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Leishmaniasis is a vector-borne disease caused by the protozoan parasite of the genus Leishmania and is transmitted by phlebotomine sand flies. There are no vaccinations for leishmaniasis, and current treatment options are limited. Modifying the gut microbiome is an innovative potential way to affect insect host behavior, development, and vector competence to interrupt pathogen transmission. This topic of the influence of microbes on sand fly development and survival is poorly studied. Previous studies demonstrated that sterile medium decreases developmental rates in *Lutzomyia Longipalpis*. However, they could still complete their development even in an axenic rearing medium, devoid of any living bacteria. It was also found that sterile medium inoculated with selected microbes enhanced larval developmental rates. My general hypothesis is that a viable bacterial community in larval media is essential for the proper development of sand fly larvae. I also hypothesize that larva fed on standard larval media would demonstrate the fastest developmental rate and highest survival rate than those fed on sterile food. To this effect, I aimed to test the effect of bacterial presence on larval development, and subsequently test the effect of larval food type on larval development. Overall, there is a statistically significant difference between the survivorship of larvae fed standard media and larvae fed sterile media, with higher developmental and survival rates in larvae that received standard media as opposed to larvae fed sterile media. Likewise, a higher developmental and survival rate was observed in larvae that were fed reinoculated media compared to larvae fed sterile media. However, when larvae were fed with attractive, conditioned media, they demonstrated lower percent survivorship than larvae fed standard media.

THE ROLE OF LARVAL REARING MEDIA TYPES IN REGULATING THE GROWTH AND DEVELOPMENT OF *PHLEBOTOMUS PAPATASI* LARVAE.

by

Nora N. Ufot

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Approved by

Dr. Gideon Wasserberg
Committee Chair

DEDICATION

I dedicate this thesis to my Lord and Savior, Jesus Christ, for the grace and wisdom to complete this work. I also dedicate this thesis to my husband, Jeremiah, thank you for your help, encouragement, and support; I appreciate you. Thank you to my son for being my biggest motivation. Thank you to my family, especially my mom for encouraging me to apply to this program in the first place. Lastly, a major thanks to my dad, my brothers, my sister, and my parents-in-laws for always supporting me throughout this journey.

APPROVAL PAGE

This thesis written by Nora N. Ufot 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

Leishmaniasis Overview

Leishmaniasis is a vector-borne disease caused by the protozoan parasite of the genus Leishmania (Kinetoplastida: Trypanosomatidae) and is transmitted by phlebotomine sand flies (Diptera: Psychodidae). The three significant forms of leishmaniasis are cutaneous, visceral, and mucocutaneous. *Leishmania major* is an agent of cutaneous leishmaniasis (CL) in humans in the Old World (Hassan et al., 2012). Cutaneous leishmaniasis is the most common form of leishmaniasis, with an incidence of 1 to 1.5 million cases annually (Murray et al., 2005). Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil, and Peru account for 90% of cutaneous leishmaniasis infections (Murray et al., 2005). Visceral leishmaniasis (VL) is the most severe and fatal form of leishmaniasis if left untreated. It is an infection of the reticuloendothelial system caused by several species of Leishmania. Most commonly, VL is caused by *L. donovani* and *L. infantum* (Ready, 2013). Anthroponotic Leishmaniasis (due to *L. donovani*) is most common in east Africa, especially Sudan, and northern India, Bihar, while zoonotic VL (due to *L. infantum*) is more widespread in the Middle East, southern Europe and South America. Visceral leishmaniasis can affect the liver, spleen, bone marrow and spread to distant lymph nodes (Murray et al., 2005). The incidence of VL is approximately 500,000 cases annually, most remarkable in Asia, the Middle East, Africa, and southern Europe (Murray et al., 2005). Mucocutaneous leishmaniasis (MCL) is the rarer form of leishmaniasis, causing partial or complete destruction of the mucous membranes of the nose, throat, and mouth (Palumbo, 2010). Mucocutaneous leishmaniasis is mainly associated with infections caused by the New World's *Le. Brazilensis* (Claborn, 2010). Mucocutaneous leishmaniasis cases occur in Latin America,

specifically in Brazil, Paraguay, Ecuador, Bolivia, Peru, Colombia, and Venezuela (Murray et al., 2005). There are no vaccinations for leishmaniasis, and current treatment options are limited.

Phlebotomine Sand Flies Overview

Phlebotomine sand flies (Diptera: Psychodidae) are the vectors of Leishmania parasites. Phlebotomine sand flies are small, about 3 mm in length, covered with setae, vary in color from pale yellow/beige to black, and their wings are angled above the abdomen when at rest (Killick-Kendrick, 1999). Sand flies are usually nocturnal and often bite at night, although a few species of sand flies bite during crepuscular daytime. Adult sand flies, both males and females consume plant sugar secretions (Maroli et al., 2013). Female sand flies are mostly anautogenous, requiring at least one blood meal to complete the development of eggs and prompt oviposition (Maroli et al., 2013). Most female sand flies are exophagic and exophilic, meaning that they bite and rest outdoors during the maturation of eggs, respectively (Killick-Kendrick, 1999). Female sand flies lay their eggs in a suitable habitat rich in organic content, such as animal excreta and dirt, offering shelter, nutrition, and hydration to their neonate larvae (Maroli et al., 2013). The life cycle of Phlebotomine sand flies is fully terrestrial. They undergo four developmental stages: egg, larvae, which involve the four instar phases, pupa, and adult (Maroli et al., 2013). Proceeding oviposition, sand fly eggs hatch within seven days, followed by larval development, which extends for about three to four weeks before pupation. Subsequently, sand flies emerge from pupa after approximately ten days (Killick-Kendrick, 1999). Adult sand flies' seasonal activity is mainly influenced by temperature and rainfall. Sand flies typically reside in areas of warmer climates such as southern Europe, Asia, Africa, and Central and South America (Maroli et al., 2013). Many sand fly species tend to rest in relatively cool and humid areas during the day, such as houses, cellars, stables, and caves (Killick-Kendrick, 1999). The emergence and increase

of leishmaniasis in the Old World and New World can be linked to anthropogenic land-use change (Wasserberg et al., 2003) and climatic changes that have favored growth in vector density (Maroli et al., 2013). Disruptions such as irrigation, dam construction, deforestation, and urbanization have resulted in changes in sand fly population densities (Maroli et al., 2013). In addition, increased human migration has facilitated the spread of infectious pathogens of both human and animal origin by introducing them into previously uninfected areas (Colwell et al., 2011).

Traditional vector control approaches include the use of insecticides. Most sand fly control measures include residual spraying, space sprays, barriers and treated netting, topical repellent, and applications in reservoir burrows (Claborn, 2010). Insecticides have been proven effective, however efficacy is decreasing due to increasing insecticide resistance and its effects on non-specifics (Hassan et al., 2012). Therefore, there is an urgent need to develop alternative, environment-friendly control measures (Warburg & Faiman, 2011). There are several alternative control measures for sand flies, such as systemic control, toxic sugar baits, and oviposition attractants. In addition, modifying the gut microbiome could be an innovative potential way to affect insect host behavior, development, and vector competence (Cansado-Utrilla et al., 2021).

The Role of the Gut Microbiome in the Growth and Development of Hematophagous Insects

A central working hypothesis is the preference-performance hypothesis (PPH). The PPH posits that natural selection selects females that make oviposition site selections that match their offspring's needs and enhance their fitness (Jaenike, 1978). The support for this hypothesis has been observed in mosquitoes. Studies show that in some mosquito species, gravid females can improve their offspring's survival and developmental rate by choosing oviposition sites that

reduce exposure to predators and increase accessibility to food sources (Wong et al., 2011). Additionally, Ponnusamy et al. 2008 demonstrated the influence of the microbial community on the oviposition site selection. It is supported that gravid females must make proper oviposition selection since the microbial community has to support their offspring's growth and development (Ponnusamy et al., 2008). Research showed that most mosquito eggs were laid in areas with more organic matter than in experimental sterile water (Ponnusamy et al., 2008).

Mosquitoes

Most work on gut microbiota in this field has been done with mosquitoes (Alacay et al., 2019). Mosquitoes are adapted to interact with bacteria, and their behaviors and biology, including oviposition site selection, oviposition stimulation, egg hatching, and larval food digestion, are frequently mediated by bacteria (Travanty et al., 2019). Travanty et al. 2019 sought to compare the effects of individual bacterial species on larval survivorship and larval development to larval performance when exposed to a diverse community of microbes. According to the findings, bacteria provide nutrition that aids larval development (Travanty et al., 2019). Furthermore, Coon et al. (2014) showed that mosquito larvae depend highly on bacteria for growth, survival, and pupation. Functional assays performed by Coon et al. 2014, showed that axenic (bacteria-free) larvae of various mosquito species failed to develop after the first instar, demonstrating that microbes are essential for survival. The mosquito species studied by Coon et al. (2014) needed living bacteria in the gut for growth. Results showed development relied on several different bacteria species, which rescued gnotobiotic larvae (meaning all the microorganisms are either known). The awareness of the gut microbiome may contribute to creating new vector control strategies (Telleria et al., 2018).

Phlebotomine sand flies

This topic of the influence of microbes on sand fly development and survival is poorly studied. However, there is substantial evidence that given the coprophagic (excrement consuming) diet of larval sand flies, it is reasonable to hypothesize that gravid females will be attracted to cues indicating the presence of organic matter from various sources (rabbit feces, rabbit chow, chicken feces, etc.) (Kakumanu et al., 2021; Marayati et al., 2015b; Nguyen et al., 2021; Peterkova-Koci et al., 2012). Several sand fly species demonstrate enhanced oviposition in response to organic material of diverse sources. For example, Schein et al. 1989 discovered that cow manure is particularly appealing to both gravid and non-gravid *Ph. papatasi* females. Also, *Ph. argentipes*, the vector of L. major in India, demonstrated strong attraction and preference to oviposit by cow and rabbit excreta (Kumar et al., 2013). Elnaiem and Ward (1992) found that oviposition sites composed of hexanal and 2-methyl-2-butanol from rabbit feces were found to enhance oviposition response in *Lutzomyia longipalpis* (Dougherty et al. 1995). These olfactory cues derived from various organic matter sources have been identified as crucial oviposition attractants and stimulants, with some of these cues provided by microbes (Kakumanu et al., 2021).

In terms of oviposition attraction, in a study conducted by Marayati et al. in 2015, a bioassay was performed to determine the attraction of *Ph. papatasi* to larval rearing media from different larval developmental stages (2nd/3rd instar and 4th instar) compared to fresh larval food, expired medium (rearing medium from which all larvae have eclosed from), and fresh rabbit feces. The results showed that gravid *Ph. papatasi* females are more attracted to larval conditioned media than to unconditioned media. Particularly, gravid females are attracted to and oviposit at the highest rate in rearing medium conditioned by second and third instar larvae. This

was followed by rearing media conditioned by fourth instar/pupae, adults, and fresh unconditioned medium (Marayati et al., 2015).

In a follow up study to Marayati et al. 2015, Faw et al. 2021 evaluated the preference of gravid sand fly for conditioned medium, showing that larval conditioned medium is still preferred over unconditioned medium, yet both are preferred over fresh medium. Based on the results, Faw et al. 2021 supported findings from Marayeti et al. 2015 that gravid females are attracted to conditioned medium, suggesting that the larvae are involved in conditioning the oviposition substrate and likely also involved in the production of oviposition attractants and stimulants. To consider the effect of bacterial community change corresponding to larval induction and medium aging, Nayma Romo-Bachara (2021), conducted a study to understand the changes in the microbial communities of the substrates. Romo-Bachara's findings confirm that bacterial community composition is dynamic over time and diverges due to the presence of larvae and then converges when larvae are gone (due to pupation and eclosion). Furthermore, aging comprises a prominent role in modifying the structure of the bacterial community.

Additionally, Kakumanu et al. (2021) obtained 12 bacterial isolates from this most attractive second/third instar rearing substrate. They conducted attraction bioassays to assess the behavioral response of gravid females to these bacterial isolates. Based on the results, these bacterial isolates were divided into four groups: highly attractive, repellent, strong response (either attraction or repellence), and no response. The study revealed that community is attractive (at low dose) and identified three species (SSI-2, SSI-9, and SSI-11) that were highly attractive at low dose.

Altogether, these studies raise the question of why are gravid sand flies attracted to these bacteria? This question brings us back to the PPH with the main support for that being

Peterkova-Koci's work. Peterkova-Koci et al. (2012) demonstrated *Lu. longipalpis* sand flies are attracted to a non-sterile medium composed of rabbit feces compared to the sterile medium. Likewise, a study by Martins et al. 2021 showed a vast number of eggs oviposited on the chicken coop substrate, demonstrating that female *Lu. longipalpis* choose oviposition sites that provide the best environment for their larvae. The females favored pots with substrates from colony food (rabbit chow/rabbit feces) and chicken shelters, which is consistent with prior research showing that hexanal and 2-methyl-2-butanol found in chicken and rabbit feces stimulated receptors in gravid *Lu. longipalpis* females. Rabbit feces has a diversified microbial community that could play an essential role in the development of sand fly larvae, as bacteria may offer required or additional nutrients (Peterkova-Koci et al., 2012). The effect of microbial communities on sand fly larval development may be due to the breakdown of rabbit feces into more consumable and absorbable nutrients or the production of additional nutrients such as vitamins and amino acids (Peterkova-Koci et al., 2012). Data showed that larvae reared in sterile media exhibited decreased developmental rates in *Lu. Longipalpis*, but they could still complete their development even in an axenic rearing medium, devoid of any living bacteria (Peterkova-Koci et al., 2012). It was also found that sterile medium inoculated with selected microbes enhanced larval developmental rates.

Study Question

In my research, I evaluated: (1) the effect of microbial presence in sand fly rearing medium on larval development. Additionally (2), I assessed the effect of larval food stage/type on larval development.

Hypotheses and Specific Aims

Based on the PPH assumption, my general hypothesis is that the behavioral oviposition attraction effect should correspond to their impact on larval growth and survival.

1. Aim 1: The effect of rearing medium sterility on larval growth and survival

With respect to the effect of overall microbial presence in sand fly rearing medium, I am hypothesizing that microbes in the rearing medium are essential for larval development. Hence, I expect that a viable microbial community in larval media is essential for the proper development of sand fly larvae. Therefore, I predicted that neonate larvae fed on larval food devoid of microbes (sterile media) will develop slower, suffer higher mortality, and exhibit lower pupal mass compared with those reared on standard larval food or sterile larval food reinoculated with microbial filtrate of standard food.

2. Aim 2: The effect of rearing medium stages on larval growth and survival

With respect to the effect of rearing medium stage, I hypothesize that gravid female's preference for larval conditioned medium should correspond to enhanced larval performance for larvae fed on this rearing medium. Hence, based on *Ph. papatasi* for larval-conditioned medium, when comparing fresh-, larval conditioned- (containing larvae reared until their $2^{nd}/3^{rd}$ instar stage), and expired (rearing medium from which all adults have eclosed) rearing medium, I predict fastest growth, highest survival, and highest pupal mass for larvae fed larval conditioned medium.

Aim 1 Approach

Producing sterile larval food

This approach involves the use of an autoclave to sterilize fresh larval media, specifically to create an axenic (microbe-free) treatment group. I successfully sterilized 0.3g of fresh larval media for 20 minutes. A sample of the media was plated on LB agar plates and incubated at 26ºC for three days as a proxy for sterility.

Producing sterile eggs

This approach involves surface sterilizing sand fly eggs. It is important to confirm surface sterilization of the eggs to create axenic neonate larvae in order to evaluate the effect of media sterility and media type on their development. The surface sterilization of sand fly eggs was performed using 100% ethanol and 2% bleach. The eggs were rinsed with sterile molecular grade water several times, and the last rinse was plated on LB agar plates, incubated at 37ºC for three days, as a proxy for sterility.

Effect of rearing medium development bioassay

I used four 24-well cell culture plates, placed a single larva was placed in each well with a small amount of media, fed once a week with one of the three food types (n=8 per food type per cell culture plate). The three types of rearing media were: standard (positive control), sterile (experiment group 1), and sterile medium inoculated with bacterial filtrate of standard medium (hereafter, reinoculated) (control group 2). Control group 2 is needed to control for autoclaving the media which could alter the composition and change attractiveness.

Aim 2 Approach

Effect of rearing medium sterility and medium stage development bioassay

I used five 24-well cell culture plates. Each of the five cell culture plates was designated for five different media types: standard, sterile, reinoculated, 2/3rd, and expired media. Based on a study conducted by Marayati et al. in 2015, gravid females are attracted to and oviposit at the highest rate in rearing media conditioned with 2/3rd instar larvae. Using a sterile paintbrush, I placed a single larva into each well of the five cell culture plates. Each neonate larva initially received a small amount of media and was fed once a week with one of the five food types (n=24 per food type per cell culture plate).

CHAPTER II: METHODS

Sand fly colony—source and maintenance

Phlebotomus papatasi sand flies are maintained at the Ecology of Infectious Disease lab at the University of North Carolina at Greensboro. The mass-rearing protocol described by Lawyer et al. (2017) is followed in rearing the sand flies. They are held in incubators at 26°C, 80 % RH. They are sugar-fed with 30 % sucrose solution. Female flies are blood-fed on live anesthetized ICR mice (Harlan) (UNCG IACUC protocol #: 20-0011, June 2020). Larvae are kept in 125 ml Nalgene jars with a layer of plaster-of-Paris on the bottom to maintain relative humidity. The larvae are fed a 1:1 ratio mixture of fresh rabbit feces with rabbit chow. The larval food is fermented in a chamber for three weeks, air-dried, and ground into powder (Lawyer et al., 2017).

Sterilization of larval rearing medium

I used the autoclave to sterilize the larval rearing medium. To secure successful sterilization, I only used small amounts of larval food in each container (125 ml Nalgene jars). Based on preliminary trials, 0.3g of rearing medium were successfully sterilized in the autoclaved for 20 minutes; therefore, this was the amount and time frame used.

Sterilization of the larvae food will be confirmed by plating the medium homogenate on LB agar plates and incubating at 37ºC for three days.

Egg sterilization

Following the sterilization of larval food, the surface sterilization of sand fly eggs is conducted. I used 100% ethanol and 2% bleach to sterilize larval eggs. From preliminary work, larval eggs were confirmed sterile when washed with 100% ethanol for 2 minutes, followed by a rinse with sterile water twice, washed with 2% bleach for 3 minutes, and rinsed six times with

sterile water. Under a sterilized hood, 20-40 sand fly eggs were washed in an Eppendorf microcentrifuge with 200 µL of 100 % ethanol for two minutes and rinsed twice with 400 µL of sterile water. The eggs were then washed with 200 µL of 2% bleach for three minutes. Subsequently, the eggs were rinsed six times with 400 µL of sterile water. Upon the final rinse, 400 µL of sterile water, was retained in the Eppendorf tubes. Sterilization of the sand fly eggs was confirmed by plating 100 µL of sterile water from the final rinse on LB agar plates and incubating at 37ºC for three days.

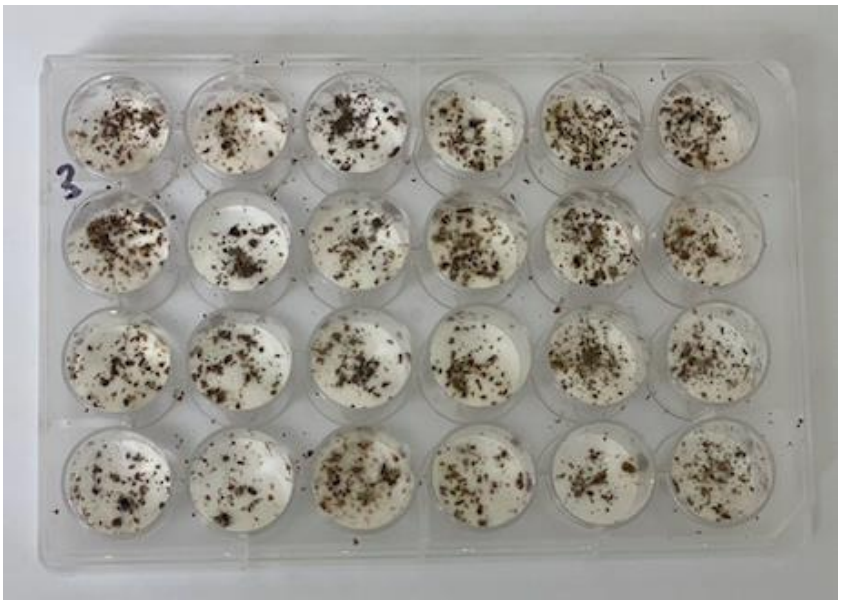
Aim 1. Effect of rearing medium sterility on larval development procedure

Four 24-well cell culture plates were prepared with a small layer of plaster-of-Paris base. A single sterile neonate larva was placed in each well using a sterile paintbrush. Each neonate larva was fed once a week (ad-libitum, approximately 0.0001g) with one of the three food types (n=8 per food type in one cell culture plate). The larvae's survivorship and growth stages were monitored every 2-3 days for 31 days.

Aim 2. Effect of rearing medium sterility and medium types on larval development procedure

Five 24-well cell culture plates were prepared with a small layer of plaster-of-Paris base saturated with water. Each plate contained a single larva in each well. Each larva was reared on five different media types: standard, sterile, reinoculated, 2/3rd conditioned, and expired media. Each larva was fed once a week with one of the five food types (n=24 per food per culture plate) and monitored every 2-3 days for survivorship and growth status until the completion of growth period (33 days). The cell culture plate that contains larvae fed sterile media was monitored and fed under a sterile hood.

Figure 1. Visual of a 24-well cell culture plate with ~0.5mm base of Paris-of-plaster. Each well contains about 0.0001g of larval media and a single neonate larva.



Larval development approach

Aim 1

I used four 24-well cell culture plates prepared with a small layer of plaster-of-Paris base (approximately 0.5 mm) and saturated with about 0.5 ml to maintain relative humidity. A single neonate larva was placed in each well. Each neonate larva initially received a small amount of media, approximately 0.0001 g, and was fed once a week with one of the three food types (n=8 per food type per culture plate) and monitored every 2-3 days. The three types of rearing media were: standard (positive control), sterile (experiment group 1), and reinoculated (control group 2). The cell culture plates were covered with parafilm, with several small puncture holes to allow airflow. The larvae in the cell culture plates were incubated at 26°C, 80 % RH. Every 2-3 days, approximately 0.5 ml of DI water was added to ensure humidity which is crucial for larval survival. I monitored their development over 31 days until they reached the pupal stage. By the

end of this growth period, the fraction of the well in which larvae reached pupation was recorded, and the pupa was weighed (using a Mettler Toledo scale) to determine its mass in milligrams. It is worth noting that the sterile media source was autoclaved weekly to ensure sterility. For the experiment of aim 1, each of the four 24-well cell culture plates contained the three treatment media types. The proximity of the three treatment groups in the same cell culture plate raised concern for cross contamination. To mitigate this issue, the experiment was revised for aim 2 experiments. Each of the five treatment media types were designated in separate 24-well cell culture plates.

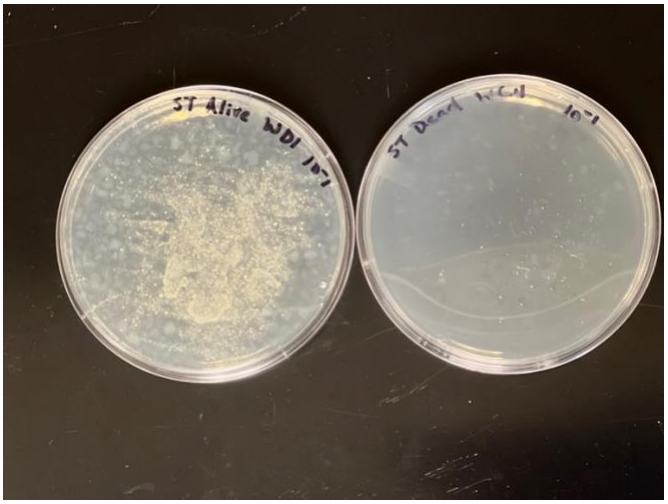
Aim 2

I used five 24-well cell culture plates prepared with a small layer of plaster-of-Paris base and saturated with small amounts of water. Each cell culture plate was designated for five different media types: standard, sterile, reinoculated, 2/3rd, and expired media. Based on a study conducted by Marayati et al. in 2015, gravid females are attracted to and oviposit at the highest rate in rearing media conditioned with 2/3rd instar larvae than the prelarval (rabbit feces, fresh larval food) or post-larval (expired colony medium) rearing media. Therefore, it is worth exploring the influence of 2/3rd rearing media and expired media on the development and survivorship of larvae. I placed a single larva into each well (24 wells per cell culture plate) of the five cell culture plates. Each neonate larva initially received approximately 0.0001g of media, and was fed once a week with one of the five food types (n=24 per food type per culture plate). The larvae were monitored every 2-3 days to evaluate survivorship and growth status, until the end of growth period, specifically for 33 days. Similar to session 1, the cell culture plates were covered with parafilm with small puncture holes and store in the incubator at 26ºC, 80% RH.

Confirmation of bacterial presence/absence in the sterilized medium

To delineate and quantify bacterial presence in the experimental medium, I conducted colony counts of larval samples from the sterile plate that survived until pupation and larval samples that did not. I pipetted 1mL of sterile, molecular grade water into each well of the sterile cell culture plate. I then sampled 100uL from 6 wells of the sterile plate (n=3 for survivors, n=3 for non-survivors) used to make serial dilutions (10^-1, 10^-2, 10^-3, 10^-4 dilutions). Each dilution, about 100uL of each, was then plated on LB agar and incubated at 30ºC for 24 hours, at which time colony counts were taken to determine if there were more or less microbes in a given treatment sample. Plates containing sterile water were labeled, sealed, and frozen at -80ºC for further quantitative analysis.

Figure 2. Visual of bacterial growth from samples of the sterile plate from wells of larvae that survived (Left) and wells of larvae that did not survived (Right).



Statistical Analyses

The analysis of development was measured by time-to-pupation of surviving larvae, growth by average pupal mass, and survival by proportion that survived of the different treatment groups. To determine the development time and survival proportion, I initially noted

the date that the experiment began. Every two to three days, I recorded the date the larvae were observed, along with the status and stage of each larva. At the end of the 4-week growth period, the fraction of the well in which larvae reached pupation was recorded, and each pupa was weighed to determine its mass. I analyzed the data in excel. I determined the developmental time by the initial date of experimental set up until the date of pupation noted. I calculated the survival proportion of larvae fed on the various diets for each time point. I also calculated the average pupa mass of larvae in each category. Using this data, I performed a survivorship analysis using the time to pupation as my response variable. A time to event variable represents the amount of time until a participant encounters an event of interest. In this case, the participant is the individual larva, and the event of interest is pupation. To depict time-to-pupation and daily survival, I created Kaplan-Meier survival curves on GraphPad Prism to plot the survivorship proportion of larvae over time for each treatment group. I analyzed time-to-pupation data using a cox proportional-hazards regression model to test the dependency of survival time on predictor variables (specifically, media types). For the cox proportional hazard regression model, the measure of the effect is the hazard rate, which is the probability of pupation, given that each larva has survived up to a specific time. Survival analyses are needed to be used for time-to-pupation as they provide an estimate of survival probability at each point in time. This is opposed to a one-way ANOVA test that tests if the mean value of a variable varies by categorical factors. I used logistic regression models to assess the effects of treatment media types, which is categorical, on the outcome variable (survival). Additionally, to evaluate the larval quality (pupa mass), I used a parametric approach (one-way ANOVA), a continuous normally distributed variable. I conducted one-way ANOVA tests to assess the effect of media sterility and media

types of pupa mass. Furthermore, post-hoc tests were conducted to identify specific differences between three or more group means when an ANOVA test is significant.

CHAPTER III: RESULTS

Aim 1. Effect of media sterility on larval development, growth, and survival

Effect on larval development

There were four 24-well cell culture plates (n=32 larvae reared for each treatment). In each cell culture plate, there were 8 replicates for each treatment media type. Based on the cox-regression analysis (Table 1) the effect of medium sterility was non-significant on larval development of all the larvae analyzed. Figure 3 shows the development time of larvae fed on the three treatment media types. The larvae fed standard media reached pupation first and larvae fed sterile media demonstrated the longest time to pupation. When looking at the development time of only the larvae that made it to pupation (i.e., excluding larvae that died earlier), there is a significant effect of media sterility on development time (Table 2, Fig. 4). The longest developmental time was recorded for larvae feeding on sterile media, of about 27 days (Fig. 4). This is opposed to larvae fed standard and reinoculated media that demonstrated shorter development time of about 25 and 24 days (Fig. 4)

Table 1. Cox proportional hazards regression model for the effect of media sterility on larval development time.

Variable:	Coefficient	Expected Coeff.	Expected Negative Coeff.	Se Coeff.	Z	P value	Lower .95	Upper .95
Sterility	-0.0337	0.9669	1.034	0.1690	-0.199	0.842	0.6942	1.347

Concordance = 0.499 (se = 0.052)
Likelihood ratio test = 0.04 on 1 df, p = 0.8
Wald test = 0.04 on 1 df, p = 0.8
Score (logrank) test = 0.04 on 1 df, p = 0.8

Figure 3. The effect of medium sterility on larval development time for larvae fed standard, sterile, and reinoculated media.

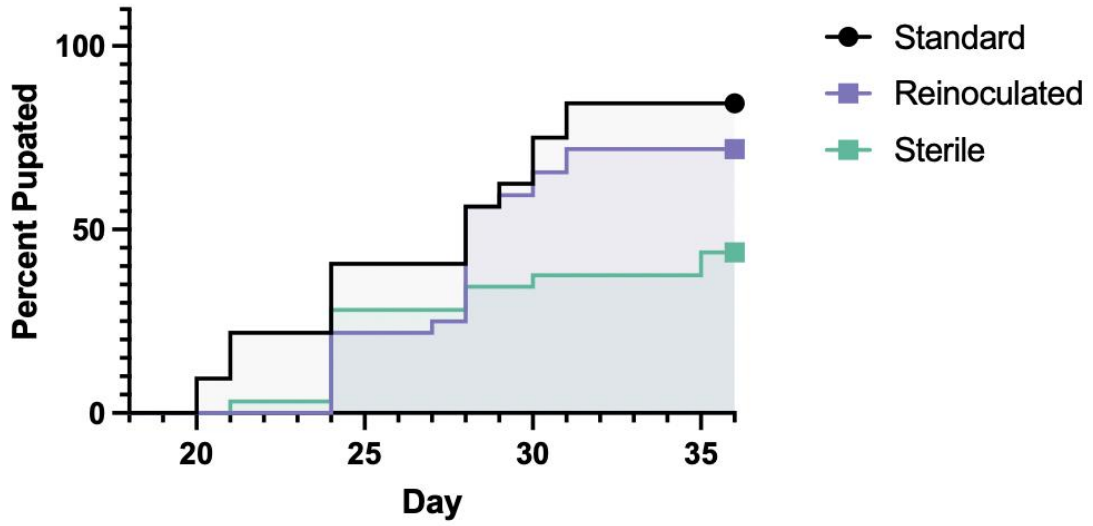
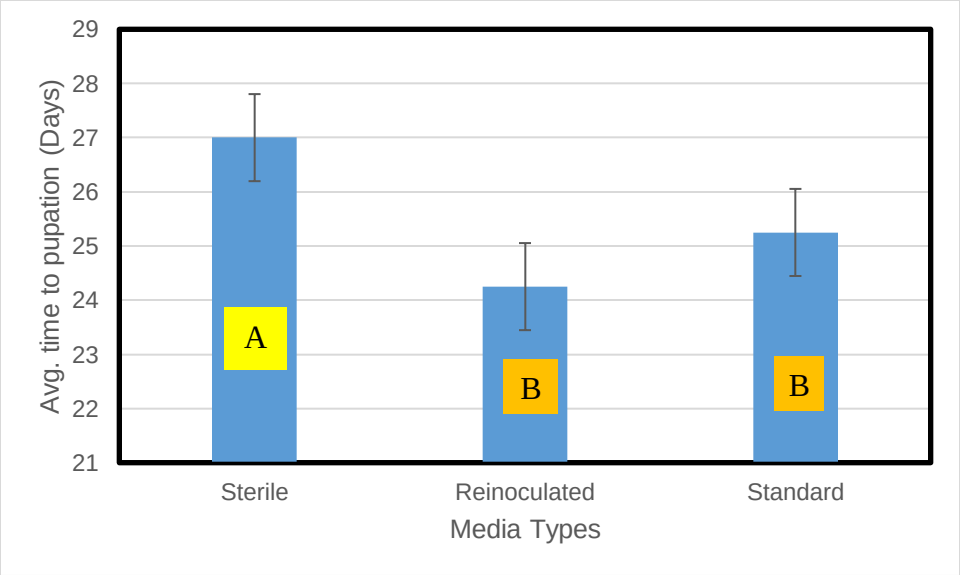


Table 2. ANOVA table of the effect of media sterility on larval development time.

	Df	Sum Sq	Mean Sq	F value	P value
Sterility	2	73.3	36.63	3.152	0.0498
Residuals	61	708.8	11.62		

Figure 4. The effect of medium sterility on average larval development time (time-to-pupation) for surviving larvae fed autoclaved larval food (sterilized), sterilized but bacterial reinoculated medium, and standard larval medium. Error bars indicate standard error. The letters (A, B, C) represent the post-hoc results of the difference between the three treatment groups.



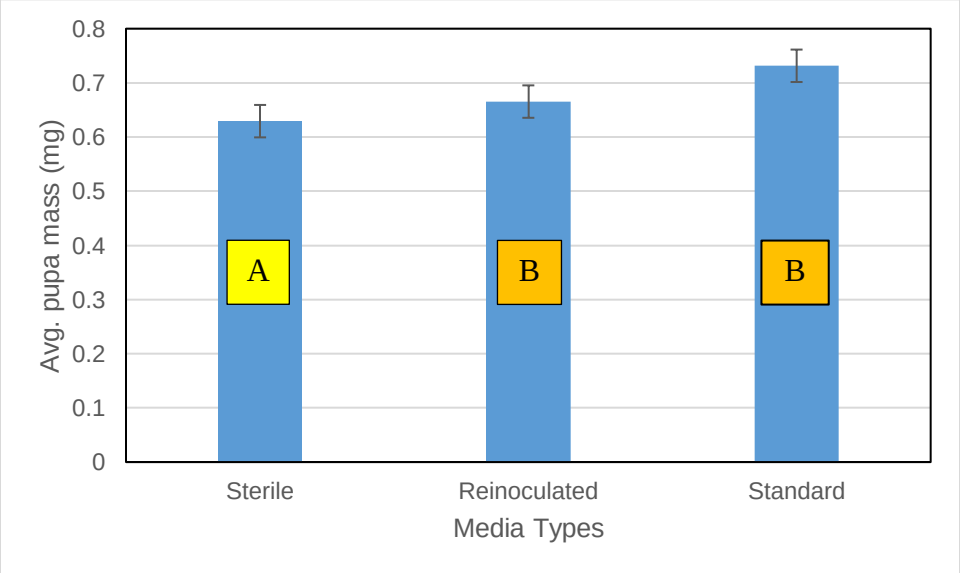
Effect on larval growth

My findings revealed a significant effect of treatment (Table 3) on the average pupae mass of larvae that survived to pupation, with the highest pupal mass for larvae fed on standard- and reinoculated media (not statistically different from one another, P=0.3484) and lower pupal mass for larvae fed on sterile medium (Fig. 5). There is a significant difference between the pupa masses of larvae fed standard media compared to larvae fed sterile media (P=0.0002), and between the pupa masses of larvae fed reinoculated and larvae fed sterile diets (P=0.0142) confirmed by one-way ANOVA test (Table 3).

Table 3. ANOVA table on the effect of medium sterility on larval growth (pupa mass) of the three treatment media types: standard, sterile, and reinoculated.

	Df	Sum Sq	Mean Sq	F value	P value
Sterility	2	1.812	0.9060	9.291	0.000213
Residuals	91	8.874	0.9075		

Figure 5. The effect of medium sterility level on pupal mass (mg) for larvae fed on standard, sterile, and reinoculated media. Error bars indicate standard error. The letters (A and B) represent post-hoc results for multiple pairwise comparison.



Effect on larval survivorship

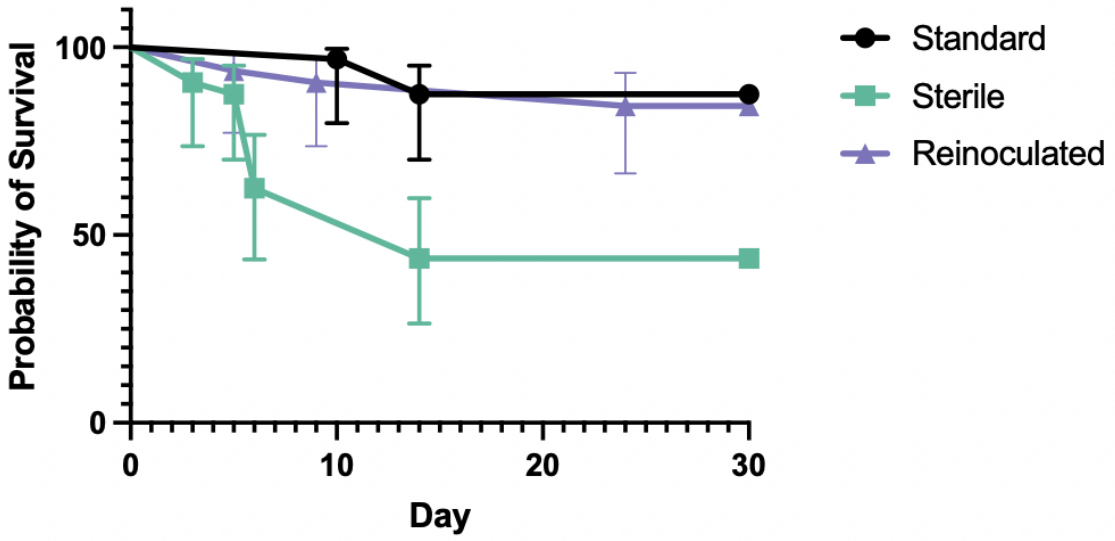
The results found a significant effect of treatment (Table 4) on proportion of survival to pupation, with highest survival for larvae feeding on standard media (84%) and reinoculated media (75%) (not statistically different from one another) and significantly lower survivorship for larvae fed on sterile medium (44%) (Fig. 6). Daily survivorship of each larva was also

evaluated with a significant decrease in survivorship of larvae fed sterile media by day 7, from 100% to 63% survivorship (Fig. 6). As demonstrated by figure 6, the larvae fed standard and reinoculated media displayed higher survivorship proportion for the duration of the experiment than larvae fed on sterile media.

Table 4. Logistic regression table of the effect of media sterility on survivorship larvae.

	Estimate	Standard Error	Z value	P value
Intercept	1.7980884	0.4340784	4.142312	<0.0001
Sterility	-0.9960148	0.3020857	-3.297127	<0.0001

Figure 6. Kaplan-Meier survival curve plotting survivorship proportion of larvae fed on three treatment media types: standard, sterile, and reinoculated media. The error bars indicate standard error.



Aim 2. Effect of medium sterility and medium stages on larval development, growth, and survivorship.

Effect on larval development

There were five total 24-well cell culture plates; one for each treatment group: standard, sterile, reinoculated, $2^{nd}/3^{rd}$, and expired media (n=24 for each media type). The results from my experiment were consistent with aim 1. Based on the cox-regression analyses (Table 5) the effect of media sterility and media stages/type on larval development rate was significant on larval development. As in the first experiment (of Aim 1), the longest time-to-pupation was observed in larvae fed on sterilized media, of about 29 days. Interestingly, this was followed by larvae fed on $2^{nd}/3^{rd}$ conditioned medium and expired media, with average developmental time of about 26 days (Fig. 7). The larvae fed standard and reinoculated media showed the shortest developmental time of about 22 days (Fig. 7). When considering the development time of only the larvae that made it to pupation (excluding larvae that died earlier), there is a highly significant effect of media sterility and media stages on development time (Table 6, Fig. 8).

Table 5. Cox proportional hazards regression model for the effect of media sterility and media stages on larval development.

Variable:	Coefficient	Expected Coeff.	Expected Negative Coeff.	Se Coeff.	Z	P value	Lower .95	Upper .95
Sterility and Stages	-0.2076	0.8125	1.231	0.0991	-2.095	0.0362	0.6691	0.9867

Concordance = 0.604 (se = 0.063)
Likelihood ratio test = 4.51 on 1 df, p = 0.03
Wald test = 4.39 on 1 df, p = 0.04
Score (logrank) test = 4.46 on 1 df, p = 0.03

Figure 7. The effect of medium sterility and media stages on larval development time for larvae fed standard, sterile, reinoculated media, 2nd/3rd, and expired media.

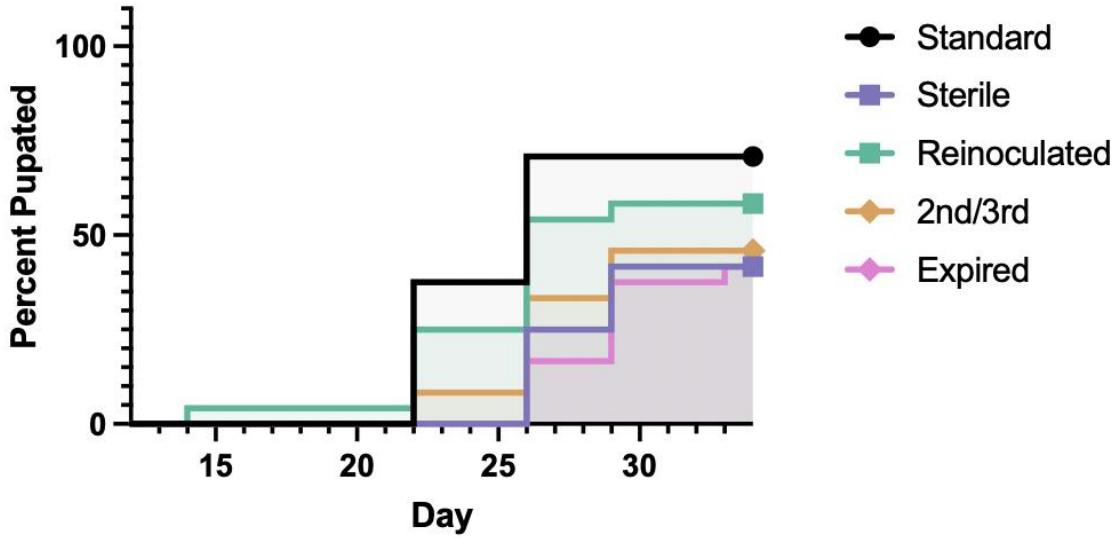
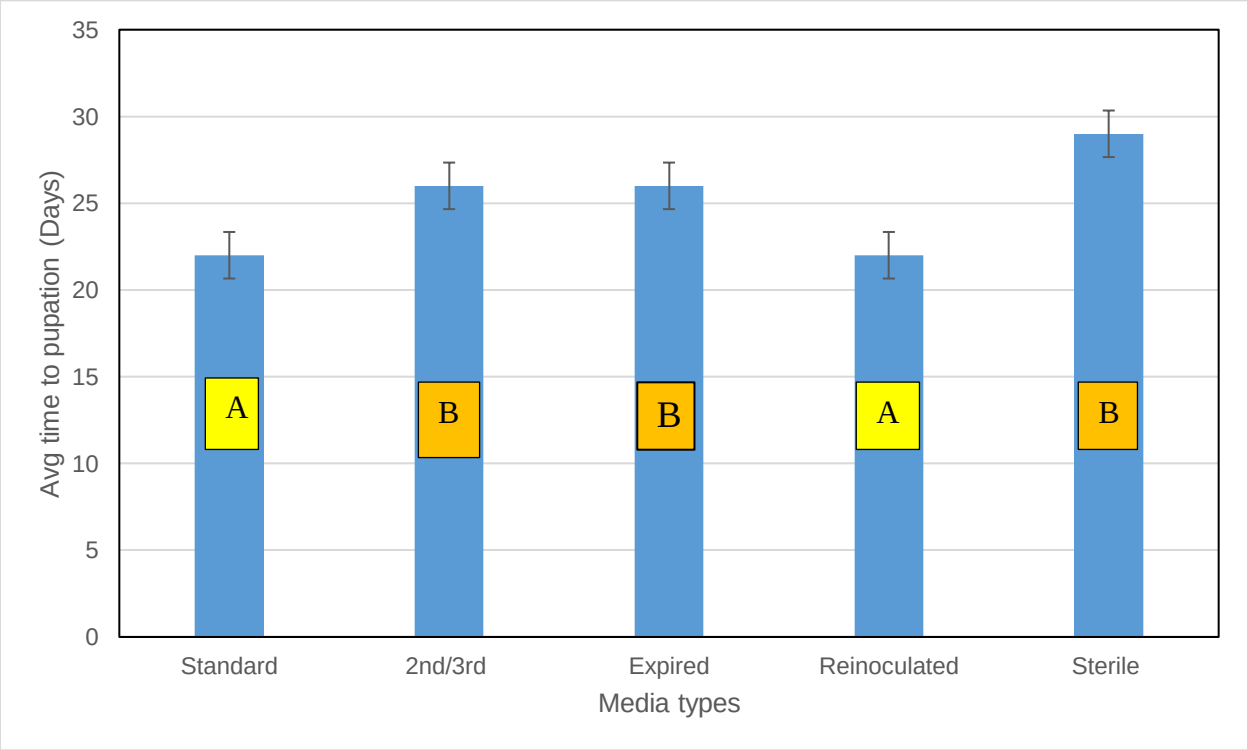


Table 6. ANOVA table on the effect of media sterility and media stages on larval development time of larvae fed five treatment media: standard, 2nd/3rd, expired, reinoculated, and sterile media.

	Df	Sum	Mean Sq	F value	P value
Sterility & stages	4	623.9	156.0	21.36	<0.0001
Residuals	58	423.5	7.3		

Figure 8. The effect of media sterility and media stages on larval development time of larvae fed standard, 2nd/3rd, expired, reinoculated, and sterile media. The error bars represent standard error. The letters (A and B) indicate the post-hoc results of the difference between the development time of the five treatment groups.



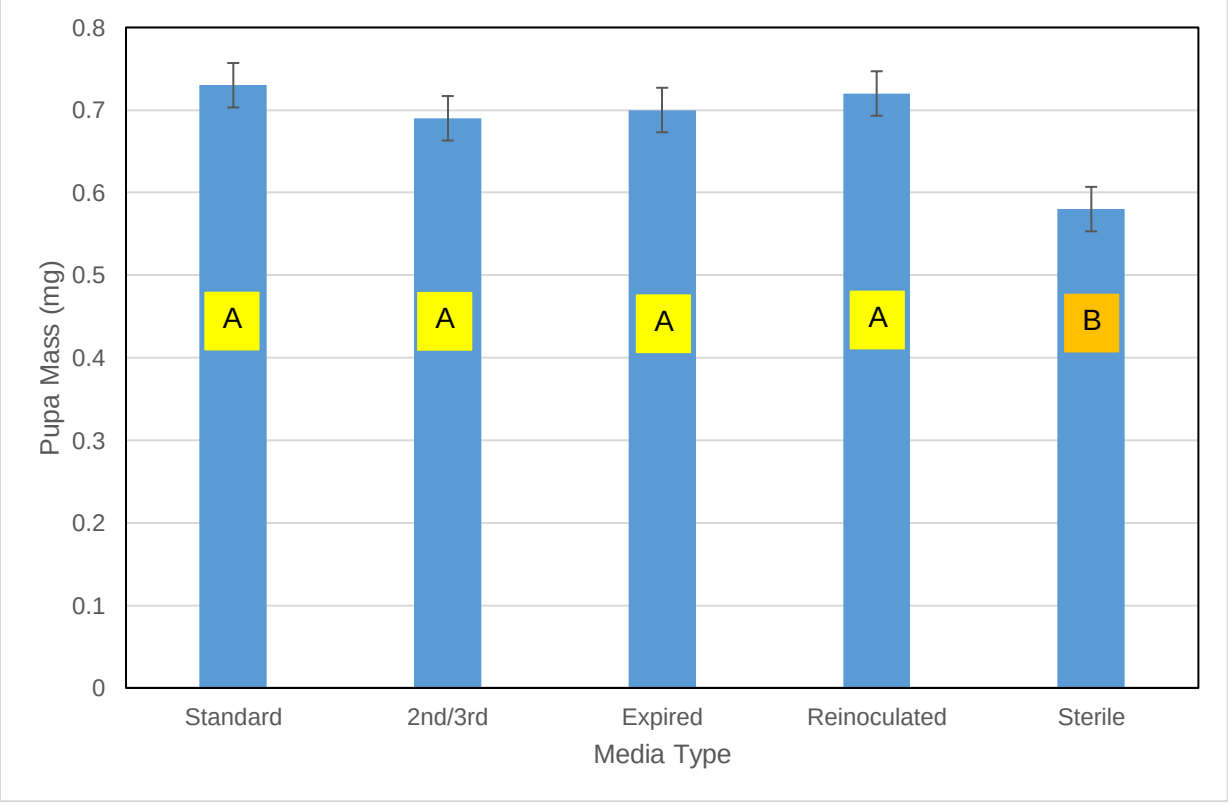
Effect on larval growth

The ANOVA results a marginally significant effect of the treatment on pupal average mass (Table 7). The main effect is due to sterile medium being substantially lower than all the rest with average pupal mass of 0.58 mg as opposed to the pupal mass of larvae fed standard media of 0.73 mg, reinoculated media of 0.72 mg, 2nd/3rd conditioned media of 0.69 mg and expired media of 0.70 mg (Fig. 9).

Table 7. ANOVA table on the effect of media sterility and media type on larval growth.

	Df	Sum	Mean Sq	F value	P value
Sterility & Stages	4	1.115	0.2787	2.331	0.0601
Residuals	114	13.632	0.1196		

Figure 9. The effect of media sterility and media stages (types) on larval growth demonstrated by average pupa masses. The treatment media types are standard, 2nd/3rd, expired, reinoculated, and sterile. The error bars indicate standard error. The letters (A and B) indicate the post-hoc results of the differences between the sterile media and the other four media types.



Effect on larval survivorship.

The logistic regression model demonstrated a marginally significant effect of media sterility and media stages on proportion of survival to pupation (Table 8, 9). The results show higher survival proportion in larvae feeding on standard (67%) and reinoculated media (58%) than larvae feeding on sterile media (42%) (Fig 10, Table 8). The analysis also shows larvae feeding on $2^{nd}/3^{rd}$ (42%) and expired media (46%) displayed similar survivorship to larvae fed sterile media (Table 9). The survival proportion of larvae feeding on $2^{nd}/3^{rd}$ media was the lowest at 42% (Fig. 10).

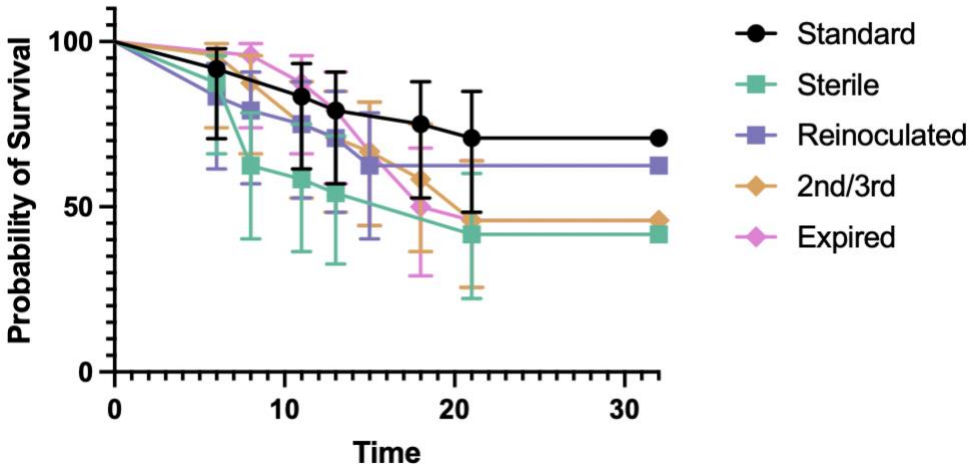
Table 8. Logistic regression table of the effect of media sterility (standard, reinoculated, and sterile media) on larval survivorship.

	Estimate	**Standard Error**	**Z value**	**P value**
Intercept	0.9550649	0.4447880	2.147236	0.03177449
Sterility	-0.2808004	0.1490743	-1.883628	0.05961537

Table 9. Logistic regression table of the effect of media stages (standard, 2nd/3rd, expired) on larval survivorship.

	Estimate	**Standard Error**	**Z value**	**P value**
Intercept	0.6890435	0.3914366	1.760294	0.07835794
Stages	-0.5146357	0.2993916	-1.718938	0.08562564

Figure 10. Kaplan-Meier survival curve plotting survivorship proportions of larvae fed five treatment media: standard, 2nd/3rd, expired, reinoculated and sterile media. The error bars represent standard error.



Confirmation of bacterial presence/absence in the sterilized medium:

The confirmation of bacteria presence/absence in sterilized media is necessary to test the effect of possible contamination of the sterile media on surviving larvae fed this treatment media. Colony counting was performed with 100uL from 6 wells for the sterile plate (n=3 for survivors, n=3 for non-survivors) used to make serial dilutions (10^-1, 10^-2, 10^-3). I found that 10^-1 and 10^-2 dilutions were too numerous to count, but 10^-3 was too few to count, making the data unquantifiable. However, upon observation, qualitatively, the sterile plate wells with living larvae had more bacteria than the sterile wells with dead larvae.

CHAPTER IV: DISCUSSION

Based on the PPH that natural selection selects females that make oviposition site selections that match their offspring's needs and enhance their fitness, I evaluated the effect of microbial presence in larval food and larval food type/stage on sand fly larvae development and survival. A study by Peterkova-Koci et al., 2012 demonstrated that most bacteria increased the rate of development of *Lu. longipalpis* sand flies compared to the sterile medium. My study aimed to confirm the effect of microbial presence on the development of *Ph. Papatasi* sand flies and examines the effects of media types on their development. Additionally, I aimed to evaluate the correlation between the most attractive $2^{nd}/3^{rd}$ instar rearing substrate found by Marayati et al. (2021) correspond to positive or negative fitness consequences in larval development.

Overall, I hypothesized that a sustainable microbial community in larval media is beneficial for the proper development (time-to-pupation) of sand fly larvae. Additionally, I hypothesized that the behavioral oviposition attraction should correspond to larval growth (pupa mass) and survival proportion. Consequently, I predicted that neonate larvae fed sterile larval food would develop slower, have higher mortality, and have lower pupal mass than those fed standard larval food or sterile larval food reinoculated with the bacterial filtrate from standard media. Regarding the effect of behavioral oviposition attraction corresponding to larval development, I predicted that larvae fed rearing medium of $2^{nd}/3^{rd}$ instar would develop faster, have less mortality, and have higher pupal mass than those fed on other diets (standard, reinoculated, or expired media).

I. Effect of bacterial presence on larval development.

The approach for this portion involved surface sterilizing sand fly eggs. Once hatched, the larvae were fed standard, reinoculated, and sterile media. It is worth noting that the sterile

media source was autoclaved weekly to ensure sterility. The plates were covered in parafilm punctured with small holes and stored in the lab's non-sterile sand fly colony incubator, raising concerns about contamination in the larvae fed sterile media reaching pupation. The proximity of the three treatment groups in the same cell culture plate might be another source of contamination.

The analysis of the effect of microbial presence on larval development shows lowest development time with larvae fed axenic (sterile) media compared to larvae fed standard and reinoculated media (27 days vs 25 and 24 days). There was no statistically significant difference effect of media sterility on development time. However, when considering survivorship, there was a higher survival proportion for larvae fed on standard and reinoculated media as opposed to sterile media (44% vs. 85% and 75%). The results confirm significant difference of media sterility on percent survivorship and pupal mass of larvae fed sterile media and larvae fed standard or reinoculated media. However, the surviving larvae fed sterile media were likely exposed to or contaminated with microbes that enhance survivorship. This was assessed by conducting colony counts of samples from the sterile cell culture plates of larvae that died and larvae that reached pupation. The data was unquantifiable, either too numerous to count or too few to count. However, qualitatively, the surviving samples from the sterile plate had more bacteria than the non-surviving sample of the sterile plate. This can be further evaluated with qualitative PCR to delineate the bacterial communities that may aid in the development and survival of these larvae.

II. Effect of larval food type on larval development.

The study by Peterkova-Koci et al. 2012 found that the living bacterial community in rabbit feces creates cues that enhance oviposition in gravid females. Peterkova-Koci et al. 2021,

demonstrated that, *Lu. longipalpis* sand flies' behavioral responses to attractive media correlate with positive fitness, consistent with the PPH. Additionally, based on Marayati et al. in 2015, gravid females are attracted to and oviposit at the highest rate in rearing media conditioned with $2^{nd}/3^{rd}$ instar larvae than the pre-larval (rabbit feces, fresh larval food) or post-larval (expired colony medium) rearing media. I sought to evaluate the correlation between the oviposition attractants and the positive or negative consequences on larval development. I predicted larvae fed $2^{nd}/3^{rd}$ media, the most attractive larvae conditioned rearing medium found by Marayati et al. 2015, would develop faster, have higher survivorship, and have a higher pupal mass than larvae fed standard, reinoculated, or expired media. However, results from my study showed neonate larvae fed $2^{nd}/3^{rd}$ media demonstrated longer development time (26 days vs 22 days) lower percent survivorship (42% vs 67%) and lower pupa mass (0.69 mg vs 0.73mg) than larvae fed standard media. These somewhat surprising findings could be explained by considering that the resources neonate larvae require for development, growth, and survival is in the process of being depleted ($2^{nd}/3^{rd}$ conditioned media) or has already been depleted (expired media). Specifically, previous studies show gravid females' oviposition site selection is mediated by the presence of eggs/early instar larvae which may indicate a viable and favorable site while the presence of older larval stages indicates the oviposition site is deleted of resources. This is an example of an ecological trap, which occurs when conflicting cues are present in a habitat, and organisms erroneously favor areas where their fitness is lower than in other available habitats (Hale & Swearer, 2016). In this case, gravid females may sacrifice potential short-term attraction benefit in terms of larval development and survivorship for long-term fitness consequence.

Some limitations of this study are I did not evaluate the assumption that larvae are born sterile. I also did not culture all of the wells of the cell culture plates; I only cultured the sterile

wells. In the future, I plan to culture all the wells or perform qPCR to obtain quantitative analysis of the level of bacteria at the end of the experiment. Also, I was not able to keep the media sterile due to the plates being covered with punctured parafilm and stored in our lab's unsterile incubator. This may be prevented with use of membrane filters on the cell culture plates to minimize exposure and contamination. Additionally, I was only able to check for bacterial presence and not other microbes via plating method. These limitations are the main reasons why I cannot conclusively establish the benefit or necessity of microbes on larval development and survival.

CHAPTER V: CONCLUSION

Overall, my study aims were to test the effect of microbial presence in sand fly rearing medium on larval development and the effect of larval food stage/type on larval development. Essentially, are microbes "beneficial" or "essential" for larval growth and survival? My findings support my hypothesis that a viable microbial community in larval media is important for the proper development of sand fly larvae. Microbial communities influence several elements of sand flies' biology, including their growth (measured as size at pupation), survival (proportion surviving to pupation), and development rate (time to pupation). The larvae fed on standard media displayed the fastest developmental rate, higher survival rate, and higher mass than those fed on sterile media. The larvae fed on reinoculated media also demonstrated faster development and higher survival rate than larvae fed sterile media confirming that the effect of sterilization is of microbial removal and not change of the autoclaving process on rearing medium chemistry. My results support previous studies that non-sterile media composed of rabbit feces has microbial community that could play an important role in the development of sand fly larvae. The study showed that *Lu. longipalpis* larvae could develop in sterile rabbit feces, but the larvae's survival rate was substantially lower and their time to reach the adult stage was significantly longer than in standard rabbit feces. This confirms the difference between the importance of microbes versus the necessity as larvae were still able to develop in sterile media.

These findings are inconclusive to my study question regarding the benefit or necessity of microbes on larval development and survival. Additionally, future studies are needed to test the effect of attractive and repellent bacteria on larval development and survivorship along with the effect of larval sterility on the offspring's fecundity.

However, my findings did not support my hypothesis that the most attractive conditioned rearing medium ($2^{nd}/3^{rd}$) would promote positive fitness consequences regarding larval development. In the context of the PPH assumption, gravid females do make oviposition site selections that match their offspring's needs. However, these attractive habitats mediated by the presence of eggs/early instar larvae, may potentially emit misguided cues that sacrifices larval performance and long-term fitness consequence.

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