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Each year, up to 1.6 million people contract leishmaniasis from the bite of a phlebotomine sand fly infected with the *Leishmania* (Kinetoplastida:Trypanosomatidae) pathogen. Therefore, the control of sand flies has been the topic of intense research for many years. Traditional control methods, such as pesticides, have not provided solace when applied singly. However, the discovery of biological compounds used by sand flies as a means of chemical communication has been applied and combined with multiple other control techniques in an integrated pest management (IPM) scheme. This study focused on the development an effective bioassay method for use in the characterization of the *Lutzomyia verrucarum* sensu stricto (ss) male sex pheromone. *Lutzomyia verrucarum* ss vectors both *Leishmania peruviana,* the causative agent of Andean cutaneous leishmaniasis and *Bartonella baciliformis,* the etiological agent of bartonellosis or Carrion's disease. As little is known about compounds that mediate intraspecific communication (pheromones) in *Lu. verrucarum* ss, this study represented an important first step towards the final goal of developing an IPM strategy for *Lu. verrucarum* ss. As *Lu. verrucarum* ss is only accessible in Peru, the closely related *Lu. longipalpis* was used in this study. This study sought to (1) develop an operative bioassay and (2) determine the biological qualities that define optimum sand fly responders in the chosen bioassay. Linear three-chamber and cage olfactometers were tested for their effectiveness as a bioassay system, and vertical and horizontal trap orientation was also evaluated for the ability to attract and capture sand flies. As the male produced sex pheromone of *Lu.*

longipalpis is known to attract unmated conspecific females, extracts of *Lu. longipalpis* males were used to gauge the responsiveness of females. Bioassays tested the effect of female response to conspecific male extracts, and categories of tested females varied in hours of male exposure (HME) (e.g., ≤ 8 HME, ≤ 24 HME, and ≤ 48 HME) as well as being blood fed or not. Additionally, the timing of male maturity was also determined. Results indicated that cage olfactometers more accurately gauged the natural behaviors of *Lu. longipalpis* females than did linear olfactometers. Horizontal and vertical traps performed equally well, and blood fed females with ≤ 24 HME were found to be the most responsive to male *Lu. longipalpis* extracts. Also, all *Lu. longipalpis* males matured by 20 hours under laboratory conditions, and maturity was found to occur in as few as 4 hours. These results not only contribute to our understanding of *Lu. longipalpis*, but have also identified responsive targets and defined appropriate methodologies for use with *Lu. verrucarum* ss.

THE ESTABLISHMENT OF A BEHAVIORAL BIOASSAY TO STUDY *LUTZOMYIA VERRUCARUM* MALE SEX PHEROMONES USING *LUTZOMYIA LONGIPALPIS* AS A MODEL SPECIES

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CHAPTER I

INTRODUCTION

Phlebotomine Sand Flies

Phlebotomine sand flies (Diptera: Pyschodidae) are the proven vectors of the leishmaniasis disease group worldwide. This multiform disease currently afflicts 12 million people in 88 countries and is the result of infection with a parasitic metacyclic protozoan belonging to the *Leishmania* genus (Kinetoplastida: Trypanosomatidae) (Alvar et al., 2006; Desjeux, 1996; Samady et al., 1996). Few other pathogens are transmitted by sand flies. Carrion's disease, caused by the α-proteobacterium *Bartonella baciliformis,* is vectored by *Lutzomyia verrucarum* sensu stricto (s.s.) in the Andean region and some arboviruses (e.g., *Phlebovirus*) are transmitted in the Old World, but none are the global burden that is the leishmaniasis group (Depaquit et al., 2010; Ready, 2013). Over 800 species of sand flies have been described, but only around 70 species have been associated with vectoring *Leishmania* (Bermudez et al., 1993; Desjeux, 2004; Murray, 2004; Ready, 2013; Samady et al., 1996; Seccombe et al., 1993). Phlebotomine sand flies are: rarely larger than 3 mm, silent fliers, covered in setae (i.e., "hairy"), and variable in color across species- nearly white to almost black. Activity patterns are normally nocturnal or crepuscular and both sexes will feed on natural sugars (e.g., plant sap or aphid honeydew), while only females are hematophagous (Killick-Kendrick, 1999). Sand flies are holometabolous insects with a life cycle consisting of four distinct larval stages,

a pupal stage, and a sexually mature adult stage. Life cycles are usually completed in 40-50 days in laboratory settings (Volf and Volfova, 2011). Sexual dimorphism is evident as males possess a slender abdomen with terminal claspers whereas the abdomen of the female is rounded with no conspicuous genital terminalia. Sandflies are predominantly found in warmer climates –with ranges extending from around 50^{o}N latitude to approximately 40^{o}S latitude (Killick-Kendrick, 1999).

In the New World, the medically important genus is *Lutzomyia.* Around 400 species of *Lutzomyia* have been identified and many species are categorized into species complexes (Bermudez et al., 1993; Killick-Kendrick, 1999; Young and Duncan, 1994). Within the *Lutzomyia* genus, the *Verrucarum* group contains at least 40 species that have been divided into series based on male genitalia morphology. The number of series and to which series individuals belong has been debated. Young and Duncan (1994) acknowledged three series, *Serrana, Townsendi, and Verrucarum,* but Galati more recently recognized a fourth, *Pia,* and a possible fifth (*Evansi*) was also postulated (Galati, 1995; Galati et al., 1995; Young and Duncan, 1994). Characteristic of individuals within species complexes, females are typically isomorphic (Cohnstaedt et al., 2011). Conversely, males can usually be distinguished by genitalia morphology, but this positive identification is many times extrapolated to identify females caught alongside them, thereby introducing blatant and unwelcome uncertainty (Cohnstaedt et al., 2011; Testa et al., 2002). There are still many cases in which this male genitalia identification method is inapplicable, however (Cohnstaedt et al., 2011; Young and Duncan, 1994). In those instances, other methods such as egg electron microscopy, isozyme analysis, nuclear and

mitochondrial based phylogenetics, and karyotyping have been applied, but even with these advanced techniques many relationships within the group are still largely uncertain (Beati et al., 2004; Escovar et al., 2002; Kreutzer et al., 1990; Sierra et al., 2000). Without appropriate identification methods, it is nearly impossible to draw solid conclusions regarding disease transmission (e.g., vector distribution, vectorial competence, etc.).

The biology and ecology of the *Verrucarum* group is highly varied and still largely unknown. *Lutzomyia verrucarum* s.l. is geographically widespread- from Mexico southward throughout Latin and South America- and contains multiple vectors of diseases such as American and Andean cutaneous leishmaniasis and bartonellosis (Young and Duncan, 1994). Most *Leishmania* transmissions from *Verrucarum* group vectors occur in rural, forested regions and areas near plantations (Alexander et al., 1995). Geographical preferences at the series level indicate that sand flies in the *Pia* and *Serrana* series inhabit lower altitudes within montane and tropical regions while lower montane areas are also inhabited by many species in the *Townsendi series.* The *Verrucarum* series is less specific and is known to occupy habitats along an altitudinal gradient -from tropical lowlands to higher montane areas (Bejarano et al., 2003). Individual species' biology and ecology show great variability throughout the group. Several species have been documented to be aggressive, anthropophilic blood-feeders including *Lu. andina, Lu. serrana, Lu. spinicrassa,* and *Lu. torvida.* Furthermore, *Lu. andina* is known to feed in both the day and night while *Lu. spinicrassa* has been documented to be naturally infected with *Leishmania* parasites. In Colombia, *Lu. youngi* can be found throughout the

year and displays a nocturnal anthropophilic feeding habit (Alexander et al., 1995). There are also species that do not seem to represent a considerable threat. *Lu. novoae* has been mostly collected from caves while *Lu. moralesi* is not known to feed on humans (Young and Duncan, 1994). *Lutzomyia verrucarum* s.s. is capable of vectoring both *Leishmania peruviana*, the causative agent of Andean cutaneous leishmaniasis and *Bartonella baciliformis,* the etiological agent of bartonellosis or Carrion's disease (Davies et al., 1993; Young and Duncan, 1994). Bartonellosis is a diphasic disease with the first phase known as Oroya fever. This acute stage is characterized by joint and muscle pain, fever, and hemolysis, and can be fatal if left untreated. The second phase is known as verruga peruana, and symptoms typically include the development of skin lesions, usually found on the head, arms, and legs. Carrion's disease can enter phase two within weeks of recovery from Oroya fever, but can also occur years later or sometimes even without the onset of phase one (Amano et al., 1997; Kosek et al., 2000; Schultz, 1968). Two outbreaks of Carrion's disease occurred in the late 1990s in areas of Peru that were not previously considered endemic for the disease, sparking a renewed medical interest (Ellis et al., 1999; Kosek et al., 2000). In Peru, *Lu. verrucarum* is highly associated with domestic areas, and is the principal endophagic (indoor-biting) sand fly found in both dry and rainy seasons. Likewise, it is most abundant in areas that receive 400-800 mm of rain annually at elevations of 1,500-3,000 m. The range of *Lu. verrucarum* is also expanding, both altitudinally and geographically (Cohnstaedt, 2009). For these reasons, *Lu. verrucarum* presents a significant risk to human health in Peru. This threat warrants a

better understanding of *Lu. verrucarum* biology if effective control efforts are to be implemented.

Leishmaniasis: Global Burden, Life Cycle, and Control Efforts

Leishmaniases account for the 9th greatest parasitic disease burden worldwide (Hotez et al., 2004; Yamey, 2002). Annually, an estimated 0.9-1.6 million people will contract leishmaniasis out of an at-risk population of 350 million (Alvar et al., 2006, 2012; Samady et al., 1996). Leishmaniasis generally affects individuals through the visceral, cutaneous, or mucocutaneous forms, but all forms affect an individual's tissues in a similar way: mononuclear phagocytic cells bearing *Leishmania* protozoans produce hyperplasially mediated histiocytomas (World Health, 1990). Leishmaniases account for 2,357,000 disability-adjusted life years (DALYs, a cumulative quantity reflecting untimely morbidity, mortality, and disability), and claim nearly 60,000 human lives each year (Hotez et al., 2004). The devastating burden of the global leishmaniases is the inspiration for those who seek to control it. The life cycle of a *Leishmania* parasite begins when the amastigote form, found within host macrophages, is ingested by a phlebotomine sandfly along with a bloodmeal. Once inside the peritrophic membrane of the sandfly, the amastigotes lyse the host macrophage and begin to develop into the larger flagellated form known as the promastigote, or leptomonad. These mobile promastigotes penetrate the peritrophic membrane and multiply through binary fission in the foregut (*Leishmania* subgenus) or midgut and hindgut (*Viannia* subgenus). After successful replication has occurred, the promastigotes produce a gel-like substance known as promastigote secretory gel (PSG) that also contains metacyclic promastigotes. Upon subsequent

feeding on a host (e.g., a human), the PSG matrix is egested into the individual through the sandfly proboscis (Bates, 2007). Macrophages and other reticuloendothelial cells phagocytize these newly acquired leptomonads, which then transform back to the amastigote stage. Within the reticuloendothelial cells the amastigotes multiply until cells are lysed, allowing them to metatastically and contiguously infect other macrophages, thereby developing histiocytomas in various tissues. The cycle reaches completion whenever this human is fed upon by another sandfly (CDC, 2010; Murray et al., 2005; Samady et al., 1996; World Health, 1990, 2014).

Epidemiologically speaking, multiple facets of the leishmanial system have been targeted by researchers in an effort to reduce the disease affliction. In some studies, the reservoir host has been the target. In Brazil, a culling regimen was instituted for domestic dogs found to be seropositive for leishmaniasis (Ashford et al., 1998). This method was controversial and short-lived; it proved to be as inefficient as it was ethically unsound (Ashford et al., 1998; Ribas et al., 2013). In a recent study, theoretical models stressed the importance of vector control for leishmaniasis- validating years of previous research while reinforcing the need for continued exploration (Ribas et al., 2013). Previous studies investigating various methods of proposed control of the hematophagus vectors of leishmaniasis (i.e., phlebotomine sand flies) target at least one element of their life cycle- primarily reproduction or foraging. Complete habitat destruction followed by continued manipulation of the targeted area proved to be very effective in the control of *Phlebotomus papatasi* - an old world vector that parasitized and lived in great gerbil (*Rhombomys opimus*) burrows (Faizulin et al., 1976). This method is severely limited

however, as habitat destruction is both labor-intensive and likely detrimental to numerous non-targeted species. In addition, larval habitat is difficult to define in many geographic regions (Killick-Kendrick, 1987). Insecticides, whether sprayed, infused, or impregnated, have been tested as a means of control. Spraying of houses or outbuildings with insecticides such as DDT is initially a successful means of control (Bray et al., 2010; Mukhopadhyay et al., 1996). However, these treatments are ephemeral and must be re-applied, thereby driving up costs significantly. Bed-netting infused with permethrin or lamdacyhalothrin has also shown potential in reducing disease transmission through vector control (Reyburn et al., 2000). This method targets sandflies that have already entered the domicile, however. Therefore, it represents the last line of defense and is unacceptable as the ideal control scheme. In other research, canid reservoir hosts have been outfitted with deltamethrin-impregnated collars or bathed in the chemical, resulting in an anti-feeding effect and increased mortality of vectors, respectively (Guanghua et al., 1994; Killick-Kendrick et al., 1997). Nonetheless, even in the most efficacious of treatments, the deleterious effect on biotic components stemming from insecticide implementation cannot be overlooked (Barker, 1958; Pimentel, 1995).

Integrated Pest Management

The amount of literature on the inefficiency and detrimental nature of pesticides is overwhelming. Pesticides are often non-selective, long-lasting pollutants, and their overuse has led to the development of many modes of resistance (e.g., metabolic, target site, and behavioral) in the arthropods that they were supposed to control (Denholm and Rowland, 1992; Logan et al., 2013; Shani, 2000). For many years, a strategy known as

integrated pest management, or IPM has shown a great deal of promise. This strategy does not seek to do away with pesticides altogether, as they can be quite useful, but rather combine multiple techniques in such a way as to minimize negative effects while maximizing safe and effective control (Shani, 2000). For several years, chemical communication has been utilized in IPM schemes. The application of intraspecific chemicals known as pheromones emerged in the early stages of IPM history. Pheromones are selective, non-contaminating chemicals that alter behavior in the receiver (Shani, 2000). In the 1960s, cotton farmers were struggling against the most damaging cotton pest of all, the pink bollworm *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) (Lykouressis et al., 2004). Larvae of the pink bollworm infest cotton plants, producing bolls, and the feeding of the pink bollworm larvae within these bolls results in high losses of cotton yield. Research on the pink bollworm eventually led to the isolation and identification of a mixture known as "gossyplure", an isomeric mixture of the female-produced sex pheromone, in 1973 (Hummel et al., 1973). In subsequent years, traps have been baited with gossyplure both in the presence and absence of insecticides and gossyplure has been systematically released over large fields. All have achieved their goal of attracting and killing male moths and disrupting natural mating, respectively, and a more environmentally-conscious reduction in the damage to cotton growth has been the outcome (Critchley et al., 1991; Gaston et al., 1977; Lykouressis et al., 2004). Since then, research on chemical communication has led to hundreds of discovered pheromones, the annual production of millions of chemical lures, and the control of insects worldwide on millions of hectares (Witzgall et al., 2010).

Lutzomyia longipalpis: Biology and Pheromones

In the New World, the most widely studied sand fly has been *Lutzomyia longipalpis.* This is primarily due to *Lu. longipalpis* being the principal vector of *Leishmania chagasi*, the etiological agent of American visceral leishmaniasis (AVL). Likewise, *Lu. longipalpis* can in many ways be seen as a generalist. Geographically, *Lu. longipalpis* has a wide but intermittent distribution from Mexico to northern Argentina. Within that range, it is locally abundant near domesticated animals in peridomestic and rural areas and can tolerate exceedingly dry conditions. Besides being the most important vector of *Leishmania chagasi, Lu. longipalpis* is known to be highly susceptible to infection with multiple other *Leishmania* parasites (Soares and Turco, 2003; Soto et al., 2001; Watts et al., 2005; Young and Duncan, 1994). These attributes, along with anthropogenic habitat manipulation (e.g., deforestation) have likely contributed to the urbanization of AVL. The expansion of AVL cases into a non-endemic urban environment has resulted in an urgency that places an increased importance of the discovery and implementation of new methods of control for the ubiquitous *Lu. longipalpis.*

Many efforts aimed at *Lu. longipalpis* control have targeted different stages of the life cycle. As alluded to earlier, vector control efforts typically target a particular life stage. The previous successes achieved in the control of other insects (e.g., pink bollworm) did not go unnoticed by sand fly researchers, and many goals have now been realized in sand fly pheromone research as well. For example, it has been documented that gravid female sand flies preferentially select an oviposition site upon recognition of

chemical cues that indicate the suitability of the site for larval development. In *Lu. longipalpis*, both host feces and conspecific eggs serve as an oviposition attractant (Andrade et al., 2008; Dougherty et al., 1995; Elnaiem and Ward, 1992). This component of sand fly biology demonstrates potential for the development of control and surveillance traps. However, during this stage in the sand fly life cycle, the females have already interacted with a host at least once. For example, these gravid sand flies may quite likely have just completed their second blood-feeding, and if infected, spread the disease. Another life stage that can be targeted is the unfed females that have not yet interacted with a host. In the wild, female *Lu. longipalpis* are known to typically mate and blood-feed simultaneously, and this is in part due to male *Lu. longipalpis* behavior. Male *Lu. longipalpis* form leks, or mating aggregations around potential hosts, and they will disperse their pheromones to attract conspecific females. It has been documented that this is an effective strategy, as female *Lu. longipalpis* are attracted to these sites based upon the recognition of host odors and male sex pheromones (Andrade et al., 2008; Bray and Hamilton, 2007a; Soares and Turco, 2003). Once a female has been attracted to lekking males, courtship behavior ensues. *Lu. longipalpis* males will flap their wings, likely producing auditory signals and/or dispersing pheromones. The female will then flap her wings as well, and mating will occur (Bray and Hamilton, 2007b). As evidenced here, mate-seeking females may be targeted with male sex pheromones. This stage is particularly important to target as it has the potential to impact the fecundity of a population following the deployment of attract-and-kill traps in the environment (Hamilton, 2008).

In New World sand flies, sex pheromones emanate from pheromone disseminating structures, which are typically located on the 3rd and 4th tergites of male *Lutzomyia* sandflies, and much research has been conducted on *Lu. longipalpis* s.l. (Hamilton, 2008). Sex pheromones from members of the *Lu. longipalpis* species complex have been characterized and, with the addition of genetic analyses, discernable population characteristics have been determined in Brazil. *Lu. longipalpis* in the first group produce copulation songs in bursts as well as twenty carbon cembrene-1 sex pheromones in Sobral, N.E. Brazil. Populations belonging to this group likely represent one species throughout their range. The second group is much more varied; sand flies in these populations emit sixteen-carbon (1*S*, 3*S*, 7*R*) 3-methyl-α-himachalene (Jacobina, Bahia State, N.E. Brazil) or (S)-9-methylgermacrene-B (Lapinha Cave, Minas Gerais, S.E. Brazil) pheromones as well as variable-pattern pulse-type copulation songs, and are likely composed of various sibling species living in sympatry (Araki et al., 2009; Hamilton et al., 1999; Spiegel et al., 2005; Tashiro, 2000) . Although *Lu. longipalpis* s.l. is now generally regarded as a species complex, there is still no general agreement on the number or distribution of sibling species within the group following over 40 years of research on the topic (Bauzer et al., 2007).

While pheromone glands of *Lu. longipalpis* from Jacobina, Brazil produce multiple compounds, (1*S*,3*S*,7*R*)-3-methyl-α-himachalene alone elicits behavioral responses and comprises 90% of gland production (Hamilton et al., 1994). Experiments conducted outdoors and in a laboratory setting have documented the attraction of *Lu. longipalpis* females to conspecific male sex pheromones, both alone and in conjunction

with host odor for (1*S*,3*S*,7*R*)-3-methyl-α-himachalene and (S)-9-methylgermacrene-B (Bray and Hamilton, 2007b; Bray et al., 2009, 2010, 2014; Morton and Ward, 1989a; Spiegel et al., 2005; Ward et al., 1993). The potential role of (1*S*,3*S*,7*R*)-3-methyl-α-himachalene as a male *Lu. longipalpis* aggregation factor has also been proposed (Spiegel et al., 2005). Male sex pheromone attract-and-kill traps show great promise to be an effective tool for monitoring and surveying populations of *Lu. longipalpis.* Firstly, the chemical components of many pheromones can be readily synthesized in the laboratory from commercially available materials (Hamilton, 2008). Secondly, pheromone traps have demonstrated the capability to be long-lasting. A study in Brazil found that *L. longipalpis* were attracted for 12 weeks to a trap that released the synthetic ±-9-methylgermacrene-B (Bray et al., 2014). Thirdly, pheromones are quite specific, therefore beneficial insects are unlikely to be harmed. It is also unlikely that resistance would develop, as this would surely lead to severe mating disruption followed by population decline and subsequent disappearance (Shani, 2000).

Goals and Hypotheses

Although much research has been conducted on *Lu. longipalpis* sex pheromones and their potential for vector control, there is a paucity of data for the medically important *Lu. verrucarum*. Although Ward et al. (1993) discovered pheromone disseminating structures in male *Lu. verrucarum*, no study has attempted to analyze or behaviorally test the male sex pheromone (Ward et al., 1993). As part of a broader collaborative project between NAMRU-6 (Naval Medical Research Unit No. 6, PI – Gissella Vasquez), UNCG (PI – Gideon Wasserberg, behavioral bioassays), and NCSU

(PI – Coby Schal, chemical extraction and electrophysiological analysis) aimed at characterizing *Lu. verrucarum* male sex pheromones (funded by the U.S. Department of Defense), the goal of the project described in this thesis was to optimize the behavioral bioassay using a closely related species (*Lu. longipalpis*) known for producing male sex pheromones (Bray and Hamilton, 2007b; Bray et al., 2009, 2010, 2014; Morton and Ward, 1989a; Spiegel et al., 2005; Ward et al., 1993). The reason for not conducting these pilot studies directly on *Lu. verrucarum* is that lab rearing of this species is very challenging. Production of this species anywhere in the world is typically very limited, which does not allow for behavioral bioassays to be conducted.

Specific Aims

In order to optimize the *Lu. longipalpis* male sex pheromone detection behavioral bioassay, my study focused on the following two specific aims:

1) Identify the Best Bioassay Apparatus and Design to Study the Response of *Lu. longipalpis* Females to Male Sex Pheromones

2) Identify the Female *Lu. longipalpis* Life Stage Most Sensitive to Male Sex Pheromones

Strategy

The development of a behavioral bioassay for the purpose of studying chemical communication in *Lu. verrucarum* was organized in such a way that the innate behavioral traits of *Lu. longipalpis* were given top priority. That is, it was necessary for the bioassay to be developed in the beginning, and subsequently refined throughout the study. The determination of biological traits that maximized female attraction to male pheromones in

bioassay respondents were dependent upon a bioassay system that facilitated natural responses in the subjects.

Specific Aim 1. Identify the Best Bioassay Apparatus and Design to Study the Response of Lu. longipalpis Females to Male Sex Pheromones

Question 1. Does *Lu. longipalpis* Female Attraction to Conspecific Male Extracts Differ Between Three-Chamber Linear and Free Flight Sticky Trap Cage Olfactometers?

Hypothesis

Lu. longipalpis female attraction to the conspecific male pheromone is affected by how easily the pheromone can be detected and assessed. Therefore, the response of *Lu. longipalpis* females to the conspecific male pheromone is expected to differ between free flight sticky trap cage and three-chamber linear olfactometers.

Rationale

Experiments in which *Lu. longipalpis* females were found to be attracted to the conspecific male pheromone typically used either a Y-tube olfactometer or a cage olfactometer to assess these behaviors (Hamilton et al., 1999a, 1999b; Morton and Ward, 1990; Ward et al., 1989). Whereas Y-tube olfactometers were composed of three distinct chambers (treatment, control, and introduction) connected via small extensions, cage olfactometers were composed of simply one large chamber. Y-tube olfactometers also possessed fans that placed introduced sand flies in the downwind direction of treatment

and control chambers while cage olfactometers did not rely on an external force to circulate air currents. I chose to compare two variations of these olfactometer types - a three-chamber linear olfactometer (i.e., a condensed Y-tube olfactometer) and a free flight sticky trap cage olfactometer to broadly determine which containment method would result in the greatest response of *Lu. longipalpis* females to male *Lu. longipalpis* pheromone extracts. The pull of odorant-containing air across the lateral chambers of the three-chamber linear olfactometer (hereafter linear olfactometer) via an external vacuum would likely allow the sand flies in the linear olfactometers to better assess the provided experimental choices from one central point, the medial chamber. However, sand flies in the free flight sticky trap cage olfactometers (hereafter cage olfactometers) are provided with a more natural range of motion and flight. Therefore, it is likely that the response of *Lu. longipalpis* females to the male pheromone will be dissimilar between olfactometer types.

Question 2. Does the Concentration of *Lu. longipalpis* Male Extract Affect the Attractive Response of Conspecific Females?

Hypothesis

As large aggregations (i.e., leks) of *Lu. longipalpis* males attract conspecific females to hosts in the wild, *Lu. longipalpis* females are expected to display higher attraction towards increasing conspecific male pheromone concentrations in linear olfactometers.

Rationale

Previous studies have documented that *Lu. longipalpis* females do in fact respond more favorably towards higher male pheromone concentrations. Ward et al. (1989) were able to double the female contacts to discs containing male extracts by increasing from one to eight *Lu. longipalpis* male pheromone equivalents (ME) (Ward et al., 1989). Bray et al. (2010) recorded a 2.8 fold increase in the number of female *Lu. longipalpis* caught in traps when the amount of male pheromone dispensed was changed from 50 ME to 500 ME in field conditions (Bray et al., 2010). However, the methodology used to survey the effects of female response to changes in pheromone concentration varied between both of the previously mentioned studies. I therefore chose to test varying concentrations of male pheromone in linear olfactometers in an attempt to standardize the results found in the literature.

Question 3. Do *Lu. longipalpis* Females Display Male-Abundance Dependent Attractive Responses to Conspecific Live Males in Linear Olfactometers?

Hypothesis

As increasing numbers of *Lu. longipalpis* females are attracted to increasing male lek sizes in natural field conditions, the attractive response of *Lu. longipalpis* females to males is expected to be positively correlated with the number of males placed in linear olfactometers.

Rationale

Previous studies have documented that the number of females visiting lekking sites is positively correlated with the number of males, or size of the lek in field settings

(Jones and Quinnell, 2002; Kelly and Dye, 1997). Jones and Quinnell (2002) also documented that females contacted and mated with a male at a faster rate when that male belonged to a larger lek in laboratory settings (Jones and Quinnell, 2002). In this study, I chose to compare how female response differed when both the number of males and the type of male containment was varied in linear olfactometers. Acknowledging the behavioral modifications of *Lu. longipalpis* females resulting from exposure to large groups of conspecific males, I predicted that *Lu. longipalpis* females would display increasingly attractive behaviors increasing male group sizes placed in linear olfactometers, regardless of the male containment type used.

Question 4. Is the Attractive Response of *Lu. longipalpis* Females to the Conspecific Male Extract Affected by Chamber Accessibility in Linear Olfactometers?

Hypothesis

Y-tube olfactometers of variable designs have been used to document the attractive effect of *Lu. longipalpis* male extracts on conspecific females. Therefore, linear olfactometers differing in chamber accessibility are not expected to affect the attractive response of *Lu. longipalpis* females to conspecific male extracts.

Rationale

Lu. longipalpis male extracts were found to significantly attract more conspecific females to the end of Y-tubes (4.5 cm diameter, 23 cm length) than the control (hexane) in a study by Ward et al. (Ward et al., 1989). A similar attractive response to *Lu. longipalpis* male extracts was observed in conspecific females by Hamilton et al. (1999) using Y-tubes of 9 mm in inside diameter and 10 cm in length (Hamilton et al., 1999a,

1999b). In this study, linear olfactometers from Specific Aim 1, Question 1 had PVC tubes (9 mm inside diameter, 6 cm in length) placed through 1 cm openings that connected the chambers of the olfactometer. In this question, I chose to test if the attractive response of *Lu. longipalpis* females to conspecific male extracts would differ if the aforementioned PVC tubes were: reduced to 3 cm in length, or removed. As *Lu. longipalpis* females were observed to be significantly attracted to conspecific male extracts in Y-tube olfactometers differing in Y-tube dimensions from previously mentioned studies, I predicted that chamber accessibility in linear olfactometers would not affect the attractive response of *Lu. longipalpis* females to conspecific male extracts.

Question 5. Does Trap Orientation Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts in Cage Olfactometers?

Hypothesis

Insects locomote towards and upon surfaces based on the orientation of the surface, and therefore *Lu. longipalpis* female attraction to the conspecific pheromone is expected to be affected by the orientation in which the pheromone is presented in.

Rationale

Esker et al. (2004) found that the number of *Chaetocnema pulicaria* (Coleoptera: Chrysomelidae) beetles captured by vertical sticky traps was significantly greater than the amount captured by horizontal traps in field conditions (Esker et al., 2004). In the insect order *Odonata*, there is evidence of variation in resting orientation preferences among taxa. Members of the suborder *Anisoptera* are known to preferentially rest vertically in their natural habitat while *Zygopterans* typically select horizontal surfaces such as rocks

for their layovers (Beutel, 2014). Moncaz et al. (2013) found that attraction to horizontal sticky traps varied at the species level for sand flies, as *Phlebotomus papatasi* were not attracted to horizontal sticky traps unless baited with CO_2, but unbaited horizontal traps captured large numbers of *P. orientalis* (Moncaz et al., 2013). Acknowledging the variability of orientation preferences in insects, the attraction of *Lu. longipalpis* females to the conspecific male produced pheromone is likely affected by the orientation in which it is presented.

Specific Aim 2. Identify the Female Lu. longipalpis Life Stage Most Sensitive to Male Sex Pheromones

Question 1. When do *Lu. longipalpis* Males Become Mature?

Hypothesis

Lu. longipalpis males cannot be considered to be sexually mature until their genitalia has rotated along the longitudinal body axis. Based on previous studies, the proportion of sexually mature males is expected to increase with time, with most maturity occurring ~24 hours post eclosion.

Rationale

Male sand flies are not mature, and therefore cannot mate, until their genitalia have rotated along the longitudinal body axis (Moncaz et al., 2012; Provost et al., 1961). The importance of genitalia rotation in sand flies is integral to the determination of virgin status in female sand flies. Although multiple studies have been conducted in which

virgin female flies were used to gauge the effect of male *Lu. longipalpis* pheromone on the behaviors of female conspecifics, the standards that defined virgin females varied considerably. Morton and Ward (1989, 1990) separated male and female *Lu. longipalpis* sand flies 0-3 hours after eclosion to achieve virgin status, while Hamilton et al. (1999) allowed male and females to remain together for up to 10 after emergence (Hamilton et al., 1999a; Morton and Ward, 1989a, 1990; Spiegel et al., 2005). However, Moncaz et al. (2012) demonstrated that in *Phlebotomus sergenti,* a Middle Eastern sand fly species, males did not possess fully rotated genitalia until 25 hours post eclosion when reared at 26^0 C in a laboratory setting (Moncaz et al., 2012). In order to empirically determine virgin/non-virgin status of *Lu. longipalpis* females, I decided to determine the length of time required for laboratory reared *Lu. longipalpis* males to become sexually mature. I predicted that *Lu. longipalpis* males reared at 26^o C would mature by 24 hours post eclosion.

Question 2. Does the Duration of Exposure of Recently Eclosed Females with Recently Eclosed Males Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts?

Hypothesis

I expect that the male sex pheromone of *Lu. longipalpis* mainly attracts conspecific virgin females for the purpose of mating. As the exposure time of *Lu. longipalpis* males and females increases, the likelihood that the females have mated also increases. Hence, I predict that *Lu. longipalpis* female attraction to conspecific male extracts is negatively correlated with female exposure time with males.

Rationale

In a study regarding *Lu. longipalpis* reproduction and behavior, researchers found that females that had mated within 24 hours were much less likely to mate again when given the opportunity (Spiegel et al., 2013). However, no studies have explicitly attempted to determine how non-virgin females react to the conspecific pheromone. Therefore, I decided to investigate how male expsoure in female *Lu. longipalpis* sand flies modifies their attraction to the conspecific male-produced pheromone in a controlled, laboratory setting. Three male exposure categories were created: ≤ 8 hours of male exposure (HME), ≤ 24 HME, and ≤ 48 HME, with male exposure defined as the amount of time that females were allowed to spend with males of the same age post eclosion. These male exposure categories were created under the assumption that *Lu. longipalpis* males do not mature until ~24 hours post eclosion (Specific Aim 2, Question 1), with ≤ 8 HME, ≤ 24 HME, and ≤ 48 HME females representing likely unmated, partially mated, and likely mated female populations, respectively. Given the intrinsic association of the male sex pheromone and the act of mating, it seems unlikely that females with increased male exposure would be attracted to the male sex pheromone. I predicted that *Lu. longipalpis* females with ≤ 8 hours of male exposure are attracted to the male pheromone whereas females with ≤ 24 and ≤ 48 hours of male exposure are not.

Question 3. Does Being Blood Fed Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts?

Hypothesis

Blood fed *Lu. longipalpis* females are typically mated and gravid and therefore are not expected to be responsive to the conspecific male pheromone. Therefore, I predict that blood fed *Lu. longipalpis* females should be less attracted to conspecific male extracts than non-blood fed females of the same age.

Rationale

Female *Lu. longipalpis* have been shown to be attracted to different semiochemicals (behavior modifying chemical compounds) at different life stages. Virgin females are attracted to male sex pheromones, foraging (blood-seeking) females are attracted to host odors (kairomones), and blood fed females are attracted to egg pheromones and fecal odors (apenumones) (Dougherty et al., 1994, 1995; Elnaiem and Ward, 1992; Hamilton et al., 1999a; Kelly and Dye, 1997; Morton and Ward, 1989a, 1990). In addition, it was found that truly virgin (separated at the pupae stage) blood fed females copulated with conspecific males only 40.3-61.0% of the time when given the opportunity (Souza et al., 2008). It is therefore highly unlikely that being blood fed would modify the behavior of likely non-virgin (≤ 24 hours of male exposure) females towards the male sex pheromone.

Question 4. Are *Lu. longipalpis* Females Truly Attracted to Conspecific Male Extracts?

Hypothesis

Under the assumption that *Lu. longipalpis* male hexane extracts contain male-produced sex pheromones and conspecific female hexane extracts do not, and that *Lu. longipalpis* females are attracted to conspecific sex pheromones, I expect that *Lu. longipalpis* females will be attracted to conspecific male hexane extracts but not conspecific female extracts.

Rationale

In studies that have documented the attractive effect of the *Lu. longipalpis* male pheromone on conspecific females, the attractive effect was confirmed by females significantly choosing hexane-based male extracts over hexane by itself (Hamilton et al., 1999a, 1999b; Morton and Ward, 1990; Ward et al., 1989). In this study, whole *Lu. longipalpis* males were extracted in hexane and used as the treatment in previous experiments while hexane itself was used as the control. In this experiment, hexane-based extracts from *Lu. longipalpis* females were used as the treatment and hexane itself the control. This experiment was used as a negative control to validate that *Lu. longipalpis* female attraction to male extracts used in this study was due to the presence of the male pheromone within the male extract. If female extracts were also found to be attractive to *Lu. longipalpis* females, then further research would need to be conducted to determine the composition of chemical compounds present in extracts of each gender.

CHAPTER II

METHODS AND MATERIALS

General Methods

Colony Maintenance

Lu. longipalpis sand flies from the Jacobina region of Brazil (hereafter LLJB) were originally colonized by Edgar Rowton (Walter Reed Army Institute of Research) and maintained in Dr. Wasserberg's laboratory. Mass-rearing of sandflies was adapted from Lawyer et al. (1991) (Lawyer et al., 1991) All sand flies were housed in Caron Incubators (Caron, Marietta, Ohio) at 26^{o} C, 80% RH, at a 12:12 light:dark cycle, with the scotophase beginning at 6 AM. Adult sand flies of both sexes were fed a saturated sugar solution and females are blood-fed on mice (UNCG IACUC Protocol 14-07) anaesthetized with a ketamine/xylazine solution. Twenty four hours post blood-feeding, sand flies were transferred to Whip-Mix® Orthodontic Plaster (Model: 5577352, Henry Schein Inc., Melville, New York) lined polycarbonate rearing jars (Nalgene™, Rochester, NY). Following a 7-day period in which females oivposited on the plaster substrate, all adult sand flies were removed via mouth aspirator and forceps. Larvae hatching from the eggs were provided with a mixture of rabbit feces and commercially available rabbit food (1:1) 2-3 times per week until pupation. Following eclosion from the pupa, adults were released from the rearing jars into 27,000 cm^3 (30 x 30 x 30 cm) polycarbonate cages as needed.

Pheromone Extracts

The extraction protocol was developed by Dr. Coby Schal and pheromones were extracted in his laboratory. Four day old male or female sandflies stored at -30º C were extracted in batches of 100. These 100 males were placed in a gas chromatography (GC) vial with lid (Thermo #C4000-1, Agilent #5182-0724). 500 µl of 99.9% hexane (Fisher #H303-1) were added to each vial and the vial was left at room temperature for 60-80 minutes. The hexane was then transferred to a conical glass vial (Chromacol #71160010745, Alltech #5128845). The sand flies were rinsed twice with 250 µl of hexane, and that solvent was added to the conical glass vials. The vials were centrifuged at 1725 rpm in a Savant SVC 100H centrifuge with 12-28 rotor for 8-10 minutes to pellet out the insect parts (e.g., scales). The supernatant was then transferred to a new GC vial with solid cap (GRACE #2109129, Fisherbrand #03-39-16). The conical vial containing residual insect parts was washed twice with 250 µl hexane, centrifuged as previously described, and the supernatant was transferred into the GC vials. The final volume was adjusted to 2,000 µl of hexane to provide a concentration of 1 male or female equivalent per 20 µl of solvent. Extracts were stored at -30º C.

Specific Aim 1, Question 1. Does *Lu. longipalpis* Female Attraction to Conspecific Male Extracts Differ Between Linear and Cage Olfactometers?

Two types of olfactometer bioassay systems were used to gauge the behaviors of female LLJB in response to LLJB male pheromone extracts. The goal was to determine which olfactometer type maximized sand fly response and produced the most consistent

and reproducible results. The protocols for experiments carried out in both olfactometer types are as follows.

Linear Olfactometers

Linear olfactometers were cylindrical apparatuses composed of three Plexiglas® chambers (9.4 cm inside diameter, 15 cm length) and two polyvinyl chloride (PVC) pipes (10.15 cm inside diameter, 2.5 cm length). The PVC pipes, responsible for connecting the lateral chambers to the medial chamber, had a white Plexiglas square inserted medially that restricted the open space to a 1 cm opening. A 6 cm tube (0.9 cm inside diameter) placed through the 1 cm opening of the Plexiglas square extended in equal parts into medial and lateral chambers, which facilitated sand fly access. Fine mesh held in place by rubber bands prevented sand fly escape from lateral chambers. In a controlled environmental room (28^{o} C, > 50% RH), olfactometers were randomly placed and rotated among replicate bioassay sessions to avoid spatial bias. Twenty female *Lu. longipalpis* were placed via aspirator into the medial chamber via a 1 cm opening, and an Airline tube (Thermo Fisher Scientific, Waltham, MA) covered by fine mesh was connected. Airline tubes were connected to a Cole-Parmer® Air Admiral® (Cole-Palmer, Vernon Hills, IL) vacuum pump (max 12 L/min) and one vacuum pump provided the suction for two olfactometers (Figure 1). Sand flies received a fifteen minute acclimation period under vacuum pressure before the addition of treatment and control apparatuses into the lateral chambers. Following placement of the treatment and control apparatuses, experiments ran under vacuum pressure for two hours unless otherwise noted. Experiments were terminated at the end of the vacuum pressure period and the number of

sand flies in each chamber were counted after olfactometers were placed in a freezer for -20^{o} C for one hour.

Cage Olfactometers

Cage olfactometers were 27,000 cm^3 (30 x 30 x 30 cm) polycarbonate cages with an attached fabric sleeve on side 1 (Figure 2) for the introduction and removal of sand flies and trapping material. Side 2 possessed a 225 cm^2 (15 x 15 cm) fine mesh window. Sticky traps were produced by heating Tangle-Trap® sticky coating (Contech Enterprises Inc., Victoria, B.C., Canada) on a hot plate until it was reduced to a liquid. An 81 cm^2 (9 x 9 cm) white copy paper square was submerged into the liquid and allowed to dry. This sticky square was then placed on a 100 cm^2 (10 x 10 cm) white foam square. An entomological needle was pushed upwards through the middle of the sticky trap so that the base of the needle rested underneath the foam square. A 1 cm^2 (1 x 1 cm) white foam square was medially pushed down the needle and on to the sticky square to add rigidity. A 2.5 cm filter paper disc was placed halfway down the needle so that it was 1 cm away from the trap and the point of the needle. Forty LLJB female sand flies were aspirated from holding cages and placed into a 125 ml Nalgene jar covered by fine mesh and held in place by rubber bands until traps were put in place. LLJB extracts of one ME (or female equivalent [see Methods section "Specific Aim 2, Question 4"]) or an equivalent amount of 99.9 % hexanes were pipetted on to the filter paper discs and, after drying for 30 seconds to sufficiently evaporate hexane, the traps were placed into the corners of the cage for a total of one treatment trap and one control trap per cage (Figure 2). The placement of treatment (LLJB extract) and control (hexane) traps was alternated for each

replicate to prevent spatial bias. The Nalgene jar was then placed at the juxtposition of sides 3, 5, and 6, the mesh was removed, and the sand flies were released in a neutral area equidistant from either trap (Figure 2). Cages were covered with black plastic sheets and all experiments were conducted in the laboratory of Dr. Gideon Wasserberg (28^{o} C, > 50% RH). Experiments began ~ 4 hours into the scotophase (dark portion of a 24 hour period) and were allowed to continue for 24 hours. The numbers of flies stuck to each trap was recorded at 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 16 hours, and 24 hours into the experiment. As sand flies are known to display crepuscular and/or nocturnal behaviors, the timing of the experiments allowed for the majority of the acitivity to occur during the beginning of the experiment (Killick-Kendrick, 1999).

Since the overall response rate in cage olfactometers was substantially higher than the linear olfactometers (See Discussion section "Specific Aim 1, Question 1"), all subsequent experiments were conducted in cage olfactometers.

Specific Aim 1, Question 2. Does the Concentration of *Lu. longipalpis* Male Extract Affect the Attractive Response of Conspecific Females?

Male extracts (ME) or an equivalent amount of 99.9 % hexanes were pipetted on to 2.5 cm filter paper discs (Thermo Fisher Scientific, Waltham, MA), serving as treatment and control apparatuses, respectively. Filter papers were allowed to dry for thirty seconds to allow for sufficient evaporation of hexane. Filter papers were placed in the base of 35 x 10 mm petri dishes (USA Scientific, Ocala, FL), and these were placed in the lateral chambers. At least 4 replicates of each of 0, 0.1, 1, 3, 6, and 9 ME were conducted, while < 4 replicates of each of 0.5, 2.5, 5, 10, 12, 15, 50, and 100 ME were

conducted June-October 2014, for a total of 57 bioassay replicates across all concentrations.

As 1 ME was found to elicit the most consistent attractive behavior in female respondents (see Results "Male Extract Concentrations"), this concentration was used for each subsequent experiment involving extracts (male or female) for both linear and cage olfactometers.

Specific Aim 1, Question 3. Do *Lu. longipalpis* Females Display Male-Abundance Dependent Attractive Responses to Conspecific Live Males in Linear Olfactometers?

In addition to testing ME concentrations, experiments were conducted using variable numbers of live males and containment methods. Treatments of 2 and 4 males contained in 30 mL vials covered with fine mesh and held in place by rubber bands (2 replicates at each level) as well as treatments of 5 and 10 males placed directly into the lateral chambers (no containment, 2 replicates at each level) were used in experiments conducted in June 2014. After results demonstrated that removing the tubes from Plexiglas squares increased the response (mean 66.65 % ± 11.62, see Results section "Specific Aim 1, Question 3"), treatments of 1, 3, 6, and 9 males contained in double layered nylon tubes (1.5 cm inside diameter, 11 cm length) were used in cage olfactometers without tubes for experiments conducted in October 2014 (2 replicates of 6 males, and 3 replicates at each other level).

Specific Aim 1, Question 4. Is the Attractive Response of *Lu. longipalpis* Females to the Conspecific Male Extract Affected by Chamber Accessibility in Linear Olfactometers?

As response rates were low (mean 20.32 % ± 3.54) when testing various ME concentrations (see Results section "Specific Aim 1, Question 4"), experiments were completed to assess the effect of tube length on sand fly response in October 2014. The accessibility between medial and lateral chambers was altered for these experiments in the following fashion: 1) tubes of 3 cm in length were placed in the 1 cm holes of Plexiglas squares so that the entire length of the tubes extended into the lateral chambers (5 replicates), and 2) tubes were completely removed from Plexiglas squares, leaving an open 1 cm hole (4 replicates). Treatment and control apparatuses were designed using the methods described in Methods section "Specific Aim 1, Question 2".

Specific Aim 1, Question 5. Does Trap Orientation Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts in Cage Olfactometers?

In these experiments, horizontal and vertical traps were constructed in the same manner, yet their positions in the cage were altered. Traps were constructed according to Methods section "Specific Aim 1, Question 1, Cage Olfactometers", but were modified in the following manner. Badge clips (Sicurix®, Atlanta, GA) clamped on to the trap were secured to the cage via white labeling tape (Fisher Scientific, Pittsburgh, PA). In vertical experiments, one trap was placed at the juxtaposition of sides 1, 2, and 6 while the other was placed at the juxtaposition of sides 2, 3, and 4 (Figure 2A). In horizontal experiments, one trap was placed at the juxtaposition of sides 1, 5, and 6 while the other

was placed at the juxtaposition of sides 3, 4, and 5 (Figure 2B). Six replicates of both orientation types were completed. LLJB females used in the experiments were 5-7 days old and had been seperated from males after ≤ 24 hours of exposure.

Overall repsonse patterns did not differ between horizontal and vertical trap orientations (see Results "Vertical and Horizontal Trap Orientation"). Therefore, all subsequent cage experiments used horizontally orinented traps as they were logistically simpler to use than vertically oriented versions.

Specific Aim 2, Question 1. When do *Lu. longipalpis* Males Become Mature?

Aging of male mosquitos via terminalia rotation has been conducted for over half a century, with one of the first records recorded in 1952 for *Aedes taeniorhynchus* (Nielsen, 1958; Provost et al., 1961). Provost et al. (1961), also working with *Ae. taeniorhynchus,* found that temperature greatly impacted the male maturity rate. They found that 95% of mosquitoes were mature by 30 hours at 36^{o} C, yet it took 42 hours for 95% of the males reared at 18^{o} C to become mature (Provost et al., 1961). This technique has been applied throughout the order Diptera, with information regarding genitalia rotation now known for species spanning multiple families (Borkent et al., 2008). As mentioned earlier, genitalia rotation data has also been recorded for sand flies, as Moncaz et al. (2012) demonstrated that *Phlebotomus sergenti* males did not become mature until 25 hours post eclosion at 26^{o} C (Moncaz et al., 2012). In my study, two methodologies, Cumulative Percent Rotation and Individual Rotation, were applied in order to determine the amount of time required for laboratory reared *Lu. longipalpis* males to become sexually mature.

Cumulative Percent Rotation

In this method, the cumulative proportion of males with rotated genitalia was evaluated with respect to time post eclosion using a series of 5 rearing cups, and over 1,000 males were surveyed. Releasing all pre-exisiting adults from rearing jars into holding cages established initial eclosion time. Afterwards, adults were released from rearing cups into holding cages at certain time intervals (2, 8, 16, 24 hours) and the cumulative proprotion of sexually mature males was determined by examining the orientation of male genitalia as detailed in Figure 5A,B,C.

Individual Rotation

In this method, 50 pupae nearing eclosion (determined by dark coloration [personal observation]) were removed from rearing cups and singly placed inside 125 ml Nalgene jars covered by fine mesh and held in place by rubber bands. The small rearing jars were then placed back into the incubator (26^{o} C, 80% RH). Every 4 hours, the jars were taken out of the incubator and each pupae was checked to determine if an adult had eclosed. If a male had eclosed, the sand fly was lightly anesthetized under a gentle stream of CO_2 and the position of genitalia orientation was determined under a dissecting microscope. These adult males were checked every four hours until genitalia was fully rotated, thereby determining the individual variability of sexual maturity.

Specific Aim 2, Question 2. Does the Duration of Exposure of Recently Eclosed Females with Recently Eclosed Males Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts?

In order to gain a bettter understanding of how being mated affects the behavior of LLJB females to the conspecific male pheromone, the amount of time that females were allowed to spend with males was used as a proxy for mating status. Results from "Cumulative Percent Rotation" and "Individual Rotation" experiments indicated low sexual maturity (~ 15%) in populations and individual flies ≤ 8 hours post eclosion (PE), ~ 80% sexually mature indivduals but ~ 35% population maturity at $8 \leq X \leq 24$ hours, and 100% sexually mature after 48 hours (See Results sections "Specific Aim 2, Question 1, Cumulative Percent Rotation, Individual Rotation"). Therefore, three male exposure categories were created: ≤ 8 hours of male exposure (HME), ≤ 24 HME, and ≤ 48 HME, with male exposure defined as the amount of time that females were allowed to spend with males of the same age post eclosion. Thirteen replicates of each category were completed using 5-7 day old LLJB females in April-May 2015.

Since females with ≤ 24 HME appeared to be the most responsive stage (See Results section "Specific Aim 2, Question 2"), subsequent bioassays used this group.

Specific Aim 2, Question 3. Does Being Blood Fed Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts?

Females with ≤ 24 HME used in this experiment were placed in an empty cage, and allowed to blood feed on a mouse (Protocol 14-07). Experiments were conducted 4 days post blood feeding, as it is known that eggs mature in sand flies 4-8 days after a

blood meal under laboratory settings (Killick-Kendrick, 1999). Females were 5-7 days post eclosion at the time of experiments. Comparisons between females used in this experiment and females with ≤ 24 HME (from Methods section "Specific Aim 2, Question 2") were made when data was analyzed.

Specific Aim 2, Question 4. Are *Lu. longipalpis* Females Truly Attracted to Conspecific Male Extracts?

In this experiment, LLJB female extracts were used as treatment and hexane as control. If females did not significantly respond to female extract-baited traps (13) while females used in previously described experiments significantly responded to male extract-baited traps, then the male pheromone was likely extracted during the extraction process. This would confirm that results inidicating female attraction in experiments using male extracts were due to the presence of the male pheromone itself. LLJB females used in the experiments were 5-7 days old and had ≤ 24 hours of male exposure. Comparisons between females used in this experiments and females with ≤ 24 HME (from Methods section "Specific Aim 2, Question 2") were made when data was analyzed.

Preference Index (PI values)

In order to reduce data and infer results for the majority of experiments, a preference index was calculated as follows:

$$PI = \frac{SF_T - SF_C}{SF_T + SF_C}$$

where SF_T is the number of sand flies in the treatment chamber for linear olfactometers or the number of sand flies stuck to the treatment trap in cage olfactometers and SF_c is the is the number of sand flies in the control chamber for linear olfactometers or the number of sand flies stuck to the control trap in cage olfactometers. This results in values ranging from -1 to 1, indicating attraction if positive and repellence if negative (Kramer and Mulla, 1979; Ponnusamy et al., 2010).

Linear Olfactometers

The calculation of a 95% confidence interval around the mean PI value was used to determine if the number of sand flies in treatment and control compartments were significantly different for 0, 0.1, 1, 3, 6, and 9 male extract (ME) concentrations, as ≥ 4 replicates were conducted for each of these concentrations (Specific Aim 1, Question 2). This method was also employed for determining significance from the experiments (4 replicates) in which tubes were removed from Plexiglas sqaures (Specific Aim 1, Question 4). Treatments were not considered significant if confidence intervals included zero. Analysis of variance (ANOVA) using PI values as the response variable was used to determine if there were significant differences among ME concentrations tested (Specific Aim 1, Question 2). As very few replicates (< 4) were conducted for each

variation of conspecific live male experiments (Specific Aim 1, Question 3), statistical analyses were not conducted. In addition, the overall low response of tube variability experiments did not allow for comparisons to be made between tube variations (Specific Aim 1, Question 4).

Specific Aim 2, Question 1, Cumulative Percent Rotation

The proportions of mature males from 2, 8, 16, and 24 hours post eclosion populations were used as the response variable in pairwise proportion tests of each post eclosion category to determine if the proportions of mature males significantly increased with time post eclosion. The number of observed males for each post eclosion category was 39, 182, 293, and 550 for post eclosion categories 2, 8, 16, and 24 hours, respectively.

Specific Aim 2, Question 1, Indiviudal Rotation

The proprotion of males maturing before 12 hours post eclosion and the proportion of males maturing after 12 hours post eclosion was tested by a proportion test to determine if the number of males maturing in one post eclosion category was significantly different than the number of males maturing in the other. The number of males maturing before 12 hours post eclosion was 11 and the number of males maturing after 12 hours post eclosion was 10.

Cage Olfactometers

As experiments began ~ 4 hours into the scotophase, the majority of sand fly movement and response was expected to occur in the beginning of the 24 hour experimental period. To test this, the traps were checked at 7 time intervals- 30 minutes,

1 hour, 2 hours, 4 hours, 8 hours, and 16 hours into the experiment, with a final check at the conclusion of the 24 hour period.

Within Experiments

Within each experiment, paired *t*-tests were used to determine if the number of sand flies stuck to treatment and control traps were significantly different at each time point. The dependent variable used in paired *t*-tests was the cumulative number of sand flies stuck to treatment and control traps at each time check.

Between Experiments

As multiple measurements were made over time, repeated measures (RM) ANOVA was used to determine if the distributions of sand flies stuck to traps: significantly increased throughout the experiment, were influenced by different levels of a factor (e.g.,vertical and horizontal trap placement), were impacted by the interaction of time and factor levels. RM ANOVA used the PI value from each time check as the dependent variable. In order to test for the final effect of the treatment in experiments, a single ANOVA was conducted between experiments using the PI value from the final (24 hour time check) as the dependent variable. If a factor was found to be significant in an ANOVA, the data from the factor was analyzed graphically or in combination with post-hoc *t*-tests. RM and single ANOVA were conducted for the following comparisons: between horizontal (6 replicates) and vertical (6 replicates) trap orientation experiments (Specific Aim 1, Question 5), between ≤ 8 (13 replicates), ≤ 24 (13 replicates), and ≤ 48 (13 replicates) hours of male exposure (HME) categories (Specific Aim 2, Question 2), between blood fed female experiments (6 replicates) (Specific Aim 2, Question 3) and

non-blood fed female experiments (13 replicates) (≤ 24 HME female experiments, Specific Aim 2, Question 2), and between female extract experiments (13 replicates) (Specific Aim 2, Question 4) and male extract experiments (13 replicates) (≤ 24 HME female experiments, Specific Aim 2, Question 2).

Data analysis was conducted using R, version 3.2.1 (The R Foundation for Statistical Computing, © 2015) and JMP®, Version 11, SAS Institute Inc., Cary, NC, 1989-2007.

CHAPTER III

RESULTS

Specific Aim 1, Question 2. Does the Concentration of *Lu. longipalpis* Male Extract Affect the Attractive Response of Conspecific Females?

Overall, response was low across all experiments (mean = 19.6 % ± 3.85 [95% CI]). Only the concentration from 1 male sand fly resulted in the significant attraction of females (mean PI value = 0.37 ± 0.32 [95% CI]) (Figure 3). However, no significant differences were found among concentrations (Table 1).

Specific Aim 1, Question 3. Do *Lu. longipalpis* Females Display Male-Abundance Dependent Attractive Responses to Conspecific Live Males in Linear Olfactometers?

Response was very low using conical vials for containment (mean = 7.29% ± 5.84 [95% CI]). In experiments using nylon tubes and no male containment, males left the treatment chambers, and the results were unsuitable for analysis.

Specific Aim 1, Question 4. Is the Attractive Response of *Lu. longipalpis* Females to the Conspecific Male Extract Affected by Chamber Accessibility in Linear Olfactometers?

In experiments in which tubes were flush against the inside of the middle chamber (i.e., all 3 cm inside the lateral compartment), response was very low (mean = 7.76% ± 8.66) while response averaged 66.65% ± 11.62 in experiments in which tubes were

removed from Plexiglas squares. Females were not attracted to the treatment of one male extract in tubeless experiments, however (mean PI value = -0.11 ± 0.41 [95% CI]).

Specific Aim 1, Question 5. Does Trap Orientation Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts in Cage Olfactometers?

Paired *t*-tests found that, for both vertical and horizontal orientation experiments, significantly more flies were stuck to treatment traps by the end of the experimental period (24 hour final time check, $P<0.05$), but not for all time checks ($P>0.05$) (Figure 4A,B).

The repeated measures (RM) ANOVA between horizontal and vertical orientation experiments found that preference for the treatment did not differ significantly between orientation (Trap Orientation, $P=0.91$) (Table 2). In addition, PI values did not significantly change across time checks (Time, $P=0.927$), and this trend was similar for females responding to vertical and horizontal trap orientations (Trap Orientation*Time, $P=0.939$) (Table 2). Likewise, the single ANOVA between horizontal and vertical orientation experiments found that final time check PI values were not significantly different from each other (Trap Orientation, $P=0.829$) (Table 3, Figure 4C).

Specific Aim 2, Question 1. When do *Lu. longipalpis* Males Become Mature?

Cumulative Percent Rotation

Significant differences were found between the proportions of mature males in each successive population of increasing age post eclosion ($P<0.05$), with the exception of the difference in mature proportions between populations of 2 and 8 hours, which was marginally significant ($0.05 < P < 0.1$). In the 2 hours post eclosion (PE) population,

15.4% of the males were mature, while the populations of 8 and 16 hours PE possessed mature male percentages of 30 and 40.3%, respectively. In the oldest age category post PE surveyed, 24 hours, 50.4% of males were observed to be mature (Figure 5C).

Individual Rotation

Only one male (4.8%) reached maturity by 4 hours PE, but between 4 and 8 hours PE another 9.5% percent matured. Thirty-eight percent of observed males matured in the > 8 and < 12 hour PE category, which brought the total mature percentage of males to 50.2% by this time. Another 38% of observed males matured in the > 12 and < 16 hours PE category, bringing the cumulative mature percentage to 90.5%. One hundred percent of observed males matured between 16 and 20 hours PE, as the last 9.5% of observed males matured during this time frame (Figure 5D). The proportion of males maturing before 12 hours PE (0.52) was not found to be significantly different from the proportion of males maturing after 12 hours PE (0.48) (Proportion test of the hypothesis that a PE category is not significantly different than the other, $P=0.83$, $X^2=0.0476$, $N=21$).

Specific Aim 2, Question 2. Does the Duration of Exposure of Recently Eclosed Females with Recently Eclosed Males Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts?

Paired *t*-tests indicated that females with ≤ 24 HME (Figure 6B) significantly chose treatment traps for all time checks ($P<0.05$), whereas females with ≤ 8 HME (Figure 6A) and ≤ 48 HME (Figure 6C) were significantly attracted to the treatment at some time points ($P<0.05$), but not by the final time check ($P>0.05$).

The RM ANOVA between male exposure experiments found that preference for the treatment did not differ significantly between male exposure categories (Male Exposure Status, *P=0.306*) (Table 4). However, PI values were found to significantly decrease across time (Time, *P<0.0001*), but this effect was not the same for all male exposure categories (Male Exposure Status*Time, *P=0.0151*) (Table 4). When graphically analyzed, females with ≤ 24 HME displayed average PI values > 0.2 for the first time check (0.5 hour), and these PI values did not appreciably change over time. Therefore, females with ≤ 24 HME chose treatment traps consistently across the experimental period (Figure 7A). Although females with ≤ 8 HME and ≤ 48 HME also displayed average PI values > 0.2 for the first time check (0.5 Hour), average PI values for both male exposure categories steadily declined throughout the experimental period to < 0.1 at the final time check (24 hour). While average PI values were still positive at the final time check for females with ≤ 8 HME and ≤ 48 HME, the proportion of females choosing the treatment decreased while the proportion of females choosing the control increased as experiments continued through the 24 hour period (Figure 7A). Output from the single ANOVA revealed that final time check average PI values were dissimilar between male exposure categories (*P=0.0447*) (Table 5). Subsequent post-hoc *t*-tests found that females with ≤ 24 HME had significantly higher (*P<0.05*) final time check average PI values than females with ≤ 8 HME and ≤ 48 HME, but females from the latter two male exposure categories did not significantly differ in final time check average PI values (*P>0.05*) (Figure 7B).

Specific Aim 2, Question 3. Does Being Blood Fed Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts?

Paired *t*-tests indicated that blood fed females significantly chose treatment traps for each time check of the experimental period ($P<0.05$) (Figure 8A). Likewise, non-blood fed females with a similar amount of male exposure (≤ 24 HME female experiments, Specific Aim 2, Question 2) also significantly chose treatment traps for each time check of the experimental period ($P<0.05$) (Figure 8B). Although females of both blood fed status significantly chose treatment traps over control traps for the entire experimental period, the average number of blood fed females stuck to treatment traps across all time checks was 27.86 ± 2.21 [95% CI], whereas the average number of non-blood fed females was 18.93 ± 1.48 [95% CI].

The repeated measures (RM) ANOVA between blood fed female and non-blood fed female experiments found that preference for the treatment did not differ significantly between blood fed status (Blood Fed Status, $P=0.182$) (Table 6). In addition, PI values did not significantly change across time checks (Time, $P=0.496$), and this trend was similar for both blood fed and non-blood fed females (Blood Fed Status*Time, $P=0.939$) (Table 6). Likewise, the single ANOVA between blood fed female and non-blood fed female experiments found that final time check PI values were not significantly different from each other (Blood Fed Status, $P=0.188$) (Table 7, Figure 8C).

Specific Aim 2, Question 4. Are *Lu. longipalpis* Females Truly Attracted to Conspecific Male Extracts?

Paired *t*-tests found that females responding to female extracts did not significantly choose treatment traps (female extract) over control traps for any time check ($P>0.05$) (Figure 9A). Conversely, females with a similar amount of male exposure (≤ 24 HME female experiments, Specific Aim 2, Question 2) responding to male extracts significantly chose treatment traps for each time check of the experimental period ($P<0.05$) (Figure 9B). In addition, the average number of females stuck to female extract treatment traps across all time checks was 17.93 ± 1.38 [95% CI], whereas the average number stuck to male extract treatment traps was 18.93 ± 1.48 [95% CI].

The repeated measures (RM) ANOVA between female extract and male extract experiments found that preference for the treatment did not differ significantly between extract gender (Extract Gender, $P=0.196$) (Table 8). In addition, PI values did not significantly change across time checks (Time, $P=0.449$), and this trend was similar for both female extract and male extract experiments (Extract Gender*Time, $P=0.112$) (Table 8). Likewise, the single ANOVA between female extract and male extract experiments found that final time check PI values were not significantly different from each other (Extract Gender, $P=0.107$) (Table 9, Figure 9C).

CHAPTER IV

DISCUSSION

Specific Aim 1, Question 1. Does *Lu. longipalpis* Female Attraction to Conspecific Male Extracts Differ Between Linear and Cage Olfactometers?

Multiple researchers have used Y-tube and cage olfactometers to document the attractive effect of *Lu. longipalpis* male sex pheromones on conspecific females. The linear olfactometer in this study was designed to represent a condensed version of the Y-tube olfactometers used by Hamilton et al. (1999) and Ward et al. (1989), whereas the cage olfactometer represented a condensed version of the cage olfactometer used by Morton and Ward (1990). As both olfactometer types used by the previously mentioned researchers allowed for the attraction of *Lu. longipalpis* females to the conspecific male pheromone to be observed, it was expected that both olfactometer types used in this study would function similarly. However, that was not the case, as female response was limited to an average of 22.95% ± 4.24 [95% CI] for all experiments in linear olfactometers. After removal of the PVC tubes in linear olfactometers, the response averaged 66.65% ± 11.62 [95% CI]. Even so, the response in cage olfactometers averaged 95.06% ± 1.44 [95% CI] for all experiments. Although other studies have demonstrated the effectiveness of Y-tube olfactometers, the linear olfactometer tested in this study was ineffective when compared to the cage olfactometer.

Specific Aim 1, Question 2. Does the Concentration of *Lu. longipalpis* Male Extract Affect the Attractive Response of Conspecific Females?

Bray et al. (2010) and Morton and Ward (1989) demonstrated that increased concentrations of male pheromone, whether extracted or synthetic, in laboratory or field settings, attracted more females than lower concentrations (Bray et al., 2010; Morton and Ward, 1989a). Due to low response, I was unable to obtain and analyze an appropriate amount of data regarding the effect of ME concentration. However, the concentration of 1 ME was found to significantly attract conspecific females (Figure 3). This result was used as the standard in all subsequent experiments, and demonstrated the effectiveness of a small ME concentration as an attractant for conspecific females in both linear and cage olfactometers. My findings support previous studies, as 1 *Lu. longipalpis* ME was found to attract conspecific females by Ward et al. (1989), although the attractive effect was more pronounced when 8 ME was used (Ward et al., 1989). The attractive effect of the *Lu. longipalpis* male pheromone on conspecific females was also reported by Bray et al. (2010) when using doses of 50 and 500 ME, with greater attraction in the latter. Although my results did not indicate that concentrations similar to those used by Morton and Ward (1989) and Bray et al. (2010) were attractive to LLJB females (i.e., 6, 9, 50, or 100 ME), this was a function of the linear olfactometer's inability to sufficiently gauge LLJB female response. LLJB female response averaged 19.6 % ± 3.85 [95% CI] in ME concentration experiments; females simply did not leave the medial chamber often. Reevaluation of ME concentration experiments should be conducted in cage

olfactometers, as they better fitted the response of LLJB females to conspecific male extracts.

Specific Aim 1, Question 3. Do *Lu. longipalpis* Females Display Male-Abundance Dependent Attractive Responses to Conspecific Live Males in Linear Olfactometers?

After observing low response to male extracts, live male experiments were implemented to help determine the cause of such limited female movement. When testing the containment method of conical vials covered in fine mesh, any useful results that could have been produced were likely eclipsed by low response (mean 7.29% ± 5.84). As stated in the results, although females did not move through the small PVC tubes, uncontained males did move throughout the chambers of olfactometers by the end of the experiment. In addition, males contained in double layered nylon tubes escaped as well. If appropriate containment methods can be implemented, the testing of variable numbers of live males in conjunction with analogous ME concentrations would be beneficial to the study, as the bait used to attract sand flies should be optimized as well.

Specific Aim 1, Question 4. Is the Attractive Response of *Lu. longipalpis* Females to the Conspecific Male Extract Affected by Chamber Accessibility in Linear Olfactometers?

This task was added secondarily after preliminary linear olfactometer experiments displayed very low response. Tubes cut in half (3 cm) and placed flush inside the medial chamber did not improve the response, but the complete removal of tubes from the 1 cm holes in the Plexiglas square raised the response rate to an average of nearly 70%. Ward

et al. (1989) used much longer and wider tubes in experiments (4.5 cm diameter, 23 cm length), but Hamilton et al. (1999) used tubes of a similar size to the ones used in this study (9 mm inside diameter, 10 cm length) (Hamilton et al., 1999a, 1999b; Ward et al., 1989). Each of the previously mentioned studies used Y-tube olfactometers and reported significant female attraction to the conspecific male pheromone. This raised the question as to why female response was so different when comparing this experiment with Hamilton et al.'s (1999) experiments, given that the tubes used in both studies were of similar dimensions. In Hamilton et al. (1999) studies, the tubes were made of glass and therefore transparent. As it is known that color affects the behavior of *Lu. longipalpis,* it is possible that the white color of the PVC tubes used in this experiment played a role in the limited movement of female subjects (Bray et al., 2010). Experiments testing the effect of using both tubeless variations and clear tubes on female response to male pheromones should be conducted to further clarify the problem of low response in linear olfactometers.

Specific Aim 1, Question 5. Does Trap Orientation Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts in Cage Olfactometers?

Multiple studies have documented resting or locomotory preferences of various insects, and it can be concluded that there is wide variation between preferences (Beutel, 2014; Esker et al., 2004; Tainchum et al., 2013). In this study, LLJB females displayed no preference for the orientation of traps (Table 2), and both orientations showcased the attractive effect of the male pheromone on female behavior that found in the literature (Figure 4A,B). These results did not support my hypothesis that the orientation in which

the male pheromone is presented in affects female attraction. Morton and Ward (1989) reported that female flies responded to pheromone extracts on the ceiling of cages, but would not respond to extracts on the floor (Morton and Ward, 1989b; Nigam and Ward, 1991). This phenomenon was not observed in this study, as female response to horizontal traps averaged 96.1% ± 4.11 [95% CI] at the end of the experimental period. In another study by Morton and Ward (1990), from which the sticky trap cage olfactometer used in this study was designed from, sticky traps were placed on the ceiling of a 216,000 cm^3 (60 x 60 x 60 cm) cage, and females were found to be significantly attracted to 8 ME baited traps. Results from this study do not refute Morton and Ward's (1990) findings, but rather suggest that both variations of horizontally oriented traps are an effective method for surveying the response of *Lu. longipalpis* females to male extracts (Morton and Ward, 1990).

Horizontally oriented traps were found to be the most practical method for surveying LLJB female behavior, as badge clips and labeling tape were required for placement of vertical traps within cages, but not necessary for placement of horizontal traps. The selection and use of horizontal traps reduced the amount of extrinsic error associated with experiments, as extraneous material inside olfactometers increased the likelihood of introducing foreign chemical compounds, thereby confounding results.

Specific Aim 2, Question 1. When do *Lu. longipalpis* Males Become Mature?

According to the literature, sand fly males do not possess rotated genitalia until ~ 24 hours post eclosion (PE), as was demonstrated with *Phlebotomus sergenti* (Moncaz et al., 2012). However, the findings from this study indicate that maturity occurs much

earlier, as mature males were observed 2-4 hours PE. By 8 hours, over 14% of observed individuals and 30% of populations were mature (Figure 5C,D). The hypothesis that LLJB males reared at 26° C mature by 24 hours PE was supported, as all observed individuals were mature by 20 hours PE. In addition, over 50% of observed individuals were mature by 12 hours and over 50% of 24 hour males were mature when observing populations. These results exemplify a distinction between the biology of Old World sand fly, *Phlebotomus sergenti,* and the New World sand fly, *Lutzomyia longipalpis.* Moncaz et al. (2012) did not observe mature *P. sergenti* until 25 hours post eclosion at 26° C, whereas the majority of LLJB males observed in this study and reared at 26° C matured in less than half of that time (Moncaz et al., 2012). This difference in maturity rates seems likely to represent fundamental differences in the biology between the two species. In addition, these results are important, as they significantly impact our understanding of sand fly biology, specifically New World species. This observed rapid male maturity rate could be an important factor in population growth and subsequent range expansion, especially as the urbanization of visceral leishmaniasis cases attributed to *Lu. longipalpis* in South America continues to increase.

Comparison of Individual Rotation and Cumulative Percent Rotation

One of the drawbacks with surveying populations is that it is impossible to remove all adult sand flies from the rearing cups at any given time. This likely did not significantly impact the calculated proportions of 8, 16, and 24 hour males, as the number of sand flies surveyed were > 100. However, as only 39 males were surveyed from the 2 hour population, older, non-removed males could have played a larger role in the

calculation of the mature proportion in the 2 hour group (Figure 5C). Individually observed males were not plagued by the error associated with "layover" males, and are therfore more trustworthy. Regardless of the erorr associated with a particular method, it is important to understand what both methods represent. Flaws considered, the cumulative percent rotation method demonstrates the variability within populations, while singular observations demonstrate variability at the indiviudal level. Therefore, both methods are important as they allow for insight into different aspects of LLJB biology.

Specific Aim 2, Question 2. Does the Duration of Exposure of Recently Eclosed Females with Recently Eclosed Males Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts?

No previous study has attempted to determine the effect of different degrees of male exposure on the documented attraction of *Lu. longipalpis* females to the conspecific male pheromone, and the understanding of the modification of this female attraction necessitates the contextualization of male exposure categories. In this study, each male exposure category represented the distinct likelihood that a female belonging to that category was mated. Females with ≤ 8 HME were considered to be likely unmated, and could also be defined as "virgin" if the definition of virginity used in Hamilton et al. (1999) were applied (Hamilton et al., 1999a). Females with ≤ 24 HME were considered partially mated, as it was assumed that male maturity occurs ~ 24 hours post eclosion. Consequently, females with ≤ 48 HME were considered to be likely mated. Results indicated that females from all male exposure categories were significantly attracted to

the male extract in the initial 2 hours of experiments (Figure 6A,B,C). As experiments continued, relatively more females with ≤ 8 HME and ≤ 48 HME chose control traps while the number of females with ≤ 24 HME consistently chose treatment traps throughout the experimental period (Figure 7A). By the end of the experiment, the number of females with ≤ 24 HME stuck to treatment traps was significantly greater than those stuck to control traps, but this significant effect was not found for females with ≤ 8 HME and ≤ 48 HME (Figure 6A,B,C). The conclusion that females with ≤ 24 HME are more attracted to male extracts than females with ≤ 8 HME and ≤ 48 HME was also supported by the single ANOVA and subsequent post-hoc *t*-tests using final time check values (Table 5, Figure 7B). The results of this study do not directly support the conclusions of other studies, as each of them found a significant attractive effect in virgin females to the conspecific male pheromone (Hamilton et al., 1999a; Morton and Ward, 1989a, 1990; Spiegel et al., 2005). However, as females with ≤ 8 HME and ≤ 48 HME in this study were found to be significantly attracted to male extracts in the beginning of the experimental period, it suggests that females differing in their duration of male exposure become habituated to the male pheromone at different rates. This may be further explained by male pheromone production, as *Lu. longipalpis* males do not typically begin production of pheromones until ~12 hours post eclosion, and this production increases for the first 3 days (Spiegel et al., 2011). Therefore, females with ≤ 8 HME were separated from males before appreciable pheromone production occurred. Being generally unaccustomed to the male pheromone, it is possible that females with ≤ 8 HME were initially inundated with the male pheromone extract in experiments, but quickly became

habituated. Females with ≤ 24 HME and ≤ 48 HME were more accustomed to the male pheromone when separated from males, but possibly differed in the proportion of mated females from each category. The majority of females with ≤ 24 HME were possibly not mated, but as they were more accustomed to the male pheromone, they habituated more slowly. Conversely, the majority of females with ≤ 48 HME were possibly mated and well accustomed to the pheromone. Females belonging to this group had the most male exposure out of all groups compared, but they also likely possessed the most variability of male exposure, as females could eclose from the pupae at any point within the 48 hour time frame of separation. Therefore, for females with ≤ 48 HME, females on the lower end of male exposure were potentially responsible for responding initially, while the majority of females with more male exposure and a larger likelihood of being mated were not as attracted to the male extracts, and therefore did not significantly choose the treatment traps in the mid-late time checks of the experimental period. In order to test these assumptions and predictions, the spermathecae of females from each exposure category could be dissected, with the proportion of females with spermatophores present being indicative of the proportion of mated females (Ilango, 2005). Thereafter, male exposure categories could be refined if necessary, and more replicates could be conducted to determine if the trends observed in this study were true for a larger sample of the *Lu. longipalpis* population.

Currently, the results from this study have led me to reject my hypothesis that the male sex pheromone of *Lu. longipalpis* mainly attracts virgin females, and conclude that

the LLJB females with ≤ 24 HME are more attracted to conspecific male extracts than any other male exposure category tested.

Specific Aim 2, Question 3. Does Being Blood Fed Affect the Attractive Response of *Lu. longipalpis* Females to Conspecific Male Extracts?

There has been no previous study that has attempted to test the effect of being blood fed on the attraction of females to the male pheromone. However, as Souza et al. (2008) demonstrated using the life stage that is responsive to the male pheromone, per the literature, virgin blood fed females copulated with males 40.3-61.0% of the time when given the opportunity (Souza et al., 2008). This would suggest that the state of being blood fed suppresses the attractive behavior in *Lu. longipalpis* females. However, results from this study suggest the opposite. Blood fed females significantly chose the treatment for all time points in the 24 hour experimental period (Figure 8A). When compared with non-blood fed females with a similar amount of male exposure (≤ 24 HME female experiments, Specific Aim 2, Question 2), more blood fed females were found to have chosen treatment traps across time points (27.86 ± 2.21 [95% CI]) than non-blood fed females (18.93 ± 1.48 [95% CI]), but preference for the treatment was not found to be significantly different between the two experiments from RM or single ANOVA (Tables 6 and 7). Nonetheless, the results from this experiment are surprising. In accordance with the natural progression of attraction to different semiochemicals at different life stages (sex pheromones and host kairomones before blood meal, egg pheromones and fecal apneumones after blood meal) and the findings from Souza et al. (2008), blood fed females were not hypothesized to be attracted to the male sex pheromone, but significant

attraction was found (Dougherty et al., 1994, 1995; Elnaiem and Ward, 1992; Hamilton et al., 1999b; Kelly and Dye, 1997; Souza et al., 2008). A possible explanation for the attractive effect seen here is that a portion of the blood fed females were virgin, and virgin females are attracted to the male pheromone, even when blood fed. In order to test this, spermathecae dissections could be conducted as described for Specific Aim 2, Question 2 to determine the virgin proportion of females with ≤ 24 HME. Thereafter, blood fed virgin females (identified through population data generated from spermathecae dissections or isolated at the pupae stage) could be used to test this hypothesis. In addition, it is possible that all females, regardless of life stage, are attracted to the male pheromone as an aggregation signal. This hypothesis could be tested with experiments for each combination of male exposure and blood fed status.

Although more blood fed females were observed stuck to treatment traps than non-blood fed females with a similar amount of male exposure, the state of being blood fed did not significantly increase attractive responses to male extracts in females with ≤ 24 HME. Regardless, results from this experiment would be more conclusive if additional replicates were conducted. Future experiments should also include tests of the *Lu. longipalpis* male sex pheromone as a potential oviposition attractant.

Specific Aim 2, Question 4. Are *Lu. longipalpis* Females Truly Attracted to Conspecific Male Extracts?

In order to answer one of the most important questions asked in this study, this experiment was conducted to determine if the pheromone extraction methodology was successful. As females with ≤ 24 HME were found to be significantly attracted to male

extracts (Specific Aim 2, Question 2), the response of females with a similar amount of male exposure to female extracts was ascertained, and comparisons were made between the two. If females were found to respond similarly to both treatments (i.e., gender extracts), then the attraction of females documented in previous experiments might not be attributed solely to the presence of the male pheromone. However, the results from this experiment were more difficult to interpret than anticipated. Females with ≤ 24 HME did not significantly choose female extracts over hexane control at any time check during the experimental period, whereas females with ≤ 24 HME significantly chose male extracts over hexane control at each time check during the experimental period (Figure 9A,B). This difference in female response would suggest that the two extracts were composed of different chemical compounds, providing evidence for the hypothesis that male extracts contained the male sex pheromone while female extracts did not. However, the difference between the averages of females stuck to treatment traps across time points for the two experiments only ranged from 0.9-1.1. In addition, RM and single ANOVA did not find that preference for the treatment was significantly different between females responding to male and female extracts (Tables 8, 9, Figure 9C). However, it should be noted that the single ANOVA using final time check PI values was conducted for the hypothesis that PI values from female extract experiments were significantly different than PI values from male extract experiments, and therefore the resulting *P* value of 0.107 represented both tails of the *F* distribution (Table 9). If the hypothesis that was tested for the single ANOVA had assumed that male extract PI values were significantly larger than female extract PI values, a one-tailed *P* value of 0.0535 indicating a marginally significant

difference between female response to male and female extracts would have been produced.

When time across the experimental period was considered in the RM ANOVA, there was no detectable difference between the response of females to female and male extracts. However, results from paired *t*-tests and the single ANOVA using final time check PI values suggested that females may respond differently to male and female extracts. Therefore, I cannot fully support my hypothesis that *Lu. longipalpis* male hexane extracts contained male sex pheromones while female hexane extracts did not. However, females with ≤ 24 HME, both blood fed and non-blood fed, were significantly attracted to male extracts, and it is likely that these extracts did contain the male pheromone. Conversely, as many females did choose female extract-baited traps across the experimental period, there was likely a recognizable chemical compound present in female extracts. Therefore, the composition of chemical compounds present in female extracts should be determined, as this would provide more conclusive results for this experiment.

Conclusion

The inability to access *Lutzomyia verrucarum* did not prevent the development of pheromone-surveying methods to be completed, as a closely related sand fly species, *Lu. longipalpis,* was used to establish appropriate methodology (Curler and Moulton, 2012).

The bioassay method chosen for the use of studying the sex pheromones of *Lu. verrucarum* was the cage olfactometer design. Cage olfactometers did not appear to impede natural locomotion patterns, but linear olfactometers better displayed the

directionality of the experimental odorant sources via an external vacuum. However, the omission of electrical devices in the cage olfactometer allowed for a utilitarian design that could be used to gauge sand fly behavior outside of laboratory settings. In addition, individual female response was likely not influenced by the response of other females as chambers served as a visual separation in linear olfactometers. In cage olfactometers, the possibility of indiviudal behavior modification increased as aggregations formed on traps, and experiments with females pre-placed on traps are being designed to test this claim. Despite the advantages and disadvantages associated with a particular bioassay, the cage olfactometer design maximized female response and produced the most consistent and reproducible results regarding *Lu. longipalpis* female behavior in this study.

The results from this study have revealed much about *Lu. longipalpis* biology, and novel results regarding locomotion and orientation, male maturity, and the biological modification of female attraction to the male sex pheromone were discovered. *Lu. longipalpis* females did not consistently move through constricted, opaque passageways, but they also did not display a preference for resting orientation. In addition, the conventional standard that male sand flies do not mature until after 24 hours post eclosion was not supported by the results of this study, as maturity was detected in as early as 4 hours post eclosion, with all individual males maturing by 20 hours post eclosion. Moreover, as virgin, non-blood fed females have been used as the model life stage in which significant attraction to the conscpecific male pheromone has been recorded, the discovery that activities such as blood feeding and increasing the male exposure of females may represent potential attractive "effect modifiers" to the male pheromone is

exciting and unexpected. As studies do not typically report field caught female characteristics, the discovery of these "effect modifiers" of female attraction stresses the importance of something as simple as the identification of common life history traits found in field caught female sand flies. An increase in the efficiency of field surveillance for phlebotomine sand flies can result from continued characteriziation of ideal targets, and this can subsequently be applied to control efforts.

The reslts from this study were intended to expedite the characterization of the alleged male produced pheromone of *Lutzomyia verrucarum.* The identification of biological attributes that are highly susceptible to pheromone-mediated behavioral manipulation as well as the establishment of an efficient bioassay method to examine these occurences were necessary first steps in the pursuit of such characterization. Although preliminary results using cage olfactometers suggest that *Lutzomyia verrucarum* do not possess male sex pheromones (Gideon Wasserberg, personal communication), it is advantageous that we develop additional insights into its biology, especially ones that pertain to its life cycle or mating procedures. As the paramount vector of medical importance in Peru, the biology of *Lutzomyia verrucarum* is still quite unknown. This study served as an initial attempt towards increasing our understanding of *Lutzomyia verrucarum* chemical communication, as the potential control of this vector of multiple pathogens through the application of such knowledge would be of great value.

Table 1. ANOVA for Male Extract Concentrations. Effect of Male Extract Concentrations on Preference Index (PI) Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Male Extract Concentrations	5	4.346	0.869	1.858	0.127
Residuals	35	16.373	0.468		

Table 2. Repeated Measures ANOVA for Vertical and Horizontal Trap Orientation. Effect of Trap Orientation, Time, and the Interaction of Both on PI Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Between Subjects					
Trap Orientation	1	0.007	0.0071	0.013	0.91
Residual Between	10	5.233	0.5233		
Within Subjects					
Time	6	0.049	0.00817	0.315	0.927
Trap Orientation*Time	6	0.0453	0.00755	0.291	0.939
Residual Within	60	1.558	0.02597		

Table 3. Single ANOVA for Vertical and Horizontal Trap Orientation. Effect of Trap Orientation on Final Time Check PI Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Trap Orientation	1	0.0022	0.0022	0.049	0.829
Residuals	10	0.4507	0.0451		

Table 4. Repeated Measures ANOVA for Male Exposure Status. Effect of Male Exposure Status, Time, and the Interaction of Both on PI Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Between Subjects					
Male Exposure Status	2	0.805	0.4025	1.225	0.306
Residual Between	36	11.827	0.3285		
Within Subjects					
Time	6	0.616	0.10273	6.485	<0.0001
Male Exposure Status*Time	12	0.409	0.03407	2.151	0.0151
Residual Within	216	3.422	0.01584		

Table 5. Single ANOVA for Male Exposure Status. Effect of Male Exposure Status on Final Time Check PI Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Male Exposure Status	2	0.2986	0.1493	3.392	0.0447
Residuals	36	1.5847	0.044		

Table 6. Repeated Measures ANOVA for Blood Fed Status. Effect of Blood Fed Status, Time, and the Interaction of Both on PI Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Between Subjects					
Blood Fed Status	1	0.773	0.7735	1.934	0.182
Residual Between	17	6.798	0.03999		
Within Subjects					
Time	6	0.0533	0.00888	0.903	0.496
Blood Fed Status*Time	6	0.0173	0.00288	0.293	0.939
Residual Within	102	1.0034	0.00984		

Table 7. Single ANOVA for Blood Fed Status. Effect of Blood Fed Status on Final Time Check PI Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Blood Fed Status	1	0.0979	0.0979	1.882	0.188
Residuals	17	0.8845	0.052		

Table 8. Repeated Measures ANOVA for Female Extracts. Effect of Extract Gender, Time, and the Interaction of Both on PI Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Between Subjects					
Extract Gender	1	1.076	1.0759	1.771	0.196
Residual Between	24	14.579	0.6075		
Within Subjects					
Time	6	0.0774	0.01291	0.969	0.449
Extract Gender*Time	6	0.1406	0.02343	1.759	0.112
Residual Within	144	1.9189	0.01333		

Table 9. Single ANOVA for Female Extracts. Effect of Extract Gender on Final Time Check PI Values.

Source	*df*	*SS*	*MS*	*F*	*P*
Extract Gender	1	0.2381	0.2382	2.804	0.107
Residuals	24	2.0387	0.085		

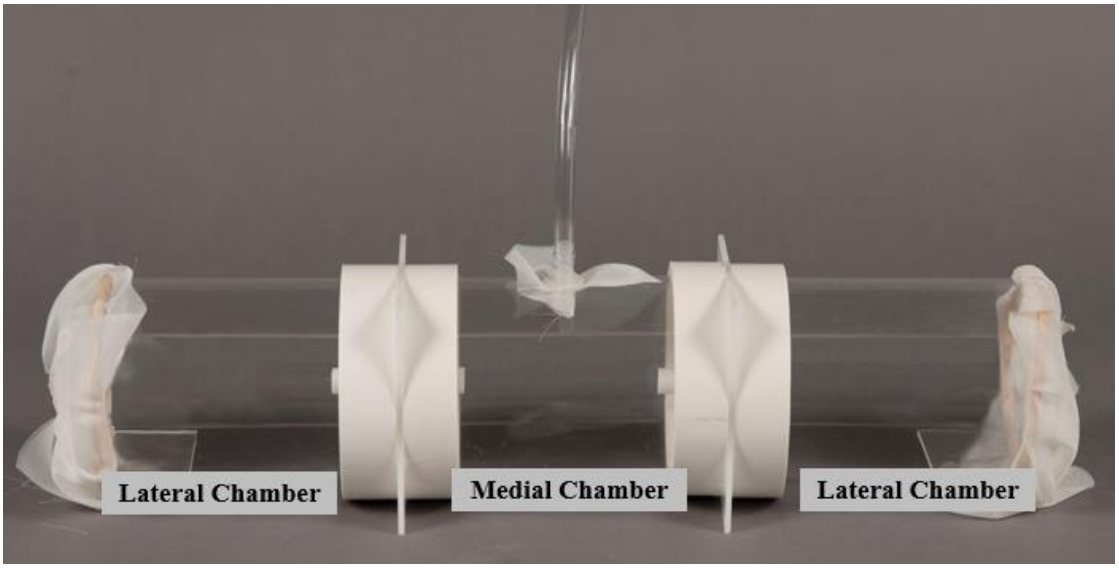

Figure 1. Linear Olfactometer. Treatment and control apparatuses were placed in lateral chambers. Airline tubing connected the medial chamber to the external vacuum pump, and odorant-containing air was pulled across lateral chambers and into the medial chamber. PVC tubes (9 mm inside diameter, 6 cm in length) can be seen extending into the left lateral and medial chambers.

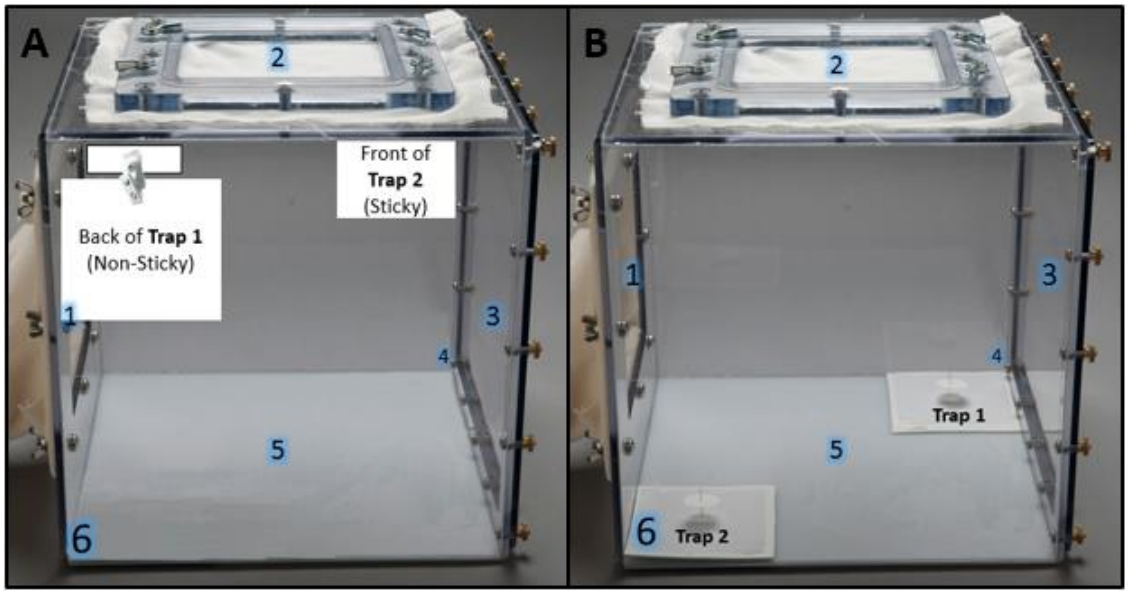

Figure 2. Cage Olfactometer. Vertically oriented trap diagram (A). Trap 1 = at the juxtaposition of sides 1, 2, and 6. Trap 2 = at the juxtaposition of sides 2, 3, and 4. Descriptions of "Non-Sticky" and "Sticky" are given to help better understand perception of the 3D cube. Both traps featured an inward-facing sticky side in experiments. Horizontally oriented trap diagram (B).

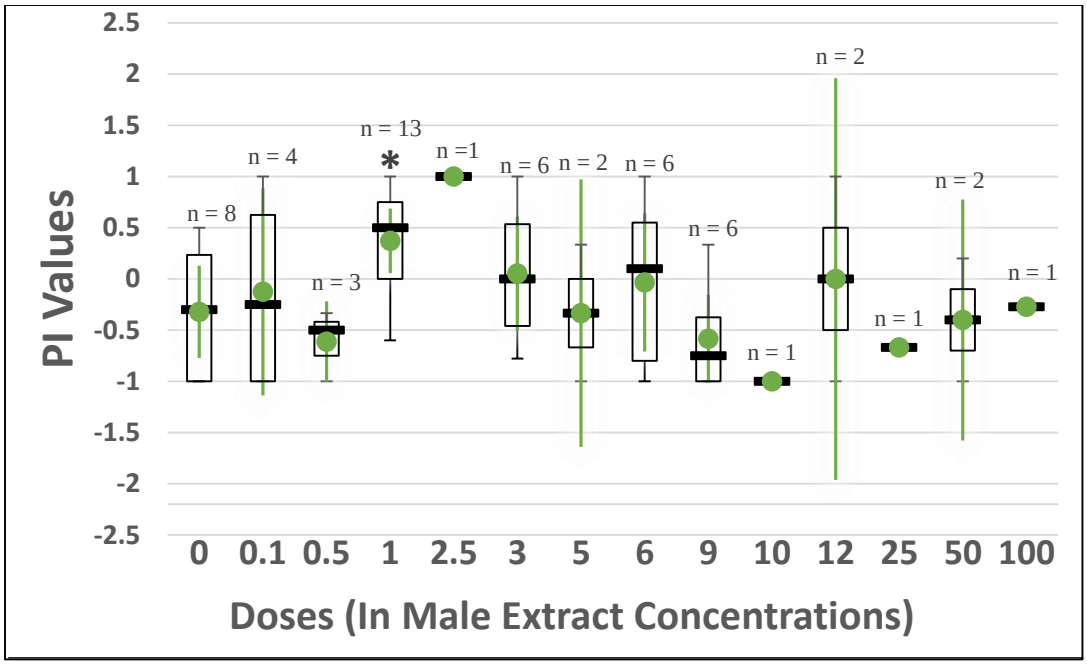

Figure 3. Box Plots of Preference (PI) Values for Male Extract Concentrations Tested. Green circles = means. Green lines = 95% CIs about the mean. * = 95% CIs that are significant (do not include zero). n = number of replicates.

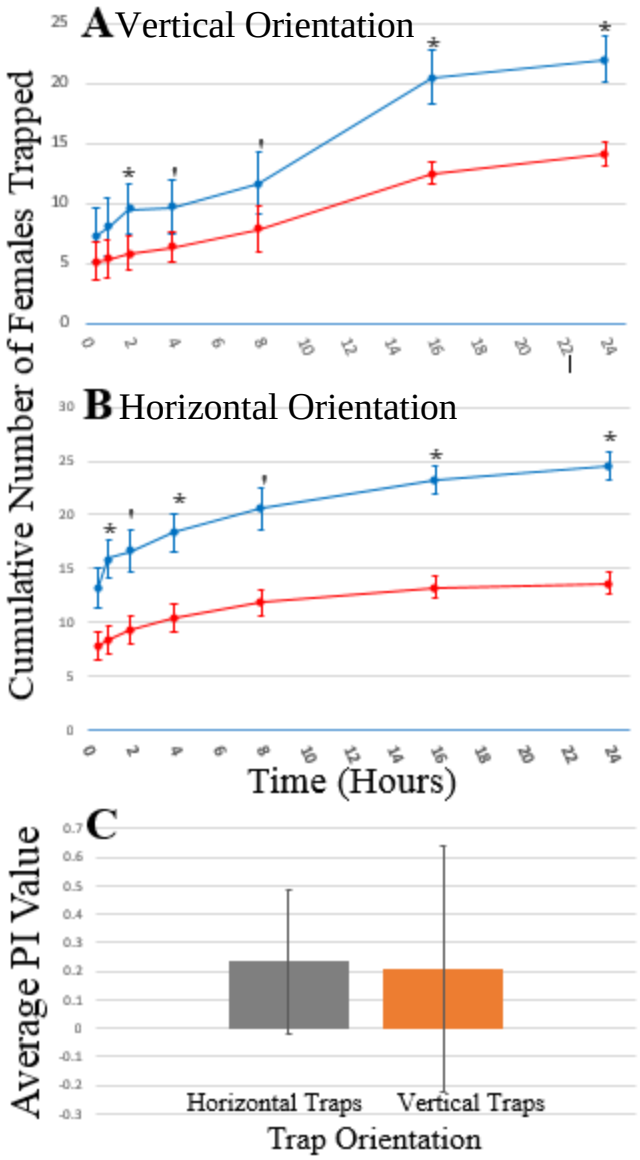

Figure 4. The Effect of Trap Orientation on Female's (≤ 24 Hours of Male Exposure) Response to Male Extracts. Panels A and B depict the change in the number of sand flies caught in treatment (filter paper with male extract, blue line) and control traps (filter paper with hexane, red line) over time for both vertical (A) and horizontal (B) trap orientations. Bars are standard error. Panel C compares the preference index of female sand flies to male extracts between the horizontal and vertical traps. Bars are 95% CI. ***P<0.0001, **P<0.01, *P<0.05, 0.1>'P>0.05.

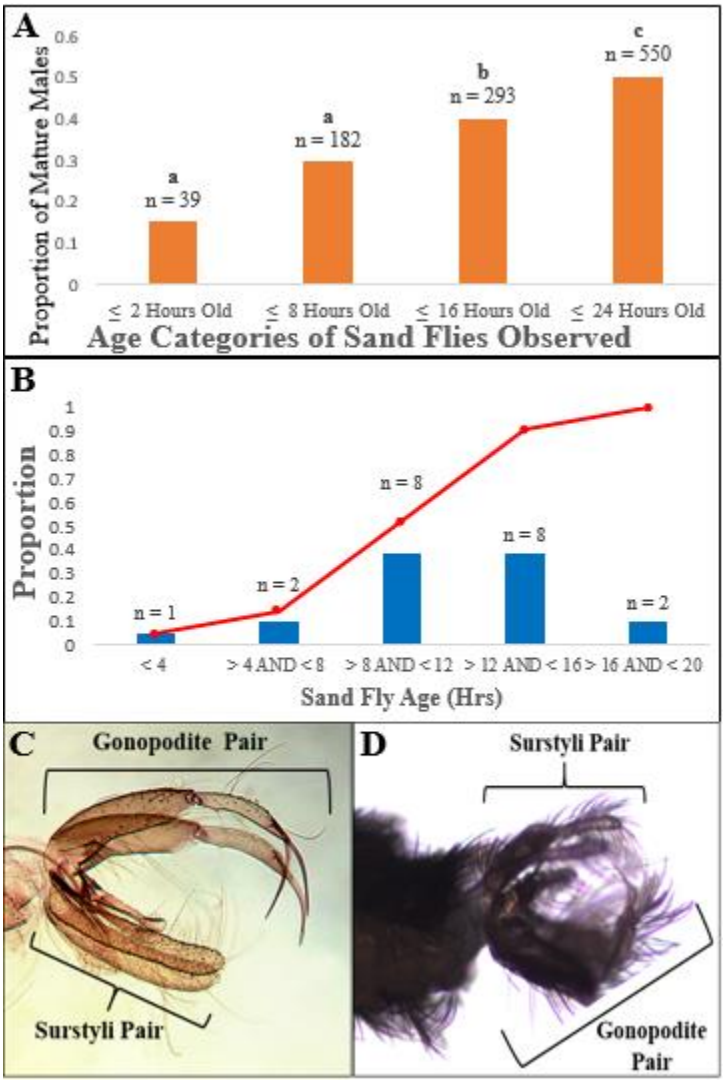

Figure 5. Change in the Proportion of Sexually Mature Males with Time since Eclosion as Determined by Releasing Flies from Rearing Cups at Different Time Intervals (A) and by Monitoring Maturation Time for Males Reared from Individual Pupae (B). Red line in Panel B depicts the cumulative proportion. Male sexual maturity was determined by orientation of male terminalia. To be categorized as sexually mature, at least one of the gonopodite pair had to be completely dorsally oriented (C). Otherwise, male was considered sexually immature (D). Panel C credited to J Stoffer, Walter Reed Biosystematics Unit: Sand Fly Taxonomy Tutorial. Categories with different letters were considered significantly different ($P<0.05$). n = Number of sand flies observed.

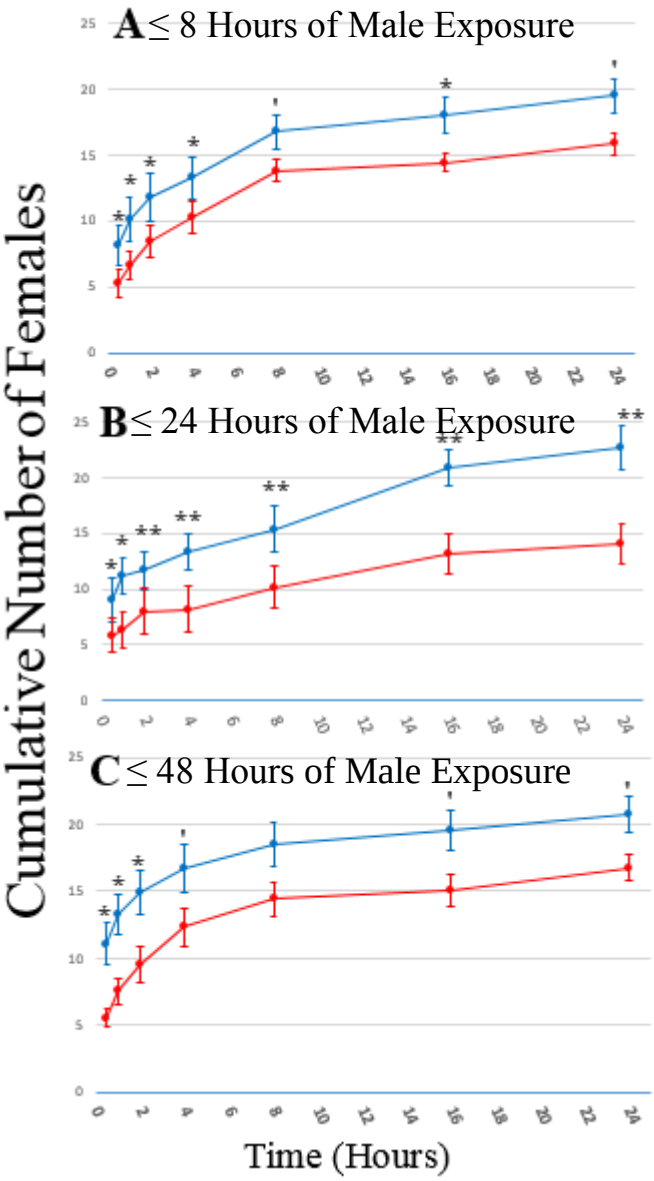

Figure 6. The Effect of Association Time of Females with Males on Female's Response to Male Extracts. Panels A, B, and C depict the change in the number of sand flies caught in treatment (filter paper with male extract, blue line) and control traps (filter paper with hexane, red line) over time for all male exposure categories. Bars are standard error. ***P<0.0001, **P<0.01, *P<0.05, 0.1>'P>0.05.

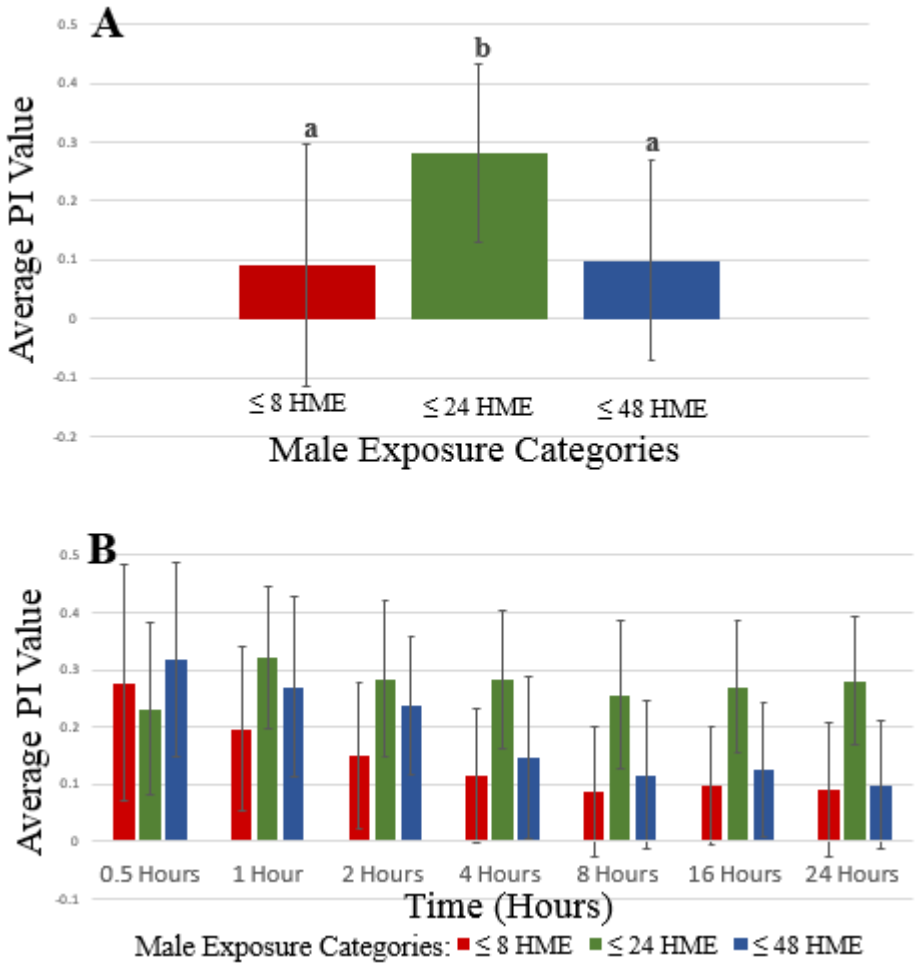

Figure 7. Comparison of the Attraction of Females of Different Male Exposure Categories to Male Extracts at the End of the Experiment (A) and Over Time (B). HME = Hours of male exposure. Categories with different letters were considered significantly different ($P<0.05$). Bars are 95% CI.

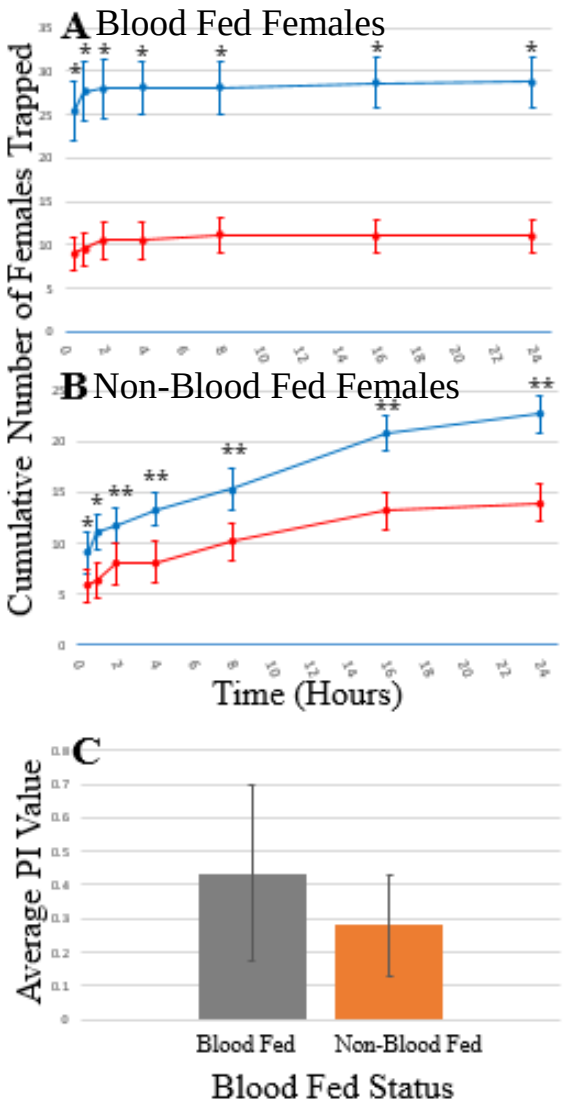

Figure 8. The Effect of Being Blood Fed on Female's (≤ 24 Hours of Male Exposure) Response to Male Extracts. Panels A and B depict the change in the number of sand flies caught in treatment (filter paper with male extract, blue line) and control traps (filter paper with hexane, red line) over time for blood fed (A) and non-blood fed females (B). Bars are standard error. Panel C compares the preference index of both blood fed and non-blood fed female sand flies to male extracts. Bars are 95% CI. ***P<0.0001, **P<0.01, *P<0.05, 0.1>'P>0.05.

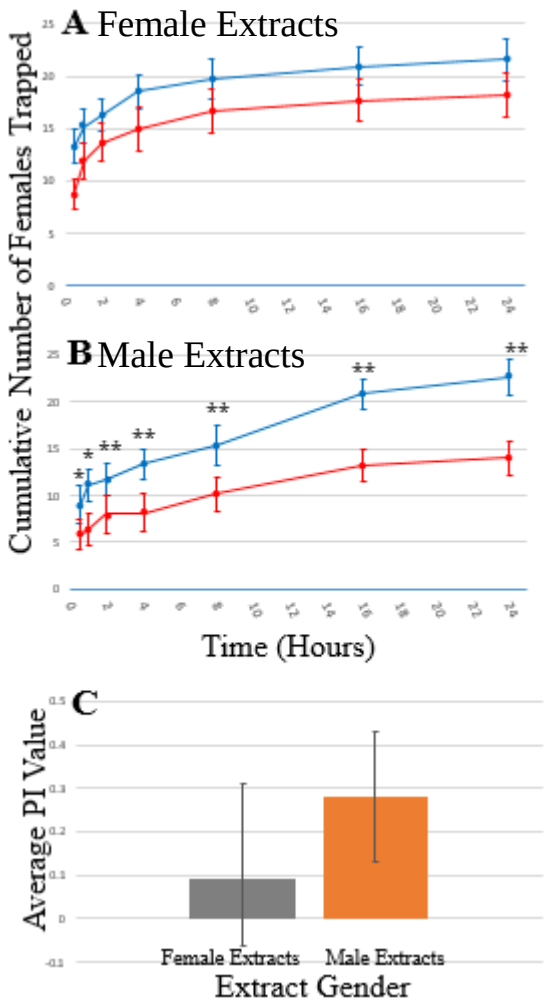

Figure 9. Comparison of the Attraction of Females (≤ 24 Hours of Male Exposure) to Male and Female Extracts. Panels A and B depict the change in the number of sand flies caught in treatment (filter paper with male extract (A) or female extract (B), blue line) and control traps (filter paper with hexane, red line) over time for extracts of each gender. Bars are standard error. Panel C compares the preference index of female sand flies to male and female extracts. Bars are 95% CI. ***P<0.0001, **P<0.01, *P<0.05, 0.1>'P>0.05.

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