IMPACT OF MYCOLACTONE PRODUCED BY *MYCOBACTERIUM ULCERANS* ON LIFE-HISTORY TRAITS OF *AEDES AEGYPTI AEGYPTI* (L.) (DIPTERA: CULICIDAE) AND RESULTING HABITAT SELECTION FOR OVIPOSITION

A Thesis

by

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ABSTRACT

Buruli ulcer (BU) is a globally recognized neglected tropical disease caused by *Mycobacterium ulcerans*. BU is the third most recurrent mycobacterial disease of humans globally after tuberculosis and leprosy. The disease results in dermal tissue necrosis exposing the tissues under the skin. Ulcers can reach 5 to 15 cm in diameter in some patients especially if they do not seek early treatment. Most cases involve individuals between the ages of 4 to 15 years. This disease was first noted in the late 1880's in Africa and has since been reported worldwide. The exact mode of transmission in BU is unclear; however, it is hypothesized contact with slow-moving rivers and associated biting aquatic insects, such as mosquitoes results in pathogen transmission. Recent research from our group demonstrated mycolactone as an attractant for adult mosquitoes seeking a blood-meal as well as oviposition sites. In this study, the impact of mycolactone (0.05 μg/mL), (0.5 μg/mL), (1.0 μg/mL) on immature life-history of *Ae. aegypti* (commonly occurs in same environment as *M. ulcerans*) was examined. We determined percent egg hatch was not significantly different across treatments. However, concentration did impact survivorship of larval mosquitoes to the adult stage. Future research will determine if development in the presence of mycolactone impacts decision-making by resulting mosquitoes seeking oviposition sites. If true, a synergistic effect with regards to the prevalence of BU and other *Ae. aegypti* associated diseases (e.g., Yellow Fever) may occur.

DEDICATION

To my lovely parents, betrothed Wedad Mawkili, family and friends. To my country Saudi Arabia and Jazan University particularly the department of biology. To my teachers for giving me the key to open all of life's doors. To whom encouraged and helped me to complete this work. To whom are might interest in my subject.

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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
GBUI	Global Buruli Ulcer Initiative
WNV	West Nile virus
HHSP	Hopkins' Host Selection Principle
DDT	dichlorodipehnyltrichlroethane
Bti	*Bacillus thuringiensis israelensis*
Bs	*Bacillus sphaericus*
RH	Relative Humidity

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Buruli ulcer (BU), also known as Bairnsdale ulcer in Australia (Quek et al. 2007), is a neglected tropical disease impacting dermal tissue of human hosts. Most cases involve individuals between the ages of 4 to 15 years (Williamson et al. 2008, Organization 2012, Vincent et al. 2014). This disease was first noted in the late 1880's in Africa and is now known to have global distribution (Williamson et al. 2008, Merritt et al. 2010). West Africa in particular is considered a highly endemic area (Fig. 1) (Johnson et al. 2005). BU was given its name in reference to a county in Uganda, where Sir Albert Cook first described ulcers consistent with *M. ulcerans* infections in 1897 in Uganda (Meyers 1995, Nakanaga et al. 2013). Since then, the WHO has developed the Global Buruli Ulcer Initiative (GBUI) that focuses on the improvement of treatment provided to people with the disease and addresses the matter of community prevention and awareness (Williamson et al. 2014).

Mycobacterium ulcerans and BU Pathology

The pathology of BU has been well studied. Early onset of BU occurs in the subcutaneous tissue layers below the skin most-often on the arms and legs (Fig. 2A) (Nakanaga et al. 2013). *M. ulcerans* is a slow-growing environmental pathogen with a doubling time of 36-48 hours depending on the growth conditions (Johnson et al. 2005). In fact, incubation for 5-8 weeks at 30–32 °C under laboratory conditions is required to obtain visible colonies (Johnson et al. 2005). The ulcers are painless; however, if not treated, the ulcers will continue to expand and could result in secondary infection, bone deformation, and osteomyelitis (Johnson et al. 2005). Though mortality is low, there is a significant amount of

morbidity associated with infection that leads to a great socioeconomic burden (Barogui et al. 2013, Yeboah Manu et al. 2013). The disease results in tissue necrosis expanding into healthy tissue resulting in damage to the dermal regions infected and exposing the tissues under the skin (Barogui et al. 2013). Ulcers can reach 5 to 15 cm in diameter in some patients especially if they do not seek early treatment. Those with larger ulcers may require surgery for treatment (Chauty et al. 2007). If not diagnosed quickly, development of ulcers could result in long and costly hospitalizations, disability or even death (Fig. 2B) (Organization 2012). The WHO and some studies recommend antibiotic treatment of early onset of BU with rifampicin in combination with streptomycin for eight weeks (Merritt et al. 2010, Organization 2012, Friedman et al. 2016). While these antibiotics are appropriate for killing *M. ulcerans* and treatment of early lesions, surgical procedures might be needed in cases

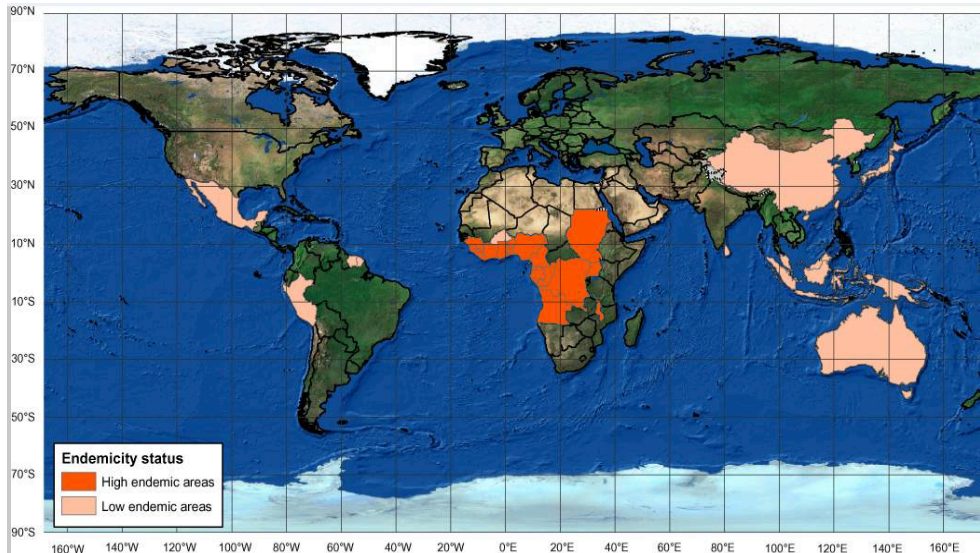

FIGURE 1. A global map representing high and low endemic areas of BUdisease as of 2005 (Johnson et al. 2005).

where the ulcers are deep, especially when the ulcers infect muscle tissues (Johnson et al. 2007, Merritt et al. 2010). However, antibiotics are often scarce and still expensive in most third world nations. Additionally, many patients cease treatment prematurely as streptomycin

requires injection by a trained administrator, and may require long distance travel to treatment centers leading to an increased familial burden (Henry and Lexchin 2002).

Epidemiology

Mycobacterium ulcerans, the causative agent of BU, is a long and slender rod-shaped acid-fast bacterium (Organization 2012). Bacteria comprising this genus are characterized by having a cell wall rich with mycolic acids, making them recalcitrant to antibiotics (Raghunathan et al. 2005). *M. ulcerans* disease is the third most recurrent mycobacterial disease of humans in the world after tuberculosis and leprosy (which cause tuberculosis and leprosy, respectively) (Johnson et al. 2005, Vincent et al. 2014). *M. ulcerans* is most commonly found in tropical subtropical countries, with West Africa being the main endemic zone as previously discussed. In West Africa, *M. ulcerans* and BU occurs most frequently in rural locations (Johnson et al. 2005). A recent study by Kenu (2014), confirmed by using

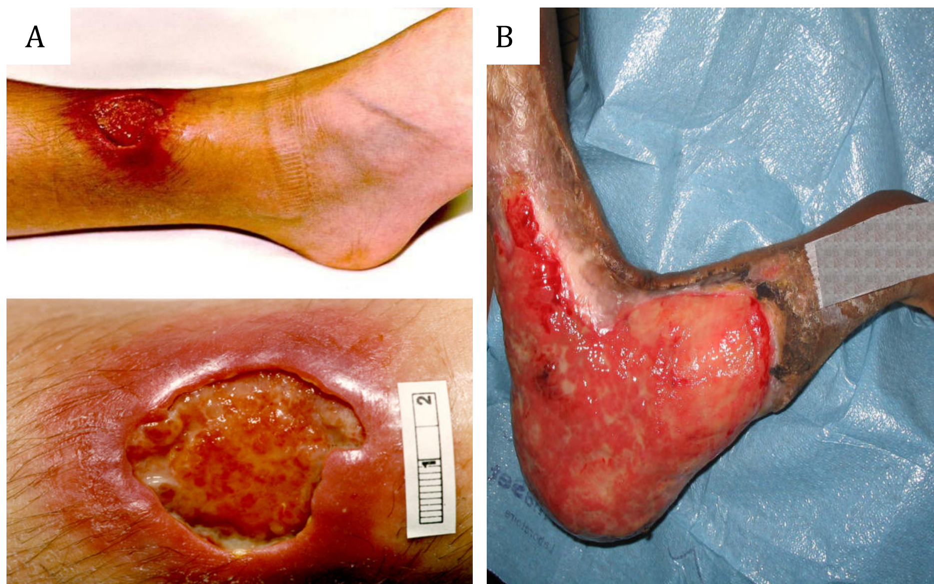

FIGURE 2.: A) A closer view, revealing deep undermining, is shown in the second panel. B) Long-Term Sequelae of *M. ulcerans* Infection (Johnson et al. 2005).

geographical information system (GIS) technology, the appearance of BU along the Densu River (Greater Accra region of Ghana) was especially high in areas where the flow of the water was slow, as well as where gentle slopes existed (Kenu et al. 2014a). In addition, a recent study by McIntosh, et al. investigated the associations between *M. ulcerans* and aquatic plant. Positive samples (ER, MU, and MPM) were found on multiple plant taxa in both lotic and lentic habitats (McIntosh et al. 2014). Slow flowing areas were determined based on prior knowledge of mountains in the region (Kenu et al. 2014a). Ultimately, Williamson (2012) reported *M. ulcerans* is often linked with a residence near an aquatic environment in Africa; however, fishermen who frequent the water for prolonged visits were not determined to be at a high risk for infection (Raghunathan et al. 2005, Pouillot et al. 2007).

Mycobacterium ulcerans and Mycolactone

Mycolactone is produced by *M. ulcerans*. Mycolactone is considered a potent cytotoxic and immunosuppressive polyketide- derived macrolide (Mve-Obiang et al. 2003, Gama et al. 2014). Some macrolides have pharmacological properties (i.e. the antibiotic erythromycin and the immunosuppressive rapamycin) (Main 2014). In 1999, mycolactone was first isolated from *M. ulcerans* and determined to be cytopathic and thus responsible for the pathology observed in the ulcers (George et al. 1999, Raghunathan et al. 2005). The discovery was an interesting one as mycolactone was a cytotoxic lipid; unique to a bacterial pathogen. In most cases, bacterial pathogens are known for producing protein-based toxins (Merritt et al. 2010).

Transmission of BU

The exact mode of transmission in BU is unclear, and is a reason BU is often referred to as a "mysterious disease"; however it is hypothesized to occur from contact with the environment such as slow-moving rivers (especially in areas prone to human-made disturbance and flooding) (Fig.3) (Johnson et al. 2005, Merritt et al. 2010, Williamson et al. 2012).

FIGURE 3. Slow-Moving Rivers: Typical Buruli ulcer riverine endemic sites in Ghana (Merritt et al. 2010).

Since the late 19th century, researchers have been attempting to determine the mode of *M. ulcerans* transmission (Johnson et al. 2005, Quek et al. 2007, Merritt et al. 2010). Transmission of the pathogen from person-to-person is not common, with only one case being reported involving a human biting another human (Merritt et al. 2010). In this case, the patient's skin surface was believed to have come into contact with the environment containing *M. ulcerans* resulting in the pathogen being driven into the skin (Merritt et al.

2010). An alternate hypothesis for a mode of transmission of *M. ulcerans* is the skin of an individual being breached by puncture and allowing contamination with the pathogen (Williamson et al. 2014).

Some researchers hypothesize that insects associated with environments containing the pathogen are potential vectors, such as black flies (Diptera: Simuliidae), mosquitoes (Diptera: Culicidae), March flies (Diptera: Tabanidae), and sandflies (Diptera: Ceratopogonidae)) (Luckhart et al. 1998, Quek et al. 2007, Merritt et al. 2010). A study reported that aquatic hemipterans, Naucoridae, infected with *M. ulcerans,* could bite mice and transmit *M. ulcerans* under laboratory conditions (Marsollier et al. 2002). In 2004, a spike in BU cases in a small Australian town near Victoria, Australia, was recorded (Quek et al. 2007). Researchers also noted high activity of *Aedes* mosquitoes at that time. Real-time PCR was used to screen 11,000 mosquitoes in the area. Results indicated a *M. ulcerans* infection rate of 4.3 per 1,000 mosquitoes (Merritt et al. 2010).

Aquatic Insects

Although BU is a "mysterious disease" and the mode of transmission is unknown, many studies have hypothesized to occur by aquatic insects (Marsollier et al. 2002, Johnson et al. 2005, Quek et al. 2007, Merritt et al. 2010, Wallace et al. 2010). BU is thought to occur from contact with aquatic environments (Johnson et al. 2005, Merritt et al. 2010, Williamson et al. 2014), and aquatic insects have been investigated as potential reservoirs or vectors. In addition, Marsollier, 2012 reported the aquatic hemipterans, Naucoridae, infected with *M. ulcerans* could bite mice and transmit the pathogens under laboratory conditions (Marsollier et al. 2002).

Recently, Wallace (2016) tested healthy mice after dipping their tails in cultures of the causative agent, *Mycobacterium ulcerans* (Wallace et al. 2016). They study exposed the tails to two species of mosquitoes, *Aedes notoscriptus* and *Aedes aegypti*. Two of 11 mice get infected after mosquitoes fed on them (Wallace et al. 2016). Therefore, mosquitoes (*Aedes notoscriptus* and *Aedes aegypti* that were used in the study*)* could act as atypical mechanical vectors of *M. ulcerans* (Wallace et al. 2016). Furthermore, Wallace et al. (2016) reported that *Aedes aegypti* is known as closely associated with humans worldwide (Wallace et al. 2016).

Aedes aegypti (Diptera: Culicidae) is the primary vector of the pathogens responsible for yellow fever and dengue fever viruses. *Aedes aegypti* adults are easily recognized by the white-banded patterns on their legs. This mosquito species has a known distribution throughout the tropical and subtropical regions of the world (Rozeboom 1960). The metamorphism in *Ae. aegypti* is a holometabolous, which characterized by complete metamorphosis. *Aedes aegypti* deposits eggs around the edges of standing, or slow-moving water sources (Rozeboom 1960). If the environment dries, the eggs are stable for months without losing vitality. Once the eggs come in contact with water, they hatch in about 2-7 days (Rozeboom 1960). Based on the preliminary experiments we did in our lab, the average in eggs hatching time was 2-6 hs (unpublished data). Larvae pass through four instars prior to becoming pupae. Pupae need 2-4 days for adult emergence at 23-27 °C. Resulting mosquitoes lay eggs inside containers holding water between 2-3 days after a blood-meal (Rozeboom 1960).

Because *Ae. aegypti* occurs in habitats endemic to *M. ulcerans*, there is a possibility that ecological interactions could occur between the two (Demangel et al. 2009). Recently, Sanders (2016) demonstrated mycolactone serves as an attractant of the mosquito, *Aedes*

aegypti aegypti (L.) the yellow fever mosquito (Diptera: Culicidae) to blood-meal sources (Sanders et al. 2016). In fact, he determined the response was dose dependent. The high dose (1.0 µg/mL) attracted mosquitoes to the blood-feeder by 29.1% compared to the control. This indicates the dose of mycolactone can serve as an attractant of mosquitoes to hosts, which can potentially result in *M. ulcerans* transmission and BU infection (Sanders et al. 2016). In contrast, low (0.05 µg/mL) and middle doses (0.5 µg/mL) served as repellents. As *Ae. aegypti* larvae develop in aquatic habitats containing *M. ulcerans*, the resulting adult mosquitoes could potentially use mycolactone as a signal for locating appropriate oviposition sites (Rozeboom 1960). But, while mosquitoes have demonstrated a preference for ovipositing in areas containing mycolactone, its impact on the development of resulting *Ae. aegypti* immature, and adults is not known.

Aedes aegypti and Pathogen Global Reliance

Mosquitoes are infected with, and transfer, pathogens during blood-feeding (Tabachnick et al. 1985, Gubler 1989, Beerntsen et al. 2000). *Aedes aegypti* (Diptera: Culicidae) is the primary vector of the pathogens responsible for yellow fever, dengue, and recently in the news, Zika (Rozeboom 1960, Nene et al. 2007, Zupanc et al. 2016).

Globally Important Viral Diseases Transmitted by *Ae. aegypti*

The virus responsible for yellow fever is an arbovirus of the *Flavivirus* genus and is transmitted in Africa by multiple species belonging to *Aedes* genus (Tabachnick et al. 1985, Gould and Solomon 2008). Thirty-four countries are either endemic for, or have regions that are endemic for yellow fever (Timoshevskiy et al. 2013). In the 17th to 19th centuries, yellow

fever was transported to many other continents, such as North America and Europe (Gould and Solomon 2008, Timoshevskiy et al. 2013) The World Health Organization reported an estimation that there are 200,000 cases of yellow fever annually, including 30,000 deaths, of which over 90% occur in Africa (Vainio and Cutts 1998, Mutebi and Barrett 2002).

Dengue on the other hand is actually considered the most important vector-borne arboviral disease of the 21st century (Gubler 2012, Timoshevskiy et al. 2013). This disease is a risk to 3.6 billion people resulting in 21,000 deaths per year in the world (Beerntsen et al. 2000, Gubler 2012). The disease became endemic in over 100 countries in Africa, West Asia and America (Gubler 2012, Halasa et al. 2012).

Since 2015, Zika virus has become a growing threat to the United States, originating from Brazil (Zupanc et al. 2016). This disease was first isolated in 1947 in Uganda and was distributed in Africa and Asia (Gould and Solomon 2008). Zika virus is transmitted to people through an infected mosquito, mainly *Ae. aegypti* (Zupanc et al. 2016). During human infection, vertical transmission, from mother to fetus, has been most frequently described, with negative outcomes in babies, presenting with congenital brain abnormalities, including microcephaly or fetal death (Zupanc et al. 2016).

Other Diseases of Human and Animals

Other viruses transmitted to humans by *Ae. aegypti* include Bwamba fever, West Nile virus (WNV), Semliki Forest virus, Bunyamwera and *Flavivirus* (Christophers 1960). In many cases these diseases are also transmitted by other *Aedes* species (Christophers 1960). For example, WNV, which causes disease primarily in children, has been isolated from the species of *Culex* and transmitted by *Ae. aegypti* (Taylor and Hurlbut 1953, Work 1955).

Furthermore, a study conducted in Colombia, Venezuelan, reported equine encephalitis transmitted by *Ae. aegypti* in 687 out of 2295 houses (Sanmartin Barberi 1954, Christophers 1960).

Aedes aegypti has been reported to transmit diseases in animals such as equine encephalitis, haemorrhagic septicaemia of buffales, fowlpox and Rift Valley fever (enzootic hepatitis) (Christophers 1960). In Africa, *Ae. aegypti* has been implicated in the transmission of equine encephalitis in animals (Christophers 1960). This vector is also responsible for transmission of equine encephalitis among birds (Kelser 1933, Christophers 1960). In Asia and sub-Saharan Africa, *Aedes aegypti* caused haemorrhagic septicaemia, a disease infecting buffaloes and cattle, particularly during the wet season (Benkirane 2002). Another viral disease in animals is fowlpox that is the worldwide disease of poultry, caused by a prototypic virus of the *Avipoxvirus* genus (Taylor and Paoletti 1988). And, Rift Valley fever, is an important disease of sheep and to a less extent of cattle in Africa (Christophers 1960). This disease causes abortion in cattle, where human could become infected by contact with infected animals (Smithburn et al. 1948, Smithburn 1949, Miller et al. 2002). In 2000, Rift Valley fever was reported for the first time in the Kingdom of Saudi Arabia and Yemen, outside Africa (Miller et al. 2002).

Vector Competency

Mosquitoes are arthropod vectors for pathogens leading to many diseases. The ability to transmit these pathogens depends on the vector competency of the species. Competency refers to the ability of the vectors to successfully acquire a disease agent microorganism (e.g., arbovirus) from the reservoir host and subsequently transmit the infectious agent to another

susceptible host (Gubler et al. 1979, Hardy et al. 1983, Collins and James 1996, Beerntsen et al. 2000, Weiss and Aksoy 2011). Vector competency depends on the ability of an infectious pathogen to survive and potentially replicate in arthropod tissue. Intrinsic and extrinsic variables have an impact on the vector competence of mosquitoes (Hardy et al. 1983, Sanders et al. 2016). If a mosquito interacts with a host that is susceptible to transmission of a particular pathogen, there are intrinsic and extrinsic factors to consider. For example, an extrinsic factor includes when mosquito will come in contact with a host that is appropriate for the pathogen or virus being transmitted; however, the intrinsic factors is the ability of the mosquito to get infected with the pathogen itself (Hardy et al. 1983). These variables play a part in order for the mosquito to be infected with the pathogen itself (Hardy et al. 1983). The impact that microbes have on the mosquito vector will influence the capability to transmit a pathogen by decreasing or increasing the ability of the vector to transmit the pathogens. A study was conducted to investigate different common gut microbes in *Anopheles albimanus* (Wiedemann) (Diptera: Culicidae) and their impact on the mosquito's vector ability using both wild and laboratory breed populations. Results showed that microorganisms in the gut of *Ae. albimanus* could diminish the ability of the vector to transmit *Plasmodium* (Weiss and Aksoy 2011).

Mosquitoes are indisputably the most important arthropod vectors of many diseases (Beerntsen et al. 2000). The maintenance and transmission of the pathogens depend on the availability of competent mosquito vectors (Beerntsen et al. 2000). *Aedes Aegypti* affects millions of people annually, and approximately 2.5 billion people are at risk of dengue, with approximately 50 million cases per year (Beerntsen et al. 2000, Nene et al. 2007). The salivary glands in mosquitoes secrete powerful antihemostatic agents that facilitate

hematophagy by counteracting the effects of vertebrate wounding responses (Beerntsen et al. 2000). In *Aedes aegypti*, these antihemostatic molecules include sialokinins, apyrases and anticoagulants that prevent platelet aggregation, vasoconstriction, and coagulation, respectively when they pierce the skin for blood-meal (James 1994, Ribeiro 1995, Beerntsen et al. 2000). Immune system molecules provide the mosquitoes with an innate defense system against invading pathogens that is both discriminatory and efficient. The figure below shows the mosquito immune responses to pathogens including melanotic encapsulation, phagocytosis, and production of antibacterial compounds and immune peptides (Fig. 4) (Paskewitz and Christensen 1996,

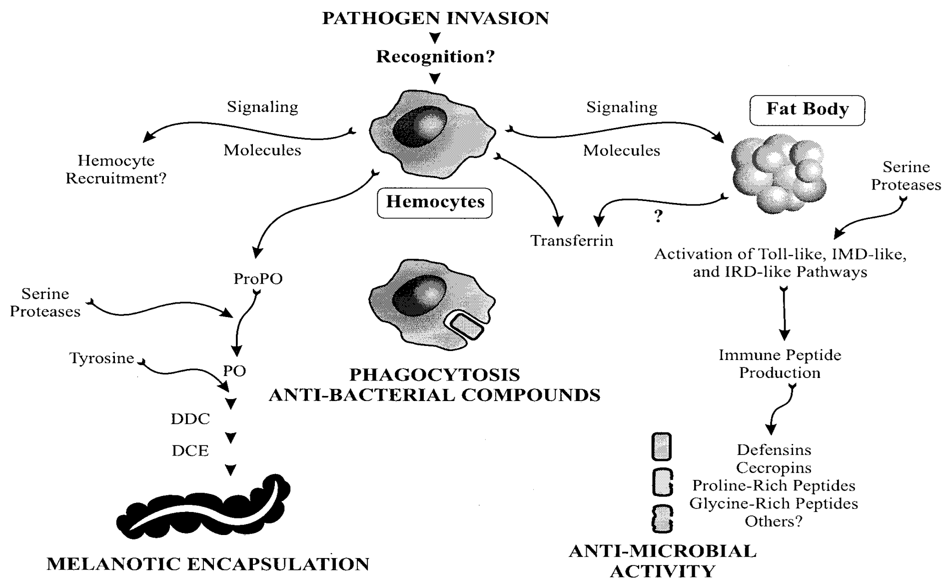

FIGURE 4. A figure shows the mosquito immune responses to pathogens contain melanotic encapsulation, phagocytosis, and production of antibacterial compounds and immune peptides

Beerntsen et al. 2000). In the female mosquito, immunocytochemistry was found to be at its highest level before and 24 h after blood-meal (Brown et al. 1999, Stanek et al. 2002).

Examples of Chemical Legacy and HHSP

Chemical legacy can be defined as changes in the chemosensory responses in insects where a new generation may inherit from parents in aspects of host association (Corbet 1985, Barron 2001). In insects, a chemical legacy from the larval environment is possible to induce a host preference in the adult (Corbet 1985, McCall and Eaton 2001). The possibility of egg-borne on semiochemicals might influence larval chemosensory development, and is important in female oviposition (Ganesan et al. 2006). For example, mosquitoes can be induced to oviposit on unmodified substrates from natural larval habitats containing live microorganisms such as bacteria. Many species of mosquitoes have shown strong oviposition preferences for sites with bacterial colonization (Corbet 1985, Trexler et al. 2003, Sumba et al. 2004, Albeny-Simões et al. 2014). Recent studies have revealed this behavior, in part, to be controlled by semiochemicals (Verhulst et al. 2009, Verhulst et al. 2011). Chemical legacy assumes the existence of chemicals at sensitive periods (i.e., adult emergence) can reduce peripheral sensitivity, causing modifications in adult behaviors (McCall and Eaton 2001).

Also, the larval chemical legacy can affect the adult oviposition-site selection, which would be of great ecological significance (Bush 1992, Barron 2001). Prior to the term chemical legacy, Hopkins' Host Selection Principle (HHSP) was proposed (Hopkins 1916, Barron 2001, Rietdorf and Steidle 2002). HHSP can be defined as oviposition site selection by an adult being partially dependent on the memory of their larval development site (Corbet 1985). For example, it has been reported that *Ae. aegypti* gains genetic preferences during the aquatic period (Barron 2001, Chow et al. 2005).

Hopkins' Host Selection Principle (HHSP) refers to the observation that many adult insects demonstrate a preference to the habitat on which they developed as larvae (Hopkins 1916, Barron 2001, Rietdorf and Steidle 2002). HHSP assumed that behavior of adult insects is conditioned by larval experience (Barron 2001, McCall and Eaton 2001, Rietdorf and Steidle 2002, Davis 2008). However, there is still very little convincing evidence for imago (pre-imagine) conditioning of host choice in insects (Surendran et al. 2012). Chow et al (2005) determined that some insects such as Culicidae (Diptera), Agromyzidae (Diptera), and Noctuidae (Lepidoptera) might gain oviposition preferences from larval-feeding experience as qualified by HHSP or through chemical legacy (Jaenike 1983, McCall and Eaton 2001, Chow et al. 2005). In mosquitoes, females use chemical cues to locate suitable water pools for oviposition that contain compounds to which they become susceptible to conditioning as their parents (McCall and Eaton 2001, Hamilton et al. 2011). Furthermore, feeding and oviposition gained from the parents, as well as adaptation to the behaviors and habitat in which they developed may also support the HHSP (Rietdorf and Steidle 2002, Hamilton et al. 2011). For example, if mosquitoes lay eggs in a habitat containing mycolactone, following these definitions and ideas, this characteristic will then impact adult decision-making with regards to oviposition in environments with or without mycolactone. However, chemical legacy and HHSP have not been explored yet with mosquitoes on mycolactone; therefore, it is the objective of this study to investigate the impacts of mycolactone on mosquito behaviors and life histories. Mosquitoes are known to be influence by chemical cues, with CO_2 being one of the most important (Verhulst et al. 2009). However more recent studies have shown that other volatiles released by bacteria can cause a change in mosquito behavior (Verhulst et al. 2009, Sanders et al. 2016).

Vector Control

Mosquito control is an important public-health practice throughout the world, especially in tropic countries where mosquitoes spread diseases, such as malaria and Zika virus. Controlling mosquitoes employs many methods, such as chemical adulticides and larvicides, non-chemical control and genetic manipulation (Raghavendra et al. 2011). Since early nineteenth century, the application of insecticides was the primary agent for the mosquito control programs in many parts of the world (Breman 2001). During the nineteenth century, many compounds were discovered, such as mercuric chloride, Paris green, phenol and cresols, naphthalene, Bordeaux mixture, rosin-fish oil soap, calcium arsenate and nicotine sulfate (Raghavendra et al. 2011). After the discovery of the insecticidal potential of dichlorodiphenyltrichloroethane (DDT) in the 20th century, a new era of vector control began.

The first synthetic organic insecticide used for effective vector control was DDT with credible success (Raghavendra et al. 2011). However, due to health and environmental effects, some countries negotiated a treaty to enact global ban for DDT use. In addition, non-chemical control methods also have been used in vector control. *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) are two bacterial species that have been widely demonstrated to be efficient against mosquitoes (Raghavendra et al. 2011). Currently, genetic manipulation, which is also called genetic modification, has been used as a vector control strategy (Raghavendra et al. 2011). However, transgenesis genetic of all the vector mosquitoes would be highly challenging. Also, the spread of transgenes through wild populations will be the limitation due to the big population (Raghavendra et al. 2011). Genetic manipulation is the direct manipulation of an organism's genome using

biotechnology (Alphey 2002). These molecular technological advances started only after the complete genome sequences were made available (Alphey 2002). Gene drive, a technique which prevents the spread of insects that carry diseases by adding, disrupting or modifying genes (Esvelt et al. 2014, Adelman and Tu 2016), was used by Hall (2015) to convert female mosquitoes into harmless males (Hall et al. 2015). Although these techniques are expensive andtime consuming, the results are very promising, and will potentially help in vector control.

Morphometric of *Aedes aegypti* Adults

The sex of the adult *Ae. aegypti* can be distinguished by the naked eye along with some microscopic characteristics. In general, the *Ae. aegypti* female is larger than the male due to the need to have blood-meal for reproduction (Christophers 1960). The other main characteristics used to distinguish between sexes are the wings, antennas and legs (Roth 1948, Christophers 1960, Gopfert et al. 1999, Sendaydiego 2013).

The wings of *Ae. aegypti* are flat, rigid structures with an intrinsic venation pattern. The venationis used to distinguish it from other species (Sendaydiego 2013). Additionally, the wings of *Ae. aegypti* are covered with scales. Gender can be differentiated based on the length of the wings. Females have a mean length of 35 mm, while in males the wing is only 25 mm (Christophers 1960) and the wing in a female is wider in proportion to that of the male. Gender can also be distinguished by the naked eye through observations of the antenna. Both sexes have a ring-like antenna with thirteen-segmented flagellum (Christophers 1960); however, males have thicker flagellum hairs (plumose) than females (Gopfert et al. 1999). Finally, three pairs of legs, the fore-leg, mid-leg and hind-leg, attach to the prothorax, mesothorax and metathorax segments, respectively (Christophers 1960) and can be used to

differentiate the sexes microscopically. Males have a hole on one side of the front and middle legs of the last tarsal segment that is used to seize the female and they have shorter front legs than the female (Roth 1948, Christophers 1960).

Objectives and Hypotheses

Objective 1:

Determine if the toxin, mycolactone, produced by *Mycobacterium ulcerans*, influences larval growth and survivorship of adult *Aedes aegypti aegypti* at different exposure concentrations (0, 0.05, 0.5 and 1.0 µg/mL).

Hypothesis 1:

H_o: *Aedes aegypti* does not demonstrate a dose response, with regard to larval growth and survivorship to the adult stage, to mycolactone.

H_a: *Aedes aegypti* does demonstrate a dose response with regard to larval growth and survivorship to the adult stage, to mycolactone.

CHAPTER II

RESEARCH, RESULTS, AND DISCUSSION

Introduction

Buruli ulcer (BU) is a globally recognized neglected tropical disease caused by *Mycobacterium ulcerans* (Johnson et al. 2005, Merritt et al. 2010) with most cases occurring in West Africa (Williamson et al. 2008, Merritt et al. 2010). Buruli ulcer is the third most recurrent mycobacterial disease of humans globally after tuberculosis and leprosy (Johnson et al. 2005, Vincent et al. 2014). Most cases involve individuals between the ages of 4 to 15 years (Williamson et al. 2008, Organization 2012, Vincent et al. 2014). This disease was first noted in the late 1880's in Africa and has since been reported worldwide (Williamson et al. 2008, Merritt et al. 2010).

Mycobaterium ulcerans produces mycolactone, which is a potent cytotoxic and immunosuppressive polyketide-derived macrolide (Mve-Obiang et al. 2003, Gama et al. 2014) responsible for manifestation of skin ulcerations, which are the primary disease symptoms. The ulcers are painless; however, if not treated, they will continue to expand and could result in secondary infection, bone deformation, and osteomyelitis (Johnson et al. 2005). Though mortality due to this disease is low, there is a significant amount of morbidity associated with secondary infection that leads to a great socioeconomic burden (Barogui et al. 2013, Yeboah Manu et al. 2013). Lastly, late diagnosis could result in long and costly hospitalizations with significant morbidity and disability (Organization 2012).

The exact mode of transmission of *M. ulcerans* is unclear; however, some hypothesize that transmission occurs through contact with environments where the pathogen is known to reside, such as slow-moving rivers (especially in areas prone to human-made

disturbance and flooding) (Johnson et al. 2005, Merritt et al. 2010, Williamson et al. 2012). Others suggest aquatic insects in these environments may serve as vectors (Marsollier et al. 2002, Johnson et al. 2005, Quek et al. 2007, Merritt et al. 2010, Wallace et al. 2010, Kenu et al. 2014b). Insects, such as black flies (Diptera: Simuliidae), mosquitoes (Diptera: Culicidae), March flies (Diptera: Tabanidae), and sand flies (Diptera: Ceratopogonidae), associated with these environments are thought to be potential vectors (Luckhart et al. 1998, Quek et al. 2007, Merritt et al. 2010). Furthermore, PCR was used to identify *M. ulcerans* in aquatic insects obtained from endemic areas in Africa (Naucoridae and Belostomatidae) (Johnson et al. 2005). In fact, under laboratory conditions, aquatic hemipterans belonging to the Naucoridae that were infected with *M. ulcerans* and allowed to bite mice were found to transmit the pathogens (Marsollier et al. 2002).

In insects, a chemical legacy from the larval environment is possible to induce a host preference in the adult (Corbet 1985, McCall and Eaton 2001). Many species of mosquitoes have shown strong oviposition preferences for sites with bacterial colonization (Corbet 1985, Trexler et al. 2003, Sumba et al. 2004, Albeny-Simões et al. 2014).

Recent studies have revealed this behavior, in part, to be controlled by semiochemicals (Verhulst et al. 2009, Verhulst et al. 2011). In addition, mosquitoes are known to be influence by chemical cues, with CO_2 being one of the most important (Verhulst et al. 2009). However more recent studies have shown that other volatiles released by bacteria can cause a change in mosquito behavior (Verhulst et al. 2009, Sanders et al. 2016). Furthermore, Davis et al. (2013) indicated that microbial volatile organic compounds (MVOCs) elicit mating and oviposition behaviors from responding insects; also, insect behaviors, especially across species, can be affected by MVOC (Davis et al. 2013).

A study by Sanders et al. (2016) demonstrated mycolactone serves as an attractant of the yellow fever mosquito, *Aedes aegypti aegypti* (L.) (Diptera: Culicidae), which inhabits many of the same locations as *M. ulcerans*, to blood-meal sources (Sanders et al. 2016). In fact, they determined the response was dose dependent with the blood-feeder treated with 1.0 µg/mL mycolactone attracting 29% more mosquitoes than a control blood-feeder. In contrast, the low (0.05 µg/mL) and middle doses (0.5 µg/mL) were repellent. With regards to the 0.5 µg/mL dose was the lowest odds of response. These results indicate the dose of mycolactone can serve as an attractant of mosquitoes to hosts, which can potentially result in *M. ulcerans* transmission and BU infection (Sanders et al. 2016). With regards to oviposition, mosquitoes demonstrated a preference for ovipositing in areas containing mycolactone at the highest concentration (Sanders et al. 2016).

Recently, Wallace et al. (2016) examined the response of mosquitoes (i.e., *Aedes notoscriptus* and *Aedes aegypti*) seeking blood-meals to the tails of healthy mice dipped in *M. ulcerans* (Wallace et al. 2016). They demonstrated mosquito feeding on contaminated sites (i.e., tails coated with *M. ulcerans*), while low (2 of 11 mice), resulted in infection (Wallace et al. 2016). Consequently, they demonstrated mosquitoes (*Ae. notoscriptus* and *Ae. aegypti* that were used in the study*)* could act as atypical mechanical vectors of *M. ulcerans* (Wallace et al. 2016). However, to date, ecological interactions between the toxin and mosquito development and corresponding behavior of resulting adults has not been examined. The objectives of the current study were to determine if immature *Ae. aegypti* development and survivorship demonstrate a dose response to mycolactone. Second, will *Ae. aegypti* adults select oviposition sites similar to those (e.g., containing specific amount of mycolactone) in which they were reared?

The objectives of the current study were to determine if immature *Ae. aegypti* development and survivorship demonstrate a dose response to mycolactone, and second, to determine whether *Ae. aegypti* adults select oviposition sites similar to those (e.g., containing specific amount of mycolactone) in which they were reared.

Materials and Methods

Insect Colonies

An *Aedes aegypti* (Liverpool strain) colony was maintained in a room at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH in a walk-in growth chamber at the Forensic Laboratory for Investigative Entomological Sciences (FLIES) Facility (Texas A&M University, College Station, TX, USA). Eggs from a Liverpool strain maintained by Dr. Michel Slotman (Texas A&M University, College Station, TX, USA) were used to initiate a colony in the FLIES Facility. Eggs were placed in 1 L of distilled water held in containers (17.5cm x 12cm x 4.5cm) at room temperature. Resulting larvae were separated into similar containers at a density of 100-200 larvae/L (Clements 1995). Larvae were provided a diet of fish food (TetraMin diet by Tetra Blacksburg, VA, USA) (Sanders 2015). Based on preliminary experiments and published literature, the food amount was changed depending on the age and number of surviving larvae, so that larvae were fed *ad libitum* to avoid overfeeding (Tsuda and Takagi 2001, Imam et al. 2014). Distilled water was added to the containers as needed. Containers were checked every 12 h for pupae. Resulting pupae were partitioned into 60 ml cups (containing 40 ml of distilled water) at a density of 50 pupae/cup. These cups were placed individually inside a cage (30 × 30 × 30 cm) and pupae monitored for adult emergence. Sugar-feeding is a necessity for survival in adult of mosquito (Sanford and Tomberlin 2011). Therefore, newly emerged adults were provided with a 10% sucrose

solution via a damp cotton ball placed on top of the cage. Mating takes place approximately within the first 2-3 h after emergence with the greatest activity of copulation usually occurring between 1600 and 1800 h (Roth 1948).

Mycolactone

Mycolactone at 1.0 µg/ml, 0.5 µg/ml, and 0.05 µg/ml was received from Dr. Heather Jordan, Mississippi State University. Mycolactone was prepared by Dr. Jordan using methods described by Mve-Obiang et al (Mve-Obiang et al. 2003) with slight modifications; specifically, *M. ulcerans* Agy99 was grown on M7H10 plates. Bacteria were scraped from the plates, dried and weighed. Mycolactone was isolated and the concentration was calculated from *M. ulcerans* cell weight and corresponded to the colony count. Previous work has shown that one cell produces approximately 1 pg of mycolactone. Concentrations were selected based on qPCR values of *M. ulcerans* from environmental samples (Williamson et al. 2012, Williamson et al. 2014, Sanders 2015). Mycolactone solutions were stored in amber vials placed in the dark at room temperature to prevent degradation due to ultraviolet light (Marion et al. 2012). Prepared mycolactone was solubilized with 95% ethanol serving as the solvent.

Blood-Feeding

The blood-feeder design was adapted from Sanders (Sanders et al. 2016). Mosquitos were 5-10-day-old and starved for 24 h prior to use in the experiments to ensure they would be receptive to blood-feeding (Sanford and Tomberlin 2011). A 4.5 x 3 x 9 cm, 45 ml cell culture flask covered with parafilm on the top surface (Corning Incorporated, NY, USA) (Fig. 6) was used as the blood-feeder. Approximately 1 ml rabbit blood (HemoStat

Laboratories, Dixon, CA, USA) was injected between the parafilm and cell culture flask. The blood-feeder was connected to a water bath maintained at approximately 37°C - 39°C in order to simulate the temperature of human blood. Mosquitoes were allowed to feed for approximately 3-4 h; females were then allowed 72 h to digest the blood (Ruktanonchai et al. 2015). After the 72 h period, a filter paper (11 cm in diameter) placed in a cup located in the corner of the cage was provided as an oviposition site. Approximately 30 ml of water was added to the cup to saturate the exposed filter paper. Females were allowed 72 h to lay eggs on the filter paper, which then was removed and dried for four days prior to storage in the incubator as previously described (Imam et al. 2014).

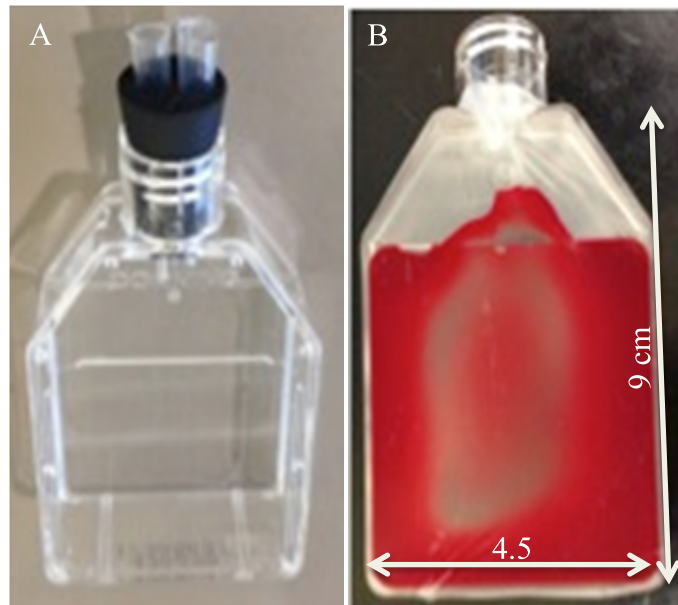

FIGURE 5. The blood-feeder (A. without blood, B, with blood) used to feed the adults *Ae. aegypti* (Liverpool strain) mosquitoes for approximately 3-4 h. The feeder is wrapped with parafilm and pumped with 1 ml of rabbit blood (Sanders 2015, Zhang et al. 2015).

Larval Growth and Survivorship to the Pupae and Adult Stage

All experiments were conducted under the laboratory conditions previously described by (Sanders et al. 2016). For the experiments, 40 *Ae. aegypti* (Liverpool strain) eggs representing multiple females from the colony were placed in a round glass jar (236 ml) (Fig. 7) (Packaging Options Direct, Louis, MO, USA) containing 55 ml of distilled water. This density was selected based on preliminary experiments, which yielded the greatest level of survivorship to the adult stage. Approximately 0.03 - 0.05 g of Tetramin fish food (Zeichner and Perich 1999) were placed in the water at the time the eggs were introduced. Tetramin served as food for the resulting mosquito larvae. Food was provided to the larvae *ad libitum* to avoid overfeeding (Tsuda and Takagi 2001, Imam et al. 2014). This feed rate was validated in the lab as well.

Mycolactone 1.0 µg/ml, 0.5 µg/ml, and 0.05 µg/ml with 95% ethanol serving as the solvent were the treatments. Two controls were used, Ethanol alone and an additional control adding nothing. The jars previously described were used as the containers for each replicate

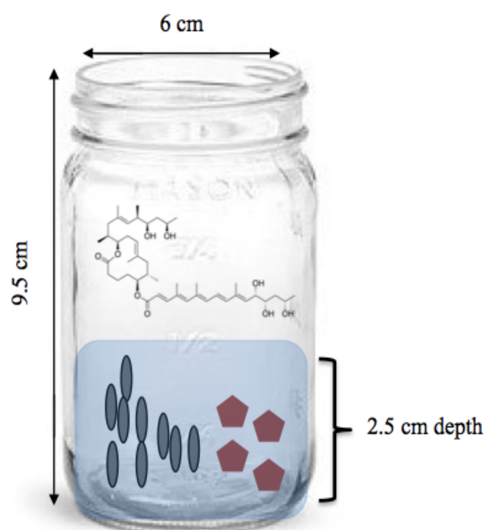

FIGURE 6. The glass container jar used in rearing larvae of *Ae. aegypti*.

during the experiment. Each mycolactone treatment was added to a glass jar prior to the introduction of mosquito eggs. Doing so would allow the eggs to be laid in a simulated mycolactone contaminated environment (Williamson et al. 2012). Each treatment was in triplicate. The glass jar was placed in a mosquito-breeder (21 x 12 cm) (BioQuip, CA, USA) to capture emerging adults. Time to pupation and emergence of adults was recorded. Resulting adults were kept in mosquito-breeders in the freezer until sex ratio of adult mosquitoes was recorded. Percent data of pupae and adults were adjusted based on number of eggs to hatch.

Statistical Analysis

Each concentration was replicated three times and analyzed using methods described by Tomberlin al. (Tomberlin et al. 2012). Data was analyzed by using commercially available statistical software (JMP® Pro 12.0.1, Cary, NC, USA) An analysis of variance was used to assess the data. Tukey's (multiple comparison procedure and statistical test) was used to separate means following a significant F test. The alpha was set at $P < 0.05$.

Results

Eggs hatch. No significant ($F = 0.781$; $df = 4, 59$; $P = 0.5444$) difference in egg hatch was determined across treatments. Furthermore, no significant ($F = 0.738$; $df = 12, 59$; $P = 0.7067$) interaction was determined between trial and treatment. However, a trial effect (table 1) was determined ($F = 9.099$; $df = 3, 59$; $P < 0.0001$). Trials 1 and 2 were significantly ($P < 0.05$) different from trials 3 and 4. The average egg hatch in trial one and two was 97.50% ± 0.01% (Figure 8a) and 80.10% ± 0.03% (Figure 8b) in trials three and four. When trials were

analyzed based on groupings from the initial analysis, significant model effects (Tables 2 and 3) still were not determined.

Survival from egg to pupal stage. Significant (F = 10.085; df = 4, 53; *P* < 0.0001) difference in survival from egg to the pupal stage was determined across treatments (Table 4). No significant (F = 1.070; df = 12, 53; *P* = 0.4142) interaction was determined between trial and treatment. Furthermore, no trial effect was determined (F = 1.436; df = 3, 53; *P* < 0.2494). The average survival of eggs to the pupal stage for the controls was 81% or greater. Survival of those exposed to the different mycolactone treatments was greatest for the middle dose (68%), which was not significantly different from the controls; however, survival when exposed to this treatment was almost double of what was observed for those assigned the high and low dose treatments (41%) (Figure 9a). Survival for those exposed to the high dose was significantly different from all other treatments and had the lowest survival 35%.

Survival from egg to adult stage. Significant (F = 10.085; df = 4, 53; *P* < 0.0001) difference in survival from egg to the adult stage was determined across treatments (Table 4). No significant (F = 1.070; df = 12, 53; *P* = 0.4142) interaction was determined between trial and treatment. Furthermore, no trial effect was determined (F = 1.436; df = 3, 53; *P* < 0.2494). The average survival of eggs to the adult stage for the controls was 81% or greater. Survival of those exposed to the different mycolactone treatments was greatest for the middle dose (68%), which was not significantly different from the controls; however, survival when exposed to this treatment was almost double of what was observed for those assigned the high and low dose treatments (41%) (Figure 9b). Survival for those exposed to the high dose was significantly different from all other treatments and had the lowest survival 35%.

Time from egg to pupal stage. Significant (F = 4.837; df = 4, 52; *P* = 0.0035) differences in development time to the pupal stage were determined across treatments (Table 5). No significant (F = 1.711; df = 12, 53; *P* = 0.1095) interaction was determined between trial and treatment. However, a trial effect was determined (F = 3.750; df = 3, 53; *P* < 0.0202). Therefore, the results for development time from egg to the pupal stage were grouped by trial. The average time from egg to pupa ± SEM is shown in (Table 7). Trials 1, 3 and 4 were significantly (*P* < 0.05) different from trials 2, 3 and 4 when thery were grouped separately. For trials 1, 3 and 4, significant (F = 6.1293; df 4, 37; *P* < 0.0016) difference in development time from egg to the pupal stage was determined across treatments. Individuals in the controls needed 7.35 day or less to become pupae. Development time for those exposed to the high dose was significantly greater (~eight days) than for individuals (~seven days) in other treatments (i.e., controls, low and middle doses). Furthermore, a significant (F = 2.7961; df = 8, 37; *P* = 0.0255) interaction was determined between trial and treatment. For trials 2, 3 and 4, treatment did not significantly (F = 1.8203; df 4, 40; *P* < 0.1552) impact development time from egg to the pupal stage. Interestingly, results were consistent with those recorded for the previous trial grouping (trials 1, 3 and 4). Those exposed to the high dose took the longest, while those assigned the middle dose were more similar to the controls.

Time from egg to adult stage. Significant (F = 5.3318; df = 4, 52; *P* = 0.0020) differences in development time to the adult stage were determined across treatments (Table 6). No significant (F = 0.8441; df = 12, 52; *P* = 0.6071) interaction was determined between trial and treatment. However, a trial effect was determined (F = 760.306; df = 3, 53; *P* < 0.0001) in development time to the adult. Therefore, the results for development time from

egg to the adult stage were grouped by trial. The average time from egg to adult ± SEM is shown in (Table 7). Trial 1 was significantly ($P < 0.05$) different from trials 2, 3 and 4. For trial 1, significant (F = 5.5869; df 4, 11; $P < 0.0243$) difference in development time from egg to the adult stage was determined across treatments. Individuals in the controls needed 9.55 day or less to become adults. Development time for those exposed to the high dose was significantly greater (~9.33 days) than for individuals (~9.12 days) in other treatments (i.e., controls, low and middle doses). For trial 3, treatment did not significantly (F = 1.9105; df 4, 10; $P < 0.2280$) impact development time from egg to the adult stage. Also, for trials 2 and 4, treatment did not significantly (F = 1.4864; df 4, 29; $P < 0.2438$) impact development time from egg to the adult stage. Furthermore, no significant (F = 0.0137; df = 4, 29; $P = 0.9996$) interaction was determined between trial and treatment.

Table 1. Analysis of Variance for egg hatch for *Ae. aegypti* eggs exposed to different concentration of mycolactone, as well as negative control and ethanol control at 25.0° ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH.

Source	Sum Of Squares	df	Mean square	F	*P*
Model	2.2230421	19	0.117002	3.3458	0.0006*
Error	1.3988062	40	0.034970		
C. Total	3.6218483	59			
Trial	1.8041918	03		17.1974	<0.0001*
Treatment	0.1092024	13		0.7807	0.5444
Trial*Treatment	0.3096479	04		0.7379	0.7067

Table 2. Analysis of Variance for egg hatch for *Ae. aegypti* eggs (trials 1 and 2) exposed to different concentration of mycolactone, as well as negative control and ethanol control at 25.0° ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH.

Source	Sum Of Squares	df	Mean square	F	*P*
Model	0.25792181	09	0.028658	1.7992	0.1313
Error	0.31856005	20	0.015928		
C. Total	0.57648187	29			
Trial	0.02385552	01		17.1974	0.2352
Treatment	0.16507339	04		0.7807	0.0678
Trial*Treatment	0.06899290	04		0.7379	0.3915

Table 3. Analysis of Variance for egg hatch for *Ae. aegypti* eggs (trials 3 and 4) exposed to different concentration of mycolactone, as well as negative control and ethanol control at 25.0° ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH.

Source	Sum Of Squares	df	Mean square	F	*P*
Model	0.1864913	09	0.020721	0.3836	0.9293
Error	1.0802461	20	0.054012		
C. Total	1.2667375	29			
Trial	0.00170734	01		17.1974	0.8607
Treatment	0.13388551	04		0.7807	0.6537
Trial*Treatment	0.05089847	04		0.7379	0.9150

Table 4. Analysis of Variance for pupae and adult emergence of *Ae. aegypti* eggs exposed to different concentration of mycolactone, as well as negative control and ethanol control at 25.0° ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH.

Source	Sum Of Squares	df	Mean square	F	*P*
Model	6.227290	19	0.327752	2.9398	0.0030*
Error	3.790593	34	0.111488		
C. Total	10.017883	53			
Trial	0.4802333	03		1.4358	0.2494
Treatment	4.4972321	04		10.0846	<0.0001*
Trial*Treatment	1.4308014	12		1.0695	0.4142

Table 5. Analysis of Variance for time to pupae for *Ae. aegypti* eggs exposed to of different concentration of mycolactone, as well as negative control and ethanol control at 25.0° ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH.

Source	Sum Of Squares	df	Mean square	F	*P*
Model	0.37955264	19	0.019976	2.8512	0.0040*
Error	0.23120771	33	0.007006		
C. Total	0.61076035	52			
Trial	0.07881210	03		3.7496	0.0202*
Treatment	0.13556272	04		4.8372	0.0035*
Trial*Treatment	0.14381549	12		1.7106	0.1095

Table 6. Analysis of Variance for time to adult for *Ae. aegypti* eggs exposed to of different concentration of mycolactone, as well as negative control and ethanol control at 25.0° ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH.

Source	Sum Of Squares	df	Mean square	F	*P*
Model	1.0085035	19	0.053079	139.6295	<0.0001*
Error	0.0125447	33	0.000380		
C. Total	1.0210483	52			
Trial	0.86707488	03		1.4358	<0.0001*
Treatment	0.00810734	04		10.0846	0.0020*
Trial*Treatment	0.00385046	12		1.0695	0.6071

Table: 7. Mean ± SEM of time (d) from egg to pupae and adult of *Ae. aegypti* exposed to of different concentration of mycolactone, as well as negative control and ethanol control at 25.0° ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH

Mean time from egg to pupae (d) Mean ± SEM		
Treatment	**Trial 1, 3 and 4**	**Trial 2, 3 and 4**
Negative control	7.35 ± 0.15 [b]	7.57 ± 0.06 [a]
Ethanol control	6.87 ± 0.20 [b]	7.57 ± 0.41 [a]
1 µg/ml	8.07 ± 0.46 [a]	8.33 ± 0.40 [a]
0.5 µg/ml	7.33 ± 0.18 [b]	7.58 ± 0.10 [a]
0.05 µg/ml	7.48 ± 0.29[a,b]	7.63 ± 0.30 [a]

Mean time from egg to adult (d) Mean ± SEM			
Treatment	**Trial 1**	**Trial 3**	**Trial 2 and 4**
Negative control	9.53 ± 0.09 [a]	13.70 ± 0.06 [a]	12.77 ± 0.05 [a]
Control (Ethanol)	9.55 ± 0.07 [a]	13.80 ± 0.00 [a]	12.63 ± 0.08 [a]
1 µg/ml	9.33 ± 0.00[a,b]	13.30 ± 0.30 [a]	12.48 ± 0.11 [a]
0.5 µg/ml	9.24 ± 0.24[a,b]	13.80 ± 0.00 [a]	12.55 ± 0.13 [a]
0.05 µg/ml	9.00 ± 0.00 [b]	13.80 ± 0.35 [a]	12.50 ± 0.13 [a]

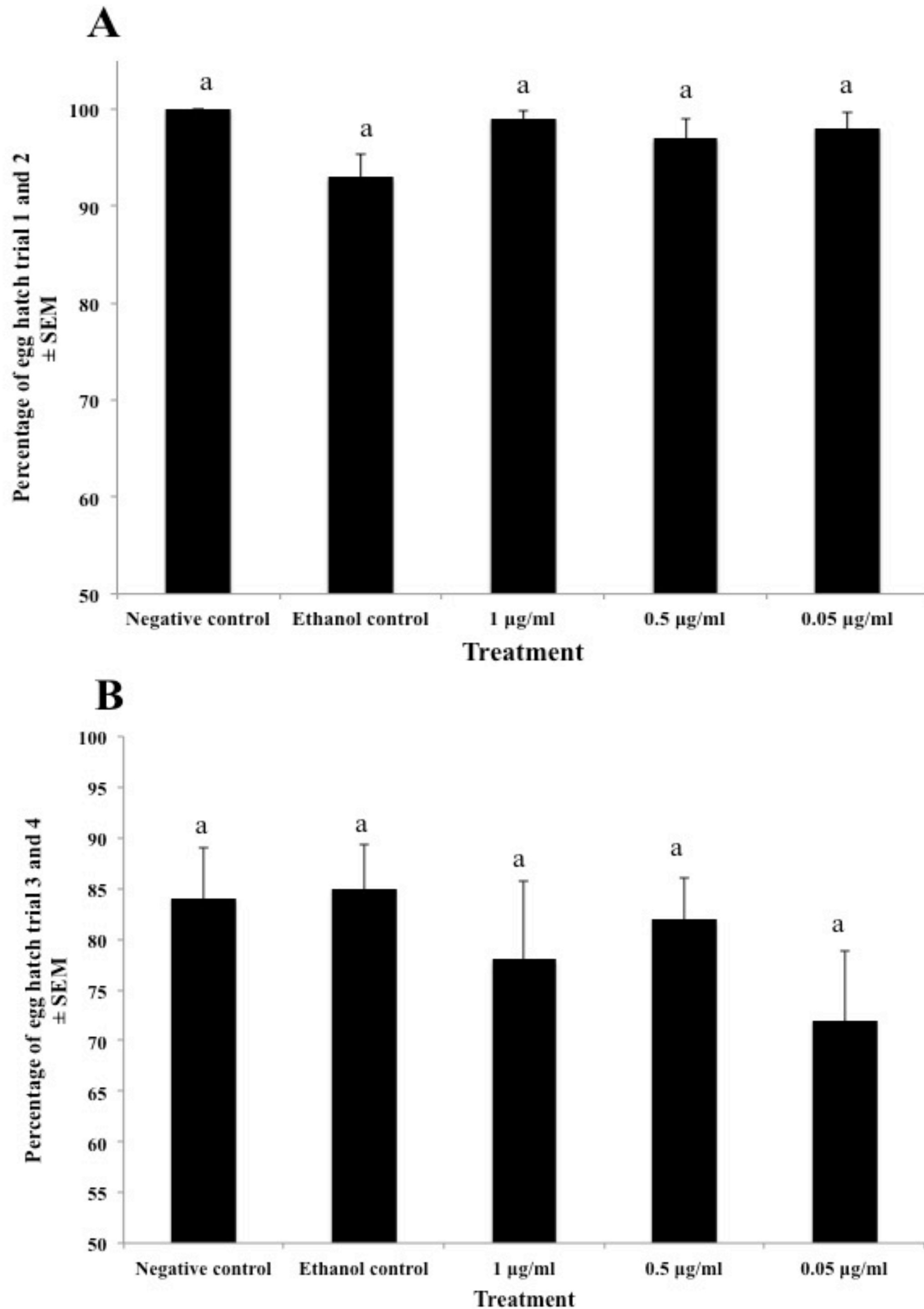

FIGURE 7. The percentage of egg hatch (n = 4[1]) of *Ae. aegypti* ± SEM exposed to three concentrations of mycolactone, as well as negative control and ethanol control at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH for (A) trials 1 and 2 for (B) trials 3 and 4 combined. [1]n = number of trials conducted.

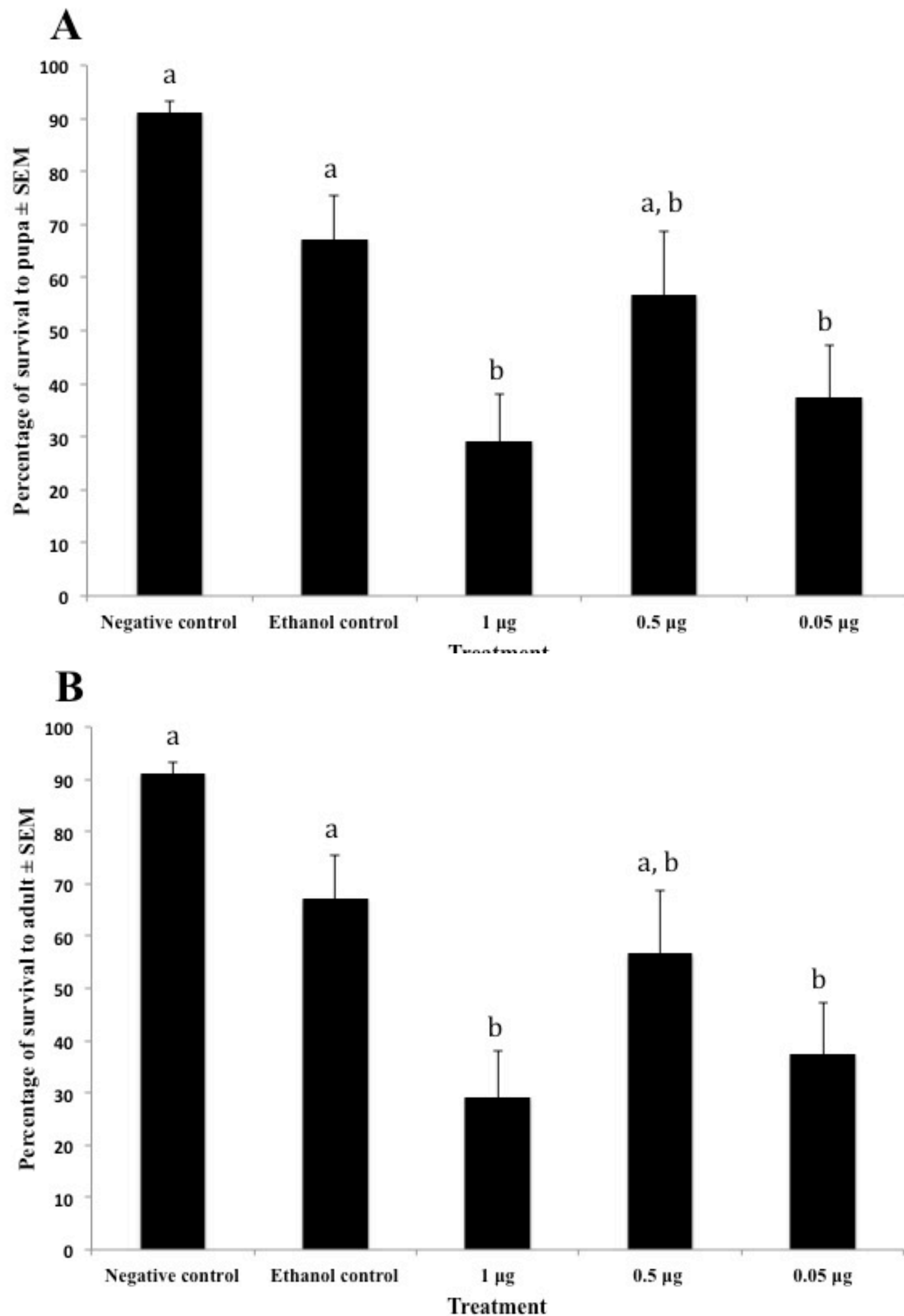

FIGURE 8. The percentage of survival from egg to the pupal and adult stages (n = 4[1]) of *Ae. aegypti* ± SEM exposed to three concentrations of mycolactone, as well as negative control and ethanol control at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH. (A) pupae (B) adults. [1] n = number of trials conducted.

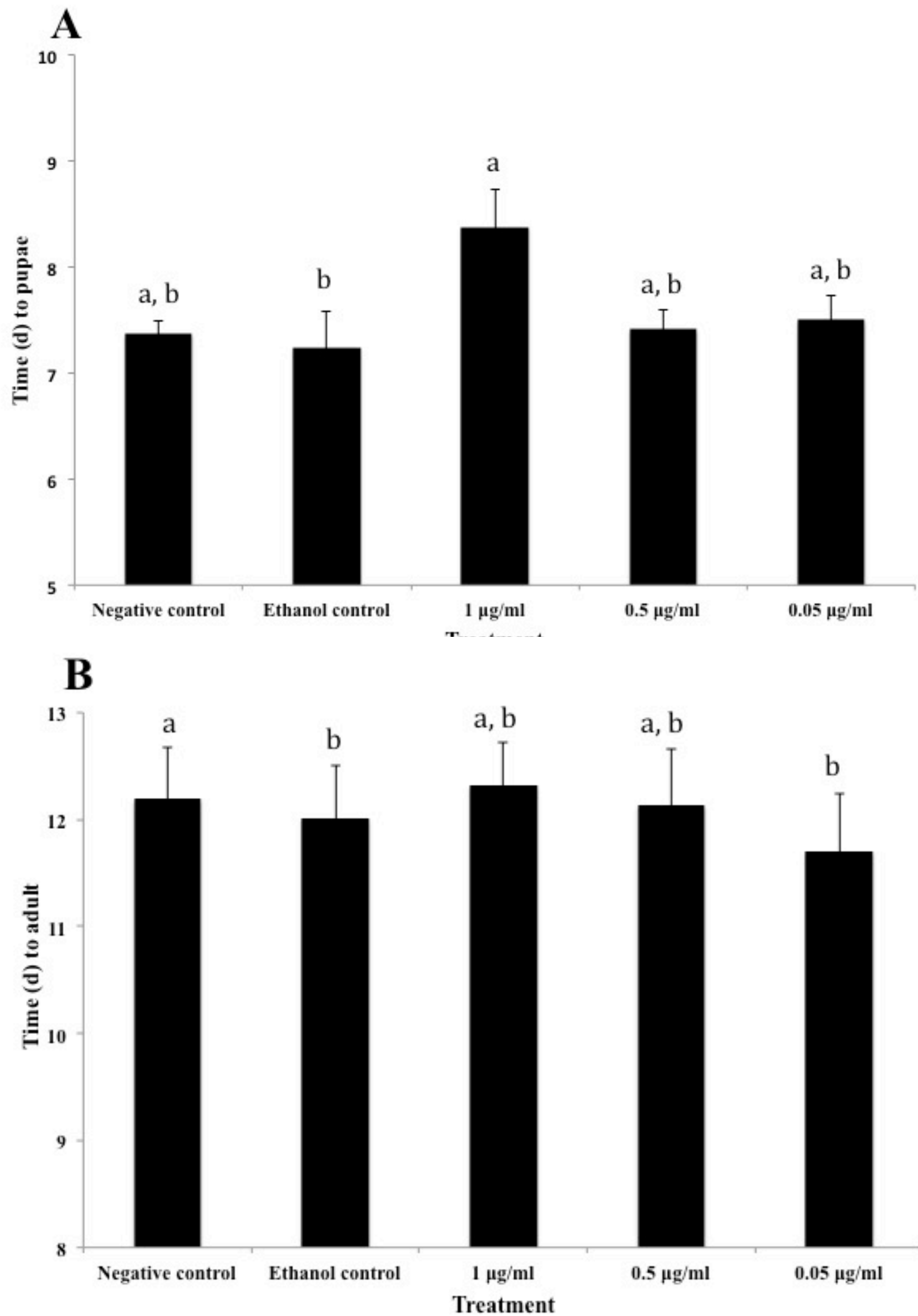

FIGURE 9. The time (d) from egg to the pupal and adult stages (n = 4[1]) of *Ae. aegypti* ± SEM exposed to three concentrations of mycolactone, as well as negative control and ethanol control at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH (A) time (d) from egg to pupae (B) to adult [1]n = number of trials conducted.

Discussion

Since the late 19th century, researchers have been attempting to determine the mode of *M. ulcerans* transmission (Johnson et al. 2005, Quek et al. 2007, Merritt et al. 2010). Some speculate the skin of an individual might be breached by sharp objects and allow contamination by the pathogen (Williamson et al. 2014). Recently, as mentioned in the introduction, Sanders et al. (2016) demonstrated mycolactone produced by *M. ulcerans* serves as an attractant of the yellow fever mosquito, *Ae. aegypti*, which inhabits many of the same locations as *M. ulcerans*, to blood-meal sources as well as oviposition sites. More recently, Wallace et al. (2016) determined the response of mosquitoes (i.e., *Ae. notoscriptus* (Skuse) and *Ae. aegypti* (L.) blood-feeding from the tails of healthy mice dipped in *M. ulcerans* resulted in infection with the pathogen in 2 of 11 cases (Wallace et al. 2016). Given the pathogen occurs in environments where *Ae. aegypti* develop and the recent discovery of *M. ulcerans* and its toxin as an attractant of adult *Ae. aegypti* to host and oviposition sites, questions regarding the ecological implications of this interaction beg to be asked, especially as related to the impact of mycolactone on mosquito biology.

The work presented here determined that mycolactone impacts larval development of *Ae. aegypti*. Furthermore, the response, while not statistically significant, varied by dose with the high and low dose reducing survivorship from egg to pupa and adult (Figures 9a-b), while the middle dose appeared to be optimal (Figure 10a). Survival of eggs to the pupal stage in the controls was above 80%, while those exposed to the high and low dose was between 35 and 40%. In contrast, those exposed to the middle dose, while still lower than the control, had a 68% survivorship.

With regards to development to the pupal stage, a similar response was observed.

Immature mosquitoes in the controls needed approximately 7.2 d to reach the pupal stage, which is similar to results from past studies conducted under similar conditions (Christophers 1960). However, those exposed to the high dose needed approximately 15% more time to reach the pupal stage (Figure 10a), while those exposed to the middle and low dose were more similar to the control. Similarly, development (Figure 10b) from egg to adult exhibited a treatment effect (Table 7, Figure 10b); however, this difference was never more than half a day and was most likely due to observer bias (e.g., observations every 24 h) rather than biological significance. Additional studies with more refined observation periods should be conducted to determine if the response is statistically different. Furthermore, fieldwork should be conducted to determine if these data are actually biologically relevant.

Throughout the course of these experiments, immature mosquitoes exposed to the middle dose, rather than the high or low dose, consistently produced similar survival rates to the adult stage as the control possibly indicating a concentration window (i.e., Goldilocks' zone) of suitability. This finding is biologically interesting as this concentration has been detected in environments where *M. ulcerans* occurs (Williamson et al. 2012). However, these results were not statistically significant thus warranting additional research on this topic.

Such a response to an abiotic factor by immature mosquitoes is not a surprise as such responses by mosquitoes has been determined for a number of abiotic conditions. For example, arthropod development optimally occurs within a given temperature range. If the temperature is too high, the immature die and if too low, development stalls. TunLin (2000) determined such a response for *Ae. aegypti* larvae (TunLin et al. 2000). Room temperatures above 30°C resulted in larval mortality, while temperatures between 20-30°C resulted in optimal development and survivorship. An additional example of such a window of optimal

response has been recorded for pH. Clark et al. (2004) determined *Ae. aegypti* larvae died when the pH of the aquatic habitat was below 4 or above 11 (Clark et al. 2004). Similar responses to biotic factors have also been recorded for mosquito larvae. Couret et al. (2014) tested the impact of four larval densities on development and survivorship. Threshold development occurred when the density was approximately 80 larvae/ 250 ml water; however, high density caused long development and mortality (Couret et al. 2014)

One explanation for the impact of mycolactone on mosquito larval development being dose dependent could relate to shifts in the availability of nutrients. As previously indicated, *M. ulcerans* occurs in lentic habitats where *Ae. aegypti* larvae also occur (Wallace et al. 2010). And, bacteria in these habitats are known to breakdown organic matter, which then serve as a primary food substrate of the mosquito larvae (Walker et al. 1988, Merritt et al. 1992, Kaufman et al. 1999). A role of mycolactone in these environments at certain concentrations it inhibits other microbes (i.e., bacteria) competing with *M. ulcerans* for similar resources. At the right concentration (i.e., low or high), mycolactone could suppress bacteria populations crucial to larval mosquito development thus impacting the availability of nutrients for mosquitoes. Such interactions have been demonstrated for other systems.

For example, bacteria use quorum sensing (QS) to coordinate gene expression according to the density of their local population (Luo et al. 2000, Miller and Bassler 2001). This process allows for coordinated physiological shifts by the bacterial population resulting in enhanced survivorship. Mycolactone could potentially disrupt this process thus reducing the likelihood of survivorship and proliferation by competing bacteria. Such QS- digesting enzymes have been found in many bacteria, such as *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Comamonas*, *Agrobacterium tumefaciens*, *Actinobacter*, *Arthrobacter*, *Klebsiella pneumonia*,

Ochrobactrum, *Microbacterium*, *Brucella melitensis* and *Ralstonia* (Leadbetter and Greenberg 2000, Dong et al. 2002, Khan and Farrand 2009, Helman and Chernin 2015).

Mycolactone could impact gut bacteria in the mosquito larvae, which play a crucial role in mosquito development. In many insects, symbiotic bacteria can play an important role. These bacteria can produce nutrients essential for arthropod development (Dale and Moran 2006, Chouaia et al. 2012). Furthermore, mycolactone could impact the beneficial bacteria to mosquito. For example, Chouaia et al. (2012) determined *Asaia* symbionts bacteria are beneficial in the development of immature *Anopheles stephensi* (L.) (Diptera: Culicidae) (Chouaia et al. 2012). Develoment of *An. stephensi* larvae reared in an habitat with rifampicin was impacted. In fact, development was delayed two to four days comparing with control (Chouaia et al. 2012). In the current study, mycolactone could have a similar impact; however, the associated bacterial community was not measured in this study.

This research showed that mycolactone impacted the survivorship of eggs to the pupal and adult stages as well as development time from egg to pupae and adults. Such results demonstrate a potential ecological link between *M. ulcerans* and *Ae. aegypti* in endemic environments where both species occur. Such results could prove crucial for understanding the etiology of the pathogen and its mode of transmission as discussed by Sanders et al. (2016) and Wallace et al. (2016). However, additional research is needed with the actual bacterium to determine if in fact its presence and ability to produce mycolactone impact mosquito development and oviposition site selection. If these results remain true, a synergism between the incidence of Buruli ulcer and yellow fever could be possible in these endemic areas.

CHAPTER III

FUTURE STUDIES, LIMITATIONS, AND CONCLUSIONS

In this study, the impacts of mycolactone on the *Ae. aegypti* life cycle was determined. Mycolactone did not impact egg hatch. However, larval development was reduced significantly by the present of mycolactone especially at the high (35%) and low (41%) doses. Interestingly, survivorship when exposed to the middle dose was similar to control (i.e., Goldilocks' zone). Furthermore, development time from egg to pupae and adult stages were impacted. A slight difference in both development times to pupae (15%) and adult stages was determined.

How mycolactone impacts mosquito development and survivorship is not known. One possibility is that mycolactone could kill bacteria in the environment essential for mosquito development. As an example, the development was delay in *An. stephensi* in the present of *Asaia* bacteria (Chouaia et al. 2012). Mycolactone produced by *M. ulcerans* could also impede communication (i.e., quorum sensing- cell-to-cell coordinated physiological shifts) of competing bacteria thus resulting in additional resources for *M. ulcerans*.

While this research is informative as to the ecology of mycolactone and its impact on *Ae. aegypti* development and behavior, future research should utilized the actual pathogen (*M. ulcerans*) in laboratory studies. Doing so will allow for experiments to be designed exploring the pathogen response to abiotic (e.g., temperature, pH) and biotic (e.g., competing bacteria) conditions, and corresponding production of mycolactone. As a result, it would tie together the pathogen responses to such environmental factors, production of mycolactone, and mosquito ecology.

Furthermore, field research is critical for accurate elucidation of these interactions. In

the lab, some insects respond in ways they otherwise they would not, such as feeding or ovipositing on items they will rarely or never be exposed to in the field. Additionally, the field conditions and experiments might not work exactly as they do in the lab. Factors such as other organisms (bacteria) might also play a role in the responses of female with mycolactone.

Additional future studies could focus on the molecular aspects of mycolactone interactions with mosquito larvae. Specifically, researchers should examine the physiological and histopathological impact of mycolactone on developing larvae. Bacteria can cause damage to midgut of larval *Ae. aegypti*. For example, a study by Singh et al. (1986) found *Bacillus thuringiensis var. israelensis* (*Bti*) ingested by *Ae. aegypti* larvae caused damage to midgut circular and longitudinal muscles (Singh et al. 1986). After six hour of *Bti* treatment, the midgut was ruptured; however, paralysis of larvae occurred at advanced stages of *Bti* poisoning (Singh et al. 1986).

Also, morphology and morphometric (i.e., length and width of wing and tibia of legs) of resulting adults should be examined. Size has been shown to correlate with fecundity (Agnew et al. 1999). Immature female *Culex pipiens* (L.) (Diptera: Culicidae) infected with the microsporidian parasite *Vavraia culicis* (Agnew et al. 1999) pupated faster and resulting adults were smaller which were less fecund (Agnew et al. 1999). Mycolactone could have the same impact on *Ae. aegypti* females.

Given mycolactone negative impact on mosquito development, it could potentially be developed as a biological control agent. Other examples of such toxins being used in integrated pest management (IPM) include, *Bti* for mosquitoes (Singh et al. 1986) that impact human and other vertebrate populations as well as herbivore populations in row crops

(Federici 2003). However, additional research is needed to determine appropriate dose that will kill mosquitoes while not impacting beneficial or non-target arthropods or vertebrates (Federici 2003, Poulin et al. 2010). Furthermore, proper application methods (e.g., encapsulation, granule, ultra-volume spray) would need to be developed as well.

Furthermore, reduced size of resulting adults could impact their ability to disperse. For example, due to the energy, larger female (3.1 mm) can disperse (0.64 km/hr) more than smaller femele (2.8 mm) (0.47 km/hr) (Briegel et al. 2001). Not only dispersal, but also the ability of locating hosts can be impacted by their size. A study by Nasci (1986) found larger females (2.64 mm) were more successfully in looking for hosts and seek for a second blood meal comparing with smaller females (2.47 mm) avaergae wing length (Nasci 1986). This inability could partially explain the endemicity of Buruli ulcer in a given location especially if adults resulting from environments contaminated with mycolactone are in fact smaller.

Other concentrations of mycolactone should be investigated. In the current study, the highest dose did not result in 100% mortality; therefore, whether increasing the dose of mycolactone would result in more mortality or not, should be considered. The higher dose should be in high concentration, meaning higher than the current dose (1.0 μg/mL).

Furthermore, behavior can be examined to see whether mycolactone affect the adult decision-making with regards to host selection. We know from previous study by Platt (1997), that female *Ae. aegypti* infected with a virus or bacterium could slow feeding or result in multiple host feedings (Platt 1997). In fact, *Ae. aegypti* females infected with dengue virus take longer to feed compared with uninfected female (Platt 1997). Slow feeding would increase the chance of the female to feed on additional hosts, in which female will transmit pathogens to numerous hosts (Platt 1997).

In summary, results determined mycolactone impacts larval survivorship and development. Larvae of *Ae. aegypti* have survived in the presence of middle does, which has been indicated in the environment, but high mortality were recorded at highest and lowest dose. Since larvae of *Ae. aegypti* have survived in the present of middle does, this could result a great activity of mosquitoes in that area of mycolactone where eventually may lead to spread of other diseases (e.g., Yellow Fever).

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