



Published in final edited form as:

J Med Entomol. 2013 July ; 50(4): 853–862.

Vector Competence of Argentine Mosquitoes (Diptera: Culicidae) for West Nile virus (Flaviviridae: Flavivirus)

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Abstract

We examined the ability of *Culex pipiens* L. complex mosquitoes from Argentina to vector West Nile virus (WNV) to assess their role in the transmission of WNV in South America. Several egg rafts of *Culex* spp. were collected from different breeding sites in the suburbs of the city of La Plata, Argentina, and a subset of each progeny was scored with morphological and genetic species indicators. Surprisingly, we did not find *Cx. pipiens* form *pipiens*, but found evidence of genetic hybrids of *Culex quinquefasciatus* and *Cx. pipiens* f. *molestus*. We then used morphological traits to create two colonies predominantly composed of one of these two taxa, although some hybrids are likely to have been included in both. These colonies were used in vector competence studies using NY99 and WN02 genotype strains of WNV obtained in New York State. As controls, we also tested colonies of U.S. *Cx. quinquefasciatus* and *Cx. pipiens* f. *molestus*. Additional *Culex* larvae from three drainage ditches near the cities of La Plata and Berisso, Argentina, were identified by morphological and high-resolution molecular markers (microsatellites) as *Cx. quinquefasciatus* Say, *Cx. pipiens* form *molestus*, and hybrids. Results indicate that Argentinian *Culex* are competent but only moderately efficient vectors of WNV and are less susceptible to this virus than comparable U.S. mosquito strains. Studies of vertical transmission of NY99 virus by *Cx. pipiens* f. *molestus* hybrids from Argentina yielded a minimal filial infection rate of 1.19 from females feeding during their second and later bloodmeals.

Keywords

Culicidae; mosquitoes; arbovirus; West Nile virus; vector competence

West Nile virus (WNV; Flaviviridae: Flavivirus) is a zoonotic pathogen, maintained in an enzootic cycle where it is transmitted between ornithophilic mosquitoes and avian hosts, but the virus also infects humans, equines, and many other vertebrates. WNV was first isolated from a febrile woman in Uganda in 1937 (Smithburn et al. 1940) and subsequently was identified as the etiologic agent of sporadic cases and major outbreaks in Africa, Europe,

Asia, Australia, and the Middle East. Following a single introduction into the United States in 1999 (Lanciotti et al. 1999), WNV rapidly expanded its range in a step-wise fashion and by 2004 was well established in North America and portions of Canada to the north (Kramer et al. 2007). Infection leads to a broad spectrum of disease ranging from fever to severe neurologic disease including meningitis, encephalitis, and acute flaccid paralysis. Severe disease is more common in older individuals, and immunocompromised hosts are at particular risk. The largest epidemics of West Nile neuroinvasive disease (WNND) in the United States and the world occurred in 2003 (2,866 cases of encephalitis, and 264 deaths) and 2012 in the United States when 2,873 cases were classified as neuroinvasive disease with 286 deaths (Centers for Disease Control and Prevention [CDC] 2012). Since 1999, an estimated 3.3 million people in the United States have been infected with WNV. This disease has also been noted in Puerto Rico, Cuba, and Argentina in addition to the continental United States and Canada in the western hemisphere (Dupuis et al. 2005, Morales et al. 2006).

The first isolation of WNV in South America was recorded in Argentina in February 2006 when it was isolated from the brains of three horses that died from encephalitis. The horses were from different farms in central Argentina (Morales et al. 2006). Phylogenetic analysis of NS5 fragments of the Argentinean viruses placed them in the North American cluster, lineage IA (Morales et al. 2006). From these three sequences, two appear to be genetically similar to NY99, showing 100% nucleotide identity and the third sequence differed by only 1 nt from WNV detected in New York (Morales et al. 2006). Seroprevalence studies performed on wild birds confirmed WNV activity between 2004 and 2005 in four different regions of Argentina (Diaz et al. 2008) and evidence of the cocirculation of St. Louis encephalitis virus (SLEV) and WNV in equines in Santa Fe province (Tauro et al. 2012).

Culex mosquito species are the predominant vectors of WNV worldwide, but the virus can infect ≈ 75 mosquito species (Sardelis et al. 2001, Turell et al. 2001, Goddard et al. 2002, Ebel et al. 2005, Turell et al. 2005, Kilpatrick et al. 2010) and >300 avian species (Komar et al. 2005, Reisen et al. 2005). The *Culex pipiens* complex in the Americas is composed of two main species, *Cx. quinquefasciatus* Say, which is adapted to warmer climates, and *Cx. pipiens* L., which is found in cooler zones. *Culex pipiens* has two known forms, form *pipiens* and form *molestus*, which differ broadly in behavior and physiology, most notably the capacity to diapause and the incidence of autogeny (summarized in Farajollahi et al. 2011). North American *Cx. pipiens* include many hybrids of the two *Cx. pipiens* forms (Fonseca et al. 2004, Strickman and Fonseca 2012), and at intermediate latitudes, hybridization between *Cx. pipiens* and *Cx. quinquefasciatus* is ubiquitous (Barr 1957, 1982; Farajollahi et al. 2011; Strickman and Fonseca 2012). In Argentina, *Cx. quinquefasciatus* occurs from the provinces of Buenos Aires and Mendoza northwards, whereas *Cx. pipiens* is found from Buenos Aires southwards to the Santa Cruz province, and hybrid forms have been found in the central area of the country (Brewer et al. 1987, Almiron et al. 1995, Humeres et al. 1998), although these findings are based exclusively on morphological identification and allozyme studies.

Vector competence is a measure of the intrinsic ability of an arthropod to become infected with a pathogen, support development or replication of the pathogen, and transmit the pathogen to a vertebrate host. Mosquito populations possess inherent differences in their ability to serve as vectors in arbovirus transmission cycles (Gubler and Rosen 1976, Jupp and Kemp 1993, Kramer et al. 1993, Vaidyanathan and Scott 2007). The vector competence of *Culex* spp mosquitoes most likely to be involved in WNV transmission in Argentina is still unknown. The purpose of the current study was to analyze the competence of *Cx. pipiens* complex mosquitoes from Argentina to begin to determine their potential importance in WNV transmission in Argentina.

Materials and Methods

Identification of Mosquitoes for Colonies

Specimens of *Culex* spp. were collected as egg rafts from different habitats such as drainage ditches and artificial containers in the suburbs of La Plata city, Buenos Aires province, Argentina, during February 2012. Egg rafts were individually hatched in tap water supplemented with finely ground guinea pig food, and reared up to the pupae stage at 26° with a photoperiod of 12:12 (L:D) h cycle in the insectary of CEPAVE (UNLP CONICET), Argentina. The fourth-instar larval exuviae were separated to assess the siphonal index (S.I.) (Brogdon 1981, 1984) under stereoscopic microscope. Pupae originally identified as *Cx. quinquefasciatus* (S.I. values of ≤ 3.40) and those belonging to *Cx. pipiens* (S.I. values of ≥ 4.30) were placed in two different cages. Emerged adults were sugar-fed for 4 d and then were blood fed to obtain eggs. Egg masses from each colony were shipped to the Arbovirus Laboratories, Wadsworth Center, NY, where two separate colonies were established. To confirm morphological identifications, genomic DNA was obtained from five individual mosquitoes and 8–10 groups of 10 individuals each, from each colony via phenol or chloroform extraction as described by Fonseca et al. (2000) followed by polymerase chain reaction (PCR) amplification of the *AceII* gene and CQ11 locus using the protocols developed by Smith and Fonseca (2004) and Bahnck and Fonseca (2006). These DNA-based rapid assays produce bands of varying sizes that are taxa specific. Hybrids will show a combination of bands from different taxa.

Mosquito rearing followed standard protocols (Gerberg et al. 1994). *Cx. pipiens* and *Cx. quinquefasciatus* from long-term colonies maintained in the insectary at Wadsworth Center, NY State Department of Health, Albany, NY, were used as controls in each vector competence experiment. All colonies were maintained with water pads and sugar cubes in 30 by 30 by 30 cm cages and held at 26°C and a photoperiod of 16:8 (L:D) h.

Field Surveys of Argentine Cx. pipiens Complex Populations. Immature stages of *Culex* mosquitoes were collected from three additional drainage ditches (Fig. 1, sites A, B, and C) located in the suburbs of La Plata city and Berisso city, Argentina, to determine the diversity of *Culex* mosquito species breeding in this area. In this geographical region, the climate is temperate (annual average temperature, from 13 to 17°C), with rainfall occurring throughout the entire year (Cabrera and Willink 1980).

Larvae from each site were transported to the insectary where they were separately reared at 26°C in trays of ≈ 300 larvae per tray, containing tap water and finely ground guinea pig food. Pupae were placed in three different cages (30 by 30 by 30 cm), one per site, for adult emergence. Twenty individual adult mosquitoes emerging from the collected immatures from each of the habitats were taken at random. These 60 mosquitoes were identified with appropriate taxonomic keys (Darsie and Mitchell 1985) using male genitalia morphology and morphometry (DV/D ratio and siphonal index; Brogdon 1981, 1984; Vinogradova 2003). Three mosquitoes from each site were also analyzed with the rapid PCR-based assays, and a subset of 22 specimens (12 from site A and 10 from site C) was genotyped with a microsatellite panel (see below for details).

Genotype Analysis

We used a panel of eight microsatellite loci that amplify consistently across all United States *Cx. pipiens* complex populations (CQ11, CQ26, CxqGT4, CxqGT6b, CxpGT4, CxpGT9, CxpGT12, CxpGT46, [Smith et al. 2005]). These same loci have been used in multiple studies of populations of the *Cx. pipiens* complex in the United States, Western Europe, northern Africa, the Middle East, Asia, and Australia (Fonseca et al. 2004, 2009; Gomes et

al. 2009; Strickman and Fonseca 2012) and therefore allow for broad comparisons. We examined the genetic ancestry of 22 specimens, 12 from La Plata and 10 from Berisso City. Microsatellite loci were amplified and sized as described in Smith et al. (2005). We assigned individuals to clusters (taxa) based on their multilocus genotypes with a maximum likelihood algorithm implemented in the program Structure 2.0 (Pritchard et al. 2000). We used 100,000 burn-in steps and 1,000,000 runs with a model of uncorrelated allele frequencies allowing admixture. In this analysis, the origin of each specimen is not disclosed, but the number of clusters (K) is decided a priori for each run. To assess the consistency of the analysis, we performed an exhaustive comparison of 10 runs scoring the similarity coefficient described in Rosenberg et al. (2002). We chose the most appropriate putative number of clusters (K = 3), by choosing the K with the highest associated ΔK (Evanno et al. 2005). To examine the ancestry of the Argentine populations, we included in the analysis several other populations: 1) specimens of *Cx. pipiens* f. *molestus* collected as larvae in midwinter in Philadelphia, PA, and Germany (specimens from these populations were brought to the laboratory, and all adult females were autogenous); 2) specimens of *Cx. pipiens* f. *pipiens* collected in Germany and the United Kingdom; and 3) specimens identified as *Cx. quinquefasciatus* from Florida and Louisiana (Fonseca et al. 2006).

Vector Competence Assays

Peroral Infection—These studies were conducted in the BSL3 insectary at the Arbovirus Laboratory, Wadsworth Center, NY State Department of Health. Vector competence assays were conducted using NY99 and WN02 genotype strains of WNV from New York (strain designations are NY99–3356 and WN02–1956). Bloodmeals containing virus were prepared using frozen virus stock in the first experiment and freshly harvested virus in the second. In the first assay, 8.5 ml of defibrinated bovine blood, 0.5 ml of 2.5% (wt:vol) sucrose, and 1.0 ml of frozen virus stock were mixed. In the second, confluent C6/36 cell monolayers in 75-cm² flasks were infected at an MOI of 0.1 and incubated at 28°C for 2–4 d. The bloodmeal was prepared by scraping infected cells into the media and mixing the suspension 1:1 with defibrinated bovine blood and 2.5% (wt:vol) sucrose.

Five- to seven-day-old female adult mosquitoes were allowed to feed on the infected bloodmeal in sausage casing for 1 hr at room temperature following the protocol of Ebel et al. (2004). Blood suspensions were frozen at –80°C for subsequent plaque assay to determine the virus titer at the time of mosquito feeding. Females that fully engorged were separated and held in 0.47-L cartons at 26°C, under a photoperiod of 16:8 (L:D) h, and provided a 10% sucrose solution ad libitum on cotton wicks until dissection. After 5, 7, 9, and 14 d after feeding on the frozen virus bloodmeal, and 7, 14, and 21 d for fresh virus bloodmeal, females were anesthetized with Triethylamine (Sigma, St. Louis, MO) and bodies, legs, and salivary secretions were obtained from each individual mosquito and processed as previously described (Ebel et al. 2004) to determine infection, dissemination, and transmission rates, respectively.

Peroral infection and transmission of WNV genotype NY99 and WN02 were evaluated in *Cx. pipiens* f. *molestus* from Argentina and *Cx. quinquefasciatus* from Argentina and United States using frozen virus; and in a second set of experiments, both species of mosquitoes from Argentina and United States were evaluated using freshly harvested virus, genotype NY99.

Vertical Transmission—Female *Cx. pipiens* f. *molestus* (Argentina) were offered a bloodmeal containing 8.5 ml of defibrinated bovine blood, 0.5 ml of 2.5% (wt:vol) sucrose, and 1.0 ml of frozen virus stock, genotype NY99. We used this genotype of WNV because it is phylogenetically close to the sequences of WNV isolated from horses in Argentina. Fully

engorged female mosquitoes were removed to a 1-liter carton and held for 4 d under the same conditions described above, after which, they were provided a dish with water for oviposition. The dead adults were collected every day and frozen at -80°C until assayed for virus. The number of infected adults was evaluated by plaque assay. Eggs rafts were hatched individually in plastic cups at 27°C , and larvae were reared in groups of 10–30 per plastic rearing container at 27°C until they reached the fourth instar. These larvae were pooled in groups of 20, frozen at -80°C , and processed for virus isolation in Vero cells to determine whether vertical transmission had occurred. After laying eggs, females were fed again using uninfected blood (9.5 ml bovine blood with 0.5 ml 50% sucrose). After feeding, a dish with water was introduced into the carton for oviposition. A second uninfected bloodmeal was offered, and mosquitoes were allowed to lay eggs again. Eggs were collected, larvae were reared, and the progeny from the second and third oviposition were processed as described.

Larval pools were ground in 1.0 ml of mosquito diluent (20% heat-inactivated fetal bovine serum [FBS] in Dulbecco's phosphate-buffered saline plus 50 $\mu\text{g}/\text{ml}$ of penicillin/streptomycin, 50 $\mu\text{g}/\text{ml}$ of gentamicin, and 2.5 $\mu\text{g}/\text{ml}$ of fungizone) using stainless steel BB's (Daisy Brand, UT) in a mixer mill and centrifuged at 2,500 rpm. Plaque assays were conducted as described in Payne et al. (2006).

Statistical Analysis

The number of mosquitoes with virus in legs (dissemination) and salivary secretion (transmission) was calculated as a proportion of the number of mosquitoes that developed body infection. The proportion of infected individuals obtained at the end of the experiment was compared between groups using χ^2 or Fisher's exact tests, as appropriate according to the sample sizes. If significant differences were observed, pair-wise comparisons using Fisher's exact test were applied, with Bonferroni correction as adjustment for multiple tests. The proportions of mosquitoes infected with fresh virus were compared with those infected with frozen virus using the same statistical analyses described previously. All analyses were performed using InfoStat version 2011 (Di Rienzo et al. 2010).

Results

Identification of Mosquitoes

Mosquitoes from Argentina instead Mosquitoes for Colonies—The separation of the F1 *Cx. quinquefasciatus* from *Cx. pipiens* was originally made based on the siphonal index, as described in Materials and Methods. After the larvae were received in the United States, the two populations from Argentina were maintained separately in the insectary, and the F2 were identified with rapid assays, as described earlier. One colony was predominantly *Cx. pipiens* f. *molestus* although a few mosquitoes exhibited *Cx. quinquefasciatus* signatures, and the other was predominantly *Cx. quinquefasciatus* with some mosquitoes also having *Cx. pipiens* f. *molestus* signature. No bands diagnostic of *Cx. pipiens* f. *pipiens* were obtained. Progeny from F3-F4 of these populations were used for vector competence studies within the first 2 mo of colonization. Because specimens with hybrid signatures were present, neither colony was composed strictly of members of one of the taxa. However, the colony that was morphologically predominantly *Cx. pipiens* f. *molestus* was and remained autogenous, a trait usually associated with *Cx. pipiens* f. *molestus*, while the predominantly *Cx. quinquefasciatus* colony never displayed this trait. For simplicity, we will refer to the *Cx. pipiens* f. *molestus*/hybrid colony as *Cx. pipiens* f. *molestus*, and the *Cx. quinquefasciatus*/hybrid colony as *Cx. quinquefasciatus*.

Field Surveys of Argentine *Cx. pipiens* Complex Populations

Cx. pipiens f. *molestus*, *Cx. quinquefasciatus*, and hybrid populations between these two species were detected in each of the three breeding sites located in suburban La Plata city and Berisso city, Buenos Aires, Argentina (Fig. 1). These sites were artificial drainage ditches with organically polluted water. At sites A (34° 51'56" S, 58° 2'1" W) and B (34° 54'59" S, 58° 0'18" W), we detected a high degree of hybridization and a preponderance of ancestry from *Cx. pipiens* f. *molestus*. At site C (34° 52'12" S, 57° 53'30" W), there were several specimens with an apparent pure *Cx. quinquefasciatus* signature although hybrids were also abundant (Fig. 2).

Vector Competence Assays

Peroral Transmission. *Cx. pipiens* f. *molestus* from Argentina (AR) (Frozen Virus)—The percent of the population of *Cx. pipiens* f. *molestus* females (AR) that became infected with WNV after feeding on infectious blood containing 1.9×10^8 PFU/ml NY99–3356 and 3.4×10^8 PFU/ml WN02–1956 were 38.1% ($N = 118$ fed females) and 43.2% ($N = 111$ fed females), respectively. Infection rates with the two viral genotypes were not significantly different (χ^2 : 0.62; $df = 1$; $P = 0.43$). Dissemination was detected in 31.1% ($N = 45$) and 12.5% ($N = 48$) of the infected females from Argentina for NY99 and WN02, respectively. Dissemination of virus varied from 6.7% (3/45) of the females that were infected by genotype NY99 on day 5 postinfection (pi), 2.2% (1/45) on day 7, 20% (9/45) on day 9, and 2.2% (1/45) on day 14. Dissemination of WNV genotype WN02 varied from 0% (0/48) on day 5, 4.2% (2/48) on day 7, 2.1% (1/48) on day 9, and 6.25% (3/48) on day 14. Virus was detected in the salivary secretions of 8.8% ($N = 45$) of infected females infected by NY99 strain. Horizontal transmission of NY99 varied from 2.2% (1/45) on day 5 pi to 4.4% (2/45) on day 9 pi and 2.2% (1/45) on day 14 pi while no transmission was detected with WN02. Significant differences between the two strains of virus in overall rate of dissemination (χ^2 : 4.7; $df = 1$; $P = 0.02$) and transmission (Irwin–Fisher bilateral: 0.54, $df = 1$, $P = 0.05$) were detected.

***Cx. quinquefasciatus* AR and the United States (Frozen Virus)**—In total, 51.6% of 93 female *Cx. quinquefasciatus* AR that fed on 6.2×10^7 PFU/ml of WNV NY99–3356 were infected as compared with 95.5% ($N = 113$) of *Cx. quinquefasciatus* (United States) that ingested blood containing the same strain of virus. Dissemination (virus-positive legs) of this strain of virus was detected in 27.1% (13/48) of the infected females from Argentina. In mosquitoes positive on day 5 pi, 15.4% of the legs were infected, 38.5% at day 7 pi, and 23% at day 9 and 14 pi (Fig. 3). In the U.S. colony, overall 47.2% (51/108) of the legs were infected, increasing from 7.8% at day 5 pi to 56.9% on day 14 pi out of the total number of infected mosquitoes (51) (Fig. 3). Virus was detected in the salivary secretions of 16.7% ($N = 48$) of infected females from Argentina and 29.6% ($N = 108$) from the United States, increasing from 12.5% day 5 to 37.5% day 9 pi (AR mosquitoes) and from 3.12% day 5 pi to 75% day 14 pi (U.S. mosquitoes) (Fig. 3).

When the female mosquitoes ingested blood containing 6.4×10^7 PFU/ml WNV WN02–1956, 17.8% ($N = 123$) of individuals from Argentina became infected, 27.3% of which had disseminated virus and 9.1% ($N = 22$) transmitted virus on days 9 and 14 pi (Fig. 3). For the U.S. colony, 51.3% ($N = 117$) were infected while 25% ($N = 60$) and 15% ($N = 60$) of these infected females also had a disseminated infection and transmitted the virus from day 7 to 14 (Fig. 3).

Significant differences were detected in the overall rate of transmission between the two virus genotypes when mosquitoes from Argentina and United States were infected per os (χ^2 : 8.42; $df = 3$; $P = 0.038$). However, pair-wise comparisons between groups (AR NY99,

U.S. NY99, AR WN02, and U.S. WN02) using Fisher's exact test with Bonferroni correction did not show significant differences. Significant differences in overall rate of infection were detected in the two virus strains between both colonies of mosquitoes (χ^2 : 143.43; df = 3; $P < 0.0001$), with significantly higher levels of infection and dissemination detected in *Cx. quinquefasciatus* from the United States infected by the strain NY99–3356.

***Cx. quinquefasciatus* and *Cx. pipiens f. molestus* AR and the United States using Fresh Virus**

—The total percent of *Cx. pipiens f. molestus* females that became infected with WNV after feeding on blood containing NY99–3356 (titer: 8.8×10^7 PFU/ml) was 30.9% of 126 fed females from Argentina and 69.4% of 36 fed females from the United States. The rates of dissemination and transmission in mosquitoes from Argentina was 64% ($N = 39$) and 38.4% ($N = 39$), respectively, with percentages increasing from day 7 to 21 while for U.S. mosquitoes, 88% ($N = 25$) had disseminated infection and 40% ($N = 25$) had detectable virus in salivary secretions, from day 7 to 14 (Fig. 4). The percent infected of *Cx. quinquefasciatus* (AR) was 4.3% ($N = 115$) after feeding on 8.8×10^7 PFU/ml of NY99, of which 40% ($N = 5$) were able to support dissemination and transmission of the virus. The mosquitoes from United States had a 86.8% ($N = 114$) infection rate, of which 76.7% had a disseminated infection and 66.6% ($N = 99$) had virus-positive salivary secretions from day 7 to 21, with variable daily rates (Fig. 4). The infection rate in *Cx. quinquefasciatus* AR was significantly different from *Cx. quinquefasciatus* United States and also from *Cx. pipiens f. molestus* (AR and the United States) (χ^2 : 177.2; df = 3; $P < 0.0001$), although no differences were detected between both species from the United States (Fig. 5). The low number of infected individuals of *Cx. quinquefasciatus* AR was insufficient for statistical analysis of dissemination and transmission rates. Despite these significant differences, there were no detectable differences in dissemination rates of this virus between both *Cx. pipiens f. molestus* AR and the United States, and *Cx. quinquefasciatus* U.S. group (χ^2 : 4.93; df = 2; $P = 0.085$). Similar transmission rates were observed in *Cx. pipiens f. molestus* AR and the United States, and they were significantly different than what was observed in *Cx. quinquefasciatus* United States (χ^2 : 12.03; df = 2; $P = 0.002$) (Fig. 5).

Mosquito Infections with Fresh and Frozen WNV NY99. *Cx. pipiens f. molestus* females (AR) that became infected with fresh WNV after feeding on blood containing genotype NY99 (titer: 8.8×10^7 PFU/ml) was compared with mosquitoes infected with frozen WNV after feeding on infectious blood containing 1.9×10^8 PFU/ml genotype NY99. No differences were detected in infection (χ^2 : 1.37; df = 1; $P = 0.24$) and transmission rates (Irwin–Fisher bilateral: 0.27, df = 1, $P = 0.15$) although significantly higher proportion of dissemination was detected in mosquitoes infected with fresh virus (χ^2 : 4.82; df = 1; $P = 0.02$).

Cx. quinquefasciatus AR that fed on 6.2×10^7 PFU/ml of frozen WNV genotype NY99 demonstrated a significantly higher proportion of infected individuals than those infected with 8.8×10^7 PFU/ml of fresh virus (χ^2 : 40.46; df = 1; $P < 0.0001$), but no differences were detected in dissemination (Irwin–Fisher bilateral: -0.01 , df = 1, $P > 0.99$) or transmission rates (Irwin–Fisher bilateral: 0.04, df = 1, $P > 0.99$). For *Cx. quinquefasciatus* United States, significantly higher rates of body (χ^2 : 5.36; df = 1; $P = 0.02$), leg (χ^2 : 7.44; df = 1; $P = 0.006$), and saliva infections (χ^2 : 15.42; df = 1; $P = 0.0001$) with frozen virus were detected.

Vertical Transmission. *Cx. pipiens f. molestus* AR—The proportion of mosquito adults infected was 65.7% (69 infected of 105 adults tested) feeding on 6.8×10^8 PFU/ml NY99 virus. After the first uninfected bloodmeal, 24 rafts with 1,200 larvae (60 pools of 20 larvae each one) were obtained with one pool positive for WNV (first or second oviposition). From the second uninfected bloodmeal, 2 pools out of 18 were positive for WNV (total of 9 rafts, 360 larvae). The third uninfected bloodmeal yielded no positive

larvae from a single pool from 1 raft, 20 larvae. The minimum filial infection rate (MFIR), that is, the minimum number of mosquitoes infected with WNV per 1,000 offspring, was 1.9.

Discussion

We collected and identified by morphological and molecular methods *Culex* mosquitoes from the suburbs of La Plata city, Argentina, and found heterogeneous populations composed of *Cx. quinquefasciatus*, *Cx. pipiens* f. *molestus*, and hybrids resulting in a complex epidemiological situation for arbovirus transmission. No pure *Cx. pipiens* f. *pipiens* were collected, nor were hybrids with *Cx. pipiens* f. *pipiens* diagnostic signatures found. Our results indicate that *Cx. quinquefasciatus* and *Cx. pipiens* f. *molestus* from temperate Argentina are competent but only moderately efficient vectors for WNV. They are less susceptible to this virus compared with the same mosquito species from the United States. *Cx. quinquefasciatus* AR had lower infection rates than the same species from the United States for both WNV genotype WN02 and NY99. *Cx. pipiens* f. *molestus* AR had also lower infection rates than U.S for WNV NY99 although similar transmission rates were observed. Therefore, even though both *Culex* species are thought to contribute to WNV transmission in Argentina, at least this population of *Cx. quinquefasciatus* could be considered an inefficient laboratory vector of the virus strains tested.

In our study, we tested two strains of WNV, NY99 and WN02. The nucleotide sequences isolated from horses in Argentina appear to be very closely related to NY99 strain in lineage 1A (Morales et al. 2006); however, the original NY99 genotype of WNV was displaced by the genotype WN02 throughout the United States by 2004 (Ebel et al. 2004, Davis et al. 2005). WN02 virus strains, compared with NY99 strains, infected a larger proportion of *Cx. pipiens* and *Culex tarsalis* and had shorter extrinsic incubation periods (Ebel et al. 2004, Moudy et al. 2007). However, it is interesting that *Cx. quinquefasciatus* did not demonstrate this trait (Vanlandingham et al. 2008). In our assay, similar rates of transmission were observed between both strains of virus for *Cx. quinquefasciatus* United States and AR, although the United States mosquitoes were also more susceptible to infection by the NY99 strain compared with WN02 strain. *Cx. pipiens* f. *molestus* AR demonstrated similar rates of infection for both strains of virus.

Significantly fewer *Cx. quinquefasciatus* mosquitoes from Argentina became infected using freshly harvested virus of strain NY99 than U.S. mosquitoes. The ability to transmit the virus was similar between *Cx. pipiens* f. *molestus* United States and AR, but 100% of the infected U.S. species transmitted 7 d earlier than the AR species. The highest level of transmission was observed in *Cx. quinquefasciatus* United States. Vertical transmission of WNV has been reported for several species of *Culex* in the United States (Baqar et al. 1993; Miller et al. 2000; Nasci et al. 2001; Dohm et al. 2002; Goddard et al. 2003; Anderson and Main 2006; Phillips and Christensen 2006; Reisen et al. 2006; Anderson et al. 2008, 2012). In our experiments, the MFIR for *Cx. pipiens* f. *molestus* was 1.19 from eggs laid during the second and later ovipositions. An MFIR of 1.9/1,000 is equal to the estimated rate in California for *Cx. pipiens* complex mosquitoes of 2.0/1,000 (Fechter–Leggett et al. 2012). Our results indicate that this mosquito species in Argentina could potentially contribute to WNV persistence through the winter by vertical transmission if they were to become infected. However, because *Cx. pipiens* f. *molestus* are autogenous and generally feed on mammals in subsequent bloodmeals (Farajollahi et al. 2011), it is unlikely they would become infected unless they contributed this trait to a hybrid population.

Cx. quinquefasciatus and *Cx. pipiens* are common mosquitoes that lay eggs in artificial containers (Almiron and Brewer 1996, Garcia et al., 2002) and drainage ditches (Campos et

al. 1993) in urban and suburban areas in Argentina. These species are numerous in locations where there are avian hosts, humans and horses. They feed primarily on birds, but also will feed on mammals and humans (Almiron and Brewer 1995). However, natural feeding patterns of these species of mosquitoes in Argentina have not been studied. Both mosquito species and intermediate forms were found in Buenos Aires province, but southward only *Cx. pipiens* has been recorded (Almiron 1995, Rossi and Vezzani 2011). Previously, no information was available about the existence of any biotypes or forms of *Cx. pipiens* in Argentina. Surprisingly, in this study, we were able to identify only *Cx. pipiens* f. *molestus* in the study area, as well as *Cx. quinquefasciatus* and hybrid populations between *Cx. quinquefasciatus* and *Cx. pipiens* f. *molestus*. This is in keeping with a recent report (Antunes De Morais et al. 2010) that hybrid forms between *Cx. quinquefasciatus* and *Cx. pipiens* occur in regions of latitudes around 34–35° S, suggesting that La Plata, Buenos Aires province, is a hybrid region. Diez et al. (2012) also extend the distribution of hybrids of *Cx. pipiens* complex in Argentina from latitudes 30° 36' S to 36° 13' S and between longitudes 57° 57' W and 64° 48' W. Future studies of distribution of both forms of *Cx. pipiens* and vector competence of hybrid populations of *Cx. pipiens* f. *molestus*, *Cx. pipiens* f. *pipiens*, and *Cx. quinquefasciatus* in Argentina need to be conducted and bloodmeal determinations made to examine host feeding preferences of hybrids.

The presence of WNV competent vectors together with the known circulation of this virus in Argentina (Morales et al. 2006, Diaz et al. 2008, Tauro et al. 2012) is a cause for concern. Knowledge of vector competence, distribution, seasonal abundance, and natural feeding patterns of these vectors can help improve arbovirus surveillance and mosquito control efforts in Argentina.

Acknowledgments

We thank Pam Chin for rearing the colonized mosquitoes used in these studies and George Condon for performing the microsatellite genotyping. We also appreciate the support of the *Fulbright Visiting Scholar Program*, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Wadsworth Center. This study was partially funded by National Institute of Health grant one C06 RR 17715-01 supporting the insectary where these studies were conducted.

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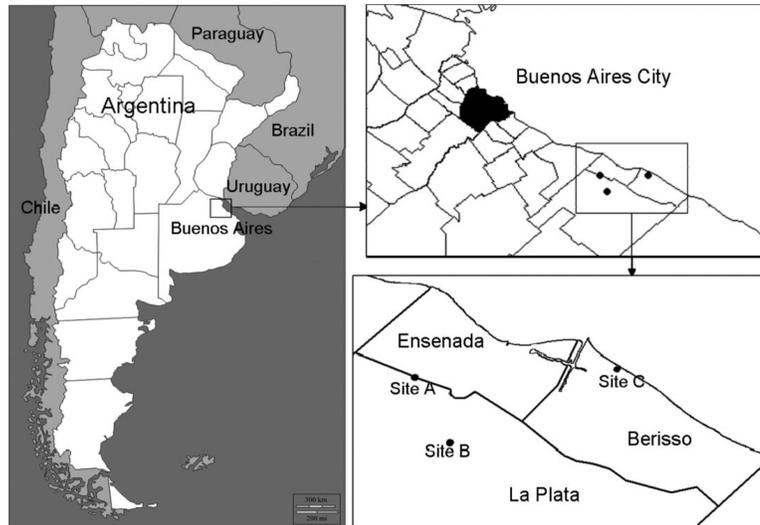


Fig. 1. Map showing the three sampling points for *Culex* mosquito immature located in Buenos Aires province, Argentina. A ($34^{\circ} 51'56''$ S, $58^{\circ} 2'1''$ W), B ($34^{\circ} 54'59''$ S, $58^{\circ} 0'18''$ W), and C ($34^{\circ} 52'12''$ S, $57^{\circ} 53'30''$).

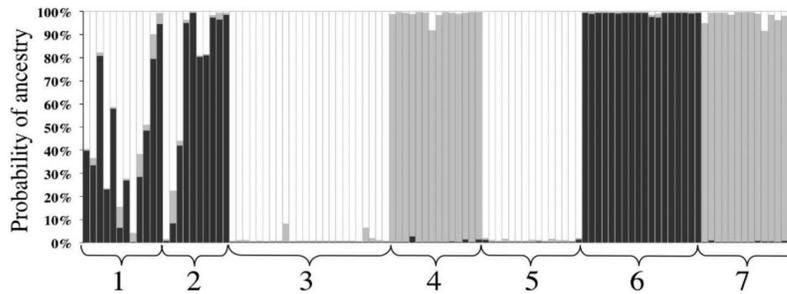


Fig. 2.

Multilocus genotype analysis of 22 specimens from Argentina based on eight microsatellite loci (2 females and 10 males from site A in Fig. 1, and 5 females and 5 males from site C). These are populations 1 and 2, respectively. For comparison, we also included specimens of *Cx. pipiens* form *molestus* from Germany and from Philadelphia (24 and 15 specimens, respectively, populations 3 and 5), *Cx. pipiens* form *pipiens* from Menstrie, Scotland, and Cambridge, United Kingdom (14 of each, population 4 and 7), and *Cx. quinquefasciatus* from Florida and Louisiana (nine of each; population 6). Each of the individuals included in the analysis is represented by a thin vertical line, partitioned into colored segments that represent the individual's probability of belonging to one of each of the genetic clusters (genetic ancestry, in the y-axis). Although the origin of each specimen is not used in the analysis, in this figure specimens were grouped by location.

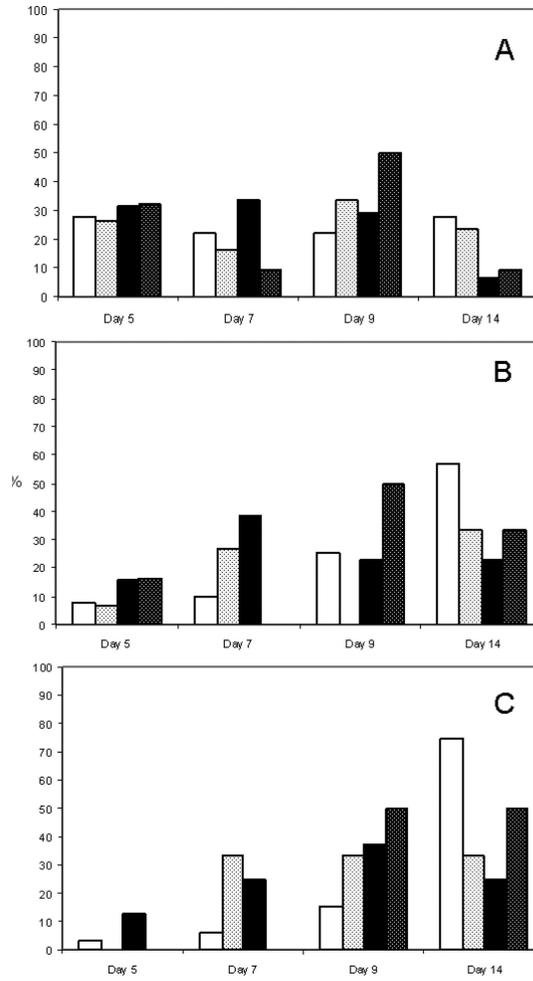


Fig. 3. Infection, dissemination, and transmission rates per day post-infection for *Cx. quinquefasciatus* orally exposed to West Nile virus (Frozen Virus). D5, 7, 9, 14 rates are percentages of mosquitoes containing virus in their bodies (A), legs (B), and saliva (C). ■ U.S. *Cx. quinquefasciatus*-NY99, □ AR *Cx. quinquefasciatus*-NY99, ▒ U.S. *Cx. quinquefasciatus*-WN02, ▓ AR *Cx. quinquefasciatus*-WN02.

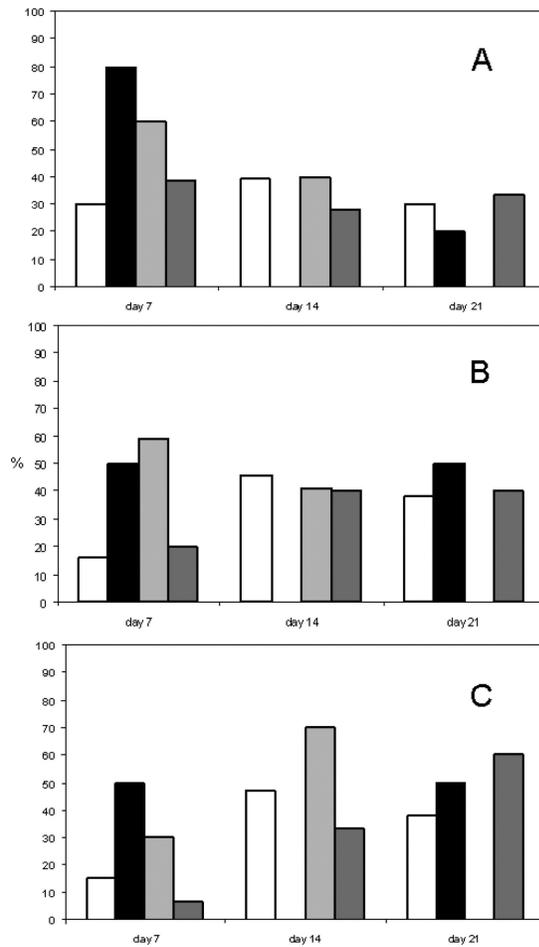


Fig. 4. Infection, dissemination, and transmission rates per day post-infection for mosquitoes orally exposed to West Nile virus genotype NY99 (Fresh Virus). D7, 14, and 21 rates are percentages of adults with infected bodies (A), infected mosquitoes with virus in legs (B), and infected mosquitoes with virus in saliva (C) for each mosquito species. □U.S. *Cx. quinquefasciatus*, ■AR *Cx. quinquefasciatus*, ■U.S. *Cx. pipiens* form molestus, ■AR *Cx. pipiens* form molestus

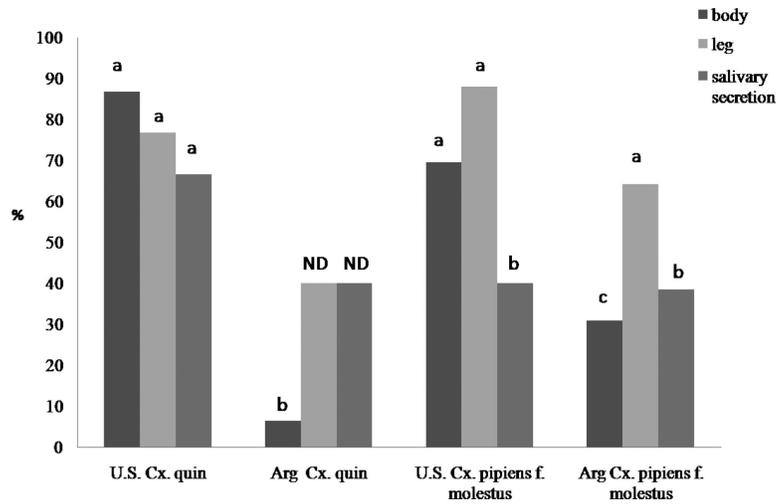


Fig. 5. Infection (body), dissemination (leg), and transmission (sal) rates for mosquitoes orally exposed to West Nile virus genotype NY99 (fresh virus) 21 d postinfection. Values followed by a common letter are not significantly different by χ^2 analysis ($P > 0.05$). U.S. *Cx. quin*, colonized *Cx. quinquefasciatus* from the United States; Arg *Cx. quin*, *Cx. quinquefasciatus* from Argentine population; U.S. *Cx. pipiens f. molestus*, colonized *Cx. pipiens* form molestus from United States; Arg *Cx. pipiens f. molestus*, *Cx. pipiens* form molestus from Argentine population.