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SCIENTIFIC NOTE

LA CROSSE VIRUS VECTOR RESTING BEHAVIORS - FIELD STUDIES WITH PROKOPAK AND RESTING SHELTER COLLECTIONS PROVIDE LOW YIELD

MARY NORDGULEN,[1] KAYLIN LEWANDOWSKI,[1] NATHAN BURKETT-CADENA,[2] DERRICK MATHIAS[2] AND BRIAN D. BYRD[1,*]

ABSTRACT. Resting adult mosquito collections provide opportunities to sample broad physiological conditions (e.g., blood-engorged, gravid, nectar-engorged, and/or parous) that yield important biological information necessary to understand vector and pathogen transmission ecology. In this study, we evaluated Prokopak aspirations of *Rhododendron* spp. and human-powered pop-up resting shelter collections at 4 residences with historical evidence of proximal La Crosse virus (LACV) transmission from May through September 2022. The goal of this study was to investigate these sampling methods in the context of LACV vector biology–focused principally on *Aedes triseriatus* (primary LACV vector) and 2 invasive species (*Ae. albopictus* and *Ae. japonicus*) that likely serve as secondary LACV vectors. Overall, 304 resting shelters and 80 Prokopak collections yielded a grand total of 33 mosquitoes, of which a third were LACV vectors (*Ae. triseriatus* [n = 1, 3.0%], *Ae. albopictus* [n = 4, 12.1%], and *Ae. japonicus* [n = 6, 18.2%]). *Anopheles punctipennis* (n = 9, 27.2%) was the most frequently collected species followed by *Culex erraticus* (n = 7, 21.2%), whereas the least frequently collected species were *Ae. triseriatus* and *Cx. pipiens* (n = 1, 3.0%). Despite substantial collection efforts, and concurrent gravid-trap evidence of LACV vectors at the collection sites, Prokopak aspiration of *Rhododendron* spp. and human-powered pop-up resting shelters did not yield a meaningful number of LACV vectors and thus, as described within, may not be useful adjuncts for the evaluation of LACV ecology and disease risk. Additional approaches to evaluate the resting behavior of these vectors in LACV endemic areas are needed.

KEY WORDS La Crosse virus vectors, *Aedes triseriatus*, *Aedes japonicus*, *Aedes albopictus*

La Crosse virus (Family *Peribunyaviridae*) is the most common cause of pediatric arboviral encephalitis in North America (Vahey et al. 2021). In North Carolina (NC), La Crosse virus (LACV) is typically the most common endemic cause of arboviral disease in humans; most cases occur in children residing within persistent geographic foci in western NC (Day et al. 2023a). Despite the long-standing public health significance of LACV disease, critical knowledge gaps remain about vector ecology, pathogen-transmission risk, and control strategies that could be addressed with more efficient methods to sample resting LACV vectors.

The eastern tree hole mosquito, *Aedes triseriatus* (Say), is the primary vector of LACV and invasive secondary vectors include *Ae. albopictus* (Skuse) and *Ae. japonicus* (Theobald) (Day et al. 2023b).

La Crosse virus can be transmitted horizontally through host feeding by an infected female, and venereally by mating. In *Ae. triseriatus*, vertical transovarial transmission leads to LACV overwintering in the mosquito egg and likely promotes interannual persistence of the virus focally (Day et al. 2023b).

Adult *Ae. triseriatus* resting behaviors are sparsely reported in the literature (Irby and Apperson 1992, Burkett-Cadena et al. 2008). *Aedes triseriatus* has been noted to rest on vegetation near larval breeding sites, on moss and lichen (Beier et al. 1982), and in swamps, woods, and swamp-wood ecotones (Irby and Apperson 1992). In a La Crosse endemic area of West Virginia, *Ae. triseriatus* population densities were greater in sugar maple and red maple habitats than in hemlock, mixed wood, or small red maple tree habitats based on ovitrap collections (Nasci et al. 2000). *Aedes albopictus* and *Ae. japonicus* are well known peridomestic species within southern Appalachia; however, their resting habits and plant interactions are poorly described in the region. Work by Urquhart et al. (2016) investigating 5 different collection methods in a shared Tennessee/NC LACV disease focus demonstrated greater collection yields for *Ae. albopictus* (6.6% of species-specific total) than *Ae. japonicus* (5.6% of species-specific total) and *Ae. triseriatus* (1.6% of species-specific total) in resting box collections; however, low sample size limited statistical inference. Taken together, the resting behaviors of *Ae. triseriatus* and the invasive secondary vectors in the LACV endemic area of western NC have not been sufficiently defined.

For this study, we investigated the resting habitats of *Ae. triseriatus* and other LACV vectors in an endemic area of western NC. Two collections methods were used, a small diameter ("Prokopak") aspirator (Vazquez-Prokopec et al. 2009) and human-powered pop-up resting shelters, as described by Burkett-Cadena et al. (2019). Study sites were located at 4 residences, 2 in Haywood County and 2 in Jackson County, NC (Fig. 1A). Each site was known to have

[1] Mosquito and Vector-Borne Infectious Disease Laboratory, College of Health and Human Sciences, Western Carolina University, Cullowhee, NC 28723.

[2] Florida Medical Entomology Laboratory, University of Florida, Vero Beach, FL 32962.

* To whom correspondence should be addressed.

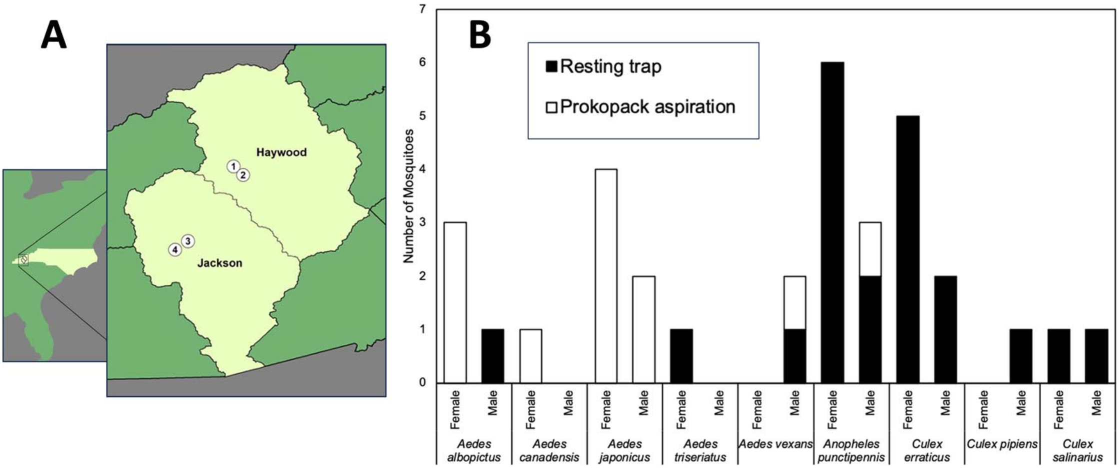

Fig. 1. A) Study locations in western North Carolina (Haywood and Jackson counties). B) Captured mosquitoes by species and collection method (black: resting trap, white: Prokopack aspiration).

or was within proximity (<300 m) to previous LACV activity (i.e., disease case investigations or detection in mosquito pools by the author) and nested within oak-hickory dominated forest habitat. Sites were residential habitats possessing native *Rhododendron* spp. plants–a dominant plant species commonly found at residences of LACV disease in western NC. Prior unpublished observations by our lab have documented *Ae. triseriatus* resting and foraging on *Rhododendron* (Byrd, unpublished data). Four human-powered pop-up resting shelters were placed at each site: two within 5 m of the house and two >15 m from the house. Shelters were placed so that they experienced the deepest shade in morning light conditions with the openings facing in a westward direction. Sites were typically sampled twice weekly from approximately 0900 to 1500 h (~81% of collections were made from 0900 to 1200) during May 31 through June 30 and August 22 through September 23, 2022. A 10- or 15-min Prokopak aspiration was performed on peridomestic *Rhododendron* spp. at each site. Subsequent collection attempts from the resting shelters occurred after a minimum of ~24 h (range 23–170 h) after the previous collection attempt. Gravid traps (Reiter-Cummings Modified Mosquito Trap, BioQuip, Rancho Dominguez, CA) infused with *Festuca* spp. hay (Sither et al. 2023) were placed at each site during the last week of the study, to confirm the presence of LACV vectors. Field collected mosquitoes were placed in individual containers, labeled by site and time of collection, and placed in a cooler with ice. Mosquitoes were returned to the laboratory, freeze-killed, and stored in a –20°C freezer. Specimens were identified on a chill table to species level using dichotomous keys found in Harrison et al. (2016). Species identification and sex for each mosquito were recorded in Excel (Microsoft, Redmond, WA). The resulting dataset was exported as .csv files for data visualization and descriptive statistics, and Z-tests of proportions in R (Version 4.2.1 (2022-06-23) and RStudio (Build 576, 2022.07.02, Package: tidyverse; R Core Team, 2021).

Mosquitoes collected by gravid traps were pooled by species, site, and date in 2 ml microcentrifuge tubes with stainless steel beads and subsequently stored at –20°C. Specimens were shipped overnight on dry ice to the Centers for Disease Control and Prevention (Division of Vector Borne Diseases, Ft. Collins, CO) and stored at –80°C. Virus testing was completed by qRT-PCR for La Crosse and Jamestown Canyon viruses (Hughes et al. 2022).

We collected 33 mosquitoes from Prokopak aspirations (n = 12 mosquitoes) and resting shelter collections (n = 21) representing three genera and nine species (Fig. 1B). *Anopheles punctipennis* (Say) (27.2%) was the most frequently collected species followed by *Cx. erraticus* (Dyar and Knab) (21.2%) and *Ae. japonicus* (18.1%), while the least frequently collected species were *Ae. triseriatus* (3%) and *Cx. pipiens* (L.) (3%). Eleven (33% of the total) primary and secondary LACV vectors were collected (Table 1).

A total of 80 Prokopak collections occurred over the study periods, with successful collections at all 4 study sites. Twelve (15%) of the total aspiration attempts resulted in the capture of at least a single mosquito. A total of 12 mosquitoes, representing 5 species, were collected by Prokopak aspiration on *Rhododendron* spp. plants (Table 1). Overall, the collection rate for Prokopak aspirations were low, and the highest collection rate occurred from August through September, with 0.08 mosquitoes collected per 5-min interval.

A total of 304 resting shelter collections (76 per site) occurred over the study periods with only 20 resting shelter collections (6.7% of total effort) resulting in the capture of at least a single mosquito. Mosquitoes were not captured by resting shelter collections at sites 1 and 2. In total, 21 mosquitoes representing seven species were collected by resting shelters (Table 1). The most

Table 1. Mosquitoes captured by collection type, Haywood County and Jackson County, NC, USA, 2022.

Species	Prokopak aspiration		Resting shelters		Total
	Females	Males	Females	Males	
Native LACV Vector					
Aedes triseriatus	0	0	1	0	1
Invasive LACV Vectors					
Ae. albopictus	3	0	0	1	4
Ae. japonicus	4	2	0	0	6
Non-LACV Vectors					
Anopheles punctipennis	0	1	6	2	9
Ae. canadensis	1	0	0	0	1
Ae. vexans	0	1	0	1	2
Culex erraticus	0	0	5	2	7
Cx. pipiens	0	0	0	1	1
Cx. salinarius	0	0	1	1	2

	Gravid Trap Collections			
	Site 1 (% Female)	Site 2 (% Female)	Site 4 (% Female)	
Ae. japonicus	39 (100%)	11 (90.9%)	13 (100%)	63
Ae. triseriatus	20 (100%)	9 (100%)	14 (100%)	43

[1] Four days of field collections (one trap per site) during the final week of the study; no successful collections were made at site 3.

common species (42.9%) was *An. punctipennis* (9 total; 66.7% female), and the second most common (33.3%) was *Cx. erraticus* (7 total; 71.4% female). These two species accounted for 76.2% of the total resting shelter collections. Two additional *Culex* species (*Cx. pipiens* [1 male], and *Cx. salinarius* (Coquillett) (2 total; 1 male, 1 female]) represented 14.3% of the collections. A single *Ae. triseriatus* (4.8% of the total), the primary vector of LACV, was collected from a resting shelter. The majority (90.5%) of mosquitoes collected by resting shelters were non-LACV vectors. Overall, female mosquitoes (61.9%) were more commonly captured by resting shelters.

The proportion of LACV vectors collected differed by the two methods (Table 1). Of the mosquitoes collected by the Prokopak, 75% were LACV (native and invasive) vectors. In contrast, only 9.5% of mosquitoes collected by the resting shelters were LACV vectors. The differences in these proportions were statistically different ($Z = 3.84$, $P < 0.001$). Overall, the resting shelters collected more *Anopheles* and *Culex* mosquitoes than the Prokopak. Although *An. punctipennis* was the most common mosquito collected, the Prokopak only captured one *An. punctipennis* male. Similarly, all *Culex* species collected during this study were collected by the human-powered resting shelters. Collectively, the proportion of *Anopheles* and *Culex* collected by the Prokopak (8.3%) was lower than the resting shelters (85.7%); this difference was statistically significant ($Z = 4.33$, $P < 0.001$). There was no statistical difference in resting shelter collections by time of day, overall number of females captured, and collections by proximity or distance to homes; however, the low number of collected mosquitoes limited the ability to make statistical inference in this study.

During the final week of the study, we collected 106 mosquitoes from gravid traps of which 40.6% ($n = 43$) were female *Ae. triseriatus*, the primary vector of LACV, and 59.4% ($n = 63$ [1 male]) were the invasive secondary LACV vector, *Ae. japonicus* (Table 1). Because traps (1 at each household) were sequentially deployed for 2–3 days at a time over the 1-week period, there were a total of 4 days of field collection efforts for gravid traps by investigators. This resulted in an average yield of 26.5 mosquitoes by field collection effort, which exceeds the total collections from resting shelter and Prokopak collections. La Crosse and Jamestown Canyon viruses were not detected by qRT-PCR analyses of the gravid trap collections.

In this study, we investigated the resting behaviors of *Ae. triseriatus*, and secondary invasive LACV vectors (*Ae. albopictus* and *Ae. japonicus*), in an endemic area in western NC using Prokopak and resting shelter collections. Of 304 resting shelter collections, 6.7% of the total effort resulted in the capture of at least a single mosquito. Our results coincide with the findings of Urquhart et al. (2016), suggesting limited collections of LACV vectors by resting traps in eastern Tennessee–a shared LACV disease focus with western NC in the Southern Appalachian region. The Prokopak collected more LACV vectors than the resting shelter; however, the single *Ae. triseriatus* (primary LACV vector) was captured by a resting shelter. The overall capture rate was low, with only 15% of total aspiration attempts resulting in the capture of at least a single mosquito. During the final week of field work, coincident with Prokopak aspirations and resting shelter collections, our gravid trap collections confirmed the presence of two LACV vectors at all sites where traps were successfully deployed.

During a 2021 field study (Byrd, unpublished data), researchers from our laboratory collected 27 times (overall rate, standardized by effort) more mosquitoes at 2 of the same study sites as ours, using a large bore "Nasci" aspirator suggesting that perhaps a larger diameter aspirator may be more effective (Byrd, unpublished data). Taken together, our Prokopak aspirations and resting shelter collections likely underestimate the presence of LACV vectors. The gravid trap was an effective tool to verify the presence of *Ae. triseriatus* and *Ae. japonicus*; however, it does not provide new information on the resting behaviors of LACV vectors. This leads us to question the effectiveness of collection methods used in this study for these specific mosquito vectors.

In future studies, investigators may wish to sample from human-powered pop-up shelters during a nocturnal period and/or prior to morning twilight, increase the number of resting shelters deployed, and consider orienting the shelters in different directions. The use of a more labor-intensive method of collection, such as a large-bore aspirator, may continue to provide some information on the resting behaviors and daily rhythms of LACV vectors. To promote effective disease prevention and control measures, there remains a critical need to further elucidate the resting behaviors of LACV vectors.

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