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Sand flies are the vectors of Leishmaniasis, which depending on the form, can be mutilating, debilitating, and/or deadly. Drawbacks of traditional vector control necessitate a more targeted control method. An alternative approach is to bring gravid females to the insecticide using an oviposition attractant. The general goal of this study was to evaluate the direct effect of conspecific stages on: (Aim 1) the oviposition response and (Aim 2) attraction of sand flies, and (Aim 3) to evaluate if these effects were dose-dependent. The general hypothesis of this study was that sand fly oviposition and attraction response will be affected by conspecific stages, and those responses will be dose-dependent. Aims 1 and 2 consisted of seven treatment groups: eggs, 1st instar larvae, 2nd/3rd instar larva, 4th instar larvae, pupae, adult males, and adult gravid females. Aim 3 treatment groups were increasing doses of eggs. All aims were tested using paired-choice bioassays. Oviposition response was measured by the number of eggs laid in the presence of treatment material and control. Attraction response was measure by the number of females stuck to baited and non-baited sticky traps. Oviposition bioassays identified a significant effect for high doses of eggs. In attraction bioassays, I found a significant negative relationship between attraction and conspecific post-hatching age, with a significant attraction to eggs. Overall, my study indicates that conspecific eggs, at particular doses, could induce elevated oviposition response as well as attraction.

OVIPOSITION SITE-SELECTION OF *PHLEBOTOMUS PAPATASI*:

THE EFFECTS OF CONSPECIFIC STAGES

by

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Approved by

_____
Committee Chair

I dedicate this thesis to my family, who has fully supported me throughout my academic career. Thank you to my father, Timothy Kowacich; my mother, Kelly Raymond; my stepfather, William Raymond; my grandparents, Robert and Jamie Freeman; and to my siblings, Heather Kowacich, Gaven Raymond, and Sydney Stienstra.

APPROVAL PAGE

This thesis written by DANNIELLE JOSEPHINE KOWACICH 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 group of parasitic vector-borne diseases caused by flagellated protozoa of the genus *Leishmania* and transmitted via a sand fly vector (Reithinger et al. 2007). The sand flies inject the pathogen into a host during blood meals (Figure 1). Promastigotes, which are the mobile form of the parasite, are phagocytized by phagocytic cells where they transform into the immobile amastigote stage. Amastigotes continue to multiply and infect other phagocytic cells. Sand flies become infected by ingesting infected cells during blood meals. In sand flies, amastigotes transform into promastigotes, further development in the gut, and migrate to the proboscis from which are then transmitted to a susceptible host during the subsequent blood-meal. Parasite species, host species/state, and other factors affect whether the infection becomes symptomatic and determine the type of leishmaniasis that results. There are three main types of Leishmaniasis: visceral, cutaneous, and mucocutaneous leishmaniasis. Visceral leishmaniasis is the deadliest form, and if left untreated it can kill up to 90% of infected individuals. The most common forms are cutaneous leishmaniasis associated with sores appearing on the skin that will start out as nodules and develop into painful ulcers

Mucocutaneous leishmaniasis presents similar symptoms to the cutaneous form but is more mutilating and disfiguring as it affects the mucous membranes of the ears, nose, and/or throat (CDC, 2013). Leishmaniasis is a widespread disease found in the tropics and subtropics, the Mediterranean area, and has recently reported in continental areas in Europe, including more than 98 countries. Most Leishmania species are zoonotic, being transferred from animals to humans, but some are anthroponotic, being transferred from human to human, and may cause epidemics. Currently, 53 *Leishmania* species have been described. There are 31 species known to be parasitic to mammals, while 20 species are pathogenic to humans. It is considered an emerging disease because of its increase in incidence and geographic distribution (Killick-Kendrick, 1999; Ferroglio et al., 2008; Akhoundi et al., 2016). All forms of the disease affect 12 million people worldwide, with 1.5–2 million added new cases annually of cutaneous, and 500,000 cases of visceral leishmaniasis. Around 50,000 deaths from the disease occur each year. Increase in worldwide incidence has been attributed to the increase of anthropogenic influences such as human migration, deforestation, urbanization, climate change, and drug resistance (Hirve et al., 2017; Oryan and Akbari, 2016). Furthermore, Leishmaniasis is considered a neglected disease with most reported cases occurring in the least developed countries with inadequate investment in research and healthcare (Alavar et al., 2006).

Leishmaniasis and Sand Flies.

In the New World, the disease is spread by sand flies of the genus *Lutzomyia*. In the Old World, it is spread by sand flies of the genus *Phlebotomus* (Reithinger et al. 2007). Like all dipterans, sand flies undergo complete metamorphosis and consist of four

complete life stages: egg, larva (4 instar stages), pupa and the adult (Figure 2). Sand flies are generally found in warm, humid parts of the world, which is essential for the larval survival but are present among a wide range of latitudes. Most species are nocturnal and rest in animal burrows, tree holes, caves, rocks and other protected habitats, including human dwellings (Claborn, 2010). Female sand flies blood-feed on a wide variety of vertebrate hosts, including humans, livestock, dogs, rodents, reptiles, amphibians, and birds. In addition to vectoring leishmaniases, sand flies also transmit other pathogens such as *Bartonella* spp., phleboviruses, certain flaviviruses, orbiviruses, and vesicular stomatitis viruses (Alexander and Maroli, 2003; Claborn, 2010). Male and female sand flies both feed on natural sources of sugar such as the sap or nectar of plants and honeydew of aphids (Killick-Kendrick, 1999). Strictly females practice reproductively obligate hematophagy relying on host blood meals for egg production. Immature stages feed on rich organic matter for survival and growth (Cameron et al., 1995). With respect to *Leishmania* spp., sand fly females are biological vectors in the sense that they are cyclopropagative, involving both amplification and developmental stage change within the sand fly to enable transmission (McHugh, 1994).

Sand Fly Control.

A significant problem with controlling sand fly populations is the lack of information on natural breeding habitats, making it difficult to target immature stages (Feliciangeli, 2004). For this reason, control via source reduction, which is altering or destroying a breeding site as to suppress juvenile stages recruitment, has not been possible in contrast to other arthropod vectors, such as mosquitoes (Baldacchino et al.,

2015; Vivero et al., 2015). As a result, adults are the primary targets of control. Several conventional methods aimed at adult stages are used for the control, including residual insecticide spraying, and personal protection using repellents or insecticide-impregnated materials (Killick-Kendrick, 1999; Bates et al., 2015). Insecticide applications have been used to target sand fly populations in endemic regions, but there are major disadvantages to the use of residual insecticides such as insecticide resistance and the harm to non-target species (Claborn, 2010). Dichlorodiphenyltrichloroethane (DDT), which was first used on a large scale for control of sand flies, has considerable environmental impacts such as long-range transport, environmental persistence, and bioaccumulation. In addition to the negative environmental impacts of DDT, there have been reports of sand fly insecticide resistance to DDT and pyrethroids and permethrins in India, Iran, Nepal, and Turkey (Ahmed et al., 2015). Currently, pyrethroid and permethrin products are mainly being used for conventional spraying (Claborn, 2010).

An alternative approach to conventional methods is to bring the vector to the insecticide rather than deliver the insecticide to the vector. This is the methodological approach for systemic control and vector control by using attractants. Systemic control impregnates insecticides into natural animal hosts using a topical or oral administration (Wasserberg et al., 2011; Mascari et al., 2013; Debrali, 2014; Gomez and Picado, 2017). This will target blood starved females, which ingest the insecticide during a blood meal, and larvae feeding on the fecal matter of the vertebrate host. Toxic sugar baits are a combination of insecticides with a sugar concentrate mix that can be applied to vegetation in endemic areas to target adult male and female sand flies feeding on a sugar

source (Bates, 2015; Fiorenzano et al., 2017). Sex pheromones and oviposition attractants are other methods previously investigated to accomplish attract-and-kill. For example, terpenes are known to be the sex pheromone associated with male *Lutzomyia longipalpus*, and there is chemical evidence that sex aggregating pheromones occur in several other *Lutzomyia* species (Spiegel et al., 2016; Gonzalez et al., 2017). Synthetic sex pheromones in concentrations like that of aggregating males were able to attract *Lutzomyia longipalpus* females in a laboratory setting as well as males and females in the field (Bray et al., 2009). Oviposition site attractants lure gravid females that have blood fed at least once and therefore have a higher chance of being infected. Furthermore, it also targets the stage responsible for population amplification (Alexander et al., 2003; Bates et al, 2015).

Oviposition Site Selection of Hematophagous Insects.

Understanding the biological factors associated with oviposition provides opportunities for the design of species-specific surveillance and control methods. Much research has been done in reference to mosquitoes. For example, *Aedes aegypti*, the vector of viruses causing yellow fever, dengue, chikungunya, and Zika, prefer to oviposit in natural and artificial containers with stagnant water; this preference has facilitated the development of toxic oviposition traps (Bentley and Day, 1989; Day, 2016). Other aspects of mosquito ecology have been investigated to further develop alternative control methods. For instance, mosquito attractants comprised of bacterial cultures have been implemented for control. Ponnusamy et al. (2016) have demonstrated in the lab and in the field an effective attractant and oviposition stimulant for *Aedes aegypti* and *Aedes*

albopictus comprised of two or more of the following bacterial species: *Lactococcus lactis, Klebsiella oxytoca, Shigella dysenteriae, Brevundimonas vesicularis*. Studies have shown that the presence of conspecific eggs and larvae in stagnant water containers attracted gravid *Ae. aegypti* and *Ae. albopictus* as well as stimulated oviposition (Allan and Kline, 1998; Wasserberg et al., 2011; Wong et al., 2011; Gonzalez et al, 2016). Furthermore, the response to the presence of conspecific stages was shown to be dose-dependent exhibiting hump-shaped relationships due to attraction at low densities but repellence at high densities due to competition (Wasserberg et al., 2011). Gonzalez et al. (2016) reported a threshold effect on oviposition response of *Aedes albopictus*, with a high response at low densities and low response at high densities, and a hump-shaped response of *Ae. aegypti* when in the presence of conspecific larvae. The range of responses indicates species-specific preferences to conspecific material.

Relatively, very little is known about oviposition behavior of sand flies. However, it is reasonable to assume that gravid flies should respond to cues in their habitat indicating a suitable oviposition site. Some environmental factors that influence oviposition site suitability include air temperature, relative humidity, soil moisture, pH, and organic matter (Chowdhury et al., 2016). In addition to environmental factors, laboratory-based experiments have also shown that pheromones emitted from conspecific material can influence oviposition response, with many responses following a density-dependent relationship (ElNaiem and Ward, 1991; Srinivansan et al., 1995). Attraction to the presence of conspecific pre-adult stages could be perceived as indicators of potential successful reproduction and therefore indicate a good oviposition site. The majority of

research studying the effects of conspecific material has been in response to eggs and relatively little has been invested in other developmental stages. For instance, the response of *Lutzomyia longipalpis* associated with conspecific eggs was investigated in the laboratory. Gravid females laid significantly more eggs when exposed to egg batches of 80 to 320. No significant response was detected with batches of 40 eggs or less indicative of a threshold-based response where an increasing number of conspecific eggs elicited a significant response (ElNaiem and Ward, 1991). The active compound in the eggs was subsequently identified as dodecanoic acid, which caused the same behavioral response in gravid female sand flies as the whole egg extract (Dougherty et al., 1992; Dougherty and Hamilton, 1997). A similar threshold response was observed with *Phlebotomus papatasi*, which was not affected by the presence of 10 – 40 eggs but laid significantly more eggs in the presence of 100 or more eggs (Srinivansan et al., 1995). Basimike et al. (1997) investigated how five different developmental stages of *Phlebotomus duboscqi* influence oviposition response by counting the number of eggs laid when in the presence of conspecific material. Treatment groups were whole crushed eggs, first and fourth instar-larvae, pupae, adult females, and adult males. Gravid *Phlebotomus duboscqi* females laid significantly more eggs on substrates baited with eggs, pupae and adult test materials compared to controls. In contrast, Schlein et al., (1990) reported that 3rd and 4th instar larvae, as well as pupae, had a strong negative effect on oviposition of *P. papatasi*. Hence, investigation of oviposition site preference with respect to all conspecific developmental stages is needed.

Laboratory studies have also shown the effects of organic matter on oviposition response and attraction. Volatile cues indicating the presence of organic matter would indicate a good oviposition site since it would indicate the presence of food for larvae. For new- and old-world sand flies there is strong evidence for organic matter of various sources eliciting oviposition responses (Doughtery et al., 1993; Wasserberg and Rowton, 2011; Kumar et al., 2013; Marayati et al., 2015). A laboratory bioassay found that gravid female *Lutzomyia longipalpis* laid significantly more eggs on substrates containing extracts of rabbit food, hay, and rabbit feces compared to the controls (Doughtery et al., 1993). Another study found significantly more eggs of *P. argentipes* oviposited on expired medium, consisting of dead flies, old unhatched eggs, larval food containing vertebrate feces, frass, and other organic matter, compared to fresh medium (Kumat et al., 2013). Similarly, the effects of frass and conspecific eggs were studied and showed that frass was a more effective oviposition stimulant compared to eggs (Wasserberg and Rowton, 2011). Interestingly, the combined effect of the two was sub-additive (Wasserberg and Rowton, 2011). Marayati et al. (2015) studied the attraction of gravid female *P. papatasi* to larval media of several developmental stages and showed sand flies are significantly attracted to and laying more eggs on rearing medium containing mostly 2nd/3rd instar and 4th instar/pupae. Yet, it is not clear if the attraction to 2nd/3rd substrate is mediated by microbial-produced kairomones or due to residual pheromone compounds produced by conspecific larvae (Marayati, 2015). **Hence, the general goal of this study was to elucidate the direct effect of different conspecific stages on the attraction and the oviposition response of *P. papatasi* females.**

Hypotheses

1. Gravid *P. papatasi* females will have a positive attraction and oviposition response to early-intermediate developmental stages that represent a relatively unused, suitable oviposition site, but will have a negative response in the presence of late larval and adult stages that are potential indicators of high intraspecific competition and/or depleted larval resources.

3. Attraction and oviposition response to conspecific eggs will exhibit a dose-dependent relationship with attraction/stimulation due to reassurance at low densities but repellence/deterrence at high densities due to potential intraspecific.

Specific Aims

Aim 1. Study the effects of conspecific stages on sand fly oviposition response.

Prediction: Gravid *P. papatasi* females will lay more eggs in the presence of early - intermediate developmental stages but will lay fewer eggs in the presence of late larval and adult stages.

Aim 2. Study the effects of conspecific stages on attraction response.

Prediction: Gravid *P. papatasi* females will be most attracted to early - intermediate developmental but will be repelled from late larval and adult stages.

Aim 3. Study the dose-dependent effects of conspecific eggs on attraction and oviposition response.

Alternative predictions:

a. A threshold effect with low to no response until exposure to a critical dose that induces a response.
b. Linear response where response increases or decreases with increased dose
c. Hump-shaped relationship with a peak response at intermediate doses.

CHAPTER II

METHODS

Approach

Two-choice bioassays were used for each aim. For aim 1, I used an oviposition bioassay consisting of oviposition pots containing sand cups (Fig. 3A) baited (treatment) or un-baited (control) with a conspecific stage (eggs, 1st instar, 2nd/3rd instar, 4th instar, pupa, adult male, adult gravid female) (Fig. 3B) to determine the stimulatory effect of conspecific stages on the oviposition response of gravid *P. papatasi*. For aim 2, I used attraction bioassays using free-flight cages and constructed sticky traps to determine the attractiveness of the seven conspecific stages to gravid female *P. papatasi* (Fig. 4). In Aims 1 and 2 equal masses of conspecific material was used. To determine the mass of conspecific material to be used, the average weight of one individual of the largest stage, 4th instar, was calculated to be 0.6 mg. Approximately 0.6 mg of each conspecific stage was weighed using an electronic balance (A&D, HR-200) while control cups consisted of empty microcentrifuge vials. After identifying eggs as the most attractive stage in aims 1 and 2, I used this in Aim 3 to evaluate if oviposition and attraction responses differ at different egg masses.

General Methods

Colony and Mass Rearing.

Phlebotomus papatasi originating from Abkük, Turkey, were obtained from the Walter Reed Army Institute of Research and maintained at the University of North Carolina Greensboro. Rearing of *P. papatasi* sand flies followed modified methods described by Walter Reed Army Institute of Research (Lawyer et al., 2017). Prior to use in the experiments, females were fed on *Mus musculus* anesthetized with isoflurane and a mixture of ketamine and xylazine (SoBran Inc. IACUC protocol number: UNC-002-2016). Newly emerged sand flies were kept in a 30x30x30 cm polycarbonate mating/feeding cage containing an approximately equal ratio of males and females. Sand flies were maintained in insect rearing chambers (Model: 6030–1, Caron®, Marietta, Ohio) at 27 °C, 80 % RH, and 12:12 light: dark cycle. Larval sand flies were maintained in 500 mL Nalgene jars (Nalgene™, Model 81063, diameter=11 cm) with a 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. Larval food was prepared by mixing fresh rabbit feces (New Zealand White strain) and rabbit chow (Purina) at a 1:1 ratio, which was fermented for 3 weeks in a dark chamber, air-dried and ground to a powder.

Sand Cups.

Sand cups were constructed and used in all experiments. They consisted of a 0.2 ml PCR tube (Axygen, Inc., Model 321-02-051) covered with a double mesh and clear rubber band to prevent visual cues. Control cups consisted of empty microcentrifuge tubes while treatment cups contained a conspecific material. Tubes were placed in 10 ml

disposable micro-beakers (ThermoFisher Scientific®, Model: 08-732-121) filled with 8 ml of autoclaved sand and 2.5 ml of DI water to keep the sand moist (Figure 3). Conspecific eggs, 1st instar larva, 2nd/3rd instar larva, 4th instar larvae, pupae used for experiments were obtained from rearing pots used for colony maintenance and were thoroughly cleaned by brushing off rearing material and debris.

Aim 1. Oviposition Bioassays.

To determine the effects of conspecific material on total oviposition response of *P. papatasi* a two-choice oviposition bioassay was used (Fig. 3A). A 2.5 cm diameter filter paper (ThermoFisher Scientific®, Model: 09-801-AA) was placed on top of the sand cups and wetted with 0.25 ml of DI water to saturate filter paper. Treatment and control sand cups were placed in 500 mL Nalgene jars (Nalgene™, Model 81063, diameter = 11 cm) covered with mesh (Fig. 3B) and assigned randomly to a location in a 55 X 40 cm plastic tubs. The experiment took place in the rearing chamber under standard conditions with 20 females per pot approximately 7 days post blood meal. The experiment ran for a three-day period. Every 24 hours 0.25 ml of DI water was added to each sand cup to keep sand and filter paper moist and fresh sugar pads (30% solution) were provided. After the 3-day period, flies were removed with mouth aspirator. The eggs laid on control and treated sand cups were counted using a dissection microscope. A total of 18 replicates per treatment set were performed.

Aim 2. Attraction Bioassays.

To evaluate the attraction of gravid *P. papatasi* females to conspecific material, I conducted a two-choice behavioral bioassay using 30x30x30 cm polycarbonate free-

flight cages consisting of a conspecific stage treatment versus a control (Figure 4). Prepared sand cups were placed in 125 mL Nalgene jars (Nalgene™, Model 1187580, diameter = 6.5 cm) with a sticky metal screen on top sprayed with adhesive (Tanglefoot®, Model 91992-MI-001) (Figure 4). The experiment took place in a climate control room at the University of North Carolina at Greensboro, Sullivan Building with humidity set to 65% RH, temperature set to 27°C, and with red light settings. Twenty Gravid female sand flies approximately 130 hours post blood meal were transferred into the free-flight cages and left for 24 hours prior to the experiment in order to get acclimated. The experiment lasted 24 hours by the end of which the number of flies attached to adhesive metal screens was counted. A total of 18 replicates per treatment set were performed.

Aim 3. Dose-Dependent Bioassays.

After determining which conspecific stage elicits the strongest positive response, I investigated whether it had a dose-dependent effect on oviposition response/attraction. Based on preliminary results, eggs were the candidate conspecific stage to be used. For all dose-dependent bioassays, a range of 5 egg masses were used as the treatment levels (0.15mg, 0.3mg, 0.6mg, 1.2mg, 2.4mg) (Mettler Toledo©, XSE105 scale info) (Table 1). Dose-dependent oviposition bioassays consisted of the same protocols from Aim 1. Dose-dependent attraction bioassays were similar to the protocols in Aim 2, but with a few modifications (based on recent results pertaining to *P. papatasi* circadian rhythm and attraction behavior) aimed at optimizing the experimental set-up. The experiment lasted 6 hours from 12:00 p.m. to 6:00 p.m. with a dim light for the first five hours and a

corpuscular light the last hour (in contrast to 24-hour red light setting in aim 2), at the end of which the number of flies attached to adhesive metal screens were counted. Also, the sticky traps consisted of clear cups rather than black cups used in the previous attraction assay. A total of 15 replicates per treatment set was performed for the oviposition assays and 10 replicates for the attraction assays.

Data Reduction and Analysis

The attractant/stimulant or repellent/deterrent response to conspecific materials was expressed as the oviposition activity index (OAI) according to Kramer and Mulla (1979):

$$OAI= (N_t – N_c)/ (N_t + N_c)$$

where N denotes the mean number of eggs laid or females stuck to sticky trap in treated (t) or control (s) substrates. Index values fall within the range of +1 to -1, with 0 indicating no preference. OAI values can be positive or negative. A positive value indicates that more eggs were laid, or females attached to the treated substrate than in the control. Conversely, more eggs lay, or females attached in the control than in the treated substrate result in a negative OAI value.

Statistical analysis of the sand fly oviposition behavior was done using a Microsoft Excel worksheet. All experiments had a pairwise choice design, where the treatment and control were on either side of the cage or pot and gravid females were given an option to choose between the two. Therefore, a paired t-test was used to test

between treatment and control cups within the oviposition jar or the holding cage. Effect of conspecific stages on OAI was analyzed using linear regression analysis. For aims 1 and 2, conspecific stage effect was tested as an ordinal variable since the stages can be categorized by time post-hatching as eggs, the four instars, and both adult sexes. I ran regression analyses separately for males and females as the oldest stage because they represent the same age group but could cause alternative responses. With dose-dependent bioassays, egg mass was used as a quantitative variable.

CHAPTER III

RESULTS

Aim 1. Oviposition Bioassays

I did not find a significant difference in the number of eggs laid in the presence of conspecific material when compared to the water control for all conspecific stages tested (egg $p = 0.82$, 1st instar larvae $p = 0.18$, 2nd/3rd instar larvae $p = 0.14$, 4th instar larvae $p = 0.70$, pupae $p = 0.92$, adult males $p = 0.79$, and adult females $p = 0.63$) (Figure 5A). Conspecific age did not have a significant effect on OAIs for either males or gravid females as the adult developmental stage (Table 1A, B) (Figure 5B,C).

Aim 2. Attraction Bioassays

I found that gravid female *P. papatasi* were significantly attracted to the baited trap containing conspecific eggs compared to the non-baited trap (paired t-test: egg $p = 0.05$). All other treatments were not significantly different from the control (paired t-test: 1st instar larvae $p = 0.17$, 2nd/3rd instar larvae $p = 0.32$, 4th instar larvae $p = 0.96$, pupae $p = 0.43$, adult males $p = 0.12$, and adult females $p = 0.36$) (Figure 6A). I identified a significant negative linear relationship between sand fly attraction (OAI) and age. This negative trend was significant for both males and females as the oldest developmental stage (Figure 6B,C and Table 2A,B). For both regressions, there was an overall attraction

to younger developmental stages, no preference for intermediate stages, and suggested repulsion to older stages.

Aim 3: Dose-Dependent Bioassays

Oviposition Response.

I identified a significant effect of conspecific eggs at 1.2 mg of conspecific eggs ($p=0.04$), and a marginally significant number of eggs laid in the presence of 0.15mg and 2.4mg of conspecific eggs ($p=0.11$; $p=0.09$, respectively). Females did not lay significantly more eggs on substrates baited with 0.3 mg and 0.6 mg of conspecific eggs ($p=0.78$; $p=0.25$) (Figure 7A). Based on the linear regression analysis I did not observe a significant linear trend with increasing egg masses and OAI ($p = 0.53$) (Table 3) (Figure 7B).

Attraction Response.

With the current number of replicates (n=12), I did not find a significant difference between the number of gravid females attached to baited sticky traps and non-baited sticky traps for all five egg masses (paired t-test: 0.15 mg $p = 0.63$; 0.3 mg $p = 0.25$; 0.6 mg $p =0.95$; 1.2 mg $p = 0.25$; 2.4 mg $p = 0.21$) (Figure 8A). Although nonsignificant, 0.3 mg of conspecific eggs elicited the highest OAI of 0.30 followed by 0.15 mg with an OAI of 0.09. High egg masses of 1.2 mg and 2.4 mg had nonsignificant negative OAIs of -0.19 and -0.21 with the intermediate egg dose of 0.6 mg having a neutral OAI of 0.01. I did not a find a significant linear trend in OAIs of gravid females exposed to increasing conspecific egg masses ($p = 0.76$) (Table 4) (Figure 8B).

CHAPTER IV

DISCUSSION

Oviposition Response and Attraction

Phlebotomine sand flies are known vectors of the protozoan parasites that cause Leishmaniasis. Currently, no vaccines are available for pathogen control, so control methods focus on reduction in sand fly populations, mainly in the form of insecticides. An alternative control method would be to bring the vector to the insecticide using an attractant (Killick-Kendrick, 1999). Oviposition attractants would be among the more effective approaches because they target gravid females, which actively transmit the disease and are responsible for population amplification. My study aimed at finding an attractive source material leading to the identification and isolation of an attractive chemical compound or mixture that could be used as bait in a lethal ovitrap. To find such a material, conspecific stages were screened for their potential to lure gravid females and elicit an elevated oviposition response. The stimulatory and attractive effects of conspecifics are well documented for hematophagous insects, particularly mosquitoes (Onyabe and Roitberg, 1997; Zettel Nalen et al., 2013, Wasserberg et al., 2014; Gonzalez et al., 2016). However, less research has been invested in the potential of sand fly conspecifics as an attractant. It is implicit to assume that natural selection has molded sand flies to be sensitive to cues indicating a suitable oviposition site that will maximize larval success. The presence of conspecific material at a potential oviposition site could

provide reassurance to gravid females that since larvae are currently present and thriving, then her offspring will thrive. In contrast, the presence of conspecifics may also signify a site with depleted resources and/or potential for competition.

I predicted that gravid *P. papatasi* females will be most attracted to early-intermediate developmental stages that represent a relatively unused, suitable oviposition site, but will be repelled from late larval and adult stages that are potential indicators of high intraspecific competition and/or depleted larval resources. My attraction assay results were consistent with this hypothesis because attraction decreased significantly with age of the conspecific stage. However, this trend was not observed for the oviposition assays of conspecific material, implicating that conspecific material can have a different effect on gravid female attraction and oviposition.

Oviposition response has two primary components, attraction and stimulation. The experimental setup of aim 1 was a combination of these components. Females could decide whether to oviposit based on the attraction cues of conspecific material and the stimulating cues present on the filter paper. None of the conspecific materials tested elicited a significant effect on oviposition response. Yet, for 1^{st} instar larvae, a strong but non-significant positive effect was observed (higher mean number of eggs in treatment compared with the control) but a negative effect of $2^{nd}/3^{rd}$ stage larvae was observed (Fig. 5).

Much of published research pertaining to the effects of conspecific material for sand flies have been in response to eggs and little has been investigated for other developmental stages. Several studies have reported *Lutzomyia longipalpis* laying

significantly more eggs in the presence of conspecific eggs compared to the control (Elnaiem and Ward, 1991; Dougherty et al., 1992; Srinivansan et al., 1995). With the initial conspecific eggs mass used in aim 1, I did not find an effect on oviposition response, but after testing a broader range of doses I did find a significant effect and marginally significant effect for higher egg masses, specifically 1.2 mg and 2.4 mg. Oviposition response to other conspecific stages in my study differs from that of Basimike et. al. (1997), who found that gravid *P. duboscqi* females preferred to oviposit on substrates baited with eggs, pupae and adult test materials compared to controls. However, my results are, tentatively, consistent with Schlein et al., (1990) who reported that 3rd and 4th instar larvae, as well as pupae, had a strong negative effect on oviposition of *P. papatasi*. Non-significant trends of my study for the $2^{nd}/3^{rd}$ instar larvae suggests a negative effect on *P. papatasi* oviposition with females laying on average 52% more eggs on control substrate compared to the baited substrate.

Few studies have focused on attraction alone as an aspect of oviposition response. Attraction response specifically pertains to long-to-medium range volatile cues the orients the flies towards the source (Day, 2016). When developing a lure-and-kill ovitrap, attraction of gravid females is an important element since it is the "lure" response. For an ovitrap to be successful its priority is to attract gravid females to the trap. Stimulation of oviposition is not necessarily as important. This study found that gravid *P. papatasi* females were attracted to early life stages, with a significant attraction to conspecific eggs compared to the water control. Attraction decreased significantly with age of the conspecific stage, so gravid females were least attracted to adults of both sexes compared

to the other stages. Marayati et al. (2015) performed a study looking at the attraction response of gravid *P. papatasi* to rearing substrate containing larvae of different stages. They reported strongest attraction to 2nd/3rd instar and 4th/pupae larval rearing medium and to substrate from which all adults have eclosed. However, this differs from the present study where 2nd/3rd instar larvae, 4th instar larvae, and pupae, did not elicit a significant effect, suggesting neutrality for these conspecific stages compared to the control. Based on my results, the observations of Marayati et al. (2015) indicate a microbiome kairomonal effect and not a conspecific pheromonal effect. My study demonstrated a direct effect of pheromonal effect emitting from conspecific eggs, identified as dodecanoic acid, which was identified in *L. longipalpis* system also as an oviposition stimulant (Dougherty et al., 1994; Dougherty and Hamilton, 1997).

When females were just given the option of flight direction to and away from a treatment source (attraction assay), there was a significant attraction to conspecific eggs with an inverse relationship between preference and age. When given the option to oviposit (oviposition response assay), no significant relationships were found with egg doses of 0.6mg. However, the results of the dose-dependent assays showed a stimulatory effect at higher egg masses (1.2 mg and 2.4 mg), indicating that the initial experiments may not have consisted of the correct dose to elicit a response. This suggests that at the correct dose, the presence of conspecific eggs has stimulatory and attractive effects on gravid female *P. papatasi*. Little research has been invested in the effects of all conspecific stages, not just eggs, and most studies have focused on overall oviposition

response without isolating attractive effects. Further analysis needs to be done with respect to these research gap to fully support the results of my study.

Dose-Dependent Bioassays

I predicted that the stimulatory or attractive response might be dose-dependent. Wasserberg et al. (2015) found that mosquitoes displayed the highest oviposition response when intermediate densities of conspecific larvae and eggs were already present. Jannat and Roithberg (2013) determined a density-dependent relationship with respect to the number of larvae present and larval death and size post-eclosure for *Anopheles gambiae s.s.* They found that increased larval densities resulted in significantly higher larval mortality rates and significantly smaller adults of both sexes. A similar study also found that larval development time decreased as conspecific density increased from zero to 80 larvae (Yoshioka et al., 2012). Because larval fitness is affected by larval density for other hematophagous insects, female sand fly attraction and oviposition to conspecific material may exhibit density-dependent trends.

With both dose-dependent attraction and oviposition bioassays, I predicted either a threshold effect, a linear effect, or a hump-shaped relationship with peak attraction/oviposition at intermediate densities. For oviposition response, I did find a significant/marginally-significant effect but only at high doses of 1.2 mg and 2.4 mg of eggs. This result is not consistent with my findings in aim 1 where I did not find a significant effect of conspecific eggs on egg deposition. However, in aim 1 I used the dose of 0.6 mg, suggesting that the dose used in Aim 1 was too low. In any case, results

from this experiment are consistent with a positive dose-effect. Studies that investigated density-dependent responses in sand flies to conspecific eggs have reported positive threshold effects (positive response to eggs, but not significant until critical dose), but none have determined if a linear relationship was present (ElNaiem and Ward, 1991; Srinivasan et al., 1995). Although I looked for linear relationships, there was no clear indication of a linear dose-response for oviposition or attraction response. There is a potential trend towards a linear relationship, but I currently cannot conclude if it is a threshold response or linear effect.

With the dose-dependent attraction bioassay no significant attraction was found across all egg-mass range and no linear relations were found. This was surprising as it contrasted with the significant attraction observed in Aim 2. Attraction bioassays with all conspecific stages (Aim 2) and the dose-dependent attraction bioassays (Aim 3) had slight design differences that could be attributed to these inconsistencies. In aim 2 the experiment ran for 24 hours and consisted of sticky traps composed of black cups. In aim 3 the experiment ran for 6 hours, specifically between 12:00 pm to 6:00 pm, which was found to be the timeframe *P. papatasi* responded best to known attractants, and consisted of sticky traps with clear cups, which would not elicit visual cues. However, results hint to a possible switch in preference from positive at doses lower than 0.60 mg and negative following it (Fig. 8). In both dose-dependent experiments, I used the same range of increasing egg mass, and although non-significant, the two assays had opposing trends. Average attraction response was positive for low to intermediate eggs densities (0.15 mg-0.60 mg) with measures in the repellency range of 1.2 mg and 2.4 mg. For the oviposition

assay, oviposition responses were in the attraction range except for 0.3 mg of eggs which results in an OAI close to 0 (OAI= -0.04). This suggests that pheromonal cues from conspecific eggs can have a different effect on gravid female orientation (attraction and repulsion) versus eliciting oviposition (stimulation or determent). Average positive oviposition responses in my study coincide with current research that overall sand flies tend to prefer to oviposit on substrate baited with conspecific eggs compared to non-baited controls (ElNaiem and Ward, 1991; Srinivasan et al., 1995; Basimike et al., 1997; Kumar et al., 2013).

CHAPTER V

CONCLUSION

In conclusion, this study found that gravid female *P. papatasi* were significantly attracted to conspecific eggs compared to other conspecific living stages (1st instar, 2nd/3rd instar, 4th instar, pupae, adult males, and adult gravid females). Attraction response followed an inverse relationship with developmental stage age. Further analysis of the dose-dependent relationship of conspecific eggs and oviposition response showed significant preference to substrates containing 1.2 mg of conspecific eggs compared to a non-baited control. I did not find significant dose-dependent trends in relation to attraction response and conspecific eggs, potentially due to small sample size.

Based on previous research and the results from this study, optimum attractive bait for a lethal ovitrap should be a semiochemical combination derived from extracts of attractive microbes present in rearing medium and conspecific eggs (Srinivasan et al., 1995; Basimike et al., 1997; Rajame et al., 1997; Kumar et al., 2013; Marayati et al., 2015). Isovaleric acid was identified as an active compound isolated from attractive microbes isolated from $2^{nd}/3^{rd}$ instar substrate (E. Hatano, unpublished). In addition, preliminary analysis suggested that dodecanoic acid is the active compound of *P. papatasi* eggs (E. Hatano, unpublished). Future analysis of isovaleric acids and dodecanoic acid should be done to evaluate the attraction and stimulatory capabilities

both individually and in combination. If either active compound has an attractive or stimulatory effect, it could be further used for developing an oviposition trap.

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APPENDIX A

TABLES

Table 1. Egg Doses. Eggs doses used for dose-dependent bioassays with average number of eggs and standard error corresponding to each dose.

Egg dose (mg)	Average number of eggs	Standard error
0.15	36	3.674234614
0.3	78.75	9.742785793
0.6	201	11.29712353
1.2	420.5	10.20110288
2.4	887.25	34.88619892

Table 2. Oviposition Response Bioassay. Simple linear regression for the effect of conspecific stages with on the oviposition attraction indices derived from number of eggs laid per filter paper with either males (A.) or gravid females (B.) as the adult stage.

A.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	0.031021	0.138149	0.224546	0.822765
Age class	-0.01773	0.035473	-0.49992	0.618165

B.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	0.007006	0.142435	0.04919	0.96086
Age class	-0.00744	0.036574	-0.20348	0.83915

Table 3. Attraction Bioassay. Simple linear regression for the effect of conspecific stages on the oviposition attraction indices derived from number of females stuck on sticky trap with either males (A.) or females (B.) as the oldest developmental stage.

A.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	0.16619	0.046498	3.574124	0.02329
Age class	-0.05314	0.015358	-3.4603	0.025811

B.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	0.154762	0.039917	3.877078	0.017886
Age class	-0.04457	0.013184	-3.38067	0.027768

Table 4. Dose-Dependent Oviposition Bioassay. Simple linear regression for the effect of increasing masses of conspecific eggs on the oviposition attraction indices derived from number of eggs laid.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	0.028961	0.168582	0.171791	0.864077
Conspecific eggs	0.054872	0.050829	1.079538	0.283902

Table 5. Dose-Dependent Attraction Bioassay. Simple linear regression for the effect of increasing masses of conspecific eggs on the oviposition attraction indices derived from females stuck on stick trap.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	0.083508	0.194303	0.429782	0.669782
Conspecific eggs	-0.16821	0.542827	-0.30988	0.758348

APPENDIX B

FIGURES

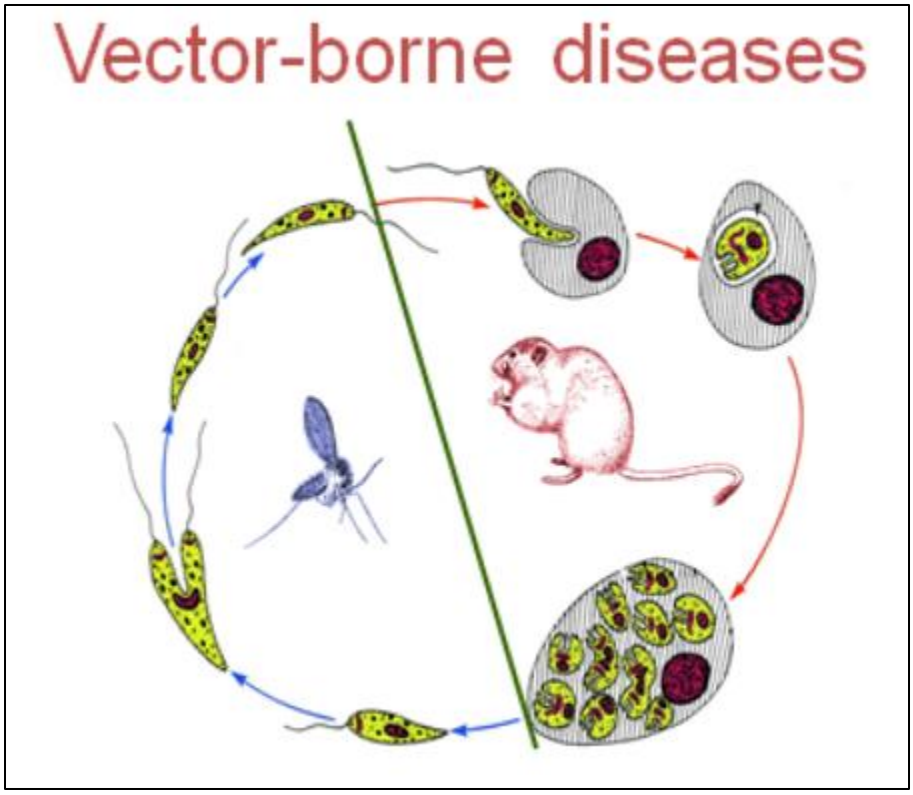

Figure 1. The Life Cycle of *Leishmania* spp. Includes a human stage where protozoa transform from flagellated promastigotes into amastigotes and multiply in number after infecting macrophages. During the sand fly stages, infected macrophages are ingested, and amastigotes transform into promastigotes and multiply to migrate to the proboscis for transmission to occur.

Figure 2. Juvenile Stages of *P. papatasi.* The four larval stages and pupal stage of P. Papatasi including 1st instar (left), 2nd instar, 3rd instar, 4th instar, and pupae (right)(Wilson, n.d.).

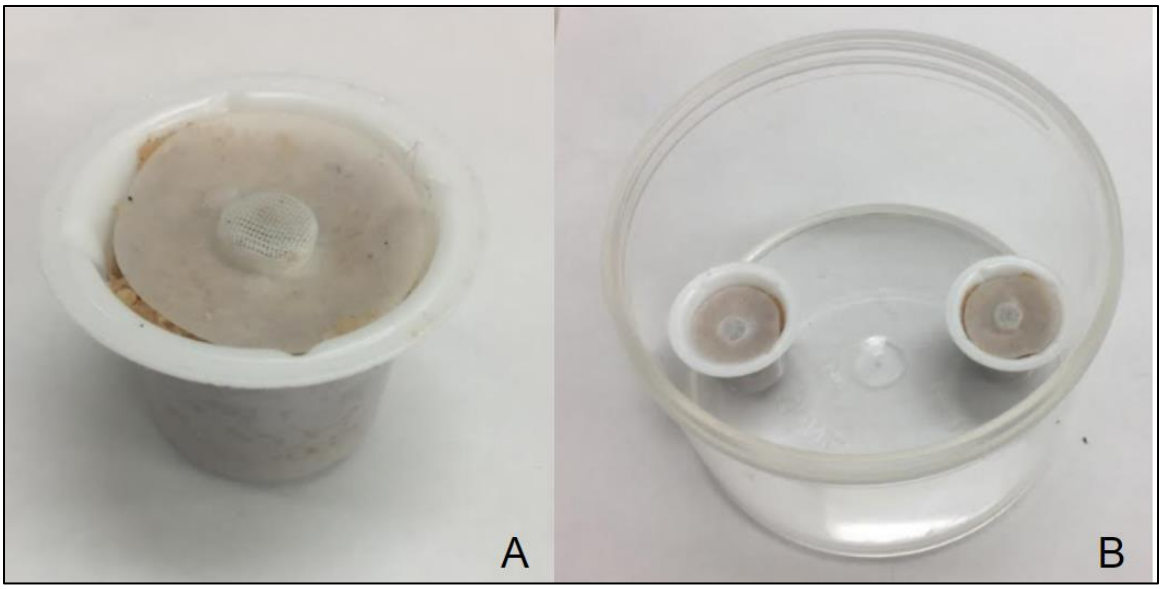

Figure 3. Oviposition Response Bioassay. A. Tube is inserted in a 10-ml disposable micro-beaker filled with 8 ml of moist autoclaved sand. B. Two-Choice behavioral bioassay cups were constructed using 500mL cups with 2.5cm filter paper discs in sand cups distributed at equal distance.

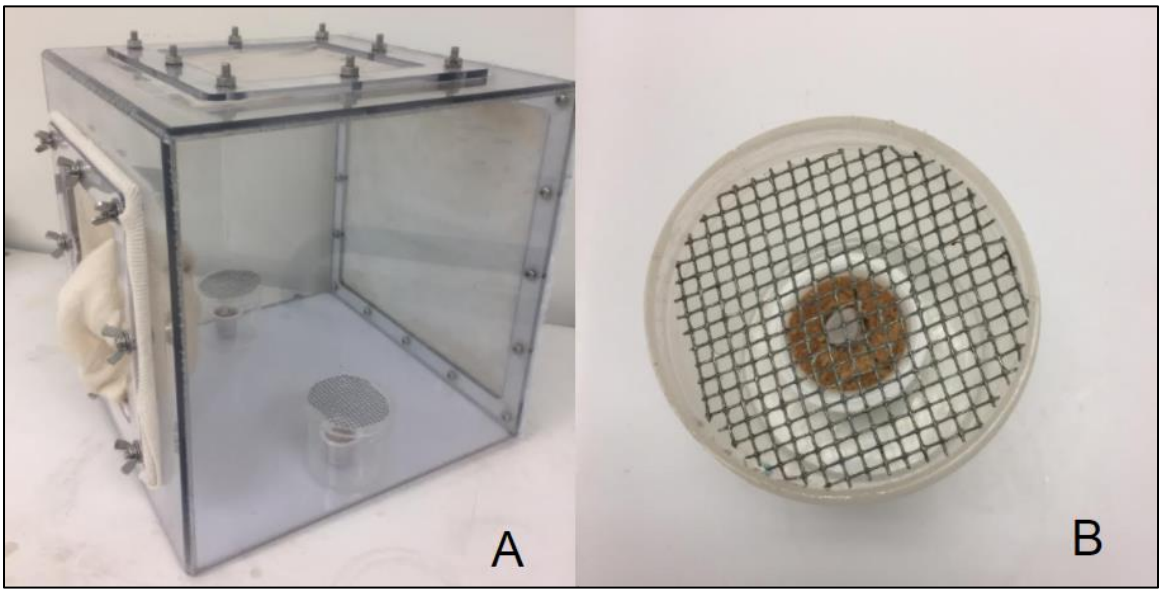

Figure 4. Attraction Bioassay. A. Free flight cages used to evaluate attraction, consisting of treatment versus control cups. B. Sticky trap used in attraction assays composed of sand cup inside a clear cup with stick metal screen.

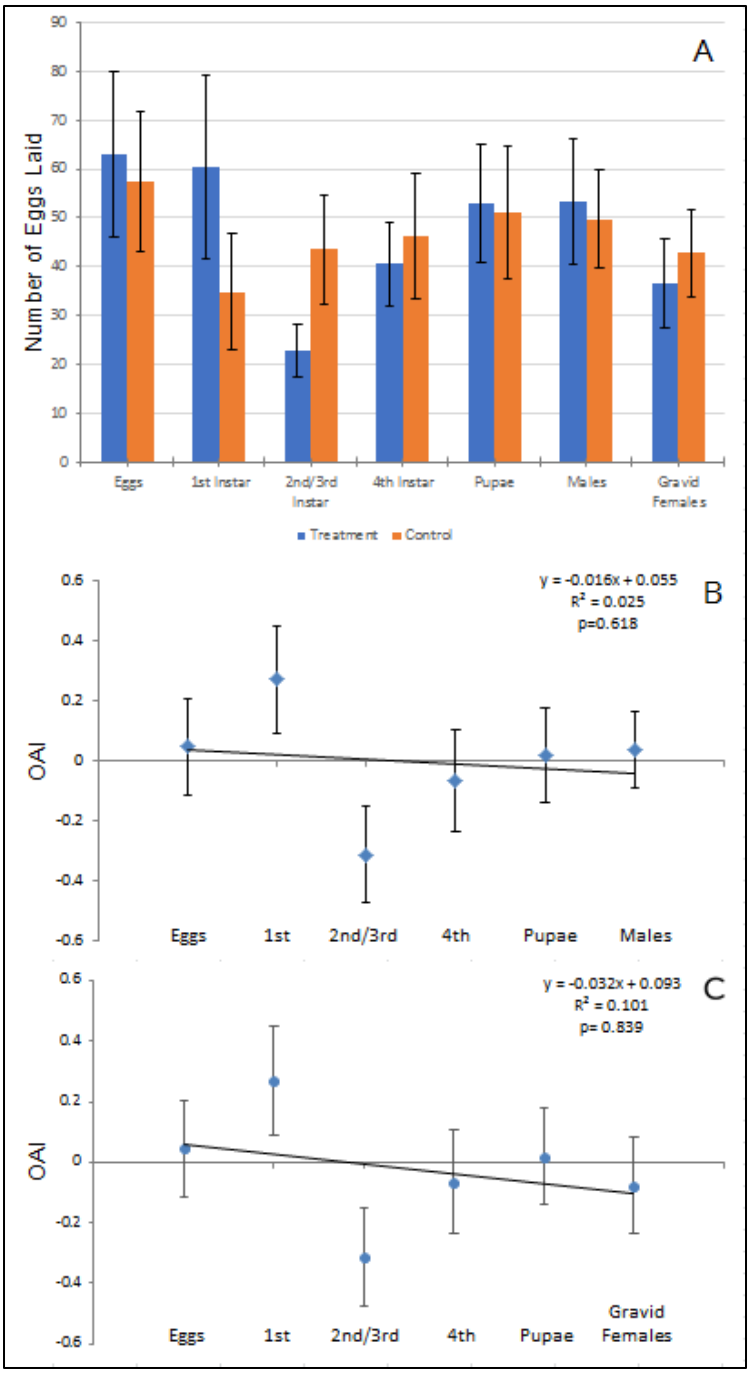

Figure 5. Oviposition Response of Gravid *P. papatasi* Females to Conspecific Stages. A. Average number of eggs laid on filter paper when in the presence of conspecific egg, larvae or adult stages. B. The effect of conspecific stage age on oviposition preference (as indicated by the Oviposition Activity Index) of gravid females with oldest stage being either males (B) or females (C). Error bars represent standard error (n=18).

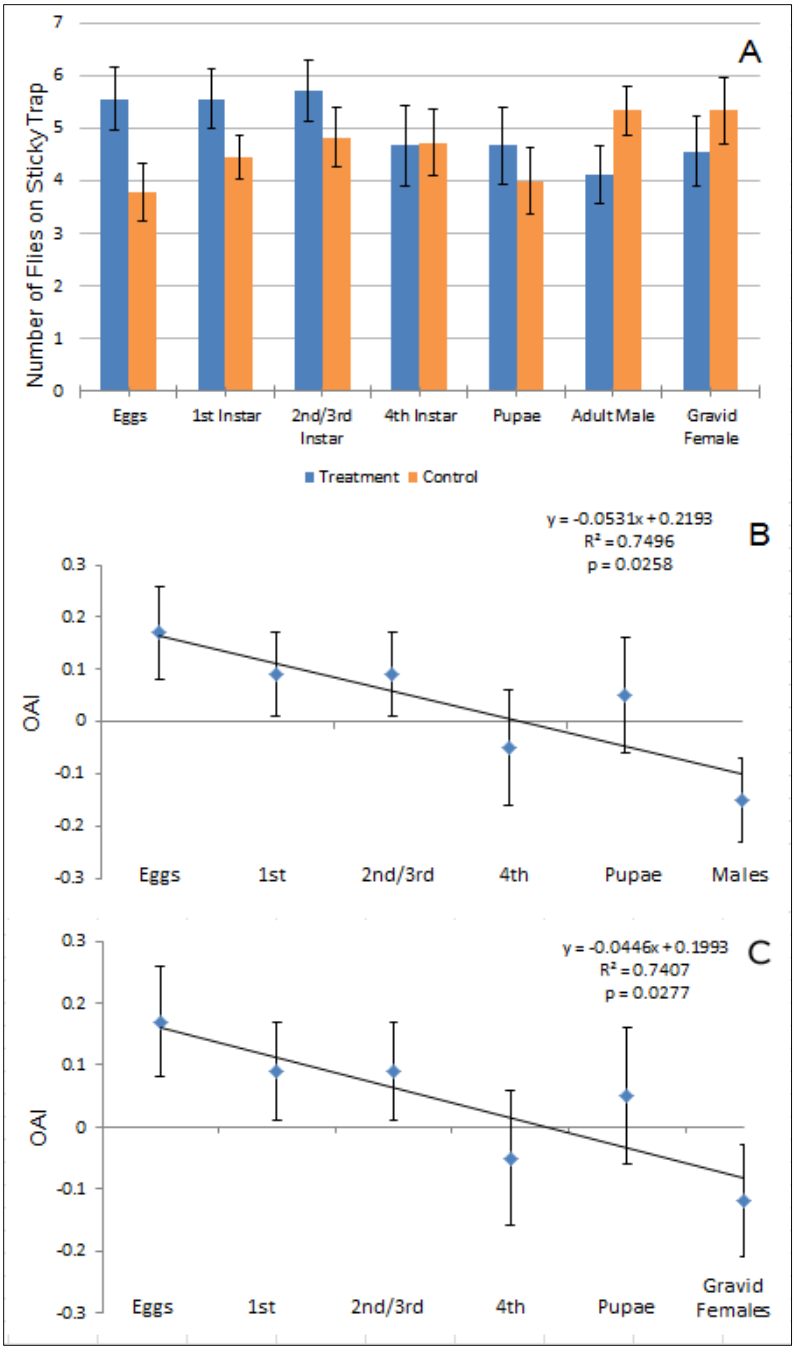

Figure 6. Attraction Response of Gravid *P. papatasi* Females to Conspecific Stages. A. Average number of females attached to sticky trap when in the presence of conspecific egg, larvae or adult stages B. The effect of conspecific stage age on attraction (as indicated by the Oviposition Activity Index) of gravid females with oldest stage being either males (B) or females (C). Error bars represent standard error (n=18).

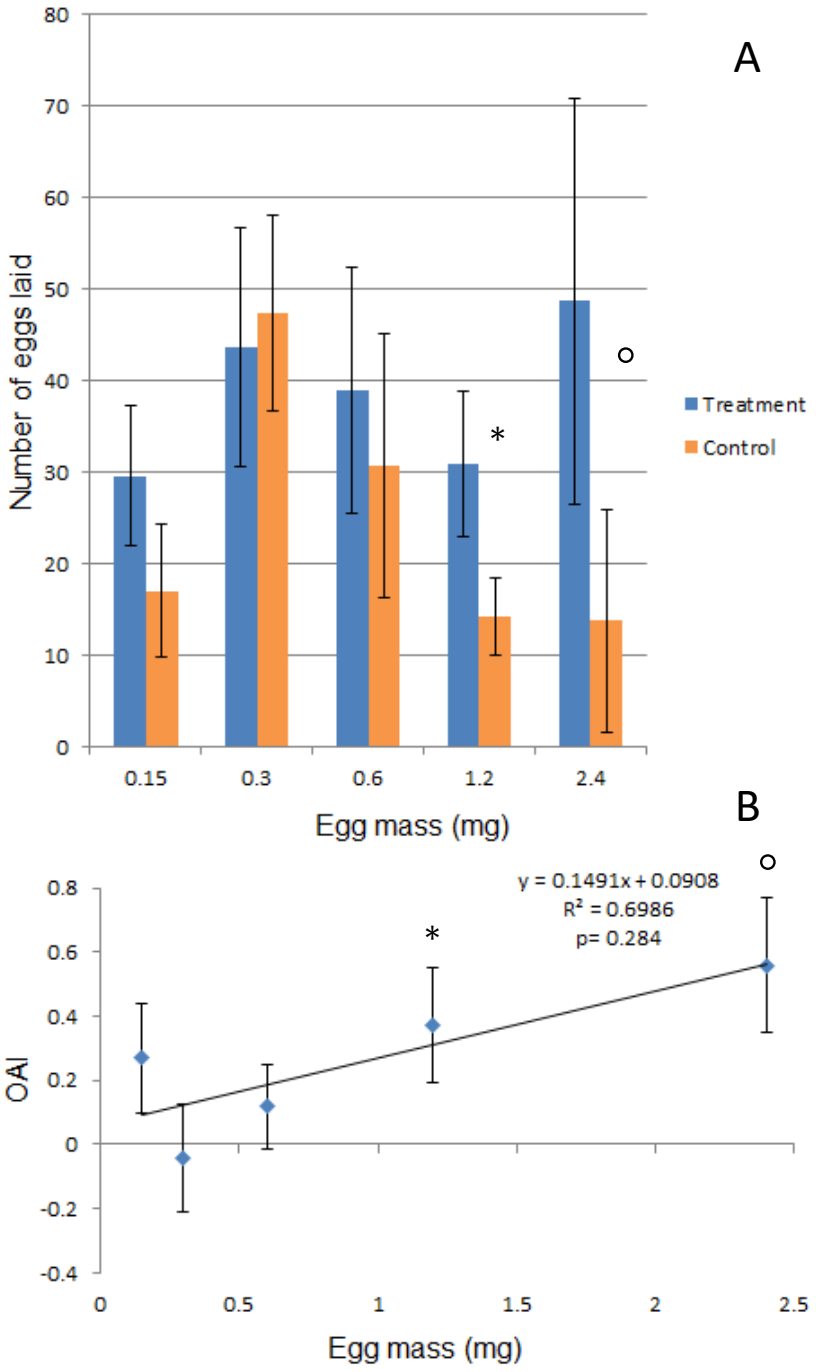

Figure 7. Oviposition Response of Gravid *P. papatasi* Females to Conspecific Eggs. A. Average number of eggs laid on filter paper when in the presence of increasing masses of conspecific eggs. B. Attraction to increasing masses of conspecific eggs, as indicated by the Oviposition Activity Index (OAI). Asterisks represent p= 0.01-0.05. Circles represent p= 0.05-0.1. Error bars represent standard error (n=15).

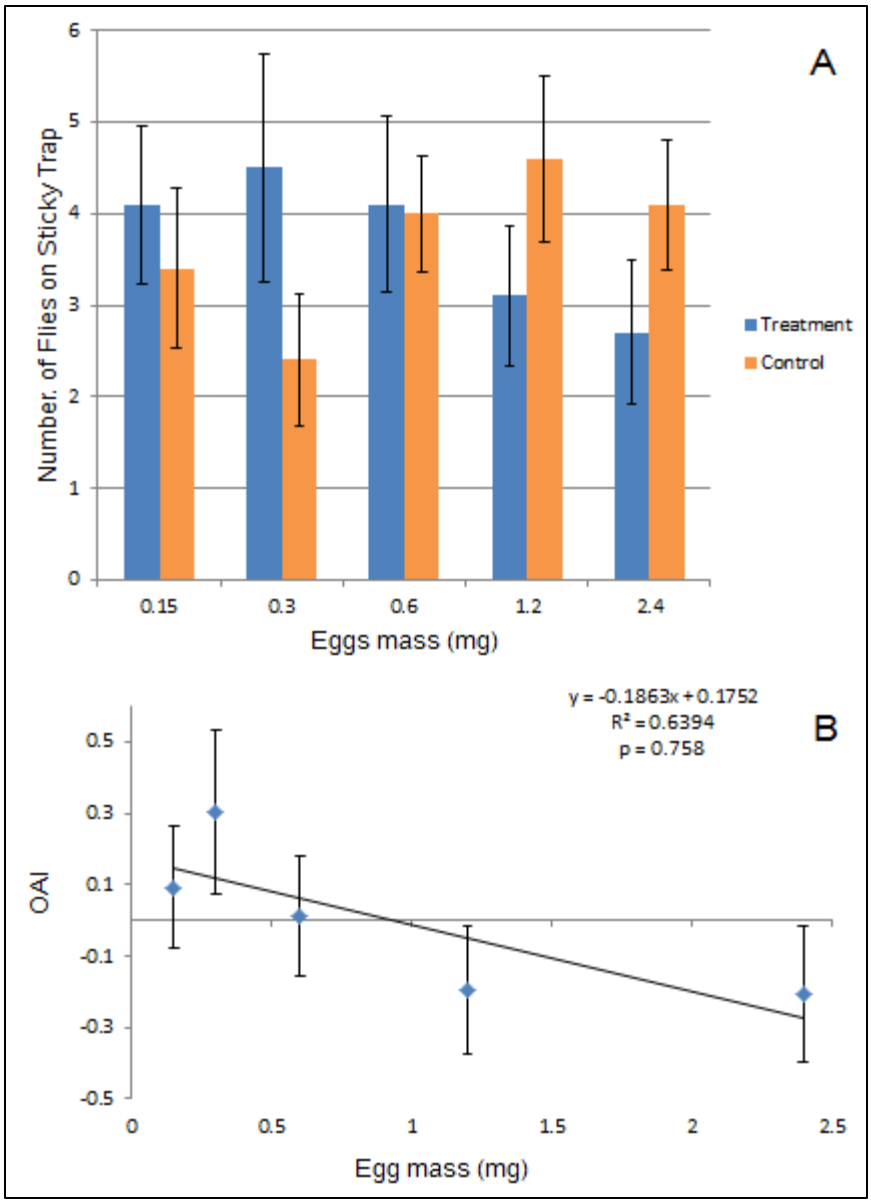

Figure 8. Attraction Response of Gravid *P. papatasi* Females to Conspecific Eggs. A. Average number of females stuck to sticky trap when in the presence of increasing masses of conspecific eggs. B. Attraction to increasing masses of conspecific eggs, as indicated by the Oviposition Activity Index (OAI). Error bars represent standard error (n=10).