EVALUATION OF THE CDC AUTOCIDAL GRAVID OVITRAP FOR THE SURVEILLANCE OF LA CROSSE VIRUS VECTORS

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LIST OF ABBREVIATIONS

Ae……..*Aedes*
AGO……..Autocidal gravid ovitrap
CDC……Centers for Disease Control and Prevention
DENv……Dengue virus
g……..Gram(s)
L……Liter(s)
LACE……La Crosse Encephalitis
LACv……La Crosse virus
OD……..Optical density
PCA……...Principal components analyses

ABSTRACT

EVALUATION OF THE CDC AUTOCIDAL GRAVID OVITRAP FOR THE SURVEILLANCE OF LA CROSSE VIRUS VECTORS

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La Crosse virus (LACv) is the most common cause of pediatric arboviral infection in North America and is endemic in western North Carolina. The virus is primarily vectored by *Aedes triseriatus* although two invasive species (*Ae. albopictus* and *Ae. japonicus*) may be important accessory LACv vectors in the Appalachian region. Trap based methods for the surveillance and control of LACv vectors remain inadequate. Thus, the evaluation of novel collection methods remains a public health priority. The Centers for Disease Control and Prevention (CDC) autocidal gravid ovitrap (AGO) is a novel trap used for the surveillance and control of *Ae. aegypti*. This report is the first evaluation of the CDC AGO within the context of surveillance for LACv vectors. In Aim 1, CDC AGOs (n=36) were deployed in a randomized complete block design for five weeks. The mean yield of LACv vectors was 0.86 mosquitoes per trap per day, and the CDC AGO was highly specific for the three targeted LACv vectors (98.7%). Furthermore, the standard CDC AGO oviposition attractant, a hay infusion, was compared with a White Oak leaf infusion. There was no significant difference in the total number of mosquitoes collected

overall by infusion type. However, the data suggest that the hay infusion is more effective for trapping *Ae. triseriatus* than the White Oak leaf infusion.

Gaining insight into the dynamic microbial communities of infusion-baited CDC AGOs in hopes of optimizing the infusions for mosquito surveillance, thus reducing public health risk of La Crosse Encephalitis (LACE) was the foundation of Aim 2. In Aim 2, microbial community metabolic profiles of the standard CDC AGO hay infusion and a White Oak leaf infusion were analyzed using Biolog EcoPlates. Principal components analyses revealed distinct separation of infusion types and exposed consistent temporal and spatial trends.

In Aim 3, CDC AGOs (n=25) were deployed in a LACE endemic area to determine the practicability of the traps and if they reduced the proportion of gravid mosquitoes. The traps produced a mean yield of 2.28 mosquitoes per trap per week, a higher trap abundance than found in Aim 1, which is a foundation for future LACv surveillance. There were no statistically significant differences in the proportions of gravid mosquitoes.

AIM 1: EFFICACY OF THE CDC AGO

Introduction

La Crosse Virus

La Crosse encephalitis (LACE) remains the most commonly reported pediatric arboviral encephalitis in North America, and the second most reported arboviral disease behind West Nile encephalitis (Gaensbauer et al., 2014, Lindsey et al., 2015). Infection by La Crosse virus (LACv: Family Bunyaviridae: California serogroup), although mostly unrecognized in humans, may lead to clinically apparent disease that is often characterized by frank encephalitis and may progress to seizures, coma, and rarely death (McJunkin et al., 1997). While the case fatality rate continues to remain low (~1%), the social and economic burden of the disease may be high (Rust et al., 1999, Utz et al., 2003, Gaensbauer et al., 2014). Recently, Gaensbauer et al. (2014) reviewed 665 pediatric cases (children < 18 years of age) of La Crosse virus disease reported during 2003-2012 to the national arboviral surveillance system (CDC ArboNET). Of these cases, the median age was 7 years and the majority of cases (82%) had an onset of illness that occurred during the months of July through September (Gaensbauer et al., 2014). Based on the available data, 97% of the cases required hospitalization; there were nine fatal cases (1.4% overall).

Although LACE was historically reported from the Midwestern region of the United States, the disease geography appears to be shifting toward the Appalachian region where, during 2003-2012, 81% of the nationally reported cases were reported from Ohio, West Virginia, North Carolina, and Tennessee (Gaensbauer et al., 2014).

However, within North Carolina the western region has long been recognized as an endemic area for La Crosse virus and associated disease (Kappus et al., 1982, Szumlas et al., 1996, Haddow and Odoi, 2009) although reports as early as 1964 reported presumptive LACE cases in NC as “California Virus Encephalitis” (Kelsey and Smith, 1978).

La Crosse Virus Transmission Cycle

La Crosse virus is maintained in an enzootic focus by transovarial, transstadial and venereal transmission mechanisms within mosquitoes, and horizontal transmission involving sciurid mammals that act as amplifying hosts (Moulton and Thompson, 1971, Pantuwatana et al., 1972, Watts et al., 1972, Watts et al., 1974, Watts et al., 1975b). *Aedes triseriatus* (Say, 1823) is the primary maintenance vector of LACv (Thompson et al., 1972, Watts et al., 1974). *Ae. triseriatus* larvae develop in tree holes and artificial containers that provide similar habitats, such as discarded tires and other water-holding containers, often in urban areas, and adults are commonly encountered in forested areas. *Ae. triseriatus* are known to feed on small mammals, such as squirrels, and humans. They are also known to exhibit transovarial transmission, which may allow them to transmit LACv without first having a blood meal.

Two invasive vectors have recently expanded to western NC (Scott, 2003, Gray et al., 2005) and co-occur with the primary LACv vector. Both are found in woodlands and urban areas, and recent evidence suggests that both invasive species, *Ae. albopictus* (Skuse, 1895) and *Ae. japonicus* (Theobald, 1901), are competent vectors that also play a role in the transmission or maintenance of the virus in some endemic areas (Tesh and Gubler, 1975, Gerhardt et al., 2001, Erwin et al., 2002, Sardelis et al.,

2002, Harris et al., 2015, Westby et al., 2015). These invasive mosquitoes may affect LACv range expansion and public health risk.

La Crosse Virus Surveillance

Presently, entomologic surveillance methods for the adult female LACv vectoring mosquitoes remain inadequate. Similarly, there is no evidence of effective trap-based methods for the control of LACv vectoring mosquitoes in the peridomestic environment in LACE endemic areas. A recent study suggested using a combination of trapping methods when sampling for LACv vectors in southern Appalachia as specific traps have different vector affinities (Urquhart et al., 2016). These diurnal *Aedes* are not readily attracted to light traps, and although attracted to CO_2-baited traps are not collected in proportions that are informative for the prediction of disease risk or population size estimates (Hoel et al., 2009). Recent efforts have demonstrated the utility of the BG-Sentinel (BGS) and infusion-baited gravid traps for the collection of all three LACv vectoring *Aedes*, but they require power sources with the addition of specialized baits or organic infusions (Urquhart et al., 2016). Furthermore, these and other methods each have inherent physiologic biases (Hoel et al., 2009, Urquhart et al., 2016, Ball and Ritchie, 2010) and may have different species-specific levels of attractiveness that have yet to be well defined. Although increasingly used to collect all three LACv vectors for entomologic and virologic surveillance, none of these methods are known to be effective at reducing local disease risk or controlling local mosquito populations.

Autocidal Gravid Ovitrap

Recently the Centers for Disease Control and Prevention (CDC) designed an improved autocidal gravid ovitrap (AGO) that uses infusion-mediated olfactory cues to

attract gravid *Ae. aegypti* and trap them on a sticky surface (Mackay et al., 2013). The CDC AGO is an affordable, low-maintenance, passive trap constructed from a black polyethylene pail that holds 10 L of water used to infuse a 30 g hay packet. Gravid mosquitoes seeking a site to oviposit are attracted to the CDC AGO by olfactory cues, but oviposition is hindered by the trap's design and the mosquitoes are subsequently caught by a sticky surface at the entrance of the trap. Barrera et al. (2014) demonstrated that the CDC AGO capture rates for *Ae. aegypti* in the field were positively correlated with BG Sentinel traps and therefore an inexpensive and useful surveillance tool. Because the CDC AGO targets and traps the gravid female, both the fertility and survival rates of the mosquito population may be simultaneously reduced. Thus the CDC AGO is also potentially useful as a control tool, acting like an environmental "sink" for both the gravid female and her offspring. To that effect, the CDC AGO was successfully used to reduce *Ae. aegypti* populations, prevent outbreaks associated with increased mosquito abundance, and produce sustained, area-wide control of the mosquito in intervention areas in Puerto Rico (Barrera et al., 2013, Barrera et al., 2014b, Barrera et al., 2014a).

It is established that baiting ovitraps with White Oak leaf infusion elicits oviposition responses by *Ae. triseriatus* and *Ae. albopictus* (Trexler et al., 1998, Beehler et al., 1992, Allan and Kline, 1995). Field observations also suggest that White Oak leaf infusion may also effectively trap *Ae. japonicus* (Byrd, personal communication). Additionally, *Ae. triseriatus* are commonly found in *Quercus* tree holes, especially White Oaks, therefore the White Oak infusions are analogous to the natural organic material

found in tree holes. White Oaks are widely distributed in the eastern and southeastern US, therefore leaves are readily available for infusion preparation.

Aim

Given that the three LACv vectors readily oviposit in standard oviposition traps, it was reasonable to assume that an infusion-baited passive “sticky trap” such as the CDC AGO may also be useful as a surveillance tool for gravid adult LACv vectors. Thus, the objectives of Aim 1 were to: 1) determine the ability of the CDC AGO to trap the three principle LACv vectors (*Ae. triseriatus*, *Ae. japonicus*, and *Ae. albopictus*), and 2) compare the attractiveness of the standard hay infusion to a White Oak leaf infusion.

Methods

Study Area

This study was conducted for five weeks during the summer of 2015 (May 24-June 27) at six peridomestic sites in two La Crosse Encephalitis endemic counties, Macon and Jackson, within western North Carolina (Table 1). The mean temperature and rainfall, factors associated with mosquito abundance, for Macon and Jackson counties in NC was high 81.5 °F, low 57.5 °F and 4.68 inches during the five weeks.

Research Design

The ability of the CDC AGO (Mackay et al., 2013, Barrera et al., 2014b) to trap LACv vectors using the CDC's standard hay infusion or a White Oak (*Quercus alba*) leaf infusion was investigated. In lieu of a 7-day old infusion, 84 g of *Q. alba* leaves, informed by the work of Trexler et al. (1998), or 30 g of hay (Barrera et al., 2014b), were placed into mesh fabric sachets and added to the CDC AGO along with 10 L of distilled water at the time of deployment. CDC AGO traps (n=36) were deployed 5 meters apart in a randomized complete block design (6 blocks with 6 traps [3 replicates per infusion type per block]) for a total of 630 trap days per infusion type across five weeks.

Mosquito Collection and Identification

Captured mosquitoes were carefully removed from the CDC AGOs sticky surface twice weekly using forceps. Specimens were counted and pooled according to species, sex, physiological status (e.g., gravid), and trap ID and block ID. Mosquitoes were morphologically identified using standard identification keys (Darsie and Ward 2005, Harrison et al., 2016) and additional taxonomic references (Carpenter and LaCasse, 1955, Savage and Smith, 1994) when key morphological characters were damaged.

Aedes triseriatus and *Ae. hendersoni* were distinguished using a previously described duplex polymerase chain reaction (PCR) assay (Wilson et al., 2014). This assay can detect the presence of both species in a single PCR reaction, which is critical for accurate identification for surveillance because these two sibling species differ in their abilities to transmit LACv. Additionally, mosquitoes that were grossly damaged were presumptively identified using rDNA ITS2 size polymorphisms (Byrd and Wesson, 2004).

Statistical Analyses

To investigate the ability of the CDC AGO to trap the three principle LACv vectors (*Ae. triseriatus*, *Ae. japonicus*, and *Ae. albopictus*), a Pearson's chi-square test was conducted in SPSS (v. 21.0, IBM Corp., Armonk, NY) to distinguish trends between the two infusion types and species.

A generalized linear mixed model (GLMM) was used to compare the attractiveness between the standard hay infusion and a White Oak infusion. Following a Poisson distribution, the counts of mosquitoes collected over the study period was used to represent the attractiveness of each infusion type to the mosquito conditional on a location within a block. The "infusion" was specified as a fixed effect, and the "block" and "trap location" were specified as random effects. The Poisson GLMM can be expressed by the following:

$$\log\{E(Y_{ij} \mid b_i, l_{ij})\} = \alpha + x_{ij}\beta + b_i + l_{ij},$$

where $b_i \sim N(0, \sigma^2_b)$ *and* $l_{ij} \sim N(0, \sigma^2_l)$ represented the random effects of block and trap location, respectively; x indicated which infusion was used, and α was the intercept.

The generalized linear mixed effect analysis was conducted in R (R Core Team, 2012) using the built-in function *glmer* in the package *lme4* (Bates et al., 2012) to indicate the relationship between the infusion type and the counts of mosquitoes of each species, *Ae. triseriatus* and *Ae. japonicus*, after controlling for block and trap location variability. *Aedes albopictus* were not included in the analysis due to low collection numbers.

Table 1: Location of study sites with mean temperature and relative humidity measurements for Aim 1

Site	County	Latitude	Longitude	Altitude (m)	Mean T AM (Range) PM (Range)	Mean RH AM (Range) PM (Range)
1	Macon	35.149417 N	83.413333 W	655	21.76 (15.78-25.67) 18.38 (12.46-21.58)	86.49 (59.38-100) 96.38 (79.24-100)
2	Macon	35.126392 N	83.372589 W	624	19.95 (16.24-26.72) 18.53 (11.83-21.75)	88.53 (65.11-100) 97.01 (83.21-100)
3	Macon	35.129206 N	83.374989 W	624	21.84 (16.39-25.47) 18.48 (11.86-21.61)	91.61 (67.53-100) 97.98 (85.64-100)
4	Jackson	35.311986 N	83.178356 W	676	22.16 (15.33-26.43) 18.23 (13.23-21.61)	82.83 (55.67-99.9) 94.60 (73.04-100)
5	Jackson	35.325944 N	83.179567 W	710	21.59 (15.19-25.73) 18.13 (13.3-21.58)	86.90 (57.82-100) 95.24 (71.52-100)
6	Jackson	--.------ N*	--.------ W*	676	22.53 (15.62-27.17) 18.31 (13.08-21.65)	80.69 (51.86-100) 94.23 (73.6-100)

AM (6:15 a.m. - 8:45 p.m.) and PM (8:45 p.m. - 6:15 a.m.)

*Location confidential due to privacy requests

Results

Vector Abundance

This experiment yielded a total of 158 mosquitoes during the five week study period, a mean yield of 0.86 mosquitoes per trap per week, and targeted the three principle LACv vectors (98.7%). *Aedes triseriatus*, the primary vector, comprised approximately half of the total mosquitoes collected (52.9%), while *Ae. japonicus* and *Ae. albopictus* represented 38.1% and 7.7% of the total collection, respectively. Approximately 90% (n=142) of the mosquitoes collected were female. Of the mosquitoes able to be assessed, excluding ones too damaged, 88% of intact mosquitoes were gravid.

No *Ae. hendersoni* were detected using a duplex PCR assay. A negative PCR result, or lack of amplification of the species-specific *Ae. hendersoni* primers, indicated the absence of *Ae. hendersoni* from the mosquito samples collected by CDC AGOs.

Infusion Comparison

Pearson's chi-square test (Figure 1 and Table 2) showed that hay tended to attract more *Ae. triseriatus* (65%) and *Ae. japonicus* (56%) than White Oak did, however hay and White Oak were less attractive to *Ae. albopictus.* Nevertheless, the Pearson's chi-square test indicated "infusion" was not statistically significantly associated with "species" (χ^2 (2) = 1.627, p = 0.443).

A Chi square analysis of infusion type by week identified similar distributions and no statistically significant difference in the number of mosquitoes (p=0.631). There were also no statistically significant differences in the number of mosquitoes collected overall by infusion type or species-specific differences in the number of mosquitoes collected

by infusion type for *Ae. japonicus* and *Ae. albopictus*. However, hay infusion seemed to be more effective for trapping *Ae. triseriatus* than the White Oak leaf infusion when controlling for block location ($p<0.05$). One limitation of our statistical analyses was that the sample size for this aim was small, which may lead to inadequate power. However, these results suggest that there is no practical difference between White Oak or hay infusions as both infusion types were able to collect the targeted LACv vectors.

Mosquito Attractiveness to Infusions

P values from the GLMM likelihood ratio tests indicated the fixed effect "infusion" had no statistically significant effects on the attractiveness to the overall abundance of mosquitos (χ^2 (1) = 3.78, p = 0.052). The hay infusion was more attractive to the mosquitoes than the White oak infusion (χ^2 (1) = 6.25, p = .012). Likewise, p values from the likelihood ratio tests indicated no statistically significant association between "infusion" and the attractiveness to *Ae. japonicus* (χ^2 (1) = 0.42, p = 0.51). Few *Ae. albopictus* were captured, thus a GLMM analysis for this species was not conducted (Table 3).

Table 2: Pearson's chi-square test of infusion by species for Aim 1

		Ae. triseriatus	*Ae. japonicus*	*Ae. albopictus*	Total
White Oak	**Count**	**28**	**23**	**5**	**56**
	% w/in Infusion	50.0	41.1	8.9	100.0
	% w/in Species	35.0	44.2	50.0	39.4
	% of Total	19.7	16.2	3.5	39.4
Hay	**Count**	**52**	**29**	**5**	**86**
	% w/in Infusion	60.5	33.7	5.8	100.0
	% w/in Species	65.0	55.8	50.0	60.6
	% of Total	3.6	20.4	3.5	60.6
Total	**Count**	**80**	**52**	**10**	**142**
	% w/in Infusion	56.3	36.6	7.0	100.0
	% w/in Species	100.0	100.0	100.0	100.0
	% of Total	56.3	36.6	7.0	100.0

Table 3: Mosquito attraction to infusion types including a Generalized Linear Mixed Model

Variable	X^2	df	p-value
Overall	3.78	1	0.052
Ae. triseriatus	6.25	1	**0.012**
Ae. japonicus	0.42	1	0.51
Ae. albopictus	---	---	---

Coefficient in bold indicates significant p-value ($p<0.05$)

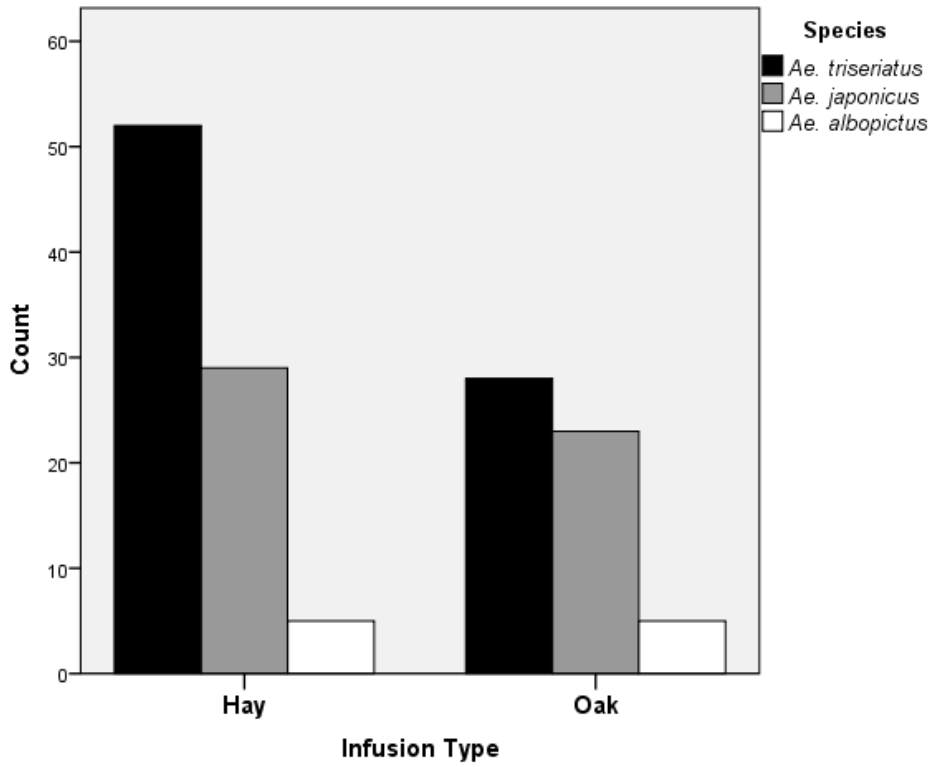

Figure 1. Trends between infusion type and species for Aim 1

Discussion

CDC AGO for La Crosse Virus Surveillance

Overall, the irregular distribution of LACv vectors and lack of sufficient surveillance and control methods for trapping adult LACv vectors are major challenges to the prevention of disease. Ideally for control, a productive trap in a LACE endemic area should target gravid mosquitoes that represent a greater virus transmission risk, and act as an entomologic sink to reduce the risk of transmission. Given that the three LACv vectors are known container-inhabiting species and readily oviposit in standard oviposition traps, it was expected that the CDC AGO would be useful for LACv surveillance. Not only do the results of this study support this assumption, but the CDC AGO was highly specific for the three targeted vectors. Additionally as supported by this study, the CDC AGO targets and eliminates the gravid female and her offspring, which were the majority (88%) of the collected mosquitoes.

The mean yield per trap was slightly lower (1.2 mosquitoes per trap per week) than observed in the Dengue AGO study (Barrera et al., 2014a, Barrera et al., 2014b). However, studies included differences in vectors, distribution, and both vector and trap densities. This study targeted *Ae. triseriatus, Ae. japonicus*, and *Ae. albopictus* while the Dengue study targeted *Ae. aegypti*. The patchy distribution of LACv vectors is inherently different than Dengue virus (DENv) vector distribution, as adult LACv vectors are typically associated with peridomestic and surrounded wooded areas and DENv vectors are well adapted to the human environment (CDC, 2016). Finally, six CDC AGOs were deployed in a LACE endemic area while three CDC AGOs were deployed in an area prone to outbreaks of *Ae. aegypti*.

A sibling species similar in appearance to *Ae. triseriatus*, *Ae. hendersoni* (Cockerell, 1918), or *Oc. hendersoni* (Reinert, 2000), is generally considered a poor LACv vector (Watts et al., 1975a, Grimstad et al., 1985, Paulson and Grimstad, 1989). *Aedes hendersoni* is susceptible to LACv infection and readily achieves a high viral dissemination rate. However, transmission of the virus is greatly reduced due to a presumptive salivary gland escape barrier (Paulson and Grimstad, 1989, Paulson et al., 1989, Paulson et al., 1992). No *Ae. hendersoni* were detected in this study using a previously described duplex PCR assay (Wilson et al., 2014).

Infusions

The results of our study suggest the two infusion types were immediately attractive and may not require an extended fermentation period as approximately 25% of the total mosquitoes collected were captured during the first week, previously referred to as the "priming week" for infusions (Figure 2).

Findings and Implications

Aim 1 was the first evaluation of the CDC AGO in the context of LACv surveillance and demonstrated the ability of the trap to collect the targeted vectors (*Ae. triseriatus, Ae. japonicus*, and *Ae. albopictus*). Although limited by sample size, we were unable to reject the null hypothesis stating that there was a difference in the overall abundance of collected mosquitoes between hay and White oak infusions. However, *Ae. triseriatus* were collected more commonly in hay-baited CDC AGOs ($p < 0.05$). Additionally, this aim provides evidence that infusions are effective without an extended fermentation period during the first week of collection. These implications may be useful for future large-scale trials to reduce LACv vectors, thus reducing public health risk of

LACE. In this context, CDC AGOs may be employed as an environmental “sink” to disrupt mosquito populations and eliminate some of the disease burden in LACE endemic areas like western NC.

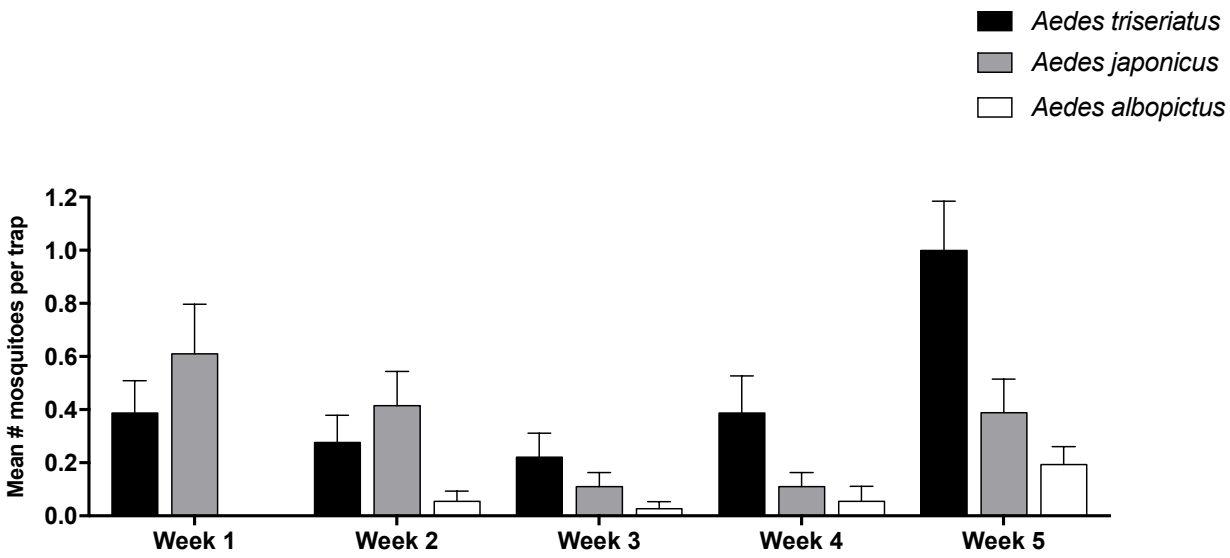

Figure 2. Species-specific mosquito collections by week for Aim 1. Mean number of mosquitoes per trap per week; error bars represent standard error.

AIM 2: MICROBIAL COMMUNITY PROFILING OF INFUSIONS

Introduction

Role of Microbial Communities in Mediating Oviposition

Immature stages of container-inhabiting mosquitoes, such as the LACv vectors (*Ae. triseriatus, Ae. japonicus, and Ae. albopictus*), live in aquatic habitats characterized by diverse microbial communities (Ponnusamy and Wesson, 2009). These microbial communities play a role in the catabolism of organic detritus, commonly plant material that accumulate in natural tree holes, that produces metabolites capable of mediating oviposition behavior of gravid mosquitoes (Ponnusamy and Wesson, 2009). Changes in microbial community structure are thought to result in variations in microbial metabolic activity and nutrients, and may correlate with mosquito attractiveness to infusion-baited traps (Beehler et al., 1992). Infusion-baited ovitraps are essentially ecosystem mesocosms that are analogous to aquatic ecosystems that naturally occur in tree holes and other container habitats of mosquitoes. Research (Trexler et al., 1998, Trexler et al., 2003, Ponnusamy et al., 2008, Ponnusamy et al., 2010) suggests oviposition is facilitated by olfactory cues from microbial metabolism of plant material, thus the recently designed CDC AGO uses hay infusion-mediated olfactory cues to attract gravid *Ae. aegypti* seeking a site to oviposit (Mackay et al., 2013).

Bacteria and bacteria-associated chemical cues are well known to mediate oviposition by *Aedes* mosquitoes (Trexler et al., 1998, Trexler et al., 2003, Ponnusamy et al., 2008, Ponnusamy et al., 2010). Similarly, the “break-down” of plant and other organic detritus by microbes in aquatic environments produces metabolites that

influence the oviposition of *Aedes* mosquitoes. Recent studies suggest that oviposition is strongly influenced by the "abundance and diversity of bacterial species, which in turn is affected by plant species…" (Ponnusamy et al., 2010). However, these influences have not been adequately determined in the context of the LACv vectors, specific gravid trap infusions, and the microbial community stability over time in these traps. Characterizing the microbial community composition of trap infusions over time may be predictive of the relative mosquito oviposition attractiveness. This information may be useful in optimizing the attractiveness of infusions to target mosquitoes throughout an entire season when the risk of virus transmission is highest.

White Oak Infusion

White Oak leaf infusions elicit oviposition responses by *Ae. triseriatus* and *Ae. albopictus* (Trexler et al., 1998, Beehler et al., 1992, Allan and Kline, 1995). In laboratory bioassays and field populations, *Ae. albopictus* were attracted to White Oak leaf infusions over a broad range of concentrations and fermentation times. *Ae. triseriatus* oviposited in only a few concentrations of older age infusions and demonstrated a significant oviposition response to ovitraps containing 7-day-old infusion (Trexler et al., 1998). Field observations also suggest that White Oak leaf infusion may also effectively trap *Ae. japonicus* (Byrd, personal communication). Additionally, *Ae. triseriatus* are commonly found in *Quercus* tree holes, especially White Oaks, therefore the White Oak infusions are analogous to the natural organic material found in tree holes. White Oaks are widely distributed in the eastern and southeastern US, therefore leaves are readily available for infusion preparation.

Aim

The objective of Aim 2 was to compare the microbial community metabolic profiles of the CDC AGO standard oviposition attractant (i.e. hay infusion) and a White Oak leaf infusion. The questions addressed by Aim 2 are: 1) is there a difference between the microbial communities of the two infusion types, and 2) are microbial community changes, or ecological succession, predictable over space and time? This aim seeks to provide insight into these dynamic microbial communities of infusion-baited CDC AGOs in hopes of optimizing the infusions for mosquito surveillance, thus reducing public health risk of LACE.

Methods

Study Area

This study was conducted for five weeks during the summer of 2015 (May 24-June 27, 2015) at six peridomestic sites in two La Crosse Encephalitis endemic counties within western North Carolina (Table 1).

Field Design

Mesh fabric sachets containing either 84 g of *Q. alba* leaves (following the methodology of Trexler et al., 1998) or 30 grams of hay (following the methodology of Barrera et al., 2014b) were added to the CDC AGO along with 10 L of distilled H_2O at the time of deployment. CDC AGOs (n=36) were deployed 5 meters apart in a randomized complete block design (6 blocks with 6 traps [3 replicates per infusion type per block]). CDC AGOs (n=14) were randomly selected using an online generator from which to obtain infusion samples to determine microbial community differences between infusions (one trap per infusion type per block). The excess amount of materials (Biolog EcoPlates; Biolog, Inc., Hayward, CA) allowed for an additional pair of trap infusions to be analyzed.

Infusion Collection

Microbial community metabolic profiles of the hay infusion and White Oak infusion were compared using a colorimetric microplate assay (Biolog microbial community analysis EcoPlates) weekly over the course of the five-week study period. The Biolog plates contain 3 replicates of 31 carbon sources[1] that may be used to

[1]Putrescine was not considered in the analyses.

"fingerprint" microbial communities (van Heerden et al., 2001, Lawley and Bell, 1998). As the microbes metabolize the unique carbon sources, a tetrazolium dye is reduced resulting in a colorimetric change that is quantifiable using a plate reader (OD_{590}). Weekly aquatic samples (~5 mL) were directly inoculated (130 microliter/well), incubated at 25°C, and then analyzed at defined time intervals (0, 48, and 72 hours) to establish community-level physiological profiles.

Statistical Analyses

The microbial community metabolic patterns are typically analyzed at defined time intervals over 2 to 5 days. Infusion optical density (OD) values at 48 hours were analyzed by principal components analyses (PCA) using the statistical software SYSTAT version 6.0 (San Jose, California) in order to 1) characterize the variation in the large, multivariate dataset, 2) determine similarity among traps and time points, and 3) eliminate the risk of losing resolution of the metabolic pattern that could arise from using later plate readings with higher OD values. PCA component loadings, or the correlations between the PCA axes and the original variables, convey how much of the variation is explained by the factor score. The higher the component loading, the stronger the correlation of the variable is to the factor score. Correlation values greater than or equal to 0.90 were recognized as the most important variables, or carbon sources, associated with the factor scores and explains much of the variation in the data. The changes observed in the "fingerprint" pattern may give insight about the microbial population changes over time. White Oak and hay infusion types were considered collectively and separately to address the questions previously listed in this aim.

Results

Principal Components Analyses

The PCA of White Oak versus hay infusion (Figure 3a), which included data from all traps and all weeks, resulted in the separation of the two infusion types. The PCA of White Oak infusion (Figure 3b), including oak traps and all weeks, revealed a successional trend, or the gradual change of a developing microbial community resulting from time and autochthonous input. Changes in the microbial community metabolic profiles progressed from the time of deployment (week 0) to an early succession community (weeks 1-2) to a later succession community (weeks 3-5). The PCA of hay infusion (Figure 3c), including hay traps and all weeks, also resulted in a similar successional trend. Individual White Oak traps weeks 0-5 (Figure 5) and individual hay traps weeks 0-5 (Figure 6) show that these temporal successions were consistent over geographical areas.

Figures 4a-f represents the progression of the five week study period and displays the separation of White Oak and hay infusion with Factor 1 accounting for the majority of the variance (mean 62.2%). The earlier weeks (weeks 0-2) show some crossover of the two infusion types before becoming more distinctly separated as the aging infusions apparently stabilized. Additionally, as infusions aged they were subject to increasing numbers of carbon source influences. On each graph, Factor 1 explains the majority of the variance accounted for from within the original variables (range 36.9%-78.6%). The percent of variation explained by Factor 1 increases as infusions age, showing a more powerful division of White Oak and hay infusions during the later weeks (weeks 3-5). It is apparent that Factor 2 did not aid strongly in explaining the

difference between infusions as the total variance explained is lower (mean 17.0%, range 7.6%-34.7%), with the exception of 34.7% for week 2.

Carbon Sources

The Biolog carbon sources that accounted for the highest percent of the variation in the PCA of White Oak versus hay infusion are shown in Table 4. There were 16 carbon sources highly correlated to Factor 1 utilized during earlier weeks (weeks 0-2), 5 of which were exclusively linked to this time frame. Complete division in carbon source utilization patterns of White Oak and hay infusion occurred during later weeks (weeks 3-5). There were 21 carbon sources strongly correlated to Factor 1 during later weeks (weeks 3-5), 10 of which were exclusively linked to this time frame. There were 11 common carbon sources correlated to Factor 1 throughout both early and late weeks. Of the carbon sources influencing older infusions, 12 were commonly utilized and are characterized as various classes of compounds, including carboxylic/ketonic acids, carbohydrates, and amino acids. Thus there was no one specific carbon source or class of carbon source influencing the analyses at 48 hours, but rather a variety of organic compounds. Itaconic acid, i-Erythritol, and γ-Hydroxybutyric acid are a few of the carbon sources useful in the distinction of older infusions and are known byproducts of fermentation.

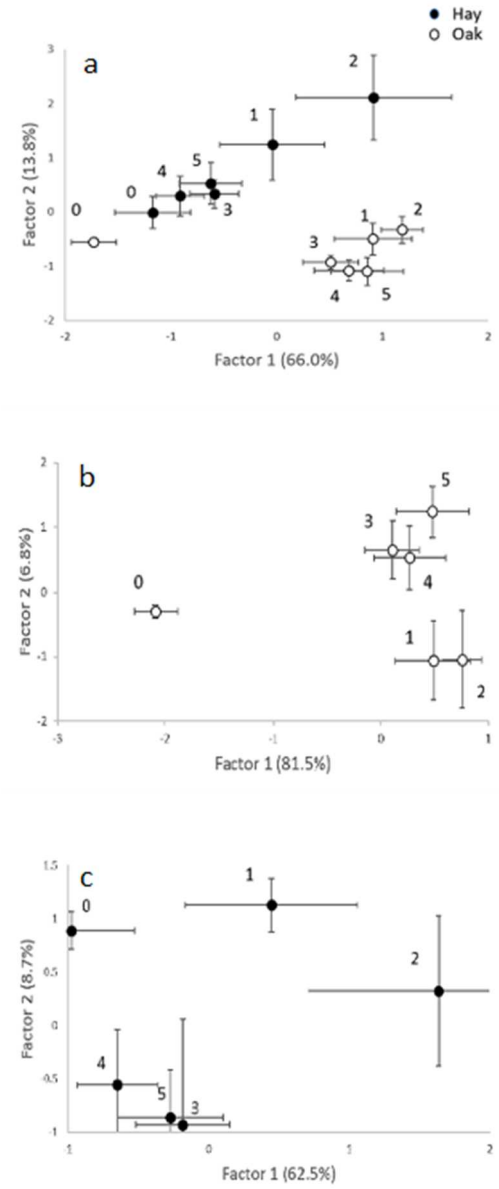

Figure 3. Principal components analyses overall trends. Hay vs. White Oak infusion OD values after 48 hour incubation for all traps for weeks 0-5 (a); White Oak infusion OD values for oak traps weeks 0-5 (b); Hay infusion OD values for hay traps weeks 0-5 (c).

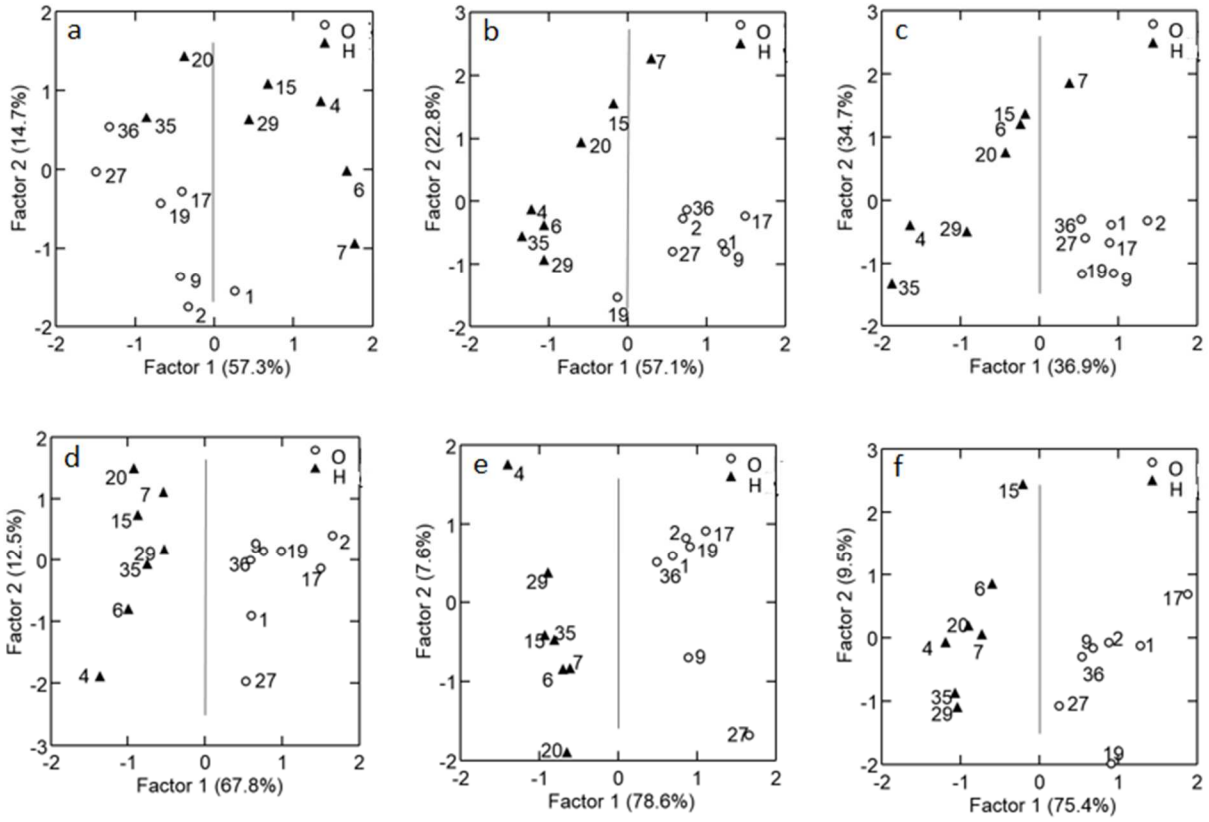

Figure 4. Principal components analyses of Hay vs. White Oak infusion OD values after 48 hour incubation for week 0 (a), week 1 (b), week 2 (c), week 3 (d), week 4 (e), and week 5 (f).

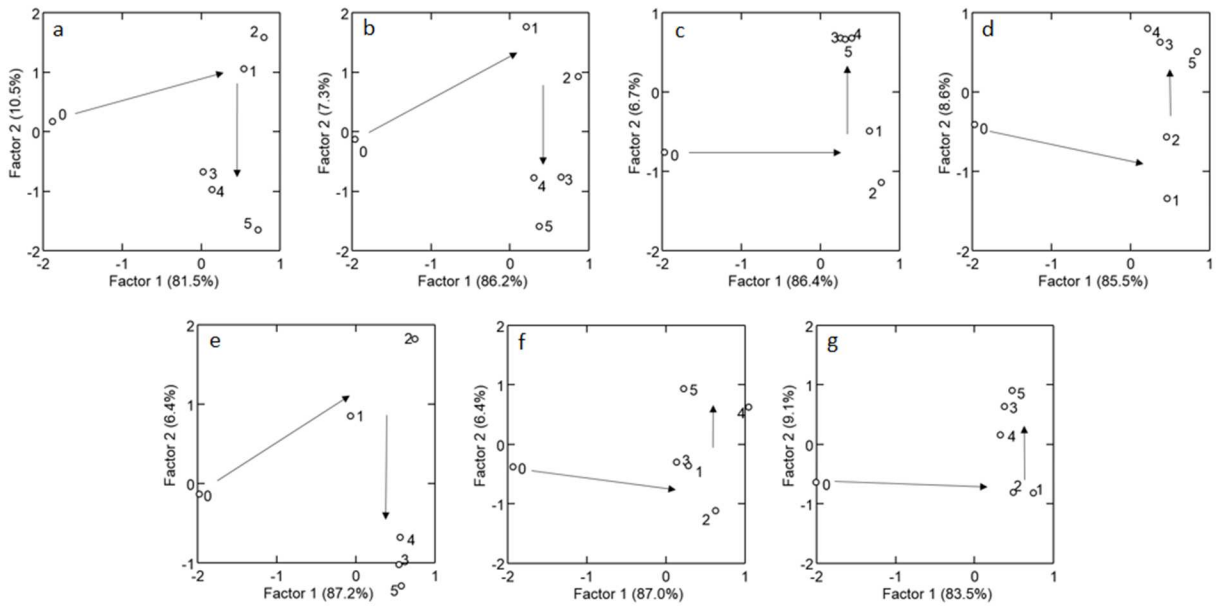

Figure 5. Principal components analyses of White Oak infusion OD values after 48 hour incubation for weeks 0-5. Each PCA graph represents a unique White Oak infusion-baited CDC AGO: Trap 1, 2, 9, 17, 19, 27, and 36 (a-f, respectively).

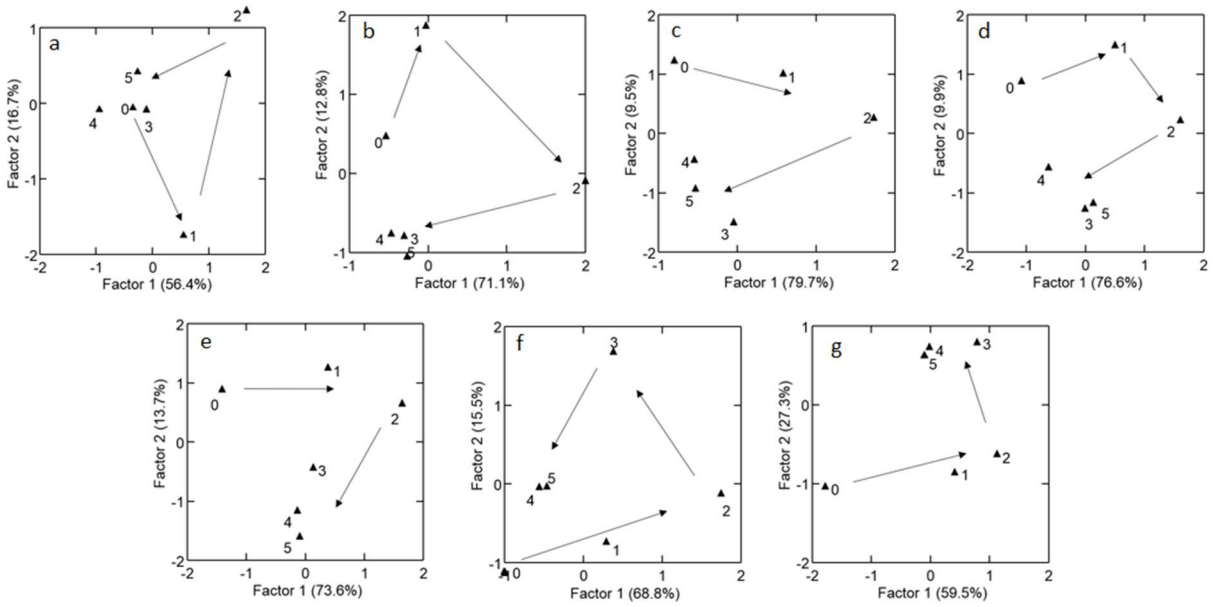

Figure 6. Principal components analyses of Hay infusion OD values after 48 hour incubation for weeks 0-5. Each PCA graph represents a unique hay infusion-baited AGO: Trap 4, 6, 7, 15, 20, 29, and 35 (a-f, respectively).

Discussion

Infusion-baited CDC AGOs

Biolog EcoPlates enable the evaluation of metabolic profiles from microbial communities that reflects the state of their activity or metabolic potential from the environment in which they were sampled (Gryta et al., 2014). Principal components analyses revealed differences in the microbial communities of hay and White Oak infusions and consistent trends, which suggest that the incubation of infusion samples in Biolog EcoPlates produced metabolic response patterns useful in characterizing microbial communities and distinguishing among samples. Additionally, the ability to rapidly visualize community profiles enabled the comparison of microbial communities across temporal and spatial scales.

Infusion-baited ovitraps have been used to monitor arbovirus vectors during disease outbreaks and routine surveillance (Barrera et al., 2014b, Tsai et al., 1989, Polson et al., 2002). Many different materials such as leaves, grass, and sod have been used to create infusions reported to be attractive to *Aedes* mosquitoes (Loor and DeFoliart, 1969, Gubler, 1971, Holck et al., 1988, Kitron et al., 1989, Allan and Kline, 1995, Lampan and Novak, 1996), however there are currently no methods for producing standardized infusions. An “Achilles heel” of infusion-based mosquito traps is that microbial communities, thus the relative attraction for mosquito oviposition, of any infusion may inherently vary over time and autochthonous input once they are deployed in the field.

Future work

The CDC AGO may be deployed in the field for up to two months with little maintenance, thus determining the stability of these trap infusions may be particularly important in eliciting mosquito oviposition. Additional evaluations, such as next generation sequencing, may enable a more comprehensive analysis of the microbial composition of trap infusions and lead to the discovery of a novel sustainable source of attractants or standardized infusion, which could increase the effectiveness of infusion-baited ovitraps such as the CDC AGO.

Table 4: Carbon sources, classes of compounds, and relative importance of each for data shown in Figures 4a-f for White Oak versus hay infusions inoculated into Biolog EcoPlates

ID	Carbon Source	Classification	Figures (correlation ≥ 0.90)					
			4a	4b	4c	4d	4e	4f
1	Water	Carbon free				X	X	X
2	D-Galactonic Acid γ-Lactone	Carboxylic/Ketonic acids	X			X	X	
3	D-Galacturonic Acid	Carboxylic/Ketonic acids		X		X	X	X
4	2-Hydroxy Benzoic Acid	Carboxylic/Ketonic acids					X	
5	γ-Hydroxybutyric Acid	Carboxylic/Ketonic acids				X	X	X
6	D-Glucosaminic Acid	Carboxylic/Ketonic acids	X			X	X	X
7	Itaconic Acid	Carboxylic/Ketonic acids				X		X
8	α-Ketobutyric Acid	Carboxylic/Ketonic acids		X		X	X	X
9	D-Malic Acid	Carboxylic/Ketonic acids	X			X*		
10	Pyruvic Acid Methyl Ester	Carbohydrates		X				
11	D-Xylose	Carbohydrates	X	X		X	X	X
12	i-Erythritol	Carbohydrates				X	X	X
13	D-Mannitol	Carbohydrates	X	X				X
14	N-Acetyl-D-Glucosamine	Carbohydrates	X					
15	D-Cellobiose	Carbohydrates	X		X*			
16	Glucose-1-Phosphate	Carbohydrates				X	X	X
17	α-D-Lactose	Carbohydrates					X	X
18	D,L-α-Glycerol Phosphate	Carbohydrates		X	X	X	X	X
19	L-Arginine	Amino acids						
20	L-Asparagine	Amino acids	X			X*		
21	L-Phenylalanine	Amino acids					X	
22	L-Serine	Amino acids		X		X	X	X
23	L-Theronine	Amino acids			X	X	X	X
24	Glycyl-L-Glutamic Acid	Amino acids			X	X	X	X
25	Tween 40	Polymers				X	X	
26	α-Cyclodextrin	Polymers					X	X
27	Phenylethyl-amine	Amines/Amides	X*		X		X	X

*Only carbon sources 9, 15, 20, and 27 were correlated ≥ 0.9 for Factor 2, and in only one case each.

AIM 3: FIELD TRIAL

Introduction

Epidemiology of La Crosse Encephalitis

Increasing urbanization and the expansion of residential housing into forests may have facilitated the emergence of LACE in the Appalachian region (Leisnham et al., 2012). This region is one of the major hotspots for LACE in the United States (Haddow and Odoi, 2009). Mosquitoes that vector LACv are container-inhabiting species and often found in wooded areas that may be close to houses, and sometimes in artificial water-holding containers discarded nearby houses, thus the peridomestic risk of LACv transmission is high (Tamini, 2011, Erwin et al., 2002). Evidence from familial cases occurring at the same residence over different years also support the claim by Erwin et al. (2002) that there are peridomestic risk factors.

Risk for LACE is greatest during the summer months (Gaensbauer et al., 2014). The warmer summer temperatures ultimately increase the abundance and distribution of vectors and hosts. Summertime is also associated with increased human activity outdoors, especially children playing in their backyards that may be adjacent to wooded areas containing host-seeking mosquitoes.

A blinded cohort study by Erwin et al. (2002) reported that children with La Crosse infection spent a greater number of daylight hours outdoors than children who were not infected, important since LACv vectors, *Ae. triseriatus* and *Ae. albopictus*, are diurnal feeders. There is some evidence (Erwin et al., 2002) that suggest *Ae. albopictus* may be a more aggressive human biter than *Ae. triseriatus*, which leads to greater

concern about the role of this species in the LACv transmission cycle, especially since recent evidence (Erwin et al., 2002) suggest the abundance may be up to three times greater around residences of LACv cases versus non-cases. Additionally, La Crosse infected children were almost four times more likely than non-infected to live in a residence with one or more tree holes nearby (within 100 meters). This finding is consistent with that of a case-control study in West Virginia that found an increased risk of LACv infection in children who lived within 90 meters of one or more tree holes (Woodruff et al., 1992).

Aim

The CDC AGO is highly specific for trapping LACv vectors (*Aedes triseriatus, Ae. japonicus*, and *Ae. albopictus*) as suggested by Aim 1 of this study. The overall goal of this thesis was to evaluate the utility of the CDC AGO as a tool in peridomestic environments where risk of virus transmission is likely highest in hopes of reducing disease. Thus, the objectives of Aim 3 were to determine: 1) the usefulness of the CDC AGO in LACE endemic areas, and 2) if the CDC AGO reduced the proportion of gravid mosquitoes.

Methods

Study Area

This study was conducted for five weeks during the summer of 2015 (August 2-September 6) at 10 peridomestic sites (5 treatment sites and 5 paired control sites) in two La Crosse Encephalitis endemic counties within western North Carolina (Table 5). Each treatment site was accompanied by a nearby (100-500 meters) paired site (separate dwelling) that served as a control.

Research Design

Mesh fabric sachets containing 30 g of hay were added to the CDC AGOs along with 10 L of distilled H_2O at the time of deployment. CDC AGOs (n=25) were deployed at least 5 meters apart around the perimeter of peridomestic residences (5 traps per treatment site). No traps were deployed at the control sites.

Mosquito Collection and Identification

Captured mosquitoes were carefully removed from the AGOs sticky surface twice weekly using forceps. Resting mosquitoes were also collected from surrounding vegetation weekly at both treatment sites and control sites using a Nasci aspirator (10 minutes per collection) to help determine the relative mosquito abundance and population structure. Specimens were enumerated and pooled according to species, sex, and physiological status (e.g., gravid), and collection method. Mosquitoes were morphologically identified using standard identification keys (Darsie and Ward, 2005, Harrison et al., 2016, Carpenter and LaCasse, 1955, Savage and Smith, 1994).

Statistical Analyses

Descriptive analyses were conducted in Excel to summarize and discover patterns in the data. A Fisher's exact test (Social Science Statistics 2016) was performed for 2x2 contingency tables of proportions of gravid mosquitoes to accommodate the small sample size this study yielded. A Z-test (Social Science Statistics 2016) was also used to determine differences between proportions of gravid mosquitoes. Two way ANOVA and a Tukey's multiple comparisons test was used to determine differences among species-specific mosquito collections by week.

Table 5: Location of study sites for Aim 3

Location	County	Latitude	Longitude
Treatment 1	Macon	--.------ N*	--.------ W*
Control 1	Macon	35.091787 N	83.167302 W
Treatment 2	Jackson	35.338512 N	83.264769 W
Control 2	Jackson	35.202056 N	83.154917 W
Treatment 3	Jackson	--.------ N*	--.------ W*
Control 3	Jackson	35.345619 N	83.202580 W
Treatment 4	Jackson	35.325704 N	83.179519 W
Control 4	Jackson	35.193517 N	83.104810 W
Treatment 5	Jackson	35.322003 N	83.234772 W
Control 5	Jackson	35.191893 N	83.14031 W

*Location confidential due to privacy requests

Results

Mosquito Abundance

Sampling yielded 285 mosquitoes collected by CDC AGOs and produced a mean yield of 2.28 mosquitoes per trap per week. The CDC AGO was highly specific for the targeted LACv vectors (99.3%). Of the total mosquitoes collected, 93.3% were female, and 86.4% of the total females collected were recognizably gravid (Table 6). Nasci collections yielded 192 mosquitoes, of which 62.0% were LACv vectors collected from both treatment sites and control sites. Of the total LACv vectoring mosquitoes, 63.0% were female, and 16% of the females were gravid (Table 7).

There were no statistically significant differences in the species-specific proportions of gravid mosquitoes as determined by a series of Fisher's exact tests and Z-tests ($p<0.05$). There was a statistically significant difference between the mean numbers of *Ae. triseriatus* collected per trap during week 1 and all other weeks ($p<0.01$) (Table 8 and Figure 7). There were no other statistically significant differences in species-specific mosquito collections by week.

Table 6: Mosquitoes collected by CDC AGOs for Aim 3

Species	n	% M (n)	% F (n)	% G (n) 95% CI
Ae. triseriatus	152	0	94.1 (143)	90.9 (130) 86.2-95.6
Ae. japonicus	56	0	100 (56)	96.4 (54) 91.5-101.3
Ae. albopictus	75	1.3 (1)	86.7 (65)	67.7 (44) 56.3-79.1
Total/LACv vectors	283	0.35 (1)	93.3 (264)	86.4 (228) 82.3-90.5
Other	0	-	-	-

M=male, F=female, G=gravid

Table 7: Mosquitoes collected by the Nasci for Aim 3

	Treatment sites				Control Sites			
Species	n	% M (n)	% F (n)	% G (n) 95% CI	n	% M (n)	% F (n)	% G (n) 95% CI
Ae. triseriatus	16	81.25 (13)	18.8 (3)	0	6	16.7 (1)	83.3 (5)	40 (2) 0-82.9
Ae. japonicus	12	25 (3)	75 (9)	22.2 (2) 0-49.4	5	0	100 (5)	40 (2) 0-82.9
Ae. albopictus	69	34.8 (24)	65.2 (45)	11.1 (5) 1.9-20.3	11	27.3 (3)	72.7 (8)	12.5 (1) 0-35.4
Total/LACv vectors	97	41.2 (40)	58.7 (57)	12.3 (7) 3.8-20.8	22	18.2 (4)	81.8 (18)	27.8 (5) 7.1-48.5
Other	42	21.4 (9)	78.6 (33)	24.2 (8) 9.6-38.8	31	22.6 (7)	77.4 (24)	29.2 (7) 11.0-47.4

M=male, F=female, G=gravid

Table 8. Two way ANOVA and Tukey's multiple comparisons test for species-specific mosquito collections by week for Aim 3

Variable		Mean Diff	95% CI	p-value
Ae. triseriatus	Week 1 v. Week 2	1.88	0.57-3.19	**0.001**
	Week 1 v. Week 3	2.32	0.61-4.02	**0.002**
	Week 1 v. Week 4	1.84	0.54-3.13	**0.001**
	Week 1 v. Week 5	1.88	0.57-3.19	**0.001**
	Week 2 v Week 3	0.44	-1.50-2.38	>0.05
	Week 2 v. Week 4	-0.04	-1.63-1.55	>0.05
	Week 2 v. Week 5	0	-1.61-1.61	>0.05
	Week 3 v. Week 4	-0.48	-2.41-1.45	>0.05
	Week 3 v. Week 5	-0.44	-2.38-1.51	>0.05
	Week 4 v. Week 5	0.04	-1.55-1.63	>0.05
Ae. japonicus	Week 1 v. Week 2	-0.24	-2.35-1.87	>0.05
	Week 1 v. Week 3	0.28	-2.91-3.65	>0.05
	Week 1 v. Week 4	-0.12	-2.32-2.08	>0.05
	Week 1 v. Week 5	0.04	-2.35-2.43	>0.05
	Week 2 v Week 3	0.52	-2.51-3.55	>0.05
	Week 2 v. Week 4	0.12	-1.85-2.09	>0.05
	Week 2 v. Week 5	0.28	-1.90-2.46	>0.05
	Week 3 v. Week 4	-0.4	-3.50-2.70	>0.05
	Week 3 v. Week 5	-0.24	-3.47-3.0	>0.05
	Week 4 v. Week 5	0.16	-2.01-2.42	>0.05
Ae. albopictus	Week 1 v. Week 2	-0.2	-1.80-1.40	>0.05
	Week 1 v. Week 3	0.64	-2.08-3.36	>0.05
	Week 1 v. Week 4	0.32	-1.61-2.25	>0.05
	Week 1 v. Week 5	0.44	-1.66-2.54	>0.05
	Week 2 v Week 3	0.84	-1.83-3.50	>0.05
	Week 2 v. Week 4	0.52	-1.34-2.38	>0.05
	Week 2 v. Week 5	0.64	-1.39-2.67	>0.05
	Week 3 v. Week 4	-0.32	-3.19-2.55	>0.05
	Week 3 v. Week 5	-0.2	-3.19-2.79	>0.05
	Week 4 v. Week 5	0.12	-2.18-2.42	>0.05

Coefficient in bold indicates significant p-value ($p<0.05$)

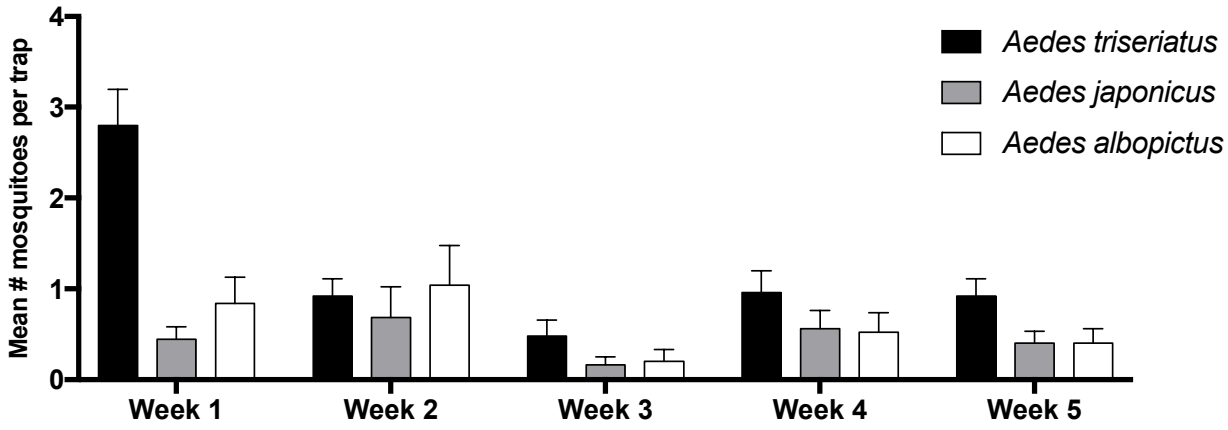

Figure 7. Species-specific mosquito collections by week for Aim 3. Mean number of mosquitoes per trap per week; error bars represent standard error.

Discussion

Utility of the CDC AGO

The CDC AGO is clearly effective at trapping LACv vectors in the peridomestic environment. The CDC AGOs were highly specific for the three targeted LACv vectors (*Ae. triseriatus, Ae. japonicus,* and *Ae. albopictus*). The CDC AGOs did not collect any other species of mosquitoes, with the possible exception of a very few individuals too damaged for identification. However, the Nasci collected other species at sites containing deployed CDC AGOs. The majority of the mosquitoes collected by the CDC AGOs were females, while the Nasci collected a higher proportion of males in addition to females. As expected, the CDC AGOs collected a higher proportion of gravid females than the Nasci samples. Additionally, there was no difference in the proportion of gravid mosquitoes between the treatment sites and control sites as measured by the Nasci aspirator.

The mean number of mosquitoes per trap per week (2.28) was higher than observed in both the Dengue AGO study (1.2 mosquitoes per trap per week [Barrera et al., 2014]) and in Aim 1 of this study (0.86 mosquitoes per trap per week). This field trial not only collected more mosquitoes than Aim 1, it collected more mosquitoes in fewer traps. Although the impact of this yield is unknown in the context of LACv control, the results are encouraging as the CDC AGOs target LACv vectors during the season LACE is highest and in a LACE endemic region.

Limitations

Control sites were chosen solely based on the proximity of each study site. Due to unconsidered factors, such as lack of vegetation or ease of access, some control

sites may have not been appropriately paired, and thus yielded fewer mosquitoes collected by the Nasci and potentially unrepresentative data. For example, one particular treatment site collected 64 mosquitoes while the paired control sited collected 4 mosquitoes. There was a pile of discarded tires nearby the residence that likely produced an abundance of mosquitoes, especially *Ae. albopictus*, which were easily collected at the treatment site but not at the control site. The tire pile was also in proximity to the control site, therefore other factors must be responsible for the small yield of mosquitoes.

Implications

The CDC AGO is highly specific and clearly effective at trapping LACv vectors in the peridomestic environment as evidenced by Aims 1 and 3 of this study. In the context of LACv surveillance, the CDC AGO may be a useful tool for future large scale trials to reduce LACv vectors and disease risk, especially at residences where children and siblings live. By “sinking” gravid mosquitoes that represent a greater risk out of the environment, it may be possible to alleviate the disease burden in LACE endemic areas.

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