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Why Mosquitoes Bite Some People More Than Others: Metabolic Correlates of Human Attraction in AEDES AEGYPTI

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WHY MOSQUITOES BITE SOME PEOPLE MORE THAN OTHERS: METABOLIC CORRELATES OF HUMAN ATTRACTION IN *AEDES AEGYPTI*

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Aedes aegypti mosquitoes are the principal vectors of two major infectious diseases that plague the developing world today: dengue fever and chikungunya, with dengue fever alone resulting in ~400 million total yearly infections, and ~24,000 deaths (Bhatt et al., 2013). Understanding the biology behind *Ae. aegypti* attraction to humans is critical for developing novel strategies to combat these diseases. Yet, even the basic act of how mosquitoes choose one human host over another is poorly understood. Many previous studies on differential attraction have focused on small, homogenous subject populations and addressed a single hypothesis. We took the opposite strategy and studied a large, diverse 150-subject cohort, capturing a multitude of variables that may be involved in host selection. Importantly, our study examined the previously unexplored possibility that mosquito preference may be correlated with differences blood metabolites between subjects. We developed the uniport olfactometer as a method for discriminating subject attraction. Within our study population we distinguished three clusters of subjects who were differentially attractive to mosquitoes. We performed metabolic profiling with subject plasma samples and acquired relative concentrations of 613 different metabolites. We also collected information pertaining to 41 other variables including demographic

information, self-reported lifestyle factors, self-reported reaction to mosquito bites, vital signs, blood type, a complete blood count panel, and clinical blood analysis. Using a variety of statistical methods for feature selection, we narrowed this list of variables and arrived at two preliminary models for mosquito attraction. These models explain 24.1% of subject variation in mosquito attraction, and approximately 19.7% of this explanatory power is due to blood metabolites alone. Metabolites within the amino acid superpathway, and specifically the histidine subpathway were negatively correlated with mosquito attraction. Conversely, molecules within the lipid metabolism superpathway, specifically long chain fatty acids and monoacylglycerols, were positively correlated with mosquito attraction. This is the first study to correlate human blood metabolomic components with selective attraction of mosquitoes to hosts. Our work establishes a framework to study the causality of these correlates, and determine the mechanisms underlying their effect on mosquito choice.

For my two handsome mischief-makers

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CHAPTER 1: INTRODUCTION

1.1 The global impact of mosquito-borne disease

Mosquitoes are deadly vectors of many of the infectious diseases that plague the developing world today. Mosquito-borne illnesses sicken and kill millions of people each year, and as a result pose a significant burden on populations, health systems, and economies in endemic countries. *Aedes aegypti* mosquitoes serve as primary vectors for three important arboviral diseases: yellow fever, dengue fever, and chikungunya (**Figure 1.1**).

Yellow fever is a mosquito-borne viral disease that has been of public health importance since its discovery in the 15th century. In the first phase of infection, it can cause headache, fever, muscle aches, and nausea. Most people will recover from this phase, but in about 15% of patients symptoms worsen to include high fever, hemorrhage, renal failure, and the jaundice for which the fever is named. Yellow fever epidemics continued to occur throughout Central and North America, killing thousands of people, until an attenuated live virus vaccine was developed in 1936 (Barnett, 2007). This vaccination has proven safe, affordable, and effective—a single dose confers life-long immunity in 99% of people (WHO, 2014). Still, an estimated 200,000 cases occur annually, causing approximately 50,000 deaths (Barnett, 2007). Although yellow fever is largely eradicated, there are no vaccinations available for the two other major diseases that *Ae. aegypti* can vector—dengue fever and chikungunya—and so they are still major public health concerns.

Dengue, or “breakbone,” fever is the most common viral mosquito-borne

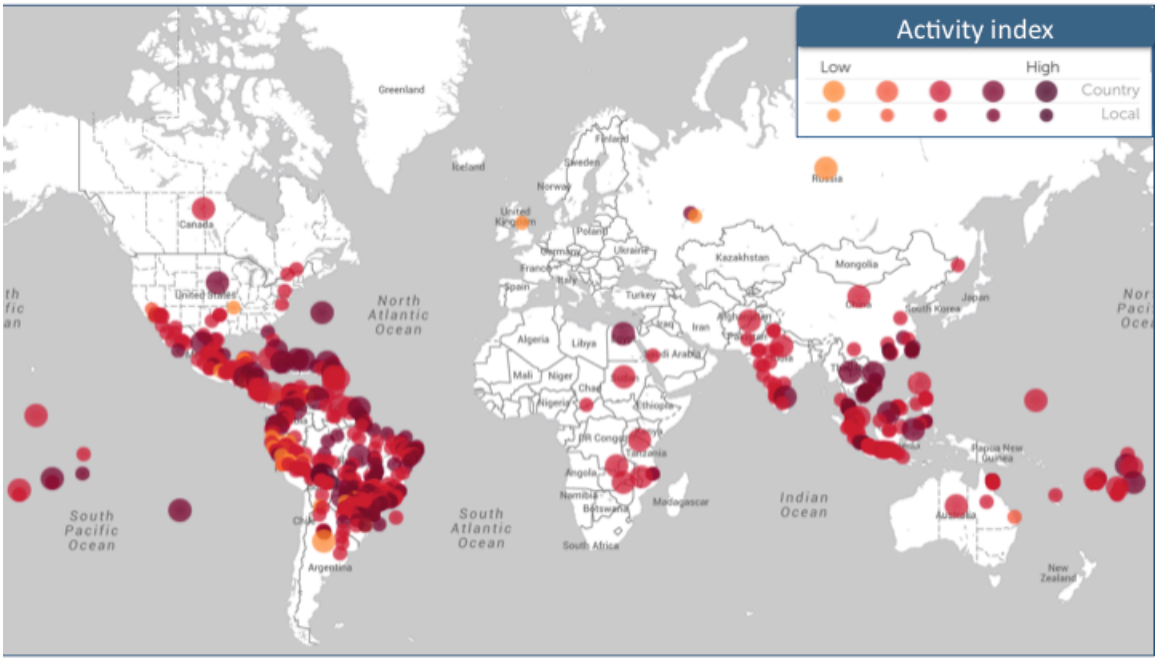


Figure 1.1 World map depicting incidence of dengue fever, chikungunya, and yellow fever in the 3 months preceding March 2015. Diseases vectored by *Aedes aegypti* mosquitoes are prevalent throughout the globe, particularly in South America and Southeast Asia. Markers correspond to reports aggregated from online news sources, eyewitness reports, expert discussions, and validated official reports. Marker size indicates country-level alerts (large circle) or state, province, and local alerts (small circle). Marker color indicates the extent of the event based on user ratings, disease importance, and the volume of news associated with the alert (Adapted from www.healthmap.org/en).

ailment, with at least 40% of the world's population living in areas with active dengue transmission (WHO, 2015a). One recently published model predicts that 390 million dengue fever infections occur annually (Bhatt et al., 2013). Dengue virus infections are acute and systemic, and are caused by four single-stranded RNA virus serotypes (Guzman et al., 2010). Though infection often does not manifest clinically, when symptoms do occur they range widely—from a mild fever with muscle aches to life-threatening dengue hemorrhagic fever or dengue shock syndrome. Exposure to one of the serotypes confers lifelong immunity to that serotype, but simultaneously increases the likelihood of developing more serious complications following infection with a second serotype. Of those patients who do die from the disease, most are children (WHO, 2015a). With the recent surge in dengue fever outbreaks—increased nearly 30-fold in the last five decades—the impetus for vaccine development is stronger than ever. Despite great effort, no effective vaccine currently exists.

Chikungunya is a lesser-known emerging tropical disease that only recently arrived in the Western hemisphere, but its potential for proliferation is vast. The virus can cause fever, headache, rash, nausea, fatigue and, most notably, severe joint pain that lasts from several days to weeks. The disease takes its name from the Makonde word *kungunyala*, meaning "that which bends up." Chikungunya is currently present in 60 countries primarily within Africa, Asia, and the Indian subcontinent, and is spreading rapidly. In December 2013, the first case of chikungunya was confirmed on the Caribbean Island of St. Martin. This was the first locally acquired case of chikungunya in the Americas, and as of

January 2015, it is suspected that there are approximately 1,135,000 cases in this region (WHO, 2015b). There are no effective vaccines to protect against chikungunya.

Because there are currently no effective treatments for dengue fever and chikungunya, strategies currently focus efforts on preventative measures such as bite prevention and mosquito population control.

1.2 *Ae. aegypti* has adapted to live in close proximity to humans throughout its lifecycle

Mosquitoes have become such dangerous arthropods because to develop and lay eggs, females must ingest a nutrient-rich meal of concentrated protein found in blood. Because a female will take multiple blood meals over the course of her reproductive lifetime, she can transmit diseases from person to person. Depending on the mosquito species, blood meals can be obtained from birds, reptiles, amphibians, or mammals, but human-preferring, or anthropophilic mosquito species such as the domestic form of *Ae. aegypti* have evolved a significant preference for humans. This strong adaptation to remain in close proximity to human hosts is reflected throughout their lifecycle (Clements, 1992b).

Like all holometabolous insects, mosquitoes go through four life stages—egg, larva, pupa, and adult (**Figure 1.2**). *Ae. aegypti* females choose to lay their eggs on damp surfaces near manmade and artificial containers. Man-made water

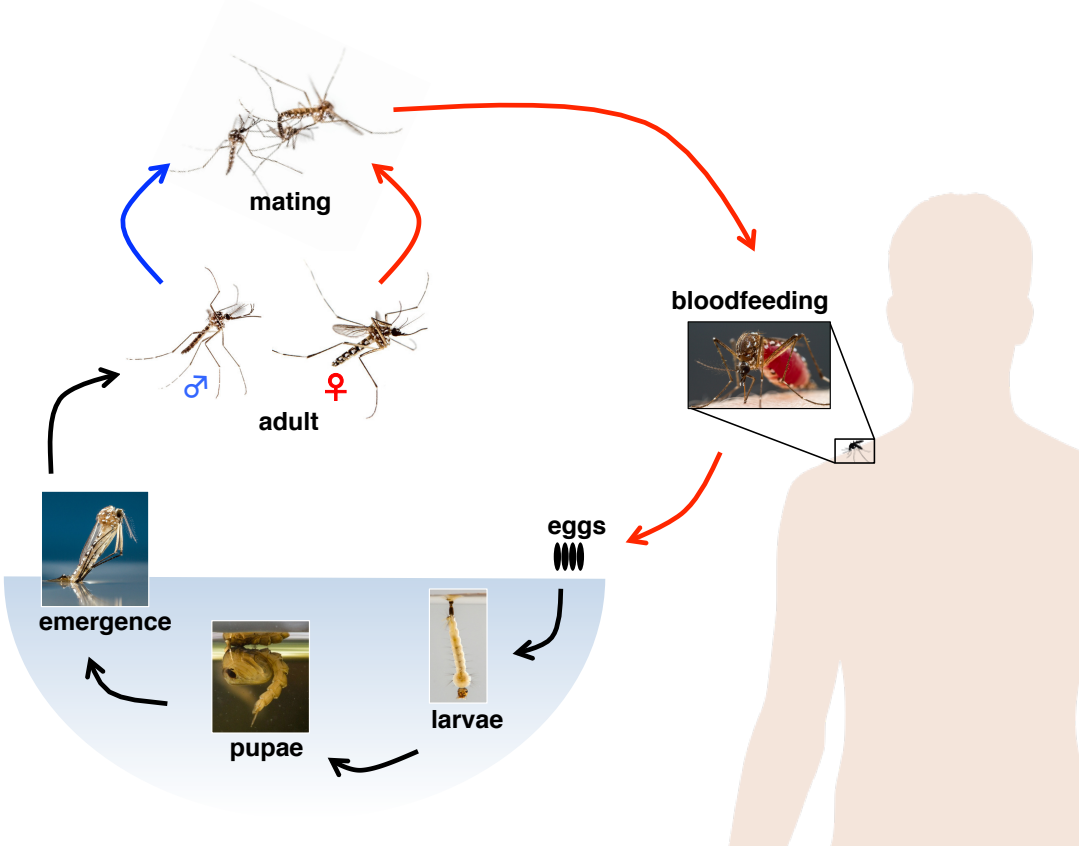


Figure 1.2 Life cycle of *Ae. aegypti*. Through the larval and pupal stages, *Aedes* offspring develop in an aquatic environment. After approximately 1 week, they emerge as adults and soon after mate. Adult males will survive on plant nectars and continue to mate, while adult females soon begin seeking out hosts from which to obtain a blood meal. When a suitable host is found, an adult female will imbibe a blood meal and use the nutrients therein to contribute to her energy stores and develop a clutch of eggs. Images © Alex Wild 2014.

sources such as rain gutters, abandoned rubber tires, and water collection containers found near human dwellings fit these criteria, and these are often host to mosquito offspring (Clements, 1992b).

After *Ae. aegypti* eggs are laid, they progress through embryonic development. If the water source dries up, the embryonated eggs can remain desiccated for months until resubmerged in water. Upon hatching, aquatic larvae feed on organic matter as they develop through four larval instars, and a pupal stage. *Ae. aegypti* mosquitoes emerge from the water as adults that survive by feeding on a combination of plant nectars (males and occasionally females) and blood (females only) (Clements, 1992b).

After female *Ae. aegypti* have reached adulthood and have mated, they enter a phase of "host-seeking," when they become strongly attracted to a combination of cues emitted by humans such as visual contrast, heat, carbon dioxide (CO_2), and human-related odors. This attraction depends on physiological factors such as age and mating status (Bowen, 1991). Once she has located a suitable host, the female ingests approximately 3-4 µL of blood which corresponds to a two-fold increase in body weight. Using the nutrients (primarily amino acids and lipids) therein, she develops her eggs and replenishes her own energy stores for survival (Clements, 1992b).

1.3 Multiple blood meals enable disease transmission

As human-adapted female mosquitoes such as *Ae. aegypti* cycle through multiple egg-laying ("gonotrophic") cycles in their lifetime—host-seeking,

obtaining a blood meal, ovipositing—the groundwork is laid for disease transmission between human hosts. Through the act of imbibing a blood meal from an infected person during a period of viremia, the female mosquito herself becomes harbor to the virus that causes the disease. The virus then survives and, if the virus reaches the salivary glands, will replicate and make the mosquito a lifelong-carrier {Clements:2012uf}. Any subsequent healthy hosts that an infected mosquito bites then become infected themselves, thus propagating the human-to-mosquito-to-human infection cycle. This situation is made more problematic by the relatively recent realization that female *Ae. aegypti* take multiple blood meals within a single gonotrophic cycle much more frequently than previously thought (Scott et al., 2000). This even further increases the opportunities to spread dangerous pathogens between humans (Scott and Takken, 2012).

1.4 Mosquitoes use multiple human-emitted cues such carbon dioxide (CO_2), heat, and odor to locate and select hosts

For effective host-seeking, anthropophilic mosquitoes use a combination of multimodal human-emitted cues that differ greatly in their spatial reach. Olfactory cues such as human odorants and exhaled CO_2 act to draw mosquitoes in at a longer range. At short-range, visual cues, body heat, moisture, and skin tastants can also be integrated to aid selecting a host and a biting site (Clements, 1992a).

1.4.1 CO_2 *both activates and attracts mosquitoes*

Each breath that we exhale contains approximately 4% CO_2, which contributes significantly to the attraction of mosquitoes to humans (Snow, 1970). This CO_2 cue serves two important roles which support mosquito host-seeking behavior: (1) it activates mosquito flight and (2) it integrates with other mosquito sensory modalities to attract mosquitoes to a host (Gillies, 1980). Male and female *Ae. aegypti* sense CO_2 via a specialized class of olfactory sensory neurons in the maxillary palp, which are tuned to detect very small changes in CO_2 levels (Grant et al., 1995). In the absence of other host cues, exposure to increases in CO_2 concentration of 0.01-0.03% above the ambient 0.04% CO_2 in air strongly stimulates mosquitoes to begin flying, and increase their movement (Eiras and Jepson, 1991). Moreover, CO_2 synergistically increases mosquito attraction when presented in combination with other sensory cues such as heat or host-related odors (McMeniman et al., 2014). For these reasons, it has long been known that CO_2 is an effective addition to mosquito traps in the field (Newhouse et al., 1966).

1.4.2 Odor cues are the most important in distinguishing between and selecting hosts

While cues such as CO_2 play a role in mosquito attraction to hosts, odor cues are species-specific (McBride et al., 2014) and—in the case of humans—individual-specific (Penn et al., 2007), allowing mosquitoes the opportunity to be selective.

Some mosquito species are generalists, attracted to a wide variety of hosts, while others are specialists. *Ae. aegypti aegypti,* the “domestic” subspecies of *Ae. aegypti,* are an example of extreme specialists, as they focus specifically on humans (Gibson and Torr, 1999). In the lab, it has been shown that this preference for humans over nonhuman animals is primarily mediated by differences in body odor components (McBride et al., 2014). Likewise, differences in volatiles released between subjects are important contributors to differential attractiveness of humans to mosquitoes (Logan et al., 2008; 2010; Qiu et al., 2006; Schreck et al., 2002; Verhulst et al., 2013).

1.4.2.1 Characteristics of typical human body odor profiles

The characteristic body odor profile of an individual is influenced by a complex interaction between their genetics, health status, and lifestyle and may convey important information about internal physiological processes. An aggregation of the literature surrounding skin volatile extractions reveals that 500 distinct VOCs (volatile organic compounds) that have been identified in skin emanations from healthy human subjects. However, it is likely that only a fraction of these are reliably present in human skin emanations and are volatile at human body temperature. The most common compounds extracted from skin are hydrocarbons, lactic acid, ketones, and aldehydes, as well as some esters and alcohols. However, despite numerous studies on human skin emanations using various techniques, very few compounds are common between reports (de Lacy Costello et al., 2014).

1.4.2.2 Body odor components most salient to mosquitoes

Much research has been focused on understanding which specific odorants may attract and repel female mosquitoes, because it allows for the development of better attractant blends for traps and safer repellent sprays. L-lactic acid has been long established as an attractive odorant for *Ae. aegypti* (Acree et al., 1968) as well as *Anopheles gambiae* mosquitoes (Dekker et al., 2002) especially when presented in combination with CO_2. Combining lactic acid with acetone synergistically enhances *Aedes* attraction to an odor-baited trap (Bernier et al., 2003). Another well-known mosquito attractant is 1-octen-3-ol, which is appealing to both *Anopheles* (Takken and Kline, 1989) and *Aedes* mosquitoes (Van Essen et al., 1994).

Using these well-known attractants in combination with experimentally-derived candidates, several groups are developing blends of human-released volatiles to better attract mosquitoes (Logan et al., 2008; Mukabana et al., 2012; Takken and Verhulst, 2013). A standard attractive blend for *An. gambiae* mosquitoes consists of ammonia, lactic acid, and tetradecanoic acid (Smallegange et al., 2005). New compounds, isovaleric acid, 4,5-dimethylthiazole, 2- methyl-1-butanol, and 3-methyl-1-butanol, were discovered from subsequent research within the same research group, though the addition of 3-methyl-1-butanol increased mosquito attraction, the addition of the other compounds to the blend produced mixed and sometimes inhibitory results (Mukabana et al., 2012). Standard attractive blends for *Ae. aegypti* have proved more difficult to formulate. Still only modestly attractive, the most effective blend

to-date is one of L-lactic acid, acetone, and dimethyl disulfide (Bernier et al., 2007).

1.4.2.3 The composition of human body odor is influenced by interactions between sweat and skin microflora

Human body odor arises from a combination of secretions from sweat glands and the volatiles released as byproducts of metabolism by our skin microflora. Differences in physical and chemical properties of the skin at various body sites help to shape unique microenvironments best suited to host particular bacterial species (Grice and Segre, 2011). Variations in temperature, moisture content, osmolarity, pH, oxygenation, nutrient availability, host immune systems, and interactions with nearby microbes all contribute to the eventual microbial composition of a particular site (Wilson, 2009).

In general, there is lower interpersonal than intrapersonal variability in bacterial profiles, and this tends to remain true over time. The variation in bacterial profiles between the same sites on the left and right side of an individual was lower than the variation between the same site on two different individuals. Even when sampled over time, variation between subjects tends to be higher than within a subject (Verhulst et al., 2010b).

Volatiles arising from a person's semi-stable skin microflora population contribute to mosquito attraction. Human sweat collected immediately upon excretion is actually odorless to humans, and only takes on its characteristic odor after incubation at temperatures permissive for bacterial growth (Shelley et al.,

1953). Induced sweating causes subjects to be more attractive to *Ae. aegypti* mosquitoes (Khan et al., 1969), while subjects who are clinically unable to sweat may be less attractive (Maibach et al., 1966b). Similarly, collected fresh human sweat is not attractive to *Anopheline* mosquitoes at first; mosquitoes only become interested in it after incubation with skin bacteria for several days (Braks and Takken, 1999). Follow-up studies revealed that volatiles released from agar plates of cultured human foot bacteria are attractive to mosquitoes in the lab (Verhulst et al., 2009), and that differences in the milieu of bacteria present on individuals may be partially responsible for differential attraction (Verhulst et al., 2011).

1.4.2.4 Human skin gland secretions interact with the microbiome to shape body odor

Humans have three types of skin glands—sebaceous, eccrine and apocrine—each with a unique bodily distribution and physiological purpose (Grice and Segre, 2011). Sebaceous glands secrete sebum, which serves to moisturize and protect the skin. This waxy substance is rich in lipids such as fatty cholesterol, esters, long-chain fatty acids, squalene, and triglycerides (Nicolaides, 1974), all of which are substrates for microbial metabolism. Eccrine and apocrine glands are sweat glands, which produce clear, odorless substances primarily composed of water and salt. Eccrine glands can also contain sodium, chloride, potassium, calcium, magnesium, lactate, ammonia, urea, bicarbonate, proteins and peptides, amino acids such as serine, ornithine, citrulline and

aspartic acid as well as some antimicrobial and immune molecules (Wilson, 2009), while apocrine sweat contains various proteins including odorant binding proteins (Jacoby et al., 2004), lipids and steroids as well as nitrogen, lactates, and various other ions (Noël et al., 2012; Wilke et al., 2007). Eccrine glands are the most abundant and found on virtually all skin. Their sweat primarily serves to aid in thermoregulation through evaporative cooling and secondarily helps to create an inhospitable environment for microorganisms by acidifying the skin (Grice and Segre, 2011). Apocrine glands are located on hairy body areas such as the armpit and groin, and they respond to emotional stimuli such as stress, pain or sexual arousal by releasing their milky secretions. Microbial metabolism of these secretions is responsible for the stereotypical odor associated with human sweat (Grice and Segre, 2011). Corynebacteria are the bacterial genus found to primarily cause the stereotypically sweaty odor and even vary in number with its intensity (Leyden et al., 1981).

1.4.2.5 Human body odor profiles are also molded by genetics, environment, health and lifestyle

An individual's unique body odor profile is influenced at least in part by genetics. In human psychophysical studies, subjects were able to match parent and offspring pairs, but not spouses, by odor alone (Porter et al., 1985). In a study of non-cohabitating twins, body odor samples from monozygotic twins were virtually indistinguishable from one another (Roberts et al., 2005). In 2009, Kuhn and Natsch investigated this similarity quantitatively in a study comparing odorant

acids in 12 pairs of twins, and found that there was a high degree of heritability (Kuhn and Natsch, 2008). Single nucleotide polymorphisms can change the intensity of body odor (Martin et al., 2010). Finally, MHC alleles can communicate genetic relatedness through changes in individual scent perhaps via immune molding of skin microbial populations (Wedekind and Füri, 1997; Wedekind et al., 1995).

Smell has also been used as a diagnostic tool for many dermatological, infectious, and metabolic diseases, which commonly result in gross, qualitative changes in body odor (Shirasu and Touhara, 2011). Recently it has been documented that humans are even able to detect more subtle signs of illness via their olfactory sense. When an immune response was elicited in healthy subjects, volunteers noticed a “more aversive” body odor compared to control subjects. The ability to detect early indicators of illness confers the potentially important evolutionary advantage of being able to avoid infected individuals (Olsson et al., 2014).

There is also some evidence of a link between diet and body odor. It has been reported that trained dogs (Hepper, 1988) and human subjects (Wallace, 1977) were able to discriminate between the body odors of monozygotic twins only when they were fed different diets. Haverik et al. (2006) investigated one specific dietary example. They selected 17 male odor donors who were fed alternatively a “meat” and “nonmeat” diet, each for 2 weeks. During the final day of each 2-week period, axillary odor was collected and its qualities rated by 30 women. Women rated male odors following the “meat” diet as significantly less

pleasant, less attractive, and more intense (Havlicek and Lenochova, 2006). A separate study investigated the influence of dietary garlic on body odor and found that garlic consumption made body odor more appealing (Fialová et al., 2012).

Body odors are also found to vary with age (Haze et al., 2001), seasons (Zhang et al., 2005), menstrual cycle (Thornhill et al., 2003), and mood (Ackerl et al., 2002; Chen and Haviland-Jones, 2000).

1.5 Anthropophilic mosquitoes exhibit differential attraction to human subjects

Perhaps because it is such an anecdotally common phenomenon, there is a rich history of folklore surrounding the question “why do mosquitoes bite some people more than others?” (**Figure 1.3**). Lay theories regarding differential mosquito attraction vary wildly, though the most commonly cited are: diet, blood sugar, gender, skin temperature, body size, and blood type. Many people believe their attractiveness to mosquitoes has altered throughout their lifetime because of changes such as pregnancy, menopause, dietary shifts, medications, or surgeries (personal observations).

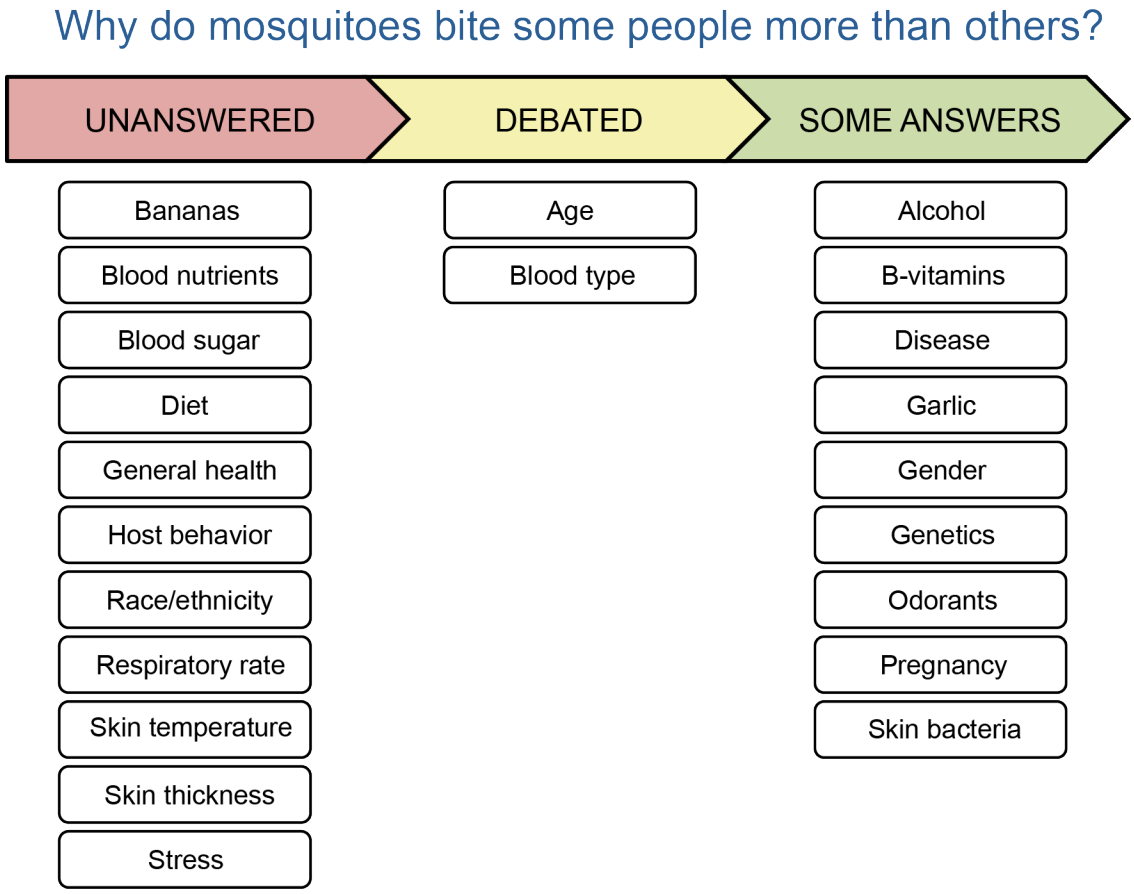


Figure 1.3 Proposed mechanisms of differential mosquito attraction to human hosts. Some of these theories have been investigated with relatively consistent results (some answers), some have been investigated with inconclusive or inconsistent results (debated), and others have not been investigated at all (unanswered). References: age (Carnevale et al., 1978; Freyvogal, 1961; Muirhead-Thomson, 1951; Spencer, 1967; Thomas, 1951), blood type (Anjomruz et al., 2014a; 2014b; Shirai et al., 2004; Thornton et al., 1976; Wood, 1976; Wood et al., 1972), pregnancy (Ansell et al., 2002; Himeidan et al., 2004; Lindsay et al., 2000), alcohol (Lefèvre et al., 2010; Shirai et al., 2002), B-vitamins (Ives et al., 2005), disease (Lacroix et al., 2005), garlic (Rajan et al., 2005), gender (Gilbert et al., 1966; Muirhead-Thomson, 1951; Qiu et al., 2006), genetics (Kirk et al., 2000; Verhulst et al., 2013), odorants (Acree et al., 1968; Logan et al., 2008; Qiu et al., 2006; Schreck et al., 2002; Verhulst et al., 2013), and skin bacteria (Verhulst et al., 2011)

Available scientific evidence does support some of these beliefs of the general public. Based on bite distribution data for several vector-borne diseases including malaria, Woolhouse et al. (1997) suggested that approximately 20% of hosts are responsible for 80% of the net transmission potential (Woolhouse et al., 1997). Similarly, another study showed that 20% of children received 80% of all malaria infections (Smith et al., 2005). Understanding the cues that mosquitoes use to identify this most-attractive fifth of the population would allow for more targeted and thus more effective disease control strategies.

Entomologists have also been intrigued by the interesting phenomenon of differential attraction and have sought to empirically prove its existence. In both laboratory and field studies using a variety of methodologies, experiments have repeatedly validated that not all humans are equally appealing to mosquitoes. Recently, Harrington et al. (2014) used human DNA fingerprinting to determine the blood meal sources of *Ae. aegypti* caught in four villages in rural Thailand. Though 66% of the people profiled were not bitten at all, 15.7% of those who were bitten were bitten 3 or more times and 3 subjects were bitten 9 times each (Harrington et al., 2014).

Many of the first published investigations of differential attraction were largely observational. Scientists visited people in regions plagued by mosquito-borne disease and recorded the number of mosquitoes biting or attempting to bite each individual person, and they found that not all people were bitten equally often (Ansell et al., 2002; Carnevale et al., 1978; Freyvogal, 1961; Muirhead-Thomson, 1951; Spencer, 1967; Thomas, 1951). However, these reports could

only suggest possible sources of this variation in appeal, as they were not controlling for any variables.

To begin to control for some of this variation, semi-field experiments were conducted wherein subjects were asked to sleep in controlled conditions, usually identical dwellings, and mosquito attractiveness was measured by collecting attracted mosquitoes and bloodfed mosquitoes from each dwelling (Himeidan et al., 2004; Knols et al., 1995; Lindsay et al., 2000; 1993). However, humans emit multiple, multimodal signals to entice mosquitoes, and these experiments could still not distinguish between them to find the causal cues.

In the laboratory, one group measured the time it took for 3 of 6 mosquitoes (50%), housed in a small cage suspended 1 cm about a subject's arm, to begin probing. Using this method, they were able to isolate differentially attractive subjects, though they were limited in their statistical power to discriminate due to the small dynamic range of the assay (Khan et al., 1969; 1965; Maibach et al., 1966b). Still other researchers found differences in the attractiveness of subjects when allowing mosquitoes access to the arms of two subjects and asking from which arm the mosquitoes would prefer to bloodfeed (Thornton et al., 1976; Wood, 1976; Wood et al., 1972). Sometimes mosquitoes were proboscis-amputated to prevent bites and only landings were scored, which likely impacted their behavior (Shirai et al., 2002; 2004).

Laboratory-based olfactometers have also been widely used in the study of differential attraction. These assays allowed comparison of mosquito attraction to the arm or hand of live hosts (Brouwer, 1959; 1960; Geier et al., 2002; Gilbert

et al., 1966; Logan et al., 2008; Qiu et al., 2006) or only body odor emanations (Logan et al., 2008; Verhulst et al., 2011). Such emanations were accumulated in a chamber (Lacroix et al., 2005; Mukabana et al., 2002; 2004), body bag (Logan et al., 2008), or collected on glass petri dishes (Schreck et al., 1982), test tubes (Geier et al., 2002), or beads (Qiu et al., 2006; Verhulst et al., 2011). Olfactometers are now the most widely used method for assessing differential mosquito attraction in the laboratory setting.

In all of these diverse experimental paradigms, subjects were differentially attractive to mosquitoes.

1.5.1 Possible mechanisms of differential attraction

1.5.1.1 Age, gender, and body size

The first published investigation of differential attraction was conducted by Muirhead-Thomson in 1951. Researchers observed 5 families in Jamaica with children of varying ages and recorded the number of *An. albimanus* mosquitoes that tried to bite each family member. In this observational study, they determined that age was a significant factor affecting attraction, as was gender, with adult males being the most attractive and small children the least (Muirhead-Thomson, 1951). Similar observational studies confirmed reports on the contribution of age in *An. gambiae* (Carnevale et al., 1978; Thomas, 1951) as well as *An. farauti* (Spencer, 1967) and *Ae. aegypti* (Freyvogal, 1961). Reports of gender differences were confirmed in *Ae. aegypti* (Gilbert et al., 1966). However, these findings may be attributable to differences in surface area due to size (Port

et al., 2009), for which they did not control. Indeed, a recent study found that the likelihood of being bitten was directly proportional to body size in a population in Peru {Liebman:2014tw}. In a later study using glass beads to collect subject emanations, no differences were found between the genders (Qiu et al., 2006).

1.5.1.2 CO_2 emissions

In *An. gambiae,* differences in CO_2 concentration in expired breath accounted for some of the variation between humans (Brady et al., 1997; Mukabana et al., 2004).

1.5.1.3 Blood type

In a well-known but controversial series of studies, variation in attractiveness to mosquitoes was been linked to blood type. The first study to report such claims was investigating the effects of skin temperature, skin pigmentation, subcutaneous fat, age, sex, nutritional status, and ABO blood group on differences in mosquito attraction between subjects. Using biting-based assays comparing subjects in a pairwise fashion, they found that subjects with blood type O were most attractive to malaria mosquitoes (Wood et al., 1972). The same group later tested the attraction of *Ae. aegypti* mosquitoes to 45 subjects and found similar results (Wood, 1976). However soon after, Thornton et al (1976) argued that the statistical approach used in the previous reports was flawed and conducted a methodologically similar experiment that showed no effect of blood type on attraction (Thornton et al., 1976). If subjects with type O

blood were more attractive to mosquitoes, one might expect to find a higher rate of mosquito-borne disease amongst that population. However, one study found fewer malaria patients with type O blood than were expected from control populations in Delhi (Madhu Gupta, 1980). More recently, type O subjects were again found slightly more attractive to proboscis-amputated *Ae. albopictus* mosquitoes than volunteers with type A blood, though the authors themselves admit to a "lack of clear preference among human blood groups exhibited in [their] study" (Shirai et al., 2004). A group in Tehran recently captured 95 human-fed *An. stephensi* mosquitoes and found that type O meals were overrepresented, although this was not a statistically significant effect (Anjomruz et al., 2014b). Most recently, the same group tested ABO group preference of *An. stephensi* in the lab and found type AB to be preferred (Anjomruz et al., 2014a). These conflicting results show that there is not yet a consensus on the relationship of blood type to mosquito attraction.

1.5.1.4 Alcohol ingestion

Three, 10-minute exposures to 35 proboscis-amputated *Ae. albopictus* mosquitoes before and within an hour after alcohol ingestion revealed that subjects were significantly more attractive to mosquitoes after alcohol ingestion. This effect was not due to increases in skin temperature or sweating (Shirai et al., 2002), and was confirmed in a later study (Lefèvre et al., 2010).

1.5.1.5 Pregnancy

Pregnancy has been shown to increase the attractiveness of women to *Anopheline* mosquitoes, but the underlying mechanisms have yet to be elucidated (Ansell et al., 2002; Himeidan et al., 2004; Lindsay et al., 2000). These authors speculated that increases in body temperature and CO_2 emissions might account for the observed increase in attraction.

1.5.1.6 Malaria infection

During the infective stage, malaria parasites increased attractiveness of asymptomatic infected individuals to *An. gambiae* mosquitoes. The authors speculate that this effect is due to changes in breath or body odor (Lacroix et al., 2005).

1.5.1.7 Odorant profiles

An early study examining attractive components of human hand washings isolated lactic acid as an important odor component in mosquito attraction to subjects. When comparing lactic acid levels amongst three study subjects who were differentially attractive, mosquito attraction seemed to scale with levels of L-lactic acid, though with such a low sample size this was not statically significant (Acree et al., 1968). After finding that differential mosquito response to humans could be largely explained by their odorants alone (Qiu et al., 2006; Schreck et al., 2002), many more groups began to investigate which volatiles were causal. By examining whole-body emanations collected from 9 subjects via

chromatography-mass spectrometry (GC-MS), five volatile compounds were identified that significantly decreased mosquito attraction: 6-methyl-5-hepten-2-one, octanal, nonanal, decanal, and geranylacetone (Logan et al., 2008). These compounds were then tested as repellents and proved somewhat effective at preventing mosquito bites (Logan et al., 2010). In a separate study, analysis of volatile profiles from highly attractive and weakly attractive subjects revealed that increased attraction was associated with odorants such as lactic acid 2-methylbutanoic acid, tetradecanoic acid, and octanal while decreased attraction was associated with higher levels of limonene, 2-phenylethanol, and 2-ethyl-1-hexanol (Verhulst et al., 2013). The results of these studies all show that volatile odorants play a critical role in mosquito attraction, though they differ as to which odorants have the biggest influence, and in what direction.

1.5.1.8 Skin microflora

Given the important role that skin bacteria play in the production of human body odor, recent work published by Verhulst et al. (2011) assessed the attractiveness of skin emanations from 48 male volunteers to *An. gambiae* mosquitoes, and examined the composition of their skin microflora. Subjects who were more attractive to mosquitoes seemed have higher levels of *Staphylococcus* spp, whereas subjects who were less attractive seemed to have higher levels of *Pseudomonas* spp. This corroborated previous reports that volatiles released by cultured *Staphylococcus epidermidis* bacteria were

appealing to malaria mosquitoes while those from *Pseudomonas aeruginosa* were unappealing (Verhulst et al., 2009; 2010a).

1.5.1.9 Genetics

HLA genes are known to cause differences in human perception of body odor (Wedekind and Füri, 1997; Wedekind et al., 1995). A recent report suggests that people carrying the HLA gene version Cw/07 may be significantly more attractive to *An. gambiae* mosquitoes, likely owing to differences in body odor profile (Verhulst et al., 2013).

1.6 Female mosquitoes require nutrients from a blood meal to reproduce and survive

1.6.1 Nutrients required for egg laying

Though female *Ae. aegypti* are able to obtain energy from plant nectars in the wild, they do not frequently do so. Instead, they appear to take frequent blood meals and use blood meal nutrients for both reproduction and the development of energy stores (Scott et al., 2000). The only essential elements required from a blood meal to produce eggs are amino acids (Dimond et al., 1956; Singh and Brown, 1957b); Ten amino acids, summarized in **Table 1**, are required for a female mosquito to mature her eggs (Dimond et al., 1956). The specific number of eggs a female mosquito is able to produce is influenced by several factors, maternal body size and nutritional condition as well as the volume and source of her blood meal (Clements, 1992b).

Table 1.1 Blood meal nutrient requirements for egg production. Table describing which nutrients obtained through a blood meal are nonessential for egg production (blue), important for egg number or survival (orange), or essential for egg production (red). (Dadd, 1985; Dimond et al., 1958; 1956; Singh and Brown, 1957a)

Amino acids	**arginine**	essential
	histidine	essential
	isoleucine	essential
	leucine	essential
	lysine	essential
	methionine	essential
	phenylalanine	essential
	threonine	essential
	tryptophan	essential
	valine	essential
	cysteine	important
	glutamic acid	important
	alanine	nonessential
	asparagine	nonessential
	aspartic acid	nonessential
	glutamine	nonessential
	glycine	nonessential
	proline	nonessential
	serine	nonessential
	tyrosine	nonessential
Vitamins		nonessential
Nucleic acid		nonessential
Sterols		nonessential
Na/K ions		important

1.6.1.1 Effects of maternal body size and nutritional reserves

In many species, maternal body size has been shown to be positively correlated with fecundity. This effect is likely due to a combination of (1) an increase in the number of ovarioles (2) greater reserves and (3) increased blood meal capacity. Large maternal body size is primarily the result of adequate larval nutrition (Blackmore and Lord, 2000; Briegel, 2003; Timmermann and Briegel, 1993). This increase in body size correlates with the development of more ovarioles, which sets the upper limit for reproductive potential (Clements, 1992b; Steinwascher, 1984). It has also been demonstrated that larger females are able to use energy from their increased maternal reserves to supplement that from an insufficient blood meal, whereas small females are not (Briegel, 1990). Finally, blood meal volume is strongly positively correlated with female size, and larger blood meals generally increase fecundity (Akoh et al., 1992; Briegel, 1990; Edman and Lynn, 1975).

1.6.1.2 Effects of blood meal size

A strong, positive correlation is known to exist between the volume of blood ingested by a female mosquito and the number of eggs she is able to lay. This relationship primarily exists for small to medium meal sizes, where most of the nutrients are diverted to egg maturation (Briegel, 1990; Jalil, 1974; Woke et al., 1956). With replete blood meals, it is thought that a female is able to mature eggs in all of the ovarioles she has available with a remaining excess of

nutrients, which she can then use to replenish maternal energy stores for survival (Briegel, 1985; Harrington et al., 2001).

1.6.1.3 Effects of blood meal source

Hosts vary greatly in the composition of their blood and therefore the quality of the meal that they provide to mosquitoes. As a result, scientists have long noted that feeding on different hosts significantly affects the female fecundity (Briegel, 1985; Chang and Judson, 1977; Harrington et al., 2001; Lea et al., 1958; Nayar and Sauerman, 1977; Phasomkusolsil et al., 2013; Spielman and Wong, 1974; Woke, 1937). In the majority of these studies, human blood was found to be the least effective for egg production as a result of its lower isoleucine content, specifically within hemoglobin (Briegel, 1985; Chang and Judson, 1977; Lea et al., 1958; Nayar and Sauerman, 1977; Spielman and Wong, 1974; Woke, 1937). However, recent evidence suggests that this effect may be attributable to the fact that all of these studies offered females sugar throughout their lifetime—a common practice in mosquito behavioral experiments (Harrington et al., 2001). When sugar is eliminated from their diet, mimicking more closely the natural behavior of *Ae. aegypti* (Costero et al., 1998; Edman et al., 1992; Scott et al., 2000), low-isoleucine human blood provided a selective advantage because it allowed for blood meal nutrients to be utilized both for egg production and synthesis of maternal energy stores. This resulted in greater survival and lifetime fecundity for the mosquito (Costero et al., 1998; Harrington et al., 2001; Naksathit and Scott, 1998).

1.6.2 Nutrients required for energy

Female mosquitoes utilize nutrients from a blood meal not only to produce eggs but also to synthesize energy reserves for survival (Briegel, 1985; Harrington et al., 2001). Lipid, glycogen and sugar are the primary forms of energy stores in the mosquito. Initial reserves are carried over from larval stages and thus can vary widely based on larval nutrition, but can be replenished following subsequent blood or sugar meals. In general, lipid stores are utilized as energy during rest while carbohydrates are utilized as energy for flight. All forms of energy—lipid, glycogen and sugar—also contribute towards egg maturation (Clements, 1992b; Foster, 1995). A recent, growing body of work suggests that for *Ae. aegypti* abstaining from sugar, feeding frequently on human blood provides an increase in these critical energy stores and is thus adaptive and advantageous (Costero et al., 1998; Harrington et al., 2001; Naksathit and Scott, 1998).

1.7 Human blood metabolome varies due to genetic and environmental factors

Metabolomics is the identification and quantification of metabolites—the dynamic end products of metabolism—in a specific biological sample. Human blood metabolomic analysis comprises low molecular weight (~50-1500 Da) molecules carried in human plasma (or serum), such as proteins and peptides, amino acids, carbohydrates, lipids, electrolytes, and waste products using GC-MS and liquid chromatography-mass spectrometry (LC-MS) methods

(Psychogios et al., 2011). Traditionally researchers have used “targeted” metabolomics, where a small panel of *a-priori*-defined metabolites is selected for detection and quantification. Increasingly an “untargeted” approach has become more common for the generation of important, unexpected insights in fields as diverse as drug discovery, disease diagnostics, the microbiome, and nutrition (Sévin et al., 2015).

A recent meta-analysis identified 4229 different metabolites in human serum and plasma samples. There was significant variability in metabolite concentrations within the human blood metabolome. Amongst healthy subjects, the average metabolite varied by +/- 50%, and many varied by as much as +/- 100% (Psychogios et al., 2011).

As is desirable for the investigation of biomarkers, between-subject variation accounts for most of the variation in metabolite concentration while within-subject variation accounts for very little. This is indicated by a high ICC (interclass correlation coefficient), which is defined as ratio of between-subject variance to total variance. An investigation of 100 subjects over the course of 4 months found the average serum metabolite to have an ICC of 0.57. This same study found that hexose, sphingolipids, glycerophospholipids, and amino acids (median ICC = 0.58, range 0.41-0.72) were metabolite classes with particularly high reliability (Floegel et al., 2011). Confirming this finding, an analysis of 159 metabolites from 20 healthy subjects, sampled after overnight fasting on three different days within a two-week period, demonstrated that there is greater

variation in metabolite concentrations between subjects than within a subject (Breier et al., 2014).

The concentration of plasma metabolites in an individual is determined by the complex interactions between genetic, environmental and physiological factors including age (Caballero et al., 1991), gender (Armstrong and Stave, 1973c; Caballero et al., 1991; Milsom et al., 1979), body mass index (BMI) (Moore et al., 2014), diet (Bergström et al., 1990; Feigin et al., 1971; McBride et al., 2007; Milsom et al., 1979), physical fatigue (Décombaz et al., 1979; Floegel et al., 2014; Rennie et al., 1981), sleep deprivation (Davies et al., 2014), circadian rhythms (Dallmann et al., 2012; Kasukawa et al., 2012; Minami et al., 2009), disease (Gowda et al., 2008; Tiziani et al., 2009), and genetics (Armstrong and Stave, 1973a; McBride et al., 2007; Paul et al., 1978).

1.7.1 The effect of genetics

There is a growing body of work suggesting that the plasma metabolome may be genetically influenced. Humans exhibit characteristic individual patterns within their amino acid profiles (Armstrong and Stave, 1973b) that remain surprisingly consistent over time (Corte and Venta, 2010; Scriver et al., 1985) and return to baseline after perturbation (Feigin et al., 1971). A twin study showed high heritability for levels of some amino acids, although the effects differed based on nutritional state {McBride:2007jd}. Genome-wide Association studies (GWAS) have suggested that 12% (Gieger et al., 2008) or even upwards of 20% (Rhee et al., 2013) of the observed variation in metabolic profiles can be

ascribed to genetic factors. A more recent GWAS identified 14 genes including 12 enzymes, a transporter, and a polycystin protein gene that significantly altered the serum metabolome in an African American population (Yu et al., 2014).

1.7.2 The effect of physiological factors

Many studies have indicated that there are diurnal variations in the plasma concentrations of various metabolites, however the details regarding which metabolites and the degree to which they vary in these reports differ (Dallmann et al., 2012; Feigin et al., 1971; 1968; Fernstrom et al., 1971; Kasukawa et al., 2012). A recent meta-analysis revealed that there are at least 37 blood metabolites that are significantly associated with BMI—among them 19 lipids and 12 amino acids (Moore et al., 2014). Likewise, changes in metabolome levels of branched chain amino acids, some fatty acids, organic acids, acylcarnitines, and phospholipids have been associated with obesity (Rauschert et al., 2014).

1.7.3 The effect of environmental factors

Components of our diet influence nutrient availability in our blood. For instance, type I diabetics require small, frequent meals to maintain their blood sugar levels. Amino acid constituents of dietary proteins make their way from the intestines to the liver and finally, into our blood streams. Thus, determining the effects of diet on metabolite levels in our blood—specifically the effects of dietary protein on plasma amino acid levels—is a line of investigation that has been open for over 50 years. Surprisingly, still, not much is known about how changes

in the diet affect the blood metabolome (Gibney et al., 2005). In a twin study, it was found that the heritability of amino acid profiles differed between fasting and non-fasting conditions, indicating a significant role of diet in shaping the metabolome (McBride et al., 2007). Several studies have found that changes in dietary protein intake can significantly alter plasma levels of certain amino acids (Adibi, 1968; Bergström et al., 1990; Fernstrom et al., 1971; Maher et al., 1984; Milsom et al., 1979; Nasset et al., 1979) though one study found no effect of dietary protein changes (Feigin et al., 1971). Though the research has been more limited, there are some reports of physical fatigue (Décombaz et al., 1979; Rennie et al., 1981; Swendseid et al., 1966) and cardiovascular health (Floegel et al., 2014) having effects on plasma amino acids. Floegel et al (2014) found that amino acid levels increased with increasing cardiovascular fitness (Floegel et al., 2014). However it appears that during times of physical fatigue or exhaustion, plasma amino acid levels may be lowered from baseline (Décombaz et al., 1979; Kingsbury et al., 1998; Rennie et al., 1981).

1.8 Our approach to studying the factors affecting differential mosquito attraction

A satisfactory understanding of differential attraction of human subjects to mosquitoes has not yet been reached, in part because the phenotype emerges from multiple complex cues emitted by humans that are each interpreted, weighed, and integrated by the mosquito, creating a multiplex behavior that is difficult to disentangle. However, another contributing factor is the prevalence of

studies within the field that use questionable methods and have small sample sizes.

In this dissertation, we take advantage of the diverse, densely populated New York City region to recruit 150 study volunteers from all three of the most prevalent racial groups to participate in an investigation of cues that drive mosquito preference for certain human hosts over others. We maximize natural human variability in our study population to survey a more diverse set of factors potentially linked to mosquito attraction, in an effort to understand which emerge as the most important contributors. In addition to collecting information pertinent to weighing in on the existing body of literature surrounding what factors influence differential attraction (**Figure 1.4**), we also gather data that allows us to assess hypotheses that have thus far remained completely unaddressed—namely, the role of the blood metabolome in influencing mosquito attraction to different hosts.

Given the importance of host blood composition to the reproductive fitness of the mosquito and the significant natural variation within human blood metabolome profiles, we investigate the idea that some human hosts may offer more nutritive blood meals for the female mosquito than others. If female mosquitoes are able to capitalize on these differences by detecting them via volatile chemical cues and choosing the most nutrient-rich meal, they could enjoy an important adaptive advantage. Here, we describe our investigation into how blood metabolome components may influence differential mosquito attraction.

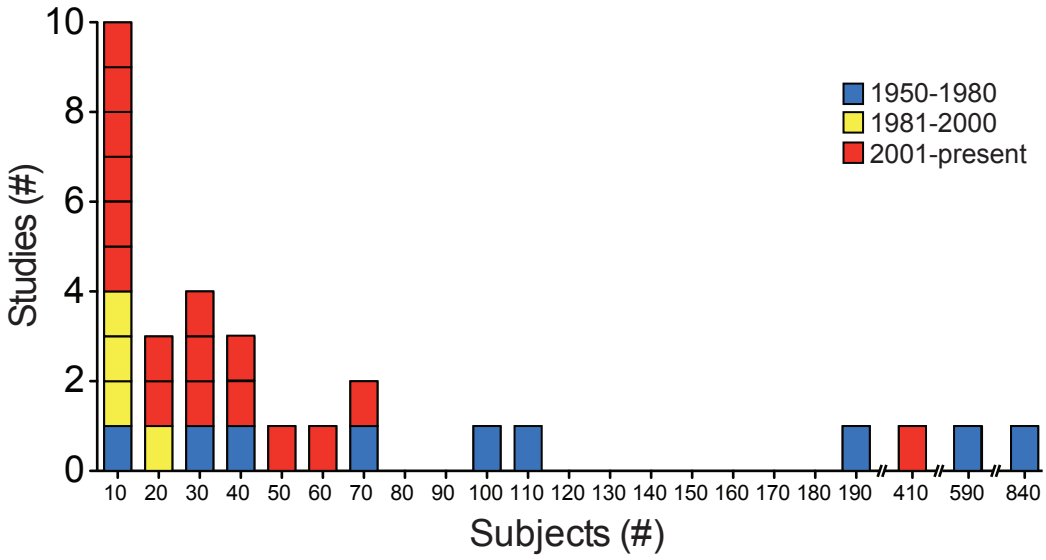


Figure 1.4 Sample size distribution of previous studies. Histogram depicting sample size distribution of previous studies investigating differential mosquito attraction to human subjects. Most studies recruited fewer than 50 subjects, and those involving more than 50 subjects were primarily conducted before the 1980s, using methods that are now outdated.

CHAPTER 2: UNIPORT OLFACTOMETER ESTABLISHED AS A METHOD TO MEASURE DIFFERENTIAL MOSQUITO ATTRACTION

2.1 Developing the uniport olfactometer assay

To begin our investigation of differential mosquito attraction to human hosts, we needed to establish a stable, reliable assay to measure mosquito attraction to volunteers. In the literature, typical assays to quantify differences in mosquito attraction to subjects range from semi-field enclosed huts connected by ventilation systems (Lacroix et al., 2005; Mukabana et al., 2002; 2004) to laboratory-based Plexiglas olfactometers (Brouwer, 1959; 1960; Geier et al., 2002; Gilbert et al., 1966; Logan et al., 2008; Qiu et al., 2006; Verhulst et al., 2011). Host stimuli range from an entire volunteer with all associated multimodal sensory cues (Himeidan et al., 2004; Knols et al., 1995; Lacroix et al., 2005; Lindsay et al., 1993; 2000; Logan et al., 2008; Mukabana et al., 2002; 2004) to odor from a specific body part collected on a glass substrate (Geier et al., 2002; Logan et al., 2008; Qiu et al., 2006; Schreck et al., 1982; Verhulst et al., 2011) (see Introduction section 1.5).

In this work, we chose to develop the uniport olfactometer as a method for assessing mosquito attraction to volunteers (**Figure 2.1 a**). This assay was adapted from previously published designs (Klowden and Lea, 1979) to accommodate the forearm of a volunteer as an odor source (Liesch et al., 2013). Using a live host as the stimulus introduces variability due to the influence of skin

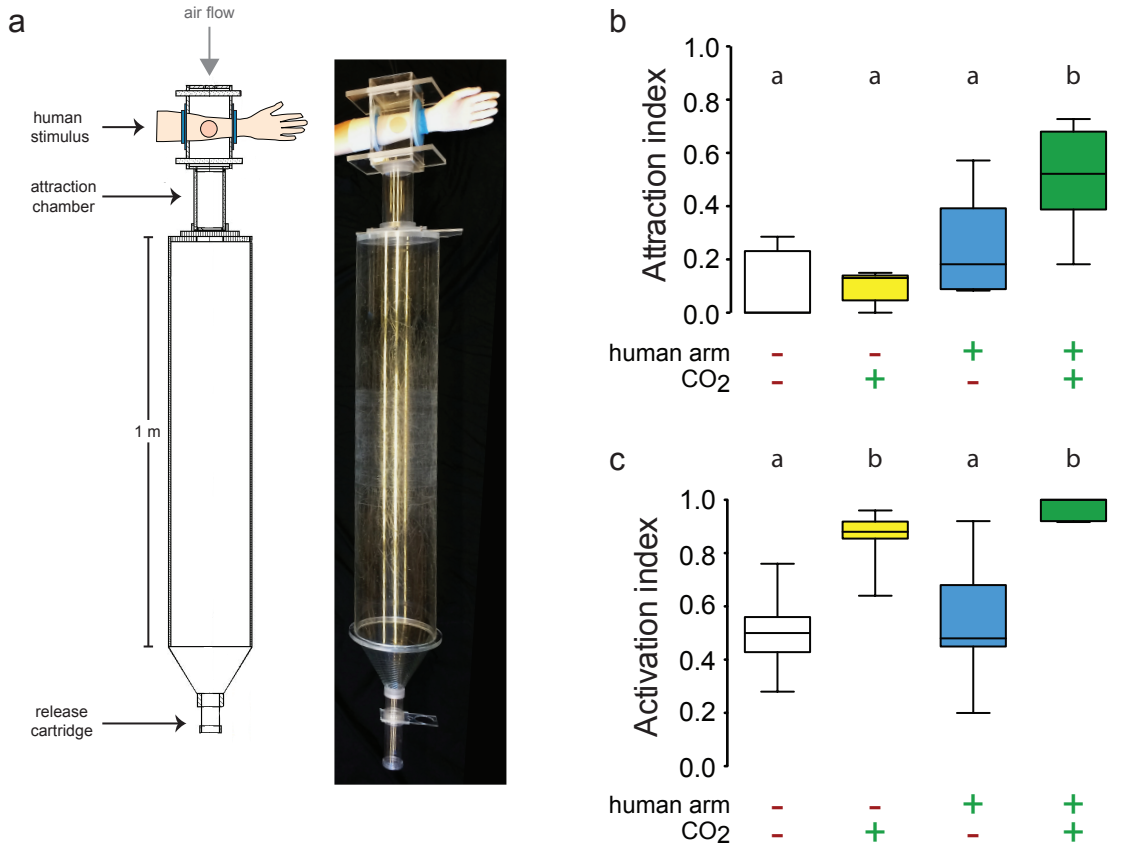


Figure 2.1 Uniport olfactometer schematic and controls. The uniport olfactometer assay measures mosquito attraction to odors arising from the forearm of different subjects. **(a)** Schematic (left) and photo (right) of the uniport olfactometer. **(b-c)** Mosquito attraction **(b)** and activation **(c)** as measured in the uniport, n=9 for each condition. Data in **b** and **c** are presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, 10-90%; outliers, black dots). Statistical comparisons made using ANOVA with post hoc Tukey's HSD test. Letters indicate statistically significant differences between groups at the level of $p<0.05$

humans on nylon stockings (McBride et al., 2014) or glass beads (Verhulst et al., 2011), which may collect only a subset of all active odorants. We controlled for the effects of skin temperature on attraction by measuring skin surface temperature immediately following every trial in the uniport. We controlled for the effects of skin surface area by covering subject forearms in nitrile gloves and exposing only a 12.56 cm^2 area of skin from which odorants could enter the assay. In the uniport assay, the extent of mosquito attraction to a stimulus is described as an attraction index, defined as the number of mosquitoes in the attraction chamber at the end of a trial divided by the total number of mosquitoes that left the release point. For the same trial, an activation index can be calculated to describe the extent of general mosquito activity and movement during the trail. Activation index is determined by dividing the number of mosquitoes that have left the release cartridge by the total number of mosquitoes in the trial.

In control experiments, we tested the attraction of mosquitoes to host-related stimuli in the presence and absence of CO_2 in the uniport olfactometer (**Figure 2.1 b,c**). Consistent with the role of CO_2 as a potent activator of mosquito attraction (Gillies, 1980), activation indices in trials where CO_2 was added to the airstream were significantly higher than those where CO_2 was not added (**Figure 2.1 c**). Mosquitoes showed minimal attraction to filtered ambient air alone, or to air with CO_2 or a host stimulus alone. However, when a host stimulus was combined with CO_2 at the same concentration as in exhaled human breath (approximately 4%), they synergistically combined to produce robust

attraction (**Figure 2.1 b**). Increasing the surface area of skin exposed in the uniport should increase the quantity of volatile odorants released to the mosquitoes, thus increasing their attraction to the stimulus. We assessed this by testing three volunteers with increasing area of skin exposed (**Figure 2.2 a**). For two of the three subjects, there were significant differences in attraction between conditions, with the smallest surface area attracting the fewest mosquitoes and the largest surface attracting the most mosquitoes. (**Figure 2.2 b**). To take advantage of the full dynamic range of mosquito attraction and minimize the potential for floor or ceiling effects, we decided to use a 12.56 cm^2 area of exposed skin, which produced intermediate levels of attraction for the control subjects (**Figure 2.2 b**). When we compared mosquito attraction to the left and right forearms of three different subjects, we found that mosquitoes were equally attracted to both the left and right arms of a given subject (**Figure 2.2 c,d**).

In natural settings, mosquitoes often encounter several potential human hosts in the same local area, and they must then make a choice of whom to bite. We investigated whether our individual measurements of attraction and subsequent rankings of subject attraction could predict the outcome of pairwise comparisons of subject attraction. First, we measured mosquito attraction to the whole arm (**Figure 2.3 a**) and a standardized area of skin (**Figure 2.3 b**) for three human volunteers individually using the uniport olfactometer. Two of the subjects, LVO-652-010 and LVO-652-006, differed significantly in their appeal to mosquitoes in both experiments, whereas LVO-652-013 exhibited an intermediate attraction index between the two (**Figure 2.3 a,b**). The ranking of

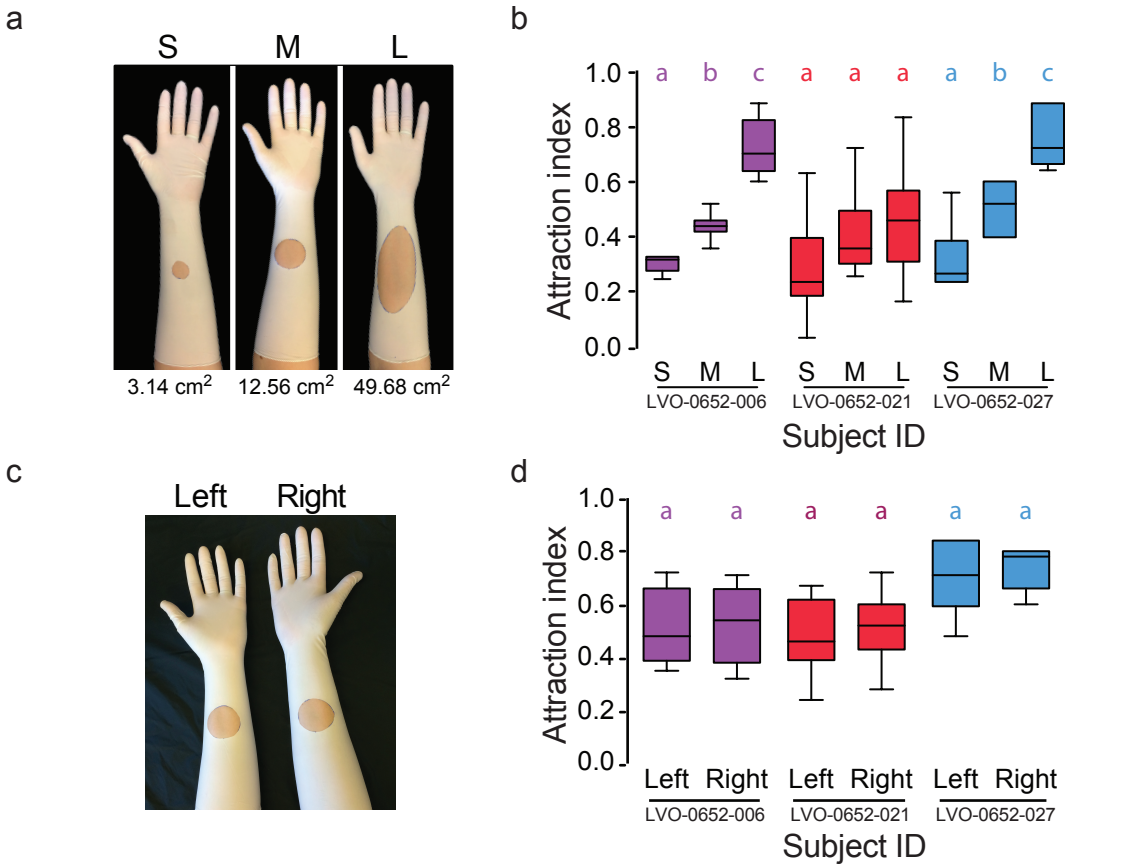


Figure 2.2 Mosquito attraction to forearms is bilaterally symmetric and increases with increasing surface area of exposed skin. (a) Photo depicting stimuli used in comparison of mosquito attraction to the same subject with different surface areas of skin exposed: small (S, 3.14 cm^2), medium (M, 12.56 cm^2), and large (L, 49.68 cm^2). **(b)** Attraction indices elicited from mosquitoes by small (S), medium (M), and large (L) areas of exposed skin, n=6 for each condition. **(c)** Photo depicting stimuli used in comparison of mosquito attraction to a 12.56 cm^2 patch of skin on the left and right forearms of the same subject. **(d)** Attraction indices elicited from mosquitoes by the left and right forearms of subjects as shown in (c), n=6 for each condition. Data in **b** and **d** are presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, 10-90%; outliers, black dots). Statistical comparisons made using ANOVA with post hoc Tukey's HSD test. Letters indicate statistically significant differences between groups at the level of $p<0.05$

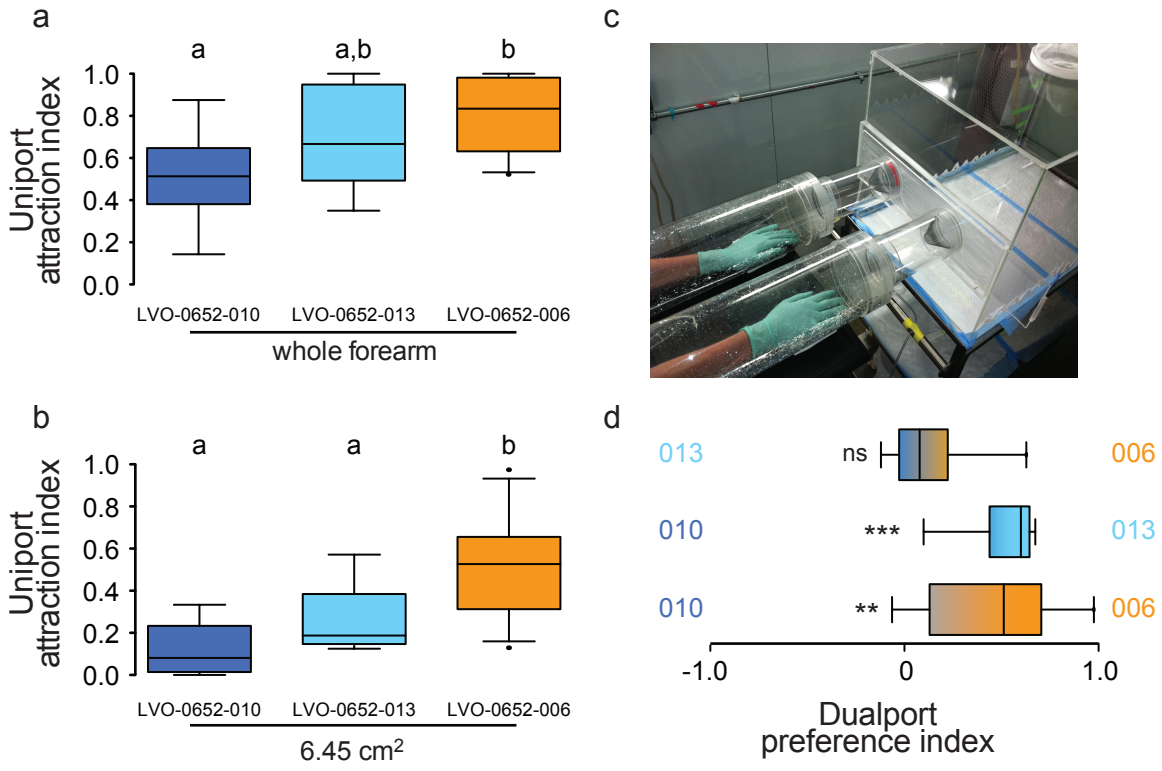


Figure 2.3 Uniport attraction rankings predict pairwise comparison outcomes. (a) Mosquito attraction to the whole forearms of three subjects measured in the uniport olfactometer, n=8 for LVO-0652-010, n=9 for LVO-0652-013, n=18 for LVO-0652-006. **(b)** Mosquito attraction to a 6.45 cm^2 patch of skin exposed on the arms of the same three subjects. n=8 for LVO-0652-010, n=7 for LVO-0652-013, n=13 for LVO-0652-006. **(c)** Photo of the arms of two human volunteers being compared in a dualport olfactometer trial **(d)** Preference index for pairwise comparisons of mosquito attraction to the whole forearms of the same three subjects in the dualport olfactometer, n=9 for each comparison. Data in **a**, **b**, and **d** are presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, 10-90%; outliers, black dots). Statistical comparisons in **a** and **b** made using ANOVA with post hoc Tukey's HSD test. Letters indicate statistically significant differences between groups at the level of $p<0.05$. Statistical analysis in **d** made by one-way student's T-test, testing if mean is significantly different from zero. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

subjects based on the individual uniport measurements was consistent between the two experiments, where 010 < 013 < 006. Then we tested these same three subjects in a pairwise fashion using a dualport olfactometer (**Figure 2.3 c**). We found that subjects 006 and 013 were more attractive than subject 010 in pairwise comparisons, as would have been predicted based on uniport measurements. Statistically, subject 013 and 006 were indistinguishable in pairwise comparisons (**Figure 2.3 d**). These data suggest that ranking individually acquired subject attraction indices do approximate outcomes of pairwise comparison tests between subjects.

2.2 Lactic acid established as a daily control for mosquito behavior

To compare the attraction of a large number of volunteers tested on different days, we needed to establish a daily uniport control to capture variation in mosquito behavioral response due to differences in rearing, changes in room conditions, and mosquito age. It has previously been shown that L-(+)-lactic acid, a monomolecular volatile odorant present in human body emanations and breath, is attractive to *Ae. aegypti* mosquitoes when presented in combination with carbon dioxide (Acree et al., 1968; Eiras and Jepson, 1991). Based on these reports, we tested the attraction of mosquitoes to 100% L-(+)-lactic acid in the uniport olfactometer to evaluate its use as a behavioral control. Mosquitoes were significantly more attracted to L-(+)-lactic acid presented in a 3.14 cm^2 dish (1 mL at 88-92% concentration) than they were to an equivalent volume of water presented in same size dish, and significantly less attracted to lactic acid than to

a human subject (**Figure 2.4**). As would be expected for a monomolecular stimulus, mosquito attraction to lactic acid was less variable than attraction to the arm of a human volunteer (**Figure 2.4**).

2.3 Pilot study validates uniport method for determining subject attraction

We then conducted a pilot study to (a) determine if the uniport assay could successfully discriminate a larger number of subjects into groups based on attraction and (b) investigate the within-subject and between-subject variability of mosquito attraction. Each subject was screened for eligibility in the study before being enrolled and scheduled, and they were recruited to reflect the demographics of New York City, as measured in the 2010 census (**Figure 2.5**). Subjects were told to follow specific instructions regarding showering and use of personal care products prior to each of their study visits in an attempt to standardize time for body odor accumulation between subjects. During each visit, subjects participated in eight olfactometer measurements. Twenty-one volunteers participated in this study, 3 of whom completed two visits and 18 of whom completed three visits (**Figure 2.6 a**). Given that we had collected multiple visits from the same subjects, we were able to estimate the between-visit variability for a subject (15.7%) and the variability between visits for different subjects (17.6%). The remaining 66.7% of variability was within-subject during a visit. Despite the

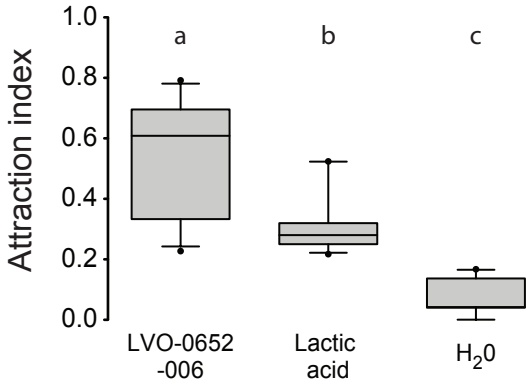


Figure 2.4 Lactic acid elicits modest levels of attraction in *Aedes aegypti*. Lactic acid, a monomolecular compound found in human sweat, produces modest levels of attraction in the uniport olfactometer. Attraction indices for a human subject (LVO-0652-006), lactic acid, and H_2O, n=11 for each condition. Data are presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, 10-90%; outliers, black dots). Statistical comparisons made using ANOVA with Tukey's HSD post-test. Letters indicate statistically significant differences between groups at the level of $p<0.05$

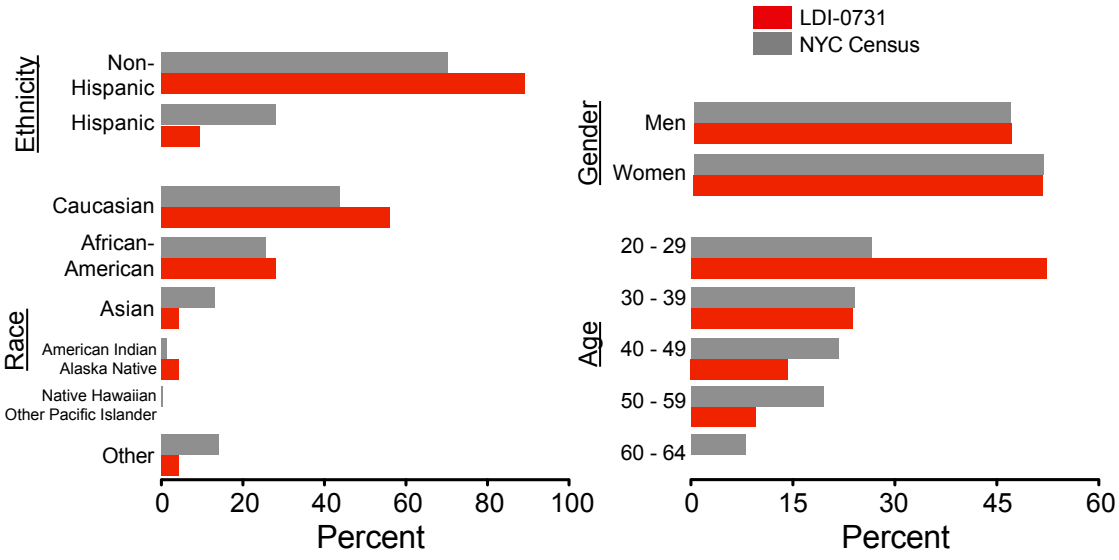


Figure 2.5. Pilot study LDI-0731 demographics. Demographic profile of subjects participating in the mosquito pilot study compared to the 2010 New York City census (n=21 subjects).

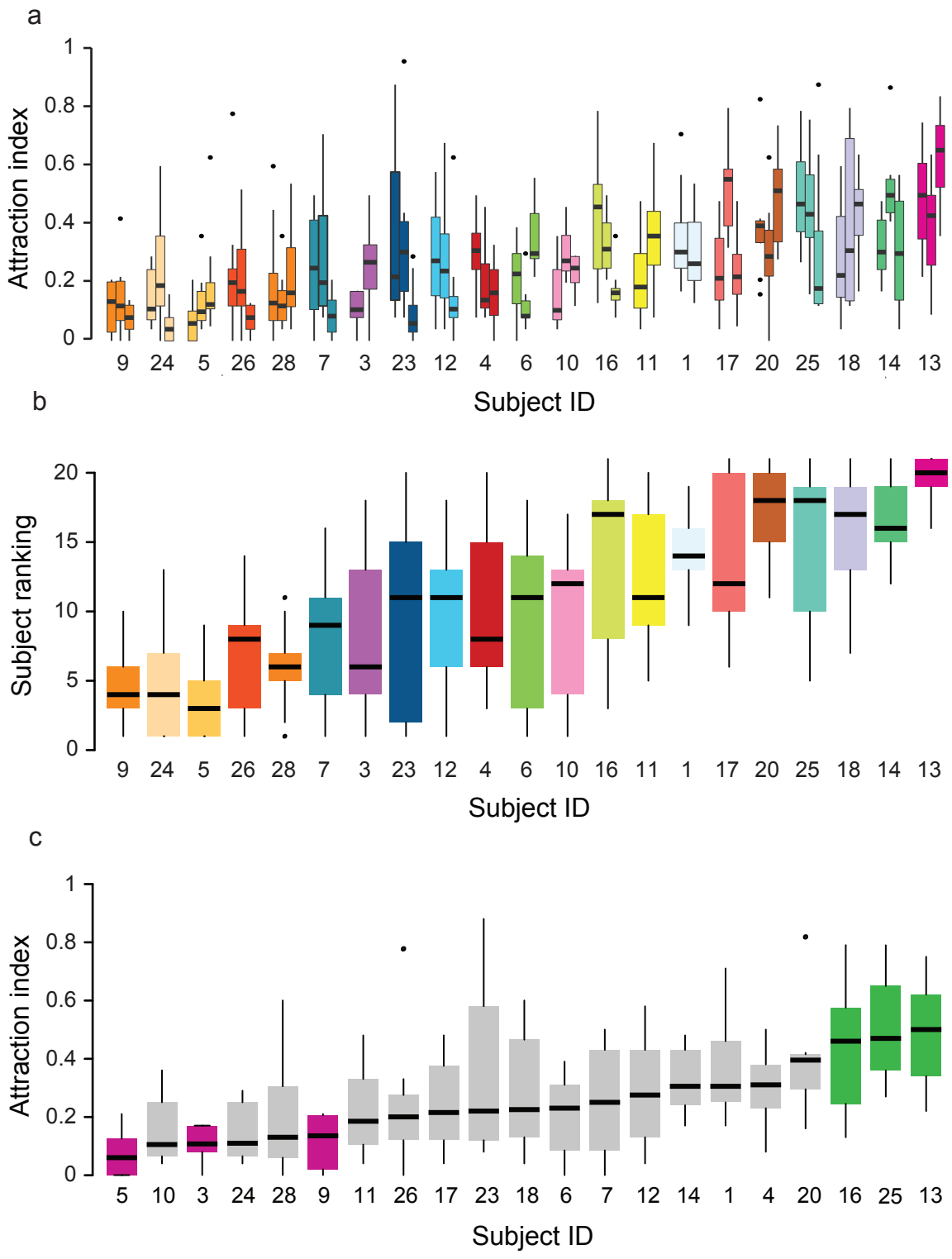

a
Attraction index
1
0.8
0.6
0.4
0.2
0
9 24 5 26 28 7 3 23 12 4 6 10 16 11 1 17 20 25 18 14 13
Subject ID
b
Subject ranking
20
15
10
5
0
9 24 5 26 28 7 3 23 12 4 6 10 16 11 1 17 20 25 18 14 13
Subject ID
c
Attraction index
1
0.8
0.6
0.4
0.2
0
5 10 3 24 28 9 11 26 17 23 18 6 7 12 14 1 4 20 16 25 13
Subject ID

Figure 2.6. Pilot study LDI-0731 subject attraction. (a) Boxplots of pilot study subject attraction index by visit, subjects arranged from left to right by ranking median of all visits combined (n=3 visits for all subjects except 1, 3, and 11 where n=2 visits. n=7-8 trials within each visit). **(b)** Boxplot of subject rankings following n=1000 simulations wherein one visit was pulled at random from each subject, then subjects were ranked by median attraction from that visit. Subjects arranged from left to right based on median attraction rankings as in (a) **(c)** Box plot of attraction index for visit 1 from all subjects, arranged by median attraction for that visit (n=7-8 trials per subject). Statistical comparisons made using ANOVA with Tukey's HSD post-test. Colors indicate statistically significant differences between magenta and green groups at the level of $p<0.05$ Data in **a** - **c** are presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, max and min; outliers, black dots).

relatively high within-subject variability, as is common in mosquito attraction studies, we were able to discriminate between subjects who were differentially attractive to mosquitoes even with only a single visit (**Figure 2.6 c**). Because we were designing a main study where subjects would participate in a single study visit, we wanted to test the stability of subject rankings after a single visit. When we selected at random one visit from each subject and ranked them based on the median of that visit and repeated this 1000 times, we found that subject attraction rankings remained relatively consistent (**Figure 2.6 b**), giving us confidence that uniport attraction is a stable trait.

2.4 Concluding remarks

These experiments validated the uniport olfactometer as a reliable assay to measure mosquito attraction to subjects. In control experiments, mosquitoes were activated by an increase in CO_2 alone and were attracted to a host when presented along with CO_2. Host attraction measurements were bilaterally consistent and dependent on the surface area of skin exposed. Lactic acid, a host-related odorant known to be attractive to *Ae. aegypti*, elicited modest and relatively stable attraction, and was therefore established as a daily behavioral control. Finally, in a pilot study of 21 subjects, each tested on three separate days, we confirmed that the uniport can segregate subjects based on differences in attraction index, despite relatively high within-subject variability. This high within-subject variability is to be expected given the complex nature of this, and other, studies of mosquito attraction to different humans, which are subject to

variation due to both mosquito behavior as well as human behavior. Possible contributors are factors such as changes in the behavioral room temperature and humidity, the accumulation of human odorants in the testing room through the day, or even the circadian effects of mosquito activity. It is also possible that human body odor profiles change over the course of the 3 hour visit, due to fasting. Volunteer body temperature or sweatiness may be influenced by repeated transitions between the hot, humid air in the behavioral testing room and the cool, dry air in the waiting room. Finally, we decided on a sample size of n=25 mosquitoes per trial, to avoid crowding within the uniport attraction chamber and due to constraints on rearing volume. This relatively small sample size per trial means that just a few mosquitoes can significantly alter the calculated attraction index. To overcome these effects, we collected 8 measurements per subject, which allowed us to more precisely narrow in on a given subject's "true" attraction index.

Despite the considerable variability in attraction, when we randomly selected one visit from each subject and used the median attraction from that visit to rank subjects from 1 to 21 one thousand separate times, we found that subject rankings remained relatively stable. This gave us confidence that though we were inviting subjects for only one visit in the main study, we would still be able to capture their relative rankings.

CHAPTER 3: DEVELOPMENT OF A TIME SERIES MODEL TO NORMALIZE SUBJECT ATTRACTION DATA

The conclusions of the studies in this thesis critically depend on the attraction data collected using the uniport olfactometer. It was not feasible to collect all of these human data within a short experimental time period, and behavioral data are inherently noisy, so we needed a method to normalize these data across all possible variables that are unlikely to impact mosquito attraction. This chapter describes the statistical model we developed to analyze all the mosquito behavior data collected in the main study LBE-0810.

Before conducting downstream analyses and interpreting subsequent results, we determined how best to make use of the parameters we collected to normalize the attraction data and allow for comparison between subjects across the course of the study. For the main mosquito study LBE-0810, volunteer attraction data were collected over the course of one calendar year. There were a variety of factors to consider, the most important among them being variation across the time of year data were collected, fluctuations in external weather conditions, fluctuations in internal room conditions, subject arm temperature, the age of the mosquitoes used for testing, and finally variation in mosquito response to our control compound, lactic acid. We hypothesized that some or all of these factors contributed experimental noise to our data.

In a preliminary analysis using a linear mixed-model to examine effects of time and period on subject attraction, with random effect of subject, we found that

subject data also showed a negative time-dependency (β=-0.019, SE=0.002, p<0.001), where attraction measured during the first trial was on average 10% higher than attraction measured during the last trial (**Figure 3.1**). Subject measurements did not differ significantly by period (in other words, between morning and afternoon sessions) (β=-0.042, SE=0.034, p=0.212).

3.1 The confounding effect of time

When we began to analyze raw subject attraction data, we also found that the month a subject was tested significantly affected their attraction index. Using a linear mixed-effects model (LME) with subject as a random intercept, we found an effect of visit month on raw subject attraction (ANOVA following LME for raw attraction by month p<0.0001) (**Figure 3.2**). Because the goal of our work was not to investigate the influence of the time of year on subject attraction, we sought to remove this effect from the data prior to further analysis.

3.2 ARIMA modeling of time series to describe the effects of time on attraction data

In order to remove the variability in attraction data that was due to time-dependency, broadly defined as month of testing, we built a model to describe the effects of time on attraction. Though the subject attraction measurements were not equally spaced over time, as is usually the case for a time series analysis, we expected that any given observation was more likely to be correlated with nearby observations than distant ones, so we treated the data as

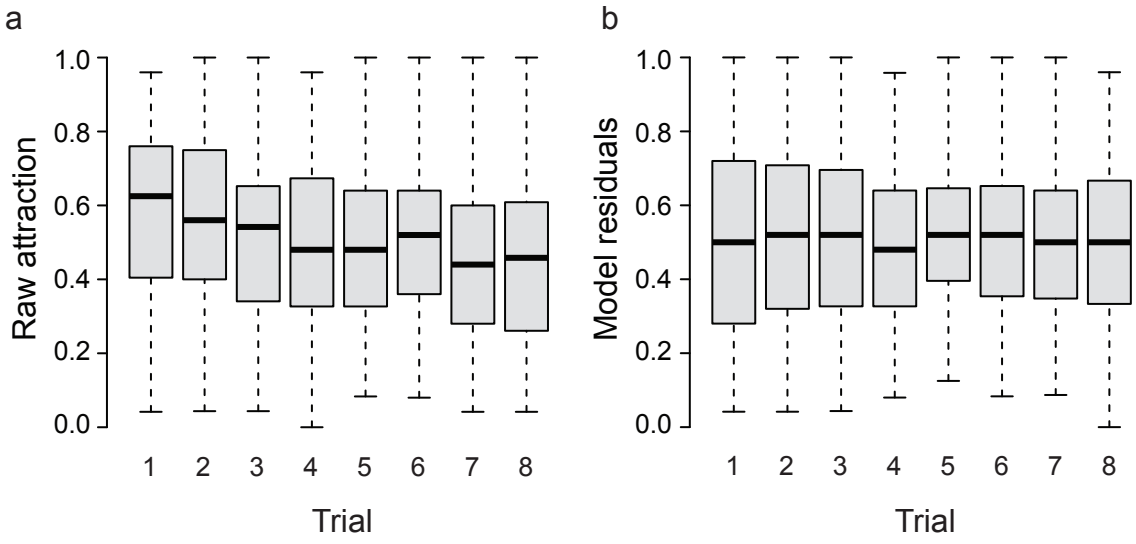


Figure 3.1 Raw attraction and model residuals by trial. (a) Raw attraction by trial across the course of the study ANOVA following a linear mixed-effects model of raw attraction by trial, with random intercept of subject, significant effect of trial $p<0.0001$ **(b)** Residuals following application of time series model by trial across the course of the study. ANOVA following linear mixed-effects model of raw attraction by trial, with random intercept of subject, no significant effect of trial $p=0.984$. Data in **a** and **b** are presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, max and min; outliers, black dots).

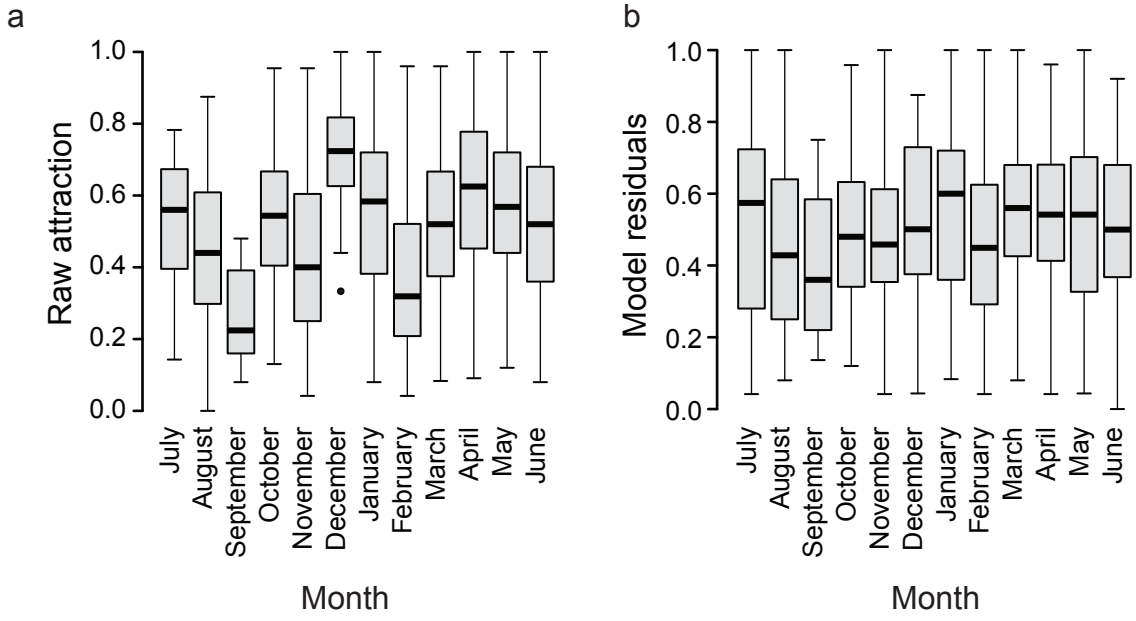


Figure 3.2 Raw attraction and model residuals by month. (a) Raw attraction by month across the course of the study. ANOVA following linear mixed-effects model of raw attraction by month, with random intercept of subject, significant effect of month $p<0.0001$ **(b)** Residuals following application of time series model by month across the course of the study. ANOVA following linear mixed-effects model of normalized residuals by month, with random intercept of subject ID, effect of month not significant $p=0.103$. Data in **a** and **b** are presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, max and min; outliers, black dots).

a time series in subsequent analyses. When we plotted raw subject attraction data over the course of the study as a time series, we noticed that there was visual evidence of a periodicity in the pattern of attraction measurements (**Figure 3.3 a**). The autocorrelation plot (**Figure 3.3 b**) and partial autocorrelation plot (**Figure 3.3 c**) for this series revealed that that the cyclical pattern that we observed by eye was detectable quantitatively. The series was stationary, and could be described by an ARMA (AutoRegressive Moving Average) process (Box et al., 2008). This process describes the dynamics of a time series as a weighted average of past values following the principle of parsimony, i.e. to mimic the time series evolution by the simplest model. The autoregressive term describes the extent to which a finite set of past measurements can predict the present observation. The moving average term addresses the extent to which a moving average of past data, with decreasing weights, is able to predict a present measurement. In our series of subject attraction data there is evidence of one autoregressive (AR(1)) and a moving average (MA(1)), meaning that a particular measurement can be predicted by a weighted average of past measurements but using a parsimonious representation with only two parameters. We used maximum likelihood methods to estimate the correct coefficients for these two terms (ar1=0.85, ma1=-0.43).

We also wanted to determine which known exogenous factors were significantly correlated with attraction, so that they could be included as regressors in the time series model (**Figure 3.4 b-g**). To assess cross-correlation differences for all time series, because not all of them were stationary. After this

transformation, we then correlated attraction with all possible explanatory variables. In the case of lactic acid, we did not have measurements collected in parallel with subject measurements—instead, we had measurements collected before and after subject trials. We therefore used the Kalman Filter to estimate values for lactic acid by exploring dynamics of bivariate time series of human and lactic acid, and using a state space model that included local level as well as cycle components (**Figure 3.5**). Weather data were downloaded from the Central Park Weather Station and time-matched with each human attraction trial. All exogenous factors with significant instantaneous correlation as measured by Spearman coefficient were retained to be included in the final model ($r_s>0.05$). This included environmental behavior room humidity, external humidity, mosquito age, and lactic acid attraction (**Figure 3.4 b-g**); the results of these tests are summarized in **Table 3.1**. Finally, we included a cycle of length 8 in the model, equivalent to the number of measurements taken for each subject. This cycle accounts for regularity at each set of 8 experiments. To incorporate all of these components at once, we chose a state space representation that includes an equation for the dynamics of attraction and other for an unobservable state that evolves as a AR(1) process.

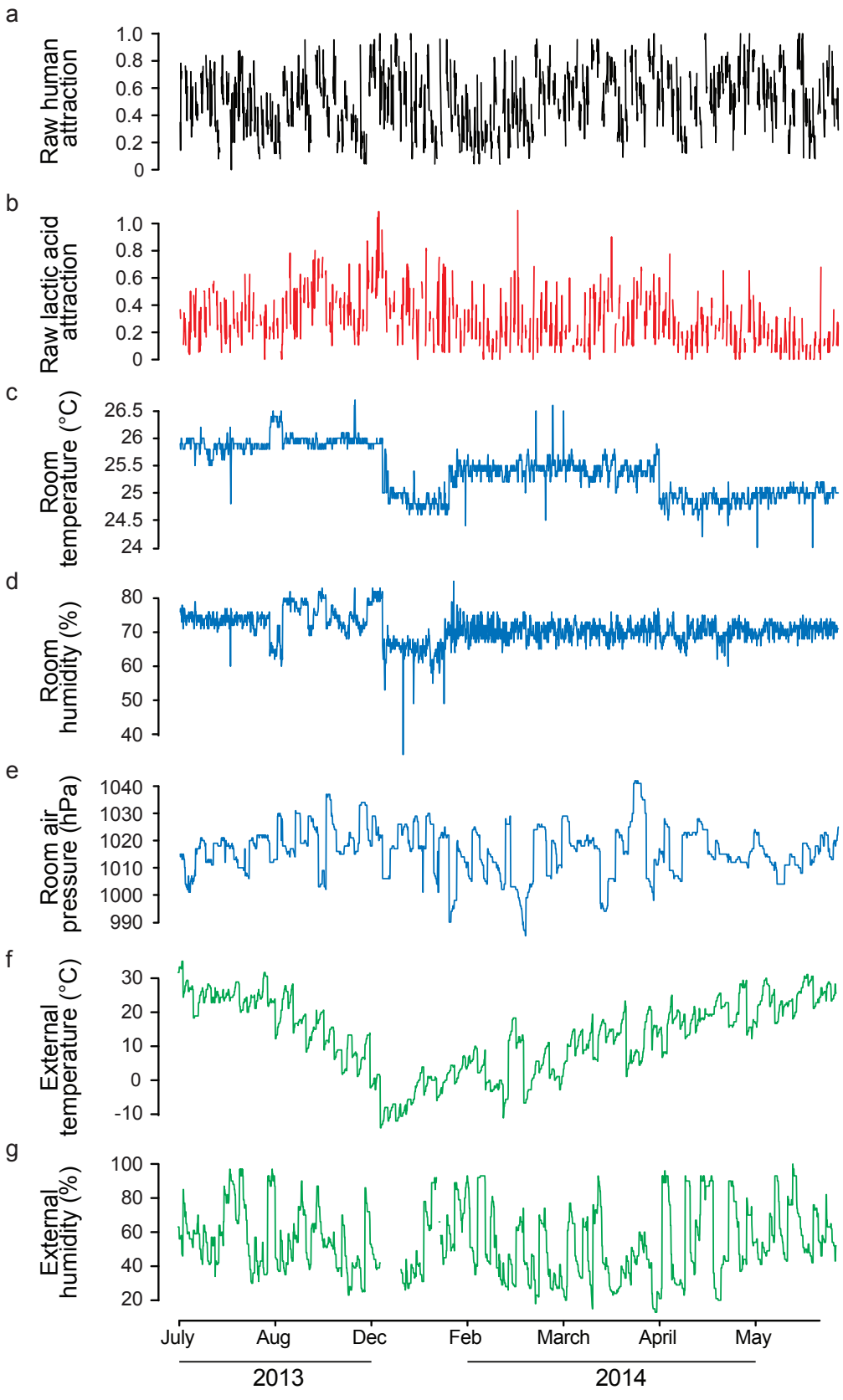

a
Raw human attraction
1.0
0.8
0.6
0.4
0.2
0
b
Raw lactic acid attraction
1.0
0.8
0.6
0.4
0.2
0
c
Room temperature (°C)
26.5
26
25.5
25
24.5
24
d
Room humidity (%)
80
70
60
50
40
e
Room air pressure (hPa)
1040
1030
1020
1010
1000
990
f
External temperature (°C)
30
20
10
0
-10
g
External humidity (%)
100
80
60
40
20
July
Aug
Dec
Feb
March
April
May
2013
2014

Figure 3.4 Time series plots of subject attraction and variables possibly contributing to time-dependency across the study
Time series plots of **(a)** Raw subject attraction **(b)** Raw lactic acid attraction **(c)** Behavioral room temperature **(d)** Behavioral room humidity **(e)** Behavioral room air pressure **(f)** External temperature and **(g)** External humidity across the course of the study

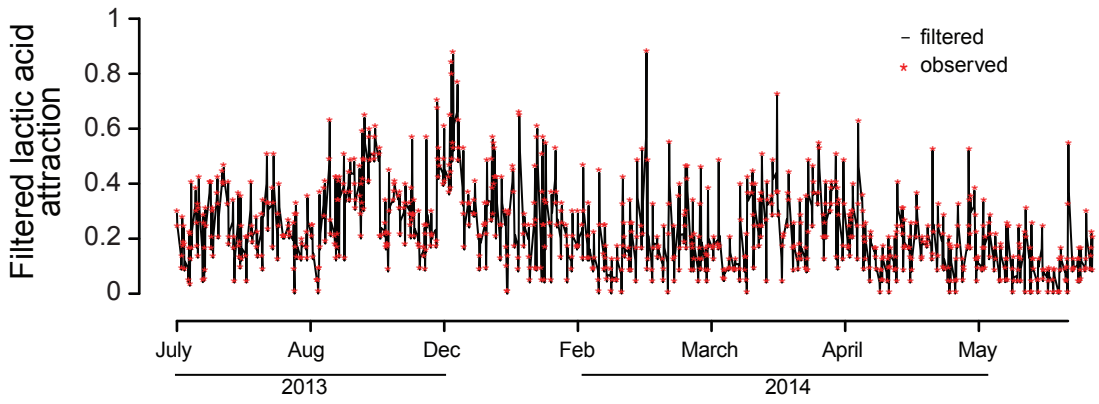


Figure 3.5. Time series plot of filtered lactic acid attraction across the study. Kalman filter estimates of lactic acid attraction across the course of the study (black) and observed lactic acid attraction (red)

Table 3.1. Correlations between possible regressors and raw subject attraction. Nonparametric (Spearman) correlation coefficients for correlation between differentiated time series of possible model regressors and differentiated time series of raw subject attraction. Correlations above 0.05 are highlighted in red: those variables were selected for inclusion as regressors in the time series model.

Variable	Correlation
Lactic acid attraction	0.083
Behavioral room temperature	0.016
Behavioral room humidity	0.052
External temperature	-0.016
External humidity	0.11
Mosquito age	0.085
Subject arm temperature	-0.003

Equation of observations: $y_t = Z\alpha_t + X_t\beta_t + \varepsilon_t$

Equation of state: $\alpha_t = T_t\alpha_{t-1} + \upsilon_t$

y_t : raw attraction

α_t : State vector that includes a term for local trend and cycle component

X_t : matrix of predictors that included : AR(1) and MA(1), external and internal weather conditions.

β_t : Regression coefficients for the matrix of predictors

ε_t : Attraction random variation

υ_t : State random variation

We fit this model to our subject attraction time series and removed its effects on the data by then working with the residuals, which were rescaled to the quantiles of the raw attraction measurements. Afterwards, we tested for autocorrelation in these normalized model residuals. We found that the cycling seen within the initial subject attraction data had indeed been removed (**Figure 3.3 d-f**). When we fit a linear mixed-effects model to this normalized attraction measurement using subject as the random intercept, we found that the effect of visit month was no longer significant (ANOVA following LME for model residuals by month p=0.103) (**Figure 3.2 b**). The effect of trial had also been removed (ANOVA following LME for model residuals by time p=0. 984) (**Figure 3.1 b**).

Application of this model removes inter-subject variability due to time-dependency and leaves inter-subject variability not due to time-dependency. For

the remainder of this thesis, we will work with the residuals from this normalization scheme, rescaled to the original quantiles of the raw attraction data, which we term “normalized attraction.” Normalized subject attraction data and raw subject attraction data correlate well (rs=0.72) (**Figure 3.6 a**) as do subject median attraction measurements (rs=0.75) (**Figure 3.6 b**).

3.3 Concluding remarks

Due to the confounding effects of time-dependency on raw subject attraction measurements, we were unable to use raw mosquito behavior data to compare subjects tested in different months. This variation in attraction across time may be partially attributable to seasonal variation in external weather conditions, which could affect either the human subjects or mosquitoes or both. For instance, subject body odor may differ in warm vs cold seasons, perhaps due to differences in the amount they sweat in different seasons. Mosquitoes may also have a biological rhythm that continues to cycle despite environmentally-stable rearing conditions, which could affect their behavior during different months. Whatever the underlying cause, the variation in attraction due to this time-dependency obscured variation due to the interesting biological phenomena that we wanted to study. We therefore removed the effects of time using a time series model, and were able to thereafter work with residuals from that model, which represented subject attraction after removal of variation due to time. With these normalized data in hand, we were now prepared to analyze correlations of attraction with other

CHAPTER 4: MOSQUITO MAIN STUDY FINDS CLUSTERS OF DIFFERENTIALLY ATTRACTIVE SUBJECTS

4.1 Study design

Having established the uniport olfactometer as a reliable tool for discriminating subjects based on mosquito attraction, we designed and conducted a large, 150-subject main study. We recruited healthy volunteers to best match the demographic composition of the New York City population (**Figure 4.1**). Volunteers were screened for eligibility before enrollment (see Materials and Methods: LBE-0810 volunteers) and each completed a screening questionnaire asking them to provide information about (1) their demographics, (2) lifestyle factors such as diet and exercise, and (3) self-reported experiences regarding their perceived attractiveness to mosquitoes as well as their perceived reaction to mosquito bites (**Appendix E**). Each subject was invited for one visit, prior to which they were required to follow personal care instructions specifying showering procedures, prohibiting scented care products, and instructing them to avoid exercise and the handling of certain pungent foods (**Appendix D**). Compliance was assessed with an electronic questionnaire on the day of their visit (**Appendix F**). If a subject met the requirements stipulated, 8 uniport olfactometer trials were conducted. The first twenty-one subjects also participated in a free-feeding assay, in which they remained immobile while 25 mosquitoes took a full blood meal from their right arm. These mosquitoes were

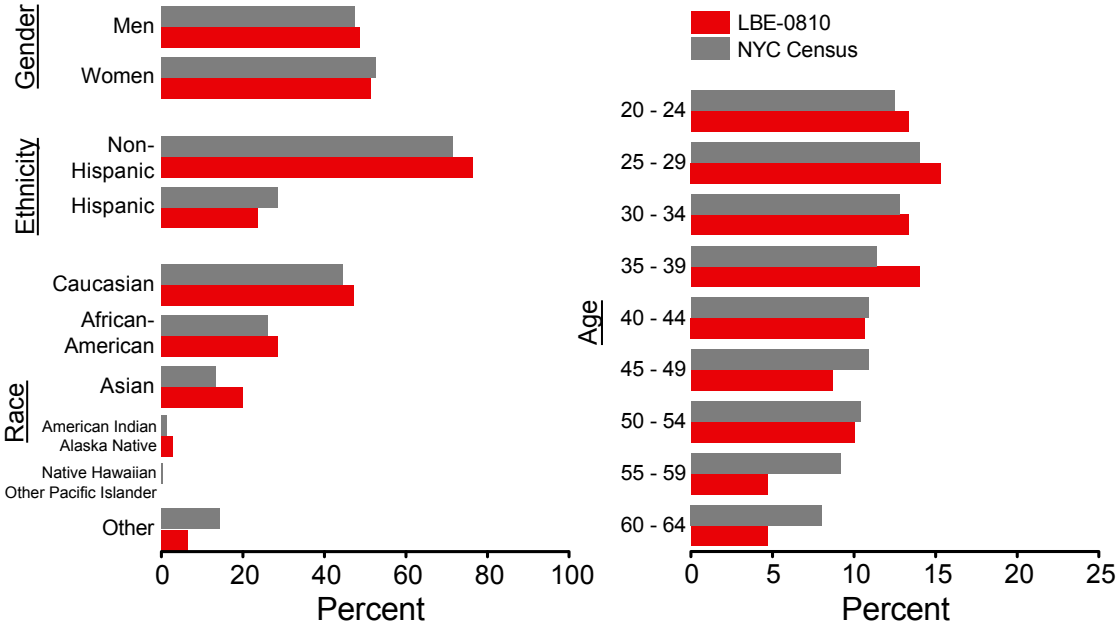


Figure 4.1 Main study LBE-0810 demographics. Demographic profile of subjects participating in the mosquito main study (n=150 subjects) compared to 2010 New York City census data.

weighed before and after the assay to determine blood meal size, then followed to assess the number of eggs each laid and how many of the eggs hatched into larvae. Following behavioral testing, subjects were escorted to the Rockefeller University Outpatient Clinic where research nurses took their vital signs and drew venous blood. These blood samples were subjected to basic clinical blood work panels, including blood type assessment, and general metabolic profiling was performed.

4.2 Main study subjects differed in normalized mosquito attraction

We began analyzing raw subject attraction data and noticed a confounding effect of visit month on mosquito attraction. We therefore developed and applied a time series model to remove variability due to time-dependency from the attraction data, as explained in Chapter 3. After normalizing attraction data, we were able to discriminate between three clusters of subjects who were significantly different from one another—low, middle, and high attractors—using k-means clustering (**Figure 4.2**). We next wanted to determine if subject self-reported attractiveness ratings agreed with our uniport measurements. First, we confirmed the reliability to self-assessment by asking subjects to rate themselves using a sliding scale from 0 to 100 from “underweight” to “overweight.” We correlated this self-assessment with measured body mass index (BMI) and found that the two correlated significantly, with a Spearman rs of 0.351 ($p<0.001$; **Figure 4.3**). We then asked whether subjects in the highly attractive cluster reported themselves as being highly attractive to mosquitoes more often than

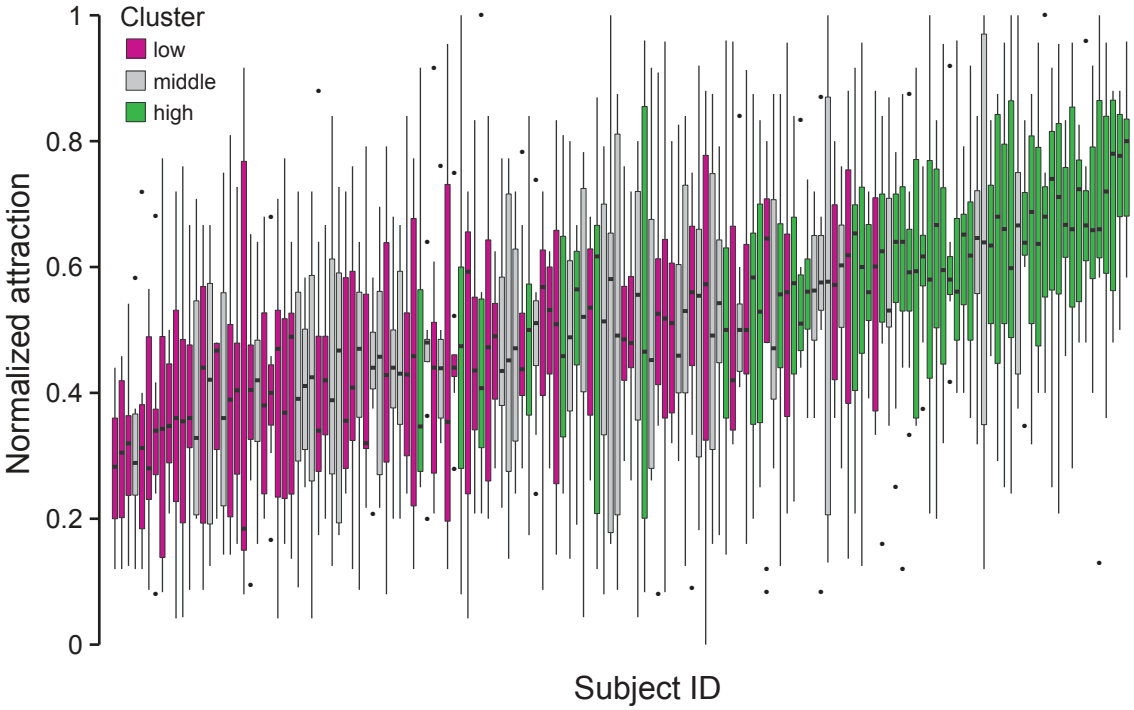


Figure 4.2 Main study LBE-0810 normalized attraction measurements by subject. Time series normalized attraction measurements plotted by subject for the main mosquito study (n=150 subjects, 8 measurements per subject). Subjects are ordered by mean, data are presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, max and min; outliers, black dots). Groups are colored by cluster following k-means clustering: low (magenta, n=56), middle (grey, n=43), and high (green, n=51). Clusters are significantly different from one another by ANOVA with Tukey HSD post test. Low vs middle $p<0.01$, low vs. high $p<0.001$, middle vs high $p<0.001$

those subjects within the lowly attractive cluster. We found that the distributions of subject responses to two separate self-assessments of attraction differed between lowly and highly attractive subjects according to a Mann-Whitney test, ($p<0.05$; **Figure 4.4**).

4.3 No fitness advantages found for mosquitoes feeding on blood from highly attractive subjects

The first 21 subjects to participate in the main mosquito study also participated in a free-feeding assay, in order to determine whether there may be a benefit to feeding on some humans over others. For anthropophilic mosquitoes maintained on a sugar-free diet in the lab, feeding on blood from their preferred hosts, humans, conferred the advantages of increased energy reserves and greater lifetime egg production as compared to feeding on guinea pig blood (Harrington et al., 2001). We were interested in determining if feeding on subjects who were more attractive to mosquitoes in the uniport olfactometer might confer an advantage to female mosquitoes. Twenty-five female mosquitoes were allowed to feed to repletion on the immobilized forearm of subjects. Mosquitoes were then followed individually to assess the weight of blood ingested, number of eggs produced, and number of larvae hatched by each female. We observed some differences in blood meal weight ingested between individuals (**Figure 4.5 a**), but when we grouped subjects who were determined to be in either the lowly-attractive or highly-attractive clusters by k-means clustering, we found that these groups did not differ significantly (**Figure 4.5 b**). We likewise found no effect on

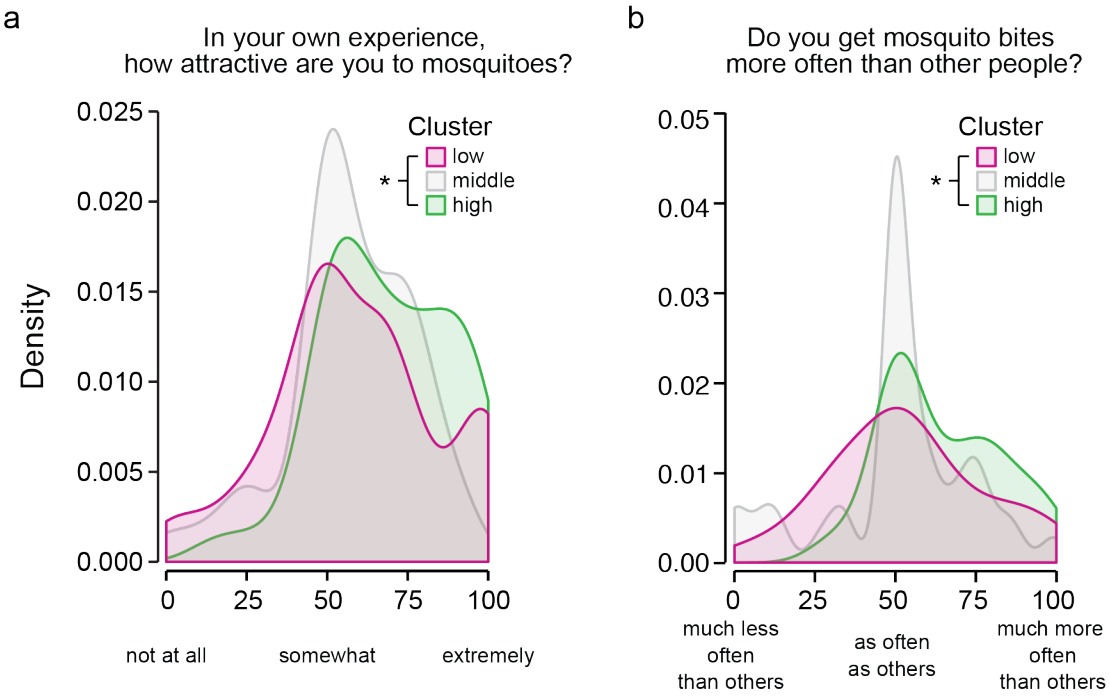


Figure 4.4 Subject self-assessment of attractiveness to mosquitoes differs by measured attraction clustering. (a) Probability density function of subject responses by attraction cluster to the question “In your own experience, how attractive are you to mosquitoes?” answered on a scale from 0 to 100 from “not at all” to “extremely.” **(b)** Probability density function of subject responses by attraction cluster to the question “Do you get mosquito bites more often than other people?” answered on a scale from 0 to 100 from “much less often than others” to “much more often than others.” Data in **a** and **b** plotted by k-means determined attraction clusters, low (magenta), middle (grey), and high (green). Statistical differences between distributions from respondents in low cluster versus high cluster determined by two-tailed Mann-Whitney test

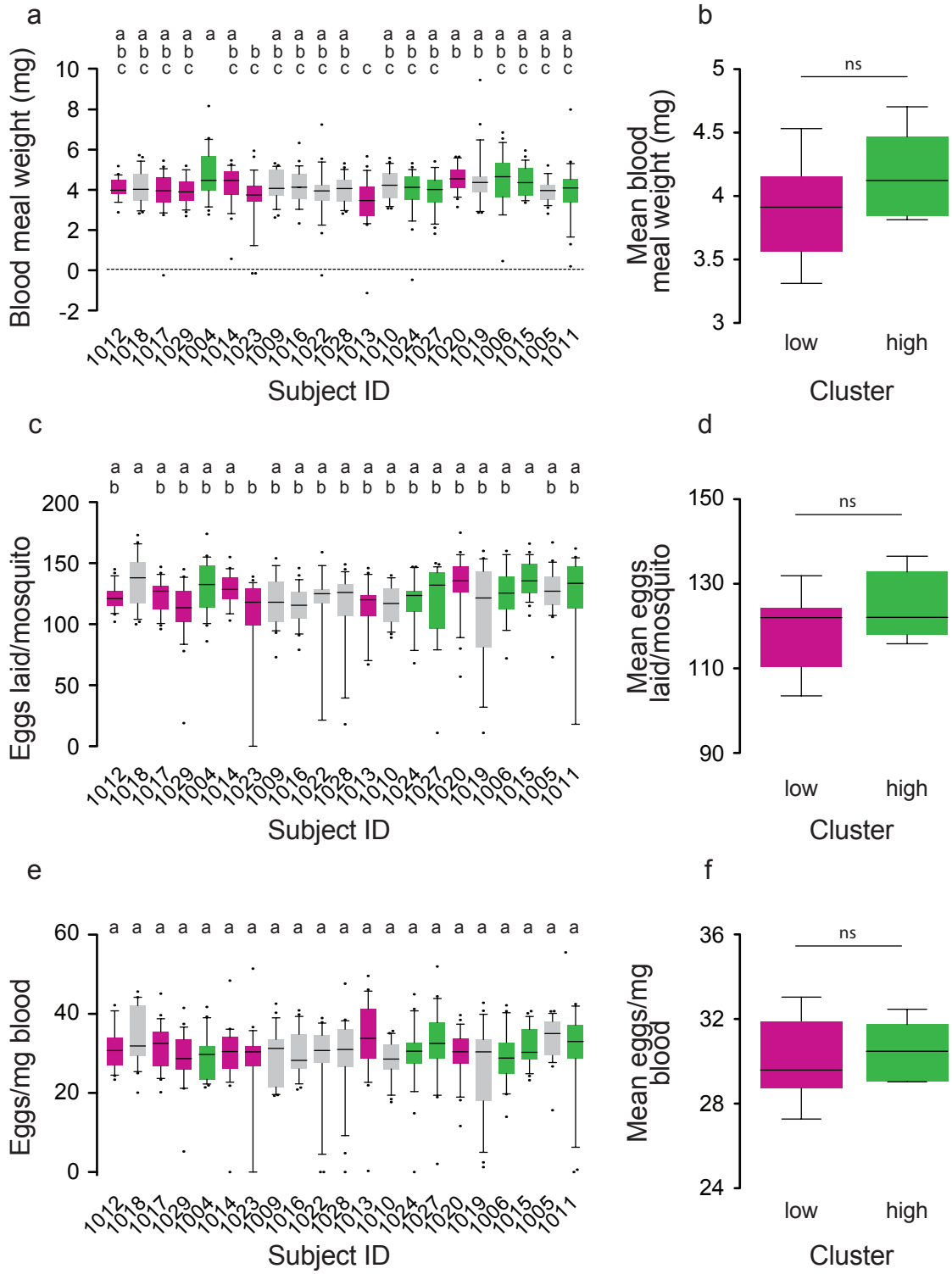

a
Blood meal weight (mg)
Subject ID
1012 1018 1017 1029 1004 1014 1023 1009 1016 1022 1028 1013 1010 1024 1027 1020 1019 1006 1015 1005 1011
b
Mean blood meal weight (mg)
ns
low
high
Cluster
c
Eggs laid/mosquito
Subject ID
d
Mean eggs laid/mosquito
ns
low
high
Cluster
e
Eggs/mg blood
Subject ID
f
Mean eggs/mg blood
ns
low
high
Cluster

Figure 4.5 Free-feeding on different subjects does not dramatically alter mosquito fecundity. (a) Weight of blood meal ingested by mosquitoes in milligrams by mosquitoes following free-feeding to repletion on different subjects, n=22-25 mosquitoes per subject **(b)** Mean weight of blood meal ingested by mosquitoes for each subject, grouped by low (magenta, n=7) and high (green, n=6) attraction clusters **(c)** Eggs laid per mosquito following free-feeding to repletion on different subjects, n=21-25 per subject **(d)** Mean eggs laid per mosquito for each subject, grouped by low (magenta, n=7) and high (green, n=6) attraction clusters **(e)** Eggs laid per milligram of blood ingested by mosquitoes following free-feeding to repletion on different subjects, n=21-24 per subject **(f)**. Mean eggs laid per milligram of blood ingested for each subject, grouped by low (magenta, n=7) and high (green, n=6) attraction clusters. Data in **a-e** presented as box plots (bounds of boxes, first and third quartiles; black line, median; whiskers, 10-90%; outliers, black dots). Colors represent cluster membership amongst full study population where magenta (low), grey (middle), and green (high). In **a**, **c**, and **e** subject boxplots arranged from highest to lowest median attraction. Statistical comparisons made using ANOVA with post hoc Tukey's HSD test. Letters indicate statistically significant differences between groups at the level of $p<0.05$. In **b**, **d**, and **f**, statistical comparisons made with two-tailed Mann-Whitney test

eggs laid per female mosquito (**Figure 4.5 c,d**), nor on eggs laid per milligram of blood ingested (**Figure 4.5 e,f**) or larva hatched (data not shown). We did see an interesting pattern in the data, where mosquitoes feeding on subjects in the highly-attractive cluster tended to take larger blood meals and lay more eggs than those fed on subjects in the lowly-attractive cluster (**Figure 4.5 b,d**), however due to a small sample size (n=6-7 per group) we had low power to detect such differences, and this was not statistically significant (p=0.18). Though these results are intriguing, due to the moderate level of discomfort endured by subjects during the free-feeding assay, we decided to cease performing this experiment with subsequent subjects.

4.4 Concluding remarks

Using the uniport olfactometer, we successfully screened 150 volunteers to determine their attractiveness to mosquitoes. Within this study population, we were able to isolate clusters of subjects who were differentially attractive to mosquitoes. We found that subjects in the highly attractive cluster more frequently self-reported as highly attractive to mosquitoes than those in the lowly attractive cluster. This suggests that mosquito attraction measured in the uniport olfactometer assay is consistent with the experiences of volunteers encountering mosquitoes in more natural settings, meaning that we are appropriately modeling real-world mosquito attraction in the laboratory.

When we allowed mosquitoes to feed to repletion on a small population of subjects, we found that there was an intriguing pattern for mosquitoes feeding on

subjects from the highly attractive cluster to take larger meals and lay more eggs than those feeding on the lowly-attractive cluster. However, this pattern was not statistically significant, possibly due to a small sample size as this portion of the study was halted prematurely due to subject discomfort. To further investigate whether such an effect exists, larger sample sizes from each of the clusters would need to be tested. Ideally, subjects from different clusters should be tested on the same day, with mosquitoes from the same cohort. To test directly for nutritional differences in blood, blood samples could be collected from volunteers and controlled volumes injected into the midgut via enema. It would also be interesting to investigate differences in the accumulation of energy reserves between mosquitoes fed on different subjects, as it has been shown previously that nutritional differences can manifest in those measurements (Harrington et al., 2001). To examine this hypothesis, we froze mosquitoes from the free-feeding assay described here directly after egg laying, and we plan to later analyze their energy stores.

CHAPTER 5: UNCOVERING METABOLIC CORRELATES OF DIFFERENTIAL MOSQUITO ATTRACTION

By the conclusion of the active enrollment stage of the main mosquito study, we had successfully screened 150 subjects to determine their attractiveness to mosquitoes. We found differences between groups of subjects who could be segregated into low-, middle-, and high-attraction clusters, and we collected data on a vast array of possible explanatory variables. To evaluate the validity in many theories of attraction (**Figure 1.3**) we obtained demographic information, self-reported lifestyle factors, self-reported reaction to mosquito bites, vital signs, blood type, a complete blood count panel, and other clinical blood work data (**Figure 5.1**). In addition, metabolic profiling was performed from subject plasma samples through Metabolon (Durham, NC) to obtain relative concentrations of 613 unique metabolites, with the goal of identifying metabolic correlates of mosquito attraction.

5.1 Gene set enrichment analysis (GSEA) shows super- and sub-pathways that are most correlated with attraction

To first look broadly towards which metabolic pathways were most likely to be important in our dataset, we employed the Gene Set Enrichment Analysis Preranked (GSEAP) method (Subramanian et al., 2005). This method is traditionally used in the analysis of genome-wide expression profiles in order to

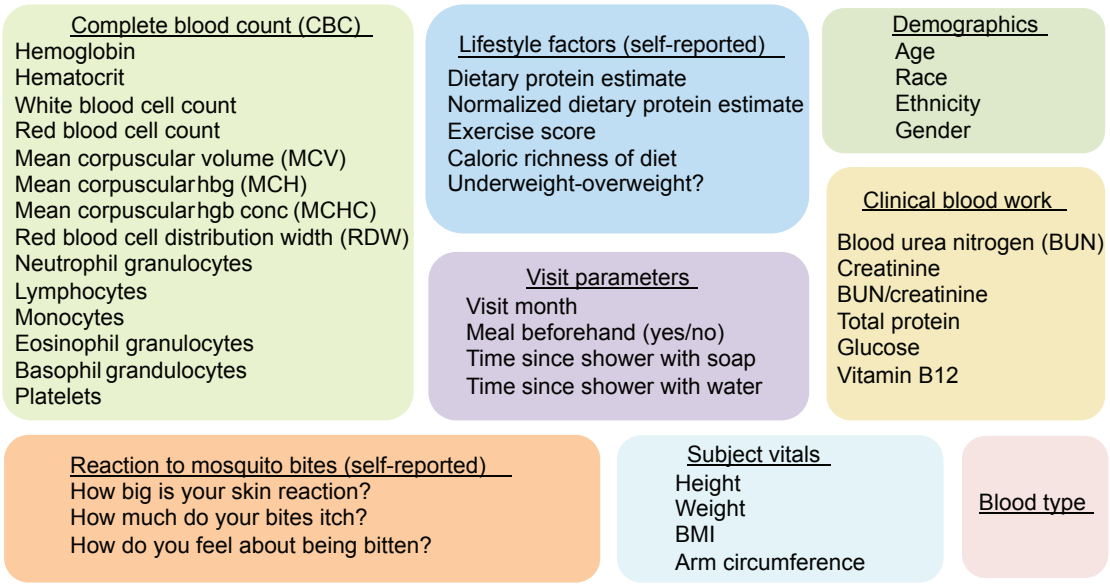


Figure 5.1 Variables collected from LBE-0810 main study subjects by category. Figure depicting variables collected from subjects in the main mosquito study which were included in the initial variable set for feature selection. Colored shapes indicate separate categories of variables. Category names are underlined. For self-reported variables, see **Appendix E** for questionnaire text.

determine if *a priori* defined gene sets, which share a common biological function, are significantly differentially expressed between groups. We applied this method to analyze our metabolomics dataset, because we were interested in determining if *a priori* defined metabolite pathways were enriched in a ranked list of variable importance determined univariately. We nonparametrically (Spearman) correlated each of the 613 original metabolites with median normalized subject attraction, giving weight to each subject based on the their variability, which we defined based on the interquartile range of their attraction measurements. We then ranked the list of metabolites based the resulting correlation coefficients from most positively correlated with attraction to most negatively correlated with attraction, and looked to see which metabolite superpathways and subpathways were most enriched in the extremes of this list. We found that the lipid superpathway was positively enriched, while the amino acid, nucleotide, and cofactors and vitamins superpathways were negatively enriched (**Figure 5.2**). When looking to more specific metabolite subpathways, we found that long chain fatty acids, monoacylglycerol, and dipeptide subpathways were positively enriched, while the histidine metabolism pathway was negatively enriched (**Figure 5.3**).

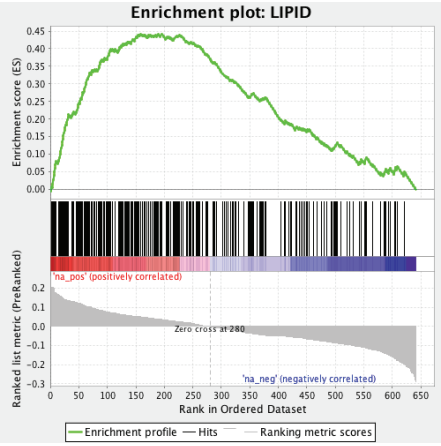
5.2 Selecting a type of model for differential attraction

Having obtained a large and complex dataset of possible effectors, we sought to establish a working model for mosquito attraction to human subjects

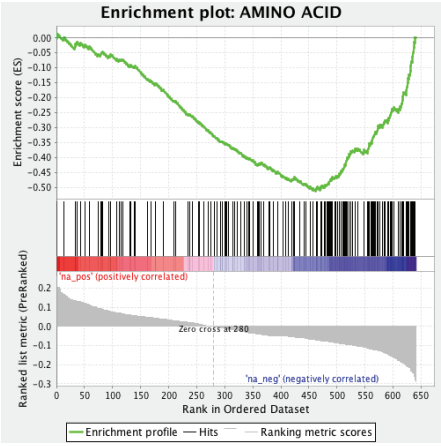
a

Superpathway	Size	ES	NES	Nominal p-value	FDR q-value	FWER p-value
Lipid	239	0.442	2.512	0.000	0.000	0.000
Amino acid	165	-0.512	-2.326	0.000	0.000	0.000
Nucleotide	36	-0.535	-1.891	0.001	0.001	0.003
Cofactors and vitamins	29	-0.522	-1.759	0.004	0.004	0.013

b



c



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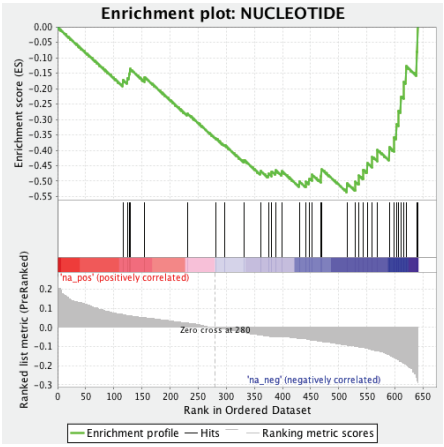


e

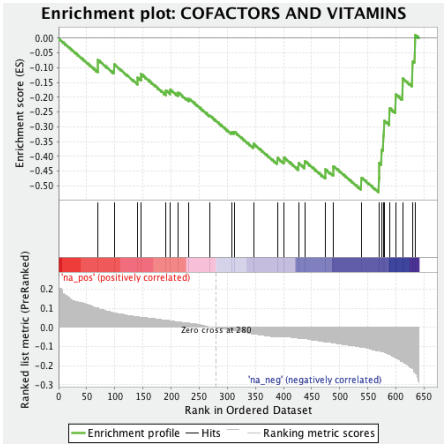
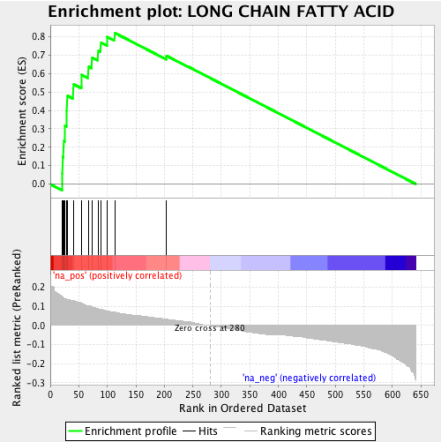


Figure 5.2 GSEA enrichment results for superpathways. (a) Superpathways that were significantly enriched in the extremes of the list of metabolites, ranked from most positive to most negative correlated with attraction, with a FWER p-value cutoff of $p<0.05$. Table lists superpathway name, number of metabolites in that superpathway (size), enrichment score (ES), normalized enrichment score (NES), nominal p-value, False Discovery Rate (FDR) q-value, and Family-Wise Error Rate (FWER) p-value. GSEA enrichment plots are shown for each significant superpathway: **(b)** lipids **(c)** amino acids **(d)** nucleotide and **(e)** cofactors and vitamins. For enrichment plots in **b** – **e**, green line shows enrichment score profile. Rastor plot illustrates positions of all metabolites within that superpathway along the ranked list. Heat map shows degree of correlation from positive correlation (red) to negative correlation (blue). Ranked list metric plot shows correlation coefficient distribution used to rank the metabolite list. Correlations between metabolites and attraction were nonparametric (Spearman) and subject contributions were weighted based on the variability in their attraction measurements defined by their interquartile range (IQR). Enrichment score (ES) reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes, Normalized enrichment score (NES) accounts for differences in metabolite pathway size and in correlations between pathways

a

Subpathway	Size	ES	NES	Nominal p-value	FDR q-value	FWER p-value
Long chain fatty acid	15	0.821	2.641	0.000	0.000	0.000
Monoacylglycerol	8	0.852	2.188	0.000	0.001	0.001
Dipeptide	9	0.742	2.001	0.001	0.006	0.018
Histidine metabolism	12	-0.681	-1.836	0.001	0.048	0.048

b



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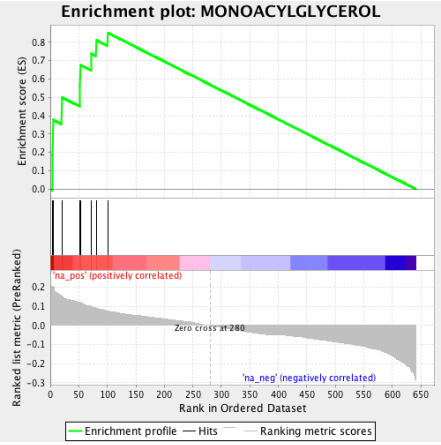
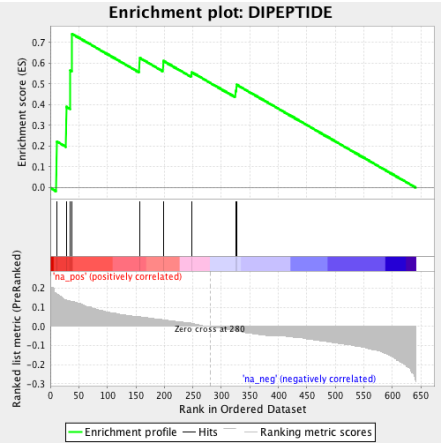


d



e

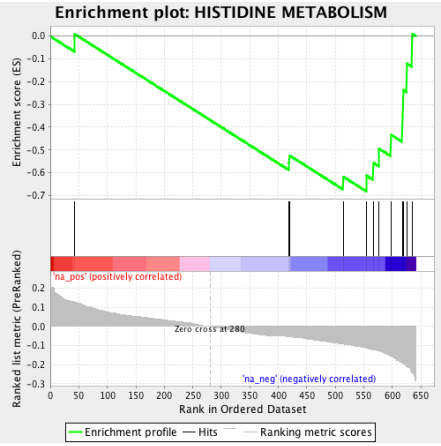


Figure 5.3 GSEA enrichment results for subpathways. (a) Subpathways significantly enriched in the extremes of the list of metabolites ranked from most positive to most negative correlated with attraction, with a FWER p-value cutoff of $p<0.05$. Table lists subpathway name, number of metabolites in that subpathway (size), enrichment score (ES), normalized enrichment score (NES), nominal p-value, False Discovery Rate (FDR) q-value, and Family-Wise Error Rate (FWER) p-value. GSEA enrichment plots are shown for each significant subpathway **(b)** long chain fatty acid **(c)** monoacylglycerol **(d)** dipeptide and **(e)** histidine metabolism. For enrichment plots in **b** – **e**, green line shows enrichment score profile. Raster plot illustrates positions of all metabolites within that subpathway along the ranked list. Heat map shows degree of correlation from positive correlation (red) to negative correlation (blue). Ranked list metric plot shows correlation coefficient distribution used to rank the metabolite list. Correlations between metabolites and attraction were nonparametric (Spearman) and subject contributions were weighted based on the variability in their attraction measurements, defined by their interquartile range (IQR). Enrichment score (ES) reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes, Normalized enrichment score (NES) accounts for differences in metabolite pathway size and in correlations between pathways

based on the variables in our study. The goal when choosing a statistical model is to identify the most parsimonious model—one that is as simple as possible while still being able to predict the outcome variable well. We decided to look first towards linear models for attraction, based on the ease of their interpretability over their nonparametric counterparts. Linear models also allow one to estimate the relative contribution of each variable in the model towards its overall explanatory power. This was appealing to us, given our expectation that mosquito attraction is likely to be a complex phenotype resulting from a combination of factors.

5.3 Reducing data dimensionality

The largest challenge in establishing such a model is the process known as dimensionality reduction—in other words, how to narrow down our list of 654 variables to a more manageable number for incorporation into a model of mosquito attraction. This process can be achieved through either feature selection (finding the most relevant subset of variables) or through feature extraction (transforming the data into fewer dimensions). In these preliminary analyses, we chose to begin with feature selection, again for ease of final model interpretability.

We made our first round of variable selections using two univariate linear modeling methods, which we implemented in parallel: linear modeling (LM) and linear mixed-effects modeling (LME) (**Figure 5.4**). Linear modeling determines how well each variable individually predicts attraction. To avoid the influence of

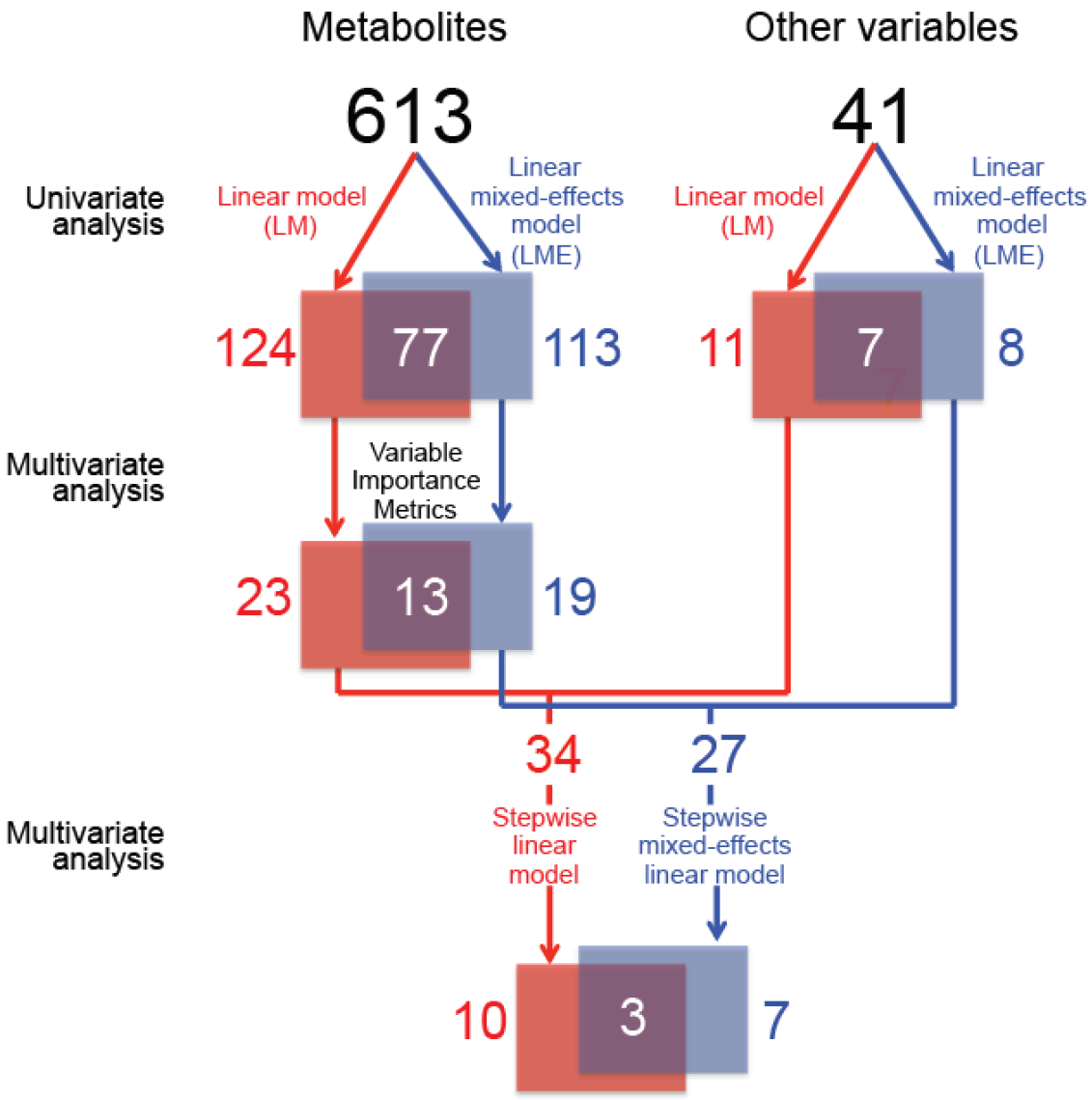


Figure 5.4 Workflow of feature selection of variables for inclusion in the final linear models for attraction. Flow chart depicting parallel processing using linear modeling (LM) and linear mixed-effects modeling (LME) for feature selection. Colors indicate two distinct selection workflows, LM (red) and LME (blue). Red (LM) and blue (LME) rectangles and numbers represent variables present following each round of selections. Numbers inside the purple box indicate number of variables overlapping between LM and LME sets at each step

outliers, we chose to use the median normalized attraction for each subject in the linear regressions. We selected any variable that, when placed in a univariate linear model for attraction, increased the fit with a significance threshold of $p<0.2$, uncorrected for multiple comparisons (**Figure 5.5 a-d**). The linear model selection narrowed the variable list to 124 metabolites and 11 other variables (**Figure 5.4**).

Whereas linear modeling determines how well each variable can predict median normalized subject attraction on its own, linear mixed-effects modeling allowed us to use all 8 normalized attraction measurements for a subject in the regression by including a random intercept term of subject. In this way, a linear mixed-effects model incorporates information about the within-subject variability in attraction measurements during the estimation process. Though there is an added benefit to incorporating this additional information, our attraction data have high within-subject variability, so we were aware that the mixed-effects modeling would likely result in overall lower estimations of fit to the data. Still, we thought it valuable to perform these two separate forms of linear modeling in parallel to gain more information about the dataset. We selected any variable that, when placed in the mixed-effects linear model for attraction, increased the fit with a significance threshold of $p<0.2$, uncorrected for multiple comparisons (**Figure 5.5 e,f**). The linear mixed-model selection narrowed the variable list to 113 metabolites and 8 other variables (**Figure 5.4**).

The metabolite lists were then of a feasible size to use multivariate analyses for further feature selection, which is more appropriate for data such as

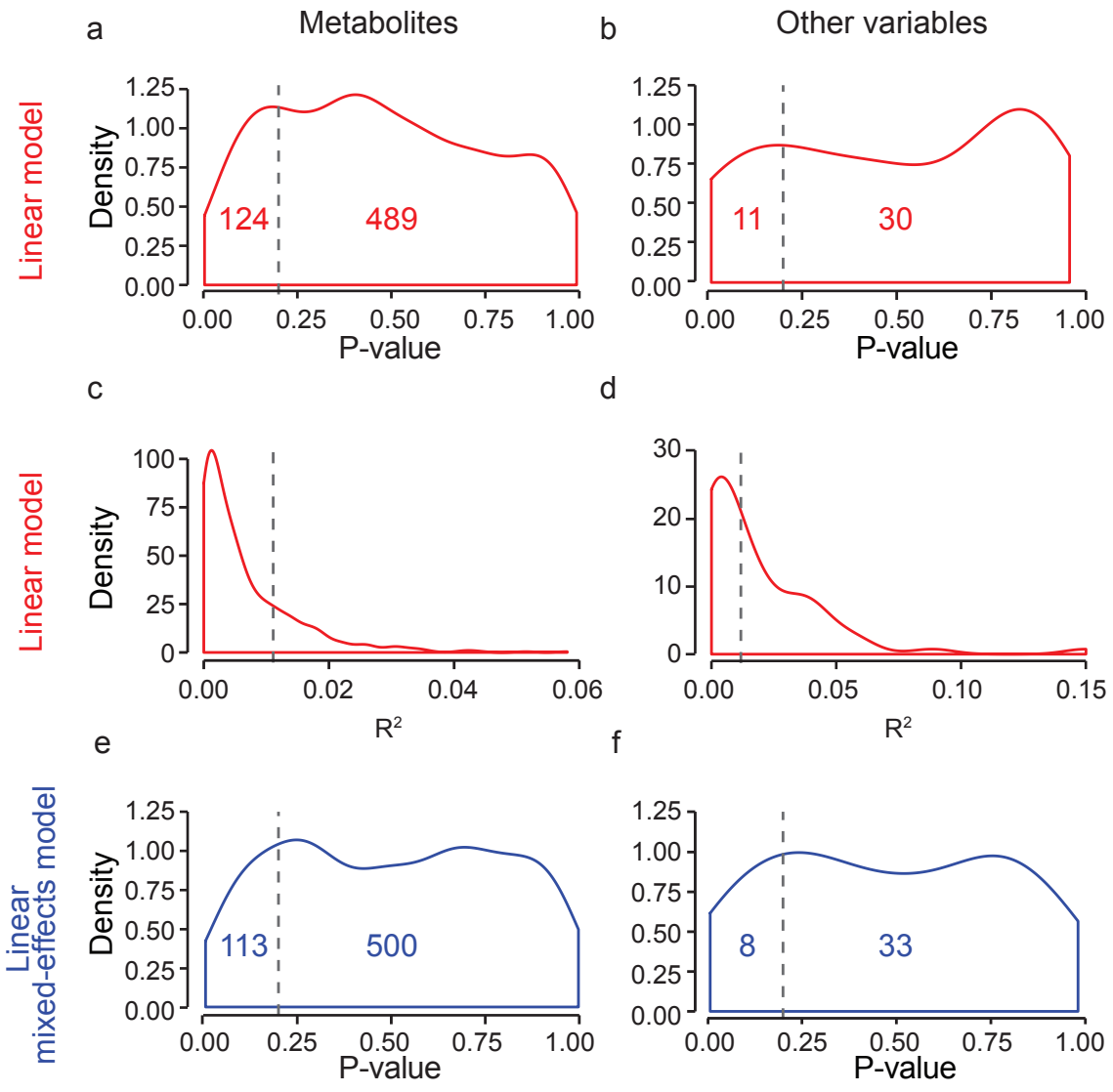

a
Metabolites
b
Other variables
Linear model
Density
1.25
1.00
0.75
0.50
0.25
0.00
124
489
11
30
0.00
0.25
0.50
0.75
1.00
P-value
c
d
100
75
50
25
0
30
20
10
0.02
0.04
0.06
0.05
0.10
0.15
R²
e
f
Linear
mixed-effects model
113
500
8
33

Figure 5.5 Probability density functions for variable p-value and R^2 distributions and cutoffs for the first round of feature selection. Probability density functions for **(a)** uncorrected p-values of all metabolites following univariate linear model fit to median normalized attraction **(b)** uncorrected p-values of all other variables following univariate linear model fit to median normalized attraction **(c)** R^2 values for all metabolites following univariate linear model fit to median normalized attraction **(d)** R^2 values for all other variables following univariate linear model fit to median normalized attraction **(e)** uncorrected p-values for all metabolites following univariate linear mixed-effects model fit to normalized attraction **(f)** uncorrected p-values for all other variables following univariate linear mixed-effects model fit to normalized attraction. In **a**-**f**, colors represent linear model (red) and linear mixed-effects model (blue) results. Grey dotted line represents cutoff point for variable selection from a total of n=613 total metabolites, n=41 other variables. In **a**, **b**, **e**, and **f**, numbers represent the number of variables on either side of the cutoff

metabolomics where variables may be correlated with one another. We calculated the median variable importance for each of the remaining variables using 10 different methods within the randomForest and caret (classification and regression training) packages in R (**Table 5.1**). Using the varImp function, the program reports an importance score for each variable, calculated differently for each algorithm employed, that characterizes the general effect of predictors on the model. The importance scores are then scaled to a maximum of 100 and can be compared across models, or, in our case, we can aggregate the results of these importance scores as a more robust measure of variable significance for further feature selection.

We plotted median variable importance for all metabolites from the narrowed lists arranged from highest to lowest, and selected a cutoff point below which the slope had consistently leveled by examining its derivative (**Figure 5.6**). For both LM and LME pipelines, this cutoff point was a variable importance of greater than 54 (n=23 metabolites from linear model pipeline and n=19 metabolites from linear mixed-effects model pipeline). These, together with the list of “other” variables narrowed through univariate methods, were included in the group of possible predictors in the stepwise algorithms to determine final models (**Table 5.2**, **Table 5.3**, **Figure 5.7**).

Table 5.1 Methods employed to evaluate variable importance for feature selection. Table of R method, general statistical approach, and importance metric determination for 10 different variable importance metrics used for feature selection.

R method	Approach	Importance metric
glmnet	Generalized Linear Model	Absolute value of the *t*-statistic
glmboost	Generalized Linear Model	Absolute value of the *t*-statistic
pls	Partial Least Squares	Contribution of the coefficients weighted proportionally to the reduction in the sums of squares
svmLinear	Support Vector Machines with Linear Kernel	Absolute value of the *t*-statistic
knn	K-nearest neighbor	Distance between the class centroid and overall centroid
cforest	Conditional Inference Random Forest	Reduction in mean squared error
ridge	Ridge Regression	Absolute value of the *t*-statistic
penalized	Penalized Linear Regression	Absolute value of the *t*-statistic
randomForest	Random Forest	Reduction in node impurity
randomForest	Random Forest	Reduction in mean squared error

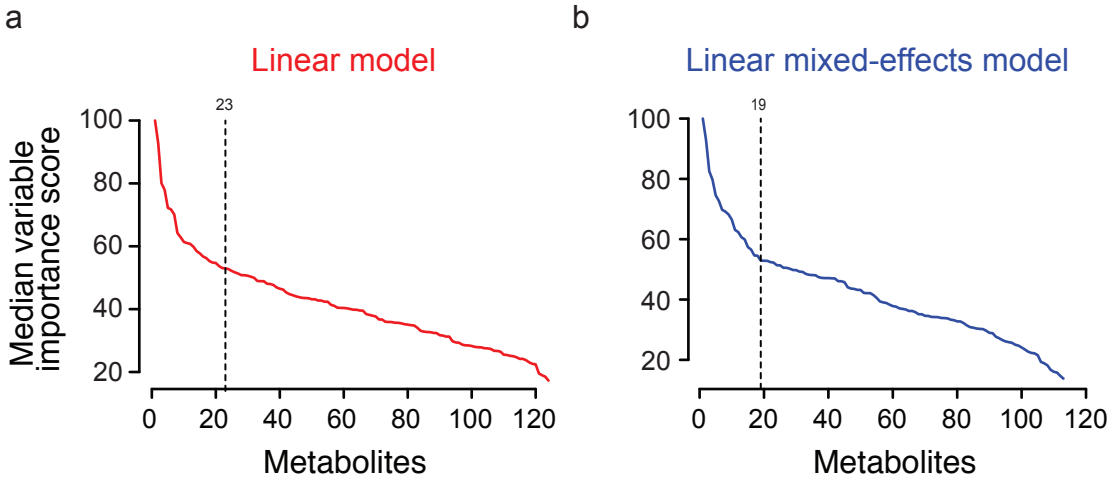


Figure 5.6 Median variable importance score for all metabolites during the second round of feature selection. (a) Plot of median variable importance score from 10 different variable importance metrics for all LM-selected metabolites (n=124) during second round of feature selection. Dotted line indicates variable selection cutoff, where the change in slope approached zero (n=23 metabolites) **(a)** Plot of median variable importance score from 10 different variable importance metrics for all LME-selected metabolites (n=113) during second round of feature selection. Dotted line indicates variable selection cutoff, where the change in slope approached zero (n=19 metabolites). Colors indicate variables from linear model (red) or linear mixed-effects model (blue) workflows.

Table 5.2 Metabolites selected for inclusion in final stepwise linear regression analysis. Median variable importance from ten multivariate variable importance measures (Imp), metabolite names, superpathways, and subpathways for metabolites selected for inclusion in final stepwise regression (n=34).

Imp	Metabolite	Superpathway	Subpathway
100.0	1-methylimidazoleacetate	Amino Acid	Histidine Metabolism
90.2	4-imidazoleacetate	Amino Acid	Histidine Metabolism
82.0	N-acetyl-aspartyl-glutamate (NAAG)	Amino Acid	Glutamate Metabolism
78.5	acetylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)
76.2	eugenol sulfate	Xenobiotics	Food Component/Plant
76.0	13-HODE + 9-HODE	Lipid	Fatty Acid, Monohydroxy
71.1	1-methylhistidine	Amino Acid	Histidine Metabolism
65.5	epiandrosterone sulfate	Lipid	Steroid
64.5	dodecanedioate	Lipid	Fatty Acid, Dicarboxylate
62.1	cis-vaccenate (18:1n7)	Lipid	Long Chain Fatty Acid
62.0	ascorbate (Vitamin C)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism
61.4	octadecanedioate	Lipid	Fatty Acid, Dicarboxylate
61.4	N-acetyl-1-methylhistidine	Amino Acid	Histidine Metabolism
61.1	N-acetylphenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism
59.9	palmitoyl-linoleoyl-glycerophosphocholine (2)	Lipid	Lysolipid
59.2	4-hydroxyhippurate	Xenobiotics	Benzoate Metabolism
58.9	S-adenosylhomocysteine (SAH)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism
58.1	carnitine	Lipid	Carnitine Metabolism
57.7	hexanoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)
55.6	alpha-hydroxycaproate	Lipid	Fatty Acid, Monohydroxy
55.4	5alpha-androstan-3beta,17beta-diol monosulfate (2)	Lipid	Steroid
55.3	urea	Amino Acid	Urea cycle; Arginine and Proline Metabolism

Table 5.3 Metabolites selected for inclusion in final stepwise linear mixed-effects regression analysis. Median variable importance from ten multivariate variable importance measures (Imp), metabolite names, superpathways, and subpathways for metabolites selected for inclusion in final stepwise regression (n=27).

Imp	Metabolite	Superpathway	Subpathway
100.0	1-methylimidazoleacetate	Amino Acid	Histidine Metabolism
98.8	4-imidazoleacetate	Amino Acid	Histidine Metabolism
82.4	N-acetyl-aspartyl-glutamate (NAAG)	Amino Acid	Glutamate Metabolism
81.7	1-methylhistidine	Amino Acid	Histidine Metabolism
75.1	ascorbate (Vitamin C)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism
75.0	eugenol sulfate	Xenobiotics	Food Component/Plant
74.1	S-adenosylhomocysteine (SAH)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism
73.0	dodecanedioate	Lipid	Fatty Acid, Dicarboxylate
68.3	N-acetylphenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism
66.1	deoxycarnitine	Lipid	Carnitine Metabolism
65.3	palmitoyl-linoleoyl-glycerophosphocholine (2)	Lipid	Lysolipid
62.8	epiandrosterone sulfate	Lipid	Steroid
59.8	4-hydroxyhippurate	Xenobiotics	Benzoate Metabolism
59.7	N-acetyl-1-methylhistidine	Amino Acid	Histidine Metabolism
59.1	hexanoylglycine	Lipid	Fatty Acid Metabolism(Acyl Glycine)
56.4	4-androsten-3alpha,17alpha-diol monosulfate (3)	Lipid	Steroid
56.3	dehydroisoandrosterone sulfate (DHEA-S)	Lipid	Steroid
56.0	propyl 4-hydroxybenzoate sulfate	Xenobiotics	Benzoate Metabolism
54.2	N-acetylcitrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism

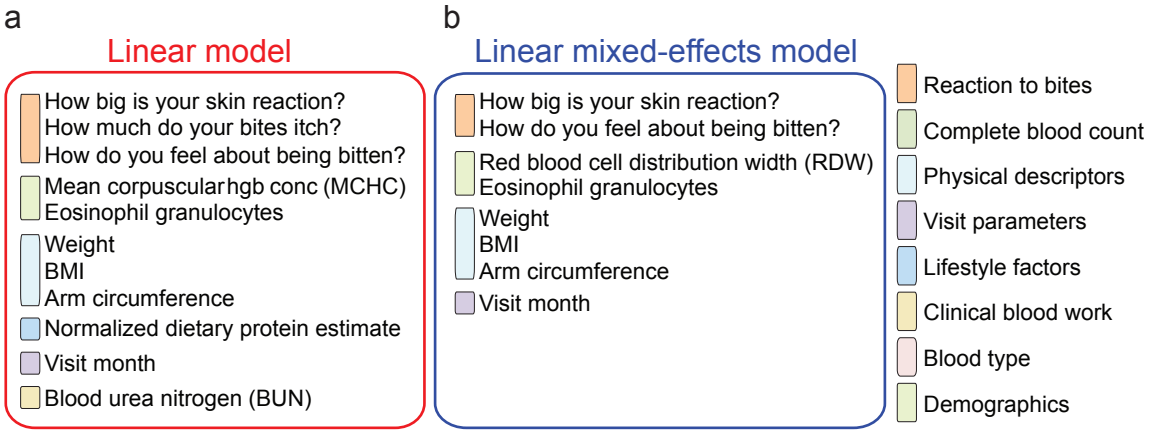


Figure 5.7 Other variables selected for inclusion in final stepwise model selections. Other variables chosen for inclusion in **(a)** stepwise linear regression (n=11) and **(b)** stepwise linear mixed-effects regression (n=8). Colored boxes denote variable categories as indicated at the far right, which correspond to Figure 5.1

5.4 Preliminary models of mosquito attraction via stepwise linear regression

Having selected a reasonable number of features to be included as possible effectors in a final model through two parallel workflows, LM and LME, we were then ready to assemble the models. We implemented stepwise regression using the R package stepAIC for both a linear model and a linear mixed-effects model, using variables from the LM and LME workflows, respectively. Forward stepwise regression begins with no variables in the model. It then identifies which variable contributes most significantly to the model fit, and adds that to the model. Then, it identifies the next most helpful variable. When a variable does not improve the fit of the model it is discarded, and this process continues until none of the remaining variables will improve the model. Backwards stepwise regression begins with a full model involving all input variables. It then removes variables that improve the model fit when deleted, until it no further variable removal improves the model. We chose to run the procedure in both directions, which results in a consensus model based on information from both forward and backward implementation.

The resulting linear model (**Table 5.4**) had an adjusted R^2 of 0. 241 (p=7.14e-06), meaning that together the variables in this model account for an estimated 24.1% of the variation in mosquito attraction to human volunteers in our study. By evaluating the fit of a linear model including only the 8 metabolites in the full model, we estimated that metabolites alone account for 19.7% of the variability in uniport attraction (adjusted R^2 of 0.1974; p=4.31e-5). The results

Table 5.4 Variables significantly contributing to a linear model of subject attraction, determined in a stepwise procedure. Table displaying the minimum set of variables necessary to best predict median subject attraction according to a stepwise linear model selection. Metabolites (grey), clinical bloodwork (green), and subject physical descriptors (blue) are organized according to absolute value of estimates, from largest to smallest. Variable R^2=0.303, adjusted R^2=0.241

Variable	Superpathway	Subpathway	Estimate	Std. Error	P-value
carnitine	Lipid	Carnitine metabolism	0.123	0.036	0.001
N-acetyl phenylalanine	Amino Acid	Phenylalanine and Tyrosine metabolism	0.0310	0.020	0.115
1-methylimidazole acetate	Amino acid	Histidine metabolism	-0.022	0.010	0.025
N-acetyl-aspartyl-glutamate	Amino acid	Glutamate metabolism	0.018	0.008	0.019
N-acetyl-1-methylhistidine	Amino acid	Histidine metabolism	-0.018	0.009	0.050
ascorbate (Vitamin C)	Cofactors and Vitamins	Ascorbate and Aldarate metabolism	0.016	0.009	0.072
Alpha-hydroxycaproate	Lipid	Monohydroxy fatty acid	0.014	0.008	0.086
eosinophils	**N/A**	**N/A**	0.010	0.005	0.062
eugenol sulfate	Xenobiotics	Food component/ plant	-0.007	0.004	0.056
Body Mass Index (BMI)	**N/A**	**N/A**	-0.005	0.002	0.018

Table 5.5 Variables significantly contributing to a mixed-effects linear model of subject attraction, determined in a stepwise procedure. Table displaying the minimum set of variables necessary to best predict subject attraction according to a stepwise selected linear mixed-effects model. Metabolites (grey), clinical bloodwork (green), and questionnaire (orange) are organized according to absolute value of estimates, from largest to smallest. Pseudo R^2=0.1292

Variable	Superpathway	Subpathway	Estimate	Std. Error	P-value
palmitoyl-linoleoyl-glycerophosphocholine (2)	Lipid	Lysolipid	0.103	0.037	0.007
1-methylimidazole acetate	Amino acid	Histidine metabolism	-0.020	0.008	0.016
4-hydroxyhippurate	Xenobiotics	Benzoate metabolism	-0.012	0.009	0.152
N-acetyl-1-methylhistidine	Amino acid	Histidine metabolism	-0.011	0.007	0.113
Skin reaction	**N/A**	**N/A**	0.010	0.0004	0.051
eugenol sulfate	Xenobiotics	Food component/ plant	-0.007	0.003	0.044
Variation in red blood cell width (RDW)	**N/A**	**N/A**	0.001	0.001	0.104

from the linear mixed-effects model are summarized in **Table 5.5.** In addition to estimating the variance due to model variables, the LME also estimates the variance due to the random intercept (in this case, subject). This makes a traditional goodness-of-fit measure difficult to obtain, so we tested adherence between the observed data and the data predicted with the model using a linear regression. The R^2 for this linear regression was 0.129—a lower value than that for the linear model likely due to the high within-subject variability in normalized attraction measurements. When comparing the models, three metabolites in particular overlapped between the linear model and the linear mixed-effects model, two of which are involved in the histidine metabolism pathway: 1-methylimidazoleacetate, N-acetyl-1-methylhistidine, and eugenol sulfate.

5.5 Concluding remarks

We began the data analysis process with a large dataset of 654 possible explanatory variables including metabolite profiling data as well as demographic information, self-reported lifestyle factors, self-reported reaction to mosquito bites, vital signs, blood type, a complete blood count panel, and other clinical blood work. We created a feature selection pipeline combining both univariate and multivariate methods to narrow the list to a more manageable size, then arrived at two preliminary linear models for mosquito attraction in our population. These are, to our knowledge, the first such models of differential mosquito attraction to human subjects. The linear model explains an estimated 24.1% of

the variability in mosquito attraction to human subjects, the majority of which (19.7%) can be attributed to the metabolites alone. This result is particularly exciting given that this study represents the first investigation into the metabolic correlates of attraction. Many of the traditional theories of mosquito attraction are not supported by our data (**Figure 1.3**). Instead, we have uncovered novel metabolic correlates that will help to shape and focus new theories of the underlying biology of differential attraction.

Two of the three metabolites common to both the LM and LME models are members of the histidine metabolism pathway, and their estimates suggest that they negatively impact attraction. This is directionally in agreement with the GSEA results, which indicated that metabolites in the histidine metabolism pathway were overrepresented in the negative extreme of a list of metabolite correlations with median normalized attraction. In addition, we see members of the lipid superpathway in both models, each with positive estimates. This is also in agreement with GSEA results, which indicate that metabolites within the lipid superpathway are positively correlated with attraction. Though the exact metabolites which are chosen within the histidine subpathway and lipid superpathway differ by model, their consistent representation suggests that they may be important contributors to the prediction of mosquito attraction.

These two models allow us to gain initial insights into the correlations between specific blood biomarkers and mosquito attraction. The analysis workflow and feature selection methods outlined here are a starting point for understanding our dataset. We acknowledge that these models represent one

approach to identifying significant correlates, and that alternative methods for statistical modeling of attraction will exist. The process of dimensionality reduction is a controversial topic in statistics, and as large datasets are becoming more commonplace, new methods and best practices are evolving constantly. There is no best answer—instead, a diversity of methods must be employed to develop many different models, and these models must then be compared based on their performance. In the future we must test these preliminary models to determine their stability and their predictive power. We also plan to explore alternative methods of feature selection and feature extraction to search for even better models of mosquito attraction from this large, foundational data set.

CHAPTER 6: DISCUSSION

The work detailed in this dissertation constitutes one of the largest human studies to date to investigate differential mosquito attraction. Moreover, this is the first investigation into how these differences in mosquito attraction may be correlated with components of the blood metabolome. We established the uniport olfactometer as a method to quantify mosquito attraction to different subjects and then used it to screen 150 volunteers. We then developed a novel application for time series methodology to normalize experimental data collected across a year-long period. Using normalized mosquito attraction measurements, we successfully discriminated clusters of subjects who were differentially attractive to mosquitoes. From these subjects, we collected demographic information, self-reported lifestyle factors, self-reported reaction to mosquito bites, vital signs, blood type, a complete blood count panel, other clinical blood work, and blood metabolic profiling. Then, using a variety of statistical methods for feature selection, we narrowed this list of variables and ultimately arrived at two tentative models for mosquito attraction. These models represent, to our knowledge, the first such models for mosquito attraction to human subjects. The preliminary linear model detailed here explains 24.1% of subject variation in mosquito attraction, and we estimate that approximately 19.7% of this explanatory power is due to blood metabolites alone – an effect more powerful in our study than many commonly cited demographic and lifestyle factors.

Many studies of mosquito attraction conducted in the past have limited their volunteer populations to include primarily one race (Brady et al., 1997; Knols et al., 1995; Lindsay et al., 1993; Logan et al., 2008; Mukabana et al., 2002; Qiu et al., 2006; Verhulst et al., 2011; 2013) and/or gender (Brady et al., 1997; Khan et al., 1965; Knols et al., 1995; Lindsay et al., 1993; Maibach et al., 1966a; Mukabana et al., 2002; Schreck et al., 2002; Shirai et al., 2002; Verhulst et al., 2011; 2013) in order to reduce demographic variability and background noise. In our study, we chose to recruit a diverse set of volunteers including three major racial groups, both genders, and a wide age range (18-65), so that we could maximize natural human variability to survey a more diverse set of factors potentially linked to mosquito attraction. These opposing strategies illustrate the trade-off that must be made when designing exploratory human studies such as this: when you include more variables, you decrease the power you have to address any of one of them individually. Given the probable complexity of the differential human attraction phenotype, and our relative lack of knowledge about its principal components, we decided to collect a large, diverse dataset from which we could extract the most important contributors. The models presented here will now serve to focus and direct future research questions to assess the causality of these correlates and the mechanisms by which they are detected by mosquitoes.

Differential mosquito attraction is a common human experience, and as a result there has been an accumulation of layperson as well as scientific theories about its underlying cause (**Figure 1.3**). We collected data to evaluate many

commonly cited theories surrounding demographics, lifestyle habits, physiology, and environment (**Figure 5.1**), and included these data in our feature selection pipeline. In our final models of attraction, none of these variables were able to predict attraction as significantly as blood metabolome components. This finding is important because it can put to rest some lingering folklore surrounding differential attraction as well work towards settling some inconsistencies regarding the influence of factors such as blood type or age. These results can also, then, stimulate new investigation of why and how differences in the blood metabolome are correlated with mosquito attraction.

Broadly speaking, there are several ways to think about the metabolic correlates of attraction we've uncovered. Female mosquitoes may be drawn to subjects with these particular blood metabolome profiles because acquiring a blood-meal with this cocktail of nutrients is directly beneficial to her or her offspring. Alternatively, these blood metabolome components may confer no direct advantage to mosquitoes, but may be correlated with other host qualities not measured here, such as specific body odor components.

Although our work linking mosquito preference to differences in the blood metabolome is correlative, these findings allow us to formulate intriguing hypotheses. The results from the GSEA analysis broadly demonstrate that metabolites within the amino acid superpathway, and the histidine subpathway in particular, are negatively correlated with mosquito attraction. Conversely, molecules within the lipid metabolism superpathway, specifically long chain fatty acids and monoacylglycerols, tend to be positively correlated with mosquito

attraction. This is directionally in agreement with our proposed linear models for mosquito attraction, which each show representation from both histidine metabolism and lipid pathway members that contribute significantly to their explanatory power (**Figure 6.1**). The correlation of mosquito attraction with members of these two particular classes of metabolites is intriguing, and taken in the context of the full models, points towards the possible role of (1) allergic responses and (2) fatty acid metabolism.

6.1 Attraction is negatively influenced by high activation of histidine pathways

Histidine is an essential amino acid for humans (Kopple and Swendseid, 1975), and importantly a precursor for histamine. Within both the linear and linear mixed-effects models of attraction we see that 1-methylimidazoleacetate and N-acetyl-1-methylhistidine adversely impact attraction. Both molecules are metabolites of histamine catabolism. 1-methylimidazoleacetate, in particular, is the main metabolite of histamine and the end product of its metabolism (Maintz and Novak). Histamine is an immune molecule released by mast cells and other immune cells in response to antigen recognition (Jutel et al., 2005). This acute inflammatory pathway is activated in response to mosquito bites (Demeure et al., 2005). If mosquitoes tend to prefer subjects who are have lower levels of histamine metabolites, and therefore likely lower histamine itself, this could mean that they are selecting subjects who have a reduced allergic response to their

Figure 6.1 Schematic diagram of attraction model findings by super- and sub-pathways. (a) Schematic of estimates for each sub- and super-pathway selected for linear model of attraction **(b)** Schematic of estimates for each sub- and super- pathway selected for mixed-effects model of attraction.

bites. This could be advantageous, as people who react less strongly to bites are less likely to notice them, and therefore allow mosquitoes to stealthily acquire bloodmeals. The defensive response mounted by humans against mosquitoes is a major factor in the mortality of females seeking a bloodmeal. Our work uncovers a possible mechanism by which mosquitoes would avoid highly immune reactive—and thus defensive—human hosts.

Interestingly, subjects presenting with insect anaphylaxis—a systemic, more extreme allergic reaction in response to insect bites—tend to show an increase in both 1-methylhistidine and 1-methylimidazoleacetate in their urine, and have an increased risk of mastocytosis (Martens-Lobenhoffer and Neumann, 1999). Mastocytosis is marked by an increase in the number of mast cells, which release histamine in response to allergens (Valent et al., 2001). In future studies it would be interesting to test the attractiveness of those who suffer from mastocytosis, because we might imagine them to be less appealing to mosquitoes. It would also be interesting to explore whether subjects who have stronger allergic responses to mosquito bites are indeed more likely to notice when they are being bitten. To assess causality, histamine levels must be manipulated within the same subjects, perhaps through dietary intervention or supplementation, and attraction tested before and after these manipulations.

6.2 Attraction is positively influenced by fatty acid metabolism pathways

The other broad theme seen throughout the data is the positive influence of lipid pathway metabolites on attraction, and more specifically, the role of long

chain fatty acids. Carnitine is the metabolite with the highest estimate of importance in the linear model for attraction, and it appears alongside a fatty acid, alpha-hydroxycaproate. Carnitine plays an essential role in lipid metabolism, helping transport fatty acids into the mitochondria where they can then be broken down to generate metabolic energy. Carnitine can be synthesized in the liver, but is also found in diets rich in protein (e.g. red meat, dairy, nuts; (Steiber et al., 2004)). Due to its role in energy metabolism, carnitine levels have been shown to increase following a lipid-rich meal (Davis et al., 1988) and decrease acutely following intense exercise (Hiatt et al., 1989). In cases of malnutrition, carnitine levels are often decreased (Khan and Bamji, 1977). In this way, carnitine may be a biomarker of dietary health.

If subjects with high levels of long chain fatty acid and carnitine levels are well-nourished, perhaps mosquitoes are more attracted to these subjects because they represent healthier meals. Certainly, when female mosquitoes acquire a blood meal, the primary nutritional requirement for egg laying is protein (Dimond et al., 1956; Singh and Brown, 1957a). In addition to egg laying, some protein is converted to energy and used to replenish maternal energy stores. It has been demonstrated that human blood meals allow for the accumulation of greater maternal energy stores than those of guinea pig, for instance, which results, resulting in greater lifetime fecundity for the mosquito (Harrington et al., 2001). When a mosquito imbibes a blood meal, she is also consuming the lipids therein, which she can presumably use directly for energy. It is possible that humans with higher plasma lipid concentrations, which correlate with higher

plasma carnitine levels, may more appealing to mosquitoes because they represent a greater energy payload, which might increase maternal survival.

It has been demonstrated that people consuming strict vegetarian diets have lower levels of plasma carnitine (Lombard et al.). It would be informative to place subjects on high-protein diets and then low-protein diets to see if their attractiveness to mosquitoes is altered. However, these dietary changes are also likely to have other metabolic consequences. To better isolate the effects of plasma carnitine specifically, mosquito attraction to vegetarians could be tested before and after L-carnitine supplementation, which has been shown to increase plasma carnitine levels (Novakova et al., 2015). To test the influence of plasma lipid concentrations on attraction, subjects could be similarly placed on high-lipid and low-lipid diets before assessing mosquito attraction.

6.3 Do blood metabolome differences between human hosts affect mosquito fitness?

If mosquito preference for certain humans over others is informed by knowledge of plasma nutrient availability, such as higher lipid content, we might expect there to be a measureable advantage to feeding on certain subjects over others. In the twenty subjects we tested in the biting assay, we did not find a relationship between subject attraction and the number of offspring produced by mosquitoes following free-feeding on that same subject (**Figure 4.5**). However, it is possible that advantages conferred by the blood of highly attractive subjects do exist, but were not obvious in this particular experimental setup. This would

particularly be the case if the nutritional advantages resulted in differences in maternal energy stores, which would increase lifetime fecundity but not egg production in a single gonotrophic cycle. Therefore the benefits of selectively targeting humans with preferred blood metabolome components might only be detected over several mosquito generations.

To explore this idea more fully, experiments should be done to (1) measure female stores of lipid, glycogen, and sugar following blood meals from different subjects and (2) establish mosquito colonies fed on blood from highly attractive and lowly attractive subjects and observe fitness effects after several generations.

6.4 If blood metabolome differences are informative, what cues do mosquitoes use to detect these differences?

If differences in the blood metabolomes of highly and lowly attractive subjects are meaningful to mosquitoes, it would be valuable to understand how they are able to sense these differences from afar. The uniport assay was designed to isolate subject odorant cues, so presumably differences in the blood metabolome would be sensed via changes in either body odorant intensity or composition. If a causative blood metabolome component is isolated, such as an increase in plasma lipids or a decrease in histidine, for example, subject body odor collections should be compared before and after interventions to alter plasma levels of these metabolites.

6.5 Other sources of variation in human attractiveness to mosquitoes

The linear models for attraction that we put forth here can account for approximately 24.1% of the variation in mosquito attraction to humans in our study. This number may shift slightly when we explore future models, however this figure likely approximates the full explanatory power of our dataset. Though this is a meaningful proportion of the variation to have accounted for, especially given that it can be attributed to novel correlates, a large percentage of variation in human attractiveness was not accounted for by any variables in our study. Our list of possible factors was not exhaustive, and it is likely that other important factors such as the composition of skin microbiota or differences in body odor profile, which have been shown to influence attraction, account for some of the remaining variation in attraction.

6.6 Investigating alternative methods for data normalization

The time series model presented here is currently the best method we have for normalizing subject attraction data collected across the course of the study. The establishment of this method represents, to our knowledge, the first implementation of time series methods on longitudinally collected data to eliminating confounding effects of differences across time due to testing across a calendar year. We plan to continue adjusting this model to pursue an optimal fit to our data. For instance, we may consider including additional regressors such as changes in room air exchange frequency, specific mosquito rearing conditions or cohorts, or even phases of the moon. By improving the fit of the time series

model, we will continue to eliminate variation in our attraction data due to factors we consider unlikely to affect mosquito attraction, so that we can better study those factors we consider most interesting.

6.7 Investigating alternative models for mosquito attraction

The two models for mosquito attraction presented in this thesis represent what are currently our best models for mosquito attraction to humans based on the variables that we measured. They are certainly reasonable models for attraction based on our data, and are likely directionally correct, but neither may yet represent the optimal model for attraction. To arrive at the model in this dissertation, we had to make decisions about how best to narrow down our large list of possible explanatory variables—a process known as feature selection. To evaluate the robustness in our feature selection, we plan to perform a bootstrap modeling experiment, where we sample from our dataset randomly with replacement and repeat our feature selection pipeline. With 1000 bootstrap resamples, we can better understand the robustness of both our data and sampling methods. Based on these results, we may need to continue to explore and integrate other methods for reducing the dimensionality of our dataset.

In the last step of our feature selection pipeline, we used stepwise regression to determine which metabolites to include in our models of attraction. Though this is a good approach to begin making exploratory models, it is not ideal because it is not able to compare all possible combinations of metabolites, and can sometimes improperly estimate variable coefficients. As we continue to

analyze this dataset, we plan to explore the possibility of alternative multivariate feature selection techniques. For example, we may use shrinkage methods such as Least Absolute Shrinkage and Selection Operator or ridge regression, which are forms of penalized regression that are particularly useful when there is multicolinearity among the regressors. These algorithms “penalize” each potential model based on the absolute size of the regression coefficients. We also plan to explore the possibility of removing colinearity within the data by first reducing its dimensionality through feature extraction techniques such as principle component analysis (PCA), then performing a regression. Though this makes interpreting the model slightly more complicated, it eliminates the possibility of overfitting and can increase the certainty of the model.

Finally, both models presented here also assume a linear relationship between explanatory variables and attraction. When using a model to understand which factors contribute to a biological phenomenon, to what degree each contribute, and in what direction, such linear methods are ideal because they allow for more straightforward biological interpretations. However, it is also possible that the relationship between our explanatory variables and attraction is not linear. To explore this possibility, we will need to apply nonlinear regression models to our data. Nonlinear models may make it more difficult to interpret the exact contributions of specific variables to mosquito attraction, but can in some cases allow for a better fit to the data with more explanatory power.

6.8 Why investigate differential mosquito attraction?

In the work presented here, we sought to harness natural variation among human subjects to understand which factors most correlate with mosquito attraction, and what information, if any, those factors are communicating to the mosquito about the host. We found, quite surprisingly, that of all the variables that we measured, blood metabolome components were most predictive of mosquito attraction. This finding opens new avenues of scientific inquiry into mosquito attraction and potentially strategies to fight mosquito-borne disease. For example, perhaps dietary changes or supplements may be meaningful –and cost effective-- methods for reducing mosquito attraction to humans. The mechanism by which differences in blood metabolites are translated into changes in body odor, perhaps through the involvement of skin bacteria, are completely unknown and warrant much further exploration.

MATERIALS AND METHODS

LDI-0731 Volunteers. All work with healthy human volunteers was approved by the Institutional Review Board of The Rockefeller University Hospital (Protocol LDI-0731). All human subjects gave their written informed consent prior to participating in these experiments. Twenty-one subjects [77 female; median age 29 (range of 21-58); 12 Caucasian, 6 African-American, 1 Asian, 1 Other; 2 Hispanic] participated in this study. Volunteers with severe insect allergies, a fear of insects, history of smoking, or history of mosquito-borne disease were excluded from participation in the study. Subjects were instructed to shower using only water at 24 hours prior to their scheduled visit. Volunteers were asked to refrain from furthering showering, vigorous physical activity, consumption of alcohol, spicy foods, garlic, onion or citrus, and use of scented personal care items for the 24-hours prior to their study visit. Compliance with these requirements was assessed using a questionnaire administered on the morning of their appointment (**Appendix C**). At the time of participation, volunteers were self-reported to be healthy. Subject oral temperature was taken directly before their visit using a single-use, disposable thermometer (Catalog #5122, Tempa•DOT™ Single-use Clinical Thermometer, 3M) and any subjects with a temperature higher than (37.7^{o}C) were rescheduled for a later date. During the study visit, volunteers refrained from eating or drinking anything besides water.

LBE-0810 Volunteers. All work with healthy human volunteers was approved by the Institutional Review Board of The Rockefeller University Hospital (Protocol LBE-0810). All human subjects gave their written informed consent prior to participating in these experiments. One hundred and fifty subjects [77 female; median age 36 (range of 18-65); 64 Caucasian, 37 African-American, 27 Asian, 12 Other; 34 Hispanic] participated in this study. Volunteers with open wounds on their forearms; using topical medications on their forearms; who wax, bleach, shave or have laser hair removal done on their forearms; using cigarettes, cigars or chewing tobacco; with severe insect allergies; with a fear of insects or their bites; with a current immunocompromising disease; with a history of smoking, drug use, or alcohol abuse; or with a history of mosquito-borne disease were excluded from participation in the study. Volunteers showered using their normal products ~48 hours (48.59 +/- 1.64, max=57.50, min=40.10) before their scheduled visit. Approximately twenty-four hours (24.50 +/- 1.62, max=33.50, min=14.10) before their scheduled visit, subjects were required to shower using only water. Volunteers were asked to refrain from furthering showering, vigorous physical activity, consumption of alcohol, spicy foods, garlic, onion or citrus, and use of scented personal care items for the 24-hours prior to their study visit. Compliance with these requirements was assessed using a questionnaire administered on the morning of their appointment (**Appendix F**). At the time of participation, volunteers were self-reported to be healthy and were not taking any prescription or over-the-counter medications or supplements. If volunteers had recently taken any over-the-counter or prescription medications or supplements,

they were scheduled for a study visit only after a medication wash-out period of at least 7 half-lives of the compound (<1% of compound in the system). Subject oral temperature was immediately taken before starting their visit using a single-use, disposable thermometer (Catalog #5122, Tempa•DOT™ Single-use Clinical Thermometer, 3M) and any subjects with a temperature higher than (37.7^{o}C) were rescheduled for a later date. During the study visit, volunteers refrained from eating or drinking anything besides water.

LDI-0731 blood sample collection. Subject whole blood samples were drawn into 10mL sodium heparin tubes (Catalog# 366480BD Vacutainer). Samples were inverted 8 times, then aliquoted. Samples for metabolomics were flash-frozen at 1 minute post blood draw, other aliquots frozen at 6 minutes 30 seconds post blood draw. Samples were stored at -80^{o}C until analysis.

LBE-0810 Blood sample collection. Blood samples were collected into 10mL sodium heparin tubes (Catalog# 366480, BD Vacutainer), then inverted 8-10 times. Whole blood samples were aliquoted and flash-frozen at 5 minutes post blood draw. To isolate plasma, remaining sample was spun down at 2500 rpms for 15 minutes at room temperature. The plasma layer was then aliquoted and flash-frozen at 20 minutes post blood draw. All samples were stored at -80^{o}C until analysis.

Mosquito rearing and maintenance. *Ae. aegypti* (Orlando strain) were reared and maintained at 25^{o}C, 70-80% relative humidity, under a 14 hr light: 10 hr dark cycle (lights on at 8 AM) as previously described (DeGennaro et al., 2013). Eggs were hatched in deoxygenated, deionized water containing powdered Tetramin tropical fish food (Tetra, Melle, Germany). At the second instar, larvae were thinned to a density of 500 larvae per 2.5 liters to prevent overcrowding. Larvae were provided with Tetramin pellets twice times daily. Adults were maintained in 28 x 28 x 28 cm cages (Catalog# 1452, Bioquip, Rancho Dominguez, CA) and given unlimited access to a 10% (w/v) sucrose solution via wick. Adult females were blood-fed on mice for stock maintenance. All blood-feeding procedures with mice were approved and monitored by The Rockefeller University Institutional Animal Care and Use Committee (IACUC, approved protocols 11487 and 14756). All behavioral experiments took place in an environmentally controlled room (Percival Scientific, Perry, IA, USA) maintained at 25^{o}C and 70-80% relative humidity.

Uniport olfactometer assay. The uniport olfactometer, modeled after one described by Klowden (Klowden and Lea, 1978), consists of a meter-long Plexiglas tube (19 cm diameter) linked on one end to an “attraction” trap (14cm long, 5 cm diameter) and a stimulus chamber (20 x 10 cm) and on the other end to a mosquito holding cartridge (World Health Organization Vector Control Research Unit in Penang, Malaysia). Walls made of white poster board surrounded the assay to reduce the influence of visual cues. Twenty-five female

Ae. aegypti mosquitoes 6-10 days post eclosion were sorted under cold anesthesia (4^{o}C) and sugar-starved, which means they were deprived of their normal food source of 10% sucrose and allowed only access to water, for approximately 20-28 hours inside mosquito holding cartridges with access to water via soaked cotton. All females used in the assay were assumed to have mated but had not taken a blood meal. At the beginning of a trial, a cartridge of mosquitoes was attached to the assay and allowed to acclimate for 5 minutes, during which time humidified room air pumped (Quite pressure pump, Catalog# 79610-81, Cole-Parmer, Vernon Hills, IL, USA) through a carbon-filter (Model# DF0070-A, Donaldson, Bloomington, MN, USA) had flowed into the system. After 5 minutes, a stimulus was inserted into the stimulus chamber: either a human forearm, covered by an arm-length nitrile glove (Catalog# BNAL, Nitritex) exposing a 12.5 cm^2 patch of skin and sealed by nitrile cuffs (Catalog# N891-N894, High Five Products, Lake Forest, IL, USA), or a 1mL aliquot of L-(+)-Lactic acid (Catalog# L6402, C.A.S. 79-33-4, Sigma Aldrich) inside the lid of a 15mL Falcon Tube (Catalog# 352096, BD Biosciences). Filtered air was then supplemented with a 10% CO_2: 90% custom air mixture (GTS-Welco, Allentown, PA, USA) to a final concentration of 5% CO_2 (as measured using CARBOCAP Hand-Held Carbon Dioxide Meter (GM70, Vaisala Inc.) via a flow-meter (Catalog# P16A1-BA0A-023-92-ST, Aalborg Instruments, Orangeburg, NY, USA). This air was then passed through the stimulus chamber, where it mixed with stimulus odors, and traveled into the body of the olfactometer at 3.8 L/min. Thirty seconds after air was supplemented with CO_2, mosquitoes were released

and allowed five minutes to fly upwind. Mosquitoes reaching the attraction chamber within the allotted time were termed “attracted,” and those who have left the holding chamber were termed “activated.” After each trial, the stimulus chamber was cleaned with 70% ethanol and dried with paper towels to remove residual odorants and a new “attraction” trap was introduced.

Free feeding assay. For each subject, two groups of 25 adult female *Ae. aegypti* mosquitoes (6-10 days post eclosion, mated, not bloodfed) were sorted under cold anesthesia (4^{o}C), placed in a holding cup (KH16A-J8000, Solo Cup Company, Lake Forest, IL, USA) and sugar starved with access to water via a soaked cotton ball for 20-28 hours before the experiment. Fasting and behavior took place in an environmentally controlled room maintained at 25^{o}C and 70-80% relative humidity. Prior to beginning the assay, one group of 25 mosquitoes were introduced into a standard 28 x 28 x 28 cm cage (Catalog# 1452, Bioquip, Rancho Dominguez, CA, USA) that had been modified to have two opposing circular openings outfitted with cotton mesh sleeves. One of the two groups of mosquitoes was allowed 5-20 minutes to acclimate inside the cage, while the other group remained in its holding cup. After acclimation, the subject, wearing a nitrile glove to protect their hand and a nitrile cuff to protect their inner elbow, inserted their arm into the cage. The arm rested on a foam cushion and was arranged such that the hand protruded from the far side of the cage and the elbow rested on the near side of the cage. The cotton sleeves were secured against the subject’s elbow and hand with rubber bands to prevent mosquito

escape and restrict biting area to the exposed forearm. Subjects were instructed to remain immobile for the entirety of the 15 minute trial, after which they removed their arm from the cage. The group of control mosquitoes were not offered a bloodmeal. Both groups were then cold anesthetized. Bloodfed mosquitoes were weighed individually, and control mosquitoes were weighed as a group. After weighing, bloodfed mosquitoes were numbered and housed individually in small holding cups (3oz Dixie cups, Dixie Consumer Products, Atlanta, GA, USA) covered with mesh. Mosquitoes were then individually followed and scored for egg laying (see Experimental Procedures: Egg laying) and hatching rate (see Experimental Procedures: Hatching rate).

Two-port olfactometer assay. The two-port olfactometer, as previously described(DeGennaro et al., 2013), consists of a large Plexiglass box (50 cm x 50 cm x 80 cm) connected to two cylindrical “attraction” traps (18 cm L x 9 cm in diameter) which were connected to two cylindrical stimulus chambers (38 cm L x 13.65 cm in diameter). On the opposing end was a box fan and filter, which were used to pull air through the stimulus chambers and into the main compartment. The main compartment was covered with white cloth to avoid the influence of external visual cues. For each trial, 50 female *Ae. aegypti* mosquitoes (5-10 days post eclosion, mated, not bloodfed) were sorted under cold anesthesia (4^{o}C) and placed in plastic cups (11.5 cm H x 11 cm in diameter) sealed with white mesh. All females used in the assay were assumed to have mated but had not taken a blood meal. Mosquitoes were sugar starved with access to water via a soaked

cotton ball for 16-24 hours before the experiment. Fasting and behavior took place in an environmentally controlled room maintained at 25^{o}C and 70-80% relative humidity. Prior to the start of the assay, mosquitoes were released into the main compartment and allowed 10-20 minutes to acclimatize. After the acclimation period, each subject introduced a forearm, covered in an arm-length nitrile glove (Catalog# BNAL, Nitritex) exposing a 12.5 cm^2 patch of skin, into the assay, the box fan was turned on, and 5% CO_2 was introduced into the stimulus ports via titration of a 10% CO_2: 90% custom air mixture (GTS-Welco, Allentown, PA, USA) using a single tube rotamer (Catalog# P16A1-BA0A-023-92-ST, Aalborg Instruments, Orangeburg, NY, USA). Mosquitoes were given 8 minutes to respond to stimulus odors and, if they choose, fly into the corresponding attraction traps. After 8 minutes, the attraction traps were sealed and the number of mosquitoes in each trap was scored. For all experiments, stimuli were alternated between ports to control for positional-bias. After each trial, the stimulus chambers were cleaned with 70% ethanol, then dried with paper towels to remove residual odorants.

Egg laying. Following acquisition of a bloodmeal by feeding directly on a human volunteer, mosquitoes were scored for egg laying. Females were allowed to recover, digest and develop their eggs for 72 hours without access to sugar and with access to water via soaked cotton. Females were then moved into plastic oviposition vials (95mm long x 25mm in diameter) each of which contained 10 mL of dH_2O and a small filter paper (Catalog# 1001-055, Whatman filter paper,

GE Healthcare, Buckinhamshire, UK) as a substrate for egg laying. Females were given 48-72 hours for egg laying, after which they were removed from the vials. Some females were frozen at -80C for energy store analysis. Egg papers were laid out to dry at 25^{o}C and 70-80% relative humidity, and when dry, egg number was counted by eye under a Nikon SMZ1500 microscope. Some egg papers were then used to score hatching rate and larval survival (see Experimental Procedures: Hatching rate and larval survival)

Hatching rate. After egg laying some egg papers were used to score hatching rate and larval survival. Dry egg papers, previously counted to determine egg number, were placed in hatching broth (deoxygenated, deionized water containing powdered Tetramin tropical fish food (Tetra, Melle, Germany) at 25^{o}C and 70-80% relative humidity. When larvae reached the second or third instar, they were manually counted.

Metabolic profiling. Global metabolic profiles were obtained for plasma from each subject using the Metabolon Platform (Metabolon) as described previously (Bridgewater BR, 2014). Samples were divided into 5 runs, balanced across age, gender, race, and raw attraction index. Briefly, Automatic MicroLab STAR system from Hamilton Company was used for sample preparation. For quality control, recovery standards were added to samples prior to extraction. Extractions were performed using an 80% (v/v) methanol/water solution. After homogenization of samples, a proprietary series of organic and aqueous extractions were carried

out in order to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions—one for liquid chromatography (LC) analysis and the other for gas chromatography (GC) analysis. Organic solvent was removed using a TurboVap (Zymark) and samples were frozen and dried under vacuum. Then, samples were prepared for either the LC/MS or GC/MS instrument. Any compounds above the detection threshold were identified by comparison to a library of purified standards or recurrent unknown entities.

Statistical analysis. Statistical analyses were performed using GraphPad Prism Software version 5.0b (GraphPad Sofrware, Inc., La Jolla, CA) and R Software Version 3.1.2 (http://www.r-project.org/).

Model for attraction. All linear models were fitted with *lm* function, and *lme* function within the *nlme* package in R using attraction as response variable and metabolites and/or questionnaires as predictors. For determining variability due to within-, between- subjects and visit components in the pilot study, a random effects model using subjects and visits as nested random effects (subject nested within visit) was fitted to raw attraction. Using the *varcomp* function within *ape* R package, we extracted the percentage of total variability due to each component.

Time series normalization. To eliminate time-series dependency due to non-observable components and exogenous factors, a state space model as

implemented at KFAS R package was fitted to the time series of human attraction using Kalman-Filter estimation. Previous to the implementation of this model, autocorrelation and partial autocorrelation plots were created using *acf* and *pacf* functions in R base package. To deal with non-observable components a local trend, a cycle of period 8 and AR(1) and MA(1) terms were added in this model. The selection of exogenous factors was carried out by evaluation of cross-correlation between attraction and exogenous time series, all differentiated. Inspection of cross-correlation plots indicated that instantaneous correlations were more important, so variables with significant Spearman correlation ($rs>0.5$) were selected for the normalization model. AR(1) and MA(1) coefficients were previously estimated by maximum likelihood using *arima* function in base R package. To avoid problems due to K-F initialization, the reversed filter was used to normalize the 16 first attraction measurements (2 subjects). Residuals from the model were normalized and rescaled to the quantiles of original attraction measurements using the *normalize.quantiles.use.target* function within the *preprocessCore* package. In the end, original range of variation was recovered but time-dependency and effects of exogenous factors were removed.

Clustering subjects by similarities in attraction. Clusters of differentially attractive subjects in the main study was determined using the *kmeans* function with Lloyd algorithm with a maximum of 700 iterations. Appropriate number of clusters was determined by the significance of their pairwise differences as evaluated by post-hoc multiple comparisons after ANOVA model.

Metabolomics analysis. For metabolomics analysis, data were normalized by Metabolon. Each value was normalized in terms of raw area counts, and then rescaled to set the median equal to 1. Missing values were then imputed with the minimum. To analyze how important metabolites were enriched in important sub/super pathways, metabolites were correlated with median attraction but with different weights for subjects according to their interquartile range (IQR) in attraction measurements. Significance for Enrichment was evaluated by an algorithm implemented in GSEA (Broad Institute, v 2.1.0) that tests if distribution of the ranks of genes in the gene set differs from a uniform distribution using a weighted Kolmogorov-Smirnov test.

Feature selection. Importance of individual metabolites/questionnaire regressors for the final model was determined by measuring variable importance according to different algorithms; random forests, glmnet, glmboost, pls, svmlinear, knn, cforest, ridge regression. Facilities within *caret* package in R were used for this goal. Top-ranked metabolites/questionnaire regressores were used as inputs for Stepwise linear and linear mixed-effects model. The final selection was done using the *stepAIC* function with the *MASS* package, by setting both backward and forward selections

APPENDIX A:

LDI-0731 Informed Consent Form

Clinical Investigation Consent Form
The Rockefeller University Hospital

1230 York Avenue
New York, New York 10065
Principal Investigator: Lindsay Dick
Phone: 212-327-6677
Fax: 212-327-7238
E-mail: Ldick@rockefeller.edu

You are being asked to join a research study, which will take place at The Rockefeller University Hospital. This form tells about the research. You should ask questions of the person who is explaining this form to you. After you feel that you understand the research, if you want to be part of the study, you will be asked to sign the form. You can always ask more questions and can later change your mind about staying in the study.

If you join the research study, you will take part for up to 4 months. The research study as a whole will last about 1 year.

About 20 people will take part in the research study. This study will involve 5 visits by you.

Title of the research study: Pilot Study: Isolation of Cues that Drive Mosquito Preference for Certain Human Hosts

I. What this research study is about, and the reason for doing this research.

The reason for doing this research is to study why mosquitoes are attracted to certain humans more than others. Female mosquitoes (such as the species *Aedes aegypti*) naturally feed on human blood as a protein source to develop their eggs. This means that after females have mated with a male and are ready to make their eggs, they are very attracted to humans. Previous studies have shown that during this time, *Aedes aegypti* female mosquitoes are more attracted to some humans than to others. It is not completely understood why the mosquitoes have this preference.

We think that female mosquitoes may target humans whose blood is particularly full of proteins or other nutrients important for producing healthy eggs and may target humans whose blood has more of these proteins and nutrients. We also think that humans may release odors that either attract or repel the mosquitoes and that these smells are produced by the bacteria that normally live on our skin and interact with our sweat. It is possible that different skin bacteria may explain differences in how people smell and how frequently they are targeted by mosquitoes.

We first need a way to find people who are frequently-targeted or rarely-targeted by mosquitoes. In this pilot study, we will determine the best way to make this distinction. We will also determine the best way to measure the health of mosquitoes' offspring and the best way to survey the types of bacteria that live on every human's skin.

Understanding why mosquitoes choose to bite particular groups of people may eventually allow us to develop new tools to reduce the spread of deadly mosquito-borne diseases such as malaria, dengue fever, yellow fever, and West Nile fever.

We are asking you to take part in this research study because you are a healthy adult between 18-65 years of age.

II. What is going to happen in this research study?

In this part, we explain the meaning of words that we are going to use to describe this study:

- "**Substances drawn from your body**" refer to **liquids** such as blood or urine. When we draw blood, take tissue, or take other substances from your body, we are taking a "**sample**."
- This is a research study and by law, we cannot tell you or your doctor the results of experimental tests.

This study will consist of 5 visits. Visits may be scheduled as soon as 1 day and as long as 3 weeks apart from one another.

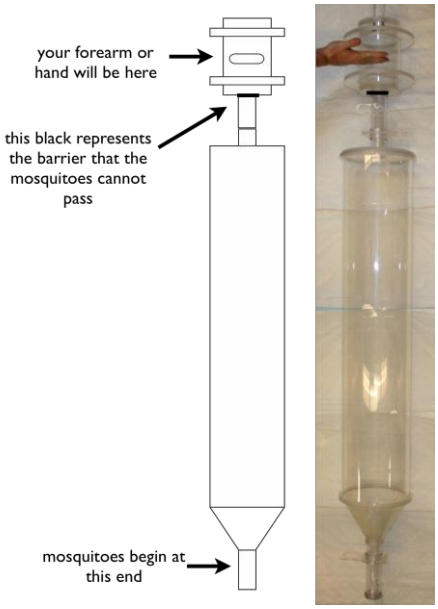
During this first visit, you will undergo a 'consent process.' During this process, the purpose of the study, what will happen in the study, any possible risks and benefits, and your right to withdraw at any time will be explained in more detail. You should ask any questions you have. You will be asked to sign an Informed Consent Form to indicate that you understand the information and are willing to take part in the study. You may still ask questions, or withdraw from the study at any time during the study. Your participation is voluntary.

During this time, you will also be asked a series of questions to determine if you are a good candidate for this study, and you will have your height, weight, blood pressure, pulse, respirations and temperature recorded. You will be asked to complete a screening questionnaire. You will also have a measurement of both mid-forearms taken with a measuring tape. You must be HIV negative to participate in this study. You will need to take a rapid HIV test to confirm you are negative. You will be asked to sign a separate consent form for HIV testing. The rapid HIV test is a swab test of your gums. These results will be determined in 20 minutes. If the results are negative, the screening will proceed. If the results are positive, you will be referred to a doctor who specializes in HIV care. Additionally, a trained nursing staff member will draw approximately 25 mL (2 tablespoons) of your blood in order to test for two forms of Hepatitis, measure your level of anemia and identify your blood type (A, B, AB, O). You must be negative for both forms of hepatitis to participate in this study. Finally, if you are a female of child-bearing age, you will be asked to provide a urine sample for a urine pregnancy test. If this result is positive, you will not be eligible to participate in this study.

If you are eligible for this study, you will be scheduled for your next study visit. On the

day of each upcoming visit, you must avoid using antibacterial soap as well as any scented personal care items such as sunscreen, body wash, body mist, cologne, or perfume. If you are not willing to follow these personal care instructions, you will not be able to participate in this study. At the end of your screening visit, you will be given directions to the Vosshall Lab where your next 3 visits will take place.

The purpose of your second visit is to determine how much mosquitoes like your smell. We will accomplish this by performing an "olfactometer test." An olfactometer is a large, enclosed plastic tube where we will put the mosquitoes. This tube is divided into two sections, which are separated by mesh screens. We will put the mosquitoes into one section of the tube, and you will put either your forearm or your hand into the other section of this tube. We will release the mosquitoes from a holding chamber at one end of the olfactometer, and they will be able to smell your skin and, if they choose, fly towards your smell. The mosquitoes cannot, however, reach your skin to bite you because there is a mesh screen blocking the way. This test does not require you to be bitten by any mosquitoes, and it is highly unlikely that you would be bitten by a mosquito. Here is a picture of the olfactometer, so that you can better understand how it works:



In the Vosshall Lab, the testing room will feel warm and humid. This room is kept at about 78% humidity and 25˚C (77˚F). You will sit in a comfortable chair where you will first have both your skin temperature taken by an infrared thermometer and oral body temperature taken by a disposable thermometer. You will also complete a visit questionnaire. You will put either your forearm or hand into the olfactometer for five minutes. Your arms will be gently supported so that you do not feel muscle tiredness from holding up your arms. During this time you will feel a gentle stream of filtered air pass over your skin. Then, the test will be over and there will be a 10-minute break, which you will spend in a waiting room. During this break, you will be asked not to eat, drink, or use tobacco products. After approximately 10 minutes, you will return to the

testing room to participate in another skin temperature and olfactometer measurement using the opposite forearm or hand.

From now on, we will use the term "**olfactometer procedure**" to refer to one measurement of your left hand or forearm and one measurement of your right hand or forearm. One olfactometer procedure is expected to take approximately 30 minutes.

During this visit, you will repeat the olfactometer procedure three more times. During your first 10-minute break, you will be asked to wear clean cloth sleeves on the skin of both of your forearms for 30 seconds to collect your unique human scent. During that time period, you may not remove the sleeves and you must not get them wet. During the remainder of your breaks, you will not be asked to wear any sleeves. By the end of the visit, both forearms and hands will be tested.

Your third visit will be identical to your second visit, with the exception that this time, during your first 10-minute break you will be asked to wear new cloth sleeves on your forearms for 5 minutes. During the remainder of your breaks, you will not be asked to wear any sleeves.

Your fourth visit will be identical to your second visit, with the exception that you will need to wear new cloth sleeves on your forearms for a total of 30 minutes. To accomplish this, you will wear the sleeves for the entirety of your first three 10-minute breaks. During the remainder of your breaks, you will not be asked to wear any sleeves.

At the end of your fourth visit, you will be given two new cloth sleeves sealed in plastic bags, which you will be asked to wear to your final visit. You should put on these sleeves 18 hours prior to your next visit. You will receive a phone call reminding you at what time you need to put on the cloth sleeves. During that time period, you may not remove the sleeve and you must not get it wet. Additionally, you must avoid using antibacterial soap and scented personal care items such as sunscreen, body wash, body mist, cologne, or perfume on the day you wear the sleeve. If you are not willing to follow these personal care instructions, you will not be able to participate in this part of the study.

At the beginning of your fifth and final study visit, we will collect the cloth sleeves from your forearms. Then, a sample of bacteria will be taken from a small area of skin on either your forearm or your hand. To collect this sample, a dry, sterile cotton swab will be rubbed across a small area of your skin for 30 seconds. Then you will participate in two olfactometer procedures. During the 10 minutes prior to each measurement, you will be instructed to wash the hand or forearm that will be placed into the olfactometer under warm tap water using unscented soap for 30 seconds. After you complete 2 olfactometer procedures, you will be directed to the Rockefeller University Hospital Outpatient Research Center (OPRC), where a trained nursing staff member will draw a 10 mL (2 teaspoons) sample of your blood. If we are unable to obtain a blood sample at this visit, we will ask you to return to the OPRC within the next few days for a repeat attempt to draw your blood. You will not be compensated for any repeat visit.

Here is a summary of all of the visits required for this study.

Visit	Description	Expected Length
1	Screening, instructions	2 hours
2	Olfactometer test, 30 second sleeves	2 hours
3	Olfactometer test, 5 minute sleeves	2 hours
4	Olfactometer test, 30 minute sleeves	2 hours
5	Return wearing 18 hour sleeves, skin swab, Olfactometer test, blood draw	2 hours

In this study, you will not receive routine care for any medical conditions you may have.

Your medical information and test results will be written in your Hospital chart. The researchers of the study may also keep separate records with information about you and your study tests.

III. What are the risks of taking part in this research study?

There may be some risks and discomforts in taking part in this study. We know that these risks and discomforts may happen during this study:

We will not intentionally let you get bitten by any mosquitoes during this course of this study, however if you accidentally get bitten, mosquito bites can lead to itching, redness, discomfort and swelling around the site of the bite. Anti-inflammatory cream (containing 1% hydrocortisone) or local anesthetic ointment or antihistamine cream will be provided upon request by the Vosshall Lab at no charge to you.

During your participation in the olfactometer test, the heat and humidity in the experimental room may cause you to feel faint. Other potential side effects from these tests include arm stiffness or discomfort from remaining immobilized for 5-minute intervals, and anxiety or panic due to the close proximity of mosquitoes and/or fear of being bitten. If you experience these side effects, you may take a break or leave at any time.

Potential side effects from wearing a cloth sleeve for up to 18 hours may be discomfort, overheating, itching, or rash.

Potential side effects associated with having your blood drawn include discomfort, pain, bleeding, bruising, nerve damage and infection at the needle site, and fainting or feeling lightheaded.

Potential side effects associated with having your skin swabbed include redness, irritation, or minor abrasions.

If you feel discomfort of any kind, you can withdraw from the study at any time. There may be other risks and discomforts that we do not know about now, but we will tell you

about them when we know.

IV. What are the benefits of taking part in this research study?

There will be no benefit to you. Instead, others may benefit in the future from what we learn from this study.

V. Who will be able to see the information learned about you in this research study?

We will keep your personal information private, and will do our best to keep this information confidential. We will listen to what you say we may do with this information, and we will follow the law. For example, by New York State law, hospitals must inform the New York State Department of Health if we find that you have a reportable communicable disease, such as a sexually transmittable disease, like chlamydia, hepatitis, gonorrhea, syphilis and HIV-1.

We will share information about you only with government agencies that oversee this research and the people at the Hospital and at The Rockefeller University in connection with their duties.

During this study, only the researchers will know that your samples came from you, because your stored samples will be identified only by a special code instead of your name. As a result, others who study your samples will not know that they came from you and will not be able to figure out that they came from you.

If the researchers publish the results of this study, they will not mention your name or other information that could identify you.

VI. What are the payment arrangements?

There is no cost to you for being in this research study.

You will be compensated for all completed visits as long as you have followed all instructions. If you leave the study early, your payment will be prorated so that you will be compensated for all completed visits up until that time.

The payment schedule is as follows:

Visit	Description	Expected Length	Payment
1	Screening, instructions	1 hour	$0
2	Olfactometer test, 30 second sleeves	2 hours	$40
3	Olfactometer test, 5 minute sleeves	2 hours	$40
4	Olfactometer test, 30 minute sleeves	2 hours	$40
5	Turn in 18 hour sleeve, skin swab, Olfactometer test, blood draw	2 hours	$60

Payment will be made to participants who fill out a form from The Rockefeller University Finance Office and are eligible for and want to receive payment.

This research involves live mosquitoes, whose behavior is influenced by the environment. If on the day of your scheduled appointment we see that this behavior is atypical, we may have to cancel your appointment and reschedule for another day. We do not expect this to occur frequently.

If research using your samples helps develop a drug or another product that is sold to the public, the drug company, the University and the researcher may share in some of the profits. For example, a cell line from your samples could be used to make a product for sale. There are no plans to pay you any money resulting from such discoveries. However, by signing this form, you do not give up any rights you may have.

VII. What happens if you don't want to stay in this study or your participation is ended?

You can choose if you want or do not want to be part of this study. If you do not join, there is no penalty and no one will hold this against you. If you decide to join this study, you may change your mind and stop taking part in the study at any time, and this will not be held against you. Information about you up to that time may stay a part of the study.

During this study, the researchers may learn new information that might make you change your mind about whether you want to stay in the study. You will be given that information promptly.

If you decide to join the study now but later want to stop, you should let the researcher know.

The researchers also may stop you from taking part in this study, even if you do not choose to stop being in it. You may be asked to leave the study if you become ill during the course of the experiment, fail to keep your appointments, or fail to follow protocol directions. Your participation may also be involuntarily terminated should the research study be cancelled by the researchers.

If you stop or if you cannot finish the study for any reason, we will pay you for the part of the study that you have finished.

VIII. Consent to the use, storage and sharing of your samples for separate research studies

May we store, use, and share your blood and/or tissue samples with other investigators at Rockefeller and elsewhere for separate studies for many years? Your samples will either be stripped of information identifying them as yours or coded (we will hold the key to the

code) so that they cannot be identified as having come from you. Other data related to your sample, but that does not identify you may accompany the samples.

Any time in the future, you may withdraw your consent to use any samples that have not already been used in research or shared. If you withdraw your consent, the remaining unused samples will be destroyed, unless the samples cannot be identified as having come from you.

Would you like us to store, use, and share your blood and/or tissue samples/associated data as described above?

Yes _____ No _____

IX. Who do you call if a medical problem results from this research study?

If you believe that this study has led to a medical problem, you should call the researcher listed below right away. The researcher will help you get appropriate, available medical care.

Name: Barbara O'Sullivan, MD
Phone No.: 212-327-8441
Cell No.: 646-772-3000
Fax No.: 212-327-8449

The Rockefeller University does not plan to pay for medical care that you may have as a result of taking part in this study at The Rockefeller University Hospital. However, you do not give up any rights you may have to seek compensation by signing this form.

X. Who do you contact if you have questions about the research study?

Please ask as many questions as you want about this research study and this consent form. If you agree to take part in this study and have questions later on, contact the following researcher:

Name: Lindsay Dick
Phone No.: 212-327-6677
Cell No.: 724-840-2293
Fax No.: 212-327-7238

If you have any concerns about your experience while taking part in this research study, you may contact The Rockefeller University Institutional Review Board (IRB) Office at (212) 327-8410, or the Office of Clinical Research at (212) 327-8408.

XI. May we have permission to contact you about future studies?

May we contact you by phone to find out if you are interested in hearing about new research studies? Contact would be made by the Rockefeller staff of the Clinical Research Support Office for Recruitment. If you decide at any time that you no longer want to be contacted, please tell us, and we will stop calling you.

Would you like us to contact you about future research studies?

Yes _____ No _____

If you say "no" to this question, this will not affect your participation in this study.

AGREEMENT TO PARTICIPATE -- SIGNATURES REQUIRED

I have read this consent form, and my questions have been answered.

A copy of this consent form will be given to you. Please keep a copy of the form as it contains important information that you may wish to refer to during the research study and thereafter.

I hereby voluntarily consent to take part in this research study.

Name of the Study Participant (Print) _____

_____ _____

Signature of Study Participant **Date (To Be Filled in by Study Participant)**

Signature of the Person Conducting the Informed Consent Discussion

I have explained the research protocol and this consent form to the participant and have answered the participant's questions about this research study and/or the consent process.

Name of Person (Print) _____

_____ _____

Signature of Person Discussing Consent **Date (To Be Filled in by Person Discussing Consent)**

APPENDIX B:

LDI-0731 Screening Questionnaire

Pilot Study: Isolation of Cues that Drive Mosquito Preference for Certain Human Hosts

Subject ID:_____ Gender: _____ Birthdate:_____

<u>Screening Questionnaire</u>. (Please circle your answers)

1. Ethnicity:

   American Indian or Alaska Native
   Asian
   Native Hawaiian or Pacific Islander
   Black or African American
   Hispanic or Latino
   White
   Other, please specify _____

2. Where were you born?

   City: _____Country: _____

3. Have you ever lived in a country other than the United States?

   No
   Yes, Countries? _____
   Time spent there? _____
   At what age? _____

4. In your own experience, how attractive are you to mosquitoes?

1	2	3	4	5
not at all		somewhat		extremely

5. If you are in a room together with a group of people, are you the first one to be bitten by mosquitoes?

   Yes / No / I don't know

6. Do you get mosquito bites more often than other people?

   More often than others
   As often as others
   Less often than others
   I don't know

7. On how many occasions do you get mosquito bites per year?

   Not even once
   One to ten times
   Ten to twenty times
   More often

8. Usually, where are you when you get bitten by mosquitoes?

   At home
   At work
   On vacation
   Somewhere else; please specify_____

9. When you do get bitten by mosquitoes, where are the bites most often located? Select no more then 2 answers.

   Face
   Neck
   Torso
   Arms
   Hands
   Legs
   Feet

10. When you do get bitten by mosquitoes, what type of skin reaction occurs most often?

    None
    I am never bitten by mosquitoes
    Red bump smaller than ¼" inch in diameter (head of pin)
    Red bump between ¼" and ½" in diameter (shirt button)
    Red bump between ½" and 1" in diameter (nickel)
    Red bump more than 1" in diameter (quarter)

11. When you do get bitten by mosquitoes, how much does the bite itch?

1	2	3	4	5
not at all		somewhat		a lot

12. Do you try and protect yourself from mosquito bites?

    No, never
    Yes, I use repellent; please specify _____
    Yes, I use a mosquito net
    Yes, I wear protective layers of clothing
    Yes, I do something else; please specify _____

13. How often do you wear perfume/aftershave?

    Never or only on special occasions
    On some days
    Once a day
    More than once a day

    If Yes, what kind? _____

14. How often do you wear deodorant?

    Never or only on special occasions
    On some days
    Once a day
    More than once a day

    If Yes, what kind? _____

15. How often do you drink alcohol?

    a. Never (Skip Part b)
       Once or twice/month
       Once or twice/week
       Almost every day
       Every day

    b. What type of alcohol do you drink most frequently?

       Beer
       Wine
       Liquor

16. How often do you eat food with garlic in it?

    Never
    Once or twice/month
    Once or twice/week
    Almost every day
    Every day

17. How often do you eat spicy foods?

    Never
    Once or twice/month
    Once or twice/week
    Almost every day
    Every day

18. Do you own any pets?

    Yes / No

    If yes, what kind(s)? _____

        Dog / cat / bird / reptile / other (please specify)

18. What is your blood type and Rh factor?

A negative	A positive
B negative	B positive
AB negative	AB positive
O negative	O positive

I do not know my blood type or Rh Factor_____

Subject initials: _____

Date: _____

Original: 02 02 11
Rev: 06-16-11

APPENDIX C:

LDI-0731 Visit Questionnaire

Pilot Study: Isolation of Cues that Drive Mosquito Preference for Certain Human Hosts

Subject ID:_____ Temp:_____

Visit # Questionnaire

1. Have you started taking any new medications (including antibiotics) since your last visit?
   ☐ Yes, _____
   ☐ No

2. Have you started taking any new vitamins/supplements since your last visit?
   ☐ Yes, _____
   ☐ No

3. Have you consumed any alcohol in the past 24 hours?
   ☐ Yes, _____
   ☐ No

4. Have you eaten any spicy food in the past 24 hours?
   ☐ Yes, _____
   ☐ No

5. Have you eaten any food with garlic or onion in the last 24 hours?
   ☐ Yes, _____
   ☐ No

6. Have you showered or gone swimming in the last 24 hours?
   ☐ Yes, _____
   ☐ No

7. Have you exercised in the past 24 hours?
   ☐ Yes, _____
   ☐ No

8. Have you used scented personal care items in the past 24 hours?
   ☐ Yes, _____
   ☐ No

9. Have you had your forearms tattooed or pierced since your last visit?
   ☐ Yes, _____
   ☐ No

10. Do you have any open cuts, wounds, or burns on your forearms today?
    - ☐ Yes, _____
    - ☐ No

11. Do you have any rashes, bites, or skin irritations on your forearms today?
    - ☐ Yes, _____
    - ☐ No

12. Have you had your forearms waxed, shaved, or bleached, since your last visit?
    - ☐ Yes, _____
    - ☐ No

(For women only)

1. Are you currently using hormonal birth control?
    - ☐ Yes, _____
    - ☐ No

2. What was the date of the first day of your last menstrual period?
    _____

    - ☐ I am post-menopausal

Subject initials: _____
Date: _____

APPENDIX D:

LBE-0810 Informed Consent Form

Clinical Investigation Consent Form
IRB Rev 2012

The Rockefeller University Hospital

1230 York Avenue
New York, New York 10065
Principal Investigator: Lindsay Lee Bellani, BS
Phone: 212-327-6677
Fax: 212-327-7238
E-mail: Lbellani@rockefeller.edu

You are being asked to join a research study, which will take place at The Rockefeller University Hospital. This form tells about the research. You should ask questions of the person who is explaining this form to you. After you feel that you understand the research, if you want to be part of the study, you will be asked to sign the form. You can always ask more questions and can later change your mind about staying in the study.

If you join the research study, you will participate for 2 study visits.
The research study as a whole will last about 2 years.

About 160 people will take part in the research study.

Title of the research study: Cues underlying the evolution of differential mosquito attraction

I. What this research study is about, and the reason for doing this research.

Female mosquitoes (such as the species *Aedes aegypti*) feed on human blood to help them make their eggs. This means that female mosquitoes are very attracted to people to obtain blood. Other research has shown that female mosquitoes are more attracted to some people than to others, but we don't completely understand why.

We think that female mosquitoes may like to bite people whose blood has more sugars or amino acids (the building blocks of proteins) that are important for producing healthy eggs. In this study, we would like to see how attracted mosquitoes are to you. Then, we want to look at the levels of sugars and amino acids in your blood, and see how these affect the health of mosquitoes.

Understanding why mosquitoes choose to bite some people may allow us to find new ways to slow the spread of diseases that mosquitoes can carry.

We are asking you to take part in this research study because you are a healthy adult between 18-65 years of age.

II. What is going to happen in this research study?

In this part, we explain the meaning of words that we are going to use to describe this study:

- "**Substances drawn from your body**" refer to **liquids** such as blood or urine. It can also mean tissues such as skin, cells and **DNA**. **Cell**s make up all parts of your body. DNA is inside all the cells of your body and carries your genetic or inherited information. When we draw blood, take tissue, or take other substances from your body, we are taking a "**sample**."

This study will consist of 2 visits.

During your first visit, you will undergo the consent process. Informed consent is a process to help you understand the purpose of the research study, what will happen in the study, possible risks and benefits, and your right to withdraw from the study at any time. All of this information will be explained to you in detail. You should ask any questions you have until you feel that you understand what is asked of you to participate. You may then want to enroll, or you may decide not to join the study. The decision to participate is entirely up to you. Even after the study has started, you may at any time ask more questions, or decide to withdraw from the study.

During this time, you will also be asked a series of questions about your current health status to determine if you are a good candidate for this study. A nursing staff member will also record your height, weight, blood pressure, pulse, respirations and temperature. You will be asked to complete a screening questionnaire about mosquito attraction and personal habits; we will also measure your mid-forearm with a measuring tape. If you are a woman of child-bearing age, you will be asked to provide a urine sample for a urine pregnancy test. If this result is positive, blood will be drawn to confirm a pregnancy. If the blood test is positive then you will not be eligible to participate in this study.

If you are confirmed eligible for this study, you will be contacted to schedule the next study visit.

Exactly 48 hours before your study visit, you must take a shower using soap. Exactly 24 hours prior to your study visit, you must then take a shower using only water. During this final shower, it is important that no soap, shampoo, baby wipes, lotions, etc. are used. During the 24 hours between your final shower and your visit, you may not shower again or go swimming. You must also avoid using any kind of soap on your arms. In addition, you must not use scented personal care items such as sunscreen, body wash, body mist, cologne, or perfume. Please also avoid vigorous physical activity (exercise), as well as consuming spicy foods, citrus fruits, garlic, and alcohol during the 24 hours before your scheduled visit. If you are not willing to follow these personal care instructions, you will not be able to participate in this study.

Your second visit will take place at the Vosshall Lab and the Rockefeller University Hospital Outpatient Unit. On this visit, we will first take your temperature, and you will be asked to complete a visit questionnaire.

Then, we will determine how much mosquitoes like your smell. We will accomplish this

by performing an "olfactometer test." An olfactometer is a large, enclosed plastic tube that is divided into two sections, which are separated by mesh screens. We will put the mosquitoes into one section of the tube, and you will put your arm into the other section of this tube. We will release the mosquitoes from a holding chamber at one end of the olfactometer, and they will be able to smell your skin and, if they choose, fly towards your smell. The mosquitoes cannot, however, reach your skin to bite you because there is a mesh screen blocking the way. This test does not require you to be bitten by any mosquitoes and it is highly unlikely that you would be bitten by a mosquito. Here is a picture of the olfactometer, so that you can better understand how it works:

For each olfactometer test, we will help you put on a long sleeve over your arm, which will have a small, hole cut from it. Then, you will be led to the testing room, which will feel warm and humid. This room is kept at about 78% humidity and 25˚C (77˚F). You will sit in a comfortable chair where you will put your arm into the olfactometer for five minutes. Your arm will be gently supported so that you do not feel muscle tiredness from holding up your arm. During the test you will feel a gentle stream of filtered air pass over your skin for 5 minutes. After the test is over, we will measure the temperature of your skin.

You will then leave the tropical room and return to the waiting room for a short (approximately 10 minute) break. While on this break you are asked not to eat, although you may only drink water. After the break, you will return to the testing room to participate in another olfactometer test. You will complete up to 10 olfactometer trials according to this procedure.

This research involves live mosquitoes, whose behavior is influenced by the environment. If on the day of your scheduled appointment we see that this behavior is abnormal, we may have to cancel your appointment and reschedule for another day. We do not expect this to occur often.

When you have completed all olfactometer tests, you will be escorted to the Rockefeller University Hospital Outpatient Research Center (OPRC), where a trained nursing staff

member may draw up to 55 milliliters (approximately 3½ tablespoons) of your blood. You do not need to fast before these bloods are drawn. Using this blood sample, we will find out your blood type (A, B, AB, or O) and Rh factor (negative or positive) and your CBC (complete blood cell count), as well as the components that make up your blood. We will also measure your blood sugar levels, total protein content, blood urea nitrogen (BUN) and creatinine levels and levels of certain vitamins (B12). We want to see if these components can explain why mosquitoes may be attracted to you or not.

In case you are accidentally bitten by a mosquito, which is unlikely, we will give you AfterBite swipe and hydrocortisone cream, which both relieve itching, which you may apply to your bites if you choose. We will also give you a mosquito bite treatment recommendation card, which will give you tips for preventing itching as well as our contact information if you have questions.

Here is a summary of the visits required for this study:

Visit	**Description**	**Approximate Length**
1	Screening, instructions	1 hour
2	Blood draw, olfactometer tests	3 hours

If we are unable to obtain a blood sample at this visit, we will ask you to reschedule for a repeat of the entire visit on another day.

- This is a research study and by law, we cannot tell you or your doctor the results of experimental tests. However, if we find anything that may be important for your health, we may suggest that you have tests done by a New York State-approved laboratory.
- If you would like, we will be able to tell you your blood type (A, B, AB, or O) and Rh factor (negative or positive), CBC (complete blood count), total protein, blood glucose, blood urea nitrogen (BUN) and creatinine levels, and vitamin B12 levels at the conclusion of the study.

In this study, you will not receive routine care for any other medical conditions you may have.

Your medical information and test results will be written in your Hospital chart. The researchers or the Sponsor of the study may also keep separate records with information about you and your study tests.

III. What are the risks of taking part in this research study?

There may be some risks and discomforts in taking part in this study. We know that these risks and discomforts may happen during this study:

During your participation in the olfactometer test, the heat and humidity in the experimental room may cause you to feel uncomfortable or faint. Other potential side

effects from these tests include arm stiffness or discomfort from remaining immobilized for 5-minute intervals.

If you are bitten by a mosquito, which is unlikely, you may experience itching, redness, discomfort and swelling around the site of the mosquito bites. These reactions are temporary, and subside quickly for most people. Anti-inflammatory cream (containing 1% hydrocortisone), local anesthetic ointment, or antihistamine cream will be provided upon request by the Vosshall Lab at no charge to you. It may be helpful to hold the affected arm under very hot water for a few seconds as a way to reduce itching and swelling without using pills or creams. You do not have to use such treatments if you do not want to.

A very rare, severe allergic reaction might occur after mosquito bites. This reaction is called "anaphylaxis". Anaphylaxis can cause problems breathing, hives and a severe drop in blood pressure. It must be treated immediately by two injections with a device called "EpiPen" into the muscle of the thigh. You will also receive immediate medical attention. Some people have a reaction called, "Skeeter syndrome". This reaction causes a large amount of swelling of the arm and hand after the mosquito bites, causes the person to feel mildly ill, and have a fever. These symptoms go away within about a week and don't require treatment.

Potential side effects associated with having your blood drawn include discomfort, pain, bleeding, bruising, nerve damage and infection at the needle site, and fainting or feeling lightheaded.

If you feel discomfort of any kind, you can withdraw from the study at any time.

There may be other risks and discomforts that we do not know about now, but we will tell you about any new information discovered which might affect your decision to participate or remain in the study.

IV. What are the benefits of taking part in this research study?

There will be no benefit to you from taking part in this study. Instead, others may benefit in the future from what we learn from this study.

V. Who will be able to see the information learned about you in this research study?

We will keep your personal information private, and will do our best to keep this information confidential. We will listen to what you say we may do with this information, and we will follow the law. For example, by New York State law, hospitals must inform the New York State Department of Health if we find that you have a reportable communicable disease, such as a sexually transmittable disease, like chlamydia, hepatitis, gonorrhea, syphilis and HIV-1.

We will share information about you only with government agencies that oversee this research and the people at the Hospital and at The Rockefeller University in connection with their duties.

During this study, only the researchers will know that your samples came from you, because your stored samples and videotaping will be identified only by a special code instead of your name. As a result, others who study your samples will not know that they came from you and will not be able to figure out that they came from you.

If the researchers publish the results of this study, they will not mention your name or other information that could identify you.

VI. What are the payment arrangements?

There is no cost to you for being in this research study.

You will be compensated $60.00 for completing the entire study, as long as you have followed all instructions. You will not be compensated for the screening visit.

Payment will be made to participants who fill out a form from The Rockefeller University Finance Office and are eligible for and want to receive payment.

If research using your samples helps develop a drug or another product that is sold to the public, the University and the researcher may share in some of the profits. For example, a cell line from your samples could be used to make a product for sale. There are no plans to pay you any money resulting from such discoveries. However, by signing this form, you do not give up any rights you may have.

VII. What happens if you don't want to stay in this study or your participation is ended?

You can choose if you want or do not want to be part of this study. If you do not join, there is no penalty and no one will hold this against you. If you decide to join this study, you may change your mind and stop taking part in the study at any time, and this will not be held against you. Information about you up to that time may stay a part of the study.

During this study, the researchers may learn new information that might make you change your mind about whether you want to stay in the study. You will be given that information promptly.

If you decide to join the study now but later want to stop, you should let the researcher know. Please call or email the researcher directly as soon as possible.

The researchers also may stop you from taking part in this study, even if you do not choose to stop being in it. You may be asked to leave the study if you become ill during the course of the experiment, fail to keep your appointments, or fail to follow protocol

directions. Your participation may also be involuntarily terminated should the research study be cancelled by the researchers, or if an adverse event occurs to you or others in the study.

If you stop or if you cannot finish the study for any reason, we will pay you for the part of the study that you have finished.

VIII. Consent to use, storage and sharing of your samples for separate research studies

The scientific value of your samples and the information obtained from them is greatly increased if we can share them with other scientists at universities and pharmaceutical companies worldwide. May we store, use, and share your blood and/or tissue samples and data with other investigators at Rockefeller and elsewhere for separate studies for many years? Your samples will either be stripped of information identifying them as yours or coded (we will hold the key to the code) so that they cannot be identified as having come from you. Other data related to your sample, but that does not identify you may accompany the samples.

Any time in the future, you may withdraw your consent to use any samples that have not already been used in research or shared. If you withdraw your consent, the remaining unused samples will be destroyed, unless the samples cannot be identified as having come from you.

Would you like us to store, use, and share your blood and/or tissue samples/associated data as described above?

Yes _____ No _____

If you say "no" to this question, this will not affect your participation in this study.

IX. Who do you call if a medical problem results from this research study?

If you believe that this study has led to a medical problem, you should call the researcher listed below right away. The researcher will help you get appropriate, available medical care.

Name: Arlene Hurley, ANP
Phone: 212-327-7433
Cell: 917-572-5017
Fax: 212-327-7373

The Rockefeller University does not plan to pay for medical care that you may have as a result of taking part in this study at The Rockefeller University Hospital. However, you do not give up any rights you may have to seek compensation by signing this form.

X. Who do you contact if you have questions about the research study?

Please ask as many questions as you want about this research study and this consent form. If you agree to take part in this study and have questions later on, contact the following researcher:

Name: Lindsay Lee Bellani
Phone: 212-327-6677
Fax: 212-327-7238
Email: Mosquitostudy@rockefeller.edu

If you have any concerns about your experience while taking part in this research study, you may contact The Rockefeller University Institutional Review Board (IRB) Office at (212) 327-8410, or the Office of Clinical Research at (212) 327-8408.

XI. May we have permission to contact you about future studies?

May we contact you by phone to find out if you are interested in hearing about new research studies? Contact would be made by the Rockefeller staff of the Clinical Research Support Office for Recruitment. If you decide at any time that you no longer want to be contacted, please tell us, and we will stop calling you.

Would you like us to contact you about future research studies?

Yes _____ No _____

If you say "no" to this question, this will not affect your participation in this study.

AGREEMENT TO PARTICIPATE -- SIGNATURES REQUIRED

I have read this consent form, and my questions have been answered.

A copy of this consent form will be given to you. Please keep a copy of the form as it contains important information that you may wish to refer to during the research study and thereafter.

I hereby voluntarily consent to take part in this research study.

Name of the Study Participant (Print) _____

_____ _____

Signature of Study Participant **Date (To Be Filled in by Study Participant)**

Signature of the Person Conducting the Informed Consent Discussion

I have explained the research protocol and this consent form to the participant and have answered the participant's questions about this research study and/or the consent process.

Name of Person (Print) _____

_____	_____
Signature of Person Discussing Consent	**Date (To Be Filled in by Person Discussing Consent)**

APPENDIX E:

LBE-0810 Screening Questionnaire

Subject ID:_____ **Study: LBE-0810**

Screening Questionnaire

1. Please specify your gender:

   ☐ Male
   ☐ Female

2. What is your date of birth?: _____ (mm/dd/yyyy)

3. What is your race?

   ☐ American Indian or Alaska Native
   ☐ Asian
   ☐ Black or African American
   ☐ Native Hawaiian or Pacific Islander
   ☐ White or Caucasian
   ☐ Other, please specify _____
   ☐ Do not wish to specify

4. What is your ethnicity?

   ☐ Hispanic or Latino
   ☐ Not of Hispanic or Latino origin
   ☐ Do not wish to specify

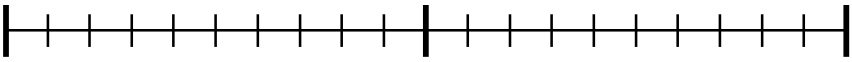
5. Where were you born?

   City, State (if applicable): _____
   Country: _____

6. Have you ever lived in a country other than the United States for more than 6 months?

☐ No
☐ Yes
Country___ _____
Time spent there_ _____
At what age_____

7. In your own experience, how attractive are you to mosquitoes?

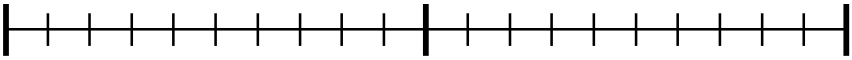


not at all somewhat extremely ☐ I don't know

8. If you are in a room together with a group of people, are you usually the first one to be bitten by mosquitoes?

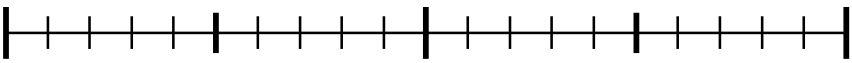
☐ Yes
☐ No
☐ I don't know

9. Do you get mosquito bites more often than other people?



Much less often than others As often as others Much more often than others ☐ I don't know

10. Approximately how many mosquito bites do you get per year?



None 10 20 30 40 or more ☐ I don't know

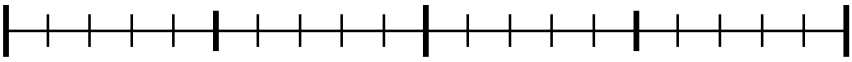
11. Usually, where are you when you get bitten by mosquitoes?

☐ At home
☐ At work
☐ On vacation
☐ Somewhere else, please specify_____
☐ I don't know

12. When you do get bitten by mosquitoes, where are the bites ***most often*** located? Select up to two answers.

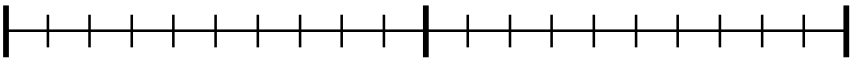
☐ Face
☐ Neck
☐ Torso
☐ Arms
☐ Hands
☐ Legs
☐ Feet
☐ I don't know

13. When you do get bitten by mosquitoes, what type of skin reaction occurs most often? *Please consult the diagram provided.*



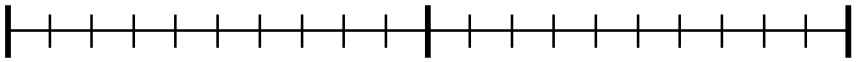
No reaction | ¼" in diameter | ½" in diameter | ¾" in diameter | 1" in diameter or larger | ☐ I don't know

14. When you do get bitten by mosquitoes, how much does the bite usually itch?



not at all | somewhat | extremely | ☐ I don't know

15. In general, how do you feel about being bitten by mosquitoes?



Not at all annoyed | Somewhat annoyed | Extremely annoyed | ☐ I don't know

16. Do you usually try to protect yourself from mosquito bites?

☐ No, never
☐ Yes, I use repellent
please specify brand or type (e.g. DEET, herbal)
_____
☐ Yes, I sleep under a mosquito net
☐ Yes, I wear protective layers of clothing
☐ Yes, I do something else
please specify _____

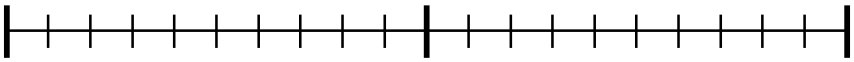
17. Do you exercise regularly?

☐ Yes
☐ No (if no, skip questions 18 and 19)

18. In an average week, how much do you exercise?

Sessions per week: _____
Minutes per session: _____

19. On average, how intense is your exercise?



Light
i.e. walking, housework, gardening

Moderate
i.e. light bicycling, power walking, yoga, dancing

Vigorous
i.e. running, rock climbing, circuit weight training

☐ I don't know

20. On average, how often do you eat each of the following?

	never	1-2x a month	1-2x a week	3-6x a week	1x per day	More than 1x a day
Dairy						
Milk	☐	☐	☐	☐	☐	☐
Cheese	☐	☐	☐	☐	☐	☐
Yogurt	☐	☐	☐	☐	☐	☐
Fruits (apples, berries, melons)	☐	☐	☐	☐	☐	☐
Grains (bread, pasta, cereal, rice)	☐	☐	☐	☐	☐	☐
Vegetables (broccoli, corn, peppers)	☐	☐	☐	☐	☐	☐
Protein foods						
Seafood/Fish	☐	☐	☐	☐	☐	☐
Red meat (beef, pork, lamb)	☐	☐	☐	☐	☐	☐
White meat (chicken, turkey)	☐	☐	☐	☐	☐	☐
Eggs	☐	☐	☐	☐	☐	☐
Soy products/Meat Alternatives	☐	☐	☐	☐	☐	☐
Beans and Peas	☐	☐	☐	☐	☐	☐
Nuts and Seeds	☐	☐	☐	☐	☐	☐

21. Are you currently vegan (no meat, seafood, eggs, or dairy)?

☐ Yes
☐ No

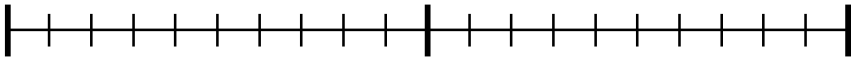
22. Do you currently follow a high protein/low carb diet (Atkins, South Beach, Dukan, Stillman diets)?

☐ Yes
☐ No

23. Do you currently add protein supplements (whey protein, amino acid powder) to your food or beverages?

☐ Yes
☐ No

24. What best describes your current diet?

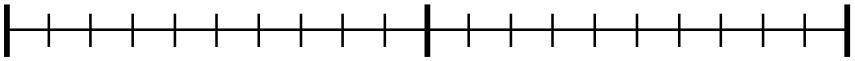


Low calorie | Moderate calorie | High calorie | ☐ I don't know

25. Is there anything else you'd like to tell us about your diet?

_____
_____
_____
_____
_____
_____
_____

26. Do you currently consider yourself:



Underweight | Just right | Overweight | ☐ I don't know

27. How often do you wear perfume/cologne?

☐ Never or only on special occasions
☐ On some days
☐ Once a day
☐ More than once a day

If Yes, what kind? _____
If Yes, where on your body do you wear it? _____

28. How often do you wear deodorant?

☐ Never or only on special occasions
☐ On some days
☐ Once a day
☐ More than once a day

If Yes, what kind? _____

29. How often do you drink alcohol?

a. ☐ Never (Skip Part b)
☐ Once or twice/month
☐ Once or twice/week
☐ Almost every day
☐ Every day

b. What type of alcohol do you drink most frequently?

☐ Beer
☐ Wine
☐ Liquor

30. How often do you eat food with garlic in it?

☐ Never
☐ Once or twice/month
☐ Once or twice/week
☐ Almost every day
☐ Every day

31. How often do you eat spicy foods?

☐ Never
☐ Once or twice/month
☐ Once or twice/week
☐ Almost every day
☐ Every day

32. Do you own any pets?

☐ Yes
☐ No

If yes, what kind(s)? *Check all that apply*

☐ Dog
☐ Cat
☐ Bird
☐ Reptile
☐ Other
Please specify_____

33. What is your blood type and Rh factor?

☐ A / Rh negative
☐ A / Rh positive
☐ B / Rh negative
☐ B / Rh positive
☐ AB / Rh negative
☐ AB / Rh positive
☐ O / Rh negative
☐ O / Rh positive

☐ I do not know my blood type or Rh Factor

Subject initials: _____

Date: _____

APPENDIX F:

LBE-0810 Visit Questionnaire

Subject ID:_____ **Study: LBE-0810**

Temperature:_____

Visit Questionnaire

1. Date of Birth: _____ (mm/dd/yyyy)

2. Have you started taking any medications (including antibiotics) since your screening visit?
   ☐ Yes
   What type(s) and when? _____
   If you know the dose, write it here:_____
   ☐ No

3. Have you started taking any new vitamins/supplements since your screening visit?
   ☐ Yes
   What type(s) and when? _____
   ☐ No

4. What have you eaten before your visit today (i.e. breakfast, lunch, snacks)?
   ☐ Meal 1
   What time? _____
   What did you eat? (please be as specific as possible)
   _____
   ☐ Meal 2
   What time? _____
   What did you eat? (please be as specific as possible)
   _____
   ☐ Meal 3
   What time? _____
   What did you eat? (please be as specific as possible)
   _____
   ☐ Meal 4
   What time? _____
   What did you eat? (please be as specific as possible)
   _____

5. Have you consumed any alcohol in the past 24 hours?
   ☐ Yes
   What type(s) and what time?
   _____
   ☐ No

6. Have you eaten any spicy food in the past 24 hours?
   ☐ Yes
   What type(s) and what time?
   _____
   ☐ No

7. Have you eaten any food with garlic or onion in the last 24 hours?
   ☐ Yes
   What type(s) and what time?
   _____
   ☐ No

8. What was the date/time of your last shower using soap?
   mm/dd/yyyy_____
   Time _____[AM/PM]

9. What was the date/time was your last shower using only water?
   mm/dd/yyyy_____
   Time _____[AM/PM]

10. Have you gone swimming in the last 24 hours?
    ☐ Yes
    What time? _____
    ☐ No

11. Have you showered or washed your arms in the last 24 hours?
    ☐ Yes
    What time and with what soaps?
    _____
    ☐ No

12. Have you exercised in the past 24 hours?
    - ☐ Yes
      What type(s) and what time?
      _____
    - ☐ No

13. Have you used scented personal care items in the past 24 hours?
    - ☐ Yes
      What type(s) and what time?
      _____
    - ☐ No

14. Have you had your arms tattooed or pierced since your screening visit?
    - ☐ Yes
      When? Where on your body is the tattoo/piercing located?
      _____
    - ☐ No

15. Do you have any open cuts, wounds, or burns on your arms today?
    - ☐ Yes
      What, where is it located, and when did it appear?
      _____
    - ☐ No

16. Do you have any rashes, bites, or skin irritations on your arms today?
    - ☐ Yes
      What, where is it located, and when did it appear?
      _____
    - ☐ No

17. Have you had your arms waxed, shaved, or bleached, or had laser hair removal treatment on your arms since your screening visit?
    - ☐ Yes
      Which and when? _____
    - ☐ No

(For women only)

1. Are you currently using hormonal birth control?
   ☐ Yes
   What type? _____
   ☐ No

2. What was the date of the first day of your last menstrual period?
   _____
   ☐ I am post-menopausal

Subject initials: _____

Date: _____

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