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Phlebotomine sand flies are the vectors of the *Leishmania* parasites as well as other bacterial and viral pathogens worldwide. Due to the variable impact of traditional vector control practices, a more ecologically-based approach was needed. The goal of this study was to develop an attractive blend that would attract gravid *Phlebotomus papatasi* female sand flies and that would be later used as a lure for a lethal oviposition trap. The general hypothesis of this study was that female sand flies have evolved to detect cues of bacterial origin indicating suitable growth habitat for its progeny. Since sand fly larvae feed on decomposing organic material of predominantly fecal origin, 5 candidate materials were tested; rabbit feces, larval food, $2^{nd}/3^{rd}$ instar rearing medium, $4^{th}$/pupae rearing medium, and expired colony medium. The total oviposition response of each of the candidates was tested using a multiple-choice bioassay, and their relative attractance was tested using a 2-choice attraction bioassay. The $2^{nd}/3^{rd}$ instar rearing medium demonstrated the highest significant effect in all bioassays and, therefore, a bacterial analysis was performed in order to culture all constituent bacterial isolates. 2-choice attraction bioassays were used to test the attractance of each of the bacterial isolates at serially-diluted concentrations. Certain bacterial isolates have demonstrated high attractance at specific concentrations, which indicates potential applications in constructing the optimal attractive bait.

Keywords: *Phlebotomus papatasi*, sand fly, leishmaniasis, oviposition, attraction

# IDENTIFYING OVIPOSITION ATTRACTANTS FROM THE LARVAL REARING MEDIUM OF *PHLEBOTOMUS PAPATASI*, THE VECTOR OF OLD WORLD ZOONOTIC CUTANEOUS LEISHMANIASIS

by

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_____
Committee Chair

This thesis is dedicated to my parents, Georges and Kinane Marayati, without whom it would not have been possible for me to complete this work. For your endless love, support, and encouragement. You are the reason why I strive to achieve my goals.

APPROVAL PAGE

This thesis written by BAHJAT FADI MARAYATI has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

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# CHAPTER I

# INTRODUCTION

## Background

### Epidemiology of Leishmaniasis.

Leishmaniasis is a cluster of diseases caused by protozoan parasites in the genus *Leishmania* (*Kinetoplastida: Trypanosomatidae*). Leishmaniasis is transmitted by the bite of a phlebotomine sand fly species (*Diptera: Psychodidae*) of the genera *Phlebotomus* (Old World) (Figure 1) and *Lutzomyia* (New World) (Antinori et al. 2012). There are three main pathogenically-distinct types of the leishmaniasis disease: cutaneous, mucocutaneous, and visceral leishmaniasis. Visceral leishmaniasis (VL), which is the most life-threatening type, is one of the top two parasitic diseases in the world, second only to malaria (Desjeux 2001). About 1.5–2 Million new cases are estimated to occur annually, but only 500,000 cases are officially reported (Desjeux 2001, Murray et al. 2005, Reithinger et al. 2007). In addition, deadly epidemics of visceral leishmaniasis occur periodically but go mostly unnoticed in spite of case–fatality rates as high as 10% (Alvar et al. 2013). In the 1990s Sudan alone suffered a crisis of 100,000 deaths due to visceral leishmaniasis (Seaman et al. 1996). Leishmaniasis has been classified by the World Health Organization as a Neglected Tropical Disease (NTD) due to incompetent health care, a high rate of under-reporting and a lack of funding for scientific research (Alvar et al. 2006, Antinori et al. 2012, Hotez et al. 2012). Most cases of leishmaniasis

occur in the poorest regions of the world, with 90% of visceral leishmaniasis cases focused in 6 endemic areas (Bangladesh, Brazil, Ethiopia, India, Nepal, and Sudan), and cutaneous leishmaniasis (CL) occurring mainly in North Africa (Algeria, Tunisia), the Middle East (Afghanistan, Iran, Saudi Arabia, and the Syrian Arab Republic), and South America (Brazil, Colombia, Peru) (WHO 2013). Leishmaniasis appears to be emerging globally due to factors such as anthropogenic land-use changes and urbanization, as well as human migration, poverty, drug resistance, and HIV co-infections (Ashford 2000, Desjeux 2001, Wasserberg et al. 2003, Norris 2004, Murray et al. 2005, Alvar et al. 2006). Cutaneous leishmaniasis (CL) (Figure 2) is the most common form of leishmaniasis and is characterized by the skin lesions and ulcerated nodules (Weina et al. 2004). CL is endemic in 70 countries worldwide. 90% of cases are concentrated in the Middle East and South America (Reithinger et al. 2007). Infections are more likely to cluster within households and are tightly linked to poverty and lack of medical attention (Yadon et al. 2003, Alvar et al. 2006). Cases of Old World CL are usually found in semi-arid and desert regions, whereas New World CL cases are mostly associated with forested areas (Reithinger et al. 2007). The unicellular flagellated parasitic protozoans of the genus *Leishmania* (*Kinetoplastida: Trypanosomatidae*) include over 21 species including the major causative agents of the Old World cutaneous leishmaniasis: *L. aethiopica, L. tropica,* and *L. major*, while *L. mexicana* and *L. brazilliensis* species complexes are more predominant in the New World (Killick-Kendrick 1990).

Life Cycle of *Leishmania* Parasites.

Unlike mosquitoes, sand flies are inaudible fliers and are 2-3 millimeter in length which allows them to penetrate through mosquito nets (Hepburn 2003), with a life cycle that takes around 5 weeks. After a blood meal, the female lays its eggs inside the burrows of the host, on organically rich moist soil, or around animal shelter in peridomestic areas (Feliciangeli 2004). Eggs hatch into the first larval stage and grow up to the fourth larval stage feeding on decaying organic matter available at the breeding site. The larvae then become pupae and then emerge as adults which feed nocturnally on sugars from a variety of sources including aphid honeydews and plant nectar (Schlein and Yuval 1987, Petts et al. 1997), and the female sand fly is hematophagous (requires a blood meal in order to lay eggs) (Young and Duran 1994). The life cycle of the *Leishmania* protozoan involves alternation between a mammalian host and a phlebotomine sand fly host. The life cycle continues as an uninfected female sand fly blood-feeding on an infected mammalian host such as rodents, hyraxes, dogs, or humans (Ashford 1996). During the blood meal, the uninfected sand fly will ingest amastigotes (the replicating non-mobile form of the protozoan) where the amastigotes develop into promastigotes (the flagellated mobile form of the protozoan) which will escape the peritrophic matrix and attach to the midgut and will then migrate to the pharynx of the host sand fly to form a bolus and block feeding. Upon a second blood meal, the bolus carrying the *Leishmania* promastigotes will be washed into the host and such promastigotes can actively invade macrophages through phagocytosis and live inside the host's own immune system where the promastigotes transform into multiplying amastigotes inside the infected macrophages. Amastigotes will

then leave the infected cells during the next blood meal to complete the cycle (Killick-Kendrick 1990, Gossage et al. 2003).

### Conventional Control Methods.

Vector or reservoir host control has been suggested to be the more effective prevention strategy (Davies et al. 2003b). In the absence of vaccination (Ali et al. 2014, Kumar and Engwerda 2014) and the limited medical treatment (Reithinger et al. 2007), and in light of high cost and serious side effects of available pentavalent antimonial-based treatments (Croft et al. 2006, Alvar et al. 2013). The current sand fly control strategies involve reservoir host population control, personal protection, and residual spraying of insecticides. Studies targeting reservoir hosts have been limited to culling regimens for domestic dogs found to be infected (Ashford et al. 1998) which was shown to be inefficient and ethically problematic (Ashford et al. 1998, Ribas et al. 2013). Bed-nets and window seals are effective and strongly recommended in areas where leishmaniasis is endemic but implementation, however, is not effective because such items are expensive and not accessible to the entire population (Davies et al. 2003a, Murray et al. 2005, Bray and Hamilton 2013). The most common approach at control is to spray the area with residual insecticides (e.g. Organophosphates and pyrethroid-based products). This is not ideal because non-target arthropods including *Apis mellifera* (honey bees) are killed (Pimentel 1995), it causes behavioral disturbances and learning disabilities in children (Eriksson 1996) and metabolic diseases such as Type 2 Diabetes

and Obesity in adults (Barker 1958, Longnecker et al. 1997, Alexander and Maroli 2003, Ruzzin 2012).

One other practical sand fly control method was performed by mechanically causing wide-spread destruction to the breeding sites in the burrows of the rodent hosts (Faizulin 1980). This is an example of source reduction which has shown great potential in reducing disease transmission (Focks et al. 2000). This approach has been applied to disrupt the malaria transmission cycle where the mosquito vector lays aquatic larvae. Successfully reducing the total population abundance and has also prolonging oviposition site-seeking time period by blood-fed mosquitos. This approach also reduces the "basic reproductive rate" ($R_o$) of malaria (Gu et al. 2006) which is a measure of the average number of secondary cases produced by an index case during its infectiousness period (Macdonald 1957).

### Ecological Control Methods.

Due to the limited effectiveness and the high public health risks that the conventional control methods have displayed, there is a need for the development of new, ecologically-based methods to control sand fly populations (Hotez et al. 2012). One of many ecological approaches has focused on the application of feed-through insecticides by which the hosts are provided with baits that contain an insecticide that is not harmful to the host but lethal to the fly upon acquiring a blood meal (Sheppard et al. 1992). This method was tested on the Syrian Hamster (*Mesocricetus auratus*) and provides evidence that the insecticide Novaluron is successful in significantly reducing egg hatching rate

and larval development of sand flies (Mascari et al. 2007). Control efforts targeting the larval instars by using feed-through insecticide techniques in the diet of the host, *Psammomys obesus* (the Sand Rat), are promising but require further assessment (Mascari et al. 2007, 2008, Wasserberg et al. 2011). Unlike mosquitoes, sand flies do not undergo aquatic larval development stages. Furthermore, the breeding sites of sand flies are cryptic and poorly described (Alexander and Maroli 2003, Feliciangeli 2004). Hence, breeding sites are often estimated by the detection of adult sand flies (Casanova 2001, Moncaz et al. 2012). For all the above reasons, the application of source reduction in sand flies has been difficult (Warburg and Faiman 2011). Due to the large endemic areas and the cryptic underground breeding sites, source reduction for sand flies has been ineffective and may also have harmful effects on other non-target species (Davies et al. 2003b, Feliciangeli 2004).

Attracting the vector to a trap which contains an attractive material mixed with lethal chemicals has been well described as a control method for vector mosquitoes and other agricultural pests, for which the term "attract-and-kill" has been introduced as an innovative and effective commercially available control method (Zeichner and Debboun 2011). Sand fly control studies applying this approach have targeted many of the weak links in the life cycle of the adult sand fly: sugar feeding stage, mating stage, blood feeding stage, and oviposition stage. Sugar is the main source of energy for flight and reproduction, both males and females sugar-feed at the adult stage (Yuval and Schlein 1986, Müller et al. 2010). The source of sugar includes a range of aphid honeydews, flower nectars, and fruits (Müller et al. 2011, Wasserberg et al. 2014b). Using an

attractive lethal sugar bait targets primarily newly hatched or the non-bloodfed females. This control method has shown promising results on mosquito species and has been suggested as a potential sand fly control method (Müller et al. 2010, Wasserberg et al. 2014b). Targeting the mating stage of the adult sand fly has focused on sex pheromones which are emitted by male sand flies to enhance mate recognition (Ward and Morton 1991, Chelbi et al. 2011). Chemical compounds of the himachalene family (1*S*,3*S*,7*R*-3-methyl-α-himachalene) have been identified as the main male sex pheromones in *Lutzomyia longapalpis*. Control efforts at the mating stage of the adult sand fly life cycle ideally target the sugar-fed, non-infective virgin females that have yet to take a blood meal (Nigam and Ward 1991, Hamilton et al. 1994, Bray et al. 2010). Another ecological control method was to target the blood-feeding females which must locate a suitable host in order to obtain a blood meal. Host odors have been tested in the lab and identified to contain a blend of carbon dioxide, octanol, lactic acid, ammonia, and caproic acid (Pinto et al. 2012). Carbon dioxide has also been used in constructing an attractive CDC-type trap in order to collect sand flies in the field (Burkett et al. 2007).

In terms of vector control, an oviposition-attractants based approach targets older females that have already acquired their first blood meal and are ready to lay their eggs and move on to their second blood meal, which is the critical stage in disease transmission. Therefore, oviposition attractants are especially effective in interrupting the disease transmission cycle in contrast to other types of semiochemicals which mainly attract young, nulliparous, non-bloodfed females (Dougherty and Hamilton 1997). Oviposition attractants are currently being tested in the field on disease vectors such as

the dengue fever mosquito vector *Aedes aegypti* (Ponnusamy et al. 2008, Barbosa et al. 2010) which have resulted in a patented oviposition trap (ovitrap) based on a chemically active ingredient which attracts egg-laying females (Wesson et al. 2011).

In the context of ecological control methods, Integrated Vector Management programs aim at deploying multiple control methods in order to reduce the vector population and disrupt the transmission cycle. The newly developed ecological control methods don't eliminate the application of the conventional control methods, such as personal protection and residual spraying. All available control methods must work in parallel in order to achieve the desirable vector control results (Croft and Hoyt 1983, Hepburn 2003, Norris et al. 2003).

### Oviposition Site Selection in Arthropods.

Oviposition Preference-Offspring Performance (P-P) theory has been suggested to explain the behavior of gravid females seeking oviposition sites (Thompson 1988). The P-P theory posits that in the absence of parental care, the female insect would evolve to select the most suitable habitat in order to increase the development and the survival of its progeny (Resetarits 1996, Nufio and Papaj 2001, Wasserberg et al. 2014a). Oviposition site selection has been the topic of discussion in various dipterans with special attention to mosquitoes as important disease vectors (Bentley and Day 1989, Vonesh and Blaustein 2010, Wong et al. 2011). A suitable oviposition site represents a site with the appropriate ambient microclimate conditions such as temperature and relative humidity, absence of predators and parasites, availability of food for the larvae,

and the presence of conspecifics which can indicate that the same site has been selected by previous females but could also indicate intraspecific competition for the food sources (Wilton 1967, Mokany and Shine 2003, Rudolf and Rödel 2005). Female mosquitoes generally orient towards attractive semiochemicals (chemical signals that are emitted by an organism for the purpose of communication; Semeion is Greek for signal) (McCall and Cameron 1995, Kelly and Dye 1997) since the survival of the progeny is the main purpose in selecting a site to lay eggs (Chapman 1985). In addition, with no parental care and no juvenile dispersal (distance travelled by larval stages), there is a great responsibility on the mother to select a suitable oviposition site in order to optimize its own fitness by enhancing the survival of its progeny (Kiflawi et al. 2003). The site selected by the mother female mosquito to lay its eggs has been demonstrated to have good correspondence with the performance of the larvae (Singer 1972, Rausher 1982). Recent studies also shed the light on the density of conspecifics (individuals of the same species such as eggs, larvae, and pupae) present at such oviposition sites (Kiflawi et al. 2003, Mokany and Shine 2003, Sumba et al. 2008). A Hump-shaped Regulation model described the cost-benefit relationship in various mosquito species, and other blood-feeding insects, where the presence of conspecific larvae indicates suitability of the oviposition site at low densities, and potential undesirable intraspecific competition at higher densities (Wasserberg et al. 2014a).

A key paper published by Dethier et al. (1960) defined the difference between an "attractant" and a "stimulant" as two important animal behavior concepts. For a certain material to be an attractant, the material has to cause the animal to make a noticeable

orientation in the directionality of the movement towards the source. However, for a material to be a stimulant it must induce a certain behavioral act (e.g. The act of feeding, mating, or oviposition) (Dethier and Browne 1960).

The biotic and abiotic environment send chemical signals, known as semiochemicals (Regnier 1971). Such semiochemicals were organized into two major classes of compounds: pheromones and allelochemicals (Nordlund et al. 1981, Vet and Dicke 1992). Pheromones are mainly intraspecific chemicals; they facilitate the chemical interactions between two individuals of the same species. Allelochemicals are interspecific chemicals; they facilitate the interaction between the organism and its abiotic environment, or other species present in that environment (Pitts et al. 2014). Allelochemicals are classified as synomones, kairomones, allomones, and apneumones. Synomones are chemicals that benefit both, the emitter and the receiver, and primary examples of synomones are the chemicals released by a plant and received by a pollinator insect where the plant gets pollinated and the insect gets a nectar award (Pitts et al. 2014). Kairomones are chemicals that only benefit the receiver, but not the emitter, such as the volatile host odors used by bloodsucking insects to locate a blood meal (Bruce et al. 2005). Allomones are compounds that benefit only the emitter, but not the receiver, and are usually used as a defense mechanism or a deterrant, such as the secretions by the social wasp *Mischocyttarus drewseni* which repels foraging ants from the stem of its nest (Whitman et al. 1990). Finally, apneumones are compounds emitted by a non-living material that can benefit a specific receiving organism but are harmful to other organisms, such as the chemical odors emitted by oatmeal which attract an

endoparasitoid (*Venturia canescens*) which invades the larval of lepidopterous pests. Insects are capable of recognizing such chemical cues using olfactory receptors (for gaseous odors, at a distance) and gustatory receptors (for solid and liquid chemicals, in close proximity) (Pitts et al. 2004). Insects exhibit olfactory sensilla on the antennae, each sensillum is arranged in a highly stereotypical fashion and detects the various semiochemicals (pheromones and allelochemicals) through specialized olfactory receptor neurons (Keil and Steinbrecht 1984). It was not long before ecologists started using such semiochemicals in biological pest and vector control programs (Borden 1989). Pheromone-based products were used to replicate the natural pheromones emitted by insects in order to interrupt reproductive activities and control population growth. One example of allelochemical-based approaches in biological control of vectors includes the sugar-based toxic baits applied in the control of mosquito species (Müller et al. 2011, Beier et al. 2012).

### Oviposition Site Selection in Sand Flies.

Gravid female sand flies do not lay their eggs indiscriminately but are hypothesized to use a range of semiochemical cues indicating an oviposition site with conditions suitable for the growth of their larvae (Killick-Kendrick 1999, Feliciangeli 2004, Moncaz et al. 2012). Work done in this field has mainly focused on *Lutzomyia longapalpis*, the vector of *Leishmania* parasites in the New World (Killick-Kendrick 1990). Behavioral screening has provided a plethora of data where conspecific eggs have been found attractive (Elnaiem and Ward 1992, Dougherty et al. 1993, 1994, Hamilton et

al. 1994, Dougherty and Hamilton 1997, Dougherty et al. 1999), whereas conspecific larvae and pupae had a repellent effect (Dougherty and Hamilton 1996). Studies using gas chromatography to analyze the oviposition pheromones secreted by the sand fly *Lutzomiya longapalpis* have established dodecanoic acid to be the primary attractive compound present (Dougherty and Hamilton 1997). In terms of allelochemicals influencing oviposition site selection, the kairomones1-octen-3-ol and 1-hexen-3-ol which have shown significant attractant effects on the abundance *Lutzomyia longapalpis* in the field (Kelly and Dye 1997, Mann et al. 2009). It has long been known that female sand flies use such kairomones to locate a suitable host for a blood meal (Nigam and Ward 1991, Dougherty et al. 1999). Studies focusing on the synergistic effect of the presence of host odor kairomones and sex pheromones simultaneously have confirmed that attractive effect of the blend is greater than each of the semiochemicals presented separately (Dougherty et al. 1999). It is important to mention that the approach using kairomones and male pheromones as bait both attract females of younger age which are yet to obtain a first blood meal, hence are less significant in the disease transmission cycle since the uninfected female sand fly will require a blood meal in order to obtain the parasite from an infected host.

### Oviposition Site Selection in *Ph. papatasi.*

Oviposition site selection of female *Phlebotomus papatasi* is poorly understood (Feliciangeli 2004). What is well known, however, is the strong ecological dependence by *Ph. papatasi* on its natural reservoir host, the Sand Rat (*Psammomys obesus*) in North

Africa and the Middle East and the Great Gerbil (*Rhombomus opimus*) in Central Asia (Ashford 1996). The moist and cool shelter provided by the burrows of these hosts present the sand fly with appropriate amounts of organic material and fecal matter which provide a desirable environment for the sand fly larvae and the abundance of reservoir hosts provides easily accessible blood meals. It is of no surprise that strong evidence has shown high abundance of *Phlebotomus papatasi* near such breeding sites (Ashford 1996, Killick-Kendrick 1999, Wasserberg et al. 2003). Adult *Ph. papatasi* have also been detected in a variety of domesticated poultry sheds and rabbit holes in inhabited areas (Feliciangeli 2004, Chelbi et al. 2008). Previous studies have demonstrated an increasing oviposition response on organic matter such as frass (solid excreta of insect larvae), but the response was sub-additive where frass alone resulted in a higher oviposition response than a combination of frass and conspecific material (Wasserberg and Rowton 2011). Other sources such as cow manure have also been shown to be a strong attractant for gravid and non-gravid females (Schlein et al. 1989). Rabbit feces have shown a strong oviposition stimulation effect (Chelbi et al. 2008).

### Microbial Ecology of Sand Flies.

The microbial ecology of sand flies is not yet understood, although it has been suggested that sand fly gut microbiome play a crucial role at the larval development stage, as well as the adult disease transmission cycle. At the larval stages, eggs hatch into larvae with sterile guts and are hence believed to acquire the majority of their gut microbiome from the environment (Peterkova-Koci et al. 2012). Peterkova-Koci et al.

(2012) also demonstrated that *Lu. longipalpis* larval development and survival rates were significantly decreased if reared using autoclaved (sterile) rabbit feces as a larval food source. In addition, the same group has also isolated the culturable bacterial isolates from rabbit feces and introduced each isolate into a sterile rabbit feces medium in order to test for individual effects of bacterial isolates. Their results suggest that bacteria of the species *Agrobacterium tumefaciens* not only significantly supported larval development, but also survived pupation and inhabited the guts of adult female sand flies (Peterkova-Koci et al. 2012). Other studies also confirm that fecal material can be made more attractive through its conditioning by microbes. *Bacillus licheniformis* and *Staphylococcus saprophyticus* were two bacterial strains isolated from the soil of a variety of sand fly breeding sites near human dwellings and cow sheds have demonstrated an enhancing effect on *Ph. papatasi* oviposition response (Radjame et al. 1997). Other important bacterial isolates from rabbit feces that had a significant effect on larval development include bacterial species of the genus *Pseudomonas* and *Enterococcus*, while isolates from the genus *Bacillus* were identified but did not have a significant effect on development of the larvae when individually added to sterile rabbit feces (Peterkova-Koci et al. 2012). Studies on the bacterial communities present in adult sand flies have identified over 100 different bacterial species from the gut microbiome (Hillesland et al. 2008) and sand flies from different geographic regions have demonstrated a wide range of variety across Old World and New World species (Sant'Anna et al. 2012). Such variation in the adult gut microbiome was attributed to the difference in ecological conditions at the breeding sites, while two bacterial species (*B.*

*pumilus* and *B. flexus*) were consistently observed across all studied breeding sites (Mukhopadhyay et al. 2012). Other studies also highlight the role of sand flies as vectors of human-related pathogenic bacteria such as *Bacillus cereus*, *Escherichia coli*, and *Staphylococcus aureus* (Hillesland et al. 2008).

**Objective**

The development of an attract-and-kill approach targeting the adult *Phlebotomus* sand fly female at the oviposition stage could potentially be effective because it attracts the gravid female to a bait which mimics the suitable environment for oviposition. Such an approach can touch upon two critical steps in the disease transmission model; reduction in the total population size by eliminating the female vector as it lays new eggs and hence killing the offspring in the process, and the selective targeting of the infected blood-fed females before spreading the infection through subsequent blood meals. This thesis project was one part of a broader research project involving a systematic integrated approach including behavioral, microbiological, and electrophysiological analyses in order to identify the optimal attractive compound(s) that drives the oviposition behavior of the sand fly. The behavioral assays played a central role in confirming the attraction of sand flies towards the compounds. The bacterial analysis performed by Dr. Charles Apperson and Dr. Loganathan Ponnusamy at North Carolina State University has established a list of the culturable microbial constituents of the attractive compounds in order to generate bacterial isolates which were sent back to our lab for behavioral testing of attraction, individually and in a mixture. In future studies, Dr. Coby Schal from North

Carolina State University will perform Gas Chromatography and Electroantennography (GC-EAD) analyses to identify any biologically-active compounds in the material suggested in this study (Figure 3). The behavioral, electrophysiological, and microbiological confirmation of such attractive compound, or blend of compounds, will be optimized to serve as an attractive lure for the potential commercial production of the desired attract-and-kill oviposition trap. Such oviposition trap can provide the basis for a novel targeted surveillance approach of sand fly populations and improve the maintenance and productivity of sand fly lab colonies which currently tend to suffer from high oviposition-related mortality of females

**Study Question**

What is the source of the olfactory cues that drive the oviposition site selection of the *Ph. papatasi* sand flies?

**General Hypothesis**

Oviposition site selection of gravid sand fly females is driven by cues indicating suitable growth substrate for its offspring. Since larval sand flies feed on decomposing organic material of predominantly fecal origin, I hypothesized that female sand flies are attracted to volatile olfactory cues indicating the presence of such a substrate. Furthermore, since organic matter decomposition is a product of bacterial activity, I hypothesized that these cues are of bacterial origin. To achieve this goal, my study focused on the following specific aims:

**Specific Aim 1**

Determine the potent source material stimulating oviposition and driving sand fly attraction from the rearing medium of different larval stages.

Study Question 1.

What source material derived from the different larval stages will elicit the highest oviposition response and attraction among gravid *Ph. papatasi* females?

Hypothesis 1.

Gravid female sand flies are differentially attracted to pre-larval fecal material as compared to larvae-conditioned rearing media which emit volatile olfactory cues indicating the presence of food for their larvae.

Prediction 1.

Material taken from colony rearing medium with successfully developing larvae will produce a different attraction and total oviposition response compared to the pre-larval and post-larval colony rearing material.

In this aim, I studied the effect of materials derived from decomposing fecal matter, which were tested for total oviposition response and relative attraction. The candidate materials chosen were all derived from rabbit feces which has previously shown a strong attractive effect (Chelbi et al. 2008, Wasserberg and Rowton 2011). The rabbit feces material was then used to make the larval food material which is, prepared by mixing a 1:1 ratio of rabbit feces and rabbit food, ground to powder, spread in a tray and

fermented inside a sealed incubator for 1 week, then flipped on the other side for another week to ensure homogenous fungal growth, then left at room temperature to dry for a week. This larval food material was then added to the coprophagic early instar larvae which used it as their primary source of food in order to grow. Early, middle, and late instar larvae required different amounts of larval food. I tested rearing media obtained from colony cups which contained larvae at the $2^{nd}/3^{rd}$ instar larval developmental stages, and also tested rearing media obtained from colony cups which contained larvae at the $4^{th}$/pupae developmental stages. Finally, I examined the effect of expired colony material which was obtained from larval colonies that were previously viable but have recently emerged into adults (eclosed) leaving an empty potential oviposition site behind. This aim was divided into 3 experiments: Experiment 1. Total oviposition response was determined using behavioral multiple-choice experiments where gravid females were introduced into large oviposition cups with all five candidate materials, plus a control. Experiment 2. The top 3 candidate materials from the multiple-choice experiments were used in paired-choice experiments where total oviposition response of each candidate is tested against a negative control. Experiment 3. The attractance of each of the candidate material was tested using a 3-chamber in-line olfactometer in which gravid females were presented with a choice to orient towards either the candidate material or a negative control.

From the attractive source material found in this specific aim, 12 Bacterial species were isolated, purified in the Apperson lab at North Carolina State University through sequencing of 16S rDNA PCR amplicons, to be used in subsequent experiments.

**Specific Aim 2**

Determine the attraction of bacterial strains isolated from the potent source material.

Study Question 2.

What bacterial strains, and what serially-diluted concentrations of such isolates will emit the chemical cues that will produce the highest attraction among gravid *Ph. papatasi* females?

Hypothesis 2.

Gravid *Ph. papatasi* are attracted to a blend of chemical cues emitted from the bacterial isolates cultured from the selected candidate source material.

Prediction 1.

Some bacterial isolates will contribute more towards the overall attractive effect of the candidate source material than others.

Prediction 2.

Attraction of the different bacterial strains is expected to be dose dependent.

Soil bacteria isolated from natural breeding habitats of *Ph. papatasi* have been known to emit odorants which attract the gravid females to lay eggs (Radjame et al. 1997). In addition, larval gut microbiome has been previously shown to play a key role in the production of semiochemicals responsible for oviposition attractance in insects (Davis

et al. 2013). Bacterial strains were isolated and cultured from the potent source material by Dr. Apperson and Dr. Ponnusamy from North Carolina State University. This aim was divided into 2 experiments: Experiment 1. An equal-ratio mixture of the culturable bacterial species isolated from the attractive material identified in Aim 1 was tested behaviorally in olfactometer bioassays to evaluate the bacterial mixture's relative attractance. Experiment 2. The attraction of gravid sand flies to volatiles of different bacterial isolates at various concentrations was evaluated behaviorally using the olfactometer bioassay, this dose response has been demonstrated in the literature and is attributed to the importance and the fine tuning of the mechanism by which a gravid female selects the optimum oviposition site (Ponnusamy et al. 2010).

# CHAPTER II

# MATERIALS AND METHODS

## Colony and Mass Rearing Protocol

*Phlebotomus papatasi* originating from Akbuk, Turkey were colonized at the Walter Reed Army Institute of Research (Silver Spring, Maryland) and maintained at the University of North Carolina in Greensboro. Rearing of *Ph. papatasi* sand flies followed the mass-rearing methods described by Lawyer et al (1991). Sand flies were maintained in incubators (Model: 6030-1, Caron®, Marietta, Ohio) at 26ºC, 80% RH, and 12:12 light:dark cycle. Flies were fed on anesthetized mice (UNCG IACUC protocol 14-07 dated 2/26/2015). Colonies were maintained in 500 mL Nalgene jars (Nalgene™, Model 81063, diameter = 11 cm) with 2.2 cm layer of Whip-Mix® Orthodontic Plaster (Model: 5577352, Henry Schein Inc., Melville, New York) on the bottom to ensure moist substrate and drainage. The rearing of one generation usually takes five weeks from egg to adult, and adult sand flies were fed using cotton soaked in 30% white table-sugar solution while larvae were fed using a specially prepared larval food which is added three times a week. The larval food mentioned here and used in the experiments was prepared by mixing rabbit feces and rabbit chow at a 1:1 ratio, fermented for 3 weeks, and ground to powder.

**Specific Aim 1**

Experiment 1. Multiple-Choice Experiments.

I conducted multiple-choice behavioral assays using 500 mL Nalgene jars (similar to the rearing jars) modified for 6-choice assays (Figure 4). Each jar was placed in water for 12 hours prior to the start of an experiment to equilibrate the moisture level of its plaster flooring. I simultaneously tested five source materials of biological origin in addition to a negative control: material obtained from colony rearing cups containing mainly $2^{nd}/3^{rd}$ instar larvae (hereafter, $2^{nd}/3^{rd}$), material obtained from colony rearing cups containing mainly $4^{th}$ instar larvae and pupae (hereafter, $4^{th}$/pupae), material from colony rearing cups which mainly contain frass from which all larvae had eclosed (hereafter, expired colony frass), larval food (hereafter, LF), rabbit feces (hereafter, RF) and a solvent (deionized water) negative control treatment (Figure 4). To minimize the potential of cross-contamination, each of these materials (1.0 mg) was placed on a filter paper disc (2.5 cm diameter) (Model: 09-801-AA, ThermoFisher Scientific®, Waltham, Massachusetts) at equal distance from the center of the cup. Three drops (~0.15 mL) of deionized water were then added to each filter paper. Each experimental session (N=9 replicate sessions conducted between 3/1/2013 and 1/3/2014) consisted of 7 oviposition jars. During the first 24 hours post blood-meal sand flies were left undisturbed in their holding cage so to not interrupt the development of the peritrophic matrix around their recently acquired blood-meal. Then, fifty gravid females were transferred into each of the 7 bioassay jars using a mouth aspirator. Jars were then returned into the rearing incubator.

To obtain a time-course of oviposition, the assays were terminated, one jar at a time, 1 to 7 days after transfer (or 2 to 8 days post blood-meal) by releasing the females into a separate holding cage. I photographed the filter papers with a T3i Canon 100 mm macro lens. Eggs laid on each filter paper were counted from high quality digital photos using the counting tools in Adobe Photoshop (Adobe Photoshop CS5 2010, Adobe™, San Jose, California).

### Experiment 2. Paired-Choice Experiments.

Based on my results from the multiple-choice experiments, I identified the top three candidate choice material: $2^{nd}$-$3^{rd}$ instar larval medium, $4^{th}$ instar larval medium, and rabbit feces organic matter. The next step was to test the top three candidates in treatment vs. control design pair-wise bioassays. Using 125mL Nalgene jars with one drainage hole at the bottom (1 cm diameter) and a 2.2 cm layer of Whip-Mix® Orthodontic Plaster (Model: 5577352, Henry Schein Inc., Melville, New York), two filter paper disc (2.5 cm diameter) (Model: 09-801-AA, ThermoFisher Scientific®, Waltham, Massachusetts) were placed at opposite ends of the cup with one filter paper having 1.0 mg of the treatment and the other serving as a negative control. 3 drops of deionized water were added to each filter paper, and 20 blood-fed gravid females (24 hours post blood meal) were introduced in each cup. Jars were left undisturbed inside the rearing incubator for 5 days, females were then released back into a separate holding cage and high definition pictures were created and the number of eggs laid on each of the filter papers was recorded using the same photography methods mentioned in Experiment 1.

Experiment 3. Olfactometer Experiments.

Attraction of gravid sand flies to various source materials was assayed with a 2-choice olfactometer. The olfactometer consisted of a cylindrical Plexiglas® apparatus made of three in-line chambers (each chamber: 9.4 cm inner diameter, 10.1 cm outer diameter, 15 cm length). A section of PVC pipe (2.5 cm length, 10.15 cm inner diameter), glued to either side of a white Plexiglas square partition (11.4 x 11. 4 cm, 3 mm thickness), and coupled the middle chamber to the outer two chambers. A hole in the center of each partition held a 6 cm long (1 cm inner diameter) tubes extending 3 cm into the central chamber and 3 cm in an outer chamber (Figure 5). In each olfactometer, source material (0.5 g) to be tested was placed on a 7.5 mL weigh boat containing 1.2 mL of orthodontic plaster and tested against a blank negative control (similar plaster-bottomed weigh boats but with 3 water droplets [ca. 0.15 mL]). In each experimental session (N=10 replicate sessions conducted between 12/4/2013 and 2/2/2014) I used six olfactometers with source materials including: $2^{nd}/3^{rd}$ medium, $4^{th}$/pupae medium, expired colony frass, LF, and RF as well as one olfactometer with blank (deionized water) controls on both sides to test for potential directionality bias. A treatment weigh boat was placed on a plastic stage at one end of the olfactometer, and the other end received a control weigh boat. The ends of the side-chambers were then covered with a fine mesh screen secured with rubber bands. Twenty gravid *Ph. papatasi* females (72 hours post blood-meal) were transferred to the middle chamber of the olfactometer. The middle chamber was then connected to a vacuum pump (Air Admiral® Cole-Parmer, Vernon Hills, IL) that delivered a total volumetric flow of 1.05 L/min. The vacuum pump

remained off for the first 60 minutes of the bioassay to allow natural diffusion and acclimation, and then the vacuum pumps were turned on for 2 hours. The olfactometer was then placed into a -20$^{o}$C freezer and the number of females in each chamber was counted. Before each bioassay, olfactometers were cleaned using an odorless cleaning detergent (RBS-35, Model: 27950, ThermoFisher Scientific, Waltham, Massachusetts). All bioassays were conducted in a controlled environment room with temperature and humidity identical to those of the rearing colony incubator (Temperature: 28$^{o}$C and Relative Humidity: 80%). Assays were conducted in the scotophase 3 – 8 hours after lights-off. The olfactometers were randomly assigned locations within the room and rotated between replicate experiments to avoid directional bias.

**Specific Aim 2**

Bacterial Culturing, Isolation, and Serial Dilutions.

In the first Experiment, an equal-ratio mixture of the culturable bacterial species from the 2$^{nd}$/3$^{rd}$ instar larval rearing medium, provided by Dr. Loganathan Ponnusamy and Dr. Charles Apperson from North Carolina State University, was tested behaviorally in olfactometer bioassays to evaluate the bacterial mixture's relative attractance. In the second Experiment of this specific aim (experiments performed between 10/13/2014 and 05/13/2015), the mixture of the culturable bacterial species from the 2$^{nd}$/3$^{rd}$ instar larval rearing medium was separated into 12 culturable individual isolated bacterial colonies (coded: SSI-1, SSI-2, SSI-3, SSI-4, SSI-5, SSI-6, SSI-8, SSI-9, SSI-10, SSI-11, SSI-13, and SSI-15. The bacterial mixture and all 12 isolates were maintained in colonies at the

lab of Dr. Charles Apperson and Dr. Loganathan Ponnusamy at North Carolina State University. Morphologically distinct colonies from culture plates were isolated, restreaked multiple times until pure bacterial isolates were obtained. Bacterial isolates were grown in 1L Erlenmeyer flasks in 200mL of TSB medium at 28$^{o}$C with continuous shaking at 150 rpm for 48 hours. The bacterial mixture was prepared by plating on Trypticase soy agar (TSA), Plate Count Agar (PCA) and Nutrient agar (NA) media. The 48 hour grown bacterial isolates were serially diluted and mixed in equal amounts before testing.

Olfactometer Bioassays.

The tests were conducted using 2-choice olfactometers (similar to the apparatus used in specific aim 1) where the different concentrations of the bacterial mixture and bacterial isolates served as the treatment and blank TSA media broth served as the negative control. All treatments and controls were placed in 30mL graduated medicine cups (Item#: 81772, Moore Medical, Connecticut). 3 olfactometers were set up per treatment concentration ($10^9$, $10^8$, and $10^7$ cells/mL in Experiment 1 in addition to $10^6$ Cells/mL only in Experiment 2). 20 gravid *Ph. papatasi* females (72 hours post blood-meal) were transferred to the middle chamber of the olfactometer. The middle chamber was then connected to a vacuum pump (Air Admiral® Cole-Parmer, Vernon Hills, IL) that delivered a total volumetric flow of 1.05 L/min. The experiment was allowed 1 hour of free diffusion (with vacuum pumps off) in order to let female sand flies acclimate to the environment, then 2 hours of vacuum pumping which will pull air through the system

from both side chambers and allow the sand flies to locate themselves and to make a choice between the treatment, middle, or control chamber. All 9 olfactometers were then placed into a -20ºC freezer and the number of females in each chamber was counted. All experiments were conducted in a walk-in environmental chamber (Hotpack, SP Scientific, Warminster, PA) at the University of North Carolina in Greensboro under standard conditions (Temperature: 28ºC and Relative Humidity: 80%).

**Statistical Analysis**

In Aim 1, oviposition time-course represents the cumulative number of eggs laid over the specified number of days and the cumulative number of eggs per female per oviposition cup was used and analysis was performed using the non-parametric Kruskal-Wallis test, followed by the non-parametric comparison for all pairs using the Steel-Dwass method. In order to compare the cumulative number of eggs among the six choices in the multiple-choice experiments within each cup and in between cups, Kruskal-Wallis was used with the treatments as variables, post hoc test was performed using the Steel-Dwass method. Kruskal-Wallis test was also used to compare between the top three candidates in the paired-choice experiments. In olfactometer experiments, an Oviposition Attraction Index (OAI) was created in order to evaluate and compare the responses of female sand flies to the treatments from different sources as opposed to a control. The index was calculated using the formula: $OAI = (N_t - N_c)/(N_t+N_c)$ where $N_t$ is the number of female sand flies found in the treatment-side chamber of the olfactometer and $N_c$ is the number of female sand flies found in the control-side chamber of the

olfactometer. This oviposition index was used in similar studies (Kramer and Mulla 1979) and can take values between -1 (most repellence) to +1 (most attraction). Kruskal-Wallis test was used to examine any significant difference in OAI across different treatments, followed by the non-parametric comparison for all pairs using the Steel-Dwass method. For the bacterial experiments in Aim 2, a t-test was used to compare the number of female sand flies in the treatment-side or control-side chambers of the olfactometer for each bacterial concentration of each bacterial isolate, and the mixture. A multiple linear regression was used to test the oviposition attraction index (OAI) across the different bacterial concentrations of each bacterial isolate. For all analyses, significance level was set at $P< 0.05$. Analysis was conducted using JMP software version 11.0 (SAS Institute Inc., Cary, NC).

# CHAPTER III

# RESULTS

## Specific Aim 1

### Preliminary Oviposition Time-Course Experiment.

There was a significant difference in the cumulative number of eggs deposited over time (Kruskal-Wallis: $\chi^2 = 35.23$ df = 6 $P < 0.0001$). Average total of eggs deposited during the first 72 hours following the blood-meal was very low (1.6 ± 1.2 eggs). The cumulative number of eggs deposited significantly increased at 96 hours following the blood-meal (Steel-Dwass: Z = 3.14 $P = 0.0273$). Cumulative number of eggs deposited demonstrated a non-significant increase between 96 hours and 192 hours after blood-meal (Steel-Dwass: Z = 2.29 $P = 0.2458$). The average number of eggs laid per female during this period (96 to 192 hours post blood-meal) was equal to 13.25 ± 2.10 (Figure 6, Table 1).

### Multiple-Choice Experiments.

Since the number of eggs laid during the first 72 hours post blood meal was low, statistical analysis was performed using data from 96 to 192 post blood-meal. There was a significant difference in the number of eggs deposited among the 6 treatments (Kruskal-Wallis: $\chi^2 = 23.62$ df = 5 $P = 0.0003$). More eggs were oviposited on each of the $2^{nd}/3^{rd}$ rearing medium, $4^{th}$/pupae rearing medium, and larval food when compared with the

water control (Steel-Dwass: Z = 3.87 $P$ = 0.0015, Z = 3.84 $P$ = 0.0016, and Z = 2.88 $P$ = 0.0449). The expired colony material showed a significant difference, albeit marginal, compared to the control (Steel-Dwass: Z = 2.77 $P$ = 0.0617). There was no significant difference between the Rabbit Feces treatment and the Expired colony rearing medium treatment when compared to the control (Steel-Dwass: Z = 2.04 $P$ = 0.3143) (Figure 7, Table 2).

Paired-Choice Experiments.

From the multiple-choice bioassays in Experiment 1, the 3 top candidate choice treatments were selected and the paired-choice bioassays were performed between 96 and 192 hours post blood meal. No significant difference across all 3 treatments was present (Kruskal-Wallis: $\chi_2$ = 2.20 df = 2 $P$ = 0.3321) (Table 3, Figure 8). There was no significant difference was between the number of eggs laid on the treatment filter paper as compared to the control filter paper in all 3 treatments (t-test: $2^{nd}/3^{rd}$: $t$ = 1.19 $P$ = 0.2536. $4^{th}$/pupae: $t$ = 0.97 $P$ = 0.3462. LF: $t$ = -1.10 $P$ = 0.2862). The $2^{nd}/3^{rd}$ treatment had an average of 49.20 ± 31.20 eggs, the $4^{th}$/pupae treatment had an average of 43.00 ± 31.20 eggs, while the LF treatment had less eggs than the control filter paper with an average of -17.12 ± 22.06 eggs.

Olfactometer Experiments.

In the olfactometer bioassays, only data from olfactometers with equal or greater than 25% response rate were selected (more than 5 out of 20 sand flies moved to either

the treatment or the control chambers). No directional bias was observed in the control olfactometers (where both side chambers had water as a negative control) with 4.33 ± 0.31 flies chose the wall-side chamber and 4.25 ± 0.51 flies chose the room-side chamber (t-test: $t = 0.60$ $P = 0.895$).

Based on the attraction preference index, there was a significant difference in attraction among treatments (Kruskal-Wallis: $\chi_2 = 32.08$ df = 5 $P < 0.0001$). Overall, response rate was 44% of flies moving to either of the side chambers, while 66% of the flies stayed in the middle chamber across all experiments. A Significantly higher response rate was observed in the olfactometers where the treatment was larval food (Steel-Dwass: Z = 2.77 $P = 0.0617$) as compared to other treatments. $2^{nd}/3^{rd}$ larval rearing medium and $4^{th}$/pupae rearing medium were the only treatments to show significant difference compared to the control (Steel-Dwass: Z = 3.63 $P = 0.0038$, and Z = 3.29 $P = 0.0124$ respectively). While larval food, rabbit feces, and expired colony rearing medium were not significantly different from the control (Steel-Dwass: Z = 1.72 $P = 0.5169$, Z = 2.35 $P = 0.1714$, and Z = 1.63 $P = 0.5785$) (Figure 9, Table 4).

**Specific Aim 2**

Testing for Sidedness Bias.

Control olfactometers used as part of all olfactometer experiments contained blank TSA media at both sides. Control olfactometers served as a control for potential biases in the directionality of the flight of the sand fly in the absence of odorants emitted by the bacterial treatment itself. Using all control olfactometers (N = 25), there was an

average of 1.95 ± 0.49 flies moving to the right-side chamber and 1.90 ± 0.50 flies moving to the left-side chamber. In accordance with the minimum percent response rate used in this study (percent response ≥ 25%) the control olfactometers with more than 25% response rate (N= 6) confirmed the no sidedness effect observed with the right-side chamber having 4.83±0.74 flies and the left-side chamber having 4.83±0.94 flies. No significant difference was observed between the right-side chamber and the left-side chamber of control olfactometers (t-test: $t$= 0.13 $P$= 0.7953) (Figure 10).

Bacterial Mixture Experiments.

In figure 11, data for the bacterial mixture was reported in terms of the percent response; black bars represent the percent of female sand flies moving to the treatment-side chamber, and gray bars represent the percentage of female sand flies moving to the control-side chamber (Error bars represent 95% confidence interval). A paired t-test was performed at each of the concentrations level and the asterisks represent any significant difference between the number of flies moving to the treatment-side chamber or the control-side chamber of that specific concentration. The differences among treatments were tested using a linear regression model and the adjusted $R^2$ was reported alongside the p-value to assess a significant difference in the OAI (Oviposition Attraction Index) across all concentrations. A post-hoc analysis using Tukey-HSD was used to establish the letter-report in order to highlight what concentrations are different among themselves (Figure 11).

The bacterial mixture was tested at 3 concentrations: $10^7$ Cells/mL (0.01X), $10^8$ Cells/mL (0.1X), $10^9$ Cells/mL (1X). The bacterial mixture demonstrated a significant negative concentration-dependent response with decreasing attraction as the bacterial concentration increases (Figure11). At the lowest bacterial concentration ($10^7$ Cell/mL), there was a marginally significant attractive effect (paired t-test: $t$= 2.43 $P$= 0.0529). The middle bacterial concentration ($10^8$ Cells/mL) showed no attractive, or repellent, effect (paired t-test: $t$ = 0.12 $P$ = 0.9087). The highest bacterial concentration ($10^9$ Cells/mL) showed a significantly repellent effect (paired t-test: $t$ = -3.04 $P$ = 0.0222). When comparing the different concentrations using the attraction index values (OAIs), $10^7$ Cells/mL showed a significantly higher attraction than $10^9$ Cells/mL (Tukey-HSD: $P$ = 0.0417). Mean OAI for $10^7$ Cells/mL was 0.60 ± 0.33, mean OAI for $10^8$ Cells/mL was -0.15 ± 0.34, and the mean OAI for $10^9$ Cells/mL was -0.52 ± 0.44.

Because of the general trend of decreasing attraction with increasing bacterial concentration, I decided to use a lower serially-diluted bacterial concentration ($10^6$ Cells/mL or 0.001X) in all subsequent bacterial isolates tested in order to detect more fine-tuned responses (Figure 11).

Bacterial Isolates Experiments.

Isolates SSI-2, SSI-9, and SSI-11 demonstrated a significant negative dose response with significant attractance at the lowest bacterial concentration (paired t-test: $t$ = 3.66 $P$ = 0.0112, $t$ = 9.15 P < 0.0001, and $t$ = 6.66 $P$ = 0.0024 respectively) and significant repellence at the highest bacterial concentration (paired t-test: $t$ = -3.49 $P$ =

0.0060, $t$ = -1.55 P < 0.0001, and $t$ = -1.32 $P$ = 0.0089 respectively) (Figures 12b, 12h, 12j). SSI-6 demonstrated the same trend of negative dose response, albeit non-significant, with significant attraction at the lowest bacterial concentration (paired t-test: $t$ = 2.55 $P$ = 0.0293) but with a non-significant repellence at the highest bacterial concentration (paired t-test: $t$ = -1.60 $P$ = 0.5725) (Figure 12f). SSI-1, SSI-3, SSI-10, and SSI-13 demonstrated the opposite effect where there was a significant positive dose response with repellence at the lowest bacterial concentration (paired t-test: $t$ = -6.70 $P$ = 0.0026, $t$ = -3.40 $P$ = 0.0192, $t$ = -1.31 $P$ = 0.2832, and $t$ = -1.98 $P$ = 0.2832 respectively) and attractance at the highest bacterial concentration (paired t-test: $t$ = 1.25 $P$ = 0.0051, $t$ = -0.45 $P$ = 0.1977, $t$ = 0.77 $P$ = 0.2846, and $t$ = 2.32 $P$ = 0.0093 respectively) (Figures 12a, 12c, 12i, 12k).

A linear regression analysis was performed on the OAI preference index values and the post-hoc test (Tukey-HSD) was used to indicate any significant difference among each of the 4 concentrations of each of the 12 bacterial isolates. Post-hoc test results are indicated by the letter report on Figure 12 (12a through 12l). Isolates SSI-2, SSI-9, and SSI-11 displayed a significantly higher OAI preference values at $10^6$ Cell/mL compared to $10^9$ Cells/mL (Tukey-HSD: $P$ = 0.0002, $P$ = 0.0001, and $P$ = 0.0001 respectively) (Figures 12b, 12h, 12j). Isolates SSI-4, SSI-5, SSI-6, and SSI-15 displayed no significant difference in OAI preference values across all concentrations (Tukey-HSD: $P$ = 0.7866, $P$ = 0.9046, $P$ = 0.0999, and $P$ = 0.9263 respectively) (Figures 12d, 12e, 12f, 12l). Isolates SSI-1, SSI-3, SSI-8, SSI-10, and SSI-13 displayed a significantly higher OAI preference

values at $10^9$ Cells/mL compared to $10^6$ Cells/mL (Tukey-HSD: $P = 0.0004$, $P = 0.0262$, $P = 0.2410$, $P = 0.0288$, and $P = 0.0037$ respectively) (Figures 12a, 12c, 12g, 12i, 12k).

# CHAPTER IV

# DISCUSSION

## Specific Aim 1

### Oviposition Time-Course.

In terms of the time-course of oviposition, *Ph. papatasi* females did not lay eggs for a period of 72 hours after the blood meal. The number of eggs laid significantly increases then shows a non-significant increasing trend for the duration of the period between 3-8 days after the blood meal (Table 1). These results are inconsistent with the literature in which a longer time period between the blood meal and oviposition was observed (Benkova and Volf 2007). The number of eggs laid per female in this study was 13.25 eggs/female. Number of eggs laid per *Ph. papatasi* female varies from one study to another and could depend on the number of generations reared in the lab since colonization (Chelbi and Zhioua 2007), time of the year during which the experiments are performed as related to the circadian fluctuation in oviposition activity (Schlein et al. 1990), but does not depend on whether the blood meal was from a human or an animal source (Harre et al. 2001). Similar laboratory studies have reported 15-20 eggs/female (Wasserberg and Rowton 2011), 15 eggs/female (Harre et al. 2001), and as high as 65 eggs/female (Chelbi and Zhioua 2007).

## Oviposition Response and Oviposition Attraction.

This study provides a foundation for a larger project which ultimately aims at the production of a commercial attract-and-kill ovitrap. The underlying concept behind such a trap is to find a material that attracts female sand flies and stimulates oviposition. To find such a material, the rearing medium of the sand fly colony was examined and a range of candidates were selected for testing. The attractive effect of rabbit feces has been well studied (Chelbi et al. 2008) and it constitutes the basis for all other candidate material tested. Sand fly larval food is prepared by mixing and fermenting rabbit feces with rabbit chow in a 1:1 ratio. Such larval food is added to the colony larvae which feed on it as they progress from early stages ($1^{st}$ instar larvae) to middle stages ($2^{nd}/3^{rd}$ instar larvae) to late stages ($4^{th}$ instar and pupae). As the larvae develop from egg to pupae, larval food is added according to a set schedule. As pupae start eclosing and adult sand flies emerge from the colony cup, larval food is not added anymore. Eventually, all adults emerge from the colony cup leaving behind expired material which represents the absence of active larvae; the presence of old digested larval food remains, as well as exuviae and dead adults.

The candidate material tested could be divided into three categories: organic material in pre-larval stages (rabbit feces, and larval food), larval developmental stages ($2^{nd}/3^{rd}$, and $4^{th}$/pupae rearing media), and a post-larval stage which represents the expired material. In the first aim of this study, the total oviposition response and the attraction bioassays both revealed a common pattern among the leading 2 candidate

materials which has ranked the 2nd/3rd instar material first followed closely by the 4th/pupae material, while the effect of rabbit feces and larval food was inconsistent in attraction bioassays and total oviposition response experiments. Such results, where pre-larval stages and post-larval stages show no significant difference in both types of bioassays, could suggest that the presence of material from live, actively metabolizing, larvae is a possible source of attraction for gravid females looking for an oviposition site to lay their eggs, since it represents an oviposition site that has been selected by previous oviposition site-seeking females and their progeny is developing successfully.

This study was inconsistent with others where fresh rabbit feces has produced a significant effect as an oviposition stimulant and an oviposition attractant in New World sand flies (Elnaiem and Ward 1992, Dougherty et al. 1993). The effect of expired colony material was also examined in the literature where an aqueous extract of old colony cup remains induced an increase in total oviposition response (Wasserberg and Rowton 2011), which was confirmed in this study as the number of eggs laid on the expired colony filter paper was significant, albeit marginal, when compared to the control. However, this study examined the effect of expired colony material a step further by differentiating between the total oviposition response (in multiple choice experiments) and attraction (in olfactometer experiments) in which expired colony material has been demonstrated to be an oviposition stimulant, but not an oviposition attractant.

Paired-Choice Experiments.

In the paired-choice experiments, the results were inconsistent with the previous multiple-choice experiments as none of the 3 treatments ($2^{nd}$/$3^{rd}$ rearing medium, $4^{th}$/pupae rearing medium, and larval food) significantly differed from the water control, and none of the treatments significantly differed among one another. This inconsistency could be attributed to methodological constraints. The main methodological difference between the multiple-choice experiments and the paired-choice experiments was the size of the experimental containers (with 500mL Nalgene cups used in multiple-choice experiments and 125mL Nalgene cups used in paired-choice experiments). Oviposition container size has been discussed in primary literature in which container volume had the most impact on oviposition rate compared to other methodological variations such as humidity, temperature, and time of day in a study by Harrington et al. (2008) on oviposition site selection in mosquitoes, while other laboratory-based oviposition response paired-choice experiments failed to highlight the increased oviposition rate on positive controls that are otherwise known to induce an increase in oviposition (Isoe et al. 1995). These results could be attributed to the restrictive oviposition cup volume since all other methodological aspects were similar: type of container used (Nalgene plastic), type of horizontal bottom surface (orthodontic plaster), the diameter of the filter paper on which the treatment and the control were placed (diameter: 2.5 cm), as well as the amount of treatment placed on the filter paper (1.0 mg).

The significant attraction towards the 2nd/3rd instar larval material (Figure 9), and the fact that the main difference between this material and other pre-larval and post-larval treatments tested is the presence of larvae, lead to the suggestion that pre-larval material (rabbit feces and larval food) is being bacterially conditioned when passing through the gut of the live, foraging, and defecating larvae at the most metabolically active larval stages of the developmental life cycle. This interpolation is supported by looking at the consistently lower oviposition response and oviposition attraction to the 4th/pupae instar larvae, which represent the developmental period where larvae start moving into the pupation stage, at which stage the pupae do not feed (Killick-Kendrick 1990).

**Specific Aim 2**

Previous studies have shown that sand flies prefer to lay eggs on the naturally bacterially active rabbit feces as opposed to its sterile (autoclaved) equivalent (Peterkova-Koci et al. 2012). They found that the presence of the diverse bacterial fauna was shown to lead to a higher larval survival rate, identifying 17 different culturable bacterial species in rabbit feces. The bacterial species isolated, then tested as a rearing medium in mixture and as individual isolates, showed that some isolates performed better than the mixture, while other isolates performed worse than the autoclaved rabbit feces (Peterkova-Koci et al. 2012). Thus, it is not surprising to find that some isolates from the 2nd/3rd larval rearing medium (which is attractive as a mixture) to be repellent or neutral when tested individually. When the solid 2nd/3rd instar material was compared to the bacterial mixture at the lowest concentration, the attractance index values for both were similar (OAI= 0.56

and OAI= 0.61 respectively, t-test: $t$= 0.18 $P$= 0.8573). Other studies found similar results where oviposition attraction of bamboo and white oak leaf infusions when tested then compared to the attractance of a mixture of all culturable isolates from each of the infusions (Ponnusamy et al. 2008).

An interesting observation in this study was that certain bacterial isolates (such as SSI-2, SSI-9, and SSI-11) have an attractive effect at low concentrations but seem to have a repellent effect at higher concentrations. This effect has been demonstrated previously and is attributed to the importance and the fine tuning of the mechanism by which a gravid female selects the optimum oviposition site (Ponnusamy et al. 2010). The use of higher dose of an attractive bacterial isolate, or mixture of isolates, could mimic the presence of a crowded environment and higher risk of potential intraspecific competition which is not suitable for the progeny.

**Conclusion**

In conclusion, this study found that the rearing medium of $2^{nd}/3^{rd}$ instar larvae is substantially more attractive than pre-larval (rabbit feces, larval food) and post-larval (expired colony) rearing media. This study further analyzed the bacterial constituents of the $2^{nd}/3^{rd}$ instar rearing medium and examined the bacterial involvement in the production of oviposition attractants. 3 bacterial isolates (SSI-2, SSI-9, and SSI-11) stood out as possible attractants at the lowest bacterial concentration. Other bacterial isolates (such as SSI-4, SSI-5, SSI-6, and SSI-15) were neutral with no significant difference in attraction among all concentrations. Still other bacterial isolates (SSI-1, SSI-3, SSI-8,

SSI-10, and SSI-13) were attractant at the highest bacterial concentration tested. In order to achieve the ultimate goal of the study, which is to construct an attractive bait for a lethal oviposition trap, a mixture of bacterial strains could be used. A combination of the most attractive bacterial isolates at the most attractive concentrations (Table 5) could include SSI-2, SSI-9, and SSI-11 at $10^6$ Cells/mL (OAI = 0.61, 0.78, 0.64 respectively). The top attractive bacterial isolates should be further analyzed, and the additive attractive effect of combining multiple bacterial strains should be examined and compared to the attractive effect of the bacterial mixture. The most repellent bacterial isolates include SSI-1 at $10^6$ Cells/mL (OAI = -0.57), as well as SSI-2 and SSI-9 at $10^9$ Cells/mL (OAI = -0.80 and -0.55, respectively). One important value of the most repellent bacterial isolates at the most repellent concentrations could have other applications, such as the construction of a repellent spray (Table 6).

Some of the limitations in this study are the use of non-infected laboratory-colonized sand flies, and the size of the olfactometers (15 cm travelling distance to each of side chambers). Future studies should include thorough testing of the different combinations of the attractive isolates, as well as scaling up the experimental design and perhaps using a wind-tunnel setting in order to better replicate attraction at a larger distance which is one of the desired characteristics of the attractive bait. Optimization and possible commercialization of such attractive bait could have substantial effects on population control and surveillance efforts on sand flies.

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APPENDIX A

TABLES

**Table 1. Oviposition Time-Course Experiment.** Kruskal-Wallis test for the effect of time period since blood-meal on the cumulative number of eggs. Mean number of eggs (± SE) represents the total number of eggs laid in all multiple-choice experiments and is cumulative over the time period between 48 and 192 hours (2 – 8 days) after the sand fly female acquires a blood meal.

| Time | Mean number of eggs (SE) | Count (N) | Score Sum | Score Mean | Standardized Score |
|---|---|---|---|---|---|
| 48 | 2.750 (2.75) | 8 | 79.500 | 9.9375 | -3.622 |
| 72 | 0.500 (0.50) | 8 | 76.500 | 9.5625 | -3.687 |
| 96 | 451.556 (259.69) | 9 | 280.500 | 31.1667 | 0.021 |
| 120 | 578.444 (210.54) | 9 | 338.000 | 37.5556 | 1.201 |
| 144 | 587.556 (226.40) | 9 | 349.500 | 38.8333 | 1.437 |
| 168 | 648.222 (223.81) | 9 | 346.000 | 38.4444 | 1.365 |
| 192 | 898.778 (247.67) | 9 | 421.000 | 46.7778 | 2.904 |

Kruskal-Wallis Test: ChiSquare = 35.2322, df = 6, $P < 0.0001$

**Table 2. Multiple-Choice Experiments.** Kruskal-Wallis test for the effect of different oviposition candidate treatments on the cumulative number of eggs oviposited per filter paper disc in 6-choice oviposition assays. Table also presents means (± SE) of number of eggs oviposited per filter paper disc for each of the treatment types. Test materials included larval rearing media of different types and stages including: fresh rabbit feces (RF), fresh larval food (LF), rearing medium containing frass of $2^{nd}$-$3^{rd}$ instar larvae ($2^{nd}$/$3^{rd}$), rearing medium containing frass of $4^{th}$ instar larvae and pupae ($4^{th}$/pupae), frass of rearing cups from which all larvae had eclosed (expired) and a negative (water) control. Significance level was set at $P > 0.05$.

| Treatment | Mean number of eggs (SE) | Count (N) | Score Sum | Score Mean | Standardized Score |
|---|---|---|---|---|---|
| Control | 25.91892 (4.68) | 37 | 2692.500 | 72.8510 | -4.009 |
| RF | 49.86486 (12.41) | 37 | 3723.500 | 100.6350 | -1.126 |
| LF | 63.43243 (12.45) | 37 | 4330.000 | 117.0270 | 0.572 |
| $2^{nd}$/$3^{rd}$ | 95.72973 (18.12) | 37 | 5108.500 | 138.0680 | 2.755 |
| $4^{th}$/pupae | 72.51351 (12.90) | 37 | 4750.000 | 128.3780 | 1.750 |
| Expired | 48.08108 (7.20) | 37 | 4145.500 | 112.0410 | 0.055 |

Kruskal-Wallis Test: ChiSquare = 23.6239, df = 5, $P = 0.0003$

**Table 3. Paired-Choice Experiments.** Kruskal-Wallis test for the effect of 2nd/3rd instar larval rearing medium (2nd/3rd), 4th/pupae rearing medium (4th/pupae), and larval food (LF) on the oviposition attraction preference index in a treatment vs. control paired-choice design. Table presents the mean oviposition attraction preference index values (±SE). Significance level was set at $P > 0.05$.

| Treatment | Treatment (SE) | Control (SE) | OAI (SE) | Count (N) | Score Sum | Score Mean | Standardized Score |
|---|---|---|---|---|---|---|---|
| 2nd/3rd | 129.91 (39.32) | 80.66 (12.45) | 0.04 (0.11) | 12 | 240.000 | 20.0000 | 0.587 |
| 4th/pupae | 143.16 (40.89) | 100.16 (16.70) | 0.03 (0.12) | 12 | 248.000 | 20.6667 | 0.856 |
| LF | 81.91 (19.09) | 126.83 (36.01) | -0.13 (0.09) | 12 | 178.000 | 14.8333 | -1.460 |

Kruskal-Wallis Test: ChiSquare = 2.2045, df = 2, $P = 0.3321$

**Table 4. Olfactometer Experiments.** Attraction of *P. papatasi* towards different treatment types in olfactometer experiments. Mean number (± SE) of *Ph. papatasi* females in the treatment, middle, and control chambers of the olfactometers and percent flies responding (Total number of flies at both the treatment and control chambers divided by total flies used) for the different treatment types. Rearing media of 2nd/3rd, 4th/pupae, and Rabbit feces (bolded) induced highest attraction. Kruskal-Wallis test results are displayed and significance level was set at $P > 0.05$.

| Treatment | Treatment (SE) | Middle (SE) | Control (SE) | OAI (SE) | % response (SE) | Count (N) | Score Sum | Score Mean | Standardized Score |
|---|---|---|---|---|---|---|---|---|---|
| Control | 4.33 (0.31) | 11.42 (0.57) | 4.25 (0.51) | 0.04 (0.07) | 43 (2.8) | 12 | 176.500 | 14.7080 | -3.507 |
| RF | 4.9 (0.18) | 12.4 (0.34) | 2.7 (0.3) | 0.30 (0.06) | 38 (17) | 10 | 321.000 | 32.1000 | 0.309 |
| LF | 6.4 (0.54) | 9.5 (0.73) | 4.2 (0.29) | 0.20 (0.03) | 53 (3.8) | 10 | 227.500 | 22.7500 | -1.534 |
| 2nd/3rd | 7.2 (0.63) | 10.9 (0.57) | 1.9 (0.23) | 0.56 (0.06) | 46 (2.8) | 10 | 505.500 | 50.5500 | 3.984 |
| 4th/pupae | 6.2 (0.61) | 11.3 (0.7) | 2.5 (0.17) | 0.41 (0.04) | 44 (3.3) | 10 | 425.500 | 42.5500 | 2.390 |
| Expired | 4.6 (0.31) | 11.9 (0.43) | 3.4 (0.34) | 0.18 (0.04) | 40 (2.1) | 10 | 174.000 | 21.7500 | -1.518 |

Kruskal-Wallis Test: ChiSquare = 32.081, df = 5, $P < 0.0001$

**Table 5. Summary of the Most Attractive Tested Bacterial Isolates Ranked by Preference Index in Descending Order.** Table shows the highest preference index recorded for each isolate and the associated concentration (in Cells/mL). Table also shows the solid source material ($2^{nd}/3^{rd}$ Source Material) and the bacterial mixture ($2^{nd}/3^{rd}$ Bacterial Mixture). Highlighted cells indicate the top 4 attractive isolates/material and their perspective concentrations, suggested to be used in future studies.

| Rank | Name | Concentration | OAI |
|---|---|---|---|
| 1 | SSI-9 | $10^6$ Cells/mL | 0.78 |
| 2 | SSI-11 | $10^6$ Cells/mL | 0.64 |
| 3 | $2^{nd}/3^{rd}$ Bacterial Mixture | $10^6$ Cells/mL | 0.61 |
| 3 | SSI-2 | $10^6$ Cells/mL | 0.61 |
| 4 | $2^{nd}/3^{rd}$ Source Material | Solid Material | 0.56 |
| 5 | SSI-1 | $10^9$ Cells/mL | 0.52 |
| 6 | SSI-13 | $10^9$ Cells/mL | 0.45 |
| 7 | SSI-6 | $10^6$ Cells/mL | 0.23 |
| 8 | SSI-3 | $10^9$ Cells/mL | 0.21 |
| 9 | SSI-10 | $10^9$ Cells/mL | 0.17 |
| 10 | SSI-15 | $10^9$ Cells/mL | 0.1 |
| 11 | SSI-5 | $10^6$ Cells/mL | -0.03 |
| 12 | SSI-4 | $10^6$ Cells/mL | -0.1 |
| 13 | SSI-8 | $10^9$ Cells/mL | -0.15 |

**Table 6. Summary of the Most Repellent Tested Bacterial Isolates Ranked by Preference Index in Ascending Order.** Table shows the lowest preference index recorded for each isolate and the associated concentration (in Cells/mL). Table also shows the bacterial mixture ($2^{nd}/3^{rd}$ Bacterial Mixture). Highlighted cells indicate the top 4 repellent isolates and their perspective concentrations, suggested to be used in future studies.

| Rank | Name | Concentration | OAI |
|---|---|---|---|
| 1 | SSI-2 | $10^9$ Cells/mL | -0.8 |
| 2 | SSI-1 | $10^6$ Cells/mL | -0.57 |
| 3 | SSI-9 | $10^9$ Cells/mL | -0.55 |
| 4 | $2^{nd}/3^{rd}$ Bacterial Mixture | $10^9$ Cells/mL | -0.52 |
| 5 | SSI-3 | $10^6$ Cells/mL | -0.44 |
| 6 | SSI-8 | $10^6$ Cells/mL | -0.42 |
| 7 | SSI-13 | $10^6$ Cells/mL | -0.33 |
| 8 | SSI-11 | $10^9$ Cells/mL | -0.32 |
| 9 | SSI-10 | $10^6$ Cells/mL | -0.27 |
| 10 | SSI-4 | $10^6$ Cells/mL | -0.1 |
| 11 | SSI-6 | $10^9$ Cells/mL | -0.05 |
| 11 | SSI-15 | $10^6$ Cells/mL | -0.05 |
| 12 | SSI-5 | $10^6$ Cells/mL | -0.03 |

APPENDIX B

FIGURES

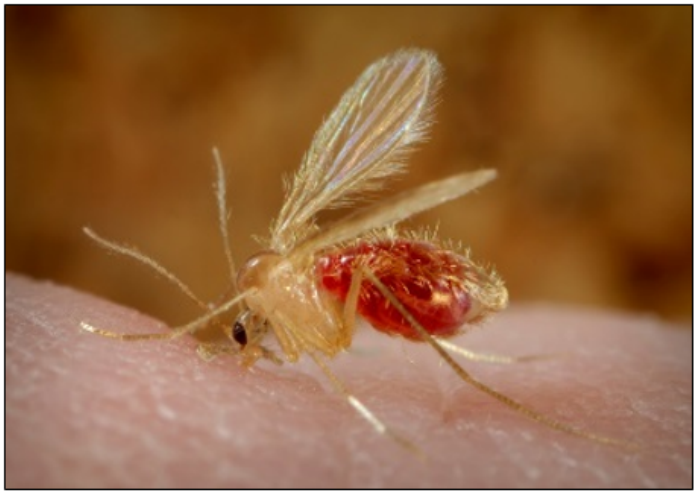

**Figure 1.** ***Phlebotomus papatasi*** **Bloodfed Female, the Main Vector of Old World** ***Leishmania major*****.**

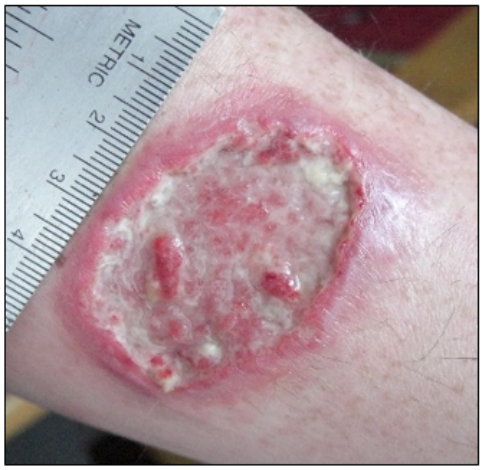


**Figure 2. Cutaneous Leishmaniasis, Symptoms are Noticeable Large Localized Skin Ulcers.**

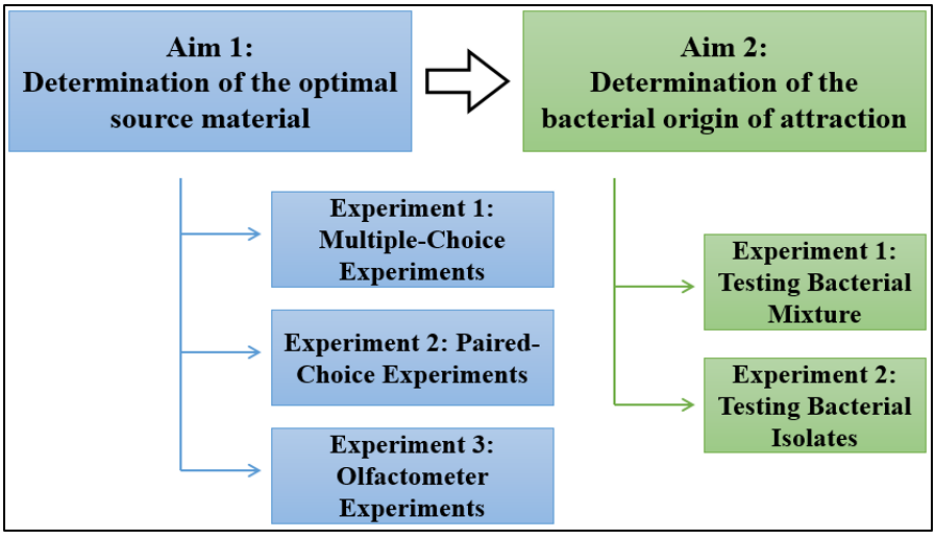


**Figure 3. Work-Flow Diagram Showing the Specific Aims of This Thesis Project**

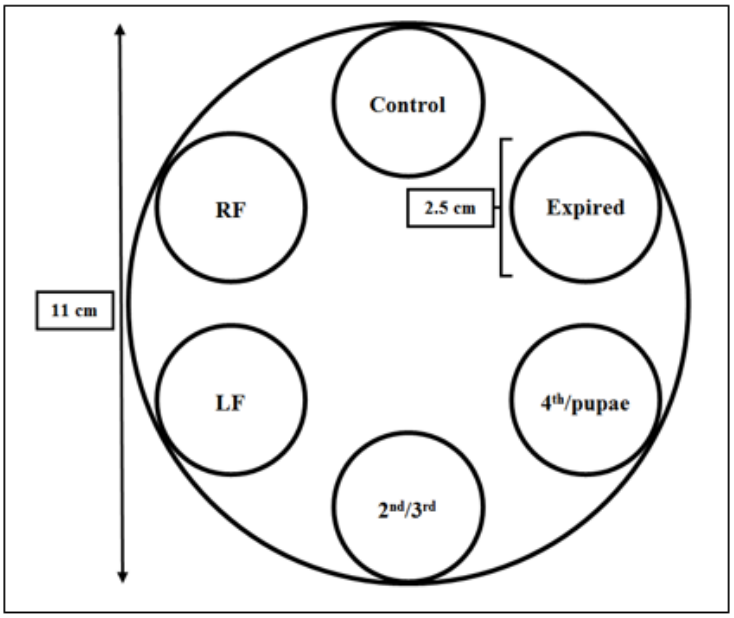


**Figure 4. Design of the Multiple-Choice Oviposition Assays.** Each assay jar was constructed of a 500 mL cup with 2.5 cm diameter filter paper discs distributed at equal distance. Six source materials were placed on the filter papers: Control (water-only); rabbit feces RF; larval food (LF); rearing medium from 2nd and 3rd larval instars (2nd/3rd); rearing medium from 4th larval instars and pupae (4th/pupae); rearing medium and frass from an expired colony (Expired).

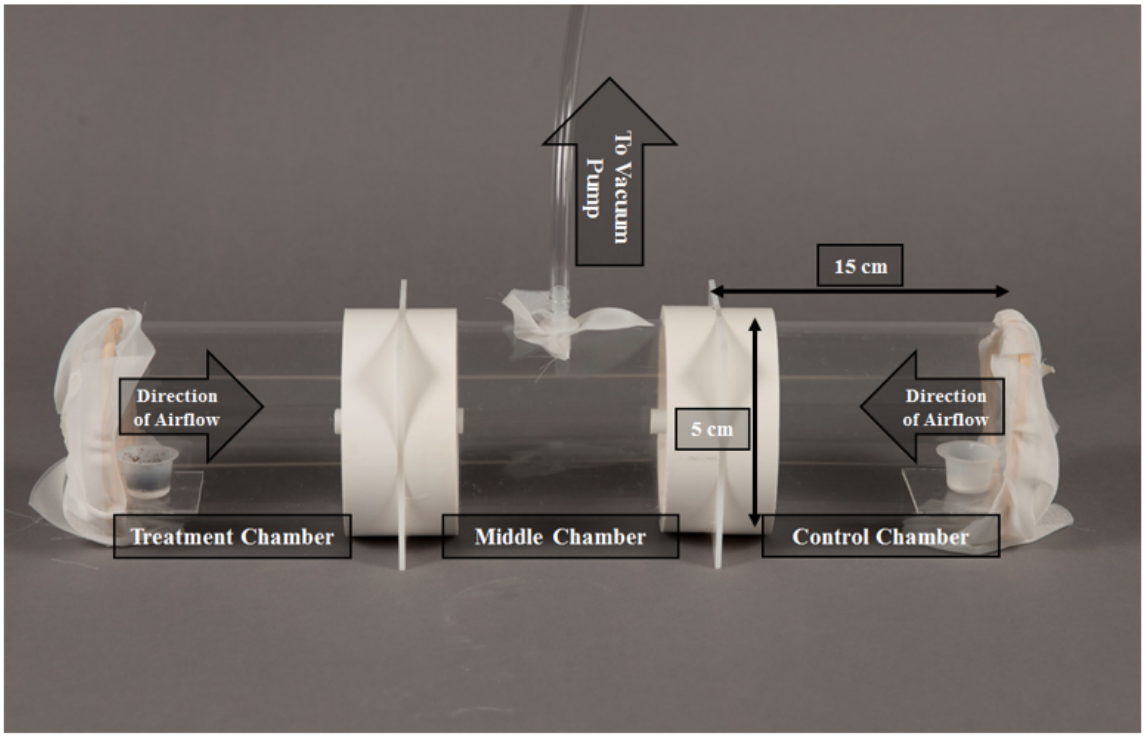


**Figure 5. Three-Chamber in-Line Olfactometer.** The olfactometer was constructed so that the vacuum pump drew air across the treatment and control cups and into the middle chamber, where 20 gravid females were introduced. Weigh boats containing test or control materials were placed on a small shelf at the end of each side chambers. Chambers were connected by 6 cm long (1 cm inner diameter) tubes extending 3 cm into both the side chamber and the central chamber.

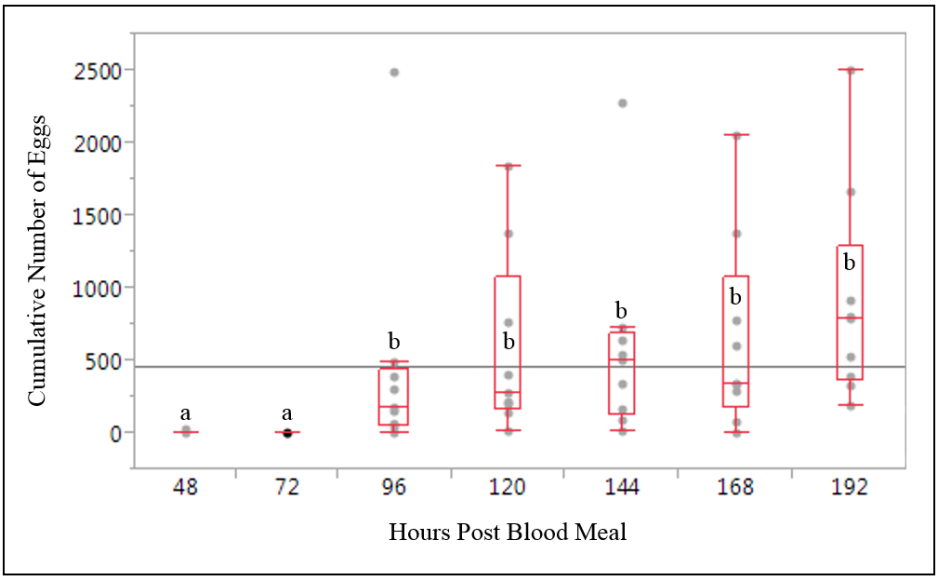


**Figure 6. Boxplot Describing Time Course of Egg Deposition Cumulative Number of Eggs per Female per Cup Over Time Since Blood Meal.** Black bars indicate median and boxes represent the second to third inter-quartile range. Different letters indicate significant difference among time periods.

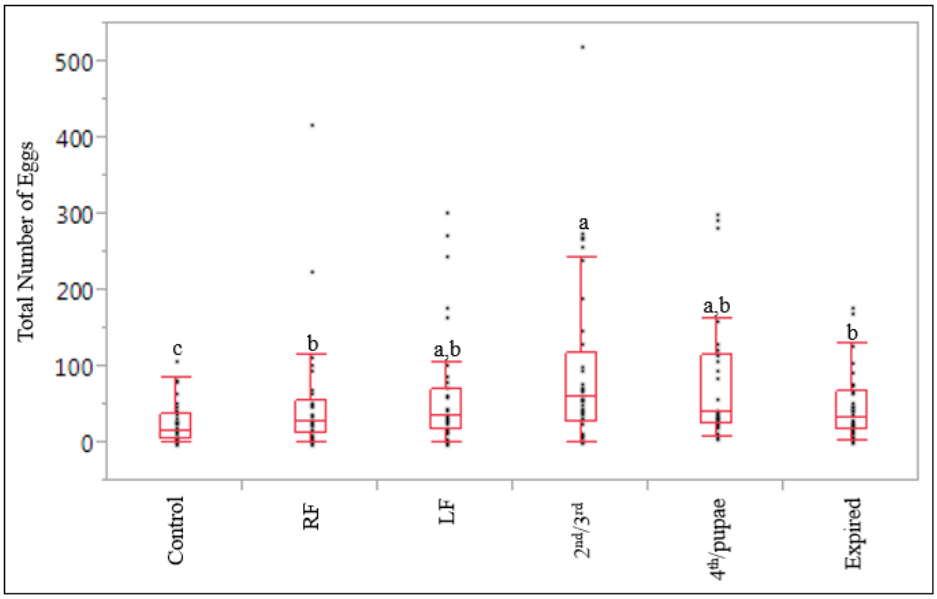


**Figure 7. Boxplot Describing the Effect of Various Rearing Media on the Cumulative Number of Eggs Oviposited per Filter Paper Disc in Multiple-Choice Oviposition Cups.** Box represents the second-to-third inter-quartile range. Letters indicate significant difference in egg numbers among treatment types.

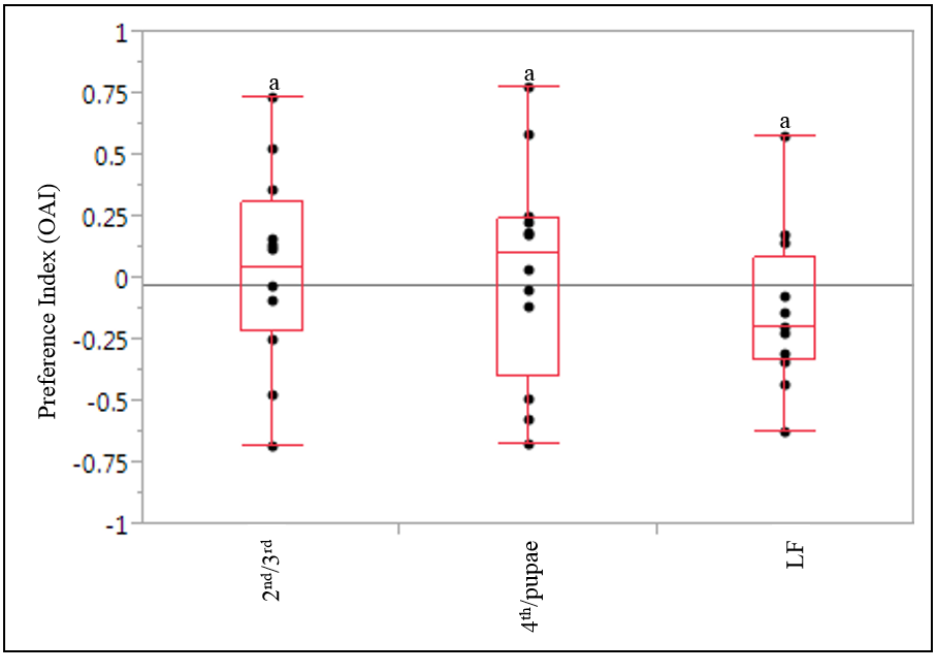


**Figure 8. Boxplot Describing the Effect of 2nd/3rd Instar Rearing Medium, 4th/Pupae Rearing Medium, and Larval Food on the Oviposition Preference Index (OAI) in Paired-Choice Oviposition Cups**. Box represents the second-to-third inter-quartile range. Similar letters indicate no significant difference in preference among treatment types.

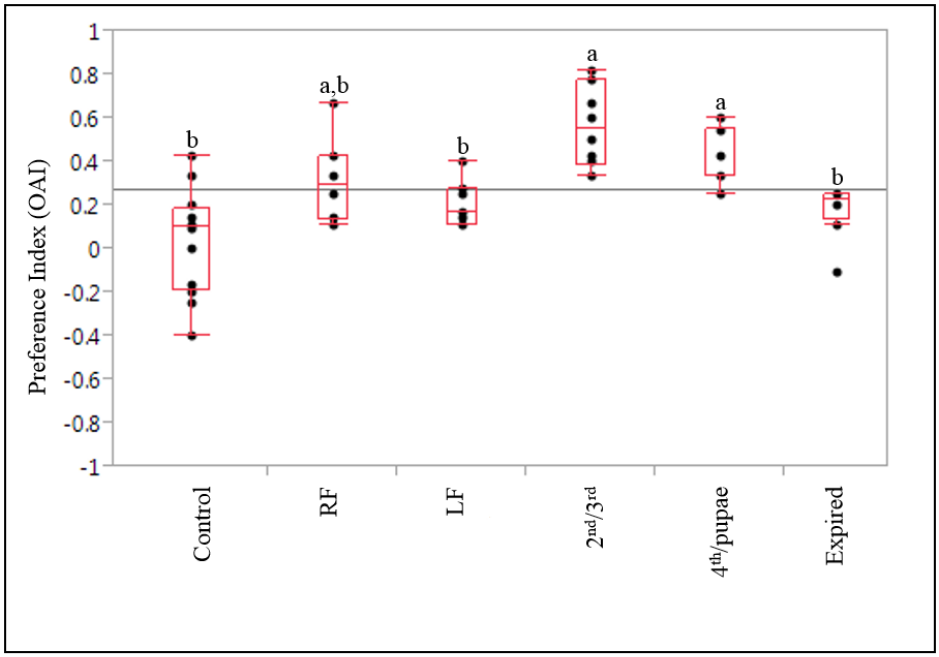


**Figure 9. Boxplot Describing the Effect of Various Rearing Media on the Oviposition Attraction Preference Index in Olfactometer Experiments.** Boxes represent the second-to-third inter-quartile range. Different letters indicate significant difference among treatment types.

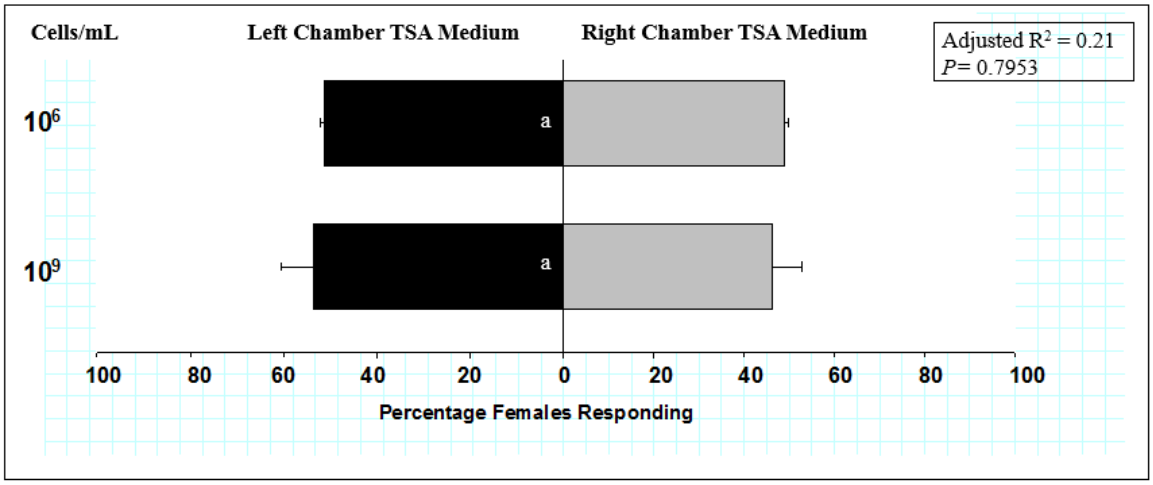


**Figure 10. Sidedness Effect in Control Experiments.** Bar graph describing the percent female sand flies found in the left chamber (black bars) compared to the percent female sand flies found in the right chamber (gray bars) of the control olfactometers, where both side chambers had blank TSA media. Letter report describes any significant difference between the olfactometers with $10^6$ and $10^9$ Cells/mL TSA media on each side. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

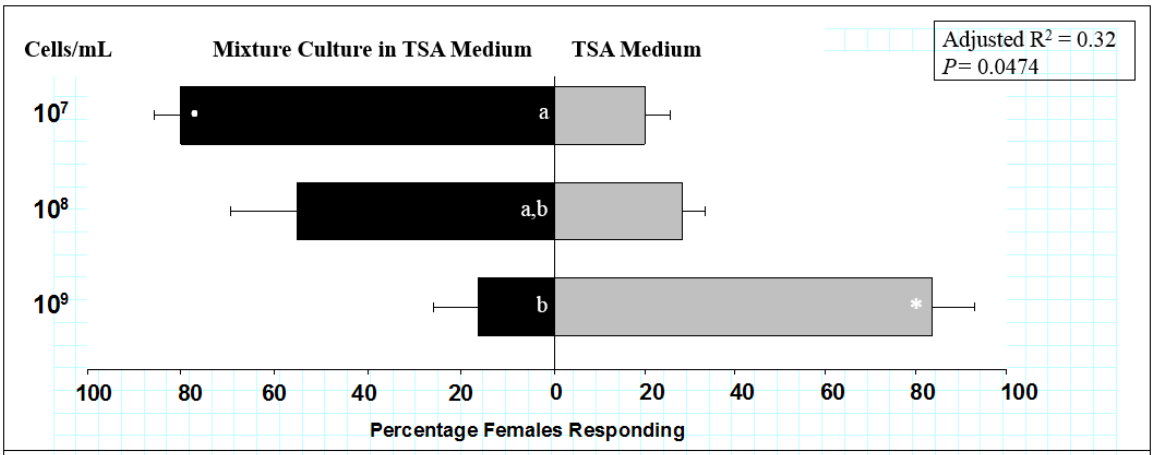


**Figure 11. Bacterial Mixture Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Bacterial mixture was tested at $10^7$, $10^8$, and $10^9$ Cells/mL. Letters report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

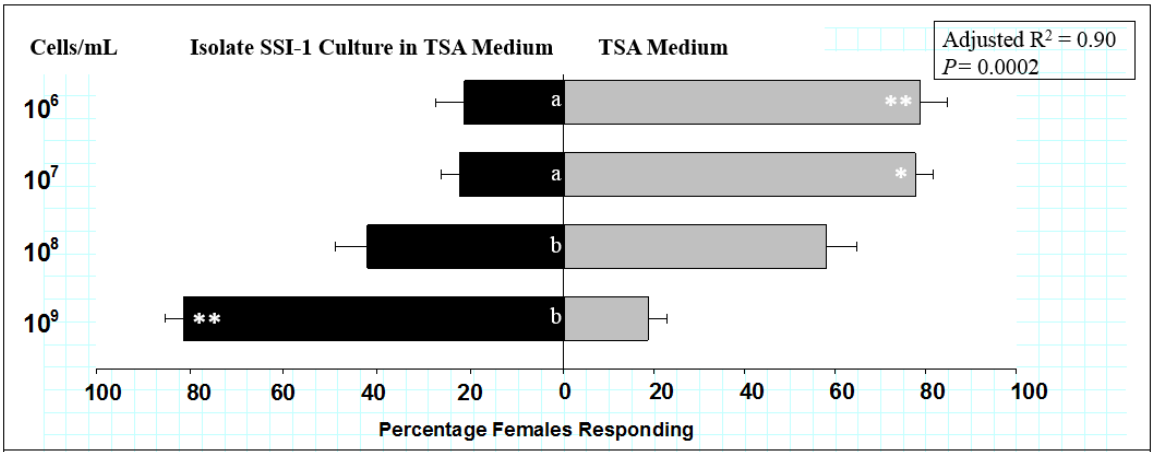


**Figure 12a. SSI-1 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

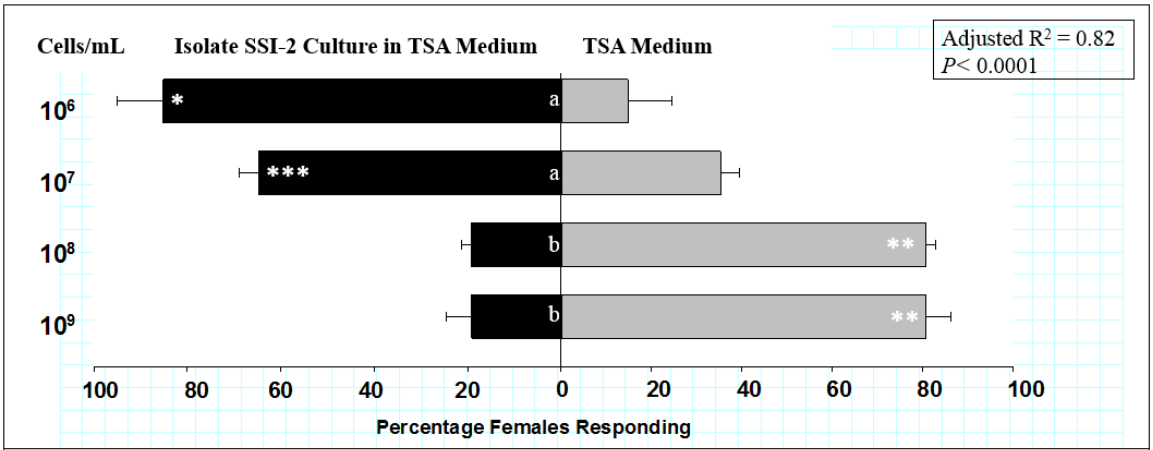


**Figure 12b. SSI-2 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

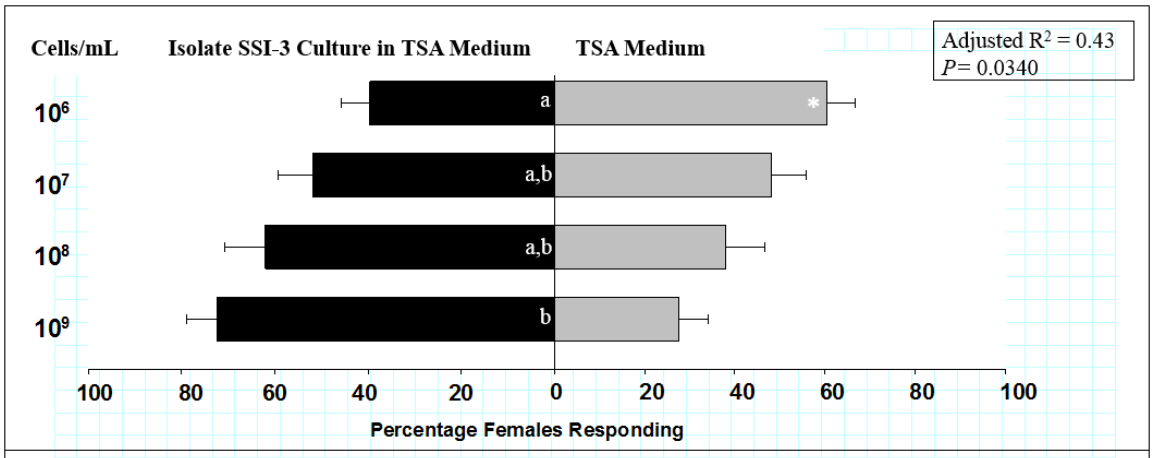


**Figure 12c. SSI-3 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

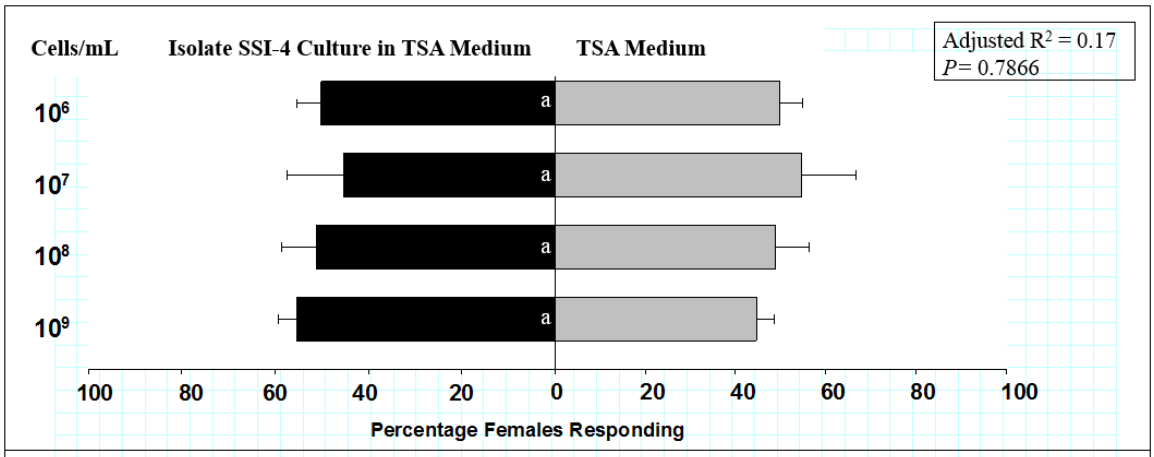


**Figure 12d. SSI-4 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

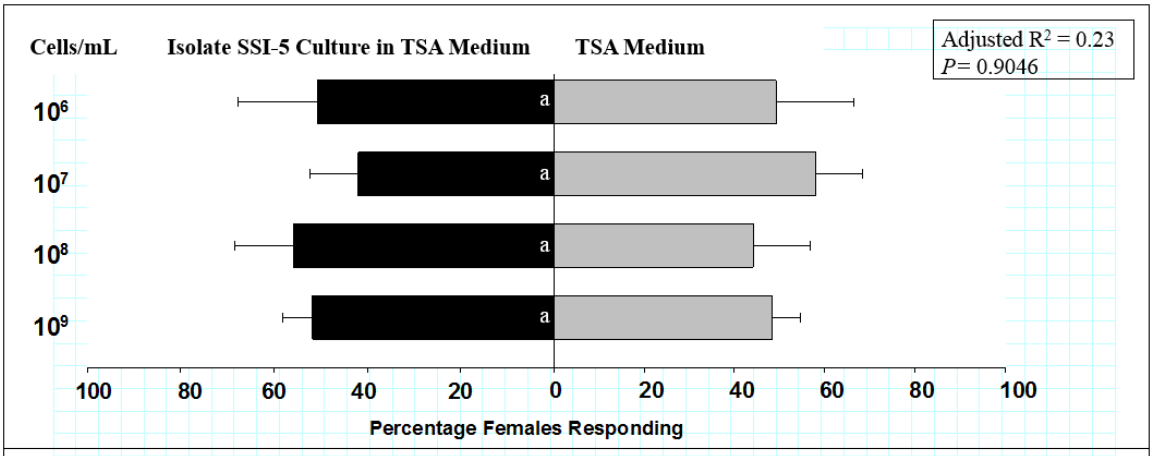


**Figure 12e. SSI-5 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

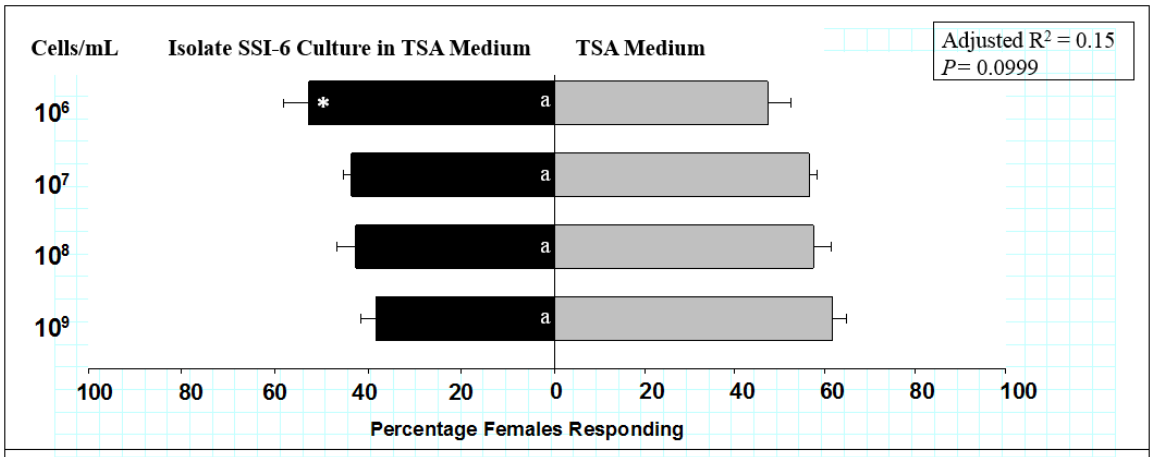


**Figure 12f. SSI-6 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

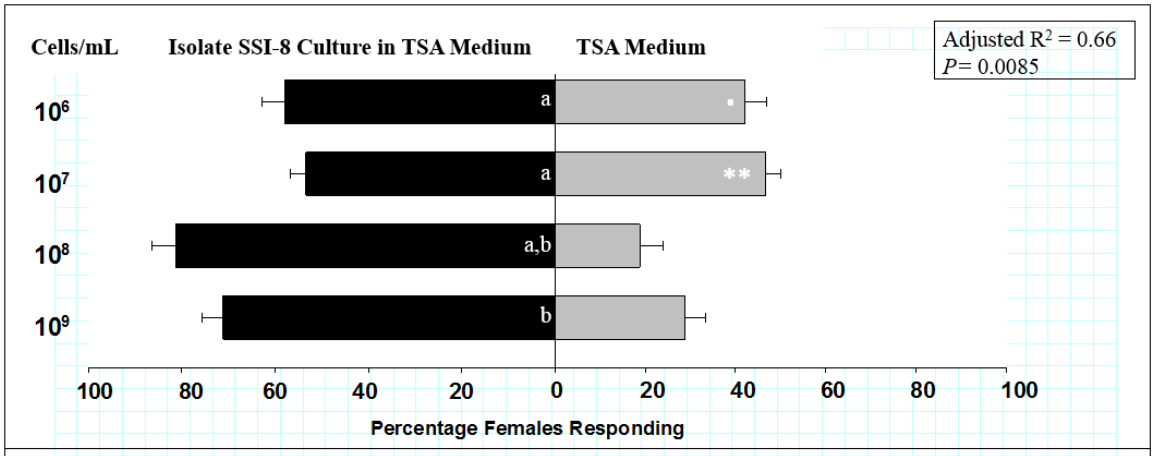


**Figure 12g. SSI-8 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

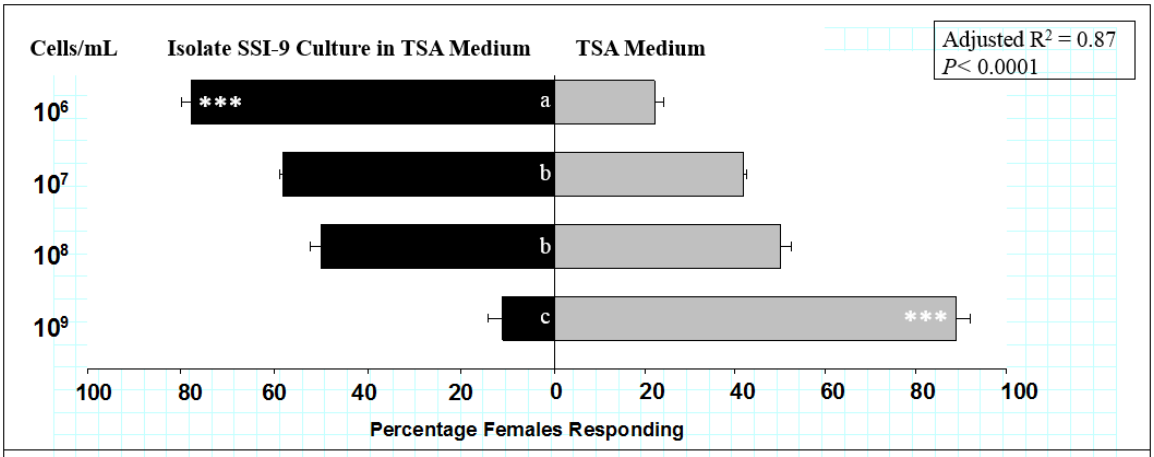


**Figure 12h. SSI-9 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

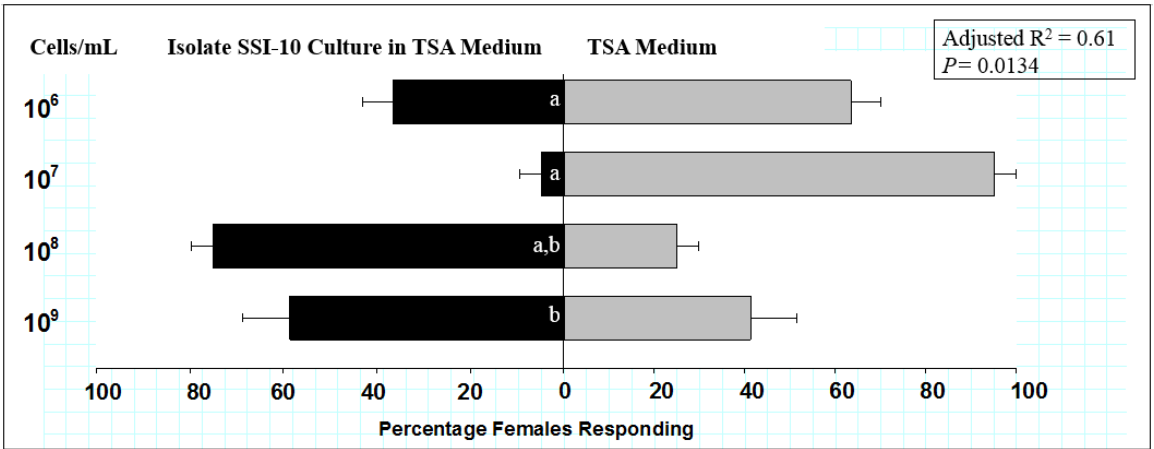


**Figure 12i. SSI-10 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

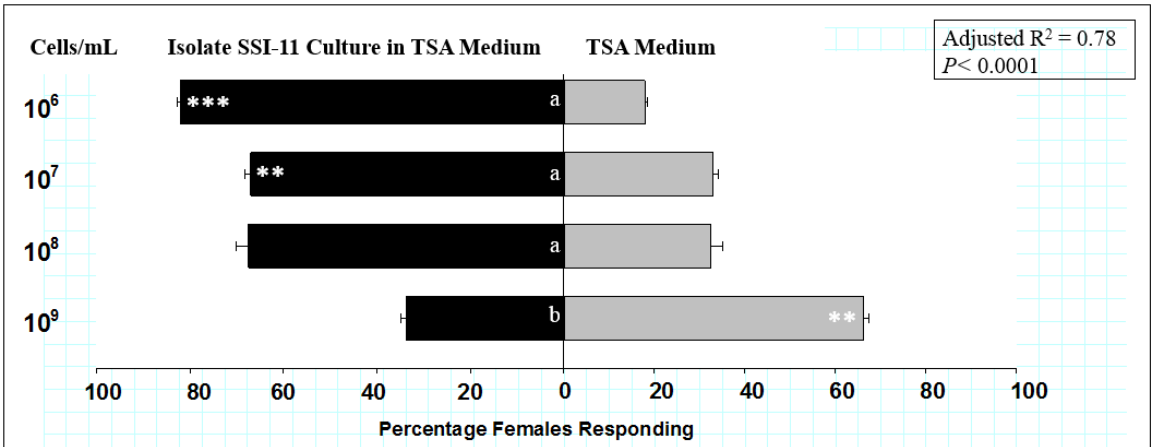


**Figure 12j. SSI-11 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

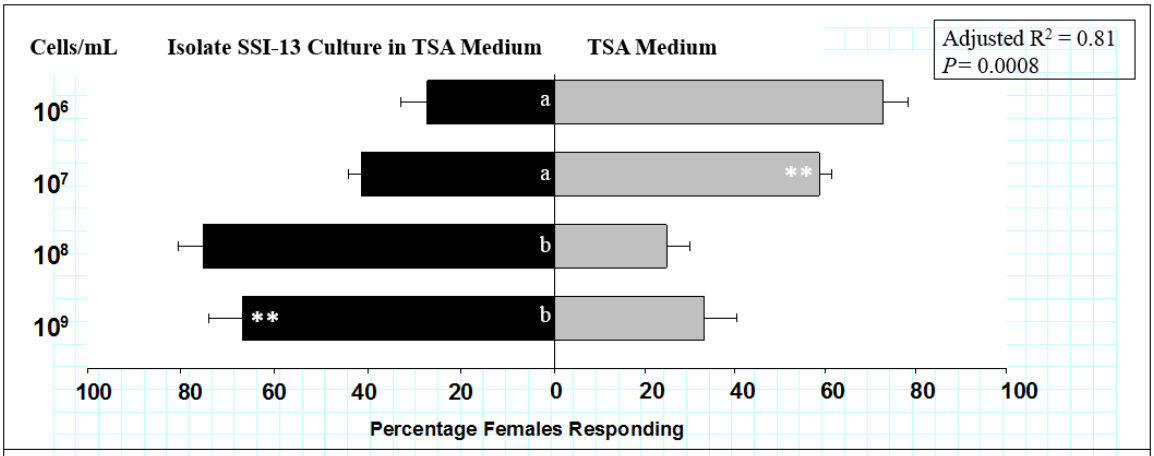


**Figure 12k. SSI-13 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.

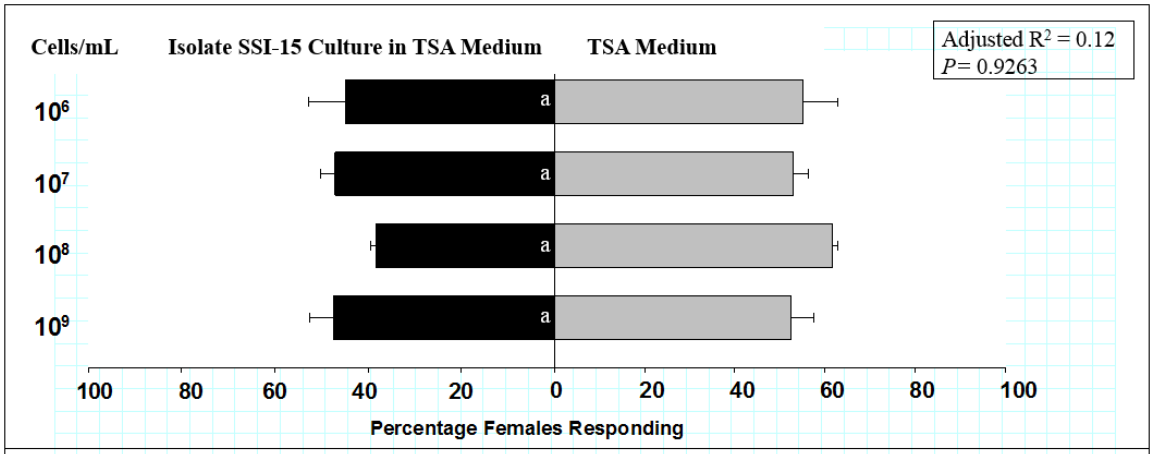


**Figure 12l. SSI-15 Bacterial Isolate Olfactometer Experiments.** Bar graphs describing the percent female sand flies found in the treatment chamber (black bars) compared to the control chamber (gray bars) of the olfactometers. Error bars show standard error on the percent response. The bacterial isolate was tested at $10^6$, $10^7$, $10^8$, and $10^9$ Cells/mL. Letter report describes significant difference in attraction among different concentrations tested. Asterisks describe the results of a paired t-test to show any significant difference between the number of females found in the treatment and the control chambers of the olfactometer. Adjusted r-squared and p-value reported from linear regression testing of OAI response among the different concentrations.