Am. J. Trop. Med. Hyg., 71(1), 2004, pp. 112–119

PREVALENCE OF WEST NILE VIRUS IN TREE CANOPY–INHABITING *CULEX PIPIENS* AND ASSOCIATED MOSQUITOES

JOHN F. ANDERSON, THEODORE G. ANDREADIS, ANDY J. MAIN, AND DANIEL L. KLINE

Department of Entomology and Department of Soil and Water, The Connecticut Agricultural Experiment Station, New Haven, Connecticut; American University in Cairo, Cairo, Egypt; U.S. Department of Agriculture, Agriculture Research Service, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, Florida

Abstract. *Culex pipiens* was the dominant mosquito captured in a West Nile virus (WNV) focus in Stratford, Connecticut. More *Cx. pipiens* were captured in Centers for Disease Control miniature light traps baited with CO_2, quail/hamster traps, and mosquito magnet experimental (MMX) traps placed in the tree canopy than in similar traps placed near the ground. Significantly more *Cx. pipiens* were captured in MMX traps placed in the canopy than in the other traps tested. Ninety-two percent and 85% of the 206 and 68 WNV isolations were from *Cx. pipiens* in 2002 and 2003, respectively; 5% and 12% were from *Cx. salinarius*. Eighty-five percent and 87% of the isolates were from mosquitoes captured in the canopy in each of the two years. The significantly larger numbers of WNV isolates from *Cx. pipiens* captured in the canopy are attributed to the significantly larger numbers of *Cx. pipiens* captured in the canopy in comparison to those captured in traps near the ground.

INTRODUCTION

West Nile virus (WNV) was initially isolated in the New World from mosquitoes and birds in the greater New York City area in 1999.[1,2] Subsequently, the virus spread and was detected in 44 states and the District of Columbia in 2002.[3] A total of 3,389 human cases were reported. Species of *Culex* are considered to be the most important vectors, though WNV has been isolated or detected from >20 species of mosquitoes in the United States.[1,2,4–9]

Surveillance of arboviruses in mosquitoes is most frequently conducted with dry ice-baited Centers for Disease Control (CDC) miniature light traps placed relatively close to the ground,[10,11] although animal baited traps also have been used.[12] However, numerous species of mosquitoes are recognized to preferentially inhabit tree canopies and/or to fly at tree canopy height,[13–15] including *Culex pipiens*.[16–19] Vertical stratification may be influenced by humidity, temperature, light,[13] and possibly by availability of hosts.[20] *Culex pipiens*, a species that preferentially feeds on birds,[21–24] is an important and competent vector of WNV in both the Old and New Worlds.[25–29] The likely importance of this species in the natural history of WNV in the northeastern United States prompted us to evaluate the prevalence of WNV-infected *Cx. pipiens* and associated species at ground and tree canopy levels using three different types of mosquito traps in a known focus for WNV in Connecticut.

MATERIALS AND METHODS

Experiments were conducted on Water Pollution Control Authority land of the Town of Stratford, Connecticut. This site (41°10′41″N, 73°07′34″W) is located adjacent to the Housatonic River where it flows into Long Island Sound and was a focal area for WNV in 2001.[6] Trapping commenced on July 8, 2002 and continued until October 17, 2002; in 2003, collections were made from May 20 through November 8. Three different types of traps placed at two different heights were evaluated in 2002; two traps were tested in 2003. Traps were replicated three times each night and were placed in a randomized design. A trap was placed near the base of a tree ~1.5 meters above the ground (ground level), and another trap of the same design was placed in the tree canopy ~7.6 meters above the ground. Tree height was ~10.7 meters.

The three types of traps tested were a CDC trap (Model 512 with an aluminum dome; John W. Hock Co., Gainesville, FL),[10] a mosquito magnet experimental (MMX) trap, (American Biophysics Corp., East Greenwich, RI),[30] and live quail or hamster traps.[12] The CDC trap uses a motor-driven rotary fan to move mosquitoes attracted by a small light and CO_2 from dry ice stored in a container above the trap to a holding net suspended beneath the trap. The MMX trap is constructed of an ~11.4-liter clear polyvinyl chloride pretzel container with a fan blowing CO_2 out the bottom and another fan providing airflow into the bottom of the trap. Carbon dioxide was supplied from a 20-lb compressed gas cylinder with a flow rate of 500 mL/min.[30] The live quail or hamster trap was a lard can or a modification (hamsters were used in place of quail on two nights).[12] The modification was a can measuring 63.5 cm long with a diameter of 34.3 cm with a screen cone leading inward into the can from each end. The quail or hamster was placed in a side door in which the bait animal was protected with a screen from feeding mosquitoes. The trap was hung in a horizontal position from the tree. The entire trap was washed using soap to remove odors whenever a different type of animal was being used. The use of quail and hamsters conformed to the guidelines approved by The Connecticut Agricultural Experiment Station's Animal Care and Use Committee. Only the MMX and CDC traps were evaluated in 2003.

Traps were operated overnight and retrieved the following morning. Captured mosquitoes were knocked down with dry ice in the field, quickly aspirated and transferred into a flat-bottomed shell vial measuring 17 × 55 mm. The vial was sealed with a rubber stopper and the juncture of the vial and stopper was wrapped with three layers of 1.9-cm wide waterproof tape. The vial was labeled and stored on dry ice until taken to the laboratory where the vial was transferred to a −80°C freezer.

Mosquitoes were identified using the key of Darsie and Ward.[31] Specimens were placed on a cold table and identified with the aid of a dissecting microscope. Female mosquitoes were grouped according to species, date, type of trap, height, and location. Numbers of mosquitoes per pool ranged from 1 to 50. Mosquitoes were kept on regular ice until processed for viruses.

For attempted isolation of viruses, mosquitoes were tritu-

rated in a 2.0-mL centrifuge tube containing a copper BB pellet and 0.5–1.25 mL of phosphate-buffered saline with 0.5% gelatin, 30% rabbit serum, and 1% 100× antibiotic-antimycotic (10,000 units/mL of sodium penicillin G, 10,000 μg/mL of streptomycin sulfate, and 25 μg/mL of amphotericin B; Invitrogen, Carlsbad, CA) in 0.85% saline. Mosquitoes were milled for four minutes in a Vibration Mill MM 300 (Retsch Laboratory, Irvine, CA) set at 30 cycles per second placed inside a biosafety hood. Samples were centrifuged at 4°C for 10 minutes at 520 × *g*. A 100-μL inoculum from each sample was placed onto a 24-hour old monolayer of Vero cells growing in a 25-cm^2 flask at 37°C in an atmosphere of 5% CO_2. Sample inoculum was added to each flask after growth medium had been decanted. The flask was rocked for five minutes, new growth medium was added, and the flask was returned to the CO_2 incubator. Cells were examined daily for cytopathogenic effect 3–7 days following inoculation.

West Nile virus was identified by a TaqMan RT-PCR assay.[32] The RNA was extracted from a 70-μL sample of infectious Vero cell growth medium using the QIAamp viral RNA mini kit protocol (Qiagen, Valencia, CA). A negative control consisting of double-processed sterile water (Sigma, St. Louis, MO) was used. The positive control was a 1:100 dilution of WNV isolated from *Culiseta melanura* (8094-01) or from *Cx. pipiens* (9837-03) in Connecticut. Viral RNA, primers, and probe were added to reagents in the TaqMan RT-PCR Ready-Mix Kit (PE Applied Biosystems, Branchburg, NJ). A 25-μL reaction volume was prepared for each isolate using 2.5 μL of viral RNA, 0.25 μL of each primer, 0.15 μL of probe, 12.5 μL of 2× buffer, 0.5 μL of RT-PCR enzyme, and 8.85 μL of water. Primers and probes (Qiagen) are identified by their genome position. Primers were WNENV-forward 1160-1180 (5′-TCAGCGATCTCTCCACCAAAG-3′) and WNENV reverse 1229-1209 (5′-GGGTCAGCACGTTTGTCATTG-3′). The probe was WNENV 1186-1207 (5′-TGCCCGACCATGGGAGAAGCTC-3′) that had the 5′ end labeled with the 6-carboxy-fluorescein (FAM) reporter dye and the 3′ end labeled with the 6-carboxy-tetramethylrhodamine (TAMRA) quencher dye. All viral isolates identified as WNV by the previously mentioned primers and probes were subjected to testing by a second set of primers and probe for confirmation. The primers were WN3′NC-forward 10,668-10,684 (5′-CAGACCACGCTACGGCG-3′) and WN3′NC-reverse 10,770-10,756(5′-CTAGGGCCGCGTGGG-3′), and WN3′NC-probe 10,691-10,714 (5′-TCTGCGGAGAGTGCAGTCTGCGAT-3′). Amplification of each sample was carried out in a Smart Cycler that was run with Smart Cycler software (Cepheid, Sunnyvale, CA). Samples were subjected to one cycle of 50°C for 20 minutes, 95°C for 10 minutes, and then 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Specimens with a cycle threshold value of <37 by the testing of both sets of primers were considered to be WNV. Cycle threshold values for the positive controls diluted 1:100 ranged between 21 and 22.

The minimum infection rate (MIR) of specific species infected with WNV was determined for *Aedes cinereus, Cx. pipiens, Cx. restuans*, *Cx. salinarius*, *Ochlerotatus sollicitans*, and *Oc. trivittatus*.[33] The total number of mosquito specimens tested equaled the number of specimens collected during the time span when WNV was isolated from mosquitoes. Individual *Cx. pipiens* collected from September 3 through October 1, 2002 were tested for WNV to determine the actual field infection rate. To ensure consistency in determining numbers of isolations of pooled mosquitoes in 2002, the individually tested specimens of *Cx. pipiens* from each trap were grouped for MIR analysis into pools of ≤50 and recorded as 1 or 0.

The Berger-Parker equation was used to provide a relative measure of dominance of each species of mosquito captured.[34] The formula for this index was $d = N_i/N$ where d was the dominant species index, N_i was the number of specimens of a specific species, and N was the total number of mosquitoes for all captured species. The index number multiplied by 100 provides the percentage composition of the total for a specific species. Mosquito and WNV data were transformed to log 10 plus 1 prior to analysis using Systat 7 (SPSS, Inc., Chicago, IL). We used analysis of variance (ANOVA) to analyze significant differences among mean numbers of *Cx. pipiens* and *Cx. salinarius* captured at different heights and in different types of traps for each night and year and for combined years. Analysis of variance was performed to assess significant differences among numbers of WNV isolates from these two species of mosquitoes by trap type and height. The Tukey honestly significant difference multiple comparison test was used to examine significant differences among trap types. Yates' corrected chi-square analysis (Systat 7) was used to compare frequencies of WNV isolates from individual mosquitoes at ground and canopy levels.

RESULTS

Mosquito diversity, abundance, and seasonality. Mosquito diversity and species dominance for 2002 and 2003 are shown in Figures 1 and 2. A total of 28,936 mosquitoes, representing 25 species, were captured on 16 nights from July 8 to October 17, 2002 in CDC, MMX, and quail/hamster traps; in 2003, 64,275 mosquitoes, representing 28 species, were captured in CDC and MMX traps set for 23 nights from May 20 through November 8. The five co-dominant species were the same for the two years. These five species (*Cx. pipiens*, *Cx. salinarius*, *Ae. cinereus*, *Ae. vexans*, and *Oc. cantator*) represented 95.4% and 91.0% of the specimens captured in 2002 and 2003. *Culex pipiens* was the dominant species and represented 66.6% and 40.2% for 2002 and 2003, respectively. *Culex salinarius* comprised 12.6% and 17.3% of the populations in 2002 and 2003, respectively.

Seasonal abundance of the dominant mosquito, *Cx. pipiens*, is shown for 2002 and 2003 (Figures 3 and 4). *Culex pipiens* were most abundant on July 9, 2002, decreased, and again increased in late August and September (Figure 3). Numbers peaked on July 22, 2003 and remained relatively abundant through September 16 (Figure 4). The second most abundant *Culex*, *Cx. salinarius*, also was most numerous on July 9, 2002, decreased to near zero in mid August, then increased and remained relatively abundant (mean ≥11) from late August through the first week in October. In 2003, *Cx. salinarius* were more abundant than in the previous year. Their numbers peaked on July 8 and August 26, 2003. Thereafter they were relatively abundant through October 7, averaging more than 30 per trap.

Effect of trap type and location on mosquito collections. Significant differences by ANOVA were noted for mean numbers of *Cx. pipiens* captured by year ($F = 42.2$, degrees of freedom [df] = 1), trap type ($F = 13.0$, df = 2), and height ($F = 45.0$, df = 1). More *Cx. pipiens* were cap-

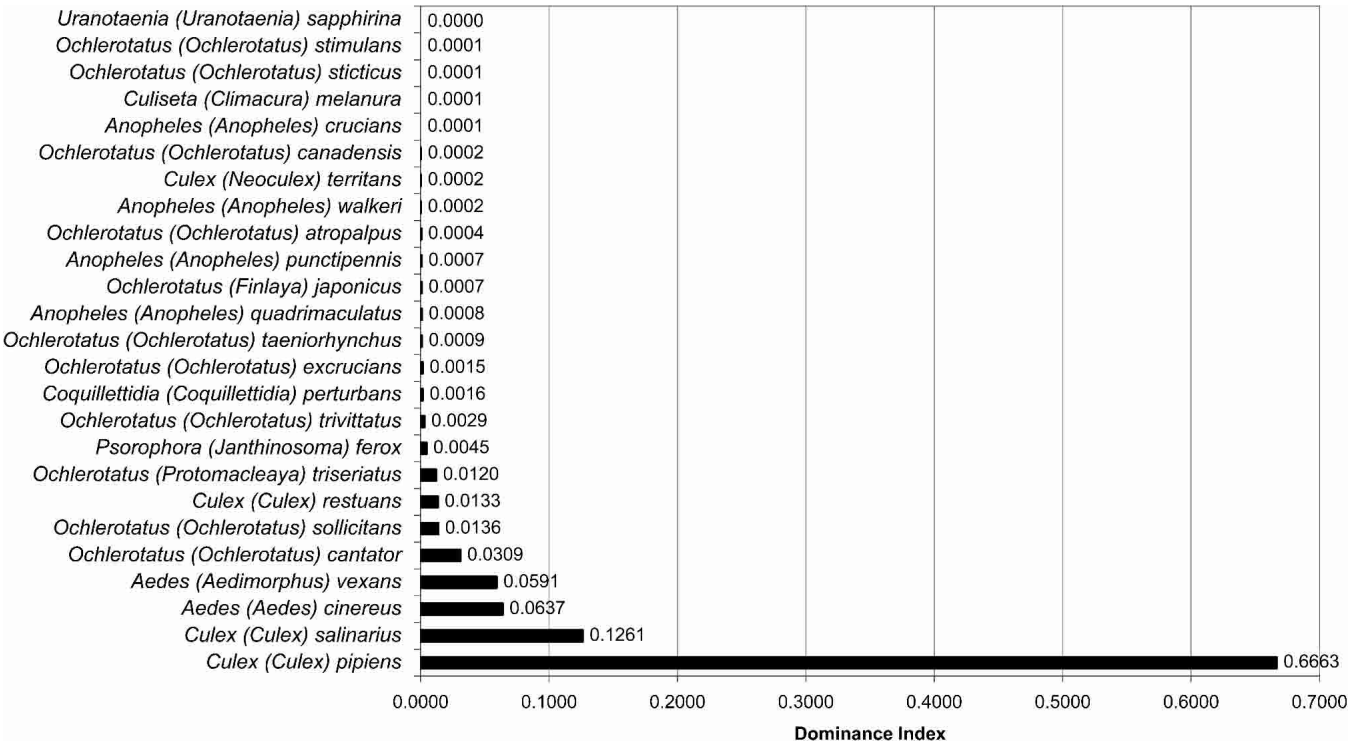

FIGURE 1. Dominant species index for adult female mosquitoes collected in Centers for Disease Control, mosquito magnet experimental, quail, and hamster traps in Stratford, Connecticut, 2002.

tured in traps placed in the canopy than in those placed near the ground (Table 1). Numbers of *Cx. pipiens* captured in quail and hamster traps were not significantly different from one another and were combined for ANOVA analysis. Significantly more *Cx. pipiens* were captured in the quail/hamster and MMX traps in the canopy than similar traps placed near the ground. The MMX traps captured significantly more *Cx. pipiens* than either the CDC or quail/hamster traps placed in the canopy. More *Cx. pipiens* were collected on 33 of 34 nights in canopy-placed MMX traps than in MMX

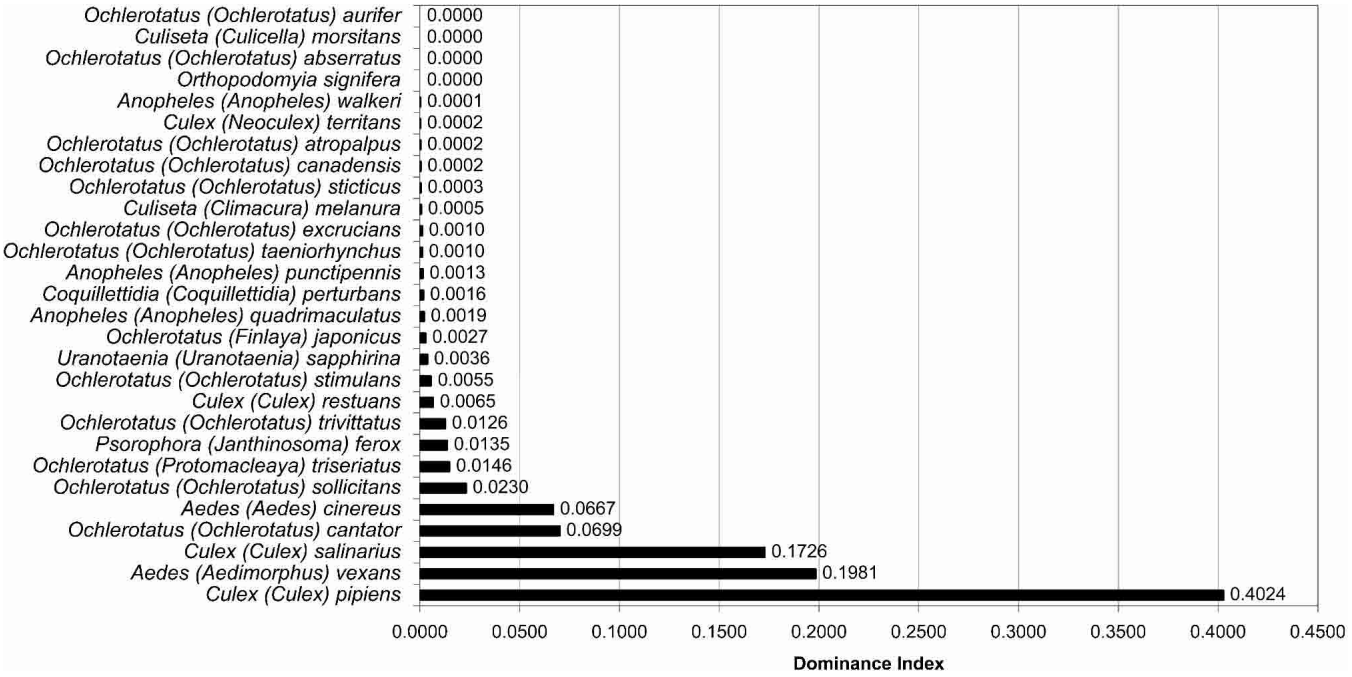

FIGURE 2. Dominant species index for adult female mosquitoes collected in Centers for Disease Control and mosquito magnet experimental traps in Stratford, Connecticut, 2003.

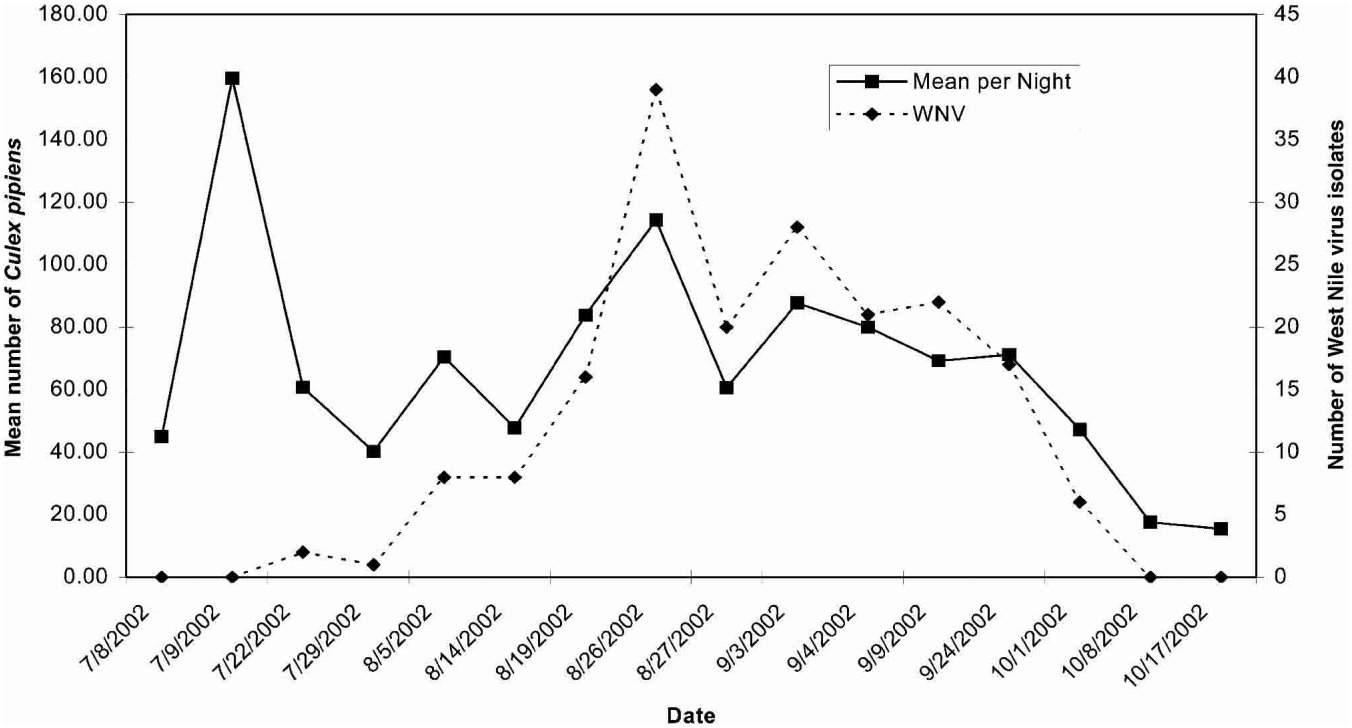

FIGURE 3. Mean numbers of *Culex pipiens* per trap per night and total West Nile virus (WNV) isolates by date, Stratford, Connecticut, 2002.

traps placed near the ground from July 8 through October 17, 2002 and from June 3 through October 27, 2003. Significant differences were recorded for 16 of the collections.

Significantly larger numbers of *Cx. salinarius* were captured in traps placed near the ground than in the canopy (Table 1). Numbers of females captured in quail/hamster traps were significantly less at ground and canopy levels than those recorded in the MMX and CDC traps, and CDC traps caught significantly fewer mosquitoes than MMX traps in the canopy (Table 1).

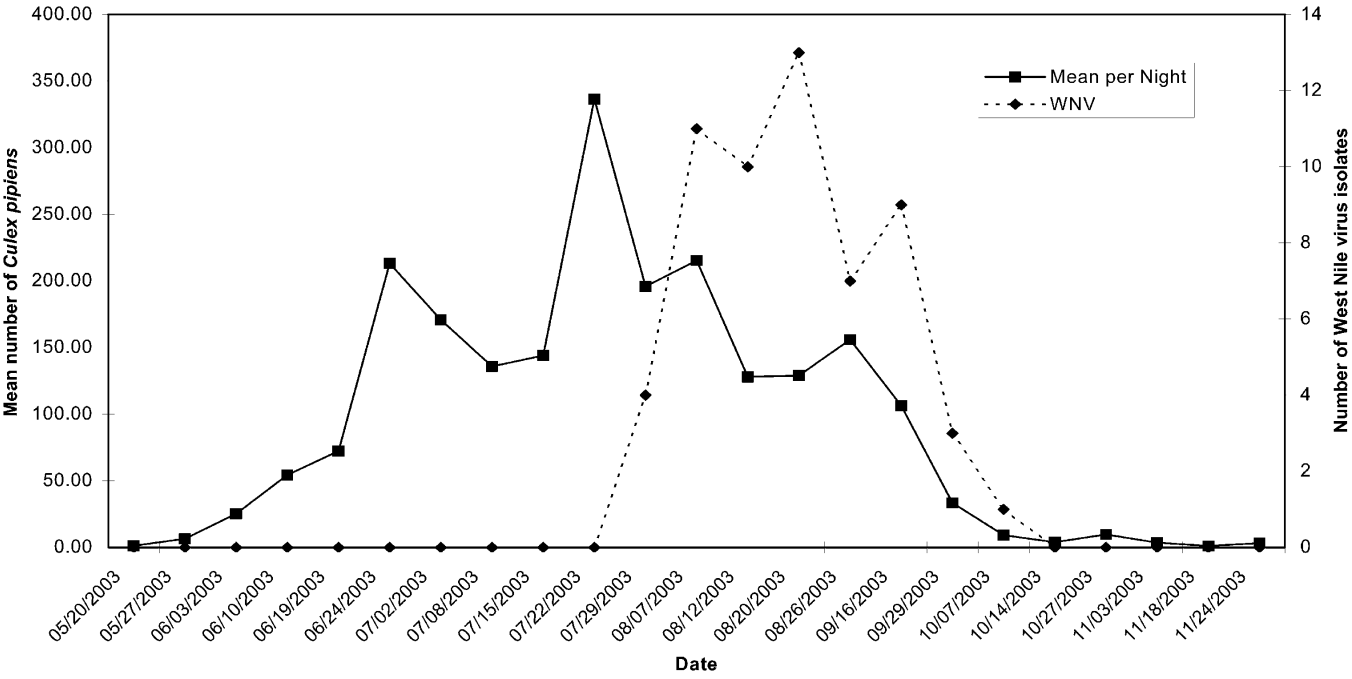

FIGURE 4. Mean numbers of *Culex pipiens* per trap per night and total West Nile virus (WNV) isolates by date, Stratford, Connecticut, 2003.

TABLE 1

Mean numbers of *Culex pipiens* and *Cx. salinarius* collected by type of trap and by height per night in Stratford, Connecticut for 2002 and 2003*

Species	Height	Trap type			
		Quail/hamster	CDC	MMX	Combined
Cx. pipiens	Ground	13.9^{aA}	23.4^{aA}	24.4^{aA}	22.2^{A}
	Canopy	32.7^{aB}	46.1^{aA}	273.0^{bB}	138.3^{B}
	Combined	23.3^{a}	34.8^{a}	148.7^{b}	
Cx. salinarius	Ground	0.5^{aA}	37.8^{bA}	48.7^{bA}	36.0^{A}
	Canopy	0.7^{aA}	14.2^{bB}	24.9^{cB}	16.0^{B}
	Combined	0.6^{a}	26.0^{b}	36.8^{b}	

* CDC = Centers for Disease Control; MMX = mosquito magnetic experimental.
Means within each row having the same lower case letter are not significantly different. Means within each column for a specific species having the same capital letter are not significantly different.

West Nile virus isolations. A total of 206 isolations of WNV were made from six species of mosquitoes in 2002 (Table 2). Sixty-eight isolations were made from four species in 2003. The cycle threshold values in the TaqMan RT-PCR assays of all isolates ranged from 10 to 18 with both sets of primers. Ninety-two and two-tenths percent and 85.3% of the isolations were from *Cx. pipiens* in 2002 and 2003, respectively. Five and three-tenths percent and 11.7% of the isolations were from *Cx. salinarius* in 2002 and 2003, respectively. Minimum infection rates for *Cx. pipiens* and *Cx. salinarius* were 18.4 and 5.0 in 2002 and 5.6 and 1.4 for 2003.

Significantly more isolations of WNV were made from *Cx. pipiens* captured in MMX traps than from *Cx. pipiens* captured in CDC and quail/hamster traps in 2002 and in CDC traps in 2003 (Table 3). West Nile virus–infected *Cx. pipiens* were identified from collections made from July 22 through October 1, 2002 (Figure 3). Eight or more isolations per night were made from August 5 through September 24. In 2003, WNV was initially cultured from *Cx. pipiens* collected on July 29 (Figure 4). Seven to 13 isolations per night were made between August 7 and September 16. A single isolation was made on October 7.

Isolations of WNV were made from *Cx salinarius* from August 19 through September 24, 2002 and from August 7 through October 7, 2003. More WNV isolates were made from *Cx. salinarius* captured in MMX traps than the other types of traps, but differences were not significant (Table 3). Infected *Cx. restuans* were obtained on August 26 and September 9, 2002 and on August 26, 2003. Vero cell cultures of WNV were made from *Oc. trivittatus* on September 24 and from *Oc. sollicitans* and *Ae. cinereus* on October 1, 2002. An isolate of WNV was obtained from *Ae. cinereus* on September 16, 2003.

Eighty-five percent of the isolates of WNV were made from mosquitoes captured in the canopy in 2002. Significantly more isolates of WNV were made from *Cx pipiens* and *Cx. salinarius* captured in traps placed in the tree canopy than in those obtained in traps near the ground (Table 4). Minimum infection rates were higher for mosquitoes captured in the canopy for both species. The single isolates of WNV from *Ae. cinereus*, *Oc. sollicitans*, and *Oc. trivittatus* and the two isolates from *Cx. restuans* were all made from mosquitoes captured in the canopy.

In 2003, 87% of the isolates were made from mosquitoes captured in the canopy. Significantly more isolations were made from *Cx. pipiens* captured in the canopy than from *Cx. pipiens* captured near the ground (Table 4). While more isolations were made from *Cx. salinarius* captured in the canopy than in traps placed near the ground, differences were not significant. *Culex pipiens* captured in the canopy had a similar MIR to those captured near the ground, but the MIR for *Cx. salinarius* captured in the canopy was higher than the MIR for *Cx. salinarius* captured near the ground. Single isolations from *Cx. restuans* and from *Ae cinereus* were from mosquitoes captured in the canopy and at ground level, respectively.

To determine actual rates of infection of *Cx. pipiens* with WNV in the canopy and near the ground, individual specimens (n = 1,081) collected on September 3, 4, 9, and 24, and October 1, 2002 at ground and canopy levels were tested for WNV. Twelve of 362 specimens collected at ground level were infected for an infection rate of 33 per 1000 mosquitoes. In the canopy, 39 of 668 specimens were infected for an infection rate of 58 per 1000 mosquitoes. Even though a larger proportion of canopy collected mosquitoes was infected, rates of infection at ground and canopy levels were not significantly different ($\chi^2 = 2.4$, df = 1).

TABLE 2

Number of West Nile virus isolations and minimum infection rates (MIRs) for six species of mosquitoes captured in Stratford, Connecticut, 2002 and 2003

Year	Species	No. of isolates	% of total isolates	MIR
2002	*Aedes cinereus*	1	0.5	0.7 (1,464)*
	Culex pipiens	190	92.2	18.4 (15,001)
	Culex restuans	2	1.0	25.4 (88)
	Culex salinarius	11	5.3	5.0 (2,268)
	Ochlerotatus sollicitans	1	0.5	2.5 (391)
	Ochlerotatus trivittatus	1	0.5	15.2 (57)
Total		206		
2003	*Aedes cinereus*	1	1.5	0.7 (1,448)†
	Culex pipiens	58	85.3	5.6 (11,730)
	Culex restuans	1	1.5	7.9 (127)
	Culex salinarius	8	11.7	1.4 (5,881)
Total		68		

* Total number of specimens tested from July 22, 2002 through October 1, 2002 is in parentheses.
† Total number of specimens tested from July 29, 2003 through October 7, 2003 is in parentheses.

DISCUSSION

West Nile virus is permanently established in the United States and will continue to be a recurring heath problem. This virus has been isolated from or detected in more than 25 species of mosquitoes, and while the importance of specific species in transmission is not clearly established, *Cx. pipiens* may be the most important enzootic vector in northeastern United States[1,2,6] and in temperate regions in the Old World.[27,29,35] *Culex pipiens* is known to feed on both mammals and birds, but in the more northern latitudes, it tends to feed on birds.[24] While it may feed preferentially on birds in northeastern United States, we easily established a laboratory colony that fed on guinea pigs (Anderson JF, Andreadis TG, unpublished data), and female *Cx. pipiens* were attracted to hamster-baited traps placed in the field. However, entry of mosquitoes into the trap without subsequent feeding does not necessarily identify the animal as a natural host.[36] Whether *Cx. pipiens* is both an enzootic and epidemic vector in northeastern United States is unknown. It is, however, the species

TABLE 3

Number of West Nile virus isolates and mininum infection rates for *Culex pipiens* and *Cx. salinarius* by trap type in Stratford, Connecticut for 2002 and 2003*

Year	Mosquito species	Trap type		
		CDC	Quail/hamster	MMX
2002	*Cx. pipiens*	52[a] (19.8) [3,924]	25[a] (18.3) [1,739]	113[b] (17.7) [9,338]
	Cx. salinarius	3[a] (3.1) [982]	2[a] (42.9) [45]	6[a] (5.0) [1,241]
2003	*Cx. pipiens*	8[a] (6.9) [1,249]		50[b] (5.4) [10,481]
	Cx. salinarius	1[a] (0.5) [2,069]		7[a] (1.9) [3,812]

* CDC = Centers for Disease Control; MMX = mosquito magnetic experimental.
Numbers within each row having the same letter are not significantly different. Minimum infection rate is in parentheses. Total number of specimens tested is in brackets.

from which most viral isolations have been made, the species from which WNV was isolated during winter,[37] and likely the most important mosquito in the natural history of this virus in northeastern United States.

Culex pipiens was the dominant species at this Stratford, Connecticut WNV focal area and the species from which 92% and 85% of the WNV isolates were made in 2002 and 2003, respectively. Peak numbers were collected at the beginning of our study in early July, 2002, but *Cx. pipiens* remained relatively abundant through September. West Nile virus was initially isolated on July 22, 2002, 13 days after *Cx. pipiens* reached its peak abundance, and was consistently isolated ($\geq$15 isolations per collecting date) from August 19 through September 24, 2002, a 36-day period when *Cx. pipiens* was relatively common. Infection rates during September 2002 at ground and canopy levels were relatively high at 33 and 58 per 1,000 specimens, respectively. *Culex salinarius* was the only other species from which a relatively large number of isolates was made. It was abundant in early July, but it was consistently collected in relative numbers in mid August through the end of September when mosquitoes of this species were infected. Minimum infection rates were highest for *Cx. restuans*, *Cx. pipiens*, and *Oc. trivittatus. Culex restuans* and *Oc. trivittatus* were captured in far fewer numbers than *Cx. pipiens* or *Cx. salinarius* during the transmission season. Their fewer numbers lessen their importance, but these species are a component of the population of vectors responsible for transferring WNV among host animals. *Culex restuans* feeds predominately on birds and is likely of some importance as an enzootic vector.[6] *Aedes cinereus*, *Oc. sollicitans*, and *Oc. trivittatus* readily feed on mammals[21–23] and could transmit the virus from birds to humans, horses, or other mammals. All species, with the exception of *Oc. trivittatus* and *Ae. cinereus,* have been shown to be susceptible and capable of transmission of WNV.[28,38]

TABLE 4

Total number of West Nile virus isolates and minimum infection rates for *Culex pipiens* and *Cx. salinarius* by trap height in Stratford, Connecticut, 2002 and 2003*

Year	Mosquito species	Trap height	
		Ground	Canopy
2002	*Cx. pipiens*	30[a] (10.6) [3,390]	160[b] (21.4) [11,611]
	Cx. salinarius	1[a] (0.7) [1,411]	10[b] (12.7) [857]
2003	*Cx. pipiens*	6[a] (5.3) [1,179]	52[b] (5.6) [10,551]
	Cx. salinarius	2[a] (0.5) [4,433]	6[a] (4.4) [1,448]

* Number within each row having the same letter are not significantly different. Minimum infection rate is in parentheses. Total number of specimens tested is in brackets.

Onset of disease in 2002 in the 17 confirmed WNV cases among Connecticut residents living within 75 km of our research site (Connecticut Department of Public Health, unpublished data) often occurred during the period of maximum WNV infection in *Cx. pipiens*. Illnesses were acquired from the third week of August into the first week of October. Twelve cases were acquired from August 23 through September 5, 2002, a 13-day interval when WNV was isolated from 20–39 pools of mosquitoes per collection date. *Culex salinarius* was also infected with WNV during this time. *Aedes cinereus*, *Oc. trivittatus*, and *Oc. sollicitans* were documented to be infected later in the season on September 24 through the first week of October. Similarly, in 2003, onset of illness of 9 of 15 WNV confirmed cases within 93 km of the Stratford, Connecticut experimental site occurred between August 19 and September 16 (Connecticut Department of Public Health, unpublished data) when 7–13 WNV isolates were made each night from *Cx. pipiens*. These relatively large numbers of WNV isolates from *Cx. pipiens* during onset of human illness may be indicative of active transmission to humans by this species. However, other species (i.e., *Cx. salinarius*) that more readily feed on humans were also infected with WNV during August and September and may be infecting humans. Certainly, these large numbers of infected *Cx. pipiens* are indicative of active transmission of virus among birds during August and September. More than 80% of the crows in 2002 tested positive for WNV from the first week of August through the first week of October (Connecticut Department of Public Health, unpublished data).

All three types of traps placed in the canopy captured more *Cx. pipiens* than similar traps placed near the ground. Differences were significant for MMX and quail/hamster traps but not for CDC traps. The capture of greater numbers of *Cx. pipiens* in all three traps when placed in the canopy suggests to us that relatively large numbers of *Cx. pipiens* inhabit the canopy. The suggestion has been made that canopy-placed CDC traps, which use light as an attractant, may attract mosquitoes to higher heights than they normally fly.[39] However, live animal traps and MMX traps that use only CO_2 as an attractant would not attract mosquitoes beyond their natural habitat.

Isolation of viruses from mosquitoes is dependent, in part, upon collection of sufficient numbers of enzootic, bridge, and epizootic species. Centers for Disease Control traps are commonly used for surveillance of mosquito-carried viruses.[10] However, unfed females represent the majority of mosquitoes attracted to CO_2 baited traps placed near the ground,[40–42] and their usefulness for detecting arboviruses may therefore be limited. However, our data clearly show that CO_2-baited

traps, particularly the MMX trap,[30] placed in tree canopies in a WNV focus captured significant numbers of infected *Cx. pipiens*.

The significantly larger numbers of WNV isolates made from *Cx. pipiens* captured in the canopy are attributed to the significantly larger numbers of *Cx. pipiens* captured in the canopy as compared with those captured in traps near the ground. The non-significant differences between the actual rates of infection in *Cx. pipiens* at canopy and ground levels support this conclusion. However, the actual infection rate and the MIR were higher in *Cx. pipiens* captured in the canopy in 2002 compared with those captured near the ground. The MIR in 2003 was similar for *Cx. pipiens* captured at both heights. The larger numbers of isolations of WNV from *Cx. salinarius* captured in the canopy were not a result of larger numbers of mosquitoes in the canopy. Fewer numbers of *Cx. salinarius* were captured in canopy-placed traps compared with traps placed near the ground, and the MIR was higher in canopy-captured mosquitoes in both years compared with mosquitoes captured near the ground. Our data confirm the reports of others that *Cx. pipiens* is prevalent in tree canopies,[16–19] but the frequent isolation of WNV from canopy-inhabiting mosquitoes is novel.

Eighty-five percent and 87% of WNV isolations were made from mosquitoes captured in trees in 2002 and 2003, respectively. Clearly, the placement of traps, particularly the MMX, in trees may be warranted for surveillance of WNV. Furthermore, mosquito control programs in northeastern United States designed to thwart an outbreak of WNV will need to recognize the relatively large numbers of infected and uninfected *Cx. pipiens* inhabiting tree canopies. For effective coverage, truck-mounted ultra low volume sprayers will need to disperse spray particles that cover space vertically from ground level to the tree canopy. Aerial application, which would deposit pesticides at both canopy and ground level, will likely be more effective.

Trivial flight movements of *Cx. pipiens*, that is flight that takes females away from their emergence site but not away from the local population,[20] have not been studied in any detail. Presumably, emerging females during summer take flight to mate, feed on both sugars and blood, find shelter, and lay eggs in suitable standing water. Flight of *Cx. pipiens* to tree canopies may account, in part, for the relatively large number of infected corvids, owls, and raptors. Both American and fish crows, (*Corvus brachyrhynchos* and *Coruns ossifragus*), raptors (i.e., red-tailed hawk [*Buteo jamaiceusis*], Cooper's hawk [*Accipiter cooperii*]), and great horned owls *(Bubo virginianus*) often lay their eggs in nests built relatively high in trees.[43] Many owls (i.e., barred owl [*Strix varia*] and Eastern screech owl [*Otus asio*]) lay their eggs in tree holes. Blue jays (*Cyanocitta cristata*) often build nests 9.1–13.9 meters off the ground. The nesting and roosting of birds in trees would seemingly enhance blood-feeding success of nighttime feeding female *Cx. pipiens* and facilitate the transfer of WNV among birds and mosquitoes.

Received January 9, 2004. Accepted for publication February 25, 2004.

Acknowledgments: We thank Michael Vasil, Terrill Goodman, Bonnie Hamid, Nicholanna Halladay, Amanda Rahmann, John Shepard, Michael Thomas, Elizabeth Alves, Laura Mickowski, Charles McGee, Susan McKnight, Lori Napolitano, Kathleen Blessing, Deborah Floch, and Kelli Jones for technical assistance. We also thank Elaine O'Keefe (Director of Health) and the Water Pollution Control Authority of Stratford, CT for enabling us to conduct this research on town property. American Biophysics Corp. (East Greenwich, RI) built the MMX trap for this study.

Financial support: The work was supported in part by Hatch Grant 763, by United States Department of Agriculture Specific Cooperative Agreement Number 58-6615-1-218, and by Laboratory Capacity for Infectious Diseases Cooperative Agreement Number U50/CCU116806-01-1 from the Centers for Disease Control and Prevention.

Authors' addresses: John F. Anderson, Department of Entomology, The Connecticut Agricultural Experiment Station, 123 Huntington Street, New Haven, CT 06511, Telephone 203-974-8440, Fax: 203-974-8502, E-mail: John.F.Anderson@po.state.ct.us. Theodore G. Andreadis, Department of Soil and Water, The Connecticut Agricultural Experiment Station, 123 Huntington Street, New Haven, CT 06511, Telephone: 203-974-8510, Fax: 203-974-8502, E-mail: Theodore.Andreadis@po.state.ct.us. Andy J. Main, American University in Cairo, 113 Kasr El-Aini, Cairo, Egypt, E-mail: andymain@aucegypt.edu. Daniel L. Kline, U.S. Department of Agriculture, Agriculture Research Service, Center for Medical, Agricultural and Veterinary Entomology, PO Box 14565, Gainesville, FL 32604, Telephone: 352-374-5855, Fax: 352-374-5933, E-mail: dkline@gainesville.usda.ufl.edu.

REFERENCES

1. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, Garmendia AE, van Kruiningen HJ, 1999. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. *Science 286:* 2331–2333.
2. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, Crise B, Volpe KE, Crabtree MB, Scherret JH, Hall RA, MacKenzie JS, Cropp CB, Panigrahy B, Ostlund E, Schmitt B, Malkinson M, Banet C, Weissman J, Komar N, Savage HM, Stone W, McNamara T, Gubler DJ, 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the Northeastern United States. *Science 286:* 2333–2337.
3. CDC, 2002. Provisional surveillance summary of the West Nile virus epidemic—United States, January-November 2002. *MMWR Morb Mortal Wkly Rep 51:* 1129–1133.
4. Nasci RS, White DJ, Stirling H, Oliver J, Daniels TJ, Falco RC, Campbell S, Crans WJ, Savage HM, Lanciotti RS, Moore CG, Godsey MS, Gottfried KL, Mitchell CJ, 2001. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. *Emerg Infect Dis 7:* 626–630.
5. White DJ, Kramer LD, Backenson PB, Lukacik G, Johnson G, Oliver J, Howard JJ, Means RG, Eidson M, Gotham I, Kulasekera V, Campbell S, The Arbovirus Research Laboratory, the Statewide West Nile Virus Response Teams, 2001. Mosquito surveillance and polymerase chain reaction detection of West Nile virus, New York State. *Emerg Infect Dis 7:* 643–649.
6. Andreadis TG, Anderson JF, Vossbrinck CF, 2001. Mosquito surveillance for West Nile virus in Connecticut, 2000: Isolation from *Culex pipiens, Cx. restuans, Cx. salinarius* and *Culiseta melanura. Emerg Infect Dis 7:* 670–674.
7. Bernard KA, Maffei JG, Jones SA, Kauffman EB, Ebel GD, Dupuis AP, Ngo KA, Nicholas DC, Young DM, Shi P-Y, Kulasekera VL, Eidson M, White DJ, Stone WB, Kramer LD, NY State West Nile Virus Surveillance Team, 2001. West Nile virus infection in birds and mosquitoes, New York State, 2000. *Emerg Infect Dis 7:* 679–685.
8. Kulasekera VL, Kramer L, Nasci RS, Mostashari F, Cherry B, Trock SC, Glaser C, Miller JR, 2001. West Nile virus infection in mosquitoes, birds, horses, and humans, Staten Island, NY, 2000. *Emerg Infect Dis 7:* 722–725.
9. Anderson JF, Vossbrinck CR, Andreadis TG, Iton A, Beckwith WH III, Mayo DR, 2001. A phylogenetic approach to following West Nile virus in Connecticut. *Proc Natl Acad Sci USA 98:* 12885–12889.
10. Sudia WD, Chamberlain RW, 1962. Battery-operated light tap, an improved model. *Mosq News 22:* 126–129.

11. Andreadis TG, Anderson JF, Tirrell-Peck SJ, 1998. Multiple isolations of eastern equine encephalitis and Highlands J viruses from mosquitoes (Diptera:Culicidae) during a 1996 epizootic in southeastern Connecticut. *J Med Entomol 35:* 296–302.
12. Bellamy RE, Reeves WC, 1952. A portable mosquito bait-trap. *Mosq News 12:* 256–258.
13. Bates M, 1949. *The Natural History of Mosquitoes*. New York: Macmillan Co.
14. Haddow AJ, Gillett JD, Highton RB, 1947. The mosquitoes of Bwamba County, Uganda, V. The vertical distribution and biting cycle of mosquitoes in rainforest, with further observations on microclimate. *Bull Entomol Res 37:* 301–330.
15. Reisen WK, Meyer RP, Tempelis CH, Spoehel JJ, 1990. Mosquito abundance and bionomics in residential communities in Orange and Los Angeles counties, California. *J Med Entomol 27:* 356–367.
16. Service MW, 1971. Flight periodicities and vertical distribution of *Aedes cantans* (Mg.), *Ae. geniculatus* (O1.), *Anopheles plumbeus* Steph. and *Culex pipiens* L. (Dipt., Culicidae) in southern England. *Bull Entomol Res 60:* 639–651.
17. Main AJ, Tonn RJ, Randall EJ, Anderson KS, 1966. Mosquito densities at heights of five and twenty-five feet in southeastern Massachusetts. *Mosq News 26:* 243–248.
18. Mitchell L, 1982. Time-segregated mosquito collections with a CDC miniature light trap. *Mosq News 42:* 12–18.
19. Novak RJ, Peloquin J, Rohrer W, 1981. Vertical distribution of adult mosquitoes (Diptera:Culicidae) in a northern deciduous forest in Indiana. *J Med Entomol 18:* 116–122.
20. Clements AN, 1999. *The Biology of Mosquitoes*. Volume 2. *Sensory Reception and Behaviour*. Oxon, United Kingdom: CABI Publishing.
21. Means RG, 1968. Host preferences of mosquitoes (Diptera:Culicidae) in Suffolk County, New York. *Ann Entomol Soc Am 61:* 116–120.
22. Tempelis CH, 1975. Host-feeding patterns of mosquitoes, with a review of advances in analysis of blood meals by serology. *J Med Entomol 11:* 635–653.
23. Magnarelli LA, 1977. Host feeding patterns of Connecticut mosquitoes (Diptera:Culicidae). *Am J Trop Med Hyg 26:* 547–552.
24. Spielman A, 2001. Structure and seasonality of Nearctic *Culex pipiens* populations. *Ann N Y Acad Scien 951:* 220–234.
25. Kitaoka M, 1950. Experimental transmission of the West Nile virus by the mosquito. *Jpn Med J 3:* 77–81.
26. Work TH, Hurlbut HS, Taylor RM, 1955. Indigenous wild birds of the Nile Delta as potential West Nile virus circulating reservoirs. *Am J Trop Med Hyg 4:* 872–888.
27. Hubalek Z, Halouzka J, 1999. West Nile fever—a reemerging mosquito-borne viral disease in Europe. *Emerg Infect Dis 5:* 643–650.
28. Turell MJ, O'Guinn ML, Oliver J, 2000. Potential for New York mosquitoes to transmit West Nile virus. *Am J Trop Med Hyg 62:* 413–414.
29. Hayes CG, 1988. West Nile viruses. Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Boca Raton, FL: CRC Press, Inc., 59–88.
30. Kline DL, 1999. Comparison of two American Biophysics mosquito traps: the professional and a new counterflow geometry trap. *J Am Mosq Control Assoc 15:* 276–282.
31. Darsie RF Jr, Ward RA, 1981. Identification and geographic distribution of mosquitoes of North America, north of Mexico. *Mosq Syst 1 (Suppl):* 1–313.
32. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, Komar N, Panella NA, Allen BC, Volpe KE, Davis BS, Roehrig JT, 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J Clin Microbiol 38:* 4066–4071.
33. Biggerstaff BJ, 2003. *PooledInfRate: A Microsoft® Excel Add-In to Compute Prevalence Estimates from Pooled Samples*. Fort Collins, CO: Centers for Disease Control and Prevention.
34. Magurran AE, 1988. *Ecological Diversity and Its Measurement*. Princeton, NJ: Princeton University Press.
35. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, Laiv L, Ungureanu A, Romanca C, Tsai TF, 1999. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. *Am J Trop Med Hyg 61:* 600–611.
36. Service MW, 1969. The use of traps in sampling mosquito populations. *Entomol Exp Appl 12:* 403–412.
37. Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey MS, Kerst AJ, Bennett P, Gottfried K, Lanciotti RS, 2001. West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. *Emerg Infect Dis 7:* 742–744.
38. Turell MJ, O'Guinn ML, Dohm B, Jones JW, 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. *J Med Entomol 38:* 130–134.
39. Service MW, 1993. *Mosquito Ecology: Field Sampling Methods*. Second Edition. London: Elsevier Applied Science.
40. Morris CD, DeFoliart GR, 1971. Parous rates in Wisconsin mosquito populations. *J Med Entomol 8:* 209–212.
41. Magnarelli LA, 1975. Relative abundance and parity of mosquitoes collected in dry-ice baited and unbaited CDC miniature light traps. *Mosq News 35:* 350–353.
42. Reisen WK, Pfuntner AR, 1987. Effectiveness of five methods for sampling adult *Culex* mosquitoes in rural and urban habitats in San Bernardino County, California. *J Am Mosq Control Assoc 3:* 601–606.
43. Pough RH, 1949. *Audubon Bird Guide: Eastern Land Birds*. Garden City, NY: Doubleday & Co.