THE EFFECT OF *MYCOBACTERIUM ULCERANS* EXOTOXIN ON HOST-SEEKING AND OVIPOSITION BEHAVIOR OF *AEDES AEGYPTI AEGYPTI* (L.) (DIPTERA: CULICIDAE)

A Thesis

by

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ABSTRACT

Buruli Ulcer (BU), an emerging tropical disease, affects thousands of individuals throughout the world with West Africa being the most devastated area. From 2005-2010 nearly 30,000 cases were reported from this region. This disease is caused by the mycobacterium, *Mycobacterium ulcerans,* which unlike other species of mycobacterium produces a mycolactone that is an immunosuppressive polyketide-derived macrolide toxin that can diffuse through plasma membranes. This disease has novel clinical symptoms that consist of a painless skin ulcer. While painless, the ulcer has negative effects, such as, bone deformation, and possible secondary infections that can often lead to death. The mode of pathogen is transmission is currently unknown. This study examined if the mycolactone serves as an attractant for host-seeking behavior, as well as oviposition site choice of the yellow fever mosquito *Aedes aegypti aegypti* (Linnaeus) (Diptera: Culicidae), a potential vector for the pathogen. The responses of adult mosquitoes to a blood-feeder treated with one of three doses (0.05 μg/mL), (0.5 μg/mL), (1.0 μg/mL) of the mycolactone or a control (95% ethanol) were examined. The same doses and control were used to examine the responses of adult mosquitoes to an oviposition substrate as well.

It was determined that mosquitoes were more attracted (29.1%) to the blood-feeder treated with the high dose (1.0 μg/mL) compared to the control. These data indicate that the mycolactone could serve as an attractant of mosquitoes to hosts, which could result in transmission of the pathogen responsible for BU, as well as enhance the transmission

of other mosquito-related pathogens such as yellow fever. It was also determined that mosquitoes were more attracted to the control (approximately 30%) compared to the low (0.05 μg/mL) or medium dose (0.5 μg/mL); however there was attraction (approximately 15%) to oviposition sites treated with the high dose. These results indicate a possible repellant effect for mosquitoes to oviposit in sites with the low and middle doses, but could be an attractant in environments with higher concentrations. Like with the attraction assay, a dose dependent response (repellence and attraction) to the mycolactone and oviposition site selection was determined. Future studies to be conducted that would provide greater insight into the role of mycolactone as an attractant would examine additional concentrations to determine the threshold that induces a shift in mosquito behavior. Additional work with the bacteria actively producing the mycolactone in combination with a host would also provide greater insight to any role in attracting potential vectors.

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NOMENCLATURE

BU	Buruli Ulcer
ER	Enyol-reductase
GIS	Geographical Information System
KR	Ketoreductase
M.ulcerans	*Mycobacterium ulcerans*
OAI	Oviposition activity index
PCR	Polymerase Chain Reaction
QS	Quorum sensing
WHO	World Health Organization

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Buruli Ulcer (BU), is a neglected tropical disease characterized by severe skin lesions in humans that are often painless (*1*). This disease was first noted in the late 1880's in Africa and has since been reported worldwide in countries such as Japan, China, and Australia (*2*). Although the disease has been identified since the late 19th century, the World Health Organization (WHO) first recognized this disease in 1998 (*3*). The WHO has since developed the Global Buruli Ulcer Initiative that focuses on not only the improvement of treatment provided to those with the disease, but also addresses the issues of community awareness and prevention (*4*). A study conducted in Ghana determining what age group is most at risk of obtaining BU demonstrated results showing that children tend to be at higher risk of obtaining the infection (*5*). The study also determined that a median age of 12 years old, and males are significantly more likely to obtain the lesions than females (*5*). This study also provided insight to the locations on the body an individual is most likely to obtain an ulcer, and determined that infections most commonly occurred on the arms and legs with a greater frequency towards the left side (*5*). It was determined that lesions were statistically more likely to be seen on the distal portion of the extremities compared to the proximal (*5*).

Mycobacterium ulcerans is the causal agent for this disease, and is most commonly found in rural locations in tropical countries, with Africa being the main endemic zone; However other endemic regions have been identified, such as rural areas

of Papua New Guinea, Malaysia, French Guiana, and Mexico (*6*). Past studies have considered proximity to stagnant or slow-flowing bodies of water as a large risk factor for Buruli Ulcer (*3, 7*). A recent study completed in the Greater Accra region of Ghana confirmed through the use of geographical information system (GIS) technology that the occurrence of BU along the Densu River was especially high in areas where the flow of the water was slow, as well as where gentle slopes existed (*7*). Slow flowing areas were determined based on prior knowledge of mountains in this region and that the water flowing from the source of the mountains is fast and slows down after entering the river (*7*). Although *M. ulcerans* is often associated with a residence near an aquatic environment in Africa, fishermen who frequent the water for prolonged visits were not determined to be at a high risk for infection (*8*).

BU Pathology

BU is the third most common mycobacterial disease worldwide behind Tuberculosis and Leprosy (*3*). *Mycobacterium ulcerans* produces a mycolactone, which has yet to be identified in other species of mycobacterium. This compound is considered a potent cytotoxic and immunosuppressive polyketide- derived macrolide (*1, 9*). Mouse fibroblast L929 cell lines were used for *in vitro* studies of this toxin. Infection with the toxin has demonstrated that the action of mycolactone consists of diffusing through the plasma membrane of the cell where the toxin is then incubated (*9-11*). Studies where cells have been incubated with the toxin have revealed early actin cytoskeleton rearrangement, cell round up, as well as detachment from the bottom of the well, resulting in apoptotic cell death (*9*).

Clinical symptoms of BU often begin with preulcerative lesions, such as nodules, that develop into deep ulcers with undermine edges that can spread and disseminate to the bone (*3*). The ulcers are more commonly found on the extremities of the body rather than the trunk. In 5-10% of patients with BU, osteomyelitis occurs, with most cases naturally accompanying a skin lesion (*3*). The lesions can be multifocal, and in several forms at one infection site. If treatment is provided during the early stages of infection, antibiotics can prove to be successful, however if left untreated the infection can result in deformities, amputations of the extremities, and loss of joint mobility (*1*).

Environmental Monitoring for BU

Detection of *M. ulcerans* in the environment has been difficult due to the slow growth rate of the bacterium making standard culturing techniques difficult. However, the DNA sequence of the *M. ulcerans* genome revealed the species has undergone reductive evolution (*12*). The DNA sequence of the genome paired with the discovery of high copy number insertion sequences in the IS*2404* and IS*2606* region provided a faster and more reliable method of detection of the bacterium [24](*12*). However, the detection of *M. ulcerans* in the environment has been based off the detection of IS*2404*, and a portion of the enyol reductase (ER) or ketoreductase (KR) domains from the polyketide synthase genes, which encode mycolactone (*mlsA, mlsB)* (*12*). The development of *M. ulcerans* PCR probes that detect the species specific gene region IS*2404* has allowed *M.ulcerans* DNA to be amplified in environmental samples including water samples, biofilms, fish, humans, and even insects (*2*). More recently the development of two TaqMan Multiplex quantitative, real times PCR has been developed as a technique for

detection for increasing the sensitivity, as well as, rapid analysis of specimens making it able to interpret both environmental and clinical samples (*13*). The use of qPCR allowed the quantification of the number of target copies making it possible to determine the difference between *M. ulcerans* and other IS*2404*-contianing mycobacteria (*13*). This development also allowed the control of PCR inhibitors, such as, humic and fulvic acids that are often seen in environmental samples (*13*). The application of qPCR has allowed the improvement on studies trying to determine the environmental reservoir of *M. ulcerans* (*13*).

Transmission of BU

The unknown mode of *M. ulcerans* transmission and its ability to be transmitted from the environment to humans is the central enigma in BU research. Many modes of transmission, such as direct contact with contaminated vegetation, insect vectors, aerosols, or the entrance of the bacterium through previous wounds have been proposed (*4*). Poor wound care is a risk factor associated with BU allowing the possibility of direct transmission; however unlike tuberculosis, person-to-person transmission is not believed to be a standard mode of transmission. A single case has been case reported involving a human biting another human (*2*). In the case of biting, the patient's skin surface was believed to have come into contact with the environment containing *M. ulcerans* and was introduced by being driven into the skin (*2*). Studies conducted on the introduction of the bacteria though a previous abrasion using a guinea pig (*Cavia porcellus*) infection model with the application of *M. ulcerans* topically applied to abrasions on the animal's skin failed to produce infection (*4*). These results showed that bacterial transmission

does not occur passively in an exposed wound. In the same study guinea pigs were injected intradermally whereby the pathogen produced ulcers, further supporting the hypothesis that biting invertebrates could serve as a mode of transmission (*4*). In a recent study conducted in Benin, Africa, the number of positive detections of *M. ulcerans* in the environment positively correlated with an increase in the prevalence of BU (*8*). In the absence of human disease, environmental pathogens will often be widespread. However, the lack of reliable positive samples from environments in which BU is absent, leads to the possibility that humans are serving as a means of geographically transporting the bacterium (*8*).

Indirect transmission of BU is speculated to occur through biting insect vectors, such as mosquitoes (Diptera: Culicidae). During a 2004 outbreak of BU in a small Australian town near Victoria, intense *Aedes* sp. activity was observed concurrently. Real-time PCR analysis allowed the environmental screening of 11,000 mosquitoes in the area, with positive MU DNA amplification results of 4.3 per 1,000 mosquitoes (*2*).

Mosquito larvae are associated with habitats that consist most often of standing or lentic water. The larvae of mosquitoes have a labral head fan that is used as a filtration feeding method by filtering particles in the water. The feeding method of mosquito larvae allows the possible consumption and concentration of *M. ulcerans* (*12*). Recent studies have examined mosquitoes as both a mechanical and biological vector of *M. ulcerans*, as well as the role mosquitoes may have in the movement of the bacterium through an aquatic food web. *Aedes aegypti* larvae were fed Australian and African strains of *M. ulcerans*, as well as *M. marinum*, which was used as a control due to its

close relation to *M. ulcerans* by sharing 98% genome similarity, to determine how long mycobacterium could be detected in the larval gut. The results showed that high levels of all bacteria were present for up to 6 days in the larval gut content; however, neither *M. ulcerans* nor *M. marinum* was detectable in the adults (*12*). These results suggest that mosquitoes are likely not serving as a biological vector. If mosquitoes are not directly transmitting the pathogen, it is possible that the attraction to the mycolactone alters their behavior to spread *M. ulcerans* to geographical regions previously not known to contain BU.

Cues Used by Mosquitoes to Locate Hosts

Many cues associated with a host are used by blood-feeding arthropods to locate them. Carbon dioxide and heat are examples of cues that are known to serve as attractants (*14*). Recent evidence indicates that host odors produced by bacteria found on the skin are also serving as cues to locate hosts. In a study using *Anopheles gambiae,* (Giles) (Diptera: Culicidae), volatiles released by four bacterial species commonly found on the human skin, with *Staphylococcus epidermidis* being the primary bacteria, were determined to be significant attractants for host-seeking behavior (*15*). Gravid female *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) females were allowed a choice test between water sources containing distilled tap water, lagoon water, and wastewater. Results suggest the mosquitoes were more inclined to oviposit their eggs in the waste and lagoon water, both of which demonstrated high bacterial content (*16*). This study also used tetracycline to treat the wastewater to determine if killing the bacteria releasing

volatiles would have an effect on mosquito behavior. The initial preference for the wastewater disappeared after antibiotic treatment (*16*).

Cues used by Mosquitoes to Locate Oviposition Sites

Oviposition is the act of depositing eggs to the species-specific biotope. The oviposition response is often influenced by resource availability, such as areas containing abundant bacteria that the larvae will feed. Oviposition site preference is often mediated by chemical cues released by the bacteria (*17*). *Aedes* spp. are characterized by laying their eggs singly on a moist substrate often adjacent to bodies of water, as well as areas known to contain floodwater (*18*). They often lay their eggs in water that is undisruptive or very slow moving. *Aedes aegypti*, which transmits the pathogen responsible for yellow fever, is in high prevalence in Africa, often in close approximation to humans (*19*). *Aedes aegypti* is sensitive to bacterial cues and tends to oviposit in areas with high bacterial densities compared to areas with low bacterial densities (*17*).

Interkingdom Communication

The exchange of chemical signals allowing bacteria to monitor their population density, as well as to regulate gene expression in a population dependent manner, is known as quorum sensing (QS) (*20*). Quorum sensing provides a cell-to-cell communication pathway in bacteria (*21*). Bacteria release chemical signal molecules known as autoinducers, which accumulate to a minimal threshold stimulatory concentration, causing an alteration in gene expression, and phenotype (*21*). Quorum sensing compounds have been used to regulate interspecies communication as well as

interkingdom communication (*20*). Quorum sensing has been observed in interactions between bacteria and eukaryotes, as well as humans and pathogens (*20*). Research examining the interkingdom interaction of the blow fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) and a mutant form of *Proteus mirabilis,* that lacks the ability to create a biofilm through swarming, a quorum sensing regulated phenotype, provided evidence that flies were more attracted to a wild-type form of the bacteria that allows swarming (*22*). Swarming in bacteria is regulated by QS (*22*). The quorum sensing volatile compounds produced by *M. ulcerans* and their possible influence on the yellow fever mosquito attraction response are unknown. This study will investigate *Ae. aegypti* adult attraction to a blood-meal and oviposition response to the mycobacterium's macrolide toxin.

Objectives and Hypotheses

Objectives:

Determine if mycolactone, a toxin produced by *Mycobacterium ulcerans*, at different concentration (95% Ethanol, 0.05 µg/mL, 0.5 µg/mL, 1.0 µg/mL) influence host attraction and oviposition responses of adult *Aedes aegypti aegypti*.

Hypothesis 1:

H_0: Mycolactone in association with a blood-meal does not differentially attract female adult *Aedes aegypti*.

H_a: Mycolactone in association with a blood meal can differentially attract female adult *Aedes aegypti*.

Hypothesis 2:

H_0: Higher concentrations of mycolactone in association with a blood-meal do not differentially attract female adult *Aedes aegypti*.

H_a: Higher concentrations of the mycolactone in association with a blood-meal can differentially attract female adult *Aedes aegypti*.

Hypothesis 3:

H_0: Mycolactone will not differentially affect the oviposition response of a gravid female adult *Aedes aegypti*.

H_a: Mycolactone can affect the oviposition response of a gravid female adult *Aedes aegypti*.

Hypothesis 4:

H_0: A high concentration of mycolactone will not differentially affect the oviposition response of a gravid female adult *Aedes aegypti*.

H_a: A high concentration of mycolactone can affect the oviposition response of a gravid female adult *Aedes aegypti*.

CHAPTER II

RESEARCH, RESULTS, AND DISCUSSION

Introduction

Buruli ulcer (BU), which is considered the third most common mycobacterial infection following tuberculosis and leprosy, is caused by *Mycobacterium ulcerans* (Actinomycetales: Mycobacteriaceae) (*23*). *Mycobacterium ulcerans* has the ability to produce a macrolide, also known as mycolactone. Mycolactone produces the novel aspect to this disease-the formation of painless nodules or papules that are found most commonly on the extremities. If left untreated, these can lead to ulcers that cover up to 15% of the body (*1, 9, 23*). There have been an increase in the incidences of BU in West Africa, and disease has also been reported in other regions of the world, such as, Australia, Japan, and China (*2, 23*). Although this disease has been described as far back as the 1880's, it wasn't until 1998 that Buruli ulcer was recognized by the World Health Organization (WHO) (*3*).

Although BU has been hypothesized to be acquired through transmission by a vector, studies have failed to incriminate a vector species and the mode of transmission remains unknown (*5*). The enigma of the mode of transmission has been studied more recently a following the discovery of PCR identified *M. ulcerans* in aquatic insects (Hemiptera: Naucoridae and Belostomatidae) collected in BU endemic regions of Africa (*6*). Researchers hypothesize that biting-water arthropods such as these could be transmitting the pathogen to humans (*24*). However, little is known about the mechanism regulating

the interaction between these insects and the host making it possible the water beetles are also attracted to the mycolactone.

A transmission model currently being investigated proposes that hosts that filter water, such as fish and mollusks, are able to concentrate the mycobacterium present from the water and then release this bacterium back into the environment allowing aquatic predators to ingest *M. ulcerans* (*24*). In a recent study conducted by Marsollier et al.2002, adult *Naucoris cimicoides* (Linnaeus) (Hemiptera: Naucoridae) were collected from wetlands and raised in aquariums that mimicked their natural environment and were fed grub worms of *Phormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) that had been injected with a concentration of 1 x 10^6 *M. ulcerans* in a volume of in order to test the possibility that this biting insect is a link in the mode of transmission (*24*). The infected and non-infected insects were then allowed to bite laboratory strain mouse tails to determine if transmission was possible, and histological sections of the salivary glands from the insects were examined (*24*). The results of this study showed that seven of the ten mice whose tails had been bitten showed positive PCR results for *M. ulcerans* DNA, as well as positive detection of the mycobacterium in the salivary gland histology (*24*).

However, the question remains, could the pathogen be transmitted by an arthropod associated with an aquatic environment that is capable of coming into contact with people removed from the water? If so, does mycolactone serve as an attractant for this vector on hosts? Recently, mycolactone was detected in *Aedes* spp. mosquitoes sampled from Point Lonsdale in Victoria, Australia (*25*). Current research has focused

on the detection of the pathogen within this potential vector; this study represents the first attempt to examine the behavioral response of adult *Ae. aegypti*, to mycolactone produced by *M. ulcerans* in regards to host seeking behavior and oviposition site selection.

Materials and Methods

Mycolactone

Mycolactone A/B was isolated and obtained from Dr. Heather Jordan at Mississippi State University who used the methods as described by A.Mve-Obiang et al.2003; however the methods were altered where *M. ulcerans Agy*99 were grown on M7H10 plates+OADC instead of in broth and the bacteria was then scraped from the plates and weighed. Dr. Jordan used the same methods for purifying the mycolactone as described by Mve-Obiang, Armand, et al. 2003, and a cytopathicity assay was conducted to confirm activity. The doses used in this study were extrapolated backwards from the weight of the cells from the colony count to assure that one cell had approximately 1 pg of mycolactone (Jordan, personal communication). Ranges of concentrations were developed off the corresponding qPCR values from environmental samples from 1 x 10^3-1 x 10^6 GU/mL. The toxin was desiccated after extraction at three concentrations of 1 ug/mL, 0.5 ug/mL, and 0.05 ug/mL, after the addition of 95% ethanol. At the time of use, 10mL of 95% ethanol was added to each vial to solubilize samples used for the study. Since the desiccant was diluted in 95% ethanol this was used as my control. Due to the negative effects of ultraviolet rays on mycolactone, amber vials were used for shipping and kept in a dark location to maintain stable concentrations.

Mosquito Colony Maintenance

Aedes aegypti eggs (Liverpool Strain) were hatched in a container with 1 L of distilled water at 25°C, 80% RH and 12:12 L:D photoperiod. Two-day-old larvae were separated into containers at a density of 100-200 larvae/L in order to provide proper conditions for larval growth (*26*). Larvae were fed a diet of 3 grams of fish food (TetraMin diet by Tetra Blacksburg, VA) that is ground down to a powder form. Feeding occurred approximately every other day to prevent bacterial contamination of the water. Upon pupation, pupae were separated into 60 mL containers containing distilled water, based on their sex at an approximate density of 50 females/cup. Females were identified based on size, with the females being larger than the male pupae, and by the characteristic that females tend to pupate later than males. Males were excluded from the study due to the lack of blood feeding behavior and oviposition (*27*). Three plastic cups containing approximately 50 female pupae were then placed inside an approximately 4.9 L grease resistant paper bucket (Solo Cup Operating Corporation, Lake Forest, IL) with mesh coverage for their emergence. Adult mosquito colonies were maintained under conditions of 25°C, 80% RH and 12:12 L:D photoperiod. Newly emerged adults were provided a 5% sucrose solution via a damp cotton ball every other day until 24 hours prior to the blood feeding experiment.

Blood -Feeder

Blood-feeders were made with a 25 cm^2 cell culture flask (Corning Incorporated, NY). In order to create a space gap to pipette the blood used in this experiment parafilm covered the surface of the blood-feeder (Figure 1). Luciano Cosme designed the blood feeders used in this study. During the blood feeding attraction trials an aliquot of 1ml of defibrinated rabbit blood (HemoStat Laboratories, Dixon, CA) was pipetted between the parafilm and the cell culture flask. A piece of Johnson & Johnson four pleated gauze (Johnson & Johnson, New Brunswick, NJ) was cut into 7.0 cm x 7.5 cm pieces which then covered the side of the blood feeder containing the parafilm. Prior to attaching the gauze to the blood-feeder, the gauze was soaked with 1 mL of the desired concentration of mycolactone.

Figure 1. The blood-feeder device developed out of a 25 cm^2 cell culture flask (Corning Incorporated, NY) used to conduct the blood-feeding behavioral assay with *Ae. aegypti.* The device is wrapped in parafilm with 1mL of defribrinated rabbit blood pipetted in a gap formed between the culture flask and parafilm.

Blood- feeding Attraction Experiment Design

A plexiglass cage (Figure 2) containing 50 female mosquitoes that had not received a blood meal and were 5-10days old was put in the incubator room under conditions previously described in the mosquito rearing section. This age group was chosen to allow mosquitoes the optimal time to develop but is limited to 10 days to ensure mosquitoes receive a blood meal before dying off. In order for the mosquitoes to acclimate to their environment they were placed in the cage 30 minutes prior to the assay. Between replications the introduction of the mosquitoes consisted of alternating from the left to right side of the cage in order to prevent any bias. Attraction to light, air flow, and CO_2 require acclimation to the environment, 30 minutes prior to the blood

feeding, to reduce the potential for positional bias (*28*). In each replicate, two blood feeders were used in order to provide a true choice between the treatments and control. This was conducted rather than just placing one blood feeder with a treatment and measuring feeding activity, because the mosquito may blood feed on the treated feeder even if not suitable to their liking in order to receive a meal to be able to become gravid. The blood feeders were placed parallel to one another on the opposite ends of the cage top (Figure 2), separated by approximately 50 cm. The two blood-feeders were connected by thin plastic tubing to a water bath set at 37°C to simulate the temperature of human blood (*29*). Each blood feeder was covered with 4- pleated gauze (Johnson & Johnson, New Brunswick, NJ) soaked with the mycolactone, or 95% ethanol as a control. An aliquot of 1 ml of rabbit blood (HemoStat Laboratories, Dixon, CA) was pipetted in the gap between the blood feeder and the parafilm as seen in Figure 1.

The numbers of mosquitoes feeding on either the treatment feeder or the control feeder were recorded using a high-definition camera (GoPro Inc., San Mateo, CA) that was mounted on the side of the plexiglass cage. The videos were reviewed to count the sum of mosquitoes present on each individual blood feeder every minute, during a 15-minute assay period, with their presence on the blood feeder being considered host-seeking activity. Each experiment was replicated four times alternating the treatment blood feeder between right and left ends of the cage top to rule out any positional effects.

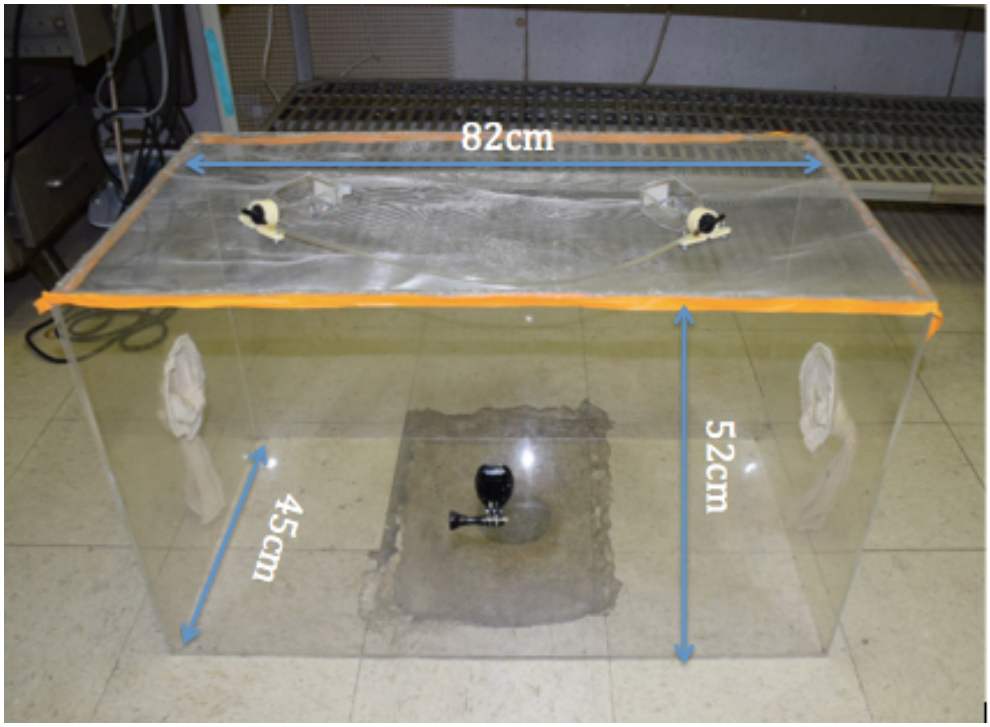

Figure 2. Plexiglass that is 82 cm x 52 cm x 45 cm in size with two circular openings on each end to allow the introduction of the *Ae. aegypti* mosquitoes for the blood-feeding behavioral assay. The blood feeding devices (figure 1) placed parallel on the top of the mesh portion of the cage 50cm apart.

Oviposition Attraction Experiment Design

Fifty female mosquitoes were given a blood meal 10 days after emerging as adults using 1 mL of rabbit blood (HemoStat Laboratories, Dixon, CA). The females were placed in a large 4.9 L grease resistant paper bucket (Solo Cup Operating Corporation, Lake Forest, IL) with adult males and were allowed to mate and become gravid. Three days after the females were blood fed they were removed from their paper emergence cup and presented with CO_2 in order to sedate the mosquitoes without killing them (*30*). Fifty-five gravid females were selected with fine point forceps and placed in a

separate paper container. The mosquitoes must be given CO_2 24 hours before the experiment is ran in order to prevent a lack of fitness or change in the physiology of the insects $C0_2$ sensing system in the adults (*31*). On the fourth day after the blood feeding the 55 mosquitoes were placed into a 61 cm x 61 cm x 61 cm wire mesh cage with two small plastic cups 5.08 cm in height placed in the cage, each containing a strip of 4 cm x 5 cm filter paper to be used as an oviposition substrate. In order for the substrate to be fit for oviposition, approximately 30 mL of water was placed in the cup to reach the bottom of the filter paper. Prior to placing the filter paper in the cups, one strip was treated with 1 mL of 95% ethanol as a control and allowed to dry. The other strip of filter paper was treated with 1 mL of mycolactone and allowed to dry. The two cups were placed in the 61 cm x 61 cm x 61 cm wire mesh cage and placed 50 cm apart at opposite corners of the cage. For each replicate, the cups were rotated clockwise to prevent the position of the cup creating a bias. The conditions of the incubator were the same as for colony maintenance. The females were allowed 48 hours to oviposit and the numbers of eggs were counted from each piece of filter paper after the assay was completed.

Statistical Analysis

Each attraction assay was replicated four times and analyzed using methods described by Tomberlin, et al. (*32*). Each attraction assay was analyzed with PROC GLIMMIX SAS 2011, a generalized linear mixed model (GLMM). The probability (P) of attraction responses by *Ae. aegypti* to the mycolactone was examined. When $P < 0.05$ the results were considered statistically significant in this experiment. The attraction

tests were also analyzed by calculating the percent response towards the treatment and the control. Replicate is significant but is included in the model as a random factor.

Results

Attraction to Blood-Feeders Treated with Mycolactone

Figure 3 shows percent attraction response by *Ae. aegypti* adults to blood-feeders treated with mycolactone at different concentrations in comparison to a control. At the lowest dose, the mosquitoes were more attracted to the control (21.8%) than the treatments. No attraction (approximately 50:50) to either treated or control blood-feeders was determined for the middle dose. However, for the high dose, more mosquitoes responded to the treated blood-feeder (29.1%) compared to the control. In comparison, from the low dose (repellence) to the high dose (attractiveness), the mosquito response shifted approximately 50%.

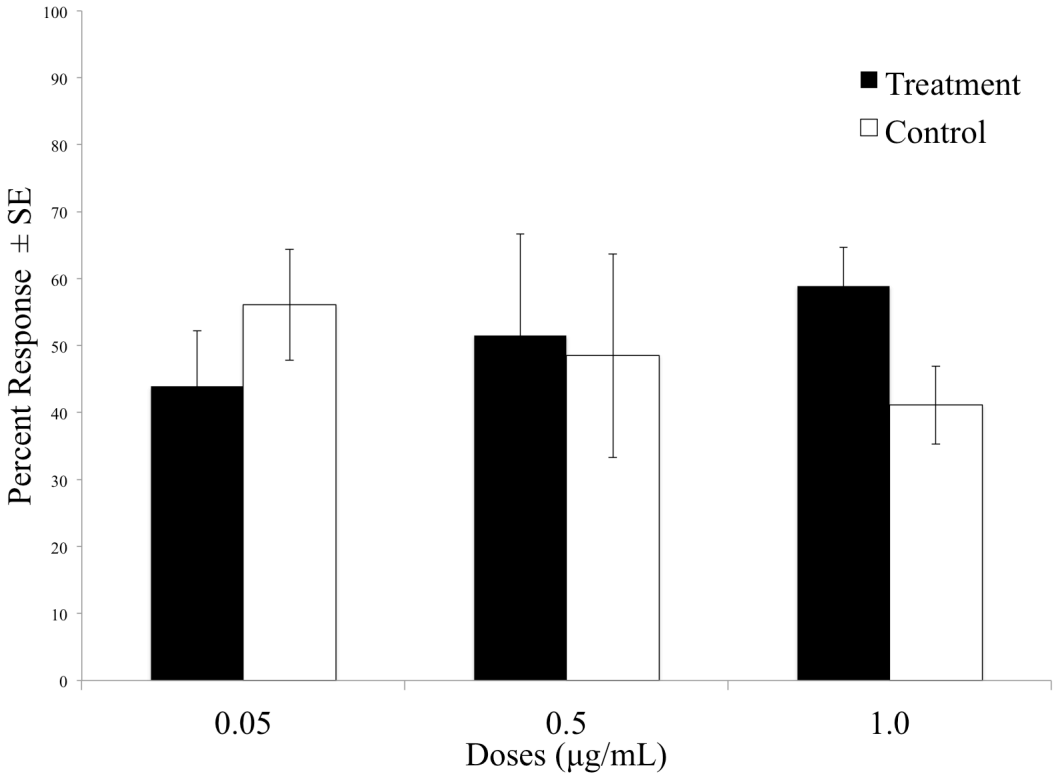

Figure 3. The average percentage response (n = 4) of 50-55 female adult *Ae. ageypti* attraction ± SE to a blood-feeding device treated with mycolactone or a control (ethanol) and located on either the left or right side of the top of a 82 cm x 45 cm x 52 cm Plexiglas cage during a 15-min time frame at approximately 25°C with a RH of 80%
n = number of trials conducted

Estimated probability values for making a host seeking choice (adjusted for replicate) are presented in Table 1. The replicate variance was significantly different from 0 and was included as a random effect in the final model. Dose ($P = 0.0287$) was a significant predictor of response. Odds of responding to the treatment were greatest (1.4328) for 1.0 µg/mL dose, while the lowest odds (0.8319) of response were for 0.05 µg/mL dose. These results mirror what was observed for percent response as presented in Figure 3.

Table 1. Estimated probability, adjusted for replicate, for the blood meal attraction assay with fixed variables related to *Ae. aegypti* choice or no choice to a blood feeder treated with mycolactone (replicates, n = 4)

Factor (Dose)	**Estimated P (SE)**	**Estimated Odds (*P*/1 - *P*)**
0.05 µg/mL	0.4541 (0.2995)	0.8319
0.5 µg/mL	0.6018 (0.2771)	1.5113
1.0 µg/mL	0.5889 (0.2781)	1.4328

(log odds (LO) = 0.2211- 0.01840 + 0 * (Conc.= 0.05 µL/mL) + 0.5970 * (Conc.= 0.5 µL/mL) + 0.5434 * (Conc.= 1.0 µL/mL)Values calculated can then be computed with *P* = exp(LO)/(1+exp(LO)).

Oviposition Preference for Sites Treated with Mycolactone

Figure 4 shows percent attraction responses by *Ae. aegypti* adults to artificial oviposition sites treated with mycolactone at different concentrations or an ethanol control. At the low and medium dose, the mosquitoes were more attracted to the control (approximately 30%) than the treatments. A slight attraction (approximately 15%) to the treated oviposition site was observed for the high dose. In comparison, from the low dose (repellence) to the high dose (attraction), mosquito response shifted approximately 45%.

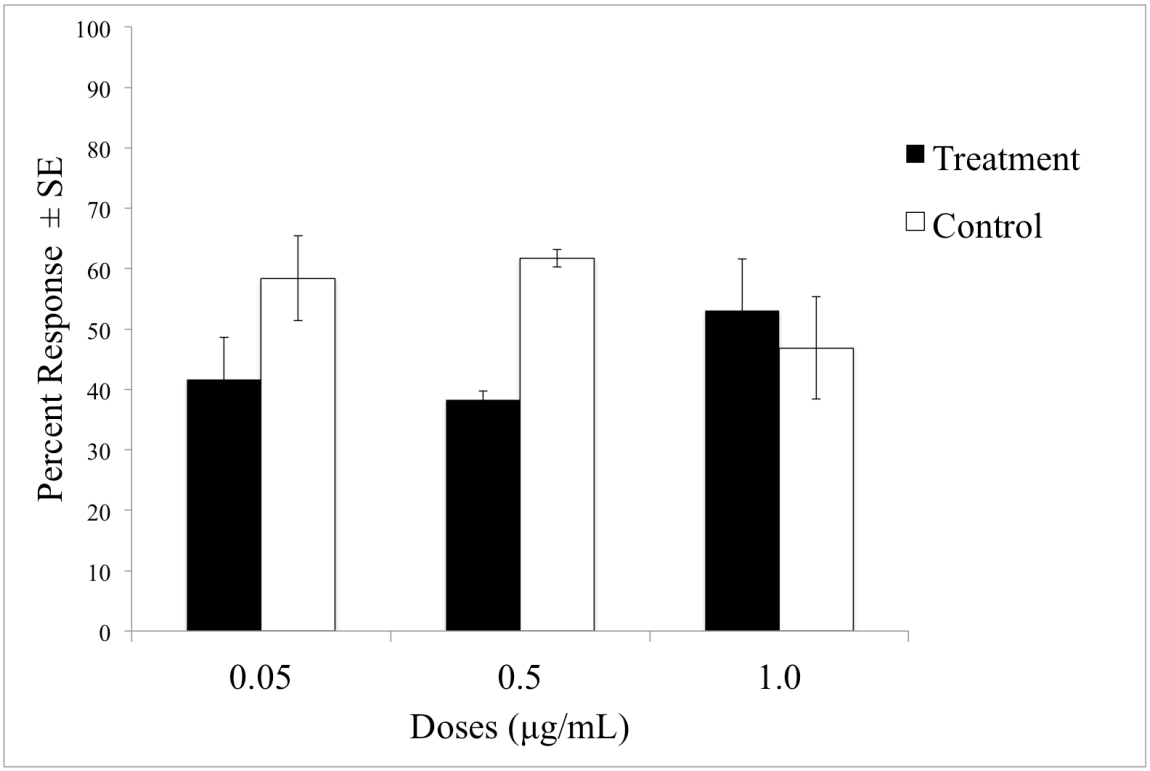

Figure 4: The average percentage response (n = 4) of 50-55 female adult *Ae. ageypti* attraction ± SE to a oviposition site treated with mycolactone or a control (ethanol) and located on either the left or right side of the top of a 82 cm x 45 cm x 52 cm Plexiglas cage during a 48 hour time frame at approximately 25°C with a RH of 80%
n = number of trials conducted

Estimated probability values for making an oviposition site choice (adjusted for replicate) are presented in Table 2. Replicate was significantly different from 0 but was included as a random effect in the final model. Dose ($P < 0.0001$) was a significant predictor of response. Odds of responding to the treatment were greatest (1.2437) for the 1.0 µg/mL dose, while the lowest odds (0.6617) of response were for the 0.5 µg/mL dose. These results mirror the observed percent response as presented in Figure 4.

Table 2. Estimated probability, adjusted for replicate, for the oviposition attraction assay with fixed variables related to *Ae. aegypti* choice or no choice to a oviposition substrate treated with mycolactone (replicates, n = 4)

Factor (Dose)	**Estimated P (SE)**	**Estimated Odds (*P*/1 - *P*)**
0.05µg/mL	0.4317 (0.1610)	0.7598
0.5µg/mL	0.3982 (0.1613)	0.6617
1.0µg/mL	0.5543 (0.1626)	1.2437

(log odds (LO) = 0.1018- 0.2746 + 0 * (Conc.= 0.05µL/mL) - 0.1383 * (Conc.= 0.5µL/mL) + 0.4927 * (Conc.= 1.0µL/mL)Values calculated can then be computed with $P = \exp(LO)/(1+\exp(LO))$.

Discussion

Ae. aegypti adults exhibited a significant level of attraction to a blood-feeder treated with mycolactone (Figure 3); furthermore, the response appeared to be dose dependent ($P = 0.0287$). *Anopheles gambiae* (Giles) (Diptera: Anopheles) have been demonstrated to be attracted to volatiles released from Limburger cheese with a positive response for female attraction (*33*). This lead to further investigation of the carboxylic

acids that were identified in Limburger cheese as attractants, to demonstrate a dose response (*33*). When the carboxylic acids were diluted with diethyl ether from 1×10^{-1} – 1×10^{-9} and attraction was tested using filter paper treated with the different concentrations within a wind tunnel, the undiluted extract acted as a repellant; however at lower doses, attraction ($P < 0.001$) increased (*33*). This study gives support to our findings that when using a higher concentration of mycolactone we see more of an impact of the toxin on the mosquito's host seeking behavior.

Analyses of the data ($P < 0.5$) indicated a replicate effect with regards to mosquito responses. Several factors could explain this observed effect in these experiments. Larval density, which was kept as consistent as possible, of *Ae. aegypti* in the rearing containers could impact adult behavior. Obviously, density dependent response with regards to larval density would impact adult emergence (*34*). Resulting food limitations, competition, and water volume all have an affect on mosquito fitness (*34*). Studies have shown that water temperature can be a determining factor in *Ae. aegypti* development causing limitations on the latitudinal and altitudinal distribution, thus making the expansion to new geographical regions difficult if the temperature is not suitable (*34*). Variation in the RH could have occurred. Relative humidity of the environment plays a large role on the oviposition site locations by causing evaporation of breeding sites when the RH is low (*34*). When examining food limitations as a fitness factor, mosquito larvae have been shown to produce larger and more adults when killing off nutritional deprived larvae occurred (*34*). These abiotic factors, which possibly varied from replications, provide a possible explanation as to why we see variance in the

replications. The possible volatile build up from the ethanol and mycolactone within the testing chamber could result in the replicate effect we see. Its possible the volatiles blend together in the cage making the choice between the two blood-feeders not as effective as a means of studying attraction. A study looking at the attraction behavior with just one blood feeder on the cage each replicate could allow informative data to compare to controls.

The observed shift (~50%) in attraction from the low dose to the high dose suggests that the high dose could be the biologically relevant dose. Unfortunately, little is known about mycolactone concentrations that occur naturally. Studies have been conducted to determine the concentration needed to cause mechanical transfer, trophic transfer and possible spread of other pathogens in association with *M. ulcerans* in endemic areas of BU (*8*). Through the use of PCR in a study conducted in Benin, Africa by Williamson et al. 2012 (*8*) using water, soil, biofilms, invertebrates, excrement, and macrophyte samples, the mean genomic units from mycolactone-producing mycobacteria were quantified (1.68×10^3 GU/mL). The quantification of the mean genomic units found in eight matrixes in the environment allows a better understanding of the environmental biological relevance of the mycolactone (*8*).

In order to determine if the highest dose is a biological relevant dose, more replications with higher and lower doses will need to be completed. The use of a living host, such as the mice, to determine whether Belostomatidae and Naucoridae are possible vectors would be beneficial (*24*). The use of a living host would alleviate some of the limitations of artificial blood-feeders that do not fully provide insight of what is

happening in the environment. The use of testing mosquito's attraction to mycolactone using a mammalian host would also allow observations to be made to help determine whether mycolactone, in combination with odors being emitted by the host, would enhance or decrease the attraction response. Another possibility is the addition of variables presented by the host.

Attractiveness of host to a mosquito is known to be influenced by chemical cues, with CO_2 being the most important; however more recent studies have shown that volatiles released by bacteria can cause a change in the behavior of the mosquito (*35*). Volatiles released by *Staphylococcus epidermidis,* which resides on human skin, can attract mosquitoes (*35*). *Anopheles gambiae,* which transmits malaria, displays variable (differential) attractiveness to humans, depending on different compositions of skin microbiota (*36*). In a study examining *An. gambiae* attraction to humans it was shown that people that are considered highly attractive have a significantly higher bacterial abundance of microbiota, however the diversity of is low (*36*). The effect of the volatiles released from a 'standard human hand' with the compound Y01 has been studied, with *Ae. aegypti* showing a high level of attraction (80%) (*37*). Further studies have been conducted using a Y-tube olfactometer and comparing the 'standard hand' (Y01) to 'test hands', containing different volatile compounds emitted from volunteers that were all white European ranging in the ages of 21-60 (*37*). The relative attraction of the mosquito to hands with the volatile compounds X04 or X08 exhibited greater effects than the volatiles found on standard the standard hand ($P = 0.002$ and $P = 0.019$ respectively), whereas, in hands with X06, Y04, Y05, and Y07 compounds, lower level of attraction

was observed compared to the standard hand ($P < 0.001$). This study demonstrates the difference of the volatiles collected can have an altered affect on the level of attractiveness based on what compounds are actually being emitted.

Aedes aegypti adults laid significantly ($P < 0.001$) more eggs in sites treated with the highest concentration (1.0 μg/mL) of mycolactone (Figure 4). However, as with the blood-feeding attraction assay, approximately 30% more eggs were deposited on the control than the treatment with the low or middle dose. These data indicate, as with attraction to a blood-meal, oviposition by *Ae. aegypti* could be repelled by the low mycolactone dose. Although no significant preference of oviposition (approximately 15%) to the sites treated with the low or middle doses was observed, an increase in oviposition preference to the site treated with the highest dose was observed. The shift in oviposition from the lowest dose to the highest dose was approximately 45%.

This compound could be an indicator that a given environment is an appropriate oviposition site for mosquitoes. *Aedes* spp. lay their eggs singly on a moist substrate often adjacent to bodies of water, as well as areas known to contain floodwater (*18*). Laboratory studies have focused on the response of gravid *Ae. aegypti* behavior when their breeding container has two dominant bacteria species *Acinitobacter calcoaceticus* (Pseudomonadales: Moraxellaceae) and *Enterobacter cloacae* (Enterobacteriales: Enterobacteriaceae) present (*38*). Mosquitoes were provided water with a bacterial suspension of each of the two bacterial species either in a screened–off inner cup or a trap cup (*38*). The oviposition activity index (OAI) which range in values from -1 to 1, where 0 indicates no response was observed for the different bacterial suspensions, and

the OAI of the screened-off inner cups was not significantly different from zero (OAI = 0.069) (*38*). However, when examining the trap cup set up, twice as many females were trapped in the cups, inferring that olfactory-mediated responses were responsible (*38*).

The overall shift in attraction from the control to the high dose mycolactone treatment for blood-feeding and oviposition behaviors provides an indication that mycolactone could be serving as a means of interkingdom communication. Research examining the interkingdom interaction between the blow fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) and a mutant form of *Proteus mirabilis,* that lacks the ability to create a biofilm through swarming, demonstrated that flies were more attracted to a wildtype form of the bacteria that allows swarming (QS response) (*22*). Sites that are known to attract mosquitoes are often ruled by a number of cues including violates emitted by oviposition sites (*22*). *M. ulcerans* can be detected in the environment often in regions near stagnant water or slow moving water, which are considered good breeding sites for *Ae. aegypti* mosquitoes, leading to the possibility that the mosquitoes are coming into contact with the mycolactone, which may be influencing them to alter their oviposition preference (*3, 18*). Although contact with mycolactone may increase host seeking behavior, this is not enough current evidence to determine if the mosquito itself is serving as the mode of transmission or as a means of geographically spreading the pathogen to new regions.

Demonstrating that mycolactone is a cue used by mosquitoes to locate hosts could have large implications with regards to refining the vector competency model. Vector competence of mosquitoes has been shown to be influenced by both intrinsic and

extrinsic factors (*39*). The extrinsic factors take into consideration if a mosquito will come in contact with a host that is suitable for the pathogen or virus being transmitted, whereas, the intrinsic factors play a role in host mosquito attraction and the ability of the mosquito to be infected with the pathogen itself (*39*). The influence microbes have on the mosquitoes' ability to transmit a pathogen provide potential to reduce the capacity of the vector, or enhance the ability to transmit the pathogen. A study examining multiple common gut microbes in *Anopheles albimanus* (Wiedemann) (Diptera: Culicidae), and their influence on the mosquito's vector capacity was conducted in both wild type laboratory and field-captured populations (*40*). This study demonstrated that microbes in the gut of *A. albimanus* could serve to reduce the capacity to vector *Plasmodium.* Commensal microbes (also referred to as secondary symbionts) have host-symbiotic relationships that in most cases enhance vector competence and originated in nature, often picked up in the environment (*40*). Significant attraction to mycolactone in host-seeking behavior could indicate that *M. ulcerans* contamination from the environment could enhance mosquito attraction to a host, thus potentially increasing the capacity of the mosquito as a vector of yellow fever or dengue fever.

The findings presented indicate that there is some attraction to mycolactone in both scenarios; however this attraction cannot be used to infer that *Ae. aegypti* are serving as the mode of transmission of MU. The study would need further testing to determine whether mosquitoes that came into contact with mycolactone and *M. ulcerans* are able to transmit the pathogen to another host. Future studies looking at the exact composition of volatiles that are being released from the mycolactone would help determine if the level

of attractiveness from the mosquito is due to a visual detection, possibly from MU found on the skin, or if the volatiles are having an impact on the change in behavior we have seen in *Ae. aegypti*.

CHAPTER III

FUTURE STUDIES & LIMITATIONS

In this study, there occurred a replication effect in both the host-seeking and oviposition assays resulted in major differences in the attraction responses per replicate. Possible factors that could have lead to the replication effect involve the effects of environmental factors, such as temperature and RH (*41*). As discussed previously, relative humidity of the environment can have detrimental effects on the oviposition site locations by causing evaporation of breeding sites when the RH is low (*34*). It is possible that the RH was not constantly at 80% causing evaporation of the oviposition water used for the oviposition site location attraction. When conducting the study I allowed 48 hours for the mosquitoes to lay their eggs, and observed visually observed slight differences in the water level when removing the filter paper in the cups. Although we were consistent in the amount of water provided the RH could be reasoning behind this. When examining food limitations as a factor in the decrease in the fitness of mosquito larvae, mosquitoes have been shown to produce larger and more adults when the larvae that have not received sufficient nutrition are removed, thus making it possible for mosquitoes not receiving enough fish food to develop to quality standards (*34*). In order to eliminate these limitations, future studies should be conducted in an environment with minimal cues available for the mosquitoes to use.

The lack of information related to the biological relevant dose is a major limitation to this study. Future studies to determine the biological relevant dose should

involve the testing of more mycolactone concentrations (both higher and lower), which could provide a threshold indicating when a response occurs. To determine if the control is serving as an attractant or deterrent conducting a study with gauze that is untreated compared to the ethanol treated gauze would be beneficial.

To better understand the mechanisms by which mycolactone may effect mosquito behavior, a study utilizing the individual volatile components from mycolactone would be beneficial; however to advance our knowledge in the understanding of the transmission of BU through mosquitoes, the use of an animal model could be essential. This would require a study that uses mice or guinea pigs that are exposed to a mosquito that has been treated with a known concentration of mycolactone and are then observed to see which of the animal models obtain BU. Using multiple known dosages in this study would allow a better insight at what concentration is needed to cause an infection when being transmitted by diseases. For regions that are endemic for BU, it would be beneficial to sample for mycolactone to determine if higher concentrations of the mycolactone in the environment correlate to an increase in the mosquito population. Lastly, a possible future study could include the examination of the mycolactone concentration of skin swabs of people who are wading through the waters where the mycolactone is determined to be present and are suitable for mosquito breeding.

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