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Quantification of intrahost bottlenecks of *West Nile virus* in *Culex pipiens* mosquitoes using an artificial mutant swarm

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Abstract

Mosquito-borne viruses are predominantly RNA viruses which exist within hosts as diverse mutant swarms. Defining the way in which stochastic forces within mosquito vectors shape these swarms is critical to advancing our understanding of the evolutionary and adaptive potential of these pathogens. There are multiple barriers within a mosquito which a viral swarm must traverse in order to ultimately be transmitted. Here, using artificial mutant swarms composed of neutral variants of West Nile virus (WNV), we tracked changes to swarm breadth over time and space in *Culex pipiens* mosquitoes. Our results demonstrate that all variants have the potential to survive intrahost bottlenecks, yet mean swarm breadth decreases during both midgut infection and transmission when starting populations contain higher levels of minority variants. In addition, WNV swarms are subject to temporal sweeps which act to significantly decrease intrahost diversity over time. Taken together, these data demonstrate the profound effects that stochastic forces can have in shaping arboviral mutant swarms.

Keywords

West Nile virus; flavivirus; *Culex pipiens*; intrahost diversity; intrahost bottlenecks; arbovirus evolution

1. Introduction

Mosquito-borne viruses are predominantly RNA viruses which exist within hosts as diverse mutant swarms. A number of studies to date have established the significant role of this swarm in viral fitness, adaptation, and pathogenesis (Ciota and Kramer, 2010), yet the specific dynamics altering swarm composition and breadth within hosts remain largely uncharacterized. There are multiple barriers a viral swarm must traverse within a mosquito in order to ultimately be transmitted, each of which could act as a significant bottleneck of genetic diversity. Viral infection in competent mosquitoes begins with acquisition of

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infectious virus during bloodfeeding and proceeds through infection of, replication within, and dissemination from the mesenteron (midgut). Secondary amplification occurs in parenteral tissues, including fat body cells and nerve tissue, allowing infection of critical target organs such as the salivary glands and, ultimately, virus transmission during subsequent bloodfeeding (Girard et al., 2004; Kramer and Ebel, 2003). *West Nile virus* (WNV, *Flaviviridae*, *Flavivirus*), the most widespread and medically important arthropod-borne virus in the United States, is transmitted primarily by *Culex* spp. mosquitoes (Kramer et al., 2007). Previous studies demonstrate that WNV exists as a diverse viral swarm in nature and that the mosquito is the primary source of this diversity (Jerzak et al., 2005). This mosquito derived diversity likely results from a combination of relaxed purifying selection and density-dependent selection needed to overcome the innate RNAi immune response (Jerzak et al., 2008; Brackney et al., 2009). Despite these findings, passage studies of WNV in *Cx. pipiens* using virus secreted in saliva resulted in a highly homogeneous viral swarm, suggesting that the diversity generated in the mosquito may often be purged by within-host bottlenecks prior to transmission (Ciota et al., 2008). Although it has been shown that *Culex* spp. mosquitoes are capable of transmitting up to 10^6 log₁₀ plaque forming units of WNV, the genetic diversity within this transmitted population has not been fully characterized (Styer et al., 2007). A recent study with *Culex quinquefasciatus* demonstrates that diversity may be maintained during transmission by this species (Brackney et al., 2011), yet *Culex* species often differ significantly in their vector competence (Kilpatrick et al., 2010), suggesting the extent of within-host bottlenecks is also likely species-specific. Here, we characterized time and tissue-dependent changes in mutant swarm breadth in *Culex pipiens* mosquitoes using an artificial swarm of neutral WNV variants that could be tracked over time and space. Our results demonstrate the extent to which the breadth of intrahost viral diversity may be limited by both temporal and spatial variation in mosquito hosts.

2. Materials and Methods

2.1. Mosquitoes

Cx. pipiens egg rafts were originally collected in Pennsylvania in 2004 (courtesy of M. Hutchinson) and subsequently colonized at the Arbovirus laboratory, Wadsworth Center. Mosquitoes were reared and maintained in 30.5 cm³ cages in an environmental chamber at 27°C, 50-65% relative humidity with a photoperiod of 16:8 (light:dark) hours. 1500-2000 adult female mosquitoes to be used for experimental infections were collected upon emergence and held in mesh top 3.8 L paper cartons and provided cotton pads with 10% sucrose ad libitum. Mosquitoes were held for 7-10 days prior to bloodfeeding.

2.2. Virus strains

The *West Nile virus* infectious clone (FL-WNV) virus was generated from an infectious cDNA clone based on New York strain 3356, isolated from an American Crow (*Corvus brachyrhynchos*) in Staten Island in 2000 (AF404756). Methods for clone manipulation and rescue of infectious WNV are as described previously (Shi et al., 2002). Insertions of silent changes in the NS1 gene of WNV, which has no known role in viral fitness in the mosquito vector, were chosen as markers for individual variants. WNV mutants were generated by site-directed mutagenesis (SDM) of the FL-WNV using the QuikChange XLII SDM kit (Stratagene, La Jolla, CA) as per the manufacturer's protocol. Mutant FL-WNV DNA was then amplified in *E. coli* and plasmid harvested by Highspeed Midiprep (Qiagen, Valencia, CA). Sequencing of NS1 mutant WNV plasmids confirmed that desired changes were engineered. All purified mutant and control FL-WNV plasmids were linearized with XbaI and transcribed using the MEGAscript kit (Invitrogen, Carlsbad, CA) supplemented with Anti-reverse cap analog (Invitrogen) and assembled as per manufacturer's protocol. Transcription reactions were incubated at 37 °C for 4 hrs. Resulting RNA was purified with

the MEGAclean kit (Invitrogen) and quantified on a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA). RNA was stored in 10µg aliquots at -80 °C. Wild-type FL-WNV RNA (variant 1) and mutant RNA including: WNV T2700C (variant 2), T2913C (variant 3), T2940C (variant 4), A2844G (variant 5), T3117C (variant 6), A2847T (variant 7) and T2910C (variant 8) were electroporated into 0.8×10^7 C6/36 mosquito cells in PBS using a GenePulser (BioRad). Transfected cells were seeded into T75 flasks and supernatants were collected from day 3 to 7 post-transfection, aliquoted and stored at -80 °C. WNV titers were quantified by plaque assay on Vero cells as previously described (Payne et al., 2006) and levels of WNV RNA were quantified by Taqman qRT-PCR (Applied Biosystems, Foster City, CA). WN S5 was created by mixing equivalent amounts (based on RNA copies) of stock of variants 1-5. Mixtures of variants 2 and 4 were used for proportion experiments and proportions were again based on RNA copy numbers. WN S8 was created by mixing equivalent amounts of stock of variants 1-8 followed by a single amplification of this mixture on mosquito cell culture (C6/36, ATCC #CRL-1660) in order to increase infectivity for feeding experiments. Exact proportions of variants used for infectious feedings were determined by molecular cloning and sequencing of experimental bloodmeals as described below.

2.3. Individual strain characterization

All individual variants were assessed for vector competence and growth kinetics, as well as genetic stability in *Cx. pipiens* mosquitoes following feeding on an infectious bloodmeal. Experimental infections were carried out as previously described (Ciota et al., 2009). Briefly, individual WNV variants were diluted to equivalent titers ($\sim 9.0 \log_{10}$ pfu/ml), mixed 1:5, virus: defibrinated goose blood (Hema Resources Inc, Aurora, OR) + 2.5% sucrose, and offered to ~ 500 female mosquitoes using a Hemotek membrane feeding system (Discovery Workshops, Accrington, UK). Following 1-hour, mosquitoes were anesthetized using CO₂ and fully engorged mosquitoes were saved and housed at 27°C for subsequent testing. Twenty-five to 50 mosquitoes per variant were tested for vector competence at days 5, 7, 10, 14 and 21 days post-feeding. Mosquitoes were incapacitated and legs were removed and placed in 1 ml mosquito diluent [MD; 20% heat-inactivated fetal bovine serum (FBS) in Dulbecco's phosphate-buffered saline (PBS) plus 50 µg/ml penicillin/streptomycin, 50 µg/ml gentamicin, and 2.5 µg/ml Fungizone]. Capillaries charged with FBS plus 50% sucrose (1:1) were used to collect salivary secretions for approximately 30 minutes followed by ejection into 0.3ml MD. Mosquitoes were then placed in individual tubes with 1.0 ml MD. All samples were held at -80°C until tested. Bodies and legs were processed separately as previously described and all samples were screened and titrated by plaque assay in duplicate on Vero cell culture. Infection rates were determined by the number of WNV positive mosquito bodies per fed mosquito, dissemination rates by the proportion of WNV positive mosquitoes with WNV positive legs, and transmission rates by the proportion of WNV positive mosquitoes with WNV positive salivary secretions. Growth kinetics were determined using viral titers of WNV positive mosquitoes on individual days. Replicative fitness refers to the slope of the best-fit log-linear line of mosquito body titers as a function of time post-feeding.

2.4. Mixed infections and sample collections

Three separate experiments utilizing artificial swarms composed of WNV variants were performed. In experiments 1 and 2, WN S5 (swarm of 5 variants) and WNV S8 (swarm of 8 variants), respectively, were diluted 1:5 in defibrinated blood plus sucrose and offered to ~ 1000 female *Cx. pipiens* mosquitoes as described above. Tissues samples were collected on days 5, 7, 10, 14, and 21 post-feeding from 12-20 mosquitoes. Collected samples included salivary secretions (SS), legs (LEG), midguts (MG), salivary glands (SG), and body remnants (REM). Dissected samples, i.e., MG, SG, and REM, were washed by passing twice

through separate clean drops of MD prior to collection. All samples were collected in MD, stored and processed as described above, and subsequently tested for WNV by plaque screen on Vero cell culture. In experiment 3, five different bloodmeals were created using proportions of variants 2 and 4 including 1:1, 2:1, 4:1, 8:1 and 16:1 and separately offered to ~500 mosquitoes. In addition to above sample collections, 5 mosquitoes/proportion were saved for testing immediately after feeding.

2.5. Molecular cloning and sequencing

In order to confirm proportion of variants in WNV positive bloodmeals and determine proportions in harvested tissues, molecular cloning and sequencing were performed on WNV positive samples. Generation and analysis of clones were performed using a modification of previous studies (Ciota et al., 2007; Ciota et al., 2009). RNA was extracted from infected specimens with QIAamp viral RNA extraction kit (Qiagen) and RT-PCR was conducted using primers designed to amplify the 3' 2201 nt of the WNV envelope (E) coding region and the 5' 3248 nt of the WNV non-structural protein 1 (NS1) coding region. RT was performed with Sensiscript RT (Qiagen) at 45 °C for 40 min followed by heat inactivation at 95 °C for 5 min. The resulting cDNA was used as a template for PCR amplification. WNV cDNA was then amplified with *Taq* polymerase (New England Biolabs, Ipswich, MA) according to the manufacturer's specifications. PCR products were visualized on a 1.5 % agarose gel and DNA was recovered by using a MinElute Gel Extraction kit (Qiagen) as specified by the manufacturer. The recovered DNA was ligated into a TOPO-TA cloning vector (Invitrogen) and transformed into One Shot TOP10 Electro-competent *E.coli* cells according to the manufacturer's protocol. Kanamycin resistant colonies were screened by direct PCR using primers specific for the desired insert and plasmid DNA was purified using a QIAprep Spin Miniprep kit (Qiagen) as specified by the manufacturer. Sequencing was performed at the Wadsworth Center Applied Genomics Technology Core using ABI 3700 and 3100 automated sequencers (Applied Biosystems). Seventeen to twenty-two clones were sequenced per sample.

2.6. Sequence and data analyses

WNV sequences were compiled, edited, and aligned using the SeqMan module of the DNASTAR software package and a minimum of two-fold redundancy throughout each clone was required for individual sequence data. As used in previous studies to quantify mutant swarm breadth, normalized Shannon entropy (S_n) was calculated based on the frequency of genotypes in populations as follows: Shannon entropy (S_n) = $\sum -i P_i \ln P_i / \ln N$, where P_i = frequency of individual genotype and N = number of clones sequenced (Ciota et al., 2007). S_n values can range from 0 (completely homogeneous) to 1 (completely heterogeneous) yet in these experiments only input haplotypes were considered and, therefore, heterogeneity could not exceed the input value in any given tissue. Statistical analyses were performed using both Microsoft Excel 2003 and GraphPad Prism version 4.00.

3. Results

3.1. Characterization of WNV variants in *Cx. pipiens* mosquitoes

The goal of these studies was to create a WNV swarm composed of variants of neutral fitness which could be tracked over time and space *in vivo* in *Cx. pipiens* mosquitoes. For this reason, all variants were individually characterized *in vivo* to confirm that no fitness changes existed relative to the FL-WNV strain (variant 1) from which they were derived. This characterization occurred over multiple experiments and included quantification of vector competence (infection, dissemination, and transmission rates), WNV titers on individual days and overall growth rates (replicative fitness, table 1). The mean replicative

fitness among variants was $0.15 \log_{10}$ pfu/mosquito/day for days 5-14 post-feeding and no statistical difference occurred among variants (linear regression analysis of log-linear slopes, $p=0.18$). Generational fluctuations in mosquito colonies are not uncommon and, in some cases, differences between individual experiments were measured in terms of both vector competence and WNV titers in mosquitoes. In all experiments, variant 1 was used as an internal control to correct for this variation. No significant differences in WNV titers on individual days were detected (t-tests, $p>0.05$), nor were significant differences in vector competence detected among variants used (Chi-squared, $p>0.05$; data not shown) within individual experiments.

In addition to confirmation of neutral fitness among variants, genetic stability was assessed by sequencing of the NS1 gene from WNV positive legs and/or salivary secretions from individual mosquitoes at days 14-21 post-feeding. All variants used for mixed feedings (variants 1-8) were confirmed to be genetically stable. A total of 3 NS1 variants created were disqualified from this study due to identification of fitness changes and/or lack of genetic stability.

3.2. Tissue-specific diversity in *Cx. pipiens* following feeding on artificial mutant swarms

WN S5 was used for the first experimental mixed feeding. Molecular cloning and sequencing confirmed that variants 1-5 were present at relatively high proportions ranging from 0.15 to 0.3 (table 1). The WNV input titer for this feeding was $8.1 \log_{10}$ pfu/ml. Overall infection rates were relatively low, 45.0%, with 27 of 60 mosquito midguts WNV positive. Of these, 15 of 27 (56.0%) had disseminated infections (WNV positive legs and/or remnants) and 6 of 27 (22.0%) had infected salivary glands. Three of 6 mosquitoes with WNV positive salivary glands also had virus in their salivary secretions, yet no salivary secretions were successfully cloned in experiment 1. WNV RNA isolated from a total of 21 tissues, including 13 midguts, 6 leg samples and 6 salivary gland samples, was cloned and sequenced from the WN S5 feeding. There were a minimum of 2 WNV positive tissues of each type from each timepoint, with the exception of day 5 post-feeding when no WNV positive legs or salivary glands were identified. Comparison of S_n values for the WN S5 bloodmeal to days 5, 7, and 10 midguts demonstrated that no substantial decrease in swarm breadth occurred with midgut infection and replication (t-test, $p>0.05$; Fig. 1). Despite this, 3 of the 7 midgut samples from days 5, 7 and 10 had lost 1 or more variants originally present in the bloodmeal, demonstrating a modest narrowing of the swarm at the population level. In addition, no significant difference in mutant swarm breadth was measured between tissue types at any individual timepoint, despite a downward trend in S_n from midguts to legs to salivary glands on day 10 (Fig. 1; t-test, $p>0.05$). Although no tissue-specific trend was measured in experiment 1, a distinct temporal trend was apparent, with a significant decrease in swarm breadth occurring from days 7-21 (Fig. 1; linear regression analysis, $r^2=0.74$, $p<0.001$; Pearson correlation, $r=-0.92$, $p=0.04$). The mean number of variants identified in day 21 tissues was 1.33 and 4 of 6 tissues contained only a single variant. No consistent trend was observed in terms of the variant which ultimately dominated, with all but variant 3 represented as a master sequence in one of the 6 day 21 tissues. This result supported the conclusion of neutral fitness among variants.

WN S8 was used for the second experimental mixed feeding. The purpose of using this strain in the follow-up experiment was (i) to more accurately represent proportions one would expect to find in a natural strain and (ii) to characterize the effects of intrahost bottlenecks on minority variants. Unlike the WN S5 population in which variants existed in roughly equivalent proportions and had no variant below 15% in the population, individual variants in the WN S8 population ranged from 52.5% (variant 1) to <2.5% (variants 6 and 7; table 1). The bloodmeal titer for this feeding was $8.7 \log_{10}$ pfu/ml. Twenty-one of 41 midguts tested (51.2 %) were WNV positive. Of these, 14 (67.0%) had disseminated

infections, 6 (29.0%) had WNV positive salivary glands, and 3 (14.0%) were capable of virus transmission. To attain more WNV positive salivary secretions in this experiment, secretions were collected from additional experimental but undissected mosquitoes at each timepoint. This provided an additional 4 WNV positive secretion samples. A total of 37 samples were successfully cloned and sequenced following the WN S8 feeding, including 13 MG, 12 LG/RM, 6 SG and 6 SS. There was some concern with the combining of legs and remnant tissues to represent disseminated virus in this experiment given the larger diversity of tissues infected in mosquito remnants, yet successful cloning of these tissues from the same mosquitoes demonstrated similar levels of swarm breadth. Unlike experiment 1, significant tissue-specific differences were identified following feeding on WN S8 (Fig. 2). Specifically, when tissues from all timepoints were combined for analyses, a significant decrease in mutant swarm breadth (S_n) was measured with WNV infection of the midgut (t-test, $p < 0.001$) and the combination of WNV infection and release from the salivary glands (Fig. 2a, SS relative to MG or LEG/RM, t-test, $p < 0.05$). Narrowing of the swarm was confirmed by quantifying the proportion of the population composed of minority sequences (Fig. 2b). Specifically, a decreasing proportion of minority sequences were identified with each successive tissue from BM to SS and individual differences were significant from BM to MG and SG to SS (chi-squared, $p < 0.05$, Fig. 2b). To assess if these decreases in swarm breadth in MG and SS were due to tissue-specific bottlenecks rather than temporal changes (i.e. downstream tissues are on average infected later), breadth in these tissues was analyzed separately on days 7, 14, and 21 (Fig. 3) and additional two-way ANOVA analyses were performed to separate the effects of tissue and time (table 2). Although statistical significance could not be achieved with data from individual days due to low sample sizes (2-4 tissues/timepoint), these results clearly demonstrate consistently narrower swarm breadth in MG samples relative to input and in SS samples relative to MG without significant temporal changes. In addition, results from ANOVA analyses demonstrate that tissue-specific differences in swarm breadth did occur independent of time both when all tissues are analyzed and when BM, MG, and SS data are considered independently (two-way ANOVA, $p < 0.05$; table 2). Despite this, a temporal decrease in mutant swarm breadth was observed in tissues responsible for secondary virus amplification (LG, RM, SG) following feeding on WN S8 (Fig. 4; linear regression analysis, $r^2 = 0.18$, $p = 0.046$; Pearson correlation, $r = -0.96$, $p = 0.04$; two-way ANOVA, $p < 0.05$; table 2). Viral titers in individual tissues were highly variable, ranging from 1.0 to 6.5 $\log_{10}$ pfu WNV/tissue (Fig. 5). The relationship between viral titers and swarm breadth was evaluated in order to test for true correlation and/or cloning bias. WNV titers did not correlate with swarm breadth (Fig. 5; Pearson correlation, $r = 0.0003$). In fact, both the highest and lowest titer samples (day 21 SG and day 11 LEG, respectively) were completely homogeneous and the most diverse sample (day 14 REM) was among the lowest titer samples.

In order to better determine the threshold input proportion for infection, experiment 3 evaluated day 5 midgut-derived WNV following feeding on five proportions of variant 2 and variant 4 ranging from 1:1 to 32:1. In addition, five time 0 samples, taken immediately after bloodfeeding, were successfully cloned and sequenced, confirming that starting proportions were maintained with ingestion and that proportions of minority variants were above the threshold for detection (data not shown). Bloodmeal titers for these feedings were comparable for each group, ranging from 8.2 to 8.4 $\log_{10}$ pfu/ml blood. Analysis of five WNV MG isolates/group demonstrated that proportions of variants on the population level are generally retained following infection and early replication (Fig. 6A). Despite this, the minority variant (variant 4) was not detected within the swarm in any of the five WNV MG isolates from group 5 (32:1), suggesting this may be a threshold at which infection is highly unlikely. Similar to WN S5 and S8 feedings, variation was seen on the individual level and the proportion of individual midgut samples which had completely lost variant 4 increased with a decreasing input BM proportion (Fig. 6B).

In addition to the midgut derived samples sequenced from experiment 3, at least 1 salivary secretion isolate from each group was successfully cloned and sequenced. These results were combined with results from experiments 1 and 2 in order to generate the probabilities of infection, transmission, and dominance given various starting proportions in the infectious bloodmeal (Fig. 7). Results for infection depict a saturation curve in which the increases in the probability of infection generally lessen with increasing proportions and the probability of infection is high (greater than 80.0%) when the variant proportion is greater than ~ 25.0% (Fig. 6A). Results for transmission were more variable, potentially due to the effects of smaller sample sizes. Despite this, a similar association was seen with the probability of transmission increasing with increased input proportions, yet with smaller effects below starting proportions of 0.25 and larger effects above that threshold relative to infection (Fig. 7A). Results for dominance demonstrate that the probability of becoming the master sequence is directly correlated with a variant's starting proportion and that, even with neutral variants, no low-end threshold was identified that would disqualify rare mutants from dominating in some mosquitoes (Fig. 7B). For example, despite being at a proportion below the level of detection (2.5%) in the WN S8 bloodmeal, variant 6 was found to dominate in two tissues tested from different mosquitoes.

4. Discussion

Stochastic events, such as intrahost bottlenecks and nonselective sweeps imposed on arboviruses in mosquito vectors could have significant effects on viral fitness and plasticity. Here, we characterized how these events shape the *West Nile virus* mutant swarm in *Cx. pipiens* mosquitoes. Specifically, using artificial mutant swarms composed of neutral fitness variants with stable mutations, we tracked swarm breadth of input variants over known barriers within the mosquito including: (i) midgut infection, (ii) midgut escape, (iii) salivary gland infection and (iv) virus transmission; and measured changes in breadth in individual tissues over time. Our results demonstrate that both time and space can contribute to significant narrowing of the WNV swarm, yet the extent of this narrowing is highly variable and largely dependent on variant proportions in infectious bloodmeals.

Although some narrowing of the WNV swarm was seen with midgut infection, no significant tissue-specific bottlenecking was measured following feeding on WN S5, demonstrating that most WNV variants which exist at relatively high proportions ($\geq 15.0\%$, table 1) are likely to survive the anatomical barriers within *Cx. pipiens*. Despite this, significant decreases in swarm breadth were measured from day 7 to 21 post-feeding (Fig. 1). The mean number of variants in all tissues 21 days after feeding on WN S5 was 1.2. This sweep was stochastic in that the variant which would ultimately dominate could not be predicted, yet selection could still play a role here if one variant were to acquire a beneficial mutation over the course of mosquito infection. Alternatively, this could be a neutral event in which the innate immune response of the mosquito overcomes infection in an increasing number of cells with time, leaving a relatively small number of cells producing virus and therefore narrowing the swarm indiscriminately. Future sequencing and phenotypic characterization of output viruses will help to clarify the mechanism at work in these temporal sweeps. A similar time-dependent decrease was seen following feeding on the WN S8 strain, yet in this case the decrease was due exclusively to temporal changes in tissues responsible for secondary virus amplification (LEG, REM, SG; Fig. 4; table 2). These results are comparable to a recent study measuring intrahost WNV nucleotide diversity in *Cx. quinquefasciatus* mosquitoes in which a general trend of decreasing nucleotide diversity was also measured from day 7 to 21 post-feeding (Brackney et al., 2011).

In contrast to these results, here we also identified significant tissue-specific bottlenecking following feeding on the WN S8 population (Figs. 2 and 3; table 2). Specifically, a

significant decrease in swarm breadth was seen with midgut infection and virus transmission and, although lacking in statistical significance, a narrowing of the swarm was also seen with salivary gland infection. Although *Cx. pipiens* and *Cx. quinquefasciatus* are sibling species which readily hybridize (Huang et al., 2011), significant variation in vector competence has been measured with these and other closely related *Culex* species (Reisen et al., 2008; Moudy et al. 2011). Such variation could explain differences in the extent of spatial bottlenecking. Additionally, what is measured in these two studies is also quite different. In the Brackney et al study (2011), overall nucleotide diversity, both that which is produced and is maintained, is measured following infection with an attenuated, highly diverse strain composed of multiple variants with potentially altered phenotypes (Fitzpatrick et al., 2010). Here, we've tracked proportions of marked input strains confirmed to have equivalent fitness. Additionally, in terms of sequence diversity, the WN S8 strain is a valid representative of what a mosquito might encounter in nature, i.e. a swarm with one dominant genotype surrounded by minority genotypes in variable proportions. This difference in methodology between these two studies could also explain differences observed in the relationship between viral titer and swarm diversity. As was shown with *Cx. quinquefasciatus*, we would anticipate that those tissues in which the level of replication was higher would also experience more mutation and higher levels of genetic diversity, yet since what we have tracked in this study are only the mutations of the variants initially present in the bloodmeal, we wouldn't necessarily expect a similar relationship with our measure of swarm breadth. Instead, the lack of a correlation between swarm breadth and WNV titer that we measured demonstrated that there was no bias for heterogeneity with high titer samples caused by our methodology (or vice versa), and that the capacity for individual tissues to produce virus had no influence on quantifying the size of the preceding bottleneck (Fig. 5). The identification of tissue-specific bottlenecks following feeding on WN S8 also contrasted with the WN S5 feeding, demonstrating, not surprisingly, that the presence of more minority variants in input strains increased the likelihood of such variants being lost throughout infection.

The most significant bottleneck identified was at the level of midgut infection (Figs. 2 and 3). The host and virus-derived factors governing WNV infection of midgut epithelial cells are not well defined, yet a previous study utilizing WNV virus-like particles in *Cx. quinquefasciatus* demonstrated that a relatively small number of cells (≤ 15) are initially infected in the posterior midgut (Scholle et al., 2004). Studies with epizootic *Venezuelan equine encephalitis virus* also demonstrated a low proportion of midgut cells infected in *Aedes taeniorhynchus* (mean of 28), yet also demonstrated that levels of susceptibility are likely both virus and vector-specific (Smith et al., 2008). Although the extent of co-infection of midgut cells is not known for WNV or any flavivirus, the availability of susceptible cells is likely to be the limiting factor determining the degree of genetic bottlenecking occurring with initial infection. Feeding on different proportions of variants 2 and 4 demonstrated that, although proportions of neutral variants in infectious bloodmeals are generally retained on the population level (Fig. 6A), even a variant comprising 50% of the viral swarm can often be lost with initial infection (Fig. 6B). In addition, these studies demonstrate that minority variants existing at proportions less than 3% rarely infect *Cx. pipiens* midguts, even at relatively high input titers. Despite this, results from the WN S8 feeding demonstrate that variants which were extremely rare in the input virus can at times persist and even dominate so input proportions are likely to be highly predictive of proportions on the population level (Fig. 7).

Following midgut infection and replication, arboviruses need to disseminate into the hemocoel to infect secondary tissues. This barrier has historically been viewed as among the most important in determining vector competence (Hardy et al., 1983). Surprisingly, neither artificial swarm used in this study identified a significant midgut escape bottleneck for

mosquitoes developing disseminated infections, as the breadth of the swarm in both midguts and parenteral tissues (LEG/REM) was similar (Figs. 1 and 2). It is clear that such a dissemination barrier exists, as many infected mosquitoes do not develop disseminated infections, yet what this result suggests is an all or nothing phenomenon rather than a case of a subset of the population reaching these secondary tissues. This implies that it is more of an immunological rather than anatomical barrier in which all variants in a neutral viral swarm that successfully infect the midgut cells and replicate are equally capable of midgut egress, yet does not rule out the possibility of strong selective pressure for individual variants in phenotypically diverse swarms.

A further narrowing of the swarm was observed with salivary gland infection and a significant decrease in swarm breadth was measured with virus transmission, supporting the idea that the infection of and egress from the salivary glands also acts as a significant anatomical barrier to WNV infection [(Chamberlain and Sudia, 1961);(Fig. 2)]. Although salivary secretion titers were generally among the lowest of tested samples, one of the six salivary secretion samples tested from the WN S8 feeding had a viral titer of $\sim 5.0 \log_{10}$ pfu/ml yet was completely genetically homogeneous. This suggests again that the measured swarm narrowing is a real phenomenon rather than the consequence of a methodology imposed bottleneck resulting from using *in vitro* transmission assays. What remains unclear is if all variants infecting the salivary glands are capable of entering the saliva and the bottleneck is during transmission or, if some of the variants getting into the glands never get out. Since all mosquitoes were dissected for these experiments, salivary secretions from individual mosquitoes could not be monitored through time, but such experiments would help to clarify this question.

Although trends are apparent with our data set, there is a large amount of variability among individuals with regard to mutant swarm breadth. For example, some midgut samples tested from early in infection contained just a single variant and some salivary gland samples contained as many as 5 variants. What we can conclude from this is that there is not a level of intrahost bottleneck that is necessarily inevitable, but that the probability of reaching each stage of infection and, ultimately, transmission certainly decreases with decreasing starting proportions (Fig. 7). Despite this, the fact that rare mutants, even with neutral fitness can occasionally dominate, stresses the need to identify minority variants in natural isolates. Incorporating selection coefficients in our predictions could help us understand the likelihood of strains with variable fitness levels surviving to transmission.

One caveat of this work is that all experimental feedings were performed with high titers of WNV in infectious bloodmeals. Although these titers are well within the range of viremia levels found in many naturally infected birds, particularly Passeriformes (Komar et al., 2003), *Culex* mosquitoes are capable of acquiring infections from lower titer bloodmeals (Anderson et al., 2010). Such infections would most likely increase the probability of tissue-specific bottlenecks; yet this may not be the case if only a subset of midgut cells are susceptible and consequently, the same limited number of cells initiate mosquito infection. Future studies infecting mosquitoes at variable doses will help to clarify this. In addition, both feeding on artificial bloodmeals and *in vitro* transmission assays, although useful for accurate quantification of input and output swarm breadth, may not be perfect representations of natural infection and transmission. Lastly, both species- and population-specific differences together with strain variation could substantially alter the dynamics of WNV infection in *Culex* mosquitoes. Despite the need to clarify such variables, these results demonstrate the profound effects that stochastic forces across time and space in mosquito vectors can have in shaping arboviral mutant swarms.

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Highlights

- An artificial mutant swarm of neutral West Nile virus variants was created.
- Changes to mutant swarm breadth in *Culex pipiens* were tracked over time and space.
- A stochastic narrowing of the mutant swarm was measured over time during infection.
- Significant decreases in swarm breadth occur with midgut infection and transmission.
- Results demonstrate the probability of variant survival during mosquito infection.

Figure 1.

Changes in mutant swarm breadth (S_n) over time and space following bloodfeeding on WN S5. Each bar represents the mean of 2-3 tissues $\pm$ SEM. No significant differences were measured between tissues on individual days (t-test, $p > 0.05$). A significant negative correlation between time and S_n was measured for all tissues from days 7 through 21 (linear regression analysis, $r^2 = 0.74$, $p < 0.001$; Pearson correlation, $r = -0.92$, $p = 0.04$).

Figure 2.

Tissue-specific differences in mutant swarm breadth in *Cx. pipiens* following feeding on WN S8. MG=midguts (n=13), LG/RM=legs or remnants (n=12), SG=salivary glands (n=6), and SS=salivary secretions (n=6). (A) Mean tissue-specific entropy (S_n $\pm$ SEM). Significant decreases in swarm breadth (*) were measured with WN S8 bloodmeal (BM) relative to MG (t-test, $p < 0.001$) and with MG relative to SS (t-test, $p = 0.03$). (B) Proportion of all sequences from bloodmeal and individual tissues identified as minority variants. Significant differences identified by Chi-squared analysis (*) were measured between BM and MG ($X^2 = 11.34$, $p = 0.02$) and SG and SS ($X^2 = 10.19$, $p = 0.03$).

Figure 3. Mutant swarm breadth in *Cx. pipiens* midguts and salivary secretions on individual days following feeding on WN S8. Bars represent mean tissue-specific entropy ($S_n \pm$ SEM, $n=2-4$ /timepoint) of midgut (MG) and salivary secretions (SS).

Figure 4.

Temporal change in WNV mutant swarm breadth (S_n) in secondary tissues following bloodfeeding on WN S8. Data points represent mean S_n of 3-7 LG (leg), RM (remnant), and SG (salivary gland) samples $\pm$ SD at 7, 11, 14, and 21 days post feeding. A significant negative correlation was measured between S_n and time (linear regression analysis, $r^2 = 0.18$, $p=0.046$; Pearson correlation, $r=-0.96$, $p=0.04$).

Figure 5. Mutant swarm breadth (S_n) and viral titers of individual *Cx. pipiens* tissues at days 5-21 following feeding on WN S8. No correlation between viral titer and S_n was measured for individual tissue types or combined data (Pearson correlation, Pearson $r = 0.0003$).

Figure 6.

Proportions of two WNV variants in *Culex pipiens* midguts (MG) following feeding on various proportions in bloodmeals (BM). A. Proportions of variant 2 and 4 clones identified from five day 5 MG samples (n=40 clones sequenced/BM and 125-200 clones sequenced/5 MG). No statistically significant differences in proportions were identified between corresponding BM and MG groups (chi-squared, $p>0.05$), yet no variant 4 clones were identified in group 5 midguts (MG5). B. Proportion of MG samples (n=5) without variant 4 (minority sequence).

Figure 7.

Predictions from combined experiments. A. The probability of infection and transmission of WNV in *Cx. pipiens* as a function of WNV variant proportion in the input mutant swarm. The probability of infection was defined as the proportion of midguts in which a variant was identified given multiple starting proportions and the probability of transmission refers to the (probability of infection)*(the proportion of individuals transmitting a variant) at each starting proportion. Lines represent best-fit non-linear relationships of association ($R^2=0.87$ and 0.83 for infection and transmission, respectively). B. The likelihood of a particular WNV variant becoming the master (dominant) sequence in all *Cx. pipiens* tissues is directly related to its starting proportion in the infectious bloodmeal (linear regression analysis, slope=1.01, $R^2=0.94$).

Table 1

Replicative fitness of individual WNV NS1 variants and proportions in artificial mutant swarms.

WNV strain	mutation	replicative fitness ¹	WN S5 proportion ²	WN S8 proportion ²
WN variant 1	FL-WNV	0.15	0.30	0.525
WN variant 2	T2700C	0.17	0.15	0.075
WN variant 3	T2913C	0.19	0.15	0.150
WN variant 4	T2940C	0.17	0.20	0.200
WN variant 5	A2844G	0.18	0.20	0.025
WN variant 6	T3117C	0.11	-	<0.025*
WN variant 7	A2847T	0.11	-	<0.025*
WN variant 8	T2910C	0.14	-	0.025

¹ Replicative fitness refers to the slope of the best fit line generated by plotting geometric means of WNV titers in 30-45 *Cx. pipiens* bodies per variant on days 5, 7, 10, and 14 post feeding on ~8.2 log₁₀ PFU virus.

² Proportions were determined by molecular cloning and sequencing of 40 clones generated from input virus.

* Variants were included in the original mixture but not identified in the input virus. Both variants were identified in individual tissues following feeding.

Results from two-way ANOVA analyses evaluating the effects of tissue and time on WNV mutant swarm breadth (S_n) following *Cx. pipiens* feeding on WN S8.

Table 2

Group	Source of variation	df	Sum of squares	Mean square	F	p-value
all tissues ¹	time	3.0	0.047	0.016	0.94	0.434
	tissue	4.0	0.328	0.082	4.93	0.004
	interaction	12.0	0.174	0.014	0.87	0.587
LG/RM, SG ²	time	3.0	0.199	0.067	3.43	0.046
	tissue	1.0	0.035	0.035	1.79	0.202
	interaction	3.0	0.031	0.010	0.53	0.667
BM, MG, SS ³	time	3.0	0.009	0.003	0.24	0.870
	tissue	2.0	0.282	0.141	10.36	0.002
	interaction	6.0	0.016	0.014	0.19	0.972

¹ Significant tissue-specific variation in mutant swarm breadth was measured independent of time

² Time-dependent variation in mutant swarm breadth independent of tissue type is measured when legs/remnants and salivary glands are analyzed separately.

³ Tissue-dependent variation in mutant swarm breadth independent of time is measured when bloodmeal, midguts, and salivary secretions are analyzed separately.