Forest Disturbance, Mosquito Vector Ecology and La Crosse Virus Dynamics in Southwestern Virginia

Maria-Richetta Camille Harris

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Dana M. Hawley, Committee Chair
Sally L. Paulson
Francois Elvinger
X.J. Meng
Robert H. Jones

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M. Camille Harris

Abstract

The influence of forest canopy disturbance (FCD) on La Crosse virus (LACV), leading cause of US pediatric arboviral encephalitis, is critical to understand in landscapes where forests are periodically harvested. Southwestern Virginia is part of an emerging focus of this interior forest bunyavirus. I investigated how the temperate forest mosquito community, LACV vectors, and the LACV amplifying vertebrate host (chipmunks) were impacted by logging. This research was conducted across an experimental FCD gradient (from least to most disturbed: contiguous control, fragmented control, clearcut, and high-leave shelterwood (SW)). Using gravid traps, I found that the mosquito community was resilient to logging with no significant difference in diversity or community composition across treatments. Mean number of female mosquitoes caught per trap-night declined with disturbance. FCD significantly affected the abundance of vector species in different ways. The primary LACV vector, *Aedes triseriatus*, and the recent invasive *Ae. japonicus* declined with logging. Other vectors (*Ae. albopictus, Ae. canadensis,* and *Ae. vexans)* thrived with logging. *Culex pipiens/restuans* was affected by disturbance but had no treatment preference. A mark-recapture study revealed that chipmunk abundance and LACV seroprevalence were greatest on the SW. In sync with *Ae. triseriatus* abundance but in contrast to the chipmunk results, mosquito LACV detection was significantly greater on unlogged sites. Surprisingly, LACV was detected in *Ae. japonicus* and *Cx. pipiens/restuans.* In a follow-up study, I isolated LACV from field-collected *Ae. japonicus*. Although LACV was previously isolated from *Cx. pipiens*, the vector competence was unknown. Therefore, I examined the vector competence of *Cx. pipiens* and *Cx. restuans*. Although poor vectors, I did detect LACV in the saliva of both species. An additional experiment found that nutritionally-stressed *Cx. restuans* were better vectors than those in the control group, indicating that environmental stressors (e.g., FCD) may alter the ability of accessory vectors to spread LACV. The influence of FCD on

LACV is complex. Because logging decreases *Ae. triseriatus* abundance, human LACV risk is likely lowered by decreased transovarial vertical transmission. However, high chipmunk seroprevalence on disturbed sites suggest horizontal transmission with accessory vectors plays a larger role in LACV risk on recently logged sites.

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List of Abbreviations

The following table describes the significance of abbreviations used throughout this dissertation. The page on which each one is first used is also given.

Abbreviation	Meaning	Page
AIC	Akaike information criterion	5
ANOVA	Analysis of variance	70
BA-1	Bovine albumin diluent 1	27
BB1	Blacksburg 1 study site	3
BB2	Blacksburg 2 study site	3
BB3	Blacksburg 3 study site	4
BLAST	Basic local alignment search tool	55
BSL-2	Biosafety level two	54
CCON	Contiguous control	4
CCUT	Clearcut	4
CDC	Centers for disease control and prevention	38
CPE	Cytopathic effects	28
C_T	Threshold cycle	27
DCLS	Virginia Division of Consolidated Laboratory Services	27
DPE	Days post-exposure	69
EU	Experimental unit	4
FCON	Fragmented control	4
GTR	General time reversible model	55
IACUC	Institutional Animal Care and Use Committee	29
LACV	La Crosse virus	22
MLE	Maximum likelihood estimation	62
MNKA	Minimum number known alive	30
NC	Newcastle study site	4
NCBI	National center for biotechnology information	58
NMDS	Non-metric multidimensional scaling	6
PERMANOVA	Permutational multivariate analysis of variance	6
PFU	Plaque-forming units	71
PRNT	Plaque-reduction neutralization test	29
RT-PCR	Reverse-transcriptase polymerase chain reaction	27
SASAB	Southern Appalachian Silviculture and Biodiversity Project	3
s.d.	Standard deviation	72
SE	Standard error	46
SW	High-leave shelterwood	4
VDGIF	Virginia Department of Game and Inland Fisheries	29
WNV	West Nile virus	75

Attributions

Dr. Dana M. Hawley, Associate Professor, Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia 24061. Dr. Hawley was my advisor and committee chair and she is a coauthor on all manuscripts in this dissertation (Chapters 1, 2, 3 and 4).

Dr. Sally L. Paulson, Associate Professor, Department of Entomology, Virginia Tech, Blacksburg, Virginia 24061. Dr. Paulson was a member of my committee and she is a coauthor on all manuscripts in this dissertation (Chapters 1, 2, 3 and 4).

Dr. Bryan L. Brown, Assistant Professor, Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia 24061. Dr. Brown, a community ecologist, is a coauthor on Chapter 1. He contributed to manuscript development and statistical analysis.

Mr. Dorian M. Jackson, undergraduate, Virginia Tech. Mr. Jackson, co-author on Chapter 4, conducted the *Culex restuans* nutritional-stress vector competence experiment.

Mr. Fan Yang, doctoral candidate, Department of Entomology, Virginia Tech, Blacksburg, Virginia 24061. Mr. Yang conducted vector competence experiments and contributed to the conceptual design of Chapter 4.

Dr. Eric J. Dotseth, State Public Health Entomologist, West Virginia Department of Health and Human Resources, Charleston, West Virginia, 25301. Dr. Dotseth contributed field and laboratory data to Chapters 3 and 4 as well as development of these manuscripts.

Dr. Paul E. Marek, Assistant Professor, Department of Entomology, Virginia Tech, Blacksburg, Virginia, 24061. Dr. Marek conducted phylogenetic analysis of the viral isolate in Chapter 3. He also contributed to manuscript development.

Dr. Steven D. Zink, Scientist, Arbovirology Lab, Wadsworth Center – New York State Department of Health, Slingerlands, NY. Dr. Zink designed the La Crosse virus primers and conducted RT-PCR of our field-collected mosquito samples in Chapter 2.

Dr. Bryan Jackson, Associate Public Health Biologist, Vector-Borne Disease Section, California Department of Public Health, Elk Grove, California, 95624. Dr. Jackson, co-author on Chapter 3, contributed to field and laboratory data as well as manuscript development.

Chapter 1

Disturbance, diversity and vectors: taxonomic response of the mosquito assemblage to logging in southwestern Virginia

M. Camille Harris, Bryan L. Brown, Sally L. Paulson, and Dana M. Hawley

Abstract

Forest disturbance may influence disease dynamics by impacting the diversity or abundance of mosquito vectors. Using an experimental field approach, we characterized mosquito communities across a gradient of forest disturbance (contiguous control, fragmented control, clearcut and high-leave shelterwood) in southwestern Virginia. From late May to September (2008-2010), 29,680 adult female mosquitoes were captured using infusion-baited gravid traps and identified morphologically to the species level. The three dominant species were *Aedes triseriatus* (55%), *Ae. japonicus* (21%), and *Culex pipiens/restuans* (20%). We found that the number of mosquitoes caught per trap-night significantly declined with the extent of forest disturbance. However, mosquito diversity and community composition were not significantly different across the treatments. The temperate forest mosquito assemblage appears to be largely resilient to logging used in even-aged silvicultural systems. Our results suggest that mosquito-borne disease risk, based on abundance, may be lower immediately following logging.

Introduction

As human populations increase, the associated demand on natural resources has resulted in landscape changes such as urbanization and logging. These anthropogenic changes often influence biodiversity (Belote et al. 2008, Duraes et al. 2013) and, in some cases, zoonotic disease risk (Keesing et al. 2006, Ezenwa et al. 2007, Pongsiri et al. 2009, Keesing et al. 2010). Several studies have examined how anthropogenic disturbance impacts the diversity of vertebrate reservoir hosts (Allan et al. 2003), and in turn, how high vertebrate diversity may decrease zoonotic disease risk when vectors feed upon a higher proportion of non-competent reservoir hosts (termed the “dilution

effect")(Schmidt and Ostfeld 2001, Ezenwa et al. 2006, Keesing et al. 2006, Allan et al. 2009). However, few studies have examined how anthropogenic changes such as forest disturbance impact the diversity of invertebrate taxa that commonly serve as disease vectors (Thongsripong et al. 2013). Using a silvicultural experimental approach which manipulated the extent of logging in otherwise similar forests, we sought to understand how logging may influence the diversity and composition of the mosquito assemblage in temperate forests. This research has implications for the dynamics of mosquito-borne avian hemoparasites (Laurance et al. 2013) and zoonotic arboviruses (Thompson et al. 1965) associated with temperate forests.

While the influence of logging on vector-borne pathogen dynamics has not been fully examined, there have been several studies on the response of invertebrates to logging. Lepidoptera abundance and diversity have been shown to decline with logging (Ghazoul 2002, Savilaakso et al. 2009), but immature Diptera are reported to have higher emergent rates and richness on logged sites (Batzer et al. 2005, Banks et al. 2007). The Order Diptera includes Culicidae mosquitoes, which can transmit disease. Timber harvesting may impact mosquito communities through multiple mechanisms. First, post-harvest changes in light levels and temperature may impact larval development (Couret et al. 2014) and the distribution of mosquito species (Berry and Craig 1984). Second, food sources for larval mosquitoes may be directly influenced by disturbance. For example, some species may thrive because increased sunlight after logging may increase algal biomass, which is consumed by larvae (Batzer et al. 2005, Banks et al. 2007). Because mosquito larvae prey on the microbes that process detritus, biomass of plant and animal detritus may impact mosquito responses to disturbance. In the short-term, clearcut and shelterwood harvests are known to increase detritus and coarse woody debris (Warren and Ashton 2014). Mosquito research in Mississippi mixed-hardwood forests has shown that plant and animal detritus is high in forested area tree holes, supporting higher larval densities, and low in urban area tires (Yee et al. 2012). Therefore, if detritus biomass or plant-animal detritus ratios (Yee et al. 2007) change post-disturbance, mosquito density may increase after logging. Third, physical removal of mature trees and an increased frequency of drying events (Kitching 2001) may limit available oviposition sites (e.g.,

tree holes) for phytotelm specialists (e.g., *Aedes*, *Orthopodomyia*). However, the desiccation-resistant eggs of such *Aedes* mosquitoes may allow them to withstand the harsh, dry conditions associated with highly disturbed forests (Sota and Mogi 1992, O'Neal and Juliano 2013). Finally, logging may indirectly impact mosquito communities by altering predator abundance and richness (Bradshaw and Holzapfel 1983, Murrell and Juliano 2013).

Here we sought to understand if even-aged forest management (single-entry and repeated-entry timber harvesting) influenced mosquito biodiversity in southwest Virginia. Because this assemblage includes the Culicine vectors of avian malaria (Kimura et al. 2010, Farajollahi et al. 2011), reptile haemogregarines (Smith et al. 1996, Harkness et al. 2010), filarial worms (Ledesma and Harrington 2011, Mehus and Vaughan 2013), and a human pediatric arbovirus (i.e., La Crosse encephalitis virus) (McJunkin et al. 1998, Haddow et al. 2011), our results have potential implications for understanding how logging affects human and wildlife disease risk. Based on the results from Culicine-dominant urban gradient studies (Johnson et al. 2008, Thongsripong et al. 2013), we predicted that intact undisturbed forest would have greater diversity and species richness with a lower abundance of disease vectors. The urban degradation gradient studies (Johnson et al. 2008, Thongsripong et al. 2013) were correlational, making it difficult to conclude whether forest disturbance or some other correlate of urbanization altered the diversity of mosquito communities. Here we use an experimental silvicultural approach, whereby forest plots were randomly assigned to one of three disturbance regimes, in order to examine how logging *per se* influences temperate mosquito communities in Appalachia.

Materials & Methods

Study Site. Our study sites in Jefferson National Forest in southwestern Virginia are part of a long-term investigation of silvicultural oak regeneration methods on biodiversity, the Southern Appalachian Silviculture and Biodiversity Project (SASAB) (Belote et al. 2008, Atwood et al. 2009, Homyack and Haas 2013). These oak-dominant (*Quercus* spp.) sites had similar overstory composition, age and topographic position (Belote et al. 2008).

Two sites used for this study (Blacksburg 1 and 2; BB1 and BB2, respectively) were located in Montgomery County, VA (37°17'35.73"N, 80°27'24.63"W (BB1); 37°18'20.35"N, 80°26'24.95"W (BB2)) while a third site (Newcastle (NC)) was located in Craig County, VA (37°27'20.78"N, 80°23'0.37"W).

Disturbance treatments. At each of the three SASAB study sites, seven two-hectare experimental units (EUs) were established with no buffer between the units. Silvicultural treatments were randomly assigned to EUs within sites using a fully randomized complete block design (Figure 1.1). For this study, three two-hectare silvicultural treatments were the focus of mosquito surveillance: repeated-entry high-leave shelterwood (SW) at 0-2 years post-disturbance; single-entry clearcut (CCUT) at 12-14 years post-disturbance; and fragmented unlogged controls (FCON) at 80-100 years old. Logging disturbance was examined on a gradient defined by the frequency of harvest and stand age: FCON>CCUT>SW. Both the CCUT and SW were harvested between 1995 and 1996 (Atwood 2008). Following the first harvest, all residual overstory stems on the SW were harvested between 2007 and 2008 (Homyack and Haas 2013), just prior to the initiation of this study. CCUT was defined as the removal of 95% of the basal overstory area while SW was defined as stand thinning with 56% of the basal area removed. FCON was defined as uncut areas with no direct timber harvesting disturbance but these sites were directly adjacent to disturbed sites with no buffer zone. These three silvicultural treatments were replicated across three study sites: BB1, BB2, and NC (Table 1.1).

Contiguous unlogged control sites. Because of the forest fragmentation created by the SASAB experimental design (Figure 1.1) and associated disturbance to FCON sites (e.g., skid trails, diffuse light from adjacent treatments), an additional non-SASAB study site (BB3) containing two contiguous controls (CCON) equivalent in size to the SASAB EUs was established for the purposes of this study. BB3 (37°18'48.59"N, 80°25'15.82"W) was also located in Jefferson National Forest (Montgomery County) for comparison to the fragmented controls (FCON) at nearby BB1 and BB2 (Table 1.1). BB3 was approximately 1.8 miles from BB2. These uncut sites were embedded within large areas

of contiguous forest that had not been recently disturbed by harvesting. These stands were dominated by oak (*Quercus alba, Q. velutina, Q. prinus)* along with yellow poplar (*Liriodendron tulipifera*). Red maple (*Acer rubrum*) and sourwood (*Oxydendrum arboretum)* were common in the midstory. The ages of the dominant and co-dominant trees in these stands were 100-130 years. Similar to the SASAB sites, the stands are on a south aspect with a moderate slope (J. Overcash, US Forest Service, pers. comm.).

Mosquito Sampling. From late May to September 2008-2010, adult mosquitoes were collected twice a week from infusion-baited gravid traps (Jackson et al. 2005). Five gravid traps were placed on each EU (Figure 1.1). A minimum 30 meter buffer zone was applied to each EU to minimize edge effects. After a minimum of 24-h storage in a -80°C freezer, mosquitoes were identified using morphological keys and pooled into groups with a maximum of fifty females by species, collection site, and date. Male mosquitoes only feed on nectar (Stone and Foster 2013) so they were not counted for this study. Female mosquitoes, which feed on plant sugars and vertebrate blood to obtain nutrients for oviposition, can transmit pathogens to animals and humans (Stone and Foster 2013). Because important adult taxonomic characters may be damaged or missing after field-collection (Saul et al. 1977, Harrington and Poulson 2008), which makes identification difficult, *Cx. restuans* and *Cx. pipiens* mosquitoes were pooled. Such pools will hereafter be referred to as *Cx. pipiens/restuans*. *Psorophora* were not identified to the species level.

Statistical Analysis. We compared mosquito community composition across forest disturbance treatments using univariate and multivariate metrics. For our univariate metrics, we evaluated three diversity indices that span values of the *q* order (Keylock 2005, Jost 2009). The *q* order is based on a diversity metric's sensitivity to common or rare species. An index with $q < 1$ is sensitive to rare species (i.e., richness). An index with $q > 1$ is most strongly influenced by common species (i.e., Simpson). Finally, the Shannon index ($q=1$) is influenced by common and rare species. Richness, Shannon and Simpson diversity indices were calculated using the vegan package of R. These univariate metrics were compared across disturbance treatments using linear mixed-effect

models (package nlme in R). The model with the lowest AIC was selected for this analysis. These models included temporal (year, Julian date) and spatial (study site location: BB1, BB2, NC, or BB3) variables as random effects with treatment (CCON, FCON, CCUT, or SW) as a fixed effect. Because BB3 did not have all the treatments, a separate analysis to compare mosquito abundance between the Montgomery County control (two replicates of CCON and FCON) treatments was conducted. We utilized a nested random effects structure with Julian date nested within year and year nested within study site location. This model (AIC: 2373) outperformed models with simpler random effects which considered location and year (2445), location and Julian date (2418), only location (2455), only year (2456), or only Julian date (2419). Mosquito community composition was examined using nonmetric multidimensional scaling (NMDS) (Kruskal 1964) and permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) in R package vegan. To examine the dissimilarity between the communities based on count data, we chose Bray-Curtis, a non-Euclidean distance measure. The appropriate number of dimensions for NMDS were chosen based on a Scree plot and associated stress values. All analyses were conducted in R version 3.00 (R Development Core Team 2013).

Results

We collected 29,680 adult female mosquitoes representing 15 species over three field seasons (2008-2010). The three dominant species were *Aedes triseriatus* (55%), *Ae. japonicus* (21%), and *Culex pipiens/restuans* (20%) (Table 1.2), all of which are in the subfamily Culicinae. Within the Culicinae (totaling 98.3% of all females), species collected represented five tribes: Aedini (78.75%), Culicini (20.97%), Mansoniini (0.01%), Orthopodomyiini (0.23%), and Toxorhynchitini (0.04%).

Disturbance had a significant effect on total mosquito abundance ($F_{3,380}$= 18.00, p<0.0001) with the lowest abundance on the shelterwood ($F_{3,380}$=17.80, p=0.04; Figure 1.2). Mosquito abundance on the CCON was not significantly different from that on the FCON ($F_{1,4}$=0.0027, p=0.96). Mosquito species richness ($F_{3,380}$ = 2.11, p=0.098), Shannon index ($F_{3,380}$ = 1.03, p=0.38) and the Simpson index ($F_{3,380}$ = 0.96, p=0.41) were

not significantly different across the treatments (Table 1.3). Although not significant, mosquito species richness tended to decline with the intensity of forest disturbance. For NMDS, two dimensions were chosen based on the Scree plot and stress values (Figure 1.3). Nonmetric multidimensional scaling of the mosquito communities suggested no significant difference between the communities across disturbance treatments (p=0.683).

Discussion

We found that the temperate forest mosquito community in southwestern Virginia was largely resilient to even-aged timber harvesting. Across a gradient of silvicultural treatments, there was no significant difference in diversity (Table 1.3) or community composition (Figure 1.3), with the exception of a trend toward lower species richness on disturbed sites. We found that our highest disturbance treatment (high-leave shelterwood) was associated with the lowest mosquito abundance (Figure 1.2). Because vector abundance is a key predictor of vector-borne disease risk (Antonovics et al. 1995, Mather et al. 1996), this result suggests that disease risk for humans and wildlife is likely to be lower immediately following a logging event.

Our results suggest that environmental conditions in closed-canopied forests are more favorable than disturbed sites for forest mosquito populations. Silvicultural treatment may impact mosquito abundance through microhabitat (e.g., sunlight levels, microbial biomass, and frequency of drying events) and macrohabitat changes (i.e., removal of overstory canopy cover and trees). It has been shown that tree density is an important factor for mosquito abundance in the Great Plains (O'Brien and Reiskind 2013). SASAB researchers have shown that partial harvesting (SW) on our study sites decreases stump sprouting, which is important for tree regeneration (Atwood et al. 2009). Because the shelterwood had the lowest tree regeneration when compared to the clearcut and control sites, our results are consistent with a role for tree density in driving mosquito abundance. Phytotelm specialists (e.g., *Ae. triseriatus*) are dominant across our sites so this tree density effect is most likely related to the presence of oviposition sites. Our result of declining abundance with disturbance is in contrast to mosquito larval studies that found the highest abundance of Culicidae on logged sites in Oregon and South Carolina (Batzer

et al. 2005, Banks et al. 2007). These contrasting results may be due to interspecific interactions at the larval stage, or may reflect idiosyncratic responses of the species present at these diverse study locations. Larval species interactions (e.g., competition and predation) are known to shape adult mosquito communities (Juliano 2009). Therefore, our results may represent the outcome of interspecific competition and predator-prey interactions occurring at the larval stage (Murrell and Juliano 2013), which we did not sample in this study. Because adult mosquitoes are the life stage capable of transmitting disease during blood-feeding, our finding of decreasing abundance of adult female mosquitoes with disturbance, while in contrast to prior larval results, is directly relevant for understanding how disease risk may change with forest disturbance.

The lower mosquito species richness observed on logged sites, although insignificant, may be a function of the loss of infrequently-caught species (Table 1.3). Because species richness is most sensitive to the presence or absence of rare species, this trend suggests that rare mosquito species were less likely to occur with higher levels of disturbance on our study sites. The overall resilience of the mosquito community to logging that we detected was not found in mosquito studies in the Peruvian Amazon and Thailand. These mosquito communities declined in diversity as the landscape was changed with urbanization (Johnson et al. 2008, Thongsripong et al. 2013). Our trend towards a decline in richness with logging was consistent, however, with those from the Amazon study that used rarefaction-based estimates of richness (Thongsripong et al. 2013). The disturbance gradient in our study was smaller than that examined in the Amazon study. Because our study specifically isolated the effects of logging, it is possible that other characteristics of urbanization explain the previously detected decreases in community diversity across that broad degradation gradient.

The mosquito assemblage resilience we observed across our gradient of forest disturbance may be related to source-sink dynamics or the storage effect. The source-sink model of metapopulation dynamics is based on habitat heterogeneity (Pulliam 1988). Persistence of insects in poor-quality sink habitats may be due to recolonization from high-quality source habitats (Frouz and Kindlmann 2001). The lower mosquito

abundance on disturbed forest suggests it may serve as a mosquito sink with higher mortality. Undisturbed forest with suitable oviposition habitat may serve as a source of emergent mosquitoes. Because the logged sites we examined were nested within a national forest, rather than an urban area, and were relatively small in size (2 ha), mosquitoes from nearby intact forest may have been able to rapidly colonize the disturbed sites. A mark-recapture study of *Culex* mosquitoes, for example, reported an average dispersal distance of 1.33 km (Ciota et. al. 2012). Second, logging treatments in our study occurred over the winter, when mosquitoes are in a dormant stage. Some species overwinter as mated adult females (e.g., *Culex*) while others are in pre-pupal stages (e.g., *Ae. triseriatus*) (Denlinger and Armbruster 2014). These mosquitoes may overwinter in tree holes or ground depressions. As such, logging most likely removed or killed many mosquitoes in diapause. However, those that remained could stay in that dormant state until environmental conditions were conducive for development (Bradshaw and Holzapfel 1984). This "storage effect" may promote mosquito community resilience. On the other hand, the observed mosquito assemblage resilience may partly be an artifact of the methods we used. Our mosquito collection methods may have hindered the detection of rare species because gravid traps are preferable for collecting generalist vector species (Reiter et al. 1986).

In summary, temperate forest mosquito communities are resilient to timber harvesting methods used in even-aged silviculture. Consistent with other studies, there was a trend of declining richness with increasing disturbance. Furthermore, the mean number of female mosquitoes caught per trap-night was lowest on the sites with the highest level of disturbance (i.e., high-leave shelterwood). This short-term decline in mosquitoes post-logging suggests mosquito-borne disease risk may also be lower at this time.

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Figure and Table Captions

Figure 1.1. Southern Appalachian Silviculture and Biodiversity Project (SASAB) disturbance treatments and mosquito sampling design. The layout for the Blacksburg 1 (BB1) study site is shown as an example, but due to randomization, the spatial arrangement of treatment assignments differed across the three SASAB study sites (see Methods). Three of the two-hectare silvicultural treatments (SW, CCUT, and FCON), highlighted in green, were used for La Crosse virus research on each SASAB site. After a minimum 30-meter buffer zone was established on each treatment, five infusion-baited gravid traps were placed as shown for mosquito collection. CCON=Contiguous control; FCON=Fragmented control, CCUT=Clearcut, and SW=High-leave shelterwood.

Figure 1.2. Mean number (±SE) of female mosquitoes caught per trap-night across four disturbance treatments, from least (CCON) to most disturbed (SW) as quantified by frequency of harvest and stand age. CCON=Contiguous control; FCON=Fragmented control, CCUT=Clearcut, and SW=High-leave shelterwood.

Figure 1.3. Ordination of mosquito communities across four different forest types. Mosquito communities did not vary significantly across treatments (p=0.68). Peach circle = Contiguous control, Grey square = Fragmented control, Purple diamond = Clearcut, and Black triangle = High-leave shelterwood.

Table 1.1. Experimental design of silvicultural disturbance treatments. The number of disturbance treatment replicates across the four study sites are shown. SASAB = Southern Appalachian Silviculture and Biodiversity Project. BB1 = Blacksburg 1, BB2 = Blacksburg 2, BB3 = Blacksburg 3, NC = Newcastle.

Table 1.2. Total female mosquito species abundance across four temperate forest treatments in southwestern Virginia. SASAB = Southern Appalachian Silviculture and Biodiversity Project. CCON=Contiguous control; FCON=Fragmented control, CCUT=Clearcut, and SW=High-leave shelterwood.

Table 1.3. Mean (±SE) species richness and diversity indices of mosquito communities across four treatments from lowest to highest disturbance in southwestern Virginia forests (2008-2010). [a]Number of sites or replicates for each habitat type. CCON=Contiguous control; FCON=Fragmented control, CCUT=Clearcut, and SW=High-leave shelterwood.

Figure 1. 1

Blacksburg Site 1
Silvicultural Treatments

1. Understory herbicide
2. Group selection
3. Clearcut (CCUT)
4. Control (undisturbed) (FCON)
5. High-leave shelterwood (SW)
6. Leave tree
7. Low-leave shelterwood

Figure 1. 2

$F_{3,380}=18.00, p<0.0001$

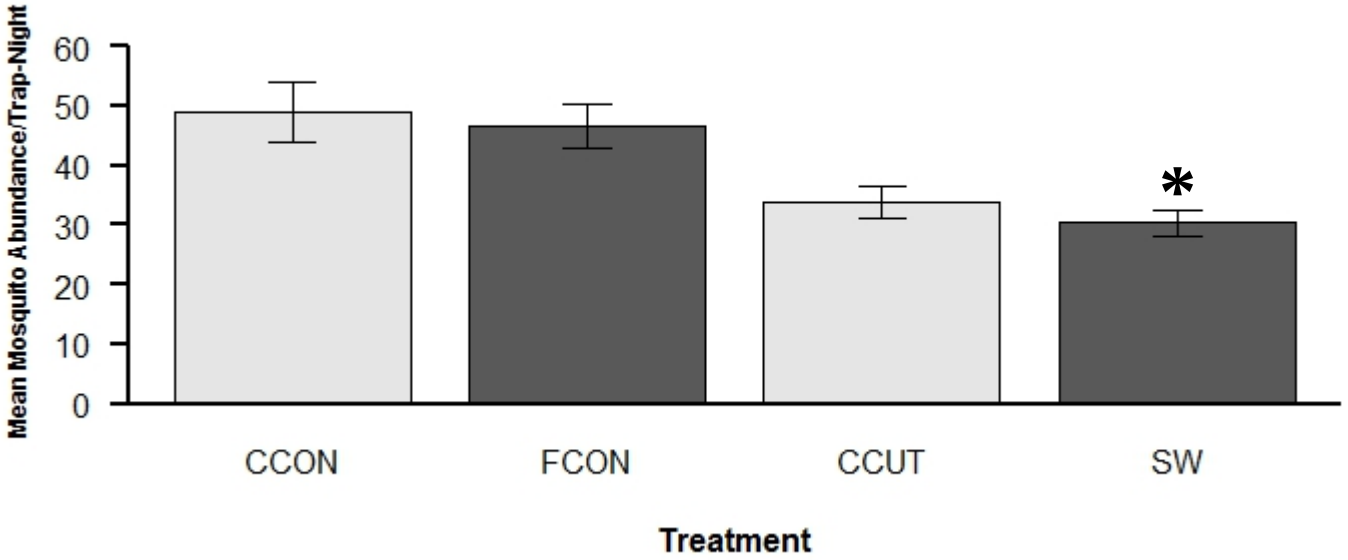

Figure 1. 3

2D Stress = 0.11

PERMANOVA: $F_{3,749}$=-57.7, p=0.68, R^2=-0.30

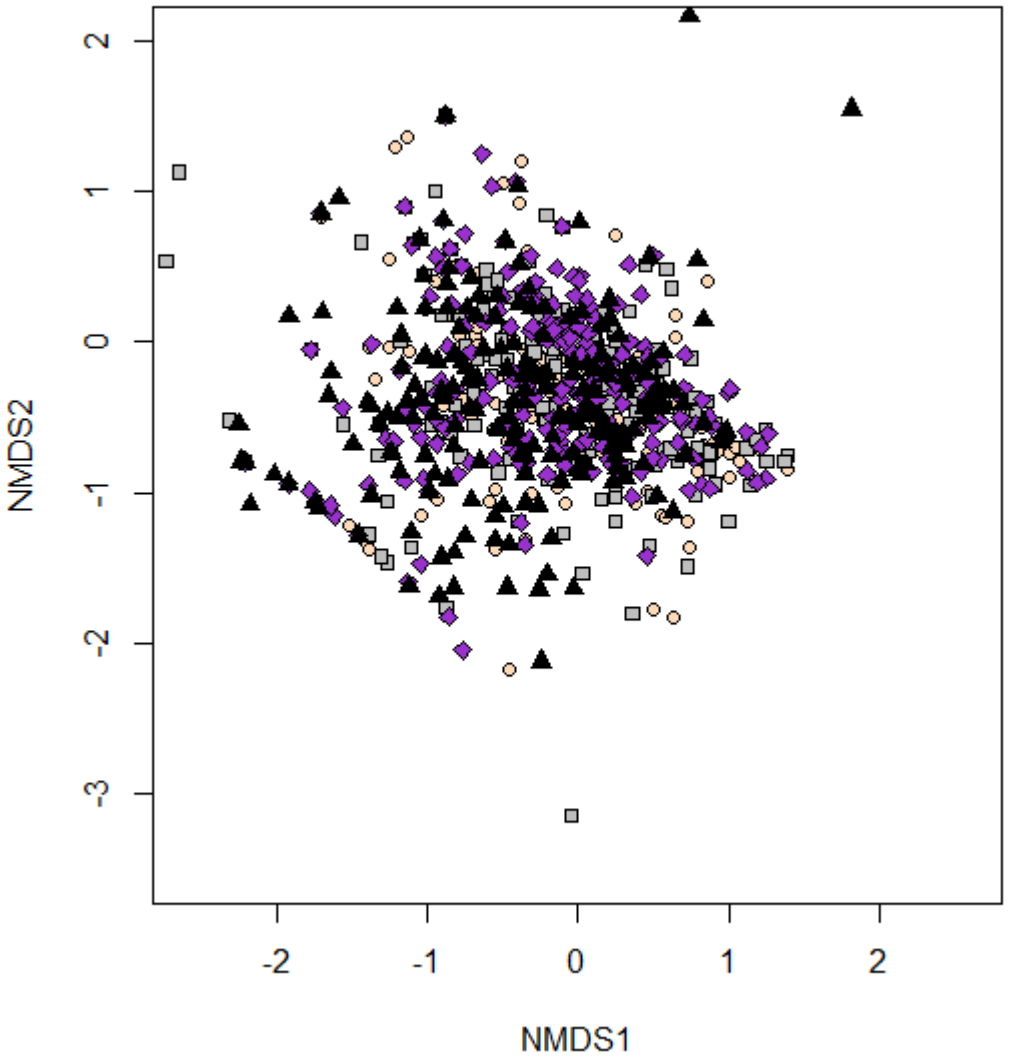

Table 1. 1

		DISTURBANCE TREATMENTS			
		Contiguous Control (CCON)	**Fragmented Control (FCON)**	**Clearcut (CCUT)**	**High-Leave Shelterwood (SW)**
		100-130 yrs.	**80-100 yrs.**	**12-14 yrs.**	**0-2 yrs.**
Study Sites	**County**		**SASAB**		
BB1	Montgomery	0	1	1	1
BB2	Montgomery	0	1	1	1
NC	Craig	0	1	1	1
BB3	Montgomery	2	0	0	0
TOTAL		**2**	**3**	**3**	**3**

Table 1. 2

		SASAB Sites			
Mosquito Species	**Contiguous Control (CCON)**	**Fragmented Control (FCON)**	**Clearcut (CCUT)**	**High-Leave Shelterwood (SW)**	**Total**
Aedes albopictus	44	38	32	77	191
Ae. canadensis	4	13	19	43	79
Ae. cinereus	0	1	0	0	1
Ae. japonicus	1618	2051	1225	1264	6158
Ae. triseriatus	3715	5659	4262	2594	16230
Ae. vexans	16	46	179	75	316
Anopheles punctipennis	29	26	33	19	107
An. quadrimaculatus	107	164	81	40	392
Coquillettidia perturbans	0	1	2	0	3
Culex erraticus	13	29	31	1	74
Cx. pipiens/restuans	1721	1402	1126	1796	6045
Ochlerotatus sticticus	1	0	1	0	2
Orthopodomyia signifera	33	16	12	7	68
Psorophora spp.	1	0	0	2	3
Toxorhynchites rutilus	2	5	2	2	11

Table 1. 3

Treatment	**N[a]**	**Richness**	**Shannon**	**Simpson**
Contiguous Control (CCON)	2	8.5 ± 0.56	0.86 ± 0.03	0.49 ± 0.01
Fragmented Control (FCON)	3	8.0 ± 0.62	0.78 ± 0.02	0.44 ± 0.01
Clearcut (CCUT)	3	8.1 ± 0.45	0.78 ± 0.02	0.45 ± 0.01
High-Leave Shelterwood (SW)	3	7.3 ± 0.55	0.80 ± 0.02	0.46 ± 0.01

Chapter 2

Influence of forest disturbance on La Crosse virus risk in southwestern Virginia

M. Camille Harris, Steven D. Zink, Sally L. Paulson, and Dana M. Hawley

Abstract

Forest disturbance has been shown to impact the dynamics of Lyme disease and human malaria but the effects of disturbance on forest arboviruses such as La Crosse virus (LACV) are currently unknown. We determined the abundance of LACV vectors and the primary amplifying vertebrate host (chipmunks; *Tamias striatus*) across a gradient of forest disturbance (contiguous control, fragmented control, clearcut and high-leave shelterwood) in southwestern Virginia. LACV surveillance was also conducted by testing collected mosquitoes for LACV nucleic acid and chipmunks for LACV-specific antibodies. Forest disturbance had a significant effect on the abundance of all LACV vectors, but in opposite directions. The abundance of *Aedes triseriatus*, the primary LACV vector, and *Ae. japonicus*, a recent invasive vector, decreased with forest disturbance. In contrast, the abundance of *Ae. albopictus*, *Ae. canadensis* and *Ae. vexans* increased with disturbance. While there was an overall disturbance treatment effect on *Culex pipiens/restuans*, no clear treatment preference was evident. Although we captured significantly more chipmunks on our most disturbed treatment (high-leave shelterwood), there was no overall treatment effect of disturbance on chipmunk abundance. LACV nucleic acid was detected in pools of *Ae. japonicus*, *Cx. pipiens/restuans* and *Ae. vexans*. There was a significant treatment effect of disturbance on LACV nucleic acid detection with most detections on the fragmented control sites. LACV antibodies were only found in chipmunks on the logged sites, but due to small sample sizes for antibody testing, this result was not statistically significant. Overall, the spatial mismatch between the detection of LACV-positive mosquitoes and LACV-specific antibodies in chipmunks suggests that while *Ae. triseriatus* transovarial vertical transmission is known to maintain the virus in undisturbed forest, horizontal transmission with sciurid rodent viral amplification may play a greater role in LACV maintenance on logged sites. The impact of forest disturbance on mosquito-borne disease risk is complicated by the varied

responses of vectors and the reservoir host. Further research is needed to understand how forest disturbance influences overall LACV risk.

Introduction

Environmental landscape changes can impact vector-borne disease dynamics by affecting the insect vectors, vertebrate hosts, or their interaction. Forest vector-borne diseases may be impacted by fragmentation, logging and deforestation. A well-researched example of the influence of forest fragmentation on vector-borne disease risk is the tick-borne Lyme disease. Forest fragments less than two hectares in size have been associated with an increased density of infected nymphs (Allan et al. 2003) due to a higher relative abundance of competent vertebrate reservoir hosts in small patches (Nupp and Swihart 1998; Krohne and Hoch 1999). Deforestation, which alters microclimatic conditions, has been shown to impact mosquito-borne human malaria. In heavily logged landscapes, *Anopheles* mosquitoes and the *Plasmodium* protozoan develop rapidly (Afrane et al. 2008). The shortened mosquito gonotrophic cycle is associated with increased human biting rates and risk of malaria (Vittor et al. 2006, Afrane et al. 2012). In contrast to work on human malaria, deforestation is associated with a decreased prevalence of avian malaria (Bonneaud et al. 2009, Chasar et al. 2009, Laurance et al. 2013), though vector abundance was not measured in these studies. Overall, research to-date suggests that the influence of forest disturbance on vector-borne diseases is likely complex, and may depend on the ecology of the vectors and reservoirs involved. The impact of forest disturbance on the dynamics of North American arboviruses such as La Crosse virus is largely unknown.

Southwestern Virginia is part of an emerging Appalachian focus of La Crosse virus (LACV) (Barker et al. 2003, Haddow et al. 2011). While most cases are subclinical, LACV can cause pediatric encephalitis (McJunkin et al. 1998). This zoonotic mosquito-borne virus is maintained in hardwood forests through *Aedes triseriatus* transovarial vertical or intergenerational transmission (Miller et al. 1977, Thompson and Beaty 1977). This is supplemented by a horizontal (i.e., intragenerational) transmission cycle between mosquitoes and sciurid rodents (especially chipmunks) (Moulton and Thompson 1971,

Gauld et al. 1974). The primary LACV vector, *Ae. triseriatus*, can overwinter the virus in tree holes (Watts et al. 1974). While the tree-hole mosquito is the primary vector, two invasive mosquitoes are now playing a larger role in the dynamics of this disease: *Ae. albopictus* (Lambert et al. 2010) and *Ae. japonicus* ((Westby et al. 2011); Chapter 3). There is also evidence that *Ae. canadensis* (Berry et al. 1986), *Ae. vexans* (Berry et al. 1983) and *Culex* mosquitoes ((Thompson et al. 1972); Chapter 4) may play a role in LACV dynamics. Thus, a large number of Culicidae vectors have been implicated in LACV dynamics. We recently showed that the temperate forest mosquito community in southwest Virginia is largely resilient to logging and associated forest fragmentation (Chapter 1). However, we found that there was an effect of logging on overall mosquito abundance (Chapter 1), suggesting population-level effects on vector species that may be critical for resulting disease risk (Antonovics et al. 1995, Mather et al. 1996). Despite the large role for Culicidae vectors in transmitting wildlife pathogens such as avian malaria and many arboviruses, including West Nile virus (Ezenwa et al. 2007), no study has yet examined how temperate forest disturbance influences Culicidae vector abundance.

There has been some prior work on how forest fragmentation affects chipmunks, the primary vertebrate reservoir of LACV, with mixed results. In oak forest in Indiana, *Tamias striatus* increased in relative abundance on clearcut sites (Kellner et al. 2013). In contrast, in West Virginia, *T. striatus* declined in response to clearcutting (Kirkland 1977). Because chipmunks act as reservoir hosts for several vector-borne diseases, including Lyme disease (Slajchert et al. 1997), babesiosis (Hersh et al. 2012), anaplasmosis (Johnson et al. 2011), West Nile virus (Platt et al. 2007) and La Crosse virus (Gauld et al. 1975), it is particularly important to understand how temperate forest logging influences chipmunk abundance and pathogen prevalence.

Here, we seek to understand how logging and associated forest disturbance impact the population dynamics of LACV vectors and chipmunks, the primary vertebrate amplifying host of LACV (Gauld et al. 1975, Patrican et al. 1985). By using silvicultural experiment field sites with randomly assigned logging treatments, we can specifically examine how forest disturbance *per se*, rather than other abiotic variables associated with

sites chosen for commercial logging, impacts mosquito vector and chipmunk populations. Based on vector ecology, we expected that mosquitoes known to favor shaded areas and tree holes (e.g., *Ae. triseriatus)* will decline in abundance with logging while those preferring sunlit areas (e.g., *Culex* mosquitoes) will increase (Crans 2004, Troyano 2009). Although there are mixed results on chipmunk responses to logging (Kirkland 1977), they are known to prefer coarse woody debris, which is found on recently logged sites (Zollner and Crane 2003, Kellner et al. 2013). Therefore, we predicted that chipmunk densities would be higher on logged sites. Because we anticipated logging would cause a decline in *Ae. triseriatus* abundance, we expected a higher prevalence of LACV antigens and antibodies on the undisturbed forest sites. However, recent models suggest that mosquito-borne disease risk assessment may be more complicated when multiple vectors are present (Lord 2010).

Materials & Methods

Study Site. Our study sites in Jefferson National Forest in southwestern Virginia are part of a long-term investigation of silvicultural oak regeneration methods on biodiversity, the Southern Appalachian Silviculture and Biodiversity Project (SASAB) (Belote et al. 2008, Atwood et al. 2009, Homyack and Haas 2013). These oak-dominant (*Quercus* spp.) sites had similar overstory composition, age and topographic position (Belote et al. 2008). Two sites used for this study (Blacksburg 1 and 2; BB1 and BB2, respectively) were located in Montgomery County, VA (37°17'35.73"N, 80°27'24.63"W (BB1); 37°18'20.35"N, 80°26'24.95"W (BB2)) while a third site (Newcastle (NC)) was located in Craig County, VA (37°27'20.78"N, 80°23'0.37"W).

Disturbance treatments. At each of the three SASAB study sites, seven two-hectare experimental units (EUs) were established with no buffer between the units. Silvicultural treatments were randomly assigned to EUs within sites using a fully randomized complete block design (Figure 1.1). For this study, three two-hectare silvicultural treatments were the focus of mosquito surveillance: repeated-entry high-leave shelterwood (SW) at 0-2 years post-disturbance; single-entry clearcut (CCUT) at 12-14 years post-disturbance; and fragmented unlogged controls (FCON) at 80-100 years old.

Logging disturbance was examined on a gradient defined by the frequency of harvest and stand age: FCON>CCUT>SW. Both the CCUT and SW were harvested between 1995 and 1996 (Atwood 2008). Following the first harvest, all residual overstory stems on the SW were harvested between 2007 and 2008 (Homyack and Haas 2013), just prior to the initiation of this study. CCUT was defined as the removal of 95% of the basal overstory area. SW was defined as stand thinning with 56% of the basal area removed in the initial establishment cut and all residual overstory removed in the final cut. FCON was defined as uncut areas with no direct timber harvesting disturbance but these sites were directly adjacent to disturbed sites with no buffer zone. These three silvicultural treatments were replicated across three study sites: BB1, BB2, and NC (Table 1.1).

Contiguous control sites. Because of the forest fragmentation created by the SASAB experimental design (Figure 1.1) and associated disturbance to FCON sites (e.g., skid trails, diffuse light from adjacent treatments), an additional non-SASAB study site (BB3) containing two contiguous controls (CCON) equivalent in size to the SASAB EUs was established for the purposes of this study. BB3 (37°18'48.59"N, 80°25'15.82"W) was also located in Jefferson National Forest (Montgomery County) for comparison to the fragmented control (FCON) at nearby BB1 and BB2 (Table 1.1). BB3 was approximately 1.8 miles from BB2. These uncut sites were embedded within large areas of contiguous forest that had not been recently disturbed by harvesting. These stands were dominated by oak (*Quercus alba, Q. velutina, Q. prinus)* along with yellow poplar (*Liriodendron tulipifera*). Red maple (*Acer rubrum*) and sourwood (*Oxydendrum arboretum)* were common in the midstory. The ages of the dominant and co-dominant trees in these stands were 100-130 years. Similar to the SASAB sites, the stands are on a south aspect with a moderate slope (J. Overcash, US Forest Service, pers. comm.).

Mosquito Sampling. From late May to September 2008-2010, adult mosquitoes were collected twice a week from infusion-baited gravid traps (Jackson et al. 2005). Five gravid traps were placed on each EU for mosquito collection (Figure 1.1). A minimum 30 meter buffer zone was applied to each EU to minimize edge effects. After a minimum of 24-h storage in a -80°C freezer, mosquitoes were identified using morphological keys

and pooled into groups with a maximum of fifty females by species, collection site, and date. Because male mosquitoes only feed on nectar (Stone and Foster 2013), they were not counted for this study. Female mosquitoes, which feed on plant sugars and vertebrate blood to obtain nutrients for oviposition, can transmit pathogens to animals and humans (Stone and Foster 2013). Because important adult taxonomic characters may be damaged or missing after field-collection (Saul et al. 1977, Harrington and Poulson 2008), which makes identification difficult, *Cx. restuans* and *Cx. pipiens* mosquitoes were pooled. Such pools will hereafter be referred to as *Cx. pipiens/restuans*. *Psorophora* were not identified to the species level.

Qualitative LACV real-time RT-PCR of 2008 Mosquito Pools. Mosquito pools from the 2008 field season were submitted to the Virginia Division of Consolidated Laboratory Services (DCLS) for virus detection. Qualitative reverse transcription-PCR (i.e., no cut-off value) was used to determine if this bunyavirus was present on our study sites. One milliliter of bovine albumin diluent (BA-1) (Nasci et al. 2002) was added to each mosquito pool. Mechanical homogenization was performed with a 4.5 mm steel bead; the resultant homogenate was centrifuged for 5 min. 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). 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 value). Samples were tested with the more sensitive primer set (LAC2364, LAC2448; Table 2.1) for two runs on an ABI PRISM 7000 system (Applied Biosystems, Inc., Foster City, CA). Samples with a crossing point value were run on a different machine (the LightCycler 2.0, Roche Diagnostics, Indianapolis, IN), re-extracted twice and run twice with both LAC2364/244 and a less sensitive primer set (LAC812, LAC881; Table 2.1). For each run, forty-five amplification cycles were performed.

Quantitative LACV real-time RT-PCR of 2009-2010 Mosquito Pools. The 2008 results led us to perform viral isolation and quantitative RT-PCR testing of isolates in

2009 and 2010. Mosquito pools were homogenized using previously described methods for LACV isolation (Gerhardt et al. 2001). Homogenate supernatant (150µl) was inoculated onto African green monkey kidney cells (Vero cells, ATCC# CCL-81), incubated at 37ºC and monitored daily for cytopathic effect (CPE). Isolates with marked CPE were harvested and submitted to the Centers for Disease Control and Prevention in Fort Collins, Colorado (Lambert et al. 2005, Lambert et al. 2010) (Chapter 3) or the Wadsworth Arbovirus Laboratories in Albany, New York for molecular testing (see Tables 2.1 and 2.2 for primers). For both laboratories, a sample was considered positive if the C_T value was ≤ 38.

Quantitative LACV Real-time RT-PCR with novel primers. Using the MagMax™ viral RNA isolation kit (Applied Biosystems, Life Technologies, Grand Island, NY) and the Freedom EVO® 150 liquid handling robotic arm (Tecan, Morrisville, NC), RNA was extracted from 100µl of the submitted cell culture isolate and eluted into 50µl of elution buffer. The 25uL reaction mix contained 0.3µl of 100µM primer and 0.3 µl of 25uM probe (Integrated DNA Technologies, Coralville, IA). The thermal cycling consisted of reverse transcription at 50ºC for 2 min, one cycle at 95ºC for 10 min to activate Taq and inactivate the reverse-transcriptase, 45 cycles at 95ºC for 10s for amplification, and 60ºC for 1 min to read the plate. Amplification and fluorescent detection were performed on the ABI 7500 real-time PCR standard system (Applied Biosystems, Inc., Foster City, CA). For each run, two no template controls were included with the samples. La Crosse virus (LACV/74/NY-M (74-32813)) stock controls were included to control for both the extraction and qRT-PCR. A sample was considered positive if the sample C_T value was ≤ 38, the positive control C_T was ≤ 38 and the negative control C_T was > 40.

Chipmunk Mark-Recapture Study. In Montgomery County (BB1, BB2 and BB3), a 7 x 7 trapping grid with a 10-meter interval was established with 3” x 3.5” x 9" large folding Sherman traps (H.B. Sherman Traps, Tallahassee, Florida). Using oats for bait, live trapping was conducted for three consecutive nights at monthly intervals. All captured chipmunks were ear-tagged to allow for mark-recapture estimates of abundance. The following morphometric data were collected from each chipmunk: age class, body

and tail length, weight and reproductive condition. Chipmunks were briefly anesthetized by a licensed veterinarian (M.C.H.) in a small tupperware container using a cotton-ball soaked with isoflurane (Baxter, Deerfield, Illinois) prior to blood collection. Blood was collected from the orbital sinus or lateral saphenous vein with collected volume not exceeding 1% of total blood volume (Parasuraman et al. 2010). All trapping and handling of small mammals was approved by the Virginia Department of Game and Inland Fisheries (VDGIF # 031626 and 038780) and the Virginia Tech Animal Care and Use Committee (IACUC# 07-083-BIOL and 10-064-BIOL).

Chipmunk plaque-reduction neutralization test (PRNT) for La Crosse virus antibodies. Blood was collected from fifty-two chipmunk captures. However, four samples were not tested because they were from recaptured chipmunks and nine had inadequate serum volume for PRNT, resulting in a total of 30 samples for testing. For recaptured chipmunks, only the last collected serum sample was tested for LACV antibodies. Although traps were placed on the contiguous control sites (BB3), no chipmunks were captured and, therefore, no blood was collected. Serum samples were heat-inactivated at 56ºC for thirty minutes to inactivate viruses and destroy complement. Using bovine albumin-1 diluent, sera were initially diluted to 1 in 10 and then titrated by two-fold serial dilutions to 1 in 320 for PRNT assays. LACV-specific neutralizing antibody titers were determined by 90% endpoint PRNT ($PRNT_{90}$). Serum-virus mixture was added to six-well plates with a confluent layer of Vero cells. A 0.5% agarose double-overlay was used and plaques were visualized with neutral red staining in the second overlay, which was applied 48 hours after the first overlay (Beaty 1995, Johnson et al. 2009). Normal guinea pig complement (S1639, Sigma-Aldrich, St. Louis, Missouri) was added to the serum-virus mixture at an 8% concentration to provide labile serum factor. Each test run was validated with a LACV-specific mouse hyperimmune polyclonal ascitic fluid positive control (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch at Galveston, Galveston, Texas), normal mouse serum negative control (M5905, Sigma-Aldrich, St. Louis, Missouri) and a LACV back-titration. Neutralizing antibody titer is expressed as the reciprocal of the

endpoint serum dilution that reduced the challenge LACV plaque count by 90% based on the back-titration.

Statistical Analysis. We compared mosquito vector abundance per trap night across forest disturbance treatments using linear mixed-effect models (package nlme). The model with the lowest AIC was selected for this analysis. The model included temporal (year, Julian date) and spatial (study site location: BB1, BB2, NC, or BB3) variables as random effects with treatment (CCON, FCON, CCUT, or SW) as a fixed effect. This model (AIC: 7081) outperformed models with simpler random effects which considered location and year (7375), location and Julian date (7109), only location (7391), only year (7402), or only Julian date (7214). We utilized a nested random effects structure with Julian date nested within year and year nested within study site location. Pearson's Chi-squared test was used to test for associations between the location of mosquito collection and detection of LACV nucleic acid.

Because of the low density of small mammals, chipmunk abundance was estimated as the minimum number known alive per hectare (MNKA) (Slade and Blair 2000). MNKA values were calculated based on treatment and year. Therefore, chipmunk abundance (MNKA) across forest disturbance treatments was compared using linear mixed-effect models with treatment as a fixed effect and year as a random effect. A nonparametric test (Pearson's Chi-squared test) was used to test for associations between the location of chipmunk capture and presence of LACV antibodies. The prevalence of LACV antibodies across the forest disturbance treatments was compared using package epiR. The LACV antibody analysis did not include the CCON treatment because no chipmunks were captured on these sites. All analyses were conducted in R version 3.00 (R Development Core Team 2013).

Results

Mosquito vector population dynamics.

***Aedes triseriatus*.** Over three field seasons, 16,230 *Ae. triseriatus* adult females were collected. There was a significant treatment effect of forest disturbance on the abundance

of the primary vector of La Crosse ($F_{3,380}$=28.1, p<0.0001). The lowest abundance was found on the SW (Figure 2.1) and the model showed a trend towards the SW being significantly different from the other treatments (t = -1.9, d.f.=380, p=0.053).

***Aedes japonicus*.** Over three field seasons, 6,158 *Ae. japonicus* adult females were collected. There was a significant disturbance treatment effect on the abundance of this invasive species ($F_{3,380}$=20.1, p<0.0001; Figure 2.1). The model parameter estimates showed that this treatment effect was driven by the two logged sites which had significantly lower abundance than the control sites (CCUT t=-2.9, d.f.=380, p= 0.0035; SW t=-2.6, d.f.=380, p= 0.0084).

***Aedes albopictus*.** Over three field seasons, 191 *Ae. albopictus* adult females were collected. There was a significant disturbance treatment effect on the abundance of this invasive species ($F_{3,380}$=6.2, p=0.0004). The SW had the greatest abundance but was not significantly different from the other treatments (Figure 2.1).

***Aedes canadensis*.** Over three field seasons, 79 *Ae. canadensis* adult females were collected. There was a significant disturbance treatment effect on the abundance of this floodwater mosquito ($F_{3,380}$=3.5, p= 0.016). Based on the model parameter estimates, the SW, which had the highest abundance, was significantly different from the other treatments (t=2.4, d.f.=380, p=0.0153) (Figure 2.1).

***Aedes vexans*.** Over three field seasons, 316 *Ae. vexans* adult females were collected. There was a significant disturbance treatment effect on the abundance of this mosquito ($F_{3,380}$=4.5, p= 0.0042). On average, this vector was most abundant on the clearcut (Figure 2.1). There was a trend toward the abundance on the CCUT being different from the other treatments (t=1.8, d.f.=380, p=0.074).

***Culex pipiens/restuans*.** Over three field seasons, 6,045 *Cx. pipiens/restuans* adult females were collected. There was a significant disturbance treatment effect on the abundance of this mosquito ($F_{3,380}$=3.2, p= 0.022) but no clear site preference.

Chipmunk Mark-Recapture Study. Fifty-two individual chipmunks were captured over three field seasons. The mean estimate of chipmunk abundance (MNKA) was 3.97 per hectare with a minimum of zero (CCON) and a maximum of 12 individuals (SW) (Figure 2.2). There was no significant difference in the MNKA across forest disturbance treatments ($F_{3,5}$=3.6, p=0.10). The model parameter estimates showed that the SW was significantly different with the highest mean MNKA (t=2.9, d.f.=5, p=0.03).

LACV mosquito surveillance. All vector mosquito pools from 2008 were qualitatively tested for LACV. In 2009 and 2010, the majority of mosquito pools (275/287, 96%) with CPE were quantitatively tested for LACV (Table 2.3). There was a significant effect of treatment on detection of LACV nucleic acid (χ^2=9.7, d.f.=3, p=0.02). The majority of positive samples (5/6, 83%) were on fragmented control sites with the exception of an *Ae. japonicus* pool on the SW. Most positive samples were from Montgomery County but there was one positive *Ae. japonicus* pool from Craig County.

In 2008, qualitative RT-PCR with the most sensitive primer set identified four LACV-positive pools on fragmented control sites (Table 2.3). Amplification was reproduced in three samples following re-extraction but was only detected in one run with the LAC2364/2448 primers for the *Ae. vexans* pool. None of the qualitative positives were confirmed with the less sensitive LAC812/LAC881 primers. Quantitative RT-PCR on the 2009-10 samples revealed no positives in 2010 but two positive *Ae. japonicus* pools in 2009 (Table 2.3).

Chipmunk plaque-reduction neutralization test (PRNT) for La Crosse virus antibodies. Sera from 38 chipmunks collected in Montgomery County (BB1 and BB2) in 2009 and 2010 was tested for LACV antibodies. PRNT (at 90% plaque reduction) confirmed the presence of serum antibodies to LACV in 5 (13%) of 38 chipmunk serum samples. All of these positive samples were from disturbed sites (SW=3, CCUT=2; Table 2.4). Prevalence of LACV in chipmunks captured on logged sites (incl. SW and CCUT) was 2.75 times (95% CI=0.17, 44.75) greater than the prevalence in chipmunks captured on the fragmented control. However, there was no significant difference when

results were compared across forest disturbance treatments (χ^2=1.376, d.f.=2, p=0.50). All titers were low (i.e., ≤ 1 in 20). Most of the positive samples were collected in June with one of the SW positives collected in late July.

Discussion

In this study, we sought to understand how forest disturbance (i.e., timber harvesting) impacted mosquito vectors and chipmunks in the context of La Crosse virus dynamics. We sampled LACV vectors and the primary reservoir host of LACV across a suite of experimentally logged forest plots. LACV vector abundance was significantly affected by forest disturbance treatments but the directionality varied with the species examined (Figure 2.1). In sync with the abundance of the primary vector, LACV nucleic acid detection was greatest on the undisturbed forest sites (Table 2.3). While the abundance of the invertebrate vectors was affected by disturbance, the generalist primary amplifying vertebrate host (chipmunks) was not significantly affected. Based on our results, we suspect that horizontal transmission with sciurid rodents plays a greater role in LACV maintenance on disturbed sites compared to undisturbed forest. However, overall risk of LACV for humans (as measured by LACV-positive mosquitoes) may be highest in unlogged forest.

Human risk for La Crosse virus is correlated with the density of *Ae. triseriatus*, the primary vector (Nasci et al. 2000). The tree-hole mosquito can maintain this bunyavirus in nature through both transovarial and venereal transmission (Pantuwatana et al. 1974, Miller et al. 1977, Thompson and Beaty 1977). Additionally, LACV can overwinter in the diapause eggs of *Ae. triseriatus* (Watts et al. 1974). As predicted, we found that the abundance of *Ae. triseriatus*, which relies largely on shaded areas and forest trees for oviposition (Haramis 1984, Nasci 1988, Barker et al. 2003), significantly declined with logging (Figure 2.1). A recent study on the same sites using oviposition data rather than adult collection also found the greatest number of *Ae. triseriatus* eggs on the FCON sites (Bova 2014). Because timber harvesting reduces overstory canopy cover and mature trees with their tree holes, resource reduction is likely the cause of the decreased density of this mosquito with forest disturbance.

Although *Ae. triseriatus* is the primary vector for LACV, invasive accessory vectors are increasingly important for LACV risk in Appalachia (Gerhardt et al. 2001, Westby et al. 2011). Two Asian invasive mosquitoes (*Ae. japonicus* and *Ae. albopictus*) have become established in the United States (Hawley et al. 1987, Moore 1999, Peyton et al. 1999, Kaufman and Fonseca 2014), including southwest Virginia (Grim et al. 2007). These species are known to be competent vectors for LACV (Tesh and Gubler 1975, Sardelis et al. 2002), capable of becoming naturally infected (Lambert et al. 2010, Westby et al. 2011). In fact, we detected LACV in three pools of *Ae. japonicus* but not in any pools of the more abundant primary vector, *Ae. triseriatus*, which outnumbered *Ae. japonicus* by 2.6 to 1. The Asian tiger mosquito, *Ae. albopictus*, is capable of transovarial LACV transmission (Tesh and Gubler 1975, Hughes et al. 2006) but the ability of *Ae. japonicus* to vertically transmit this virus is unknown. Our research shows that invasive vector abundance is differentially affected by logging, which may have important implications for LACV risk. The Asian rock pool mosquito (*Ae. japonicus*) significantly declined with logging in our study (Figure 2.1). In contrast, *Ae. albopictus*, known to thrive in sunlit urban areas (Barker et al. 2003, Troyano 2009), significantly increased with logging, having the highest abundance on the shelterwood (Figure 2.1). These results highlight the need for further LACV surveillance and research regarding these invasive vectors.

Accessory LACV vectors include well-established and native mosquitoes in addition to the recent Asian invasive species. Therefore, we also examined the influence of logging on *Ae. canadensis, Ae. vexans,* and *Culex* species. La Crosse virus has been isolated from field-collected *Ae. canadensis* (Masterson et al. 1971, Berry et al. 1983, Berry et al. 1986, Nasci et al. 2000). Although considered a poor LACV vector experimentally (Watts 1973), the floodwater mosquito has been shown to play a role in LACV dynamics in Ohio and West Virginia (Berry et al. 1986, Nasci et al. 2000). We found that the abundance of *Ae. canadensis* is impacted by logging with the highest abundance found on the shelterwood (Figure 2.1). Although LACV was previously detected in this vector in our region (Jackson 2009), LACV was not detected in the *Ae. canadensis* pools on our study sites. However, the low LACV infection rates previously reported (Berry et al.

1983, Nasci et al. 2000) suggest that it would be difficult to detect this bunyavirus from our small sample size.

The role of *Ae. vexans*, another floodwater mosquito, in current LACV dynamics is unknown. This species has been shown to be a competent LACV vector but transmission rates were low (Watts 1973). While there have been reports of LACV isolations from this species in the past (Sudia et al. 1971), the current role of *Ae. vexans* in LACV dynamics in emerging Appalachian foci is unknown. Our research shows that logging does significantly impact *Ae. vexans* populations and the highest abundance was found on the clearcut (Figure 2.1). Because this species prefers to oviposit in unshaded areas for flooding of eggs laid in ground depressions (Crans 2004), the higher abundance on the clearcut is not unexpected. Similar to another floodwater mosquito (i.e., *Ae. canadensis*), logged sites with decreased canopy cover are associated with a greater abundance of this vector. Although LACV was detected in a pool of *Ae. vexans*, the high C_T value and lack of repeatability indicate that this result should be interpreted with caution. Further surveillance and research is needed to understand the role of this species in LACV dynamics.

The last LACV accessory vectors we examined were *Culex pipiens/restuans*. Inability to morphologically differentiate the *Culex* species prevented a clear determination of how logging impacts *Cx. restuans* and *Cx. pipiens* specifically. Based on previous research in southwestern Virginia and West Virginia, however, we know that *Cx. restuans* is dominant over *Cx. pipiens* throughout the season (Joy and Sullivan 2005, Jackson and Paulson 2006), and thus our data are most likely to reflect patterns attributable to *Cx. restuans*. Our findings suggest that logging has an impact on *Culex* abundance but no treatment preference was evident. This is not surprising because these species can oviposit in a variety of habitats (Crans 2004). Although known to be ornithophilic, there is evidence that *Culex* mosquitoes will feed on mammals (Hamer et al. 2009) and may play a role in LACV dynamics (Thompson et al. 1972) (Chapter 4). Logging does impact *Culex* mosquito abundance and these vectors may play an accessory role in LACV dynamics.

La Crosse virus nucleic acid detection was significantly affected by disturbance treatment. In fact, 83% of positive mosquito pools were from control sites (Table 2.3) while only one positive pool was detected on the SW. In agreement with our predictions and previous work, *Ae. triseriatus* density is the best correlate for human LACV risk in terms of LACV-positive mosquito pools (Nasci et al. 2000). Unlogged forest had the highest abundance of *Ae. triseriatus* and the greatest detection of LACV.

Although vector abundance has been shown to be most important for LACV risk (Nasci et al. 2000), the presence of vertebrate reservoir hosts is also critical for horizontal transmission (Gauld et al. 1975). Thus, we examined the abundance of the primary vertebrate reservoir- chipmunks- across forest disturbance treatments. While the white-footed mouse (*P. leucopus*), the primary reservoir for Lyme disease, is known to increase in abundance with forest fragmentation (Nupp and Swihart 1998), the response of *T. striatus* varies (Kirkland 1977, Nupp and Swihart 2000, Kellner et al. 2013). Our results indicate that chipmunk abundance (as measured by MNKA) on the sites with the greatest disturbance (SW) was significantly higher than on the other treatments (Figure 2.2). Shelterwood harvests may be associated with increased coarse woody debris, an important resource for rodents to avoid predation (Zollner and Crane 2003). In fragmented forest, the increased proportion of edge habitat and decreased granivore competition allows *T. striatus* with its small home range (0.2 hectares) to thrive (Nupp and Swihart 2000). Although not measured in this study, the previous year's pulsed resources (e.g., oak mast) are known to impact rodent populations (McShea 2000, Clotfelter et al. 2007), and oak mast is likely to vary with the extent of forest disturbance. It is important to note that our methods for measuring chipmunk abundance have limitations. MNKA is known to underestimate populations (Slade and Blair 2000) so a more extensive mark-recapture study might better elucidate the impact of logging on these sciurid rodents.

In addition to examining chipmunk abundance, we asked whether the likelihood of reservoir exposure to LACV varied with forest disturbance in our study. Once a chipmunk is infected with LACV by a mosquito, it maintains a viremia for an average of

2-3 days (Pantuwatana et al. 1972, Patrican et al. 1985). After the short viremia, chipmunks develop a lifelong immunity evident by antibody levels (Moulton and Thompson 1971). Although we found no significant difference in the presence of LACV antibodies across the forest treatments, likely due to small sample sizes, the prevalence rate was over 2 times greater for chipmunks captured on the logged sites when compared to the controls. These prevalence values were lower than what has been previously reported for chipmunks in endemic areas where end of season prevalence rates ranged from 55-100% (Gauld et al. 1974). Feeding behavior of mosquitoes has to be considered alongside amplifier host antibody prevalence in order to determine resulting risk for LACV horizontal transmission. Generalist vectors such as *Culex* spp. may take more bloodmeals from the abundant rodents on disturbed sites. Alternatively, mammalophilic mosquitoes like *Ae. triseriatus* may dilute LACV by taking a higher proportion of non-amplifier (e.g., deer) bloodmeals on disturbed sites (Wright and DeFoliart 1970, Nasci 1985). On our sites, there was no difference in deer abundance based on fecal pellet surveys (C.L. Squibb, unpublished data), but feeding behavior of vectors may be influenced by habitat in addition to simply the abundance of potential bloodmeal hosts. Bloodmeal analysis is needed to determine whether mosquito feeding behavior varied across forest treatments.

Overall, the impact of logging on LACV dynamics appears to be complicated. LACV vectors showed mixed responses to forest disturbance. The primary vector, *Ae. triseriatus,* and *Ae. japonicus*, the species for which we detected several LACV-positive pools, showed a general decline with logging. In contrast, chipmunk reservoirs tend to increase in abundance with logging and the only seropositive chipmunks were on disturbed sites. Thus, the vectors and reservoirs appear to show contrasting responses to logging in our system. Prior work indicates that the rate of *Ae. triseriatus* vertical transmission determines the amount of horizontal transmission necessary to maintain LACV in the environment (Miller et al. 1977). Logging, which decreases the abundance of *Ae. triseriatus,* may lead to a decrease in transovarial transmission but an increase in the amount of horizontal LACV transmission. Because logging tends to be associated with an increased density of rodents, the role of chipmunks and mammalophilic

accessory vectors (e.g., *Ae. albopictus*) may become more important on logged sites. For Lyme disease and human malaria, forest disturbance has been shown to have a clear directional effect on disease risk (Allan et al. 2003, Afrane et al. 2012). La Crosse virus is unique in its ability to be maintained through vertical and horizontal transmission. This epidemiologic complexity along with the increasing role of accessory vectors, makes the impact of forest disturbance on LACV dynamics complex. The vectors, vertebrate amplifying hosts and their interaction (i.e., bloodmeal analysis) must be examined to better ascertain how forest disturbance affects LACV risk.

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Figure and Table Captions

Figure 2.1. Mean number (± SE) of female mosquito vectors (*Aedes triseriatus, Aedes japonicus, Aedes albopictus, Aedes canadensis, Culex* spp., and *Aedes vexans)* caught per trap-night across forest disturbance treatments, from least (CCON) to most disturbed (SW) as quantified by frequency of harvest and stand age. CCON=Contiguous control; FCON=Fragmented control, CCUT=Clearcut, and SW=High-leave shelterwood.

Figure 2.2. Mean *Tamias striatus* abundance (minimum number known alive per hectare (MNKA) ± SE) across forest disturbance treatments, from least (CCON) to most disturbed (SW) as quantified by frequency of harvest and stand age. CCON=Contiguous control; FCON=Fragmented control, CCUT=Clearcut, and SW=High-leave shelterwood.

Table 2.1. Primers used for amplification of La Crosse virus (LACV).

Table 2.2. Primers designed for La Crosse virus M segment amplification.

Table 2.3. La Crosse virus nucleic acid detection results from mosquitoes collected in southwestern Virginia (2008-2010). Detections were from pools or groups of up to fifty female mosquitoes of the same species caught on the same day and collection site.

Table 2.4. La Crosse virus antibody prevalence in chipmunks (*n*=38) based on plaque-reduction neutralization testing across forest disturbance treatments, from least (CCON) to most disturbed (SW) as quantified by frequency of harvest and stand age. A sample was defined as positive if there was a 90% reduction in plaques when compared to the LACV back-titration. CCON=Contiguous control; FCON=Fragmented control, CCUT=Clearcut, and SW=High-leave shelterwood.

Figure 2. 1

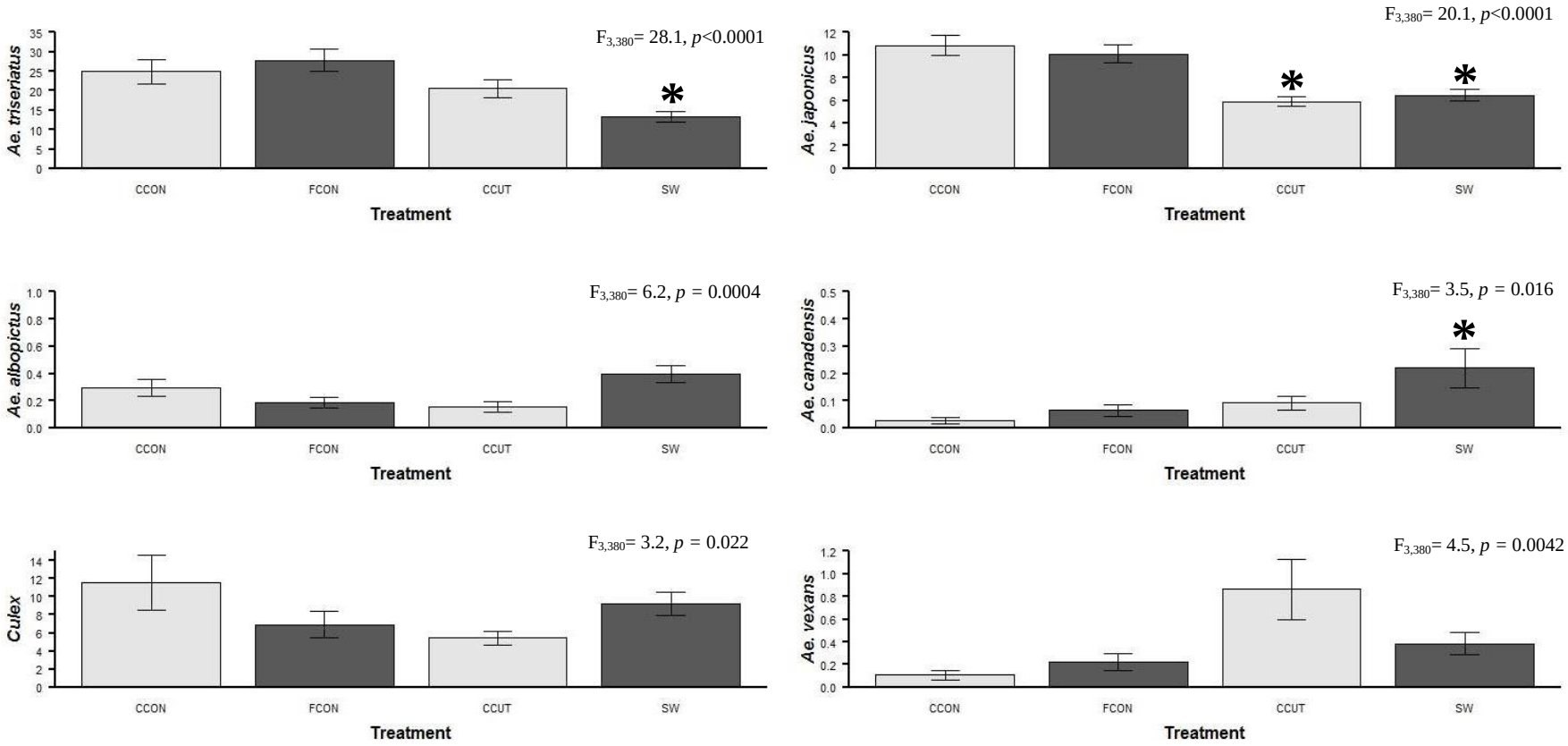
Ae. triseriatus
$F_{3,380}$= 28.1, p<0.0001
Ae. japonicus
$F_{3,380}$= 20.1, p<0.0001
Ae. albopictus
$F_{3,380}$= 6.2, p = 0.0004
Ae. canadensis
$F_{3,380}$= 3.5, p = 0.016
Culex
$F_{3,380}$= 3.2, p = 0.022
Ae. vexans
$F_{3,380}$= 4.5, p = 0.0042
CCON
FCON
CCUT
SW
Treatment

Figure 2. 2

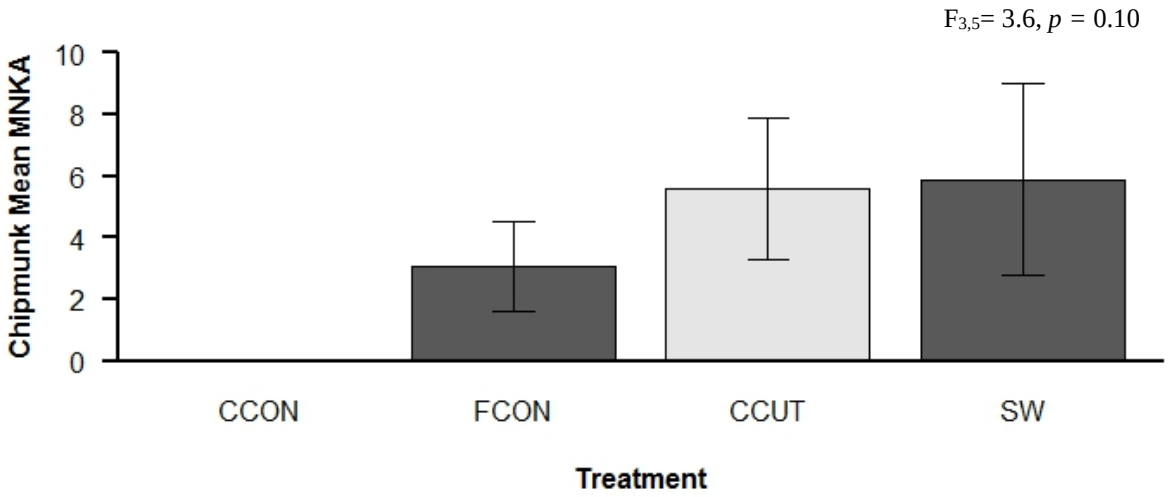
$F_{3,5}$= 3.6, p = 0.10
Chipmunk Mean MNKA
10
8
6
4
2
0
CCON
FCON
CCUT
SW
Treatment

Table 2. 1

State	Year	Laboratory	Primer/Probe Name	LACV M segment primer/probe sequence (5'→ 3')	Source
VA	**2008**	VA DCLS	LAC836 LP1 LAC812 LF1 LAC881 LR1 LAC2387 LP2 LAC2364 LF2 LAC2448 LR2	CATCCATTCACAGAGTGTGGCACGC TGCAAGCTATGCGGCCTAGT AGCGAGCACCACAGACACAA AATGGGCCAAGTGTGTATAGGAAACCATCA CAATAATTGCGTGTGGTGAACC GACCGATCAGTGCTAGATTGGAA	(R. Lanciotti, CDC, pers. comm.)
VA	**2009**	CDC		AGTAGTGTACTACC TTRAARCADGCATGGAA	(Lambert et al. 2010)

Table 2. 2

LACV Primer/Probe	Location in M Segment (GU206142)	LACV M segment primer/probe sequence (5'→ 3')	Primer Size (bp)
F Primer	817	CTATGCGGCCTAGTGTATC	19
R Primer	872	GGAAGTATCATAGCGAGCACC	21
Probe	844	CY5-CACAGAGTGTGGCACGCATTGTGTC-3BHQ_2	25

Table 2. 3

Mosquito Species	Total Pools Tested (Year)	LACV Positive pools	Mean C_T Value	Pool Size	Treatment	County	Month	Year
Ae. triseriatus	59 (2008) 12 (2009) 11 (2010)	0						
Ae. japonicus	53 (2008) 27 (2009) 16 (2010)	3	38 14 23	22 3 50	FCON SW FCON	Montgomery Montgomery Craig	July July July	2008 2009 2009
Ae. albopictus	10 (2008) 1 (2009)	0						
Cx. pipiens/restuans	64 (2008) 1 (2009) 3 (2010)	2	42 42	3 7	FCON FCON	Montgomery Montgomery	August July	2008 2008
Ae. vexans	18 (2008)	1	44	2	FCON	Montgomery	August	2008

Table 2. 4

FOREST DISTURBANCE TREATMENT	**LACV Ab NEGATIVE**	**LACV Ab POSITIVE**	**SEROPREVALENCE**
FCON	7	0	0%
CCUT	12	2	14%
SW	14	3	18%

Chapter 3

Detection and isolation of La Crosse virus in field-collected *Aedes japonicus japonicus* (Diptera: Culicidae) in the Appalachian Region

M. Camille Harris, Eric J. Dotseth, Bryan T. Jackson, Sally L. Paulson, Paul E. Marek and Dana M. Hawley

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Abstract

We report La Crosse virus detection and isolation in *Aedes japonicus* mosquitoes in the Appalachian region, an emerging geographic focus of La Crosse encephalitis. As the leading cause of arboviral pediatric encephalitis in the USA, detection of this virus in an invasive vector species is of significant public health importance.

Introduction

La Crosse encephalitis virus (LACV), a California serogroup bunyavirus, is the major cause of pediatric arboviral encephalitis in the United States (US) (1). Since its 1963 discovery in Wisconsin, LACV has been identified in 32 states within the contiguous US (2). The Appalachian Mountains are part of an emerging focus of LACV (3). *Aedes triseriatus*, the primary vector of LACV, is present in southwestern Virginia and West Virginia but we have recently noted the emergence of two invasive congeners: *Aedes albopictus* (3) and *Aedes japonicus* (4). Both have been shown to be competent experimental LACV vectors (5, 6). While LACV has been isolated from *Ae. albopictus* (7, 8), it has only been detected in *Ae. japonicus* in Tennessee (9). Here we report the isolation of LACV from *Ae. japonicus* adults in southwestern Virginia and seven independent field detections of LACV from *Ae. japonicus* eggs and adults (Virginia and West Virginia). These findings suggest a potential role of this invasive vector in Appalachian LACV dynamics (Figure 3.1).

The Study

In 2005, mosquito eggs were collected weekly in Wise County, Virginia using ovitraps. Larvae were reared to adults in a BSL-2 insectary at 24°C, 75% RH, and 16L:8D photoperiod. In 2008 and 2009, adult mosquitoes were collected weekly from infusion-baited gravid traps in Montgomery County, VA. Mosquitoes from all years were identified to species using morphology and grouped in pools of ≤50 individuals according to species, location, and collection date. Adults were stored at -80°C until viral testing. Reverse transcription-PCR was used for 2005 and 2008 LACV detection (Table 3.1). In 2009, mosquito pools were homogenized using previously described methods for LACV isolation (7). Homogenate supernatant (150µl) was inoculated onto Vero cells, incubated at 37ºC and monitored daily for cytopathic effect (CPE). Isolates with marked CPE were harvested and submitted to the Centers for Disease Control and Prevention in Fort Collins, Colorado, USA, for RT-PCR (8) (Table 3.1).

In 2013, mosquito surveillance was conducted as part of the West Virginia Department of Health and Human Resources Mosquito Surveillance Program. Using gravid traps, CO_2 emitting light traps, and BG Sentinel traps baited with octenol lures, adult mosquitoes were collected weekly from counties with high (Nicholas, Fayette, Raleigh) and low (Kanawha, Jackson, Wood) human incidence of LACV (as defined in (10)). Specimens were pooled by species, county, and collection date for RT-PCR (Table 3.1) at the WV Office of Laboratory Services using previously described methods (11).

LACV was detected in an *Ae. japonicus* pool, collected as eggs, in August 2005 from Wise County, VA. LACV was also detected in one *Ae. japonicus* pool from Montgomery County, VA in July 2008 (Table 3.2). LACV RNA was detected in five separate WV *Ae. japonicus* pools, representing three counties and four months (Table 3.2).

We successfully isolated LACV from a 2009 *Ae. japonicus* pool collected in Montgomery County, VA (Table 3.1). This pool was sent to the CDC where it was successfully amplified via RT-PCR.

Nucleotide sequencing and a BLAST query were performed on the amplified cDNA. LACV medium (M) segment was used to infer phylogeny (Figure 3.2). Coding sequences were aligned in Mesquite version 2.75 with Opalescent version 2.10 (12, 13). Phylogenetic trees for the polyprotein genes were estimated using a maximum likelihood based method and assuming a general time reversible (GTR) model with gamma-distributed rate heterogeneity of nucleotide substitution GTR + Γ in RAxML version 8.0.0 (14). Support values for each clade were generated in RAxML by using 1000 rapid bootstrap replicates. The VA 2009 *Ae. japonicus* isolate is within the previously described Lineage I, which includes Midwestern and Appalachian isolates (15).

Conclusions

This is the first known report of LACV isolation in field-collected *Ae. japonicus* in Appalachia. Furthermore, the large number of LACV detections we observed highlight the need for local clinicians to consider this arbovirus as a differential diagnosis for pediatric encephalitis cases. Our detection of LACV in *Ae. japonicus* collected as eggs in 2005 and the ability of LACV to be transmitted transovarially in *Ae. triseriatus* suggests that future research should examine the ability of LACV to be vertically-transmitted by this invasive vector. The detection of LACV in both low and high human LACV incidence WV counties suggests mosquito control efforts for LACV should include populations of *Ae. japonicus* in addition to native *Ae. triseriatus*. In this Appalachian LACV focus, *Ae. japonicus* may play an important role in the maintenance, transmission and range expansion of LACV.

Acknowledgements

We thank Dr. Amy Lambert of the CDC for conducting RT-PCR and sequencing of the Virginia 2009 isolate, Dee Petit and Andrew Luna of VA-DCLS for technical assistance, and Nate Lambert, Allen Patton, Bonnie Fairbanks, Jennifer Miller and Laila Kirkpatrick for field and lab assistance. In West Virginia, Christi Clark, Chris Boner, and Lindsay Kuncher of the WV Department of Health & Human Resources Office of Laboratory Services provided laboratory assistance and field surveillance was conducted by Kristin Alexander, Stephen Catlett, Hannah Cavender, Robert Deneer, Jennifer Beamer Hutson, Mickey King-Fowler, Dustin Mills, Daniel Payne, and Courtney Stamm. Mosquito

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Figure and Table Captions

Figure 3.1. Locations of La Crosse virus (LACV) detections and isolation from *Aedes japonicus* pools. The red star represents the location of the *Aedes japonicus* LACV isolate and the green stars represent locations of *Aedes japonicus* LACV detections. Pink counties represent the location of human LACV cases. Yellow counties have historically had LACV activity but no positive reports occurred in 2013. Figure of 2013 human cases of LACV adapted from United States Geological Survey website (http://diseasemaps.usgs.gov/lac_us_human.html).

Figure 3.2. Phylogeny of La Crosse virus based on the M segment of the viral polyprotein gene. State of isolate origin, isolation year, mosquito or vertebrate isolate source and the NCBI accession numbers are listed for each isolate within the tree. The scale bar represents the number of nucleotide substitutions per site. The 2009 Virginia isolate (NCBI accession no. XXXXXXXX) groups with Lineage 1 viruses in Appalachia. *Ae*., *Aedes*; AL, Alabama; CT, Connecticut; GA, Georgia; MN, Minnesota; MO, Missouri; NC, North Carolina; NY, New York; OH, Ohio; *Ps*., *Psorophora*; TN, Tennessee; TX, Texas; VA, Virginia; WI, Wisconsin; and WV, West Virginia.

Table 3.1 Primers used for amplification and sequencing of La Crosse virus (LACV)

Table 3.2. La Crosse virus (LACV) detections in *Aedes japonicus* pools from Virginia and West Virginia

Figure 3. 1

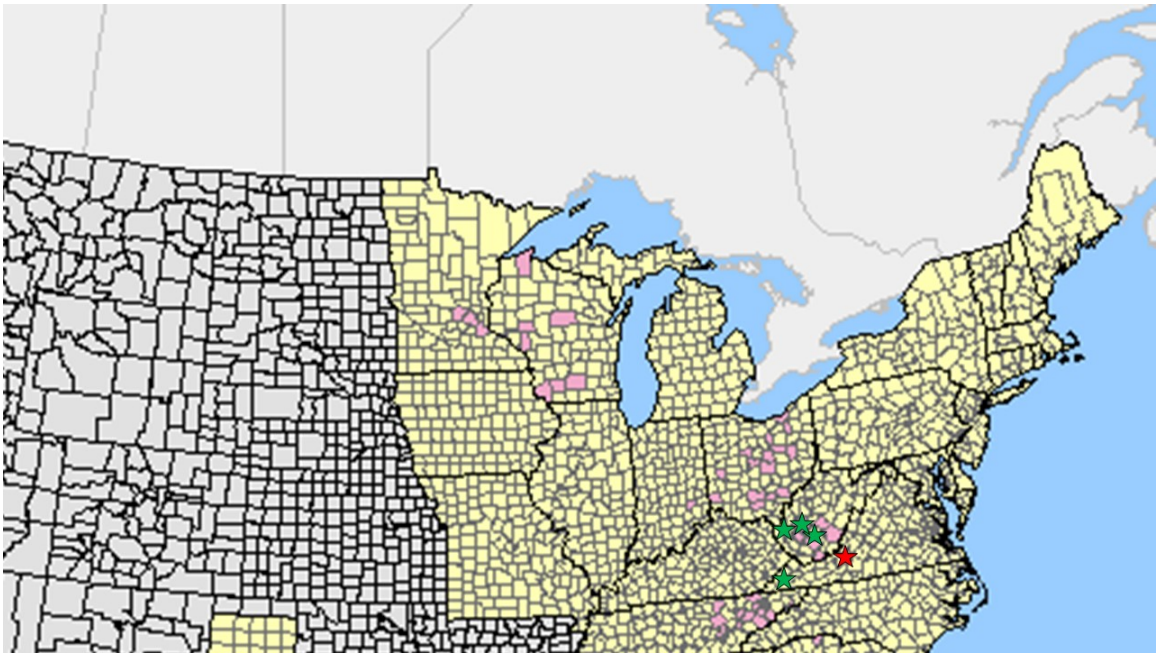

Figure 3. 2

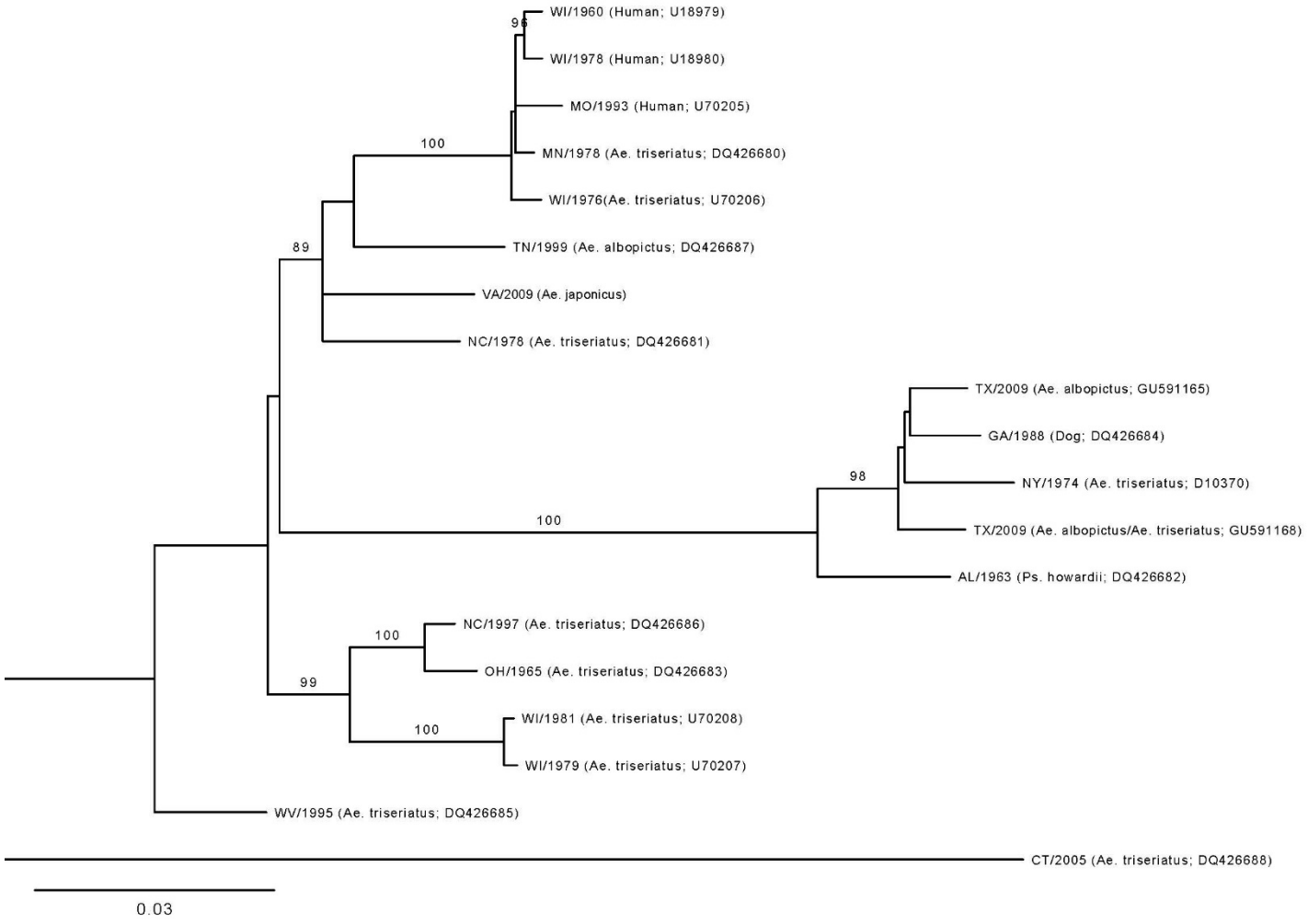
WI/1960 (Human; U18979)
96
WI/1978 (Human; U18980)
MO/1993 (Human; U70205)
100
MN/1978 (Ae. triseriatus; DQ426680)
WI/1976(Ae. triseriatus; U70206)
89
TN/1999 (Ae. albopictus; DQ426687)
VA/2009 (Ae. japonicus)
NC/1978 (Ae. triseriatus; DQ426681)
TX/2009 (Ae. albopictus; GU591165)
GA/1988 (Dog; DQ426684)
98
NY/1974 (Ae. triseriatus; D10370)
100
TX/2009 (Ae. albopictus/Ae. triseriatus; GU591168)
AL/1963 (Ps. howardii; DQ426682)
NC/1997 (Ae. triseriatus; DQ426686)
100
OH/1965 (Ae. triseriatus; DQ426683)
99
WI/1981 (Ae. triseriatus; U70208)
100
WI/1979 (Ae. triseriatus; U70207)
WV/1995 (Ae. triseriatus; DQ426685)
CT/2005 (Ae. triseriatus; DQ426688)
0.03

Table 3. 1

State	Year	LACV M segment primer sequence (5'→ 3')	Source
VA	**2005, 2008**	LAC2364 CAATAATTGCGTGTGGTGAACC LAC2448 GACCGATCAGTGCTAGATTGGAA	(R. Lanciotti, pers. comm.)
VA	**2009**	AGTAGTGTACTACC TTRAARCADGCATGGAA	(8)
WV	**2013**	LAC935 TATAAAAGCCTAAGAGCTGCCAGAGT LAC1018c GACCAGTACTGCAGTAATTATAGACAAT	(11)

Table 3. 2

Life stage collected	Pool size	Trap type	County, State	Collection month and Year	LACV detection method	CT values	MLE (95% CL)
Eggs	9	Ovitrap	Wise, VA	Aug 2005	RT-PCR	38.04	8.59 (0.54 - 41)
Adults	22	Gravid Trap	Montgomery, VA	July 2008	RT-PCR	37.57	4.51 (0.26 - 22)
Adults	3	Gravid Trap	Montgomery, VA	July 2009	Isolation, RT-PCR	14	0.23 (0.01-1.11)
Adults	36	Multiple Adult Traps	Fayette, WV	June 2013	RT-PCR	37.66	13.41 (5.18-29.14)
Adults	1	Multiple Adult Traps	Cabell, WV	July 2013	RT-PCR	34.72	13.41 (5.18-29.14)
Adults	15	Multiple Adult Traps	Fayette, WV	Aug 2013	RT-PCR	37.35	13.41 (5.18-29.14)
Adults	2	Multiple Adult Traps	Fayette, WV	Aug 2013	RT-PCR	34.64	13.41 (5.18-29.14)
Adults	1	Multiple Adult Traps	Kanawha, WV	Sept 2013	RT-PCR	37.43	13.41 (5.18-29.14)

Chapter 4

La Crosse virus field detection and vector competence of *Culex* mosquitoes

M. Camille Harris, Fan Yang, Dorian M. Jackson, Eric J. Dotseth, Sally L. Paulson, and Dana M. Hawley

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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. In order to assess vector competence, we offered LACV blood meals to field-collected *Cx. restuans* Theobald, *Cx. pipiens* L., and *Aedes triseriatus* (Say). Both *Culex* species were susceptible to infection and dissemination. 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 to *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 become infected, disseminate and transmit LACV. These species should be considered potential LACV vectors and controlled in LACV-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

(Gerhardt et al. 2001, Aspen et al. 2003, Smith and Fonseca 2004, Byrd et al. 2009). 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 (Turell et al. 2010), 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 (Hardy et al. 1983), disseminating through the hemocoel after surmounting the midgut escape barrier to infect other tissues, and prevailing over the salivary barriers to be orally transmitted (Tabachnick 2013).

La Crosse virus (LACV), a California serogroup *Orthobunyavirus*, remains a major cause of pediatric arboviral encephalitis in the United States (McJunkin et al. 1998, Reimann et al. 2008, Haddow et al. 2011a). Since its 1964 isolation in Wisconsin (Thompson et al. 1965), LACV has been identified in thirty-two states within the contiguous US (CDC 2011). The Appalachian region, where this study was performed, is part of an emerging focus of LACV (Barker et al. 2003, Haddow et al. 2011b). This arbovirus is maintained in hardwood forests primarily through *Aedes triseriatus* transovarial vertical transmission (Miller et al. 1977, Thompson and Beaty 1977). In fact, the primary LACV vector can overwinter the virus in tree holes (Watts et al. 1974b). The rate of *Ae. triseriatus* vertical transmission determines the amount of horizontal transmission necessary to maintain LACV in the environment (Miller et al. 1977). Horizontal transmission between *Ae. triseriatus* and sciurid rodents (i.e., chipmunks, squirrels) (Beaty and Calisher 1991) is supplemented by *Ae. triseriatus* venereal transmission (Thompson and Beaty 1977).

Vector competence studies have identified the capacity for other mosquito species to serve as accessory vectors of LACV: *Ae. albopictus, Ae. aegypti* (Hughes et al. 2006), and *Ae. japonicus* (Sardelis et al. 2002).Virus isolation from field-collected *Ae. albopictus* mosquitoes has confirmed their potential to serve as vectors in the

field (Gerhardt et al. 2001, Lambert et al. 2010). Although vector competence research revealed poor virus multiplication in *Ae. canadensis* (Watts 1973), field research has shown they may serve as accessory vectors of LACV (Berry et al. 1986, Nasci et al. 2000). LACV was first isolated from field-collected *Culex pipiens* in Wisconsin in 1967 (Thompson et al. 1972) 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 *restuans* prior to 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 d post infection (Tesh and Gubler 1975, Harbach 2012). These results suggest that LACV-positive field samples from *Cx. pipiens* may represent true infection versus virus-positive vertebrate blood-meals and/or mis-identification. Here we examine the vector competence of *Culex* mosquitoes, for which we and others have documented LACV in the field (Thompson et al. 1972).

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. Because nutritionally-stressed larvae are known to impact the LACV vector competence for *Ae. triseriatus* (Grimstad and Haramis 1984, Paulson and Hawley 1991), we also conducted a companion experiment to determine if *Cx. restuans* mosquitoes, dominant over *Cx. pipiens* in southwestern Virginia and West Virginia (Joy and Sullivan 2005, Jackson and Paulson 2006), are more efficient vectors when larvae are resource-limited.

Materials & Methods

Appalachian field testing for La Crosse virus.

Virginia Mosquito Collection. In 2008, adult mosquitoes were collected weekly from infusion-baited gravid traps (Jackson et al. 2005) at oak-dominant sites in Jefferson National Forest (Belote et al. 2008) in Montgomery County, VA, USA. After a minimum of 24-h storage in a -80°C freezer, mosquitoes were pooled into

groups of up to fifty females by species, collection site, and date. Because important adult taxonomic characters may be damaged or missing after field-collection (Saul et al. 1977, Harrington and Poulson 2008), *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 RT-PCR of Virginia Mosquito Pools. Mosquito pools were submitted to the Virginia Division of Consolidated Laboratory Services (DCLS) for virus detection. Qualitative reverse transcription-PCR (i.e., no cut-off value) was used to determine if this bunyavirus was present on our study sites. One milliliter of bovine albumin diluent (BA-1) (Nasci et al. 2002) 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 min. 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). 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 value). Samples were tested with the more sensitive primer set (LAC2364, LAC2448; Table 4.1) for two runs on an ABI PRISM 7000 system (Applied Biosystems, Inc., Foster City, CA). Samples with a crossing point value were run on a different machine (the LightCycler 2.0, Roche Diagnostics, Indianapolis, IN), re-extracted twice and run twice with both LAC2364/244 and a less sensitive primer set (LAC812, LAC881; Table 4.1). For each run, forty-five amplification cycles were performed.

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 (Joy and Sullivan 2005). Samples were collected weekly from counties with high (Nicholas, Fayette, Raleigh) and low human incidence of LACV (Kanawha, Jackson, Wood) (as previously defined) (Haddow et al. 2011a). 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 with octenol lures were used to capture adult mosquitoes. Trap site selection was based on habitat suitability for the vectors of West Nile virus 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 due to difficulty in differentiating field-damaged *Culex restuans* from *Culex pipiens* and efforts to conserve laboratory resources. Although *Culex pipiens/restuans* was the most active *Culex* mosquito group, other *Culex* species (i.e. *Culex 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 three minutes. The QIAamp RNeasy Mini kit (Qiagen, Valencia, CA) 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). Polymerase chain reactions were run using the ABI 7500 FAST Real-Time PCR system (Applied Biosystems). Biosearch Technologies provided the primers (LAC935, LAC1018c) and Taqman probe (LAC963) (Lambert et al. 2005). Forty amplification cycles were performed. Samples with a C_T value $\leq$40 were considered positive.

La Crosse virus vector competence experiment.

Mosquito Collection and Rearing for Vector Competence Study. Culex egg rafts were collected using oviposition traps and *Ae. triseriatus* eggs were collected using ovitraps placed on the campus of Virginia Tech, Blacksburg, VA (Barker et al. 2003, Jackson et al. 2005). To-date, La Crosse virus has not been detected in *Ae. triseriatus* (50 eggs) or *Culex* (30 egg rafts) at this collection site (Yang, unpublished data). In addition, *Ae. triseriatus* egg LACV-infection rates (0.15/1,000) are very low in Montgomery County, VA (Jackson 2009).

Eggs were reared to adults in an insectary (24°C, 75% relative humidity, and 16:8 [L:D] hr) as previously described (Jackson et al. 2012). Approximately 24 hr after placement in the environmental chamber, newly hatched larvae were morphologically identified to species (Andreadis 2005). Larvae were reared at a density of 250 larvae per container (33x17.5x11cm) in 1600 mL deionized water and fed *ad libitum* bovine liver powder solution (7.5g/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 d old post-emergence.

Larval Nutritional Stress Experiment. Larvae that were confirmed to be *Cx. restuans* were distributed into two plastic shoe boxes (33x17.5x11cm) containing 1600 mL of deionized water and approximately 200 larvae. The larvae were fed a 500 mL/7.5g bovine liver powder (MP Biomedicals, Solon, OH) solution. The control group was fed 45 mL of the bovine liver powder solution while the nutritionally stressed larvae in the other container were only fed 15 mL of the liver powder. Thus, the nutritionally stressed group was always fed one-third the amount of nutrients that the control group received (Paulson and Hawley 1991). Five days after the initial feeding, larvae were fed a second time with 30 mL of liver powder given to the control group and 10 mL of 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*. In order to determine if nutritional stress influenced adult size, wings were measured to assess body size (McCombs 1980). Wing length was measured using the Dino-Lite digital microscope (AM-4113ZTL, Worchester, PA).

Virus. The La Crosse virus strain used for this study (VA0921075, Passage 12) was isolated from *Ae. triseriatus* collected in Duncan Gap, VA in 1999 (Barker et al. 2003). The isolate was maintained in the laboratory by alternate passage through *Ae. triseriatus* mosquitoes and African green monkey kidney (Vero) cells. Prior to this study, the isolate had been passaged through Vero cells ten times and *Ae. triseriatus* twice. The viral stock titer was 5.3×10^7 plaque-forming units (PFU)/mL.

Vector Competence. Forty-eight hr before artificial bloodmeal feeding, week-old mosquitoes were transferred to one-liter 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 of 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 bloodmeal 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 (Gargan et al. 1983).

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 d post-exposure (dpe), respectively. Females were immobilized by chilling on ice or exposure to triethylamine (Kramer et al. 1990). 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 (Aitken 1977, Boromisa et al. 1987), (Dodson et al. 2011). After 30 minutes, tube contents were expelled into 0.3mL 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 (5000 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 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. The percentages of mosquitoes tested that contained virus in their abdomen were considered infected. Those with disseminated infections had virus in their legs and abdomen. Those with virus-positive salivary expectorate were capable of transmitting the virus. 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 in R, which is based on the modified Wald method (Agresti and Coull 1998). Wing length measurements were analyzed by using one-way analysis of variance (ANOVA). All analyses were conducted in R version 3.0.0 (R Development Core Team 2013).

Results

Qualitative Detection of LACV RNA in Virginia mosquito pool samples.

1,071 adult *Cx. pipiens/restuans* collected from Montgomery Co, VA in 2008 were combined into 64 pools and analyzed for LACV. Two field-collected pools of *Culex pipiens/restuans* mosquitoes collected from this population in July ($n_{\text{mosquitoes}}$=7) and August ($n_{\text{mosquitoes}}$=3) had high C_T values, suggesting low concentrations of LACV. Out of four replicate runs with the LAC2364/2448

primers, a positive signal was obtained from the *Cx. pipiens/restuans* pools in three (July C_T values: 43, 40, 42) or two (August 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 CDC have been improved upon (Lambert et al. 2005). The bias-corrected MLE infection rate for LACV-infected *Culex pipiens/restuans* in Montgomery Co., VA was estimated to be 1.8 (95% CL = 0.3 – 6) infected mosquitoes per 1,000 specimens (Biggerstaff 2006).

Quantitative Detection of LACV RNA in West Virginia mosquito pool samples. In 2013, 13,363 adult *Culex* mosquitoes from WV were combined into 388 mosquito pools and analyzed for LACV. LACV was detected in six of these 388 mosquito pools (Table 4.2), which were collected in late August through early September. The bias-corrected MLE LACV infection rate for *Culex* spp. in WV was 0.4 (95% CL = 0.2-0.9) infected mosquitoes per 1,000 specimens (Biggerstaff 2006). LACV-positive *Culex* mosquitoes were collected in urban, and peridomestic habitat (Joy and Sullivan 2005) 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 (Mutebi et al. 2012), so multiple groups were offered blood meals (Table 4.3). Three groups of *Cx. restuans*, the dominant *Culex* spp. on our study sites, two groups of *Cx. pipiens* and one group of *Ae. triseriatus* were offered LACV bloodmeals for this study. Prior to conducting statistical analyses across species, the proportion of infected and uninfected across the different species groups were compared. There was no significant difference between the three *Cx. restuans* groups in terms of the percentage of infected (p=0.11), disseminated (p=0.17), or transmitting (p=0.17) mosquitoes. For *Cx. pipiens*, there was no significant difference between the groups in terms of infected (p=0.24), disseminated

(p=0.12) or transmitting (p=1.0) mosquitoes. Based on these results and in order 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 infected mosquitoes (p=0.36; Table 4.3). *Ae. triseriatus* had a significantly greater percentage of infected, disseminated and transmitting mosquitoes when compared to *Cx. restuans* (p <0.001) and *Cx. pipiens* (p <0.001).

***Culex restuans* Nutritional Stress Experiment.** Based on wing length, larvae reared under nutritionally stressed conditions were significantly smaller (mean wing length = 3.30mm, s.d.= 0.16 mm) compared to those reared under control nutritional conditions (mean wing length = 3.41mm, s.d. =0.21 mm; $F_{1,58}$=5.3; p=0.025). Although not statistically significant, larvae reared under control conditions (mean days to pupation = 9.5; mean days to emergence = 11) pupated and emerged sooner than the nutritionally-stressed larvae (mean days to pupation = 10.5; mean days to emergence = 12.5).

Virus titers of blood meals for the control and nutritionally-stressed groups were 4.1 x 10^6 and 8.5 x 10^6 PFU/mL, respectively. Nutritionally-stressed mosquitoes (n=28) were more likely to become infected (p<0.001), disseminate LACV (p<0.001) and have virus-positive expectorate (p=0.02) compared to the control mosquitoes (n=31). There was no evidence of transmission based on salivary expectorate testing for the control group but a small percentage were infected (6%) and disseminated (6%) LACV (Table 4.4). LACV-positive expectorate was evident in the nutritionally-stressed group (18%). This group also had a significantly higher percentage of mosquitoes that were infected (54%) and disseminated the virus (43%).

Discussion

Our results indicate that *Culex restuans* and *Cx. pipiens* are both susceptible to LACV infection, but they are not as permissive to LACV as the primary vector

Aedes 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 transmission (Hughes et al. 2006). *Aedes canadensis* has also been found to have low LACV transmission efficiency with 25-27% transmission rates (Watts 1973). Finally, *Aedes japonicus*, a more recent invading vector, is also less permissive to LACV oral infection with estimated transmission rates ranging from 0-88% (Sardelis et al. 2002). 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 (Nasci et al. 2000). In Ohio, LACV was isolated more often from *Ae. canadensis* than *Ae. triseriatus* (Berry et al. 1986). 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 (Gerhardt et al. 2001, Lambert et al. 2010). Additionally, LACV has been detected (Westby et al. 2011) and isolated from field-collected *Ae. japonicus* (Chapter 3).

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 their contribution to LACV dynamics is small. Bloodmeal 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) (Pantuwatana et al. 1972). 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 4.3) 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 (Grimstad and Walker 1991), in North American strains of *Ae. aegypti* and *Ae. albopictus* with dengue-2 virus (Alto et al. 2008), in *Cx. p. pipiens* with West Nile virus (Vaidyanathan et al. 2008) and in *Ae. aegypti* infected with Sindbis virus (Muturi et al. 2011). 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 our positive control vector, *Ae. triseriatus* (Table 4.3), and is similar to that reported for *Ae. albopictus* (41%) (Hughes et al. 2006). There are other studies, however, that have not found this connection. Large, not small, *Ae. aegypti* were more competent for Ross River virus (Nasci and Mitchell 1994) and chikungunya virus (Westbrook et al. 2010). In fact, large Thailand strains of *Ae. aegypti* were more likely to be infected with dengue-2 virus in a different study (Sumanochitrapon et al. 1998). No correlation between body size and vector competence has been reported for *Cx. tarsalis* infected with West Nile virus (Dodson et al. 2011), western equine encephalitis virus or St. Louis encephalitis virus (Reisen et al. 1997). 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.

Based on our laboratory results, we suspect that our LACV-positive field samples were most likely due to true infections with LACV. Although we did not find *Culex* species (in the absence of larval nutritional stress) to be very permissive to LACV, low vector competence alone does not preclude an important role for *Culex* species. If the relative abundance of a secondary vector, even with low vector competence, is higher than *Ae. triseriatus* (Gerhardt et al. 2001), that species may play a significant role in LACV dynamics in the field. At our WV sampling sites, *Culex* mosquitoes had higher relative abundance than *Aedes* species late in the season when they were found infected with LACV (Dotseth, unpublished data). In southwestern VA, however, *Ae. triseriatus* remained the dominant species on the collection sites (Table 1.2). 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 (Means 1968, Wright and Defoliart 1970, Hamer et al. 2009), the primary amplifying vertebrate hosts for LACV. The degree to which *Cx. pipiens* populations are mammalophilic versus ornithophilic can vary at both small (Means 1968) and large geographic scales (Kilpatrick et al. 2007, Huang et al. 2009). 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 (Kothera et al. 2012), but Virginia and West Virginia are within the hybridization zone of *Culex p. pipiens* and *Cx. p. quinquefasciatus* (Barr 1957, Huang et al. 2011). Previous research suggests *Cx. pipiens* and *Cx. fatigans (=Cx. p. quinquefasciatus)* may be infected with LACV (Thompson et al. 1972, Tesh and Gubler 1975). Recent work suggests hybrids of *Cx. pipiens* and *Cx. quinquefasciatus* have enhanced transmission of WNV (Ciota et al. 2013). Their vector competence 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 (Ciota et al. 2011) in contrast to *Ae. triseriatus* which overwinters in pre-pupal stages (Watts et al. 1974a, McGaw et al. 1998), 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 (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. Although artificial containers have been associated with a higher risk of human LACV cases (Hedberg et al. 1985, Woodruff et al. 1992, Haddow et al. 2011b), our results suggest that pest managers in LACV-endemic areas should also control *Culex* breeding sites (e.g., stagnant pools of water) that are not conducive to container-breeding *Aedes* species. Overall, we recommend that future mosquito LACV surveillance, especially in emerging regions, should include *Culex* and not just *Aedes* mosquitoes.

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Figure and Table Captions

Table 4.1. Primers used for amplification of La Crosse virus (LACV).

Table 4.2. Detection of La Crosse virus in West Virginia *Culex* spp. by RT-PCR.

Table 4.3. Relative vector competence of *Culex restuans, Culex pipiens* and *Aedes triseriatus* for La Crosse virus following oral exposure. Mosquitoes were collected at ten and fourteen days post-exposure. If virus was recovered from the abdomen but not the legs or expectorate, the mosquito was considered infected. 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.

Table 4.4. Effect of larval nutritional stress on *Culex restuans* vector competence for La Crosse virus following oral infection. Vector competence of nutritionally-stressed mosquitoes was compared to that of control groups. Mosquitoes were collected at ten and fourteen days post-exposure. If virus was recovered from the abdomen but not the legs or expectorate, the mosquito was considered infected. 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.

Table 4. 1

State	Year	Primer/Probe Set	LACV M segment primer sequence (5'→ 3')	Source
VA	2008	LAC836 LP1	CATCCATTCACAGAGTGTGGCACGC	(R. Lanciotti, CDC, pers. comm.)
		LAC812 LF1	TGCAAGCTATGCGGCCTAGT	
		LAC881 LR1	AGCGAGCACCACAGACACAA	
		LAC2387 LP2	AATGGGCCAAGTGTGTATAGGAAACCATCA	
		LAC2364 LF2	CAATAATTGCGTGTGGTGAACC	
		LAC2448 LR2	GACCGATCAGTGCTAGATTGGAA	
WV	2013	LAC 935	TATAAAAGCCTAAGAGCTGCCAGAGT	(Lambert et al. 2005)
		LAC 1018c	GACCAGTACTGCAGTAATTATAGACAAT	
		LAC 963 probe	TGTGCAAGTCGAAAGGGCCTGCA	

Table 4. 2

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

Table 4. 3

Species and Group Number	**Bloodmeal LACV Titer**	**Days post-exposure (DPE)**	**Sample Size (*n*)**	**% Infected (95% C.I.)**	**% Disseminated (95% C.I.)**	**% Transmitting (95% C.I.)**
Cx. restuans 1	2.9 x 10^7	10	16	37% (0.2-0.6)	31% (0.1-0.6)	31% (0.1-0.6)
Cx. restuans 2	1.8 x 10^7	10	5	0% (0-0.5)	0% (0-0.5)	0% (0-0.5)
Cx. restuans 3	8.5 x 10^6	10	12	0% (0-0.3)	0% (0-0.3)	0% (0-0.3)
Cx. restuans 1	2.9 x 10^7	14	14	0% (0-0.2)	0% (0-0.2)	0% (0-0.2)
Cx. restuans 2	1.8 x 10^7	14	7	14% (0-0.5)	0% (0-0.4)	0% (0-0.4)
Cx. restuans 3	8.5 x 10^6	14	13	8% (0-0.4)	8% (0-0.4)	8% (0-0.4)
Total *Cx. restuans*			**67**	**13% (0.1-0.2)**	**9% (0-0.2)**	**9% (0-0.2)**
Cx. pipiens 1	1.3 x 10^7	10	13	23% (0.1-0.5)	8% (0-0.4)	8% (0-0.4)
Cx. pipiens 2	1.3 x 10^7	10	21	19% (0.1-0.4)	14% (0-0.4)	9% (0-0.3)
Cx. pipiens 1	1.3 x 10^7	14	18	0% (0-0.2)	0% (0-0.2)	0% (0-0.2)
Cx. pipiens 2	1.3 x 10^7	14	18	33% (0.2-0.6)	17% (0-0.4)	0% (0-0.2)
Total *Cx. pipiens*			**70**	**20% (0.1-0.3)**	**10% (0-0.2)**	**4% (0-0.1)**
Ae. triseriatus	1.4 x 10^7	10	21	62% (0.4-0.8)	57% (0.4-0.7)	57% (0.4-0.7)
Ae. triseriatus	1.4 x 10^7	14	21	62% (0.4-0.8)	33% (0.2-0.5)	24% (0.1-0.5)
Total *Ae. triseriatus*			**42**	**67% (0.5-0.8)**	**48% (0.3-0.6)**	**40% (0.3-0.5)**

Table 4. 4

Treatment Group	**Sample Size (*n*)**	**Days post exposure (DPE)**	**% Infected (95% C.I.)**	**% Disseminated (95% C.I.)**	**% Transmitting (95% C.I.)**
Control	16	10	12% (0-0.4)	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**		**6% (0-0.2)**	**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	100% (0.7-1.0)	86% (0.6-1.0)	36% (0.2-0.6)
Total Nutritionally-Stressed	**28**		**54% (0.4-0.7)**	**43% (0.3-0.6)**	**18% (0.1-0.4)**

Chapter 5

Summary and Conclusions

Summary

Landscape changes can impact vector-borne disease dynamics (Allan et al. 2003, Afrane et al. 2012) but the influence on mosquito-borne viruses is unknown. Because temperate forests provide the backdrop for La Crosse virus (LACV) transmission, I sought to understand how disturbance in the form of even-aged timber harvest (i.e., clearcut and high-leave shelterwood) influenced the mosquito community and LACV dynamics by examining the influence of disturbance on mosquito community composition (Chapter 1), mosquito population abundance (Chapter 2), and vertebrate reservoir host abundance (Chapter 2). Unexpectedly, LACV surveillance during the first field season identified viral nucleic acid in a recently established invasive species (*Aedes japonicus*) and *Culex pipiens/restuans* mosquito pools (Chapter 3). The latter pools included a native vector, *Cx. restuans*, and a well-established invasive species (*Cx. pipiens*). There is a gap in the literature regarding the vector competence of these *Culex* mosquitoes, so I conducted laboratory experiments to determine if LACV could be transmitted by these species (Chapter 4). Because nutritionally-stressed *Ae. triseriatus* are known to be better LACV vectors (Paulson and Hawley 1991), I also examined how such resource limitation influenced the vector competence of *Cx. restuans* (Chapter 4).

Does forest disturbance influence the mosquito community?

Overall, the temperate forest mosquito community is resilient to forest disturbance in southwestern Virginia. I found no difference in mosquito diversity on undisturbed forest compared to disturbed sites. However, it is interesting to note that there was a trend toward lower mosquito species richness on the disturbed sites. While logging did not significantly affect the community, I found that it was associated with a significant decline in total mosquito abundance. This decline in vector abundance is evidence that population-level effects and mosquito-borne disease risk may be lower immediately following a logging event, which I examined further in Chapter 2.

Does forest disturbance influence La Crosse virus dynamics?

For LACV dynamics, the impact of forest disturbance is complex because of the vertebrate amplifying hosts and multiple vectors, which are affected in different ways. *Aedes triseriatus* abundance significantly declined with forest disturbance, suggesting that LACV vertical transmission may be lower on logged sites. In contrast, chipmunk abundance was significantly higher on the high-leave shelterwood sites. In line with the mosquito abundance results, LACV detection in mosquitoes was greatest on undisturbed sites but LACV antibody prevalence in chipmunks was greatest on logged sites. This differential response of the primary vector and primary amplifying host is further complicated by secondary vectors. The invasive *Ae. japonicus* declined with logging but *Ae. albopictus, Ae. canadensis* and *Ae. vexans* increased in abundance with logging. While there was a disturbance treatment effect on *Cx. pipiens/restuans,* no clear treatment preference was evident. Overall, these results suggest that the impact of forest disturbance on LACV is complex.

Does the invasive species Aedes japonicus play a role in La Crosse virus dynamics in Appalachia?

It's been suggested that landscape disturbance opens the door for invasive species (i.e., passenger model) (MacDougall and Turkington 2005, Jakubowski et al. 2010). Contrary to this, we found that the recently established invasive *Ae. japonicus* declined in abundance with logging. However, it may be that invasive mosquitoes on the logged sites were able to play a larger role in LACV dynamics. While *Ae. japonicus* has been proven experimentally to be a competent LACV vector (Sardelis et al. 2002), field evidence is sparse (Westby et al. 2011). During the first field season, I detected LACV in an *Ae. japonicus* pool on undisturbed forest. This detection was followed-up by an isolation of the virus and additional field detections (Chapters 2 and 3). Phylogenetically, the isolate was within LACV Lineage I, which includes Midwestern and Appalachian isolates (Armstrong and Andreadis 2006). While the isolation was from a high-leave shelterwood site, the three detections were from undisturbed forest. Based on my results,

it is clear that *Ae. japonicus* is important for LACV dynamics in Appalachia. The impact of forest disturbance on the role of *Ae. japonicus* in LACV dynamics is not yet clear.

Are Culex mosquitoes serving as accessory vectors for La Crosse virus in Appalachia? While LACV has previously been isolated from the well-established invasive *Cx. pipiens* (Thompson et al. 1972), the detection of viral nucleic acid in *Culex* mosquitoes on our sites was surprising. During field collections (Chapters 1 and 2), I could not distinguish between *Cx. pipiens* and *Cx. restuans* and therefore did not know which of these two mosquito species was responsible for the LACV detection. Therefore, I took the opportunity to fill in the literature gap regarding vector competence of the native *Cx. restuans* and invasive *Cx. pipiens*. Because it has been suggested that nutritional stress may impact vector competence, I also designed a companion experiment to examine how nutritional stress might influence the competence of our more dominant *Culex* species: *Cx. restuans* (Jackson and Paulson 2006). These primarily ornithophilic mosquitoes (Hamer et al. 2009) appear to be poor vectors of LACV based on high C_T values and low percentages of infected, disseminated and transmitting mosquitoes. Resource-limited *Cx. restuans*, however, had percentages of infected, disseminated and transmitting mosquitoes that were comparable to the primary LACV vector (*Ae. triseriatus*). While their role in LACV dynamics is likely to be small, *Culex* mosquitoes can develop disseminated infections and transmit LACV.

Conclusions

Based on my dissertation research, I encourage physicians, public health officials, entomologists and veterinarians in Montgomery and Craig counties to heighten their consideration of the local risk of La Crosse virus. Public health officials and entomologists should monitor for LACV and that monitoring should be extended to potential secondary vectors such as *Ae. japonicus*, which may play an increasingly important role in LACV dynamics. Medical practitioners should include it as a differential diagnosis in children who could be exposed to this rural encephalitis (McJunkin et al. 2001). Veterinarians should also be aware of this zoonotic disease for which canine patients can seroconvert and develop disease (Tatum et al. 1999, Troyano

2009). As a veterinarian, I examined this arbovirus from a One Health approach (Scotch et al. 2009, Anholt et al. 2012). LACV, the leading cause of arboviral pediatric encephalitis in the USA, is a zoonotic disease that can be affected by landscape changes because of the impact on ectothermic vectors, generalist rodent amplifying vertebrate hosts and their interaction. My dissertation research on La Crosse virus suggests that when invertebrate vectors and vertebrate amplifying hosts are impacted by environmental changes, the results can directly affect human and animal well-being by influencing zoonotic disease risk.

Future Directions

My dissertation research led to many questions regarding the influence of forest disturbance on the mosquito community and LACV dynamics in Appalachia.

Future Directions to understand how forest disturbance impacts the mosquito community

Mosquito community: predation risk

Toxorhynchites rutilus, a predator of mosquito larvae, was detected in very small numbers across all treatments in Montgomery County (Chapter 1). Predation is known to limit mosquito population dynamics (Juliano 2007). However, due to low collection numbers, I was unable to elucidate the impact of these and other predators (e.g. *Corethrella appendiculata*) on the mosquito community. A study to determine how forest disturbance impacts vector predation risk should be conducted.

Forest Disturbance and canopy-level mosquito communities

I examined the mosquito communities at the basal level using gravid traps and found them to be resilient to forest disturbance. Because logging directly impacts the overstory (Belote et al. 2008), future research should examine the mosquito assemblage at canopy-level. This community may be more sensitive to logging.

Future Directions to understand how forest disturbance impacts LACV dynamics

Feeding behavior of LACV vectors

While I detected LACV in *Ae. japonicus* and *Culex* mosquitoes, the impact of these results on LACV dynamics has not yet been fully elucidated. Are LACV infections wasted on these vectors or are they spreading the virus to naïve rodents and humans? If horizontal transmission appears to be more important on disturbed sites, are *Ae. triseriatus* feeding on sciurid rodents more? In order to answer these questions, molecular bloodmeal analyses (Molaei et al. 2006, Hamer et al. 2009) in addition to LACV surveillance should be conducted on individual mosquitoes collected on disturbed and undisturbed forest sites.

Microclimate changes and emergence of LACV vectors

Because I focused on adult mosquitoes, I was unable to elucidate if vectors on disturbed sites emerged sooner than those on undisturbed forest sites. Research should be done to examine the presence of pupae (Haramis 1984) across these sites to determine if the primary or secondary LACV vectors emerge from diapause eggs sooner because of the warmer microclimate present on the disturbed sites (Harpole and Haas 1999).

Tree holes, LACV and resource limitation

Nutritionally-stressed *Ae. triseriatus* (Paulson and Hawley 1991) and *Cx. restuans* (Chapter 4) are better LACV vectors. In a student project (Dudzinsky, unpublished data) that examined *Ae. triseriatus* and *Ae. japonicus* wing size across my study sites in August 2010, there was no difference in wing size across the disturbance treatments. Despite a decline in abundance with logging, these results suggest that *Ae. triseriatus* and *Ae. japonicus* mosquitoes on the disturbed sites were able to meet their nutritional needs based on adult wing size. Such monitoring should be extended for a season and combined with LACV surveillance to determine if forest disturbance can result in nutritionally-stressed larvae and smaller adult vectors that are more likely to transmit LACV.

Future Directions to understand the role of secondary vectors in LACV dynamics

Role of secondary vectors in LACV vertical transmission and overwintering

I have shown that *Ae. japonicus* and *Culex* mosquitoes may play a role in horizontal transmission but it is unknown if transovarial transmission and overwintering can occur in these species. The latter would be intriguing for *Culex* mosquitoes which overwinter as adults (Ciota et al. 2011) in comparison to *Ae. triseriatus* and *Ae. japonicus* that overwinter as eggs or larvae (Watts et al. 1974, Kaufman and Fonseca 2014).

Culex genetics, blood-feeding behavior and LACV vector competence

There is evidence of a seasonal switch in blood-feeding behavior of *Cx. pipiens* (Kilpatrick et al. 2006) but Virginia lies within the hybrid zone of *Cx. pipiens* (Huang et al. 2011). Genetics may impact the LACV competence of Appalachian *Culex pipiens.* It may also affect their feeding preferences (Huang et al. 2009). While my vector competence experiments answered the first question of whether it is possible for *Culex* to transmit LACV, further experiments are warranted to understand viral titers that develop in these species and to determine the barriers to LACV dissemination through parenteral infections (e.g., midgut versus salivary gland barriers).

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