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Many environments are either spatially or temporally stochastic, meaning organisms have had to develop evolutionary progeny risk-spreading strategies to deal with such uncertainty. One such evolutionary strategy is skip oviposition (laying eggs in more than one site) strategies, advantageous life-history strategies where individuals are not putting all eggs in one basket. This study's broader goal was to investigate the existence of skip oviposition in *Phlebotomus papatasi* female sand flies. My specific goals were: (Aim 1) study the effect of the number of oviposition sites on skip oviposition behavior, (Aim 2) study the effect of resource variability on skip oviposition behavior, (Aim 3) study the effect of spatial scale on skip oviposition behavior, and (Aim 4) study the effect of conspecific females on oviposition behavior. The general hypothesis is that gravid *Phlebotomus papatasi* may employ skip oviposition due to the ephemeral nature of oviposition sites. However, the existence of skip oviposition may vary based on the heterogeneity of oviposition sites inside rodent burrows, the spatial distance between sites, and the female sand fly population around each site. A series of bioassays were conducted using solitary gravid females exposed to varying numbers of oviposition sites and varying quality sites within small (container) and medium (free-flight cage) scales. At the medium scale, ten gravid females were exposed to a varying number of oviposition sites of equal quality. I found that skip oviposition was common at the small container scale (jar) but less so at the free-flight cage scale. Specifically, with respect to Aim 1, I found no significant difference in eggs laid between available sites,

and there was a fixed egg clutch size. In respect to Aim 2, I found that female sandflies titrated eggs' distribution in a dose-dependent manner, showing a positive relation between eggs laid in sites and increasing habitat quality. In respect to Aim 3, I found that females showed the same patterns as seen on the smaller scale but at a reduced rate. In respect to Aim 4, I found that females were stimulated to lay more eggs when in other females' presence at the medium scale. The next crucial step in this experimental set-up is to evaluate if the same oviposition patterns happen within a natural environment and not just within a laboratory.

EVIDENCE OF SKIP OVIPOSITION IN *PHLEBOTOMUS PAPATASI* SAND FLIES

by

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Approved by

_____
Committee Chair

I dedicate this thesis to my family, who have fully supported me throughout my academic career. Thank you to my father Bryan McLaughlin, and to my grandparents, Tolley and Shirley McLaughlin.

APPROVAL PAGE

This thesis written by LEXUA GRACE MCLAUGHLIN 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

Oviposition Site Selection of Hematophagous Insects.

For insects that lack parental care and where larval dispersal is limited, the oviposition-site selection is a critical fitness-enhancing decision and therefore has implications on the distribution, abundance, and dynamics of insect populations (Aberu et al., 2015; Wasserberg et al., 2013; Wasserberg et al., 2014). Understanding the factors affecting oviposition-site selection in hematophagous insects may provide opportunities for developing new control approaches. Source reduction is one form of such control that relies on the knowledge of oviposition and larval habitats. For example, this approach is used to control mosquito populations by draining or filling larval habitats, thus making them unavailable for oviposition (Baldacchino et al., 2015; Yohannes et al., 2005). Another effective method of control discovered through oviposition behavior research is the addition of natural enemies such as *Bacillus thuringiensis israelensis* (BTI) or mosquitofish to larval habitats to reduce mosquito abundance (Becker, 1997; Bence, 1988). Research studies on the physical and chemical characteristics of mosquito oviposition behaviors have facilitated the use and success of source reduction and natural enemies' addition to larval habitats. Oviposition behavior can be affected by factors such as food availability, conspecific competition, bacteria, and predator risk are a few

physical and chemical aspects of mosquito oviposition behaviors that have been studied (Bentley and Day, 1989; Day 2016).

Larval Habitat Quality in Mosquitoes

The quality of larval habitats is an essential determining factor in the oviposition-site selection of gravid mosquitoes. The quality of larval habitats is determined by several factors, including the absence of competitors or predators and food availability (Blaustein et al., 2004; Reiskind and Wilson, 2004; Takken and Knols, 1999). Intraspecific competition has been the focus of many studies, and results have varied on the effects of conspecific competition on oviposition site choice. Studies have shown a positive oviposition response to lower densities and a negative response in higher densities when presented with varying densities of conspecific immature stages. The interactions of a positive relationship between an individual's enhanced fitness and the increasing density of conspecifics and intraspecific competition could explain this type of oviposition response. This interaction should result in a hump-shaped relationship between conspecific density and oviposition rate (Wasserberg et al. 2014). The hump-shaped relationship is an outcome of the trade-off between the opposing forces of conspecific immature densities and intraspecific competition with positive effects of oviposition occurring with more eggs or larvae present at low densities and negative effects of intraspecific competition occurring at higher densities (Wasserberg et al. 2014). Food availability is another important factor in oviposition-site selection, as it directly relates to larval growth and survival (Yoshioka et al., 2012). Wasserberg et al. (2013) showed that gravid female *Aedes albopictus* oviposited more eggs on sites with organic matter

present relative to the control sites in a positive, dose-dependent manner (Wasserberg et al., 2013). The effects of predation risk on oviposition behavior has been studied in various ways in gravid female mosquitoes. Blaustein et al. (2004) showed that gravid *Culiseta longiareolata* and *Chaoborus crystallinus* avoided sites with predators while gravid females of *Chironomus riparius* and *Chaoborus flavicans* did not. Unfortunately, all of these studies are based on multiple female groupings and do not observe gravid females' oviposition behavior on an individual level.

Bet-Hedging and Skip Oviposition in Mosquitoes.

When searching for oviposition sites, insects are confronted with heterogeneous environments that can be spatially and temporally stochastic. Under such conditions, bet-hedging has shown to be an adaptive strategy used by insects (Edgerly et al., 1998; Hopper, 1999; Khatchikian et al., 2010; Olofsson et al., 2009). Bet-hedging is the theory that refers to how individuals should optimize their fitness in a variable and unpredictable environment. An individual can optimize their fitness in stochastic environments by spreading the risk of progeny loss through ovipositing eggs in multiple oviposition sites rather than just a single site (Olofsson et al., 2009). Employing skip oviposition allows insects such as *Aedes spp.* to use bet-hedging by ovipositing their eggs in multiple sites rather than using batch oviposition, the act of ovipositing in one site (Day, 2016; Edgerly et al., 1998). Bet hedging through skip oviposition is associated with a tradeoff between spreading the risk of progeny loss and increased risk of female adult mortality when ovipositing eggs in multiple sites (Edgerly et al., 1998; Khatchikian et al., 2010). Bet-hedging is advantageous in increasing the chances of progeny survival when an

environment's biotic factors (e.g. competition, predation, resource levels) and abiotic factors (e.g. drying, flooding, desiccation) are varying and unpredictable (Blaustein and Schwartz, 2001; Edgerly et al., 1998; Erich et al., 2015). However, spreading the risk is less profitable in predictable environments (Edgerly et al., 1998; Aberu et al., 2015; Harrington and Edman, 2001). A stable, predictable environment would be more of an ideal environment for batch oviposition, being able to oviposit all eggs at once, with the benefit of less energy cost to adult mosquitoes and reduced risk of mortality due to the female having to travel less distance before ovipositing (Edgerly et al., 1998; Aberu et al., 2015; Harrington and Edman, 2001). Gravid females of many *Aedes spp.* oviposit their eggs singularly and are more likely to employ skip oviposition by ovipositing in multiple sites. Gravid females of *Culex spp.* oviposit their eggs through batch oviposition, laying their eggs as a batch in one site (Day, 2016). Studies observing the effects of intraspecific competition and organic matter availability via single female mosquito oviposition bioassays have shown female mosquitoes exhibiting skip oviposition (Nazni et al., 2016; Trexler et al., 1998; Williams et al., 2008). In both Nazni et al. (2016) and Williams et al. (2008), it was observed that while single *Aedes aegypti* gravid females prefer to oviposit the majority of their eggs in sites with medium conspecific egg densities with 11-38 eggs, females would oviposit remaining eggs in other sites with conspecifics of a lower or higher egg density equally (Nazni et al., 2016; Williams et al., 2008). Another study investigating the effects of varying organic matter on oviposition selection with single gravid female *Aedes albopictus* and *Aedes triseriatus* showed the females employing skip oviposition (Trexler et al., 1998). When in the presence of 60% and 30% organic matter

infusions (respectively), females preferred to oviposit in organic matter infused sites but still oviposited in other plain water sites as well (Trexler et al., 1998). Gravid female *Aedes spp.* often use skip oviposition to their advantage, but there is a limit to how many sites they can reach depending on the female's physiological state. A laboratory bioassay showed that *Aedes aegypti* females of a larger size could visit more oviposition sites than smaller females, especially if given a sucrose solution as a nutritional resource (Tsunoda et al., 2013).

Applications of Skip Oviposition for Vector Control

While there are benefits that skip oviposition offers to mosquitoes, this behavior can also be exploited for insecticide dissemination among visited oviposition sites, specifically in natural or artificial containers. Mosquito vectors can be more spatially distributed since females can lay eggs at multiple sites with large distances between each site, making the auto-dissemination of insecticides an effective means of disease vector management by exploiting skip oviposition behavior (Reiter, 2007; Suman et al., 2014). Auto-Dissemination is a novel strategy that exploits multiple oviposition sites in vectors that use skip oviposition. Auto-Dissemination is a pull (attraction and transfer) and push (dispersal and transfer to target habitats) strategy that uses insect growth regulators (IGR), such as pyriproxyfen (PPF, or a juvenile hormone analog), placed in an artificial resting spot for adult mosquitoes where the IGR will stick to the adults. Then IGR will fall into every site visited and therefore is disseminated through multiple sites. Once in the site, the IGR acts as an insecticide and reduces adult eclosion in larvae by inhibiting larval development. An added advantage to this strategy is that all of this occurs without

harming the adult mosquito and preventing travel to multiple oviposition sites (Gaugler et al., 2012; Caputo et al., 2012). In one study in New Jersey, USA, several *Ae. albopict*us hot spots were determined, and auto-dissemination stations were set up using PPF powder. Results showed an overall significant reduction in egg and larval population in all sites and a significantly higher pupal mortality (between a rate of 70-100% pupal mortality) (Unlu et al., 2017).

Oviposition Site Selection in Sand Flies

In sand flies, worldwide vectors of leishmaniasis, the issue of skip oviposition has never been studied. Leishmaniasis is a worldwide vector-borne disease found in arid, tropical regions and subtropical regions, transmitted by sand flies of the genus *Lutzomyia* (New World) and *Phlebotomus* (Old World) (Reithinger et al. 2001). Leishmaniasis has three different forms, including Visceral Leishmaniasis (VL), which is often fatal when untreated, Cutaneous Leishmaniasis (CL), which causes painful lesions on the skin, and Mucocutaneous (MCL), which causes painful lesions in mucous membranes (CDC, 2013). Unfortunately, leishmaniasis is considered a neglected disease due to most cases not being reported in the least developed countries with little investment in research and healthcare (Alavar et al., 2006). With no known cure for the etiologic agent discovering ways to control sand fly population, and reduce exposure to sand fly bites, is the most effective means of disease prevention (Antinori et al., 2012; Claborn, 2010). Little is known about sand flies' oviposition and breeding habits despite being a known disease vector of leishmaniasis (Feliciangeli, 2004). *Phlebotomine* sand flies typically require relatively warm, moist environments. Animal burrows often provide such an environment

but can also be found in tree holes, caves, rocks, and other protected habitats, including human dwellings (Claborn, 2010). Like mosquitos, studies have been done investigating the effects of conspecific competition and organic matter, or larval food, availability on oviposition site selection in sand flies (Kumar et al., 2013; Peterkova-Koci, 2012; Srinivansan et al., 1995; Wasserberg and Rowton, 2011). In one study with *Phlebotomus argentipes*, it was shown that more eggs were oviposited on used larval rearing medium, which consisted of dead flies, old unhatched eggs, larval food containing vertebrate feces, frass, and other organic matter, compared to fresh medium (Kumar et al., 2013). Bacteria in the microbial community of vertebrate feces seem to play an important role in sand fly oviposition selection and larval development (Peterkova-Koci, 2012). A laboratory bioassay on the significance of bacteria to sand fly oviposition found that *Rhizobium radiobacter* bacteria not only acting as a good oviposition attractant to gravid female *Lutzomyia longipalpis* but also promoted larval development (Peterkova-Koci, 2012). Wasserberg and Rowton (2011) showed *Lutzomyia longipalpis* and *Phlebotomus papatasi* females preferring to oviposit on sites with expired organic matter mediums or frasses and sites with conspecific eggs (Wasserberg and Rowton, 2011). Another study focused on the effects of conspecific eggs showed *Ph. Papatasi* preferring to oviposit in sites with high conspecific egg densities with 100 or more eggs while avoiding sites of low conspecific egg densities with 40 eggs or below (Srinivansan et al., 1995). In our lab so far, the effects of visual cues, the presence of conspecific eggs, and larval rearing medium on *Ph. papatasi* oviposition behavior have been studied. What has been found with the studies is that oviposition-site color, lighting level, and photoperiod play

important roles in guiding the oviposition behavior of *phlebotomine* sand flies (Shymanovich et al., 2019). *Phlebotomine* sand flies have also been shown to be attracted to oviposition sites containing conspecific egg densities and conspecific larval densities of the 1^{st} and $2^{nd}/3^{rd}$ instar stages (Kowacich et al., 2020). Marayati et al. (2015) showed that gravid females are more attracted to oviposition sites with rearing mediums exposed to $2^{nd}/3^{rd}$ and 4^{th} instar/pupae. However, all these studies were done using groups of females. So, at this point, we still do not know if these patterns are produced by single females disseminating their egg clutch among several oviposition sites or by different numbers of females depositing their entire egg clutch among alternative oviposition sites. Hence, there is an important need to evaluate sand flies' oviposition behavior from the perspective of the individual gravid female. Furthermore, the spatial dynamics of sand fly's oviposition is completely unknown and warrants research.

Study Goal and General Hypothesis

In this study, my general goal was to evaluate the spatial dynamics of *Ph. papatasi* sand fly's oviposition behavior. Specifically, I wanted to evaluate if sand flies exhibit skip oviposition or, alternately, they employ batch oviposition behavior. I hypothesized that *Ph. papatasi* female sand flies are likely to employ skip oviposition to reduce the risk of progeny loss due to biotic factors (e.g. competition, predation, resource levels) or stochastic abiotic effect (e.g. flooding, desiccation). This could occur on two different scales, between burrows, and within burrows. Female sand flies may skip oviposit between burrows to spread the risk of losing progeny in the event of one burrow

flooding or desiccating. The occurrence of skip oviposition within burrows would likely occur between lavatory sites, areas where rodent inhabitants defecate, where proper resources are available to larvae once hatched. To investigate this possible phenomenon, I conducted a series of oviposition bioassays using single gravid females presented with a number of oviposition sites varying in their quantity, quality, and distance. My **general hypothesis** was that gravid *Ph. papatasi* will employ skip oviposition on a small scale, possibly on a larger scale, with a varying number of eggs per site based on the number of oviposition sites and resource levels present.

Specific Aims

1) **Study the effect of the number of oviposition sites available on skip oviposition behavior.**

Hypothesis. I hypothesize that number of available oviposition sites would not affect a female's egg clutch size and that eggs will be distributed randomly among the available sites. I studied this effect by introducing single females into a small arena containing one, two, or four identical oviposition sites and compared the mean number of eggs laid and the variance in eggs laid among the oviposition sites.

Predictions:

i. Gravid females will exhibit skip oviposition, and the number of oviposition sites used will increase with the number of oviposition sites available.

ii. Gravid females will exhibit skip oviposition with fixed egg clutch sizes.

2) **Study the effect of the resource level on skip oviposition behavior.**

Hypothesis. Female sand flies will likely bias their number of eggs according to resource level and oviposit more eggs in oviposition sites with higher organic matter content to ensure better larvae survival chances. However, with more oviposition sites available female sand flies may experience difficulty differentiating between sites. I studied this effect by presenting individual gravid females with two or four oviposition sites containing varying concentrations of aqueous organic matter extracts.

Predictions:

i. Gravid females will exhibit skip oviposition and titrate egg deposition in a dose-dependent manner, favoring higher organic matter levels.

ii. Gravid females' ability to differentiate site quality will decrease with increasing oviposition site availability.

3) **Study the effect of the scale: comparison of the effect of oviposition sites number and resource level on oviposition patterns between the small container (jar) scale and the medium free-flight cage scale.**

Hypothesis. With *Ph. papatasi* sandflies typical environment being burrows inhabited by varying species of rodents, the question of how extensive the behavior of skip oviposition may be in terms of the distance between oviposition sites on the scale of with-in or between different burrow systems. Gravid females will still show an oviposition response, but it is hard to say how distance will affect the response. Gravid females may have a decreased ability to differentiate between the quality of sites with more distance between sites, thus causing a variation in oviposition behavior when comparing a small

distance between sites and larger ones. I studied this effect by having varying distances between the oviposition sites. For Aim 3.1, single females were placed in a free-flight cage (medium scale arena) containing one, two, or four identical oviposition sites. The mean number of eggs laid, and the number of eggs laid among the oviposition sites were compared to those measured in Aim 1 completed within a 500 mL Nalgene jar (small scale arena). For Aim 3.2, free-flight cages contained two and four sites of varying organic matter content available to a single female sand fly. The mean number of eggs laid, and the number of eggs laid among the oviposition sites were compared to those calculated in Aim 2 completed within a 500 mL Nalgene jar (small scale arena).

Predictions:

i. Gravid females placed within a small-scale arena will exhibit skip oviposition and will exhibit skip oviposition at a reduced rate in the medium scale arena.
ii. Gravid females placed within a medium arena will titrate egg deposition in a dose-dependent manner, favoring higher organic matter levels, like in the small-scale arena but at a reduced rate.
iii. Gravid females' ability to differentiate site quality will decrease with larger distances between sites.

4) Study the effect of conspecific females.

Hypothesis. In previous studies, it has been demonstrated that the presence of other adult female sand flies has a negative effect on the attraction of an oviposition site (Kowacich et al., 2020). However, it has not been studied how different numbers of conspecific females present may affect the per capita oviposition rate. It is hard to say how the per

capita oviposition rate may be affected since studies showed the negative effect of conspecific females on attraction. However, the presence of oviposition site seeking conspecific females may act to stimulate, particularly with larger distances between sites, because these females' presence would indicate a preferable oviposition site possibly due to certain pheromones released. I studied this effect by introducing a group of ten gravid female sand flies into a free-flight cage containing one, two, or four identical oviposition sites. The mean per capita oviposition rate was measured and then compared to the mean per capita from cages set for Aim 3.1 where only a single female sand fly was present.

Predictions:

i. Ten gravid females placed within a medium scale arena will have a greater per capita oviposition rate than medium scale arenas containing individual gravid females.

CHAPTER II

METHODS

General Methods

Insects and Colony Maintenance

We used *Phlebotomus papatasi* sand flies originating from Abkük, Turkey, and maintained at the University of North Carolina in Greensboro. Rearing of *Ph. papatasi* sand flies followed the mass-rearing methods described by Modi and Rowton (Modi et al., 1999), and flies were blood-fed on live anesthetized ICR mice. Sandflies were maintained in incubators (Caron®, Marietta, OH, USA) at 26 °C, 80 % RH, and 12:12 light: dark cycle. Colonies were maintained in 500 mL Nalgene jars with a 2.2 cm layer of WhipMix® Orthodontic Plaster on the bottom to ensure moist substrate and drainage. Larval food was prepared by mixing fresh rabbit feces and rabbit chow (Purina) at a 1:1 ratio and fermented for three weeks in a dark chamber, airdried, and ground to a powder.

Basic Experimental Setting

Oviposition Jar Arena. In this experimental set-up a small-scale distance between sites is established. A 10 mL disposable micro-beaker (sand cup) was filled with 8 mL of autoclaved sand and 3 mL of DI water to keep sand moist and placed in 500 mL Nalgene jars (Fig. 1). A 2.5 cm diameter filter paper was placed on the sand in each cup and saturated with 50 μL of either plain DI waters or a treatment solution. The Nalgene jars

were covered with a mesh and placed in 55 x 40 cm plastic tubs. The experiments took place in incubators at 26℃, 80 % RH, and 12:12 light: dark cycle. One female was added per pot around seven days post blood meal (PBM), added via mouth aspirator. After four days, and again three days later, 50 μL of DI water or treatment solution was added to sand cups to keep filter paper moist. Fresh sugar pads were provided with a 30 % sugar solution to provide nutrition for the gravid females. After a 12-day period, flies were removed with mouth aspirators.

Free Flight Arena. A 30x30x30 polycarbonate free-flight cage can be used as a medium-scale distance between sites. The same method we used in the oviposition jar arena for setting up the sand cup oviposition sites will be replicated in free-flight cages (Fig. 2). One single gravid female (Aim 3) or ten gravid females (Aim 4) around seven days after PBM was released into each free-flight cage and left for 24 hours to get acclimated. The experiments took place in a climate control room at the University of North Carolina at Greensboro with humidity set to 65% RH and temperature set to 27℃. After two days, and each day after 50 μL of DI water or treatment solution was added to sand cups to keep sand and filter paper moist. Fresh sugar pads were provided with a 30 % sugar solution to provide nutrition for the gravid females. After a 7-day period, flies were removed with mouth aspirators.

Aqueous Extract of Expired Organic Matter

The aqueous extract was made using larval rearing media from which all adults have enclosed (hereafter, FRASS). It was formed by mixing 1g of expired organic matter in 10 mL DI water for 10 minutes and allowed to settle for 10 minutes, forming a 1X concentration. A serial dilution using the supernatant was then used to obtain concentrations of 0.1X and 0.01X.

Aim-Specific Methods

Aim 1- The Effect of the Number of Oviposition Sites on Skip Oviposition Behavior

To determine the effect of the number of oviposition sites available on skip oviposition behavior in *Ph. papatasi,* one-choice, two-choice, and four-choice oviposition bioassays were used. The sand cups for all oviposition bioassays were placed with-in Nalgene jars (Figure 1 A, B, C). DI water was used to saturate and re-saturate the sand cup filter papers. I conducted four such experimental sessions with ten replicate jars in each session. The eggs laid in the cups were then manually counted using a dissection microscope.

Aim 2- The Effect of Resource Level on Skip Oviposition Behavior

To determine the effect of varying organic matter concentrations on the oviposition response of gravid *Ph. papatasi,* two-choice and four-choice oviposition bioassays were used. The sand cups were placed within Nalgene jars (Figure 1 B, C). FRASS aqueous extracts of three different concentrations and DI water were used to saturate and re-saturate the treatment and control filter papers, respectively. I conducted

five such experimental sessions with ten replicate jars in each session (two-choice required ten replicates of each treatment vs. control). Then the eggs laid in control and treatment cups were manually counted using a dissection microscope.

Aim 3.1- The Effect of Number of Oviposition Sites on Skip Oviposition at the Free-Flight Cage Scale

To determine the effect of varying distances between a varying number of available oviposition sites on the oviposition response in gravid *Ph. papatasi*, one-choice, two-choice, and four-choice oviposition bioassays were used once again. In all oviposition bioassays, 0.1g of FRASS was placed under the sand cups' filter paper and placed within free flight cages (Figure 2 A, B, C). The FRASS aqueous water extract was not used due to preliminary results showing an absence in oviposition response. DI water was used to saturate and re-saturate the sand cup filter papers. I conducted one such experimental session with nine replicate cages. The eggs laid in the cups were then manually counted using a dissection microscope.

Aim 3.2- The Effect of Resource Level on Skip Oviposition at the Free-Flight Cage Scale

To determine the effect of varying distances between oviposition sites of varying resource amounts on the oviposition response in gravid *Ph. papatasi* two-choice and four-choice oviposition bioassays were used once again. In all oviposition bioassays, 0.5 g, 0.1 g, or 0.01 g of FRASS was placed under the treatment sand cups' filter paper, and 0 g was placed under the filter paper of control sand cups. The sand cups were then placed with-in free flight cages (Figure 2 B, C). DI water was used to saturate and re-saturate the

filter paper. I conducted one such experimental session with nine replicate cages (two-choice requires nine replicated of each treatment vs. control). The eggs laid in control and treatment cups were manually counted using a dissection microscope. The Aim 1 and Aim 2 oviposition bioassays were used for comparison as a small-scale distance between oviposition sites versus medium-scale.

Aim 4- The Effect of Conspecific Females

To determine the effect of varying conspecific females on the oviposition response in gravid *Ph. papatasi,* one-choice, two-choice, and four-choice oviposition bioassays were used once again. In all oviposition bioassays, 0.1g of FRASS was placed under the sand cups' filter paper and placed within free flight cages (Figure 2 A, B, C). DI water was used to saturate and re-saturate the sand cup filter papers. I conducted one such experimental session with six replicate cages. The eggs laid in the cups were then manually counted using a dissection microscope.

Data Analysis

Egg clutch size was calculated by adding the number of eggs laid in all sand cups inside a jar or cage. Per-capita oviposition rate (for cages with ten females' or cages from Aim 3.1) was calculated by dividing the total number of eggs laid on sand cups in a cage by the number of females present. Skip oviposition was determined as a jar or cage containing two or more oviposition cups in which single females laid eggs in more than one oviposition cup. The level of preference for OM over control was calculated as an oviposition preference index, which is calculated by dividing the number of eggs laid in the treatment cups by the total number of eggs laid in control and treatment cups

combined. Given that “preference” is a proportion, we analyzed the data using weighted logistic regression, with the total number of eggs laid in the treatment and control cups combined as the weighting factor. The independent variable (Aim 2 using aqueous OM dilution level or Aim 3 using solid OM amounts) was log-transformed for mitigating statistical leverage effects (In Aim 2 there was a + 2 offset to avoid negative values). Statistics of the sand fly oviposition behavior was calculated using R-Studio. In the two-choice oviposition bioassay, a paired t-test was used between the two sites to assess any difference between control and treatment oviposition cups. In the four-choice oviposition bioassay, a one-way ANOVA test was conducted, followed by Tukey's posthoc tests to compare the mean number of eggs between all four cups. The results from the paired t-test and Tukey’s posthoc test will then be compared to determine the effect of increasing the number of available oviposition cups on skip oviposition behavior. A linear regression analysis was then calculated based on the sum of the eggs laid by a single female sand fly compared to the varying number of cups available to determine how the number of cups available affects the number of eggs laid in total by individual gravid females. Results from all tests will be compared between the small scale and medium scale to determine the effect of scale on skip oviposition behavior.

CHAPTER III

RESULTS

Aim 1 - The Effect of the Number of Oviposition Sites on Skip Oviposition Behavior

In 19 jars (47.5%), no eggs were laid, due females dying or were not gravid, thus creating experimental artifacts. Skip oviposition was exhibited in remining jars of the two-choice bioassays in 13 out of 21 jars (61.9%), and in 8 jars eggs were laid in one cup (38.1%). In the four-choice bioassay, skip oviposition was exhibited in 15 out of 40 jars (37.5%), and in 14 jars (35%), no eggs were laid. Among jars in which eggs were laid, 46.1% of the jars eggs were laid in one cup, in 23.2% of the jars, eggs were laid in two cups, in 11.5% of the jars, eggs were laid in three cups, and in 19.2% of jars, eggs were laid in all four cups. In the two-choice bioassays, I compared the number of eggs laid by a single gravid female *Ph. papatasi* between two available oviposition cups within a jar using a paired t-test to test for sidedness by comparing the number of eggs laid in cups A and B. I did not find a difference in the mean number of eggs laid by a single gravid female *P. papatasi* between cups A and B ($t = 1.4433$, $df = 15$, $p = 0.1695$), showing no evidence of sidedness when female's oviposit eggs (Figure 3A). In the four-choice bioassays, I compared the number of eggs laid by a single gravid female *Ph. papatasi* between the four available oviposition cups within a jar using a one-way ANOVA to test for sidedness by comparing the number of eggs laid in cups A, B, C, and D. I did not find

a significant difference in the number of eggs laid by a single gravid female Ph. papatasi between cups A, B, C, or D (F-value = 1.3176, p = 0.1695), showing no evidence of sidedness when female's oviposit eggs (Figure 3B). To test the effect of the number of oviposition sites on the total number of eggs laid by a single female, I ran a linear regression analysis between the sum of eggs laid in all sites available in a jar and the number of sites available. I did not find a significant linear trend with increasing the total sum of eggs and the number of available oviposition sites (t = 0.203, p = 0.84) (Figure 3C and Table 1A). I did, however, find using a simple linear regression that the average number of eggs laid in cups did significantly decrease as the number of sites available increased (t = -3.900 p = 0.000167) (Figure 3D). In summary, the results gathered here showed that gravid female sand flies do exhibit skip oviposition, but do not show sidedness when oviposition sites are of equal quality. I also determined that the number of sites available does not affect the number of total eggs laid by individual females.

Aim 2- The Effect of Resource Level on Skip Oviposition Behavior

Skip oviposition was exhibited in two-choice bioassays with 86 out of 109 jars (78.9%). It was notable that the degree of skip oviposition decreased as the FRASS concentration increased (85.7%/14.3%, 78.0%/22.0%, and 70.5%/29.5% for 0.01X/Water, 0.1X/Water, and 1X/Water, respectively). Similarly, in four-choice bioassays with 24 out of the 33 jars (73%). There was a marginally significant difference in the egg clutch sizes in both two-choice and four-choice bioassays with OM presence compared to Aim 1 egg clutch sizes (t = -1.9464, df = 15, p-value = 0.07059; t = -2.196, df = 16, p-value = 0.04318, respectively) (Figure 5). However, though the presence of

OM does seem to stimulate greater egg clutch sizes, in two-choice bioassays, it was noticed that as the FRASS extract concentration decreased, the egg clutch size did as well (33.4±2.15 eggs, 31.03±4.31 eggs, and 26.74±3.15 eggs for 1X, 0.1X, and 0.01X respectively). In the two-choice bioassays, I compared the number of eggs laid by a single gravid female *Ph. papatasi* between treatment and control oviposition cups within a jar using a paired t-test comparing oviposition stimulation caused by aqueous FRASS extracts. I found that single gravid female *Ph. papatasi* laid more eggs in cups treated with varying concentrations of aqueous FRASS extract. Single female sand flies laid significantly more eggs in sand cups treated with 1X FRASS over the control sand cup ($t = -2.2391$, $df = 33$, $p = 0.03201$) but did not show the same oviposition stimulation in cups with 0.1X and 0.01X FRASS ($t = -1.0687$, $df = 32$, $p = 0.2932$; and $t = 1.4335$, $df = 41$, $p = 0.1593$, respectively). This indicated that gravid females are stimulated to oviposit in cups of higher resource levels, such as 1X FRASS sites (Figure 4A). In four-choice bioassays, I compared the number of eggs laid by a single gravid female *Ph. papatasi* between four available oviposition cups within a jar using a one-way ANOVA to test for oviposition stimulation caused by aqueous FRASS extracts. There was a significant difference in the number of eggs laid between cups of varying FRASS treatments ($F = 6.7309$, $p = 0.0002976$). Single female sand flies favored 1X FRASS over water, 0.1X, and 0.01X based on a Tukey's posthoc test ($t = 3.931$, $df = 128$, $p = 0.0008$; $t = -3.609$, $df = 128$, $p = 0.0025$; and $t = -3.385$, $df = 128$, $p = 0.0052$, respectively) (Figure 4B). In the two-choice bioassays, an oviposition preference index using a linear logistic regression showed a significant linear trend between the proportion of the number of eggs laid

within treatment cups and FRASS concentration ($t = 3.401$, $p = 0.000671$) (Figure 4C and Table 2A). In summary, the results gathered here showed that females were stimulated to oviposit in higher resource quality cups but also laid eggs in cups of poor quality, supporting the theory of bet-hedging and spreading the risk of progeny loss.

Aim 3.1- The Effect of Number of Oviposition Sites on Skip Oviposition at the Free-Flight Cage Scale

In free-flight cages, individual gravid female *Ph. papatasi* did not oviposit at all when one or two cups were available. However, when cages contained four oviposition cups, they laid eggs in two out of nine cages. In these cages, skip oviposition was exhibited, with one cage having 65 eggs distributed 50, 7, 7, and 1 in four cups, and another cage had 31 eggs distributed 20, 11, 0, and 0 among four cups. Based on a one-way ANOVA, there was no significant difference in the number of eggs laid between the oviposition cups ($F = 0.0462$, $p = 0.7251$), indicating no evidence of sidedness here as well (Figure 6B). To test the effect of the number of oviposition cups on the total number of eggs laid by a single female, I ran a linear regression analysis between the sum of eggs laid in cups and the number of cups available. I found a marginally significant linear trend with increasing the total sum of eggs and the available oviposition cups ($t = 1.897$, $p = 0.0695$), indicating that the proximity to the oviposition cue source may play an important role at larger distances between cups (Figure 6D and Table 1B). In summary, my results gathered in this section showed that skip oviposition was existent at the free-flight cage scale but at a reduced rate compared to that within the oviposition jar scale. Gravid female sand flies also do not show sidedness when oviposition cups are of equal

quality but at a reduced rate in free-flight cages. However, unlike bioassays in jars, the number of cups available does seem to affect the number of total eggs laid by individual females in a free-flight cage.

Aim 3.2- The Effect of Resource Level on Skip Oviposition at the Free-Flight Cage Scale

Skip oviposition was existent but on a reduced scale when compared to the oviposition jars in Aim 2. Only one out of 27 cages in the two-choice bioassay, skip oviposition occurred (3.7%). Similarly, in the four-choice bioassays, 3 out of 9 cages skip oviposition occurred (33.3%). In two-choice bioassays, I compared the number of eggs laid by a single gravid female *Ph. papatasi* between treatment and control oviposition cups within a free-flight cage using a paired t-test to test for oviposition stimulation caused by varying amounts of solid FRASS. I found that single gravid female *Ph. papatasi* laid more eggs in cups treated with varying amounts of FRASS but at a lower rate, compared with Nalgene jars. The single female sand flies showed no significant difference in the amount of eggs in sand cups treated with 0.5 g, 0.1 g, or 0.01 g of FRASS over the control sand cup ($t = -1.6053$, $df = 8$, p-value = 0.1471; $t = -0.60999$, $df = 8$, p-value = 0.5588; and $t = -1.2217$, $df = 8$, p-value = 0.2566, respectively)(Figure 8A). However, the mean number of eggs laid in FRASS cups (6.11 ± 2.81) was significantly higher than in the control cups (0.4 ± 0.4) ($z=2.299$, $p = 0.02$). In four-choice bioassays, I compared the number of eggs laid by a single gravid female *Ph. papatasi* between four available sand cups within a free-flight cage using a one-way ANOVA to test for oviposition stimulation caused by varying solid FRASS amounts. There was a

marginally significant difference in the number of eggs laid between cups of varying FRASS treatments (f = 2.3601, p = 0.08993). However, unlike what was seen in the four-choice bioassays in jars, female sand flies were only stimulated mostly in cups of the highest resource level, 0.5 g of FRASS, based on a Tukey's posthoc test (t = 2.484, p = 0.0819) (Figure 8B). An oviposition preference index using logistic regression showed no significant linear trend between the proportion of the number of eggs laid within treatment cups and FRASS amounts (z = 1.412, p = 0.158) (Figure 8C and Table 2B). In summary, the results gathered here showed that females exhibited skip oviposition at the free-flight cage scale but at a reduced rate when compared to the oviposition jar scale. The mean number of eggs laid was significantly higher in cups with FRASS (particularly 0.5 g when within a four-choice bioassay). However, there did not appear to be any significant dose-dependent titration of eggs distributed as seen in Aim 2 at the oviposition jar scale.

Aim 4 – The Effect of Conspecific Females.

When comparing cages with individual or ten gravid females present, cages containing one and two cups individual females did not lay eggs, cages with ten gravid females did lay eggs (Figure 6A, C). There was a marginally significant difference between per capita oviposition rates found in the two-choice bioassays within single and ten gravid female cages (t = -2.2727, df = 5, p = 0.0722). There also seemed to be a marginally significant positive correlation between the per capita oviposition rates and the number of oviposition cups (r^2 = 0.2037, p-value = 0.06) (Figure 7). In summary, the

results gathered in this section suggest that conspecific females may stimulate per capita oviposition rates with a positive correlation to increasing number of oviposition sites.

CHAPTER IV

DISCUSSION

When a hematophagous insect oviposits its eggs, it can do that either through batch oviposition, with all eggs laid at once in one site. Alternatively, it could exhibit skip oviposition, laying a few eggs in different sites. Skip oviposition is a form of bet-hedging by "not putting one's eggs in one basket," thus spreading the risk of progeny loss. This study's main goal was to determine if gravid *Ph. papatasi* female sand flies exhibit skip oviposition behavior, which has previously not been studied in sand fly research. The effects of the number of oviposition sites present, resource level, and distance between sites on any skip oviposition behavior were examined in this study.

The Effect of Number of Oviposition Sites

There is no research about skip oviposition behavior in relation to sand flies, a known behavior observed in female *Aedes spp.* and *Anopheles spp.* mosquitoes. Skip oviposition is used as a means of bet-hedging: spreading the risk of progeny loss in unpredictable environments by ovipositing their eggs in multiple sites rather than in one site (Day, 2016; Edgerly et al., 1998; Hopper, 1999; Olofsson et al., 2009). To find if gravid female sand flies exhibit skip oviposition, several experiments were conducted where single female sand flies were placed under different conditions to test for skip oviposition behavior and see how certain environmental factors affect this behavior's presence. In this study's first aim, I evaluated if single *Ph. papatasi* females distribute

their eggs among several oviposition sites of equal quality. If they do, how do they distribute their eggs: equally, randomly, or by exhibiting some location effect (i.e., “sidedness”). I also evaluated if the number of oviposition sites available affects females' reproductive output in terms of the total number of eggs they lay across all available oviposition sites.

In the first aim of my study, I showed that female *Ph. papatasi* do exhibit skip oviposition behavior under certain conditions. This behavior was observed within the Nalgene jar scale (small arena) when single gravid female sand flies were presented with a varying number of equal quality oviposition sites. The females distributed their eggs among the sites without any noticeable side preference (“sidedness”). I also observed that the individual females' egg clutch size was not affected by the number of oviposition sites present. However, when the number of oviposition sites increased, the number of eggs per oviposition site decreased, indicating a fixed mean egg clutch size distributed over more oviposition sites. These results are consistent with my predictions. We can now answer whether sand flies exhibit skip oviposition behavior from what we have seen in this aim. However, this aim also showed how female sand flies oviposit a fixed mean egg clutch and distribute them without any apparent preference through all available sites if they are of equal quality. Much like skip oviposition, the effect of the number of same quality oviposition sites available on oviposition behavior has not been studied in sand flies on both an individual and group level. Further research could be devoted to this effect in female sand flies of either experiments with individuals and larger groups to possibly determine what is the average individuals' fixed egg clutch size and what is the

average per capita oviposition rates of individuals or within larger groups. This information could be used as a comparison to egg clutch sizes and per capita oviposition rates from other studies focusing on different environmental effects on oviposition behavior such as conspecific eggs or larvae or the presence of organic matter, in either varying amounts or types (Kumar et al., 2013; Peterkova-Koci, 2012; Srinivansan et al., 1995; Wasserberg and Rowton, 2011).

The Effect of Resource Level in Oviposition Sites

There have been many studies completed on how oviposition behaviors in sand flies are affected by organic matter. Fecal matter of various rodent species has shown to be a strong attractant and stimulant to ovipositing female sand flies as seen in laboratory studies such as Elnaiem and Ward (1992), Marayati et al. (2015), and Rama et al. (2014), where sites with rabbit feces were most favored. These studies involved the use of large groups of female sand flies and thus did not allow for the observation of how individual female sand flies respond to the presence of organic matter. This means that it is unknown whether organic matter stimulates individual females to oviposit more eggs or if it stimulates more females to oviposit as a whole. In the second aim of my study, I completed a similar set of experiments as my first aim; however, rather than having similar quality oviposition sites, I varied the quality of the oviposition sites with aqueous extracts of FRASS concentrations of 1X, 0.1X, and 0.01X using either paired choice or four choice experiments. I predicted that single gravid female sand flies would oviposit more eggs in sites of higher organic matter concentration, the number of eggs laid in each site will decrease with the increasing number of sites while still favoring higher organic

matter sites, and as the number of sites available increase the female's ability to differentiate between sites organic matter quality will decrease.

The results showed that individuals still exhibited skip oviposition, but there was a clear preference for high organic matter concentration sites. When only two sites were presented, there was a clear linear trend between the increase in the proportion of the number of eggs laid within sites and increasing aqueous FRASS concentrations. This indicates that individual females titrate eggs' distribution within sites in a dose-dependent manner or habitat quality. However, it was notable that the highest organic matter concentration was favored when four sites were presented. Still, individuals did not seem to differentiate much between the three remaining sites of varying organic matter concentrations. Overall, when given varying oviposition sites of different qualities, individual female sand flies still laid eggs in both low- and high-quality oviposition sites. This behavior is consistent with the idea of bet-hedging with females not "putting all their eggs in one basket" and laying their eggs in multiple sites but favoring sites of higher quality (Olofsson et al., 2009).

The Effect of Scale and Conspecific Females

While skip oviposition was exhibited in individual gravid female sand flies within the small arena setting of a Nalgene jar, the question of how this is related to "real world" environments arose when begin experiments based within the free-flight cage scale. My results showed that gravid female sand flies could titrate and lay their eggs individually but at lower rates. The results also show that females can determine the quality of available sites and allocate their progeny to higher-quality sites but not in a dose-

dependent manner. This skip oviposition behavior found within the small and medium arena could be represented in the real world as within-burrow heterogeneity. For example, a female sand fly might distribute eggs among potentially suitable oviposition sites such as rodent lavatories within animal burrows (Claborn, 2010; Shenbrot et al., 2002). However, with this idea established, the next question becomes, “could there be skip oviposition at a larger scale”? My study's third aim observed how the distance between oviposition sites affected oviposition behavior in female sand flies by completing the same experiments done in aim 1 and 2 but within a medium-size arena: a free-flight cage. Unfortunately, my aim 3 needed many preliminary iterations, and certain changes were added to get usable data to work within my study. Preliminary experiments showed that the aqueous organic matter concentrations were not strong enough to stimulate an oviposition response because none of the female sand flies oviposited their eggs within the available oviposition sites. I also found that there needs to be at least 0.1 g of solid organic matter present to stimulate an oviposition response. Lastly, due to preliminary results of low egg counts or the absence of ovipositioning, even with a stimulus, the effect of having more than one female sand fly (conspecific females) present on the oviposition response within the free-flight cage was evaluated and set as my Aim 4.

In my Aim 3.1, I predicted that there would be a variation in oviposition behavior by individual female sandflies within the free-flight cage, likely a reduced rate in oviposition due to sandflies having more difficulty differentiating or locating sites. My results did show that individual females exhibited skip oviposition at this scale but at a

substantially reduced rate when presented with a varying number of same quality sites. In cages with 1-2 cups, no oviposition occurred; however, in two out of nine cages with four oviposition sites, the females did oviposit and exhibited skip oviposition. This occurrence would indicate that proximity to the oviposition cue source might play a role in the females' oviposition behavior. This would also be consistent with the linear relation found between the increasing number of eggs laid and the number of sites available to individual gravid females. In my Aim 3.2, I predicted that females would still favor sights of higher organic matter amounts and that females would have a more challenging time differentiating between sites with greater distance between them. First, I found that skip oviposition was exhibited when single females were presented with different sites of varying organic matter amounts of 0g, 0.01g, 0.1g, and 0.5g. It was notable that the oviposition rate and skip oviposition were at a substantially lower rate within the free-flight cage once again. However, unlike within the small arena, females did lay the majority of their eggs within higher-quality sites, but there was no apparent dose-dependent manner when the eggs were distributed.

My study's final aim focused on the effect of conspecific females when, as stated previously, individual females placed within free-flight cages showed low or no oviposition rates. I hypothesized that when more female sand flies are present, they will be able to locate oviposition sites more efficiently to oviposit since conspecific females can act as an attractant to preferable oviposition sites or enhance their acuteness to oviposition cues. I also predicted that in the presence of more conspecific females, the per capita oviposition rate would increase. My results also showed that there was a higher

rate of oviposition in all oviposition bioassays in the presence of multiple conspecific gravid females. In cages containing 2 oviposition sites, there were higher per-capita oviposition rates with ten females compared to cages containing a single female. There was also a significant trend between the per capita oviposition rate increase and the increasing number of oviposition sites available. The reason for this response is not yet known. It might be due to other gravid females' presence that stimulates one another through certain chemical stimuli to lay eggs at located sites, also called “group oviposition” (Browne et al. 1969). Females may also be stimulated by chemical stimuli resultant of conspecific eggs, which are known to stimulate oviposition in sand flies (Dougherty et al. 1992, Kowacich et al. 2020).

Study Limitations and Future Studies

Based on this study's results, these experiments could be improved by completing more sessions in all the experiments conducted for my study to get more data. It would also be beneficial to conduct the same Aim 1 experiment with multiple female groups like those completed within the free-flight cage in Aim 3.1. Another possible future aspect to analyze is developing an experiment that tests these same methods but on a larger scale to determine if skip oviposition occurs on an inter-burrow scale.

The results of this study also have implications in terms of the design of bioassays evaluating oviposition cues. Overall, my results suggest that if one were trying to decipher a behavioral mechanism in terms of experimental design, single-female experiments are more efficient over group experiments due to being free of any effects caused by group interactions (Okal et al. 2015). On the other hand, if the experiment's

goal requires a high throughput screening, group bioassays are more beneficial with less overall variability and require less time. This study could not address what sort of skip oviposition behaviors may occur on an inter-burrow scale, only on an intra-burrow scale, since we have yet to develop an experimental design to simulate an inter-burrow scale. We did show that gravid female sand flies can exhibit skip oviposition at both the small and medium scales under laboratory conditions. However, it is still unclear if the same patterns can be found in a natural environment or at what scale. Understanding this part of sand fly oviposition behavior is important in terms of control because if sand flies are capable of ovipositing in different burrows, then there is a potential for the autodissemination control method being useful in controlling sand flies that are known for having highly cryptic breeding sites (Gaugler et al., 2012; Caputo et al., 2012; Feliciangeli, 2004).

CHAPTER V

CONCLUSION

In conclusion, this study found that gravid female *Ph. papatasi* do exhibit skip oviposition both at small and medium scales, though the medium-scale had a reduced oviposition rate. Single female sand flies also distribute their eggs equally among the same quality sites with a fixed egg clutch size. However, when sites are of varying quality within a small arena, female sand flies titrate their eggs' distribution in a dose-dependent manner, favoring the higher quality sites but still ovipositing in low-quality sites supporting the theory of bet-hedging. Within a medium arena, the female sand flies will favor higher-quality sites but do not show the same dose-dependent distribution. Finally, there seems to be an indication that group ovipositioning may play an important role in larger distances between oviposition sites. However, we have yet to test this theory within a small arena and only within the medium arena. Still, it is yet to be determined if this occurs on a smaller scale and required further analysis.

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APPENDIX A

TABLES

Table 1. Oviposition Bioassay (Aim 1 and 3.1). Linear regression analysis testing the effect of the number of oviposition sites on the total number of eggs laid by a single gravid female in all oviposition sites combined within a small (Nalgene jar) (A) and medium (free flight cage) (B) sized arenas. Table C exhibits the same relations as in B but for a group of 10 females.

A.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	1.14700	0.14312	8.014	7.56e-10
Sites	0.01012	0.04994	0.203	0.84

B.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	-5.333	5.314	-1.004	0.3251
Sites	3.810	2.008	1.897	0.0695

C.

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	1.583	21.066	0.075	0.9410
Sites	16.107	7.962	2.023	0.0601

Table 2. Oviposition Bioassay (Aim 2 and 3.2). Logistic linear regression analysis testing for the effect of varying levels of organic matter in oviposition sites available on the proportion of eggs laid by a single gravid female in treatment oviposition sites over control sites combined within a small (Nalgene jar) (A) and medium (free flight cage) (B) sized arenas.

A.

	Coefficients	*Standard Error*	*z-vlaue*	*P-value*
Intercept	0.22659	0.05527	4.099	4.14e-05
Log (OM)	0.14606	0.04294	3.401	0.000671

B.

	Coefficients	*Standard Error*	*z-vlaue*	*P-value*
Intercept	3.2371	0.5310	6.096	1.09e-09
Log (OM)	0.2695	0.1909	1.412	0.158

APPENDIX B

FIGURES

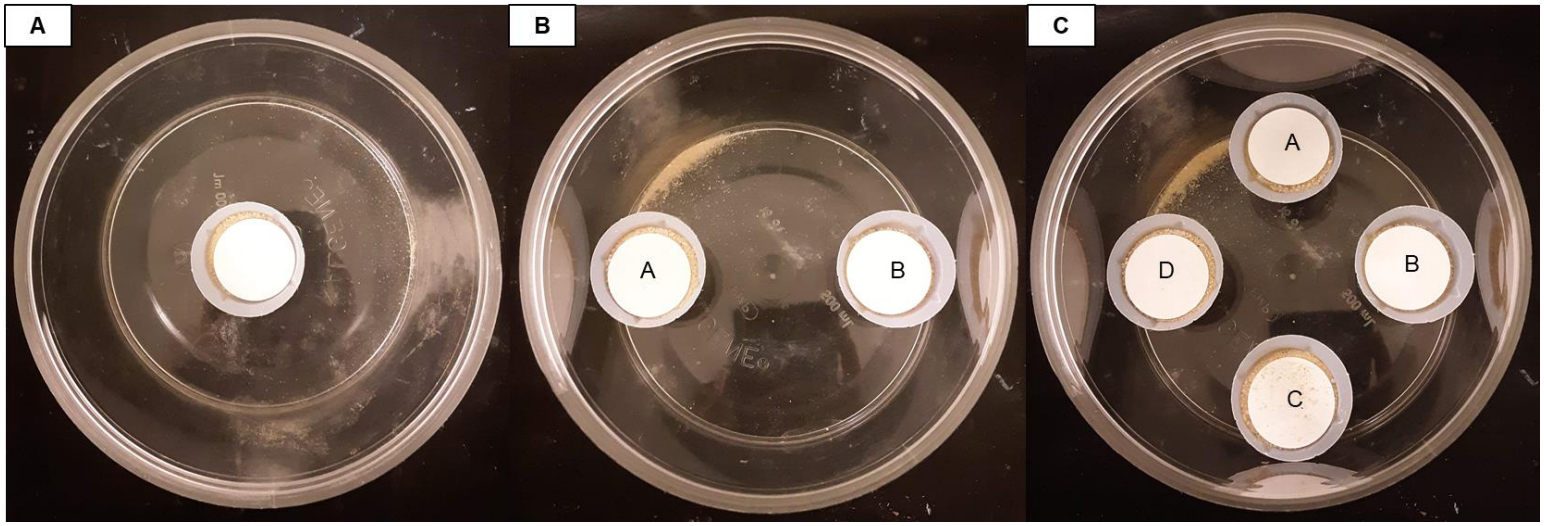

Figure 1. Small Scale Oviposition Bioassays. Small scale oviposition bioassays were constructed using a 500mL Nalgene jars with white 2.5cm filter paper discs in 10 mL plastic sand cups. A. One-Choice. B. Two-Choice consisting of treatment versus control cups. C. Four-Choice consisting of three different treatment versus a control cups.

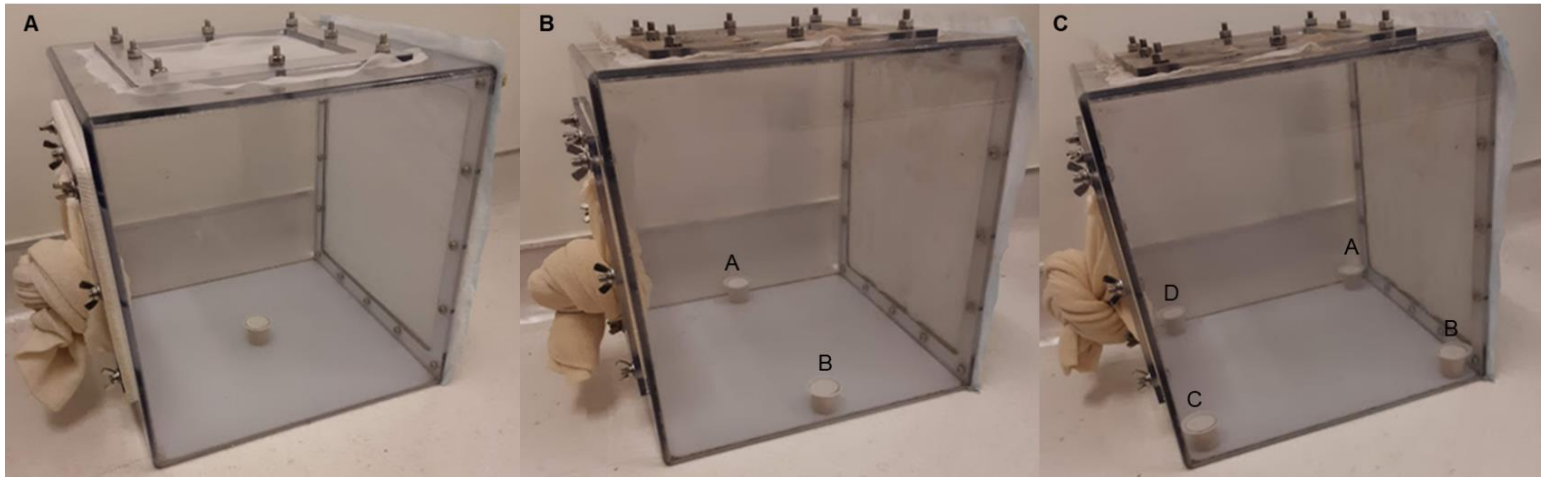

Figure 2. Medium Scale Oviposition Bioassays. Medium-scale oviposition bioassays were constructed using 30x30x30 polycarbonate free-flight cages with white 2.5cm filter paper discs in a 10 mL plastic sand cup. A. One-Choice. B. Two-Choice consisting of treatment versus control cups. C. Four-Choice consisting of three different treatment versus a control cups.

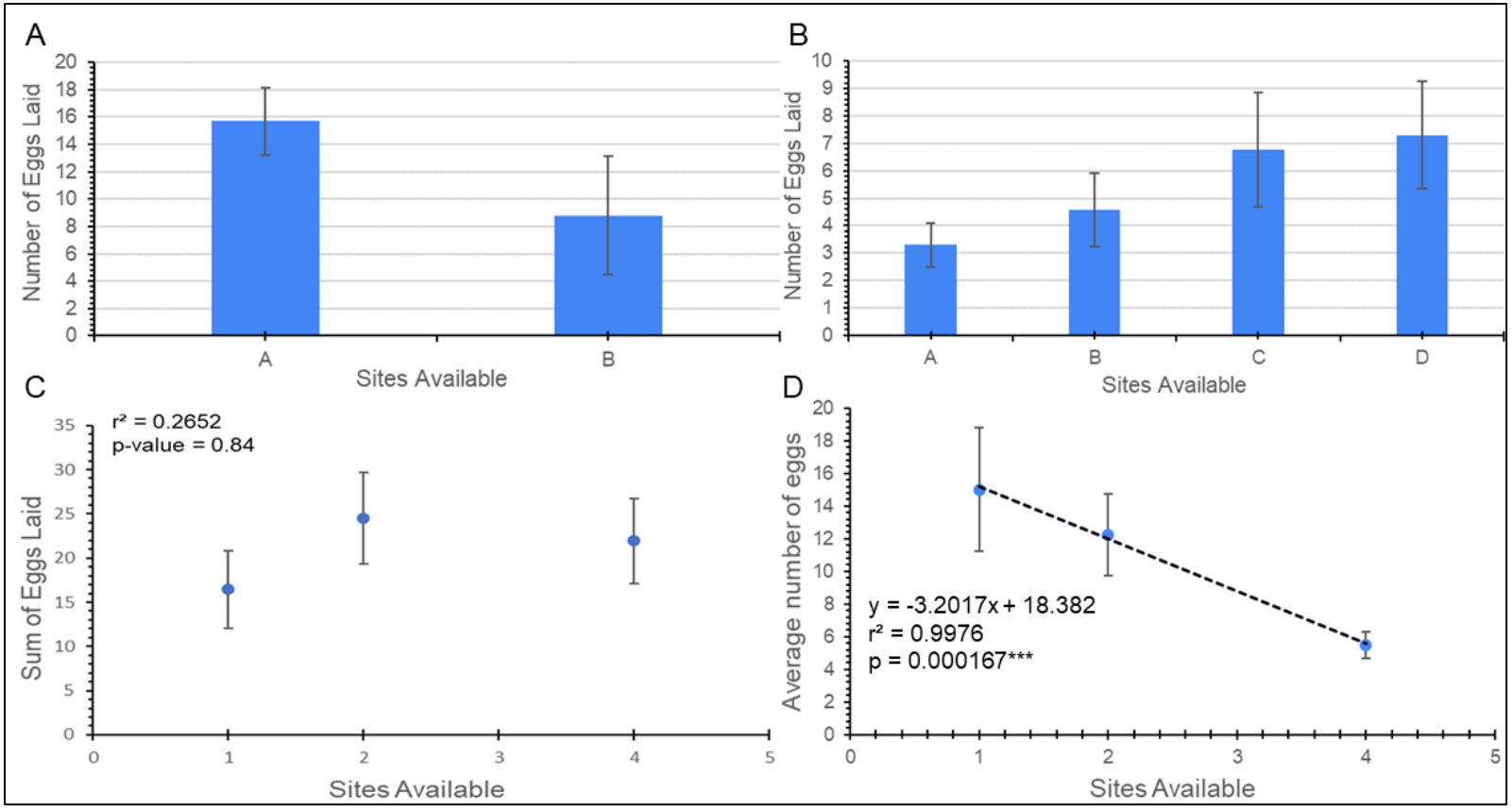

Figure 3. Oviposition Response of Single Gravid *Ph. papatasi* Female to Different Sites at the Nalgene Jar Scale. Average number of eggs laid on filter paper of the same quality oviposition sites within Nalgene jars. A. Two-choice oviposition bioassays with cups A and B: paired t-test (p = 0.1695). B. Four-choice oviposition bioassays with cups A, B, C, and D: one-way ANOVA (p = 0.2764). C. Simple linear regression between the number of oviposition sites available and the total number of eggs laid across all oviposition sites within Nalgene jars (p = 0.84). D. Simple linear regression between the average number of eggs laid in sites and the number of oviposition sites available within Nalgene jars (p = 0.000167***. Error bars represent standard error. (* p <0.05, ** p <0.01, *** p <0.001)

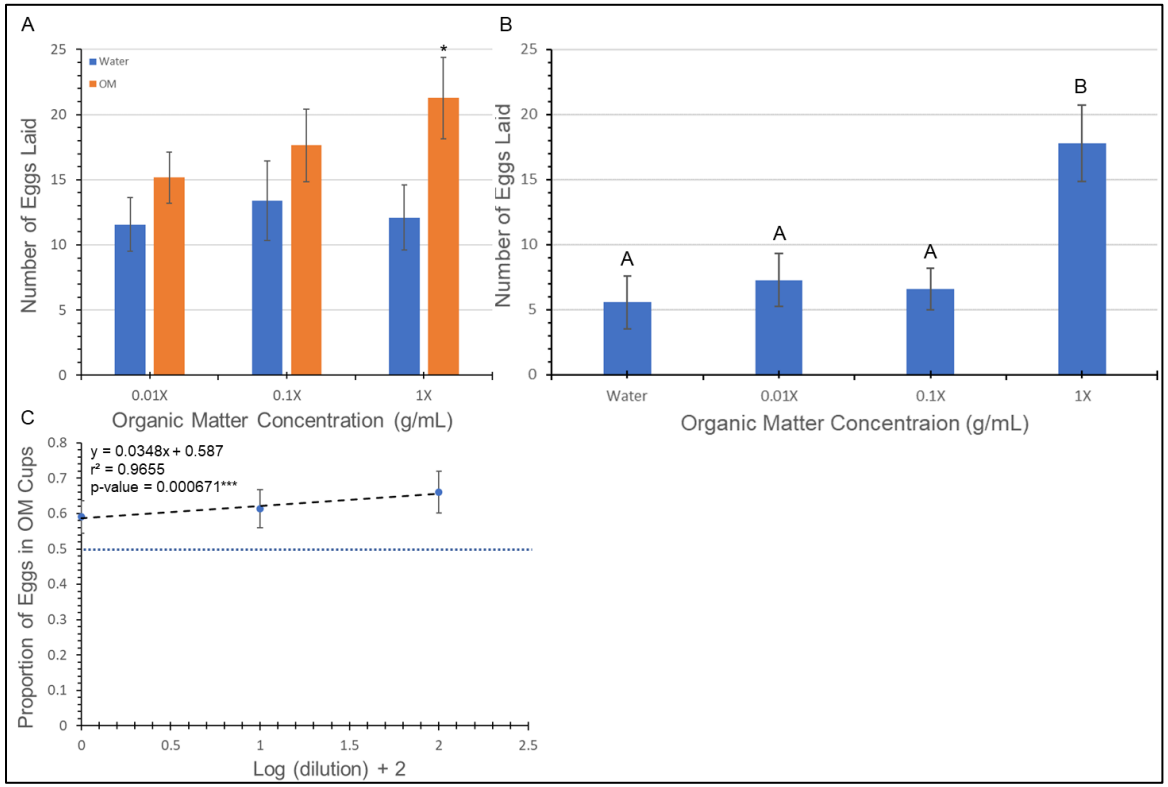

Figure 4. Oviposition Response of Single Gravid *Ph. papatasi* Female to Organic Matter Concentrations at the Nalgene Jar Scale. Average number of eggs laid on filter paper with varying aqueous FRASS concentrations within oviposition sites within a within Nalgene jars. A. Two-choice oviposition bioassay with cups A (0.01X, 0.1X, or 1X) versus B (Water): paired t-test ($p = 0.1593$, $p = 0.2932$, $p = 0.03201^{*}$, respectively). B. Four-choice oviposition bioassays with cups A (Water), B (1X), C (0.1X), and D (0.01X): one-way ANOVA ($p = 0.0002976^{***}$); post hoc (B-C $p = 0.0025^{**}$, B-D $p = 0.0052^{**}$, B-A $p = 0.0008^{***}$). C. Oviposition response to increase organic matter concentrations (log-transformed), as indicated by the preference index ($p = 0.000671^{***}$), with dashed line at 0.5 representing neutral choice line: above = preference, below = repellence. Error bars represent standard error. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

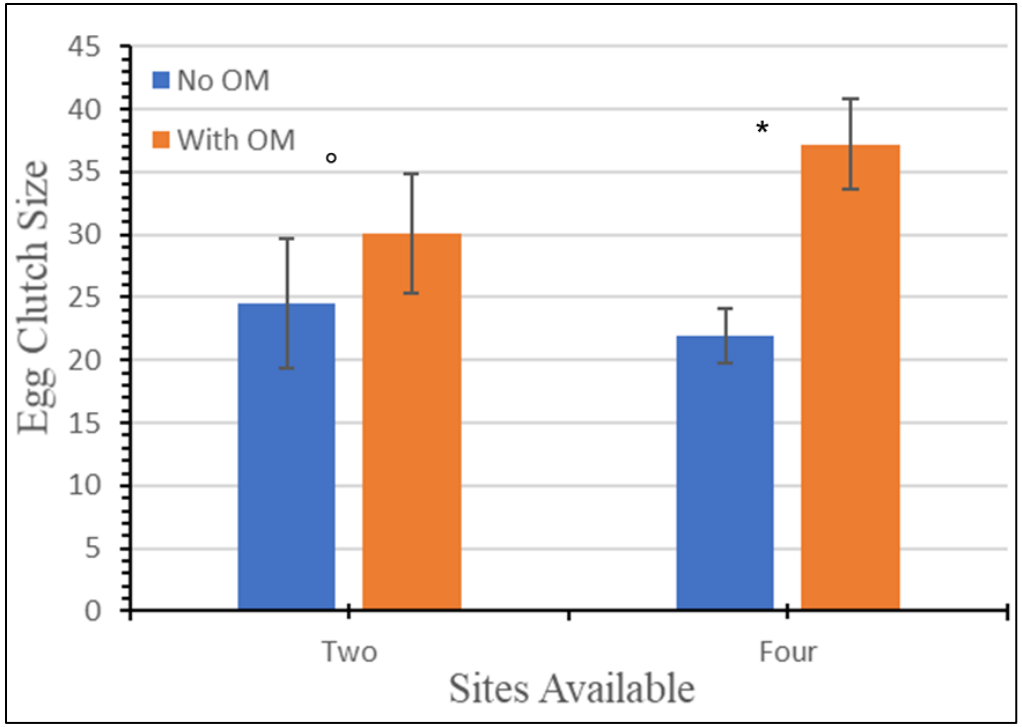

Figure 5. Average Egg Clutch Sizes of Single Gravid *Ph. papatasi* Female with and without Organic Matter presence at the Nalgene Jar Scale. Average egg clutch size laid on filter paper within Nalgene jars either with or without organic matter (aqueous extract of expired organic matter) present: paired t-test between Aim 1 and Aim 2 two-choice and four-choice bioassays ($p = 0.0706$ °, $p = 0.0432$ *, respectively). Error bars represent standard error. (° $0.1 < P < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

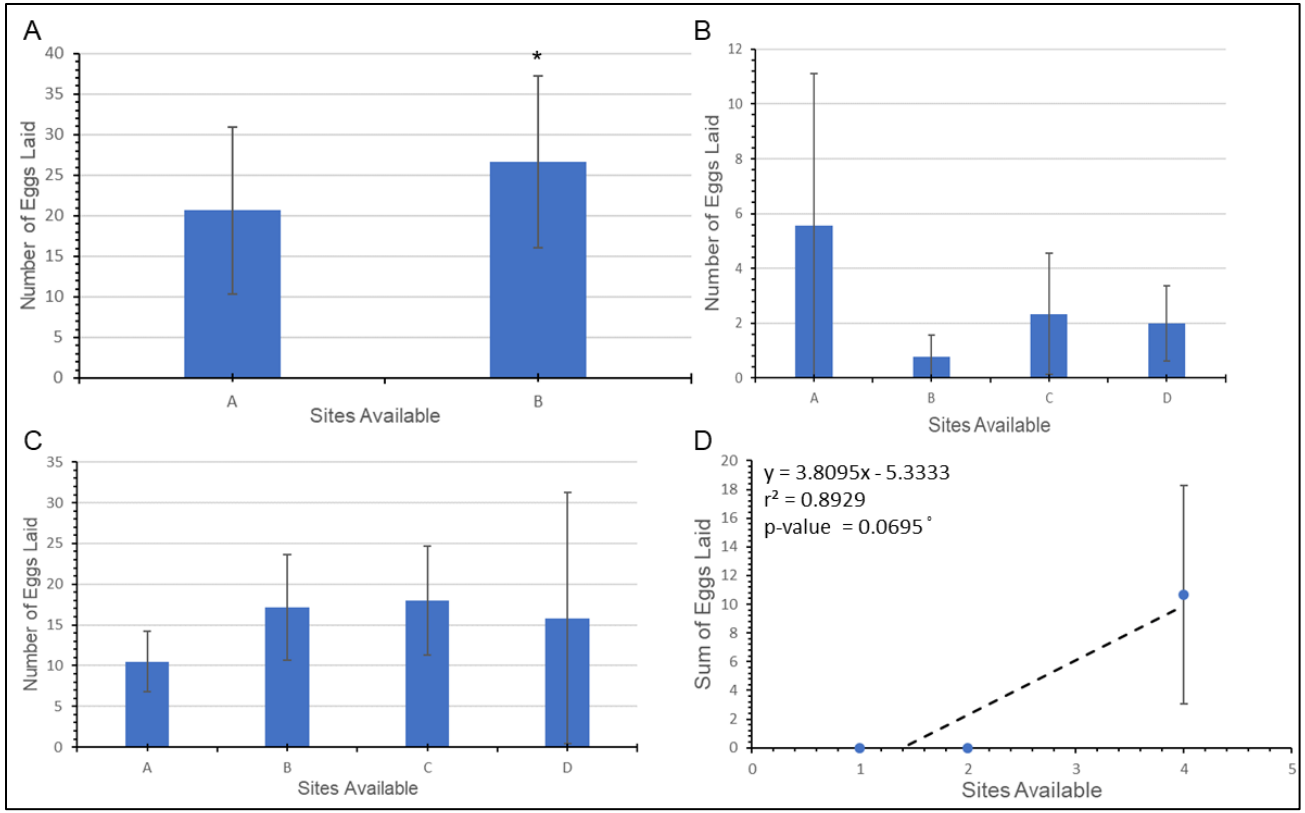

Figure 6. Oviposition Response of Gravid *Ph. papatasi* Females to Different Sites at the Free-Flight Scale. Average number of eggs laid on filter paper of the same quality oviposition sites within free-flight cages. A. Two-choice oviposition bioassay with ten gravid females with cups A and B: paired t-test ($p = 0.02838^{*}$). B. Four-choice oviposition bioassay with a single gravid female: one-way ANOVA ($p = 0.7251$). C. Four-choice oviposition bioassay with ten gravid females: one-way ANOVA ($p = 0.9385$). D. Simple linear regression between the number of oviposition sites available and Sum of eggs laid with a single gravid female ($p = 0.0695^{\circ}$). Error bars represent standard error. ($^{\circ}$ $0.1 < p < 0.05$, * $p < 0.05$)

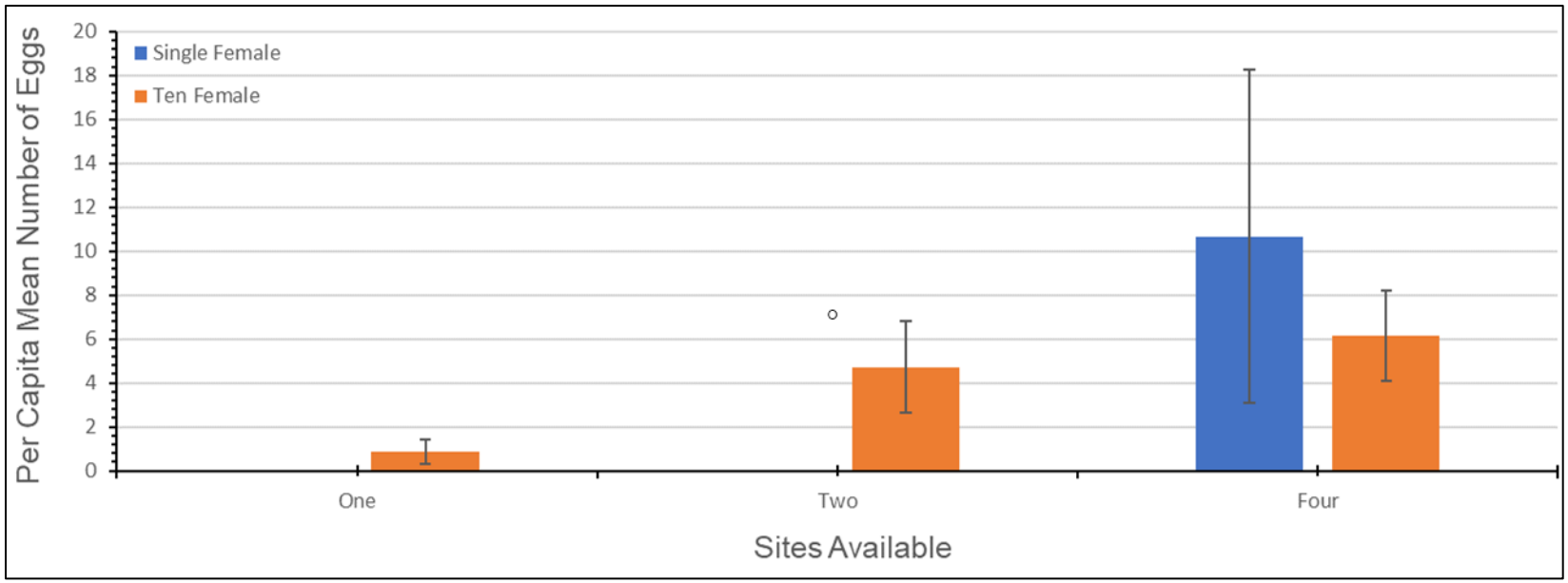

Figure 7. Oviposition Per-capita Response of Gravid *Ph. papatasi* Females to Different Sites at the Free-Flight Scale. Average Per-capita of the mean number of eggs laid on filter paper within the same quality sites in free-flight cages of either single or ten gravid females: paired t-test between one-choice, two-choice, and four-choice bioassays ($p = 0.1928$, $p = 0.0722^{\circ}$, $p = 0.4367$, respectively). Error bars represent standard error. ($^{\circ}$ $0.1 < P < 0.05$)

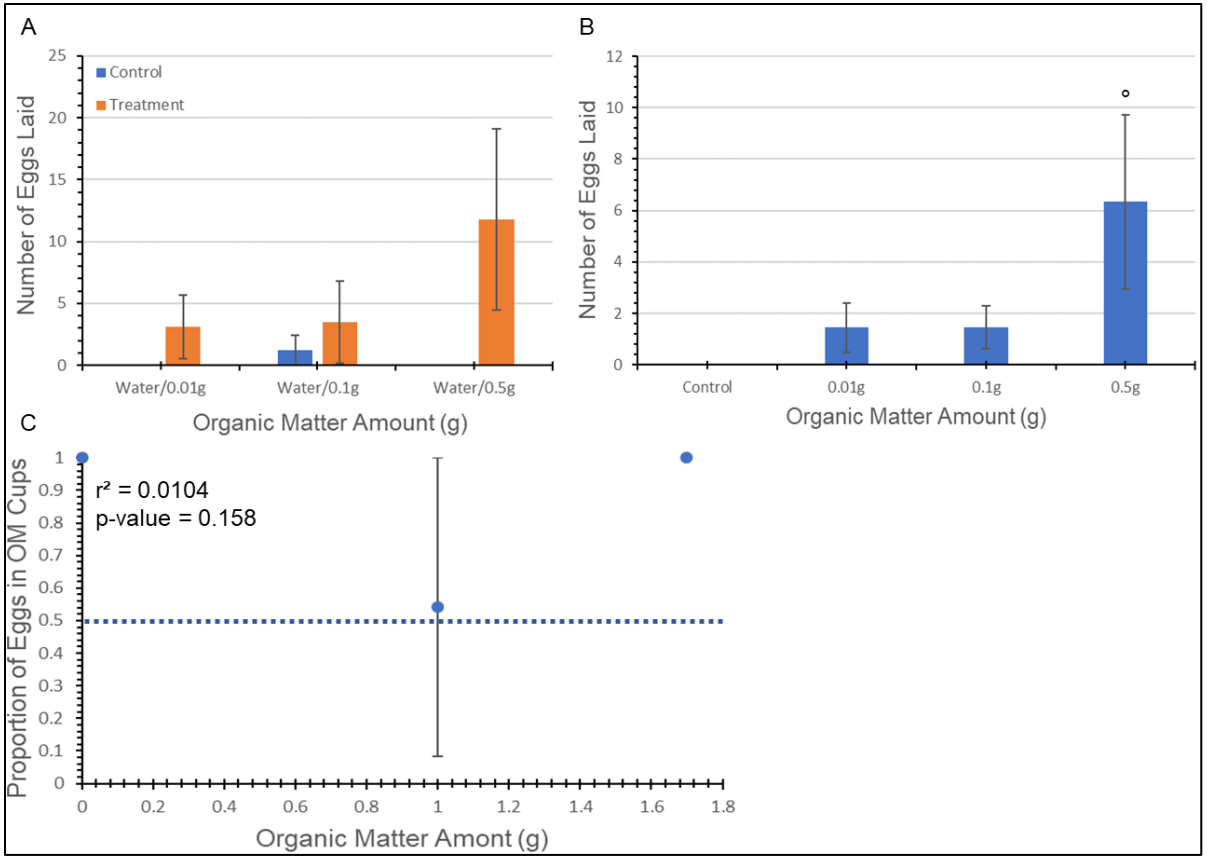

Figure 8. Oviposition Response of Single Gravid *Ph. papatasi* Female to Organic Matter Amounts at the Free-Flight Scale. Average number of eggs laid on filter paper with varying solid FRASS amounts within oviposition sites within free-flight cages. A. Two-choice oviposition bioassay with cups A (0.01 g, 0.1 g, or 0.5 g) versus B (Water): paired t-test (p = 0.2566, p = 0.5588, p = 0.1471, respectively). B. Four-choice oviposition bioassays with cups A (Water), B (0.5 g), C (0.1 g), and D (0.01 g): one-way ANOVA (p = 0.08993°); post hoc (B-A p = 0.0819°). C. Oviposition response to increase organic matter concentrations (log-transformed), as indicated by the preference index (p = 0.158), with dashed line at 0.5 representing neutral choice line: above = preference, below = repellence. Error bars represent standard error. (° 0.1 < p < 0.05)