ABSTRACT

REINBOLD-WASSON, DREW DAVID. Container Breeding *Aedes* spp. (Diptera: Culicidae) Oviposition Behavior as a Target for Surveillance, Control, and Improvements in Trap Design. (Under the direction of Dr. Michael Reiskind).

Mosquito-borne arboviruses (i.e., dengue, chikungunya, Zika, and yellow fever) will continue as a public health threat for the foreseeable future across tropical and subtropical regions of the world. Dengue fever is endemic to at least 100 countries, with an estimated 390 million cases occurring each year. This threat, in large part, is due to anthropophilic container-breeding mosquito species *Aedes aegypti* (Linnaeus), the yellow fever mosquito, and *Aedes albopictus* (Skuse), the Asian tiger mosquito. The close association of these mosquito species and human populations increases the risk of vector-borne disease transmission. Disease risk relates to vectorial capacity or the average number of future infectious mosquito bites arising from feeding on a single host. Generally, it requires two mosquito bites to transmit disease to humans, as a mosquito lay eggs in between bites, the gravid condition is, therefore, a logical target for disease surveillance and control efforts. My goal was to take on three issues related to oviposition behavior. Chapter 2 observes patterns of local oviposition distribution by *Ae. albopictus* mosquitoes at a fine (10m) and coarse (100m) scale by examining arrays of ovitraps sampled over two weeks in 2017 and 2018. This research found *Ae. albopictus* has a clear preference for oviposition in forested habitat. In 2018, I sampled oviposition sites pre- and post-Hurricane Florence finding significant increases in *Aedes albopictus* population; however, no change in oviposition site preferences. Fine-scale ovitrap grids provided evidence high amounts of “skip-oviposition” or the laying of eggs in multiple containers during a single gonadotrophic cycle within the sampled *Ae. albopictus* population. Chapter 3 continues the exploration of skip-oviposition behavior of *Aedes aegypti, Ae. albopictus*, and *Aedes triseriatus* using individual
oviposition trials and assessing resulting egg spreading. In studying, I make use of an aggregation statistic, the index of dispersion, to enhance the description of egg spreading behavior. These studies found *Ae. aegypti* and *Ae. albopictus* spread eggs significantly more than *Ae. triseriatus*. Oviposition site “quality” modifies egg contribution per site, which means low “quality” sites increase egg spreading behavior. However, it also found that temperature and photoperiod do not appear to change skip-oviposition behavior in *Ae. albopictus*. In Chapter 4, I developed and assessed a cost-effective, multipurpose, 6-volt mosquito trap integrating features of both a host-seeking and gravid mosquito traps for the collection of undamaged live specimens; a Multifunctional Mosquito Trap (MMT). This project was born from my own experiences sampling for vectors of disease in remote locations such as the Middle East and islands in the Pacific Ocean. I took advantage of advances in 3D printing technology, which reduce cost while allowing the construction of novel trap components. Field evaluations found the MMT performed as well or better than comparable commercial traps. This project demonstrates an easy to construct, inexpensive, and versatile mosquito trap, potentially useful for the surveillance of multiple mosquito species and other hematophagous insects by incorporating various attractants into the MMT.
Container Breeding *Aedes* spp. (Diptera: Culicidae) Species Oviposition Behavior as a Target for Surveillance, Control and Improvements in Trap Design

by

Drew David Reinbold-Wasson

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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APPROVED BY:

_______________________________  _______________________________
Dr. Michael Reiskind                Dr. Wes Watson
Committee Chair

_______________________________  _______________________________
Dr. Coby Schal               Dr. Kevin Gross
DEDICATION

I dedicate this to my Family (Peggy, Joseph, and Georgieana), without their constant love and support, I would never have commissioned as an Officer in the US Army, nor dared to continue my education in medical entomology.
BIOGRAPHY

Drew David Reinbold-Wasson was born July 13, 1977, in Sedro Woolley, Washington, to teachers Bernard and Mary Reinbold. He grew up in rural Oregon on a small cattle farm with his older brother Kirk and identical twin brother Keith. He graduated with honors from Clatskanie High School in 1995. Post high school, Drew attended Oregon State University graduating with a Bachelor of Science in Zoology in 1999. He then accepted a research technician position at the University of North Carolina for two years before attending an MS program at Washington State University. After completing a Master of Science in Zoology in 2005, Drew enlisted in the US Army as a Medical Laboratory Technician (68K) later, 2010, commissioning as Medical Service Corp Officer specializing in Medical Entomology (72B). After almost 15 years of service, Drew has been stationed across the United States (Maryland, Colorado, Texas, Washington, Washington DC). Before attending NC State, he was stationed overseas in Tokyo, Japan, for two years and will follow completion of this program conducting vector-borne pathogen research in Tbilisi, Georgia. He deployed to Kuwait, 2013-2014, providing medical entomology expertise and preventive medicine support to military units across the Middle East, including Qatar and the United Arab Emirates.

MAJ Reinbold-Wasson’s awards and decorations include the Superior Unit Award, Meritorious Service Medal (3), Overseas Service Ribbon (2), Global War on Terrorism Expeditionary Medal, The Norwegian Foot-March Skill Insignia (Road Marching Badge), Army Commendation Medal (3), Army Achievement Medal (2), the Army Good Conduct Medal, the National Defense Service Medal, Global War on Terrorism Service Medal, the Noncommissioned Officers Professional Development Ribbon, and the Army Service Ribbon.

Drew and his wife Peggy, have two children; son, Joseph, and daughter, Georgieana.
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CHAPTER 1

Introduction

Two mosquitoes (Diptera: Culicidae) of the subgenus *Stegomyia* (Theobald), *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse), are globally important vectors of arbovirus causing sporadic, epidemic, and endemic viral diseases, including dengue fever virus (Bhatt et al., 2013), chikungunya virus (Wahid, Ali, Rafique, & Idrees, 2017), Zika virus (Bogoch et al., 2016), and yellow fever virus (Oliveira Silva et al., 2020). Successful invasive of *Ae. aegypti* and *Ae. albopictus* preceded the global expansion of many of these arboviruses (Kraemer et al., 2015). *Aedes aegypti* originated in Africa, now has a worldwide tropical and subtropical distribution through human trade and movement, and is broadly associated with human development (Brown et al., 2014). A more recent invasive species originating from Asia, *Aedes albopictus* has a worldwide distribution in temperate and subtropical regions due to the international tire trade (Hawley, 1988). As arbovirus vectors, it is essential to understand the distribution of each species within local environments. *Aedes aegypti* and *Aedes albopictus* inhabit similar environmental niches, including the use of small natural or artificial water containers as oviposition sites. Host-seeking adults from both species exhibit limited dispersal from larval habitat, so the availability of artificial containers often dictates distribution and risk of pathogen transmission.

*As Aedes aegypti and Aedes albopictus are competent vectors for many arboviruses* (Gratz, 2004; Souza-Neto, Powell, & Bonizzoni, 2019). As competent disease vectors, both mosquito species can transmit diseases between human hosts. The association of high mosquito density and human population creates a situation where a single infectious human can give rise to a large number of mosquito infections, leading to an increase in human cases of the disease. The average number of future infectious bites from vectors feeding on a single infected human
per day is known as vectorial capacity \((V)\) and calculated with the following formula (Brady et al., 2016; Mullen & Durden, 2019):

\[
V = \frac{ma^2bp^n}{-\ln(p)}
\]

Vectorial capacity accounts for \((m)\) number of mosquitoes per human, \((a)\) human-biting rate, which describes as the mosquito biting rate multiplied by the portion feeding on humans, \((b)\) vector competence or the ability for a species of mosquito to pass a particular pathogen to a host, \((p)\) the probability of mosquito daily survival, \((n)\) extrinsic incubation rate or how long it takes a mosquito to become infectious to humans after acquiring a pathogen. Many aspects of the vectorial capacity formula are important and worth study; however, in terms of mosquito surveillance and control, one aspect is of specific interest, \((a)\) the human biting rate. Vectorial capacity simplifies human-biting by squaring \((a)\), which just means two human blood meals must occur for disease transmission. After the first human blood meal, a mosquito undergoes egg development and oviposition, before taking as second human blood meal. Container \textit{Aedes} species oviposition is a logical target for disease surveillance and control, due to the higher probability gravid mosquitoes are positive for a vector-borne disease.

Container \textit{Aedes spp.} place eggs above the water line in water-filled containers. The inundation of containers initiates egg hatching along the oviposition substrate. Once hatched, larvae develop by feeding on available organic material in their container habitats. Before adult emergence, mosquito larvae undergo four larval instars and an active pupal stage. Post-emergence, adults mate and fed on local sugar sources (i.e., fruits and flowers) (Clements, 1999). For the most part, \textit{Ae. aegypti} and \textit{Ae. albopictus} are anautogenous, meaning they require a blood meal to produce the first batch of eggs. As adults, these mosquitoes stay close to larval habitats, preferring to acquire blood meals from animals or humans who transverse or live locally to the
oviposition habitat (Clements, 1999; Hawley, 1988). In effect, they are “ambush” feeders, generally seeking blood meals during daylight hours. Sugar intake drives early egg follicle development to a previtellogenic resting stage, where eggs remain arrested until the mosquito takes a blood meal (Clements, 1999). Post-blood meal Aedes aegypti and Aedes albopictus females find an appropriate resting location (i.e., foliage or urban harborage) as they undergo further egg development. Egg follicles continue development due to blood meal acquisition, vitellogenesis leading to maturation of oocytes (Clements, 1999). After blood meal engorgement and mating, gravid females develop a sensitivity to volatile oviposition site attractants (Clements, 1999). Egg maturation entices a now gravid female mosquito to begin the search for an oviposition site, with egg fertilization occurring from stored sperm as the egg passes through the oviduct.

Mosquitoes utilize a combination of factors for oviposition site selection, including physical, visual, and chemical cues (Clements, 1999). Container breeding Aedes species preferentially oviposit in small (<1 L) to medium (1 L to 5 L) water containers (Reiskind & Zarrabi, 2012). Physical cues influence gravid container Aedes spp. orientation on oviposition sites, which include local scale oviposition site elevation (Chadee, 1991), the site material (Oliva, Correia, & Albuquerque, 2014), and oviposition site habitat (Clements, 1999). Visual cues play an important role in gravid mosquito oviposition site selection. A known visual cue for container Aedes spp. is “lightness” or reflectance of an oviposition site, which describes the mirror-like shine from water in the environment (Allan, Day, & Edman, 1987; Bentley & Day, 1989; Clements, 1999). Mirror surfaces elicited oviposition behaviors in Aedes aegypti, including attempts to oviposit (Kennedy, 1942). The visual “chromaticity” cue describing the oviposition site color is a significant oviposition factor in container Aedes spp. mosquitoes (Allan
et al., 1987). *Aedes aegypti* and *Aedes albopictus* show a marked preference for black colored oviposition sites over other colors (Hoel et al., 2011; Pavlovich & Rockett, 2000). Oviposition site chemical cues can both attract and repel gravid container *Aedes* species mosquitoes (Clements, 1999). Container *Aedes* species cue in on chemical odors such as site plant organic content (Ponnusamy et al., 2010; Trexler, Apperson, & Schal, 1998), bacterial community of an oviposition site (Ponnusamy, Schal, Wesson, Arellano, & Apperson, 2015), or chemicals such as methyl propanoate (Clements, 1999). Ultimately, mosquito oviposition site selection is a combination of species-specific site preferences, the “quality” of an oviposition site, the availability of oviposition sites within an environment, and the current physiological “need” of an individual mosquito to lay eggs (i.e., can she wait or does she need to lay them now).

Studies suggest a variety of cues are important in oviposition choice, including the identity and concentration of abiotic and biotic inputs (Bentley & Day, 1989; Clements, 1999; Day, 2016). Oviposition cues which increase the number of eggs laid in a site are known as oviposition stimulants such as; the organic composition of the oviposition media (Trexler et al., 1998), the inorganic composition of the media (cite), oviposition substrate composition, and presence of conspecific eggs or larvae (Chadee, Corbet, & Greenwood, 1990; Davis, Kaufman, Hogsette, & Kline, 2015; Shragai, Harrington, Alfonso-Parra, & Avila, 2019). Oviposition cues known as oviposition deterrents decrease egg-laying at oviposition sites, which include predator fish (Cavalcanti Pamplona, Luciano de Goes, Alencar, Lima, & Heukelbach, 2009), pathogens such as *Plagiorhchis elegans* (Zahiri & Rau, 1998), or lack of larval nutritional content (Wong, Stoddard, Astete, Morrison, & Scott, 2011). As a gravid mosquito locates an oviposition site, she flies into the container and lands directly on the water surface with all six legs assessing odor, gustatory, and tactile oviposition cues (Clements, 1999). If there is a strong enough
deterrent at the oviposition site, such as the presence of a larval predator, she will fly off and seek a new site. However, if this site meets a minimum acceptable “quality,” she continues to the container wall for oviposition. Once in position, she proceeds to lay eggs singly between 10-50 mm above the waterline along the container wall substrate, moving over a few millimeters for each egg. Much like environmental cues that influence oviposition site selection, the mosquito’s choice to oviposit after reaching a site is likely affected by a combination of inputs from both stimulants, deterrents, and the physiological state of the mosquito.

While oviposition stimulants and deterrents modify egg contribution to a specific container, container breeding Aedes mosquitoes also exhibit a behavior where before laying her entire egg batch an individual Aedes will stop and move to a new container (Chadee et al., 1990; Fay & Perry, 1965; Oliva et al., 2014). The behavior of laying eggs in multiple containers during a single gonotrophic cycle is known as “skip-oviposition” (Mogi & Mokry, 1980). Laboratory studies provide direct evidence of this behavior for Aedes aegypti, Aedes albopictus, and Aedes triseriatus (Corbet & Chadee, 1993; Davis et al., 2015; Santos de Abreu, Filipe Vieira, Morais, Ribeiro, & Eiras, 2015; Trexler et al., 1998). Indirect ovitrap data suggests other container breeding Aedes species display skip-oviposition behavior such as Aedes polynesiensis (Rozeboom, Rosen, & Ikeda, 1973) and Aedes japonicus (Balestrino et al., 2016).

In my dissertation, I tackle three issues related to oviposition behavior. In Chapter 2, I assess the patterns of local oviposition distribution by Aedes albopictus mosquitoes at a fine (10m) and coarse (100m) scale by examining arrays of ovitraps sampled over two weeks. In Chapter 3, I explore the skip-oviposition behavior of Aedes aegypti, Aedes albopictus, and Aedes triseriatus using individual oviposition trials and assessing resulting egg spreading. In Chapter 4, I developed a novel 3D printed mosquito trap that can collect both gravid and host-seeking
mosquitoes; furthermore, I evaluated this trap against five different types of commercial mosquito trap.

Chapter 2:

It is essential to understand local *Ae. albopictus* distribution in order to attribute health risks of given locations. Oviposition traps (ovitraps) are an effective means of determining distribution providing information on species presence or absence, and relative concentration of container breeding *Aedes* species mosquito populations. In 2017 and 2018, I conducted a field study of local *Ae. albopictus* distribution using a coarse-scale ovitrap grid (100 m between ovitraps) and a fine-scale ovitrap grid (10 m between ovitraps) using a corporate campus in Research Triangle Park, North Carolina. The goal is to conduct comparisons between within and between two scales of oviposition surveillance for *Aedes albopictus*. In 2018, by chance, I collected data pre- and post- Hurricane Florence landfall for the fine-scale ovitrap grid. There are no previous reports of the impact of hurricanes on *Aedes albopictus* populations.

Chapter 3:

*Aedes aegypti* and *Aedes albopictus* egg spreading among multiple containers or “skip-oviposition” is well documented (Chadee et al., 1990; Corbet & Chadee, 1993; Davis et al., 2015; Trexler et al., 1998). However, many previous studies focus on using skip-oviposition behavior to assess the “quality” of a single oviposition site among multiple sites (Corbet & Chadee, 1993; Davis et al., 2015). This chapter aims to assess skip-oviposition behavior in response to a multiple cup oviposition environment were all sites are of equal “quality” with three species of *Aedes* mosquito: *Ae. aegypti*, *Ae. albopictus* and *Ae. triseriatus*. In this study, I
measure differences between species and their response to an enriched oviposition treatment and an unenriched oviposition site treatment. Finally, I assess how fall temperature and light condition diapause egg-laying inducing conditions influence skip-oviposition behavior in *Ae. albopictus*. My goal in these studies is to enhance knowledge of skip-oviposition behavior and improve assessment of this behavior in laboratory environments.

Chapter 4:

Mosquito surveillance activities employ a variety of traps depending on mosquito species and condition: host-seeking or gravid. Additionally, mosquito surveillance tends to be limited to available commercial traps, later modified with attractants to suit specific surveillance requirements (i.e., targeting specific species or large collection numbers). The goal of this project is to develop a cost-effective, self-supporting, multipurpose, 6-volt mosquito trap integrating features of both host-seeking and gravid mosquito traps. This project takes advantage of 3D printing technology, allowing for specific trap components which are difficult to produce with conventional construction techniques. Finally, I assess this multipurpose trap against standard commercially available traps in three limited field evaluation comparison studies for gravid *Ae. albopictus*, host-seeking *Ae. albopictus*, and total host-seeking mosquitoes.
REFERENCES


Day, J. F. 2016. Mosquito oviposition behavior and vector control. Insects, 7(65)


Shragai, T., L. Harrington, C. Alfonso-Parra, and F. Avila. 2019. Oviposition site attraction of Aedes albopictus to sites with conspecific and heterospecific larvae during an ongoing invasion in Medellin, Colombia. Parasites & Vectors, 12(1), 455.


CHAPTER 2

Local Scale Oviposition Pattern of *Aedes albopictus* (Skuse) (Diptera: Culicidae) and Response to Hurricane Florence, in Wake County, North Carolina

ABSTRACT

The mosquito *Aedes albopictus* (Skuse) vectors arbovirus diseases such as dengue, chikungunya, and Zika. As a globally invasive mosquito is a significant health threat to humans living near *Ae. albopictus* populations where these diseases are prevalent. It is essential to understand the local distribution of *Ae. albopictus* in assessing health risks to associated human communities. A container breeding mosquito, *Ae. albopictus* is closely associated with its oviposition habitat, small natural or artificial water containers. Therefore, oviposition traps (ovitraps) are useful in conducting surveillance. In 2017, we conducted a two-week field study of local *Ae. albopictus* distribution using both a coarse-scale ovitrap grid (65 ovitraps with 100 m separated traps) and a fine-scale ovitrap grid (a 121 ovitrap square with 10 m separated traps) across a corporate campus in Research Triangle Park, North Carolina. The fine-scale ovitrap grid was repeated in 2018 for three sample weeks collecting oviposition data pre- and post-Hurricane Florence landfall. ArcGIS inverse distance weighting overlays of *Ae. albopictus* oviposition activity highlight higher ovitrap egg densities and higher probabilities of ovitrap occupation in forest sections of the ovitrap grids. In contrast, ovitraps placed in the open sections of the ovitrap grids had lower probabilities of egg occupation and lower densities,
suggesting observed open section results were due to skip-oviposition behavior *Ae. albopictus*. There was a 3-fold increase in *Ae. albopictus* oviposition activity 21 days following the 2018 landfall of Hurricane Florence. However, there was no observable change in habitat preference, suggesting that significant rainfall events increase *Ae. albopictus* population through large scale hatching but do not modify local distribution. This study implies that the placement of ovitraps in the local environment is critical for both surveillance and control. Ovitrap grids are a potentially useful tool in assessing local *Ae. albopictus* distributions, identifying both areas of high mosquito activity and cryptic oviposition.
INTRODUCTION

Mosquito-borne arboviruses (i.e., dengue, chikungunya, Zika, and yellow fever) will continue as a public health threat for the foreseeable future across tropical and subtropical regions of the world (Kraemer et al., 2019; Messina et al., 2019; World Health Organization, 2017). Dengue fever is endemic to at least 100 countries, with an estimated 390 million cases occurring each year (Bhatt et al., 2013; Quam, Sessions, Kamaraj, Rocklov, & Wilder-Smith, 2016). This threat, in large part, is due to anthropophilic container-breeding mosquito species *Aedes aegypti* (L.), the yellow fever mosquito, and *Aedes albopictus* (Skuse), the Asian tiger mosquito (Kraemer et al., 2019; Lwande et al., 2020; Messina et al., 2019). Both species are adapted to human activities and distributed worldwide (Lwande et al., 2020). Their close association with human hosts increases risk for mosquito-borne pathogen introduction and transmission within a local environment, as each species vectors multiple viruses (Ali, Wagatsuma, Emch, & Breiman, 2003; Brady & Hay, 2020; Honorio et al., 2009; Lin, Wen, Teng, & Chang, 2016; Wen, Lin, Teng, & Chang, 2015).

The local distribution of these species can affect pathogen transmission. A study in Bangladesh found 56% of the variance in human dengue case clusters explained by clusters of *Ae. albopictus* (Ali et al., 2003). Similarly, local *Ae. albopictus* populations transmitted dengue fever virus from an urban green space of Yoyogi Park, Tokyo, Japan, contributing to 160 locally acquired human cases (Quam et al., 2016). Risk is directly associated with local *Ae. albopictus* distribution in close association with human populations, i.e., an individual’s risk of pathogen exposure increases the more they interact with *Ae. albopictus* populations. Therefore, it is important to understand local *Ae. albopictus* distributions to assess risk to human populations.
Introduced to the United States in the mid-1980s, *Aedes albopictus* preferentially distributes into forested areas of urban, suburban, and rural habitats, and is uncommon if vegetation is removed (Hawley, 1988). Studies of *Ae. albopictus* distribution found a preference for forested border habitat commonly associated with human disturbance (Ali et al., 2003; Barker, Brewster, & Paulson, 2003; Cianci et al., 2015; Hawley, 1988; Paupy, Delatte, Bagny, Corbel, & Fontenille, 2009; Reiskind, Griffin, Janairo, & Hopperstad, 2017). The local distribution of this species is closely associated with forested larval habitat (Hawley, 1988; Lacroix, Delatte, Hue, & Reiter, 2009). This species lays eggs singly in small water containers, both natural and man-made, by placing eggs along the container’s substrate above the waterline (Hawley, 1988). Female *Ae. albopictus* use a wide range of containers but prefer black ovitraps with rugulose paper liners (Yap, Lee, Chong, Foo, & Lim, 1995). Adult dispersal from larval habitats is limited, between 50-250m (Hawley, 1988; Medeiros-Sousa, Fernandes, Ceretti-Junior, Bruno Wilke, & Marrelli, 2017; Silver, 2008; Vavassori, Saddler, & Muller, 2019). Additionally, *Ae. albopictus* limits dispersal along forested habitat, again in close association with their larval habitat (Lacroix et al., 2009). This species utilizes many different types of water-filled oviposition sites (i.e. tree holes, bamboo, flower pots, tires, rain barrels, etc.) contributing to its ability to invade and distribute in novel local environments, as artificial and natural container habitats are ubiquitous (Hawley, 1988; Paupy et al., 2009).

The oviposition trap or “ovitrap” was first described in 1965 as a method of surveillance for *Aedes aegypti* (Fay & Perry, 1965). A useful tool in studying container breeding mosquitoes, it provides specific information regarding mosquito behavior, activity, and distribution through the accumulation of eggs over time. In general, the ovitrap is a small water-filled container with an oviposition substrate such as a wooden paddle or paper lining. Ovitraps are also effective for
the surveillance of *Ae. albopictus* because it uses similar oviposition habitats as *Ae. aegypti* (Hawley, 1988; Hoel et al., 2011). Ovitraps are useful for a wide range of surveillance applications: population monitoring, understanding species distribution, and habitat association. For population monitoring, ovitraps are an inexpensive and effective method for determining the presence or absence of dengue vectors *Ae. aegypti* and *Ae. albopictus* (Focks, 2003). A North Carolina statewide survey assessed the distribution of *Ae. albopictus*, *Ae. triseriatus*, *Ae. japonicus* and *Ae. hendersoni* all species of container *Aedes* (Reed et al., 2019). Ovitraps are effective tools in spatial-temporal monitoring of container breeding *Aedes* species mosquito distribution. Ovitrap surveillance of *Ae. albopictus* in urban Raleigh, NC, highlighted areas of high and low population distribution (Richards, Apperson, Ghosh, Cheshire, & Zeichner, 2006).

Another study using ovitrap surveillance found *Ae. albopictus* was active from February to December in Rome, Italy, with peak oviposition activity at the end of August (Toma, Severini, Di Luca, Bella, & Romi, 2003). Ovitrap studies are also useful in determining habitat preferences for different species of container breeding *Aedes* species. A recent study in Rome, Italy, identified a significant positive association of *Ae. albopictus* egg accumulation in ovitraps with their placement in vegetated sites (Cianci et al., 2015). In Rio de Janeiro, Brazil, ovitraps collected *Aedes aegypti* and *Ae. albopictus* eggs in higher abundance near dwellings, indicating these species are closely associated with houses in the rural environment (Lourenco-de-Oliveira, Castro, Braks, & Lounibos, 2004). Ovitraps are thus a versatile tool for the surveillance and research of container breeding *Aedes* species.

In 2017 and 2018, I studied the distribution of egg-laying of an *Ae. albopictus* population inhabiting a corporate campus in Research Triangle Park, North Carolina. I aimed to assess how multiple scales of ovitrap surveillance describe the local *Ae. albopictus* population’s distribution.
I used two scales of ovitrap grids: a coarse-scale ovitrap grid with 100 m ovitrap separations, and a fine-scale ovitrap grid with 10 m ovitrap separations installed in the southern portion of the campus. By chance, a major weather event, Hurricane Florence, affected the Raleigh, NC area during the 2018 sample periods, allowing for the assessment of a hurricane event on oviposition distribution. My final goal was to assess how nutrient availability altered *Ae. albopictus* oviposition distribution in fine-scale ovitrap grid.
MATERIALS & METHODS

Surveillance Study:

I conducted oviposition trap (ovitrap) surveillance from August 31 to September 15 of 2017 at the Research Triangle Institute (RTI) International campus located in Research Triangle Park, North Carolina. I placed a campus-wide coarse-scale grid of 65 ovitraps (100 m trap separations) and a fine-scale square grid of 121 ovitaps (10 m trap separation) in the southern stormwater management section (Figure 2.1, 2.2). From 7 September to 5 October 2018, I repeated the fine-scale ovitrap grid surveillance. Table 2.1 lists sample periods for coarse-scale and fine-scale ovitrap grids.

I selected coordinates for a coarse-scale ovitrap sites in the ArcGIS program (ESRI, 2011). Then I transferred the coordinates for each proposed site into a Trimble June 3B GPS (Trimble Navigation Limited, Westminster, CO, USA) (Figure 2.1). A Trimble GPS allowed accurate ovitrap placement throughout the RTI campus. I did not place ovitrap proposed sites that fell within construction zones, over buildings, or similar large obstructions (Figure 2.1). If a tree or similar small obstruction interfered with ovitrap placement, then the trap was placed one meter to the west of the obstruction. Selected sites and coordinates were later transferred from the GPS unit to the ArcGIS map to mark sites. This method created a continuous 100 m separated ovitrap grid for a two-week sampling of container breeding mosquito activity.

For the fine-scale ovitrap grid, I used the Trimble GPS to locate the northwestern corner. I ran a transect east to west with a 100 m measuring tape, placing an ovitrap every 10 m (Figure 2.3). Then I ran a north to south transect, again setting an ovitrap every 10 m. I repeated this method until I created a square grid of ovitraps with eleven ovitraps per transect in either direction. A forested border bisected the fine-scale ovitrap grid; therefore, a portion of the
ovitraps are in an open section and a portion in a forested section (Figure 2.2). For the second year, 2018, I adjusted ovitrap grid placement based on analysis from the previous year, shifting more ovitraps into the forested portion of the study site (Figure 2.2). September 7 to 11, 2018 sample period for 2018 was interrupted by Hurricane Florence, forcing early collection of ovitrap samples.

I used 475 ml black cup ovitraps for this study. Each ovitrap contained 300 ml of oviposition media (either tap water or oak leaf infusion) and a 7 cm x 25 cm strip of heavyweight seed germination paper (Anchor Co. St. Paul, Minnesota, US) for oviposition. I attached each ovitrap to a wooden stake suspended three inches above the ground (Figure 2.4). Ovitraps received a weep hole at the 300 mL fill point to prevent over-filling from rainwater. I attached ovitraps to the north side of the surveillance stake during placement. I inspected ovitraps every seven days collecting oviposition paper and media from each ovitrap. Oviposition paper was hung dried for 24 hours, then examined and recorded for mosquito eggs under a dissecting microscope. After egg counting, I maintained the oviposition papers at 27 °C in plastic containers with wet paper towels. For species confirmation, I hatched collected eggs by submerging each oviposition paper in individual containers containing 500 ml of water with four pellets of fish food (Wardly Pond pellets, Hartz Mountain Corporation, Secaucus, NJ, USA) in an incubator set to 27 °C and 14:10 light:dark photoperiod. The fish food to encourage hatching in *Ae. albopictus*. In 2017, I attempted hatching for all oviposition papers. However, in 2018 I randomly selected 20% of oviposition papers from each sample period for hatching. I reduced conformation hatching due to the large increase in eggs collected, and the previous year collection was 100% *Ae. albopictus*. All hatched larvae developed to fourth-instar were then immersed in boiling water and preserved in ethanol for later identification.
From September 21 to 28, 2018, I used an oak leaf infusion to assess the effect of attractive media on oviposition distribution within the fine-scale grid. I made an oak leaf infusion with tap water incubated for four days with a 16 g/L bundle of dried willow oak (*Quercus phellos*) leaves, 0.3 g/L brewer’s yeast (CAT NO: 903312, MP Biomedicals, Solon, OH, USA), and 0.3 g/L egg albumin (CAT NO: A388-500, Fisher Scientific, USA) (Obenauer, Kaufman, Allan, & Kline, 2009; Reiskind & Janairo, 2018). The concentrated 16 g/L oak leaf infusion was diluted 1:4 at the start of the sample period with tap water for a final concentration of 4 g/L oak leaf infusion.

I collected oviposition media from each ovitrap at the end of the sample periods using 532 ml Whirl-Pak® sample bags (Nasco, Madison, WI, USA). To estimate water volume, I weighed the remaining oviposition media from each sample period approximating water volume loss by converting weight in grams to milliliters for water. I incubated the Whirl-Pak® sample bags at room temperature for five days, collecting and identifying any larvae that developed.

Species Identification:

Container *Aedes* species identification used species egg morphology (Bova, Paulson, & Paulson, 2016). Egg morphology is sufficient due to low container *Aedes* species diversity in Wake County, North Carolina. All larvae were identified using common *Aedes* species larval identification key (Farajollahi & Price, 2013).

Temperature and Photoperiod:

I placed Hobo Pendant® Temperature/Light 8K Data Loggers model UA-002-08 (Onset Computer Corporation, Bourne, MA, USA) into ten of the 121 ovitraps in the sample grid during
the 2018 sample season. The data loggers were programmed to record temperature (°C) and light (Lux/ft²) every 15 minutes. As a limited resource, I randomized the placement of data loggers among ovitraps in the fine-scale grid.

Drone Surveillance:

* *Aedes albopictus* oviposition distribution study occurred in conjunction with RTI’s drone surveillance program. This program conducted drone flights over the RTI-International campus during ovitrap surveillance. Attached to the drone was a high-resolution camera that took multiple still images later stitched together by RTI-International personnel. I imported the high-resolution image map as a layer in ArcGIS. Figures 2.1 and 2.2 show the difference between Ersi USGS satellite map and the high-resolution map produced by drone flights (ESRI, 2011).

Data Analysis:

I considered two outcomes for each sampling period: ovitrap egg occupancy, defined by the presence of at least one *Aedes albopictus* egg and egg density (eggs/ovitrap/day), accounting for one short sampling period, less than seven days, immediately before Hurricane Florence.

I conducted all spatial pattern analyses in ArcGIS 10.5.1 (ESRI, 2011). Research Triangle Institute International provided a mosaic high-resolution map layer from drone imagery conducted during the 2017 sample periods (1-15 September 2017). I imported the drone map into ArcGIS as a background layer for maps presented in this chapter. Inverse distance weighted (IDW) analysis was applied to all sample periods using ovitrap egg occupation, egg density (eggs/ovitrap/day), and daily ovitrap water loss (Getis & Ord, 1992; Lin et al., 2016). Ovitrap egg occupation maps displayed IDW color ramping with ten equal distance separations between
0 and 1. Egg density and daily water loss maps reflect IDW color ramping determined by ¼ standard deviation separations from the mean recorded value. Therefore, the number of separations can differ based on the applied data. Inverse weighted color ramp maps display consistent colors regardless of the measured variable: blue (low) to red (high).

Data analysis conducted in R (R Core Team, 2017). Each sample period ovitrap egg occupation was analyzed with the binomial family glm() function, relative to two environmental factors: ovitrap daily water loss and ovitrap location in regards to the forested tree line (Bartlett-Healy, Healy, & Hamilton, 2011; Reiskind et al., 2017). Ovitrap daily water loss is directly relevant to a mosquito’s oviposition choice. *Aedes albopictus* prefers vegetative habitats; therefore, distance from the forested tree line is a logical choice for comparisons. I conducted multinomial regression to assess repeated egg occupation outcomes over the sampling period (i.e., 0, 1, 2, or 3 occupations) using the package nnet (Venables & Ripley, 2002) with multinomial probability graphs produced using the package effects (J. Fox & Hong, 2009; J. Fox 1947- & Weisberg, 2019). The multinomial approach allows the probing of ovitrap occupancy consistency to gain better insight into oviposition behaviors. I conducted negative binomial regression on egg density with the glm.nb() function in the package MASS (Venables & Ripley, 2002), producing graphs with the package ggplot2 (Wickham, 2009).
RESULTS

I collected 18,733 eggs during five collection weeks from two years over seven sample periods (Table 2.2). *Aedes albopictus* eggs occupied 531 of 735 (72.24%) ovitraps. Ovitrap egg count data shows a negative binomial distribution with a median of 22 eggs per occupied ovitrap (Figure 2.5). In 2017, fine-scale ovitrap grid mean egg density in occupied traps decreased from 3.5 ± 0.38 (SEM) eggs/ovitrap/day to 2.06 ± 0.29 (SEM) eggs/ovitrap/day. In contrast, the 2018 fine-scale ovitrap grid mean egg density in occupied ovitraps increased from 3.07 ± 0.38 (SEM) eggs/ovitrap/day pre-Hurricane Florence to 9.7 ± 0.63 (SEM) eggs/ovitrap/day 14 to 21 days post-Hurricane Florence.

Ovitrap occupation and egg density is dependent on ovitrap water loss and position within the grid with regards to the vegetative tree line. Additionally, Ovitraps positioned within the forested sections of ovitrap grids experienced lower daily water loss (Figure 2.6). Therefore, collinearity exists between daily ovitrap water loss and ovitrap position distance from the tree line. I measured the collinearity with the variance inflation factor (VIF), which resulted in a VIF of 1.272, low enough to ignore the effect of collinearity on the variance of the coefficient.

Patterns of Ovitrap Occupancy and Egg Density:

Coarse-scale and fine-scale IDW overlays of the ovitrap grids exhibit high and low areas of ovitrap egg occupation, egg density, and daily water loss (Figure 2.7). *Aedes albopictus* eggs occupied a majority of ovitraps in the coarse-scale grid throughout the RTI campus. However, many occupied ovitraps had low egg density, with only a few traps receiving higher amounts of eggs (Figure 2.7). Similar to the coarse-scale ovitrap grid, the fine-scale grid, in both 2017 and 2018, found *Ae. albopictus* eggs were occupying a majority of ovitraps. There is a clustering of...
Ovitrap Occupation:

The 2017 and 2018 fine-scale ovitrap grid displayed similar increased odds of ovitrap egg occupation by *Ae. albopictus* (Odds = 1.074, $Z_{238} = 5.870$, $p$-value < 0.0001; Odds = 1.078, $Z_{353} = 6.533$, $p$-value < 0.0001; respectively) in relation to the distance from the tree line (Table 2.3). However, the coarse-scale ovitrap grid did not identify a significant relationship between ovitrap placement concerning the distance to a forested tree line. All sample grids and periods showed a non-significant trend of odds reduction in ovitrap egg occupation with an increase in ovitrap
daily water loss. Three weeks post-Hurricane Florence, the likelihood of ovitrap egg occupation was much greater (Odds 26.94, Z_{353} = 4.687, p-value < 0.0001) when controlling for the effects of distance from the forested tree line and ovitrap daily water loss. A univariate binomial regression shows a trend where the probability of ovitrap egg occupation increases with a reduction in distance from the forested tree line (Figure 2.9). The probability of ovitrap egg occupation reduces as daily ovitrap water loss increases (Figure 2.10).

Multinomial regression analysis indicates the probability of repeated ovitrap egg occupation by *Ae. albopictus* is higher in the forest section as compared to the open section (Figure 2.11). In the 2017 and 2018 fine-scale ovitrap grids, repeated ovitrap egg occupation is the highest probable outcome for the forested section extending approximately 10 m into the open section (2.11). Forested sections of the coarse-scale and fine-scale grids show a higher probability of repeated ovitrap occupation; however, results varied between landscape scales and sample periods (Table 2.4). Ovitraps with lower daily water loss also indicate a higher probability of repeated occupation during the 2017 and 2018 sample periods (Figure 2.12). Odds of repeated ovitrap occupation showed a non-significant trend of decreasing odds as daily ovitrap water loss increases, again results varied between landscape scales and sample periods (Table 2.4).

Egg Density:

Ovitraps egg density was higher in forested sections for all sample periods and landscape scales. (2017 Coarse Scale: Z_{126} = 3.256, p-value = 0.001; 2017 Fine Scale: Z_{242} = 3.256, p-value < 0.0001; 2018 Fine Scale: Z_{353} = 8.160, p-value < 0.0001) (Table 2.5, Figure 2.13). When controlling for distance from the forested tree line and sample period, daily ovitrap water loss did
not significantly affect ovitrap egg density (Table 2.5). However, the trend is for lower ovitrap egg density when there are higher rates of daily ovitrap water loss (Figure 2.13). The second week of sampling in 2017 indicates lower ovitrap egg density than the previous sample period of the coarse-scale and fine-scale ovitrap grid; in this comparison, only the fine-scale ovitrap grid had significantly average trap egg density ($Z_{242} = -2.415$, $p$-value = 0.016). Three-weeks post-Hurricane Florence found a significantly higher average ovitrap egg density in the fine-scale grid as compared to the pre-hurricane sample period ($Z_{353} = 8.893$, $p$-value < 0.0001).

2018 Fine Scale Grid Temperature and Illumination:

Ovitrap water temperature ($\degree$C) and illumination (Lum/ft$^2$) increased from 0700 until approximately 1400, at which time both measures peaked regardless of location in the sample grid. By 1900 illumination was approximately 0 Lum/ft2 across all sensors. Both temperature and illumination are higher in open sections than forested sections of the fine-scale ovitrap grid. The center of the open area showed the highest temperature and illumination measurements as compared to measurements closer to the tree line. In contrast, temperature and illumination measurements within the forest section were relatively equal. The sample period before Hurricane Florence was significantly ($F = 11.71$, $p$-value = 0.0009) warmer (26.3 $\degree$C average temperature) than the final sample period in 2018 (24.2 $\degree$C average temperature). Average daytime light levels did not significantly vary between sample weeks.
DISCUSSION

In this study, we investigated the utility of ovitrap grids at two local landscape scales to observe *Ae. albopictus* distribution at a corporate campus in Research Triangle Park, NC. Both landscape-scale ovitrap grids indicated *Ae. albopictus* mainly prefer to lay eggs in ovitraps located in the forest sections of the campus. The coarse-scale grid was informative at the local population level; identifying locations within the central RTI-International campus where *Ae. albopictus* was unknown to occupy. However, it was less informative regarding specific habitat use within the multiple campus environments. The intention was to observe local *Ae. albopictus* distribution using a fine-scale ovitrap grid within a small specified habitat using dense 100m by 100m square ovitrap grid. Ovitrap egg occupation was consistent throughout the forested sections of the fine-scale ovitrap grid and less consistent in the open sections. Forested sections of the grid contained higher ovitrap egg density; however, the distribution of ovitraps with high egg density remained patchy within the forest, throughout all study periods. A potential factor for the patchy oviposition distribution in the forest is dry temporary stream beds located in the southeastern section of the ovitrap grid (Figure 2.2). This study also found a relationship between ovitrap *Ae. albopictus* egg density and the forested tree line dividing open and forest habitat within the sampling grids. Ovitraps with higher egg densities occurred farther into the forest, and ovitraps located in the open sections of the fine-scale ovitrap grid had lower egg densities. Ovitrap occupation results suggest the probability of egg occupation in a dense array of ovitraps is sensitive to gravid *Ae. albopictus* population, which increases as influenced by Hurricane Florence and decreases as conditions become less favorable, as seen in the second 2017 sample period.
Impact of Hurricane Florence:

Hurricane Florence impacted the study area from 12-15 September 2018 inundating Raleigh, NC, with 6.98” of rain (NOAA/NWS). The average September monthly total rainfall for Raleigh, NC, is 4.26” (NOAA/NWS). There is limited information on hurricane response in mosquitoes. In 2018, I found an average egg density of 1.78 ± 0.26 egg/ovitrap/day in the four-day study period before Hurricane Florence, which is comparable to the 2017 fine-scale ovitrap grid results (Table 2). Egg density averaged from only occupied sites is possibly a better measure of mosquito behavior due to the estimated difference in the number of ovipositing female *Ae. albopictus* in the pre- and post- Hurricane Florence. *Aedes albopictus* ovitrap egg density averaged for only occupied ovitraps adjusts pre-Hurricane Florence average ovitrap egg density to 3.07 ± 0.38 (SEM) eggs/ovitrap/day and post-Hurricane Florence average ovitrap egg density to 9.7 ± 0.63 (SEM) eggs/ovitrap/day. This result represents a 3.16-fold increase in average ovitrap egg density by female *Ae. albopictus* population. Furthermore, segregating average ovitrap egg density by the open section and forest section of the ovitrap grid finds the open section ovitraps only experienced a 2.51-fold increase in average egg density. In contrast, the pre- and post-hurricane comparison found the forest section increased by 3.52-fold in average ovitrap egg density. A clear indication of *Ae. albopictus*’ preference for accumulating eggs in oviposition sites situated in a forested habitat (Cianci et al., 2015; Hawley, 1988; Lourenco-de-Oliveira et al., 2004; Reiskind et al., 2017).

The massive influx of rainwater from Hurricane Florence likely facilitated the hatching of *Ae. albopictus* eggs present in this study location. *Aedes albopictus* lay desiccant resistant eggs accumulating into an “egg bank,” which hatch after water inundation by rain events (Hawley, 1988). In a recent study in Puerto Rico, researchers using CDC autocidal gravid traps (AGO)
reported a significant increase in female *Ae. aegypti* across all study sites five weeks post-Hurricane Maria (Barrera et al., 2019). In contrast, *Ae. taeniorhynchus* population crashed in the wake of Hurricane Wilma (2005) and Hurricane Irma (2018) (Lucas, Watkins, Phillips, Appazato, & Linn, 2019). The difference is likely due to *Aedes taeniorhynchus* dependence on coastal salt-marsh habitat, which is readily disturbed by hurricane events and the influx of freshwater. Container breeding mosquitoes such as *Ae. albopictus* and *Ae. aegypti* are well prepared to increase population in response to hurricane events. Another way to view these data is during typical late summer and fall weather conditions, approximately one-third of *Ae. albopictus* eggs are hatching at any given time in local oviposition sites, suggesting the egg bank may build up during the spring and summer season to take advantage of inundation events.

Skip-Oviposition:

Container breeding mosquitoes such as *Ae. albopictus* display an oviposition behavior known as “skip-oviposition,” where they lay eggs in multiple containers during a single gonotrophic cycle (Davis, Kaufman, Hogsette, & Kline, 2015; Mogi & Mokry, 1980). While unlikely a factor in the coarse-scale ovitrap grid due to the 100 m distance between ovitraps, this behavior potentially affected results in the fine-scale ovitrap grid studies. Under constant laboratory conditions, *Ae. albopictus* lays eggs in an average of five ovitraps (Reinbold-Wasson 2020). This information indicates a single gravid *Ae. albopictus* mosquito could account for multiple ovitrap occupations in the fine-scale ovitrap grid. Field studies show that skip-oviposition behavior can affect estimates of mosquito populations and demonstrate egg accumulation for preferred locations (Briggs & Osenberg, 2019). Laboratory skip-oviposition studies found the oviposition media affected the proportion of *Ae. albopictus* eggs contributed to
each container per individual oviposition event, 34% for tap water, and 48% for oak leaf infusion (Reinbold-Wasson 2020). This information allows estimation of the number of eggs which constitute the 1st oviposition event, a 2nd oviposition event, and on down until the eggs from the average individual *Ae. albopictus* are exhausted (Table 2.6). Table 2.6 displays the estimation of the number of eggs laid during the 2nd skip-oviposition event is relatively close, between 19-21 eggs regardless of proportional oviposition of eggs. This estimation is close to the median amount of eggs (22) identified in the total egg distribution of occupied ovitraps (Figure 2.5). This estimation allows for the identification of ovitraps with egg totals, which potentially represent a 2nd or more skip-oviposition event. In applying a maximum of 21 eggs per ovitrap, finds the 2017 coarse-scale and fine-scale and 2018 pre-Hurricane fine-scale ovitrap grids captured potential skip-oviposition behavior, due to relatively low levels of total eggs per ovitrap during these surveillance periods. Further, categorizing ovitraps into open and forest sections found 83% of occupied ovitraps in the open sections, and 67% of occupied ovitraps in the forest sections contained 21 or less eggs, providing evidence that ovitraps outside of the forest are likely serving as the 2nd or greater skip-oviposition sites. In contrast, the first oviposition sites are probably occurring within the forest sections of the ovitrap grids. This result was changed post-Hurricane Florence with 26.5% of occupied ovitraps in open sections and 9.9% of occupied ovitraps in forest sections containing 21 or fewer eggs. This result was likely due to the significant increase in *Ae. albopictus* females following Hurricane Florence, with multiple females skip-ovipositing in many ovitraps. It also suggests, smaller total populations of *Aedes albopictus* assist in capturing skip-oviposition behavior in the field, as larger populations dilute the ability to detect individual oviposition events.
Implications for Mosquito Control:

This study emphasized the role of forested areas as a refuge for *Ae. albopictus*. The placement of oviposition sites farther into a forest increases the probability of ovitrap *Ae. albopictus* egg occupation and increase egg density. Forested habitats decrease illumination, temperature, and container water loss, further enhancing oviposition site usability and longevity. In contrast, open habitat oviposition sites experience heat extremes during the day and high levels of site water loss. The ovitrap grid study indicates a large reduction in the probability of continued *Ae. albopictus* egg occupation and lower egg density even oviposition sites only 10 m from a forest border into an open habitat.

The effectiveness of mosquito surveillance and control traps is dependent on placement within the environment. Ovitraps are common tools for both surveillance and control of *Ae. albopictus*. Autocidal Gravid Oviposition Traps (CDC AGO) and Biogents Gravid *Aedes* Trap (GAT) are oviposition kill traps, both of which display limited success in “trap out” studies (Johnson, Ritchie, & Fonseca, 2017). These results emphasize the importance of applying ovitrap control measures inside forested areas. *Aedes albopictus* in this study showed a preference for initial oviposition in forest habitat, followed by the female moving to open habitat seeking oviposition sites. Therefore, oviposition “trap and kill” traps such as the AGO or GAT positioned outside of forested habitat may only be of limited control effect as an average of 34% to 48% eggs of any give female have likely already oviposited in a forest site. Autodissemination traps such as the In2care rely on the skip-oviposition behavior of adult *Ae. albopictus* to distribute larvicide to additional oviposition sites (Buckner, Williams, Marsicano, Latham, & Lesser, 2017). These traps face the same challenge of attracting gravid female *Aedes* species mosquitoes as the oviposition “trap and kill” traps. It is relatively unknown if female
Aedes move back and forth between open and forested habitat during oviposition. This study suggests egg accumulation in open habitats by Ae. albopictus is the result of 2\textsuperscript{nd} or greater skip-oviposition events. Autodissemination traps placed within the forest border, approximately 10 m, will increase the likelihood of larvicide dissemination to oviposition sites within the forest and open habitats, thereby enhancing the control effect of any given trap.

This study found a rapid increase in local Ae. albopictus populations in response to hurricane events. Future hurricane planning should account for this rapid increase in mosquito populations, specifically container breeding vector mosquitoes. Rapid response should include tip and toss measures along with larvicide applications as hurricane events lead to a mass hatching in container breeding species. Quickly applied control efforts could blunt the impact of hurricanes on vector mosquito populations.
REFERENCES


Day, J. F. 2016. Mosquito oviposition behavior and vector control. Insects, 7(65),


Mann, H. B., and D. R. Whitney. 1947. On a test of whether one of 2 random variables is stochastically larger than the other. Annals of Mathematical Statistics, 18(1), 50-60.


Table 2.1. Ovitrap Sample periods for 2017 and 2018

<table>
<thead>
<tr>
<th>Year</th>
<th>Week</th>
<th>Sample Period</th>
<th>Ovitrap Grid</th>
<th>Oviposition Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>1</td>
<td>31 AUG - 7 SEP 2017</td>
<td>Fine</td>
<td>Tap Water</td>
</tr>
<tr>
<td>2017</td>
<td>1</td>
<td>1 - 8 SEP 2017</td>
<td>Coarse</td>
<td>Tap Water</td>
</tr>
<tr>
<td>2017</td>
<td>2</td>
<td>7-14 SEP 2017</td>
<td>Fine</td>
<td>Tap Water</td>
</tr>
<tr>
<td>2017</td>
<td>2</td>
<td>8-15 SEP 2017</td>
<td>Coarse</td>
<td>Tap Water</td>
</tr>
<tr>
<td>2018</td>
<td>1</td>
<td>7-11 SEP 2018</td>
<td>Fine</td>
<td>Tap Water</td>
</tr>
</tbody>
</table>

Wake County, North Carolina - Hurricane Florence 14 SEP 2018

<table>
<thead>
<tr>
<th>Year</th>
<th>Week</th>
<th>Sample Period</th>
<th>Ovitrap Grid</th>
<th>Oviposition Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>2018</td>
<td>2</td>
<td>21-28 SEP 2018</td>
<td>Fine</td>
<td>4 g/L Oak Leaf Infusion</td>
</tr>
<tr>
<td>2018</td>
<td>3</td>
<td>28 SEP - 5 OCT 2018</td>
<td>Fine</td>
<td>Tap Water</td>
</tr>
</tbody>
</table>

Table 2.2. Results of coarse-scale and fine-scale ovitrap grid sampling from 2017 and 2018.

<table>
<thead>
<tr>
<th>Sample Date</th>
<th>Sites (Percent Occupied)</th>
<th>Total Eggs</th>
<th>Egg density eggs/ovitrap/day (SEM)</th>
<th>Daily Water Loss mL/ovitrap/day (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017 Coarse Scale</td>
<td>65 (69.2%)</td>
<td>658</td>
<td>1.45 ± 0.3</td>
<td>26.73 ± 1.51</td>
</tr>
<tr>
<td>8-15 SEP</td>
<td>65 (43.1%)</td>
<td>472</td>
<td>1.04 ± 0.29</td>
<td>28.03 ± 1.24</td>
</tr>
<tr>
<td>2017 Fine Scale</td>
<td>121 (71.1%)</td>
<td>2106</td>
<td>2.49 ± 0.3</td>
<td>14.57 ± 0.60</td>
</tr>
<tr>
<td>7-14 SEP</td>
<td>121 (71.1%)</td>
<td>1240</td>
<td>1.46 ± 0.22</td>
<td>17.16 ± 0.52</td>
</tr>
<tr>
<td>2018 Fine Scale</td>
<td>121 (57.8%)</td>
<td>860</td>
<td>1.78 ± 0.26</td>
<td>5.64 ± 0.27</td>
</tr>
<tr>
<td>7-11 SEP 2018</td>
<td>121 (86.8%)</td>
<td>5862</td>
<td>6.92 ± 0.63</td>
<td>5.63 ± 0.31</td>
</tr>
<tr>
<td>21-28 SEP 2018</td>
<td>121 (91.7%)</td>
<td>7535</td>
<td>8.9 ± 0.2</td>
<td>15.74 ± 0.53</td>
</tr>
</tbody>
</table>
Table 2.3. Binomial regression using ovitrap occupation for 2017 coarse-scale and fine-scale ovitrap grids and 2018 fine-scale ovitrap grid.

<table>
<thead>
<tr>
<th>Sample Date</th>
<th>Odds</th>
<th>2.5</th>
<th>97.5</th>
<th>Z-value</th>
<th>p-value</th>
<th>AIC</th>
<th>Pseudo-$R^2$ (McFadden)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2017 Coarse Scale</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>3.559</td>
<td>0.983</td>
<td>12.889</td>
<td>1.934</td>
<td>0.053</td>
<td>169.724</td>
<td>0.093</td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.014</td>
<td>0.997</td>
<td>1.030</td>
<td>1.616</td>
<td>0.106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.986</td>
<td>0.944</td>
<td>1.029</td>
<td>-0.645</td>
<td>0.519</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.320</td>
<td>0.152</td>
<td>0.676</td>
<td>-2.987</td>
<td>0.003***</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2017 Fine Scale</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>3.582</td>
<td>1.485</td>
<td>8.642</td>
<td>2.840</td>
<td>0.005***</td>
<td>246.447</td>
<td>0.181</td>
</tr>
<tr>
<td>Distance</td>
<td>1.074</td>
<td>1.049</td>
<td>1.100</td>
<td>5.870</td>
<td>0.000***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.986</td>
<td>0.938</td>
<td>1.036</td>
<td>-0.573</td>
<td>0.567</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>1.027</td>
<td>0.546</td>
<td>1.932</td>
<td>0.082</td>
<td>0.934</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2018 Fine Scale</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>1.189</td>
<td>0.630</td>
<td>2.243</td>
<td>0.533</td>
<td>0.594</td>
<td>258.624</td>
<td>0.328</td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.078</td>
<td>1.054</td>
<td>1.102</td>
<td>6.533</td>
<td>0.000***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.954</td>
<td>0.883</td>
<td>1.031</td>
<td>-1.193</td>
<td>0.233</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>8.888</td>
<td>4.042</td>
<td>19.546</td>
<td>5.434</td>
<td>0.000***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 3</td>
<td>26.936</td>
<td>6.796</td>
<td>106.757</td>
<td>4.687</td>
<td>0.000***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4. Multinomial regression for 2017 coarse-scale and fine-scale ovitrap grids and 2018 fine-scale ovitrap grid.

<table>
<thead>
<tr>
<th>Sample Ovitrap Grid</th>
<th>Odds</th>
<th>Z-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2017 Coarse Scale</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One Event</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.008</td>
<td>0.561</td>
<td>0.574</td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.933</td>
<td>-1.410</td>
<td>0.158</td>
</tr>
<tr>
<td>Two Events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.016</td>
<td>1.004</td>
<td>0.315</td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.929</td>
<td>-1.428</td>
<td>0.153</td>
</tr>
<tr>
<td><strong>2017 Fine Scale</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One Event</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.163</td>
<td>3.839</td>
<td>0.000***</td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>1.078</td>
<td>1.126</td>
<td>0.26</td>
</tr>
<tr>
<td>Two Events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.194</td>
<td>4.454</td>
<td>0.000***</td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.979</td>
<td>-0.315</td>
<td>0.753</td>
</tr>
<tr>
<td><strong>2018 Fine Scale</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One Event</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.129</td>
<td>1.052</td>
<td>0.293</td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.796</td>
<td>-1.132</td>
<td>0.257</td>
</tr>
<tr>
<td>Two Events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.394</td>
<td>2.710</td>
<td>0.007***</td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.813</td>
<td>-0.934</td>
<td>0.350</td>
</tr>
<tr>
<td>Three Events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>1.358</td>
<td>2.504</td>
<td>0.012**</td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.906</td>
<td>-0.452</td>
<td>0.651</td>
</tr>
</tbody>
</table>
Table 2.5. Negative binomial regression by egg density for 2017 coarse-scale and fine-scale ovitrap grids and 2018 fine-scale ovitrap grid.

<table>
<thead>
<tr>
<th>Sample Date</th>
<th>Estimate</th>
<th>S.E.</th>
<th>Z-value</th>
<th>p-value</th>
<th>AIC</th>
<th>Pseudo-(R^2) (McFadden)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2017 Coarse Scale</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.281</td>
<td>0.543</td>
<td>0.517</td>
<td>0.605</td>
<td>375.747</td>
<td>0.028</td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>0.025</td>
<td>0.008</td>
<td>3.256</td>
<td>0.001***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>0.002</td>
<td>0.019</td>
<td>0.095</td>
<td>0.924</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>-0.418</td>
<td>0.333</td>
<td>-1.254</td>
<td>0.210</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2017 Fine Scale</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.788</td>
<td>0.251</td>
<td>3.139</td>
<td>0.002***</td>
<td>863.43</td>
<td>0.061</td>
</tr>
<tr>
<td>Distance</td>
<td>0.044</td>
<td>0.006</td>
<td>7.543</td>
<td>0.000***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>-0.011</td>
<td>0.015</td>
<td>-0.718</td>
<td>0.473</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>-0.431</td>
<td>0.179</td>
<td>-2.415</td>
<td>0.016**</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2018 Fine Scale</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.390</td>
<td>0.138</td>
<td>2.827</td>
<td>0.005***</td>
<td>1879.454</td>
<td>0.079</td>
</tr>
<tr>
<td>Distance from Tree Line</td>
<td>0.025</td>
<td>0.003</td>
<td>8.160</td>
<td>0.000***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily Water Loss</td>
<td>-0.12</td>
<td>0.013</td>
<td>-0.871</td>
<td>0.384</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>1.386</td>
<td>0.138</td>
<td>10.063</td>
<td>0.000***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 3</td>
<td>1.692</td>
<td>0.190</td>
<td>8.893</td>
<td>0.000***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6. Table representing the estimation of the average amount of eggs expected for each skip-oviposition event. Average starting egg total for Ae. albopictus based on laboratory results from skip-oviposition studies (see Chapter 3).

<table>
<thead>
<tr>
<th>Skip-Oviposition Estimation</th>
<th>34% Skip</th>
<th>40% Skip</th>
<th>48% Skip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Eggs</td>
<td>85</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Ovitrap #1</td>
<td>28.90</td>
<td>34</td>
<td>40.8</td>
</tr>
<tr>
<td>Ovitrap #2</td>
<td>19.07</td>
<td>20.4</td>
<td>21.21</td>
</tr>
<tr>
<td>Ovitrap #3</td>
<td>12.59</td>
<td>12.24</td>
<td>11.03</td>
</tr>
<tr>
<td>Ovitrap #4</td>
<td>8.31</td>
<td>7.34</td>
<td>5.74</td>
</tr>
<tr>
<td>Ovitrap #5</td>
<td>5.48</td>
<td>4.41</td>
<td>2.98</td>
</tr>
<tr>
<td>Ovitrap #6</td>
<td>3.62</td>
<td>2.64</td>
<td>1.55</td>
</tr>
<tr>
<td>Ovitrap #7</td>
<td>2.39</td>
<td>1.59</td>
<td>0.81</td>
</tr>
<tr>
<td>Ovitrap #8</td>
<td>1.58</td>
<td>0.95</td>
<td>0.42</td>
</tr>
</tbody>
</table>
Figure 2.1. Research Triangle Institute (RTI) International corporate campus. (A) RTI campus study site represented using a USGS map available in ESRI ArcGIS (ESRI, 2011). (B) Campus map overlay stitched together from multiple high-resolution images taken by a drone-mounted camera. (C) Coarse-scale ovitrap grid. Yellow sites represent placed ovitraps; pink sites represent proposed ovitrap positions not placed due to ongoing construction activity or hard surface obstruction (i.e., road, buildings, other structures). (D) Fine-scale ovitrap study area in the southern section of the RTI campus.
Figure 2.2. Research Triangle Institute campus stormwater mitigation area. (A) Map of study site without ovitrap grid represented using USGS map available in the Esri ArcGIS program (ESRI, 2011). (B) A map of the study site produced by the RTI-International drone program during the 2017 study using high-resolution photography. High-resolution images were stitched together, following multiple overflights from the RTI drone. Yellow points represent ovitraps placed during 2017 fine-scale ovitrap sample periods. Blue points represent ovitraps placed during 2018 fine-scale ovitrap sample periods. Blue lines represent dry stream beds that fill during significant scale rain events.

Figure 2.3. Diagram of ovitrap placement plan for ovitrap grid with 10 m separations.
Figure 2.4. Oviposition trap affixed to a survey stake. Ovitraps are each provisioned with an oviposition paper and 300 mL of oviposition media (tap water or oak leaf infusion).

Figure 2.5. Histogram represents all occupied ovitraps; the x-axis bin width is five eggs and starts with ovitraps containing one to five eggs. The blue dash line highlights the median egg amount per ovitrap.
Figure 2.6. Scatter plot showing the correlation between daily ovitrap water loss and ovitrap position distance from the forested tree line. The plot shows Pearson's correlation coefficient values as calculated in R. All sample periods demonstrated a negative correlation of ovitraps placed in open sections as compared to forest sections of the ovitrap grid. Zero on the plot x-axis is the location of the tree line. Positive values are the distance in meters an ovitrap was placed into a forested section of the ovitrap grid, whereas negative values are the distance in meters from the tree line into the open sections of the ovitrap grids.
<table>
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<th></th>
<th>Ovitrap Occupation</th>
<th>Egg Density (eggs/day)</th>
<th>Water Loss (mL/day)</th>
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Figure 2.7. Inverse distance weighted overlays from ArcGIS for coarse and fine ovitrap grids. Color ramping low (blue) and high (red). Ovitrap occupation color ramp uses equal separations between 0 and 1. Egg density and daily water loss color ramps by 1/4th standard deviation separations.
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Figure 2.10. Individual binomial regression lines showing the probability of ovitrap occupation as ovitrap daily water loss increases.
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Figure 2.13. Negative binomial regression of average egg density compared to both distance from the forested tree line and daily water loss.
CHAPTER 3
Skip-Oviposition Behavior Comparisons Among Container Breeding Aedes spp. Mosquitoes
(Diptera: Culicidae)

ABSTRACT

Container Aedes species mosquitoes vector many human diseases (i.e., dengue, chikungunya, Zika, or yellow fever). Invasive and native container Aedes spp. utilize small water containers as oviposition and larval habitat. One behavioral adaptation to variable oviposition environments is for females to lay eggs in multiple sites during each gonotrophic cycle, known as “skip-oviposition,” this behavior has been documented for several container breeding Aedes species mosquitoes. This study examined skip-oviposition behavior in three container Aedes spp. by testing the following hypotheses. H$_1$: Container Aedes species mosquitoes adapted to utilizing temporal urban habitats have high rates of skip-oviposition behavior. H$_2$: Container breeding Aedes species mosquitoes engage in spread more egg clutches when oviposition sites are nutritionally poor. H$_3$: Aedes albopictus is known to aggregate eggs in fall conditions decreasing skip-oviposition behavior due to diapause inducing fall temperature and photoperiod. Eight-cup test arena’s assessed individual Aedes aegypti (L.), Aedes albopictus (Skuse), and Aedes triseriatus (Say) female mosquitoes for skip-oviposition using the index of dispersion (ID), an aggregation statistic. Index of dispersion scales the egg spreading using a mean-variance ratio between eight oviposition cups converting skip-oviposition into a single
statistic between 0 (egg aggregation in one container) and 1 (equal egg spreading among all containers). Under nutritionally enriched oviposition media (oak leaf infusion) conditions invasive *Ae. aegypti* and *Ae. albopictus* spread eggs significantly more (KW $\chi^2_5 = 47.8$, $p$-value < 0.0001) than native *Ae. triseriatus*. Egg spreading increased among containers for *Ae. aegypti* and *Ae. albopictus* when using nutritionally unenriched (tap water) oviposition media; conversely, *Ae. triseriatus* responded to this treatment by simply retaining eggs. *Aedes albopictus* did not modify skip-oviposition behavior when exposed to fall-like environmental conditions (reduced temperature and shortened photoperiod). These results suggest approximately 90% of female *Ae. aegypti* and 95% of female *Ae. albopictus* skip-oviposit their eggs under “preferred” conditions, with the multiple oviposition site usage increasing under “non-preferred” oviposition sites. In contrast, *Ae. triseriatus* skip-oviposits less frequently, aggregating a greater proportion of their eggs in a few oviposition sites. This study suggests that further understanding of skip-oviposition behavior is needed to make the best use of autodissemination trap technology, in which skip-ovipositing females spread a potent larvicide among oviposition sites within the environment.
INTRODUCTION

Invasive and native container breeding *Aedes* mosquitoes are important vectors of mosquito-borne viruses (Lounibos, 2002). Anthropophilic invasive *Aedes* mosquitoes include *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse), which are the main vectors of dengue, chikungunya, and Zika virus in tropical and sub-tropical regions worldwide (Bonizzoni, Gasperi, Chen, & James, 2013; Leta et al., 2018). Native container breeding *Aedes* mosquitoes are also important vectors of disease, with *Aedes triseriatus* (Say) the primary vector of La Crosse virus within the United States (Borucki, Kempf, Blitvich, Blair, & Beaty, 2002; Miller, Defoliart, & Yull, 1978). The worldwide success of container-breeding *Aedes* mosquitoes is associated with laying of desiccation-resistant eggs in small water-filled containers such as natural tree holes, rock pools, tires or other artificial containers that can be easily transported by human activity (Bentley & Day, 1989; Day, 2016; Reiter, 2007). Furthermore, most container breeding *Aedes* species mosquitoes will readily take blood meals from human hosts (Lounibos, 2002). *Aedes triseriatus* remains a concern for invasion into Europe due to the presence of similar favorable breeding habitats as in its native range in the United States, which facilitated the invasion of another container breeding native North American mosquito, *Aedes atropalpus* (Coquillett), in the late 1990s (Medlock et al., 2012; Schaffner, Medlock, & Van Bortel, 2013).

Invasive mosquito species undoubtedly adapt to new habitats using multiple adaptive strategies (Day, 2016). One theory in evolution involves the development of risk aversion traits known as bet-hedging (Slatkin, 1974). The underlying effect of bet-hedging traits is the decrease in mean fitness through less variation in fitness (Childs, Metcalf, & Rees, 2010; Simons, 2011). Bet-hedging traits, therefore, increase the geometric mean fitness, i.e., long-term or generational fitness, of a population at the potential cost of an individual’s fitness. For example: in *Aedes*
species, not all eggs hatch in response to a favorable water inundation event, a trait known as installment hatching (Gillett, 1955). *Aedes* eggs that do not hatch during the first inundation represent an insurance policy, preventing complete clutch failure in a variable environment. A specific bet-hedging risk aversion strategy, known as diversifying bet-hedging, hypothesizes that individuals avoid risk by “not placing all their eggs in one basket” (Olofsson, Ripa, & Jonzen, 2009). Individual container breeding *Aedes* mosquitoes exhibit the diversifying bet-hedging strategy by laying eggs among multiple small containers, a behavior known as “skip-oviposition” (Davis, Kaufman, Hogsette, & Kline, 2015; Fay & Perry, 1965; Rey & O’Connell, 2014; Swan, Lounibos, & Nishimura, 2018; Trexler, Apperson, & Schal, 1998). Skip-oviposition behavior increases the likelihood of survival by spreading the risk of clutch failure among multiple sites. This spreading of risk increases the likelihood of partial clutch failure; in exchange, there is a reduction in the risk of complete clutch failure.

The term “skip-oviposition” coined to describe the behavior of *Wyomia smithii* female mosquitoes distributing eggs among multiple pitcher plants (Mogi & Mokry, 1980). Skip-oviposition now broadly describes the mosquito behavior of distributing eggs among multiple oviposition sites during a single gonotrophic cycle (Day, 2016; Harrington & Edman, 2001; Mogi & Mokry, 1980). *Aedes aegypti, Ae. albopictus* and *Ae. triseriatus* exhibit skip-oviposition behavior, i.e., egg spreading, in laboratory studies (Corbet & Chadee, 1993; Davis et al., 2015; Swan et al., 2018; Trexler et al., 1998). *Aedes aegypti* spread their eggs among multiple oviposition containers (Reiter, 2007). Skip-oviposition behavior potentially contributes to the global invasion success of *Ae. albopictus* through international trade (Hawley, 1988). *Aedes triseriatus* expanded its southern range in Mexico due to human activity and the use of man-made containers (Sanchez-Trinidad et al., 2014).
Container choice due to the quality of oviposition sites for *Ae. aegypti* and *Ae. albopictus* is a repeating focus for oviposition studies. Implicitly, these studies use skip-oviposition behavior as a tool for assessing habitat preference by comparing counts of eggs in one container versus others (Corbet & Chadee, 1993). Oviposition site “quality” is a variable definition that includes differences in nutrition, conspecific larval, conspecific eggs, type of site, or site color (Bentley & Day, 1989). Individual *Ae. albopictus* increase egg aggregation in response to one “preferred” oak leaf infused water cup as compared to seven other water only containers (Trexler et al., 1998). In the same study, *Aedes triseriatus* individuals preferentially aggregated eggs in the oak leaf infusion cup; however, they did not modify already limited egg spreading behavior (Trexler et al., 1998). Trexler et al. 1998 highlight using skip-oviposition behavior as a method to assess specific habitat preferences and differences between species. The presence or absence of conspecific individuals also modifies the oviposition behavior of both *Ae. aegypti* and *Ae. albopictus*. *Aedes albopictus* displays a preference for “quality” oviposition sites with uncrowded larval conditions (Davis et al., 2015). Similarly, *Aedes aegypti* individuals prefer to aggregate eggs in oviposition cups containing lower conspecific egg density conditions (Chadee, Corbet, & Greenwood, 1990; Nazni, Bandara, Azahari, Craig, & Lee, 2016). Type of oviposition container can also function as a “quality” measure in skip-oviposition studies. A container-specific study compared flowerpots, coconut shells, and clear plastic cups, finding that *Ae. aegypti* spread eggs to previously avoided clear plastic cup once it was painted black (Oliva, Correia, & Albuquerque, 2014). The previous examples focus on oviposition site choice when there is a difference among multiple containers. However, there is a lack of information on the underlying mechanisms of skip-oviposition choice.
Few studies specifically address fundamental behavioral mechanisms associated with risk aversion egg spreading in container breeding *Aedes* species mosquitoes. A consistent observed oviposition behavior is individual *Ae. aegypti* and *Ae. albopictus* aggregate a larger proportion of eggs into a “favorite” cup (Oliva et al., 2014; Santos de Abreu, Filipe Vieira, Morais, Ribeiro, & Eiras, 2015). Individuals from both species display this behavior by the aggregation of 40% or more of their eggs in one cup when presented with an identical multiple oviposition cup environment (Davis et al., 2015; Oliva et al., 2014; Santos de Abreu, Filipe Vieira et al., 2015). *Aedes aegypti* and *Ae. albopictus* respond to a larger amount of oviposition sites by increasing the number of cups occupied and subsequently decreasing average egg contribution to each cup (Santos de Abreu, Filipe Vieira et al., 2015; Swan et al., 2018). These observations suggest *Ae. aegypti* and *Ae. albopictus* are potentially conducting more skip-oviposition egg clutch events than available oviposition sites, hence a corresponding increase in cup occupation with the addition of more cups. Furthermore, *Ae. aegypti* spreads eggs more equally than *Ae. albopictus*, even as both species utilize the same number of oviposition sites (Rey & O’Connell, 2014; Swan et al., 2018). There is no indication either age or gonotrophic cycle modifies skip-oviposition behavior, as individual *Ae. aegypti* females consistently spread eggs through five gonotrophic cycles (Oliva et al., 2014). However, there is evidence that physiological condition affects behavioral mechanisms. *Aedes aegypti* held three days post blood meal without an oviposition site, forced egg retention, significantly reduced skip oviposition behavior when presented with a multiple oviposition site test arena (Chadee, 1997).

Skip-oviposition is a targeted behavior for control using larvicide autodissemination traps, potentially controlling *Ae. aegypti, Ae. albopictus*, and possibly additional container breeding *Aedes* species (Buckner et al., 2017). There is little knowledge of the behavioral
ecology of skip-oviposition in container _Aedes_ species mosquitoes. Therefore, a greater understanding of the behavioral mechanisms of skip-oviposition may provide insights into how to enhance container breeding mosquito control.

Few studies compare skip-oviposition behavior among species of container breeding _Aedes_ mosquito. This study reports single gonotrophic cycle skip-oviposition patterns of three species of container breeding _Aedes_ mosquito: _Ae. aegypti_, _Ae. albopictus_ and _Ae. triseriatus_. The skip-oviposition test arenas are similar to previous studies with each arena containing eight identical oviposition sites (Chadee et al., 1990; Trexler et al., 1998). All oviposition cups in a test arena received either nutritionally enriched (oak leaf infusion) oviposition media or unenriched (tap water) oviposition media when assessing differences in egg spreading between container _Aedes_ species. Previous laboratory assessments of _Ae. albopictus_ found this species spreads eggs in summer conditions, and aggregates eggs under fall conditions (Fonseca, Kaplan, Heiry, & Strickman, 2015). This study assessed individual diapause egg-laying induce _Ae. albopictus_ to assess skip-oviposition under fall conditions, as Fonseca et al. 2015 used cages of multiple _Aedes albopictus_, making it difficult to determine the effect of skip-oviposition behavior in their results. These studies assess the following hypothesis:

H₁: Container _Aedes_ species mosquitoes adapted to utilizing temporal urban habitats have high rates of skip-oviposition behavior.

H₂: Container _Aedes_ species mosquitoes increase skip-oviposition when oviposition sites are of poor “quality.”

H₃: _Aedes albopictus_ decreases skip-oviposition behavior due to diapause inducing fall temperature and photoperiod.
Each of these hypotheses generates a clear prediction assessed through arena studies in the laboratory. The larger goal is to further understanding of skip-oviposition behavior; predict the following outcomes:

P1: Skip-oviposition, as measured by egg spreading among multiple containers, will be higher in the urban adapted mosquitos, *Ae. aegypti* and *Ae. albopictus*, than the forest preferring *Ae. triseriatus* regardless of media “quality.”

P2: Unenriched (tap water) poor “quality” oviposition media will increase egg spreading over enriched (oak leaf infusion) good “quality” oviposition media with all species: *Ae. aegypti*, *Ae. albopictus* and *Ae. triseriatus*.

P3: *Ae. albopictus* will increase egg aggregation, conversely decrease egg spreading in response to a diapause inducing fall condition.
MATERIALS & METHODS

Mosquito Rearing and Colony Maintenance:

For this series of oviposition studies, I reared multiple species of container breeding *Aedes* mosquitoes obtained from colonies located at the North Carolina State University (NCSU) Biological Resources Facility (BRF). Three container breeding mosquito species used in skip-oviposition experimental trials: *Ae. aegypti*, *Ae. albopictus* and *Ae. triseriatus*. The *Aedes aegypti* colony, in the laboratory since 2014, was derived from field material collected in Florida and Arizona. The *Aedes albopictus* colony is from locally collected populations in Wake County, North Carolina. Each year, individuals from *Ae. albopictus* eggs field-collected in Wake County were introduced into this colony to reflect the current field population. The recent laboratory colony, 2018, of *Aedes triseriatus* colony, originated from local populations in Wake, New Hanover, and Nash Counties, North Carolina, with F5-F8 generations used in this study.

Our laboratory maintains *Ae. aegypti* and *Ae. albopictus* in Bugdorm-1 adult cages (Insect Rearing Cage, Dimensions: W30 cm x D30 cm x H30 cm, MegaView Science Co., Ltd., Taiwan). Larger collapsible adult cages (Dimensions: 46 cm x 46 cm x 46 cm, Bioquip Products, California, US) facilitate colony maintenance for *Ae. triseriatus* as this species displays improved mating success in the expanded area. For skip-oviposition experiments, the smaller Bugdorm-1 adult cage worked well for *Ae. triseriatus* colonies as the smaller cage did not affect the single generation mating success of this species.

Adult mosquitoes from each species were hatched and reared in identical environmental conditions to limit the influence of environmental effects between species for skip-oviposition testing (100 larvae/L, 0.6 g fish food). The mosquito rearing facility maintained a constant temperature of 27 °C with 14:10 light:dark photoperiod and average relative humidity of 52%.
Model DS1923-F5# iButton® data loggers (Maxim Integrated Products, Wisconsin, US) monitored temperature and humidity within the BRF mosquito rearing room. Adult female mosquitoes are fed weekly with defibrinated bovine blood (HemoStat Laboratories, California, US) in hog casing (The SausageMaker, Inc, New York, US) covered small petri dish. A HeatMax Hothands hand warmer (Kobayashi Consumer Products LLC, Georgia, US) warms the bovine blood dish for 10 min, at which point the dish is inverted and placed on the top of the adult cage for mosquito feeding. An oviposition cup lined with heavy weight seed germination paper (Anchor Co. St. Paul, Minnesota, US) was placed in the adult cages to collect eggs weekly for laboratory experiments and propagation of each colony.

All hatching for container *Aedes* species hatched for skip-oviposition experimental trials occurred in modified Rubbermaid® Egg Keeper (Rubbermaid Inc, North Carolina, US). Modified) larvae trays each containing 1 L tap water and 0.6 g ground fish food (Wardley Pond Pellets, Hartz Canada Inc., Ontario, Canada) (Figure 3.1). Two days post-hatching, I separated larvae into new trays with a concentration of 100 larvae per tray. I observed each tray for pupal development five days to seven days post-hatching. If pupae were present, then they were collected in a small water container and placed in the Bugdorm-1 adult cages for adult emergence. Emerged adults fed from sugar feeders containing a 10% sucrose solution in a 120 mL lidded cup with a 100 mm filter wick (Tidi Products Neenah, Wisconsin, US) extending 25 mm outside of the cup, inserted into the center of the lid.

Experimental Procedures:

Container *Aedes* species adult testing arenas, Bugdorm-1 adult cages, contained eight identical black plastic 473 mL oviposition cups and a sugar feeder. Skip-oviposition studies
used oviposition cups filled with 250 mL of oviposition media (either all cups had enriched or unenriched media), and each cup had a 7 cm x 25 cm strip of seed germination paper (oviposition paper). Upon paper insertion, oviposition media covered half of the paper inside the cup. Each oviposition paper had the assigned cage numbers (1 through 24) and cup positions (A to H, see Figure 3.2) written on it. Cup placement was identical for each trial cage, beginning with the placement of cup A in the front left corner of the test arena, then moving clockwise around the arena, ending with cup H (Figure 3.2). Mosquitoes fed off of centrally located 30 mL plastic cup sugar feeders containing 20 mL of a 10% sucrose solution, covered by a 50 mm x 50 mm square of Bemis™ Parafilm™ (Thermo Fisher Scientific), with a 25 mm filter wick inserted into the parafilm.

All skip-oviposition behavioral trials took place in two incubators (Model DR-36VL, Percival, Perry, Iowa, US). Each incubator holds twelve cages, three shelves of four cages, allowing the assessment of 24 individual female mosquitoes per experimental trial (Figure 3.3). Incubators were set to the temperature and photoperiod as required for each specific experimental trial. Relative humidity remained high, 87% ± 2.5%, throughout each trial, due to the large volume of oviposition media totaling 24 L per incubator.

Oviposition media for skip-oviposition experimental series included a nutritionally “enriched” oak leaf infusion media and a nutritionally “unenriched” media (tap water). Tap water incubated in a water cooler at room temperature one day before skip-oviposition trials served as unenriched oviposition media. Previous experiments indicate that enriched oak leaf infusion (OLI) is an attractive oviposition water for *Ae. aegypti, Ae. albopictus* and *Ae. triseriatus* mosquitoes (Ponnusamy et al., 2010; Trexler et al., 1998). A single 90L batch of concentrated 16 g/L OLI reduced variation in oviposition media used for subsequent skip-
oviposition experiments. To make 90L of OLI, I used a 32-gal Rubbermaid trash bin (Rubbermaid Inc, North Carolina, US) filled with 90 L of tap water and equal amounts of brewer’s yeast (MP Biomedicals, cat no. 903312, Fisher Scientific), 27 g (0.3 g/L), and egg albumin (Fisherbrand™ Albumin from Eggs (Powder) cat no. A388-500, FisherChemical), 27 g (0.3 g/L) (Obenauer et al., 2009; Reiskind & Janairo, 2018). Locally collected dried willow oak leaves (Quercus phellos) in fine mesh cloth created a bundle,1440 g (16 g/L), immersed for infusion in the water mixture. Willow oak leaf infusion is a readily available resource that when nutritionally enriched successfully used in Ae. albopictus larval studies in our laboratory (Reiskind & Janairo, 2018). The willow oak leaf bundle was held down by a smaller trash bin inserted above the infusion. The infusion incubated at room temperature for five days, then one-quart Ziploc plastic bags each received a 250 mL aliquot of OLI. The 16 g/L OLI aliquots froze at -20 °C in 6-quart plastic containers to prevent media leakage from the Ziploc bags. The day before skip-oviposition experiments thawed 16 g/L OLI aliquots mixed with tap water created a working concentration of 2 g/L OLI oviposition media.

On the morning of the start of experimental trials, I assembled test arenas (Figure 3.2) with both oviposition cups (Figure 3.4) and sugar feeders. Following which, I blood-fed each group of mosquitoes per protocol. Post-blood-feeding, I aspirated female mosquitoes from adult rearing cages into small transport containers, observing each mosquito to ensure they contained a blood meal. I then transported the small containers of mosquitoes in a Styrofoam cooler from the NCSU BRF to NCSU Method Building One for experimental trials. For trails, test arenas received an individual mosquito randomly assigned via the random() function in Microsoft Excel. Trials began once test arenas received mosquitos and placed back in incubators.
Experimental trials ended after six days with the collection of individual female mosquitoes and oviposition papers. Sacrificing mosquitoes in a -20 °C freezer, I later dissected for wing length measures and assessment of egg retention. I removed the right wing each female mosquito, left wing if the right wing was damaged, then mounted it on a microscope slide measuring the distance from the axillary incision to the end of the R₄+₅ vein to nearest 0.001 mm excluding the fringe scales with a microscope camera and software (Model MU1803-CK, AM Scope, California, US), (Armbruster & Hutchinson, 2002). Following wing removal, I dissected each female, counting any retained eggs. Finally, recording egg count from each of the eight oviposition papers for the individual tested mosquito. Six *Ae. albopictus* females are excluded from correlations by wing length due to damaged wings; however, included in remaining analysis.

*Aedes* Species Skip-Oviposition Assessment:

Three experimental series in assessed skip-oviposition behavior in container breeding *Aedes* species.

**Aedes** species comparison: the first series of experiments assessed differences in skip-oviposition behavior between *Ae. aegypti*, *Ae. albopictus* and *Ae. triseriatus* using 2 g/L OLI oviposition media and incubators set to 27 °C and 14:10 photoperiod. The eight oviposition cups each contained equal amounts of 2 g/L OLI oviposition media to assess differences in container *Aedes* species response.

Unenriched oviposition media assessment: the second series of experiments assessed differences in skip-oviposition behavior between *Ae. aegypti*, *Ae. albopictus* and *Ae. triseriatus* when using unenriched (tap water) oviposition media to enriched (2 g/L OLI) oviposition media and
incubators set to 27 °C and 14:10 photoperiod. The eight oviposition cups in each test arena contained an equal volume of either all unenriched oviposition media or enriched 2 g/L OLI oviposition media.

**Egg diapause induced *Aedes albopictus* assessment:** the third series of skip-oviposition experiments utilized 2 g/L OLI oviposition media in all test arena oviposition cups and *Ae. albopictus* species mosquitoes. This experiment compared individual *Ae. albopictus* reared and tested at 27 °C and 14:10 photoperiod and 21 °C and 10:14 photoperiod, an environmental condition that produces diapause egg-laying adult females (Fonseca et al., 2015). Only *Ae. albopictus* known to diapause in the egg stage, whereas *Ae. aegypti* is not known to diapause and *Ae. triseriatus* diapauses during both egg and larvae stages, creating a logistical difficulty in producing diapause egg-laying adult females.

Synchronized egg hatching and larval rearing produced same-day adult emergence of all three *Aedes* species. Synchronized hatching also produced *Aedes albopictus* in diapause conditions for adult emergence at the same time as non-diapause condition individuals. Skip oviposition assessment used 5-7 day post emerged adults allowing time for in cage mating and pre-blood meal egg development. For synchronizing adult emergence, egg hatching occurred over three days: day one, *Ae. triseriatus*, day two *Ae. albopictus*, day three *Ae. aegypti*. *Ae. albopictus* hatched in diapause-inducing conditions four days before *Ae. albopictus* in non-diapause inducing conditions allowed synchronized adult emergence. A skip-oviposition assessment over six days allowed *Ae. triseriatus* to oviposit undisturbed during the testing as this species takes longer to develop and lay eggs than either *Ae. aegypti* or *Ae. albopictus*. Similarly, the diapause egg-laying *Ae. albopictus* adults required a more extended oviposition period, eleven days, due to egg development delay from reduced temperature.
Skip-Oviposition Metrics:

Skip-oviposition assessment used two metrics comparing each species and treatment combination. The first is the count of occupied oviposition cups, which is the primary method of skip-oviposition comparison in previous studies (Chadee, 1997; Davis et al., 2015; Oliva et al., 2014; Santos de Abreu, Filipe Vieira et al., 2015; Swan et al., 2018; Trexler et al., 1998). For the second metric, an aggregation statistic known as the index of dispersion provided a single useful statistic for analysis of skip-oviposition behavior (Fisher, Thornton, & MacKenzie, 1922; Walker, 1999; Young & Young, 1998). The index of dispersion (ID) is a variance-to-mean ratio, initially proposed by Fisher et al. in 1922. Walker 1999 recommended a modification for more complicated data sets resulting in an ID score scaled from zero to one. The following formula calculates the index of dispersion:

\[
Index \text{ of dispersion} = \frac{K(N^2 - \sum f^2)}{N^2(K - 1)}
\]

Where \( K \) is the number of oviposition cups, \( N \) is the total number of eggs (N), and \( f \) is the number of eggs in each oviposition cup. In the case of skip-oviposition data, the following formula calculates the identical ID value:

\[
Index \text{ of dispersion} = \frac{K - \frac{\text{variance} \ (f)}{\text{mean} \ (f)^2}}{K}
\]

Index of dispersion acts to modify discrete cup counts placing the index values into a continuous scale from zero to one allowing for more sophisticated statistical analysis.

Oviposition cups grouped and reorganized from highest egg count (i.e., “favorite” cup) to lowest egg count, 1 to 8, assess’ the pattern of distribution for each female mosquito (Oliva et al., 2014). Species oviposition pattern estimated by averaging total eggs for each cup group, one through eight, then calculated the error around each cup group in the standard error of the mean.
(SEM). This method also produces an average index of dispersion score for each Aedes species and treatment combination.

Data analysis:

Data analysis conducted in R 3.6.2 (R Core Team, 2017) using the PMCRMplus package (Pohlert, 2019) and produced all graphs using the ggplot2 package (Wickham, 2009). A total of 41 mosquitoes failed to complete skip-oviposition assessment, therefore excluded from the analysis, 26 who retained eggs, and 15 who failed to produce eggs (Table 1). The distribution of skip-oviposition ID score is not normal; therefore, it requires a non-parametric Kruskal-Wallis test to assess differences in skip-oviposition behavior between species for occupied cups and ID. The Steel-Dwass-Critchlow-Fligner test (Steel-Dwass test) followed significant results as a post hoc test of multiple comparisons (Critchlow & Fligner, 1991; Dwass, 1960; Kruskal & Wallis, 1952; Steel, 1959). A Mann-Whitney U-test compared two treatment groups of *Ae. albopictus* (diapause induced and enriched 2 g/L OLI oviposition water) using the wilcox.test() function in R (Mann & Whitney, 1947). For statistically interesting non-significant Steel-Dwass test results, a post hoc Mann-Whitney U-test provided additional comparison (Table 3.2).
RESULTS

Of the 192 individual *Aedes* mosquitoes assessed for skip-oviposition behavior, 151 mosquitoes successfully completed skip-oviposition trials (Table 3.1). A significantly larger proportion, 35.4%, \( (\chi^2 = 32.89, p\text{-value} < 0.0001) \) of *Ae. triseriatus* retained eggs as compared to *Ae. aegypti* and *Ae. albopictus*. A majority, 73%, of all tested *Aedes* mosquitoes failing to develop eggs were *Ae. albopictus*. However, due to *Ae. albopictus* comprising 50% of all tested mosquitoes there was no proportional difference among the three *Aedes* species \( (\chi^2 = 2.76, p\text{-value} = 0.2521) \) in failure to develop eggs.

*Aedes* mosquito wing length did not influence skip-oviposition behavior \( (ID) \) for all treatments (Figure 3.5). Additionally, an individual’s total eggs did not influence skip-oviposition behavior \( (ID) \) in *Ae. aegypti* and *Ae. triseriatus* (Figure 3.6). However, *Ae. albopictus* showed a slightly positive correlation between total eggs and skip-oviposition behavior \( (ID) \) under enriched (2 g/L OLI) oviposition media and unenriched (tap water) oviposition media \( (t_{50} = 2.14, p\text{-value} = 0.038 \text{ and } t_{10} = 2.29, p\text{-value} = 0.045, \text{ respectively}) \). In contrast, there was a slight negative correlation in the same comparison for diapause induced *Ae. albopictus* \( (t_{13} = -2.23, p\text{-value} = 0.044) \) (Figure 3.6).

*Ae. aegypti* and *Ae. albopictus* with enriched (2 g/L OLI) oviposition media conditions occupied more cups and displayed a greater \( ID \) than *Ae. triseriatus* (cup occupation: KW \( \chi^2_{5} = 43.8, p\text{-value} < 0.0001, ID \text{ KW } \chi^2_{5} = 47.8, p\text{-value} < 0.0001 \) ) (Table 3.2). A post hoc comparison found slightly increased egg spreading \( (ID) \) in *Ae. aegypti* over *Ae. albopictus* \( (ID \text{ MW, W} = 1087.5, p\text{-value} = 0.0389) \), but no difference in cup occupation \( (\text{MW, W} = 917, p\text{-value} = 0.5925) \) (Table 3.2, Figure 3.7). *Aedes albopictus* increases egg spreading in response to unenriched oviposition media treatment as compared to enriched (2 g/L OLI) oviposition media.
but did not increase cup occupation (Table 3.2, Figure 3.8). Conversely, *Ae. aegypti* increase cup occupation in response to unenriched oviposition media treatment (MW, \( p \)-value = 0.01) but did not increase in ID (Table 3.2, Figure 3.8).

Species distributions reveal a proportion of individuals from each species aggregated all eggs in one container under enriched (2 g/L OLI) oviposition media treatment (Figure 3.7). A significantly higher proportion of *Ae. triseriatus* individuals aggregated eggs in one cup (\( \chi^2 = 11.02, p \)-value = 0.004) over *Ae. aegypti* and *Ae. albopictus*. Under unenriched oviposition media conditions, neither *Ae. aegypti* nor *Ae. albopictus* aggregated all eggs in a single cup (Figure 3.8). *Aedes albopictus* under diapause and non-diapause inducing conditions show similar distributions in cup occupation and ID score (Figure 3.9).

There was no arena cup positional effect on the probability of cup occupation or individual egg totals for *Ae. aegypti* or *Ae. triseriatus* (*Ae. triseriatus* in unenriched water conditions not tested). The same analysis found *Ae. albopictus* to have no difference in the probability of cup occupation nor individual cup egg totals. However, *Ae. albopictus* under enriched (2 g/L OLI) oviposition media treatment contributed significantly more eggs (\( F_{1,310} = 5.836, p \)-value = 0.0163) to corner cups, finding the average eggs per corner cup was 12.94 eggs, as opposed to middle cups where the average eggs per middle cup was 9.04 eggs.

Similar to Olivia et al. 2014, reorganizing cups for individual mosquito grouping from the highest egg total to lowest found the average number of eggs per cup by species and treatment (Table 3.2). This process identified a pattern of oviposition for each *Aedes* species in which each cup received an average percentage of eggs. *Aedes aegypti* and *Ae. albopictus* show a higher mean percentage of eggs per oviposition cup (43% and 48%, respectively) under enriched (2 g/L OLI) oviposition media conditions than unenriched oviposition media reduced
egg contribution in both species (33% and 34%, respectively). In contrast, *Ae. albopictus* in the diapause egg-laying inducing environmental condition did not modify the mean percentage of eggs (48%) contributed to each oviposition cup when compared to the non-diapause egg-laying inducing conditions.
DISCUSSION

This study assessed the skip-oviposition behavior of *Ae. aegypti*, *Ae. albopictus* and *Ae. triseriatus* under laboratory conditions. Skip-oviposition behavioral experiments used test arenas consisting of small cages with eight identical oviposition sites, similar to previous studies (Chadee et al., 1990; Chadee, 1997; Trexler et al., 1998). The resulting skip-oviposition behavior supports the first hypothesis, clearly showing urban adapted *Ae. aegypti* and *Ae. albopictus* spread eggs more than forest preferring *Ae. triseriatus* under identical oviposition conditions. The difference in egg spreading is less defined between *Ae. aegypti* and *Ae. albopictus*. A direct species comparison suggests each species uses a similar number of oviposition cups; however, *Ae. aegypti* spreads eggs more evenly among cups than *Ae. albopictus* resulting in significantly higher ID values. *Aedes aegypti* and *Ae. albopictus* both increased egg spreading in response to a nutritionally unenriched (tap water) oviposition media supporting the second hypothesis. The media “quality” comparison supports the idea of *Ae. aegypti* and *Ae. albopictus* aggregate more eggs under “preferred” conditions, increasing egg spreading under “non-preferred” conditions. *Aedes triseriatus* simply retained eggs in response to the nutritionally unenriched oviposition media treatment, resulting in a sample size that was too small to make substantiated inference about modifications in *Ae. triseriatus* skip-oviposition behavior.

*Aedes albopictus* females were previously found in laboratory settings to spread eggs under summer conditions and aggregate eggs in fall conditions in response to containers with conspecific eggs (Fonseca et al., 2015). These results suggest *Ae. albopictus* skip-oviposition behavior should be lower in fall conditions, i.e., lower temperature and shorter day length. However, there is a lack of support for the third hypothesis as *Ae. albopictus* did not modify
skip-oviposition behavior under diapause inducing fall conditions. One reason for a difference in findings could be due to Fronseca et al. 2015 assessing groups of gravid *Ae. albopictus* as opposed to individuals. This study does not account for the effect of multiple mosquitoes ovipositing in the same environment. Additionally, Fronseca et al. 2015 only allowed five days for oviposition by diapause egg-laying induced gravid *Ae. albopictus*, this study found *Ae. albopictus* require at least eleven days for all individuals to complete a gonotrophic cycle under diapause inducing conditions, 21 °C and a 10:14 photoperiod.

The index of dispersion provides a sensitive single variable for statistical analysis adaptable for the multiple permutations of skip-oviposition studies in which individual mosquitoes aggregate or spread eggs during a single gonotrophic cycle. Previous studies used occupied cups to assess differences in skip-oviposition (Santos de Abreu, Filipe Vieira et al., 2015; Swan et al., 2018; Trexler et al., 1998). If only counting cup occupation for this study, one would have concluded there was no difference between *Ae. aegypti* and *Ae. albopictus* egg spreading behavior. The ID score showed *Ae. aegypti* spreads eggs more evenly between the same number of cups as *Ae. albopictus*. As the ID statistic is sensitive to egg distribution differences, it is well suited for comparative analysis between species and oviposition treatments. Additionally, the index of dispersion could potentially assist with the development of a skip-oviposition behavior predictive model.

While more individual *Ae. albopictus* did not develop eggs during skip-oviposition assessment, proportionally this result was no different than *Ae. aegypti* or *Ae. triseriatus*. One of three issues likely led to the lack of egg development: the mosquito possibly did not mate, the mosquito did not ingest a complete blood meal for egg development, or the mosquito expired before egg development. There were also differences in the average number of eggs *Aedes*
groups developed between enriched and unenriched oviposition media treatment. This result was likely due to skip-oviposition assessments occurring with different rearing groups during different times of the year. A majority of enriched (OLI) oviposition media assessments occurred during the fall of 2018, whereas unenriched oviposition media assessments happened during the summer of 2019.

Behavioral influences of cup position within a test arena are always a concern. Previous studies address this by rotating cups every twenty-four hours throughout the experiment (Nazni et al., 2016; Oliva et al., 2014). Cup rotation is potentially ineffective for two reasons. If using a continuous rotating table, a new unmeasured behavioral could effect could be introduced during skip-oviposition assessments. *Ae. aegypti* and *Ae. albopictus* generally oviposit in the last four hours of the photoperiod, rotating oviposition sites every twenty-four hours does not change positional effects of the test arena, it just masks them from the observer. Other methods can address the position effects, for example, clear acrylic cages, random cup placement within a cage (if testing different cups), and patterned cup placement patterns (Corbet & Chadee, 1993; Davis et al., 2015; Santos de Abreu, Filipe Vieira et al., 2015; Swan et al., 2018; Trexler et al., 1998). This study used a static cup position, finding only *Ae. albopictus* under enriched (OLI) oviposition media conditions displayed a bias for corner cups, likely due to the shaded corners (Figure 3.2). *Aedes albopictus* preference for corner cups highlights a limitation in using small test arenas for skip-oviposition studies. A goal for future studies of container *Aedes* species skip-oviposition behavior should be to create a purely neutral test cage. In light of a cup position effect, this study potential has a reduction in measured *Aedes albopictus* skip oviposition behavior under some assessment conditions.
This study provides insight into the component mechanisms of skip-oviposition between container breeding *Aedes* species mosquito. When a gravid female mosquito reaches the first oviposition site, she assesses the site and decides to lay no eggs, a proportion of eggs, or all eggs. In *Ae. aegypti* and *Ae. albopictus*, only a small percentage (9% and 5.7%, respectively) aggregate all their eggs. Chadee 1990, found a similar result in *Ae. aegypti* reporting 10% aggregated all eggs in one container with 200 mL nutritionally unenriched water. A larger percentage (32%) of *Ae. triseriatus* aggregated all eggs in the same condition. Trexler et al. 1998 found a similar result, with 70% of tested *Ae. triseriatus* occupying 1 or 2 oviposition sites. These data suggest individual container *Aedes* mosquitoes lay a mean proportion of eggs per oviposition event based on her current egg total (Table 3.3). It is unknown what mechanism allows the mosquito to “feel” done and move on to another site. Another relatively unknown factor is how many skip-oviposition events occur during a normal gonotrophic cycle. The results suggest *Ae. aegypti* and potentially *Ae. albopictus* skip-oviposit at a higher rate than measured due to high ID distribution, which is towards the maximum of an eight skip-oviposition assessment (Figure 3.7, 3.8). Previous studies support this assessment as cup occupation increases in response to increases in cup number, but few studies have utilized more than eight cups in a test arena (Santos de Abreu, Filipe Vieira et al., 2015; Swan et al., 2018). An eight-cup oviposition test arena may not be enough sites to accommodate egg spreading of *Ae. aegypti*; therefore, one could potentially identify a measurable difference in skip-oviposition between *Ae. aegypti* and *Ae. albopictus* by increasing the number of cups in oviposition assessments. There is potential for cup re-visitation during skip-oviposition assessments. Chadee 1990 found *Ae. aegypti* avoided ovipositing into containers that received eggs from the first gonotrophic cycle during her second gonotrophic cycle. It is relatively unknown if individual mosquito’s visit the same site
multiple times during a gonotrophic cycle. A preliminary skip-oviposition assessment using sensor-equipped oviposition cups indicate that re-visitation does occur. In this assessment, a single gravid *Ae. albopictus* visited two sites eight times, moving back and forth between cups (unpublished observation). Oviposition re-visitation remains an open question, as this proof of concept test was unable to confirm egg-laying occurred during any given visitation, the sensors only counted the mosquito’s movement into an out of the oviposition cups.

The oviposition pattern revealed through grouping oviposition cups by egg totals suggests a specific order to oviposition events. Previously, *Aedes aegypti* exhibited the same oviposition pattern with a “favorite” cup and specific order to the subsequent egg amounts (Oliva et al., 2014). The “favorite” cup could simply represent the first oviposition event. The proportion of eggs an individual female mosquito contributes to the “favorite” cup is likely incumbent on her assessment of the container’s “quality.” As the gonotrophic cycle continues, this mosquito visits more oviposition sites with fewer and fewer eggs to contribute to any given container. This idea is easier to visualize by regrouping oviposition cups from highest to lowest egg totals, as shown in Table 3.3. In identical oviposition habitat conditions as presented in this study, the proportion of eggs laid per skip-oviposition is assumed to be consistent. The assumptions of the “favorite” cup as initial oviposition site and proportional egg contributions based on habitat “quality” in subsequent oviposition events provide structure to observed oviposition patterns in *Ae. aegypti, Ae. albopictus* and *Ae. triseriatus*. However, this insight remains an assumption without supporting data from a direct observational skip-oviposition study.

This study provides new insight into skip-oviposition behavior unearthing aspects of this behavior, which are not well understood. Future studies should include direct observation
technology to document each skip-oviposition event accurately. Current technology is sufficient to create skip-oviposition test arenas with camera-equipped oviposition sites to facilitate direct observation. Skip-oviposition assessments should expand to include other container breeding Aedes species mosquitoes. For example, Aedes japonicus (Theobald) is a recent invasive mosquito, is now found throughout the eastern United States, and the Australian Aedes notoscriptus (Skuse) now found in California (Kampen & Werner, 2014; Peterson & Campbell, 2015). Continuation of skip-oviposition assessments further enhances understanding of this behavior as an adaptive bet-hedging trait potentially assisting with the invasion of new habitats. Indeed, comparing invasive populations of these species to native populations in their ancestral range could provide evidence for the importance of skip-oviposition in invasions. Finally, future studies should account for container numbers when assessing skip-oviposition behavior. While logistically challenging, correct assessment may recommend larger test arenas with greater numbers of oviposition sites, as these metrics suggest a limitation of Ae. aegypti and Ae. albopictus skip-oviposition behavior due to not enough oviposition sites.

Implications for control:

Autodissemination traps as a more modern method of mosquito control take advantage of container breeding Aedes species mosquitoes’ propensity to oviposit in multiple containers. Traps such as the In2care coat an ovipositing mosquito larvicide, i.e., pyriproxyfen (Buckner et al., 2017). The treated mosquito then distributes larvicide to additional oviposition sites though skip-oviposition behavior, achieving mosquito control through repeated distribution of larvicide. This method allows for the treatment of cryptic oviposition sites, which are typically not easy to find or treat. This study suggests that autodissemination traps should use oviposition media of
limited nutritional quality to reduce the proportion of eggs contributed to trap and increase dissemination in the environment. The goal of the autodissemination trap is to be the first site visited by skip-ovipositing mosquitoes, enhancing larvicide distribution. These results indicate *Ae. aegypti* would distribute larvicide slightly more than *Ae. albopictus*, and it is unlikely an autodissemination trap will be useful in the control of *Ae. triseriatus* due to limited skip-oviposition.
REFERENCES


Mann, H. B., and D. R. Whitney. 1947. On a test of whether one of 2 random variables is stochastically larger than the other. Annals of Mathematical Statistics, 18(1), 50-60.


Table 3.1. Total *Aedes* mosquitoes a used in a skip-oviposition evaluation and the mean number of eggs oviposited for each mosquito group. Note, *Aedes triseriatus* response was insufficient for analysis as only three individuals completed the skip oviposition assessment in unenriched water treatment.

<table>
<thead>
<tr>
<th>Species Treatment</th>
<th>Complete Oviposition</th>
<th>Retained Eggs</th>
<th>No Egg Development</th>
<th>Total Tested (Percent Complete)</th>
<th>Mean Eggs (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes aegypti</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched OLI Water</td>
<td>33</td>
<td>2</td>
<td>1</td>
<td>36 (91.7%)</td>
<td>70.27 (3.89)</td>
</tr>
<tr>
<td>Unenriched Water</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>12 (91.7%)</td>
<td>109.27 (5.55)</td>
</tr>
<tr>
<td><em>Aedes albopictus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched OLI Water</td>
<td>52</td>
<td>1</td>
<td>7</td>
<td>60 (86.7%)</td>
<td>85.77 (3.39)</td>
</tr>
<tr>
<td>Unenriched Water</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12 (100%)</td>
<td>103.92 (7.9)</td>
</tr>
<tr>
<td>Diapause Induced</td>
<td>15</td>
<td>5</td>
<td>4</td>
<td>24 (62.5%)</td>
<td>108.2 (6.59)</td>
</tr>
<tr>
<td><em>Aedes triseriatus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched OLI Water</td>
<td>25</td>
<td>10</td>
<td>1</td>
<td>36 (69.4%)</td>
<td>84.28 (4.99)</td>
</tr>
<tr>
<td>Unenriched Water</td>
<td>3</td>
<td>7</td>
<td>2</td>
<td>12 (33%)</td>
<td>69.33 (1.2)</td>
</tr>
</tbody>
</table>
Table 3.2. Post hoc tests following Kurskal-Wallis test. $p < 0.05^*$, $p < 0.01^*$*, $p < 0.001^{***}$

<table>
<thead>
<tr>
<th>Skip-Oviposition Treatment Comparisons</th>
<th>Oviposition Cups</th>
<th>Index of Dispersion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steel-Dwass Test</td>
<td>Mann Whitney Test</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>W</td>
</tr>
<tr>
<td>Enriched (OLI) Media</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ae. aegypti – Ae. albopictus</em></td>
<td>0.9945</td>
<td>917</td>
</tr>
<tr>
<td></td>
<td>0.5925</td>
<td></td>
</tr>
<tr>
<td><em>Ae. aegypti – Ae. triseriatus</em></td>
<td>0.0000***</td>
<td>698</td>
</tr>
<tr>
<td></td>
<td>0.0000***</td>
<td></td>
</tr>
<tr>
<td><em>Ae. albopictus – Ae. triseriatus</em></td>
<td>0.0000***</td>
<td>1087</td>
</tr>
<tr>
<td></td>
<td>0.0000***</td>
<td></td>
</tr>
<tr>
<td>Unenriched (Tap Water) Media</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ae. aegypti – Ae. albopictus</em></td>
<td>0.5861</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>0.1133</td>
<td></td>
</tr>
<tr>
<td><em>Ae. aegypti – Ae. triseriatus</em></td>
<td>0.0485*</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>0.0055**</td>
<td></td>
</tr>
<tr>
<td><em>Ae. albopictus – Ae. triseriatus</em></td>
<td>0.2898</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>0.0434*</td>
<td></td>
</tr>
<tr>
<td>Enriched vs. Unenriched Media</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ae. aegypti</em></td>
<td>0.1</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>0.01**</td>
<td></td>
</tr>
<tr>
<td><em>Ae. albopictus</em></td>
<td>0.7318</td>
<td>232.5</td>
</tr>
<tr>
<td></td>
<td>0.1665</td>
<td></td>
</tr>
<tr>
<td><em>Ae. triseriatus</em></td>
<td>0.9997</td>
<td>33.5</td>
</tr>
<tr>
<td></td>
<td>0.7897</td>
<td></td>
</tr>
<tr>
<td>Diapause Inducing Conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ae. albopictus</em></td>
<td>297.5</td>
<td>0.1459</td>
</tr>
<tr>
<td></td>
<td>353</td>
<td>0.583</td>
</tr>
</tbody>
</table>
Table 3.3. Skip-oviposition pattern for *Aedes aegypti*, *Aedes albopictus*, and *Aedes triseriatus* in response to enriched (2 g/L OLI) oviposition media, unenriched (tap water) oviposition media, and diapause inducing conditions. The presented data are the average egg totals with the standard error of the mean (SEM) for each oviposition cup reordered from highest to lowest egg totals for each treatment and species combination. The calculated index of dispersion is from cup averages. The percent estimate shows the estimated proportion of eggs deposited per oviposition cup to achieve the calculated ID.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Total Eggs (SEM)</th>
<th>Cup 1 (SEM)</th>
<th>Cup 2 (SEM)</th>
<th>Cup 3 (SEM)</th>
<th>Cup 4 (SEM)</th>
<th>Cup 5 (SEM)</th>
<th>Cup 6 (SEM)</th>
<th>Cup 7 (SEM)</th>
<th>Cup 8 (SEM)</th>
<th>ID</th>
<th>Percent Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes aegypti</em></td>
<td>Enriched OLI Media (n = 33)</td>
<td>70.27 (3.89)</td>
<td>31.48 (3.09)</td>
<td>15.12 (1.50)</td>
<td>10.3 (1.07)</td>
<td>6.33 (0.67)</td>
<td>3.54 (0.51)</td>
<td>2.09 (0.43)</td>
<td>0.91 (0.26)</td>
<td>0.48 (0.17)</td>
<td>0.82</td>
<td>43%</td>
</tr>
<tr>
<td></td>
<td>Unenriched Media (n = 11)</td>
<td>103.91 (7.9)</td>
<td>34.58 (3.33)</td>
<td>24.17 (1.87)</td>
<td>18.5 (1.73)</td>
<td>11.42 (1.56)</td>
<td>6.83 (1.57)</td>
<td>5.0 (1.47)</td>
<td>3.0 (1.16)</td>
<td>0.42 (0.34)</td>
<td>0.90</td>
<td>33%</td>
</tr>
<tr>
<td><em>Aedes albopictus</em></td>
<td>Enriched OLI Media (n = 52)</td>
<td>85.77 (3.39)</td>
<td>43.15 (2.26)</td>
<td>18.86 (1.27)</td>
<td>10.75 (0.89)</td>
<td>5.91 (0.59)</td>
<td>3.55 (0.45)</td>
<td>2.0 (0.35)</td>
<td>1.11 (0.28)</td>
<td>0.19 (0.08)</td>
<td>0.77</td>
<td>48%</td>
</tr>
<tr>
<td></td>
<td>Unenriched Media (n = 12)</td>
<td>109.27 (5.54)</td>
<td>38.54 (2.77)</td>
<td>25.64 (2.26)</td>
<td>17.27 (2.27)</td>
<td>11.55 (1.59)</td>
<td>7.36 (1.13)</td>
<td>5.36 (1.03)</td>
<td>3.09 (0.61)</td>
<td>0.45 (0.25)</td>
<td>0.89</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>Diapause Induced (n = 15)</td>
<td>108.2 (6.58)</td>
<td>55.0 (6.43)</td>
<td>22.93 (2.84)</td>
<td>13.2 (0.86)</td>
<td>7.93 (0.99)</td>
<td>4.47 (0.83)</td>
<td>2.33 (0.60)</td>
<td>1.13 (0.46)</td>
<td>0.8 (0.36)</td>
<td>0.77</td>
<td>48%</td>
</tr>
<tr>
<td><em>Aedes triseriatus</em></td>
<td>Enriched OLI Media (n = 25)</td>
<td>84.28 (4.99)</td>
<td>62.92 (5.47)</td>
<td>11.52 (2.48)</td>
<td>4.44 (1.30)</td>
<td>2.56 (0.92)</td>
<td>0.84 (0.49)</td>
<td>0.56 (0.39)</td>
<td>0.36 (0.32)</td>
<td>0.04 (0.04)</td>
<td>0.46</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>Unenriched Media (n = 3)</td>
<td>69.33 (2.08)</td>
<td>60.33 (14.4)</td>
<td>5.67 (7.37)</td>
<td>2.0 (2.64)</td>
<td>1.0 (1.73)</td>
<td>0.33 (0.57)</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
<td>0.27 (0)</td>
<td>0.27</td>
<td>87%</td>
</tr>
</tbody>
</table>
FIGURES

Figure 3.1. Equipment for mosquito rearing. (A) Larval tray, Rubbermaid egg keeper, modified for use with rearing mosquito larva. (B) Bugdorm-1 for use with adult populations of container breeding *Aedes* species mosquitoes.

Figure 3.2. Position of oviposition cups in the eight-cup testing arena. (A) Diagram of cup placement and sugar source. (B) Bugdorm-1 provisioned with oviposition cups, oviposition paper, and sugar source.
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Figure 3.5. Scatter plot displaying Pearson correlation coefficient (R) evaluating the correlation between wing length and index of dispersion for three species of container breeding *Aedes* mosquito.
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Figure 3.7. Histograms displaying the distribution of skip-oviposition behavior scores (ID and cup occupation) for *Aedes aegypti*, *Aedes albopictus*, and *Aedes triseriatus* in response to enriched (2 g/L OLI) oviposition media. Oviposition cup occupation lists the number of cups occupied cups per individual, whereas ID is the calculated score specific to an eight-cup test arena. The vertical line in each distribution highlights the median value.
Figure 3.8. Histograms displaying the distribution of skip-oviposition behavior scores (ID and cup occupation) for *Aedes aegypti*, *Aedes albopictus*, and *Aedes triseriatus* in response to unenriched (tap water) oviposition media. Oviposition cup occupation lists the number of cups occupied cups per individual, whereas ID is the calculated score specific to an eight-cup test arena. The vertical line in each distribution highlights the median value.
Figure 3.9. Histograms displaying the distribution of skip-oviposition behavior scores (ID and cup occupation) for *Aedes albopictus* reared and assessed under two environmental conditions, summer-like (27 °C, 14:10 L:D photoperiod) and fall-like (21 °C, 10:14 L:D photoperiod). Both *Aedes albopictus* groups assessed with enriched (2 g/L OLI) oviposition media. The vertical line in each distribution highlights the median value.
CHAPTER 4

Development and Validation of a 3D-Printed Multifunctional Trap for Surveillance of Mosquitoes (Diptera: Culicidae)

ABSTRACT

An essential component of vector-borne disease monitoring programs is mosquito surveillance. Surveillance efforts employ a variety of collection traps depending on species of mosquito and targeted conditions, i.e., host-seeking, resting, or gravid. Surveillance activities often utilize commercial traps, later modified to accept specific mosquito species attractants. The advent of widely available and affordable 3D printing technology allows the construction of novel trap designs and components. The study goal was to developed and assessed a cost-effective, multipurpose, 6-volt mosquito trap integrating features of both a host-seeking and gravid mosquito traps for the collection of undamaged live specimens; a Multifunctional Mosquito Trap (MMT). Three separate tests assessed the multipurpose functionality of the MMT in comparison to commercial traps: gravid *Aedes albopictus*, host-seeking *Aedes albopictus*, and total number of host-seeking mosquitos regardless of species. A nine-day split-plot study with 72-hour sample periods compared the MMT, Frommer Updraft Gravid Trap, and Gravid *Aedes* Trap (GAT) provisioned with an oviposition attractant (4 g/L oak leaf infusion) in the collection of gravid *Aedes albopictus* females. A sixteen-day comparison study between the MMT and BG-Sentinel 2 (BG2) trap tested differences in field collections of host-seeking *Aedes*
*albopictus* females while using the BG-lure attractant. The third assessment compared the MMT against the Centers for Disease Control (CDC) light trap (without light) using a dry ice attractant for collection of total host-seeking female mosquitoes in a six-day 24-hour sample period study. Field evaluations found the MMT performed as well or better than comparable commercial traps. This project demonstrates an easy to construct, inexpensive, and versatile mosquito trap, potentially useful for the surveillance of multiple mosquito species and other hematophagous insects by incorporating various attractants into the MMT.
INTRODUCTION

Active mosquito surveillance is an essential component of vector-borne disease control programs. As a pillar of the Global Vector Control Response 2017-2030, vector surveillance, and monitoring assists in reducing vector-borne disease (World Health Organization, 2017). A key component of successful monitoring programs is the use of surveillance traps near at-risk human populations (Roiz et al., 2018). Often the type of trap deployed is driven by the need to collect as many mosquitoes as possible, specifically targeting the known vector species (Silver, 2008). A diversity of mosquito surveillance traps are available for the collection of large numbers of mosquitoes through the use of a variety of attractants including light, odor, visual stimuli, thermal, movement, or sound (Hoel et al., 2011; Kline, 2002; Silver, 2008; Sudia & Chamberlain, 1962). We can broadly divide the multitude of mosquito surveillance traps into two categories: suction traps and non-suction traps.

Suction surveillance traps feature an in-line electric suction fan designed to pull mosquitoes into a collection chamber. The most widely used suction trap is the Centers for Disease Control light trap (CDC-LT) originally developed in the 1960s (Silver, 2008; Sudia & Chamberlain, 1962). The CDC-LT features an incandescent light source as the main attractant; however, it is routine to augment this trap with a CO₂ attractant in the form of dry ice or from a CO₂ compressed gas cylinder (Silver, 2008). The BG-Sentinel (BGS) (Biogents AG, Regensburg, Germany) is another suction trap that has become the “gold-standard” for species-specific traps (Kroeckel, Rose, Eiras, & Geier, 2006). Originally designed to target Aedes aegypti through visual and chemical cues, it has also proven effective in the collection of Aedes albopictus (Lacroix, Delatte, Hue, Dehecq, & Reiter, 2009; Meeraus, Armistead, & Arias, 2008; Ritchie et al., 2006). The CDC-LT and BGS mainly target host-seeking adult mosquitoes.
Gravid female mosquitoes have also been a target of surveillance with suction traps. The CDC Gravid Trap designed to capture gravid female Culex mosquitoes does so by placing an updraft fan intake chamber directly above a tray of water (Reiter, 1987). The updraft suction fan design pulls mosquitoes up into a collection chamber and is an improvement over downdraft suction fan designs because the latter pull mosquitoes past the fan blade, potentially degrading mosquito catch quality for identification (Wilton & Fay, 1972; Wilton, 1975). Additionally, many mosquito species respond to suction fan air current by flying upward, potentially reducing mosquito catch in downdraft suction designs (Silver, 2008; Wilton, 1975). An updraft suction fan does not reduce total mosquito catch even when fan power is low, whereas downdraft suction fans lose catch if similarly underpowered (Wilton, 1975).

Non-suction traps, commonly known as passive traps, are generally designed to be visually stimulating and lure mosquitoes into collection chambers with odor attractants, then collect mosquitoes with sticky cards or kill them with a pesticide. Their main advantage is the ability to collect mosquitoes over long periods, i.e., days to weeks, and have more commonly been used to target egg-laying, gravid mosquitoes (Barrera et al., 2014; Johnson et al., 2015). The CDC autocidal gravid oviposition trap (AGO) and the gravid Aedes trap (GAT) (Biogents AG, Regensburg, Germany) attract gravid container-breeding Aedes mosquitoes into collection chambers with water-filled bases, killing the mosquitoes with sticky cards (Barrera et al., 2014; Mackay, Amador, & Barrera, 2013; Ritchie et al., 2014). In addition to surveillance, AGOs and GATs have been used for control, but with limited success (Johnson et al., 2017). Passive traps can also target host-seeking mosquitoes for disease surveillance. The Sentinel Mosquito Arbovirus Capture Kit (SMACK), utilizing a CO₂ attractant, identified Ross River virus and Barmah Forest virus in samples collected from North Queensland, Australia (Johnson et al.,
Another example of a host-seeking passive trap is the Silva trap, which performs as well as a CDC-LT but without the need for a suction fan (Silva, Costa-Neta, de Sousa de Almeida, M, de Araujo, & Aguiar, 2019).

In remote locations, a lack of access to surveillance traps inspired the building of light traps out of local materials (Silver, 2008). Local resourced light traps were able to accomplish mosquito surveillance but did so without standardization. One method to assist in building consistent traps is to use 3D-printed components. In recent years, 3D-printing technology has greatly increased in availability and affordability for many different applications. In the case of mosquito traps, this technology allows for the development of prototype components, which allows researchers to rapidly modify traps to suit their needs (Hoshi et al., 2019). Printing a 3D trap similar to the CDC-light trap reduces approximate per trap cost from $106 to $12.97 (Hoshi et al., 2019). Thermoplastics used in 3D printing are relatively inexpensive, reducing per trap costs for surveillance programs after the initial purchase of 3D printers (Hoshi et al., 2019). Different plastics are available for 3D printing, depending on the application. Polylactic acid (PLA) is an inexpensive plastic made from renewable sources (i.e., corn starch, sugar cane); however, it degrades rapidly with environmental exposure. This plastic is common for building prototype parts due to its low cost, but long-term usage trap usage requires environmentally durable 3D printable plastics. Two printable plastics that are durable to environmental exposure: acrylonitrile butadiene styrene (ABS) and glycol modified polyethylene terephthalate (PETG). As a structural material, PETG prints better is stronger, more durable, and has greater color availability. However, ABS is less expensive and lighter weight. A large benefit to 3D printed traps is the rapid replacement of parts damaged during trap operation. Also, the flexibility of 3D printing allows rapid modifications of trap design (Hoshi et al., 2019). Overall, there is a lack of
published literature about 3D printed mosquito surveillance traps. This deficit represents an opportunity for researchers to develop and widely distribute novel trap designs that can be economically produced by local surveillance agencies.

No trap targets both gravid and host-seeking mosquitoes. This study presents an adaptable trap design built using 3D printing technology and conventional construction techniques, which can target gravid or host-seeking mosquitoes. The goal was to design, build, and assess an inexpensive, easy to construct, resilient, 6-volt multipurpose trap, able to collect gravid or host-seeking mosquitoes during 24-hour sample periods; a “Multifunctional Mosquito Trap,” or MMT.
MATERIALS & METHODS

Multifunctional Mosquito Trap Design:

The multifunctional mosquito trap (MMT) uses an updraft suction collection chamber inspired by the Frommer updraft gravid trap (Model 1917, John W. Hock Company) in combination with a 5-gallon bucket base similar to the CDC AGO. A novel intake manifold integrates both trap elements (Figure 4.1). This intake manifold consists of a 3D printed intake component attached to a commercially available Viagrow 6-inch mesh pot bucket lid insert (Hydo Generation Inc., Atlanta, GA, USA).

The 3D components initially designed in Tinkercad software (Autodesk Inc, San Rafael, CA, USA) then imported into Cura software (Fargo Additive Manufacturing Equipment 3D, Fargo, ND, USA) for printing on a Lulzbot Mini 2.0 3D printer (Fargo Additive Manufacturing Equipment 3D, Fargo, ND, USA). 3D printing of trap components used either black or white PETG 3D filament (Esun, Shenzhen, China) depending on the part.

Appendix A shows trap construction, component list, and step-by-step assembly instructions. I used a band saw to cut the ABS pipe components, though a hand saw with a miter box can accomplish the same cuts. Additional recommended tools include power drill, hot glue gun, soldering iron, wire stripper/cutter, and utility knife.

Smoke emitters assessed airflow in the assembled MMT. Emitters placed inside the trap reservoir and near the intake manifold showed air suction tracing up from the reservoir and in from the side of the top into the intake, then through the collection chamber flowing out sideways from the rain hood (Figure 4.2). The small (under the intake manifold) and large (base) reservoirs can accommodate a large variety of attractants. This trap functions by attracting
mosquitoes into the intake manifold; subsequently, they are drawn into the collection chamber by the updraft suction fan (Figure 4.2).

Field Trapping Evaluations:

I assessed the multifunctional mosquito trap for the collection of gravid *Aedes albopictus* mosquitoes, host-seeking female *Aedes albopictus*, and total host-seeking female mosquitoes in three separate field evaluations against standard single function commercial traps. A field evaluation for the collection of gravid *Aedes albopictus* mosquitoes compared the MMT to the Frommer updraft gravid trap (Frommer-GT), and GAT (Figure 4.3). Another field evaluation compared host-seeking *Aedes albopictus* collections of the MMT and BG-Sentinel 2 trap (BG2) using the BG-lure (Biogents, Regensburg, Germany) chemical attractant (Figure 4.3). The final field evaluation compared total female mosquito collections between the MMT and CDC-LT using dry ice as an attractant (Figure 4.4).

I selected three field sites for gravid *Aedes albopictus* collection on North Carolina State University (NC State) property in Wake County, NC. At each of the three field sites received three traps (one MMT, one Frommer-GT, and one GAT) along the sites tree line with 30 meters separation between traps. Over nine days, I rotated traps at each site every 72-hours. A previous sampling using host-seeking BG2 traps with BG-Lure found *Aedes albopictus* at all sites. Two sites, Site 1 (8758338°35'18.8406"W 4262783°19'23.9109"N) and Site 2 (8759453°0'56.8645"W 4262616°6'22.7069"N), are located at the NC State Lake Wheeler Road Field Laboratory, a 1,500-acre property, and another sample site, Site 3 (8760444°8'39.4755"W 4271500°26'39.6741"N), was near Method Road Buildings (Figure 4.5, 4.6). A 4 g/L oak leaf infusion (OLI) prepared five days before initiation of the field evaluation provided an attractant.
for gravid *Aedes albopictus* mosquitoes (Obenauer et al., 2009; Reiskind & Janairo, 2018). I made OLI by mixing in a water cooler 320 g (16 g/L) of locally collected dried willow oak leaves (*Quercus phellos*) bundled in a fine mesh cloth, 4.8 g (0.3 g/L) of brewer’s yeast (MP Biomedicals, Cat no. 903312, Fisher Scientific), and 4.8 g (0.3 g/L) of egg albumin (Cat no. A388-500, FisherChemical) into 20 L of tap water, then incubating the 16 g/L oak leaf infusion for five days. Finally, diluting the OLI 1:4 in tap water resulting in a 4 g/L oak leaf infusion on the day of use. For field evaluation set-up the Frommer-GT and GAT received 3 L of 4 g/L OLI, the MMT due to a more extensive reservoir base received 6 L of the same oviposition attractant.

I inspected gravid traps daily, replacing batteries and collection chambers for the Frommer-GT and MMT during each visit. For the GATs, collection and replacement of sticky cards occurred on 72-hour intervals, rotating all traps at each site during this inspection. For each rotation, I mixed OLI from each trap with 6 L of excess OLI maintain at room temperature in our laboratory. Then I reallocated the mixed OLI into each trap; 3 L for the Frommer-GT and GAT, 6L for the MMT. Following each collection period, I identified mosquitoes from each trap and dissected females to determine gravid status. The number of collected gravid female *Ae. albopictus* was the measured response during each 72-hour sample period, due to the difficulty and expense of replacing sticky cards for the GATs.

I conducted a 16-day field evaluation at the NC State Lake Wheeler Field Laboratory Site 1 (Figure 4.6) to assess the MMT for collecting of host-seeking *Aedes albopictus*. In this assessment, I placed an MMT and BG2 along the tree line with a 60 m separation between traps. I provisioned each trap with a BG-lure attractant and conducted two eight-day 24-hour sample periods with trap rotations every four days. I inspected each trap daily, collecting 24-hour catch
samples and replacing batteries. In this case, I used the number of female *Ae. albopictus* collected in each 24-hour sample period as the response variable.

For the third field evaluation, I used a dry ice attractant to assess the effectiveness of the MMT at collecting host-seeking mosquitoes regardless of species. I compared the MMT to the CDC-LT without a light bulb, which is a standard set up for collecting large numbers of host-seeking mosquitoes (Silver, 2008). Again, trap assessment took place at Site 1, located at the Lake Wheeler Field Laboratory (Figure 4.6). I placed each trap with 60 m separations in new locations within Site 1. Each morning I inspected traps, provisioning each with approximately 3.5 lbs. of dry ice, collecting mosquito catch, and replacing batteries. I tied the CDC-LT overhanging tree branches for sampling with the intake positioned approximately 1.5 m above the ground. In contrast, the MMT, when positioned in the environment, has an intake at 0.38 m high, which does not require hanging to function. I collected mosquitoes daily over six 24-hour sample periods, and I switched traps positions after three sample days; therefore, each trap had equal surveillance time at both locations within Site 1. In this field evaluation, I measured the response in total female mosquitoes collected.

Mosquito processing:

Mosquitoes were frozen upon collection, later identified under a dissecting microscope (Model SZ61, Olympus, Shinjuku, Japan) using *The Mosquitoes of the Mid-Atlantic Region: An Identification Guide* (Harrison, Byrd, Sither, & Whitt, 2016). Dissection of mosquitoes assessed gravid status for all female mosquitoes collected during gravid trapping tests.
Data analysis:

Data analysis conducted in R (R Core Team, 2017) and figures produced using the package ggplot2 (Wickham, 2009). A split-plot ANOVA model analysis using the linear mixed effect models package lmerTest (Kuznetsova, Brockhoff, & Christensen, 2017) compared gravid *Ae. albopictus* collections. Trap comparison for BG-lure (BG2 vs. MMT) or dry ice (CDC-LT vs. MMT) as attractants compared total host-seeking female mosquito collections using pairwise t-tests (t.test in R) for (R Core Team, 2017).
RESULTS

There was no difference among traps for gravid *Ae. albopictus* ($F_{2,16} = 0.711$, $p$-value = 0.506). This field evaluation collected an average of $4.48 \pm 0.96$ gravid *Ae. albopictus* mosquitoes per 72-hour sample period per trap (Figure 4.7). When removing the largest outlier for the Frommer-GT data set, 25 gravid female *Ae. albopictus*, the cumulative trap average count reduced to $3.69 \pm 0.58$ gravid *Ae. albopictus* mosquitoes. When put in perspective, this assessment collected three to four gravid *Ae. albopictus* mosquitoes per 72-hour sample period per trap (regardless of type). There was a significant difference between sample sites ($F_2 = 5.615$, $p$-value = 0.014), as expected; therefore, the sample site is a blocking factor in statistical analysis. There was no difference among locations within each sample site ($F_2 = 0.585$, $p$-value = 0.569) or interactions between location and trap ($F_2 = 0.965$, $p$-value = 0.453).

Host-seeking female *Ae. albopictus* field collections did not differ between BG2 and MMT traps using BG-lure (Figure 4.8; $t_{15} = 1.377$, $p$-value = 0.189). When I group all collected mosquitoes, there was an average of $28 \pm 4.44$ female *Ae. albopictus* mosquitoes per 24-hour sample period regardless of the trap.

In conducting a trap field evaluation using dry ice, I found no difference ($t_5 = -2.183$, $p$-value = 0.081) in total female mosquito collections between the CDC-LT and MMT (Figure 4.8). However, after removing a single sample period outlier from each trap type; the MMT collected an average of $109.8 \pm 13.71$ mosquitoes per 24-hour sample period, significantly more ($t_4 = -7.703$, $p$-value = 0.002) than the CDC-LT which collected an average of $25.4 \pm 13.82$ female mosquitoes per 24-hour sample period. A majority (92.42%) of collected mosquitoes were *Ae. albopictus* regardless of trap when using a dry ice attractant, likely due to the use of only one survey location, which previously demonstrated a heavy *Ae. albopictus* density.
DISCUSSION

The Multifunctional Mosquito Trap performed as well or better than comparable single function traps. For most of the tests, there was no significant difference between traps, and most of the point estimates (mean mosquitoes captured) were similar, suggesting increasing the sample size would not likely result in statistical significance. The one exception was comparing the MMT to the CDC-LT, in which the MMT outperformed the CDC-LT in collecting host-seeking female mosquitoes when removing one outlier from each trap. However, over 90% of the mosquitoes caught in that field experiment were *Ae. albopictus*; it would be a benefit to further compare the CDC-LT and MMT under other common settings for pest mosquito monitoring, including flood water and saltmarsh habitats. Likewise, it would also be important to compare the MMT to the BGS and gravid traps in a more tropical, *Aedes aegypti*-dominated landscape.

Mosquito surveillance traps represent a significant expense for mosquito control and surveillance activities. The cost of producing the Multifunctional Mosquito Trap is $31.60, not including the cost of batteries and chargers. A cost savings compared to the direct purchase of commercial traps BG2 $213 and CDC-LT $106, similar to that noted by Hoshi et al. 2019 for a 3D print light trap. Surveillance programs already using CDC-LT would only need to produce the MMT as this trap uses the same gel-cell rechargeable 6V battery. Additional cost savings advantage can also be realized in the repair of printed components as they degrade from use, versus purchasing brand new CDC-LT or BGS. There is a caveat to cost savings, Hoshi et al. 2019 and this study only include material costs and do not account for the total cost of producing commercial taps (i.e., materials, manufacturing, rent, salaried employees, patient protection, shipping, taxes, and of course profit).
The multifunctional mosquito trap displays several advantages as a multipurpose surveillance trap. Much like the BG2, it breaks down into components for transportation and storage, it is easily placed in the environment and supplemented with multiple attractants. Attractants in the MMT can be used singly or in combination due to multiple reservoirs incorporated into the design. The large reservoir base while only used to hold attractive oviposition media (Reiskind, Greene, & Lounibos, 2009; Trexler et al., 1998) in this study, can hold a wide range of bulky attractant material such as rodent litter (Le Goff et al., 2017) or bottles of CO₂-producing yeast-sugar mixtures (Jerry, Mohammed, & Mohammed, 2017). The smaller reservoir accommodates self-contained attractants such as BG-Lure as demonstrated in this study, octanol cartridges, or similar small volume odor attractants. As an adaptable trap, the MMT’s 3D printed intake manifold can be modified to accept new attractants which do not physically fit into the current trap design.

The updraft suction design used in the MMT is similar to previously utilized updraft designs (Kimsey & Chaniotis, 1984; Silver, 2008; Wilton & Fay, 1972). Updraft suction designs collect mosquitoes as they fly upward in response to the air current (Wilton & Fay, 1972). Additionally, the robust ABS plastic collection chamber protects specimens both in the field and during transportation, similar to the Silva trap (Silva et al., 2019). The mesh nets utilized in downdraft traps potentially damage specimens leading to difficulties in species identification. Specimens collected in the MMT can be used for disease detection and in further live insect studies.

Like many suction traps, a 6V battery powers the MMT for 24-hr mosquito sampling. Electric power represents a general limitation to suction traps. The MMT does use 6V instead of 12V batteries, which are lighter, charge faster, and transportable on passenger airplanes.
Trap durability is another limitation, as suction traps often damaged during deployment. While there was no testing for MMT environmental resilience, it does utilize a 25% rectilinear infill support structure for trap components in the PETG 3D printing process. The infill support structure creates durable components while reducing the volume of thermoplastic used in each print (Fernandez-Vicente, Calle, Ferrandiz, & Conejero, 2016). Additionally, printing 3D objects takes training and experience to become proficient at printing the 3D components for the MMT. Finally, there is a production limitation is the length of time 3D components take to print. For example, a single MMT intake takes 13 hours to print on one printer. I predict as 3D printing becomes more widely available, faster, and simpler to implement, many of these limitations will be alleviated.

The current assessed MMT design does not include a light source for attracting mosquitoes during operation at nighttime, which could be a limitation in specific settings. The CDC-LT is often run without lights to avoid bycatch (Hoekman et al., 2016; O'Brien & Reiskind, 2013). Future refinements to the MMT include adding a light source; however, if an immediate light source solution is critical, then merely adding a LED flashlight to the MMT would provide a temporary solution.

This study demonstrates the MMT as an adaptable multipurpose suction trap useful in mosquito surveillance. As a multipurpose trap, the MMT potentially reduces trap cost for surveillance programs due to the low production cost and ability to target multiple species as gravid or host-seeking mosquitoes. Since completion of this study, I developed an improved version of the MMT, removing the unnecessary central support column to reduce weight and cost (see design in Appendix B). Future improvements include incorporation of a 6V LED light strip featuring UV and color lights for specific mosquitoes, a smaller pore size insect screen, and
a component to increase “lightness” or reflectance resembling liquid water on the surface of the trap. The addition of these features will potentially expand the range of hematophagous insects collected by this trap. Additionally, future studies will include surveillance for a broader range of mosquito species, biting midges (*Ceratopogonidae*), black flies (*Simuliidae*), sand flies (*Phlebotominae*), and horn flies (*Haemotobia irritans*).

As a 3D printed trap, publishing the MMT 3D component files and construction instructions allows others to make this trap. The MMT demonstrated the ability to collected I mosquitoes using multiple attractants, an ability to be appreciated in remote locations with limited access to commercial surveillance traps, and no access to common attractants such as dry ice. In the future, undoubtedly, other trap designs will be forthcoming; however, much like the MMT, they will need to be assessed for effectiveness in the surveillance of mosquitoes. This study provides a tested trap design readily adaptable to the specific needs of many different insect surveillance applications.
REFERENCES


ESRI. 2011. ArcGis desktop: Release 10.5.1 [computer software]. Redlands, CA: Environmental Systems Research Institute:


Figure 4.1. A diagram of the major components of the Multifunctional Mosquito Trap. Insect screen separates the intake manifold from the base reservoir. 3D model created with Blender software (Community, 2018).
Figure 4.2. (A) A 3D generated model of the Multifunctional Mosquito Trap (MMT). (B) A cross-sectional model of the MMT show insect access and updraft suction into the collection chamber; a small reservoir holds BG-Lure (attractant) cartridge. (C) A diagram of airflow (blue) from the outside environment, the updraft fan assembly pulls air from the trap base passed attractants (yellow) as assessed by a smoke test. 3D models created with Blender software (Community, 2018).
Figure 4.3. (A) Gravid *Aedes* Trap with sticky card and 4 g/L oak leaf infusion attractant. (B) Frommer Updraft Gravid Trap with 4 g/L oak leaf infusion attractant. (C) Multifunctional Mosquito Trap configured for use with 4 g/L oak leaf infusion and BG-lure attractants. (D) BG-Sentinel 2 with BG-lure attractant.
Figure 4.4. (A) CDC Light Trap without light set up with dry ice container. (B) Multifunctional Mosquito Trap set up with dry ice container.
Figure 4.5. Three forested study sites located in Raleigh, NC. Map produced using ArcGIS software (ESRI, 2011).
Figure 4.6. An area map showing locations of forest study sites. Field evaluations compared trap collections of gravid *Aedes albopictus*, host-seeking *Aedes albopictus*, and total host-seeking mosquitoes conducted at Site 1. Map produced using ArcGIS software (ESRI, 2011).

Figure 4.7. Boxplot of gravid *Aedes albopictus* collections comparing three trap types, Frommer Updraft Gravid Trap (Frommer-GT), Gravid Aedes Trap (GAT), and Multifunctional Mosquito Trap (MMT). The black line represents the median collected mosquitoes per 72-hour sampling period for each trap type (nine sample periods per trap). Whiskers are 1.5 x interquartile range (IQR). Red * represent outliers data points. Letters describe significant differences between traps.
Figure 4.8. Boxplot comparisons of host-seeking mosquito collections by trap type. Left figure: Sixteen 24-hour sample period comparison between BG-Sentinel 2 (BG2) and Multifunctional Mosquito Trap (MMT) using BG-Lure attractant. Right figure: Six 24-hour sample period comparison between CDC light trap (CDC-LT) and Multifunctional Mosquito Trap (MMT) using Dry Ice attractant. The black line represents the median collected mosquitoes per 24-hour sampling period for each trap type (nine sample periods per trap). Whiskers are 1.5 x interquartile range (IQR). Red * represent outliers data points. Letters describe significant differences between traps.
CHAPTER 5
Conclusion

In this dissertation, I studied container breeding *Aedes* mosquito oviposition as a behavior and a target for surveillance. In chapter 2, observational oviposition studies using arrays of ovitraps provided evidence of *Ae. albopictus* distribution within a local environment, oviposition site preferences within specific habitats, and skip-oviposition behavior. In chapter 3, I continued to investigate skip-oviposition behavior identifying differences among species and the effect of universal oviposition media “quality”. In chapter 4, I used this opportunity to develop a multipurpose trap with 3D printing technology to target both gravid mosquitoes and host-seek mosquitoes.

In chapter 2, the original was to observe local oviposition distribution of *Aedes albopictus* in conjunction with the Research Triangle Institute (RTI) International drone surveillance program. Ovitrap grid arrays “digitized” the local environment providing information on oviposition habitat preference of *Ae. albopictus*. This approach, which places ovitraps independent of known mosquito preferred habitat, is useful in showing both positive and negative sample space. Negative samples provide context to positive samples. Often in sampling programs, place ovitraps in specific environmental locations, which are likely to produce definite *Ae. albopictus* presence results. In this study, I was able to use inverse distance weighted maps to observed oviposition distribution in correlation with high-definition maps of the local environment. These maps provided valuable insight into how mosquitoes oviposit in the environment when there is a high density of oviposition sites. There are significant drawbacks to ovitrap array studies. They are very labor-intensive, I discovered something that looks straight forward on a piece of paper is an entirely different situation once you get on the
ground. This technique removes large amounts of mosquito eggs and can only be utilized for a short period before it likely traps out the local population. My study only conducted a couple of weeks of sampling, to short of time to trap out and affect the population. In this project, I observed many oviposition papers with one or two eggs, which led to the next chapter on skip-oviposition.

My third chapter started with skip-oviposition observations from the field. These observations sparked an interest in the behavior of laying eggs in multiple oviposition sites. A literature review found limited knowledge on the behavior, which mostly focuses on oviposition site preference. Through my experiments in skip-oviposition, I’ve developed a sense that skip-oviposition is well ingrained in a few species of container Aedes species mosquito. As a candidate bet-hedging behavior, skip-oviposition likely assists with the successful invasion of novel environments as it spreads the risk of egg batch failure among multiple egg clutches. Future studies of this behavior should include direct observation of each oviposition event, to provide greater insight into skip-oviposition. Direct observation information, combined with the use of the index of dispersion, could potentially lead to the development of a skip-oviposition prediction model. This model could assist with predictions for the use of larvicide autodissemination traps, enhancing the effectiveness of this control measure on container Aedes species.

The fourth chapter, for me, was indeed a passion project born from the frustrations of field surveillance. In my experience conducting insect surveillance in America, the Middle East, and Western Pacific, the rule is you never have the right tool for the job. There are many limitations to traveling abroad for mosquito surveillance. First, the best mosquito attractant, dry ice, while commonly available in the US, is not available anywhere else. Electric power may or
may not be available and likely not in the correct voltage for your on hand equipment. Finally, air travel places restrictions on battery size, which limits the ability to commercially transport the “gold standard” in Aedes species surveillance the Biogents Sentinel-2 (BG2) as it runs on a larger 12V battery. So with these criteria in mind, I set out to develop a simple, robust trap useful in many different settings. The result is a novel 3D printed trap dubbed the “Multifunctional Mosquito Trap.” There are many advantages to a 3D printed traps, in particular for my interest is the ability to construct and repair traps on-site. The current version reported in my dissertation works well but could, of course, use improvements. I plan on incorporating a light source (UV), reduce the insect screen pore size, and add a “lightness” element to the top of the trap lid to simulate a water-filled container. Fortunately, I can continue to develop this trap with future studies. However, I hope to publish this design for researchers to use in many different applications in the surveillance of small flying insect pest species.

Mosquito surveillance and control efforts target container Aedes species mosquitoes as they are significant vectors of many human pathogens (i.e., dengue, chikungunya, yellow fever, Zika). My dissertation provided insight into aspects of container Aedes spp. mosquito oviposition behavior as a target for surveillance to enhance control activities. Impartial fine-scale ovitrap surveillance provides information on the local distribution of vector Aedes species. These studies of skip-oviposition behavior potentially enhance understanding of how this behavior works, as well as how it can impact control efforts. Finally, from my experience with 3D trap design, I recommend surveillance and control agencies embrace 3D printing technology as it can reduce cost while enhancing efforts.
APPENDICES
Appendix A: Instructions for the construction and assembly of the Multifunctional Mosquito Trap Mark.V.

Appendix A – Figure A. Multifunctional Mosquito Trap (MMT) Mark.V.
• 3-inch ABS Schedule 40 Cellular core DWV pipe (Charlotte Pipe and Foundry Company, Charlotte, NC, USA)
• 3-inch ABS connectors (NIBCO, USA)
• Viagrow 6-inch mesh pot bucket lid insert (Hydo Generation Inc., Atlanta, GA, USA)
• Standard black 5-gallon bucket (Leaktite, Maine, USA)
• Fiberglass insect screen 18 x 16 (Phifer, Tuscaloosa, Alabama, USA)
• RF-500TB-14415 6-volt DC electric motor (Amazon, USA) 2700-3000 RPM
• 16 Gage flexible electric wire
• AMP female disconnects (Tyco Electronics, Fuquay-Varina, NC, USA)
• White and Black PETG 2.85 mm 3D filament (Esun, Shenzhen, China)
• Band saw or hand saw with miter box
• Hot glue gun
• Power drill
• Soldering Iron
• Wire cutter and stripper
• Utility Knife
### MMT Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black 5-Gal Bucket</td>
<td>1</td>
<td>$3</td>
</tr>
<tr>
<td>3-Inch ABS Pipe</td>
<td>400 mm</td>
<td>$2</td>
</tr>
<tr>
<td>3-Inch ABS Coupling</td>
<td>2</td>
<td>$10</td>
</tr>
<tr>
<td>Viagrow 6-Inch Mesh Bucket Lid Insert</td>
<td>1</td>
<td>$2.30</td>
</tr>
<tr>
<td>2.85mm PETG Black Filament</td>
<td>322 g</td>
<td>$10</td>
</tr>
<tr>
<td>6V DC Motor (RF-500TB-14415)</td>
<td>1</td>
<td>$4</td>
</tr>
<tr>
<td>Black Rain Hood (9-inch Diameter Frisbee)</td>
<td>1</td>
<td>$1</td>
</tr>
<tr>
<td><strong>Miscellaneous Parts:</strong></td>
<td></td>
<td>$2</td>
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<tr>
<td>Insect Screen (Fiberglass)</td>
<td>1 ft&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Screws + Nuts</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>16g Wire (Red/Black)</td>
<td>2 x 3 ft</td>
<td></td>
</tr>
<tr>
<td>Female Terminal Connectors</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Hot Glue</td>
<td>As Needed</td>
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**Total Materials Cost:** $37.30

Appendix A – Figure C. Estimated cost of MMT Mark.V production.
Appendix A – Figure D. MMT Mark.V component list.
Appendix A – Figure E. Insect screen cut-out patterns for construction of the MMT.
Appendix A – Figure F. Collection chamber assembly instructions.

1 x 3-inch ABS pipe (160 mm Length)

1 x Insect Screen Cone

1 x 3-inch ABS pipe (5 mm Length)

1 x Insect Screen 89 mm diameter

1 x 3-inch ABS pipe connector

Insert and glue insect screen cone into ABS pipe connector followed by 5 mm ABS pipe

Once glue dries combine both pieces by slipping top screened connector onto bottom chamber to fully assemble the collection chamber

Glue insect screen cone into ABS pipe by the 3-inch base

Assembled Collection Chamber
Appendix A – Figure G. MMT Mark.V intake manifold assembly instructions.
Appendix A – Figure H. Support column assembly instructions.

1 x 3-inch ABS pipe (76.2 mm Length)
1 x 3-inch ABS pipe (5 mm Length)
1 x Insect Screen 89 mm diameter
1 x 3-inch ABS pipe connector
1 x 3-inch ABS pipe (146 mm Length) 8 x 20 mm holes

Insert and glue insect screen cone into ABS pipe connector followed by 5 mm ABS pipe

Once glue dries combine the three pieces by slipping the top 3-inch ABS pipe onto the screened connector then inserting the bottom 3-inch ABS pipe

Drill 8 20 mm holes into the base of the 146 mm length 3-inch ABS pipe

Assembled Support Column
Appendix A – Figure I. Updraft fan assembly instructions.

3 x #8-32 ½ inch screw and wing nut
1 x #8-32 ½ inch screw and nut

PETG 3D print: Fan Motor Mount

RF-500TB-14415 6-volt DC motor

PETG 3D print: Fan Blade

PETG 3D print: Rain Hood Mount

Rain Hood: 9-inch diameter frisbee

16-gauge Red Insulated wire

16-gauge Black Insulated wire

After securing fan motor use screws/wing nuts to attach rain hood mount and rain hood (drill small hole in rain hood)

Use 2 mm drill bit clean out 3D printed fan then slide onto DC motor

Clean out printed holes for rain hood mount, motor mount and wire insertions. Then slide in motor with fan and secure with side screw/nut

Once assembled, solder red and black 16 gauge wires to fan motor to produce an updraft towards rain hood

Assembled Fan Assembly
Appendix A – Figure J. 5-gallon bucket base assembly instructions.
Appendix A – Figure K. Final assembly instructions for the MMT Mark.V.
Appendix B: Instructions for the construction and assembly of the Multifunctional Mosquito Trap Mark.VI.

Appendix B – Figure A. Multifunctional Mosquito Trap (MMT) Mark.VI.
• 3-inch ABS Schedule 40 Cellular core DWV pipe (Charlotte Pipe and Foundry Company, Charlotte, NC, USA)
• 3-inch ABS connectors (NIBCO, USA)
• Viagrow 6-inch mesh pot bucket lid insert (Hydo Generation Inc., Atlanta, GA, USA)
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• 16 Gage flexible electric wire
• AMP female disconnects (Tyco Electronics, Fuquay-Varina, NC, USA)
• White and Black PETG 2.85 mm 3D filament (Esun, Shenzhen, China)
• Band saw or hand saw with miter box
• Hot glue gun
• Power drill
• Soldering Iron
• Wire cutter and stripper
• Utility Knife

Appendix B – Figure C. Construction tool list.
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<tr>
<th>MMT Components</th>
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<th>Cost</th>
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</tr>
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<td>3-Inch ABS Pipe</td>
<td>250 mm</td>
<td>$1.30</td>
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<tr>
<td>3-Inch ABS Coupling</td>
<td>1</td>
<td>$5</td>
</tr>
<tr>
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<td>$2.30</td>
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<tr>
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<td>357 g</td>
<td>$10</td>
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<tr>
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<td>$4</td>
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<td>$1</td>
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Appendix B – Figure C. Estimated cost of MMT Mark.VI production.
Appendix B – Figure D. MMT Mark.VI component list.
Appendix B – Figure E. Insect screen cut-out patterns for construction of the MMT.
Appendix B – Figure F. Collection chamber assembly instructions.

1 x 3-inch ABS pipe (160 mm Length)

1 x Insect Screen Cone

1 x 3-inch ABS pipe (5 mm Length)

1 x Insect Screen 89 mm diameter

1 x 3-inch ABS pipe connector

Insert and glue insect screen cone into ABS pipe connector followed by 5 mm ABS pipe

Once glue dries combine both pieces by slipping top screened connector onto bottom chamber to fully assemble the collection chamber

Glue insect screen cone into ABS pipe by the 3-inch base

Assembled Collection Chamber
Appendix B – Figure G. MMT Mark.VI intake manifold assembly instructions.
Appendix B – Figure H. Updraft fan assembly instructions.
Appendix B – Figure I. Final assembly instructions for the MMT Mark.VI.