To the Graduate Council:

I am submitting herewith a thesis written by De'Jun Stanich entitled " Epidemiology of La Crosse Encephalitis in Eastern Tennessee, 2000 and 2001." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Reid R. Gerhardt, Major Professor

We have read this thesis
And recommend its acceptance:

Jerome Grant

Carl Jones

Accepted for the Council:

Vice Provost and Dean of Graduate Studies

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EPIDEMIOLOGY OF LA CROSSE ENCEPHALITIS IN EASTERN TENNESSEE, 2000 AND 2001

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

De'Jun Stanich
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Dedication

This thesis is dedicated to my family, my mother, DiAnna Stanich, my father, Stanley Stanich Jr., and my two siblings, Crystal'Ann Stanich and Milana Stanich, who have been the inspiration to help me complete all the requirements in preparing this thesis. Each family member has encouraged and promoted my development with their spiritual and financial help, which I will be forever grateful. DiAnna Stanich has been an incessant source of help by directing my activities to enable me to reach my goals. Stanley Stanich has encouraged my aspirations with his financial and motivational help. Friendship and kind words of support from Crystal'Ann Stanich have provided the strength and backbone to my well being. A constant source of insects as well as letters of direction were provided by Milana Stanich and was fortifying to my spirit.

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Abstract

Peridomestic landscape factors were investigated in 2000 and 2001 at 24 La Crosse (LAC) encephalitis and 79 control sites in an eleven county region in eastern Tennessee to determine the factors that place residents in these counties at risk for LAC encephalitis. Data collected included the condition of the home and yard, distances from the home to the road and tree line, presence and number of artificial containers and tree holes, and the habitat type of the surrounding area. Oviposition traps and CO2-baited Centers for Disease Control (CDC) miniature traps (lights removed) were deployed at each residence. Adult mosquitoes reared from the collected egg strips from the oviposition traps were identified to species, sexed, counted and placed into lots of <-50 individuals, and frozen at –80 °C. The lots were sent to the CDC Division of Vector-Borne Infectious Diseases (DVBID) in Fort Collins, Colorado, for virus isolation. Results were compared between LAC encephalitis and control sites to identify any possible differences that were present.

LAC encephalitis sites commonly had more disposable containers, and had one or more tree holes present on the premises in comparison to control sites. LAC encephalitis sites were commonly in mature and successional forests; whereas, most control sites were in the suburbs. Most of the LAC encephalitis cases were in close proximity to the forest (less than 15.2 m), and most control cases were farther than 30.4 m away from the forest. "Moderately maintained" homes were more commonly encountered at LAC encephalitis sites than at control sites. *Aedes albopictus* female larvae and pupae were more likely to

be encountered at the LAC encephalitis sites than at the control sites in tree holes or artificial containers. The overall mosquito burden (larvae/pupae collected and host-seeking adults from adult traps) for *Ae. albopictus* was statistically higher at the LAC encephalitis sites than at the control sites.

Mean elevations, mean permanent containers, all yard conditions, and mean tree holes at LAC encephalitis or control sites were not significantly different, and no arboviruses were isolated from mosquitoes collected or reared from any of the sites.

A survey evaluating receptacle type, type of substrate, amount of water, color of water, and amount of organic material in receptacles was recorded at 65 sites (56 controls and 9 LAC encephalitis sites) in 2001. Bird baths, plastic tarps, paint jugs (3.8-Liter), bowls, pans, and lids were common at control sites. More tree holes and permanent containers were found on control premises. Sunlit and partially shaded containers were more plentiful at control sites. Containers with no water, and ¼, ½ and completely full of water were predominately observed at control sites. Containers at control sites contained clear water, had less organic matter, and had a higher percentage of mosquitoes present.

Tires were common around LAC encephalitis sites. Greater percentages of disposable containers were located on LAC encephalitis premises. Totally shaded containers were encountered mainly at LAC encephalitis sites. Most of the containers found on LAC encephalitis premises were ¾ full. Containers from LAC encephalitis sites were mostly light yellow in color. LAC encephalitis sites had higher percentages of no mosquitoes present. Containers with various substrates (leaves, grass, soil, etc.) were predominantly encountered at LAC encephalitis sites.

Mixed substrates were the most predominant substrate type contained in all containers holding *Ae. albopictus* larvae that developed to adults in the laboratory. Permanent containers and tree holes holding *Ae. albopictus* larvae were most frequently encountered in completely shaded conditions. Disposable containers with *Ae. albopictus* larvae present in them were found in both sunlit and completely shaded conditions. Completely shaded permanent containers were the most commonly found combination containing *Ae. albopictus* larvae.

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Chapter I

i. La Crosse Encephalitis in Tennessee

Only nine cases of La Crosse (LAC) encephalitis were reported to the Tennessee Department of Health from 1964 to 1996 in Tennessee (Jones et al. 1999). However an average of ten cases per year were recorded from 1997 to 1999 in the eastern region of Tennessee (Jones et al. 1999). During the summer of 2000, 17 cases were confirmed as LAC encephalitis based on a fourfold increase in the titer number of virus specific antibodies in the patients' blood (Smith 2001). The dramatic escalation of cases of LAC encephalitis in combination with the isolation of La Crosse virus (LACV) and Jamestown Canyon Virus (JCV) from the Asian tiger mosquito, *Aedes albopictus* (Suske) in Tennessee (Gottfried et al. 2002, Gerhardt et al. 2001), has heightened public health officials' awareness of vector-borne diseases. Smith (2001) investigated various regional landscape ecology features in eastern Tennessee to elucidate the factors that would predispose children to LAC encephalitis. Further investigations into the variables that contribute to the relative risk of contracting LAC encephalitis in virus endemic areas are essential to educate the public as well as public health officials.

ii. Container-Inhabiting Mosquitoes

The two mosquito species that are suspected vectors of LACV in Tennessee are the eastern tree hole mosquito, *Ochlerotatus =(Aedes) triseriatus* (Say) (Watts et al. 1972) and *Ae. albopictus* (Gerhardt et al. 2001, Erwin et al. 2002). The larvae of both species usually develop in temporary water found in various artificial and natural containers.

Ochlerotatus triseriatus is a native, diurnal species that completes immature development in natural cavities of trees as well as artificial containers, (tires, buckets, bird baths) (Hedberg et al.1985) and is frequently encountered in the woodlands of eastern North America. *Ochlerotatus triseriatus* is a low-density mosquito that has a flight range of 425 m or more (Scholl et al. 1979a), and inhabits a wide area from New Brunswick to Florida, west to central Texas and the lower Great Lakes region (Walker 1992). This mosquito overwinters in the egg stage and is believed to be a primary vector of LAC virus in some areas due to repeated LACV isolations (Pantuwatana et al. 1972, Thompson et al. 1972, Watts et al. 1972, Clark et al. 1982, Nasci et al. 2000). It bites humans as readily as the LACV maintenance hosts (e.g. the eastern gray squirrel, *Sciurus carolensis* (Gmelin), western fox squirrel, *Sciurus niger* (L.), chipmunk, *Tamias striatus* (Illiger)) red fox (*Vulpes fulva*) (L.), and gray fox (*Urocyon cinereoargenteus*) (Schreiber) (Loor and DeFoliart 1970) (Moulton and Thompson 1971). La Crosse Virus is transovarially transmitted from the female mosquito of *Oc. triseriatus* to her offspring (Watts et al. 1973, Miller et al. 1977). Transovarially infected males of *Oc. triseriatus*, which do not take blood meals are capable of infecting females with LACV via venereal transmission. (Watts et al. 1973, Thompson and Beaty 1978). Sudia et al. (1971) established that *Oc. triseriatus* fed on viremic hosts in the lab attained a 100% rate of infection of LACV after a seven-day incubation period, and a transmission rate of 50% after 21 days.

Aedes albopictus is indigenous to the tropical forests of Southeast Asia and found throughout the northern distribution of Asia (Smith 1956, Nawrocki and Hawley 1987). In 1985, *Ae. albopictus* became established in Houston, Texas (Sprenger and

Wuithiranyagool 1986) and is currently distributed throughout most of the southeastern United States (Moore and Mitchell 1997) including all the counties in Tennessee (Moore 1998). Despite having a short range of flight (<525 m) (Niebylski and Craig 1994) and a non-migrating nature, dispersal via used tires has facilitated the spread of *Ae. albopictus* (Reiter and Sprenger 1987). Twenty-two arboviruses have been isolated from *Ae. albopictus* world-wide; however, eight different viruses have been found in the United States: Cache Valley Virus (CV), JCV, Eastern Equine Encephalomyelitis Virus (EEE), Keystone Virus (KEYV), Tensaw Virus (TENV), Potosi Virus (POTV), West Nile Virus (WNV), and LACV (Moore and Mitchell 1997, Gottfried et al. 2002, Mitchell et al. 1999, Gerhardt et al. 2001, Nasci 2001, personal communication and www.sporthorsemedicine .com/mosquito_specs_.htm, Savage 2001, Savage 2001). In 1999, Gerhardt et al. (2001) isolated LACV from *Ae. albopictus* adults reared from eggs collected at a human LAC encephalitis case site in Anderson County, Tennessee. They postulated that the increase in LAC encephalitis might be associated with the expanding range of *Ae. albopictus*.

Aedes albopictus is an opportunistic feeder that displays aggressive blood feeding habits which, combined with the numerous isolates of various arboviruses, suggests that it may be an effective arbovirus vector (Hawley 1988, Niebylski and Craig 1994). Tennessee Cooperative Extensive Service has received numerous accounts of *Ae. albopictus* being a nuisance to the general public. This species has a broad tolerance for larval development habitat, and may be found in mountains, up to 1667 m (Szumlas et al. 1996a,b) and lowland forests, (Swanson et al. 2000) and also in suburban and urban areas (Hawley 1988).

iii. Mosquito Container Survey

Investigation of type and frequency of containers has helped categorize the mosquito species suited for developing in these specialized niches. In small areas of Sarasota and Tallahassee, Florida, observations of various artificial container types (bird bath, 18.9-Liter paint bucket, 18.9-Liter jug, animal trough, bowl, etc) have shown that three predominant mosquito species inhabit these niches. In a 10-block area from the Osprey/Orange area of Sarasota, Florida, the yellow fever mosquito, *Aedes aegypti* (L.) and the southern house mosquito, *Culex pipiens quinquefasciatus* (Say) were the predominant species found. From a 12-block area of Pontiac/Meridian in Tallahassee, Florida, *Ae. albopictus* and *Cx. pipiens quinquefasciatus* were the most commonly encountered species in containers in August and in October (Schreiber et al. 1992). A survey examining the water-holding containers within open areas of Chungho, Taiwan identified trash containers as the most common site in which *Ae. albopictus* larvae and pupae dwell. Water storage containers for gardening and flower vases were other sites employed by *Ae. albopictus* larvae and pupae for development in open areas of Chungho, Taiwan (Teng et al. 1999). *Aedes albopictus* were collected from peridomestic containers in small numbers in a 1990 survey in Western North Carolina and slowly becoming established in this area (Szumlas et al. 1996a).

Ochlerotatus triseriatus was found in 2.3% of the artificial container collections from the Tallahassee site. *Ochlerotatus triseriatus* only existed in "old containers with much decomposed leaf material in them" (Schreiber et al. 1992). *Ochlerotatus triseriatus* larvae and pupae inhabited tree holes, tires, paint cans, buckets and various other containers in Ohio (Berry 1984). 89% of mosquito collections from tree holes and

artificial containers in central Missouri had *Oc. triseriatus,* which were predominantly collected from "artificial containers containing water and leaf litter" in central Missouri, 1986 and 1988 (Debboun and Hall 1992). *Ochlerotatus triseriatus* larvae were collected from tires, metal containers, plastic containers, and fiberglass troughs from LAC encephalitis case sites in Minnesota (Hedburg et al. 1985). The most abundant species collected from peridomestic containers in a 1990 survey of a LACV-Endemic area of western North Carolina was *Oc. triseriatus* (Szumlas et al. 1996a).

Results of an investigation conducted in central Missouri from 1986 to 1988 showed that *Oc. triseriatus* selected tree holes from any tree species, and preferred sunlit tree holes and artificial containers ranging from 0 to 5 m above ground level. *Ochlerotatus triseriatus* inhabited tree holes with pH values ranging from 7.0 to 8.9. The conductivity of water in tree holes with mosquito larvae present ranged from 430 to 2,852 microhms. In artificial containers, *Oc. triseriatus* was found in a wider pH range of 6.0 to 8.2, and the conductivity ranged from 156 to 1163 microhms (Debboun and Hall 1992). *Ochlerotatus triseriatus* are significantly predisposed to shaded habitats in Michigan (Wilmont et al. 1992). *Ochlerotatus triseriatus* larvae were recovered from shaded tires and other containers in West Virginia as opposed to sunlit containers (Joy and Hildreth-Whitehair 2000).

In Tallahassee and Sarasota, Florida, *Ae. albopictus* larvae primarily inhabited medium-size containers (tires, animal troughs, bowls, small buckets, 3.8-liter paint cans, 3.8-liter bottles, jugs, and large vases) and sporadically were found inhabiting large containers (bird baths, wheel barrows, boat hulls, 18.9-liter paint buckets, garbage cans, 189.3-liter drums, and 18.9-liter jugs). *Aedes albopictus* collections from Tallahassee

and Sarasota, Florida, were also from cylinders (18.9-liter paint bucket, garbage can, 189.3-liter drum, 3.8-liter paint can, small bucket, and 3.2-Liter can). Narrow-mouthed containers (18.9-liter Jug, 3.8-liter bottle, jug, large vase, drink bottle, vase) found in a study in Sarasota and Tallahassee, Florida, also contained sparse numbers of *Ae. albopictus* immatures (Schreiber et al. 1992).

Artificial containers at LAC encephalitis sites in West Virginia were correlated with increased *Oc. triseriatus* egg density in ovitraps at these sites and the presence of tree holes significantly increased disease risk for LAC encephalitis in West Virginia (Odds ratio=8.5) (Woodruff et al. 1992). Trash-type containers were the most commonly used niche for *Oc. triseriatus* larvae and pupae in about 70% of the LAC encephalitis cases in Ohio (Berry 1984).

iv. La Crosse Virus and Encephalitis

LACV is endemic to the north central and eastern United States and has been the most medically important of all the California serogroup viruses within the United States (Johnson et. al. 1990). Detection of LAC encephalitis is determined by demonstrating a fourfold change in virus-specific immunoglobin G (IgG) antibody titer levels between acute and convalescent sera (Beaty et al.1982). This disease causes a wide range of symptoms from a persistent headache and vomiting to extreme rare cases that end in death (<1%) (Erwin et al. 2002). Most (94%) of the LAC viral infections have an onset in late July, August, and September in children <15 years of age (Kappus et al. 1983, Erwin et al. 2002). In some areas of Indiana 12.8% of the residents tested positive for viral antibodies for LACV (Grimstad et al. 1984) and, in western North Carolina, 9.6% of the residents in the Cherokee Indian Reservation were positive for the LACV antibody

(Szumlas et al. 1996b). These findings indicate that LAC encephalitis may be more prevalent than previously thought. For every 26 reported LAC encephalitis cases, 1,571 asymptomatic cases are probably not diagnosed (Kappus et al. 1983, Grimstad et al. 1984). The natural habitat of mosquitoes is slowly diminishing due to depletion of forested habitats and more people are living in woodland areas; thus, these factors may cause a potential increase in viral transmission to humans (Sudia et al. 1971).

v.-Peridomestic Landscape Factors Affecting La Crosse Encephalitis and Mosquito Distribution

In an endemic area of West Virginia, LACV and *Oc. triseriatus* were found in mixed northern hardwoods, hemlock mixed with hardwoods, and small-diameter red maples, but higher *Oc. triseriatus* populations were associated with mixed sugar/red maple habitats (Nasci et al. 2000).

Children in homes that had ≥10 tires had a threefold increase risk of contracting LAC encephalitis in West Virginia (Odds ratio 3.2). Another statistically proven risk of contracting LAC encephalitis in West Virginia occurred around residences that had ≥6 other types of artificial containers (Odds ratio 4.1) (Woodruff et al. 1992).

Close proximity of the house to the forest edge may also increase LAC encephalitis risk in West Virginia (Odds ratio 3.2). (Woodruff et al. 1992). LAC encephalitis sites in rural and suburban areas of Ohio were typically in "close" proximity to the wood lot (Berry 1984).

vi. Research Objectives

The reason this study was conducted was to identify risk factors for LAC encephalitis in eastern Tennessee. The introduction of *Ae. albopictus*, various landscape

features, and the impacts of human interaction were analyzed to determine the factors that contribute to the increase in LAC encephalitis. In order to carry out this objective, mosquitoes were collected from various traps and larval habitats found at LAC encephalitis and control premises to find infected mosquitoes.

Chapter II

A Case Comparison Study of La Crosse Encephalitis in Eastern Tennessee

i. Abstract

Entomological and environmental inspections were made of the residences or areas frequented by 103 children diagnosed with central nervous system infections (CNSI) in eastern Tennessee in 2000 and 2001. Those cases that had a fourfold increase in LAC specific immunoglobin between acute and convalescent serologies were designated as La Crosse (LAC) encephalitis cases and those cases without such a fourfold increase in LAC specific immunoglobin between acute and convalescent serologies were classified as control cases. Data collected included the location, elevation, condition of the home and yard, distances from the home to the road and tree line, presence and number of artificial containers and tree holes, and the habitat type of the surrounding area. Oviposition traps and carbon dioxide baited Centers for Disease Control (CDC) mininiature traps (lights removed) were deployed at each residence. Adult mosquitoes reared from oviposition traps and containers holding mosquito larvae and pupae were processed for virus isolation. Results were compared between LAC encephalitis and control sites to identify any possible differences. La Crosse encephalitis sites had three times more disposable containers ($P=0.002$), and twice as many had one or more tree holes present ($P=0.013$) than control sites. Mature and successional forests most commonly surrounded LAC encephalitis sites ($P=0.013$); however, control sites were most commonly situated in suburban habitats ($P=0.020$). Most of the homes of LAC encephalitis cases (70%) were in close proximity (less than 15.2 m) to the forest ($P<0.01$), and 70% of the control homes were farther than 30.4 m from the forest

(P<0.01). "Moderately maintained" homes were four times more likely to be encountered at LAC encephalitis sites than at control sites, but yard conditions of LAC encephalitis and control sites were not significantly different (P>0.05).

Oviposition activity for *Ae. albopictus* and *Oc. triseriatus* did not significantly differ between the LAC encephalitis and the control sites (P>0.05). *Aedes albopictus* eggs collected from control and LAC encephalitis sites had an average rearing success ranging from 3.7 to 25.8% and for *Ochlerotatus triseriatus* it ranged from 0.89 to 18.55%. *Aedes albopictus* female larvae and pupae were fifty times more likely to be encountered at the LAC encephalitis site than at the control site in tree holes or containers (RR=50.44), but no difference with *Oc. triseriatus* was found. The overall mosquito burden (larvae/pupae collected and host-seeking adults from adult traps) for *Ae. albopictus* (P=0.007) but not for *Oc. triseriatus* was statistically higher at the LAC encephalitis sites than at the control sites.

No significant (P>0.05) differences were found between mean elevations, permanent containers, and tree holes at LAC encephalitis or control sites, and no arboviruses were isolated from mosquitoes collected or reared from any of the sites.

ii. Introduction

La Crosse virus (LACV) is endemic to the north central and eastern United States and has been the most medically important of all the California serogroup viruses within the United States (Johnson et. al. 1990). La Crosse Virus is transmitted by two suspected mosquito vectors (*Ae. albopictus* and *Oc. triseriatus*) in Tennessee (Gerhardt et. al. 2001, Erwin et al. 2002). Symptoms of LAC encephalitis range from severe headache, vomiting, and seizures to rare cases of death (<1%) (Erwin et. al. 2002).

An endemic focus of LAC encephalitis has occurred throughout the Midwestern states especially Ohio, Wisconsin, Minnesota, Illinois, West Virginia. In West Virginia, the presence of tree holes, ≥10 tires, ≥6 other types of artificial containers, and close proximity of the house to the forest edge were found to be risk factors for LAC encephalitis (Woodruff et. al. 1992). High population densities of *Oc. triseriatus* may increase the risk of severe disease in LAC encephalitis patients in West Virginia. La Crosse virus and *Oc. triseriatus* were both found at all sites studied in Nicolas County, West Virginia. Higher *Oc. triseriatus* populations were associated with mixed red (*Acer rubrum* L.) and sugar maple (*Acer saccharum* Marsh) habitats in Nicholas County, West Virginia (Nasci et al. 2000). La Crosse encephalitis in Illinois is typically associated with homes built on "edges of gullies and ravines, where hardwoods and tree holes are common, and where tires and other discarded containers can be found" (Kitron et al. 1997). In Minnesota, artificial containers may have been a risk factor for LAC encephalitis patients (Hedberg et al. 1985). In Ohio, LAC encephalitis sites were in "close" proximity to the forest (Berry 1984).

The most abundant species collected from peridomestic containers in a 1990 LACV endemic area of western North Carolina was *Oc. triseriatus* (Szumlas et al. 1996a). Trash-type containers were the most common larval habitat for *Oc. triseriatus* larvae and pupae in about 70% of the LAC encephalitis cases in Ohio (Berry 1984). *Ochlerotatus triseriatus* larvae were collected from tires, metal containers, plastic containers and fiberglass troughs from LAC encephalitis case sites in Minnesota (Hedberg et. al. 1985).

Prevalence of human LAC encephalitis has increased in Tennessee in the past five years in comparison to the previous thirty years (1963 to 1996); only nine cases were reported to the Tennessee Department of Health. From 1997 to 1999, averages of ten cases in eastern Tennessee were reported to the Tennessee Department of Health (Jones et al. 1999). During 2000 and 2001, averages of fifteen cases of LAC encephalitis were reported to the Tennessee Department of Health. Knowledge of the peridomestic risk factors that increase the likelihood of contracting La Crosse Encephalitis is essential to help reduce disease risk and adequately inform the public, and public health officials. The objective of this research was to determine the factors (elevation, artificial and natural containers, yard condition, etc.) that place eastern Tennessee residents at risk for LAC encephalitis.

iii. Materials and Methods

Disease surveillance: Active surveillance for potential cases of LAC encephalitis was performed by the East Tennessee Regional Health Department primarily at a large childrens' hospital in eastern Tennessee, which is the primary pediatric referral center in the region. Patients that met the following criteria were enrolled into the surveillance system:

- Age 6 months to 30.
- Physician-diagnosed febrile central nervous system infection (CNSI).
- Alternative diagnosis for the illness not immediately available.

The parent or sole custodian of each patient was asked for permission to conduct an environmental and entomological survey of the area surrounding their house or any other site where the child spent a several (≥5 hours per day) hours outdoors during the three

weeks prior to the onset of symptoms (Erwin et al. 2002). In the cases where there were divided times, the site where the child had spent the most daylight time was selected. Site surveys were executed promptly by one of two individuals (average 3 days) once the patient was enrolled into the surveillance system, and preceding the confirmation of the type of infection. Anthony B. Smith conducted the site surveillance for 2000 of this research (Smith 2001, Erwin et al. 2002), and in 2001 De'Jun Stanich performed all the site surveillance. Performing site evaluations before the determination of LAC encephalitis infection was done to ensure that the persons conducting the survey would not be influenced by knowledge of the source of the CNSI.

Site evaluation: For 2000, a total of 38 sites (15 LACE and 23 Controls), and in 2001, a total of 65 sites (9 LACE and 56 Controls) were evaluated. The coordinates (latitude and longitude) and elevation in meters of the survey sites were ascertained by using a hand-held GeoExplorer3® produced by Trimble. Environmental assessment included the following; condition of the home and yard: (1=well-maintained, 2=moderately well maintained, and 3=poorly maintained), distance of the home to the nearest tree line and road (1= ≥ 31 m from the tree line or road, 2=15-30.9 m, and 3=0-14.9 m). Other parameters assessed were: the presence and number of artificial containers and natural containers (tree holes), general type of habitat encompassing the site (suburban [residential area on the outskirts of any city or large town] urban [residence in a densely populated city or city], cultivated pasture, unmanaged pasture, successional forest, mature forest, nursery, riparian, annual crop, permanent crop, or fallow).

Larval mosquito collections: At each site, all artificial and natural containers on the premises were examined to determine whether there was any mosquito larvae or pupae present. All mosquito larvae and/or pupae were collected from the containers, transported to the laboratory, and reared to adults in Mosquito Breeders® (Bioquip, Gardena, CA.). The mosquito adults that emerged as adults were identified to species and counted, but the larvae and pupae collected from artificial and natural containers were not counted.

Oviposition activity: *Ochlerotatus triseriatus* and *Ae. albopictus* eggs were collected for two to three weeks at each site in the study with up to 30, 473 mL black 10 m apart (interior and exterior) plastic cups (ovitraps) that were mounted on trees (Loor and DeFoliart 1969). These black plastic cups were filled with 400 mL of tap water (Gottfried 1999) and had two drainage holes just below the rim that allowed drainage of excess water. Ovitraps were mounted on the north side of trees about 0.5 m from the ground (Loor and DeFoliart 1969). A paper clip was used to attach a 5 cm x 24.5 cm strip of 76 lb. seed germination paper (Anchor Paper Company, Saint Paul, MN) to the plastic cup to provide mosquito oviposition substrate (Steinly et al. 1991). Seed germination paper was replaced with new strips weekly when strips were collected. They were placed in plastic bags for transportation to the laboratory, and held in a humidifying chamber while the strips were still moist for four days. At this time, the eggs were counted, identified to species (Pratt and Kidwell 1969) and reared to adults (Gerhardt et al. 2001).

Rearing: Eggs from ovitraps were placed in a solution of 2.0-Liters of water and 5 g of dried liver powder in plastic dish pans. Liver powder was mixed 24 hours prior to

submerging ten egg strips to ensure the water was deficient in dissolved oxygen to induce hatching (Gottfried 1999). Pans containing liver powder were placed in rearing chambers at 28±1 °C. Once pupae were observed within the pans, they were transferred into Mosquito Breeders®. Emergence number for adults of each species was determined by dividing the total number of adults emerged by the total number of eggs counted on the oviposition strips for each species (Gottfried 1999).

Host-seeking adult collections: A dry-ice baited Center for Disease Control (CDC) non-lighted trap was deployed at each site to attract host-seeking mosquitoes for a twenty-four hour period. Dry ice liberated from an open nozzle of a 1.89-liter water cooler was hung on any convenient object (tree limb, fence post) adjacent to the trap (Gottfried 1999). Mosquitoes collected from these traps were transported in an ice chest to the laboratory, counted and identified to species, and placed into pools in the same manner as the reared mosquitoes.

Processing for virus isolation: All collected and reared adults were sorted by species and sex and placed into lots of ≤50 individuals/lot. These pools were placed in 12 x 75 mm (5 ml) conical screw cap tubes (Fisherbrand #02681339) and stored and transported at –80° C to the CDC Division of Vector-Borne Infectious Diseases (DVBID) in Fort Collins, Colorado for processing and virus isolation (Gottfried et al. 2002, Gerhardt et al. 2001). DVBID personnel conducted processing and virus isolation.

Statistical analysis: All factors were recorded and entered into the computer database program, EPI-INFO (1990) for LAC encephalitis and control sites. Convalescent titers were used to establish a final diagnosis for each child as LAC encephalitis or a control case. Once the final diagnosis was established, surveillance data

at LAC encephalitis and control cases were entered into and analyzed by EPI-INFO Version 6®, an epidemiological statistical program from the CDC and the World Health Organization (WHO), to determine what, if any, differences existed between LAC encephalitis and control sites (EPI-INFO 1990). All comparisons were analyzed using an alpha (α) level of 0.05. Discrete data were compared for LAC encephalitis and control sites using chi-square analysis, and continuous data were compared utilizing ANOVA analysis. Furthermore, all data that were not considered normally distributed by Bartlett's test for homogeneity of variance were subsequently analyzed and corrected for normality by the Kruskal-Wallis test for LAC encephalitis and control sites. Relative Risk (RR), a "risk ratio calculated by dividing the incidence in individuals exposed '(LAC encephalitis)' to a risk factor by incidence in nonexposed individuals '(control)'", was assessed to identify any factors that cause individuals to be more susceptible to LAC encephalitis (Smith 1995).

iv. Results

2000 Results

Introduction: In 2000, a total of 38 sites were evaluated by Anthony Smith and in 2001 (Smith 2001, Erwin et al. 2002), a total of 65 sites were evaluated by De'Jun Stanich. The data were analyzed separately for 2000, 2001 and the combined data for 2000 and 2001. The distribution of LAC encephalitis and control cases in 2000 and 2001 are displayed in Figures 1 and 2.

Overall: A total of 38 sites were surveyed in 2000 with sites ranging in distribution from the Cumberland Plateau to near the North Carolina border (Smith 2001). Fifteen children met the case definition for LAC encephalitis and twenty-three

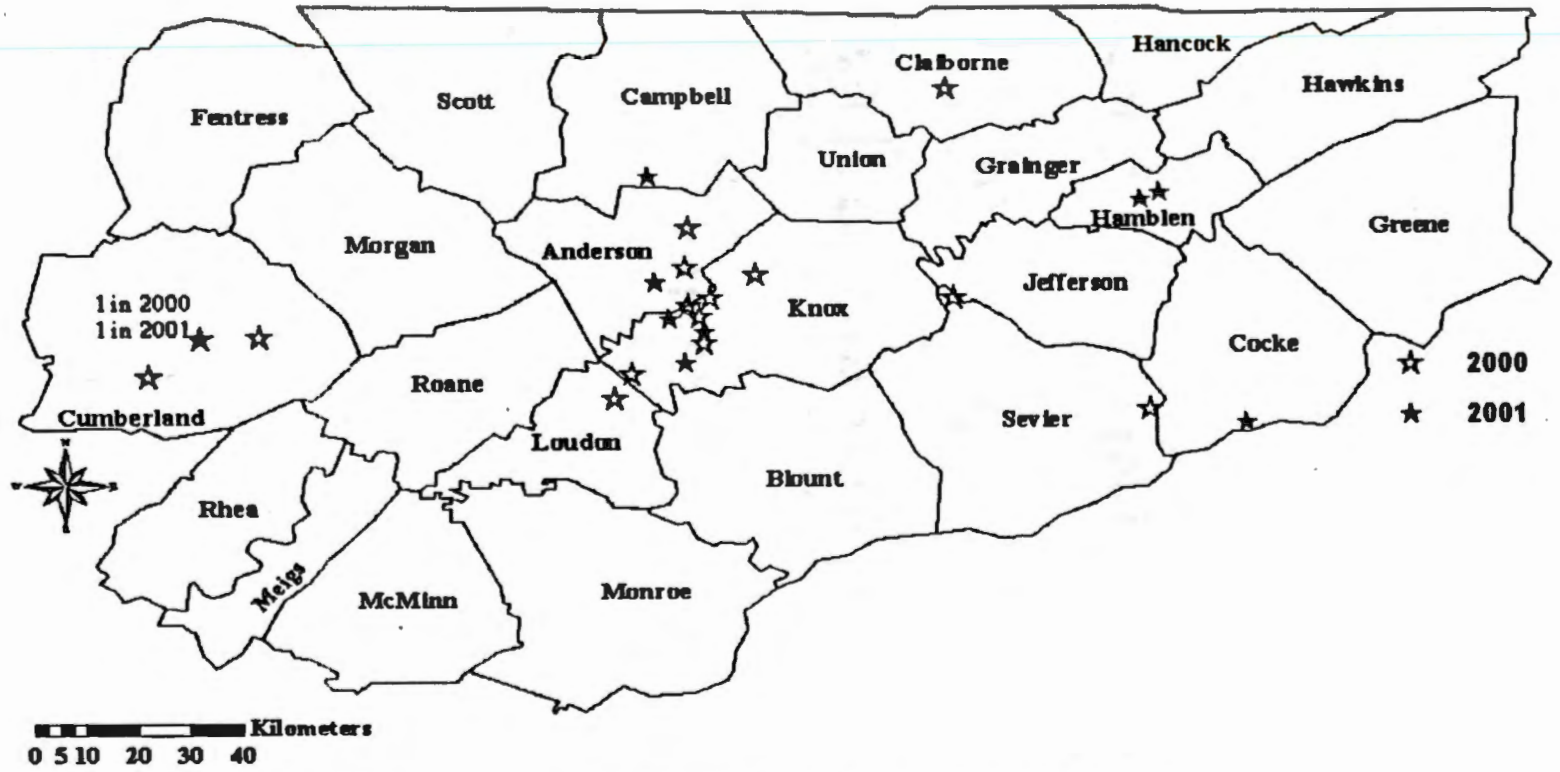


Figure 1. Distrubution of La Crosse encephalitis cases in eastern Tennessee, 2000 and 2001.

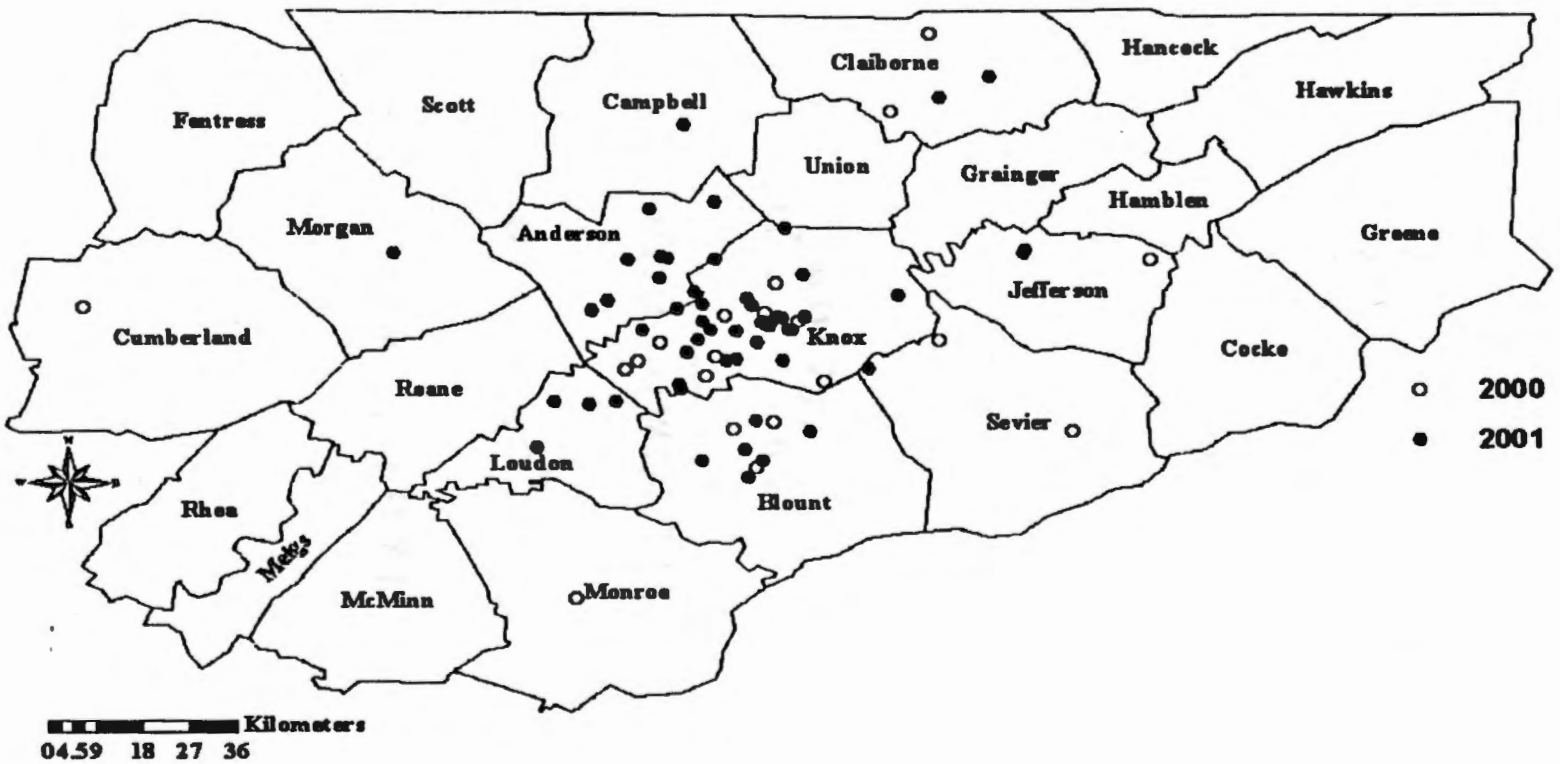


Figure 2. Distribution of control cases in eastern Tennessee, 2000 and 2001.

children did not meet the criteria for LAC encephalitis. The homes of these children were designated as either LAC encephalitis and control sites, respectively (Smith 2001).

Elevation: Elevations were not significantly different (P=0.14) between LAC encephalitis (388.2 m) and control sites (346.7 m) (Smith 2001). The elevations ranged from 535.3 m to 245.1 m at LAC encephalitis sites. The elevations at control sites ranged from 515.1 m to 0 m at control sites.

Artificial and natural containers: The average numbers of permanent (12.5 and 9.7, P=0.77) and disposable containers (99.1 and 84.2, P=0.68) were not significantly different between LAC encephalitis and control sites (Smith 2001). Mean numbers of tree holes were not significantly different (P=0.38) when compared between LAC encephalitis (2.2) and control sites (1.6). When comparing none and one or more treeholes at LAC encephalitis sites there was almost a twofold increase in relative risk at LAC sites when compared to control cases (Table 1) (RR=1.93, P=0.028) (Erwin 2002).

Surrounding habitat: Suburban habitats surrounded both LAC encephalitis and control sites. However, LAC encephalitis sites were five times more likely to be in successional forests than were control sites (Table 1) (RR=5.25, P=0.034) (Smith 2001).

Forest and road distances: There were no significant differences in distance from road and forest-line to the LAC encephalitis and control homes (Table 1) (Smith 2001). Most LAC encephalitis residences were in close proximity (< 15.2 m) to the forest, yet most control sites were at least 30.4 m from the forest. Most control and LAC encephalitis residences were in close proximity (< 15.2 m) to the road.

House and yard condition: Yard and house conditions were statistically the same at LAC encephalitis and control sites (Table 1) (Smith 2001, Erwin et al. 2002). Most

Table 1. Tree hole, house condition, yard condition, distance from forest, distance from the road, and type of surrounding habitat for LAC encephalitis (LACE) and control sites in eastern Tennessee, 2000 (Erwin et al. 2002).

	LACE n=15		CONTROL n=23		
Variables	**Number**	**%**	**Number**	**%**	**P**
Tree hole					
(1 or more)	13	86.67	10	43.48	0.028*
House Condition					
Well maintained	7	46.67	14	60.87	0.598
Moderately maintained	6	40.00	3	13.04	0.115
Not well maintained	2	13.33	6	26.09	0.439
Yard Condition					
Tidy	5	33.33	11	47.83	0.583
Moderately Tidy	7	46.67	6	26.09	0.338
Untidy	3	20.00	6	26.09	1.000
Distance from forest					
Forest > than 30.4 m	3	20.00	12	52.17	0.100
Forest 15.2-30.4 m	3	20.00	4	17.39	1.000
Forest < than 15.2 m	9	60.00	7	30.43	0.142
Distance from road					
Road > than 30.4 m	5	33.33	5	21.74	0.472
Road 15.2-30.4 m	3	20.00	2	8.70	0.364
Road < than 15.2 m	7	46.67	16	69.57	0.283
Surrounding Habitat					
Suburban	9	60.00	16	69.57	0.790
Successional Habitat	6	40.00	2	8.70	0.034*

* Significant at the 0.05 level

LAC encephalitis residences were moderately tidy; whereas, most control cases were tidy. LAC encephalitis and control homes were both predominantly well maintained.

Oviposition activity: A mean of 4.4 *Ae. albopictus* eggs per trap day was collected from both the LAC encephalitis sites and control sites (Table 2) (P=0.31). Mean numbers of *Oc. triseriatus* eggs per trap per day were 9.2 at LAC encephalitis sites, and 7.7 at control sites (Table 2) (P=0.56) (Smith 2001).

Rearing success: Adult *Ae. albopictus* were reared from 25.8% of the eggs collected at LAC encephalitis sites, and 20.8% of eggs collected from control sites (Smith 2001). Overall, *Oc. triseriatus* had a low rearing success with 3.1% of LAC encephalitis collected eggs and 0.9% of control sites collected eggs resulting in adults (Smith 2001).

Larval mosquito collection: Although no significant difference existed, 9.07 *Ae. albopictus* adults from larva/pupal collections were collected from LAC encephalitis sites, and 2.19 adults from larva /pupal collections from control sites. Control sites averaged 7.43 *Oc. triseriatus* adults from larva/ pupal collections, but no *Oc. triseriatus* adults were recovered from larva/pupal collections at LAC encephalitis sites; however, no statistical significance was detected (Smith 2001).

Host-seeking adult collections: An average of 6.9 *Ae. albopictus* females were collected from adult traps at LAC encephalitis sites and 3.3 at control sites, but no significant differences were noticed (Table 2). The mean numbers (0.4) of *Ae. albopictus* males were significantly (P=0.046) higher at LAC encephalitis sites than at control sites (Table 2) (Smith 2001). An average of 1.6 and 2.5 *Oc. triseriatus* females were collected

Table 2. Comparison of the mean numbers of mosquito eggs, larvae, host-seeking adults, adults reared from eggs collected from ovitraps, and overall mosquito burden at La Crosse encephalitis (LACE) and control sites in eastern Tennessee 2000.

Variables	LACE	Control	P
Aedes albopictus			
Eggs/trap day collected	4.4	4.4	0.31
Adult females reared from oviposition strips	37.2	38.5	0.90
Adult males reared from oviposition strips	37.9	29.0	0.42
Adult females collected†	6.9	3.2	0.26
Adult males collected†	0.4	0.0	0.046*
Adults reared from larvae†	9.1	2.2	0.08
Overall Mosquito Burden (ave. of all†)	17.4	5.4	0.013*
Ochlerotatus triseriatus			
Eggs/trap day collected	9.2	7.7	0.56
Adult females reared from oviposition strips	7.9	2.8	0.16
Adult males reared from oviposition strips	11.8	4.5	0.29
Adult females collected†	1.6	2.7	0.53
Adult males collected†	0.0	0.0	-
Adults reared from larvae†	0.0	7.4	0.30
Overall Mosquito Burden (ave. of all†)	1.7	10.1	0.22

* Significant at the 0.05 level

† Mosquito population categories used to calculate overall mosquito burden

from adult traps at LAC encephalitis and control sites respectively, but no significant differences were observed. No *Oc. triseriatus* males were collected from LAC encephalitis sites or control sites (Table 2) (Smith 2001).

Overall mosquito burden: The overall mosquito burden (average of all adult reared from larvae from containers and host-seeking adults collected) for *Ae. albopictus* was threefold higher at LAC encephalitis sites than at control sites (RR=3.19) (P=0.013). Although not significantly different, control sites were six times more likely to have higher overall mosquito burden of *Oc. triseriatus* than LAC encephalitis sites. (Table 2) (Erwin et al. 2002).

Virus isolations: A total of 4,145 mosquitoes in 167 pools were sent to CDC in Fort Collins for virus isolation: 3,541 (1,848 female and 1,693 male) *Ae. albopictus* and 604 (290 female and 314 male) *Oc. triseriatus.* There were no successful isolations of any arboviruses from any of these mosquitoes.

Other Mosquitoes: Other mosquitoes [*Culex pipiens pipiens* (L.), *Cx. pipiens/restuans* (L. and Theobald), *Cx. spp.* and *Orthopodomyia signifera* (Coquillett)] were collected from CDC non-lighted carbon dioxide baited traps and tested for viruses during this investigation. However, no viruses were isolated from these mosquitoes (Smith 2001).

2001 Results

Overall: During 2001, 65 sites were inspected throughout eastern Tennessee extending from the Kentucky and North Carolina borders to the Cumberland Plateau. Fifteen children tested positive for LAC encephalitis using acute and convalescent

serologies; however, only nine families agreed to participate in this investigation, and the remaining 56 children were not LAC encephalitis cases (controls) based on convalescent titers. The LAC encephalitis and control cases where surveillance was conducted are illustrated in Figures 1 and 2.

Elevation: Average elevation was 399.52 m at LAC encephalitis sites and 301.74 at control sites, yet no statistical significance (P=0.16) was found. The elevation at LAC encephalitis sites ranged from 296.4 m to 522.7 m. Control sites ranged in elevation from 28.8 m to 436.2 m.

Artificial and natural containers: The mean numbers of permanent (2.4 and 1.3, P=0.13) and disposable (28.1 and 0.3, P=0.13) containers were not significantly different between LAC encephalitis and control sites, respectively. Similarly, the mean numbers of tree holes at LAC encephalitis (3.3) and control (1.0) sites were not significantly different (P=0.90). Also the presence of one of more tree holes versus zero holes at LAC encephalitis sites were not significantly (P= 1.00) different from control sites (Table 3).

Surrounding habitat: Control sites were three times more likely to be surrounded by suburban habitats (70%) than LAC encephalitis sites (Table 3) (RR=3.05) (P=0.021). LAC encephalitis sites were almost four times more likely to be found in mature and successional forests than control sites (Table 3) (RR=3.73) (P=0.005). One other LAC encephalitis case site was located in an urban setting (11%). The remaining control sites were found in urban settings (12%) and cultivated pastures (18%).

Forest and road distances: The distance to the road at all distances was not significantly different at the LAC encephalitis or the control sites (Table 3). The distance to the forest was almost seven times more likely to be greater than 30.4 m away from the

Table 3. Tree hole, house condition, yard condition, distance from the forest, distance from the road, and type of surrounding habitat for LAC encephalitis (LACE) and control sites in eastern Tennessee for 2001.

Variables	LACE n=9		CONTROL n=56		P
	Number	%	Number	%	
Tree hole					
(1 or more)	2	22.22	15	26.79	1.00
House Condition					
Well maintained	6	66.67	52	92.86	0.049*
Moderately maintained	2	22.22	3	5.36	0.13
Not well maintained	1	11.11	1	1.79	1.00
Yard Condition					
Tidy	7	77.78	40	71.43	0.46
Moderately Tidy	0	0.0	15	26.79	1.10
Untidy	2	22.22	1	1.79	0.048*
Distance from forest					
Forest > than 30.4 m	1	11.11	43	76.79	0.000**
Forest 15.2-30.4 m	0	0.0	9	16.07	0.33
Forest < than 15.2 m	8	88.89	4	7.14	0.000**
Distance from road					
Road > than 30.4 m	2	22.22	15	26.79	0.32
Road 15.2-30.4 m	3	33.33	19	33.93	1.00
Road < than 15.2 m	4	44.44	22	39.29	1.00
Surrounding Habitat					
Suburban	2	22.22	38	67.86	0.021*
Successional and Mature Forest	6	66.66	10	17.86	0.005**

* Significant at the 0.05 level
** Significant at the 0.01 level

forest at control sites (RR=6.91) (P<0.01); whereas, LAC encephalitis sites were 12 times more likely to be less than 15.2 m away from the forest (RR=12.41) (P<0.01) (Table 3).

House and yard condition: A "not well maintained" home was only observed at one control site and no significant differences existed between the LAC encephalitis and control sites (Table 3). A "well maintained" home was 1.4 times more likely to be encountered at a control site rather than a LAC encephalitis site (RR=1.39) (P=0.049) (Table 3). An "untidy" yard was more than twelve times more likely to be seen at a LAC encephalitis site than at a control site (Table 3) (RR=12.41) (P=0.048).

Oviposition activity: A mean of 1.1 and 1.6 *Ae. albopictus* eggs per trap day were collected from LAC encephalitis and control sites, respectively (P=0.70) (Table 4). Likewise, *Oc. triseriatus* mean numbers of eggs per trap day were 4.7 and 5.2 eggs per trap day at LAC encephalitis and control sites, respectively. No significant differences (P=0.42) in egg densities were noticed between the control and LAC encephalitis sites (Table 4).

Rearing success: The rearing success for *Ae. albopictus* was 3.68% from eggs collected at LAC encephalitis sites and 8.87% for those reared from control sites had a rearing success of 8.87%. Reared *Ae. albopictus* females (P=0.39) and males (P=0.80) from oviposition strips were not significantly different between LAC encephalitis and control sites (Table 4).

Overall, *Oc. triseriatus* had a rearing success of 18.55% at LAC encephalitis sites, and 5.73% at control sites. From LAC encephalitis sites, the average numbers of *Oc. triseriatus* females and males reared successfully from eggs were 1.1 and 0.7, while

Table 4. Comparison of the mean numbers of mosquito eggs, larvae, host-seeking adults, adults reared from eggs collected from ovitraps, and overall mosquito burden at La Crosse encephalitis (LACE) and control sites in eastern Tennessee 2001.

Variables	LACE	CONTROL	P
Aedes albopictus			
Eggs /trap day	1.1	1.6	0.70
Adult females reared from oviposition strips	28.9	18.5	0.39
Adult males reared from oviposition strips	34.3	20.4	0.80
Adult females collected †	2.4	3.4	0.80
Adult males collected †	0.3	0.4	0.14
Adults reared from larvae (female) †	3.0	0.9	0.08
Adults reared from larvae (male) †	2.9	0.6	0.46
Overall Mosquito Burden (ave. of all†)	2.2	1.3	0.37
Ochlerotatus triseriatus			
Eggs /trap day	4.7	5.2	0.42
Adult females reared from oviposition strips	1.1	3.8	0.51
Adult males reared from oviposition strips	0.7	2.6	0.90
Adult females collected†	0.2	0.5	0.94
Adult males collected†	0.0	0.0	-
Adults reared from larvae (female) †	0.2	0.2	0.34
Adults reared from larvae (male) †	0.4	0.2	0.29
Overall Mosquito Burden (ave. of †)	0.2	0.2	1.00

† Mosquito population categories used to calculate overall mosquito burden

control sites averaged only 3.8 females and 2.5 males. No significant differences were found among these comparisons (Table 4).

Larval mosquito collection: Numbers of male and female *Ae. albopictus* reared from larvae collected at control and LAC encephalitis sites were not significantly (P=0.08 and P=0.46) different, respectively (Table 4). Similarly, numbers of male and female *Oc. triseriatus* reared from larvae collected from control and LAC encephalitis sites were not significantly (P=0.30 and P=0.35) different, respectively (Table 4).

Host-seeking adult collections: Male and female *Ae. albopictus* adults collected from adult traps at LAC encephalitis and control sites (P=0.80 and P=0.14, respectively) were not significantly different from one another (Table 4). Female *Oc. triseriatus* adults collected from adult traps at LAC encephalitis and control sites were not significantly different (P=0.94). No males of *Oc. triseriatus* were collected from the CDC non-lighted carbon dioxide baited traps.

Overall mosquito burden: The overall "mosquito burden" was not significantly (P=0.38) different for *Ae. albopictus* between the control and LAC encephalitis sites (Table 4). The overall "mosquito burden" was not statistically (P=1.0) different for *Oc. triseriatus* between the control and LAC encephalitis sites (Table 4).

Virus isolations: A total of 6,131 mosquitoes in 475 pools were sent to the CDC Fort Collins for virus isolation (2,829 male and 2,600 female *Ae. albopictus,* 296 male and 406 female *Oc. triseriatus).* No isolations of LAC virus were found.

Other mosquitoes: *Anopheles punctipennis* (n=10) (Say), *Cx. pipiens pipiens* (n=21) (L.), *Cx. pipiens/restans* (n=36) (Theobald), the floodwater mosquito, *Aedes vexans* (103) (Meigen), and *Psorophora ferox* (n=14) (von Humboldt), species were also

collected from carbon dioxide non-lighted CDC traps. All of these mosquito species were tested for all arboviruses in this investigation. No viruses were isolated from these mosquitoes.

Combined 2000 and 2001 Results.

Overall: The 2000 and 2001 case data in eastern Tennessee evaluated by Brent Smith (2001) and De'Jun Stanich were combined to identify any risk factors that contributed to a LAC encephalitis case. Twenty-four LAC encephalitis participants and seventy-nine control participants agreed to be involved in this study. The geographic distribution of both the control and the LAC encephalitis sites ranged from the Cumberland Plateau to the northern border of Tennessee close to Kentucky and the North Carolina border (Figure 1 and 2).

Elevation: Average elevation at the 24 LAC encephalitis sites in 2000 and 2001 was 392.43 m and at the 79 control sites was 310.71 m, yet no statistical (P=0.096) significance was found. Although there was higher elevations usually at LAC encephalitis sites than control sites, not all LAC encephalitis sites were at high elevations.

Artificial and natural containers: The average number of disposable containers were almost three times as common at LAC encephalitis sites (72.5) than at control sites (24.7). (RR=2.93)(P=0.002). Permanent container mean numbers of were not significantly (P=0.07) different when compared between control (3.73) and LAC encephalitis sites (8.71). The mean numbers of tree holes were also not significantly (P=0.24) different between LAC encephalitis (2.5) and control sites (1.2). However, LAC encephalitis sites were almost twice as likely to have one or more tree holes than control sites (RR=1.97) (P=0.013) (Table 5).

Table 5. Tree hole, house condition, yard condition, distance from the forest, distance from the road, and type of surrounding habitat for LAC encephalitis (LACE) and control sites in eastern Tennessee for 2000 and 2001 combined.

	LACE n=24		CONTROL n=79		
Variables	**Number**	**%**	**Number**	**%**	**p**
Tree hole (1 or more)	15	62.50	25	31.65	0.013*
House Condition	13	54.17	66	83.54	0.198
Well maintained	8	33.33	6	7.59	0.003*
Moderately maintained	3	12.50	7	8.86	0.694
Not well maintained					
Yard Condition					
Tidy	12	50.00	51	64.56	0.297
Moderately Tidy	7	29.17	21	26.58	0.989
Untidy	5	20.83	7	8.86	0.144
Distance from forest					
Forest > than 30.4 m	4	16.67	55	69.62	0.000**
Forest 15.2-30.4 m	3	12.50	13	16.46	0.757
Forest < than 15.2 m	17	70.83	11	13.92	0.000**
Distance from road					
Road > than 30.4 m	7	29.17	20	25.32	0.911
Road 15.2-30.4 m	6	25.00	21	26.58	0.911
Road < than 15.2 m	11	45.83	38	48.10	0.969
Surrounding Habitat					
Suburban	9	37.5	54	68.35	0.013*
Successional and Mature Forest	10	41.66	13	16.45	0.021*

* Significant at the 0.05 level
** Significant at the 0.01 level

Surrounding habitat: Most control sites (68%) were in suburban habitats in comparison to LAC encephalitis sites (P=0.013). Higher percentages of LAC encephalitis case sites were found in mature and successional forests (42%) (P=0.020) (Table 5). The remaining LAC encephalitis cases lived adjacent to suburban (38%), urban (4%) and cultivated pastures (8%).

Forest and road distances: No statistical differences were found when comparing the distance from the road in any category (Table 5). When comparing the forest distance, control sites were four times more likely to be farther than 30.4 m from the forest (RR=4.18)(P<0.01), and LAC encephalitis sites were five times more likely to be less than 15.2 m from the forest (P<0.01) (Table 5).

House and yard condition: Analysis with EPIINFO indicated that no significant difference occurred between LAC encephalitis and control cases for all three of the yard conditions (Table 5). "Moderately maintained" houses were four times more likely at LAC encephalitis sites than at control sites (RR=4.39) (P=0.003) (Table 5). There were no statistical differences when comparing "well maintained" (P= 0.20) or "not well maintained" (P=0.69) homes at LAC encephalitis and control sites (Table 5).

Oviposition activity: Ae. albopictus were collected from every site (103) where oviposition traps were placed; whereas, *Oc. triseriatus* eggs were collected from 98 (95%) sites. Each seed germination substrate strip placed at the LAC encephalitis and control sites averaged 2.76 *Ae. albopictus* eggs per day, respectively; whereas, every substrate strip placed at the control sites averaged 3.02 *Ae. albopictus* eggs per day. These two groups did not differ statistically (P=0.59) (Table 6). The average number of

Table 6. Comparison of the mean numbers of mosquito eggs, larvae, host- seeking adults, adults reared from eggs collected from ovitraps, and overall mosquito burden at La Crosse encephalitis (LACE) and control sites in eastern Tennessee in 2000 and 2001 combined.

Variables	LACE	CONTROL	P
Aedes albopictus			
Eggs /trap day	2.7	3.0	0.281
Adult females reared from eggs	34.0	22.2	0.531
Adult males reared from eggs	37.9	22.3	0.352
Adult females collected†	5.2	6.5	0.200
Adult males collected†	0.3	0.3	0.075
Adults reared from larvae (female) †	4.5	0.1	0.004**
Adults reared from larvae (male) †	3.3	0.8	0.083
Overall Mosquito Burden (ave. of all†)	13.4	8.4	0.007**
Ochlerotatus triseriatus			
Eggs /trap day	7.0	6.5	0.587
Adult females reared from eggs	5.3	3.4	0.560
Adult males reared from eggs	7.1	2.9	0.682
Adult females collected†	1.1	1.1	0.185
Adult males collected†	0.0	0.0	-
Adults reared from larvae (female) †	0.1	1.3	0.672
Adults reared from larvae (male) †	0.2	0.9	0.567
Overall Mosquito Burden (ave. of all†)	1.3	3.2	0.302

** Significant at the 0.01 level

† Mosquito population categories used to calculate overall mosquito burden

Oc. triseriatus eggs per trap day was not significantly different between LAC encephalitis (6.96) and control (6.47) sites (Table 6).

Rearing success: The rearing success for *Ae. albopictus* was 14.71% from the eggs on the oviposition strips collected at LAC encephalitis sites and 14.84% at control sites. *Aedes albopictus* eggs collected from control sites had a rearing success of 14.84% (Table 6). Overall, *Oc. triseriatus* had a rearing success of 10.85% at LAC encephalitis sites and 3.31% at control sites (Table 6).

Larval mosquito collection: Aedes albopictus averaged 3.93 (range 0-51) larvae/pupae from LAC encephalitis sites, and an average 0.43 (range 0-22) larvae/pupae were collected from control sites. *Aedes albopictus* female adults reared from larvae and pupae (4.54) were 51 times more likely to be encountered at LAC encephalitis sites (4.5) than at control sites (0.1) (RR=51.24, P=0.004) (Table 6). This trend was not true for *Oc. triseriatus* larvae and pupae. On average, more female (1.29) and male (0.87) *Oc. triseriatus* adults reared from larva/pupal were collected from control sites than from LAC encephalitis sites (0.08 and 0.17, respectively), yet no statistical significance was found (Table 6).

Host-seeking adult collections: An average of 5.2 *Ae. albopictus* females and 0.3 males were collected from the adult traps of LAC encephalitis sites and similar numbers (6.4 and 0.3, respectively) were collected from control sites. No significant differences were found when comparing these means. A mean of 1.1 *Oc. triseriatus* female adults were collected with adult traps from both LAC encephalitis sites and control sites. No significant differences were observed between these groups. *Ochlerotatus triseriatus* males were not collected from any adult traps (Table 6).

Overall mosquito burden: The overall "mosquito burden" (larvae/pupae collected and host-seeking adults from adult traps) for *Ae. albopictus* was statistically higher (mean=13.38) at the LAC encephalitis sites than at the control sites (mean=8.39) (RR=1.59) (P=0.007462) (Table 6). Overall "mosquito burden" means for *Oc. triseriatus* were 1.33 at LAC encephalitis sites and 3.24 at control sites, and they were not significantly (P=0.30) different (Table 6).

v. Discussion

Introduction: In eastern Tennessee, Brent Smith (2001) conducted an entomological and environmental evaluation of 15 LAC encephalitis and 23 control sites in 2000. Smith found that LAC encephalitis sites were five times more likely to be in successional forests than were control sites. In addition, LAC encephalitis sites had a higher overall mosquito burden for *Ae. albopictus* and contained male mosquitoes from the CDC carbon dioxide traps from the LAC encephalitis sites. In 2001, De'Jun Stanich conducted the entomological and environmental surveillance in eastern Tennessee and found that LAC encephalitis sites were found primarily in suburban and successional and mature forests, were in close proximity to the forest (< than 15.2 m), and predominantly either had tidy or untidy yard conditions.

Elevation: Elevations at LAC encephalitis or control sites were not significantly different in eastern Tennessee. Previous studies have suggested that elevation does not limit incidence of *Oc. triseriatus* and *Ae. albopictus*. For example, *Oc. triseriatus* has been found at all elevations in West Virginia, although LAC encephalitis cases are currently only confined to the south-central portion of West Virginia (Joy and Hildreth-

Whitehair 2000). *Aedes albopictus* was found in forested habitats that varied in elevation from 450 to 1,800 m in Thailand (Scanlon and Esah 1965).

Artificial and natural containers: Similar to LAC encephalitis sites in West Virginia (Woodruff et al. 1992), the presence of tree holes also increased LAC encephalitis disease risk in eastern Tennessee. No statistical significance was found for discarded tires and non-tire artificial containers in West Virginia (Woodruff et al. 1992), although disposable containers (i.e. trash, tires, plastic tarps, and other easily discarded items) increased LAC encephalitis risk to eastern Tennessee residents. *Ochlerotatus triseriatus* larvae were recovered from tires, metal containers, plastic containers, and fiberglass water troughs at LAC encephalitis sites in Minnesota (Hedberg et al. 1985). *Ochlerotatus triseriatus* primarily inhabited tree holes, tires, paint cans, buckets, and various other containers at LAC encephalitis sites in Ohio (Berry 1984). All container types found in this study contained *Ae. albopictus*. Endemic areas for LAC encephalitis in western North Carolina were surveyed and *Oc. triseriatus* were most commonly found in peridomestic containers (Szumlas et al. 1996a).

Surrounding habitat: La Crosse encephalitis residents were situated around mature and successional forests; however, control sites were most commonly situated around suburban habitats in eastern Tennessee. Residential areas of Chungho, Taiwan, were the most common habitat that *Ae. albopictus* preferred for development (Teng et al. 1999). *Aedes albopictus* displays a predilection for colonizing rural areas in southwest Florida (Hornby et al. 1994) and readily colonizes containers in the North Carolina mountains (Szumlas et al. 1996a,b), woodlands in Illinois (Swanson et al. 2000), and urban and suburban habitats of Illinois (Kitron et al. 1998). *Ochlerotatus triseriatus*

mainly inhabits the deciduous forests in North America (Sinsko and Craig 1979) and will also readily disperse across wet meadows, old fields, and open yards in southwestern areas in Wisconsin (Mather and DeFoliart 1984).

Forest and road distances: Close proximity of the house to the forest edge seems to increase LAC encephalitis risk in West Virginia (Woodruff et al. 1992) and in eastern Tennessee. LAC encephalitis sites in rural and suburban communities in Ohio also were in "close" proximity to woodlots (Berry 1984).

House and yard condition: Yard conditions were not significantly different between LAC encephalitis and control cases in West Virginia or in eastern Tennessee. The condition of the home was also not a significant factor that contributed to a LAC encephalitis case in West Virginia (Woodruff et al. 1992), yet "moderately maintained" homes in eastern Tennessee were more commonly at LAC encephalitis sites rather than control sites.

Oviposition activity: Eggs of *Ae. albopictus* were collected from every site (103) where oviposition traps were placed; yet *Oc. triseriatus* eggs were only collected from 95 sites (92%) for 2000 and 2001. Similar results were found in South Carolina in a container survey where *Ae. albopictus* were found at all trap sites and 80.5% of the sites had *Oc. triseriatus* on the premises (Richardson et al. 1995). Eggs of *Oc. triseriatus* were found at all LAC encephalitis and control sites in West Virginia (Nasci et al. 2000). Oviposition activity for *Oc. triseriatus* did not significantly differ between the LAC encephalitis and the control sites in eastern Tennessee and in West Virginia (Woodruff et al. 1992). Oviposition traps employed in a survey in South Carolina yielded *Ae. albopictus, Oc. triseriatus, Cx. restuans, Cx. pipiens pipiens, Cx. territans* (Walker),

Toxorhynchites rutilus septemtrioalis (Dyar and Knab), and *Orthopodomyia signifera*. The most common species was *Ae. albopictus* (89.2%), and 6.5% of the mosquitoes collected were *Oc. triseriatus* in Clemson, South Carolina (Richardson et al. 1995). Ovitrap collections in West Virginia during 1996 yielded 317,257 mosquito eggs from which 73,984 mosquitoes were reared to the adult stage. The predominant species was *Oc. triseriatus* (99.32%) and the other was *Ae. hendersoni* Cockerell (0.68%) (Nasci et al. 2000). On average, one endemic LAC encephalitis site in West Virginia had 10.4 *Oc. triseriatus* eggs collected per ovitrap per day and another endemic LAC encephalitis site had 52.6 (Nasci et al. 2000).

Rearing success: *Aedes albopictus* eggs collected from control sites had an average rearing success ranging from 8.8 to 20.8 % in 2000 and 2001. The average rearing success for *Ae. albopictus* eggs collected at LAC encephalitis sites ranged from 3.7 to 25.8%. *Ochlerotatus triseriatus* had an incredibly low average rearing success ranging from 3.14 to 18.55% at LAC encephalitis sites, and control sites had similar average low rearing success ranging from 0.89 to 5.73% during 2000 and 2001. One reason rearing success could have been low is that the oviposition strips were left in the vehicle all day long, and the plastic bags that these strips were placed in were very warm when they were brought into the laboratory. Barrera (1996) also had low emergence levels of *Oc. triseriatus* adults (11.0%). Barrera (1996) observed many pupae of *Oc. triseriatus* die before adult emergence. High larval mortality may be an additional cause for this low rearing success (Hawley 1988). Successful hatching of 62.1% of all *Aedes* species (*Ae. albopictus, Oc. triseriatus,* and *Ae. aegypti*) was conducted in a Florida study in 1992 (Hornby et al. 1994); however, in Tennessee in 2000 and 2001, rearing success

fluctuated between 3.14 to 25.8% for *Oc. triseriatus* and *Ae. albopictus*. Mass rearing of eggs collected in Illinois from soaked balsa wood oviposition strips yielded a 95% hatch rate during the first flooding. "Good" adult emergence rates also occurred in this study in 1979 (Clark et al. 1982).

Larval mosquito collection: Aedes albopictus female larvae and pupae were fifty times more likely to be encountered at LAC encephalitis sites than at control sites in tree holes or containers, yet this was not true for *Oc. triseriatus*. From Peoria County, Illinois, during 1997, *Oc. triseriatus* were collected from tires and tree holes, and both *Oc. triseriatus* and Ae. *albopictus* were recovered from vases (Swanson et al. 2000).

Host-seeking adult collections: Aspiration sampling in western North Carolina in 1989 and 1990 predominantly collected *Oc. triseriatus* (Szumlas et al. 1996a). No *Ae. albopictus* were collected from western North Carolina because it was slowly moving in and had not been established yet. Landing and biting rates of *Ae. albopictus* were the most intense in the forest edge in Missouri (Niebylski and Craig 1994). Landing collection in West Virginia yielded 1,055 *Oc. triseriatus* and 1,863 *Ae. canadensis canadensis* (Theobald) mosquitoes throughout the 1996 season (Nasci et al. 2000). No male adult *Oc. triseriatus* were collected during the study in eastern Tennessee, 2000 and 2001, yet adult males of *Ae. albopictus* were collected. One plausible explanation that *Ae. albopictus* males were collected for this study in CDC carbon dioxide traps is that males of *Ae. albopictus* readily disperse to hosts that females utilize as a blood meal to attempt to mate with females (Gubler and Bhattacharya 1972).

Overall Mosquito Burden: The overall mosquito burden (larvae/pupae collected plus host-seeking adults from adult traps) for *Ae. albopictus* was significantly higher at

the LAC encephalitis sites than at the control sites, but this trend was not true for *Oc. triseriatus*. *Ochlerotatus triseriatus* was the dominant species encountered in tree holes and artificial containers in central Missouri (Debboun and Hall 1992). *Ochlerotatus triseriatus* larvae were frequently collected from tree holes in a survey conducted in West Virginia in 2000 (Joy and Hildreth-Whitehair 2000) and *Oc. triseriatus* population densities were highest in LAC endemic regions of West Virginia where artificial containers were present (Nasci et al. 2000).

vi. Summary

La Crosse encephalitis disease risk is increased when one or more tree holes are on the premises, and disposable containers are abundant throughout the yard. Close proximity (< than 15.2 m) to the forest as well as living in mature and successional forests seems to increase LAC encephalitis risk. Well maintained and moderately maintained houses were also common at LAC encephalitis residences. These factors may be useful to understand the epidemiology of LAC encephalitis.

Chapter III.

An Evaluation of Artificial and Natural Containers in 2001 of La Crosse Encephalitis and Control Sites in Eastern Tennessee.

i. Abstract

Bird baths, plastic tarps, 3.8-liter paint jugs, bowls, pans, and lids were common among control sites, yet tires were common around LAC encephalitis sites. Disposable containers were more likely to be found on LAC encephalitis premises. Totally shaded containers were encountered mainly at LAC encephalitis sites. Most of the containers found on LAC encephalitis premises were ¾ full. Containers from LAC encephalitis sites were mostly light yellow in color. Containers with various substrates (leaves, grass, soil, etc) were twice as likely to be encountered at LAC encephalitis sites than at control sites. LAC encephalitis sites had higher percentages of no mosquitoes present.

More tree holes and permanent containers were found on control premises. Sunlit and partially shaded containers were more plentiful at control sites. Containers with no water, and ¼, ½ and completely full of water were predominantly observed at control sites rather than LAC encephalitis sites. Containers at control sites contained water that was clear in color. Containers at control sites had less organic matter than LAC encephalitis sites. Control sites had a higher percentage of containers with mosquitoes present within containers.

Aedes albopictus (Skuse) and *Ochlerotatus = (Aedes) triseriatus* (Say) were the two mosquitoes collected from artificial and natural containers. *Oc. triseriatus* were found predominantly in permanent containers. Mixed substrates were the most predominant substrate type in all containers holding *Ae. albopictus* larvae that developed

to adults in the laboratory. Permanent containers and tree holes holding *Ae. albopictus* larvae were most frequently encountered in completely shaded conditions. Disposable containers with *Ae. albopictus* larvae present in them were found in both sunlit and completely shaded conditions. Completely shaded permanent containers were the most commonly found combination containing *Ae. albopictus* larvae.

ii. Introduction

Aedes albopictus and *Oc. triseriatus* are the primary container-dwelling mosquitoes in eastern Tennessee, and both have been implicated as vectors of La Crosse Virus (LACV). The primary vector of LACV is *Oc. triseriatus* due to repeated isolations of LAC virus from this mosquito (Pantuwatana et al. 1972, Thompson et al. 1972, Watts et al. 1972, Clark et al. 1982). Another suspected vector of LACV is *Ae. albopictus* due to the recent isolation of LACV in Anderson County, Tennessee (Gerhardt et al. 2001) and because *Ae. albopictus* was found at every LAC encephalitis site. (Erwin et al 2002). A partial synopsis of the types of containers utilized by *Ae. albopictus* and *Oc. triseriatus* are presented in Tables 7 and 8, respectively. Schreiber et al. (1992) conducted a peridomestic container block survey in Tallahassee, Florida, and categorized containers according to Table 9 (except for plastic tarps). This research was undertaken to determine the types, size, amount of water, color of water, and organic content of containers at LAC encephalitis and control sites in eastern Tennessee in 2001.

iii. Materials and Methods

An evaluation was conducted to examine all containers (tree hole or containers) capable of holding water from June to October 2001 at 65 sites (56 Control sites and 9 LAC encephalitis sites) in eastern Tennessee (Figures 1 and 2). These sites were

Table 7. Documentation of types of containers where *Aedes albopictus* has been found.

Container	Place	Source
Cemetery flower vases	McLennan County, Texas	Duhrkopf 1994
Plastic cups, used tires	Sarawak, Malaysia	Seng and Jute 1994
Used tires, water drums	Fiji	Kay et al. 1995
Used tires	Albania	Adhami and Murati 1987
Various containers	Sao Paulo State, Brazil	Chiaravalloti et al. 1996
Trash receptacles, water storage containers, flower vases	Chungho, Taiwan	Teng et al. 1999
Medium-size containers, large containers, cylindrically shaped containers, narrow-mouthed containers	Tallahassee, Florida	Schreiber et al. 1992

Table 8. Documentation of types of containers where *Ochlerotatus triseriatus* have been found.

Container Type	Place	Source
Cemetery flower vases	McLennan County, Texas	Duhrkopf 1994
Artificial containers	Tallahassee, Florida	Schreiber et al. 1992
Tree holes, tires, paint cans, buckets, other containers	Ohio	Berry 1984
Tires, metal containers	Minnesota	Hedberg et al. 1985
Tree holes, artificial containers, plastic containers, fiberglass troughs	Central Missouri	Debboun and Hall 1992
Tree holes, shaded tires, manmade Containers	West Virginia	Joy and Hildreth-Whitehair 2000

Table 9. A classification scheme for artificial water-holding containers in a La Crosse Encephalitis survey of eastern Tennessee, summer and fall 2001. (modified from Schreiber et al. 1992).

	SIZE OF CONTAINER		
SHAPE OF CONTAINER	***Large***	***Medium***	***Small***
Open	1. Bird bath, wheel barrow, boat hull, plastic tarp *	4. Animal trough, frying pan, bowl	8. Bowl, pan, lids
Cylinder	2. 18.9-liter paint bucket, garbage can, 208.2-liter drum	5. 3.8-liter paint can, small bucket	9. 3.2-liter can
Narrow-Mouthed	3. 18.9-liter jug	6. 3.8-liter bottle, jug, large vase	10. Drink bottle, vase
Tire		7. All tires considered medium size	

* Common larval habitat encountered in eastern Tennessee

enrolled by contacting patients or parents of patients that were age 6 months to 30 years, had a febrile central nervous system infection, and no alternative diagnosis for another illness was available. A LAC encephalitis case was found by a fourfold increase in virus specific immunoglobin between acute and convalescent serologies and all other cases that did not meet these criteria are hereafter referred to as control cases. The container type, substrate type, shade condition, water level, shade of water, amount of organic matter, and presence of mosquitoes were assessed to determine the most common types and conditions of containers at LAC encephalitis and control sites. Containers from which adult mosquitoes were reared were further examined to determine what conditions enabled these mosquitoes to complete development.

Artificial and natural container types: Each container that was found was placed into one of ten categories (Table 9) as modified from Schreiber et al. 1992. Tree holes were not taken into account in that research. Thus, each tree hole and container found in this study was also segregated into one of three different types of containers holding water [disposable containers (trash, old tires, plastic traps, and other easily discarded items), permanent containers (flower pot reservoirs, bird baths, toys, water bowls, wheel barrow, watering can, rain barrel, and buckets), and tree holes] (Smith 2001).

Substrate type and amount of organic matter: Each container was examined to identify which of these substrate types were found (1=none, 2=grass only, 3=leaves only, and 4=mixed). The amount of organic material (none, less than ¼ full, ¼- ¾ full, more than ¾ full) observed in each container was also recorded.

Shade conditions: Similarly, recognition of one of the following three shade conditions was noted for each container (1=sunlit, 2=partially shaded, and 3=completely

shaded). These shade conditions were all monitored between 9:00 and 10:00 am standard eastern time for consistency.

Water level and color: Amount of water in each container was recorded according to these groupings: 1=no water, 2=1/4 full, 3=1/2 full, 4=3/4 full, and 5=full. The color of the water (1=clear, 2=light yellow, 3=light green, 4=dark brown) also was reported for each container.

Mosquitoes presence: The presence or absence of mosquitoes larvae, the species and, numbers of each species that survived to adulthood also was noted for each container. All pupae and larvae were not counted for this investigation. In fact, many pupae died while they were developing and while they were trying to emerge as adults.

Statistical analysis: The comparison of all container types, and substrate type, shaded conditions, level and color of water, amount of organic matter, the presence or absence of mosquitoes within each container at LAC encephalitis and control sites was analyzed using chi-square tests in EPI-INFO Version 1990. Another analysis considering only the containers with immature mosquitoes that emerged as adults in laboratory conditions were entered into SAS 8.2 and comparatively analyzed with ProcFreq. Container type and mosquito species, container type and shade conditions using the chi-square tests were compared between LAC encephalitis and control sites. Since multiple comparisons utilizing these data were assessed using Chi-square tests for association, Bonnferroni techniques (Lentner and Bishop 1986) for adjustment of the alpha level were employed. Bonnferroni adjusts the probability for multiple comparisons by dividing the alpha level (0.05) by the number of contrasts being compared. Variable alpha levels were employed ranging from 0.01 to 0.0056 to detect a significant difference when several

contrasts were made. A frequency test was done using SAS 8.2 for the most common substrate found in all container types. These two different comparisons using the ProcFreq 8.2 Version of SAS (SAS 2000) were utilized to determine the frequency of both containers and substrate type, and containers and shade conditions. Relative Risk (RR), a "risk ratio calculated by dividing the incidence in individuals exposed to a risk factor by incidence in nonexposed individuals"(Smith 1995), was compared for various variables between LAC encephalitis and control sites by dividing the highest percentage by the lowest percentage (i.e., clear water 62.88/7.30=8.61).

iv. Results and Discussion

Comparison of all containers at LAC encephalitis and control sites:

Artificial and natural container types: Numbers of bird baths, plastic tarps, 3.8-liter paint jugs, bowls, pans, and lids were significantly greater at control sites; whereas, only tires were significantly greater at LAC encephalitis sites (Table 10). Significantly greater percentages of tree holes ($P< 0.001$) and permanent containers ($P< 0.001$) were found in control sites, yet LAC encephalitis sites had significantly ($P< 0.001$) greater percentages of disposable containers (Table 11). Accumulated tires, a disposable container, have been directly associated with human cases of LAC encephalitis cases in Wisconsin (Parry 1983).

Substrate Type: No substrate within all artificial and natural containers were found 18 times more often at control sites than LAC encephalitis sites (RR= 18.67) ($P< 0.001$) (Table 12). A greater percentage of control sites had artificial and natural containers with grass in them ($P< 0.001$) (Table 12). Control and LAC encephalitis sites

Table 10. Numbers and proportions of container type at LAC encephalitis (LACE) and control sites in eastern Tennessee, 2001.

	LACE			Control			
Container Type	Numbers	Numbers of LACE sites with Various containers	% of Total	Numbers	Numbers of LACE sites with various containers	% of Total	P
Bird bath	2	2	0.68	26	19	25.74	0.000****
Plastic Tarp	1	1	0.34	6	5	5.94	0.001****
18.9-Liter jug	0	0	0.00	1	1	0.99	0.256
Animal trough	0	0	0.00	3	1	2.97	0.016
3.8-Liter paint jug	0	0	0.00	26	9	25.74	0.000****
Tires	250	1	85.32	5	1	4.95	0.000****
Bowl, pan, lids	39	2	13.31	34	4	33.66	0.000****
Drink bottles, vase	1	1	0.34	0	0	0.00	1.000
Total	293	7	100.00	101	30	100.00	

**** Significant at the 0.006625 alpha level

Table 11. Numbers and proportions of tree holes and containers at LAC encephalitis (LACE) and control cases in eastern Tennessee, 2001.

	LACE			Control			
Container Type	Numbers	Numbers of LACE sites with various artificial and natural containers	% of Total	Numbers	Numbers of Control sites with various artificial and natural containers	% of Total	P
Disposable container	251	2	82.84	23	8	16.43	0.000***
Permanent container	22	6	7.26	67	28	47.14	0.000***
Tree holes	30	2	9.90	51	20	36.17	0.000***
Total	303	10	100.00	141	56	100.00	

*** Significant at the 0.01667 level

Table 12. Substrate types contained within containers at LAC encephalitis (LACE) and control sites.

	LACE		Control		
Substrate Type	**Number**	**% of Total**	**Number**	**% of Total**	**P**
None	7	2.33	43	43.43	0.000*
Grass	0	0.00	6	6.06	0.000*
Leaves	1	0.33	1	1.01	0.434
Various	291	96.68	44	44.44	0.000*
Others	2	0.66	5	5.05	0.011
Total	301	100.00	99	100.00	

* Significant at the 0.01 level

were not significantly different when artificial and natural containers with leaves and containers with other substrate types were compared between the sites (P=0.434211) (Table 12). Containers with various substrates (leaves, grass, soil, etc.) were twice as likely to be encountered at LAC encephalitis sites than at control (RR=2.18) (Table 12).

Shade conditions: Sunlit containers were thirty-nine times more likely to be found at control sites than LAC encephalitis sites (RR=39.66) (P< 0.001) (Table 13). Partially shaded containers were almost five times more likely to be found at control sites than LAC encephalitis sites (RR=4.99) (P< 0.001) (Table 13). However, totally shaded containers were five times more likely to be encountered at LAC encephalitis sites than control sites (RR=5.86) (P< 0.001) (Table 13).

Water level and color: Containers with no water, ¼ full of water, ½ full of water, and full of water were predominantly observed at control sites than at LAC encephalitis sites (P< 0.001) (Table 14). Eighty-nine percent of the containers at LAC encephalitis sites were ¾ full, whereas only three percent of the containers were ¾ full at control sites (P< 0.001) (Table 14). A greater percentage of the containers at control sites had water that was clear rather than at the LAC encephalitis sites (P< 0.001) (Table 15). Of the containers found at LAC encephalitis sites, most were light yellow (P< 0.001) (Table 15).

Organic material and amount of organic matter: A higher percentage of containers at control sites had no organic matter than at LAC encephalitis sites (P< 0.001) (Table 16). Containers that were less than ¼ full of organic matter present were commonly encountered at LAC encephalitis sites rather than control sites (P< 0.001)

Table 13. Shade conditions within containers at LAC encephalitis (LACE) and control sites.

	LACE		Control		
Shade Conditions	**Number**	**% of Total**	**Number**	**% of Total**	**P**
Sunlit	5	1.66	65	65.66	0.000***
Partially shaded	11	3.64	18	18.18	0.000***
Shaded completely	286	94.70	16	16.16	0.000***
Total	302	100.00	99	100.00	

*** Significant at the 0.0167 level

Table 14. Water level within containers at LAC encephalitis (LACE) and control sites.

	LACE		Control		
Water Level	**Number**	**% of Total**	**Number**	**% of Total**	**P**
No water	20	6.64	36	36.36	0.000*
1/4 full	6	1.99	42	42.42	0.000*
1/2 full	3	1.00	8	8.08	0.001*
3/4 full	268	89.04	3	3.03	0.000*
Full	4	1.33	9	9.09	0.001*
Total	301	100.00	98	100.00	

* Significant at the 0.01 level

Table 15. Water color within containers at LAC encephalitis (LACE) and control sites.

	LACE		Control		
Water Color	Number	% of Total	Number	% of Total	P
Clear	22	7.31	61	62.89	0.000**
Light Yellow	258	85.71	3	3.09	0.000**
Light green	1	0.33	0	0.00	1.000
Brown	0	0.00	1	1.03	0.243
Total	281	100.00	65	100.00	

** Significant at the 0.0125 level

Table 16. Amount of organic matter within containers at LAC encephalitis (LACE) and control sites.

	LACE		Control		
Organic Matter Level	Number	% of Total	Number	% of Total	P
No Organic Matter	9	3.16	41	70.69	0.000**
Less than ¼ full	276	96.84	8	13.79	0.000**
¼ to ¾ full	0	0.00	6	10.34	0.000**
More than ¾ full	0	100.00	3	5.17	0.014
Total	285	0.00	58	100.00	

** Significant at the 0.0125 level

(Table 16). Control sites also had more containers that were ¼ to ¾ full of organic matter than LAC encephalitis sites ($P < 0.001$) (Table 16).

Mosquito presence: Control sites contained a higher percentage of containers with mosquitoes present that emerged as adults from larval collections ($P < 0.003$) and mosquitoes present that did not emerge as adults from larval collections ($P < 0.001$) within containers; however, LAC encephalitis sites contained higher percentages of no mosquitoes present ($P < 0.001$) (Table 17).

Comparison of all containers that had adult mosquitoes emerge in laboratory conditions and the attributes in these containers.

Artificial and natural container types: *Aedes albopictus* was the most frequently encountered species collected in tree holes (67%,n=4), permanent containers (68%, n=70), and disposable containers (94%, n=64) (Table 18). *Aedes albopictus* preferred inhabiting earthen cement pots, plant pots, molluscan shells, bamboo stumps, and tree holes in an urban garden study conducted in Calcutta city from October 1995 to September 1996 (Ray and Tandon 1999). *Aedes albopictus* readily utilized all container types to continue immature development on a temperate island of Japan, yet the most common site of immature development was in cemetery stone vases (Sota et al. 1994). *Aedes albopictus* was discovered in mango tree holes, an ovitrap, and a tire in Fiji (Laille et al. 1990). In Sarawak, a study conducted from 1989 to 1992 revealed *Ae. albopictus* preferred plastic cups and used tires outside, and ant traps and flower vases inside to undergo immature development (Seng and Jute 1994). *Ochlerotatus triseriatus* was found in low frequencies in tree holes (33%, n=2), permanent containers (13%, n=13),

Table 17. Presence or absence of mosquitoes in containers at LAC encephalitis (LACE) and control sites.

	LACE		Control		
Mosquito Presence	**Number**	**% of Total**	**Number**	**% of Total**	**P**
Present	3	1.00	7	7.22	0.003*
Absent	287	95.35	75	77.32	0.000*
Present none emerged as adults from larvae	11	3.65	15	15.46	0.000*
Total	301	100.00	97	100.00	

* Significant at the 0.01667 level

Table 18. Frequency of containers that held *Aedes albopictus*, and *Ochlerotatus triseriatus*.

Mosquito Species	**Tree hole**		**Permanent container**		**Disposable Container**		
	Number	**% of Total**	**Number**	**% of Total**	**Number**	**% of Total**	**Total**
Ae. albopictus	4	3	70	51	64	46	138
Oc. triseriatus	2	11	13	68	4		
Total	6		83		68	21	19

and disposable containers (6%, n=4). *Ochlerotatus triseriatus* larvae were recovered from tires, metal containers, plastic containers, and fiberglass water troughs at LAC encephalitis sites in Minnesota (Hedberg et al. 1985). *Ochlerotatus triseriatus* primarily inhabited tree holes, tires, paint cans, buckets, and various other containers at LAC encephalitis sites in Ohio (Berry 1984). No association was found between *Ae. albopictus* and *Oc. triseriatus* with container type (P=0.0543). In McLennan County, Texas, cemetery flower vases (permanent container) examined from June to December 1992 yielded *Ae. albopictus*, *Oc. triseriatus*, *Oc. hendersoni* Cockerell, and *Ae. epactius* Dyar and Knab consistently throughout the summer, yet no *Ae. aegypti* were found (Duhrkopf 1994). *Aedes aegypti*, *Ae. albopictus*, *Ae. pseudoscuttelaris* Theobald, and *Ae. polynesiensis* Marks larvae were all collected primarily from discarded tires and water drums in Fiji in 1992 (Ray and Tandon 1999).

Substrate type and containers: Permanent containers (30%) and disposable containers (30%) with mixed substrates were the most commonly encountered type of habitat for *Ae. albopictus* larvae that emerged as adults in the laboratory (Table 19). Mixed substrates were the only substrate type found in tree holes (Table 19). Sixty-five percent of all artificial and natural containers had mixed substrates in them. Other containers found that contained larvae had no substrate (4%, n=4), grass (10%, n=10), and other substrates (sand, tree bark, pieces of twigs) (21%, n=22). *Ochlerotatus triseriatus* larvae commonly develop in tree holes containing decaying vegetation in Michigan (Wilmont et al. 1992). *Ochlerotatus triseriatus* were predominantly collected from artificial containers containing water and leaf litter in central Missouri (Debboun and Hall 1992).

Table 19. The various substrate types located in containers that produced adult mosquitoes.

Container Type	No substrate		Grass Substrate		Mixed Substrate		Other		
	Number	% of C. T.	Number	% of C. T.	Number	% of C. T.	Number	% of C. T.	
Tree hole	0	0	0	0	3	10	0	0	
Permanent container	3	10	3	10	9	30	3	10	
Disposable container	0	0	0	0	9	30	0	0	
Combined Total (C. T.)									30

Shade conditions and containers: Permanent containers holding *Oc. triseriatus* and *Ae. albopictus* larvae were most frequently encountered in completely shaded conditions (Table 20). *Ochlerotatus triseriatus* larvae commonly inhabit shaded areas (Jenkins and Carpenter 1946, Barton 1978, Wilmont et al. 1992, Joy and Hildreth-Whitehair 2000). All tree holes holding mosquito larvae were only found in completely shaded conditions (Table 20). Disposable containers with *Ae. albopictus* larvae present were found in both sunlit (20%) and completely shaded conditions (10%) (Table 20). Disposable containers had mosquito larvae were predominantly found in sunlit conditions (61%) (Table 21). Permanent containers (97%) containing *Ae. albopictus* larvae were commonly found in completely shaded conditions (Table 21). Sunlit disposable containers, partially shaded permanent containers, and completely shaded containers held *Ae. albopictus* larvae (Table 21), although greater numbers were found in completely shaded permanent containers (Table 21).

v. Summary

Numbers of bird baths, plastic tarps, 3.8-liter paint jugs, bowls, pans, and lids were predominantly found at control sites. Significantly greater percentages of tree holes ($P < 0.001$) and permanent containers ($P < 0.001$) were found at control sites. Most control sites had artificial and natural containers with no substrate and grass. Partially shaded and sunlit artificial and natural containers were predominantly found at control sites. Containers with no water, ¼ full of water, ½ full of water, and full of water were predominantly observed at control sites. A greater percentage of the containers at control

Table 20. Containers holding mosquito larvae are illuminated by various shade conditions.

Container Type	Sunlit		Partially shaded		Completely shaded		
	Number	% of C. T.	Number	% of C. T.	Number	% of C. T.	
Tree hole	0	0	0	0	3	10	
Permanent container	3	10	3	10	12	40	
Disposable container	6	20	0	0	3	10	
Combined Total (C.T.)							30

Table 21. Shade conditions found within containers holding mosquito larvae.

Shade Conditions	Tree hole		Permanent container		Disposable container		
	Number	% of Total	Number	% of Total	Number	% of Total	Total
Sunlit	0	0	0	0	39	100	39
Partially Shaded	0	0	2	100	0	0	2
Completely Shaded	4	4	68	70	25	26	97

sites had water that was clear. A higher percentage of containers at control sites had no organic matter, and were ¼ to ¾ full of organic matter. Control sites contained a higher percentage of containers with mosquitoes present that emerged as adults from larval collections and mosquitoes present that did not emerge as adults from larval collections within containers.

Only tires were predominantly found at LAC encephalitis sites. LAC encephalitis sites had significantly ($P < 0.001$) greater percentages of disposable containers. Most LAC encephalitis sites had containers with various substrates (leaves, grass, soil, etc.). Totally shaded containers were more likely to be encountered at LAC encephalitis sites. Most containers at LAC encephalitis sites were ¾ full. Of the containers found at LAC encephalitis sites, most were light yellow. Containers that were less than ¼ full of organic matter present were commonly encountered at LAC encephalitis sites. LAC encephalitis sites contained higher percentages of no mosquitoes present.

Aedes albopictus was the most frequently encountered species collected in all natural and artificial containers, yet *Oc. triseriatus* was found in low frequencies in natural and artificial containers. Permanent containers and disposable containers with mixed substrates were the most commonly encountered type of habitat for *Ae. albopictus* larvae that emerged as adults in the laboratory. Grass, no substrate, and other substrates (sand, tree bark, pieces of twigs) were all commonly found in natural and artificial percentage of containers with mosquitoes present that emerged as adults from larval collections and mosquitoes present that did not emerge as adults from larval collections within containers.

Only tires were predominantly found at LAC encephalitis sites. LAC encephalitis sites had significantly ($P < 0.001$) greater percentages of disposable containers. Most LAC encephalitis sites had containers with various substrates (leaves, grass, soil, etc.). Totally shaded containers were more likely to be encountered at LAC encephalitis sites. Most containers at LAC encephalitis sites were ¾ full. Of the containers found at LAC encephalitis sites, most were light yellow. Containers that were less than ¼ full of organic matter present were commonly encountered at LAC encephalitis sites. LAC encephalitis sites contained higher percentages of no mosquitoes present.

Aedes albopictus was the most frequently encountered species collected in all natural and artificial containers, yet *Oc. triseriatus* was found in low frequencies in natural and artificial containers. Permanent containers and disposable containers with mixed substrates were the most commonly encountered type of habitat for *Ae. albopictus* larvae that emerged as adults in the laboratory. Grass, no substrate, and other substrates (sand, tree bark, pieces of twigs) were all commonly found in natural and artificial containers that held larvae. Permanent containers and tree hole holding *Oc. triseriatus* and *Ae. albopictus* larvae were primarily encountered in completely shaded conditions and a few were found in partially shaded conditions. Disposable containers that held mosquito larvae, especially *Ae. albopictus* were in both sunlit, and completely shaded conditions.

IV. Conclusions

i. Case comparison study

The high overall mosquito burden for *Ae. albopictus* at LAC encephalitis sites suggests that this mosquito is more common at LAC encephalitis residences and is also a vector for La Crosse Virus (LACV). Eliminating sites where container-dwelling immature mosquitoes can develop will help to reduce mosquito populations. Thus, placing all artificial containers, especially disposable containers (e.g., pools, trash, easily discarded items), upside down to eliminate water from collecting can be an effective method of reducing mosquitoes. Also, plastic tarps covering equipment were a common source of immature mosquito development; therefore, straightening out these plastic tarps so no small cavities form to catch and hold water would prevent sites where mosquitoes could develop in. In addition, filling up all tree holes (e.g., natural containers) up to five meters with sand will prevent the immature mosquitoes from developing. Permanent containers that hold water purposely (e.g., bird baths) should be cleaned and replenished with fresh water weekly.

ii. Further Research Direction

When conducting this type of research, oviposition strips should be removed from the vehicle and placed in the shade while other research is conducted to ensure that the eggs don not desiccate or overheat. An oviposition attractant could also be added to the tap water (e.g., tannic acid) in the oviposition traps to increase the number of eggs deposited on the oviposition substrate.

Further research to compare artificial and natural containers should be conducted within the same time period (e.g., 9:00 to11:00 am) to ensure that data are consistent

when shade condition is measured. When possible, a device that measures shade should be used.

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Vita

De'Jun Melona Stanich was born in Tucson, Arizona, on November 27, 1976. She graduated from Lake Worth High School, Lake Worth, Florida, in June 1995 and from The University of Tennessee in Chattanooga, Tennessee in December 2000 with a Bachelors of Science in Biology.

In January 2001, she entered the department of Entomology and Plant Pathology at The University of Tennessee in Knoxville, Tennessee. She worked as a graduate research assistant with Dr. Reid R. Gerhardt. In December 2002, she received the Master of Science degree with a major in Entomology. She will pursue a career in biological research.

The author is a member of Tennessee Entomological Society, Gamma Sigma Delta Agricultural Honor Society, Institute Women's Association, and the Graduate Student Association for the Entomology and Plant Pathology department in Knoxville, Tennessee.

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