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Aquatic Stage Mosquito Biocontrol: Effects of Entomopathogenic Fungi Blastospores and Conidia on *Aedes aegypti* Pupae

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AQUATIC STAGE MOSQUITO BIOCONTROL: EFFECTS OF ENTOMOPATHOGENIC FUNGI BLASTOSPORES AND CONIDIA ON *AEDES AEGYPTI* PUPAE

A Thesis

by

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ABSTRACT

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Fungal entomopathogens present an opportunity to produce mosquito controls on-site, from remote locations to developing communities. Locally produced biopesticides have the potential to empower communities to develop internal disease vector control initiatives to protect local public health. We tested the effectiveness of the conidia solution and blastospore solution of *Metarhizium anisopliae* ESALQ 9, *Metarhizium anisopliae* ESALQ 1037, *Isaria fumosorosea* Apopka 97, and *Isaria fumosorosea* Ifr 9901 in controlling *Aedes aegypti* pupae. We observed that entomopathogenic fungi liquid blastospore solution induce mortality in *Aedes aegypti* pupae at a significantly greater rate than conidia solution and control treatment. However, further analysis of testing methods used in this study reveal that results may be an inaccurate representation of the fungi's effect on mosquito pupae.

DEDICATION

I would like to dedicate this thesis to my brother, Marcelo, who generously lent me his computer after I broke both of mine so that I may finish my research. I would also like to dedicate this thesis to mi Madre, Ana Isabel, who pushed me to finish this document at every opportunity she found. From you, I am. I would also like to dedicate this thesis to mi Padre, Mario Alberto, who lifts me up in any path I choose to follow. From you, I am. I would also like to dedicate this thesis to God. The God of Nanak. The God of Moses. The God of Abraham. The God of Isaac. The god of Jacob. The God of Elijah. The God of Israel. Thank you for making an environment so vast and mysterious, that we practice science in order to understand it piece by piece and live better by its side day by day. He is. I am.

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CHAPTER I

INTRODUCTION

Ecology of *Aedes aegypti*

The mosquito (Diptera: Culicidae) is considered the most dangerous animal to humans in the world (Gates 2015). Historian Timothy Winegard, author of the book "The Mosquito: A Human History of Our Deadliest Predator," argues that mosquito-vectored disease is responsible for killing as many as 52 billion people over the course of human existence, roughly half of all people who have ever lived (Winegard 2019).

Worldwide, mosquitoes are responsible for about one to two million deaths per year (Carabello 2014). The most vulnerable populations to the mosquito disease vector are often found in developing communities with limited access to markets and servicing agencies that provide mosquitocide protection for public health (WHO 2017).

According to the European Centre for Disease Prevention and Control, the *Aedes aegypti* mosquito is predominantly responsible for vectoring yellow fever virus, dengue virus,

chikungunya virus and ZIKA virus (ECDC 2016). The World Health Organization also identifies *Aedes aegypti* as a vector for Lymphatic filariasis and Rift Valley fever (WHO 2018).

The goal of this study is to illuminate novel approaches to mosquito biocontrol, crafting the tools to empower local communities to take their public health into their own hands.

The Globalization of *Aedes Aegypti*

In 2018, researchers at Yale University, Dr. Jeffrey R Powell, Dr. Andrea Gloria-Soria, Dr. Panayiota Kotsakiozi attempted to recreate the last 600 years of *Aedes aegypti'*s migration history. Researchers reviewed studies on the population genetics of *Aedes aegypti* as well as findings of epidemiological records of disease occurrences transmitted by *Aedes aegypti*. Researchers argue that most of *Aedes aegypti's* great migrations were concurrent with great human migrations across history.

Aedes aegypti shares an evolutionary history in Africa with a familiar native African vertebrate host, humans. The wild African *Aedes aegypti formosus* subspecies started to become domesticated as it came into contact with humans and their dwellings -- using humans as a primary blood source, and human-made water containers for oviposition and larval development. *Aedes aegypti aegypti,* the domesticated subspecies outside of Africa, split from *Aedes aegypti formosus* 400-550 years ago with the rise of transatlantic shipping by Europeans. Starting in the 16th century, ships originating in Europe would stop in West Africa to load native Africans for the slave trade prior embarking on their two to four month Atlantic crossing. In their stop in

West Africa, ships would also resupply with fresh water from coastal villages, which likely included *Aedes aegypti* eggs and larvae. Since these eggs were from mosquito populations that lived alongside humans in villages, they were likely preadapted to breed in human-made water storage containers, like those present on the European ships.

The first reports of yellow fever in the Americas was in 1648 in La Habana, Cuba and Yucatan, México. The yellow fever virus is also native to Africa. Since the yellow fever virus remains infectious in human carriers for only seven to ten days, adult mosquitoes rarely live more than one month, and vertical transmission of yellow fever virus from adult mosquito to egg cytoplasm is very rare, it is very likely that multiple mosquito generations and yellow fever transmission cycles occurred during the two to four months of the transatlantic trip.

Ships from the New World navigating back to their European ports of origin introduced *Aedes aegypti* to the Mediterreanean region around 1800, where it was established until about 1950. The Suez Canal opened in 1869, and with it, *Aedes aegypti* was introduced into Asia, establishing itself by the 1870s, then onto Australia (1877) and the South Pacific (1904). Today, we can find *Aedes aegypti* in most tropical and subtropical climates across the globe (Powell et al. 2018).

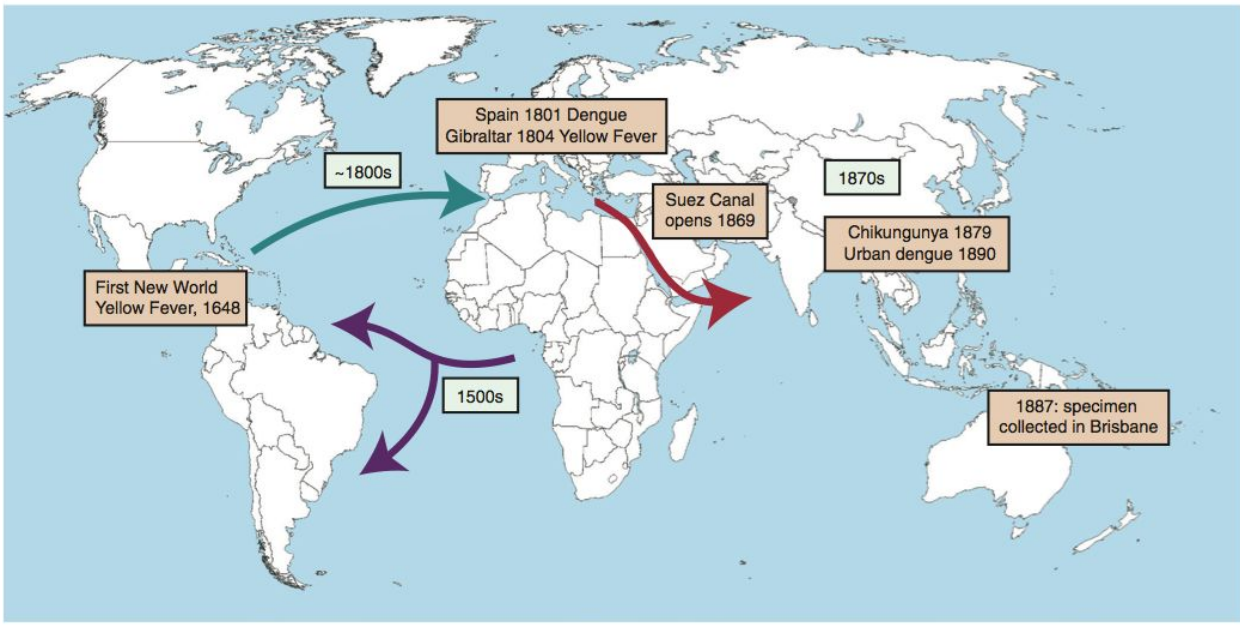

Figure 1.01 Summary of the history of *Aedes aegypti* over the last 600 years. The proposed routes of movement are shown by the arrows with proposed dates. Major epidemiological events are noted in boxes. The times are consistent with dates of separation of mosquito populations estimated from genetic data. Image from "Recent History of *Aedes aegypti*: Vector Genomics and Epidemiology Records," by J.R. Powell et al. 2018 *BioScience,* 68(11), 85.

Aedes aegypti adapts well to human environments. In human environments, *Aedes aegypti* is excellent at seeking out dark, secluded resting spots as well as suitable microenvironments, like discarded containers, in which to lay eggs. *Aedes aegypti* eggs are resistant to desiccation, and only hatch when conditions are suitable for larval mosquitoes to develop into adults. Both *Aedes*

aegypti adults and eggs are well evolved to survive a two to four month trip in a ship with humans.

Aedes aegypti eggs are especially well suited for traveling long distances because they have the capacity to remain viable for long periods of time, hatching only when conditions are just right for larval development. Dr. Muniaraj Mayilsamy and his team, of the Vector Control Research Centre Field Station in Nadu, India, experimented with an extreme case, in which they tested *Aedes aegypti* egg viability after a period of 1,889 days (5.15 years). The oviposition paper strips holding the eggs were air dried, placed inside a ziplock polythene bag, and stored inside a thermocol box under room temperature (24–38°C) and humidity between 55% and 60%. Dr. Mayilsamy calculated an initial egg viability at 88.54%, and after 5.15 years, 4.89% of the mosquito eggs still remained viable (Mayilsamy et al. 2019).

Local Mosquito Vectored Disease

Texas. According to the Texas Department of Health and Human Services, in 2017, Texas reported 43 cases of dengue, 15 cases of chikungunya, and 55 cases of ZIKA. In 2017, all 43 cases of dengue were travel related, with the majority of cases reported travel to Mexico (42%) and India (35%). Of the 55 ZIKA cases in 2017 Texas reported 55 ZIKA cases: 5 (9%) were locally-acquired cases transmitted by mosquitoes, 1 (2%) was a congenital disease case whose mother traveled during pregnancy, and 49 (89%) of the cases were travel-related. Furthermore, in 2017, two of the Rio Grande Valley counties made onto to the list of highest reported ZIKA

cases: Cameron ([14 cases] 25%), Harris ([11 cases] 20%), Hidalgo ([8 cases] 15%), and Bexar ([4 cases] 7%) (Texas Health and Human Services 2018).

In 2018, Texas reported 20 cases of dengue, 7 of chikungunya, and 4 of ZIKA. Of the 20 cases of dengue, 19 were travel associated, and 1 case was acquired locally in Starr County. Texas Health and Human Services argues that this local transmission event likely resulted from increased dengue transmission in the neighboring Mexican states of Tamaulipas and Nuevo Leon during the fall of 2018. The majority of travel-associated dengue cases reported travel to Mexico (25%), India (25%), and Southeast Asia (20%). Of the seven chikungunya cases, six were confirmed to be travel-associated, and one case where travel status was not confirmed because the case was lost to a follow-up, and zero locally-acquired cases. Travel-associated cases reported travel to India (50%), Aruba (17%), Mexico (17%), and the Philippines (17%). All of the 4 ZIKA cases in 2018 were travel associated. Cases reported travel to Belize ([2 cases] 50%), India ([1 case] 25%), and Mexico ([1 case] 25%) (Texas Health and Human Services 2019).

In 2019, Texas reported 59 cases of dengue, 12 cases of chikungunya, and 2 cases of ZIKA. Counties with highest concentration of dengue were Dallas (11 cases), Harris (8 cases), Travis (6 cases), Collin (6 cases), and Bexar (5 cases). For chikungunya, the counties with the highest concentration were Dallas (2 cases), Lubbock (2 cases), and Travis (2 cases). County information was not provided for the 2 ZIKA cases (Texas Health and Human Services 2020).

Tamaulipas. Tamaulipas, the Mexican state sharing a border with Texas along the Rio Grande River for 370 km also experiences its share of arbovirus cases. In 2017, Tamaulipas reported 528 cases of dengue, 288 cases of ZIKA, and 3 cases of chikungunya. In 2017, Reynosa, the city bordering Hidalgo County and the Rio Grande Valley Metro area had the highest concentration of dengue (83 cases), with Ciudad Victoria, the capital city 319 km from the Texas border, led with 241 reported cases of ZIKA (Laboratorio Estatal de Salud Pública, 2017).

In 2018, Tamaulipas reported 485 cases of dengue, 636 of ZIKA, and 0 of chikungunya. With its reported 636 cases of ZIKA, Tamaulipas was the state with the highest concentration of ZIKA cases in all of Mexico for 2018 (Secretaria de Salud 2018).

In 2019, Tamaulipas reported 400 cases of dengue, 0 of ZIKA, and 0 of chikungunya. In 2019, Tamaulipas reported 372 cases of dengue. The highest concentrations of dengue in 2019 were in Tampico (101 cases), Reynosa (91 cases), Altamira (72 cases), Matamoros (43 cases), and Ciudad Victoria (27 cases) (Secretaria de Salud 2019; Hernandez 2020).

Table 1.01 Reported number of arbovirus cases in Texas, USA and Tamaulipas, Mexico. Laboratorio Estatal de Salud Pública 2017[1]; Secretaria de Salud 2018[2]; Secretaria de Salud 2019[3]; Texas Health and Human Services 2018[4]; Texas Health and Human Services 2019[5]; Texas Health and Human Services 2020[6]. Reported arbovirus cases are generally higher in Tamaulipas than in Texas.

	Texas Cases	**Tamaulipas Cases**

2017		
ZIKA	55[4]	288[1]
Dengue	43[4]	528[1]
Chikungunya	15[4]	3[1]
2018		
ZIKA	4[5]	636[2]
Dengue	20[5]	485[2]
Chikungunya	7[5]	0[2]
2019		
ZIKA	2[6]	0[3]
Dengue	59[6]	400[3]
Chikungunya	12[6]	0[3]

Economic Drivers of Arbovirus Infection Rates. In 1999, a collaboration of researchers from the Centers for Disease Control and Prevention, City of Laredo Health Department, Secretaria de Salud de México, University of Texas, Texas Department of Health in Laredo, Texas, and the Secretaría de Salud de Tamaulipas en Nuevo Laredo, Tamaulipas, Mexico, decided to answer the question if there was a higher density of urban dengue on the Mexican city of Nuevo Laredo than the Texas city Laredo, and if so, why? Researchers analyzed blood samples from 516 people

(228 in Laredo, 288 in Nuevo Laredo), for anti-dengue immunoglobulin M (IgM) by performing a IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA), and for anti-dengue IgG by IgG-ELISA and mixed dengue antigens.

Researchers found that IgM seropositivity was lower in Laredo (1.3%; 95% confidence interval, CI 0 to 3%) than in Nuevo Laredo (16%; CI 12% to 20%). IgG seropositivity was also lower in Laredo (23%; CI 17% to 28% vs. 48%; CI 41% to 55%). Even though researchers found more prevalence of dengue on the Mexican than on the Texan side, mosquito-infested containers were more abundant on the Texas side of the border: the Breteau Index (the number of infested containers per 100 houses) was 91 in Laredo versus 37 in Nuevo Laredo, which according to researchers puts Laredo, Texas, on par with the Breteau Index during major dengue epidemics in Puerto Rico, 7 times higher than the level the World Health Organization considers "high risk."

When looking at development in both cities, researchers found that 82% of homes in Laredo had central or room air-conditioning, versus 24% in Nuevo Laredo. In Laredo, evaporative coolers (a low-technology air-conditioning device that cools and humidifies air by drawing it from outdoors through a continually wetted screen) were less prevalent, a greater proportion of houses had intact screens in Laredo, the average distance between houses was greater in Laredo, and fewer persons lived in each house in Laredo, Texas.

In their statistical analysis, IgM seropositivity was significantly associated with five variables: absence of air- conditioning, fewer room air-conditioning units, the presence of an evaporative cooler, no travel outside the Laredo/Nuevo Laredo area, and shorter distances to neighboring houses. IgG seropositivity was significantly associated with absence of central air-conditioning,

fewer room air-conditioning units, smaller plot size, a history of crossing the border during the previous 3 months, a greater number of occupants per household, and a shorter distance to neighboring houses.

Researchers estimated that 55% of cases of dengue in Nuevo Laredo would not have occurred if all households in Nuevo Laredo had air-conditioning. Researchers concluded that differences in prevalence of urban dengue between Laredo and Nuevo Laredo were due mostly to economic factors, which led to a difference in lifestyle (Reiter et al. 2003).

Aedes aegypti Physical Traits

Aedes aegypti adults range in size from 4-7mm. *Aedes aegypti's* defining trait are the lyre shaped white scales on the dorsal surface of the thorax. White basal bands that look like stripes are present on each tarsal segment of the hind legs. Their abdomens are generally dark brown, and may also possess white scales. Females are generally larger than males. The main difference between sexes is that females have short, sparse hairs on their antennae, where males have plumose antennae. Males feed primarily on nectar, and females predominantly consume blood, though they can feed on nectar. Both male and females have a dark proboscis or tongue (Samaroo 2015)

Distribution

Aedes aegypti distribution is restricted due to their intolerance of temperate winters and arid heatwaves. Temperatures below 10˚C and above 44˚C results in the death of *Aedes aegypti* larvae (Womack, 1993). *Aedes aegypti* makes its habitat mainly in tropical, subtropical, and mild temperate environments across the globe (Samaroo 2015).

With climate change bringing warmer temperatures to temperate areas, researchers predict that *Aedes aegypti* will have a greater habitat range to expand into in the coming years (Khormi 2014).

Figure 1.02 Global distribution of *Aedes aegypti.* Researchers argue that this range will be altered with climate change. Image from "Climate Change and the Potential Global Distribution of *Aedes aegypti* Spatial Modelling Using GIS and CLIMEX Figures," by H.M. Khormi et al., 2017, Geospatial Health, 8(2), 407.

Habitat & Activity

Aedes aegypti tends to lay its eggs on the walls of containers that have the capacity to collect enough water for eggs to hatch and develop from larvae to pupae and into adult mosquitoes. The eggs can survive desiccation for months, and hatch only after they are submerged in water, usually after a rain (Samaroo 2015).

Aedes aegypti prefers clean water found in many types of domestic containers inside or near human dwellings. Water quality affects the productivity of a potential mosquito breeding habitat. Typically, greater numbers of mosquitoes are produced in bodies of water with poor circulation, higher temperatures and higher organic content. When not feeding or mating, and especially in the warmest parts of the day, *Aedes aegypti* like to rest in cool shaded undisturbed areas (Nazri et al. 2013; Chadee 2013; Focks et al. 1993; Murrell & Steven 2008; Samaroo 2015).

Food and Feeding. Both sexes feed on sugar-containing fluids, like nectar from flowers and fruits. Likewise, during larval development, both sexes feed on organic particulate matter present in the water (Zettel and Kaufman 2013; Samaroo 2015).

Female mosquitoes, on the other hand, feed on blood to provide the necessary nutrients for egg production. In a study where wild mosquitoes were aspirated, identified, and analyzed from 5 different study sites in Thailand, Cornell University researchers found that female *Aedes aegypti* adult mosquitoes fed almost exclusively on humans. Humans represented 99% (658 / 664) of the

meals of female mosquitoes that had fed on a single host species Furthermore, humans represented 97% (86 / 88) of the meals of female mosquitoes that had fed on multiple blood hosts, which included at least one human host. *Aedes aegypti* fed on a lower frequency of other hosts including bovine, swine, cat, rat, and chicken which represented <1% of bloodmeals (Ponlawat & Harrington, 2005; Kanazawa University 2019).

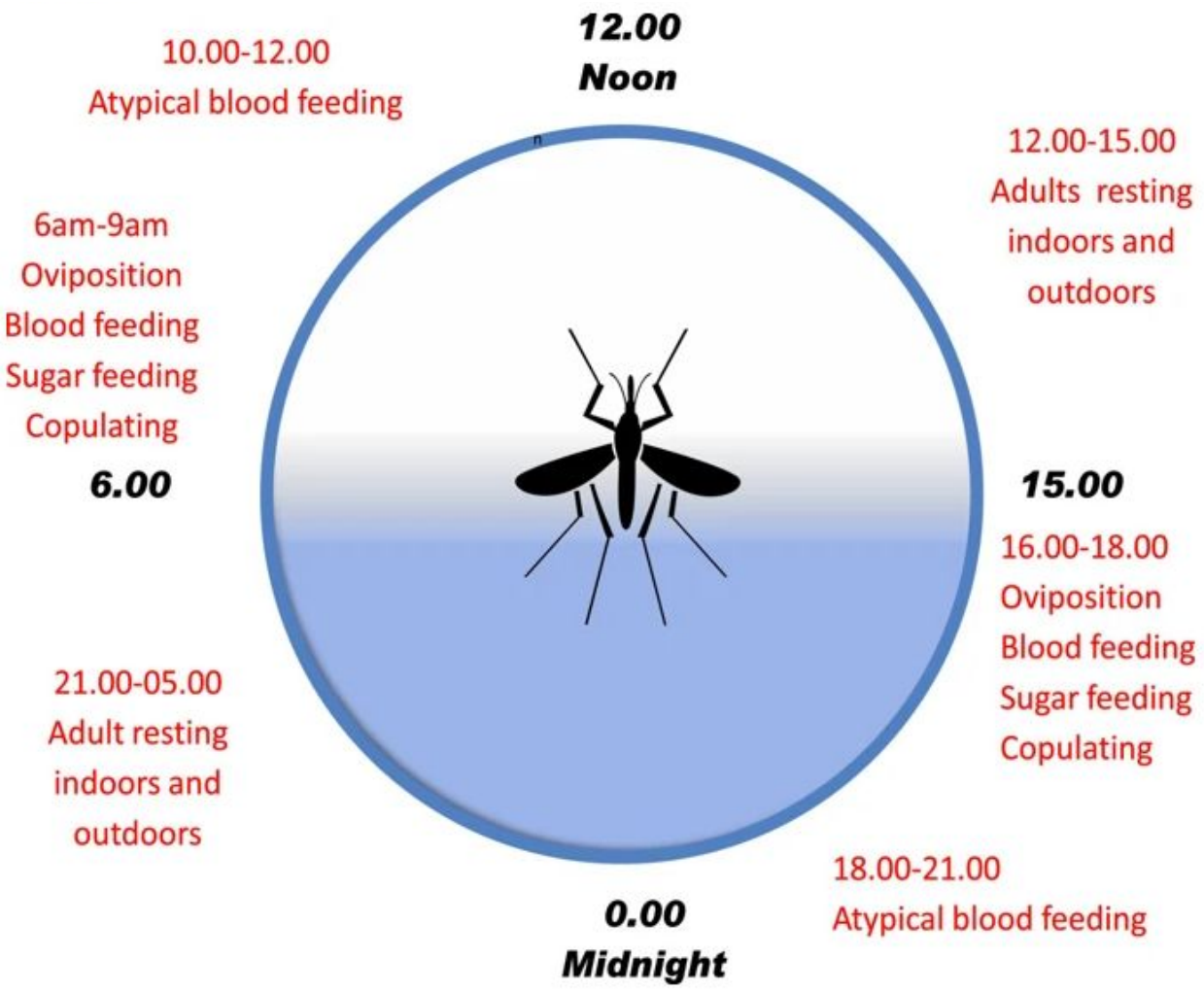

Figure 1.03 Circadian rhythm of *Aedes aegypti* observed in Trinidad. Image from "Resting behaviour of *Aedes* aegypti in Trinidad: with evidence for the re-introduction of indoor residual spraying (IRS) for dengue control," by D. D. Chadee, 2013, *Parasites and Vectors,* 6(255), 5.

Oviposition Behavior. The mosquito lays her eggs approximately 3 days after a blood meal. Females can produce about 100-200 eggs per batch, and can lay about 5 batches of eggs --

500-1000 total eggs in her lifetime. However, the size of the blood meal usually determines the quantity of eggs produced.

Aedes aegypti does not lay its eggs contiguously. Instead, *Aedes aegypti* disperses the laying of a clutch over hours or days, over two or more sites, depending on the availability of suitable substrates (Samaroo 2015).

Life Cycle

Aedes aegypti is holometabolous in its metamorphosis, with a life cycle consisting of an egg, larva, pupa, and adult stage. The entirety of the aquatic cycle can occur in roughly 7-8 days, with water temperature and food supply playing a role in the speed of development (Samaroo 2015).

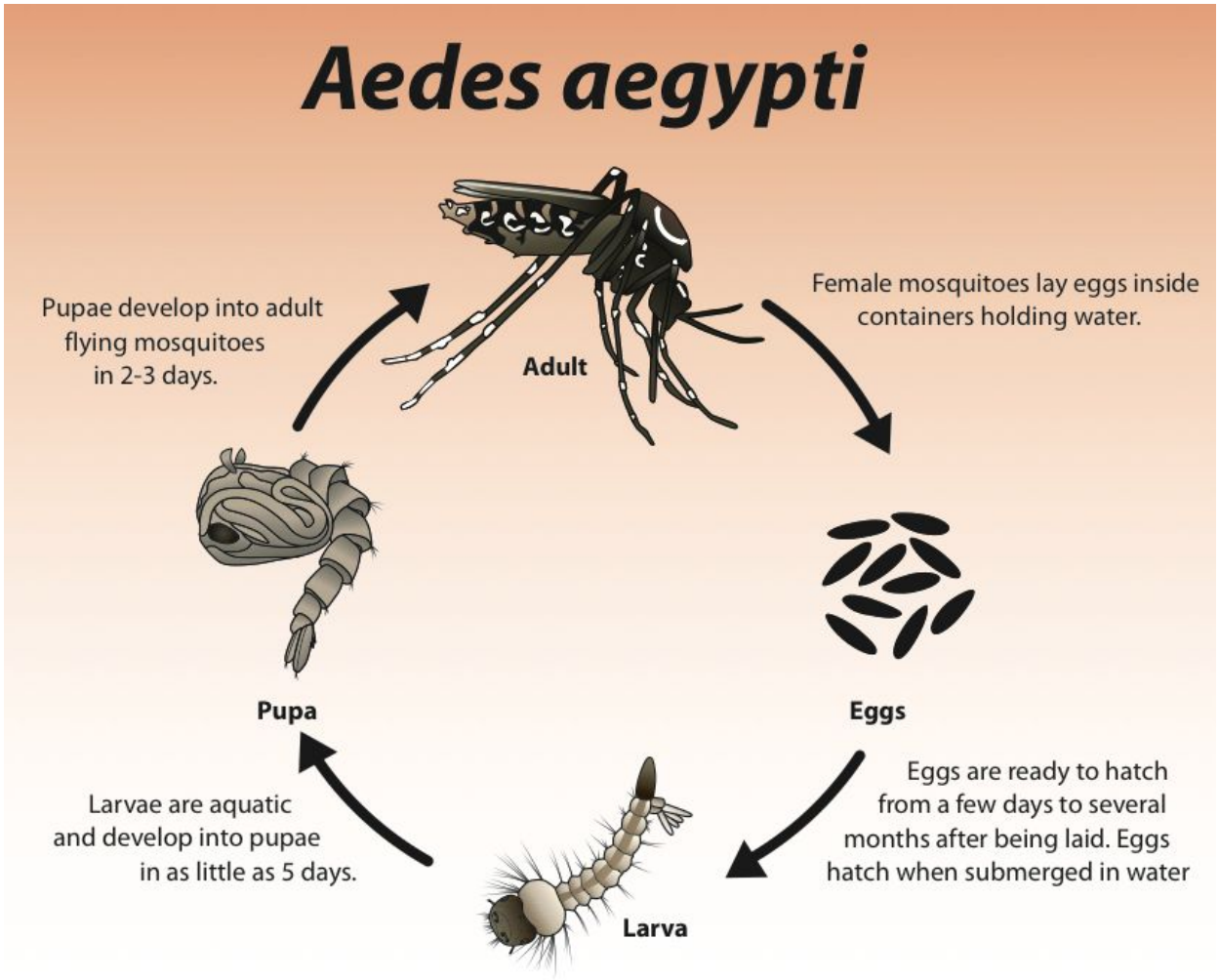

Figure 1.04 Life cycle of an *Aedes aegypti* mosquito. Image from "Mosquito Life Cycle," by CDC, 2019.

Larval Stage. Larvae feed on organic matter in their water habitat, like algae and other microscopic organisms. Most of the larval stage of *Aedes aegypti* is spent at the surface of the water, though larval mosquitoes will swim to the bottom of their habitat if disturbed or when feeding (Nelson 1984).

Larvae are often found around homes in shaded puddles, tires, or within any object holding water. The larvae pass through four instars in their development. Males develop faster than females, so males generally pupate earlier. If temperatures are cool, *Aedes aegypti* can remain in the larval stage for months, as long as both water and food supply is sufficient (Foster & Walker 2002).

Pupal Stage. After the fourth instar, *Aedes aegypti* enter the pupal stage. Mosquito pupae differ from other holometabolous insects in that mosquito pupae respond to stimuli and are mobile.

Pupae do not feed, and take approximately two days to develop. Adults emerge by ingesting air to expand the abdomen thus splitting open the pupal case, and emerging head first.

This is the final stage in the aquatic portion of *Aedes aegypti* life cycle, the last stage where one can eradicate this pest before it develops the capacity for disease transmission. Because mosquito pupae do not feed, popular commercial mosquito biopesticide *Bacillus thuringiensis* var israelensis, which requires ingestion for it to work, is ineffective on pupal stage mosquito (University of Florida 2017; EPA 2018).

Entomopathogenic fungi, another class of commercial biopesticides, however, are capable of killing mosquitoes in their final aquatic pupal stage (Carolino et al. 2019).

Ecology of Entomopathogenic Fungi

Mosquito Biocontrol Candidates

Entomopathogenic fungi like commercially available *Beauveria bassiana* (*Bb*), *Metarhizium anisopliae (Ma)* and *Isaria fumosorosea (If)* are pathogenic to a variety of hosts, including members in the order *Diptera*, like the Yellow-fever Mosquito, *Aedes aegypti* (Alkhaibari et al. 2016; Geetha et al. 1999; Darbro et al. 2011).

Habitat and Distribution

Fungal entomopathogens are globally distributed in almost all terrestrial ecosystems. Diversity is at its highest in the tropical forests, but fungal entomopathogens are also found in extreme habitats such as in the high Arctic tundra and Antarctica (Bridge & Worland 2004; Tosi et al. 2004; Eilenberg 2002; Vega et al. 2012).

Life Cycle

Parasitic Cycle. In their natural soil ecosystems, fungal spore adhesion onto a host cuticle usually results from passive mechanisms. Insects usually randomly encounter aerial conidia in the soil, and spores adhere to the insect host.

Both conidia and blastospores are covered in a mucous layer which aids the spore in adhering to the host cuticle. The outer mucous layer is composed mainly of glycoproteins that prevent spore desiccation, as well as defend against toxic polyphenols possibly present on the host.

Second, the conidia or blastospore germinates and penetrates the insect integument via a germ tube. Certain glycoproteins in the mucous layer serve an enzymatic function, aiding in the dissolution of the host cuticle and the subsequent uptake of nutrients necessary for germination.

Finally, the fungus enters the insect body, colonizes the host's haemocoel, generally resulting in insect death. The fungus continues saprophytic growth until sporulation (Samson et al. 1988; Alkhaibari et al. 2016).

Saprophytic Cycle. Entomopathogenic fungi, like *Beauveria* and *Metarhizium* species, do not require an insect host to complete their life cycle. They have the capacity for saprophytic growth on decaying organic matter. These fungi's capacity for saprophytic feeding allows them to be reared in a lab on artificial substrates (Ortiz-Urquiza et al. 2014).

Cultivation

Entomopathogenic fungi in the phylum *Ascomycota* like *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* can be reproduced asexually to produce mainly conidia or blastospores. Entomopathogenic fungal spores can be cultivated on cellulose-based substrate

through solid state fermentation (SSF) to primarily produce aerial conidia, or through liquid state fermentation (LSF) to primarily produce liquid blastospores (Sahayaraj 2008).

Solid-state fermentation (SSF) involves the cultivation of microorganisms on moist solid substrates. SSF is a popular production method since most fungi sporulate well on solid substrates. On the other hand, liquid-state fermentation (LSF) involves the cultivation of microorganisms in nutrient broth substrates (Ooijkaas 2000).

Conidia. Both conidia and blastospores are haploid cells that are genetically identical to the parent isolate. Conidia are uniform shaped, hydrophobic spores which are produced in 12–20 days through SSF or agar plate. Conidia are produced through a process of mitosis that happens at the ends of specialized strands of reproductive hyphae. While both *Bb* and *If* produce condia alternately on an extending tip of a conidiophore, *Ma* produces conidia in chains from phialides. Aerial conidia are more durable than blastospores. However, blastospores have been observed to be more virulent against aquatic stage mosquito hosts (Deacon 2005; Alkhaibari et al. 2016).

Blastospores. On the other hand, blastospores are thin-walled, pleomorphic, hydrophilic spores produced within 2–3 days in LSF. Blastospores are produced through the process of budding. In this process, a bud develops on the surface of a strand of hyphae, with the cytoplasm of the bud being continuous with that of the parent cell. Next, the nucleus of the parent cell divides, one of the daughter nuclei migrates into the bud, and the other remains in the parent cell. The parent cell

is capable of producing several buds over its surface by continuous synthesis of cytoplasm and repeated nuclear divisions. After a bud develops to a certain point, and even before it is severed from the parent cell, it is capable of budding via the same process and producing a chain of cells. Eventually, the individual buds pinch off the parent cell and become individual blastospores. Blastospores germinate in the same way as conidia, producing a germ tube, which develops into a new hypha (Alkhaibari et al. 2016; Vega et al. 2012).

Cultivation on Agricultural Byproducts. In a study screening agricultural products and byproducts for the production of entomopathogenic fungal spores and biomass, researchers successfully cultivated *Beauveria bassiana, Paecilomyces fumosoroseus,* and *Verticillium lecanii* conidia through SSF on rice, wheat, sorghum, pearl millet, raghi, maize, carrot seeds, jack seeds, ladies fingers, and rice husk. Researchers also successfully cultivated blastospores through LSF on coconut water, rice cooked water, rice wash water, and wheat wash water.

Researchers inoculated 100g of rice with 1 ml of spore suspension and 100 ml of rice cooked water with 1 ml of spore suspension, and observed that *Beauveria bassiana* produced 11.24×10^8 conidia per gram of rice after 14 days incubation in SSF, and 10.21×10^8 blastospores produced per gram of rice cooked water after 7 days incubation in LSF (Sahayraj et al. 2008).

Entomopathogenic Fungi and Mosquito Control

Entomopathogenic fungi are considered a viable ecological pesticide alternative for controlling both aerial and aquatic stage mosquitoes. Commercially available entomopathogenic fungi like *Metarhizium anisopliae, Beauveria bassiana, and Isaria fumosorosea* have been observed to significantly control mosquitoes in their aerial adult stage as well as their aquatic larval and pupal stages (Scholte 2004; Prasad & Veerwal 2012; Bukhari et al. 2011).

Aquatic Stage Mosquito Control

As recommended by the Environmental Protection Agency (EPA), it is beneficial to control mosquitoes in their aquatic stages because it provides an opportunity to control the organism before it develops the capacity to vector diseases in the surrounding community (EPA 2018).

Review of Lethal Spore Concentrations

The following is a summary of findings across research of spore concentration (spores/ml) and mortality rate of several entomopathogenic fungi on aquatic stage mosquitoes. In these studies, spore concentrations range from 1×10^5 to 4.8×10^{10} and mortality rates range from 0% - 100%.

Table 1.02 Summary of lethal concentrations of entomopathogenic fungi formulations on aquatic stage mosquitoes. From Prasad et al. 2013; Alkhaibari et al. 2016; Bitencourt et al. 2018; Benserradj et al. 2014; Rashed et al. 2013; Geetha et al. 1999; & Sani et al. 2016. Spore concentrations range from 1 x 10^5 to 4.8 x 10^{10} spores/ml and mortality rates for aquatic stage mosquitoes range from 0% - 100%.

Researchers	**Year**	**Fungus**	**Spore Type**	**Host**	**Host Stage**	**Concentration (spores/ml)**	**Time**	**Mortality Rate**
Prasad et al.	2012	*Beauveria bassiana*	Conidia	*Anopheles stephensi*	Pupae	4.8 x 10^{10}	7 days	83%
Prasad et al.	2012	*Beauveria bassiana*	Conidia	*Anopheles stephensi*	Pupae	2.56 x 10^{10}	7 days	79%
Prasad et al.	2012	*Beauveria bassiana*	Conidia	*Anopheles stephensi*	Pupae	1.92 x 10^{10}	7 days	64%
Prasad et al.	2012	NA	NA	*Anopheles stephensi*	Pupae	Control (0.01% Tween 80)	7 days	3%
Alkhaibari et al.	2016	*Metarhizium anisopliae ARSEF 4556*	Conidia	*Aedes aegypti*	Larvae	1 x 10^7	5 days	100%
Alkhaibari et al.	2016	*Metarhizium anisopliae ARSEF 4556*	Blastospores	*Aedes aegypti*	Larvae	1 x 10^7	2 days	100%

Alkhaibari et al.	2016	*Metarhizium anisopliae ARSEF 4556*	NA	*Aedes aegypti*	Larvae	Control (0.01% Tween 80)	7 days	2%
Bitencourt et al.	2018	*Beauveria bassiana CG 479*	Conidia	*Aedes aegypti*	Larvae	1×10^7	7 days	43%
Bitencourt et al.	2018	*Beauveria bassiana CG 479*	Blastospores	*Aedes aegypti*	Larvae	1×10^7	7 days	58%
Bitencourt et al.	2018	NA	NA	*Aedes aegypti*	Larvae	Control (0.01% Tween 80)	7 days	0%
Benserradj et al.	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1×10^5	5 days	40%
Benserradj et al.	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1×10^6	5 days	48%
Benserradj et al.	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1×10^7	5 days	76%
Benserradj et al.	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1×10^8	5 days	88%
Benserradj et al.	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1×10^9	5 days	96%

Benserradj et al.	2014	NA	NA	*Culex pipiens*	Larvae	Control (0.01% Tween 80)	5 days	0%
Rashed et al.	2013	*Aspergillus niger*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	68%
Rashed et al.	2013	*Aspergillus ochraceus*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	61%
Rashed et al.	2013	*Aspergillus parasiticus*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	29%
Rashed et al.	2013	*Beauveria bassiana*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	40%
Rashed et al.	2013	*Candida sp.*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	81%
Rashed et al.	2013	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	32%
Rashed et al.	2013	*Penicillium citrinum*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	67%
Rashed et al.	2013	*Penicillium stoloniferum*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	33%
Rashed et al.	2013	NA	NA	*Culex pipiens*	Larvae	Control	14 days	0%

Geetha et al.	1999	*Beauveria bassiana*	Conidia	*Culex quinquefasciatus*	Larvae	1 x 10^8	5 days	84%
Geetha et al.	1999	NA	NA	*Culex quinquefasciatus*	Larvae	Control	5 days	0%
Geetha et al.	1999	*Beauveria bassiana*	Conidia	*Anopheles stephensi*	Larvae	1 x 10^8	5 days	60%
Geetha et al.	1999	NA	NA	*Anopheles stephensi*	Larvae	Control	5 days	0%
Geetha et al.	1999	*Beauveria bassiana*	Conidia	*Aedes aegypti*	Larvae	1 x 10^8	5 days	0%
Geetha et al.	1999	NA	NA	*Aedes aegypti*	Larvae	Control	5 days	0%
Sani et al.	2016	*Metarhizium anisopliae*	Conidia	*Culex quinquefasciatus*	Larvae	1 x 10^6	5 days	60%
Sani et al.	2016	*Metarhizium anisopliae*	Conidia	*Culex quinquefasciatus*	Larvae	1 x 10^7	5 days	80%
Sani et al.	2016	*Metarhizium*	Conidia	*Culex*	Larvae	1 x 10^8	5	90%

		anisopliae		*quinquefasciatus*			days	
Sani et al.	2016	NA	NA	*Culex quinquefasciatus*	Larvae	Control	5 days	0%

Conidia Applied to Aquatic Stage Mosquitoes

In aquatic application, conidia, which are naturally hydrophobic are mixed with a surfactant so that they can mix in the water. The surfactant is usually a 0.01% Tween 80 solution, though oil formulations are sometimes also used. Other studies do not mention the use of a surfactant, like research Dr. Prasad and Dr. Veerwal in MohanLal Sukhadia University in Udaipur, India, which tested *Beauveria bassiana* SSF conidia against *Anopheles stephensi* mosquito pupae. These researchers observed that when treated with 3.5 ml of a 6.4×10^{11} conidia/ml solution in 50 ml of water (final dose final dose 4.8×10^{10} conidia/ml), pupal mortality in the sample population was 83.33%. Mortality was 81.11% when pupae were treated with 2.5 ml of the 6.4×10^{11} conidia/ml solution (final dose 3.2×10^{10} conidia/ml), 78.88% when treated with 2.0 ml (final dose 2.56×10^{10} conidia/ml), and 64.44% with 1.5 ml (final dose 1.92×10^{10} conidia/ml), compared to 3.33% mortality in the control treatment (Prasad & Veerwal 2012).

A final spore concentration of 4.8×10^{10} conidia/ml is relatively high spore concentration compared to other larval studies. Dr. O. Benserradj and his team tested the effect of a range of spore concentrations *Metarhizium anisopliae* on *Culex pipiens* mosquitoes. They tested spore concentrations of 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 conidia/ml. At 96 hours, he found that a spore concentration of 1×10^5 induced 40% mortality, 1×10^6 induced 40% mortality, 1×10^7 induced 40% mortality, 1×10^8 induced 40% mortality, and 1×10^9 induced 40% mortality (Benserradj et al. 2014). Most studies use 1×10^7 as the spore concentration in testing (Alkhaibari et al. 2016; Bitencourt et al. 2018; Carolino et al. 2019).

Pathogenicity of Condia: A Million Ways to Die. In the research literature, there is debate as to the mechanisms behind how conidia cause death for aquatic stage mosquitoes. Dr. Cynthia M. Lacey and her team at the University of Florida examined the mechanisms behind which entomopathogenic fungi induce mortality in mosquito larvae. Dr. Lacey noted that when the *Culex quinquefasciatus* larvae broke the surface water tension with their air-intake perispiracular valves, floating *Metarhizium anisopliae* conidia adhered to the inside surface of the larvae's valves. Those spores subsequently germinated and proceeded to invade the siphon tip tissue, extending into and blocking the trachea, resulting in suffocation and death. Dr. Lacey also noted that conidia that had sunk to the bottom of the containers were ingested by the *Culex* larvae, occluding their gut, inducing mortality within 6 - 24 HR after ingestion (Lacey et al. 1988).

In 2013, Dr. Butt from Swansea University and his team used electron spectroscopy to observe how *Metarhizium anisopliae* interacts with *Aedes aegypti* larvae. For easier identification, his

team used a green fluorescence protein transformed strain of *Metarhizium,* which appeared green in images. Dr. Butt noted that *Metarhizium anisopliae* killed *Aedes aegypti* larvae without adhering to the larvae's cuticle. They observed that conidia ingested by the larvae failed to germinate inside of the larvae. The larvae were instead able to expel ingested conidia in fecal pellets, at least until death. Dr. Butt and his team observed that *Metarhizium anisoplia*e upregulated genes associated with early pathogenic response (proteinases Pr1 and Pr2, and adhesins, Mad1 and Mad2) in the presence of larvae, but the established infection process observed in terrestrial hosts did not progress, and insecticidal destruxins were not detected. Dr. Butt and his team concluded that larval mortality was induced by *Metarhizium anisopliae* proteases triggering stress-induced apoptosis in the larvae, which ultimately led to death.

In all, entomopathogenic conidia have been observed inducing death in mosquito larvae by adherence, germination, penetration, and colonization of the hemocoel by the fungus, by releasing toxins inside of the larval gut, and by releasing toxins in the water around the larvae (Lacey et al. 1988; Butt et al. 2013).

Blastospores in Aquatic Application

Blastospores of entomopathogenic fungi cultivated through LSF have been observed to induce greater mortality rates in larval stage mosquitoes than aerial condia cultivated through SSF. Electron microscopy research by Dr. Alkhaibari from Swansea University in UK and his

associates comparing how blastospore and conidia solutions of *Metarhizium anisopliae* affect *Aedes aegypti* larvae reveals the mechanisms behind the increased virulence of blastospores in aquatic environments (Alkhaibari et al. 2016). These mechanisms may be able to translate into an increased pathogenicity for pupal stage mosquitoes.

Blastospore Solutions. Researchers have observed that when treated with blastospore solutions entomopathogenic fungi induce greater mortality rates on larval stage mosquitoes than when treated with aerial conidia solutions. In 2016, researchers observed that *Metarhizium anisopliae* blastospores caused 100% mortality 2 days post inoculation on *Aedes aegypti* larvae, while wet and dry conidia caused 100% mortality at 5 days post inoculation (Alkhaibari et al. 2016).

Mechanisms Behind Mortality. Whereas traditional conidia solutions of *Metarhizium anisopliae* have difficulty adhering to aquatic hosts, temperature scanning electron microscopy images of *Metarhizium anisopliae* blastospores on *Aedes aegypti* larvae reveal that blastospores adhere to host at a higher rate than conidia because they produce an enveloping water insoluble mucilage. Blastospores also generally germinate faster than condia (2-8 hrs versus 12–24 hrs) which reduces exposure to environmental stressors, as well as gives the host less time to mobilize defense mechanisms.

When comparing pathogenicity of *Metarhizium anisopliae* blastospores to wet and dry conidia solutions, Dr. Alkhaibari observed that at a consistent final dosage of 1×10^7 conidia/ml in 100

ml of distilled water, LSF blastospores of *Metarhizium anisopliae* ARSEF 4556 "were significantly more virulent against *Ae*. *aegypti* larvae than either the wet ($\chi^2 = 49.13$, pairwise $P<0.001$) or dry ($\chi^2 = 55.32$, $P<0.001$) conidial formulations...[with] no significant difference in survival between the wet and dry conidia ($\chi^2 = 0.568$, $P = 0.451$). Blastospores caused 100% mortality 2 days post inoculation (LT50 = 0.92 days), while wet and dry conidia caused 100% mortality at 5 days post inoculation with LT50 values of 2.52 and 2.76 days respectively" (Alkhaibari et al. 2016).

Mucilage Adhesion. Alkhaibari observed that blastospores of *Metarhizium anisopliae* "adhered to almost any part of the mosquito larval cuticle…. Low temperature-scanning electron microscopy (SEM) showed that the blastospores were often covered with copious mucilage which was present in sheet, reticulate and strand form. The mucilage appeared to be water insoluble since it was present when larvae were recovered from water. Mucilage strands were extruded at the fungus-cuticle interface and were particularly abundant at blastospore apices. Mucilage strands were strong as they resisted destruction when samples were plunged in the preparatory pre-cooled nitrogen slush and their structure was not affected by the solvents used during sample preparation for transmission electron microscopy. In thin sections, mucilage appeared as an amorphous, non-uniform, matrix of fibrils that coated the relatively thin cell wall but also extended beyond the blastospores" (2016).

Rather than observing pathogenicity only from SSF conidia adhering to the larval cuticle surface, researchers observed that mortality was due to stress induced in the larva's gut by spore-bound proteases on the surface of ingested conidia (Alkhaibari et al. 2016; Butt 2013; Greenfield 2014).

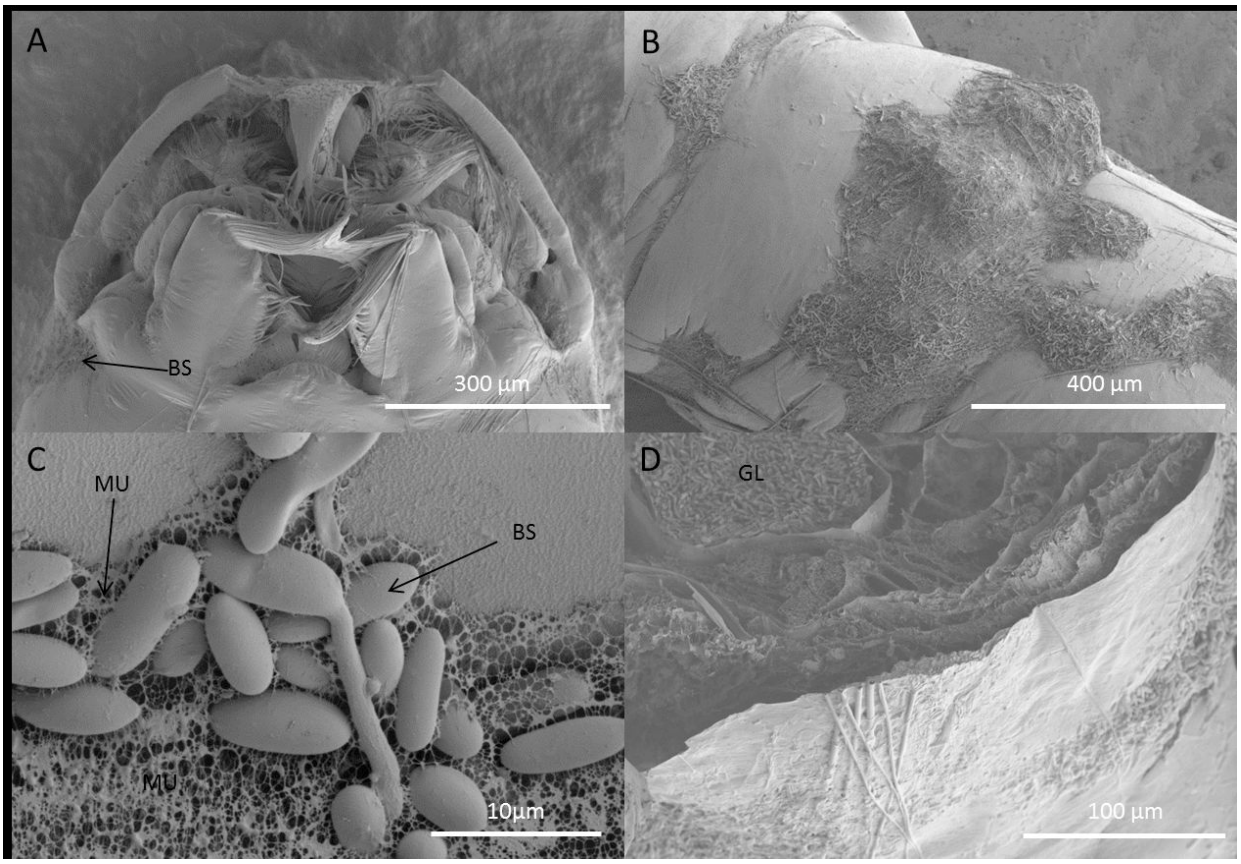

Figure 1.05 Scanning electron microscopy of *Aedes aegypti* larvae with *Metarhizium anisopliae* blastospores. Larvae were inoculated with 1 x 10^7 blastospores/ml and prepared for SEM 20 hrs post inoculation. (A) Head of *Aedes* aegypti larvae showing blastospores (BS) attached to the surface of the cuticle. (B) Blastospores (BS) at different stages of germination attached to the surface of the abdomen. (C) Germinating and nongerminating blastospores surrounded by a

mucilaginous matrix (M). (D) Cross section of infected larvae showing blastospores of *Metarhizium anisopliae* occluding the gut lumen (GL). Image from "*Metarhizium brunneum* Blastospore Pathogenesis in *Aedes aegypti* Larvae: Attack on Several Fronts Accelerates Mortality." by A. M. Alkhaibari, PLoS Pathog, 12(7).

Quicker Death. Dr. Alkhaibari's observation of the capacity of blastospores to adhere to almost every part of the mosquito larval cuticle, brings promise to the employment of entomopathogenic blastospores for controlling mosquito pupae. Further, the observation that blastospores caused 100% mortality 2 days post inoculation, while conidia caused 100% mortality at 5 days post inoculation add to this promise. The major obstacle to fungal pathogenicity in aquatic stage mosquitoes is the relatively short pupal development time. Mosquitoes only remain in their pupal form for 1 to 4 days depending on the temperature, so a quick infection is necessary for effective control (AMCA 2018).

Fungal Frontiers in Research

In September of 2019, a team of researchers from the State University of North Fluminense and the Federal University of Roraima in Brazil published novel research by asking the question: will *Metarhizium anisopliae* induce greater mortality for *Aedes aegypti* pupae when applied as conidia or as blastospores? This is the first research looking at the effect of entomopathogenic fungi blastospores on mosquito pupae.

In this study, pupae were obtained by collecting eggs using oviposition traps deployed in an urban environment. *Metarhizium anisopliae* conidia were produced using solid media and blastospores were produced by inoculating conidia in liquid culture. Mosquito larvae were inoculated with a final spore concentration of 1×10^7 spores/ml. Researchers observed that *Metarhizium anisopliae* blastospores were more virulent against *Aedes aegypti* pupae than conidia. They observed that 100% of pupae died within 24 hours of exposure to *Metarhizium anisopliae* blastospores. Researchers noted that large quantities of mucilage were produced by the blastospores in the presence of the pupae, which aided in adhesion to the pupae's cuticle. Researchers also observed conidiogenesis on the pupal cuticle in blastospores treatments. On the other hand, researchers observed that conidial suspensions resulted in 57% survival at 24 hours and 23% at 48 hours for *Aedes aegypti* pupae. However, researchers also observed that a proportion of the adults, which emerged from pupae exposed to conidia, also succumbed to fungal infection (Carolino et al. 2019).

This study aims to replicate these findings and further investigate the observed increased pathogenicity of entomopathogenic fungi on mosquito hosts.

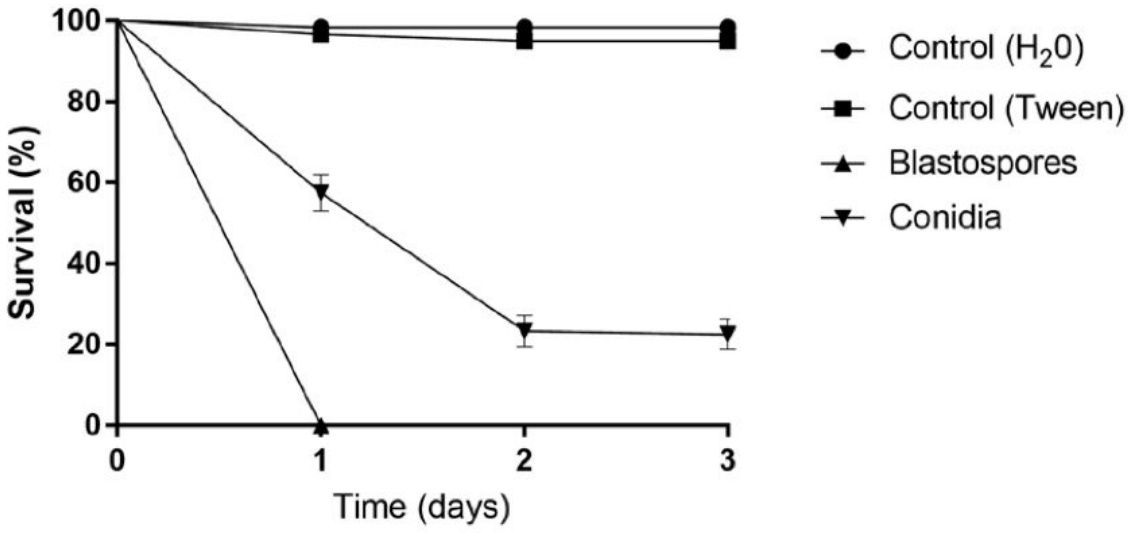

Figure 1.06 Survival curves of *Aedes* aegypti pupae following exposure to blastospore and conidial suspensions (1 x 10^7 spores/ml) during 3 days. Control groups were exposed to distilled water or Tween 20 (0.01%). Image from "*Aedes* aegypti are Highly Susceptible to Infection by *Metarhizium anisopliae* Blastospores," by A. T. Carolino et al. 2019, *Journal of Pure and Applied Microbiology*, 13(3), 1631.

Figure 1.07 *Metarhizium anisopliae* blastospores infecting *Aedes aegypti* pupae, adhering to the host cuticle with copious amounts of mucilage (A and B). After infection by blastospores, conidial sporulation was observed on dead pupae (C). Pupae infected with *Metarhizium anisopliae* conidia showed no mucilage production and no sporulation was observed on the insect integument (D-F). Image from "*Aedes aegypti* are Highly Susceptible to Infection by *Metarhizium anisopliae* Blastospores," by A. T. Carolino et al. 2019, *Journal of Pure and Applied Microbiology*, 13(3), 1631.

CHAPTER II

HYPOTHESES, MATERIALS, AND METHODS

Research Questions and Hypotheses

Research Questions

In this study, the question being posed is:

- Are entomopathogenic fungi spore solutions more effective in controlling pupal stage *Aedes aegypti* when they are applied as a conidia solution or as a blastospore solution?

To answer this question:

- Six entomopathogenic fungi, two varieties from three different species were screened for their effectiveness in killing *Aedes aegypti* pupae when applied as a conidia solution and as a blastospore solution:
    - *Metarhizium anisopliae* ESALQ9 (*Ma* E9)
    - *Metarhizium anisopliae* ESALQ1037 (*Ma* E1037)
    - *Beauveria bassiana* GHA (*Bb* GHA)

- *Beauveria bassiana* ANT03(*Bb* ANT03)
- *Isaria fumosorosea* Apopka97 (*If* A97)
- *Isaria fumosorosea* Ifr 9901 (*If* 9901)
- Control 0.01% Tween 80 solution

For each of the entomopathogenic fungi varieties being tested, the following three research questions are being asked:

1. Is pupal mortality in the **Blastospores** treatment significantly different from mortality in the **Control** treatment?
2. Is pupal mortality in the **Conidia** treatment significantly different from mortality in the **Control** treatment?
3. Is pupal mortality in the **Blastospores** treatment significantly different from mortality in the **Conidia** treatment?

Hypothesis

Liquid blastospore solution of all tested species of entomopathogenic fungi will induce mortality in *Aedes aegypti* pupae at a significantly greater rate than their conidial solutions and control treatment, respectively.

Pupal Mortality Rates = PMR

$$Control_{PMR} < Conidia_{PMR} < Blastospores_{PMR}$$

For all screened fungi species and varieties.

H_0 (Null Hypothesis):

- Null Hypothesis 1: Pupal mortality in the **Blastospore** treatment is not significantly different from pupal mortality in the **Control** treatment.

- Null Hypothesis 2: Pupal mortality in the **Conidia** treatment is not significantly different from pupal mortality in the **Control** treatment.

- Null Hypothesis 3: Pupal mortality in the **Blastospore** treatment is not significantly different from pupal mortality in the **Conidia** treatment.

H_A (Alternative Hypothesis):

- Hypothesis 1: Pupal mortality in the **Blastospore** treatment is significantly different from pupal mortality in the **Control** treatment.

- Hypothesis 2: Pupal mortality in the **Conidia** treatment is significantly different from pupal mortality in the **Control** treatment.

- Hypothesis 3: Pupal mortality in the **Blastospore** treatment is significantly different from pupal mortality in the **Conidia** treatment.

Methods

Testing Fungal Entomopathogens for Virulence Against *Aedes aegypti* Pupae

The following fungal strains were tested for pathogenicity in mosquito pupae. Spore samples of fungal strains were provided by the Dr. Daniel Flores lab at USDA AHPIS:

Table 2.01 Species of entomopathogenic fungi being screened in this study, where they were sourced, and the abbreviations that will be used for them in this text.

Species	**Strain**	**Source**	**Abbreviation**
Metarhizium anisopliae	ESALQ 9	Metaril	*Ma E9*
Metarhizium anisopliae	ESALQ 1037	Metaril	*Ma E1037*
Beauveria bassiana	GHA	Botanigard	*Bb GHA*
Beauveria bassiana	ANT03	Bioceres	*Bb ANT03*
Isaria fumosorosea	Apopka 97	PFR97	*Is A97*
Isaria fumosorosea	Ifr 9901	NoFly WP	*Is 9901*

Preparing Spore Formulations. Fungal strains were cultivated as conidia on potato dextrose agar (PDA), and blastospores on potato dextrose broth (PDB). Spores were collected, and spore viabilities were calculated by creating a 100x dilution of the collected spores, smearing a PDA agar plate with the dilution, incubating the plate for 18 hours, cutting a 3 mm x 3 mm square of agar, dying the square with Trypan Blue, counting viable and unviable spores under the microscope, and calculating the total observed viable spores divided by total observed spores. Spore counts were subsequently done with a haemocytometer. Spore solutions were diluted to a

final test concentration of 1 x 10^8 viable spores/ml by adding 0.01% Tween 80 solution. The fungal pathogens employed in this study have no recorded secondary effects on mammals (Scholte 2004).

Rearing *Aedes aegypti* Pupae for Testing

Aedes aegypti pupae used for testing were sourced from an F1 generation colony, reared and bred in the lab with guidance from the Dr. Christopher Vitek lab at UTRGV. An F1 generation *Aedes aegypti* colony was started from mosquito larvae sourced from standing water in residential homes around the Rio Grande Valley, and identified under a microscope as adults. No IACUC approval is necessary for testing on arthropods (Cornell 2020).

Figure 2.01 Larvae freshly retrieved from one of the collections sites. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

Wild collected larvae were reared to adulthood and identified as adults under a microscope. Positively identified *Aedes aegypti* adults were placed back into the insect rearing cage, starved for 24 hrs, blood fed, and oviposition traps were placed in rearing cages to collect F1 generation of eggs. Eggs were hatched in an oxygenated nutritive broth, and fed liver powder until pupation was observed. Pupae were collected to be used in testing.

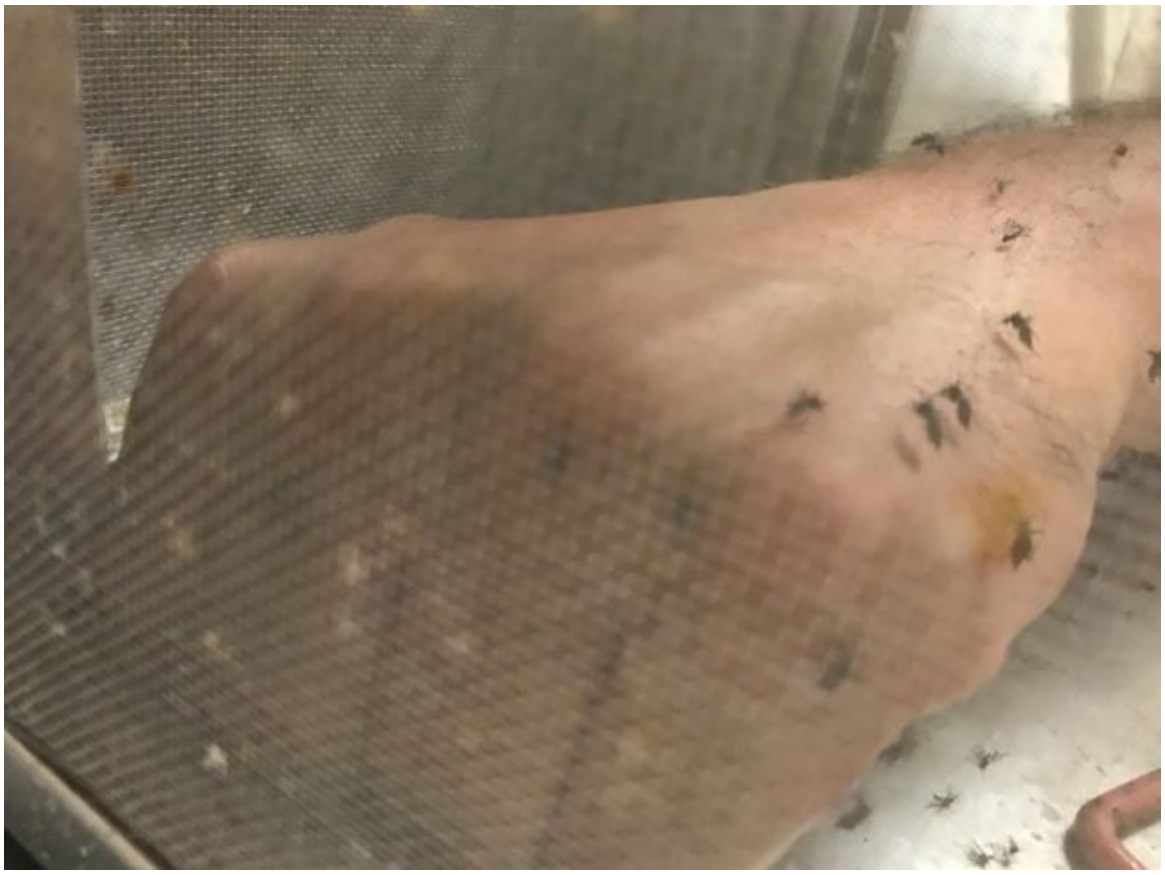

Figure 2.02 Aedes aegypti adult female mosquitoes blood feeding on this researcher. Male mosquitoes lived in the same cage, for mating, and were fed with sucrose solution. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

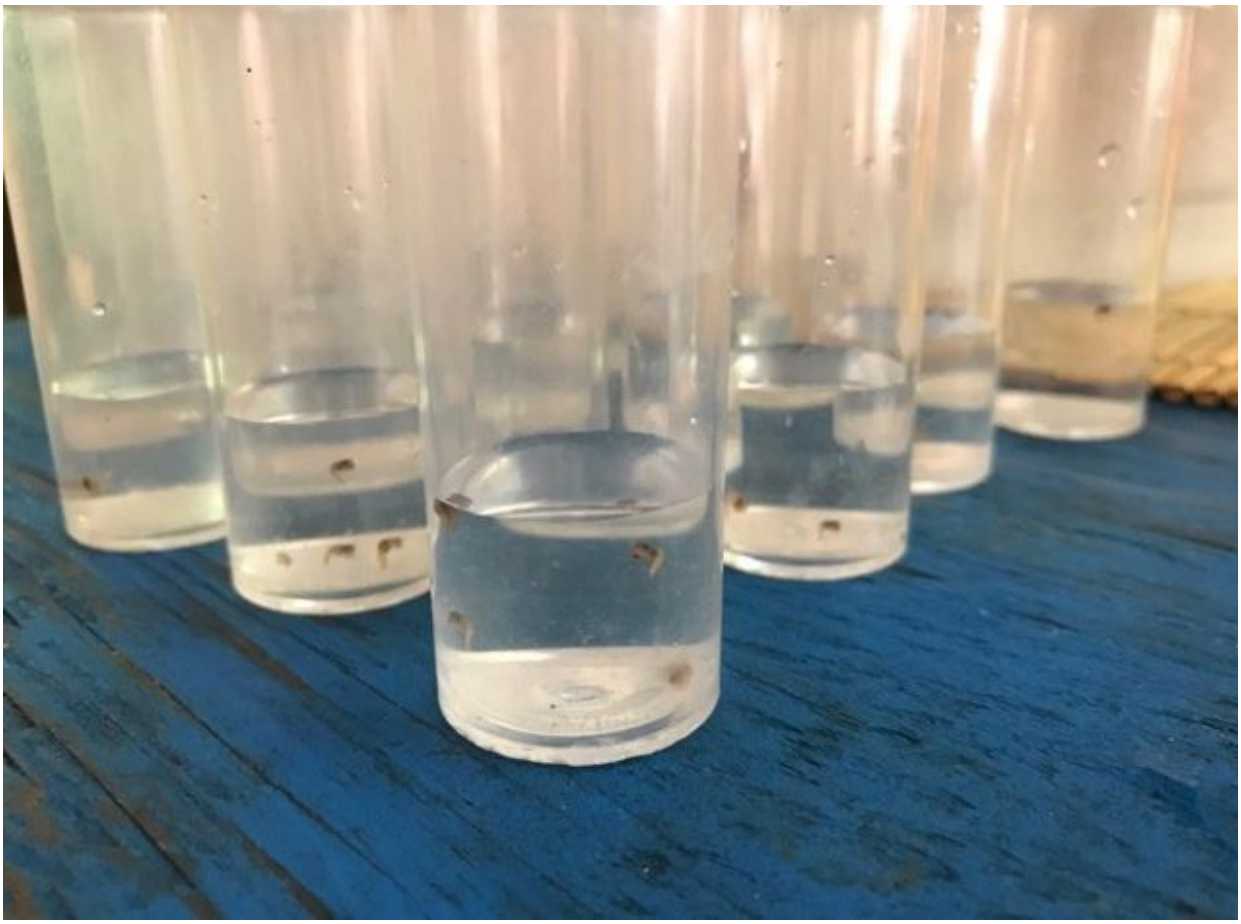

Figure 2.03 *Aedes aegypti* pupae prepared for testing. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

Applying Conidiospore & Blastospore Treatments on *Aedes aegypti* Pupae

Treatment Application. Collected pupae were placed in 25 ml plastic containers containing 9 ml of tap water. Five pupae were placed per container.

1 ml of 1 x 10^8 spores/ml solution was applied to 9 ml of tap water in treatment conditions, for a final concentration of 1 x 10^7 viable spores/ml per treatment, based on Alkhaibari et al. (2016). Treatments were replicated six times for a total of 30 samples tested per condition.

Figure 2.04 *Aedes aegypti* pupae post inoculation. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

Monitoring. Pupae were determined to be deceased if they showed no signs of movement when the sample is disturbed. Pupae that showed even the slightest observable jerking movements

were counted as living. Samples were monitored every 12 hours. Quantity of observed deceased pupae along, observed time of mortality, observed time of adult emergence was recorded. Monitoring continued for 3 days.

Data Collected. The following data was collected for each of the strains tested. *Beauvaria bassiana* was excluded from data collection because samples were contaminated repeatedly when rearing *Beauveria bassiana* fungus in the lab.

Figure 2.05 *Aedes aegypti* pupae killed by *Metarhizium anisopliae* E9 blastospores. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

Table 2.02 Average of mortality data across all conditions. Average mortality and average adult emergence were calculated across all conditions, N = 6. A greater percent mortality for pupae was recorded in the blastospore treatment than in the conidia treatment across all strains of fungi tested.

Metharizhium anisopliae E9	**AVERAGE %Mortality**	**AVERAGE %Adult Emergence**
Ma E9 Conidia	3% (SD = 7.45%)	97% (SD =7.45%)
Ma E9 Blastospores	20% (SD = 16.32%)	80% (SD = 16.32%)
Metharizhium anisopliae E1037		
Ma E1037 Conidia	0% (SD = 0%)	100% (SD = 0%)
Ma E1037 Blastospores	17% (SD = 13.74%)	83% (SD = 13.74%)
Isaria fumosorosea A97		
If A97 Conidia	7% (SD = 14.91%)	93% (SD = 14.91%)
If A97 Blastospores	57% (SD = 21.34%)	43% (SD = 21.34%)
Isaria fumosorosea 9901		
If 9901 Conidia	0% (SD = 0%)	100% (SD = 0%)
If 9901 Blastospores	53% (SD = 31.97%)	47% (SD = 31.97%)
Control		
Control TOTAL	0% (SD = 0%)	100% (SD = 0%)

CHAPTER III

STATISTICAL ANALYSIS OF DATA

Illustrating Data

Survivorship Curves

Data was illustrated as Kaplan-Meyer survivorship curves for each of the mosquito pupae population across treatment conditions.

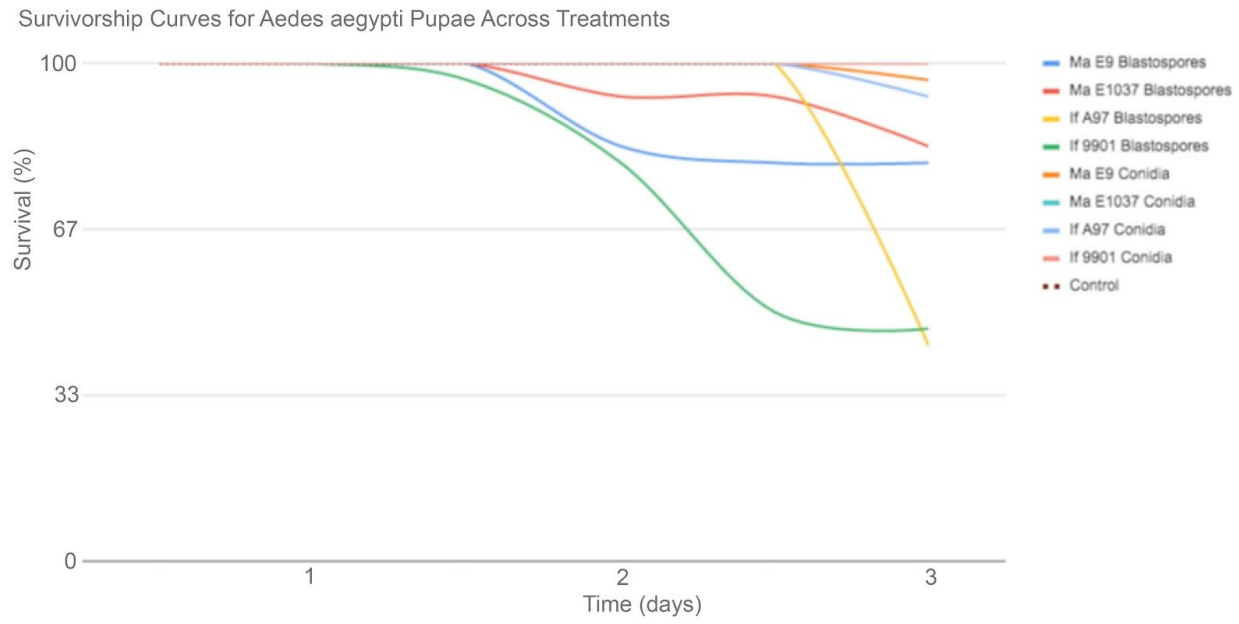

Figure 3.01 Survivorship curves for *Aedes aegypti* pupae when treated with *Ma* E9 blastospores and conidia, *Ma* E1037 blastospores and conidia, *Is* A97 blastospores and conidia, *Is* 9901 blastospores and conidia, as well as a control 0.01% Tween 80 solution.

The four lower curves are the mosquito pupae treated with blastospores, they start to separate from the conidia and control conditions around 36 hours to 48 hours. The five curves from the top are the mosquito treated with conidia as well as the control Tween 80; however, only three curves are visible on the survivorship plot because two treatments -- *If* 9901 Conidia and *Ma* E1037 Conidia overlap with the Control curve since no deaths were observed in these three conditions.

Log-rank Test

A Log-rank test was utilized to analyze if differences observed between curves are statistically significant. Log rank tests were not performed for *If* 9901 Conidia nor *Ma* E1037 Conidia, since no deaths were observed in these conditions, like the control condition. The Log-rank tests if differences observed between two or more entire mortality curves are statistically significant. To perform the log rank test, data was first coded into a Google Sheets Spreadsheet, recording if each individual pupae in each treatment condition experienced a (1) death, (2) what time in the study the death was recorded, and (3) which treatment condition the pupae was subjected to. Table 3.01 demonstrates how to code data in preparation for the Kaplan-Meyer analysis and Log rank test.

Table 3.01 Example table for coding data for Kaplan-Meyer analysis and Log-rank test.

Pupae	**Time**	**Death Event**	**Group**
1	Amount of hours	Binary code	Enter treatment condition
2	elapsed in trial	1 for death	code here
Each pupae gets	until death	0 for no death	(MAE9C, IFA97B, etc.)
its own cell	is recorded		
for example...			
27	48H	1	IF9901B
28	60H	1	IF9901B
29	72H	0	IF9901B
30	72H	0	IF9901B

Analyses were conducted with the help of an open sourced tool created and shared by Croatian Hematologist, Dr. Marko Lucijanić, who programmed a Microsoft Excel spreadsheet for analyzing the effects of several variables on patients' mortality over a duration. The spreadsheet allows for Kaplan-Meyer survivorship analysis, survivorship curves, as well as Log rank test for calculating statistical significance in observed outcome differences over a period of time. A link to his Excel sheet is provided in the works cited.

Dr. Lucijanić's spreadsheet also calculates the P-value and Hazard Ratio. The P-value determines the probability -- assuming the null hypothesis were true -- of observing a more extreme test statistic in the direction of the alternative hypothesis than the one observed. At a 0.05 significance, to reject the null hypothesis, the calculated P-value must be less than 0.05. A P-value less than 0.05 indicates there is less than a 5% probability the null hypothesis is correct and the observed results are random (McLeod 2019).

A hazard ratio is the ratio of the (risk of an outcome in one group) / (risk of an outcome in another group) at a given interval of time. In our study, the hazard ratio refers to risk of mosquito pupae death in one group versus risk of mosquito pupae death in the comparison group. A hazard ratio of 1 means lack of association, a hazard ratio greater than 1 suggests an increased risk, and a hazard ratio below 1 suggests a smaller risk. The reciprocal value that accompanies the hazard ratio is (1 / hazard ratio), which equals the hazard ratio if the groups were switched in order in the comparison (Toledo 2018; Brody 2016).

In analysis, a log-rank hazard ratio is used when there are deaths observed in both groups, Mantel-Haenszel hazard ratio is instead used when there are no deaths observed in one of the groups being compared.

Table 3.02 Log-rank test results determine whether differences observed between mortality curves is statistically significant, P-value, and the Log-rank or Mantel-Haenszel Hazard Ratio with its reciprocal value.

MA E9	**Blasto vs Control**	**Conidia vs Control**	**Blasto vs Conidia**
Reject H(0)	**YES**	NO	**YES**
P value	0.01	0.15	0.04
Log-rank Hazard Ratio (HR)	8.63	7.65	6.36
Reciprocal Value (1 / HR)	0.12	0.13	0.16
MA E1037			
Reject H(0)	**YES**	NO	**YES**
P value	0.02	na	0.02
Mantel-Haenszel haz. Ratio (HR)	8.18	na	8.18
Reciprocal Value (1 / HR)	0.12	na	0.12
IF A97			
Reject H(0)	**YES**	NO	**YES**
P value	<0.001	0.15	<0.001
Log-rank Hazard Ratio (HR)	15.55	7.65	0.10
Reciprocal Value (1 / HR)	0.06	0.13	9.70
IF 9901			
Reject H(0)	**YES**	NO	**YES**
P value	<0.001	na	<0.001
Mantel-Haenszel haz. Ratio (HR)	11.85	na	11.85
Reciprocal Value (1 / HR)	0.08	na	0.08

In all, we observed that across the tested fungi species and varieties:

PMR Blastospore > PMR Control & PMR Blastospore > PMR Conidia

However, we also observed that

PMR Control = PMR Conidia

CHAPTER IV

RESULTS, CONCLUSIONS, IMPLICATIONS, & FURTHER RESEARCH

Results

Blastospores versus Control

Even though the 100% mortality rates by blastospores observed in other research were not replicated, *Aedes aegypti* pupae mortality rates were observed to be significantly greater than the control condition across all blastospore treatments.

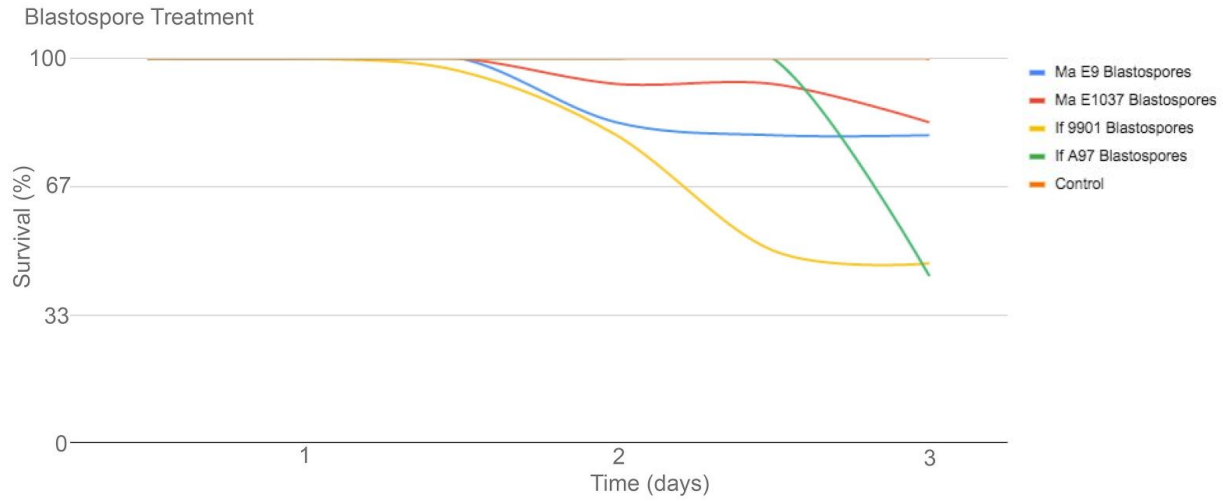

Figure 4.01 Survivorship curves of *Aedes aegypti* pupae exposed to *Ma* E9 blastospores, *Ma* E1037 blastospores, *If* 9901 blastospores, *If* A97 blastospores, and control 0.01% Tween 80 solution.

Conidia versus Control

In contrast to mortality rates observed in other research, in this preset study, *Aedes aegypti* pupae mortality rates were not significantly different than the control condition (0% mortality) across all conidia treatments. Observed mortality rates for conidia treatments ranged between 0% - 7%.

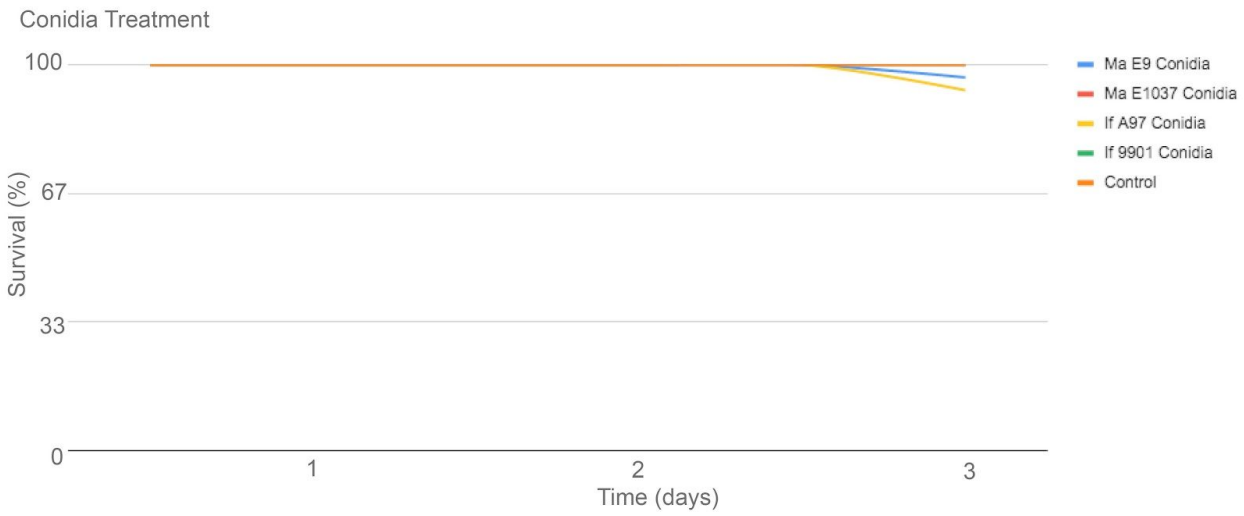

Figure 4.02 Survivorship curves of *Aedes aegypti* pupae exposed to *Ma* E9 conidia, *Ma* E1037 conidia, *If* 9901 conidia, *If* A97 conidia, and control 0.01% Tween 80 solution.

Blastospores versus Conidia

In concordance with other research, *Aedes aegypti* pupae mortality rates were observed to be significantly greater when treated with blastospores, than when treated with conidia, across all fungi tested.

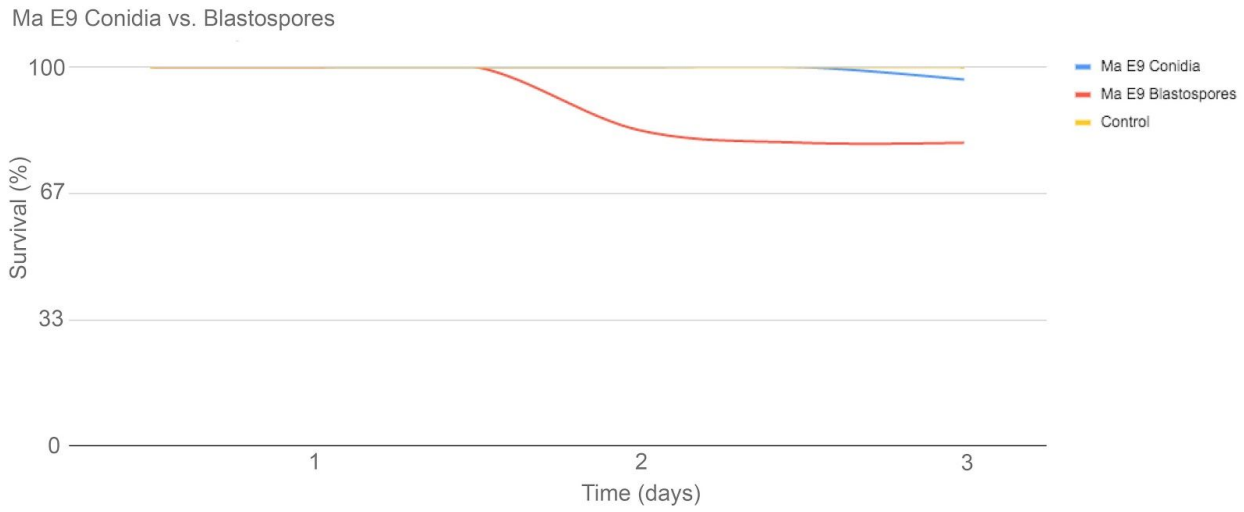

Figure 4.03 Survivorship curves of *Aedes aegypti* pupae exposed to *Ma* E9 conidia, blastospores, and a control 0.01% Tween 80 solution.

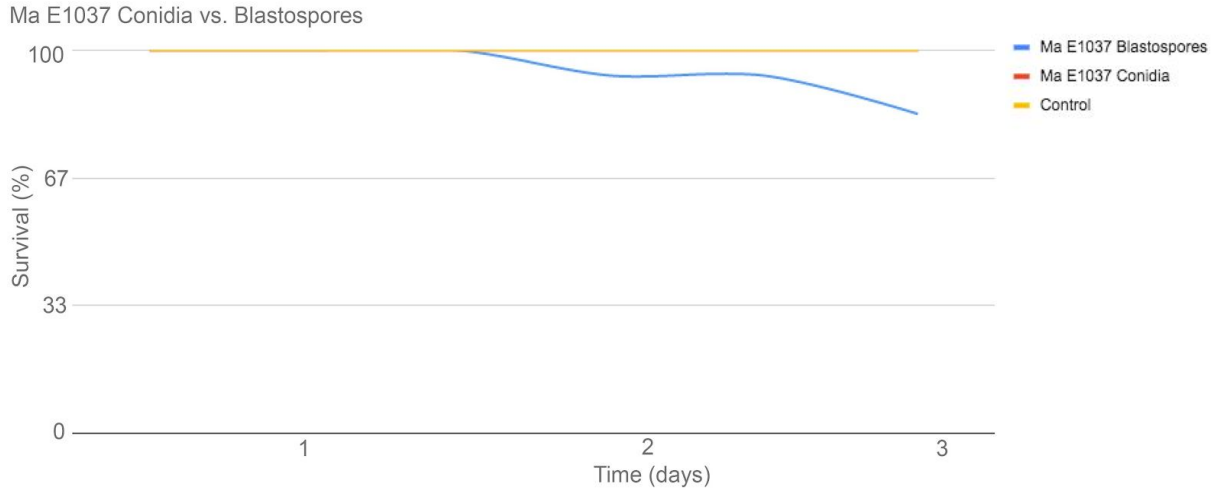

Figure 4.04 Survivorship curves of *Aedes aegypti* pupae exposed to *Ma* E1037 conidia, blastospores, and a control 0.01% Tween 80 solution.

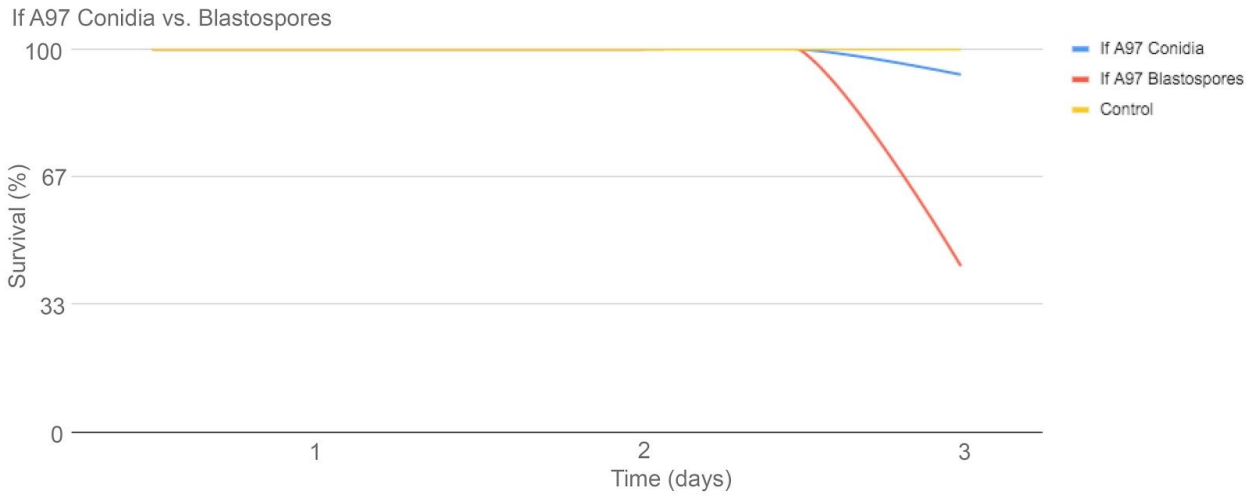

Figure 4.05 Survivorship curves of *Aedes aegypti* pupae exposed to *If* A97 conidia, blastospores, and control 0.01% Tween 80 solution.

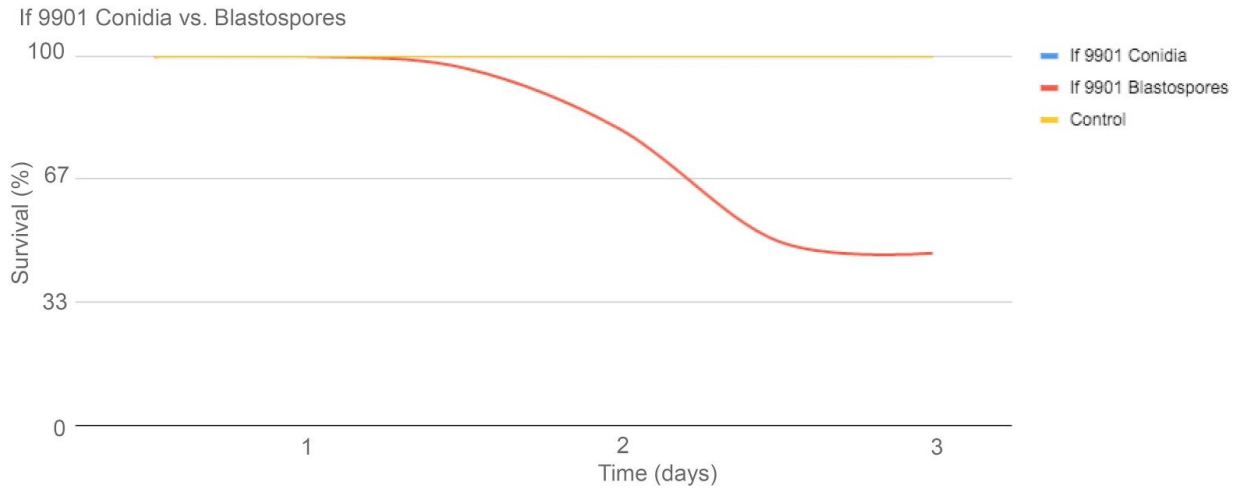

Figure 4.06 Survivorship curves of *Aedes aegypti* pupae exposed to *If* 9901 conidia, blastospores, and control 0.01% Tween 80 solution.

Implications, Limitations, and Further Research

Implications

The goal of this study was to illuminate novel approaches to aquatic stage mosquito control. The protocol outlined in this study calls for cultivated conidia to be run through a LSF process to produce blastospores, so that they may be more effective in controlling mosquito pupae.

For communities, developing an entomopathogenic fungal production method can be used to accomplish more than one goal, from general insect control, to drought protection, and even soil disease prevention (Dara 2019). These same cultivation protocols and methods can be employed to produce edible mushrooms on agricultural waste products.

Furthermore, the potential of entomopathogenic fungi to control mosquito pupae make it a potential candidate to supplement *Bacillus thuringiensis israelensis* (*Bti*) biopesticide applications for mosquito larvae. *Bti* has long been used successfully worldwide as a biological pest control agent against mosquito larvae. *Bti* bacteria produce protein crystals which are toxic to mosquito larvae (Canada 2011). Mortality rates for mosquito larvae have been observed at between 69%-98%, which can be sustained for up to 90 days, and do not deter mosquito oviposition in the area (Thavara et al 2004; Santos 2003). However, *Bti* formulations are ineffective against mosquito pupae, because the larvae cease feeding upon pupation (EPA 2018). Including blastospore solutions of entomopathogenic fungi in *Bti* applications may result in a better-rounded aquatic mosquito targeted biopesticide.

The observations in this study provide evidence for the importance of cultivating and preparing an entomopathogenic fungus in specific fashion so that it performs in the conditions you are introducing it to.

Limitations

Conidia. In this study, we observe that conidia did not have a significant effect on the mortality rates of mosquito pupae. Over the 72 hours, the conidiospores were observed to sink to the bottom of the 50 ml container, while the pupae remained floating on the surface of the water. Lacey et al. (1988) mentioned that mosquito larvae were observed to ingest conidia that had sunk to the bottom of the container, which was the pathway to pathogenicity. Mosquitoes do not feed in their pupal stage, which could be a reason for why there was little effect observed in these conditions. Though it may not be a sufficient reason, since Carolino et al. 2019 recorded 77% mortality rate for *Aedes aegypti* pupae 48 hours post inoculation with *Metarhizium anisopliae* conidia.

Possible Asphyxiation by Biological Mat. In all of the blastospore treatments, after the 72H observation, a thin biological mat was observed growing across the surface of the water. It is possible that this biological mat could have asphyxiated the mosquito pupae in the 72H of data collection. Dissecting the mosquito pupae under an electron microscope could give more insight into the causes of death, though it is possible that internal colonization by the fungus followed

death by asphyxiation. Another method that would help determine if the pupae was asphyxiated due to an external biological mat and not due to internal parasitism by the fungi would be to perform a surface sterilization of the dead pupae to remove any microorganisms from the exterior of the pupae, place the pupae on a PDA plate, incubate it, and observe if any mycosis develops. If any mycelial growth is observed developing from the pupae after a surface sterilization, the death of the pupae would probably have been affected by fungal infection. If no mycelial growth is observed on the pupae after the surface sterilization, it would suggest that the pupae died from external factors, possibly asphyxiation.

Though the biological mat was not analyzed, and it is impossible to tell precisely what microorganism contributed to its growth, there is a possibility that the mat was comprised fungal mycelium which had formed on the surface of the liquid inside of test containers, since blastospore treatments were all diluted in a potato dextrose nutrient broth, not in a 0.01% Tween 80 solution. Fungal blastospores had nutrition available with or without mosquito pupae, where conidia did not. This extra nutrition could have been a driver for mycelium to keep growing to the point of developing thin mycelial mat on the surface of the liquid, or it could have aided in the fitness of the blastospores. Contamination from bacteria or any other microorganism, which were introduced by way of the pupae or the air, could have also possibly led to the development of a biological mat on the surface of the test liquid.

In the collection process of wild mosquito larvae for rearing the F1 generation for testing, biological mats were observed forming on the surface of the collected stagnant water on several occasions. Within days, these biological mats asphyxiated whole collection batches, leading to a

mass death of around 200-300 individual larvae. A biological mat thick enough to cut off air supply to the mosquito pupae could have played a role in these observed results.

Furthermore, the possibility of death due to asphyxiation by biological mat leads this researcher to question the validity of the studies testing entomopathogenic fungi blastospores in small containers, where a biological mat could possibly be formed across the whole top of the liquid, blocking pupal access to oxygen. Wide containers would make it a greater challenge for the microorganisms to develop a mat across the top of the liquid. The following table is a summary of container size used in studies testing the effects of entomopathogenic fungi formulations on aquatic stage mosquitoes. Container size ranged from 10ml - 250ml for non semi-field condition lab testing for entomopathogenic fungi.

Table 4.01 Summary of container size used in studies testing the effects of entomopathogenic fungi formulations on aquatic stage mosquitoes. Prasad et al. 2013, Alkhaibari et al. 2016, Bitencourt et al. 2018, Benserradj et al. 2014, Rashed et al. 2013, Geetha et al. 1999, & Sani et al. 2016.

Researchers	**Test Container Size**	**Year**	**Fungus**	**Spore Type**	**Host**	**Host Stage**	**Concentration (spores/ml)**	**Time**	**Mortality Rate**
Prasad et al.	50 ml	2012	*Beauveria bassiana*	Conidia	*Anopheles stephensi*	Pupae	4.8×10^{10}	7 days	83%

Prasad et al.	50 ml	2012	*Beauveria bassiana*	Conidia	*Anopheles stephensi*	Pupae	2.56 x 10^{10}	7 days	79%
Prasad et al.	50 ml	2012	*Beauveria bassiana*	Conidia	*Anopheles stephensi*	Pupae	1.92 x 10^{10}	7 days	64%
Prasad et al.	50 ml	2012	NA	NA	*Anopheles stephensi*	Pupae	Control (0.01% Tween 80)	7 days	3%
Alkhaibari et al.	100 ml	2016	*Metarhizium anisopliae ARSEF 4556*	Conidia	*Aedes aegypti*	Larvae	1 x 10^{7}	5 days	100%
Alkhaibari et al.	100 ml	2016	*Metarhizium anisopliae ARSEF 4556*	Blastospores	*Aedes aegypti*	Larvae	1 x 10^{7}	2 days	100%
Alkhaibari et al.	100 ml	2016	*Metarhizium anisopliae ARSEF 4556*	NA	*Aedes aegypti*	Larvae	Control (0.01% Tween 80)	7 days	2%
Bitencourt et al.	10 ml	2018	*Beauveria bassiana CG 479*	Conidia	*Aedes aegypti*	Larvae	1 x 10^{7}	7 days	43%
Bitencourt et al.	10 ml	2018	*Beauveria bassiana CG 479*	Blastospores	*Aedes aegypti*	Larvae	1 x 10^{7}	7 days	58%

Bitencourt et al.	10 ml	2018	NA	NA	*Aedes aegypti*	Larvae	Control (0.01% Tween 80)	7 days	0%
Benserradj et al.	50 ml	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1 x 10^5	5 days	40%
Benserradj et al.	50 ml	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1 x 10^6	5 days	48%
Benserradj et al.	50 ml	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1 x 10^7	5 days	76%
Benserradj et al.	50 ml	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1 x 10^8	5 days	88%
Benserradj et al.	50 ml	2014	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	1 x 10^9	5 days	96%
Benserradj et al.	50 ml	2014	NA	Na	*Culex pipiens*	Larvae	Control (0.01% Tween 80)	5 days	0%
Rashed et al.	10 ml	2013	*Aspergillus niger*	Conidia	*Culex pipiens*	Larvae	3.65 x 10^5	14 days	68%
Rashed et al.	10 ml	2013	*Aspergillus ochraceus*	Conidia	*Culex pipiens*	Larvae	3.65 x 10^5	14 days	61%
Rashed et al.	10 ml	2013	*Aspergillus parasiticus*	Conidia	*Culex pipiens*	Larvae	3.65 x 10^5	14 days	29%

Rashed et al.	10 ml	2013	*Beauveria bassiana*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	40%
Rashed et al.	10 ml	2013	*Candida sp.*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	81%
Rashed et al.	10 ml	2013	*Metarhizium anisopliae*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	32%
Rashed et al.	10 ml	2013	*Penicillium citrinum*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	67%
Rashed et al.	10 ml	2013	*Penicillium stoloniferum*	Conidia	*Culex pipiens*	Larvae	3.65×10^5	14 days	33%
Rashed et al.	10 ml	2013	NA	NA	*Culex pipiens*	Larvae	Control	14 days	0%
Geetha et al.	250 ml	1999	*Beauveria bassiana*	Conidia	*Culex quinquefasciatus*	Larvae	1×10^8	5 days	84%
Geetha et al.	250 ml	1999	NA	NA	*Culex quinquefasciatus*	Larvae	Control	5 days	0%
Geetha et al.	250 ml	1999	*Beauveria bassiana*	Conidia	*Anopheles stephensi*	Larvae	1×10^8	5 days	60%

Geetha et al.	250 ml	1999	NA	NA	*Anopheles stephensi*	Larvae	Control	5 days	0%
Geetha et al.	250 ml	1999	*Beauveria bassiana*	Conidia	*Aedes aegypti*	Larvae	1×10^8	5 days	0%
Geetha et al.	250 ml	1999	NA	NA	*Aedes aegypti*	Larvae	Control	5 days	0%
Sani et al.	20 ml	2016	*Metarhizium anisopliae*	Conidia	*Culex quinquefasciatus*	Larvae	1×10^6	5 days	60%
Sani et al.	20 ml	2016	*Metarhizium anisopliae*	Conidia	*Culex quinquefasciatus*	Larvae	1×10^7	5 days	80%
Sani et al.	20 ml	2016	*Metarhizium anisopliae*	Conidia	*Culex quinquefasciatus*	Larvae	1×10^8	5 days	90%
Sani et al.	20 ml	2016	NA	NA	*Culex quinquefasciatus*	Larvae	Control	5 days	0%

Further Research

Replicate of Present Study. Conducting a replicate of this study where pupae are all placed in a larger container and treated together, rather than divided into smaller containers, would help reduce the possibility of a biological mat forming on the surface of the liquid and affecting the results of the study. Furthermore, to ensure that calculated spore viabilities remain consistent until testing, an additional dose dilution should be prepared to be plated for viability at the same time that spores are applied to pupae. It is possible that some spore viability was lost during the transportation of spores solutions from the USDA labs in McCook, TX, to the UTRGV labs in Edinburg, TX. Additionally, instead of placing the pupae in tap water for testing, a replicate of this study should place the pupae in RO water, to reduce the possibility of the chemicals and contaminants in the tap water affecting the results of the study.

Semi-field Conditions. Conducting this study under semi-field conditions, would reveal more evidence pertaining to the effectiveness of entomopathogenic fungi when controlling mosquito pupae. Semi-field conditions would help answer the question if entomopathogenic fungi will remain pathogenic with additional variables like UV exposure and water contaminants.

Dr. Tullu Bukhari and her team at Wageningen University asked these very questions in their 2011 study, and tested conidia spore dilutions mixed with different carriers to observe which formulations would induce the greatest mortality on mosquito larvae. The test containers used in this study were plastic trays (25 × 25 × 8 cm) and were filled with 1000 ml of tap water (Bukhari

et al. 2011). Tests performed in larger containers with more variables added to the study, using stagnant water collected from the field, for example, would reveal a more accurate representation of the fungi's performance of aquatic stage mosquitoes in the field.

Cost-effective Cultivation. The next step in this line of research would be to test if entomopathogenic fungi can be produced in a cost-effective manner on agricultural waste, and to test if the agriculture-waste produced spores are effective in controlling aquatic mosquitoes.

A preliminary test was conducted where unviable birdseed that had been eaten by a small beetle in the family Tenebrionidae, genus *Tribolium* was autoclaved for 20 min at 15psi and subsequently inoculated with 1ml of *If* A97 blastospore solution. The insect contaminated bird seed was used as a substrate to test if entomopathogenic fungi cultivated can be cultivated on grains that have been damaged by insects. Full colonization of the birdseed by the fungus was observed in 19 days. These promising preliminary results should be examined in future studies.

Figure 4.07 Unviable birdseed fully colonized by *Isaria fumosorosea* PFR97. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

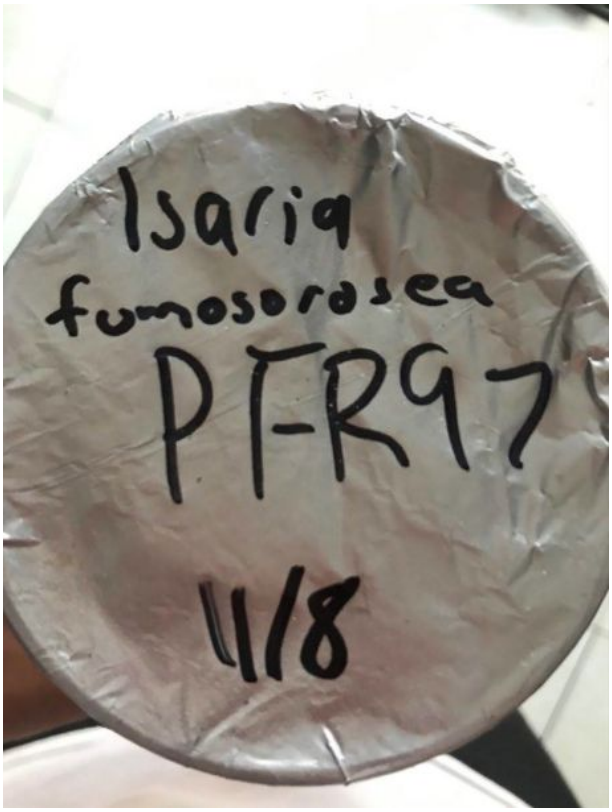

Figure 4.08 Full colonization was observed 19 days post inoculation. Photo by Ricardo Alberto Ramirez Garcia-Roja

Figure 4.09 Unviable birdseed was a mix of millet and milo, sunflower seeds were removed prior to autoclaving. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

Accounting would also be necessary for costing out the production of entomopathogenic fungi from supplies to application, so that we can really see if entomopathogenic fungi is economically more advantageous than importing organic or chemical pesticides.

Additional Fungal Candidates for Aquatic Application. The *Beauveria bassiana*, *Isaria fumosorosea*, and *Metarhizium anisopliae* tested in this study are all naturally found in terrestrial

habitats, and have been cultivated to be adapted for aquatic application. Aquatic fungi that are natural parasites of aquatic stage *Aedes aegypti* and other species of mosquitoes should also be considered as candidates for use in mosquito biocontrol. In a study where fungi was isolated and identified from water samples collected from *Aedes* mosquito breeding containers in seven distinct districts of Bangkok, Thailand, researchers were able to isolate 21 different strains of fungi -- among them *Metarhizium anisopliae* and *Penicillium citrinum*, which have both been tested on aquatic stage mosquitoes (Wasinpiyamongkol & Kanchanaphum 2019).

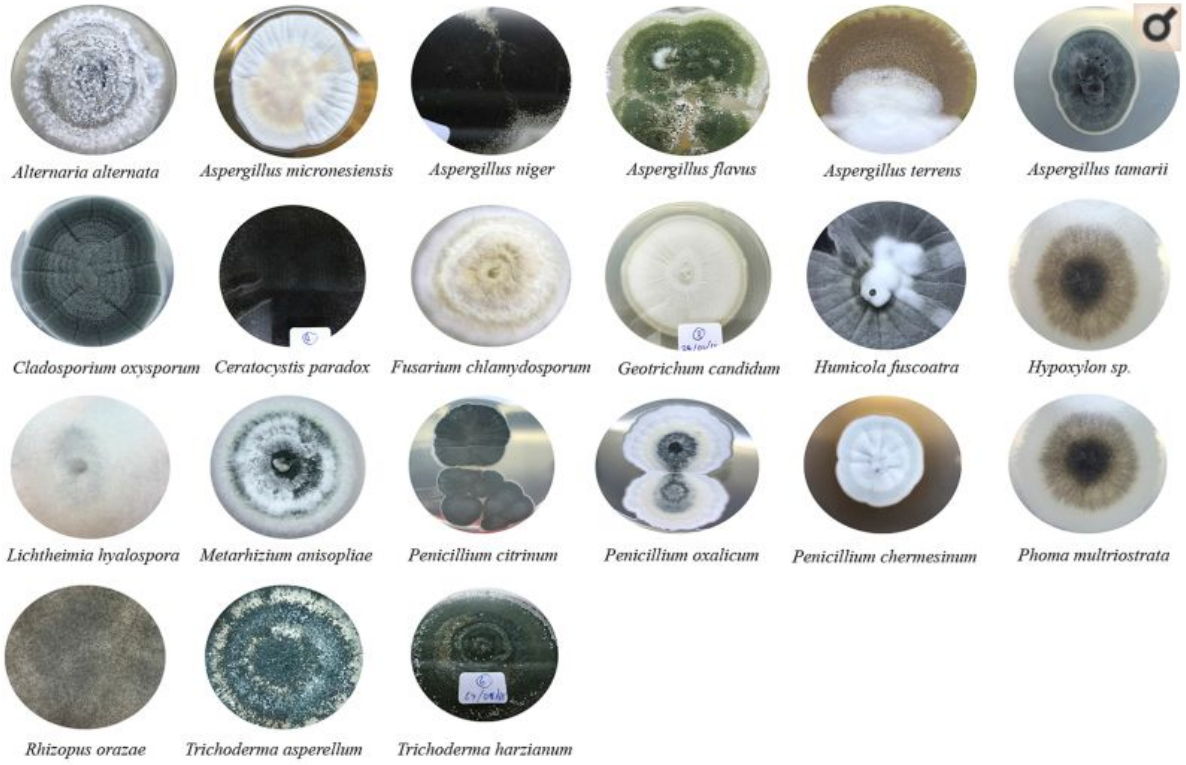

Figure 4.10 Colony and conidia of fungi isolated from water in mosquito breeding sites throughout Bangkok, Thailand, on PDA agar medium. Image from "Isolating and identifying fungi to determine whether their biological properties have the potential to control the population

density of mosquitoes," by Ladawan Wasinpiyamongkola and Panan Kanchanaphumb, 2019, Heliyon. 2019 Aug; 5(8): e02331.

Penicillium citrinum was also isolated from *Aedes* larvae collected from natural and artificial mosquito breeding sites in the states of Amazonas and Rondônia in Brazil, along with nine other species of fungi (Pereira et al 2009). In a laboratory study, a conidial suspension of 1 x 10^6 spores/ml of *Penicillium citrinum* induced 100% mortality in third-instar larvae within 2 hours of application (Maketon 2013).

Fungi of the genus *Coelomomyces* should also be considered for use in aquatic stage mosquito biocontrol. *Coelomomyces* have flagellate zoospores, which aid in their mobility in aquatic environments. These fungi can be found on all continents, except Antarctica, and are listed as parasites of the aquatic stage *Culex*, *Culiseta*, *Aedes*, *Anopheles*, *Psorophora*, and *Uranotaenia* mosquitoes, the most common hosts being *Anopheles* followed by *Aedes* and *Culex* aquatic stage mosquitoes (Scholte 2004). Fungi that are naturally occurring in aquatic environments and are natural parasites of the target species of mosquito are excellent candidates for testing aquatic stage mosquito biocontrol. Strains of fungi that are isolated from aquatic environments may be able to perform better at killing aquatic stage mosquitoes than fungi that have been cultivated to adapt to aquatic environments, like was done in this present study.

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APPENDIX

APPENDIX

Materials Employed in this Study

Materials and protocols were provided by the Dr. Racelis Lab as UTRGV, Dr. Vitek Lab at UTRGV, and Dr. Flores Lab at USDA APHIS.

Entomopathogenic Fungi Spore Cultivation

Potato Dextrose Agar (PDA) Method. The following materials were used to cultivate conidia of the entomopathogenic fungi employed in this study on PDA media:

1. Potato Dextrose Agar Powder
2. 500 ml Conical Flask
3. Petri Dishes
    a. 100 mm x 15 mm
    b. 60 mm x 15 mm
4. Tween 80
5. Reverse Osmosis (RO) Water
6. Sterile Cotton Swabs
7. Vortex Mixer
8. Plate Spreaders
9. Petri Dish Turntable
10. 5 ml Microtubes
11. 1.5 ml Microtubes
12. Metal Blades
13. Laminar Flow Hood
14. Latex Gloves

15. Autoclave
16. Incubation Chamber
17. Ethynol
18. Wipes
19. Glass Spheres
20. Micropipette with Sterile Tips
21. *Ma* E9 conidia isolated from Metaril
22. *Ma* E1037 conidia isolated from Metaril
23. *Bb* GHA conidia isolated from Botanigard
24. *Bb* ANT03 conidia isolated from Bioceres
25. *If* 9901 conidia from NoFly WP
26. *If* Apopka97 conidia from PFR9

Potato Dextrose Broth (PDB) Method. The following materials were used to cultivate blastospores of the entomopathogenic fungi employed in this study through liquid state fermentation on PDB media:

1. Potato Dextrose Broth Powder
2. 500 ml Conical Flask
3. Reverse Osmosis (RO) Water
4. Vortex Mixer
5. 5 ml Microtubes
6. 1.5 ml Microtubes
7. Laminar Flow Hood
8. Latex Gloves
9. Shaker
10. Ethynol
11. Wipes
12. Autoclave
13. Micropipette with Sterile Tips
14. *Ma* E9 conidia isolated from Metaril
15. *Ma* E1037 conidia isolated from Metaril

16. *Bb* GHA conidia isolated from Botanigard
17. *Bb* ANT03 conidia isolated from Bioceres
18. *If* 9901 conidia from NoFly WP
19. *If* Apopka97 conidia from PFR97

Spore Counting and Quality Control. The following materials were used to count entomopathogenic fungi spores and calculating viability prior to employing spore dose dilutions in this study:

1. Distilled Water
2. Vortex Mixer
3. Haemacytometer
4. Microscope
5. Cotton Swab
6. Petri Dishes
7. PDA Agar
8. RO Water
9. 500 ml Conical Flask
10. Autoclave
11. Trypan Blue
12. Tally Counter

Dose Dilutions. The following materials were used to prepare spore dose dilutions for this study:

1. Micropipette with sterile tips
2. RO Water
3. Tween 80
4. Vortex Mixer
5. Scientific Calculator

Mosquito Rearing

Wild Larvae Collection. The following materials were used to collect wild larvae in residential areas around the Lower Rio Grande Valley:

1. Metal Strainer with Handle
2. 750 ml clear plastic containers with lid

Incubating into Adulthood. The following materials were used to incubate wild larvae in lab at UTRGV until they emerged as adult

1. 32 oz Mosquito Breeder Chamber
2. 50% Sucrose Solution
3. Deionized Water
4. Glass Vials
5. Aluminum Vial Holder
6. Cotton
7. Insect Rearing Cage

Identifying Adults' Species. The following materials were used to identify adult mosquitoes emerged in lab from wild caught larvae:

1. Small Insect Aspirator
2. Plastic Vial with Lid
3. Freezer
4. Flat Ice Tray
5. Crushed Ice
6. 60x15 mm Petri Dishes
7. Microscope
8. Forceps

Blood Feeding. The following materials were used to blood feed lab hatched *Aedes aegypti* adults.

1. Researcher's Arm
2. Timer
3. Oviposition Trap
4. Ziploc bag

Egg Hatching. The following materials were used for hatching *Aedes aegypti* eggs in this study:

1. Tap Water
2. Difco Nutrient Broth
3. Aerator
4. Magnetic Stir Bar
5. Mixer
6. 1000 ml Beaker
7. Flat Tray with Loose Fitting Lid
8. Deionized Water
9. Pipette

Rearing F1 Pupae for Testing. The following materials were used for rearing F1 generation *Aedes aegypti* pupae for testing in this study:

1. 750 ml clear plastic container with lid
2. Tap Water
3. Liver Powder
4. Pipette

Protocols

Rearing *Aedes aegypti* Pupae

***Aedes* Collection Sources.** Wild *Aedes* larvae were harvested from a breeding site identified in McAllen, Texas, with GPS coordinates 26.171938, -98.226106 as well as 26.237208, -98.221886.

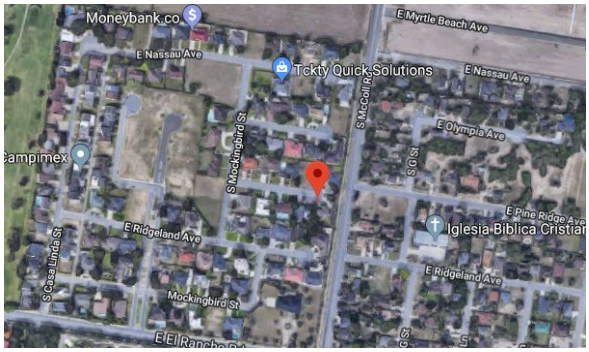

Figure A.01 *Aedes* aegypti larvae collection site. GPS: 26.171938, -98.226106. Image from www.google.com/maps.

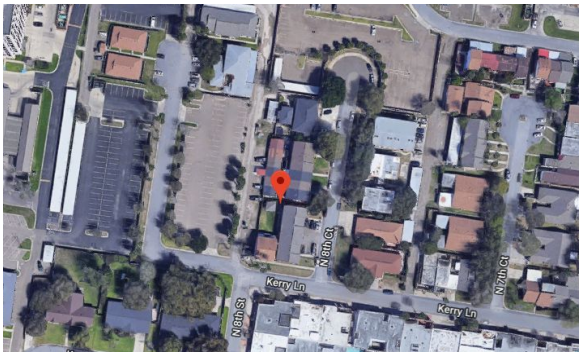

Figure A.02 *Aedes* aegypti larvae collection site. GPS: 26.237208, -98.221886. Image from www.google.com/maps.

Collected mosquito larvae were reared to adulthood, identified as *Aedes aegypti* under a microscope, and bred into an F1 generation of pupae for testing in this study. No IACUC approval is necessary for the application of fungal pathogens on arthropods (Cornell 2020).

Collecting Larvae. Standing water was emptied through a mesh strainer, larvae were caught in the strainer. Water from a standing water source with residue and plant matter was placed into a

750 ml container, to provide larvae with nutrition during their growth. A mosquito breeding chamber was screwed onto the container holding larvae to collect emerged adult mosquitoes.

Figure A.03 Larvae freshly retrieved from one of the collections sites. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

Transporting Larvae. Larvae were collected from the breeding site and transported to UTRGV College of Sciences, where they were incubated at room temperature, around 24° C and observed daily.

Preparing Colonies for Adult Emergence. Immediately after transport, deceased larvae and pupae were discarded from the colony. Water and sucrose solution was placed on the net of 750 ml container, so that mosquitoes had nutrition available upon emergence.

Identifying Emerged Adults under a Microscope. Mosquitoes were aspirated from the cage into a 50 ml vial. Vial was placed into a freezer for 20 seconds to stun the mosquitoes. Petri dishes were placed on a tray of ice to keep mosquitoes stunned. Adult mosquitoes were identified under a microscope. Positively identified *Aedes aegypti* adults were placed back into the insect rearing cage.

Breeding Mosquitoes. Sucrose solution was removed 24 hours prior to feeding to starve the mosquitoes, and get them ready for blood feeding the next day. An oviposition trap was placed inside of the insect rearing cage.

Arm was inserted into the insect rearing cage with a closed fist, to avoid getting bites on sensitive portions of the hands. Adult mosquitoes were allowed to feed for 20 minutes.

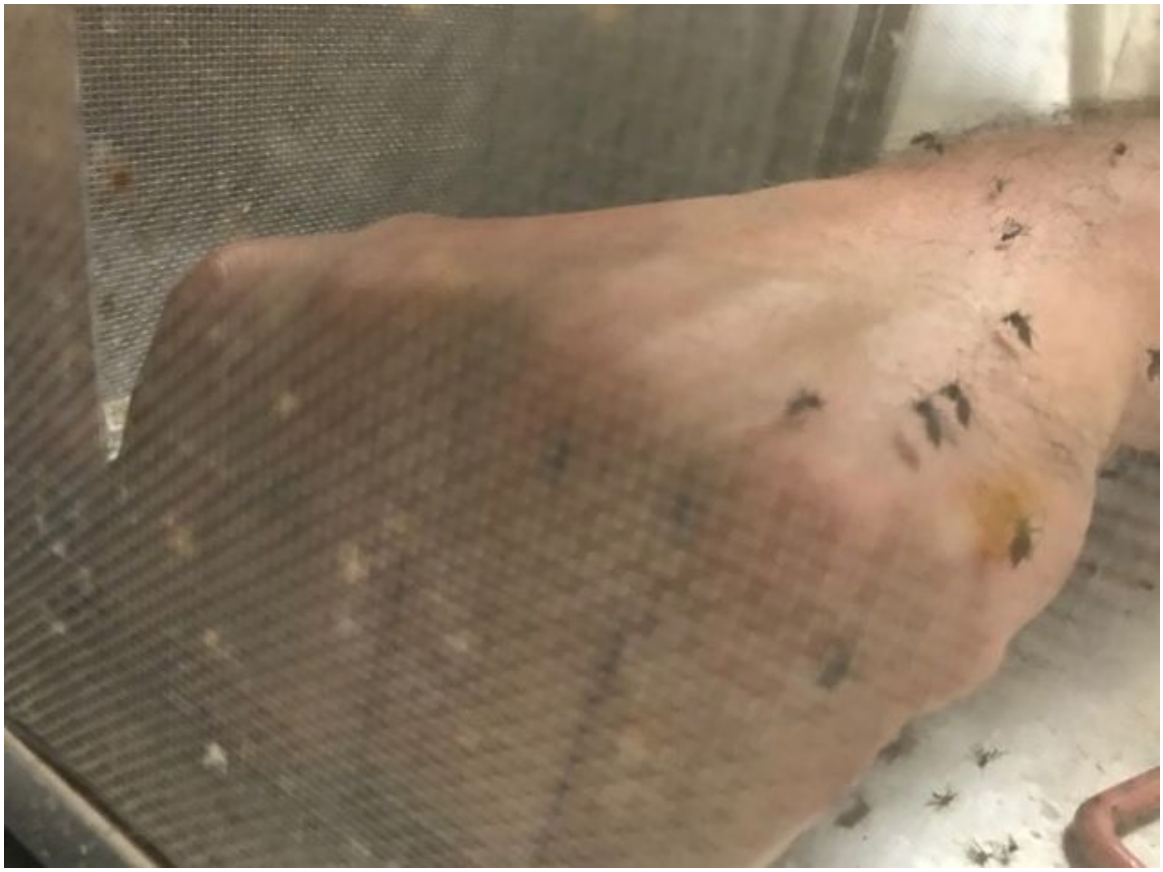

Figure A.04 *Aedes aegypti* adult female mosquitoes blood feeding on this researcher. Male mosquitoes lived in the same cage, for mating, and were fed with sucrose solution. Photo by Ricardo Alberto Ramirez Garcia-Rojas.

After 1 week, paper in the oviposition trap was collected, inspected for *Aedes aegypti* eggs, and placed inside a Ziploc bag until ready to hatch.

Hatching Mosquito Eggs. Nutrient broth was prepared to submerge the oviposition strips into. Broth was mixed with a vortex mixer and was oxygenated for 1 hour. Oviposition strips were submerged into oxygenated nutrient broth.

Collecting Hatched Larvae. After 48 hours, broth with hatched larvae was run through a Britta coffee filter cup with mesh net. The collected larvae were placed into a larvae rearing tray.

Feeding F1 Generation Larvae. Larvae were fed liver powder on a daily basis during incubation.

Observation Schedule & Collecting Pupae for Testing. Larvae were kept in incubation until pupation was observed. Collected pupae were used for testing.

Incubating larvae were inspected for pupation every 12 hours. Pupae were collected with a dropper and moved into another 50 ml container with 9 ml of DO water. Five pupae were placed per container in preparation for treatments.

Collected pupae were placed in corresponding treatment conditions within 30 minutes of being collected. All specimens were tested within 12.5 hours of pupation. All incubation and treatments were conducted inside an insect rearing cage at room temperature (about 24^{o} C).

Cultivating Entomopathogenic Fungi Conidia on Potato Dextrose Agar990

The fungal pathogens employed in this study have no recorded secondary effects on mammals (Scholte 2004).

Preparing PDA Agar. 24 petri dishes with 10 ml of PDA Agar (18 g Potato Dextrose Agar powder, 500 ml reverse osmosis (RO) water) were autoclaved.

Laminar flow hood was turned on and left running for 5 minutes to help clear out any contaminants. This and all subsequent transfers were done under a laminar flow hood.

Cultivating Conidia. 0.125 grams of conidiospore powder from:

- *Metarhizium anisopliae* E1037
- *Metarhizium anisopliae* E9
- *Isaria fumosorosea* APOPKA97
- *Isaria fumosorosea* IFR9901
- *Beauveria bassiana* GHA
- *Beauveria bassiana* ANT03

was mixed into 2 ml of 0.1% Tween 80 and RO H_20 solution.

PDA 60x15 mm petri dishes were inoculated with 125 µl of spore solution for each of the 6 strains. Plates were incubated at 29.4° C.

Plates were inspected after 8 days to collect spore powder from any confluently sporulated plates. Plates without confluent sporulation were left to incubate for an additional 8 days.

Incubating Inoculated Plates. Plates were incubated at 29.4° C in an atmospheric growth chamber until confluent sporulation.

Collecting Spores From Plates. Conidia produced in samples were harvested using a spatula and a cotton swab, placed into a 0.1% Tween 80 solution, spore counted, and refrigerated.

Quality Control of Spores. 100 µl of harvested spore solution was placed into 900 µl of 0.01% Tween 80 solution to make a 10X dilution of the original harvested stock. The 10X dilution was spread on a PDA petri dish plate using a sterile cotton swab, and left to incubate at 29.4° C in an incubation chamber for 16-18H.

After incubation, a 3mm x 3mm square of agar was cut from the petri dish, placed on a microscope slide, and a drop of Trypan Blue was placed on top of the piece of agar, and spread with a glass cover slip.

Under a microscope, total viable spores were counted. Viable spores appear dyed blue and have an observable germ tube coming out of the spore, other non-dyed spores and dyed spores without a germ tube were counted as dead spores. Spore viability was calculated by total viable spores divided by total spores counted.

Calculating Spore Counts with Haemocytometer. 100 µl of harvested spore solution was placed into 900 µl of 0.01% Tween 80 solution to make a 10X dilution of the original harvested stock. 100 µl of 10X dilution was placed into 900 µl of 0.01% Tween 80 solution to make a 100X dilution of the original harvested stock.

10 µl of 100X dilution were placed into the haemocytometer slide and analyzed under the microscope. Spore counts were multiplied by spore viability to get a final calculation of viable spore concentration of 1×10^8 viable spores/ml. Spore dilutions were made by adding 0.01% Tween 80 solution.

Conidia Treatment Dose Dilution. The conidia solution was diluted down to 1 ml of 1×10^8 viable spores/ml of RO water and 0.01% Tween 80.

Cultivating Entomopathogenic Fungi Blastospores on Potato Dextrose Solution

Preparing PDB. We prepared 6 potato dextrose solutions (6 g Potato Dextrose Powder, 250 ml RO water) in six 500ml conical flasks, one for each fungal strain being screened. The mouths of the flasks were covered with aluminum foil, and the flasks and medium were autoclaved for 20 min at 15 psi.

Inoculating PDB. 0.125 g of spore powder from each strain was placed inside of nutrient broth.

Incubating PDB. Flasks were placed on a shaker, at a rate of 115 RPH, and incubated for 3 days.

Quality Control of Spores. 100 µl of harvested spore solution was placed into 900 µl of 0.01% Tween 80 solution to make a 10X dilution of the original harvested stock. The 10X dilution was spread on a PDA petri dish plate using a sterile cotton swab, and left to incubate at 29.4° C in an incubation chamber for 16-18H.

After incubation, a 3mm x 3mm square of agar was cut from the petri dish, placed on a microscope slide, and a drop of trypan blue was placed on top of the piece of agar, and spread with a glass cover slip.

Under a microscope, total viable spores were counted, Viable spores appear dyed blue and have an observable germ tube coming out of the spore, other non-dyed spores and dyed spores without a germ tube were counted as dead spores. Spore viability was calculated by total viable spores divided by total spores counted.

Calculating Blastospore Counts with Haemocytometer. 100 µl of harvested spore solution was placed into 900 µl of 0.01% Tween 80 solution to make a 10X dilution of the original harvested stock. 100 µl of 10X dilution was placed into 900 uL of 0.01% Tween 80 solution to make a 100X dilution of the original harvested stock.

10 µl of 100X dilution was placed into the haemocytometer slide and analyzed under the microscope. Spore counts were multiplied by spore viability to get a final calculation of viable spore concentration of 1×10^8 viable spores/ml. Spore dilutions were made by adding 0.01% Tween 80 solution.

Blastospore Treatment Dose Dilution. The liquid blastospore solution was diluted down to 1 ml of 1×10^8 viable spores/ml in RO water and 0.01% Tween 80.

Research Questions:

Broken Down for Statistical Analysis

Metarhizium anisopliae E9:

1. Is pupal mortality in the ***Metarhizium anisopliae*** **E9 Blastospores** treatment significantly different from mortality in the **Control** treatment?
2. Is pupal mortality in the ***Metarhizium anisopliae*** **E9 Conidia** treatment significantly different from mortality in the **Control** treatment?
3. Is pupal mortality in the ***Metarhizium anisopliae*** **E9 Blastospores** treatment significantly different from mortality in the ***Metarhizium anisopliae*** **E9 Conidia** treatment?

Metarhizium anisopliae E1037:

1. Is pupal mortality in the ***Metarhizium anisopliae*** **E1037 Blastospores** treatment significantly different from mortality in the **Control** treatment?
2. Is pupal mortality in the ***Metarhizium anisopliae*** **E1037 Conidia** treatment significantly different from mortality in the **Control** treatment?
3. Is pupal mortality in the ***Metarhizium anisopliae*** **E1037 Blastospores** treatment significantly different from mortality in the ***Metarhizium anisopliae*** **E1037 Conidia** treatment?

Beauveria bassiana GHA:

1. Is pupal mortality in the ***Beauveria bassiana*** **GHA Blastospores** treatment significantly different from mortality in the **Control** treatment?
2. Is pupal mortality in the ***Beauveria bassiana*** **GHA Conidia** treatment significantly different from mortality in the **Control** treatment?
3. Is pupal mortality in the ***Beauveria bassiana*** **GHA Blastospores** treatment significantly different from mortality in the ***Beauveria bassiana*** **GHA Conidia** treatment?

Beauveria bassiana ANT03:

1. Is pupal mortality in the ***Beauveria bassiana*** **ANT03 Blastospores** treatment significantly different from mortality in the **Control** treatment?
2. Is pupal mortality in the ***Beauveria bassiana*** **ANT03 Conidia** treatment significantly different from mortality in the **Control** treatment?
3. Is pupal mortality in the ***Beauveria bassiana*** **ANT03 Blastospores** treatment significantly different from mortality in the ***Beauveria bassiana*** **ANT03 Conidia** treatment?

Isaria fumosorosea IFR9901:

1. Is pupal mortality in the ***Isaria fumosorosea*** **IFR9901 Blastospores** treatment significantly different from mortality in the **Control** treatment?
2. Is pupal mortality in the ***Isaria fumosorosea*** **IFR9901 Conidia** treatment significantly different from mortality in the **Control** treatment?
3. Is pupal mortality in the ***Isaria fumosorosea*** **IFR9901 Blastospores** treatment significantly different from mortality in the **Control** treatment?

Isaria fumosorosea Apopka97:

1. Is pupal mortality in the ***Isaria fumosorosea*** **Apopka97 Blastospores** treatment significantly different from mortality in the **Control** treatment?
2. Is pupal mortality in the ***Isaria fumosorosea*** **Apopka97 Conidia** treatment significantly different from mortality in the **Control** treatment
3. Is pupal mortality in the ***Isaria fumosorosea*** **Apopka97 Blastospores** treatment significantly different from mortality in the ***Isaria fumosorosea*** **Apopka97 Conidia** treatment?

Collected Data

Survivorship data for all conidia and blastospore treatments on *Aedes aegypti* pupae.

Metarhizium anisopliae E9 Conidia

Table A.01 Mortality data for ***Metarhizium anisopliae* E9 Conidia** applied to *Aedes aegypti* pupae.

	12H Living	12H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
Ma E9 1	5	0	0	5	0	0	5	0	0	5	0	1	5	0	1	5	0	5	20 %	80 %
Ma E9 2	5	0	0	5	0	0	5	0	0	5	0	2	5	0	5	5	0	5	0 %	100 %
Ma E9 3	5	0	0	5	0	0	5	0	0	5	0	4	5	0	5	5	0	5	0 %	100 %
Ma E9 4	5	0	0	5	0	0	5	0	0	5	0	3	5	0	5	5	0	5	0 %	100 %
Ma E9 5	5	0	0	5	0	0	5	0	0	5	0	2	5	0	4	4	1	4	0 %	100 %
Ma E9 6	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	0 %	100 %
Ma E9 AVG																			3 %	97 %

Metarhizium anisopliae E9 Blastospores

Table A.02 Mortality data for ***Metarhizium anisopliae* E9 Blastospores** applied to *Ae. aegypti* pupae.

	12H Living	12H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
Ma E9 1	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	0 %	100 %
Ma E9 2	5	0	0	5	0	0	5	0	1	5	0	3	5	0	5	5	0	5	0 %	100 %
Ma E9 3	5	0	0	5	0	0	5	0	0	3	2	2	3	2	2	3	2	3	40 %	60 %

Ma E9 4	5	0	0	5	0	0	5	0	0	5	0	1	4	1	1	4	1	4	20 %	80 %
Ma E9 5	5	0	0	5	0	0	5	0	0	4	1	2	4	1	4	4	1	4	20 %	80 %
Ma E9 6	5	0	0	5	0	0	5	0	1	3	2	3	3	2	3	3	2	3	40 %	60 %
Ma E9 AVG																			20 %	80 %

Table A.03 Summary of mortality data for ***Metarhizium anisopliae* E9 Conidia and Blastospores** applied to *Ae. aegypti* pupae.

	AVERAGE % Mortality	AVERAGE % Adult Emergence
Ma E9 Conidia	3 %	97 %
Ma E9 Blastospores	20 %	80 %
Control	0%	100%

***Metarhizium anisopliae* E1037 Conidia**

Table A.04 Mortality data for ***Metarhizium anisopliae* E1037 Conidia** applied to *Aedes aegypti* pupae.

	12H Living	12 H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
Ma E1037 1	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	0 %	100 %
Ma E1037 2	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	0 %	100 %
Ma E1037	5	0	0	5	0	0	5	0	0	5	0	0	5	0	0	5	0	5	0 %	100 %

3																				
Ma E1037 4	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	0 %	100 %
Ma E1037 5	5	0	0	5	0	0	5	0	0	5	0	2	5	0	2	5	0	5	0 %	100 %
Ma E1037 6	5	0	0	5	0	0	5	0	0	5	0	4	5	0	4	5	0	5	0 %	100 %
Ma E1037 AVG																			0 %	100 %

Metarhizium anisopliae E1037 Blastospores

Table A.05 Mortality data for ***Metarhizium anisopliae* E1037 Blastospores** applied to *Aedes aegypti* pupae.

	12H Living	12 H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
Ma E1037 1	5	0	0	5	0	0	5	0	0	5	0	0	5	0	3	5	0	5	0 %	100 %
Ma E1037 2	5	0	0	5	0	0	5	0	0	4	1	0	4	1	3	4	1	4	20 %	80 %
Ma E1037 3	5	0	0	5	0	0	5	0	0	5	0	0	5	0	2	4	1	4	20 %	80 %
Ma E1037 4	5	0	0	5	0	0	5	0	2	5	0	2	5	0	5	4	1	4	20 %	80 %
Ma E1037 5	5	0	0	5	0	2	5	0	0	4	1	0	4	1	4	3	2	3	40 %	60 %
Ma E1037 6	5	0	0	5	0	0	5	0	0	5	0	0	5	0	2	5	0	5	0 %	100 %
Ma E1037 AVG																			17%	83%

Table A.06 Summary of mortality data for ***Metarhizium anisopliae*** **E1037 Conidia and Blastospores** applied to *Ae. aegypti* pupae.

	AVERAGE % Mortality	AVERAGE % Adult Emergence
Ma E1037 Conidia	0 %	100 %
Ma E1037 Blastospores	17%	83%
Control	0%	100%

Isaria fumosorosea **A97 Conidia**

Table A.07 Mortality data for ***Isaria fumosorosea*** **A97 Conidia** applied to *Aedes aegypti* pupae.

	12H Living	12 H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
If A97 1	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	3	2	3	40%	60%
If A97 2	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	5	0	5	0 %	100 %
If A97 3	5	0	0	5	0	0	5	0	0	5	0	0	5	0	4	5	0	5	0 %	100 %
If A97 4	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	5	0	5	0 %	100 %
If A97 5	5	0	0	5	0	0	5	0	2	5	0	2	5	0	5	5	0	5	0 %	100 %
If A97 6	5	0	0	5	0	0	5	0	0	5	0	3	5	0	5	5	0	5	0 %	100 %
If A97 AVG																			7%	93%

***Isaria fumosorosea* A97 Blastospores**

Table A.08 Mortality data for ***Isaria fumosorosea* A97 Blastospores** applied to *Aedes aegypti* pupae.

	12H Living	12 H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
If A97 1	5	0	0	5	0	0	5	0	0	5	0	4	5	0	4	4	1	4	20%	80%
If A97 2	5	0	0	5	0	0	5	0	0	5	0	2	5	0	2	2	3	2	60%	40%
If A97 3	5	0	0	5	0	0	5	0	0	5	0	0	4	1	3	3	2	3	40%	60%
If A97 4	5	0	0	5	0	0	5	0	1	5	0	1	1	4	1	1	4	1	80%	20%
If A97 5	5	0	0	5	0	0	5	0	1	1	4	1	1	4	1	1	4	1	80%	20%
If A97 6	5	0	0	5	0	0	5	0	2	5	0	2	2	3	2	2	3	2	60%	40%
If A97 AVG																			57%	43%

Table A.09 Summary of mortality data for ***Isaria fumosorosea* A97 Conidia and Blastospores** applied to *Ae. aegypti* pupae.

	AVERAGE % Mortality	AVERAGE % Adult Emergence
If A97 Conidia	7%	93%
If A97 Blastospores	57%	43%
Control	0%	100%

Isaria fumosorosea 9901 Conidia

Table A.10 Mortality data for ***Isaria fumosorosea* 9901 Conidia** applied to *Aedes aegypti* pupae.

	12H Living	12 H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
If 9901 1	5	0	0	5	0	0	5	0	0	5	0	0	5	0	2	5	0	5	0 %	100 %
If 9901 2	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	0 %	100 %
If 9901 3	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	0 %	100 %
If 9901 4	5	0	0	5	0	0	5	0	2	5	0	2	5	0	5	5	0	5	0 %	100 %
If 9901 5	5	0	0	5	0	0	5	0	0	5	0	2	5	0	5	5	0	5	0 %	100 %
If 9901 6	5	0	0	5	0	0	5	0	4	5	0	4	5	0	5	5	0	5	0 %	100 %
If 9901 AVG																			0%	100%

Isaria fumosorosea 9901 Blastospores

Table A.11 Mortality data for ***Isaria fumosorosea* 9901 Blastospores** applied to *Aedes aegypti* pupae.

	12H Living	12 H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
If 9901 1	5	0	0	5	0	0	5	0	3	5	0	5	5	0	5	5	0	5	0 %	100 %
If 9901 2	5	0	0	5	0	0	5	0	0	5	0	2	3	2	2	2	3	2	60 %	40 %
If 9901 3	5	0	0	5	0	0	4	1	0	3	2	0	3	2	1	3	2	3	40 %	60 %

If 9901 4	5	0	0	5	0	0	5	0	0	3	2	0	0	5	0	0	5	0	100 %	0 %
If 9901 5	5	0	0	5	0	0	5	0	1	4	1	1	1	4	1	1	4	1	80 %	20 %
If 9901 6	5	0	0	5	0	0	5	0	0	4	1	1	3	2	3	3	2	3	40 %	60 %
If 9901 AVG																			53%	47%

Table A.12 Summary of mortality data for ***Isaria fumosorosea* 9901 Conidia and Blastospores** applied to *Ae. aegypti* pupae.

	AVERAGE % Mortality	AVERAGE % Adult Emergence
If 9901 Conidia	0%	100%
If 9901 Blastospores	53%	47%
Control	0%	100%

Control

Table A.13 Mortality data for **Control - 0.01% Tween 80 and water solution** applied to *Aedes aegypti* pupae.

	12H Living	12 H Dead	12H Emerged Adults	24H Living	24H Dead	24H Emerged Adults	36H Living	36H Dead	36H Emerged Adults	48H Living	48H Dead	48H Emerged Adults	60H Living	60H Dead	60H Emerged Adults	72H Living	72 H Dead	72H Emerged Adults	% Mortality	% Adult Emergence
Control 1	5	0	0	5	0	0	5	0	0	5	0	1	5	0	5	5	0	5	0 %	100 %
Control 2	5	0	0	5	0	3	5	0	3	5	0	3	5	0	5	5	0	5	0 %	100 %
Control 3	5	0	0	5	0	0	5	0	0	5	0	3	5	0	3	5	0	5	0 %	100 %
Control 4	5	0	0	5	0	0	5	0	0	5	0	0	5	0	3	5	0	5	0 %	100 %
Control 5	5	0	0	5	0	0	5	0	0	5	0	2	5	0	2	5	0	5	0 %	100 %
Control 6	5	0	0	5	0	0	5	0	2	5	0	2	5	0	5	5	0	5	20 %	80 %

Control AVG																			0%	100%

Table A.14 Summary of mortality data for **Control - 0.01% Tween 80 and water solution** applied to *Aedes aegypti* pupae.

	AVERAGE % Mortality	AVERAGE % Adult Emergence
Control	0%	100%

BIOGRAPHICAL SKETCH

Ricardo Alberto Ramirez Garcia-Rojas graduated from Harvard University in Cambridge, Massachusetts in 2013 with a B.A. in Psychology with a Secondary Field in Studio Filmmaking. Determined to create value from perception, Ricardo worked as an advertising consultant. Unfulfilled in his pursuits, Ricardo returned to the Rio Grande Valley to concentrate on his first love, food. While constructing community gardens for the Boys & Girls Club of Edinburg, Ricardo would seek advice from Dr. Alexis E. Racelis, who instead went on to invite him to seek the mastery and skill he thirsted for in the Agriculture, Environment, and Sustainability Sciences Program at the University of Texas Rio Grande Valley. While in the program, Ricardo, led by a tenacious curiosity, focused his time, art, and research on applied mycology.

Ricardo and his brother Marcelo will be putting up their first fungal mycelium piece, *El Templo del Nanacatl*, in Fiddler's Green Amphitheater in September 2020 (si Dios quiere). The COVID-19 pandemic raised uncertainty with our installation date; however, we are happy and blessed that we are still healthy in our wait. Ricardo completed the Master of Science degree in Agricultural, Environmental, and Sustainability Sciences in August 2020.

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