FAW, LINDSEY RAE, M.S. The Effects of Microbes and Larval Conditioning on *Phlebotomus papatasi* Oviposition Site Selection. (2020)
Directed by Dr. Gideon Wasserberg. 48 pp.

Leishmaniases are a group of neglected, vector-borne diseases vectored by sand fly species. *Phlebotomus papatasi* is incriminated as the vector of *Leishmania major* parasites, the causative agent of cutaneous leishmaniasis (CL), a disfiguring disease that leaves persistent wounds. The lack of a vaccine and the lack of effective population control calls for novel control methods. One method is that of oviposition-site attractants which operate by bringing the vector to the insecticide rather than vice-versa. Previous studies have shown that larval rearing medium conditioned by foraging larvae (conditioned medium) is the most attractive source material to gravid sand flies and it is suspected to be mediated by the microbial composition. Conditioned medium is representative of a suitable place for larval development and gravid females will be attracted to conditioned medium compared to unconditioned medium. We also hypothesize that gravid females will be attracted to medium with bacteria present. With respect to oviposition, there is no evidence for a preference to conditioned medium. With attraction, there is a significant trend for the preference to conditioned medium. Temporal effects are evident for attraction and oviposition and indicate that bacterial communities in the medium change over time.

THE EFFECTS OF MICROBES AND LARVAL CONDITIONING ON *PHLEBOTOMUS PAPATASI* OVIPOSITION SITE SELECTION

by

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A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2020

Approved by

_____
Committee Chair

I dedicate this thesis to my family and friends who have supported me through my entire academic career. Thank you to my parents, Bobby and Sharon Faw; my brother and sister-in-law, Wesley and Selena Faw; my nephew, Colton Faw; and countless friends along the way.

APPROVAL PAGE

This thesis written by LINDSEY RAE FAW 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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ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my incredible advisor, Dr. Gideon Wasserberg, for giving me the opportunity to work in your lab. My research was supported by his NIH RO1 grant AI123327-01. I want to thank my committee, Dr. Kasie Raymann and Loganathan Ponnusamy for their extremely valuable input and time spent on my project. I want to thank Nayma Romo Bechara who was integral in design and preparation of this project. I also want to thank all other graduate and undergraduate lab members who are integral in maintaining our thriving colony and aiding me along the way. Lastly, I want to thank the late Matthew Miller, who contributed heavily to preliminary work on this project.

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CHAPTER I

INTRODUCTION

Background

Leishmaniasis Epidemiology. Leishmaniases are a group of neglected, vector-borne diseases found in warm, tropical, semi-arid, and arid environments around the world that are caused by *Leishmania* parasitic protozoans (Antinori et al., 2012; Maroli et al., 2013; Hotez et al., 2012). Leishmaniasis comprises three common forms: visceral, cutaneous, and mucocutaneous. Visceral leishmaniasis (VL), the deadliest form when left untreated, causes fever, swelling of the spleen and liver, weakness, and extreme weight loss (Marray et al., 2005). Cutaneous Leishmaniasis (CL) causes persistent skin lesions and leads to disfiguring scars (Murray et al., 2005; Maroli et al., 2013). Mucocutaneous leishmaniasis (MCL) causes persistent lesions in the mucosal lining of the mouth, nose, and lymph nodes of the body and can lead to the destruction of the mucosal membranes (Marray et al., 2005). Leishmaniases are endemic in more than 98 countries on 5 continents with a total of 350 million people at risk, and there are 12 million new cases of infection per year, although likely underestimated (Akhoundi et al., 2016; Alvar et al., 2012; Antorini et al., 2012; Hotez et al., 2012). Due to underfunding, underreporting, the absence of proper healthcare, and the lack of a vaccine, leishmaniases are considered neglected tropical diseases (Alvar et al.,

2012; Antinori et al., 2012; Hotez et al., 2012). Additionally, there has been an increase in incidence and geographic distribution that are believed to be associated with anthropogenic influences (Chalgraf et al., 2018; Akhoundi et al., 2016; Bates et al., 2015; Gonzalez et al., 2010; Wasserberg et al., 2003). Due to the rising leishmaniasis infections, it is of great importance to produce alternative methods for control.

Sand Flies as Vectors of Leishmaniasis. Sand flies are the vectors of leishmania parasites and are present in both the old and new world. In the new world, sand flies of the genus *Lutzomyia* are incriminated as vectors of leishmaniases and the genus *Phlebotomine* in the old world (Killick-Kendrick, 1999). Sand flies are often found in or near habitats and dwellings of their reservoir hosts (mammals such as canids, rodents, marsupials, hyraxes, etc.) (Maroli et al. 2013; Claborn, 2010). This provides requirements for a warm and humid microclimate and shelter from the sun during the day (Akhoundi et al., 2016; Claborn, 2010). Female sand flies typically require blood meals for egg production (Maroli et al. 2013; Claborn, 2010). Therefore, disease agent acquisition and transmission are directly associated with blood-feeding (Claborn, 2010). Once sand flies are gravid, there is a two-part process to egg-laying, including finding a suitable oviposition site and egg deposition (oviposition-site attraction and oviposition, respectively). Relatively, little is known about sand fly preferences for attraction and oviposition.

Vector control is a major component of leishmaniasis disease prevention. Current methods are generally based on insecticide spraying; however, insecticides have unforeseen consequences such as insecticide resistance, insecticides work better in urban areas than in rural settings, and insecticides affect both target and nontarget species (Claborn, 2010; Bates et al., 2015). Therefore, there is great interest in alternative sand fly control methods. An innovative approach is using attraction-based control methods, such as oviposition-site attractants, bringing the vector to the insecticide rather than bringing the insecticide to the vector. Oviposition-site attractants offer a targeted approach to population control by using known attractants to lure gravid insects to a simulated suitable oviposition site impregnated with a lethal insecticide (Bentley & Day 1989; Ponnusamy et al., 2008). Oviposition-site attractants target gravid females that have had at least one blood-meal, which are responsible for population amplification and are more likely to be infected with Leishmania parasites compared to younger, nonblood-fed females (Bates et al., 2015; Marayati et al., 2015).

Oviposition Site Selection in Hematophagous Insects. Based on the "Preference-Performance Hypothesis" (PPH), insects without parental care should oviposit in a way that optimizes larval growth and, therefore the fitness of the mother (Jaenike, 1978). Oviposition site selection in mosquitoes and other species lacking parental care is essential for the developmental success of offspring. Oviposition site selection in hematophagous insects is a critical fitness

determining factor (Bentley & Day, 1989). Variables such as predation risk, the abundance of food, and the presence of conspecifics are known to influence the oviposition-site selection in hematophagous insects (Wasserberg et al., 2014; Bentley & Day 1989). Across many taxa of hematophagous insects, the presence of conspecifics enhances the attraction and oviposition behavior (Kowacich et al., 2020; Day 2016; Kumar et al., 2013; Wasserberg and Rowton 2011; Basimike 1997; Dougherty et al., 1993; Bentley and Day 1989). Conspecifics-related cues may indicate suitable oviposition sites based on the success of previous larvae (Wong et al., 2011).

Oviposition Site Selection in Mosquitoes. In mosquitoes, the evaluation of oviposition sites suitability was shown to be based on factors such as color of the substrate, presence of organic matter, color of the oviposition container, the presence of conditioned water (water in which conspecific larvae were reared), and the presence of conspecific eggs (see review in Day, 2016). In mosquitoes, the development of the larvae is altered by interactions within the species and between species, and predator avoidance. (Wong et al., 2011; Yoshioka et al., 2012).

Conspecifics: *Aedes albopictus* mosquitoes oviposit most of their eggs in locations that correlated with high larval fitness (indicated by mortality rate, time-to-pupation, and time-to-emergence). High fitness substrates in this study were characterized by the presence of low-medium density of conspecific larvae (Yoshioka et al., 2012). However, overcrowding of larvae that created

competitive pressure between larvae decreased larval fitness with slowed growth, high mortality, and decreased fecundity (Wong et al., 2016; Yoshioka et al., 2012). This creates a hump-shaped relationship between the number of larvae and the success of the larvae (Wasserberg et al., 2014).

In *Ae. triseratus, Ae. aegypti,* and *Ae. altropalpus* gravid females were more attracted to conspecifics and specifically larval rearing water from conspecific instars (Bentley & Day, 1989; Zahiri & Rau, 1997; Trexler et al., 2003; Wong et al., 2011; Day, 2016). Larval rearing water was still attractive after sterilization, which indicates that the larvae contribute to the attractive qualities of the water by altering the water itself (Zahiri & Rau, 1997). Additionally, in field studies of *Ae. albopictus* mosquitoes, larval rearing water was still favored and is conserved in both the natural world and in the laboratory (Wong et al., 2011).

Organic Matter and Bacteria: Organic matter has shown to be attractive to mosquitoes. In *Aedes aegypti,* more eggs were consistently laid in the presence of organic matter consisting of bamboo leaf infusions and white oak leaf infusions was shown to elicit an oviposition response in comparison to water controls (Ponnusamy et al., 2008). It was also shown that bacteria isolated from the bamboo and white oak leaves were also attractive compared to water controls (Ponnusamy et al., 2008).

Mosquitoes rely on their microbiome for development, survival, and pupation (Coon et al., 2015; Sharma et al., 2013). In a study conducted by Coon et al., (2015), the gut microbiome was shown to be variable between species, but

most, and possibly all, mosquito species were unable to develop without bacteria present. For example, surface-sterilized *Ae. aegypti, An. gambiae,* and *Ae. atropalpus* mosquitoes that were fed sterilized food and placed in sterile water hatched but were unable to live past five days without microbes (Coon et al., 2015). When reinoculated with bacteria, the larvae were rescued and able to develop normally (Coon et al., 2015). In a study by Sharma et al., (2013), sterile females experienced low fecundity, follicular resorption, and reduced blood meal digestion than those who were not sterile (Sharma et al., 2013). It is suggested that the inability of the females to digest the blood meal may explain the reduced egg production and oviposition (Sharma et al., 2013). The presence of the microbiome in mosquitoes also affects the vectorial capacity, (Weiss & Aksoy 2011; Sharma et al., 2013; Mustafa et al., 2016; Kelly et al., 2017; Telleria et al., 2018; Trexler et al., 2003). Therefore, the microbiome could provide a novel biocontrol for vectors. For example, the introduction of *Wolbachia* bacteria in *Ae. aegypti* that inhibits transmission of the dengue virus. (Weiss & Aksoy 2011; Mustafa et al., 2016; Fraser et al., 2017).

The presence of bacteria in larval rearing medium is also known to influence oviposition site selection. In a study with *Ae. aegypti* mosquitoes, bacterial isolates derived from bamboo and white-oak leaves were used in oviposition bioassays. The mosquitoes laid significantly more eggs (greater than 90% of eggs) in the presence of the bacterial isolates than in water controls (Ponnusamy et al., 2008). Chemicals found in the bacterial isolates, specifically

carboxylic acids and methyl esters, were shown to be oviposition kairomones with most of the eggs being laid (greater than 90% of eggs) in the cultured isolates compared to sterile medium (Ponnusamy et al., 2008). In another study with *Ae. albopictus,* gravid females laid significantly more eggs in microbiologically baited substrates in which the bacteria were isolated from an oviposition site, similar to that of Ponnusamy et al., 2008 (Trexler et al., 2003). However, some bacteria were attractive while others were repulsive, indicating that although an effect is present, not all have the same effect (Trexler et al., 2003). These experiments indicate that the presence of bacteria in medium is an important part of oviposition site selection. The attraction to bacteria is suggested to be due to the microbial requirements for larval development (Trexler et al., 2003; Coon et al., 2015).

Oviposition Site Selection in Sand Flies. Some studies with sand flies where shown to be consistent with the PPH (Peterkova-Koci et al., 2012; Marayati et al., 2015; Kowacich et al., 2020). In sand flies, it is known that factors including air and soil temperature, relative humidity, pH, color of substrate, and the presence of organic materials affect sand fly activities (Shymanovich et al., 2019, Chowdhury et al., 2016). It is also known that sand flies prefer darker oviposition sites compared to lighter oviposition sites (Shymanovich et al., 2019).

Conspecifics: There is some research on the effects of conspecifics on oviposition site selection in other sand flies species. In a study with *Lu. longipalpis*, conspecific eggs were shown to be attractive and an oviposition

stimulant (Dougherty and Hamilton, 1997, Dougherty et al., 1993). Additionally, an oviposition stimulant, dodecanoic acid, was isolated from the surface of eggs and was shown to be an effective oviposition stimulant (Dougherty et al., 1993). In *Sergentomyia* sand flies, there were more eggs laid in the presence of eggs and pupae for *Se. shwetzi* and no significant differences in *Se. imgrami* (Basimike, 1997). For *Ph. duboscqi,* sand flies laid more eggs in the presence of crushed eggs (Basimike, 1997). Basimike, 1997 also showed an increase in the number of eggs laid in the presence of pupae (Basimike, 1997).

In *Ph. papatasi*, conspecific materials were shown to be attractive to gravid sand flies (Kowacich et al., 2020). Traps with conspecific eggs were significantly more attractive to and simulative than traps without eggs (Kowacich et al., 2020). Gravid sand flies were attracted to eggs and juveniles (Kowacich et al., 2020). The youngest were the most simulative while the oldest juveniles were repellent (Kowacich et al., 2020). It was thought that this may suggest that the presence of young larvae indicates unexploited resources, whereas older larvae indicate exploited resources (Kowacich et al., 2020). There is also a hump-shaped dose-dependent response to conspecific eggs with a positive linear relationship for oviposition. There is a preference for larger egg masses for oviposition and preference for smaller egg masses for attraction (Kowacich et al., 2020). Kowacich et al., 2020, also confirmed the use of dodecanoic acid as a oviposition stimulant (Kowacich et al., 2020).

Organic Matter and Bacteria: Sand flies are coprophagic and therefore females are expected be attracted to site containing food for their larvae, i.e. organic matter. In sand flies, like in mosquitoes, organic matter (rabbit feces, rabbit chow, and chicken feces) elicited a positive oviposition response (Doughtery et al., 1993). For example, in *Lutzomyia longipalpis* sand flies, Dougherty showed that *Lutzomyia longipalpis* were stimulated by kairomones present in organic matter (consisting of rabbit feces, rabbit chow, and chicken feces) (Dougherty et al., 1993). This was confirmed again in *Lutzomyia longipalpis* and also shown in *Phlebotomus papatasi* (Wasserberg & Rowton, 2011).

It is expected that sand flies also rely on their microbiome for development, survival, and pupation, although there is little known about this process in sand flies. Sand fly larvae acquire their gut microbiome from their environment, i.e., their larval food and breeding areas (Mukhopadhyay et al., 2012; Telleria et al., 2018). Adults can also acquire some bacteria in infected natural sugar food sources and blood meal sources (Mukhopadhyay et al., 2012). It is known that the microbiome of the environment plays a role in influencing the oviposition response of sand flies from the new world (Peterkova-Koci et al., 2012). Specifically, *Lutzomyia longipalpis* sand flies show a clear preference to non-sterilized rabbit feces compared to sterilized rabbit feces (Peterkova-Koci et al., 2012). Additionally, *Lu. longipalpis* larvae reared in sterile medium had significantly higher mortality and longer developmental time (Peterkova-Koci et

al., 2012). It was also shown that the attraction to conspecific conditioned medium (medium in which conspecific larvae were reared in) is attractive in *Ph. papatasi* sand flies (Marayati et al., 2015). As larvae forage and defecate into the medium, it is hypothesized that the gut microbiome alters the composition of the medium (Marayati et al., 2015). Based on this hypothesis, twelve bacterial isolates were isolated conditioned medium and some were shown to be attractive while others were repellent (Kakumanu et al., 2020).

Effect of Microbiome on Vector Competence. In sand flies, the presence of the microbiome is important not only to the development of larvae but also to vector competence (Monteiro et al., 2017; Kelly et al., 2017; reviewed in Telleria et al., 2018). The *Leishmania* parasite promastigote form resides within the gut of the sand fly and must be able to proliferate in the gut in the presence of the microbiome of the sand fly (Kelly et al., 2017). From this, it was shown that the microbiome of the sand fly is a critical factor for the growth of *leishmania* parasites and the ability to differentiate into an infective state (Kelly et al., 2017). When the gut microbiome was removed with the use of antibiotics, it inhibited the ability of the *Leishmania* parasites to develop into infectious states (Kelly et al., 2017). Therefore, alterations to the gut microbiome of sand flies could provide a potentially novel control mechanism. Since sand flies can also gain their microbiota through transstadial passage from mothers to offspring, it is possible to introduce bacteria, like that in mosquitoes, that can inhibit vector competence of the sand fly to control disease (reviewed in Telleria et al., 2018).

Previous Studies. In a study by Marayati et al., 2015, a bioassay was conducted to determine the attraction of *Ph. papatasi* to larval rearing media from different larval developmental stages ($2^{nd}/3^{rd}$ instar and 4^{th} instar) compared to fresh larval food, expired medium (rearing medium in which all larvae have eclosed from),, and fresh rabbit feces. The conditioned media were those in which larvae were reared. It was shown that *Ph. papatasi* was more attracted to the larval conditioned media than to that of unconditioned media (Marayati et al., 2015). Specifically, the gravid females were more attracted to larval rearing medium that contained larvae reared up until the $2^{nd}/3^{rd}$ instar compared to media of other stages or expired medium (medium in which all flies have already eclosed) (Marayati et al., 2015). It was hypothesized that this relationship is due to the high bioactivity of $2^{nd}/3^{rd}$ instar larvae and their contributions to the activation of the microbiome of the substrate. However, it was unclear whether this attraction to $2^{nd}/3^{rd}$ larval rearing substrate is indeed due to its conditioning by foraging and defecating larvae or, alternatively, simply due to aging of the rearing medium.

In a preliminary study to differentiate between these two competing hypotheses, the late Matthew Miller (a former EHS student in the Wasserberg lab) conditioned one fresh larval food jar with 9000 1^{st} instar larvae and another larval food jar was left to age, with no larvae, for the same amount of time. Attraction of gravid sand flies to the larval conditioned versus the unconditioned larval food was then evaluated weekly over 5 weeks. From this, it was shown that

conditioned larval medium was more attractive than aged food and was remained attractive until all pupae had eclosed. Once all the larvae had eclosed, the attraction to the conditioned medium disappeared and even became slightly (although non-significantly) repellent (M. Miller, unpublished data).

A metagenomic analysis of the conditioned medium reared to the 2nd/3rd instar and unconditioned media showed there was little difference in the microbiome of the conditioned medium compared with the unconditioned medium. The main difference appeared to be associated with the time-effect with microbiome of both conditioned and aged substrate substantially different in comparison with that of the baseline fresh unconditioned media (M. Miller, unpublished data). Considering there was only one replicate of the conditioned or aged media, it would be beneficial to repeat the experiment to see if the pattern is maintained. Furthermore, the oviposition response was not assessed in that study and I believe this should be integral to understanding oviposition site selection as well. However, while it is still unknown what is driving this attraction in sand flies, it is hypothesized that the microbiome plays an important role.

Study Question

In this study, I evaluated if larval conditioning of rearing substrate (by larval foraging and defecating) enhances gravid sand fly's preference for the conditioned medium. Additionally, I evaluated the role of microbes in affecting the oviposition site selection of gravid sand flies.

General Hypotheses

Microbes are assumed to be essential for the development of sand fly larvae and therefore I hypothesize that: (1) gravid females will select oviposition sites containing microbes, i.e. conditioned medium. (2) larval gut microbiome enriches rearing substrate in a manner that drives gravid female attraction to larval-conditioned medium.

Specific Aims

Aim 1: The Effect of Larval Food Conditioning on Sand Fly Oviposition Site Preference. I evaluated the effects of larval conditioning on sand fly attraction and oviposition response, two distinct components of oviposition behavior, using two media: one inoculated with sand fly eggs and reared for 3 weeks (at which larvae reach their 2^{nd}-3^{rd} instar stage) and one with unconditioned rearing medium aged for the same amount of time. The conditioned and unconditioned medium was used in a paired-choice bioassay for attraction and oviposition. For attraction, the response variable was the number of flies caught on a constructed sticky trap with conditioned medium compared to a sticky trap with unconditioned medium. For oviposition, the response variable was the number of eggs laid in an oviposition pot with conditioned medium compared to an oviposition pot with unconditioned medium (Figure 1).

Prediction 1 - Attraction: Gravid sand flies will be attracted to larval food conditioned by foraging larvae compared to the unconditioned but aged rearing medium.

Prediction 2 - Oviposition: Gravid sand flies will oviposition more eggs on larval rearing medium conditioned by foraging larvae compared to compared to the unconditioned but aged rearing medium.

Aim 2: The Effect of Microbes on Sand Fly Oviposition Site Preference. I evaluated the effects of microbial presence in larval rearing medium on oviposition site selection using a bacterial addition experiment in which I tested the effect of microbe filtrate inoculation on oviposition site preference of gravid sand flies. Autoclaved fresh larval food was used as a base for inoculation of $2^{nd}/3^{rd}$ instar conditioned medium bacterial filtrates with buffer and another of just buffer (Figure 2A).

Prediction 1 - Attraction: Gravid sand flies will be more attracted to sterile medium inoculated with $2^{nd}/3^{rd}$ instar microbial filtrate than medium inoculated with buffer alone.

CHAPTER II

METHODS

General Methods

Colony and Mass Rearing. *Phlebotomus papatasi* sand flies originating from Abkuk, Turkey were maintained following methods described by Lawyer et al. (2017). The flies were blood-fed at SoBran Biosciences in Greensboro, NC on live anesthetized ICR mice (SoBran Inc. IACUC protocol number: UNC-002-2016). Sand flies were maintained in incubators at 26°C and 85% relative humidity and reared under a 12:12 hrs. light: dark reverse photoperiod. Larvae were fed fresh larval food comprised of rabbit feces and rabbit chow while adults were given sugar water (as outlined in Lawyer et al., 2017).

Attraction Bioassays. Larval rearing medium (conditioned as treatment and unconditioned as control) was placed into a constructed sticky trap consisting of a micro-beaker with 10mL of sand, 0.10g (±0.03) of medium (an optimal amount as determined by Marayati et al., 2015), and 2mL of deionized water. Each micro-beaker was placed into a 125mL Nalgene jar with a sticky mesh affixed on top, (Figure 3A) and placed into a 12in x 12in x 12in free flight cage (Figure 3B) along with 20 gravid sand flies. The free-flight cages were placed in an environmental control room at a constant temperature of 26°C and relative humidity of 65%. The environmental control room is maintained at a

14:10 light: dark photoperiod with crepuscular periods between 5:00 – 6:00 and between 18:00 - 19:00. Flies were introduced into free-flight cages 24 hours in advance of experiments. The flies were able to select a site between the hours of 12:00 and 18:00, the optimum time for attraction (Shymanovich et al., 2019). The number of flies caught on the sticky traps was counted as a measure of attraction response.

Oviposition Bioassays. The oviposition bioassays consisted of a paired design of one treatment (conditioned medium) and one control (unconditioned medium) micro-beaker in a single 500mL Nalgene jar with 20 gravid sand flies (Figure 3C). Micro-beakers consisted of 10mL of sand, 0.01g of medium 2mL of deionized water, and a filter paper on top (Figure 3D). The Nalgene jars were placed in an incubator at a constant temperature of approximately 26°C and relative humidity of 85%. Flies were sugar-fed daily. The jars were removed after 3 days, and the number of eggs laid on the filter paper were counted as a measure of oviposition response.

Bioassay Development

To begin bioassay development, I started with 4oz mason jars (Figure 4A). The mason jars were chosen due to the chemically inert properties of glass. Since we know that volatiles are important for oviposition, eliminating that factor was important for controlling for confounding variables.

Larval Conditioning.

i. Preliminary Assessment of Mason Jars: Using 4oz mason jars (Kerr), 1200 larvae were reared with 4g of conditioned or unconditioned larval rearing medium. (Figure 4A). A 4mm hole was created in the center of the aluminum lid using a nail to puncture the aluminum and covered it with gene tape. This hole was intended to be used for the head space collection of volatiles using Solid Phase Microextraction (SPME) (to be done by Eduardo Hatano from NCSU). Unfortunately, larvae did not live to the 2nd/3rd larval instar which is believed to be related to the lack of proper airflow. Therefore, no attraction or oviposition experiments were conducted.

ii. Mason Jars with Mesh Coverings: Using the same 4oz mason jars, 1200 larvae and 4g of larval rearing medium were introduced for rearing. However, instead of the aluminum lids, mesh was used to maintain improved air flow. Unfortunately, again, larvae did not survive to the 2nd/3rd larval instar. All larvae have decomposed by the due time suggesting that, again, air flow was insufficient and that larvae have suffocated. This time, the food being added at once was considered to be the culprit of suffocation. Therefore, no attraction or oviposition experiments were conducted.

iii. Mason Jars with Mesh Top and Gradual Feeding: Using the same methods as above, 1200 larvae were reared with larval food added gradually. Once hatched, the first-week larvae were fed fresh larval food five days a week by adding small amounts each time (approximately 1 gram) and for second week

larvae, a larger amount (approximately 2 grams) was added twice a week. In week 3, larvae have reached the 2nd/3rd larval instar stage, at which point the larvae were removed from the medium using a soft paintbrush and the medium was used for attraction bioassays.

iv. Mason Jars with Eggs: Given the difficulty in counting larvae, I preferred to try applying the same conditioning effect by starting the experiment at the egg stage. This is easier because the egg number could be estimated by their weight and eliminated the need for lengthy separation of larvae from medium once the larvae had hatched. Using the same methods as above, 1200 eggs were introduced and once hatched larvae, were fed gradually as described above. After reaching the 2nd/3rd larval instar stage, the medium was separated from the larvae and was used for attraction bioassays.

v. Nalgene Jar Assessment: Due to unsatisfactory results that were unable to emulate previous results using the mason jars, we reverted to using 500mL plastic Nalgene jars as Matthew Miller did previously. To obtain larval conditioned medium, 9000 *Ph. papatasi* eggs were reared in 500mL Nalgene jars with a plaster of Paris bottom to maintain moisture within the jars (Figure 4B). Once hatched, the first-week larvae were fed fresh larval food five days a week and twice a week for second-week larvae. Unconditioned medium jars (control jars) were reared under the same conditions but in the absence of larvae. After two weeks, when the larvae reached the 2nd/3rd instar development stage, the larvae were removed, and the conditioned medium was used for oviposition and

attraction bioassays. These methods were determined to be the best method of larval conditioning.

Bacterial Inoculation. Substrate sourced from conditioned medium was suspended in phosphate-buffered saline (PBS) in an Eppendorf tube. The mixture was then homogenized using a pestle and vortexed to mix. The tube was then centrifuged at 5000rpm for 3 minutes. The supernatant was then removed and 0.5mL was added to 0.1g of sterilized medium. The inoculated medium was vortexed to mix. The inoculated medium was then added to a sticky trap. Another sticky trap was created using sterile medium (autclaved in an Eppendorf tube for one hour) with buffer alone as a control (Figure 2A).

Statistical Analyses

Descriptive statistics were conducted in a Microsoft Excel worksheet. Because a paired cup design was used, treatment and control responses were analyzed using paired t-tests. ANOVA analyses were completed using R statistical software.

CHAPTER III

RESULTS

Aim 1. The Effect of Larval Conditioning

Medium Conditioning Using Mason Jars.

Attraction Bioassays: Larvae in the experimental pots survived through their three weeks of rearing, resulting in stage distribution being approximately half second and half third instar. No significant difference was found in the number of flies trapped in sticky traps baited with larval conditioned medium (2.17±0.88) versus sticky traps baited with non-larval conditioned medium (3.33±1.36) (Figure 5). The majority of the flies, 60.6%, were attracted to the unconditioned medium compared conditioned medium percentage of 39.4%.

Oviposition Bioassays: There was no statistically significant difference between the number of eggs laid on filter papers baited with conditioned (32.45±10.26) versus unconditioned (36.87±11.66) media (Paired t = 0.05, n = 10, *P* = 0.4946). The highest quantity of eggs, 46.1%, were laid on unconditioned medium followed by 40.5% on the conditioned medium, and 13.4% in water. However, there was a marginally significant difference in the number of eggs laid on medium compared with water; conditioned medium (t = 6.36, n = 10, *P* = 0.077), unconditioned medium (t = 6.81, n = 10, *P* = 0.015) (Figure 6).

Medium Conditioning Using Nalgene Jars.

Attraction Bioassays: The multiple regression model (Table 1) shows that there was a significant difference in the number of flies caught over time. After three weeks, the number of flies caught increased compared to baseline numbers. There was also a significant difference in the number of flies caught in conditioned medium compared to unconditioned medium. There was no evidence of an interaction effect of time and conditioning (t = -1.07, *P* = 0.29). Data for each trial individually is shown in Table 2.

Baseline and Temporal Effects. Before conditioning, there was no significant difference in the attraction response between the to-be-conditioned medium (0.9 ± 0.21) and to-be-unconditioned (0.6 ± 0.14) media (Paired t = 1.09, n = 20, *P* = 0.2088) (Figure 7). After aging, both conditioned medium (t = 6.30, n = 32, $P = 9.943*10^{-8}$) and unconditioned medium (t = 2.94, n = 32, $P = 2.484*10^{-5}$) showed a significant increase in the number of flies caught compared to baseline numbers. There is significant evidence that the number of flies caught increases with time (Table 1). After 15 days, it is estimated that the mean number of flies caught increases by an average of 2.34 ± 0.36 flies (Table 1). The effect size for conditioned medium was 303.33% increase with aging and a 326.67% increase for unconditioned (Table 3).

Treatment Effects. A significant difference (Paired t = 2.02, df = 32, *P* = 0.0432) was found in number of flies trapped in sticky traps baited with larval conditioned medium (3.63 ± 0.64) versus sticky traps baited with non-larval

conditioned medium (2.56 ± 0.45) (Table 3) (Figure 7). The majority of flies, 58.6%, were attracted to conditioned medium. Conditioning increases the number of flies by an average of 0.77 ± 0.35 (Table 1).

Oviposition Bioassays: The multiple regression model (Table 4) shows that there was a significant difference in the number of eggs laid over time. After three weeks, the number of eggs laid increased compared to baseline numbers. There was no significant effect of medium conditioning on the number of eggs laid (Table 4). There was no evidence of an interaction effect of time and conditioning (t = 0.38, *P* = 0.71). Data for each trial individually is shown in Table 5.

Baseline and Temporal Effects. There was also no significant difference in the oviposition response for to-be conditioned (82.6 ± 19.47 eggs) and to-be unconditioned (115.7 ± 27.27 eggs) media (Paired t = 1.22, n = 20, *P* = 0.2623) (Table 4) (Figure 8). After aging, conditioned medium showed a significant increase in the number of eggs laid (t = 2.30, n = 32, *P* = 0.026) while unconditioned showed a non-significant trend of increase (t = 1.65, n=32, *P* = 0.190). There is significant evidence that the number of eggs increases with time. After 15 days, it is estimated that the mean number of eggs laid increases by an average of 49.80 ± 21.75 flies (Table 4). The effect size for conditioned medium was 76.76% increase with aging and a 41.50% increase for unconditioned (Table 6).

Treatment Effects. There was no statistically significant difference between the number of eggs laid on filter papers baited with conditioned (146 ± 25.81) versus unconditioned (163.7 ± 28.94) media (Paired t = 0.62, n = 64, *P* = 0.5873) (Table 5) (Figure 8).

Aim 2. The Effect of Bacterial Inoculation

Due to the COVID-19 pandemic, I was unable to conduct the bacterial inoculation experiment to completion. However, I was able to generate methods and design a future experiment. Firstly, I was able to create a methodology to complete the experiment. Secondly, I was able to confirm that autoclaving was an effective method of sterilization. Thirdly, I was able to confirm that inoculation of medium was effective, and the bacteria were able to grow on sterile medium.

Confirmation of Sterilization.

Based on an agar streak plate of autoclaved media, it was confirmed that autoclaved media had far less bacterial colonies than that of fresh larval food (Figure 9A&B). This data was used to confirm the sterilization by autoclave method that will be used in a future bacterial inoculation experiment.

Inoculation of Sterile Medium.

A sample of autoclaved media was reinoculated with a bacterial filtrate from 2nd/3rd larval instar media to ensure that the autoclaved media is still viable for bacterial growth. Based on the agar plate and the growth shown, after 24 hours the bacteria were able to grow on the autoclaved medium (Figure 9C).

These methods will be used in a future bacterial inoculation experiment to determine the role of bacteria in attraction and oviposition behavior.

CHAPTER IV

DISCUSSION

My study aimed at determining if foraging larvae alter the larval rearing substrate in a manner that makes it more alluring to oviposition site seeking females. Based on the “Preference-Performance Hypothesis” (PPH), gravid sand flies should select the oviposition site that enhance the growth and survival of their progeny thereby enhancing their own the fitness (Jaenike, 1978). It was suggested that rearing substrate that had been conditioned by foraging larvae would indicate a suitable place for larvae to grow and provide reassurance to the mothers that their larvae will be successful. Previous research by Marayati et al., 2015, showed that the conditioned medium is indeed attractive and oviposition stimulating for gravid sand fly females (Marayati et al., 2015). My study aimed at ascertaining whether the conditioning was the attractive element or if aging alone was also attractive.

It is suspected that microbiome is a driving force of the attraction to conditioned medium. Conditioned medium could provide a good source for essential gut microbiota in the larvae. It is hypothesized that the larval gut microbiome is essential for the development of larvae (Peterkova-Koci et al., 2012). In *Lutzomyia* sand flies, the microbiome and larval development has been shown to the linked (Peterkova-Koci et al., 2012). It was shown that most

bacteria increased the rate of development compared to sterile medium (Peterkova-Koci et al., 2012). However, this has yet to be explored in old-world *Phlebotomine* species. In addition to reconfirming the attraction to conditioned medium, my study aimed to also test oviposition responses to conditioned medium and the growth and development of larvae exposed to medium in the presence of bacteria. Unfortunately, the latter was not accomplished due to the covid-19 issue.

Effect of Larval Conditioning

Originally, I started the experiment using glass mason jars due to their chemical inertness. However, due to little success, we reverted to using plastic Nalgene jars. At first, it is likely that the aluminum lids and the addition of three grams of food at once suffocated the larvae that were unable to survive. For the following trials, the inability to sustain larvae to the 2nd/3rd instar is likely related to the number of larvae. In the mason jars, 1200 larvae were used whereas in the Nalgene jars 9000 larvae were used. There is also the likelihood of plaster inconsistencies based on the structure of the bottom of the mason jar compared to that of the Nalgene jars. Since we use Nalgene jars in colony maintenance and know of their effectiveness, we decided that the use of the Nalgene jars would be best.

I predicted that conditioned medium would elicit a stronger attraction and oviposition response than unconditioned, but aged medium. Based on my

results, I confirmed this relationship for attraction but not for oviposition. A previous study of the circadian rhythms showed that attraction and oviposition operated as separate events at different times during the day (Shymanovich et al., 2019). Therefore, it stands to reason that different processes are responsible for the behaviors and would create a difference in response. However, it is unclear at this point as to what those processes are, and more experiments are needed to decipher these responses.

After three weeks of rearing the media (either unconditioned or conditioned), the number flies caught increased significantly from baseline. For oviposition, the number of eggs laid also increased. This suggests that, irrespective of larval conditioning,medium aging has a meaningful impact on attraction and oviposition. Additionally, the number of eggs laid in conditioned and unconditioned medium was marginally significant from the eggs laid in water alone, which reconfirms that organic matter is always attractive.

Effect of Bacterial Inoculation

The attraction to conditioned medium is believed to be related to the microbial community present. Peterkova-Koci et al., 2012 demonstrated that sterile medium was less attractive than non-sterile medium (Peterkovi-Koci et al., 2012). It was also shown that larvae raised in sterile medium, developed slower and experienced a higher mortality rate than those fed non-sterile medium (Peterkova-Koci et al., 2012). Non-axenic medium (medium with bacteria)

enhances larval growth and survival and therefore makes ecological sense (based on the PPH) that it would be attractive.

However, it is important to characterize the bacteria present in the conditioned and aged media. Characterization will allow for a more in-depth analysis of how each bacterium alters the oviposition site selection and how they affect larval development. Peterkova-Koci et al., 2012 were able to characterize the bacteria present in rabbit feces and its effects on *Lutzomyia longipalpis.* From the bacterial characterization, it was shown that different bacteria affected the development of larvae differently (Peterkova-Koci et al., 2012). Most species of bacteria allowed for shorter development time than sterile medium. In a previous study from our lab, the culturable bacteria present in larval conditioned rearing medium was characterized. Of the twelve bacteria isolates that were found in the medium, some were shown to be very attractive to gravid females while others were shown to be repellent (Kakumanu et al., 2020)). This data confirms the influence of microbial in oviposition-site selection. It was however, not determined if any of these were also oviposition stimulants. My study aimed to remedy this issue by monitoring larval development and survival when introduced to media with microbes. Unfortunately, due to research restriction as the result of a global pandemic, I was unable to confirm the role of the microbes in oviposition site selection. Nonetheless, in this study, I developed a methodology that will aid in this process for future studies.

Matthew Miller's previous metagenomic work showed that there was little difference in the bacterial community of conditioned and unconditioned medium. Because there was little difference, it seems that the changes in the microbial community are temporal effects instead of conditioning effects. This study shows that the temporal changes in the microbial community are attractive when aged to 2-3 weekes irrespective of whether it was conditioned by larvae or not. The next step in this process would be to determine what changes occur in the microbial community over the three-week rearing period and how that effects larval development. Since we know that some of the bacterial present in the medium are attractive, a bacteria could be isolated and used as an oviposition-site attractant for sand fly control or used to reduce the vectorial capacity, or the daily rate at which new infections can arise from a current infection, of sand flies to limit disease spread (Dye 1986).

CHAPTER V

CONCLUSION

For insects without maternal care, oviposition-site selection comprising of attraction and oviposition is the most important decision that gravid females can make for optimizing fitness. Based on this study, there is a very important temporal effect on larval rearing medium bacterial community succession on oviposition-site selection. Results of my study suggest that over time, the bacterial community changes in a way that becomes more attractive to gravid sand flies. Although conditioning may be an important aspec, my results suggests that aging is a more important factor in changing the bacterial community structure. These changes in the bacterial community could prove useful for the characterization of an oviposition-site attractant for population control.

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APPENDIX A

TABLES

Table 1. Linear Regression Model for Attraction. Linear regression model for the effect of larval conditioning and medium aging time on number of flies trapped to a sticky trap.

	Coefficient	Std. Error	T-value	P-value
Intercept:	0.2654	0.3278	1.115	0.2677
Time:	2.3438	0.3551	6.600	1.93*10^-9 ***
Conditioning:	0.7692	0.3455	2.226	0.0282 *

Table 2. Effect of Larval Conditioning on Attraction. Effect of conditioning on the number of flies trapped on a sticky trap for trial 1, trial 2, and combined.

	Conditioned Mean	Unconditioned Mean	P-value
Trial 1:	2.67 ± 0.77	2.167 ± 0.63	0.53
Trial 2:	4.20 ± 0.94	2.80 ± 0.63	0.048 *
Combined:	3.63 ± 0.64	2.56 ± 0.45	0.043

Table 3. Effect of Aging Time on Attraction.

	Baseline	After 3 weeks	P-value	Effect Size
Conditioned:	0.90 ± 0.21	3.63 ± 0.64	$9.95*10^{-8}$ ***	303.33%
Unconditioned:	0.60 ± 0.14	2.56 ± 0.45	$2.48*10^{-5}$ ***	326.67%

Table 4. Linear Regression Model for Oviposition. Linear regression model for the effect of larval conditioning and medium aging time on number of eggs laid on medium.

	Coefficient	Std. Error	T-value	P-value
Intercept:	116.89	20.30	5.759	9.95*10-8 ***
Time:	49.80	21.75	2.289	0.0242 *
Conditioning:	-23.66	20.88	-1.133	0.2601

Table 5. Effect of Larval Conditioning on Oviposition. Effect of conditioning on the number of eggs laid on medium or trial 1, trial 2, and combined.

	Conditioned Mean	Unconditioned Mean	P-value
Trial 1:	160.25± 35.83	159.90 ± 35.75	0.99
Trial 2:	122.25 ± 35.29	170.08 ± 49.10	0.31
Combined:	146.00 ± 25.81	163.72 ± 28.94	0.59

Table 6. Effect of Aging Time on Oviposition.

	Baseline	After 3 weeks	P-value	Effect Size
Conditioned:	82.60 ± 19.47	146.00 ± 25.81	0.026 **	76.76%
Unconditioned:	115.70 ± 27.27	163.72 ± 28.94	0.19	41.50%

APPENDIX B

FIGURES

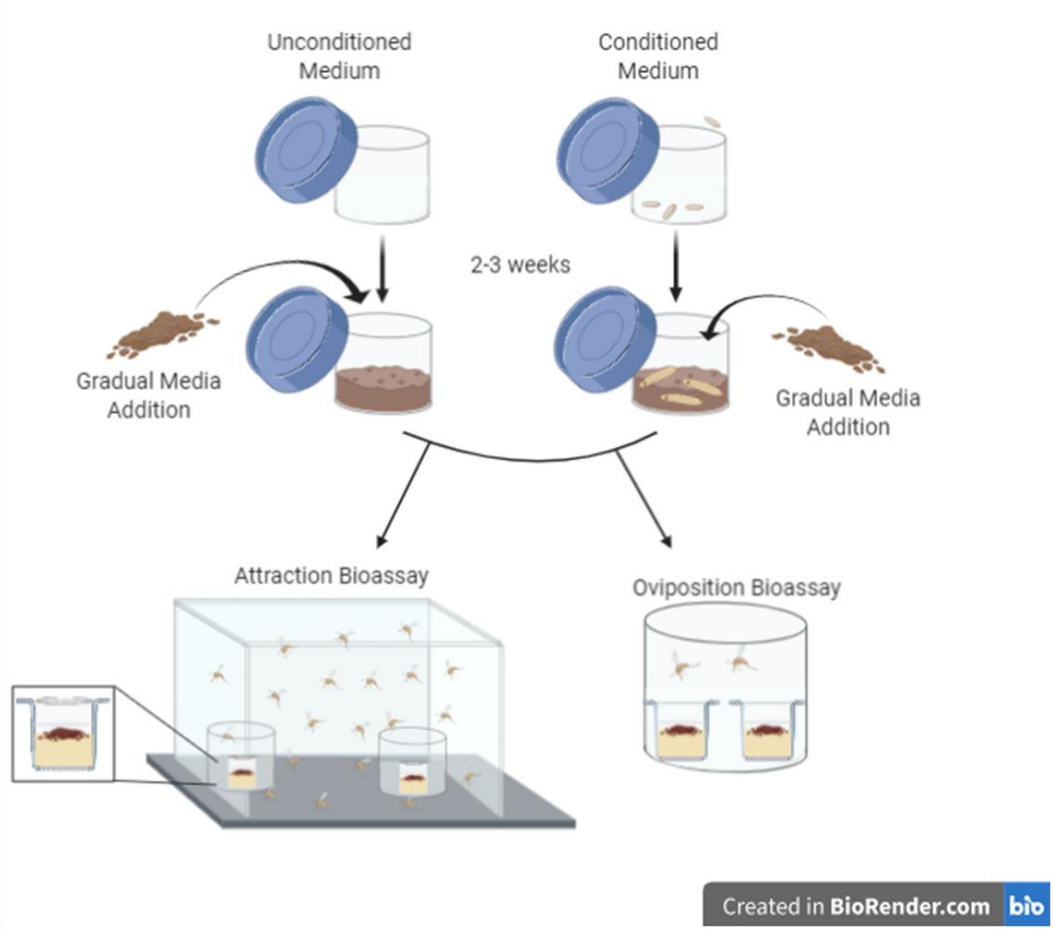

Figure 1. Experimental Approach for Larval Conditioning. Eggs are introduced to a rearing container to be reared for 3 weeks. Another rearing container is reared for 3 weeks but in the absent of larvae. Medium is gradually added to both containers for 3 weeks. After rearing, the larvae are removed from the medium and the medium is used in attraction and oviposition bioassay.

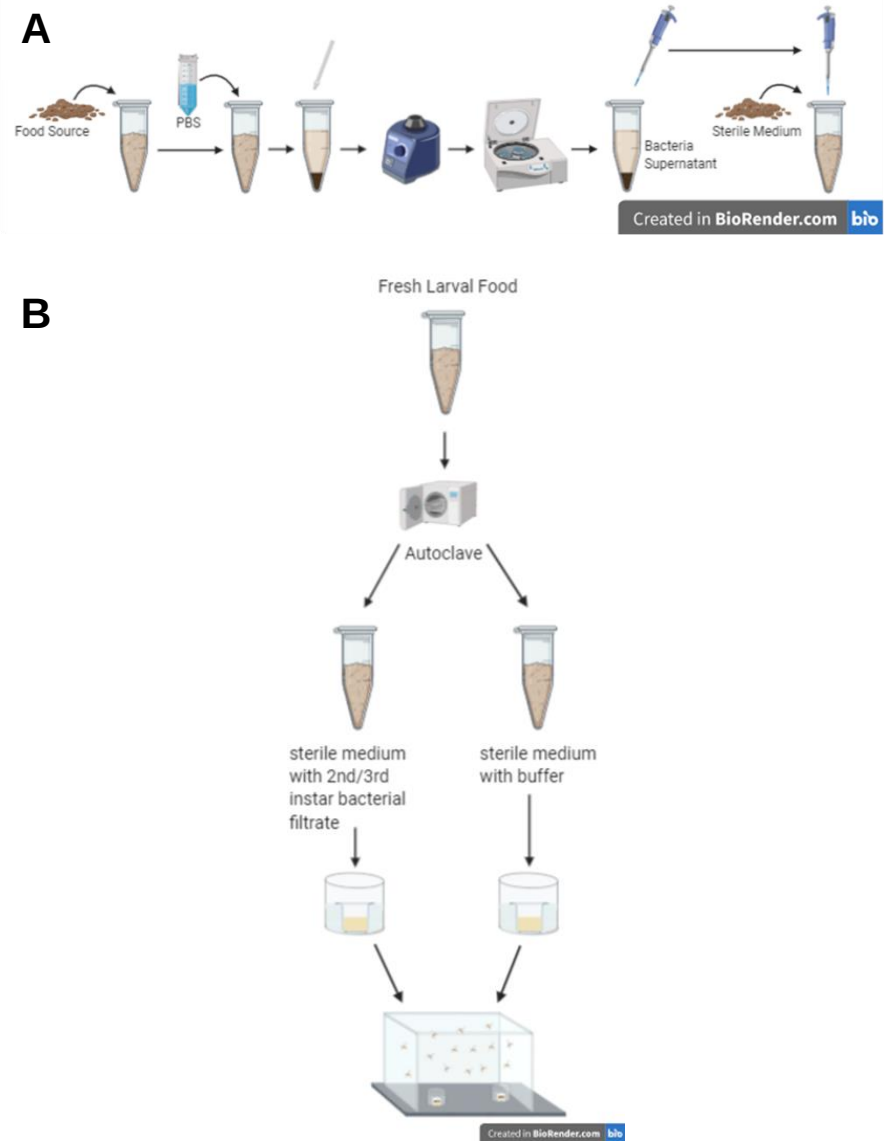

Figure 2. Experimental Approach for Substrate Inoculation. A) Bacterial inoculation of medium methods. A food source (2^{nd}/3^{rd} instar medium) is added to an Eppendorf tube, mixed with PBS and homogenized using a pestle. The tube is then vortex and then centrifuged. After centrifugation, the bacterial supernatant is added to sterile medium and is then ready to be used in bioassays. B) Experimental approach for substrate inoculation. Fresh larval food is autoclaved in Eppendorf tubes. A bacterial filtrate is added to the treatment tubes. Buffer (same as that used in the bacterial inoculated medium) is added to the control tubes.

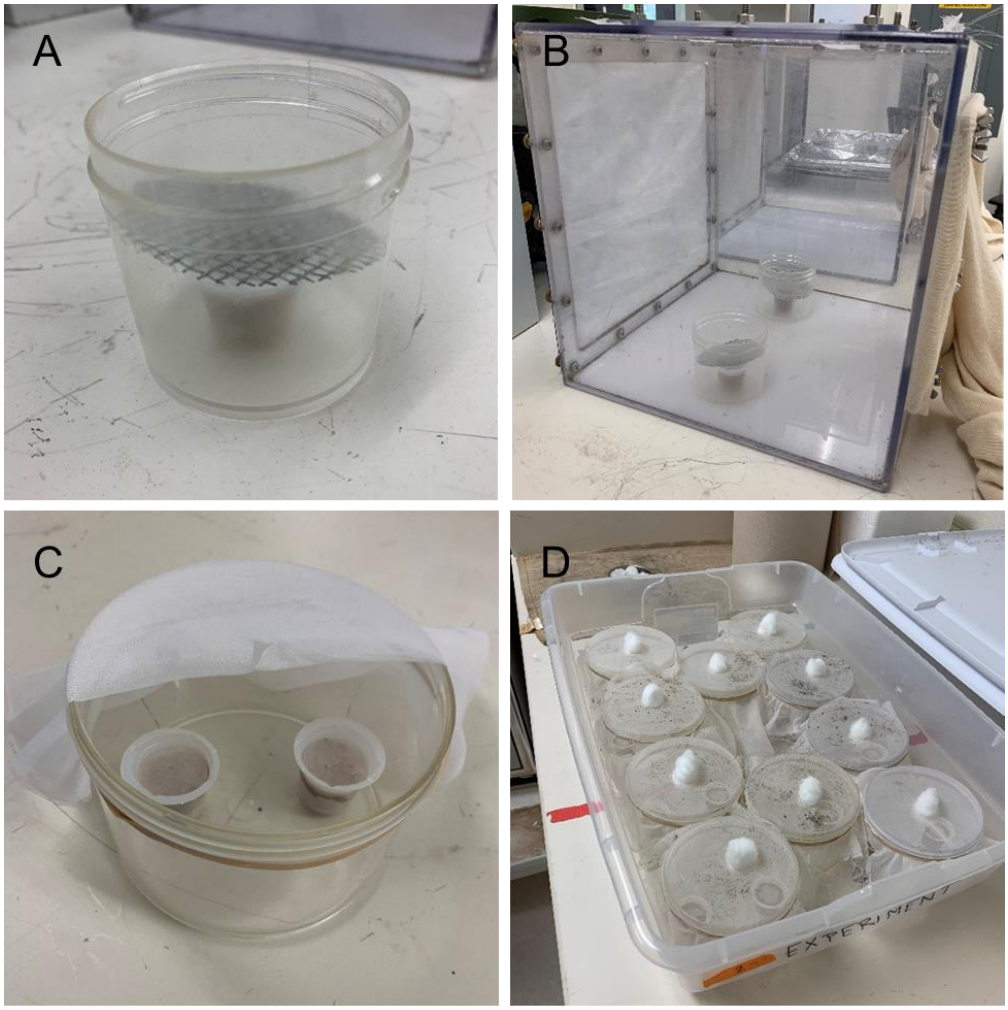

Figure 3. Attraction and Oviposition Bioassays. Attraction Bioassay: A) Constructed sticky trap consisting of a 125mL Nalgene jar and a metal sticky mesh. Inside is a micro-beaker with sand, medium, and a filter paper. B) Paired design of sticky traps in a free-flight cage with 20 flies. One of the pair has conditioned medium and the other had unconditioned medium. Oviposition Bioassay. C) Oviposition jar with mesh affixed with a rubber band and 20 flies. Inside are two micro-beakers with sand. One of the sand cups has conditioned medium and the other with unconditioned medium. D) Multiple oviposition jars in a large tub to be put into the incubator.

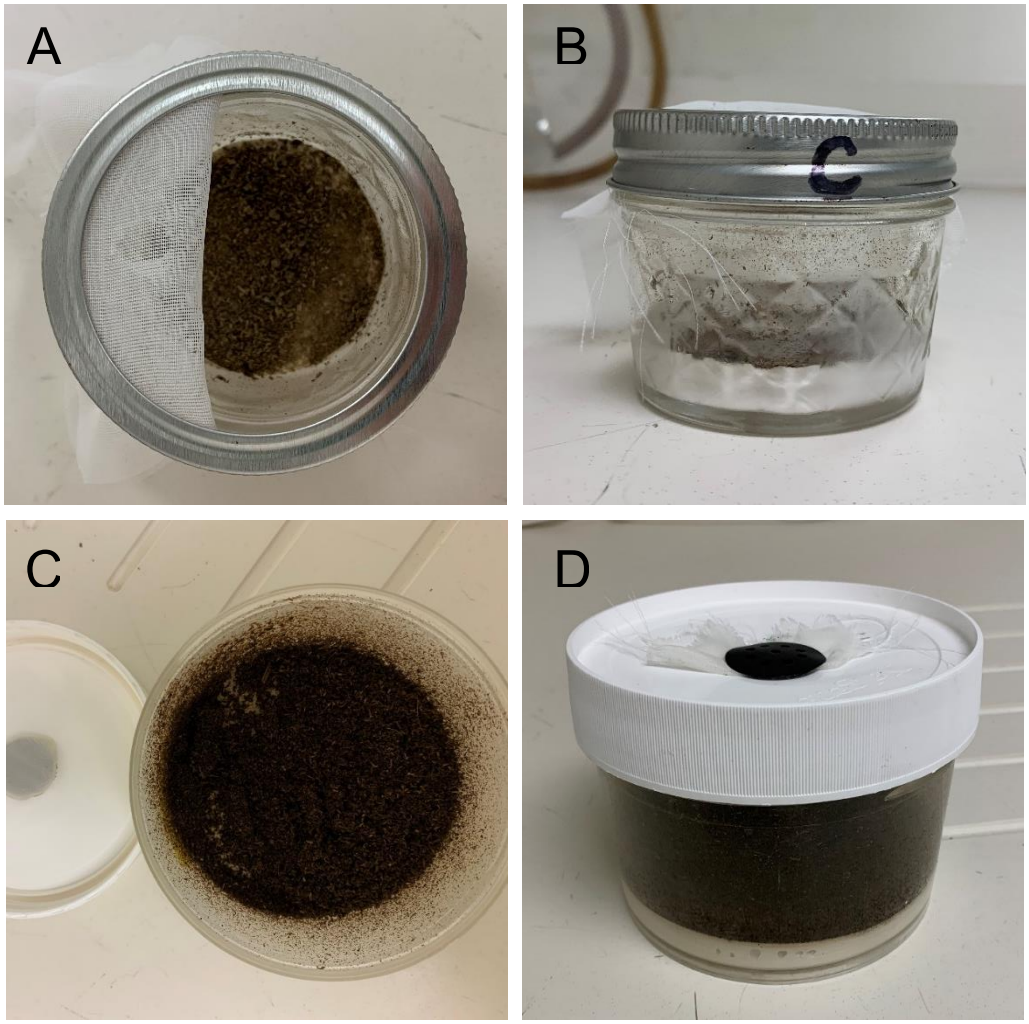

Figure 4. Larval Conditioning Methods. A) and B) Mason jar conditioning. C) and D) Nalgene jar conditioning.

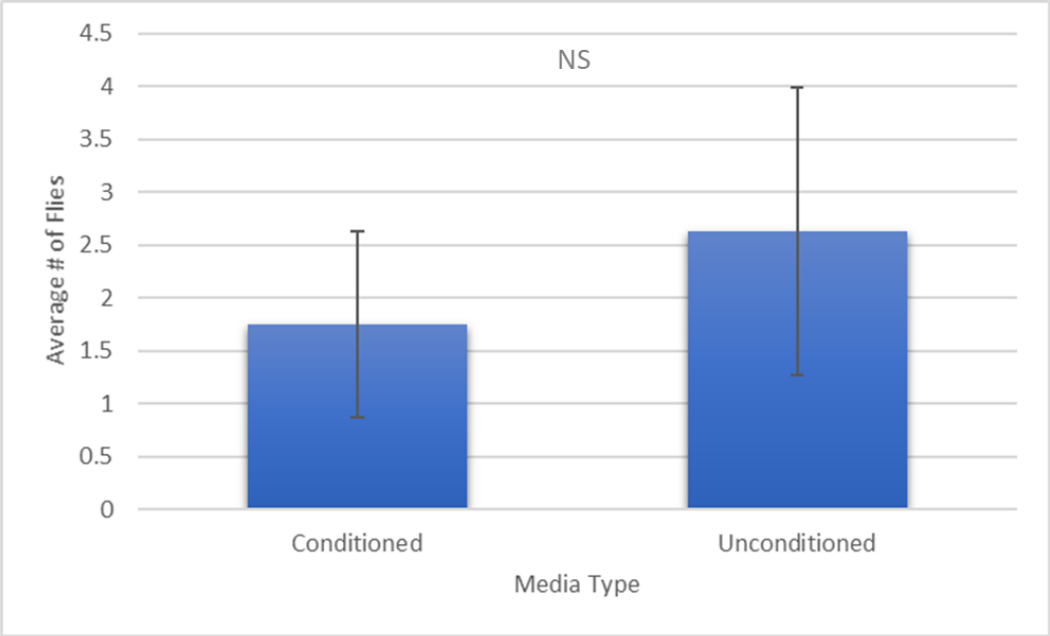

Figure 5. Effects of Conditioning with Mason Jars on Attraction.

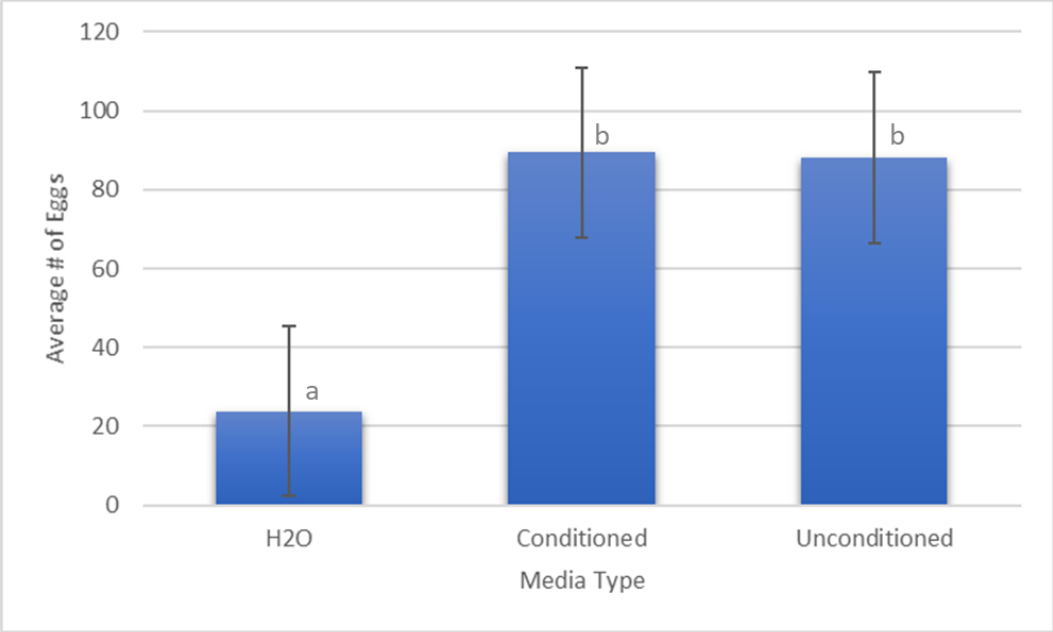

Figure 6. Effects of Conditioning with Mason Jars on Oviposition. Letters indicate significance at the 0.05 level.

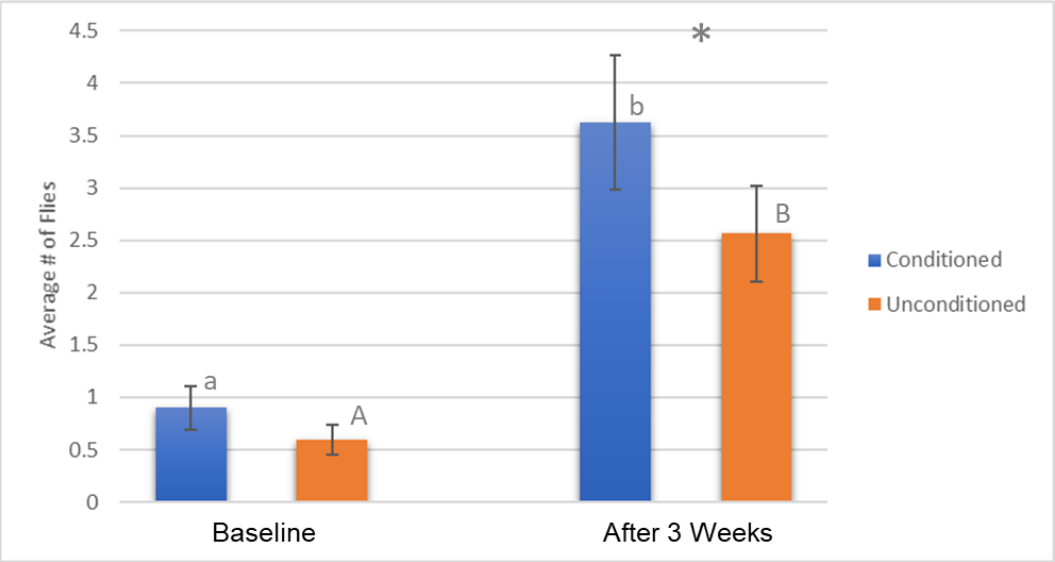

Figure 7. Effect of Conditioning and Aging on Attraction. The effect of larval conditioning and medium aging time on the number of flies trapped on a sticky trap. Asterisks indicate significance at the 0.05 level between conditioned and unconditioned medium. Letters indicate significance at the 0.05 level between baseline and after 3 weeks.

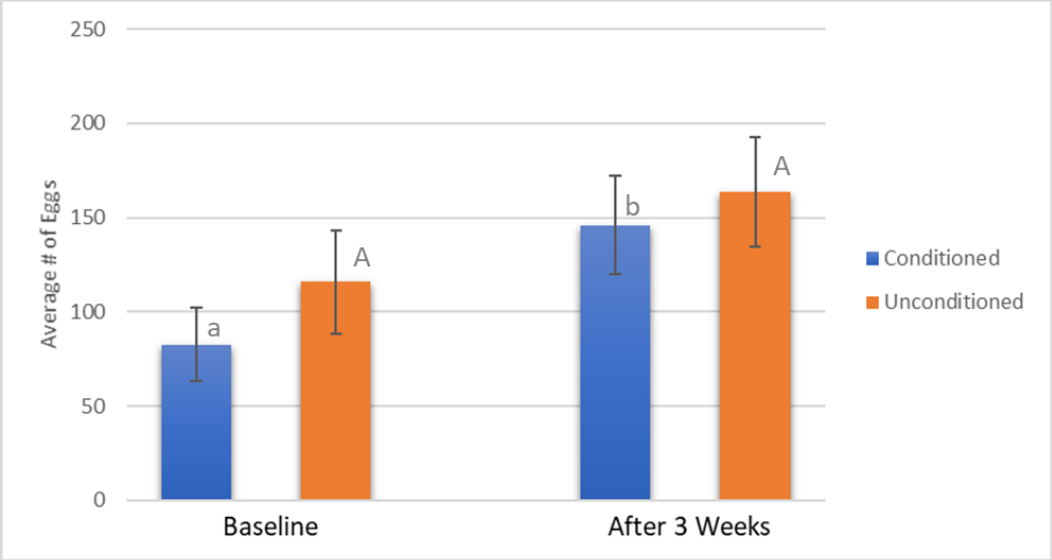

Figure 8. Effect of Conditioning and Aging on Oviposition. The effect of larval conditioning and medium aging time on the number of eggs laid Asterisks indicate significance at the 0.05 level between conditioned and unconditioned medium. Letters indicate significance at the 0.05 level between baseline and after 3 weeks.

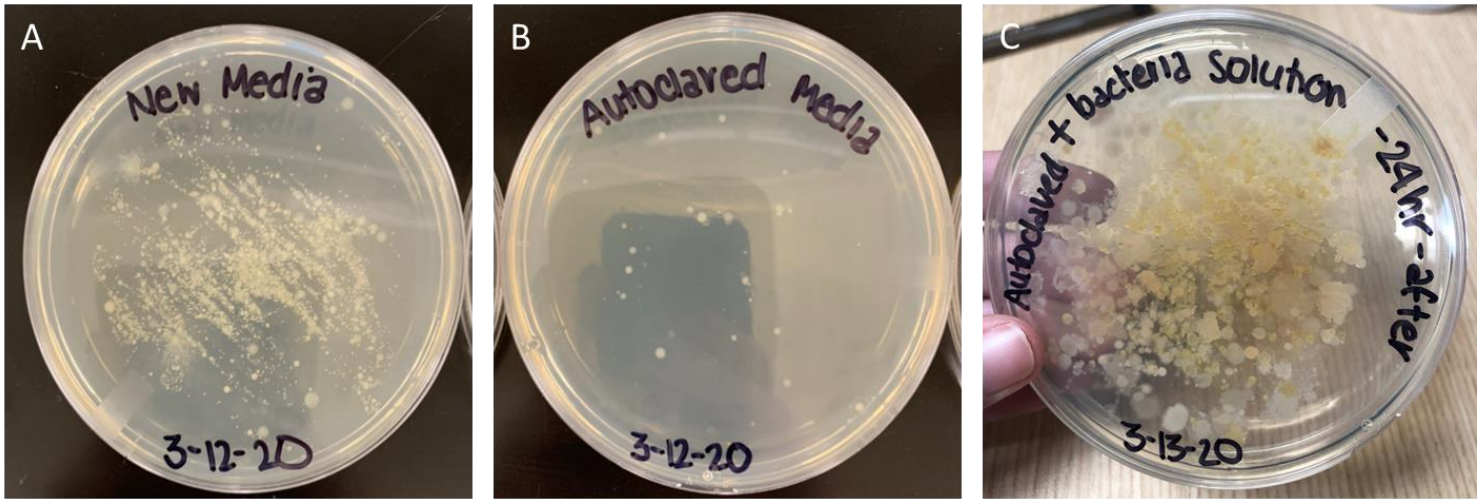

Figure 8. Bacterial Inoculation Results. Agar plates with filtrates from A) fresh larval food, B) autoclaved fresh larval food confirming the effectivity of autoclaving in substantially reducing the bacterial community. C) Confirmation of reinoculated sterile medium producing bacteria