JOURNAL OF THE AMERICAN MOSQUITO CONTROL ASSOCIATION

Mosquito News

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Journal of the American Mosquito Control Association, 26(3):257–264, 2010

STUDIES ON HIBERNATING POPULATIONS OF *CULEX PIPIENS* FROM A WEST NILE VIRUS ENDEMIC FOCUS IN NEW YORK CITY: PARITY RATES AND ISOLATION OF WEST NILE VIRUS

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ABSTRACT. A 3-year study was undertaken to examine the parity status, survival, and prevalence of West Nile virus (WNV) in overwintering populations of *Culex pipiens pipiens* collected from a hibernaculum located in a WNV endemic region in New York City. Nearly 6,000 females were collected from December through April. Parity rates were highest among females collected in December and January, ranging from 12.3% to 21.9%, depending on the year. In each year of the study, the proportion of parous females declined significantly during the course of the winter; the percentage of parous females found in April ranged from 0.9% to 10%. Results provide unequivocal evidence that parous *Cx. p. pipiens* females from this region of the northeastern US enter hibernacula in the fall in comparatively high proportions not previously recognized for this species, and while these females experience significant mortality during the winter, some survived to April to emerge in the spring. The absence of any detectible blood remnants in overwintering females reaffirms that blood feeding does not occur among diapausing females during the winter. The possibility that a portion of the diapausing population may be autogenous as a result of hybridization with sympatric belowground populations of *Cx. p. pipiens* "form molestus" is discussed. A single isolation of WNV was obtained in Vero cell culture from a pool of 50 females collected on January 11, 2007, representing an infection prevalence of 0.07% in the overwintering population in 2007 (n = 1,370 mosquitoes, 33 pools). No isolations of WNV were made from mosquitoes collected in 2008 (n = 1,870 mosquitoes, 190 pools) or 2009 (n = 1,767 mosquitoes, 184 pools). Findings provide further evidence for local overwintering of WNV in diapausing *Cx. p. pipiens*, albeit at very low rates, consistent with the paucity of WNV-positive mosquitoes detected in June and early July despite the emergence of females from hibernacula in early May in this region.

KEY WORDS *Culex pipiens*, parity rates, overwintering, hibernacula, New York City

INTRODUCTION

It is generally acknowledged that in cool-temperate regions of the world, aboveground populations of anautogenous *Culex pipiens pipiens* L. overwinter in natural and manmade shelters as nonblooded, nulliparous, inseminated females (Service 1969, Hayes 1973, Slaff and Crans 1977, Sulaiman and Service 1983, Jaenson 1987, Onyeka and Boreham 1987, Vinogradova 2000). Parous females enter hibernacula on occasion in the fall but purportedly experience high mortality and have not been shown to survive the winter (Slaff and Crans 1977, Jaenson 1987, Onyeka and Boreham 1987). Nevertheless, evidence of blood feeding in *Cx. p. pipiens* prior to hibernation and presumed overwintering was reported in 1 study (Jumars et al. 1969) but appears to be exceedingly rare.

Culex p. pipiens is also recognized as the primary vector of West Nile virus (WNV) in north temperate regions (Hubalek and Halouzka 1999, Hayes et al. 2005). Based upon the detection of virus in hibernating females (Nasci et al. 2001, Bugbee and Forte 2004, Farajollahi et al. 2005), this species is thought to serve as a natural overwintering host responsible for initiating transmission of the virus in the spring. The manner in which prehibernating females become infected with WNV in the fall before entering hibernacula, however, has not been entirely resolved. Given that the vast majority of females that enter diapause do not apparently take a blood meal (Eldridge 1987, Mitchell 1988), infection presumably occurs through vertical transmission of the virus from infected females to their progeny. Vertical transmission of WNV by *Cx. p. pipiens* has been demonstrated in the laboratory (Dohm et al. 2002, Goddard et al. 2003, Anderson et al. 2008) and by the isolation of virus from field-collected males, nulliparous females, and adults reared from field-collected larvae (Anderson and Main 2006, Anderson et al. 2006, Reisen et al. 2006). Anderson and Main (2006) have further documented horizontal transmission of WNV by a vertically infected female that had been in diapause for more than 5½ months, therein demonstrating that vertically infected *Cx. p. pipiens* females that enter diapause in the fall are able to initiate infection the following spring. The alternative mechanism, wherein older prehibernating females that had previously acquired an infectious blood meal undergo diapause and survive the winter, albeit unlikely, remains an open question requiring

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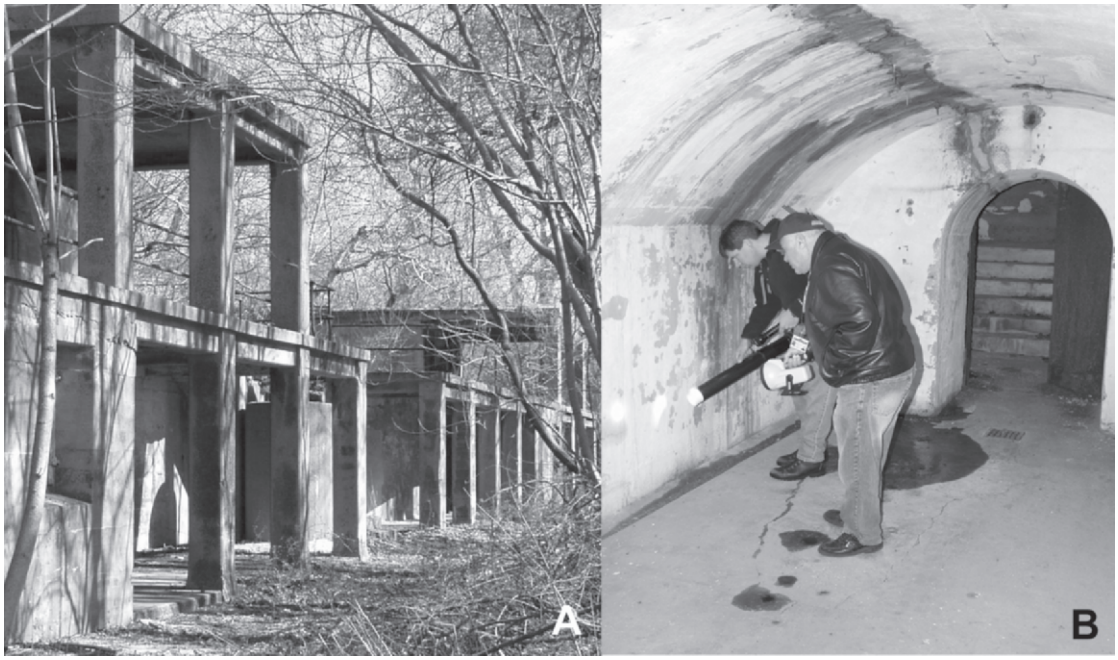

Fig. 1. *Culex pipiens pipiens* overwintering hibernaculum at historic Ft. Totten in Queens, New York City. (A) Outside entrance to concrete structure. (B) Primary collection site showing aspiration of resting mosquitoes from walls and ceilings.

unequivocal evidence of blood feeding and/or parity in overwintering females that emerge in the spring.

In January 2007, we initiated a 3-year study to examine the parity status, survival, and prevalence of WNV in overwintering populations of *Cx. p. pipiens* from a hibernaculum located in a WNV endemic region in Queens, New York City. The results of that investigation are reported herein.

MATERIALS AND METHODS

Study area and mosquito collection

Mosquitoes were collected over 3 successive winters from an abandoned concrete structure associated with the battery at historic Ft. Totten in Queens, New York (40°47′43″N, 73°41′46″W) (Fig. 1A). The site is located in the immediate vicinity where the initial outbreak of WNV occurred in 1999 (Asnis et al. 2000) and has remained a focal area for recurring virus activity ever since. Mosquitoes were collected on 2 occasions, January and April, during the 1st year (2007), and once a month from December through early April in the following winters, 2007–08 and 2008–09. Mosquitoes were located with a flashlight and were mostly found resting on visibly damp white plastered walls and gray concrete ceilings in several cavernous rooms (approximately 6 m long × 3.5 m wide × 3 m high) that were below ground level and farthest from the entrances to the structure (Fig. 1B).

Mosquitoes were collected from resting sites with a battery-powered backpack aspirator (model 1412, John W. Hock Company, Gainesville, FL) and a small handheld battery-powered aspirator (Hausher's Machine Works, Trenton, NJ), placed in small holding cages (15 cm × 22 cm × 26 cm), supplied with a 10% sucrose solution, and transported alive to the laboratory. In the laboratory, mosquitoes were transferred into larger cages (30.5 cm^3) covered with moist cotton batting and a plastic covering to maintain high humidity and supplied with a 10% sucrose solution. In an effort to enhance the likelihood of detecting live virus, caged mosquitoes were held at 24°C–26°C under natural photoperiod for a minimum of 6 days and a maximum of 29 days before dissection with virtually no mortality (Dohm and Turell 2001).

Mosquito identification and dissection methods

All mosquitoes were identified based on diagnostic morphology (Andreadis et al. 2005, Darsie and Ward 2005). Confirmation of the identity of *Cx. p. pipiens* as diapausing "form pipiens" rather than nondiapausing "form molestus" was determined through genetic analysis of a cohort of 50 females collected in January 2007 using 12 microsatellite markers (Huang et al. 2008).

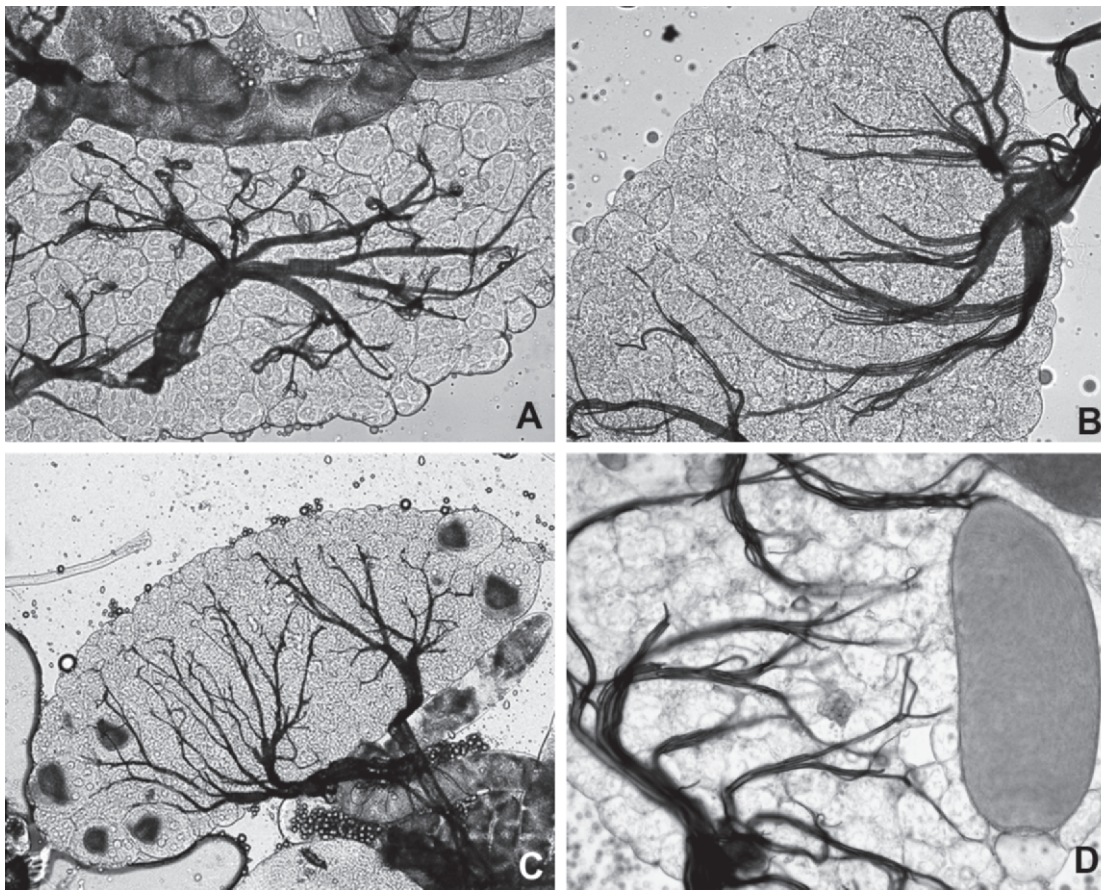

Fig. 2. Portions of ovaries from overwintering *Cx. pipiens pipiens*. (A) Nulliparous ovary with tightly coiled tracheolar skeins. (B) Parous ovary with uncoiled tracheolar skeins. (C) Parous ovary with stage-II follicles containing yolk. (D) Parous ovary with fully formed egg.

Ovarian dissections were conducted under a stereomicroscope with live females that were immobilized by chilling. The ovaries were removed from each female, placed in a drop of saline solution on a microscope slide, and examined with a Zeiss Axioplan 2 Imaging microscope. Parity status was determined by examining the terminations of the fine tracheoles covering the ovaries (Detinova 1962). The presence of tightly or slightly loosened coiled tracheolar ''skeins'' was considered an indication of nulliparity (Fig. 2A), while ovaries with stretched and uncoiled tracheoles were classified as parous (Fig. 2B). Individuals containing a few uncoiled tracheoles, but with most still coiled in skeins were designated as nullipars. The spermathecae were additionally examined for the presence of live sperm, and digital images of selected ovaries were recorded for documentation and further evaluation.

Mosquitoes were separated by parity status for virus isolation and testing. The thorax, head, and abdominal remnants were placed in 2-ml microcentrifuge tubes in pools of 50 or fewer individuals during the 1st year and 10 or fewer individuals thereafter, and immediately frozen at −80°C for later processing.

Differences in monthly parity rates were analyzed by chi-square analysis using Yates's correction for continuity (SPSS 2003).

Virus isolation and identification

A copper BB and 1–1.5 ml of PBS-G were added to each microcentrifuge tube, and mosquitoes were homogenized using a Vibration Mill MM300 (Retsch Laboratory, Irvine, CA) set at 25 cycles per second for 4 min. The mosquito homogenates were centrifuged at 4°C for 7 min at 520 × *g*, and 100 μl of the supernatant were added to a monolayer of confluent Vero cells growing in 25-cm^2 tissue-culture flasks. Cells were maintained at 37°C, 5% CO_2 and monitored daily for cytopathic effect from day 3 to day 7.

Table 1. Parity rates among overwintering *Culex pipiens* populations collected from a hibernaculum in Queens, New York, during the winters of 2006–07, 2007–08, and 2008–09.

	2006–07		2007–08		2008–09	
Collection month	No. examined	% parous[1]	No. examined	% parous[1]	No. examined	% parous[1]
December	—	—	584	21.4a	541	16.8a
January	513	12.3a	302	21.9a	364	8.0b
February	—	—	269	13.0b	303	8.9b
March	—	—	323	11.8b	435	8.4b
April	219	0.9b	300	10.0b	215	9.3b

[1] Parity rates within each column followed by a different letter are significantly different ($P < 0.01$, chi-square analysis using Yate's correction for continuity [SPSS 2003]).

Ribonucleic acid (RNA) was extracted from infected cell cultures using the viral RNA kit (Qiagen, Valencia, CA) and tested for WNV by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) assays (Lanciotti et al. 2000). All of the mosquitoes collected during the 2nd year of sampling (2007–08) were rescreened by molecular methods in addition to cell-culture assay. For this procedure, 70 µl of the mosquito homogenates were directly processed for RNA isolation and screened for WNV by real-time RT-PCR as previously described.

Meteorological data

Climatological data were obtained from National Oceanic and Atmospheric Administration–Climatological Data Center publications for New York (http://www7.ncdc.noaa.gov/IPS/cd/cd.html). Monthly average temperatures and deviations from normal were derived from the official recording station located at La Guardia airport, which was located approximately 8 km from Ft. Totten.

RESULTS

Mosquito collections and identification

In total, 5,955 overwintering mosquitoes were collected over the 3-year period from resting sites within the concrete hibernaculum at Ft. Totten. With the exception of 1 female *Uranotaenia sapphirina* (Osten Sacken) and another female *Anopheles quadrimaculatus* Say collected in December 2008 and February 2009, respectively, all specimens were identified as female *Cx. p. pipiens*. No males were detected, nor were any other resident *Culex* species found (*Culex erraticus* Dyar and Knab, *Culex restuans* Theobald, *Culex salinarius* Coquillett, or *Culex territans* Walker).

Identification of overwintering *Cx. p. pipiens* mosquitoes as diapausing "form pipiens" was confirmed through comparative microsatellite analysis of 50 females collected from the hibernaculum in January 2007. No significant differences were found with aboveground populations of *Cx. p. pipiens* that were collected from Connecticut, New Jersey, and Massachusetts from June through October. However, significant genetic differences were found with nondiapausing populations of *Cx. p. pipiens* "form molestus" that were similarly collected in January from the underground sewer system in Manhattan, New York City (Huang et al. 2008).

Parity status

Ovarian dissections were conducted on 4,368 *Cx. p. pipiens*, and both nulliparous and parous females were identified in each monthly collection (Table 1). Nulliparous females were readily identified by the presence of ovarian tracheoles with tightly coiled or slightly loosened skeins (nodules) at the tips. Egg follicles were observed in Dentinova's stage I or I–II (Fig. 2A). Tracheoles in parous females by contrast were unraveled and clearly arranged as straight or very loosely curled threads (Figs. 2B, C, D). Egg follicles were mostly in stage I or I–II, but in some females, stage-II follicles with yolk granules in the protoplasm of the oocytes were occasionally seen (Fig. 2C). More rarely, parous females with fully formed eggs in stage V were detected (Fig. 2D) (collected on January 11, 2007, April 3, 2007, January 6, 2009, February 27, 2009, April 22, 2009). Mature sperm cells were universally observed within the ruptured spermatheca of all dissected females, regardless of parity status, and none of the mosquitoes contained visible evidence of blood or blood remnants.

Parity rates were highest among females collected during the beginning of the winter (December and January) and ranged from 12.3% to 21.9% depending on the year (Table 1). In each year of the study, the proportion of parous females declined significantly ($P < 0.01$) during the course of the winter. The percentage of parous females found in April ranged from a low of 0.9% in 2007 to a high of 10% in 2008.

The decline and presumed mortality in the number of parous females found in 2007 were coincident with an abnormally cold month of February (−2.9°C below normal) following 4 straight months of higher than normal tempera-

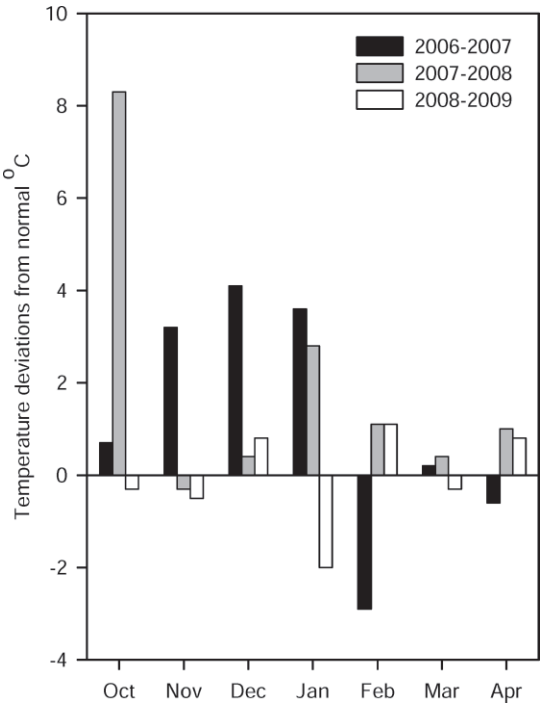

Fig. 3. Monthly mean temperature deviations from normal recorded at La Guardia Airport, Queens, New York.

tures (Fig. 3). Average monthly temperatures recorded during the 2007–08 season were mostly at or above normal, while the winter of 2008–09 was characterized by an abnormally cold month of January (−2.0°C below normal), which was coincident with a significant reduction in the number of parous females that were found during that month (16.8% to 8.0%).

Virus isolation and identification

In total, 3,722 (325 pools) nulliparous, 555 (59 pools) parous, and 730 (23) *Cx. p. pipiens* females of unknown parity status were assayed for virus growth in Vero cell culture (Table 2). No viral isolations were obtained from any females for which parity status had been previously determined, nor were any of the mosquitoes collected during the 2nd year of the study (December 18, 2007 through April 15, 2008) positive for WNV following direct testing of the pools by RT-PCR. However, 1 isolation of WNV in Vero cell culture was obtained from a pool of 50 females that were collected on January 11, 2007, but were not dissected and for which parity status was not determined. WNV infection was independently confirmed by re-isolation of the virus in cell culture and by direct testing of the positive mosquito pool by real-time RT-PCR, which yielded Ct values of 34.4 and 35.1 with 2 different WNV primer sets (Lanciotti et al. 2000).

DISCUSSION

With this investigation, we provide unequivocal evidence demonstrating that parous *Cx. p. pipiens* females from this region of the northeastern US enter hibernacula in the fall in comparatively high proportions not previously recognized for this species. We further document that while parous females experience significant mortality during the winter, as noted by others (Slaff and Crans 1997, Jaenson 1987, Onyeka and Boreham 1987), in certain years, some survive to April and presumably emerge in the spring. These findings run counter to the generally accepted view that only non-blood-fed nulliparous *Cx. p. pipiens* individuals undergo reproductive diapause and enter hibernation (Vinogradova 2000). However, they are consistent with the observations of Jumars et al. (1969), who reported successful overwintering of parous *Cx. p. pipiens* individuals from a similar abandoned concrete and brick bunker site located at Fort DuPont in Delaware City, Delaware (39°35′22″N, 75°34′03″W).

It is generally presumed that in Nearctic regions, diapausing populations of *Cx. p. pipiens* from northern latitudes are anautogenous and must acquire a blood meal to produce eggs. It is logical to infer then that parous females found in the overwintering population in the current investigation most likely blood fed prior to entering the hibernaculum in the fall, unless a portion of the population is autogenous. The absence of any detectible blood remnants in overwintering females reaffirms that blood feeding does not likely occur among diapausing females during the winter and early spring in the hibernacula (Mitchell 1988). Autogenous populations have been identified in North America (Richards 1941; Wray 1946; Rozeboom 1951; Spielman 1964, 1971; Kent et al. 2007; Huang et al. 2008; Mutebi and Savage 2009) but only among nondiapausing *Cx. p. pipiens* "form molestus," which are confined to enclosed spaces in urban subterranean habitats such as sewer systems and flooded basements. Populations of these 2 physiological biotypes are for the most part reproductively isolated due to differences in their breeding sites (Rozeboom and Gilford 1954; Spielman 1964, 2001). This view is consistent with comparative microsatellite analyses showing that the 2 biotypes are genetically distinct entities (Kent et al. 2007, Huang et al. 2008). However, evidence of "molestus" genetic ancestry among a small portion of the aboveground population (Fonseca et al. 2004, Kent et al. 2007, Kilpatrick et al. 2007, Huang et al. 2008, 2009), and the documentation of occasional episodes of inter-

Table 2. West Nile virus (WNV) isolation data from overwintering *Culex pipiens pipiens* populations collected from a hibernaculum in Ft. Totten, Queens, NY.

Collection date	No. days held at 24°C–26°C	No. mosquitoes and (pools) tested	Maximum pool size	Parity status	No. WNV-positive pools
Jan. 11, 2007	6–12	450 (9)	50	nulliparous	0
Jan. 11, 2007	6–12	63 (4)	23	parous	0
Jan. 11, 2007	27	638 (13)	50	undetermined	1
Apr. 3, 2007	29	217 (6)	50	nulliparous	0
Apr. 3, 2007	29	2 (1)	2	parous	0
Dec. 18, 2007	16–21	459 (46)	10	nulliparous	0
Dec. 18, 2007	16–21	125 (13)	10	parous	0
Jan. 23, 2008	13–15	236 (24)	10	nulliparous	0
Jan. 23, 2008	13–15	66 (6)	10	parous	0
Feb. 20, 2008	15	234 (24)	10	nulliparous	0
Feb. 20, 2008	15	35 (4)	10	parous	0
Mar. 18, 2008	16–21	285 (29)	10	nulliparous	0
Mar. 18, 2008	16–21	38 (4)	10	parous	0
Apr. 15, 2008	15	270 (27)	10	nulliparous	0
Apr. 15, 2008	15	30 (3)	10	parous	0
Apr. 15, 2008	15	92 (10)	10	undetermined	0
Dec. 16, 2008	21–23	450 (45)	10	nulliparous	0
Dec. 16, 2008	21–23	91 (10)	10	parous	0
Jan. 22, 2009	12–13	334 (34)	10	nulliparous	0
Jan. 22, 2009	12–13	29 (4)	10	parous	0
Feb. 26, 2009	12	276 (29)	10	nulliparous	0
Feb. 26, 2009	12	27 (4)	10	parous	0
Mar. 24, 2009	14–15	316 (32)	10	nulliparous	0
Mar. 24, 2009	14–15	29 (4)	10	parous	0
Apr. 21, 2009	14	195 (20)	10	nulliparous	0
Apr. 21, 2009	14	20 (2)	10	parous	0

breeding where sympatric populations coexist in the northeastern US (Spielman 1971, 2001) suggest some level of gene flow and possible hybridization between the 2 biotypes (Kent et al. 2007). The degree to which hybridization occurs where populations of these 2 biotypes are sympatric in nature and whether or not specific genes for autogeny may be expressed in aboveground populations are intriguing questions that remain to be explored. Autogeny has been reported in aboveground populations of *Cx. p. pipiens* from southern Europe (Gomes et al. 2009) and the Middle East (Nudelman et al. 1988).

Our isolation of live WNV from 1 pool of *Cx. p. pipiens* collected in January of 2007 represented an infection prevalence of approximately 0.07% (n = 1,370 mosquitoes, 33 pools) in the overwintering population during the 1st year. No isolations of WNV were made from mosquitoes collected in 2008 (n = 1,870 mosquitoes, 190 pools) or 2009 (n = 1,767 mosquitoes, 184 pools). The low prevalence of WNV infection found in 2007 and absence of detectable virus in overwintering populations in 2008 and 2009 were surprising in light of the levels of WNV activity detected in Queens County, New York City, during the active transmission season. Fourteen confirmed human cases and 129 WNV-positive mosquito pools from *Cx. p. pipiens* and *Cx. p. pipiens–Cx. restuans* (mixed) were documented from July through October, 2006–08 (CDC Epi-X Forum, Vector-Borne Diseases). However, the prevalence rate of WNV infection found in 2007 was comparable to rates observed in overwintering *Cx. p. pipiens* collected from the same site in New York City in 2000 (0.04% live virus, 0.1% PCR positive, n = 2,383 mosquitoes) (Nasci et al. 2001); New Jersey in 2001–03 (0.08% PCR positive, n = 1,324 mosquitoes) (Farajollahi et al. 2005); and Pennsylvania in 2003 (0.2% PCR positive, n = 501) (Bugbee and Forte 2004). It may be of significance to note that in all instances, including the present, the detection of virus was from overwintering mosquitoes collected in January or February.

Unfortunately, we are unable to shed any further insight into the mechanism(s) by which overwintering mosquitoes become infected with WNV in the fall, since the parity status of mosquitoes in the WNV-positive pool was not determined. Certainly, vertical transmission remains a highly plausible explanation, as demonstrated by Anderson and Main (2006), but the contribution of prehibernating parous females that acquired an infectious blood meal and survived the winter remains unresolved. In either event, our findings do provide additional evidence for local overwintering of WNV in diapausing *Cx. p. pipiens*, albeit at very low rates. This is consistent with the paucity of WNV-positive mosquitoes typically detected in June and early July (Andreadis et al. 2004), despite the

emergence of females from hibernacula in early May in this region (Farajollahi and Crans 2003).

ACKNOWLEDGMENTS

We wish to thank Michael Thomas, John Shepard, and Shannon Finan from The Connecticut Agricultural Experiment Station for technical assistance in collection, identification, and processing of mosquito specimens. We also wish to acknowledge Zahir Shah, Mohamed Soliman, and Varner DeMary from the New York City Department of Health & Mental Hygiene for assistance in collection of mosquitoes from Ft. Totten. This research was supported in part by grants to TGA from the Centers for Disease Control and Prevention (U50/CCU116806-01-1) and the US Department of Agriculture (58-6615-1-218, CONH00768, and CONH00773).

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