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# THE OLFACTORY AND MICROBIAL BASIS OF MOSQUITO ATTRACTION TO HUMANS

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To: Dean Michael R. Heithaus
College of Arts, Sciences, and Education

This dissertation, written by John S. Castillo, and entitled The Olfactory and Microbial Basis of Mosquito Attraction to Humans, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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DEDICATION

This dissertation is dedicated to my anchors my mother and my father. Your constant sacrifice over the years has always inspired me to seek more to think outside of the box and to never give up. Without your exemplary leadership this would not be possible.

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ABSTRACT OF THE DISSERTATION

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Mosquitoes infect hundreds of millions of people every year with a variety of deadly viruses, making them the most dangerous animal on earth. However, humans are not equally attractive to mosquitoes, leaving some individuals more vulnerable to mosquito borne-illness than others. The infectious behavior of the mosquito is dependent on multiple sensory cues, with odor amongst the most crucial cues for human host detection. Human odor is strongly influenced by an individual's skin microbiome, as the human body would be largely odorless if not for the volatile organic compounds produced by the commensal bacteria on the human skin. Multiple studies have shown that skin microbiota play an important role in generating volatile compounds from sweat. Using a uniport olfactometer to measure mosquito attraction of *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* mosquitoes. 119 human subjects were assayed and the microbiome and volatilome of each subject was sampled in the same session to capture an odor and microbial profile for each individual. To determine what strains of bacteria were associated with high attraction and low attraction individuals we used random

forests as well as a mixed effects model. We identified chemical compounds and strains of bacteria that are differentially abundant between subjects. By examining the interaction of attraction, volatilome, and microbiome across subjects, this study aims to determine how the bacteria on our skin affect mosquito attraction.

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| | |
|---|---|
| *Ae.* | *Aedes* |
| *An.* | *Anopheles* |
| ASV | Amplicon Sequence Variant |
| $CO_2$ | Carbon dioxide |
| *Cx.* | *Culex* |
| *C.* | *Corynebacterium* |
| DEET | N, N-Diethyl-meta-toluamide |
| EAG | Electroantennography |
| GRs | Gustatory Receptors |
| IRs | Ionotropic Receptors |
| LH | Lateral horn |
| MB | Mushroom body |
| *orco* | Olfactory receptor co-receptor |
| ORN | Olfactory receptor neuron |
| ORs | Odorant receptor |
| *P.* | *Pseudomonas* |
| *S.* | *Staphylococcus* |
| Spp. | Species |
| QIIME | Quantitative Insights Into Microbial Ecology |

## CHAPTER 1: Introduction

"Be persistent like a mosquito, at the end you will get your bite."

- Bangambiki Habyarimana

Human skin secretions are a contributor to human odor that attract mosquitoes and may explain why some people are bitten more frequently than others. Due to their effectiveness as vectors which results in mortality, incapacity, and economic losses, mosquitoes are widely regarded as the most hazardous disease-transmitting vectors to humans. Mosquitoes are efficient vectors for a range of human borne pathogens including Dengue viruses (DENV), Chikungunya virus (CHIKV), and Zika virus (ZIKV). Nearly half of the world is susceptible to DENV, and there has been a resurgence of both ZIKV and CHIKV over the last ten years (Christofferson 2016, Adams et al., 2019). Olfaction is essential to numerous stages of the mosquito's life cycle, including reproduction and blood consumption. This sensory system enables mosquitoes to detect various aspects of their environment, including potential sustenance sources and oviposition sites. The human host is an integral component of the life cycle of anthropophilic mosquitoes such as *Aedes aegypti*. The occurrence of human host preference in mosquitoes is not common; it has occurred in only a couple hundred out of approximately 3500 mosquito species, including *Aedes aegypti* sister species *Aedes albopictus*, malaria vector *Anopheles gambiae*, and West Nile Virus vector *Culex quinquefasciatus* (Takken & Knols, 2009, Fang 2010). *Aedes aegypti* has come to flourish in tropic and subtropic environments throughout the world while *Aedes albopictus* flourishes in cooler climates, but their habitats are expanding due to rising global temperatures (Kraemer et al., 2015). Anthropophilic mosquitoes are extremely attracted to human scent, but human scent is a complex odor space comprised of over 1,000 volatile organic compounds (VOCs) (Drabińska et al., 2021). It is difficult to determine which components of the

odor plume are responsible for mosquito attraction due to the complexity of the odor plume (Amann et al., 2014). Isolating the components of human odor that attract mosquitoes could lead to novel strategies in combatting vector borne illness.

Humans are odorless if not for the skin bacteria that breakdown amino acids and lipids, from body secretions, into volatile short chain carboxylic acids (Shelley et al., 1953, James et al., 2004). Human attraction to mosquitoes thus is directly related to our skin microbiome and individuals that are favored result in higher risk of contracting vector-borne disease. Additionally age, sex, pregnancy, personal hygiene, and parasite infection are variables that have been shown to affect a person's attractiveness to mosquitoes (Muirhead-Thomson 1951, Port et al., 1980, Lindsay et al., 2000, Robinson et al., 2018). Olfactometry has been thoroughly used to elucidate various components of human odor that attract mosquitoes (Bernier et al., 2003, Liesch et al., 2013, DeGennaro et al., 2013, McBride et al., 2014, McMeniman et al., 2014, Basrur et al., 2020). Preference for human hosts over other vertebrates is determined by neural changes that involve central brain circuits and modifications in the confirmation of odor specific olfactory receptors (Bohbot et al., 2010, DeGennaro et al., 2013, McMeniman et al. 2014, McBride et al., 2014).

This introduction seeks to present the current knowledge on the topic of mosquito olfactometry, olfaction and the progress made in understanding how human skin commensals influence human odor and thus mosquito attraction behavior.

**1.1 Using Olfactometers to study mosquito behavior**

Olfaction is central to many stages of the mosquito life cycle, particularly in reproduction and blood feeding. This sensory system allows the mosquito to detect different aspects of their environment, including food sources and possible oviposition destinations. For anthropophilic mosquitoes such as Aedes aegypti, the human host is a critical part of the life cycle.

Anthropophilic mosquitoes are highly attracted to human scent, but human scent is a complex odor space comprised of over 1,000 volatile organic compounds (VOCs) (Drabińska et al., 2021). Due to the intricacy of human odor dispersion, it is difficult to determine which components are essential for mosquito attraction (Amann et al., 2014).

Olfactometry has been extensively used to study mosquito behavior in different ecological contexts (Willis 1947, Acree et al., 1968, Feinsod & Spellman, 1979, Eiras & Jepson 1994, Klowden 1996, Bernier et al., 2003, Liesch et al., 2013, DeGennaro et al., 2013, McBride et al., 2014, McMeniman et al., 2014, Basrur et al., 2020). Single choice olfactometers can be used to identify ecologically-relevant compounds that influence mosquito behavior. Early examples of olfactometer experiments studied live human odor and were used to isolate mosquito host-seeking cues such as CO2 and temperature (Willis 1947). Interestingly, the mosquito attractant lactic acid was identified in a single choice olfactometer assay (Acree et al., 1968). Simple innovations such as using an airstream to stimulate mosquito flight upwind and the introduction of a CO2 plume drastically increase mosquito activity in the olfactomer assay.

Most of the research on mosquito attraction and repulsion has been conducted using olfactometers, which are devices designed to introduce olfactory stimuli under controlled conditions and ascertain a behavioral response. The Y-tube and dual-port olfactometers are comparable in that they permit researchers to compare mosquito attraction to two distinct stimuli, such as carbon dioxide, lactic acid, or human hosts (Acree et al., 1968, Braks & Takken 1999, Geier & Boeckman 1999). Geometric differences between Y-tube and dual-port olfactometers may influence the distribution of odors and presentation of stimuli in these two assay types (Geier & Boeckman, 1999). Early olfactometer experiments were critical for the establishment of mosquito olfaction as a field (Willis 1947, Acree et al., 1968, Feinsod & Spellman, 1979, Klowden 1996) but involved highly complex setups that imposed challenges for experimental

replication. Modern single-choice uniport olfactometers made improvements in usability and were constructed using acrylic material that make them highly transparent for the detection of insect location, easy to fabricate, and simple to assemble (Liesch et al., 2013).

Researchers (Otienoburu et al., 2011, Busula et al., 2015, and van Loon et al., 2015) have used mesocosms, which are enclosed, mid-sized natural settings that allow mosquitoes to be studied in a controlled setting. These studies show that mosquitoes are attracted to mixtures of human odor and plant volatiles. These mesocosm studies are valuable because they have shown that mosquitoes can detect low concentrations of compounds when nectar and host seeking (Torr et al., 2008; van Loon et al., 2015); however, mesocosm experiments are conducted at greater distances than olfactometer experiments. The application of uniport olfactometry to the study of ecologically significant volatile organic compounds provides a potent method to quantitatively test attraction at a range of up to 1 m and evaluate olfactory cues in isolation.

### 1.2 Mosquito Olfaction

Over 3,500 mosquito species have been documented globally, encompassing both blood-feeding and non-blood-feeding species (Clements, 1999; Lehane, 2005). Host choice by blood-feeding mosquitoes is exceptionally broad, with female mosquitoes feeding on humans, non-human primates, other mammals, birds, amphibians as well as invertebrates (Clements, 1999; Harris et al., 1969; Tempelis, 1975; Reeves et al., 2018; Verhulst et al., 2018). Mosquitoes locate and distinguish among potential hosts based predominantly on the detection of olfactory signals from host secretions (Cardé, 2015; McBride, 2016; Takken and Knols, 1999; Takken and Verhulst, 2013; Tchouassi et al., 2022). Many mosquito species do not demonstrate a preference for specific hosts, but feed opportunistically across a host range available in their habitat (Lyimo and Ferguson, 2009; Takken and Verhulst, 2013).

Anthropophilic female mosquitoes require vertebrate blood consumption to obtain the nutrients necessary for embryo development (Detinova, 1949). This sequence of alternate feeding and behavior enables mosquitoes to seek a new human host, thereby facilitating the transmission of pathogens such as dengue viruses (DENV), chikungunya virus (CHIKV), and Zika virus (ZIKV), among others (Patterson et al., 2016). Female mosquitoes traverse their complex environment by incorporating a variety of sensory inputs and rely predominantly on their chemosensory systems to find potential hosts. These chemosensory systems facilitate the interpretation of external information from the periphery by directly connecting to the central nervous system, which coordinates survival-related behaviors such as obtaining a blood meal (McIver 1987, Su et al., 2012). Females utilize chemosensory appendages such as the antenna and maxillary palp to locate a suitable host (Raji & DeGennaro, 2017). The antenna and maxillary palp are covered with sensory hairs known as sensilla, which contain insect olfactory sensory neurons (OSNs) that have a direct association with the antennal lobe in the brain and, in most circumstances, adhere to the one-receptor-one-neuron rule (Nakagawa & Vosshall, 2008, Carey et al., 2010). Recent data reveals that mosquitoes have a more intricate olfactory system than other insects giving them the ability to recognize hosts with greater sensitivity (Herre et al., 2022). OSNs connect to interneurons and projection neurons inside the brain, which transmit information to higher brain locations including the mushroom bodies and Kenyon cells of the lateral horn (Heisenberg 2003). These neurons house sensory receptors that are responsible for distinguishing between vertebrate species and sensing numerous characteristics of the mosquito host.

Body odor (produced by the microbiota on our epidermis [Shelley et al., 1953; Verhulst et al., 2011]) and carbon dioxide are important mosquito attractants that signal the presence of host. In the absence of carbon dioxide, loss of odorant receptor (OR) and ionotropic receptor (IR)

function in female mosquitoes reduces host attraction (DeGennaro et al., 2013, Raji et al., 2019). Carbon dioxide (CO2) is an important cue because it signals that a vertebrate may be nearby; it activates the female mosquito to switch from nectar-seeking to host-seeking (Dekker et al., 2005). Female mosquitoes use gustatory receptors (GRs) Gr1, Gr2, and Gr3 to detect CO2 emitted by a vertebrate host approximately 10–20 ms after exposure (McMeniman et al., 2014; van Breugel et al., 2015). Females approach the host and use IRs Ir21a and Ir25a, as well as the transient receptor potential channel A1 (TRPA1), to detect heat (Corfas & Vosshall, 2015; Greppi et al., 2020). Finally, if all the necessary host cues are confirmed, the female lands and uses an array of receptors in her legs and proboscis to initiate blood-feeding (Sparks et al., 2013, Jung et al., 2015, Jove et al., 2020). Neurons expressing OR receptors recognize a variety of volatile organic compounds (VOCs), including terpenes, aldehydes, esters, alcohols, aromatics, and ketones, whereas the more conserved IRs have been shown to respond to carboxylic acids and amines (Carey et al., 2010, Pitts et al., 2017; Raji et al., 2019). Several of these chemical classes have been identified in the emanations of host organisms (Bernier et al., 1999, 2000, Zhao et al., 2022). Although many discoveries about the function of chemosensory receptors in host-seeking have been made, the components of human odor that attract mosquitoes are still immensely unknown.

By determining which components of human host odor influence mosquito attraction, we can identify novel repellents and attractants to manipulate mosquito behavior while reducing the spread of vector-borne diseases.

### 1.3 The human skin microbiome and its influence on human odor

Odors are among the most crucial sensory cues for mosquitoes to differentiate and detect potential human hosts. Human odor is derived from biotransformations of odorless skin secretions, into volatile organic compounds (VOC's), by cutaneous bacteria of the human skin

microbiome (HSM) (Shelley et al, 1953, Leyden et al., 1981). The HSM is a dynamic often protective landscape of many different bacterial groups dominated by *Cutibacterium* spp., *Staphylococcus* spp., and *Corynebacterium* spp in healthy individuals (Byrd et al., 2018). To persist in such an algid, acidic, and dry environment, the resident microbiota of our skin has adapted to utilize our sweat, sebum, and the stratum corneum as resources (Scharschmidt & Fishbach, 2013). The relative abundance of bacterial taxa associated with moist, arid, and sebaceous microenvironments varies according to the physiology of the skin site, as determined by sequencing surveys of healthy adults (Costello et al., 2009, Grice et al., 2009). Sebaceous sites are dominated by lipophilic *Cutibacterium* species, whereas bacteria that flourish in humid environments, such as *Staphylococcu*s and *Corynebacterium* species, were more prevalent in moist areas, such as the elbow creases and the soles of the feet (Grice & Segre 2011). Despite persistent environmental changes, longitudinal sampling revealed that cutaneous microbial communities were largely stable over the course of a two-year study (Oh et al., 2016). Based on strain and single-nucleotide-level analyses, stability was determined by the maintenance of strains over time, as opposed to the reacquisition of common species from the environment (Oh et al., 2016). Individuals were found to be colonized by distinct multi-phyletic communities of *Cutibacterium acne*s and *Staphylococcus epidermidis* (*S. epidermidis*) strains at different body sites (Oh et al., 2016). Furthermore, *S. epidermidis* isolates from a commensal clade exhibited site specificity for human feet (Oh et al., 2016). Not only are the HSM's of individuals stable over time but the diversity of species on the HSM is correlated with health, signaling the importance of the immune system in maintaining healthy populations of bacteria (Byrd et al., 2018).

Correlations exist between high microbial population densities in the axilla and odor, with individuals who are considered to have malodor housing a greater proportion of lipophilic

and nonlipophilic *Corynebacterium* in their axilla (Leyden et al., 1981). An extensive population of human skin bacteria expands on the secretions of the eccrine, apocrine, and sebaceous glands and breaks down precursors into $C_2$-$C_{18}$ fatty acids (Preti et al., 1987, Parekh 2002). Skin bacteria, most notably of the *Corynebacterium* and *Staphylococcus* genus, convert volatile short-chain carboxylic acids from lipids and amino acids in sweat (Natsch et al., 2003, James et al., 2004). Studies have shown that there are 26 different carboxylic acids which are secreted from human sweat as precursors and broken down by the Corynebacteirum enzyme N-acyl-glutamine-aminoacylase, contributing to axillary odor (Natsch et al., 2006).

Given that the bacteria from our HSM contribute dramatically to our bodily odors, and mosquitoes rely heavily on their olfactory systems to find human hosts, the bacterial species of an individual's HSM could determine whether they are more prone to mosquito bites. Thus, it is important to determine what bacterial populations contribute to an individual's mosquito attraction.

**1.4 The role of bacteria on mosquito attraction behavior**

The concentration and chemical composition of human odor is associated with the bacteria on human skin with individuals that have a larger microbial population density of *Corynebacterium* spp. exhibit more intense odor (Rennie et al., 1991). Human odor is a strong attractant to mosquitoes over the odor of other vertebrates (DeGennaro et al., 2013). Bacteria was first shown to influence mosquito behavior when scientists showed that a broth culture of Bacillus cereus derived from human skin was attractive to *Ae. aegypti* (Shreck and James 1968). Multiple studies have demonstrated that skin bacteria from our sweat affect mosquito attraction (Meijerink et al., 2000, Bernier et al., 2000, Verhulst et al., 2009, Verhulst et al., 2010, Verhulst et al., 2011). The role of bacteria in mosquito attraction was further confirmed when scientists showed that fresh sweat sterilized using a bacterial filter and incubated was less attractive to *An.*

*gambiae* mosquitoes than incubated sweat (Braks et al. 1999). Furthermore, incubated broth of bacterial strains *Staphylococcus epidermidis* and *Corynebacterium minutissimum* have been demonstrated to attract *An. gambiae* (Verhulst et al., 2010). This group additionally showed that mosquitoes were more attracted to subjects with reduced microbial diversity and higher levels of *Staphylococcus* spp., but were less attracted to individuals with higher levels of *Pseudomonas* spp. (Verhulst et al., 2011). Individuals that were in the poorly attractive group had 3.1 fold more Pseudomonas spp. than their highly attractive counterparts (Verhulst et al., 2011). In a recent study containing only post-menopausal females, *Staphylococcus* spp. and *Streptococcus* spp. is more commonly found on individuals that are highly attractive to *An. coluzzi* while subjects in the poorly attractive group contained *Finegoldia* spp (Showering et al., 2022). The species of bacteria on one's skin as well as the diversity of species can influence mosquito attraction of humans which additionally, is influenced by mosquito species (Busula et al., 2017).

Many of the studies establishing the HSM as modulators of mosquito preference involved using *An. gambiae* with fewer studies exploring the role of bacteria in attraction in *Ae. albopictus* (Michalet et al, 2019). For *Ae. albopictus*, studies using bacterial isolates showed 3 distinct species of bacteria namely *Staphylococcus saprophyticus*, *Kytococcus sedentarius*, and *Kocuria rhizophila* to be significantly attractive to mosquitoes compared to the luria broth (LB) control plate (Michalet et al., 2019). In this same study, scientists suggested bacterial species *Staphylococcus hominis* and *Corynebacterium tuberculostearicum* to be repellent (Michalet et al., 2019). Less is understood about the role the HSM has in host preference for the highly anthropophilic ZIKA vector *Ae. aegypti* and WNV vector *Cx. quinquefasciatus* which is known to commonly feed on birds among other available species (Syed and Leal 2009, Garcia-Rejon et al., 2010, Spanoudis et al., 2020). Identifying which bacterial strains are most appealing to *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* mosquitoes can increase our

understanding of which skin microbiome components affect mosquito attraction and thus determine which populations are more susceptible to mosquito bites.

It is important to study the link between skin bacteria and mosquito attraction and to pinpoint the volatile chemicals generated by skin bacteria that serve as mosquito attractants. Modulating the HSM to reduce the odors that attract mosquito bites could be a long term approach to combatting vector borne illness transmitted by mosquitoes.

**1.5 Dissertation objectvies and organization**

Chapter 1 introduces the primary topics of the dissertation, namely the use of olfaction in mosquito behavior studies and what is known about the relationship between the microbiome and mosquito attraction. In addition, the current knowledge of mosquito olfaction and bacteria that attract or repel mosquitoes has been reviewed. Chapter 1 also suggests ways forward for novel probiotic vector control strategies. The second chapter provides a comprehensive method for constructing a modern uniport olfactometer for behavioral analysis of mosquito host detection. In addition, this methods paper offers insight into novel studies employing olfactometry, such as the use of males and the evaluation of plants' attractiveness to other insects. Chapter 2 is a reprint of the published method in *Cold Spring Harbor Laboratory Protocols*. In Chapter 3, we give a detailed method on our process of quantifying an olfactometer experiment using *Ae. aegypti*. This methods paper contains the necessary information for individuals to perform, analyze and quantify a reproducible behavioral experiment to study mosquito olfaction using a uniport olfactometer. This chapter is a reprint of the published method in *Cold Spring Harbor Laboratory Protocols*. Chapter 4 investigates mosquito attraction of three vector species *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* to 119 human subjects and identifies a link between the HSM and the VOC's that make up their odor profile. Chapter 5 analyzes the attraction of mosquito species *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* to four common species of bacteria found on human skin

and reports the behavioral responses mosquitoes have to different bacterial community models.

All chapters are summarized in chapter 6. Research gaps and future directions are also discussed.

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# CHAPTER 2: Building a Uniport Olfactometer to Assess Mosquito Responses to Odors

## 2.1 ABSTRACT

The uniport olfactometer behavioral assay is currently one of the most reliable single-choice methods to use to study mosquito attraction to olfactory stimuli. It allows for the reproducible calculation of mosquito attraction rate to human hosts or to other olfactory stimuli. Here, we present the design of our modified uniport olfactometer. Consistent carbon-filtered air flows through the assay, creating positive pressure that reduces odor contamination from the room. It includes a precision-milled white acrylic base to facilitate easy setup and consistent placement of the component parts. Our design can be made by a commercial acrylic fabricator or an academic machine shop. This olfactometer is designed to assess the responses of mosquitoes but could be applied to other insects that fly upwind toward an odor stimulus. In a companion protocol, we detail how to perform the experiments with mosquitoes by using the uniport olfactometer.

## 2.2 MATERIALS

### 2.2.1 Reagents

Compressed $CO_2$ cylinder

Thin mesh wrap (For Attraction Trap Cylinder)

Latex gloves

### 2.2.2 Equipment

Uniport olfactometer components (multiple custom parts constructed from acrylic)

- Stimulus box (length = 25.4cm, height = 25.4cm, breadth = 20.32cm, two entry circles on each side with 13cm diameter)
- Attraction trap cylinder (length = 15.24cm, diameter = 8.255cm)
- Activated chamber (length = 74.93cm, diameter = 13cm)
- Activated chamber circular entry plate (diameter 13cm with 5.08cm hole in the center)
- Activated chamber circular exit plate (diameter 13cm with 8.255cm hole in the center)

Flowmeter for air (King Instrument Company, 7520 series, 2-24 SCFH)

Flowmeter for $CO_2$ (Model VFA-1, 0.1-1 SCFH, Dwyer Instruments, Inc)

Vacuum pump (oil-free, model used: Gast DOA-P704-AA high-capacity vacuum pump, with gauge, regulator, and relief; 1.1 cfm, 25.5" Hg, 115 VAC)

Erlenmeyer flask (2000ml)

$CO_2$ tank

$CO_2$ regulator

WHO tubes (length = 13cm, diameter = 5cm) (World Health Organization Vector Control Research Unit, Penang, Malaysia).

Activated charcoal (CAS #7440-44-0) packed in a media canister with barbed fittings (W.A. Hammond Hammond Drierite™ Gas Drying Unit)

2x ¼" NPT x ¼" Barbed fittings

2x 1/8" NPT x ¼" Barbed fittings

¼" ID Vinyl Tygon tubing (To connect air and $CO_2$ to the stimulus box)

## 2.3 METHODS

### 2.3.1 Fabricating the Olfactometer

1. Contact a local acrylic fabricator to assist you in the construction of the uniport olfactometer according to the plans outlined in figure 1.
2. It is critical to assess that the parts fit together correctly and sit properly in the CNC-milled base.
3. It is recommended that multiple uniports are fabricated at the same time to reduce costs and increase experimental output.
4. Multiple uniport olfactometers can be used at the same time if you have sufficient space.

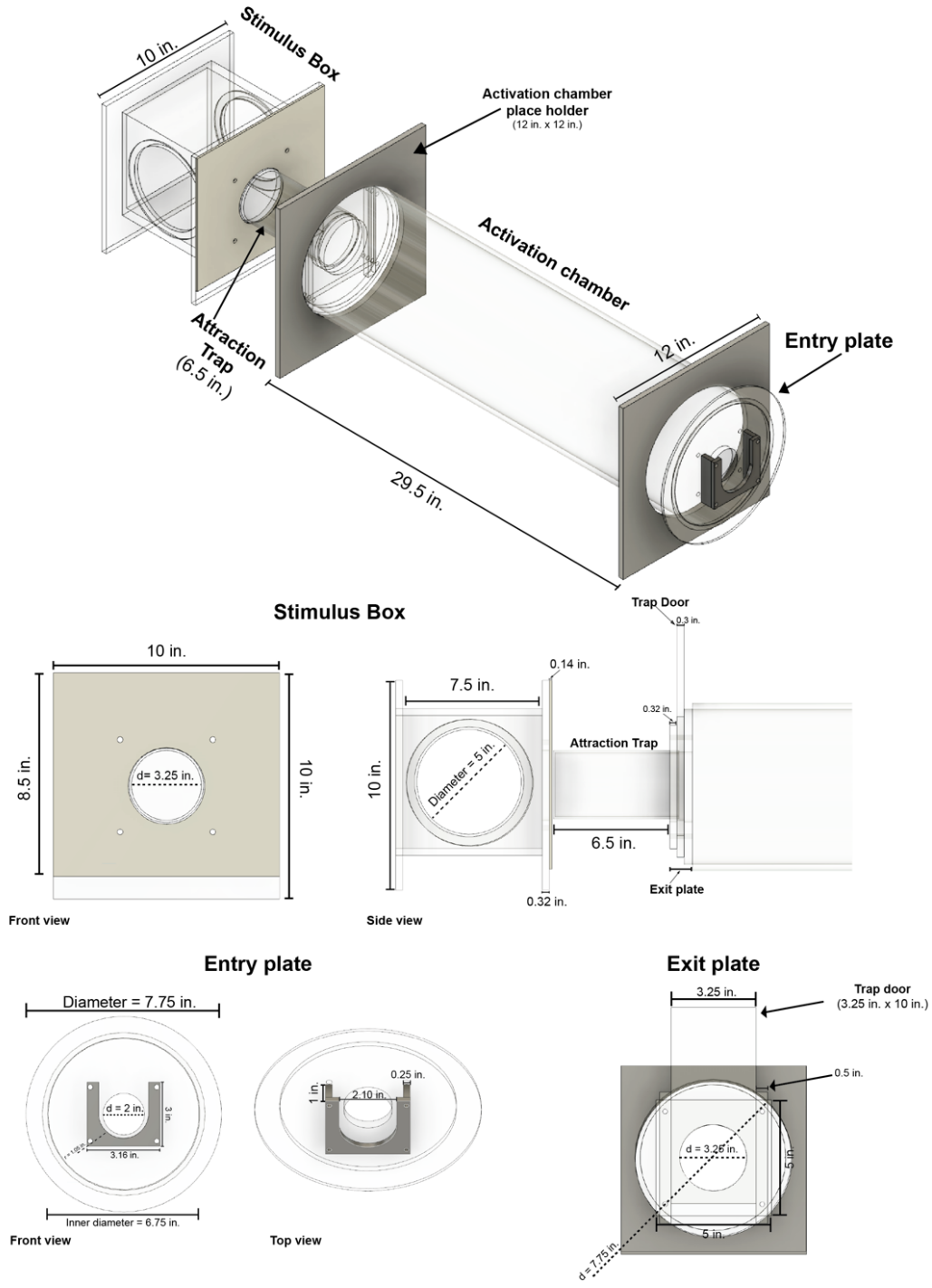


**Figure 1. Plans for fabricating the uniport olfactometer.** All components presented above are made of clear acrylic except for a white acrylic face plate for the stimulus chamber. This removable component of the stimulus chamber was designed to obscure mosquitoes from being able to view items placed in the stimulus chamber from a distance.

### 2.3.2 Assembling the Olfactometer

1. Place olfactometer base (Fig.2) placed on a sturdy table within a humidified and temperature-controlled room.


**Figure 2. The olfactometer base.** The base is made from computer numerical control (CNC) milled white acrylic (24-inch width x 48-inch length x 0.75 inch thick) with 12-inch slots milled to hold the olfactometer component in fixed positions. 6-inch risers hold the stimulus chamber in position but allow for sliding the chamber for easy disassembly. This is designed to hold the olfactometer components securely together, preventing any changes to the olfactometer component alignment to maintain consistency between trials. It is best to bring your olfactometer to the CNC service provider to assure a good fit.

2. Place the activated chamber (Fig.3) on olfactometer base so that it is held in place by slots on the base.

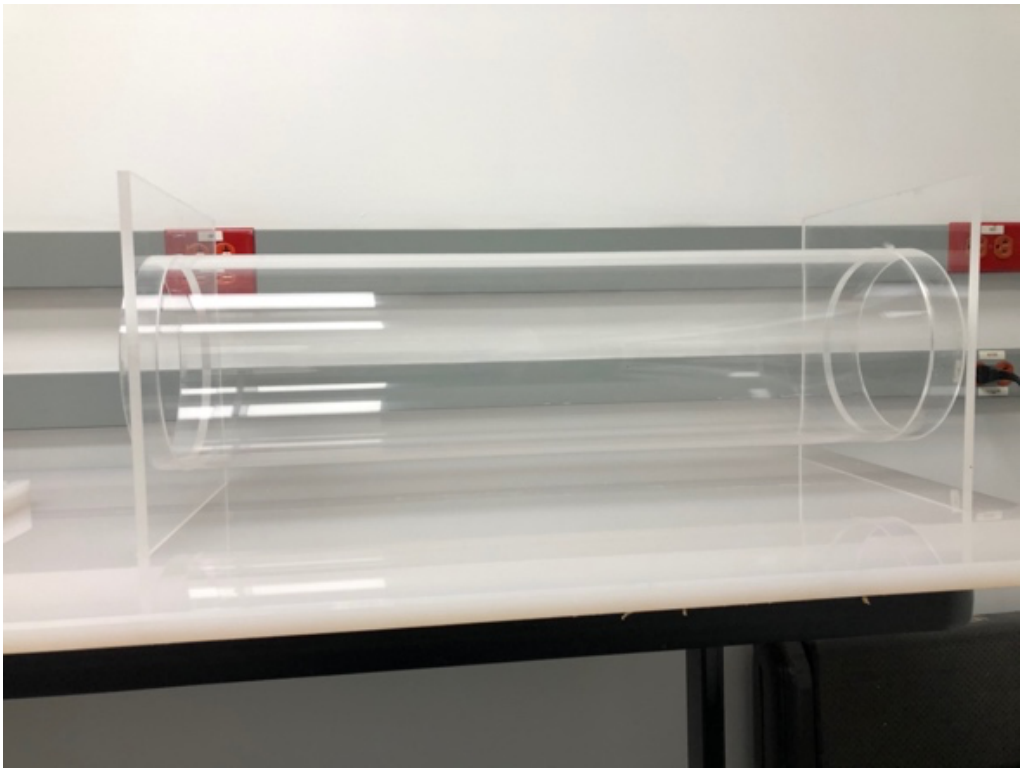

**Figure 3. The activated chamber.** The chamber is securely fitted to the CNC-milled olfactometer base.

3. Fit the activated chamber circular entry and exit plates (Fig. 4) to the respective ends of the activated chamber.

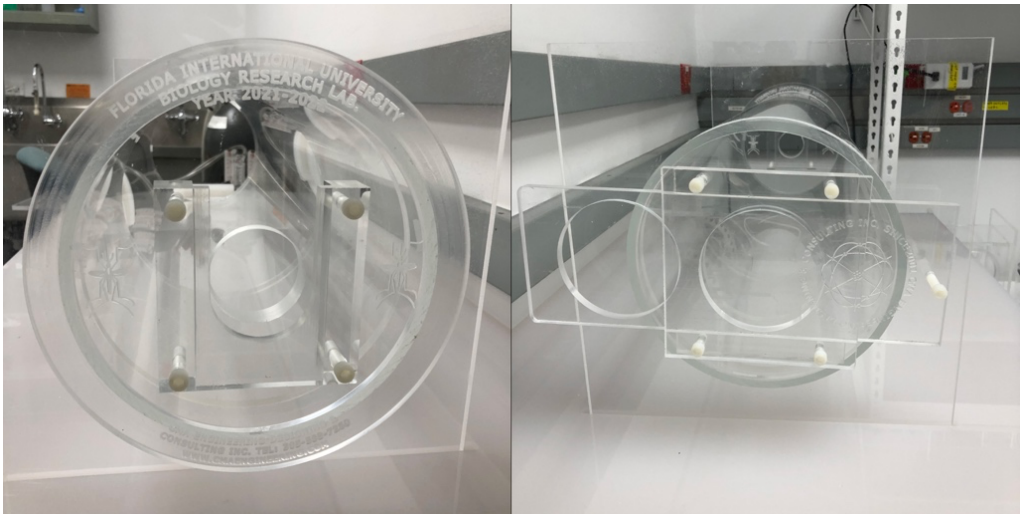


**Figure 4. Activated chamber entry (left) and exit plates (right)**

4. Attach attraction trap to circular exit plate with gate closed.

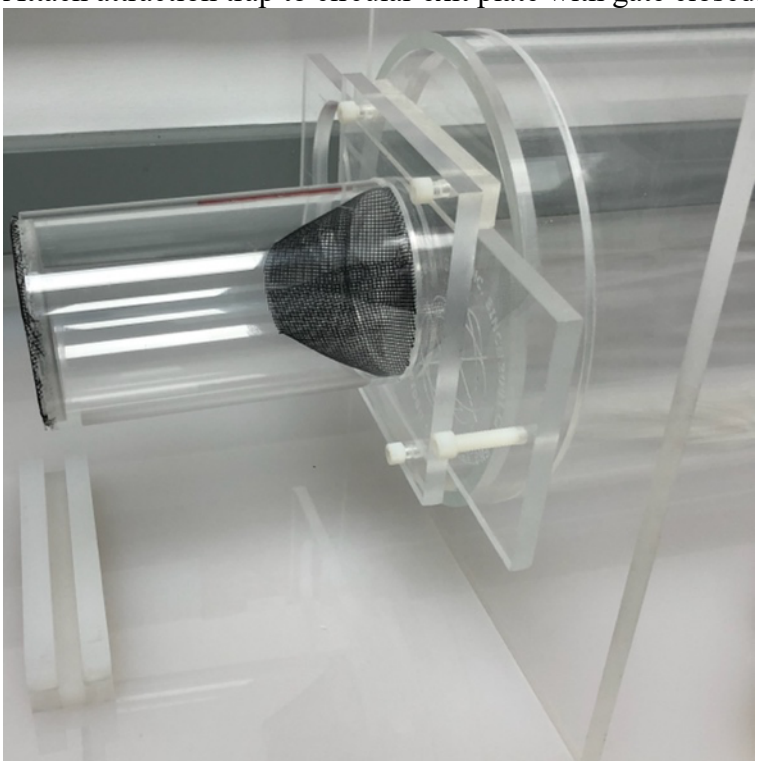

**Figure 5.** Attraction trap attached to circular exit plate.

5. Attach the stimulus box to attraction trap. Use latex gloves to seal the large circular arm entries.

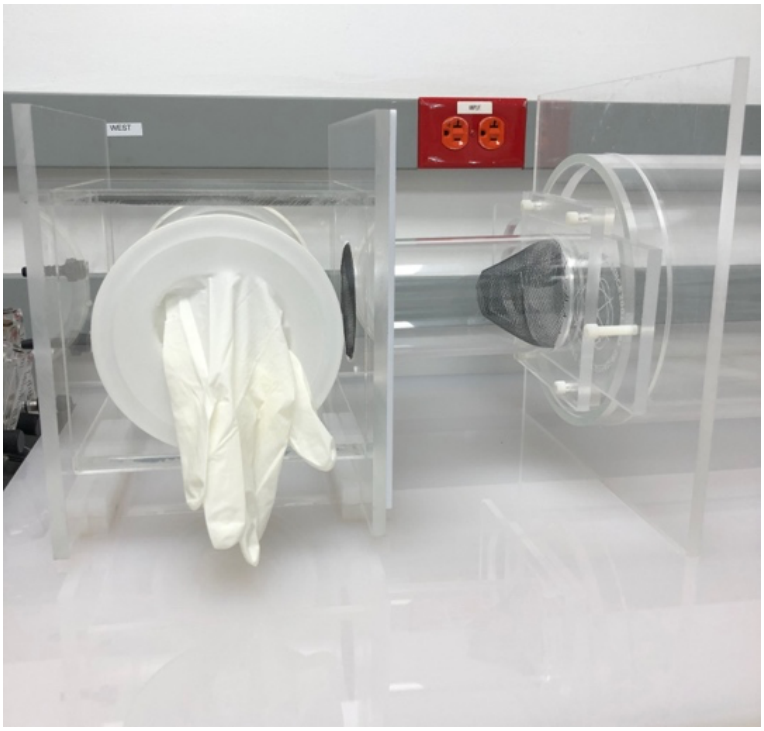

**Figure 6.** Sealed stimulus box attached to attraction trap.

6. Confirm the olfactometer is sealed by lightly pulling on the left latex glove. The right latex glove should move, indicating the olfactometer is airtight.

7. The vacuum pump should be placed on the floor near the olfactometer.

8. Using Tygon tubing, the positive pressure outlet of the vacuum pump is connected to a canister filled with activated charcoal.

9. The charcoal filtered air is in turn connected via Tygon tubing to a 25 cm stainless steel tube in a stoppered 2000 mL Erlenmeyer flask containing 1000 mL of deionized water. It is important that the 25cm stainless steel tube be below water level in the Erlenmeyer flask to ensure humidity in the system.

10. The outlet of the Erlenmeyer flask is then connected to the 2-24 SCFH flowmeter via Tygon tubing.

11. Connect the $CO_2$ tank to a 0.1-1 SCFH flowmeter.

12. Screw ¼" NPT-to-barb fittings into stimulus box and connect the Tygon tubing from the $CO_2$ and airstream flowmeters and the assay is ready to begin.

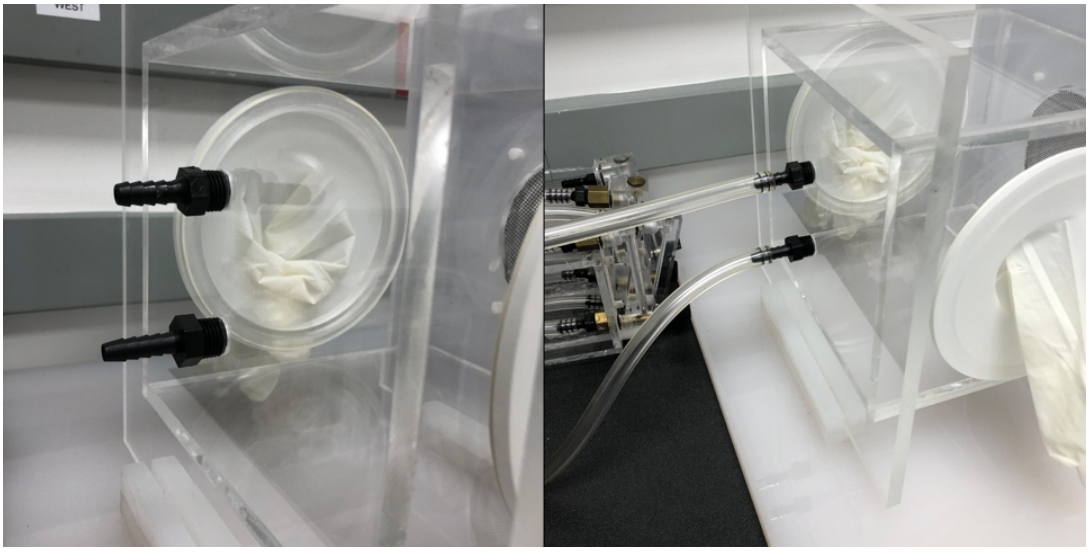

**Figure 7.** Closeup of NPT to barbed fittings screwed into stimulus box (left). Tygon tubing attached to barb fittings (right).

## 2.4 DISCUSSION

### 2.4.1 Fabricating a Uniport Olfactometer

We recommend using a local acrylic fabricator to construct the component parts of the olfactometer from cast acrylic (Liesh et al., 2013). We also recommend that multiple olfactometers be constructed at the same time to reduce the cost per olfactometer and allow for continued olfactometry when one is being used or cleaned. Please see figure 1 for the dimensions to fabricate the component parts of the uniport olfactometer.

### 2.4.2 Assembling the Olfactometer

The olfactometer should be assembled in a climate-controlled room atop a sturdy table or benchtop (McIndoo 1926). The climate of the behavior room should resemble rearing conditions, 25˚C-27˚C with relative humidity of 50-70% is appropriate if using *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* mosquitoes (Willis et al., 1947, Raji et al., 2019) . Olfactometer parts should be thoroughly cleaned and dried before and after each use. It is best practice to first assemble the activated chamber as it is the largest component of the olfactometer,

and once it is properly inserted into the olfactometer base then the other components can be efficiently attached. Following the placement of the activated chamber, connect the entry and exit plates to the respective ends of the chamber. Allow adequate space on the table for the attraction trap and stimulus box. Prior to connecting the stimulus box, place latex gloves onto the entry ports and screw in both ¼" NPT male x ¼" barbed fittings. Once the stimulus box is connected to the attraction trap connect two lengths of Tygon tubing onto the barb fittings at the end of the stimulus box. The vacuum pump should have the media canister with activated charcoal and the Erlenmeyer flask with water added in series before attaching to the air flowmeter. The Tygon tubing from the stimulus box should then be connected to the $CO_2$ and air flowmeters which are connected to the $CO_2$ cylinder and the humidified and carbon-filtered air, respectively. To verify that the olfactometer is air sealed turn on the air pump and release $CO_2$ from the cylinder. Latex gloves should fill with air if the system is properly sealed.

## 2.5 REFERENCES

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## CHAPTER 3: Quantifying Mosquito Attraction using a Uniport Olfactometer

### 3.1 ABSTRACT

Female mosquitoes respond to the world around them by using chemosensory organs, such as their antenna, to detect volatile compounds emitted from a vertebrate host. These chemosensory systems facilitate the interpretation of external stimuli from the periphery by connecting to the central nervous system and eliciting behaviors necessary for survival, such as obtaining a blood meal. This innate behavior leads to the transmission of pathogens, including dengue virus, chikungunya virus, and Zika virus. Olfaction is a primary sense mosquitoes use to differentiate between vertebrate hosts and studying it can lead to novel strategies to reduce the risk of disease. In this protocol, we present an olfactory-driven behavioral assay using a uniport olfactometer that measures mosquito attraction rate to a specific stimulus. We include details of the behavioral assay and data analysis, as well as how to prepare the mosquitoes before their introduction into the olfactometer. This uniport olfactometer behavioral assay is currently one of the most reliable methods to study mosquito attraction to a single stimulus.

### 3.2 MATERIALS

**It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.**

#### 3.2.1 Reagents

*Ae. aegypti* mosquitoes (newly hatched larvae)

$H_2O$ (deionized)

Odor stimulus (see Steps 10–16)

Sucrose solution (10% w/v autoclaved in deionized $H_2O$)

Unscented dish soap

### 3.2.2 Equipment

Cotton balls

Environmentally controlled room

Latex gloves

Mouth aspirator with HEPA filter (John W. Hock Company model 612)

Portable vacuum with disposable bags (for cleaning olfactometer)

Rearing cage (BugDorm1, MegaView Science Co., Ltd., Taiwan)

Polycarbonate Food Pan 12.75 in length x 10.38 in width x 4.00 in height (Carlisle #10221B, Oklahoma City, OK, USA)

Scissors

Small paintbrush

Timer

Uniport olfactometer (fully assembled)

WHO tubes (length = 13 cm, diameter = 5 cm; World Health Organization Vector Control Research Unit).

## 3.3 METHOD

### 3.3.1 Preparing Mosquitoes for Behavior Trials

*Maintain mosquitoes in an environmentally controlled room at 25-27°C with 50-75% relative humidity on a 14:10 light dark cycle. These conditions and other rearing information apply to* Aedes aegypti *mosquitoes. If another species is used, different conditions may be needed.*

1. To ensure you have healthy and active female *Aedes aegypti* mosquitoes for the assay, rear larvae in polycarbonate pans (12.75 in length x 10.38 in width x 4.00 in height) at a density of 250 larvae per 1,200 mL of $H_2O$.
2. Once mosquitoes have emerged as adults, provide *ad libitum* access to a 10% sucrose solution in their rearing cage.
3. Maintain adult females and males together for a minimum of 7 d post-eclosion so that they are given sufficient time to mate.

*Females no older than 21 d should be used in the assay. Ideally, they should be between 7 and 14 d old.*

4. 12–16 h before the assay, cold anesthetize female mosquitoes (4°C) until they are knocked down (approximately 5 min), resting motionless on the floor of the cage. Sort gently using a small paintbrush into groups of 30. Collect 30 mosquitoes with a mouth aspirator and place them into WHO tubes.

*Cold anesthetization makes mosquitoes less active and facilitates their transfer into the WHO tubes. Mosquitoes may perish due to stress from cold anesthetization. Gently graze the anesthetized mosquitoes with small paintbrush to assure they are alive. If mosquitoes do not retract their legs to their original position, they are likely dead and should be discarded. Mosquitoes should not be kept at 4°C for longer than 1 h. Allow mosquitoes to recover for at least 12–16 h before experimentation.*

5. Immediately after placing mosquitoes into the WHO tubes (Step 4), provide mosquitoes with access to a cotton ball soaked in deionized $H_2O$ so that they do not desiccate. This 12-16 h water-only starvation period is intended to increase mosquito activity.

### 3.3.2 Performing the Host-Seeking Experiment with the Uniport Olfactometer

6. Turn on the air pump and open the $CO_2$ regulator valve connected to the uniport olfactometer setup.

7. Check flowmeters and the regulator and adjust air flow to 18 to 24 standard cubic feet per h (SCFH) and $CO_2$ flow to 0.2 to 0.4 SCFH.
8. Open the sliding gate on the WHO tube to allow $CO_2$ and air to pass through the attraction trap and into the activated chamber.

*$CO_2$ exhalation is characteristic of living vertebrates and can be modeled by releasing an airstream of $CO_2$ into the uniport which creates an attractive plume (Dekker et al., 2005, van Breugel et al., 2015). $CO_2$ along with filtered air act as a stimulus for the mosquito to leave the unactivated chamber, but both cues alone are not sufficient to trap more than 20% of mosquitoes in the activated chamber (Fig. 1C).*

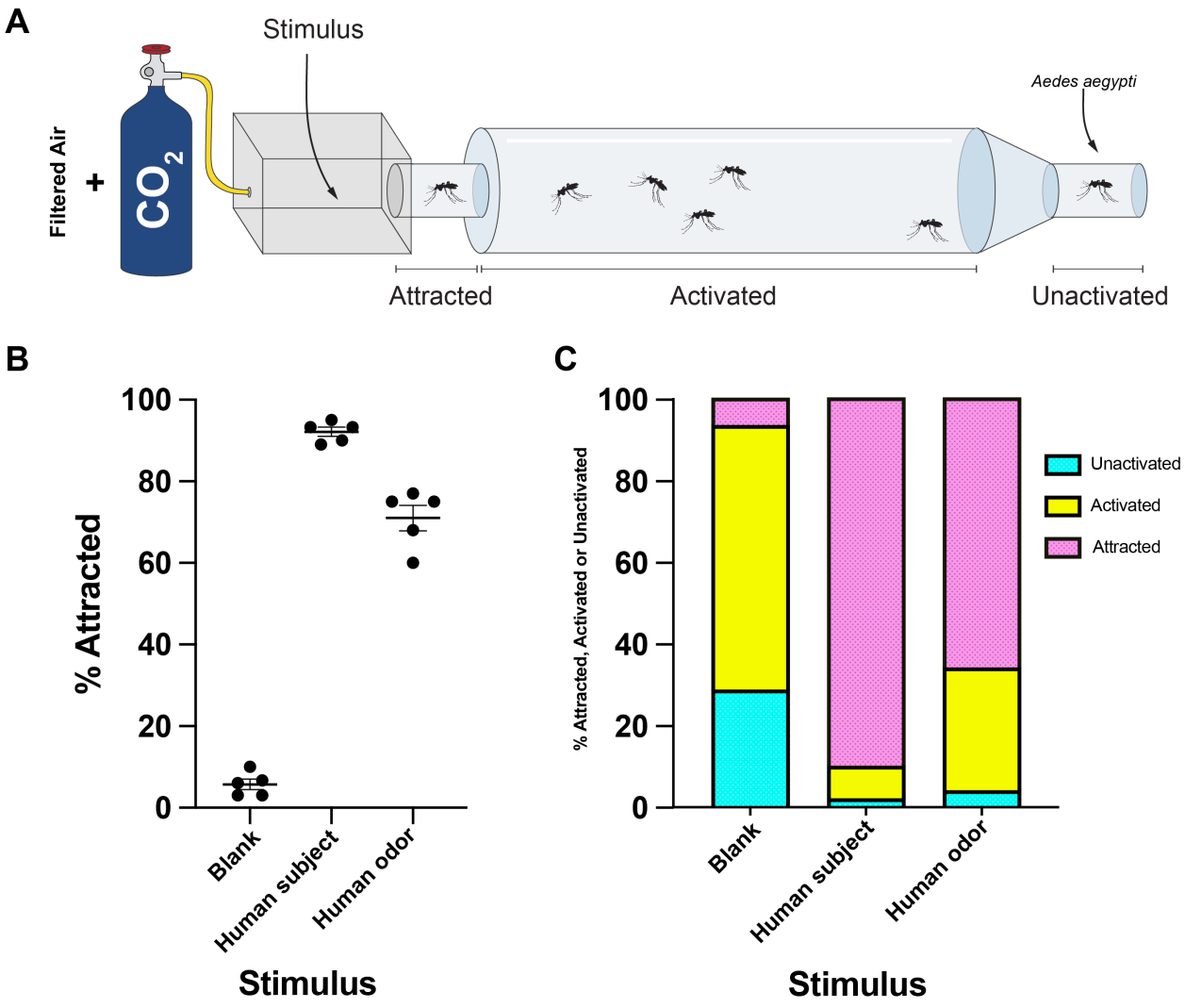


**Figure 8. *Aedes aegypti* responses to attractive stimuli in the uniport olfactometer.** (A) Schematic of the uniport olfactometer. Mosquitoes are released from the unactivated chamber (WHO tube) and fly upwind, entering the activation chamber. Mosquitoes may remain in the activated chamber, or may continue on to the attraction chamber. After eight minutes have elapsed, the assay is scored by counting mosquitoes in each chamber to identify unactivated, activated and attracted counts and then dividing by the total number of mosquitoes assayed in Fig. 1B and 1C. (B) Shows *Ae. aegypti* responses to a blank trial ($CO_2$ + air), a human subject (forearm in the olfactometer stimulus box), and human odor (nylon sleeve in olfactometer stimulus box). Dots represent trials with mean and SEM plotted for each condition. (C) Shows the average percentage of female mosquitoes attracted (magenta), activated (yellow), or unactivated(cyan) of the 5 trials in (B). Each trial was performed with 30 female mosquitoes.

9. Before running an experiment, perform a blank trial with the latex gloves sealing the entry circles of the stimulus box. If attraction is higher than 20%, clean the stimulus box and attraction trap with unscented dish soap and rinse thoroughly, with a final rinse with deionized water and dry thoroughly. Confirm the $CO_2$ level is 2,250–2,750 ppm and then perform another blank trial.

*This step ensures that the system was thoroughly cleaned before the experiment.*
*Attraction higher than 20% indicates that contaminating odors are present within the system or $CO_2$ concentration may be erroneously high.*

#### *3.3.2.1 For inanimate objects*

10. Immediately after completing a successful blank trial (with ≤ 20% attraction), remove the latex glove and insert the object (i.e., odor stimulus on small platform) into the stimulus box.

11. Place the inanimate object in the center of the stimulus box, elevated sufficiently so that it is in the path of the $CO_2$ and filtered airstream.

#### *3.3.2.2 For human subjects*

12. Immediately after completing a successful blank trial (with ≤ 20% attraction), cut one of the latex gloves with sharp scissors to remove four fingers, leaving the thumb intact.
13. Instruct the subject to sit in a comfortable position with their fist and forearm resting on the far and near edges of the entry ports, respectively.

14. Place the WHO tube with mosquitoes onto the activated chamber circular entry plate.
15. Release mosquitoes from the WHO tube by gently sliding open the door.
16. Set a timer for 8 min.

*Eight minutes provides enough time for mosquitoes to respond to the new environment and make a decision.*

### 2.3.3 Calculating the Response Rate in the Assay

17. Once the 8 min allotted for the experiment is concluded, close the gate at the exit plate and at the entrance of the WHO tube.
18. Perform counts of all unactivated mosquitoes in the WHO tube release chamber, in the activated chamber, and in the attraction trap.

*Mosquitoes that do not leave the WHO tube release chamber during the experiment are scored as unactivated. Those that leave the WHO tube but never enter the attraction trap are scored as activated, and those that enter the attraction trap are scored as attracted.*

*Occasionally, mosquitoes will be killed during handling and will remain in the WHO tube. Any dead mosquitoes should be subtracted from the number of mosquitoes from the total number assayed. Frequently observing dead mosquitoes (i.e., more than 10% in an assay) is cause for concern and signals the need for careful review of rearing practices and handling procedures to ensure that mosquitoes are in optimal condition for behavioral assessment. Normally, airflow*

*and $CO_2$ are enough to get most mosquitoes activated. Additional odor cues may be required to get mosquitoes into the attraction trap.*

19. To calculate the percentage of mosquitoes attracted, divide the total number of mosquitoes used in the assay trial by the number of mosquitoes in attraction trap.

20. To calculate the percentage of mosquitoes activated, add the total number of mosquitoes in the activated chamber and the attraction trap and divide by the total number of mosquitoes in the assay.

**2.3.4 Cleaning the Olfactometer**

21. Clean the olfactometer immediately after an experiment by using a portable vacuum. Begin by slowly inserting the vacuum head into the activated chamber, using suction to remove mosquitoes that are in the chamber. Repeat this process for mosquitoes in the WHO tube. Freeze and autoclave the vacuum bags containing mosquitoes for disposal.

22. To clean the olfactometer between subjects, isolate the stimulus box and wash with unscented dish soap and deionized $H_2O$, rinsing thoroughly with deionized $H_2O$.

*Avoid using solvent-based cleaning products such as those containing ethanol, as they will damage the acrylic.*

23. To determine whether the assay equipment is clean, perform a blank trial (no stimulus) with only $CO_2$ and air. If mosquito attraction is higher than 20%, clean the stimulus box again with soap and water and perform another blank.

## 2.4 DISCUSSION

Mosquitoes use their sense of smell to find a suitable host, and the proper physiological conditions must be met for them to initiate host-seeking and blood-feeding. Initially, a female mosquito nectar-seeks to obtain energy stores which can be critical for her survival and fecundity (Briegel 1990, Foster 1995). The female mosquito is not attracted to a human arm for 24 h after eclosure, and it is therefore crucial that she obtain a sugar meal during this period (Jones & Pilitt, 1973). The quality of this sugar meal influences her ability to host-seek (Jones & Pilitt, 1973). Additionally, when mosquitoes are given sugar *ad libitum* before a blood meal, they show a significant decline in blood feeding and host-seeking when compared to mosquitoes given access to only water (Jones & Madhukar 1976). Females are also reduced in their ability to host-seek for at least 48 h once they obtain a blood meal (Klowden & Blackmer, 1987). Female mosquitoes have a refractory period where they cannot be inseminated for 48 h posteclosion (Lea 1968). When working with different mosquito species it is important to adjust the room temperature, humidity and light cycle conditions to be ecologically relevant and ensure increased activity. Randomizing trials by running controls throughout the experiment when using different stimuli in the olfactometer is a good practice to reduce extraneous results. Starving mosquitoes on water and providing a well humidified room can create the proper environment for mosquito participation. If mosquito participation is low, make certain that $CO_2$ levels in the olfactometer do not exceed 5,000 ppm as over exposure to $CO_2$ can anesthetize mosquitoes (Williams 1946). These factors have influenced the design of our protocol, and using our methods, we have achieved high response rates to both humans and human odor with *Aedes aegypti* females (Fig. 1B). We have also achieved positive results with other mosquito species such as *Aedes albopictus*, *Anopheles gambiae*, and *Culex quinquefasciatus.*

Additionally, our uniport olfactometer has a stimulus chamber that allows for the inclusion of a larger region of the human body, increasing stimulus signal. Our design uses positive air pressure and carbon-filtration to remove extraneous odors that may be present in the room from the stimulus being studied, providing the ability to focus on mosquito host-seeking attractants and repellents that are placed in the assay. To occlude visual cues from the stimulus chamber, a white acrylic face plate was placed on its front. The dimensions of the activation chamber provide sufficient space so that the assay does not force a choice. Additionally, to increase the structural integrity of the olfactometer, our design includes a full-length baseplate into which the assay components fit, eliminating leaks due to assembly irregularities and movement of olfactometer components when removing mosquitoes between trials.

## 2.5 CONCLUSION

A repeatable, quantitative method to assess attraction to human hosts, host analogs, and other attractants is critical for the study of mosquito host-seeking behavior. This updated uniport olfactometer assay simplifies the collection of quantitative attraction data. Using the experimental setup described here can reduce variables when assaying mosquito attraction. A key advantage of this uniport design lies in its exclusion of extraneous odors from the assay itself, as the system is under constant positive pressure provided by filtered air and bottled $CO_2$. The single-choice host-seeking assay presented has been used to analyze female mosquito responses to human subjects, to human odor trapped on nylon sleeves, and to individual compounds found on human skin.

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# CHAPTER 4: Species-specific mosquito preferences for the bacteria and odor of individual humans

## 4.1 INTRODUCTION

Mosquito species are responsible for transmitting pathogens that cause the death of over 400,000 people annually and this number is expected to increase with rising global temperatures (Franklinos et al., 2019). These vectors for diseases such as dengue, malaria, Zika and chikungunya proliferate in developing countries due to living conditions, such as standing water and lack of air conditioning, that give way to mosquito reproduction (Gallup & Sachs, 2001). *Ae. aegypti* are opportunistic feeders in nature and have thrived in parallel to human expansion during colonialism evolving to feed exclusively on human hosts (McBride 2016). The mosquitoes *Ae. aegypti* and *Ae. albopictus* can transmit various arboviruses including Chikungunya virus, Dengue virus and Zika virus which result in millions of infections and thousands of deaths per year (Paupy et al., 2009, Bhatt et al 2013, Michalet et al., 2019, Chouin-Carneiro et al., 2020). The southern house mosquito *Culex quinquefasciatus* is a vector of West Nile Virus (WNV) and various other filarial nematodes putting populations at risk of disease in various urban settings (Bartholomay et al., 2010, Bhattacharya et al., 2016). These urban species proliferate by inhabiting containers that are used by humans with *Ae. albopictus* inhabiting cooler climates (Eisen & Moore, 2013). Although *Ae. albopictus* and *Cx. quinequefasciatus* thrive in urban environments, there are numerous studies that report these species to be less anthropophilic than *Ae. aegypti*, with *Cx. quinquefasciatus* often preferring avian hosts to mammalian hosts (Bogh et al., 1998, Sawabe et al., 2010, Muñoz et al., 2011). Mosquitoes use a multimodal approach to find a copacetic host which involves the detection of $CO_2$, odor, and heat (Raji & DeGennaro, 2017, Greppi et al., 2020). Out of these known host-seeking cues human odor varies vastly from person to person and could be the reason why mosquitoes prefer some human hosts over others. Interestingly, humans would be close to odorless if not for the

commensal organisms inhabiting our skin (Shelley et al., 1953). While previous work has demonstrated higher and lower attraction across human hosts, the patterns of attraction between individual humans and multiple species has not been examined (Verhulst et al., 2011, Showering et al., 2022). Previous studies have shown that incubated bacterial strains *Staphylococcus epidermidis* and *Corynebacterium minutissimum* were attractive to *Anopheles gambiae* (Verhulst et al., 2010). More recent studies have shown *Ae. albopictus* to be less attracted to subjects whose microbiome contained *Corynebacterium* spp. giving implications that mosquito species have adapted to respond to cues that are strain dependent (Michalet et al., 2019). Identifying what strains of bacteria are highly attractive to *Aedes aegypti (Ae. aegypti), Aedes albopictus (Ae. albopictus) and Culex quinquefasciatus* (*Cx. quinquefasciatus*) mosquitoes can expand our knowledge on what components of the skin microbiome influence mosquito attraction. Due to the importance of skin microbiota on human odor profiles, we hypothesized that different human subjects would contain different species of bacteria that influence their odor profiles and influence their overall mosquito attraction across different mosquito species. To characterize the human odor and skin microbial components that promote mosquito attraction, we designed a human study that successfully recruited and tested 119 diverse subjects from our community in Miami, Florida. We compared each subject's mosquito attraction rates, odor profile, and skin commensals with those of others to provide insights into the key chemicals and bacteria that enable mosquitoes to select a host. Our study collected data on each subject's attraction to *Ae. aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus*, species specific resolution of their skin microbiota, and the volatiles emitted from their skin.

## 4.2 METHODS

### 4.2.1 Recruitment

All research in this study was reviewed and approved by the Florida International University Instutional Biosafety Committee and Institutional Review Board. In order to characterize the signatures of human odor that drive mosquito attraction, we recruited and successfully assayed 119 subjects between the ages of 18-59 (Figure 9D). Subjects self-reported their race and ethnicity, with White Hispanic/LatinX subjects making up 42.01%, 21.01% identified as White non-Hispanic, 15.29% as Black, 14.29% as Multiracial, and 6.27% as Asian (Figure 9C). The sex of subjects was also self-reported, with 50.85% male subjects and 49.15% female subjects (Figure 9C & 9D).

### 4.2.2 Mosquito rearing

*Aedes aegypti* (Orlando strain), *Aedes albopictus* (MRA-804 strain), and *Culex quinquefasciatus* (JHB strain) mosquitoes were reared and maintained at 25-28˚C, 75% relative humidity under a 14:10 light-dark cycle (lights on at 8 am for *Aedes* genus lights on at 1pm for *Culex* species). Mosquito eggs were hatched in deionized, deoxygenated water containing dissolved tablets of Tetramin tropical fish food (Catalog#16152, Tetra, Melle, Germany), to feed the emerged larvae. Adult mosquitoes were given ad libitum access to 10% sucrose solution. About 1 to 2-week-old adult females were fed on defibrinated sheep blood to generate eggs. Before behavioral assays, 7 to 21 day old sugar-fed mosquitoes were sorted 30 per tube and sexed under hypothermic (4˚C) conditions and fasted for 20 hours on water.

### 4.2.3 Assessing the attractiveness of human subjects

Mosquitoes were placed in a custom-made uniport olfactometer to analyze their behavioral attraction (Raji et al., 2019, Castillo et al., 2023). The uniport olfactometer consists of a large plexiglass tube (75cm long and 13cm wide) connected to a small cylindrical cage (13cm

long and 5cm in diameter) that contains the mosquitoes prior to the experiment. At the far end of the plexiglass tube connected to the stimulus chamber, the left arm of the human subject is inserted in an enclosed space with dimensions of 25 cm by 20 cm by 13 cm. In the stimulus chamber, carbon-filtered, humidified air and CO2 can combine with odorants to attract mosquitoes that have been released from a trap. Acrylic flowmeter Model VFA-4- SSV (Dwyer Instruments Inc., IN, USA) set to 3 SCFH was used to measure the CO2 release rate in the stimulus chamber. The final concentration for $CO_2$ in the assay was maintained at 2500-2700ppm by a carbon dioxide monitor (Catalog#CO2-100, Amprobe). Whereas, air flow rate was set at 21 standard cubic feet per hour by an air flowmeter (King Instruments CA, USA). The sealed design of the uniport, air filtration, and the positive pressure caused by air circulation in the apparatus will isolate the assay from all possible environmental scents.

Prior to experimentation, subjects were instructed to shower the night before their visit to prevent the use of skin products such as deodorants, antiperspirants, perfume, or cologne. Uniport olfactometry was performed during subject visits for both *Aedes aegypti* and *Ae. albopictus*, in triplicate for each species, to determine attraction. To quantify *Cx quinquefasciatus* attraction, subjects wore nylon stockings over their arms for 12-16 hours to collect odor. The stockings were worn following their shower the evening before the study and were collected at the beginning of their visit and stored at -20°C until use in the olfactometer.

Each olfactometer experiment is conducted for eight minutes and contains 30 females of each species. Each participant underwent six trials of olfactometer assays, three with *Ae. aegypti* and three with *Ae. albopictus*. *Cx. quinquefasciatus* are night biting mosquitoes and trials were performed under moon light (lux 0.05-0.1) conditions using nylon socks in the olfactometer rather than a live human. Before each human experiment, a blank (no human subject or nylon sleeve) trial is conducted with each species. During the course of the investigation, the mosquito

species is randomized. All mosquitoes were subjected to behavioral experiments only once before being sacrificed.

### 4.2.4 Profiling body odor collection

To identify volatile organic compounds (VOCs) emanating from the epidermis, we extracted volatiles from the atmosphere of each participant's left arm. The left arm was placed in a nylon bag (Toppits, Cofresco Frishhalteprodukte GmbH&Co., Minden, Germany), and their volatiles were collected for 90 minutes on Tenax adsorbent fibers by circulating ultra zero grade air (Airgas) at a rate of 400 mL $min^{-1}$ per minute into the top of the bag and simultaneously applying a vacuum (GAST, Model DOA-P704-AA, Michigan, USA) of 200 mL $min^{-1}$ to the back of the stainless steel thermal desorption tubes filled with 200 mg of Tenax TA.

Using a thermodesorption unit and GCMS, we were able to characterize human volatile profiles. VOCs were desorbed from the Tenax tubes for 10 minutes at 250 °C and captured in a sorbent trap chilled with liquid nitrogen at -110 °C. Compounds were desorbed from this trap during secondary desorption at 40 °C $s^{-1}$ and afterwards at 280 °C for 10 min, and later transferred in split mode to a non-polar gas-chromatography (GC) column (RXI-5ms 30 m×0.25 mm×1.00 μm). The chromatographic procedure was conducted at a carrier gas flow rate of 1 mL $min^{-1}$. The temperature of the GC oven was programmed to rise from 40 °C (5 min hold time) to 280 °C (8 min hold time) at a rate of 5 °C $min^{-1}$. The temperature of the MS transfer line was set to 280 °C. The electron beam energy was set to 70 eV, and the ion source temperature was set to 250 °C. The mass spectrometer scanned m/z 35–400 at a rate of 4.7 scans $s^{-1}$. Helium gas was used for desorption and chromatographic analyses. GC–MS data were processed using the MetAlign–MSClust software pipeline (Lommen and Kools, 2012). In brief, MetAlign corrects the baseline and eliminates the noise of each GC–MS output file. Subsequently, it aligns the

individual mass peaks in all chromatograms (Lommen, 2009). MSClust then clusters the aligned mass peaks so that mass spectra of putative compounds are reconstructed (Tikunov et al., 2012).

### 4.2.5 Sampling the human skin microbiome

Following behavioral analysis, the subject rested their arm atop a table covered with a sterile surgical drape. A total of four samples were collected from the underside of the subject's arm, each comprising a 5 x 5 cm area. The sampling sites are located on the distal volar forearm (beginning just above the wrist crease), mid volar forearm, proximal volar forearm, and the antecubital fossa.

For each sample site, two sterile double swabs (BD BB CultureSwab EZ II) were used to collect samples. Each swab moistened with a sterile aliquot of SCF-1 buffer (50 mM Tris buffer [pH 7.6], 1 mM EDTA [pH 8.0], and 0.5% Tween-20) and vigorously swabbed against the skin for two minutes, rolling swabs to collect along the entire flocked surface. Swabs from each sample site clipped with sterilized cutters into a 25 ml conical tube containing 1 ml of DNA/RNA Shield (Zymo Research, California, USA) and mixed. Blank samples were collected for each subject by waving swabs in the air for two minutes, then collected and processed identically in DNA/RNA Shield.

For extraction, samples were first vortexed for 1 minute to displace cells from the swab to the DNA/RNA Shield. The suspension was then be transferred to a BashingBead Lysis Tube containing 0.1 & 0.5 mm glass beads (Zymo Research, California, USA). Bacterial cells were then concentrated by centrifugation and lysed in the ZR BashingBead™ Lysis tube (0.1 & 0.5 mm beads) containing BIOMICS® Lysis Solution using the Omni Bead Rupto-24 Elite (Omni International, Kennesaw, GA) (velocity of 6 m/s for 1 minute, 5 minutes rest, cycle is repeated three times for a total of 3 minutes of bead beating). DNA was extracted from preserved subject

samples with bead beating using the ZymoBIOMICS DNA Microprep Kit (Zymo Research) with bead beating for 1 minute 6 m/s lysis followed by a 5 minute rest phase, repeated a total of three beating cycles using a BeadBead Ruptor Elite bead mill homogenizer (Omni) to facilitate unbiased microbial lysis. DNA samples were amplified with indexed 27F (5'GCATC/barcode/AGRGTTYGATYMTGGCTCAG3') and 1492R (5'GCATC/barcode/RGYTACCTTGTTACGACTT3') primers,with 16 base pair barcodes used in unique combinations to identify libraries. Libraries were constructed in a 25 µl reaction volume with 2.5 µM of each primer and 12.5 µ KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems). Libraries were then amplified using the following program: a 95 °C initial denaturation for three minutes, followed by 28 cycles of amplification with 95 °C denaturation for 30 seconds, 57 °C annealing for 30 seconds, and 30 second extention at 95 °C. Libraries were purified on AMPure PB beads (Pacific Biosciences), quantified via Qubit dsDNA HS (Thermo Fisher) and normalized prior to pooling. SMRTbell libraries were constructed from amplicon pools and sequenced for a 15-hour movie duration on the Sequel II Platform (Pacific Biosciences). Human subject skin microbiomes were characterized with full-length (V1-V9) 16S amplicon sequencing using PacBio HiFi sequencing. Previous mosquito/host microbiome investigations used short fragment Illumina 16S sequencing, however our method catches all the hypervariable areas of 16S, vastly improving the assay's taxonomic accuracy. Demultiplexed libraries were denoised to amplicon sequence variants (ASVs) using DADA2 (Callahan et al. 2019) and analyzed using QIIME 2 (Bolyen et al. 2019), with classification performed using VSEARCH (Rognes et al. 2016) in combination with the Silva 138 99% OTUs full-length sequences database. The ASV method begins by identifying which precise sequences were read and how often each precise sequence was read. These data are then combined with an error model for the sequencing run, allowing the comparison of similar readings to determine the

probability that a particular read at a particular frequency is not due to sequencer error (Nearing et al., 2018). Essentially, this generates a p-value for each exact sequence, where the null-hypothesis is equivalent to that exact sequence being the result of sequencing error (Callahan et al., 2019). This resulted in the species-level identification of nearly all major taxa present in the samples.

### 4.2.6 Statistical analysis

**Identifying High and Low Attraction Subjects**

High and low attraction individuals were identified for *Ae. aegypti, Ae. albopictus*, and *Cx. quinquefasciatus* by normalizing mean attraction within species using the Ordered Quantile (ORQ) normalization transformation and then identifying the high and low attraction subjects falling outside of an 80% confidence interval for each vector species. This gave us 13 High Attraction subjects and 12 Low Attraction subjects for *Ae. aegypti,* 12 High and Low Attraction subjects for *Ae. albopictus*, and 12 High and Low Attraction subjects for *Cx. quinquefasctus.* We then compared both high and low attraction subject groups with a t-test and both groups significantly differed from each other ($p>0.0001$) (Figure 1B, Figure 1D, Figure 1F).

**Identification of microbial components**

The microbiome data were analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME2) (version 2019.1) program. The sequences were demultiplexed with DADA2 and the reads were truncated to eliminate low-quality scores. The feature table, phylogeny data, and metadata table were then imported for analysis into MicrobiomeAnalyst (Dhariwal et al. 2017) . Based on the mean abundance of operational taxonomical units (OTUs), samples were filtered

for low abundance (10%) and variability using an inter-quantile range assessment. The data were normalized by rarifying them to the minimum library size.

**Mixed-effects models to identify unique features**

Data were analyzed using open source statistical software, R v.3.5.0. Mosquito attraction was compared to the subjects headspace and microbiome by fitting linear mixed-effects models using the package lme4 (Bates et al., 2014), with subject being the random effect. To prepare the mixed model, headspace GC-MS peak area was normalized by the median and log transformed using MetaboAnalyst 4.0 (Chong et al., 2018). This gave a series of 36 chemical compounds to 109 subjects, data was not collected for 10 subjects, hence sparse principal component analysis (SPCA) was used to reduce the dimensionality of this data set using the R package 'sparsepca' (Zou et al., 2006, Guerra-Urzola et al., 2021) . We used a scree plot, a line plot of the eigenvalues of principal components in our analysis, and found that 70% of our data was explained using 8 dimensions in our analysis. A scree plot always displays the eigenvalues in descending order, from the largest to smallest. The scree test identifies the "elbow" of the graph where the eigenvalues appear to level off, and components to the left of this point are retained as significant. Human skin microbiome normalized read counts were rarefied to the smallest library using MicrobiomeAnalyst 2.0 after noise/redundancy filtering using DADA2 which gave us 211 amplicon sequence variants (ASV's) for 113 subjects, 6 of which no amplification occurred. A non-negative matrix factorization (NMF) was used on the microbiome data to reduce the dimensionality of this continuous data (Gajoux and Seoighe, 2010). A scree test identified that 80% of the variance in our data was explained by 4 dimensions. The headspace data reduced to 8 dimensions from the SPCA and the microbiome data in 4 dimensions from the NMF were ran against the attraction data with each subject included as a random effect in the model.

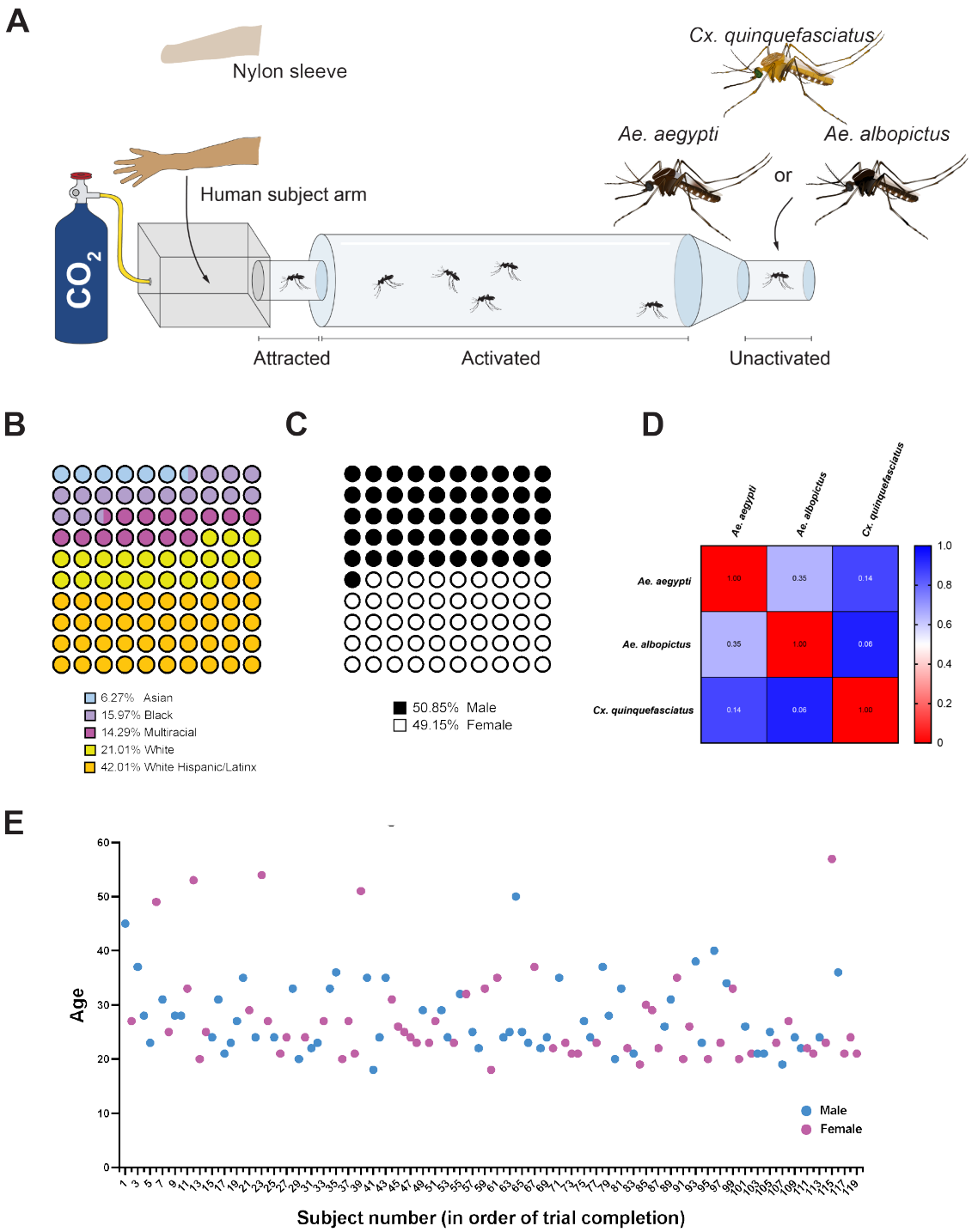


**Figure 9 Human study conceptualization and design**. (A) Schematic of the uniport olfactometer used for quantification of mosquito attraction to subject arms in of *Ae. aegypti* and *Ae. albopictus* or nylon sleeves in *Cx. quinquefasciatus* experiment. (B) Shows the self-identified racial make-up of the 119 subjects in the study. (C) Sex breakdown up of study participants. (D) Pearson's-r correlation coefficient comparing attraction rates of mosquito species. The closer the r value is to 1 the stronge the correlation. (E) Age distribution of study participants.

## 4.3 RESULTS

### 4.3.1 Mosquito species prefer different human subjects

To investigate the human odor and skin microbial components that promote mosquito attraction, we designed a human study that successfully recruited and tested 119 diverse subjects from our community in Miami, Florida (Figure 9B). Previous studies have placed strict limitations on the recruitment of their subjects often reducing their subject demographics to all male studies or entirely post-menopausal female studies (Verhulst et al., 2011, Showering et al., 2022). For this study, our goal was to recruit subjects whose sex was equally male and female and we wanted to ensure the cohort be from diverse backgrounds .

While previous work has demonstrated higher and lower attraction across human hosts, the patterns of attraction between individual humans and multiple species had not been examined. For the first time, we profiled a diverse human population against multiple mosquito species to understand the underlying nature of attraction variation in human hosts across vector species. From our attraction data distribution we calculated relative attraction as the number of mosquitoes that entered the attraction trap divided by the total (n=30). High and low attraction groups were identified by normalizing mean attraction within species using the Ordered Quantile (ORQ) normalization transformation and then selecting subjects falling outside of an 80% confidence interval for each vector species. This gave us 13 High Attraction subjects and 12 Low Attraction subjects for *Ae. aegypti* (Fig. 10A), 12 High and Low Attraction subjects for *Ae. albopictus* (Fig. 11A), and 12 High and Low Attraction subjects for *Cx. quinquefasctus* (Fig 12A) these same subjects were elected for the analysis of skin microbiome and headspace analysis. A non-parametric t-test showed that there were significant differences in attraction between the high and low groups for each species (Fig. 10B, 11B and 12B). We found that there

was detectable correlation in high and low attraction individuals across *Ae. aegypti, Ae. albopictus*, and *Cx. quinquefasciatus* (Fig 9D).

### 4.3.2 Differences in the microbiome of high and low attraction subjects

We identified skin microbiome patterns associated with high- and low attraction subjects across *Ae. aegypti, Ae. albopictus*, and *Cx. quinquefasciatus*, with each species having its own unique signature. Examination of differences between the high and low attraction communities to *Aedes aegypti* identifies several species with differential abundance between the populations. Using relative abundance as the detection threshold we find differences in the abundance of different species for high attraction subjects (Fig 10C) compared to low attraction subjects (Fig 10D). The differences pertain mainly to high attraction groups containing larger amounts of *Staphylococcus hominis* and *Corynebacterium tuberculostearicum* (Fig 10C). At the species level, we find that *Staphylococcus hominus* are associated with higher attraction (Wilcoxon test, FDR adjusted p- value < 0.05) (Fig. 10F). Using Random Forest machine learning, we also identify *Pseudomonas oryzihabitans* and *Mycobacterium phocaicum* as candidate species for low attraction (Fig.10E). Additionally, using mixed-model analysis we found 4 Amplicon Sequence Variants (ASV's) that contribute significantly to low attraction in *Ae. aegypti* (Fig. 17). The species breakdown of these ASV's are 2 *Cutibacterium acnes* species, 1 *Staphylococcus epidermidis* species and 1 *Micrococcus luteus* species of bacteria. Subjects who were in the high attraction group had half as much read counts as their low attraction counterparts for these significant ASV's (Fig. 17).

The differences between the high and low attraction communities to *Aedes albopictus* identified several species with differential abundance between high and low attraction groups. The core microbiomes of subjects who are of low attraction to *Ae. albopictus* exhibited larger amounts of *Cutibacterium acnes* as well as lower abundance of *Staphylococcus epidermidis* (Fig

11C & 11D). At the species level, we find that *Gemella morbillorum, Mogibacterium diversum* and *Streptococcus intermedius* are associated with higher attraction (Wilcoxon test, FDR adjusted p- value < 0.05) (Fig. 11F). Whereas, *Staphylococcus ureilyticus* and *Dermatophilacae spp.* were significantly abundant in low attraction subjects (Fig. 11F). Using Random Forest machine learning, we also identify *Streptococcus spp.* to be candidate species for high attraction mainly *Streptoccocus intermedius* while *Corynebacterium kefirresidentii* are candidate species for low attraction (Fig.11E).

The high and low attraction microbial communities for *Cx. quinquefasciatus* revealed a number of species whose abundance varied between the two groups. The core microbiomes of individuals with high attraction to *Cx. quinquefasciatus* contained more *Cutibacterium acnes* as well as *Staphylococcus epidermidis* (Fig. 12C). While individuals that were low attraction contained higher amounts of *Staphylococcus hominis* relative to *Staphylococcus epidermidis* and higher abundance of *Micrococcus luteus* (Fig. 12D). At the species level, *Streptoccocus oralis* and *Corynebacterium spp.* are associated with high attraction (Wilcoxon test, FDR adjusted p-value 0.05) (Figure 12F). In contrast, *Staphylococcus warneri* were substantially more prevalent in subjects with low attraction (Figure 11F). Random Forest machine learning identified *Staphylococcus warneri* and *Actinomyces oris* as candidate species for low attraction, whereas Corynebacterium *gottigense, Peptoniphilus lacydonensis and Dermabacter vaginalis* are candidate species for high attraction (Fig.12E).

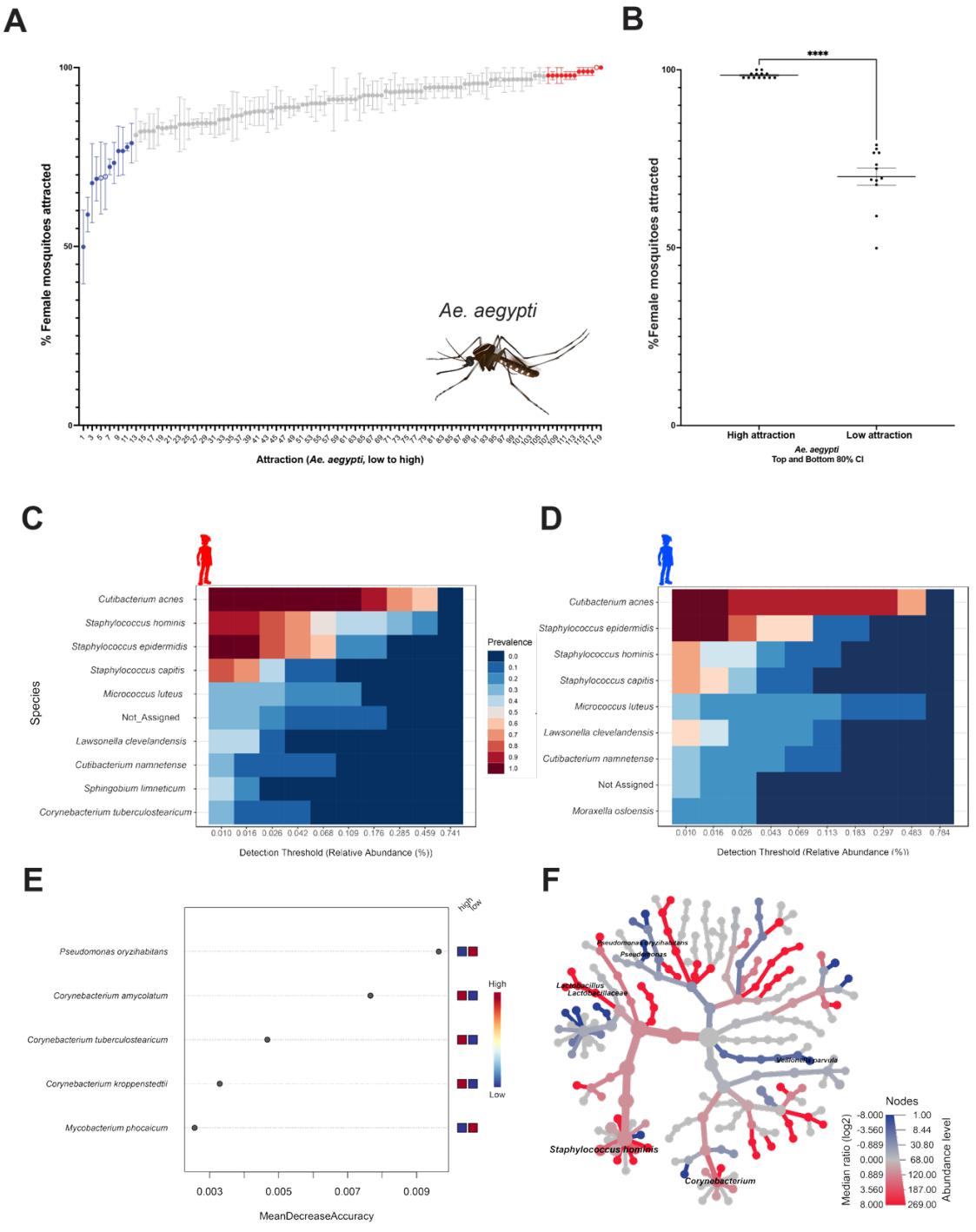


**Figure 10. *Ae. aegypti* High attraction subjects differ in their microbiome from Low attraction subjects.** (A) Shows behavioral responses of all 119 subjects to *Ae. aegypti.* Red dots reflect high attraction subjects while lue dots represent low attraction subjects. (B) Non-parametric t-test of high and low attraction groups. ($p < 0.001$). (C) Core microbiome of high attraction individuals based on relative abundance. (D) Core microbiome of low attraction individuals based on relative abundance. (E) The mini heatmap to the right of the plot indicates whether the taxa are higher (red) or lower (blue) in our high attraction or low attraction groups. (F) Heat tree representing a Wilcoxon rank sum test. Red nodes represent abundance in high

attraction subjects while blue nodes represent abundance in low attraction subjects. Labeled nodes represent taxa that are significantly abundance with a p-value cutoff at 0.05.

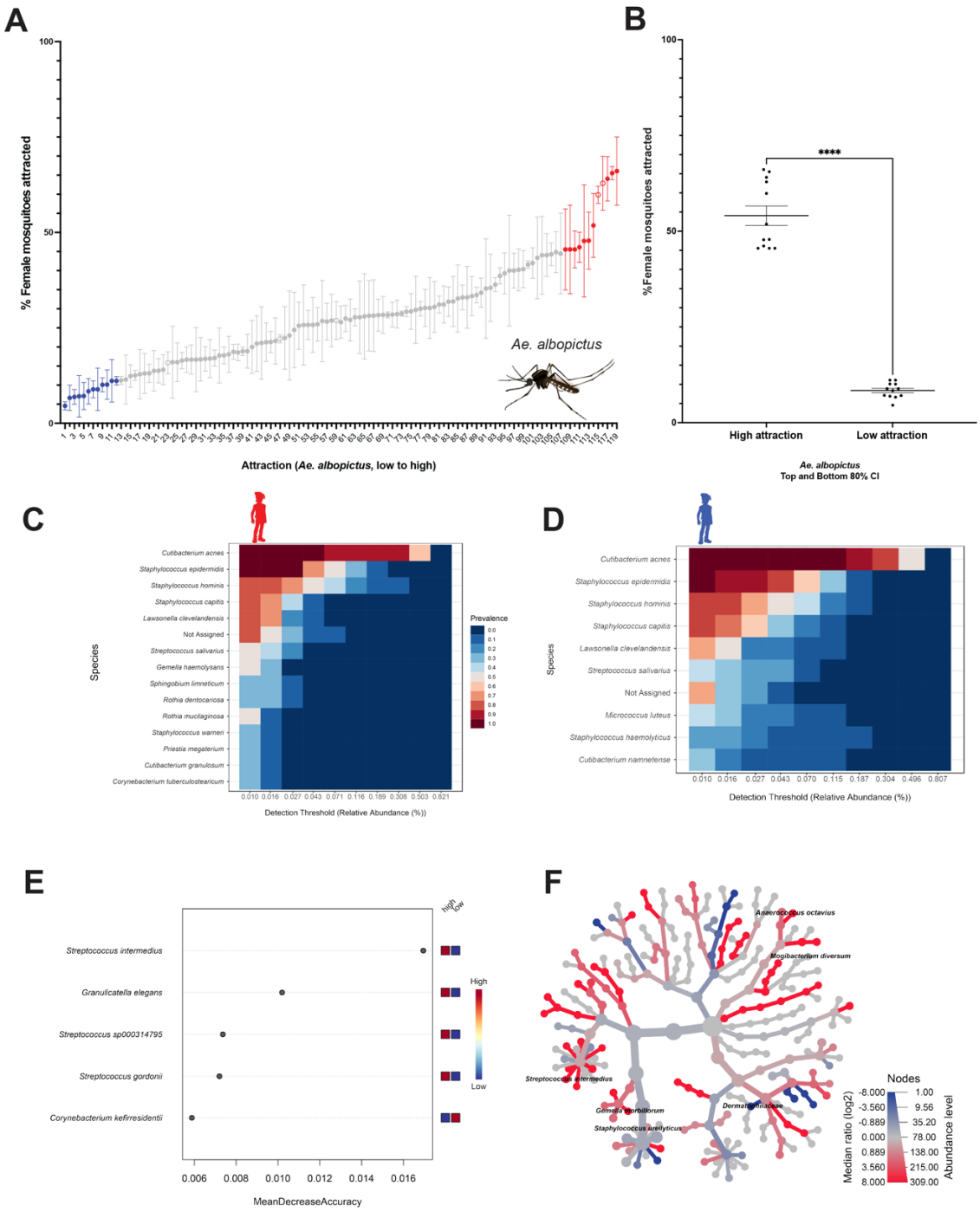


**Figure 11 *Ae. albopictus* High attraction subjects contain differential abundance for key bacteria compared to low attraction subjects.** (A) Shows behavioral responses of all 119 subjects to *Ae. albopictus.* Red dots reflect high attraction subjects while lue dots represent low attraction subjects. (B) Non-parametric t-test of high and low attraction groups. ($p < 0.001$). (C) Core microbiome of high attraction individuals based on relative abundance. (D) Core microbiome of low attraction individuals based on relative abundance. (E) The mini heatmap to the right of the plot indicates whether the taxa are higher (red) or lower (blue) in our high attraction or low attraction groups. (F) Heat tree representing a

Wilcoxon rank sum test. Red nodes represent abundance in high attraction subjects while blue nodes represent abundance in low attraction subjects. Labeled nodes represent taxa that are significantly abundance with a p-value cutoff at 0.05.

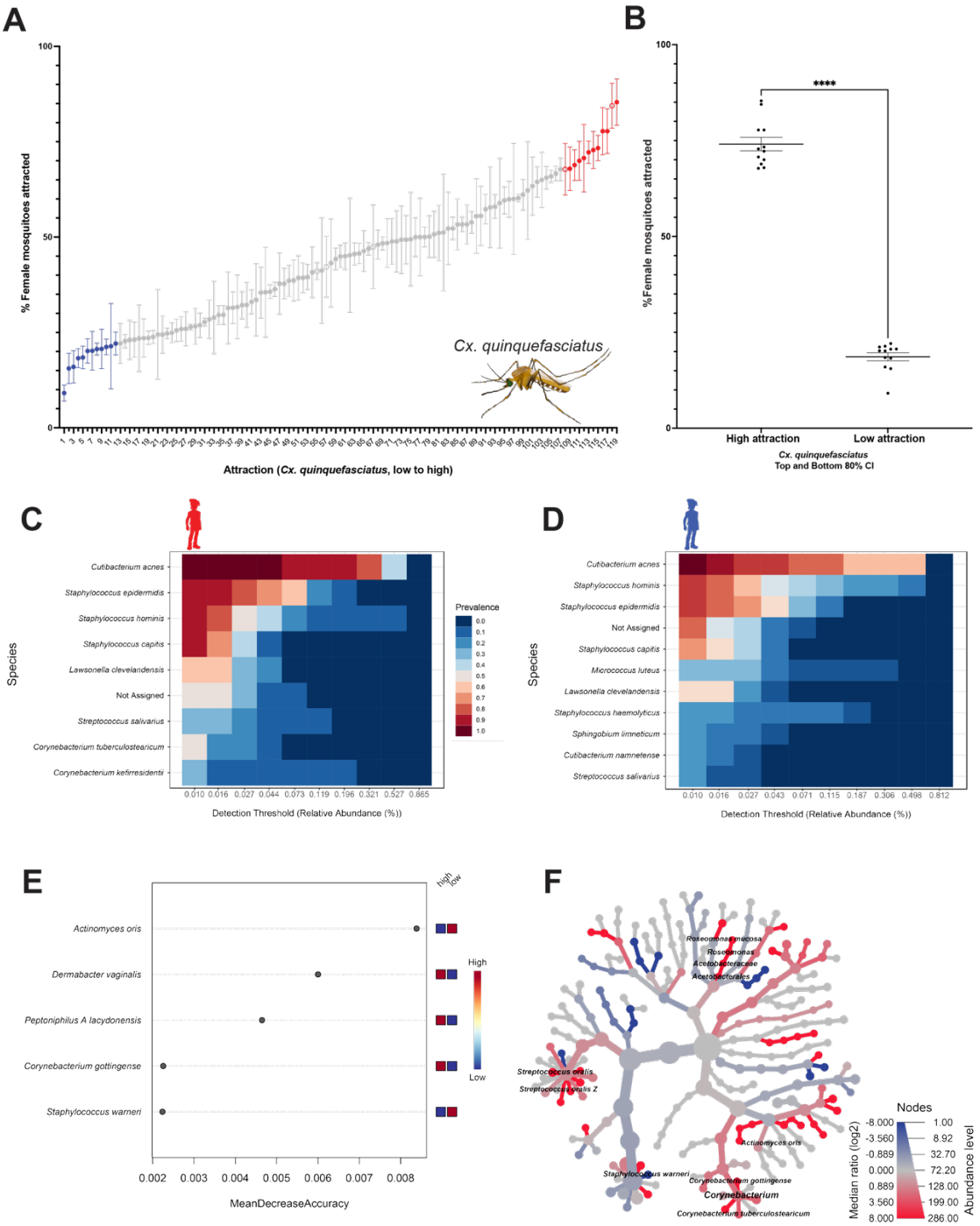


**Figure 12. *Cx. quinquefasciatus* microbial components that modulate mosquito attraction**. (A) Shows behavioral responses of all 119 subjects to *Cx. quinquefasciatus*.. Red dots reflect high attraction subjects while lue dots represent low attraction subjects. (B) Non-parametric t-test of high and low attraction groups. ($p < 0.001$). (C) Core microbiome of high attraction individuals based on relative abundance. (D) Core microbiome of low attraction individuals based on relative abundance. (E) Random forest machine learning. The mini heatmap to the right of the plot indicates whether the taxa are higher (red) or lower (blue) in our high attraction or low attraction groups. (F) Heat tree representing a Wilcoxon

rank sum test. Red nodes represent abundance in high attraction subjects while blue nodes represent abundance in low attraction subjects. Labeled nodes represent taxa that are significantly abundance with a p-value cutoff at 0.05.

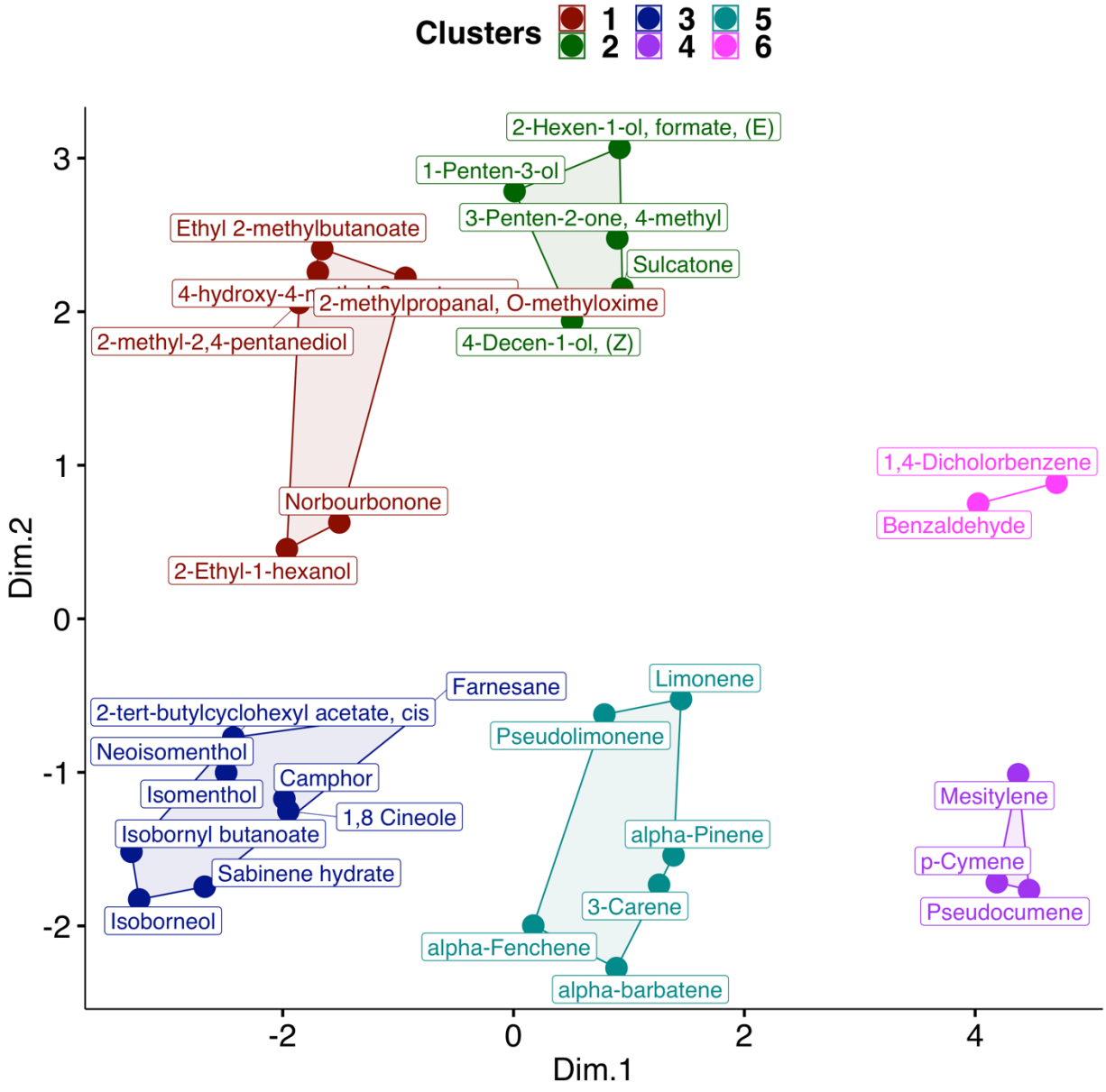


**Figure 13. Dynamic headspace compounds cluster into 6 different groups**. K-means clustering takes into account atomic structure information and partitions these observations into k number of groups. The closer the chemicals are in the cluster the more similar their atomic structure.

### 4.3.3 VOC's involved in attraction to mosquitoes

The dynamic headspace of human subjects was collected and grouped based on behavioral attraction. This resulted in 36 chemical compounds which cluster into 6 different groups by k-means clustering (Fig. 13). Clusters 1 and 2 contain compounds with functional groups that are alcohols, aldehydes or ketones (Fig 13). While clusters 3,4 and 5 contain terpenes and terpene derivatives that are often cyclical in their structure. Cluster 6 contains compounds that have a benzene ring such as benzaldehyde and 1,4 Dichlorobenzene.

Only camphor and isobornyl butanoate, out of the 10 most informative volatiles regarding *Ae. aegypti* attraction, are more abundant in the high attraction group than the low attraction group (Fig. 14). These two related compounds, a terpene and a terpene derivative, may derive from a biosynthetic pathway that is enriched in the microbiomes of subjects with high attraction. The attraction patterns of *Ae. aegypti* are driven by higher levels of low attraction volatiles, whereas the converse is true for *Ae. albopictus*: the ten most explanatory volatiles are more prevalent in high attraction subjects than in low attraction subjects. Among the more abundant chemical species in the high attraction group is sulcatone, a behaviorally active compound that, depending on its concentration, can be associated with either mosquito attraction or repellency (McBride, 2016). Mixed-model analysis showed that Benzaldehyde, Camphor and 3-Carene contribute significantly to the modulation of attraction for *Ae. albopictus* mosquitoes with Benzaldehyde and Camphor more abundant in high attraction subjcets while 3-Carene was more abundant in low attraction subjects.

The dynamic headspace collection also shows that alpha-pinene and pseudolimonene concentrations are elevated in *Cx. quinquefasciatus* subjects with low attraction (Fig. 16). Using a mixed-model we also found that Pseudocumene, Limonene, and Mesitylene were significant in determining *Cx. quinquefasciatus* attraction. Pseudocumene and Limonene are more abundant in

the headspace of low attraction subjects for this species while mesitylene abundance is the same in both high attraction and low attraction subjects (Fig. 16). Alpha-pinene has been shown to repel the domestic housefly, whereas pseudolimonene has not yet been shown to have any effect on insects (Haselton et al., 2015). Both of these are compounds worthy of further investigation as potential repellents for *Culex* species.

Overall, the proliferation of compounds more prevalent in subjects with low attraction may indicate that low attraction may be caused by the presence of chemical volatilome species as opposed to their absence.

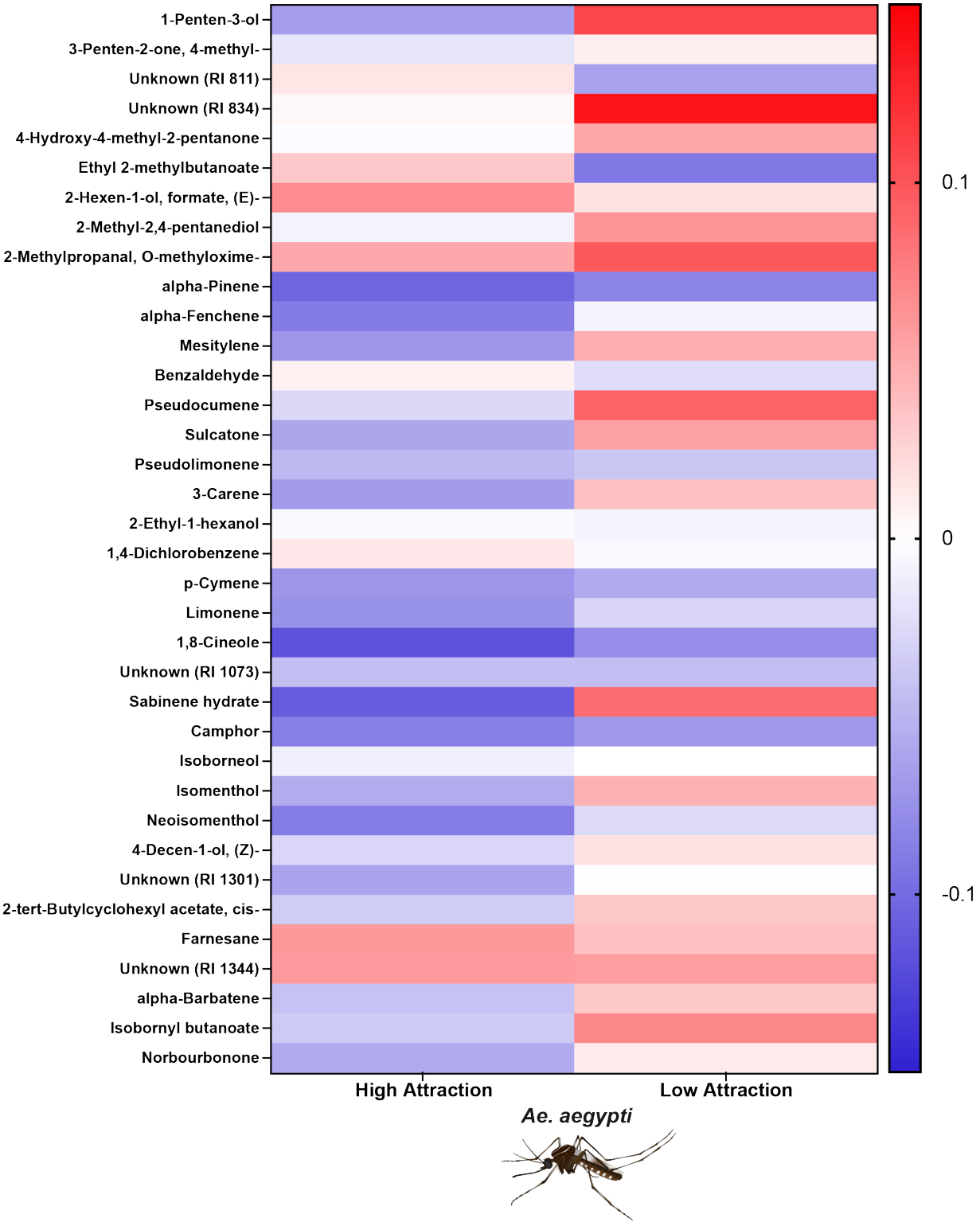


**Figure 14. Differences in chemical headspace between high and low attraction individuals.** Gas Chromatography-Mass Spectrometry was performed after thermodesorption of Tenax-TA tubes. High and low attraction groups were separated by mosquito behavioral response.

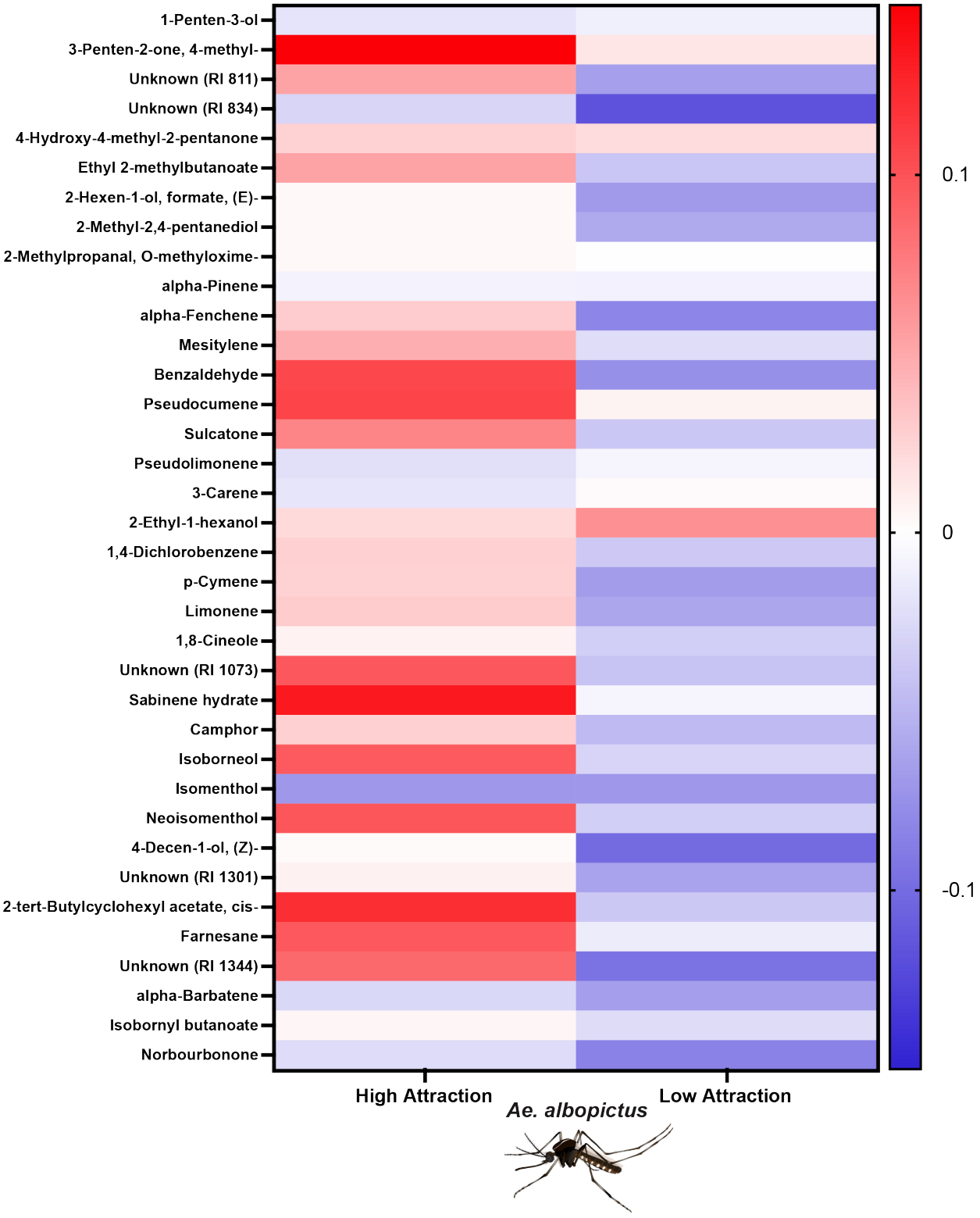


**Figure 15 Differences in chemical headspace between high and low attraction individuals in *Ae. albopictus.*** GC-MS results of subjects who were high or low attracction to *Ae. albopictus.*

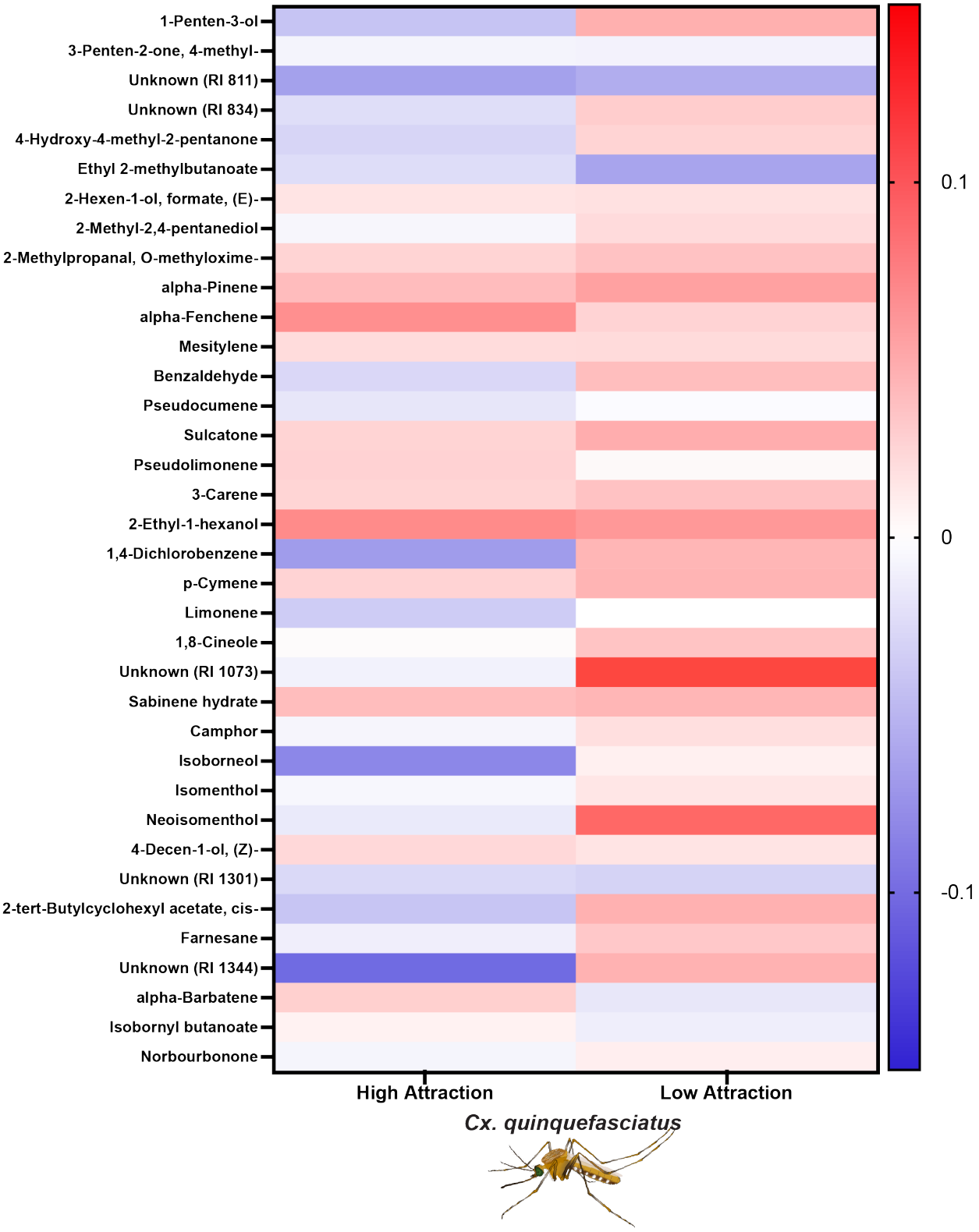


**Figure 16 Differences in chemical headspace between high and low attraction individuals in *Cx. quinquefasciatus.*** GC-MS results of subjects who were high or low attraction to *Cx. quinquefasciatus.*

## 4.4 DISCUSSION

Previous studies have analyzed the microbiome of subjects and compared to their attraction in *Anopheles* mosquitoes (Verhulst et al., 2011, Showering et al., 2022), but this is the first study to show the comparison between host-preference, subject microbiome and odor profile. We hypothesized that the differences in chemical components as well as specific species of bacteria in *Ae. aegypti* versus *Ae. albopictus* and *Cx. quinquefasciatus* are the result of underlying host preferences for these species. Specifically, *Ae. aegypti* is the most anthropophilic of these species (McBride et al. 2014). *Cx. quinquefasciatus* has an avian host preference and is more promiscuous regarding vertebrate hosts than *Ae. aegypti*, even though it also shows a high degree of anthropophily in urban environments (Garcia-Rejon et al. 2010). *Ae. albopictus*, a peridomestic mosquito, is the least anthropophilic of these three vector species (Cebrián-Camisón et al. 2020).

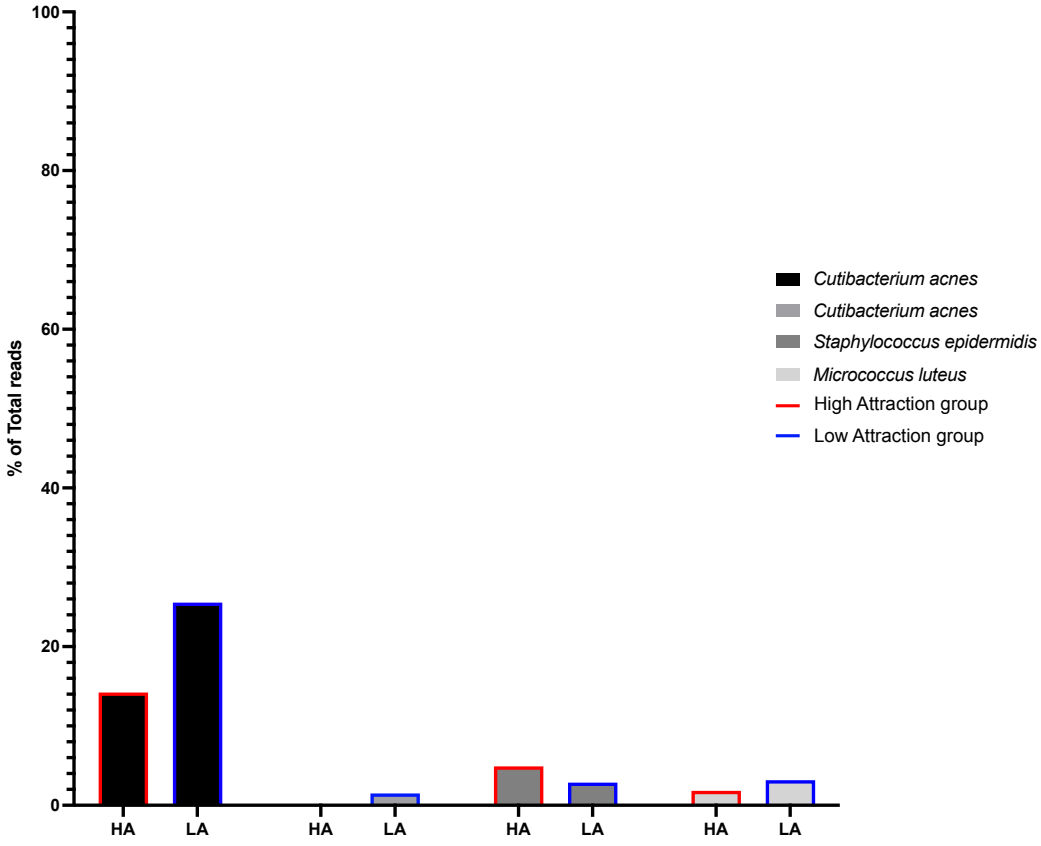


**Figure 17 Average total read counts of 4 significant ASV's from Mixed model.** Average total read count of significant ASVs between high attraction (HA) and low attraction (LA) groups.

While our initial investigation sought to identify conserved host attraction cues across vector species, our findings indicate that attraction in *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* is driven by separate and distinct volatilome cues, each with its own microbiome signatures associated with attraction. Although common microbial and volatile cues may exist, our analyses have not yet identified a common core of microorganisms or volatiles associated with vector species' host preference. Despite the fact that all three species are capable of feeding on humans, only one, *Ae. aegypti*, is specialized to human hosts. This suggests that a concealing repellent strategy may be able to identify compounds that protect humans from the most lethal, human-host-specialized mosquitoes. DeGennaro et al. (2015) found that masking

alone would not result in a universal insect repellent comparable to DEET, which has significant implications for repellent design.

This study is the first study to analyze the differences in mosquito host preference to all three of these species as well as to such a large subject cohort. These findings suggest that efforts to modify the epidermis microbiome should focus on the production of active repellents rather than masking attractants. Our research also suggests that this can be achieved by combining low-attraction microbes with the engineered expression of repellent compounds in a probiotic mélange of bacteria. Our research illustrates how microbes interact to produce conditions that attract mosquitoes particularly modulating the mosquito host seeking response. Additionally, we can test repellent candidates using our random forests machine learning results (Fig. 10E,11E &12E). The Random Forests algorithm uses an ensemble of classification trees (forest), with class prediction based on the majority vote of the ensemble. As the forest is built, it can provide an unbiased estimate of the classification errors by aggregating cross-validation results using bootstrapped samples. In addition, the algorithm also measures the importance of each feature based on the increase of the classification errors when it is permuted. To comprehend how these microorganisms influence mosquito attraction, additional research on microbial community interaction and competition is required. This work would benefit both the engineering of skin commensals and the understanding of how differences in the skin microbiome underlie differences in the mosquito attraction rate of individual subjects.

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# CHAPTER 5: MOSQUITO ATTRACTION TO IN-VITRO SKIN BACTERIA COMMUNITIES USING OLFACTOMETRY

## 5.1 ABSTRACT

Human skin microorganisms contribute significantly to the production of volatiles that attract mosquitoes. Using some of the most prevalent human skin bacterial species, we created *in-vitro* community models to determine if increased single species bacteria could modulate the attractiveness of humans to female *Ae. aegypti, Ae. albopictus* and *Cx.quinquefasciatus* mosquitoes and how the interactions of these bacteria affects overall attractiveness. *Ae. aegypti* mosquitoes were highly attracted to sweat plates of *Staphylococcus epidermidis* as well as optimized ratios of bacteria but overall complexity of the model alone does not attract the species. *Ae. albopictus* mosquitoes are less anthropophilic than *Ae. aegypti* and showed a low attraction to all plates regardless of ratio alluring to lower attraction of these skin commensals in the species. *Cx quinquefasciatus* mosquitoes responded strongly to single species sweat plates of *Staphyloccocus epidermidis, Corynebacterium minutissimum* and *Brevibacterium epidermidis.* Our data demonstrates, for example, that an in vitro community model reflecting the skin composition of highly attractive individuals to *An. gambiae* was more attractive to the anthropophilic *Ae. aegypti*. When different bacterial species were plated in different ratios the effects on mosquito responses varied significantly, indicating that specific bacteria community composition modulate attraction and that mosquito responses to skin volatiles is highly dependent on ratios of bacteria on skin that contribute to overall human odor.

## 5.1 INTRODUCTION

Our skin is home to billions of bacteria, fungi, and viruses, which live in distinct communities that make up the skin microbiome (Grice and Segre, 2011). Similar to the microbes in our gut, the epidermis microbiota plays a crucial role in protecting us from invading pathogens and boosting our immune system (Byrd et al., 2018, Callewaert et al., 2021). The cutaneous microbiota also produces odors that distinguish us from one another and could modulate attraction to bloodsucking insects (Verhulst et al., 2011, Busula et al., 2017). Among these arthropods are mosquitoes, which rely primarily on olfaction, specifically perception of volatile organic compounds (VOC's), to locate their hosts (Cardé 2015, Martinez et al., 2021). However, other sensory signals are thought to be essential in triggering the host-seeking behavior of these insects. Carbon dioxide ($CO_2$) emitted by humans and other animals stimulates the seeking behavior of mosquitoes (McMeniman et al., 2014, Dickinson et al., 2015). The mosquitoes then use chemosensory, visual, and thermal cues to locate their host (Raji and DeGennaro, 2017). Mosquitoes use human body odors as their primary sensory cue to distinguish hosts. These insects rely on their sophisticated sensory system, which consists of olfactory sensory neurons located in hair-like sensilla on the antennae, maxillary palps, and proboscis to detect and respond to odors in their environment (Hill and Ignell, 2021). After locating a host, mosquitoes can feed on blood and transmit pathogens. Malaria, Dengue virus, West Nile virus, and ZIKA virus disease can all be transmitted by mosquitoes (Weaver et al., 2018, Dahmana and Mediannikov, 2020). Current options for repelling mosquitoes from humans rely primarily on the frequent reapplication of topical repellents such as DEET, picaridin, and plant oils (DeGennaro, 2015). DEET has been the gold standard for decades, despite the fact that it can cause skin irritation, dissolve polymers in high concentrations, and emanate a strong odor (DeGennaro, 2015, Dennis et al., 2019).

Our objective was to use *in-vitro* community models to investigate the effect of human skin bacteria on mosquito attraction under laboratory controlled coniditions. Using four of the most prevalent bacterial species on human skin, we developed *in-vitro* community models to determine whether and how microbial diversity affect the behavior of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*. *In vitro* community models reflecting the skin composition of high and low attraction individuals based on attraction to *An. gambiae* (Verhulst et al., 2011, Verhulst et al., 2013) were developed and used to examine whether and how such mixtures influence the behavior of these three vector species.

## 5.2 METHODS

### 5.2.1 Mosquito rearing

*Aedes aegypti* (Orlando strain), *Aedes albopictus* (MRA-804 strain), and *Culex quinquefasciatus* (JHB strain) mosquitoes were reared and maintained at 25-28˚C, 75% relative humidity under a 14:10 light-dark cycle (lights on at 8 am for *Aedes* genus lights on at 1pm for *Culex* species). Mosquito eggs were hatched in deionized, deoxygenated water containing dissolved tablets of Tetramin tropical fish food (Catalog#16152, Tetra, Melle, Germany), to feed the emerged larvae. Adult mosquitoes were given ad libitum access to 10% sucrose solution. About 1 to 2-week-old adult females were fed on defibrinated sheep blood to generate eggs. Before behavioral assays, 7 to 21 day old sugar-fed mosquitoes were sorted 30 per tube and sexed under hypothermic (4˚C) conditions and fasted for 20 hours on water.

### 5.2.2 Behavior assay to test mosquito attraction

Mosquitoes were placed in a custom uniport olfactometer to analyze their behavioral attraction to bacterial sweat plates (Raji et al., 2019, Castillo et al., 2023). The uniport olfactometer consists of a large plexiglass tube (75cm long and 13cm wide) connected to a small cylindrical cage (13cm long and 5cm in diameter) that contains the mosquitoes prior to the

experiment. At the far end of the plexiglass tube connected to the stimulus chamber, the left arm of the human subject is inserted in an enclosed space with dimensions of 25 cm by 20 cm by 13 cm. In the stimulus chamber, carbon-filtered, humidified air and CO2 can combine with odorants to attract mosquitoes that have been released from a trap. Acrylic flowmeter Model VFA-4- SSV (Dwyer Instruments Inc., IN, USA) set to 3 SCFH was used to measure the CO2 release rate in the stimulus chamber. The final concentration for $CO_2$ in the assay was maintained at 2500-2700ppm by a carbon dioxide monitor (Catalog#CO2-100, Amprobe). Whereas, air flow rate was set at 21 standard cubic feet per hour by an air flowmeter (King Instruments CA, USA). The sealed design of the uniport, air filtration, and the positive pressure caused by air circulation in the apparatus will isolate the assay from all possible environmental scents.

Each behavior trial lasts for 8 minutes and 30 female mosquitoes of the respective species are tested. After the 8 minutes have passed attraction is scored as percent of female mosquitoes in the attraction trap compared to the total. Female mosquitoes are then euthanized through a hepa filtered vacuum (Model SCAE0, Miele, Gütersloh, Ostwestfalen-Lippe, Germany).

**5.2.3 Methods for in-vitro community models**

In-vitro community models were grown in Sweat media or Brain-Heart Infusion (BHI) media. Sweat media was prepared by adding 20.9 g $L^{-1}$ MOPS (Thermo Fisher, BP308500), 1 g $L^{-1}$ yeast extract (BP1422-2), 2 g $L^{-1}$ NaCl (BP3581), 0.65 mg $L^{-1}$ cod methyl ester fatty acids (Sigma-Aldrich, C5650-5G), 0.1 g $L^{-1}$ Tween 80 (Thermo Fisher, BP338-500), 1 µL of lactic acid (Sigma-Aldrich, C.A.S. 79-33-4) and 7.5 g $L^{-1}$ of Tryptic Soy Broth (TSB) (Sigma-Aldrich, 22092-500G) to deionized water while stirring, and media was then autoclaved for 35 min at 121° C. The magnetic bar was kept in the media bottle during autoclaving, so that media could be stirred following the autoclaving process. Tween 80 and cod fatty acids separate phases at high temperatures, thus the media needed to be homogenized while cooling down. Final pH of

the media was 6.0. BHI was prepared by suspending 52 g in 1 L of purified water, followed by autoclaving for 35 min at 121 °C. In both cases, we used 1.5 % of agar (Sigma-Aldrich, 05039-500G) when preparing solid media.

Stocks of *Staphylococcus epidermidis* (ATCC #12228, Manassas, VA, USA), *Pseudomonas aeruginosa* (9027-MINI-PACK, ATCC, Manassas, VA, USA), *Corynebacterium minutissimum* (ATCC #23348, Manassas, VA, USA) and *Brevibacterium epidermidis* (ATCC #35514, Manassas, VA, USA) were prepared according to the ATCC protocol. BHI media was used to prepare 20% glycerol bacteria stocks.

Monocultures of each of the bacteria species were cultured in BHI at 30 ° C, shaking at 220 rpm for 24 h. To ensure equal ratios being plated monocultures of each of the bacteria were standardized by diluting in BHI media to 1 O.D using the NanoDrop 2000 Spectrophotometer (ThermoFisher, ND2000CLAPTOP, USA). For the olfactometer assays, single species previously cultivated as monocultures were added to a sweat media agar plate to form the different human skin *in vitro* bacterial communities. We examined six *in vitro* community models in total composed of up to four bacteria species that were tested in a competitive environment. *Staphylococcus epidermidis* was the reference control grown as a single model. Double was composed of *S. epidermidis, P. aeruginosa* in 1:1 ratio; Triple was composed of *S. epidermidis, P. aeruginosa, C. minutissimum* in 1:1:1 ratio; quadruple was composed of *S. epidermidis, P. aeruginosa, C. minutissimum, B. epidermidis* in 1:1:1:1 ratio; the High Attraction (HA) community was composed of *S. epidermidis, P. aeruginosa, C. minutissimum, B. epidermidis* in 3:3:2:8 ratio and the Low Attraction (LA) community was composed of the same four species but in 1:6:1:8 ratio, both based on the ratios of these skin bacteria determined for individuals that were highly or poorly attractive to *An. gambiae* (Verhulst et al. 2011). A total of

100 µL of the bacterial inoculum was applied on sweat media agar plates and incubated at 37 °C for 24 h prior to the bioassay.

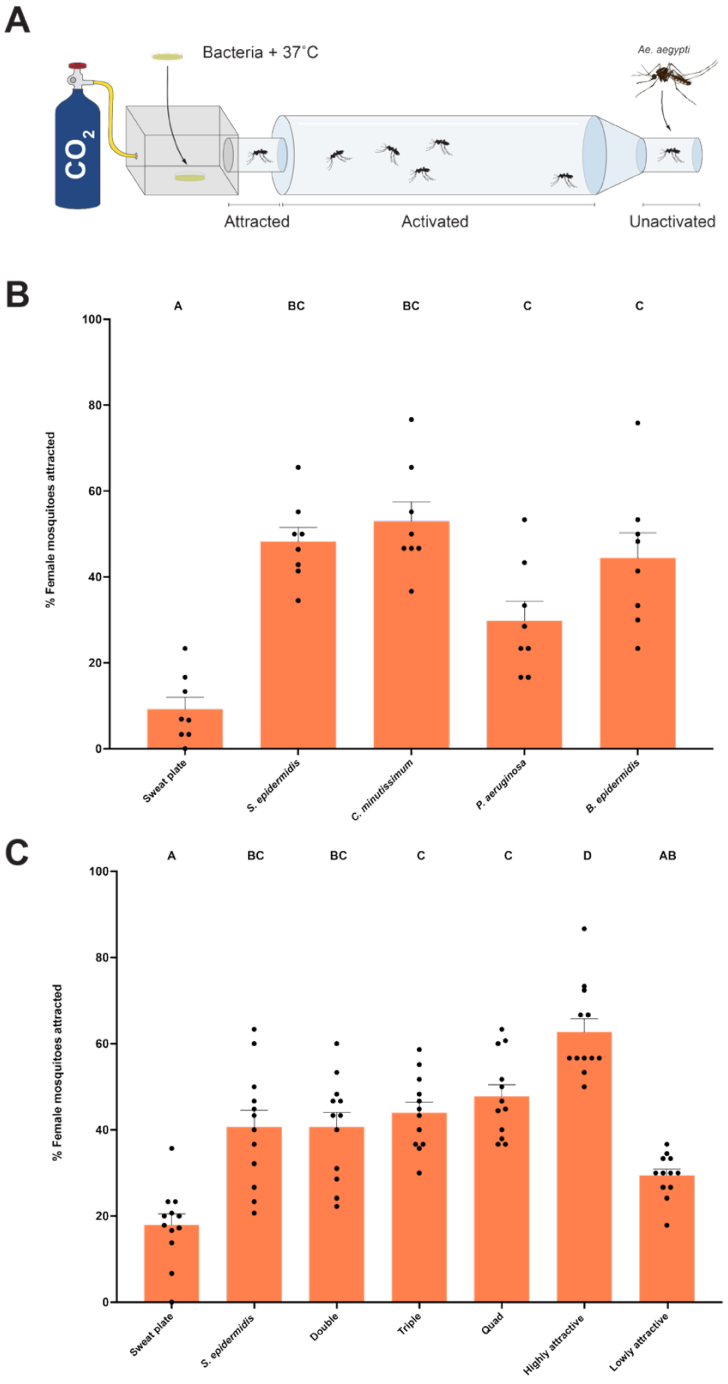


**Figure 18 *Ae. aegypti* prefer bacterial communities with a specific ratio that reflects a high attraction individual.** (A) Shows the schematic of our behavior experiment. Bacteria are placed in the olfactometer on a hot plate at 37˚C. (B) Shows the percentage of *Ae. aegypti* females response to single species of bacteria. A One-way ANOVA was used to explore differences between bacterial species with letters representing significantly different groups ($p < 0.05$). (C) Shows *Ae. aegypti* response to in-vitro community models. A One-way ANOVA was used to determine significantly different groups.

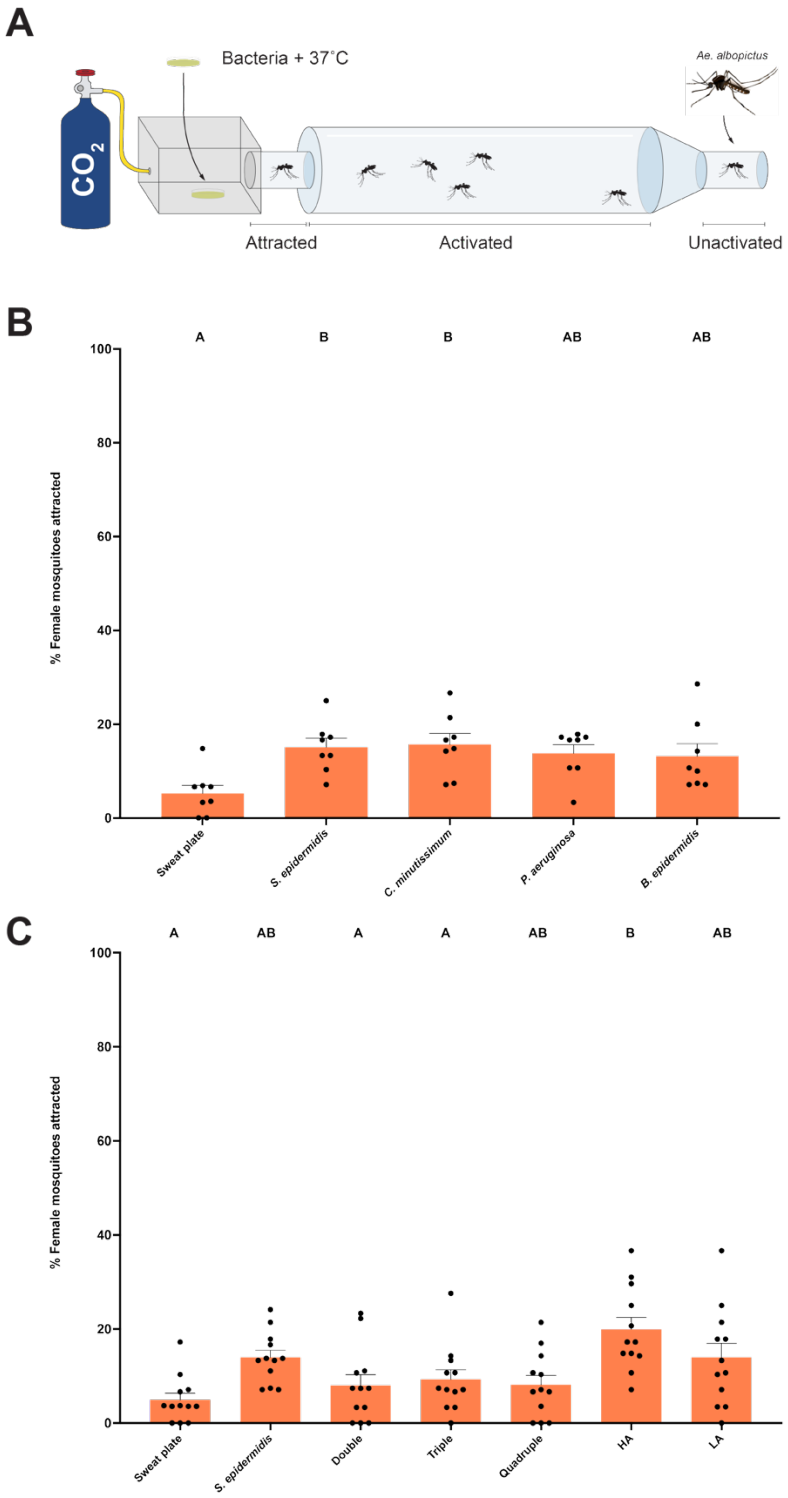


**Figure 19 *Ae. albopictus* shows reduced attraction for single species and in-vitro community models.** (A) Shows the schematic of our behavior experiment. Bacteria are placed in the olfactometer on a hot plate at 37˚C. (B) Shows *Ae. albopictus* response to single species of bacteria on sweat plates. A one-way ANOVA determined no significant difference between the bacteria species with *S. epidermidis* and *C. minutisimum* significantly different than the control sweat plate. (C) *Ae. albopictus* does not discriminate between plates containing *in-vitro* communities. Letters represent significantly different groups based on a one-way ANOVA.

## 5.4 RESULTS AND DISCUSSION

### 5.4.1 *Ae. aegypti* show robust response to human skin commensals

Female *Ae. aegypti* were attracted to 3 species of bacteria equally with reduced attraction to *P. aeruginosa* as determined by our one-way ANOVA (Fig. 18, $p < 0.01$). Incubated broth cultures of *S. epidermidis* and *C. minutissimum* have been shown to attract *An. gambiae* compared to their non-incubated cultures (Verhulst et al., 2010). In our olfactometer assay *S. epidermidis* and *C. minutissimum* are more attractive than the control sweat plate and *P. aeruginosa* but not significantly different from *B. epidermidis* (Fig. 18A & 18B).

Using four of the most abundant human skin bacterial species, we created *in vitro* community models to assess whether increased microbial biodiversity could reduce human attractiveness to *Ae. aegypti* females. We did not detect differences between more complex bacterial models, but the composition of the bacterial community could increase or decrease mosquito attraction (Fig. 18C). For instance, female mosquitoes preferred the *in vitro* community model reflecting the skin composition of a high attraction individual to *An. gambiae* than the community model reflecting the skin composition of a low attraction individual to *An. gambiae* (Fig. 18C, ANOVA, $p < 0.001$).

This result could be due to *Ae. aegypti* showcasing a high level of anthropophilic behavior and their ability to modulate host-preference based on a minimal amount of host odor cues (De Obaldia et al., 2022). *C. minutissimum* has been shown to breakdown compounds from our sweat to produce axillary malodour (James et al., 2004) while *S. epidermidis* breaks down components from sweat and produces volatile fatty acids that have been implicated in mosquito attraction studies (James et al., 2004, Raji et al., 2019, De Obaldia et al., 2022). Thus, 3 out of

the 4 species of bacteria that have been shown to modulate *An. gambiae* attraction present sufficient odor cues to drive attraction of *Ae. aegypti* to human hosts.

### 5.4.2 *Ae. albopictus* does not discriminate between human skin commensals on sweat plates

*Ae. albopictus* is known to feed on vertebrate hosts depending on their habitat and availability (Fikrig et al., 2023). When presented with the single species community model, *Ae. albopictus* attraction did not exceed 30% for any of the bacterial species (Fig. 19). A one-way ANOVA showed differences in responses to *S. epidermidis* and *C. minutissimum* compared to the control sweat plate but no significant difference between any of the single species plates (Fig. 18A). The community model data showed that *Ae. albopictus* do not typically discern between more complex communities that have been shown to attract *An. gambiae* (Fig. 19B). The greatest differences for this mosquito species were between the HA community model which was significantly different from the double and triple models as well as the control sweat plate (Fig. 19B, $p < 0.05$). Vertebrate hosts house different bacterial populations and therefore the reduced attraction in our study could be due to the overall general host preference often exhibited by *Ae. albopictus* (Fikrig & Harrington, 2021). Previous studies have shown that *Ae. albopictus* are attracted to bacterial cultures when compared to luria broth controls namely to *Kocuria rhizophila*, this species of bacteria was not studied in this assay (Michalet et al., 2019).

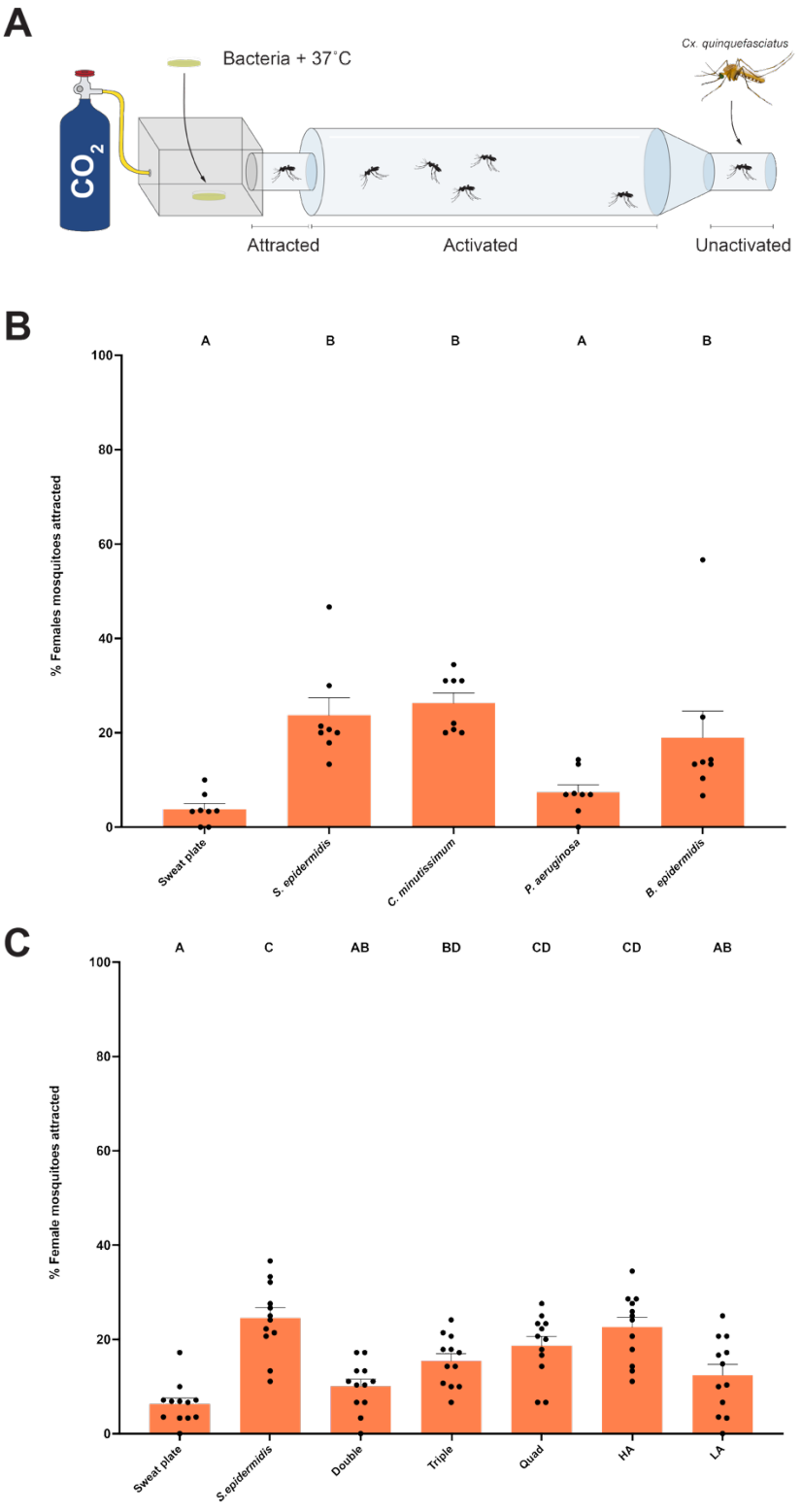


**Figure 20 *Cx. quinquefasciatus* response to *in-vitro* community models shows reduced attraction for *P.aeruginosa.*** (A) Shows the schematic of our behavior experiment. (B) Response to single species models. (C) Response to community models. Letters demean significantly different groups based on ANOVA.

### 5.4.3 *Cx. quinquefasciatus* attraction is reduced when *P. aeruginosa* is present

When presented with 4 abundant species of bacteria found on human skin *Cx. quinquefasciatus* did not show a significant preference for any species but was significantly reduced in their attraction to *P. aeruginosa* (Fig. 20B, $p < 0.01$). The community models showed a significant difference between the high attraction and low attraction models (Fig. 20B, ANOVA, $p < 0.05$). *S. epidermidis* alone was more attractive than the control sweat plate as well as the double, triple and low attraction models (Fig. 20B). *S. epidermidis* could potentially drive *Cx. quinquefasciatus* preference as its reduction leads to lower attraction in some models.

Host choice by blood-feeding mosquitoes is exceptionally broad, with female mosquitoes feeding on humans, non-human primates, other mammals, and birds among other species (Clements, 1999; Verhulst et al., 2018; Spanoudis et al., 2020). *Cx. quinquefasciatus* is known to feed on birds and this preference is distinguishable to the odor profiles based on specific species (Spanoudis et al., 2022). Given this capability *Cx. quinquefasciatus* has been implicated in the transmission of both bird and mammalian disease (Negi & Verma, 2018). *Cx. quinquefasciatus* feeds mostly on birds with this study being the first to show its attraction to bacterial models. The microbiome of birds has been shown to be influenced by the uropygial glands along with their plumage that could influence mosquito attraction (Maraci et al., 2018). Due to their extended surface, feathers provide an excellent surface for scents to emanate from a bird; however, to the best of our understanding, no study has examined specific bird bacterial components and their influence on *Cx. quinquefasciatus* attraction. A study linking the bacterial communities of birds with *Cx. quinquefasciatus* attraction is necessary to further understand what microbial cues attract this vector species.

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## CHAPTER 6: RESEARCH GAPS AND FUTURE DIRECTIONS

Due to the significant role mosquitoes play in the transmission of diseases, the relationship between mosquitoes and human odor has been thoroughly investigated. Mosquitoes are highly effective vectors for a variety of human-borne pathogens, including, among others, Dengue virus, Chikungunya virus, and Zika virus (Franklinos et al., 2019). In light of the emergence and resurgence of these diseases over the past decade, it is crucial to comprehend the mechanisms underlying mosquito attraction to human odor.

Anthropophilic mosquitoes, such as *Aedes aegypti* and *Aedes albopictus*, have a significant attraction to human odor, which consists of more than 1,000 volatile organic compounds (Dràbinska et al., 2021). Due to the complexity of the human odor plume, identifying the specific components responsible for mosquito attraction has proved to be a challenging task. The breakdown of amino acids and lipids by skin microorganisms into volatile short-chain carboxylic acids is a significant contributor to the human fragrance that attracts mosquitoes, according to research (James et al., 2004). Moreover, age, sex, personal hygiene, pregnancy, and parasite infection have been shown to impact a person's attractiveness to mosquitoes. Pregnant women, for example, are more alluring to mosquitoes due to the increased temperature of their skin and the presence of carbon dioxide in their breath (Ansell et al., 2002). Malaria-infected individuals have been found to be more appealing to mosquitoes, which may contribute to the disease's spread (Busula et al., 2017).

Olfactometry has been extensively used to identify the numerous mosquito-attracting components of human odor. Neural changes involving central brain circuits and modifications in the affirmation of odor-specific olfactory receptors determine the preference for human hosts over those of other vertebrates (Raji & DeGennaro, 2017). The application of olfactometry and

the identification of the specific components of human odor that attract mosquitoes could result in the development of novel strategies for combating vector-borne disease.

Chapters 2 and 3 of this dissertation established a rigorous and reproducible method for studying mosquito olfaction using an olfactometer (Castillo et al., 2023). Chapter 4 aimed to investigate the microbiome, odor profile, and host preference of three species of mosquitoes: *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*. The study hypothesized that differences in the chemical components and specific species of bacteria in these mosquitoes were related to their host preferences. While previous studies have compared the microbiomes of subjects to their attraction in *Anopheles* mosquitoes, this study is the first to examine the relationship between microbiome, odor profile, and host preference. The results suggest that attraction in these mosquitoes is driven by separate and distinct volatilome cues, each with its own microbiome signatures associated with attraction. We also propose a way to test repellent candidates using machine learning results. The study emphasizes the need for further research on microbial community interaction and competition to understand how differences in the skin microbiome underlie differences in mosquito attraction rates.

Chapter 5 investigated the role of different bacterial species in attracting female *Aedes aegypti, Aedes albopictus* and *Cx. quinquefasciatus* mosquitoes to human hosts. We used olfactometer assays to evaluate the attractiveness of various bacterial cultures to mosquitoes and found that female *Ae. aegypti* were equally attracted to three species of bacteria, while their attraction to *Pseudomonas aeruginosa* was reduced. Using *in-vitro* community models of human skin bacterial species we assessed whether increased microbial biodiversity could reduce human attractiveness to mosquitoes. We found that the composition of the bacterial community could increase or decrease mosquito attraction, with female *Ae. aegypti* preferring the community model reflecting the skin composition of a high attraction individual to *An. gambiae*. This

suggests that certain bacterial species present sufficient odor cues to drive attraction of *Ae. aegypti* to human hosts. However, *Ae. albopictus* did not show a discernible preference for any single species of bacteria, suggesting that their attraction to bacterial cultures may be influenced by overall host preference. *Cx. quinquefasciatus* exhibited no discernible preference for any of the four tested bacterial species, but was substantially less attracted to *P. aeruginosa*. It was discovered that *S. epidermidis* alone was more attractive than the control sweat plate but not significantly different to other community models. While it is known that *Cx. quinquefasciatus* feeds primarily on birds, no study has examined the effect of specific bacterial components from birds on mosquito attraction. A study linking the bacterial communities of birds with the attraction of *Cx. quinquefasciatus* would be necessary to better understand the microbial signals that attract this vector species. This study highlights the potential role of human skin microbiota in shaping mosquito behavior and provides insights into how microbial biodiversity can influence mosquito attraction to humans.

In conclusion, it is essential to comprehend the mechanisms underlying mosquito attraction to human scent in order to prevent the spread of vector-borne diseases. The use of olfactometry, microbiome sequencing and machine learning algorithms has yielded promising results in identifying the mosquito-attracting components of human odor. These findings could pave the way for the creation of innovative strategies to combat vector-borne diseases and enhance public health.

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