Journal of the American Mosquito Control Association, 17(2):137–141, 2001

SCIENTIFIC NOTE

OCHLEROTATUS J. JAPONICUS IN FREDERICK COUNTY, MARYLAND: DISCOVERY, DISTRIBUTION, AND VECTOR COMPETENCE FOR WEST NILE VIRUS[1]

MICHAEL R. SARDELIS[2] AND MICHAEL J. TURELL[3]

Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702-5011

ABSTRACT. *Ochlerotatus japonicus japonicus* is reported for the 1st time south of the Mason–Dixon Line, in Frederick County, Maryland. Fifty-seven oviposition trap samples were collected throughout the county between June 30 and August 24, 2000. From 971 larvae reared from the oviposition traps, 5 species were identified: *Ochlerotatus triseriatus* (45%), *Oc. j. japonicus* (43%), *Aedes albopictus* (7%), *Culex pipiens* (4%), and *Toxorynchites ritulus septentrionalis* (<1%). *Ochlerotatus j. japonicus* was found widely distributed over the area sampled. This is the 1st record of *Ae. albopictus* in the county as well. Vector competence studies indicated that *Oc. j. japonicus* is an efficient laboratory vector of West Nile (WN) virus. Depending on the viral titer at time of feeding, the estimated transmission rates for *Oc. j. japonicus* for WN virus were 2–4 times higher than that for *Cx. pipiens.* Studies of the viral titer in mosquitoes over time showed that titers in the bodies of infected *Oc. j. japonicus* reached their peak ($\sim 10^{6.5}$ plaque-forming units/mosquito) between 7 and 11 days after taking an infectious blood meal, and that virus became detectable in the legs (an indicator of disseminated infection) as early as 3 days after taking an infectious blood meal.

KEY WORDS *Ochlerotatus j. japonicus,* Maryland, distribution, West Nile virus, vector competence

The subspecies *Ochlerotatus japonicus japonicus* (Theobald) was reported for the 1st time in the USA in New Jersey and New York in the late summer of 1998 (Peyton et al. 1999). This mosquito has since been found in Connecticut (Andreadis, unpublished data), Ohio (Restifo, unpublished data), and Pennsylvania (Pagac, unpublished data). *Ochlerotatus japonicus* sensu lato (s.l.) is native to Japan, Korea, Taiwan, and southern China (Tanaka et al. 1979). Its distribution and bionomics in the USA are still largely unknown. This species breeds in natural and artificial containers and is generally found associated with wooded areas. Within its native range, *Oc. japonicus* s.l. is active primarily during the daytime (Tanaka et al. 1979). Little is known about the feeding preference of *Oc. japonicus* s.l. In Japan, it was reported to bite humans as well as birds (Tanaka et al. 1979). In the laboratory, *Oc. japonicus* s.l. readily fed on birds and mice (Miyagi 1971).

The public health importance of *Oc. j. japonicus* in the USA has not been studied in detail. Takashima and Rosen (1989) reported that this species was able to transmit Japanese encephalitis virus in the laboratory, and Turell et al. (2001) found a New Jersey strain of *Oc. j. japonicus* to be an efficient laboratory vector of West Nile (WN) virus. West Nile virus was detected in *Oc. j. japonicus* captured in New York in 2000 (Centers for Disease Control and Prevention 2000).

On June 8, 2000, mosquito larvae were collected from tires in an automobile salvage yard in Frederick, Frederick County, in western Maryland (39°23′33″N, 77°23′55″S). The tires (~70) were piled in a shaded area and the majority contained leaf litter. The entire contents of 8 tires were collected and taken to the laboratory, where the mosquito larvae were separated from the debris and reared to adults. Of the 687 specimens collected, 508 (74%) were *Oc. j. japonicus,* 165 (24%) were *Ochlerotatus triseriatus* (Say), and 14 (2%) were *Culex pipiens* L. The *Oc. j. japonicus* specimens were confirmed by taxonomists at the Walter Reed Biosystematics Unit (WRBU), Museum Support Center, Smithsonian Institution, Washington, DC, and voucher specimens were provided to WRBU. Specimens from this collection were subsequently used in a study of the population genetics of *Oc. j.*

[1] The views of the authors do not necessarily reflect the position of the Department of Defense or the Department of the Army. In conducting research using animals, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals,* as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication 86-23, revised 1996). The facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

[2] Division of Tropical Public Health, Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814.

[3] Department of Vector Assessment, Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick, MD 21702-5011.

Table 1. Summary of the oviposition trap survey done in Frederick County, Maryland, between June 30 and August 24, 2000.

Species or species combination	*n*	% of total	Ovitraps[1]	
			No. positive	% positive
Ochlerotatus triseriatus	437	45	25	45
Ochlerotatus j. japonicus	422	43	25	45
Aedes albopictus	68	7	6	11
Culex pipiens	43	4	1	2
Toxorhychites rutilus septentrionalis	1	<1	1	2
Total	971	100	44	80
Oc. triseriatus and *Oc. j. japonicus*	38 (54)[2]	39[3]	10	18
Ae. albopictus and *Oc. triseriatus*	28 (54)	3	2	4
Oc. j. japonicus and *Ae. albopictus*	13 (92)	1	1	2
Oc. j. japonicus, Oc. triseriatus, and *Ae. albopictus*	0	0	0	0

[1] Fifty-seven oviposition traps were set at 57 different sites.
[2] Percentage of the 1st species listed in the combination.
[3] Percent of total of species combinations calculated using the total for the entire collection (971).

japonicus (Fonseca et al. 2001). When the operator of the salvage yard was asked about the origin of the tires, he indicated that all the tires were removed from rims of cars in his salvage yard.

To survey the distribution of *Oc. j. japonicus* in Frederick County, Maryland, oviposition traps (Zeichner and Perich 1999) were set throughout the county between June 30 and August 24, 2000. Each ovitrap consisted of a black cup (473-ml capacity) filled with 250 ml of dechlorinated tap water. A velore ovistrip (25 × 11 mm) was affixed to the side of the cup by a paper clip to serve as the oviposition substrate. The traps were placed in sites that were at least partially shaded (e.g., the base of a tree) and just into the tree line of the road that was used to access the area. Seven days later, the traps were returned to the laboratory and checked for the presence of larvae or eggs. Larvae were transferred to containers containing dechlorinated tap water, provided ground catfish chow for nutrition, and reared at 26°C, 80–85% relative humidity, and 16:8 h light:dark photoperiod. Eggs on ovistrips were flooded with dechlorinated tap water on the day they were collected and the larvae were reared as described above. Voucher specimens, 4th-stage larvae, and adults were preserved in 80% ethanol or pinned for later identification. A subsample of the larvae was allowed to pupate, and adults were identified after emergence.

Mosquito eggs or larvae were collected from 80% (44 of 57) of the oviposition traps. A total of 971 mosquitoes was collected and identified. *Ochlerotatus triseriatus* and *Oc. j. japonicus* accounted for 88%, and *Aedes albopictus* (Skuse), *Cx. pipiens,* and *Toxorynchites rutulus septentrionalis* (Dyar and Knab) accounted for the other 12% of these specimens (Table 1). The location of oviposition traps in our study is shown in Fig. 1. *Ochlerotatus j. japonicus* and *Oc. triseriatus* were collected across the entire sampling area. In contrast, *Ae. albopictus* was collected in the vicinity of the city of Frederick and the southern border towns of Point of Rocks and Brunswick.

Because of recent interest in WN virus and the need to elucidate the role newly invasive mosquito species may play in the epidemiology of WN virus in the eastern USA, we conducted laboratory studies of the vector competence of Maryland-collected *Oc. j. japonicus* for WN virus. Additionally, a study was done to evaluate viral replication and dissemination in these mosquitoes over time.

The *Oc. j. japonicus* used in the vector studies were reared from larvae collected at the original discovery site and from eggs collected during the countywide ovitrapping. The immature stages of mosquitoes were handled and reared as described above. Four- to 10-day-old adult mosquitoes were used in the susceptibility, transmission, or viral growth studies.

The WN virus strain (Crow 397–99) used was isolated from a dead crow found in Bronx, NY, during an epizootic in 1999 (Turell et al. 2000) and had been passaged once in Vero cell culture. Viral stock suspensions, triturated mosquito suspensions, and chicken blood samples were tested for infectious virus by plaque assay on Vero cells as described by Gargan et al. (1983), except that the 2nd overlay, containing neutral red stain, was added 2 days after the 1st overlay.

One-day-old chickens (*Gallus gallus*) were inoculated subcutaneously with 0.1 ml of a suspension containing $10^{4.2}$ plaque-forming units (PFU) of WN virus and mosquitoes were allowed to feed on them 1 or 2 days later. Immediately after mosquito feeding, a 0.1-ml blood sample was obtained from the jugular vein of each chicken and diluted in 0.9 ml of diluent (10% heat-inactivated fetal bovine serum in medium 199 with Earl's salts, $NaHCO_3$, and antibiotics) plus 10 units of heparin per milliliter to determine the viremia at the time of mosquito feeding. Engorged mosquitoes were transferred to 3.8-liter cardboard cartons with netting over the open

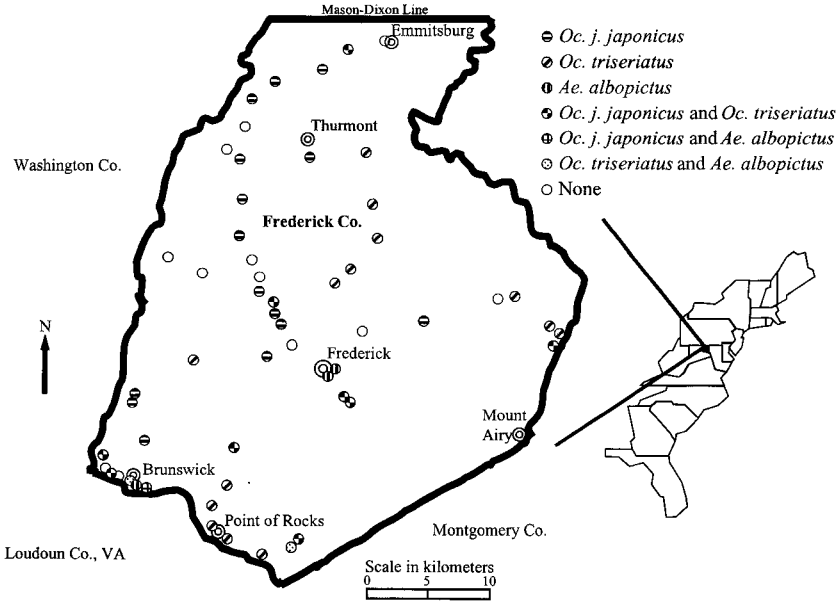

Fig. 1. Map of Frederick County, Maryland, showing locations of oviposition traps and *Ochlerotatus* and *Aedes* mosquitoes captured at each site; based on a single survey using 57 oviposition traps between June 30 and August 26, 2000.

end and maintained at 26°C. Four days after the infectious blood meal, an oviposition substrate was added to each cage. After 12–14 days, the mosquitoes were killed and their legs and bodies were triturated separately in 1 ml of diluent and frozen at −70°C until assayed for virus. Presence of virus in a mosquito's body indicated infection, whereas virus in the legs indicated that the mosquito had a disseminated infection (Turell et al. 1984).

To determine transmission rates, some of the mosquitoes that had taken an infectious blood meal were individually allowed to refeed on a 1- to 2-day-old chicken 12 or 13 days after the initial infectious blood meal. In addition to the orally exposed mosquitoes, mosquitoes intrathoracically inoculated (Rosen and Gubler 1974) 6–8 days previously with 0.3 μl of a suspension containing $10^{4.2}$ PFU of WN virus/ml were allowed to feed on individual chickens. The presence of virus in the blood of a chicken 24–48 h after mosquito feeding was used to indicate viral transmission. The proportion of mosquitoes with a disseminated infection that transmitted virus by bite (T(d)) was determined for orally exposed and inoculated mosquitoes. These percentages were used to calculate an overall T(d) percentage, which was then multiplied by the dissemination rate to obtain an estimated transmission rate.

To evaluate viral growth and dissemination over time, mosquitoes were fed on a viremic chicken and held at 26°C. Samples of 3–5 mosquitoes were assayed, leg and bodies separately, for virus immediately after the infectious blood meal and on days 1, 3, 5, 7, 11–12, and 14 after blood feeding.

Ochlerotatus j. japonicus was susceptible to infection with WN virus at both of the viral titers tested (Table 2). Data for *Cx. pipiens,* the suspected vector in New York (Centers for Disease Control and Prevention 1999), is included for comparison. The proportion of *Oc. j. japonicus* infected with WN virus significantly increased with the viral titer of the blood meal ($\chi^2 = 10.5$, df = 1, $P = 0.001$), whereas for *Cx. pipiens,* the titer of the blood meal did not significantly affect the rate of infection (Fisher's exact test, $P = 0.544$). *Ochlerotatus j. japonicus* was significantly less susceptible to oral infection than was *Cx. pipiens* at the low titer range (Fisher's exact test, $P = 0.038$); however, at the high titer range, no significant difference was found in oral susceptibility to infection between the 2 species ($\chi^2 = 0.0$, df = 1, $P = 0.996$). The proportion of *Oc. j. japonicus* developing a disseminated infection was significantly higher than that of *Cx. pipiens* at each of the viral titers tested ($\chi^2 = 6.3$, df = 1, $P = 0.012$ and $\chi^2 = 44.8$, df = 1, $P < 0.001$ at the low and high titer ranges, respectively).

Nearly all (97% or 29 of 30) *Oc. j. japonicus* with a disseminated infection transmitted WN virus by bite. This included 10 of 11 orally exposed and 19 of 19 inoculated individuals. Thus, route of infection did not significantly affect transmission rates (Fisher's exact test, $P = 0.367$), and the overall T(d) percentage for *Oc. j. japonicus* in our study, 97%, was virtually identical to the 100% (6

Table 2. Oral susceptibility to and transmission of West Nile virus by a Maryland strain of *Ochlerotatus j. japonicus*.

Species	Virus titer at time of feeding[1] (log_{10} PFU/ml)	No. tested	Infection rate[2]	Dissemination rate[3]	Estimated transmission rate[4]
Oc. j. japonicus	6.0 ± 0.5	92	57	56	54
	7.0 ± 0.4	83	80	77	75
Culex pipiens[5]	6.0 ± 0.5	17	82	23	20
	7.0 ± 0.4	78	79	24	21

[1] Titer in chickens (*Gallus gallus*) inoculated 24 or 48 h previously with a New York stain of West Nile virus (Crow 397-99) (PFU, plaque-forming units).

[2] Percentage of mosquitos with virus in their body.

[3] Percentage of mosquitos with virus in their legs.

[4] An estimate of the percentage of mosquitos that will transmit virus by bite 12–13 days after an infectious blood meal and storage at 26°C, calculated by multiplying percent dissemination times the percentage of mosquitoes with disseminated virus that transmitted virus [T(d)]. Overall T(d) percentages were previously determined to be 97% for *Oc. j. japonicus* and 88% for *Cx. pipiens*.

[5] *Culex pipiens* data at the lower titer range from D. Dohm (unpublished data) and at the higher titer range from Turell et al. (2001).

of 6) rate reported for New Jersey *Oc. j. japonicus* by Turell et al. (in press).

Based on the proportion of mosquitoes that developed a disseminated infection and the overall T(d) percentage, *Oc. j. japonicus* was more efficient at transmitting WN virus than was *Cx. pipiens*: more than 2 times more efficient at the lower viral dose ($10^{6.0\pm0.5}$), and nearly 4 times more efficient at transmitting virus at the higher viral dose ($10^{7.0\pm0.4}$; Table 2).

For mosquitoes that fed on chickens with a mean viremia level of $10^{6.5}$ PFU/ml of blood, viral titers in both species generally increased from day 3 to 11, with titers reaching nearly 10^7 PFU per body for *Oc. j. japonicus* and approximately $10^{6.5}$ PFU per body for *Cx. pipiens* (Table 3). Disseminated infections were detected 3 days after the infectious blood meal in *Oc. j. japonicus,* but not until days 11–12 in *Cx. pipiens* (Table 3).

This is the 1st report of *Oc. j. japonicus* south of the Mason–Dixon Line and it indicates that this species' range in the USA is expanding. Previous published reports and recent informal reports indicate that it is also found in New York, New Jersey, Connecticut, Pennsylvania, and Ohio. Although *Oc. j. japonicus* is generally described as a northern-climate species within its native range in Japan, it has been reported as far south as 33°N, in Chejudo Island, Republic of Korea (Tanaka et al. 1979). Thus, based solely on climatic information, *Oc. j. japonicus* may expand its range as far south as Jacksonville, FL, in the USA.

Given its distribution within Frederick County and its abundance relative to *Oc. triseriatus,* the introduction of *Oc. j. japonicus* apparently occurred before this year's discovery. Surveys in Connecticut looking specifically for *Oc. j. japonicus* found it to be widespread and breeding in areas away from tire dumps (Andreadis, unpublished data). *Ochlerotatus j. japonicus* has been present in Connecticut for between 2 and 11 years, based on the reevaluation of adult collections from recent years and from a 1989 survey of tire-breeding mosquitoes (Andreadis 1989). Thus, based on the data from this and the Connecticut studies, *Oc. j. japonicus* has been in Frederick County, Maryland, for at least a few years. Analysis of the data from our study also seems to suggest that local expansion of the range of *Oc. j. japonicus* is not driven only by the movement of infested, used automobile tires.

Table 3. Viral titers[1] over time in the bodies and legs of a Maryland strain of *Ochlerotatus j. japonicus* after oral exposure to a West Nile virus–infected chicken with a viremia of $10^{6.5}$ plaque-forming units (PFU)/ml of blood.

Species	Part assayed	Days after oral exposure: 0	1	3	5	7	11–12	14
Oc. j. japonicus	Body	3.1–4.0 (3/3)	0[2]–3.1 (1/3)	3.8–4.7 (3/3)	4.2–4.9 (3/3)	4.2–6.9 (3/3)	6.0–7.0 (5/5)	6.0–6.8 (3/3)
	Leg	0 (0/3)	0 (0/3)	0–2.7 (2/3)	2.1–2.9 (3/3)	0–5.7 (2/3)	3.7–5.4 (5/5)	4.1–5.3 (3/3)
Cx. pipiens[3]	Body	3.9–4.0 (3/3)	NT	3.0–4.0 (3/3)	3.6–4.2 (3/3)	3.9–4.3 (3/3)	0–6.6 (5/6)	2.6–6.4 (6/6)
	Leg	0 (0/3)	NT	0 (0/3)	0 (0/3)	0 (0/3)	0–4.2 (2/6)	0–4.5 (3/6)

[1] Log_{10} PFU/ml of body or leg suspension. Range (number of mosquitoes with virus in the respective part assayed/number assayed) NT, not test.

[2] A viral titer of zero (0) indicates that virus was not present or that the viral titer was below the detection limit of the assay.

[3] *Culex pipiens* data from D. Dohm (unpublished data).

The distribution of *Oc. j. japonicus* seemed to be associated with *Oc. triseriatus.* Seventeen percent of the ovitraps contained both species (Table 1) and the distribution of positive ovitraps for either species were interwoven (Fig. 1). This overlap of the 2 species would be likely, given that they both breed in containers. In the USA, *Oc. j. japonicus* has been collected in a broad variety of container types, such as tin cans, water dishes for potted plants, concrete rainwater drainage forms, buckets, pans, and 3.8-liter milk jugs (Sardelis, unpublished data). Thus, the likelihood for this species to come in contact with humans may be similar to that of *Oc. triseriatus.* To date, no studies have been published on biting preference of *Oc. j. japonicus* in the USA. Whether some type of competition will occur between these 2 species for breeding sites and survival also remains to be shown.

Although the focus of this study was not on *Ae. albopictus,* some items of information regarding this species are noteworthy. This is the 1st record of *Ae. albopictus* in Frederick County, Maryland. The collection of *Ae. albopictus* in the city of Frederick and in 2 other small towns in the county indicates that potential exists for this species to become a nuisance to residents.

This study showed that the Maryland strain of *Oc. j. japonicus* has the potential to serve as a WN viral vector, based on its susceptibility to infection, and its ability to transmit WN virus efficiently. These data are in agreement with a similar study by Turell et al. (2000), who studied a New Jersey strain of *Oc. j. japonicus.* Additionally, the high relative efficiency of transmission and the shorter extrinsic incubation period for *Oc. j. japonicus* compared to *Cx. pipiens* may have an important bearing on the epidemiology of WN virus. However, in the absence of information on the survivorship, host preference, and abundance of *Oc. j. japonicus* in the USA, making an accurate prediction on the possible impact of this newly invasive species is difficult.

Given the widening distribution and apparent relative abundance of *Oc. j. japonicus* in the USA, it is important to evaluate its potential to become involved in transmission of other arboviruses, such as eastern equine encephalitis, St. Louis encephalitis, and La Crosse encephalitis viruses.

We thank D. Dohm for the use of his unpublished data; R. Leon, M. O'Guinn, L. Wasieloski, and K. Kenyon for their critical reading of the manuscript; and M. Delgado and D. Schachner for their assistance with the mosquito rearing. This research is part of the doctoral dissertation of Michael R. Sardelis.

REFERENCES CITED

Andreadis TG. 1989. A survey of mosquitoes breeding in used tire stockpiles in Connecticut. *J Am Mosq Control Assoc* 4:256–260.

Centers for Disease Control and Prevention. 1999. Outbreak of West Nile–like viral encephalitis—New York, 1999. *Mor Mortal Wkly Rep* 48:845–849.

Centers for Disease Control and Prevention. 2000. West Nile activity—New York and New Jersey, 2000. *Mor Mortal Wkly Rep* 49:640–642.

Fonseca DM, Campbell S, Crans WJ, Mogi M, Miyagi I, Toma T, Bullians M, Andreadis TG, Berry RL, Pagac B, Sardelis MR, Wilkerson RC. 2001. *Aedes (Finlaya) japonicus* (Diptera: Culicidae) a newly recognized mosquito in the USA: first analyses of genetic variation in the US and putative source populations. *J Med Entomol* 38:135–146.

Gargan TP II, Bailey CL, Higbee GA, Gad A, El Said S. 1983. The effect of laboratory colonization on the vector pathogen interaction of Egyptian *Culex pipiens* and Rift Valley fever virus. *Am J Trop Med Hyg* 32:1154–1163.

Miyagi I. 1972. Feeding habits of some Japanese mosquitoes on cold-blooded animals in the laboratory. *Trop Med* 14:203–217.

Peyton EL, Campbell SR, Candeletti TM, Romanowski M, Crans WJ. 1999. *Aedes (Finlaya) japonicus japonicus* (Theobald), a new introduction into the United States. *J Am Mosq Control Assoc* 15:238–241.

Rosen L, Gubler D. 1974. The use of mosquitoes to detect and propagate dengue viruses. *Am J Trop Med Hyg* 23: 326–335.

Takashima I, Rosen L. 1989. Horizontal and vertical transmission of Japanese encephalitis virus by *Aedes j. japonicus* (Diptera: Culicidae). *J Med Entomol* 26:454–458.

Tanaka K, Mizusawa K, Saugstad ES. 1979. A revision of the adult and larval mosquitoes of Japan (including the Ryukyu Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae). *Contrib Am Entomol Inst (Ann Arbor)* 16:1–987.

Turell MJ, Gargan TP II, Bailey CL. 1984. Replication and dissemination of Rift Valley fever virus in *Culex pipiens. Am J Trop Med Hyg* 33:176–181.

Turell MJ, O'Guinn M, Oliver J. 2000. Potential for New York mosquitoes to transmit West Nile virus. *Am J Trop Med Hyg* 33:176–181.

Turell MJ, O'Guinn M, Dohm DJ, Jones JW. 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. *J Med Entomol* 38:130–134.

Zeichner BC, Perich MJ. 1999. Laboratory testing of a lethal ovitrap for *Aedes aegypti. Med Vet Entomol* 13: 234–238.