Factors influencing sugar feeding in invasive mosquitoes

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Abstract

Phytophagy (*i.e.*, feeding on plant-derived materials) is an essential component of mosquito biology. Yet, it has been historically neglected as most research effort has been concentrated on host-seeking behavior and pathogen transmission. As mosquitoes are the deadliest animals on earth and because challenges, such as the rise of insecticide resistance, arise, there is an urgent need for developing effective and ecologically friendly disease vector control strategies. It is therefore important to deepen our understanding of mosquito phytophagy and, consequently, its potential to develop novel vector control methods. Particular major disease vectors are *Ae. aegypti* and *Ae. albopictus*, which are spreading rapidly through the US, in part due to climate change. Herein, we first examine the effect of temperature on *Ae. aegypti* sugar-feeding behavior as well as overall locomotive activity and survival, using total carbohydrate assays and actometer experiments. An optimum temperature range for mosquito activity is proposed and discussed in the context of global warming. We then observe the tentative benefit provided by city-planted ornamental flowers to *Ae. aegypti* and *Ae. albopictus* living in heavily-populated, urban areas. Mosquito sugar-feeding activity and, subsequently, sugar consumption were tested for eleven commonly-planted ornamentals. Additionally, scents were collected from the headspace of each ornamental, and volatile composition was analyzed and discussed as potential cues that could mediate mosquito-plant interactions.

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General Audience Abstract

Sugar-feeding is an important, but generally neglected, aspect of mosquito biology, affecting, for example, their survival, metabolism, and ability to lay eggs. While females need blood to mature their eggs, males feed exclusively on sugar, further highlighting the importance of this food source. Mosquitoes use several cues to locate flowers to feed on, including the plant scent. *Aedes aegypti* and *Aedes albopictus* are two urban species that are invasive to the US and are the vectors of several deadly pathogens including dengue, Zika and chikungunya. It is thus to study any aspect of their biology that could lead to the development of new tools to limit their propagation. Because of the nutrients provided to mosquitoes by flowering plant species, considering the dynamic ecological relationship between human, plant, and mosquito in urban, heavily populated areas is critical. Additionally, how temperature is mediating each of these interactions is important to understand and keep in consideration. Here, we first examine the effect of temperature on sugar-feeding, activity and survival in *Ae. aegypti*, and discuss our results in the context of potential changes in temperature caused by climate change. We also provide some insights on the role that ornamental flower species play in urban areas in the ability of these mosquito species to thrive, by attracting mosquitoes to areas where they will have access to a high number of human blood meals.

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General Introduction

Disease vector mosquitoes are the deadliest animal on Earth, infecting and killing millions of humans globally every year, and putting many more at risk for contracting diseases (CDC, 2019). Species such as *Aedes aegypti* and *Aedes albopictus* play a particularly large role in the context of disease transmission. Diseases known to be carried and transmitted by these two species include dengue, chikungunya, Zika, and yellow fever (Wilder-Smith et al. 2017). This contributes to their potent lethality to humans, as vaccines and, in some cases, treatments are not available. Given their high degree of adaptability and critical disease relevance, it is important to understand these species' trends in global distribution. As of 2015, both species have been determined to have an established presence in every continent except Antarctica, and the areas they occupy are predicted to be continually expanding (Kraemer et al. 2015).

Image 1 ***Aedes aegypti.*** Accessed from www.cdph.ca.gov

Both *Ae. aegypti* and *Ae. albopictus* exhibit a high degree of anthropophily (*i.e.*, preferentially feeding on humans), with *Ae. aegypti* having shown a strong preference for human blood over blood from other warm-blooded vertebrates (Ponlawat et al. 2005) (Images 1 & 2). Female mosquitoes are the sole sex requiring a blood-meal, and use the proteins blood contains to develop eggs and carry out oviposition (Christophers, 1960). Less widely understood, albeit just as important, is their acquisition of energy via carbohydrate (sugar) intake from varying sources. Sugar-feeding affects many aspects of both the male and female mosquito adult life cycle; males feed solely on sugar sources (Foster, 1995), highlighting the importance of this behavior. It is worth mentioning that some species of mosquito, such as *Toxorhynchites* spp., are incapable of

feeding on blood as adults and exclusively receive energy from plant-derived sugar sources (Collins et al. 2000).

Throughout the history of mosquito research, research on phytophagy in many mosquito species, relative to hematophagy, has been comparatively neglected. Plant sugar sources, however, are a necessity for a successful mosquito life cycle (Peach et al. 2019). Flying, host-seeking, and oviposition are all aspects of their lives that are affected by receiving energy from plants, particularly in how successful they are at carrying out these activities (Foster, 1995; Stone et al., 2009). The impact that sugar-feeding has on mosquitoes' lives has led to propositions for vector surveillance and control via sugar-feeding exploitation, such as the use of toxic sugar baits (TSBs) (Hall-Mendelin et al. 2010, Ferguson et al. 2010). Additionally, the effect sugar-feeding has on the ability of mosquitoes to successfully vector their associated pathogens has begun to be studied, and an inverse relationship between the two has been proposed. Specifically, human biting rates decrease when mosquitoes have access to sugar, and some evidence has suggested the composition of plant nectar can impede parasite development within the mosquito (Stone and Foster, 2013).

Image 2 ***Aedes albopictus.*** Accessed from www.cdph.ca.gov

Several different sources of energy provided by plants have been confirmed for mosquitoes, although floral nectar appears to be the most commonly utilized (Clements, 1999; Foster, 1993; Peach et al. 2019). Other sources include extrafloral nectaries, such as nectar excreted from leaves or petioles, which *Anopheles gambiae* and *Culex* spp. have been confirmed to feed on (Gary Jr. and Foster, 2004). Field studies have reported *Anopheles* spp., *Culex* spp., *Aedes* spp., *Psorophora* spp., and *Culiseta*

spp. feeding on damaged fruits such as apples, grapes, and peaches (Joseph 1970). A less common and effective, yet still viable, source of fuel can come from plant juices; *Ae. albopictus* has been documented feeding on lucky bamboo, a plant lacking any inflorescence (Qualls et al. 2013). Cases of sugar-feeding on honeydew, the sugary liquid produced by aphids after consuming plant sap, have been documented for multiple species of mosquito, including *Ae. aegypti* (Gary Jr. and Foster 2004; Peach et al., 2019). In the same vein, female *Malaya* spp. are known to consume *Crematogaster* spp. ant regurgitate, demonstrating the remarkable variability in which different species of mosquito are able to acquire plant-provided energy (Farquharson, 1918). Lastly, occurrences of *Toxorhynchites rutilus septentrionalis* feeding on black oak tree sap have been reported (Collins et al. 2000).

Locating a host to obtain a blood-meal is known to be driven by several factors, including olfaction, vision, heat, and the presence or absence of carbon dioxide (CO_2) (van Breugel et al., 2015). Less information is available regarding how the location of sugar sources is carried out, but olfaction seems to be an important cue the mosquitoes use to locate the plants (Lahondère et al., 2020). There is sufficient evidence from both field and laboratory experiments that suggests that mosquitoes are indeed responsive to volatile compounds present in the scent profiles of certain plant species (Clements, 1999). It is understood that mosquitoes perceive odor through reception of odor molecules via sensilla located on their antennae, which house odorant receptors that are attuned to specific odorants (Zwiebel, 2004). Experiments using electroantennograms coupled with gas chromatography, in conjunction with behavioral choice assays, have been used on multiple occasions to determine what kind of response a volatile compound from a given flower, fruit, or plant evokes for mosquitoes. Examples of compounds that have been experimentally assayed for Aedine species responses include lilacs, nonanal and benzaldehyde, from the

Platanthera obtusata and *Silene otites* flowers, respectively (Lahondère et al., 2020; Jhumer et al. 2007). *Ae. aegypti* have been shown to be responsive to compounds like (E)-2-hexen-1-ol and (E) linalool oxide from *Pithecellobium dulce* (Barredo and DeGennaro, 2020). Varying forms of hydrocarbon esters have been found to contribute to a major portion of fruit scent compositions, but more work needs to be done to determine if these compounds drive mosquito response (Pachuwah, 2016).

Olfaction is just one of the components required for effective mosquito-host and mosquito-plant locating. Visual cues, particularly color and contrast, have been identified as important for successful mosquito foraging. Pale-colored flowers have been thought to be generally attractive to mosquitoes, because of their higher visibility at lower levels of light, relative to other vegetative and inflorescent colors (Foster and Hancock, 1994). However, *Ae. aegypti* have exhibited responsiveness to several different colors in artificial flower experiments, such as blue, yellow and red (Dieng et al., 2018). Additionally, *An. gambiae* were shown to have the differential ability to associate specific visual patterns with increased nectar availability, suggesting the capacity of mosquitoes to learn and remember flower species providing a larger source of food and energy (Bernáth et al., 2016). Lastly, *Cx. pipiens* was determined to perceive and utilize ultraviolet (UV) light cues produced by *Tanacetum vulgare,* and *Hieraciumm lachenalii* as a means to help them locate these plants (Peach et al. 2019).

During the process of host-seeking, mosquitoes use host-provided CO_2 plumes to begin tracking their host at long distance, and subsequently use visual, olfactory and thermal cues to determine a suitable landing location to feed (van Breugel et al., 2015). This principle could also potentially be applied to sugar-foraging, due to the presence of microorganisms on plants. These microorganisms produce small amounts of CO_2 and heat, which could collectively be used by

mosquitoes to help identify promising nectar sources (Herrera and Pozo, 2010; Smallegange et al. 2010). Additionally, as microorganisms consume nectar they produce semiochemicals as a byproduct of metabolism and fermentation (Rering et al. 2018). These semiochemicals could be used by mosquitoes to help analyze the quality of nectar from a given plant, but sufficient experimental evidence has yet to be provided.

After successful identification and subsequent landing onto a plant providing a potential nectar source, a mosquito will begin to probe the inflorescence of the plant using its proboscis. The carbohydrates that are present in plant nectar are the monosaccharides fructose and glucose and their disaccharide sucrose. Because glucose is a compound also present in blood, females rely on neurons located on their stylet to distinguish between components exclusive to nectar and blood (Jové et al. 2020). Mosquitoes have been found to sense plant-derived carbohydrates using sensilla located on their labella and legs (Kessler et al. 2015). Upon sensillar perception of these carbohydrates, the labella will open and allow for nectar uptake. Nectar will presumably reach gustatory receptors located on the mosquito's mouthparts, which help to initiate sucking and continual consumption of sugars (Pappas and Larsen, 1978). Depending on the current physiological needs of a given mosquito, the fate and final destination of nectar carbohydrates can vary.

Generally, ingested sucrose, glucose and fructose will be used immediately for powering flight activity or will be converted to glycogen, trehalose or triacylglycerols (Clements, 1999). The lattermost of these end products is stored in the fat body and is used as energy reserves for survival, rather than for locomotion (van Handel, 1984). Lipids have also been shown to be used for facilitating oviposition, although these lipids seem to be acquired from blood, rather than sugar meals (van Handel, 1993). This demonstrates the important role that carbohydrate-derived fatty

acids play in overall mosquito survival. Interestingly, *Toxorhynchites* spp. mosquitoes acquire all of the nutrients they need for a full life cycle, namely proteins for oviposition, without taking a blood meal. These nutrients are instead obtained during the larval stage via the killing and consumption of other mosquito and arthropod larvae (Schiller et al., 2019). This prepupal killing behavior, in addition to their adult sugar diet, has led to several proposed applications for using *Toxorhynchites* spp. as a possible biocontrol agent.

Additionally received from nectar sugar-feeding are the glucose-based polysaccharides trehalose and glycogen. Trehalose is the main carbohydrate molecule found in mosquito hemolymph, where it can be quickly delivered to different types of tissues as needed upon introduction of different stresses. It has a high versatility and has been characterized for multiple mosquito functions, including fuel for flight muscles and breakdown into glucose molecules during dehydration (Clements, 1999; Hagan et al. 2018). It has also been proposed as an energy source for *Plasmodium falciparum* parasites that have infected *An. gambiae* mosquitoes (Liu et al. 2013). Glycogen received from nectar-feeding is used for long-term energy storage, and is deposited in either the fat body or flight muscles (Clements, 1999). Mosquitoes beginning to diapause will show a preference for finding sugar meals over blood meals, so that they can accumulate a sufficient amount of glycogen and triacylglycerols to survive overwintering (Chang et al. 2016).

The destination of a meal within a mosquito upon ingestion will differ depending on whether the meal is composed of blood or sugar. Blood meals will almost exclusively travel directly to the midgut for immediate digestion (Day, 1954). Alternatively, sugar meals are delivered to the ventral diverticulum (crop), where they will be stored until the mosquito's physiological needs necessitates their digestion (Calkins et al. 2017). When needed, nectar solutions are transferred from the crop to the midgut, where α-glucosidases have been shown to

aid in the breakdown of the carbohydrates that compose these solutions (Souza-Neto, 2007). Depending on whether the meal is blood or nectar, females are able to activate one of two separate feeding mechanisms. As previously mentioned, stylet neurons exclusive to female mosquitoes are able to identify the presence of blood, whereas sugar meals are recognized upon contact with the labella and do not require use of the stylet (Jové et al. 2020). These exclusive mechanisms and digestive routes allow quick distinction and proper nutrient allocation for each encountered meal.

Because of the impact mosquitoes have on disease transmission and human mortality, several mosquito abatement programs have been developed and established with varying strategies and effectiveness. Commonly used control strategies include application of insecticides and larvicides to areas with high mosquito prevalence, and the personal use of repellents. Looking at *Ae. aegypti* and *Ae. albopictus* specifically, these strategies have recently had limited effectiveness in preventing epidemics, in part because of increased insecticide resistance, lack of sufficient coverage, and failure to implement strategies on a large scale (Achee et al. 2019). Additionally, the negative impact that chemical-based strategies have on the environment and non-target organisms is of increasing concern (Fiorenzano et al. 2017).

A promising and increasingly popular strategy that has arisen as a potential solution for the aforementioned issues is the use of Toxic Sugar Baits (TSBs), or Attractive Toxic Sugar Baits (ATSBs) (Image 3). These baits take advantage of mosquitoes' natural attraction to plant-based sugar sources by combining a sugar solution with a toxin that will kill the baited mosquito upon consumption (Fiorenzano et al. 2017). ATSBs further cultivate these traps by adding a plant-sourced attractant intended to lead mosquitoes away from feeding on their natural sugar sources.

Several field studies have proven these baits to be an effective control strategies, eradicating hundreds of mosquitoes over large spans of land in high disease risk areas (Beier et al. 2012).

As it stands in the field currently, an improved understanding of different possible attractants will further optimize the effectiveness of these traps. Different species of mosquito will respond to different volatile compounds. Looking at *Ae. aegypti* and *Ae. albopictus* specifically, L-lactic-acid and 1-octen-3-ol are effective attractants in ATSBs (Scott-Fiorenzano, 2017). Many potential volatile phytochemicals exist and could serve as attractants. Indeed, multiple Aedine species have exhibited an attraction for benzenoids and terpenes originating from flower scents, such as *o*-cresol and β-myrcene and (E)-β-ocimene, respectively (Nyasembe and Torto, 2014; Nyasembe et al. 2018).

Image 3 Attractive Toxic Sugar Bait. Towels are soaked with the attractant and insecticide cocktail. The bowl collects any runoff from the towel for reuse. Image modified from Stewart et al. (2013).

An important aspect of mosquito biology to consider when developing tools for disease-vector control, including ATSBs, is their sensitivity to the varying temperatures they experience throughout a 24-hour cycle. In the context of blood-feeding, changing temperatures affect biting rates and, consequently, the ability of an infected mosquito to vector its associated disease from host-to-host (Suh et al. 2019). Many mosquito species tend to forage for blood meals at dusk and dawn, and the same is true for locating sugar meals. Crepuscular sugar-feeding has been recorded in the field from both males and females in multiple species, including *Ae. taeniorhynchus*, *Ae. vexans*, *Ae. trivittatus*, and *An. implexus* (Haeger, 1955; Yee et al. 1992; McCrae et al. 1976). However, this is not a ubiquitous characteristic, as day-active and nocturnal

feeding species exist (Foster, 1995). Because mosquitoes are ectotherms (*i.e.*, cold-blooded), they are extremely susceptible to variations in environmental temperatures despite the fact that some species are known to regulate their body temperature during blood-feeding via evaporative cooling of droplets of mixed urine and blood (Lahondère and Lazzari, 2012).This is particularly important in the context of climate change, which may alter mosquito life traits and processes, such as rate of development and survival (Paaijmans et al. 2013). Included in these affected processes is sugar-feeding and metabolism, which will be expanded in detail herein this body of work (Upshur et al. 2019).

Temperature also has a major effect on the parasitic organisms and arboviruses vectored by mosquitoes. Upon imbibement of blood from a host infected with *Plasmodium falciparum*, gametocytes will become active via a process known as exflagellation within the mosquito's gut. At temperatures of 30°C and above, this developmental process is inhibited, thereby decreasing vector capacity (Ogwan'g et al. 1993). Being within an optimum temperature range also affects the Extrinsic Incubation Period (EIP) of the parasites, or how fast the parasite is able to complete development within the mosquito (Paaijmans et al. 2011). In terms of arboviruses, viral replication has generally been shown to have an inverse relationship with environmental temperatures, requiring longer EIPs at lower temperatures (Hardy et al. 1983). With this given information, it is important to consider climate change in the context of global parasite and arbovirus distribution.

Sugar-feeding in all mosquito species is a relatively understudied and poorly understudied process. The implications this behavior has on mosquito biology, physiology and overall fitness, and therefore vector capacity, cannot be overshadowed. Based on preceding information, the current study herein aims to answer the following questions:

- How does temperature and access to sugar affect *Ae. aegypti* activity and survival?

- What are the effects of temperature on total carbohydrate content in *Ae. aegypti*?
- Can experimental evidence be provided for whether *Ae. aegypti* and *Ae. albopictus* are attracted or repelled to certain ornamental flowers?
- What volatile compounds emitted from these flowers are these species responsive to?
- Are volatile compounds emitted from these flowers driving attraction or repellency?
- Can plant-derived volatile compounds be used to further optimize ATSBs or develop new attractive traps?

This thesis is organized into two separate chapters with a universal focus on mosquito phytophagy. The first chapter examines the role that temperature and sugar access has on *Ae. aegypti* activity, survival, and sugar content using actometer and total carbohydrate analyses. The second chapter focuses on sugar feeding on ornamental plants. Preferences of *Ae. aegypti* and *Ae. albopictus* for eleven different species of ornamental plant species were observed, and their capacity to uptake nectar from the flowers of these species are presented. In addition, volatile compounds present in the scent profiles of each of these ornamental species are reported and discussed in the context of the literature available on mosquito olfaction and the role plant volatiles play in mediating plant-mosquito interactions.

Chapter 1: Temperature and Sugar Feeding Effects on the Activity of a Laboratory Strain of *Aedes aegypti*

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Abstract: *Aedes aegypti* is an invasive mosquito species that is expected to expand its global distribution through climate change. As poikilotherms, mosquitoes are greatly affected by the temperature of the environment which can impact host-seeking, blood-feeding, and flight activity as well as survival and ability to transmit pathogens. However, an important aspect of mosquito biology on which the effect of temperature has not been investigated is water and sugar-feeding and how access to a sugar source might affect the insect's activity and survival under different thermal conditions. To close this knowledge gap, we relied on actometer experiments to study the activity of both female and male Ae. aegypti at 20°C, 25°C, and 30°C, providing either water or 10% sucrose to the insects. We then measured the total carbohydrate contents of alive mosquitoes using the anthrone protocol. Survival was assessed and compared between all groups. Results from this study will inform on the thermal biology of Ae. aegypti mosquitoes and how access to sugar affects their activity.

Keywords: actometer; abiotic factors; total carbohydrates; disease vector insects; invasive species; mosquito

1. Introduction

The average global surface temperature is projected to increase throughout the 21st century (Coffel et al. 2017). This might lead to longer infection seasons and expansion of multiple vectors' geographic distributions, resulting in an increase of vector-borne disease risk. Regions at risk include much of Africa and Central and South America (Pachauri et al. 2014), as well as North America (Reidmiller et al. 2018). A disease vector insect of particular concern is *Aedes aegypti* (Linnaeus in Hasselquist, 1762), which can transmit dengue, chikungunya, Zika, and yellow fever (Wilder-Smith et al. 2017). Chikungunya and dengue are of growing global public health concern as a consequence of their recent geographical spread (Weaver, 2014). *Ae. aegypti* distribution is currently the widest ever recorded (Kraemer et al. 2015). It has expanded widely Northward in the United States since 1995 (Hahn et al. 2017) and is projected to expand substantially around the world (Capinha et al. 2014). Moreover, the number of people at risk is expected to significantly increase, with Australian, European, and North American populations expected to have the largest proportional increase in exposure (63–80% by 2061–2080) (Monaghan et al. 2018).

As mosquitoes are poikilotherms and thus greatly affected by temperature, the changes in distribution of *Ae. aegypti* due to climate change (Kraemer et al. 2015; Monaghan et al. 2018; Khormi and Kumar, 2018) requires further investigation about the effects of temperature on *Ae. aegypti* physiology and behavior. The effects of temperature on some aspects of *Ae. aegypti* host-seeking, blood-feeding, and flight activity have been characterized (as reviewed in (Reinhold et al. 2018), as well as its effects on reproductive activity and survival (de Almeida Costa et al. 2010). However, the effect of temperature on water intake and sugar-feeding, a behavior to which both male and female *Ae. aegypti* have an evolutionary commitment (Foster, 1995), has not been previously investigated.

Despite the fact that water does not provide nutrients, water uptake is a common behavior in both female and male mosquitoes allowing them to avoid dehydration and death, in particular under dry conditions and in the absence of other water sources such as nectar or blood (Benoit and Denlinger, 2007; Benoit et al. 2010). It is essential to maintain their water balance and it has been shown to be critical for minimizing dehydration in diapausing mosquitoes (Benoit and Denlinger, 2007). Water is imbibed in small volume and passed into the midgut while nectar is usually stored in the dorsal diverticula and is transferred to the crop as needed (Friend, 1978; Friend et al. 1988; Friend et al. 1989; Schmidt and Friend, 1991).

Sugar-feeding is an important and physiologically relevant behavior for both male and female mosquitoes as their fitness depends on having a diet optimal in quality and quantity (House, 1961). Energy from sugar-feeding is essential for mosquito flight energy (Foster, 1995; Nayar and van Handel 1971), flight duration and distance (Briegel et al. 2001), host-seeking, blood-feeding, maintenance, and reproductive success (Foster, 1995; Nayar and Van Handel 1971). Mosquitoes obtain sugar by feeding on a wide range of carbohydrate sources including floral and extrafloral nectar, rotting or decaying fruit, tree sap, and honeydew (Foster, 1995; Clements, 1999; Nielsen and Greve, 1950; Haeger, 1955; Lahondere et al. 2020). Additionally, mosquitoes have been known to get sugar through ant regurgitation (Farquharson, 1918; Farquharson et al. 1922; James, 1914). The consumed sugar can diffuse as trehalose in hemolymph and spread through tissues to reach muscles needed for flight (Clements, 1999). Energy derived from these sugars also impacts how persistent females are at obtaining blood (Foster, 1995; Dittmer et al. 2019) and helps to sustain them in the absence of blood meals (Briegel et al. 2001).

Sugar-feeding is known to be affected by weather (e.g., temperature, humidity), season, and locality (e.g., tropical vs. temperate climate) (Foster, 1995) and is thus likely to be affected by

abiotic factors such as temperature. The effect of temperature on blood-feeding is complex as the temperature of the food source, environment, and convection currents from the food source, combined with the environmental temperature, likely affect feeding due to their general effects on the mosquito's activity (Christophers, 1960). The proposed thermal optimum for biting and blood-feeding has been generally stated to be between 26 and 35°C and is humidity-dependent (Christophers, 1960). However, the effects of temperature and optimum range for sugar-feeding as well as how temperature possibly impact plant cues (e.g., olfactory), detection, and integration in mosquitoes have yet to be investigated.

Understanding the effects of temperature on sugar-feeding as the global surface temperature is projected to change is particularly important as the Toxic Sugar Bait technique (TSBs) is emerging as a control strategy to target nectar-seeking mosquitoes (Airs et al. 2019). The use of TSBs is gaining attention as insecticide resistance rises among mosquito populations (Liu, 2015; Qualls et al. 2015; Russell et al. 2013). Moreover, the World Health Organization has urged vector control programs to develop novel strategies for integrated mosquito management (IMM) that are cost-effective, sustainable, and environmentally friendly (Fiorenzano et al. 2017; WHO, 2012). Fiorenzano et al. (Fiorenzano et al. 2017) recently highlighted that sugar-baiting has been effective in controlling multiple mosquito species including major disease vectors such as *Ae. aegypti* (Khallaayoune et al. 2013), *Culex pipiens* (Khallaayoune et al. 2013), *Ae. albopictus* (Revay et al. 2014; Junnila et al. 2015; Qualls et al. 2012; Naranjo et al. 2013), *Anopheles gambiae* (Qualls et al. 2015; Muller et al. 2010; Stewart et al. 2013), and *Culex quinquefasciatus* (Khallaayoune et al. 2013; Qualls et al. 2012; Stewart et al. 2013; Muller et al. 2010), with low impacts on non-target arthropods. A recent study confirmed that sugar feeding is a common behavior of *Ae. aegypti* females in urban areas and suggested that TSBs on plants could be a

potentially effective control strategy (Qualls et al. 2016). Given both the projected expansion of *Ae. aegypti* due to climate change and the potential use of TSB as a control strategy, understanding how temperature affects sugar-feeding in *Ae. aegypti* is a knowledge gap that needs to be filled.

2. Materials and Methods

2.1. Insects

The strain of *Ae. aegypti* mosquitoes used was Rockefeller (MR-734, MR4, AATCC® , Manassas, VA, USA). Larvae were reared in 26 × 35 × 4 cm covered trays that were filled with deionized water with about 200 larvae per tray. The trays were kept in a climatic chamber at 26 °C ± 0.5 °C and 60 ± 10% humidity under light:dark cycles of 12 h:12 h. The diet of the larvae consisted of Hikari Tropic First Bites (Petco, San Diego, CA, USA). For the experiment, around 120 pupae were placed into mosquito breeding containers (BioQuip, Rancho Dominguez, CA, USA—1425, 1425DG) on the day of pupation and until emergence. No sugar was provided to the recently emerged adults before the and until emergence.

2.2 Actometer Experiments

Actometer setup. Mosquito activity was measured using an actometer (Model LAM25, TriKinetics Inc, Waltham, MA, USA) (Figure 1A). Cotton plugs (Genesee Scientific, Morrisville, NC, USA, Cat. 49-102) were cut in half and pierced to fit a plastic transfer pipette (Fisherbrand. Waltham, MA, USA, Cat. 13-711-7M) through. The plastic transfer pipette was then filled with either 10% sucrose (Sigma Aldrich, CAS #57-50-1) solution or DI water depending on which variable was being tested. The water group also serves as a control for a possible effect of humidity on mosquito behavior and thus allowed us to decouple the effect of access to a source of food from

the impact of humidity on the general activity. A cotton ball was then rolled up and placed into the pipette bulb so that half of it was in the liquid being tested, and the other half was left outside so the mosquito could have access to it. This technique allowed for the cotton to stay humid for the whole experiment (*i.e.*, 7 days). Then, 1-day-old unfed mosquitoes were collected (32 males or 32 females). After being stored at 4°C for approximately 5 min, the mosquito container was placed on ice to further prevent the mobility of the mosquitoes. One mosquito was placed in each glass tube and the cotton plug and pipette bulb apparatus were placed to prevent the mosquito from escaping. The tubes were then placed in the actometer which was then placed in the climatic chamber at either 20, 25, or 30 degrees Celsius (°C) (Figure 1B). The relative humidity in the tubes was 80 ± 10%. Each of the twelve conditions was conducted in duplicates (n = 64 mosquitoes per group). The mosquito activity (i.e., the number of beam crossing per 10 min intervals) was recorded for 7 consecutive days using the DAMSystem3 Software (Trikinetics, Waltham, MA, USA). After seven days, the mosquitoes were collected by briefly placing the tubes on ice to anesthetize the individuals and placed in 1.7 microliter tubes for storage at −70°C until the total carbohydrate content assays were conducted.

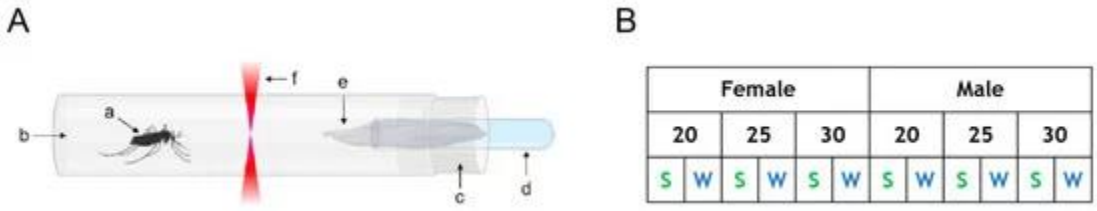

Figure 1. Actometer Experimental Setup. (A) Schematic of the setup for monitoring the mosquito activity. A mosquito (a) is placed in a glass tube (b) closed by a cotton plug (c) pierced in its center to fit a cut transfer pipette (d) that is filled with either a 10% sucrose solution or DI water. A cotton mesh (e) is inserted in the transfer pipette and provides access to the solution and maintains a high RH (Relative Humidity) in the tube for the entire experiment. The actometer can hold

32 tubes. (B) Table summarizing the twelve different conditions tested. Numbers indicate temperature in °C. S: sugar, W: water.

Data analysis. The activity of the 64 mosquitoes for each condition was analyzed through William's mean, which is used to support datasets with zero values (Haddow, 1960). Insects that died during the course of the 7-day experiments were recorded and their activity was analyzed and included in the analysis until the last movement was detected by the actometer. Lighting change was accounted for as well through the exclusion and interpolation of the arithmetic mean right before and after the lights were turned off. This helped to avoid bias associated with the increase in activity when the lights were turned off (Eilerts et al. 2018; Gentile et al. 2006). The activity of the different groups was compared time point by time point using a pairwise Student's t-test; p-values were adjusted for multiple comparisons with the Bonferroni method using R (R Core Team, 2016). Normality was assessed through a Shapiro–Wilk test.

2.3 Total carbohydrates content assays

Total carbohydrates contents were measured using the method described by van Handel (van Handel, 1985) (Figure 2). Briefly, anthrone (Sigma-Aldrich CAS #90-44-8) reagent was prepared by combining 150 mL water in a 1-L Erlenmeyer flask on ice with 380 mL sulfuric acid (Fisher CAS #7664-93-9), in which 750 mg of anthrone was then dissolved. Each mosquito analyzed was placed in a culture glass tube (Sigma-Aldrich C1048-72EA) that was filled to a 5 mL mark with anthrone reagent and then crushed with a glass rod. The sample was heated for 17 min at 92 °C in a dry bath, then cooled before being vortexed for 15–20 s. A sample without a mosquito was prepared additionally as a blank for the spectrophotometer (Perkin Elmer Lambda 20 UV/Visible Spectrophotometer). The optical density (OD) of each sample was then determined

at 625 nm. For samples with an OD625 above one, 200 µL of sample was diluted with 800 µL anthrone reagent that had been heated as above, giving a dilution factor of 5. The carbohydrate content was quantified using the OD values and a calibration line that had been created by performing the above procedure with samples containing 25, 50, 100, 150, and 200 µg of glucose solution. This assay was performed with both male and female *Ae. aegypti* mosquitoes that were 7 days old and alive at the end of the actometer experiments.

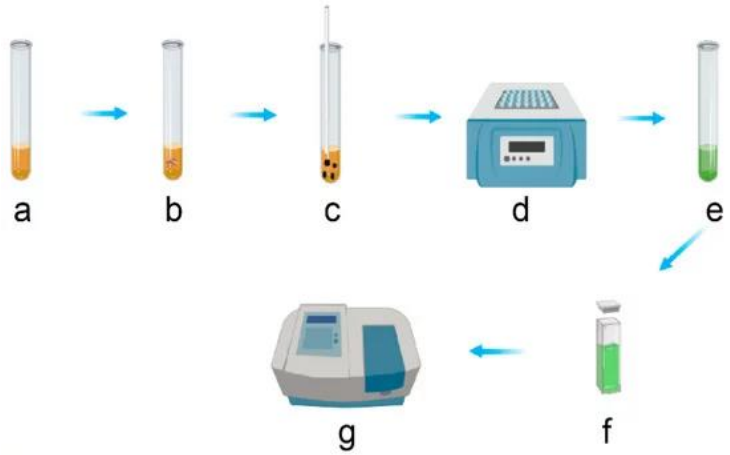

Figure 2. Total Carbohydrate Analyses Schematic. The different steps used for the total carbohydrate contents analysis. (a) 5 mL of cold anthrone reagent is placed in a test tube; (b) A single mosquito is added to the anthrone and (c) crushed. (d) The mixture is heated at 92 °C for 17 min and (e) cooled down before being (f) transferred to a cuvette. (g) The absorbance is read using a spectrophotometer and later converted to sugar content.

Data analysis. Normality was assessed through a Shapiro–Wilk test. A two-way ANOVA followed by a Tukey HSD post hoc test was used to assess differences between groups using R.

3. Results

3.1. Actometer Experiments

Activity. In both females and males, the activity increased with temperature (pairwise *t*-test with Bonferroni correction, for all comparisons: $p < 0.001$) (Figure 3). In females, the water-fed groups were significantly more active than the sugar-fed group at all three tested temperatures (*t*-test, for all comparisons: $p < 0.001$). However, in males, access to a sugar source increased activity at 20 and 25°C but not at 30°C. Overall, water-fed females tended to have a higher flying activity than water-fed males (*t*-tests, for all comparisons: $p < 0.001$). However, when provided with sucrose, males were more active at 20 and 25°C (*t*-tests, for all comparisons: $p < 0.001$) compared to females, but not at 30°C (*t*-test, $p = 1$). Interestingly, we noticed some nocturnal activity in both females and males under all conditions.

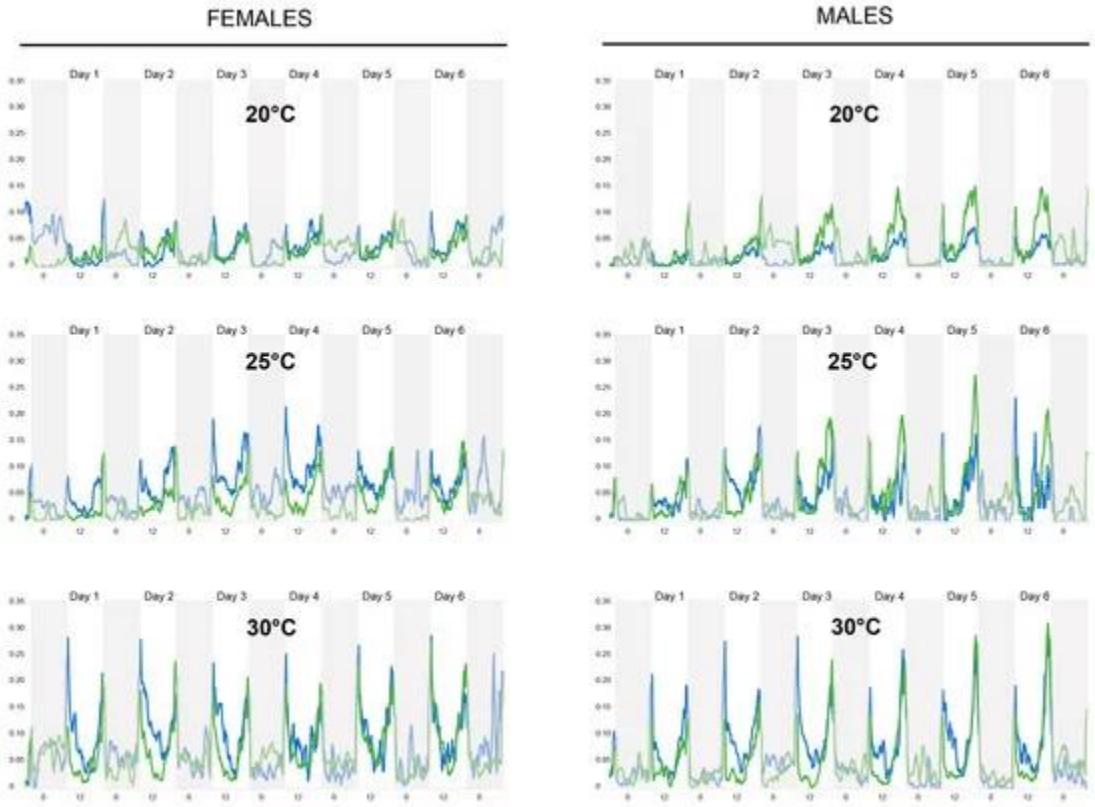

Figure 3. Actometer Activity. Activity results for females (left) and males (right) at the three different temperatures tested (20, 25, and 30°C). Blue lines exhibit results for the water-fed mosquitoes while green lines exhibit results for the sucrose-fed mosquitoes. Each line is the average activity (Williams' mean) (y-axis) of 64 mosquitoes. Grey vertical bars indicate nighttime and white vertical bars indicate daytime. The x-axis represents time: 0 = midnight, 12 = noon.

Survival. In both females and males, sucrose increases the survival of mosquitoes compared to the water groups (Figure 4A). However, females and males did not show difference in their survival. We then performed Log-rank tests comparing survival curves (Harrington and Fleming, 1982) built on our Kaplan–Meier estimates of the survival probability (Kaplan and Meier, 1958) (Figure 4B). We noted a significant difference in survival between the different treatments for both females and males (Log-rank test, $p < 0.001$).

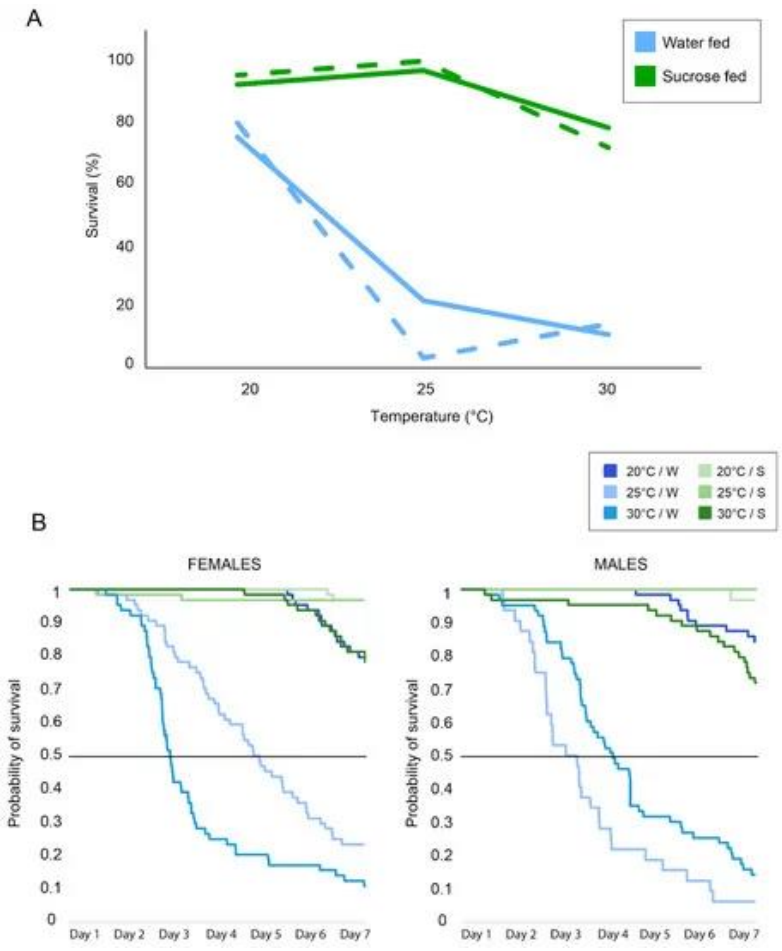

Figure 4. Actometer Survival. (A) Survival (in percentages) after the seven-day actometer experiments at different temperature regimes for females (plain lines) and males (dashed lines) when fed with sucrose (in green) or maintained on water (in blue). (B) Raw (Kaplan–Meier) survival data throughout the course of the seven-day experiments for the different conditions tested in females (left) and males (right). The black line indicates 50% of mosquito mortality.

In females, access to sugar had a significant positive impact on survival (Log-rank tests, for all comparisons $p < 0.001$). A strong effect of temperature on survival was noted in the water groups (Log-rank tests, for all comparisons $p < 0.001$). However, access to sucrose minimized the effect of temperature between groups maintained at 20°C and 25°C (Log-rank test $p = 1$) but not for the other groups. Both female groups with access to sugar at 20°C and 25°C had significantly higher survival rates (92.19% and 96.88%, respectively) than the sugar-fed female group at 30°C

(78.13%) (Log-rank test, $p = 0.001$ and $p = 0.002$, respectively). In males, no significant difference between sucrose and water-fed groups was found at 20°C (Log-rank test $p = 0.07$), while at 25°C and 30°C, sugar access significantly increased the mosquito survival (Log-rank test, $p < 0.001$). Temperature had a significant impact on survival for both the sucrose groups and water groups (Log-rank test, for all comparisons $p < 0.001$). For water-fed males, the 20°C group had the highest survival rate (79.69%), followed by the 30°C group (14.06%) and the 25°C group (3.13%). For the sugar-fed males, the 25°C group had the highest survival rate (100.00%), followed by the 20°C group (79.69%) and the 30°C group (71.88%).

The average number of days lived, including mosquitoes that survived during the whole experiment and ones that did not, was higher for females which had access to sugar (20°C: 6.51 days; 25°C: 6.38 days; 30°C: 6.27 days) compared to the water-fed groups (2°C: 6.35 days; 25°C: 4.2 days; 30°C: 2.88 days) (t-test, for all comparisons, $p < 0.001$). This was also observed for males (sucrose groups: 20°C: 6.5 days; 25°C: 6.06 days; 30°C: 6.54 days; water groups: 20°C: 6.24 days; 25°C: 3.67 days; 30°C: 2.78 days) (t-test, for all comparisons, $p < 0.001$).

3.2. Total Carbohydrates Content Assays

Upon generation of total carbohydrate contents using van Handel's method (Figure 2), any statistical variance between differing experimental groups was sought out using a two-way ANOVA, followed by a Tukey HSD post hoc test. A summary of how each variable or combination thereof plays a role in affecting the overall carbohydrate content of *Ae. aegypti* is displayed in Table 1. Each individual variable (i.e., temperature, food source, and sex) had a statistically significant effect on carbohydrate concentrations (for all comparisons, $p < 0.001$). In

addition, these three variables in conjunction with one another were shown to also affect carbohydrate levels ($p = 0.004$).

Factors	Df	Sum sq	Mean Sq	F value	Pr(>F)	Significance
Temperature	2	121442	60721	37.876	6.09E-16	***
Foodsource	1	110499	110499	68.926	1.18E-15	***
Sex	1	49724	49724	31.016	4.38E-08	***
Temperature:Foodsource	2	10465	5232	3.264	0.03914	*
Temperature:Sex	2	1628	814	0.508	0.6022	
Foodsource:Sex	1	6	6	0.004	0.9522	
Temperature:Foodsource:Sex	2	17728	8864	5.529	0.00424	**
Residuals	455	729438	1603			

Table 1. Total Carbohydrate Analyses ANOVA Analysis. Summary table for the two-way ANOVA analysis of the impact of the access to sugar, sex, and temperature on the total carbohydrate concentrations in *Aedes aegypti* mosquitoes.

Following the ANOVA, we performed a Tukey HSD post hoc test to determine the statistical differences between each of the 12 experimental groups (Figure 5).

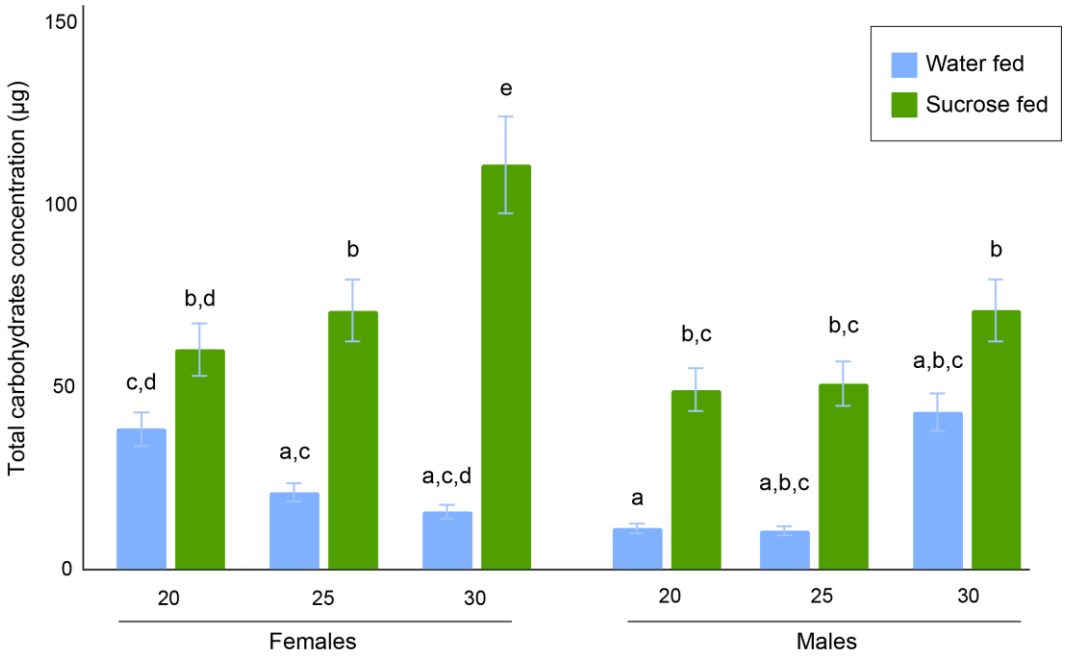

Figure 5. Total Carbohydrate Concentrations. Bar plot of the total carbohydrate contents in females and males. Bars indicate the standard error of the mean. Letters above bars denote statistical differences between groups. (Females—20 °C WF: n = 48, SF: n = 58; 25 °C WF: n = 14, SF: n = 61; 30 °C WF n = 7, SF n = 50; Males—20 °C WF n = 50, SF: n = 60; 25 °C WF n = 2, SF n = 64; 30 °C WF n = 9, SF n = 25). WF = water-fed; SF = sucrose-fed.

In females, the 30°C sugar-fed group had the highest relative sugar concentration (108.01 ± 9.33 µg) and was statistically different from the 20°C sugar-fed group (60.10 ± 4.98 µg) and 25°C sugar-fed group (70.85 ± 6.77 µg) ($p < 0.01$). However, no significant difference was detected between the 20°C and 25°C sugar-fed groups ($p = 0.94$). In addition, the 25°C sugar-fed group was significantly different from the three water-fed female groups ($p < 0.01$ for 20°C and 25°C and $p = 0.02$ for 30°C), for which sugar concentrations were 38.59 ± 6.42 µg, 20.9 ± 5.47 µg, 15.61 ± 2.79 µg, respectively.

In males, the 20°C and 25°C sugar-fed groups had sugar contents of 49.12 ± 2.86 µg and 50.77 ± 3.62 µg, respectively, and no significant difference was found ($p = 1$). The 30°C sugar-

fed male group had a higher sugar content (71.10 ± 6.33 µg) but was not significantly different than the 20°C or 25°C groups ($p = 0.16$ and $p = 0.24$, respectively). Each of the sugar-fed groups had a significantly ($p < 0.01$) higher sugar content than the 20°C water-fed group (11.02 ± 0.66 µg). Statistical differences were not observed between the sugar-fed and water-fed groups when compared with the 25°C (10.36 ± 3.79 µg) and 30°C (42.90 ± 13.67 µg). This may be due to the small sample sizes due to high mortality in these two groups ($n = 2$ for the 25C water-fed males and $n = 9$ for the 30°C males).

When comparing females and males, we found that the 20°C water-fed males and females were significantly different ($p = 0.0281$). The 25°C water-fed female and male groups were not significantly different ($p = 0.15$), although this may be related to the small sample size of the 25°C male water-fed group ($n = 2$). The 30°C water-fed female and male groups were also not significantly different, but this may also be due to small sample sizes. The sugar content of the 20°C sugar-fed females was not significantly different than the 20°C sugar-fed males ($p = 0.96$), and the 25°C sugar-fed females' sugar content was also not significantly different than the 25°C sugar-fed males' sugar content ($p = 0.15$). A significant difference ($p < 0.01$) was found between the 30°C sugar-fed females and 30°C sugar-fed males.

4. Discussion

The daily patterns of activity and survival results from the actometer experiments provide essential insights into how females and males are affected by temperature and how this effect is mediated by access to sugar. In the present study, we show that sugar deprivation increases activity in females at all tested temperatures while sugar deprivation only increases males' activity at 30°C and decreases it at 20°C and 25°C. It is worth mentioning that the activity results for the females

at 30°C and for the males at 25°C and 30°C may be influenced by the low number of surviving mosquitoes in those groups. Overall, males had a higher level of activity when they had access to sugar. This can be explained by the fact that males rely entirely on sugar feeding to sustain their metabolism and have lower energetic reserves compared to females (Foster, 1995; Van Handel, 1965). Males also take smaller sugar meals and are required to seek for nectar more often than females, which need carbohydrates, but can also rely on blood as a source of water and nutrients (Foster, 1995; Gary and Foster, 2006). In the absence of sugar, the females may have increased activity because of their higher nutrient pools carried over from the larval stage that can be used as a source of energy for flight (van Handel, 1965).

Our results show that access to sugar improved survival for both females and males across all three tested temperatures. Interestingly, most females (~75%) and males (~80%), when maintained at 20°C, were able to survive the whole experiment (7 days) without access to sugar, thus highlighting their resilience and tolerance to an environment with limited resources. This indicates that under cool temperatures, mosquitoes can easily survive without access to nectar by decreasing their general activity and use stored energy reserves while waiting for more favorable conditions to return. Higher temperature had an overall negative impact on survival rates, although access to sugar minimized this effect. These results agree with Costa et al. (2010), who found that survival rates decreased as temperature increased from 25°C to 30°C and 35°C. The seemingly optimum temperature range around 25°C indicates that in the face of climate change, regions with temperatures nearing closer to 30°C may experience declining *Ae. aegypti* populations and associated diseases, while areas with temperature averages rising to around 25°C may see increases in *Ae. aegypti* populations and disease.

Our actometer data clearly show the two peaks of activity (i.e., at dawn and dusk) that have been previously reported in this species (Christophers, 1960). Interestingly, we also show that the mosquitoes (both females and males) were active at night. *Ae. aegypti* has been classically considered a day-active species, but our data indicate that nocturnal activity also occurs. This is consistent with previous reports of nectar-feeding activity during the night or at dusk/dawn in several mosquito species, both diurnal and nocturnal (Clements, 1999; McCrae et al. 1976; Yuval et al. 1994; Smith and Gadawski, 1994). Indeed, sugar-feeding in wild mosquitoes has been observed to have diel periodicities, suggesting that an endogenous rhythm underlies this behavior, although it has been found that sugar-feeding is also dependent on the time in relationship to the time of sunrise and sunset (Clements, 1999; Grimstad and DeFoliart, 1974).

The total carbohydrates assay indicates that the sugar-fed mosquitoes consumed the most at 30°C, as both females and males had the highest sugar content among all groups. This agrees with previous findings that insect sugar consumption increases with temperatures between 20°C and 30°C (Lemoine et al. 2014). This also fits with the actometer results that show that both sexes had the highest activity at higher temperatures because higher activity would likely indicate more frequent visitation of the sugar source, and the higher activity would be enabled by the higher energy consumption. This positive relationship may be caused by the increase in metabolism that occurs at elevated temperatures (Berrigan and Partridge, 1997; Gillooly et al. 2001; Klespsatel et al. 2019; Vorhees et al. 2012) and triggers an increase in the mosquito activity which enables them to eat enough to upkeep with their metabolism. This would agree with a recent observation by Klepsatel et al. (2019) who reported a positive correlation between metabolism and temperature, as well as food consumption and temperature in *Drosophila*. Conversely, lower temperatures led

to lower activity, likely because the metabolic rates were lower, thus the mosquitoes did not need to eat as much to meet their metabolic energy demands.

Toxic sugar baits have emerged as an important tool for controlling mosquito populations and may prove to be particularly useful for *Ae. aegypti* and other disease vectors in light of this positive relationship between sugar-feeding and temperature and the projected global warming temperature increases. Rising temperatures will likely raise metabolic rates and affect levels of water and thus cause higher sugar consumption. Furthermore, understanding how much sugar is consumed at different temperatures, which, as shown here, influences flying activity, could be a consideration when determining dose concentrations of the sugar baits. Determining a precise toxin concentration for the TSBs based on fluctuating weekly environmental temperatures may prove to make them even more cost-effective and powerful. TSBs during the warmest months could be more cost-effective and use lower doses because the temperature should cause the mosquitoes to eat more, thus a lower toxin concentration would be needed. Additionally, using projected global temperature changes to determine what geographic regions will have optimum sugar-feeding conditions can inform on what areas may benefit the most from TSB use.

This study sheds light on the combinatorial effects of constant temperature, sugar availability, and activity in an important disease vector mosquito species. The next step is to conduct assays with fluctuating temperatures (i.e., cooler nights and warmer days) to reflect current natural settings and predicted ones to observe the potential impact on the mosquito activity in the presence or absence of a sugar source. This will lead us to have a better understanding of how global warming might affect general mosquito activity and survival, and consequently its effect on their global distribution and population dynamics. This is critical to predict so that regions most likely to have high population densities are equipped with supplies to control and mitigate

their populations and accompanying diseases. As sugar sources are variable throughout the year (at least in temperate regions) (reviewed by (Foster, 1995)), it also appears essential to get a better knowledge of the potential sources of nectar that mosquitoes feed on in the field. As climate evolves, certain plants might have a longer/shorter blooming seasons which might in return affect mosquito population dynamics. Additionally, as the effects of temperature on *Aedes* survivability and oviposition have been found to be humidity level dependent (de Almeida Costa et al. 2010; Schmidt et al. 2018), future assays could investigate the impact of humidity on sugar-feeding as well. Understanding this factor's effect will enable a more global understanding of climate change's potential effect on mosquito population distribution and season duration as global warming is projected to change global humidity levels (Coffel et al. 2017). Future related work could also investigate the effects of dehydration on sugar-feeding as drought frequency is predicted to increase with global warming (Dai, 2013), and dehydrated mosquitoes have been shown to have heightened activity levels and blood-feeding behavior (Hagan et al. 2018). Finally, we conducted this work using a well-established line of *Ae. aegypti* that has been maintained for many generations under laboratory conditions. It would thus be interesting to compare the present results with data from field-caught mosquitoes. Overall, determining how changing temperatures and humidity will affect *Ae. aegypti* behavior (including sugar-feeding) is crucial for understanding how population distribution and dynamics will be affected.

5. Conclusions

Combining actometer experiments and calorimetric assays, we studied the impact of temperature and access to sugar on *Ae. aegypti* mosquitoes' activity and survival. We show that access to sugar increases survival in both females and males and that activity was also correlated

with access to sugars. The temperature had a strong effect on the general activity in both sexes and on carbohydrates consumption and storage. This study is the first to assess the combined effects of temperature and access to a sugar source on females and males *Ae. aegypti* mosquitoes' daily activity patterns. It is of particular importance in the context of climate change and the emergence of new control tools such as the Toxic Sugar Baits. This study constitutes the first step of investigation on the extent to which temperature, and in particular future climate, might affect mosquito distribution and how access to sugar might contribute to their overall fitness.

Author Contributions

Conceptualization, C.L.; data curation, I.F.U., E.A.B., C.H. and C.L.; funding acquisition, C.L.; investigation, I.F.U., E.A.B., C.H. and C.L.; methodology, C.L.; project administration, C.L.; supervision, C.L.; validation, C.L.; visualization, C.L.; Writing—Original draft, I.F.U., E.A.B. and C.L.; Writing—Review and editing, I.F.U., E.A.B. and C.L.

Master's candidate Irving Forde Upshur specifically contributed the following: conducting total carbohydrate analyses and organizing data, writing methods and results, and reviewing/editing full document.

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Chapter 2: Sugar-Feeding by Invasive Mosquito Species on Ornamental Plants

by Irving Forde Upshur, Aley Savory and Chloé Lahondère

In preparation for the *Journal of Medical Entomology*

Abstract: Feeding on plant-derived sugars is an essential component of mosquito biology that affects key aspects of their lives such as survival, metabolism, and reproduction. Mosquitoes locate plants to feed on using olfactory and visual cues. *Aedes aegypti* and *Aedes albopictus* are two mosquito species invasive to the US, and are vectors of diseases such as dengue fever, chikungunya, and Zika. These species live in urban, heavily-populated areas, where they have a high accessibility to human hosts as well as to plants in backyards and town landscapes. Therefore, it is important to understand what plants might attract / repel mosquitoes to inform citizens and authorities accordingly. Here, we observe *Ae. aegypti* and *Ae. albopictus* sugar-feeding behavior with eleven different commonly-planted ornamental plant species. We then assessed feeding activity using the anthrone method. The composition of volatiles in the headspace of each plant was then examined using gas-chromatography mass-spectroscopy, and how these volatiles may be mediating feeding interactions is discussed.

Keywords: *Aedes aegypti*, *Aedes albopictus*, phytophagy, behavior, olfaction, gas-chromatography

1. Introduction

Phytophagy, the act of feeding on plants, is important for many insect species, including blood-sucking mosquitoes. Acquiring carbohydrates is essential for both male and female mosquitoes and can in some species constitute the sole source of food for adults (*e.g.*, *Toxorhynchites* spp.). Males feed exclusively on plant-derived sugars and recently-emerged females tend to seek sugar before taking their first blood-meal for egg production and oviposition (Foster, 1995). In addition to carbohydrates, it has also been determined that mosquitoes are acquiring vitamins, amino acids and salts from plant nectar (Baker and Baker, 1973; Nicolson and Thornburg, 2007; Rivera-Perez et al., 2017).

To locate a sugar meal, mosquitoes are driven by several cues, including vision and olfaction (*e.g.*, plant-emitted semiochemicals) (Nyasembe and Torto, 2014). Volatile odorant molecules are perceived via odorant receptors present on the antennae. These odor receptors are fine-tuned to be specifically receptive to certain volatiles (Nyasembe et al. 2012). The information is then processed in the brain and, depending on the type of chemical, the behavioral output changes. Some chemicals will elicit an attractive or repellent response from the mosquito, while others will not elicit a response (*i.e.*, indifference) (Lahondere et al. 2020). Compounds classified as terpenes and benzenoids are common in flower scent profiles and drive mosquito attraction. Mosquitoes have been shown to be attracted to flower semiochemicals like linalool, (Z)-3-hexen-1-ol, and benzaldehyde and repelled by compounds such as β-myrcene and limonene (Nyasembe and Torto, 2014; Jaleta et al. 2016). Building on a more comprehensive understanding of which plant-emitted semiochemicals are attractive and repellent to mosquitoes can lead to the development of new and efficient disease vector control tools. It is even more important as we are

facing mosquito insecticide resistance worldwide, which challenges current control efforts (Liu et al., 2015).

A promising mosquito control strategy is the use of toxic sugar baits (TSBs) containing mixtures of attractive odorants and toxic compounds and taking advantage of mosquitoes' natural requirement to feed on sugar. These traps bypass pre-existing problems that conventional control strategies have faced, such as insecticide resistance, and have been shown to have a low impact on non-target organisms like plant pollinators and mosquito predators (Gu et al. 2020; Khallaayoune et al. 2013). Briefly, a chosen insecticide is mixed with sugar in water and supplemented with an attractive volatile compound sourced from a plant, after which the mixture can be applied to a plant, fruit or bait-station (Revay et al. 2015). Because of the relatively limited knowledge of volatile attractants in the field, more studies of TSBs have been observed through application to known attractive plants and fruits (*e.g.*, guava, mango, banana) rather than baits (Meza et al. 2020). Examples of compound classes used as oral insecticides include pyrethroids, organophosphates, and neonicotinoids, while examples of attractive compounds include eugenol and garlic oil (Fiorenzano et al. 2017). Successful TSB use has been observed on *Aedes aegypti*, *Aedes albopictus*, *Culex pipiens*, *Culex quinquefasciatus*, and *Anopheles gambiae* (Fiorenzano et al. 2017).

Among current disease vectors that are of particular concern are *Ae. aegypti* and *Ae. albopictus* mosquitoes. These species are responsible for spreading dengue, chikungunya and Zika viruses, all of which still have frequent incidence globally as vaccines and / or treatments remain unavailable (Leta et al. 2018). According to a recent study by Leta et al. (2018), a total of 215 countries and territories exhibit environments that are suitable for *Ae. aegypti* and *Ae. albopictus* habitation. In the context of climate change and global warming, the geographic distribution of

these two species might shift and potentially spread diseases in new areas (Ryan et al. 2019). As mosquitoes are poikilotherms (*i.e.*, cold blooded animals), physiological, biological, and behavioral processes are highly impacted and dependent on the environmental temperature. Furthermore, *Ae. aegypti* and *Ae. albopictus* are characterized by a high ecological fitting, meaning that they can adapt to novel environments easily, which further contributes to their high invasivity (Reinhold et al. 2018).

Sugar feeding in mosquitoes is relatively understudied compared to host-seeking and blood-feeding behavior, as pathogens are transmitted to humans and animals when the insects bite. Yet it appears crucial to study this behavior, as it bears the potential for the development of new tools for vector surveillance and control. Resources that mosquitoes use in populated urban areas to obtain a sugar meal and how invasive species adapt to local ornamental plants remain poorly understood. Some ornamental plants (*e.g.*, *Ligustrum quihoui*, *Pittosporum tobira*, *Loropetalum chinense*) have been shown to increase survivorship in *Ae. albopictus*, and could therefore contribute to their ability to successfully transmit disease by increasing their overall fitness (Tian et al. 2019). In addition, the distribution of a population of *An. gambiae* male mosquitoes in Western Burkina Faso was shown to be influenced by the presence of absence of ornamentals (Gouagna et al. 2010). Ornamental plant abundance has been shown to directly affect the population distributions of both *Ae. aegypti* and *Ae albopictus* during field experiments in southern Mexico and Guangzhou, China, respectively (Martinez-Ibarra et al. 1997; Li et al. 2014). When tested for the presence of sugar in their crop, individuals from both species exhibited a higher proportion of sugar feeding when collected from an urban area with a higher amount of blooming ornamental plants, compared to urban areas with little or no ornamental plant presence. This prompts the hypothesis that certain ornamental species, by providing nectar to the mosquitoes,

might greatly influence their fitness and could consequently increase the risk of disease transmission in heavily populated areas.

The present study aims to get a better understanding of sugar feeding preferences in *Aedes aegypti* and *Ae. albopictus* in the US. Indeed, these two species originally from Asia and Africa have been able to adapt to the US and other parts of the world where the plants they encounter are different from their native areas. We first examined mosquito landing and feeding preference on different ornamental plant species that are commonly found in nurseries and backyards. These plant species varied in flower shape, size, color, scent, and nectar contents, so that the possible effect of sugar availability on preference may be observed. We then analyzed the scent profile of each of these plants by gas-chromatography coupled with mass-spectrometry to identify chemicals emitted by the plants that the mosquitoes might use to locate them or could repel them. Our next steps consist in conducting electro-antennograms coupled with gas-chromatography to identify chemicals that are detected by the mosquitoes and olfactometer assays to test single chemicals and blends to attract or repel these invasive species. This work improves our understanding of the elusive mosquito-plant relationship, and brings insights on the development of new and efficient control tools such as attractive baits or repellents.

2. Materials and Methods

2.1 Insects

The Rockefeller *Ae. aegypti* strain (MR-734, MR4, AATCC®, Manassas, VA, USA) and the ATM95 *Ae. albopictus* strain (ATM-NJ95, AATCC®, Keyport, NJ, USA) were used for this study. Larvae were reared in 26 x 35 x 4 cm covered trays that were filled with deionized water. The trays were kept in a climatic chamber at 26°±0.5°C and 60 ± 10% humidity under light:dark

cycles of 12h:12h. The diet of the larvae consisted of Hikari Tropic First Bites (Petco, San Diego, CA, USA). For the experiment, around 100 pupae were placed into mosquito breeding containers (BioQuip, Rancho Dominguez, CA, USA—1425, 1425DG) on the day of pupation. Upon emergence, female and male mosquitoes were starved for 1-2 days before being individually selected with forceps. To do so, the containers were placed in a cool environment to immobilize mosquitoes and isolated for the following plant visitation assays.

2.2 Plant visitation assays

Plant Visitation. Two BugDorm-1 insect cages (BugDorm, DP1000) were placed on top of a 26 x 35 x 4 cm tray containing water to help prevent desiccation. These cages were then placed in a secondary larger acrylic cage which further provided a warm and humid environment for the mosquitoes. Each assay was conducted with both mosquito species to allow for better comparisons between the two species (*e.g.*, survival, feeding). Within each cage, a GoPro camera (Hero5 Black), a water-containing cup covered by a wet paper towel, and the flower of interest were placed (Fig. 6). Eleven ornamental plants were tested: Wave Petunia, Red Impatiens, Marigold, Celosia, Butterfly Bush, Guara, Lantana, Mexican Heather, Scaevola, Goldenrod, and Yarrow. In addition, an iButton (Maxim, DS1923) was programmed and

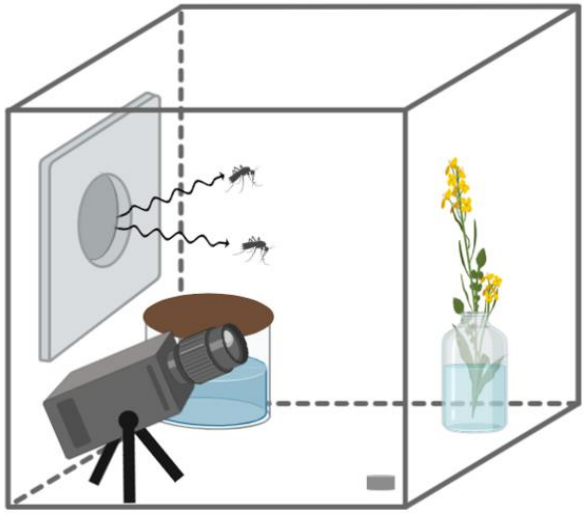


Figure 6. Plant Visitation Setup. The standard layout for a plant visitation assay. Mosquitoes are released after all elements are setup in the cage. Elements include ornamental flower, GoPro aimed at flower, water cup topped with soaked paper towel, and iButton temperature/humidity recording device.

added to the cage to record humidity and temperature throughout the assays (Table 2). Ten females and ten males of *Ae. aegypti* and *Ae. albopictus* were released into these cages at the end of the day between 4:30 and 5:30 pm, which has been previously reported as a peak sugar-feeding activity time for *Ae. aegypti* (Gillett et. al, 1962).

Plant Species	Mosquito Species	Average Temperature	Average RH
Wave Petunia	*Ae. aegypti*	N/A	N/A
Wave Petunia	*Ae. albopictus*	19.315	83.335
Red Impatiens	*Ae. aegypti*	19.25	83.48
Red Impatiens	*Ae. albopictus*	N/A	N/A
Marigold	*Ae. aegypti*	19.325	81.315
Marigold	*Ae. albopictus*	19.513	79.99
Celosia	*Ae. aegypti*	19.337	76.03
Celosia	*Ae. albopictus*	19.517	81.883
Butterfly Bush	*Ae. aegypti*	19.347	74.327
Butterfly Bush	*Ae. albopictus*	19.397	75.507
Guara	*Ae. aegypti*	19.233	80.4
Guara	*Ae. albopictus*	19.49	88.153
Lantana	*Ae. aegypti*	19.577	82.267
Lantana	*Ae. albopictus*	19.477	76.13
Mexican Heather	*Ae. aegypti*	19.06	74.113
Mexican Heather	*Ae. albopictus*	19.373	73.553
Scaevola	*Ae. aegypti*	19.5	69.76
Scaevola	*Ae. albopictus*	19.593	70.637
Goldenrod	*Ae. aegypti*	19.833	83.223
Goldenrod	*Ae. albopictus*	19.373	75.37
Yarrow	*Ae. aegypti*	19.365	74.32
Yarrow	*Ae. albopictus*	19.247	70.487

Table 2. iButton Data. Average temperature and relative humidity data across all ornamental and mosquito species, as recorded by the Maxim iButton during the experiments.

In the following morning, between 9:00 and 10:00 am, alive mosquitoes were collected using a Bug Vacuum (Redeo, XCQ-B), sorted by sex, and stored for future analysis at -70 ºC. Dead mosquitoes were tallied and removed before beginning another assay. Three replicates for

both mosquito species were conducted for each plant, totaling six assays per plant. Because of the large size of the plants, stems of Butterfly Bush, Guara, Lantana, Mexican Heather, Scaevola and Yarrow were cut to fit within the visitation assay cages. The scent profiles from cut flowers were compared with those of intact plants using GC-MS to ensure that no difference was noticed that could potentially affect the mosquitoes' landing and feeding behavior.

Video analysis. Video recordings for each assay were limited to around two hours after the assay started to ensure that the peak of sugar feeding activity observed in these mosquitoes (as described above) was included. Due to the consistent timing of initializing the assays, videos generally captured footage between 4:30 p.m. and 7:30 p.m. Each video was analyzed by counting the total number of mosquito landings and feedings on the flower of interest. Landings were defined as a mosquito flying to the flower and subsequently idling on it for any amount of time. A single feeding was defined as the vertical movement of a mosquito's head in a flower (*i.e.*, probing), which is characteristic motion of nectar-feeding. Additionally, the sex of each mosquito landing or feeding was indicated when possible.

2.3 Scent Collection and GC-MS analyses

Scent Collection. To collect the headspace of each plant species, the inflorescence of the plant was enclosed in a nylon oven bag (Reynolds Kitchens, USA) that was tight around the stem. Two tygon tubes (Cole-Parmer, USA) were connected on one side to a diaphragm air pump (Gast, Benton Harbor, MI, USA), while the other side was inserted at the small opening of the bag. Air flow was then initiated by connecting the pump to a 6V battery (Power-Sonic Batteries, USA). One tube pumped ingoing air into the bag through a charcoal filter cartridge (1 L/min.), which

served to remove any contaminants from the pump or the surrounding environment. The other tube pulled air out of the bag (1 L/min.) through a headspace trap composed of a borosilicate Pasteur pipette (VWR, Radnor, PA, USA) containing 100 mg of Porapak powder Q 80-100 mesh (Waters Corporation, Milford, MA, USA). After a minimum of 24 hours of headspace collection, traps were eluted with 600 μL of 99% purity hexane (227064-2L, Sigma Aldrich, Saint-Louis, MO, USA). The samples were sealed and stored in 2 mL amber borosilicate vials (VWR, Radnor, PA) with Teflon-lined caps (VWR, Radnor, PA) and were subsequently stored at −70°C until analysis by GC-MS. For headspace controls, samples were taken concurrently from empty oven bags. For each plant species, 12 collection replicates were performed, 2 of them were from cut stems to assess for any potential difference in scent emission between intact and cut flowers used for the plant visitation assays.

GC-MS Analysis. An aliquot of 20 μL was pipetted from each plant scent sample from the preceding method and placed into a vial with an insert (VWR, Radnor, PA) to be used with the GC-MS (Trace 1310, Thermo Fisher Scientific) equipped with a 30 m column (I.D. 0.25 mm, #36096-1420, Thermo Fisher Scientific). Helium was used as the carrier gas at a constant flow of 1 cc/min. After each sample was prepared for the species of interest, they were loaded into the machine using an autosampler (TriPlus RSH, Thermo FIsher Scientific). Generally, 14-16 samples were prepared, including a hexane blank, an "empty bag" control, the inflorescence replicates for each plant, and cut inflorescence, if applicable. The oven temperature was set at 45°C, held for 4 minutes followed by a heating gradient ramping to 230°C, held for 6 minutes (total run: 28.5 min.).

Chromatogram peaks were integrated using the Chromeleon software MS quantitative processing method (Thermo Fisher Scientific) and tentatively identified using the online NIST

library. Major peaks found with consistently high abundances across multiple samples for each ornamental were then recorded for comparison across ornamental species. An internal standard, made of different concentrations of heptyl acetate (Sigma-Aldrich, CAS #112-06-1, USA), was added to each sample to calculate the concentrations of each compound based on a calibration curve. External synthetic standards, if available commercially, will be used in the near future to confirm the chemical identity as in Lahondère et al. (2020).

2.4. Carbohydrate content assays

Nectar detection in the mosquito crop. Carbohydrate contents were measured by sequentially using the cold and warm anthrone methods described by van Handel (1985). First, mosquitoes were crushed with a glass rod in culture glass tubes (Sigma-Aldrich C1048-72EA) containing 300 μL of cold anthrone reagent to detect for fructose consumption, as fructose is a monosaccharide that would only be present in the mosquito if it fed on nectar. The anthrone reagent was prepared by combining 150 ml water in a 1 liter Erlenmeyer flask on ice with 380 ml sulfuric acid (Fisher CAS #7664-93-9), in which 750 mg of anthrone (Sigma-Aldrich CAS #90-44-8) was then dissolved. The samples were kept idle at room temperature (25°C) for 30 minutes and compared against a negative control and positive control containing a mosquito which was fed with a 20% fructose solution.

Quantitative carbohydrate assays. Following this, glass tubes were filled with anthrone reagent to a 5 mL mark, heated for 17 min at 92°C in a dry bath, and then cooled before being vortexed for 15-20 sec. A sample without a mosquito was prepared additionally as a control and blank for the spectrophotometer (Perkin Elmer Lambda 20 UV/Visible Spectrophotometer). The

optical density (OD) of each sample was then determined at 625 nm. For samples with an OD_{625} above one, 200 μL of sample was diluted with 800 μL anthrone reagent that had been heated as above, giving a dilution factor of 5. The carbohydrate content was quantified using the OD values and a calibration line that had been created by performing the above procedure with samples containing 25, 50, 100, 150, and 200 μg of glucose solution. An ANOVA was used to compare the carbohydrate contents between the different groups using the software R (R Development Core Team, 2017).

3. Results

3.1 Plant Visitation Assays

Landings: *Ae. aegypti* exhibited a higher number of visitations for the Guara, Mexican Heather, Goldenrod, and Yarrow ornamental species, indicating a species-dependent preference for these plants (Figure 7). Goldenrod had the highest number of landings from *Ae. aegypti* (20 total), while Red Impatiens and Marigold were visited by only one male (*i.e.*, M) and one female (*i.e.*, F), respectively. The variation in landings between male and female *Ae. aegypti* across all eleven ornamental plant species is low, with Butterfly Bush (0M, 5F), Mexican Heather (7M, 2F), and Goldenrod (13M, 17F) being the exceptions. The differences between male and female landings for the remaining eight plant species were no more than +/- 2 mosquitoes. Overall, less total landings were observed from *Ae. aegypti* across all eleven different ornamental plant species we tested, compared to *Ae. albopictus*.

Variation in landings between male and female *Ae. albopictus* was much higher than the variation in *Ae. aegypti* (Figure 7). Females landed more on Wave Petunia (11M, 19F), Marigold (8F, 16M), Lantana (1M, 7F), and Scaevola (2M, 7F). Conversely, males landed more on Celosia

(19M, 5F), Butterfly Bush (5M, 1F), Goldenrod (11M, 3F), and Yarrow (11M, 3F). No variation in sex was seen with Guara and Mexican Heater; both species were visited by one male and one female *Ae. albopictus*. These two plant species also had the lowest total landings from *Ae. albopictus*, while Wave Petunia (11M, 19F) had the most.

Landings from mosquitoes whose sex could not be determined were omitted from these results, but do not affect the cumulative trend of the data. Red impatiens and *Ae. albopictus* data is not present in these results, as a colony had not been established at the point that Red Impatiens was in the lab.

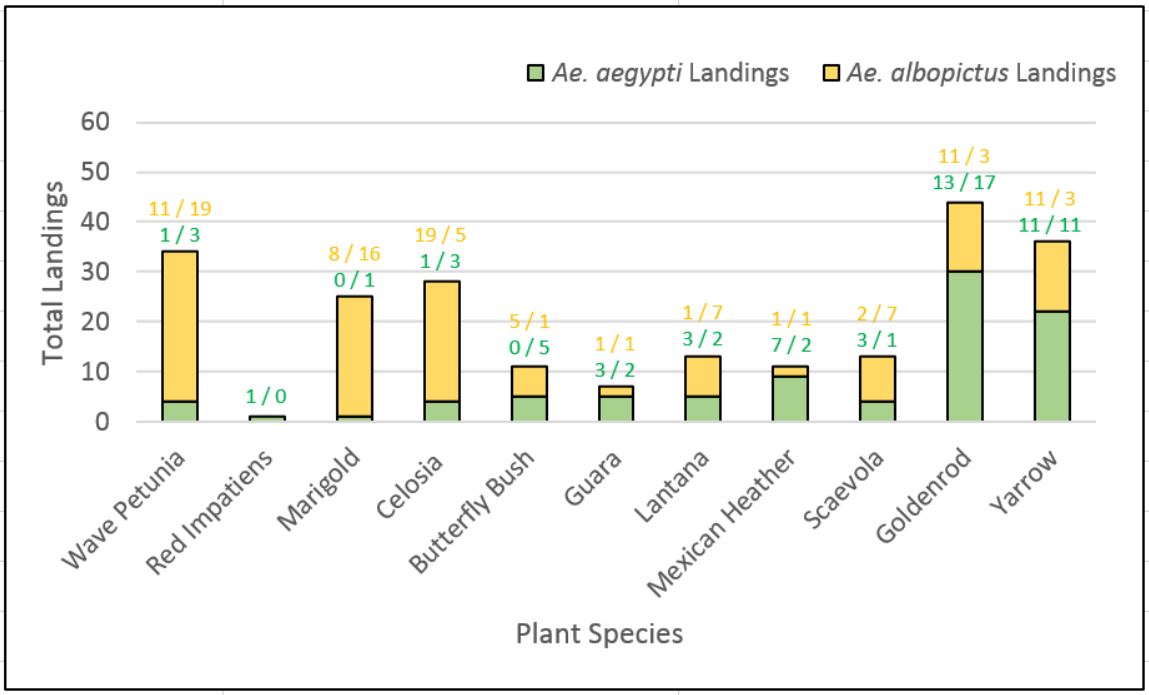


Figure 7. Plant Visitation Landings. Total number of landings by either *Ae. aegypti* (green) or *Ae. albopictus* (yellow) on the eleven different tested ornamental flower species. Landings are counted as any time a mosquito is seen idling on any part of the flower Landings by males and females are represented by numbers above the bars, and are color-coded by species; males are to the left of the slash while females are to the right. Unknown landings by either species are omitted from this graph.

Feedings: Feedings from *Ae. aegypti* were seen on five ornamental plant species, including Butterfly Bush, Guara, Lantana, Goldenrod, and Yarrow (Figure 8). Across all eleven species, only Goldenrod had more feedings from *Ae. aegypti*, compared to *Ae. albopictus*; Goldenrod also had the most feeding of the five plants fed on by *Ae. aegypti*. Most feeding was seen from females, with Butterfly Bush (0M, 4F), Guara (0M, 1F) and Lantana (0M, 1F) having only females feed and Goldenrod (2M, 7F) having significantly more feedings from females. Males fed more on Yarrow (2M, 1F) than females, but only by one mosquito. Red Impatiens was not fed on by *Ae. aegypti*, coinciding with the low number of landings seen in Figure 7. Across all eleven ornamental species, more feedings were observed from *Ae. albopictus*.

Ae. albopictus mosquitoes were seen feeding on eight of eleven ornamental species. In contrast to *Ae. aegypti*, more total feeding events were observed from male *Ae. albopictus* compared to females. Celosia (4M, 0F), Butterfly Bush (4M, 1F), Goldenrod (5M, 1F), and Yarrow (6M, 0F) all had a greater number of males feeding. The remaining four plants, Marigold (2M, 4F), Guara (0M, 1F), Lantana (1M, 3F), and Scaevola (0M, 3F), saw more female feedings, but the discrepancy between sex was much smaller. No feeding from either mosquito species were seen on Wave Petunia and Mexican Heather, the latter synchronizing with the low landing activity seen in the preceding subsection (Figure 7). Goldenrod and Yarrow were tied at six feedings for most feedings seen from *Ae. albopictus*.

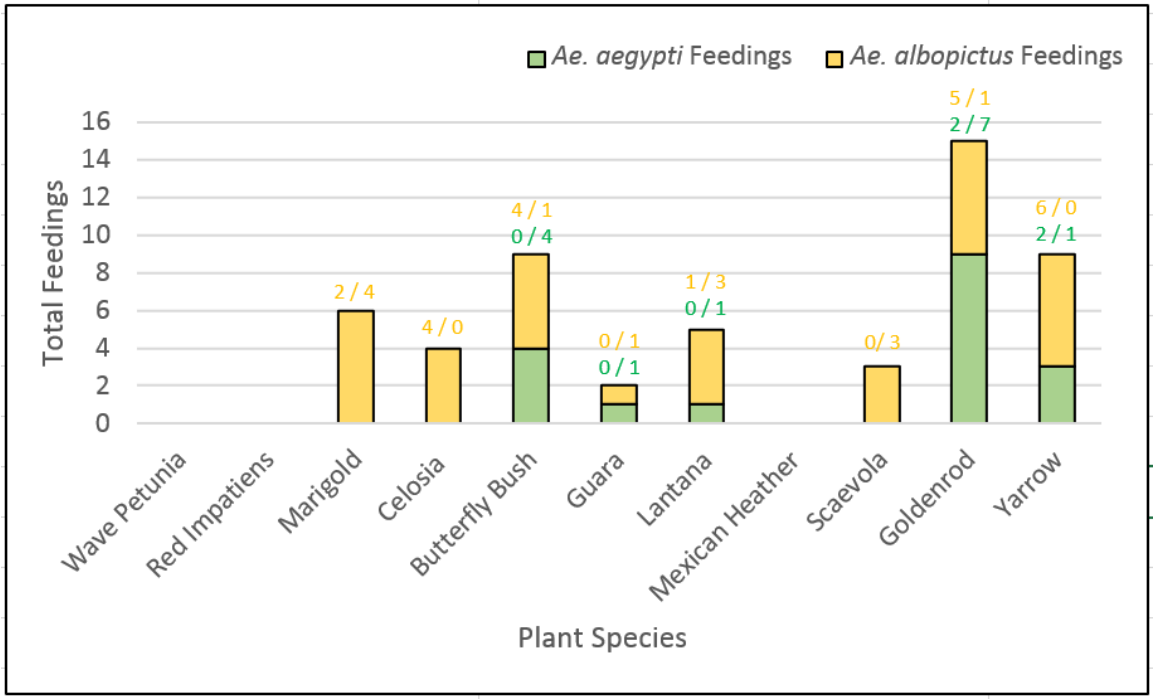


Figure 8. Plant Visitation Feedings. Total number of feedings by either *Ae. aegypti* or *Ae. albopictus* on the eleven different tested ornamental flower species. Feedings were defined by the vertical head movement characteristic of nectar-feeding. Feedings by males and females are represented by numbers above the bars, and are color-coded by species; males are to the left of the slash while females are to the right. Unknown feedings by either species are omitted from this graph.

Survival: *Ae. aegypti* survival rates, measured as an average across the three visitation assay replicates for each ornamental species, are represented in Figure 9. Across nine of eleven ornamental species, *Ae. aegypti* females exhibited a higher average survival rate than males, with the exception of a shared 97% and 80% average survival rate for Celosia and Lantana, respectively. Both males and females survived well in assays (97%) with the Celosia. The lowest survival rates from both sexes was observed with the Yarrow plant, with a 47% and 63% survival rate from males and females, respectively.

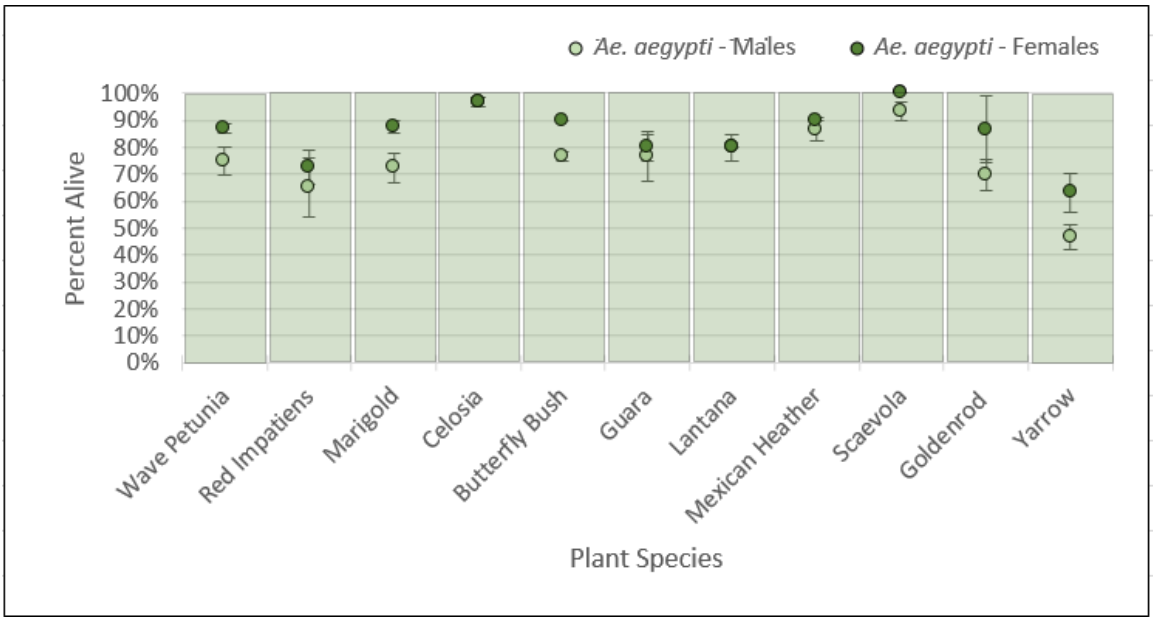


Figure 9. *Ae. aegypti* Plant Visitation Survival. Percentage of *Ae. aegypti* collected alive from the plant visitation assays and frozen down for later sugar analysis. Percentages are represented as averages of the three replicates (n = 30 for each data point) performed for each plant species. Survival is represented over the 16-hour timespan of the assay (5 p.m. – 9 a.m.).

Similarly, average survival rates of *Ae. albopictus* for each ornamental species are represented in Figure 10. In all cases, females survived at a higher rate than males. The variation in survival between *Ae. albopictus* females and males was generally greater than the variation between females and males in *Ae. aegypti*. Survival rates for females and males were highest with Celosia (97%) and Scaevola (80%), respectively; in contrast, lowest rates occurred with Marigold (63%) and Lantana (30%) for females and males, respectively.

Red Impatiens data is absent from these results, as a colony had yet to be established in the lab at the time of the test.

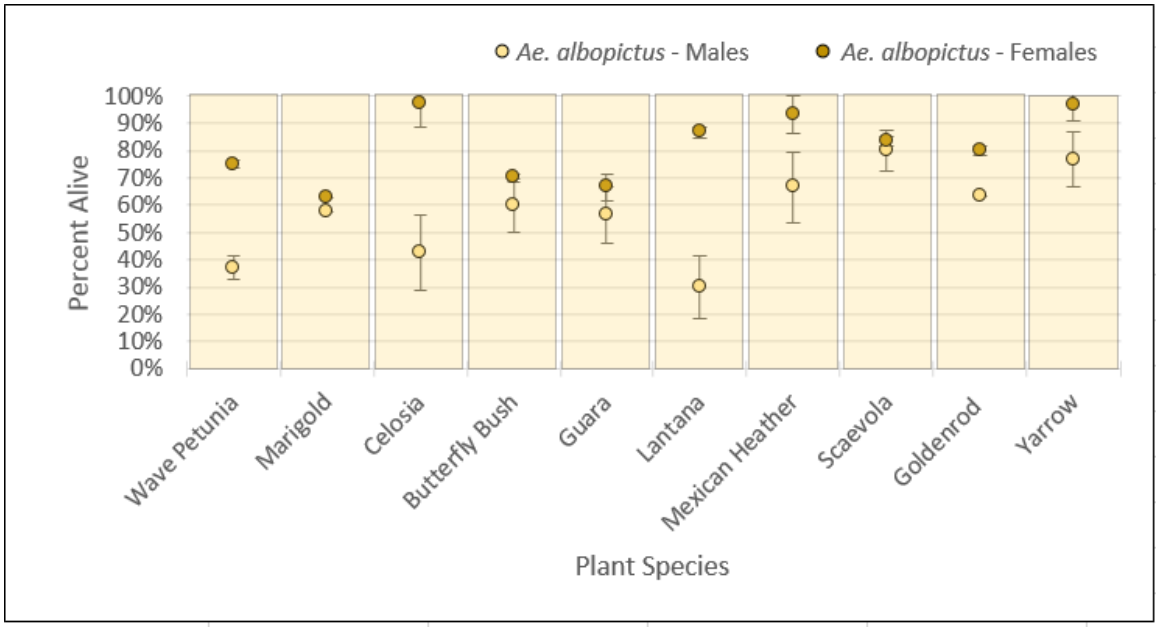


Figure 10. *Ae. albopictus* Plant Visitation Survival. Percentage of *Ae. albopictus* collected alive from the plant visitation assays and frozen down for later sugar analysis. Percentages are represented as averages of the three replicates (n = 30 for each data point) performed for each plant species. Survival is represented over the 16-hour timespan of the assay (5 p.m. – 9 a.m.).

3.2 Carbohydrate content assays

Qualitative Analysis. Mosquitoes that produced a positive result in the cold anthrone test indicated a confirmed fructose consumption. Of all mosquitoes that were alive at the end of the plant visitation assays, the percentage of mosquitoes that produced a positive test result are represented in Figure 11. *Ae. albopictus* produced a higher number of positive tests overall compared to *Ae. aegypti*. A preference for ornamentals between species can be observed, with only *Ae. albopictus* testing positive from assays with Scaevola, Goldenrod, and Yarrow. Marigold and Mexican Heather had no positive tests from either species.

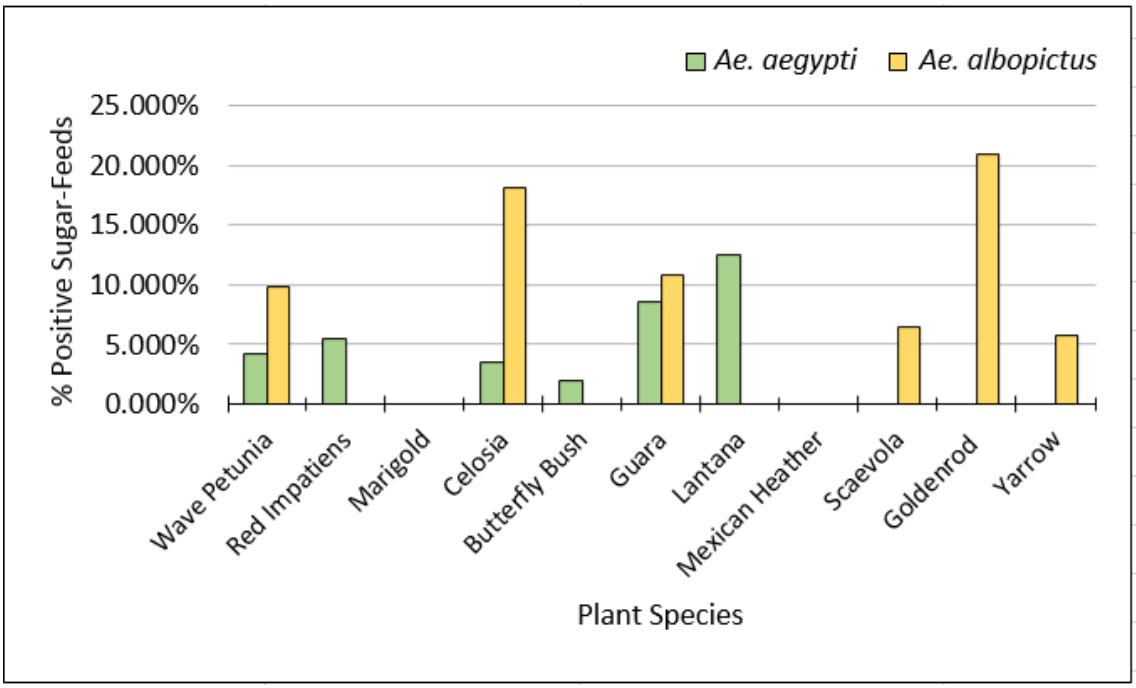


Figure 11. Qualitative Sugar Analysis. Percentage of *Ae. aegypti* and *Ae. albopictus* that tested positive for the consumption of fructose. Only mosquitoes that were alive from the plant visitations assays were tested.

Quantitative Analysis. The total amount of carbohydrates within the fructose-positive mosquitoes were quantified using the warm anthrone method (Figure 12). Both species were able to obtain the highest concentration of sugars from the Wave Petunia (201 ∓100.42 µg in *Ae. aegypti*; 167.64 ∓124.89 µg in *Ae. albopictus*). One *Ae. aegypti* mosquito (2%) tested positive for fructose consumption from the Butterfly Bush, but had a 140.71 µg of total carbohydrates content. The large amount of carbohydrates in this single mosquito may be representative of a large amount of glycogen stored before feeding, as glycogen is one of the carbohydrates detected by the anthrone method. Additionally, a large proportion (20.93%) of *Ae. albopictus* tested positive for fructose consumption from Goldenrod, and had a relatively low amount of total carbohydrates (59.26 µg) compared to other mosquito groups. No differences were found between groups which is explained by the low number of samples (ANOVA, $p = 0.219$, $Df = 11$; F value = 1.385).

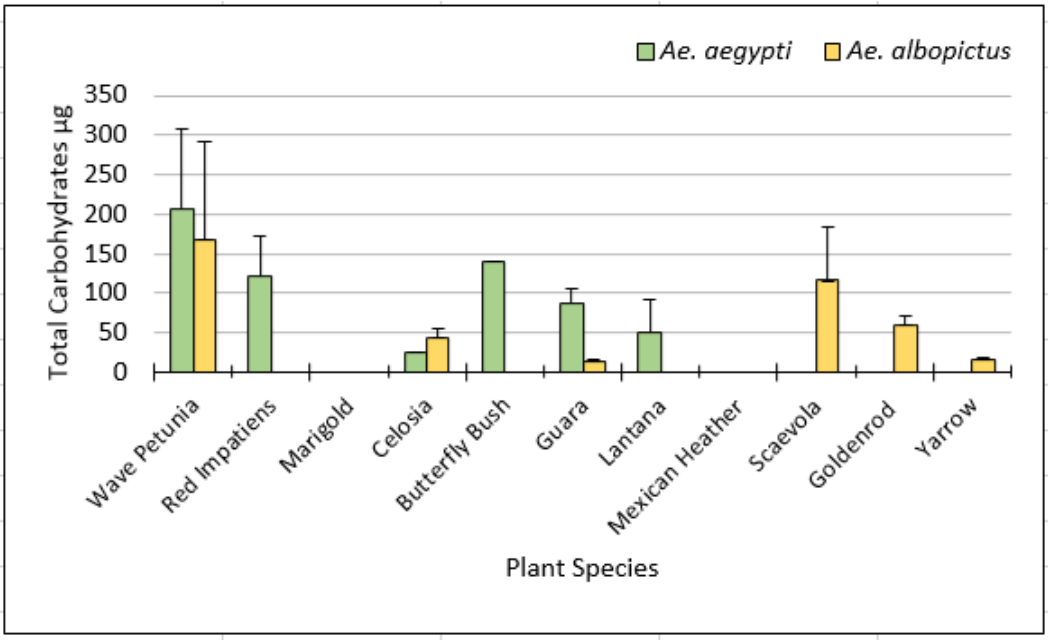


Figure 12. Quantitative Sugar Analysis. Total amount of carbohydrates in micrograms in *Ae. aegypti* and *Ae. albopictus* for each plant species. Values are represented as averages (+/- standard errors) amongst all mosquitoes from a given species that tested positive for fructose in the preceding qualitative analysis. Absorbances were converted to carbohydrate concentration using a glucose standard curve generated beforehand.

The total carbohydrate concentrations between mosquitoes testing positive for fructose consumption and mosquitoes testing negative were compared as well. These data are represented in Figure 13 and 14 for *Ae. aegypti* and *Ae. albopictus*, respectively. Concentrations from negative-testing mosquitoes were consistently lower than their positive-testing counterparts. However, due to the low number of samples for "positive" mosquito groups, we could not demonstrate the higher average contents in carbohydrates in the "positive" groups statically (for all comparisons, Student *t*-test, $p > 0.05$).

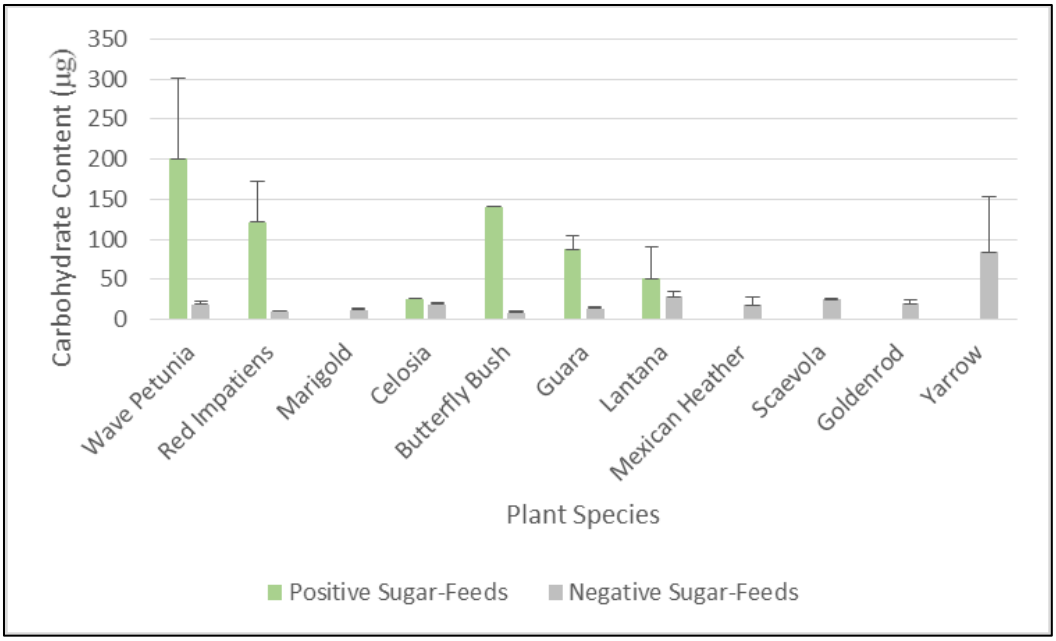


Figure 13. *Ae. aegypti* Total Carbohydrate Concentrations. Total amount of carbohydrates in *Ae. aegypti* positive for fructose, compared to *Ae. aegypti* negative for fructose. Values are represented as averages (+/- standard errors).

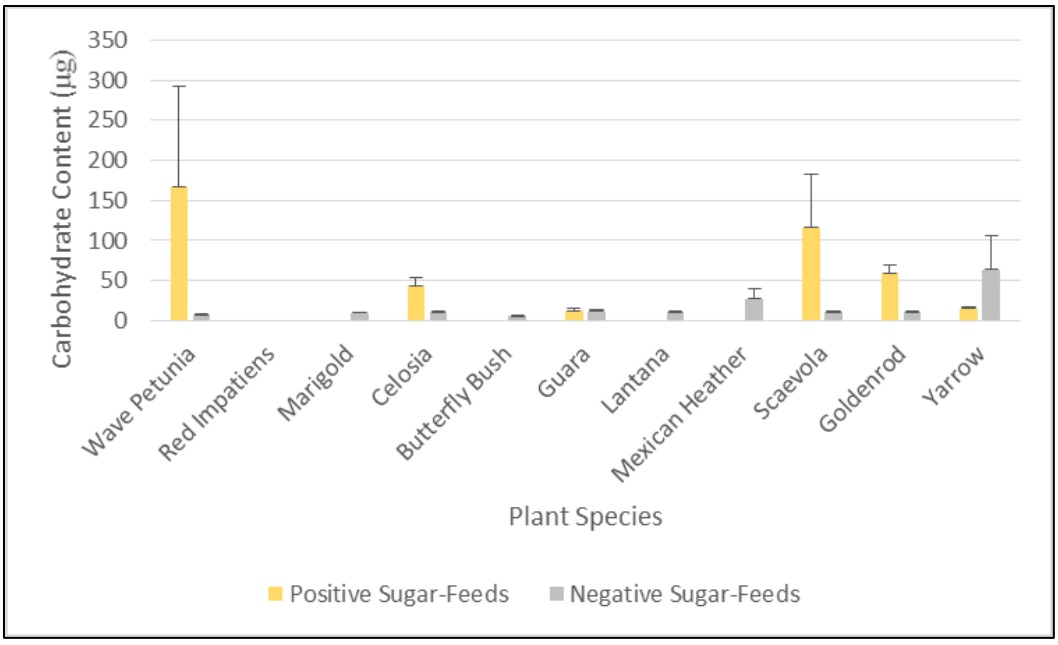
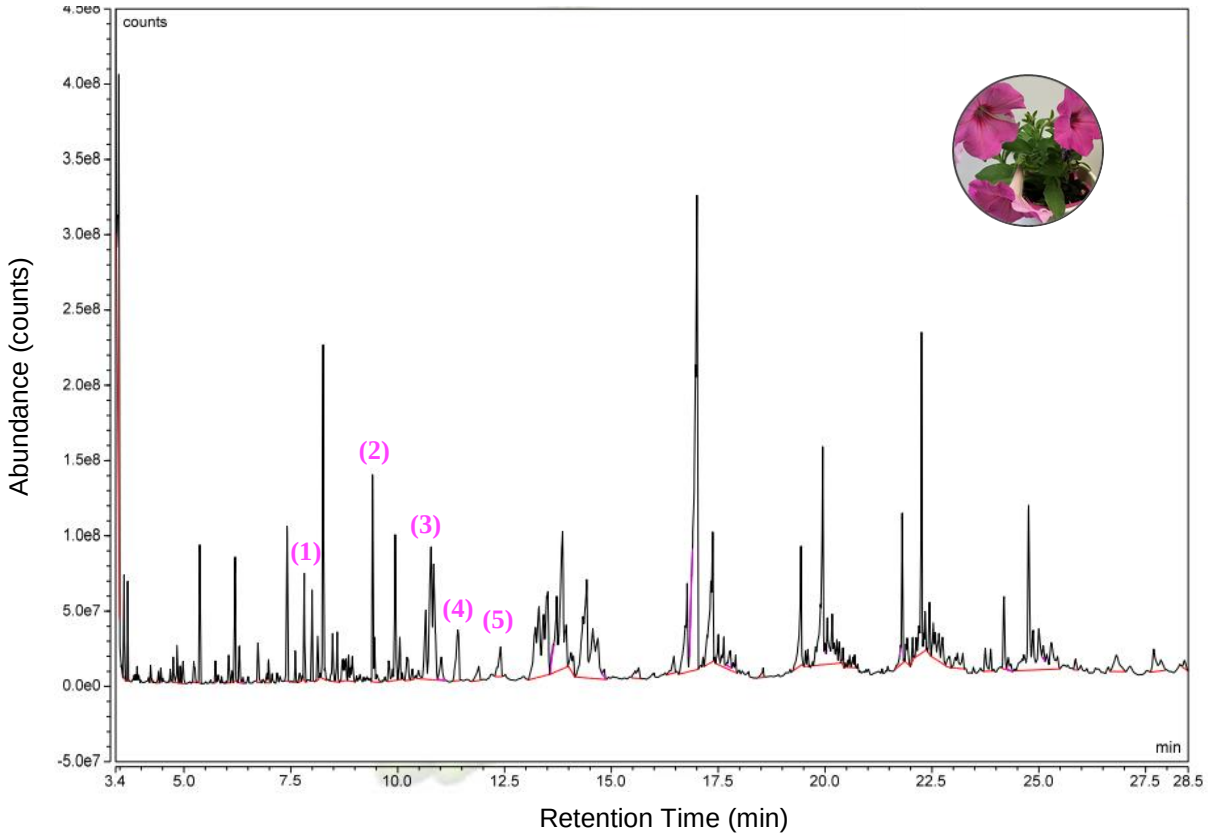


Figure 14. *Ae. albopictus* Total Carbohydrate Concentrations. Total amount of carbohydrates in *Ae. albopictus* positive for fructose, compared to *Ae. albopictus* negative for fructose. Values are represented as averages (+/- standard errors).

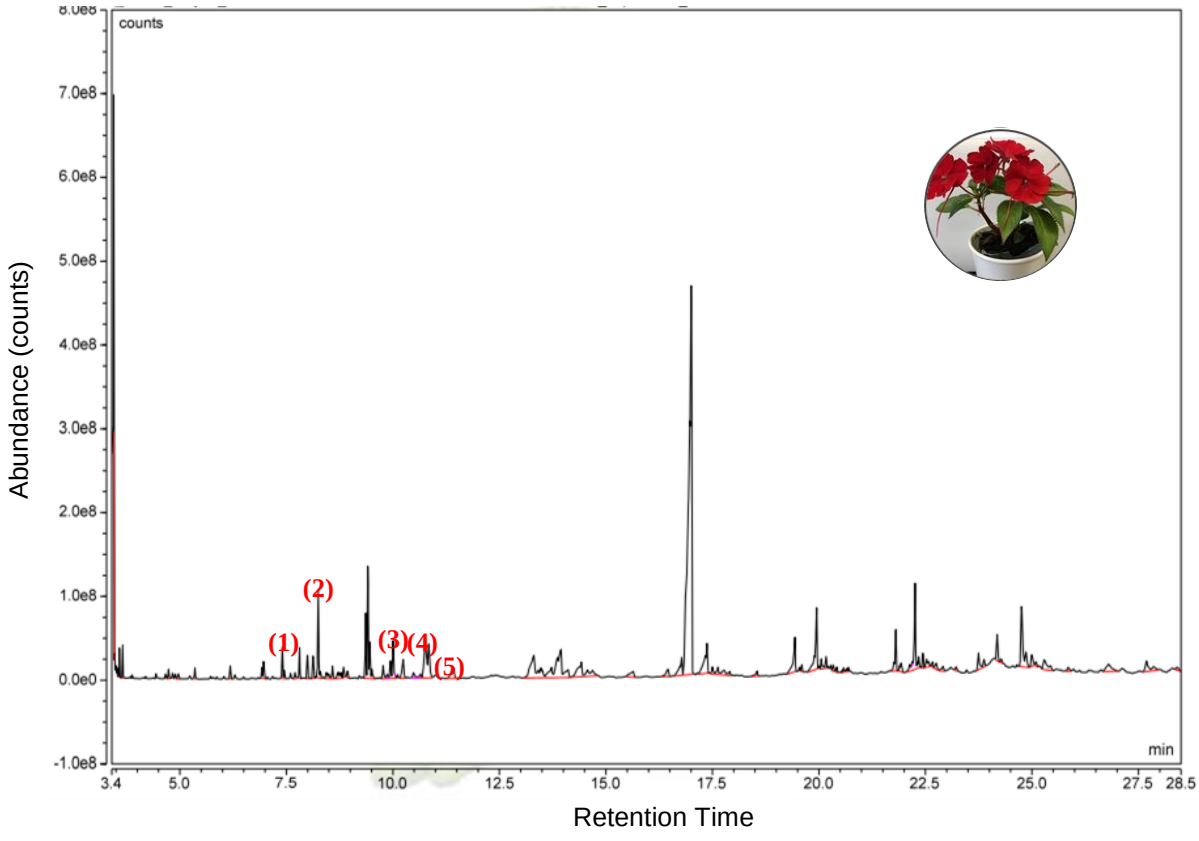
3.3 GC-MS Analysis of Plant Odor

Scent samples for each ornamental species were analyzed and the peak area abundances of these chemicals, as given by the respective chromatogram, are represented in Figures 15-25.



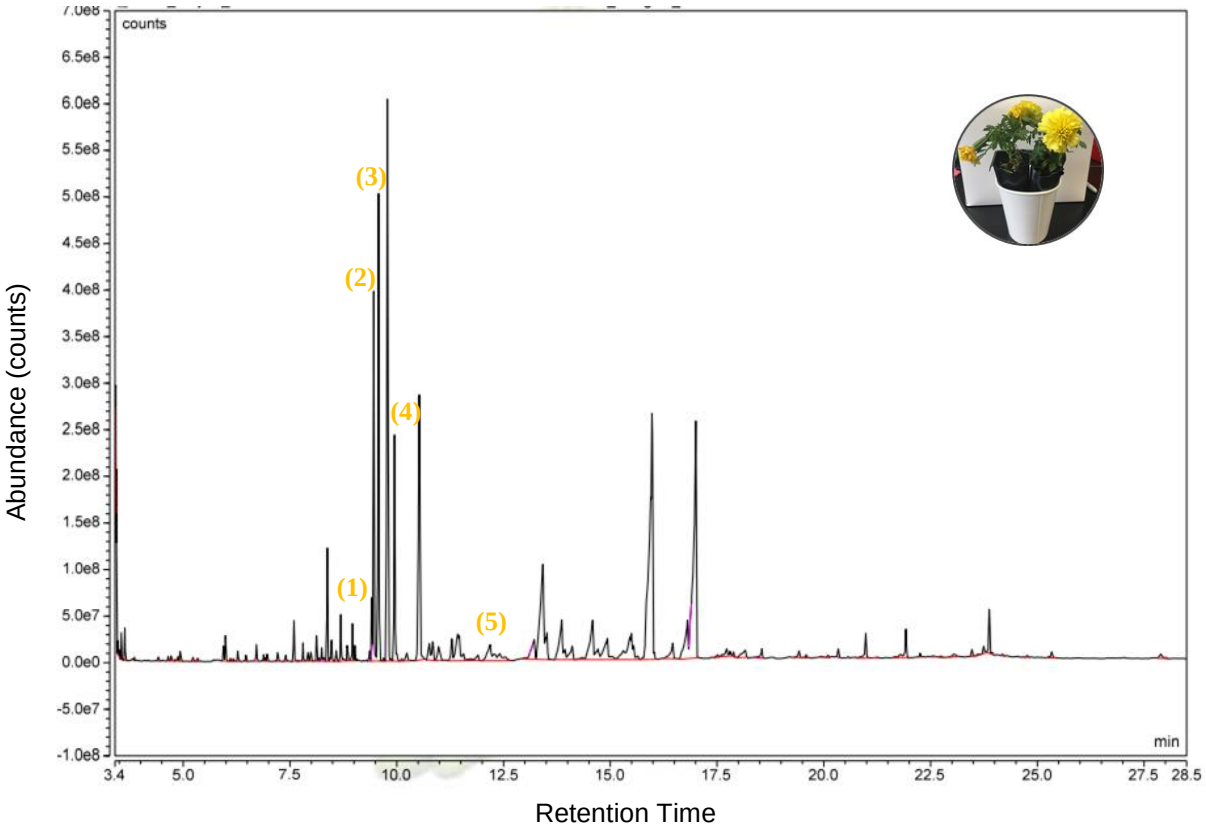
Peak Number	Retention Time	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	81372.76444	0.74%
	4.9105	3-Hexanol	184763.5732	1.68%
	6.278	1-Hexanol	447922.4677	4.07%
	6.944	Heptanal	169627.0732	1.54%
	7.604	a-Pinene	220165.2971	2.00%
1	8.135	Benzaldehyde	2096539.405	19.06%
	8.3117	1-Heptanol	238472.0132	2.17%
2	8.965	Octanal	572637.9759	5.21%
3	9.468	D-Limonene	521866.6132	4.74%
	9.5056	Benzyl Alcohol	181558.8398	1.65%
	10.223	1-Octanol	176126.2673	1.60%
4	10.846	Nonanal	5367669.574	48.79%
5	12.4169	Methyl salicylate	741995.9962	6.74%
				100%

Figure 15 Wave Petunia Chromatogram. Sample Wave Petunia chromatogram with the most abundant peaks labeled 1-5. In total, 8 scent samples and 13 volatiles are represented in the table.



Peak number	Retention Time	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	68754.36593	0.55%
	4.9105	3-Hexanol	67789.96365	0.54%
	4.931	Hexanal	36211.10895	0.29%
	6.278	1-Hexanol	27516.16533	0.22%
	6.934	2-Heptanol	334638.4482	2.65%
	6.944	Heptanal	43092.3211	0.34%
	7.604	a-Pinene	56574.70953	0.45%
1	8.135	Benzaldehyde	586443.164	4.65%
	8.4957	1-Octen-3-ol	120318.3837	0.95%
	8.965	Octanal	282221.2461	2.24%
2	9.468	D-Limonene	1739115.115	13.79%
	9.5056	Benzyl Alcohol	230986.7327	1.83%
	9.5191	Eucalyptol	123226.5444	0.98%
	9.6926	Benzene acetaldehyde	32181.7209	0.26%
	9.778	β-Ocimene	540685.9497	4.29%
3	10.0124	β-Terpinene	2363690.965	18.74%
4	10.781	Linalool	2645300.454	20.97%
5	10.846	Nonanal	3315854.902	26.29%
				100%

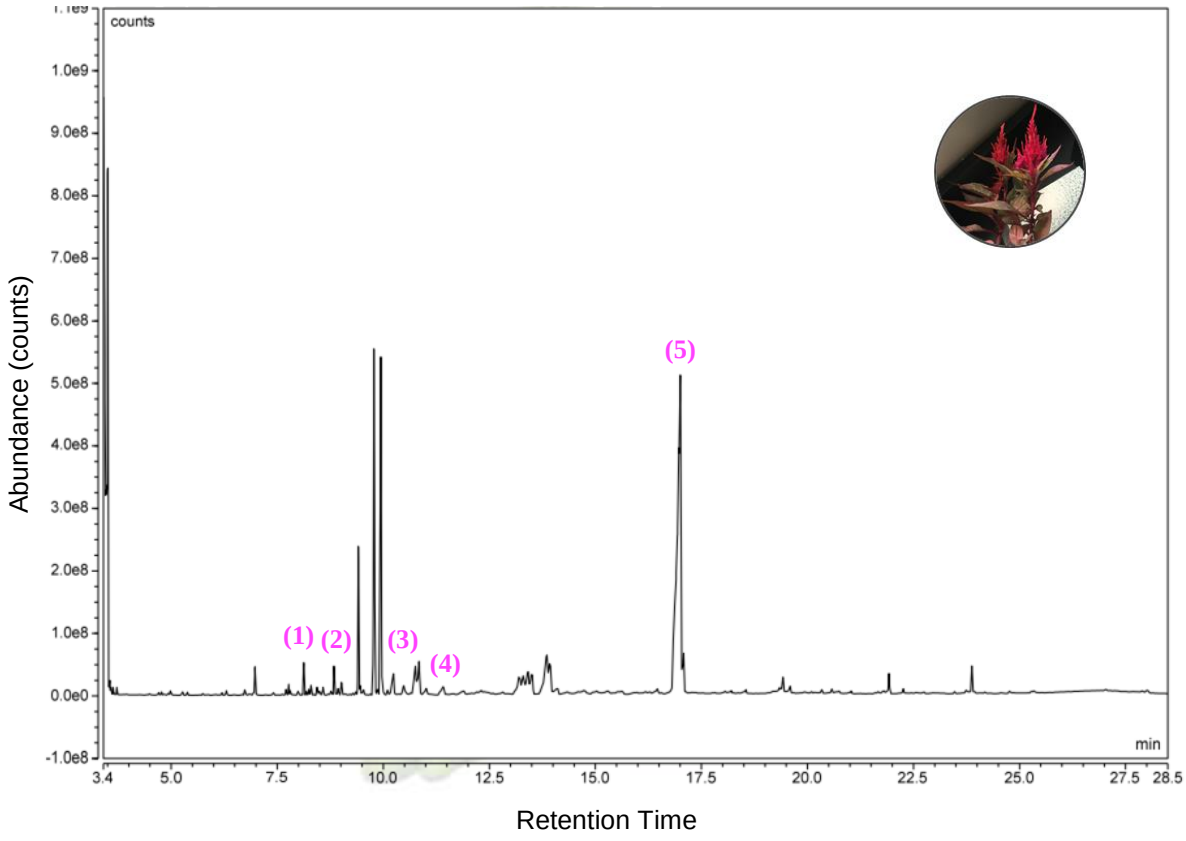
Figure 16 Red Impatiens Chromatogram. Sample Red Impatiens chromatogram with the most abundant peaks labeled 1-5. In total, 6 scent samples and 18 volatiles are represented in the table.



Peak Number	Retention Time	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	89840.30252	0.23%
	4.9105	3-Hexanol	71879.56923	0.18%
	4.931	Hexanal	1180105.585	2.97%
	5.999	3-Hexen-1-ol	308515.0404	0.78%
	6.278	1-Hexanol	129134.5092	0.32%
	6.686	2-Heptanone	520030.2127	1.31%
1	8.135	Benzaldehyde	2303599.721	5.79%
	8.965	Octanal	43369.86634	0.11%
	8.713	β-Myrcene	1281146.342	3.22%
	9.053	a-Phellandrene	611714.0871	1.54%
2	9.468	D-Limonene	12384733.6	31.15%
	9.5056	Benzyl Alcohol	46422.43969	0.12%
	9.5191	Eucalyptol	384847.59	0.97%
3	9.5701	trans-β-Ocimene	12199951.34	30.69%
4	9.6926	Benzene acetaldehyde	2814221.894	7.08%
	10.781	Linalool	1535582.586	3.86%
5	10.846	Nonanal	1882705.338	4.74%

	10.9782	Phenylethyl Alcohol	884511.1001	2.22%
	12.4169	Methyl salicylate	1082291.52	2.72%
				100%

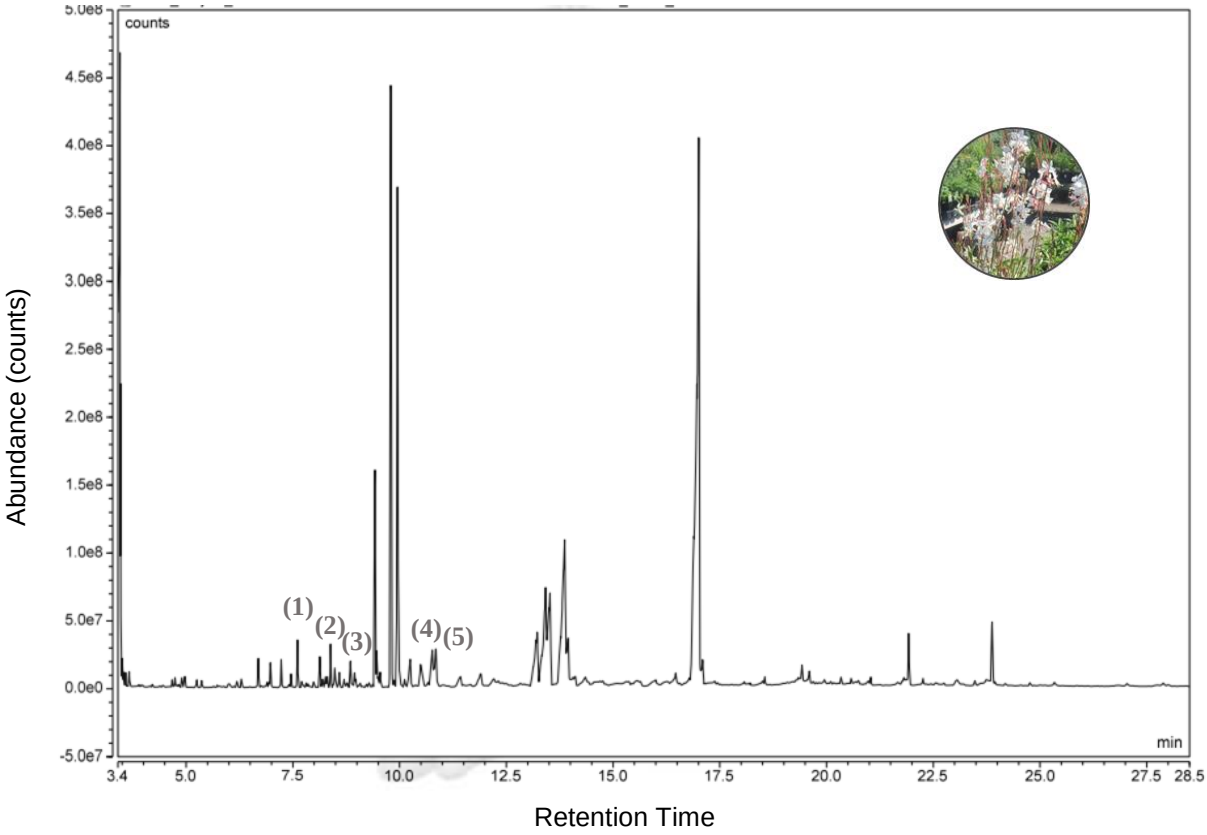
Figure 17 Marigold Chromatogram. Sample Marigold chromatogram with the most abundant peaks labeled 1-5. In total, 10 scent samples and 19 volatiles are represented in the table.



Peak Number	Retention Time	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	77219.21743	0.66%
	4.9105	3-Hexanol	76124.30364	0.66%
	4.931	Hexanal	171881.0838	1.48%
	6.278	1-Hexanol	222558.3511	1.92%
	6.944	Heptanal	14977.60578	0.13%
	7.217	Anisole	12403.93848	0.11%
1	8.135	Benzaldehyde	706148.0717	6.08%
	8.4957	1-Octen-3-ol	164326.2448	1.41%
	8.965	Octanal	182920.131	1.58%

2	9.053	a-Phellandrene	570057.6592	4.91%
	9.4647	Limonene	240237.3209	2.07%
3	10.781	Linalool	1972345.907	16.98%
4	10.846	Nonanal	1932711.189	16.64%
	12.465	L-a-Terpineol	160324.3311	1.38%
5	17.0867	β-Bisabolene	5109621.848	44.00%
				100%

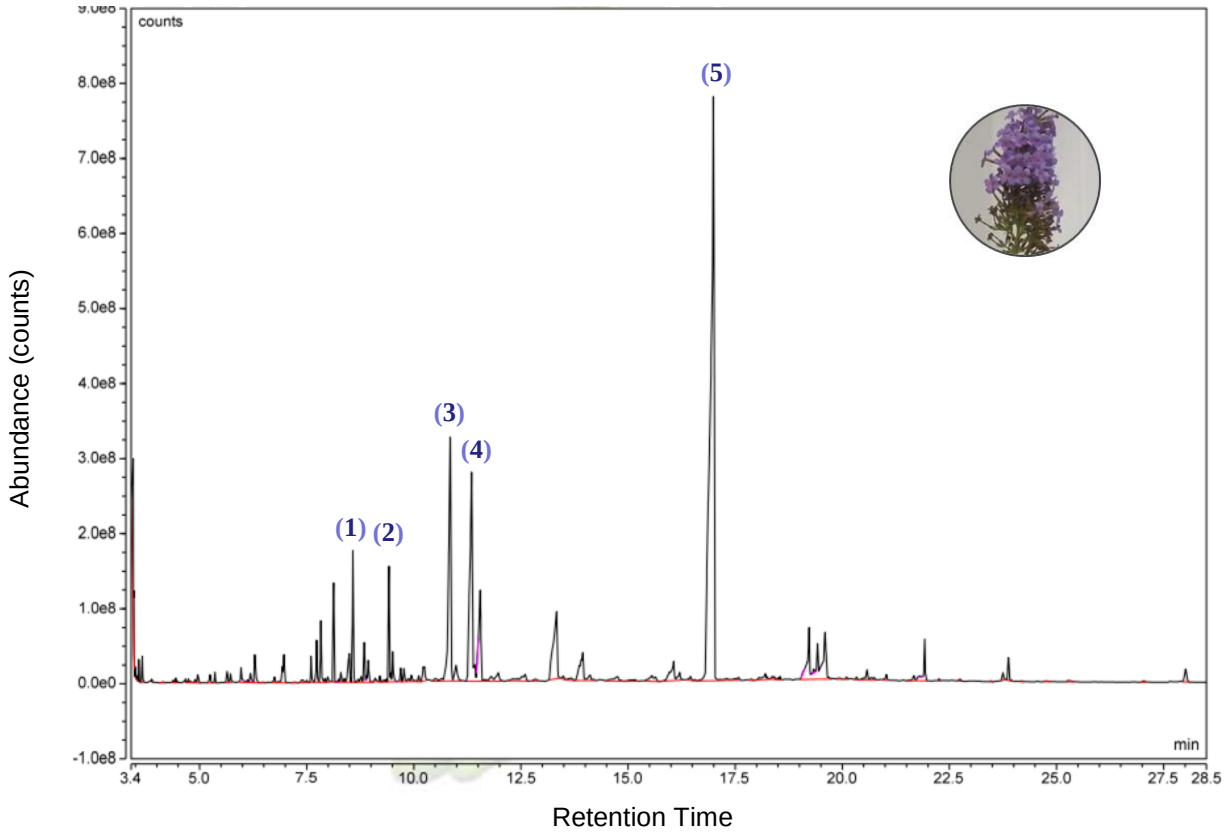
Figure 18 Celosia Chromatogram. Sample Wave Petunia chromatogram with the most abundant peaks labeled 1-5. In total, all 12 scent samples and 15 volatiles are represented in the table.



Peak Number	Retention Time	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	220771.0209	3.03%
	4.9105	3-Hexanol	284803.3594	3.90%
	6.278	1-Hexanol	250319.6252	3.43%
1	6.686	2-Heptanone	498110.8323	6.83%
	6.944	Heptanal	139387.5798	1.91%

	7.217	Anisole	260641.4749	3.57%
2	8.135	Benzaldehyde	471960.4201	6.47%
	8.713	β-Myrcene	30545.73354	0.42%
3	8.965	Octanal	332704.158	4.56%
	9.5056	Benzyl Alcohol	197484.285	2.71%
	9.519	D-Limonene	245944.2314	3.37%
4	10.781	Linalool	1214661.139	16.65%
5	10.846	Nonanal	2950911.551	40.45%
	10.9782	Phenylethyl Alcohol	196636.9468	2.70%
				100%

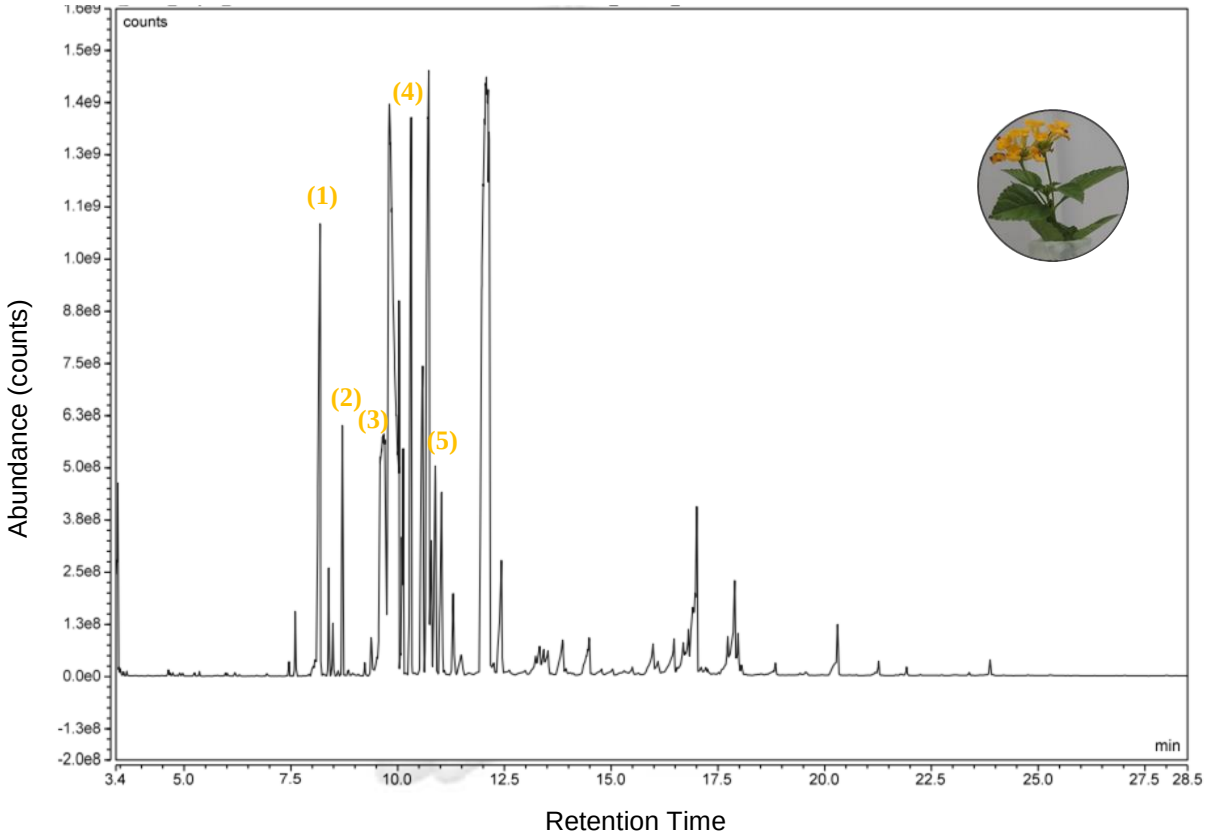
Figure 19 Guara Chromatogram. Sample Guara chromatogram with the most abundant peaks labeled 1-5. In total, 7 scent samples, 2 cut-inflorescence samples, and 14 volatiles are represented in the table.



Peak Number	Retention Index	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	85161.18067	0.23%
	4.9105	3-Hexanol	50243.21873	0.13%

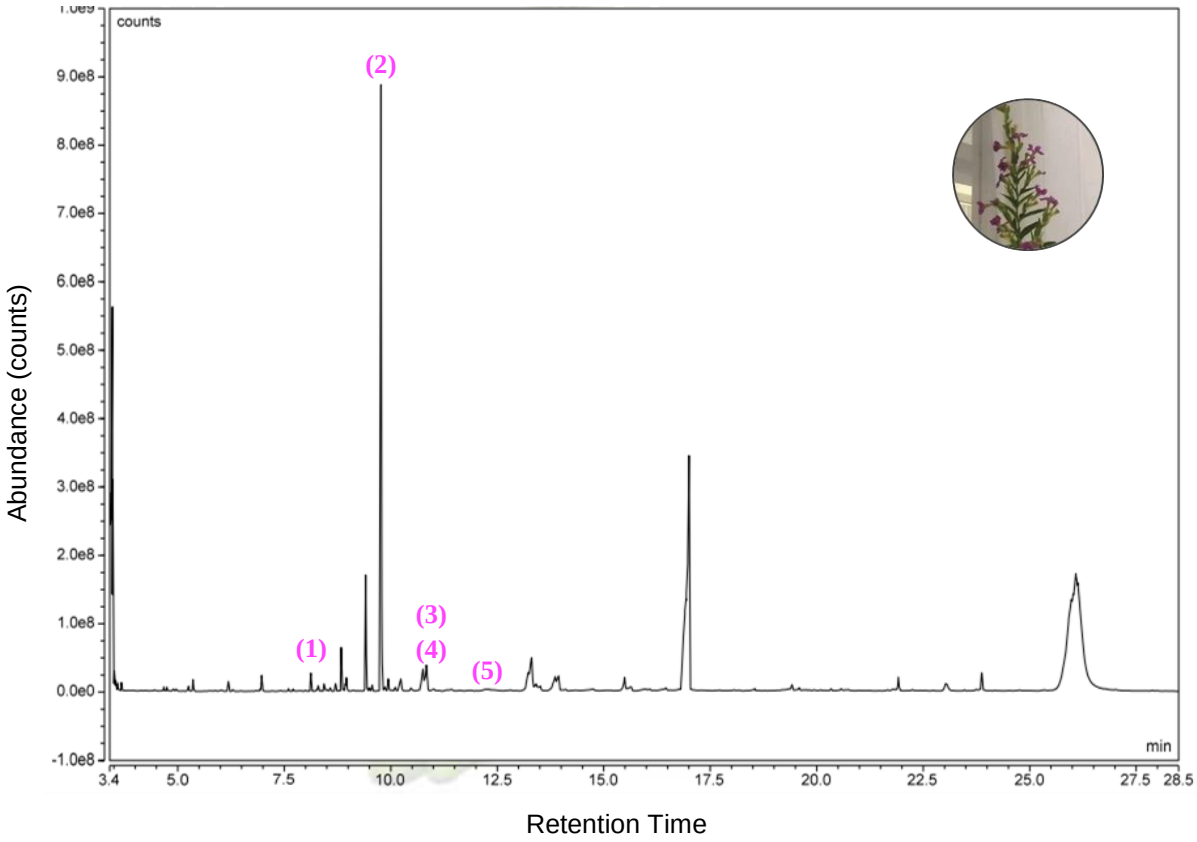
	4.931	Hexanal	107191.1085	0.29%
	6.278	1-Hexanol	1062254.745	2.85%
	6.944	Heptanal	127061.153	0.34%
1	8.135	Benzaldehyde	2720119.225	7.29%
	8.4957	1-Octen-3-ol	1095321.085	2.94%
	8.965	Octanal	256516.804	0.69%
2	9.5056	Benzyl Alcohol	1744141.387	4.68%
	9.519	D-Limonene	61295.91866	0.16%
	9.5701	trans-β-Ocimene	49602.31129	0.13%
	9.6926	Benzene acetaldehyde	159981.8938	0.43%
	10.781	Linalool	569141.2779	1.53%
3	10.846	Nonanal	3749209.057	10.05%
4	10.9782	Phenylethyl Alcohol	1614505.976	4.33%
5	16.910	a-Farnasene	27818767.49	63.93%
				100%

Figure 20 Butterfly Bush Chromatogram. Sample Butterfly Bush chromatogram with the most abundant peaks labeled 1-5. In total, 5 scent samples, 2 cut-inflorescence samples, and 16 volatiles are represented in the table.



Peak Number	Retention Time	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	168554.2544	0.11%
	4.9105	3-Hexanol	229686.3234	0.15%
	4.931	Hexanal	327333.74	0.21%
	6.686	2-Heptanone	53541.73739	0.03%
	6.944	Heptanal	95594.86373	0.06%
1	8.13	Benzaldehyde	41313422.54	26.30%
	8.594	3-Octanone	665029.0525	0.42%
2	8.713	β-Myrcene	10824814.09	6.89%
	8.819	3-Octanol	321016.5528	0.20%
	8.965	Octanal	210403.0387	0.13%
	9.5056	Benzyl Alcohol	651775.3173	0.41%
	9.519	D-Limonene	1448608.317	0.92%
3	9.778	β-Ocimene	62401276.66	39.73%
4	10.118	Terpinene	15422136.3	9.82%
5	10.781	Linalool	14299383.06	9.10%
	11.659	Lilac aldehyde C/B	264600.2195	0.17%
	12.4169)	Methyl salicylate	8369003.836	5.33%
				100%

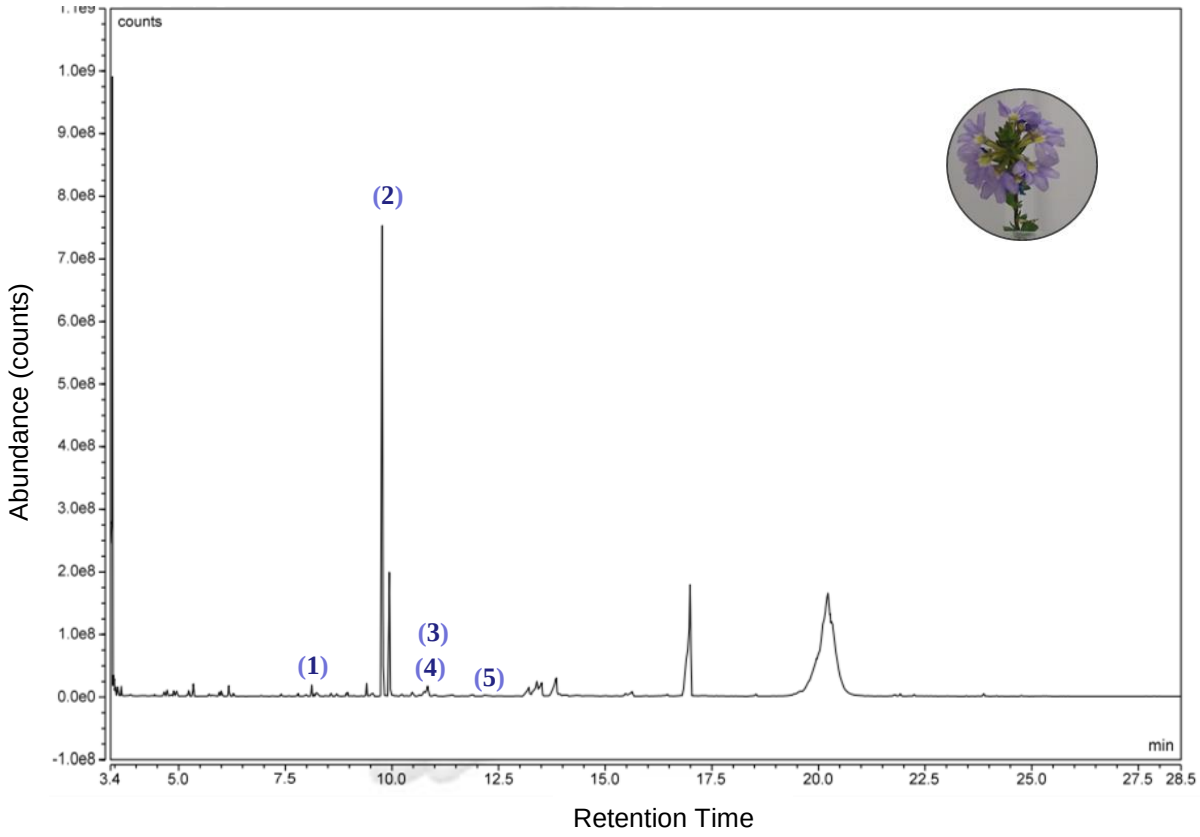
Figure 21 Lantana Chromatogram. Sample Lantana chromatogram with the most abundant peaks labeled 1-5. In total, 10 scent samples, 2 cut-inflorescence samples, and 17 volatiles are represented in the table.



Peak Number	Retention Index	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	201281.9662	0.64%
	4.9105	3-Hexanol	121869.5446	0.38%
	6.278	1-Hexanol	22438.42359	0.07%
	6.944	Heptanal	81039.27929	0.26%
	7.217	Anisole	56494.14972	0.18%
1	8.135	Benzaldehyde	864833.1289	2.73%
	8.4957	1-Octen-3-ol	42477.66474	0.13%
	8.965	Octanal	177127.3843	0.56%
	9.5056	Benzyl Alcohol	90009.73184	0.28%
	9.519	D-Limonene	113342.9268	0.36%
2	9.778	β-Ocimene	26297328.7	83.05%
	9.6926	Benzene acetaldehyde	34423.58092	0.11%
	10.118	Terpinene	41778.8457	0.13%

3	10.781	Linalool	1096742.293	3.46%
4	10.846	Nonanal	1784577.371	5.64%
5	12.4169	Methyl salicylate	637606.5587	2.01%
				100%

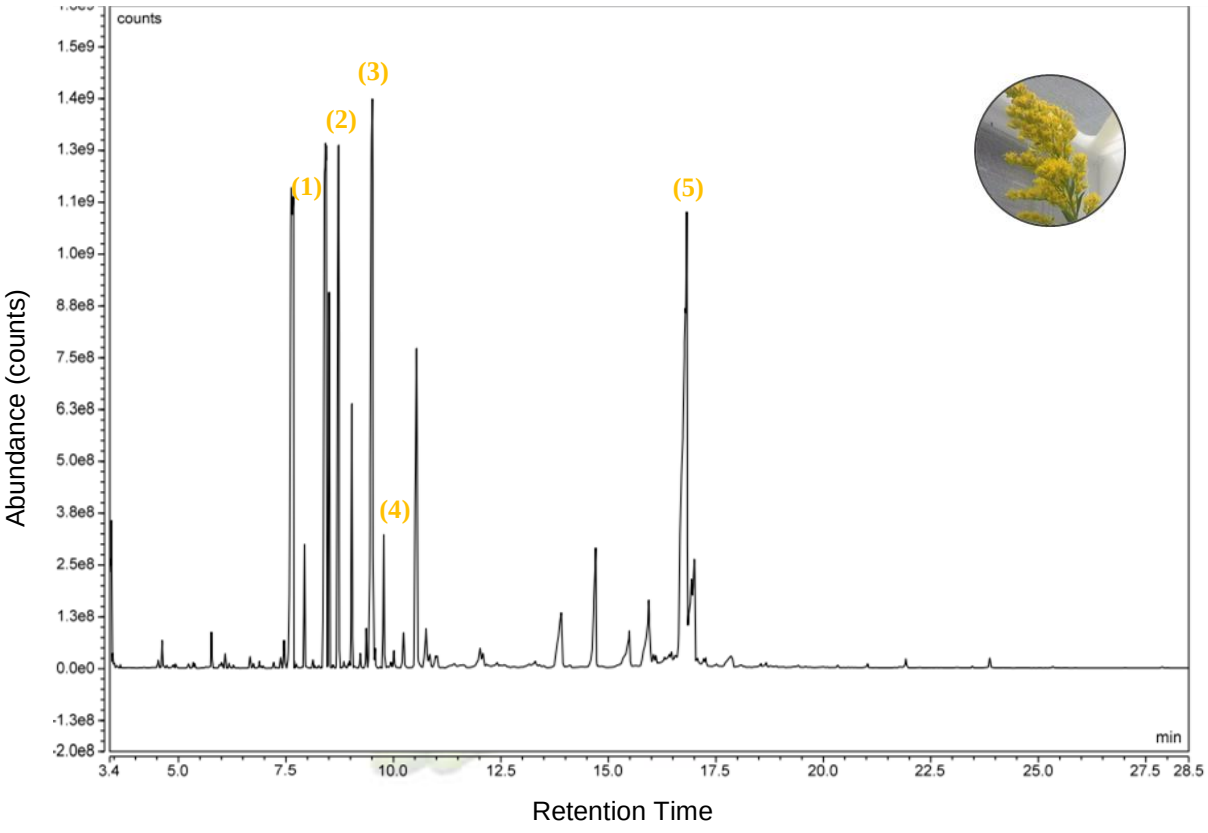
Figure 22 Mexican Heather Chromatogram. Sample Mexican Heather chromatogram with the most abundant peaks labeled 1-5. In total, 9 scent samples, 2 cut-inflorescence samples, and 16 volatiles are represented in the table.



Peak Number	Retention Time	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	115114.0427	0.75%
	4.9105	3-Hexanol	180179.7213	1.18%
	6.278	1-Hexanol	264446.6014	1.72%
	6.944	Heptanal	71383.21301	0.47%
	7.217	Anisole	94493.02946	0.62%
	7.598	a-Pinene	133737.2323	0.87%
1	8.135	Benzaldehyde	587476.1016	3.83%
	8.965	Octanal	106835.2973	0.70%
	9.5056	Benzyl Alcohol	162522.3737	1.06%

	9.519	D-Limonene	80354.68154	0.52%
	9.6926	Benzene acetaldehyde	22025.07729	0.14%
2	9.778	β-Ocimene	10448113.74	68.14%
3	10.781	Linalool	671141.193	4.38%
4	10.846	Nonanal	1813306.71	11.83%
5	12.4169	Methyl salicylate	583058.8236	3.80%
				100%

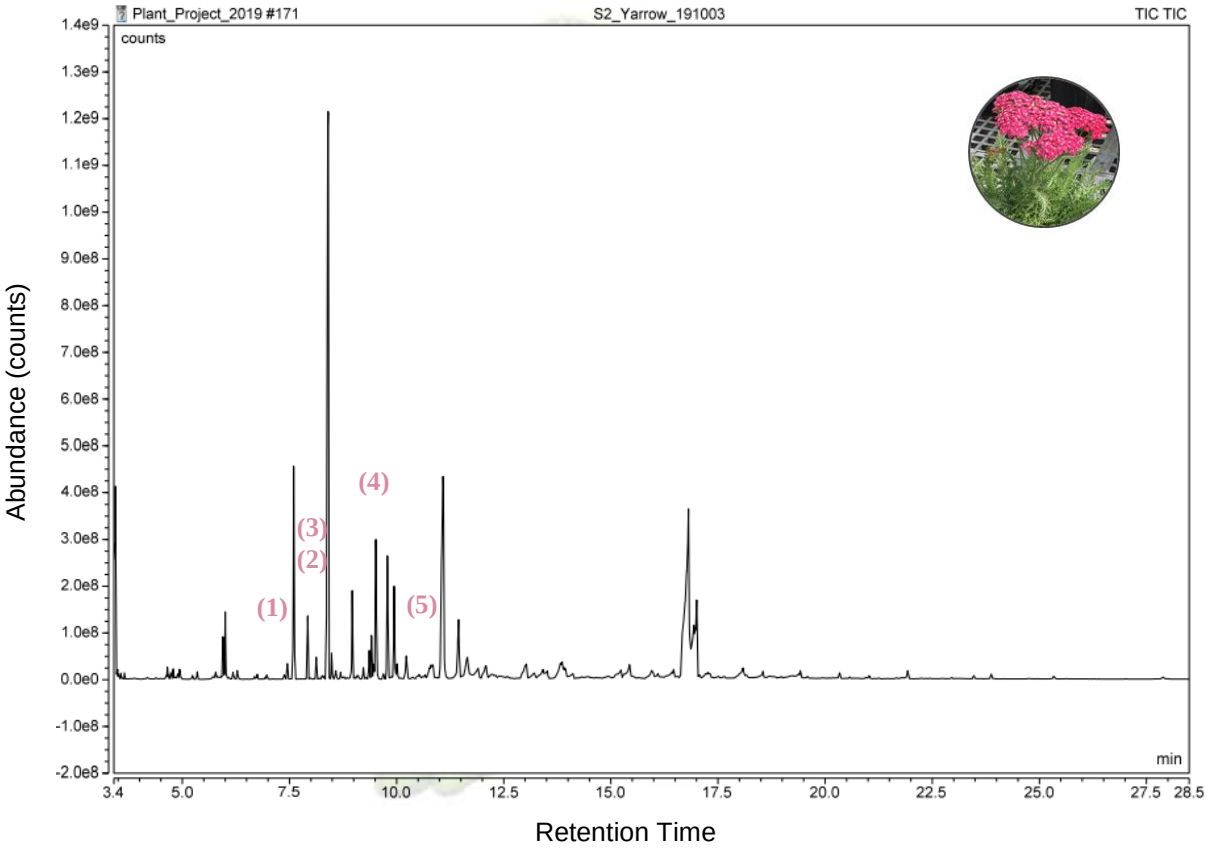
Figure 23 Scaevola Chromatogram. Sample Scaevola chromatogram with the most abundant peaks labeled 1-5. In total, 7 scent samples, 2 cut-inflorescence samples, and 15 volatiles are represented in the table.



Peak Number	Retention Time	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	20866.09671	0.01%
	4.931	Hexanal	473615.2342	0.17%
	6.278	1-Hexanol	179675.4791	0.07%
	7.217	Anisole	378567.383	0.14%
1	7.951	Camphene	17936922.61	6.60%
	8.135	Benzaldehyde	1021714.255	0.38%

2	8.747	β-Myrcene	62220262.69	22.88%
	8.965	Octanal	363047.0555	0.13%
	9.053	a-Phellandrene	23624978.98	8.69%
3	9.519	D-Limonene	62096592.94	22.84%
4	9.778	β-Ocimene	15553293.8	5.72%
	10.781	Linalool	12071857.41	4.44%
	10.846	Nonanal	2839550.929	1.04%
	11.625	trans-Verbenol	2990546.662	1.10%
	12.4169	Methyl salicylate	1381554.135	0.51%
	13.788	Bornyl acetate	5700627.379	2.10%
5	16.7502	Germacrene D	63050998.69	23.19%
				100%

Figure 24 Goldenrod Chromatogram. Sample Goldenrod chromatogram with the most abundant peaks labeled 1-5. In total, 12 scent samples and 17 volatiles are represented in the table.



Peak Number	Retention Index	Chemical Name	AVG ABUNDANCE	%
	4.649	3-Hexanone	264125.3264	2.54%
	4.9105	3-Hexanol	141539.3262	1.36%
	4.931	Hexanal	347383.5293	3.34%
	6.944	Heptanal	81594.05025	0.79%
1	7.598	3-Carene	860551.6372	8.29%
2	7.951	Camphene	1134487.88	10.92%
3	8.135	Benzaldehyde	737313.7801	7.10%
	8.747	β-Myrcene	192522.1557	1.85%
	8.965	Octanal	234858.5994	2.26%
	9.461	D-Limonene	195664.9998	1.88%
4	9.5191	Eucalyptol	3029019.382	29.17%
	9.6926	Benzene acetaldehyde	266000.5918	2.56%
	10.781	Linalool	437136.8493	4.21%
5	10.846	Nonanal	2308750.685	22.23%
	11.655	Camphor	154280.0444	1.49%
				100%

Figure 25 Yarrow Chromatogram. Sample Yarrow chromatogram with the most abundant peaks labeled 1-5. In total, 9 scent samples, 2 cut-inflorescence samples, and 15 volatiles are represented in the table.

Among these volatiles, we found terpenoids including linalool and aliphatics such as nonanal in abundance across several ornamental species, including Wave Petunia, Red Impatiens, Celosia, Butterfly Bush, Guara, Mexican Heather, Scaevola and Yarrow. Nonanal, in particular, was the volatile with the highest relative abundance in Wave Petunia (48.79%), Red Impatiens (26.29%) and Guara (40.45%). Other aliphatic compounds, like hexanol, 3-hexanone, and heptanal, were present in small concentrations in almost every ornamental. In Lantana, Mexican Heather and Scaevola, terpenoic ß-ocimene comprised the majority of the scent compositions, with a relative peak area abundance of 39.73%, 83.05%, and 68.14%, respectively. Aromatic benzaldehyde and terpenoic limonene were found ubiquitously across all eleven ornamental species at varying concentrations. Benzaldehyde, however, had high relative peak area abundances in Wave Petunia (19.06%) and Lantana (26.30%), while limonene exhibited the highest relative abundance of Marigold's scent (31.15%) and composed 22.84% of the peak abundance in Goldenrod.

Terpenoids were the most prominent class of volatile compounds present in the scent samples. Some less common terpenoid volatiles were unique to certain ornamental species, yet still displayed a major relative peak abundance in their associated plant scent. ß-bisabolene (44.00%) and germacrene D (23.19%) were present only in the Celosia and Goldenrod scents, respectively, yet exhibited the highest relative peak area abundances for both ornamentals. α-farnesene was abundant only in Butterfly Bush, making up 63.93% of the total scent's peak area abundance. Eucalyptol was present in minute concentrations in Red Impatiens (0.98%) and Marigold (0.97%) but had the highest relative peak area abundance in Yarrow making up to

29.17% of the total scent. Camphene was found with a moderate abundance in Goldenrod (6.60%) and Yarrow (8.29%). Similarly, α-phellandrene exhibited moderate abundances in the scents of Marigold (1.54%), Celosia (4.91%) and Goldenrod (8.69%).

4. Discussion and Perspectives

This work sheds light on the interaction between *Ae. aegypti* and *Ae. albopictus* mosquitoes and ornamental plants and provides some insights into the relatively understudied field of mosquito phytophagy. Using plant visitation assays, the frequency of mosquito landings and feedings on eleven different common ornamental plants was assessed and compared between the two invasive mosquito species. Additionally, the presence of fructose, a sugar found in nectar, in the mosquitoes' gut was determined using the anthrone method (van Handel, 1985). Lastly, volatile compounds for each ornamental plant's scent profile were identified using GC-MS.

When assessing the relationship between either *Ae. aegypti* and *Ae. albopictus* and a given plant species, it is important to consider how the results from each experiment are intertwined. Starting with Wave Petunia, neither mosquito species were observed feeding on the flower, but individuals of both species tested positive for nectar consumption and received the highest amount of carbohydrates relative to the other ornamentals. This could be attributed to feeding occurring outside of the time that the GoPro was recording, potentially due to the daily variation in volatile emission in Petunias, which emit stronger scent at night to attract their pollinators: moths (Fenske et al. 2015). Moreover, it is possible that the mosquitoes obtained carbohydrates by feeding on extra floral nectar or from the stem / leaves of the plant (Foster, 1995; Clements, 1999). *Ae. aegypti* had low landing and positive fructose tests with Red Impatiens, but the few fructose-positive mosquitoes contained the third-highest amount of total carbohydrates. Both Wave Petunia and Red Impatiens contained high relative peak area abundances of nonanal, a volatile that has been shown

to synergize with CO_2 and attract *Cx. pipiens* mosquitoes (Syed and Leal, 2009). It is possible that nonanal plays a bigger role in attraction for *Ae. albopictus* compared to *Ae. aegypti*, as the majority of landings on Wave Petunia were seen from *Ae. albopictus*, whereas very few *Ae. aegypti* were observed landing on the two plant species. Plant visitation assays with Red Impatiens and *Ae. albopictus* are required to further provide evidence for this.

Marigold has been previously labelled as a mosquito-repellent plant. The scent of Marigold contained a high abundance of limonene, which has been defined in the literature as an insect repellent, thus supporting the prior labelling of this plant as mosquito-repellent (Maia and Moore, 2011). However, several landings and feedings were observed from *Ae. albopictus*, indicating the plant is not inherently repellent to this mosquito species. Furthermore, neither mosquito species were positive for any sugar consumption from Marigold, indicating they were not able to receive nectar when seen feeding, or the amount they did receive was negligible and / or rapidly metabolized. Celosia, similar to Marigold, saw landing and feeding activity almost exclusively from *Ae. albopictus*. This may be in part due to the fact that β-bisabolene dominated the relative peak area abundances in Celosia, which is a volatile that was determined as a constituent in essential oils that were known to be repellent to *Ae. aegypti* (Boonyuan et al. 2014). Almost 20% of *Ae. albopictus* in plant visitation assays with Celosia tested positive for fructose consumption, but contained a low concentration of total carbohydrates, potentially indicating low nectar accessibility.

Close to equivalent counts of landings and feedings (near five each) were observed from both *Ae. aegypti* and *Ae. albopictus* with the Butterfly Bush. A previously conducted experiment observed a tendency for *Ae. albopictus* to oviposit in water buckets near flowering Butterfly Bushes, proposing an attraction to this ornamental (Davis et al. 2016). The data here helps support

this claim. However, *Ae. albopictus* seemed to have a harder time obtaining carbohydrates from Butterfly Bush, as only *Ae. aegypti* tested positive for fructose consumption. In addition, α-farnesene was a prominent volatile in the scent profile of Butterfly Bush. In experiments testing compounds' repellency to *Ae. aegypti*, β-farnesene was shown to exhibit a low degree of repellence (Cantrell et al. 2018). This provides some insights into the potentially attractive qualities of farnesene. This work, to our knowledge, is the first to examine the relationship between any species of mosquito and Guara. A relatively small number of landings and feedings were seen from both species, and no one species clearly favored Guara over the other. Additionally, close to 10% of both mosquito species tested positive for fructose, but *Ae. aegypti* showed a greater concentration of total carbohydrates. The highest peak area abundances in Guara's scent profile were given by nonanal and linalool, the latter of which has been characterized as repellent to mosquitoes (Muller et al. 2009). This may explain why this ornamental was seemingly not as attractive as Wave Petunia, which also had a high relative nonanal abundance.

Lantana has been tested with *Anopheles* spp. and defined as mosquito-repellent in the literature (Sambali et al. 2011). We identified β-ocimene as the most abundant volatile in the Lantana's headspace, which has been shown to elicit dose-dependent repellent responses in *Ae. aegypti* mosquitoes (Dube et al. 2011). This repellent quality is put into question by the moderate number of landings on this ornamental seen from both mosquito species, and four attempted feedings from *Ae. albopictus*. This could be attributed to Lantana being the only choice for the mosquitoes and was fed on out of necessity to survive (*i.e.*, avoid desiccation / dehydration). Choice experiments using the Y-maze olfactometer assay (see below) are required to further define the valence of the chemicals emitted by this plant. It should also be noted that although *Ae.*

albopictus was observed feeding several times, only *Ae. aegypti* tested positive for the presence of carbohydrates, even though only one *Ae. aegypti* was seen feeding.

β-ocimene is a compound with a high degree of ubiquity in plant scents and is an important mediator for pollinator interactions (Farré-Armengol et al. 2017). In a review by Farré-Armengol et al. (2017), β-ocimene was observed in 75% of 63 plant families that were represented in the study. The Mexican Heather and Scaevola ornamentals fit this observation, with β-ocimene exhibiting the highest relative peak area abundances in both plant scents and potentially playing a role in facilitating the pollination of these plants by bees and butterflies. *Ae. aegypti* were seen landing nine times on Mexican Heather, but neither species was observed feeding or tested positive for nectar consumption. Scaevola showed similar results, but with *Ae. albopictus* being slightly more active. Additionally, the few *Ae. albopictus* mosquitoes testing positive for fructose gave the second-highest concentration of total carbohydrates for *Ae. albopictus*. More testing is needed to determine if the bulk of the carbohydrates here were indeed received from the plant. Furthermore, more evidence is required to confirm or deny the potentially mosquito-repellent qualities of these two ornamentals.

Occurrences of mosquitoes visiting Goldenrod have been recorded as early as 1907 (Knab, 1907), and *Cx. pipiens* has been proposed as a potential pollinator for this plant (Peach and Gries, 2016). High numbers of landings and feedings were seen from both *Ae. aegypti* and *Ae. albopictus* on Goldenrod in the present study; indeed, this plant had the highest number of both landings and feedings from *Ae. aegypti*, compared to the other tested ornamentals. Interestingly, close to 25% of *Ae. albopictus* tested positive for fructose consumption, yet no *Ae. aegypti* gave positive tests. Germacrene D was present in high abundance in all Goldenrod scent samples. This volatile was shown to be present at moderately low concentrations that were found repellent by *Ae. aegypti*

(Azeem et al. 2019). However, it is likely this volatile does not elicit dose-dependent repellent responses and is not repellent on its own, considering the high activity of *Ae. aegypti* here. In addition, the valence of a single volatile may be changed when interacting with concurrent volatiles in the same blend or scent, which may be the case here.

While there are many variations in coloration and taxonomy, Yarrow has been generally defined as a mosquito repellent flower (Tunón et al. 1994). However, a high number of *Ae. aegypti* landings and *Ae. albopictus* feedings occurred during the plant visitation assays. Even with this high number of *Ae. albopictus* feedings, not much nectar feeding was apparent from the total carbohydrate assays. Eucalyptol had the highest peak area abundance in Yarrow, and has been shown elicit repellent responses in mosquitoes in other members of the Asteraceae plant family, such as Fitch's spikeweed (Klocke et al. 1986)

Nectar composition and availability are important factors to consider when analyzing the results obtained in the present study. Traditionally, plants modify the chemical composition of their nectar to serve as rewards for specific pollinators that they share a symbiotic relationship with (Chalcoff et al. 2006). Because mosquitoes are generalist pollinators, it is unlikely that any of these ornamentals are developing their nectar for mosquito attraction specifically (Peach and Gries, 2019). The concentrations and composition of compounds within the nectar of the eleven ornamentals still plays a part in how attractive the given plant is to the mosquito, but future work using liquid-chromatography mass-spectroscopy (LC-MS) (see General Discussion and Perspectives) is needed to further our understanding of the amount of nutrients the mosquitoes obtain by visiting the plants. Because of the multi-cue approach mosquitoes take to locate a feed on a sugar source, the effect of flower color should also be considered. As it stands in the literature at the current time of writing, mosquitoes seem to exhibit a slight preference for lighter-colored

flowers, and *Ae. aegypti* have been shown to be sensitive to ultraviolet and yellow-green wavelength ranges (Foster, 1995; Clements, 1999; Peach et al. 2019). This preference seems to be supported here, with pink and, particularly, yellow flowers (*e.g.*, Wave Petunia, Marigold, Goldenrod) getting consistently higher activities from *Ae. aegypti* and *Ae. albopictus*, while the purple flowers (*e.g.*, Mexican Heather, Scaevola) had relatively lower activities from mosquitoes. Lastly, the morphology of the plant's flowers and how well the mosquito's proboscis can probe them and receive nectar is an important aspect of this relationship and could provide a physical restriction to an otherwise attractive flower. For example, Goldenrod's tube-shaped flowers were likely easy to access and were part of the reason why such a high proportion of *Ae. albopictus* were positive for fructose. In contrast, the wide, circular shape of Marigold's flowers may have provided an inhibition to sugar access for both species.

Experiments to directly follow the results presented in this section include determining volatile emission rate via internal standards, gas-chromatography electroantennograms GC-EAGs, and y-maze/olfactometer experiments. Each of these is described in detail in the following "*General Discussion and Perspectives*" section of this thesis.

Important note: Some of this work was intended to be carried out before the end of these thesis graduate studies, but was inhibited due to the outbreak of covid-19 during the 2020 spring semester. This created a constraint that put a halt to research in the lab. Experiments planned for the 2020 spring semester affected by the pandemic were gas-chromatography electroantennograms (GC-EAGs) and running GC-MS samples with internal and external standards.

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General Discussion and Perspectives

The data presented here help to build on our limited understanding of mosquito phytophagy. The mosquito-plant relationship is multi-faceted, and can be affected by multiple variables. We first looked at temperature and its effect on *Ae. aegypti* sugar-feeding activity, behavior and how an abiotic factor, such as temperature, impacts survival. Using actometer experiments and warm anthrone assays, an optimum temperature range for efficient sugar-feeding, and consequently blood-feeding, was determined. Because of the mosquito's physiological dependence on environmental temperatures, they will be affected by variations in climate caused by global warming. Within the next century, climate change is predicted to put close to a billion new people at risk for diseases transmitted by *Ae. aegypti* and *Ae. albopictus* (Ryan, 2019). This makes it crucial to understand how climatic temperatures and humidity will modify the feeding activity of mosquitoes, in order to best control the transmission of vectored diseases. By expanding on the work presented in chapter one and using it in conjunction with predictive climate modeling and ATSB placement in high-risk areas, precautions can be made against times when vector disease transmission risk will be maximized.

In the second part of this work, we turned to ornamental plants and their role in providing sugar to two invasive mosquito species: *Ae. aegypti* and *Ae. albopictus,* which live in urban areas in close association with their human hosts. Evidence for sugar-feeding activity on eleven different ornamentals was provided using plant visitation and cold and warm anthrone assays. Additionally, we used gas-chromatography mass-spectroscopy to analyze the volatile composition of the headspace of each of the ornamental species, and discussed the roles these volatiles might play in mediating mosquito-plant interactions. The data presented in chapter two of this work can be shared with city officials and the general public to inform them of ornamental plants that may or

may not be attracting mosquitoes to the public areas they are being planted. Specifically, Goldenrod and Yarrow had the highest amount of feeding and landing activity from both mosquito species; similarly, Butterfly Bush was seen fed on multiple times by both species. Conversely, Marigold and Mexican Heather had relatively low feeding and landing activity from both species and did not seem to provide any (or a limited amount of) carbohydrates to the mosquitoes. In all, the work from chapter 2 shows that *Ae. aegypti* and *Ae. albopictus* can make use of energy provided to them from ornamental plant sugars to help establish their presence in urban, heavily populated areas in the US.

ATSBs are a new and rapidly developing tool for vector surveillance and control. These baits are an appealing solution because of their minimized impact on the environment and relative ease to create, distribute, and setup. However, more applied work is necessitated for these traps before they can be widely distributed to high disease-risk areas. At the time of writing, dissertation research based on the results presented in chapter two to optimize the efficacy of ATSBs is planned for the next few years. After determination of volatiles and volatile blends that *Ae. aegypti* and *Ae. albopictus* find irrefutably attractive in the lab using y-maze/olfactometer experiments, these blends will be tested in the field on local populations in the New River Valley to determine their effectiveness for use with ATSBs. Furthermore, "cocktails" with varying concentrations and compositions of volatile blends and insecticides can be tested in the field to minimize negative effects on the environment and non-target organisms and to maximize the attraction of invasive mosquito species.

Several experiments will be conducted in the future, tentatively as part of dissertation research, based on the work presented in the preceding sections, including running all of the GC-MS samples with an internal standard. Using this method, the emission rate for all volatiles of

interest for each of the different eleven ornamentals will be determined. Briefly, a calibration curve is built using known concentrations of a chemical. A known concentration of this compound that will not interfere with the concentrations or stability of volatiles within the scent is added to an aliquot of the sample. The sample is then run with the GC-MS using the same technique as described previously, and a new chromatogram containing a new internal standard peak is generated. Because the concentration of the standard is known, the abundance of its associated peak can be compared to other peaks representing known volatiles of interest, and emission rates can be identified. In order to accurately identify the compounds emitted by the plants, external synthetic standards, if available commercially, can be injected in the GC-MS and their ion profiles be compared with the peaks of interest in the plant chromatogram. From here, important volatiles in each plant's scent profile can be confirmed, and a list of volatile compounds to test in subsequent electroantennography experiments can be created. We have started this part of the work using heptyl acetate as the internal standard and will resume once access to the laboratory is restored. This will allow us to statistically compare the scent of the different plants (as in Lahondère et al., 2020) and possibly determine trends that could indicate / predict if a plant might be attractive or repellent to the mosquitoes based on the composition of its scent.

Electroantennography (EAG) is a method by which the electrical activity of an insect's antennae, when exposed to a volatile compound of choice, can be recorded. Whether or not the insect is detecting a volatile or blend thereof can be determined by the presence or absence of a deflection in the electrical signal (see Fig. 26). Combining this technique with gas-chromatography (*i.e.*, GC-EAG) allows for the antennae to be exposed to all volatiles within a given scent profile, one by one. Deflections can be attributed as responses to individual components of the scent profile, based on the time at which they are observed. In the context of this thesis, GC-EAGS will

be used with *Ae. aegypti* and *Ae. albopictus* and the scents extracted from the eleven different ornamental plants to provide evidence as to which volatiles these aedine species are responsive to. Using information produced from the previously mentioned emission rates and GC-EAGs, EAGs can then be used with compounds and blends to further identify and confirm the specific components of each scent that are eliciting antennal responses. Moreover, we can test various concentrations of a given chemical or blend.

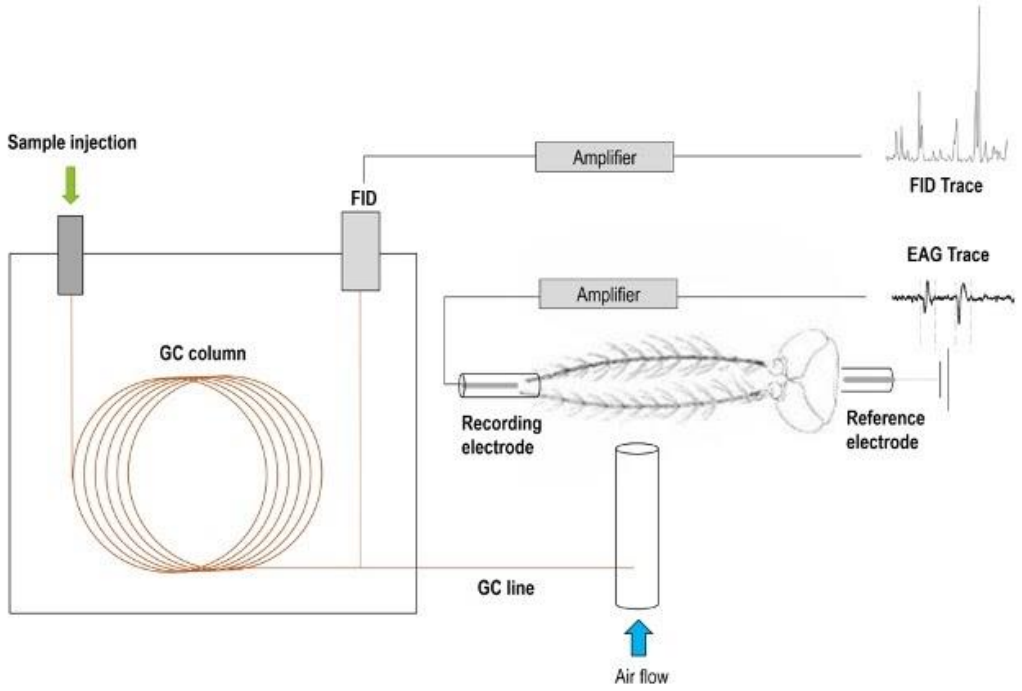

Figure 26. Electro-antennogram coupled with Gas-Chromatography (GC-EAGs) Schematic. A mosquito head is mounted between two electrodes (*i.e.*, reference and recording electrodes) which are connected to an amplifier to record the electrical activity in the antennae. The head is bathed in an airflow that carries the volatiles released by the GC. The injected plant headspace is split between the mosquito head and the FID which is connected to a computer that records the chromatogram trace. If the antennae are detecting a chemical, a deflection can be observed on the EAG trace.

From here, Y-maze olfactometer (see Fig. 27) experiments would be conducted to assess whether the responses given by these compounds (at a given concentration) are leading to attraction, repellency, or indifference in the mosquitoes. Mosquitoes would be released on one side

of the maze, while the other sides on the opposite end of the maze would be supplemented with the volatile compound of interest and a negative or positive control (*i.e.*, the solvent of the chemical). The activity of the mosquitoes within the maze would indicate the valence of a given volatile or blend of volatiles. For instance, the choice of mosquitoes to gather at the side of the maze containing a volatile rather than a negative control would imply attraction for the volatile. This technique could also be performed using two different ornamentals to determine if there is a mosquito preference between flowers, which could not be determined using the single-ornamental plant visitation assays.

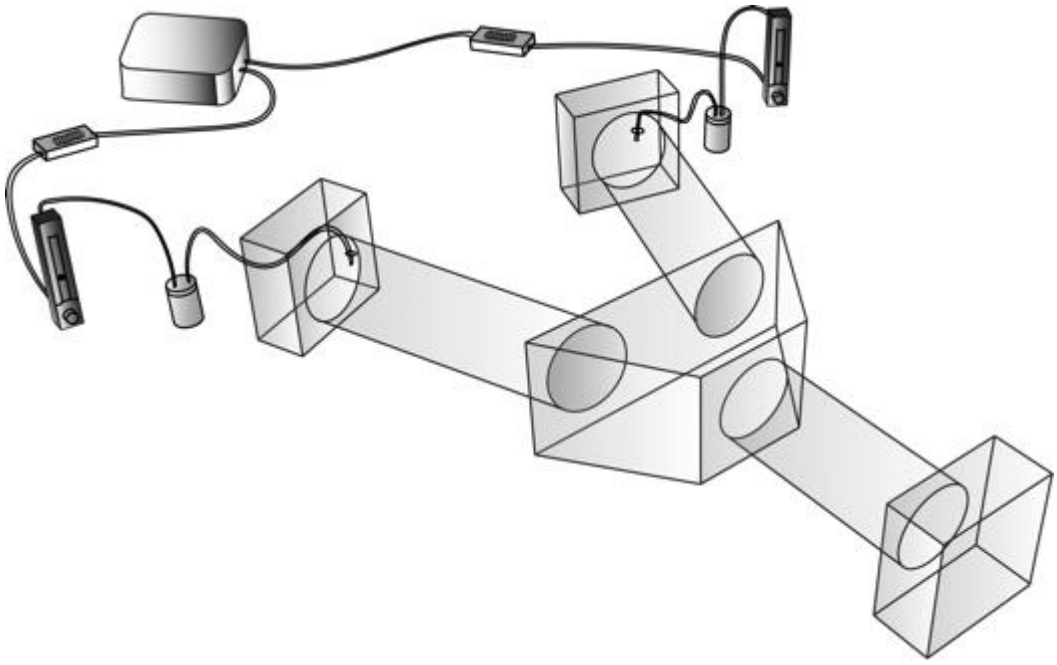

Figure 27. Y-Maze Olfactometer Schematic. A mosquito is released in the front chamber and has to fly upwind and choose between one of the two olfactometer arms, each carrying a different chemical, blend of chemicals or control. Air flow is created by two fans at the rear end of each olfactometer tube. The chemicals of interest are released inside the olfactometer via a pump and tubing connected to a vial containing the chemical(s) (modified from Vinauger *et al.*, 2018).

In addition to the aforementioned experiments planned to be conducted in the Lahondère lab, collaborative projects can further develop our understanding of olfaction and mosquito

phytophagy and open new applications for the use of this information. A relatively understudied dynamic of mosquito phytophagy is the role that nectar composition, in conjunction with olfactory and visual cues, has in guiding the mosquito's choices when searching for plant sugar. Using liquid-chromatography mass-spectroscopy techniques in collaboration with Dr. Kylie Allen's (Department of Biochemistry, Virginia Tech) lab, the chemical components of nectar from varying flower species, including the tested eleven ornamentals, can be determined and assessed. Applying the information of phytophagy and olfaction beyond ATSBs, the remote application of attractant-containing traps delivered via unmanned systems has also been proposed in collaboration with Dr. David Schmale's (Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech) lab.

Numerous experiments based on the work in chapter two can be conducted in the future. These include "field" observations of interactions between these ornamentals and local mosquitoes in areas, such as residential backyards, where mosquitoes would come upon the ornamentals naturally. This would help to circumvent the main shortcoming of the plant visitation assays, namely that the mosquitoes are provided with only one ornamental species to land or feed from, which is not likely what they would experience in a "real-life" environment. In addition, an in-depth analysis of the role that *Ae. aegypti* and *Ae. albopictus* may have in pollinating these ornamentals can be assessed. As mentioned above, an NMDS analysis of the GC-MS data can be performed to compare plant scent profiles to tentatively determine their role in attraction/repellency.

Finally, the data presented in chapter two of this work can be shared with city officials and the general public to inform them of ornamental plants that may or may not be attracting mosquitoes to the public areas they are being planted. Specifically, Goldenrod and Yarrow had the

highest amount of feeding and landing activity from both mosquito species; similarly, Butterfly Bush was seen fed on multiple times by both species. Conversely, Marigold and Mexican Heather had relatively low feeding and landing activity from both species, and did not seem to provide any carbohydrates to the mosquitoes. More work needs to be done as to how the activities of mosquitoes will change when exposed to the choice of several different species of flowers, as they would be in a city garden. In addition, each mosquito species might respond differently to the plants; therefore, expanding this work to more invasive mosquito species and more ornamentals would provide valuable insight into mosquito-plant interactions. Knowledge generated by this work will also inform the development of mosquito control tools including Toxic Sugar Baits (see above), and will build upon the current knowledge in the field of volatiles known to elicit responses in invasive *Ae. aegypti* and *Ae. albopictus*.

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