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The incidence of La Crosse encephalitis (LACE) (a mosquito-borne pediatric neuroinvasive infectious disease) has increased in the Appalachian region. A causal association between anthropogenic landscape change and LACE emergence has been hypothesized, but has not been studied. This potential association was the major impetus for my dissertation.

My specific goals were to:

(1.) Characterize, ecologically, the forest-to-field ecotone in a LACE endemic area and describe the distribution of La Crosse virus (LACV) mosquito vectors along it.

(2.) Determine the effects of experimental larval habitat supplementation on the ecology of LACV vectors along forest-to-field ecotones.

(3.) Determine if the vertical distribution of LACV vector differs along a forest-to-urban ecotone within an urban landscape

(4.) Determine if the vertical distribution of LACV vector differs along a forest-to-field ecotone and if experimental larval habitat supplementation affects this vertical distribution.

I determined the natural distribution of the LACV mosquitoes in the western North Carolina along forest-to-field ecotones (Aim 1) using oviposition cups and by trapping adult host-seeking and resting mosquitoes. In the second year I introduced 9 tires (Aim 2) in either the forest or the field habitats along the ecotones to determine the effect of artificial larval habitat supplementation on the ecology of these mosquitoes. I also deployed oviposition cups along three heights: 0, 4.5, and 9 m in each habitat along the forest-to-field ecotone (Aim 4) and in an urban landscape (Aim 3) to explore the vertical distribution of LACV mosquitoes along a gradient of anthropogenic land use intensity and determine the effect of container introduction along this neglected spatial dimension.

Aedes triseriatus was the most abundant mosquito in the Appalachian sites followed by *Ae. japonicus* and *Ae. albopictus.* Oviposition activity decreased along the ecotone, from forest to field habitats, with *Aedes triseriatus* mostly found in the forest and *Ae. japonicus* peaking at the edge and *Aedes albopictus* with relatively higher abundance in the field. Tires were associated with a higher abundance of *Ae. triseriatus* and *Ae. albopictus*, but also with a decrease in *Ae. japonicus*. The effect of tire introduction persisted throughout all three years of the experiment. *Ae. triseriatus* was most commonly present in tires in the forest habitat and *Ae. albopictus* the tires in the field, while *Ae. japonicus* and *Ae. hendersoni* did not show a significant difference between forest and field tires. Species specific parity rates did not differ among habitats and were not impacted by tire addition. Also, *Ae. triseriatus* mosquitoes collected from the forest habitat were larger than those collected at the edge habitat. In terms of tire introduction effects, only *Ae. japonicus* was significantly affected with individuals at the control sites being larger than those collected at the tire introduction sites.

In the urban landscape study (Aim 3), I only collected *Ae. albopictus*, *Ae. triseriatus*, and *Ae. hendersoni* eggs. Even though all three species mostly exploited the shaded forest and park habitats, only *Ae. albopictus* was found in the urban campus habitats of UNCG. All three mosquito species in the forest and park habitats mostly oviposited at ground level, a surprising finding for *Ae. hendersoni*, which typically oviposits at the canopy level.

Along the forest-to-field ecotones (Aim 4), the vertical distribution in the control plots was consistent with previous studies, with *Ae. triseriatus* exhibiting no clear vertical affinity while *Ae. hendersoni* showing clear preference for ovipositing at the canopy level. Both invasive species (*Ae. albopictus* and *Ae. japonicus*), exhibited a clear preference for ovipositing at ground level. Tire introductions to either the field or the forest habitat resulted in a shift in the vertical habitat use pattern for all four species: *Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus*, substantially enhanced their affinity to oviposition at ground level, while *Ae. hendersoni* reduced its affinity towards the canopy level and laid relatively more at mid-level heights.

These findings highlight the well-known importance of container control for the purpose of source reduction and provide a broader framework of understanding regarding the scope, scale, and heterogeneity associated with these anthropogenic changes and their impact on LACV entomological risk.

EXPERIMENTAL LANDSCAPE EPIDEMIOLOGY OF LA CROSSE VIRUS IN THE SOUTHERN APPALACHIAN MOUNTAINS

by

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To my wife, Haruna Schwarz, for her incredible patience and unbending support. I could not have done it without you. Thank you.

APPROVAL PAGE

This dissertation written by Marcelo Schwarz Giribaldi has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

During the last few decades, the emergence of previously unknown pathogens and the resurgence of previously controlled infectious diseases have resulted in substantial economic losses and increased human morbidity and mortality (Daszak et al., 2001a; Gratz, 1999; Gubler, 1996, 1998a, 2002; Gupta et al., 2012; Jones et al., 2008b; Meentemeyer et al., 2012; Morse, 1995; Myers and Patz, 2009b; Patz et al., 2008; Rosenberg et al., 2018; Washer, 2010; Woolhouse and Gowtage-Sequeria, 2005). Nearly 60% of all human pathogens and approximately 75% of recent emerging infectious diseases are considered zoonoses (Bueno-Marí et al., 2015; Cunningham, 2005); zoonotic diseases are caused by pathogens transmitted from a vertebrate animal host to humans (Bueno-Marí et al., 2015; Cunningham, 2005; Jones et al., 2008b). Typically, the emergence of zoonotic diseases is associated with the exposure of humans to novel pathogens cycling in natural, often complex, ecological systems. Hence, understanding the underlying processes associated with the transmission of these pathogens necessitates the application of an ecological approach. We may apply such an approach to investigate the interactions between both vectors and hosts (e.g., location and frequency of contact, etc.) and their environment (e.g., habitat preferences, predator-prey interactions, etc.). These complex systems can cycle in endemic (i.e., constant presence and usual prevalence) or epidemic (i.e., increased presence or outbreak) states in wildlife with limited or no public health recognition because humans do not represent an essential component of the sylvan pathogen transmission cycle and are commonly the result of proximity to its wildlife cycle (e.g., "passive exposure" events). Thus, understanding the ecology of these systems is essential for identifying interventions for controlling and preventing human exposure.

Human-induced land-use changes, which are often associated with encroachment into pristine environments and with habitat modifications that facilitate pathogen transmission, are key factors influencing the emergence of zoonotic diseases (Daszak et al., 2001a; Despommier et al., 2006a; Meentemeyer et al., 2012; Patz et al., 2000, 2004, 2008; Woolhouse and Gowtage-Sequeria, 2005). These changes (e.g., logging, agriculture, urbanization, etc.) can lead to fragmentation, which is defined by Wilcove et al (1986) as a process by which "a large expanse of habitat is transformed into a number of smaller patches of smaller total area, isolated from each other by a matrix of habitats unlike the original" (Wilcove et al., 1986). Fragmentation commonly results in habitat loss, and it changes the properties of remaining habitats by altering their configurations often leading to connectivity loss or isolation of patches (Fahrig, 2003). Thus, one of the consequences of fragmentation is the development of ecotones (Despommier et al., 2006b; Farina, 2006a; Kark, 2007). Farina (2006) defines ecotones as "special areas in which different types of habitats meet and where ecological processes are strongly influenced by the co-occurrences of different land attributes" (Farina, 2006a). Ecotones can be the result of natural- or human-induced disturbance regimes (Farina, 2006a; Harris, 1988; Riser, 1995). Generally, the edges (i.e., boundaries between habitats) of natural ecotones are "soft" because they are characterized by relatively larger transition zones (i.e., ecotones) which allow for a greater degree of organism flux, whereas human-induced edges tend to be "hard" in the sense that they are commonly characterized by abrupt transitions (i.e., ecotones) with sharp gradients of environmental variables (Farina, 2006a). Nevertheless, the degree of "softness" or "hardness" of such edges is scale-dependent and can only be characterized within a species-specific context (Farina, 2006a; Walker et al., 2003). The movement of organisms across edges and into adjacent habitats is regulated by environmental variables, both biotic and abiotic (Farina, 2006a; Murcia, 1995; Walker et al., 2003). Species sensitive to microclimatic factors may perceive an edge as an impermeable barrier preventing movement between neighboring habitats (Farina, 2006a). Conversely, organisms may move across an edge but could find that this new transitional habitat (i.e., ecotone) contains novel stressors that are detrimental to their survival and/or fecundity (e.g., predators, limited breeding or development opportunities). Thus, ecotones represent important sites for studying the interactions of organisms with their environment and for identifying biotic and abiotic variables that may enable or limit their movement and persistence across the landscape. Ecotones are important for the study of ecology at-large, as they allow us to determine the biotic consequences of habitat fragmentation at a species or community level, and for disease ecology specifically given the potential for pathogen spillover and disease control applications (Bolger et al., 1997).

Ecotones and disease ecology.

Ecotones may enhance the dispersal of pathogens, vectors, and hosts, providing more opportunities for human "spill-over" events, including host switching (i.e., a change in the host specificity of a pathogen). They can facilitate human contact with animals carrying pathogens in previously unperturbed ecosystems. For example, in French Guiana the risk of rabies virus has likely increased on the edge between forest and peri-urban areas because vampire bats (i.e., rabies virus main reservoir) may shift blood-feeding hosts to cattle and humans due to lack of sufficient hosts that are commonly found in pristine forests (Thoisy et al., 2016). Also, the Yellow Fever virus is believed to have switched from a non-human enzootic primate cycle, characterized by transmission between canopy dwelling monkeys and forest mosquitoes, to humans by shifting from a forest-based enzootic cycle to domestic and peri-domestic mosquito species such as *Aedes aegypti* establishing both a zoonotic (animal-mosquito-human) and finally an anthroponotic cycle (human-mosquito-human) in South America (Despommier et al., 2006b). Such a change is often associated with an epidemiological transition from episodic events due to sporadic exposure events, to continuous endemic zoonotic spill-over from natural systems into human systems, to epidemic/pandemic outbreaks due to adaptation of the pathogen to be human-to-human transmitted (Woolhouse et al., 2005). For example, in the Yellow fever system described above, ecotones developed from human settlements and agriculture (e.g., banana plantations) may have facilitated this epidemiological shift from sylvatic (forest-based) to peridomestic settings by bringing into close proximity the anthropophilic ("human-liking") *Aedes* mosquitoes, which flourished in the settlements and banana plantations, with the primates that resided in the forest and served as natural reservoirs for Yellow Fever (Despommier et al., 2006b). Ecotones can also enhance fitness of the vector by allowing for more favorable conditions such as better larval habitats or foraging opportunities. In western Kenya, newly created farmland, adjacent to forest and swamp, enhanced the abundance of the vector of the Malaria parasite in the area, *Anopheles gambiae*, and shortened its development time (Munga et al., 2006). Aquatic habitats with increased nutrient levels and temperatures are believed to be responsible for this (Munga et al., 2006). Ecotones can be sites of high species diversity and/or abundance (Harris, 1988). For example, Lounibos (1981) studied the habitat segregation of African treehole mosquitoes along ecotonal sites in Kenya, collecting species from 4 genera, including 14 *Aedes spp.*, 3 *Culex spp.*, 3 *Eretmapodites spp.*, and 2 *Toxorynchites* spp. In this study, the highest mean number of mosquito species per trap was collected in ecotonal sites, characterized by mixed grasses, shrubbery and trees, compared to forested and rural sites (i.e., village) (Lounibos, 1981). As the examples above demonstrate, ecotones may represent high risk areas for disease risk by increasing the likelihood for host-switching and providing vectors with novel habitat and foraging opportunities (Engering et al., 2013; Woolhouse et al., 2005).

Anthropogenic environmental effects and invasive vector species.

Invasive species may also enhance pathogen transmission risk. The introduction of invasive vector or hosts species is commonly facilitated by increased global trade and travel as well as by the ability of some invasive species to proliferate in novel environments characterized by high levels of human land-use intensity. For example, the invasive *Aedes albopictus* and *Ae. japonicus* were introduced to the USA with the importation of used tires from Asia (Hawley et al., 1987). The close affiliation of *Aedes albopictus* with humans, their dwellings, and refuse enabled the rapid spread and establishment of this mosquito species across several states in the south and east United States (Benedict et al., 2007). Likewise, the invasive *Aedes japonicus*, having become established in North America and Central-Western Europe, may be reducing native mosquito populations in certain areas while increasing in its own abundance (Andreadis and Wolfe, 2010; Andreadis et al., 2001; Bartlett-Healy et al., 2012; Kampen and Werner, 2014; Peyton et al., 1999). *Aedes japonicus* is a competent vector of several pathogens, such as West Nile Virus and La Crosse Virus, and could be increasing the risk of transmission for these diseases due to its anthropophilic nature (Bartlett-Healy et al., 2012; Sardelis et al., 2002; Turell et al., 2001; Westby et al., 2011).

Conceptual framework.

Zoonotic systems are intricate and dynamic due to the varied ecological interactions between environment, hosts, pathogens, reservoirs, and vectors.

Given the complexity and multi-dimensionality of vector-borne zoonotic diseases and the impact of anthropogenic environmental change on them, I am presenting below (Fig. 1) a conceptual model that would serve as a conceptual framework for my dissertation. It is based on the concept of “disease nidality” first proposed by Pavlovsky (1966) (Pavlosky, 1966) and further developed to the concept of “landscape epidemiology” by Reisen (2009) (Reisen, 2009). The transmission of vector-borne pathogens requires a reservoir host (organism responsible for the long-term maintenance of the pathogen), an etiologic agent (the pathogen), and a vector (the organism that transmits the pathogen from an infectious to a susceptible host) (Clements, 2012; Rockett, 2009). The ecological niches of each one is represented by a circle (Fig. 1). In zoonotic diseases, the reservoir host is a non-human animal, whereas in anthroponotic diseases humans are the reservoir. Mosquitoes, ticks, fleas, sand flies and black flies are all well-known vectors of parasites, viruses and bacteria. The ecological niche of a vector-borne disease system (i.e., disease niche) can be modeled conceptually as the nexus of the ecological niches of the host, a disease agent, and a vector (Clements, 2012; Pavlosky, 1966; Rockett, 2009). In order for a pathogen to persist in an environment, all of its disease niche components must be present (Reisen, 2009). Nevertheless, the mere presence of the niche components are often not enough for the pathogen to spread and persist within the host population. The environment may mediate transmission by enhancing or limiting the interactions of the niche components, leading to either epidemic or endemic states, respectively, or to the complete loss of the pathogen in the ecosystem. For example, high temperatures (e.g., >28° C) reduce the efficiency of fleas in the transmission of *Yersinia pestis* as the bacterium cannot block the digestive track of the flea, preventing regurgitation (Hinnebusch et al., 1998). Similarly, high and low temperatures may also negatively affect the survival and biting frequency of mosquitoes as well as the development rate of the pathogen (Afrane et al., 2012). Also, extended dry periods may trigger aestivation in *Anopheles* mosquitoes, thus, reducing their activity and contact with host and pathogen (Lehmann et al., 2010). These changes to the environment are often derived from human activities (e.g., land use change). Moreover, warmer temperatures, a consequence of global climate change, have been associated with broader distributions of vectors. For example, the distribution of *Anopheles* mosquitoes is likely increasing as they move upwards in altitude in the highlands of Ethiopia and South America, formerly sub-optimal landscapes due to lower temperatures (Siraj et al., 2014).

From the perspective of a conceptual model, natural and human factors affect the disease niche components in multiple ways. These interactions are represented by arrows in **Figure 1.1**. For example, human settlements (anthropogenic environment) in sylvatic areas (natural environment) may expose humans to a zoonotic disease system. This may be followed by an increase in the density of tree holes leading to more larval habitats (natural risk factor) or recreational activities taking place in an endemic habitat providing increased blood-feeding opportunities for mosquitoes (human-behavioral risk factor). Moreover, the interactions between the natural and anthropogenic factors themselves can further impact the disease niche components, this is represented by cogwheels in **Figure 1.1**. For example, the introduction of artificial containers (human risk factor) coupled with precipitation and input of leaf litter (natural risk factor) may increase the abundance of a vector by creating an optimal larval habitat for the mosquito and, thus, enhance pathogen transmission.

These alterations can vary in scale, from small scale effects, such as the introduction of tires in a forest, to large scale effects, such as changes to the landscape through habitat fragmentation. Therefore, the alterations may involve top-down effects, such as those of the large-scale natural environment on the nested, smaller scale human environment. However, the alterations may also result in bottom-up effects of the smaller scale components on the large-scale environment, or on effects of one niche component (e.g., vector) on another (e.g., host). For example, hunting of a predator (e.g., fox) may increase the abundance of a reservoir host (e.g., deer mice, reservoir host of the Lyme disease spirochete *Borrelia burgdorferi*), thus having cascading effects on the local ecosystem. Collectively, these components (i.e., host, pathogen, vector, and the environment) drive transmission risk. Thus, to prevent pathogen transmission and mitigate or predict infection risk we must recognize the ecological complexities of these systems and identify the interactions between human and natural risk factors as well as their effects on the disease niche at multiple scales. An ecological approach to studying emerging vector-borne zoonotic diseases is therefore essential.

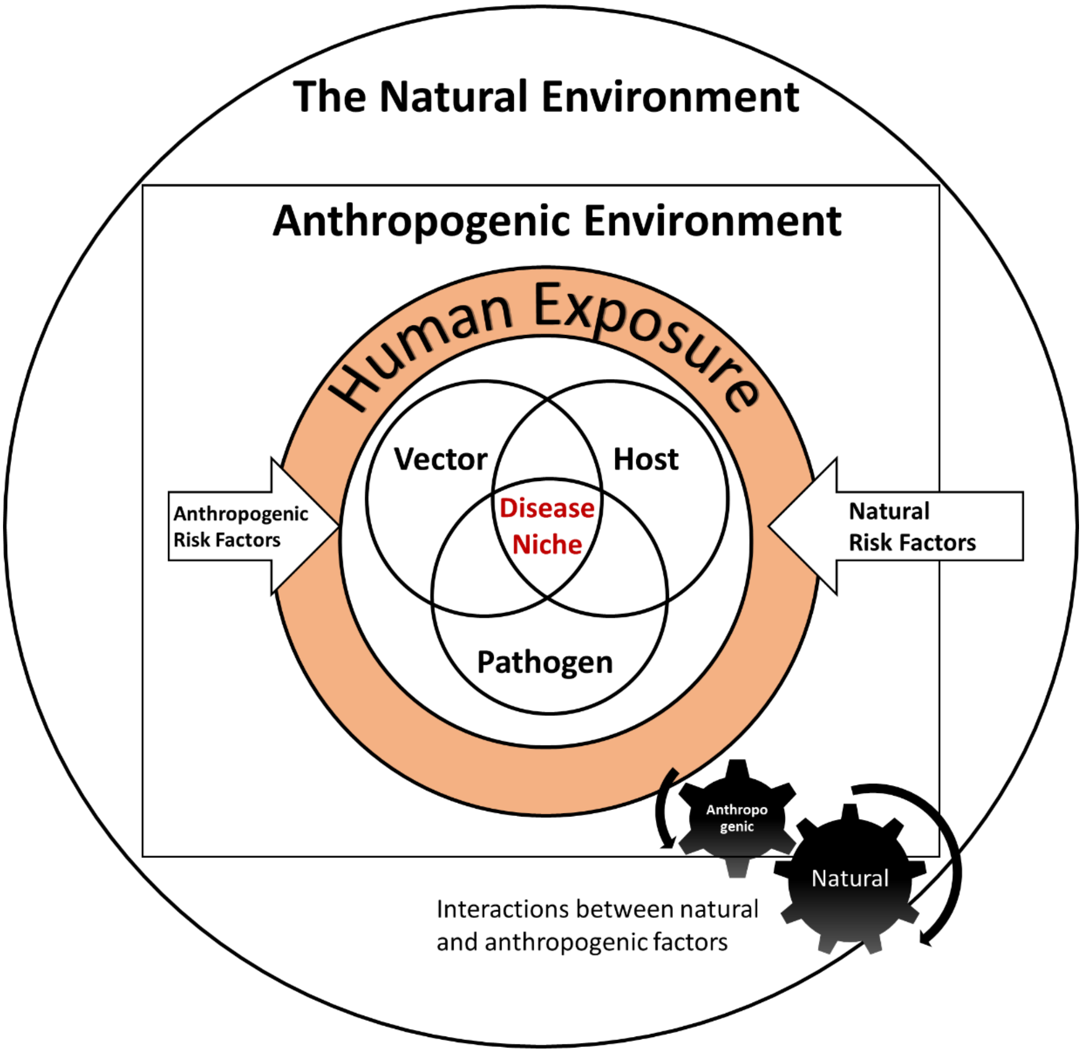

Figure 1.1. Conceptual model of a vector-borne disease niche with human environment nested within natural environment. Large circle represents the natural environment and the square represents the human-derived environment nested within it (e.g., grazing field) in which human exposure may place. Cogwheels and arrows represent bi-directional interactions of natural and anthropogenic risk factors affecting the components of the disease niche system.

The focus of this study is limited to the La Crosse disease system, however, the approach utilized may be applied to other systems of contemporary importance (e.g., West Nile virus, Ross River Fever, Lyme Disease, Eastern Equine and Japanese Encephalitis).

La Crosse Encephalitis Study System.

La Crosse Encephalitis (LACE) is one of the most important mosquito-borne diseases in the United States (Utz et al., 2005). It is a pediatric disease, mainly affecting children under the age of 16, in which patients may develop aseptic meningitis or meningoencephalitis (McJunkin et al., 2001). Nevertheless, subclinical or mild infections are much more common (Rust et al., 1999). There is currently no vaccine available (CDC [Centers for Disease Control and Prevention], 2011), hence, prevention of exposure is currently the most effective mode of control. The mean cost per LACE case, based on both direct and indirect medical costs, was estimated at approximately $35,000,while the projected cost of lifelong neurologic sequelae in 5 severe cases can range between $48,000 and $3.1 million; these estimates are in 2003 U.S. dollars (Utz et al., 2003). The disease was found to significantly affect the social life and self-esteem of the patient and results in the loss of approximately 13-14% of productive life years (Utz et al., 2005). Approximately 30 to 180 cases of the disease are reported every year in the United States (Rust et al., 1999). However, the true incidence of the disease is unknown and there may be as many as 300,000 human exposures annually (Utz et al., 2003). Given the likely under-reporting of this disease, its economic and social burden in endemic areas may be grossly under-estimated.

Description of La Crosse transmission cycle and disease niche

The La Crosse disease niche is composed by the La Crosse virus (pathogen), its mosquito vectors (*Aedes triseriatus*, *Ae. japonicus*, *Ae. albopictus*), and the vertebrate host species (Sciurid mammals). It is one of the most ecologically complex vector-borne disease systems with three transmission routes (horizontal, including venereal, and vertical,), a primary native vector species, two invasive species able to transmit the virus, and a number of amplifying sciurid hosts.

Pathogen. The La Crosse virus (LACV) is a negative-sense RNA virus (Family: Bunyaviridae, Genus: *Bunyavirus*) and is part of the California (CAL) serogroup, which includes the pathogens responsible for California encephalitis, Jamestown Canyon, Snowshoe Hare, and others (Borucki et al., 2002; Calisher, 1994).

Mosquito vectors

Native mosquitoes. *Aedes (Ochlerotatus) triseriatus*, the eastern tree hole mosquito, is the primary LACV vector and is commonly found in deciduous oak and hickory forests (Rust et al., 1999). This species is frequently found in sympatry with its sibling species, the arboreal *Aedes hendersoni*, in LACV endemic areas (Nasci, 1982; Novak et al., 1981; Scholl and DeFoliart, 1977; Sinsko and Grimstad, 1977; Truaian and Craig, 1968). Both readily oviposit in tree-holes, competing in larval stages although also displaying distinct preferences along the vertical gradient (i.e., height), thus potentially showing some degree of niche partitioning along a vertical axis (Nasci, 1982; Novak et al., 1981; Scholl and DeFoliart, 1977; Sinsko and Grimstad, 1977; Truaian and Craig, 1968).

Invasive mosquitoes. Although *Ae. triseriatus* is understood to be the primary vector of LACV, recently introduced mosquitoes (*Aedes albopictus* and *Aedes japonicus*) are thought to be playing an increasing role in the transmission of the virus (Leisnham and Juliano, 2012a; Sutherland, 2008). The ecology of *Ae. triseriatus* may be influenced by resource competition from both of these secondary vectors, as they have been shown to oviposit in the same larval habitats and laboratory experiments have found *Ae. albopictus* larvae to outcompete *Ae. triseriatus* under low resource conditions (Bevins, 2008; Leisnham and Juliano, 2012a; Swanson et al., 2009). Moreover, the apparent geographic shift of the disease is concurrent with the introduction and spread of both invasive mosquito species, however, it is currently unclear whether their introduction has had any effects on the ecology of *Ae. triseriatus* or on LACV transmission risk in the area (Leisnham and Juliano, 2012a; Peyton et al., 1999; Swanson et al., 2009). *Aedes albopictus* is a container (i.e., natural and artificial) mosquito, commonly associated with peri-domestic and urban environments (Moore and Mitchell, 1997). This aggressive day-time biter is believed to have been introduced into the United States from Asia with the importation of used tires, as early as 1987 in Houston, Texas (Hawley et al., 1987; Moore and Mitchell, 1997; Sprenger and Wuithiranyagool, 1986). It is now found throughout most of the eastern and southern United States, and as far west as California (Hahn et al., 2017). This mosquito is of considerable public health importance as it can vector several pathogens including La Crosse, Zika, chikungunya, dengue, the flaviviruses for Japanese encephalitis, West Nile and Yellow fever, among others (Benedict et al., 2007; Wong et al., 2013). Likewise, established populations of the invasive "rock pool" mosquito, *Aedes japonicus*, were first seen in New York and New Jersey in 1998 (Kampen and Werner, 2014; Peyton et al., 1999). The importation of used tires is also suspected for the introduction of this species (Andreadis et al., 2001; Kampen and Werner, 2014). It is now widely distributed throughout the eastern United States and it is also found in the states of Oregon, Washington and Hawaii, as well as in Canada (Quebec and Ontario provinces) (Harris et al., 2015a; Kampen and Werner, 2014; Kaufman and Fonseca, 2014; Riles et al., 2017; Thielman and Hunter, 2006). This mosquito is also of great public health importance as it can vector several arboviruses including La Crosse and Japanese encephalitis, dengue, chikungunya, and West Nile virus, among others (Harris et al., 2015a; Sardelis et al., 2002; Schaffner et al., 2011; Turell et al., 2001; Westby et al., 2015). By 2003, both invasive species were found in Appalachia (i.e., West Virginia), a region endemic for LACV and *Ae. triseriatus*, likely competing with the native mosquito in artificial containers (Joy and Sullivan, 2005a).

Host species

Amplifying hosts. Sciurid mammals, such as the Eastern grey squirrel (*Sciurus carolinesis*), Eastern fox squirrel (*Sciurus niger*), Eastern chipmunk (*Tamias striatus*) and woodchucks (*Marmota monax*) amplify the virus by developing high levels of the presence of the virus in their bloodstream (i.e., viremia), they are therefore regarded as “amplifying hosts” of the virus (Amundson et al., 1985; Clements, 2012). The red fox (*Vulpes fulva*) has also been shown to develop high LACV viremia(Amundson et al., 1985). Although they may not be considered reservoir hosts (i.e., population in which a pathogen can be maintained permanently (Haydon et al., 2002)) due to their short-lasting viremias, these species are believed to play an important role in the horizontal transmission cycle of the virus because of their close association with its primary vector *Ae. triseriatus* and the high levels of viremias they develop, which last approximately 2.5 days (ranging from 1-4 days) with subsequent recovery (Clements, 2012; Osorio et al., 1996). In contrast, larger mammals (e.g., deer and humans) do not develop high enough viremias to transmit the LACV back to its mosquito vectors. In these cases, the larger mammals are known as “dead-end hosts” and they may reduce the transmission of the virus in endemic areas where the virus cycles (Rust et al., 1999). The virus does not appear to exert any adverse effects on the mosquito vectors

(Patrican and DeFoliart, 1985) or the amplifying hosts (Borucki et al., 2002). The enzootic system of this disease is restricted to transmission between the mosquito vectors and amplifying hosts, commonly in or near forested areas (Rust et al., 1999).

Reservoir hosts. The mosquito vectors would likely be considered the functional reservoir hosts of the virus because it can overwinter in infected mosquito eggs (Borucki et al., 2002; Harris et al., 2015b; Watts et al., 1975). The role of vertical transmission in overwintering eggs is of particular importance in enabling the virus to persist through the winter months given the short-lasting viremias in small mammal amplifying hosts (e.g., sciurids) (Amundson et al., 1985; Osorio et al., 1996).

LACV transmission routes. There are 3 possible routes for the transmission of the virus: horizontal, vertical and venereal (**Figure 1.2**).

- **Vertical transmission.** All 4 mosquito species have been shown to be able to transmit the virus transovarially, from female to offspring (Harris et al., 2015a; Paulson and Grimstad, 1989; Westby et al., 2015). Although transovarial rates for *Ae. japonicus* are currently not known, studies have shown relatively high rates for *Ae. triseriatus* (i.e., 30-71%) and *Ae. albopictus* (i.e., 52%) (Hughes et al., 2006; Lambrechts and Scott, 2009; Miller et al., 1977; Patrican and DeFoliart, 1985; Watts et al., 1975), as well as lower rates for *Ae. hendersoni* (i.e., 25%) (Paulson and Grimstad, 1989). Overwintering of the virus is maintained by diapausing eggs (i.e., eggs under dormant state during adverse climatic conditions (Clements, 2012)) that retain the virus through the winter, which then replicates in the spring and summer in embryo and ovarian tissues without producing deleterious effects on the vector (Borucki et al., 2002).

- **Horizontal transmission.** LACV can be transmitted horizontally in two ways:
  - The LACV is transmitted between the mosquito vectors and amplifying hosts through blood feeding (Borucki et al., 2002; Rust et al., 1999).
  - The LAC virus can also be transmitted venereally from *Aedes triseriatus* males to females during mating (Thompson and Beaty, 1977). Males acquire infections through vertical transmission (Fig. 1.2).

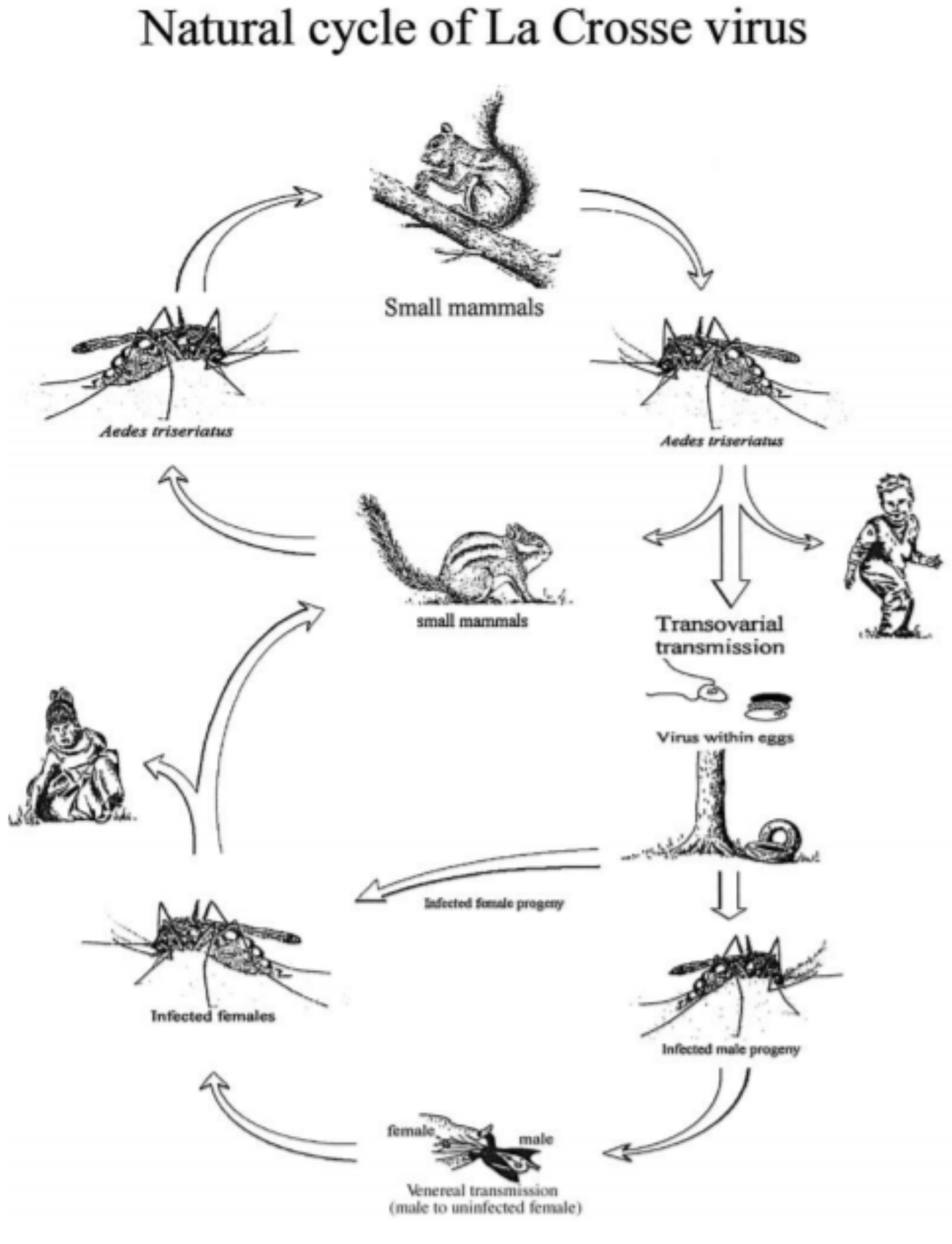

Figure 1.2. Natural transmission cycle of La Crosse virus (Borucki et al., 2002).

Vector competence of LACV vectors. The native (*Ae. triseriatus* and *Ae. hendersoni*) and invasive (*Ae. albopictus* and *Ae. japonicus*) mosquitoes differ in their ability to transmit the virus following exposure. Paulson et al. (1989), Paulson et al. (1992), and Harris et al. (2015) studied the LACV vector competence (i.e., ability to acquire, maintain and transmit the virus once exposed) of *Ae. triseriatus* and sibling species *Ae. hendersoni* in the laboratory. They found the transmission rate (i.e., probability of transmission given contact) of *Ae. triseriatus* to be 37-40% (Harris et al., 2015b; Paulson et al., 1989), mediated primarily by the midgut escape barrier (i.e., virus is ingested but midgut epithelium tissue prevents it from infecting haemocoel (Franz et al., 2015; Paulson et al., 1989), and a transmission rate of only 7-8% (Paulson et al., 1989, 1992) for sibling *Ae. hendersoni*, limited significantly by the salivary gland escape barrier (i.e., salivary glands are infected but virus cannot be transmitted orally (Franz et al., 2015; Paulson et al., 1992). Watts et al. (1973) and Sardelis et al., (2002), described *Ae. triseriatus* having a higher vector competence for LACV (i.e., 70-100%) than those cited above, also based on laboratory studies (Sardelis et al., 2002; Watts et al., 1973). In addition, Sardelis et al. (2002) estimated rates for *Ae. japonicus* to be 35-88% (Sardelis et al., 2002) and found these to be slightly lower, although comparable to those of *Ae. triseriatus*. Moreover, in a laboratory study Grimstad et al., (1989) found *Ae. albopictus* LACV transmission rates of 17-47% (Grimstad et al., 1989). The LACV primary vector, *Ae. triseriatus*, and the two invasive vectors (i.e., *Ae. japonicus* and *Ae. albopictus*) are considered competent vectors, however, due to *Ae. hendersoni*'s relatively low LACV transmission rates (7-8%), this native species is commonly not regarded as an important vector (Paulson and Grimstad, 1989).

Landscape change and LACV emergence in Appalachia.

The La Crosse (LAC) disease system provides an opportunity to study the effect of human land use on a zoonotic vector-borne disease. Although the LAC enzootic system appears to cycle primarily in sylvatic forested habitats, it is believed that anthropogenic effects, such as deforestation, habitat encroachment (e.g., human settlements in sylvatic areas), as well as the recent introduction of invasive mosquito species might underlie LACV emergence in Appalachia (Bevins, 2008; Gubler, 1998a; Leisnham and Juliano, 2012a).

Study region: western North Carolina

The climate of the western North Carolina region (WNC) is characterized by wet spring and summer months, and relatively dry fall and winter months (NEMAC, 2016), while the topography is characterized by mountain peaks and wide valleys, ranging in elevation from 500 to 2,000 meters (NEMAC, 2016). The precipitation varies considerably within the region given the topography, commonly averaging anywhere from 900 to 2500mm per annum (NEMAC, 2016). The temperatures are relatively mild year-round, commonly between 0 C and 5 C in the winter season (day-time) and seldom surpassing 30 C in the summer (day-time), however, these also vary based on altitude (NEMAC, 2016). The landscape of the region is primarily rural, a patch mosaic composed predominantly of forested patches (i.e., mixed hardwoods deciduous forest) but also hosts large open patches such as low intensity development, pasture and grassland (**Figure 1.3**) (NEMAC, 2016). However, forest fragmentation in private land is increasing and is expected to increase further due to human development pressures (Stein et al., 2005). Thus, ecotones are likely becoming a more common feature in the region.

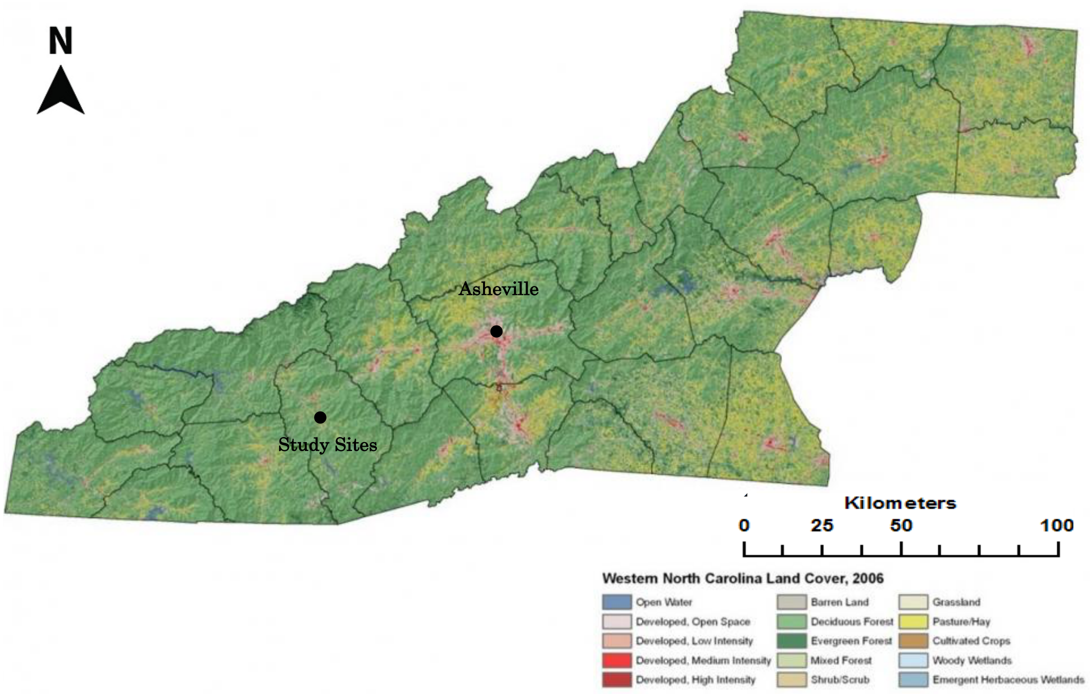

Figure 1.3. Land cover map of western North Carolina, 2006 (NEMAC, 2016). The region is dominated by deciduous forest land cover, however, developed and pasture lands are commonplace likely resulting in the formation of numerous ecotones.

Over the last 4 decades WNC has experienced robust population growth and a considerable increase in human land-use per capita (i.e., development) (Vogler et al., 2010). From 1976 to 2006, the population of the 19 counties that compose the western NC region grew by 42% and is projected to increase by an additional 25% between 2006 and 2030 (Vogler et al., 2010). Moreover, the human footprint (i.e., developed land in acres per capita) increased from 0.06 in 1976 to 0.30 in 2006, and is projected to increase to 0.39 in 2030 (**Figure 1.4**) (Vogler et al., 2010).

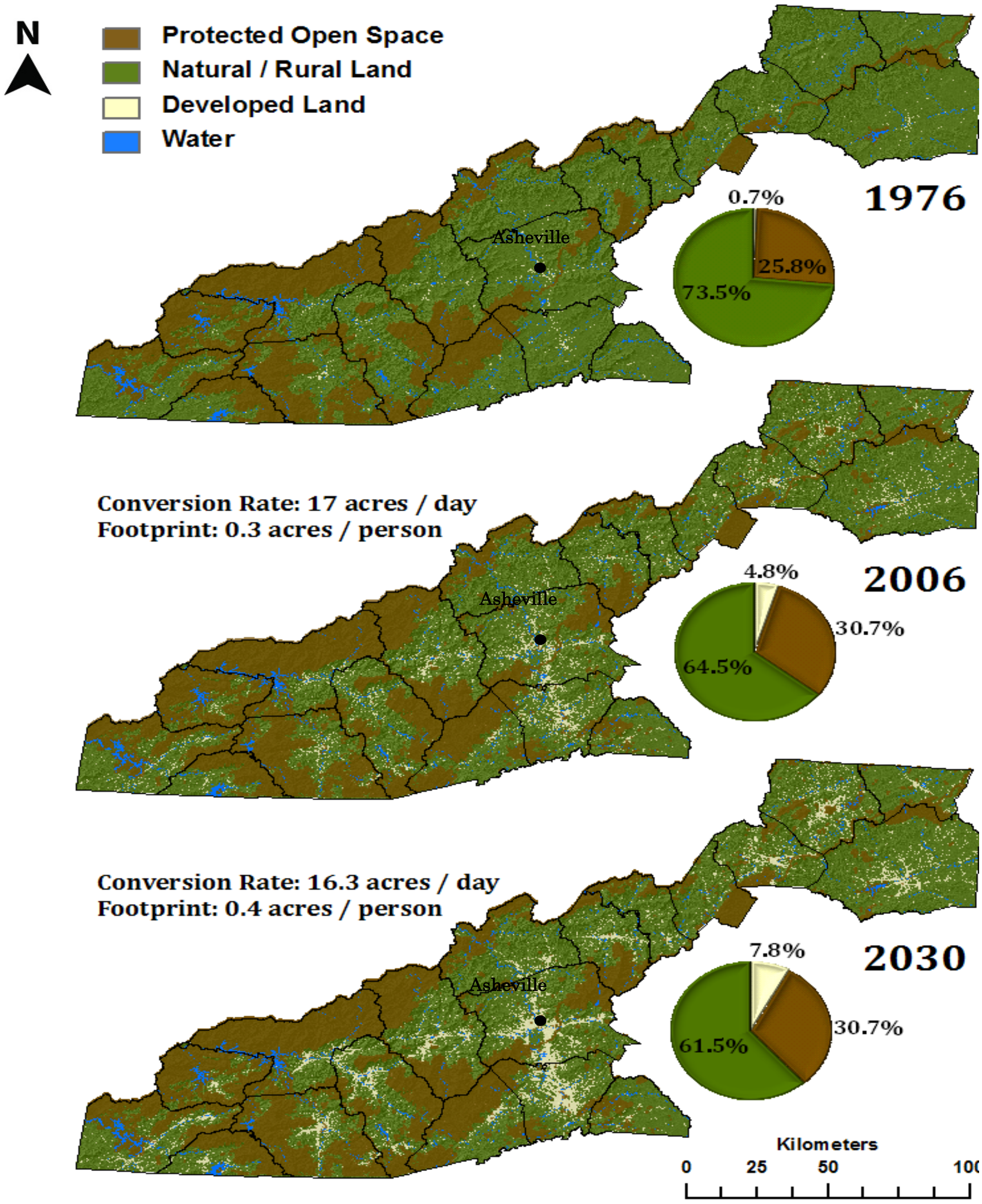

Figure 1.4. Land-use change in western North Carolina from 1976 to 2006 and 2030 (projected) (Vogler et al., 2010).

LACV emergence in the Appalachian region.

The reported LACE incidence has shifted geographically within the past three decades, from upper Midwestern states (i.e., Illinois, Indiana, Minnesota, Ohio, and Wisconsin) to the Appalachian region (i.e., North Carolina, South Carolina, Tennessee, Virginia, and West Virginia) **(Figure 1.5)** (Leisnham and Juliano, 2012a).

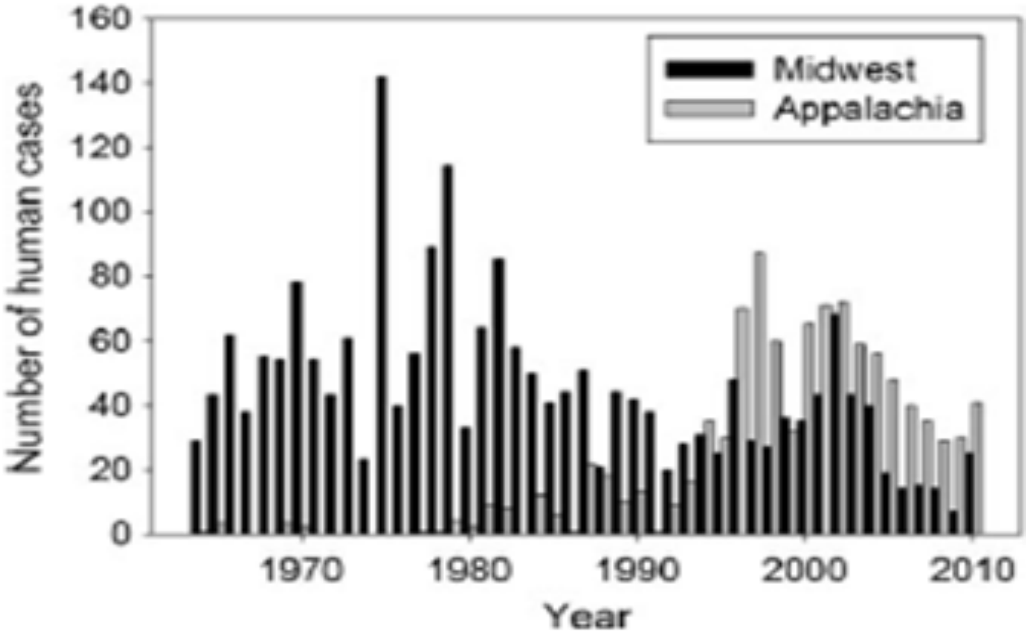

Figure 1.5. Number of cases with CAL serogroup viral disease in Midwestern (Ohio, Wisconsin, Minnesota, Illinois) and Appalachian (West Virginia, North Carolina, Tennessee) states of the United States (Leisnham and Juliano, 2012a).

In North Carolina, the number of neuroinvasive reported cases has increased considerably within the last three decades, from 32 reported cases between 1990 and 1999 to 145 between 2000 and 2009 and 190 between 2009 and 2018 (CDC [Centers for Disease Control and Prevention], 2011, 2019). Virtually all the LACE cases in NC originate in the western region of the state (**Figure 1.6**) (CDC [Centers for Disease Control and Prevention], 2019; United States Geological Survey, 2013). Moreover, La

Crosse virus (LACV) exposure, which includes all previous symptomatic and asymptomatic infections and is determined by testing for the neutralizing antibody to LACV, was found to increase directly with age, highlighting the endemic nature of the disease in the area (Szumlas et al., 1996a).

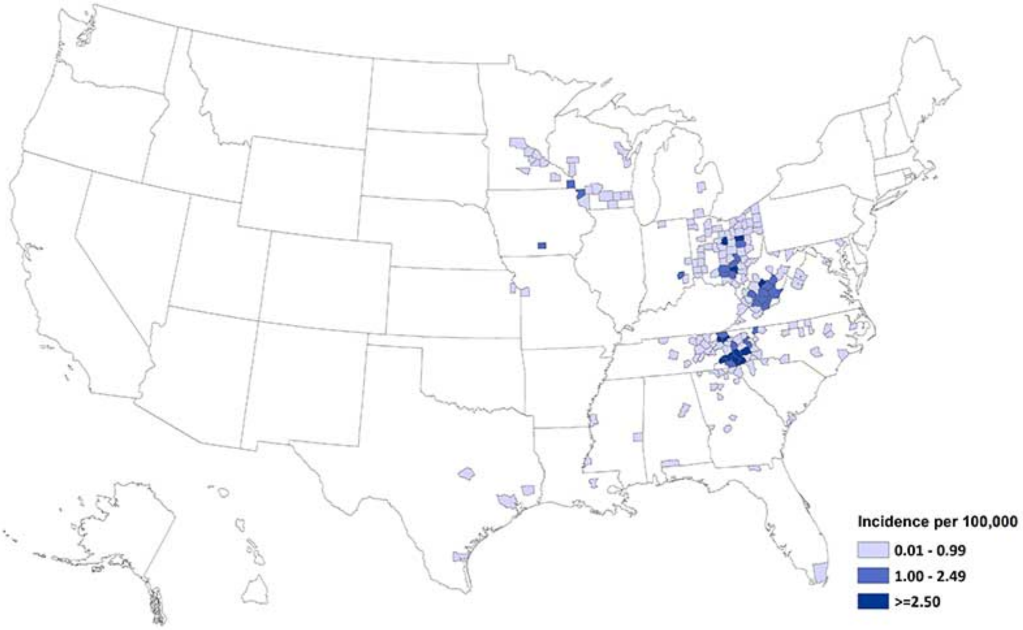

Figure 1.6. La Crosse virus neuroinvasive disease average annual incidence by county of residence, 2009–2018 (CDC [Centers for Disease Control and Prevention], 2019).

The ecology of LACV and anthropogenic effects in the southern Appalachian region

In a previous study by our group (Tamini et al 2021) a comparative ecological approach was applied to evaluate the effect of anthropogenic disturbance on the ecology of LACV vectors. Specifically, the group assessed differences in vector species abundance and environmental variables between six residential habitats of historical LACE cases and adjacent forest patches. The study found higher LACV vector abundance as well as blood-feeding and parity rates in the peridomestic habitats of sites with higher levels of anthropogenic disturbance (i.e., artificial containers) compared to adjacent forest habitats (Tamini et al., 2021). However, in sites with low number of artificial containers, they found higher mosquito abundance in the adjacent forest habitats compared to the peridomestic habitats. Higher densities of artificial containers in peri-domestic habitats adjacent to forest habitat appear to have an important effect on *Ae. triseriatus* abundance in residential habitats, which may result in an increase in LACV transmission risk. The study, however, was limited in its scope to forest-covered sites and small-scale anthropogenic disturbance (i.e., artificial containers). Nonetheless, it underscores the need to study the impact of local and broader scale anthropogenic land-use change and disturbance regimes on the LACV system. In my study, I therefore evaluated the effect of land-use change at a larger spatial scale (i.e, forest to agricultural land) and the smaller-scale anthropogenic effect of introduced artificial containers (i.e., tires).

Several studies have cited the need to better understand the ecology of LAC virus (LACV) vectors following the establishment of the two invasive species, *Ae. albopictus* and *Ae. japonicus*, as well as the effects of anthropogenic land use change on the system (Bevins, 2008; Gratz, 2004a; Leisnham and Juliano, 2012a; Woodruff et al., 1992). The ecological complexity of the LAC system together with the anthropogenic modification of the native environment highlights the need for an ecologically-based approach in studying this system.

General goals and aims

The general goal of this study was to determine the consequences of anthropogenic land-use change in North Carolina, both large-scale and small-scale, on

LACV ecology and risk. In order to achieve this goal, I focused on the following aims:

Aim 1. Characterize the distribution of LACV vectors along forest-to-field ecotones (i.e., large-scale anthropogenic disturbance) and their association with key environmental variables.

Aim 2. Determine the effects of tire introduction (i.e., small-scale anthropogenic effects) on the distribution patterns and community structures of LACV vectors along forest-to-field ecotone.

Aim 3. Compare the horizontal and vertical distribution of LACV vectors along a gradient of anthropogenic land-use intensity (i.e., urban, park, forest) within an urban landscape (i.e., large-scale anthropogenic effects).

Aim 4. Compare the vertical distribution of LACV vectors along forest-to-field ecotones and determine the effect of tire introduction on these distributions. (i.e., combination of small scale and large-scale anthropogenic effects).

Dissertation organization

The study was organized into the following chapters corresponding with the aims above:

[Chapter II (corresponding to Aim 1):] Characterization of the distribution of LACV mosquito vectors along forest-to-field ecotones.

The region of western North Carolina has been experiencing substantial change in its landscape structure, driven largely by the expansion of human development and agriculture. The region is composed of a landscape mosaic characterized primarily by forested patches but with ever increasing open patches (e.g., pasture, grassland, etc.), which will continue to give rise to human-derived forest-to-field ecotones. The effect of these ecotones on both native and invasive LACV vectors is not clearly understood.

Differences in species abundance along an ecotone are hypothesized to be determined by variations in environmental resources and environmental conditions along this gradient. However, the specific environmental factors that determine the abundance of LACV vectors along forest-to-field ecotones are unknown. Although *Ae. triseriatus* is known to be sylvatic, the habitat preferences of the recently invasive *Ae. albopictus* and *Ae. japonicus* are poorly understood. I sought to determine the habitat affinities of the three species and their level of tolerance with respect to sub-optimal habitats along the ecotone. Therefore, species-specific relative abundance patterns (as determined by oviposition patterns and collection of resting adults) and a range of pertinent environmental variables were measured along forest-to-field ecotones to test the hypotheses that *the three LACV vectors cluster differently along the ecotone, however, overlapping primarily in the edge habitat thus leading to greater diversity (i.e., "edge effect") and elevated LACV transmission risk in the edge due to higher LACV vector abundance.*

Predictions.

1. *Aedes triseriatus* will display an affinity for the forest habitats and will be found in lower numbers in the field habitats.
2. *Aedes japonicus* will display an affinity for the edge habitats while still being commonly found in the forest.
3. *Ae. albopictus* will display an affinity for the field habitats and will be found in lower numbers in the forest habitat.

4. All three LACV vectors will be positively associated with tree cover (e.g., tree density).

[Chapter III.] The effect of tire introduction on the distribution of LACV vectors along forest-to-field ecotones.

The distribution and community structure of LACV vectors vary as a result of habitat preferences and, likely, competitive interactions. Moreover, the effects of larval habitat supplementation (i.e., artificial containers) on LACV vectors' distribution and abundance are unclear. Thus, I introduced tires along the forest-to-field ecotones to study the consequences of artificial breeding site supplementation on mosquito abundance and distribution, determine the spatial and temporal scales associated with this effect, and the impact on LACV risk to humans. I hypothesized that *given the limited availability of breeding sites,* **(i)** *tire introduction will result in an increase in the mosquito abundance,* **(ii)** *change mosquito species distribution, and* **(iii)** *enhance mosquito performance (measured in terms of body size and longevity) of all container-breeding mosquitoes.*

Predictions.

1. The abundance of *Aedes triseriatus*, *Ae. japonicus* and *Ae. albopictus* will increase following the introduction of tires in a given habitat (i.e, intra-habitat effect), as well as in adjacent habitats (i.e., inter-habitat effect).
2. The introduction of tires will be associated with increased body size and longevity for all three LACV vectors.

[Chapter IV.] Vertical distribution of native and invasive container-inhabiting *Aedes* mosquitoes along a gradient of anthropogenic land-use intensity within an urban landscape.

Abiotic (e.g., temperature, relative humidity) and biotic factors (plant cover, interspecific competition) may influence the horizontal and vertical distribution of mosquito species. Moreover, interactions of species with their environment may differ depending on both small-scale dynamics and the landscape at-large (Figure 1.1). Chapters 2,3 and 5 seek to characterize the ecology of LACV vectors in a natural environment composed of forests impacted by medium-to-low-scale anthropogenic land-use intensity (i.e., forest-to-field ecotones and artificial containers), a common feature of the landscape in the western North Carolina region. Nevertheless, urban environments are growing in the region at-large and globally. In cities, the anthropogenic environment is dominant and the natural environment is nested within it (i.e., large-scale anthropogenic land use intensity). It is, thus, important to understand the horizontal and vertical distribution of LACV vectors across a gradient of anthropogenic land-use intensity levels within the urban matrix, to determine if and how oviposition patterns differ between rural (i.e., forest-to-field ecotones; Chapters 2,3, and 5) and urban landscapes.

I hypothesized that *the habitat context within which a tree is located influences the local-scale effects of biotic interactions and/or abiotic factors resulting in different patterns of vertical distributions of local container-breeding mosquitoes along the tree's vertical dimension.*

Predictions.

1. *Aedes albopictus* will be more common in habitats with high anthropogenic land use intensity (i.e., campus) and will be more selective in its vertical habitat selection towards ovipositing at ground level in all habitats.
2. *Aedes triseriatus* will be more common in low anthropogenic land use intensity habitats (i.e., forest) and a generalist in its vertical habitat selection.
3. *Aedes hendersoni* will be more common in low anthropogenic land use intensity habitats (i.e., forest) and more selective in its vertical habitat selection towards the canopy.

[Chapter V.] The effect of habitat type (i.e., forest, edge, field) and tire introduction on the vertical distribution of LACV vectors along forest-to-field ecotones.

The community structure of LACV vectors may be impacted by *Ae. hendersoni*, a sympatric sibling species to *Ae. triseriatus*. A relatively poor LACV vector, the native *Ae. hendersoni* primarily resides in arboreal canopies. The vertical distribution of this new mosquito assemblage (i.e., native and invasive mosquitoes) has not yet been described. Moreover, the effect of larval habitat supplementation on their distribution along a vertical dimension is unknown.

Ecotones represent good models for studying the vertical distribution patterns of LACV vectors and how these may change along habitat types as well as in response to anthropogenic larval habitat supplementation. Therefore, the study sites were used to test the hypotheses that **(i)** *the vertical distribution of Ae. triseriatus differs between the habitats of the forest-to-field ecotones being more selective of higher elevations in the field and edge habitats but more generalist in the forest as* *leaf litter is expected to be in high abundance at all elevations, however, the vertical distribution of Ae. hendersoni, and the invasives (i.e., Ae. japonicus and Ae. albopictus) does not change, remaining most abundant at higher elevations and ground level, respectively; and that* **(ii)** *larval habitat supplementation at ground level alters the vertical distribution of the native mosquitoes, shifting their oviposition activity to higher elevations, likely due to increased competition with Ae. albopictus* and *Ae. japonicus as the abundance of these anthropophilic species is expected to increase primarily at ground level given their known affinity for artificial containers.*

Predictions.

1. *Aedes triseriatus* will be more abundant at higher elevations in the field and edge habitats but its abundance will not vary greatly in the forest habitat. However, the distribution of *Ae. triseriatus* will shift to higher elevations with larval habitat supplementation in the forest habitat, likely due to increased competition with *Ae. albopictus* and *Ae. japonicus*.
2. *Aedes hendersoni* will remain most abundant at higher elevations in all habitats along the ecotone. Larval habitat supplementation at ground level will reduce the abundance of this mosquito, likely due to increased competition at higher elevations with *Ae. triseriatus*.
3. *Aedes albopictus* and *Ae. japonicus* will remain selective towards ground level in all habitats along the ecotone. Larval habitat supplementation at ground level will increase their abundance but it will not change their vertical distribution bias towards ground level.

General Methods

The study took place in the Tuckaseegee valley of western North Carolina between August 2011 and September 2014. In this 4-year study, the first year constituted a baseline to evaluate the habitat effect prior to tire introduction. In the subsequent three years, following tire introduction in the summer of year 2, changes in vector mosquito community where monitored. The tire introduction methods are described in Chapter 3 and those regarding egg collections along a vertical gradient in Chapter 5.

In addition, the habitat effect on the distribution of LACV vectors was also studied within a landscape characterized by higher levels of anthropogenic land use intensity (i.e., university campus in the city of Greensboro, NC). The sites and methodology for this study are described further in Chapter 4.

Study sites: Forest-to-Field ecotones in western North Carolina.

Six sites located in the Tuckaseegee valley of western North Carolina within an area of 1.6 km^2 were used in this study. For the selection criteria the sites had to comprise forest-to-field ecotones, with forest and field patches of at least 200m in length as measured from the edge and a minimum width of 200m. Each site selected must have been at least 100m from the nearest study site. Two parallel sampling transects were deployed in each site. The transects were 40m apart and extended 100m from forest edge to the interior of the mixed-hardwood forest and a 100m from the edge into open field areas (e.g., grazing fields) (**Figure 1.7**).

Figure 1.7. Forest-to-field ecotone sites in the Tuckasegee valley (western NC) with parallel sampling transects [coordinates= 35.283275, -83.140272].

Figure 1.7 depicts the study sites (e.g., AB, CD, EF, etc.). Each site is designated by the type of treatment it received: along with the tire introduction Control = no tires, Tires in the field, tires in forest (to be discussed further in Chapter 3) and **figure 1.8** illustrates examples of the habitats along the ecotone.

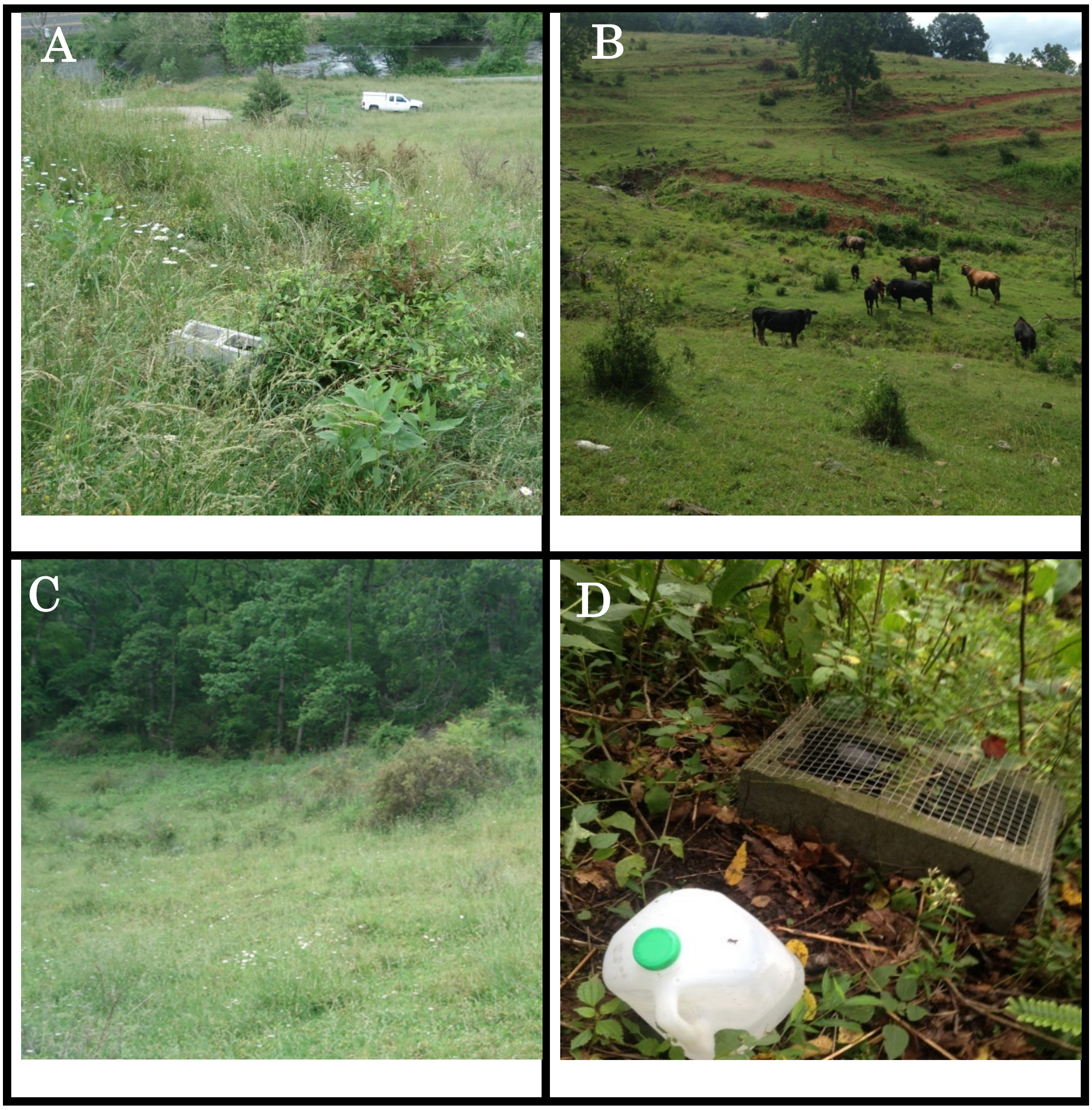

Figure 1.8. Pictures of study sites and oviposition traps (ovitraps). **A.** Field habitat (Site CD); **B.** Field habitat (Site EF); C. Forest edge habitat (Site CD); **D.** Ovitrap in Forest habitat with water jug for recharging cup and wire mesh to prevent rummaging by wildlife (Site AB).

Mosquito sampling methods

1. Oviposition trap transects

In this study we employed oviposition traps (i.e., "ovitraps") to collect the eggs of container-breeding mosquitoes (e.g., *Aedes spp.*). Ovitraps are widely used in mosquito surveillance efforts and research to survey the abundance and oviposition activity of container-breeding mosquitoes given their efficacy, simplicity and low-cost

(Bellini et al., 1996; Chanampa et al., 2018; Gopalakrishnan et al., 2012; Hoel et al., 2011; Melo et al., 2012). The ovitraps used in my study were composed of black plastic cups lined with a seed germination paper used as substrate for the deposition of eggs and filled with water to attract ovipositing females (Bellini et al., 1996; Chanampa et al., 2018; Hoel et al., 2011).

Each transect consisted of 15 ovitraps. The ovitraps were spaced at a distance of 10m within 40m of the edge and 20m apart when beyond 40m from edge (**Figure 1.9**). Higher resolution of sampling stations in the proximity to the edge were due to the potential higher variability around the edge habitat.

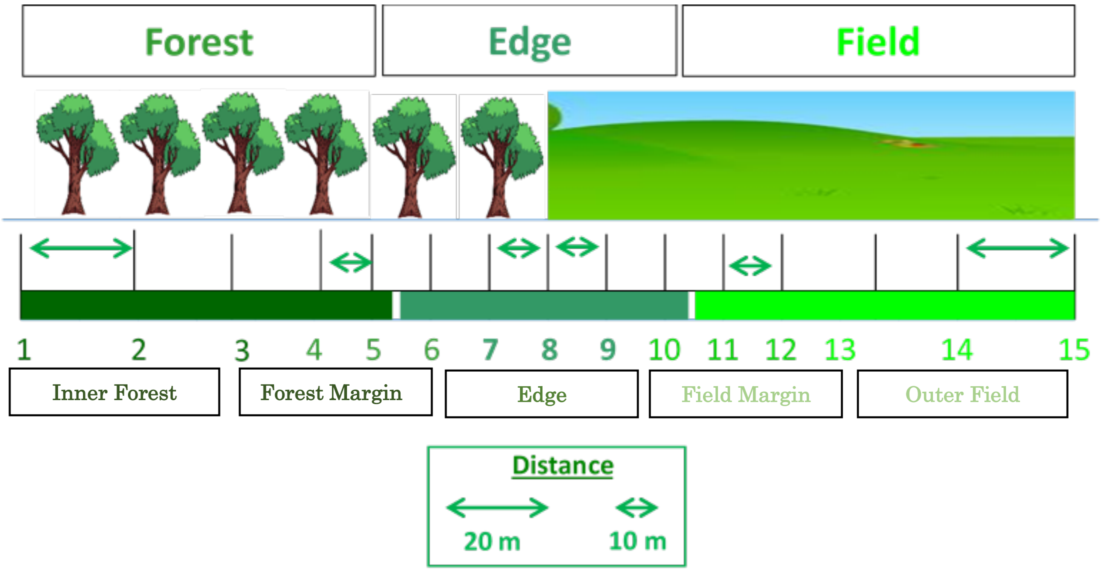

Figure 1.9. Illustration of a sampling transect containing 15 ovitrap stations along forest-to-field ecotone and 2 habitat designations applied in the study i. ‘Forest, Edge, Field’ and ii. ‘Inner Forest, Forest Margin, Edge, Field Margin, Outer Field’.

The ovitraps consisted of 480mL plastic cups lined with germination paper (ovistrips) which serve as a substrate for the deposition of mosquito eggs and mimic the appearance of tree-holes (**Figure 1.10**). These cups were filled with ca.250mL of water to attract female oviposition and punctured to create a drainage hole to prevent overflow due to precipitation.

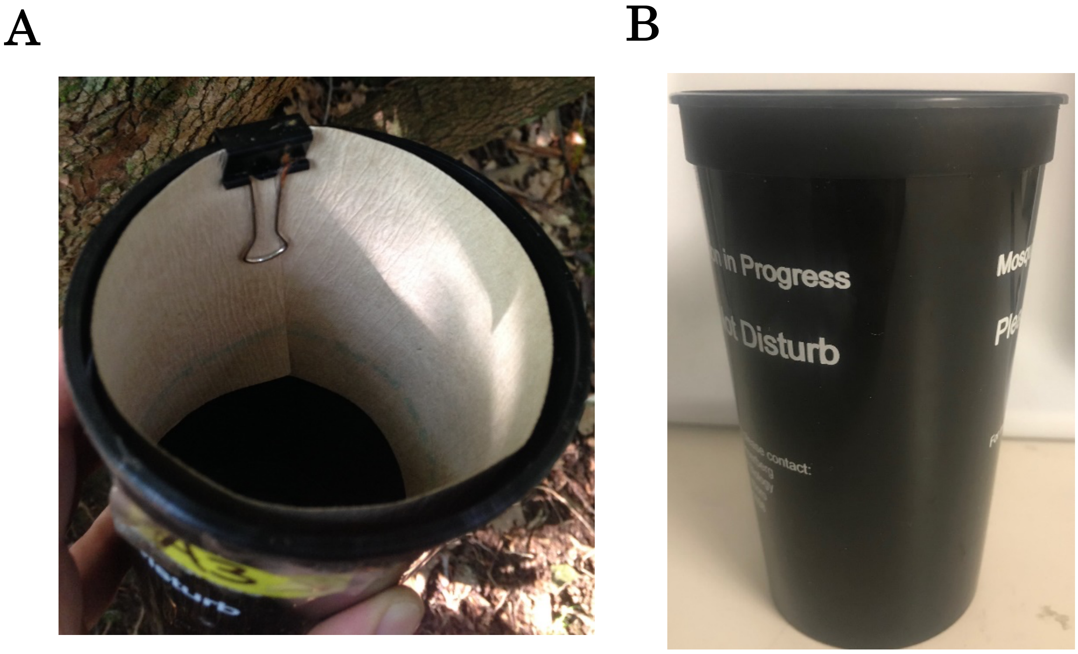

Figure 1.10. A. Ovitrap lined with germination paper and secured with metal clip; **B.** 480mL Plastic cup used as ovitrap.

After collection, the eggs on each strip were counted visually using a dissection microscope. Subsequently, the strips were flooded with dechlorinated tap-water in plastic 2800mL sized trays ("Larval tray", BIOQUIP– Cat No. 1426B), with bovine liver powder (MP Biomedicals, LLC. – Cat No. 900396) provided to serve as a food resource substrate (i.e., enhanced bacterial growth) for hatched larvae, and placed in an incubator at 28°C and 80% relative humidity for a one-week period (**Figure 1.11A**). Ovistrips with zero egg counts were not flooded, however, these were incorporated in the count data as true zeros. Fourth instar larvae and pupae were then transferred to emergence cups ("Mosquito breeder"; BIOQUIP – Cat No. 1425DG)

(**Figure 1.11B-C**). Once eclosed, adult mosquitoes were freeze-killed and identified to species and sex under a dissecting microscope.

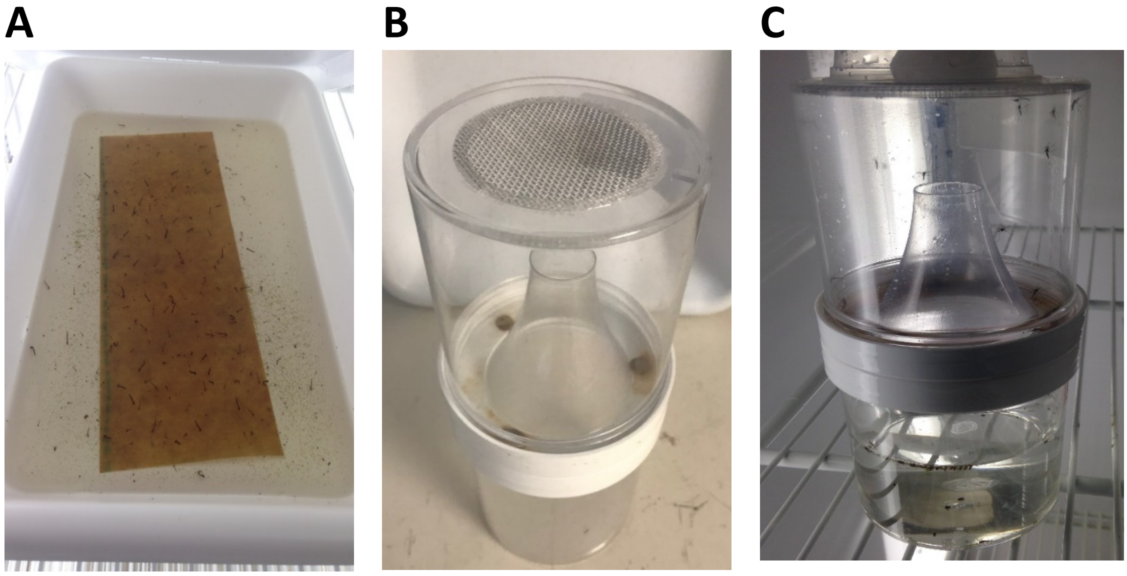

Figure 1.11. A. 2800-mL plastic tray with reared larvae in an incubator; **B.** Empty emergence cup (bottom: larvae and pupae holding section, top: funnel and emergence section); **C.** Emergence cup in use with pupae (bottom) and recently emerged adults (top).

2. **Vertical oviposition traps**

Within each sampling replicate, ovitraps were positioned at three heights: 0 meters (tree-base level), 4.5 meters (mid-tree level), and 9 meters (canopy level) above ground. The ovitraps were placed along the trunk of mature trees (hardwood trees >25 cm diameter) using a pulley system. These methods are further described in Chapter 4 and Chapter 5.

3. **Resting adult mosquitoes: Nasci aspirator.**

The Nasci-aspirator is a mobile aspirator tool for sampling resting mosquitoes in vegetation (Nasci, 1981a). It is cylindrical and measures approximately 100cm in length and 40cm in diameter (**Figure 1.12A**). The aspirator makes use of an electric fan and is powered by a 12V battery. It draws air thereby pulling adult mosquitoes into a catch bag when placed in proximity to vegetation and shaded areas where specimens are commonly found resting. The aspiration was performed by moving the machine in an up-and-down motion from the chest down to knee-height while sweeping vegetation and likely resting habitat (**Figure 1.12B**).

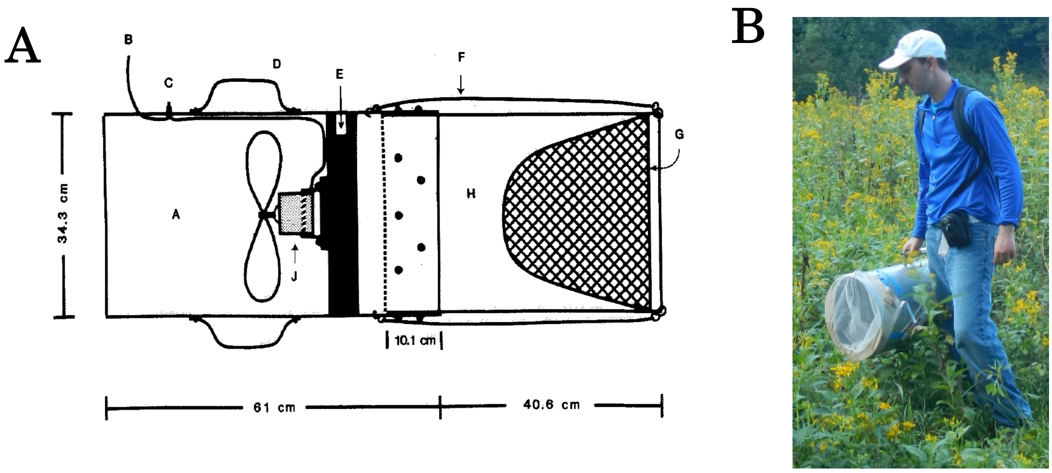

Figure 1.12. A. Diagram of original Nasci aspirator (1981) (Nasci, 1981a); **B.** Nasci aspirator in use.

The transect collections were standardized in 15-minute intervals, with each interval consisting of one of the five areas designed for the sites (i.e., outer-field [Field II], field-margin [Field I], edge, forest-margin [Forest I], and inner-forest [Forest II]). The collector walked in a standardized zig-zag manner (**Figure 1.13**) from one transect to another for 15 minutes per area (i.e, 5 collection areas per site), placing the area-specific catch-bags in a cooler with dry ice to preserve the specimens for transport.

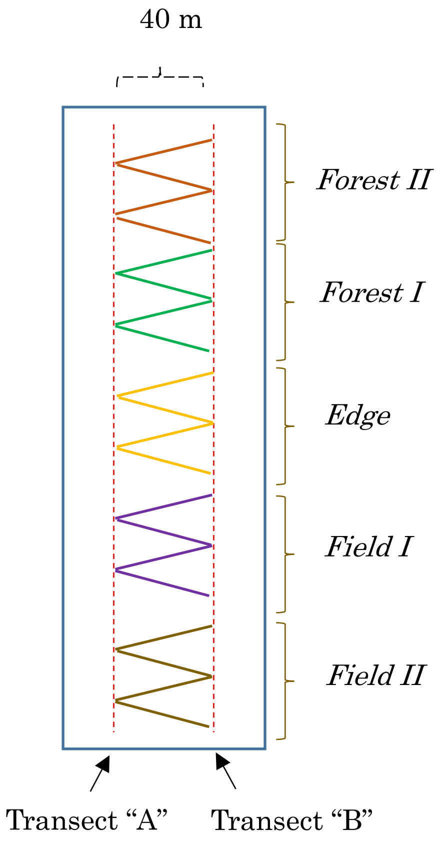

Figure 1.13. Nasci aspiration collection scheme. Each site was divided into 5 sections as described above. Collector walked in zig-zag manner for 15 minutes.

4. **Host-seeking mosquitoes:**
    a. **BG sentinel traps.** These traps were used in Year 1 of this study prior to the introduction of tires (i.e., baseline period). Further described in Chapter 2.
    b. **Human Landing Catch (HLC method).**

The Human Landing Catch (HLC) is an effective method for collecting *Aedes*, *Anopheles*, and *Culex* mosquitoes seeking a blood meal (Barnard et al., 2014; Govella et al., 2011; Haddow et al., 2009). The host-seeking behavior of LACV vectors was assessed by sampling mosquitoes landing on 2 seated adult human volunteers. The two collectors worked in shifts of 20 minutes per ecotone habitat (i.e., Forest, Edge, Field) between afternoon and early-evening time. Two sessions of each site and habitat sampled (e.g., AB-Forest) were conducted on a given day. The earlier session took place from 13:20 to 17:30 (earliest = 13:20 to 13:40 h) and the second from 17:30 to 21:00 (latest = 20:36 to 20:56 h). Mosquitoes were collected with an aspirator when landing on exposed skin, primarily lower limbs. Collections were placed in catchment containers and then in a cooler with dry ice for transport. Collected specimens were identified to species and sex in the laboratory.

Molecular identification of adult *Ae. triseriatus* and *Ae. hendersoni*.

The two native *Aedes* species are difficult to distinguish visually, particularly at the adult stage. Moreover, these sister species are found in sympatry in NC. Nevertheless, it is important to distinguish between these species due to their different abilities to transmit the virus (i.e., *Ae. triseriatus* is regarded as a primary LACV vector and *Ae. hendersoni* is considered a non-competent vector). Therefore, in Chapter 4 we employed a duplex PCR method, described by Wilson et al. (2014) (Wilson et al., 2014), to accurately identify vertical mosquito collections determined by microscopy to belong to the *Ae.triseriatus/Ae. hendersoni* group.

Specimen dissections and processing

Adults collected using either of the above two methods were used for determination of body size by measuring wing size and parity statis by dissecting their ovaries.

1. **Wing size**

The size of adult specimens was determined by measuring wing length. After species and sex identification in the laboratory, the wings of each *Aedes spp.*

specimen were detached from the thorax and placed on double-sided tape which was then attached to a microscope glass slide. The wings were labeled in accordance with the identification number assigned to the specimen. An image of the wings was then focused using a stereomicroscope (Olympus SZ61) and captured using a 1.3 megapixel microscopy camera attached to the microscope (Infinity Capture software, Infinity 1-1M camera, Lumenera Corporation, Ottawa, Canada). After software calibration, the wings were measured using the Infinity Analyze software package. A line was drawn from the axillary incision to the apical margin, excluding the fringe scales, this produced a value for length (micrometers) (Nasci, 1986; Schneider et al., 2004). If different, the values of each pair of wings were averaged.

2. Parity status.

Female *Aedes spp.* Mosquitoes' ovaries were dissected, following methods described by Detinova (1945 and 1962) (Detinova, 1945, 1962) and Meadows (1968) (Meadows, 1968), to determine parity status of both resting and host-seeking collections. Parity status, nulliparous (i.e., not laid eggs) or parous (i.e., laid eggs) is determined by examination of the terminal tracheoles in the ovaries. Coiled tracheoles (i.e.,tracheole skeins) are characteristic of nulliparous females (**Figure 1.14A**), whereas distended tracheoles describe parous females (**Figure 1.14B**). The method is based on the tracheal system of the ovaries during the first gonotrophic cycle, in which the terminal nodes of the tracheoles permanently uncoil with egg maturation (Beklemishev et al., 1959; Detinova, 1945). Thus, a female with one or more oviposition events will possess distended (i.e., net-like) tracheoles.

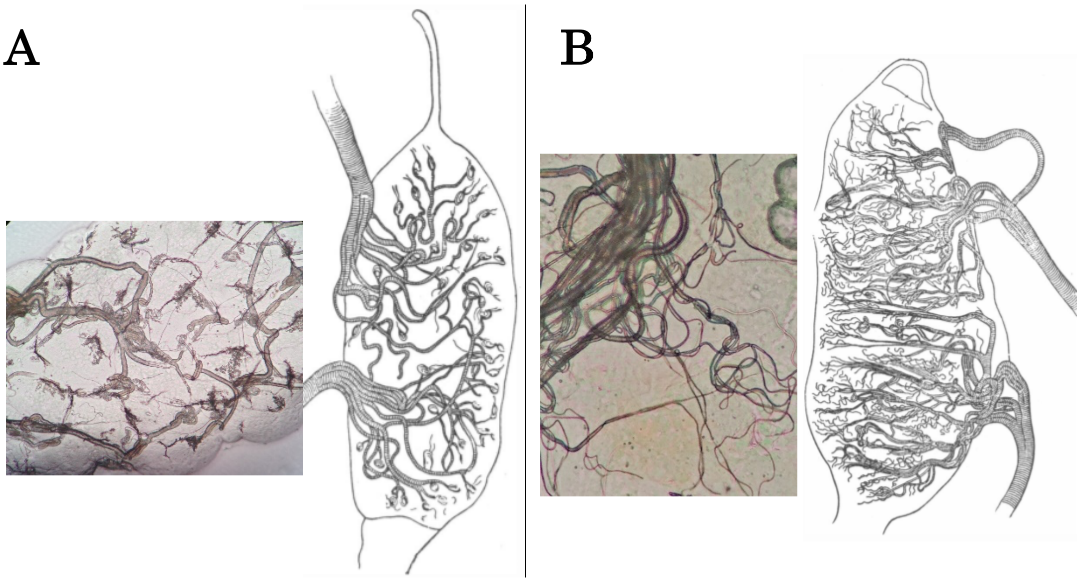

Figure 1.14. Parity dissections. **A.** nulliparous female with coiled terminal nodes; **B.** parous female with distended tracheoles (Detinova, 1945).

Parity status is useful in describing pathogen transmission risk as most females, with the exception of vertical and venereal transmission, will first become infected through an initial blood feeding event from an infected host and must then oviposit in order to reach the end of the gonotrophic cycle. Only a second blood feeding may allow for the transmission of the pathogen to another host. Parity status may also be used as a proxy for age, given that a female must have gone through at least one gonotrophic cycle in order to reach parity. The relative age-structure of a population can, therefore, be estimated based on the proportion of parous females in that population.

3. Testing for LACV infection

Resting and host-seeking female collections were screened for LACV using methods described by (Gerhardt et al., 2001) as well as (Ksiazek and Yuill, 1977) and (Kuno, 1998; Kuno et al., 1996). Vero cell culture wells were inoculated with 100-µl of mosquito specimen BA-1 homogenate, incubated for 7 days, and checked daily for cytopathic effects. Samples showing evidence of cytopathic effects were then tested for LACV using reverse transcriptase PCR.

CHAPTER II

MOSQUITOES ON THE EDGE: CHARACTERIZATION OF THE FOREST-TO-FIELD ECOTONE AND THE DISTRIBUTION OF LACV MOSQUITO VECTORS ALONG IT

Introduction

A common feature of anthropogenic fragmentation of natural forests is an increase in the relative amount of edge habitats (Farina, 2006b; Pfeifer et al., 2017). This results in the formation of ecotones, which are defined as ‘a zone of transition between adjacent ecological systems, having a set of characteristics uniquely defined by space and time scales and by strength of the interactions between adjacent ecological systems’ (Holland et al., 1991). Ecotones play an important role in the emergence of zoonotic and vector borne diseases infectious diseases because they represent a transition zone between natural and anthropogenic habitats where animal-human contact is facilitated (Despommier et al., 2006b; Hassell et al., 2017). The emergence and re-emergence of vector-borne diseases can often be linked to human land use changes such as deforestation, agriculture, and urbanization (Diuk-Wasser et al., 2020; Leisnham and Juliano, 2012a; Mayi et al., 2019; Reisen, 2010; Steiger et al., 2016). With tick borne diseases, for example, forest fragmentation, was suggested to increase disease risk to humans through reduction of host diversity (the effect known as the “dilution effect”) (Ostfeld and Keesing, 2000) and increased exposure to recreational human activity (Connally et al., 2009). With mosquito borne diseases, emergence patterns can be associated with land-use changes such as deforestation, agriculture intensification, and urbanization (Steiger et al., 2016). Newly available habitats for mosquitoes, such as irrigation systems, dams, and other water-holding bodies, have also enabled mosquitoes to spread into previously uninhabitable areas (Multini et al., 2020). A classic example of such effect is the transmission of Yellow fever, which is maintained through three transmission cycles: sylvatic, intermediate, and urban. The sylvatic cycle occurs in the rainforest, involving sylvatic *Aedes africanus* or mosquitoes of the genus *Haemagogus* and *Sabethes* in South America. The intermediate cycle in the forest-savanna ecotone has peridomestic anthropophilic Aedes spp. such as *A. furcifer, A. taylori, A. luteocephalus*, and *A. simpsoni* that act as "bridging vectors" driving a zoonotic spill-over from the simian reservoir host to rural humans. Humans, in turn, may introduce the pathogen into the urban cycle, where humans act as the reservoir host with *Aedes aegypti* and *Aedes bromeliae* as the main vectors (Gardner and Ryman, 2010; Silva et al., 2020).

The region of western North Carolina has been undergoing considerable change during the past two decades due to increased population growth and human land use (Vogler et al., 2010). This regional development is creating a new type of landscape mosaic composed of forest patches, open field patches, and urban patches of various kinds (e.g., residential, commercial, etc.) (NEMAC, 2016). Thus, although the landscape remains primarily rural, dominated by deciduous forest patches, human development pressures are increasing landscape fragmentation and the abundance of field patches (Stein et al., 2005; Vogler et al., 2010). These recent changes to the landscape have resulted in an increase in the prevalence of forest-to-field ecotones in the region. The consequences of this landscape change on the LACV vectors remain unclear. It is, thus, important to determine habitat affinities of LACV vectors in this patch mosaic, including their tolerance of sub-optimal habitats.

Prior to the introduction of the invasive *Ae. albopictus* and *Ae. japonicus* mosquitoes, the native *Ae. triseriatus* was considered (and may still be) the most abundant mosquito in the western North Carolina area and the key vector of LACV (Szumlas et al., 1996b, 1996c, 1996a). *Aedes triseriatus* breeds in tree holes and artificial containers (Barker et al., 2003a, 2003b; Beehler et al., 1992). The development of its larvae (i.e., oviposition, growth rate survival, adult mass,) is strongly affected by leaf detritus and, thus, by canopy density (Kling et al., 2007a). High abundance of Oak leaf litter is strongly associated with larval development which feed on microorganisms in the biofilm of the leaf surface (Walker et al., 1997). In both laboratory and field investigations, Trexler et al. (1998) found that *Ae. triseriatus* laid significantly more eggs in traps with oak leaf infusions compared to traps with water (Trexler et al., 1998). Moreover, Joy and Hildreth-Whitehair (2000) trapped this mosquito in West Virginia across 15 larval habitat types, both natural and artificial, and found an overwhelming proportion of larvae in shaded compared to sunlit habitats. For example, 69.9% of shaded tires contained *Ae. triseriatus* compared to only 50.8% of sunlit tires. With other man-made containers, they found *Ae. triseriatus* in 52.6% of containers in shaded areas compared to only 24.0% in sunlit ones (Joy and Hildreth-Whitehair, 2000). Nonetheless, this mosquito is commonly found also in open fields adjacent to forested areas (DeFoliart and Lisitza, 1980; Mather and DeFoliart, 1984a). Utilization of this sub-optimal habitat was enhanced when field patches contained isolated trees or tires (Lampman et al., 1997a; Mather and DeFoliart, 1984a). Moreover, in a study by Barker et al (2003), *Ae. triseriatus* appeared to show no oviposition (i.e., egg-laying) preference between yards and adjacent forests at a LACE case site (i.e., homes of infected human cases). Therefore, although *Ae. triseriatus* is a sylvatic species which most commonly breeds in hardwoods (Barker et al., 2003b), its presence is not limited to wild forests (Mather and DeFoliart, 1984b). It is often found in peri-domestic areas within, or adjacent to, forested sites, where it inhabits artificial containers (Mather and DeFoliart, 1984b).

The two invasive species, *Aedes albopictus* and *Aedes japonicus* may colonize natural larval habitats, however, they thrive in artificial containers and peri-domestic environments (Bartlett-Healy et al., 2012; Bevins, 2007). *Aedes albopictus*, native to southeast Asia, was introduced to the continental US in 1985 in imported used automobile tires and now exists in more than 25 states mainly in southern and mid-

Atlantic states (Gratz, 2004b; Hahn et al., 2017). It is an aggressive, diurnal, anthropophagic species with a strong affinity for anthropogenic habitats (Gratz 2004). It is a competent vector for at least 22 arboviruses, notably dengue and chikungunya and La Crosse viruses (Gratz, 2004b; Grimstad et al., 1989). *Aedes japonicus* is also known to exploit tree holes, bamboo stumps, and catch basins (Andreadis et al., 2001; Bevins, 2007; Tsuda et al., 1994). The larvae are most commonly found in habitats with decaying organic matter, such as leaf detritus (Andreadis et al., 2001; Tanaka et al., 1979). *Aedes japonicus* regularly oviposits in rock pools, both sunlit and shaded, although it appears to prefer shade, as well as forested and bushy habitats in general (Bevins, 2007; Sota et al., 1994; Tanaka et al., 1979). Byrd et al (2019) surveyed riverine rockpools in the southern Appalachian Mountains and found that *Ae. japonicus* was highly abundant in rockpools with water cooler than 17°C.

Both *Ae. albopictus* and *Ae. japonicus* are competent vectors of LACV (Bara et al., 2016; Bevins, 2008; Gratz, 2004b; Paulson et al., 1989). Thus, their coexistence with *Ae. triseriatus* in LACE endemic areas may be increasing transmission risk to humans by virtue of their more anthropophilic nature. In a recent study, a former student of Wasserberg and Byrd, evaluated if and how residential scale anthropogenic effects (within a forested landscape in the southern Appalachian region) impact the ecological processes associated with LACV transmission (Tamini et al., 2021). They applied a comparative ecological approach to evaluate how anthropogenic land-use differences affect mosquito vector abundance, community structure, gonotrophic status, and blood-meal accessibility within and between peridomestic and matched forest habitats. As expected, *Ae. triseriatus* was more sylvatic while *Ae. japonicus* was more common in the peridomestic backyard of local inhabitants. Surprisingly, *Ae. albopictus* was quite uncommon in that study site. The most significant finding of this study was that the abundance, gravidity and parity rates of both species were strongly associated with levels of peridomestic habitat up-keep. Specifically, both species were more abundant in peri-domestic areas containing high number of artificial containers but were more common in forested areas where peridomestic yards contained low number of artificial containers. Similarly, gravidity, parity, and blood-feeding rates were higher in peridomestic habitats with high number of artificial containers but higher in the forest habitats for sites containing low number of containers (Tamini et al., 2021). Results of this study indicate that anthropogenic influences can modify mosquito's habitat use patterns in a manner that might increase entomological risk to resident humans.

That study (Tamini et al., 2021), however, was limited to forest covered sites. **The general goal of my study** here, was to bridge this gap and evaluate patterns of distribution and habitat use of these mosquito species along a broader spatial scale which incorporates the regionally characteristic human-induced spatial heterogeneity, which includes: forest patches, open fields, and the transition zone (ecotone) between them. By establishing a sampling transect going from the inner forest, through the edge, and into the adjacent open cattle pasture field, I quantified mosquito oviposition activity and resting adult distribution. Using CO_2-baited BG Sentinel traps, I also characterize adult mosquito's host seeking behavior and measured their wing size to determine size differences among habitats.

My specific aims were to:

1. **(1)** Characterize the forest-to-field ecotone in terms of biotic and a-biotic factors.
2. **(2)** Characterize the distribution of *Ae. triseriatus*, *Ae., japonicus*, and *Ae. albopictus* along this ecotone.
3. **(3)** Characterize the host seeking behavior and fitness attributes (body size) of these mosquitoes along this forest-to-field ecotone.

Predications:

Aim 1. Ecotone characteristics.

Three alternative hypotheses were suggested: **a**) a "soft edge" with a smooth transition in environmental variables along the gradient; **b**) a "hard edge" with an abrupt transition and no intermediate habitat between forest and field; and **c**) an "gradual edge" with a transitional habitat.

I **predicted** that the forest-field ecotone would be characterized by a gradual edge habitat (hypothesis C) because the edge zone (on the field side) is typically covered with Blackberry (*Rubus fruticosus*) and other shrubby thickets, which are expected to make the transition at the edge more gradual.

Aim 2. Mosquito distribution along the ecotone.

Based on published information about these mosquito's habitat affinities, I predicted that in terms of both oviposition activity and resting adult distribution:

(1) The native *Ae. triseriatus* would be most abundant in the forest habitat, *Ae. japonicus* in the edge habitat, and *Ae. albopictus* in the field habitat.

(2) I also predict that some overlap in their distribution should occur with *Ae. triseriatus* spilling into the field habitat, *Ae. albopictus* spilling into the forest habitat, and *Ae. japonicus* spilling into both field and forest habitat from the edge habitat.

Aim 3. Host seeking behavior and fitness attributes. I predicted that host seeking behavior and wing size would correspond to the species' habitat affinities with *Ae. triseriatus* most abundant and larger in the forest, *Ae. japonicus* in the edge, and *Ae. albopictus* in the field.

Novelty:

Most previous studies have characterized mosquito distribution in either forest habitats or in urban/rural environments. But no study on this system yet exists that characterizes the entire ecotonal distribution of these mosquitoes.

Significance:

A clear understanding of habitat use by LACV vectors would better inform public health efforts by characterizing LACE risk along the forest-to-field ecotone. Distinct habitat use of the three species would denote a risk broadly distributed along the ecotone. However, if all species cluster similarly (i.e., forest habitat), risk would be focused, denoting potential LACV transmission hot-spots as well as habitats with lower risk (e.g., field habitat).

Methods

Design of sampling plots

Six sampling plots located on privately-owned land and characterized by forest-to-field ecotones were selected for this study (see Chapter I). Two hundred meters long ovipot transect extended 100 meters from the forest edge into the forest and 100 meters into the field with ovitraps located at 10 meter intervals between 0 – 40 m and at 20 m intervals between 40 and 100 m to allow for finer resolution near the edge where we expected a greater degree of change in biotic and abiotic variables to occur. I also categorized this gradient into 5 distinct habitat classifications: three ovitraps were located in the forest interior (hereafter “Inner forest”) between 60 m and 100m from the forest edge, 3 were located at 20 to 40 meters from the forest edge (hereafter “Forest margins”). The “forest edge” habitat included an ovitrap located at the forest edge and 10 meters to either side. The next three were located between 20 – 40 meters from the edge into the field side (hereafter “field margins”), and last three at 60-80 meters from the forest edge (hereafter “outer field”) (Figure 2). Resting adult mosquitoes were trapped using a Nasci aspirator (see Chapter I) at a coarser resolution based on the 5 habitat categories described above, with each habitat type sampled for 15-minutes in each plot.

Ecotone characterization in terms of environmental variables

At each station along the ecotonal transects, I measured the following environmental variables: temperature, relative humidity, tree density, tree diameter at breast height (DBH), nearest neighbor distance to nearest tree, canopy cover, degree of leaf litter, and degree of undergrowth.

Temperature and relative humidity. Relative humidity and temperature readings were taken using a pocket weather meter (Kestrel® 3000). The meter was held above each ovitrap and allowed to calibrate for approximately 30 seconds before data collection. Values used for each station were calculated as deviation from the mean for that particular transect.

Tree density and nearest tree distance. The nearest tree distance was measured from each ovitrap to the closest tree in a 360° direction. Tree density was measured by surveying the vicinity of each ovitrap. Specifically, the area around each ovitrap encompassed 10 meters from the ovitrap along the transect as well as 4.5 meters from the ovitrap in each direction perpendicular to the transect. In addition, given their relative proximity, I combined the tree count for both transects at each ovitrap in a given plot (e.g., A13 and B13). Thus, the total unit area for the tree density measure was 360 m^2.

Tree diameter at breast height (DBH). A Biltmore stick was used to measure the diameter of trees at breast height. The stick was held at a right angle to the axis of the tree, approximately 130 cm from its base and approximately 60 cm from the eye of the volunteer. All the trees within 10m of a particular transect station were measured and mean was calculated.

Canopy cover. Canopy cover was determined using a GRS Densitometer^{TM} (Geographic Resource Solutions). The densitometer was held directly above each ovitrap station and directed vertically towards the canopy with the crosshairs positioned within the sighting (Stumpf, 1993). The degree of canopy cover (i.e., shade) was noted according to 3 levels: 0 (<33% cover), 1 (33-66% cover), and 2 (>66% cover).

Leaf cover and undergrowth vegetation. Leaf cover and undergrowth vegetation (i.e., low shrub and herbaceous cover) was determined using a GRS Densitometer™. The densitometer was held above each ovitrap and directed vertically towards the ground. The amount of leaf detritus and undergrowth vegetation was noted according to 3 levels: 0 (<33% cover), 1 (33-66% cover), and 2 (>66% cover).

Mosquito trapping methods

Mosquito egg collection using ovitraps. Ovitraps were used to collect eggs by attracting ovipositing females (see Chapter I).

Ovistrips were collected and replaced approximately once a week from August 12th to September 19th during 2011 and from May 5th to June 27th during 2012 for a total of 14 sampling sessions.

Resting mosquito collections using Nasci aspirator. A Nasci-aspirator was used to collect mosquitoes resting in the vegetation (see Chapter I). This took place once, on June 24th, 2012. This was conducted at the 5-habitat resolution as described above. Each habitat type was sampled for 15-minutes in each plot.

Host-Seeking mosquito collections using the BG-Sentinel. CO_2-baited BG-Sentinel traps are effective for the collection of host-seeking container-inhabiting *Aedes* mosquitoes (Maciel-de-Freitas et al., 2006; Williams et al., 2006). These traps measure 36cm in diameter and are 40cm tall. Female mosquitoes are attracted by carbon dioxide delivered through a dry ice cooler located approximately 20cm above the trap. An electrical fan draws carbon dioxide and approaching mosquitoes into a catch bag (Figure 2.1). Commercial lures may also be added to the traps, however, these were not used in this project due to potential differential species-specific attraction. One BG-Sentinel was placed at each major ecotone habitat (i.e., field, edge, and forest) per site. Traps were deployed for 48 hours, allowing for two catch-bag collections, one per 24-hour period. The traps were activated in the afternoon hours of the first day by connecting the fan to a 12V battery and placing a cooler with dry-ice above the trap. In the morning of the second day, the catch-bag in each trap was collected, placed in a dry ice cooler to preserve specimens, and replaced. The BG-Sentinel was then re-activated by replacing the 12V battery and replenishing the dry ice cooler. Lastly, in the morning of the third day, the catch bags were collected again and placed in dry ice. The BG-Sentinel traps were then disassembled. Host-seeking trapping took place in June 14-16 and June 21-23.

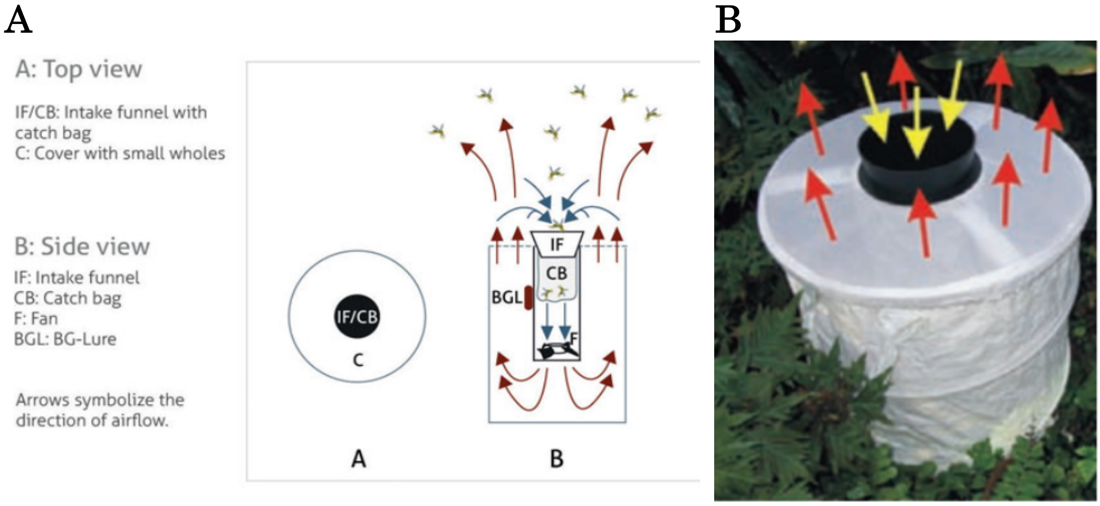

Figure 2.1. BG Sentinel Trap*. **A.** Diagram of BG-Sentinel trap. Blue arrows denote incoming air, red arrows denote outgoing air (Biogents, 2019a). **B.** Picture of BG-Sentinel trap. Arrows denote incoming (yellow) and outgoing (red) air (Biogents, 2019b).*BG-Lure was not used in this study. A cooler with dry ice was placed approx. 20cm above intake funnel as a carbon dioxide source to attract host-seeking females.

Adult mosquito specimen processing

Wing size. The wings of adult specimens collected from the Nasci aspirator and BG-Sentinel traps were pulled gently from its base and measured using a 1.3 megapixel microscopy camera attached to a microscope and the Infinity Analyze software package to determine their size (see Chapter I).

Parity rates. The ovaries of adult specimens collected from the Nasci aspirator and BG-Sentinel traps were dissected to determine parity status (i.e., non-parous or parous). Parity status can be used as a proxy for physiological age (see Chapter I).

Data reduction and statistical analysis

Environmental variables. Linear regression was used to analyze associations between environmental variables and distance from forest edge. The 100-meters section of our transects starting at the edge in the direction of the forest was labeled with negative distance from the edge in meters (i.e., -10m to -100m), and the edge to field direction is labeled with a positive distance from the edge (i.e., 10m to 100m, etc.).

Eggs. Given the non-normal distribution of the eggs as count data (Shapiro-Wilk=0.606, df=4,594, $P<0.001$), a Negative Binomial Regression generalized linear model was used. For the model selection we set our level of significance at $p<0.05$. Linear and second-order polynomial regressions were used to study the distribution of eggs regarding distance from the forest edge.

BG sentinels. Fisher's Exact Test was used to analyze differences in adult host-seeking abundance between ecotone habitats.

Wings. I conducted one-way ANOVA tests for difference in size of adult *Aedes spp.*, as measured by wing size. This analysis was employed on the resting adult and host-seeking collections.

Parity. I sought to conduct a Fisher's Exact Test to test for associations between parity status and habitat, however, the sample size for parous mosquitoes was too small (n=1).

Results

I. Ecotone characterization.

Microclimate. In terms of temperature and relative humidity, a clear threshold response is evident with sharp transition between the forest and the field habitats (Fig. 2.2A, B). Relative humidity exhibits a mirror image of these trends with humidity high and fairly constant at the forest habitat ($R^2 = 0.001$, $P = 0.823$) (Fig. 2.2A). It then decreases sharply at the edge (b = -0.252, $R^2 = 0.390$, $P < 0.001$) and remains fairly constant in the field (b = -0.19, $R^2 = 0.007$, P = 0.556). With respect to temperature (Fig. 2.2B), no significant linear trend was observed at the forest habitat ($R^2 = 0.002$, P = 0.201). However, a significant increase occurred at the edge habitat ($R^2 = 0.399$, $P < 0.001$). At the field habitat a weak but significant increase was observed ($R^2 = 0.111$, P<0.001). These two variables (temperature and RH) were highly colinear (Spearman's rho correlation = -0.679, $P < 0.001$).

Tree characteristics. 'Tree density' exhibits a decreasing significant linear trend (b = -0.609, $R^2 = 0.429$, $P < 0.001$) all the way through the forest and into the edge (-100 to +10). Once in the field habitat, tree density remains fairly constant with respect to distance from the edge, showing a slight decrease between the field margin and outer field habitats (b = -0.135, $R^2 = 0.205$, P <0.001) (Fig. 2.2C). 'Distance to nearest tree' and 'canopy cover' were highly colinear (Spearman's rho correlation = -0.651, P < 0.001). Canopy cover was constant all across the forest habitat (b = 0.000, $R^2 = 0.001$, P = 0.0.785), but then drops precipitously (b = -0.031, $R^2 = 0.370$, $P < 0.001$) at the edge habitat, and continues to decrease gradually (b = -0.010, $R^2 = 0.117$, $P < 0.001$) in the field habitat (Fig. 2.2F). 'Distance to nearest tree' exhibits a mirror image of 'canopy cover', with constant low inter-tree distance all across the forest habitat (b = 0.003, $R^2 = 0.003$, P = 0.117). Beyond the edge (0 to 100), inter-tree distance gradually increases (b = 0.207, $R^2 = 0.282$, $P < 0.001$) with distance from the edge. DBH, as a measure of tree size, increased gradually from the forest to the edge (b = 0.047, $R^2 = 0.077$, P = 0.05). At the field habitat, though, no linear trend ($R^2 = 0.0007$, P = 0.447) was observed with mean DBH being highly variable across that section of the ecotone (Fig. 2.2D).

Undergrowth and leaf litter characteristics. Given that trees are the source of leaf litter, leaf litter cover was clearly highest in the forest habitat (Fig. 2.2G). No linear trend occurred at this habitat with respect to distance to the edge (b = -0.004, R^2 = 0.045, P = 0.108) (-100 to -20). At the edge habitat, however, leaf cover drops sharply (-20 to +20) (b = -0.046, R^2 = 0.537, P < 0.001). It then remained constant and low in the field habitat (b = -0.001, R^2 = 0.003, P = 0.655). Degree of plant undergrowth, which included shrubs and grasses, was fairly low and constant in the forest habitat (b = -0.004, R^2 = 0.025, P = 0.235), but then increased significantly at the edge (b = 0.033, R^2 = 0.315, P < 0.001) and remains approximately constant across the field habitat (b = -0.004, R^2 = 0.027, P = 0.207).

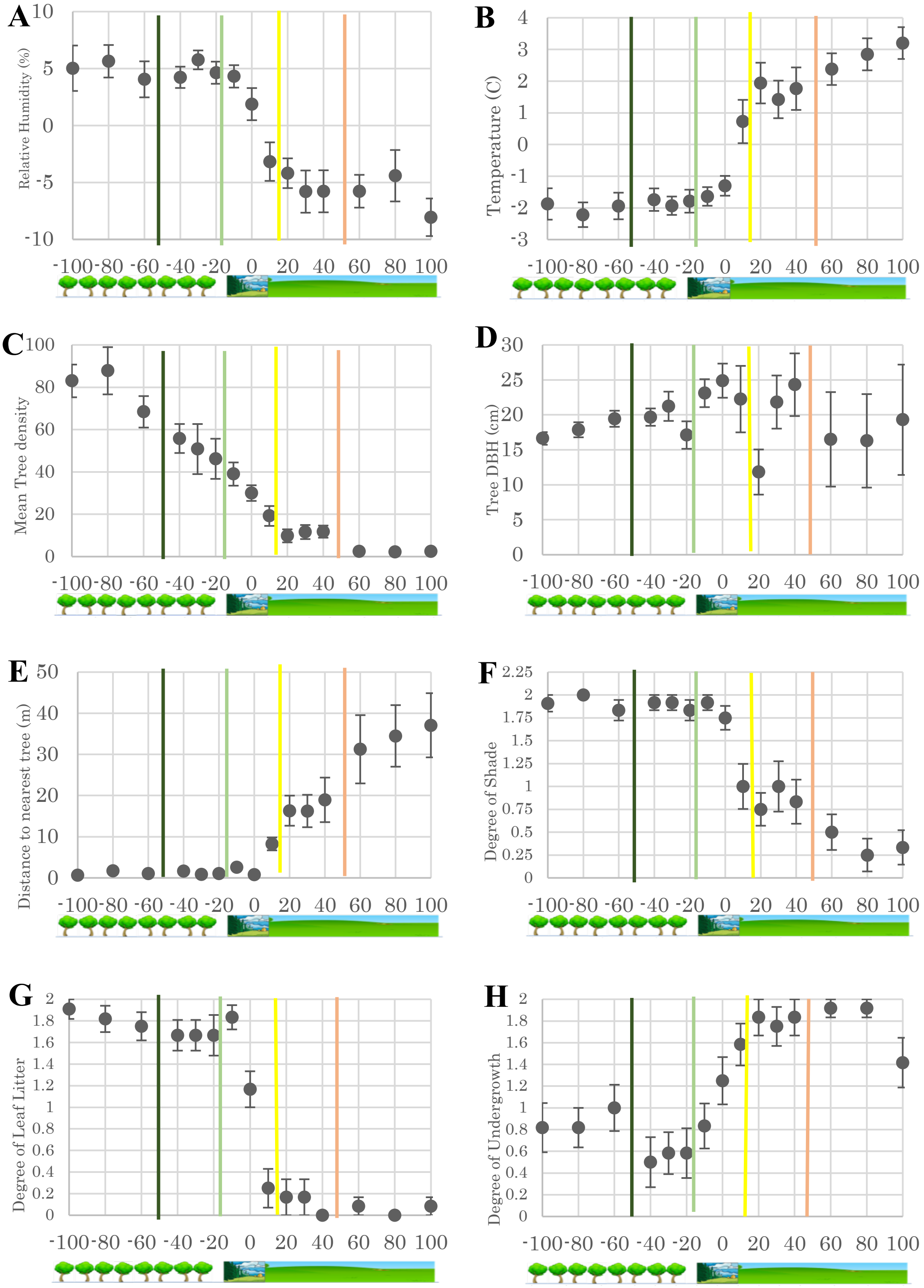

Figure 2.2. Habitat characteristics of forest-to-field ecotones. A) Relative humidity (%) deviance from the mean for each site; B) Temperature (C) deviance from the mean for each site; C) Tree density; D) Mean tree diameter at breast height (cm); E) Distance to nearest tree; F) Degree of shade (0/1/2); G) Degree of leaf litter (0/1/2); H) Degree of undergrowth (0/1/2).

II. Mosquito distribution along the forest-to-field ecotone

1. Oviposition activity

A total of 101,040 eggs were collected throughout the study with *Aedes triseriatus* comprising the majority of this collection (92%), followed by *Ae. japonicus* (5%), and *Ae. albopictus* (3%). Mean density of *Aedes triseriatus* eggs decreased linearly along the forest-to-field ecotone (Fig. 2.3C) ($y = -0.483X+88$, $P < 0.0001$, $R^2 = 0.828$). Mean density of *Aedes japonicus* eggs exhibited hump-shaped relationship along the forest-to-field ecotone, initially increasing when moving from the inner forest towards the edge, peaking at the edge, and then decreasing in the field with distance from the edge (Fig. 3.3E). ($y = 2.374+0.022X-0.0002X^2$, $R^2 = 0.506$). The linear term was not statistically significant ($P = 0.198$) but the second-order term was ($P = 0.044$). Yet, this model was substantially better than a competing linear model ($\Delta AIC = 2.3$ compared with the linear model). With *Aedes albopictus*, egg distribution did not differ along the forest-to-field ecotone ($P = 0.26$) (Fig. 2.3G) no linear trend was observed.

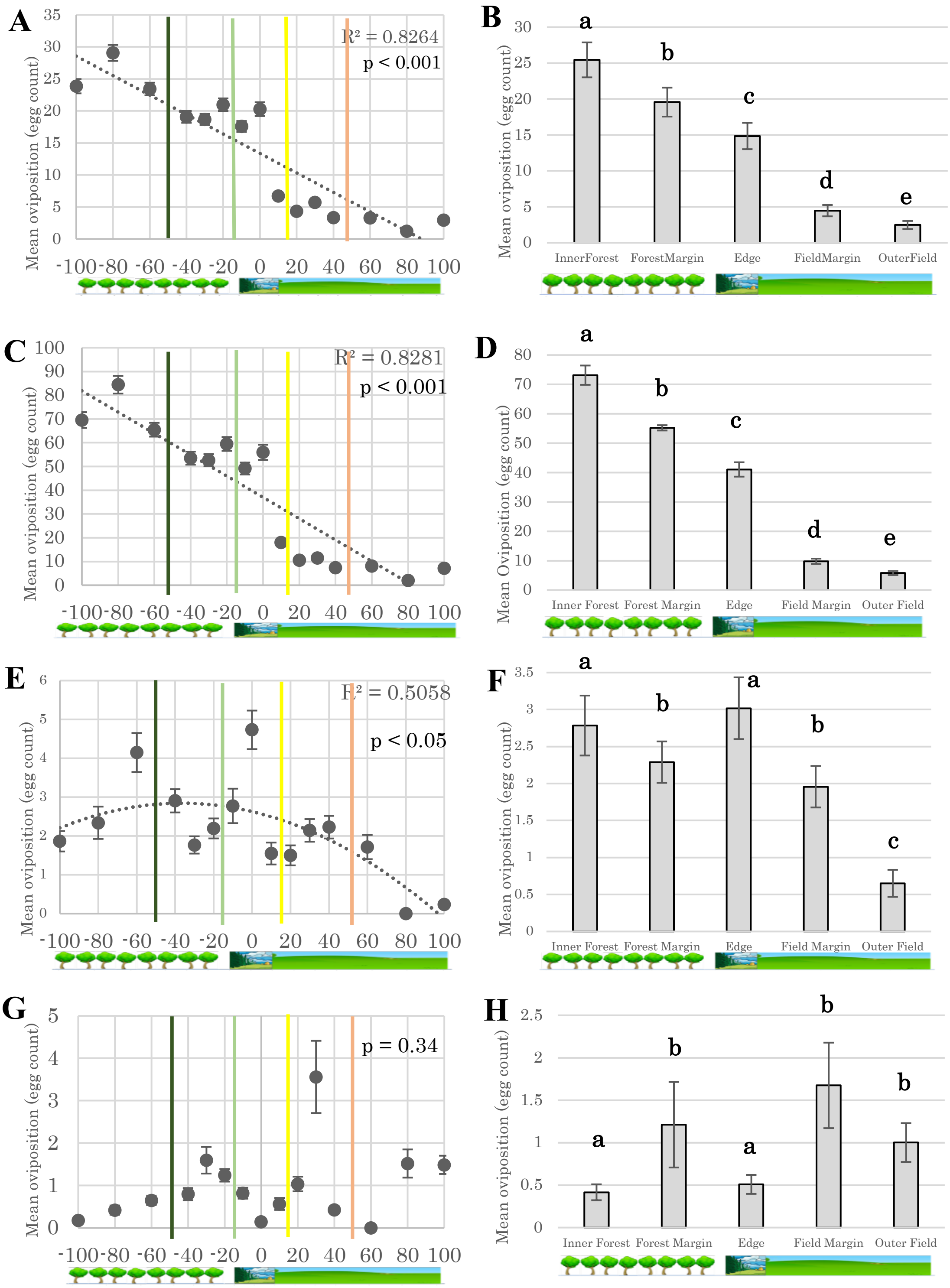

Figure 2.3. Distribution along forest-to-field ecotones by ovitrap (meters from edge) and habitat. *Aedes spp.* oviposition by ovitrap (A) and habitat (B); *Ae. triseriatus* oviposition by ovitrap (C) and habitat (D); *Ae. japonicus* oviposition by ovitrap (E) and habitat (F); *Ae. albopictus* oviposition by ovitrap (G) and habitat (H).

2. The effect of environmental variables on oviposition distribution.

The eight environmental variables measured were found to be highly colinear with one another (Table 2.1). The key structural driver of this forest-to-field ecotone are trees. We therefore tested two unique tree characteristics: tree density and tree size (determined by DBH), as potential explanatory variables driving mosquito distribution. We evaluated if our a-priori designation of the five sub-habitats (inner-forest, forest margin, edge, field margin, outer field) is consistent with mosquito distribution and if the effect of environmental variables differs among these habitat classifications.

Table 2.1. Spearman Rho Correlation Matrix of environmental variables along forest-to-field ecotones.

Correlations

			Leaf litter	Canopy cover	Under-growth	Distance to tree	Relative humidity	Tempe-rature	Tree density	DBH
Spearman's rho	Leaf litter	Correlation Coefficient	1.000	.736**	-.667**	-.632**	.631**	-.727**	.745**	-0.026
		Sig. (2-tailed)		0.000	0.000	0.000	0.000	0.000	0.000	0.751
	Canopy cover	Correlation Coefficient	.736**	1.000	-.576**	-.651**	.451**	-.693**	.723**	0.076
		Sig. (2-tailed)	0.000		0.000	0.000	0.000	0.000	0.000	0.357
	Undergrowth	Correlation Coefficient	-.667**	-.576**	1.000	.550**	-.284**	.547**	-.653**	0.054
		Sig. (2-tailed)	0.000	0.000		0.000	0.000	0.000	0.000	0.514
	Distance to tree	Correlation Coefficient	-.632**	-.651**	.550**	1.000	-.421**	.645**	-.683**	-.234**
		Sig. (2-tailed)	0.000	0.000	0.000		0.000	0.000	0.000	0.004
	Relative humidity	Correlation Coefficient	.631**	.451**	-.284**	-.421**	1.000	-.679**	.500**	0.081
		Sig. (2-tailed)	0.000	0.000	0.000	0.000		0.000	0.000	0.343
	Temperature	Correlation Coefficient	-.727**	-.693**	.547**	.645**	-.679**	1.000	-.700**	-0.106
		Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000		0.000	0.202
	Trees	Correlation Coefficient	.745**	.723**	-.653**	-.683**	.500**	-.700**	1.000	-0.019
		Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	0.000		0.821
	DBH	Correlation Coefficient	-0.0263	0.076	0.054	-.234**	0.081	-0.106	-0.019	1.000
		Sig. (2-tailed)	0.751	0.357	0.514	0.004	0.343	0.202	0.821	

**. Correlation is significant at the 0.01 level (2-tailed).

Aedes triseriatus. The best model was found to be the saturated model (ΔAIC = 231 with respect to the next best model) (Table 2.2). The effect of habitat category was highly significant, with egg density of *Ae. triseriatus* being highest in the inner forest and then decreasing significantly at each subsequent habitat category (Fig. 2.3D). The effect of tree density by itself, in this model, was not statistically difference as it was probably encompassed by the effect of habitats. However, interestingly, we found a significant_statistical "tree density x Habitat" interaction (Table 2.2). The effect of tree density on oviposition activity was positive and significant in the edge (b = 0.73, P = 0.041) and field margin (b = 0.618, P = 0.046) habitats while in the forest interior the positive effect was not significant (b = 0.31, P = 0.230). The exceptions were the 'forest margin' habitat where a non-significant negative trend was observed (b = -0.06, P = 0.755) and the outer field that was characterized by on order of magnitude larger effect of tree density compared with the other habitats (b = 1.97, P = 0.002). The effect of DBH on oviposition was significant with an, overall, positive effect (b = 0.23, P < 0.001) (Table 2.2). Yet, as indicated by the significant "DBH x Habitat" interaction, this effect differed among the five habitats, with a positive effect in all four habitats except for the edge habitat where a non-significant negative trend was observed (b = -0.19, P = 0.745). The positive effects were significant in the field margin (b = 0.03, P < 0.001) and outer field habitats (b = 0.02, P < 0.001). Interestingly, there were also a statistical 2-way interactions between tree density and DBH (Table 2.2) suggesting that the effect of tree density differs among areas with large tree compared to areas with small, possibly young, trees. Also, we found a significant "tree density x DBH x Habitat" three-way interaction (Table 2.2) suggesting that the degree of effect modification of tree size on the effect of tree density could differ among the habitats.

Table 2.2. Negative binomial model testing the effect of habitat, tree density, and tree DBH on *Aedes triseriatus* distribution along the forest-to-field ecotone.

	Wald Chi-square	**df**	**P**
Intercept	339.303	1	0.001
Habitat	510.612	4	0.001
Trees	0.506	1	0.477
DBH	49.985	1	0.001
Habitat x Trees	48.045	4	0.001
Habitat x DBH	109.727	4	0.001
Trees x DBH	15.052	1	0.001
Habitat x Trees x DBH	87.359	4	0.001

Aedes japonicus. The best model included the three main effects of: Habitat', 'Tree density' and 'DBH' and all 2-way interactions (ΔAIC = 150 compared to second-best model) (Table 2.3). Highest oviposition activity occurred at the edge habitat (3.25±0.17), followed by the forest habitats (inner forest = 3.00±0.16; forest margin = 2.17±0.12), and lowest in the field habitats (field margin = 2.11±0.12; outer field = 0.70±0.05), particularly the outer field habitat (Figure 2.3F). This is consistent with the hump-shaped trend previously described (Fig. 2.3E). Here, 'tree density' exhibited an overall significant positive effect (b = 0.02, P < 0.001). However, as indicated by the "tree density x habitat" interaction (Table 2.3) this effect of tree density differed among the ecotonal habitats. Even though the effect of tree density on *Ae. japonicus'* oviposition activity in any specific habitat was not statistically significant, the effect size of tree density on oviposition activity (as reflected by the slope of the habitat-specific regression line) appears to be gradually increasing when moving from the forest and out to the field. Specifically, weakest effect was observed at the 'inner forest' habitat where a negative non-significant effect was observed (b = -0.03, P = 0.399). Then, it switches to become weakly positive at the forest margin (b = 0.03, P = 0.445), edge (b = 0.07, P = 0.286) and the 'field margins' (b = 0.08, P = 0.304) habitats. Similarly to what was observed with *Ae. triseriatus*, oviposition activity increased at the 'outer field' with an increase in tree density, however, this effect was not significant (b = 0.22, P = 0.280). As with *Ae. triseriatus*, DBH had an, overall, significant positive effect (Table 3) on *Ae. japonicus* oviposition activity (b =

0.03, $P < 0.001$). However, as reflected by the significant “DBH x Habitat” interaction (Table 2.3), this effect tended to differ between the habitats. It tended to have (non-significant) negative effects at the forest margins (b = -0.118, P =0.302) and the edge habitats (b = -0.030, P = 0.785) but a positive (non-significant) effect in the inner forest (b = 0.099, P =0.756), outer field (b = 0.024, P = 0.269), and the field margins (b = 0.110, P = 0.50) habitats. As indicated by the significant “DBH x tree density” interaction (Table 2.3), the effect of tree density appears to differ among sites characterized by large versus small trees.

Table 2.3. Negative binomial model testing the effect of habitat, tree density, and tree DBH on *Aedes japonicus* distribution along the forest-to-field ecotone.

	Wald Chi-square	**df**	**P**
Intercept	73.915	1	<0.001
Habitat	132.461	3	<0.001
Trees	27.706	1	<0.001
DBH	35.888	1	<0.001
Habitat x Trees	73.078	3	<0.001
Habitat x DBH	117.499	3	<0.001
DBH x Trees	50.754	1	<0.001

Aedes albopictus. The best model for this species included the three main effects of 'Habitat', 'Tree density' and 'DBH' and all 2-way interactions (ΔAIC = 6) (Table 2.4). *Aedes albopictus* oviposition activity was highest in the field habitat (1.51±0.79), particularly, the 'field margin' habitat (Fig. 2.2H), followed by the 'forest margin', and 'outer field' habitats (Fig. 2.2H). Lowest oviposition activity was recorded at the inner forest and edge habitats (Fig. 2.2H). In contrast with *Ae. triseriatus* and *Ae. japonicus* the overall effect of tree density was negative (b = -0.01, P < 0.001). However, as the Tree density x Habitat' interaction indicates this effect was not consistent across all habitats. The effect was positive, but not significant, in the inner forest (b = -0.009, P = 0.306), negative and suggestive at the forest margin (b = -0.037, P = 0.062), not significant at the edge (b = 0.003, P = 0.887), and negative but not significant at the field margin habitat (b = -0.12, P = 0.419).

However, in contrast, in the outer-field habitat the effect of tree density was strongly positive (b = 0.11, P < 0.001). As with the other mosquito species, the effect of DBH was overall significantly positive (b = 0.04, P < 0.001). However, as the significant DBH x habitat interaction indicates, this effect differs among the habitats. The effect of DBH tended to be negative at the inner forest (b = -0.032, P =0.668), positive at the edge (b = 0.029, P = 0.331), field margin (b =0.050, P = 0.637), and outer field (b = 0.021, P = 0.458) habitats, but was particularly strong and significant at the forest margin habitat (b = 0.315, P = 0.002). As with the other two mosquito species, here we also observed a significant 'DBH x tree density' interaction suggesting a differential effect of tree density for areas with trees of different sizes.

Table 2.4. Negative binomial model testing the effect of habitat, tree density, and tree DBH on *Aedes albopictus* distribution along the forest-to-field ecotone.

	Wald Chi-square	**df**	**P**
Intercept	34.454	1	0.001
Habitat	59.621	3	0.001
Trees	18.799	1	0.001
DBH	13.083	1	0.001
Habitat x Trees	131.055	3	0.001
Habitat x DBH	130.321	3	0.001
DBH x Trees	14.950	1	0.001

3. Distribution of adult resting mosquitoes along the forest-to-field ecotone

Resting adults. A total of only 37 adult mosquitoes were collected. This small sample size precluded conducting any meaningful statistical analysis. The majority of these specimens were collected in the inner forest (78%) and the rest were collected in the forest margin (13%) and edge (8.1%) habitats. None were collected in the field habitat. Unfortunately, the collected samples were very much bitten up so identification to the species level was not possible.

III. Host seeking behavior and fitness attributes.

Host seeking females. A total of 33 female mosquitoes were collected in dry-ice baited BG sentinel traps, comprising 16 *Ae. triseriatus* and 17 *Ae. japonicus*. No *Ae. albopictus* were found. Both species exhibited an un-even distribution among the three main ecotonal habitats. *Aedes triseriatus* exhibited a significant bias

(X^2=17.375, df=2, p<0.001) towards the forest habitat and was completely absent from the field habitat (Fig. 2.4). In contrast, *Ae. japonicus* biased significantly its activity towards the edge habitat (X^2=10.706, df=2, p<0.01), with much fewer mosquitoes caught in the forest or field habitats (Fig. 2.4).

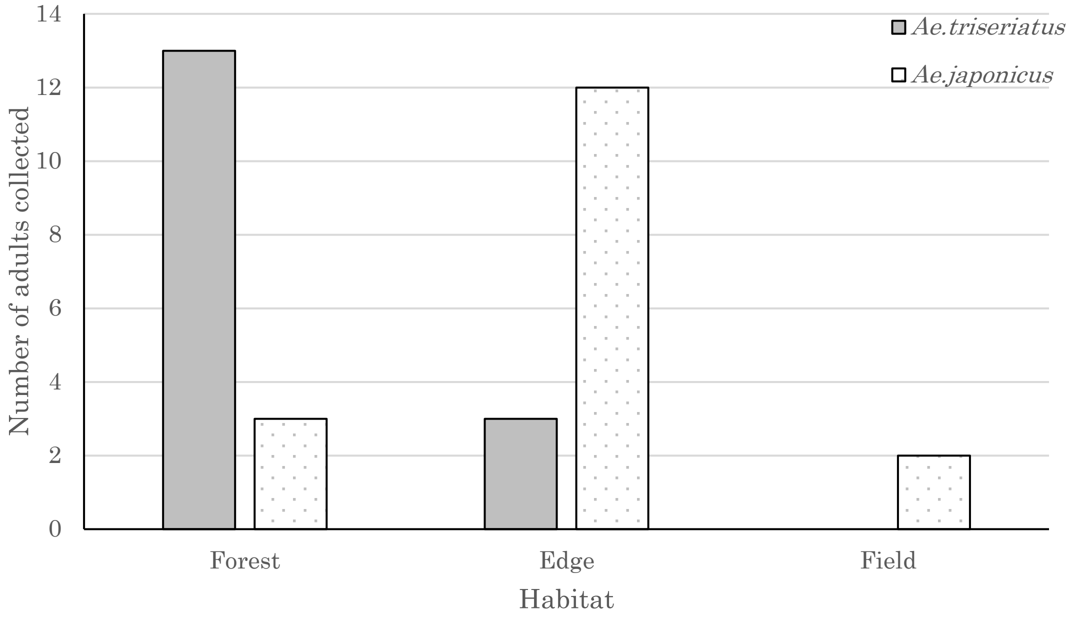

Figure 2.4. Host-seeking *Aedes spp.* mosquitoes' abundance by ecotone habitat, trapped using BG Sentinel traps on June 2012.

Wing size analysis of host seeking mosquitoes. For *Ae. triseriatus*, wing size did not differ significantly between the forest and the edge habitats (mean±SE; Forest= 3887.45±229.08µm; Edge= 4066.50±423.19µm) (Table 2.5).

Table 2.5. One-way ANOVA for *Aedes triseriatus* wing size by habitat (forest, edge, field).

	Sum of Squares	df	Mean Square	F	P
Between groups	78142.736	1	78142.736	0.454	0.512
Within groups	2410809.876	14	172200.705		
Total	2488952.611	15			

For *Ae. japonicus*, wing size in the edge habitats (4008.43±173.80µm) was significantly lower than that of mosquitoes collected at the forest habitat (4412.08±246.58µm) but significantly higher than that in the field habitat (3334.62±1265.44µm) (Table 2.6).

Table 2.6. One-way ANOVA for *Aedes japonicus* wing size by habitat forest, edge, field).

	Sum of Squares	df	Mean Square	F	P
Between groups	1395742.444	2	697871.22	4.968	0.023
Within groups	1966591.843	14	140470.846		
Total	3362334.288	16			

Discussion

The region of western North Carolina has been undergoing considerable change due to increased population growth and human land use change (NEMAC, 2016; Vogler et al., 2010). Thus, although the landscape remains primarily rural, dominated by a temperate hardwood deciduous forests, human development pressures are increasing landscape fragmentation and field patches in particular are becoming a common feature (Stein et al., 2005; Vogler et al., 2010). These changes resulted in an increase in the prevalence of forest-to-field ecotones in the region. The consequences of this landscape change on the LACV vectors remain unclear. The goal of my study was to address this question by: (1) characterizing the forest-to-field ecotone in terms of major environmental variables, (2) describe the distribution of LACV vectors, and their determining factors, along this ecotone, (3) characterize exposure risk along this ecotone by describing the distribution of host seeking mosquitoes along this ecotone.

Ecotone characterization

The forest-to-field ecotone is, by definition, determined by tree cover which affects a variety of biotic and biotic variables. When looking at three cover characteristics, which included tree density, distance to nearest tree, and canopy cover, I found that tree density decreases gradually within the forest habitat when moving from the inner forest towards the edge, and then continues to decrease but more sharply in the edge. In the field this linear trend stops, however tree density appears slightly to be higher in the field margins compared with the outer forest which has the lowest tree density. Nearest neighbor distance and canopy cover look like mirror images of one another with constant low nearest neighbor distance throughout the forest habitat and then sharp increase at the edge followed by gradual increase beyond the edge and into the field. Canopy cover is consistently high throughout the forest all the way to the forest edge and then drops sharply at the edge, which remains low in the field with average cover higher in the field margin than the outer field. Degree of undergrowth is inversely correlated to canopy cover with low coverage throughout the forest and then sharp increase in the edge habitat and remains high throughout the field with some decrease at the farthest part of the field. This undergrowth consisted mainly of spiny shrubs in the form of Black raspberry (*Rubus occidentalis*) and grasses. Shrubs were more common in the field margin and grasses more in the outer field. It is possible, therefore, that these shrubs may provide some more favorable microclimate conditions that buffers the edge effect. Leaf litter, that is known to affect oviposition site quality of tree hole breeding mosquitoes (Kling et al., 2007a), was also strongly correlated to canopy cover, with high coverage in the forest and low in the field. Interestingly, tree size, as reflected by DBH, does not seem to change along the ecotone, although it tends to be much more variable in the field. These plant-related characteristics has a strong effect on abiotic conditions such as RH and temperature. Mean temperatures are consistently low in the forest and then increase at the edge and remains high in the field. Yet, it is suggested that temperatures are lower at the field margins compared with the outer field. Similarly, RH is consistently high in the forest habitat and then drops sharply at the edge and remains relatively low in the field habitat and being lowest at the farthest point in the field. These results suggest that the forest habitat is relatively constant in terms of its biotic and abiotic conditions. However, the edge, appears overall to be quite “hard” with sharp transitions with respect to most variables measured. The field habitat, overall, appears less hospitable for mosquitoes in terms of higher temperature and lower RH. However, the field habitat appears to be a bit more heterogeneous with the “forest margin” being, potentially, more hospitable with somewhat higher tree and shrub coverage and slightly lower temperatures and higher RH compared with the outer field.

Mosquito distribution along the ecotone.

The three LACV vector species appear to have quite distinct habitat affinities. *Aedes triseriatus,* which was, numerically (according to oviposition activity), the dominant species in this system (92%) is clearly a sylvatic species, preferring the denser inner parts of the forest. It then drops sharply at the edge and remains fairly low as you move away into the outer field. In contrast *Ae. japonicus* appears to have an affinity to the edge habitat. It exhibits a hump-shaped distribution along the ecotone initially increasing gradually when moving from the inner forest towards the edge, peaking at the edge area, and then dropping sharply when moving outward into the field. *Aedes albopictus* appears to be the least sylvatic among the three species, showing the highest degree of tolerance towards the warmest and driest field habitat. *Aedes albopictus* has been commonly associated with anthropogenic environments and artificial containers (Gratz, 2004b; Manica et al., 2016), whereas *Aedes japonicus* appears to prefer shade and forested or bushy environments (Andreadis et al., 2001; Tanaka et al., 1979). In contrast, *Aedes triseriatus* is well-known to be associated with forests given its affinity for shaded environments (Joy and Hildreth-Whitehair, 2000; Szumlas et al., 1996c; Trexler et al., 1998; Walker et al., 1997). It is also known to be strongly affected by the presence of leaf detritus at the larval stages (Kling et al., 2007a).

The effect of environmental variables of mosquito distribution.

The effect of tree density. I used tree density as the main variable characterizing this ecotone. With *Ae. triseriatus*, there is a clear and significant positive effect of tree density on *Ae. triseriatus* abundance. Furthermore, the effect of tree density differs among the different sub habitats, with strongest effect exhibited in the outer field habitat. This effect makes ecological sense because in the least hospitable habitat the dependency of the sylvatic *Ae. triseriatus* on tree cover is expected to be the strongest. In a similar way, with *Ae. japonicus*, the effect of tree density is consistent with the hump-shaped distribution of this species. In the inner forest, the effect of tree cover is not significant and even tends to be negative. It then becomes slightly positive in the forest margin, edge, and field margin. However, as with *Ae. triseriatus*, the effect of tree density is the strongest in the outer field habitat, which is the least favorable habitat for this species. With *Ae. albopictus*, the effect of tree density is exactly opposite, with a general negative effect of tree density on *Ae. albopictus* abundance when measured across the ecotone. Yet, in the outer field habitat, as with the other species, tree density had a positive effect.

The effect of tree size (DBH). Tree DBH is often thought to be correlated with the occurrence of tree holes (Bennett et al., 1994; Blakely and Didham, 2008; Fan et al., 2011) and therefore, we expected it to be positively correlated with mosquito abundance. Overall, for *Ae. triseriatus* our results were consistent with this hypothesis, with a positive effect in all four habitats except for the edge habitat. With *Ae. japonicus,* the effect of DBH varied more substantially across the ecotonal habitats with no effect or slightly negative effects in the forest margins and the edge habitats and a positive effect in the inner forest, outer field, and the field margins habitats. The cause for this habitat-specific effect on both species is not clear and requires further study. Another interesting phenomenon that was observed with both *Ae. triseriatus* and *Ae. japonicus* was a significant “DBH-by-tree density” interaction, suggesting that the effect of tree density differ among sites characterized by large versus small trees. The cause of this phenomenon is also unclear and requires further study. As with the other mosquito species, the effect of DBH on *Ae. albopictus* was overall positive but this effect also differed among the habitats with negative effect at the inner forest and positive at all other habitats and a particularly strong at the forest margin habitat. As with the other two mosquito species, here we also observed a significant DBH x tree density interaction suggesting a differential effect of tree density for areas with trees of different sizes.

With resting adult mosquitoes, relatively few specimens were collected and none of them were identifiable to the species level. The majority of them were collected in the forest habitat, which would suggest that the majority of these were *Ae. triseriatus*.

Host seeking behavior and body size patterns along the ecotone

Based on CO_2 baited BG sentinel trapping, only *Ae. triseriatus* and *Ae. japonicus* were collected. Their distribution is consistent with the oviposition activity distribution, although here their distribution appears to be much more skewed towards their preferred habitat. *Aedes triseriatus* was trapped mainly at the forest habitat, much less in the edge habitat, and was completely absent from the field habitat. *Aedes japonicus* host seeking activity also was, on the other hand, highly biased towards the edge habitat, with much fewer collected in the forest and field habitats. This result suggest that human LACV exposure risk is highly habitat specific, with highest risk at the forest habitat were *Ae. triseriatus* is more prevalent and were it mostly forages. Yet, in the edge habitat where most human activity typically takes place, *Ae. japonicus* appears to most commonly be found and given the fact that it is at least as competent (Paulson et al., 1989) or even more LACV competent (Bara et al., 2016) than *Ae. triseriatus* it is suggested that exposure risk might also be high in that habitat. Based on wing size analysis of the mosquitoes collected using this method, no difference was found for *Ae. triseriatus*. For *Ae. japonicus*, however, mosquitoes collected at the forest habitat tended to be the largest, followed by the edge habitat, with smallest mosquitoes collected in the field habitat. This may suggest that in terms of fitness, optimal conditions for larval growth may occur in the forest habitat, but due to *Ae. triseriatus* dominance in the forest habitat, *Ae japonicus* might be pushed to mainly utilizing the edge habitat (Ingrassia, 2007). The smallest sized *Ae japonicus* individuals were collected at the field habitat which is consistent with its lowest affinity to this habitat.

Conclusion

The forest-to-field ecotone, as a typical landscape characteristic of the local landscape, appears to be characterized by a fairly "hard edge" (Farina, 2006b; Riser, 1995; Walker et al., 2003) with a fairly abrupt transition from the forest to the field. Yet, probably due to the age of this disturbance, a lot of undergrowth in the form of shrubs had the time to develop at or close to the edge and possibly somewhat buffer this transition. Indeed, in terms of mosquito distribution, all three mosquitoes occurred in all three habitats albeit with unique and distinct habitat affinities. These results suggest that the current prevalent landscape mosaic characterized by forest and field patches, and with an edge habitat connecting them is consistent with the distribution of the three LACV competent Aedine species: with *Ae. triseriatus* being potentially the key vector in forest patches, *Ae. albopictus* in field patches, and *Ae. japonicus* the edge habitat. This means that currently there are no habitats that are relatively "safe" from LACV risk and the public should adjust their protective behavior accordingly.

CHAPTER III

THE EFFECT OF LARVAL HABITAT SUPPLEMENTATION (I.E., ARTIFICIAL CONTAINERS) ON THE ECOLOGY OF LACV VECTORS ALONG FOREST-TO-FIELD ECOTONES

Introduction

Human environmental change is a driving force in the emergence of zoonotic diseases (Daszak et al., 2001b; Jones et al., 2008a; Myers and Patz, 2009a; Patz et al., 2004). As described by Pavlosky (1966), the natural nidality of disease (i.e., Disease Niche) encompasses specific abiotic and biotic conditions (e.g., climate, vegetation, soil) where host, vector and pathogen interact (Pavlosky, 1966). Therefore, natural and anthropogenic factors may reduce or enhance disease risk.

The resurgence of vector-borne diseases is considered a global health problem driven in large part by anthropogenic factors such as human-induced habitat changes (Gubler, 1998b; Harrus and Baneth, 2005; Jones et al., 2008a). The availability and abundance of natural (e.g., tree holes, rock pools) or artificial containers has been shown in many studies to be strongly associated with the abundance of container-inhabiting mosquitoes (Andreadis, 1988; Joy and Sullivan, 2005b; Kaufman et al., 2005; Kling et al., 2007b; Lampman et al., 1997b; McMahon et al., 2008; Qualls and Mullen, 2006; Yee, 2008). It is reasonable, therefore, to assume that the availability of containers as mosquito ovipositing sites are a major factor limiting these mosquitoes' abundance and distribution.

Discarded tires tend to collect rainwater, which remains shaded enabling water to remain available for an extended time and accumulate organic matter in the form of leaf detritus. Hence, used tires provide an optimal larval habitat (Yee, 2008). La Crosse virus (LACV) vectors *Ae. triseriatus* and *Ae. albopictus* are commonly the most abundant and often collected mosquitoes from tires in North-Central and Southern USA, respectively (Yee, 2008). The degree of shade has been described as a particularly important factor affecting the community composition of tires (Yee, 2008). Other variables such as leaf detritus, tire orientation, and proximity to peri-domestic environments have also been found to affect larval communities (Kling et al., 2007b; McMahon et al., 2008; Yee, 2008). Nevertheless, these were all short-termed observational studies monitoring mosquito populations in sites where tires pre-existed (e.g., tire dumps) and comparing mosquito abundance to sites where tires were absent. Surprisingly, with the exception of Ho et al. (1989)'s *Aedes spp.* competition study (Ho et al., 1989), to our knowledge no controlled study on the effect of larval habitat supplementation on mosquitoes has been performed. Furthermore, the habitat context (e.g., forest-to-field ecotones) of this effect is poorly understood.

In this work, we conducted a long-term (4 years) experimental study considering the effect of larval habitat supplementation (i.e., artificial container introduction) on the abundance, distribution, and community structure of LACV mosquitoes. Our general hypothesis, in this context, is that larval habitat supplementation should enhance mosquito abundance within the habitat with supplementation (i.e., intra-habitat) and, possibly, beyond the habitat (i.e., inter-habitat). This should have significant implications on mosquito community structure due to its effect on the interactions between native and invasive species (Yee, 2008).

The specific impacts of contemporary human-induced environmental changes on La Crosse Encephalitis (LACE) disease risk remains unclear. Similarly, environmental manipulations are uncommon in the experimental study of disease ecology. Here we apply an ecological experimentation (i.e., larval habitat supplementation along forest-to-field ecotones) to test the effects of human environmental change on a disease system by comparing species-specific abundance and distribution, as well as differences in community structure and fitness across experimental treatments and ecotones.

Methods

Overall strategy. In this 4-year study, the first year constituted a baseline to evaluate the habitat effect prior to tire introduction (*Chapter II*). We then monitored changes in vector mosquito community in the subsequent three years, following supplementation of larval habitats in the summer of year 2 (i.e., June 27th, 2012). One site was incorporated between years 1 and 2. Therefore, in order to the test for differences between baseline and treatment period as well as control and treatment sites, the baseline period in this analysis will only correspond to the months of May and June 2012, it will not include Year 1 (i.e., 2011) data.

Supplementation of larval habitats. Following the one-year period of baseline sampling (i.e., “pre-manipulation”), 9 tires were introduced to either the forest section (2 replicate sites) or the field section of the plot (2 replicate sites) (Fig. 3.1). Two sites did not receive the tire treatment and, thus, served as control sites.

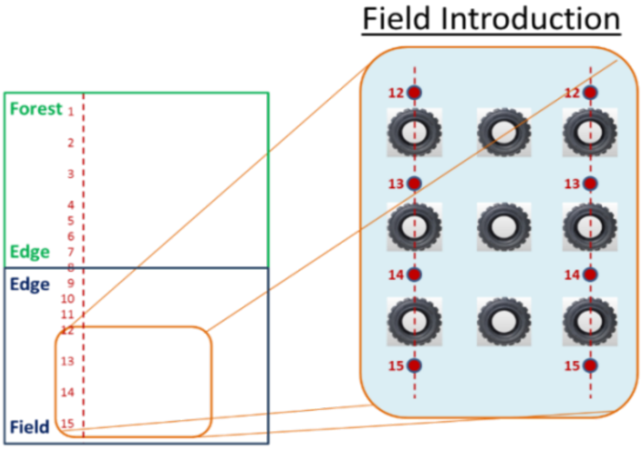

Figure 3.1. Example of tire introduction in field habitat. Numbers 1-15 refer to oviposition traps (i.e., ovitraps) along the transect.

Within the study plots, we assessed relative mosquito abundance using oviposition traps, resting adult abundance using Nasci aspirator sampling and host-seeking adults using the 'human landing catch' aspiration technique.

Table 3.1. Study design table, includes all sampling procedures.

	Control	Forest tire introduction	Field tire introduction
Plots	AB and GH	CD and EF	IJ and KL
Baseline (2012)	**Egg collections:** 2012/05/01, 2012/05/09, 2012/05/16, 2012/05/30, 2012/06/06, 2012/06/13, 2012/06/20, 2012/06/27		
	Resting adult collections: 2012/06/24 (GH), 2012/06/26 (AB),	**Resting adult collections**: 2012/06/24 (EF), 2012/06/26 (CD),	**Resting adult collections**: 2012/06/24 (KL), 2012/06/26 (IJ),
Treatment year 1 (2012)	**Egg collections:** 2012/07/22, 2012/07/29, 2012/08/05, 2012/08/12, 2012/08/19, 2012/08/26		
	Resting adult collections: 2012/08/01 (GH), 2012/08/08 (AB), 2012/09/09	**Resting adult collections:** 2012/08/01 (EF), 2012/08/08 (CD), 2012/09/09	**Resting adult collections:** 2012/08/01 (KL), 2012/08/08 (IJ), 2012/09/09
Treatment year 2 (2013)	**Egg collections:** 2013/06/17, 2013/07/13, 2013/08/17, 2013/09/14		
	Larval tire collection: 2013/06/17, 2013/07/13, 2013/08/11, 2013/09/08		
	Resting adult collections: 2013/06/23, 2013/07/12, 2013/08/11, 2013/09/09		
Treatment year 3 (2014)	**Egg collections:** 2014/05/29, 2014/08/13. 2014/09/17		
	Larval tire collections: 2014/06/27, 2014/08/28, 2014/09/24		
	Resting adult collections: 2014/06/07 (GH), 2014/06/08 (AB), 2014/07/01 (AB), 2014/07/02 (GH), 2014/07/15 (GH), 2014/07/16 (AB) 2014/08/21, 2014/09/10 (GH), 2014/09/17 (AB)	**Resting adult collections:** 2014/06/07 (EF), 2014/06/08 (CD), 2014/07/01 (CD), 2014/07/02 (EF), 2014/07/15 (EF), 2014/07/16 (CD) 2014/08/21, 2014/09/10 (EF), 2014/09/17 (CD)	**Resting adult collections:** 2014/06/07 (KL), 2014/06/08 (IJ), 2014/07/01 (IJ), 2014/07/02 (KL), 2014/07/15 (KL), 2014/07/16 (IJ) 2014/08/21, 2014/09/10 (KL), 2014/09/17 (IJ)
	Human Landing Catch collections: 2014/08/17, 2014/09/20, 2014/09/21	**Human Landing Catch collections:** 2014/08/04 (EF), 2014/08/05 (EF), 2014/08/16 (CD), 2014/08/30 (EF), 2014/09/19 (CD), 2014/09/20 (CD), 2014/09/21 (CD)	**Human Landing Catch collections:** 2014/08/04 (KL), 2014/08/05 (KL), 2014/08/16 (IJ), 2014/08/30 (KL), 2014/09/19 (IJ), 2014/09/20 (IJ), 2014/09/21 (IJ)

Egg collections using oviposition traps. LACV vectors' eggs were collected using oviposition traps (i.e., ovitraps) placed on two parallel transects (15 per transect) along the ecotone, for each of the 6 sites. Oviposition strips were deployed for 7 days each sampling session before collection. These were then taken to our laboratory to determine egg counts and rear adults for identification. A total of 8 sampling sessions were conducted prior to larval habitat supplementation.

Resting adult collections using the Nasci aspirator. A Nasci aspirator was used to sample resting mosquitoes in vegetation (Nasci, 1981b). Each site was divided into a total of five areas, two in the field, the edge, and two in the forest. The aspirator was used in a standardized manner for 15 minutes per area. Catchments for the field habitats (Field I & Field II) and forest habitats (Forest I & Forest II) were processed separately. However, the field habitat collections as well as the forest collections were averaged for data analysis. Three sampling sessions took place in 2012, one before container introduction (06/24/2012) and two after (08/01/2012 and 09/09/2012). In addition, four sampling sessions were conducted in 2013 and six in 2014.

Host-Seeking mosquito collections by the Human Landing Catch (HLC) method. The host-seeking behavior of LACV vectors was assessed by sampling mosquitoes landing on 2 adult human volunteers between August 4th and September 21st.

All adult collections were identified based on described morphological features at our laboratory using a dissecting microscope. Wings were then detached and sizes were determined using microscopy camera and software. In addition, parity dissections were performed on females with flat-abdomens to determine parity status (i.e., nulliparous or parous).

La Crosse virus screening. Adult collections (i.e., resting and host-seeking) were screened for La Crosse virus infection using methods described by Gerhardt et al (2001), Ksiazek and Yuill (1977), Kuno (1998) and Kuno et al (1996) (Gerhardt et al., 2001; Ksiazek and Yuill, 1977; Kuno, 1998; Kuno et al., 1996).

Data reductions and statistical analysis.

The species-specific egg abundance was inferred by multiplying the fraction of adult species emerging from each flooded ovistrip by the total number of eggs on that strip. Given the non-normal distribution of the eggs as count data, a negative binomial regression generalized linear model was used. We first tested the full model with interactions including ‘habitat’, ‘species’, and ‘treatment’ (i.e., container introduction), to test for significant differences between species and to evaluate if abundance is associated with habitat supplementation within (intra-) and beyond (inter-) treated habitat, even after controlling for ecotone habitat.

In addition, one-way ANOVA tests for difference in size of adult *Aedes spp.*, as measured by wing size, were conducted after testing and meeting normality assumptions. This analysis was employed on the resting adult and host-seeking collections. Moreover, a logistic regression model was used to test for the effect of habitat, treatment and time of collection (i.e., session) on parity status of resting and host-seeking adults.

Lastly, the Mann-Whitney U test (non-parametric) was used to test for significant differences between larvae abundance in forest and field habitat introduced containers (i.e., supplemented larval habitats). Larvae counts in these containers had a non-normal distribution, thus, this rank-based test was used.

Results

Baseline stage

I. Oviposition activity

General.

Table 3.2. Total number of eggs collected and distribution of species between habitats during baseline period.

	Ae. triseriatus	*Ae. japonicus*	*Ae. albopictus*	Total eggs collected
Forest	61.4%	40.9%	100.0%	53,136
Edge	35.1%	40.0%	0%	31,282
Field	3.4%	19.1%	0%	3,875
Total eggs collected	82,930	5,352	10	88,293

Overall oviposition activity. In the baseline stage (that comprised 8 trapping sessions in May and June 2012) we wanted to confirm that the study sites do not differ from one another. We therefore tested for difference in oviposition activity, using NB regression, among sites designated to be control sites and the sites designated to be treatment sites: 'forest tire introduction' and 'field tire introduction' sites. This was done for the entire plot and also for each of the three main ecotonal habitats: forest, edge, and field. No significant difference was found between the plots, neither at the entire plot scale (Fig. 3.2A) or the habitat specific scale for the 'forest' and 'edge' habitats (Fig. 3.2B,C). In the field sites, the sites designated to the ‘control’ sites actually had marginally significantly higher oviposition activity (Fig. 3.2D).

Species specific oviposition activity

Aedes triseriatus. No significant difference was found among the plots at the entire plot scale (Fig. 3.3A). Similarly, no significant difference was found among the plots at the ‘forest’ and ‘edge’ habitats (Fig. 3.3B,C). In the field sites, the sites designated to the ‘control’ sites actually had marginally significantly higher oviposition activity (Fig. 3.3D).

Aedes japonicus. Significant differences were found among the plots at the entire plot scale, with highest oviposition activity actually occurring in the plots designated to be the ‘control’ plots (Fig. 3.4A). Similarly, a marginally significant difference was found at the ‘forest’ habitat for plots designated to be ‘control’ plots (Fig. 3.4B). No significant differences were found in the other habitats (Fig. 3.4C,D).

Aedes albopictus. Very few *Ae. albopictus* were found during this baseline period and hence no inference can be made regarding pre-existing differences among plots (Fig. 3.5).

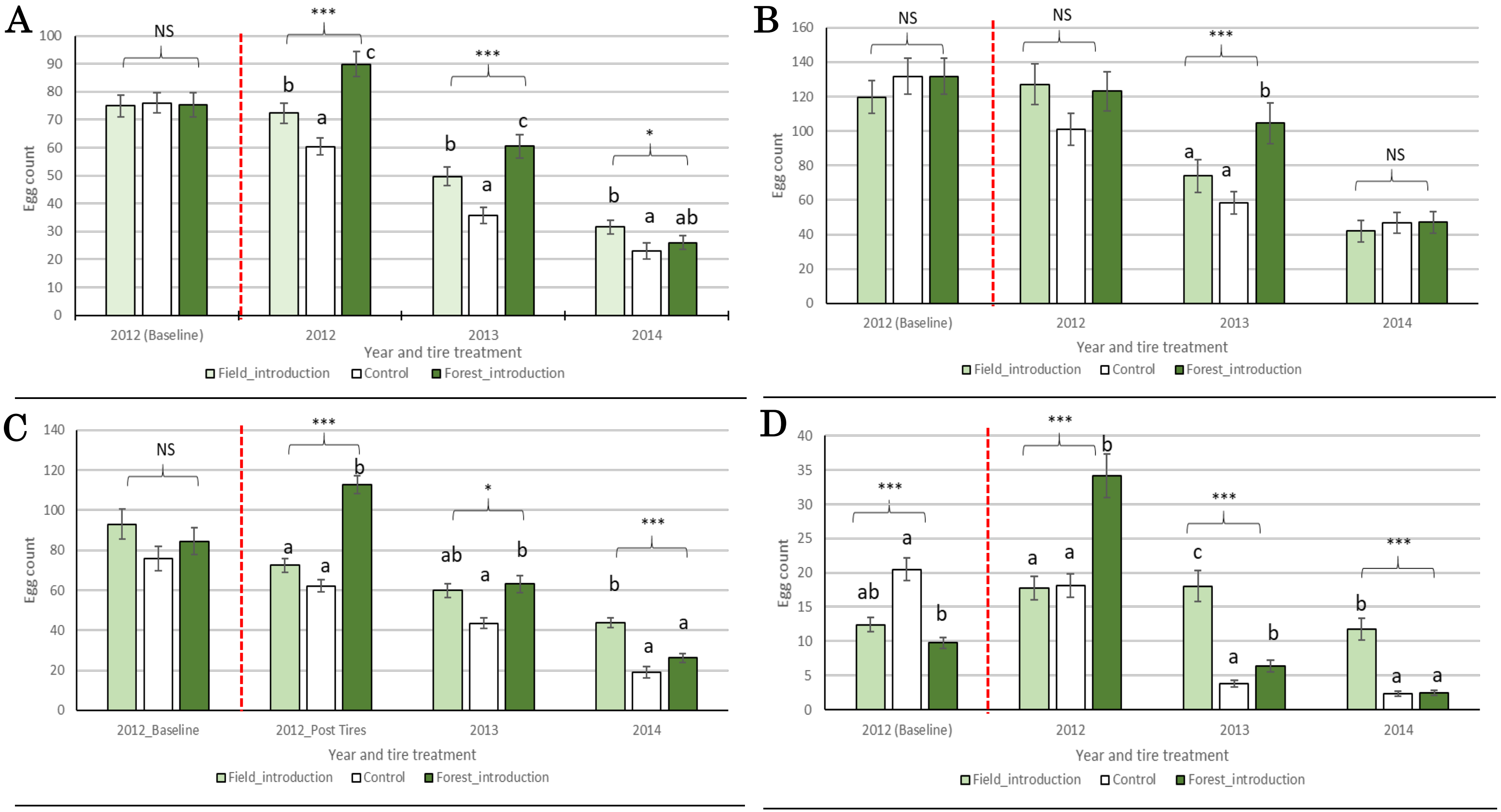

Figure 3.2. *Aedes spp.* oviposition along ecotones before/after tire introduction. Bars=SE; *=p<0.05; **=p<0.01; ***=p<0.001. Letters denote significance based on p<0.05 for Post-hoc comparisons with Bonferroni corrections.

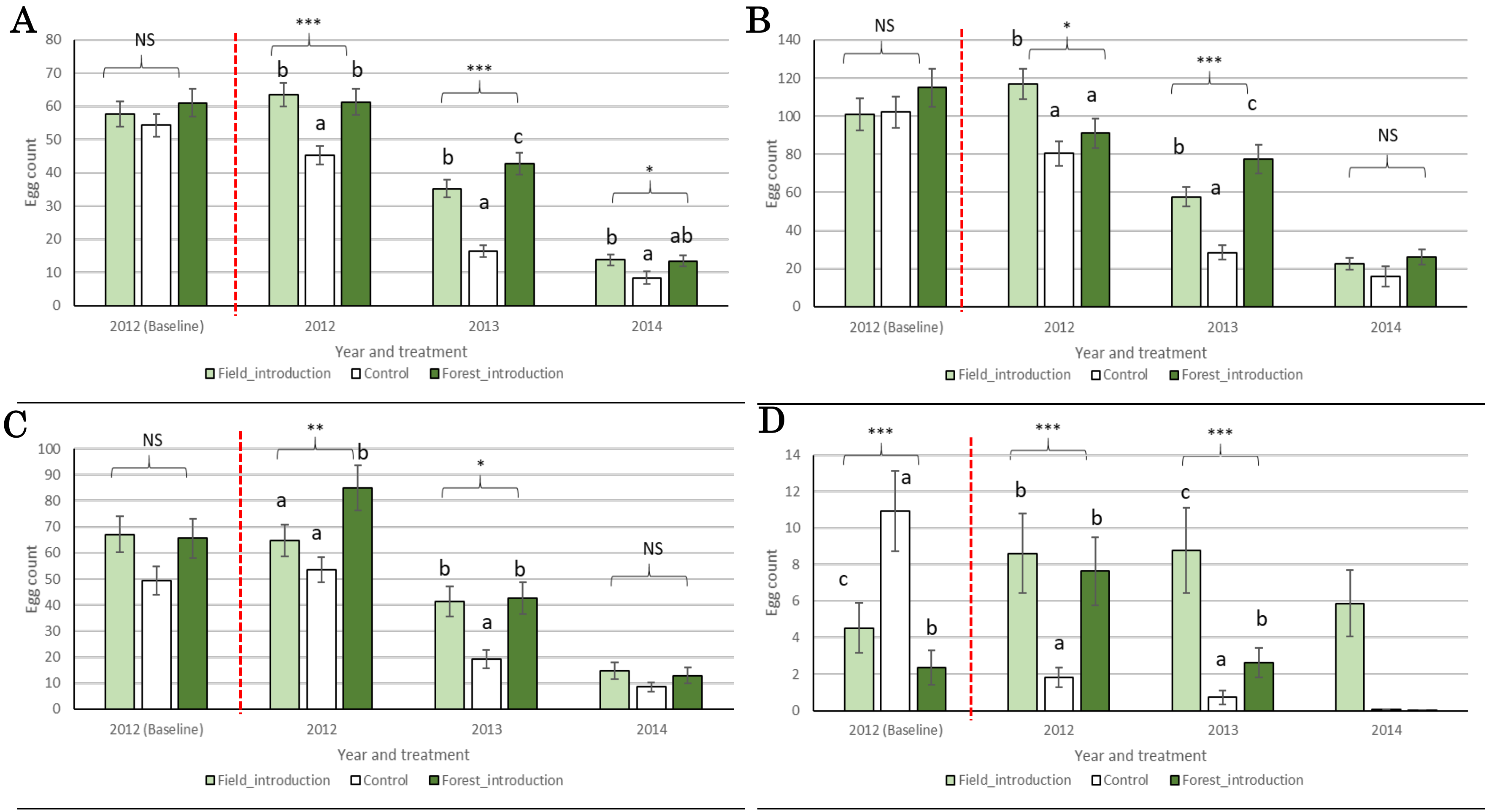

Figure 3.3. *Aedes triseriatus* oviposition along ecotones before/after tire introduction. Bars=SE; *=p<0.05; **=p<0.01; ***=p<0.001. Letters denote significance based on p<0.05 for Post-hoc comparisons with Bonferroni corrections.

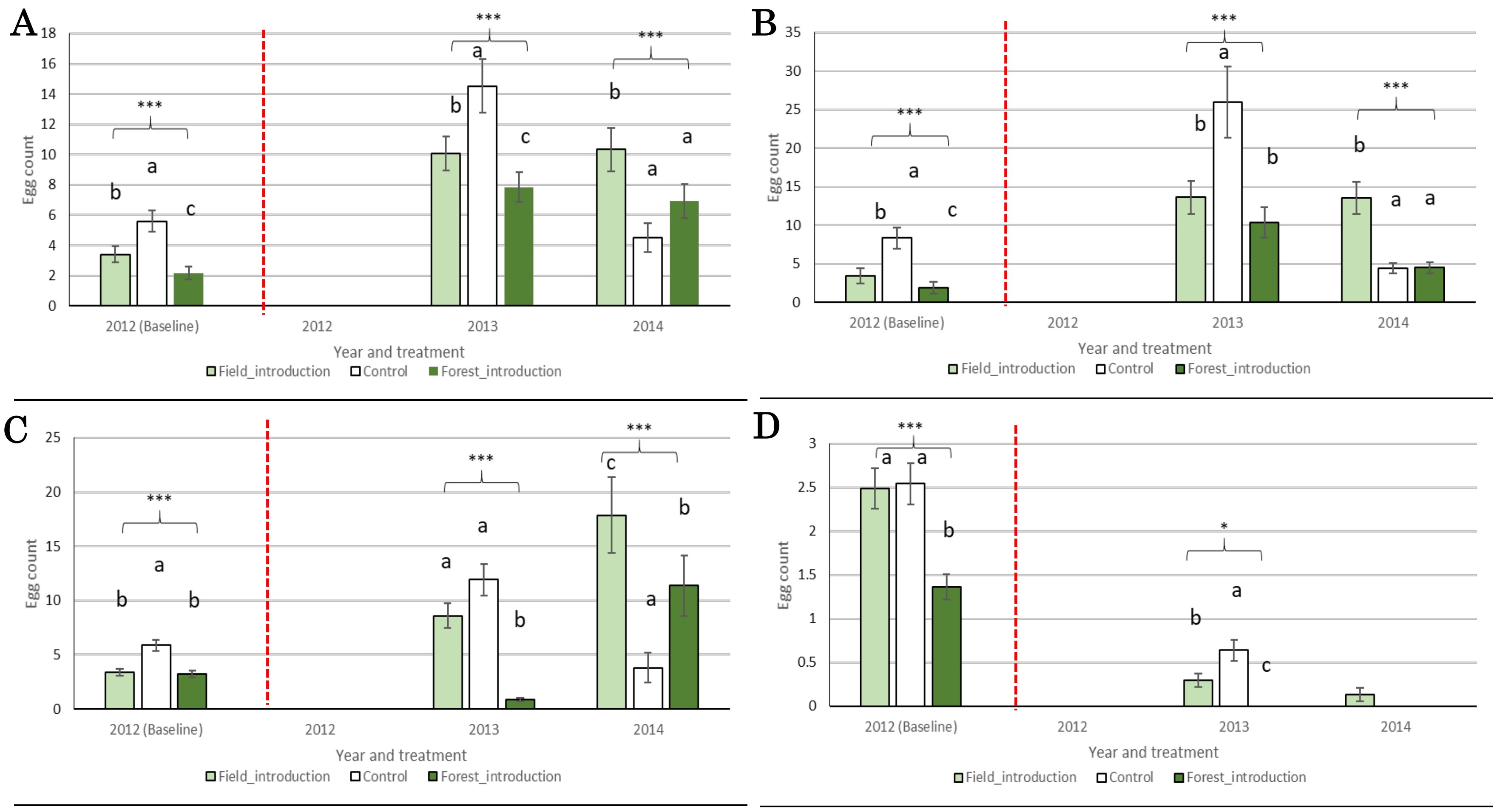

Figure 3.4. *Aedes japonicus* oviposition along ecotones before/after tire introduction. Bars=SE; *=p<0.05; **=p<0.01; ***=p<0.001. Letters denote significance based on p<0.05 for Post-hoc comparisons with Bonferroni corrections.

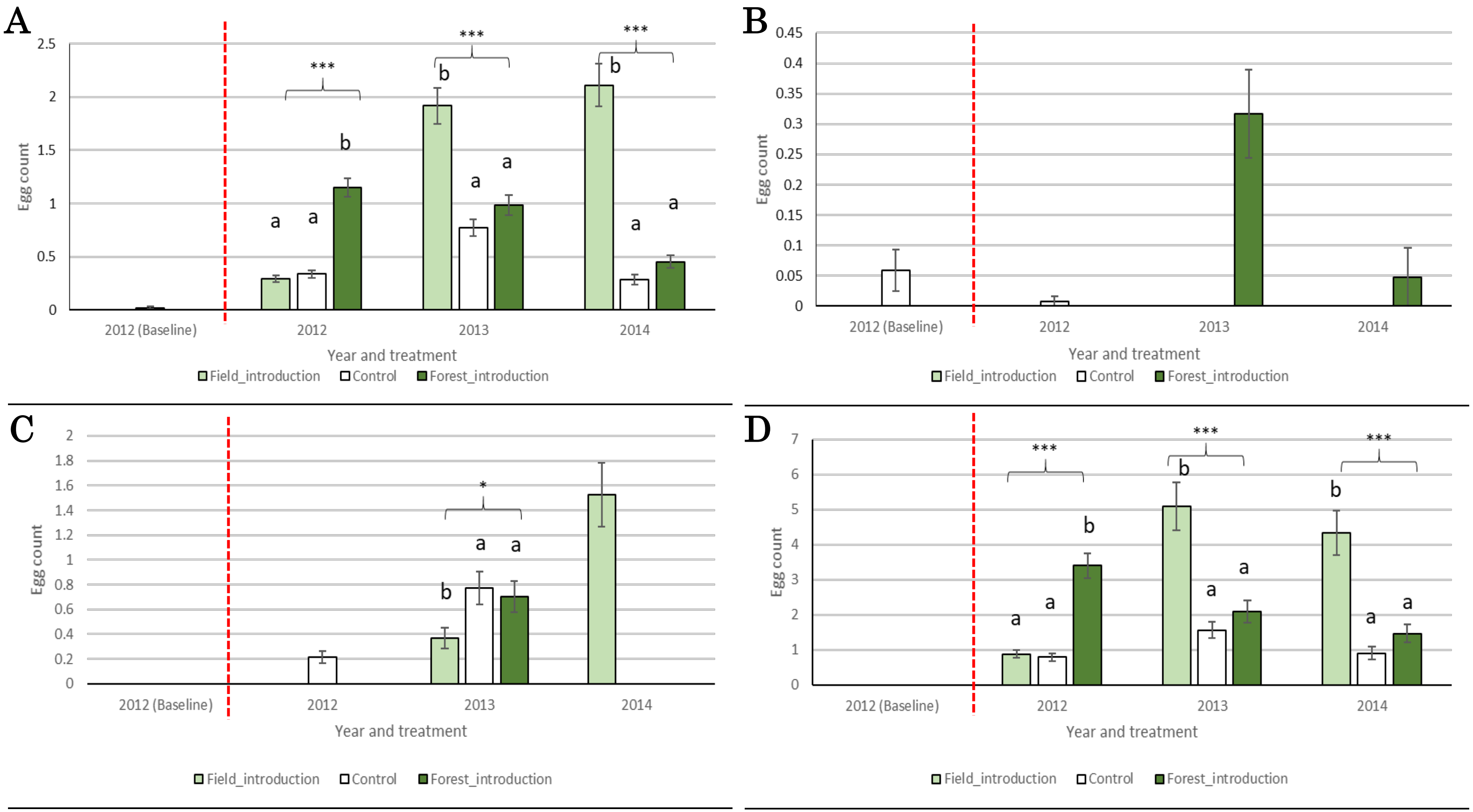

Figure 3.5. *Aedes albopictus* oviposition along ecotones before/after tire introduction. Bars=SE; *=p<0.05; **=p<0.01; ***=p<0.001. Letters denote significance based on p<0.05 for Post-hoc comparisons with Bonferroni corrections.

Resting adult mosquitoes (Nasci trapping)

Overall resting adult distribution. No significant differences were found in the baseline period between species (W = 3.409, df = 2, P = 0.182) or treatment plots (W = 3.905, df = 2, P = 0.142). The number of resting adults collected during this period was fairly small (Table 3.3).

Table 3.3. Total number of resting *Aedes spp.* adults collected and distribution of species between habitats during baseline period.

	Ae. triseriatus	*Ae. japonicus*	*Ae. albopictus*	Total adults collected
Forest	100%	100%	0%	15
Edge	0%	0%	100%	2
Field	0%	0%	0%	0
Total adults collected	6	9	2	17

Aedes triseriatus. No significant difference (W = 0.797, df = 2, P = 0.671) was found among the plots at the entire plot scale although there was a trend of most mosquitoes caught in plots designated to be ‘field tire introduction’ and least in plots designated to be ‘forest tire introduction’ (Fig. 3.6A). Similar pattern was observed in the forest habitat (Fig. 3.6B). No *Ae. triseriatus* adults were trapped in the other habitats during this baseline period (Fig. 3.6C,D).

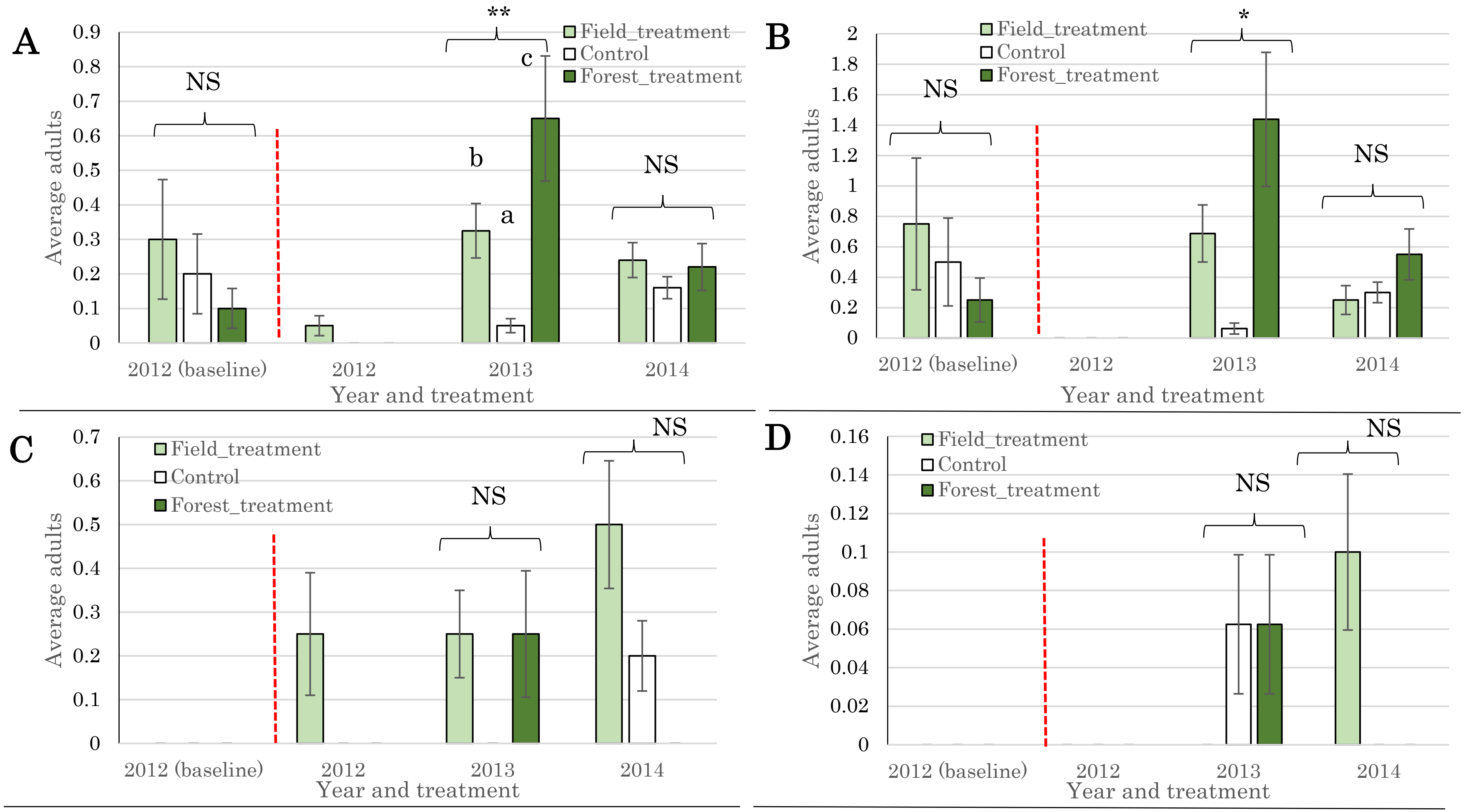

Figure 3.6. *Aedes triseriatus* resting adult (Nasci) collections. A. Overall; B. Forest habitat; C. Edge habitat; D. Field habitat. Bars=SE; *=p<0.05; **=p<0.01;. Letters denote significance based on p<0.05 for Post-hoc comparisons with Bonferroni corrections.

Aedes japonicus. No significant difference (W = 1.891, df = 2, P = 0.388) was found among the plots at the entire plot scale although there was a trend of most mosquitoes caught in plots designated to be 'field tire introduction' and least in plots designated to be 'control' plots (Fig. 3.7A). This pattern is driven by *Ae. japonicus'* distribution in the forest habitat because none were trapped in any of the other habitats (Fig. 3.7).

Aedes albopictus. At the baseline stage *Ae. albopictus* adults were caught only in the edge habitat and in this case only in plots designated to be 'field tire introduction' (Fig. 3.8).

Summary.

All in all, based on both oviposition activity data and resting adult distribution the pre-treatment baseline distribution of all three mosquito species does not seem to differ among plots designated to receive tire introduction in the forest, the field, or the controls. In other words, there does not seem to be any evidence for pre-existing differences among the plots that might bias our inference regarding the effect of the tire introduction treatments.

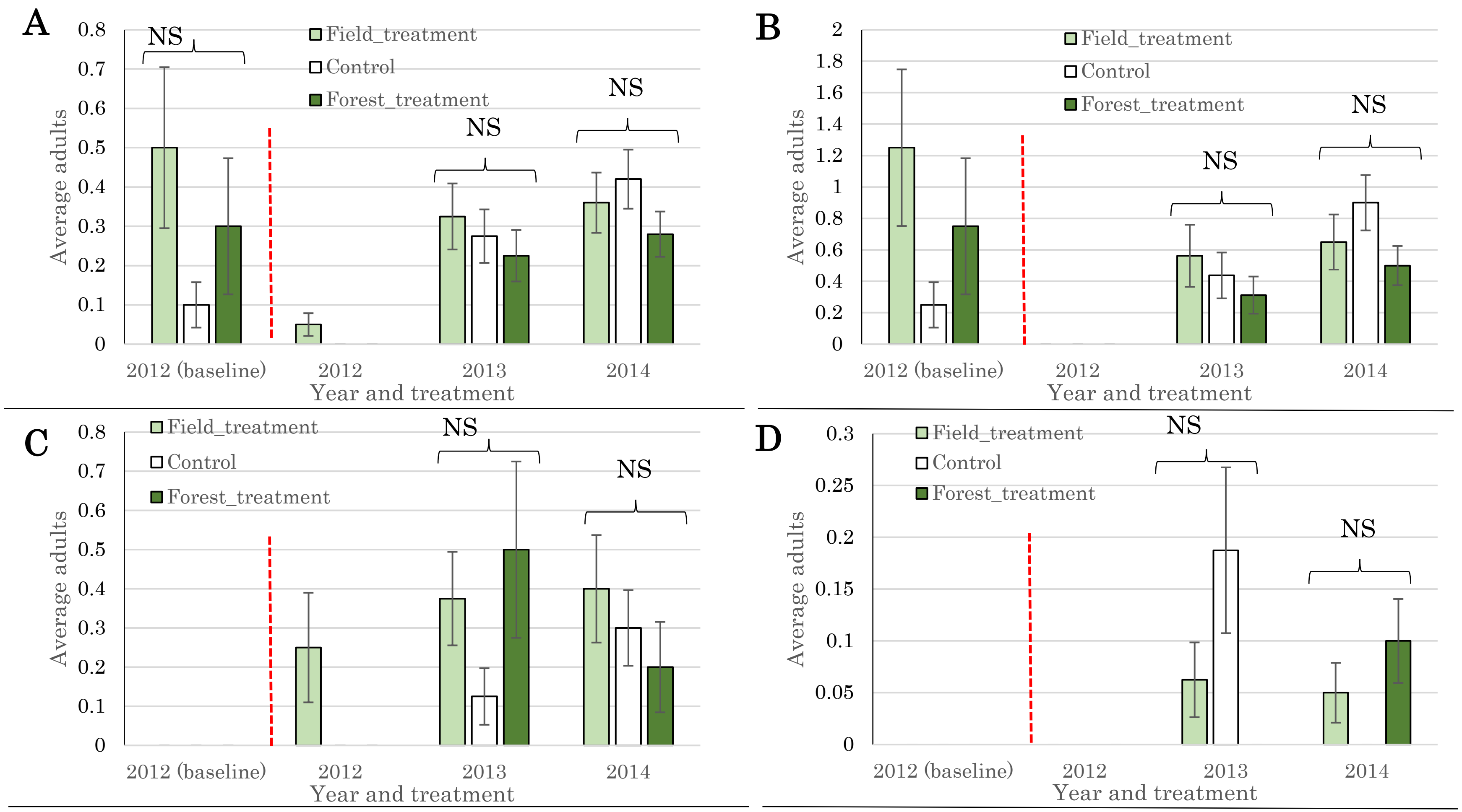

Figure 3.7. *Aedes japonicus* resting adult (Nasci) collections. A. Overall; B. Forest habitat; C. Edge habitat; D. Field habitat. Bars=SE; *=p<0.05; **=p<0.01. Letters denote significance based on p<0.05 for Post-hoc comparisons with Bonferroni corrections.

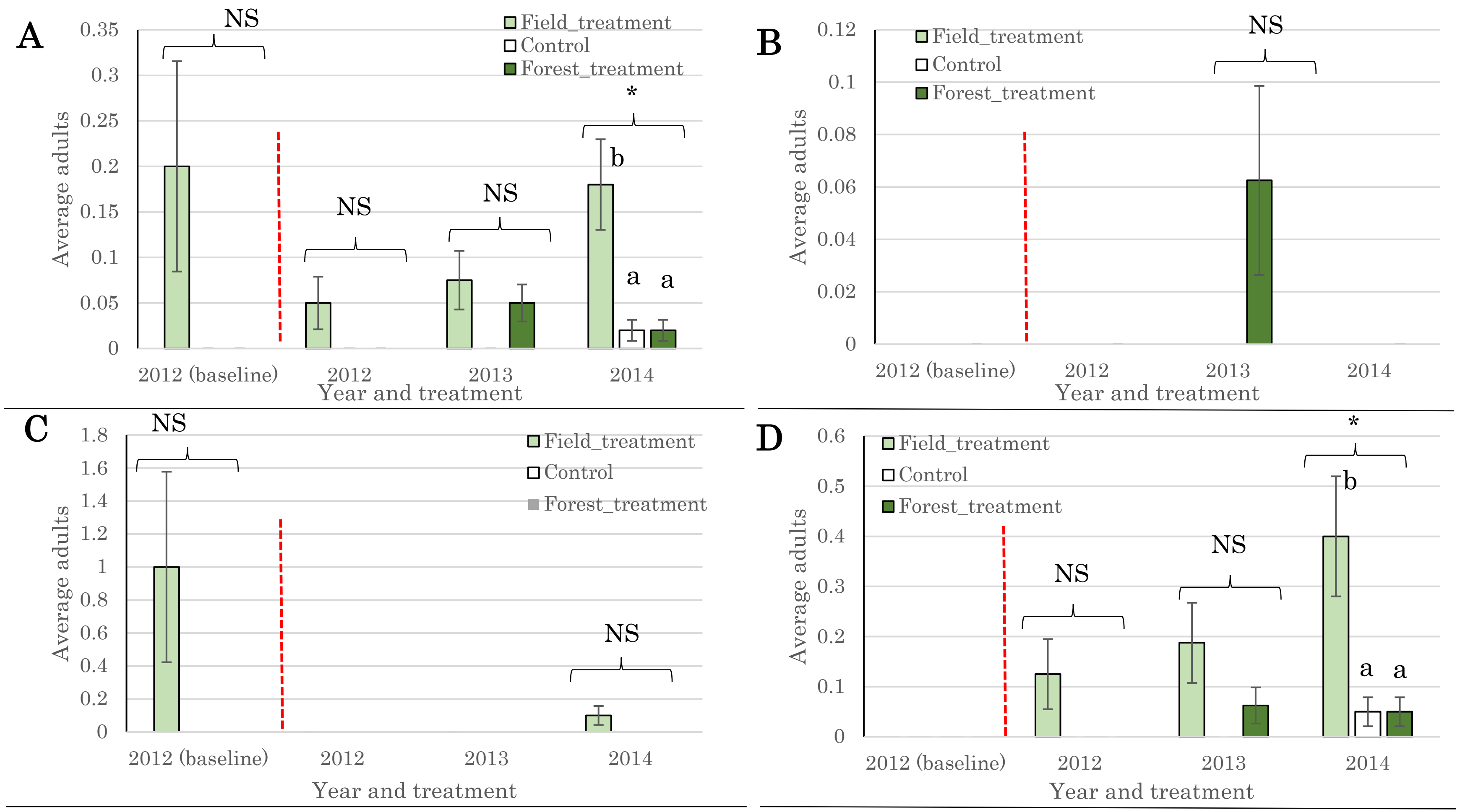

Figure 3.8. *Aedes albopictus* resting adult (Nasi) collections. A. Overall; B. Forest habitat; C. Edge habitat; D. Field habitat. Bars=SE; *=p<0.05; **=p<0.01. Letters denote significance based on p<0.05 for Post-hoc comparisons with Bonferroni corrections.

Experimental stage

Oviposition activity

Table 3.4. Four-way negative binomial model depicting the effects of Species, Habitat, Treatment, Year and their respective interactions on oviposition activity. This saturated model was the best models (∆AIC=179 compared to closest model without a 4-way interaction).

	Wald Chi-square	**df**	**P**
Intercept	367.521	1	<0.001
Species	778.199	2	<0.001
Habitat	369.841	2	<0.001
Tires	52.544	2	<0.001
Year	98.736	2	<0.001
Species x Habitat	877.875	4	<0.001
Species x Tires	168.818	4	<0.001
Species x Year	226.159	3	<0.001
Habitat x Tires	73.462	4	<0.001
Habitat x Year	31.758	4	<0.001
Tires x Year	101.199	4	<0.001
Species x Habitat x Tires	35.564	5	<0.001
Species x Habitat x Year	57.882	4	<0.001
Species x Tires x Year	140.755	4	<0.001
Habitat x Tires x Year	79.432	6	<0.001
Species x Habitat x Tires x Year	12.943	2	0.002

AIC = 24741.133

A total of 125,988 eggs were collected during the experimental stage (July 2012-September 2014). A clear effect of ‘species’ was detected with *Ae. triseriatus* being, clearly, the dominant species (39.0±1.8), followed by *Ae. japonicus* (4.8±0.5), and *Ae. albopictus* (1.2±0.2) (Table 3.4). Also, we found a significant effect of ‘habitat’ with highest number of eggs laid in the forest habitat (90.1±4.0), followed by the edge (62.0±3.7), and the field (15.3±1.3) habitats (Table 3.4). Furthermore, as indicated by the 'species x habitat' interaction with *Ae. triseriatus* dominant in the forest habitat (67.3±3.7) followed by the edge (44.8±3.4) and field (4.5±1.0) habitats, *Ae. japonicus* also dominant in the forest (7.3±1.2) followed by the edge habitat (6.9±1.0) and field (0.2±0.1) habitats, and *Ae. albopictus* most common in the field habitat (2.6±0.5) followed by the edge (0.8±0.4) and forest (0.2±0.1) habitats. 'Year' also had a significant effect, with 2012 being the most productive year (19.14±1.15), followed by 2013 (14.67±1.07), and 2014 (6.69±0.73). Most important, though, was the overall significant effect of treatment (Table 3.4). In contrast with the baseline period where the plots did not differ from one another, following tire introduction, oviposition activity increased significantly at the tire-introduction plots in comparison with the control plots. This effect was the strongest at the field tire addition' plots (17.0±1.2), followed by the 'forest tire addition' plots (16.7±1.3), 'with lowest overall ovipositon activity in the control plots (11.4±0.9) (Fig. 3.2A). As the significant 'treatment x year' indicates, the effect of the treatment differed between the years, with strong and significant effects for both the forest tire-introduction and the field tire-introduction in years 2012 and 2013 and only a significant field tire-introduction effect in 2014 (Fig.

3.2). Furthermore, as indicated by the significant ‘habitat x treatment’ interaction (Table 3.4), the effect of the treatment differed among the habitats. The effect of ‘treatment’ was significant in the forest (W = 6.96, df = 2, P = 0.031) and the field (W = 6.96, df = 2, P = 0.031) habitats, and marginally significant in the edge habitat (W = 4.461, df = 2, P = 0.092). In the forest (in comparison with the control plots), the within-habitat effect of tire addition was significant (99.85±7.25 vs 75.39±6.43, respectively; P = 0.012). But surprisingly, also the between-habitat effect of tire introduction to the field had a significant effect on oviposition activity in the forest habitat (95.56±7.25 vs 75.39±6.43, respectively; P = 0.034) (Fig. 3.2B). The effect of within-habitat tire introduction was more pronounced in year 2 of the experiment, but the effect of tire introduction to either habitat disappeared in year 3 of the experiment (Fig. 3.2B). In the edge habitat (in comparison with the control), the effect of tire introduction was significant only for plots where tires were introduced to the forest (49.23±7.61 vs 31.73±4.27, respectively; P = 0.032) but not for plots where tires were introduced to the field (41.04±5.29 vs 31.73±4.27, respectively; P = 0.168) (Fig.3. 2C). This effect was most pronounced in the first year (2012) but then disappeared in years two and three (Fig. 3.2C). In the field habitat, the surprising effect was that, in the first year, the between-habitat effect of tire introduction to the forest outweighed the within-habitat effect of tire introduction to the field (Fig. 3.2D). But then, in the subsequent years, the local effect predominated (Fig. 3.2D).

Species-specific effects

Given the significant statistical interactions of ‘species’ with ,’treatment’, ‘habitat’, and ‘year’ (Table 3.4), I analyzed the effects of these variables for each species separately.

Aedes triseriatus. The overall effect of tire addition was highly significant (Table 3.5). In all three years of the experiment oviposition activity was higher in the treatment plots compared with the control plots, although this effect decreased slightly in the third year (Fig. 3.3A). The effect of the tire addition treatment was also habitat specific (Table 3.5). In the forest habitat, the effect of the treatment was significant ($W = 29.682$, $df = 2$, $P < 0.001$). The inter-habitat effect of tire introduction to the field was, in the first year, the strongest (Fig. 3.3B). In the second year, the within-habitat effect was stronger, and in the third year the effect of treatment was not significant (Fig. 3.3B). In the edge habitat, the effect of treatment was significant ($W = 23.391$, $df = 2$, $P < 0.001$) in years 1 and 2 but not in year 3 (Fig. 3.3C). The effect of tire introduction to the forest habitat had the greater effect in year 1 but then in years 2 and 3 the effect of tire introduction to the forest or the field was similar (Fig.

3.3C). In the field habitat, the plots designated to be the control plots had a pre-existing higher mosquito abundance. Yet, following the tire introduction these relations flipped completely (Fig. 3.3D). Specifically, there was a significant treatment effect in years 1, 2 , and 3, with a significant within- and between-habitats effects in year 1 and only a within-habitat effect in years 2 and 3 (Fig. 3.3D).

Table 3.5. Three-way negative binomial model testing the effects of Habitat, Treatment, and Year on oviposition *Aedes triseriatus* oviposition activity. This model was the best model (ΔAIC=212 compared to closest model without interaction).

	Wald Chi-square	**df**	**P**
Intercept	1048.055	1	<0.001
Habitat	2672.569	2	<0.001
Tires	289.399	2	<0.001
Habitat x Tires	215.766	2	<0.001
Year	1045.034	2	<0.001

AIC = 16532.672

Aedes japonicus. Significant effects of treatment, habitat, year, and treatment x habitat interactions were detected. In year 1, no *Ae. japonicus* were trapped. In the second year, the effect of the treatment was highly significant but in an opposite direction to what was expected: the control plots had significantly more mosquitoes than any of the treatment plots (Fig. 3.4A). In year 3, the effect of treatment was again significant, but the relations have flipped, with the control plots having lowest oviposition activity and the field tire introduction treatment having the largest impact

(Fig. 3.4A). The habitat specific effects are consistent. The negative effect of the treatment in year 2 is driven by the treatment's effect in the forest habitat (Fig. 3.4B). In the edge and field habitats the effect of treatment was not significant (Fig. 3.4C,D). In contrast, the positive effect of the treatment in year 3 was mainly driven by *Ae. japonicus'* response at the edge habitat, where tire introduction to the field had the larger effect (Fig. 3.4C).

Table 3.6. Three-way negative binomial model testing the effects of Habitat, Treatment, and Year on oviposition *Aedes japonicus* oviposition activity. This model was the best model among all competing models (ΔAIC=35 compared to closest model without interaction).

	Wald Chi-square	**df**	**P**
Intercept	1095.007	1	<0.001
Habitat	715.484	2	<0.001
Tires	265.096	2	<0.001
Habitat x Tires	71.683	3	<0.001
Year	1095.231	1	<0.001

AIC = 4921.264

Aedes albopictus. Here too a significant treatment and treatment x habitat interaction, and year were detected. In year 1, a strong effect of tire introduction to the forest was detected. Yet, in years 2 and 3, the tire introduction effect was mainly driven via tire introduction to the forest. (Fig. 3.5A). Aedes albopictus was very uncommon in the forest habitat. The positive 'forest-tire-introduction' effect described in year 1 was mainly exhibited at the field habitat, suggesting a strong inter-habitat effect (Fig.

3.5D). In 2013, the significant treatment effect at the edge habitat, was actually a negative effect with reduced oviposition activity in the treatment plots (Fig. 3.5C). Yet, in the field habitat a positive treatment effect was detected mainly due to a within-habitat effect of tire introduction to the field (Fig. 3.5D). Finally, in year 3, *Ae. albopictus* were detected only at the field habitat, where a significant effect of treatment was detected with oviposition activity highest in the field-tire-introduction plots (within-habitat effect) followed by a weaker marginally significant (P = 0.07) effect of forest-tire-introduction effect (Fig. 3.5D).

Table 3.7. Three-way negative binomial model testing the effects of Habitat, Treatment, and Year on oviposition *Aedes albopictus* oviposition activity. This model was the best model (ΔAIC=80 compared to closest model without interaction).

	Wald Chi-square	**df**	**P**
Intercept	50.165	1	<0.001
Habitat	501.628	2	<0.001
Tires	35.681	2	<0.001
Habitat x Tires	49.445	3	<0.001
Year	49.989	1	<0.001

AIC = 4017.029

II. Resting adult mosquito distribution

General. A total of *329* resting adult mosquitoes were collected. In contrast with the oviposition activity were *Ae. triseriatus* dominated (86.6%), here the relative abundance of it and of *Ae. japonicus* was similar, with the latter being slightly more abundant (Fig. 9). In addition we also caught *Aedes vexans*, *Culex spp.*, among others.

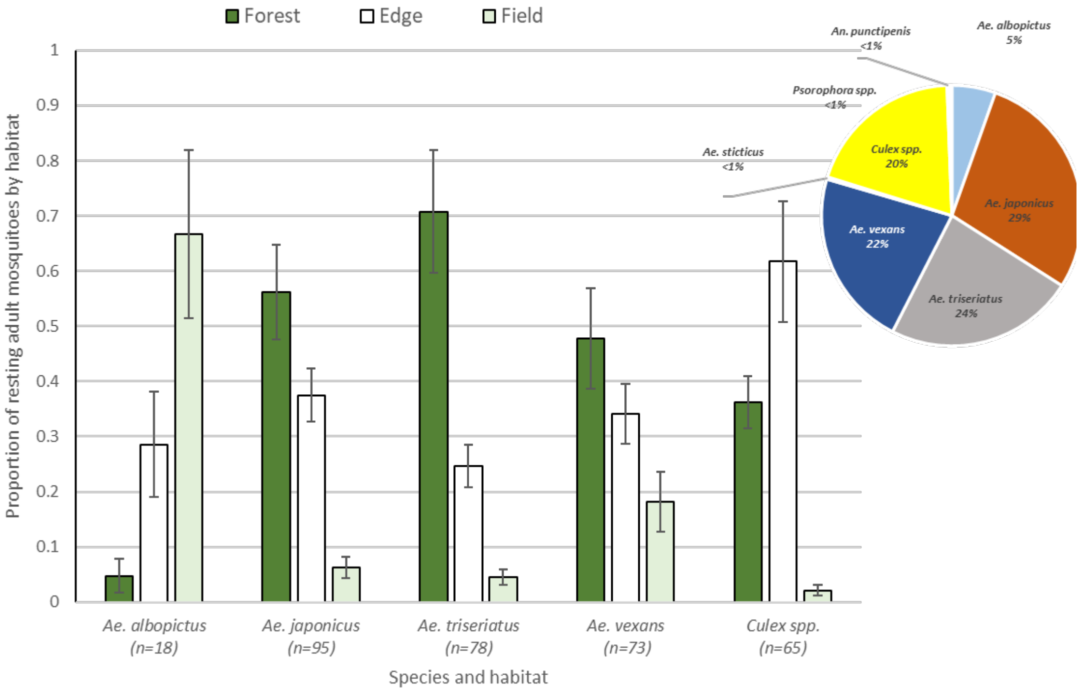

Figure 3.9. Proportion of each resting adult mosquito species by habitat (Nasci).

Table 3.8. Four-way negative binomial model testing the effects of Habitat, Treatment, Species, and Year on resting *Aedes spp.* adult abundance. This model was the best model (∆AIC=4 compared to closest model).

	Wald Chi-square	**df**	**P**
Intercept	161.442	1	<0.001
Habitat	3.366	2	0.186
Tires	9.500	2	0.009
Species	19.228	2	<0.001
Habitat x Species	27.896	4	<0.001
Tires x Species	16.639	4	0.002

AIC = 833.671

Species-specific analysis

Aedes triseriatus. Tire introduction had an overall marginally significant effect (Table 3.9). This effect was only significant in year 2 of the experiment (barely any *Ae. triseriatus* were collected in year 1). In this year (year 2), the major impact was due to tire introduction to the forest (Fig. 3.7A). The same pattern, yet even more pronounced, was exhibited when looking at the effect of the treatment on number of resting adult *Ae. triseriatus* mosquitoes in the forest habitat. A similar within-habitat effect was exhibited also in year 3 (Fig. 3.7B). In the edge and field habitat only very few adults were collected precluding any meaningful statistical analyses (Fig. 3.7C,D). A significant effect of habitat was found with *Ae. triseriatus* most common in the forest habitat. Also, a significant effect of tires x year was detected with a large increase in year 2 and a subsequent drop in number collected in year 3.

Table 3.9. Three-way negative binomial model testing the effects of Habitat, Treatment, and Year on abundance of resting *Aedes triseriatus* adults. This model was the best model (∆AIC=20 compared to the closest model habitat and tire simple effects).

	Wald Chi-square	df	P
Intercept	75.504	1	<0.001
Habitat	26.373	2	<0.001
Tires	8.485	2	0.014
Year	0.013	1	0.910
Tires * Year	7.687	4	0.052

AIC = 309.014

Aedes japonicus. With *Ae. japonicus* no significant effect of treatment was observed (W=1.141, df =2 , P = 0.565). The only significant effect was that of habitat (W=28.299, df = 2, P < 0.001) with highest number collected in the forest habitat (0.47±0.07), followed by the edge (0.26±0.07), and the field (0.05±0.02) habitats (Fig. 3.8).

Table 3.10. Three-way negative binomial model testing the effects of Habitat, Treatment, and Year on abundance of resting *Aedes japonicus* adults. This model was the best model (DAIC=3 compared to closest model).

	Wald Chi-square	df	P
Intercept	102.745	1	<0.001
Habitat	28.299	2	<0.001

AIC = 388.750

Aedes albopictus. Despite relatively low numbers, significant 'habitat' (W=9.022, df=2, P = 0.011) and 'treatment' (W=8.439, df =2 , P = 0.015) effects were detected. This mosquito was mainly prevalent in the field habitat (0.0.07±0.03), followed by the edge (0.01±0.01), and forest (0.00±0.00) habitats. With respect to the effect of treatment, tire introduction to the field had the strongest effect (0.05±0.03), followed by the forest-tire-introduction (0.01±0.01), and control (0.00±0.00) (Fig. 3.9).

Table 3.11. Three-way negative binomial model testing the effects of Habitat, Treatment, and Year on abundance of resting *Aedes albopictus* adults. This model was the best model (DAIC=4 compared to closest model).

	Wald Chi-square	**df**	**P**
Intercept	54.516	1	<0.001
Habitat	9.022	2	0.011
Tires	8.439	2	0.015

AIC = 112.986

III. Mosquito larval distribution in the tires

A total of 2261 larval mosquitoes were collected from all tires: 725 in the forest tires and 1536 in field tires. A total of 1363 were collected in year 2 and 898 in year 3. Five container breeding mosquitoes were collected in both habitats, which in addition to *Ae. triseriatus* (N = 719), *Ae. japonicus* (N = 560), and *Ae, albopictus* (N = 946), also included *Ae. hendersoni* (N = 26) and *Toxoronchytes rutilus* (N = 10). *Ae. albopictus* larvae was significantly (U = 3,683.00, P < 0.001) more abundant in the field tires (8.96±2.7) compared to forest tires (1.29±0.08)). *Ae. japonicus* exhibited no significant differences between the two habitats (U=5,345.500, P = 0.691) but tended to be more common in the field tires (4.32±1.8) compared to forest tires (2.6±0.3), whereas *Ae. triseriatus* larvae were significantly more common (U=7,182.500, P<0.001) in the forest tires (5.88±0.6) compared to the field tires (2.78±0.9) (Fig. 10).

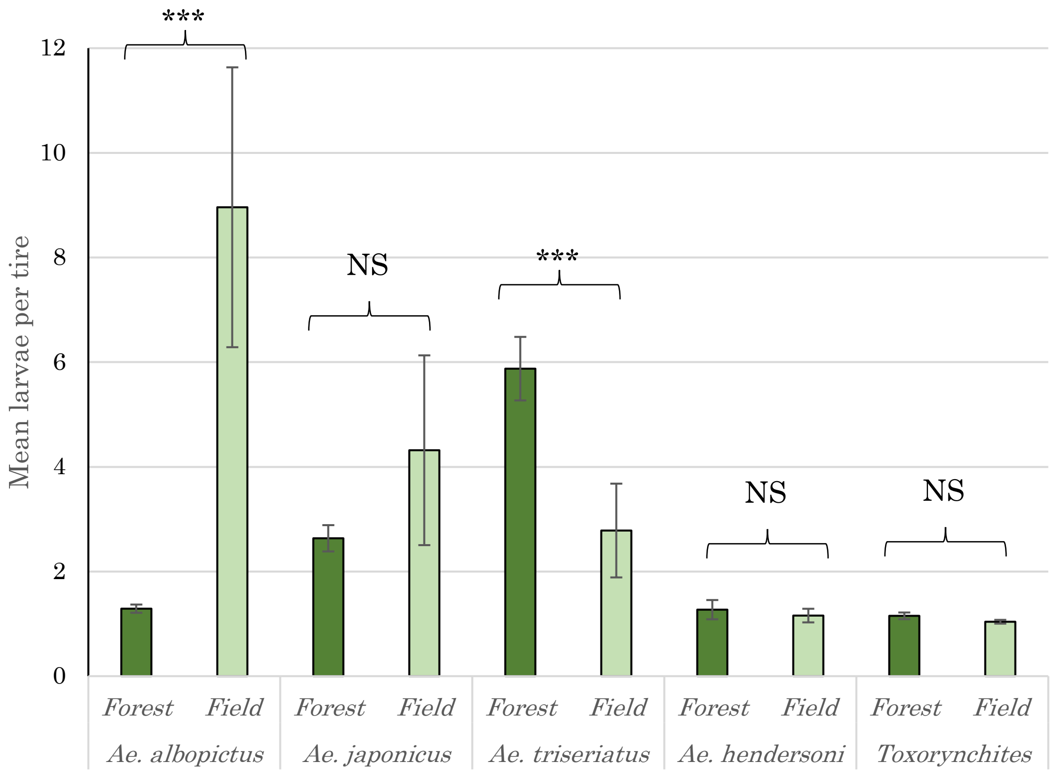

Figure 3.10. Mean *Aedes spp.* and *Toxorynchites rutilus* larvae collected from forest and field tires. Bars = SE. ***= P < 0.001, NS = No significant difference.

IV. Host seeking patterns using landing and biting method

A total of 386 host-seeking mosquitoes were collected. We found a significant effect of 'species' with *Ae. japonicus* being the most common species (3.00±0.4), followed by *Ae. triseriatus* (0.81±0.1), and *Ae. albopictus* (0.48±0.1). Also, a significant effect of habitat was found with highest host-seeking activity detected in the forest habitat (3.00±0.4), followed by the edge (0.86±0.1), and the field (0.40±0.1) habitats. A significant ‘habitat x species’ interaction was detected (Table 3.12), consistent with the species-specific habitat affinities of these species: *Ae. triseriatus* and *Ae. japonicus* were mainly host seeking in the forest habitat (*Ae. triseriatus*: 1.87±0.4, *Ae. japonicus*: 6.46±1.3) followed by the edge habitat (*Ae. triseriatus*: 0.47±0.2, *Ae. japonicus*: 1.71±0.4). *Aedes triseriatus* was completely absent from the field habitat, while *Ae. japonicus* was very sparse there. In contrast, *Ae. albopictus* was most active in the field habitat (1.10±0.4), followed by the edge (0.20±0.1) and the forest (0.13±0.1). Finally, as indicated by the significant three-way interaction (Table 3.12) the effect of the tire addition treatment appeared to differ among species and habitats (Fig. 3.11). For *Ae. triseriatus*, treatment effect was significant only in the forest habitat, with local tire effect having a positive effect on number of host-seeking mosquitoes (Fig. 3.11). With *Ae. japonicus*, treatment was also significant in the forest habitat, although here, surprisingly, local tire addition resulted in reduced host seeking activity. In contrast, with *Ae. albopictus* the effect of the treatment was only significant in the field habitat with a strong local effect of tire addition in that habitat (Fig. 3.11). There appears also to be a slight spill-over effect into the edge habitat with field-tire addition affecting *Ae. albopictus* activity in the edge.

Table 3.12. Three-way negative binomial model testing the effects of Habitat, Treatment, and Year on abundance of host-seeking *Aedes spp.* adults. This model was the best model (DAIC = 17 compared to closest model without three-way interaction).

	Wald Chi-square	**df**	**P**
Intercept	3.484	1	0.062
Habitat	9.352	2	0.009
Species	10.933	2	0.004
Tires	0.184	2	0.912
Habitat * Species	27.894	3	<0.001
Habitat * Species * Tires	19.916	9	0.018

AIC = 623.706

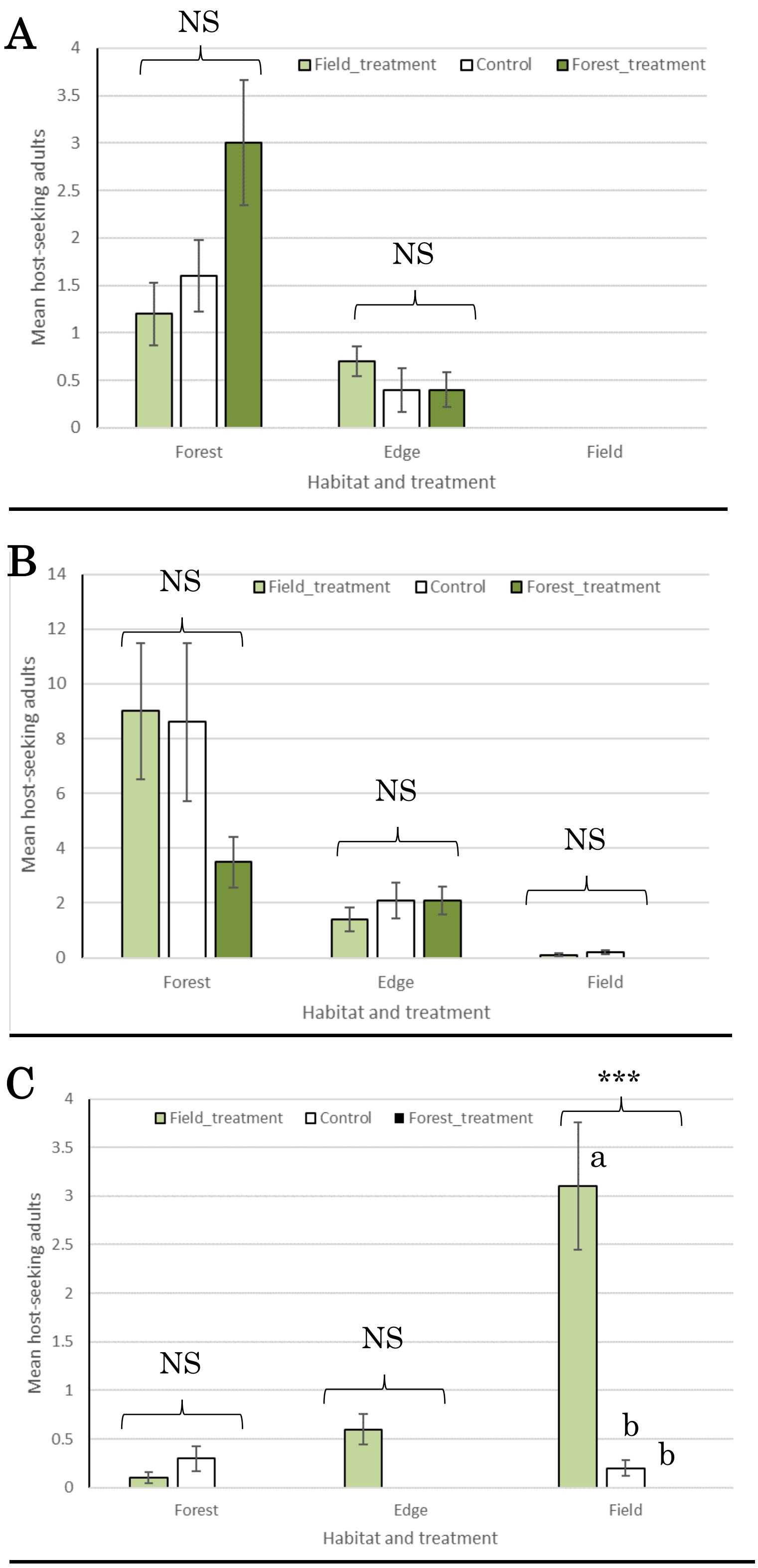

Figure 3.11. Host-seeking *Aedes spp.* abundance by habitat and tire-introduction treatment. A. *Aedes triseriatus*; B. *Aedes japonicus*; C. *Aedes albopictus*. Bars = SE.

V. Parity patterns

We used a logistic regression analysis to test for the effects of habitat, treatment, and their interaction on the proportion of parous females of each species.

Resting adults collection. Given that sample size was too small for *Ae. triseriatus* (N = 20), *Ae. japonicus* (N = 15) and *Ae. albopictus* (N = 8) no analysis could be done.

Host seeking mosquitoes. Here, in addition to testing (using logistic regression) for the effects of habitat, treatment, and their interaction, we also tested for the effect of time comparing between the afternoon session (13:20 - 17:30) and the dusk session (17:30 – 21:00). For *Ae. triseriatus*, neither habitat (P = 0.720), treatment (P = 0.732) nor time (P=0.460) had a significant effect. Similarly, none of these factors had a significant effect on proportion parity of *Ae. japonicus* (habitat effect: P = 0.988, treatment effect P = 0.089, time effect: P = 0.082).

VI. Wing size patterns

For resting adult mosquito collections, two-way ANOVA was used to analyze the effect habitat, treatment and their interaction. For host seeking mosquito collections, three-way ANOVA was used to analyze the effect habitat, treatment, time, and their interactions.

Resting adult collection. For *Ae. triseriatus* (N=77) no significant effect was found for treatment. But a marginally significant effect of habitat was found (F = 2.728, P = 0.087) with wing size of mosquitoes collected in the field (3311±287µm) significantly lower than that of mosquitoes collected in the forest (4000±91µm) or the edge (4049±234µm) habitats. For *Ae japonicus*, a significant effect of habitat was found (F = 3.573, P = 0.046), with wing size of mosquitoes collected at the edge habitat (4055±137µm) significantly higher than that of mosquitoes collected at the forest (3808±104µm) or the field (3251±275µm) habitats. This pattern is consistent with this species' numerical affinity to the edge habitat. For *Ae. albopictus*, significant (or marginally significant) effects were found for the effects of 'habitat' (F = 4.667, P = 0.041) and 'treatment' (F = 4.928, P = 0.054). Wing size was highest for mosquitoes collected at the edge habitat (3378±168um) and lowest for mosquitoes collected at the forest habitat (2984±168µm), with intermediate size for mosquitoes collected at the field habitats (3000±66µm).

Host seeking mosquitoes. *Aedes triseriatus* (N=73) mosquitoes were collected only at the forest and edge habitats. A significant effect of 'habitat' was found (Table 13a) with significantly larger wing size for mosquitoes collected in the forest habitat (3728±52µm) compared with the edge habitat (3491±102µm) (Table 13a). For *Ae.*

japonicus significant ‘treatment’ and ‘habitat’ effect were found (Table 3.13b). Surprisingly, wing size of mosquitoes collected in the control plots (3887±90μm) was significantly larger than that of mosquitoes collected in the forest-treatment (3697±102μm) or the field-treatment plots (3724±93μm). The effect was consistent across all three habitats (Fig. 3.12). Nonetheless, this effect is consistent with the negative effect of the treatment on *Ae. japonicus* in the field (Fig. 3.12). Also, consistent with the general affinity of *Ae. japonicus* for the edge habitat, wing size was larger for mosquitoes collected in the edge habitat (4005±58μm) compared with those collected in the forest (3866±32μm) or the field (3438±250μm) (Fig. 3.12). With *Ae. albopictus*, only the effect of ‘time’ was significant (Table 3.13c), with larger wing size for mosquitoes collected at the afternoon (2961±81μm) compared with those collected at dusk (2717±87μm).

Table 3.13. ANOVA test for *Aedes spp.* wing size along forest-to-field ecotone.

A. *Aedes triseriatus*	**Tests of Between-Subjects Effects**				
Dependent Variable:			**Wing_size (µm)**		
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept	621193524.36	1	621193524.36	3987.03	0.000
Habitat	664021.96	1	664021.96	4.26	0.043
Error	11062049.46	71	155803.51		
Total	999965355.46	73			

B. *Aedes japonicus*	**Tests of Between-Subjects Effects**				
Dependent Variable:			**Wing_size (µm)**		
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept	353553680.500	1	353553680.500	1898.524	0.000
Habitat	1409402.162	2	704701.081	3.784	0.024
Tires	1950305.581	2	975152.790	5.236	0.006
Error	49349763.801	265	186225.524		
Total	4169893409.707	270			

C. *Aedes albopictus*	**Tests of Between-Subjects Effects**				
Dependent Variable:			**Wing_size (µm)**		
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept	344841431.653	1	344841431.653	2294.047	0.000
Time	634524.327	1	634524.327	4.221	0.046
Error	6163125.620	41	150320.137		
Total	355403038.538	43			

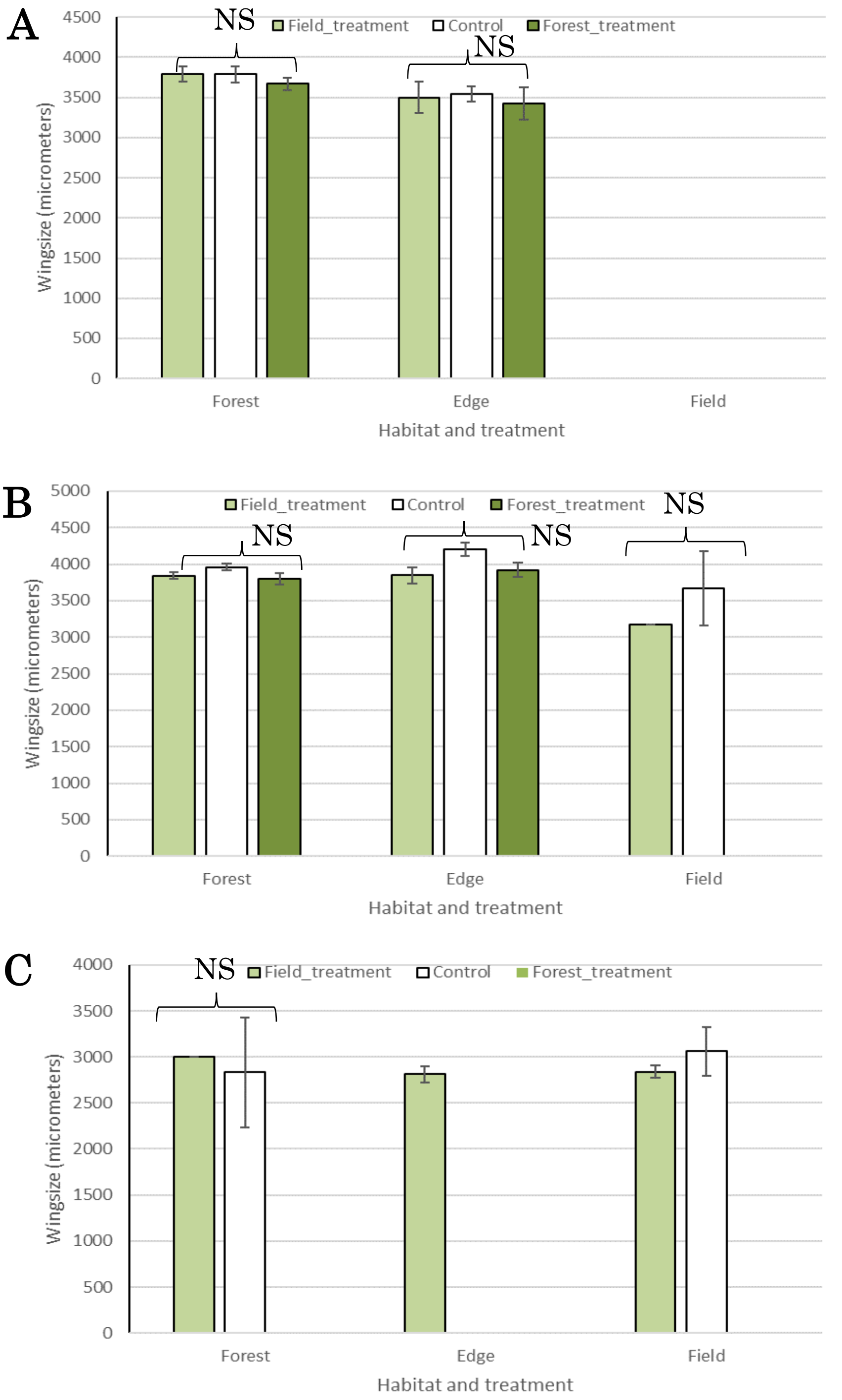

Figure 3.12. Host-seeking *Aedes spp.* wing size along forest-to-field ecotones. A. *Aedes triseriatus*; B. *Aedes japonicus*; C. *Aedes albopictus*. Bars = SE; NS = No significant difference.

VII. Infection patterns

A total of 529 adult specimens (resting = 143; host-seeking = 386) were screened for LACV infection. One pool corresponding to an *Ae. japonicus* specimen collected while resting in the forest habitat on September 10, 2014 showed signs of cytopathic effects (CPE) in vero cell culture. However, a subsequent Bunyavirus-specific PCR test did not confirm this result, LACV infection was, thus, rejected.

Discussion

In the context of Pavlovsky's “landscape epidemiology” conceptual framework, Reisen (2010) has proposed that the characteristics of the landscape mosaic could have important implications for vector-borne disease dynamics, distribution, and emergence (Pavlosky, 1966). These types of questions have been studied extensively in urban landscapes exhibiting heterogeneity in transmission potential along environmental gradients, such as the availability of vegetated areas such as parks, cemeteries, and backyards or along anthropogenic gradients often associated with socio-economic status (LaDeau et al., 2015; Parham et al., 2015; Reisen, 2010). However, these kinds of studies are relatively uncommon in rural landscapes. In the context of LACV transmission system, studies have been conducted within either forested landscapes (Szumlas et al., 1996b; Tamini et al.,

2021) or within relatively urbanized landscapes (Rowe et al., 2020). However, no study have yet evaluated drivers of LACV entomologic risk across the forest-to-field ecotone, which is most characteristic feature of the landscape in the LACV endemic region of the southern Appalachian mountains. Furthermore, the role of artificial containers, particularly used tires, in enhancing transmission risk of this or other mosquito-borne disease systems is well recognized (LaDeau et al., 2015; Leisnham and Juliano, 2012b; Vezzani, 2007; Whiteman et al., 2020; Yee, 2008). Nonetheless, all studies on the effect of artificial containers and on mosquito distribution and abundance have relied solely on naturally occurring containers and no study yet exists that studied the effect of artificial containers on mosquito communities experimentally.

The goal of my study was to evaluate, experimentally, the effect of artificial breeding site addition (in the form of used tires) on the distribution, abundance, dynamics, demography, community composition, and fitness of LACV vectors and its implications for LACV infection risk. Specifically, for sampling sites extending the forest-to-field ecotone, I added an array of 9 tires, either at the forest side or the field side and evaluated over three years the effect of this addition on the abundance, distribution, and demography of the mosquitoes along this forest-to-field ecotone. We also evaluated the scales of the tire introduction effect both in space and in time.

The effect of tire introduction on mosquito oviposition activity along the forest-to-field ecotone.

Baseline stage.

Initially, at the baseline stage, I established that the plots to be designated to be the treatment plots were not different from the plots designated to be the control plots. This was confirmed, with a few exceptions, based on oviposition activity and resting adults both at the entire plots scale as well as for the habitat-specific scale. The exception was the field habitat where the to-be control plots had a trend of higher numbers, which then flipped to be the lowest in the experimental stage. At the species level, this was confirmed for *Ae. triseriatus* but could not be confirmed for *Ae. albopictus* that was absent at the baseline phase. For *Ae. japonicus*, the plots designated to be the 'control' plots had higher abundance, a fact that needs to be taken into account when interpreting the results for this species.

Experimental stage.

Overall, habitat-specific, and species-specific effects. At the experimental stage, a strong effect of tire addition on overall mosquito oviposition activity was detected, with higher oviposition activity in the treatment plots compared with the control plots. This was the case also at the habitat-specific scale. This effect was significant for *Ae. triseriatus* and *Ae. albopictus* at the plot and habitat scales. In contrast, the effect of tire addition was negative on *Ae. japonicus*. This was the case at the entire plot scale and in the forest habitat. This effect was not significant at the edge and field habitats. Yet, as mentioned above, this observation should be taken with caution given that in the baseline stage the to-be control plots had an overall higher abundance, an effect that might have been carried over into the experimental stage. It needs to be highlighted though that this effect was observed only in the second year of the experiment (in year 1, no *Ae. japonicus* were collected). In the third year, this effect disappeared in the forest habitat and, actually, completely flipped in the edge habitat, with treatment effect being highly significant, with tire addition to the field having the strongest positive impact followed by that of tire addition to the forest. There was no effect in the field habitat, where *Ae. japonicus* was relatively scarce throughout all three years of the experiment.

Temporal scale aspects of tire introduction effect. The effect of tire introduction on mosquito oviposition activity was quite instantaneous. In the second half of the summer of 2012, shortly after the introduction of the tires, oviposition activity levels in the experimental plots increased significantly. This effect persisted into the second year of the experiment but was reduced in year 3 of the experiment. This pattern is mainly driven by the dynamics in the forest habitat. In the edge and the field habitats treatment effect remained persistent across all three years of the experiment. These patterns concerning overall oviposition activity are mainly driven by *Ae. triseriatus*, which was the most common species. For *Ae. japonicus* it is not clear if tire introduction had an effect in the first two years of the experiment. However, in the third year a clear and strong positive effect was detected, predominantly due to its response in the edge habitat. With *Ae. albopictus*, the effect of tire addition was also immediate, with a strong effect in year 1 in the forest and field habitats and a consistent effect in the subsequent years, mainly in the edge and field habitats.

Spatial scale aspects of tire introduction effect. The tire array was introduced in experimental plots either in the extreme side of the field or the extreme side of the forest. This enabled us to assess the spatial scale of the effect of this intervention. Looking at the overall oviposition activity, in the forest habitat, local tire addition had a significant effect of increased oviposition activity. Surprisingly, also tire addition to the field resulted in increased oviposition activity in the forest habitat, suggesting that even a sub-optimal habitat could act as a source of dispersers to adjacent habitats. In year 1 the effect of tire addition to either habitat was more or less even, but in year 2 the local effect was dominant. Mosquito response at the edge habitat is interesting because it indicates the relative magnitude of a habitat-specific anthropogenic effect (in the form of artificial container addition) on mosquito abundance at the edge. In year 1 the forest introduction was the most impactful, in year 2 forest and field tire introduction had a similar effect, and in year 3 field tire introduction had a greater effect. In the field, tire introduction to the forest (between-habitat effect) had the greatest impact in year 1. This, however, flipped in favor of dominance of a within-habitat effect (tire addition to the field) in years 2 and 3. Breaking this into the species-specific effects reveals that for *Ae. triseriatus*, in the forest habitat, tire addition to the field (the between-habitat effect) had the greater effect. This switched to a greater within-habitat effect in year 2 and possibly year 3. At the edge habitat, the forest tire addition had the greater impact in year 1 and then the impact of tire addition to the field or to the forest was more or less similar in years 2 and 3. In the field habitat, local tire addition had a strong impact already in year 1. Yet, a significant between-habitat effect was detected as well. The within-habitat effect remained strong also in years 2 and 3 while the between-habitat effect gradually receded. With *Ae. japonicus*, in the forest habitat the main effect was the between-habitat effect and that just came about in year 3. In the field habitat, tire introduction did not have an effect in either habitat. Yet, at *Ae. japonicus'* preferred habitat of the edge habitat, both tire addition treatments had a significant effect, albeit only in year 3. With *Ae. albopictus*, tire addition effects appear to be mainly local and in the edge habitat the main contributor was tire introduction to the forest in year 2 and tire introduction to the field in year 3.

Can mosquito larval distribution in the tires explain tire introduction effects? The field tires were dominated with *Ae. albopictus* larvae (41.84%), followed by *Ae. triseriatus* (31.80%) and *Ae. japonicus* (24.77%). Surprisingly, also *Ae. hendersoni* larvae (2.07%) were found there in addition to *Toxorynchites rutilus* (0.44%). The dominance of *Ae. albopictus* in field tires is consistent with the local-scale effect observed in years 2 and 3 in the field but not with the between habitat effect observed in year 1. Also, its dominance could partially explain the edge pattern where a strong field-tire effect was observed in year 3 but is not consistent with the strong forest-tire effect observed in year 2. In the forest tires, *Ae. triseriatus* was the dominant species (69.79%). Hence the significant local effect of tire introduction to the forest, was consistent with that. Surprisingly, we also observed a between-habitat effect of field tire introduction in year 1 despite the fact that *Ae. triseriatus* larvae were not very common there. Nonetheless, the significant local effects within the field suggests that this tire addition was still impactful locally and possibly at the between habitats scale. It is also interesting to note that within the field habitat, a significant between-habitat effect was detected, which is consistent with *Ae. triseriatus'* dominance in the forest tires. With *Ae. japonicus* no significant difference was found in the number of larvae in the field versus the forest tires although quantitatively it appeared to be more common in the field tires. Indeed, in the 3rd year of the study when positive treatment effects were detected in the edge habitat, the dominant effect was that of field tire addition.

The effect of tire introduction on the distribution of resting adults along the forest-to-field ecotone.

In the baseline stage, no pre-treatment effect was observed for *Ae. triseriatus*, although abundance trended towards plots designated to be field-tire introduction. A strong local effect of tire introduction to the forest was observed in year 2 and less so in year 3, which is consistent with the prevalence of this species in the forest tires. In the edge and field habitat, in the baseline stage, no *Ae. triseriatus* mosquitoes were detected. Yet, in year 3 in the field, a strong local effect was detected. In the edge habitat, field-tire introduction had a strong effect in years 1, 2, and 3. These results suggest that *Ae. triseriatus* mosquitoes produced in the field tires tend to move as adults into the more shaded adjacent edge or forest habitats. This could also, partially, explain the cross-habitat effects detected with the oviposition pattern. With resting adult *Ae. japonicus*, no significant effect of treatment was observed at any of the habitats. With *Ae. albopictus*, as with the oviposition activity, tire addition effects were mainly local both at the forest and the field habitats, while in the edge it was mostly absent. This result is consistent with the relatively low dispersal capacity and site fidelity characteristic of *Ae. albopictus* (Lacroix et al., 2009; Marini et al., 2010).

The effect of tire introduction on the host seeking activity of females along the forest-to-field ecotone.

As with resting adult mosquitoes, but in contrast with oviposition activity, *Ae. japonicus* was the more common species. Similarly, habitat use of the species was consistent with that of resting adults, with both *Ae. triseriatus* and *Ae. japonicus* mostly using the forest habitat followed by the edge habitat, and *Ae. albopictus* mainly using the field habitat. The distribution of host seeking females was quite consistent with the distribution of resting adults but more extreme. In other words, the relative representation of host seeking *Ae. japonicus* in the forest habitat (78%) was greater than its representation in resting adults at the same habitat (56%). This might hint that *Ae. japonicus* is relatively more anthropophilic and a more aggressive human feeder than *Ae. triseriatus*. The effect of tire addition was species- and habitat-specific. Specifically, tire addition to the forest resulted in significant increase in the host seeking behavior of *Ae. triseriatus* in that habitat. Surprisingly, the same treatment resulted in significant reduction in the host seeking activity of *Ae. japonicus*. Tire addition to field resulted in a significant enhancement of *Ae. albopictus* activity in that habitat. Lastly, *Ae. albopictus'* host-seeking activity was significantly higher in the late afternoon hours compared to early evening. Indeed this mosquito is known to be active during daylight (Delatte et al., 2010; Kamgang et al., 2012). It may therefore increase the risk of LACV transmission at that time.

Qualitative effects of tire introduction on female parity and adult size.

The distribution patterns described above that were based on oviposition activity, resting adults, and host seeking can be thought as addressing the quantitative aspects of habitats and artificial containers. However, parity and body size patterns can be perceived as addressing the qualitative aspects of these effects as they measure aspects of species performance in terms of physiological age (parity) and fitness (body size) (Blackmore and Lord, 2000; Haramis and Foster, 1990; Landry et al., 1988; Xue et al., 2010). In terms of parity, no differences were found for either species, not in terms of habitat or in terms of tire introduction effect. This result is somewhat surprising as often patterns of abundance and distribution tend to be consistent with patterns of parity. A classic example of that is the study of Tamini et al (2021), who conducted an observational study in which he evaluated if peridomestic conditions affect La Crosse virus entomological risk in the area of the Maggie Valley of southwestern NC. In that study they found that in sites with few artificial containers in the peridomestic habitat mosquito abundance (mainly *Ae. triseriatus* and *Ae. japonicus*) was higher in the forest habitat compared with the peridomestic habitat but in sites where the peridomestic habitat contained a lot of artificial container mosquito abundance was higher at the perideomestic habitat. However, they also reported that patterns of parity rate and gravidity rate was consistent with these abundance patterns (Tamini et al., 2021).

Parity rate is mainly driven by factors affecting female's daily survival rate (Dye, 1992). So, it is possible that the adults are less sensitive to the habitat conditions as they are able to regulate the location of their shelter. However, body size is mainly dictated by the quality of the larval habitat (Araújo et al., 2012; Marini et al., 2010; Shapiro et al., 2016; Yan et al., 2021). For both *Ae. triseriatus* and *Ae. japonicus*, body size trends are consistent with its quantitative habitat affinities suggesting that larval rearing conditions are relatively favorable, in a species-specific manner, in those habitats. The exception was *Ae. albopictus*, which based on the resting adults' data, exhibited larger body size in the edge habitat whereas its abundance tend to always be higher in the field habitat. This pattern, however, was not exhibited when analyzing the host seeking data which was more comprehensive.

In terms of the effect of the tire introduction, no significant effect was observed for either *Ae. triseriatus* or *Ae. albopictus*. This result is somewhat surprising because we would expect tire addition to enhance the overall breading conditions of larvae in that habitat. Furthermore, tires are assumed to be an ideal larval habitat because it provides both shelter in terms of shade and acts as an excellent collector of leaf litter and other organic waste that improve larval habitat (Yee, 2008). Only with *Ae. japonicus* we found a significant effect of the tire introduction. But in that case the effect was, surprisingly, negative with mosquitoes in the control plots being on average larger than those in the treatment plots. Yet, this result is consistent with the negative effect of tire addition on this species' oviposition activity which was consistently higher in the control plots and in terms of adult distribution, which was reduced in the forest habitat following tire introduction. These results suggest that *Ae. japonicus* might be a weaker competitor than the native *Ae.*

triseriatus in the forested areas or the invasive *Ae. albopictus* in the field habitat. Indeed, most studies on this topic have found this species to be a weaker (or not stronger) competitor than other local species (Kaufman and Fonseca, 2014). It was also found to be more strongly affected by intraspecific competition compared to native species, which may influence its lower competitive capacity (Hardstone and Andreadis, 2012), however, it was found to be a weaker competitor than *Ae. albopictus* (Armistead et al., 2008).

Conclusion

The LAC system may serve as a model for understanding existing and novel risk factors regarding vector-borne diseases in general, including the role of human induced environmental change on the incidence of these diseases. We found evidence to suggest that human-induced habitat fragmentation and supplementation (i.e., artificial container introduction) alters the distribution and abundance of mosquito vectors and may, therefore, enhance transmission risk.

Study limitations. This study makes inferences regarding the effects of larval habitat supplementation by comparing control sites and sites in which containers were introduced either into the forest or field habitat. Although our study surveyed egg and adult mosquito abundance for three years, a fairly extended period of time, we employed just 2 replicate sites per treatment. Future studies should consider employing more sites as well as sites located several kilometers apart, to ensure independence between plots.

Future directions. Further research is required to better understand and quantify absolute risk associated with anthropogenic factors such as differences in blood-feeding preferences and LACV incidence in amplifying hosts in response to human-induced land-use change. Moreover, given the significant overlap in distribution of the three LACV vectors in the edge habitat and the understanding that edges have played an important role in the transmission of enzootic pathogens to humans, future studies should focus on this habitat. In particular, they should evaluate the effect of container introduction on LACV vectors' abundance, fitness, and rates of LACV infection.

Public Health Applications. We have shown that artificial containers can have far reaching spatial (between habitats) and temporal (across years) effects on vector abundance and, by extension, disease risk. We recommend that public health efforts target artificial containers both in terms of active removal and educating the public about the risks, in particular those beyond peri-domestic areas which are likely overlooked.

CHAPTER IV

HORIZONTAL DISTRIBUTION AFFECTS THE VERTICAL DISTRIBUTION OF NATIVE AND INVASIVE CONTAINER-INHABITING *AEDES* MOSQUITOES WITHIN AN URBAN LANDSCAPE

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ABSTRACT: The vertical dimension constitutes an important niche axis along which mosquitoes may adjust their distribution. Here, we evaluated whether the vertical distribution of container-inhabiting *Aedes* mosquitoes differs along a gradient of anthropogenic land-use intensity within an urban landscape. Using a pulley system, we hung oviposition cups at three heights (ground level, 4.5, and 9 m) and in three habitats: forest, park, and a built environment. We hypothesized that mosquito abundance and diversity would be highest in the least disturbed forest habitat, decrease in the park, and be lowest at the UNC-Greensboro campus. We also expected *Aedes albopictus* (Skuse) and *Ae. triseriatus* (Say) to mainly oviposit at ground level and *Ae. hendersoni* (Cockerell) at canopy height. *Aedes albopictus* was the most common species (68.8%) collected in all three habitat types and was the only species found in the built environment. In that habitat, *Ae. albopictus* exhibited a bimodal distribution with the lowest activity at the intermediate height (4.5 m). *Aedes triseriatus* (28.9%) did not differ in egg abundance between the forest and park habitats but did exhibit diverse vertical habitat use while avoiding the canopy in the park habitat. *Aedes hendersoni* (2.3%) was the most sylvatic species and oviposited only at ground level. Our results indicate that the vertical distribution of mosquitoes is affected by the type of habitat in which they occur, and that this variation could be driven via local-scale modification of microclimatic factors.

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Introduction

Urban environments are among the fastest growing habitats on earth, with potentially severe consequences for biodiversity loss as well as human health (Seto et al. 2012). Vector-borne diseases are on the rise (Rosenberg et al. 2018), with particular concern due to the emergence or resurgence of diseases such as chikungunya, dengue, La Crosse encephalitis, and Zika, as a consequence of globalization and urbanization (Foley et al. 2005, Weaver et al. 2018,). In the context of Pavlovsky's "landscape epidemiology" conceptual framework, Reisen (2010) has proposed that the characteristics of the landscape mosaic could have important implications for vector-borne disease emergence (Pavlovsky 1966). Within this framework, the disease niche occurs when and where the niches of the pathogen, vector, and host(s) occur within a permissive environment (Reisen 2010). Urban landscapes represent unique mosaics of habitats, with heterogeneity in transmission potential varying along environmental gradients, such as the availability of vegetated areas such as parks, cemeteries, and backyards or along anthropogenic gradients often associated with socio-economic status (LaDeau et al. 2015, Parham et al. 2015, Reisen 2010). These types of heterogeneities have the potential to affect availability of larval sites, reservoir host availability, microclimate (e.g., urban heat island effect), and vector longevity, all of which affect the vectorial capacity of pathogens (Becker et al. 2014, Dowling et al. 2013, Maeda et al. 2018, Parham et al. 2015). In addition, these factors were shown to affect the degree of exposure of humans to vectors by affecting behavioral aspects such as recreational habits or time spend outdoors, use of repellents or bed-nets, as well as passive exposures related to things such as availability of window screens, presence of artificial containers, and others (Degarege et al. 2019, Lockaby et al. 2016, Ostfeld 2010, Parham et al. 2015). These factors were shown to play important roles in a wide variety of diseases such as West-Nile virus, chikungunya, Zika, dengue, Lyme disease, malaria, leishmaniasis, Chagas disease, and others (Hotez et al. 2012, Lenk et al. 2018, Parham et al. 2015, Reisen 2010, Weaver et al. 2018).

Mosquito movement and distribution occurs both in the vertical and horizontal dimensions. However, most studies on the ecological factors affecting mosquito distribution and abundance describe the horizontal distribution of mosquitoes based on sampling at, or near, ground level (Reiskind and Lounibos 2013, Swanson et al. 2000). The vertical niche dimension of mosquito activity is a relatively neglected aspect in our understanding of mosquito ecology (Chadee 2004, Fitzgerald and Livdahl 2019, Huestis et al. 2019, Jayathilake et al. 2015, Liew and Curtis 2004, Mercer et al. 2010, Obenauer et al. 2009). With treehole mosquitoes, the vertical niche axis is particularly ecologically relevant. For example, at sites where *Aedes triseriatus* (Say) (Diptera: Culicidae) and *Ae. hendersoni* (Cockerell) were found in sympatry, *Ae. triseriatus* oviposited in greater abundance at lower elevations while *Ae. hendersoni* oviposited almost exclusively at higher elevations (Sinsko and Grimstad 1977). Other studies have shown similar results consistent with vertical niche partitioning, suggesting that *Ae. triseriatus* prefers to oviposit at ground level and *Ae. hendersoni* at the canopy (Loor and DeFoliart 1970, Scholl and DeFoliart 1977). In contrast, Novak et al. (1981) collected *Ae. triseriatus* and *Ae. hendersoni* adults primarily from the forest canopy (21 m) compared to ground level (2 m). Recently, Fitzgerald and Livdahl (2019) demonstrated that the reported differences in *Ae. triseriatus* and *Ae. hendersoni* vertical stratification oviposition patterns may be, in part, explained by interspecific competition. This is supported by the convergence of habitat use in allopatric populations and divergence in areas where the two species occur in sympatry. Interestingly, the western tree hole mosquito, *Aedes sierrensis* (Ludlow), which currently exists in the absence of other competing Aedine species, does not appear to segregate oviposition activity along the vertical axis (Mercer et al. 2010).

Factors that influence the vertical stratification of mosquitoes and their oviposition behavior are complex. Species interactions, abiotic environmental factors, and nutrient levels are commonly suggested as elements that influence vertical oviposition behaviors (Copeland and Craig 1992, Fitzgerald and Livdahl 2019). Similarly, in urban settings, the built environment often provides novel ecological opportunities for niche partitioning along the vertical niche dimension. Chadee (2004) studied the oviposition activity of *Ae. aegypti* (Linnaeus) at five elevations between 0 – 60 m above ground in the urban Port of Spain, Trinidad. He collected the highest number of *Ae. aegypti* eggs at elevations between 13 and 24 m (Chadee 2004). Jayathilake et. al (2015) studied the vertical distribution of *Ae. aegypti* and *Ae. albopictus* along high-rise buildings in an urban setting in Colombo, Sri Lanka. They observed differential vertical distribution, with *Ae. albopictus* laying eggs exclusively between ground level and 6.1 m heights and *Ae. aegypti* laying eggs at all elevations with no clear vertical pattern (Jayathilake et al. 2015). Williges et al. (2014) also found *Ae. albopictus* ovipositing primarily at ground level when compared to 1, 2, 3, and 4 m heights in a wooded residential neighborhood in New Jersey (Williges et al. 2014). In contrast, Liew and Curtis (2004) found that when both gravid *Ae. albopictus* and *Ae. aegypti* were released at 12th story height in a vacant condominium building in Singapore, neither species showed a tendency for movement to lower levels, suggesting that *Ae. albopictus* can also use high-elevation habitats (Liew and Curtis 2004).

Invasive mosquitoes are known to influence the geographical distribution range and habitat use of native mosquito species along horizontal axes at both regional and local scale. For example, it has been well documented that the invasion of *Ae. albopictus* have affected the distribution of *Ae. aegypti* in Florida (Livdahl and Willey 1991, Reiskind and Lounibos 2013) with potential implications to risk of arboviruses such as dengue, Chikungunya, and Zika viruses (Lwande et al. 2019). With *Ae. triseriatus*, there has been no evidence of an effect of *Ae. albopictus* on *Ae. triseriatus* distribution, which is consistent with modeling results by Lidvahl and Willey (1991) who predicted that these two species can coexist in sylvatic environments where treeholes constitute the main larval habitat but not in peridomestic environments where tires are the main larval habitat *Ae. albopictus* is predicted to drive *Ae. triseriatus* to local extinction (Livdahl and Willey 1991). It is not well understood, however, if and how invasive mosquitoes influence the vertical distribution of native mosquito species. Furthermore, whereas the horizontal and, to a lesser extent, the vertical distribution of mosquitoes have been studied, as reviewed above, the interaction of the two axes remains relatively poorly understood.

Only a few studies to date have addressed the question of the interaction between the horizontal and the vertical distribution of mosquitoes (Laporta and Sallum 2014, Medeiros-Sousa et al. 2019, Obenauer et al. 2009). Studies in the Atlantic forest of Brazil have investigated patterns of species co-occurrence along natural and anthropogenic gradients and how these affect horizontal and vertical distribution as well mechanisms of coexistence and mosquito community structure (Laporta and Sallum 2014, Medeiros-Sousa et al. 2019). Laporta and Sallum (2014) demonstrated that the coexistence of *Wyeomyia. muehlensi* and *Wy. Quasilongirostris* is enabled via vertical habitat partitioning only at an ecotonal scrub-forest ecotone but not in any of the other distinct habitats (Laporta and Sallum 2014). Furthermore, Medeiros-Sousa et al. (2019) showed that in anthropogenically disturbed forests, the malaria vector *Anopheles cruzii,* which typically inhabits the canopy, tends to increases its activity at ground level. In North America, Obenauer et al. (2009) reported that in the forest habitat, *Ae. triseriatus* oviposited predominantly in cups at 6 m height (four times more than at 1 m), while *Ae. albopictus* oviposited approximately at similar rates at both heights (slightly more at 1 m). However, in suburban areas, both species increased substantially their preference towards ovipositing at 1 m (Obenauer et al. 2009).

In our current study, we asked: does the vertical distribution of immature stages of native and invasive container-breeding mosquitoes differ along a gradient of urbanization within an urban landscape? We hypothesized that the habitat context within which a tree is located would influence the local-scale effects of abiotic factors or biotic interactions, resulting in different patterns of vertical distributions of local container-breeding mosquitoes along the tree's vertical dimension. We sampled in three habitats within the campus of the University of North Carolina at Greensboro: a built environment in the campus (representing high urbanization level), a golfing park (representing intermediate urbanization level), and an undisturbed remnant deciduous forest (representing low disturbance level) (Figure 4.1).

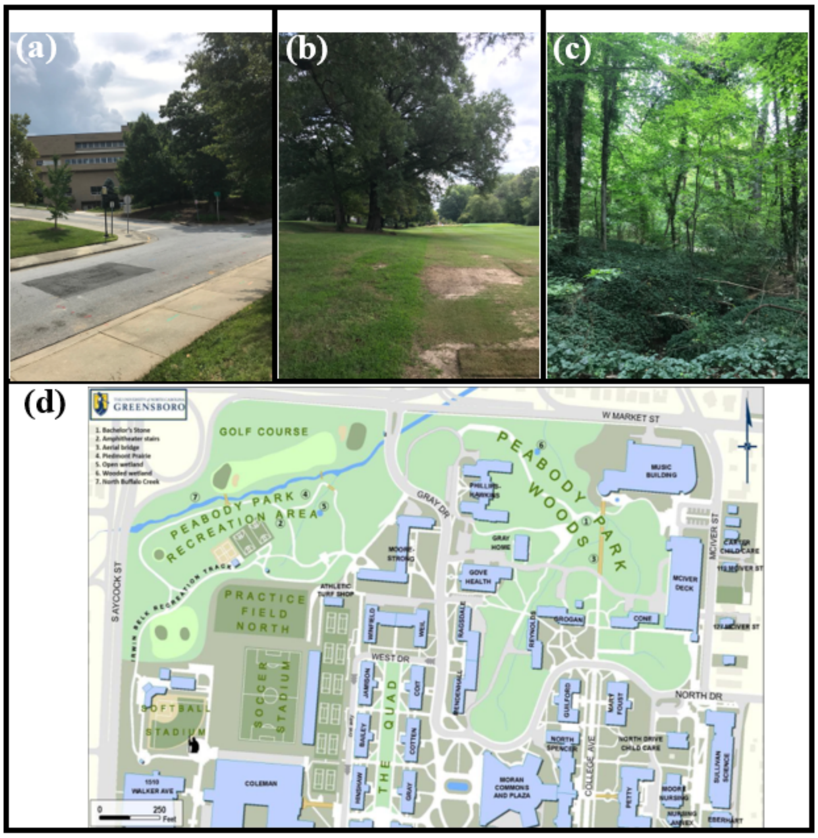

Figure 4.1. Representative pictures of the three study habitats: (a) Campus, (b) Park, (c) Forest. The "forest habitat" sampling sites were located within the Peabody Park Woods and the "park habitat" sampling sites were located within the Peabody Park Recreation Area (d). "Campus habitat" sampling sites were located within the built environment of the UNC-Greensboro campus (d). Sampling sites depicted as black triangles (d).

We used oviposition cups (ovicups) placed at 0 m (representing ground level habitat), 4.5 m (representing mid-tree level habitat below the canopy level), and 9 m (representing the canopy habitat) (Figure 4.2). Based on mosquitoes known preferences for shaded environments (Reiskind et al. 2017, Scholl and DeFoliart 1977), we expected our collections to be most abundant in the forest habitat, followed by the park, and in least abundance in the campus habitats. We also expected *Ae. albopictus* to be the more common species in the more urban campus habitat and *Ae. triseriatus* to be relatively more common in the forested habitat. We also expected *Ae. albopictus* to be relatively selective towards ovipositing at the ground level, while *Ae. triseriaus* was expected to be more generalist in its vertical habitat selection and *Ae. hendersoni* was expected to be relatively selective towards the canopy habitat (Fitzgerald and Livdahl 2019, Scholl and DeFoliart 1977).

Materials and methods

Study sites

The study was conducted at the University of North Carolina at Greensboro (UNCG) campus (Figure 4.1) from August 31st to October 25th, 2013. We sampled weekly for eight weeks in three habitat categories. The built habitat within the UNCG campus was characterized by university buildings, parking lots, and small, highly managed, lawn patches (Figure 4.1a). The park habitat was a recreational park comprised of a golf course, walking trails, and scattered large oak trees (Figure 4.1b). The forested habitat was a hickory-oak forested area in the northern section of the campus (Figure 4.1c). Three trees, spaced at least 100 m apart, were selected for each habitat to serve as replicates for the vertical ovicup arrays (Figure 4.1d).

Vertical oviposition cup system

Within each sampling replicate, ovicups were positioned at three heights: 0, 4.5, and 9 m above ground. Sampling heights were selected to represent three distinct vertical habitats: tree base, tree trunk below canopy level, and canopy, respectively (Figure 4.2a). Ovicups were 480 ml black plastic drinking cups lined with seed germination paper (76#, Anchor Paper, St. Paul, MN) and filled with approximately 250 ml of water. The seed germination paper covered approximately 70% of the surface area within the cups and served as an oviposition substrate (ovistrips). We used a paper clip to secure the ovistrips to the inner wall of the cups. A drainage hole was punched two-thirds up the cup to maintain water levels and prevent overflow due to precipitation. We positioned the ovicups along the trunks of mature trees (hardwood trees ≥25 cm diameter), using a pulley system (Figure 4.2b) that enabled us to collect and replace the ovistrips weekly and add water as needed.

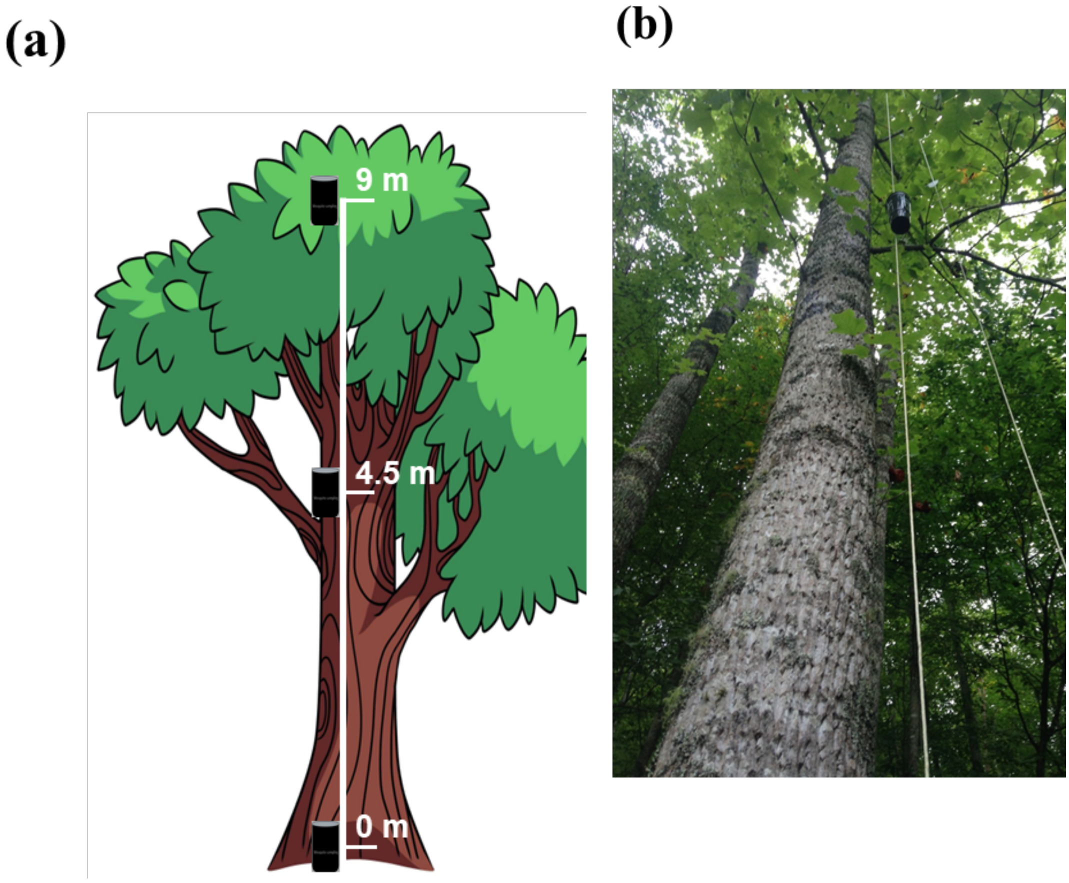

Figure 4.2. (a) Schematic drawing representing the three vertical habitats sampled along a tree using oviposition cups. (b) Pulley system used for vertical placement of ovicups along the tree trunk. Photo depicts an ovicup suspended at mid-tree elevation of 4.5 m.

Mosquito enumeration and identification

The number of eggs on each ovistrip was counted using a stereomicroscope. The ovistrips were flooded with dechlorinated tap-water in plastic 1-gallon sized trays containing bovine liver powder (Nasco, Fort Atkinson, WI) provided to serve as a food resource substrate for hatched larvae. Ovistrips with no eggs were not flooded, however, the zero observations were incorporated in the dataset. Trays containing ovistrips were then placed in a rearing chamber (Model: 6030–1, Caron, Marietta, Ohio) at 28º C and 80% relative humidity for a one-week period. Fourth instar larvae and pupae were then transferred to “mosquito breeder” emergence containers (BioQuip Products, Rancho Dominquez, CA). Once eclosed, adult mosquitoes were freeze-killed, enumerated, and identified morphologically using a dichotomous identification key (Harrison et al. 2016). Morphological identification of *Ae. triseriatus* and *Ae. hendersoni* for a subset of collections (n=265) was confirmed using a species-specific duplex PCR assay (Wilson et al. 2014).

Data analysis

The species-specific egg numbers laid on each ovistrip was inferred by multiplying the fraction of emergent adults of each species by the total number of eggs on that ovistrip (hereafter, ‘inferred eggs numbers’). Given the non-normal distribution of the eggs as count data, a Poisson regression generalized linear model (GLM) (using Log link function) was used for statistical analyses. To account for the clustered nature of the data, given that each tree has three ovicups (i.e., 0, 4.5, and 9 m), we used a random-intercept GLM model with “TREE” as the clustering factor. We first tested the effect of habitat and ovicup height on the overall number of eggs and then analyzed all species-specific effects using the inferred eggs numbers.

Analyses were done using STATA (StataCorp 2007). We also qualitatively analyzed patterns of species co-occurrence by calculating the frequency of ovistrips in which more than one species occurred and how this was distributed with respect to horizontal and vertical habitats.

Results

Trap collections

A total of 3,219 mosquito eggs were collected from 27 ovicups throughout the eight-week sampling period, comprising 216 separate weekly collections. With regard to horizontal habitats, most eggs were collected from the forest habitat compared to the park and the campus habitats (Table 4.1). With regard to vertical habitats, most eggs were laid at the tree-base level, followed by mid-tree and canopy levels (Table 4.1). With regard to species, among all hatched eggs, the majority were *Ae. albopictus* followed by *Ae. triseriatus* and *Ae. hendersoni* (Table 4.1).

Table 4.1. Summary table describes the distribution of eggs collected in oviposition cups during this study. The first column summarizes the total number of eggs laid in the horizontal habitat (campus, park, forest), as well as the range of eggs laid per oviposition cup within each horizontal habitat. The second column summarizes the total number of eggs laid in the vertical habitat (tree base: 0 m, mid-tree: 4.5 m, canopy: 9 m), as well as the range of eggs laid per oviposition cup within each vertical habitat. The third column summarizes the distribution of hatched eggs by species.

Habitat	**Total no. eggs (%)**	**Egg range**	**Height**	**Total no. eggs (%)**	**Egg range**	**Species**	**No. eggs (%)**
Campus	335 (10.4)	0-62	Tree-base	2,240 (69.6)	0-160	*Ae. albopictus*	586 (68.8)
Park	1,199 (37.3)	0-188	Mid-tree	563 (17.5)	0-188	*Ae. triseriatus*	246 (28.9)
Forest	1,685 (52.3)	0-158	Canopy	416 (12.9)	0-66	*Ae. hendersoni*	19 (2.3)
Total	3,219		Total	3,219		Total eggs hatched:	851 (26.4)

Overall oviposition activity: effect of habitat, ovicup height, and sampling week

A significant effect of habitat ($z = 5.43$, $P < 0.001$), week ($z = -0.243$, $P < 0.001$), and habitat-by-height interaction ($z = -2.64$, $P = 0.008$), but not of height ($z = -0.93$, $P = 352$), was found. Highest number of eggs was laid in the forest habitat (mean±SE: 23.84±2.55), followed by the park (16.73±2.51), and substantially less in the campus habitat (4.86±2.23). Vertical oviposition patterns differed among the three horizontal habitats with preference for the ground level in the forest and park but not in the campus habitat. Temporal variations in mosquito oviposition activity were, indeed, significant with the earlier (and warmer) four weeks of the study exhibiting higher oviposition activity than the later (and cooler) four (weeks 1-4 = 24.95±0.45; weeks 5-8= 5.86±0.85).

Mosquito species horizontal habitat use

A significant 'species' effect was found with *Ae. albopictus* being the most abundant species (8.50±1.11), followed by *Ae. triseriatus* (3.05±0.94) and *Ae. hendersoni* (0.63±0.53) (Table 4.2). In addition, a significant species-by-habitat interaction was found comparing habitat use of *Ae. albopictus* with that of *Ae. hendersoni* but not when comparing habitat use of *Ae. albopictus* with that of *Ae. triseriatus* (Table 4.2). Least favorable for all three mosquito species was the campus habitat, with *Ae. triseriatus* and *Ae. hendersoni* absent from it altogether (Figure 4.3). Yet, *Ae. hendersoni* was highly specific for the 'forest' habitat, while *Ae. triseriatus* occurred in the forest and park habitat quite equally (Figure 4.3). *Aedes albopictus* was the only species found in the campus habitat (Figure 4.3).

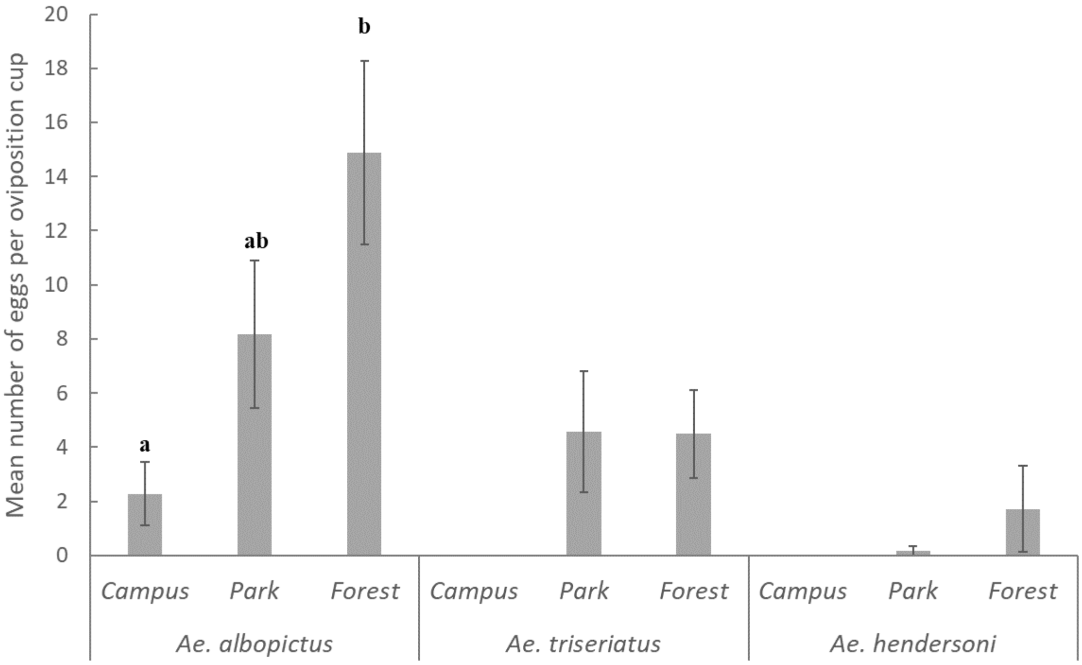

Figure 4.3. Horizontal distribution of oviposition activity of *Ae. albopictus*, *Ae. triseriatus*, and *Ae. hendersoni* among the campus, park, and forest habitats. Error bars = SE. Letters denote significant differences as per post-hoc test with Bonferroni correction in mean oviposition between habitats for each species.

Mosquito species vertical habitat use

Aedes albopictus. 'Habitat' had a significant effect (coef. = 1.65, SE = 0.49, z = 3.38, $P = 0.008$), with highest abundance in the forest habitat. 'Height' also had a significant effect (coef. = -0.23, SE = 0.02, z = -10.49, $P < 0.0001$) with highest number of eggs laid at the tree-base level. However, as indicated by the significant 'Habitat-by-height' interaction (coef. = -0.06, SE = 0.014, z = -4.27, $P < 0.0001$)), vertical distribution of *Ae. albopictus* differed among the three habitats. In the 'forest' habitat, it exhibited a clear preference for the tree-base level with oviposition activity being lowest (although not significantly different from canopy level) at mid-tree level (Figure 4.4A). In the ‘park’ habitat, *Ae. albopictus* also exhibited significant preference for ovicups at the tree-base level with a gradual linear decrease in oviposition activity with ovicup height (Figure 4.4A). In contrast, in the ‘campus’ habitat, oviposition activity was non-existent at mid-tree level and did not differ significantly between tree-base and canopy levels (Figure 4.4A).

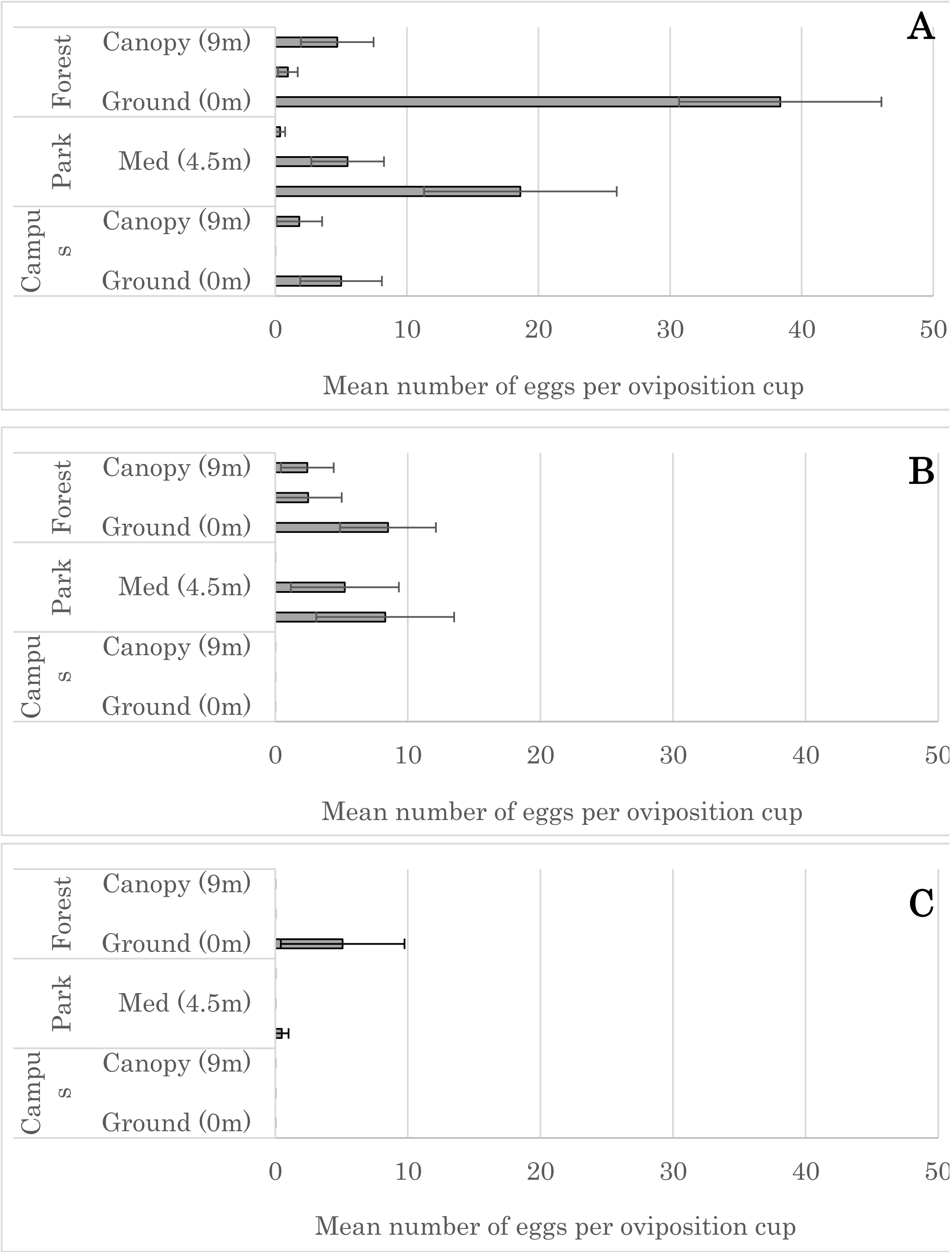

Figure 4.4. Vertical distribution patterns of *Ae. albopictus* (A), *Ae. triseriatus* (B), and *Ae. hendersoni* in the three habitats: Campus, Park, and Forest. Error Bars = SE. Letters denote significant differences in mean oviposition between heights for each habitat.

Aedes triseriatus. Given that *Ae. triseriatus* was absent from the 'campus' habitat, this analysis was limited to the 'forest' and 'park' habitats. 'Habitat' did not have a significant effect (coef. = -0.18, SE = 1.15, z = -0.15, *P* = 0.878). 'Height' had a significant effect (coef. = -0.32, SE = 0.043, z = -7.40, *P* < 0.0001) with highest number of eggs laid at the tree-base level.

As indicated by the significant 'habitat-by-height' interaction (coef. = 0.076, SE = 0.027, z = -2.89, *P* = 0.004) vertical distribution of *Ae. triseriatus* differed between these two habitats. In the 'forest' habitat, *Ae. triseriatus* occurred at all three elevations but was most abundant at the tree-base level. In contrast, at the 'park' habitat, *Ae. triseriatus* occurred quite equally at tree-base and mid-tree elevations but was completely absent from the canopy level (Figure 4.4B).

Aedes hendersoni. 'Habitat' was the only significant factor for this species (coef. = 3.29, SE = 1.66, z = 1.98, *P* = 0.048), with highest abundance in the forest habitat (Figure 4C). The effect of 'Height' was not significant (coef. = -5.74, SE = 7762.4, z = 0.00, *P* = 0.999) despite the fact that *Ae. hendersoni* oviposited only in cups at tree-base level. This was due to the 0-inflated nature of this data (Figure 4.4C).

Co-habitation patterns

In the campus habitat, as described above, only *Ae. albopictus* occurred. In the park habitat, 79% of ovistrips had a single species. Only three ovistrips contained more than one species: two with *Ae. albopictus* and *Ae. triseriatus* and one with *Ae. triseriatus* and *Ae. hendersoni.* For the forest habitat, 87.2% (n = 48 ovicups) of all ovistrips collected contained a single species. Of the remaining seven ovicups, 7.3% included ovistrips where *Ae. albopictus* and *Ae. triseriatus* co-occurred and 5.5% where all three species co-occurred. *Ae. albopictus* and *Ae. hendersoni* or *Ae. triseriatus* and *Ae. hendersoni* never co-occurred. Five out of the six co-occurrence incidents occurred at ground level and one at 9 m.

Discussion

The horizontal oviposition-site habitat selection of the three mosquito species (*Ae. albopictus*, *Ae. hendersoni*, and *Ae. triseriatus*) found in this study was strongly associated with tree cover. The highest oviposition activity occurred in the forest habitat followed by the park and the lowest activity was observed in the built environment of the campus. The three species exhibited a range of habitat selectivity, with *Ae. hendersoni* highly selective towards the forest habitat, *Ae. triseriatus* utilizing both the forest and the park habitat, and *Ae. albopictus* utilizing all three habitats. A similar pattern was observed by Reiskind et al. (2017), with host-seeking mosquitoes sampled along forest-to-field ecotones in central North Carolina. In their study, all three species (*Ae. albopictus*, *Ae. triseriatus,* and *Ae. hendersoni*) were more abundant in the deciduous forest habitat and least abundant in the open field; *Ae. albopictus* was most abundant near the forest edge. These results are consistent with the widely accepted view of *Ae. albopictus* as highly adapted to anthropogenic environments (Bartlett-Healy et al. 2012, Becker et al. 2014, Dowling et al. 2013). In contrast, *Ae. hendersoni* is known as a sylvatic species and typically occurs only in deciduous and mixed forests (Fitzgerald and Livdahl 2019). *Aedes triseriatus* is known to be a relatively sylvatic species but also common in peridomestic habitats nested within forested environments (Leisnham and Juliano 2012). Consistent with these studies, we found *Ae. hendersoni* to be the most selective towards the 'forest' habitat, while *Ae. triseriatus* was more of a habitat generalist occupying any habitat with moderate to high tree coverage.

All three mosquito species found in this study exhibited an observable degree of preference towards ovipositing at the ground level, but this varied with respect to their degree of selectivity. *Aedes hendersoni* was the most selective, ovipositing only at the ground level. *Aedes albopictus* was highly selective towards the ground level at the forest habitat, but its degree of selectivity decreased at the park habitat and even more at the campus habitat. *Aedes triseriatus* was the least selective towards the ground level and readily laid eggs at mid-elevation as well as in the canopy within the forest habitat. Based on previous studies, we expected that *Ae. hendersoni* would be found more frequently at higher elevations (Fitzgerald and Livdahl 2019, Scholl and DeFoliart 1977). However, we found *Ae. hendersoni* to be restricted to the ground level. The reason for this difference is not clear but may be associated with differences within the surrounding urban matrix. Whereas all previous studies on the vertical distribution of *Ae. triseriatus* and *Ae. hendersoni* were conducted in a relatively pristine deciduous forest, our study sites were nested within the urban landscape of downtown Greensboro. It is possible that microclimatic conditions or other biotic factors (e.g., blood-feeding hosts) associated with *Ae. hendersoni*'s affinity towards the canopy are absent when forest patches are nested within an urban matrix. The generality of this observation requires further empirical validation.

Another interesting observation was the bimodal vertical distribution of *Ae. albopictus* at the forest and campus habitats. The lowest abundance of *Ae. albopictus* eggs was collected at 4.5 m above ground. Traps at this elevation were more exposed and tended to lack foliage cover (Figure 4.2B) possibly resulting in lower levels of leaf-litter deposition and relative-humidity, factors known to adversely affect *Ae. albopictus* activity (Crepeau et al. 2013). Particularly interesting is the fact that the selectivity of *Ae. albopictus* for the ground level substantially decreased at the campus environment. It is possible that the lack of the microclimatic buffering effect of forest undergrowth and reflection of heat from adjacent impervious surfaces (e.g., building walls, pavement, asphalt) makes the ground level habitat much less attractive in terms of high temperature and low relative humidity.

Interspecific competition has often been used to explain the difference in the vertical distribution of *Ae. triseriatus* and *Ae. hendersoni* with the former tending to occur more at the ground level and the latter more at the canopy level (Fitzgerald and Livdahl 2019, Loor and DeFoliart 1970, Scholl and DeFoliart 1977). Copeland and Craig (1992) confirmed that when the two occurred in sympatry, *Ae. triseriatus* was indeed the better competitor. A recent study by Fitzgerald and Livdahl (2019) provided a strong support for this hypothesis. They observed that in allopatric populations both species tended to oviposit at ground level, but in sympatric populations *Ae. triseriatus* tended to mostly oviposit at ground level and *Ae. hendersoni* at the canopy level (Fitzgerald and Livdahl 2019). In our study, the opposite pattern was observed, with *Ae. hendersoni* solely utilizing the ground elevation. Yet, the generality of this observation should be taken with caution given the low sample size of *Ae. hendersoni* and the relatively short duration of the study. In contrast to many previous studies showing *Ae. triseriatus* to mostly oviposit at ground level (Loor and DeFoliart 1970, Scholl and DeFoliart 1977), in our study we found *Ae. triseriatus* to be more of a generalist. It did exhibit a slight preference for the tree-base, but this was weak and not statistically significant. This divergent pattern might be associated with the dominance of *Ae. albopictus* in this system. Indeed, Novak et al. (1993) demonstrated that larvae of the invasive *Ae. albopictus* were competitively dominant over larvae of *Ae. triseriatus* (Novak et al. 1993), although Livdahl and Willey's (1991) model predicted the two should be able to coexist locally within treeholes. Indeed, the forest habitat where *Ae. albopictus* was numerically the most abundant was the only habitat where *Ae. triseriatus* also oviposited at the canopy level. This observation is consistent with that of Obenauer et al. (2009) who observed that in a suburban habitat both species exhibited oviposition selectivity towards the ground level, but in the forest habitat *Ae. triseriatus* increased its selectivity towards the canopy.

At the micro-scale of the oviposition cup, mosquito species co-occurrence patterns indicated that in most cases (ca. 80%) oviposition cups were utilized by a single species only, suggesting intentional avoidance by gravid females of oviposition sites previously used by a different species as a possible way of avoiding inter-specific larval competition. In the few cases where different mosquito species co-occurred, it was mainly *Ae. albopictus* together with *Ae. triseriatus.* All co-occurrence cases occurred at the ground level and at the forest and park habitats. In these forested habitats, all three species appear to stably coexist, despite a shared preference for the ground level, whereas in the campus habitat only *Ae. albopictus* occurred. These observations are in agreement with the model of Livdahl and Willey (1991), which predicted that *Ae. albopictus* could locally coexist with *Ae. triseriatus* in forested habitats with tree holes as oviposition sites but not in more anthropogenically modified environments where artificial containers are the main oviposition sites. The shared vertical and horizontal habitat use patterns observed here suggest that interspecific competition probably plays a relatively minor role in determining the horizontal and vertical habitat use patterns in this system. It is more likely that innate adaptations to different abiotic conditions underlie the mosquitoes' habitat preferences and that variation in microclimatic conditions that occur along the urban gradient could modify these patterns of habitat use.

Overall, given the short duration, limited spatial scope of our study, and lack of data on adult vertical and horizontal distribution, caution should be taken with respect to inference about the role of interspecific competition in driving the horizontal and vertical distribution patterns of the mosquito species in this ecological system. Yet, the time of this study (late summer and early fall) is typically the period of the year when mosquito abundance peaks in this area (Wasserberg et al. 2013) and therefore reflects the time when competition is expected to be the most intense.

In terms of public health implications, many previous studies suggested that increases in the degree of anthropogenic land-use change and level of urbanization are often associated with increases in human exposure to mosquito-borne pathogens (Leisnham et al. 2009, Lenk et al. 2018, Medeiros-Sousa et al. 2019). In contrast, our study suggests that an increase in urbanization might decrease exposure to mosquito bites, both in terms of decreased abundance as well as in terms of a shift of biting activity away from the ground level. These results may suggest that the "heat-island effect," often described as facilitating of transmission of mosquito-borne pathogens in urban areas (LaDeau et al. 2015), might have an upper limit when thermal conditions become too harsh. Results of our study may also have public health implications for disease systems such as La Crosse encephalitis, where the mosquito species studied here play a major role in virus transmission in endemic regions.

CHAPTER V

THE EFFECT OF HABITAT AND TIRE INTRODUCTION ON THE VERTICAL DISTRIBUTION OF LA CROSSE VIRUS MOSQUITO VECTORS

Introduction

The distribution of LACV vectors has mainly been studied by sampling done at ground level (Andreadis et al., 2001; DeFoliart and Lisitza, 1980; Joy and Hildreth-Whitehair, 2000; Lampman et al., 1997b; Mather and DeFoliart, 1984b; Tanaka et al., 1979; Tsuda et al., 1994).However, their vertical distribution has been relatively neglected and remains an unclear, yet important, aspect of the disease system (Chadee, 2004; Dao et al., 2014; Jayathilake et al., 2015; Liew and Curtis, 2004; Loor and DeFoliart, 1970; Mercer et al., 2010; Novak et al., 1981; Obenauer, 2009; Scholl and DeFoliart, 1977; Sinsko and Grimstad, 1977). Most work on this topic has been done about the system of

Aedes triseriatus (Say) (Diptera: Culicidae) and *Ae. hendersoni* (Cockerell). It was found that when in sympatry, *Ae. triseriatus* oviposited in greater abundance at lower elevations while *Ae. hendersoni* oviposited almost exclusively at higher elevations

(Sinsko and Grimstad 1977). Other studies have shown similar results consistent with vertical niche partitioning, suggesting that *Ae. triseriatus* prefers to oviposit at ground level and *Ae. hendersoni* at the canopy (Loor and DeFoliart 1970, Scholl and DeFoliart

1977). In contrast, Novak et al. (1981) collected *Ae. triseriatus* and *Ae. hendersoni* adults primarily from the forest canopy (21 m) compared to ground level (2 m). Recently, Fitzgerald and Livdahl (2019) demonstrated that the reported differences in *Ae. triseriatus* and *Ae. hendersoni* vertical stratification oviposition patterns may be, in part, explained by interspecific competition. This was supported by the convergence of habitat use towards lower elevations in allopatric populations and divergence in areas where the two species occur in sympatry (Fitzgerald and Livdahl 2019).

The main invasive species to LACV endemic areas are *Ae. albopictus* or *Ae. japonicus* (Leisnham and Juliano 2012). As shown in my study (see, previous chapters) and by others (Haddow et al. 2009, Leisnham and Juliano 2012, Westby et al. 2015, Rowe et al. 2020, Tamini et al. 2021), *Ae. albopictus* tends to occur in warmer open fields and in more urbanized environments whereas *Ae. japonicus* prefers cooler and shaded environments with a strong affinity to forest-field edge habitat. Relatively little amount of information is available about the vertical distribution of *Ae. albopictus* and no published information is available about the vertical distribution of *Ae. japonicus*. Jayathilake et. al (2015) studied the vertical distribution of *Ae. aegypti* and *Ae. albopictus* along high-rise buildings in an urban setting in Colombo, Sri Lanka. They observed differential vertical distribution, with *Ae. albopictus* laying eggs exclusively between ground level and 6.1 m heights and *Ae. aegypti* laying eggs at all elevations with no clear vertical pattern (Jayathilake et al. 2015). Williges et al. (2014) also found *Ae. albopictus* ovipositing primarily at ground level when compared to 1, 2, 3, and 4 m heights in a wooded residential neighborhood in New Jersey (Williges et al. 2014). As described in the previous chapter, in the area of UNCG campus *Ae. albopictus* exhibited a clear preference for ground level in the forest and the park habitats, but in the campus habitat it exhibited a bimodal distribution ovipositing only at the ground level and higher canopy and absent from the middle height. The vertical distribution of *Ae. japonicus* has not yet been studied.

Dr. Brian Byrd’s group recently investigated species-specific oviposition patterns within the Western Carolina University campus and the vicinity (unpublished data). *Aedes hendersoni* most commonly oviposited at higher elevations (i.e., 6m and

9m) but, nevertheless, also ovipositing over 30% of its eggs at lower elevations (0m and 3m). The oviposition rate of *Ae. hendersoni* at ground level was higher in forested sites compared to urban sites. In that study, *Ae. triseriatus* did not display any significant differences in oviposition along the vertical gradient. Both invasive species, *Ae. albopictus* and *Ae. japonicus*, appeared to most commonly oviposit at ground and middle levels (i.e., 0m and 3m) with less than 25% of their egg collections from higher elevations.

As I demonstrated in the previous chapter (Chapter IV), the vertical distribution of native and invasive mosquitoes differs within an urban landscape when comparing between a built environment (UNCG campus), recreational park (golf course), and a relatively undisturbed natural remnant forest. Here, my goal was to determine if the vertical distribution of native and invasive container-breeding mosquitoes differed along the forest-to-field ecotones in a rural landscape of the southern Appalachian Mountains. However, more than that, I wanted to evaluate if an experimental addition of artificial containers could affect the vertical distribution of these mosquitoes along the forest-to-field ecotone. I appended this experiment to the already running experiment on the effect of tire addition (Chapter III) by using the same vertical distribution sampling method described in Chapter IV in order to compare the vertical distribution of the mosquitoes in each of the three ecotonal habitats (forest, edge, field) for study plots containing or not containing experimentally introduced tires. I hypothesized that in the experimental plots, tire addition will modify the natural abundance and distribution patterns of the local mosquitoes. This in turn, was expected to affect the vertical distribution of the local mosquitoes.

Methods

Study sites. The study was conducted in the Tuckasegee valley located in Jackson County, western North Carolina, USA (35°16'52.9"N, 83°08'22.8"W; elevation: 660m above sea level). We deployed traps in 6 sampling plots on privately-owned land, each characterized by a forest-to-field ecotone. The forest sections were composed of mixed hardwoods (e.g., Hickory-Oak) and the field sections were used as grazing land for cattle. The 6 study sites were all located within a ca.1.60 square kilometers area. On each plot we placed oviposition traps (i.e., ovitraps) along a tree in 3 habitats, the ecotone edge, the forest (i.e., 100 meters from edge) and the field (i.e., 100 meters from edge) (Fig. 5.1A). Three ovitraps were placed in each habitat along a vertical axis, for a total of 9 ovitraps per site and 54 ovitraps throughout all 6 sites. We collected eggs from ovitraps a total of 9 times, seven times between July and

September of Year 1 (i.e., 2012) and twice between July and August of Year 2 (i.e., 2013).

Vertical oviposition trap system. We positioned oviposition traps at three levels along a vertical axis, including: ground level, 4.5 meters (i.e., “mid”), and 9 meters above ground (i.e., “high”), using a pulley system (Fig. 5.1B-C), that enabled us to collect and replace the ovistrips as well as water on a weekly basis.

Artificial container introduction. A month prior to the start of this study, 9 tires were introduced to either the forest section (2 replicate sites) or the field section of the plot (2 replicate sites) (Fig. 5.1). Two sites did not receive the tire treatment and, thus, served as control sites.

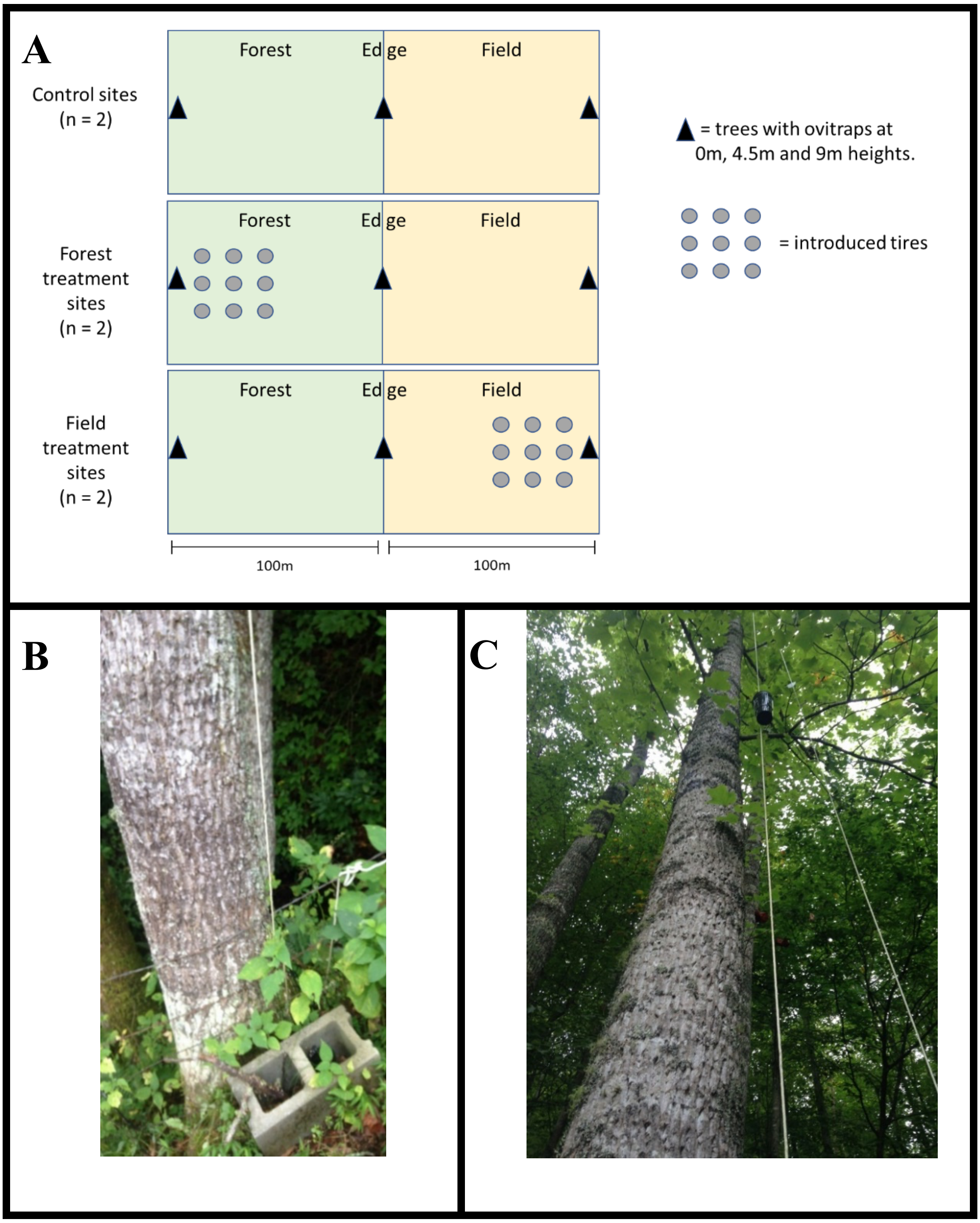

Figure 5.1. A. Schematic of study plots with control sites and sites with introduction of tires in field or forest habitat. Triangles denote the location of trees in each of the 3 habitats (forest, edge, field) along which the ovitraps were placed at ground (0m), medium elevation (4.5m) and canopy (9m); **B.** Cinder block at ground level used to secure pulley and place ground level ovitrap; **C.** Pulley system for vertical placement of oviposition traps Ovitrap at 4.5 meters above ground

Lab methods. The processing of ovistrips and hatching of eggs followed methods described in Chapter I. Once at the fourth instar stage, larvae were killed by submerging in hot (i.e., 80-100 Celsius) water and then immediately transferred to a 70-80% ethanol solution. Preserved larvae were then identified to species using an identification guide for the mid-Atlantic region. To distinguish between sister siblings *Ae. triseriatus* and *Ae. hendersoni*, particular attention was given to the length ratio and branch number of the 1-X setae as well as branching of 1-S setae (Harrison et al., 2016).

Data reductions and statistical analysis. The species-specific egg number laid on each ovistrip was inferred by multiplying the fraction of emergent adult of each by the total number of eggs on that ovistrip (hereafter, 'inferred egg count'). Given the non-normal distribution of the eggs as count data (Shapiro-Wilk test: $P < 0.001$), a negative binomial regression generalized linear model was used.

I first tested the effect of habitat, elevation, and tire treatment on the 'overall number of eggs' and then analyzed all species-specific effects separately using the inferred eggs numbers. Analyses were done using SPSS 25 (IBM Corporation).

Results

A total of 38,820 mosquito eggs were collected from 54 ovitraps across 9 separate collections, 7 weekly collections from July 28th to September 9th, 2012 and 2 collections from 2013, July 13th and August 17th. More eggs were collected from sites with tires in the forest habitat (i.e., forest treatment) compared to control sites (i.e., no tires) and sites with tires in the field habitat (i.e, field treatment) (Table 1). The majority (66%) of eggs collected were *Aedes triseriatus* (n = 21,854), followed by *Ae. japonicus* (20%) (n = 6,624), with *Ae. albopictus* (8.3%) (n = 2,762) and *Ae. hendersoni* (6%) (n=1,917) being the least common (Table 5.1).

Table 5.1. The effect of tire introduction on overall and species-specific oviposition activity

	Control (±SE)	Field treatment (±SE)	Forest treatment (±SE)
Overall	77.1±5.4^{ab}	61.9±4.6^{a}	108.7±8.1^{b}
Aedes triseriatus	43.4±3.4^{ab}	33.6±2.6^{a}	62.4±4.9^{b}
Aedes japonicus	13.3±2.3^{ab}	8.0±1.7^{a}	21.0±2.9^{b†}
Aedes albopictus	3.7±0.7	4.2±0.9	9.8±1.8
Aedes hendersoni	7.7±1.4^{a}	1.8±0.5^{b†}	2.7±0.5^{ab}

Significance is based on $P < 0.05$ for Post-hoc comparisons with Bonferroni corrections (t-Test: Two-Sample Assuming Equal Variances).
† = denotes suggestive ($0.05 < P < 0.10$) but not significant (after Bonferroni correction) differences.

Species specific analysis vertical distribution

Aedes triseriatus.

I found a significant effect of habitat, height, tires and all interactions (AICΔ=41 compared to closest model with no 3-way interaction) (Table 5.2). Interestingly, the highest number of eggs were laid in the field habitat (31.74±3.2), followed by the forest (26.42±2.7), and the edge (21.68±2.2) habitats. Moreover, there was a negative association with height. We collected more eggs at ground level (50.0±5.3), followed by medium height (34.8±3.2) and canopy (11.13±1.1). Tire introduction to the forest had the higher overall impact on mean, per-plot, oviposition activity compared with the control. Surprisingly, tire introduction to the field resulted in non-significant reduction in mean oviposition activity compared with the control (Fig. 5.2).

Table 5.2. GLM Negative Binomial regression model for *Aedes triseriatus* oviposition activity.

Test of Model Effects

	Wald Chi-Square	**df**	**Sig.**
(Intercept)	2472.521	1	.000
Habitat	14.249	2	.001
Height	111.119	2	.000
Tires	27.826	2	.000
Habitat x Height	71.089	4	.000
Habitat x Tires	52.356	4	.000
Height x Tires	48.479	4	.000
Habitat x Height x Tires	46.620	8	.000

AIC = 2809.00

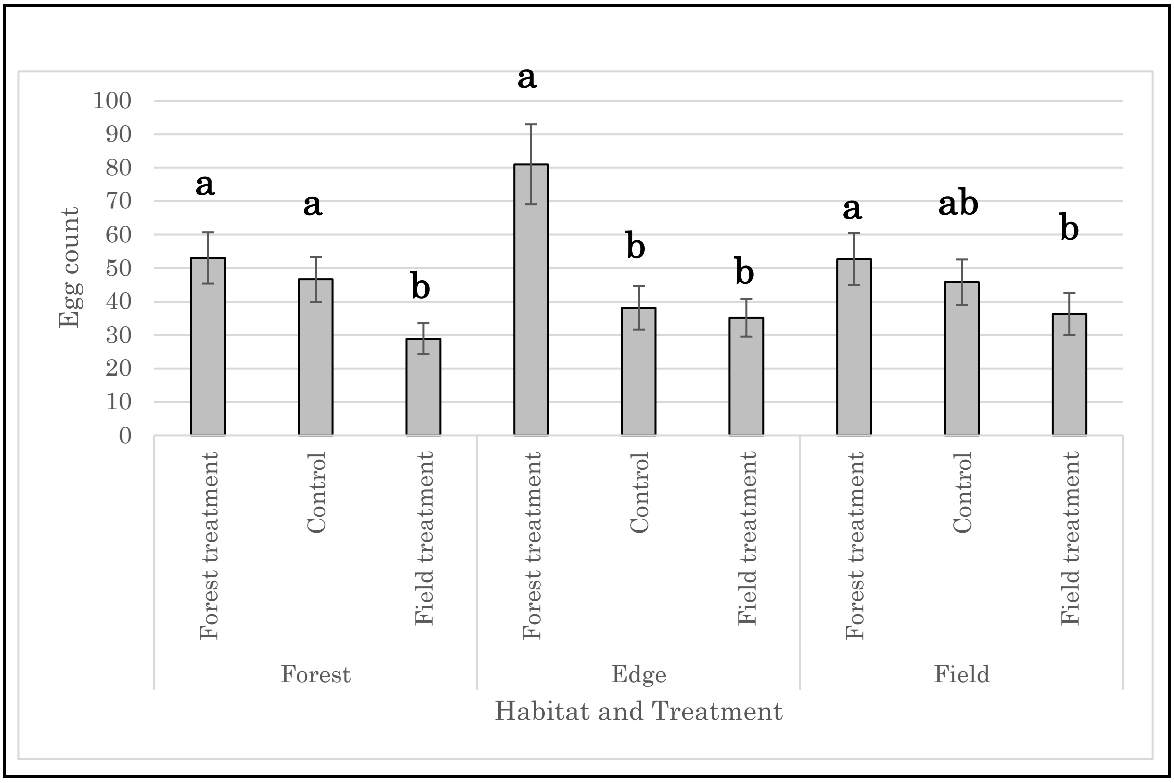

Figure 5.2. *Aedes triseriatus* oviposition activity by habitat and tire introduction treatment. Significance is based on p<0.05 for Post-hoc comparisons with Bonferroni corrections (t-Test: Two-Sample Assuming Equal Variances).

As indicated by the 'Habitat x Height' interaction, vertical distribution of *Ae. triseriatus* differed among the habitats. This pattern is particularly clear in the 'control' plots where vertical distribution exhibited no clear pattern in the forest or the field habitat but a clear preference for ground level was exhibited in the edge habitat (Fig. 5.3A). However, the tire introduction treatment appears to sharply modify the vertical distribution of *Ae. triseriatus*, as indicated by the significant 'height x tire' interaction (Table 5.2), with a relatively less distinct vertical distribution in the control plots but a strong preference for ground level at both forest- and field-treatment sites

(Fig. 5.2). The significant three-way interaction of “habitat, height, and tire” suggest that the effect of tire introduction on the vertical distribution differs among the habitats. Indeed, although tire addition enhanced the vertical patterning of oviposition activity distribution in both forest-tire addition and field-tire addition plots, the habitat-specific vertical pattern appeared to differ. For example, in the forest treatment sites, vertical distribution was significant in the forest and edge habitat but not in the field habitat (although the general pattern was similar) while in the field introduction treatment vertical distribution was significant in all three habitats (Fig. 5.3).

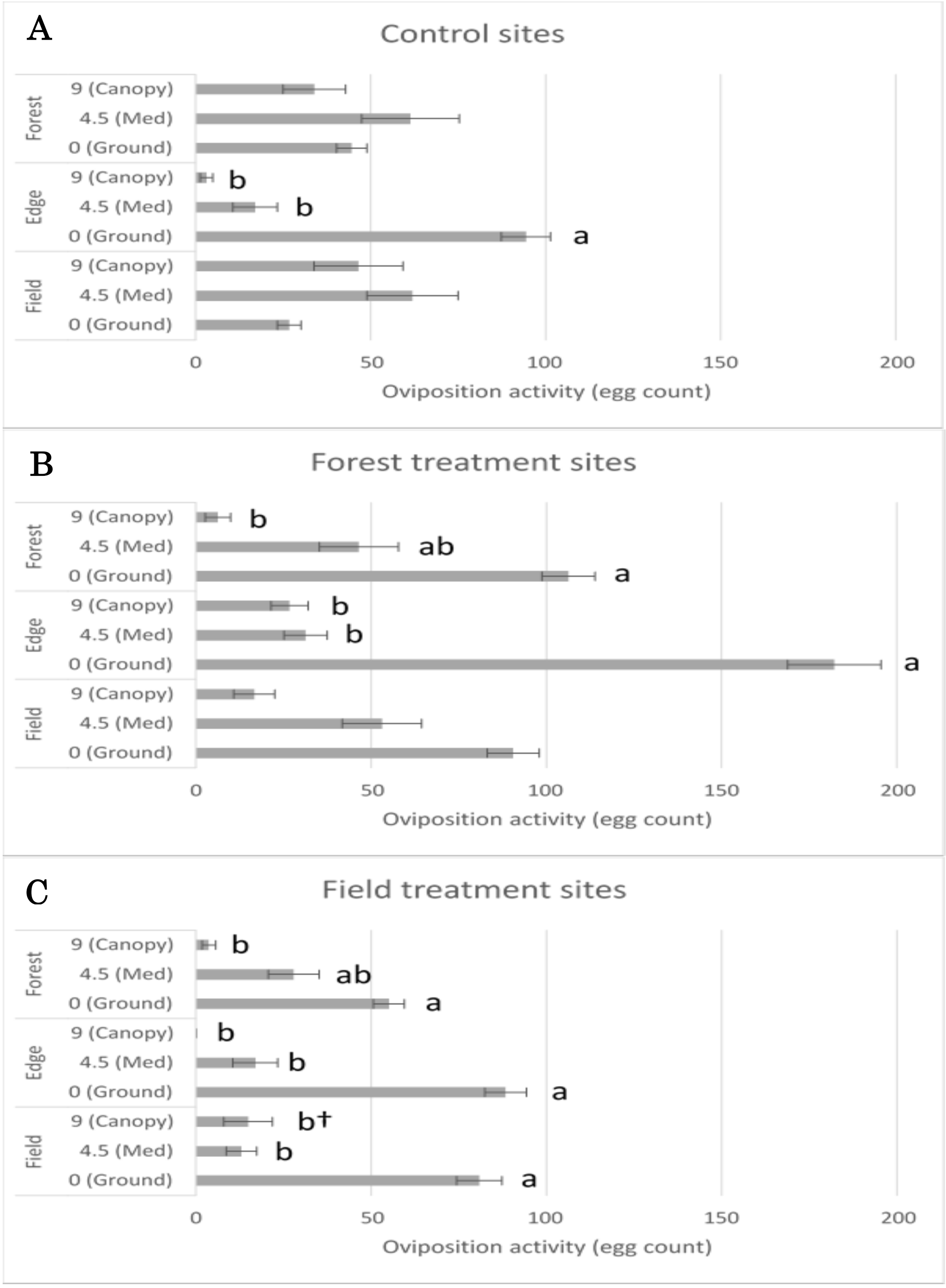

Figure 5.3. *Aedes triseriatus* oviposition activity by tire introduction treatment. A. Control sites; B. Forest tires treatment sites; C. Field tires treatment sites. Significance is based on P < 0.05 for Post-hoc comparisons with Bonferroni corrections (t-Test: Two-Sample Assuming Equal Variances).

Aedes hendersoni.

I found a significant effect of habitat, height, tires, habitat-by-tires and height-by-tires interactions, as well as of sampling month (ΔAIC=246 compared to closest model without 'month' factor) (Table 5.3). *Aedes hendersoni* was most abundant in the forest habitat (6.47±1.9), followed by the field (4.76±1.7), and the edge (3.12±2.1). It exhibited a clear and significant average affinity for the height elevation of 9 m (6.53±2.0), followed by 4.5 m (4.52 ±1.5) and ground level (3.16±2.1). Tire introduction actually had a significant negative effect on *Ae. hendersoni* oviposition activity both at forest- (3.03±1.0) and field-tire introduction plots (2.17±1.1) compared with the control plots (9.06±3.0). The 'habitat x height' interaction was not statistically significant consistent with the fact that, overall, *Ae. hendersoni* exhibited general affinity for higher elevations in all habitats (Fig. 5.4). Most important, as indicated by the 'tire x height' interaction, was the finding that tire introduction affects the vertical distribution of this species. In the control plots, *Ae. hendersoni* exhibited a clear preference for the height elevation (9 m) in both the forest and field habitat and, surprisingly, preference for the ground habitat in the edge habitat. In contrast, in the treatment plots of both treatments there appears to be increased affinity for the medium height. This pattern is particularly strong in the forest treatment plots and less so (and mainly in the edge habitat) in the field treatment plots.

‘Habitat x tire’ was also significant. The effect of tire introduction in the forest had a significant negative effect on *Ae. hendersoni* oviposition activity in both forest and edge habitats but not in the field, however, tire introduction in the field had a significant negative effect in the field and forest habitats but not in the edge habitat (Fig. 5.4). Month effect was also significant, with *Ae. hendersoni* more common in the early summer than later periods (July: 9.81±1.6; August: 3.14±0.7; September: 1.15±0.3).

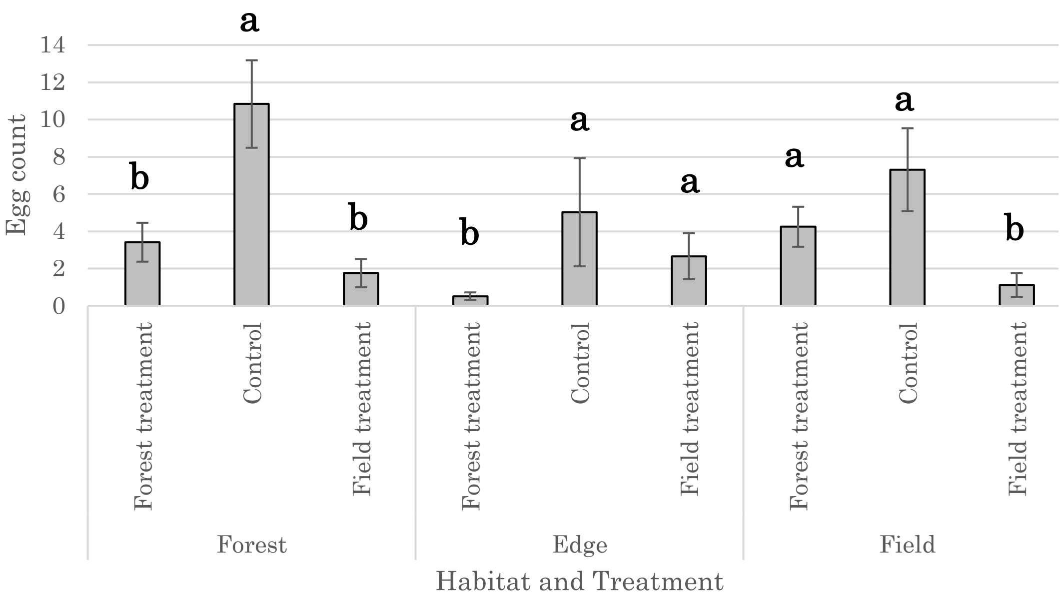

Figure 5.4. *Aedes hendersoni* oviposition activity by habitat and tire introduction treatment. Significance is based on $P < 0.05$ for Post-hoc comparisons with Bonferroni corrections (t-Test: Two-Sample Assuming Equal Variances).

Table 5.3. GLM Negative Binomial regression model for *Aedes hendersoni* oviposition activity.

Test of Model Effects

	Wald Chi-Square	**df**	**Sig.**
(Intercept)	.001	1	.971
Habitat	9.319	2	.009
Height	69.982	2	.000
Tires	32.995	2	.000
Habitat x Tires	71.953	4	.000
Height x Tires	108.497	4	.000
Month	163.686	2	.000

AIC = 1,008.334

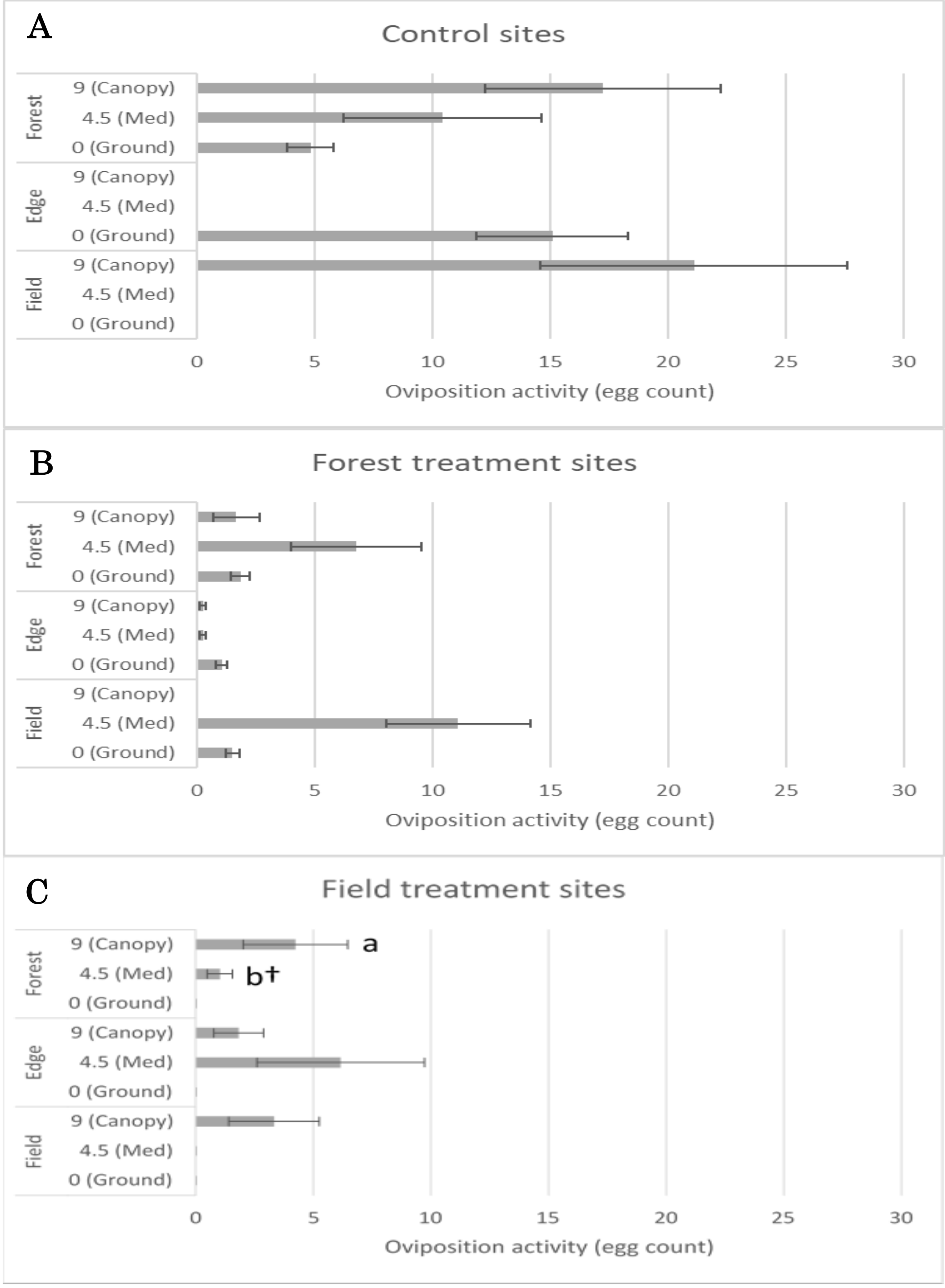

Figure 5.5. *Aedes hendersoni* oviposition activity by tire introduction treatment. A. Control sites; B. Forest tires treatment sites; C. Field tires treatment sites. Significance is based on P < 0.05 for Post-hoc comparisons with Bonferroni corrections. † = denotes suggestive (0.05 < P < 0.10) but not significant (after Bonferroni correction) differences.

Aedes albopictus

The best model (ΔAIC=15 compared to closest model without 'month' factor) included significant effects of: habitat, height, tires, habitat-by-height, habitat-by-tires, and height-by-tire, height-by-tires interactions, and sampling month (Table 5.4). *Aedes albopictus* was, on average, most abundant in the field habitat (9.28±3.4), followed by the edge (7.10±2.2) and forest habitats (3.80±1.5). It also tended to oviposit mostly at ground level (17.90±4.1), much less at mid-elevation (2.00±1.0) and at high elevation (0.41±0.4). Tire introduction in the forest resulted in a significant increase in *Ae. albopictus* oviposition activity (9.78±1.8) compared with control (3.65±0.75) and field treatment (4.18±0.9). As indicated by the Habitat x Height interaction, it tended to be less ground-specific in the forest habitat compared with the edge and the field habitats (Fig. 5.6). However, tire introduction (as supported by the 'Habitat x Tires' interaction) appears to have strengthened this species' affinity to the ground level in comparison with the control (Fig. 5.7). The introduction of tires in the forest had a significant positive effect on *Ae. albopictus* oviposition compared to control. There was also a significant positive effect of tires introduced in the forest on *Ae. albopictus* oviposition activity in the edge habitat. However, tire introduction had a significant negative effect in the forest habitat (Fig. 5.7). The month effect was also significant, with *Ae. albopictus* displaying a hump-shaped distribution, peaking in August (9.06±2.6) and less abundant in July (3.21±1.4) and September (5.2±1.9).

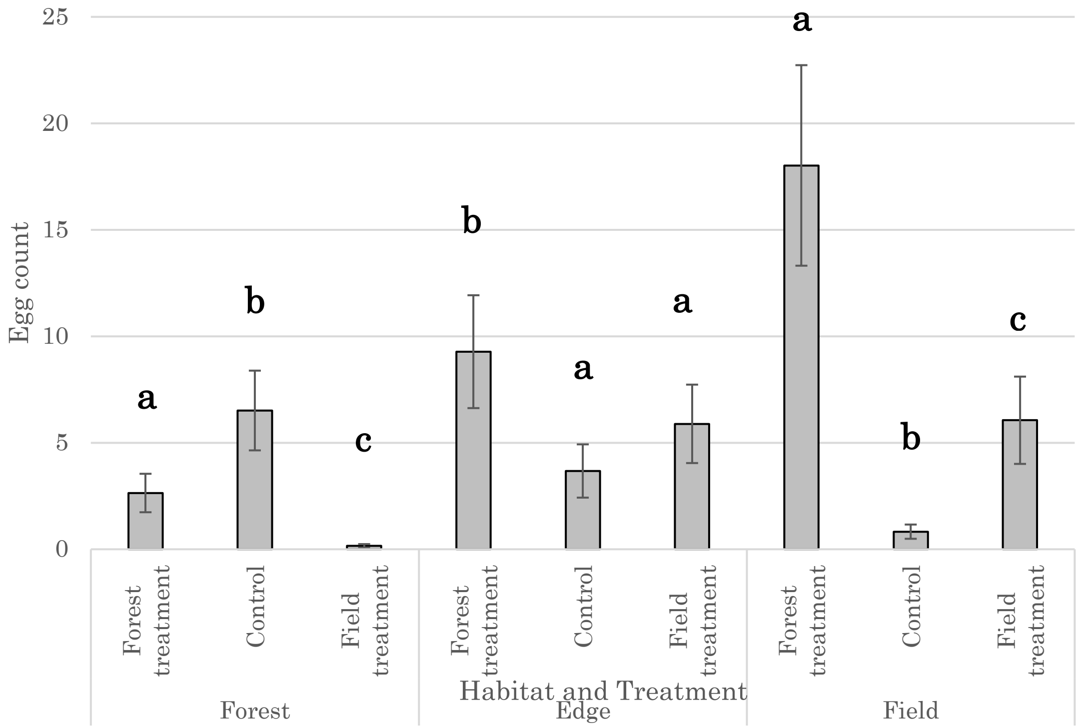

Figure 5.6. *Aedes albopictus* oviposition activity by habitat and tire introduction treatment. Significance is based on P < 0.05 for Post-hoc comparisons with Bonferroni corrections (t-Test: Two-Sample Assuming Equal Variances).

Table 5.4. GLM Negative Binomial regression model for *Aedes albopictus* oviposition activity.

Test of Model Effects

	Wald Chi-Square	**df**	**Sig.**
(Intercept)	0.109	1	.741
Habitat	39.513	2	.000
Height	41.889	2	.000
Tires	21.846	2	.000
Habitat x Height	45.190	1	.000
Habitat x Tires	23.939	2	.000
Height x Tires	8.371	1	.004
Month	16.979	2	.000

AIC = 591.891

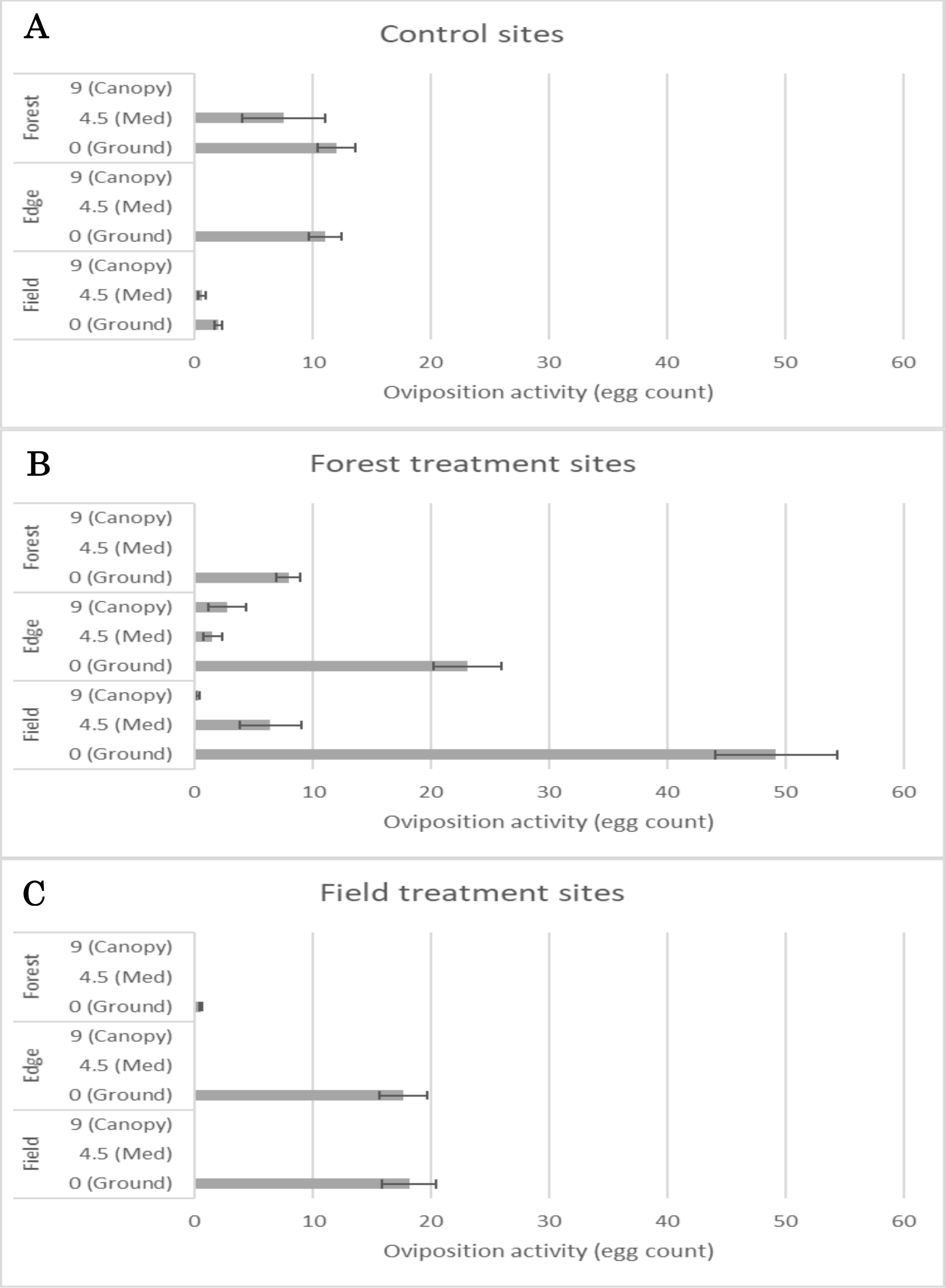

Figure 5.7. *Aedes albopictus* oviposition activity by tire introduction treatment. A. Control sites; B. Forest tires treatment sites; C. Field tires treatment sites. Significance is based on $p<0.05$ for Post-hoc comparisons with Bonferroni corrections (t-Test: Two-Sample Assuming Equal Variances).

Aedes japonicus

The best model (ΔAIC=184 compared to the closest model with 'month' factor) included all main effects of habitat, height, and tire introductions and a 'habitat x tire' interaction (Table 5.5). As described in previous chapters, oviposition activity of this species was highest at the edge (24.18±5.4), followed by the forest (13.26±4.1) and the field (4.51±2.1) habitats. Most outstanding was its almost absolute use of the ground level (41.90±6.7), with very low use of mid elevation (0.82±0.7) and no use of the higher elevation (Fig. 5.9). Tire introduction resulted in significant increase in oviposition activity in the forest-introduction plots (21.00±5.0) in comparison to field-introduction (8.00±3.0) or the control (13.25±4.2). Tire introduction to the forest resulted in an increase in oviposition activity in both forest and field habitats compared with the control. The introduction of tires in the field resulted in an increase in *Ae. japonicus* oviposition activity in the field compared with the control but also a decrease in the edge habitat (Fig. 5.8).

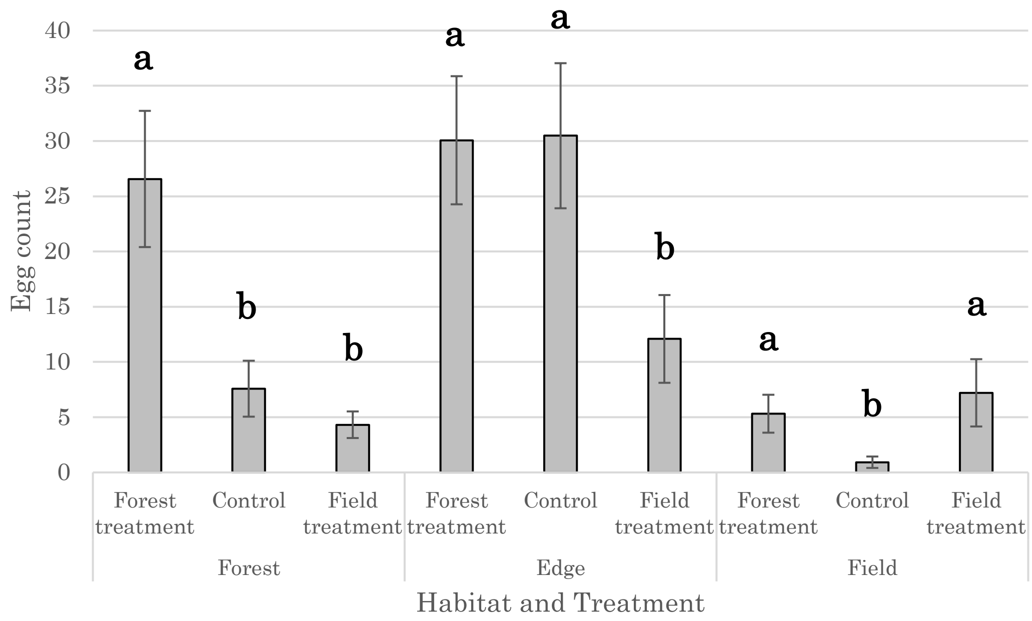

Figure 5.8. *Aedes japonicus* oviposition activity by habitat and tire introduction treatment. Significance is based on P < 0.05 for Post-hoc comparisons with Bonferroni corrections (t-Test: Two-Sample Assuming Equal Variances).

Table 5.5. GLM Negative Binomial regression model for *Aedes japonicus* oviposition activity.

Test of Model Effects

	Wald Chi-Square	**df**	**Sig.**
(Intercept)	.960	1	.327
Habitat	133.641	2	.000
Height	246.836	1	.000
Tires	86.648	2	.000
Habitat x Tires	55.320	4	.000

AIC = 1,069.504

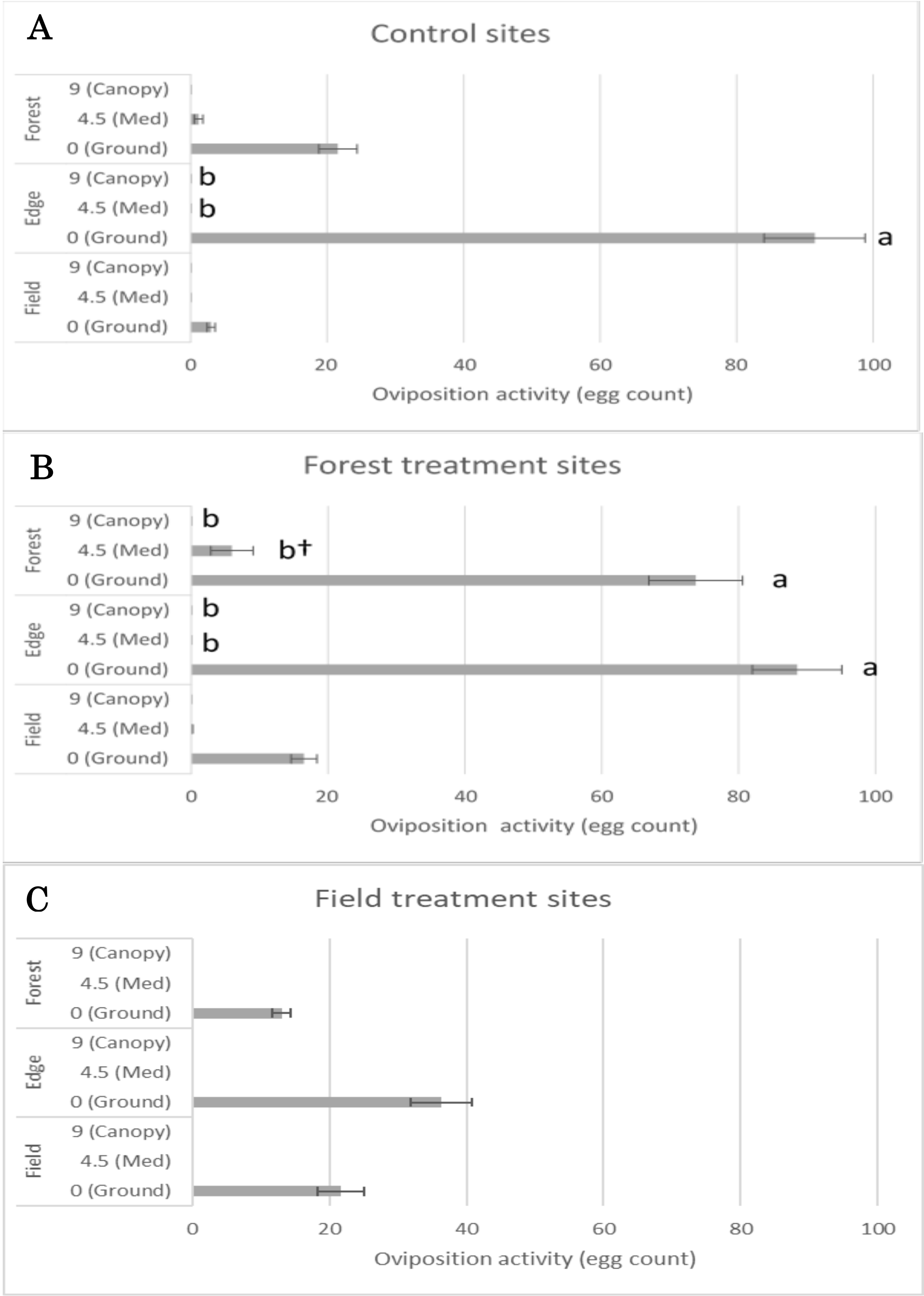

Figure 5.9. *Aedes japonicus* oviposition activity by tire introduction treatment. A. Control sites; B. Forest tires treatment sites; C. Field tires treatment sites. Significance is based on $P < 0.05$ for Post-hoc comparisons with Bonferroni corrections. † = denotes suggestive ($0.05 < P < 0.10$) but not significant differences.

Discussion

The effect of horizontal distribution on vertical distribution along the forest-to-field ecotone.

As in our previous study (Chapter III) on the effect of horizontal distribution on vertical distribution along an urban-to-forest ecotone at the UNCG campus, our results here exhibited that, for some of our study species, vertical distribution differed among the ecotonal habitats studied here. A significant 'habitat-by-height' interaction was exhibited for *Ae. triseriatus* and *Ae. albopictus* but not for *Ae. japonicus*. As shown in the control plots, *Ae. triseriatus* did not exhibit any particular vertical pattern in the forest or the field habitat but exhibited a clear vertical pattern in the edge where it was most abundant at ground level, followed by mid-level, and canopy levels. With *Ae. hendersoni*, even though the 'habitat-by-height' interaction was not significant (probably due to small sample size), this species exhibited a clear preference for the canopy level at the forest and field habitat but utilized only the ground level in the edge habitat. However, in general, these two species (*Ae. triseriatus* and *Ae. hendersoni*) exhibited vertical partitioning fairly similar to what has been reported in the past with the former being relatively generalist with slight affinity to ground level and the latter preferring the higher elevations (Fitzgerald and

Livdahl, 2019; Novak et al., 1981; Obenauer et al., 2009; Scholl and DeFoliart, 1977; Sinsko and Grimstad, 1977). With *Ae. albopictus*, similar to *Ae. triseriatus*, in the control plots equally using the ground and middle elevations (with a slight preference for the ground level), but completely absent from the canopy elevation, in the forest and field habitat. However, it exclusively used the ground level in the edge habitat. Similar to the pattern at the UNCG campus (chapter IV), lowest affinity for ground level was observed in the open field habitat. In contrast to all other species, in the control plots, *Ae. japonicus* used almost exclusively the ground level. It is interesting to note that in the edge habitat this affinity was particularly strong. This strong affinity for oviposition on the ground level at the edge habitat by all species is a novel and unique observation. The cause of this pattern is far from being clear. Trees used in this experiment were located either in the inner forest, the outer field, or the very edge of the forest facing the field. In the inner forest abiotic conditions are probably favorable at all elevations while in the outer field condition are probably unfavorable at all elevations. In contrast, trees located at the very edge of the forest may face relatively dry conditions at the mid- and canopy elevations. In contrast, as described in chapter 2, the edge and the adjacent field margins areas are characterized by highest degree of undergrowth plant cover, which may provide relatively favorable microclimatic conditions in terms of lower heat and higher relative humidity. This is, obviously, a speculation and more research should follow in order to evaluate the repeatability of this pattern and elucidate its cause.

The effect of tire introduction on the vertical distribution of LACV mosquitoes along the forest-to-field ecotone.

Aedes triseriatus and *Ae. albopictus* exhibited a significant 'tire-by-height' statistical interactions, which support the assertion that tire introduction affects the vertical distribution of these mosquitoes. For both species, tire addition at either the forest or field habitat caused a switch in their vertical habitat use from being slight or indistinguishable in the control plots to being strongly biased towards ground level at the experimental plots. This was particularly clear at the forest and field habitats and less so in the edge habitat. With *Ae. hendersoni* a similar, yet non-significant, trend was observed. In the control plots, as expected, this species exhibited a preference for ovipositing at the canopy level at the forest and field habitats. Yet, in the experimental plots the degree of this preference appears to have decreased while preference for mid-elevation appears to have increased. Furthermore, tire introduction resulted in an overall negative effect on *Ae. hendersoni*'s oviposition activity compared with the control plots, suggesting possibly an in-direct inter-specific effect. Yet, such a possibility is inconsistent with the trends *Ae. triseriatus* exhibited, which given its increased preference for ground level would be expected to actually enhance *Ae. hendersoni*'s canopy level habitat use. Clearly, this question also requires further assessment. With *Ae. japonicus*, tire addition treatment did not affect its vertical habitat use which was consistently and strongly biased towards ground level use. Yet, tire addition resulted in decrease in *Ae. japonicus*' abundance at the field treatment habitat, which might have been driven due to the increase in *Ae. albopictus*' abundance in those plots.

Comparison of my results with findings from other groups working in this area

Brian Byrd's group (Western Carolina University) have been working on a similar topic of vertical distribution of LACV vectors in and around the University's campus. Their results have not been published yet but have been presented in a poster at the Entomological Society of America national conference (Riles et al., 2012). Similar to my findings, they showed that, the invasive species *Ae. albopictus* and even more *Ae. japonicus* preferred laying eggs at or close to the ground level while the native species (*Ae. triseriatus* and *Ae. hendersoni*) exhibited either no vertical preference or preference for the canopy levels, respectively. This pattern, with respect to *Ae. triseriatus* being a vertical generalist and *Ae. albopictus* being ground level specialist, were also observed in our UNCG verticals study. *Aedes japonicus* was not detected in that study. Surprisingly, *Ae. hendersoni* in that study showed non-significant preference for the ground level. Fitzgerald and Livdahl (2019) studied the vertical distribution of *Ae. triseriatus* and *Ae. hendersoni* and found the former ovipositing primarily at ground level and the latter at the canopy. Interestingly, in allopatric sites, the proportion of *Ae. triseriatus* oviposition activity showed an intermediate height preference and that of *Ae. hendersoni* showed lower habitat preferences (Fitzgerald and Livdahl, 2019). Obenaeur et al (2009) surveyed the oviposition activity of *Ae. triseriatus* and *Ae. albopictus* in Florida and found *Ae. triseriatus* exhibiting preference for higher elevations (6m) and *Ae. albopictus* preferring the lower elevations (1m) (Obenauer et al., 2009). Williges et al (2014) also found *Ae. albopictus* primary ovipositing at ground level. These studies support my findings concluding that *Ae. albopictus* prefers to oviposit at ground level, *Ae. hendersoni* is most commonly found at higher elevations, and that the oviposition of *Ae. triseriatus* may vary, potentially due to competitive pressures and habitat characteristics.

Study limitations

Conclusions regarding the effect of treatment were determined based on comparisons between control sites (i.e., no tires) and treatment sites (i.e., tires). Although I established a baseline for these sites in chapter 2, I do not have baseline data for the specific locations of the vertical traps deployed in this study comparing oviposition activity prior to the introduction of tires. Inferences regarding the effect of tire introduction should acknowledge this limitation. In addition, collections from the tires (described in chapter 3) showed the presence of the *Toxorhynchites rutilus* mosquito in these sites, a highly effective predator of *Aedes spp.* larvae (Campos and Lounibos, 2000; Kesavaraju and Juliano, 2004). The extent to which the abundance and distribution of LACV vectors was mediated by this predator is unknown and should be explored in future studies. Moreover, ovitraps were placed along 3 trees in each plot (one per habitat). However, given the experimental design, the study is limited to 2 replicates per habitat and treatment. The data may, therefore, be heavily influenced by characteristics specific to the trees on which the ovitraps were deployed.

Implications of our results and future directions

This study is the first to evaluate, experimentally, factors that could potentially affect the vertical distribution of the oviposition activity of mosquitoes. Specifically, it tested the effect of artificial containers on the vertical distribution of LACV mosquitoes. Assuming our findings are consistent and representative (see study limitations section), our findings suggest the introduction of artificial containers may influence the oviposition activity of LACV vectors, in particular *Ae. triseriatus* and *Ae. albopictus* by either attracting females to oviposit at ground level and/or by enhancing hatching success. Moreover, the introduction of tires was associated with a significant increase in abundance of both *Ae. triseriatus* and *Ae. albopictus*. Therefore, artificial containers likely increase the risk of LACV transmission in endemic areas, such as forests and adjacent fields. Public health initiatives should therefore prioritize educating the public and target refuse in LACV endemic areas.

CHAPTER VI

GENERAL DISCUSSION

In this study, I investigated the effect of two types of typical anthropogenic effects, forest fragmentation and larval habitat supplementation through the introduction of artificial containers, that could affect the risk of LACE emergence in the south Appalachian region. First (Aim 1), I characterized the distribution of LACV vectors along forest-to-field ecotones. Then, in Aim 2, by introducing tires to the forest or the field habitats of this ecotone, I tested the effect of this disturbance on the distribution and abundance (based on oviposition activity and resting adult distribution), behavior (i.e., host seeking adults), and performance (i.e., parity rate, body size) of these LACV vectors. The vertical distribution of container breeding mosquitoes is a neglected, yet important aspect of their biology. Furthermore, it is not known if this vertical distribution differs among typical habitats and whether and how native and invasive mosquito species differ in that respect. I evaluated this question in two types of landscapes: an urban landscape in a forest-park-campus ecotone, conducted at the University of North Carolina at Greensboro (UNCG) campus area (Aim 3) and within a rural landscape of the Appalachian mountains along forest-to-field ecotones (Aim 4). In the latter aim, I also evaluated the effect of tire introduction (as described in Aim 2) on the vertical distribution.

Results summary.

In **Aim 1**, I showed that forest fragmentation and the formation of a new type of landscape composed of fields, forest patches, and edges has a determining effect on the identity of LACV vectors, with *Ae. triseriatus* mostly occurring in forest patches, *Ae. albopictus* in the field patches, and *Ae. japonicus* in the edge habitats. Furthermore, tree density and tree size had, generally, positive effects on the abundance of these species. However, the type and degree of this effect varied across the different ecotonal habitats. Given that all three species are competent LACV vectors and each was found to be most abundant at different habitats along the ecotone, this indicates that no place within this rural landscape is "safe" and that residents and visitors to these areas should always be vigilant and use the necessary personal protection measures to reduce their levels of exposure. Yet, a greater entomologic risk occurs in forest or edge habitats where mosquito activity and abundance appear to be elevated.

Following the introduction of tires (**Aim 2**), oviposition activity substantially increased at both the forest-tire introduction and field-tire introduction sites.

Specifically, tires were associated with a higher abundance of *Ae. triseriatus* and *Ae. albopictus*, but also with a decrease in *Ae. japonicus*. Interestingly, the scale of the tire introduction effect was not limited to the habitat to which it was introduced, but also had effects on the distribution and abundance of mosquitoes at other habitats along this ecotone. In terms of the temporal scale, tire introduction had quite an immediate effect with mosquito distribution and abundance affected within a few weeks following tire introduction. The effect of tire introduction persisted throughout all three years of the experiment, although the effect appeared to be diminishing in the third year. When exploring the identity and abundance of mosquitoes in the tires, we found that *Ae. triseriatus* was dominant in tires in the forest habitat (*Ae. triseriatus* [N=506] > *Ae. japonicus* [N=167] > *Ae. albopictus* [N=29] > *Ae. hendersoni* [N=15]) and *Ae. albopictus* dominated the tires in the field (*Ae. albopictus* [N=917] > *Ae. japonicus* [N=393] > *Ae. triseriatus* [N=213] > *Ae. hendersoni* [N=11]). In contrast, *Ae. japonicus* and *Ae. hendersoni* were inferior to *Ae. triseriatus* (forest) and *Ae. albopictus* (field), which reinforces the findings that the tires have a negative effect on the absolute abundance of these two species (chapters III and V). Species specific parity rates did not differ among habitats and was not impacted by tire addition. In terms of body size, *Ae. triseriatus* mosquitoes collected from the forest habitat were larger than those collected at the edge habitat. In terms of tire introduction effects, only *Ae. japonicus* was significantly affected. However, interestingly, this effect was negative with *Ae. japonicus* collected at the control sites being larger than those collected at the tire introduction sites.

At the UNCG campus area (**Aim 3**), the anthropophilic *Ae. albopictus* was the most abundant mosquito, followed by *Ae. triseriatus*, and *Ae. hendersoni*. Even though all three species mostly exploited the shaded forest and park habitats, only *Ae. albopictus* was found in the urban campus habitats of UNCG. All three mosquito species in the forest and park habitats mostly oviposited at ground level. This is often the reported pattern for *Ae. albopictus* and *Ae. triseriatus*. However, this pattern was surprising for *Ae. hendersoni*, which typically oviposits at the canopy level. Most interesting was the observation that in the urban habitat of the UNCG campus, *Ae. albopictus* lost its preference for the ground level and oviposited either at the ground level or the canopy level but not in the middle, most exposed, height.

Along the forest-to-field ecotone in the southern Appalachian mountains (**Aim 4**), the vertical distribution in the control plots was consistent with previous studies, with *Ae. triseriatus* exhibiting no clear vertical affinity while *Ae. hendersoni* showing clear preference for ovipositing at the canopy level. Both invasive species

(*Ae. albopictus* and *Ae. japonicus*), exhibited a clear preference for ovipositing at ground level, with the latter being particularly selective for this vertical habitat. Tire introductions to either the field or the forest habitat, resulted in a shift in the vertical habitat use pattern for all four species: *Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus*, substantially enhanced their affinity to oviposition at ground level, while *Ae. hendersoni* reduced its affinity towards the canopy level and laid relatively more at mid-level heights.

Context-dependent and scale-dependent responses of LACV vectors to anthropogenic effects

In the field of ecology in general (Chamberlain et al., 2014) and mosquito ecology in particular (Juliano, 2009), it is becoming increasingly clear that ecological processes and species interactions are context-dependent. For example, in a meta-analysis, Chamberlain et al. (2014) evaluated if the strength or the sign of the interaction changes as conditions change. Out of 70 predation experiments, they found that the sign of the interaction changed with conditions in 45% of the experiments. An even higher proportion of competition experiments found sign changes, and a still greater proportion of mutualism experiments found effect size changes (60%). With larval mosquitoes, the relative impacts of competition and predation commonly change across a gradient of habitat size, type, and permanence (Juliano, 2009).

My study can be used as an example for such a situation and how that could potentially result in changes to entomologic risk to humans. This is contextualized within the conceptual model described in the general introduction. The disease niche of vector borne diseases is determined by a sub-set of conditions enabling the overlap of the niches of the host, vector, and pathogen. Furthermore, the environmental conditions often determine the strength of these interactions and thereby the rates of pathogen transmission within that enzootic system (Reisen, 2010). These, in turn, determine the degree of entomologic risk (described by the orange ring surrounding the basic ecological niche) (Fig. 6.1). Small scale anthropogenic modifications such as a peridomestic habitat in the woods (Tamini et al., 2021) or artificial container introduction (this study) act as “effect modifiers” modifying the currently existing “natural environment” in a manner that might enhance the naturally occurring transmission rates (Fig. 6.1, left panel). However, in areas dominated by man-made environments (Fig. 6.1, right panel), the “disease niche” is often contained within a small “pocket” of a natural (or semi-natural) patch, with the broader scale anthropogenic landscape determining the environmental conditions of the "natural" remnant patch within it (Fig. 6.1).

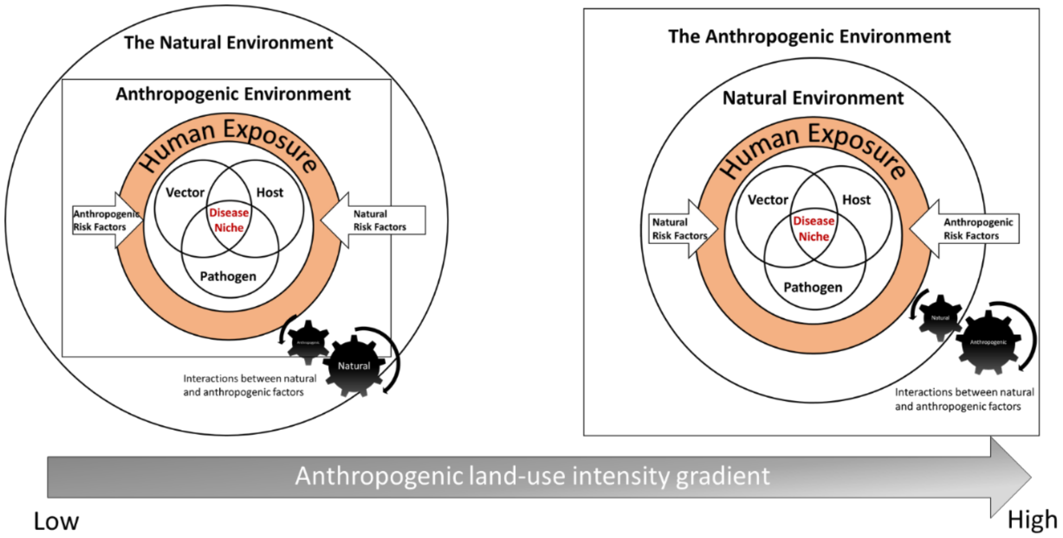

Figure 6.1. Conceptual model of context-dependent vector-borne disease niches. Circles represent the natural environment and squares the anthropogenic environment. In areas characterized by low anthropogenic land-use intensity (left), the anthropogenic environment (e.g., house) is nested within the larger-scale natural environment (e.g., rural landscape, forest). In areas characterized by high anthropogenic land-use intensity (right), the natural environment (e.g., remnant forest) is nested within the larger-scale anthropogenic environment (e.g., city). Cog wheels and arrows represent bi-directional interactions of natural and anthropogenic risk factors affecting the components of the disease niche system.

In my study, several of my findings can fit this framework. One is the **habitat-specific effect of environmental variables**. For example, I found that the effect of tree density was relatively weak in the forested habitats but (for most species) it was strongest and positive in the most open parts of the “outer-field” habitat. A similar effect was with DBH .

Second, was **the effect of tire addition**. I showed that, tire addition in the “natural” forest habitat (corresponding to left panel of Fig. 6.1) contributed mostly to an increase in the abundance of *Ae. triseriatus* but tire addition to the “anthropogenic” field habitat resulted mostly in increase to *Ae. albopictus* abundance. These effects had both local-scale (within habitat) and ecotonal-scale (between habitats) effects. In addition, I found that these responses resulted in a decrease in the abundance of *Ae. hendersoni* and *Ae. japonicus* (edge and field habitats only), which might reflect an indirect effect of the tire addition due to enhanced competitive effect of *Ae. triseriatus* and *Ae. albopictus* on these species. These effects were mostly “quantitative” in terms of an effect on species abundance but also partially “qualitative” in terms of the effect on *Ae. japonicus’* body size, however, no effect on the parity rate of any of the other species. This qualitative response of *Ae. japonicus* was actually negative with mosquitoes being smaller in the treatment habitats than in the control pots.

Third, was **the effect of habitat on vertical distribution**. This can be discussed at two levels: effect of habitat type within a landscape type (i.e., rural: forest, edge, field; urban: campus, field, forest) and comparison of this effect between two different landscapes (i.e., urban and rural). In the rural sites the four species showed the expected vertical distributions, according to the literature, in both the forest and field habitats with *Ae. albopictus* and *Ae. japonicus* common at lower elevations, *Ae. hendersoni* preferring the canopy, and *Ae. triseriatus* being fairly generalist. However, in the edge habitat all four species were found primarily at ground level. In contrast, although the vertical distribution of *Ae. triseriatus* remained fairly similar in the urban landscape compared to the rural landscape, that of *Ae. hendersoni* changed considerably, as it was found ovipositing exclusively at ground level. The vertical distribution of *Ae. albopictus* also differed in the urban landscape with this mosquito more readily ovipositing at higher elevations.

Novelty and significance of this study

This study is novel because it evaluates, simultaneously, the role of two types of anthropogenic effects: forest fragmentation and introduction of artificial containers. Moreover, it applies an experimental approach to evaluate these effects and it does that over an extended temporal scale (3 years) and across two distributional dimensions: horizontal and vertical. It also incorporates a comparative approach by comparing these patterns of vertical distribution between two different types of ecotones in two different regions. Forest fragmentation and the formation of a new type of landscape comprised of fields, forest patches, and edges has a determining effect on the identity of LACV vectors, with *Ae. triseriatus* decreasing in abundance with distance from the forest, *Ae. albopictus* most commonly found in the field, and *Ae. japonicus* in the edge habitats.

Particularly important is the impact of larval habitat supplementation. In this study, I showed that artificial containers (tires) have both local-scale and larger ecotone-scale impacts and that this impact can happen over a short time but persist for a long period if the containers are not removed. Finally, artificial tire introduction could also impact the levels of human exposure by enhancing ground-level activities of most mosquito species in areas with artificial containers. These findings highlight the well-known importance of container control for the purpose of source reduction but also provide a broader framework of understanding regarding the scope, scale, and heterogeneity associated with these anthropogenic changes and their impact on LACV entomological risk.

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