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Characterization of mosquito-adapted West Nile virus

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Abstract

West Nile virus (WNV), a mosquito-borne flavivirus, has significantly expanded its geographical and host range since its 1999 introduction into North America. The underlying mechanisms of evolution of WNV and other arboviruses are still poorly understood. Studies evaluating virus adaptation and fitness in relevant *in vivo* systems are largely lacking. In order to evaluate the capacity for host-specific adaptation and the genetic correlates of adaptation *in vivo*, this study measured phenotypic and genotypic changes in WNV resulting from passage in *Culex pipiens* mosquitoes. An increase in replicative ability of WNV in *C. pipiens* was attained for the two lineages of WNV tested. This adaptation for replication in mosquitoes did not result in a replicative cost in chickens, but did decrease cell-to-cell spread of virus in vertebrate cell culture. Genetic analyses of one mosquito-adapted lineage revealed a total of nine consensus nucleotide substitutions with no accumulation of a significant mutant spectrum. These results differed significantly from previous *in vitro* studies. When St Louis encephalitis virus (SLEV), a closely related flavivirus, was passaged in *C. pipiens*, moderately attenuated growth in *C. pipiens* was observed for two lineages tested. These results suggest that significant differences in the capacity for mosquito adaptation may exist between WNV and SLEV, and demonstrate that further comparative studies in relevant *in vivo* systems will help elucidate the still largely unknown mechanisms of arboviral adaptation in ecologically relevant hosts.

INTRODUCTION

West Nile virus (WNV; genus *Flavivirus*, family *Flaviviridae*) is a mosquito-borne virus that was introduced into the USA in the New York City area in 1999. The virus has been highly successful in establishing itself, with continued expansion of both its geographical and its host range (Davis *et al.*, 2005; <http://www.cdc.gov/ncidod/dvbid/arbor>). Worldwide, WNV has infected over 75 species of mosquito (Higgs *et al.*, 2004) and over 300 species of birds (Marra *et al.*, 2003), with an expanding range now spanning across the USA and into Canada, Mexico and Central and South America (Austin *et al.*, 2004; Cruz *et al.*, 2005; Dupuis *et al.*, 2005; Elizondo-Quiroga, 2005; Granwehr *et al.*, 2004; Lanciotti *et al.*, 1999; Morales, 2006). St Louis encephalitis virus (SLEV) is a flavivirus that is genetically and antigenically highly similar to WNV (Monath & Heinz, 1996). It was first recognized in 1933 in St Louis, MO, USA, when a large outbreak occurred. A second major outbreak occurred in 1974–1975 in the Mississippi River valley (Chamberlain, 1980). Since then, SLEV, unlike WNV, has caused only intermittent outbreaks (Chandler *et al.*, 2001; Day & Stark, 2000), which generally remain isolated with limited levels of activity (Monath &

Heinz, 1996; Reisen, 2003; <http://www.cdc.gov/ncidod/dvbid/arbor>). Evolutionary pressures on these viruses are applied by the ornithophilic mosquito and avian hosts that sustain their transmission cycles (Kramer & Bernard, 2001), yet the underlying mechanisms of arboviral evolution in nature are still poorly understood. Evaluating the selective pressures that drive arbovirus adaptation and identifying the genetic correlates of success in disparate environments are crucial for predicting the ability of these and other potentially important vector-borne viruses to thrive in new and dynamic environments.

With a few exceptions, such as duck hepatitis B virus (Lenhoff *et al.*, 1998) and foot-and-mouth disease virus (Carrillo *et al.*, 1998), passage studies evaluating virus adaptation and fitness in relevant *in vivo* systems are largely lacking. These studies are essential if the selective forces that shape arbovirus evolution are to be characterized accurately. In order to evaluate the capacity for host-specific adaptation and the genetic correlates of adaptation *in vivo*, we measured both phenotypic and genotypic changes in WNV resulting from passage in *Culex pipiens* mosquitoes. Specifically, virus growth kinetics and vector competence in *C. pipiens* were measured for WNV before and after passage, and alterations in both consensus sequences and mutant swarm diversity were identified. To our knowledge, this is the first study using a relevant *in vivo* host to fully characterize adapting populations of arboviruses. Many previous studies have also attempted to evaluate the constraints of cycling on evolution and host-specific adaptation; however, these studies exclusively used cell-culture systems (Chen *et al.*, 2003; Ciota *et al.*, 2007a; Cooper & Scott, 2001; Holland *et al.*, 1991; Novella *et al.*, 1999a, b; Weaver *et al.*, 1999; Zarate & Novella, 2004). In order to evaluate the cost of *in vivo* mosquito adaptation to replication in disparate hosts, we evaluated viraemia in chickens and viral spread *in vitro* of mosquito-adapted WNV strains. Our previous studies using *in vitro*-adapted virus strains also suggested that WNV and SLEV may differ significantly in how each adapts (Ciota *et al.*, 2007a, b, c). Specifically, SLEV cell-culture adaptations were found to be much more species-specific than WNV, and this corresponded to substantial differences in intrahost genetic diversity and adaptability to new hosts (Ciota *et al.*, 2007c). In order to begin to evaluate whether similar differences occur *in vivo*, we also compared the phenotypic changes in these two viruses following mosquito passage. The results presented here suggest that significant differences in the capacity for *in vivo* adaptation may exist between replicating populations of WNV and SLEV. This demonstrates that further comparative studies in relevant *in vivo* systems are warranted to help elucidate the still largely unknown mechanisms of adaptation and evolution of arboviruses.

METHODS

Mosquitoes

C. pipiens egg rafts were originally collected in Pennsylvania in 2004 (courtesy of Michael Hutchinson, Pennsylvania State University, PA, USA) and shipped to the insectary at The Arbovirus Laboratories, Wadsworth Center, for colonization. Mosquitoes were reared and maintained in 12" × 12" × 12" cages in an environmental chamber at 27 °C, 70% relative humidity, with a photoperiod of 16 : 8 h (light : dark). Adult female mosquitoes used for experiments were kept in mesh-top 3.8 l paper cartons and provided with cotton pads soaked in 10% sucrose *ad libitum*. Mosquitoes were kept for 4–7 days before being transferred to 0.6 l cups for experimental infections in the BSL-3 facility.

Chickens

Specific-pathogen-free chicken eggs (*Gallus gallus*) were obtained from Sunrise Farms (Catskill, NY, USA) and hatched in an incubator (G.Q.C.) at The Arbovirus Laboratories. Newborn chickens were transferred 3–12 h post-hatching to the BSL-3 animal facility in

preparation for virus inoculations. Chickens were separated by experimental group and housed in metal cages with individual light sources and daily fresh food and water and resting pads.

Viruses and mosquito passage

Biological clones of WNV (WNV UNP) and SLEV (SLEV UNP) were produced by plaque purification and amplification in Vero cells as described previously (Ciota *et al.*, 2007a). WNV UNP was derived from WNV NY003356, a primary isolate from an American crow that was collected in 2000 in Staten Island, NY, and prepared by one round of amplification in Vero cells (Ebel *et al.*, 2001). SLEV UNP was derived from the SLEV Kern 217 strain isolated in 1989 from *Culex tarsalis* from Kern County, CA, and passaged twice in Vero cells (obtained from Dr William Reisen, University of California at Davis, USA; Kramer & Chandler, 2001). Sequential passage of viruses in *C. pipiens* mosquitoes was completed for two separate lineages (L1 and L2) of WNV and SLEV by intrathoracic (IT) inoculation of approximately 10 p.f.u. in 0.1 µl mosquito diluent [MD: 20% heat-inactivated fetal bovine serum (FBS) in Dulbecco's PBS plus 50 µg penicillin/streptomycin ml⁻¹, 50 µg gentamicin ml⁻¹ and 2.5 µg Fungizone ml⁻¹]. Approximately ten mosquitoes were inoculated for each passage, and capillary-tube transmission assays were used to collect salivary secretions at 7 or 14 days post-inoculation (p.i.), basically as described previously (Aitken, 1977) with modifications (Ebel *et al.*, 2005). Briefly, mosquitoes were incapacitated with triethylamine (Sigma-Aldrich), their legs were removed and placed in 1 ml MD, and the mosquito proboscis was carefully inserted into a capillary tube containing FBS diluted with an equal volume of 50% sucrose. Salivation was allowed to proceed for approximately 30 min and the solution was ejected into 0.3 ml MD. In order to ensure sufficiently high viral titres for successful passage, salivary secretions from four mosquitoes were pooled and used for inoculation of the next group. A total of 19 passages was completed in this manner. In order to establish a stock of the passaged virus at a sufficient viral titre to be able to conduct replicate blood feeding experiments at mosquito passage 20 (MP20), 20 (WNV) or ten (SLEV) mosquitoes were inoculated and whole mosquitoes were pooled in 1 ml MD at 7 days p.i. Virus populations were labelled as UNP for unpassaged biological clones as described above and in Ciota *et al.* (2007a), and as MP_n for virus passaged in *C. pipiens* *n* times. L1 and L2 refer to the two replicates of mosquito passage. Virus stocks and virus-positive mosquito bodies, legs and salivary secretions were stored at -80 °C until subsequent testing.

ID₅₀ and virus growth curve assays in mosquitoes

Female *C. pipiens* were infected by IT inoculation (Rosen & Gubler, 1974) for determination of the 50% infectious dose (ID₅₀) and growth of individual virus strains. The ID₅₀ for each WNV strain was determined by IT inoculation of ten mosquitoes per dilution using tenfold increasing concentrations of virus from 0.1 p.f.u. and screening for infection by plaque assay in Vero cell cultures at 7 days p.i. Calculations of ID₅₀ were done using the Reed–Muench formula. Inoculations for growth curve assays were done with 10–20 times the ID₅₀ and viral titre was determined for eight to ten mosquitoes per time point harvested on days 1–7, 14 and 21 p.i. For both assays, mosquitoes were frozen individually at -80 °C at appropriate time points in 2 ml microcentrifuge tubes filled with 1 ml MD plus one 5 mm metal bead (Daisy). Samples were thawed and homogenized for 30 s at 24 Hz in a Mixer Mill MM301 (Retsch). Debris was then pelleted by centrifugation at 6000 g for 5 min and the supernatant was titrated or screened by plaque assay in duplicate in Vero cells as described previously (Payne *et al.*, 2006).

Vector competence

Infection, dissemination and transmission rates were determined as described previously (Ebel *et al.*, 2005). Briefly, 7-day-old female *C. pipiens* were deprived of sucrose for 48 h and then offered a cotton pad saturated with a 1 : 10 mixture of the appropriate virus : defibrinated goose blood (Hema Resource) with a 2.5% final sucrose concentration. After 1 h of exposure to the blood meal, mosquitoes were sedated with CO₂ and fully engorged mosquitoes were transferred to 0.6 l cartons and reserved for experimental testing. On days 5, 7, 9 and 14 post-feeding, 50 mosquitoes from each sample group were incapacitated and their legs removed and placed in 1 ml MD. Capillaries charged with FBS plus 50% sucrose (1 : 1) were used to collect salivary secretions for approximately 30 min, at which time the mixture was ejected into 0.3 ml 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 described above, and all samples were screened or titrated by plaque assay in duplicate in Vero cells. Virus-positive legs indicated virus egress from the mosquito midgut and spread into the haemolymph and parenteral tissues (dissemination). Virus-positive salivary secretions indicated that virus had infected the salivary glands and been released into the salivary secretions so that it was capable of being transmitted to another host (transmission). Blood-meal titres for each experiment (i.e. the concentration of virus in the blood meals offered to experimental mosquitoes) were also determined by plaque assay.

Chicken viraemia experiments

One- to 2-day-old chickens were inoculated subcutaneously with approximately 10 p.f.u. WNV UNP, WNV MP20 L1 (lineage 1) or WNV MP20 L2 (lineage 2) in a volume of 100 µl. Experiments for separate lineages were conducted separately. Five chickens per virus and two mock-inoculated chickens, i.e. inoculated with animal diluent (PBS plus 1% FBS) alone, were housed separately in adjacent cages and distinguished by coloured leg bands. Chickens were bled from the brachial vein and 50–100 µl blood was collected by capillary action in serum separator tubes on days 1–5 p.i. Chickens were monitored for signs of illness and were euthanized using 100 µl Sleepaway (Fort Dodge Animal Health) followed by cervical dislocation upon completion of the experiment. Blood was centrifuged at 5000 g for 10 min and the serum was removed, diluted 1 : 10 in BA-1 [Hanks' M-199 salts, 0.05 Tris/HCl (pH 7.6), 1% BSA, 0.035 g sodium bicarbonate l⁻¹, 100 U penicillin ml⁻¹, 100 mg streptomycin ml⁻¹, 1 mg Fungizone ml⁻¹] and stored at -80 °C until tested. Levels of viraemia were determined by plaque titration using Vero cells. All animal use was approved by the Wadsworth Center Institutional Animal Care and Use Committee (06-355).

Fluorescent focus assays and foci size measurement

Fluorescent focus assays were conducted as described previously using insect C6/36 cells and Vero cells (Payne *et al.*, 2006). Cell monolayers were inoculated with tenfold serial dilutions of virus in a final volume of 50 µl. Virus adsorption was allowed to proceed for 1 h at 37 °C (Vero) or 28 °C (C6/36), with rocking of the slides every 15 min. An overlay of minimal essential medium, 5% FBS and 0.8% carboxymethyl cellulose (ICN Biomedicals) was added following adsorption. The infected monolayer was incubated at 37 °C for 24 h (Vero) or 28 °C for 72 h (C6/36) and the overlay medium was removed from the wells and replaced with cold PBS. After 5 min incubation on ice, the PBS was removed and the cells were fixed for 10 min in ice-cold absolute methanol (Sigma-Aldrich) and then washed with PBS. Slides were incubated with a primary anti-WNV monoclonal antibody (5H10; Invitrogen) diluted in PBS containing 0.2% BSA (PBS/BSA) for 1 h at room temperature and then washed three times with PBS/BSA. Antibody-labelled cells were incubated for 30 min with a secondary antibody conjugated with FITC (KPL) diluted 1 : 50 in PBS/BSA, washed three times with PBS/BSA and mounted in anti-fading Vectashield Mounting Medium (Vector Laboratories). A Zeiss Axiovert 25 microscope, equipped with a Fluor 10×

objective and FITC filter sets was used for evaluation. Images were captured using a Zeiss AxioCam MRC digital camera and AxioVision software. The sizes of foci were measured using AxioVision software by creating a best-fit circle around the fluorescent signal and recording the diameter. Ten foci were chosen at random for measurement. Focus diameter measurements ranged from 59 to 238 μm in Vero cells and from 100 to 436 μm in C6/36 cells. This process was repeated several times to ensure unbiased estimates.

Sequencing

Determination of the full-genome sequences of WNV UNP and WNV MP20 L2 were completed as described previously (Ciota *et al.*, 2007a). RNA was extracted from WNV using RNeasy spin columns (Qiagen) according to the manufacturer's protocol. Primers for WNV were designed from GenBank sequence accession no. AF260967. One-step RT-PCR (Qiagen) was conducted using primers that generated nine overlapping PCR products. Reverse transcription reactions were carried out at 50 °C for 30 min, followed by inactivation of the transcriptase at 95 °C for 15 min. Amplification was then carried out for 40 cycles of 94 °C for 20 s, 55 °C for 30 s and 72 °C for 2 min, with final elongation at 72 °C for 10 min. PCR products were visualized on a 1.5% gel and the bands were then allowed to run through 1% NuSieve GTG low-melting-point agarose (Cambrex). Sequencing was performed at the Wadsworth Center Molecular Genetics Core with ABI 3700 automated sequencers (Applied Biosystems) using overlapping primers to give a minimum of twofold redundancy. Sequences were compiled and edited using DNASTAR.

High-fidelity RT-PCR, cloning and sequencing

Production and analysis of clones was performed basically as described previously (Ciota *et al.*, 2007b; Jerzak *et al.*, 2005). RNA was extracted from infected specimens using RNeasy spin columns (Qiagen) and RT-PCR was conducted using primers designed to amplify the 3' 1311 nt of the WNV envelope coding region and the 5' 3248 nt of the WNV non-structural protein 1 (NS1) coding region (forward primer WNV1311: 5'-ATGCGCCAAATTTGCCTGCTCTAC-3'; reverse primer WNV3248: 5'-ATGGGCCCTGGTTTTGTGTCTTGT-3'). Reverse transcription of 5 μl RNA was performed using Sensiscript reverse transcriptase (Qiagen) at 45 °C for 40 min. Reverse transcription reactions were followed by heat inactivation at 95 °C for 5 min. The resulting cDNA was used as template for PCR amplification. WNV cDNA was then amplified with a 'high-fidelity' protocol using *PfuUltra* (Stratagene), according to the manufacturer's specifications. Amplification was carried out for 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 4 min, followed by one cycle at 72 °C for 10 min. PCR products were visualized on a 1.5% agarose gel and DNA was recovered using a MinElute Gel Extraction kit (Qiagen) as specified by the manufacturer. The recovered DNA was ligated into the cloning vector pCR-Blunt II-TOPO (Invitrogen) and transformed into One Shot TOP10 Electrocomp *E. coli* cells (Invitrogen) according to the manufacturer's protocol. Kanamycin resistance was used for initial detection of transformed colonies. Colonies were then screened by direct PCR using primers specific for the desired insert. Plasmid DNA was purified using a QIAprep Spin Miniprep kit (Qiagen) as specified by the manufacturer. Sequencing was carried out using five pairs of overlapping WNV primers together with T7 and SP6 primers. Sequencing was performed at the Wadsworth Center Molecular Genetics Core using ABI 3700 and 3100 automated sequencers (Applied Biosystems). Between 20 and 26 clones were sequenced per sample.

Data analysis

Sequences were compiled and edited using the SeqMan module of the DNASTAR software package, with a minimum of twofold redundancy throughout each clone required for sequence data to be considered complete. Between 20 and 26 clones from each individual

sample were aligned using MEGALIGN within DNASTAR. The consensus sequence for each sample was determined and the sequence of each clone was compared with the consensus sequence of the population. The percentage of nucleotide mutations (total number of mutations/total number of bases sequenced) was used as an indicator of genetic diversity. Statistics were performed using Microsoft Excel 2003 and GraphPad Prism version 4.03.

RESULTS

Virus growth kinetics in *C. pipiens*

In order to determine whether adaptation for replication in *C. pipiens* resulted from mosquito passage, growth of WNV and SLEV before (UNP) and after (MP20 L1 and MP14/MP20 L2) IT inoculation of 10–20 times the ID₅₀ of individual virus strains was compared (Figs 1 and 2). ID₅₀ values were similar for all strains tested for each virus, ranging from 0.6 to 1.0 p.f.u. (individual data not shown). Actual inoculum titres for all viruses were quantified by plaque assay and determined to be similar (within 5 p.f.u.). Results for WNV indicated adaptation resulting from passage (Fig. 1). Although initial growth rates appeared to be similar for both strains, significantly higher titres were seen on days 2, 4, 7 and 14 p.i. for WNV MP20 L1 (*t*-test, *P*<0.05) and days 5, 6, 7, 14 and 21 p.i. for WNV MP20 L2 (*t*-test, *P*<0.05) relative to WNV UNP. Titre differences between WNV MP20 L2 and WNV UNP peaked at day 7 p.i. at greater than 1.7 log₁₀ p.f.u. per mosquito, whereas differences between WNV MP20 L1 never exceeded 0.7 log₁₀ p.f.u. (day 4 p.i.) and were just 0.5 log₁₀ p.f.u. at day 7 p.i. The biological significance is uncertain given these small differences, despite statistical significance at some time points. WNV MP14 L2 demonstrated growth that was intermediate to WNV UNP and WNV MP20 L2, which corresponded to a mixture of small and average Vero plaque sizes observed with this strain. Unlike WNV, SLEV MP20 strains demonstrated moderately attenuated growth in *C. pipiens* following passage (Fig. 2). SLEV MP20 L1 titres were significantly lower than SLEV UNP on days 2, 3, 5 and 6 p.i. (*t*-test, *P*<0.05), after which titres remained equivalent for the remainder of the experiment. Similarly, SLEV MP20 L2 titres were significantly lower on days 1–4 p.i. (*t*-test, *P*<0.05) and were statistically equivalent from day 5 to 21 p.i. All experiments generating virus growth curves in mosquitoes were repeated at least once and similar results were obtained (data not shown).

Vector competence of *C. pipiens*

Infection, dissemination and transmission rates were determined in *C. pipiens* mosquitoes for *C. pipiens*-passaged (MP L2) WNV and WNV UNP. Blood-meal titres of WNV MP20 L2 and WNV UNP used in feeding experiments were 6.7 log₁₀ p.f.u. ml⁻¹ and 7.4 log₁₀ p.f.u. ml⁻¹, respectively. Despite the lower blood-meal titre, infection rates overall were significantly higher (Fisher's exact test, *P*<0.001) for WNV MP20 L2 (74 %) compared with WNV UNP (41 %; Table 1). Significantly higher infection rates were also measured for WNV MP20 L2 on days 5, 7 and 9 post-feeding (Fisher's exact, *P*<0.005), but lacked statistical significance on day 14. Dissemination and transmission rates overall and on individual days were low for both viruses, and no significant differences in dissemination or transmission rates between WNV MP20 L2 and WNV UNP were identified on any individual day. Given the relatively low blood-meal viral titres, these rates were expected (Ciota *et al.*, 2007c). Cell-culture amplification could correct this problem, but was not utilized in this case due to the potential genetic alterations that could result from this process.

Virus spread

In order to evaluate the effect of mosquito adaptation on WNV spread *in vitro*, fluorescent focus assays and subsequent foci measurements were taken before and after mosquito

adaptation in C6/36 and Vero cells. The results indicated a significant decrease in foci size resulting from mosquito adaptation in Vero cell culture, but not in C6/36 cell culture (Fig. 3; *t*-test, $P < 0.001$). Specifically, the mean focus size for ten foci chosen at random decreased from 134.1 μm for WNV MP1 L2 to 92.2 μm for WNV MP20 L2 in Vero cells, but remained statistically equivalent (173.5–201.2 μm , $P = 0.24$) in C6/36 cells. WNV MP20 was compared with WNV MP1 rather than WNV UNP to control for incorporation of host factors resulting from a single passage. The results correlated with observations of small plaque sizes for mosquito-passaged WNV in Vero cells (not shown). Foci experiments were repeated three times and multiple sets of ten were counted for each replicate. The dataset shown in Fig. 3 is a representative set.

Chicken viraemia

One- to 2-day-old chickens were inoculated subcutaneously with 5–15 p.f.u. WNV UNP, WNV MP20 L1 or WNV MP20 L2 in a volume of 100 μl . WNV MP20 L1 and MP20 L2 were compared with WNV UNP in separate experiments (Fig. 4). Inoculum titres of the UNP and MP virus strains were within 2 p.f.u. for each individual experiment. All chickens in both experiments became viraemic. Growth kinetics were similar for WNV UNP and WNV MP20 for both lineages, but statistically significant differences (*t*-test, $P < 0.05$) did occur at one time point for L1 and at two time points for L2. WNV MP20 L1 demonstrated significantly lower viraemia titres on day 1 p.i. (*t*-test, $P = 0.01$), but displayed higher viraemia titres on days 3–5. The differences on days 3–5 p.i. were not statistically different due to large SD values and small sample sizes. WNV MP20 L2 displayed significantly higher titres on days 1 (*t*-test, $P = 0.02$) and 3 (*t*-test, $P = 0.003$) p.i, but the kinetics overall were similar. For WNV MP20 L2, the small plaque size phenotype observed in Vero cell culture remained throughout the viraemia experiment. WNV MP20 L1 Vero plaque size was also generally smaller relative to WNV UNP (not shown). L1 experimental results were slightly more variable than L2 results and the viraemia kinetics for WNV UNP were slightly different for the two experiments (Fig. 4). Variation between chicken experiments is not uncommon, but this could partially be attributed to slightly more variation in hatching time and, therefore, age at the time of inoculation. Chickens in the L2 experiment were 24–36 h old, whereas chickens in the L1 experiment were 24–48 h old. Chickens of various hatch times were distributed equally among groups so that no age bias was given to any one experimental group.

Genetic diversity

Analysis and comparison of 20–26 clones per virus of WNV nt 1315–3245 were used to evaluate the genetic diversity of WNV resulting from mosquito passage. This region included the majority of both the envelope and NS1 coding regions. WNV UNP, WNV MP20 L1 and WNV MP20 L2 were highly homogeneous in the region analysed, indicating little to no accumulation of diversity resulting from mosquito passage (Table 2). In order to evaluate whether selection potentially contributed to the lack of genetic diversity in WNV MP20 L2, an intermediate passage, WNV MP10 L2, was also tested. This virus displayed equivalent titres to WNV UNP on day 7 p.i. in *C. pipiens* (data not shown) and lacked the small plaque phenotype observed with subsequent adapted passages. WNV MP10 L2 was also found to be highly homogeneous, indicating that homogeneity probably existed throughout mosquito passages.

Full-genome sequencing

In order to determine genetic correlates of mosquito adaptation and the small plaque/foci phenotype, full-genome analysis of WNV MP20 L2 was undertaken and the results compared with the sequence of WNV UNP (Table 3). The virus used for analysis consisted of a pool of 20 mosquitoes. The lack of genetic diversity in this population indicated that

pooling had little effect on the results (Table 2). A total of nine nucleotide substitutions, three in the structural genes and six in the non-structural (NS) genes, were identified as resulting from passaging (Table 3). Six nucleotide changes resulted in amino acid substitutions. Of these changes, four were fairly conservative changes and two were non-conservative. Mutation G1873A resulted in a glycine to serine amino acid change in the envelope protein and mutation T6550C resulted in a tyrosine to histidine amino acid change in the NS4A protein. The most mutated region was the NS5 coding region, with three nucleotide substitutions.

DISCUSSION

Many previous studies have utilized passage in cell-culture systems to clarify the capacity for adaptation and evolution of arboviruses (Chen *et al.*, 2003; Ciota *et al.*, 2007a; Cooper & Scott, 2001; Holland *et al.*, 1991; Novella *et al.*, 1999a, b; Weaver *et al.*, 1999; Zarate & Novella, 2004), yet *in vivo* passage studies in biologically relevant hosts are largely lacking. For this reason, the evolutionary pressures acting on these viruses are still poorly understood. Specifically, the capacity for further adaptation, the genetic correlates of adaptation and the evolutionary constraints of host cycling are not well described for arboviruses. Here, we characterized the phenotypic and genotypic alterations in WNV resulting from passage in *C. pipiens* mosquitoes. *C. pipiens* is the predominant vector for WNV in the northern USA and Europe, and is a sibling species of another *Culex* mosquito, *Culex pipiens quinquefasciatus*, which also dominates the WNV transmission cycle (Turell *et al.*, 2001a, b, 2005). An increase in replicative ability for WNV in *C. pipiens*, determined by evaluation of post-inoculation growth kinetics before and after passage, was attained for the two lineages of WNV tested (Fig. 1). In order to best describe the correlates of this adaptation, the lineage demonstrating the most significant gains in replicative ability (WNV MP20 L2) was chosen for further characterization. This strain was also found to be more infectious than WNV UNP following mosquito feeding on infectious blood meals, although this did not translate to differences in dissemination and transmission (Table 1). This may be partially explained by the fact that blood-meal titres were considerably lower (fivefold) for WNV MP20 L2. Generation of chicken viraemia curves for WNV UNP, WNV MP20 L1 and WNV MP20 L2 also suggested that adaptation to mosquitoes did not result in a replicative cost in the avian environment (Fig. 4). In fact, multiple time points for both WNV MP20 L1 and WNV MP20 L2 displayed higher viraemia titres, and statistical significance (*t*-test, $P < 0.05$) was measured on days 1 and 3 p.i. for WNV MP20 L2, which was also highly adapted to mosquitoes (Fig. 1; Table 1). It has been proposed that the relative lack of evolution observed with most arboviruses is a result of the evolutionary constraints imposed on the virus due to the need to replicate in disparate hosts and that this constraint may lead to fitness trade-offs in each host (Scott *et al.*, 1994). Many *in vitro* studies testing this hypothesis have provided inconsistent results (Ciota *et al.*, 2007a, b; Greene *et al.*, 2005; Novella *et al.*, 1999a; Weaver *et al.*, 1992); thus, evaluating the constraints of host switching in relevant hosts *in vivo* is required for an accurate assessment of the relevance of this concept in nature. Fitness trade-offs as a result of evolutionary constraint imply that an adaptation to one host, particularly adaptation obtained through exclusive passage in one host, would generally result in a replicative cost in the disparate host. With no substantial attenuation measured in viraemia growth kinetics or peak viraemia before and after mosquito adaptation (Fig. 4), the results presented here do not support this idea. Species-specific differences in viraemia levels clearly exist among avian hosts (Komar *et al.*, 2003), so the possibility that differences could be seen in other bird species cannot be ruled out. Furthermore, analysis of infectious foci sizes *in vitro* did indeed reveal a host-specific effect on virus spread (Fig. 3). WNV foci sizes were significantly smaller in Vero cells following passage in mosquitoes, and this attenuation was not observed when measured in C6/36 mosquito cell culture. This indicates that factors other than virus growth kinetics

may be important to study in order to fully characterize the phenotypic cost of host-specific adaptation, and highlights the difference between *in vitro* and *in vivo* phenotypic studies.

Previous *in vitro* studies with mosquito-cell-adapted WNV also found no replicative cost in avian cells (Ciota *et al.*, 2007a). Although the *in vivo* data presented here are generally consistent with these *in vitro* studies, genetic analyses revealed that *in vitro*- and *in vivo*-adapted populations are very different in terms of the breadth of the mutant spectra accumulated during passage (Table 2). In previous *in vitro* studies, the capacity for cell-culture-adapted WNV to replicate efficiently in disparate hosts was attributed to the highly diverse and therefore adaptable population attained in passage (Ciota *et al.*, 2007b). Here, through analyses of genetic diversity of the same region, we found that WNV remained highly homogeneous with passage in mosquitoes (Table 2). This result suggests that genetic heterogeneity, even following host-specific adaptation, is not required for replicative success in disparate environments. This emphasizes the plasticity and robustness of WNV and is consistent with the levels of replicative success observed with multiple hosts in the laboratory and in nature (Higgs *et al.*, 2004; Marra *et al.*, 2004; <http://www.cdc.gov/ncidod/dvbid/arbor>). This result of genetic homogeneity following passage also contrasts with a similar laboratory study in which WNV accrued significant heterogeneity with passage in *C. pipiens* (Jerzak *et al.*, 2005). The only considerable difference in passage methodology between these studies was that salivary secretions, as opposed to whole mosquito bodies, were used for each passage here. This suggests that significant genetic bottlenecks may have occurred at the well-documented salivary gland infection and escape barriers (Grimstad *et al.*, 1985; Kramer *et al.*, 1981; Woodring *et al.*, 1996) of these inoculated mosquitoes. More substantial studies evaluating the effect of salivary as well as midgut barriers are necessary to evaluate fully the extent of genetic bottlenecks within the mosquito; however, this result suggests that an extensive mutant swarm may rarely be transmitted to a vertebrate host and, therefore, that interhost quasispecies dynamics may potentially be less significant for WNV than intrahost quasispecies dynamics (Ciota *et al.*, 2007b; Jerzak *et al.*, 2005, 2007). This is a crucial distinction when evaluating the implications of mutant swarm diversity in virus evolution.

Analysis of full-genome sequencing also revealed that significantly more consensus genetic change occurred following 20 passages in *C. pipiens* mosquitoes compared with changes previously identified following 40 passages in mosquito cell culture with the same virus strain (Ciota *et al.*, 2007a; Table 3). Of the nine substitutions identified here, six were non-synonymous changes. Although all mutations could potentially be phenotypically important, two resulted in non-conservative amino acid changes. The first, G1873A, resulted in a glycine (non-polar) to serine (uncharged polar) substitution at aa 303 of the envelope glycoprotein. This is a potential candidate for conferring WNV adaptation to the mosquito, given the well-documented role of the flavivirus envelope protein in assembly, binding and replication (Chambers *et al.*, 1990; Scherret *et al.*, 2001; Shirato *et al.*, 2004). Another potentially significant change was T6550C, resulting in a tyrosine (uncharged polar) to histidine (charged polar) amino acid substitution in the NS4A polypeptide. NS4A has yet to be implicated as having any specified role in virus replication in the mosquito, but multiple mutations in NS4 have been identified previously in mosquito-cell-adapted WNV and SLEV (Ciota *et al.*, 2007a).

Differences in adaptation *in vitro* have been reported previously for WNV and SLEV following mosquito-cell passage (Ciota *et al.*, 2007c). Here, we demonstrated that significant differences in the capacity for adaptation to mosquitoes *in vivo* also exist between these two closely related flaviviruses. Surprisingly, passage of SLEV in *C. pipiens* resulted in somewhat attenuated growth in *C. pipiens*, with significantly lower titres measured early for both lineages relative to SLEV UNP (Fig. 2). A possible explanation for this exists in our

passage methodology. By using salivary secretions from a single day and then diluting and inoculating just 10 p.f.u. for each subsequent passage, we imposed a significant bottleneck, which SLEV may not have been able to overcome. The fact that WNV did not merely overcome this bottleneck, but also adapted further to *C. pipiens*, suggests a difference in phenotypic robustness between these two viruses and that SLEV probably resides at near-maximum fitness in this host, whilst WNV most likely has the potential for further fitness gains in mosquito vectors. As these viruses are likely to be subjected to bottlenecks in nature within and between hosts, and more substantially between seasons, these differences could play a significant role in the variable levels of activity and geographical range observed with WNV and SLEV in nature.

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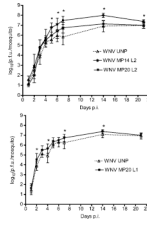


Fig. 1. Virus growth kinetics of WNV in IT-inoculated *C. pipiens* mosquitoes before (UNP) and after (MP) passage in *C. pipiens*. Individual points represent mean titres \pm SD of eight to ten mosquitoes. An asterisk indicates significantly higher titres for MP20 strains relative to UNP virus (*t*-test, $P < 0.05$).

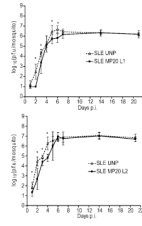


Fig. 2.

Virus growth kinetics of SLEV in IT-inoculated *C. pipiens* mosquitoes before (UNP) and after (MP) passage in *C. pipiens*. Individual points represent mean titres \pm SD of eight to ten mosquitoes. An asterisk indicates significantly higher titres for SLEV UNP relative to MP20 strains (*t*-test, $P < 0.05$).

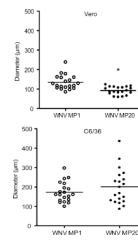


Fig. 3. Foci size of WNV MP1 L2 and WNV MP20 L2 identified by fluorescent focus assay in Vero or C6/36 cell culture. Vero measurements were taken at 24 h p.i. and C6/36 measurements at 48 h p.i. Significantly lower foci sizes were found for MP20 relative to MP1 in Vero cell culture, indicated by an asterisk (t -test, $P < 0.001$).

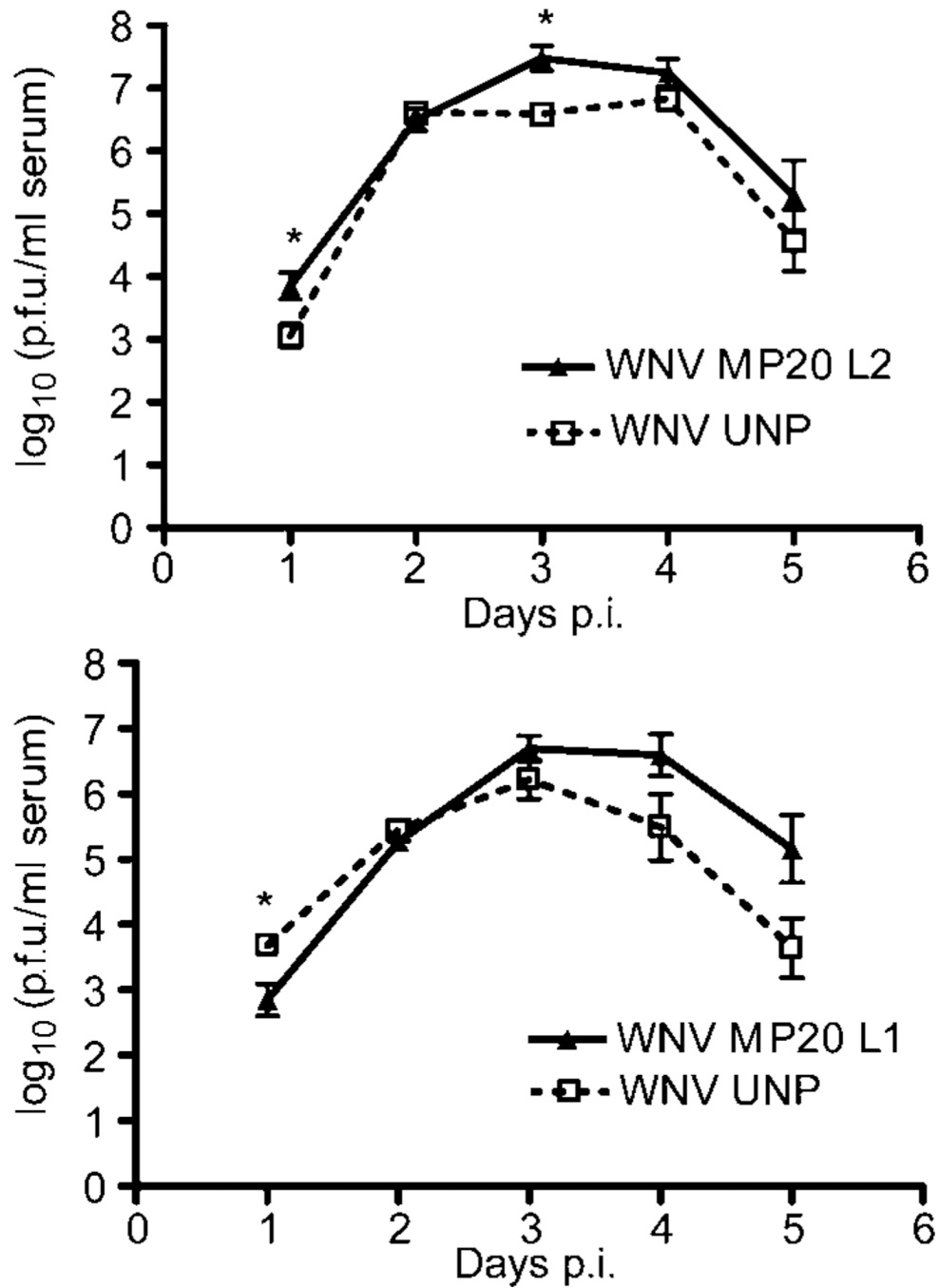


Fig. 4. WNV viraemia in 1–2-day-old chickens following inoculation of WNV before (UNP) and after (MP) *C. pipiens* passage. Individual points represent the mean titres \pm SD of five chickens. Statistically significant differences are indicated by an asterisk (*t*-test, $P < 0.05$).

Table 1

Vector competence of *C. pipiens* mosquitoes 5–14 days after feeding on infectious blood meals containing WNV UNP or WNV MP20 L2

WNV strain	Day post-feeding	No. infected (%) [*]	% Infected and disseminated	% Infected and transmitting
UNP	5	22 (44)	7	0
MP20	5	44 (88)	9	0
<i>P</i> value		<0.001 [†]	0.323	–
UNP	7	21 (42)	14	0
MP20	7	36 (72)	11	3
<i>P</i> value		0.004 [†]	0.669	1.000
UNP	9	15 (30)	20	0
MP20	9	34 (68)	9	0
<i>P</i> value		<0.001 [†]	0.353	–
UNP	14	23 (46)	17	0
MP20	14	33 (66)	30	6
<i>P</i> value		0.069	0.355	0.507
UNP	All	81 (41)	NA	NA
MP20	All	147 (74)	NA	NA
<i>P</i> value		<0.001 [†]	–	–

* 50 mosquitoes tested per time point for both UNP and MP20.

[†] Statistically significant (Fisher's exact test).

Table 2Nucleotide diversity of nt 1315–3245 for WNV before (UNP) and after (MP) passage in *C. pipiens*

Virus	No. of clones (total nt sequenced)	% Nt diversity* (no. of mutations)
WNV UNP	20 (38 620)	0.005 (2)
WNV MP10 L2	26 (50 206)	0.008 (4)
WNV MP20 L2	26 (50 206)	0.004 (2)
WNV MP20 L1	25 (48 275)	0.002 (1)

* Nt diversity, total nucleotide changes relative to consensus/total number of nucleotides sequenced.

Table 3

Nucleotide and amino acid changes identified in WNV MP20 L2 relative to WNV UNP by full-genome analysis

Position (nt)	Region	Nt substitution	Aa substitution
232	C	T→C	F→L
537	prM	G→A	None
1873	Envelope	G→A	G→S
4874	NS3	A→G	K→R
6550	NS4A	T→C	Y→H
7245	NS4B	T→C	None
7950	NS5	T→C	None
8877	NS5	G→T	E→D
10196	NS5	G→A	S→N