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La Crosse Virus Field Detection and Vector Competence of *Culex* Mosquitoes

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Abstract. La Crosse virus (LACV), a leading cause of arboviral pediatric encephalitis in the United States, is emerging in Appalachia. Here, we report field and laboratory evidence that suggest LACV may be using *Culex* mosquitoes as additional vectors in this region. This bunyavirus was detected by reverse-transcriptase polymerase chain reaction in two pools of *Culex* mosquitoes in southwestern Virginia and in six pools in West Virginia. To assess vector competence, we offered LACV blood meals to field-collected *Culex restuans* Theobald, *Cx. pipiens* L., and *Aedes triseriatus* (Say). Both *Culex* species were susceptible to infection. LACV-positive salivary expectorate, indicative of the ability to transmit, was detected in a small proportion of *Cx. restuans* (9%) and *Cx. pipiens* (4%) compared with *Ae. triseriatus* (40%). In a companion study of *Cx. restuans* only, we found that adults derived from nutritionally stressed larvae were significantly more likely to disseminate and transmit LACV. Our results indicate a potential role of *Culex* spp. in LACV dynamics that should be explored further in endemic areas.

INTRODUCTION

Arbovirus surveillance of field-collected mosquitoes is an important aspect of public health and mosquito control programs. However, virus-positive field samples from previously undocumented vector species can be challenging to interpret for two reasons. First, virus-positive field samples from unexpected species may result from incorrect morphologic species identification or species cross-contamination, both of which can be determined using molecular assays.[1–4] Second, positive samples may reflect virus that is simply present in the midgut of a mosquito following a blood meal on an infected vertebrate host without the subsequent infection and dissemination that is necessary for vector competence,[5] thus having no epidemiologic significance. Experimental assessment of vector competence is therefore imperative to determine if the virus is capable of overcoming the midgut infection barrier,[6] disseminating through the hemocoel after surmounting the midgut escape barrier to infect other tissues, and prevailing over the salivary barriers to be orally transmitted.[7]

La Crosse virus (LACV), a California serogroup *Orthobunyavirus*, remains a major cause of pediatric arboviral encephalitis in the United States.[8–10] Since its 1964 isolation in Wisconsin,[11] LACV has been identified in 32 states within the contiguous United States.[12] The Appalachian region, where this study was performed, is part of an emerging focus of LACV.[13,14] This arbovirus is maintained in hardwood forests largely through *Aedes triseriatus* transovarial vertical transmission.[15,16] In fact, the primary LACV vector can overwinter the virus in tree holes.[17] However, vertical transmission alone is insufficient to maintain LACV in nature,[15] and thus horizontal transmission between mosquito vectors and sciurid rodents (i.e., chipmunks, squirrels)[18] is essential for LACV to persist in the environment and to maintain its ability to amplify successfully in vertebrate hosts. Results from a model parameterized with experimental infection data indicate that horizontal transmission between sciurid rodents and mosquitoes likely accounts for approximately 50% of female mosquito infections with LACV.[15] Finally, horizontal transmission of LACV can also occur via *Ae. triseriatus* venereal transmission,[16] but this route is thought to represent a small contribution to LACV transmission.

Vector competence studies have identified the capacity for other *Aedes* species to serve as vectors of LACV: *Ae. albopictus*, *Ae. aegypti*,[19] and *Ae. japonicus*.[20] Virus isolation from field-collected *Ae. albopictus* mosquitoes has confirmed their potential to serve as vectors in the field.[1,21] Although vector competence research revealed poor virus multiplication in *Ae. canadensis*,[22] field research has shown that they may serve as accessory vectors of LACV.[23,24] In addition to other *Aedes* species, LACV was first isolated from field-collected *Culex pipiens* in Wisconsin in 1967,[25] and here we document multiple field-collected pools of *Cx. pipiens*/*restuans* mosquitoes that were LACV-positive in the Appalachian region. Although LACV dissemination and transmission had not been assessed in *Cx. pipiens* or *Cx. restuans* before this study, Tesh and Gubler infected *Cx. fatigans* (*Cx. p. quinquefasciatus*) with LACV by intrathoracic inoculation and isolated virus from whole body plaque assays 8–10 days postinfection.[26,27] These results suggest that LACV-positive field samples from *Cx. pipiens* may represent true infection versus virus-positive vertebrate blood meals and/or misidentification. Here we test this by assaying the LACV vector competence of *Cx. pipiens* and *Cx. restuans*, for which we and others have documented LACV in the field.[25]

The purpose of this study was to determine if *Cx. restuans* and *Cx. pipiens* can become orally infected with LACV, disseminate the virus, and transmit it. Furthermore, because nutritionally stressed larvae are known to impact *Ae. triseriatus* vector competence for LACV,[28,29] we also conducted a companion experiment to determine if *Cx. restuans* mosquitoes, dominant over *Cx. pipiens* in southwestern Virginia and West Virginia,[30,31] are more efficient vectors when larvae are resource limited.

METHODS

Appalachian field testing for LACV. *Virginia mosquito collection.* In 2008, adult mosquitoes were collected weekly from infusion-baited gravid traps[32] at oak-dominant sites in Jefferson National Forest[33] in Montgomery County, VA.

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After a minimum of 24-hour storage in a −80°C freezer, mosquitoes were identified and pooled into groups of up to 50 females by species, collection site, and date. Because important adult taxonomic characters may be damaged or missing after field collection,[34,35] *Cx. restuans* and *Cx. pipiens* mosquitoes were pooled. Such pools will hereafter be referred to as *Cx. pipiens*/*restuans*.

RNA extraction and qualitative real-time reverse transcription polymerase chain reaction of Virginia mosquito pools. Mosquito pools from 2008 were submitted to the Virginia Division of Consolidated Laboratory Services for virus detection. Qualitative reverse transcription polymerase chain reaction (qRT-PCR) (i.e., no cutoff value) was used to determine if this bunyavirus was present on our study sites. Of bovine albumin diluent (BA-1),[36] 1 mL was added to each mosquito pool. Mechanical homogenization of pooled mosquitoes was performed with a 4.5-mm steel bead; the resultant homogenate was centrifuged for 5 minutes at 13,500 rpm. Viral RNA was extracted from the supernatant of the homogenized mosquito pools with the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RT-PCR targeting the M segment of LACV was conducted with the QuantiTect probe RT-PCR Kit (Qiagen) using two primer-probe sets (Table 1). We present the threshold cycle (C_T), defined as the amplification cycle at which the fluorescence increased above the threshold value (i.e., crossing point cycle). For each run, 45 amplification cycles were performed. Samples with a crossing point on the first run underwent a total of six RT-PCR assays on two machines (ABI PRISM 7000 [Applied Biosystems, Inc., Foster City, CA] and LightCycler 2.0 [Roche Diagnostics, Indianapolis, IN]) with four independent DNA extractions.

West Virginia mosquito surveillance. Mosquito surveillance was conducted from May 22, 2013 through September 25, 2013 as part of the West Virginia Department of Health and Human Resources Mosquito Surveillance Program. The 56 collection sites spanned the eastern, western, and central regions of West Virginia.[30] Samples were collected weekly from counties with high (Nicholas, Fayette, and Raleigh) and low human incidence of LACV (Kanawha, Jackson, and Wood) as previously defined.[14] Samples were also collected on a semi-regular basis in additional counties by the state health department or weekly by local health agencies. Infusion-baited gravid traps, carbon dioxide–emitting light traps, and BG Sentinel traps (Bio-Quip Products, Rancho Dominguez, CA) with octenol lures were used to capture adult mosquitoes. Trap site selection was based on habitat suitability for the vectors of West Nile virus (WNV) and LACV as well as ease of accessibility. Traps were either placed in an open area or within the transitional zone between open area and deep forest cover. Specimens of the same genus, collecting locality, and collecting date were placed in the same pool and tested for LACV. *Culex* species from the same survey site and collection date were tested together because of difficulty in differentiating field-damaged *Cx. restuans* from *Cx. pipiens* and efforts to conserve laboratory resources. Although *Cx. pipiens*/*restuans* was the most active *Culex* mosquito group, other *Culex* species (i.e., *Cx. erraticus*) were incorporated in the *Culex* mosquito pools.

RNA extraction and quantitative real-time RT-PCR of West Virginia mosquito pools. Mosquitoes were tested for LACV using real-time RT-PCR. Mechanical homogenization of mosquitoes was performed with two copper beads in each pool and lysed in guanidine isothyiocyanate–containing RNA lysis buffer (RLT from RNeasy kit, Qiagen). The homogenate was centrifuged at 17,000 rpm for 3 minutes. The QIAamp RNeasy Mini Kit (Qiagen) was used to isolate viral RNA from the resultant homogenate. Real-time RT-PCR was used to detect LACV using AgPath-ID One-Step RT-PCR with detection enhancer (Applied Biosystems). PCRs were run using the ABI 7500 FAST Real-Time PCR system (Applied Biosystems). Biosearch Technologies (Novato, CA) provided the primers (LAC935, LAC1018c) and Taqman probe (LAC963).[38] Forty amplification cycles were performed. Samples with a C_T value ≤ 40 were considered positive.

LACV vector competence experiment. *Mosquito collection and rearing for vector competence study. Culex* egg rafts were collected using oviposition traps on the campus of Virginia Tech, Blacksburg, VA.[32] *Aedes triseriatus* eggs were collected using ovitraps placed in forested areas near the road at the entrance to Jefferson National Forest in Montgomery County, VA.[13] Collected mosquitoes were not tested for LACV before this study. However, LACV has not been detected in *Ae. triseriatus* (50 eggs) or *Culex* (30 egg rafts) at these collection sites (Fan Yang, unpublished data). In addition, *Ae. triseriatus* egg LACV-infection rates (0.15/1,000) are very low in Montgomery County (Bova J, unpublished data).[39]

Eggs were reared to adults in an insectary (24°C, 75% relative humidity, and 16:8 light:dark [L:D] hour) as previously described.[40] Approximately 24 hours after placement in the environmental chamber, newly hatched larvae were morphologically identified to species.[41] Larvae were reared at a density of 250 larvae per container (33 × 17.5 × 11 cm^3) in 1,600 mL deionized water and fed ad libitum bovine liver powder solution (7.5 g/500 mL). Adults were provided with 10% sucrose water on a cotton pledget ad libitum. The field-collected *Cx. pipiens*, *Cx. restuans*, and *Ae. triseriatus* were used for experimentation at 7–10 days old postemergence.

TABLE 1
Primers used for amplification of LACV

State	Year	Primer/probe set	LACV M segment primer sequence (5(→ 3′)	Source
Virginia	2008	LAC836 probe	CATCCATTCACAGAGTGTGGCACGC	(Robert S. Lanciotti, CDC, personal communication)
		LAC812 LF1	TGCAAGCTATGCGGCCTAGT	
		LAC881 LR1	AGCGAGCACCACAGACACAA	
		LAC2387 probe	AATGGGCCAAGTGTGTATAGGAAACCATCA	
		LAC2364 LF2	CAATAATTGCGTGTGGTGAACC	
		LAC2448 LR2	GACCGATCAGTGCTAGATTGGAA	
West Virginia	2013	LAC963 probe	TGTGCAAGTCGAAAGGGCCTGCA	[37]
		LAC935	TATAAAAGCCTAAGAGCTGCCAGAGT	
		LAC1018c	GACCAGTACTGCAGTAATTATAGACAAT	

LACV = La Crosse virus.

Larval nutritional stress experiment. Larvae that were confirmed to be *Cx. restuans* were distributed into two plastic shoe boxes ($33 \times 17.5 \times 11$ cm^3) containing 1,600 mL deionized water and approximately 200 larvae. The larvae were fed a 500 mL/7.5 g bovine liver powder (MP Biomedicals, Solon, OH) solution. The control group was fed 45 mL bovine liver powder solution while the nutritionally stressed larvae in the other container were only fed 15 mL liver powder. Thus, the nutritionally stressed group was always fed one-third the amount of nutrients that the control group received.[29] Five days after the initial feeding, larvae were fed a second time with 30 mL liver powder given to the control group and 10 mL liver powder given to the nutritionally stressed group. Containers were checked daily for pupae that were placed in a separate container until their emergence. As above, adult mosquitoes were fed a 10% sucrose solution on a cotton pledget ad libitum. To determine if nutritional stress influenced adult size, wings were measured to assess body size.[42] Wing length was measured using the Dino-Lite digital microscope (AM-4113ZTL; Big C, Torrance, CA).

Virus. The LACV strain used for this study (VA0921075) was isolated from *Ae. triseriatus* collected in Duncan Gap, VA, in 1999.[13] The isolate was maintained in the laboratory by 12 alternate passages through *Ae. triseriatus* mosquitoes and African green monkey kidney (Vero) cells. The viral stock titer was 5.3×10^7 plaque-forming units (PFU)/mL.

Vector competence. Forty-eight hours before artificial blood meal feeding, week-old mosquitoes were transferred to 1-L cages with mesh screening on top and only offered deionized water on a cotton pledget. Female mosquitoes were separated into species-specific groups of 30–50 mosquitoes. They were allowed to engorge overnight on an artificial blood meal offered on a cotton pledget. The blood meal contained 1 mL LACV mixed with 9 mL of pre-warmed rabbit or chicken blood (Lampire Biological Products, Pipersville, PA) and 10% sucrose. The blood meal returned to ambient temperature overnight. At the beginning of the feeding period, at least 0.2 mL of each blood meal was saved for viral titer determination. Virus titer was determined by using standard plaque assays on Vero cells with a series of 10-fold serial dilutions performed in duplicate six-well plates.[43]

Groups of blood-fed mosquitoes were transferred to 0.7 L (1-pint) cages and maintained on 10% sucrose for 2 weeks. Half of the mosquitoes from each group were collected at 10 and 14 days postexposure (DPE), respectively. Females were immobilized by chilling on ice or exposure to triethylamine.[44] To assess transmission potential, their salivary expectorate was collected by inserting their proboscis into a capillary tube filled with a 1:1 mixture of 10% sucrose and fetal bovine serum.[45–47] After 30 minutes, tube contents were expelled into 0.3 mL of BA-1 diluent and stored at −80°C along with abdomen and legs for later virus testing. Abdomen and legs were homogenized separately with one steel BB followed by centrifugation (5,000 rpm for 3 minutes). Mosquitoes were tested for infection by plaque assays. Wells were scored as positive or negative depending on the presence or absence of plaques, respectively. If virus was recovered from the abdomen but not from the legs or expectorate, the mosquito was considered to have a non-disseminated infection limited to the midgut. If virus was recovered from the abdomen and legs, it was classified as a disseminated infection. Mosquitoes with a virus-positive expectorate were classified as transmitting.

Statistics. Fisher's exact test was used to compare the percentage of infected, disseminated, and transmitting mosquitoes between the three vector species and treatment groups. Confidence intervals for these rates were calculated using package PropCIs (various confidence interval methods for proportions) in R (R package version 0.2-5, Ralph Scherer, Hannover, Germany), which is based on the modified Wald method.[48] Wing length measurements were analyzed by using one-way analysis of variance. All analyses were conducted in R version 3.0.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/) (R Development Core Team, 2013).

RESULTS

Qualitative detection of LACV RNA in Virginia mosquito pool samples. Of the adult *Cx. pipiens/restuans* collected from Montgomery County in 2008, 1,071 were combined into 64 pools and analyzed for LACV. Two field-collected pools of *Cx. pipiens/restuans* mosquitoes collected from this population in July ($n_{\text{mosquitoes}} = 7$) and August ($n_{\text{mosquitoes}} = 3$) were weakly positive for the presence of LACV M segment RNA. Out of four runs with the more sensitive LAC2364/2448 primers, a positive signal was obtained from the *Cx. pipiens/restuans* pools in three (C_T values: 43, 40, and 42) or two (C_T values: 44, 41) runs for the July and August pools, respectively. None of the qualitative positives were confirmed with the less sensitive LAC812/LAC881 primers. These early LACV primer sets from the Centers for Disease Control and Prevention have since been improved on.[37] The bias-corrected maximum likelihood estimate (MLE) infection rate for LACV-infected *Cx. pipiens/restuans* in Montgomery County was estimated to be 1.8 (95% confidence level [CL] = 0.3–6) infected mosquitoes per 1,000 specimens.[49]

Quantitative detection of LACV RNA in West Virginia mosquito pool samples. In 2013, 13,363 adult *Culex* mosquitoes from West Virginia were combined into 388 mosquito pools and analyzed for LACV. LACV was detected in six of these 388 mosquito pools (Table 2), which were collected in late August through early September. The bias-corrected MLE LACV infection rate for *Culex* spp. in West Virginia was 0.4 (95% CL = 0.2–0.9) infected mosquitoes per 1,000 specimens.[49] LACV-positive *Culex* mosquitoes were collected in urban and peridomestic habitat[30] in the Central Allegheny plateau (Kanawha County), Ohio River lowland (Jackson and Cabell Counties), and the Alleghany highlands (Berkeley County).

Vector competence. Virus titers of blood meals ranged from 4.1×10^6 to 2.9×10^7 PFU/mL. *Culex* feeding success was low, as has been previously noted,[50] so multiple groups were offered blood meals (Table 3). Three groups of *Cx. restuans*,

TABLE 2
Detection of LACV in West Virginia *Culex* spp. by RT-PCR

County	Collection date	C_T values	Number of mosquitoes in pool
Kanawha	8/27/2013	34	1
Kanawha	8/29/2013	38	3
Berkeley	8/30/2013	34	2
Jackson	9/4/2013	37	3
Cabell	9/11/2013	33	8
Kanawha	9/18/2013	39	8

LACV = La Crosse virus; RT-PCR = reverse transcription polymerase chain reaction.

TABLE 3
Relative vector competence of *Culex restuans*, *Cx. pipiens*, and *Aedes triseriatus* for LACV following oral exposure

Species and group number	Blood meal LACV titer	DPE	Sample size (*n*)	% Non-disseminated (95% CI)	% Disseminated (95% CI)	% Transmitting (95% CI)
Cx. restuans 1	2.9×10^7	10	16	12 (0–0.4)	31 (0.1–0.6)	31 (0.1–0.6)
Cx. restuans 2	1.8×10^7	10	5	0 (0–0.5)	0 (0–0.5)	0 (0–0.5)
Cx. restuans 3	8.5×10^6	10	12	0 (0–0.3)	0 (0–0.3)	0 (0–0.3)
Cx. restuans 1	2.9×10^7	14	14	0 (0–0.2)	0 (0–0.2)	0 (0–0.2)
Cx. restuans 2	1.8×10^7	14	7	14 (0–0.5)	0 (0–0.4)	0 (0–0.4)
Cx. restuans 3	8.5×10^6	14	13	0 (0–0.3)	8 (0–0.4)	8 (0–0.4)
Total *Cx. restuans*	–	–	67	5 (0–0.1)	9 (0–0.2)	9 (0–0.2)
Cx. pipiens 1	1.3×10^7	10	13	15 (0–0.4)	8 (0–0.4)	8 (0–0.4)
Cx. pipiens 2	1.3×10^7	10	21	5 (0–0.3)	14 (0–0.4)	9 (0–0.3)
Cx. pipiens 1	1.3×10^7	14	18	5 (0–0.3)	0 (0–0.2)	0 (0–0.2)
Cx. pipiens 2	1.3×10^7	14	18	17 (0–0.4)	17 (0–0.4)	0 (0–0.2)
Total *Cx. pipiens*	–	–	70	10 (0.1–0.2)	10 (0–0.2)	4 (0–0.1)
Ae. triseriatus	1.4×10^7	10	21	10 (0–0.3)	57 (0.4–0.7)	57 (0.4–0.7)
Ae. triseriatus	1.4×10^7	14	21	33 (0.2–0.5)	33 (0.2–0.5)	24 (0.1–0.5)
Total *Ae. triseriatus*	–	–	42	21 (0.1–0.4)	45 (0.3–0.6)	40 (0.3–0.5)

CI = confidence interval; DPE = days postexposure; LACV = La Crosse virus.
Mosquitoes were collected at 10 and 14 DPE. If virus was recovered from the abdomen but not from the legs or expectorate, the mosquito was considered to have a non-disseminated infection. If virus was recovered from the abdomen and legs, it was classified as a disseminated infection. Mosquitoes with virus-positive salivary expectorate were classified as transmitting.

the dominant *Culex* spp. on our study sites, two groups of *Cx. pipiens*, and one group of *Ae. triseriatus* were offered LACV blood meals for this study. Before conducting statistical analyses across species, the proportion of non-disseminated infections, disseminated infections, and transmitting mosquitoes across the replicate groups within species were compared. There was no significant difference between the three *Cx. restuans* groups in terms of the percentage of non-disseminated ($P = 0.40$), disseminated ($P = 0.17$), or transmitting ($P = 0.17$) mosquitoes. For *Cx. pipiens*, there was no significant difference between the groups in terms of the percentage of non-disseminated ($P = 1.0$), disseminated ($P = 0.12$), or transmitting ($P = 1.0$), indicating that the small differences in viral titers of the blood meals did not influence our results. Therefore, to maximize statistical power, groups were pooled within species for statistical comparisons. Both *Cx. restuans* and *Cx. pipiens* were susceptible to infection with LACV, and there was no significant difference between the percentage of non-disseminated infections ($P = 0.33$), disseminated infections ($P = 1.0$), or transmitting mosquitoes ($P = 0.32$; Table 3). *Aedes triseriatus* had a significantly greater percentage of non-disseminated ($P = 0.01$), disseminated ($P < 0.001$), and transmitting ($P < 0.001$) mosquitoes when compared with *Cx. restuans*. There was no significant difference between non-disseminated infections from *Ae. triseriatus* and *Cx. pipiens* ($P = 0.10$). However, there was a significantly greater percentage of *Ae. triseriatus* disseminated ($P < 0.001$) and transmitting ($P < 0.001$) mosquitoes when compared with *Cx. pipiens*.

***Cx. restuans* nutritional stress experiment.** Based on wing length, larvae reared under nutritionally stressed conditions were significantly smaller (mean wing length = 3.30 mm, standard deviation [SD] = 0.16 mm) compared with those reared under control nutritional conditions (mean wing length = 3.41 mm, SD = 0.21 mm; $F_{1,58} = 5.3$; $P = 0.025$).

Virus titers of blood meals for the control and nutritionally stressed groups were 4.1×10^6 and 8.5×10^6 PFU/mL, respectively. There was no significant difference in non-disseminated infections between the groups ($P = 0.10$). However, nutritionally stressed mosquitoes were more likely to have disseminated infections ($P = 0.002$) and virus-positive expectorate ($P = 0.02$) compared with the control mosquitoes. There was no evidence of transmission based on salivary expectorate testing for the control group but a small percentage had disseminated LACV infections (6%) (Table 4). In contrast, LACV-positive expectorate was evident in the nutritionally stressed group (18%).

DISCUSSION

Our results indicate that *Cx. restuans* and *Cx. pipiens* are both susceptible to LACV infection, but they are not as permissive to LACV as the primary vector *Ae. triseriatus*. Our results concur with other laboratory studies in that *Ae. triseriatus* demonstrated greater vector competence for LACV than other species. *Aedes albopictus* and *Ae. aegypti* have been shown to be less permissive to LACV than *Ae. triseriatus* in terms of oral infection and vertical

TABLE 4
Effect of larval nutritional stress on *Culex restuans* vector competence for LACV following oral infection

Treatment group	Sample size (*n*)	DPE	% Non-disseminated (95% CI)	% Disseminated (95% CI)	% Transmitting (95% CI)
Control	16	10	0 (0–0.2)	12 (0–0.4)	0 (0–0.2)
Control	15	14	0 (0–0.2)	0 (0–0.2)	0 (0–0.2)
Total control	31	–	0 (0–0.1)	6 (0–0.2)	0 (0–0.1)
Nutritionally stressed	14	10	7 (0–0.3)	0 (0–0.3)	0 (0–0.3)
Nutritionally stressed	14	14	14 (0–0.4)	86 (0.6–1.0)	36 (0.2–0.6)
Total nutritionally stressed	28	–	11 (0–0.3)	43 (0.3–0.6)	18 (0.1–0.4)

CI = confidence interval; DPE = days postexposure; LACV = La Crosse virus.
Vector competence of nutritionally stressed mosquitoes was compared with that of control groups. Mosquitoes were collected at 10 and 14 DPE. If virus was recovered from the abdomen but not from the legs or expectorate, the mosquito was considered to have a non-disseminated infection. If virus was recovered from the abdomen and legs, it was classified as a disseminated infection. Mosquitoes with virus-positive salivary expectorate were classified as transmitting.

transmission.[19] *Aedes canadensis* has also been found to have low LACV transmission efficiency with 25–27% transmission rates.[22] However, a more recent invading vector, *Ae. japonicus*, has been shown to have nearly identical transmission rates as *Ae. triseriatus*.[20] Although *Ae. triseriatus* is the most efficient transmitter of LACV, this bunyavirus appears to be making use of several accessory vectors. *Aedes canadensis* has been shown to have field infection rates greater than *Ae. triseriatus* in West Virginia.[24] In Ohio, LACV was isolated more often from *Ae. canadensis* than *Ae. triseriatus*.[23] This pathogen also appears to be taking advantage of recent biotic invasions. LACV has been isolated from *Ae. albopictus* in regions where this species is competing with the major LACV vector.[1,21] In addition, LACV has been detected[51] and isolated from field-collected *Ae. japonicus*.[52]

Our results demonstrated experimentally that *Culex* spp. are capable of transmitting this arbovirus and may serve as additional vectors of LACV. Because these were newly colonized *Culex* strains, rather than established laboratory colonies, these results are likely to be more representative of field vector competence. However, their poor vector competence, low field infection rates, and high C_T values suggest that their contribution to LACV dynamics may be small. Blood meal viral titers in this study (i.e., 10^6–10^7 PFU/mL) were equivalent or higher than the maximum LACV viremia levels that sciurid rodents are known to develop (10^6 PFU/mL).[53] Yet *Culex* vector competence was still quite low, suggesting that *Culex* species may not play a very large role in LACV dynamics. However, in our companion experiment, the percentage of disseminated infections of *Cx. restuans* increased to 43% (within the range of *Ae. triseriatus*; Table 3) when larvae were nutritionally stressed, suggesting that under some environmental conditions, *Culex* species may play a significant role in LACV dynamics.

Depending on the virus-vector system, there is evidence that larval nutritional stress may affect the ability of adult mosquitoes to serve as arboviral vectors. Stress at the larval stage resulting in smaller adult body size has been associated with higher infection and transmission rates in *Ae. triseriatus* with LACV,[54] in North American strains of *Ae. aegypti* and *Ae. albopictus* with dengue-2 virus,[55] in *Cx. p. pipiens* with WNV,[56] and in *Ae. aegypti* infected with Sindbis virus.[57] Our findings agree with these in that larval nutritional stress and smaller adult *Cx. restuans* were more likely to transmit LACV than larger adults. In fact, the percentage of disseminated infections in our nutritionally stressed *Cx. restuans* (43%) falls within the confidence interval detected for the primary LACV vector, *Ae. triseriatus* (Table 3), and is similar to that reported for *Ae. albopictus* (41%).[19] There are other studies, however, that have not found this connection. Large, not small, *Ae. aegypti* were more competent for Ross River virus[58] and chikungunya virus.[59] In fact, large Thailand strains of *Ae. aegypti* were more likely to be infected with dengue-2 virus in a different study.[60] No correlation between body size and vector competence has been reported for *Cx. tarsalis* infected with WNV,[47] western equine encephalitis virus, or St. Louis encephalitis virus.[61] Extrinsic factors (i.e., changes in the abiotic environment and interspecific interactions) may influence adult body size and vector competence depending on the species and virus. In the case of *Cx. restuans*, our results specifically suggest that larval nutritional conditions may influence the ability of this species to serve as vectors for LACV. Further study should investigate the mechanism underlying this result.

On the basis of our laboratory results, we suspect that our LACV-positive field samples were most likely due to true infections with LACV. We did not find *Culex* species (in the absence of larval nutritional stress) to be very permissive to LACV. The ornithophilic feeding preferences of *Culex* mosquitoes may also prevent them from playing a major role in LACV dynamics. However, *Cx. restuans* and *Cx. pipiens* will engorge on sciurid rodents,[62–64] the primary amplifying vertebrate hosts for LACV. The degree to which *Cx. pipiens* populations are mammalophilic versus ornithophilic can vary at both small[62] and large geographic scales.[65,66] The latter was suspected to be due to introgression of the underground *Cx. pipiens* form *molestus*, an aggressive human biter and mammalophilic mosquito. Limited hybridization occurs between *Cx. p. pipiens* f. *molestus* and the *Cx. p. pipiens* f. *pipiens*,[67] but Virginia and West Virginia are within the hybridization zone of *Cx. p. pipiens* and *Cx. p. quinquefasciatus*.[68,69] Recent work suggests hybrids of *Cx. pipiens* and *Cx. quinquefasciatus* have enhanced transmission of WNV[70] and research suggests both *Cx. pipiens* and *Cx. fatigans* (*Cx. p. quinquefasciatus*) may be infected with LACV.[25,26] The vector competence of *Cx. p. pipiens*/*quinquefasciatus* hybrids for LACV, however, is unknown. Therefore, genetic studies combined with blood meal analyses and vector competence experiments are needed to further characterize *Cx. pipiens* L. complex populations in Appalachia and their potential to serve as vectors of arboviruses with mammalian reservoirs.

There are still many questions regarding the vectorial capacity of *Cx. restuans* and *Cx. pipiens* for LACV. First, future vector competence studies comparing oral and parenteral infections of these *Culex* species would help elucidate potential barriers to LACV dissemination and transmission. Second, the role of *Culex* species, which overwinter as adults[71] in contrast to *Ae. triseriatus* that overwinters in prepupal stages,[17,72] in contributing to LACV overwintering is particularly important to examine. It is interesting to note that in West Virginia, some of the LACV-positive *Culex* and LACV human cases were near abandoned or empty homes (Eric J. Dotseth, personal observation), which could serve as overwintering hibernacula. Arboviral surveillance of *Culex* emerging from hibernacula in LACV-endemic areas should be conducted to test this hypothesis. Artificial containers conducive to container-breeding *Aedes* have been associated with a higher risk of human LACV cases.[14,73,74] However, our results suggest that pest managers in LACV-endemic areas should control *Culex* breeding sites for WNV and LACV control. We recommend additional research to elucidate the role of *Culex* mosquitoes in LACV dynamics.

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