ABSTRACT

Title of Thesis:	FIELD TESTING THE ATTRACTANCY AND TOXICITY OF THE PRO-FRAGRANCE COMPOUND, OKOUMAL, TO GRAVID *AEDES* MOSQUITOES AND THEIR OFFSPRING LARVAE
	Maya Hamanaka Babu, Master of Science, 2020
Thesis Directed By:	Paul Leisnham, Associate Professor Department of Environmental Science and Technology

Mosquitoes are among the most prominent and medically important insects in the world, causing substantial public health concerns in many regions. *Aedes* spp. mosquitoes are arguably the most important invasive species in the United States and worldwide. Oviposition traps are typically baited with plant infusions that release a suite of volatile compounds that attract gravid female mosquitoes, but plant infusions require weekly maintenance and are difficult to standardize. The overall goal of my thesis was to evaluate the efficacy of Okoumal, a pro-fragrance compound, at attracting ovipositing gravid adult *Aedes* mosquitoes and act as a toxin to their larvae offspring using field and laboratory trials. My results indicate that although Okoumal is toxic to *Aedes* larvae, there is little evidence of it being an attractant to oviposition gravid female *Ae. albopictus* casting doubt of its use as a bait in mosquito surveillance and control.

FIELD TESTING THE ATTRACTANCY AND TOXICITY OF THE PRO-FRAGRANCE COMPOUND, OKOUMAL, TO GRAVID *AEDES* MOSQUITOES AND THEIR OFFSPRING LARVAE

by

Maya Hamanaka Babu

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science

2020

Advisory Committee:

Dr. Paul T. Leisnham, Chair
Dr. Mitchell Pavao-Zuckerman
Dr. Lance Yonkos

Acknowledgements

I would like to thank my thesis advisor, Dr. Paul Leisnham, and my committee members Dr. Mitchell Pavao-Zuckerman and Dr. Lance Yonkos for providing valuable advice on my experimental designs and statistical analysis, and for their continued support.

I am grateful to my lab mates Debasmita Patra, Sarah Rothman, Kaitlin Saunders, Megan Saunders, and Cameron Smith who provided support and encouragement, helped collect field samples, helped sort and identify mosquitoes, and helped maintain mosquito populations for experimental trials.

Finally, I am very thankful for my parents, Kaladi and Kimie Babu, and friends for supporting me along the way and pushing me to the finish line.

Table of Contents

Acknowledgements ii
Table of Contents iii
List of Tables iv
List of Figures v
Chapter 1: General Introduction 1
Mosquito-Borne Vector Diseases: A Public Health Concern 1
Important Invasive Aedes in America 3
Mosquito Control and Surveillance 5
Integrated Pest Management at the Larval Stage 10
Okoumal: A Pro-Fragrance Chemical Attractant 12
Project Goals and Summary 13
References 15
Chapter 2: Investigating the Pro-Fragrance, Okoumal, as an Attractant to Gravid Female Mosquitoes Using Field and Laboratory Trials 27
Abstract 27
Introduction 28
Materials & Methods 34
Laboratory Oviposition Choice Assay Methods 34
Dose-Response Oviposition Field Study 36
Statistical Analyses 37
Laboratory Oviposition Choice Assay Statistical Analysis 37
Dose-Response Oviposition Field Study Statistical Analysis 38
Results 39
Laboratory Oviposition Choice Assay 39
Dose-Response Oviposition Field Study 39
Discussion and Conclusions 39
List of Figures 47
References 55
Chapter 3: Testing the Potential of the Pro-Fragrance Compound, Okoumal, as a Toxin to *Aedes aegypti* Larvae Using Dose-Response Laboratory Experiments 63
Abstract 63
Introduction 64
Materials & Methods 68
Statistical Analyses 71
Results 72
Discussion and Conclusions 72
List of Tables 80
List of Figures 83
References 95
Chapter 4: General Conclusions 102
References 106

List of Tables

Table 3.1 Summary Least squares linear models for estimated finite……..... 81
rate of increase and proportion survivorship of female and male *Ae. aegypti* in response to the independent variables Okoumal dose and soaking time

Table 3.2 Summary Least squares linear models for development time……..... 82
and body size of female and male *Ae. aegypti* in response to the independent variables Okoumal dose and soaking time

List of Figures

Figure 2.1 30 cm^3 enclosures for laboratory oviposition assay……..... 48

Figure 2.2 Okoumal pad lures used for the field oviposition study……..... 49

Figure 2.3 Oviposition trap used in field trials……..... 50

Figure 2.4 Percentage of eggs laid in control versus treatment cups……..... 51
for Trial 1

Figure 2.5 Percentage of eggs laid in control versus treatment cups……..... 52
for Trial 2

Figure 2.6 Least square means ± standard error for total mosquito eggs……..... 53
collected based on Okoumal dose in summer field trials

Figure 2.7 Least square means ± standard error for *Ae. albopictus* eggs……..... 54
collected based on Okoumal dose in summer field trials

Figure 3.1 Treated Okoumal-soaked woodchips…….....……..... 86

Figure 3.2 Cups filled with distilled water, *Ae. aegypti* larvae, larval……..... 87
food, and woodchips

Figure 3.3 Mean ± SE *Ae. aegypti* per capita rate of population change……..... 88
to varying concentrations of Okoumal

Figure 3.4 Mean ± SE *Ae. aegypti* female survivorship exposed to……. 89
varying concentrations of Okoumal

Figure 3.5 Mean ± SE *Ae. aegypti* male survivorship exposed to…… 90
varying concentrations of Okoumal

Figure 3.6 Mean ± SE *Ae. aegypti* female development time exposed……... 91
to varying concentrations of Okoumal

Figure 3.7 Mean ± SE *Ae. aegypti* male development time exposed……..... 92
to varying concentrations of Okoumal

Figure 3.8 Mean ± SE *Ae. aegypti* female body size exposed……..... 93
to varying concentrations of Okoumal (immediate and delayed
addition of larvae) in dose-response laboratory experiments

Figure 3.9 Mean ± SE *Ae. aegypti* male body size exposed to……..... 94
varying concentrations of Okoumal (immediate and
delayed addition of larvae) in dose-response laboratory
experiments

Chapter 1: General Introduction

Mosquito-Borne Vector Diseases: A Public Health Concern

Vector-borne diseases have had an overwhelming impact on public and animal health throughout history. According to the World Health Organization (WHO), 17% of infectious diseases are vector-borne, resulting in more than 700,000 human deaths annually and can cause substantial economic impacts on livestock (WHO, 2017). Infectious disease vectors can circulate solely among animals, solely among humans, or from animals to humans (WHO, 2017). Mosquitoes are among the most prominent and medically important insect disease vectors around the world, causing substantial public health concerns in many regions. For example, malaria is a disease transmitted by mosquitoes in the genus *Anopheles* and is endemic in much of Sub-Saharan Africa (Miller et al., 2002).

Due to limited medical capabilities and poor mosquito control, mosquito-human contact and resultant malarial rates remain high in many areas of the world (Miller *et al.,* 2002). It is estimated that there are over 200 million cases of malaria and nearly 500,000 malaria-related deaths worldwide, making malaria the most medically important mosquito-borne disease on earth (WHO, 2017). Malaria was endemic throughout the United States from the 1600s to the mid-1900s (Gubler et al., 2001). Today, about 1700 cases of malaria are diagnosed each year in the United States, typically occurring in travelers returning from areas where malaria is prominent (Centers for Disease Control, 2018).

Another important disease spread by mosquitoes is dengue, an arthropod-borne virus (Family: *Flaviviridae,* genus: *Flavivirus)* native to Africa now vectored by numerous species in many places worldwide in the genus *Aedes*. In Southeast Asia, the Pacific, and the Americas, there are about 50 million reported cases of dengue infections each year (Guzman *et al.* 2010). Approximately 2-5 billion people live in areas where dengue is endemic (Guzman and Kouri, 2002). Dengue was thought to have been under control in the Americas after its principal vector, the invasive *Aedes aegypti* (L.) mosquito from Africa, was eliminated in the 1950s (Guzman et al., 2010). But since the 1980s, it has reemerged as a threat after the reinvasion and rapid spread of *Aedes* mosquitoes from Japan (Bouri et al., 2012).

In addition to dengue and malaria outside their historical ranges, mosquito invasions have caused the emergence of other novel disease threats around the globe, including chikungunya, Zika, and the West Nile arboviruses that were previously poorly known. Chikungunya (Family: *Togaviridae,* genus: *alphavirus)* is spread by invasive *Aedes* mosquitoes in mainly tropical regions, and the first chikungunya-related epidemic occurred during 1952-1953 in Tanzania (Pialoux et al., 2007). More recently, the chikungunya virus arrived in the Americas in 2013 (Flores and O'Neill, 2018). Another emerging threat is Zika (Family: *Flaviviridae,* genus: *flavivirus)*. Zika was first reported in Uganda in 1947, but recent outbreaks have occurred in 2015 and 2016 where *Aedes* mosquitoes have invaded and long been established (Messina et al., 2016). Two counties in Florida reported 321 cases of Zika in 2016 (Likos et al., 2016). Recent modeling deems Zika and chikungunya a threat to temperate regions in the United States due to high density populations and rising temperatures (Manore,

2017). In the Northeastern United States, the West Nile virus is the main novel disease threat (Mackenzie, 2004). The first reported cases of West Nile in the Western Hemisphere occurred in New York City in late 1999, where it was rapidly spread by *Culex* mosquitoes, which are native to Africa and Europe (Nash et al., 2001). Currently, the West Nile Virus has spread to all continents except Antarctica (Weaver and Reisen, 2010).

Important Invasive *Aedes* in America

As non-native species spread into new areas, they often alter diseases by disrupting existing host-pathogen interactions or by introducing novel disease syndromes (Lounibos, 2002). Increasing anthropogenic changes, including globalization and the increase in commercial trade and travel, have increased the probability of non-native mosquitoes and their associated pathogenic agents to spread to more distant locations around the world (Kyle and Harris, 2008). Climate change and land-use modifications have further helped arriving non-native species establish and spread once they arrive in a new region (Tatem et al*.,* 2006).

Ae. aegypti and *Aedes albopictus* (Skuse) are arguably the most important invasive species in the United States and worldwide. *Ae. aegypti,* more commonly known as the yellow fever mosquito, is the primary vector of dengue, yellow fever, and chikungunya virus transmissions (Guzman and Kouri, 2002). *Ae. aegypti* is more anthropophilic than other mosquito species with more catholic feeding patterns, thus, posing a higher risk of spreading disease (McMeniman et al., 2009). *Ae. aegypti* was

first introduced to the Western Hemisphere during the 1800s since colonies were transported in water containers on ships (Reiter, 2001; Slosek, 1986). *Ae. aegypti* are commonly found in Asia, South America, and Africa (Nene et al., 2007). As of 2017, *Ae. aegypti* has been detected in 124 locations within California (Porse et al., 2018). *Ae. aegypti* utilize water-filled containers for their developmental stages (eggs, larvae, and pupae) (Reiter, 2001). Controlling *Ae. aegypti* can be difficult since it likes to be in close proximity to humans and readily locates open containers for its larvae (Gibbons and Vaughn, 2002). *Ae. aegypti* larvae and indoor adults are typically controlled by the use insecticides, however, they have developed a resistance to insecticides, making it difficult to control their populations (Vontas et al*.,* 2012).

The Asian tiger mosquito, *Ae. albopictus*, is native to Southeast Asia and has invaded Africa, Asia, and the Americas over the past 40 years (Gratz, 2004). *Ae. albopictus* was first detected in the continental United States in the mid-1980s and rapidly spread throughout the southeastern part of the United States (Benedict et al., 2007). The species was most likely introduced to these areas via dormant eggs on tires transported by cargo ships during the early 20th century (Gratz, 2004). *Ae. albopictus* was discovered breeding in California in 2001 (Benedict et al., 2007). Today, *Ae. albopictus* is regularly found as far north as Connecticut, Philadelphia, and New York City. Surveillance of *Ae. albopictus* is important to better understand the breadth of the impact this species has had on public health outbreaks. *Ae. albopictus* is a known vector for at least 22 different arboviruses, most notably dengue, which deems a serious public health concern (Gratz, 2004). *Ae. albopictus* eggs are also desiccation-resistant, which may have also facilitated the spread of this

species and the species is also known to outcompete other mosquito species it interacts with (Juliano et al., 2002).

Ae. aegypti has seen a widespread disappearance in the southeastern part of the United States due to being outcompeted by *Ae. albopictus* (Lounibos, 2002). When a species establishes in a new area, it typically interacts with native residents that are ecologically similar (Reiskind and Lounibos, 2013). This is what happened when *Ae. albopictus* first invaded Florida. During the 1980s in the southeastern part of the United States, *Ae. albopictus* were superior to *Ae. aegypti* larvae in resource competition, thus, displacing the latter (Lounibos et al., 2002). Studies have shown that *Ae. albopictus* outcompetes *Ae. aegypti* in resource competition, inhibits the hatching of *Ae. aegypti* eggs and have caused sterility in the yellow fever mosquito due to interspecific mating (Lounibos, 2002). In parts of North America, Central Africa, and Brazil, there are still some urban and suburban areas in which *Ae. aegypti* and *Ae. albopictus* coexist and share the same habitat; however, it is thought that eventually *Ae. aegypti* will be displaced by *Ae. albopictus* (Paupy et al., 2009).

Mosquito Control and Surveillance

With few vaccines available, minimizing human exposure to and managing their populations of vector species, including *Ae. aegypti* and *Ae. albopictus,* remain the primary methods for reducing their pestiferous and disease burdens. Accurate surveillance and trapping of focal vector species are fundamental to effective disease management. Mosquito monitoring is a vital part of an integrated mosquito management program. Monitoring populations of vector species can help predict

transmission risks in space and time and inform public health and mosquito control intervention efforts (Juliano and Lounibos, 2005).

Trapping host-seeking adult female mosquitoes is a common approach to monitor and surveil vector populations because it targets the life-stage of direct public health importance. However, adult trapping is typically labor intensive and expensive. Adult trapping requires using live humans, animals, or chemical (e.g, CO_2, human pheromones) and physical (e.g., color contrasts) cues as bait, and mechanical, often motorized, methods of capturing lured individuals (Silver, 2008). Such methods are often beyond means of many mosquito management programs or restrict the distribution and intensity of trapping (Silver, 2008).

In order to obtain the necessary nutrients necessary for egg production, females require a blood meal from a vertebrate host (Mullen & Durden, 2009; Takken et al., 2013). Females search for an attractant that helps induce oviposition when looking to lay eggs. This oviposition behavior occurs since the mosquito's sensorial system is complex and consists of thermoreceptors, chemoreceptors, mechanoreceptors, and hygroreceptors (Navarro-Silva et al., 2009). This complex system can detect a wide breadth of volatile compounds that inform location of food, presence of mating partners, or sites suitable for oviposition (Luntz, 2003; Navarro-Silva et al., 2009). Specialized setae known as olfactory and gustatory sensilla are connected to these receptors by neurons (Navarro-Silva et al., 2009). Olfactory sensilla occurs in pairs and can be found on the head, antennae palpus, and female ovipositors (Hallem et al., 2006; Navarro-Silva et al., 2009).

When seeking out a blood meal, females have the potential to spread disease between their hosts, which deems surveillance of mosquitoes in the egg-laying stage important. An approach to monitor mosquito activity is to target gravid (i.e., egg-laying) females by using oviposition traps that collect the female or her oviposited eggs. Ovitraps are often black cups with tapered sides that are filled with tap water and are generally left in the field for a week (Reiter et al., 1991). Typically, a coarse material, such as seed germination paper (e.g., Ritchie et al., 2003) or Masonite paddle (e.g., Leisnham and Juliano, 2009), is placed in each ovitrap to provide a surface onto which gravid females can oviposit and for those eggs to be easily collected by investigators.

Ovitraps provide a cheap and simple tool that is easily replicable for surveilling many vector species (Silver, 2008). Comparing the number of eggs among systematically positioned ovitraps is often used as a convenient approach of relative mosquito activity in space and time and varying scales, including among landscapes, neighborhoods within cities, and among wet and dry seasons between weeks (Silver, 2008). Another benefit of ovitrapping is that it may indicate areas of high oviposition activity where management efforts can focus on controlling immature stages (eggs, larvae) in nearby aquatic habitats. Immature mosquitoes are often easier and more efficient to control compared to adults since they are typically concentrated in discrete water bodies (Floore, 2006). Having knowledge of mosquito breeding sites and their oviposition behavior is a good method to both control and monitor these insects (Pates and Curtis, 2005).

To improve collections, oviposition traps are usually baited with chemical cues that are attractive to ovipositing females. The most common bait used in oviposition traps is plant infusion water that provides complex combinations of organic materials, which have variable compositions (Clements, 1999; Silver, 2008). The organic volatiles that are produced in nutrient-rich habitats i.e., decaying plant material, cue female oviposition behavior and provide microbes on which larvae can feed, which is what females are seeking. Plant infusion water is usually made days before ovitraps are deployed in the field, involving seeping plant material (e.g., hay, leaf litter) in water, and then baiting a known amount in water-filled oviposition traps (Ponnusamy et al., 2010; Silver, 2008). Usually, oviposition activity (i.e., numbers of females ovipositing eggs) is monitored indirectly by enumerating numbers of oviposited eggs after ovitraps have been deployed by a specific length of time in the field. Ovitraps are usually retrieved within seven days to avoid egg hatching and development to adulthood (Silver, 2008).

Some ovitraps are designed to also kill the visiting female and her eggs. These traps can use varied mechanical means, including adhesive surfaces (e.g., sticky trap, Kröckel et al., 2006), traps designed to prevent females from exiting after ovipositing (e.g., GAT traps, Ritchie et al., 2014), or motorized fans that blow females into a collection bag. With using sticky ovitraps, more information can be obtained about the number of egg-laying mosquito populations (Kröckel et al., 2006). Another such similar trap is the CDC autocidal gravid ovitrap (AGO). An AGO prevents mosquito eggs from hatching larvae by either chemical or mechanical means (Barrera et al., 2014). AGO's typically deliver increased intensity of olfactory and visual cues to

gravid females (Barrera et al., 2014). Because ovitraps are relatively easy to make, they provide a key tool in which to detect gravid female mosquito populations, even in low density populations (Silver, 2008).

Despite their wide-use and advantages over other forms of mosquito surveillance, there are limitations to current ovitrapping approaches and technology. Using plant infusion water as a bait within traps can be logistically challenging when deploying large numbers of traps since plant infusion bait typically needs to be recharged on a weekly basis. Chemical and mechanical approaches to kill visiting females or their offspring (e.g., autocidal traps) usually add a considerable cost per ovitrap and require regular upkeep. There remains considerable scope to improve the effectiveness of ovitrapping approaches.

Ovipositing *Aedes* are often attracted to aquatic development habitats by semiochemicals which are signaling chemicals that incite behavior in organisms (Kline, 2007). The use of semiochemicals in traps that rely on odors for mosquito control and surveillance is a relatively new and viable technique used by vector management programs (Wooding et al., 2020). There are over 100 semiochemicals identified as being effective mosquito attractants, but the implementation of these chemicals can be difficult since there is a variation in how different species of mosquitoes respond to odors and sometimes the semiochemicals need to be a complex blend of chemicals to be effective (Wooding et al., 2020).

Volatiles, such as semiochemicals, need to come in contact with specialized olfactory receptors (ORs) in order to elicit mosquito behavior (Leal et al., 2013). These ORs are located on the dendrites of olfactory receptor neurons (ORN) in the

chemosensory appendages on a mosquito's head (Leal et al., 2013). ORs form ion channels with the coreceptor, Orco, which was first discovered in the fruit fly (Larsson et al., 2004; Choo et al., 2018). Semiochemicals are then activated by OR-Orco complexes that signal a transmission to the brain that could lead to a behavioral response by the mosquito (Choo et al., 2017). Odorant-binding proteins (OBPs) solubilize, bind, and deliver odorant molecules to ORs and are involved in the first steps of odorant reception (Deng et al., 2013; Choo et al., 2018). The first insect OBP was discovered in *Antheraea polyphemus,* the giant moth, and the first mosquito OBP was found in the antennae of a female *Culex quinquefasciatus* (Vogt & Riddiford, 1981; Deng et al., 2013). To date, 34 classic OBPs have been identified in *Ae. aegypti*, however, *Ae. albopictus* have few reported classic OBPs (Deng et al., 2013). Semiochemicals play an important role in vector management strategies, and these semiochemicals are typically discovered by using laboratory assays (Choo et al., 2018).

Integrated Pest Management at the Larval Stage

Mosquito populations are largely regulated at the immature stage (e.g., mosquito larvae or mosquito pupae). Eliminating the number of water-filled containers (known as source reduction) that mosquitoes can utilize to breed and develop can greatly help manage *Aedes* mosquitoes. With source reduction, the general population needs to be educated on how to be effective in reducing habitats as well as be motivated to implement these practices (Dowling et al., 2013). Since the primary source of *Aedes* breeding sites are containers in residential homes, education

about source reduction is of the utmost importance in order to eliminate water containers (Fonseca et al., 2013). Many public schools in the United States provide educational programs to educate children on how their families can prevent mosquito proliferation at their homes (Rose, 2001). Source reduction can be difficult to manage and control for public health agencies since many of these artificial containers are found on private, residential property. This makes it difficult to manage whether or not residents are controlling and reducing the numbers of artificial or water filled containers on their properties.

Removing water-filled containers that could potentially host mosquito larvae is typically the first step in source reduction but if water in a container cannot be emptied or removed, larvicides are used (Marcombe et al., 2014). If an outbreak of a mosquito-borne disease occurs or if adult mosquitoes become a serious issue, insecticides, known as adulticides are used to target the adults (Marcombe et al., 2014). Adulticides are typically applied at ultra-low volumes during the night-time when people are indoors to limit human exposures (Rose, 2001). Insecticide-based interferences have controlled invasive *Aedes* mosquitoes for quite some time, but the mosquitoes have started to develop a resistance to the current insecticides used (Vontas et al., 2012).

In addition to killing mosquitoes in their current ranges, repellents are commonly used as a way to avoid being bitten. The most common insect repellent is diethyltoluamide (DEET), which has been used globally since 1957 and is effective against many different species of mosquitoes (Fradin and Day, 2002). Mosquito control and surveillance can be controversial, especially when it uses biological

controls or pesticides since it can raise human health and environmental concerns (Dowling et al., 2013). There remains a need to further investigate synthetic materials that can effectively control mosquitoes without compromising human or environmental health.

Okoumal: A Pro-Fragrance Chemical Attractant

The United States Department of Agriculture-Agricultural Research Service (USDA-ARS) has a long history of research on mosquito trapping methods and chemical attractants under the umbrella of its National Program 104: Medical, Veterinary, and Urban entomology (USDA-ARS 2019). Recently, the USDA-ARS has been exploring the potential of pro-fragrance compounds with core structures containing acetals and ketals of oxygenated sesquiterpenes at attracting *Aedes* mosquitoes. At least one study has shown that an example of these compounds can trigger the same neuron receptors in adult females as CO_2 and preliminary data from laboratory trials have shown that examples of these compounds are as or more attractive to host-seeking females than CO_2 (Tauxe et al., 2013).

The USDA has recently patented a chemical compound called [2,4-Dimethyl-2-(5,5,8,8-tetramethyl-6,7-dihydronaphthalen-2-yl)-1,3-dioxolane], commonly called Okoumal (Vigon; East Stroudsburg, PA), as an attractant to host-seeking female mosquitoes (USPTO, 2016). Okoumal is a synthetic chemical compound that has a woody and amber aroma (National Center for Biotechnology Information, 2020). Subsequent observations in the lab and in an initial field experiment have shown that the compound, Okoumal may not only be attractive to host-seeking individuals, but

also be effective in attracting ovipositing *Aedes* females (USPTO, 2016; Saunders and Leisnham, 2018). Pro-fragrance compounds, including Okoumal, are less volatile than many other fragrant attractants (e.g., CO_2), which potentially makes this substance long-lasting and more field stable. Okoumal and its derivatives as mosquito attractants have been accepted as a patent and field trials as needed to further explore their use in field conditions.

Okoumal, which is commercially available, is also a known toxin to aquatic organisms, making it a good candidate for being a toxin to mosquito larvae (NCBI, 2020). Since Okoumal was an attractant to gravid females in preliminary field trials and it is a known toxin to aquatic organisms, it is a good candidate to be used in a "bait and kill" trap (Saunders & Leisnham, 2018; NCBI, 2020). For the "bait and kill" trap, a gravid female mosquito would be attracted to an oviposition trap baited with Okoumal where she would lay her eggs on a seed germination paper (Ong & Jaal, 2015). Once the female's eggs hatch into larvae in the Okoumal-baited traps, they would not survive due to the toxicity of Okoumal.

Project Goals and Summary

The overall goal of my thesis is to test the efficacy of Okoumal in oviposition traps that target *Aedes* mosquitoes. To address this goal, I will undertake two research questions that field test the attractancy of Okoumal to gravid adult *Aedes* females and its toxicity to their larval offspring. In Chapter 2, I will test the attractancy of Okoumal to adult females in a laboratory oviposition choice assay and test the minimum effective Okoumal dose to attract adult females in a field oviposition

survey. In Chapter 3, I will test the toxicity of Okoumal to newly hatched *Aedes* larvae with a laboratory-based dose-response trial.

References

Barrera, R., M. Amador, V. Acevedo, B. Caban, G. Felix, and A. J. Mackay. 2014. Use of the CDC autocidal gravid ovitrap to control and prevent outbreaks of *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology 51: 145-154.

Benedict, M. Q., R. S. Levine, W. A. Hawley, and L. P. Lounibos. 2007. Spread of the tiger: Global risk of invasion by the mosquito *Aedes albopictus*. Vector-Borne and Zoonotic Diseases 7: 76-85.

Bouri, N., T. K. Sell, C. Franco, A. A. Adalja, D. A. Henderson, and N. A. Hynes. 2012. Return of epidemic dengue in the United States: implications for the public health practitioner. Public Health Reports 127: 259-266.

CDC. 2018. About malaria.

Choo, Y. M., P. X. Xu, J. K. Hwang, F. F. Zeng, K. M. Tan, G. Bhagavathy, K. R. Chauhan, and W. S. Leal. 2018. Reverse chemical ecology approach for the identification of an oviposition attractant for *Culex quinquefasciatus*. Proceedings of the National Academy of Sciences of the United States of America 115: 714-719.

Clements, A. 1999. The biology of mosquitoes, vol. 2, CABI International.

Deng, Y. H., H. Yan, J. B. Gu, J. B. Xu, K. Wu, Z. J. Tu, A. A. James, and X. G. Chen. 2013. Molecular and functional characterization of odorant-binding

protein genes in an invasive vector mosquito, *Aedes albopictus*. Plos One 8: 11.

Dowling, Z., S. L. Ladeau, P. Armbruster, D. Biehler, and P. T. Leisnham. 2013. Socioeconomic status affects mosquito (Diptera: Culicidae) larval habitat type availability and infestation level. Journal of Medical Entomology 50: 764-772.

Floore, T. G. 2006. Mosquito larval control practices: past and present. Journal of the American Mosquito Control Association 22: 527-533.

Flores, H. A., and S. L. O'Neill. 2018. Controlling vector-borne diseases by releasing modified mosquitoes. Nature Reviews Microbiology 16: 508-518.

Fonseca, D. M., I. Unlu, T. Crepeau, A. Farajollahi, S. P. Healy, K. Bartlett-Healy, D. Strickman, R. Gaugler, G. Hamilton, D. Kline, and G. G. Clark. 2013. Area-wide management of *Aedes albopictus*. Part 2: Gauging the efficacy of traditional integrated pest control measures against urban container mosquitoes. Pest Management Science 69: 1351-1361.

Fradin, M. S., and J. F. Day. 2002. Comparative efficacy of insect repellents against mosquito bites. New England Journal of Medicine 347: 13-18.

Gibbons, R. V., and D. W. Vaughn. 2002. Dengue: an escalating problem. British Medical Journal 324: 1563-1566.

Gopalakrishnan, R., M. Das, I. Baruah, V. Veer, and P. Dutta. 2012. Studies on the ovitraps baited with hay and leaf infusions for the surveillance of dengue vector, *Aedes albopictus* in northeastern India. Tropical Biomedicine 29: 598-604.

Gratz, N. G. 2004. Critical review of the vector status of *Aedes albopictus*. Medical and Veterinary Entomology 18: 215-227.

Grill, C. P., and S. A. Juliano. 1996. Predicting species interactions based on behaviour: Predation and competition in container-dwelling mosquitoes. Journal of Animal Ecology 65: 63-76.

Gubler, D. J., P. Reiter, K. L. Ebi, W. Yap, R. Nasci, and J. A. Patz. 2001. Climate variability and change in the United States: Potential impacts on vector- and rodent-borne diseases. Environmental Health Perspectives 109: 223-233.

Guzman, M. G., and G. Kouri. 2002. Dengue: an update. Lancet Infectious Diseases 2: 33-42.

Guzman, M. G., S. B. Halstead, H. Artsob, P. Buchy, F. Jeremy, D. J. Gubler, E. Hunsperger, A. Kroeger, H. S. Margolis, E. Martinez, M. B. Nathan, J. L. Pelegrino, S. Cameron, S. Yoksan, and R. W. Peeling. 2010. Dengue: a continuing global threat. Nature Reviews Microbiology: S7-S16.

Hallem, E. A., A. Dahanukar, and J. R. Carlson. 2006. Insect odor and taste receptors, pp. 113-135, Annual Review of Entomology, vol. 51. Annual Reviews, Palo Alto.

Juliano, S. A. 1998. Species introduction and replacement among mosquitoes: Interspecific resource competition or apparent competition? Ecology 79: 255-268.

Juliano, S. A., and L. P. Lounibos. 2005. Ecology of invasive mosquitoes: effects on resident species and on human health. Ecology Letters 8: 558-574.

Juliano, S. A., G. F. O'Meara, J. R. Morrill, and M. M. Cutwa. 2002. Desiccation and thermal tolerance of eggs and the coexistence of competing mosquitoes. Oecologia 130: 458-469.

Kline, D. L. 2007. Semiochemicals, traps/targets and mass trapping technology for mosquito management. Journal of the American Mosquito Control Association 23: 241-251.

Krockel, U., A. Rose, A. E. Eiras, and M. Geier. 2006. New tools for surveillance of adult yellow fever mosquitoes: comparison of trap catches with human landing rates in an urban environment. Journal of the American Mosquito Control Association 22: 229-238.

Kyle, J. L., and E. Harris. 2008. Global spread and persistence of dengue, pp. 71-92, Annual Review of Microbiology, vol. 62. Annual Reviews, Palo Alto.

Larsson, M. C., A. I. Domingos, W. D. Jones, M. E. Chiappe, H. Amrein, and L. B. Vosshall. 2004. Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron 43: 703-714.

Leal, W. S. 2013. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annual Review of Entomology, Vol 58 58: 373-391.

Leisnham, P. T., and S. A. Juliano. 2009. Spatial and temporal patterns of coexistence between competing *Aedes* mosquitoes in urban Florida. Oecologia 160: 343-352.

Likos, A., I. Griffin, A. M. Bingham, D. Stanek, M. Fischer, S. White, J. Hamilton, L. Eisenstein, D. Atrubin, P. Mulay, B. Scott, P. Jenkins, D. Fernandez, E. Rico, L. Gillis, R. Jean, M. Cone, C. Blackmore, J. McAllister, C. Vasquez, L. Rivera, and C. Philip. 2016. Local mosquito-borne transmission of Zika Virus - Miami-Dade and Broward Counties, Florida, June-August 2016. MMWR-Morbidity and Mortality Weekly Report 65: 1032-1038.

Lounibos, L. P. 2002. Invasions by insect vectors of human disease. Annual Review of Entomology 47: 233-266.

Lounibos, L. P., S. Suarez, Z. Menendez, N. Nishimura, R. L. Escher, S. M. O'Connell, and J. R. Rey. 2002. Does temperature affect the outcome of larval competition between *Aedes aegypti* and *Aedes albopictus*? Journal of Vector Ecology 27: 86-95.

Luntz, A. J. M. 2003. Arthropod semiochemicals: mosquitoes, midges and sealice. Biochemical Society Transactions 31: 128-133.

Mackenzie, J. S., D. J. Gubler, and L. R. Petersen. 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nature Medicine 10: S98-S109.

Manore, C. A., R. S. Ostfeld, F. B. Agusto, H. Gaff, and S. L. LaDeau. 2017. Defining the risk of Zika and Chikungunya virus transmission in human population centers of the Eastern United States. PLOS Neglected Tropical Diseases 11: 19.

Marcombe, S., A. Farajollahi, S. P. Healy, G. G. Clark, and D. M. Fonseca. 2014. Insecticide resistance status of United States populations of *Aedes albopictus* and mechanisms involved. PLOS One 9: 10.

McMeniman, C. J., R. V. Lane, B. N. Cass, A. W. C. Fong, M. Sidhu, Y. F. Wang, and S. L. O'Neill. 2009. Stable introduction of a life-shortening Wolbachia infection into the mosquito *Aedes aegypti*. Science 323: 141-144.

Messina, J. P., M. U. G. Kraemer, O. J. Brady, D. M. Pigott, F. M. Shearer, D. J. Weiss, N. Golding, C. W. Ruktanonchar, P. W. Gething, E. Cohn, J. S. Brownstein, K. Khan, A. J. Tatem, T. Jaenisch, C. J. L. Murray, F. Marinho, T. W. Scott, and S. I. Hay. 2016. Mapping global environmental suitability for Zika virus. Elife 5: 19.

Miller, L. H., D. I. Baruch, K. Marsh, and O. K. Doumbo. 2002. The pathogenic basis of malaria. Nature 415: 673-679.

Mullen, G., and L. Durden. 2009. Medical and Veterinary Entomology, Academic Press.

Nash, D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M. Sherman, S. Wong, M. Layton, G. L. Campbell, J. T. Roehrig, D. J. Gubler, W. J. Shieh, S. Zaki, P. Smith, and W. N. O. R. Working. 2001. The outbreak of West Nile virus infection in the New York City area in 1999. New England Journal of Medicine 344: 1807-1814.

National Center for Biotechnology Information. PubChem Database. CID=3034278, h. p. n. n. n. g. c. d. L. a. o. J., 2020).

Navarro-Silva, M. A., F. A. Marques, and J. E. Duque. 2009. Review of semiochemicals that mediate the oviposition of mosquitoes: a possible sustainable tool for the control and monitoring of Culicidae. Revista Brasileira De Entomologia 53: 1-6.

Nene, V., J. R. Wortman, D. Lawson, B. Haas, C. Kodira, Z. J. Tu, B. Loftus, Z. Y. Xi, K. Megy, M. Grabherr, Q. H. Ren, E. M. Zdobnov, N. F. Lobo, K. S. Campbell, S. E. Brown, M. F. Bonaldo, J. S. Zhu, S. P. Sinkins, D. G. Hogenkamp, P. Amedeo, P. Arensburger, P. W. Atkinson, S. Bidwell, J. Biedler, E. Birney, R. V. Bruggner, J. Costas, M. R. Coy, J. Crabtree, M.

Crawford, B. deBruyn, D. DeCaprio, K. Eiglmeier, E. Eisenstadt, H. El-Dorry, W. M. Gelbart, S. L. Gomes, M. Hammond, L. I. Hannick, J. R. Hogan, M. H. Holmes, D. Jaffe, J. S. Johnston, R. C. Kennedy, H. Koo, S. Kravitz, E. V. Kriventseva, D. Kulp, K. LaButti, E. Lee, S. Li, D. D. Lovin, C. H. Mao, E. Mauceli, C. F. M. Menck, J. R. Miller, P. Montgomery, A. Mori, A. L. Nascimento, H. F. Naveira, C. Nusbaum, S. O'Leary, J. Orvis, M. Pertea, H. Quesneville, K. R. Reidenbach, Y. H. Rogers, C. W. Roth, J. R. Schneider, M. Schatz, M. Shumway, M. Stanke, E. O. Stinson, J. M. C. Tubio, J. P. VanZee, S. Verjovski-Almeida, D. Werner, O. White, S. Wyder, Q. D. Zeng, Q. Zhao, Y. M. Zhao, C. A. Hill, A. S. Raikhel, M. B. Soares, D. L. Knudson, N. H. Lee, J. Galagan, S. L. Salzberg, I. T. Paulsen, G. Dimopoulos, F. H. Collins, B. Birren, C. M. Fraser-Liggett, and D. W. Severson. 2007. Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science 316: 1718-1723.

Ong, S. Q., and Z. Jaal. 2015. Investigation of mosquito oviposition pheromone as lethal lure for the control of *Aedes aegypti* (L.) (Diptera: Culicidae). Parasites & Vectors 8: 7.

Pates, H., and C. Curtis. 2005. Mosquito behavior and vector control, pp. 53-70, Annual Review of Entomology, vol. 50. Annual Reviews, Palo Alto.

Paupy, C., H. Delatte, L. Bagny, V. Corbel, and D. Fontenille. 2009. *Aedes albopictus*, an arbovirus vector: from the darkness to the light. Microbes and Infection 11: 1177-1185.

Pialoux, G., B. A. Gauzere, S. Jaureguiberry, and M. Strobel.

2007. Chikungunya, an epidemic arbovirosis. Lancet Infectious Diseases 7: 319-327.

Ponnusamy, L., D. M. Wesson, C. Arellano, C. Schal, and C. S. Apperson.

2010. Species composition of bacterial communities' influences attraction of mosquitoes to experimental plant infusions. Microbial Ecology 59: 158-173.

Porse, C. C., S. Messenger, D. J. Vugia, W. Jilek, M. Salas, J. Watt, and V. Kramer. 2018. Travel-associated Zika Cases and Threat of Local Transmission during global outbreak, California, USA. Emerging Infectious Diseases 24: 1626-1632.

PT, S. M. a. L. October 2018. Testing an easily deployable mosquito attractant: promising results from initial field trials. Society of Vector Ecology 48t^{h} Annual Conference at Yosemite National Park.

Reiskind, M. H., and L. P. Lounibos. 2013. Spatial and temporal patterns of abundance of *Aedes aegypti* L. (*Stegomyia aegypti*) and *Aedes albopictus* (Skuse) *Stegomyia albopictus* (Skuse) in southern Florida. Medical and Veterinary Entomology 27: 421-429.

Reiter, P. 2001. Climate change and mosquito-borne disease. Environmental Health Perspectives 109: 141-161.

Reiter, P., M. A. Amador, and N. Colon. 1991. Enhancement of the CDC ovitrap with hay infusions for daily monitoring of *Aedes-aegypti* populations. Journal of the American Mosquito Control Association 7: 52-55.

Ritchie, S. A., S. Long, A. Hart, C. E. Webb, and R. C. Russell. 2003. An adulticidal sticky ovitrap for sampling container-breeding mosquitoes. Journal of the American Mosquito Control Association 19: 235-242.

Ritchie, S. A., T. S. Buhagiar, M. Townsend, A. Hoffmann, A. F. van den Hurk, J. L. McMahon, and A. E. Eiras. 2014. Field validation of the gravid *Aedes* trap (GAT) for collection of *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology 51: 210-219.

Rose, R. I. 2001. Pesticides and public health: Integrated methods of mosquito management. Emerging Infectious Diseases 7: 17-23.

Saunders, M., and P. Leisnham. October 2018. Testing an easily deployable mosquito attractant: promising results from initial field trials. Society of Vector Ecology 48t^{h} Annual Conference at Yosemite National Park.

Silver, J. B., M. W. Service, and SpringerLink (Online service). 2008. Mosquito ecology field sampling methods, pp. xxi, 1494 p. Springer, Dordrecht, the Netherlands.

Slosek, J. 1986. *Aedes aegypti* mosquitoes in the Americas: A review of their interactions with the human population. Social Science & Medicine 23: 249-257.

Takken, W., and N. O. Verhulst. 2013. Host preferences of blood-feeding mosquitoes, pp. 433-+. In M. R. Berenbaum (ed.), Annual Review of Entomology, Vol 58, vol. 58. Annual Reviews, Palo Alto.

Tatem, A. J., S. I. Hay, and D. J. Rogers. 2006. Global traffic and disease vector dispersal. Proceedings of the National Academy of Sciences of the United States of America 103: 6242-6247.

USDA-ARS. 2019. National Program 104: Veterinary, Medical, and Urban Entomology Strategic Division.

Vogt, R. G., and L. M. Riddiford. 1981. Pheromone binding and inactivation by moth antennae. Nature 293: 161-163.

Vontas, J., E. Kioulos, N. Pavlidi, E. Morou, A. della Torre, and H. Ranson. 2012. Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. Pesticide Biochemistry and Physiology 104: 126-131.

Weaver, S. C., and W. K. Reisen. 2010. Present and future arboviral threats. Antiviral Research 85: 328-345.

WHO. 2017a. Vector-borne diseases.

WHO. 2017b. World Malaria Report 2017. World Malaria Report 2017: 1-208.

Wooding, M., Y. Naude, E. Rohwer, and M. Bouwer. 2020. Controlling mosquitoes with semiochemicals: a review. Parasites & Vectors 13: 20.

Chapter 2: Investigating the Pro-Fragrance, Okoumal, as an Attractant to Gravid Female Mosquitoes Using Field and Laboratory Trials

Abstract

Mosquitoes are among the most medically important insects in the world, causing substantial public health concerns in many regions. *Aedes* spp. mosquitoes are arguably the most important invasive species in the United States and worldwide and can collectively vector a range of viruses, including West Nile, dengue, and Zika. Ovipositing *Aedes* are often attracted to aquatic development habitats by semiochemicals (signaling chemicals that incite behavior in organisms). Oviposition traps are typically baited with plant infusions that release a suite of volatile compounds that attract gravid female mosquitoes, but plant infusions require weekly maintenance and are difficult to standardize. Many past compounds that have been used to attract and trap mosquitoes are highly volatile under field conditions, making it difficult to achieve a slow release and sustain their efficacy over time. Recently, the USDA-ARS has been exploring the pro-fragrance compound, Okoumal to attract *Aedes* mosquitoes. The goal of this study was to evaluate the efficacy of Okoumal, at attracting ovipositing gravid adult *Aedes* mosquitoes using field and laboratory trials. Oviposition traps baited with varying doses of Okoumal were compared in a replicated Latin-square design in the field and an oviposition choice assay compared four Okoumal doses with control treatments in laboratory enclosures. Across all comparisons, there was no evidence of Okoumal attractancy to *Aedes albopictus*.

These results cast doubt on Okoumal's use as a bait in mosquito surveillance and control. Future studies should investigate the efficacy of other pro-fragrance compounds at attracting gravid *Aedes* mosquitoes.

Introduction

Vector-borne diseases have had an overwhelming impact on public and animal health throughout history (World Health Organization, 2017). According to the World Health Organization, 17% of infectious diseases are vector-borne, resulting in more than 700,000 human deaths annually and substantial economic impacts on livestock (WHO, 2017). Infectious disease vectors can circulate among animals, among humans, or from animals to humans (WHO, 2017). Mosquitoes are among the most prominent and medically important insect disease vectors around the world, causing substantial public health concerns in many regions (Silver, 2008).

A female mosquito typically requires a blood meal before she is able to lay eggs and this biting behavior is what transmits disease (Ariani et al., 2015). Typically, a blood meal from a host allows a mosquito to lay more eggs with higher viability rates (Ariani et al., 2015). In addition to biting hosts and moderating immediate disease transmission, female mosquitoes are fundamentally important in determining the growth of mosquito populations and resultant long-term disease dynamics. By selecting favorable oviposition sites where there are more resources and fewer predators, females can help their offspring survive and develop through to adulthood (Wong et al., 2011). The oviposition behavior of female mosquitoes is a vital life

history trait under strong selection pressure and individual females are influenced by numerous environmental cues, such as rainfall rates, humidity, and temperature when choosing oviposition sites (Bentley & Day, 1989). In addition to environmental cues, ovipositing females are also influenced by chemical, visual, and olfactory cues associated with the container habitats they utilize (Bentley & Day, 1989).

Aedes albopictus (Skuse), the Asian tiger mosquito, is the most common mosquito found in urban areas in the northeastern part of the United States (Moore, 1999; Gratz, 2004). Native to Asia, *Ae. albopictus* has invaded numerous regions worldwide and was first detected in the continental United States in the mid-1980s when eggs and larvae were transported on tires on ships (Tatem, Hay, & Rogers, 2006; Benedict et al., 2007). *Ae. albopictus* is adapted to extreme climates and environments, such as cold temperatures, by laying desiccation-resistant diapausing eggs that can survive droughts and cold winters which has helped in its spread and invasion (Paupy et al., 2009). *Ae. albopictus* is one of the most commonly studied container-breeding mosquito species because of its extensive spread and its major public health importance (Lounibos, 2002). *Ae. albopictus* mosquitoes are known to lay their eggs in artificial containers found in urban residential areas.

In order to obtain the necessary nutrients necessary for egg production, females require a blood meal from a vertebrate host (Mullen & Durden, 2009; Takken et al., 2013). When looking to lay eggs, a female looks for an attractant that helps induce oviposition. This oviposition behavior occurs since the mosquito's sensorial system is complex and consists of thermoreceptors, chemoreceptors, mechanoreceptors, and hygroreceptors (Navarro-Silva et al., 2009). This complex

system can detect a wide breadth of volatile compounds that inform location of food, presence of mating partners, or sites suitable for oviposition (Luntz, 2003; Navarro-Silva et al., 2009). Specialized setae known as olfactory and gustatory sensilla are connected to these receptors by neurons (Navarro-Silva et al., 2009). Olfactory sensilla occurs in pairs and can be found on the head, antennae palpus, and female ovipositors (Hallem et al., 2006; Navarro-Silva et al., 2009).

When seeking out a blood meal, females have the potential to spread disease between their hosts, which deems surveillance of mosquitoes in the egg-laying stage important. An approach to monitor mosquito activity is to target gravid (i.e., egg-laying or ovipositing) females by using oviposition traps that collect the female or her eggs. Ovitraps are often black cups with tapered sides that are filled with tap water and are left in the field for a week (Reiter et al., 1991). Typically, a coarse material, such as seed germination paper (e.g., Ritchie et al., 2003) or Masonite paddle (e.g., Leisnham and Juliano, 2009), is placed in each ovitrap to provide a surface onto which gravid females can oviposit and for those eggs to be easily collected by investigators.

Ovitraps provide a cheap and simple tool that is easily replicable for monitoring many vector species (Silver, 2008). Oviposition traps are commonly baited with attractants to ovipositing females to improve collections. The most common bait is plant infusion water that provides complex combinations of organic materials, which have variable compositions (Clements, 1999). The organic volatiles that are produced in nutrient-rich habitats i.e., decaying plant material, cue female oviposition behaviour and provide microbes on which larvae can feed, which is what

females are seeking. Plant infusion water is usually made days before ovitraps are deployed in the field, involving seeping plant material (e.g., hay, leaf litter) in water, and then baiting a known amount in water-filled oviposition traps (Silver, 2008; Ponnusamy et al., 2010).

Ovipositing *Aedes* are often attracted to aquatic development habitats by semiochemicals which are signaling chemicals that incite behavior in organisms (Kline, 2007). The use of semiochemicals in traps that rely on odors for mosquito control and surveillance is a relatively new and viable technique used by vector management programs (Wooding et al., 2020). Mosquitoes have highly sophisticated olfactory systems that have hundreds of receptor proteins (Ray, 2015).There are over 100 semiochemicals identified as being effective mosquito attractants, but the implementation of these chemicals can be difficult since there is a variation in how different species of mosquitoes respond to odors and sometimes the semiochemicals need to be a complex blend of chemicals to be effective (Wooding et al., 2020). Semiochemicals play an important role in vector management strategies, and these semiochemicals are typically discovered by using laboratory assays (Choo et al., 2017).

Usually, oviposition activity (i.e., numbers of females ovipositing eggs) is monitored indirectly by enumerating numbers of oviposited eggs after ovitraps have been deployed by a specific length of time in the field (Silver, 2008; Leisnham & Juliano, 2009). To avoid egg hatching and development to adulthood, ovitraps are usually retrieved within seven days (Silver, 2008). Despite their wide-use and advantages over other forms of mosquito surveillance, there are limitations to current

ovitrapping approaches and technology. Using plant infusion water as a bait within traps can be logistically challenging when deploying large numbers of traps and plant infusion bait typically needs to be recharged on a weekly basis. Chemical and mechanical approaches to kill visiting females or their offspring (e.g., autocidal traps) usually add a considerable cost per ovitrap and require regular upkeep. There remains considerable scope to improve the effectiveness of ovitrapping approaches in order to effectively monitor and survey important *Aedes* mosquito vectors.

Pro-fragrance compounds are compounds that emit fragrances in a volatile form when it undergoes one or more chemical transformations (Dykstra et al., 2009). This means that pro-fragrance compounds could potentially be used as an alternative to plant infusions. A few studies have shown that pro-fragrance compounds can trigger the same neuron receptors in adult females as CO_2 and preliminary data from laboratory trials have shown that examples of these compounds are as or more attractive to host-seeking females than CO_2 (Tauxe et al., 2013).

The USDA has recently patented a chemical compound called [2,4-Dimethyl-2-(5,5,8,8-tetramethyl-6,7-dihydronaphthalen-2-yl)-1,3-dioxolane], commonly called Okoumal, as an attractant to host-seeking female mosquitoes (USPTO, 2016). Okoumal has a core structure containing acetals and ketals of oxygenated sesquiterpenes, and it has shown to be an attractant to *Aedes* mosquitoes (USPTO, 2016; Saunders & Leisnham, 2018). Okoumal is a chemical compound, with a woody aroma, that is commercially available and used in the fragrance industry. Pro-fragrance compounds, such as Okoumal, can be less volatile than other fragrant attractants, which potentially makes this substance long-lasting and more field stable.

In this chapter, I tested the effect of Okoumal on the oviposition of *Ae. albopictus* at two spatial scales in both a laboratory oviposition choice assay and dose-response field oviposition study. The laboratory choice assay explored whether Okoumal would attract a gravid female if she had a choice to oviposit in containers that were close together. In contrast, while the field experiment gave a female the choice between the Okoumal-baited ovitraps and other potential habitats in the area, it tested the effect of Okoumal at a much larger scale. In both the laboratory choice assay and the field study, oviposition activity was measured as the number of eggs laid by female mosquitoes. Number of eggs laid by the female was chosen to calculate oviposition behavior rather than the number of females or oviposition events since *Aedes* species are known to have skip oviposition behavior (Davis et al., 2015). Skip oviposition behavior occurs when a female chooses to lay her eggs in batches across different oviposition sites rather than laying all eggs in one location (Davis et al., 2015). By skip ovipositing, the female mosquito lessens the potential for the offspring to compete with each other for resources (Davis et al., 2015).

The goal of this chapter was to test whether Okoumal is an effective attractant to gravid *Aedes*. I performed both field and laboratory tests since I wanted to see the impacts of Okoumal attractancy effects at two different spatial scales. By calculating oviposition activity, I was able to see whether a mosquito was laying more eggs in the control or treatment oviposition traps. This first step allowed me to analyze whether Okoumal may or may not be an effective attractant to gravid female mosquitoes.

Materials & Methods

Laboratory Oviposition Choice Assay Methods

Replicate trials were conducted using 30 cm^3 enclosures (Fig. 1). Enclosures consisted of white polyester mesh netting (BioQuip Bug Dorm, Rancho Dominguez, California). For each trial, four enclosures were individually housed in four separate incubators to maintain independence. Each incubator was set at 25° C and 16:8 h light-dark cycle to mimic summer field conditions. Within each enclosure, two identical black oviposition cups were positioned in opposite corners. Each oviposition cup was filled with 400ml of rested tap water and lined with seed germination paper to provide a substrate on which female *Ae. albopictus* could oviposit eggs. One oviposition cup in each enclosure was randomly assigned as the treatment cup and received either a high (30 mg) or low dose (10 mg) of Okoumal. Doses were determined based on the range of those eliciting host-seeking feeding responses to *Ae. aegypti* mosquitoes in a prior enclosed laboratory experiment (USPTO, 2016). For each treatment cup, Okoumal was pipetted onto a 20 mm disk filter paper and paper-clipped to the cup side facing outward. The other oviposition cup in each of the four enclosures was assigned as the control oviposition cup and received a filter paper disk without the Okoumal dose. Two of the four enclosures in each trial were randomly assigned to have treatment cups with high doses while the other two enclosures received treatment cups with low doses.

Adult female *Ae. albopictus* in the laboratory assay were from $F_{1\text{-}2}$ colonies at the University of Maryland that had been established from field populations in Baltimore, Maryland. *Ae. albopictus* females were blood-fed to repletion on day 1 of

the trial and each enclosure received one blood-fed individual. One blood-fed female was released into a cage rather than a cohort so that female oviposition behavior would not be influenced by that of other females. *Ae. albopictus* mosquitoes prefer to lay eggs in containers with high conspecific larvae since the presence of larvae may indicate reliable food sources (Shragai et al., 2019). After 7 days, oviposition cups were retrieved from the cages and egg papers were stored under humid conditions for five days to ensure embryonation until they were hatched. Egg hatching was stimulated by flooding papers in a nutrient broth solution made with lactalbumin. Numbers of viable eggs (after hatching) on egg papers were counted and recorded rather than female visitations since *Ae. albopictus* are known to have skip oviposition behavior (Davis et al., 2015). Four replicate trials were conducted (i.e., blocks) over four weeks. For each trial, new oviposition cups were used, and all enclosures were cleaned with non-scented cleaner to ensure no Okoumal contamination among trials. High and low doses were rotated around each enclosure between trials to prevent confounding Okoumal dose with enclosure and incubator set up.

Because preliminary analyses indicated little attractancy to Okoumal on *Ae. albopictus* (see Results), we repeated the assay using a lower pair of Okoumal doses (1 mg and 3 mg) along a range that had shown attractancy to host-seeking females in prior laboratory trials (USPTO, 2016). The procedures for this repeated assay were the same as the first assay with higher Okoumal doses, except that Okoumal was mixed in an acetone solution to more accurately deliver the compound at the smaller doses. Control cups only received acetone without Okoumal. Overall, the two

oviposition-choice assays both compared two doses with controls (no Okoumal) across four replicate blocks.

Dose-Response Oviposition Field Study

A dose-response oviposition field study was conducted along the Paint Branch River in College Park, Maryland, USA (lat.: 38.99, lon.: -76.94) known to have populations of *Ae. albopictus* (Saunders & Leisnham, 2018). The study was conducted in summer (June-August) 2019, during peak *Ae. albopictus* activity in the region (Dowling et al., 2013). The study was conducted using oviposition traps consisting of standard 600 ml black plastic cups. For this study, Okoumal was delivered via pad lures, a synthetic inert lure produced from dental cotton rack and polytube by the Invasive Insect Biocontrol & Behavior Laboratory at the USDA Beltsville Agricultural Research Center (Fig. 2; Verhulst et al., 2016). Pad lures were placed in holed 15ml tubes and fixed on the outer perimeter of the oviposition cup with a rubber band. A 4x4 Latin square (Okoumal doses: 0 mg, 300 mg, 600 mg, and 900 mg) was randomly established and replicated across five sites. At each site, four oviposition traps were secured in at ground level and sheltered from direct sunlight and wind, which are conditions favorable for *Ae. albopictus* (Fig 3; Silver, 2008).

Traps were > 50 meters apart at each site, and sites were at least 150 meters apart to maintain spatial independence. *Ae. albopictus* are known to be poor flyers and can disperse as far as a few hundred meters from their breeding site but usually the dispersal distance is much less (Marini et al., 2019; Vavassori et al., 2019). At

each site, Okoumal treatment doses were randomly assigned to traps. Traps were serviced and Okoumal lures rotated every 7 days over a four-week sampling period for a total of 20 replicates per treatment dose (5 blocks x 4 weeks) and 80 total observations (5 x 4 x 4 treatment doses). During trap servicing, the contents of each experimental cup, including seed paper and any prematurely hatched larvae, were taken to the laboratory for processing. A new experimental cup was inserted into each holder cup, and the rotated Okoumal lure was fastened to the holder cup.

In the laboratory, seed germination papers were stored under humid conditions for five days to ensure egg embryonation, after which time they were flooded in a nutrient broth solution (lactalbumin powder: diluted 1:10 with distilled water) to hatch eggs. Collected and hatched larvae were raised using ideal conditions until they developed to late (3rd or 4th) instars where they were counted and identified to species level using a mosquito identification key (Darsie & Richard, 2016).

Statistical Analyses

Laboratory Oviposition Choice Assay Statistical Analysis

The oviposition activity index (OAI) was calculated to evaluate the response of gravid females to Okoumal (Ponnusamy et al., 2010). The OAI standardizes data by converting the number of eggs laid in the treatment cup to a proportion after correcting for the number of eggs laid in the control cup (Ponnusamy et al., 2010). For each trial, the OAI was calculated for each replicate as follows:

$$OAI = \frac{N_t - N_c}{N_t + N_c}$$

where N_t is the number of eggs laid in the treatment cup and N_c is the number of eggs laid in the control cup. For each female, an OAI greater than 0 indicates she oviposited more eggs in the treatment cup whereas an OAI less than 0 indicates she oviposited more eggs in the control cup (Ponnusamy et al., 2010). Wilcoxon Signed Rank Tests were used to test the hypothesis that the median OAI was significantly different than 0 for each dose. Kruskal-Wallis tests were used to test whether there was a difference in number of eggs laid in treatment cups between high and low dose conditions. All analyses were conducted using the JMP statistical program (JMP, 15.0. SAS Institute Inc., Cary, NC). For all analyses, experiment-wise $\alpha = 0.05$.

Dose-Response Oviposition Field Study Statistical Analysis

The effects of treatment (Okoumal dose) was tested on the numbers of *Ae. albopictus* viable (hatched) eggs using a generalized linear model (negative binomial) mixed model. Week, site, and trap location nested in site were included as random factors. Generalized linear mixed models with the same structure were also used to test the relationships of Okoumal on *Ae. japonicus* and *Ae. triseriatus* that were also collected but because these species were only collected in a small proportion of traps (see Results), a binomial link function was used to test the occurrence of oviposition. All analyses were conducted using the PROC GLIMMIX procedure on SAS statistical analysis software (SAS Institute 9.4, Cary, NC). For all analyses, experiment-wise $\alpha = 0.05$.

Results

Laboratory Oviposition Choice Assay

The OAI did not differ from 0 under any of the doses across both oviposition choice assays (W-values= -11.0-6.0, p-values= 0.1406-1.000; Fig. 4; Fig. 5), indicating no oviposition preference between treatment and control cups. There were also no differences in the percentage of eggs oviposited between higher and lower doses for either assay (z=0, p=1 and z=-0.64, p=0.5203, respectively; Figs. 4- 5).

Dose-Response Oviposition Field Study

A total of 3,057 mosquitoes were collected across the four trapping weeks, consisting primarily of 2,596 (85.0%) *Ae. albopictus*, 226 (7.4%) *Ae. japonicus,* and 232 (7.6%) *Ae. triseriatus*. Out of the 80 total observations, 77 contained *Ae. albopictus*, while 18 and 20 contained *Ae. japonicus* and *Ae. triseriatus*, respectively. There was no relationship between Okoumal dose on abundances of total mosquitoes ($F_{3,54}$=1.67, p=0.1835) (Fig. 6) or *Ae. albopictus* ($F_{3,54}$=1.21, p=0.3159) (Fig. 7). Nor were there any relationships of Okoumal on likelihood of *Ae. japonicus* ($F_{3,54}$=1.67, p=0.1869) and *Ae. triseriatus* ($F_{3,54}$=2.15, p=0.1044) oviposition.

Discussion and Conclusions

The choice of an oviposition site by adult female mosquitoes is under strong selection pressure, and individuals use a variety of chemical, environmental, and

olfactory cues to find favorable conditions (Navarro-Silva et al., 2009). Mosquito attractants have been used to manage mosquito populations by attracting adult females and subsequently trapping them or their offspring (Okumu et al., 2010; Andersen & Davis, 2014; Wooding et al., 2020). Preliminary studies have shown that the chemical compound Okoumal might be an attractant to both host-seeking and gravid *Aedes* females (USPTO, 2016; Saunders & Leisnham, 2018). The main goal of this chapter was to build on these preliminary studies and test Okoumal more rigorously using both field and laboratory trials. In this chapter, I investigated the attractancy of Okoumal to gravid *Ae. albopictus* females at two different spatial scales in both a laboratory oviposition choice assay and a dose-response field study but found little evidence that oviposition activity increased compared to control treatments.

It is often unclear at what distance a chemical compound might have semiochemical effects on a mosquito species (Wooding et al., 2020). Because of this, studies are often conducted at varying spatial scales. Laboratory-based oviposition choice studies are a cost-effective approach to test the effects of chemical compounds on adult females seeking a habitat to lay eggs (Ponnusamy et al., 2010). In this study, a laboratory choice assay explored whether Okoumal would attract gravid females that were given a choice to oviposit in containers that were close together, with doses ranging from 1 to 30 mg. This range of doses is similar to those that had been found to have been attractive to *Ae. albopictus* in a preliminary oviposition field study, which observed the greatest response at 50 mg among traps that were set 10 m apart in a single-location Latin Square gridded design (Saunders and Leisnham, 2018).

Saunders and Leisnham (2018) compared varying doses of Okoumal made from serial dilutions with deionized water over only one week in September, which is at the end of the summer period when mosquitoes are active. Saunders and Leisnham (2018) conducted their study when female mosquitoes were more likely seeking overwintering sites and laying diapause eggs (Roiz et al., 2011; Caminade et al., 2012). This could explain differences since my study was conducted under typical summer conditions (June-August). Once a female locates a potential oviposition site, she uses physical and chemical cues to evaluate water quality (Albeny-Simoes et al., 2014; Day, 2016; Segev et al., 2017). Thus, by adding Okoumal dilutions directly to water in an oviposition trap, Okoumal could have a chemotactile effect on egg-laying behavior. However, this is unlikely given that *Ae. albopictus*, like all *Aedes* mosquitoes, oviposit eggs on the sides of containers and rarely contacts the water surface when ovipositing (Silver, 2008). Further, Okoumal is hydrophobic and therefore its direct addition to traps through serial dilutions is likely to affect the consistency of its effect. The oviposition choice assay in this study used lures on the side of oviposition traps that could more easily be standardized and thus represented a more rigorous small-scale test of Okoumal attractancy to *Ae. albopictus* than Saunders and Leisnham (2008).

Perhaps the most compelling preliminary data demonstrating a semiochemical effect of Okoumal were from laboratory trials conducted by the Invasive Insect Biocontrol and Behavior Laboratory at USDA-ARS on *Ae. aegypti* (USPTO, 2016). In this study, the likelihood of host-seeking *Ae. aegypti* to take a blood meal were affected by Okoumal exposure. There might be a number of reasons why I did not see

similar effects of Okoumal on oviposition behavior in my choice assay here. First, the laboratory trials on *Ae. aegypti* used an experimental apparatus (i.e., Klun & Debboun (K&D) module, Klun & Debboun, 2000) to expose replicated cohorts of host-seeking *Ae. aegypti* to varying doses of just-treated Okoumal cloth strips placed over collagen membrane strips covering blood meals. Mosquito cohorts were either housed in 20 cm^3 enclosures directly in touch with the blood meal or in a wind tunnel that used human breath to attract them to the blood source. These experimental conditions were more likely to test Okoumal's efficacy as a stimulant to elicit the desired female behavior of taking a meal from a present blood source (USPTO, 2016). In contrast, for the choice assays in this study, I exposed free flying adult females to Okoumal versus control treatments to test the ability of Okoumal to attract gravid mosquitoes.

The second reason why I did not see similar semiochemical effects of Okoumal might be because I used *Ae. albopictus* instead *of Ae. aegypti*. *Ae. albopictus* and *Ae. aegypti* utilize similar container habitats in the field, frequently cooccur across numerous conditions in the field, and are generally considered ecologically similar urban mosquito species (Sharma et al., 2008). Prior laboratory studies have shown *Ae. albopictus* and *Ae. aegypti* to be attracted to similar semiochemicals that elicit oviposition, such as p-Cresol, which is a derivative of phenol (Wooding et al., 2020). Nevertheless, there may be key differences in the responses of each species to Okoumal that explain the lack of an effect of Okoumal on *Ae. albopictus* behavior in my assay compared to the positive effect of Okoumal observed on *Ae. aegypti*. Third, I used number of viable eggs laid in order to evaluate oviposition site selection. Even though eggs laid accurately indicate the oviposition

site, it says little about the attractants that result in oviposition at one site and act as a repellent or deterrent that prevented another oviposition site (Day, 2016).

For the field oviposition study, I used much greater doses of Okoumal (300-900 mg), to test the attractancy of Okoumal over a larger spatial scale that mimics the typical set-up of oviposition traps in the field which seek to attract mosquitoes away from surrounding habitat. Studies have shown that some sites such as water-filled artificial containers, tree holes, and flooded ditches can be detected by a female's olfactory signals of up to several meters when females are in flight looking to lay eggs (Day, 2016). As with the oviposition choice assay, I found no evidence of Okoumal attractancy to *Ae. albopictus* or co-occurring species, *Ae. japonicus* and *Ae. triseriatus* in the field oviposition study.

The lack of attractancy of Okoumal in both the choice assay and field experiment is likely underpinned by the interaction of the compound with the olfactory physiology of *Ae. albopictus*. Volatiles, such as semiochemicals, need to come in contact with specialized olfactory receptors (ORs) in order to elicit mosquito behavior (Leal et al., 2013; Wooding et al., 2020). Mosquitoes use olfactory receptor neurons (ORN) to detect attractants (Leal et al., 2013; Choo et al., 2018). The lack of attractancy could be that there was not enough Okoumal compound triggering the ORN or there could have been too much compound triggering receptors leading to disorientation and this may have offset any attractancy of the compound experienced when the mosquito was further away (Leal et al., 2013). In addition, maybe there was too much Okoumal triggering the ORN that elicited a repellency effect and offset attractancy (Hao et al., 2013; Ong et al., 2015; Choo et al., 2018).

It is also possible that Okoumal may repel mosquito oviposition at higher doses and have little effect at lower doses. In the laboratory oviposition assay, there seemed to be an apparent trend of decreasing OAI with increasing dose, possibly indicating a repellency effect of Okoumal at higher doses (Pearson correlation: $r = -0.37$, $p=0.0699$). The correlation between dose and OAI was likely nonsignificant due to a low sample size. Some studies have shown that spatial repellents and repellent-treated can impair and deter mosquito oviposition behavior (Bibbs & Kaufman, 2017). Over short distances some semiochemicals may act as stimulants or deterrents but over long distances, they may act as repellents or attractants (Cameron & Lorenz, 2013). In addition to repellents, mosquitoes also become disoriented by pyrethroid volatiles (Bibbs & Kaufman, 2017). Our findings are consistent with those in other studies that have found weaker or opposite effects of compounds at higher doses (Hao et al., 2013; Ong & Jaal, 2015). For example, one study has found that caproic acid, a known mosquito oviposition pheromone, attracted more gravid mosquitoes at 1 ppm (OAI= 0.32) compared to the control and the higher treatment dose of 100 ppm (OAI= 0.09) (Ong & Jaal, 2015). The specific biological mechanisms for weak attractancy or repellency of insects to compounds at higher doses is poorly understood but is thought to be related to an oversaturation of chemoreceptors resulting in a threshold effect (Hao et al., 2013).

Another study tested the effectiveness of the synthetic pheromone erythron-6-acetoxy-5-hexadecanolide as an attractant to *Culex quinquefasciatus* and found it was an attractant at most doses (Barbosa et al., 2007). However, at the highest dose of 1.56μg the assay had a negative OAI value (-0.82), which indicates a repellency

effect (Barbosa et al., 2007). Barbosa et al. (2007) believes the repellency effect at the highest dose occurred because the high pheromone might signal to the female that it is not a viable oviposition location since there might be possible resource competition with larvae already present in the area. It is possible, however, if I had used higher or lower doses, we may have seen different effects. Carbon dioxide is a commonly used compound to attract host-seeking female mosquitoes. One study conducted by Takken and Kline (1989) found that even when CO2 concentrations were increased from 200 to 1,000 cc/min., there was not a significant increase in the numbers of mosquitoes that were trapped.

To our understanding, this is the first study to rigorously test the effects of Okoumal as an attractant to gravid *Aedes* females. Okoumal is one of many pro-fragrance compounds available. For example, the chemical compounds Versalide and Phantolide have similar molecular weights and chemical structures to Okoumal and could be tested to see whether it is an attractant to female mosquitoes (National Center for Biotechnology Information, 2020). In addition to testing other synthetic chemicals similar to Okoumal, future studies should look at investigating other species of mosquitoes. In the oviposition field study, I was also able to test the responses of *Ae. triseriatus* and *Ae. japonicus* in addition to *Ae. albopictus*, but these species were in relatively low abundance and I was restricted to exploring only modeling the likelihood of an oviposition event.

The use of oviposition attractants can enhance the ability of ovitraps to detect mosquito populations and can help reduce and control localized populations of mosquitoes. Chemical cues play a vital role in the selection of oviposition sites for

female mosquitoes and when applied properly, could help aid in mosquito control and surveillance. Semiochemicals could be used as a tool in trapping methods to better detect mosquito populations and capture target mosquito species. Okoumal is known to be toxic to aquatic organisms but its toxicity has yet to be tested in addition to its attractancy in water (NCBI, 2020). As a first step, I tested Okoumal's attractancy out of water but did not find significant results. There is still a need to find an attractant that can replace plant infusions.

List of Figures

1. **Figure 1:** 30 cm^3 enclosures used in laboratory dose-response trials. Enclosures were housed in an incubator and had two oviposition cups (a control and treatment cup) in the corners of the enclosures. Filter papers were paper clipped to the outside of oviposition cups.
2. **Figure 2:** Okoumal cotton pad lures used for the field oviposition study. Okoumal cotton pads were placed inside 15ml holed tubes.
3. **Figure 3:** Black oviposition traps lined with seed germination paper and Okoumal lures rubber banded on outside of trap used in summer (June-August) 2019 field trials in an urban forested area.
4. **Figure 4:** Percentage of eggs laid in control versus treatment (10 and 30 mg of Okoumal) oviposition cups for Trial 1 including OAI values.
5. **Figure 5:** Percentage of eggs laid in control versus treatment (1 and 3 mg of Okoumal) oviposition cups for Trial 2 including OAI values.
6. **Figure 6:** Least square means ± standard error for total mosquito eggs collected based on Okoumal dose in summer (June-August) 2019 field trials in an urban forested area.
7. **Figure 7:** Least square means ± standard error for *Ae. albopictus* eggs collected based on Okoumal dose in summer (June-August) 2019 field trials in an urban forested area.

Figures

Figure 1.

Figure 2.

Figure 3:

Figure 4:

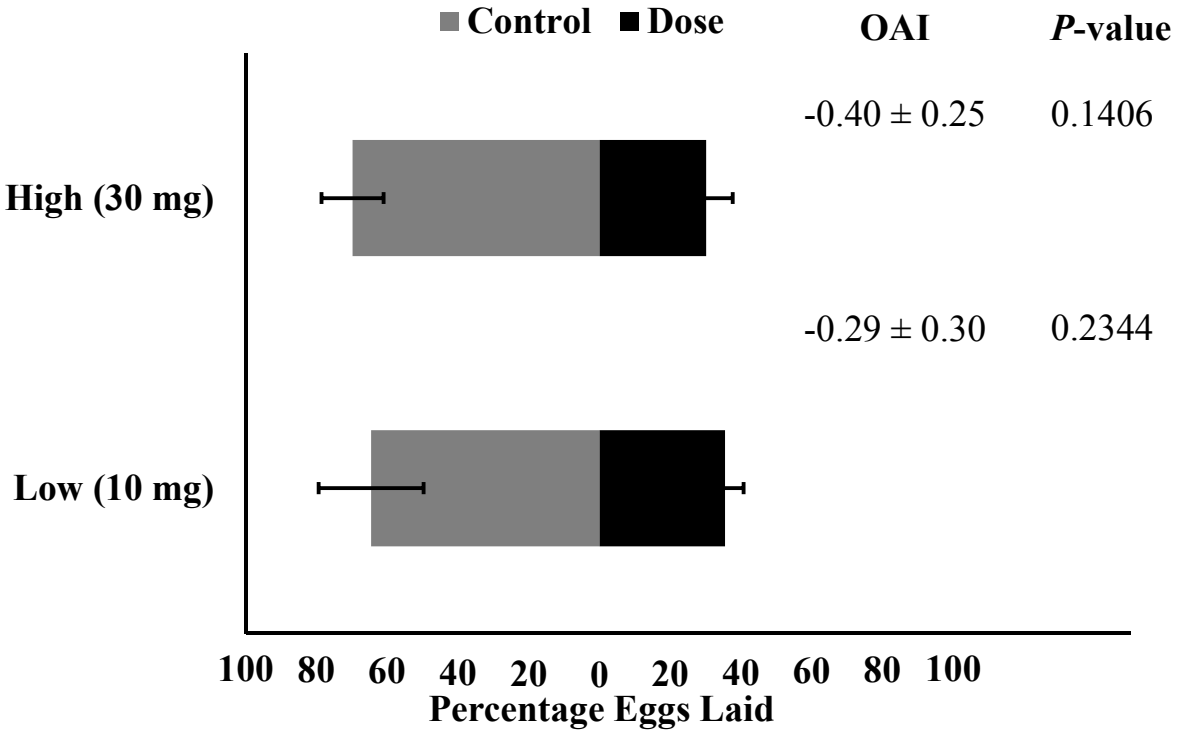

Control
Dose
OAI
P-value
-0.40 ± 0.25
0.1406
High (30 mg)
-0.29 ± 0.30
0.2344
Low (10 mg)
100 80 60 40 20 0 20 40 60 80 100
Percentage Eggs Laid

Figure 5:

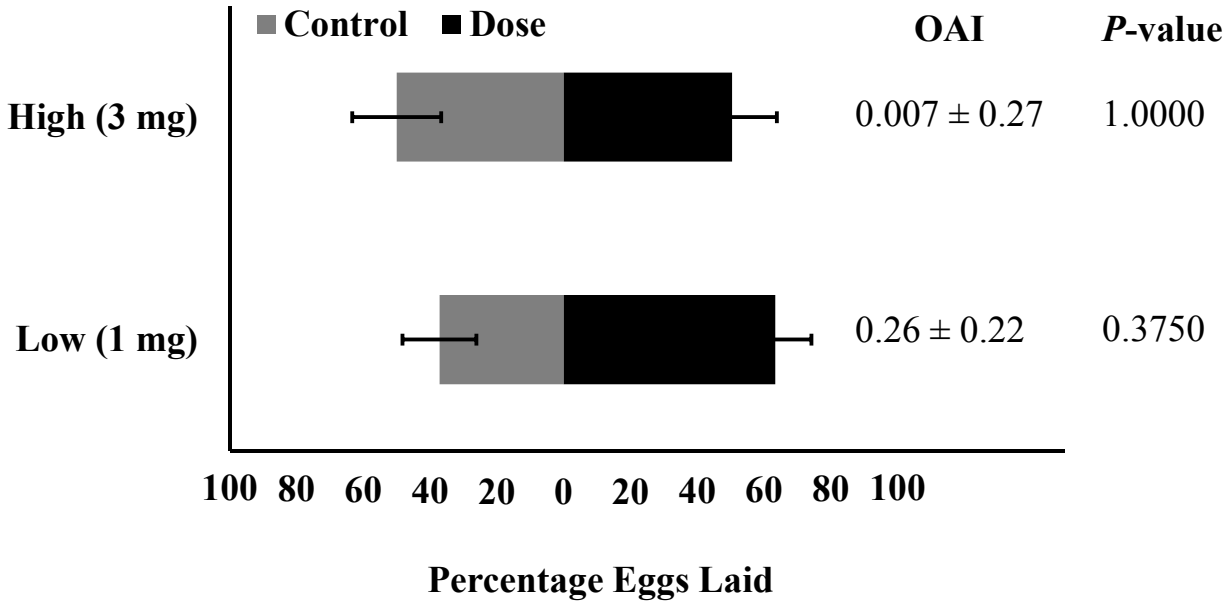

Control
Dose
OAI
P-value
High (3 mg)
0.007 ± 0.27
1.0000
Low (1 mg)
0.26 ± 0.22
0.3750
100 80 60 40 20 0 20 40 60 80 100
Percentage Eggs Laid

Figure 6:

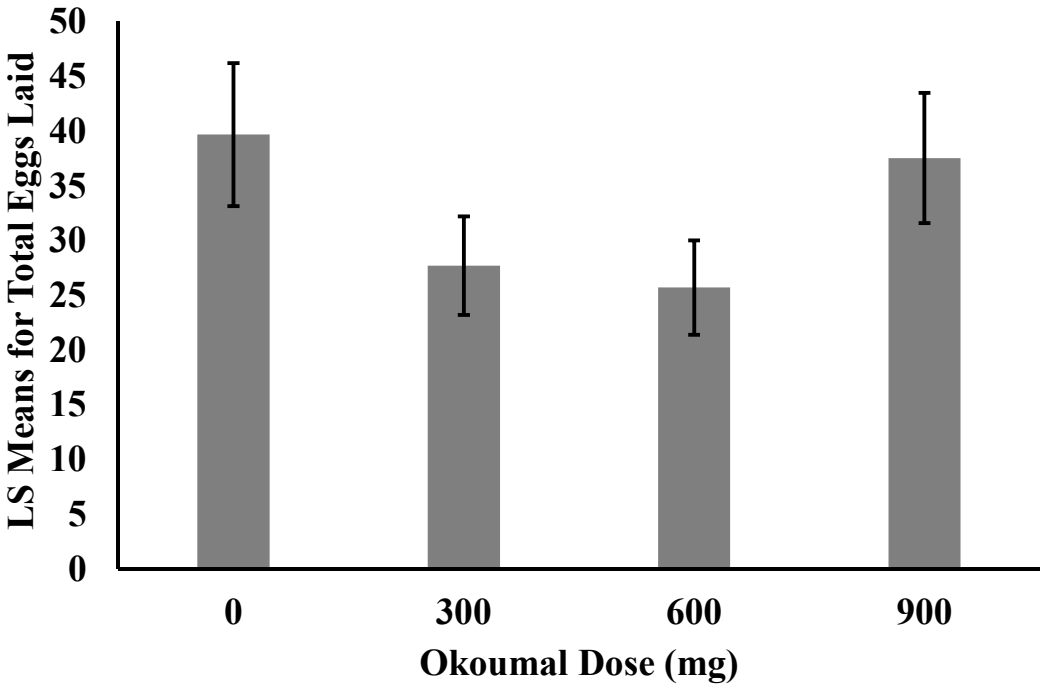
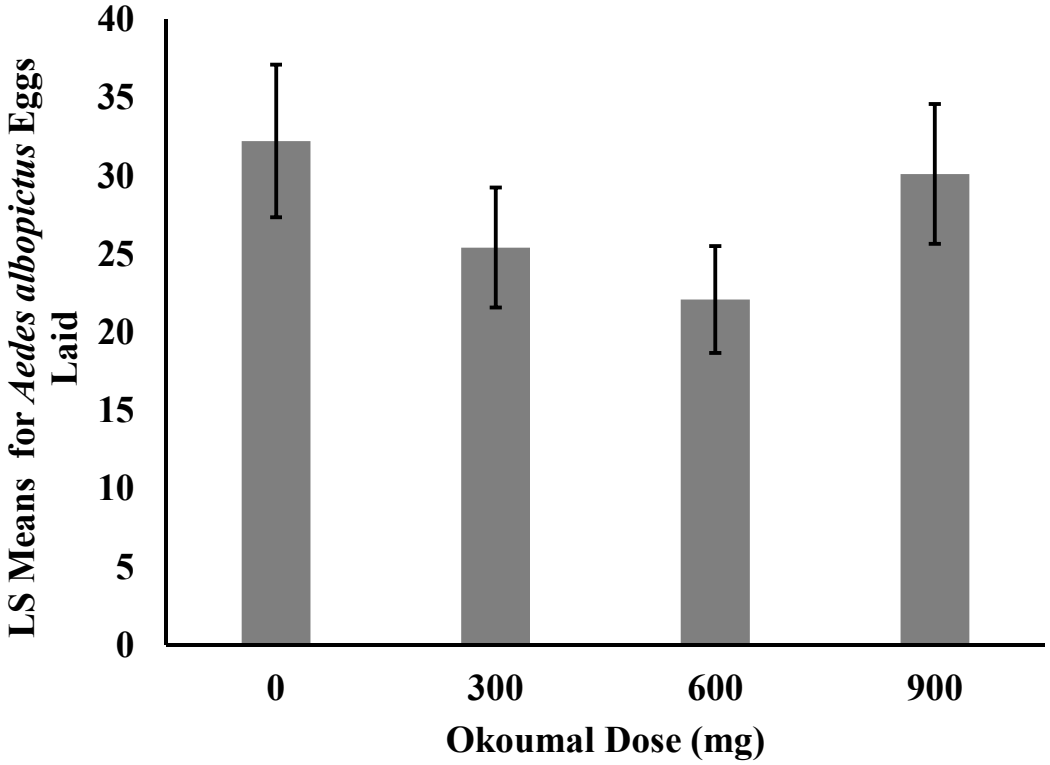

LS Means for Total Eggs Laid
50
45
40
35
30
25
20
15
10
5
0
0
300
600
900
Okoumal Dose (mg)

Figure 7:


LS Means for Aedes albopictus Eggs Laid
40
35
30
25
20
15
10
5
0
0
300
600
900
Okoumal Dose (mg)

References

Albeny-Simoes, D., E. G. Murrell, S. L. Elliot, M. R. Andrade, E. Lima, S. A. Juliano, and E. F. Vilela. 2014. Attracted to the enemy: *Aedes aegypti* prefers oviposition sites with predator-killed conspecifics. Oecologia 175: 481-492.

Anderson, E. M., and J. A. Davis. 2014. Field evaluation of the response of *Aedes albopictus* (Stegomyia albopicta) to three oviposition attractants and different ovitrap placements using black and clear autocidal ovitraps in a rural area of Same, Timor-Leste. Medical and Veterinary Entomology 28: 372-383.

Ariani, C. V., S. C. L. Smith, J. Osei-Poku, K. Short, P. Juneja, and F. M. Jiggins. 2015. Environmental and genetic factors determine whether the mosquito *Aedes aegypti* lays eggs without a blood meal. American Journal of Tropical Medicine and Hygiene 92: 715-721.

Barbosa, R. M. R., A. Souto, A. E. Eiras, and L. Regis. 2007. Laboratory and field evaluation of an oviposition trap for *Culex quinquefasciatus* (Diptera: Culicidae). Memorias Do Instituto Oswaldo Cruz 102: 523-529.

Benedict, M. Q., R. S. Levine, W. A. Hawley, and L. P. Lounibos. 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector-Borne and Zoonotic Diseases 7: 76-85.

Bentley, M. D., and J. F. Day. 1989. Chemical ecology and behavioral-aspects of mosquito oviposition. Annual Review of Entomology 34: 401-421.

Bibbs, C. S., and P. E. Kaufman. 2017. Volatile pyrethroids as a potential mosquito abatement tool: a review of pyrethroid-containing spatial repellents. Journal of Integrated Pest Management 8.

Cameron, M., and Lorenz. 2013. Biological and Environmental Control of Disease Vectors, CABI.

Caminade, C., J. M. Medlock, E. Ducheyne, K. M. McIntyre, S. Leach, M. Baylis, and A. P. Morse. 2012. Suitability of European climate for the Asian tiger mosquito *Aedes albopictus*: recent trends and future scenarios. Journal of the Royal Society Interface 9: 2708-2717.

Choo, Y. M., P. X. Xu, J. K. Hwang, F. F. Zeng, K. M. Tan, G. Bhagavathy, K. R. Chauhan, and W. S. Leal. 2018. Reverse chemical ecology approach for the identification of an oviposition attractant for *Culex quinquefasciatus*. Proceedings of the National Academy of Sciences of the United States of America 115: 714-719.

Clements, A. 1999. The Biology of Mosquitoes, vol. 2, CABI International.

Darsie, R., and R. Ward. 2016. Identification and Geographical Distribution of the Mosquitoes of North America, North of Mexico University Press of Florida.

Davis, T. J., P. E. Kaufman, J. A. Hogsette, and D. L. Kline. 2015. The effects of larval habitat quality on *Aedes albopictus* skip oviposition. Journal of the American Mosquito Control Association 31: 321-328.

Day, J. 2016. Mosquito Oviposition Behavior and Vector Control. Insects 7.

Dorm, B. B., Rancho Dominguez, California.

Dowling, Z., P. Armbruster, S. L. LaDeau, M. DeCotiis, J. Mottley, and P. T. Leisnham. 2013. Linking mosquito infestation to resident socioeconomic status, knowledge, and source reduction practices in suburban Washington, DC. EcoHealth 10: 36-47.

Dykstra, R. R., G. S. Miracle, and L. M. Gray inventors. 2009. Photo-labile pro-fragrance conjugates.

Hallem, E. A., A. Dahanukar, and J. R. Carlson. 2006. Insect odor and taste receptors, pp. 113-135, Annual Review of Entomology, vol. 51. Annual Reviews, Palo Alto.

Hao, H. L., J. C. Sun, and J. Q. Dai. 2013. Dose-dependent behavioral response of the mosquito *Aedes albopictus* to floral odorous compounds. Journal of Insect Science 13: 8.

JMP, S. I. I., Cary, NC.

Klun, J. A., and M. Debboun. 2000. A new module for quantitative evaluation of repellent efficacy using human subjects. Journal of Medical Entomology 37: 177-181.

Leal, W. S. 2013. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annual Review of Entomology, Vol 58 58: 373-391.

Leisnham, P. T., and S. A. Juliano. 2009. Spatial and temporal patterns of coexistence between competing *Aedes* mosquitoes in urban Florida. Oecologia 160: 343-352.

Lounibos, L. P. 2002. Invasions by insect vectors of human disease. Annual Review of Entomology 47: 233-266.

Luntz, A. J. M. 2003. Arthropod semiochemicals: mosquitoes, midges and sealice. Biochemical Society Transactions 31: 128-133.

Marini, F., B. Caputo, M. Pombi, M. Travaglio, F. Montarsi, A. Drago, R. Rosa, M. Manica, and A. della Torre. 2019. Estimating spatio-temporal dynamics of *Aedes Albopictus* Dispersal to guide control interventions in case of exotic arboviruses in temperate regions. Scientific Reports 9: 9.

Moore, C. G. 1999. *Aedes albopictus* in the United States: Current status and prospects for further spread. Journal of the American Mosquito Control Association 15: 221-227.

Mullen, G., and L. Durden. 2009. Medical and Veterinary Entomology, Academic Press.

National Center for Biotechnology Information. PubChem Database. CID=3034278, h. p. n. n. n. g. c. d. L. a. o. J., 2020).

Navarro-Silva, M. A., F. A. Marques, and J. E. Duque. 2009. Review of semiochemicals that mediate the oviposition of mosquitoes: a possible sustainable tool for the control and monitoring of Culicidae. Revista Brasileira De Entomologia 53: 1-6.

Okumu, F. O., G. F. Killeen, S. Ogoma, L. Biswaro, R. C. Smallegange, E. Mbeyela, E. Titus, C. Munk, H. Ngonyani, W. Takken, H. Mshinda, W. R. Mukabana, and S. J. Moore. 2010. Development and field evaluation of a synthetic mosquito lure that is more attractive than humans. PLOS One 5: 7.

Ong, S. Q., and Z. Jaal. 2015. Investigation of mosquito oviposition pheromone as lethal lure for the control of *Aedes aegypti* (L.) (Diptera: Culicidae). Parasites & Vectors 8: 7.

Paupy, C., H. Delatte, L. Bagny, V. Corbel, and D. Fontenille. 2009. *Aedes albopictus*, an arbovirus vector: From the darkness to the light. Microbes and Infection 11: 1177-1185.

Ponnusamy, L., N. Xu, K. Boroczky, D. M. Wesson, L. Abu Ayyash, C. Schal, and C. S. Apperson. 2010. Oviposition responses of the mosquitoes *Aedes*

aegypti and *Aedes albopictus* to experimental plant infusions in laboratory bioassays. Journal of Chemical Ecology 36: 709-719.

PT, S. M. a. L. October 2018. Testing an easily deployable mosquito attractant: promising results from initial field trials. Society of Vector Ecology 48t^{h} Annual Conference at Yosemite National Park.

Ray, A. 2015. Reception of odors and repellents in mosquitoes. Current Opinion in Neurobiology 34: 158-164.

Roiz, D., M. Neteler, C. Castellani, D. Arnoldi, and A. Rizzoli. 2011. Climatic factors driving invasion of the tiger mosquito (*Aedes albopictus*) into New Areas of Trentino, Northern Italy. PLOS One 6: 8.

SAS Institute 9.4, C., NC. SAS Institute 9.4, Cary, NC.

Saunders, M., and P. Leisnham. October 2018. Testing an easily deployable mosquito attractant: promising results from initial field trials. Society of Vector Ecology 48t^{h} Annual Conference at Yosemite National Park.

Segev, O., R. Verster, and C. Weldon. 2017. Testing the link between perceived and actual risk of predation: mosquito oviposition site selection and egg predation by native and introduced fish. Journal of Applied Ecology 54: 854-861.

Sharma, K. R., T. Seenivasagan, A. N. Rao, K. Ganesan, O. P. Agarwal, R. C. Malhotra, and S. Prakash. 2008. Oviposition responses *of Aedes aegypti* and *Aedes albopictus* to certain fatty acid esters. Parasitology Research 103: 1065-1073.

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: 10.

Silver, J. B., M. W. Service, and SpringerLink (Online service). 2008. Mosquito ecology field sampling methods, pp. xxi, 1494 p. Springer, Dordrecht, the Netherlands.

Takken, W., and D. L. Kline. 1989. Carbon-dioxide and 1-octen-3-ol as mosquito attractants. Journal of the American Mosquito Control Association 5: 311-316.

Takken, W., and N. O. Verhulst. 2013. Host preferences of blood-feeding mosquitoes, pp. 433-+. In M. R. Berenbaum (ed.), Annual Review of Entomology, Vol 58, vol. 58. Annual Reviews, Palo Alto.

Tatem, A. J., S. I. Hay, and D. J. Rogers. 2006. Global traffic and disease vector dispersal. Proceedings of the National Academy of Sciences of the United States of America 103: 6242-6247.

Tauxe, G. M., D. MacWilliam, S. M. Boyle, T. Guda, and A. Ray. 2013. Targeting a dual detector of skin and co2 to modify mosquito host seeking. Cell 155: 1365-1379.

USPTO inventor. Submitted December 30, 2016. Development of a novel type of chemical bait for mosquitoes. U.S. Provisional Patent Applications Serial #621440518.

Vavassori, L., A. Saddler, and P. Muller. 2019. Active dispersal of *Aedes albopictus*: a mark-release-recapture study using self-marking units. Parasites & Vectors 12: 14.

Verhulst, N. O., B. T. Weldegergis, D. Menger, and W. Takken. 2016. Attractiveness of volatiles from different body parts to the malaria mosquito *Anopheles coluzzii* is affected by deodorant compounds. Scientific Reports 6.

WHO. 2017. Vector-borne diseases.

Wong, J., S. T. Stoddard, H. Astete, A. C. Morrison, and T. W. Scott. 2011. Oviposition site selection by the dengue vector *Aedes aegypti* and its implications for dengue control. PLOS Neglected Tropical Diseases 5: 12.

Wooding, M., Y. Naude, E. Rohwer, and M. Bouwer. 2020. Controlling mosquitoes with semiochemicals: a review. Parasites & Vectors 13: 20.

Chapter 3: Testing the Potential of the Pro-Fragrance Compound, Okoumal, as a Toxin to *Aedes aegypti* Larvae Using Dose-Response Laboratory Experiments

Abstract

Mosquitoes are among the most prominent and medically important insects in the world, causing substantial public health concerns in many regions. *Aedes* spp. mosquitoes are arguably the most important invasive species in the United States and worldwide and can collectively vector a range of viruses, including West Nile, dengue, and Zika. The most common approach to reduce numbers of mosquito larvae has been through the reduction of aquatic habitat or the use of larvicides. However, habitat reduction and larviciding can be difficult to implement for mosquito control agencies. Recently, the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) has been exploring the potential of various pro-fragrance compounds, such as Okoumal, to attract *Aedes* females and kill their offspring larvae. The overall objective of this study was to evaluate the efficacy of Okoumal to act as a toxin to *Aedes* larvae. I conducted dose-response trials to test the efficacy of Okoumal as a toxin to *Ae. aegypti* larvae and found that higher Okoumal concentrations resulted in lower *Ae. aegypti* per capita rate of population change and survival, and longer development times. Okoumal's negative effects on *Ae. aegypti* were reduced when larvae were exposed to applications that had been sitting in habitats for two weeks. The results indicate early promise that Okoumal is toxic to

Aedes mosquito larvae and negatively affects the population performance of the species, but that its negative effects may be reduced two weeks after application.

Introduction

Vector-borne diseases have had an overwhelming impact on public and animal health throughout history. According to the World Health Organization (WHO), 17% of infectious diseases are vector-borne, resulting in more than 700,000 human deaths annually and substantial economic impacts on livestock (WHO 2017). Infectious disease vectors can circulate solely among animals, solely among humans, or from animals to humans (WHO 2017). Mosquitoes are among the most prominent and medically important insect disease vectors around the world, causing substantial public health concerns in many regions.

The processes that occur at developmental (eggs, larval) life stages of mosquitoes can predict the distribution and abundances of adult mosquitoes (Juliano, 2009). Larval densities in developmental habitats, such as artificial water-filled containers, are influenced by many biotic and physical factors, such as temperature, environmental toxins, predation, and resource competition (Clements, 1999). Almost all literature on how mosquitoes respond to environmental toxins is focused on commercial or chemicals that are easily obtainable to act as insecticides or larvicides to control for vector mosquito species (Floore, 2006).

Aedes aegypti (L)*,* more commonly known as the yellow fever mosquito, is the primary vector of dengue, yellow fever, and chikungunya virus transmissions (Guzman and Kouri 2002). *Ae. aegypti* is more anthropophilic than other mosquito

species with more catholic feeding patterns, thus, posing a higher risk of spreading disease (McMeniman et al., 2009). *Ae. aegypti* was first introduced to the Western Hemisphere on cargo ships in the 1800s (Reiter 2001; Slosek 1986). *Ae. aegypti* are commonly found in Asia, South America, and Africa (Nene et al., 2007). *Ae. aegypti* utilize water-filled containers for their developmental stages (eggs, larvae, and pupae) (Reiter 2001). Controlling *Ae. aegypti* can be difficult due to its liking of being close to humans as well as how *Ae. aegypti* readily locates open containers for its larvae (Gibbons and Vaughn 2002). *Ae. aegypti* larvae and indoor adults are typically controlled by insecticides, however, they have developed a resistance to insecticides, making it difficult to control their populations (Vontas et al., 2012).

Mosquito populations are largely regulated at the immature stage (e.g., mosquito larvae or mosquito pupae). Eliminating the number of water-filled containers (known as source reduction) that mosquitoes can utilize to breed and develop can greatly help manage *Ae. aegypti.* With source reduction, the general population needs to be educated on how to be effective in reducing habitats as well as be motivated to implement these practices (Dowling et al., 2013). Since the primary source of *Ae. aegypti* containers are in residential homes, education about source reduction is of the utmost importance in order to eliminate water containers (Fonseca et al., 2013). The World Health Organization (WHO) recommends source reduction to control urban mosquito vector species since it can be a cost-effective approach (WHO, 1997). Source reduction can be difficult to manage and control for public health agencies since many of these artificial containers are found on private, residential property. This makes it difficult to manage whether or not residents are

controlling and reducing the numbers of artificial or water filled containers on their properties.

Removing water-filled containers that could potentially host mosquito larvae is typically the first step in source reduction but if water in a container cannot be emptied or removed, larvicides are used (Marcombe et al., 2014). Organophosphates such as temephos (a chemical compound that has a low toxicity level to mammals) or bacterial toxins, such as *Bacillus thurungiensis* var. *israelensis (Bti),* can easily be deployed into mosquito breeding sites, targeting mosquito larvae to kill them (Vontas et al., 2012). Insecticide-based interferences have controlled invasive *Aedes* mosquitoes for quite some time, but the mosquitoes have started to develop a resistance to the current insecticides used, so there is a need to investigate other chemicals that could potentially be toxicants to mosquito larvae (Vontas et al., 2012).

Mosquito control and surveillance can be controversial, especially when it uses biological controls or larvicides since it can raise human health and environmental concerns (Dowling et al., 2013). With globalization and urbanization occurring at a rapid pace, invasive mosquito species are more readily able to spread into new areas and cause prolific vector-borne disease cases worldwide, and effective management and control strategies need to be employed to better eradicate this insect since they are of public health concern. The USDA has recently patented a chemical compound called [2,4-Dimethyl-2-(5,5,8,8-tetramethyl-6,7-dihydronaphthalen-2-yl)-1,3-dioxolane], commonly called Okoumal, as a mosquito attractant (USPTO, 2016). Okoumal has a core structure containing acetals and ketals of oxygenated sesquiterpenes, and it has shown to be an attractant to host-seeking *Aedes* mosquitoes

in laboratory trials and an attractant to gravid females in field trials (USPTO, 2016; Saunders & Leisnham, 2018). Okoumal is a chemical compound that is commercially available. It is used in the fragrance industry and has a woody aroma.

In this study, the effects of Okoumal on the survival and development of *Ae. aegypti* was examined using controlled laboratory dose-response study. Okoumal, a pro-fragrance compound, is known to be toxic to aquatic organisms (National Center for Biotechnology Information, 2020). In this study, we will examine the effects of Okoumal and investigate the per capita rate of population change (r, Goldberg & Fleetwood, 1987). By calculating the finite rate of population change (λ'), the population performance of mosquito populations can be estimated from laboratory experiments. λ is a composite measure of population performance that incorporates survivorship, development time, and fecundity, and is superior to only looking at survivorship when determining population-level effects of environmental conditions on species (Livdahl and Sugihara, 1984).

Calculating λ' helps account for nonlinear interactions between different fitness parameters and is a more biologically sound measure of population performance (Leisnham and Juliano, 2009). Prior studies that investigated λ' produced different conclusions for survival and λ' of a species, highlighting the importance of calculating λ' for this study (Livdahl and Sugihara, 1984; Leisnham and Juliano, 2009). Experiments that consider only individual fitness parameters yield limited inference about population performance (Kesavaraju et al., 2014). By calculating λ', I was able to determine the varying effects Okoumal had on mosquito

ecology. This toxicity experiment was the first step needed in order to test the efficacy of Okoumal as being a mosquito larval toxin.

Materials & Methods

Laboratory-based dose-response experiments were performed to test the effect of Okoumal on important fitness parameters and overall population performance on *Ae. aegypti* larvae. Standardized 10x5x5 mm cubic teak woodchips (Fig. 1; Chemveda, Hyderabad, India) were chosen as a substrate to deploy Okoumal, following prior studies that have used similar materials in attractive lures for mosquitoes and is also a natural carrier material (Rapley et al., 2009). Treated woodchips were prepared by the Invasive Insect Biocontrol and Behavior Laboratory at USDA-ARS in Beltsville, Maryland. They were treated by soaking teak woodchips in an Okoumal: hexane solution of known concentration to saturation, and then exposed to slow evaporation to yield a substrate whose Okoumal concentration could be easily manipulated, easily stored, and straightforwardly deployed in the field.

Treated woodchips were prepared to have an Okoumal concentration of 200 mg Okoumal per 1 g woodchips, which will allow expected high doses from a reasonable number of woodchips. In each single-species experiment, a total of 78 800 mL cups received 750 mL of distilled water and 30 woodchips. The proportion of treated: control (i.e., untreated) chips in each cup (0:30, 2:28, 5:25, 10:20, 20:10, 30:0) was altered to vary the dose of Okoumal while controlling for woodchip number. Ten woodchips weigh 1.36 ± 0.03 grams. Therefore, the proportions of

treated: control woodchips in these experiments will yield the following concentrations: 0mg/L, 72 mg/L, 180 mg/L, 360 mg/L, 720 mg/L, and 1080 mg/L.

Each cup was randomly assigned a concentration, with fourteen replicate cups for concentrations 0mg/L, 72 mg/L, 180 mg/L, 360 mg/L, 720 mg/L, and eight replicates for concentration 1080 mg/L. All cups were provisioned with 1 mg of 1:1 bovine liver and lactalbumin powder (diluted 1:10) and housed in an incubator at 25°C and 16:8 h light-dark cycle. Lactalbumin powder was added into beakers in order to limit resource competition and mimic field conditions. Two days after the cups were set up, newly hatched larvae were added. *Ae. aegypti* eggs were sourced from $F_{1\text{-}2}$ colonies in the Leisnham Lab at the University of Maryland and were hatched in nutrient broth solutions.

Within 24 hours, 20 first instar larvae were randomly assigned to half of the replicate cups of each Okoumal concentration cup. After 14 days, newly hatched larvae were added to the second half of the cups to constitute a second run of the experiment that would test the effect of Okoumal after woodchips had soaked for two weeks. All cups were checked daily and pupae were removed from cups and placed into individual vials until they emerged into adults. Every seven days after the start of the experiment, 1 mg of 1:1 bovine liver and lactalbumin liver powder (diluted 1:10) were added to cups weekly to mimic regular nutrient additions to habitats in the field and limit resource competition. Once pupae emerged into adults, the date of emergence were recorded and adults were dried (>24 h, 50 °C), sexed, and had their wing-lengths measured.

Once pupae emerged into adults, the date of emergence was recorded and adults were dried (>24 h, 50 °C), sexed, and had their wing-lengths measured. For each cup, proportion survivorship, median female development time, and median female mass was calculated. These fitness parameters were used to calculate λ', a composite index of population performance based on *r',* which estimates the realized per capita rate of population change ($dN/N\,dt = r$, the exponential growth rate) for each replicate cohort (Livdahl and Sugihara, 1984):

$$\lambda' = \exp\left[\frac{\ln\left[(1/N_0)\sum_x A_x f(w_x)\right]}{D + \left[\sum_x xA_x f(w_x) \Big/ \sum_x A_x f(w_x)\right]}\right]_0$$

where N_0 is the initial number of females (assumed to be 50% per cup), x is the median time to eclosion (measured in days), A_x is the median number of females eclosing on day x, w_x is the median body size on day x, and $f(w_x)$ is a function describing size dependent fecundity for each species, estimated from the mean wing length on day x,wx of female mosquitoes (Livdahl and Sugihara 1984). The function for *Ae. aegypti* is $f(wx) = (1/2)\exp[4.5801 + 0.8926(\ln wx)] - 1$ (Grill and Juliano 1996). D is the mean days it takes for an adult mosquito to mate, blood feed, and oviposit, and is estimated at 12 days for *Ae. Aegypti* (Grill and Juliano 1996).

Keeping the numbers of woodchips constant across all doses controlled for any effects that woodchips themselves might have on the survival and development of *Ae. aegypti* larvae. Nevertheless, it is still important to determine if woodchips had

any effect on larval to more accurately assess the impacts of Okoumal. Therefore, in addition to the trials above, we conducted a separate experiment to test for the effects of woodchips on Ae. aegypti survival and development. Eight replicate cups received 20 newly hatched (<24 h) first-instar *Ae. aegypti* larvae and exposed to the same controlled conditions as in the trials above. However instead of receiving varying proportions of treated: control woodchips, 4 cups received 30 control (i.e., untreated) woodchips and 4 cups received no woodchips. After seven days, numbers of surviving larvae and the mean instar of survivors was calculated for each cup. I found no effect of woodchips on either the survival (t-test: t=-0.63, p=0.5504, df=6) or mean instar (t-test: t=-0.15, p=0.8890, df=6), suggesting that woodchips were an appropriate substrate for the dose-response trials.

Statistical Analyses

We tested the effect of Okoumal (0, 300, 600, 900 mg), soaking time (immediate, delayed), and their interaction on proportion survivorship (male and female separately), wing length (male and female separately), development time (male and female separately) using linear models with a Gaussian distribution with the PROC GLIMMIX procedure (SAS Institute 9.4, Cary, NC). λ' did not meet normality or homogeneity of variances despite transformations; therefore, we tested for effects of Okoumal, soaking time, and their interaction using a randomization test (Cassell, 2011). In all models, dose was treated as categorical since preliminary data analysis saw a nonlinear relationship of each response with dose. Post hoc tests for different concentration levels of Okoumal were conducted using a Bonferroni

correction for delayed and immediate soaking times. For all analyses, experiment-wise $\alpha = 0.05$.

Results

There were significant main effects of both dose and soaking time, as well as significant interactions between dose and soaking time for λ', female survivorship and male survivorship, female and male development time, and female body size (Tables 1 & 2, Figs 3 – 8). λ,' male survivorship, and female survivorships all decreased with increasing Okoumal dose, especially when larval cohorts were exposed to Okoumal that had been recently added than Okoumal compared to Okoumal that had been sitting for two weeks (Figs. 3-5). Female and male development time was also longer in cups where Okoumal had been recently added at all by one Okoumal dose in which there were survivors (Figs. 6-7). Development time did not vary with Okoumal dose for either sex in cups in which Okoumal had been sitting (Table 2; Figs. 6-7). Female body size did not vary with Okoumal dose or sitting time, except at 180 mg/L, where it was lower for larval cohorts in cups where Okoumal had been recently added (Fig. 8). There was no effect of dose, sitting time, or their interactions for male body size (Table 2; Fig. 9).

Discussion and Conclusions

The cooccurrence of invasive mosquito species and human populations has become more commonplace with anthropogenic changes and the increasing spread of

this pestiferous species worldwide. Due to the importance of mosquitoes to public health, it is vital to study this vector in order to control and monitor its spread. There is a need for a synthetic chemical that is easily deployable and that can act as both an attractant and toxin to mosquito species. The results of this dose-response study clearly showed that higher Okoumal concentrations resulted in lower Ae. aegypti per capita rate of population change and survival, and longer development times. This study showed that Okoumal can act as an important toxin.

Okoumal effects on *Ae. aegypti* larvae may be through both direct and non-direct pathways. Okoumal may be directly lethal to larvae to negatively affect survival rates, or it might negatively affect survival indirectly by reducing microbial food resources. Likewise, Okoumal may indirectly have non-lethal effects on mosquito body size and development time, and selection for larvae that have specific genotypes, such as smaller individuals that develop more quickly. Possibly indirect effects include availability on microbial food. The availability of food can influence possible mechanisms by which Okoumal could affect different fitness parameters in unison. In this study, treatments with low survival rates had higher median body sizes and faster development times, suggesting that either surviving larvae had more food resources that promoted growth and development or that inherently larger and faster developing larvae were more likely to survive. Since Okoumal is a known toxin to aquatic organisms, I wanted to test the efficacy of it being a toxin to mosquito larvae (NCBI, 2020). However, if Okoumal were added to habitats in the field, it could impact non-target organisms. In order for Okoumal to be an effective toxin targeting only mosquito larvae, it would need to be added to artificial container habitats in

urban areas that have known concentrations of mosquito larvae and no other organisms present. Okoumal should not be added to large bodies of water since there might be negative effects on non-target organisms.

Okoumal effects on *Ae. aegypti* larvae may be through both direct and non-direct pathways. Okoumal may be directly lethal to larvae to negatively affect survival rates, or it might negatively affect survival indirectly by reducing microbial food resources. Likewise, Okoumal may indirectly have non-lethal effects on mosquito body size and development time, and selection for individuals that have specific genotypes (smaller mosquitoes developed quicker). Indirect effects included availability on microbial food. The availability of food can influence possible mechanisms by which Okoumal could affect different fitness parameters such as survival, body size, development time, and λ'. Treatments with low survival rates had higher median body size and faster development times. Since Okoumal is a known toxin to aquatic organisms, I wanted to test the efficacy of it being a toxin to mosquito larvae (NCBI, 2020). However, if Okoumal were added to habitats in the field, it could impact non-target organisms. In order for Okoumal to be an effective toxin targeting only mosquito larvae, it would need to be added to artificial container habitats in urban areas that have known concentrations of mosquito larvae and no other organisms present. Okoumal should not be added to large bodies of water since there might be negative effects on non-target organisms.

There was not a significant main effect based on dose for male body size. In this laboratory experiment, the mean λ'was over 1.0, which indicates that there was a positive population growth from 0 to 180 mg/L of Okoumal but both λ' and survival

steeply declined to zero from 360 to 1080 mg/L of Okoumal (Fig. 9). This result combined with the strong association between *Ae. aegypti* and Okoumal concentrations in the laboratory, suggests that *Ae. aegypti* is likely to be negatively affected by Okoumal under many field conditions. Variations in λ'of *Ae. aegypti* as a result of Okoumal doses appears to be primarily driven by survival. Interestingly, however, despite a steep decline in mean *Ae. aegypti* survival from 180 mg/L and 1080 mg/L of Okoumal, *Ae. aegypti* λ' remained unchanged (Fig. 3; Fig. 4). This result was probably due to a small increase in female and male body size from 72 to 180 mg/L of Okoumal (Fig. 7; Fig. 8).

Previous studies looking at the toxicological effects of compounds to mosquito species typically look at survival which gives limited inference on population performance for multiple generations. By calculating the finite rate of population change (λ'), the population performance of mosquito populations can be estimated from laboratory experiments. λ' incorporates survivorship, development time, and fecundity, and is superior to only looking at survivorship when determining population-level effects of environmental conditions on species. Although there were similar relationships of Okoumal dose and soaking time with both *Ae. aegypti* λ' and female survival in my study here, prior studies investigating have shown varying conclusions for these two variables, reiterating the importance of calculating λ' in order to better understand environmental impacts on populations (Livdahl & Sugihara, 1984). Since λ' accounts for non-linear interactions between body size, development time, and survival, it is a more meaningful measurement of population

performance compared to investigating each of these fitness parameters individually (Livdahl & Sugihara, 1984; Villena et al., 2017).

Moreover, the body size of emerging adult females is an important parameter to investigate in addition to survival since the adult life-stage is of public health importance and body size has been shown to affect a female mosquito's vectorial capacity. For example, a recent study has shown that smaller female Aedes mosquitoes have a reduced likelihood of transmitting dengue virus since they have a shorter lifespan and live out the incubation period to be infectious (Juliano et al., 2014). For many adult mosquito species, a larger adult body size is also related to the survival and dispersal, which are two additional attributes that likely affect disease dynamics (Hawley, 1985; Briegel & Timmermann, 2001; Smith et al., 2012; LaDeau et al., 2015).

The results of this study suggest that with higher doses of Okoumal, body size decreases which may alter disease transmission since smaller females were produced and do not transmit diseases as effectively as larger mosquitoes. Since Okoumal seems to be an effective toxin to *Ae. aegypti* larvae, there could be a potential for it to be used in "bait and kill" traps. In Chapter 2, I tested the efficacy of Okoumal as an attractant to gravid female *Aedes* mosquitoes using both field and laboratory trials. However, from this trial conducted in Chapter 2, I did not see any effect of Okoumal as an attractant to *Aedes* mosquitoes. Due to the lack of an attractancy effect of Okoumal, it is not appropriate to be used in a "bait and kill" trap, even though it is toxic to *Aedes* larvae. Although Okoumal was a toxin to mosquito larvae, it may have degraded over time due to volatilization effects. Due to the volatilization effects of

Okoumal, it might not be effective in "bait and kill" traps since the efficacy of it as a toxin also degrades over time. Other materials need to be investigated that can act as both an attractant and toxin to mosquitoes to aid in mosquito control and surveillance.

Plant infusions are commonly used in oviposition traps in order to enhance the attractancy effect of the trap (Silver, 2008). There is a potential for Okoumal to be added to oviposition traps baited with plant infusion to act as an effective "bait and kill" trap. However, plant infusions are messy and require maintenance, so there is a need to find a synthetic material that can be used in a "bait and kill" trap (Silver, 2008). Prior studies have investigated the potential of natural toxins to be used in "bait and kill" traps which could be a potential substitute for Okoumal.

A study conducted by Carrieri et al. (2009) investigated the use of a known mosquito larval toxin, *Bacillus thurungiensis* var. *israelensis (Bti)* in oviposition traps. From this study, they saw that *Bti* was an attractant to ovipositing *Ae. albopictus* mosquitoes and was also acting as a toxin to the offspring larvae (Carrieri et al., 2009). This study also saw that *Bti* (B_{14}) baited oviposition traps collected about 17.4% more eggs compared to the control traps used in the study (Carrieri et al., 2009). *Bti* could potentially be used in "bait and kill" traps because it is not a toxin to humans, deeming it potentially safer than other insecticides and larvicides that are used in mosquito control (WHO, 2009). A study conducted by Ponnusamy et al. (2008) found that cues associated with microorganisms found in plant infusions directed gravid *Ae. aegypti* to deposit more than 90% of their eggs into the ovitraps enriched with microbes.

Although *Bti* can potentially be used in "bait and kill" traps, it may be difficult for mosquito control agencies to use in developing countries since it is expensive to implement (Federici et al., 2003). Another mosquito larvae toxin that has been investigated by researchers is Spinosad, an insecticide based on chemicals found in the bacterial species *Saccharopolyspora spinosa* (Thomposon et al., 2000). Spinosad is an insecticide that is naturally derived (Perez et al., 2007). A study conducted by Perez et al. (2007) saw that there was a weak but significant attractancy effect of Spinosad for gravid *Ae. aegypti* at a concentration of 20 ppm but not at 5 ppm.

Future studies can investigate compounds similar to Okoumal to be used in "bait and kill" traps. For example, the chemical compounds Versalide and Phantolide have similar molecular weights and chemical structures to Okoumal and could be tested to see whether it is an attractant to female mosquitoes (NCBI, 2020). In addition to testing other synthetic chemicals similar to Okoumal, future studies should look at investigating other species of mosquitoes since I only investigated *Ae. aegypti* larvae in this study. In addition to testing different species, future studies can complement the laboratory trials with field trials to see whether there is an effect in field conditions.

To our understanding, this is the first study to test the effects of Okoumal on the population performance of mosquitoes. The goal of this study was to test the efficacy of Okoumal to act as a toxin to *Ae. aegypti* mosquito larvae, and I saw that with an increasing dose of Okoumal, there was a decrease in both male and female survivorship, indicating that Okoumal was a toxin to larvae. From this study, I was able to see that population performance was heavily driven by both male and female

survivorship. I also saw that with higher doses of Okoumal, female body size decreased which is important since smaller females are less likely to transmit diseases (Hawley, 1985; Briegel & Timmermann, 2001; Smith et al., 2012; LaDeau et al., 2015). Although Okoumal was a toxin to larvae, there is still a need to find a synthetic attractant to be used in a "bait and kill" trap to enhance mosquito control and surveillance strategies.

List of Tables

1. **Table 1:** Least squares linear models for estimated finite rate of increase and proportion survivorship of female and male *Ae. aegypti* in response to the independent variables Okoumal dose and the time.
2. **Table 2:** Least squares linear models for development time and wing length of female and male *Ae. aegypti* in response to the independent variables Okoumal dose and the time.

Tables

Table 1. Least squares linear models for estimated finite rate of increase and proportion survivorship of female and male *Ae. aegypti* in response to the independent variables Okoumal dose and the time.

	Estimated finite rate of increase, λ'			Survivorship					
				Female			*Male*		
Source	df	F	P	df	F	P	df	F	P
Okoumal dose	5, 62	15162.8	**<0.0001**	5, 62	39.26	**<0.0001**	5, 60	39.07	**<0.0001**
Soaking time	1, 62	20066.7	**<0.0001**	1, 62	66.56	**<0.0001**	1, 60	28.97	**<0.0001**
Okoumal dose*Exposure	5, 62	6834.2	**<0.0001**	5, 62	5.65	**0.0002**	5, 60	5.87	**0.0002**

Table 2. Least squares linear models for development time and wing length of female and male *Ae. aegypti* in response to the independent variables Okoumal dose and the time.

	Development Time						Body Size					
	Female			Male			Female			Male		
Source	df	F	P	df	F	P	df	F	P	df	F	P
Okoumal dose	4, 46	9.74	**<0.0001**	4, 43	19.97	**<0.0001**	4, 46	11.26	**<0.0001**	4, 43	0.88	0.4858
Soaking time	1, 46	54.95	**<0.0001**	1, 43	130.57	**<0.0001**	1, 46	44.98	**<0.0001**	1, 43	1.59	0.2141
Okoumal dose * Exposure	2, 46	21.29	**<0.0001**	3, 43	23.88	**<0.0001**	2, 46	28.09	**<0.0001**	3, 43	1.96	0.1341

List of Figures

1. **Figure 1:** Treated Okoumal-soaked teak woodchips used in laboratory dose-response trials.
2. **Figure 2:** Cups filled with 750 ml distilled water, 20 *Ae. aegypti* larvae, lactalbumin larval food, and Okoumal teak woodchips.
3. **Figure 3.** Mean ± SE *Ae. aegypti* per capita rate of population change to varying concentrations of Okoumal (immediate and delayed addition of larvae). Inset graph shows the variation among Okoumal concentrations 0 to 720 mg/L. Markers that do not share the same letter show significant differences ($P<0.05$). Lowercase letters denote differences for immediate addition of larvae and uppercase letters denote differences for delayed addition of larvae.
4. **Figure 4:** Mean ± SE *Ae. aegypti* female survivorship exposed to varying concentrations of Okoumal (immediate and delayed addition of larvae) in dose-response laboratory experiments. Markers that do not share the same letter show significant differences ($P<0.05$). Lowercase letters denote differences for immediate addition of larvae and uppercase letters denote differences for delayed addition of larvae.
5. **Figure 5:** Mean ± SE *Ae. aegypti* male survivorship exposed to varying concentrations of Okoumal (immediate and delayed addition of larvae) in dose-response laboratory experiments. Markers that do not share the same letter show significant differences ($P<0.05$). Lowercase letters denote

differences for immediate addition of larvae and uppercase letters denote differences for delayed addition of larvae.

6. **Figure 6:** Mean ± SE *Ae. aegypti* female development time exposed to varying concentrations of Okoumal (immediate and delayed addition of larvae) in dose-response laboratory experiments. Markers that do not share the same letter show significant differences ($P<0.05$). Lowercase letters denote differences for immediate addition of larvae and uppercase letters denote differences for delayed addition of larvae.
7. **Figure 7:** Mean ± SE *Ae. aegypti* male development time exposed to varying concentrations of Okoumal (immediate and delayed addition of larvae) in dose-response laboratory experiments. Markers that do not share the same letter show significant differences ($P<0.05$). Lowercase letters denote differences for immediate addition of larvae and uppercase letters denote differences for delayed addition of larvae.
8. **Figure 8:** Mean ± SE *Ae. aegypti* female body size exposed to varying concentrations of Okoumal (immediate and delayed addition of larvae) in dose-response laboratory experiments. Markers that do not share the same letter show significant differences ($P<0.05$). Lowercase letters denote differences for immediate addition of larvae and uppercase letters denote differences for delayed addition of larvae.
9. **Figure 9:** Mean ± SE *Ae. aegypti* male body size exposed to varying concentrations of Okoumal (immediate and delayed addition of larvae) in dose-response laboratory experiments. Markers that do not share the same

letter show significant differences ($P<0.05$). Lowercase letters denote differences for immediate addition of larvae and uppercase letters denote differences for delayed addition of larvae.

Figures

Figure 1:

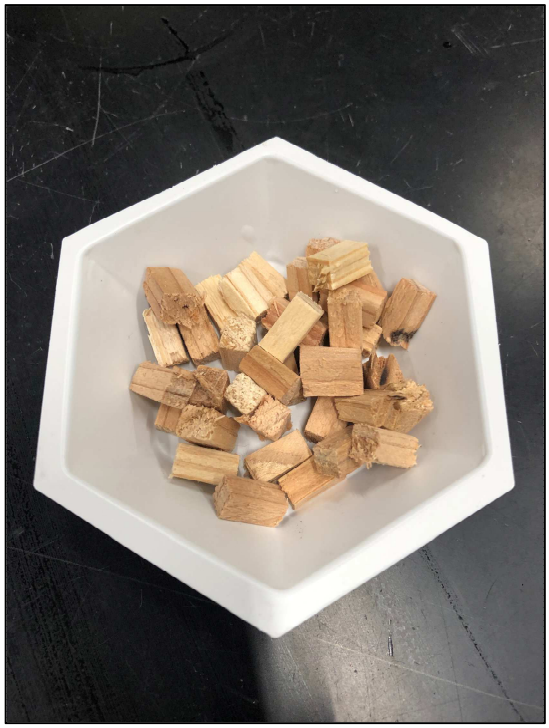


Figure 2:

Figure 3.

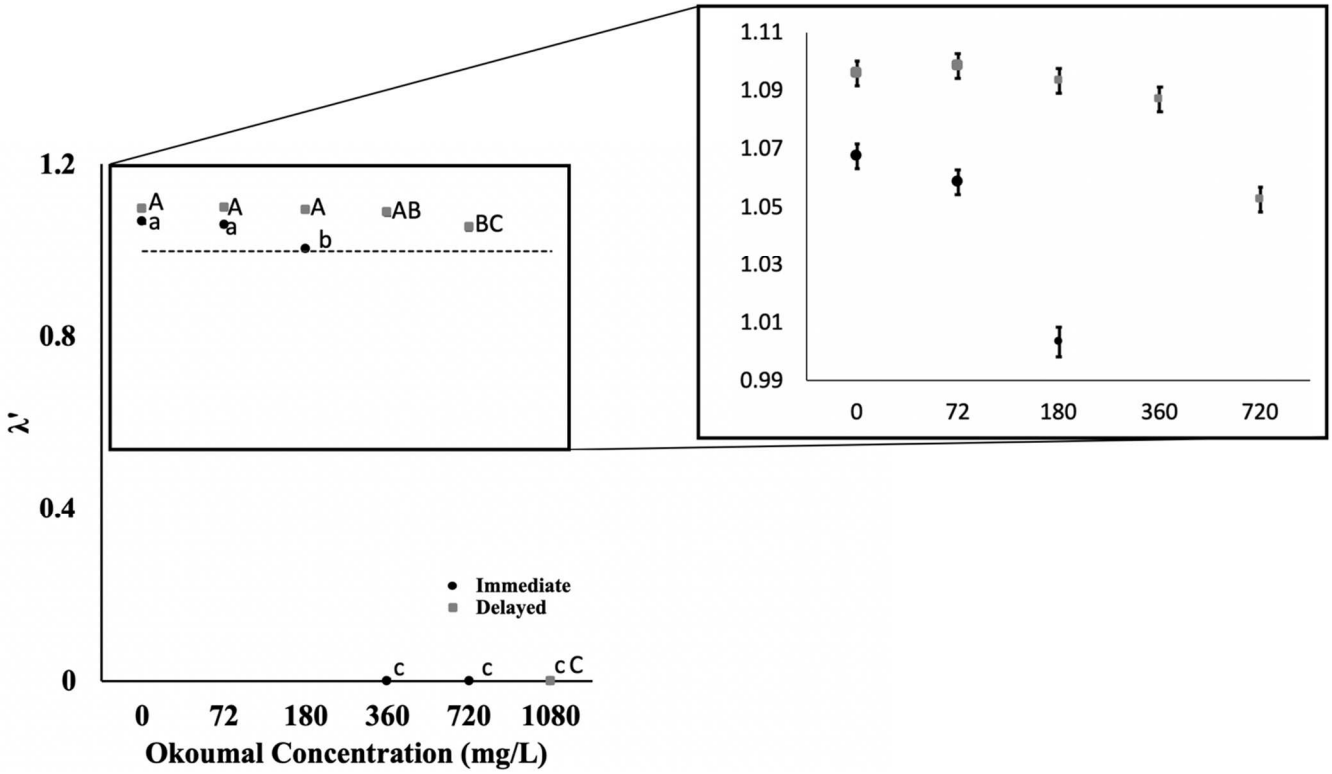

1.11
1.09
1.07
1.05
1.03
1.01
0.99
0
72
180
360
720
1.2
0.8
0.4
0
λ'
A
a
A
a
A
b
AB
BC
Immediate
Delayed
c
c
c C
0
72
180
360
720
1080
Okoumal Concentration (mg/L)

Figure 4:

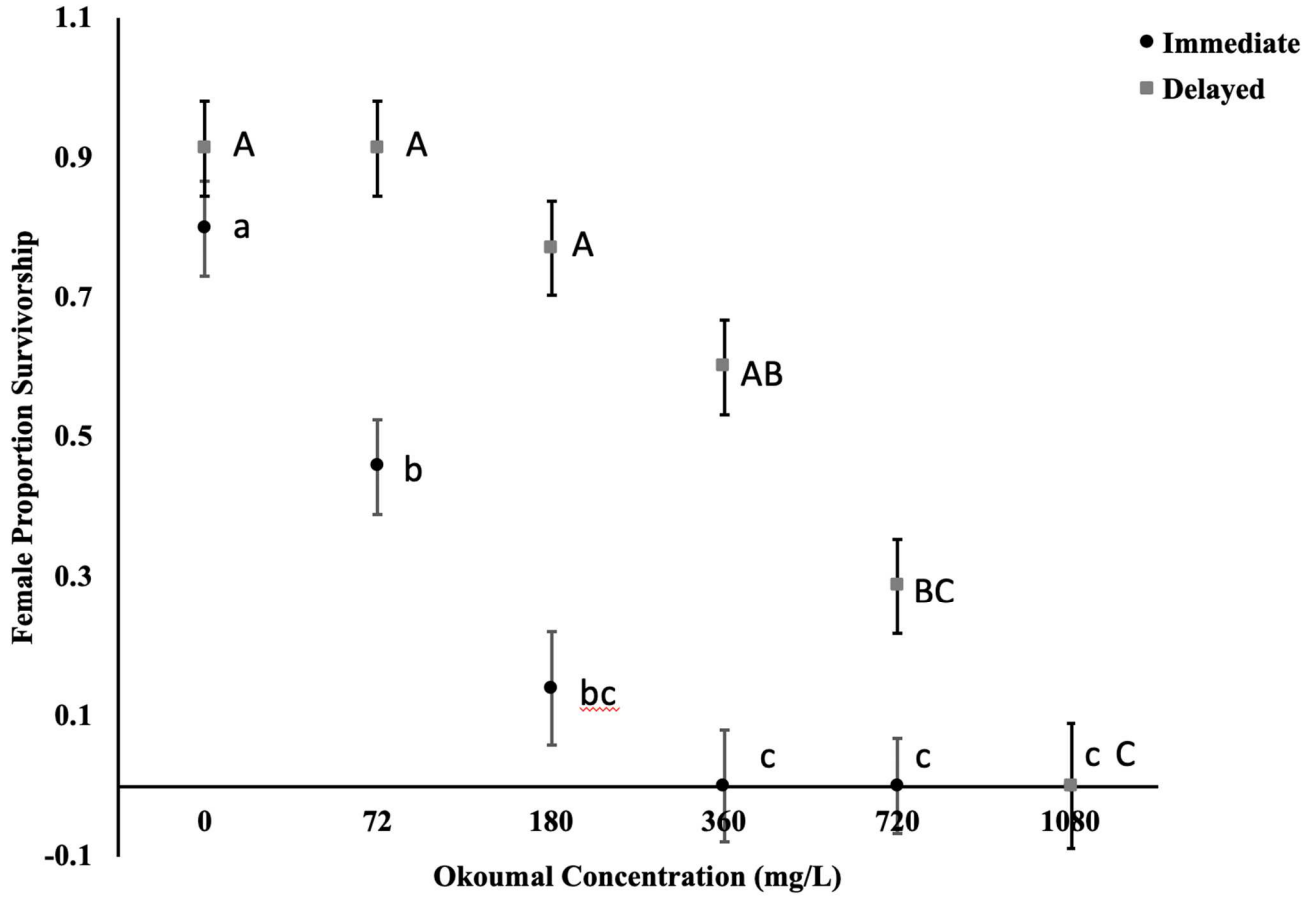

1.1
0.9
0.7
0.5
0.3
0.1
-0.1
Female Proportion Survivorship
Immediate
Delayed
A
A
A
AB
BC
a
b
bc
c
c
c C
0
72
180
360
720
1080
Okoumal Concentration (mg/L)

Figure 5:

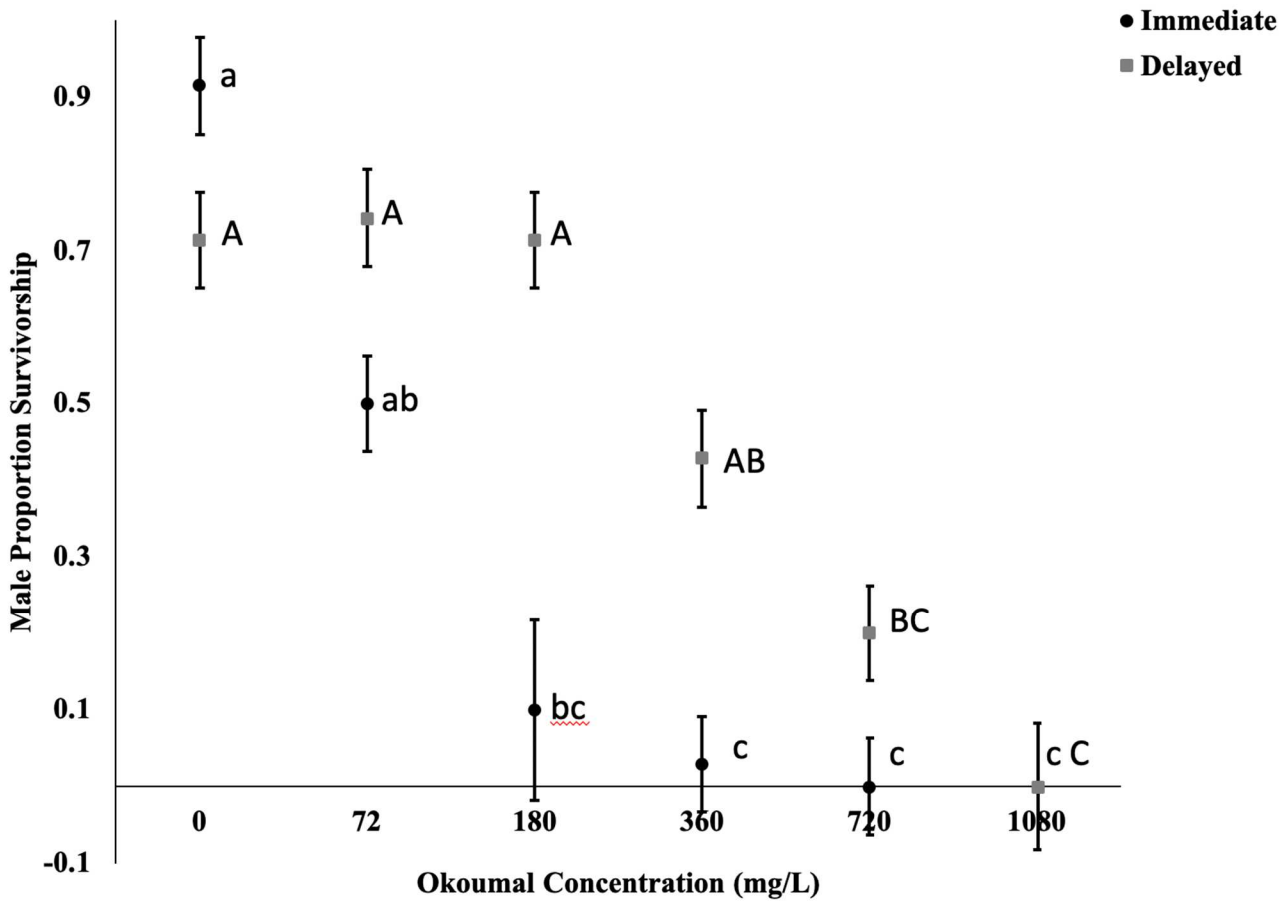

Immediate
Delayed
Male Proportion Survivorship
0.9
0.7
0.5
0.3
0.1
-0.1
a
A
A
A
ab
AB
BC
bc
c
c
c C
0
72
180
360
720
1080
Okoumal Concentration (mg/L)

Figure 6:

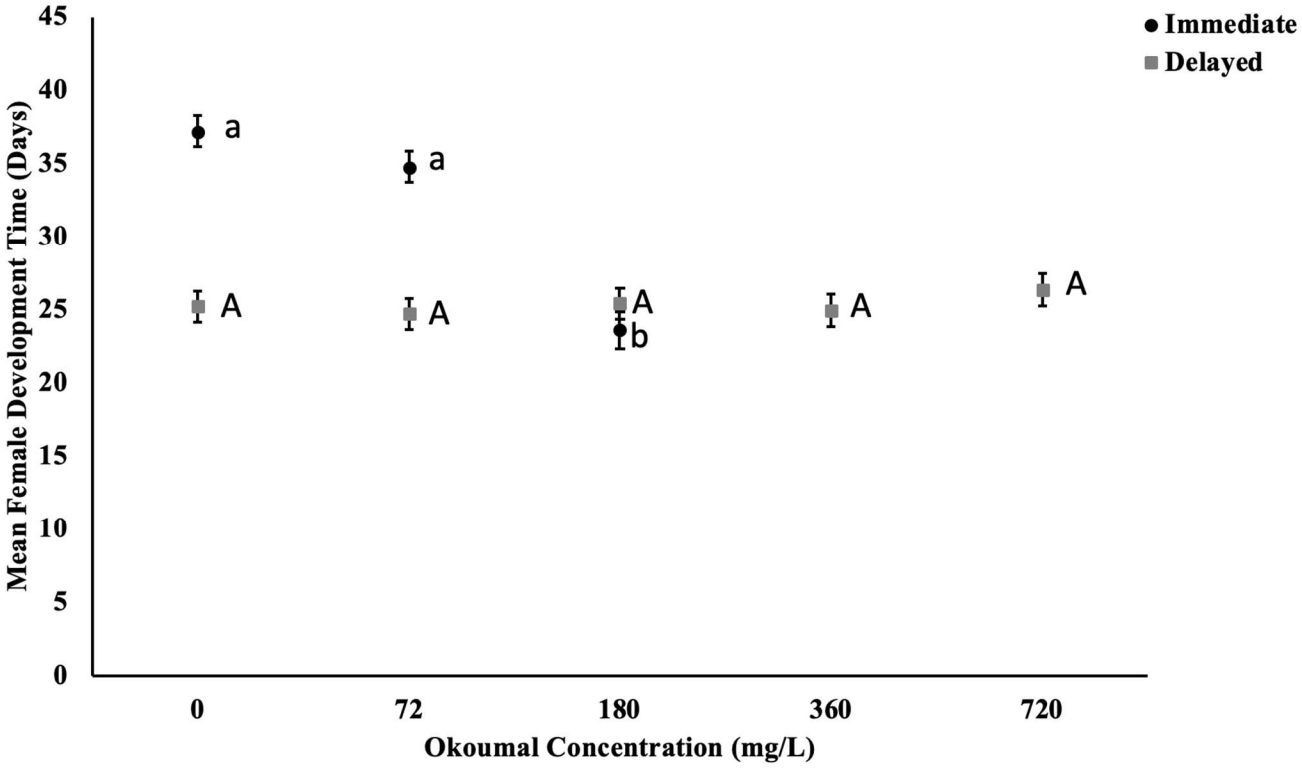

Immediate
Delayed
Mean Female Development Time (Days)
45
40
35
30
25
20
15
10
5
0
a
a
A
A
A
b
A
A
0
72
180
360
720
Okoumal Concentration (mg/L)

Figure 7:

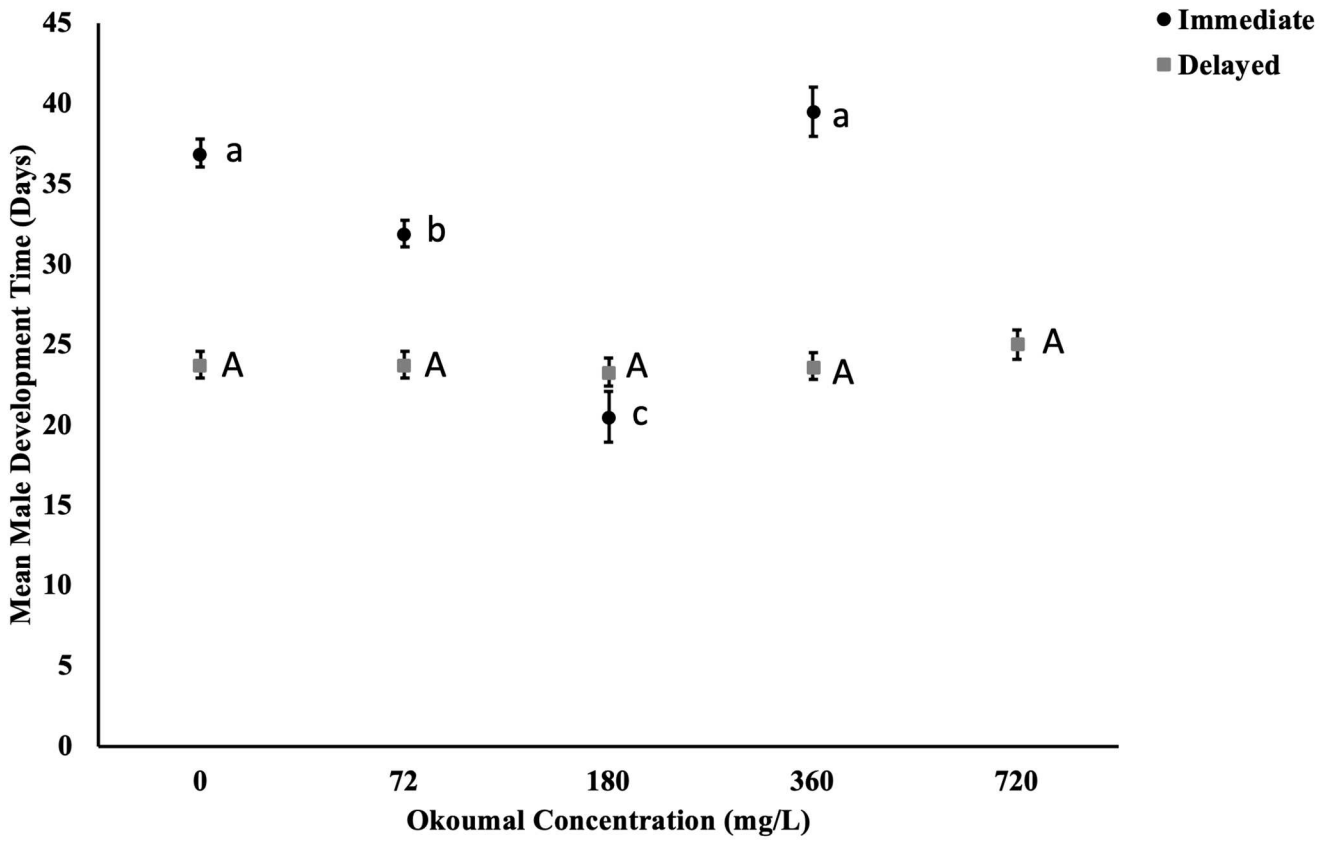

Immediate
Delayed
Mean Male Development Time (Days)
45
40
35
30
25
20
15
10
5
0
a
b
c
a
A
A
A
A
A
0
72
180
360
720
Okoumal Concentration (mg/L)

Figure 8:

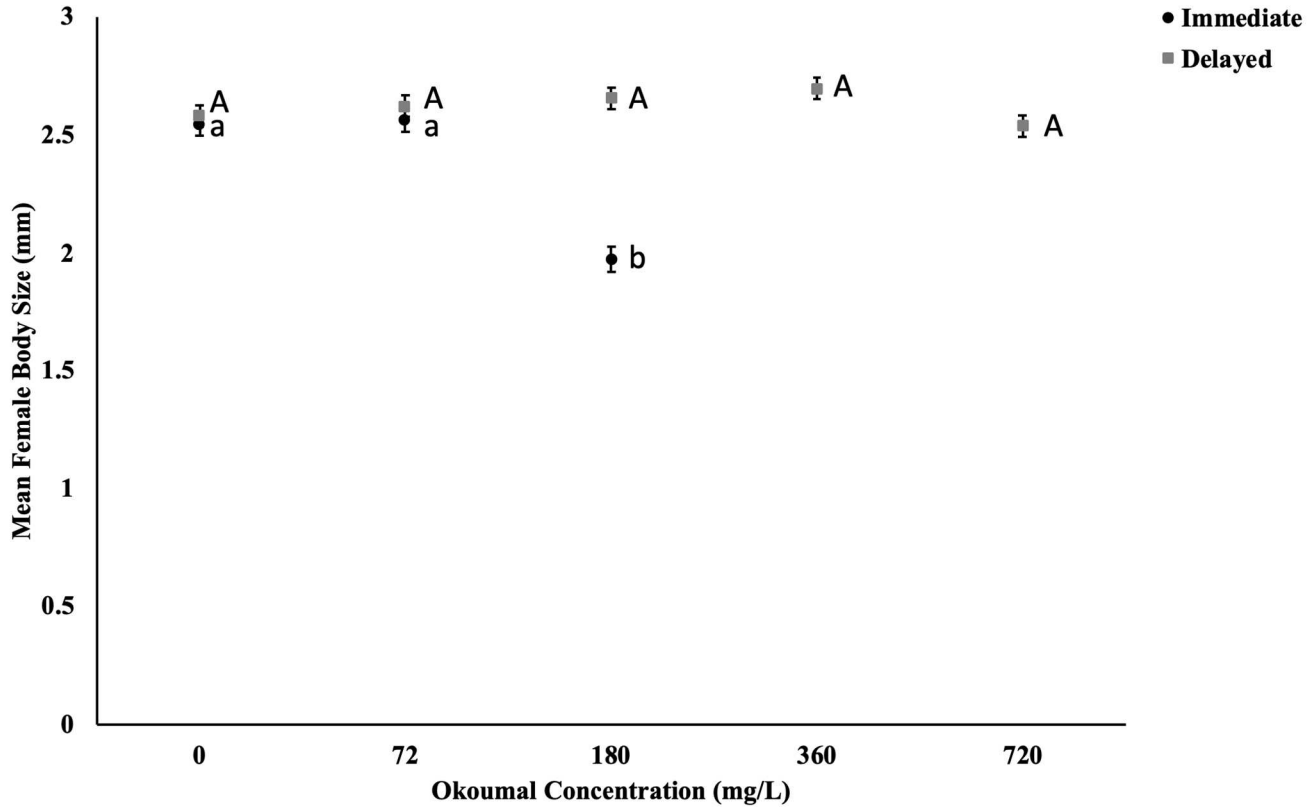
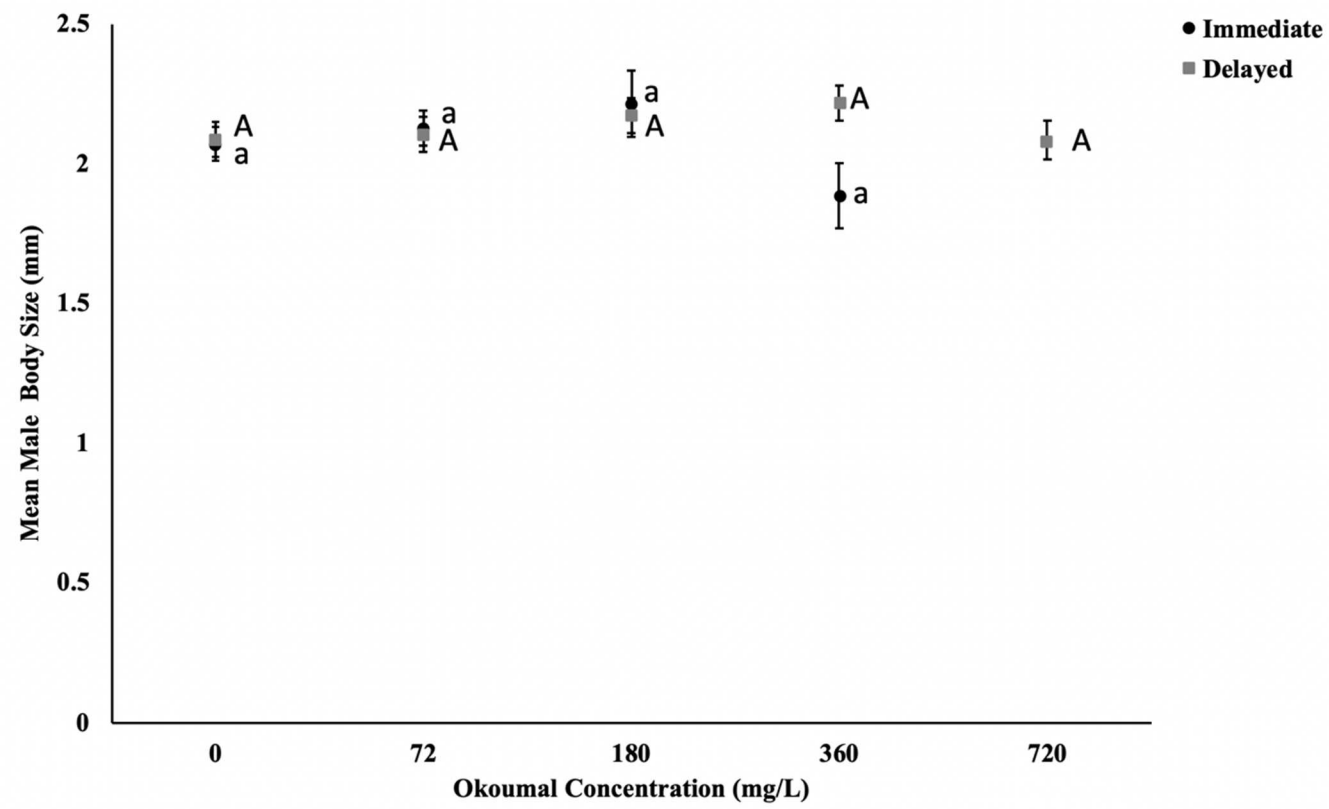

Immediate
Delayed
Mean Female Body Size (mm)
3
2.5
2
1.5
1
0.5
0
A
a
A
a
A
b
A
A
0
72
180
360
720
Okoumal Concentration (mg/L)

Figure 9:


Immediate
Delayed
Mean Male Body Size (mm)
2.5
2
1.5
1
0.5
0
A
a
a
A
a
A
A
a
A
0
72
180
360
720
Okoumal Concentration (mg/L)

References

Briegel, H., and S. E. Timmermann. 2001. *Aedes albopictus* (Diptera: Culicidae): physiological aspects of development and reproduction. Journal of Medical Entomology 38: 566-571.

Carrieri, M., A. Masetti, A. Albieri, B. Maccagnani, and R. Bellini. 2009. Larvicidal activity and influence of *Bacillus thuringiensis* var. *israelensis* on *Aedes Albopictus* oviposition in ovitraps during a two-week check interval protocol. Journal of the American Mosquito Control Association 25: 149-155.

Cassell, D. L. 2011. A Randomization-test wrapper for SAS PROCs; SAS Institute Inc.: Cary, NC, USA.

Clements, A. 1999. The Biology of Mosquitoes, vol. 2, CABI International.

Dowling, Z., P. Armbruster, S. L. LaDeau, M. DeCotiis, J. Mottley, and P. T. Leisnham. 2013. Linking mosquito infestation to resident socioeconomic status, knowledge, and source reduction practices in suburban Washington, DC. EcoHealth 10: 36-47.

Federici, B. A., H. W. Park, D. K. Bideshi, M. C. Wirth, and J. J. Johnson. 2003. Recombinant bacteria for mosquito control. Journal of Experimental Biology 206: 3877-3885.

Floore, T. G. 2006. Mosquito larval control practices: past and present. Journal of the American Mosquito Control Association 22: 527-533.

Fradin, M. S., and J. F. Day. 2002. Comparative efficacy of insect repellents against mosquito bites. New England Journal of Medicine 347: 13-18.

Gibbons, R. V., and D. W. Vaughn. 2002. Dengue: an escalating problem. British Medical Journal 324: 1563-1566.

Goldberg, D., and L. Fleetwood. 1987. Competitive effect and response in four annual plants. Journal of Ecology 75: 1131–1143.

Guzman, M. G., and G. Kouri. 2002. Dengue: an update. Lancet Infectious Diseases 2: 33-42.

Hawley, W. A. 1985. The effect of larval density on adult longevity of a mosquito, *Aedes-sierrensis* - Epidemiological Consequences. Journal of Animal Ecology 54: 955-964.

Jorgense, S. 2008. Biodegradation. Encyclopedia of Ecology: 366-367.

Juliano, S. A. 2009. Species interactions among larval mosquitoes: context dependence across habitat gradients. Annual Review of Entomology 54: 37-56.

Juliano, S. A., G. S. Ribeiro, R. Maciel-De-Freitas, M. G. Castro, C. Codeco, R. Lourenco-de-Oliveira, and L. P. Lounibos. 2014. She's a femme fatale:

low-density larval development produces good disease vectors. Memorias Do Instituto Oswaldo Cruz 109: 1070-U1112.

Kesavaraju, B., P. T. Leisnham, S. Keane, N. Delisi, and R. Pozatti. 2014. Interspecific Competition between *Aedes albopictus* and *A. sierrensis*: Potential for Competitive Displacement in the Western United States. PLOS One 9.

LaDeau, S. L., B. F. Allan, P. T. Leisnham, and M. Z. Levy. 2015. The ecological foundations of transmission potential and vector-borne disease in urban landscapes. Functional Ecology 29: 889-901.

Leisnham, P. T., and S. A. Juliano. 2009. Spatial and temporal patterns of coexistence between competing *Aedes* mosquitoes in urban Florida. Oecologia 160: 343-352.

Livdahl, T., and G. Sugihara. 1984. Non-linear interactions of populations and the importance of estimating per capita rates of change. Journal of Animal Ecology 53: 573-580.

Marcombe, S., A. Farajollahi, S. P. Healy, G. G. Clark, and D. M. Fonseca. 2014. Insecticide resistance status of United States populations of *Aedes albopictus* and mechanisms involved. PLOS One 9: 10.

McMeniman, C. J., R. V. Lane, B. N. Cass, A. W. C. Fong, M. Sidhu, Y. F. Wang, and S. L. O'Neill. 2009. Stable introduction of a life-shortening Wolbachia infection into the mosquito *Aedes aegypti*. Science 323: 141-144.

National Center for Biotechnology Information. PubChem Database. CID=3034278, h. p. n. n. n. g. c. d. L. a. o. J., 2020).

Nene, V., J. R. Wortman, D. Lawson, B. Haas, C. Kodira, Z. J. Tu, B. Loftus, Z. Y. Xi, K. Megy, M. Grabherr, Q. H. Ren, E. M. Zdobnov, N. F. Lobo, K. S. Campbell, S. E. Brown, M. F. Bonaldo, J. S. Zhu, S. P. Sinkins, D. G. Hogenkamp, P. Amedeo, P. Arensburger, P. W. Atkinson, S. Bidwell, J. Biedler, E. Birney, R. V. Bruggner, J. Costas, M. R. Coy, J. Crabtree, M. Crawford, B. deBruyn, D. DeCaprio, K. Eiglmeier, E. Eisenstadt, H. El-Dorry, W. M. Gelbart, S. L. Gomes, M. Hammond, L. I. Hannick, J. R. Hogan, M. H. Holmes, D. Jaffe, J. S. Johnston, R. C. Kennedy, H. Koo, S. Kravitz, E. V. Kriventseva, D. Kulp, K. LaButti, E. Lee, S. Li, D. D. Lovin, C. H. Mao, E. Mauceli, C. F. M. Menck, J. R. Miller, P. Montgomery, A. Mori, A. L. Nascimento, H. F. Naveira, C. Nusbaum, S. O'Leary, J. Orvis, M. Pertea, H. Quesneville, K. R. Reidenbach, Y. H. Rogers, C. W. Roth, J. R. Schneider, M. Schatz, M. Shumway, M. Stanke, E. O. Stinson, J. M. C. Tubio, J. P. VanZee, S. Verjovski-Almeida, D. Werner, O. White, S. Wyder, Q. D. Zeng, Q. Zhao, Y. M. Zhao, C. A. Hill, A. S. Raikhel, M. B. Soares, D. L. Knudson, N. H. Lee, J. Galagan, S. L. Salzberg, I. T. Paulsen, G. Dimopoulos, F. H. Collins, B. Birren, C. M.

Fraser-Liggett, and D. W. Severson. 2007. Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science 316: 1718-1723.

Ney, R. 1995. *Fate and Transport of Organic Chemicals in the Environment: A Practical Guide*, 2nd ed., Government Institutes, Inc.: Rockville, MD.

Perez, C. M., C. F. Marina, J. G. Bond, J. C. Rojas, J. Valle, and T. Williams. 2007. Spinosad, a naturally derived insecticide, for control of *Aedes aegypti* (Diptera: Culicidae): efficacy, persistence, and elicited oviposition response. Journal of Medical Entomology 44: 631-638.

Ponnusamy, L., N. Xu, S. Nojima, D. M. Wesson, C. Schal, and C. S. Apperson. 2008. Identification of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by *Aedes aegypti*. Proceedings of the National Academy of Sciences of the United States of America 105: 9262-9267.

Rapley, L. P., P. H. Johnson, C. R. Williams, R. M. Silcock, M. Larkman, S. A. Long, R. C. Russell, and S. A. Ritchie. 2009. A lethal ovitrap-based mass trapping scheme for dengue control in Australia: II. Impact on populations of the mosquito *Aedes aegypti*. Medical and Veterinary Entomology 23: 303-316.

Reiter, P. 2001. Climate change and mosquito-borne disease. Environmental Health Perspectives 109: 141-161.

SAS Institute 9.4, C., NC. SAS Institute 9.4, Cary, NC.

Silver, J. B., M. W. Service, and SpringerLink (Online service). 2008. Mosquito ecology field sampling methods, pp. xxi, 1494 p. Springer, Dordrecht, the Netherlands.

Slosek, J. 1986. *Aedes aegypti* mosquitoes in the Americas: A review of their interactions with the human population. Social Science & Medicine 23: 249-257.

Smith, D. L., K. E. Battle, S. I. Hay, C. M. Barker, T. W. Scott, and F. E. McKenzie. 2012. Ross, Macdonald, and a theory for the dynamics and control of mosquito-transmitted pathogens. PLOS Pathogens 8: 13.

Thompson, G. D., R. Dutton, and T. C. Sparks. 2000. Spinosad - a case study: an example from a natural products discovery programme. Pest Management Science 56: 696-702.

USPTO inventor. Submitted December 30, 2016. Development of a novel type of chemical bait for mosquitoes. U.S. Provisional Patent Applications Serial #621440518.

Villena, O. C., I. Terry, K. Iwata, E. R. Landa, S. L. LaDeau, and P. T. Leisnham. 2017. Effects of tire leachate on the invasive mosquito *Aedes albopictus* and the native congener *Aedes triseriatus*. Peer J 5: 15

Vontas, J., E. Kioulos, N. Pavlidi, E. Morou, A. della Torre, and H. Ranson. 2012. Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. Pesticide Biochemistry and Physiology 104: 126-131.

WHO. 1997. Dengue hemorrhagic fever: diagnosis, treatment, prevention and control, Geneva: World Health Organization.

WHO. 2009. *Bacillus thuringiensis israelensis (Bti)* in drinking-water background document for development of WHO guidelines for drinking-water quality.

WHO. 2017. Vector-borne diseases.

Chapter 4: General Conclusions

In this study, my aim was to contribute to the scientific understanding of chemicals that potentially could aid mosquito control agencies with management strategies. The USDA recently patented a chemical compound called [2,4-Dimethyl-2-(5,5,8,8-tetramethyl-6,7-dihydronaphthalen-2-yl)-1,3-dioxolane], commonly called Okoumal, as a mosquito attractant (USPTO, 2016). Okoumal has shown to be an attractant to host-seeking *Aedes aegypti* mosquitoes in laboratory trials and an attractant to gravid *Aedes* females in field trials (USPTO, 2016; Saunders & Leisnham, 2018). For my thesis work, I wanted to further test the efficacy of Okoumal as being an attractant to gravid *Aedes* mosquitoes as well as being a toxin to *Ae. aegypti* larvae.

In chapter two, I investigated the attractancy of Okoumal to gravid *Aedes albopictus* females at two different spatial scales in both a laboratory oviposition choice assay and a dose-response field study but found little evidence that oviposition activity increased compared to control treatments. The lack of attractancy of Okoumal in both the choice assay and field experiment is likely underpinned by the interaction of the compound with the olfactory physiology of *Ae. albopictus*. Volatiles, such as semiochemicals, need to come in contact with specialized olfactory receptors (ORs) in order to elicit mosquito behavior (Leal et al., 2013; Wooding et al., 2020). Mosquitoes use olfactory receptor neurons (ORN) to detect attractants (Leal et al., 2013; Choo et al., 2018). The lack of attractancy could be that there was not enough Okoumal compound triggering the ORN or there could have been too much compound triggering receptors leading to disorientation and this may have offset any

attractancy of the compound experienced when the mosquito was further away (Leal et al., 2013). In addition, maybe there was too much Okoumal triggering the ORN that elicited a repellency effect and offset attractancy (Hao et al., 2013; Ong et al., 2015; Choo et al., 2018). Our findings are consistent with those in other studies that have found weaker or opposite effects of compounds at higher doses (Hao et al., 2013; Ong & Jaal, 2015). For example, one study found that caproic acid, a known mosquito oviposition pheromone, attracted more gravid mosquitoes at 1 ppm (OAI= 0.32) compared to the control and the higher treatment dose of 100 ppm (OAI= 0.09) (Ong & Jaal, 2015). The specific biological mechanisms for weak attractancy or repellency of insects to compounds at higher doses is poorly understood but is thought to be related to an oversaturation of chemoreceptors resulting in a threshold effect (Hao et al., 2013).

In chapter three, I investigated the potential of Okoumal as an effective toxin to *Ae. aegypti* mosquito larvae. The results of my thesis work indicate early promise that Okoumal may be an effective toxin to *Ae. aegypti* mosquito larvae. Okoumal had both direct and indirect toxicity effects on *Ae. aegypti* larvae. Okoumal had direct lethality effects impacting larval survival rates, non-lethal effects on mosquito body size and development time, and selection for individuals that have specific genotypes (smaller mosquitoes developed quicker). Indirect effects included availability on microbial food. The larvae that did survive were larger and developed more quickly. The results of chapter three suggest that with higher doses of Okoumal, body size decreases which may alter disease transmission since smaller females were produced and do not transmit diseases as effectively as larger mosquitoes. Although Okoumal

was a toxin to mosquito larvae, it is possible it degraded over time due to volatilization effects. Due to the potential bacterial degradation and volatilization effected, Okoumal did not work as effectively when soaking in an environment for a longer period of time. Other materials need to be investigated that can act as both an attractant and toxin to mosquitoes to aid in mosquito control and surveillance.

Through the research I conducted, Okoumal may not be effective to be used in "bait and kill" traps. My thesis work findings might inform control since agencies could potentially add Okoumal to artificial container habitats that are known to have mosquito larvae concentrations. Control agencies will need to ensure that the containers they place Okoumal, or another chemical compound similar to it, is where the majority of ovipositing females are located. This is important since there is a connection between individual mosquito habitats and regional populations. Many control strategies call for an "all out" campaign where all aquatic habitats are treated with larvicides, but this can be difficult for resource-deprived communities to implement (Gu et al., 2008). Some mosquito container habitats may be inaccessible or cryptic to locate which can make control challenging.

Studies, however, have shown that not all mosquito habitats need to be treated in order for relative abundances in an area to decline (Gu et al., 2008; Bayoh et al., 2010). For example, a study in Kenya investigated the relationship between the abundance of *Anopheles* mosquitoes and insecticide-treated bed nets and concluded that if a high proportion of households in a community have permethrin-treated bed nets, the abundances of *Anopheles* declines (Bayoh et al., 2010). Researchers from this study also noted that larger containers produced more adults (Bayoh et al., 2010).

Another case study conducted in University Park, Maryland, using citizen science techniques, investigated the relationship between the use of gravid autocidal traps in residential yards and abundances of *Ae. albopictus* mosquitoes (Johnson et al., 2018). This study concluded that the threshold to have abundances of *Ae. albopictus* to decrease is to have about 80% of residents participate in order to achieve significant control (Johnson et al., 2018).

Since Okoumal is toxic to aquatic organisms, it should not be added to major waterways since it could have negative effects on non-target organisms (National Center for Biotechnology Information, 2020). Since Okoumal is not effective as an attractant, it can be paired with other known attractants, such as plant infusion. By pairing Okoumal with plant infusion in an oviposition trap, researchers would be able to attract gravid females to the trap and kill offspring larvae that hatch from collected eggs. This can be a useful strategy for mosquito control because using plant infusion in water only attracts gravid females and does not act as a toxin but with adding Okoumal to the trap, it would easily kill the offspring mosquito larvae. My results suggest that Okoumal is not an effective attractant to gravid female mosquitoes but is an effective toxin to mosquito larvae. Future research can investigate other synthetic materials that can be easily deployable in the field and be used in "bait and kill" traps.

References

Bayoh, M. N., D. K. Mathias, M. R. Odiere, F. M. Mutuku, L. Kamau, J. E. Gimnig, J. M. Vulule, W. A. Hawley, M. J. Hamel, and E. D. Walker. 2010. Anopheles gambiae: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. Malaria Journal 9: 12.

Choo, Y. M., P. X. Xu, J. K. Hwang, F. F. Zeng, K. M. Tan, G. Bhagavathy, K. R. Chauhan, and W. S. Leal. 2018. Reverse chemical ecology approach for the identification of an oviposition attractant for *Culex quinquefasciatus*. Proceedings of the National Academy of Sciences of the United States of America 115: 714-719.

Gu, W. D., J. Utzinger, and R. J. Novak. 2008. Habitat-based larval interventions: A new perspective for malaria control. American Journal of Tropical Medicine and Hygiene 78: 2-6.

Hao, H. L., J. C. Sun, and J. Q. Dai. 2013. Dose-dependent behavioral response of the mosquito *Aedes albopictus* to floral odorous compounds. Journal of Insect Science 13: 8.

Johnson, B. J., D. Brosch, A. Christiansen, E. Wells, M. Wells, A. F. Bhandoola, A. Milne, S. Garrison, and D. M. Fonseca. 2018. Neighbors help neighbors control urban mosquitoes. Scientific Reports 8: 6.

Leal, W. S. 2013. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annual Review of Entomology, Vol 58 58: 373-391.

National Center for Biotechnology Information. PubChem Database. CID=3034278, h. p. n. n. n. g. c. d. L. a. o. J., 2020).

Ong, S. Q., and Z. Jaal. 2015. Investigation of mosquito oviposition pheromone as lethal lure for the control of *Aedes aegypti* (L.) (Diptera: Culicidae). Parasites & Vectors 8: 7.

Saunders, M., and P. Leisnham. October 2018. Testing an easily deployable mosquito attractant: promising results from initial field trials. Society of Vector Ecology 48t^{h} Annual Conference at Yosemite National Park.

USPTO inventor. Submitted December 30, 2016. Development of a novel type of chemical bait for mosquitoes. U.S. Provisional Patent Applications Serial #621440518.

Wooding, M., Y. Naude, E. Rohwer, and M. Bouwer. 2020. Controlling mosquitoes with semiochemicals: a review. Parasites & Vectors 13: 20.