BLOOD-MEAL PREFERENCE OF *AEDES AGEYPTI* (DIPTERA: CULICIDAE) TO RESOURCES TREATED WITH THE TOXIN PRODUCED BY *MYCOBACTERIUM ULCERANS*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Blood-Meal Preference of *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) to Resources Treated with the Toxin Produced by *Mycobacterium ulcerans*

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Mycolactone is a unique toxin produced by *Mycobacterium ulcerans* (MU), the causative agent of Buruli ulcer. The primary vector for Buruli ulcer (BU) is still unknown; however, some hypothesize mosquitoes, such as *Aedes aegypti*, could be the culprit due to their high vector competence with other pathogens and association with environments endemic to BU. Through a simple attraction test, supplemented with Evans Blue dye in blood to distinguish blood meal sites, the affinity for *A. aegypti* to take a blood meal successfully from a mycolactone saturated area was evaluated and these results show that there is a higher attraction for the control blood meal site in the lowest and highest dose, 0.05 μg/mL and 1.0 μg/mL respectively, and a higher attraction for the treatment blood meal site in the mid-level dose of 0.5 μg/mL. These results thus explore the possibility of interkingdom communication between mycolactone and *A. aegypti*. Additionally, this research will test the possibility that *A. aegypti* could help in spreading Buruli ulcer or causing secondary infection in people infected with BU with another disease in which they are the primary vector.

DEDICATION

To my mother, all I wish is to make you proud.

ACKNOWLEDGMENTS

I want to thank Dr. Tomberlin for finding something special in me and taking me on this journey into research. I could not have asked for a better project to be selected for and the amount of support and help available from you is highly reassuring when my path becomes less clear.

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CHAPTER I

INTRODUCTION

Rationale

Buruli ulcer (BU) is a problem as it is seen to be increasing in occurrence and may soon rival its *Mycobacterium* relatives, leprosy and tuberculosis [1]. BU is detrimental in physical aspects to those without early detection; therefore it is important that all aspects of its causative bacteria, *Mycobacterium ulcerans* and its product (Mycolactone), are researched to gain a better understanding of the disease [2]. It is currently unknown exactly how BU is spread or what the primary vector is, although there have been studies that have shown that proximity to a water source, in most cases a slow moving river expresses a higher incidence of BU cases [3]. This environment of a slow moving river also proves to be an optimal location for mosquitoes to oviposit. The purpose of this research is to determine the role of the mosquito, *A. aegypti* (Linnaeus), (Diptera: Culicidae) in transmission cycle of BU as it is known to be a competent vector and due to its affinity for ovipositing on substrate that will be flooded as seen in places with high incidence of BU. Furthermore, *A. aegypti* is a confirmed vector for dengue fever, and if they are differentially attracted to successfully take blood meals from mycolactone saturated areas, this could result in those infected with BU to also contract dengue fever [4].

Background

Mycobacterium ulcerans is a causative agent of a disease called Buruli ulcer (BU). This microbe is part of the Mycobacterium family that is associated with fungi. This disease causes ulcers on

the skin, which is most closely characteristic of its relative *Mycobacterium leprae* where lesions are formed on the surface of the skin. While similar to *M. leprae* in the aspect that it causes problems on the skin's surface, *M. ulcerans* also produces a toxin called mycolactone that acts as an analgesic in the open ulcer formed by the bacterium. This toxin is unique to *M. ulcerans* as it is only found in etiological variants of the bacterium but not in any other *Mycobacterium spp*. Furthermore, the biological purpose of this compound is not known. Some speculate that it serves as an interkingdom signal that regulates attraction of potential dispersal agents, such as mosquitoes (Diptera: Culicidae). For that reason, an attraction test of *A. aegypti* to this toxin is under question to determine if there is interkingdom communication.

Buruli ulcer can, like *Mycobacterium tuberculosis* and *Mycobacterium leprae*, be treated with antibiotics within the early stages of the disease. Those that are diagnosed with BU must be diagnosed early if they hope to gain treatment without risk of permanent disfigurement. While there are antibiotic treatments available they require long amounts of time of administration to be effective and is costly which is not economical to those diagnosed with BU.

CHAPTER II

METHODS

Mosquito colony maintenance

Methods were adapted from the work of Michael Sanders (Sanders 2015). *A. aegypti* eggs (Liverpool Strain) were reared to the adult stage for use in these experiments. Eggs were hatched in a six quart container with 1 L distilled water in an incubator at 25°C, 80% RH, and 12:12 L:D. Larval populations were limited to approximately 100-200 larvae per container and were given approximately 3 g fish food (TetraMin diet by Tetra Blacksburg, VA) on alternating days. Pupae were separated from the larval containers and placed in 60 mL cup at a density of 50/cup. These containers were placed in a grease resistant paper bucket approximately 4.9 L in size (Solo Cup Operating Corporation, Lake Forest, IL) covered with mesh to prevent emergent adults from escaping and stored in the incubator previously described. Emergent adult mosquitoes were provided with a cotton ball dampened daily with a 5% sucrose solution. Emergent mosquitoes were knocked down with CO_2 48 hours prior to experimental trials and approximately 50 females were separated into each container with access to a cotton ball dampened with 5% sucrose solution. Mosquitoes were aged to 5-10-d-old when utilized for experimental purposes. Upon reaching the appropriate age, the cotton ball was removed approximately 24 hours before they were provided a blood meal.

Blood-feeder

The blood-feeder consisted of a 25 cm^2 cell culture flask (Corning Incorporated, NY) covered with parafilm creating a pocket on the outside of the flask into which 2 mL of defibrinated rabbit blood (HemoStat Laboratories, Dixon, CA) was pipetted. The top of the blood-feeder was connected to a rubber stopper with valves in the stopper for in going and outgoing water. The ingoing and outgoing valves were connected via a thin plastic tube that recycled the water through a water bath - creating a closed circuit - set to 37°C simulating human body temperature. (Figure 1) The blood-feeder was then placed face down onto a 7.0 cm x 7.5 cm piece of Johnson & Johnson four pleated gauze (Johnson & Johnson, New Brunswick, NJ) treated with 1 mL of the desired concentration of mycolactone or ethanol.

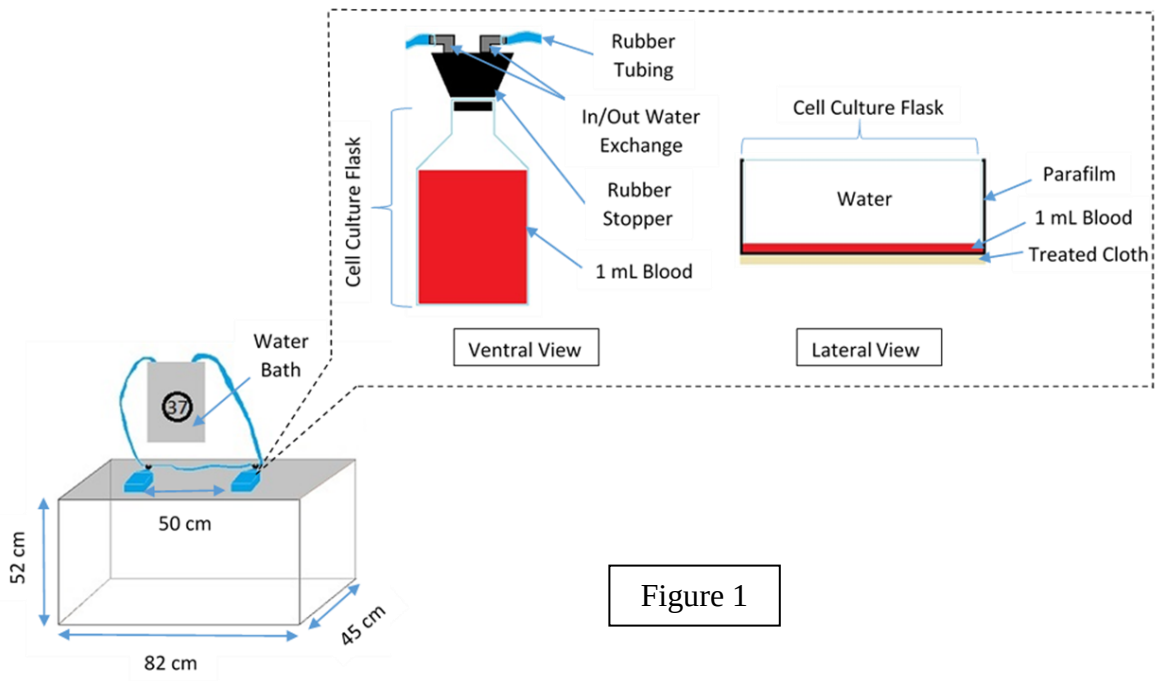


Figure 1

Experimental design

This design is based on Xinyang et al. (2015) [5]. Approximately 50 adult female mosquitoes 5-8-d old that had not been previously blood fed and deprived of sucrose for 24 hours were released into a plexiglass cage in the incubator room as previously described in the mosquito rearing section. The female mosquitoes were released within the cage from alternating sides each replicate 30 minutes prior to blood-feeding as an acclimation period to ensure enough time to eliminate positional bias. Two blood-feeders were used in each replicate placed face down 50 cm apart parallel to each other on top of gauze with the desired treatment concentration of mycolactone and the other the control – 95% ethanol - on the cage. One feeder was filled with 2 mL of rabbit blood mixed with Dulbecco's Phosphate Buffer Saline (DPBS) (Sigma Aldrich, Milwaukee, WI) at a 1:1 ratio and the other blood-feeder with 2 mL of rabbit blood treated with Evans Blue dye (Sigma Aldrich, Milwaukee, WI) mixed with DPBS at a 200 μg/kg concentration in the blood at a 1:1 ratio to allow for appropriate color distinction of blood-meal sites [6]. The use of Evans Blue dye created a visible color difference between the pure and treated blood which was then used to determine blood feeding location of each female adult mosquito. Three replicates were performed with each concentration of mycolactone performed once each time and in the same manner for each replicate. The blood-feeder treated with the Evans Blue dye was switched each replicate to ensure that the dyed blood would not be associated exclusively with either control or treatment gauzes. The female mosquitoes were allowed to blood feed for 15 minutes before the blood-feeder was removed. The mosquitoes were knocked down with CO_2 and then the engorged mosquitoes were separated from those that had not taken a blood meal. The engorged mosquitoes were then separated based upon the blood color found within the abdomen.

CHAPTER III

RESULTS

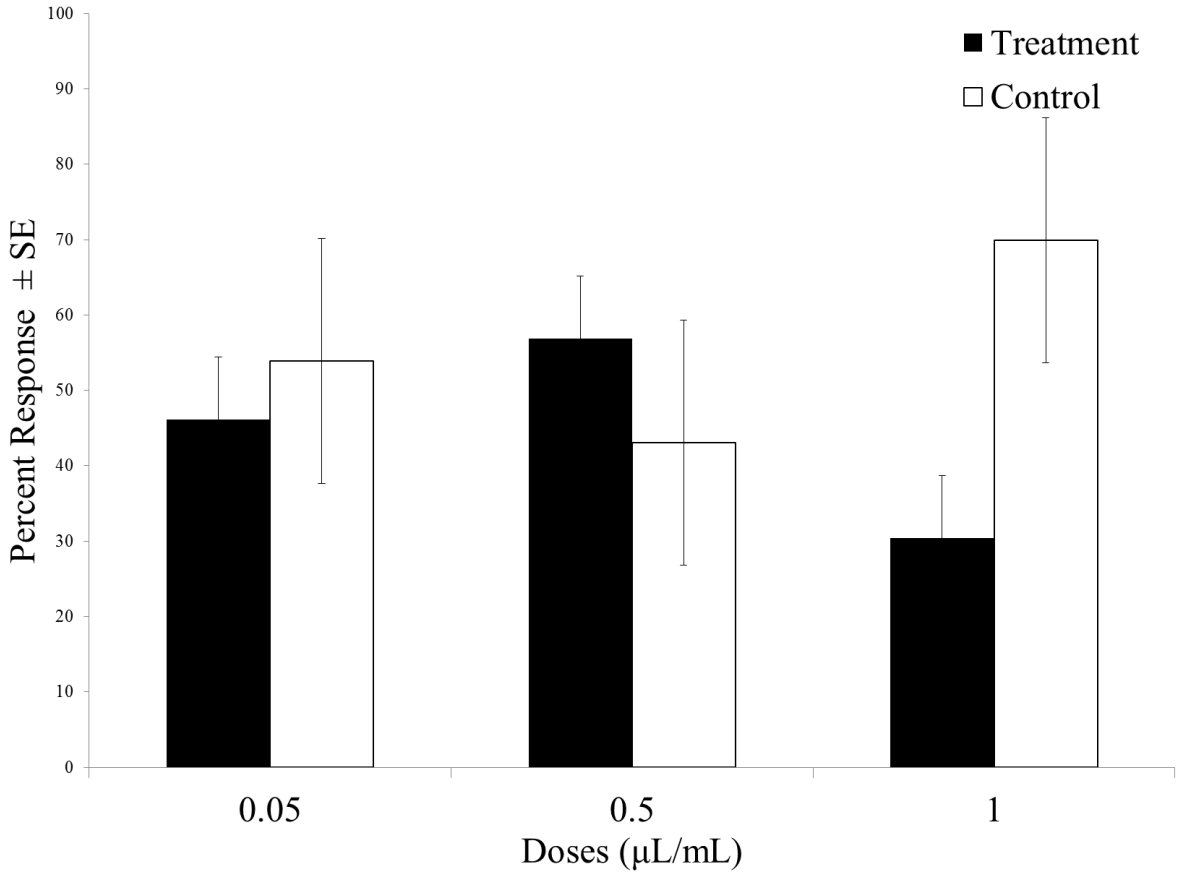
Determination of blood meal

At the culmination of each of trial engorged mosquitoes were separated based upon the color of the blood in their abdomen to determine blood meal source. Most of the mosquitoes were able to be easily separated based upon visual determination of color due to the large color differential between the blood treated with DPS and Evans Blue dye, but there were cases when the mosquitoes did not take a large blood meal at which time it was more difficult to make a clear distinction. Although a sample size of approximately 50 adult female mosquitoes were used at the end of the fifteen-minute blood feeding period in some trials as few as only four had taken a blood meal which made each mosquito statistically relevant. Results of this research were then determined through statistical analysis based upon those that had actually taken a blood meal.

Percent Response to each blood meal site

For the low dose of 0.05 μg/mL it was found that there was a 46.1% response for the mosquitoes to take a blood meal from the blood meal site treated with mycolactone which is 7.8% below the response to the control blood meal site which had a response of 53.9%. The middle dose of 0.50 μg/mL dose shows an opposite trend as the mycolactone treated blood meal had a 56.9% response for the mosquito to take a blood meal as opposed to the lower response of 43.1% to the control blood site. Again, like the 0.05 μg/mL dose there is not an extremely large difference in the response to either treated or control blood meal site. The highest dose of mycolactone at 1.0 μg/mL shows quite a significant difference in the choice of blood meal site as the control had a

69.9% response as opposed to the 30.4% response seen in the treatment blood meal site. All of these statistics are seen displayed in the graph below.



CHAPTER IV

DISCUSSION

Attraction vs. successful blood meal

These trials were based on a previously performed experiment still pending in publication (Sanders 2015) using a simple binary choice set up to determine simple attraction of *A. aegypti* to the toxin mycolactone produced by MU. The results were calculated based on the amount of mosquitoes visually seen on each of the two blood-feeders each minute, one treated with mycolactone and one with control. Attraction was calculated based solely on the number of mosquitoes counted on the feeder as blood feeding could not be determined from the footage. The information gathered from this current research performed aims to strengthen the implications by determining the site where the female *A. aegypti* actually takes a blood meal. It was believed that the results of this experiment would parallel that of the simple attraction tests previously performed and outcome for the 0.05 μg/mL of 95% ethanol vs. a control of 95% ethanol show that there is a similarity of attraction as the treatment site was almost just as attractive a blood meal site for the mosquitoes as the control treated blood-feeder. The 0.50 μg/mL of 95% enthanol vs. the control of 95% ethanol based off visual analysis determined that there was a slightly greater attraction for the treated blood feeders which is congruent with the attraction seen in the previous experimental data. The last dose of 1.00 μg/mL of 95% ethanol vs. the control of 95% ethanol as was more pronounced than that of the previous two doses with a significantly large affinity of the mosquitoes to take blood meals from the control blood meal site. This was determined by the visual discrimination of the blood meal observed in the abdomen of engorged mosquitoes. The results obtained from this study are not exactly congruent

with the previous performed study, but both research results offer insightful knowledge about the chances of *A. aegypti* being differentially attracted to mycolactone.

Implications

The implications of the results reached by this research point to the *A. aegypti* mosquito as not too likely to be differentially attracted to take a blood meal from a site that is saturated with mycolactone at low and high doses. The results for the middle dose however, at 0.5 μg/mL, show that they are more attracted to take blood meals from this site which is somewhat worrisome as the middle dose is what is most closely related to the dose found on a person with an open ulcer created by MU. Further research should be performed to strengthen these findings and the use of spectrophotometry in these future trials would be helpful in providing definitive results of the location from which a blood meal is taken.

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