STAPHYLOCOCCUS EPIDERMIDIS AND *MYCOBACTERIUM ULCERANS* IMPACT ATTRACTION OF AEDES AEGYPTI (L.) (DIPTERA: CULICIDAE) TO A BLOOD-FEEDING SOURCE

A Dissertation

By

DONGMIN KIM

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Jeffery K. Tomberlin
Committee Members,	Tawni L. Crippen
	Heather R. Jordan
	Craig J. Coates
	Gabriel L. Hamer
Head of Department,	Pete D. Teel

August 2019

Major Subject: Entomology

ABSTRACT

Aedes aegypti, is a vector of pathogens transmitting many infectious diseases, such as Zika, Dengue, and Yellow Fever. Skin-inhabiting bacteria, such as *Staphylococcus epidermidis*, produce specific volatile organic compounds (VOCs) which participate in attracting mosquitoes to human hosts. However, little is known about the ecology of the bacteria and its role in mosquito behavior; specifically whether bacterial communication through quorum sensing (QS) modulates mosquito behavior.

This study determined if the QS system could be manipulated chemically (e.g., quorum sensing inhibitor (QSI), C-30, furanone) to suppress mosquito responses. The results showed disrupting QS by *S. epidermidis* with an inhibitor (QSI) reduced the VOC composition by 35.0% and suppressed mosquito attraction by 55.1%. To explore further questions, mycolactone as a potential QS compound produced by the environmental pathogen, *Mycobacterium ulcerans*, regulating mosquito behavior was determined. A blood-feeder treated with *M. ulcerans* wildtype elicited a 126.0% and 171.0% greater attraction than *M. ulcerans* mutant or control, respectively. In terms of polymicrobial interactions, the addition of *M. ulcerans* to *S. epidermidis* resulted in 23.7% and 72.1% greater mosquito attraction than *S. epidermidis* and *M. ulcerans* alone. Most interestingly, *S. epidermidis* concentrations are typically low on the extremities.

In this study, an interdisciplinary approach was used to elucidate the ecological ramifications associated with interkingdom cross-talk between bacteria and eukaryotic. This knowledge can lead to the development of a new class of odor-masking or

inhibitory compounds as it is thought to be the less selective pressure than pre-existing pesticide or repellent use, which can be exploited in the protection from mosquito bites, aiming at compounds that reduce the production of attractive volatiles on the human skin. It has a broad range of potential applications in agriculture, medicine, pest-management, and etc.

DEDICATION

I would like to dedicate this work to my mother Mrs. Park Sun-ae whose dreams for me have resulted in this achievement and without her loving upbringing and nurturing, I would not have been where I am, or what I am, today. Had it not been for my mother's unflinching insistence and support, my dreams of excelling in education would have remained mere dreams. I thank my mother with all my heart, and I know she is my guardian angel. This one is for you mom!

ACKNOWLEDGEMENTS

I would like to thank God Almighty for giving me the strength, knowledge, ability, and opportunity to undertake this research study and to persevere and complete it satisfactorily. Without His blessings, this achievement would not have been possible.

First and foremost, in my journey towards this degree, I have found a teacher, a friend, an inspiration, a role model and a pillar of support as my guide, my advisor, Dr. Jeffery Tomberlin allowing me to work in the F.L.I.E.S. Facility. He has been there providing his heartfelt support and guidance at all times and has given me invaluable guidance, inspiration, and suggestions in my quest for knowledge. Without his able guidance, this dissertation would not have been possible, and I shall eternally be grateful to him for his assistance.

I would also like to thank my other committee members, Dr. Tawni Crippen, Dr. Heather Jordan, Dr. Craig Coates, Dr. Gabriel Hamer, and Dr. Jonathan Cammack for their expertise, support, and suggestions for my research.

I would like to give special thanks to Dr. Tawni Crippen, for her guidance and support throughout this process and for allowing me to work in her lab at USDA. I could not have asked for a better friend and mentor and am thankful for each and every opportunity she presented to me. Her constant support, guidance, and motivation were invaluable. I cannot thank her enough for these experiences and the personal growth you have facilitated over the years.

I owe special thanks to Dr. Heather Jordan, in particular, for teaching me microbiology. Your willingness to always look over my preliminary exam, manuscripts, and offer constructive comments, suggestions, and perspectives was crucial to this process. I am so blessed to have had your experience and advice throughout this endeavor.

I would also like to express my gratitude to Dr. Pablo Delclos for taking the time to teach me how to use R, especially when he was so busy with his own work. I owe him so much for all the hours that he spent with me and it is tremendously appreciated.

Special thanks to Brittny jones, Dr. Travis Rusch, and Chelsea Holcomb. They are great friends, lab mates, and researchers. Thank you for all the moments we have shared, all the shared dreams and wishes, secrets, laughter, and tears, and above all, for your friendship. Each precious second will be treasured in my heart forever.

I would like to give special thanks to Dr. Dong-kyu Lee and Dr. Hyesook Kang, who have all made a tremendous contribution in helping me reach this stage in my life and supported me emotionally and financially. I always knew that you believed in me and wanted the best for me. Without their able guidance, this study would not have been possible and I shall eternally be grateful to them for assistance.

I would like to say my sincere appreciation to Dr. Jimmy K. Olson, who, although no longer with us. He guided and encouraged me to pursue this degree. Without him, I would not have had the courage to embark on this journey in the first place.

I would like to give thanks to Rebecca Hapes, Dr. Hojun Song, and Dr. Laura Ripley for their unconditional support.

Thanks to my lab mates at the F.L.I.E.S. Facility for their support and being a wonderful group of scientists and researchers. I am so fortunate to have gone through this with such a knowledgeable group of individuals. This would not have been possible without their unwavering and unselfish love and support given to me at all times.

I would also like to say a heartfelt thank you to my Dad and brother for always believing in me and encouraging me to follow my dreams.

Finally, I would like to thank the Department of Entomology at Texas A&M University and all my friends.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a dissertation committee consisting of Professor Jeffery K. Tomberlin (major advisor), Craig J. Coates (committee member), Gabriel L. Hamer (committee member) of the Department of Entomology, and Assistant Professor Heather R. Jordan (committee member) of the department of Biological Sciences, Mississippi State University, and Dr. Tawni L. Crippen (committee member) of Agricultural Research Service, USDA.

Transcriptome Analysis portions of Chapter II, primarily in Methods and Results, were written with Dr. Heather R. Jordan (the department of Biological Sciences, Mississippi State University).

All other work conducted for the dissertation was completed by the student independently.

This research was supported in part from funds provided by Texas A&M AgriLife Insect Disease Program.

NOMENCLATURE

AGR	Accessory Gene Regulator
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
BU	Buruli Ulcer
CFU	Colony Forming Unit
CLSA	Closed-Loop Stripping Analysis
F.L.I.E.S.	Forensic Laboratory for Investigative Entomological Sciences
GC-MS	Gas Chromatography-Mass Spectrometry
GLMM	Generalized Linear Mixed Model
HSD	Honest Significant Difference
ISA	Indicator Species Analysis
MB	Middle Brook
MSA	Mannitol Salt Agar
MU	*Mycobacterium ulcerans*
NIST	National Institute of Standards and Technology
NMDS	Non-Metric Multidimensional Scaling
OADC	Oleic Albumin Dextrose Catalase
OD	Optical Density
PBS	Phosphate Buffered Saline
PERMANOVA	Permutational Multivariate Analysis of Variance

QS	Quorum Sensing
QSI	Quorum Sensing Inhibitor
RPKM	Reads Per Kilobase of transcript per Million
SE	*Staphylococcus epidermidis*
Spp.	Species
USDA	United States Department of Agriculture
VOC	Volatile Organic Compound

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Overview

Accumulating evidence suggests the global climate (i.e., conditions measured over 30 years or longer) is changing as a result of human activities; most importantly, greenhouse gases being produced through the use of fossil fuels (McMichael et al., 2004). Insects are ectothermic creatures with relatively short generation times. Their physiologies and resultant fitness are strongly influenced by the microclimate that they experience. Insects could, therefore, be sensitive to climate change at the population level over short time periods (Parmesan, 2009). Direct effects of thermal evolution, also indirect effects mediated through evolved changes in life history, particularly in voltinism (number of generations per year), may affect the vulnerability to pesticides (Dinh Van et al., 2014).

Worldwide, mosquitoes (Diptera: Culicidae) are potential vectors of pathogens that cause infectious diseases such as malaria, dengue, chikungunya, West Nile fever and Japanese encephalitis, that affect both humans and animals (WHO, 2014a). In terms of morbidity and mortality, mosquitoes are considered the most dangerous animals confronting mankind (Spielman, 2001). Humans are perpetually at high risk of becoming infected by a mosquito-borne disease. For instance, an estimated 3.3 billion people in 97 countries and territories are at risk of malaria (WHO, 2014b). Mosquitoes have greatly increased, not only in the tropic areas but also as far north as the Palearctic region, including most of Europe. The spread in space and time of many vector-borne diseases is strongly influenced by environmental factors (landscape,

location, and abundance of hosts and vectors, etc.) and climate (temperature, humidity, etc.) that influence population dynamics of the vector and the reservoir hosts of pathogens (Boukraa, 2016).

Aedes aegypti (Linnaeus) (Diptera: Culicidae) has a known distribution throughout the tropical regions of the world (Christophers, 1960). *Aedes aegypti* maintains close association with human populations and is the principal vector of the etiological agents of yellow fever and dengue fever (Tomori, 2004; WHO, 2002) as well as the chikungunya fever epidemics in countries in the Indian Ocean area (Ligon, 2006) and as noted more recently, Zika virus (Chouin-Carneiro et al., 2016; Marchette et al., 1969). Dengue virus is vectored chiefly by *Ae. aegypti* that thrives in the human-modified peri-urban habitats of the developing tropical world. The range of Dengue virus has expanded considerably in recent years, resulting in 50 to 100 million infections annually, with thousands of deaths, mostly from its severe form, dengue hemorrhagic fever (Halstead, 2007). Despite an effective vaccine, yellow fever also remains a disease burden in Africa and parts of South America, with ~200,000 cases per year resulting in ~30,000 deaths (Tomori, 2004). The rapid and highly successful adaptation to human habitats and its subsequent spread is proof of the adaptive flexibility this genetic variation confers. This adaptability presents a challenge to control populations of *Ae. aegypti* in efforts to decrease their impact on human health (Gloria-Soria et al., 2016).

Insecticides have been used to control agricultural and medical pests, including vectors of human pathogens. House residual spraying and insecticide treated bed nets have successfully controlled anthropophilic and endophagic vectors (WHO, 2006). However, there are certain undesirable effects (e.g., environmental pollution, insecticide resistance) of pesticide usage

which cannot be ignored (Jeyaratnam, 1990). Health effects of insecticides can cause both acute and chronic problems. Although everyone is at risk from exposure when insecticides are applied. The most vulnerable groups are children, pregnant women, the elderly, and people with compromised immune systems (Perry Jr, 1998). All insecticides are also associated with some risk of harm to the environment (Stahl, 2002) and select for resistance in insect vector populations, depending on the volume and frequency of applications (Hemingway & Ranson, 2000). Therefore, the development of novel vector-control strategies has been identified as a top priority within the global health community (WHO, 2002).

Backgrounds

Pathogen transmission occurs due to mosquitoes, in many cases, needing a vertebrate blood meal to finish their gonotrophic reproductive cycle (Pitts et al., 2004). Mosquito host-seeking behavior is heavily influenced by the chemical molecules in the environment (Sugiharto et al., 2016). Volatile organic compounds (VOCs) including organic atmospheric trace gasses other than carbon dioxide and monoxide (Kesselmeier & Staudt, 1999) of human origin are the principal cues used by female mosquitoes during their nocturnal and diurnal activity (Orsborne et al., 2016) for blood (Takken, 1991; Takken & Knols, 1999).

The odor complex contains host-specific chemicals allowing the mosquito to efficiently forage complex landscapes (Gillies, 1988). There is indeed a clear overlap among the kairomones to which mosquito species respond: carbon dioxide, ammonia, lactic acid, and other aliphatic carboxylic acids play a role in the host-seeking process (Smallegange & Takken, 2010).

With the exception of carbon dioxide, these compounds are present on human skin and in associated sweat (Braks et al., 2001; Cork & Park, 1996).

The skin microbiota plays an important role in human odor production, especially in the production of the characteristic odor from sweat which serves as a cue for mosquitoes (Verhulst et al., 2010). Many of the *biogenic volatile organic compounds* (biogenic VOCs) emitted by the human body, to which mosquitoes respond, are produced by bacteria (Dejong & Knols, 1995). Many groups of saturated, unsaturated, and oxygenated derivatives are included within this classification of VOCs (Kesselmeier & Staudt, 1999). For many VOCs, specific odorant receptors are known to exist on the mosquito antennae (Carey et al., 2010; Syed & Leal, 2009) and tarsi (Bentley & Day, 1989) that are specific for odors associated with hosts (Lemasson et al., 1997) and appropriate oviposition sites (Takahashi & Davis, 1981).

Biological interactions are the effects organisms in a community have on one another. In nature, no organism exists in absolute isolation, and thus every organism must interact with the environment and other organisms (Elton, 1968). Advances in the field of cell-cell communication in bacteria have now shown that many bacteria communicate. They use secreted chemical molecules to coordinate the behavior of the group. This process, quorum sensing (QS), regulates gene expression and ultimately, phenotypes, in response to fluctuations in cell-population density (Miller & Bassler, 2001). Quorum sensing traditionally was thought to be a mechanism of intraspecific communication.

However, emerging research has shown QS in interspecies communication, and such systems have been shown in a wide variety of bacteria (Lowery et al., 2008). According to Tomberlin et al. (2012), VOCs to which primary colonizers of vertebrate carrion, such as blow

flies (Diptera: Calliphoridae), respond are the same signaling molecules produced and utilized for QS by bacteria found on the resource. This principle has also been determined for mosquitoes. A blood-feeder treated with wildtype *Staphylococcus epidermidis* / tryptic soy broth (TSB) attracted 74% of *Ae. aegypti* compared to the *agr*- strain (accessory gene regulator for quorum sensing) of *S. epidermidis* / TSB ($P \leq 0.0001$) (Zhang et al., 2015).

Furthermore, QS associated molecules are also known to reduce competition with other bacteria. The *agr* system of staphylococci is a QS system, which controls the expression of exoproteins and surface proteins in a growth phase-dependent manner (Peng et al., 1988). QS is used by the *agr* system between different staphylococcal species, which has proven to be an efficient inhibitor of the *agr* response of *Staphylococcus aureus* strain Newman (Otto et al., 1999). According to (Henke & Bassler, 2004; Williams et al., 2007), QS has been selected for because it optimizes population growth or survival. In particular, a large body of evolutionary theory, and empirical studies on a range of other organisms, has shown that cooperation and communication can generate conflicts of interest between individuals (Frank, 1998; Hamilton, 1964; Lehmann & Keller, 2006; Smith & Harper, 2003).

With regards to *S. epidermidis*, one potential bacteria competitor is *Mycobacterium ulcerans*. This species is a non-spore-forming, aerobic and acid-fast bacillus, and the causative agent of an infectious disease of the skin and underlying soft tissues, known as, Buruli ulcer (BU). This disease has a known distribution in Africa, the Americas, Asia, and the Western Pacific. Most cases occur in tropical and subtropical regions except in Australia (Marsollier et al., 2002), China, and Japan (WHO, 2015). *M. ulcerans* is genetically very close to the typical intracellular parasites *Mycobacterium tuberculosis* and *Mycobacterium marinum* (Stinear et al.,

2000), and has greater than 98% nucleotide sequence similarity with *M. marinum,* indicating *M. ulcerans* evolved from *M. marinum* (Stinear et al., 2000; Stinear et al., 2007; Yip et al., 2007). Understanding the life history traits of *M. ulcerans* within its natural aquatic ecosystems, and the preventive and therapeutic tools for reducing the incidence of the disease are still very limited (Duker et al., 2004; Johnson et al., 2005; Sizaire et al., 2006).

M. ulcerans, unlike other species of mycobacterium, produces 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 sometimes lead to death. The mode *M. ulcerans* transmission to human is currently unknown (Mve-Obiang et al., 2003).

Research into interspecies competitive strategies has revealed diverse mechanisms by which bacterial species can coexist with, or dominate, other organisms competing for the same pool of resources (Hibbing et al., 2010). Although it is well established that single-species populations of bacteria can evolve over time, relatively few studies have examined the potential for co-evolution in mixed species environments (Boles et al., 2004; Rainey & Travisano, 1998). In the macroecological world, co-evolution between competitors, between pathogens and hosts, and between mutualists from different species has been repeatedly observed (Chisholm et al., 2006). Co-evolution of two bacterial species has recently been directly demonstrated for a commensal interaction in which one organism, *Pseudomonas putida,* depends on the partner organism *Acinetobacter* spp. strain C6 in order to grow on benzyl alcohol as a sole carbon source (Christensen et al., 2002; Hansen et al., 2007). Typically, the product of one conversion step is

the substrate for the next organism in the chain; each organism lives off the waste product(s) of its predecessor. Their defining characteristic as it relates to syntrophy is that in many of these associations, the producer is critically dependent on the activities of the consumer (Schink & Stams, 2006): degradation of short chain volatile fatty acids like propionate and butyrate is only sustainable if the electrons produced in the process are removed by other organisms (Dolfing, 2013).

Bacteria also engage in intraspecies competition and can participate in cooperative behaviors. In complex communities, these intraspecies processes can influence interspecies interactions, including facilitating competitive strategies that require cooperation between individuals (Hibbing et al., 2010). For instance, even for a simple ecosystem consisting of only two species, there are six possible distinct types of interaction including neutralism, commensalism, amensalism, competition, mutualism, and parasitism (Faust & Raes, 2012). The social interactions of bacteria alter the physiology, gene expression, and survival of individual cells and also enable the collective behaviors of populations, therefore significantly impacting the dynamics and functionality of an entire community (Blanchard & Lu, 2015).

Some researchers hypothesize insects associated with environments containing *M. ulcerans* may potentially serve as vectors (i.e., black flies (Diptera: Simuliidae), such as mosquitoes (Diptera: Culicidae), March flies (Diptera: Tabanidae), and sandflies (Diptera: Ceratopogonidae) (Luckhart et al., 1998; Quek et al., 2007; Singh et al., 2019; Wallace et al., 2010). A study reported the aquatic hemipterans Naucoridae infected with *M. ulcerans* could bite mice and transmit the pathogens under laboratory conditions (Marsollier et al., 2002). In 2004, an increase in BU cases in a small Australian town near Victoria in Australia was recorded.

Researchers also noted high activity of *Aedes* mosquitoes at that time. Real-time PCR was used to screen 11,504 mosquitoes in the area. Results indicated a *M. ulcerans* infection rate of 4.3 per 1,000 mosquitoes (Johnson et al., 2007). According to (Wallace et al., 2010), *Ae. aegypti*, *Ae. albopictus*, *Ochlerotatus triseriatus*, and *Culex restuans* larvae readily ingest wildtype *M. ulcerans*, isogenic toxin-negative mutants, and *M. marinum* isolates and remain infected throughout larval development. However, in that study, *M. ulcerans* was not recovered from adult mosquitoes.

Recently, Sanders et al. (2017) demonstrated mycolactone serves as an attractant of the mosquito, *Aedes aegypti* to blood-meal sources. In fact, he determined the response was dose dependent. The high dose (1.0 μg / mL) of mycolactone 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. These results suggest that arthropods utilizing resources in an *M. ulcerans* positive environment have evolved highly sensitive sensory systems allowing for the quick discovery, colonization, and utilization of these ephemeral resources (Dethier, 1948; Spivak et al., 1991).

The objectives of this research are to determine quorum-sensing (QS)-mediated, microbe-microbe interactions impact mosquito host-seeking behavior. Our model system will include *S. epidermidis*, a normal skin commensal and opportunistic skin pathogen and *M. ulcerans*, an invasive skin pathogen. As discussed above, both of these independently produce compounds that are attractive to mosquitoes. This work will determine the impact of mixed species of *S. epidermidis* and *M. ulcerans* on the production of compounds and mosquito attraction.

This research could lead to a better understanding of tropical interactions between human skin commensals and invasive microbial species such as the pathogen *M. ulcerans* and its VOCs resulting in synergistic or antagonistic responses of higher organisms, (i.e., *Ae. aegypti*) that utilize the ecosystem (human). Identifying specific mechanisms could lead to applications for novel trapping devices or repelling. Finally, this work will lead to a fundamental understanding of whether inter-kingdom signaling is a mechanism for regulating vector attraction and pathogen transmission. The research objectives are as follows:

Objectives

1. Determine the impact of quorum sensing inhibitor (QSI) in regulating *S. epidermidis* and *Ae. aegypti* interactions. The following subobjectives will be measured:

    a. VOC emissions from *S. epidermidis* with or without QSI.

        H_o: There will be no observable difference in the VOC community on *S. epidermidis* with or without QSI.

        H_a: There will be observable difference in the VOC community on *S. epidermidis* with or without QSI.

    b. Host-seeking behavior of *Ae. aegypti* to blood-feeders treated with or without QSI on *Staphylococcus epidermidis.*

        H_o: There will be no observable difference in host-seeking behavior of *Ae. aegypti* to blood-feeders treated with or without QSI on *S. epidermidis.*

H_a: There will be observable difference in host-seeking behavior of *Ae. aegypti* to blood-feeders treated with or without QSI on *S. epidermidis.*

2. To determine the effect of *M. ulcerans* mycolactone productivity on the host-seeking behaviors of *Ae. aegypti.*

H_o: There will be no observable difference in host-seeking preference of *Ae. aegypti* between a blood-feeder treated with mycolactone active wildtype and mutant of *M. ulcerans.*

H_a: There will be observable difference in host-seeking preference of *Ae. aegypti* between a blood-feeder treated with mycolactone active wildtype and mutant of *M. ulcerans.*

3. Determine the synergistic effect on host-seeking preference of *Ae. aegypti* by microbial communities comprised of *M. ulcerans* and/or *S. epidermidis*. The following subobjectives will be measured:

a. Host-seeking behavior of *Ae. aegypti* to blood-feeders treated with or without *S. epidermidis* and/or *M. ulcerans*.

H_o: There will be no observable difference in host-seeking behavior of *Ae. aegypti* to blood-feeders treated with or without *S. epidermidis* on *M. ulcerans.*

H_a: There will be observable difference in host-seeking behavior of *Ae. aegypti* to blood-feeders treated with or without *S. epidermidis* on *M. ulcerans*.

b. Host-seeking behavior of *Ae. aegypti* to blood-feeders treated with *M. ulcerans* and *S. epidermidis* at different concentrations.

H_o: There will be no observable difference in host-seeking behavior of *Ae. aegypti* to blood-feeders treated with *M. ulcerans and S. epidermidis* at different concentrations.

H_a: There will be observable difference in host-seeking behavior of *Ae. aegypti* to blood-feeders treated with *M. ulcerans and S. epidermidis* at different concentrations.

References

Bentley MD & Day JF (1989) Chemical ecology and behavioral aspects of mosquito oviposition. Annual review of entomology 34: 401-421.

Blanchard AE & Lu T (2015) Bacterial social interactions drive the emergence of differential spatial colony structures. BMC systems biology 9: 59.

Boles BR, Thoendel M & Singh PK (2004) Self-generated diversity produces "insurance effects" in biofilm communities. Proceedings of the National Academy of Sciences of the United States of America 101: 16630-16635.

Boukraa S (2016) Global changes, human activities and (re-) emerging of mosquito-borne pathogens: diversity, ecology and control of potential vectors: Université de Liège, Liège, Belgique.

Braks MAH, Meijerink J & Takken W (2001) The response of the malaria mosquito, *Anopheles gambiae,* to two components of human sweat, ammonia and L-lactic acid, in an olfactometer. Physiological Entomology 26: 142-148. doi:10.1046/j.1365-3032.2001.00227.x.

Carey AF, Wang G, Su C-Y, Zwiebel LJ & Carlson JR (2010) Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464: 66-U77. doi:10.1038/nature08834.

Chisholm ST, Coaker G, Day B & Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124: 803-814.

Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D, Dupont-Rouzeyrol M, Lourenco-de-Oliveira R & Failloux AB (2016) Differential Susceptibilities of *Aedes aegypti* and *Aedes albopictus* from the Americas to Zika Virus. PLoS Negl Trop Dis 10: e0004543. doi:10.1371/journal.pntd.0004543.

Christensen BB, Haagensen JA, Heydorn A & Molin S (2002) Metabolic commensalism and competition in a two-species microbial consortium. Applied and environmental microbiology 68: 2495-2502.

Christophers S (1960) *Aedes aegypti* (L.) the yellow fever mosquito: its life history, bionomics and structure. Rickard.

Cork A & Park K (1996) Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. Medical and veterinary entomology 10: 269-276.

Dejong R & Knols BGJ (1995) Selection of biting sites on man by 2 malaria mosquito species. Experientia 51: 80-84.

Dethier VJ (1948) Chemical insect attractants and repellents. Soil Science: 65: 204.

Dinh Van K, Janssens L, Debecker S & Stoks R (2014) Temperature-and latitude-specific individual growth rates shape the vulnerability of damselfly larvae to a widespread pesticide. Journal of Applied Ecology 51: 919-928.

Dolfing J (2013) Syntrophic propionate oxidation via butyrate: a novel window of opportunity under methanogenic conditions. Applied and environmental microbiology 79: 4515-4516.

Duker AA, Carranza EJ & Hale M (2004) Spatial dependency of Buruli ulcer prevalence on arsenic-enriched domains in Amansie West District, Ghana: implications for arsenic mediation in *Mycobacterium ulcerans* infection. International Journal of Health Geographics 3: 1.

Elton CS (1968) Animal Ecology. Great Britain. William Clowes and Sons Ltd.

Faust K & Raes J (2012) Microbial interactions: from networks to models. Nature Reviews Microbiology 10: 538-550.

Frank SA (1998) Foundations of social evolution. Princeton University Press.

Gillies MT (1988) Anopheline mosquitos: vector behaviour and bionomics. In: Wernsdorfer WH and McGregor I (eds) Malaria - principles and practice of malariology. Churchill Livingstone, Edinburgh, UK,: pp 453-485.

Gloria-Soria A, Ayala D, Bheecarry A, Calderon-Arguedas O, Chadee DD, Chiappero M, Coetzee M, Elahee KB, Fernandez-Salas I, Kamal HA, Kamgang B, Khater EI, Kramer LD, Kramer V, Lopez-Solis A, Lutomiah J, Martins A, Jr., Micieli MV, Paupy C, Ponlawat A, Rahola N, Rasheed SB, Richardson JB, Saleh AA, Sanchez-Casas RM, Seixas G, Sousa CA, Tabachnick WJ, Troyo A & Powell JR (2016) Global genetic diversity of *Aedes aegypti*. Mol Ecol 25: 5377-5395. doi:10.1111/mec.13866.

Halstead SB (2007) Dengue. The Lancet 370: 1644-1652.

Hamilton WD (1964) The genetical evolution of social behaviour. II. Journal of theoretical biology 7: 17-52.

Hansen SK, Rainey PB, Haagensen JA & Molin S (2007) Evolution of species interactions in a biofilm community. Nature 445: 533-536.

Hemingway J & Ranson H (2000) Insecticide resistance in insect vectors of human disease. Annual review of entomology 45: 371-391.

Henke JM & Bassler BL (2004) Bacterial social engagements. Trends in cell biology 14: 648-656.

Hibbing ME, Fuqua C, Parsek MR & Peterson SB (2010) Bacterial competition: surviving and thriving in the microbial jungle. Nature Reviews Microbiology 8: 15-25.

Jeyaratnam J (1990) Acute pesticide poisoning: a major global health problem. World Health Stat Q 43: 139-144.

Johnson PD, Azuolas J, Lavender CJ, Wishart E, Stinear TP, Hayman JA, Brown L, Jenkin GA & Fyfe JA (2007) *Mycobacterium ulcerans* in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. Emerging infectious diseases 13: 1653.

Johnson PD, Stinear T, Pamela L, Pluschke G, Merritt RW, Portaels F, Huygen K, Hayman JA & Asiedu K (2005) Buruli ulcer (*M. ulcerans* infection): new insights, new hope for disease control. PLoS Med 2: e108.

Kesselmeier J & Staudt M (1999) Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. Journal of atmospheric chemistry 33: 23-88.

Knols BGJ (1996) Odour-mediated host-seeking behaviour of the Afro-tropical malaria vector *Anopheles gambiae* Giles.

Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, Moore CG, Carvalho RG, Coelho GE & Van Bortel W (2015) The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. Elife 4: e08347.

Lehmann L & Keller L (2006) The evolution of cooperation and altruism–a general framework and a classification of models. Journal of evolutionary biology 19: 1365-1376.

Lemasson JJ, Fontenille D, Lochouarn L, Dia I, Simard F, Ba K, Diop A, Diatta M & Molez JF (1997) Comparison of behavior and vector efficiency of *Anopheles gambiae* and *An. arabiensis* (Diptera: Culicidae) in Barkedji, a Sahelian area of Senegal. Journal of Medical Entomology 34: 396-403.

Ligon BL (2006) Reemergence of an unusual disease: the chikungunya epidemic, Vol. 17: Seminars in pediatric infectious diseases (ed. Elsevier, pp. 99-104.

Lowery CA, Dickerson TJ & Janda KD (2008) Interspecies and interkingdom communication mediated by bacterial quorum sensing. Chemical Society Reviews 37: 1337-1346.

Luckhart S, Vodovotz Y, Cui L & Rosenberg R (1998) The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. Proceedings of the National Academy of Sciences 95: 5700-5705.

Marchette N, Garcia R & Rudnick A (1969) Isolation of Zika virus from *Aedes aegypti* mosquitoes in Malaysia. American Journal of Tropical Medicine and Hygiene 18: 411-415.

Marsollier L, Robert R, Aubry J, Saint André J-P, Kouakou H, Legras P, Manceau A-L, Mahaza C & Carbonnelle B (2002) Aquatic insects as a vector for *Mycobacterium ulcerans*. Applied and environmental microbiology 68: 4623-4628.

McMichael AJ, Campbell-Lendrum D, Kovats S, Edwards S, Wilkinson P, Wilson T, Nicholls R, Hales S, Tanser F & Le Sueur D (2004) Global climate change.

Miller MB & Bassler BL (2001) Quorum sensing in bacteria. Annual Reviews in Microbiology 55: 165-199.

Mve-Obiang A, Lee RE, Portaels F & Small P (2003) Heterogeneity of mycolactones produced by clinical isolates of *Mycobacterium ulcerans*: Implications for virulence. Infection and immunity 71: 774-783.

Orsborne J, DeRaedt Banks S, Hendy A, Gezan SA, Kaur H, Wilder-Smith A, Lindsay SW & Logan JG (2016) Personal Protection of Permethrin-Treated Clothing against *Aedes aegypti*, the Vector of Dengue and Zika Virus, in the Laboratory. PloS one 11: e0152805. doi:10.1371/journal.pone.0152805.

Otto M, Süßmuth R, Vuong C, Jung G & Götz F (1999) Inhibition of virulence factor expression in *Staphylococcus aureus* by the *Staphylococcus epidermidis* agr pheromone and derivatives. FEBS letters 450: 257-262.

Parmesan C (2009) Climate and species\'range. Nature 382: 765; 766.

Peng H, Novick R, Kreiswirth B, Kornblum J & Schlievert P (1988) Cloning, characterization, and sequencing of an accessory gene regulator (agr) in *Staphylococcus aureus*. Journal of bacteriology 170: 4365-4372.

Perry Jr GF (1998) Human Health Effects of Pesticides: LWW.

Pitts RJ, Fox AN & Zwiebel LJ (2004) A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America 101: 5058-5063. doi:10.1073/pnas.0308146101.

Quek TY, Athan E, Henry MJ, Pasco JA, Redden-Hoare J, Hughes A & Johnson PD (2007) Risk factors for *Mycobacterium ulcerans* infection, southeastern Australia. Emerging infectious diseases 13: 1661-1666.

Rainey PB & Travisano M (1998) Adaptive radiation in a heterogeneous environment. Nature 394: 69-72.

Sanders M, Jordan H, Serewis-Pond C, Zheng L, Benbow M, Small P & Tomberlin J (2017) *Mycobacterium ulcerans* toxin, mycolactone may enhance host-seeking and oviposition behaviour by *Aedes aegypti* (L.)(D iptera: C ulicidae). Environmental Microbiology 19: 1750-1760.

Schink B & Stams A (2006) The prokaryotes: an evolving electronic resource for the microbiological community.

Singh A, McBride WJH, Govan B, Pearson M & Ritchie SA (2019) A survey on *Mycobacterium ulcerans* in Mosquitoes and March flies captured from endemic areas of Northern Queensland, Australia. PLoS neglected tropical diseases 13: e0006745.

Sizaire V, Nackers F, Comte E & Portaels F (2006) *Mycobacterium ulcerans* infection: control, diagnosis, and treatment. The Lancet infectious diseases 6: 288-296.

Smallegange RC & Takken W (2010) Host-seeking behaviour of mosquitoes: responses to olfactory stimuli in the laboratory, Vol. 2: Olfaction in Vector-Host Interactions (ed. by W Takken & BGJ Knols), pp. 143-180.

Smith JM & Harper D (2003) Animal signals. Oxford University Press.

Spielman A (2001) Mosquito: The story of man's deadliest foe. Hachette Books.

Spivak M, Conlon D & Bell WJ (1991) Wind-guided landing and search behavior in fleshflies and blowflies exploiting a resource patch (Diptera: Sarcophagidae, Calliphoridae). Annals of the Entomological Society of America 84: 447-452.

Stahl A (2002) The Health Effects of Pesticides Used for Mosquito Control. Citizens Environmental Research Institute and Citizens Campaign for the Environment.

Stinear TP, Jenkin GA, Johnson PD & Davies JK (2000) Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. Journal of bacteriology 182: 6322-6330.

Stinear TP, Seemann T, Pidot S, Frigui W, Reysset G, Garnier T, Meurice G, Simon D, Bouchier C & Ma L (2007) Reductive evolution and niche adaptation inferred from the genome of

Mycobacterium ulcerans, the causative agent of Buruli ulcer. Genome research 17: 192-200.

Sugiharto VA, Grieco JP, Murphy JR, Olsen CH, Colacicco-Mayhugh MG, Stewart VA, Achee NL & Turell MJ (2016) Effects of Preexposure to DEET on the Downstream Blood-Feeding Behaviors of *Aedes aegypti* (Diptera: Culicidae) Mosquitoes. Journal of Medical Entomology: tjw066.

Syed Z & Leal WS (2009) Acute olfactory response of *Culex* mosquitoes to a human- and bird-derived attractant. Proceedings of the National Academy of Sciences of the United States of America 106: 18803-18808. doi:10.1073/pnas.0906932106.

Takahashi FT & Davis EE (1981) Humoral alteration of chemoreceptor sensitivity in *Aedes aegypti* following a blood meal. Scientific Papers of the Institute of Organic and Physical Chemistry of Wroclaw Technical University: 969-974.

Takken W (1991) The role of olfaction in host-Seeking of mosquitoes-A review. Insect Science and Its Application 12: 287-295.

Takken W & Knols BGJ (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes. Annual review of entomology 44: 131-157. doi:10.1146/annurev.ento.44.1.131.

Tomberlin JK, Crippen TL, Tarone AM, Singh B, Adams K, Rezenom YH, Benbow ME, Flores M, Longnecker M & Pechal JL (2012) Interkingdom responses of flies to bacteria mediated by fly physiology and bacterial quorum sensing. Animal Behaviour 84: 1449-1456.

Tomori O (2004) Yellow fever: the recurring plague. Critical reviews in clinical laboratory sciences 41: 391-427.

Verhulst NO, Takken W, Dicke M, Schraa G & Smallegange RC (2010) Chemical ecology of interactions between human skin microbiota and mosquitoes. Fems Microbiology Ecology 74: 1-9. doi:10.1111/j.1574-6941.2010.00908.x.

Wallace JR, Gordon MC, Hartsell L, Mosi L, Benbow ME, Merritt RW & Small PL (2010) Interaction of *Mycobacterium ulcerans* with mosquito species: implications for transmission and trophic relationships. Applied and environmental microbiology 76: 6215-6222.

WHO (2002) Dengue and Dengue Haemorrhagic Fever. World Health Organization, Geneva.

WHO (2006) Malaria vector control and personal protection. World Health Organization technical report series 936, 1.

WHO (2014a) A global brief on vector-borne diseases.

WHO (2014b) World malaria report 2014. Geneva: WHO; 2014.

WHO (2015) Buruli Ulcer Report. World Health Organization.

Williams P, Winzer K, Chan WC & Camara M (2007) Look who's talking: communication and quorum sensing in the bacterial world. Philosophical Transactions of the Royal Society B: Biological Sciences 362: 1119-1134.

Yip MJ, Porter JL, Fyfe JA, Lavender CJ, Portaels F, Rhodes M, Kator H, Colorni A, Jenkin GA & Stinear T (2007) Evolution of *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria from a common *Mycobacterium marinum* progenitor. Journal of bacteriology 189: 2021-2029.

Zhang X, Crippen TL, Coates CJ, Wood TK & Tomberlin JK (2015) Effect of Quorum Sensing by *Staphylococcus epidermidis* on the Attraction Response of Female Adult Yellow

Fever Mosquitoes, *Aedes aegypti aegypti* (Linnaeus)(Diptera: Culicidae), to a Blood-Feeding Source. PloS one 10: e0143950.

CHAPTER II

QUORUM SENSING INHIBITOR APPROACHES TO UNRAVEL THE BIOLOGICAL FUNCTION OF INTERKINGDOM SIGNALING MOLECULE IN STAPHYLOCOCCUS EPIDERMIDIS

Synopsis

Aedes aegypti, is a vector of pathogens transmitting many infectious diseases, such as Zika, Dengue, and Yellow Fever. Skin-inhabiting bacteria, such as *Staphylococcus epidermidis*, produce specific volatile organic compounds (VOCs) which participate in attracting mosquitoes to human hosts. Several studies have investigated microbial VOCs enroot to developing mosquito control methods; however, information about bacterial ecology and its role in mosquito behavior is lacking, specifically, whether bacterial communication through quorum sensing (QS) modulates VOC production that affects mosquito behavior. This study demonstrated disrupting QS by *S. epidermidis* with an inhibitor (QSI) reduced the VOC composition by 35.0% and suppressed mosquito attraction by 55.1%. RNASeq data underscored the regulation of metabolism and stress response of *S. epidermidis* and resulting protein transport of secondary metabolites potentially leading to altered VOCs production. This interdisciplinary study provides the first evidence of interkingdom cross-talk between bacteria and eukaryotic organisms that can be altered by QS manipulation.

Introduction

Hematophagous arthropods transmit approximately 17% of pathogens responsible for all infectious human diseases globally (Ponnusamy et al., 2008). The mosquito *Ae. aegypti* (Linnaeus) (Diptera: Culicidae) is a key vector due to its high degree of anthropophilic (i.e., host preference for humans over animals) and endophilic (i.e., strong association with human communities) behaviors (Harrington et al., 2001; Takken & Knols, 1999). It is well established that mosquitoes use visual stimuli (Muir et al., 1992), carbon dioxide (Gillies, 1980), heat (Davis & Sokolove, 1975) and chemosensory receptors (e.g., antenna (Carey et al., 2010; Syed & Leal, 2009), maxillary palps (Athrey et al., 2017; Lu et al., 2007), and tarsi (Bentley & Day, 1989)) to locate hosts. But recently, a connection has been made between a number of primary olfactory cues for mosquitoes that have been demonstrated to emanate from microbes associated with a host's skin (Braks et al., 2000; Braks & Takken, 1999).

Human skin is an ecosystem consisting of multiple niches occupied by complex fungal and bacterial communities (Grice et al., 2008). Many host associated factors such as topographical location, age, immune status, and sex modulate the composition and function of these communities. This impacts microbial community structure and in turn, its associated VOCs production (Hsiao et al., 2013; Sharon et al., 2010). Many VOCs associated with bacterial metabolic activity were historically hypothesized to be waste products or evolutionary leftovers (Davies, 1990; Haslam & Haslam, 1985), which will not link and enhance the fitness of the producer (Firn & Jones, 2000). However, the microbial utilization of these secondary metabolic pathways allows broader access to alternative nutrient biosynthesis; a good strategy for establishment in a new environment or response to stress (i.e., stringent response; ppGpp),

facilitating persistent survival (Breitling et al., 2013; Ochi, 1987). Thus this study was initiated to more fully delineate the link between the ecology of host-microbial communities and associated mosquito responses.

Microbial community VOCs serving as cues regulating mosquito responses to potential hosts are quite diverse (e.g., alcohols, hydrocarbons, ketones, and short-chain fatty acids) (Korpi et al., 2009; Schulz & Dickschat, 2007; Verhulst et al., 2010; Verhulst et al., 2011) and vary with microbial species (Fischer et al., 1999; Korpi et al., 2009). *Staphylococcus epidermidis* (SE), a predominant commensal bacterium primarily colonizing human epithelia, axillae, head, and nares (Kloos & Musselwhite, 1975; Noble, 2004) convert odorless sweat, secreted from human skin glands (e.g., sebaceous, apocrine, eccrine), into unique distinctive odors (Schulz & Dickschat, 2007; Verhulst et al., 2010; Verhulst et al., 2011). Such specific microbial VOCs provide distinct information to insects foraging for resources (Liu et al., 2016), but the mechanism of specific VOCs on mosquito responses has not been fully described.

We hypothesize microbial VOC production is tightly linked with bacterial quorum sensing (QS), which coordinates community responses by regulating cellular phenotypical and physiological characteristics (e.g., *agr* system in Staphylococci and *fsr* system in Enterococci). These responses are related to a host of functions, such as symbiosis (Lupp et al., 2003), virulence (Ji et al., 1995; Vuong et al., 2000), conjugation (Dunny et al., 1978), antibiotic production (Bainton et al., 1992), and biofilm formation (Davies et al., 1998; Kong et al., 2006; Zhu & Mekalanos, 2003) in response to confinement in particular niches (Haas et al., 2002). Bacterial QS elicits either an inductive or inhibitory effect for regulating biological activities and

ecological fitness in conspecific and heterospecific environments (He et al., 2012; Rasmussen & Givskov, 2006b).

Interkingdom interactions, such as between animalia and prokaryote, mediated by QS compounds, have been identified for several systems (Joint et al., 2002; Lee et al., 2015; Ma et al., 2012; Mathesius et al., 2003; Tomberlin et al., 2012; Zhang et al., 2015). Molecules produced from QS machinery have been shown to modulate host immunomodulatory effects (Chhabra et al., 2003; Ritchie et al., 2003), and QS-mimic compounds can regulate virulence factors to promote host growth (Hartmann et al., 2014) and systemic resistance (Zhou et al., 2003) against multiple pathogens. Some QS molecules (e.g., indole) provide distinct types of information to augment multicellular organismal behaviors and responses (e.g., vector host preference, oviposition site selection) (Liu et al., 2016; Tomberlin et al., 2012; Zhang et al., 2015). Previous research in this lab has demonstrated differential detection and response by mosquitoes to wildtype SE and QS inhibited (Agr-) mutant-SE (Zhang et al., 2015). Similar research has been conducted on blow flies (Diptera: Calliphoridae), that responded differentially to microbial VOCs produced by wild-type *P. mirabilis* and its *rfaL* QS-mutant (Ma et al., 2012) while attempting to locate larval resources. These studies demonstrate the affiliation of volatile QS compounds as active participants providing eukaryotic organisms (i.e., plants, arthropods) with the capability of interacting with their landscapes to manipulate behaviors for biological fitness in stochastic ecosystems.

The inhibition of bacterial QS circuits (i.e., furanones) has been proposed as a potential strategy to mitigate bacterial pathogenicity (Hentzer et al., 2003) and biofilm formation (Balaban et al., 2007; He et al., 2012; O'Loughlin et al., 2013). However, the mosquitoes' genomic

responses to quorum quenching molecules and resulting downstream impacts on their behavior are not known. This study links QS pathways within human dermal bacteria, its mRNA expression, and the volatiles it produces, with the decision-making processes of mosquitoes that could modify their recognition of hosts as blood-meals.

Materials and Methods

Mosquito Colony

Aedes aegypti (Liverpool strain) was maintained in a colony held in an environmental chamber (25.0 ± 0.5℃, 65.0 ± 5.0% RH, and a photoperiod of 12:12 (L:D) h) at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S. Facility) at Texas A&M University, College Station, Texas, USA. Mosquito larvae (~l,000) were reared in enamel pans (25 cm x 35 cm x 5 cm) containing 1.5 L of reverse osmosis (RO). Larvae were fed a diet of fish food, TetraMin™ (Tetra, Virginia, USA) on a standardized mosquito rearing schedule (Gerberg et al. 1994). Pupae were collected daily and placed in 50 ml cup of RO water at a density of 100/cup. Containers were partitioned into groups of three and placed into 30.5 cm x 30.5 cm x 30.5 cm aluminum screened wire mesh cages (BioQuip Products Inc., California, USA) for adult eclosion. Emergent adults were provided *ad libitum* with a 10% sucrose solution placed on absorbent cotton rolled in cotton-muslin gauze cloth and inserted in a 50 ml glass bottle placed inside each adult cage. Blood-feeding of 5-8 d old mosquitoes was performed using an artificial membrane. Forty-eight hours after blood-feeding, a 2 x 5 cm filter paper placed in a 50 ml black cup containing 30 ml of RO water was provided as an oviposition site in each cage. Females were allowed to deposit eggs in the container for three days. Filter paper containing eggs were removed from the

container and placed on a shelf in the incubator room, allowed to air dry, and then stored at room temperature until use.

Bacteria Preparation for Mosquito Behavior Assay

Staphylococcus epidermidis (1457) strain (Kies et al., 2003) was grown on mannitol salt agar (MSA; Neogen Corp. 2008) at 37℃ for 48 h then isolated onto a blood agar plates and incubated overnight at 37°C. For use in the mosquito behavior experiment, an inoculum of 10^8 CFU/ml ($2.1 \pm 0.6 \times 10^8$ cfu/ml) in phosphate buffered saline (PBS) was used.

Experiment Design for Mosquito Behavior Assay

Experiments were a modification of previously described methods (Zhang et al., 2015). Two hours prior to each trial, 50 mated female mosquitoes (3-5 d-old) that had never been offered a blood meal, were collected using a battery powered aspirator (Hausherrs Machine Works Co., New Jersey, USA). Mosquitoes were released into a clear Plexiglas cage (82 x 52 x 45 cm) with a wire mesh top and allowed to acclimate at a temperature of 24.0 ± 1.0℃ and a relative humidity of 65.0 ± 5.0%. Experiments were performed 30 min after sunrise (chamber at 12:12 L:D), which corresponded to the normal biting activity of *Ae*. *aegypti* (Yasuno & Tonn, 1970).

Blood-feeders were individually constructed of a 25-ml sterile tissue culture flask (Corning Inc., New York, USA) tightly wrapped with parafilm secured with cellophane tape. A 1 ml aliquot of defibrinated rabbit blood (HemoStat Laboratories, California, USA) was injected into the space between the culture flask and parafilm. A 5.0 x 5.0 cm piece of 100% cotton gauze (Dynarex Co., South Carolina, USA) was placed to absorb the inoculum over the parafilm and

secured with two rubber bands. Four blood-feeders were placed equal distance horizontally and vertically (24 cm) apart in a square pattern, gauze side down on the wire mesh top while connected to a water bath (Thermo Fisher Scientific, Connecticut, USA) and maintained at 37℃ (Zhang et al., 2015). Each feeder was inoculated with 1 ml of either 10^7 cfu/ml SE, 50 μM/ml QSI, furanone C-30 ((Z-)-4-Bromo-5-(bromomethylene) -2(5H)-furanone; Sigma-Aldrich Corp., Missouri, USA), 10^7 cfu/ml SE + 50 μM/ml QSI, furanone C-30 (SE + QSI) in PBS, or only PBS as a control.

For each experiment, four trials were performed in succession by rotating each of the four treatments to each of the four different corner locations initially assigned by a random number generator and rotated clockwise across trials to prevent positional bias. All equipment was cleaned with 3% Lysol and 95% ethanol treatments between trials. During the experiments, mosquito landing activity at each blood-feeder was recorded, with no one present in the room during the trial, with a camera (2160p / 30fps, LG, Korea) mounted on the outside of the cage. Total number of mosquitoes responding by landing and touching each blood-feeder (response) was determined for each minute for a 15 min assay period. Each experiment was replicated three times (12 trials total).

Statistical Analysis for Mosquito Behavior Assay

The odds ratios of choosing a particular treatment applied in a blood-feeder were tested and plotted using in R version 3.4.3, the DescTools package (https://cran.r-project.org/web/packages/DescTools/index.html). In order to determine whether the response by *Ae. aegypti* to the different treatments was significantly different ($P \leq 0.05$) across treatments

and trials, the data were analyzed using analysis of covariance (ANCOVA) with JMP® statistical software version 13 (SAS Institute Inc., North Carolina, USA), using a generalized linear mixed model (GLMM).

Experiment Design for VOCs Collection Assay

Bacterial volatiles were analyzed from the following samples: 1) 0.5 ml of 10^7 cfu/ml SE 2) each 0.5 ml of 50 μM/ml QSI and 10^7 cfu/ml SE 3) 0.5 ml of 50 μM/ml QSI 4) 0.5 ml of PBS. A protocol was modified from Groenhagen et al. (2013) (Groenhagen et al., 2013) designed to improve filtration in the incoming air. Each sample was transferred to a 12 ml amber glass bottle and VOCs were collected by the closed-loop-stripping-analysis (CLSA) technique at room temperature. Before every headspace sampling from treatment, the apparatus was thoroughly cleaned with dichloromethane (CH_2Cl_2) and autoclaved at 121℃ for 15 min. Each amble glass bottle was placed in a 7.5 x 11 cm (O.D x H) glass filtering jar (Kimble Chase, Vineland, New Jersey, USA) with a ground flat glass and sealed with a parafilm. The rubber stopper on the top of the glass filtering jar was equipped with one hole and inserted with a volatile trap packed with approximately 30.0 mg of Hayesep® Q porous polymer (Volatile Assay Systems, New York, USA) connecting vacuum pump (Rocker, Scientific Co., Ltd., New Taipei City, Taiwan) with Tygon® tubing (Saint-Gobain S.A., Pennsylvania, USA). The tooled hose was connected with 3 cm of Tygon® tubing piece inserted with a bacterial filter (Midwest Supplies, Minneapolis, USA, 0.2 μm pore size) and a 14.6 cm carbon filtered pipet (Marineland, Cincinnati, USA) to purify incoming air. Samples from each treatment were obtained by running the apparatus at 1 L min^{-1} for 1 h, respectively.

GC-MS Analysis

GC-MS analyses were carried out on Agilent 6890 gas chromatograph with an Agilent 5973 mass selective detector (Agilent Technologies, Santa Clara, CA, USA) by the Environmental Research Group at Texas A&M University in College Station, Texas. The GC was programmed as follows: 5 min at 50℃, increasing at 5℃/min^{-1} to 320℃, and operated in split/splitless mode: 60 s at 250℃. The carrier gas was Helium at 1.2 ml min^{-1}. Candidate identification of compounds was made by matching comparison of mass spectra with the mass spectra fragmentation patterns in the National Institute of Standards and Technology (NIST) 05 mass spectra library for peaks observed in the chromatograms.

Statistical Analysis for VOCs

The GC-MS data were processed to estimate the percentage of the area of each compound in every sample across treatments including control. In order to determine difference among volatile profiles, permutational multivariate analysis of variance (PERMANOVA) was tested using the Adonis function in R version 3.4.3, the vegan package (http://CRAN.R-project.org/package=vegan). VOC profiles were analyzed using non-metric multi-dimensional scaling (NMDS) based on the Bray-Curtis distance matrix to minimize the complex data of the area percentages into a two-dimensional space. Indicator species analysis was conducted to identify the compounds as influential species that may or may not related to among each group. The reliability of stress values was set at less than 0.2. Compound abundance was also compared using a two-way ANOVA using with JMP® statistical software version 13 (SAS Institute Inc.,

North Carolina, USA), Tukey-Kramer Honest Significant Difference (HSD). Significant levels were set at $P \leq 0.05$.

Experiment Design for Transcriptome Analysis Assay

Three, three-mL frozen bacterial suspensions per treatment or control were centrifuged at 7000xg at 4°C for 5 minutes. The culture supernatant was removed and total RNA was isolated from the bacterial pellet using Trizol® (Thermo Fisher Scientific, Massachusetts, USA), following the manufacturer's instructions. Following this, RNA was treated with Turbo DNAse (Thermo Fisher Scientific) according to the manufacturer's instructions to remove trace DNA. RNA quality was analyzed by agarose gel electrophoresis and RNA concentrations were determined using Qubit 2.0 (Thermo Fisher Scientific). All samples were stored at -80°C until further processing for library preparation. Total RNA libraries were created using the NEBNext® Ultra™ RNA Library Prep Kit and NEBNext® Multiplex Oligos (Dual Index Primers) (New England Biolabs, Massachusetts, USA) for Illumina® (Illumina, Inc. California, USA) and associated protocols. High-throughput RNA sequencing was performed by St. Jude Children's Research Hospital on an Illumina HiSeq2000 with 2 X 100bp PE (paired end) read lengths.

Statistical Analysis for Transcriptome

Sequences were initially trimmed by the sequencing facility using TrimGlare v0.4.2 (Krueger & Galore, 2015), but a more stringent quality trimming was also performed using default parameters within the Qiagen CLC Workbench 12.0 (https://www.qiagenbioinformatics.com/)

following QC analysis of sequence reads. Resulting high-quality reads were aligned to the *Staphylococcus epidermidis* (SE) 1457 genome (downloaded from the NCBI database using accession numbers CP020462 and CP020463 corresponding to the SE genome and plasmid, respectively).

RNASeq data were mapped with the following parameters: (a) maximum number of allowed mismatches was set at 2, with insertions and deletions set at 3; (b) Length and similarity fractions were set to 0.8, with autodetection for both strands; (c) minimum number of hits per read was set to 10. Gene expression values were reported as reads per kilobase of transcript per million (RPKM) mapped reads. Treatment reads with an absolute fold change of 1.4 with p-value less than or equal to 0.5 were considered as significant. Following this, transcripts were further annotated into pathways by linking protein ID with potential conserved domains and protein classifications archived within the Conserved Domain Database (Marchler-Bauer et al., 2016), and by using KEGG and STRING databases (Jensen et al., 2008; Kanehisa et al., 2015). A heat map of expression values was created in the CLC workbench measuring Euclidean distance with average cluster linkage. Gene expression was filtered by statistics with minimum absolute fold change set at 1.5 and *P*-value set at ≤ 0.05 for expression between SE wild-type and QSI treatments.

Results

Mosquito Behavior

Odds ratio analysis indicates mosquito response to the blood-feeder treated with SE (3.58) relative to the PBS control was significantly greater than for treatment with SE+ QSI (1.24) or

QSI (0.89) relative to the control (Figure II.1). While treatments ($F_3 = 167.7555$; $P \leq 0.0001$) and time ($F_1 = 7.2914$; $P \leq 0.0071$) significantly impacted mosquito response, no significant treatment (SE, SE + QSI, QSI, and PBS) and time interaction ($F_3 = 0.6574$; $P \leq 0.5785$) was measured. In general, mosquitoes spent 2.36, 2.83, and 2.55 more time on the blood-feeder treated with SE than with SE + QSI, QSI, and PBS, respectively. Mosquito response to the SE treated blood-feeders was greater than the other three treatments at every time point over the 15 min experimental period (Figure II.2). SE alone accounted for 46.1% of the total responses and the addition of QSI to SE accounted for 19.6% of the recorded responses; or 55.1% fewer responses than SE alone (Figure II.3). The QSI treatment alone was equally as attractive as the SE + QSI, as well as the PBS control indicating the QSI was not repellent. In fact, no significant difference was measured between SE + QSI, QSI, and PBS over time. Mosquito responses to SE + QSI accounted for 20.1% which was 8.5% greater than QSI and PBS, individually.

VOCs

A total of twenty-six compounds were identified from the headspace volatiles among samples by comparing experimental mass spectra with the NIST14 Mass Spectral Library in Table 1. *Staphylococcus epidermidis* had a mean of 16.00 ± 1.53 compounds compared to 12.00 ± 1.00 with QSI application. The mean of compounds detected from each QSI and control is 13.33 ± 0.33 and 13.67 ± 0.88, respectively. The number of compounds in SE was increased by 28.57% compared to control, whereas the number of compounds in SE + QSI was reduced by 35.29% compared to SE. Excluding octane which was used as an internal standard, only nine VOCs were shared by all treatments: Furfural, Benzene, 1,3-dimethyl, Benzaldehyde, Phenol, Nonanal,

Benzothiazole, 2,5 Cyclohexadiene, Butylated Hydroxytoluene, and Diethyl Phthalate. Unique compounds based on relative frequency and abundance across treatments were 4,7-Methano-1H-indene, Pregnane-3 (Pregnane-3 20-dione), Morphine, Heneicosane, Heptadecane 9-octyl, Tetracosane, and Lanosta (20.Xi.-Lanosta-7, 9(11)-diene-3.beta.,18, 20-triol) in Table 2. Relative abundance and quantity range percentages for each treatment are summarized in Table 2, organizing VOCs by retention times. The differential VOC profiles across treatments were statistically determined by (ANOSIM, R = 0.4134, $P \leq 0.037$). The stress value representing the accuracy in spatially similarity/dissimilarity was 0.1046.

Transcriptome

Sequencing and trimming yielded an average fragment and read length of 132 and 1,249,203, respectively. Altogether, 65 genes were differentially regulated between SE+ QSI and SE. Twenty-nine genes were significantly up-regulated when SE was treated with a QSI. A heat map of the top 59 significantly expressed genes from SE with and without QSI is shown in Figure II.4. Of the significantly up-regulated genes, four were involved in environmental information processing, and included genes encoding for bacterial secretion, lipoprotein export, a hexose-6-phosphate: phosphate antiporter, and for membrane transport. Seven were involved in genetic information processing; including for translation, chaperones and folding catalysts, replication, repair, and (d)NTP-pool sanitation involving *gyrB, dnaB, ung*, and a gene encoding a putative YabN. Twelve were found to be involved in metabolism including riboflavin metabolism, carbohydrate metabolism, nitrogen or urea metabolism, glycan biosynthesis, and metabolism and lipid metabolism. Four of these twelve were genes encoding for amino acid transport and

metabolism. Three significantly up-regulated genes were classified as participating in signaling and cellular processes, including genes encoding a multidrug efflux transporter, a nucleoside transporter, and a gene involved in cell wall metabolism. Finally, one gene from the SE plasmid p1457 was up-regulated whose function is currently unknown.

Thirty-six genes were significantly down-regulated when SE was treated with a QSI. Five genes were associated with environmental information processing pathways, all involved in response to various stresses such as general stress response or to alkaline shock. Ten were involved in genetic information processing with one involved in translation, one in the biosynthesis of the modified nucleoside, queuosine tRNA, and three with stress response. Involvement in stress response included *per*, a gene peroxide-responsive transcriptional repressor, *spxA*, a transcription factor that may function to reduce growth and development processes during periods of stress, and *clpB*, involved in cell recovery from heat, oxidative and other stress. Also, *dps*, a stationary phase nucleoid protein that sequesters iron and protects DNA from damage was also down-regulated.

Five significantly down-regulated genes were associated with metabolism including three for energy metabolism, one for riboflavin metabolism, and one encoding a protein arginine kinase associated with general metabolism. Six significantly down-regulated genes were associated with signaling and cellular processes including one encoding a serine protease, one encoding a zinc metallopeptidase, one encoding a M50 family peptidase, one encoding a RidA family reactive intermediate/imine deaminase. Genes also down-regulated and associated with signaling and cellular processes included F0F1 ATP synthase subunit alpha, thought to be involved in cell motility, intracellular trafficking, secretion, and vesicular transport, and *spoVG*,

involved in regulation of cell wall metabolism, and plays a role in sporulation and other functions in other organisms. Also identified were two genes encoding two hypothetical proteins and three down-regulated genes that were not associated with any of the above pathways, including a gene encoding a predicted lipoprotein, one encoding a putative gas vesicle protein, and one encoding a transmembrane protein.

Discussion

Quorum sensing molecules produced via the human commensal bacteria, SE functions as a cue, possibly a signal, for mosquitoes with regards to locating hosts. We determined *Ae. aegypti* attraction to blood-feeders treated with SE with or without the QSI, furanone, resulted in 55.1% lower attraction to the blood-feeder when compared with SE without QSI treatment. Furthermore, the application of QSI reduced SE VOC profiles by 35.3% and many of those compounds are in fact associated with quorum sensing in bacteria, such as morphine (Babrowski et al., 2012) and tetracosane (LewisOscar et al., 2018). Furthermore, transcriptome analysis indicated treatment with the QSI shifted responses to increase stress responses, as well as interfere with metabolism and protein synthesis. To our knowledge, this study provides the first evidence of interkingdom cross-talk between bacteria (i.e., *S. epidermidis*) and eukaryotic organisms (i.e., *Ae. aegypti*), that can be altered through manipulation of the bacterial QS pathway.

The ecological ramifications associated with bacterial interactions as related to mosquito attraction are partially known. Bacteria interact with each other and their surroundings (Rasmussen & Givskov, 2006a) through a number of means, including chemical communication (i.e., QS), by which bacteria respond to population density of conspecifics or heterospecifics (He

et al., 2012). Quorum sensing systems serve as a mechanism regulating microbial phenotype and genotype expression. In some instances, other prokaryotic and eukaryotic organisms eavesdrop on this information as a means to interpret their environment (He et al., 2012). Since QS mechanisms are widespread in both prokaryotic and single-celled eukaryotic organisms (e.g., fungi) (De Sordi & Mühlschlegel, 2009), it is not surprising that communication through QS has extended to the relationship between bacteria and higher eukaryotic organisms. For example, it is highly probable that certain densities of bacteria, which is key to initiating QS responses, are monitored by other organisms and that information is utilized for their own purposes.

As previously mentioned, QS molecules, such as indole, for example, provide distinct types of information to differentiate eukaryotic organisms (e.g., vector host preference, oviposition site selection) (Tomberlin et al., 2012; Zhang et al., 2015). It has been postulated that QS molecules, as elicitors produced by the bacterium, also induce a receptor present in host cells (Hughes & Sperandio, 2008; Ritchie et al., 2003). For example, QS molecules (i.e., homoserine lactones or autoinducing peptides) influences host immune responses (cytokine and nonspecific immune activity (Ritchie et al., 2003)), such as lymphocyte and tumor necrosis factor (Hughes & Sperandio, 2008). Mota and Cornelis (2005) showed that QS molecules could induce apoptosis in various host cell types. The ability of bacteria to communicate and behave as for social interactions as a multi-cellular organism significantly impacts other organisms (Tomberlin et al., 2012; Zhang et al., 2015). Data from this work has provided further evidence that QS molecules play an important role in mediating interactions between bacteria and eukaryotes.

Because of the close ties between microbes and their hosts, VOCs could be indicators of host nutritional value or other ecologically relevant information. Smeekens et al. (2014)

determined during disease, specific immune responses (e.g., cytokine) in patients decreased, or shifted, predominant dermal bacterial populations (Firmicutes: *S. epidermidis*). Therefore, shifted VOC compositions (or concentrations) or loss of a signature chemical resulting from impacted host conditions may induce mosquito host preferences as such information could increase the likelihood of securing a blood-meal or reduce the likelihood of being killed by the host.

The interdisciplinary study presented here is the first to demonstrate that disrupting a QS circuit within bacteria associated with specific hosts suppresses mosquito attraction. Thus as all living organisms ranging from plants (rhizosphere and phyllosphere) to human beings (skin surface and gut) are evolutionarily associated with microbes, modulation of physical properties within the bacterial environment, such as QS induced bacterial VOCs, opens a new realm of possibilities with regards to management of medical and veterinary vectors, and agricultural pests.

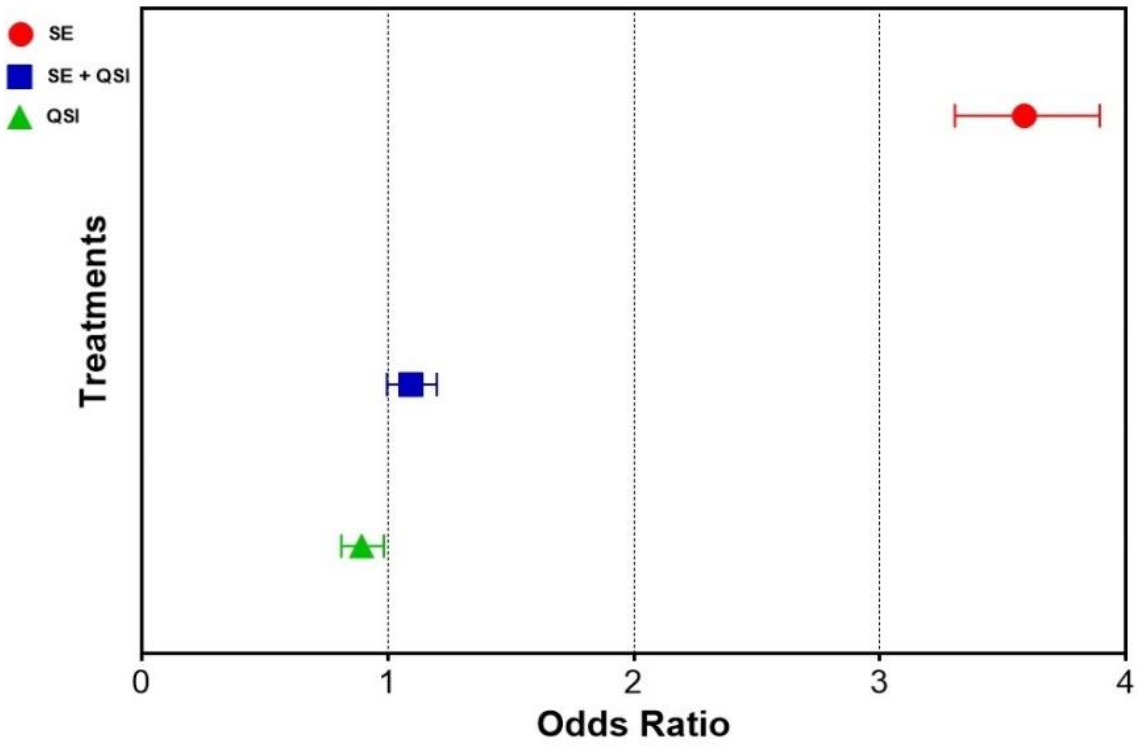


Figure II.1. The odds ratios of 3-5 d-old female *Ae. aegypti* mosquito responses to blood-feeders treated with SE (*S. epidermidis*), SE + QSI, QSI, versus PBS control placed equal distance horizontally and vertically (24 cm) apart on the top of an 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos conducted at 24 ℃ and 65% RH.

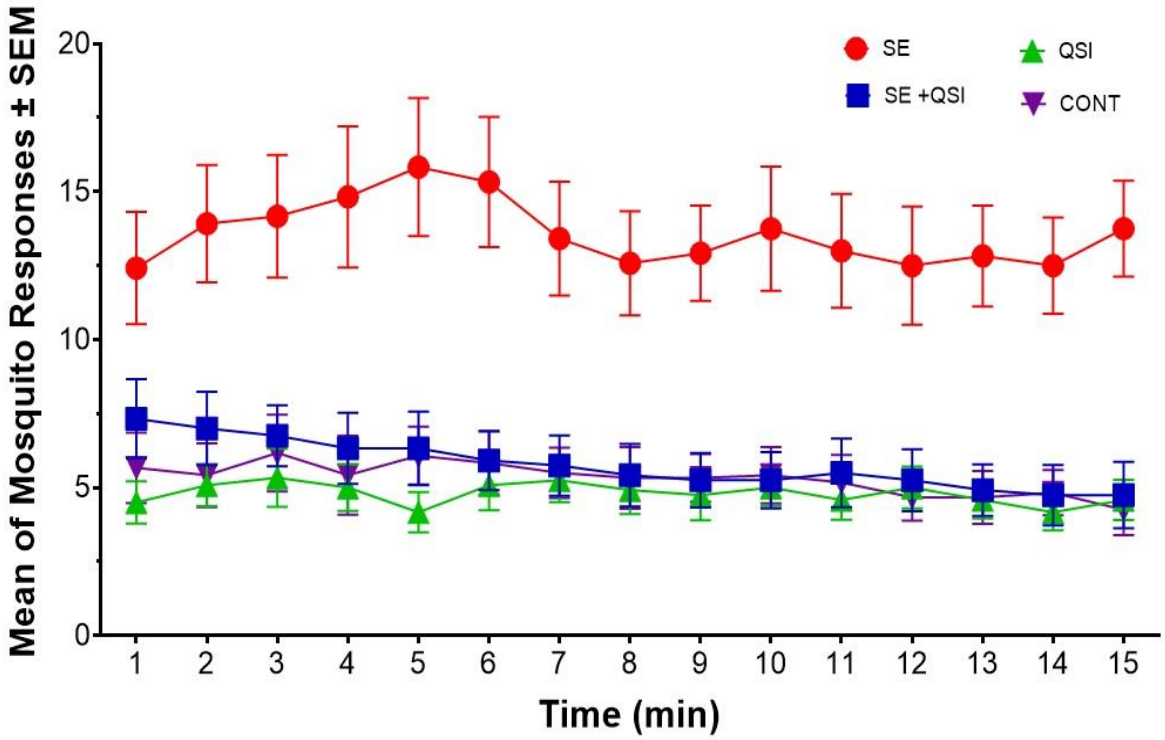


Figure II.2. Mean number of 3-5 d-old female *Ae. aegypti* mosquito responses per minute ± SEM to blood-feeders treated with SE (*S. epidermidis*), SE + QSI, QSI, and PBS (CONT) placed equal distance horizontally and vertically (24 cm) apart on the top of a 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos at 24 ℃ and 65% RH.

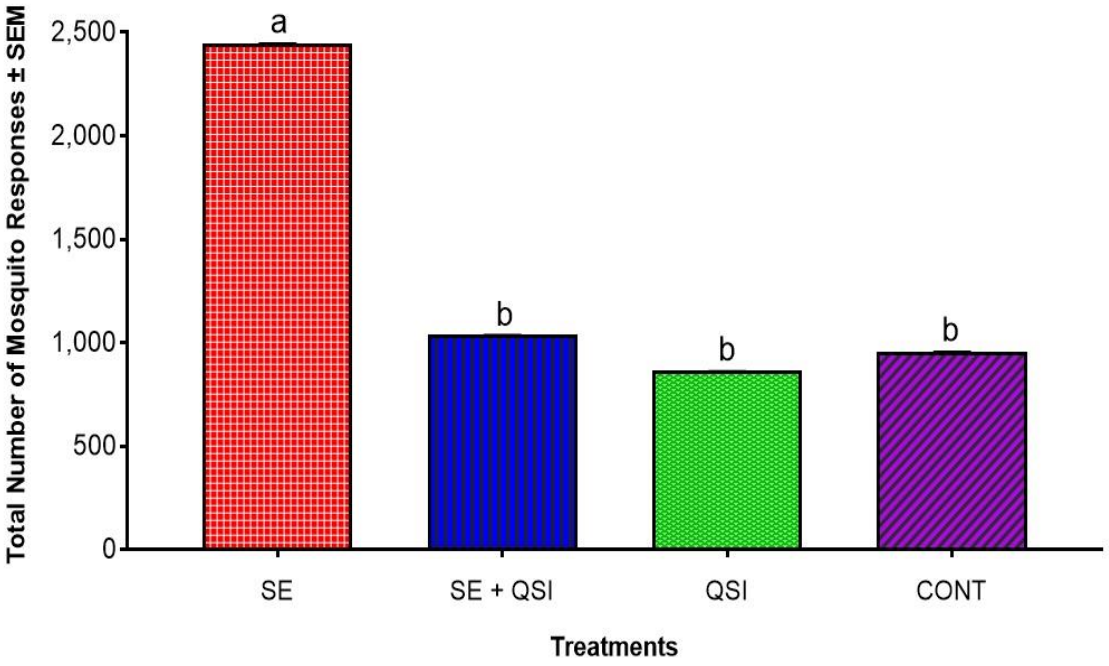


Figure II.3. Total number of 3-5 d-old female *Ae. aegypti* mosquito responses to blood-feeders treated with SE (*S. epidermidis*), SE + QSI, QSI, and PBS (CONT) placed equal distance horizontally and vertically (24 cm) apart on a 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos at 24 ℃ and 65% RH.

a-b: The same letter is not significantly different ($P \leq 0.05$).

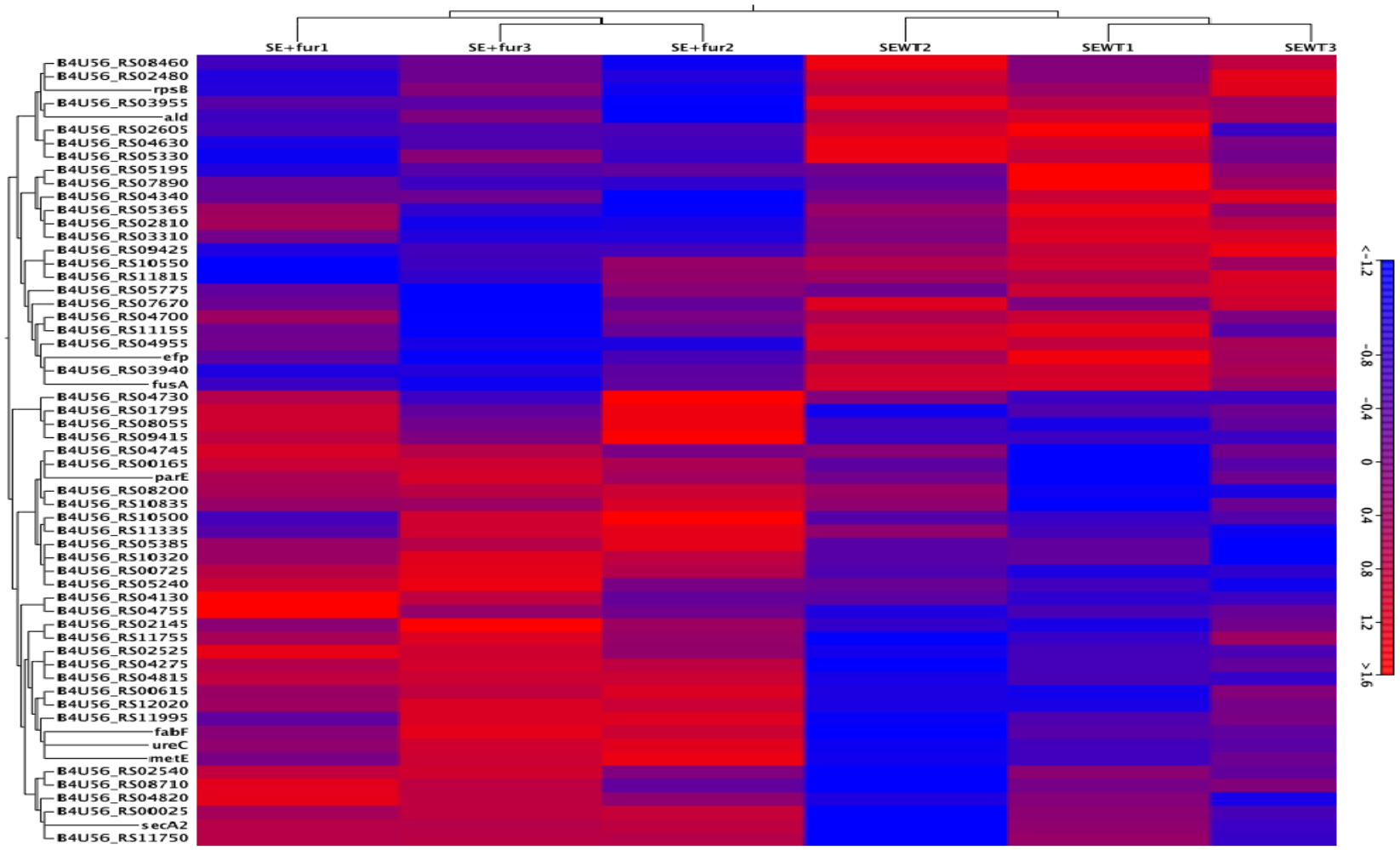


Figure II.4. A heat map of the top 59 significantly expressed genes from triplicate samples of *S. epidermidis* with QSI (SE+fur) and without (SEWT) QSI treatments for 15 min at 24 ℃ and 65% RH.

Table II.1. Total compounds identified using GC-MS emitted from *S. epidermidis* with or without QSI, QSI, and control. Trial was conducted at 25 ± 0.5℃ with 65 ± 5.0% RH.

#	Compound	Identified				Retention Time (min)	Class
		SE[a)]	SE + QSI[b)]	QSI	CONT[c)]		
1	Octane	+[d)]	+	+	+	6.18	Alkanes
2	Furfural	+	+	+	+	7.38	Furan Alcohols Furans Ethers
3	Benzene, 1,3-dimethyl	+	+	+	+	8.87	
4	1-Heptene,4-methyl	+	+			11.77	
5	4,7-Methano-1H-indene	+				11.86	
6	Benzaldehyde	+	+	+	+	12.39	Benzenoids Alcohols Ketones Aldehydes
7	Phenol	+	+	+	+	12.94	Alcohols Benzenoids
8	Benzene,1,3-dichloro		+	+	+	14.34	
9	4Cyanocyclohexene		+	+	+	14.45	
10	Acetophenone		+	+		16.08	Benzenoids Ketones
11	Nonanal	+	+	+	+	17.33	Aldehydes
12	Benzothiazole	+	+	+	+	21.26	Benzenoids Thiazole Sulfur compound

Table II.1. Continued.

#	Compound	Identified				Retention Time (min)	Class
		SE[a)]	SE + QSI[b)]	QSI	CONT[c)]		
13	Pentadecane			+	+	25.88	Acids Carboxylic Acids
14	Hexacosane			+		25.99	Alkanes
15	2,5 Cyclohexadiene	+	+	+	+	27.69	
16	Butylated Hydroxytoluene	+	+	+	+	28.69	Benzenoids Alcohols
17	Diethyl Phthalate	+	+	+	+	30.71	Alcohols
18	Pregnane-3[e)]	+				37.89	
19	Morphine	+				40.65	
20	Octacosane	+		+		42.16	Alkanes
21	Heneicosane	+				45.64	Alkanes
22	Heptadecane 9-octyl	+				48.25	
23	Tetracosane	+				49.79	Alkanes
24	Lanosta[f)]	+				49.85	
25	Hexadecanoic acid				+	50.79	

Table II.1. Continued.

#	Compound	Identified				Retention Time (min)	Class
		SE[a)]	SE + QSI[b)]	QSI	CONT[c)]		
26	Octadecanoic acid.2	+			+	53.53	

[a)] SE: *Staphylococcus epidermidis* 1457

[b)] QSI: Quorum Sensing Inhibitor, (Z-)-4-Bromo-5-(bromomethylene) -2(5H)-furanone

[c)] CONT: Phosphate buffered saline (PBS)

[d)] Compound present

[e)] Pregnane-3: pregnane-3 20-dione

[f)] Lanosta: 20.Xi.-Lanosta-7, 9(11)-diene-3.beta., 18, 20-triol

Table II.2. Compounds based on relative frequency and abundance only emitted from *S. epidermidis*. Trial was conducted at 25 ± 0.5℃ with 65 ± 5.0% RH.

#	Compounds	Retention Time (min)	Relative Abundance* (Mean ± SEM) SE[a)]	Quantity Range (%)	Class	Biological Relevance
1	4,7-Methano1Hindene[b)]	11.86	0.0005 ± 0.0001	87 ~ 95		Antibiotic tolerance
2	Pregnane-3[c)]	37.89	0.8623 ± 0.4731	91		N/A
3	Morphine[b)]	40.65	0.7712 ± 0.4045	90 ~ 95		QS (Virulence)
4	Heneicosane	45.64	0.6563 ± 0.6472	95	Alkanes	Attraction for oviposition
5	Heptadecane 9-octyl	48.25	1.5494 ± 0.8586	89 ~ 96		Human breath
6	Tetracosane	49.79	1.0052 ± 0.9457	97	Alkanes	Attraction for oviposition, Host-seeking, and QS (Biofilm)
7	Lanosta[d)]	49.85	0.6936 ± 0.6395	97		N/A

* The most abundant Octane as an internal standard is assigned 1 and the others assigned a fractional percent of that value.

a) SE: *Staphylococcus epidermidis* 1457

b) Significant indicator compounds ($P \leq 0.05$)

c) Pregnane-3: pregnane-3 20-dione

d) Lanosta: 20.Xi.-Lanosta-7, 9(11)-diene-3.beta., 18, 20-tri

References

Athrey G, Cosme LV, Popkin-Hall Z, Pathikonda S, Takken W & Slotman MA (2017) Chemosensory gene expression in olfactory organs of the anthropophilic *Anopheles coluzzii* and zoophilic *Anopheles quadriannulatus*. BMC genomics 18: 751.

Babrowski T, Holbrook C, Moss J, Gottlieb L, Valuckaite V, Zaborin A, Poroyko V, Liu DC, Zaborina O & Alverdy JC (2012) *Pseudomonas aeruginosa* virulence expression is directly activated by morphine and is capable of causing lethal gut derived sepsis in mice during chronic morphine administration. Annals of surgery 255: 386.

Bainton NJ, Stead P, Chhabra SR, Bycroft BW, Salmond G, Stewart GS & Williams P (1992) N-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochemical Journal 288: 997-1004.

Balaban N, Cirioni O, Giacometti A, Ghiselli R, Braunstein JB, Silvestri C, Mocchegiani F, Saba V & Scalise G (2007) Treatment of *Staphylococcus aureus* biofilm infection by the quorum-sensing inhibitor RIP. Antimicrobial agents and chemotherapy 51: 2226-2229.

Bentley MD & Day JF (1989) Chemical ecology and behavioral aspects of mosquito oviposition. Annual review of entomology 34: 401-421.

Braks MA, Scholte EJ, Takken W & Dekker T (2000) Microbial growth enhances the attractiveness of human sweat for the malaria mosquito, *Anopheles gambiae* sensu stricto (Diptera: Culicidae). Chemoecology 10: 129-134.

Braks MA & Takken W (1999) Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae* sensu stricto. Journal of chemical ecology 25: 663-672.

Breitling R, Ceniceros A, Jankevics A & Takano E (2013) Metabolomics for secondary metabolite research. Metabolites 3: 1076-1083.

Carey AF, Wang G, Su C-Y, Zwiebel LJ & Carlson JR (2010) Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464: 66-U77. doi:10.1038/nature08834.

Chhabra SR, Harty C, Hooi DS, Daykin M, Williams P, Telford G, Pritchard DI & Bycroft BW (2003) Synthetic analogues of the bacterial signal (quorum sensing) molecule N-(3-oxododecanoyl)-L-homoserine lactone as immune modulators. Journal of medicinal chemistry 46: 97-104.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW & Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280: 295-298.

Davies J (1990) What are antibiotics? Archaic functions for modern activities. Molecular microbiology 4: 1227-1232.

Davis EE & Sokolove PG (1975) Temperature responses of antennal receptors of the mosquito, *Aedes aegypti*. Journal of comparative physiology 96: 223-236.

De Sordi L & Mühlschlegel FA (2009) Quorum sensing and fungal–bacterial interactions in *Candida albicans*: a communicative network regulating microbial coexistence and virulence. FEMS yeast research 9: 990-999.

Dunny GM, Brown BL & Clewell DB (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proceedings of the National Academy of Sciences 75: 3479-3483.

Firn RD & Jones CG (2000) The evolution of secondary metabolism–a unifying model. Molecular microbiology 37: 989-994.

Fischer G, Schwalbe R, Möller M, Ostrowski R & Dott W (1999) Species-specific production of microbial volatile organic compounds (MVOC) by airborne fungi from a compost facility. Chemosphere 39: 795-810.

Gillies M (1980) The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): a review. Bulletin of Entomological Research 70: 525-532.

Grice EA, Kong HH, Renaud G, Young AC, Bouffard GG, Blakesley RW, Wolfsberg TG, Turner ML & Segre JA (2008) A diversity profile of the human skin microbiota. Genome research 18: 1043-1050.

Groenhagen U, Baumgartner R, Bailly A, Gardiner A, Eberl L, Schulz S & Weisskopf L (2013) Production of bioactive volatiles by different *Burkholderia ambifaria* strains. Journal of chemical ecology 39: 892-906.

Haas W, Shepard BD & Gilmore MS (2002) Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature 415: 84-87.

Harrington LC, Edman JD & Scott TW (2001) Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood? Journal of Medical Entomology 38: 411-422.

Hartmann A, Rothballer M, Hense BA & Schröder P (2014) Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. Frontiers in plant science 5: 131.

Haslam E & Haslam E (1985) Metabolites and metabolism. Clarendon Press GB-Oxford.

He Z, Wang Q, Hu Y, Liang J, Jiang Y, Ma R, Tang Z & Huang Z (2012) Use of the quorum sensing inhibitor furanone C-30 to interfere with biofilm formation by *Streptococcus mutans* and its luxS mutant strain. International journal of antimicrobial agents 40: 30-35.

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z & Kristoffersen P (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. The EMBO journal 22: 3803-3815.

Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, Codelli JA, Chow J, Reisman SE & Petrosino JF (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 155: 1451-1463.

Hughes DT & Sperandio V (2008) Inter-kingdom signalling: communication between bacteria and their hosts. Nature Reviews Microbiology 6: 111.

Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, Doerks T, Julien P, Roth A & Simonovic M (2008) STRING 8—a global view on proteins and their functional interactions in 630 organisms. Nucleic acids research 37: D412-D416.

Ji G, Beavis RC & Novick RP (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proceedings of the National Academy of Sciences 92: 12055-12059.

Joint I, Tait K, Callow ME, Callow JA, Milton D, Williams P & Cámara M (2002) Cell-to-cell communication across the prokaryote-eukaryote boundary. Science 298: 1207-1207.

Kanehisa M, Sato Y, Kawashima M, Furumichi M & Tanabe M (2015) KEGG as a reference resource for gene and protein annotation. Nucleic acids research 44: D457-D462.

Kies S, Vuong C, Hille M, Peschel A, Meyer C, Götz F & Otto M (2003) Control of antimicrobial peptide synthesis by the agr quorum sensing system in *Staphylococcus epidermidis*: activity of the lantibiotic epidermin is regulated at the level of precursor peptide processing. Peptides 24: 329-338.

Kloos WE & Musselwhite MS (1975) Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. Applied microbiology 30: 381-395.

Kong K-F, Vuong C & Otto M (2006) *Staphylococcus* quorum sensing in biofilm formation and infection. International Journal of Medical Microbiology 296: 133-139.

Korpi A, Järnberg J & Pasanen A-L (2009) Microbial volatile organic compounds. Critical reviews in toxicology 39: 139-193.

Krueger F & Galore T (2015) A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files.

Lee J-H, Wood TK & Lee J (2015) Roles of indole as an interspecies and interkingdom signaling molecule. Trends in microbiology 23: 707-718.

LewisOscar F, Nithya C, Alharbi SA, Alharbi NS & Thajuddin N (2018) In vitro and in silico attenuation of quorum sensing mediated pathogenicity in *Pseudomonas aeruginosa* using *Spirulina platensis*. Microbial pathogenesis 116: 246-256.

Liu WQ, Longnecker M, Tarone AM & Tomberlin JK (2016) Responses of *Lucilia sericata* (Diptera: Calliphoridae) to compounds from microbial decomposition of larval resources. Animal Behaviour 115: 217-225. doi:10.1016/j.anbehav.2016.03.022.

Lu T, Qiu YT, Wang G, Kwon JY, Rutzler M, Kwon H-W, Pitts RJ, van Loon JJ, Takken W & Carlson JR (2007) Odor coding in the maxillary palp of the malaria vector mosquito *Anopheles gambiae*. Current Biology 17: 1533-1544.

Lupp C, Urbanowski M, Greenberg EP & Ruby EG (2003) The *Vibrio fischeri* quorum-sensing systems ain and lux sequentially induce luminescence gene expression and are important for persistence in the squid host. Molecular microbiology 50: 319-331.

Ma Q, Fonseca A, Liu W, Fields AT, Pimsler ML, Spindola AF, Tarone AM, Crippen TL, Tomberlin JK & Wood TK (2012) *Proteus mirabilis* interkingdom swarming signals attract blow flies. The ISME journal 6: 1356.

Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, Chitsaz F, Derbyshire MK, Geer RC & Gonzales NR (2016) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic acids research 45: D200-D203.

Mathesius U, Mulders S, Gao M, Teplitski M, Caetano-Anollés G, Rolfe BG & Bauer WD (2003) Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. Proceedings of the National Academy of Sciences 100: 1444-1449.

Mendki M, Ganesan K, Shri P, Suryanarayana M, Malhotra R, Rao K & Vaidyanathaswamy R (2000) Heneicosane: an oviposition-attractant pheromone of larval origin in *Aedes aegypti* mosquito. Current Science 78: 1295-1296.

Mota LJ & Cornelis GR (2005) The bacterial injection kit: type III secretion systems. Annals of medicine 37: 234-249.

Muir LE, Kay BH & Thorne MJ (1992) *Aedes aegypti* (Diptera: Culicidae) vision: response to stimuli from the optical environment. Journal of Medical Entomology 29: 445-450.

Muller JF, Ghosh S, Ikuma K, Stevens AM & Love NG (2015) Chlorinated phenol-induced physiological antibiotic resistance in *Pseudomonas aeruginosa*. FEMS microbiology letters 362.

Navarro-Silva MA, Marques FA, Duque L & Jonny E (2009) Review of semiochemicals that mediate the oviposition of mosquitoes: a possible sustainable tool for the control and monitoring of Culicidae. Revista Brasileira de Entomologia 53: 1-6.

Noble WC (2004) The skin microflora and microbial skin disease. Cambridge University Press.

O'Loughlin CT, Miller LC, Siryaporn A, Drescher K, Semmelhack MF & Bassler BL (2013) A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. Proceedings of the National Academy of Sciences 110: 17981-17986.

Ochi K (1987) Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: significance of the stringent response (ppGpp) and GTP content in relation to A factor. Journal of bacteriology 169: 3608-3616.

Pachuwah P (2016) The role of floral and fruit scent compounds as mosquito attractants: developing new methods for monitoring mosquito populations.

Phillips M, Herrera J, Krishnan S, Zain M, Greenberg J & Cataneo RN (1999) Variation in volatile organic compounds in the breath of normal humans. Journal of Chromatography B: Biomedical Sciences and Applications 729: 75-88.

Ponnusamy L, Xu N, Nojima S, Wesson DM, Schal C & Apperson CS (2008) Identification of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by *Aedes aegypti*. Proceedings of the National Academy of Sciences 105: 9262-9267.

Rasmussen TB & Givskov M (2006a) Quorum-sensing inhibitors as anti-pathogenic drugs. International Journal of Medical Microbiology 296: 149-161.

Rasmussen TB & Givskov M (2006b) Quorum sensing inhibitors: a bargain of effects. Microbiology 152: 895-904.

Ritchie AJ, Yam AO, Tanabe KM, Rice SA & Cooley MA (2003) Modification of in vivo and in vitro T-and B-cell-mediated immune responses by the *Pseudomonas aeruginosa* quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. Infection and immunity 71: 4421-4431.

Schulz S & Dickschat JS (2007) Bacterial volatiles: the smell of small organisms. Natural product reports 24: 814-842.

Sharon G, Segal D, Ringo JM, Hefetz A, Zilber-Rosenberg I & Rosenberg E (2010) Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. Proceedings of the National Academy of Sciences: 201009906.

Smeekens SP, Huttenhower C, Riza A, Van De Veerdonk FL, Zeeuwen PL, Schalkwijk J, Van Der Meer JW, Xavier RJ, Netea MG & Gevers D (2014) Skin microbiome imbalance in patients with STAT1/STAT3 defects impairs innate host defense responses. Journal of innate immunity 6: 253-262.

Syed Z & Leal WS (2009) Acute olfactory response of *Culex* mosquitoes to a human- and bird-derived attractant. Proceedings of the National Academy of Sciences of the United States of America 106: 18803-18808. doi:10.1073/pnas.0906932106.

Takken W & Knols BGJ (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes. Annual review of entomology 44: 131-157. doi:10.1146/annurev.ento.44.1.131.

Tomberlin JK, Crippen TL, Tarone AM, Singh B, Adams K, Rezenom YH, Benbow ME, Flores M, Longnecker M & Pechal JL (2012) Interkingdom responses of flies to

bacteria mediated by fly physiology and bacterial quorum sensing. Animal Behaviour 84: 1449-1456.

Torres-Estrada JL, Meza-Alvarez RA, Cruz-Lopez L, Rodriguez MH & Arredondo-Jimenez JI (2007) Attraction of gravid *Anopheles pseudopunctipennis* females to oviposition substrates by *Spirogyra majuscula* (Zygnematales: Zygnmataceae) algae under laboratory conditions. Journal of the American Mosquito Control Association 23: 18-23.

Verhulst NO, Andriessen R, Groenhagen U, Kiss GB, Schulz S, Takken W, Van Loon JJ, Schraa G & Smallegange RC (2010) Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria. PloS one 5: e15829.

Verhulst NO, Qiu YT, Beijleveld H, Maliepaard C, Knights D, Schulz S, Berg-Lyons D, Lauber CL, Verduijn W & Haasnoot GW (2011) Composition of human skin microbiota affects attractiveness to malaria mosquitoes. PloS one 6: e28991.

Vuong C, Saenz HL, Götz F & Otto M (2000) Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. Journal of infectious diseases 182: 1688-1693.

Yasuno M & Tonn RJ (1970) A study of biting habits of *Aedes aegypti* in Bangkok, Thailand. Bulletin of the World Health Organization 43: 319.

Zhang X, Crippen TL, Coates CJ, Wood TK & Tomberlin JK (2015) Effect of Quorum Sensing by *Staphylococcus epidermidis* on the Attraction Response of Female

Adult Yellow Fever Mosquitoes, *Aedes aegypti aegypti* (Linnaeus)(Diptera: Culicidae), to a Blood-Feeding Source. PloS one 10: e0143950.

Zhou H, Yao F, Roberts DP & Lessie TG (2003) AHL-deficient mutants of *Burkholderia ambifaria* BC-F have decreased antifungal activity. Current microbiology 47: 0174-0179.

Zhu J & Mekalanos JJ (2003) Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. Developmental cell 5: 647-656.

CHAPTER III

EFFECT OF MYCOLACTONE BY MYCOBACTERIUM ULCERANS ON THE ATTRACTION RESPONSE OF FEMALE ADULT YELLOW FEVER MOSQUITOES, AEDES AEGYPTI (DIPTERA: CULICIDAE) TO A BLOOD-FEEDING SOURCE

Synopsis

Mycolactone produced by the environmental pathogen *Mycobacterium ulcerans* functions as a cue, or potentially interkingdom signal, for mosquitoes with regards to locating hosts and oviposition sites. Little is known about the mosquitoes' responses directly to *M. ulcerans*. A triple-choice assay was conducted to determine the host-seeking preference of *Ae. aegypti* compared between *M. ulcerans* wildtype (mycolactone active) and mutant (mycolactone inactive). Results demonstrated mosquito response to a blood-meal treated with *M. ulcerans* wildtype was 126.0% and 171.0% greater than *M. ulcerans* mutant and control, respectively. This study is the first to link mycolactone as a cue regulating interkingdom interactions between viable *M. ulcerans* and *Aedes aegypti*. These data provide insight as to the ecological implications in terms of disease prevalence and pathogen dispersal.

Introduction

Mosquitoes are responsible for the transmission of many pathogens including viruses, parasitic protozoans, and filariae, which cause infectious disease in humans; such as dengue fever, yellow fever, and malaria, respectively (Kettle, 1984). More than three billion people are at risk to these pathogens (Becker et al., 2010). Recent evidence suggests mosquitoes could also serve as the vector of *Mycobacterium ulcerans* (Wallace et al., 2010).

Mycobacterium ulcerans is a pathogenic bacteria associated with a skin disease, Buruli ulcer (BU) prevalent in tropical and subtropical regions (Marsollier et al., 2002; WHO, 2015). Buruli ulcer cases have been reported in at least 31 countries (Merritt et al., 2005) but West African countries such as Benin, Cote d'Ivoire, and Ghana are considered an endemic area (Van der Werf et al., 2005). The BU is associated with extensive necrosis and minimal inflammatory response (Sarfo et al., 2016). It could lead to extensive ulcerations that cover 15% of the skin surface (George et al., 1999). Recent evidence indicates mosquitoes could be serving as a mechanical vector for the pathogen (Wallace et al., 2010; Wallace et al., 2017). However, the exact relationship between *M. ulcerans* and mosquitoes has not truly been elucidated.

A number of environmental cues are used by mosquitoes to locate hosts. In addition to vision (Muir et al., 1992), abiotic factors such as carbon dioxide (Gillies, 1980), and heat (Davis & Sokolove, 1975) play a role; however, mosquitoes are known to heavily rely on olfactory sensory discriminating chemical cues associated with hosts (Takken & Knols, 1999). As determined previously, microbes associated with human

skin convert odorless human skin residues (i.e., sweat) to aliphatic and aromatic carboxylic acids (Bosch et al., 2000; Cork & Park, 1996). It has been demonstrated that organic atmospheric trace gases, including volatile organic compounds (VOCs) of human origin, are the principal cues used by female mosquitoes for host blood acquisition (Takken, 1991; Takken & Knols, 1999). A potential cue for attracting mosquitoes to individuals with *M. ulcerans* present is the toxin it produces, mycolactone.

A recent study by Sanders et al. (2017) demonstrate mycolactone serves as an attractant for yellow fever mosquito, *Aedes aegypti aegypti* (L.) (Diptera: Culicidae). This species commonly occurs in areas endemic to BU disease and mosquito larvae share an environment, most lentic (Raghunathan et al., 2005; Williamson et al., 2008) or vegetation (McIntosh et al., 2014) in standing water associated with BU disease. The result showed mosquito response to a blood-feeder treated with a high dose (1.0 μg/mL) of mycolactone was greater by 29.1 % than control group. However, this relationship extends beyond attraction to a blood-meal: mosquitoes also showed a preference for oviposition in areas containing mycolactone at the highest dose (Sanders et al., 2017). Furthermore, Mashlawi (2017) demonstrated mosquitoes reared in water with mycolactone were more likely to oviposit in similar habitats.

As previously mentioned, a biological role of mycolactone with mosquitoes has been partially proposed. However, the mosquitoes' responses directly to viable *M. ulcerans* and its resulting impact on mosquito behavior are not known. Here, we show a triple-choice assay to determine host-seeking preference of *Ae. aegypti* of *M. ulcerans*, and the potential role of mycolactone in shaping the response. This work could provide

greater understanding of the mechanisms driving ecological interactions regulating engagements between an environmental pathogen *M. ulcerans* and mosquito, with implications for transmission.

Materials and Methods

Mycobacterium ulcerans wildtype (1615) and mycolactone mutant (*M. ulcerans* 1615::*TN118*) (Marsollier et al., 2007) were used for the experiments. *M. ulcerans* 1615::*TN118* produces neither the core nor the side chain of mycolactone due to an insertion in mup045, has been well characterized, and is easily visualized by the lack of pigmentation (George et al., 1999; Mosi et al., 2012). Both were grown on Middlebrook (MB) 7H10 agar (Difco Labs, Maryland, USA) supplemented with 10% (v/v) oleic acid, albumin, dextrose supplement (OADC) with or without the antibiotic hygromycin (Sigma-Aldrich Corp., Missouri, USA) at 32℃ for 6 to 8 weeks. Four sterilized 25 mm (dia.) filter disks (Whatman, Maidstone, UK) were placed on top of the agar prior to complete solidification in a 100mm bacteriological plate and 100µl of *M. ulcerans* in PBS at an optical density (OD_{600}) = 1.2 was spread directly onto the plate. After 6-8 weeks, the filter paper was aseptically removed from the plate and used for the experiment (Figure III.1).

Aedes aegypti (Liverpool strain) was maintained in a colony held in an environmental chamber (25.0 ± 0.5℃, 65.0 ± 5.0% RH, and a photoperiod of 12:12 (L:D) h) at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S. Facility) at Texas A&M University, College Station, Texas, USA. Mosquito larvae

(~l,000) were reared in enamel pans (25 cm x 35 cm x 5 cm) containing 1.5 L of reverse osmosis (RO). Larvae were fed a diet of fish food, TetraMin™ (Tetra, Virginia, USA) on a standardized mosquito rearing schedule (Gerberg et al., 1994). Pupae were collected daily and placed in 50 ml cup of RO water at a density of 100/cup. Containers were partitioned into groups of three and placed into 30.5 cm x 30.5 cm x 30.5 cm aluminum screened wire mesh cages (BioQuip Products Inc., California, USA) for adult eclosion. Emergent adults were provided *ad libitum* with a 10% sucrose solution placed on absorbent cotton rolled in cotton-muslin gauze cloth and inserted in a 50 ml glass bottle placed inside each adult cage. Blood-feeding of 5-8 d-old mosquitoes was performed using an artificial membrane. Forty-eight hours after blood-feeding, a 2 x 5 cm filter paper placed in a 50 ml black cup containing 30 ml of RO water was provided as an oviposition site in each cage. Females were allowed to deposit eggs in the container for three days. Filter paper containing eggs were removed from the container and placed on a shelf in the incubator room, allowed to air dry, and then stored at room temperature until use.

Experiment Design

Experiments were a modification of methods previously described (Zhang et al., 2015). Host-seeking preference of *Ae. aegypti* by comparison between *M. ulcerans* wildtype (1615), mutant (TN:118) and PBS inoculated filter was determined by aggregation behavior of *Ae. aegypti* to blood-feeders treated with *M. ulcerans* wildtype or mutant inoculated filter. Briefly, 2 h prior to each trial, 50 mated female mosquitoes (3-5 d old)

that had never received a blood meal, were collected using an aspirator (Hausherrs Machine Works Co., New Jersey, USA). Mosquitoes were released into a clear Plexiglas cage (82 x 52 x 45 cm) with an aluminum wire mesh top and allowed to acclimate at 24.0 ± 1.0℃, relative humidity of 65.0 ± 5.0%. Experiments were performed 30 min after sunrise (chamber at 12:12 L:D), which corresponds to the normal biting activity of *Ae*. *aegypti* (Yasuno & Tonn, 1970).

Blood-feeders were individually constructed of a 25-ml sterile tissue culture flask (Corning Inc., New York, USA) tightly wrapped with parafilm secured with cellophane tape. A 1 ml aliquot of defibrinated rabbit blood (HemoStat Laboratories, California, USA) was injected into the space between the culture flask and parafilm. A 5.0 x 5.0 cm autoclaved piece of 100% cotton gauze (Dynarex Co., South Carolina, USA) was placed over the parafilm. A disk grown with *M. ulcerans* (wildtype or mutant) or a sterile PBS control 25 mm disk was inserted between cotton gauze layer and the parafilm. Three blood-feeders, connected to a water bath (Thermo Fisher Scientific, Connecticut, USA) and maintained at 37℃, were placed in parallel, 16.5 cm apart, gauze side down on the wire mesh top (Sanders et al., 2017). For each experiment, three trials were performed in succession by rotating each of the three treatments to each of the three different locations initially assigned by a random number generator and rotated clockwise across trials to prevent positional bias. All equipment was cleaned with 3% Lysol then 95% ethanol between trials. During the experiments, mosquito landing activity at each blood-feeder was recorded with a camera (2160p / 30fps, LG, Korea) mounted on the outside of the cage. The total number of mosquitoes responding by landing and touching each blood-

feeder (response) was determined for each minute over a 15 min assay period. Each experiment was replicated three times (9 trials total).

Statistical Analysis

The odds ratios of choosing a particular treatment applied in a blood-feeder were tested and plotted using in R version 3.4.3, the DescTools package (https://cran.r-project.org/web/packages/DescTools/index.html). All data were tested for normal distribution with JMP® statistical software version 13 (SAS Institute Inc., North Carolina, USA), using the Shapiro–Wilk test. In order to determine whether the mosquito response rate was significantly different among treatments and nine trials, the data were analyzed using analysis of covariance (ANCOVA). A comparison of mosquito responses across treatments was conducted with using a generalized linear mixed model (GLMM). The probability (*P*) of response (attraction) by *Ae. aegypti* to the different treatments was examined for significant difference ($p \leq 0.05$).

Results

Mosquito responses between *M. ulcerans* wildtype and mutant were significantly different. Odds ratio analysis indicated mosquito response to the blood-feeder treated with *M. ulcerans* wildtype was significantly greater (4.77) relative to the control, or *M. ulcerans* mutant (1.25), relative to the control (Figure III.2). **A** significant interaction among treatment types (*M. ulcerans* wildtype, *M. ulcerans* mutant, and control) was

detected over time (F_2 = 288.1908; $p \leq 0.0001$). No significant trial and time effect interaction (F_1 = 1.9856; $p \leq 0.1596$) were found.

The mean number of mosquito responses to blood-feeders with different treatments was compared (Figure III.3). Blood-feeder treated with *M. ulcerans* wildtype elicited a greater response (55.2% in total number of response) at all time points than *M. ulcerans* mutant and control. Mosquito responses to *M. ulcerans* wildtype and mutant were significantly different over the 15-min period; response to *M. ulcerans* wildtype was significantly greater than *M. ulcerans* mutant at 2 (97.0%), 3 (104.8%), 4 (103.4%), 5 (105.8%), 6 (112.2%), 7 (146.3%), 8 (137.4%), 9 (137.2%), 10 (156.3%), 11 (130.3%), 12 (133.1%), 13 (159.3%), 14 (122.0%), and 15 (148.7%) min post exposure. The peak mean number (± SE) of mosquitoes responding to *M. ulcerans* wildtype occurred at 10 min (36.4 ± 3.3) and for *M. ulcerans* mutant and control at 11 min (14.7 ± 1.7) and 5 min (12.2 ± 2.1), respectively (Figure III.3). The *M. ulcerans* wildtype treatment elicited the lowest number of responses by mosquitoes to the blood-feeders at 1 min (15.7 ± 3.6). Response to *M. ulcerans* mutant and control were lower at 1 min (9.2 ± 2.1) and (7.7 ± 1.6), respectively. The control blood-feeder, treated with PBS, elicited the lowest mosquito response at all of time points.

Total mosquito responses to the treatments were quantified. The total number of attraction responses (7,207) from the 9 trials (over the 15 min experimental period) to *M. ulcerans* wildtype, *M. ulcerans* mutant, and control treatments were 3,980 (55.2%), 1,761 (24.4%), and 1,466 (20.3%), respectively (Figure III.4). A significant difference (F_2 = 234.3004; $p \leq 0.0001$) was measured between *M. ulcerans* wildtype and other

treatments over the 15 min experiment period. A significant difference was found between *M. ulcerans* wildtype and mutant over time, as the total number of responses to *M. ulcerans* wildtype was 126.0% greater than to *M. ulcerans* mutant. Total mosquito responses to *M. ulcerans* wildtype were 171.5% greater than control. Mosquito responses to *M. ulcerans* mutant and control were not significantly different ($p \leq 0.0537$) over the 15 min period (Figure III.4).

Discussion

Mycolactone produced by *M. ulcerans* functions as a cue, or potentially an interkingdom signal, for mosquitoes with regards to locating hosts. We determined *Ae. aegypti* attraction to blood-feeders treated with *M. ulcerans* wildtype, resulted in 126% greater attraction to the blood-feeder when compared with *M. ulcerans* mutant (mycolactone inactive) treatment. To our knowledge, this study provides the first evidence of biological relevance between viable *M. ulcerans* and *Ae. aegypti*, and that such interaction can be amplified through the secondary metabolite mycolactone.

With regards to *M. ulcerans*, mycolactone has unique molecular properties such as low vapor pressure and high boiling points (Sanders et al., 2017), suggesting its potential to produce candidate VOCs regulating mosquito behavior as related to host-seeking or oviposition. As previously mentioned, mosquitoes are primarily guided to locate a suitable host by VOCs produced by bacteria. For example, *Staphylococcus epidermidis*, associated with the human skin microbiota, are biological mediators of mosquito attraction and blood-feeding (Verhulst et al., 2010; Verhulst et al., 2011).

Verhulst et al. (2009) demonstrated *S. epidermidis* on blood agar plates was more attractive to *Anopheles gambiae* (Diptera: Culicidae) *sensu stricto,* a vector for the malaria parasite, than sterile blood agar plates. These results likely explain why washing feet with anti-bacterial soap results in *An. gambiae* shifting in blood feeding to other body parts (Dejong & Knols, 1995); indicating an interaction between the mosquito and human skin microbiota.

Mycolactone controls cell membranes on the host which facilitates environmental persistance of *M. ulcerans* (Sarfo et al., 2016) ; but how it facilitates this function is not fully understood. Bacteria interact with each other through specific communications pathways (i.e., autoinducer in quorum sensing (QS)). Such responses to these compounds are tightly linked with bacterial density (Rasmussen & Givskov, 2006). In fact, QS from bacteria elicit host (e.g., humans, plants, other multicellular organisms) responses (Dudler & Eberl, 2006). Mycolactone could function as quorum sensing (QS) antagonist (Dr. Heather Jordan, unpublished data) or regulator to biological activities, such as symbiosis (Lupp et al., 2003), virulence (Ji et al., 1995; Vuong et al., 2000) or conjugation (Dunny et al., 1978). Such ability could allow *M. ulcerans* to become established and persist within a given environment (e.g., water or human skin).

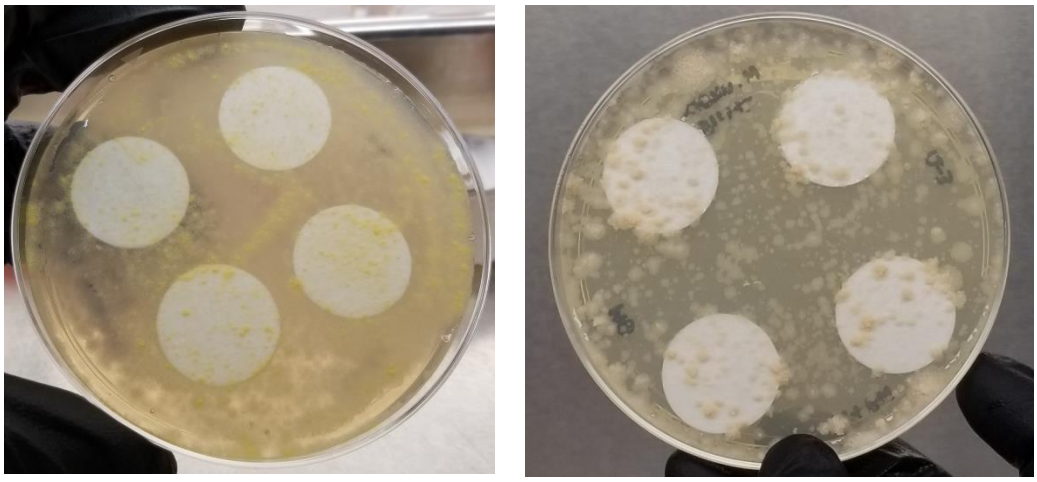
The ability of bacteria to QS has been demonstrated to regulate mosquito attraction to a blood-meal. Zhang et al. (2015) determined mosquitoes are more attracted (74.0%) to wildtype *S. epidermidis* (i.e., commensal on human skin; able to QS) than accessory gene regulator (*agr*) mutant *S. epidermidis* (unable to QS). Similar to this study, adult blow fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) response to *P.*

mirabilis, a bacteria associated with carrion (i.e., source for larval development), was partly tied to QS (Tomberlin et al., 2012). This evidence suggested there may be widespread interkingdom interactions between mosquitoes and microbes that have biological and ecological importance within an environment.

While results were consistent across experiments, limitations of the current study were identified. Our approach when examining *M. ulcerans*-mosquito interactions was to use a single bacteria species and determine its impact on the mosquito of interest. Such an approach is known to be limiting in terms of deciphering the true ecological relevance of bacterial interactions with mosquitoes, as the behavior of the bacteria in isolation can be quite different than in the community mixtures typically encountered in a complex and dynamic ecosystem (e.g., human skin). Furthermore, morphological and physiological differences between *M. ulcerans* wildtype and mutant may have an influence on mosquito behavior, particular host-seeking behavior; mycolactone is UV active and *M. ulcerans* mutant lacks pigmentation and is not UV active (Sarfo et al., 2010). The role of color vision in mosquito host-seeking behavior has been ascertained with the exception of a few studies (Bidlingmayer & Hem, 1979; Gillies & Wilkes, 1982), showing *Ae. aegypti* had relatively poor acuity but is capable of specific wavelength discrimination (323 nm ~ 621 nm) (Muir et al., 1992). To determine their impact on bacteria-mosquito interactions and reduce the variability these factors should be examined in greater detail.

Results from these experiments could be used to develop novel methods for shifting VOCs profiles by manipulating the mycolactone system of host-associated

invasive bacteria, resulting in reduced mosquito attraction. In addition to host-seeking behavior, previous research also demonstrated mosquitoes preferred to oviposit on substrates containing a high concentration of mycolactone, rather than sterilized substrates (Mashlawi, 2017), indicating that mycolactone potentially plays an important role in the biology and community ecology of the interaction in its natural environment. A recent study showed specific bacteria-associated VOCs (e.g., indole and skatole) function as both possible host-seeking attractant and oviposition stimulants for mosquitoes (Carey et al., 2010; Qiu et al., 2006; Syed & Leal, 2009). Based on these previous studies, mycolactone potentially associated with QS systems of the *M. ulcerans* leading to shifting VOCs production should be explored for the potential development of new and effective methods for mosquito control and suppressing pathogen transmissions.



(a) **(b)**

Figure III.1. Morphological features of *Mycobacterium ulcerans*; (a) wildtype (1615) and (b) mutant (TN:118)

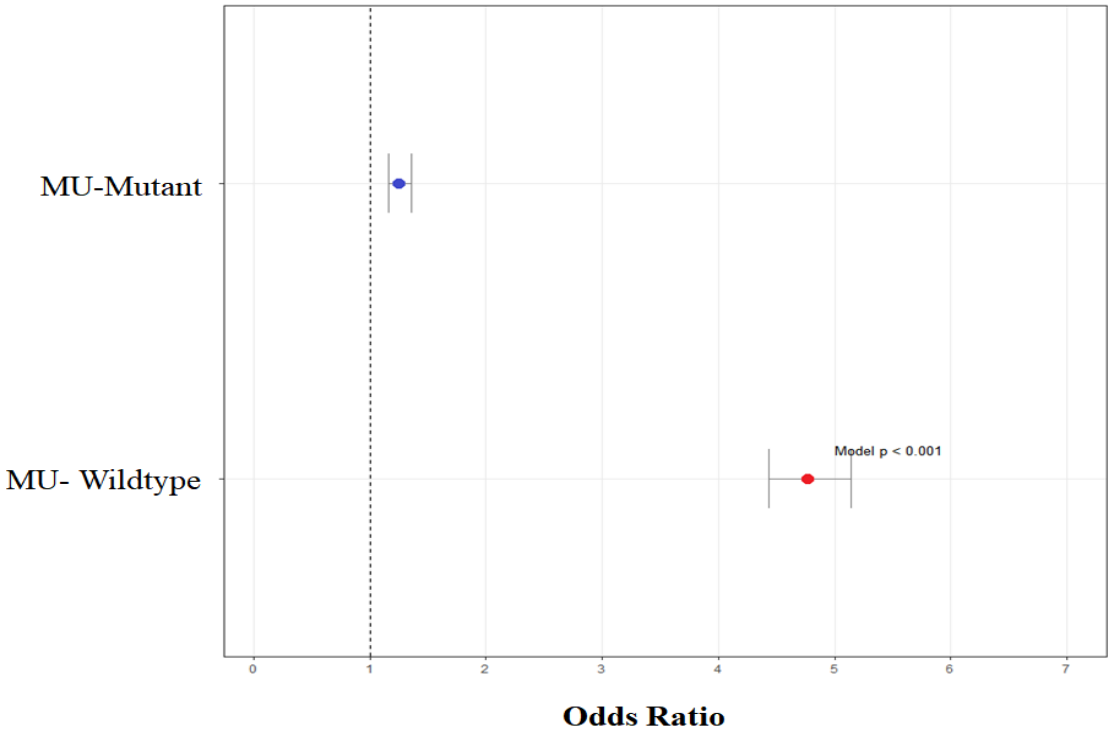


Figure III.2. The odds ratios of 3-5 d-old female *Ae. aegypti* mosquito responses to blood-feeders treated with MU (*M.ulcerans*) wildtype, MU mutant, versus PBS control placed in parallel, 16.5 cm apart on the top of an 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during triplicate trials of 15 min with 50 mosquitos conducted at 24 ℃ and 65% RH.

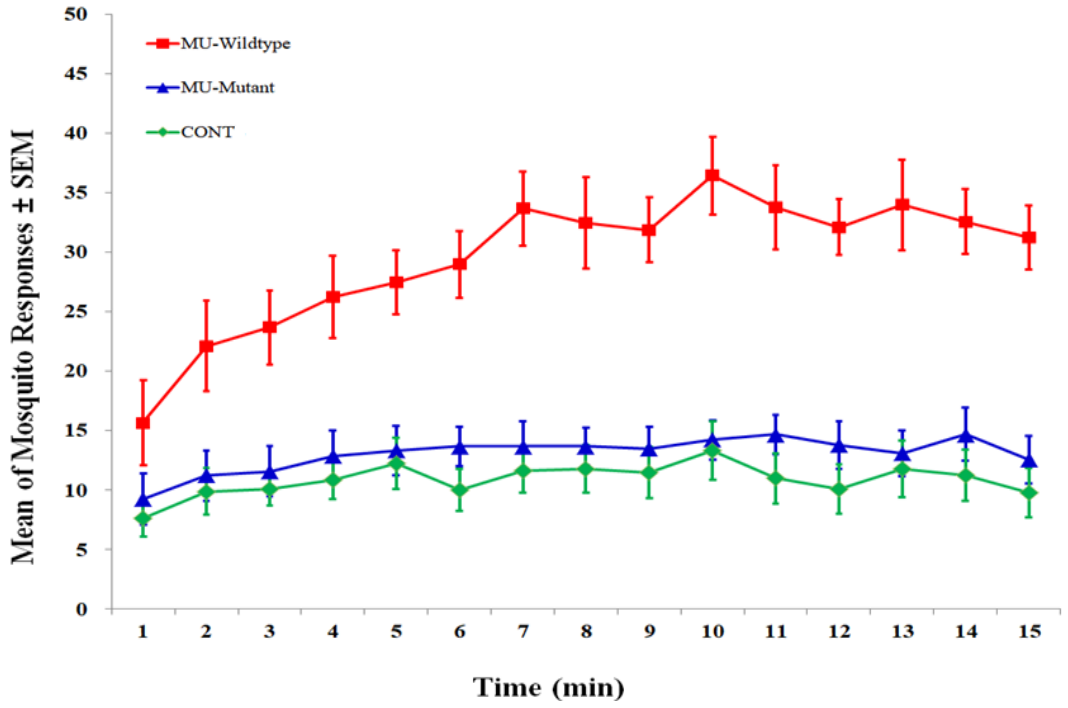


Figure III.3. Mean number of 3-5 d-old female *Ae. aegypti* mosquito responses per minute ± SEM to blood-feeders treated with MU (*M.ulcerans*) wildtype, MU mutant, and PBS (CONT) placed in parallel, 16.5 cm apart on the top of an 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during triplicate trials of 15 min with 50 mosquitos conducted at 24 ℃ and 65% RH.

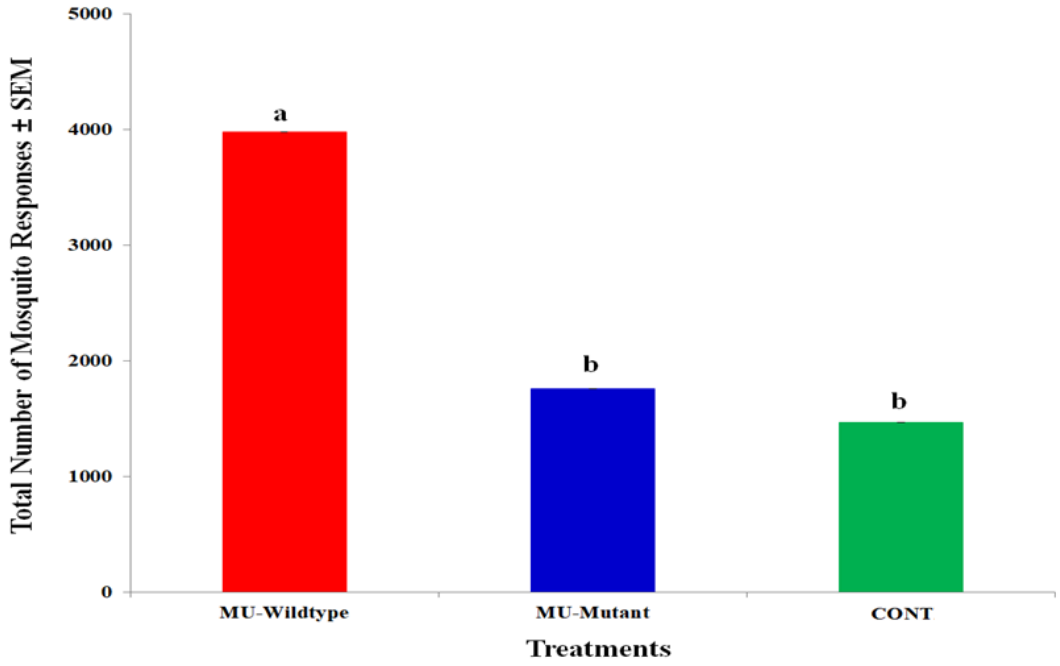


Figure III.4. Total number of 3-5 d-old female *Ae. aegypti* mosquito responses to blood-feeders treated with MU (*M.ulcerans*) wildtype, MU mutant, and PBS (CONT) placed in parallel, 16.5 cm apart on the top of an 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during triplicate trials of 15 min with 50 mosquitos conducted at 24 ℃ and 65% RH.

a-b The same letter is not significantly different ($p \leq 0.05$).

References

Becker N, Petrić D, Boase C, Lane J, Zgomba M, Dahl C & Kaiser A (2010) Mosquitoes and their control. Springer.

Bidlingmayer W & Hem D (1979) Mosquito (Dipteral Culicidae) flight behaviour near conspicuous objects. Bulletin of Entomological Research 69: 691-700.

Bosch OJ, Geier M & Boeckh J (2000) Contribution of fatty acids to olfactory host finding of female *Aedes aegypti*. Chemical Senses 25: 323-330.

Carey AF, Wang G, Su C-Y, Zwiebel LJ & Carlson JR (2010) Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464: 66.

Cork A & Park K (1996) Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. Medical and veterinary entomology 10: 269-276.

Davis EE & Sokolove PG (1975) Temperature responses of antennal receptors of the mosquito, *Aedes aegypti*. Journal of comparative physiology 96: 223-236.

Dejong R & Knols BGJ (1995) Selection of biting sites on man by 2 malaria mosquito species. Experientia 51: 80-84.

Dudler R & Eberl L (2006) Interactions between bacteria and eukaryotes via small molecules. Current opinion in biotechnology 17: 268-273.

Dunny GM, Brown BL & Clewell DB (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proceedings of the National Academy of Sciences 75: 3479-3483.

George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R & Small P (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283: 854-857.

Gerberg EJ, Barnard DR & Ward RA (1994) Manual for mosquito rearing and experimental techniques. American Mosquito Control Association, Inc.

Gillies M (1980) The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): a review. Bulletin of Entomological Research 70: 525-532.

Gillies M & Wilkes T (1982) Responses of host-seeking Mansonia and Anopheles mosquitoes (Diptera: culicidae) in west Africa to visual features of a target. Journal of Medical Entomology 19: 68-71.

Ji G, Beavis RC & Novick RP (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proceedings of the National Academy of Sciences 92: 12055-12059.

Kettle DS (1984) Medical and veterinary entomology. Croom Helm Ltd.

Lupp C, Urbanowski M, Greenberg EP & Ruby EG (2003) The *Vibrio fischeri* quorum-sensing systems ain and lux sequentially induce luminescence gene expression and are important for persistence in the squid host. Molecular microbiology 50: 319-331.

Marsollier L, André JPS, Frigui W, Reysset G, Milon G, Carbonnelle B, Aubry J & Cole ST (2007) Early trafficking events of *Mycobacterium ulcerans* within Naucoris cimicoides. Cellular microbiology 9: 347-355.

Marsollier L, Robert R, Aubry J, Saint André J-P, Kouakou H, Legras P, Manceau A-L, Mahaza C & Carbonnelle B (2002) Aquatic insects as a vector for *Mycobacterium ulcerans*. Applied and environmental microbiology 68: 4623-4628.

Mashlawi AMH (2017) Impact of Mycolactone Produced by *Mycobacterium ulcerans* on Life-History Traits of *Aedes aegypti* aegypti (L.)(Diptera: Culicidae) and Resulting Habitat Selection for Oviposition.

McIntosh M, Williamson H, Benbow ME, Kimbirauskas R, Quaye C, Boakye D, Small P & Merritt R (2014) Associations between *Mycobacterium ulcerans* and aquatic plant communities of West Africa: implications for Buruli ulcer disease. EcoHealth 11: 184-196.

Merritt RW, Benbow ME & Small PL (2005) Unraveling an emerging disease associated with disturbed aquatic environments: the case of Buruli ulcer. Frontiers in Ecology and the Environment 3: 323-331.

Mosi L, Mutoji NK, Basile FA, Donnell R, Jackson KL, Spangenberg T, Kishi Y, Ennis DG & Small PL (2012) *Mycobacterium ulcerans* causes minimal pathogenesis and colonization in medaka (Oryzias latipes): an experimental fish model of disease transmission. Microbes and infection 14: 719-729.

Muir LE, Kay BH & Thorne MJ (1992) *Aedes aegypti* (Diptera: Culicidae) vision: response to stimuli from the optical environment. Journal of Medical Entomology 29: 445-450.

Qiu YT, Van Loon JJ, Takken W, Meijerink J & Smid HM (2006) Olfactory coding in antennal neurons of the malaria mosquito, *Anopheles gambiae*. Chemical Senses 31: 845-863.

Raghunathan PL, Whitney EA, Asamoa K, Stienstra Y, Taylor Jr TH, Amofah GK, Ofori-Adjei D, Dobos K, Guarner J & Martin S (2005) Risk factors for Buruli ulcer disease (*Mycobacterium ulcerans* infection): results from a case-control study in Ghana. Clinical Infectious Diseases 40: 1445-1453.

Rasmussen TB & Givskov M (2006) Quorum sensing inhibitors: a bargain of effects. Microbiology 152: 895-904.

Sanders M, Jordan H, Serewis-Pond C, Zheng L, Benbow M, Small P & Tomberlin J (2017) *Mycobacterium ulcerans* toxin, mycolactone may enhance host-seeking and oviposition behaviour by *Aedes aegypti* (L.)(D iptera: C ulicidae). Environmental Microbiology 19: 1750-1760.

Sarfo FS, Phillips R, Wansbrough-Jones M & Simmonds RE (2016) Recent advances: role of mycolactone in the pathogenesis and monitoring of *Mycobacterium ulcerans* infection/Buruli ulcer disease. Cellular microbiology 18: 17-29.

Sarfo FS, Phillips RO, Rangers B, Mahrous EA, Lee RE, Tarelli E, Asiedu KB, Small PL & Wansbrough-Jones MH (2010) Detection of Mycolactone A/B in *Mycobacterium ulcerans*–Infected Human Tissue. PLoS neglected tropical diseases 4: e577.

Syed Z & Leal WS (2009) Acute olfactory response of Culex mosquitoes to a human- and bird-derived attractant. Proceedings of the National Academy of Sciences: pnas. 0906932106.

Takken W (1991) The role of olfaction in host-seeking of mosquitoes: a review. International Journal of Tropical Insect Science 12: 287-295.

Takken W & Knols BG (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes. Annual review of entomology 44: 131-157.

Tomberlin JK, Crippen TL, Tarone AM, Singh B, Adams K, Rezenom YH, Benbow ME, Flores M, Longnecker M & Pechal JL (2012) Interkingdom responses of flies to bacteria mediated by fly physiology and bacterial quorum sensing. Animal Behaviour 84: 1449-1456.

Van der Werf TS, Stienstra Y, Johnson RC, Phillips R, Adjei O, Fleischer B, Wansbrough-Jones MH, Johnson PD, Portaels F & van der Graaf WT (2005) *Mycobacterium ulcerans* disease. Bulletin of the World Health Organization 83: 785-791.

Verhulst NO, Andriessen R, Groenhagen U, Kiss GB, Schulz S, Takken W, Van Loon JJ, Schraa G & Smallegange RC (2010) Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria. PloS one 5: e15829.

Verhulst NO, Beijleveld H, Knols BG, Takken W, Schraa G, Bouwmeester HJ & Smallegange RC (2009) Cultured skin microbiota attracts malaria mosquitoes. Malar J 8: 302.

Verhulst NO, Qiu YT, Beijleveld H, Maliepaard C, Knights D, Schulz S, Berg-Lyons D, Lauber CL, Verduijn W & Haasnoot GW (2011) Composition of human skin microbiota affects attractiveness to malaria mosquitoes. PloS one 6: e28991.

Vuong C, Saenz HL, Götz F & Otto M (2000) Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. Journal of infectious diseases 182: 1688-1693.

Wallace JR, Gordon MC, Hartsell L, Mosi L, Benbow ME, Merritt RW & Small PL (2010) Interaction of *Mycobacterium ulcerans* with mosquito species: implications for transmission and trophic relationships. Applied and environmental microbiology 76: 6215-6222.

Wallace JR, Mangas KM, Porter JL, Marcsisin R, Pidot SJ, Howden B, Omansen TF, Zeng W, Axford JK & Johnson PD (2017) *Mycobacterium ulcerans* low infectious dose and mechanical transmission support insect bites and puncturing injuries in the spread of Buruli ulcer. PLoS neglected tropical diseases 11: e0005553.

WHO (2015) Buruli Ulcer Report. World Health Organization.

Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, McIntosh MD, Quaye C, Ampadu EO, Boakye D & Merritt RW (2008) Distribution of *Mycobacterium ulcerans* in Buruli ulcer endemic and non-endemic aquatic sites in Ghana. PLoS neglected tropical diseases 2: e205.

Yasuno M & Tonn RJ (1970) A study of biting habits of *Aedes aegypti* in Bangkok, Thailand. Bulletin of the World Health Organization 43: 319.

Zhang X, Crippen TL, Coates CJ, Wood TK & Tomberlin JK (2015) Effect of Quorum Sensing by *Staphylococcus epidermidis* on the Attraction Response of Female Adult Yellow Fever Mosquitoes, *Aedes aegypti* aegypti (Linnaeus)(Diptera: Culicidae), to a Blood-Feeding Source. PloS one 10: e0143950.

CHAPTER IV

SYNERGISTIC EFFECTS OF MYCOBACTERIUM ULCERANS AND STAPHYLOCOCCUS EPIDERMIDIS ON AEDES AEGYPTI (DIPTERA: CULICIDAE) HOST-SEEKING BEHAVIOR

Synopsis

The human dermis is an ecosystem consisting of multiple niches occupied by complex microbial communities producing unique volatile organic compounds (VOCs), which play an important role in attracting mosquitoes. Little is known about the effect of polymicrobial interactions on mosquito behavior. A four-choice assay was conducted to determine the interaction effect of the pathogenic bacterium, *Mycobacterium ulcerans* with the dermal commensal bacterium, *Staphylococcus epidermidis* at different concentrations (0, 10^3, 10^5, and 10^7) on *Aedes aegypti* attraction to a blood-meal. Results demonstrate that adding *M. ulcerans* to *S. epidermidis* resulted in 23.7% and 72.1% greater mosquito attraction than *S. epidermidis* and *M. ulcerans* alone. *M. ulcerans* with *S. epidermidis* 10^3 cfu/ml (35.9%) was significantly more attractive than the control (21.5%), or *S. epidermidis* at 10^5 (24.1%) and 10^7 (18.5%) cfu/ml. This study is the first to demonstrate the synergistic effects on host-seeking preference of *Ae. aegypti* by polymicrobial communities comprised of *M. ulcerans* and *S. epidermidis*, which could provide insight as to the mechanisms regulating *M. ulcerans* dispersal as well as serve as a mode of infection.

Introduction

Mechanisms regulating host-seeking behavior by anautogenous female mosquitoes (Diptera: Culicidae) are well documented (Bowen, 1991; Zwiebel & Takken, 2004). In fact, host-seeking behavior is comprised of intricate interactions among multiple cues ranging from visual (Takken, 1991) to thermal (Klun et al., 2013) stimuli; however, olfaction serves as a primary detection system implemented by mosquitoes to locate and select a host (Basáñez et al., 2007; Michael et al., 2001; Scott et al., 2000) and under the right circumstances, transmit pathogens, such as Dengue virus (Bhatt et al., 2013), Yellow fever virus (Jentes et al., 2011), Chikungunya virus (Leparc-Goffart et al., 2014), and Zika virus (Musso & Gubler, 2015). With regards to many of these pathogens, the yellow fever mosquito *Aedes aegypti* (Linnaeus) (Diptera: Culicidae), a highly anthropophilic (i.e., host preference for humans over animals) and endophilic (i.e., strong association with human communities) species (Harrington et al., 2001), is the primary vector. Recent evidence suggests the same could be true for *Ae. aegypti* and *Mycobacterium ulcerans*, the causative agent of Buruli ulcer (BU) (Johnson et al., 2007; Johnson & Lavender, 2009; Wallace et al., 2010).

The human dermis is an ecosystem consisting of multiple niches occupied by a complex microbial community (Grice et al., 2008). Host-associated microbiome composition is partly regulated by any number of internal or external factors, with slight alterations in abiotic or biotic conditions leading to shifts in community structure (well described in the Anna Karenina effect) (Zaneveld et al., 2017). From a biotic perspective, microbes present on the skin are constantly shifting due to immigration, emigration,

establishment, or even extinction (Rosenthal et al., 2011). Abiotic factors such as temperature, pH, the degree of UV exposure and nutrient contents are also important for shaping skin microbial community (Egert & Simmering, 2016; Grice et al., 2008).

An adaptive strategy used by microbes to persist in such stochastic environments (i.e., human dermis) is through the emission of volatile organic compounds (VOCs) produced via metabolic activity (Breitling et al., 2013; Ochi, 1987) as signals or cues. In essence, mosquitoes coming in contact with hosts potentially move microbes from one environment (i.e., host) to another, thus enhancing survivorship. Certain types of viruses are transmittable through mechanical transmission by mosquitoes. For example, *Ae. aegypti* is associated with mechanical transmission of myxoma virus (Fenner et al., 1952). Otake et al. (2002) also demonstrated *Aedes vexsans* (Meigen) (Diptera: Culicidae) could serve as a mechanical vector to carry porcine reproductive and respiratory syndrome virus to naïve pigs under experimental conditions.

Bacterial VOCs are chemically diverse and are as comparably complex as biochemical products (e.g., enzymes, hormones) in eukaryotes (Kai et al., 2009). Approximately 300-400 VOCs profiles are known to emanate from bacteria associated with a human host (Lin et al., 2002), and VOCs compositions can be species-specific in response to environmental circumstances (Wheatley, 2002). In some instances, these VOCs serve as indicators of environmental quality (*sensu lato*, public information; Valone (1989)). For example, female mosquitoes utilize VOCs produced from host-associated bacteria as a means to locate and evaluate human hosts as food substrates (Schulz & Dickschat, 2007; Verhulst et al., 2010; Verhulst et al., 2011).

Numerous VOCs have been identified as attractants or activators (i.e., causing high excitation but lower attraction) for female mosquitoes (e.g., 1-octen-3-ol, lactic acid, and acetone) based on both laboratory and field tests (Hall et al., 1984). The level of attraction resulting from these compounds is related to concentration and their presence in combination with others (Bernier et al., 2003; Steib et al., 2001; Verhulst et al., 2011). Of course, as previously mentioned, VOCs profiles are regulated by bacteria associated with the host and their associated composition. In fact, composition of VOCs produced by microbial communities differ from that of bacteria grown in monocultures (Tyc et al., 2015). For example, approximately 67.7% greater number of volatile compounds was detected in the pairwise combinations of two bacteria (i.e., *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80) than individual species (Tyc et al., 2015). Unfortunately, as related to mosquito attraction as related to microbial VOCs, most studies worked with single bacteria species (Verhulst et al., 2009) in culture, or with synthetic blends of these VOCs (Verhulst et al., 2010).

These experiments raise two important questions; 1) how would the introduction of a pathogenic bacterium on a host skin environment impact mosquito attraction by the associated microbial community, and 2) does mosquito attraction change depending on the status of the host microbial community (concentration or abundance) at the time the pathogen was introduced? To explore these questions under laboratory conditions, an environmental pathogen and skin commensal need would need to be identified as a model. To address these questions, the pathogen, *M. ulcerans*, and the skin commensal, *S. epidermidis* were selected.

Mycobacterium ulcerans is the etiological agent of BU, a skin disease mainly with individuals residing in tropical and subtropical regions (Marsollier et al., 2002; WHO, 2015). *Mycobacterium ulcerans* produces a destructive polyketide derived macrolide toxin, mycolactone, resulting in a host immunosuppressive response (George et al., 1999) and lesion formation (Johnson et al., 2005). Recent evidence indicates BU transmission is potentially due to mosquito blood-feeding (Johnson et al., 2007; Lavender et al., 2011). Furthermore, the mycolactone produced by *M. ulcerans* has been determined to enhance *Ae. aegypti* attraction (Sanders et al., 2016). However, it is not known if *M. ulcerans* impacts mosquito responses to hosts.

Staphylococcus epidermidis on healthy individuals occupies 65-90% of the normal flora on the dermis (Bauman et al., 2017; Nilsson et al., 1998). It is the most frequently isolated species from human epithelia, axillae, head, and nares (Kloos & Musselwhite, 1975). *Staphylococcus epidermidis* can be highly beneficial as it provides the first line of defense against potential pathogens by serving as a physical barrier (e.g., different pH than surrounding environment, (Braks & Takken, 1999), maintenance of nutritional homeostasis, as well as healthy competition through bacteriocin production (Cotter et al., 2013). This species is a well-known producer of a large spectrum of characteristic volatile organic compounds (VOCs) (e.g., short-chain volatile fatty acids) (Schulz & Dickschat, 2007; Verhulst et al., 2010; Verhulst et al., 2011) resulting from specific metabolism or metabolic pathway (Kai et al., 2009). These odorous compounds are believed to contribute to the distinctive olfactory signature of humans for mosquitoes (Smallegange et al., 2005).

With these bacteria as our model, we investigated how interactions by *M. ulcerans* and *S. epidermidis* at different concentrations impact mosquito attraction to a blood-meal. This work could provide greater understanding of the mechanisms regulating interactions between human skin commensals and an environmental pathogen, *M. ulcerans*.

Materials and Methods

Mycobacterium ulcerans wildtype (1615) strain (Marsollier et al., 2007) was grown on Middlebrook (MB) 7H10 agar (Difco Labs, Maryland, USA) supplemented with 10% (v/v) oleic acid, albumin, dextrose, catalase supplement (OADC) at 32℃ for 6 to 8 weeks. Four sterilized 25 mm (dia.) filter disk (Whatman, Maidstone, UK) were placed on top of the agar prior to complete solidification in a 100mm bacteriological plate and 100µl of *M. ulcerans* in PBS at an optical density (OD_{600}) = 1.2 was spread directly onto the plate. After 6-8 weeks, the filter paper was sterilely removed from the plate and used for the experiment.

Staphylococcus epidermidis wildtype (1457) strain (Kies et al., 2003) was grown on mannitol salt agar (MSA; Neogen Corp. 2008) at 37℃ for 48 h then isolated onto a blood agar plates and incubated overnight at 37°C. For use in the mosquito behavior experiment, an inoculum of 10^8 cfu/ml ($6.7 \pm 1.3 \times 10^8$ cfu/ml) in phosphate buffered saline (PBS) was used. For use with *M. ulcerans*, *S. epidermidis* inoculums of 10^8 ($4.4 \pm 1.8 \times 10^8$ cfu/ml), 10^6 ($5.2 \pm 3.4 \times 10^6$ cfu/ml), and 10^4 ($2.3 \pm 0.3 \times 10^4$ cfu/ml) cfu/ml in PBS were used.

Aedes aegypti (Liverpool strain) was maintained in a colony held in an environmental chamber (25.0 ± 0.5°C, 65.0 ± 5.0% RH, and a photoperiod of 12:12 (L:D) h) at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S. Facility) at Texas A&M University, College Station, Texas, USA. Mosquito larvae (~l,000) were reared in enamel pans (25 cm x 35 cm x 5 cm) containing 1.5 L of reverse osmosis (RO). Larvae were fed a diet of fish food, TetraMin™ (Tetra, Virginia, USA) on a standardized mosquito rearing schedule (Gerberg et al. 1994). Pupae were collected daily and placed in 50 ml cup of RO water at a density of 100/cup. Containers were partitioned into groups of three and placed into 30.5 cm x 30.5 cm x 30.5 cm aluminum screened wire mesh cages (BioQuip Products Inc., California, USA) for adult eclosion. Emergent adults were provided *ad libitum* with a 10% sucrose solution placed on absorbent cotton rolled in cotton-muslin gauze cloth and inserted in a 50 ml glass bottle placed inside each adult cage. Blood-feeding of 5-8 d-old mosquitoes was performed using an artificial membrane. Forty-eight hours after blood-feeding, a 2 x 5 cm filter paper placed in a 50 ml black cup containing 30 ml of RO water was provided as an oviposition site in each cage. Females were allowed to deposit eggs in the container for three days. Filter paper containing eggs were removed from the container and placed on a shelf in the incubator room, allowed to air dry, and then stored at room temperature until use.

Experiment Design

Experiments were a modification of methods previously described (Zhang et al., 2015). Briefly, 2 h prior to each trial, 50 mated female mosquitoes (3-5 d old) that had never received a blood meal, were collected using an aspirator (Hausherrs Machine Works Co., New Jersey, USA). Mosquitoes were released into a clear Plexiglas cage (82 x 52 x 45 cm) with an aluminum wire mesh top and allowed to acclimate at 24.0 ± 1.0℃, relative humidity of 65.0 ± 5.0%. Experiments were performed 30 min after sunrise (chamber at 12:12 L:D), which corresponds to the normal biting activity of *Ae*. *aegypti* (Yasuno & Tonn, 1970).

Blood-feeders were individually constructed of a 25-ml sterile tissue culture flask (Corning Inc., New York, USA) tightly wrapped with parafilm secured with cellophane tape. A 1 ml aliquot of defibrinated rabbit blood (HemoStat Laboratories, California, USA) was injected into the space between the culture flask and parafilm. A 5.0 x 5.0 cm autoclaved piece of 100% cotton gauze (Dynarex Co., South Carolina, USA) was placed over the parafilm and a sterilized 25 mm disk or a disk grown with *M. ulcerans* was inserted between cotton gauze layer and the parafilm. Four blood-feeders, connected to a water bath (Thermo Fisher Scientific, Connecticut, USA) and maintained at 37℃, were placed equal distance horizontally and vertically (24 cm) apart in a square pattern, gauze side down on the wire mesh top (Sanders et al., 2016). For each experiment, four trials were performed in succession by rotating each of the four treatments to each of the four different corner locations initially assigned by a random number generator and rotated clockwise across trials to prevent positional bias. All equipment was cleaned with 3%

Lysol then 95% ethanol between trials. During the experiments, mosquito landing activity at each blood-feeder was recorded with a camera (2160p / 30fps, LG, Korea) mounted on the outside of the cage. Total number of mosquitoes responding by landing and touching each blood-feeder (response) was determined for each minute for a 15 min assay period. Each experiment was replicated three times (12 trials total).

Host-seeking preference of *Ae. aegypti* by microbial communities comprised of *M. ulcerans* and/or *S. epidermidis* was determined. We measured aggregation behavior of *Ae. aegypti* to blood-feeders treated with *M. ulcerans* and 100 µl of *S. epidermidis* at either 10^4, 10^6, 10^8 CFU/ml in PBS or 100 µl of PBS as a control. Further controls tested included measuring host-seeking behavior of *Ae. aegypti* to blood-feeders treated with or without *S. epidermidis* and/or *M. ulcerans*. Each feeder was inoculated with 100 ml of 10^8 cfu/ml *S. epidermidis*, PBS on a *M. ulcerans* inoculated filter, 10^8 cfu/ml *S. epidermidis* + *M. ulcerans* inoculated filter, or only PBS as a control.

Statistical Analysis

The odds ratios of choosing a particular treatment applied in a blood-feeder were tested and plotted using in R version 3.4.3, the DescTools package (https://cran.r-project.org/web/packages/DescTools/index.html). All data were tested for normal distribution with JMP® statistical software version 13 (SAS Institute Inc., North Carolina, USA), using the Shapiro–Wilk test. In order to determine whether the mosquito response rate was significantly different among treatments and twelve trials, the data were analyzed using analysis of covariance (ANCOVA). A comparison of mosquito responses

across treatment was conducted with using a generalized linear mixed model (GLMM). The probability (*P*) of response (attraction) by *Ae. aegypti* to the different treatments was examined for significant difference ($p \leq 0.05$).

Results

Pure versus mixed M. ulcerans and S. epidermidis treatments

Mosquito response to *M. ulcerans* with or without *S. epidermidis* differed. Odds ratio analysis indicated mosquito response to the blood-feeder treated with *S. epidermidis* + *M. ulcerans* (1.38) relative to the control was significantly greater than for treatments with *S. epidermidis* (0.64) or *M. ulcerans* (0.29) relative to the control (Figure IV**.**1). Significant interaction among treatment types (*S. epidermidis*, *M. ulcerans*, *S. epidermidis* + *M. ulcerans*, and Control) was detected over time ($F_3 = 30.1284$; $p \leq 0.0001$). No significant trial ($F_1 = 2.1686$; $p \leq 0.1413$) or time effects ($F_1 = 2.6260$; $p \leq 0.1056$) were found.

The mean number of mosquito responses to blood-feeders with different treatments was compared (Figure IV**.**2). Blood-feeder treated with *S. epidermidis* + *M. ulcerans* elicited the greater (37.2% in total number of response) at all time points than *S. epidermidis*, *M. ulcerans*, and Control. Mosquito responses to *S. epidermidis* + *M. ulcerans* and *S. epidermidis* were not significantly different overall across the 15 min period; however, mosquito responses to blood-feeder with *S. epidermidis* + *M. ulcerans* was significantly greater at 4 (29.7%), 5 (26.8%), 10 (24.3%), 11 (29.4%), 12 (31.9%), 13 (5.2%), and 14 (38.4%) min post exposure than *M. ulcerans*. The peak mean number (± SE) of mosquitoes response to *S. epidermidis* + *M. ulcerans* occurred at 11 min (23.1

± 3.3) and for *S. epidermidis* and *M. ulcerans* at 13 min (19.1 ± 2.6) and 7 min (13.7 ± 2.2), respectively (Figure IV.2). The *S. epidermidis* + *M. ulcerans* treatment elicited the lowest number of responses by mosquitoes to the blood-feeders at 1 min (14.9 ± 1.9), and *S. epidermidis* and *M. ulcerans* at 2 min (14.0 ± 1.5) and 15 min (10.0 ± 1.8), respectively. The control blood-feeder, treated with PBS, elicited the lowest mosquito response at all of time points.

Total mosquito responses to the treatments were quantified. The total number of attraction responses (9,819) from the 12 trials (over the 15 min experiment period) to *S. epidermidis*, *M. ulcerans*, *S. epidermidis* + *M. ulcerans*, and Control treatments were 2,951 (30.1%), 2,124 (21.6%), 3,654 (37.2%), and 1,090 (11.1%), respectively (Figure IV.3). A significant difference ($F_3 = 128.0977$; $p \leq 0.0001$) was measured between *S. epidermidis* + *M. ulcerans* and other treatments over the 15 min experiment period. *S. epidermidis* alone accounted for 30.1% of the total responses and the addition of *M. ulcerans* to *S. epidermidis* accounted for increase of 7.2% of the recorded responses; or 23.7% greater responses than *S. epidermidis* alone. Total mosquito responses to *S. epidermidis* + *M. ulcerans* were 72.0% greater than *M. ulcerans*.

M. ulcerans with S. epidermidis at different concentrations

Mosquito response to *M. ulcerans* with varying concentrations of *S. epidermidis* differed. Odds ratio analysis indicated mosquito response to the blood-feeder treated with *M. ulcerans* and *S. epidermidis* 10^3 (2.45) relative to the control was significantly greater than for treatments with *M. ulcerans* with *S. epidermidis* 10^5 (1.20) or *S. epidermidis* 10^7

(1.39) relative to the control (Figure IV.4). Significant interaction among treatment types (*M. ulcerans* with *S. epidermidis* 10^3, 10^5, 10^7 cfu /ml, and Control) was detected over time ($F_3 = 30.1284$; $p \leq 0.0001$). No significant trials by time interaction ($F_1 = 2.5549$; $p \leq 0.1104$), or location ($F_1 = 1.1082$; $p \leq 0.2928$) interactions were found.

The mean number of mosquito responses to blood-feeders with different treatments was compared (Figure IV.5). Blood-feeder treated with *M. ulcerans* with *S. epidermidis* at 10^3 cfu/ml elicited the greatest response (35.9% in total number of responses); however, mosquito responses to *M. ulcerans* with *S. epidermidis* 10^3 cfu/ml and 10^7 cfu/ml were significantly different only at the 10 min time point; *M. ulcerans* with *S. epidermidis* 10^3 cfu/ml was 139.4% greater responses than *M. ulcerans* with *S. epidermidis* 10^7 cfu/ml. The peak mean number (± SE) of mosquitoes response to *M. ulcerans* with *S. epidermidis* 10^7 cfu /ml occurred at 7 min (7.4 ± 1.7) and for with *S. epidermidis* 10^3, 10^5 cfu/ml, and Control at 10 min (13.2 ± 2.7), 9 min (9.1 ± 1.6), and 3 min (8.0 ± 2.1), respectively (Figure IV.5). Control and *M. ulcerans* with *S. epidermidis* 10^7 cfu/ml elicited the lowest number of responses at 1 min (4.8 ± 1.1) and (5.7 ± 1.6), whereas the lowest responses for *S. epidermidis* 10^5 and 10^7 cfu/ml, were recorded at 2 min (8.4 ± 2.8), and 15 min (5.3 ± 1.2), respectively. The *M. ulcerans* with *S. epidermidis* 10^7 cfu/ml treatment elicited a lower mosquito response than other treatments at the most of time points.

Total mosquito responses to the treatments were quantified. The total number of attraction responses (5,591) from the 12 trials (over the 15 min experimental period) to *M. ulcerans* with *S. epidermidis* 10^3, 10^5, 10^7 cfu/ml, and Control blood-feeder were

2,008 (35.9%), 1,345 (24.1%), 1,034 (18.5%), and 1,204 (21.5%) respectively (Figure IV.6). Responses to *M. ulcerans* with *S. epidermidis* 10^3 cfu/ml treated blood-feeders were significantly higher than that to the other three treatments. A significant difference ($F_3 = 22.8847$; $p \leq 0.0001$) was measured between *M. ulcerans* with *S. epidermidis* 10^3 cfu/ml and other treatments. *M. ulcerans* with *S. epidermidis* 10^3 cfu/ml alone counted for 35.9% of the total responsed and for increases of 11.8%, 17.4%, and 14.4% of the recorded responses at *M. ulcerans* with *S. epidermidis* 10^5, 10^7 cfu/ml, and Control.

Discussion

We determined interactions between the environmental microbial pathogen *M. ulcerans* and the human commensal microbe *S. epidermidis*, enhanced mosquito attraction to a blood-meal. In fact, mosquito response was greater for bacterial combinations (23.7% and 72.1%, respectively) rather than the individual bacteria. This research is critical for understanding the epidemiology of *M. ulcerans* as related to infection of hosts and its potential dispersal into the surrounding environment.

This research also demonstrated mixed culture of bacteria can in some instances lead to greater attraction rather than individual microbes. Similar results for mosquito attraction have been recorded for other combinations of bacteria associated with the human body. Verhulst et al. (2010) showed a blend obtained from a bacteria combination (*Bacillus subtilis*, *Brevibacterium epidermidis*, *Corynebacterium minutissimum*, and *Staphylococcus epidermidis*) induced mosquito attractions but no mosquito response in single bacterium (*Pseudomonas aeruginosa*) observed. Ponnusamy

et al. (2010) showed gravid *Ae. aegypti* responses to plant infusions were most affected by bacterial community diversity and abundance. A previous similar study demonstrated polymicrobial community rather than a single species, differentially influenced the oviposition responses by the black soldier fly, *Hermetia illucens* (L.), (Diptera: Stratiomyidae) (Zheng et al., 2013). However, as related to mosquitoes, a direct mechanism (e.g., VOCs) regulating mosquito attraction has not been thoroughly investigated.

The human body is an ecosystem consisting of multiple niches occupied by complex bacterial communities (Grice et al., 2008); so, it is not a surprise that mixtures elicit greater responses than individual bacterial in colony. The interaction between microbes as well as between a human host and associated bacteria play pivotal roles in regulating pathogen transmission (ASF, 2008); such changes through these interactions involve shifts in host metabolisms (Cho & Blaser, 2012) as well as host signaling pathways (Hughes & Sperandio, 2008). As suggested by our work, polymicrobial interactions, especially between *M. ulcerans* and *S. epidermidis*, could serve as a mechanism allowing for detection and determination of suitable hosts by vectors, such as the yellow fever mosquito, *Ae. aegypti*. If true, this model serves as a unique system for deciphering the mechanisms regulating the transmittal of two pathogens within a given ecosystem.

In addition, shifting the *S. epidermidis* concentration in combination with a set concentration of *M. ulcerans* also impacted mosquito response. The lowest concentration *S. epidermidis* (10^3 cfu/ml) resulted in a greater level of enhanced responses (35.9%)

than that to the 10^5 (24.1%) or 10^7 (18.5%) cfu/ml. It should be noted, *S. epidermidis* vary in concentrations at different locations on a human host. For example, *S. epidermidis* is a predominant species in nares and head ranging from 90 to 100% of the staphylococci; however, relatively lower composition from 10 to 45% of those isolated from the legs and arms (Kloos & Musselwhite, 1975), which could be indicative of low concentration on the extremities. This discovery, though novel in the BU epidemiology field, should not come as a surprise considering the number of patients, showing legs and especially arms, were significantly more infected with BU than other body regions (Hospers et al., 2005). In our study, particularly, the following question how *M. ulcerans* connected with certain dermatological disorders (i.e., Buruli ulcer) interacting with different level of exposure of *S. epidermidis* impact mosquito host-seeking behavior might be answered.

The attraction of mosquitoes to hosts could serve as a mechanism for infection as discussed in the introduction, as well as dispersal of *M. ulcerans* to new habitats; something that has not considered previously. Such interactions are known to occur. Lacroix et al. (2005) showed the malaria parasite (*Plasmodium falciparum*) shifted host traits (VOCs), which enhanced vector attraction. It is theoretically possible for a mosquito to be a mechanical vector (Chamberlain & Sudia, 1961) for *M. ulcerans* under favorable circumstances (McIntosh et al., 2014; Raghunathan et al., 2005; Williamson et al., 2008); high concentration of bacteria in peripheral areas of the infected host. Gravid *Ae. aegypti* females prefer oviposition sites (Allan & Kline, 1995; Sant'ana et al., 2014) highly overlapping with habitats, most lentic water (Raghunathan et al., 2005;

Williamson et al., 2008), vegetation (McIntosh et al., 2014), or biofilm (Williamson et al., 2012) commonly colonized by *M. ulcerans* (Wallace et al., 2010).

As previously mentioned, volatiles from microbes serve as attractants for mosquitoes seeking a blood-meal (Verhulst et al., 2010; Verhulst et al., 2011) or an oviposition site (Mokany & Shine, 2003; Orr & Resh, 1992; Ponnusamy et al., 2011). Therefore, increased contact between a vector (e.g., mosquito) and a host (e.g., human), could provide greater dispersal opportunities for *M. ulcerans* to the environment or vice versa. For example, *Francisella tularensis*, a Gram-negative, intracellular, zoonotic bacterium is mechanically dispersed on mosquito mouthparts to small mammals (Abu Kwaik & Akimana, 2011). If true, such shifts in VOCs by microbial interactions could indicate the relationship is much closer than previously thought, as the compounds could serve as a signal rather than a cue (Tomberlin et al., 2017).

While results were consistent across experiments, limitations were identified. The mosquito colony used in this research is genetically limited due to avoiding the introduction of new gene stock (i.e., Liverpool strain, inbreeding depression). Such a bottleneck selects for tendencies to engage in chemosensory genes (Anton et al., 2003; Zwiebel & Takken, 2004) which leads to mosquito host preferences atypical of wild populations (Athrey et al., 2017). Furthermore, the system employed in this study used bacteria cultured on an artificial diet which is quite different than human skin. Furthermore, gene expression by the microbes as well as the host and subsequent influence on VOCs production and associated mosquito responses should be investigated to truly appreciate the interactions between bacteria and host. In order to fully dissect the

mechanisms regulating mosquito response to *M. ulcerans* and *S. epidermidis*, communities would need to be investigated within the context of natural environments.

The ecological ramifications associated with bacterial interactions as related to mosquito attraction are partially known. As microbes have a conserved or integral adaptation of interacting with the host to occupy their ecological niches, a mosquito appears to utilize highly sensitive olfactory systems to detect microbial metabolites for the quick discovery of ephemeral resources (i.e., human host) (Schulz & Dickschat, 2007; Verhulst et al., 2010; Verhulst et al., 2011). This ability could also provide dispersal opportunities as a mechanical or biological vector for microbes. This unique interaction allows both microbes (Fenner et al., 1952; Otake et al., 2002) and mosquitoes (Schulz & Dickschat, 2007; Verhulst et al., 2010; Verhulst et al., 2011) to elicit for maximizing biological activities and ecological fitness in conspecific and heterospecific environments. Mechanisms employed by the pathogen to manipulate its vector behavior and increase its potential transmission is not fully understood but our study showed the *M. ulcerans* interacting with *S. epidermidis* appeared to influence the parameters (e.g., mosquito attraction) that are most critical for transmission and fitness in several vector-borne pathogens (e.g., Yellow Fever, and Dengue).

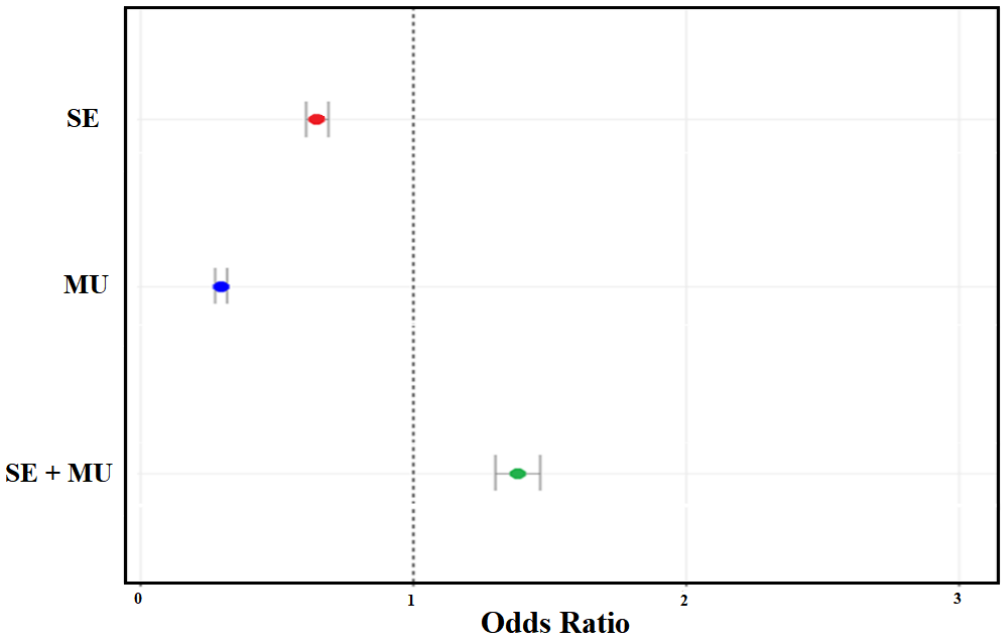


Figure IV.1. The odds ratios of 3-5 d-old female *Ae. aegypti* mosquito responses to blood-feeders treated with SE (*S. epidermidis*), MU (*M. ulcerans*), SE + MU, versus PBS control placed equal distance horizontally and vertically (24 cm) apart on the top of an 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos conducted at 24 ℃ and 65% RH.

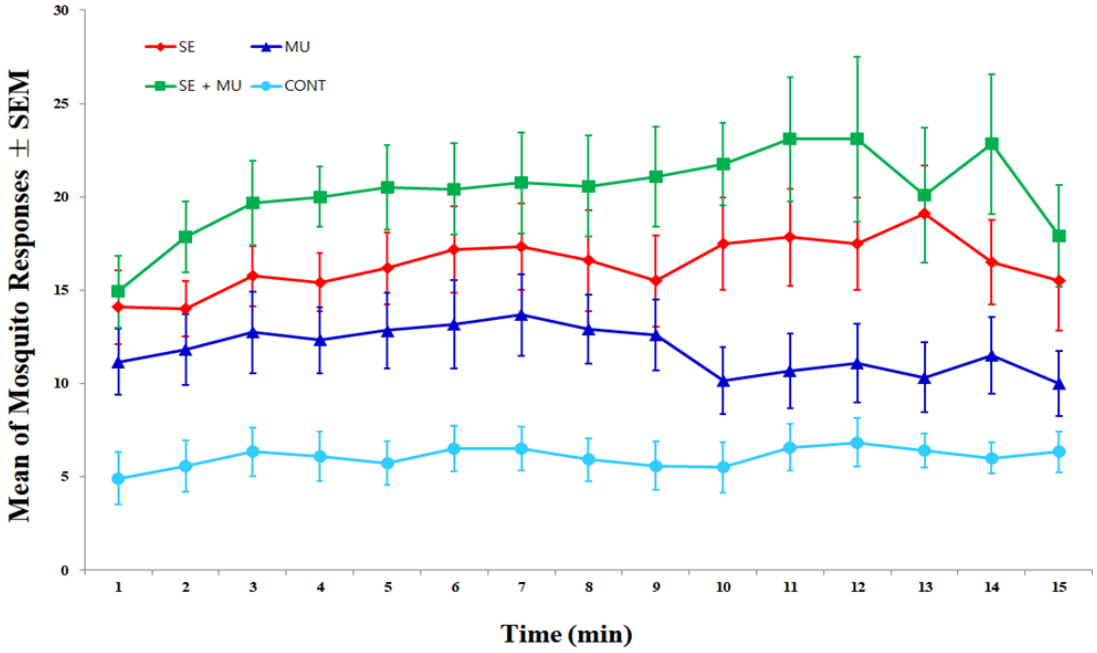


Figure IV.2. Mean number of 3-5 d-old female *Ae. aegypti* mosquito responses per minute ± SEM to blood-feeders treated with SE (*S. epidermidis*), MU (*M. ulcerans*), SE + MU, and PBS (CONT) placed equal distance horizontally and vertically (24 cm) apart on the top of a 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos at 24 ℃ and 65% RH.

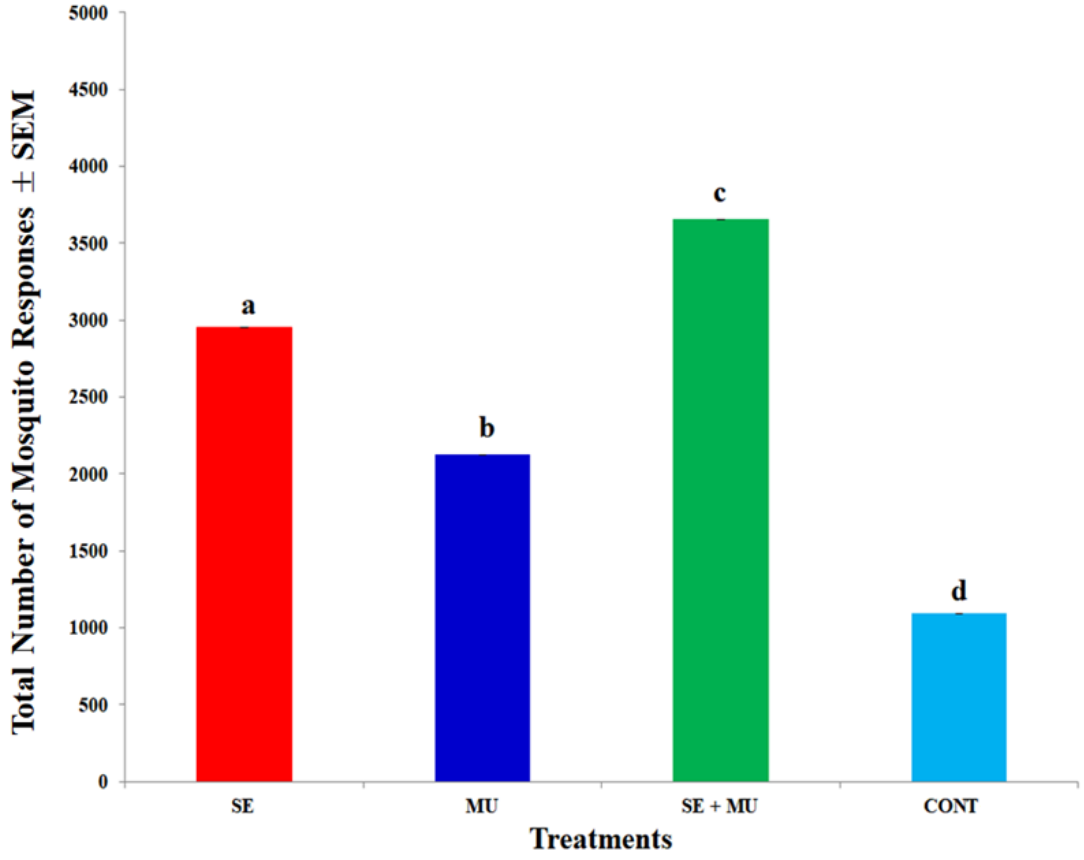


Figure IV.3. Total number of 3-5 d-old female *Ae. aegypti* mosquito responses to blood-feeders treated with SE (*S. epidermidis*), MU (*M. ulcerans*), SE + MU, and PBS (CONT) placed equal distance horizontally and vertically (24 cm) apart on a 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos at 24 ℃ and 65% RH.

a-d: The same letter is not significantly different ($p \leq 0.05$).

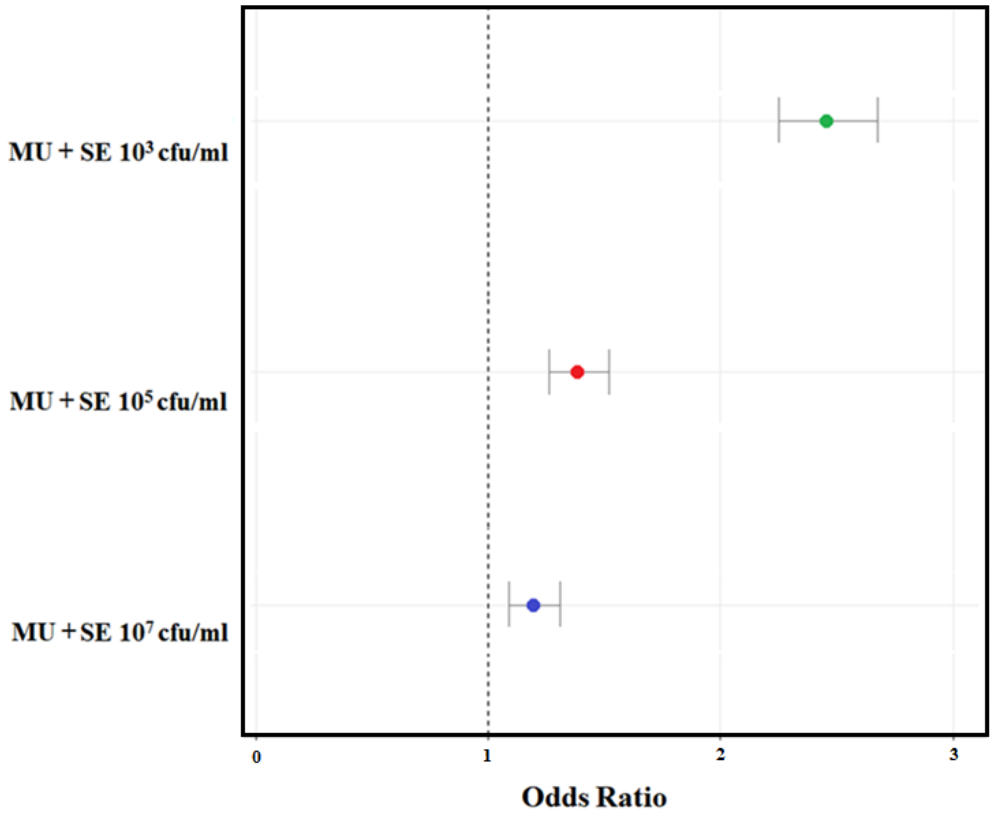


Figure IV.4. The odds ratios of 3-5 d-old female *Ae. aegypti* mosquito responses to blood-feeders treated with MU (*M. ulcerans*) and SE (*S. epidermidis*) at different concentrations (10^3, 10^5, 10^7 cfu/ml), versus PBS control placed equal distance horizontally and vertically (24 cm) apart on the top of an 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos conducted at 24 ℃ and 65% RH.

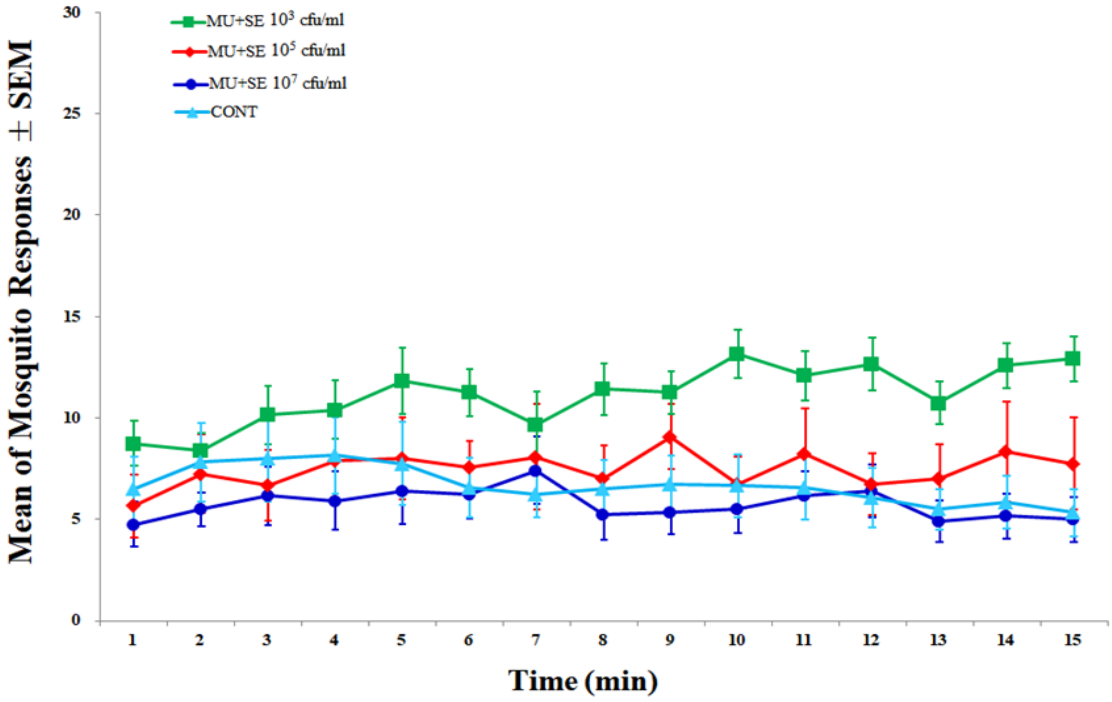


Figure IV.5. Mean number of 3-5 d-old female *Ae. aegypti* mosquito responses per minute ± SEM to blood-feeders treated with MU (*M. ulcerans*) and SE (*S. epidermidis*) at different concentrations (10^3, 10^5, 10^7 cfu/ml), and PBS (CONT) placed equal distance horizontally and vertically (24 cm) apart on the top of an 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos conducted at 24 ℃ and 65% RH.

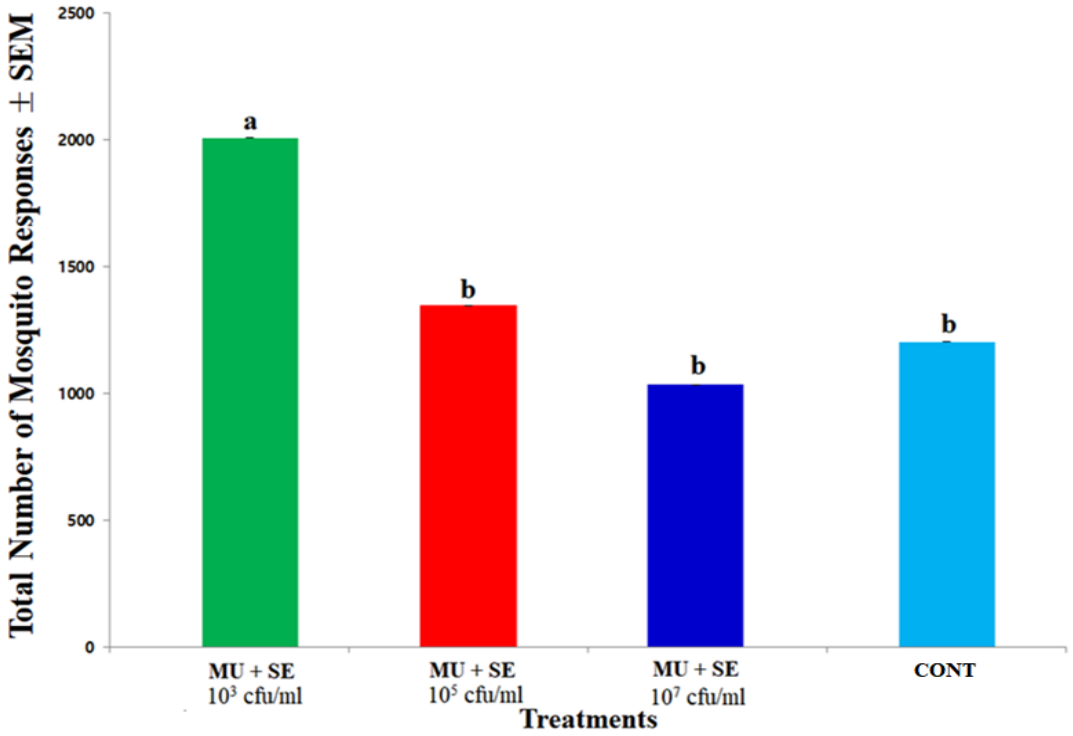


Figure IV.**6.** Total number of 3-5 d-old female *Ae. aegypti* mosquito responses to blood-feeders treated with MU (*M. ulcerans*) and SE (*S. epidermidis*) at different concentrations (10^3, 10^5, 10^7 cfu/ml), and PBS (CONT) placed equal distance horizontally and vertically (24 cm) apart on the top of an 82 cm (L) x 45 cm (W) x 52 cm (H) Plexiglas cage during quadruplicate trials of 15 min with 50 mosquitos conducted at 24 ℃ and 65% RH.

a-b: The same letter is not significantly different ($p \leq 0.05$).

References

Abu Kwaik Y & Akimana C (2011) Francisella–arthropod vector interaction and its role in patho-adaptation to infect mammals. Frontiers in microbiology 2: 34.

Allan SA & Kline DL (1995) Evaluation of organic infusions and synthetic compounds mediating oviposition in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). Journal of chemical ecology 21: 1847-1860.

Anton S, van Loon JJ, Meijerink J, Smid HM, Takken W & Rospars J-P (2003) Central projections of olfactory receptor neurons from single antennal and palpal sensilla in mosquitoes. Arthropod structure & development 32: 319-327.

ASF M (2008) Humans have ten times more bacteria than human cells: How do microbial communities affect human health?

Athrey G, Cosme LV, Popkin-Hall Z, Pathikonda S, Takken W & Slotman MA (2017) Chemosensory gene expression in olfactory organs of the anthropophilic *Anopheles coluzzii* and zoophilic *Anopheles quadriannulatus*. BMC genomics 18: 751.

Basáñez M-G, Razali K, Renz A & Kelly D (2007) Density-dependent host choice by disease vectors: epidemiological implications of the ideal free distribution. Transactions of the Royal Society of Tropical Medicine and Hygiene 101: 256-269.

Bauman RW, Machunis-Masuoka E & Cosby CD (2017) Microbiology with diseases by body system. Pearson.

Bernier UR, Kline DL, Posey KH, Booth MM, Yost RA & Barnard DR (2003) Synergistic attraction of *Aedes aegypti* (L.) to binary blends of L-lactic acid and acetone, dichloromethane, or dimethyl disulfide. Journal of Medical Entomology 40: 653-656.

Bhatt S, Gething PW, Messina JP, Farlow AW, Moyes CL, Myers MF, Brady OJ, Drake JM, Wint GRW, Hay SI, Brownstein JS, Hoen AG, Sankoh O, George DB, Jaenisch T, Simmons CP, Farrar JJ & Scott TW (2013) The global distribution and burden of dengue. Nature. doi:10.1038/nature12060.

Bowen M (1991) The sensory physiology of host-seeking behavior in mosquitoes. Annual review of entomology 36: 139-158.

Braks MA & Takken W (1999) Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae* sensu stricto. Journal of chemical ecology 25: 663-672.

Breitling R, Ceniceros A, Jankevics A & Takano E (2013) Metabolomics for secondary metabolite research. Metabolites 3: 1076-1083.

Chamberlain RW & Sudia WD (1961) Mechanism of transmission of viruses by mosquitoes. Annual review of entomology 6: 371-390.

Cho I & Blaser MJ (2012) The human microbiome: at the interface of health and disease. Nature Reviews Genetics 13: 260.

Cotter PD, Ross RP & Hill C (2013) Bacteriocins—a viable alternative to antibiotics? Nature Reviews Microbiology 11: 95-105.

Egert M & Simmering R (2016) The microbiota of the human skin: Microbiota of the Human Body. Springer, pp. 61-81.

Fenner F, Day M & Woodroofe GM (1952) The mechanism of the transmission of myxomatosis in the European rabbit (Oryctolagus cuniculus) by the mosquito *Aedes aegypti*. Australian Journal of Experimental Biology and Medical Science 30: 139-152.

George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R & Small P (1999) Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283: 854-857.

Grice EA, Kong HH, Renaud G, Young AC, Bouffard GG, Blakesley RW, Wolfsberg TG, Turner ML & Segre JA (2008) A diversity profile of the human skin microbiota. Genome research 18: 1043-1050.

Hall D, Beevor P, Cork A, Nesbitt BF & Vale G (1984) 1-Octen-3-ol. A potent olfactory stimulant and attractant for tsetse isolated from cattle odours. International Journal of Tropical Insect Science 5: 335-339.

Harrington LC, Edman JD & Scott TW (2001) Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood? Journal of Medical Entomology 38: 411-422.

Hospers IC, Wiersma IC, Dijkstra PU, Stienstra Y, Etuaful S, Ampadu EO, van der Graaf WT & van der Werf TS (2005) Distribution of Buruli ulcer lesions over body surface area in a large case series in Ghana: uncovering clues for mode of

transmission. Transactions of the Royal Society of Tropical Medicine and Hygiene 99: 196-201.

Hughes DT & Sperandio V (2008) Inter-kingdom signalling: communication between bacteria and their hosts. Nature Reviews Microbiology 6: 111.

Jentes E, Poumerol G, Gershman M, Hill D, Lemarchand J, Lewis R, Staples J, Tomori O, Wilder-Smith A & Monath T (2011) Informal WHO Working Group on Geographic Risk for Yellow Fever. The revised global yellow fever risk map and recommendations for vaccination, 2010: consensus of the Informal WHO Working Group on Geographic Risk for Yellow Fever. Lancet Infect Dis 11: 622-632.

Johnson PD, Azuolas J, Lavender CJ, Wishart E, Stinear TP, Hayman JA, Brown L, Jenkin GA & Fyfe JA (2007) *Mycobacterium ulcerans* in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. Emerging infectious diseases 13: 1653.

Johnson PD & Lavender CJ (2009) Correlation between Buruli ulcer and vector-borne notifiable diseases, Victoria, Australia.

Johnson PD, Stinear T, Pamela L, Pluschke G, Merritt RW, Portaels F, Huygen K, Hayman JA & Asiedu K (2005) Buruli ulcer (M. ulcerans infection): new insights, new hope for disease control. PLoS medicine 2: e108.

Kai M, Haustein M, Molina F, Petri A, Scholz B & Piechulla B (2009) Bacterial volatiles and their action potential. Applied microbiology and biotechnology 81: 1001-1012.

Kies S, Vuong C, Hille M, Peschel A, Meyer C, Götz F & Otto M (2003) Control of antimicrobial peptide synthesis by the agr quorum sensing system in *Staphylococcus epidermidis*: activity of the lantibiotic epidermin is regulated at the level of precursor peptide processing. Peptides 24: 329-338.

Kloos WE & Musselwhite MS (1975) Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. Applied microbiology 30: 381-395.

Klun JA, Kramer M & Debboun M (2013) Four simple stimuli that induce host-seeking and blood-feeding behaviors in two mosquito species, with a clue to DEET's mode of action. Journal of Vector Ecology 38: 143-153.

Lacroix R, Mukabana WR, Gouagna LC & Koella JC (2005) Malaria infection increases attractiveness of humans to mosquitoes. PLoS biology 3: e298.

Lavender CJ, Fyfe JA, Azuolas J, Brown K, Evans RN, Ray LR & Johnson PD (2011) Risk of Buruli ulcer and detection of *Mycobacterium ulcerans* in mosquitoes in southeastern Australia. PLoS neglected tropical diseases 5: e1305.

Leparc-Goffart I, Nougairede A, Cassadou S, Prat C & De Lamballerie X (2014) Chikungunya in the Americas. The Lancet 383: 514.

Lin H-J, Lin Y-H & Chen K-S (2002) Microbe-mediated method and apparatus for attracting mosquitoes: Google Patents.

Marsollier L, André JPS, Frigui W, Reysset G, Milon G, Carbonnelle B, Aubry J & Cole ST (2007) Early trafficking events of *Mycobacterium ulcerans* within Naucoris cimicoides. Cellular microbiology 9: 347-355.

Marsollier L, Robert R, Aubry J, Saint André J-P, Kouakou H, Legras P, Manceau A-L, Mahaza C & Carbonnelle B (2002) Aquatic insects as a vector for *Mycobacterium ulcerans*. Applied and environmental microbiology 68: 4623-4628.

McIntosh M, Williamson H, Benbow ME, Kimbirauskas R, Quaye C, Boakye D, Small P & Merritt R (2014) Associations between *Mycobacterium ulcerans* and aquatic plant communities of West Africa: implications for Buruli ulcer disease. EcoHealth 11: 184-196.

Michael E, Ramaiah K, Hoti S, Barker G, Paul M, Yuvaraj J, Das P, Grenfell B & Bundy D (2001) Quantifying mosquito biting patterns on humans by DNA fingerprinting of bloodmeals. The American journal of tropical medicine and hygiene 65: 722-728.

Mokany A & Shine R (2003) Oviposition site selection by mosquitoes is affected by cues from conspecific larvae and anuran tadpoles. Austral Ecology 28: 33-37.

Musso D & Gubler DJ (2015) Zika virus: following the path of dengue and chikungunya? The Lancet 386: 243-244.

Nilsson M, Frykberg L, Flock J-I, Pei L, Lindberg M & Guss B (1998) A fibrinogen-binding protein of *Staphylococcus epidermidis*. Infection and immunity 66: 2666-2673.

Ochi K (1987) Metabolic initiation of differentiation and secondary metabolism by Streptomyces griseus: significance of the stringent response (ppGpp) and GTP content in relation to A factor. Journal of bacteriology 169: 3608-3616.

Orr B & Resh V (1992) Influence of *Myriophyllum aquaticum* cover on Anopheles mosquito abundance, oviposition, and larval microhabitat. Oecologia 90: 474-482.

Otake S, Dee SA, Rossow KD, Moon RD & Pijoan C (2002) Mechanical transmission of porcine reproductive and respiratory syndrome virus by mosquitoes, *Aedes vexans* (Meigen). Canadian journal of veterinary research 66: 191.

Ponnusamy L, Böröczky K, Wesson DM, Schal C & Apperson CS (2011) Bacteria stimulate hatching of yellow fever mosquito eggs. PloS one 6: e24409.

Ponnusamy L, Wesson DM, Arellano C, Schal C & Apperson CS (2010) Species composition of bacterial communities influences attraction of mosquitoes to experimental plant infusions. Microbial ecology 59: 158-173.

Raghunathan PL, Whitney EA, Asamoa K, Stienstra Y, Taylor Jr TH, Amofah GK, Ofori-Adjei D, Dobos K, Guarner J & Martin S (2005) Risk factors for Buruli ulcer disease (Mycobacterium ulcerans infection): results from a case-control study in Ghana. Clinical Infectious Diseases 40: 1445-1453.

Rosenthal M, Goldberg D, Aiello A, Larson E & Foxman B (2011) Skin microbiota: microbial community structure and its potential association with health and disease. Infection, Genetics and Evolution 11: 839-848.

Sanders M, Jordan H, Serewis-Pond C, Zheng L, Benbow M, Small P & Tomberlin J (2016) *Mycobacterium ulcerans* toxin, Mycolactone May Enhance Host-Seeking and Oviposition Behavior by *Aedes aegypti* (L.)(Diptera: Culicidae). Environmental Microbiology.

Sant'ana AL, Roque RA & Eiras AE (2014) Characteristics of grass infusions as oviposition attractants to Aedes (Stegomyia)(Diptera: Culicidae). Journal of Medical Entomology 43: 214-220.

Schulz S & Dickschat JS (2007) Bacterial volatiles: the smell of small organisms. Natural product reports 24: 814-842.

Scott TW, Amerasinghe PH, Morrison AC, Lorenz LH, Clark GG, Strickman D, Kittayapong P & Edman JD (2000) Longitudinal studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: blood feeding frequency. Journal of Medical Entomology 37: 89-101.

Smallegange RC, Qiu YT, van Loon JJ & Takken W (2005) Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae* sensu stricto (Diptera: Culicidae). Chemical Senses 30: 145-152.

Steib BM, Geier M & Boeckh J (2001) The effect of lactic acid on odour-related host preference of yellow fever mosquitoes. Chemical Senses 26: 523-528.

Takken W (1991) The role of olfaction in host-Seeking of mosquitoes-A review. Insect Science and Its Application 12: 287-295.

Tomberlin JK, Crippen TL, Wu G, Griffin AS, Wood TK & Kilner RM (2017) Indole: An evolutionarily conserved influencer of behavior across kingdoms. Bioessays 39: 1600203.

Tyc O, Zweers H, de Boer W & Garbeva P (2015) Volatiles in inter-specific bacterial interactions. Frontiers in microbiology 6: 1412.

Valone TJ (1989) Group foraging, public information, and patch estimation. Oikos: 357-363.

Verhulst NO, Andriessen R, Groenhagen U, Kiss GB, Schulz S, Takken W, Van Loon JJ, Schraa G & Smallegange RC (2010) Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria. PloS one 5: e15829.

Verhulst NO, Beijleveld H, Knols BG, Takken W, Schraa G, Bouwmeester HJ & Smallegange RC (2009) Cultured skin microbiota attracts malaria mosquitoes. Malar J 8: 302.

Verhulst NO, Qiu YT, Beijleveld H, Maliepaard C, Knights D, Schulz S, Berg-Lyons D, Lauber CL, Verduijn W & Haasnoot GW (2011) Composition of human skin microbiota affects attractiveness to malaria mosquitoes. PloS one 6: e28991.

Wallace JR, Gordon MC, Hartsell L, Mosi L, Benbow ME, Merritt RW & Small PL (2010) Interaction of *Mycobacterium ulcerans* with mosquito species: implications for transmission and trophic relationships. Applied and environmental microbiology 76: 6215-6222.

Wheatley R (2002) The consequences of volatile organic compound mediated bacterial and fungal interactions. Antonie Van Leeuwenhoek 81: 357-364.

WHO (2015) Buruli Ulcer Report. World Health Organization.

Williamson HR, Benbow ME, Campbell LP, Johnson CR, Sopoh G, Barogui Y, Merritt RW & Small PL (2012) Detection of *Mycobacterium ulcerans* in the

environment predicts prevalence of Buruli ulcer in Benin. PLoS neglected tropical diseases 6: e1506.

Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, McIntosh MD, Quaye C, Ampadu EO, Boakye D & Merritt RW (2008) Distribution of *Mycobacterium ulcerans* in Buruli ulcer endemic and non-endemic aquatic sites in Ghana. PLoS neglected tropical diseases 2: e205.

Yasuno M & Tonn RJ (1970) A study of biting habits of *Aedes aegypti* in Bangkok, Thailand. Bulletin of the World Health Organization 43: 319.

Zaneveld JR, McMinds R & Thurber RV (2017) Stress and stability: applying the Anna Karenina principle to animal microbiomes. Nature microbiology 2: 17121.

Zhang X, Crippen TL, Coates CJ, Wood TK & Tomberlin JK (2015) Effect of Quorum Sensing by *Staphylococcus epidermidis* on the Attraction Response of Female Adult Yellow Fever Mosquitoes, *Aedes aegypti* aegypti (Linnaeus)(Diptera: Culicidae), to a Blood-Feeding Source. PloS one 10: e0143950.

Zheng L, Crippen TL, Holmes L, Singh B, Pimsler ML, Benbow ME, Tarone AM, Dowd S, Yu Z & Vanlaerhoven SL (2013) Bacteria mediate oviposition by the black soldier fly, *Hermetia illucens* (L.),(Diptera: Stratiomyidae). Scientific reports 3: 2563.

Zwiebel L & Takken W (2004) Olfactory regulation of mosquito–host interactions. Insect biochemistry and molecular biology 34: 645-652.

CHAPTER V

CONCLUSIONS

Overview

The Human Microbiome Project explores human microbiota as related in part to associated health (Grice et al., 2008) and behavior (Dinan et al., 2015). And at a finer scale, the human body itself is a complex ecosystem providing trillions of microbes with diverse habitats (i.e., glands, organs) and conditions (i.e., temperature and moisture) in which to exist (Kearney et al., 1984; Marples). However, these interactions should not be limited to what occurs on, or in, the body as these interactions perturbate throughout the surrounding ecosystem (Ezenwa & Williams, 2014; Hyland & Cryan, 2016).

Secondary metabolites produced by these microbes serve as a mechanism regulating community richness, diversity, and function (Ryan & Dow, 2008). In particular, signaling molecules (i.e., autoinducing peptides (AIP)), through mechanisms such as quorum sensing (QS) modulate coordinated activities by these microbes (Miller & Bassler, 2001; Ng & Bassler, 2009; Rutherford & Bassler, 2012), which influences phenotypical and physiological characteristics (e.g., *agr* system in Staphylococci (Vuong et al., 2003) and *fsr* system in Enterococci (Sifri et al., 2002)) contributing to symbiosis (Nealson & Hastings, 1979), virulence (Ji et al., 1995), conjugation (De Kievit & Iglewski, 2000), antibiotic production (Ryan & Dow, 2008), and biofilm formation (Davies et al., 1998).

Interkingdom interactions regulated by QS compounds have been identified for a number of systems (Lee et al., 2015; Tomberlin et al., 2017; Yang et al., 2005; Zhang et al., 2015). These unique interactions serve as a mechanism regulating engagements with eukaryotes (i.e., plants, (in) vertebrates, and arthropods) within an environment. In fact, QS molecules impact higher life behavioral responses, such as host-seeking behavior by insects (Tomberlin et al., 2012; Zhang et al., 2015).

Previous research on this topic has been conducted in the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S.) Facility at Texas A&M University. Initial efforts by previous researchers in the FLIES Facility demonstrated the blow fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) respond to QS by-products produced by microbes associated with decomposing carrion (i.e., source for larval development), and these responses are partially governed by the physiological state (e.g., sex determinant, ovarian status) of the fly (Tomberlin et al., 2012).

This research was expanded to the yellow fever mosquito *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) response to blood-feeders treated with *Staphylococcus epidermidis* (i.e., commensal on human skin). Through this work, it was determined that mosquito response was impacted by the ability of the bacteria to QS (Zhang et al., 2015). Specifically, they demonstrated mosquitoes are more attracted (74.0%) to the wildtype *S. epidermidis* (able to QS) than a mutant *S. epidermidis* (unable to QS) (Zhang et al., 2015). This research was the first to tie together microbial physiology and ecology with mosquito responses to potential blood-meals (Zhang et al., 2015). My research built on these previous results by determining if the QS system could be manipulated chemically

(e.g., quorum sensing inhibitor (QSI), C-30, furanone) to suppress mosquito responses (see chapter II). To explore further questions, mycolactone as a potential QS compound produced by the environmental pathogen, *Mycobacterium ulcerans*, regulating mosquito behavior was determined (see chapter III). Furthermore, the synergistic effect on the host-seeking preference of *Ae. aegypti* by polymicrobial communities comprised of *M. ulcerans* and the skin commensal, *S. epidermidis* as well as at different concentrations were examined in my work (See chapter IV).

Findings

In chapter II (subobjective-1), I determined the impact of C-30 furanone (QSI) on VOC production by *S. epidermidis.* The number of compounds in *S. epidermidis* treated with the QSI was reduced by 35.3%. Seven compounds were unique and only produced by *S. epidermidis*. The compound, 4,7-Methano1Hindene, may contribute to microbial antibiotic resistance (Muller et al., 2015). Heneicosane, another compound identified, plays a role in mosquito oviposition stimulant (Mendki et al., 2000; Navarro-Silva et al., 2009). I also identified two compounds associated with QS. Morphine plays a key role in a variety of functions associated with the virulence factor of *Pseudomonas aeruginosa* (Babrowski et al., 2012). The other compound, tetracosane, partially mediates biofilm formation and is also an attractant for mosquitoes seeking a blood-meal (Pachuwah, 2016) as well as an oviposition site (Torres-Estrada et al., 2007). A final compound identified, heptadecane 9-octyl, is found in the breath of humans (Phillips et al., 1999), and could be a possible mosquito attractant as human breath is a known attractant. The

compounds, pregnane-3 20-dione and lanosta, were identified as indicators of mosquito response; however, their ecological relevance is not known at this time.

Through this study (chapter II), both qualitative and quantitative differences in VOC profiles were determined for the wildtype *S. epidermidis* as well as when its QS system was disrupted with the QS inhibitor, C-30 furanone. Several identified volatiles were shown to be associated with the microbial QS system and possibly regulating mosquito behavior (Host-seeking and oviposition). This discovery may lead to deciphering bacteria communication within and between species basis of important aspects for arthropod physiological responses and behaviors.

In chapter II (subobjective-2), I determined the effect of disruption of QS by *S. epidermidis* on mosquito attraction with the QS inhibitor, C-30 furanone. In terms of mosquito responses, a blood-feeder inoculated with *S. epidermidis* attracted 3X as many mosquitoes as the blood-feeder treated with *S. epidermidis* and QSI. The QSI treatment alone was no different than what was observed for the control blood-feeder. This study provides the evidence interkingdom cross-talk between bacteria (i.e., *S. epidermidis*) and eukaryotic organism (i.e., *Ae. aegypti*) and such interactions can be altered through manipulation of bacterial QS.

For Chapter III, I determined the host-seeking preference of *Ae. aegypti* to *M. ulcerans* wildtype (mycolactone active) as well as a *M. ulcerans* mutant (mycolactone inactive). A blood-feeder treated with *M. ulcerans* wildtype elicited a 126.0% and 171.0% greater attraction than *M. ulcerans* mutant or control, respectively. This work could provide insight of the mechanism in ecological interaction regulating engagements via

mycolactone between an environmental pathogen *M. ulcerans* and mosquito within an ecosystem.

For Chapter IV, I examined the interactions between the environmental pathogen, *M. ulcerans* and the predominant bacteria on human skin, *S. epidermidis,* and resulting impact on mosquito attraction. The following subobjectives were examined; 1) how would the introduction of a pathogenic bacterium on a host skin environment impact mosquito attraction by the associated microbial community, and 2) does mosquito attraction change depending on the status of the host microbial community (concentration or abundance) at the time the pathogen was introduced. The result in Subobjective-1 showed the addition of *M. ulcerans* to *S. epidermidis* resulted in 23.7% and 72.1% greater mosquito attraction than *S. epidermidis* and *M. ulcerans* alone. In Subobjective-2, I determined the concentration of *S. epidermidis* in the presence of *M. ulcerans* also impacted mosquito attraction to blood-feeders. The total number of mosquito response to *M. ulcerans* with *S. epidermidis* 10^3 cfu/ml (35.9%) was significantly greater than that to the 10^5 (24.1%), 10^7 (18.5%) cfu/ml, and control (21.5%). As *S. epidermidis* concentrations can vary across the human dermis landscape (Kloos & Musselwhite, 1975), understanding how *M. ulcerans* interacts with these varying concentrations and the impact on mosquito attraction is critical. Most interestingly, *S. epidermidis* concentrations are typically low on the extremities; as previously pointed out, I determined mosquito attraction to *M. ulcerans* was greatest when it was mixed with *S. epidermidis* as the lowest concentrations. This discovery could link mosquitoes as potential vectors (Wallace et al., 2010) for *M. ulcerans* as *Ae.*

aegypti is most attractive to the extremities of people and *M. ulcerans* infections sites are typically on the extremities as well.

This finding is key for understanding (i) how *M. ulcerans* become established within the human host as the incidence of BU disease (ii) how *M. ulcerans* relate to maintaining ecological stability with other microbes (iii) how polymicrobial interactions play a role for interkingdom communication with mosquito (i.e., potential carrier). Finally, this research could be a fundamental background for an epidemiological phenomenon that broad range of mosquito-borne disease (e.g., Yellow fever and Dengue fever) outbreak occurred in BU endemic area of West Africa.

Limitations and Future Works

While data presented in Chapter II provide definitive evidence the synthetic furanone (i.e., QSI), impacts VOC compositions that regulate the response of mosquitoes to blood-feeders with *S. epidermidis* present, additional research is still needed. Potential directions for future research includes, but is not limited to; 1) direct applications to host skin to suppress microbial combination and resulting mosquito attraction, or 2) potential repellents in spray or emission sites for repelling mosquitoes. A recent study showed there are possible scenarios of QSI resistance following; (1) induced higher autoinducer production, (2) modified autoinducer production, and (3) mutation with higher affinity to autoinducers (García-Contreras et al., 2013). Future studies should examine the selective pressure and induction of resistance on microbial communities resistance mediated by

QSI. With the advent of different biochemical and molecular techniques for resistance-gene frequency estimation may become more feasible.

Furthermore, my research explored these interactions under set conditions. And, it is known olfactory thresholds of mosquito response are significantly influenced by environmental factors such as temperature and humidity (Dethier & Chadwick, 1948). For example, the activity of antennal receptor neurons on *Ae. aegypti* tended to be optimal at 26 to 28 ℃ (Davis & Sokolove, 1975) and humidity has been reported as a factor to regulate mosquito attraction (Price et al., 1979). Future research should examine these environmental factors in greater detail to determine their impact on bacteria-mosquito interactions and reduce variability. This would be especially important in areas where extremely high day-time temperatures prevail (e.g., West Africa).

Evidence presented in Chapter III demonstrates mycolactorne produced by the environmental pathogen *M. ulcerans* plays an important role in the host-seeking preference of *Ae. aegypti* mosquito. While the results from my study are informative, limitations were determined. First and foremost, conducting research with a single bacteria species (i.e., *M. ulcerans*) is a limitation, and I am not sure these results translate more "natural" conditions where microbes exist in complex communities (e.g., human skin) should be done with caution. Furthermore, future research should determine how morphological and physiological difference between *M. ulcerans* wildtype and mutant influence on mosquito host-seeking behavior. As previously mentioned in Chapter III, a pigmentation difference between *M. ulcerans* wildtype and mutant was

observed, which could influence mosquito responses (Muir et al., 1992). Furthermore, mycolactone produced *M. ulcerans* wildtype is UV active (Fidanze et al., 2001); so, mosquito response could be due to visual cues in conjunction with olfactory sensory should be examined in future studies.

While the study presented in Chapter VI provides definitive evidence mosquito attraction depends on the status of the host microbial community (composition from subobjective-1 and concentration from subobjetive-2), additional research is still needed. As stated repeatedly in previous chapters as well as here, this research needs to be replicated with polymicrobial interactions to determine the impact on resulting VOC compositions, which are primary cues used by mosquitoes to locate hosts. Most studies on microbial VOC profiling have been conducted with monocultures of already well-described bacterial genera (Tyc et al., 2015). In fact, it is not well known on how inter-specific interactions affect VOC composition or concentration. Therefore, further work should be conducted to investigate a change in VOC compositions as well as concentrations from polymicrobial interactions. Furthermore, the potential to integrate experiment approaches with gene expression by polymicrobial interaction which subsequently shifts VOC production should be appreciated for understanding to explore fundamental ecological, biological, and epidemiological questions about these unique interkingdom interactions: vector-host-microbe that potentially lead to suppressing pathogen transmissions.

Discussion

Inhibition of bacterial QS is growing and fascinating filed (Geske et al., 2008). Understanding of biosynthesis, reactivity, and degradation of QS signals could be a key to reveal the mechanism of inter/intraspecific or interkingdom communication between microbe on skin and mosquito. This study (Chapter II) efforts focus on inhibition of the QS pathway of certain skin bacteria (i.e., *S. epidermidis*) may reduce a person's attractiveness to mosquitoes. The possible assumption is QSI interference with bacteria communication reduces mosquito attraction potentially by masking the host from the mosquito or by indicating a poor quality resource. This knowledge can lead to the development of a new class of odor-masking or inhibitory compounds as it is thought to be the less selective pressure than pre-existing pesticide or repellent use, which can be exploited in the protection from mosquito bites, aiming at compounds that reduce the production of attractive volatiles on the human skin. It has a broad range of potential applications in agriculture, medicine, pest-management, and etc.

Recently, the biological role of mycolactone produced by *M. ulcerans* with mosquitoes has been partially proposed (Mashlawi, 2017; Sanders et al., 2017). Data collected during the study presented in Chapter III provided the first to evidence mycolactone as a cue regulating interkingdom interactions between viable *M. ulcerans* and *Aedes aegypti*. The results I presented elucidated a key role of mycolactone, considering a potential QS signaling molecule are not only directly recognized by a mosquito, and consequently modulate their behavior. This study has benefited substantially from the introduction and application of ecological relevance in terms of

disease prevalence and pathogen dispersal. This evidence may have shed light on the true origins of small molecules associated with interkingdom interactions between mosquitoes and microbes that have biological and ecological importance within an environment.

To my knowledge, these data (Chapter VI) are the first to demonstrate the biological relevance of polymicrobial interaction how related to mosquito attraction. This approach can generate a better understanding of human skin ecosystem responses by invasive microbial species and result in responses of higher organisms attempting to increase the ecological fitness for survival. It is testable predictions about how specific microbial interactions influence VOC productions and how individual components in response to ecological characteristics that affect mosquito behavior. These predictions are amenable to testing in the lab and applicable in a field setting.

Many studies focused primarily on bacteria as a sinister role by investigating antibiotic resistance genes and their molecular epidemiological aspects (Bayliss et al., 2017; Salyers et al., 2004). However, the bacterial VOCs are belonging to chemically diverse classes (e.g., alkenes, ketones, pyrazines) and highly specialized in multiple tasks to maintain their biological functions (Schulz & Dickschat, 2007). As mentioned above, they play an important role in interkingdom interaction by acting as single or mixture compounds comparable in complexity to that known as a cue, or potentially interkingdom signal for arthropods. In this study, an interdisciplinary approach was used to elucidate the ecological ramifications associated with bacterial interactions as related

to mosquito attraction. We hoped our results help explain the potential for behavior to influence variation in arthropod community structure.

References

Babrowski T, Holbrook C, Moss J, Gottlieb L, Valuckaite V, Zaborin A, Poroyko V, Liu DC, Zaborina O & Alverdy JC (2012) *Pseudomonas aeruginosa* virulence expression is directly activated by morphine and is capable of causing lethal gut derived sepsis in mice during chronic morphine administration. Annals of surgery 255: 386.

Bayliss SC, Verner-Jeffreys DW, Bartie KL, Aanensen DM, Sheppard SK, Adams A & Feil EJ (2017) The promise of whole genome pathogen sequencing for the molecular epidemiology of emerging aquaculture pathogens. Frontiers in microbiology 8.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton Jt & Greenberg E (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280: 295-298.

Davis EE & Sokolove PG (1975) Temperature responses of antennal receptors of the mosquito, *Aedes aegypti*. Journal of comparative physiology 96: 223-236.

De Kievit TR & Iglewski BH (2000) Bacterial quorum sensing in pathogenic relationships. Infection and immunity 68: 4839-4849.

Dethier V & Chadwick L (1948) Chemoreception in insects. Physiological reviews 28: 220-254.

Dinan TG, Stilling RM, Stanton C & Cryan JF (2015) Collective unconscious: how gut microbes shape human behavior. Journal of psychiatric research 63: 1-9.

Ezenwa VO & Williams AE (2014) Microbes and animal olfactory communication: where do we go from here? Bioessays 36: 847-854.

Fidanze S, Song F, Szlosek-Pinaud M, Small P & Kishi Y (2001) Complete structure of the mycolactones. Journal of the American Chemical Society 123: 10117-10118.

García-Contreras R, Maeda T & Wood TK (2013) Resistance to quorum quenching compounds. Applied and environmental microbiology: AEM. 02378-02313.

Geske GD, O'Neill JC & Blackwell HE (2008) Expanding dialogues: from natural autoinducers to non-natural analogues that modulate quorum sensing in Gram-negative bacteria. Chemical Society Reviews 37: 1432-1447.

Grice EA, Kong HH, Renaud G, Young AC, Bouffard GG, Blakesley RW, Wolfsberg TG, Turner ML & Segre JA (2008) A diversity profile of the human skin microbiota. Genome research 18: 1043-1050.

Hyland NP & Cryan JF (2016) Microbe-host interactions: Influence of the gut microbiota on the enteric nervous system. Developmental biology 417: 182-187.

Ji G, Beavis RC & Novick RP (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proceedings of the National Academy of Sciences 92: 12055-12059.

Kearney J, Harnby D, Gowland G & Holland K (1984) The follicular distribution and abundance of resident bacteria on human skin. Microbiology 130: 797-801.

Kloos WE & Musselwhite MS (1975) Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. Applied microbiology 30: 381-395.

Lee J-H, Wood TK & Lee J (2015) Roles of indole as an interspecies and interkingdom signaling molecule. Trends in microbiology 23: 707-718.

Marples M The Ecology of the Human Skin (Charles C Thomas, Bannerstone House, Springfield, Illinois, 1965). A seminal and comprehensive work of classical dermatological microbiology.

Mashlawi AMH (2017) Impact of Mycolactone Produced by *Mycobacterium ulcerans* on Life-History Traits of *Aedes aegypti* aegypti (L.)(Diptera: Culicidae) and Resulting Habitat Selection for Oviposition.

Mendki M, Ganesan K, Shri P, Suryanarayana M, Malhotra R, Rao K & Vaidyanathaswamy R (2000) Heneicosane: an oviposition-attractant pheromone of larval origin in *Aedes aegypti* mosquito. Current Science 78: 1295-1296.

Miller MB & Bassler BL (2001) Quorum sensing in bacteria. Annual Reviews in Microbiology 55: 165-199.

Muir LE, Kay BH & Thorne MJ (1992) *Aedes aegypti* (Diptera: Culicidae) vision: response to stimuli from the optical environment. Journal of Medical Entomology 29: 445-450.

Muller JF, Ghosh S, Ikuma K, Stevens AM & Love NG (2015) Chlorinated phenol-induced physiological antibiotic resistance in *Pseudomonas aeruginosa*. FEMS microbiology letters 362.

Navarro-Silva MA, Marques FA, Duque L & Jonny E (2009) Review of semiochemicals that mediate the oviposition of mosquitoes: a possible sustainable tool for the control and monitoring of Culicidae. Revista Brasileira de Entomologia 53: 1-6.

Nealson K & Hastings JW (1979) Bacterial bioluminescence: its control and ecological significance. Microbiological reviews 43: 496.

Ng W-L & Bassler BL (2009) Bacterial quorum-sensing network architectures. Annual Review of Genetics 43: 197-222.

Pachuwah P (2016) The role of floral and fruit scent compounds as mosquito attractants: developing new methods for monitoring mosquito populations.

Phillips M, Herrera J, Krishnan S, Zain M, Greenberg J & Cataneo RN (1999) Variation in volatile organic compounds in the breath of normal humans. Journal of Chromatography B: Biomedical Sciences and Applications 729: 75-88.

Price GD, Smith N & Carlson DA (1979) The attraction of female mosquitoes (*Anopheles quadrimaculatus* Say) to stored human emanations in conjunction with adjusted levels of relative humidity, temperature, and carbon dioxide. Journal of chemical ecology 5: 383-395.

Rutherford ST & Bassler BL (2012) Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harbor perspectives in medicine 2: a012427.

Ryan RP & Dow JM (2008) Diffusible signals and interspecies communication in bacteria. Microbiology 154: 1845-1858.

Salyers AA, Gupta A & Wang Y (2004) Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in microbiology 12: 412-416.

Sanders M, Jordan H, Serewis-Pond C, Zheng L, Benbow M, Small P & Tomberlin J (2017) *Mycobacterium ulcerans* toxin, mycolactone may enhance host-seeking and oviposition behaviour by *Aedes aegypti* (L.)(Diptera: Culicidae). Environmental Microbiology 19: 1750-1760.

Schulz S & Dickschat JS (2007) Bacterial volatiles: the smell of small organisms. Natural product reports 24: 814-842.

Sifri CD, Mylonakis E, Singh KV, Qin X, Garsin DA, Murray BE, Ausubel FM & Calderwood SB (2002) Virulence effect of *Enterococcus faecalis* protease genes and the quorum-sensing locus fsr in *Caenorhabditis elegans* and mice. Infection and immunity 70: 5647-5650.

Tomberlin JK, Crippen TL, Tarone AM, Singh B, Adams K, Rezenom YH, Benbow ME, Flores M, Longnecker M & Pechal JL (2012) Interkingdom responses of flies to bacteria mediated by fly physiology and bacterial quorum sensing. Animal Behaviour 84: 1449-1456.

Tomberlin JK, Crippen TL, Wu G, Griffin AS, Wood TK & Kilner RM (2017) Indole: An evolutionarily conserved influencer of behavior across kingdoms. Bioessays 39.

Torres-Estrada JL, Meza-Alvarez RA, Cruz-Lopez L, Rodriguez MH & Arredondo-Jimenez JI (2007) Attraction of gravid *Anopheles pseudopunctipennis* females to oviposition substrates by *Spirogyra majuscula* (Zygnematales: Zygnmataceae)

algae under laboratory conditions. Journal of the American Mosquito Control Association 23: 18-23.

Tyc O, Zweers H, de Boer W & Garbeva P (2015) Volatiles in inter-specific bacterial interactions. Frontiers in microbiology 6: 1412.

Vuong C, Gerke C, Somerville GA, Fischer ER & Otto M (2003) Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. Journal of infectious diseases 188: 706-718.

Wallace JR, Gordon MC, Hartsell L, Mosi L, Benbow ME, Merritt RW & Small PL (2010) Interaction of *Mycobacterium ulcerans* with mosquito species: implications for transmission and trophic relationships. Applied and environmental microbiology 76: 6215-6222.

Yang F, Wang L-H, Wang J, Dong Y-H, Hu JY & Zhang L-H (2005) Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. FEBS letters 579: 3713-3717.

Zhang X, Crippen TL, Coates CJ, Wood TK & Tomberlin JK (2015) Effect of Quorum Sensing by *Staphylococcus epidermidis* on the Attraction Response of Female Adult Yellow Fever Mosquitoes, *Aedes aegypti* aegypti (Linnaeus)(Diptera: Culicidae), to a Blood-Feeding Source. PloS one 10: e0143950.