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Olfactory Behavioral Responses of Mosquito Vectors to Select Attractants and Floral Scents as Related to Circadian Rhythms and Photoperiod Regimes

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OLFACTORY BEHAVIORAL RESPONSES OF MOSQUITO VECTORS TO SELECT ATTRACTANTS AND FLORAL SCENTS AS RELATED TO CIRCADIAN RHYTHMS AND PHOTOPERIOD REGIMES

by

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ABSTRACT

OLFACTORY BEHAVIORAL RESPONSES OF MOSQUITO VECTORS TO SELECT ATTRACTANTS AND FLORAL SCENTS AS RELATED TO CIRCADIAN RHYTHMS AND PHOTOPERIOD REGIMES

Bernadette A. Ferraro
Old Dominion University, 2020
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This dissertation discusses mosquito behavioral activities involving circadian rhythms defined as insect sensitivity to select of chemical volatiles that vary throughout the 24-hour day. Circannual rhythms occur over seasons varying in photoperiod, defined as the seasonal cycle of light and darkness. These cycles can be endogenously controlled by circadian clocks. The impetus for this research was initiated when reading about the neglected temporal dimension in the context of insect chemical ecology, including insect olfaction. It was proposed that sensitivity to odors does not change in general, but specific sensitivities could vary according to time of day. Chemical scents emitted by host or conspecifics could change according to time of day or season. The serious omission of not including the temporal dimension in the correct timing of assays can lead to false or misleading results upon interpretation of data.

The two mosquito species that I studied were male and female *Culex restuans* and *Aedes albopictus*. These species were selected because they differ in both circadian rhythms and photoperiod. *Aedes albopictus* is diurnal and is active primarily in the summer. *Culex restuans* is crepuscular/nocturnal and is active primarily in the spring and fall. The inclusion of male mosquitoes has been considered an important first step in the study of male mosquito behavioral ecology, as there is a gender imbalance when researching and studying mosquitoes.

The measuring instruments utilized to conduct my entomology experiments were a flexible dual port olfactometer, and laboratory constructed photoperiodic light regimes. The four chemicals used to test olfactory mosquito responses were acetone, carbon dioxide, lactic acid, and octanol. The floral scents tested for *Culex restuans* included Clethra, Fennel, and Oregano Plants, and Mountain Mint. *Aedes albopictus* scents tested were Rose absolot, Lavendar Oil, Sweet Orange, and Cilantro and Mountain Mint (flowers and leaves).

The results of these entomology experiments revealed the following information: Although there were differences between species and between female and male mosquito response to select odorants, several female and male mosquitoes of both species did respond positively to specific host odors and floral scents. Neither males nor females of *Aedes albopictus* demonstrated significant preferences for either the host odors or the floral scents tested. However, *Culex restuans* females significantly preferred the host odors of carbon dioxide and lactic acid treatments to control filtered air. In contrast, *Culex restuans* males did not show a positive response to either chemical. Future research into the chemical ecology of male and female mosquitoes will be helpful in investigating these differences.

This dissertation is dedicated to my late parents, Dominic and Josephine Ferraro, who spent their lifetime in devotion to me. They were all that is good in me. I always considered them to be “the wind beneath my wings,” enabling me to aspire and to fly to the highest of heights. They will always be remembered and loved with deepest affection.

Your Loving Daughter,

Bernadette A. Ferraro

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Respectfully,

Bernadette A. Ferraro

NOMENCLATURE

AFR	Air filtration rate
FA	Filtered air
LUX	Light level measured in lux
NP	North Port of the olfactometer
RH	Relative humidity
SP	South Port of the olfactometer

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Background Information and Significance

Mosquitoes have been placed in the kingdom Animalia, phylum Arthropoda, class Insecta, order Diptera, and family Culicidae (Edwards 1932, Eldridge and Erdman 2004, Mattingly 1971). Insects have been present on earth since the Devonian Period approximately 400 million years ago. However, flies and particularly mosquitoes in the Order Diptera, appeared some 200 million years ago during the Triassic Period. Despite their small size and delicate body structures, mosquito remains have been found in preserved fossil specimens. Fossilized remains similar morphologically to the species *Culex* evolved during the Eocene Epoch of the Quarternary Period approximately 40 to 60 million years ago (Eldridge and Erdma 2004). The first *Aedes* fossils were found by Edwards in 1932. These *Aedes* fossils were determined to be 30 million years old and dated back to the Middle Oligocene Epoch. Most recent data have catalogued 3,200 mosquito species worldwide of which approximately 150 to 200 species have been documented in the United States (Becker et al. 2003). In addition, 50 mosquito species, have been reported in Virginia by the Virginia Mosquito Control Association in 2003.

Excluding Antartica, mosquitoes can be found worldwide in all tropical and temperate zones. Very few mosquitoes serve as vectors for the transmission of pathogenic diseases such as malaria, dengue, yellow fever, West Nile Virus (WNV), and the repertoire of encephalitis viruses (Becker et al. 2003, Eldridge and Erdman 2004). Of species that are pathogenic hematophagous insects, it is the female mosquito that requires the blood proteins necessary to trigger ovarian activity, synthesize yolk, and develop eggs (Klowden 1995). However, the ability of female

mosquitoes to acquire a rich source of blood protein for reproductive purposes requires several morphological adaptations. Waage (1979) suggested that these morphological adaptations could have evolved from two possible routes. First, insects that preyed upon other insects, as well as plant feeding insects, had mouthparts and digestive tracts anatomically and functionally suited for puncturing and consuming tissues. These adaptations allowed them to shift to blood meals from a vertebrate host. Second, blood feeding without any anatomical and physiological pre-adaptations could have gradually evolved, initially feeding on organic matter and sloughed host skin, finally consuming live skin and blood. Klowden (1995) stated that the hematophagy in mosquitoes most likely developed via the first route.

Hematophagous insects in general, especially mosquitoes, have influenced the course of evolution, and the transmission of pathogenic diseases via arthropod borne vectors (Curtis 1996). A prominent example of vector borne disease is the mosquito species *Anopheles gambiae*, which transmits malaria via the parasite *Plasmodium falciparum*. The global malaria statistics are staggering. According to the World Health Organization (WHO) estimates, 500 million people worldwide are infected at any given time with the malarial parasite (Gibson 1996). About 120 million new cases of malaria reportedly occur each year, with children among its most vulnerable victims. Malaria causes about 1 million deaths per year among children in Africa. However, due to the ease of international travel, an increasingly globalized economy along with population and climatic changes, malaria and other vector borne pathogens associated with mosquitoes are no longer endemically confined to the tropical areas of the world. Transportation of these vectors to new environments, with favorable habitats for reproduction facilitate explosive numerical growth, compounded by encroaching pesticide resistance, places every

human being inhabiting the earth at increased risk for contracting arthropod borne diseases (Crans 1994, Eldridge and Erdman 2004, Lehane 1996, Gibson 1996).

Stages of Development in Mosquitoes

During their development, mosquitoes undergo four distinct and separate life stages. These four stages include egg (rafts in *Culex restuans* or single egg depositions in *Aedes albopictus*), larva, pupa, and mature adult stage (McIver 1980, Clements 1999, Crans 2004).

Egg Rafts

Females from different mosquito species can lay their eggs in a variety of aqueous habitats. In the species *Culex,* female mosquitoes can lay their egg rafts nocturnally on the surfaces of fresh or stagnant waters. Characteristically, these mosquitoes lay their eggs one at a time, sticking them together to form a raft. Among *Culex*, the number of eggs comprising a raft are species specific with *Culex pipiens* containing 100 or more eggs per raft (Gladney and Turner 1969). The dimensions of the *Culex* raft are 6.35 mm in length, and 4.75 mm in width (Carpenter and LaCasse 1955, Gladney and Turner 1969, Crans 2004).

Individual Egg Depositions

In general, *Aedes* mosquitoes lay their eggs individually on moist substrates surrounding tree holes or near salt marshes that are flooded by water (O'Meara 1992). A good example of this is *Aedes albopictus,* also known as the Asian tiger mosquito. The dimensions of *Aedes albopictus* ranges from 2 to 10 mm in length depending upon the food supply in a stagnant pool of water, and the numbers of larvae present in an aqueous environment.

Larval Stage

All mosquitoes require water to complete their life cycle. The optimal conditions for egg hatchings encompass an aqueous environment along with a rich supply of oxygen (Gladney and Turner 1969, Crans 2004). The larval stage is characterized by much activity, with young larvae commonly known as “wigglers”. Larvae attach to the water’s surface to obtain oxygen via a specialized tube known as a siphon. Larvae have voracious appetites, and feed on algae, microorganisms, organic matter, and in the case of *Toxorhynchitis rutilus*, other larvae. Larvae molt four times, with instars being designated as stages between molts. When larvae are at their fourth instar, they are approximately 6.35 mm in length, and enter the pupal stage (Crans 2004).

Pupal Stage

Morphologically, the pupa is shaped like a comma, whereby the head, or cephalothorax contains the mouthparts, legs and wings of the adult mosquito form. The tail of the comma encompasses the future abdomen of the mosquito, along with its sclerotized segments.

Pupae are buoyant and are known as tumblers. Like larvae, they must live in water to survive. When they float to the water’s surface, they draw oxygen through two tubes known as trumpets. However, in contrast to larvae, they do not eat. When metamorphosis is completed, the pupal case splits. Upon emergence from the pupal case, the young adult mosquito rests upon the water’s surface, to dry in preparation for flight. The completion of this process may take minutes, hours, or approximately two days (Crans and McNelly 1997, Eldridge and Erdman 2004).

The Adult Stage

The adult, or fourth stage of the mosquito life cycle is demarcated by well-defined

anatomical regions comprising the head, thorax, and abdomen.

Mosquito Olfaction

For mosquitoes, olfaction is the overriding sensory modality. In females, olfaction is used for host location in anticipation of a blood meal, and oviposition site selections for egg depositions in an optimal aqueous environment that could ensure survival into adulthood (Becker et al. 2003, Why and Walton 2020). In both male and female mosquitoes, olfaction is also used to identify nectar sources provided by flowers and fruits (Muller et al. 2011). The sugars supplied by these nectar sources enable mosquitoes to fly short distances (Grimsted and DeFoliart 1974, Foster and Hancock 1994). In addition, odors emanating from pools of stagnant waste, provide mosquitoes with a source of water to prevent dessication and death via dehydration. The primary source of chemical information for mosquitoes is through their antennae.

Male Mosquito Antennae

There are whorls of stout fibrillae in the basal 12 segments of the antennae. The fibrillae are longer at the base, shorter at the top, and are defined as plumose (Steinbrecht 1996, Clements 1999).

Adult mosquitoes are defined as sexually dimorphic in that male and female antennae of the same species demonstrate strikingly different morphologies. The antennae of male mosquitoes are defined as plumose and possess numerous thick bristles. McIver (1980) stated that the antennae of both sexes collectively contain 13 flagellar segments that are set in a globular pedicel and are attached to the head by a ring- shaped scape. In male mosquitoes, the basal 12 segments are characterized by whorls of stout fibrillae that decrease in length distally

along the shaft of the antennae. Although male mosquitoes have the same structural type of sensillae as female mosquitoes, these sensillae are limited to the terminal two flagellar segments. In addition, male mosquitoes have significantly fewer chemosensory sites on their sensillae for odorant attachment. Rather, male antennae appear to respond to specific numerical vibrations produced by the wing beats of their female conspecifics, thereby initiating behaviors associated with mating (Clements 1999).

Female Mosquito Antennae

The antennae of female mosquitoes are pilose, less dense, with fewer setae than their male counterparts. However, female antennae have more sites for odorant deposition. Approximately 90% of female sensillae are olfactory, and chemosensitive to odorants, while 10% are thermosensitive to changes in relative humidity (RH). The antennae of female mosquitoes' function to identify nectar sources for nutrition, and subsequent flight energy, for detection of their male counterparts, to host location for blood meals to develop their eggs, and to find oviposition sites (pools of stagnant water) for multiple egg depositions to complete their life cycle (Clements 1999).

Mosquito Species Under Study

Culex restuans

This species has yellowish white to golden brown scales throughout the body. *Culex restuans* is a medium sized mosquito measuring 4 to 10 mm in length. Its head is characterized by yellowish-white to golden-brown scales. Dorsally, these scales are dark brown, while laterally, they are yellowish white. The coloration of the thoracic scales ranges from light brown to reddish brown. Dark brown to bronze scales characterize the abdomen, and the legs have

bronze to a blue-green reflected coloration (Carpenter and LaCasse 1955, Slasf and Apperson 1989).

Mosquitoes are considered weak fliers. The wing length of *Culex restuans* is 4.0 to 4.4 mm, and it has a flight distance of 1 to 2 miles. *Culex restuans* is similar to *Culex pipiens* and is commonly associated with it. However, *Culex restuans* can be identified, and differentiated from *Culex pipiens*, by observing the head capsule after the single hairs on the air tube have emerged. The habitats of *Culex restuans* have included ditches, stagnant pool waters, and water placed in artificial containers. This mosquito species has two peaks of activity with the first peak in the spring-early summer, and the second peak in the late summer-early autumn. Although *Culex restuans* has a predilection for primarily avian hosts, it has been known to attack man. Diseases known to be transmitted by *Culex restuans* include avian malaria *(Plasmodium vivax*), filiariasis, West Nile Virus (WNV), Japanese Encephalitis (JE), St. Louis Encephalitis (SLE), and Eastern Equine Encephalitis (EEE). Of the 3,200 mosquito species documented in the scientific literature, 1,216 are of the *Culicine* species. The eggs of *Culex restuans* are oviposited as rafts, with the raft numbers being specific to the species. In terms of its circadian rhythms, *Culex restuans* prefer to fly and feed at night, and are therefore classified as nocturnal (Carpenter and LaCasse 1955, Crans 1994).

Aedes albopictus

There are alternating black and white stripings on the legs of this mosquito, and the distinctive solitary white line that starts between the eyes and ends on the dorsal side of the thorax.

This species is characterized by black and white striped legs, and a small black and white body. An additional characteristic marking includes a solitary white line of tightly bound scales, found initially between the eyes, and proceeds down the dorsal side of the thorax. The length of the *Aedes albopictus* mosquito varies from 2 to 10 mm, depending on the food supply in a stagnant pool of water, and density of the larval population. In the mature female, prospective hosts are identified by a plethora of odorants that include carbon dioxide, ammonia, fatty acids, lactic acid, carbonaceous organic substances emanating from the host, climatic conditions (humidity), and visual recognition. The average blood meal is 2 microliters (µl). *Aedes albopictus* are aggressive and vigorous biters. They attack birds and mammals (including human beings). Their pathogenic and viral transmissions are associated with Yellow Fever, Dengue Fever, Eastern Equine Encephalitis (EEE), St. Louis Encephalitis (SLE), and *Dinofilaris immitis*, the parasite that causes heartworm in cats and dogs. Most recently, *Aedes albopictus* has been the secondary purveyor of the Zika virus, which is believed to be linked to microcephaly in newborn infants (Shragai et al. 2017). Moreover, in adults, the Zika virus has been demonstrated to be sexually transmissible between partners, and to cause Guillame-Barre Syndrome, a demyelinating disease of the nerves (Shragai et al. 2017).

Although these mosquitoes were originally endemic to the tropical and subtropical regions of Southeast Asia, they have since adapted to the more temperate regions of the world (Bonizzoni et al. 2013). In 1985, *Aedes albopictus* were first discovered in a shipment of tires in the port of Houston, Texas. They have since migrated across the southern United States, and up the east coast as far as Maine. Their competition with other mosquito species has been attributed to their reproductive biology of successfully depositing scores of eggs, and their ability to adapt

to various environments. In terms of their circadian rhythms, *Aedes albopictus* flies and feeds in the daytime (diurnal), and sometimes at dusk and dawn (crepuscular).

Mosquitoes rely on several senses in order to find nectar sources, distinguish mates, locate hosts, and seek oviposition sites including vision (Allan et al. 1987), vibration and olfaction. Olfaction predominates as a sensory modality. In females, olfaction is used for host location in anticipation of a blood meal, and oviposition site selections for egg depositions in an optimal aqueous environment that could ensure survival into adulthood (Becker et al. 2003, Why and Walton 2020). In both male and female mosquitoes, olfaction is also used to identify nectar sources provided by flowers and fruits. The sugars supplied by these nectar sources enable mosquitoes to fly short distances (Grimsted and DeFoliart 1974, Foster and Hancock 1994). In addition, odors emanating from pools of stagnant waste, provide mosquitoes with a source of water to prevent dessication and death via dehydration.

Behavioral Studies Involving Mosquitoes and Other Arthropods

The idea for my prospective research project on mosquitoes evolved as a result of reading two scientific papers. The first paper written by Lazzari et al. (2004) was entitled: The Chemical Ecology of Insect Vectors – The Neglected Temporal Dimension. The second scientific document was from Chapter 30 in an extensive two volume work on mosquitoes written by Clements (1999) entitled Adult Circadian Rhythms. With this in mind, I would like now to present some salient points raised by these articles, and to discuss how they served to establish the significance of my research project.

It has been well established that many aspects of insect physiology and behavior vary with time and are synchronized with environmental cycles. In discussing the neglected temporal

dimension in the context of insect chemical ecology, and the overriding sensory modality of insect olfaction Lazzari et al. (2004) state that the interpretation of experimental results could be compromised, or even misleading unless experiments were repeated at different times of the circadian cycle. Hence, the insects' sensitivity to unitary, binary, or synergistic blends of chemical volatiles can vary throughout the 24-hour diel and be endogenously controlled by circadian clocks.

Therefore, with regard to olfaction, and insect behavioral responses to olfactory cues permeating the environment, Lazzari et al. (2004) proposed two hypotheses. Firsts ensitivity to odors does not change in general, but *specific* sensitivities could vary according to the temporal allocation by which a particular arthropod behavior is associated. Second, chemical scents emitted by hosts or conspecifics, could change according to the time of day (diurnal, crepuscular, and nocturnal).

The serious omission of not including the temporal dimension in scientific experimentation, along with its implications in the correct timing of its assays for subsequent interpretation of data, was illustrated by a survey undertaken by Lazzari et al. (2004) regarding 70 recent articles on the chemical ecology of insect vectors. The results of this survey revealed that 60% of the articles made no mention of the time of day of the experiments, 13% reported only a broad reference to the day or night according to the diurnal or nocturnal habits of the animals studied, and 2% indicated times outside the activity period of the surveyed arthropods. Only 24% reported explicit times within the temporal window of activity of the surveyed insects. However, the insects cited in this temporal dimension survey included: tsetse flies (Van Der Goes Van Naters 1998) the fruit fly *Drosophila melanogaster* (Krishnan 1999, Tanoue 2004), the blood sucking bug *Triatoma infestans* (Barrozzo and Lazzari 2004, Barrozzo et al. 2004), and

the cockroach *Leucophaea maderae* (Page and Koelling 2003), but not mosquitoes. Because mosquito species differ so greatly in their circadian and circannual patterns, it is especially important to consider how their natural activities patterns will influence bioassay results.

Photoperiods and Circadian Rhythms

In an effort to apply salient points raised by Lazzari's recent survey of mosquitoes, I found that in the publications I have read on the subject of olfactometric results of mosquito attractants, repellents, or floral scents there was no mention of the 24-hour diel cycle that would classify a particular mosquito species slated for study as diurnal, crepuscular, or nocturnal. While attention was given to precise temperature, relative humidity (RH), and air stream flow in olfactometers, no mention was made about lux intensities utilized in their experiments, or the time of day with reference to particular mosquito activity (Bernier et al. 2003, Cork 1996, Dekker et al. 2001, Shirai et al. 2001). For example, Bernier et al. (2003) in publishing their results on the synergistic attraction of *Aedes aegypti* females to binary blends of L- lactic acid and acetone, and dichloromethane, or diethylsulfide made no mention of the time of day of the experiment or photoperiod regime. In contrasting studies, Shirai et al. (2001) reported that L-lactic acid, a known mosquito attractant, also exhibited properties of relative and absolute repellency when *Aedes albopictus* females were exposed to samples of human arm and mouse skin. In this study, as in other studies cited, the experimental time of day, and timing of assays were not reported.

Therefore, studies that include only temperature, an important entraining agent, but not light, also an important entraining agent, present only half the research picture. Interpretation of the data derived from laboratory experiments on circadian rhythmicity will be focused on

mosquito behavior, and olfactory responses to known attractants and floral scents. Although mosquito eyesight has poor resolution, it is sensitive to light conditions whether natural in the field, or simulated in the laboratory, under different lux intensities. Enhanced light sensitivity might allow mosquitoes to follow the host odor plumes even under low lighting conditions by the process of optomotor pneumotaxis, this activity being defined as movement in response to learned behavior.

Both the scientific literature survey conducted by Lazzari et al. (2004), and my readings on mosquito olfactory behavioral responses (Lutz et al. 2017) suggest a neglected temporal dimension involving studies of circadian rhythmicity in insect vectors in general, and mosquitoes in particular. Therefore, in my research project, the two entraining agents of temperature and light will be included. It is expected that this will yield new scientific information, and a more comprehensive understanding of circadian rhythmicity as it applies to the 24-hour diel, along with concomitant mosquito olfactory responses in the presence of known attractants and floral scents. Of the 3,200 mosquito species that have been documented and classified (Becker et al. 2003), the most comprehensive work on these arthropods has predominantly involved *Culex pipiens*, *Anopheles gambiae*, and *Aedes aegypti*. In my research project, olfactory behavioral responses under simulated light conditions will include studies on the *Culex restuans* and *Aedes albopictus* mosquito species. The information gleaned from these experimental studies could provide the prospect of *new* scientific information on circadian rhythms in response to timed exposure of selected attractants and floral scents. Moreover, in all papers I have read thus far, olfactometric experiments on *Culicine, Anopheline*, and *Aedes* mosquitoes have been focused exclusively on the females of their respective species, *without* reference to their male conspecifics. By rearing mosquitoes from single egg depositions (*Aedes albopictus*) and egg rafts

(*Culex restuans*) to adults under laboratory conditions, I acquired a greater number of male mosquitoes than if caught by CDC traps in the field. Based upon my preliminary readings, there is a clear-cut sexual imbalance when studying mosquitoes (Ferguson et al. 2005). Scientific information on male ecology and olfactory behavioral responses related to circadian rhythms are generally lacking. Therefore, my research project will provide an important first step in studying male mosquito olfactory behavioral responses to various odorants.

How Circadian Rhythms Affect Feeding and Parasite Cycles in Mosquitoes

Circadian rhythms are defined as 24-hour rhythmic cycles that are regulated by an organism's internal molecular clock. Circadian rhythms are further refined by the diel, that regulates the alternate light:dark (L:D) cycle (Clements 199, Rund et al. 2011, Das and Dimopoulos 2008).

In terms of their behavior and physiology, the mosquito's daily rhythmic patterns have encompassed such pre-adult actions as: larval development, pupation, and eclosion (Chiba 1974, Beck 1980). Adult circadian rhythms have included mating swarms at dusk and dawn (crepuscular patterns), nocturnal flight activities, oviposition, and the dual behaviors of feeding patterns which have included sugar imbibing for species survival, flight endurance in both males and females, and mosquito–host interactions, for the acquisition of blood meals as per egg development and maturation in the female alone (Cork 1996, Manda et al. 2007).

In studies on the aspects of plant feedings and preferences involving *Anopheles gambiae*, Manda et al. (2007) conducted follow-up studies to determine if there was a relationship between the mosquito's food preferences, its survival, and fecundity. In this investigation, six species of plants were selected for study and evaluation. The plants species were *Hamelia patens,*

Parthenium hysterophorus, Ricinus communis, Senna didymobotrya, and *Tacoma stans*. There was one less preferred species, *Lantana camara*. The results of these studies demonstrated that the first four plant species were preferred by the *Anopheline* mosquitoes of both genders. Moreover, it was shown that mosquitoes who preferred *Hamelia patens, Parthenium hysterophorus, Ricinus communis*, and *Senna didymobotrya* lived longer and in the case of females, oviposited more eggs. Although knowledge of plant-mosquito interactions is considered fragmented by entomologists, this study can contribute important information.

Five-hundred million people world-wide are infected at any given time with the malarial parasite, *Plasmodium falciparum*. Moreover, internationally 120 million *new* cases of malaria have been documented via WHO epidemiological reports each year. Therefore, given the increasing resistance to long term pesticide usage, and its deleterious effects on the environment, concomitant with its carcinogenic potential in terms of human health, it becomes imperative to research *other* options in the quest to implement effective vector control program strategies, and to reduce the transmissions of such pathogens as malaria (*Anopheles gambiae*) mosquitoes, and yellow fever (*Aedes aegypti*) mosquitoes.

In elegantly designed experiments conducted by Lang et al. (2018), pre-measured amounts of (0.1 mg, 0.3 mg, 0.5 mg, and 1.0 mg) of high quality and medium quality dried fish food were fed daily to *Aedes aegypti* larvae. The objectives of these research studies were to determine if the *quantity* of larval fish food influenced specific *post eclosion* behavioral traits in transgenically-reared male *Aedes aegypti* mosquitoes.

The results of these series of experiments, revealed the following information: First, adult male body size was correlated to be *directly* related to the amount and quality of fish food

ingested during larval pre-eclosion. Second, larger adult male *Aedes aegypti* mosquitoes survived longer, and behaviorally engaged in increased post eclosion swarming activities. Third, although larger males engaged in increased swarming activities, it did not necessarily mean that they were successful at copulating with their female conspecifics. This appeared to be largely due to female *Aedine* copulation preferences. Fourth, male fitness in terms of *competiton* was of importance in these experiments, as they determined if laboratory–reared transgenic mosquitoes would be able to compete with their wild counterparts in the field, and therefore be used for the implementation of effective mosquito and vector control strategies. Fifth, these fitness, swarming, and pre-copulation studies provided opportunities for entomologists to learn more about the behavioral ecology of male mosquitoes.

In studies reflecting the molecular analysis of photic inhibition of blood feeding in *Anopheles gambiae*, Das and Dimopoulos (2008), reported the following: First, *Anopheles gambiae* mosquitoes exhibited an endophilic, nocturnal feeding behavior pattern. Second, the blood-feeding behavior associated with female *Anopheline* mosquitoes was under circadian control. Third, when short light pulses of 2 to 5-minute durations were applied in the dark phase, feeding was momentarily inhibited in a clock *independent* manner. Fourth, when light pulses were applied for longer durations (1 to 2 hours), they created a phase advance of blood feeding in a clock *dependent* manner. Fifth, short and long light pulses created inhibition of blood feeding via the circadian clock. Sixth, these unknown mechanisms may involve the chemosensory system. Seventh, the chemosensory system may include the antennae, head, and select parts of the *Anopheline* body. Eighth, external factors such as light, temperature, relative humidity, food, and mosquito–host interactions influence the circadian clocks. Ninth, in terms of chemosensory genes, there are dichotomous functions: in the female *Anopheles* mosquito, the odorant-binding

proteins focus primarily on the activities of host-seeking and blood feeding. In the male, odorant binding proteins may encompass pheromones, select odorants for mating, and nectar feeding. The odorant binding proteins of the male *Anopheline* mosquito, may not be influenced or regulated by the same entraining agents, or feeding mechanisms as those of the female.

The discovery of rhythm genes for vision and olfaction will provide deeper understanding about the phenomenon of light transduction in the compound eye of *Anopheles gambiae*, as well as more information regarding odorant-binding proteins and olfactory co-receptors. In addition to providing research insights into how this mosquito's sensory modalities regulate olfaction, this information may also provide insights into *Anopheline* host-seeking behavior (Rund et al. 2011).

LD (Light:Dark) Photoperiod Regimes

Most experimental investigations of mosquitoes using acoustic or photoelectric actography methods have involved studies of flight activity periodicities (Taylor and Jones 1969, Clements 1999). Comparison of actograph records of female mosquito populations in different physiological states have provided insights into the effects on flight periodicity.

In my experiments involving the effects of periodicity on the olfactory responses to different photoperiod regimes, the instrument used was a two-port olfactometer, designed to specification by Bernadette A. Ferraro and made by Scientific Glass of Virginia. The olfactometer was used to determine how light and periodicity affected olfactory responses in DD = Constant Darkness, 16L:8D = Summer), 12L:12D = Spring/Fall, and LL = Constant Light. Adult males and adult non-blood fed females of *Culex restuans* and *Aedes albopictus* mosquitoes were adults were subjected to the same photoperiod regimes.

In conclusion, with increasing resistance to insecticides, and their potentially deleterious effects both environmentally and in regard to human health concerns, there is an impetus to research mosquito behaviors in order to interrupt vector transmissions.

Photoperiod Regimes

Photoperiod is defined as the seasonal cycle of light and darkness, which affects the behavioral and physiological functions of all living organisms (Beck 1980). Experiments conducted by Haddow et al. (1961) under controlled laboratory conditions demonstrated that daily activity rhythms were at least partially endogenous, with and related to photoperiod. Mosquitoes and other hematogenous insects feed at certain times of the day (diurnal),) dawn or dusk (crepuscular) and night (nocturnal). It is therefore, these feeding habits that determine the time and place for the transmission of vector-borne diseases such as yellow fever (*Aedes aegypti*), and malaria (*Anopheles gambiae*).

Other aspects of insect photoperiodism include photoperiodic entrainment of mating behavior, locomotion rhythms, oviposition in females, and feeding behavior patterns among male and female mosquitoes (Gillett et al. 1962). In terms of photoperiodic feeding behavior in females, Haddow (1961) reported that although multiple studies were undertaken in the field on this subject, a paucity of work had been conducted under controlled laboratory conditions. Mosquitoes in general required space for normal flight activity, and confinement produced limitations upon experimental accuracy. In contrast, field studies in the environment of the tropical forest produced a wealth of information about feeding behavior patterns in mosquitoes. It was reported by Haddow (1956) and Haddow and Gillett (1958) that different mosquito species fed at different heights in the forest canopy. Moreover, different mosquito species were

observed to be biting at different times with their overall biting patterns forming a bimodal crepuscular pattern. Gillett (1961) reported that *Aedes aegypti* also displayed a crepuscular biting rhythm with the most active biting periods recorded at dawn and prior to sunset at dusk. It was concluded that circadian periodicity and photoperiodic entrainment may play a role in determining insect responsiveness, and the predilection for feeding at certain times of the day may be controlled by underlying physiological functions that control the threshold of the insect's responsiveness.

In a comparative study on the nocturnal behavior of *Aedes aegypti* and *Aedes* a*lbopictus,* Kawada et al. (2005) reported that the nocturnal behavior of non-blood fed females from these species were studied utilizing an automatic recording device equipped with a photoelectric sensor. In addition, CO_2, heating, and contrasting black and white colors were used as attractive cues for mosquitoes. The results included the following: First, *Aedes aegypti* was more sensitive to light than *Aedes albopictus*. Second, the threshold for light intensity that stimulated nocturnal host-seeking behavior was less than (<) 0.1 lux (approximately 0.01-foot candle) in *Aedes aegypti* and greater than (>) 10 lux (approximately 1-foot candle) in *Aedes albopictus***.** Third, complete darkness during daytime de-activates the host-seeking activity. Fourth, findings suggest that visual cues are indispensable for host-seeking behavior.

Description of Research Project

Insects possess an acute sense of smell. In mosquitoes, growing evidence suggests that olfaction is the overriding sensory modality utilized for host location, host recognition, selection of oviposition sites, and aggregation of conspecifics (McIver 1980, Bowen 1999, 1996, Cork 1996, Pickett and Woodcock 1996). In mosquitoes as in other arthropods, olfactory cues that

permeate the environment are initially detected by insects' antennae before being transported to, and processed by, the higher brain centers. As the functional diversity of mosquito olfactory sensilla is greater than the diversity and variation of sensillar morphological types (Steinbrecht 1996), behavioral studies utilizing olfactometry will be implemented.

Recent studies indicate that sensitivity to odors might be influenced by circadian rhythmicity, as some mosquito species restrict their activities to specific times of the day, dawn or dusk, or night (Haddow 1956, Clements 1999, Lazzari et al. 2004). Seasonality and light:dark (LD) cycles might also play a role (Clements 1999). Chemical volatiles were tested in adult male and adult non-blood fed female *Culex restuans* and *Aedes albopictus* mosquitoes to evaluate orientation responses, gender and species differences in sensory attraction to select olfactory odors under various lighting conditions, and light:dark cycles. The mosquito species tested for olfactometric studies were the crepuscular/nocturnal *Culex restuans* and the diurnal *Aedes albopictus*. Mosquito species were tested under different seasonal photoperiod regimes including summer with 16L:8D, spring/fall with 12L:12D, and free running DD and LL for both *Culex restuans* and *Aedes albopictus*. This research project on circadian rhythms and photoperiodic regimes as it is applied to mosquito attractants and floral scents was designed to facilitate greater understanding of the role olfaction plays in host-seeking behavior of mosquito vectors and provide opportunities for chemical manipulation of mosquito behavior.

Broad Objectives

The long term objectives of this research project were to investigate olfactory behavioral responses as influenced by circadian rhythmicity and light:dark cycles in mosquitoes. These

behavioral studies were conducted utilizing olfactometry under laboratory constructed simulated lighting conditions and light:dark cycles.

Rationale

The olfactory system of mosquitoes is important because antennal sensillae play a major role in insect survival, host location, host recognition, selection of oviposition sites, and aggregation of conspecifics. Adult male and non-blood fed female *Culex restuans* and *Aedes albopictus* mosquitoes were tested. They depend primarily on olfactory sensillae in behaviorally responding to a variety of environmental cues (Beaty and Marquardt 1996, Goddard 2003). A series of experiments were repeated at different times of the circadian cycle and under different light:dark conditions to determine if mosquito behavioral responses to selected chemical volatiles were be maintained during the day with response to odorant intensities constant over time, or were observed only during a narrow temporal window. Circadian studies of mosquito olfaction involving temporal component data can contribute to a better understanding of vector responses to select odorants.

Hypotheses

1. Behavioral differences exist among male and non-blood fed female *Culex restuans* and male and non-blood fed female *Aedes albopictus* mosquitoes because these species are active at different times during the 24-hour diel cycle.

2. Mosquitoes that are active during different times of the year differ in their olfactory responses, with most activities being expected under the light regimes of their natural activity patterns. Mosquito activities vary according to species, gender, age, physiological state, mating, feeding, and oviposition when exposed to different laboratory constructed photoperiods/light regimes.

CHAPTER II

METHODS

Mosquitoes

In preparation for studies of circadian rhythms and photoperiod regimes, an insectary was constructed in the Entomology Laboratory on the campus of Old Dominion University in Norfolk, Virginia. Incubators were designated as continuously dark (DD), 12L:12D (spring/fall), 16L:8D (summer), and continuously light (LL). In order to attract female mosquitoes to potential oviposition sites, barrels of odiferous stagnant water were placed at selected locations in Norfolk, Virginia. Barrels of stagnant water samples were then collected and transferred to the ODU campus. Egg rafts of *Culex* species and individual eggs of *Aedes* species were then separated and placed into separate mosquito breeders (Bioquip, Rancho Dominguez, California), containing 200 ml. of deionized water (dH_2O). Larvae were fed dried fish food (Tetramin, Hartz Mountain Company, Secaucus, New Jersey) every other day between the hours of 9: 30 am to 12:30 pm respectively. Pupae were then removed and placed into *new* mosquito breeders until they eclosed into adults. These mosquitoes were then identified according to gender and species. Adult male and non-blood fed female *Culex restuans* and *Aedes albopictus* mosquitoes were given diets consisting of 10% sucrose solution for a period of three to ten days to ensure survival.

Laboratory Equipment and Supplies

Olfactometer

To initiate olfactory behavioral studies of adult male and non blood-fed female *Culex restuans* and *Aedes albopictus* mosquitoes based on circadian rhythms and photoperiod regimes,

a flexible dual–port olfactometer was designed by Bernadette A. Ferraro and built to specification by Scientific Glass of Virginia (Fig. 1). Functionally, the staging area at the entrance served as a site whereby adult male and non-blood fed female *Culex restuans,* and adult male and non-blood fed female *Aedes albopictus* mosquitoes were introduced into the experimental chamber for the purpose of acclimatization. The period of mosquito acclimatization prior to experimentation was 5 to10 minutes. The ports at the end of left (north port) and right (south port) arms were used alternatively as control and treatment ports. Each olfactometric experiment lasted 60 to 120 minutes. At the conclusion of each experiment, the deceased mosquitoes were retrieved. Olfactometric site locations whereby dead mosquitoes were found were so noted and documented. Both North and South ports including the midsection stem and entrance were flushed for 10 minutes with filtered air, thereby creating a new preparatory environment for the implementation of a new experiment with a new batch of mosquitoes. Air was delivered to each port though the university air supply system, and on the average, air filtration rates (AFR) were between 75 – 100 ml/sec. In *Culex restuans*, most olfactometric experiments involving known attractants were conducted utilizing multiple replicates. However, those experiments involving floral scents had smaller numbers of replicates. While odorants involving *Culex restuans* mosquitoes were tested utilizing only unitary chemicals with filtered air as a control, two mosquito behavioral studies involving *Aedes albopictus,* involved choices between acetone and lactic acid, and octenol and lactic acid.

Chemical Odorants

To study and interpret the olfactory behavioral responses of adult male and non-blood fed female *Culex restuans* and *Aedes albopictus*, specific chemicals selected for experimentation were classified as either host odors or floral scents. Of the two mosquito species investigated,

four chemicals were chosen for the host odor behavioral studies that included 10 µl of acetone, carbon dioxide, lactic acid, and octenol. For the floral scents, natural odorants selected for prospective mosquito studies included 10 µl of the clethra plant, fennel plant, mountain mint, and the oregano plant in *Culex restuans*. However, natural floral scents selected for *Aedes albopictus* mosquito studies included 10 µl of rose absolot, lavender oil, sweet orange, cilantro (flowers and leaves), and mountain mint (flowers and leaves). In all experiments, both adult male and non-blood fed female *Culex restuans* mosquitoes and *Aedes albopictus* species were tested separately in the olfactometer to evaluate their behavioral responses as per their odorant preferences, non preferences, or attraction to filtered air.

Environmental Laboratory Settings

To maintain the consistency of the ODU Entomology Laboratory, the following environmental conditions were maintained for both male and female *Culex restuans* and *Aedes albopictus* mosquitoes. For each experiment, laboratory environmental conditions were recorded for Relative Humidity (RH), temperature in °C, Air Filtration Rates (AFR) in ml/sec, and Lux (x 100) light intensity. Photoperiod regimes were maintained in incubators with light settings assigned to the following: Incubator # 1 (DD constant darkness), Incubator #3 (16L:8D summer photoperiod), Incubator #4 (12L:12D fall or spring photoperiod), and Incubator # 5 (LL constant light) for male and female *Culex restuans* mosquitoes. However, for male and female *Aedes albopictus* mosquitoes, adjusted light settings were assigned the following order: Incubator # 1 (DD constant darkness), Incubator #4 (16L:8D summer photoperiod), Incubator # 5 (12L:12D fall or spring photoperiod), and Incubator #6 (LL constant light), respectively.

Culex restuans Experiments

Filtered Air Experiments with *Culex restuans* mosquitoes

In order to evaluate for bias for either the North or South port in the flexible dual port olfactometer, a series of four mosquito experiments utilizing only filtered air as odorants was devised. These experiments were done so that no misleading test results would be incorporated into this study.

Experiment 1 was conducted July 22, 2010. Three males and one female *Culex restuans* mosquitoes were subjected to filtered air from both ports simultaneously. The photoperiod of the test mosquitoes was continous darkness at DD, and the lux was 502 x 100. The movement of filtered air 90 ml/sec. The RH was 51%, and the temperature was recorded at 25.5 °C. The Start Time for Experiment 1 was documented at 9:55 am, and the Finish Time was 10:55 am.

Experiment 2 was conducted July 22, 2010. Experiment 2 consisted of no males and three female *Culex restuans* mosquitoes being tested utilizing only filtered air. The photoperiod tested mosquitoes reared at 16L:8D and the lux was set at 502 x 100. The RH was 51% with the AFR designated at 90 ml/sec. The temperature was 25.5°C. The Start Time was 11:10 am. Experiment 2 was concluded at 11:55 am.

Experiment 3 was conducted July 21, 2010. In Experiment 3, three males and one female of *Culex restuans* were tested utilizing filtered air, under 12L:12D photoperiodic conditions. The lux was 75 x 100, and the RH was 45%. The AFR was 100 ml/sec. The temperature was 24.5° C. Experiment 3 commenced at 10:50 am and concluded at 11:50 am.

Experiment 4 was conducted July 21, 2010. Experiment 4 was the final experiment utilizing filtered air in both ports. In this experiment, four males and one female *Culex restuans* mosquitoes were tested. The photoperiod regime for test mosquitoes was LL. The lux intensity

was 535 x 100, and the RH was 50%. The AFR was 100 ml/sec. The temperature was 24.5°C. The Start Time for Experiment 4 was 12:00pm and its Finish Time was 12:40 pm.

Host Odor Experiments with *Culex restuans* Mosquitoes

Host odors studied included: acetone 99.5% (ACROS), carbon dioxide (CO_2) (Eckerd Antacid), L-(+)-Lactic Acid High Purity (Spectrum), and octanol (1-octan-3-ol) (BioQuip Products). Filtered air was utilized as the control agent in all unitary olfactometric tests involving *Culex restuans* mosquitoes.

Acetone is a ketone with the chemical formula of $(CH_2)_2$ CH). It has chemical qualities that make it miscible with water. In addition, acetone functions well as a solvent. In human beings, acetone can be found in blood and urine (Lehninger 2013). In these research experiments involving *Culex restuans*, acetone was utilized in a unitary capacity.

Experiment 1 with acetone was conducted August 16, 2010. In Experiment 1, five *Culex restuans* males were exposed to 10µl of acetone in the North Port, with filtered air as the control agent in the South Port. Mosquitoes from photoperiod 12L:12D (spring/fall) were tested. The temperature was 21.5 °C. The Relative Humidity (RH) was 46%, while the lux range was 15-17 lux x 100. The Air Filtration Rate (AFR) was 90 ml/sec. The start time for testing observations commenced at 9:45 am.

Experiment 2 was conducted August 16, 2010. In Experiment 2, five *Culex restuans* females were exposed to 10 µl of acetone in the South Port, with filtered air as the control agent in the North Port. Mosquitoes from photoperiod 12L:12D were tested. The temperature was 21.5 °C. The Relative Humidity (RH) was 46%, while the lux range was 15-17 lux x 100. The Air Filtration Rate (AFR) was 90 ml/sec. The start time for testing observations commenced at 11:10am.

Chemically, carbon dioxide (CO_2), is a naturally occurring gas consisting of two oxygen atoms covalently bound to one carbon atom. Among entomologists, carbon dioxide is considered to be a common attractant for insects in general, and for mosquitoes in particular (Kline et al. 1990, Takken and Kline 1989, Takken 1991, Constantini 1996, Cork 1996).

In these series of experiments with carbon dioxide, 14 tests were undertaken utilizing CO_2 as the chemical treatment. Five ml of dH_2O was added to a tablet consisting of aspirin 325 mg, sodium bicarbonate 1916 mg., and citric acid 1,000 mg to create CO_2 vapor. Filtered air located in the opposite port of the olfactometer, served as an agent of control

Experiment 1 was conducted July 29, 2010.In Experiment 1, two tablets placed in 5ml dH_2O were utilized as the source of CO_2. This experiment contained adult *Culex restuans* mosquitoes consisting of seven males and two females from photoperiod DD (constant darkness). The RH was 48%, air filtration rate (AFR) was set at 88 ml/sec., the lux was 65 x 100, and the temperature was 25.5 °C. Start time for experimental observations and documentation began at 11:05 am. This study was concluded at 12 noon.

Experiment 2 was conducted August 3, 2010. In Experiment 2, two tablets along with 5 ml of dH_2O, were used to study the behavioral effects of vaporized CO_2 on nine adult *Culex restuans* mosquitoes (six males and three females) from photoperiod LL (constant light). The RH was 48%, AFR was 90 ml/sec, the lux was 505 x 100, and the temperature was 23.0 ° C. Start time was 10:20 am, and conclusion time was 11: 45 am.

Experiment 3 was conducted August 3, 2010. In Experiment 3, two tablets were used as the CO_2 equivalent, along with 5 ml of dH_2O. The total number of *Culex restuans* mosquitoes consisted of 11 males and 8 females. The photoperiod was 16L:8D (summer setting). The laboratory rnvironment included an RH at 48%, and an AFR at 90 ml./sec. The lux was 454 x

100, and the temperature was 25.5°C. The start time of this experiment was 10:45 am, and its conclusion recorded at 12:15 pm.

Experiment 4 on carbon dioxide was conducted July 30, 2010. In Experiment 4, two tablets and 5 ml (dH_2O) were used. There were nine males and four female *Culex restuans* mosquitoes from photoperiod 16L:8D. The RH was 48%, AFR was 90 ml/sec, lux was 70 x 100, and temperature was 24.0 °C. The start time was 11:21 am, and the finish time was 12:05 pm.

Experiment 5 on carbon dioxide was conducted August 4, 2010. In Experiment 5, four males and eight female *Culex restuans* mosquitoes from photoperiod 16L:8D were tested behavior with two tablets plus 5 ml of dH_2O. The RH was 48%, AFR was 95 ml/sec, the lux was 580 x 100, and the temperature was 24.0 °C. Start time of this experiment was 10:00 am, and it was completed at 12: 55 pm.

Experiment 6 on carbon dioxide was conducted August 5, 2010. In Experiment 6, two tablets plus 5 ml of dH_2O were used to test the behavioral effects of CO_2 on three male and six female *Culex restuans* mosquitoes. The photoperiod was 12L:12D designation. The RH was 48%. The AFR was 98 ml/sec., and the lux was 575 x 100. The temperature was 24.0°C. Start time for the experiment commenced at 11:40 am, and it was concluded at 1:15 pm.

Experiment 7 on carbon dioxide was conducted July 26, 2010. Experiment 7 was conducted using five male and two female *Culex restuans* mosquitoes from photoperiod 12L:12D with the North Port containing filtered air, and the South Port containing CO_2. For 7-1, the RH was 48%. The lux was 440 x 100, and the temperature was 26.0 °C. The start time for 7-1 and 2 of Experiment 7 was 10:18 am and it concluded at were 11:45 am.

Experiment 8 was conducted August 12, 2010. In Experiment 8, two tablets plus 5 ml of dH_2O were tested for the effects of CO_2 in the South Port on three male and eight female *Culex restuans* mosquitoes from photoperiod 12L:12D. The RH was 48%, and the AFR was set at 92 ml/sec. The lux was 65 x 100. The temperature was 24.5 °C. Start time was 10:00 am, and the conclusion of Experiment 8 was 11:00 am.

Experiment 9 was conducted July 28, 2010. In Experiment 9, CO_2 from two tablets and 5 ml dH_2O. was in the North Port. The number of adult *Culex restuans* mosquitoes consisted of five males and four females from photoperiod LL (constant light). The lux was 508 x 100. Air Filtration Rate (AFR) was 91 ml/sec. The temperature was 22.5 ° C. Start time was 10:00 am, and the conclusion was 11:00 am.

Experiment 10 on carbon dioxide was conducted August 2, 2010. In Experiment 10, four adult *Culex restuans* mosquitoes (two males and two females) from photoperiod LL (constant light) were placed in the dual port olfactometer along with two tablets plus 5 ml of dH_2O in the South Port. The lux was 508 x 100. The AFR was 91ml/sec. The temperature was 22.5 °C, and RH was 48%. Start time was 10:20 am, and it concluded at 12:50 pm.

Experiment 11 on carbon dioxide was conducted August 3, 2010. In Experiment 11, 11 adult *Culex restuans* mosquitoes consisting of seven males and four females from photoperiod DD were subjected to two tablets and 5 ml dH_2O in the North Port. The lux was 85 x 100. The RH was 48%, and the AFR was 91 ml/sec. The temperature was 24.0 °C. The start time for Experiment 11 was 10:30 am, and it concluded at 12:30 pm.

Experiment 12 on carbon dioxide was conducted August 6, 2010. Experiment 12 tested two tablets plus 5 ml of dH_2O in the North Port. The lux was 528 x 100. The RH was 48%, and

the AFR was 91 ml/sec. The temperature was 24.0 °C. This experiment involved nine males and seven females *Culex restuans* from photoperiod 16L: 8D. The start time for this experiment was 10:55 am, and its conclusion was 12:30 pm.

Experiment 13 on carbon dioxide was conducted August 9, 2010. Experiment 13 consisted of two tablets plus 5 ml dH_2O to create carbon dioxide in the North Port. The photoperiod was 12L:12D. The lux was 550 x 100. The RH was 48% and the AFR was 95 ml/sec. The temperature was 24.5 °C. This experiment tested 13 adult mosquitoes that included six males and seven females from photoperiod 12L:12D.. The start time for this project was 10:45 am, and the finish time was 12:45 pm.

Experiment 14 on carbon dioxide was conducted August 10, 2010. In Experiment 14, CO_2 in the North Port was tested on nine male and eight female adult *Culex restuans* mosquitoes from photoperiod LL (constant light). The lux was 540 x 100. The RH was 48%, and the AFR was 95 ml/sec. The temperature was 24.5°C. Start time was 10:10 am, and the conclusion was 12:10 pm.

Lactic acid is a carboxylic acid with the chemical formula of $C_3\ H_6\ O_3$. Chemically, lactic acid expresses itself as a chiral, and has two optical isomers which includes L+LA or S – LA. The other chiral is a mirror image of D – (-) LA or R – LA. The concentration of lactic acid in the blood lactate is usually 1 to 2 m/mol/L at rest, and 20 m/mol/L upon exercise. However, it is L-LA that is the major component in material isolated from humans that actively serves as an attractant to the yellow fever mosquito, *Aedes aegypti* (Acree et al.1988, Bernier et al. 2003).

Five experiments involving lactic acid exposure of adult males and non-blood fed female *Culex retuans* mosquitoes were undertaken.

Experiment 1 was conducted August 27, 2010. In Experiment 1, 10 µl of lactic acid was placed in the North Port. The lux was 38 x 100. The RH was 68%, and the AFR was 95 ml/sec. The temperature was 24.0°C. The experiment consisted of nine males and two *Culex restuans* females from photoperiod 12L:12D. The start time was 11:44 am, and the experiment was concluded at 1:30 pm.

Experiment 2 was conducted August 23, 2010. Experiment 2 consisted of 10 µl of lactic acid in the North Port. The lux was 33 x 100. There were 18 adult *Culex restuans* mosquitoes that included 13 males and five females from photoperiod 16L:8D (summer setting). The RH was 72%, and the AFR was 98ml/sec. The temperature was 24.0°C. The start time was 10:55 am, and the experiment was finished at 12:30 pm.

Experiment 3 was conducted August 24, 2010. Experiment 3 consisted of 10 µl of lactic acid in the South Port. Seventeen adult *Culex restuans* mosquitoes consisting of 12 males and five females from photoperiod 12L:12D (spring/fall) were tested. The lux was 47 x 100. The RH was 48%, and the AFR was 89 ml/sec. The temperature was 23.5°C. The start time was 10:42 am, and the finish time was 12:42 pm.

Experiment 4 was conducted August 24, 2010. Experiment 4 consisted of 10 µl of lactic acid in the North Port. Twenty-three adult *Culex restuans* mosquitoes consisting of 20 males and three females kept in constant darkness, photoperiod DD, were tested. The lux was 40 x 100. The RH was 48%, and the AFR was 90 ml/sec. The temperature was 22.5°C. Start time was 10:11 am and the experiment was concluded at 11:50 am.

Experiment 5 on lactic acid was conducted August 26, 2010. Ten µl of lactic acid in the South Port was used in Experiment 5 to study the effects on *Culex restuans* behavior. Three

males and two females from photoperiod LL (constant light) were tested. The lux was 478 x 100. The RH was 78%, and the AFR was 90 ml/sec. The temperature was 23.0 °C. The start time of the experiment was 11:05 am, and it was concluded at 12 noon.

Octanol is a volatile compound that can be isolated from many sources such as fungi, plants, and even the breath of oxen (Dikjstra and Wilken 1976, Hall et al. 1984). However, Takken and Kline (1989) were the first entomologists to report its function as a mosquito attractant. To study the effects of octenol as a mosquito attractant, they constructed a series of field studies depicting a wide variety of ecological habitats (Kline 1994). Chemically, octenol is defined as an 8-carbon monounsaturated alcohol. Characteristically, octenol also has an asymmetric center, along with 2 optical isomers, and a terminal double bond. Octenol has been documented in the scientific literature to be an effective mosquito attractant when acting either in a unitary capacity, or synergistically with (CO_2) or lactic acid. Kline (1994) also noted that attractant reactivity may be species specific for adult mosquitoes (Dijkstra and Wilken 1976, Hall et al. 1984, Kline 1994).

Experiment 1 on octanol was conducted August 19, 2010. In Experiment 1, 10 µl of octanol was placed in the North Port. Five adult *Culex restuans* mosquitoes consisting of one male and four females were tested from photoperiod DD. The lux was 22 x 100. The RH was 48%, and the AFR was 100 ml/sec. The temperature was 23.5 °C. This experiment commenced at 11:00 am and was concluded at 1:00 pm.

Experiment 2 was conducted August 16, 2010. In Experiment 2, 10 µl of octanol placed in the South Port. Thirty adult *Culex restuans* mosquitoes consisting of 12 males, and 18 females from photoperiod 16L:8D (summer setting) were tested. The lux of 36 x 100. The RH was 48%,

and the AFR was 90 ml/sec. The temperature was 24.5 °C. The start time was 11:40 am, and the finish time was 12:47 pm.

Experiment 3 was conducted August 19, 2010. In Experiment 3, 10 µl of octanol was placed in the South Port. The lux was 22 x 100. The RH was 48% and the AFR was 90 ml/sec. The temperature was 23.5 ° C. Five adult *Culex restuans* mosquitoes, consisting of two males and three females from photoperiod LL (constant light) were tested. This experiment commenced at 11:00 am and was concluded at 1:00 pm.

Floral Scent (Clethra Plant, Fennel Plant, Mountain Mint, and Oregano Plant) Experiments with *Culex restuans* Mosquitoes

The efficacy of natural floral scents, and their subsequent behavioral effects on male and female *Culex restuans* mosquitoes were tested. In these series of experiments, four natural floral scents were selected. They were as follows: Clethra Plant (*Clethra alnilfolia*), Fennel Plant (*Faeniculum vulgare*), Mountain Mint (*Pynanthemum virginianum*), and the Oregano Plant (*Plectranthus amboinicus*).

Experiment 1 on clethra was conducted July 12, 2010. This experiment tested the leaves of the clethra plant (*Clethra alnilfolia*) in the North Port. In this experiment, four adult *Culex restuans* males from photoperiod 16L:8D (summer setting) were tested. The RH was 48%, and the AFR was 100 ml/sec. The lux was 87.5 x 100, and the temperature was 24.5 °C. The start time was 11:24 am and the conclusion of this experiment was at 1:00 pm.

Experiment 1 on fennel was conducted July 15, 2010. In Experiment 1, the fennel plant (*Foeniculum vulgare*) was placed in the South Port. There wer five adult male *Culex restuans* mosquitoes from photoperiod 16L:8 D (summer setting). The RH was 51%, and the AFR was 90

ml/sec. The lux was 42 x 100, and the temperature was 23°5 C. Experiment 1 commenced at 10:00 am and was concluded at 11:40 am.

Experiment 2 on fennel was conducted July 19, 2010. In Experiment 2, fennel plant was placed in the North Port. The fennel plant was tested on four adult *Culex restuans* mosquitoes consisting of two males and two females from photoperiod DD (constant darkness). The RH was 51%, and the AFR was 92 ml/sec. The lux was 40 x 100, and the temperature was 24.5° C. The experiment began at 11:15 am and was concluded at 12:05 pm.

Experiment 1 on mountain mint was conducted July 9, 2010. Mountain mint (*Pyenanthemum virginianum*) placed in the South Port was tested on four male adult *Culex restuans* mosquitoes from photoperiod 16L:8D (summer setting). The RH was 57%, the AFR was 97 ml/sec. The lux was 540 x 100. The temperature was 23.5 ° C. The start was 11:00 am, and the finish was 12 noon.

Experiment 1 on oregano was conducted July 7, 2010. Oregano contains the compound carvacrol, which is toxic to mosquitoes. In Experiment 1, the oregano plant (*Plectranthus amboinious*) placed in the South Port was tested on four adult *Culex restuans* mosquitoes consisting of three males and one female from photoperiod 16L: 8D (summer setting). The RH was 59%, the AFR was 93 ml/sec. The lux was 490 x 100. The temperature was 24.0 °C. The start time was 10:00 am, and the finish time was 12:24 pm.

Experiment 2 on oregano was conducted July 14, 2010. In Experiment 2, an oregano plant placed in the North Port was tested. In this experiment, four adult male mosquitoes from photoperiod 16L:8D (summer setting) were tested. The RH was 54%, and the AFR was 95

ml/sec. The lux was 484 x 100. The temperature was 24.5 ° C. The start time was 10:40 am, and the conclusion of this experiment was at 12:10 pm.

Aedes albopictus Experiments

Filtered Air (FA) Experiments with *Aedes albopictus* Mosquitoes

In trials involving filtered air (FA), adult males and non-blood fed adult female *Aedes albopictus* mosquitoes were tested to evaluate any bias for a particular port in the dual port olfactometer. In these trials, FA alone was delivered in both North and South Ports to determine if mosquitoes preferred either port.

Experiment 1 was conducted November 13, 2013. Two adult *Aedes albopictus* mosquitoes consisting of one male and one female from photoperiod 16L:8D (summer setting) were placed in a dual port olfactometer, whose North and South ports were infused with (FA). The relative humidity (RH) was 67%. The Air Filtration Rate (AFR) was 92 ml/sec. The temperature was 21° C. This experiment commenced at 2:13 pm and finished at 2:28 pm.

Experiment 2 on filtered air was conducted November 13, 2013. The trial involved one male and one female *Aedes albopictus* mosquito from photoperiod 16L:8D (Summer). The RH was 66%. The AFR was 92 ml/sec. The temperature was 21° C. The start time was 2:37 pm and the finish time was 2:52 pm.

Experiment 3 on filtered air was conducted November 13, 2013. For Experiment 3, the behavioral responses of one male and one female *Aedes albopictus* from photoperiod LL (constant light) were tested. The RH was 66%. The AFR was 92 ml/sec. The temperature was 21° C. The experiment started at 3:01 pm and finished at 3:16 pm.

Host Odor Experiments with *Aedes albopictus* Mosquitoes

Experiment 1 on acetone was conducted October 28, 2014. In this research investigation, 13 adult *Aedes albopictus* mosquitoes consisting of three males and 10 females from photoperiod 16L:8D (summer) were tested and were placed inside the main entrance of the olfactometer. Deposition of mosquitoes at the main entrance occured at 1:23 pm so that they would become acclimated to their new environment. At 1:31 pm, 10 µl of acetone was placed in the olfactometer's North Port. The lux was 006 x 100, the RH was 39%, and the AFR was 50 ml/sec. The temperature inside the olfactometer was 22 °C. This experiment was finished at 3:05 pm.

Experiment 2 on acetone was conducted October 30, 2014. In Experiment 2, seven *Aedes albopictus* adult mosquitoes from photoperiod 16L:8D (summer) were tested. Four males and three females were deposited inside the entrance of the dual port olfactometer at 1:24 pm. Ten µl of acetone were placed in the olfactometer's South Port at 1:30 pm. The lux was 292 x 100, the RH was 34%, and the AFR was 72 ml/sec. The temperature inside the olfactometer was 23 °C. Experiment 2 was concluded at 1:00 pm on November 1, 2014.

Experiment 3 on acetone was conducted November 4, 2014. In Experiment 3, adult *Aedes albopictus* mosquitoes comprising 16 males and 13 females from photoperiod LL (constant light) were tested with 10 µl of acetone in the olfactometer's North Port. The lux was 91 x 100, the RH was 20%, the AFR was 60 ml/sec, and the temperature was 24 °C. This experiment was initiated at 1:38 pm, with 10 µl acetone being introduced at 1:44 pm into the North Port. The finish time of Experiment 3 was at 3:17 pm.

Experiment 4 on acetone was conducted November 6, 2014. In Experiment 4, 28 *Aedes albopictus* mosquitoes, 16 males and 12 females from photoperiod LL (constant light) were

tested with 10 µl of acetone in the olfactometer's South Port. The lux was 005 x 100. The RH was 43%, the AFR was 59 ml/sec, and the temperature inside the olfactometer tube was 24°C. Experiment 4 began with the process of mosquito acclimatization at 1:30 pm. Ten µl acetone was infused at 1:35 pm, and the finish time was 3:06 pm.

Experiment 5 on acetone was conducted November 11, 2014. Experiment 5 contained nine adult *Aedes albopictus* mosquitoes, one male and eight females, from photoperiod 12L:12D (fall/spring). Ten µl of acetone was infused in the olfactometer's North Port (NP). The lux was 005 X 100, the RH was 44%, the AFR was 60ml/sec, and the temperature was 22 °C. At 1:55 pm, mosquitoes were deposited in the main entrance of the olfactometer, and after a 5-minute interval, acetone was added in the North Port. Experiment 5 was completed at 3:25 pm.

Experiment 6 on acetone was conducted December 4, 2014. In Experiment 6, adult *Aedes albopictus* mosquitoes comprising two males and four females from 12L:12D photoperiod regime were tested. Ten µl of acetone was placed in the olfactometer's South Port (SP). The lux was 008 x 100 and the RH was 16%. The AFR was 57 ml/sec, and the temperature was 22 °C. Mosquitoes acclimated from 1:40 pm – 1:45 pm and then acetone was placed into the South Port (SP). At 12 pm, Experiment 6 was officially concluded.

In the following experiments, mosquitoes were given a choice between acetone in one treatment port and lactic acid in the other treatment port.

Experiment 1 was conducted November 18, 2014. In Experiment 1, adult *Aedes albopictus* mosquitoes (six males and 18 females) from photoperiod LL (constant light) were tested in this choice between acetone and lactic acid. Ten µl of acetone and 10 µl of lactic acid were placed in the olfactometer's North and South Ports, respectively. The lux was007 x 100, the

RH was 11%. The AFR was 54 ml/sec, and the temperature was 21° C. Mosquito acclimatization at the olfactometer's main entrance occurred from 1:35 pm to 1:40 pm, and then infusions of 10 µl of acetone and 10 µl of lactic acid were placed in their respective ports.

Experiment 2 was conducted November 20, 2014. Experiment 2 consisted of 23 adult mosquitoes, five males and 18 females, from photoperiod 16L:8D. The odorants utilized in Experiment 2 were 10 µl of lactic acid (North Port), and 10 µl of acetone (South Port). The start time for this experiment was 1:37 pm with the introduction of 23 *Aedes albopictus* mosquitoes into the flexible dual port olfactometer. Lactic acid and acetone were added at 1:45 pm, after an acclimatization period for the *Aedes albopictus* mosquitoes. The lux was 007 x 100, the RH was 11%, the AFR was 47 ml/sec, and the temperature within the flexible dual port olfactometer was 22°C. The finish Time for Experiment 2 was was 3:14 pm.

Experiment 3 was conducted November 13, 2014. Experiment 3 contained 19 *Aedes albopictus* mosquitoes,18 females and one male, from photoperiod LL (constant light). The lux was 006 x 100, and the RH was 26%. The AFR was 60 ml/sec. The temperature was 22°C. Lactic acid occupied the North Port, while acetone was assigned to the South Port. The start time was 1:30 pm when the mosquitoes were introduced into the flexible dual port olfactometer. The selected odorants were then added at 1:37 pm after mosquito acclimatization.

In order to determine response when exposed to gaseous carbon dioxide (CO_2), adult male and female *Aedes albopictus* mosquitoes were studied via a series of nine trials of approximately 15 minutes in duration.

Experiment 1 on carbon dioxide was conducted January 14, 2013. One male *Aedes albopictus* mosquito from 16L:8D photoperiod (summer) was tested and allowed to acclimatize

for 5-minutes beginning 1:46 pm, and then carbon dioxide was added to the North Port. The RH was 27%, the AFR was 88 ml/sec, and the temperature was 20°C. The experiment concluded at 2:01 pm.

Experiment 2 on carbon dioxide was conducted January 14, 2013. In Experiment 2, an adult *Aedes albopictus* male mosquito from photoperiod LL was placed in the dual port olfactometer. Carbon dioxide was then infused by the addition of 3 ml dH_2O to one tablet in one port and filtered air control in the other port. The RH was 67%. The AFR was 87 ml/sec, and the temperature was 21°C. The start time was 3:01pm, and the finish time was 3:16 pm.

Experiment 3 on carbon dioxide was conducted November 21, 2013. In Experiment 3, one adult male and one adult female *Aedes albopictus* mosquitoes from 16L:8D photoperiod were deposited in the olfactometer. The lux was 974 x 100. The RH was 44%, and the AFR was 87 ml/sec. The temperature inside the olfactometer was 23°C. At 1:58 pm mosquitoes were added. Then 3 ml of (dH_2O) and a tablet was added at 2:08 pm. The finish time was 2:13 pm.

Experiment 4 on carbon dioxide was conducted November 21, 2013. In Experiment 4, one adult female *Aedes albopictus* mosquito from photoperiodic regime 16L:8D (summer) was placed in the main entrance of the olfactometer and allowed to acclimatize for five minutes before infusion of gaseous CO_2. The lux was 990 x 100. The RH was 45%, and the temperature was 24°C. The AFR was 84.5 ml/sec. The start time for Experiment 4 was 2:45 pm and the finish time was 3:00 pm.

Experiment 5 on carbon dioxide was conducted January 23, 2014. Mosquitoes from LL (constant light) were tested. A singular adult *Aedes albopictus* female mosquito was tested under lux of 1125 x 10. The RH was 25%, and the AFR was 90 ml/sec. The temperature was 12°C. The

experiment started at 11:23 am, and the infusion of CO_2 into its respective treatment port occured at 11:43 am. Experiment 5 was concluded at 12:03 pm.

Experiment 6 on carbon dioxide was conducted February 6, 2014. In Experiment 6, two adult female *Aedes albopictus* mosquitoes from photoperiod regime 16L:8D (summer) were tested with CO_2 infusions in the North Port. The lux was 519 x 100, the RH was 29%, and the AFR was 97 ml/sec. The temperature was 18.5°C. Experiment 6-1 began at 10:50am and was finished at 11:11am.

Experiment 7 on carbon dioxide was conducted February 6, 2014. In Experiment 7, one female *Aedes albopictus* from a photoperiod of constant darkness (DD) was tested. There was a lux of 224 x 100, and an RH of 27%. The internal temperature of the olfactometer was 23.5°C. The start time for Experiment 7 was 11:20am, followed by the infusion of gaseous CO_2 in one port at 11:25am. By 11:40 am, the study was concluded.

Experiment 8 on carbon dioxide was conducted February 6, 2014. In Experiment 8, one *Aedes albopictus* female from the spring/fall photoperiod of 12L:12D was tested. For approximately 5-minutes, this female was allowed to acclimatize from 11:43 am to 11:48 am before exposure to CO_2 infusion. The lux was 224 x 100, and the RH was 27%. The temperature was 23.5°C. This study finished at 12:04 pm.

Experiment 9 on carbon dioxide was conducted February 21, 2014. In Experiment 9, eight female *Aedes albopictus* mosquitoes from photoperiod 12L:12D (spring/fall) were deposited in the olfactometer's main entrance. There was a lux of 740 x 100, the RH was 34%, the AFR was 92 ml/sec, and the temperature at 21.5°C. The start time was 10:50am and carbon dioxide was added to the North Port at 10:55 am, and the stop time was 12:02 pm.

Experiment 1 with lactic acid was conducted June 2, 2014. In Experiment 1 of the lactic acid series, one male and 12 female *Aedes albopictus* mosquitoes from photoperiod 12L:12D (spring/fall) were positioned at the main entrance of the dual port olfactometer. After acclimatization for 5 minutes, 10µl of lactic acid (LA) was placed in the North Port of the olfactometer. The lux was 540 x 100, the RH was 26%, the AFR was 90 ml/sec, and the temperature was 22°C. Experiment 1 began at 10:45am and was concluded at 12:10pm.

Experiment 2 with lactic acid was conducted June 5, 2014. Ten adult *Aedes albopictus* mosquitoes consisting of one male and nine females from the photoperiod 12L:12D were transferred from the incubator to the olfactometer. Ten µl of LA deposited in the instrument's South Port were added after 5-minutes. The lux was 855 x 100. Relative humidity was 61%. The AFR was 95 ml/sec, and the temperature was 23°C. Start time was 10:36 pm.

Experiment 3 with lactic acid was conducted June 9, 2014. Experiment 3 consisted of mosquitoes from LL (constant light). A total of 15 *Aedes albopictus* mosquitoes were placed in the olfactometer at 11:26 am and allowed to acclimatize to their new environment. At 11:33 am, 10 µl of LA was deposited in the North Port. The lux was 750 x 100. The RH was 52%, and the AFR was 95 ml/sec. The temperature was 22°C. Experiment 3 was concluded at 1:05 pm.

Experiment 4 on lactic acid was conducted June 12, 2014. Twenty-four *Aedes albopictus* mosquitoes from photoperiod LL consisting of 19 females and five males were deposited in the main entrance of the dual port olfactometer. The lux was 466 x 100, the RH was 65%, the AFR was 92 ml/sec, and the temperature was 24°C. Experiment 4 commenced at 10:45 am, with the initial placement of mosquitoes in the olfactometer. Then 10 µl of LA was put in the South Port, and behavioral responses were recorded. By 12:22 pm, Experiment 4 was concluded.

Experiment 5 with lactic acid was conducted June 16, 2014. In Experiment 5, there were 22 mosquitoes, nine males and 13 females, from photoperiod 12L:12D (spring/fall) tested. The lux was 512 x 100 was utilized, the RH was 52%, the AFR was 94 ml/sec, and the temperature was 24°C. This experiment began at 12:20pm and was concluded at 2:01pm.

Experiment 6 on lactic acid was conducted June 19, 2014. In Experiment 6, nine males and six six female *Aedes albopictus* from photoperiod 16L:8D (summer), were tested with 10 µl lactic acid in the South Port of the olfactometor. The lux was 723 x 10, the RH was 47%, the AFR was 93 ml/sec, and the temperature was 26°C. Experiment 6 began at 11:15 am and concluded at 12:59 pm.

Experiment 7 on lactic acid was conducted June 23, 2014. Five male and ten female *Aedes albopictus* mosquitoes from 16L:8D photoperiod (summer) were tested with 10 µl lactic acid. The lux was 198 x 100, the RH was 37%, the AFR was 99 ml.sec, and the temperature was 22°C. Experiment 7 began at 12:19pm, with 10 µl of lactic acid added to the olfactometer's North Port at 12:25 pm. It ended at 2:01pm.

Experiment 8 on lactic acid was conducted June 26, 2014. Fifteen adult *Aedes albopictus* mosquitoes consisting of four males and 11 females, were subjected to an LL constant lighting photoperiod before being transferred to the dual port olfactometer. The lux was 176 x 100. The RH was 53%. The AFR was 97 ml/sec, and the temperature was 24°C. Experiment 8 commenced at 10:39 am with 10 µl of lactic acid added to the olfactometer's North Port and ended at 12:26 pm.

Experiment 9 with lactic acid was conducted July 1, 2014. Experiment 9 contained a total of 13 *Aedes albopictus* mosquitoes, five males and eight females from 16L:8D photoperiodic

regime. Then 10 µl of lactic acid were added to the North Port of the olfactometer at 11:14 am, five minutes after mosquitoes were introduced. The lux was 400 x10, the RH was 48%, and the AFR was 82 ml/sec. The temperature was 24°C. Experiment 9 was completed at 1:03 pm.

Experiment 10 with lactic acid was conducted July 3, 2014. Adult *Aedes albopictus* mosquitoes consisting of four males and eight females from photoperiod 12L:12D were exposed to 10 µl of lactic acid added to the South Port of the olfactometer at 11:27 am. The lux from Experiment 10 was 34 x 100. The RH was 51%, the AFR was 70 ml/sec, and the temperature was 27°C. Experiment 10 began at 11:20 am and ended at 1:02 pm.

Experiment 11 on lactic acid was conducted July 7, 2014. A total of 11 *Aedes albopictus* mosquitoes consisting of five males and six females from photoperiod 12L:12D were tested. Experiment 11 began at 10:42 am. At 10:51 am, 10 µl of lactic acid was deposited in the olfactometer's North Port. The lux was 196 x 100. The RH was 55% and the AFR was 61 ml/sec. This experiment came to its conclusion at 12:25 pm.

Experiment 12 with lactic acid was conducted July 14, 2014. In Experiment 12, eleven male mosquitoes, and five female *Aedes albopictus* mosquitoes from 16L:8D photoperiod (summer) were exposed to 10 µl of lactic acid. This experiment began at 10:50 am, and the lactic acid was deposited in the South Port (SP) seven minutes later. The lux was 103 x 100. The RH was 46%, the AFR was 73 ml/sec, and the temperature was 24°C. By 12:24 pm, Experiment 12 was concluded.

Experiment 13 with lactic acid was conducted July 15, 2014. Nineteen *Aedes albopictus* mosquitoes, 12 males and seven females fromphotoperiod 16L:8D (Summer) were tested. At 10:38 am, 10 µl of lactic acid was deposited in the olfactometer's North Port. The lux was 103 x

100, the RH was 60%, and the AFR was 80 ml/sec. The temperature was 25°C. At 12:29 pm, Experiment 13 was concluded.

Experiment 14 with lactic acid was conducted February 27, 2014. Two males and 16 females of *Aedes albopictus* from 12L:12D photoperiod were tested. 10 µl of lactic acid was added to the olfactometer's South Port at 11:05 am. The lux was 103 x 100. The RH was 27%, and the temperature was 19°C. The AFR was 91 ml/sec. Experiment 14 concluded at 12:01 pm.

Experiment 15 on lactic acid was conducted March 6, 2014. At 10:55 am, Experiment 15 commenced with a total of 22 adult *Aedes albopictus* mosquitoes, six males and sixteen females, from photoperiod LL. At 11:06 am, 10 µl of lactic acid was added to the olfactometer's North Port (NP). The lux was 750 x 100. The RH was 29%. The AFR was 85 ml/sec, and the temperature was 18°C. By 12:04 pm, this study was concluded.

Experiment 16 with lactic acid was conducted March 20, 2014. Three males and seven females of *Aedes albopictus* from 16L:8D photoperiod were tested. The mosquitoes were put in the olfactometer's main entrance at 11:20 am. After a 5-minute period of acclimatization, 10 µl of lactic acid was added to the South Port at 11:25 am. The lux was 178 x 100. The RH, AFR, and temperature were 40%, 85 ml/sec. and 21°C, respectively. At 12:03 pm, Experiment 16 was concluded.

Experiment 17 on lactic acid was conducted March 27, 2014. Seven *Aedes albopictus* mosquitoes (four males and three females) from photoperiod 16L:8D were tested. Experiment 17 began at 11:21 am. This was followed by10 µl lactic acid deposition in the olfactory North Port at 11:27 am. The lux was 141 x 100. The RH was 26%, and the AFR was 89 ml/sec. The temperature was 21°C. By 12 noon, Experiment 17 was concluded.

Experiment 18 on lactic acid was conducted April 17, 2014. Experiment 18 consisted of eleven *Aedes albopictus* females from a rooftop greenhouse with naural photoperiod. At 11:36 am, the mosquitoes were transferred to the olfactometer. At 11:41 am, 10 µl of lactic acid were deposited in the olfactometer's South Port. The lux was 705 x 100 and the AFR was 96 ml/sec. The RH was 32%. The finish time for Experiment 18 was 12:01 pm.

Experiment 19 with lactic acid was conducted April 24, 2014. In Experiment 19, four *Aedes albopictus* male mosquitoes and eight female mosquitoes were collected from DD (constant darkness) photoperiod. The lux and RH were 223 x 100, and 27%, respectively. The AFR was 94 ml/sec. The temperature was 21.5°C. Experiment 19 commenced at 10:39 am. After a 5-minute acclimatization period, 10 µl of lactic acid was added to the South Port, and olfactory activities observed and recorded. At 11:52 am, experimentation was finalized.

Experiment 20 with lactic acid was conducted April 17, 2014. In Experiment 20, eleven *Aedes albopictus* mosquito females were tested. At 11:36 am, the mosquitoes were transfered to the olfactometer and subjected to 10 µl of lactic acid in the South Port at 11:35 am. The lux was 705 x 100, the RH was 32%, and the AFR was 96 ml/sec. The temperature was 21.5°C. At 11:55 am, Experiment 20 was finalized.

Experiment 21 on lactic acid was conducted June 2, 2014. Thirteen adult *Aedes albopictus* mosquitoes consisting of one male and twelve females from photoperiod 12L:12D (spring/fall) were tested. At 10:45 am, thirteen mosquitoes were transferred from the incubator environment to the olfactometer. At 10:51 am, 10 µl of lactic acid was introduced into the olfactometer's North Port (NP). The lux was 540 x 100. The RH, AFR, and temperature were 26%, 92 ml/sec. and 22 °C, respectively. The finish time for Experiment 21 was 12:07 pm.

Experiment 22 on lactic acid was conducted June 5, 2014. Adult *Aedes albopictus* mosquitoes consisting of one male and seven females from 12L:12D photoperiod were tested. Ten µl of lactic acid were deposited in the olfactometer's South Port at 10:41 am. The lux was 855 x 100, the RH was 61%, and the AFR was 95 ml/sec. The temperature was 23°C. Finalization of Experiment 22 occurred at 1:07 pm.

Experiment 23 on lactic acid was conducted June 9, 2014. Twenty *Aedes albopictus* mosquitoes, five males and fifteen females, from photoperiod LL. The lux was 950 x 100. The RH and AFR were 52%, and 95 ml/sec, respectively. The temperature was 22°C. Experiment 23 commenced at 11:26 am, and 10 µl of lactic acid ws introduced into the North Port at 11:33 am. Finish time for Experiment 23 was 1:05 pm.

Experiment 24 with lactic acid was conducted June 16, 2014. In Experiment 24, thirteen *Aedes albopictus* females and eight males from photoperiod LL were tested. The lux was 510 x 100. The RH was 52%. The AFR and temperature were 94 ml/sec and 24°C, respectively. The start time began at 12:20 pm. At 12:25 pm, 10 µl lactic acid was put into the North Port's olfactometer. The finish time for Experiment 24 was 2:01 pm.

Experiment 25 with lactic acid was conducted July 1, 2014. Experiment 25 commenced with a total of ten *Aedes albopictus* mosquitoes, six males and four females, from photoperiod 16L:8D. The lux was 400 x 100. The RH, AFR, and temperature were 48%, 84 ml/sec, respectively, and the temperature was 24°C. After olfactometric acclimatization for five minutes, mosquitoes were exposed to 10 µl of lactic acid which was deposited into the North Port at 11:14 am. At 12:13 pm, this study was concluded.

Experiment 26 with lactic acid was conducted July 7, 2014. Experiment 26 contained a total of nine adult *Aedes albopictus* mosquitoes, four males and five females, from photoperiod 12L:12D. The nine mosquitoes were deposited into the olfactometer at 10:42 am and allowed to acclimate. Ten µl of lactic acid were then deposited into the North Port at 10:52 am. The lux, RH, and AFR were 1961 x 100, 55%, and 43 ml/sec respectively. The temperature was 24°C. The finish time for Experiment 26 was 12:25 pm.

Experiment 27 on lactic acid was conducted July 10, 2014. Four female *Aedes albopictus* mosquitoes from photoperiod DD were tested. FA was infused into the North Port of the olfactometer, while 10 µl of lactic acid were deposited in the South Port. The lux was 1717 x 100. The RH was 60%, and the AFR was 84 ml/sec. The temperature was 23°C. At 12:29 pm, Experiment 27 was concluded.

Experiment 28 on lactic acid was conducted July 14, 2014. Fourteen *Aedes albopictus* mosquitoes consisting of nine males and five females from 16L:8D photoperiod were tested in the dual port olfactometer. The lux was 327 x 100, while the RH was 59%. The AFR was 79 ml/sec, and the temperature was 25°C. The start time was 10:33 am, and the inclusion of 10 µl of lactic acid in the North Port commenced at 10:38 am. The finish time was 11:57 am.

Experiment 29 on lactic acid was conducted July 17, 2014. Experiment 29 of the lactic acid series began at 10:50 am when 14 *Aedes albopictus* mosquitoes, six males and eight femalesfrom photoperiod 12L:12D were tested. They were exposed to 10 µl lactic acid deposited in the olfactometer's South Port seven minutes later. The lux was 1843 x 100. The RH was 43%, and the AFR was 73 ml/sec. The temperature was 24°C. The finish time for Experiment 29 was 12:29 pm.

Experiment 30 on lactic acid was conducted March 6, 2014. Six males and thirteen female *Aedes albopictus* mosquitoes from 16L:8D photoperiod were tested with 10 µl lactic acid deposited in the olfactometer's North Port. The lux was 725 x 100 and the RH was 29%. The AFR was 85 ml/sec, and the temperature was 18°C. This experiment commenced at 10:55 am and was concluded at 11:55 am.

Experiment 1 on octanol was conducted July 21, 2014. This experiment consisted of 24 *Aedes albopictus* mosquitoes, nine males and 16 females, from photoperiod LL. The lux was 180 x 100. The RH was 62%, the AFR was 65 ml/sec, and the temperature was 23°C. The start time for Experiment 1 of the octanol series was 10:50 am, and the introduction of 10 µl of octenol in the North Port was recorded at 10:57 am. The finish time for Experiment 1 was 12:27 pm.

Experiment 2 on octanol was conducted July 24, 2014. There were 19 *Aedes albopictus* mosquitoes, with nine males and 10 females from photoperiod LL. The lux was 465 x 100. The RH and AFR were 64% and 70 ml/sec, respectively. The temperature was 24°C. Start time for Experiment 2 was 10:55 am and was followed by the deposition of 10 µl of octanol in the South Port of the olfactometer. Finish time was 12:33 pm.

Experiment 3 on octanol was conducted April 26, 2014. For Experiment 3, a total of 11 *Aedes albopictus* mosquitoes consisting of one male and 10 females from photoperiod 16L:8D were tested. The lux was 383 x 100. The RH was 48%, and the AFR was 66 ml/sec. The temperature was 25°C. The start time was 10:56 am, and 10 µl of octanol was deposited in the North Port at 11:00 am. Finish time was 12:32 pm.

Experiment 4 on octanol was conducted July 31, 2014. In Experiment 4, eight female *Aedes albopictus* mosquitoes from photoperiod 12L:12D were tested. The lux wast 30 x 10. The

RH was 48%, and the AFR was 61 ml/sec. The temperature was 23°C. The start time for Experiment 4 began at 10:37 am, and the octanol test odorant was placed in the olfactometer's South Port at 10:43 am. The finish time was 12:17 pm.

Experiment 5 on octanol was conducted August 4, 2014. Experiment 5 included a total of 35 *Aedes albopictus* mosquitoes consisting of 15 males, and 20 females from LL photoperiod. The lux was 4 x 100. The RH was 65%, the AFR was 62 ml/sec, and the temperature was 22°C. Start time was 10:33 am. Octanol was placed in the North Port at 10:47 am. The finish time was 12:21 pm.

Experiment 6 with octanol was conducted August 18, 2014. Experiment 6 consisted of 24 *Aedes albopictus* mosquitoes, 10 males and 14 females from photoperiod LL. The lux was 4 x 100. The RH was 54%, the AFR was 52 ml/sec, and the temperature was 23°C. This experiment began officially at 10:35 am when the mosquitoes were transferred to the main entrance of the dual port olfactometer for the purpose of acclimatization. At 10:47 am, 10 µl of octanol was added to the South Port of the olfactometer. By 12;18 pm, Experiment 6 was concluded.

Experiment 7 with octanol was conducted September 9, 2014. Seven *Aedes albopictus* mosquitoes, two males and five females, from photoperiod 12L:12D were tested. The lux was 302 x 100. The RH was 68%, and the AFR was 73 ml/sec. The temperature was 22°C. Experiment 7 began at 1:42 pm. At 1:47 pm, 10 µl of octanol was placed in the North Port of the olfactometer. The finish time was 3: 18pm.

Experiment 8 with octanol was conducted December 2, 2014. Sixteen *Aedes albopictus* mosquitoes comprised Experiment 8, which consisted of three males and 13 females from photoperiod 16L:8D. The lux was 007 x 100 and the RH was 27%. However, the AFR was 65

ml/sec, and the temperature was 22°C. Afternoon experimentation began at 1:32 pm, followed by the introduction of 10 µl of octanol in the South Port five minutes later. Experiment 8 was concluded at 3:10 pm.

Experiment 9 with octanol was conducted November 29, 2014. In Experiment 9, seven *Aedes albopictus* male and eight female mosquitoes from photoperiod 16L:8D were tested. The lux was 006 x 100. The RH was 37%, and the AFR was 71 ml/sec. The temperature was 22°C. The start time was 1:35 pm, and the introduction of 10 µl of octanol was placed in the North Port at 1:40 pm. At 3:08 pm, Experiment 9 was concluded.

Experiment 10 on octanol was conducted October 23, 2014. In the final octenol investigative series, 18 *Aedes albopictus* mosquitoes consisting of three males and 15 females from photoperiod 16L:8D were tested. The lux, RH, and AFR were 005 x 100, 31%, and 45 ml/sec, respectively. The temperature was 23°C. Experimentation began at 1:20 pm and followed at 1:25 pm with the infusion of 10 µl of octenol in the olfactometer's South Port. The finish time for Experiment 10 was 2:57 pm.

The following experiments gave mosquitoes a choice between lactic acid in one treatment port and octanol in the other treatment port.

Experiment 1 was conducted August 4, 2014. This study involved two male and two female *Aedes albopictus* mosquitoes from photoperiod LL. Ten µl octanol was placed in the North Port and 10 µl lactic acid was placed in the South Port. The lux was 4 x 100. The temperature was 22°C. The RH was 62%, and the AFR was 62 ml/sec. The start time for Experiment 1 was 10:33 am. After eight minutes, octanol and lactic acid were added into their respective ports at 10:41 am. The finish time was 11:47 am.

Experiment 2 was conducted August 4, 2014. This experiment tested 15 male and 20 female *Aedes albopictus* mosquitoes from photoperiod LL. The lux was 4 X100. The RH was 65%, the AFR was 62 ml/sec, and the temperature was 22° C. Experiment 2 commenced at 10:33 am, with the addition of 10 µl octanol (North Port) and 10 µl lactic acid (South Port) at 10:41 am. The conclusion was at 12:21 pm.

Experiment 3 was conducted August 7, 2014. In Experiment 3, 21 adult *Aedes albopictus* mosquitoes, eight males and 13 females from photoperiod LL, were introduced into the dual port olfactometer. Then 10 µl lactic acid (North Port) and 10 µl octanol (South Port) were added. The lux was 44 x 100. The RH was 50%, and the AFR was 60 ml/sec. The temperature was 25° C. Experiment 3 commenced at 10:47 pm, along with the introduction of octanol (South Port), and lactic acid (North Port) at 11:07 am. This experiment was concluded at 12:37 pm.

Experiment 4 was conducted August 7, 2014. Experiment 4 was a behavioral study that consisted of seven males and 11 female *Aedes albopictus* mosquitoes from photoperiod LL. Jn this experiment, the lux was 8 x 100 and the RH was 53%. Tthe AFR was 60 ml/sec. The temperature was 24°C. This study started at 10:47 am and progressed with the inclusion of 10 µl lactic acid (North Port), and 10 µl octanol (South Port) at 10:57 am. At 12:40 pm, this experiment was concluded.

Experiment 5 was conducted August 11, 2014. Experiment 5 consisted of four males and four female *Aedes albopictus* from photoperiod 16L:8D. The lux was 6 x 100. The RH was 59%, and the AFR was 55 ml/sec. The temperature was 23° C. Experiment 5 commenced at 10:48 am, and the inclusion of the 10 µl octanol in the North Port and 10 µl lactic acid in the South Port was recorded at 11:00am. Experiment 5 was concluded at 12:40pm.

Experiment 6 was conducted August 14, 2014. Experiment 6 had 12 female *Aedes albopictus* from photoperiod 12L:12D. The lux was 6 x 100, the RH was 41%. The AFR was 55 ml/sec, and the temperature was 22° C. Experiment 6 commenced at 10:52 am, and 10 µl lactic acid (South Port) and 10 µl octanol (North Port) was introduced at 11:00 am. Experiment 6 was finished at 12:30 pm.

Experiment 7 was conducted August 21, 2014. Experiment 7 consisted of four males and seven females from photoperiod 12L:12D. The lux was 40 x 100. The RH was 65%, and the temperature was 24° C. Experiment 7 began at 11:00 am and was followed at 11:07 am by the inclusion of 10 µl lactic acid in the South Port and 10 µl octanol in the North Port. At 12:41 pm, Experiment 7 was concluded.

Experiment 8 was conducted September 4, 2014. Experiment 8 consisted of three males and seven females of *Aedes albopictus* from photoperiod 16L:8D. The lux was low at 6 x 100. However, the RH was elevated at 78%. In addition, the AFR was elevated at 72 ml/sec, along with increased temperature at 25° C. Experiment 8 began at 1:23 pm, and the addition of octanol and lactic acid was at 1:33 pm. Experiment 8 concluded at 3:04 pm.

Floral Scent (Rose absolot, Lavender Oil, Sweet Orange, Cilantro, Mountain Mint) Experiments with *Aedes albopictus* Mosquitoes

Experiment 1 with Rose absolot was conducted September 11, 2014. In this investigation, 25 *Aedes albopictus* mosquitoes consisting of 10 males and 15 females from photoperiod LL were tested. At 1:35 pm, Experiment 1 of the floral scent series commenced. At 1:40 pm, 10 µl of Rose absolot was added to the olfactometer's South Port. The lux was 7 x 100, an RH of 57%, and the AFR was 74 ml/sec. The temperature was 24°C. This experiment concluded at 3:11 pm.

Experiment 2 with Rose absolut was conducted September 16, 2014. Experiment 2 included 33 *Aedes albopictus* mosquitoes with 17 males and 16 females from photoperiod LL. At 1:35 pm, 10 µl of Rose absolot was deposited in the North Port. The lux was 6 x 100, the RH was 48%, and the AFR was 79 ml/sec. The temperature was 23°C. At 3:02 pm, Experiment 2 of the Rose absolot series was concluded.

Experiment 1 on lavender oil was conducted September 18, 2014. Forty-two *Aedes albopictus* mosquitoes consisting of 33 males and nine females were tested for their behavioral responses to lavender oil. These mosquitoes were transferred from Incubator 5 and allowed to acclimatize in the olfactometer's main entrance. Experiment 1 of the Lavender Oil series commensed at 2:03 pm. At 2:08 pm, 10 µl of lavender oil was introduced into the olfactometer's South Port (SP). Both the lux and RH were low at 7 x 100 and 36% respectively. However, the AFR was high at 96 ml/sec. The temperature was 22°C, and the *Aedes albopictus* were reared in an (LL) photoperiodic regime. The finish time for this experiment was 3:34 pm.

Experiment 2 was conducted September 25, 2014, in the lavender oil series. Experiment 2 consisted of 13 *Aedes albopictus* mosquitoes, seven males and six females from photoperiod LL. The lux was 7 x 100, and the RH was 64%. The AFR was 57 ml/sec. The temperature was 21°C. The start time for this experiment was 3:46 pm. At 3:52 pm, 10 µl of Lavender Oil was placed into the North Port. The finish time for this experiment was 5:23 pm.

Experiment 1 was conducted September 30, 2014 on sweet orange oil. This experiment tested 38 *Aedes albopictus* mosquitoes, 26 males and and 12 females from photoperiod LL. The lux, RH, and AFR were 6 x 100, 47%, and 57 ml/sec., respectively. The temperature was 21°C.

This experiment commenced at 1:28 pm, and 10 µl of sweet orange oil was introduced into the South Port at 1:33 pm. At 3:03 pm, the experiment was concluded.

Experiment 1 was conducted October 2, 2014 on cilantro flowers. The first cilantro flower experiment used 23 *Aedes albopictus* mosquitoes, 15 males and eight females from photoperiod LL. The lux was 40x10, and the RH was 51%. The AFR and temperature were 53 ml/sec and 22°C, respectively. The start time was 1:40 pm, and at 1:45 pm, cilantro flowers were introduced into the olfactometer's North Port. At 3:17 pm, this experiment was concluded.

Experiment 2 was conducted October 7, 2014 on cilantro flowers. The experiment included 13 males, and eight females of *Aedes albopictus* mosquitoes from photoperiod LL. The lux was 5 x 10, and the RH was 39%. The AFR was 54 ml/sec., and the temperature was 22°C. The experiment commenced at 1:33 pm with the introduction of 10 Cilantro flowers into the olfactometer's South Port at 1:42 pm. The finish time for this experiment was 3:17 pm.

Experiment 1 was conducted October 2, 2014, on cilantro leaves. In Experiment 1, there were 13 *Aedes albopictus* males and eight females from LL photoperiod**.** This experiment commenced at 1:33 pm. By 1:42 pm, cilantro leaves were introduced into the North Port of the olfactometer. The lux, RH, and AFR were 7 x 100, 38%, and 55 ml/sec, respectively. The temperature was 22°C. The experiment was concluded at 3:17 pm.

Experiment 2 was conducted October 7, 2014, on cilantro leaves. In Experiment 2, 15 *Aedes albopictus* male mosquitoes and eight female *Aedes albopictus* mosquitoes from photoperiod LL were tested at 1:42 pm and were subjected to cilantro leaves that were placed in the olfactometer's South Port. The deposition of the leaves occurred at 1:42 pm. The lux was 40 x 10, the RH was 48%, and the AFR was 55 ml/sec. The finish time was 3:17 pm.

Experiment 1 was conducted October 16, 2014, on mountain mint flowers in one port and mountain mint leaves in the other. *Aedes albopictus* mosquitoes consisting of five females from 16L:8D photoperiod were tested. The lux was 7 x 100, the RH was 36%, and the AFR was 52 ml/sec. The temperature was 24 °C. Start time for this experiment was 1:40 pm. At 1:45 pm, mountain mint flowers were introduced into the North Port of the olfactometer. The finish time was at 3:15 pm.

Experiment 2 was conducted October 16, 2014, on mountain mint leaves and simultaneously flowers were tested in the other port. In Experiment 2, five *Aedes albopictus* mosquito females from photoperiod 16L:8D were utilized. The lux, RH, AFR and temperature were 7 x 100, 36%, 52 ml/sec, and 24 °C, respectively. Start time for this experiment was 1:40 pm. At 1:45 pm, mountain mint leaves were introduced into the South Port of the olfactometer. The finish time for this experiment was recorded at 3:15 pm.

Fig. 1. Flexible Dual Port Olfactometer. This measuring instrument is designed to observe and document mosquito behavior. Note important component parts: staging area, stem or midpiece (flexible portion), and left and right arms for the placement of control agent and treatment odorant. The olfactometer was built to specification for Bernadette A. Ferraro by Scientific Glass, Virginia.

CHAPTER III

RESULTS

The following results were obtained by observations of mosquito behaviors within the confines of a flexible dual port olfactometer in the Old Dominion University Entomology Laboratory in Norfolk, Virginia.

Experiments with *Culex restuans* Mosquitoes

Filtered Air Experiments with *Culex restuans* Mosquitoes

The four experiments included a total number of 16 *Culex restuans* mosquitoes, 10 male mosquitoes and six females (Table 1). These mosquitoes were observed to fly to both North and South Ports, which both contained filtered air. The results of these tests indicated that there were no preferences for either port conducted on the mosquitoes tested from the photoperiodic regimes of DD, 16L:8D, 12L:12D, and LL.

Host Odors Experiments with *Culex restuans* Mosquitoes

For the acetone experiments, of the 10 *Culex restuans* mosquitoes tested with acetone and filtered air, none visited the acetone port, and two females but no males visited the filtered air control port (Table 2). There were not enough replicates to perform statistical analyses, but these preliminary results suggest there is no attraction to acetone.

For the carbon dioxide (CO_2) experiments, significantly more *Culex restuans* females visited the CO_2 treatment port than the filtered air control port over the fourteen experimental trials with carbon dioxide (Sign Test, 13 differences, x = 1, P = 0.002) (Fig. 2). Overall, there were 38 females in the treatment CO_2 port and eight females in the filtered air control port (Table 3). There was no significant difference in the number of *Culex restuans* males visiting the two ports (Sign Test, 6 differences, x = 2, P = 0.344) (Zar 1999). Nineteen males visited the CO_2 treatment port, and 18 males visited the filtered air control port (Table 3).

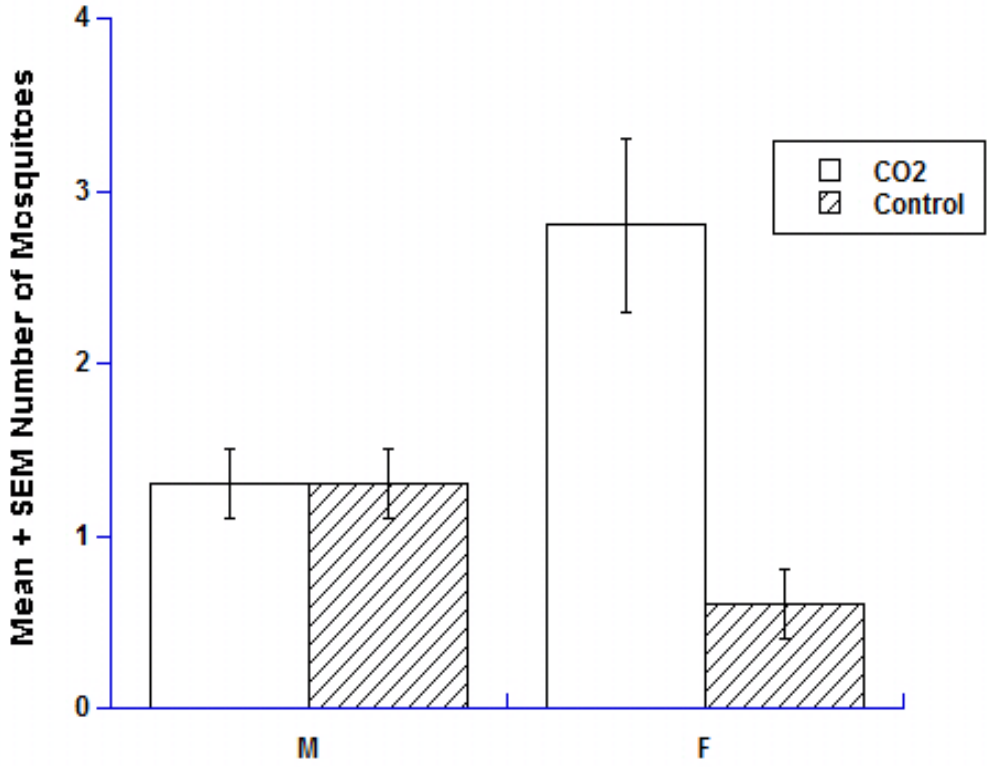

Fig. 2. Mean ± SEM *Culex restuans* mosquito males (M) and females (F) that chose the CO_2 treatment port or the filtered air control port. Females chose the carbon dioxide port significantly more often than the filtered air control port (Sign Test, 13 differences, x = 1, P = 0.002).

For the lactic acid experiments, significantly more *Culex restuans* females visited the lactic acid port than the filtered air control port (Sign Test, 5 differences, x = 0, P = 0.031). In these five olfactometric experiments, 12 female mosquitoes visited the lactic acid port, while only two females visited the filtered air control port (Table 4). In contrast, more males visited the control port with filtered air than the lactic acid port, but the difference was not significant (Sign Test, 5 differences, x = 1, P = 0.188). Twelve males visited the lactic acid port, and 21 males visited the control port with filtered air (Fig. 3).

For the octanol (1-octan-3-ol) experiments, results revealed that six female *Culex restuans* mosquitoes visited the octanol port, and 14 females visited the filtered air control port (Table 5). Five male *Culex restuans* mosquitoes visited the octenol port, and four males visited the filtered air control port.

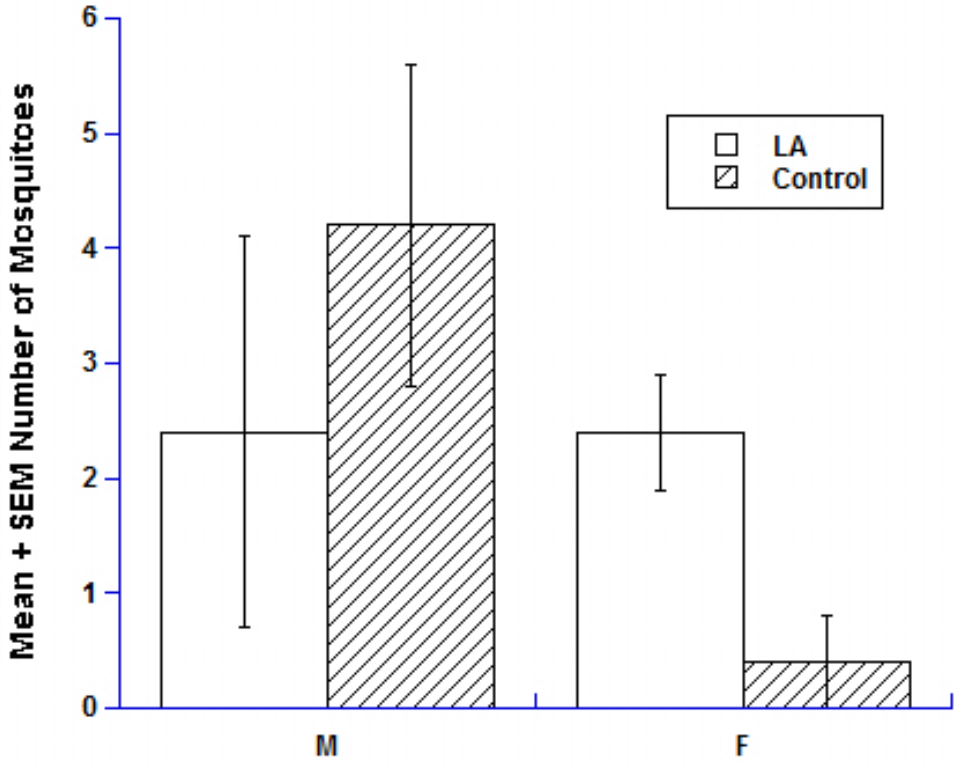

Fig. 3. Mean ± SEM *Culex restuans* mosquito males (M) and females (F) that chose the Lactic Acid (LA) treatment port or the filtered air control port. Females chose the Lactic Acid port significantly more often than the filtered air control port (Sign Test, 5 differences, x = 0, P = 0.031).

Floral Scent Experiments with *Culex restuans* Mosquitoes

For the clethra plant (*Clethra alnifolia*) experiment, only one experiment with four male *Culex restuans* mosquitoes was undertaken to test mosquito preferences for the Clethra plant. One mosquito visited the port containing the Clethra plant and one male *Culex restuans* mosquito traveled to the filtered air control port. These results indicate that Clethra was not highly attractive to *Culex restuans* and no further studies were warranted.

For the fennel plant (*Foeniculum vulgare*) experiments, two olfactometric experiments were conducted to evaluate mosquito response to the fennel plant. Overall, 11 male and two female *Culex restuans* mosquitoes were tested. Three males visited the Fennel port and three males visited the filtered air control port. No females visited the Fennel port and one female visited the filtered air control port. These results did not indicate a strong attraction of *Culex restuans* mosquitoes to fennel, and no further studies were warranted.

In the mountain mint (*Pycnanthemum virginianum*) experiment, this one experiment involving four male *Culex restuans* mosquitoes, no males visited either the mountain mint port or the filtered air control port. These results did not indicate an attraction to mountain mint and no further studies were warranted.

Two experiments tested *Culex restuans* response to the oregano prelant (*Plectranthus amboinious*). Overall, seven males and one female were tested with oregano. Three males visited the oregano port and three males visited the filtered air control port. The solitary female visited the oregano port. These results did not indicate a strong response to oregano and no further studies were warranted.

Table 1. *Culex restuans* mosquito responses to filtered air in both ports by males (M) and females (F)

Experiment	Number	North Port	South Port	Photo-period
1	3 M 1 F	0 M 1 F	1 M 0 F	DD
2	0 M 3 F	0 M 2 F	0 M 1 F	16L:8D
3	3 M 1 F	1 M 0 F	2 M 0 F	12L:12D
4	4 M 1 F	3 M 0 F	1 1 F	LL

There were no differences in the numbers of male and female *Culex restuans* mosquitoes visiting the South and North ports of the olfactometer when filtered air flowed through both ports (Table 1). These results demonstrated that mosquitoes were not biased to visit or avoid a specific port depending on location.

Table 2. *Culex restuans* mosquito responses to acetone treatment port and filtered air control port by males (M) and females (F)

Experiment	Number	Acetone	Filtered Air (Control)	Photo-period
1	5 M	0 M	0 M	12L:12D
2	5 F	0 F	2 F	12L:12D

There was not a strong response by *Culex restuans* mosquitoes to visit either the treatment port with acetone or the filtered air control port as evidenced by the low numbers entering the ports (Table 2).

Table 3. *Culex restuans* mosquito responses to CO_2 treatment port and filtered air control port by males (M) and females (F)

Experiment	Number	CO_2	Filtered Air Control	Photo-period
1	7 M 2 F	1 M 1 F	1 M 1 F	DD
2	6 M 3 F	1 M 1 F	1 M 1 F	LL
3	11 M 8 F	1 M 1 F	3 M 0 F	16L:8D
4	9 M 4 F	2 M 1 F	2 M 2 F	16L:8D
5	4 M 8 F	2 M 5 F	2 M 2 F	16L:8D
6	3 M 6 F	2 M 4 F	1 M 1 F	12L:12D
7	5 M 2 F	1 M 2 F	1 M 1	12L:12D

Table 3. Continued

Experiment	Number	CO_2	Filtered Air Control	Photo-period
8	3 M 8 F	0 M 2 F	0 M 0 F	12L:12D
9	5 M 4 F	1 M 1 F	1M 0 F	LL
10	2 M 2 F	0 M 1 F	1 M 0 F	LL
11	7 M 4 F	2 M 3 F	1 M 0 F	DD
12	9 M 7 F	3 M 5 F	2 M 0 F	16L:8D
13	6 M 7 F	1 M 6 F	0 M 0 F	12L:12D
14	9 M 8 F	2 M 6 F	2 M 1 F	LL

Female *Culex restuans* mosquitoes significantly preferred visiting the carbon dioxide treatment port over the filtered air control port (Sign Test, 13 differences, x = 1, P = 0.002). Male *Culex restuans* mosquitoes did not differ significantly in their visits to either the treatment or control port (Sign Test, 6 differences, x = 2, P = 0.344) (Table 3).

Table 4. *Culex restuans* mosquito responses to lactic acid treatment port and filtered air control port by males (M) and females (F)

Experiment	Number	Lactic Acid	Filtered Air Control	Photo-period
1	9 M 3 F	1 M 2 F	2 M 0 F	12L:12D
2	13 M 5 F	1 M 3 F	2 M 2 F	16L:8D
3	12 M 5 F	1 M 4 F	6 M 0 F	12L:12D
4	20 M 3 F	9 M 1 F	9 M 0 F	DD
5	3 M 2 F	0 M 2 F	2 M 0 F	LL

Female *Culex restuans* mosquitoes visited the treatment port with lactic acid significantly more often than they visited the filtered air control port port (Sign Test, 5 differences, $x = 0$, $P = 0.031$). Male *Culex restuans* mosquitoes did not differ significantly in their visits to either the treatment port or the control port (Table 4).

Table 5. *Culex restuans* mosquito responses to octanol treatment port and filtered air control port by males (M) and females (F)

Experiment	Number	Octanol	Filtered Air Control	Photo-period
1	1 M 4 F	0 M 1 F	0 M 2 F	DD
2	12 M 18F	5 M 3 F	2 M 11 F	16L:8D
3	2 M 3 F	0 M 2 F	2 M 1 F	LL

Neither male nor female *Culex restuans* mosquito showed a strong response to either the treatment port containing octanol or the filtered air control port (Table 5).

Experiments with *Aedes albopictus* Mosquitoes

Filtered Air Experiments with *Aedes albopictus* Mosquitoes

Studies involving *Aedes albopictus* mosquito responses to filtered air encompassed three trials with one male and one female in each trial. After mosquitoes were placed in the dual port olfactometer, both north and south ports were then infused with filtered air. Neither male nor female *Aedes albopictus* mosquitoes visited either port (Table 6), indicating that there was no bias in port choice based on port location.

Host Odor Experiments with *Aedes albopictus* Mosquitoes

In the acetone experiments, a total of 43 male *Aedes albopictus* mosquitoes were selected for the six experiments (Table 7). Of the 43 male mosquitoes, only eight visited the acetone port, and four males visited the filtered air control port. These results were not significantly different (Sign Test, 3 differences, $x = 0$, $P > 0.05$). Of the 52 female *Aedes albopictus* mosquitoes tested, nine visited the acetone port and 12 visited the filtered air control port. These results were not significantly different (Sign Test, 5 differences, $x = 2$, $P = 0.500$).

These three experiments on acetone and lactic acid offered mosquitoes a choice between acetone in one port and lactic acid in the other port. A total of 12 males and 54 females *Aedes albopictus* mosquitoes were tested in these experiments (Table 8). Two males visited the acetone port and one male visited the lactic acid port. Eleven females visited the acetone port and 17 females visited the lactic acid port. There were not enough replicates for statistical analyses, but these preliminary results indicated that there was no preference for one chemical over the other by either males or females.

In Experiments 1-9 on carbon dioxide (CO_2), mosquitoes were given a choice between a carbon dioxide treatment port and a control port with filter air. Only three male *Aedes albopictus* mosquitoes were tested overall, and none chose either the carbon dioxide port or the filtered air control port (Table 9). Thirteen females were tested, and two chose the carbon dioxide port and three chose the filtered air control port. These results did not show a significant attraction to carbon dioxide.

In the lactic acid (LA) experiments, the behavioral studies involving choices between 10 µl of lactic acid in the treatment port and filtered air in the control port for male and female *Aedes albopictus* mosquitoes consisted of 30 experiments. In these experimental series, there was a total of 137 male and 274 female mosquitoes tested. Thirty-four of these males visited the lactic acid port, and 45 males visited the filtered air control port (Table 10a and Table 10b). These results were not significantly different (Sign Test, 19 differences, $x = 6$, $P = 0.084$). For females, 80 visited the lactic acid port and 67 visited the filtered air control port. These results were not significantly different (Sign Test, 26 differences, $x = 10$, $P > 0.212$).

There were 11 experiments with octanol to test male and female *Aedes albopictus* mosquitoes with octanol in the treatment and filtered air in the control port. The total number of males tested was 50. There were 13 males that visited the octenol port and 12 males who visited the filtered air control port (Table 11). These numbers were not significantly different (Sign Test, 8 differences, $x = 4$, $P = 0.637$).

For *Aedes albopictus* females, the total number tested in these 11 experiments was 130. There were 28 females that visited the treatment port with octenol, and 35 females that visited

the filtered air control port. These numbers were not significantly different (Sign Test, 10 differences, x = 4, P = 0.377).

In Experiments 1-8 on octanol and lactic acid, there were a total of 119 *Aedes albopictus* mosquitoes tested. male attraction to a combination of octanol + lactic acid, had a collective total of 23 mosquitoes (Table 12). Of the 43 male *Aedes albopictus* mosquitoes tested, 15 visited the octanol port and seven visited the lactic acid port. These numbers were not significantly different (Sign Test, 7 differences, x = 2, P = 0.227).

Of the 76 female *Aedes albopictus* mosquitoes tested, 22 visited the octanol port and 19 visited the lactic acid port. These numbers were not significantly different (Sign Test, 7 differences, x = 3, P = 0.500).

Floral Scent Experiments with *Aedes albopictus* Mosquitoes

In the first part of Experiment 1 involving Rose absolot, there were 25 *Aedes albopictus* mosquitoes. Of the male mosquitoes that constituted the first part of this experiment, no male responded to Rose absolot. Eight males did not respond to this floral scent or FA, while only one mosquito was attracted to FA.

Experiment 2 with Rose absolut had a total of 17 males. In this experiment, there was a demonstrated attraction to the scent of Rose absolot. Of the 14 males studied under conditions of observed frequencies, five preferred Rose absolot, eight did not respond, and one was attracted to (FA). It is interesting to denote, that Experiment 1 was conducted under 1:35 minutes, while Experiment Two was undertaken at 1:25 minutes duration. Yet both experiments yielded different responses as per preferences to Rose absolot. The photoperiod regime in both experiments was LL.

In Experiment 1 with lavender oil, the distribution of nine females collectively was three females displaying (+) preference, three females yielding non-preference, and three females attracted to (FA) in a 1:1:1 ratio. In the second part of this experiment, there were 15 females with an arithmetical distribution of 7 females showing (+) preference for this botanical scent, 6 displaying non-preference, and 2 females gravitating towards (FA). Female conspecifics in Experiment 1 totaled nine mosquitoes, with four, the greatest number, demonstrating a preference for lavender oil. No response was made by two female *Aedes albopictus* mosquitoes located in the cage and main tube areas respectively. The (FA) source located in the North Port, attracted three females.

In Experiment 2 on lavender oil, the South Port (SP) contained lavender oil. The numerical total of adult *Aedes albopictus* mosquitoes that constituted Experiment 2 was 13. The presence of (FA) in the North Port (NP) attracted six male mosquitoes and the treatment port for lavender oil attracted two mosquitoes. The results of Experiment 2 on lavender oil showed of the six females studied, none displayed a response and were in the areas constituting the cage, main tube, ring before the stocking, and tube before the stocking. There were no female mosquitoes preferring lavender oil, and no females gravitated towards (FA) located in its South Port source**.**

Experiment 1 on sweet orange consisted of only one singular study of this odorant, regarding olfactorial behavioral responses of both male and female *Aedes albopictus*. In this experiment, five males showed preference for sweet orange, while 17 males exhibited no particular preference for this scent. Four males were attracted to (FA). This experiment was conducted under the photoperiodic regime of continuous light. Collectively, there were 12 females tested in Experiment 1. Five females demonstrated preference for sweet orange, while only three females showed no preference or attraction for this odorant. Four females gravitated

towards (FA). It should be noted, that although this was a small experiment, females showed a slightly greater olfactory response to sweet orange than their male conspecifics. In contrast, male counterparts had more than five times greater non-preferential olfactory expression. However, despite the small replicate pool of Experiment 5, both male and female *Aedes albopictus* mosquitoes were equally attracted to (FA).

In Experiment 1 on cilantro flowers, a total of 15 males in the classification of cilantro (flowers), had the following arithmetical bbreakdown: preference for cilantro was three, no response to cilantro flowers was nine, and attraction to (FA) was 3 in a 1:3:1 ratio. In Experiment 1, two females exhibited (+) preference for cilantro (flowers), while non-preference was slightly elevated at three, and attraction for (FA) was three.

Experiment 2 on cilantro flowers contained at a total of 13 males with a slightly higher preference for cilantro flowers with four, no response at six and (FA) having a numerical attraction of three. In Experiment 2, the female preference for cilantro flowers was two mosquitoes and no response by five mosquites. The attraction for (FA) was one. The collective total of male *Aedes albopictus* mosquitoes under study in Experiments 1 and 2 for Cilantro flowers was 28, while the collective number for female *Aedes albopictus* mosquitoes was 16.

For the cilantro leaf Experiment 1with *Aedes albopictus* mosquitoes, there was a collective total of 21 mosquitoes tested for preference to cilantro (leaves) placed initially in the South Port of the olfactometer. Nine males exhibited no response and three males showed a preference for cilantro (leaves).

In Experiment 2 on cilantro leaves, 15 males exhibited the following numerical breakdown: no response was dominant with seven males not attracted to either the North Port (leaves), or South Port (flowers).

In Experiments 1 and 2 on mountain mint, the two experiments were conducted simultaneously in the *Aedes albopictus* series. This mosquito study contained only five females and zero males. The female mosquitoes observed did not show a predilection or attraction to mountain mint whether it involved flowers or leaves.

Table 6. *Aedes albopictus* mosquito responses to filtered air in both North and South ports by males (M) and females (F)

Experiment	Number	North Port	South Port	Photo-period
1	1 M 1 F	0 M 0 F	0 M 0 F	16L:8D
2	1 M 1 F	0 M 0 F	0 M 0 F	16L:8D
3	1 M 1 F	0 M 0 F	0 M 0 F	LL

There were no visits by either male or female *Aedes albopictus* mosquitoes to either the North Port or the South Port of the olfactometer when filtered air flowed through both ports (Table 6). These results indicated that *Aedes albopictus* mosquitoes did not have a bias for visiting either port depending on location.

Table 7. *Aedes albopictus* mosquito responses to acetone treatment port or filtered air control port by males (M) and females (F)

Experiment	Gender	Acetone	Filtered Air Control	Photo-period
1	3 M 10 F	1 M 2 F	0 M 1 F	16L:8D
2	4 M 3 F	1 M 1 F	0 M 0 F	16L:8D
3	16 M 13 F	1 M 0 F	1 M 4 F	LL
4	17 M 12 F	3 M 3 F	3 M 1 F	LL
5	1 M 8 F	0 M 1 F	0 M 4 F	12L:12D
6	2 M 6 F	2 M 2 F	0 M 2 F	12L:12D

There were no significant differences in the number of visits by *Aedes albopictus* mosquito males (Sign Test, 3 differences, $x = 0$, $P > 0.05$) or females (Sign Test, 5 differences, $x = 2$, $P = 0.500$) to the treatment port containing acetone or the filtered air control port (Table 7),

Table 8. *Aedes albopictus* mosquito responses to acetone and lactic acid treatment ports by males (M) and females (F)

Experiment	Gender	Acetone	Lactic Acid	Photo-period
1	6 M 18 F	1 M 4 F	1 M 9 F	LL
2	5 M 18 F	1 M 4 F	0 M 8 F	16L:8D
3	1M 18F	0 M 3 F	0 M 0 F	LL

Visits to the acetone treatment port and the lactic acid treatment port were similar for male and female *Aedes albopictus* mosquitoes (Table 8). There were too few replicates for statistical analysis, but the results did not indicate that one chemical was preferred over the other by either males or females.

Table 9. *Aedes albopictus* mosquito responses to carbon dioxide (CO_2) treatment port or filtered air control ports by males (M) and females (F)

Experiment	Number	CO_2	Filtered Air Control	Photo-period
1	1 M 0 F	0 M 0 F	0 M 0 F	16l:8D
2	1 M 0 F	0 M 0 F	0 M 0 F	LL
3	1 M 0 F	0 M 0 F	0 M 0 F	16L:8D
4	0 1 F	0 M 0 F	0 M 0 F	16L:8D
5	0 M 1 F	0 M 0 F	0 M 0 F	LL
6	0 2 F	0 M 0 F	0 M 0 F	16L:8D
7	0 M 1 F	0 M 0 F	0 M 0 F	DD
8	0 M 1 F	0 M 0 F	0 0 F	12L:12D
9	0 M 8 F	0 M 2 F	0 M 3 F	12L:12D

There was not a strong response by either males or females of *Aedes albopictus* mosquitoes to either the carbon dioxide treatment port or the filtered air control port (Table 9). Not enough mosquitoes responded to conduct a statistical analysis.

Table 10a. *Aedes albopictus* mosquito responses to lactic acid treatment port or filtered air control port by males (M) and females (F) in Experiments 1-15

Experiment	Number	LacticAcid	Filtered Air Control	Photo-period
1	1 M 12F	1 M 3 F	0 1 F	12L:12D
2	1 M 9 F	0 M 3 F	0 M 2 F	12L:12D
3	5 M 15 F	0 M 1 F	1 M 10 F	LL
4	5 M 17Fe	1 M 2F	2 M 3 F	LL
5	9 M 13 F	2 M 8 F	2 M 3 F	12L:12D
6	9 M 6 F	2 M 1 F	2 M 0 F	16L:8D
7	5 M 10 F	2 M 5 F	0 M 0 F	16L:8D
8	4 M 11 F	2 M 8 F	1 M 1 F	LL
9	5 M 4 F	1 M 1F	2 M 1 F	16L:8D
10	4 M 6 F	0 M 2 F	2 M 1 F	12L:12D
11	4 M 5 F	0 M 2 F	2 M 0 F	12L:12D
12	9 M 5 F	3 M 1 F	4 M 3 F	12L:12D
13	10 M 5 F	3 M 1 F	5 3 F	16L:8D

Table 10a. Continued

Experiment	Number	Lactic Acid	Filtered Air Control	Photo-period
14	3 M 16 F	0 M 9 F	2 M 4 F	12L:12D
15	5 M 17 F	2 M 2 F	1 M 0 F	LL

The results for Table 10a and Table 10b were combined for statistical analyses on the 30 experiments on lactic acid. The results of the analyses are presented on the next page.

Table 10b. *Aedes albopictus* mosquito responses to lactic acid treatment port or filtered air control port by males (M) and females (F) in Experiments 16-30

Experiment	Number	Lactic Acid	Filtered Air Control	Photo-period
16	3 M 7 F	0 M 0 F	0 M 2 F	16L:8D
17	4M 3 F	1 M 9 F	3 M 0 F	16L:8D
18	0 M 11F	0 M F	0 M 0 F	Green House
19	4M 8F	0 M 1F	2 M 3F	DD
20	2 M 16F	0 M 9 F	0 M 0 F	LL
21	0 M 4 F	0 M 3F	0 M 1 F	NA
22	6 M 7 F	2 M 1F	2 M 4 F	12L:12D
23	3 M 15F	M 1 F	0 M 7 F	LL
24	5 M 13F	1 M 5F	2 M 5 F	LL
25	6 M 4 F	3 M 1 F	1 M 1 F	16L:8D
26	4 M 5 Fe	0 M 3 F	2 M 0 F	12L:12D
27	0 M 4 F	0 M 1 F	0 M 0 F	DD
28	9 M 5F	3 M 1 F	4 M 3 F	16L:8D

Table 10b. Continued

Experiment	Number	Lactic Acid	Filtered Air Control	Photo-period
29	6 M 8 F	2 M 2 F	2 M 4 F	12L:12D
30	6 M 13 F	3 M 1 F	1 M 0 F	LL

The results for the lactic acid experiments with *Aedes albopictus* mosquitoes were combined for Table 10a and Table 10b to test whether there were more visits to either the lactic acid treatment port or the filtered air control port. There was no difference in visits to either port for males (Sign Test, 19 differences, $x = 6$, $P = 0.084$) or for females (Sign Test, 26 differences, $x = 10$, $P > 0.212$).

Table 11. *Aedes albopictus* mosquito responses to octanol treatment port or filtered air control port by males (M) and females (F)

Experiment	Number	Octanol	Filtered Air Control	Photo-period
1	9 M 10 F	0 M 1 F	4 M 4F	LL
2	9 M 10F	5 M 4F	1 M 3F	LL
3	1 M 10F	1 M 2F	0 M 3F	16L:8D
4	0 M 8 F	0 M 1F	0 M 3F	12L:12D
5	0 M 18F	0 M 2F	M 3F	12L:12D
6	10 M 14F	5 M 8F	2 M 4F	LL
7	2 M 5 F	0 M 2F	2 M 2F	12L:12D
8	3 M 13F	0 M 1F	0 M 7 F	16L:8D
9	7M 8 F	0 M 1 F	1 M 0 F	16L:8D
10	3 M 15F	0 M 0F	1 M 2F	16L:8D
11	6 M 19F	2 M 6F	1 M 4F	16L:8D

There were no significant differences for *Aedes albopictus* males in the number of visits they made to the octanol treatment port or the filtered air control port (Sign Test, 8 differences, x = 4, P = 0.637) (Table 11). There was also no significant difference in visits to either port by by *Aedes albopictus* females (Sign Test, 10 differences, x = 4, P = 0.377).

Table 12. *Aedes albopictus* mosquito responses to octanol or lactic acid treatment ports by males (M) and females (F)

Experiment	Number	Octanol	Lactic Acid	Photo-period
1	2 M 2F	2 M 2 F	0 M 0F	LL
2	15 M 20Fe	4 M 4F	1 M 5F	LL
3	8 M 13 F	3 M 5 F	2 M 3F	LL
4	7 M 11F	3 M 5 F	1 M 1 F	LL
5	4 M 4 F	1 M 1 F	2 M 1 F	16L:8D
6	0 M 12F	0 M 3F	0 M 4F	12L:1D
7	4M 7F	0 M 1 F	1 M 2F	12L:12D
8	3 M 7F	2 M 1 F	0 M 3F	16L:8D

There were no significant differences in the number of visits that male *Aedes albopictus* mosquitoes made to either the lactic acid treatment port or the octanol treatment port (Sign Test, 7 differences, $x = 2$, $P = 0.227$). Females also showed no significant difference in visits (Sign Test, 7 differences, $x = 3$, $P = 0.500$) (Table 12).

CHAPTER IV

DISCUSSION

Photoperiod and Circadian Rhythms

The seasonal photoperiods involved in this phase of experimentations encompassed constant darkness (DD), 16 L:8D (Summer), 12L:12D (Spring/Fall), and constant light (LL). Olfaction is the overriding sensory and behavioral modality of mosquities, including male and female *Culex restuans* mosquitoes. However, it can be postulated that although in olfactometric studies, *observed* behavior may appear to be the same, the *mechanisms* involved may be different when there is a transition between the 24-hour diel, and seasonal changes. A good example of this might be after the ingestion of a blood meal or change in seasonal nectar feedings among males and females in field studies. Carpenter and LaCasse (1955) reported that *Culex restuans* mosquitoes had two peaks of activity, with the first peak occurring in the spring-early summer, and the second peak in the late summer-early fall (autumn).

The rearing of adult *Aedes albopictus* mosquito colonies under 24-hour circadian rhythms, and designated seasonal photoperiod regimes began with individualized egg depositions being placed in four incubators at the Old Dominion University Entomology Laboratory located in Norfolk, Virginia. Incubator One was designated as (DD photoperiod regime), Incubator Three was (16L:8D) or summer, Incubator Four was (12L:12D) classified as spring/fall, and Incubator Five was “free running constant light” at (LL). In the second part of this discussion, circadian rhythms and photoperiodic studies involving non-blood fed female and male *Aedes albopictus* were compared with their male and female *Culex restuans* counterparts. The compilation of data was derived from 81 experiments utilizing Filtered Air Tests, in addition

to known attractants (Acetone, Acetone + Lactic Acid, Carbon Dioxide, Lactic Acid, Octenol, and Octenol and Lactic Acid) and floral scents that included (Rose absolot, Lavender Oil, Sweet Orange, Cilantro flowers and leaves and Mountain Mint flowers and leaves.

Clements (1999) and Taylor and Jones (1969) stated that constant darkness (DD), is a free – running rhythm. Moreover, they observed from kymographic experimentation, that the level of flight activity in female *Aedes egypti* mosquitoes was low in constant darkness, but that the cycle itself was continuous with a period of about 22.5 hours. In addition, Taylor and Jones (1969) reported that male adults were also less active but followed a similar behavioral pattern as their female conspecifics. In other entomological investigations, (Haddow et al. 1961), (Gillett et al. 1962), and (Gillett 1961) concluded that *Aedine* activity being a cyclical process, was controlled by an endogenous rhythm. The results of the five olfactometric (DD) experiments conducted in the ODU Entomology Laboratory revealed the following information: In the constant dark reactions, adult male and female *Culex restuans* mosquitoes were observed to have the lowest flight activity. A plausible explanation for this low flight activity can perhaps be provided in scientific publications by Lanciani (1992) and Lanciani and Edwards (1993), whereby the effects of photoperiod on wing length and wing area were studied in the mosquito species *Anopheles quadrimaculatus*. It was reported that those mosquito offspring reared under shorter photoperiods (8L:16D), had disproportionately greater wing length, wing area, and body weight than those mosquitoes reared under longer photoperiods (16L:8D). Therefore, given these results gleaned by observational studies and quantitative documentation may suggest that disproportionately long wing length and wing area may contribute to decreased flight activity in *Anopheles quadrimaculatus*. Although the focus of this research paper is confined to olfactory responses in *Culex restuans*, and resultant mosquito behavior with regard to exposure to selected

chemical odorants, perhaps wing length and wing area could be considered in terms of decreased flight activity under photoperiodic conditions of constant darkness. In terms of mosquito reproduction, the (DD) offspring produced were the lowest in number, with only a total of 57 progeny being split into 41 males and 16 females.

In studies conducted by Lanciani (1992) and Lanciani and Edwards (1993), it was demonstrated that male and female *Anopheles quadrimaculatus* mosquitoes reared under 16L:8D photoperiod regime had shorter wing length, wing area, and body weight. The results of these studies suggest that a decrease in wing length, wing area, and body weight could facilitate increased flight activity since the wings are not disproportionately lengthened, and there is less body weight to hamper flight towards prospective odorants. In addition, although *Anopheles quadrimaculatus* are nocturnal, as is the *Culex restuans* species in this research study, it should be noted that with regard to wing length, wing area, and body weight, there may be diversity of behavioral responses as per flight activities, and the pursuit of attractive odorants in other mosquito species yet to be studied.

In the present study, the phase setting effects of the 12L:12D photoperiod regime included abrupt transitions of light – on at 10:00 pm, and light – off at 10:00 am, for those *Culex restuans* mosquito experiments cited in this current study. It is also interesting to denote, that in earlier research investigations conducted on *Aedes aegypti*, A study discovered that the photoperiod ranges of (12L:12D) to (16L:8D) had endogenous phased peaks of light-on and light-off that approximately coincided with each other. This was attributed this to insufficient summer temperature in this particular *Aedine* species with widespread distribution. Taylor and Jones (1969) reported that 24.8° C was the minimum temperature that coincided with geographical latitude, where there would be a maximum of 16 hours of daylight. Moreover,

Taylor and Jones (1968) stated that prospectively, the combination of species distribution is limited by summer day lengths (circadian rhythms) and latitude (exogenous factor). Therefore, it is these factors that control the timing of flight activity in this species of mosquito. In summation, in terms of circadian rhythms and photoperiod regimes, flight activity is correlated with light duration, in the Taylor Jones (1969) experiments that were set at 70 lux, in a 24-hour time period.

In retrospect, it does seem plausible to suggest that the (12L:12D) and (16L:8D) photoperiods do coincide in the *Culex restuans* mosquitoes under study. Both (12L:12D) and (16L:8D) are controlled endogenously and are not free-running rhythms like (DD) and (LL). In terms of their reproduction, (12L:12D) photoperiod regime had the second highest number of offspring produced (42 males, 36 females) as cited in Table 4-3 in this current study. By comparison, (16L:8D) produced (92 males and 74 females).

Clements (1999), defined free-running rhythms such as (DD and LL), as conditions of oscillators running at their natural frequencies under constant conditions, when not entrained by environmental time cues. In this section, the behavioral and olfactometric effects of LL will be discussed by presenting select research publications (Jones 1976, Taylor and Jones 1969, Chiba 1990, Clements 1999), and comparing them with the results compiled in this current study.

In undertaking research investigations into the persistence of LL in the mosquito, *Culex pipiens fatigans* (Wied), Jones (1976) reported that although constant light (LL) was a free-running rhythm, behavioral rhythms such as flight activity, cannot be negated, as a weak cyclical activity still exists under LL conditions. Moreover, it was observed that the LL cycle was unimodal, with a temporal period of approximately 26 hours duration. In additional investigative

studies, Jones (1976) cited the behavioral similarities between *Anopheles gambiae* and its nocturnal counterpart, *Culex pipiens fatigans* (Wied). In citing the similarities between these two Dipterans, Jones (1976) stated that the flight activities of *Culex pipiens fatigans* (Wied) and *Anopheles gambiae* were similar. These similarities in flight activities also apply to male and female mosquitoes in these respective species under study. However, the differences between *Culex pipiens fatigans* (Wied) and *Anopheles gambiae* involves in what is defined as different threshold sensitivities. In *Culex pipiens pallens* (Wied), the oscillator in 50 lux was persistent in this species, but not in *Anopheles gambiae*. For further clarification and understanding of this circadian data, Truman's Hypothesis can be applied, which states that circadian clocks can be divided into two types. In Type 1, light seems to act directly on the oscillator, stopping it in constant light (LL). In Type 2, the photoreceptor and oscillator are separate, therefore the rhythm is free running in LL. Hence, in daily behavioral activities – ie. circadian activities, the second part of Truman's Hypothesis applies in this dipteran behavioral scenario.

In documenting the circadian rhythms of mosquito behaviors (Chiba 1974), stated that the diel rhythm could be demonstrated by holding mosquitoes in constant light. These constant light (LL) conditions enabled the rhythm to escape environmental controls, thus creating a free-running rhythm to emerge with its own circadian period. This circadian period is weaker when compared to other non free-running rhythms. However, it is independent, and its molecular mechanisms, and how they effect mosquito olfactorial responses to odorants either in unitary or binary blends have yet to be elucidated.

Studies conducted on *Culiseta incidens* exhibited what transpired when those mosquitoes were subjected to a LL regime. It was noted, that when the period of onset was lengthened by 0.42 hours, it created a day length greater than 24 hours. When the offset was shortened, the

active phase was reduced daily. This created the formation of a V-shaped pattern. Under these illuminatory circumstances, with the increase in light intensity under the (LL) regime, the ontowards effect on the rhythmicity becomes either faint or disappears.

Under the above cited conditions, Aschoff's circadian rule applies which states that the free-running period lengthens with an increase in light intensity. Where there is a transfer from (DD) to (LL) there is a coexistence of nocturnality to diurnality. The concepts of nocturnality and diurnality provides the basis for the two – oscillator model. With regard to the two – oscillator model, it is plausible to inquire of the location of the oscillator. In a paper published by Page and Koelling (2003), it was reported that the oscillator was located in the optic lobe. However, when the mosquito *Culex pipiens pallens* was deprived of major portions of its optic lobe, only a weak circadian rhythm was detectable and entrainable. Hence, it would appear that for a mosquito to elicit olfactorial responses to select odorants, the presence of the correct wavelength to stimulate differential light intensities under (LL) conditions would be required.

Taylor and Jones (1968) reported on the results of scientific data compiled on the circadian rhythm of flight activities in the diurnal mosquito, *Aedes aegypti* regarding the phase-setting effects of light-on and light-off. The results of this scientific data are as follows: In the circadian rhythm whereby the *Aedes aegypti* mosquitoes were reared under constant conditions, such as constant light (LL), there is an increase in the level of flight activity. This cycle encompasses a period of about 26 hours. The first peak manifests itself approximately 13 hours after light-on designated as normal time. Light-on and light-off have phase-setting effects on the rhythms' activities. The temporal duration for light-off is 22-23 hours, while the time for light-on is 13-14 hours. The free-running period for (DD) is 22.5 hours, and for constant light (LL), it is 26 hours. In investigating the separate effects of light-on and light-off, light-activated

experimentation, was conducted on the diurnal mosquito, *Aedes aegypti*. Taylor and Jones (1969) obtained the following data from these light-activated experiments. There were kymographic peaks of activity that included the light-on time period of 13-14 hours, and the light-off time period of 26 hours. The free-running rhythm in (LL) was 26 hours. Those female *Aedes aeygpti* mosquitoes reared under the LD 4:20 regime, and then transferred to constant light (LL) exhibited peaks of activity that were not very distinct. Those female *Aedes aegypti* mosquitoes reared under the LD 20:4 regime, and transferred to LL conditions, caused the first peak in LL to emerge 11-12 hours after light-on. However, beyond the first peak, subsequent peaks were more diffuse and less distinctive to identify when mosquito populations were viewed collectively. Moreover, this lack of peak distinctiveness became more pronounced when mosquito activities were viewed based on the flight activities of individual mosquitoes.

How does flight activity apply to olfaction? How is it relevant to this research study on olfaction in male and female *Culex restuans* mosquitoes? Flight activity applies to olfaction, because it is directly linked to behavioral responses (ie) these responses being mating among male and female conspecifics, feeding to include imbibing of sugar contents of specific flowers for nutrition, and flight energy in both males and females, specifically for hematophagous behavior among female mosquitoes for the purposes of egg development, and oviposition for egg maturation into adults to perpetuate the species. All these basic behaviors, and more specific behaviors are conducted and expressed at different times during the diel cycle. Olfaction is relevant to this particular study because it is intended to elucidate what specific behaviors may be expressed at different times of the day.

Of interest in this discussion section, is the phenomenon of free-running rhythms which occur under (DD) and (LL) conditions. In comparing both (DD) and (LL) regimes, there are both

similarities and differences whose subtleties may have significantly divergent effects in terms of male and female mosquito responses to select odorants, and their olfactorial behavioral responses.

How do the results of this study correlate with the phase-setting experiments conducted by Taylor and Jones (1969)? Although olfactory results in this study were based on behavioral responses of male and female *Culex restuans* mosquitoes to select odorants in a dual port olfactometer rather than in results obtained via kymographic studies, the "equalization" of female mosquitoes in the olfactometer to the preferential status of CO_2, is similar to results obtained when (20L:4D) is changed to (LL) in the kymograph. It was noted that in the kymographic recordings the first peak was distinct, but subsequent peaks were less distinct, and ultimately more diffuse. Therefore, peak diffuseness in the kymograph could be considered as analogous to equalization in the olfactometer.

What is the relationship as per odorant effectiveness under (DD) and (LL) conditions? Is it plausible to consider that odorant binding receptors (obr) are fewer in number, and less complex than those receptors associated with (12L:12D) and (16L:8D) photo regimes? In addition, are fewer receptor numbers equivalent to less binding time for the odorant? Will physiological zero time be significantly shorter than binding times under (12L:12D) and (16L:8D) conditions? How will these odorant binding receptors be similar or different in male and female *Culex restuans* mosquitoes investigated in this study? How will there be similarities and differences in the approximately 3,200 species of mosquitoes cataloged?

Under (LL) conditions, the constancy of light exposure prior to any phase-setting applications, would it be plausible to consider that constant light eventually shortens the odorant

binding capacity, and therefore leads to more diffuse peaks collectively, and in terms of studying individual mosquitoes kymographically, or in recorded observations in the olfactometer? How are the morphologies of the recepticles and geometric patterns of the odorant binding protein? How do they differ in various species? Lastly, what are the mechanisms whereby the repressor of olfactory responses in (DD) and (LL) are manifest?

The seasonal photoperiods involved in this phase of experimentations in the present study encompassed (DD), 16 L:8D (Summer), 12L:12D (Spring/Fall), and (LL). Olfaction is the overriding sensory and behavioral modality of mosquities, including male and female *Culex restuans* mosquitoes. However, it can be postulated that although in olfactometric studies, *observed* behavior may appear to be the same, the *mechanisms* involved may be *different* when there is a transition between the 24-hour diel, and seasonal changes. A good example of this might be after the ingestion of a blood meal or change in seasonal nectar feedings among males and females in field studies. Carpenter and LaCasse (1955) reported that *Culex restuans* mosquitoes had two peaks of activity, with the first peak occurring in the spring-early summer, and the second peak in the late summer-early autumn.

The rearing of adult *Aedes albopictus* mosquito colonies under 24-hour circadian rhythms, and designated seasonal photoperiod regimes began with individualized egg depositions being placed in four incubators at the Old Dominion University Entomology Laboratory located in Norfolk, Virginia. Incubator One was designated as (DD photoperiod regime), Incubator Three was (16L:8D) or summer, Incubator Four was (12L:12D) classified as spring/fall, and Incubator Five was “free running constant light” at (LL). In the second part of this discussion, circadian rhythms and photoperiodic studies involving non-blood fed female and male *Aedes albopictus* were compared with their male and female *Culex restuans* counterparts.

The compilation of data was derived from 81 experiments utilizing Filtered Air Tests, in addition to known host odors (Acetone, Acetone and Lactic Acid, Carbon Dioxide, Lactic Acid, Octanol, and Octanol and Lactic Acid) and floral scents that included (Rose absolot, Lavender Oil, Sweet Orange, Cilantro flowers and leaves and Mountain Mint flowers and leaves.

Mosquito Response to Odorants

Host Odors

Acetone (CH_3COCH_3) is the simplest example of a ketone. It is defined as an organic compound containing a carbon atom connected to an oxygen atom by a double bond along with 2 carbon atoms (Porter and Kaplan 2011). In terms of its molecular mass, acetone weighs 58.08 g/mole. Small amounts of acetone are produced in the human body by the process of decarboxylation of ketone bodies. In diabetics, there is an increase in acetone production, which combined with CO_2 can provide the foundation for mosquito attraction towards a prospective host. In a paper published by Bernier et al (2003), on the synergistic attraction of 6 to 8 day old *Aedes aegypti* (L) female mosquitoes, it was postulated that the synergistic blend of (L-LA + acetone), in *Aedes aegypti* female mosquitoes produced an attraction similar to the synergistic behavioral blend of (CO_2 + acetone), whereby the role of CO_2 served primarily as an activator, and secondarily as an attractant.

In related video studies on the effects of acetone, octenol, and phenols on tsetse flies ie *Glossina pallidipes* (Austen), Brady and Griffiths (1993) observed and reported that no odors other than CO_2, or combinations of CO_2 with other chemicals activated and stimulated upwind flight by tsetse flies. However, when acetone or octenol were released in a unitary capacity, rather than in a synergistic blend, no upwind attraction or movements were elicited in the tsetse

flies. In this study, there were 3 observational field studies associated with the video. They are as follows: downwind flight, upwind flight, and crosswind flight. The results of these flight investigations concluded that the mixture of 4:1:8 associated with the octanol/phenol combination is an effective stimulus for upwind orientation in tsetse flies analogous to the effect of CO_2. However, acetone independently may produce a lesser response. It was suggested that acetone may be more involved in the potentiation of visual responses in the identification of prospective hosts for the acquisition of blood sources.

Based on the reports published by Bernier et al. (2003) on *Aedes aegypti* females, and Brady and Griffiths (1993) on *Glossina pallidipes* (Austen), it would appear to suggest that acetone used in a unitary capacity, is ineffective for an olfactorial response. In terms of dosage, Bernier et al (2003) in combining acetone, dimethyldisulfide, and dichloromethane used 400 µl of the chemical, while the acetone evaluation conducted in the present study utilized only 10 µl of acetone. Hence, it would appear that dosage and synergistic blends (acetone + L-LA) or (acetone +CO_2), are required to evoke an olfactometric response in dipterans. Moreover, Brady and Griffiths (1993) utilized a synergistic mixture of 4:1:8 when combining the effects of acetone, octenol, and phenols in tsetse flies. Age can be an important factor in olfactometric responses to the chemicals under investigation, as there must be a correlation between increased age and olfactorial prowess. The ages of the female *Aedes aegypti* mosquitoes were 6 to 8 days old. In terms of acclimation time, *Aedes aegypti* mosquitoes were retained in the olfactometer's holding area for 1 hour, while the *Culex restuans* were given 10 minutes to acclimate to the olfactometer's environment in the present study. To date, no studies have been conducted on the molecular masses of various odorants utilized in olfactometric studies. Perhaps if undertaken, insights can be gained by studying the nature of the bondings, and how these bonds interact with

other chemicals. What role do wind currents play in the dissipation and attachment of acetone, CO_2, and lactic acid molecules to their receptors? Perhaps acetone alone in a unitary capacity, does not evoke an olfactometric response because acetone receptors might be fewer in number, smaller in size, and their responses to the chemical may change during the course of the 24-hour diel. Responses to acetone may differ according to species, as *Aedes aegypti* mosquitoes are diurnal, and *Culex restuans* are nocturnal. Hence, with diurnality and nocturnality, there may be differences in receptor shape, number, and distribution, and therefore in terms olfactory response. It is interesting to denote, that in both Bernier et al (2003) and Brady and Griffiths (1993) scientific studies, there was no mention of time of day, and LD photoperiod regimes in their respective publications.

It could be postulated that the lack of response of the male and female *Culex restuans* mosquitoes to acetone could be explained via the chemical nature of the odorant itself. In these two experiments, 10 µl of acetone were used only in a unitary capacity. However, in a paper published by Bernier et al. (2003), they postulated that 400 µl of acetone would have a greater olfactorial response if utilized in a synergistic blend along with lactic acid. It was further observed that acetone would have the same excitatory effect as CO_2 combined with lactic acid (LA). For when acetone combines synergistically with LA, this odorant will be expressed at a level greater than or equal to lactic acid + CO_2.

In recent molecular based studies utilizing microarray techniques on *Anopheles gambiae*, Rund et al. (2011), reported that in a 24-hour period, rhythmic genes along with their full complements, were responsible for the production of two clocks. The first clock involved the criteria of constant environmental temperature and light conditions. The second clock encompassed the light/dark (LD) cycle that will alternate between excitatory and inhibitory

(refractoriness) state in an organism's endogenous circadian clock. Hence, with regard to comparing male and female responses to CO_2, perhaps females have a greater number of excitatory receptors for CO_2, while males have less excitatory receptors. However, males may possess a greater number of inhibitory receptors, or receptors that have a prolonged refractory period, thereby accounting for a greater incidence of non-preference for CO_2 among male *Culex restuans* mosquitoes.

Carbon dioxide (CO_2) is defined as a chemical compound composed of two oxygen molecules *covalently* bonded to a single carbon atom. Carbon dioxide is produced by all animals, plants, fungi, and microorganisms during the process of respiration. In particular, it is utilized by plants during the process of photosynthesis. The molar mass of CO_2 is 44.0095 g/mol. Its molecular shape is linear (O=C=O) (Porter and Kaplan 2011).

Despite the abundance of papers published on CO_2, the sequences of the host-finding abilities of this odorant in mosquitoes, is still poorly understood. In a comprehensive review of the entomological literature, on the host-finding abilities of CO_2, Gillies (1980) defined the dual terminology and roles of activator and attractant in mosquito behavioral responses. In addition, the contrasting results of various research investigations utilizing CO_2 were presented in this review.

In his paper, Gillies (1980) referred to activation as the initiation of flight activity. This was followed by orientation to the chemical stimulant whereby CO_2 was emitted by the combination of the host's breath, and the warm convection currents provided by the host's skin. The mechanism of attraction was defined as the culmination of two behavioral responses that included kinesis + optomotor anemotaxis.

However, the expression of CO_2 in olfactometric experimentation exhibited a variety of effects. In the laboratory, if the diameter of the Y-shaped olfactometer was too small, with stem 8.6 mm and arms 6.0 mm, the architectural conditions would be considered too confining, and therefore limit the olfactorial responses (Gillies 1980). In 1968, Mueller reported that CO_2 was repellent at 1%-33%, but a higher level was attractive at 50%. Hence, the effect of CO_2 concentration demonstrated the duality of either a (+) or (-) response when exposed to a targeted mosquito population. Studies on the effects of synergism showed that when CO_2 was absent, the effects of other odorants present such as lactic acid and octenol were significantly reduced. Moreover, when CO_2 was utilized in a singular capacity, and subsequently removed from the host's expired breath, the numbers of mosquitoes that were attracted to the host were also reduced.

Takken and Kline (1989) presented a paper whereby they studied the olfactory behavioral responses of mosquitoes to CO_2 and octenol under field conditions. The sites under study were located in the environs of Snake Bight and Lower Swanee reserves in Florida. At the Snake Bight sight, when CO_2 was utilized, the catch of *Aedes taniorhynchus* increased two-fold, while the populations of *Anopheles atropos* and *Anopheles crucians* increased ten fold. When only octenol was used, the number of mosquitoes caught in baited traps were not to be significantly different than those caught with standard traps. However, the mosquito species caught only when octenol was given, included a higher number of *Aedes taniorhynchus, Aedes crucians*, *Aedes quadrimaculatus* (Say), and *Wyomia mitchelli*. It was also found that when both CO_2 and octenol were used synergistically, there was a two-fold increase in the number of mosquitoes caught in the traps. It was concluded, based on the data compiled in this study that CO_2 serves as an attractant, and responds to warm convection currents emitted by the host.

In a paper evaluating the effects of CO_2, 1-octan-3-ol, and lactic acid used as baits in mosquito magnet pro-traps for *Aedes albopictus,* (Hoel et al. 2007) reported that few known attractants were considered effective in both laboratory studies and field tests. It was communicated, that the olfactorial behavioral responses of these three odorants by *Aedes albopictus* has not been definitively evaluated, or well documented in the entomological literature. Subsequent field studies on the efficacy of baited traps revealed the following: When octenol was employed in a unitary capacity, there appeared to be a decrease in trap collections of *Aedes albopictus*. The combination of octanol and lactic acid however, had a synergistic effect, suggesting that *Aedes albopictus* responded to lactic acid as an odorant. The combination of CO_2 + lactic acid + octenol resulted in the greatest *Aedes albopictus* collection in the field. Hence, the results of these entomology field studies utilizing baited traps established that CO_2 was the universal attractant in insects in general and in mosquitoes in particular and that its presence greatly enhanced the synergistic effects of other chemical odorants that influenced mosquito behavioral responses.

In contrasting studies conducted on the hematophagous bug, *Triatoma infestans* which is the purveyor of Chagas Disease in southern South America, Barrozzo and Lazzari (2004) studied the behavioral olfactory responses to CO_2 and other host odors. In this paper, the authors demonstrated the presence of two endogenously controlled temporal windows during the time period of the scotophase or dark period of the diel cycle. These two subdivision cycles of the scotophase included dusk (which encompassed the active host-seeking phase), and dawn (which included the phenomenon of refuge searching speculatively against predation of the species). With experiments set at 12L:12D, and the early and late scotophase set at 12:00 – 17:00 hours, and 19:00 – 0:00 hours respectively, the following results were obtained: The threshold level for

(+) locomotor responses of *Triatoma infestans* to threshold levels was set at 300-400 ppm. When combined with L- lactic acid at 100 µg, the synergistic effects along with 300µg of CO_2 were recorded. At 100 µg, 1-octen-3-ol was interpreted as an orientation odorant for *Triatoma infestans*. The inclusion of 100 µg of D –lactic acid elicited no locomotor responses in the *Triatome*. These premiere olfactorial series of experiments conducted by Barrozo and Lazzari (2004) demonstrated that CO_2 + L- lactic acid + 1-octen-3-ol functioned as attractants in Triatomes. Most importantly, it was established that sensitivity to CO_2 varied throughout the course of the 24-hour day.

In an entomology paper published by Barrozzo et al. (2004), it was the intent to investigate the existence of a daily 24-hour rhythm as per the locomotor sensitivity and olfactorial responsiveness of CO_2 in *Triatoma infestans*. In addition, it was the intent of this investigation to determine if the rhythm was under exogenous or endogenous control.

In these experiments, the 4^{th} instar larvae were reared under a 12L:12D photoperiod regime. However, as adults the *Triatoma infestans* were kept in constant darkness (DD), and subjected to the CO_2 gas. Interpretation of these results by Lazzari (1992) demonstrated that CO_2 observations recorded 2 bursts of locomotor activity: one in the early evening (dusk), and a similar amount of activity in the late evening (dawn). These results indicated that locomotor activity was under the influence of an endogenous oscillator. In contrast, when *T. infestans* insects were tested under the conditions of constant light (LL), the activation and attraction towards CO_2 yielded a (-) response. The results of these entomology experiments revealed that: The responsiveness towards to a chemical cue such as CO_2 was the result of a circadian (endogenous rhythm) in these hematophagous insects. The locomotor activity in *T. infestans* was classified as bimodal.

In retrospect, regarding the circadian experiments conducted on *Culex restuans* mosquitoes, and the insect *Triatoma infestans*, perhaps the following points can be considered. In terms of comparative nutritional status, male and female *Culex restuans* mosquito larvae were reared on fish flakes (Tetramin) every other day, while third and fourth instar larvae from *T. infestans* were fed weekly on hens until molting. Adult *Culex restuans* mosquitoes were fed on 10% sucrose solution, while adult *T. infestans* were starved for a period of 10 to 20 days until entomology experiments were initiated (Barrozo et al. 2004). In this regard, does starvation improve the ability to respond to CO_2? In terms of age comparisons, the *T. infestans* were 10 to 20 days old upon experimentation, while the *Culex restuans* mosquitoes tested were post eclosion. Does age modify olfactorial responses? Do older insects have a more sophisticated olfactory system in terms of structure and physiological response? The experiments of Barrozo and Lazzari (2004) established that CO_2 exposure of *Triatoma infestans* was classified as bimodal with pulsating bursts of CO_2 emissions. Is the emission of pulsated CO_2 gas more effective in eliciting an oriented response of *T. infestans* to the odorant, as compared to the movement of mosquitoes in steady stream currents in an olfactometer? Regarding olfactory sensitivity in *Culex restuans* mosquitoes, how does constant darkness (DD) laboratory conditions compare with (DD) conditions in *T. infestans*? Specifically, (DD) conditions in the *Culex restuans* mosquitoes under study revealed according to gender, that female mosquitoes showed a greater (+) preference for CO_2, while their male conspecifics displayed a greater tendency towards non-preference. In the *T. infestans* studies, gender was not specified. It should also be considered, that the *Culex restuans* under study, excepting the P_1 generation, were non-blood fed. What would be the correlation between blood-feeding, and olfactorial sensitivity to host odors such as a designated activator or attractant as CO_2?

It should be noted that the lack of repsonse to carbon dioxide from the singular *Aedes albopictus* male mosquito tested under controlled laboratory conditions should be considered a microcosm of this phenomenon that reflects the pattern of carbon dioxide field studies. That is, when carbon dioxide traps are constructed under field conditions, there is a paucity of male mosquito presence. Instead, the predominant population found in these carbon dioxide traps are female mosquitoes from a variety of species (Steinbrecht 1996). Therefore, the very presence of male mosquitoes in the carbon dioxide field traps may be attributed to the following possibilities: coincidence, the pursuit of females for courtship and reproductive purposes, the lack of carbon dioxide receptors in the male mosquito antennnae, and if carbon dioxide receptors are present, they lack the *critical* threshold number of receptors to elicit behavioral response(s) to the presencee of the odorant at least in olfactometric testing involving male *Aedes albopictus*. Lastly, it should also be considered that there are 3,200 species of mosquitoes. Although the vast majority of these species have yet to be tested and their physiological and genetic mechanisms elucidated, there could be a possibility that in some of these dipteran species, there is a critical threshold number whereby carbon dioxide does eleicit behavioral responses from some species of male mosquitoes.

Culex restuans mosquitoes had an increase in attraction among females to carbon dioxide. The test was approximately 1.5 to 2 hours in length. The photoperiod imposed was (16L:8D), and *Culex restuans* are designated as nocturnal mosquitoes.

Lactic acid, a 3-carbon organic acid is produced by anaerobic respiration. It is the end-product of glycolysis, which provides energy anaerobically in skeletal muscles during heavy exercise. In a study, lactic acid appeared to have no significant effects on the olfactorial behavioral responses in male *Culex restuans* mosquitoes. For under the LL photoperiod regime,

no preferential responses were observed and recorded in Experiment 5. These data contrast with the numerical data received under DD photoperiod conditions of a free-running rhythm. For lactic acid, in the DD designated free-running rhythm, nine males were attracted to lactic acid, while LL showed no attraction to lactic acid in the olfactometric environment. More observational examples of lactic acid attractiveness in olfactometric experimentation includes the 16L:8D Experiment 2, whereby only one male *Culex restuans* displayed any preferential attraction to lactic acid, and in Experiments 1 and 3 under photoperiod 12L:12D, whereby one male mosquito in Experiment 1, and one male mosquito in Experiment 3 exhibited a (+) preference for lactic acid respectively.

Lactic acid (LA) is a chemical that plays a role in several biochemical processes. It is a carboxylic acid with the molecular formula $C_3H_6O_3$. Lactic acid is a chiral and has two optical isomers. They are as follows: L- (+) – LA or (S) – LA. Its mirror image is known as: D (-) – LA, or R-LA. Of the 2 isomers, it is L- (+) – LA that is biologically the most important. Lactic acid is found in human blood with the concentration of blood lactate usually at 1-2 mmol/L at rest, and 20 mmol/L during exercise. The molar mass of lactic acid is 90.08 g/mol. The molecular formula of lactic acid is $C_3H_6O_3$ (Porter and Kaplan 2011).

In investigating lactic acid sensitive receptors on the antennae of *Aedes aegypti* females, Davis and Sokolove (1976) reported that of the four morphological receptors, it was the sensilla basiconica (A_3 cells), that were sensitive to lactic acid. To further elucidate their findings, Davis and Sokolove (1976) examined 136 grooved pegs from the antennae of 77 adult female *Aedes aegypti* mosquitoes. The results of these experiments revealed the following information: There were two types of afferent neurons that had an effect on the most prominent behavior, namely

host–seeking. These two neurons classified as either excitatory or inhibitory, were found in the same sensilla.

In addition, behavioral experiments conducted on CO_2, revealed no CO_2 sites were present on the sensilla basiconica, but sites for CO_2 were present on the maxillary palps (Kellogg 1970, Zwiebel and Takken 2004). Moreover, when CO_2 was used in a unitary capacity, it elicited no behavioral response from *Aedes aegypti* either as an activator or as an attractant. However, when CO_2 was combined with LA, it created a *synergistic* effect of central origin (Acree et al. 1968). The conclusions drawn by this microelectrode study on lactic acid were: It is a chemical that is a product of human sweat. It is a mosquito attractant. The sensilla basiconica (A_3) cells are *specific* for the lactic acid receptor.

In contrasting olfactometric studies, on female *Anopheles gambiae* a nocturnal mosquito, Braks et al. (2001), analyzed the two components from human sweat – ammonia and lactic acid. The goals enacted in this study, were to investigate fresh and incubated sweat samples, and to analyze and interpret the two odorants conducive to attracting 4 to 8-day old *Anopheles gambiae* mosquitoes.

In comparing the effects of these two odorants, it was found that selective removal of lactic acid from sweat samples did not affect its attractiveness in *Anopheles gambiae*. However, in contrast, *Aedes aegypti* a diurnal mosquito, had a stronger predilection for L-lactic acid (L–LA). In terms of analyses of ammonia (NH_3), Braks et al. (2001) provided a premiere report of how *Anopheles gambiae* responded to this chemical. It was so noted, that in *Anopheles gambiae*, (NH_3) was an important kairomone, while in *Aedes aegypti*, it was L-LA, with the presence of NH_3 serving only to potentiate the attraction of L-LA in this mosquito species.

In this scientific paper, 5 to 14-day old mosquitoes were researched via a dual port olfactometer to determine and evaluate the degree of anthropophily in female *Anopheles gambiae*. In these series of experiments, Dekker at al. (2002) examined sweat and sebaceous glands for the presence of L-LA receptors, as their differential levels of L-LA on human skin correlated with the differential attractiveness of mosquitoes towards their prospective hosts. Tests conducted on *Anopheles gambiae* revealed that the combination of CO_2 + acetone + NH_3 along with L-LA *collectively* played a role in host – finding. In addition, the concept of host – finding encompassed receptor distribution for L-LA, quantification of receptors, compartmentalization of receptor function(s), and the nature of excitation and inhibition of the (A_3 cells). In these studies, *Anopheles gambiae* exhibited differential levels of attractiveness on the skin. In contrast, the behavioral response in *Aedes aegypti* showed a strong synergism that existed between CO_2 and L-LA, while in *Anopheles gambiae*, the synergistic attraction between CO_2 and L-LA, was only slightly more than the effect of these odorants separately.

Bowen et al. (1994) conducted investigative research on the behavioral physiology of LA sensitive receptors in the mosquito, *Aedes altropalpus*. This mosquito species under these studies were classified as autogenous. The LA sensitive receptors, located in the mosquitoes' antennae, are controlled by peripheral receptors which function in the control of mosquito – host responsiveness. Electrophysiological studies undertaken on 158 antennal sensilla from 44 *Aedes altropalpus* female mosquitoes revealed the following information: Only the sensilla basiconica (A_3 cells), were sensitive in LA receptors. Sensitivity to LA sensitive receptors, were age dependent in the autogenous *Aedes altropalpus*. Low sensitivity to LA excited cells occurred when *Aedes altropalpus* mosquitoes were less than 12 hours old. However, when the ages of these dipterans reached 12 to 24 hours, there was high sensitivity in these LA receptors. In

contrast, electrophysiological studies conducted on the anautogenous and diurnal mosquito, *Aedes aegypti* revealed that that spike potential in this species of similarities, was non existant. However, despite contrasts regarding the autogenous *Aedes altropalpus*, and the anautogenous *Aedes aegypti*, there were comparative similarities: Gravid females of both species were non–host responsive, because the receptors were downloaded. Parous mosquitoes of both species were host responsive. Non responsiveness was primarily age related as mosquitoes less than 12 hours old had low sensitivity of receptors.

In consideration of L-LA, its receptor, and the olfactometric results of experimentation on male and female *Culex restuans* mosquitoes, there are some questions that come to mind. Namely, how is LA effective or expressed at different times of the day? Does a free running (DD) photoperiod influence preference? Is this preference gender-related? Logically, since *Culex restuans* is behaviorally active at night, one would think more females would show a greater (+) preference for LA than their male conspecifics. How greatly does a free-running photoperiod influence mosquito behavior, and ultimately the mechanisms involved in host-seeking? The results of the opposite free-running rhythm (LL) was examined in the experiments conducted in the present study. It is interesting to denote, that of the female mosquitoes detected, both showed a preference for LA, while in contrast, of the three males tested, none displayed any attraction for LA. The results of this limited study would appear to suggest that light plays an important role in mosquito response to LA, as more females were attracted to LA under 16L:8D and 12L:12D photoperiod regimes.

Regarding the scientific publication by Braks et al. (2001) on two components of human sweat – LA and NH_3, it was interesting to denote that when LA was selectively removed from sweat samples in *Anopheles gambiae* (a nocturnal mosquito), the degree of attractiveness to LA

was not diminished, while in *Aedes aegypti* (a diurnal mosquito), the diminution of LA did affect attractiveness. Therefore, what roles do nocturnality and diurnality play in the sensitivity and expression of the LA receptor of these respective dipterans? Are the morphologies of the nocturnal receptor for LA different than the diurnal LA receptor? Or, are these receptors the same or similar, but their expressivity is influenced by the amount of light in the photoperiod regime?

Dekker et al. (2002) discussed the concept of the L-LA receptor, and differential attractiveness. In terms of the circadian rhythm in a 24-hour day, what would constitute, or be the hourly change(s) that would culminate in the behavioral expression involving host-seeking? How would these hourly sequences be different in diurnal mosquitoes as opposed to nocturnal mosquitoes? In addition, it should be noted, that although the mosquito under study (*Culex restuans*) in this research project is nocturnal, it is *Culicine* as opposed to the nocturnal mosquito frequently under study – *Anopheles gambiae*. In this regard, how would the differential attractiveness differ in these 2 nocturnal dipterans? Lastly, in terms of autogeny and anautogeny as discussed by Bowen et al. (1994), what would be the olfactory behavioral expressiveness of non-blood fed *Culex restuans* mosquitoes in this project as opposed to blood fed *Culex restuans* mosquitoes?

A paper published by Vinauger et al. (2014), addressed the issue of how insects such as *Aedes aegypti* female mosquitoes can recognize and learn how to differentiate between various olfactory chemical stimuli. The methodologies employed in the Vinauger experiments, were two-fold. *Aedes aegypti* mosquitoes were separated into two groups. In Group One, mosquitoes were trained individuall*y*, whereby an odorant such as LA was defined as the conditioned stimulus (CS). The odorant LA, then was reinforced with a thermalized blood stimulus so as to

train the mosquito to anticipate a blood meal. The second group was defined as *groups* of mosquitoes exposed to the same stimuli. The results of these experiments were as follows: Mosquitoes could learn to associate and differentiate between LA and thermalized blood. Lactic acid plus octenol were readily learned. Mosquitoes could not be trained with chemical agents such as β myrcene and benzyl alcohol. Cyclohexamide functioned to affect long term memory in mosquitoes by disrupting it. This is crucial, because it would affect the most important aspect of mosquito behaviors, that is, host-seeking and host selection. It appeared that the results of the cyclohexamide reactions, will prospectively provide insights into heterogeneous biting patterns, as female *Aedes aegypti* mosquitoes do not bite their prospective hosts all at the same time. Heterogenous biting patterns are important behavioral aspects of the *Aedes aegypti* female mosquitoes, as they correlate directly with temporal and circadian disease transmissions in propsective hosts.

As expected, in female *Aedes albopictus*, the attraction towards the port containing lactic acid was nearly threefold as compared to their male conspecifics. The cumulative number counted for preference was 29 *Aedes albopictus* females. Those who showed no response totaled 50 adult mosquitoes, while 27 females were attracted to the port ontaining filtered air. The values received for filtered air in females were three times the values of their male counterparts.

It has been firmly established in the entomological literature, that lactic acid is a well-known mosquito attractant particularly among female mosquitoes due to the presence of lactic acid receptors on their antennae (Bowen et al. 1994, Dekker et al. 2002). Lactic acid is chemically defined as a carboxylic acid. Davis (1976) published a premiere paper in which he reported that he had located receptors for L-LA in *Aedes aegypti* male mosquitoes. However, subsequent experiments involving comparative spike discharge patterns in males, found that

these results did not elicit an attraction for L-LA when compared to their *Aedes aegypti* female counterparts. For in researching male *Aedes aegypti* antennae, Davis (1976) found that while both blunt-tipped sensilla trichodea Type II (A2 –II), were present in both male and female *Aedes aegypti* antennae, but there were decided gender differences as per their responses and functions. In male Aedes aegypti, the (A2 – II) chemosensory cells responded to plant nectar, while in *Aedes aegypti* females, these same cells functioned as oviposition site attractants. McIver and Siemicki (1979) reviewed the fine structure of antennal sensillae of male *Aedes aegypti* and noted that certain male mosquitoes possessed host-finding behavior. Their function was to bring their female conspecifics closer to prospective hosts, for mating and blood meals to develop their eggs. Moreover, the percentage breakdown of antennal morphological types, and correlative functions include, 91% possess olfactory receptors, 7% mechanoreceptors, and 2% thermoreceptors.

With acetone, it could be in the nature of the chemical itself (a simple ketone), that when used in a singular capacity, acetone does not evoke olfactory sensations in this species of mosquitoes.

In Experiment 5, and the behavioral reactions of male *Aedes albopictus* mosquitoes, it can be inferred that the attraction in males to acetone is not strong, unlike its preference as demonstrated in Experiment 6. However, in contrast, female *Aedes albopictus* mosquitos demonstrated an alternate behavioral pattern. The limited numerical data presented in Experiments 5 and 6, suggest that acetone was a weak attractant among the repertoire of odorants responsible for eliciting behavioral responses in female Aedine mosquitoes. Another important factor when considering receptors is age, which is directly related to the ability to function effectively when a mosquito is exposed to an odorant under (12L:12D) photoperiodic conditions**.**

However, a question does come to mind, and that is: What are the physiological roles played by acetone and lactic acid in the process of mosquito response? Acetone is considered a weak attractant. Therefore, perhaps the greater role of acetone is that of a potentiator. Acetone may serve as a weak attractant in mosquitoes due to the paucity of acetone receptors on their antennae. Also, their numbers may be gender specific (less receptor numbers in the male, more receptor sites for females. What is the role of lactic acid? Perhaps in the acetone + lactic acid combination, it is lactic acid that serves in an attractant capacity, while acetone serves as its potentiator. Given the chemical designations of acetone as the simplest ketone, and lactic acid as a carboxylic acid, perhaps it is the nature of their bonding capacities which contributes to the magnitude of the overall mosquito olfactorial response.

Octanol, also known as 1-octan-3-ol, is derived from many sources in nature. In humans, octenol originates from two sources, human breath and the emanations from human sweat. Octanol can be found primarily in plants and fungi. This odorant has also been isolated in animals such as from the breath of oxen. Functionally, octanol is a chemical that serves as an attractant for hematophagous biting insects such as mosquitoes. Chemically, octanol is an 8-carbon monounsaturated alcohol, that contains 2 optical isomers with a terminal double bond (R) – (-) octanol, and (S) – (+) – octanol. The molecular formula for octenol is $C_8H_{16}O$. The molecular mass of octenol is 121.21204 g/mol (Kline 1994, Porter and Kaplan 2011).

In the publication entitled Olfactory Attractants For Mosquito Surveillance and Control, 1-Octan-3-ol, Kline (1994) stated that the purpose of this field study was to evaluate and review current knowledge pertaining to 35 mosquito species, and their behavioral responses to octanol. The data from these field studies included three salient points: CO_2 elicited a (+) response in all 35 mosquito species tested. The combination of CO_2 plus octanol is synergistic. Of the seven

Culex species tested, the response to CO_2 or octenol alone was minimal. Based upon the results of these field studies, it was concluded that: CO_2 appears to be the universal attractant in mosquitoes, while octenol is a selective attractant. It was suggested that the prevailing synergism between CO_2 and octanol could provide the foundation for the development of safer mosquito control strategies.

In a report published by Takken and Kline (1989) entitled: Carbon Dioxide and I–Octan–3–ol As Mosquito Attractants, it was so noted that in field studies conducted utilizing CO_2 at the Snake Bight and Lower Swanee locations in Florida, mosquitoes attracted to this odorant included those from the *Aedes, Anopheles, Wyomeii*, and *Culex* species. This indicated that CO_2 alone served in the dual capacities as a dipteran activator, and flight orientor. In addition, the presence of CO_2 in these field studies documented a two-fold selective increase in the presence of *Aedes taniorhynchus*, and a ten-fold increase in *Anopheles atropos* and *Anopheles crucians*.

In contrast, when only octanol was utilized, significant numbers of the *Aedes* species were represented. These *Aedes* apecies included *Aedes taniorhynchus*, *Aedes crucians*, and *Aedes quadrimaculatus* (Say). It was observed that there was a paucity of representative *Culex* species responding to the presence of octenol in this field study. This was attributed to the fact that most *Culex* species are ornithophilic. Hence, since *Culex* mosquito species are ornithophilic, and therefore have a predilection for birds, this explains the facilitation and transmission of West Nile Virus (WNV) in the avian population to humans.

In comparing the field study experiments of Kline (1994) and Takken and Kline (1989) to the olfactometric experiments of the current study, it is interesting to denote, that while seven *Culex* species were captured in their bait-traps, *Culex restuans*, the mosquito under study here

was not among the seven *Culex* species represented in the corresponding field experiments. The results of these experiments lend further credence that octanol alone, or in synergistic combination with CO_2 is generally minimally responsive to the octenol odorant in *Culicine* dipterans.

Floral Scents

Plant flowers may serve as attractants via color, shape, and odor as well as a primary source of food. Accessory structures such as leaves, may serve as repellents (Das et al. 2003), thereby yielding a duality of functions. In addition, it might be worthwhile to conduct research investigations into the molecular weight/mass of these florals as well as the types of bonds that constitute them. For including specific extraneous factors such as wind speed, temperature, or internal factors such as time of day, may contribute to greater understanding of how odorants interact with antennal receptors to elicit olfactory behavioral responses.

Since commercial synthetic pesticides have over time created resistance in hematophagous insects such as mosquitoes, it has become feasible to explore other options in the areas of vector control. In this regard, essential oils from a wide variety of plants are currently being researched for their potential repellent properties. It has been suggested that these repellent properties are attributed to the presence of one or more volatile monoterpinoid substances (Trongtokit et al. 2005). Therefore, in this section, field studies and laboratory studies utilizing olfactometric behavioral observations on select mosquito species will be presented.

In premiere field studies, at three site locations, Grimstad and DeFoliart (1974) observed nectar feeding preferences of Wisconsin mosquito species that included male and female *Anopheles quadrimacultaus* and *Aedes vexans*. The primary purpose of this field study was to

determine which plant species served as select nectar sources for mosquitoes during a 24-hour day. It was documented, that of the 23 plant species represented at the Sandhill Game Conservation Area, Curtis Prairie, and Point Beach sites, four plant species were favored by the mosquitoes under study. These four plant species included: Ox-eye daisy (*Chrysanthemum*), yarrow (*Achillea millefolium*), common milkweed (*Asclepia syriaca*), and goldenrod (*Solidago spp*.). It was observed that feeding preferences in the field were not the result of randomized floral selections. Rather, floral selections by mosquitoes were based upon definitive preferences that included the following criteria: olfactory attractiveness, a particular floral scent, floral colors with highest preference for white, yellow, and pink colored flowers, age, visual attractiveness as per floral size, shape, and prevailing climatic conditions in general. In terms of nectar preferences, studies have demonstrated a decided gender preference for the types of nectar imbibed by mosquitoes. For males, it is nectar concentration that contains 10% sucrose as part of their diet and that fulfills their nutritional requirements. For females, it is 10% glucose solutions, that promotes glycogen storage, flight energy, and longevity. Unanswered in this field study on floral preferences and feeding habits in select mosquito species, is the crucial point at which a preferred flower would become unattractive to a feeding mosquito, due to a decrease in sugar concentration, remains unresolved.

A review of field studies compiled by Foster and Hancock (1994) on nectar –related olfactory and visual attractants for mosquitoes confirmed the following: Both male and female mosquitoes feed on plant sugars with specific preferences that activate and elicit chemosensory and behavioral responses in dipterans. In terms of visual attractants, entomologists in field observations have noticed that mosquitoes have a predilection for lighter colored flowers, in particular, those flowers exhibiting white coloration, as these flowers contain the highest

percentage of nectar, and mixtures of floral scents (Grimstad and DeFoliart 1974). In addition to floral scents, Bowen (1999) demonstrated that the fructose contents of raw fruits attracted feedings in both lab and field studies conducted on *Culex tarsalis* and *Culex pipiens* mosquitoes respectively. Kline et al. (1990) in employing honey-extracted-bait-suction traps succeeded in capturing, 2,773 *Aedes taniorhynchus* female mosquitoes. Although these figures obtained in the honey-baited trap experiments did not equal the numbers of mosquitoes captured utilizing CO_2 (11,700), it can be postulated that as more sophisticated and targeted floral attractants are elucidated via phytochemical research, equilibration of nectar-feeding attractants, and kairomone attractants will be equally valid in both laboratory and field research studies in medical entomology.

In a paper published on the Comparative Repellency of 38 Essential Oils Against Mosquito Bites by Trongtokit et al. (2005) three mosquito species consisting of *Aedes aegypti, Culex quinquefasciatus*, and *Anopheles dirus* were subjected to laboratory testing. The methodologies utilized in these tests included concentrations of undiluted essential oils, as well as lesser concentrations of diluted essential oils such as those diluted with alcohol solvent at 70%, 50%, and 10% concentrations. During the experimentation, it was so noted and recorded that the lengths of protection time against mosquito bites were decreased with decreasing concentrations of diluted essential oils. A concentration of essential oils diluted to 50% exhibited a range of 50 to 80 minutes of protection, while 10% concentration provided a protection time of only 30 minutes or less. However, of the 38 undiluted essential oils researched, the most promising prospective repellents included the plants of: *C. nardus, P. cablin, S. aromaticum*, and *Z. limonella*, which provided two hours of complete repellency in human subjects, and was most effective in the *Aedes aegypti* mosquito. In summation, it was suggested that further investigative

studies into the development of phytochemicals should be encouraged, so that targeted formulations as per better solvents could contribute to the efficacy of the finished product (Burfield and Reekie 2005). Moreover, in addition to extensive laboratory investigations providing new and insightful information as per mosquito behavior during the course of a 24 hour day, the utilization of phytochemicals should also be applied in field studies, so that additional information could be gleaned to develop a safer, more non-resistant, environmentally friendly repellent.

Evaluations of the protective and repellent effects of 41 essential oils, and their synthetic counterparts were documented in *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* (Amer and Mehlhorn 2006). The results of these phytochemical experiments revealed that five essential oils provided eight hours of protection and 100% repellency. These oils were identified as follows: Litsea (*Litsea cubeba*), Cajeput (*Melaleuca leucadendium*), Niaouli (*Melaleuca quinquenervia*), violet (*Viola odorata*), and Catnip (*Niepeta cataria*). Like the previous report published by Trongtokit et al. (2005) and Amer and Mehlhorn (2006) also confirmed that repellency against mosquitoes increased with increasing concentrations of the 41 oils under study. However, it should be noted that in the research paper of Amer and Mehlhorn (2006), not only were the 41 essential oil extracts tested singly, but 11 oil mixtures from this group were tested, and their repellent properties were compared against their synthetic chemical counterparts Moreover, the testing times of the mosquitoes under study were documented. *Aedes aegypti* mosquitoes were tested between 8:00 – 16:00 hours. While *Anopheles stephensi* and *Culex quinquefasciatus* were tested between 16:00 – 24:00 hours. The inclusions for time periods for testing are important because: The circadian rhythms and photoperiods may help to delineate an important phytochemical adjuvant in the effective treatment of mosquito-host interactions.

Aedes aegypti are diurnal and arthropophilic, while *Anopheles stephensi* and *Culex quinquefasciatus* are nocturnal, and in terms of host predilection, mammophilic and ornithophilic respectively. This aspect may provide valuable insights as per mosquito behavior. A more comprehensive understanding of circadian rhythms and photoperiod regimes in these vector-borne mosquitoes, may ultimately lead to the development of a more sophisticated, and targeted essential oil extracts utilized either in a unitary capacity, or in synergistic blends (Canyon et al. 2005). Sophisticated and targeted essential oils may further delineate the biological concepts of protection and repellency, thus developing a phytochemical whereby precise formulations, select solvents, and fixatives can be utilized to ultimately provide an essential oil product that will have a high degree of efficacy, low host toxicity, non—resistance through subsequent dipteran generations, and is environmentally safe.

In this study, there are four floral species under study that may prospectively serve as mosquito attractants via their floral scents (Sukumar et al. 1991). They may also serve as repellents via the essential oils that compose these phytochemicals. The floral species under study included the Clethra plant (*Clethra anilfolia*), Fennel plant (*Foeniculum vulgare*), Mountain Mint (*Pynanthemum virginianum*), and Oregano plant (*Plectranthus amboinius*). In ongoing botanical research investigations, essential oils extracted from plants via the processes of distillation and targeted utilization of solvents, may facilitate the delineation of protection times, and potential repellency effects against mosquitoes and their intended hosts.

Unlike conditions of field studies involving select species of mosquitoes, visual attractants such as white, pink, and yellow flowers are absent in the current study, as there was only a specific amount (10 µl) of measured odorant present in the olfactometer, along with the control port containing filtered air (Grimstad and DeFoliart 1974, Foster and Hancock 1994).

Although olfaction is the overriding sensory modality in mosquitoes, vision is also an important modality in the selection processes of dipterans as per floral size, morphology, and color which is lacking in the confines of the olfactometer. Climactic conditions such as relative humidity (RH) in the olfactometer remains in a steady state, unlike field conditions whereby climatic conditions may vary over a 24-hour period. Perhaps it is this variation in climatic conditions which makes the nectar contained in the floral scent more attractive and appealing to both male and female mosquitoes for feeding, nutrition, and longevity. The Clethra plant is characterized by yellow clove-scented flowers, and dark green-scented leaves. This plant is known to attract butterflies, bees, and mosquitoes.

In the current study, the results suggest that the Fennel plant may function as a mosquito repellent in general. Moreover, the Fennel plant has powerful monoterpinoids, which contain the major components of anethole ($C_{10}H_{12}O$), fenchone ($C_{10}H_{16}O$), flavonoids, and limonene ($C_{10}H_{16}$) (Phytochemicals 2009). The Fennel plant is characterized by yellow flowers and feathery leaves. It is rich in antioxidants and is possessed of antibacterial properties.

Four male *Culex restuans* mosquitoes were utilized in Experiment 1 involving Mountain Mint. In this experiment, all four male mosquitoes tested, showed no response to Mountain Mint under 16L:8D photoperiodic conditions. It should be noted however, that given the size of this experiment, larger studies are needed to definitively determine protection times (via number of skin applications, and quantification of evaporation times of the chemical), including the repellency qualities of this essential oil. Moreover, the qualities of protection time and repellency should be evaluated and tested in terms of mosquito species that are diurnal, crepuscular, and nocturnal as Rutledge et al. (1983) reported that the patterns of sensitivity to repellent compounds varied between mosquito genera. In addition, Nicolaides et al. (1968) and Cockcroft

et al. (1998) have suggested that tests conducted on the repellency effects of essential oils, should be undertaken using human subjects. For other subjects, such as animals or artificially created membranes, may not yield the correct research data necessary to determine the efficacy of repellents applied to the human skin, and their evaporation rates.

In terms of its chemical constituents, mountain mint (*Pycnanthemum virginianum*) is composed of 80% pulegon, 10% methone, 3-5% limonene, and 2% menthol. However, other species of Mountain Mint may have different percentages of these same chemical constituents as 44% methone and 27% pulegon have been reported for these phytochemicals. Mountain Mint flowers are characterized by white coloration, with a sprinkling of tiny purple spots. It has been known to attract bees, wasps, butterflies, and moths. Based upon the limited olfactometric experiments conducted here, it might act as a repellent to *Culex restuans* male mosquitoes.

It can be concluded that the role of floral scents should be further delineated to include differential repellency ie strong, medium, and weak repellents, their effects on mosquitoes in terms of host-seeking, and how floral scent pathways evoke a variety of olfactory behavioral expressions in male and female *Culex restuans*.

Chemically, oregano contains two volatile oils (El Babili et al. 2011). These oils are carvacol and thymol that have specific functions. Carvacol is defined as a terpinoid with anti-helminithic properties and serves to eliminate parasites and fungal infections. Thymol, in contrast, is a strong natural antiseptic and fungicidal agent.

In the case of mountain mint, although the number of females was small, the results strongly suggest that under (16L:8D) photoperiodic regime, that no preference for this odorant exists. Instead, three of the five females observed, chose the control port containing filtered air,

while the remaining two female *Aedes albopicus* mosquitoes showed no response to Mountain Mint. By increasing insect numbers, and including male *Aedes albopictus* conspecifics in the experiment, the potential for Mountain Mint as a repellent should be pursued and delineated.

Filtered Air Experiments

Neither *Culex restuans* nor *Aedes albopictus* showed a preference for a specific port when both ports had filtered air flow through them during experiments. These results indicated that there was no bias for a given port that would interfere with experimental data obtained with host odor or floral scent trials. Experiment 3 consisted of only one male and one female *Aedes albopictus* mosquitoes that were subjected to (LL) photoperiodic lighting conditions. The behaviors observed, mirrored the movements of those mosquitoes subjected to (16L:8D) photoperiodic regime. That is, no flight movements were observed beyond the confines of the stem and staging areas of the olfactometer. The interpretation of these data also suggests that the feasibility and validity of experimental results have been established, and that further olfactometric testing with known attractants and floral scents are applicable under controlled laboratory conditions. These studies were conducted utilizing filtered air tests for two male and two female *Aedes albopictus* mosquitoes. In both experiments, neither the two male nor two female *Aedes albopictus* mosquitoes moved beyond the stem and staging areas of the flexible dual port olfactometer. Although these experiments were brief in terms of time duration, it appears that both male and female *Aedes albopictus* mosquitoes did not demonstrate *any* predilection for either the North or South Ports of the olfactometer. These results established the validity of pursuing further testing of prospective odorants.

CHAPTER V

CONCLUSIONS

The present study investigated two mosquito species, *Aedes albopictus* and *Culex restuans*, that differed in activity patterns both in the diel cycle and throughout the year. *Aedes albopictus* is a diurnal species that seeks hosts and floral rewards during the day. It is primarily active in the summer. In contrast, *Culex restuans* is a crepuscular/nocturnal species that seeks hosts and visits flowers from dusk through dawn. It is primarily active during the spring and fall seasons. Both species were kept in incubators and subjected to a variety of photoperiods, including summer with lights on for 16 hours and off for 8 hours (16L:8D), and fall/spring with lights on for 12 hours and off for 12 hours (12L:12D). They were also kept in incubators with constant light (LL) or constant dark (DD).

Both female and male mosquitoes of both species were tested for response to host odors (acetone, carbon dioxide, lactic acid, octanol) in a dual-port oflactometer that allowed a choice between treatment as a choice chemical treatment in one port or filtered air in the opposite control port. For *Aedes albopictus*, some trials involved a choice between two chemicals (acetone and lactic acid, or lactic acid and octanol). Male mosquitoes have received little experimental attention until recently, and there is not much information in the scientific literature about their behavior and chemical ecology.

Among the 3,200 mosquito species, there might be a differential atttraction for odorants. That is to state whether the species is diurnal, nocturnal, or crepuscular, the environmental influences including RH, AFR, temperature, exposure time, circadian rhythms, and photoperiod regimes might all factor into the ability to respond. Olfaction may be influenced by multiple

factors such as gender, age, physiological state, mating, feeding, and oviposition in both the 24-hour diel, and seasonal changes/photoperiods. Light, and its duration under the 24-hour diel, and seasonal changes, is an important, and powerful entraining agent. It complements the internality of the circadian clock, with the externality of the 24-hour diel, and seasonal photoperiodic changes. In prospective publications about entomological experiments, it is important to record the time of day under which the experiments were conducted (Ferguson et al. 2005). This will facilitate greater knowledge about the behavioral expressivity under which the dipteran is being studied. It will also contribute to the reproducibility, interpretation, and validity of results.

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Presentations

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Honors and Awards

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