SHORT COMMUNICATION

Detection of West Nile Viral RNA from an Overwintering Pool of *Culex pipens pipiens* (Diptera: Culicidae) in New Jersey, 2003

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ABSTRACT In total, 1,324 *Culex pipiens pipiens* L. female mosquitoes were collected at Ft. Hancock, Monmouth County, New Jersey, from January to March 2001–2003. Mosquitoes were held in an insectary at 27°C and a photoperiod of 16:8 (L:D) h for 6 to 21 d after which they were tested in 34 pools. West Nile viral RNA was detected in one pool by a TaqMan reverse transcription-polymerase chain reaction assay; however, infectious virus could not be isolated using either Vero cell plaque assay or C6/36 mosquito cells. Twenty females dissected in January and March 2003 confirmed ovarian diapause status. We suggest that the mode of infection in this pool of overwintering females may have been due to vertical (transgenerational) transmission.

KEY WORDS West Nile virus, *Culex pipiens pipiens*, overwintering, viral RNA detection

ALTHOUGH WEST NILE VIRUS (Flaviviridae, *Flavivirus*) (WNV) has been isolated from a variety of mosquito species in the United States, it is primarily maintained in an enzootic cycle between *Culex* mosquitoes and avian amplifying hosts (Komar 2003), much like its antigenically related New World counterpart St. Louis encephalitis virus (Flaviviridae, *Flavivirus*) (SLEV) (Mitchell et al. 1980). Surveillance efforts in the northeastern United States have shown that the majority of WNV-positive mosquito pools are derived from *Culex pipiens pipiens* L. and *Culex restuans* Theobald (Andreadis et al. 2001, Nasci et al. 2001a). Laboratory trials have documented the efficiency of *Culex* mosquitoes as competent horizontal and vertical vectors of WNV in North America (Turell et al. 2001, Goddard et al. 2002).

Because there is a clear affiliation between WNV and *Culex* mosquitoes during the warm season, one may expect the virus to persist in the primary vector population during the winter. However, numerous attempts at recovering flaviviruses from diapausing mosquitoes in the northeastern United States have resulted in disappointing outcomes, with only a few documented isolations on record. These include the isolation of SLEV from single pools of overwintering *Cx. p. pipiens* collected in Ft. Washington, MD, and Ft. Mifflin, PA (Bailey et al. 1978), and the detection of WNV in two pools of *Cx. p. pipiens* and one pool of unidentified *Cx.* species from Ft. Totten, NY (Nasci et al. 2001b). The difficulty in obtaining winter isolates of flaviviruses in mosquitoes may be because it is difficult to collect enough overwintering mosquitoes for virus isolation attempts (Rosen 1987) or because the virus is extremely temperature sensitive (Dohm and Turell 2001). It is interesting to note that all positive isolations in the past were recovered from mosquitoes hibernating inside abandoned quarters of former military forts where it is possible for researchers to collect large numbers of mosquitoes for winter isolation attempts. The isolation of SLEV was made from mosquitoes that were held in an insectary for >15 d and allowed to blood feed on a chicken (Bailey et al. 1978). Isolation of infectious WNV from Ft. Totten was obtained only after testing almost the entire volume of the original mosquito homogenate in cell culture (Nasci et al. 2001b).

The current study documents the infection of WNV in diapausing *Cx. p. pipiens* collected at an abandoned military fort in Monmouth County, New Jersey, during January 2003.

Materials and Methods

Collection Site and Specimen Collections. Overwintering mosquitoes were collected from January to March in 2001, 2002, and 2003 from Ft. Hancock (40° 4645′ N, 74° 0007′ W). The abandoned fort is located in Gateway National Park, situated on the Sandy Hook Peninsula of Monmouth County. The majority of mosquitoes were hand aspirated and placed on dry ice immediately for transport back to the laboratory. On three different occasions, a portion of the collections were kept alive, transported in small

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Table 1. Female *Cx. p. pipiens* mosquitoes collected during the overwintering period from Ft. Hancock, New Jersey, 2001–2003

Yr	No. collected	No. incubated (d)[a]	No. pools tested	RNA-positive WNV pools (%)[b]	MFIR[c] (95% CI)
2001	201	68 (6)	7	0	0
2002	341	211 (8)	9	0	0
2003	782	645 (15)	13	1 (0.16)	1.28 (0.07–6.06)
		137 (21)[d]	5	0	
Totals	1,324	1,061	34	1 (0.08)	0.76 (0.04–3.57)

[a] Mosquitoes were brought to the laboratory alive and kept at 27°C and a photoperiod of 16:8 (L:D) h.
[b] Tested by TaqMan RT-PCR.
[c] MFIR, minimum field infection rate per 1000 specimens tested.
[d] This group incubated for 15 d, allowed to blood feed, and further incubated for 6 d.

cages, and then placed into a larger cage with a 10% sucrose solution and held at a temperature of 27°C, with a photoperiod of 16:8 (L:D) h. Mosquitoes were held for 6, 8, and ≥15 d in 2001, 2002, and 2003, respectively. In conducting this research, we followed guidelines set forth in the Guide for the Care and Use of Laboratory Animals as approved by the Animal Use Committee of Rutgers University under protocol No. 86-129.

Virus Detection. Mosquito pools were initially tested at the New Jersey Department of Health and Senior Services (NJDHSS), Trenton, NJ, for West Nile viral RNA by a TaqMan reverse transcription-polymerase chain reaction (RT-PCR), by using previously established procedures (Lanciotti et al. 2000). Nucleic acid amplification and detection of West Nile viral RNA in the extracted material was done using an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) with TaqMan reagents. All samples were screened using primer/probe set WN3′NC (forward, 5′-CAGACCACGCTACGGCG-3′ and reverse, 5′-CTAGGGCCGCGTGGG-3′), and all screen positives were retested using a unique primer/probe combination WNENV (forward, 5′-TCAGCGATCTCTCCACCAAAG-3′ and reverse, [5′-GGGTCAGCACGTTTGTCATTG-3′) as reported and described by Lanciotti et al. (2000). No live virus isolation attempts were conducted on any of the samples at the NJDHSS laboratory.

Conformational testing of positive pools was conducted at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (CDC), Ft. Collins, CO, by using the above-mentioned methods with the exception of using a Bio-Rad iCycler (Bio-Rad, Hercules, CA) for real-time analysis. Positive pools were tested with a Vero cell plaque assay and also inoculation of C6/36 *Aedes albopictus* (Skuse) cells (Beaty et al. 1989) to isolate any infectious virus. In addition, a species-specific PCR assay was used to confirm the morphological identification of pooled mosquitoes from 2003 (Aspen et al. 2003).

Dissection Methods. Dissections were conducted under a stereomicroscope by using the ovariolar separation technique of Hoc and Schaub (1996). One ovary was immediately removed and allowed to air dry to be examined for parity by using Detinova's method of ovarian tracheation (Detinova 1962). The second ovary from each female was further teased apart to expose individual follicles and examined under 400× magnification by using phase contrast microscopy. Five ovarioles from each female were selected for examination and measurement. The developmental stage of the primary follicle was recorded using Christophers and Mer stages (Clements 2000). In addition, the lengths of the primary and secondary follicles were measured for five ovarioles by using an ocular micrometer, and a mean value was recorded. Measurements were used to calculate the ratio of the primary to secondary follicle for determination of ovarian diapause induction (Spielman and Wong 1973). Finally, one to three spermathecae were transferred to a drop of Hayes saline and examined at 200–400× magnification for presence of sperm.

Dissections were only conducted on mosquito collections from 2003. Two experiments were performed. In experiment 1, mosquitoes from the overwintering site were brought back to the laboratory alive and held for 15 d before being tested for presence of WNV. Ten mosquitoes were retained for dissections before the incubation period, and 10 were dissected post incubation period. Experiment 2 entailed collection and holding of mosquitoes once again for 15 d; however, they were then allowed to blood feed on a restrained Japanese quail and further held for 6 d to allow for bloodmeal digestion and oviposition. Ten mosquitoes were retained for dissections before incubation, 13 were dissected post incubation, and five were dissected post blood feeding and oviposition.

Results

In total, 1,324 diapausing *Cx. p. pipiens* were collected from Ft. Hancock during the winters of 2001–2003. West Nile viral RNA was detected by TaqMan RT-PCR in one pool of 50 *Cx. p. pipiens* collected on 18 January 2003 and incubated for 15 d (Table 1). The sample initially was tested at NJDHSS and exhibited a cycle threshold (CT) value of 26 for the WN3′NC primer/probe set. The sample then was reextracted and tested for WN3′NC and WNENV, resulting in CT values of 30 and 32, respectively. Confirmation testing at CDC resulted in CT values of 31 and 25 for WN3′NC and WNENV primer/probe sets in that order. Although the sample was positive by TaqMan RT-PCR at both agencies, no plaques were produced in Vero cells on several attempts. Inoculation onto C6/36 cells

Table 2. Ovarian follicle physiological stage, mean primary follicle size, and primary/secondary follicular ratio of *Cx. p. pipiens* females collected during the overwintering period from Ft. Hancock, New Jersey 2003

	Exp 1[a] (18 Jan. 2003)		Exp 2[b] (31 Mar. 2003)		
	Pre	Post	Pre	Post	Fed
No. collected	645			137	
No. dissected	10	10	10	13	5
Follicle stage	Ia	Ib–IIb	Ia	Ib–IIb	Ib–IIa
1° follicle length, μm (range)	48.3 (43.5–54.0)	103.7 (73.5–135.0)	49.9 (45.8–52.0)	73.3 (61.0–95.0)	72.9 (59.5–83.5)
1°/2° ratio[c] (range)	1.37 (1.30–1.50)	2.21 (1.77–3.05)	1.35 (1.23–1.47)	2.25 (2.00–2.61)	2.16 (1.89–2.34)

[a] Mosquitoes were brought to the laboratory alive and incubated at 27°C and a photoperiod of 16:8 (L:D) h for 15 d. Pre, before incubation; Post, postincubation.

[b] Mosquitoes incubated for 15 d, given a bloodmeal, and further incubated for 6 d to allow for oviposition. Fed, postblood feeding and oviposition.

[c] Length of the primary (1°) follicle divided by the length of the secondary (2°) follicle.

and RT-PCR testing of two aliquots yielded CT values between 37 and 40. This result was ascribed to detection of residual West Nile viral RNA from the original mosquito pool inoculum rather than being an indication of virus replication in the C6/36 cells. Overall, West Nile viral RNA was detected in 0.13% of the mosquitoes collected in 2003. No virus was detected in the samples collected in 2001 and 2002 (Table 1).

Dissections were performed on a small portion of mosquitoes collected during the 2003 overwintering period. In experiment 1, ten mosquitoes were dissected immediately after collection from the field. All exhibited 100% insemination and nulliparity and were determined to be in a state of ovarian diapause (Table 2). Ten mosquitoes also were dissected after the 15-d incubation period. These mosquitoes also exhibited 100% insemination and nulliparity; however, follicular morphometrics indicated that they had terminated ovarian diapause (Table 2).

In experiment 2, ten mosquitoes collected from overwintering sites were once again dissected before incubation. All mosquitoes were inseminated and nulliparous and were determined to be in ovarian diapause (Table 2). After a 15-d incubation period, 13 mosquitoes were dissected and were all found to be inseminated and nulliparous. Ovaries had resumed development, indicating the females had terminated diapause (Table 2). Five mosquitoes were examined 6 d after blood feeding and ovipositing and exhibited 100% insemination and parity (Table 2).

Twelve pools of mosquitoes from 2003 were tested by a PCR assay to confirm the morphological identification of specimens (Aspen et al. 2003). All samples morphologically identified as *Cx. p. pipiens* were verified as being *Cx. p. pipiens* by using molecular techniques.

Discussion

West Nile viral RNA was detected in *Cx. p. pipiens* during the winter at Ft. Hancock, supporting an earlier study in the northeastern United States where WNV was detected in three pools of *Cx. p. pipiens* taken from hibernaculae (Nasci et al. 2001b). Bailey et al. (1978) were successful in isolating the closely related SLEV from two pools of overwintering *Cx. p. pipiens* collected in Maryland and Pennsylvania. All three of the above-mentioned experiments subjected mosquitoes taken from hibernation to an incubation period ranging from 1 to 20 d before pooling for arbovirus isolation attempts. Numerous researchers have shown that environmental temperature has a direct effect on infection and transmission rates of flaviviruses in experimentally infected mosquitoes, with a general increase in both rates as the holding temperature increases (Takashi 1976, Reisen et al. 1993, Dohm et al. 2002a). Furthermore, Dohm and Turell (2001) have experimentally shown that incubation of mosquitoes at 26°C after exposure to lower overwintering temperatures greatly enhances detection of WNV in *Cx. p. pipiens.* In our study, we were successful in discovering evidence of WNV in overwintering mosquitoes during 2003 when mosquitoes were held in an insectary for a period of 15 d before testing.

Our failure to detect infectious virus from the West Nile viral RNA-positive pool remains a mystery. Although the CT values obtained by both diagnostic laboratories indicated that the sample was strongly positive (based on a 45 cycle amplification threshold) by TaqMan RT-PCR procedures, we were unsuccessful on several attempts to isolate live WNV. We cannot blame inactivation of virus on collection or storage, because a cold chain was maintained in all steps of the procedure, and the sample was thawed minimally for extraction purposes.

The small numbers of dissections performed on mosquitoes taken from hibernation during 2003 indicated that *Cx. p. pipiens* were in a state of ovarian diapause when collected from the field. Ovarian diapause is determined by the Christopher's stage of follicular development, the size of the primary follicle, and the size ratio between the primary and secondary follicle (Eldridge 1987). Although we dissected only a small portion of mosquitoes, we encountered only nullipars, those that had not taken a blood meal and successfully oviposited. Previously, Slaff and Crans (1977) showed that the majority, if not all, of overwintering *Cx. p. pipiens* mosquitoes in New Jersey are nulliparous. They suggested that small numbers of parous mosquitoes may enter the hibernaculae but are not likely to survive the overwintering period.

If overwintering mosquitoes are predominantly unfed and nulliparous, they would not have been exposed to an arbovirus through the horizontal transfer of the pathogen from a viremic vertebrate to a susceptible vector. However, an exception has been argued, citing the possibility of gonotrophic dissociation, the capability of some mosquitoes to use blood as an energy source rather than for egg development. Although this hypothesis has been experimentally validated for *Cx. p. pipiens* (Eldridge 1968, Eldridge and Bailey 1979, Bailey et al. 1982), it is a more common phenomenon in *Anopheles* species (Washino 1977). Some researchers also have argued against the occurrence of gonotrophic dissociation in wild *Culex* mosquitoes (Mitchell and Briegel 1989a). For example, Mitchell (1981, 1983) has indicated that host seeking and blood feeding are regulated independently as distinct steps in the life cycle of a mosquito and that the only means by which the host-seeking step may be bypassed is through the placement of a suitable host within proximity of the mosquito, an occurrence that is highly unlikely to occur in nature. Furthermore, Mitchell and Briegel (1989b) have experimentally shown that such force-fed *Cx. p. pipiens* females do not use blood for lipogenesis as previously thought. If gonotrophic dissociation is rare or nonexistent for *Cx. p. pipiens*, then the only other possible means by which a truly overwintering mosquito may become infected is through vertical transmission.

Vertical transmission, or transgenerational transmission, is the passage of a pathogen from an infected female to her offspring. Evidence for vertical transmission has steadily been growing over the past few decades and may provide a fitting explanation for overwintering infections in mosquitoes. For example, vertical transmission has been documented in the laboratory for WNV (Baqar et al. 1993, Turell et al. 2001, Dohm et al. 2002b, Goddard et al. 2003), and there is also field evidence of vertical transmission of WNV in Africa for *Culex univittatus* Theobald mosquitoes, the primary enzootic and epizootic vectors of the disease in that region (Miller et al. 2000). Although most studies advocate that vertical transmission rates generally range between 0.1 and 1.0%, Rosen (1987) reports that a considerable difference may occur in progeny of different females of the same species, approaching 20% or greater at times. Additionally, transmission rates vary considerably among different species (Rosen et al. 1989), different populations of the same species (as shown by Goddard et al. 2003 for WNV in two populations of *Cx. tarsalis* Coquillett in California), different viral strains (Rosen et al. 1989) and environmental conditions (Dohm et al. 2002b). As evidence for vertical transmission of flaviviruses continues to grow, it stands to reason that this mechanism may be an important factor in local trans-seasonal virus maintenance and that in North America, *Culex* mosquitoes may serve as the primary vector and reservoir of WNV.

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