Habitat Preferences and Phenology of *Ochlerotatus triseriatus* and *Aedes albopictus* (Diptera: Culicidae) in Southwestern Virginia

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ABSTRACT Recently, the number of reported human cases of La Crosse encephalitis, an illness caused by mosquito-borne La Crosse virus (LAC), has increased in southwestern Virginia, resulting in a need for better understanding of the virus cycle and the biology of its vectors in the region. This study examined the spatial and temporal distributions of the primary vector of LAC, *Ochlerotatus triseriatus* (Say), and a potential secondary vector, *Aedes albopictus* (Skuse). Ovitrapping surveys were conducted in 1998 and 1999 to determine distributions and oviposition habitat preferences of the two species in southwestern Virginia. Mosquitoes also were collected for virus assay from a tire dump and a human La Crosse encephalitis case site between 1998 and 2000. *Oc. triseriatus* and *Ae. albopictus* were collected from all ovitrap sites surveyed, and numbers of *Oc. triseriatus* eggs generally were higher than those of *Ae. albopictus*. Numbers of *Oc. triseriatus* remained high during most of the summer, while *Ae. albopictus* numbers increased gradually, reaching a peak in late August and declining thereafter. In Wise County, relative *Ae. albopictus* abundance was highest in sites with traps placed in open residential areas. Lowest numbers of both species were found in densely forested areas. Ovitrapping during consecutive years revealed that *Ae. albopictus* was well established and overwintering in the area. An oviposition comparison between the yard and adjacent forest at a human La Crosse encephalitis case site in 1999 showed that *Ae. albopictus* preferentially oviposited in the yard surrounding the home, but *Oc. triseriatus* showed no preference. LAC isolations from larval and adult collections of *Oc. triseriatus* females from the same case site indicated the occurrence of transovarial transmission.

KEY WORDS *Ochlerotatus triseriatus*, *Aedes albopictus*, La Crosse virus, arbovirus, oviposition, encephalitis

LA CROSSE ENCEPHALITIS IS the most common and important pediatric mosquito-borne disease in the USA (Rust et al. 1999), with cases distributed mainly in the eastern and midwestern states. The principal vector of La Crosse virus (LAC) is *Ochlerotatus triseriatus* (Say), the eastern treehole mosquito (Berry et al. 1974, Pantuwatana et al. 1974, Watts et al. 1974). As its common name indicates, this mosquito develops in treeholes and is encountered commonly throughout the forested areas of the eastern and midwestern United States.

In its natural cycle, LAC is amplified in a mammalian reservoir host such as the eastern chipmunk (*Tamias striatus*) (Gauld et al. 1975) or the gray squirrel (*Sciuris carolinensis*) (Ksiazek and Yuill 1977). Infected female *Oc. triseriatus* also are capable of transmitting LAC transovarially to their progeny (Watts et al. 1973, Miller et al. 1977). LAC overwinters in transovarially infected eggs, and infected larvae hatch the following spring (Balfour et al. 1975). Humans are tangential hosts for LAC, and *Oc. triseriatus*-human contact has increased because of humans populating forested areas and *Oc. triseriatus* breeding in artificial containers near homes.

Aedes albopictus (Skuse), another mosquito that has spread throughout the eastern United States, also breeds in containers and commonly inhabits areas near human dwellings. *Ae. albopictus* is a competent experimental vector of LAC, with infection and oral transmission rates equal to or higher than those for *Oc. triseriatus* (Grimstad et al. 1989, Cully et al. 1992). The rates for transovarial transmission of LAC in *Ae. albopictus*, however, are lower than those for *Oc. triseriatus* (Tesh and Gubler 1975). Recently, LAC also has been isolated from field-collected *Ae. albopictus* in Tennessee, further implicating this species as a potential secondary vector in the LAC cycle.

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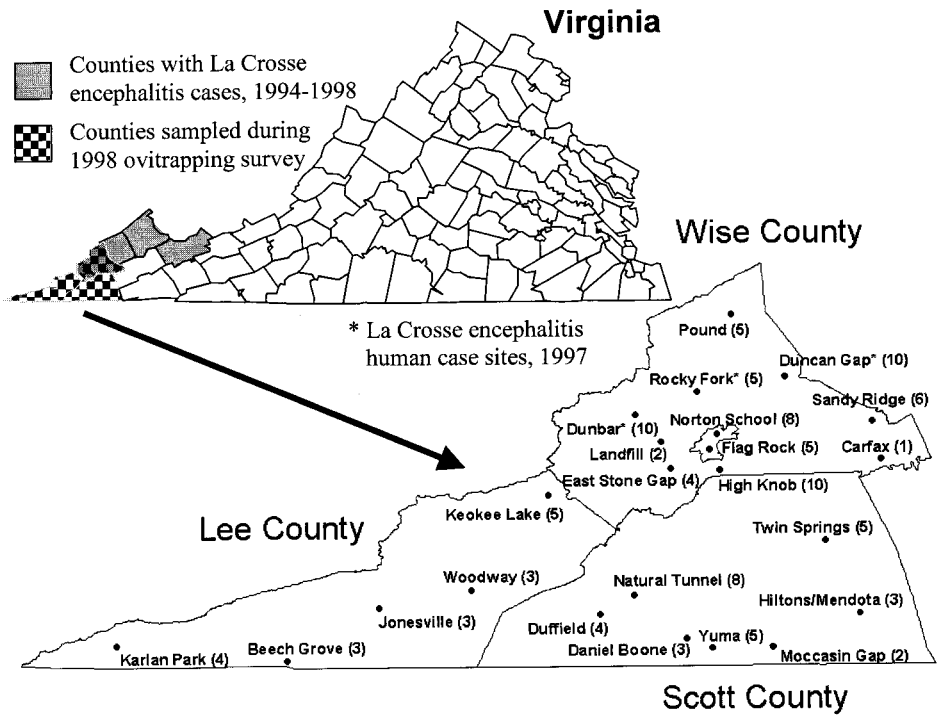

Fig. 1. Virginia counties with confirmed human La Crosse encephalitis cases, 1994–1998, and 1998 collection sites showing numbers of ovitraps per site in parentheses.

From 1975 through 1993, only one case of La Crosse encephalitis was reported from the state of Virginia. However, between 1994 and 1998, CDC recorded a total of 13 cases of La Crosse encephalitis from areas in southwest Virginia in the Appalachian Mountains (Fig. 1). In 1997, five cases were reported from Wise County in western Virginia, comprising disease incidence of ≈13 per 100,000; three of the five cases were from a small cluster of homes in a single community within the county. Because of these recent La Crosse encephalitis cases, southwest Virginia has emerged as a significant focus of LAC activity. This study examined the spatial and seasonal distributions of *Oc. triseriatus* and *Ae. albopictus* as measured by oviposition activity in southwestern Virginia. Additionally, mosquitoes from two sites in Wise County were collected and tested for LAC infection.

Materials and Methods

Study Areas. This study was conducted in Wise, Scott, and Lee Counties, which form the western tip of Virginia (Fig. 1) in the Appalachian Mountains. Landcover in the area consists largely of Appalachian mixed hardwoods that include oak-hickory, maple-beech-birch, and white pine-hemlock forest types (Johnson 1992). Forest stands are interrupted by small towns and large cleared areas resulting from strip mining for coal or occasional pastureland. Elevations range from ≈400 m to 1287 m, with most residential areas between 400 m and 800 m. Wise County was chosen for study because of recent human La Crosse encephalitis cases, and two adjacent counties, Lee and Scott, were chosen because they, along with Wise County and the city of Norton, make up the Lenowisco Health district, facilitating cooperation among the county health departments.

Mosquito Sampling. The ovitrap design consisted of a 450 ml black plastic cup nailed to a tree or post, with drain holes approximately halfway up each side. Strips of seed germination paper, ≈5 cm wide, served as the oviposition substrate, or ovistrips (Steinly et al. 1991). The ovistrips were collected and replaced each week, and remaining water and organic matter were replaced with fresh water to bring the level up to the drain holes. After collection, the eggs were brought to the lab and examined under a dissecting microscope to count and identify species (Linley 1989a, b). Eggs from the year 2000 collections at Duncan Gap were stored in an insectary at 24°C, 80% RH, and 16:8 (L:D) photoperiod. Eggs were hatched and reared to adults according to the methods of Munstermann and Wasmuth (1985). Adult mosquitoes were sorted by species and sex and stored at −70°C for later virus assay.

Adult mosquitoes were collected approximately every 2 wk from June through October 1998 and weekly from 28 May through 1 October 1999 in shrubby areas bordering the Wise County Landfill's tire dump and in forested areas surrounding the human case site in Duncan Gap. Adult host-seeking mosquitoes were collected using a flashlight-type aspirator. For a 30-min collection period, all mosquitoes approaching or land-

ing on the human collector were aspirated. Live specimens were transported to the lab in cages constructed from 3.6 liter plastic buckets (Munstermann and Wasmuth 1985) with a wet paper towel draped over the top to increase humidity. Mosquitoes were frozen live at −70°C and were later sorted and identified by species and sex over wet ice, then stored at −70°C until virus testing was performed.

Virus Assay of Mosquitoes. Mosquitoes were pooled in groups of 50 or fewer specimens according to species and sex. Pools were prepared for assay according to the methods of Nasci et al. (2000) and tested for virus by plaque assay on Vero cells (Beaty et al. 1995) using methyl cellulose (0.8% wt:vol) as the overlay and staining with crystal violet in buffered formalin (0.5 g crystal violet/500 ml buffered formalin). Virus isolates were confirmed as LAC by reverse transcriptase polymerase chain reaction (RT-PCR), followed by genomic sequencing performed on an ABI Prism 377 sequencer (Applied Biosystems/PerkinElmer, Foster City, CA) according to the recommendations of the manufacturer. Previously published L-segments (LCL80C, LCL199V) (Kuno et al. 1996) were used to amplify viral RNA by RT-PCR for sequencing.

Mosquito Distribution Survey. To determine the regional distribution and relative oviposition activity of *Oc. triseriatus* and *Ae. albopictus*, ovitraps were placed at 11 sites in Wise County, five sites in Lee County, and seven sites in Scott County. Three of the sites in Wise County were near La Crosse encephalitis human case sites from 1997. The number of ovitraps per site varied according to the nature of the habitat and ranged from 1 to 10 (Fig. 1). Collections were made every 7–12 d from 8 June through 3 October 1998.

Wise County Collections during 1999 and 2000. In 1999, ovitraps were set out at two of the sites from the previous year, Duncan Gap and Rocky Fork. The Duncan Gap site was associated with three human La Crosse encephalitis cases, and the Rocky Fork site was associated with one case, all from 1997. Rocky Fork, an open area without adjacent forest, was selected because it had a high proportion of *Ae. albopictus* collected from the five ovitraps at the site during 1998. Six ovitraps were placed at Rocky Fork during 1999. At Duncan Gap, casual observations from the previous year suggested that *Ae. albopictus* preferentially oviposited in ovitraps located in the yard as compared with the forested area surrounding the home. To compare the oviposition preferences of the two species at this site, five ovitraps were placed in the yard immediately surrounding the home and five ovitraps were placed in the adjacent forest. Ovistrips at all sites were collected and replaced weekly, and the eggs were counted and identified to species (Linley 1989a, b). Our intent was to hatch the eggs and rear them for virus isolation, but after an insectary failure, the eggs were found to be inviable. In 2000, eggs were again collected weekly from 10 ovitraps at the Duncan Gap site. These eggs were reared to adults, identified to species, and tested for the presence of LAC. Mosquitoes tested for virus were collected during the periods from 20 June through 18 July 2000 and 15 August through 18 September 2000.

Statistical Analysis. The mean numbers of eggs per species collected at the Rocky Fork site were compared by *t*-test (GraphPad Software, Inc., 1994), and differences between the mean numbers of eggs per species collected between habitat types for the Duncan Gap site were analyzed using a generalized linear models analysis of variance (ANOVA) (GLM ANOVA, Hintze 1998). Before analysis, Rocky Fork egg counts were subjected to a square-root transformation of $\sqrt{(y+0.1)}$. However, logarithmic transformations of $\log[(y+0.01)*100]$ were performed on Duncan Gap counts because square-root transformations of the counts did not provide sufficient equalization of variances between test groups.

Results

Mosquito Distribution and Phenology. In 1998, a total of 114 ovitraps distributed among 23 sites in southwestern Virginia were sampled repeatedly throughout the season (Fig. 1). *Oc. triseriatus* and *Ae. albopictus* were found at each of the sites surveyed, and *Oc. triseriatus* was abundant in most of the areas studied in southwestern Virginia (Fig. 2). Although *Ae. albopictus* was also present at every site, its relative abundance was generally lower than that of *Oc. triseriatus*. *Oc. triseriatus* comprised 90.1% of all mosquitoes collected during this period, with *Ae. albopictus* comprising the remaining 9.9%.

In Wise County, the oviposition activity of *Oc. triseriatus* increased steadily through June (Fig. 3), peaking in late June/early July (43.7 eggs/trap-day) and again with three peaks of similar intensity at the end of July through late August (46.0, 44.0, and 46.4 eggs/trap-day, respectively); oviposition then declined through the last collection date in early October. Oviposition by *Ae. albopictus* gradually increased to a peak (9.6 eggs/trap-day) in late August and declined thereafter.

Oviposition Habitat Preferences. The relative oviposition activity of the mosquito species varied among the Wise County sites in 1998 (Fig. 4). Although statistical comparisons were not made because of the wide range of sample sizes obtained from the survey sites, ovitraps from open, unforested sites had a higher proportion (25.9–44.2%) and greater intensity (62.0–86.7 eggs/trap-wk) of *Ae. albopictus* eggs than those from densely forested surroundings (0.1–0.3%, 0.1–0.2 eggs/trap-wk). Locations in which ovitraps were near edges of forested areas, such as forest surrounding a residential area, had intermediate *Ae. albopictus* egg numbers (4.6–36.5 eggs/trap-wk) and relative abundance (1.7–20.5%). Numbers of *Oc. triseriatus* were lowest (53.7–77.9 eggs/trap-wk) in densely forested sites, but no clear pattern was evident for the other two categories (131.7–458.4 eggs/trap-wk in forest edge; 78.3–248.2 eggs/trap-wk in open areas).

Of the 1998 Wise County collections, the highest prevalence of *Ae. albopictus* was found at the Rocky Fork site. However, this site was only intermediate in

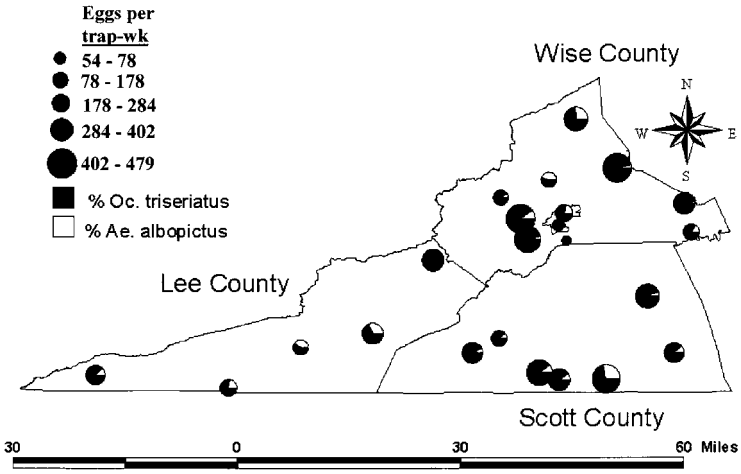

Fig. 2. Percentages of *Oc. triseriatus* and *Ae. albopictus* by site for ovitrap collections between 8 June and 3 October 1998. Size of circle indicates mean total egg count per trap-wk for the collection season.

total mosquito abundance, as measured by the mean number of eggs per ovitrap-wk (Fig. 4). During 1998, the mean density of eggs per ovitrap and relative abundance of *Ae. albopictus* and *Oc. triseriatus* were equivalent (Fig. 5a) ($P > 0.4$), and ovitrap data from 1999 showed a similar pattern (Fig. 5b) ($P > 0.5$).

The Duncan Gap site yielded the highest mean total of mosquitoes per ovitrap-wk for the Wise county sites, but the relative abundance of *Ae. albopictus* was intermediate (Fig. 4). In 1999, equal numbers of ovitraps were placed in the yard and in the surrounding forest. Analysis of oviposition counts revealed that *Ae. albopictus* laid a significantly higher number of eggs in the yard versus the forest ovitraps (Fig. 6) ($F = 22.69$, df = 1, $P = 0.001$). The difference between the two habitat types was greatest during late July and early August, when *Ae. albopictus* populations were highest. *Oc. triseriatus* showed no preference between yard and forest over the collection period (Fig. 6) ($F = 1.29$, df = 1, $P > 0.2$). During collection week 31, the number of *Oc. triseriatus* collected in the yard ovitraps was much higher than the number in the forest traps, but this difference was not significant because of the large amount of variation associated with the *Oc. triseriatus* counts for this date (YARD: Sample mean = 1134, SE = 841; FOREST: Sample mean = 313, SE = 73).

LAC Infection Rates. The species, stages, and numbers of mosquitoes assayed for virus are shown in Table 1. Of these mosquitoes, two pools of *Oc. triseriatus* tested positive for LAC by plaque assay and RT-PCR. When base sequences of the 251-bp S-segment amplicons were compared with published LAC genome sequences (GenBank AF025479), homology

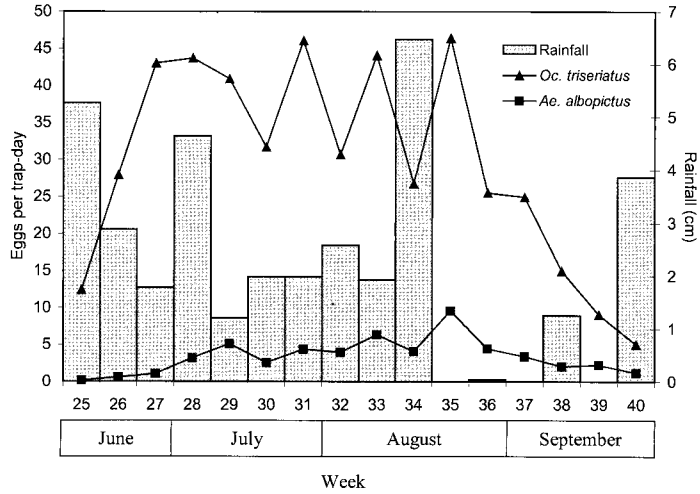

Fig. 3. Mean weekly egg counts per trap-day for all Wise County ovitrap collections between 8 June and 3 October 1998.

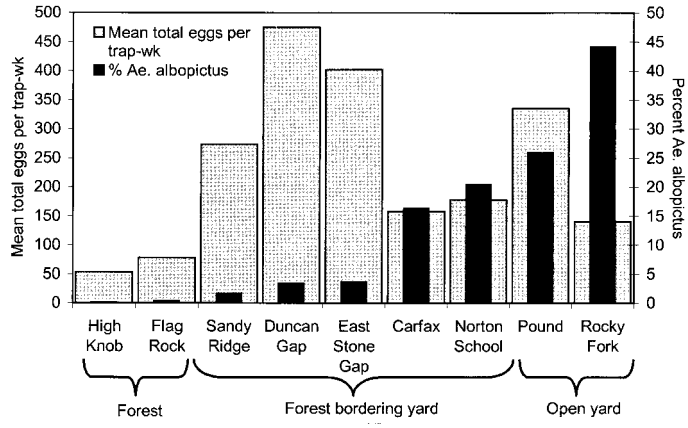

Fig. 4. Seasonal averages of relative *Ae. albopictus* egg abundance and total eggs per trap-wk for both species for Wise County collection sites showing differences in species composition among oviposition habitats. Collections were made weekly from 8 June through 3 October 1998. Dunbar and Landfill sites were excluded from the figure because they failed to fit into one of the three site openness categories.

was 100%, confirming the product was LAC. Both of these pools were collected from the Duncan Gap site that was associated with three human cases during 1997. LAC-positive specimens were obtained from larval and adult collections taken in June 1998 and July 1999, respectively. This represents the first isolation of LAC from field-collected mosquitoes in Virginia.

Discussion

Previous studies in a LAC-endemic area in western North Carolina have reported that 80.9% of mosquitoes collected from peridomestic containers (Szumlas et al. 1996a) and >98% of those collected in an ovitrapping survey (Szumlas et al. 1996b) were *Oc. triseriatus.* Our ovitrap collections in southwest Virginia during 1998 yielded a similarly high relative abundance of *Oc. triseriatus* (90.1%), but the overall percentage of *Ae. albopictus* found in our study was higher (9.9%) than those previously reported by Szumlas and others (<1%) (1996a, b). In a more recent study, Jones et al. (1999) also reported similar species composition in an emerging LAC focus in Tennessee (80% and 20% *Oc. triseriatus* and *Ae. albopictus*, respectively). *Ae. albopictus* was collected from the Rocky Fork (Fig. 5) and Duncan Gap human case sites during 1998 and 1999, and similar numbers of this species were found in both years at Rocky Fork, indicating the ability of *Ae. albopictus* to become established and overwinter in southwestern Virginia. This was not unexpected, as other workers (Hawley et al. 1989, Swanson et al. 2000) have previously reported *Ae. albopictus* overwintering in colder climates in Indiana and Illinois.

Neither of the two species was evenly distributed within the three counties studied, indicating that levels of risk for La Crosse virus transmission probably vary within the region. Because the numbers of mosquitoes assayed from 1998 and 1999 collections with LAC-positive mosquitoes are low, exact minimum field infection rates (MFIRs) cannot be determined. During 2000, when larger numbers of mosquitoes were collected, none of the 8,785 *Oc. triseriatus* tested were shown to be positive by plaque assay, so MFIRs presumably are low (<1/1,000) for this area, although rates in particular foci may be higher. These probable low MFIRs are in agreement with those for *Oc. triseriatus* reported previously from nearby LAC-endemic areas in western North Carolina (0.97/1,000 [Kappus

Table 1. Mosquito species collected in Wise County, VA, 1998–2000, and tested for virus

Mosquito species	1998 collections		1999 Adult collections		2000 Egg collections[a]	Total No. Tested
	Duncan Gap case site[b]	Wise Co. Tire Dump	Duncan Gap case site	Wise Co. Tire Dump	Duncan Gap case site	
Oc. triseriatus	308[c]	13	229[d]	7	8,785	9,342
Oc. atropalpus	0	27	0	2	0	29
Oc. trivittatus	8	2	0	0	0	10
Ae. albopictus	28	368	147	857	69	1,469
Ae. vexans	67	0	13	0	0	80

[a] Eggs were hatched and reared to adults before testing.
[b] Includes season-long adult landing collections and 2 larval collections during June 1998.
[c] LAC was isolated from 1 pool of 12 females collected as larvae in June 1998.
[d] LAC was isolated from 1 pool of 2 females collected as adults in July 1999.

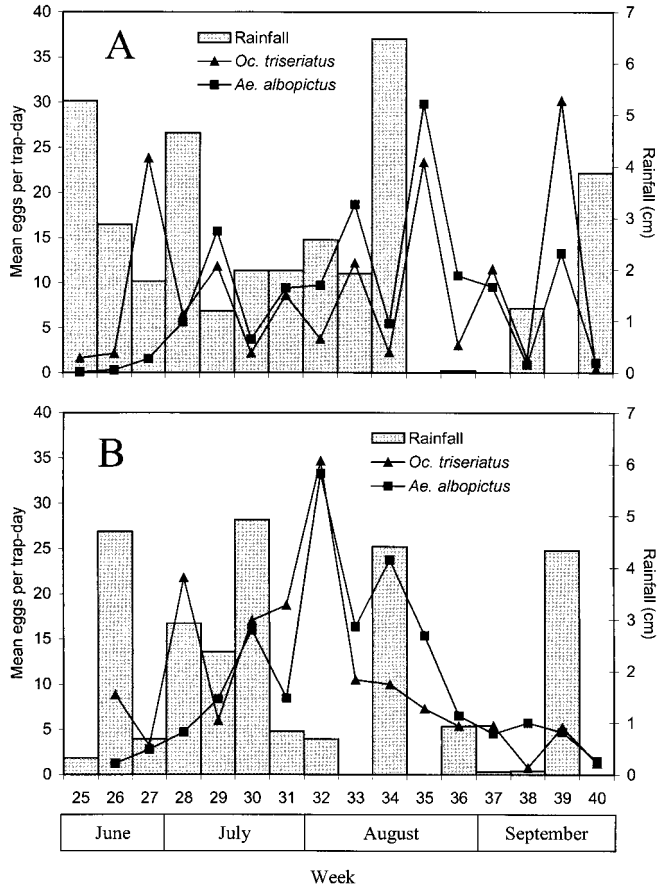

Fig. 5. Mean egg numbers per trap-day for ovitrap collections from Rocky Fork human La Crosse encephalitis case site. Eggs were collected weekly between 8 June and 3 October 1998 (A), and again the following year between 18 June and 1 October 1999 (B). Numbers displayed in figure are back-transformed from means of $\sqrt{(Y+0.1)}$ transformed data used for statistical comparisons.

et al. 1982] and 0.26/1,000 [Szumlas et al. 1996a]) and West Virginia (0.4–7.5/1,000 [Nasci et al. 2000]).

These data suggest that the typical enzootic cycle of LAC is occurring in Wise County, with *Oc. triseriatus* serving as the sole or, at least, the principal vector of the virus. Virus testing of several mosquito species—mainly *Oc. triseriatus* and *Ae. albopictus*—yielded two isolates of LAC from collections near the Duncan Gap human La Crosse encephalitis case site. The first isolate was obtained from a larval collection of *Oc. triseriatus* in 1998, indicating that transovarial transmission of LAC is occurring in this site. The second isolate was obtained from *Oc. triseriatus* adults in the same site the following year. This isolate proved that the virus could persist in the local mosquito population through winter, barring the unlikely chance of a reintroduction of virus into the population from a distant source. These data, combined with three human LAC infections at or near this site during 1997, suggest that the virus was able to persist in a small focus for at least three consecutive seasons. Chipmunks and squirrels are common in Wise County's deciduous forests, so horizontal transmission to and from these potential reservoir hosts probably supplements vertical transmission.

No evidence from this study showed that *Ae. albopictus* has been involved in transmission of LAC in Wise County. However, most of the *Ae. albopictus* tested to date have been collected from the Wise County tire dump, not from a human case site. Because the LAC-positive *Oc. triseriatus* specimens have been collected from a human case site, further sampling for *Ae. albopictus* will be necessary in these areas to determine the extent of involvement of *Ae. albopictus* in LAC transmission. It seems unlikely that *Ae. albopictus* would primarily be responsible for maintenance of LAC in southwest Virginia, given its recent introduction into the area and its lower transovarial transmission rates compared with *Oc. triseriatus* (Tesh and Gubler 1975). *Ae. albopictus* feeds on a wide variety of mammalian, avian, and reptilian hosts, including humans and sciurids (Niebylski et al. 1994), and natural transovarial transmission of LAC by *Ae. albopictus* recently has been found in endemic areas in Tennes-

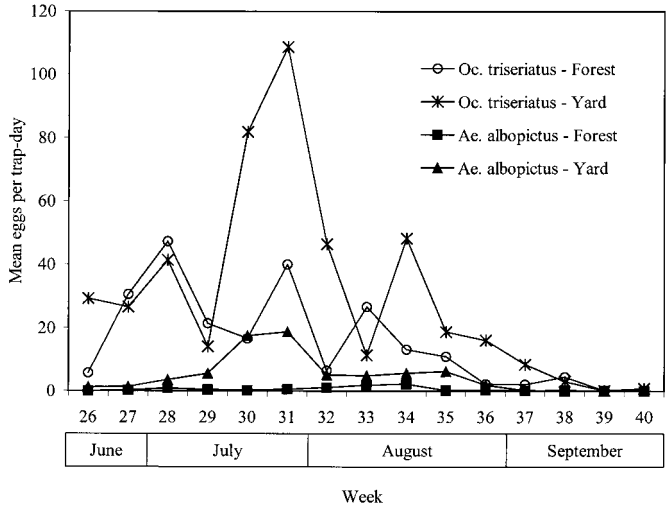

Fig. 6. Geometric means of *Ae. albopictus* and *Oc. triseriatus* egg counts per trap-day at the Duncan Gap human La Crosse encephalitis case site showing numbers for yard and forest. Eggs were collected weekly from 18 June through 1 October 1999.

see and North Carolina (Gerhardt et al. 2001), showing that this species is capable of vertical LAC transmission in nature. It is capable of horizontal transmission to vertebrate hosts but probably is not an efficient vector, given its catholic blood feeding habits. *Ae. albopictus* seems more likely than *Oc. triseriatus* to use breeding areas in the cleared areas around homes, which increases the chance for *Ae. albopictus* to come into contact with humans, and thus it may serve as a bridge vector, providing a link between viremic reservoir hosts and humans.

In conclusion, this study demonstrated that *Oc. triseriatus* is abundant in LAC-endemic areas studied in southwestern Virginia, and transovarial transmission of LAC was found in *Oc. triseriatus* near at least one La Crosse encephalitis human case site. *Ae. albopictus* is also present, and our collections have shown that it is now established and overwintering in southwestern Virginia. Oviposition intensity and relative abundance varied for *Oc. triseriatus* and *Ae. albopictus* based on characteristics of the oviposition habitat.

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