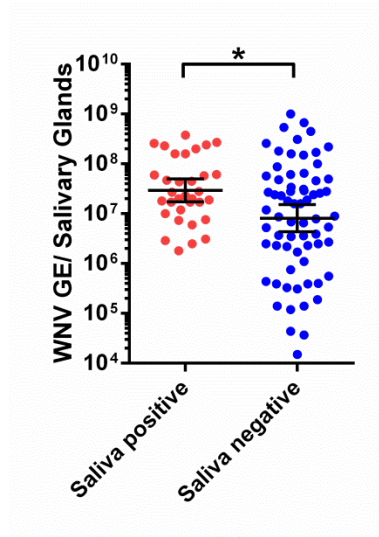


Figure 3.2. Viral load is related to the salivary gland escape barrier. Comparison of WNV genome equivalents (GE) per set of salivary glands from paired saliva WNV positive and negative samples (geometric mean with 95% confidence interval, $*p < 0.05$, Mann-Whitney test).

Intrahost WNV population structure is mosquito species-dependent

Three mosquitoes from each species that had detectable WNV RNA in all four compartments were used to assemble three biological replicates of each tissue per species. WNV RNA was examined using NGS to define species- or tissue-dependent impacts on virus mutational diversity. Approximately 6% of > 22 million reads obtained from each specimen aligned to WNV, resulting in > 12,000× coverage depth across the viral genome (**Figure 3.3**). However, the coverage depth from one biological replicate of *Cx. pipiens* salivary glands and all three *Cx. pipiens* saliva was much lower (< 100×) precluding viral population analysis. Analysis of the remaining samples was limited to the protein coding sequence (nucleotide positions 97-10,395) due to large variation in the sequencing coverage of the untranslated regions (presumably caused by secondary structures in these regions).

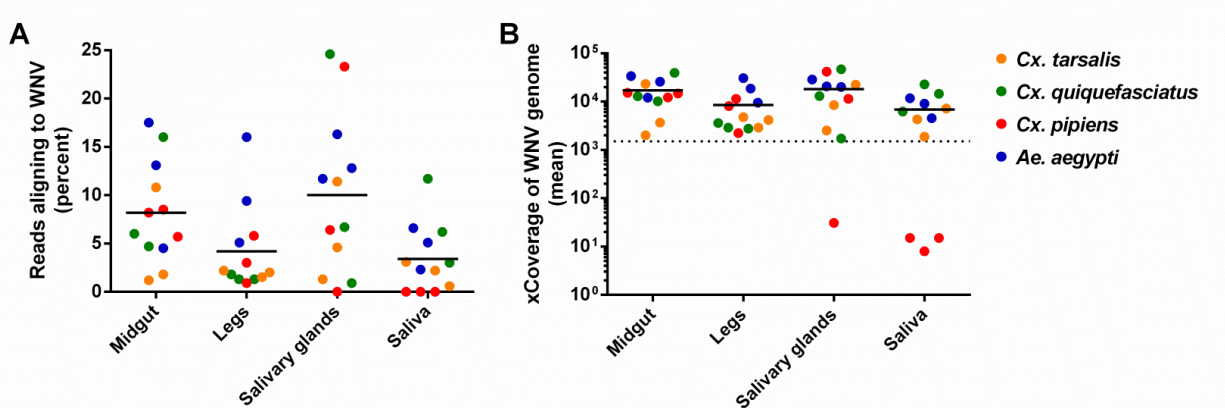


Figure 3.3. Percent reads and coverage of the WNV genome. (A) Mosquito tissues and saliva were randomly amplified and sequenced on the Illumina HiSeq2500, averaging ~23 million 100-nt reads per library and were aligned to the WNV genome. (B) Mean fold coverage of the WNV genome (average number of nucleotides sequenced per site). The minimum coverage for intrahost viral population analysis used in this study was 1500× (dashed line).

iSNVs from each biological replicate were combined by species and tissue to assess their genome positions and frequencies (Figure 3.4, Table S2). As expected, the relatively homogenous input WNVic population diversified within each mosquito species (i.e. iSNVs reached higher frequency, Figure 3.4). However, the number of iSNV sites that reached HF (> 0.034 , upper 5% of a gamma distribution), changed the consensus sequence (frequency > 0.5), and went to fixation (frequency = 1) were mosquito species- and tissue- dependent (Table 3.2). The most HF iSNVs, consensus changes, and fixations were found in *Cx. quinquefasciatus* and *Cx. tarsalis* tissues, however most of the consensus sequence changes detected in the salivary glands of *Cx. tarsalis* were not found in the saliva (Table 3.3). In addition, none of the observed consensus changes (Table 3.3) resemble known WNV lineage-defining mutations (e.g. V to A substitution at envelope codon position 159 that place it in the “WN02” lineage [102]).

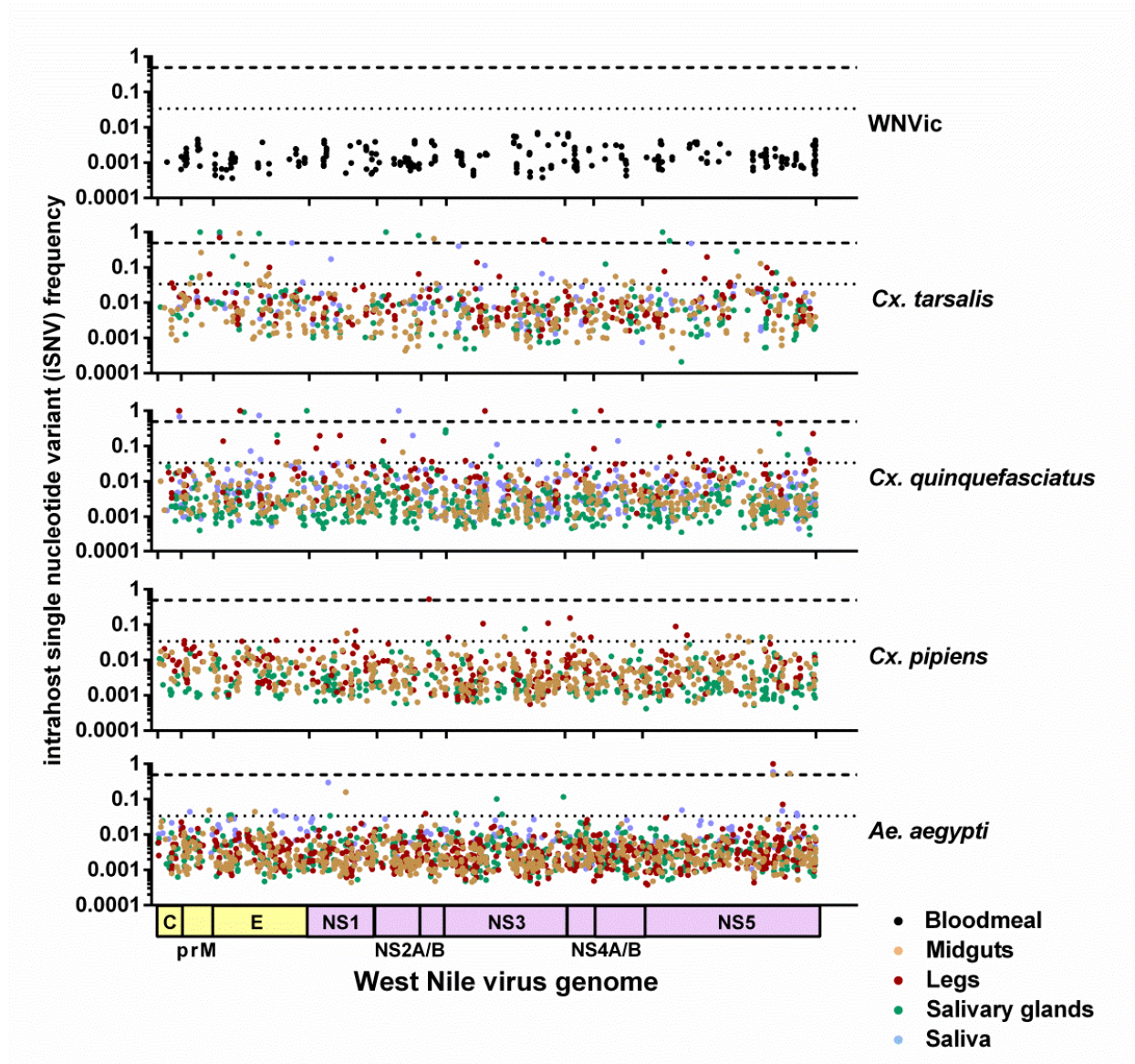


Figure 3.4. More high frequency single nucleotide variants are generated during virus replication in *Cx. quinquefasciatus* and *Cx. tarsalis*. iSNVs from each biological replicate ($n = 3$) were plotted by their position on the WNV genome and their frequency in each mosquito species and tissue. The WNV genome consists of three structural protein coding regions (shown in yellow), capsid (C), premembrane (prM), and envelope (E), and seven nonstructural (NS) protein coding regions (shown in purple). The cut-offs for high frequency iSNVs (0.034, dotted line) and consensus sequence changes (0.5, dashed line) are shown. iSNV sites are categorized by frequency in **Table 3.2** and all consensus sequence changes are listed in **Table 3.3**.

Table 3.2. Categorization of WNV iSNV sites by frequency.

Mosquito species	Tissue	Total variant sites from the combined biological replicates			
		iSNV ^a	HF iSNV ^b	Consensus changes ^c	Fixations ^d
<i>Cx. tarsalis</i>	M	295	20	2	0
	L	197	16	2	0
	SG	211	13	7	3
	S	105	9	1	0
<i>Cx. quinques</i>	M	411	5	0	0
	L	159	23	5	4
	SG	512	17	6	3
	S	305	14	7	5
<i>Cx. pipiens</i>	M	419	5	0	0
	L	240	13	1	0
	SG	339	2	0	0
	S	N.D. ^e	N.D.	0	0
<i>Ae. aegypti</i>	M	600	5	1	0
	L	578	3	1	1
	SG	494	8	1	1
	S	144	8	1	0
WNVic	BM	286	0	0	0

^a All intrahost single nucleotide variant (iSNV) sites detected in the WNV coding sequence.

^b High frequency (HF) includes all iSNV sites with a frequency > 0.034. See materials and methods for calculation.

^c Consensus changes include all iSNV sites with a frequency > 0.5. For one *Cx. pipiens* salivary gland and all *Cx. pipiens* saliva, consensus changes were assessed using low-coverage assemblies (Figure 3.3).

^d Fixations include all iSNVs that completely replaced the consensus nucleotide, frequency = 1.

^e Not determined (N.D.) due to insufficient coverage depth.

Cx. quinques, *Cx. quinquefasciatus*; WNVic, West Nile virus infectious clone; M, midgut; L, legs; SG, salivary glands; S, saliva.

Table 3.3. WNV consensus changes during systemic mosquito infection.

Mosquito species	Tissue (replicate)	Genome position	nt change	Coding region	S/N	aa change	iSNV freq
<i>Cx. tarsalis</i>	M (B)	1375	TC	envelope	N	Y427H ^b	0.94
		4418	AG	NS2B	N	E1441G ^c	0.65
	L (A)	1066 ^a	AT	envelope	N	M324L	0.71
		6139	TC	NS3	N	Y221H ^b	0.60
	SG (A)	759	AG	prM	S		1
		1066 ^a	AT	envelope	N	M324L	1
		1681	TC	envelope	S		0.92
		3668	GT	NS2A	N	G1191V ^d	1
		4179	TC	NS2A	S		0.82
		7996	GA	NS5	N	G2634S ^e	1
		8105	CG	NS5	N	T2670S	0.57
	S (A)	1066 ^a	AT	envelope	N	M324L	0.86
<i>Cx. quinques</i>	L (A)	434 ^a	TC	capsid	N	V113A	1
	L (B)	428 ^a	TC	capsid	N	I111T ^f	1
	L (C)	1383 ^a	GA	envelope	S		1
		5214 ^a	AC	NS3	S		0.99
		7029 ^a	GA	NS4B	S		1
	SG (A)	434 ^a	TC	capsid	N	V113A	1
	SG (B)	428 ^a	TC	capsid	N	I111T ^f	1
		1447	GA	envelope	N	A161T ^f	0.91
		6620	TG	NS4A	N	T2175R ^g	0.98
		6624	GA	NS4A	S		0.98
	SG (C)	2427	AG	envelope	S		1
	S (A)	434 ^a	TC	capsid	N	V113A	0.69
	S (B)	428 ^a	TC	capsid	N	I111T ^f	1
		1682	TC	envelope	N	L529S ^f	0.74
		3866	TA	NS2A	N	F1257Y	1
	S (C)	1383	GA	envelope	S		1
		5214	AC	NS3	S		1
		7029	GA	NS4B	S		1
<i>Cx. pipiens</i>	L (B)	4341	TC	NS2B	S		0.53
<i>Ae. aegypti</i>	M (C)	9982	CT	NS5	S		0.53
	L (A)	9723 ^a	CT	NS5	S		1
	SG (A)	9723 ^a	CT	NS5	S		1
	S (A)	9723 ^a	CT	NS5	S		0.60

^a iSNV was detected in multiple tissues.

^b Amino acid side chain change from hydrophobic to positive charged.

^c Amino acid side chain change from negative charged to special case.

^d Amino acid side chain change from special case to hydrophobic.

^e Amino acid side chain change from special case to polar uncharged.

^f Amino acid side chain change from hydrophobic to polar uncharged.

^g Amino acid side chain change from polar uncharged to positive charged.

nt, nucleotide; S/N, synonymous or nonsynonymous mutation; aa, amino acid; iSNV freq, intrahost single nucleotide frequency; *Cx. quinques*, *Cx. quinquefasciatus*; M, midgut; L, legs; SG, salivary glands; S, saliva.

Intrahost viral population structure was further assessed using several genetic diversity indices: genetic richness (number of unique iSNV sites per million WNV reads, **Figure 3.5A**), complexity (the proportion of different variants in a mutant spectrum, estimated by the Shannon entropy, **Figure 3.5B**), distance (iSNVs and amino acid substitutions per coding sequence, **Figure 3.5C-3D**), and divergence (accumulation of independent mutations between two isolated populations, estimated by F_{ST} [400], **Figure 3.5E**, **Figure 3.6**). Despite similar levels of richness and complexity (**Figure 3.5A-B**, $p > 0.05$ by Kruskal-Wallis -Dunn's corrections), WNV populations in *Cx. quinquefasciatus* and *Cx. tarsalis* contained greater genetic diversity at both the iSNV (**Figure 3.5C**) and amino acid levels (**Figure 3.5D**), and diverged further from the input WNV as compared to WNV in *Cx. pipiens* and *Ae. aegypti* (**Figure 3.5E**, **Figure 3.6**). The increased genetic diversity in *Cx. quinquefasciatus*, and to a lesser extent *Cx. tarsalis*, was largely due to increased accumulation of HF iSNVs (including consensus changes and fixations, **Figure 3.4**).

iSNVs generated during replication in all mosquito species seemed to be distributed uniformly across the viral coding sequence (**Figure 3.4**). To investigate this further, we combined the WNV populations from each tissue to determine whether particular WNV genomic regions were more diverse (**Figure 3.6**). Nucleotide and amino acid diversities were greatest in *Cx. quinquefasciatus* and *Cx. tarsalis*, and were uniformly distributed across the protein coding sequence. In *Cx. quinquefasciatus*, the capsid coding region was more diverse due to a relatively small number of HF iSNVs (**Table 3.3**).

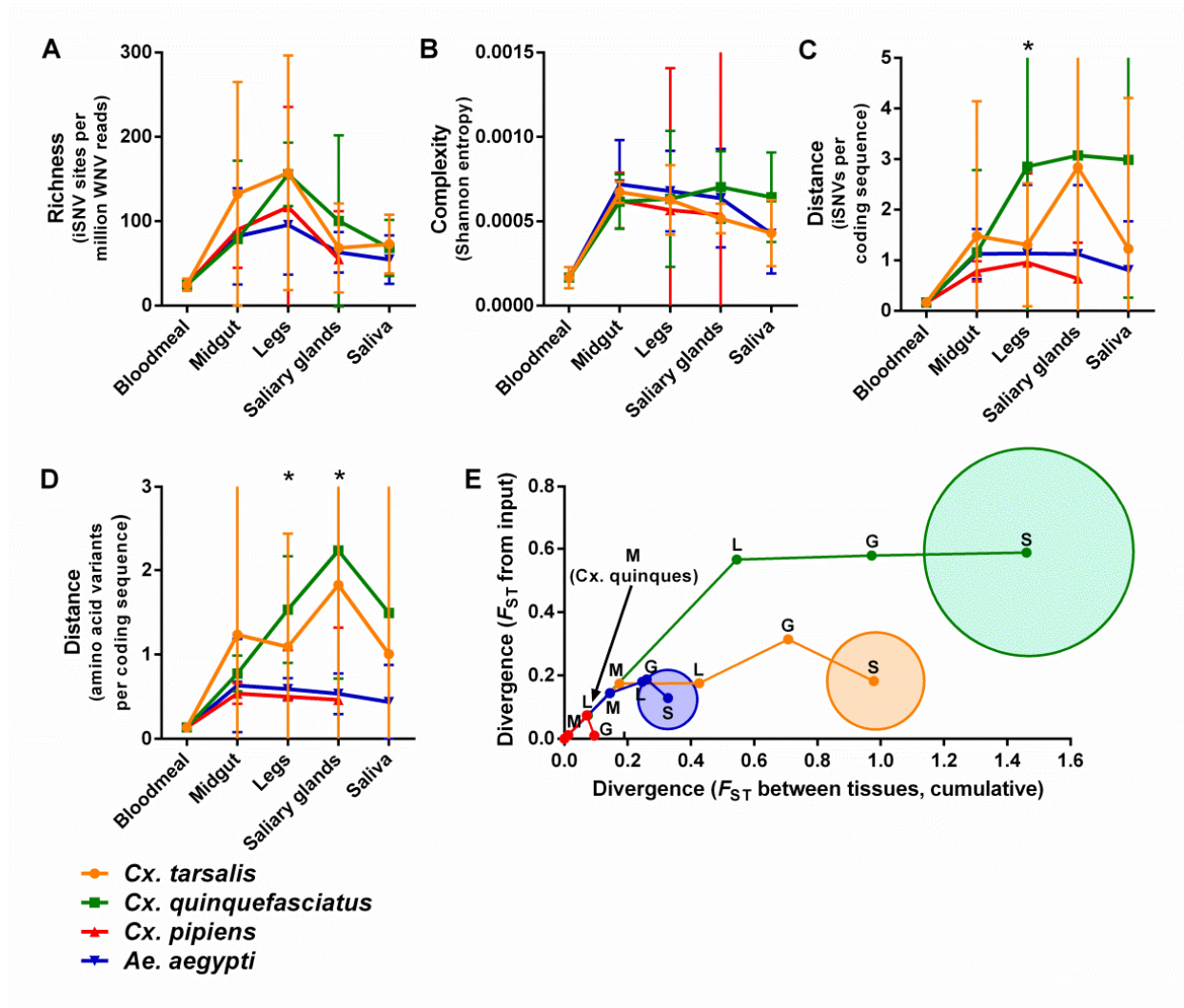


Figure 3.5. Diversification of WNV during systemic infection of mosquitoes. (A-D) Intrahost genetic diversity was characterized by measuring (A) richness, (B) complexity (the proportion of different variants in a mutant spectrum), (C) iSNV distance, and (D) amino acid distance. Data shown as means with 95% confidence intervals (*, $p < 0.05$). (E) Mean genetic divergence (accumulation of independent mutations between two isolated populations) of each population from the input virus (y-axis), between tissues (x-axis, cumulative), and among biological replicates (circles, shown to axis scale). Individual comparisons are shown in **Figure 3.6**.

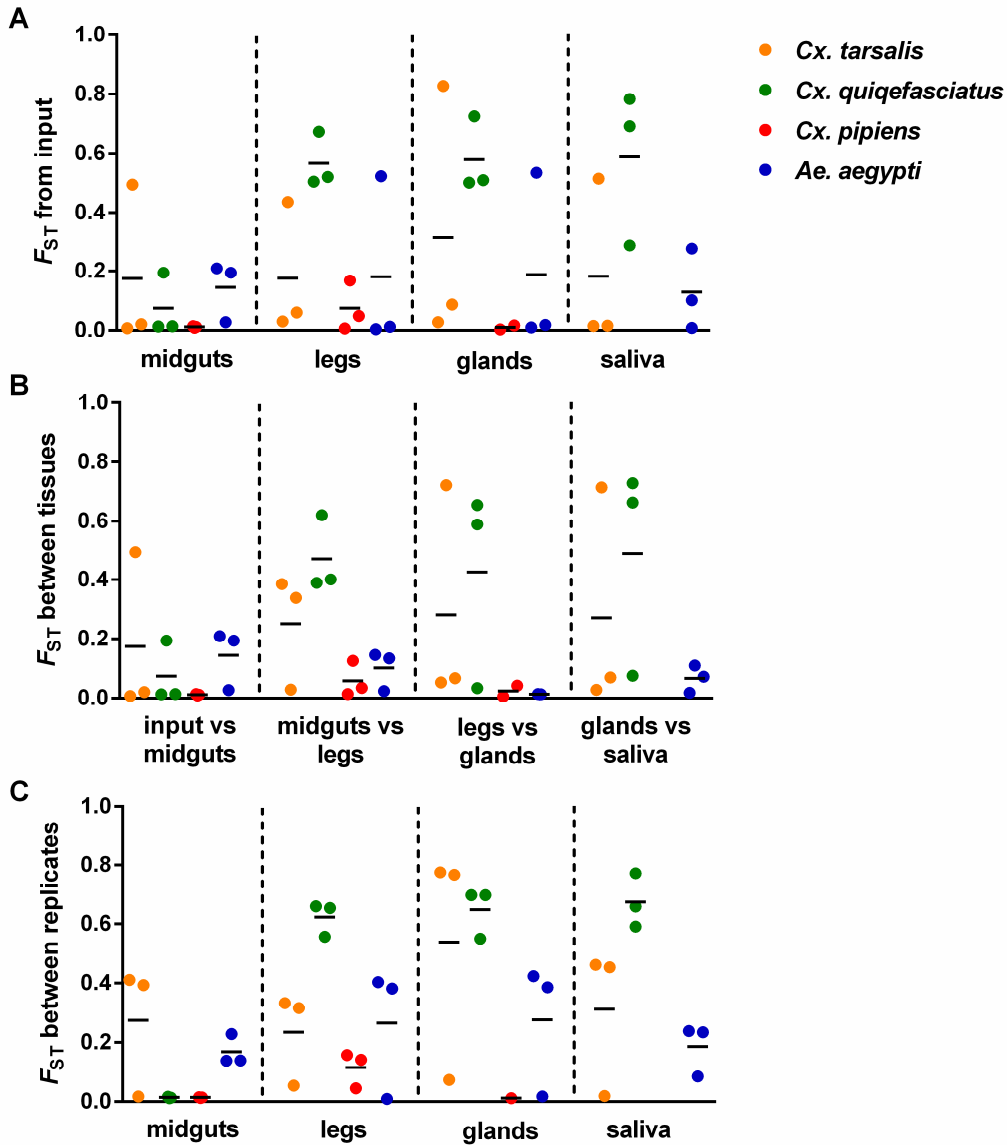


Figure 3.6. Genetic divergence between viral populations. (A-C) Genetic divergence (F_{ST}) of (A) each population from the input virus, (B) between tissues (matched within same mosquito), and (C) among biological replicates (within a tissue). The bars represent the means which were used to create **Figure 3.5E**. F_{ST} was calculated using equations 2-4 [400].

Viral population declines and expansions during systemic mosquito infection

To assess population bottlenecks during mosquito infection we tracked the spread of individual unique iSNVs (**Figure 3.7A**) and the 30 most common predicted haplotypes

(conservatively predicted using only HF iSNVs, **Figure 3.7B**) during systemic spread between mosquito tissues. Most input (e.g. < 5% in saliva, **Figure 3.8A**) and locally-derived (**Figure 3.7A**) unique iSNVs were not transferred between tissues. For example, > 90% of iSNVs detected in the saliva were not detected in other tissues. In addition, the original WNVic input haplotype was dominant in most tissues, except in *Cx. quinquefasciatus*. In this species a new dominant haplotype was detected in the hemolymph (**Figure 3.7B**). These observations led us to hypothesize that bottlenecks within mosquitoes limit haplotype spread. We thus evaluated intra-tissue viral demographics using Tajima's D (i.e. comparison of pairwise mismatches and segregating sites) [404] and Harpending's raggedness index (i.e. the distribution of pairwise mismatches) [405,406]. D values were consistently negative and not significantly different among species, tissues, or replicates (mean = -0.73, $p > 0.05$ by Kruskal-Wallis-Dunn's corrections); and the distributions of pairwise mismatches (**Figure 3.7C**) revealed several multimodal curves. Phylogenies reconstructed from predicted haplotypes were also consistent with population declines and expansions (**Figure 3.7D**). In tissues with extensive diversification, the trees demonstrated strong spatial structure (clades corresponded to intra-tissue populations) and star-like branching topologies (see biological replicates A and B in *Cx. tarsalis* and A, B, and C in *Cx. quinquefasciatus*). Together, these results suggest periodic population declines followed by expansions and are consistent with sequential population bottlenecks and recoveries involving founder's effects.

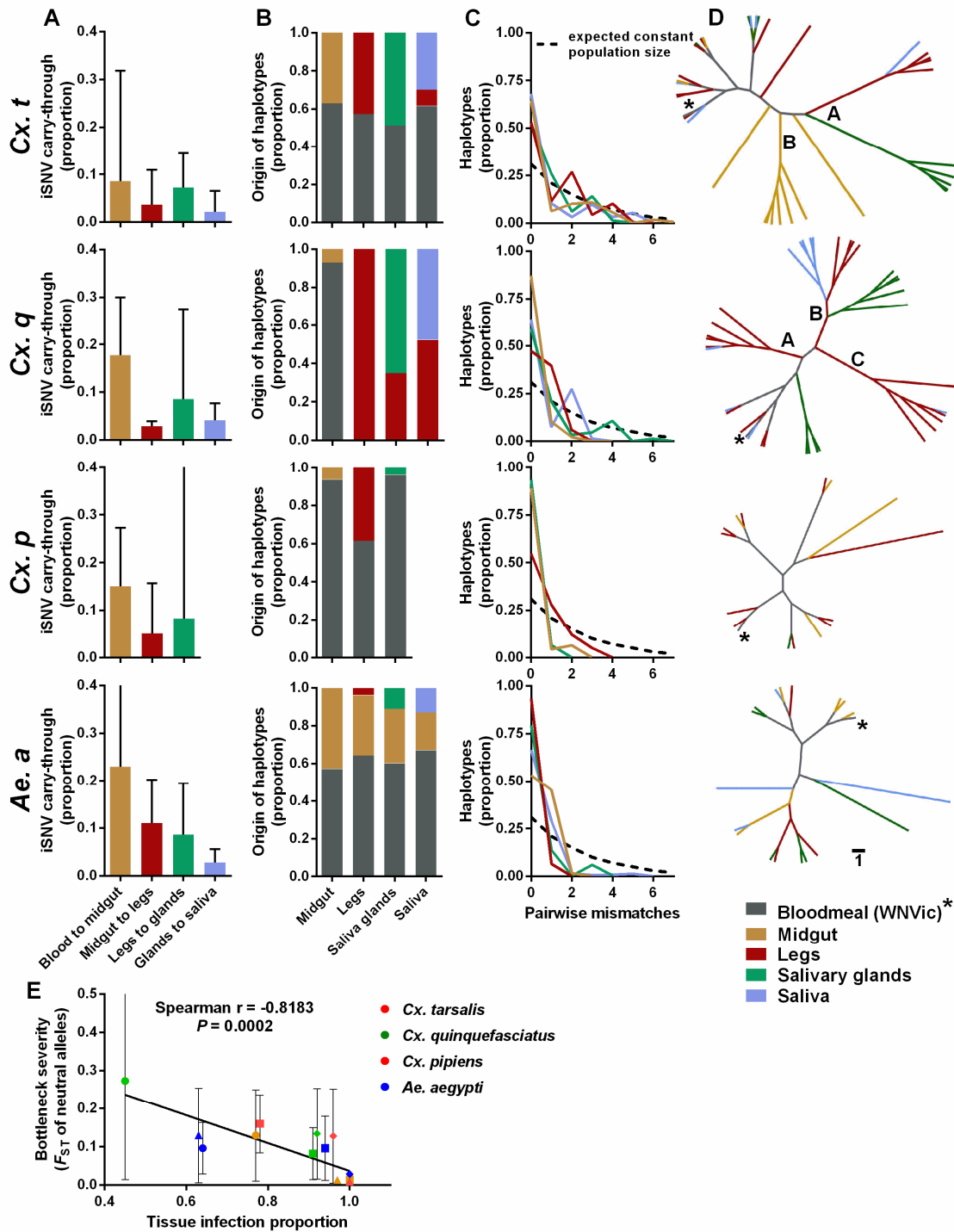


Figure 3.7. Recovery of viral genetic diversity during intra-tissue population expansions following bottlenecks. (A) Proportion of iSNVs found in the subsequent viral population (e.g. carry-through from bloodmeal to the midgut) (mean with 95% confidence interval). (B) Haplotypes were predicted from each viral population were characterized by compartment of origin (e.g. haplotype in a saliva population was originally detected in the legs). The average composition of haplotype origins is shown for each tissue. (C) The distribution of pairwise

mismatches between haplotypes. Shown are the average mismatch distributions for each tissue and the expected distribution for a constant population size (dashed line). “Ragged” lines indicate recent population expansions following declines. **(D)** Phylogenies of all of the predicted haplotypes from each species. The letters represent distinct clades from biological replicates (A, B, and C) and the asterisk marks the input virus branch. **(E)** Genetic divergence of neutral alleles was calculated to determine the amount of genetic variance between populations caused by bottlenecks and drift. Larger F_{ST} values indicate a more severe bottleneck (smaller N_e).

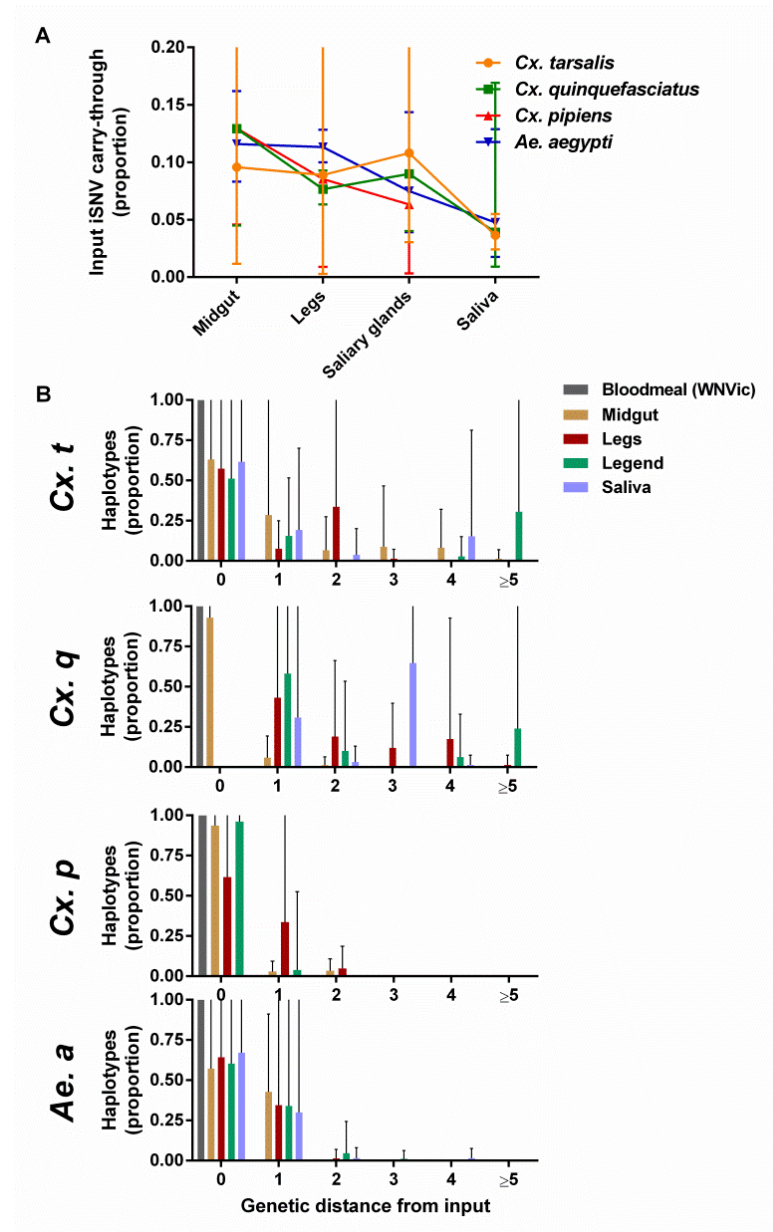


Figure 3.8. Carry-through of input iSNVs and genetic distances of predicted viral haplotypes. **(A)** Proportion of input iSNVs found in subsequent tissues. **(B)** Predicted WNV haplotypes were sorted by genetic distance (the number of nucleotide differences or Hamming distance) from the dominant WNVic (input virus) haplotype (mean with 95% confidence interval).

The number of founder WNV genomes in the midgut from the bloodmeal was calculated to determine the population bottleneck size at the point of initial mosquito infection (i.e. N_e). We estimated N_e by the genetic variance within and between populations caused by drift by calculating F_{ST} from neutral alleles (third codon synonymous iSNVs not predicted to co-occur on haplotypes with nonsynonymous mutations, **Table 3.4**) [400,408]. Between 52 and 129 WNV genomes initiated mosquito midgut infection and were not significantly different among species ($p > 0.05$ by Kruskal-Wallis-Dunn's corrections). N_e calculations between the other tissues produced highly variable results and could not be confidently estimated in this study (data not shown). However, we were able to determine the relative bottleneck severity between tissues and discovered a correlation with the anatomical barrier strength (**Figure 3.7E**).

Table 3.4. The effective population size (N_e) estimated between the bloodmeal and midgut WNV populations using genetic variance (F_{ST}).

Species	<u>F'_{ST} between replicates^a</u>			<u>N_e at the midgut infection barrier^b</u>				
	A-B	A-C	B-C	A-B	A-C	B-C	Mean	95% CI ^c
<i>Cx. tarsalis</i>	0.010	0.017	0.022	119	64	48	77	1-170
<i>Cx. quinques</i>	0.011	0.009	0.013	104	130	87	107	54-161
<i>Cx. pipiens</i>	0.013	0.012	0.010	86	87	90	100	52-127
<i>Ae. aegypti</i>	0.314	0.324	0.006	3	3	229	90	1-401
Combined							90	52-129

^a Calculated using equations 2-4 [400] and only the third codon synonymous iSNVs not predicted to co-occur on haplotypes with nonsynonymous mutations (see Tables S4-S7).

^b Calculated using equation 5 [408]. F_{ST} between the input bloodmeal replicates is 0.001.

^c 95% confidence intervals (CI) calculated from the standard deviation between replicates. *Cx. quinques*, *Cx. quinquefasciatus*.

The phylogenies also showed that some prevalent *de novo* haplotypes that were transferred between tissues acquired additional, locally derived, secondary mutations (**Figure 3.7D**). Populations containing these haplotypes were also the most divergent (**Figure 3.8**). Haplotypes in the salivary glands and saliva with mutations shared with haplotypes arising in the

midguts or legs acquired on average $2.5\times$ more locally derived mutations than haplotypes without common mutations (2.4 compared to 0.9, $p < 0.05$ - Mann-Whitney test).

Intrahost purifying selection is host-dependent

Selection becomes the predominant force changing variant frequencies as N_e increases. We estimated the rates of intra-tissue N_e expansion and N_e at the time of sequencing using GE as a proxy. Viral GE increased at faster rates during replication in *Cx. tarsalis* and *Cx. quinquefasciatus* midguts (**Figure 3.1D**) even though the number of GEs in the midguts at the time of sequencing were not significantly different between species (**Figure 3.1C**, $p > 0.05$), and these differences may also influence selection.

Viral iLVs (including single and double nucleotide insertions and deletions) in the coding sequence are predicted to be deleterious and thus rapidly removed by selection. Therefore we first assessed the level of purifying selection by measuring the accumulation of HF iLVs per coding sequence (**Figure 3.9A**). The input WNVic did not contain any HF iLVs, therefore all HF iLVs must have accumulated during replication in mosquitoes. We did not detect species or tissue-dependent effects on viral iLVs accumulation ($p > 0.05$, Kruskal-Wallis-Dunn's corrections). Next we calculated the ratio of d_N to d_S substitutions per coding sequence site (d_N/d_S) using both iSNVs and iLVs. Viral populations from the species in which WNV diverged the most, *Cx. tarsalis* and *Cx. quinquefasciatus*, had higher d_N/d_S ratios (**Figure 3.9B**) and d_N rates (**Figure 3.9C**) compared to *Cx. pipiens* and *Ae. aegypti*, but similar d_S rates (**Figure 3.9D**). These data suggest that slight differences in mosquito-specific selection may lead to higher population divergence. Specifically, more controlled viral replication during infection of *Cx.*

pipiens and *Ae. aegypti* midguts (**Figure 3.1D**), is likely apparent in our data as stronger purifying selection (**Figure 3.9B**).

In addition, these data, combined with previously reported data on intrahost population dynamics of WNV in various avian species (Chapter 2), demonstrate that purifying selection is weaker in mosquitoes compared to birds.

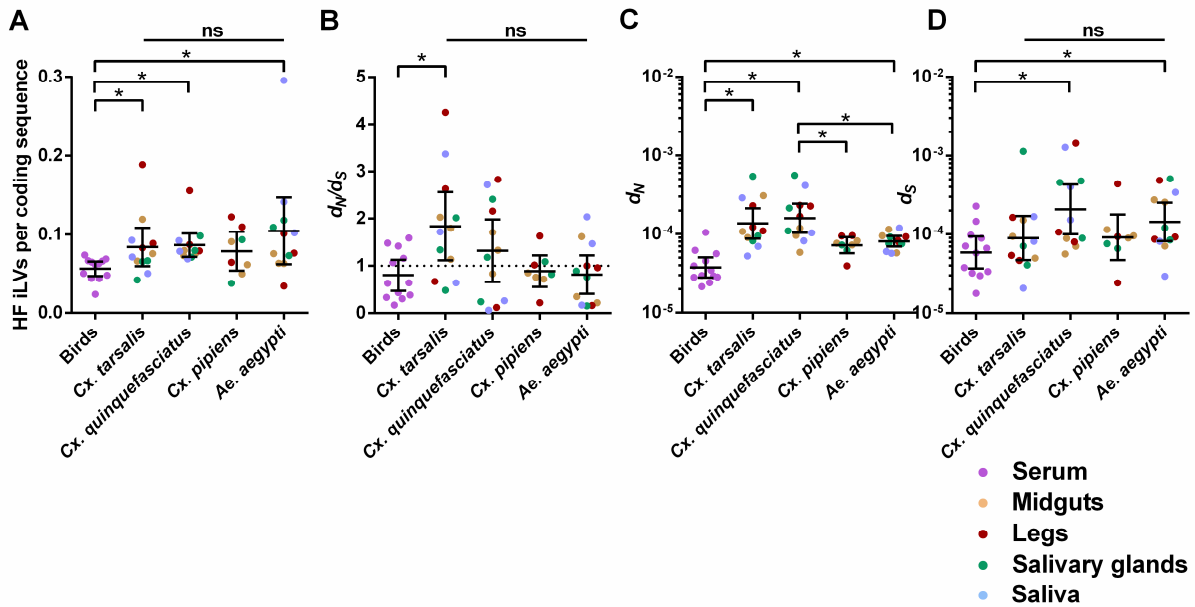


Figure 3.9. Host-specific strength of purifying selection *in vivo*. (A-D) The strengths of purifying selection were compared between mosquito species and between mosquitoes and birds. Wild caught birds (American crows, house sparrows, and American robins) and 2 days old chicks ($n = 3$ for each species) were previously inoculated with the same WNVic used in this study and the serum was sequenced at 3 days post infection (Chapter 2). (A) Accumulation of potentially deleterious mutations was estimated by the number of high frequency iLVs. (B) The ratios of nonsynonymous (d_N) to synonymous (d_S) substitutions per nonsynonymous and synonymous coding sequence site, respectively (d_N/d_S , > 1 [dotted line] weak purifying selection, < 1 strong purifying selection), (C) the d_N rates, and (D) the d_S rates were used to infer the strength of purifying selection. All data were summarized using the mean (A, B) or geometric mean (C, D) and the 95% confidence interval from each tissue and biological replicate per host (*, $p < 0.05$; ns, not significant).

Fitness of saliva WNV populations is lower in avian cells relative to the input virus

The fitness of WNV within mosquito saliva was estimated using two methods. First, relative fitness was measured using *in vitro* competition against a genetically marked reference virus (**Figure 3.10A**, **Figure 3.11A**). The saliva-derived viruses were mixed 1:1 with the reference virus and added to a monolayer of chicken DF-1 cells. WNV in mosquito saliva was consistently displaced by the reference virus during direct competition. WNV recovered from *Cx. quinquefasciatus* represented only 10-20% of the total following 8 days of competition and was significantly lower than the proportion of the WNVic during competition ($p > 0.05$, Mann-Whitney test). WNV from *Cx. tarsalis*, *Cx. pipiens*, and *Ae. aegypti* saliva was undetectable by 2 days post infection. However, the range of high accuracy for the quantitative sequencing assay is 0.1 to 0.9 [171], therefore we can only determine that the undetectable competitor WNV is < 10%. Second, we calculated the ratios of viral GE to PFUs to determine if lower relative fitnesses were due to losses in infectivity (**Figure 3.10B**). We found no significant differences among species or in comparison to the input WNV ($p > 0.05$, Kruskal-Wallis-Dunn's corrections).

The competitive fitness data suggests that several mutations with decreased relative fitness are incorporated into the WNV populations during systemic mosquito infection and are transmitted in the expectorated saliva. Therefore we tested the relative fitness of the highest frequency nonsynonymous mutation detected in each sequenced saliva sample by engineering the mutations into the WNVic (**Table 3.1**). Five of the nine mutations lowered the fitness in DF-1 cells relative to the WNVic and the remaining four had no effect (**Figure 3.10C**, **Figure 3.11B**). A I449T mutation to the WNV nonstructural protein 3 (NS3) derived from *Cx. tarsalis* also had severe replication defects in DF-1 cells (**Figure 3.10D**), while mutation NS5-W808C

from *Ae. aegypti* had diminished replication in both avian (DF-1) and mosquito cells (*Ae. albopictus* clone C6/36) relative to the WNVic (Figure 3.11C). These data are consistent with the findings that ~60% of SNVs are predicted to be deleterious or lethal [411].

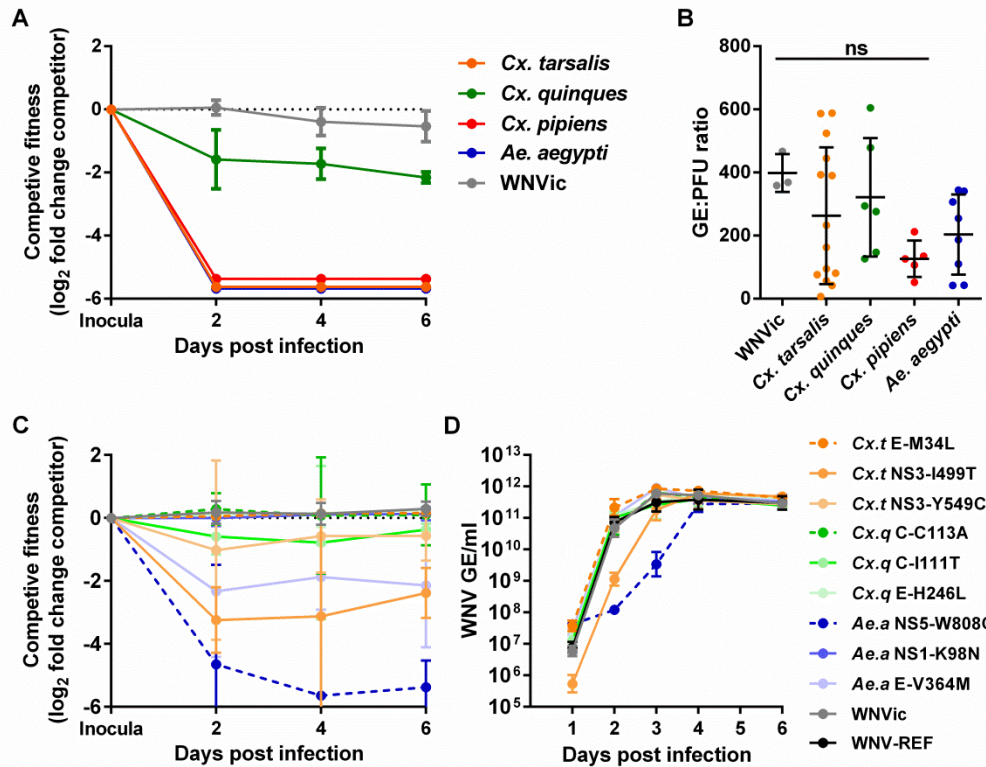


Figure 3.10. Viral populations in mosquito saliva have a lower fitness in bird cells relative to the input virus. (A) Competitive replicative fitness in DF-1 cells of the WNVic and WNV recovered from mosquito saliva (competitors) compared to a WNV reference (WNV-REF) during co-infection. The proportion of the competitor genotypes from the DF-1 supernatants were determined by quantitative sequencing (Figure 3.11A) and were normalized by the fold change (log₂ transformed) from the inoculum. Values below the dotted line at 0 represent samples with decreased competitive fitness compared to WNV-REF. The proportion of competitor from saliva samples that went to extinction (proportion = 0) was reset to 0.01 for fold change calculations. *Cx. quinques*, *Culex quinquefasciatus*. (B) WNV GE:PFU ratios from the bloodmeal and all saliva samples containing virus were calculated to determine if differences in relative fitness were due to differences in infectivity (mean with 95% confidence interval; ns, not significant). (C-D) The highest nonsynonymous variant detected in each recovered saliva sample was engineered into the WNVic to determine the fitness of mosquito-derived mutations (Table 3.1). (C) Competitive fitness of the mutants relative to the WNVic during DF-1 cell infection was determined as described in (A) (Figure 3.11B). (D) Replicative fitness of the mutants compared to the WNVic and WNV-REF during DF-1 cell infection was determined by qRT-PCR ($n = 4$ for each virus). Replication fitness in *Ae. albopictus* clone C6/36 cells is shown in Figure 3.11C. *Cx.t*, *Culex tarsalis*; *Cx.q*, *Cx. quinquefasciatus*; *Ae.a*, *Aedes aegypti*.

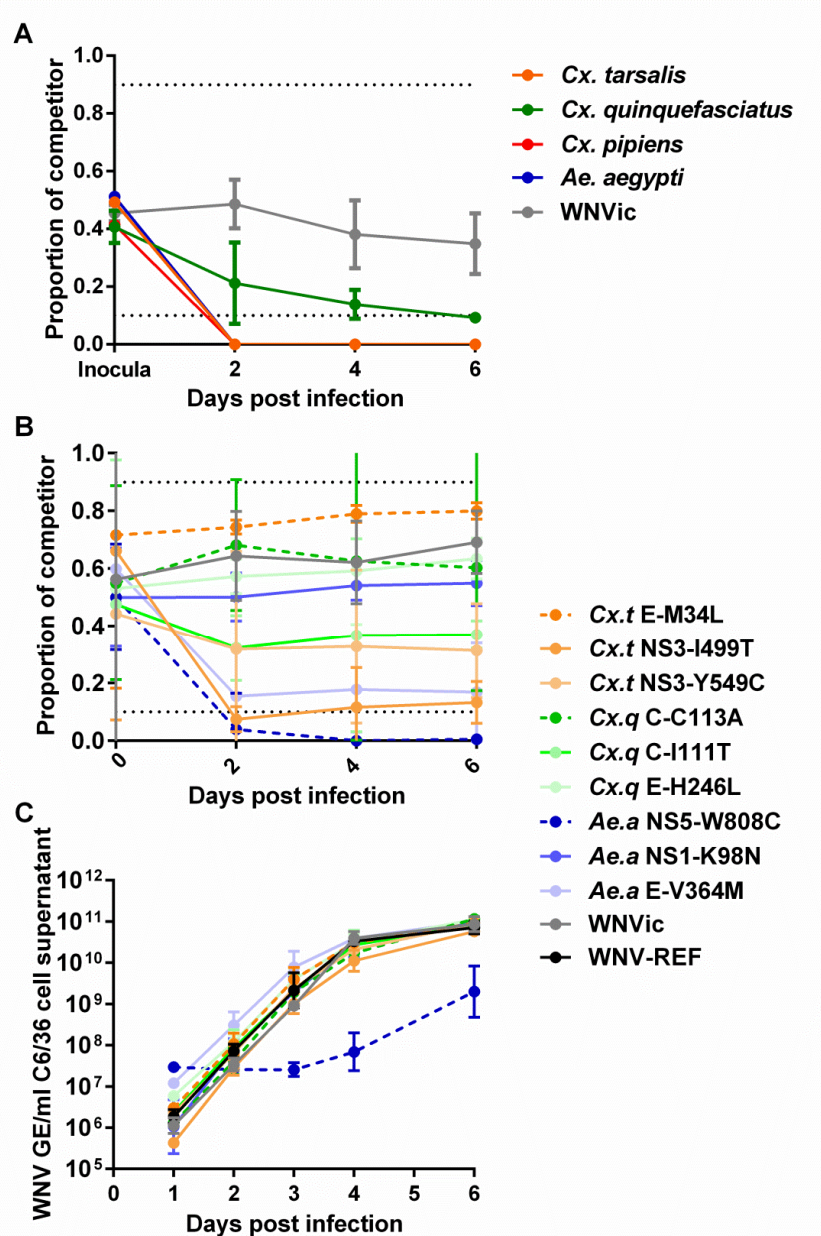


Figure 3.11. Competitive fitness of viral populations recovered from mosquito saliva and mosquito-derived mutations relative to the input virus. (A-B) Competitive replicative fitness in chicken fibroblasts (DF-1 cells) of the WNV infectious clone (WNVic) and (A) WNV recovered from mosquito saliva and (B) mosquito-derived mutations (competitors) compared to a WNV reference (WNV-REF) during co-infection. The proportion of the competitor genotypes from the DF-1 supernatants were determined by quantitative sequencing [386] and the dotted lines at 0.1 and 0.9 indicate the range of high accuracy [171]. The mosquito-derived mutations were the highest nonsynonymous variant detected in each recovered saliva sample and engineered into the WNVic (Table 3.1). (C) Replicative fitness of the mutants compared to the WNVic and WNV-REF during *Aedes albopictus* clone C6/36 cell infection was determined by qRT-PCR targeting the WNV envelope coding sequence ($n = 4$ for each virus). *Cx.t*, *Culex tarsalis*; *Cx.q*, *Cx. quinquefasciatus*; *Ae.a*, *Aedes aegypti*.

Discussion

Species-dependent impacts on viral genetic diversity

Using NGS we characterized WNV populations during systemic mosquito infection from individuals of four important WNV vectors. Our data confirm that replication in mosquitoes promotes WNV diversification [137,161,164]. Importantly, however, our data also show that the degree of viral divergence is species-dependent. Our first observation was that a much greater number of WNV consensus mutations occurred during replication in the tissues of *Cx. quinquefasciatus* and *Cx. tarsalis* as compared to *Cx. pipiens* and *Ae. aegypti*. Measures of genetic distance from the input virus also demonstrated that WNV replication in *Cx. quinquefasciatus* and *Cx. tarsalis* produced twice as many iSNVs compared to *Cx. pipiens* and *Ae. aegypti*. The nucleotide diversity from *Cx. pipiens* naturally infected with WNV [137] and field-derived *Ae. aegypti* experimentally infected with DENV [160] were similar to our intrahost WNV data from the same species (~1 per genome). However our calculations of WNV nucleotide diversity during replication in *Ae. aegypti* were about 4× lower than what was reported during CHIKV infection [117]. These results suggest that the evolutionary outcomes of RNA virus transmission by an arthropod are the product of specific virus-vector interactions that influence genetic drift and selection.

Repeated stochastic reductions in genetic diversity

Vector competence is largely determined by barriers to infection and escape from key mosquito tissues, principally the midgut and salivary glands. These anatomical barriers impose bottlenecks as arboviruses spread in mosquito tissues [292,295]. Several aspects of our data suggest that RNA viruses undergo stochastic reductions in genetic diversity in mosquitoes. First,

the majority of the iSNVs and novel haplotypes generated within one tissue were not detected in the subsequent tissue (e.g. iSNVs generated in the midguts were rarely detected in the legs). Second, Tajima's D (negative values), Harpending's raggedness (multimodal mismatch distributions), and the phylogenies (spatial structure and star-like topologies) suggest that genetic bottlenecks arise when new tissues are colonized [404-406,412].

The magnitude of these bottlenecks is however dependent upon the virus diversity, and amount of virus in the bloodmeal, and virus-vector pairing. We exposed mosquitoes to a high dose ($\sim 2 \times 10^8$ PFUs) of virus containing one dominant haplotype and several very low frequency variants. Ciota et al. found that high frequency haplotypes (≥ 0.15) are more likely to survive the bottlenecks within *Cx. pipiens* [292]. The same was true of WNV infection in *Cx. tarsalis* and *Ae. aegypti* wherein the input haplotype was dominant in all tissues, but not in *Cx. quinquefasciatus* in which the input haplotype went to extinction in the legs. Forester et al. determined the bottleneck severity is inversely proportional to amount of virus in the bloodmeal [295]. Therefore transmission cycles involving vertebrate hosts with high peak viremia, such as birds infected with WNV [176], may have less severe midgut bottlenecks compared to cycles involving hosts that develop lower viremias, such as rodents infected with some subtypes of Venezuelan equine encephalitis virus [413]. The high dose that we used for this experiment is representative of the dose present in natural avian bloodmeals; therefore the observation that midgut infection has the weakest bottleneck (52-129 founder genomes) among the barriers tested likely reflects natural WNV transmission. Moreover, the midgut infection bottleneck size is also dependent upon the virus strain and mosquito species pair. For example, Gutierrez et al. calculated about a 6 $\times$ greater number of founder genomes with enzootic VEEV strain paired with *Cx. taeniopus* ($N_e \sim 520$) than with an epizootic strain paired with *Ae. taeniorhynchus* ($N_e \sim 83$)

[296]. Our data demonstrate that population bottlenecks occur across a range of mosquito vectors of WNV and that bottleneck severity (i.e. small N_e) may be related to the strength of the anatomical barrier (small proportion of infected tissues). Specifically, within *Cx. quinquefasciatus* we predict that the least severe bottlenecks occur during midgut infection (~100% infection rate) and the strongest during egress into the saliva (~45%).

Recovery of genetic diversity during population expansions

Following stochastic reductions in genetic diversity, we observed rapid recovery in virus population size and diversity during expansions in the next tissue/compartiment that was likely promoted by RNAi [164,165]. However, our data does not support that differences in RNAi targeting were responsible for the differences in intra-tissue divergence detected among species. RNAi promotes diversification by selecting for rare haplotypes until they are no longer rare. This diversifying selection is best measured by Shannon entropy where genetic complexity is the greatest when the frequencies of two alleles at a locus are both 0.5 and complexity decreases as one allele becomes more dominant. Therefore diversifying selection as imposed by RNAi will act to increase genetic complexity more so than divergence. During mosquito infection, WNV complexity was not significantly different among species while more variants trended towards fixation during replication in *Cx. quinquefasciatus* and *Cx. tarsalis*. This suggests that bottlenecks and selection are more likely responsible for the species differences in viral divergence than RNAi.

Stochastic forces alter the genetic composition of the viral populations as they pass through the mosquito anatomical barriers, but as the population expands, even weak selection may play a role in WNV replication. All viral populations studied developed large numbers of

deleterious mutations (i.e. iLVs), but our measures of d_N/d_S suggest that selection may effect intra-tissue divergence. Specifically, the mosquito species with the most intra-tissue divergence, *Cx. quinquefasciatus* and *Cx. tarsalis*, also had the highest nonsynonymous mutation rates (d_N) despite *Cx. pipiens* and *Ae. aegypti* having similar d_S values. These variations appear to be due to differences in the strength of purifying selection and may be mediated by mosquito's ability to control WNV replication. Pressure against viral replication removes the least fit genomes first (e.g. nonsynonymous mutations with lowered fitness), and control of replication, such as in *Cx. pipiens* and *Ae. aegypti* midguts, lowers d_N . Thus, the comparatively rapid rates of WNV population expansion within *Cx. tarsalis* and *Cx. quinquefasciatus* may facilitate higher genetic divergence because there is less pressure from purifying selection.

Haplotypes arising *de novo* in mosquitoes were more likely to accumulate further mutations than the input haplotype ($\sim 2.5\times$). In fact, the intra-tissue populations with the most genetic diversity (especially within *Cx. quinquefasciatus*) appear to have been seeded by a haplotype that arose within mosquitoes (rather than the input WNV haplotype). We hypothesized that the mutations arising on *de novo* haplotypes could have 1) decreased the replication fidelity or 2) helped the virus to explore an adaptive landscape. Increased viral genetic diversity has been shown to be beneficial in mosquitoes [171], possibly by providing a mechanism for escaping RNAi [164,165] or by cooperative interactions between haplotypes and viral proteins [414]. However, decreased fidelity haplotypes often have lower relative fitness through accumulation of deleterious mutations [266,269]. *De novo* haplotypes increased in frequency and persisted in multiple tissues but did not cause an accumulation of iLVs compared to the other species; therefore it is not likely that these mutations caused changes in replication fidelity. Multi-step pathways that cause accumulations of viral mutations have been discovered in other viral

systems [415,416], including arboviruses [115,117]. The principle of a multi-step pathway is that even weak selection for a beneficial mutation allows a network of secondary adaptive mutations. Overall, our results suggest that multi-step adaptive pathways may arise during a single systemic mosquito infection.

Lower relative fitness in avian cells

Repeated reductions in genetic diversity may lead to the accumulation of mutations that confer low fitness [122,272]. As has been suggested for DENV [160], the immediate deleterious effects of bottlenecks and high mutation rates appeared to be avoided by WNV through the rapid recovery of viral genetic diversity during intra-tissue replication. However, rapidly expanding populations and high MOIs may allow mosquito tissues to tolerate new mutations, which could be either costly or beneficial in a new environment [402]. Surprisingly, we detected a severely lower competitive fitness of the saliva derived WNV populations relative to the input virus in avian cells. Several changes to the viral population structure may account for the relative fitness declines in the absence of notable consensus changes in the recovered saliva populations. All viral populations in our studies accumulated abundant iLVs (> 0.1 per coding sequence, all frame-shifting) and nonsynonymous mutations (> 0.5 per amino acid sequence), most of which are predicted to be lethal or deleterious [411]. In fact, five of most frequent nonsynonymous mutations detected from the nine recovered saliva populations engineered into the WNVic decreased the relative fitness of the virus. Furthermore, limiting our analysis to the protein coding sequence likely missed potentially important mutations to the untranslated regions that may have negatively influence RNA structure and fitness [417]. However, the viral genetic diversity detected in mosquito saliva may have benefits not measured in our experiments that could

facilitate rapid adaptation in new environments. In addition, the mechanisms of fitness recovery in a highly purifying avian environment require further study.

Arbovirus transmission cycles lead to slow rates of evolution [148]. A common explanation for this is that mutations occurring in mosquitoes are deleterious in vertebrates (and vice versa), leading to fitness trade-offs. However, this hypothesis has been debated due to conflicting results [140,142,157]. These data, combined with our previous studies [141,418], support a fitness trade-off in birds but not in mosquitoes. The difference between studies may be partially attributable to methods for measuring relative fitness (competitive vs replicative), MOI differences (low MOIs to allow for variants to reach their true fitness levels [121]), and the replication environment (*in vivo* vs *in vitro*). These differing results may also represent the complex nature of virus-host interactions. For example, flavivirus-*Culex*-bird and alphavirus-*Aedes*-rodent cycles may fundamentally differ in their evolutionary dynamics.

Conclusions

In addition to RNAi, inter-tissue bottlenecks and intra-tissue selection pressures can significantly alter viral populations. Our data demonstrate that *Cx. quinquefasciatus* may be significant drivers of WNV divergence and are more likely to transmit virus with consensus sequence changes compared to other mosquito species examined. We previously demonstrated in wild birds that WNV disease-susceptibility was negatively associated with maintaining viral fitness (Chapter 2). Taken together, we hypothesize that transmission cycles involving *Cx. quinquefasciatus* and American robins (disease resistant) would be more likely to produce novel WNV genotypes while maintaining high viral fitness than transmission cycles involving *Cx. pipiens* and American crows (disease susceptible). In addition, we have outlined the stochastic

and deterministic forces that continuously shape viral populations (**Figure 3.12**). At anatomical barriers, viral populations undergo population bottlenecks that greatly reduced genetic diversity through drift and founder's effects. A small virus population seeds subsequent tissues and then rapidly expands. Population fluctuations and genetic diversity led to tissue-specific viral haplotypes distinct from the input virus population. The impacts of repeated bottlenecks on the virus populations are important for two main reasons. 1) The high variance in variant frequencies detected among the mosquito-borne viruses should allow the populations to explore very different adaptive landscapes [97,98], such as would be expected between mosquitoes and birds. 2) However, genetic drift coupled with weak purifying selection in mosquitoes may also lead to the accumulation of deleterious mutations (i.e. mutational load). Therefore, the collective fitness of the expectorated WNV from mosquitoes started at a point of lower relative fitness (i.e. in a fitness landscape valley) than the input viruses during avian cell infection. Thus, most of the mosquito-derived viruses were rapidly removed by strong purifying selection and/or were displaced by the more fit input viruses during mass selection. The observed lower fitness relative to the input virus is reminiscent of that predicted to occur as a result of Muller's ratchet [271], which has been observed to result in virus fitness declines *in vitro* [122,272], but not previously *in vivo*. Taken together, our results illustrate the irony of arthropod transmission, and may explain why arboviruses have low long-term rates of amino acid substitution compared to other host-specific RNA viruses [148].

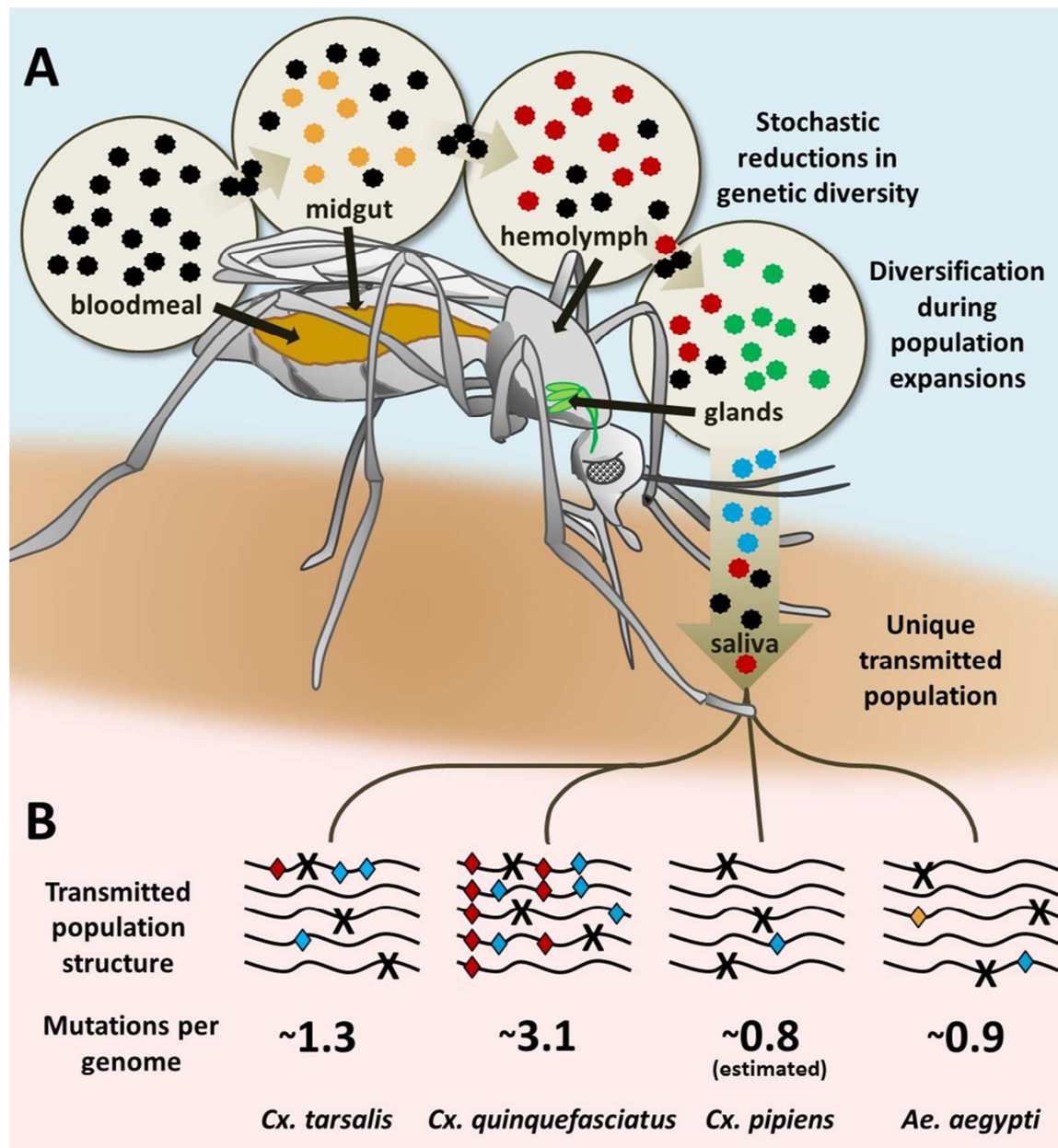


Figure 3.12. Impacts of anatomical barriers and mosquito species on viral population structure. (A) Mosquitoes feed upon a bloodmeal containing a relatively homogenous WNV population than seeded infection in the midgut epithelial cells. Within the tissue, the viruses rapidly diversified during a phase of population expansion and weak purifying selection. Only a few viruses escaped and seeded infection in the next set of cells, reducing genetic diversity. The cycling of stochastic reductions and rapid diversification led to unique subpopulations in each tissue and transmitted in the saliva. (B) The genetic diversity of the transmitted viral populations is dependent upon the vector species, but all accumulate potentially deleterious mutations such as frame-shifting insertions and deletions and low fitness amino acid substitutions (mutations per genome represent both iSNVs and HF iLVs). The virus and mutation colors represent tissue of origin (bloodmeal = black, midgut = orange, hemolymph (legs) = red, salivary glands = green, saliva = blue) and “X” represents predicted deleterious mutations.

Chapter 4: Region specific patterns of West Nile virus population structure, injury, and interferon-stimulated gene expression in the brain from a fatal case of encephalitis

Introduction

West Nile virus (WNV) is a mosquito-borne virus that can result in human neuroinvasive disease (WNND) [419]. Postmortem studies from patients with WNV encephalitis reveal characteristic neuronal loss and glial nodules in the grey matter of the thalamus, medulla, pons, midbrain, basal ganglia, and anterior horn of the spinal cord [420]. However, cortical neurons do not display pathologic injury. In mice, regional neuronal susceptibility is in part due to type I interferon-dependent restriction of WNV infection through the induction of interferon-stimulated genes (ISGs) [373,421]. In human cases, the underlying distribution of ISG expression, WNV quantitative loads, viral genetic diversity, and the degree to which they associate with central nervous system (CNS) injury are not known. Accordingly, we examined these factors in distinct brain regions of a patient with WNND. Our data suggest that thalamic regions displaying neuropathologic and neuroimaging evidence of injury also exhibit high ISG expression and viral loads associated with increased WNV amino acid diversity. In comparison, cortical neuronal regions exhibit unexpectedly high viral loads but minimal injury, decreased ISG expression, and lower viral amino acid diversity.

Materials and methods

Tissue collection and immunohistochemistry

CNS tissue was placed in RNAlater (LifeTechnologies) or 10% formalin for downstream analysis and total RNA isolated using the High Pure Viral RNA kit (Roche). Fixed brain tissue

samples were prepared for immunohistochemistry (IHC) analysis with an additional step of three subsequent 7 min treatments of 0.1% sodium borohydride in PBS to remove background autofluorescence. Tissues were labeled with rabbit monoclonal antibody against cleaved-caspase 3 (CC3, Cell Signaling #9664) and mouse monoclonal antibody to WNV envelope (ATCC, clone E18, VR-1611) at a dilution of 1:100 overnight at 4 °C. TritC conjugated Goat anti-IgG (Jackson ImmunoResearch) was used for secondary staining. Coverslips were mounted with ProLong Gold antifade reagent (Life Technologies), images obtained using an Olympus VS120 Virtual Slide system, and analyzed using Olympus VS-Desktop software. Secondary only labeling controls were used to calibrate exposures for each tissue type. All research has been reviewed and approved by the University of Colorado Institutional Biosafety Committee and Colorado Multiple Institutional Review Board (COMIRB).

RNA isolation and sequencing

WNV RNA copies per mg of tissue were determined from total RNA isolated from each indicated CNS region using previously described primers and qRT-PCR [388]. Ribosomal depleted (RiboMinus, Waltham, MA) total RNA from each brain region was prepared for next-generation sequencing (NGS) using the Ovation RNA-Seq System V2 and Ultralow Library kits (NuGEN, San Carlos, CA), as described in Chapter 2, on the NextSeq 500 platform (Illumina; sequenced at Colorado State University IDRC Genomics Core). The 150 nucleotide (nt) paired-end reads were demultiplexed using BaseSpace (Illumina). To determine ISG expression, reads were aligned to human *Stat1* (GenBank accession no. NM_007315), *Rsd2* (NM_080657), *Ifi27* (NM_001288957), *Irg1* (NM_001258406), *Irf1* (NM_002198), *Oas1* (NM_016816), and *Ifit1* (NM_001548) using MOSAIK [390].

To obtain the consensus WNV sequence from this patient and to define viral genetic diversity, WNV reads from the frontal cortex were assembled using Trinity [389] to create a reference sequence (KT020853) for guided assembly from each region using MOSAIK. Intra-tissue WNV minority nucleotide variants were analyzed using Vphaser2 [391].

Results

Case report

A 51 year-old female presented with 2 days of increasing altered mental status and decreasing responsiveness. Illness was preceded by 4 days of fever, nausea, and diarrhea. The patient's past medical history was remarkable for a history of rheumatoid arthritis treated with methotrexate and prednisone, last on treatment 1 year prior to presentation. The patient was evaluated in the emergency room, intubated for airway protection, and lumbar puncture performed to obtain cerebral spinal fluid (CSF), which exhibited 163 white blood cells/ml with a differential of 99% lymphocytes. Magnetic resonance imaging (MRI) revealed increased signal in the insula, medial temporal lobe, medial left thalamus, and left cerebral peduncle (**Figure 4.1A-B**). CSF was positive for WNV IgG (1.82, normal <1.29 IV) and IgM (8.74, normal <0.89 IV; Focus Diagnostics ELISA), but CSF and serum were negative for WNV RNA by RT-PCR (ARUP Laboratories, Roche Molecular Systems Inc.). With these data, the patient was diagnosed with WNV encephalitis. During hospitalization in the intensive care unit, the patient was weaned from sedation, remained comatose, and had an electroencephalogram (EEG) showing diffuse slowing. The patient did exhibit brain stem function but minimal peripheral responses with a physical exam consistent with loss of lower motor neuron function in all four extremities. MRI of the spine exhibited no spinal cord lesions. Despite aggressive supportive care, the patient

passed away following a cardiac arrest at day 11 of hospitalization. Autopsy was initiated 22 hours and 30 minutes after death and found no evidence of myocardial infarction or coronary artery disease. Brain tissue was collected at time of autopsy.

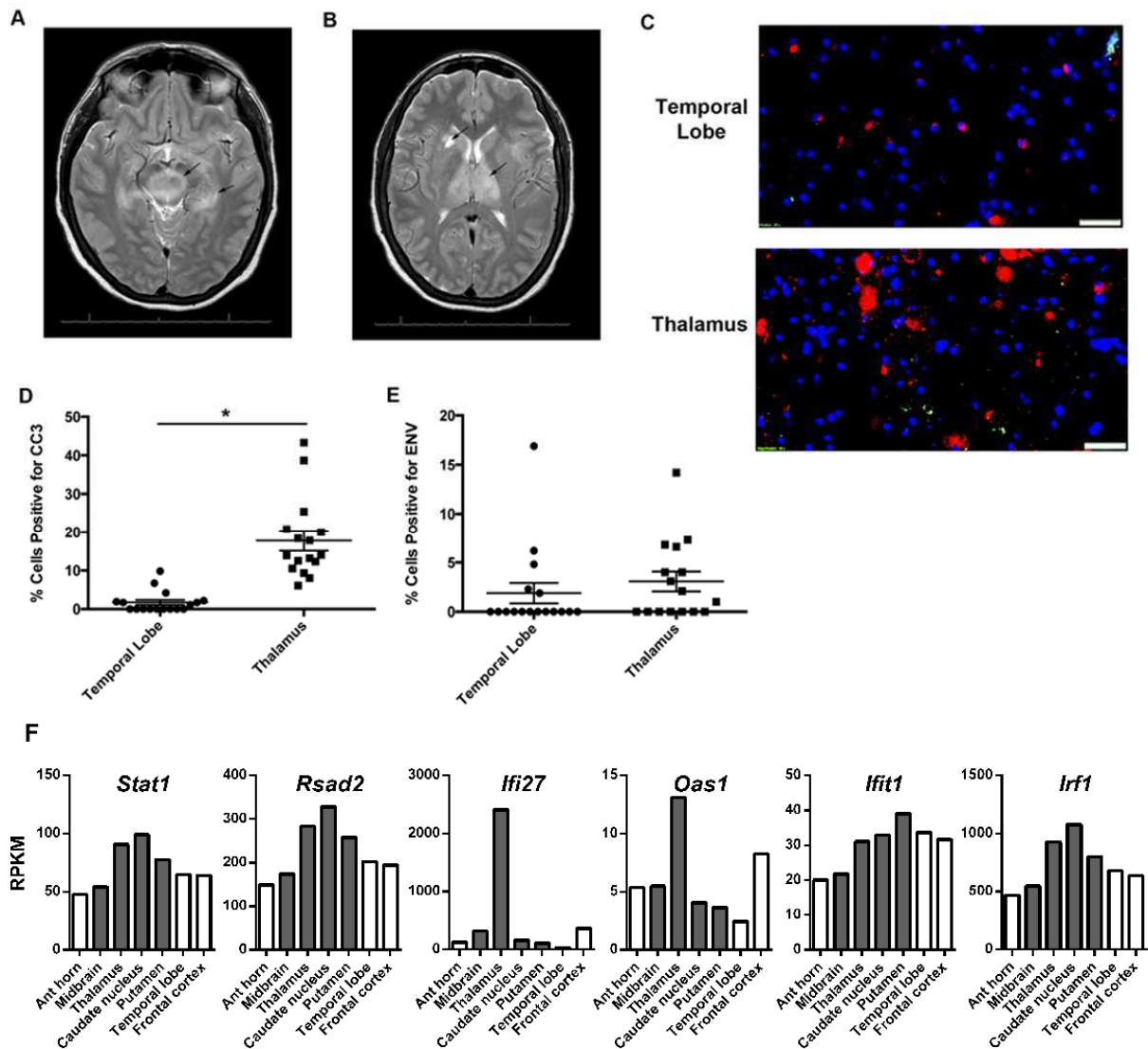


Figure 4.1. Regional MRI injury patterns correlate with apoptosis and ISG expression. MRIs (T2 sequences) showing increased signal intensity in the (A) midbrain substantia nigra and left mesial temporal lobe (arrows) and (B) the thalamus and right caudate nucleus (arrows). (C) IHC staining for cleaved-caspase 3 (cy3, red) and WNV envelope antigen (TRITC, green) from indicated brain regions. Bar=50μm. Percent of cells per high-power field positive for (D) cleaved-caspase-3 (CC3) and (E) WNV envelope antigen. * $p < 0.0001$, unpaired T-test. (F) Reads per kilobase per million mapped reads (RPKM) to ISGs from different brain regions were determined by next-generation sequencing. Grey bars indicate brain regions with neuronal injury.

Brain injury and ISG expression

MRI evidence of injury in the thalamus correlated with a 10-fold increase ($p < 0.0001$) in expression of cells positive for a marker of apoptosis (CC3) when compared to an uninjured region in the temporal lobe (17.8 ± 2.6 vs 1.7 ± 0.67 , mean positive cells per HPF $\pm$ SEM, **Figure 4.1C-D**). CC3 expression was not correlated with WNV envelope antigen expression (**Figure 4.1E**). Using NGS of whole tissue RNA, we determined the relative expression of ISGs found to be important in murine neuronal control of WNV [352] between indicated regions of the CNS during acute human WNV encephalitis (**Figure 4.1F**). We found that, in general, ISG expression was the highest in the subcortical tissues of the thalamus and basal ganglia (caudate nucleus and putamen) that also exhibit injury.

Viral loads

As detailed above, CSF was positive for WNV IgG and IgM but negative for WNV RNA. However, the frontal cortex, thalamus, and anterior horn of the spinal cord exhibited in excess of 1×10^7 WNV RNA copies per mg of tissue; whereas, the midbrain, caudate nucleus, putamen, and temporal lobe all exhibited less than 1×10^6 WNV RNA copies per mg of tissue (**Figure 4.2A**). From NGS, each tissue yielded 25-33 million sequencing reads and 0.04% (frontal cortex) to 0.00004% (temporal lobe) of these aligned to WNV genetic sequences. WNV population size (WNV RNA copies) directly correlated with the WNV sequencing coverage as previously described (**Figure 4.2B**) (Chapter 2). The consensus WNV genome sequence obtained from this patient belonged to the WN02 genotype (**Figure 4.3**); however, the WNV consensus sequence contained several novel amino acid substitutions found mostly within the viral nonstructural proteins (**Figure 4.2C, Table 4.1**).

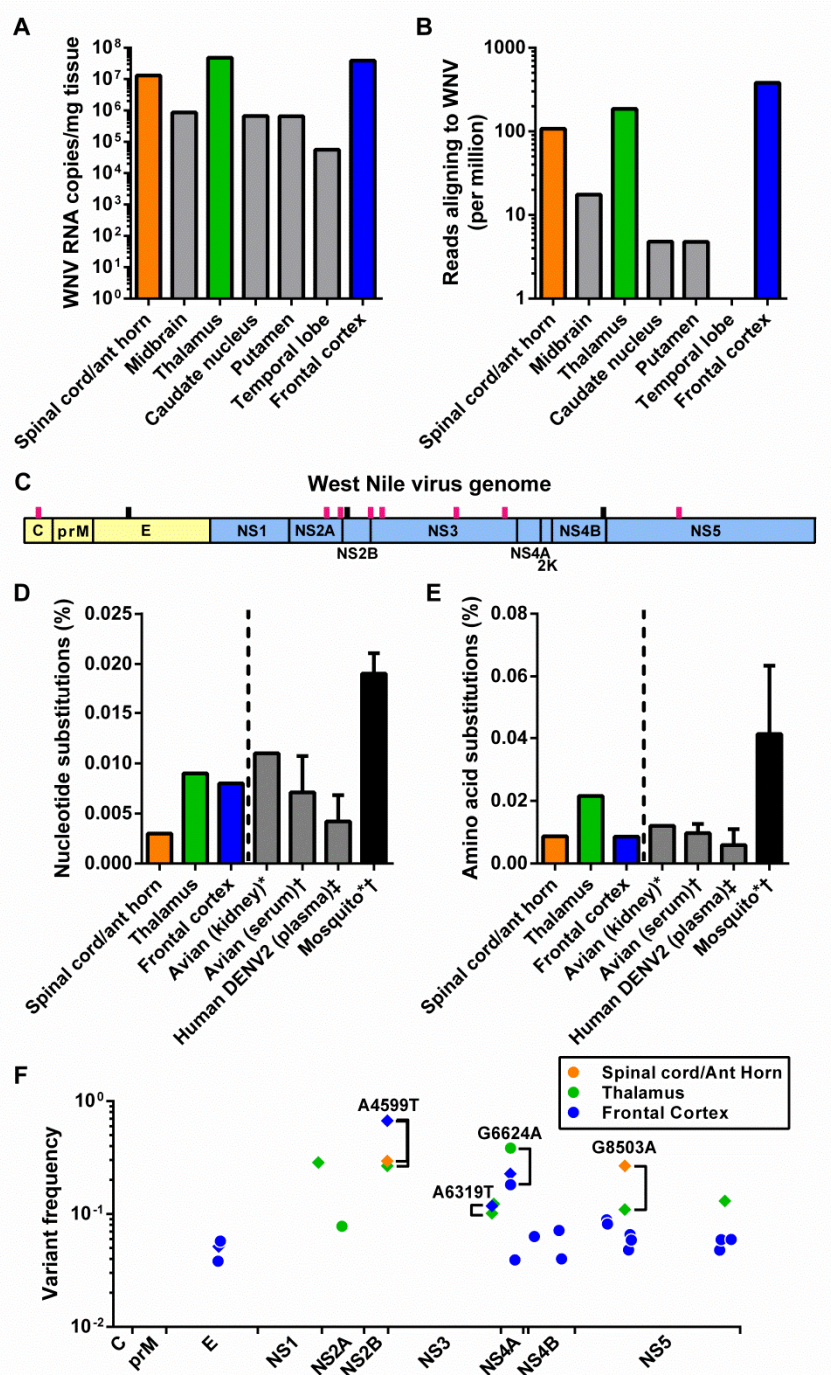


Figure 2. Variations of WNV copies and population structure among brain regions. (A) WNV RNA was quantified from different brain tissues, prepared for next-generation sequencing, and (B) aligned to the WNV genome (displayed as number of aligning reads per million reads sequenced). The colored bars represent tissues with enough WNV coverage for subsequent population genetic analysis. (C) The consensus WNV sequences from each tissue were analyzed and each line represents an amino acid change compared to the prototype strain NY99 (pink lines represent novel mutations, see also Table 1). The percent of all sequenced WNV (D) nucleotides and (E) amino acids with substitutions were compared to published reports of WNV and dengue

virus in other vertebrate samples and WNV in mosquitoes. (F) Individual intra-tissue WNV variants were plotted across the genome. Diamonds represent amino acid substitutions and circles represent silent mutations. *data adapted from Chapter 2; † data adapted from reference [137]; ‡ data adapted from reference [422].

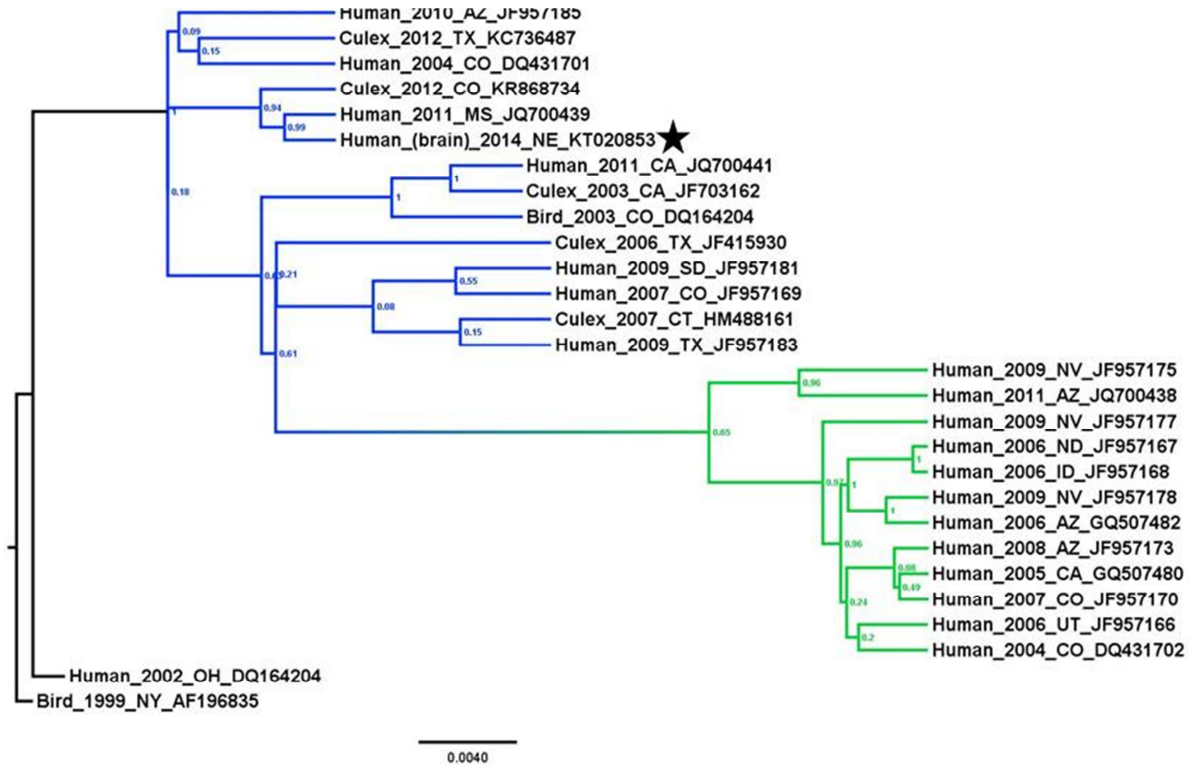


Figure 4.2. WN02 genotype WNV recovered from the patient's brain. Phylogenies were constructed using the Bayesian Markov chain Monte Carlo method in BEAUti and BEAST (v1.8) [407] with a HKY substitution and gamma site heterogeneity model and a lognormal relaxed molecular clock. Star = brain sequence, black = NY99 genotype, blue = WN02 genotype, green = SW03.

Table 1: WNV amino acid substitutions compared to the prototype strain NY99.

Strain (source, year, state)	Pairwise identity	Nt changes	WNV genome nucleotide position										
			268	1442	4025	4208	4294	4599	4749	5717	6350	7635	8621
NY99 (flamingo, 1999, NY)			G	T	G	A	A	A	C	G	T	A	A
04-214CO (human, 2004, CO)	99.8	25	●	C	●	●	●	●	●	●	●	●	●
CO5-07 (human, 2007, CO)	99.6	41	●	C	●	●	●	●	●	●	●	●	G
BSL6-11 (human, 2011, MS)	99.4	61	●	C	●	●	G	●	T	●	●	G	●
AVA1202600 (<i>Culex</i> , 2012, TX)	99.4	64	●	C	●	●	●	●	●	●	●	●	●
FtC-3699 (<i>Culex</i> , 2012, CO)	99.4	64	●	C	●	●	●	●	●	●	●	●	●
BSL2-10 (human, 2010, AZ)	99.3	68	●	C	●	●	●	●	●	●	●	G	G
<i>Spinal cord/ant horn</i>	99.2	85	A	C	A	●	G	●/T	A	●	●	G	T
<i>Midbrain*</i>			A	C	A	●	G	●/T	--	T	●	G	T
<i>Thalamus</i>	99.2	85	A	C	A	●	G	●/T	A	●	●	G	T
<i>Caudate*</i>			A	--	A	T	--	●/T	A	●	C	G	T
<i>Putamen*</i>			A	--	A	●	--	--	--	--	●/C	--	--
<i>Frontal cortex</i>	99.2	86	A	C	A	●	G	T/●	A	●	●	G	T
Protein aa #			A58T	V159A	R167K	N228I	I26V	Q94H	F46L	G369C	H580Q	I240M	K314M
Protein			C	E	NS2A	NS2A	NS2B	NS2B	NS3	NS3	NS3	NS4B	NS5

Nt, nucleotide; aa, amino acid; C, capsid; E, envelope; NS, nonstructural; ●, ancestral nucleotide.

*, could not determine pairwise identity or the number of nucleotide differences due to incomplete coverage of the coding sequence.

Intratissue WNV genetic diversity

Due to low sequencing coverage of WNV from some tissues, only the cortex, thalamus, and anterior horn regions were analyzed for WNV population diversity. The percent of nucleotides and amino acids with substitutions found within these brain regions was comparable to that previously reported for WNV and dengue virus isolated from other vertebrate tissues, but less than from mosquitoes (**Figures 4.2D-E**) [137,422](Chapter 2). The thalamic and cortical tissues contained similar nucleotide diversity, but most of the WNV variants detected in the cortex were silent mutations (i.e. do not change the amino acid sequence) (**Figure 4.2F**). The thalamus contained considerably more WNV amino acid substitutions in comparison to the cortex. The intra-tissue WNV nucleotide variants tended to be concentrated in the nonstructural genome regions, except for three envelope variants found in the cortex (**Figure 4.2F**). Four nucleotides were maintained as minority variants between at least two different regions, but the majority of the nucleotide variants were found only in a single brain region (**Figure 4.2F**). We found additional evidence for region-specific differences in WNV populations by comparing the partial consensus sequences from all of the brain tissues obtained (**Table 4.1**).

Discussion

Neuronal injury is independent of viral replication

The presented clinical symptoms and injury patterns, defined by neuroimaging and immunohistochemistry, in the subcortical grey matter of the thalamus, basal ganglia, and midbrain were representative of a typical brain associated with WNV encephalitis [423,424]. To our surprise, we found high viral loads in the frontal cortex despite showing little injury, while regions exhibiting injury exhibited highly variable viral loads. These data suggest that in human

WNND, neuronal injury may be independent of viral replication and injury in the brain may be due to regional factors.

Differential ISG expression is associated with neuronal injury

Postmortem studies have shown that injury patterns correlate with inflammatory infiltrates in the brain [420]; suggesting that inflammation and innate neuronal subtype susceptibility to injury are important determinants of disease. The ISGs *Irf1*, *Irg1*, *Ifi27*, and *Rsad2* were shown to inhibit WNV replication in cortical neurons and they were more highly expressed in the cerebellum compared to the cortex of mice [352]. In this patient, we also show that expression of *Irf1*, *Ifi27*, and *Rsad2*, in addition to *Stat1*, *Oas1*, and *Ifit1*, are regionally heterogeneous, with the high levels generally found in the subcortical regions of the brain. Thus, it may be that regional differences in ISG expression may contribute to the regional patterns of neuronal injury.

WNV selection is stronger in injured regions

Our data on region-specific patterns of WNV genetic diversity further suggest that WNV encounters different selective pressures and/or stochastic bottlenecks (i.e. random selection of viral variants) as it spreads throughout brain. As with injury, the regional differences in immune responses and susceptibility of neuronal subtypes may contribute to the strength of selection. For example, WNV populations in the thalamus exhibited an increased frequency of amino acid substitutions when compared to the cortex, suggesting an association between increased ISG expression and selection in the CNS. Although consensus amino acid differences between the WNV populations of the cortex and the thalamus were minimal, an expanding body of evidence

demonstrates that minority variants may directly and significantly influence WNV phenotype [131,168].

Future directions

This work is from a single patient; therefore, the findings are hypothesis generating and need to be verified in experimental models. However, the confluence of human clinical data and timely acquisition and analysis of human brain tissue is difficult to complete for this sporadic infection. In this one host, we used NGS to show evidence of intra-regional variation in viral populations and ISG expression, which was similar to the findings presented from a mouse model [352]. Still, other hosts (or patients) with differing ISG patterns may exhibit different regional viral genetic variation. Thus, further studies of minority WNV variants and immune activation in the human CNS will be needed to identify mechanisms of viral selection that are associated with injury and disease.

Conclusions

We report the first comprehensive study of WNV variation within a human case of viral encephalitis and describe possible associations between viral nucleotide substitutions, neuroradiographic and pathologic injury patterns, and ISG expression. These data should guide future investigations into the role of inflammatory responses in selective viral pressures and injury patterns in the brain following viral infections.

Chapter 5: Concluding Remarks

Application of new technology to West Nile virus evolution

Arboviruses such as West Nile virus (WNV), chikungunya virus (CHIKV), and Zika virus are emerging and re-emerging threats to human health worldwide. Genetic complexity of viruses within hosts contributes to their ability to rapidly adapt to new environments [49-51], cause disease [131-134], and evade antiviral defenses [425]. It is therefore critical that we develop a more detailed understanding of the virus-host interactions that influence viral population structures and fitness. Previous *in vitro* studies provided the framework for the viral quasispecies theory and many other aspects of intrahost virus evolution [49-51,121-123], but the challenge of today is to apply these concepts to more natural settings. Advancements in molecular technology, particularly next-generation sequencing (NGS), helped to reveal details about the evolutionary dynamics of viral populations during intrahost dissemination, transmission, and disease outbreaks at incredible resolution [117,139,158,160,378,426,427]. Applying these advancements to experimental evolutionary studies of arboviruses within ecologically relevant hosts will help to unravel the complexities of the transmission cycles and predict evolutionary patterns.

The work contained in this dissertation sought to describe the formation of WNV populations during infection of important avian hosts (Chapter 2), mosquito vectors (Chapter 3), and a human brain from a fatal case of encephalitis (Chapter 4). Taken together, one conclusion is fairly obvious: WNV evolution is highly dependent upon the host environment. There are major differences between the WNV population structures formed within mosquitoes and birds, but also subtle differences with significant consequences between species. It demonstrates that

WNV and other arbovirus populations constantly encounter unique pressures that alter its evolutionary trajectory. Fitting together these complicated pathways will help us to create a more refined model of arbovirus evolution.

Selection vs genetic drift

We first describe that the dominant WNV sequence is commonly maintained during wild bird infection. In fact, in the 45 birds analyzed (3 species, 5 passages, and 3 replicates/passage), only 14 mutations arose to > 50% frequency in the serum and only three were nonsynonymous. The lack of genetic shuffling shows that selection rather than random genetic drift is the prevailing force in birds. This also indicates that there are not severe population bottlenecks between the site of infection (subcutaneous layer of the breast plate) and the serum. Similarly, we did not find evidence for bottlenecks among the anterior horn of the spinal cord, thalamus, and frontal cortex regions in the human brain. Perhaps this indicates that within vertebrates, WNV populations remain large enough to be constantly molded by natural selection (or remain too large to be affected by drift). Mosquitoes, on the contrary, impose several population bottlenecks during systemic WNV infection. The bottlenecks prevented most of the viral variants from passing between tissues and compartments, forcing the viruses to evolve in isolation and creating unique subpopulations. Random sampling of variants at tissue barriers can redistribute the WNV mutant spectra, further increasing the differences between subpopulations. Therefore, genetic drift is much stronger in mosquitoes than in vertebrates.

The rarity of high frequency amino acid substitutions detected in birds demonstrates that 1) the dominant WNV sequence used to initiate infection sits at a high fitness peak and 2) purifying selection is very strong. Confined by selection, WNV could only accumulate a small

amount of genetic diversity during avian infection which was similar between bird species (as measured by genetic complexity [Shannon entropy] and distance). As predicted [131,137,161], WNV populations in mosquitoes were not confined by strong purifying selection and accumulated significantly more genetic diversity than from birds (**Figure 5.1**). The increased genetic diversity in mosquitoes is likely due to a combination of genetic drift and RNA interference (RNAi)-mediated diversifying selection [164,165]. As with birds, genetic diversity, as measured by richness and complexity, was similar between mosquito species. Even though we detected bird and mosquito species-dependent impacts on a few mutations that arose to high frequency, these data advocate for a universal set of rules that govern intrahost genetic diversity. One broad hypothesis is that there is an equilibrium between viral diversification and host restriction within an insular replication environment (like within a mosquito or bird). This was borrowed from a similar type of governance formulated by MacArthur and Wilson to understand the regulation of animal species on an island [428]. They proposed that where the rates of immigration (viral diversification) and extinction (host restriction) intersected would represent an equilibrium and the total number of species (viral variants) present within the island (host or tissue). Furthermore, the equilibrium can change and is set by island size and distance from mainland. In our case, the equilibrium is likely set by specific virus and host factors, meaning that the equilibrium is higher in mosquitoes than birds but may not be the same between different arboviruses. It is possible that many different patterns follow similar rules; however, this requires direct testing for intrahost viral genetic diversity.

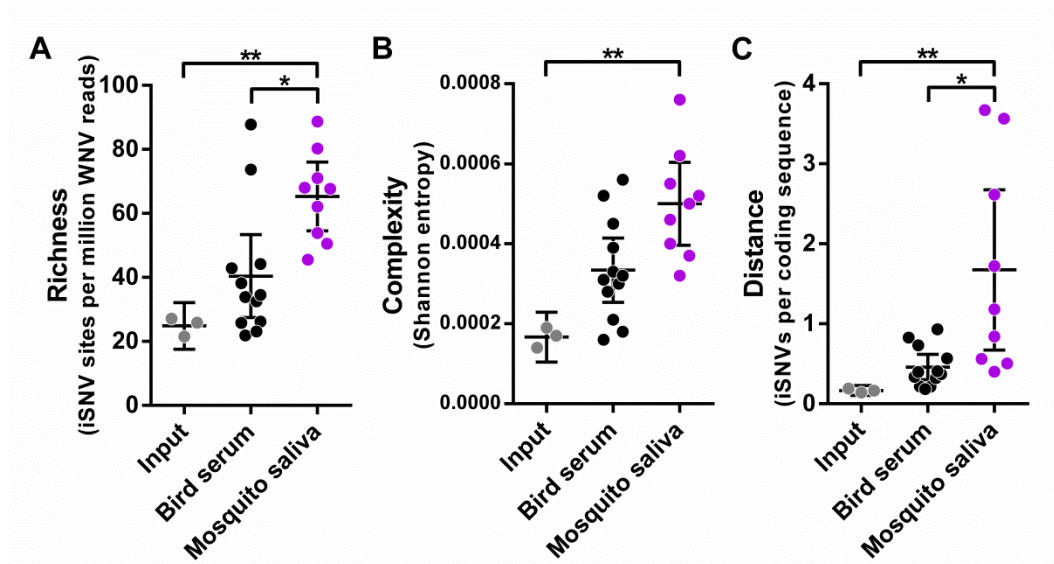


Figure 5.1. Genetic diversity is greater in mosquito saliva than bird serum. Viral intrahost genetic diversity from bird serum (Chapter 2) and mosquito saliva (Chapter 3) was characterized by measuring (A) richness, (B) complexity (the proportion of different variants in a mutant spectrum), and (C) iSNV distance. Data shown as means with 95% confidence intervals (*, $p < 0.05$, **, $p < 0.01$ by Kruskal-Wallis-Dunn's corrections).

Virus-host interactions that drive viral adaptation

There were some very interesting differences between the WNV population structures among the infected crows, sparrows, and robins. In the birds most susceptible to disease (crows, 100% mortality rate), we detected more unique variant loci (genetic richness) and deleterious mutations (frame-shifting insertions and deletions). In the bird least susceptible to disease (robins, 0% mortality), new variants and haplotypes arose to higher frequency. Many of these high frequency WNV mutations found in robins altered the amino acid sequence and were not found in the other bird species. This suggests that there may be some weak positive selection occurring in robins, and it may be associated with a more robust antiviral response that limits viral replication and susceptibility to disease. A similar trend was found in the different brain regions. The thalamus, which expressed the highest levels of interferon-stimulated genes, also had the highest levels of amino acid substitutions among the regions analyzed. The changes to

the WNV amino acid sequence were mostly found in the nonstructural protein coding regions that are important for viral counter-defenses to the host innate immune response. The data presented here fit the predictions described by Grenfell et al. that during infection of susceptible hosts (and tissues), viral adaptation increases (commonly estimated by amino acid changes) with the strength of the immune response [333]. The scale obviously tips when the immune response is too strong, but since viral replication was still occurring, we assume the strength did not progress much beyond “moderate”.

During infection of mosquitoes, we also found differences in WNV population structure among the species associated with rate of WNV population expansion. Purifying selection was stronger in mosquitoes better at controlling the rates of intra-tissue WNV population expansion (i.e. within *Cx. pipiens* and *Ae. aegypti* midguts). The presence of severe bottlenecks, however, randomly altered the distribution of variants and made it difficult to detect positive selection. Therefore, we can suggest an association between viral replication control, possibly mediated by a stronger innate immune response, and the level of purifying selection within mosquitoes, but not positive selection.

Interestingly, no mutations to the protein coding sequence were reproducible at high frequencies among any of the sequenced samples. This is somewhat surprising because the WNV that was used in Chapters 2 and 3 was from the NY99 genotype that was quickly displaced by WN02 [101], suggesting there was room for adaptation that was not explored during our experiments. Even more surprising was that the WN02 mutation to the envelope protein, A159V, was never detected in any of our samples, even at low frequencies. However, there were mosquito- and bird-specific mutations that occurred in the 3' stem loop (3'SL) of the untranslated region (UTR) that were highly reproducible. Analyses of both UTRs were not

included in the research chapters because not every sample had enough coverage at the extreme ends of the genome to enable proper population genetic statistical analysis. Yet, in the samples with sufficient coverage, we consistently detected distinct UTR mutations in 3-15% of the haplotypes (**Figure 5.2**). In birds, there were three U-to-C mutations at sites 10956, 10957, and 10959 all found on the same haplotype that “loosened” the 3'SL. Conversely in mosquitoes, there were four single nucleotide mutations and one insertion co-occurring on the same haplotype made the entire stem a perfect double-stranded RNA match. Why and how these haplotypes form is not known. Intermediates with only some of the described mutations were never found, so perhaps these arise via host RNA editing similar to APOBEC3G editing of HIV [429]. If these mutations are arising in birds via RNA editing, then by what mechanism? U-to-C editing is known to occur, however it is very rare [430]. Perhaps a more logical explanation is that adenosine deaminases may be performing the more common A-to-I editing on the negative viral RNA strand [431] and then being copied as U-to-C changes on the positive strand. However, it is not even clear at this point if they provide a virus or host advantage. One thing that is clear though is that they were never found at higher than 15-16% frequency; therefore, some force is limiting their accumulation. Further experimentation is necessary to determine how these haplotypes are infective on their own, if long distance *cis* acting RNA-RNA interactions required for negative-strand synthesis are impaired [245-248], or if they can somehow present themselves as a decoy to maintain fitness of the population (a true quasispecies trait).

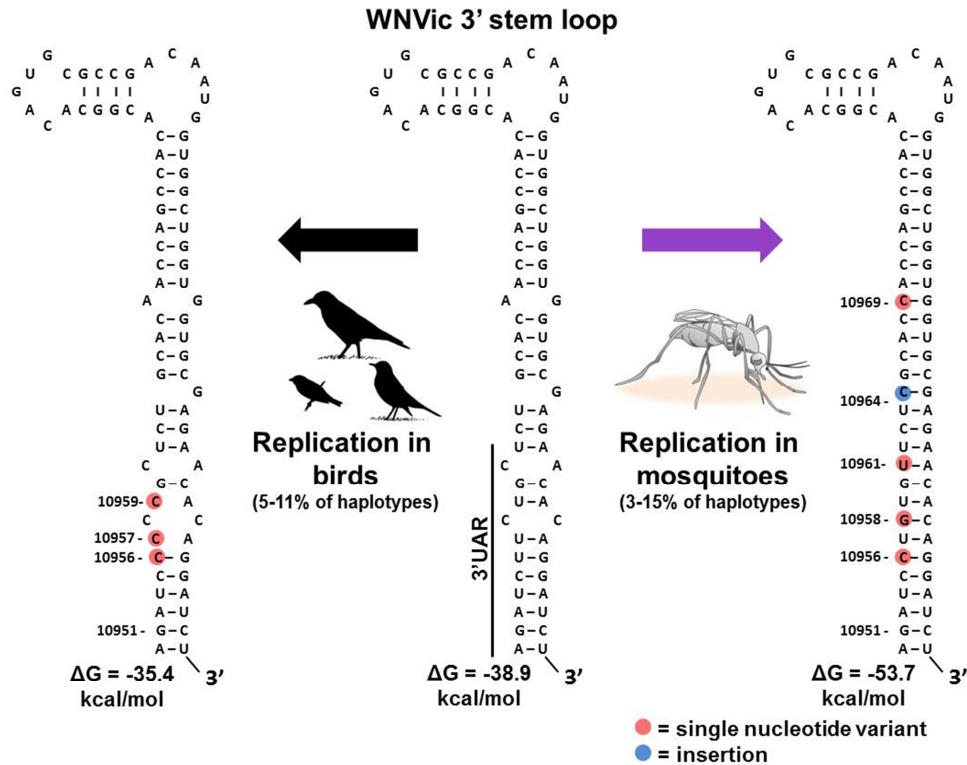


Figure 5.3. Species-dependent alterations to the WNV 3' stem loop. Mutations co-occurring on the same sequences were detected during WNV replication in birds (Chapter 2) and mosquitoes (Chapter 3), and were not found in the opposite host or the input WNV infectious clone (WNVic). Changes to Gibbs free energy (ΔG) was calculated using Mfold [432].

Fitness trade-off hypothesis

A significant consequence of WNV replication in crows is lower relative fitness in chicks compared to WNV derived from sparrows and robins. We believe this to be a product of the large WNV population sizes generated during infection of crows, potentially leading to increases in intrahost MOI and thereby complementation. Indeed, WNV populations recovered from crow serum contained more genetic load (deleterious mutations such as insertions and deletions) and evidence for weaker purifying selection compared to the other species, supporting the MOI-complementation hypothesis. Populations carrying extra genetic load would need to be purged in the next host, as was seen in our fitness competitions in chicks. However, the replication environments in the different birds did not significantly alter the relative fitness of the WNV

populations when transmitted to mosquitoes. Combined with previous data [141,171], it appears that mosquitoes are very tolerant of several different WNV population structures, again indicating that purifying selection is weak. Then it should not be surprising that a replication environment dominated by weak purifying selection, genetic drift, and RNAi-dependent diversification (which does not act to maintain amino acid integrity) would lead to severe relative fitness declines in hosts dominated by selection – birds. As a result there is not a fitness trade-off in mosquitoes, but a severe fitness trade-off in birds. Furthermore, this conclusion is independent of the bird and mosquito species involved in transmission.

Refining the transmission model

The data presented in this dissertation can be used to create a more detailed map of the WNV demographic changes as they travel through mosquitoes, birds, and occasionally into a human brain. From an ingested bloodmeal, the viral population faces many barriers before it can infect a vertebrate again. First, only a few viruses within the population will seed infection in the midgut [293,294], though the exact number of viruses are estimated to range from one to thousands [292,295,296]. The population bottleneck severity will determine the amount of genetic diversity randomly lost from the bloodmeal to the midgut; however, genetic diversity is rapidly recovered during intratissue population expansion by mutation, diversifying selection from RNAi [164,165], weak purifying selection, and sometimes, given the correct circumstances, positive selection [117]. WNV populations that make it to the saliva must endure these cycles of random genetic reductions and recoveries at each additional anatomical barrier (midgut escape, salivary gland infection, and release into the saliva). How far the viral population diverged from its starting point in the bloodmeal is dependent on mosquito species-dependent factors – possibly

including bottleneck severities, RNAi targeting, selective sweeps, and intrahost MOIs. Moreover, the genetic diversity accumulated within mosquitoes may paradoxically provide advantages in divergent fitness landscapes.

The WNV population in the mosquito saliva contains a high proportion of low fitness and deleterious variants; therefore the 10^4 - 10^6 PFU founding WNV population transmitted to birds [291] will undergo a significant reduction in genetic diversity through immediate purifying selection as infection is established. The pathway of infection in birds is likely similar to mammals. Primary replication occurs in the fibroblasts, keratinocytes, and Langerhans cells near the site of infection [173,338]. Birds, however, lack lymph nodes, so perhaps the infected Langerhans cells travel to dermal lymphoid nodules [433] to seed primary viremia and visceral-organ dissemination [173,340,341]. Our data suggests that the viral populations do not encounter severe population bottlenecks during this process, but the selective constraints limits the amount and type of genetic diversity that accumulates (most are synonymous mutations). The strengths of purifying selection are predicted to lessen as the population size, MOIs, and intracellular complementation increases (deleterious mutations are harbored by high fitness variants within the same cells). Nonetheless, the viral population that reaches sufficient blood titers for transmission back to mosquitoes contains less deleterious mutations than the population transmitted to the bird, allowing it to have greater fitness. The cost of switching between stochastic (mosquitoes) and deterministic (birds) pressures during this arbovirus cycle is probably the cause of their apparent slow rates of evolution.

What happens when the WNV population spills over into humans is not entirely clear. If the bird data gives us any indication, then the population likely undergoes initial purifying selection then establishes systemic infection without many dramatic changes to the population.

What variants make it beyond the blood brain barrier (BBB) is another mystery. Jerzak et al. showed the high amount of WNV genetic diversity accumulated during passage in mosquitoes decreased the lethality of a homogenous starting population [131]. Therefore, the master WNV sequence can lose pathogenesis when it is mixed with variants containing random mutations, either because it is easier for the host to clear the infection or the variants cannot pass through the BBB. On the contrary, Vignuzzi et al. demonstrated that more diverse polio virus populations are better at crossing the BBB than homogenous ones [132]. These population-dependent impacts on neuroinvasion suggest that there could be a stochastic and/or deterministic bottleneck at the BBB. The variants that make it into the central nervous system, however, looked as if they could move freely between the different regions without encountering bottlenecks. This was supported by 1) identical mutations between tissues sequenced at similar frequencies and 2) similar levels of viral RNA extracted between the regions. Thus, once virus is in the brain, injury to specific regions is more likely to result from host- rather than virus-dependent factors.

What can we predict about WNV evolution?

Unlike Stapleford et al. who experimentally reproduced the emergence of a recent epidemic strain of CHIKV [132], we did not detect positive selection for any mutation with emergence potential. Therefore, we cannot predict any specific details about further adaptive potential of WNV in North America. We can, however, make predictions about fitness and population structure during different transmission cycles involving the mosquitoes and birds studied within this dissertation. For example, a transmission cycle involving *Culex quinquefasciatus* (pushes WNV to diverge the furthest) and American robins (maintains the highest fitness, selects for variants to reach higher frequency) will result in faster rates of WNV

evolution (i.e. more consensus sequence changes) while maintaining higher fitness than any other combination of vectors and hosts examined. On the other side, a transmission cycle involving *Cx. pipiens* (pushes WNV to diverge the least) and American crows (maintains lower fitness, keeps mutations at low frequency) will result in slower rates of WNV evolution while maintaining lower fitness (accumulate more deleterious mutations).

We can make further generalized predictions about the role of birds by saying that any species that cannot control infection (i.e. produce very high viremia and often succumb to infection similar to American crows, such as the common grackle [176]) will push WNV populations into a lower fitness landscape by preserving more deleterious mutations. Whereas susceptible birds that can control infection and limit viremia (e.g. mourning doves and Eurasian collared-doves [176,227]) will drive selection for highly fit variants. Thus, perhaps we can use simple viremia studies to make broad inferences about evolution in birds, though knowing more details about the virus-host interactions will help to refine these extrapolations.

In mosquitoes, however, it is much more difficult to make predictions about difference species. Genetic complexity, which is most likely influenced by RNAi, was not significantly different among tissues and species. This is not to diminish the role of RNAi in generating viral genetic diversity during systemic mosquito infection, but suggests that RNAi targeting is not so different between these species (*Cx. tarsalis*, *Cx. quinquefasciatus*, *Cx. pipiens*, and *Ae. aegypti*) to significantly alter the evolutionary trajectory of the WNV infectious clone. We did discover that aspects of vector competence may directly influence intra-mosquito WNV evolution. First, mosquitoes that allow for more rapid viral replication will have weaker purifying selection than from mosquitoes that are better at controlling WNV replication. Second, we found an association between the dissemination rate and the bottleneck size between two mosquito tissues. For

example, we expect to find a more severe population bottleneck at the midgut escape barrier when there is a 50% compared to a 75% dissemination rate. Together, we can use these data to construct a rational hypothesis towards why WNV diverged approximately 3× more during replication in *Cx. quinquefasciatus* than within the other species. WNV reaches peak titers during replication in *Cx. tarsalis* and *Cx. quinquefasciatus* faster than in *Cx. pipiens* and *Ae. aegypti*, allowing the accumulation more nonsynonymous mutations. In addition, the anatomical barriers are more severe within *Cx. quinquefasciatus* than *Cx. tarsalis* which act to randomly alter the variant distribution and lead to further divergence. Therefore, it is possible to use basic PCR or plaque assays to estimate the rate of WNV evolution, but compared to birds, it will take analyzing several different tissues and time points. However, more detailed studies are necessary to discover the mechanisms that govern vector competence in order to really know what drives viral evolution within mosquitoes. Even so, the results presented in this dissertation represent a major advancement in the understanding and prediction of WNV evolution during transmission.

References

1. Gubler DJ (1997) Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. In: Gubler DJ, Kuno, G, editor. *Dengue and Dengue Hemorrhagic Fever*. New York: CAB International. pp. 1-22.
2. Weaver SC, Vasilakis N (2009) Molecular evolution of dengue viruses: Contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. *Infection Genetics and Evolution* 9: 523-540.
3. Bryant JE, Holmes EC, Barrett ADT (2007) Out of Africa: A molecular perspective on the introduction of yellow fever virus into the Americas. *PLoS Pathog* 3: 668-673.
4. Haddow AJ (1969) Natural History of Yellow Fever in Africa. *Proceedings of the Royal Society of Edinburgh Section B-Biology* 70: 191-&.
5. Spielman A, D'Antonio M (2001) *Mosquito : a natural history of our most persistent and deadly foe*. New York: Hyperion. xix, 247 p. p.
6. Ross R (1897) On some Peculiar Pigmented Cells Found in Two Mosquitos Fed on Malarial Blood. *Br Med J* 2: 1786-1788.
7. Reed W, Carroll J, Agramonte A (1901) The etiology of yellow fever. An additional note. *Journal of the American Medical Association* 36: 431-440.
8. Gubler DJ (1998) Resurgent vector-borne diseases as a global health problem. *Emerg Infect Dis* 4: 442-450.
9. Gubler DJ (1994) Perspectives on the prevention and control of dengue hemorrhagic fever. *Gaoxiong Yi Xue Ke Xue Za Zhi* 10 Suppl: S15-18.
10. Gubler DJ (2002) Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol* 10: 100-103.
11. Halstead SB (2007) Dengue. *Lancet* 370: 1644-1652.
12. Messina JP, Brady OJ, Pigott DM, Golding N, Kraemer MU, et al. (2015) The many projected futures of dengue. *Nat Rev Microbiol* 13: 230-239.
13. Kramer LD, Styer LM, Ebel GD (2008) A global perspective on the epidemiology of West Nile virus. *Annu Rev Entomol* 53: 61-81.
14. Musso D, Cao-Lormeau VM, Gubler DJ (2015) Zika virus: following the path of dengue and chikungunya? *Lancet* 386: 243-244.
15. Weaver SC, Lecuit M (2015) Chikungunya virus and the global spread of a mosquito-borne disease. *N Engl J Med* 372: 1231-1239.
16. Robertson SE, Hull BP, Tomori O, Bele O, LeDuc JW, et al. (1996) Yellow fever: a decade of reemergence. *JAMA* 276: 1157-1162.
17. Eldridge BF (2005) Mosquitoes, the Culicidae. In: Marquart WC, editor. *Biology of Disease Vectors*. 2nd ed. Burlington, MA: Elsevier Academic Press. pp. 95-112.
18. Borkent A (2005) The Biting Midges, the Ceratopogonidae (Diptera). In: Marquart WC, editor. *Biology of Disease Vectors*. 2nd ed. Burlington, MA: Elsevier Academic Press. pp. 113-126.
19. Munstermann LE (2005) Phlebotomine Sand Flies, the Psychodidae. In: Marquart WC, editor. *Biology of Disease Vectors*. 2nd ed. Burlington, MA: Elsevier Academic Press. pp. 141-152.

20. Klompen H (2005) Ticks, the Ixodida. In: Marquart WC, editor. *Biology of Disease Vectors*. 2nd ed. Burlington, MA: Elsevier Academic Press. pp. 45-56.
21. Hoff GL, Trainer, D. O. (1973) Arboviruses in Reptiles: Isolation of a Bunyamwera Group Virus from a Naturally Infected Turtle. *Journal of Herpetology* 7: 55-62.
22. Shortridge KF, Ng MH, Oya A, Kobayashi M, Munro R, et al. (1974) Arbovirus Infections in Reptiles - Immunological Evidence for a High Incidence of Japanese Encephalitis-Virus in Cobra Naja-Naja. *Trans R Soc Trop Med Hyg* 68: 454-460.
23. Klenk K, Snow J, Morgan K, Bowen R, Stephens M, et al. (2004) Alligators as West Nile virus amplifiers. *Emerg Infect Dis* 10: 2150-2155.
24. Calisher CH, Karabatsos, N. (1988) Arbovirus serogroups: definition and geographic distribution; Monath TP, editor. Boca Raton, FL: CRC Press. 19-57 p.
25. Karabatsos N (1985) *International Catalogue of Arboviruses*. San Antonio, TX: Am Soc Trop Med Hyg.
26. Van Regenmortel MHV, Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeogh, D. J., Pringle, C. R., Wickney, R. B. (2000) Seventh report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press. 1162 p.
27. Weaver SC, Reisen WK (2010) Present and future arboviral threats. *Antiviral Res* 85: 328-345.
28. Gritsun TS, Lashkevich VA, Gould EA (2003) Tick-borne encephalitis. *Antiviral Res* 57: 129-146.
29. Grard G, Moureau G, Charrel RN, Lemasson JJ, Gonzalez JP, et al. (2007) Genetic characterization of tick-borne flaviviruses: New insights into evolution, pathogenetic determinants and taxonomy. *Virology* 361: 80-92.
30. Ebel GD (2010) Update on Powassan Virus: Emergence of a North American Tick-Borne Flavivirus. *Annu Rev Entomol* 55: 95-110.
31. Constantine DG, Woodall DF (1964) Latent Infection of Rio Bravo Virus in Salivary-Glands of Bats. *Public Health Reports* 79: 1033-1039.
32. Hoshino K, Isawa H, Tsuda Y, Yano K, Sasaki T, et al. (2007) Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. *Virology* 359: 405-414.
33. Weaver SC, Winegar R, Manger ID, Forrester NL (2012) Alphaviruses: Population genetics and determinants of emergence. *Antiviral Res* 94: 242-257.
34. Nasar F, Palacios G, Gorchakov RV, Guzman H, Da Rosa APT, et al. (2012) Eilat virus, a unique alphavirus with host range restricted to insects by RNA replication. *Proc Natl Acad Sci U S A* 109: 14622-14627.
35. Linn ML, Gardner J, Warrilow D, Darnell GA, McMahon CR, et al. (2001) Arbovirus of marine mammals: a new alphavirus isolated from the elephant seal louse, *Lepidophthirus macrorhini*. *J Virol* 75: 4103-4109.
36. McLoughlin MF, Graham DA (2007) Alphavirus infections in salmonids - a review. *Journal of Fish Diseases* 30: 511-531.
37. Steele KE, Reed, D. S., Glass, P. J., Hart, M. K., Ludwig, G. V., Pratt, W. D., Parker, M. D., Smith, J. D. (2007) Alphavirus Encephalitis. In: Dembek ZF, editor. *Medical Aspects of Biological Warfare*. Washington, DC: Office of The Surgeon General. pp. 241-270.
38. Suhrbier A, Jaffar-Bandjee MC, Gasque P (2012) Arthritogenic alphaviruses-an overview. *Nature Reviews Rheumatology* 8: 420-429.

39. Beaty BJ, Sundin DR, Chandler LJ, Bishop DHL (1985) Evolution of Bunyaviruses by Genome Reassortment in Dually Infected Mosquitos (*Aedes-Triseriatus*). *Science* 230: 548-550.
40. Briese T, Calisher CH, Higgs S (2013) Viruses of the family Bunyaviridae: Are all available isolates reassortants? *Virology* 446: 207-216.
41. Reese SM, Blitvich BJ, Blair CD, Geske D, Beaty BJ, et al. (2008) Potential for La Crosse virus segment reassortment in nature. *Virology Journal* 5.
42. Laughlin LW, Meegan JM, Strausbaugh LJ, Morens DM, Watten RH (1979) Epidemic Rift-Valley Fever in Egypt - Observations of the Spectrum of Human Illness. *Trans R Soc Trop Med Hyg* 73: 630-633.
43. Hoffmann B, Scheuch M, Hoper D, Jungblut R, Holsteg M, et al. (2012) Novel Orthobunyavirus in Cattle, Europe, 2011. *Emerg Infect Dis* 18: 469-472.
44. Nunes MRT, da Rosa APAT, Weaver SC, Tesh RB, Vasconcelos PFC (2005) Molecular epidemiology of group C viruses (Bunyaviridae, Orthobunyavirus) isolated in the Americas. *J Virol* 79: 10561-10570.
45. Lambert AJ, Lanciotti RS (2009) Consensus Amplification and Novel Multiplex Sequencing Method for S Segment Species Identification of 47 Viruses of the Orthobunyavirus, Phlebovirus, and Nairovirus Genera of the Family Bunyaviridae. *J Clin Microbiol* 47: 2398-2404.
46. Tesh RB (1988) The Genus Phlebovirus and Its Vectors. *Annu Rev Entomol* 33: 169-181.
47. Savage HM, Godsey MS, Lambert A, Panella NA, Burkhalter KL, et al. (2013) First Detection of Heartland Virus (Bunyaviridae: Phlebovirus) from Field Collected Arthropods. *American Journal of Tropical Medicine and Hygiene* 89: 445-452.
48. Honig JE, Osborne JC, Nichol ST (2004) The high genetic variation of viruses of the genus Nairovirus reflects the diversity of their predominant tick hosts. *Virology* 318: 10-16.
49. Steinhauer DA, Domingo E, Holland JJ (1992) Lack of Evidence for Proofreading Mechanisms Associated with an Rna Virus Polymerase. *Gene* 122: 281-288.
50. Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, et al. (1982) Rapid Evolution of Rna Genomes. *Science* 215: 1577-1585.
51. Domingo E, Sabo D, Taniguchi T, Weissmann C (1978) Nucleotide sequence heterogeneity of an RNA phage population. *Cell* 13: 735-744.
52. Ribeiro JMC (1995) Blood-Feeding Arthropods - Live Syringes or Invertebrate Pharmacologists. *Infectious Agents and Disease-Reviews Issues and Commentary* 4: 143-152.
53. Kuno G, Chang GJJ (2005) Biological transmission of arboviruses: Reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends. *Clinical Microbiology Reviews* 18: 608-+.
54. Smithburn KC, Hughes, T. P., Burke, A. W., Paul, J. H. (1940) A Neurotropic Virus Isolated from the Blood of a Native of Uganda. *American Journal of Tropical Medicine* 20: 471-472.
55. Hayes CG (2001) West Nile virus: Uganda, 1937, to New York city, 1999. *West Nile Virus: Detection, Surveillance, and Control* 951: 25-37.
56. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI, et al. (1998) West Nile encephalitis epidemic in southeastern Romania. *Lancet* 352: 767-771.

57. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, et al. (1999) Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* 286: 2333-2337.
58. Pesko KN, Ebel GD (2012) West Nile virus population genetics and evolution. *Infect Genet Evol* 12: 181-190.
59. Kilpatrick AM (2011) Globalization, land use, and the invasion of West Nile virus. *Science* 334: 323-327.
60. Ross RW (1956) The Newala epidemic. III. The virus: isolation, pathogenic properties and relationships to the epidemic. *Journal of Hygiene* 54: 177-191.
61. Chretien JP, Anyamba A, Bedno SA, Breiman RF, Sang R, et al. (2007) Drought-associated Chikungunya emergence along coastal East Africa. *American Journal of Tropical Medicine and Hygiene* 76: 405-407.
62. Hochedez P, Jaureguiberry S, Debruyne M, Bossi P, Hausfater P, et al. (2006) Chikungunya infection in travelers. *Emerg Infect Dis* 12: 1565-1567.
63. Parola P, de Lamballerie X, Jourdan J, Rovey C, Vaillant V, et al. (2006) Novel chikungunya virus variant in travelers returning from Indian Ocean islands. *Emerg Infect Dis* 12: 1493-1499.
64. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, et al. (2007) Chikungunya virus in US travelers returning from India, 2006. *Emerg Infect Dis* 13: 764-767.
65. Rezza G, Nicoletti L, Angelini R, Romi R, Finarelli AC, et al. (2007) Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* 370: 1840-1846.
66. Leparc-Goffart I, Nougaiere A, Cassadou S, Prat C, de Lamballerie X (2014) Chikungunya in the Americas. *Lancet* 383: 514-514.
67. Kendrick K, Stanek, D., Blackmore, C. (2014) Notes from the Field: Transmission of Chikungunya Virus in the Continental United States — Florida, 2014. *Morbidity and Mortality Weekly Report* 63: 1137-1137.
68. Diaz-Gonzalez EE, Kautz TF, Dorantes-Delgado A, Malo-Garcia IR, Laguna-Aguilar M, et al. (2015) First Report of Aedes aegypti Transmission of Chikungunya Virus in the Americas. *Am J Trop Med Hyg* 93: 1325-1329.
69. Fisher M, Staples, E. (2014) Notes from the Field: Chikungunya Virus Spreads in the Americas — Caribbean and South America, 2013–2014. *Morbidity and Mortality Weekly Report* 63: 500-501.
70. PAHO (2015) Chikungunya.
71. Charlwood JD, Alecrim WA (1989) Capture-Recapture Studies with the South-American Malaria Vector Anopheles-Darlingi, Root. *Annals of Tropical Medicine and Parasitology* 83: 569-576.
72. Reisen WK, Lothrop HD (1995) Population Ecology and Dispersal of Culex Tarsalis (Diptera, Culicidae) in the Coachella Valley of California. *J Med Entomol* 32: 490-502.
73. Venkatesan M, Rasgon JL (2010) Population genetic data suggest a role for mosquito-mediated dispersal of West Nile virus across the western United States. *Mol Ecol* 19: 1573-1584.
74. Hamer GL, Kitron UD, Goldberg TL, Brawn JD, Loss SR, et al. (2009) Host Selection by Culex pipiens Mosquitoes and West Nile Virus Amplification. *American Journal of Tropical Medicine and Hygiene* 80: 268-278.

75. Kent R, Juliusson L, Weissmann M, Evans S, Komar N (2009) Seasonal Blood-Feeding Behavior of *Culex tarsalis* (Diptera: Culicidae) in Weld County, Colorado, 2007. *J Med Entomol* 46: 380-390.
76. Brusatte SL, O'Connor JK, Jarvis ED (2015) The Origin and Diversification of Birds. *Current Biology* 25: R888-R898.
77. Grimaldi DA, Engel, M. S. (2005) *Panorpida: Antliophora and Amphiesmenoptera. Evolution of the Insects*. New York, NY: Cambridge University Press. pp. 468-547.
78. Levinson RS, Strauss JH, Strauss EG (1990) Complete Sequence of the Genomic Rna of Onyong-Nyong Virus and Its Use in the Construction of Alphavirus Phylogenetic Trees. *Virology* 175: 110-123.
79. Strauss JH, Strauss EG (1994) The Alphaviruses - Gene-Expression, Replication, and Evolution. *Microbiological Reviews* 58: 491-562.
80. Weaver SC, Hagenbaugh A, Bellew LA, Netesov SV, Volchkov VE, et al. (1993) A Comparison of the Nucleotide-Sequences of Eastern and Western Equine Encephalomyelitis Viruses with Those of Other Alphaviruses and Related Rna Viruses. *Virology* 197: 375-390.
81. Garmashova N, Gorchakov R, Volkova E, Paessler S, Frolova E, et al. (2007) The old world and new world alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J Virol* 81: 2472-2484.
82. Owen J, Moore F, Panella N, Edwards E, Bru R, et al. (2006) Migrating birds as dispersal vehicles for West Nile virus. *Ecohealth* 3: 79-85.
83. Komar N, Clark GG (2006) West Nile virus activity in Latin America and the Caribbean. *Revista Panamericana De Salud Publica-Pan American Journal of Public Health* 19: 112-117.
84. Malkinson M, Banet C, Weisman Y, Pokamunski S, King R, et al. (2002) Introduction of West Nile virus in the Middle East by migrating white storks. *Emerg Infect Dis* 8: 392-397.
85. Rappole JH, Derrickson SR, Hubalek Z (2000) Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerg Infect Dis* 6: 319-328.
86. Tabachnick WJ (1991) Evolutionary genetics of arthropod-borne diseases. The yellow fever mosquito. *American Entomologist* 37: 14-24.
87. Cathey JT, Marr JS (2014) Yellow fever, Asia and the East African slave trade. *Trans R Soc Trop Med Hyg* 108: 252-257.
88. Dick OB, San Martin JL, Montoya RH, del Diego J, Zambrano B, et al. (2012) The History of Dengue Outbreaks in the Americas. *American Journal of Tropical Medicine and Hygiene* 87: 584-593.
89. Galbraith SE, Barrett, A. B. (2009) Yellow Fever. In: Barrett AD, Stanberry, L. R., editor. *Vaccines for Biodefense and Emerging and Neglected Diseases*. Amsterdam: Academic Press. pp. 754-789.
90. Romano APM, Costa ZGA, Ramos DG, Andrade MA, Jayme VD, et al. (2014) Yellow Fever Outbreaks in Unvaccinated Populations, Brazil, 2008-2009. *PLoS Negl Trop Dis* 8.
91. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2013) The global distribution and burden of dengue. *Nature* 496: 504-507.
92. Bowden SE, Magori K, Drake JM (2011) Regional differences in the association between land cover and West Nile virus disease incidence in humans in the United States. *Am J Trop Med Hyg* 84: 234-238.

93. Kilpatrick AM, Meola MA, Moudy RM, Kramer LD (2008) Temperature, viral genetics, and the transmission of West Nile virus by *Culex pipiens* mosquitoes. *PLoS Pathog* 4.
94. Gubler DJ, Reiter P, Ebi KL, Yap W, Nasci R, et al. (2001) Climate variability and change in the United States: Potential impacts on vector- and rodent-borne diseases. *Environmental Health Perspectives* 109: 223-233.
95. Shaman J, Day JF, Stieglitz M (2005) Drought-induced amplification and epidemic transmission of West Nile Virus in southern Florida. *J Med Entomol* 42: 134-141.
96. Rueda LM, Patel KJ, Axtell RC, Stinner RE (1990) Temperature-Dependent Development and Survival Rates of *Culex-Quinquefasciatus* and *Aedes-Aegypti* (Diptera, Culicidae). *J Med Entomol* 27: 892-898.
97. Fisher RA (1930) The genetical theory of natural selection. Oxford,: The Clarendon press. xiv, 272 p. p.
98. Wright S (1977) Evolution and the Genetics of Populations. Chicago: University of Chicago Press.
99. Ebel GD, Dupuis AP, 2nd, Ngo K, Nicholas D, Kauffman E, et al. (2001) Partial genetic characterization of West Nile virus strains, New York State, 2000. *Emerg Infect Dis* 7: 650-653.
100. Anderson JF, Vossbrinck CR, Andreadis TG, Iton A, Beckwith WH, et al. (2001) A phylogenetic approach to following West Nile virus in Connecticut. *Proc Natl Acad Sci U S A* 98: 12885-12889.
101. Beasley DWC, Davis CT, Guzman H, Vanlandingham DL, da Rosa APAT, et al. (2003) Limited evolution of West Nile virus has occurred during its southwesterly spread in the United States. *Virology* 309: 190-195.
102. Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD (2004) Genetic and phenotypic variation of West Nile virus in New York, 2000-2003. *Am J Trop Med Hyg* 71: 493-500.
103. Moudy RM, Meola MA, Morin LL, Ebel GD, Kramer LD (2007) A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes. *Am J Trop Med Hyg* 77: 365-370.
104. Macdonald G (1961) Epidemiologic Models in Studies of Vector-Borne Diseases. *Public Health Reports* 76: 753-764.
105. Davis CT, Ebel GD, Lanciotti RS, Brault AC, Guzman H, et al. (2005) Phylogenetic analysis of North American West Nile virus isolates, 2001-2004: evidence for the emergence of a dominant genotype. *Virology* 342: 252-265.
106. Brault AC, Huang CY, Langevin SA, Kinney RM, Bowen RA, et al. (2007) A single positively selected West Nile viral mutation confers increased virogenesis in American crows. *Nat Genet* 39: 1162-1166.
107. Duggal NK, Bosco-Lauth A, Bowen RA, Wheeler SS, Reisen WK, et al. (2014) Evidence for co-evolution of West Nile Virus and house sparrows in North America. *PLoS Negl Trop Dis* 8: e3262.
108. Armstrong PM, Vossbrinck CR, Andreadis TG, Anderson JF, Pesko KN, et al. (2011) Molecular evolution of West Nile virus in a northern temperate region: Connecticut, USA 1999-2008. *Virology* 417: 203-210.
109. Langevin SA, Bowen RA, Reisen WK, Andrade CC, Ramey WN, et al. (2014) Host competence and helicase activity differences exhibited by West Nile viral variants expressing NS3-249 amino acid polymorphisms. *PLoS One* 9: e100802.

110. Zou G, Puig-Basagoiti F, Zhang B, Qing M, Chen LQ, et al. (2009) A single-amino acid substitution in West Nile virus 2K peptide between NS4A and NS4B confers resistance to lycorine, a flavivirus inhibitor. *Virology* 384: 242-252.
111. Mertens E, Kajaste-Rudnitski A, Torres S, Funk A, Frenkiel MP, et al. (2010) Viral determinants in the NS3 helicase and 2K peptide that promote West Nile virus resistance to antiviral action of 2',5'-oligoadenylate synthetase 1b. *Virology* 399: 176-185.
112. Kraemer MUG, Golding N, Brady OJ, Messina JP, Smith DL, et al. (2015) The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. *Tropical Medicine & International Health* 20: 38-38.
113. Tsetsarkin KA, Vanlandingham DL, Mcgee CE, Higgs S (2007) A single mutation in chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathog* 3: 1895-1906.
114. Tsetsarkin KA, Chen RB, Leal G, Forrester N, Higgs S, et al. (2011) Chikungunya virus emergence is constrained in Asia by lineage-specific adaptive landscapes. *Proc Natl Acad Sci U S A* 108: 7872-7877.
115. Tsetsarkin KA, Chen RB, Yun RM, Rossi SL, Plante KS, et al. (2014) Multi-peaked adaptive landscape for chikungunya virus evolution predicts continued fitness optimization in *Aedes albopictus* mosquitoes. *Nature Communications* 5.
116. Tsetsarkin KA, Weaver SC (2011) Sequential Adaptive Mutations Enhance Efficient Vector Switching by Chikungunya Virus and Its Epidemic Emergence. *PLoS Pathog* 7.
117. Stapleford KA, Coffey LL, Lay S, Borderia AV, Duong V, et al. (2014) Emergence and Transmission of Arbovirus Evolutionary Intermediates with Epidemic Potential. *Cell Host Microbe* 15: 706-716.
118. Ebel GD (2014) Toward an Activist Agenda for Monitoring Virus Emergence. *Cell Host Microbe* 15: 655-656.
119. Holmes EC (2013) What can we predict about viral evolution and emergence? *Curr Opin Virol* 3: 180-184.
120. Woolhouse ME, Haydon DT, Antia R (2005) Emerging pathogens: the epidemiology and evolution of species jumps. *Trends Ecol Evol* 20: 238-244.
121. de la Torre JC, Holland JJ (1990) RNA Virus Quasispecies Populations Can Suppress Vastly Superior Mutant Progeny. *J Virol* 64: 6278-6281.
122. Duarte E, Clarke D, Moya A, Domingo E, Holland J (1992) Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet. *Proc Natl Acad Sci U S A* 89: 6015-6019.
123. Batschelet E, Domingo E, Weissmann C (1976) The proportion of revertant and mutant phage in a growing population, as a function of mutation and growth rate. *Gene* 1: 27-32.
124. Eigen M (1971) Selforganization of Matter and Evolution of Biological Macromolecules. *Naturwissenschaften* 58: 465-&.
125. Eigen M, Schuster P (1979) The hypercycle, a principle of natural self-organization. Berlin ; New York: Springer-Verlag. viii, 92 p. p.
126. Eigen M (1996) On the nature of virus quasispecies. *Trends Microbiol* 4: 216-218.
127. Eigen M (1993) Viral quasispecies. *Sci Am* 269: 42-49.
128. Holland JJ, Delatorre JC, Steinhauer DA (1992) Rna Virus Populations as Quasi-Species. *Curr Top Microbiol Immunol* 176: 1-20.
129. Llaure AS, Andino R (2010) Quasispecies theory and the behavior of RNA viruses. *PLoS Pathog* 6: e1001005.

130. Andino R, Domingo E (2015) Viral quasispecies. *Virology* 479-480: 46-51.
131. Jerzak GV, Bernard K, Kramer LD, Shi PY, Ebel GD (2007) The West Nile virus mutant spectrum is host-dependant and a determinant of mortality in mice. *Virology* 360: 469-476.
132. Vignuzzi M, Stone JK, Arnold JJ, Cameron CE, Andino R (2006) Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* 439: 344-348.
133. Borderia AV, Lorenzo-Redondo R, Pernas M, Casado C, Alvaro T, et al. (2010) Initial fitness recovery of HIV-1 is associated with quasispecies heterogeneity and can occur without modifications in the consensus sequence. *PLoS One* 5: e10319.
134. Lancaster KZ, Pfeiffer JK (2011) Mechanisms controlling virulence thresholds of mixed viral populations. *J Virol* 85: 9778-9788.
135. Woolhouse ME, Taylor LH, Haydon DT (2001) Population biology of multihost pathogens. *Science* 292: 1109-1112.
136. Liu WJ, Rourke MF, Holmes EC, Aaskov JG (2011) Persistence of multiple genetic lineages within intrahost populations of Ross River virus. *J Virol* 85: 5674-5678.
137. Jerzak G, Bernard KA, Kramer LD, Ebel GD (2005) Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection. *J Gen Virol* 86: 2175-2183.
138. Bonneau KR, Mullens BA, MacLachlan NJ (2001) Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle, and *Culicoides sonorensis*. *J Virol* 75: 8298-8305.
139. Parameswaran P, Charlebois P, Tellez Y, Nunez A, Ryan EM, et al. (2012) Genome-wide patterns of intrahuman dengue virus diversity reveal associations with viral phylogenetic clade and interhost diversity. *J Virol* 86: 8546-8558.
140. Vasilakis N, Deardorff ER, Kenney JL, Rossi SL, Hanley KA, et al. (2009) Mosquitoes Put the Brake on Arbovirus Evolution: Experimental Evolution Reveals Slower Mutation Accumulation in Mosquito Than Vertebrate Cells. *PLoS Pathog* 5.
141. Deardorff ER, Fitzpatrick KA, Jerzak GV, Shi PY, Kramer LD, et al. (2011) West Nile virus experimental evolution in vivo and the trade-off hypothesis. *PLoS Pathog* 7: e1002335.
142. Coffey LL, Vasilakis N, Brault AC, Powers AM, Tripet F, et al. (2008) Arbovirus evolution in vivo is constrained by host alternation. *Proc Natl Acad Sci U S A* 105: 6970-6975.
143. Smith-Tsurkan SD, Wilke CO, Novella IS (2010) Incongruent fitness landscapes, not tradeoffs, dominate the adaptation of vesicular stomatitis virus to novel host types. *J Gen Virol* 91: 1484-1493.
144. Cooper LA, Scott TW (2001) Differential evolution of eastern equine encephalitis virus populations in response to host cell type. *Genetics* 157: 1403-1412.
145. Wilson DS, Yoshimura, J. (1994) On the coexistence of specialists and generalists. *American Naturalist* 144: 692-707.
146. Kassen R (2002) The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology* 15: 173-190.
147. Holmes EC (2003) Patterns of intra- and interhost nonsynonymous variation reveal strong purifying selection in dengue virus. *J Virol* 77: 11296-11298.

148. Woelk CH, Holmes EC (2002) Reduced positive selection in vector-borne RNA viruses. *Molecular Biology and Evolution* 19: 2333-2336.
149. Weaver SC, Rico-Hesse R, Scott TW (1992) Genetic diversity and slow rates of evolution in New World alphaviruses. *Curr Top Microbiol Immunol* 176: 99-117.
150. Jenkins GM, Rambaut A, Pybus OG, Holmes EC (2002) Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. *Journal of Molecular Evolution* 54: 156-165.
151. Ciota AT, Kramer LD (2010) Insights into arbovirus evolution and adaptation from experimental studies. *Viruses* 2: 2594-2617.
152. Holmes EC (2003) Error thresholds and the constraints to RNA virus evolution. *Trends Microbiol* 11: 543-546.
153. Weaver SC, Brault AC, Kang W, Holland JJ (1999) Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. *J Virol* 73: 4316-4326.
154. Coffey LL, Vignuzzi M (2011) Host alternation of chikungunya virus increases fitness while restricting population diversity and adaptability to novel selective pressures. *J Virol* 85: 1025-1035.
155. Novella IS, Hershey CL, Escarmis C, Domingo E, Holland JJ (1999) Lack of evolutionary stasis during alternating replication of an arbovirus in insect and mammalian cells. *Journal of Molecular Biology* 287: 459-465.
156. Ciota AT, Jia YQ, Payne AF, Jerzak G, Davis LJ, et al. (2009) Experimental Passage of St. Louis Encephalitis Virus In Vivo in Mosquitoes and Chickens Reveals Evolutionarily Significant Virus Characteristics. *PLoS One* 4.
157. Ciota AT, Lovelace AO, Jia Y, Davis LJ, Young DS, et al. (2008) Characterization of mosquito-adapted West Nile virus. *J Gen Virol* 89: 1633-1642.
158. Sessions OM, Wilm A, Kamaraj US, Choy MM, Chow A, et al. (2015) Analysis of Dengue Virus Genetic Diversity during Human and Mosquito Infection Reveals Genetic Constraints. *PLoS Negl Trop Dis* 9: e0004044.
159. Lin SR, Hsieh SC, Yueh YY, Lin TH, Chao DY, et al. (2004) Study of sequence variation of dengue type 3 virus in naturally infected mosquitoes and human hosts: implications for transmission and evolution. *J Virol* 78: 12717-12721.
160. Sim S, Aw PP, Wilm A, Teoh G, Hue KD, et al. (2015) Tracking Dengue Virus Intra-host Genetic Diversity during Human-to-Mosquito Transmission. *PLoS Negl Trop Dis* 9: e0004052.
161. Jerzak GV, Brown I, Shi PY, Kramer LD, Ebel GD (2008) Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching. *Virology* 374: 256-260.
162. Brackney DE, Scott JC, Sagawa F, Woodward JE, Miller NA, et al. (2010) C6/36 *Aedes albopictus* cells have a dysfunctional antiviral RNA interference response. *PLoS Negl Trop Dis* 4: e856.
163. Scott JC, Brackney DE, Campbell CL, Bondu-Hawkins V, Hjelle B, et al. (2010) Comparison of dengue virus type 2-specific small RNAs from RNA interference-competent and -incompetent mosquito cells. *PLoS Negl Trop Dis* 4: e848.
164. Brackney DE, Beane JE, Ebel GD (2009) RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification. *PLoS Pathog* 5: e1000502.

165. Brackney DE, Schirtzinger EE, Harrison TD, Ebel GD, Hanley KA (2015) Modulation of Flavivirus Population Diversity by RNA Interference. *J Virol* 89: 4035-4039.
166. Ciota AT, Lovelace AO, Jones SA, Payne A, Kramer LD (2007) Adaptation of two flaviviruses results in differences in genetic heterogeneity and virus adaptability. *J Gen Virol* 88: 2398-2406.
167. Brackney DE, Pesko KN, Brown IK, Deardorff ER, Kawatachi J, et al. (2011) West Nile virus genetic diversity is maintained during transmission by *Culex pipiens quinquefasciatus* mosquitoes. *PLoS One* 6: e24466.
168. Ciota AT, Ngo KA, Lovelace AO, Payne AF, Zhou Y, et al. (2007) Role of the mutant spectrum in adaptation and replication of West Nile virus. *Journal of General Virology* 88: 865-874.
169. Prasad AN, Brackney DE, Ebel GD (2013) The role of innate immunity in conditioning mosquito susceptibility to West Nile virus. *Viruses* 5: 3142-3170.
170. Blair CD, Olson KE (2015) The Role of RNA Interference (RNAi) in Arbovirus-Vector Interactions. *Viruses-Basel* 7: 820-843.
171. Fitzpatrick KA, Deardorff ER, Pesko K, Brackney DE, Zhang B, et al. (2010) Population variation of West Nile virus confers a host-specific fitness benefit in mosquitoes. *Virology* 404: 89-95.
172. Aaskov J, Buzacott K, Thu HM, Lowry K, Holmes EC (2006) Long-term transmission of defective RNA viruses in humans and *Aedes* mosquitoes. *Science* 311: 236-238.
173. Samuel MA, Diamond MS (2006) Pathogenesis of West Nile virus infection: a balance between virulence, innate and adaptive immunity, and viral evasion. *J Virol* 80: 9349-9360.
174. Keller BC, Fredericksen BL, Samuel MA, Mock RE, Mason PW, et al. (2006) Resistance to alpha/beta interferon is a determinant of West Nile virus replication fitness and virulence. *J Virol* 80: 9424-9434.
175. Kleinschmidt-DeMasters BK, Beckham JD (2015) West Nile Virus Encephalitis 16 Years Later. *Brain Pathology* 25: 625-633.
176. Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, et al. (2003) Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerg Infect Dis* 9: 311-322.
177. Bernard KA, Maffei JG, Jones SA, Kauffman EB, Ebel G, et al. (2001) West Nile virus infection in birds and mosquitoes, New York State, 2000. *Emerg Infect Dis* 7: 679-685.
178. Turell MJ, Dohm DJ, Sardelis MR, O Guinn ML, Andreadis TG, et al. (2005) An update on the potential of North American mosquitoes (Diptera : Culicidae) to transmit West Nile virus. *J Med Entomol* 42: 57-62.
179. Zompi S, Harris E (2012) Animal models of dengue virus infection. *Viruses* 4: 62-82.
180. Ziegler SA, Lu L, da Rosa AP, Xiao SY, Tesh RB (2008) An animal model for studying the pathogenesis of chikungunya virus infection. *Am J Trop Med Hyg* 79: 133-139.
181. Reisen WK (2013) Ecology of West Nile virus in North America. *Viruses* 5: 2079-2105.
182. Brinton MA (2014) Replication Cycle and Molecular Biology of the West Nile Virus. *Viruses-Basel* 6: 13-53.
183. Jupp PG, McIntosh BM, Blackburn NK (1986) Experimental assessment of the vector competence of *Culex* (*Culex*) *neavei* Theobald with West Nile and Sindbis viruses in South Africa. *Trans R Soc Trop Med Hyg* 80: 226-230.

184. Jupp PG, McIntosh BM (1970) Quantitative experiments on the vector capability of *Culex* (*Culex*) *univittatus* Theobald with West Nile and Sindbis viruses. *J Med Entomol* 7: 371-373.
185. Taylor RM, Work TH, Hurlbut HS, Rizk F (1956) A study of the ecology of West Nile virus in Egypt. *Am J Trop Med Hyg* 5: 579-620.
186. Balenghien T, Vazeille M, Grandadam M, Schaffner F, Zeller H, et al. (2008) Vector competence of some French *Culex* and *Aedes* mosquitoes for West Nile virus. *Vector Borne Zoonotic Dis* 8: 589-595.
187. Kay BH, Fanning ID, Carley JG (1984) The vector competence of Australian *Culex annulirostris* with Murray Valley encephalitis and Kunjin viruses. *Aust J Exp Biol Med Sci* 62 (Pt 5): 641-650.
188. Paramasivan R, Mishra AC, Mourya DT (2003) West Nile virus: the Indian scenario. *Indian Journal of Medical Research* 118: 101-108.
189. Ilkal MA, Mavale MS, Prasanna Y, Jacob PG, Geevarghese G, et al. (1997) Experimental studies on the vector potential of certain *Culex* species to West Nile virus. *Indian Journal of Medical Research* 106: 225-228.
190. Akhter R, Hayes CG, Baqar S, Reisen WK (1982) West Nile virus in Pakistan. III. Comparative vector capability of *Culex tritaeniorhynchus* and eight other species of mosquitoes. *Trans R Soc Trop Med Hyg* 76: 449-453.
191. Turell MJ, O'Guinn ML, Dohm DJ, Jones JW (2001) Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. *J Med Entomol* 38: 130-134.
192. Sardelis MR, Turell MJ, Dohm DJ, O'Guinn ML (2001) Vector competence of selected North American *Culex* and *Coquillettidia* mosquitoes for West Nile virus. *Emerg Infect Dis* 7: 1018-1022.
193. Ebel GD, Rochlin I, Longacker J, Kramer LD (2005) *Culex restuans* (Diptera: Culicidae) relative abundance and vector competence for West Nile Virus. *J Med Entomol* 42: 838-843.
194. Andreadis TG, Anderson JF, Vossbrinck CR (2001) Mosquito surveillance for West Nile virus in Connecticut, 2000: isolation from *Culex pipiens*, *Cx. restuans*, *Cx. salinarius*, and *Culiseta melanura*. *Emerg Infect Dis* 7: 670-674.
195. Jupp PG, McIntosh BM (1970) Quantitative experiments on the vector capability of *Culex* (*Culex*) *pipiens fatigans* Wiedemann with West Nile and Sindbis viruses. *J Med Entomol* 7: 353-356.
196. Kay BH, Fanning ID, Carley JG (1982) Vector competence of *Culex pipiens quinquefasciatus* for Murray Valley encephalitis, Kunjin, and Ross River viruses from Australia. *Am J Trop Med Hyg* 31: 844-848.
197. Goddard LB, Roth AE, Reisen WK, Scott TW (2002) Vector competence of California mosquitoes for West Nile virus. *Emerg Infect Dis* 8: 1385-1391.
198. Kilpatrick AM, Kramer LD, Campbell SR, Alleyne EO, Dobson AP, et al. (2005) West Nile virus risk assessment and the bridge vector paradigm. *Emerg Infect Dis* 11: 425-429.
199. Molaei G, Andreadis TG (2006) Identification of avian- and mammalian-derived bloodmeals in *Aedes vexans* and *Culiseta melanura* (Diptera: Culicidae) and its implication for West Nile virus transmission in Connecticut, U.S.A. *J Med Entomol* 43: 1088-1093.

200. Gibbs SE, Wimberly MC, Madden M, Masour J, Yabsley MJ, et al. (2006) Factors affecting the geographic distribution of West Nile virus in Georgia, USA: 2002-2004. *Vector Borne Zoonotic Dis* 6: 73-82.
201. Amore G, Bertolotti L, Hamer GL, Kitron UD, Walker ED, et al. (2010) Multi-year evolutionary dynamics of West Nile virus in suburban Chicago, USA, 2005-2007. *Philos Trans R Soc Lond B Biol Sci* 365: 1871-1878.
202. Bertolotti L, Kitron UD, Walker ED, Ruiz MO, Brawn JD, et al. (2008) Fine-scale genetic variation and evolution of West Nile Virus in a transmission "hot spot" in suburban Chicago, USA. *Virology* 374: 381-389.
203. Fauver JR, Pecher L, Schurich JA, Bolling BG, Calhoun M, et al. (2015) Temporal and Spatial Variability of Entomological Risk Indices for West Nile Virus Infection in Northern Colorado: 2006-2013. *J Med Entomol*.
204. Schurich JA, Kumar S, Eisen L, Moore CG (2014) Modeling *Culex tarsalis* abundance on the northern Colorado front range using a landscape-level approach. *J Am Mosq Control Assoc* 30: 7-20.
205. Ezenwa VO, Godsey MS, King RJ, Gupitill SC (2006) Avian diversity and West Nile virus: testing associations between biodiversity and infectious disease risk. *Proc Biol Sci* 273: 109-117.
206. Kunkel KE, Novak RJ, Lampman RL, Gu W (2006) Modeling the impact of variable climatic factors on the crossover of *Culex restuans* and *Culex pipiens* (Diptera: culicidae), vectors of West Nile virus in Illinois. *Am J Trop Med Hyg* 74: 168-173.
207. Ruiz MO, Chaves LF, Hamer GL, Sun T, Brown WM, et al. (2010) Local impact of temperature and precipitation on West Nile virus infection in *Culex* species mosquitoes in northeast Illinois, USA. *Parasit Vectors* 3: 19.
208. DeGroot JP, Sugumaran R, Brend SM, Tucker BJ, Bartholomay LC (2008) Landscape, demographic, entomological, and climatic associations with human disease incidence of West Nile virus in the state of Iowa, USA. *Int J Health Geogr* 7: 19.
209. Reisen WK, Fang Y, Martinez VM (2005) Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. *J Med Entomol* 42: 367-375.
210. Brault AC, Langevin SA, Ramey WN, Fang Y, Beasley DW, et al. (2011) Reduced avian virulence and viremia of West Nile virus isolates from Mexico and Texas. *Am J Trop Med Hyg* 85: 758-767.
211. Lopez CM, Cienfuegos S, Dacal V, Vazquez L, Panadero R, et al. (2010) Efficacy of anthelmintic control programs against natural *Muellerius capillaris* infection in sheep in the north-west of Spain. Effect on blood gases and pH in venous blood samples. *Parasite* 17: 167-171.
212. Reisen WK, Hahn DC (2007) Comparison of immune responses of brown-headed cowbird and related blackbirds to west Nile and other mosquito-borne encephalitis viruses. *J Wildl Dis* 43: 439-449.
213. Guerrero-Sanchez S, Cuevas-Romero S, Nemeth NM, Trujillo-Olivera MT, Worwa G, et al. (2011) West Nile virus infection of birds, Mexico. *Emerg Infect Dis* 17: 2245-2252.
214. Perez-Ramirez E, Llorente F, Jimenez-Clavero MA (2014) Experimental infections of wild birds with West Nile virus. *Viruses* 6: 752-781.

215. Mahmood F, Chiles RE, Fang Y, Barker CM, Reisen WK (2004) Role of nestling mourning doves and house finches as amplifying hosts of St. Louis encephalitis virus. *J Med Entomol* 41: 965-972.
216. McLean RG, Shriner RB, Kirk LJ, Muth DJ (1989) Western equine encephalitis in avian populations in North Dakota, 1975. *J Wildl Dis* 25: 481-489.
217. Boyle DB, Dickerman RW, Marshall ID (1983) Primary viraemia responses of herons to experimental infection with Murray Valley encephalitis, Kunjin and Japanese encephalitis viruses. *Aust J Exp Biol Med Sci* 61 (Pt 6): 655-664.
218. Nemeth N, Young G, Ndaluka C, Bielefeldt-Ohmann H, Komar N, et al. (2009) Persistent West Nile virus infection in the house sparrow (*Passer domesticus*). *Arch Virol* 154: 783-789.
219. Swayne DE, Beck JR, Smith CS, Shieh WJ, Zaki SR (2001) Fatal encephalitis and myocarditis in young domestic geese (*Anser anser domesticus*) caused by West Nile virus. *Emerg Infect Dis* 7: 751-753.
220. Wunschmann A, Ziegler A (2006) West Nile virus-associated mortality events in domestic Chukar partridges (*Alectoris chukar*) and domestic Impeyan pheasants (*Lophophorus impeyanus*). *Avian Dis* 50: 456-459.
221. Styer LM, Bernard KA, Kramer LD (2006) Enhanced early West Nile virus infection in young chickens infected by mosquito bite: effect of viral dose. *Am J Trop Med Hyg* 75: 337-345.
222. Melles S, Glenn SM, Martin K (2003) Urban Bird Diversity and Landscape Complexity: Species-Environment Associations Along a Multiscale Habitat Gradient. *Ecology and Society* 7.
223. Savage HM, Aggarwal D, Apperson CS, Katholi CR, Gordon E, et al. (2007) Host choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the *Culex pipiens* complex, from Memphis and Shelby County, Tennessee, 2002-2003. *Vector Borne Zoonotic Dis* 7: 365-386.
224. Hamer GL, Kitron UD, Brawn JD, Loss SR, Ruiz MO, et al. (2008) *Culex pipiens* (Diptera: Culicidae): a bridge vector of West Nile virus to humans. *J Med Entomol* 45: 125-128.
225. Molaei G, Andreadis TG, Armstrong PM, Anderson JF, Vossbrinck CR (2006) Host feeding patterns of *Culex* mosquitoes and West Nile virus transmission, northeastern United States. *Emerg Infect Dis* 12: 468-474.
226. Kilpatrick AM, Daszak P, Jones MJ, Marra PP, Kramer LD (2006) Host heterogeneity dominates West Nile virus transmission. *Proc Biol Sci* 273: 2327-2333.
227. Panella NA, Young G, Komar N (2013) Experimental infection of Eurasian collared-dove (*Streptopelia decaocto*) with West Nile virus. *J Vector Ecol* 38: 210-214.
228. Day JF, Curtis GA (1989) Influence of Rainfall on *Culex nigripalpus* (Diptera: Culicidae) Blood-Feeding Behavior in Indian River County, Florida. *Annals of the Entomological Society of America* 82: 32-37.
229. Kilpatrick AM, Kramer LD, Jones MJ, Marra PP, Daszak P (2006) West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. *PLoS Biol* 4: e82.
230. Diuk-Wasser MA, Molaei G, Simpson JE, Folsom-O'Keefe CM, Armstrong PM, et al. (2010) Avian communal roosts as amplification foci for West Nile virus in urban areas in northeastern United States. *Am J Trop Med Hyg* 82: 337-343.

231. Kale HW, Edman JD, Webber LA (1972) EFFECT OF BEHAVIOR AND AGE OF INDIVIDUAL CICONIIFORM BIRDS ON MOSQUITO FEEDING Mosquito News 29.
232. Blackmore JS, Dow RP (1958) Differential Feeding of *Culex tarsalis* on Nestling and Adult Birds. Mosquito News 18: 15-17.
233. Edman JD, Webber LA, Schmid AA (1974) Effect of host defenses on the feeding pattern of *Culex nigripalpus* when offered a choice of blood sources. J Parasitol 60: 874-883.
234. Paz S, Albersheim I (2008) Influence of warming tendency on *Culex pipiens* population abundance and on the probability of West Nile fever outbreaks (Israeli Case Study: 2001-2005). Ecohealth 5: 40-48.
235. Delatte H, Gimonneau G, Triboire A, Fontenille D (2009) Influence of temperature on immature development, survival, longevity, fecundity, and gonotrophic cycles of *Aedes albopictus*, vector of chikungunya and dengue in the Indian Ocean. J Med Entomol 46: 33-41.
236. Dohm DJ, O'Guinn ML, Turell MJ (2002) Effect of environmental temperature on the ability of *Culex pipiens* (Diptera: Culicidae) to transmit West Nile virus. J Med Entomol 39: 221-225.
237. Flamand A (1980) Rhabdovirus genetics. In: Bishop DHL, editor. Rhabdovirus. Boca Raton: CRC Press. pp. 115-139.
238. Davis CW, Nguyen HY, Hanna SL, Sanchez MD, Doms RW, et al. (2006) West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. J Virol 80: 1290-1301.
239. Chu JJ, Ng ML (2004) Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. J Virol 78: 10543-10555.
240. Chu JJ, Leong PW, Ng ML (2006) Analysis of the endocytic pathway mediating the infectious entry of mosquito-borne flavivirus West Nile into *Aedes albopictus* mosquito (C6/36) cells. Virology 349: 463-475.
241. Heinz FX, Allison SL (2000) Structures and mechanisms in flavivirus fusion. Adv Virus Res 55: 231-269.
242. Ivanyi-Nagy R, Lavergne JP, Gabus C, Ficheux D, Darlix JL (2008) RNA chaperoning and intrinsic disorder in the core proteins of Flaviviridae. Nucleic Acids Res 36: 712-725.
243. Pong WL, Huang ZS, Teoh PG, Wang CC, Wu HN (2011) RNA binding property and RNA chaperone activity of dengue virus core protein and other viral RNA-interacting proteins. FEBS Lett 585: 2575-2581.
244. Nowak T, Farber PM, Wengler G (1989) Analyses of the terminal sequences of West Nile virus structural proteins and of the in vitro translation of these proteins allow the proposal of a complete scheme of the proteolytic cleavages involved in their synthesis. Virology 169: 365-376.
245. Alvarez DE, De Lella Ezcurra AL, Fucito S, Gamarnik AV (2005) Role of RNA structures present at the 3'UTR of dengue virus on translation, RNA synthesis, and viral replication. Virology 339: 200-212.
246. Corver J, Lenches E, Smith K, Robison RA, Sando T, et al. (2003) Fine mapping of a cis-acting sequence element in yellow fever virus RNA that is required for RNA replication and cyclization. J Virol 77: 2265-2270.
247. Khromykh AA, Meka H, Guyatt KJ, Westaway EG (2001) Essential role of cyclization sequences in flavivirus RNA replication. J Virol 75: 6719-6728.

248. Lo MK, Tilgner M, Bernard KA, Shi PY (2003) Functional analysis of mosquito-borne flavivirus conserved sequence elements within 3' untranslated region of West Nile virus by use of a reporting replicon that differentiates between viral translation and RNA replication. *J Virol* 77: 10004-10014.
249. Basu M, Brinton MA (2011) West Nile virus (WNV) genome RNAs with up to three adjacent mutations that disrupt long distance 5'-3' cyclization sequence basepairs are viable. *Virology* 412: 220-232.
250. Mackenzie JM, Khromykh AA, Jones MK, Westaway EG (1998) Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. *Virology* 245: 203-215.
251. Westaway EG, Mackenzie JM, Khromykh AA (2002) Replication and gene function in Kunjin virus. *Curr Top Microbiol Immunol* 267: 323-351.
252. Miller S, Kastner S, Krijnse-Locker J, Buhler S, Bartenschlager R (2007) The non-structural protein 4A of dengue virus is an integral membrane protein inducing membrane alterations in a 2K-regulated manner. *J Biol Chem* 282: 8873-8882.
253. Miller S, Sparacio S, Bartenschlager R (2006) Subcellular localization and membrane topology of the Dengue virus type 2 Non-structural protein 4B. *J Biol Chem* 281: 8854-8863.
254. Kamer G, Argos P (1984) Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Res* 12: 7269-7282.
255. Ray D, Shah A, Tilgner M, Guo Y, Zhao Y, et al. (2006) West Nile virus 5'-cap structure is formed by sequential guanine N-7 and ribose 2'-O methylations by nonstructural protein 5. *J Virol* 80: 8362-8370.
256. Wengler G (1993) The NS 3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. *Virology* 197: 265-273.
257. Wengler G (1991) The carboxy-terminal part of the NS 3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. *Virology* 184: 707-715.
258. Gorbalenya AE, Donchenko AP, Koonin EV, Blinov VM (1989) N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. *Nucleic Acids Res* 17: 3889-3897.
259. Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CK, et al. (2009) Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell Host Microbe* 5: 365-375.
260. Miorin L, Romero-Brey I, Maiuri P, Hoppe S, Krijnse-Locker J, et al. (2013) Three-dimensional architecture of tick-borne encephalitis virus replication sites and trafficking of the replicated RNA. *J Virol* 87: 6469-6481.
261. Dokland T, Walsh M, Mackenzie JM, Khromykh AA, Ee KH, et al. (2004) West Nile virus core protein; tetramer structure and ribbon formation. *Structure* 12: 1157-1163.
262. Modrich P, Lahue R (1996) Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu Rev Biochem* 65: 101-133.
263. Sanjuan R, Nebot MR, Chirico N, Mansky LM, Belshaw R (2010) Viral mutation rates. *J Virol* 84: 9733-9748.
264. Duffy S, Shackelton LA, Holmes EC (2008) Rates of evolutionary change in viruses: patterns and determinants. *Nat Rev Genet* 9: 267-276.

265. Snapinn KW, Holmes EC, Young DS, Bernard KA, Kramer LD, et al. (2007) Declining growth rate of West Nile virus in North America. *J Virol* 81: 2531-2534.
266. Coffey LL, Beeharry Y, Borderia AV, Blanc H, Vignuzzi M (2011) Arbovirus high fidelity variant loses fitness in mosquitoes and mice. *Proc Natl Acad Sci U S A* 108: 16038-16043.
267. Beaucourt S, Borderia AV, Coffey LL, Gnadig NF, Sanz-Ramos M, et al. (2011) Isolation of fidelity variants of RNA viruses and characterization of virus mutation frequency. *J Vis Exp*.
268. Rozen-Gagnon K, Stapleford KA, Mongelli V, Blanc H, Failloux AB, et al. (2014) Alphavirus mutator variants present host-specific defects and attenuation in mammalian and insect models. *PLoS Pathog* 10: e1003877.
269. Van Slyke GA, Arnold JJ, Lugo AJ, Griesemer SB, Moustafa IM, et al. (2015) Sequence-Specific Fidelity Alterations Associated with West Nile Virus Attenuation in Mosquitoes. *PLoS Pathog* 11: e1005009.
270. Van Slyke GA, Ciota AT, Willsey GG, Jaeger J, Shi PY, et al. (2012) Point mutations in the West Nile virus (Flaviviridae; Flavivirus) RNA-dependent RNA polymerase alter viral fitness in a host-dependent manner in vitro and in vivo. *Virology* 427: 18-24.
271. Muller HJ (1964) The Relation of Recombination to Mutational Advance. *Mutat Res* 1: 2-9.
272. Chao L (1990) Fitness of RNA virus decreased by Muller's ratchet. *Nature* 348: 454-455.
273. Novella IS, Reissig DD, Wilke CO (2004) Density-dependent selection in vesicular stomatitis virus. *J Virol* 78: 5799-5804.
274. Froissart R, Wilke CO, Montville R, Remold SK, Chao L, et al. (2004) Co-infection weakens selection against epistatic mutations in RNA viruses. *Genetics* 168: 9-19.
275. Garcia-Arriaza J, Manrubia SC, Toja M, Domingo E, Escarmis C (2004) Evolutionary transition toward defective RNAs that are infectious by complementation. *Journal of Virology* 78: 11678-11685.
276. Moreno IM, Malpica JM, Rodriguez-Cerezo E, Garcia-Arenal F (1997) A mutation in tomato aspermy cucumovirus that abolishes cell-to-cell movement is maintained to high levels in the viral RNA population by complementation. *J Virol* 71: 9157-9162.
277. Ke R, Aaskov J, Holmes EC, Lloyd-Smith JO (2013) Phylodynamic analysis of the emergence and epidemiological impact of transmissible defective dengue viruses. *Plos Pathogens* 9: e1003193.
278. Combe M, Garijo R, Geller R, Cuevas JM, Sanjuan R (2015) Single-Cell Analysis of RNA Virus Infection Identifies Multiple Genetically Diverse Viral Genomes within Single Infectious Units. *Cell Host Microbe* 18: 424-432.
279. Franz AW, Kantor AM, Passarelli AL, Clem RJ (2015) Tissue Barriers to Arbovirus Infection in Mosquitoes. *Viruses* 7: 3741-3767.
280. Klowden MJ (2013) *Physiological Systems in Insects*. San Diego, CA: Academic Press.
281. Houk EJ, Hardy JL (1979) In vivo negative staining of the midgut continuous junction in the mosquito, *Culex tarsalis* (Diptera: Culicidae). *Acta Trop* 36: 267-275.
282. Weaver SC, Scott TW, Lorenz LH, Repik PM (1991) Detection of eastern equine encephalomyelitis virus deposition in *Culiseta melanura* following ingestion of radiolabeled virus in blood meals. *Am J Trop Med Hyg* 44: 250-259.
283. Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol* 32: 1295-1309.

284. Girard YA, Klingler KA, Higgs S (2004) West Nile virus dissemination and tissue tropisms in orally infected *Culex pipiens quinquefasciatus*. *Vector-Borne and Zoonotic Diseases* 4: 109-122.
285. Hardy JL, Houk EJ, Kramer LD, Reeves WC (1983) Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annu Rev Entomol* 28: 229-262.
286. Stark KR, James, A. A. (1996) The salivary glands of disease vectors. In: Beaty BJ, Marquardt, C., editor. *The Biology of Disease Vectors*. Niwot, CO: University Press of Colorado. pp. 333-348.
287. Girard YA, Popov V, Wen J, Han V, Higgs S (2005) Ultrastructural study of West Nile virus pathogenesis in *Culex pipiens quinquefasciatus* (Diptera : Culicidae). *J Med Entomol* 42: 429-444.
288. Salazar MI, Richardson JH, Sanchez-Vargas I, Olson KE, Beaty BJ (2007) Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes. *BMC Microbiol* 7.
289. Takahashi M, Suzuki K (1979) Japanese encephalitis virus in mosquito salivary glands. *Am J Trop Med Hyg* 28: 122-135.
290. Whitfiel.Sg, Murphy FA, Sudia WD (1971) Eastern Equine Encephalomyelitis Virus - an Electron Microscopic Study of *Aedes-Triseriatus* (Say) Salivary Gland Infection. *Virology* 43: 110-&.
291. Styer LM, Kent KA, Albright RG, Bennett CJ, Kramer LD, et al. (2007) Mosquitoes inoculate high doses of West Nile virus as they probe and feed on live hosts. *PLoS Pathog* 3: 1262-1270.
292. Ciota AT, Ehrbar DJ, Van Slyke GA, Payne AF, Willsey GG, et al. (2012) Quantification of intrahost bottlenecks of West Nile virus in *Culex pipiens* mosquitoes using an artificial mutant swarm. *Infection Genetics and Evolution* 12: 557-564.
293. Smith DR, Adams AP, Kenney JL, Wang E, Weaver SC (2008) Venezuelan equine encephalitis virus in the mosquito vector *Aedes taeniorhynchus*: infection initiated by a small number of susceptible epithelial cells and a population bottleneck. *Virology* 372: 176-186.
294. Scholle F, Girard YA, Zhao Q, Higgs S, Mason PW (2004) trans-Packaged West Nile virus-like particles: infectious properties in vitro and in infected mosquito vectors. *J Virol* 78: 11605-11614.
295. Forrester NL, Guerois M, Seymour RL, Spratt H, Weaver SC (2012) Vector-Borne Transmission Imposes a Severe Bottleneck on an RNA Virus Population. *PLoS Pathog* 8.
296. Gutierrez S, Thebaud G, Smith DR, Kenney JL, Weaver SC (2015) Demographics of Natural Oral Infection of Mosquitos by Venezuelan Equine Encephalitis Virus. *J Virol* 89: 4020-4022.
297. Mayr E (1942) *Systematics and the origin of species from the viewpoint of a zoologist*. New York,: Columbia University Press. xiv, 334 p. incl. illus. (incl. maps) tables, diags. p.
298. Forrester NL, Coffey LL, Weaver SC (2014) Arboviral bottlenecks and challenges to maintaining diversity and fitness during mosquito transmission. *Viruses* 6: 3991-4004.
299. Kramer LD, Ciota AT (2015) Dissecting vectorial capacity for mosquito-borne viruses. *Curr Opin Virol* 15: 112-118.
300. Dickson LB, Sanchez-Vargas I, Sylla M, Fleming K, Black WCt (2014) Vector competence in West African *Aedes aegypti* Is Flavivirus species and genotype dependent. *PLoS Negl Trop Dis* 8: e3153.

301. Karpf AR, Lenches E, Strauss EG, Strauss JH, Brown DT (1997) Superinfection exclusion of alphaviruses in three mosquito cell lines persistently infected with Sindbis virus. *J Virol* 71: 7119-7123.
302. Zou G, Zhang B, Lim PY, Yuan Z, Bernard KA, et al. (2009) Exclusion of West Nile virus superinfection through RNA replication. *J Virol* 83: 11765-11776.
303. Bolling BG, Olea-Popelka FJ, Eisen L, Moore CG, Blair CD (2012) Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex pipiens* laboratory colony and effects of co-infection on vector competence for West Nile virus. *Virology* 427: 90-97.
304. Jupatanakul N, Sim S, Dimopoulos G (2014) The insect microbiome modulates vector competence for arboviruses. *Viruses* 6: 4294-4313.
305. Beerntsen BT, James AA, Christensen BM (2000) Genetics of mosquito vector competence. *Microbiol Mol Biol Rev* 64: 115-137.
306. Blair CD, Olson KE (2015) The role of RNA interference (RNAi) in arbovirus-vector interactions. *Viruses* 7: 820-843.
307. Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler JL (2006) Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. *Nat Immunol* 7: 590-597.
308. Keene KM, Foy BD, Sanchez-Vargas I, Beaty BJ, Blair CD, et al. (2004) RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 101: 17240-17245.
309. Myles KM, Wiley MR, Morazzani EM, Adelman ZN (2008) Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proc Natl Acad Sci U S A* 105: 19938-19943.
310. Sanchez-Vargas I, Scott JC, Poole-Smith BK, Franz AW, Barbosa-Solomieu V, et al. (2009) Dengue virus type 2 infections of *Aedes aegypti* are modulated by the mosquito's RNA interference pathway. *PLoS Pathog* 5: e1000299.
311. Ratcliff F, Harrison BD, Baulcombe DC (1997) A similarity between viral defense and gene silencing in plants. *Science* 276: 1558-1560.
312. Pusch O, Boden D, Silbermann R, Lee F, Tucker L, et al. (2003) Nucleotide sequence homology requirements of HIV-1-specific short hairpin RNA. *Nucleic Acids Res* 31: 6444-6449.
313. Westerhout EM, Ooms M, Vink M, Das AT, Berkhout B (2005) HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res* 33: 796-804.
314. Konishi M, Wu CH, Kaito M, Hayashi K, Watanabe S, et al. (2006) siRNA-resistance in treated HCV replicon cells is correlated with the development of specific HCV mutations. *J Viral Hepat* 13: 756-761.
315. Cullen BR, Cherry S, tenOever BR (2013) Is RNA interference a physiologically relevant innate antiviral immune response in mammals? *Cell Host Microbe* 14: 374-378.
316. LaDeau SL, Kilpatrick AM, Marra PP (2007) West Nile virus emergence and large-scale declines of North American bird populations. *Nature* 447: 710-713.
317. Wheeler SS, Barker CM, Fang Y, Armijos MV, Carroll BD, et al. (2009) Differential Impact of West Nile Virus on California Birds. *Condor* 111: 1-20.
318. Ward MP, Beveroth TA, Lampman R, Raim A, Enstrom D, et al. (2010) Field-based estimates of avian mortality from West Nile virus infection. *Vector Borne Zoonotic Dis* 10: 909-913.

319. George TL, Harrigan RJ, LaManna JA, DeSante DF, Saracco JF, et al. (2015) Persistent impacts of West Nile virus on North American bird populations. *Proc Natl Acad Sci U S A* 112: 14290-14294.
320. Nemeth NM, Thomsen BV, Spraker TR, Benson JM, Bosco-Lauth AM, et al. (2011) Clinical and pathologic responses of American crows (*Corvus brachyrhynchos*) and fish crows (*C. ossifragus*) to experimental West Nile virus infection. *Vet Pathol* 48: 1061-1074.
321. Brault AC, Langevin SA, Bowen RA, Panella NA, Biggerstaff BJ, et al. (2004) Differential virulence of West Nile strains for American crows. *Emerg Infect Dis* 10: 2161-2168.
322. McClure CJ, Rolek BW, McDonald K, Hill GE (2012) Climate change and the decline of a once common bird. *Ecol Evol* 2: 370-378.
323. Saracco JF, Royle JA, DeSante DF, Gardner B (2010) Modeling spatial variation in avian survival and residency probabilities. *Ecology* 91: 1885-1891.
324. Bradley CA, Gibbs SE, Altizer S (2008) Urban land use predicts West Nile virus exposure in songbirds. *Ecol Appl* 18: 1083-1092.
325. Wheeler SS, Vineyard MP, Woods LW, Reisen WK (2012) Dynamics of West Nile virus persistence in House Sparrows (*Passer domesticus*). *PLoS Negl Trop Dis* 6: e1860.
326. Wheeler SS, Langevin SA, Brault AC, Woods L, Carroll BD, et al. (2012) Detection of persistent west nile virus RNA in experimentally and naturally infected avian hosts. *Am J Trop Med Hyg* 87: 559-564.
327. Reisen WK, Fang Y, Lothrop HD, Martinez VM, Wilson J, et al. (2006) Overwintering of West Nile virus in Southern California. *J Med Entomol* 43: 344-355.
328. VanDalen KK, Hall JS, Clark L, McLean RG, Smeraski C (2013) West Nile virus infection in American Robins: new insights on dose response. *PLoS One* 8: e68537.
329. Boyle DB, Marshall ID, Dickerman RW (1983) Primary antibody responses of herons to experimental infection with Murray Valley encephalitis and Kunjin viruses. *Aust J Exp Biol Med Sci* 61 (Pt 6): 665-674.
330. Hoebe K, Janssen E, Beutler B (2004) The interface between innate and adaptive immunity. *Nat Immunol* 5: 971-974.
331. Pesko KN, Fitzpatrick KA, Ryan EM, Shi PY, Zhang B, et al. (2012) Internally deleted WNV genomes isolated from exotic birds in New Mexico: function in cells, mosquitoes, and mice. *Virology* 427: 10-17.
332. Van Valen L (1973) A new evolutionary law. *Evol Theory* 1: 1-30.
333. Grenfell BT, Pybus OG, Gog JR, Wood JL, Daly JM, et al. (2004) Unifying the epidemiological and evolutionary dynamics of pathogens. *Science* 303: 327-332.
334. Williamson S (2003) Adaptation in the env gene of HIV-1 and evolutionary theories of disease progression. *Molecular Biology and Evolution* 20: 1318-1325.
335. Wolinsky SM, Korber BT, Neumann AU, Daniels M, Kunstman KJ, et al. (1996) Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. *Science* 272: 537-542.
336. Bakonyi T, Ferenczi E, Erdelyi K, Kutasi O, Csorgo T, et al. (2013) Explosive spread of a neuroinvasive lineage 2 West Nile virus in Central Europe, 2008/2009. *Vet Microbiol* 165: 61-70.
337. Frost MJ, Zhang J, Edmonds JH, Prow NA, Gu X, et al. (2012) Characterization of virulent West Nile virus Kunjin strain, Australia, 2011. *Emerging Infectious Diseases* 18: 792-800.

338. Lim PY, Behr MJ, Chadwick CM, Shi PY, Bernard KA (2011) Keratinocytes are cell targets of West Nile virus in vivo. *Journal of Virology* 85: 5197-5201.
339. Johnston LJ, Halliday GM, King NJ (2000) Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus. *J Invest Dermatol* 114: 560-568.
340. Samuel MA, Whitby K, Keller BC, Marri A, Barchet W, et al. (2006) PKR and RNase L contribute to protection against lethal West Nile Virus infection by controlling early viral spread in the periphery and replication in neurons. *Journal of Virology* 80: 7009-7019.
341. Ben-Nathan D, Huitinga I, Lustig S, van Rooijen N, Kobiler D (1996) West Nile virus neuroinvasion and encephalitis induced by macrophage depletion in mice. *Arch Virol* 141: 459-469.
342. Sejvar JJ (2014) Clinical manifestations and outcomes of West Nile virus infection. *Viruses* 6: 606-623.
343. Busch MP, Wright DJ, Custer B, Tobler LH, Stramer SL, et al. (2006) West Nile virus infections projected from blood donor screening data, United States, 2003. *Emerg Infect Dis* 12: 395-402.
344. Daffis S, Suthar MS, Gale M, Diamond MS (2009) Measure and Countermeasure: Type I IFN (IFN-alpha/beta) Antiviral Response against West Nile Virus. *Journal of Innate Immunity* 1: 435-445.
345. Schoggins JW, Rice CM (2011) Interferon-stimulated genes and their antiviral effector functions. *Current Opinion in Virology* 1: 519-525.
346. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, et al. (2011) A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* 472: 481-485.
347. Szretter KJ, Brien JD, Thackray LB, Virgin HW, Cresswell P, et al. (2011) The interferon-inducible gene viperin restricts West Nile virus pathogenesis. *Journal of Virology* 85: 11557-11566.
348. Perelygin AA, Scherbik SV, Zhulin IB, Stockman BM, Li Y, et al. (2002) Positional cloning of the murine flavivirus resistance gene. *Proc Natl Acad Sci U S A* 99: 9322-9327.
349. Pichlmair A, Lassnig C, Eberle CA, Gorna MW, Baumann CL, et al. (2011) IFIT1 is an antiviral protein that recognizes 5'-triphosphate RNA. *Nat Immunol* 12: 624-630.
350. Brien JD, Daffis S, Lazear HM, Cho H, Suthar MS, et al. (2011) Interferon regulatory factor-1 (IRF-1) shapes both innate and CD8(+) T cell immune responses against West Nile virus infection. *Plos Pathogens* 7: e1002230.
351. Cheriya V, Leaman DW, Borden EC (2011) Emerging roles of FAM14 family members (G1P3/ISG 6-16 and ISG12/IFI27) in innate immunity and cancer. *J Interferon Cytokine Res* 31: 173-181.
352. Cho H, Prohl SC, Szretter KJ, Katze MG, Gale M, Jr., et al. (2013) Differential innate immune response programs in neuronal subtypes determine susceptibility to infection in the brain by positive-stranded RNA viruses. *Nat Med* 19: 458-464.
353. van Boxel-Dezaire AH, Rani MR, Stark GR (2006) Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* 25: 361-372.
354. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, et al. (2005) Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23: 19-28.

355. Hoenen A, Liu W, Kochs G, Khromykh AA, Mackenzie JM (2007) West Nile virus-induced cytoplasmic membrane structures provide partial protection against the interferon-induced antiviral MxA protein. *Journal of General Virology* 88: 3013-3017.
356. Fredericksen BL, Gale M, Jr. (2006) West Nile virus evades activation of interferon regulatory factor 3 through RIG-I-dependent and -independent pathways without antagonizing host defense signaling. *Journal of Virology* 80: 2913-2923.
357. Szretter KJ, Daniels BP, Cho H, Gainey MD, Yokoyama WM, et al. (2012) 2'-O methylation of the viral mRNA cap by West Nile virus evades ifit1-dependent and -independent mechanisms of host restriction in vivo. *Plos Pathogens* 8: e1002698.
358. Daffis S, Szretter KJ, Schriewer J, Li J, Youn S, et al. (2010) 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature* 468: 452-456.
359. Wilson JR, de Sessions PF, Leon MA, Scholle F (2008) West Nile virus nonstructural protein 1 inhibits TLR3 signal transduction. *Journal of Virology* 82: 8262-8271.
360. Guo JT, Hayashi J, Seeger C (2005) West Nile virus inhibits the signal transduction pathway of alpha interferon. *Journal of Virology* 79: 1343-1350.
361. Liu WJ, Wang XJ, Mokhonov VV, Shi PY, Randall R, et al. (2005) Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. *Journal of Virology* 79: 1934-1942.
362. Laurent-Rolle M, Boer EF, Lubick KJ, Wolfenbarger JB, Carmody AB, et al. (2010) The NS5 protein of the virulent West Nile virus NY99 strain is a potent antagonist of type I interferon-mediated JAK-STAT signaling. *Journal of Virology* 84: 3503-3515.
363. Munoz-Jordan JL, Laurent-Rolle M, Ashour J, Martinez-Sobrido L, Ashok M, et al. (2005) Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *Journal of Virology* 79: 8004-8013.
364. Suen WW, Prow NA, Hall RA, Bielefeldt-Ohmann H (2014) Mechanism of West Nile virus neuroinvasion: a critical appraisal. *Viruses* 6: 2796-2825.
365. Hasebe R, Suzuki T, Makino Y, Igarashi M, Yamanouchi S, et al. (2010) Transcellular transport of West Nile virus-like particles across human endothelial cells depends on residues 156 and 159 of envelope protein. *BMC Microbiol* 10: 165.
366. Garcia-Tapia D, Loiacono CM, Kleiboeker SB (2006) Replication of West Nile virus in equine peripheral blood mononuclear cells. *Vet Immunol Immunopathol* 110: 229-244.
367. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, et al. (2004) Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 10: 1366-1373.
368. Arjona A, Foellmer HG, Town T, Leng L, McDonald C, et al. (2007) Abrogation of macrophage migration inhibitory factor decreases West Nile virus lethality by limiting viral neuroinvasion. *J Clin Invest* 117: 3059-3066.
369. Daffis S, Samuel MA, Suthar MS, Gale M, Jr., Diamond MS (2008) Toll-like receptor 3 has a protective role against West Nile virus infection. *J Virol* 82: 10349-10358.
370. Lazear HM, Daniels BP, Pinto AK, Huang AC, Vick SC, et al. (2015) Interferon-lambda restricts West Nile virus neuroinvasion by tightening the blood-brain barrier. *Sci Transl Med* 7: 284ra259.
371. Samuel MA, Wang H, Siddharthan V, Morrey JD, Diamond MS (2007) Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis. *Proc Natl Acad Sci U S A* 104: 17140-17145.

372. Hunsperger EA, Roehrig JT (2006) Temporal analyses of the neuropathogenesis of a West Nile virus infection in mice. *J Neurovirol* 12: 129-139.
373. Samuel MA, Diamond MS (2005) Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. *J Virol* 79: 13350-13361.
374. Armah HB, Wang G, Omalu BI, Tesh RB, Gyure KA, et al. (2007) Systemic distribution of West Nile virus infection: postmortem immunohistochemical study of six cases. *Brain Pathology* 17: 354-362.
375. Sejvar JJ (2007) The long-term outcomes of human West Nile virus infection. *Clin Infect Dis* 44: 1617-1624.
376. Erickson AK, Pfeiffer JK (2013) Dynamic viral dissemination in mice infected with yellow fever virus strain 17D. *J Virol* 87: 12392-12397.
377. Li Q, Zhou L, Zhou M, Chen Z, Li F, et al. (2014) Epidemiology of human infections with avian influenza A(H7N9) virus in China. *N Engl J Med* 370: 520-532.
378. Gire SK, Goba A, Andersen KG, Sealfon RS, Park DJ, et al. (2014) Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. *Science* 345: 1369-1372.
379. Farci P, Strazzer R, Alter HJ, Farci S, Degioannis D, et al. (2002) Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. *Proc Natl Acad Sci U S A* 99: 3081-3086.
380. Essajee SM, Pollack H, Rochford G, Oransky I, Krasinski K, et al. (2000) Early changes in quasispecies repertoire in HIV-infected infants: correlation with disease progression. *AIDS Res Hum Retroviruses* 16: 1949-1957.
381. Dawson JR, Stone WB, Ebel GD, Young DS, Galinski DS, et al. (2007) Crow deaths caused by West Nile virus during winter. *Emerging Infectious Diseases* 13: 1912-1914.
382. Langevin SA, Brault AC, Panella NA, Bowen RA, Komar N (2005) Variation in virulence of West Nile virus strains for house sparrows (*Passer domesticus*). *American Journal of Tropical Medicine and Hygiene* 72: 99-102.
383. Komar N, Panella NA, Langevin SA, Brault AC, Amador M, et al. (2005) Avian hosts for West Nile virus in St. Tammany Parish, Louisiana, 2002. *American Journal of Tropical Medicine and Hygiene* 73: 1031-1037.
384. Shi PY, Tilgner M, Lo MK, Kent KA, Bernard KA (2002) Infectious cDNA clone of the epidemic west nile virus from New York City. *J Virol* 76: 5847-5856.
385. Langevin SA, Bowen RA, Reisen WK, Andrade CC, Ramey WN, et al. (2014) Host Competence and Helicase Activity Differences Exhibited by West Nile Viral Variants Expressing NS3-249 Amino Acid Polymorphisms. *Plos One* 9.
386. Hall GS, Little DP (2007) Relative quantitation of virus population size in mixed genotype infections using sequencing chromatograms. *J Virol Methods* 146: 22-28.
387. Smith DR, Carrara AS, Aguilar PV, Weaver SC (2005) Evaluation of methods to assess transmission potential of Venezuelan equine encephalitis virus by mosquitoes and estimation of mosquito saliva titers. *American Journal of Tropical Medicine and Hygiene* 73: 33-39.
388. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, et al. (2000) Rapid detection of west nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J Clin Microbiol* 38: 4066-4071.

389. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol* 29: 644-652.
390. Lee WP, Stromberg MP, Ward A, Stewart C, Garrison EP, et al. (2014) MOSAIK: a hash-based algorithm for accurate next-generation sequencing short-read mapping. *PLoS One* 9: e90581.
391. Yang X, Charlebois P, Macalalad A, Henn MR, Zody MC (2013) V-Phaser 2: variant inference for viral populations. *BMC Genomics* 14: 674.
392. Guo Y, Li J, Li CI, Long J, Samuels DC, et al. (2012) The effect of strand bias in Illumina short-read sequencing data. *Bmc Genomics* 13: 666.
393. Gregori J, Salicru M, Domingo E, Sanchez A, Esteban JI, et al. (2014) Inference with viral quasispecies diversity indices: clonal and NGS approaches. *Bioinformatics*.
394. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, et al. (2012) VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* 22: 568-576.
395. Topfer A, Zagordi O, Prabhakaran S, Roth V, Halperin E, et al. (2013) Probabilistic inference of viral quasispecies subject to recombination. *J Comput Biol* 20: 113-123.
396. Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25: 1451-1452.
397. Nei M, Gojobori T (1986) Simple Methods for Estimating the Numbers of Synonymous and Nonsynonymous Nucleotide Substitutions. *Mol Biol Evol* 3: 418-426.
398. Fu YX, Li WH (1993) Statistical tests of neutrality of mutations. *Genetics* 133: 693-709.
399. Fay JC, Wu CI (2000) Hitchhiking under positive Darwinian selection. *Genetics* 155: 1405-1413.
400. Fumagalli M, Vieira FG, Korneliussen TS, Linderoth T, Huerta-Sanchez E, et al. (2013) Quantifying population genetic differentiation from next-generation sequencing data. *Genetics* 195: 979-992.
401. Li DS, Lott WB, Lowry K, Jones A, Thu HM, et al. (2011) Defective Interfering Viral Particles in Acute Dengue Infections. *Plos One* 6.
402. Stern A, Bianco S, Yeh MT, Wright C, Butcher K, et al. (2014) Costs and benefits of mutational robustness in RNA viruses. *Cell Rep* 8: 1026-1036.
403. Beyer HM, Gonschorek P, Samodelov SL, Meier M, Weber W, et al. (2015) AQUA Cloning: A Versatile and Simple Enzyme-Free Cloning Approach. *PLoS One* 10: e0137652.
404. Tajima F (1989) Statistical-Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism. *Genetics* 123: 585-595.
405. Harpending HC, Sherry ST, Rogers AR, Stoneking M (1993) The Genetic-Structure of Ancient Human-Populations. *Current Anthropology* 34: 483-496.
406. Rogers AR, Harpending H (1992) Population-Growth Makes Waves in the Distribution of Pairwise Genetic-Differences. *Molecular Biology and Evolution* 9: 552-569.
407. Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian Phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* 29: 1969-1973.
408. Monsion B, Froissart R, Michalakis Y, Blanc S (2008) Large Bottleneck Size in Cauliflower Mosaic Virus Populations during Host Plant Colonization. *PLoS Pathog* 4.
409. Jukes TH, Cantor, C.R. (1969) Evolution of protein molecules; H.N. M, editor. New York: Academic Press. 71-123 p.

410. Kryazhimskiy S, Plotkin JB (2008) The Population Genetics of dN/dS. *Plos Genetics* 4.
411. Sanjuan R, Moya A, Elena SF (2004) The distribution of fitness effects caused by single-nucleotide substitutions in an RNA virus. *Proc Natl Acad Sci U S A* 101: 8396-8401.
412. Slatkin M, Hudson RR (1991) Pairwise Comparisons of Mitochondrial-DNA Sequences in Stable and Exponentially Growing Populations. *Genetics* 129: 555-562.
413. Carrara AS, Gonzales G, Ferro C, Tamayo M, Aronson J, et al. (2005) Venezuelan equine encephalitis virus infection of spiny rats. *Emerg Infect Dis* 11: 663-669.
414. Ciota AT, Ehrbar DJ, Van Slyke GA, Willsey GG, Kramer LD (2012) Cooperative interactions in the West Nile virus mutant swarm. *BMC Evol Biol* 12: 58.
415. Agudo R, Ferrer-Orta C, Arias A, de la Higuera I, Perales C, et al. (2010) A Multi-Step Process of Viral Adaptation to a Mutagenic Nucleoside Analogue by Modulation of Transition Types Leads to Extinction-Escape. *PLoS Pathog* 6.
416. Borderia AV, Isakov O, Moratorio G, Henningsson R, Aguera-Gonzalez S, et al. (2015) Group Selection and Contribution of Minority Variants during Virus Adaptation Determines Virus Fitness and Phenotype. *PLoS Pathog* 11: e1004838.
417. Kieft JS, Rabe JL, Chapman EG (2015) New hypotheses derived from the structure of a flaviviral Xrn1-resistant RNA: Conservation, folding, and host adaptation. *RNA Biol* 12: 1169-1177.
418. Grubaugh ND, Smith DR, Brackney DE, Bosco-Lauth AM, Fauver JR, et al. (2015) Experimental evolution of an RNA virus in wild birds: evidence for host-dependent impacts on population structure and competitive fitness. *PLoS Pathog* 11: e1004874.
419. Beckham JD, Tyler KL (2012) Neuro-intensive care of patients with acute CNS infections. *Neurotherapeutics* 9: 124-138.
420. Armah HB, Wang G, Omalu BI, Tesh RB, Gyure KA, et al. (2007) Systemic distribution of West Nile virus infection: postmortem immunohistochemical study of six cases. *Brain Pathol* 17: 354-362.
421. Lazear HM, Pinto AK, Vogt MR, Gale M, Jr., Diamond MS (2011) Beta interferon controls West Nile virus infection and pathogenesis in mice. *J Virol* 85: 7186-7194.
422. Romano CM, Lauck M, Salvador FS, Lima CR, Villas-Boas LS, et al. (2013) Inter- and Intra-Host Viral Diversity in a Large Seasonal DENV2 Outbreak. *PLoS One* 8.
423. Ali M, Safriel Y, Sohi J, Llave A, Weathers S (2005) West Nile virus infection: MR imaging findings in the nervous system. *AJNR Am J Neuroradiol* 26: 289-297.
424. Petropoulou KA, Gordon SM, Prayson RA, Ruggieri PM (2005) West Nile virus meningoencephalitis: MR imaging findings. *AJNR Am J Neuroradiol* 26: 1986-1995.
425. Gerrish PJ, Garcia-Lerma JG (2003) Mutation rate and the efficacy of antimicrobial drug treatment. *Lancet Infect Dis* 3: 28-32.
426. Henn MR, Boutwell CL, Charlebois P, Lennon NJ, Power KA, et al. (2012) Whole genome deep sequencing of HIV-1 reveals the impact of early minor variants upon immune recognition during acute infection. *PLoS Pathog* 8: e1002529.
427. Andersen KG, Shapiro BJ, Matranga CB, Sealfon R, Lin AE, et al. (2015) Clinical Sequencing Uncovers Origins and Evolution of Lassa Virus. *Cell* 162: 738-750.
428. MacArthur RH, Wilson EO (1967) The theory of island biogeography. Princeton, N.J.,: Princeton University Press. xi, 203 p. p.
429. Sadler HA, Stenglein MD, Harris RS, Mansky LM (2010) APOBEC3G contributes to HIV-1 variation through sublethal mutagenesis. *J Virol* 84: 7396-7404.

- 430. Sharma PM, Bowman M, Madden SL, Rauscher FJ, 3rd, Sukumar S (1994) RNA editing in the Wilms' tumor susceptibility gene, WT1. *Genes Dev* 8: 720-731.
- 431. Danecek P, Nellaker C, McIntyre RE, Buendia-Buendia JE, Bumpstead S, et al. (2012) High levels of RNA-editing site conservation amongst 15 laboratory mouse strains. *Genome Biol* 13: 26.
- 432. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31: 3406-3415.
- 433. Igyarto BZ, Lacko E, Olah I, Magyar A (2006) Characterization of chicken epidermal dendritic cells. *Immunology* 119: 278-288.