OVIPOSITION AND HOST-SEEKING BEHAVIORAL RESPONSES OF

SIMULIUM VITTATUM (DIPTERA: SIMULIIDAE)

TO POTENTIAL ATTRACTANTS IN LABORATORY BIOASSAYS

by

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(Under the Direction of Raymond Noblet)

ABSTRACT

The oviposition and host-seeking behavioral responses of *Simulium vittatum* (Diptera: Simuliidae) to putative oviposition pheromones in or on conspecific eggs and potential host-seeking attractants were investigated in the laboratory. A series of behavioral bioassays was conducted to observe the oviposition responses of gravid *S. vittatum* females when presented freshly oviposited conspecific eggs, extracts of conspecific eggs, identified chemical compounds from extracts of conspecific eggs, and potential host-seeking attractants. The response of gravid *S. vittatum* females demonstrated that factors on freshly oviposited conspecific eggs were an oviposition stimulant but not a long range volatile attractant. Host-seeking responses of black flies to 1-octen-3-ol, carbon dioxide, and other compounds in a Y-tube olfactometer indicated the potential of using specific blends of host-seeking attractants to bait a black fly trap and provide a more effective method to monitor transmission of *Onchocerca volvulus*.

INDEX WORDS: Black flies, Simuliidae, *Simulium vittatum*, *Simulium damnosum* complex, *Simulium ochraceum* complex, oviposition, pheromones, host-seeking, onchocerciasis

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DEDICATION

I dedicate this thesis to my parents, Tommy W. McGaha Sr., Deloris P. Madewell, and James P. Madewell, who have continuously believed in me and pushed me to pursue my dreams. I feel as though they are always with me despite the miles of distance between us. For this reason, I owe all of my accomplishments to them. Shirley Abbott, a famous writer, once wrote, "We all grow up with the weight of history on us. Our ancestors dwell in the attics of our brains as they do in the spiraling chains of knowledge hidden in every cell of our bodies." This quotation resonates with me as the influence of my family is abundantly apparent throughout my life and work.

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CHAPTER 1

INTRODUCTION

Problem Statement

Black flies (Diptera: Simuliidae) have long been recognized to be economic and medically important pests worldwide. Some African and Latin American species of the genus *Simulium* are vectors of *Onchocerca volvulus*, the causative agent of onchocerciasis which is also known as river blindness. Onchocerciasis eliminating agencies monitor black populations in endemic areas so that the agencies can either continue medically treating the human population or declare the area cleared of the disease. The currently used method of capturing black flies employs human-baited collections. Using a human as bait may be considered a questionable or unethical practice because the human used as bait may be or may become infected with *O. volvulus* during the collection process. If a trap could be developed using an attractant specifically for black flies, then agencies could monitor onchocerciasis more efficiently and safely.

Previous research on the oviposition and host-seeking behaviors of black flies has led recent researchers to investigate the idea that the oviposition and host-seeking behaviors of black flies are mediated by attractive volatile chemical compounds. Through the use of colonized black flies and laboratory bioassays, black fly attractants could be identified for potential use on a baited trap or to improve existing trap designs.

Hypothesis and Research Objectives

The main focus of this research was to identify attractants for *Simulium vittatum* to establish a black fly specific attractant or blend of attractants to be used as bait on a black fly trap. The hypothesis of this study was that volatile chemical compounds are associated in mediating the oviposition and host-seeking behaviors of colonized *S. vittatum*. This hypothesis was investigated by addressing the following objectives:

A1. To determine the extent to which *Simulium vittatum* gravid females prefer to oviposit on a substrate that has fresh conspecific eggs.

A2. To determine and identify the factors responsible for *Simulium vittatum* gravid females' preference to oviposit on a substrate with fresh conspecific eggs.

B. To determine the extent to which a Y-tube olfactometer can be used to identify potential attractants to parous *Simulium vittatum* females.

CHAPTER 2

LITERATURE REVIEW

Life Cycle of Black Flies

The life cycle of black flies (Diptera: Simuliidae) revolves around moving freshwater. Wherever moving freshwater is found in the wild, black flies will most likely be present (Adler & McCreadie 1997). Black flies are holometabolous insects. The cycle begins when a female oviposits onto a moist substrate that is in or near flowing freshwater. Black fly eggs are susceptible to desiccation, so choosing a suitable substrate is crucial for the adult female. Larvae hatch from the eggs and use silk to adhere to substrates in moving water. The larvae attach to silk pads spun on substrates by hooklets on their prolegs (Adler et al. 2004).

Black fly larvae are often described as indiscriminant filter feeders which will intake particles ranging from 0.09 to 350 μm from the water (Wotton 1976, Chance 1977). The diet of the larvae can vary but mostly consists of bacteria, diatoms, and vegetative debris. The number of larval instars varies from species to species but the most common number of larval instars is 6 or 7 (Alvan-Aguilar & Hamada 2003). After the final instar, a larva becomes an immobile pupa. A silk cocoon encases the pupa and attaches it to a substrate in the water. Eventually adults will emerge from the pupae, most often during morning hours (Davies 1950). Male adults will emerge before females due to the faster larval development of males. Both male and female adults feed on nectar, but the females of most species will also require a blood meal for egg development. Anautogenous species require a blood meal before the female can fully develop its ovaries, but autogenous species do not require an initial blood meal for their first batch of eggs

but will need a blood meal to develop the second batch. Female black flies will travel 15 to 500 kilometers to find a host (Garms et al. 1979, Moore & Noblet 1974).

Economic and Medical Importance of Black Flies

Many species of black flies are major pests of livestock and humans. A golf club in South Carolina was estimated to lose $27,202 per year, which also impacts the economy of South Carolina itself, due to pest black fly species in the *Simulium jenningsi* group (Gray et al. 1996). Pest black fly populations have led some government agencies to spend considerable money to control the black fly population. The Negro River Valley, a major agricultural area in Argentina, is troubled by high levels of black fly activity, and controlling the black fly population in this area would cost an estimated $1,623,360 per year (Gray et al. 1999). Since the recent evidence that black flies have the potential to spread vesicular stomatitis virus (VSV), economic costs of VSV can be associated with economic impact of black flies (Smith et al. 2009). An epidemic of VSV in the United States in 1995 caused $50-100 million loss in the cattle industry (Bridges et al. 1997).

The medical importance of black flies cannot be ignored considering the involvement the flies have in transmitting *Onchocerca volvulus,* the causative agent of onchocerciasis. Onchocerciasis is present in Mexico, Guatemala, Ecuador, Columbia, Venezuela, Brazil, Yemen, and 28 African countries (Mackenzie et al. 2012). The primary vectors are the *Simulium ochraceum* complex in Latin America and the *Simulium damnosum* complex in Africa. The transmission of the parasite from the fly to a human victim can result in severe itching, visual impairment, and blindness (Enk et al. 2003). Recent estimates indicate that more than 102 million people are at risk of infection in Africa, which is where 99% of cases exist (World Health Organization 2011, Higazi et al. 2011). With only an estimated 500,000 people at risk, cases of

onchocerciasis are less prevalent in Latin America than Africa with only 3 foci suspected to have ongoing transmission in Venezuela and Brazil (Cupp et al. 2011). In 2008, it was estimated that 25.7 million people were infected with *O. volvulus* and 256,000 were blind, 746,000 had impaired vision, and 4.2 million suffered from severe itching (Crump et al. 2012). Many organizations such as the Onchocerciasis Control Programme (OCP), Onchocerciasis Elimination Programme for the Americas (OEPA), and African Programme for Onchocerciasis Control (APOC) have been formed to control and end the threat of onchocerciasis. It has been proposed that onchocerciasis will be under control worldwide in 2025 (Crump et al. 2012).

Black Fly Olfaction

Physiology of black flies relative to olfaction can influence behavior such as oviposition and host-seeking. The head of black flies contains many sensory organs, but this review will focus on the antenna and maxillary palp. In contrast to the plumose antenna of a female mosquito, a female black fly antenna is setaceous and resembles an inverted cone. The antenna of a black fly usually consists of a scape, pedicel, and 7-9 flagellomeres and can have 800-1800 sensilla depending on the species (Mercer & Mciver 1973b). Sensilla on the antenna could be used for chemoreception, mechanoreception, and other sensory modes, but the physiology of these is poorly understood. A more understood sensory organ of black flies is the maxillary palp. Each maxillary palp consists of 5 segments with a distinctly swollen 3rd segment. This 3rd segment harbors a sensory vesicle called the Lutz's organ, which is used to detect host odors such as CO_2 (Mercer & Mciver 1973a). In comparison to the Lutz's organ of black flies, the maxillary palp of mosquitoes houses a similar sensory organ called a capitate peg. The mosquito capitate peg consists of 3 neurons sensitive to host odors such as CO_2 and 1-octen-3-ol (Grant & O'Connell 1996). Biting midges (Diptera: Ceratopogonidae) also possess a CO_2-sensitive organ

which is similar to the mosquito capitate peg and Lutz's organ in black flies (Grant & Kline 2003).

Oviposition by black flies involves many olfaction sensilla on the entire body. Antenna and maxillary palp sensilla have many different sensilla that could aid in oviposition. Other sensory organs on the head include the labium, which contain chemoreceptors and mechanoreceptors, and the labella which contains water, salt, and sugar sensitive sensilla (Angioy et al. 1982, Sutcliffe & Mciver 1982). Campaniform sensilla on spurs from the middle and hind tibiae can detect pressure from a substrate when the black fly is at rest (Sutcliffe & McIver 1976). Observations of ovipositing black flies using their forelegs to tap around a potential oviposition substrate suggests sensilla are present to aid in gathering external stimuli (Golini & Davies 1975).

Oviposition Behavior of Black Flies

Behavioral strategies of black fly oviposition have evolved uniquely for different species according to their habitat and other selective pressures. The oviposition behavioral strategies varies among species: 1) ovipositing directly into the water while flying and 2) ovipositing onto a substrate from the water either while flying or landed (Golini & Davies 1987). A majority of the species that oviposit while landed are in the genus *Simulium* (Adler et al. 2004). Of the species which oviposit while landed on a substrate, some aggregately deposit eggs in masses which has been described as "communal oviposition", a term coined by Muirhead-Thompson (1956) while observing *Simulium damnosum* Theobald. This communal oviposition behavior seemed to be linked with the eggs already present on the substrate. Substrates with freshly deposited eggs that were 1-day old induced more oviposition from gravid female *Simulium reptans* more than when substrates lacked eggs (Coupland 1991, 1992) . Even though communal

oviposition was occurring, some regarded the communal behavior to be coincidental due to a lack of suitable oviposition habitat; however, other scientists have observed communal oviposition when abundant suitable oviposition substrate was available (Davies 1962, Crosskey 1990).

Others have proposed that communal oviposition can be mediated by the eggs themselves via pheromones (McCall 1995). McCall et al. (1994) and Rodriguez-Perez et al. (2003) used a binary choice bioassay design to observe the oviposition choice of *Simulium damnosum* and *Simulium ochraceum* complexes. In a later study, the oviposition pheromone of the *S. damnosum* complex was demonstrated to be volatile (McCall 1995). Experiments with the *S. ochraceum* complex never demonstrated volatility but demonstrated a preference gravid females to oviposit on a substrate with fresh conspecific eggs (Rodriguez-Perez et al. 2003).

Oviposition pheromones from eggs of other dipteran species have been previously documented in mosquitoes and sand flies (Osgood 1971, Elnaiem & Ward 1991). Identification of oviposition pheromones was successful for mosquitoes of *Culex* (6-acetoxy-5-hexandecanolide) and the sand fly, *Lutzomyia longipalpis* (dodecanoic acid); however, the black fly oviposition pheromone structure was never identified (Laurence 1982, Laurence 1985, Dougherty & Hamilton 1997, McCall 1997).

Host-seeking Behavior of Black Flies

Unlike mosquitoes, one of the most medically important Dipterans, host-seeking behavior of black flies is poorly understood. Most of the research concerned with black fly host-seeking behavior has been generated from field studies with baited traps. One attractant commonly used in field experiments is carbon dioxide. Field studies using traps baited with carbon dioxide have attracted *S. slossanae, S. damnosum* complex, *S. arcticum* complex, and *S. ochraceum* complex

(Kim & Merritt 1987, Moore & Noblet 1974, Sutcliffe et al. 1994, Sutcliffe et al. 1995, Thompson 1976b). Carbon dioxide has also been shown to have a synergistic effect on attracting host-seeking black flies when combined with other host-seeking attractants, such as human breath, crude cattle extract, and 1-octen-3-ol (Atwood & Meisch 1993, Sutcliffe et al. 1994, Thompson 1976b). 1-octen-3-ol, a known mosquito host-seeking attractant, is attractive on traps in the field (Atwood & Meisch 1993). 1-octen-3-ol is a component of cattle and human odor (Cook et al. 2011).

Although this field research is valuable to understanding the host-seeking behavior of black flies, a controlled study in a laboratory with more precise experiments is needed. Only one study has been published on the host-seeking behavior of black flies in a laboratory setting using a Y-tube olfactometer (Opoku 2008). This study determined that *Simulium ornatum* was attracted to low concentrations of carbon dioxide, 1-octen-3-ol, and humidified air. A Y-tube olfactometer can be used to accurately identify specific concentrations of a chemical compound or substance that are attractive to a given insect species. Several attractants of haematophagous Diptera other than black flies have been investigated and successfully identified with a Y-tube olfactometer; these included several species of mosquitoes and biting midges (Blackwell et al. 1996, Bosch et al. 2000, Cook et al. 2011, Geier & Boeckh 1999, Geier et al. 1999).

Field Trapping of Black Flies

In this review of black fly field traps, two categories of traps will be discussed: 1) non-baited traps and 2) baited traps. Non-baited traps, traps without a volatile attractant, relied mostly on visual and location attraction cues to attract black flies. Light traps have been successful when positioned over or near oviposition sites (Kyorku 1982). Sticky aluminum panels have been used to capture gravid females when positioned on the stream banks where

oviposition sites are located (Bellec 1976). The same aluminum trap by Bellec (1976) was modified to float and possessed sticky plastic strips which allowed the trap to imitate oviposition substrate more efficiently (Bellec et al. 1984). This floating raft strategy was also effective in England (Hansford & Ladle 1979). Plexiglass coated with an adhesive was successful when suspended over a stream (Adler et al. 1983). For monitoring up- and down-stream flight, a Malaise trap was used (Adler et al. 1983). Suction traps were efficient (Johnson et al. 1982). Imitations of whole hosts as well as a body part, such as an ear, coated with adhesive were also used successfully (Anderson & Yee 1995, Schmidtmann, 1987).

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CHAPTER 3

OVIPOSITION RESPONSE OF *SIMULIUM VITTATUM* TO CONSPECIFIC EGGS IN LABORATORY BIOASSAYS

Introduction

Black fly (Diptera: Simuliidae) oviposition behavior has been observed and investigated in the wild, but is poorly understood. The *Simulium damnosum* complex, *Simulium reptans*, and *Simulium verecundum* gravid females have been separately observed in the wild to oviposit in groups on a single substrate, producing aggregations of fertile eggs (Coupland 1991, 1992; Golini & Davies 1975; Muirhead-Thompson 1956). Muirhead-Thompson (1956) while observing *S. damnosum* complex termed this behavior "communal oviposition". After observing *S. reptans*' preference to oviposit on a substrate with fresh eggs rather than a substrate without fresh eggs, Coupland (1991, 1992) suggested that the preference may be mediated by an oviposition pheromone. However, prior to postulations of Coupland (1991,1992), Davies (1962) suggested that aggregated oviposition was due to a lack of suitable substrates when he observed oviposition in different species of black flies, some with and without communal oviposition behavior. To clarify this issue, laboratory investigations were conducted to determine the behavioral basis for the phenomenon of communal black fly oviposition.

A binary choice chamber bioassay was used to demonstrate that gravid *S. damnosum* and *Simulium ochraceum* complexes preferred to oviposit on a substrate with fresh conspecific eggs rather than a control substrate without eggs when presented two choices (P. J. McCall et al. 1994, Rodriguez-Perez et al. 2003). Further research with *S. damnosum* complex led to the conclusion

that oviposition preference is mediated by a volatile emitted from fresh eggs and originating in the ovaries (McCall 1995, McCall, et al. 1997). However, the oviposition pheromone was never identified. If a black fly oviposition pheromone could be identified and synthesized in the laboratory, it could be used as an attractant on traps, which could replace the current scenario in which humans are used to collect black flies for monitoring for the presence of *Onchocerca volvulus,* the causal agent of onchocerciasis, a devastating infectious disease transmitted by some species of black flies.

The objective of this study was to determine the extent to which colonized *Simulium vittatum* can be used as a research model to investigate oviposition behavioral responses and to identify the oviposition pheromone. If behavioral responses of *S. vittatum* are similar to the responses of *S. damnosum* and *S. ochraceum* complexes when given the chance to oviposit on a substrate with eggs or a substrate with no eggs, then further studies with *S. vittatum* could be performed in efforts to identify the oviposition pheromone. Several bioassays - binary choice chamber, oviposition attraction tower, and oviposition attraction single chamber - were used to assess the behavior of gravid *S. vittatum* when presented fresh conspecific eggs.

Materials and Methods

Binary Choice Chamber Bioassay

***Simulium vittatum* adults.** Experiments were conducted with gravid female *S. vittatum* (= cytospecies IS-7) adults. The adults were reared in colony at the University of Georgia and 3-day old or older adults were prepared for oviposition as described by Gray and Noblet (1999). Females used in the binary choice chamber bioassays were first anesthetized with carbon dioxide gas and then placed on a cold plate (Cryolizer 2-800, Kemtec Science Inc., Hanover, PA, USA) for selection, which was based on eye morphology so no males were selected, with a modified

pipette aspirator (Figure 3.1). Gravid females used in the experiments were collected in a modified pipette and sealed with cotton and tape and placed into the pre-oviposition chamber until needed in the binary choice chamber bioassays.

***Simulium vittatum* eggs.** Fresh, 30- and 60-minute old, conspecific eggs were used in the binary choice chamber bioassays as the test treatment. To produce test treated substrates with fresh eggs, one carton of adult flies were prepared for oviposition and placed into an oviposition chamber as described in Gray and Noblet (1999). Whatman grade 1 filter paper (7.0 cm, GE Healthcare, Little Chalfont, UK) was used as the substrate in the oviposition chamber, which was then be placed in the binary choice chamber as the test treatment after eggs were deposited on the filter paper by the flies. The number of eggs on the filter paper used as the test treatment was not quantified. The objective of the of the oviposition process prior to the bioassay was to produce the largest amount of eggs on the filter paper in an effort to expose the flies to the highest possible concentration of hypothesized factors on the eggs.

Bioassays. The binary choice chamber (BCC) bioassay was used to assess the behavior of gravid black flies when given two choices, a control and a test treatment. This bioassay was inspired by previous experiments which involved observing the oviposition behavior of *S. damnosum* and *S. ochraceum* (P. J. McCall et al. 1994, Rodriguez-Perez et al. 2003). The bioassay assessed communal behavior by comparing the number of flies that oviposited on the control to a similar substrate with conspecific eggs. In later experiments, the number of flies that were actually observed on the filter papers with the control and test treatments was also compared.

The BCC was a modified 27.9 x 16.8 x 13.7 cm polystyrene clip box (Sterilite Corporation, Townsend, MA, USA). To allow an entry point for the flies to be aspirated into the

BCC, a 2.5 x 2.5 cm square shaped opening was cut and lined with two 3.8 x 3.8 cm squares of dental dam (Coltene/Whaledent Inc., Mahwah, NJ, USA) that were alternately sliced in the middle. Where the treated filter papers are placed during the experiment, Two 4.5 cm holes, which were 13.0 cm apart in relation to their centers, were cut from the bottom of the BCC. The front of the BCC was removed and replaced with plastic mesh, which had 1.0 cm vertical slits in a perpendicular position to the center of the holes, to allow removal of the flies during the experiment.

A wooden light box with a polyurethane coated surface served as the base for the BCC. The wooden box was 73.0 x 30.5 x 12.7 cm with two sets of paired holes; each pair of holes represented a place for one BCC (Figure 3.2). The wooden box was lined with aluminum foil to more evenly distribute light through the holes of the BCC. The light source inside the box was a 55.9 cm T8 fluorescent plant growth light (Utilitech, distributed by Good Earth Lighting Inc., Wheeling, IL, USA) which rested on a sheet of aluminum foil. Light intensity was measured with a photometer (Photometer1, Quantum Instruments Inc., Hauppauge, NY, USA) to ensure equal intensity emitted from each hole in the BCC. A 30.5 x 21.6 cm rectangular divider was placed in the middle between the two BCCs to prevent light contamination between the chambers.

A larger BCC (68.6 x 25.4 x 25.4 cm), which was seven times larger in volume than the previously described smaller BCC, was constructed of clear acrylic plastic. The expanded area of the BCC was used to assess the oviposition behavior of the flies in a larger environment (Figure 3.3). In comparison to the smaller BCC, the larger BCC contained the same sized entry point for the flies and holes for the treated filter papers. The larger BCC also contained a plastic mesh front side which contained slits for fly removal during the experiment. In contrast the

smaller BCC, the holes in the larger BCC were 46.4 cm apart. The same light box as described for the smaller BCC was used for the larger BCC with the exception that the inner middle holes were covered which allowed only light from the outer holes to be present for the BCC bioassay.

The bioassay was conducted between 12:00 and 17:00 hours in a room with no lights. Experimental room temperature averaged 24.0 ºC (measured with Kestrel 3500 Pocket Weather Meter, Birmingham, MI, USA). Once the test treatment (freshly deposited eggs on a piece of filter paper) and a control (wet filter paper with no eggs) were placed over the appropriate holes in the two different BCCs used in the experiments, gravid female black flies previously selected and held in a modified pipette, were aspirated into the appropriate BCCs.

Experiments 1 and 2 were conducted in the small BCC, with the age of the eggs and duration of each replicate varying. Experiment 1 was conducted with 60-minute old eggs and the duration of each replicate was 60 minutes. To provide moisture for oviposition, 1.0 ml of deionized water was pipetted onto each filter paper every 15 minutes. Experiment 2 was conducted with 30- and 60-minute old eggs and the duration of each replicate was 20 minutes. In the 20 minute experiment, 1.0 ml of deionized water was applied only once at 10 minutes onto each filter paper. Ovipositing flies were aspirated from the bioassay chambers to reduce the contamination resulting from the addition of fresh eggs on the control substrate or the test substrate with eggs. Experiments 1 and 2 were replicated 10 times; a new group of 20-gravid females was used in each replicate.

Experiment 3 was conducted in the large BCC with 30-minute old eggs and each replicate was 30 minutes in duration. An application of 1.0 ml of water was pipetted onto each filter paper every 10 minutes. A new group of 40-gravid female flies was used in 5 replicates

with the large BCC. In all experiments, flies were immediately aspirated from the BCC through the slits in the mesh screen when they started to oviposit.

Experiment 4 was conducted in the small BCC and consisted of 30-minute old eggs as the control and hexane-washed 30-minute old eggs as the test. The control eggs were collected and used in the experiment as described before in experiments 1, 2, and 3. Hexane-washed eggs consisted of fresh 30-minute old eggs which were rinsed three times with 5.0 ml of hexane. Once rinsed, the hexane-washed eggs were held for the hexane to evaporate until the hexane was no longer emitting from the substrate.

Statistical analysis. The oviposition responses, number of flies ovipositing on the substrates, were compared using the Wilcoxon signed-ranks test. The attraction responses, number of flies located on the substrates, in experiments 2 and 3, respectively, were compared using a paired t-test. Significance was demonstrated if probability levels were equal to or less than 0.05 when control and test values were compared. Statistical analysis was performed using GraphPad software (Prism 6, GraphPad Software, La Jolla, CA, USA).

Oviposition Attraction Bioassays

***Simulium vittatum* adults.** *S. vittatum* were reared as previously stated in the BCC bioassays. Once adults emerged and were collected, they were allowed to mate and subsequently incubated in a climate-controlled room at 25 ºC and 85 % relative humidity for at least 3 days. After incubation, females were gravid and ready to oviposit. Gravid females used in this study were not anesthetized which is contrary to the gravid females in the BCC experiments. Experimental gravid females were collected from a modified mosquito breeder (Bioquip, Inc., Rancho Dominguez, CA, USA) with a modified pipette aspirator (Figure 3.4). After 20 females were aspirated into the pipette, it was sealed with cotton and tape prior to the experiment.

***Simulium vittatum* eggs.** Fresh, 30-minute old eggs were used in this study as the test treatment. Twenty gravid *S. vittatum* females were aspirated into a 60 x 15 mm disposable Falcon petri dish (BD, Franklin Lakes, NJ, USA), which was lined with cotton on the bottom with a piece of 5.5 cm Whatman grade 1 filter paper (GE Healthcare, Corning, NY, USA) on top. Eight ml of deionized water were applied to the filter paper and cotton (U.S. Cotton, Gastonia, NC, USA) before the flies were aspirated inside the petri dish. Thirty minutes of oviposition was allowed inside the petri dish, which produced sufficient numbers of eggs on the filter paper. The filter paper with freshly oviposited eggs was used as the test treatment in this study. The number of eggs on the filter paper used as the test treatment was not quantified. The objective of the of the oviposition process prior to the bioassay was to produce the largest amount of eggs on the filter paper in an effort to expose the flies to the highest possible concentration of hypothesized factors on the eggs.

Bioassays. The oviposition attraction (OA) bioassays were used to assess the behavior of gravid female black flies when given one choice, either a control or test treatment, filter paper without eggs or filter paper with eggs, respectively. The oviposition attraction tower (OAT) consisted of a neutral chamber, test chamber, and base (Figure 3.5). The neutral and test chambers were modified 20.3 x 20.3 x 20.3 cm collapsible aluminum cages (Bioquip, Rancho Dominguez, CA, USA). The neutral chamber was lined with black plastic on the left, right, and top sides, and the front side was lined with clear plastic. The bottom was not lined but a wooden platform covered half of the bottom which allowed the flies to be aspirated into the neutral chamber without being directly aspirated into the test chamber. The neutral chamber rested on top of the test chamber which was lined with black plastic on the left, right, and back sides, and the front was lined with clear plastic. The bottom and top of the test chamber were not lined.

The bottom of the test chamber rested on top of the base. The base was a 25.4 x 25.4 x 34.3 cm wooden stand with a 5.5 cm hole in the top platform which holds a 60 x 15 mm petri dish lid. On the bottom platform of the base, a 7.5-W light (Feit Electric Inc., Pico Rivera, CA, USA) in a standard socket base was placed in the middle. The base with the light inside was covered with two black cloths to prevent any light from exiting (Figure 3.5). Light was directed through the hole in the top platform, and intensity was measured with a photometer (Photometer1, Quantum Instruments Inc., Hauppauge, NY, USA).

The oviposition attraction single chamber (OASC) bioassay was identical to the tower except it did not have a neutral chamber but only a test chamber with the top covered with black plastic (Figure 3.6). In the OASC bioassay, the option for the substrate to be covered with a mesh screen was available by using the bottom of a 60 x 15 mm petri dish which had the bottom removed and replaced with mesh screen (Figure 3.6). The mesh screen prevented flies from coming into direct contact with the substrate, but allowed air in proximity to the eggs to escape into the test chamber.

All experiments were conducted in a room without any external lights. The only light source for each bioassay was the light source at the bottom of the base. Temperatures were on average 20-21 Cº. Ten repetitions were conducted each time as a set with a couple of individuals observing the behavior of the flies. Of the 10 bioassays, 5 were of the test sample and 5 were of the control sample. Three sets of bioassays were conducted for each experiment, resulting in 15 repetitions for each treatment. In each repetition a naïve group of 20 gravid females was used. Each repetition was conducted for 20 minutes.

Experiment 1 and 2 were conducted in the OAT bioassay. Filter paper with 30-minute old conspecific eggs was used as the test treatment and a piece of filter paper without eggs was

used as the control. The filter paper was used as bait at the bottom of the test chamber in a petri dish which rested on the top of the base. In the beginning of each repetition, 20 gravid females were released via aspirator in the neutral chamber in the direction of the wooden platform. In the OAT bioassay, the filter paper was not covered with a mesh screen. The distance from the bottom opening of the neutral chamber to the filter paper was 20.3 cm. In experiment 1, the focus was measuring the time it took the first fly to land on the substrate and how long it took the fly to oviposit after it landed on the substrate. Experiment 2 focused only on measuring the number of flies that were attracted to the substrate.

Experiments 3 and 4 were conducted in the OASC bioassay. Thirty-minute old conspecific eggs on filter paper were used as a test treatment and a piece of filter paper without eggs was used as the control. The filter paper was used as bait at the bottom of the test chamber in a petri dish which rested on the top of the base. Without a neutral chamber, the flies were aspirated directly into the test chamber. The direction of aspiration was not directly at the filter paper but to a corner of the test chamber. Experiment 3 did not have a mesh screen cover over the filter paper, which allowed the flies to come into direct contact with the test filter paper, but experiment 4 had a mesh screen over the filter paper so the flies could not come into direct contact with the filter paper but could still be exposed to any volatiles emitting from the filter paper.

Statistical analysis. An unpaired t-test was used to compare the attraction responses (number of flies attracted to the substrate) to the substrate without eggs and the substrate with eggs. Statistical analysis was performed using GraphPad software (Prism 6, GraphPad Software, La Jolla, CA, USA).

Results

Binary Choice Chamber Bioassay

Experiment 1. Sixty-minute old *S. vittatum* eggs on wet filter paper were compared to a control consisting of a piece of wet filter paper without eggs in the small BCC. Ten replicates were conducted, and the number of flies that oviposited on a substrate with eggs was compared to a control. Out of the 200 flies used in the experiment, 63 (31.5%) oviposited either on the control or test substrate, and a significant number (Wilcoxon signed-ranks test, $P \leq 0.01$) of the flies that oviposited selected the substrate with fresh eggs (Table 3.1). Most of the oviposition activity was observed during the first 20 minutes of the experiment.

Experiment 2. Thirty- and sixty-minute old *S. vittatum* eggs on wet filter paper were compared to a control consisting of a piece of wet filter paper without eggs in the small BCC. In 10 replicates, 5 replicates for each egg age, the number of flies that oviposited and number of flies actually on the substrates were compared.

A total of 15 (15.0%) out of the 100 tested flies oviposited either on the substrate with the control substrate with no eggs or the test substrate with 30-minute old eggs. The substrate with 30-minute old eggs was selected for oviposition significantly (Wilcoxon signed-rank test, $P \leq 0.05$) more than a control with no eggs. The replicates with the 60-minute old eggs yielded 10 (10.0%) ovipositing flies on either substrate out of 100 flies tested, and as with the 30-minute old eggs, the substrate with the 60-minute old eggs was oviposited upon significantly more (Wilcoxon signed-ranks test, $P \leq 0.05$) than the control substrate. The number of oviposition flies on the substrates of 30- and 60-minute old eggs was compared and no significant difference was found. When replicates of the 30- and 60-minute old eggs treatments were combined and

analyzed, a more significant response (Wilcoxon signed-ranks test, $P \leq 0.005$) was apparent for the substrate with eggs over the substrate with no eggs (Table 3.1).

Significant (paired t-test, $P \leq 0.05$) attraction to the test substrate over the control substrate was only observed at 6-8 and 19 minutes for the 30-minute old egg replicates, and 6-11, 13,14,16,18, and 19 minutes for the 60-minute old egg replicates (Table 3.2). Attraction of gravid flies to the substrate with the 30- and 60-minute old eggs compared to the control was also significant (paired t-test, $P \leq 0.05$) at 6-14 and 16-20 minutes after the beginning of the experiment (Figure 3.7). No significant difference was found between the attraction responses to the 30- and 60-minute old eggs.

Experiment 3. In a larger BCC, 30-minute old *S. vittatum* eggs on wet filter paper were compared to a control consisting of a piece of wet filter paper without eggs in the large BCC. The number of flies that oviposited and number of flies actually on the substrates were compared (Table 3.1). A total of 18 (9.0%) flies oviposited from the 200 flies tested in the experiment. The number of flies that preferred to oviposit on a substrate with fresh conspecific eggs was marginally more but not significantly more (Wilcoxon signed-ranks test, $P > 0.05$) than the control with no eggs. The attraction of gravid females to the substrate with conspecific eggs was not significant (paired t-test, $P > 0.05$) when compared to the control at 10 minutes after the beginning of the experiment (Table 3.2).

Experiment 4. In 4 replicates, a total of 80 flies were used and 39 (48.75%) chose to oviposit on either the control or test substrate. Unwashed 30-minute old eggs induced more flies to oviposit and attracted more flies than the substrate with hexane-washed 30-minute old eggs. The oviposition response was not significant (Wilcoxon signed-ranks test, $P > 0.05$), but significant (paired t-test, $P \leq 0.05$) attraction was only present at 12-20 minutes (Figure 3.8).

Oviposition Attraction Bioassays

Experiment 1. The amount of time until the first fly exited the neutral chamber and landed on the substrate at the bottom of the test chamber of the OAT was not significantly different (unpaired t-test, $P > 0.05$) between the two treatments of a substrate with no eggs and a substrate with fresh eggs. However, a significant (unpaired t-test, $P \leq 0.05$) response was observed between the two treatments in the time the fly required to oviposit after landing on the substrate (Table 3.3).

Experiment 2. The number of flies attracted to a piece of filter paper with 30-minute old conspecific eggs was compared to the number of flies attracted to a piece of filter paper with no eggs in the OAT bioassay. After observing 15 repetitions with a total of 300 gravid females being tested, no significant (unpaired t-test, $P > 0.05$) attraction to the substrate with eggs was observed with only an average of 19.0 ± 2.8% (mean ± SEM) of the tested flies being attracted to the substrate. The control substrate with no eggs produced similar results to the test substrate attracting 15.0 ± 2.5% flies (Table 3.4).

Experiment 3. The number of flies attracted to a piece of filter paper with 30-minute old conspecific eggs was compared to the number of flies attracted to a piece of filter paper with no eggs in the OASC bioassay, in which the substrates were not covered with a mesh screen. A significant (unpaired t-test, $P \leq 0.05$) number of flies, with an average of 12.3 ± 2.2% (mean ± SEM), was more attracted to the filter paper with conspecific eggs than the control filter paper without eggs, which attracted 4.0 ± 1.2% (Table 3.4).

Experiment 4. The number of flies attracted to a piece of filter paper covered with a mesh screen with 30-minute old conspecific eggs was compared to the number of flies attracted to a piece of filter paper with no eggs covered with a mesh screen in the OASC bioassay. Even

though more flies on average were attracted to the mesh screen over the filter paper with eggs, 15.3 ± 3.3%, than the mesh screen over the filter paper without eggs, 9.7 ± 2.9%, the difference was not significant (unpaired t-test, $P > 0.05$) (Table 3.4).

Discussion

The BCC bioassay indicated that gravid *S. vittatum* prefer to oviposit on a substrate with 30- or 60-minute old eggs rather than a substrate with no eggs. Previous experiments with *S. damnosum* and *S. ochraceum* complexes gave similar results (McCall et al. 1994, Rodriguez-Perez et al. 2003). This is important because both species are important vectors of *O. volvulus*. During the BCC experiments, the location of the flies was recorded to determine whether or not the eggs would attract flies to land on a substrate. Although on average more flies were observed on the substrates with eggs, this observation was after the first 5 minutes of the experiment. During the first 5 minutes, flying and landing behaviors of the flies appeared random and not oriented to a specific substrate. The flies would randomly land on a substrate; however, it seemed that when a fly landed on a substrate with eggs, the fly prolonged its stay and eventually oviposited. To determine if the fly was attracted to eggs or chemicals associated with the eggs, the OA bioassays were developed. The OASC bioassay indicated that *S. vittatum* females were not attracted by volatiles from conspecific eggs but did indicate a stimulation effect from the conspecific eggs when the flies were on the oviposition substrate and in close proximity or direct contact with the eggs. In contrast to the hypothesis that chemicals associated with fresh black fly eggs are volatile attractants for gravid black fly females, this study demonstrated chemical factors associated with fresh eggs only induced a gravid female black fly to oviposit on a substrate. These results are contrary to the results of a previous study with *S. damnosum* complex in which the flies were attracted to the volatiles of conspecific eggs (McCall 1995).

These findings are not promising for onchocerciasis control and prevention programs seeking a more efficient monitoring method via pheromone-baited traps.

Suggested factors influencing the selection of an oviposition site by gravid female black flies in the wild include air current over water, substrate color and reflectance, and tactile characteristics of the substrate (Bellec 1976; Coupland 1991, 1992; Golini & Davies 1975). These natural factors are not easily replicated in laboratory experiments. In the oviposition bioassays of this study, several characteristics of a natural black fly oviposition site were not included, such as running water, substrate color, and environmental conditions.

A concern in conducting experiments using a colonized strain and comparing it to a wild form, even of the same species, is that it may not be a valid comparison due to inbreeding and selection for laboratory conditions. The colony of *S. vittatum* has been in culture since 1981. Selection pressure related to mating and oviposition behaviors may have occurred since the flies were colonized. Oviposition in the UGA colony occurs in a small oviposition chamber (Gray & Noblet 1999). The constricted environment in the colony oviposition chamber may select for females that might not respond in a similar way in the wild. Mating behavior of the colonized flies may also be selected differently than wild flies due to the flies being restricted in a small tube to mate, which is quite different than the spacious environment of a mating swarm in the wild. In a previous study, genetic variations have been found between the *S. vittatum* colony and original-wild populations; therefore, a comparative behavioral study would be warranted to ensure the behavior of the colonized *S. vittatum* can be a true model for wild black flies (Brockhouse & Adler 2002).

Communal oviposition has been observed in the field in many different species (Adler et al. 2004). It has been hypothesized that black flies communally oviposit to prevent desiccation, predation, and parasitism (Adler et al. 2004, P. J. McCall et al. 1994). Along with the benefits of communal oviposition come disadvantages such as possible suffocation of the eggs on the inside

of communally oviposited egg masses (Imhof & Smith 1979). Fungal infection can also make communal oviposition an unfavorable phenomenon (Hywel-Jones & Ladle 1986). Communal oviposition may either benefit or harm the chances of survival for eggs in clumped masses.

Other species belonging to the order Diptera (*Culex* spp. and *Lutzomyia longipalpis*) also communally oviposit (Elnaiem & Ward 1991, Osgood 1971). Through laboratory experimentation, an aggregation pheromone was determined to be a factor in oviposition behavior for *Culex* mosquitoes and the sand fly, *L. longipalpis* (Dougherty & Hamilton 1997, Laurence et al. 1985). During previous investigations with the mosquito, sand fly, and black fly oviposition pheromones, non-polar solvents were successful in extracting the substance from the eggs (Elnaiem & Ward 1991, McCall 1995, Starratt & Osgood 1973). In this study with *S. vittatum* IS-7, preliminary results demonstrated that gravid females preferred to oviposit on a substrate with fresh eggs rather than a substrate with hexane-washed eggs when given the two choices in the BCC indicating a possible role for a volatile or contact attractant associated with *S. vittatum* eggs. Further work is needed to validate the significance of this preference.

Tables and Figures

Table 3.1. The oviposition response of adult gravid female *Simulium vittatum* to conspecific eggs in the binary choice chamber (BCC) bioassays. * Indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$ (Wilcoxon matched-pairs signed rank test).

BCC experiment	N	Age of eggs (minutes)	Duration of experiment (minutes)	Oviposition response (mean ± SEM)	
				Control	Test
Experiment 1	10	60	60	0.8 ± 0.6	5.7 ± 1.0 **
Experiment 2	5	30	20	0.6 ± 0.4	2.4 ± 0.6 *
	5	60	20	0.2 ± 0.2	1.8 ± 0.6 *
	10	30 and 60	20	0.4 ± 0.2	2.1 ± 0.4 **
Experiment 3	5	30	30	0.4 ± 0.2	3.2 ± 0.9
Experiment 4	4	30	20	7.3 ± 1.7	2.5 ± 0.3

Table 3.2. The attraction response of adult gravid female *Simulium vittatum* to conspecific eggs in the small and large binary choice chamber (BCC) bioassays at 10 minutes. * Indicates $P \leq 0.01$ and ** indicates $P \leq 0.001$ (paired t-test).

BCC experiment	N	Age of eggs (minutes)	Attraction response (mean ± SEM)	
			No eggs present	Eggs present
Experiment 2	5	30	1.2 ± 0.4	3.0 ± 0.6
	5	60	0.8 ± 0.2	3.2 ± 0.2 **
	10	30 and 60	1.0 ± 0.3	3.1 ± 0.4 **
Experiment 3	5	30	2.8 ± 0.9	5.2 ± 1.3

Table 3.3. The attraction response of adult gravid female *Simulium vittatum* to conspecific eggs in experiment 1 of the oviposition attraction (OA) bioassays. * Indicates $P < 0.05$ (unpaired t-test).

		Response time in minutes (mean ± SEM)	
Response	N	No eggs present	Eggs present
Initial landing	5	6.0 ± 3.0	2.6 ± 0.4
On substrate before oviposition	5	14.8 ± 3.9	3.8 ± 1.4 *

Table 3.4. The attraction response of adult gravid female *Simulium vittatum* to conspecific eggs in the oviposition attraction (OA) bioassays. * Indicates $P < 0.01$ (unpaired t-test). Abbreviations for OA bioassay descriptions are as follows: oviposition attraction tower (OAT) and oviposition attraction single chamber (OASC).

			Attraction response (mean ± SEM)	
OA experiment	N	OA bioassay description	No eggs present	Eggs present
Experiment 2	15	OAT	3.0 ± 0.5	3.8 ± 0.5
Experiment 3	15	OASC (no screen)	0.8 ± 0.2	2.5 ± 0.4 *
Experiment 4	15	OASC (screen)	1.9 ± 0.6	3.1 ± 0.7

Figure 3.1. Modified pipettes for aspiration.

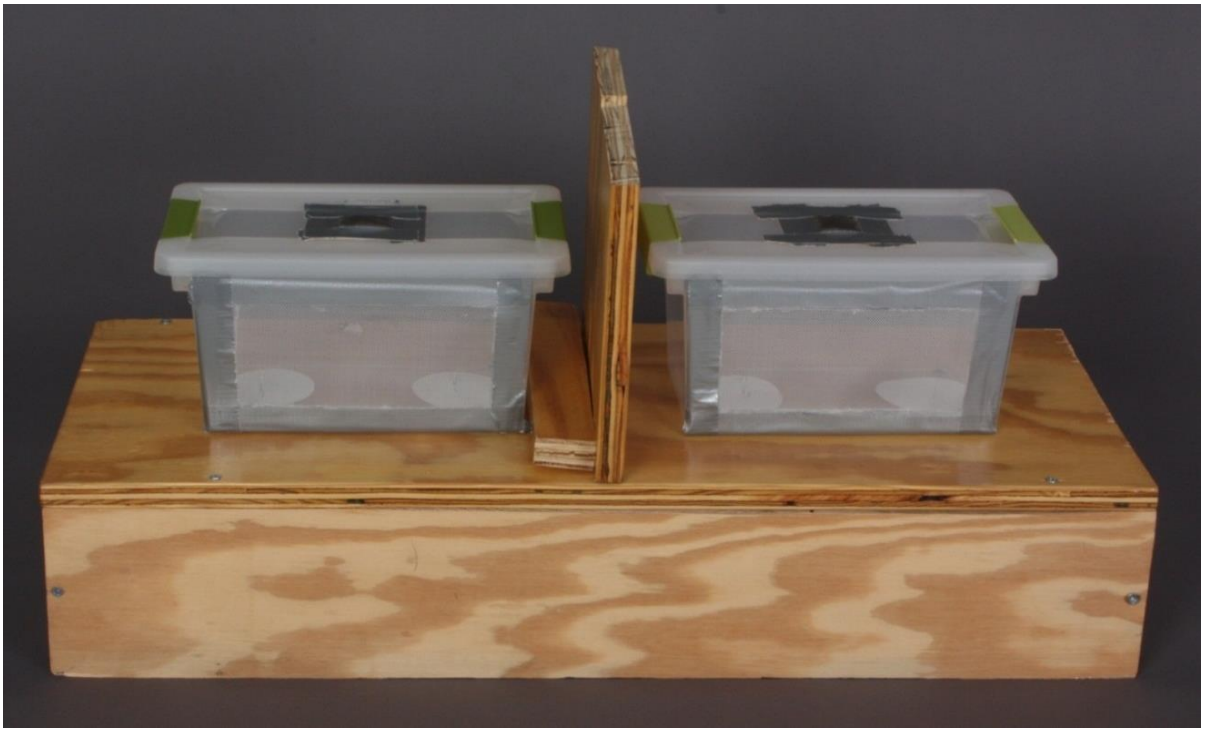

Figure 3.2. A pair of binary choice chambers separated by a piece of plywood on a light box.

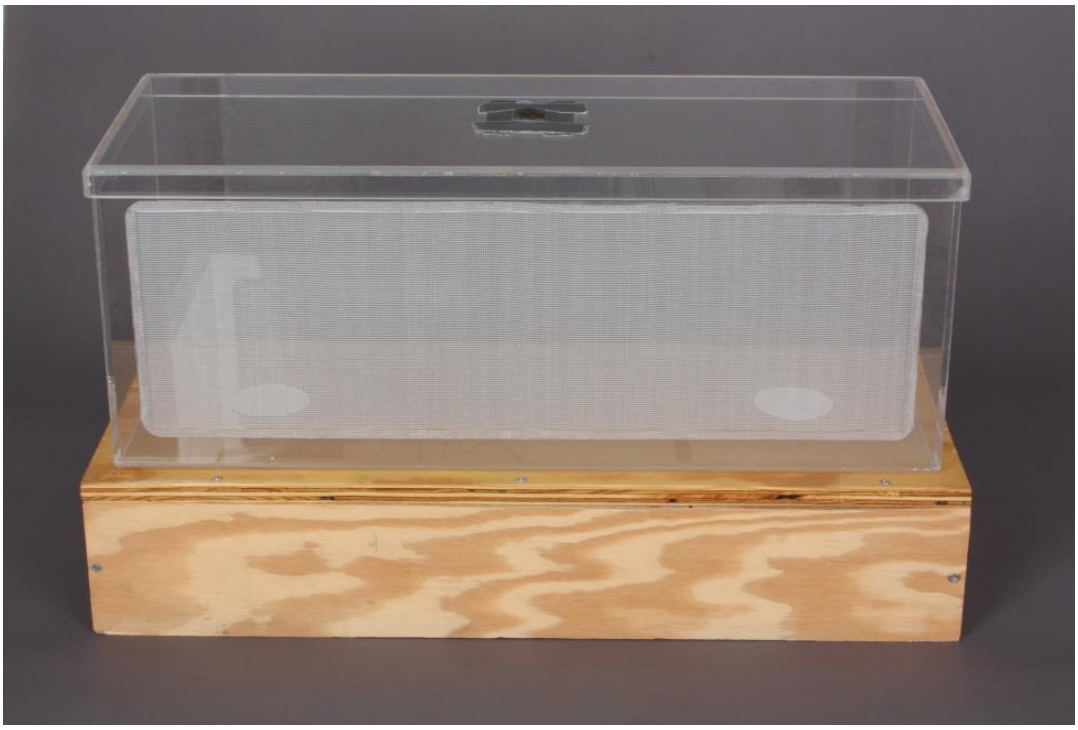

Figure 3.3. Large binary choice chamber on a light box.

Figure 3.4. Modified mosquito breeder with modified pipette aspirator inserted into it.

Figure 3.5. (Left) Oviposition attraction tower (OAT) with wooden base uncovered, exposing light. (Right) OAT with wooden base covered.

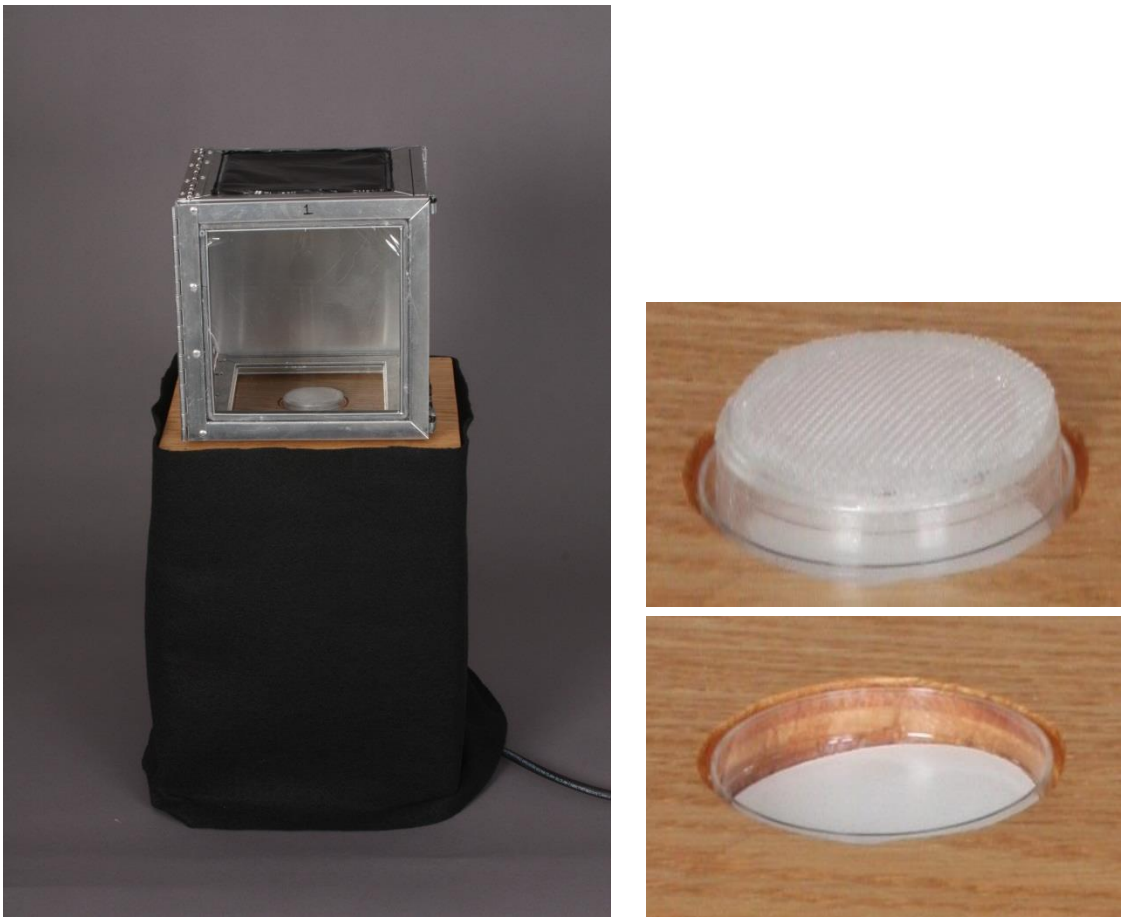

Figure 3.6. (Left) Oviposition attraction single chamber (OASC) with wooden base covered. (Right top) OASC with substrate covered with a mesh screen. (Right bottom) OASC with substrate uncovered.

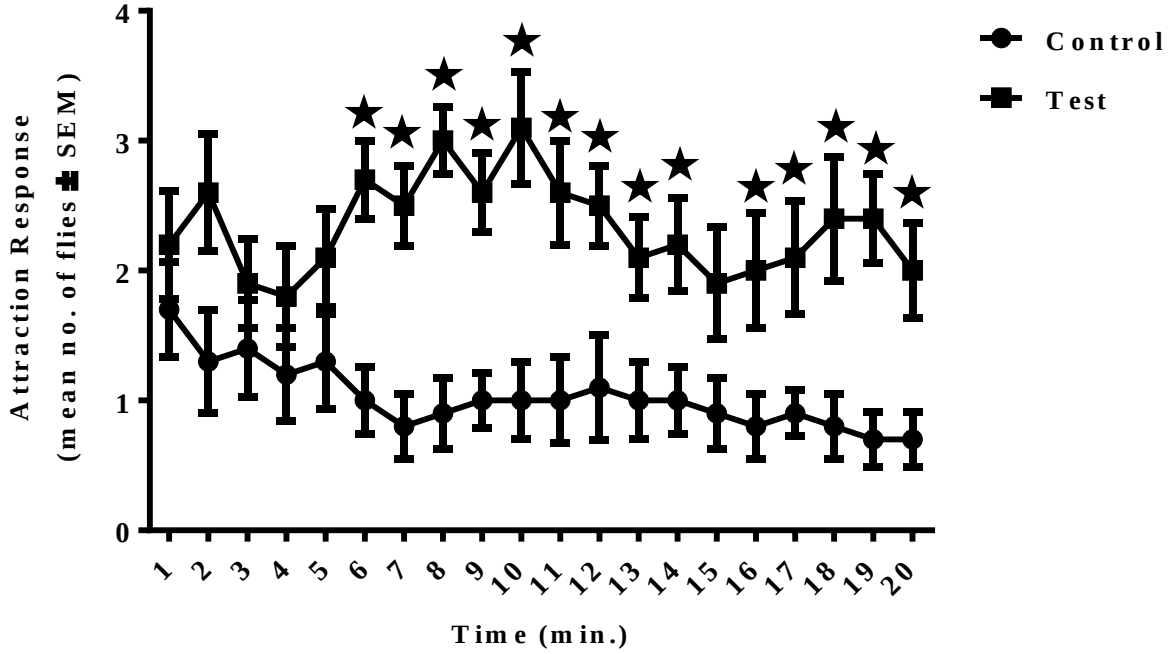

Figure 3.7. Attraction response of *Simulium vittatum* to 30- and 60-minute old eggs. The control treatment of no eggs is denoted by line with circles and the test treatment of 30- and 60-minute old eggs is denoted by line with squares. A significant response is indicated by a black star $P \leq 0.05$, paired t-test.

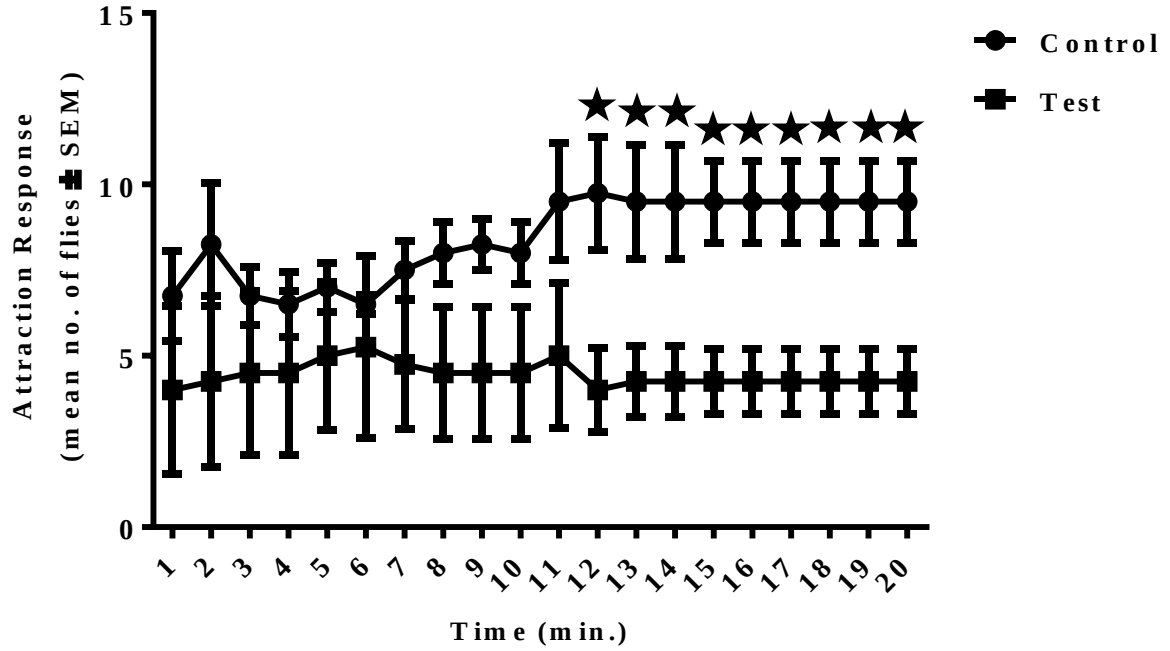

Figure 3.8. Attraction response of *Simulium vittatum* to hexane washed eggs. The control treatment of unwashed eggs is denoted by line with circles and the test treatment of hexane washed eggs is denoted by line with squares. A significant response is indicated by a black star $P \leq 0.05$, paired t-test.

CHAPTER 4

OVIPOSITION RESPONSE OF *SIMULIUM VITTATUM* TO EXTRACTS AND POTENTIAL OVIPOSITION STIMULANTS ISOLATED FROM CONSPECIFIC EGGS

Introduction

Our current understanding of communal oviposition black flies has been gained by observing ovipositing females in the wild and in the laboratory (Golini & Davies 1975, McCall et al. 1994, Muirhead-Thompson 1956, Rodriguez-Perez et al. 2003). An oviposition pheromone has been proposed to be a factor in this event (Coupland 1992). Previous laboratory studies with the *Simulium damnosum* complex have demonstrated attraction of gravid females to fresh eggs and mature-ovary volatiles (McCall 1995, P. J. McCall et al. 1997). However, the volatile attractants from the fresh eggs and mature ovaries were not identified.

In the previous chapter, the binary choice chamber (BCC) and oviposition attraction (OA) bioassays demonstrated that *Simulium vittatum* responded similarly to fresh conspecific eggs as *Simulium damnosum* and *Simulium ochraceum* complexes. Although the behavior was similar, the experiments with the BCC and OA did not provide any evidence of a volatile pheromone. Instead, the BCC and OA study demonstrated that *S. vittatum* would randomly land on a substrate, control or test treated, and would prolong its stay with a more likely chance of oviposition when it landed on a substrate with fresh eggs. These behavioral observations led to the thought that the factor responsible for communal oviposition of *S. vittatum* and other species demonstrating this behavior is non-volatile and requires contact by the gravid female.

To determine if chemicals associated with fresh conspecific eggs influence *S. vittatum* oviposition, a new bioassay, the petri dish bioassay was developed to measure the oviposition response of a single gravid female black fly when exposed to a stimulus such as an extract or chemical compound. This study used the petri dish bioassay and chemical analysis via gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) to observe the oviposition response to chemical factors associated with fresh eggs, and to identify the chemical factors that elicited an active response.

Materials and Methods

***Simulium vittatum* adults.** Experiments were conducted with gravid female *S. vittatum* (= cytospecies IS-7) adults. The flies were reared in colony from egg to emergence at the University of Georgia as described by Gray and Noblet (1999). After emergence, the flies were allowed to mate before being stored in an incubator for 5 days at 21.0 ºC and 90.0 % relative humidity.

To determine the number of days post-emergence at which the fly would contain mature ovaries, dissections of adult females at different post-emergence days (0-5) were performed. Before the use of the phase/contrast microscope to observe the follicular development of the eggs, dissections were made under the magnification of a stereomicroscope (RZ Series, Meiji Techno America, San Jose, CA, USA). During dissections the contents of the fly were in a solution of Pringle's saline on a glass slide (Pringle 1938). Criteria used to determine follicular development of eggs in this study were established in a previous study of black fly oogenesis (Cupp & Collins 1979).

***Simulium vittatum* egg extracts.** Fresh 30-minute old eggs were obtained by allowing 5-day old *S. vittatum* adults to oviposit on a small square of green linen (Jo-Ann's Fabric Shops,

Athens, GA, USA) in an oviposition chamber for 30 minutes. Fresh 30-minute old eggs were removed from the linen with a razor and placed into a 2.0-ml amber glass vial (Wheaton Science Products, Millville, NJ, USA). Immediately, the weight of the vial with eggs was measured with a scale to determine the mass of the eggs. The weight of the vial without eggs was measured beforehand. After recording the mass of the eggs, 1500 µl of the selected solvent: water, methanol, or hexane was added. Once the solvent was applied, the vial was agitated on a vortex (Maxi Mix II, Barnstead Thermolyne, Dubuque, IA, USA) for 15 seconds then placed in a freezer at -60 ºC for 24 hours. After being frozen, the vial was allowed to thaw and the supernatant was removed and placed in a new vial. The new vial was immediately used or capped with N_2 and stored at -60 ºC.

Chemicals. Pure solvents, methanol and hexane (≥98.5%; Thermo Fisher Scientific, Fair Lawn, NJ, USA), and deionized water, were used in creating the extracts from fresh eggs. Identified compounds from novel peaks of the active extracts were purchased for bioassay experiments (Alfa Aesar, Ward Hill, MA, USA): 1-octadecanol (97%), 1-tetradcanol (>97%), 1-pentadecene (97%), 1-hexadecene (94%), 1-tridecene (97%), and (Sigma-Aldrich, St. Louis, MO): 1-tridecanol (97%).

Behavioral bioassays. The petri dish (PD) bioassay experiments were conducted in 60 x 15 mm Pyrex glass petri dishes (Corning Inc., Corning, NY, USA), which was used upside down for the purpose of this bioassay. The petri dish was lined with a circular layer of cotton (U.S. Cotton, Gastonia, NC, USA), and on top of the cotton a piece of 55 mm Whatman grade 1 filter paper (GE Healthcare, Little Chalfont, UK) was placed, which acted as the substrate the flies would come into contact with during the bioassay. Immediately before a bioassay, 40 µl of a control or test sample was applied to the filter paper in the petri dish. After the solvent had

evaporated, 5.0 ml of deionized water was pipetted onto the treated filter paper. The wet, treated filter paper was then covered with the top of the petri dish. A single gravid female fly was aspirated into the petri dish at the beginning of the bioassay. Twenty prepared petri dishes, 10 controls and 10 test samples, were used in a single set. Each experiment consisted of 6 sets. During the bioassay, the petri dishes were placed on a light box, which contained wooden sides and a transparent Plexiglas top (Figure 4.1). Three fluorescent lights (Utilitech, distributed by Good Earth Lighting Inc., Wheeling, IL, USA) were mounted inside the light box. A black cloth covered the petri dishes on the light box. All experiments were conducted in a climate controlled room with temperatures at 23-26 ºC and relative humidity at 75-90 %. Each set was allowed 30 minutes of oviposition time before the results were recorded.

Petri dish bioassay experiment 1. In PD bioassay experiment 1, the oviposition response of gravid *S. vittatum* was observed when presented with a wet substrate impregnated with an egg extract or solvent only. Three types of extract solvents were used (deionized water, methanol, and hexane) and their controls were the solvent alone.

Petri dish bioassay experiment 2. PD bioassay experiment 2 was conducted with identified chemical compounds from the active extracts as the test stimulus. Different dilutions (1:1000, 1:100, 1:10, and 1:1) were tested for each chemical compound.

Statistical analysis. The number of flies that oviposited when exposed to the control and the extract was recorded after 30 minutes for each replicate. All replicate values of the control and test responses were summed for each treatment. A Fisher's exact test was used to statistically analyze the sums of the control and test responses for a test stimulus and its respected control stimulus. A test stimulus was considered an oviposition inducer (active) if the probability value for the Fisher's exact test was ≤ 0.05.

To compare the test responses of active stimuli, the difference value of each active stimulus was analyzed with an unpaired t-test. The difference value for a single replicate of a test stimulus was calculated with the formula:

$$D = T - C$$

where *D* denotes the difference value, *T* denotes the number of flies that oviposited for the test stimulus, and *C* denotes the number of flies that oviposited for the control stimulus. Statistical analyses were performed using GraphPad software (Prism 6, GraphPad Software, La Jolla, CA, USA). The raw data (number of ovipositing flies) was transformed into percentages for graphing purposes. All statistical analysis was performed on the raw data.

Chemical analysis. Gas chromatography (GC) was used to initially screen active extracts and identify any novel peaks in the extract sample, compared to the solvent control, which could be potential oviposition stimulatory compounds. The initial GC screenings were conducted on a Varian 3800 GC (Agilent Technologies, Santa Clara, CA, USA). The Varian 3800 GC was equipped with a Varian 8400 AutoSampler and CP-8410 AutoInjector (Agilent Technologies, Santa Clara, CA, USA). The column used was a 30 m x 0.25 mm internal diameter with a 0.25 µm phenyl arylene polymer film with a 10 m DuraGuard (DB-5MS, Agilent Technologies, Santa Clara, CA, USA). A 10-µl injection was initiated in split-less mode at 80 Cº and then increased at a rate of 6.0 Cº/min. until it reached 220 Cº, which it then held for 10 min.

Once a novel peak was identified in the extract sample, a second screening with gas chromatography-mass spectrometry (GC-MS) was conducted using a Hewlett Packard 6890 GC, which was coupled to a Hewlett Packard 5973 MS (Hewlett Packard, Palo Alton, CA, USA). The column used was a 30 m x 0.25 mm internal diameter with a 0.25 µm (5%-phenyl)-methylpolysiloxane film (HP-5, Hewlett Packard, Palo Alton, CA, USA). A 10-µl sample was injected via HP 7673 Injector (Hewlett Packard, Palo Alton, CA, USA). Initial temperature was 35 Cº with a 1.0 min. hold, and then with a 6.0 Cº/min. rate, the temperature reached 260 Cº, which then held for 12 min. Chemical species were identified by matching the mass spectrum to known chemical species stored in the computer library, Fatty Acid Methyl Ester (FAME) Database.

Results

Ovary dissections. Follicular development stages of *S. vittatum* eggs from the ovary dissections are shown in figure 4.2. Ovaries of females at 0-2 days post-emergence consisted of eggs with a stage N, I, II, and III follicle. Females at 3-4 days post-emergence contained eggs primarily with a stage IV follicle but some possessed a stage V follicle. Eggs with a stage V follicle were consistently present in 5 days post-emergence flies; therefore, 5 days post-emergence flies were used for the behavioral bioassays.

Petri dish bioassay experiment 1. Gravid female *S. vittatum* were significantly ($P < 0.05$, Fisher's exact test) induced to oviposit more when exposed to the test treatments of water and hexane extracts (50.0 ± 5.2 % and 51.7 ± 4.8 %, respectively) in comparison to their controls (18.3 ± 3.1 % and 30.0 ± 4.5 %, respectively). The methanol extract (30.0 ± 9.3 %) did not significantly ($P > 0.05$, Fisher's exact test) stimulate the flies to oviposit more than its control (28.3 ± 5.4 %).

Chemical analysis of active extracts from conspecific eggs. Figure 4.3 shows the initial GC analysis of the water and hexane extracts. A peak with a retention time of 23.524 min. was present in the water extract sample but not for the deionized water sample. Further analysis with GC-MS identified 1-octadecanol and 1-tetradecanol within the novel peak from the water extract.

Initial GC analysis of the hexane extract found a peak with a retention time of 13.989 min. in the hexane extract sample but not for the hexane solvent sample. Further analysis with GC-MS identified: 1-pentadecene, 1-hexadecene, and 1-tridecene within the novel peak from the hexane extract.

Petri dish bioassay experiment 2. In figure 4.4, the oviposition responses to the identified chemical compounds of the active water extract and their controls are shown. None of the test compounds induced significantly more oviposition than its respective control.

Two compounds identified from the active hexane extract, 1-pentadecene (1:100) and 1-tridecene (1:10), induced significantly ($P < 0.05$, Fisher's exact test) more oviposition than their controls (Figure 4.5). The mean difference percentages of the 1:100 dilution of 1-pentadecene (31.7 ± 8.7 %) and 1:10 dilution of 1-tridecene (30.0 ± 6.8 %) were not significantly ($P > 0.05$, unpaired t-test) different.

Discussion

Extracts and individual chemical compounds when in direct contact with gravid *S. vittatum* induce more oviposition than a control exposure of solvent only. Crude water and hexane extracts of fresh conspecific eggs induced oviposition, but only compounds identified from the hexane extract (1-pentadecene and 1-tridecene) elicited a response similar to that induced by the crude extracts. In a previous study, 1-pentadecene has been demonstrated in the

beetle *Tribolium confusum* to be a potential aggregation pheromone due to the attractive behavior of the beetle to the substance (Verheggen et al. 2007). Another tenebrionid, *Parastizopus transgariepinus*, uses 1-tridecene as a male-produced sex pheromone (Barrozo & Lazzari 2004).

The hydrophobic characteristic of the compounds identified from the non-polar hexane extract would be plausible that they adhere more strongly to the surface of the black fly egg in running water in comparison to the polar compounds identified from the water extract. This adherence to the surface of the eggs would allow the flies to come into contact with the compound(s) while physically sensing the potential oviposition substrate. Golini and Davies (1975) observed a tactile investigative behavior by black flies at an oviposition site. The black flies would land on a potential oviposition substrate and tap their forelegs around the surface and then fly away. This behavior could represent the black fly using chemoreceptors on its forelegs to determine if it had landed on a suitable oviposition substrate. Although it is tempting to compare the results of this study to wild oviposition behavior such a comparison may not be valid. The flies from this study have been colonized since 1980. Selective pressures on the population differ from the wild and could influence the behavior of the current generation of flies. However, the results from this study supported the hypothesis that the factors located on fresh eggs, which elicited an oviposition response, need to come in direct contact with the fly for the response to occur.

Tables and Figures

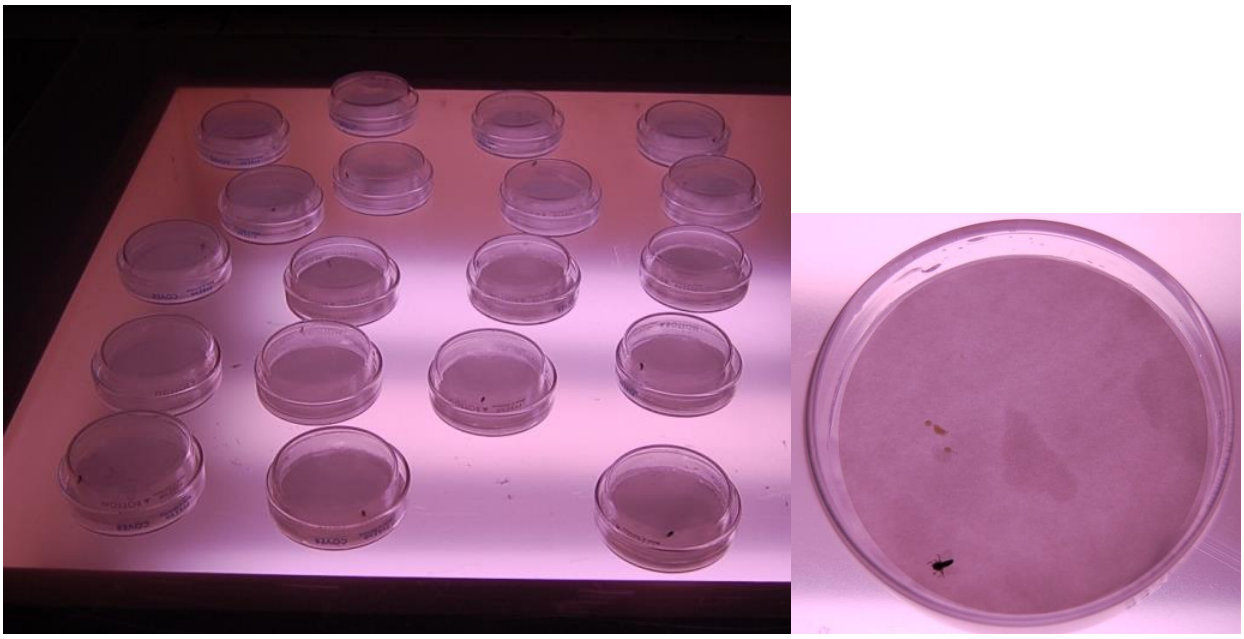

Figure 4.1. (Left) Petri dishes on light box. (Right) Recently oviposited eggs on filter paper in a petri dish.

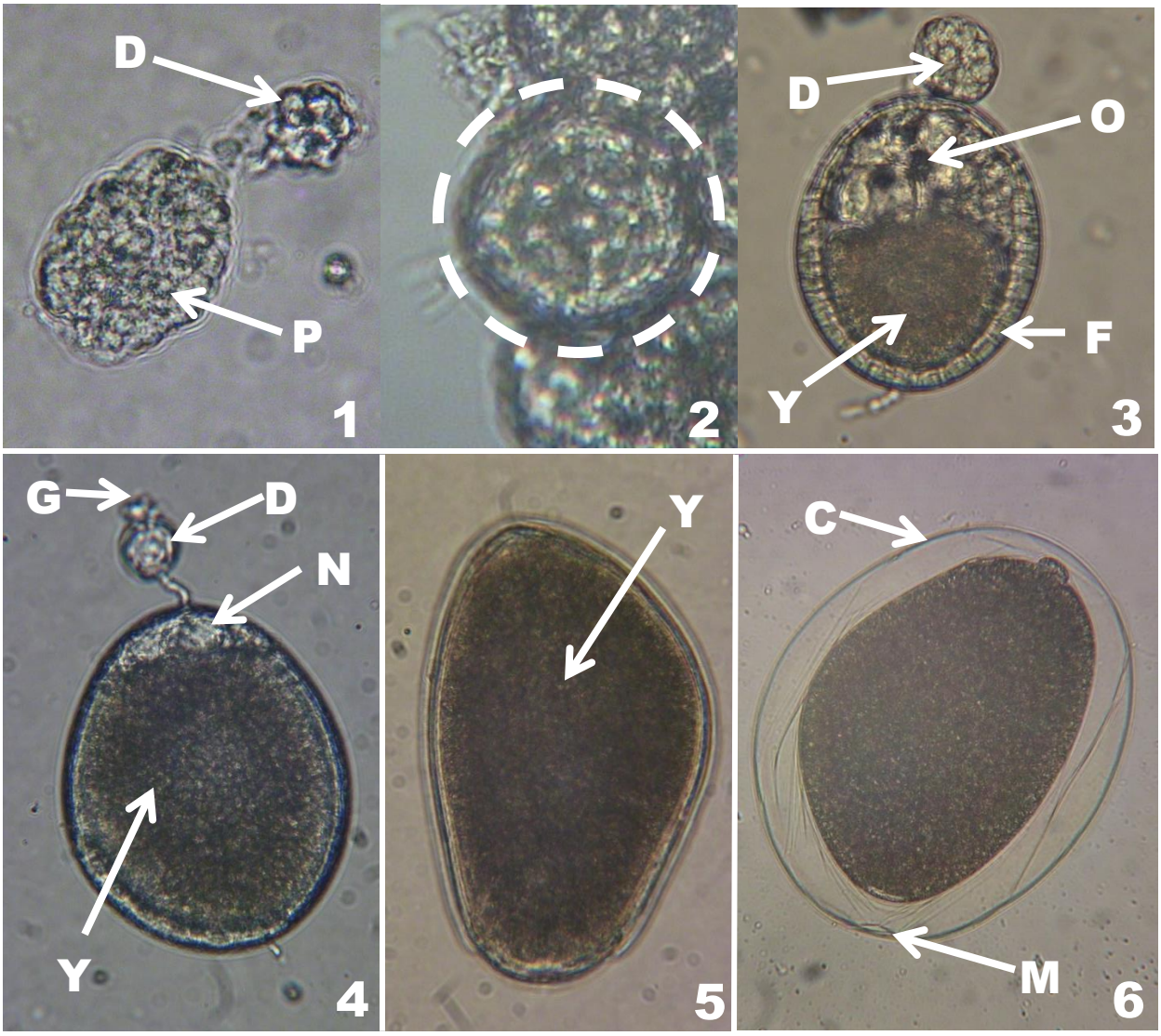

Figure 4.2. Oogenesis in *Simulium vittatum*. 1. Stage N. Undifferentiated proximal follicle (P) and distal follicle (D). 2. Stage I follicle. Follicular space occupied by an oocyte, nurse cells, and previtellogenic yolk granules (inside dashed circle). 3. Stage II follicle. Half of follicular space is occupied by yolk (Y) and the other half a developing oocyte (O). A follicular epithelium (F) is now present. 4. A stage III follicle. Nurse cells (N) are located at the posterior of the follicular space while yolk (Y) occupies the remainder. The distal follicle (D) and germarium (G) are present. 5. Stage IV follicle. Nurse cells are no longer visible and yolk (Y) is predominantly visible in the follicular space. 6. Stage V follicle. A distinct chorion (C) and micropyle (M) are visible.

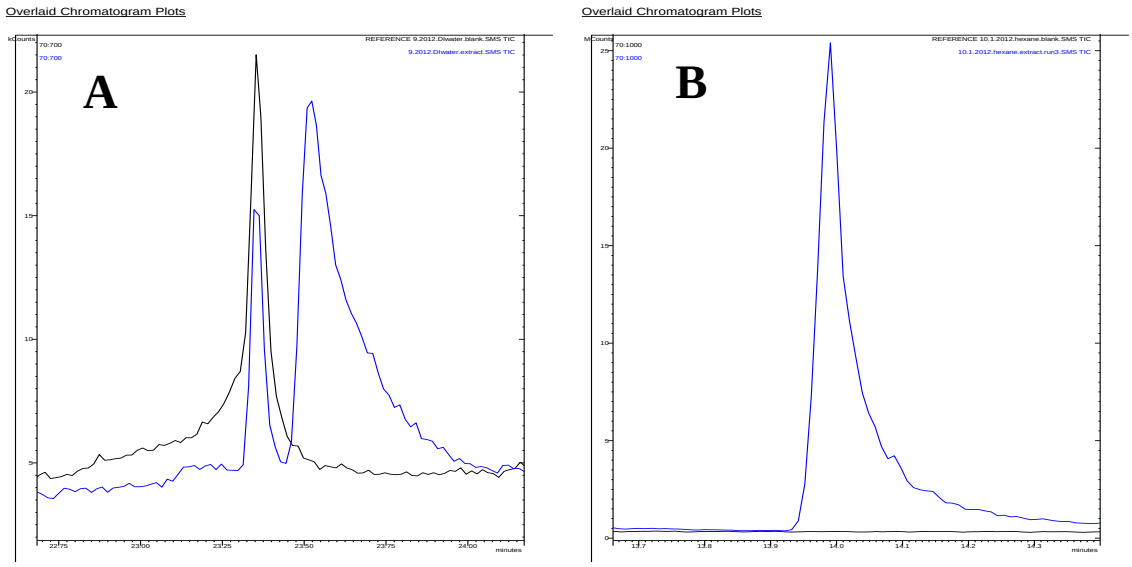

Figure 4.3. Gas chromatography chromatograms of *S. vittatum* extracts and their respected controls of solvent only. A. Chromatograph of water extract of fresh eggs (blue line) and control sample of deionized water (black line). B. Chromatograph of hexane extract of fresh eggs (blue line) and control sample of hexane (black line).

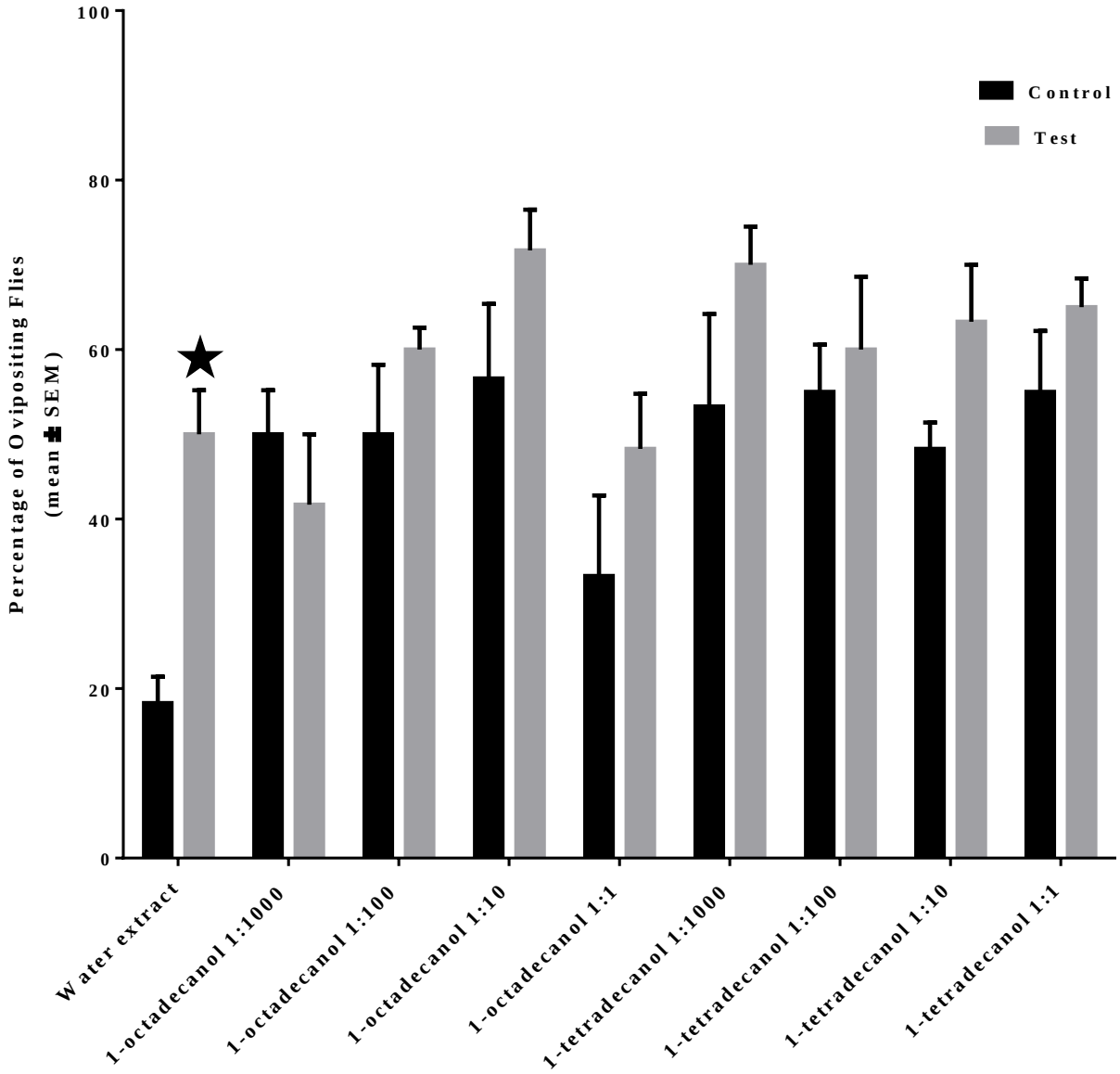

Figure 4.4. Oviposition responses of gravid *S. vittatum* to a water extract of fresh eggs and compounds identified in the extract. A black star located above the test response of a treatment indicates a significant ($P < 0.05$, Fisher's exact test) difference between the test and control responses for the treatment.

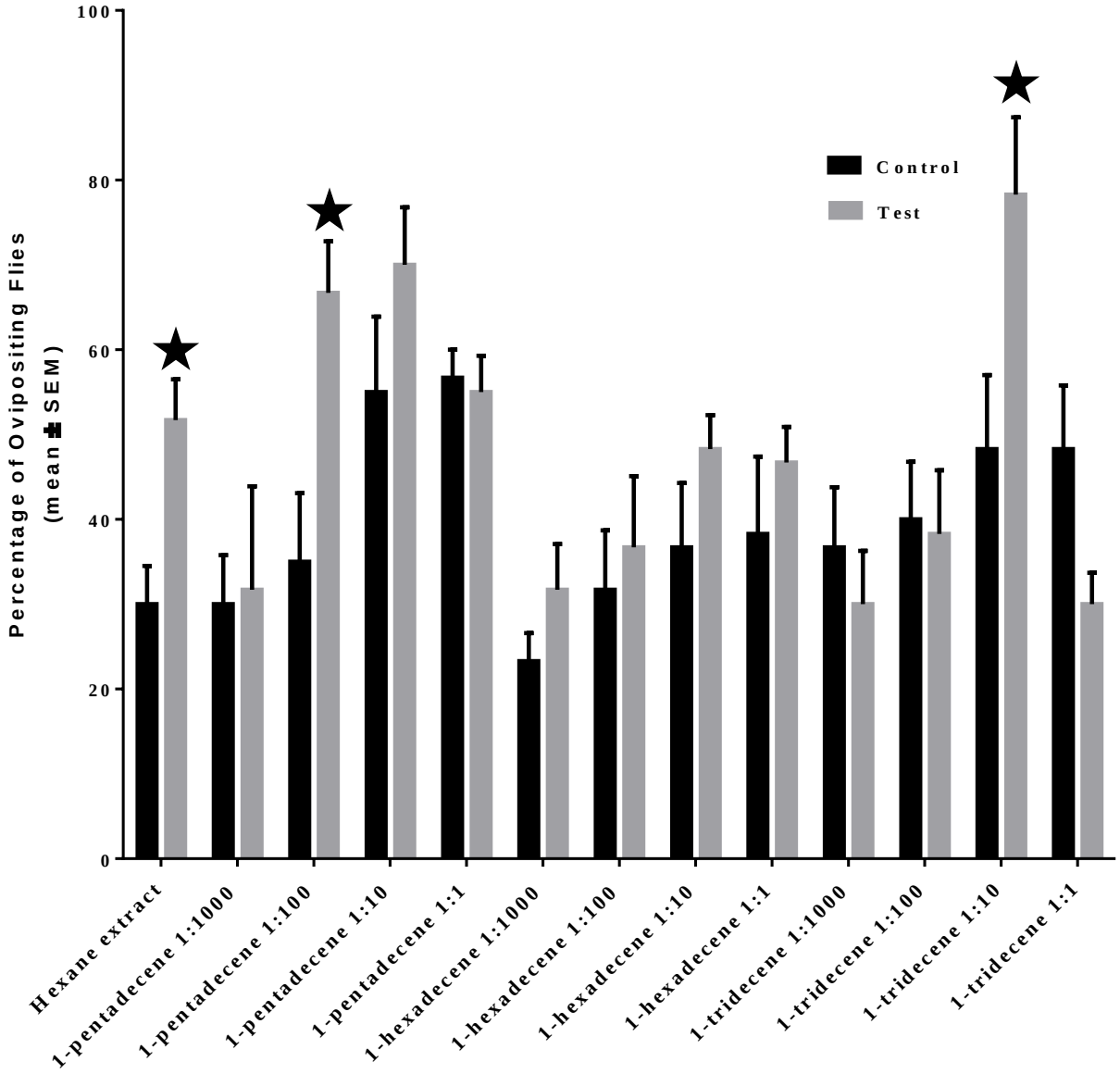

Figure 4.5. Oviposition responses of gravid *S. vittatum* to a hexane extract of fresh eggs and compounds identified in the extract. A black star located above the test response of a treatment indicates a significant ($P < 0.05$, Fisher's exact test) difference between the test and control responses for the treatment.

CHAPTER 5

HOST-SEEKING BEHAVIORAL RESPONSE OF *SIMULIUM VITTATUM*, THE *SIMULIUM OCHRACEUM* COMPLEX, AND *SIMULIUM DAMNOSUM* COMPLEX TO POTENTIAL ATTRACTANTS IN A Y-TUBE OLFACTOMETER

Introduction

Although most black fly species are considered haematophagous nuisance pests, some species in the genus *Simulium* can transmit debilitating disease agents to livestock as well as humans (Gray et al. 1996, Enk et al. 2003, Mead et al. 2009). Onchocerciasis, also known as river blindness, is a devastating human disease associated with black flies. The causal agent, a nematode parasite, *Onchocerca volvulus*, is primarily transmitted by *Simulium* species in Latin America and Africa (Katabarwa et al. 2013). Tremendous efforts have been conducted by health organizations which have allowed transmission of *O. volvulus* to be interrupted in endemic areas (Cupp et al. 2011). To determine if interruption has occurred, monitoring of the black fly population in endemic areas for the presence of *O. volvulus* enables the organizations to provide organized medical treatments and vector control (Higazi et al. 2011).

Host-seeking behavior of black flies has been investigated in numerous field trials, which focused on identifying what attracts a host-seeking black fly to its host. Unlike the mosquito, the black fly is poorly understood in the area of host-seeking behavior. One attractant commonly used in field experiments is carbon dioxide. Field studies using traps baited with carbon dioxide have attracted *S. slossanae*, *S. damnosum* complex, *S. arcticum* complex, and *S. ochraceum* complex (Kim & Merritt 1987, Moore & Noblet 1974, Sutcliffe et al. 1994, Sutcliffe et al. 1995,

Thompson 1976b). Carbon dioxide also has a synergistic effect in attracting host-seeking black flies when combined with other host-seeking attractants, such as human breath, crude cattle extract, and 1-octen-3-ol (Atwood & Meisch 1993, Sutcliffe et al. 1994, Thompson 1976b). 1-octen-3-ol, a known mosquito host-seeking attractant, is attractive on traps in the field (Atwood & Meisch 1993). 1-octen-3-ol is a component of cattle and human odor (Cook et al. 2011).

Although field research is valuable to help better understand the host-seeking behavior of black flies, controlled laboratory studies would allow more precise experiments. Only one study has been published on host-seeking behavior of a black fly in a laboratory setting using a Y-tube olfactometer (Opoku 2008). This laboratory study demonstrated that *S. ornatum* was attracted to low concentrations of carbon dioxide, 1-octen-3-ol, and humidified air. A Y-tube olfactometer can be used to identify specific concentrations of a chemical compound or substance that are attractive to a given insect species. Several attractants of haematophagous Diptera other than black flies have been investigated with a Y-tube olfactometer and subsequently identified (Blackwell et al. 1996, Bosch et al. 2000, Cook et al. 2011, Geier & Boeckh 1999, Geier et al. 1999).

The objective of this study was to demonstrate the behavioral responses of host-seeking black flies to potential volatile attractants in a Y-tube olfactometer, and to identify volatile compounds most attractive to host-seeking black flies.

Materials and Methods

Host-seeking female black fly adults. Experiments were conducted with parous female *S. vittatum* (= cytospecies IS-7) adults reared in colony at the University of Georgia as described by Gray and Noblet (1999). Parous flies were generated by allowing the flies to oviposit in an oviposition chamber. Subsequently, an aspirator was used to collect the parous flies from the

oviposition chamber and transfer them into a paper carton, with a piece of cotton saturated with 10% sugar solution on the screened lid and stored in a rearing chamber. Conditions in the rearing chamber were 21 ºC and 90 % relative humidity. Experimental parous females were aspirated from the paper carton and transferred to a transparent holding container, modified mosquito breeder (Bioquip, Inc., Rancho Dominguez, CA, USA), for precise aspiration. A modified pipette aspirator was used to transfer the experimental group of parous females to the bioassay.

Experiments with vector species were conducted with wild caught *S. ochraceum* and *S. damnosum* complexes in Union Juarez, Chiapas, Mexico and Banfora, Burkina Faso, respectively. The flies were collected at breeding sites by collectors using human volunteer as bait. Once the fly landed on the human bait, a collector would capture the fly in a vial and store it in a cooler. After collection, the flies were transported to the experimental room, where the flies were allowed to acclimate to the temperature.

Y-tube olfactometer. Behavioral bioassays were conducted in a Y-tube olfactometer, which was modified for conducting experiments with black flies from a previous Y-tube olfactometer used with mosquitoes (Geier & Boeckh 1999). The Y-tube was fabricated by the University of Georgia Instrument Shop from transparent acrylic tubing with an internal diameter of 3.81 cm. The Y-tube olfactometer (Figure 5.1) consisted of four sections: release chamber, Y-split chamber, stimulus arm trap chambers, and stimulus chambers. The release chamber contained an aspirator entry point on the ventral side, a fabric mesh screen on the dorsal side, and a metal mesh rotating door in the apical side. The mesh partitions permitted air flow through the release chamber and the rotating door was used to release the flies after acclimation in the Y-split chamber, which connects the release chamber to the stimulus arm trap chambers. Each stimulus

arm chamber contains a rotating metal mesh screen door, which closes after the test run to block any flies from entering or exiting the stimulus arm chamber. A stimulus chamber is connected to the apical end of the stimulus arm trap chamber, which is separated by a fabric mesh screen. The stimulus chamber is accessible by a sliding door. During the acclimation and test run periods, a black cloth covered the Y-tube olfactometer completely, except the apical end of the stimulus chambers. The opening in the black cloth allowed fluorescent light (Utilitech, distributed by Good Earth Lighting Inc., Wheeling, IL, USA) to enter the distal end of the stimulus chambers. This light source was required to initiate activation of the flies. Preliminary experiments without the presence of light at the distal end of the Y-tube olfactometer demonstrated that flies would not participate even in the presence of a proven attractant. Air was supplied into the distal end of the stimulus chambers. An air pump (Greentrees Hydroponics, Vista, CA, USA) was used to pump air, 25-26 ºC, through a hydrocarbon filter (LabClear, Diamond Tool and Die, Inc., Oakland, CA, USA). Filtered air was pumped through an acrylic humidifier (University of Georgia Instrument Shop, Athens, GA, USA) before the air-line was split into 2 separate lines, which entered flow meters (Brooks Instruments, Hatfield, PA, USA) to provide regulated air flow to the stimulus chambers. Air entering the stimulus chambers was supplied at a flow rate of 1.0 lpm for *S. vittatum* and 1.5 lpm for *S. ochraceum* and *S. damnosum* complexes.

Chemicals. Liquid test and control chemicals were introduced into the Y-tube by injecting 20 µl of the test or control solution onto a piece of 2.5-cm Whatman grade 1 filter paper (GE Healthcare, Little Chalfont, UK). The solvent was allowed 20 seconds to evaporate before the filter paper was placed in the stimulus chamber, where a clamp held the filter paper in place. Filter paper was prewashed with hexane before the experiment.

The selection of test chemicals to be used for *S. ochraceum* and *S. damnosum* was based on data from electroantennogram (EAG) studies (N. Burkett-Cadena and R. Young, personal communication, February and April 2013).

Behavioral bioassays. Experimental trials in the Y-tube olfactometer consisted of two phases: a 10-minute acclimation phase, which allowed flies in the release chamber to be exposed to clean air, and a 20-minute test phase for *S. vittatum* and a 1-minute test phase for the *S. ochraceum* complex and *S. damnosum* complex. After each experimental run the flies were returned to the release chamber or discarded. The control and test stimulus chambers were alternated after each replicate. A block of testing consisted of four experimental runs in which two groups of 20 flies were used. A single group of flies was only tested twice before being discarded. A control run with clean air as the stimulus for each Y-tube arm was performed before each block of testing to ensure that no bias was present. After each block of testing, the Y-tube olfactometer was cleaned using water and detergent (Alconox: precision powdered detergent, Alconox, Inc., White Plains, NY, USA), and then rinsed with ethanol.

Statistical analysis. Activation (number of flies that exited the release chamber) was measured for each replicate only with *S. vittatum*. An arcsine transformation was performed on the proportions of flies activated. The transformed means ± SEM were calculated for the activation responses of the test treatments and were compared to the activation response to air by using a one-way ANOVA test followed by Dunnett's multiple comparisons test.

Attraction (number of flies that were trapped on the side of a stimulus treatment) was measured for each replicate of *S. vittatum, S. ochraceum* complex, and *S. damnosum* complex tests. The percentage of test attraction for a replicate was calculated using the formula:

$$\text{Test Attraction} = (T \times 100) / (T + C)$$

where *T* denotes the number of flies trapped on the test stimulus side and *C* denotes the number of flies trapped on the control stimulus side. The percentage of test attraction for each treatment was averaged. The same calculation was performed to find the control attraction response for each treatment. An arcsine transformation was performed on the proportions of flies attracted. The transformed mean of the control and test attraction responses were compared using a paired t-test, and the test attraction responses for all the treatments were compared using a one-way ANOVA test followed by the Tukey *post hoc* test (Geier & Boeckh 1999, Geier et al. 1999). Statistical analyses were performed using GraphPad software (Prism 6, GraphPad Software, La Jolla, CA, USA).

Results

***Simulium vittatum* bioassays.** Activation and attraction responses to 1-octen-3-ol (6-6000 µg), CO_2, and CO_2 added with 1-octen-3-ol (15% and 600 µg, respectively) are shown in table 1. An increasing activation response from host-seeking *S. vittatum* was observed with increasing concentrations of 1-octen-3-ol as the test stimulus. The 600- and 6000-µg test treatments of 1-octen-3-ol activated significantly more flies (58.7 ± 4.3, $P < 0.05$ and 73.7 ± 5.1, $P < 0.0005$, respectively) than the activation response for air. No significant difference was indicated between the activation responses of the 600- and 6000-µg test treatments of 1-octen-3-ol. The attraction response increased with the increase of 1-octen-3-ol concentration, but the highest and most significant attraction response for 1-octen-3-ol was at 600 µg (88.7 ± 3.8, paired t-test, $P = 0.016568$). A less attractive but significant response was observed with 1-octen-3-ol at 6000 µg (71.7 ± 3.3, paired t-test, $P = 0.008$). No significant difference was

indicated between the attraction responses of the 600- and 6000-µg test treatments of 1-octen-3-ol.

A 15% CO_2 mixture with air significantly activated more flies (55.0 ± 6.8, $P < 0.05$) than the activation response for air, but the test treatment did not attract significantly more flies than its control of air. When 1-octen-3-ol (600 µg) was added with the 15% CO_2 mixture with air, the activation response (95.0 ± 3.5, unpaired t-test, air $P < 0.05$) was significantly more than the activation responses for air, CO_2, and 1-octen-3-ol (6, 60, 600, 6000 µg). An attraction response of 82.8 ± 6.2 was achieved by the mixture and was significantly more attractive ($P = 0.048$) than its control attraction response; however the test attraction response was not more significant than the other significantly attractive test treatments (Table 5.1).

Responses of activation and attraction to chemical compounds at different dilutions (1:1000, 1:100, 1:10) versus a hexane control are shown in figures 5.2 and 5.3. 1-octen-3-ol (1:10), heptanoic acid (1:10), hexanoic acid (1:10), isobutyric acid (1:1000, 1:100, 1:10), dihydrocarvone (1:10), 2-decanone (1:10), and *m*-cresol (1:10) elicited a significant activation response in comparison to the activation response of air. 1-octen-3-ol (1:100) was the only compound to elicit a significant attraction response from the compounds tested shown in figure 5.3.

***Simulium ochraceum* complex bioassays.** Activation responses of *S. ochraceum* complex were high (>85%) for every test treatment as well as a control run with air versus air. In table 5.2, the test attraction responses of host-seeking *S. ochraceum* complex are shown to potential host-odor attractants which were tested at a dilution of 1:100. No attraction was exhibited by the flies when exposed to air or CO_2. Significant attraction was observed to

dihydrocarvone (76.5 ± 3.9, $P < 0.05$, paired t-test), acetophenone (64.4 ± 3.8, $P < 0.05$, paired t-test), and 1-octen-3-ol (70.7 ± 2.5, $P < 0.005$, paired t-test).

***Simulium damnosum* complex bioassays.** Activation responses of *S. damnosum* complex were high (>85%) for every test treatment as well as a control run with air versus air. In table 5.3, the test attraction responses of host-seeking *S. damnosum* complex are shown to potential host-odor attractants. No attraction occurred with the controls of air or hexane. A dose-dependent test attraction response was observed when exposed to 1-octen-3-ol and heptanoic acid, which both elicited a significant response ([1:100], 74.3 ± 5.2, $P < 0.05$ and [1:100], 80.0 ± 0.0, $P < 0.005$, respectively). No attraction was observed to hexanal.

Discussion

Laboratory experiments with a Y-tube olfactometer have demonstrated dose-dependent responses of host-seeking female *S. vittatum*, *S. ochraceum* complex, and *S. damnosum* complex to several chemical compounds which have been identified from hosts of the species. Among the chemicals, 1-octen-3-ol was the most consistently attractive compound among the three species. The highly volatile compound 1-octen-3-ol has been identified in odors from cattle, host of *S. vittatum*, and human, a host of *S. ochraceum* and *S. damnosum* complexes (Cork & Park 1996, Hall et al. 1984). However, the range of behavioral responses to 1-octen-3-ol (1:1000, 1:100, 1:10) was more sensitive with *S. vittatum* and *S. damnosum* complex than *S. ochraceum* complex, which may be due to the host specificity of the species.

Other activating compounds for host-seeking *S. vittatum* (2-decanone and *m*-cresol) have been identified from the volatiles of cattle (Oyarzun et al. 2009). In the same study, *m*-cresol was attractive to the horn fly, *Haematobia irritans*. *Triatoma infestans*, a vector of *Trypanosoma cruzi* (the causative agent of Chagas' disease), is attracted to 1-octen-3-ol and isobutyric acid

(Barrozo & Lazzari 2004, Guerenstein & Guerin 2001). Similar to responses of the black flies in the current study, *T. infestans* demonstrated a dose-dependent attraction response to 1-octen-3-ol, which was only found attractive at an intermediate dose (Barrozo & Lazzari 2004). Isobutyric acid along with hexanoic acid was isolated from volatiles of human foot odor (Caroprese et al. 2009).

Activation responses of the different species were difficult to compare due to the higher levels of flight activity in *S. ochraceum* and *S. damnosum* complexes, which were collected directly from the field versus *S. vittatum*, taken from a laboratory colony. In a Y-tube olfactometer control run (air vs. air), *S. vittatum* had an average activation response of 25% after 20 minutes of exposure compared to *S. ochraceum* and *S. damnosum* complexes which exhibited an average activation of 90% after 1 minute. The increase in activation between the species may be due to light sensitivity or some unknown physiological factor controlling behavior. Black flies are photopositive, and in a related study, Y-tube olfactometer experiments with host-seeking *Culicoides* used a light source at the distal side of the olfactometer to induce flight activity towards the arms (Blackwell et al. 1994, Blackwell et al. 1996). In preliminary experiments in the current study, almost no activation was achieved when a light source was not present. Higher activation responses from the wild *S. ochraceum* and *S. damnosum* complexes may be due, at least in part to circadian rhythm and host-seeking behavior activity peaks.

Response differences to volatile host-seeking attractants can occur between *Simulium* species as we have learned in this study. 1-octen-3-ol, acetophenone, and tetradecanoic acid were attractive to *S. ochraceum* complex, but *S. vittatum* was only attracted to 1-octen-3-ol. This difference in attraction responses between *S. vittatum* and *S. ochraceum* complex could be due to various factors. *S. vittatum* is autogenous and does not require a blood-meal for its ovaries to

mature and produce the first eggs as compared to *S. ochraceum* complex, which is anautogenous and must have a blood-meal before developing an initial batch of eggs. An anautogenous species would need highly developed and sensitive receptor neurons in its sensilla for immediate host-seeking identification, whereas an autogenous species would not require its host-seeking abilities to be so finely tuned. In a physiological comparison study of sensitivity to CO_2 in receptor neurons of the mosquito maxillary palp, *Culex molestus*, an autogenous species, exhibited less sensitivity to CO_2 than *Culex pipiens*, an anautogenous species (Grant & O'Connel 2010). If receptor neuron sensitivity to host volatile attractants can vary between autogenous and anautogenous species of mosquitoes, then it could be the case for black flies. A second factor focuses on the age of the flies which could have resulted in different responses. Since *S. vittatum* were reared in a colony and used after initial oviposition, their age ranged between 10 and 17 days old prior to experimentation, but the ages of the wild *S. ochraceum* and *S. damnosum* complexes were not known. Davis (1984) demonstrated higher sensitivity to host odors in the grooved peg sensilla of *Aedes aegypti*, which was age-dependent until 100 hours post-emergence, and Grant & O'Connell (2007) found young female mosquitoes possessed receptor neurons less sensitive to host odors than older females.

Distribution, quantity, and type of chemoreceptors on the sensory organs (antenna, maxillary palps, labrum, labium, and labella) of the flies could also impact the preference of a host. Upon noticing the number of sensilla on a single antenna from ornithophilic (~1800) and non-bloodsucking (~800) species of black flies, it is worth postulating that "more is better" (Mercer & Mciver 1973b). Specialized sensory organs also come into play with host-seeking. Ornithophilic species have larger Lutz's organs, a sensory vesicle found in the swollen 3rd segment of the maxillary palps which detects CO_2 similarly to the capitate pegs of mosquitoes

and biting midges, than mammalophilic and non-bloodsucking black flies (Mercer & Mciver 1973a). These varying morphological factors could impact the results when comparing the responses of *S. vittatum* and *S. ochraceum* and *S. damnosum* complexes to the same chemical compound.

Tables and Figures

Table 5.1. Behavioral responses of host-seeking *S. vittatum* to 1-octen-3-ol and CO_2. Values followed by a different letter in the activation row are significantly different (one-way ANOVA). Significant attraction to a test stimulus compared to its control was denoted by asterisks (* $P < 0.05$ and ** $P < 0.005$, paired t-test).

Response (% ± SE)	Test Treatments						
			1-octen-3-ol (µg)				
	Air	CO_2	6.0	60	600	6000	CO_2 + 600
Activation	25.0 ± 3.5 a	55.0 ± 6.8 b	21.3 ± 3.7 a	30.0 ± 8.7 a	58.7 ± 4.3 b	73.8 ± 5.1 c	95.0 ± 3.5 c
Attraction	50.0 ± 28.9	55.0 ± 11.0	37.5 ± 23.9	60.4 ± 21.3	88.7 ± 3.8 *	71.7 ± 3.3 **	82.8 ± 6.2 *

Table 5.2. Behavioral responses of host-seeking *S. ochraceum* complex to potential host odor attractants (1:100). Significant attraction to a test stimulus compared to its control was denoted by asterisks (* $P < 0.05$ and ** $P < 0.005$, paired t-test).

Test Treatment	Test Attraction Response (% ± SEM)
Air	49.5 ± 3.8
CO_2	53.8 ± 4.5
1-octen-3-ol	70.7 ± 2.5 **
3-octanol	52.5 ± 5.2
Hexadecanoic acid	53.2 ± 8.3
Octanoic acid	37.7 ± 6.1
Acetophenone	64.4 ± 3.8 *
6-methyl-3-hepten-2-one	52.7 ± 8.9
Dihydrocarvone	76.5 ± 3.9 *

Table 5.3. Behavioral responses of host-seeking *S. damnosum* complex to potential host odor attractants. Significant attraction to a test stimulus compared to its control was denoted by asterisks (* $P < 0.05$ and ** $P < 0.005$, paired t-test).

Test Treatment	Dilution	N	Test Attraction Response (% ± SEM)
Air	Pure	4	52.1 ± 2.1
Hexane	Pure	4	50.0 ± 5.6
1-octen-3-ol	1:1000	4	66.7 ± 5.6
	1:100	4	74.3 ± 5.2 *
	1:10	4	59.1 ± 8.1
Hexanal	1:1000	2	46.4 ± 3.6
	1:100	2	45.0 ± 5.0
	1:10	2	37.5 ± 12.5
Heptanoic acid	1:1000	2	58.3 ± 8.3
	1:100	2	80.0 ± 0.0 **
	1:10	2	52.8 ± 30.6

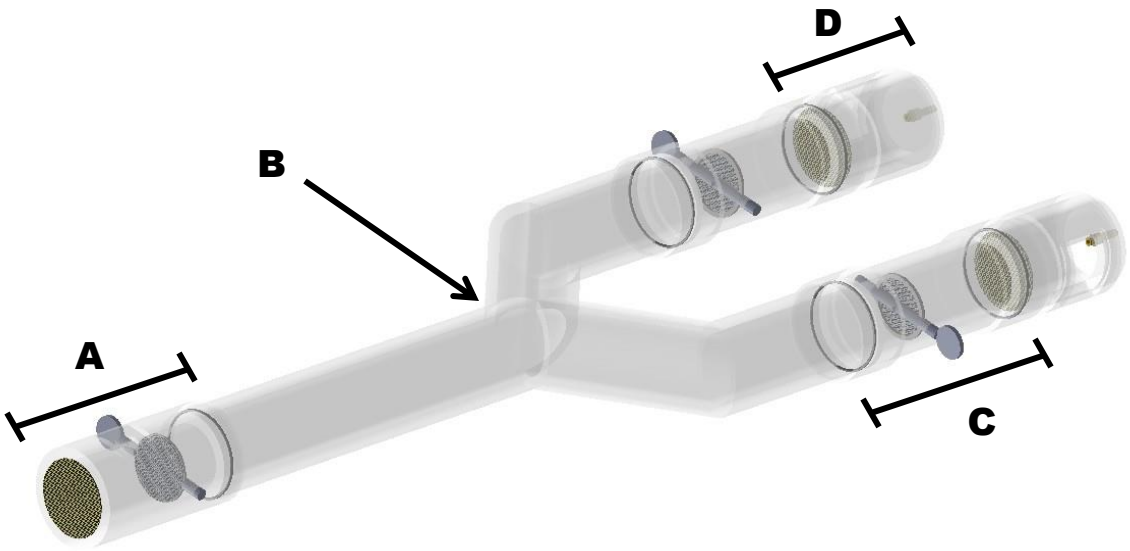

Figure 5.1. Animated picture of Y-tube olfactometer: A) release chamber, B) Y-split chamber, C) stimulus arm trap chamber, and D) stimulus chamber.

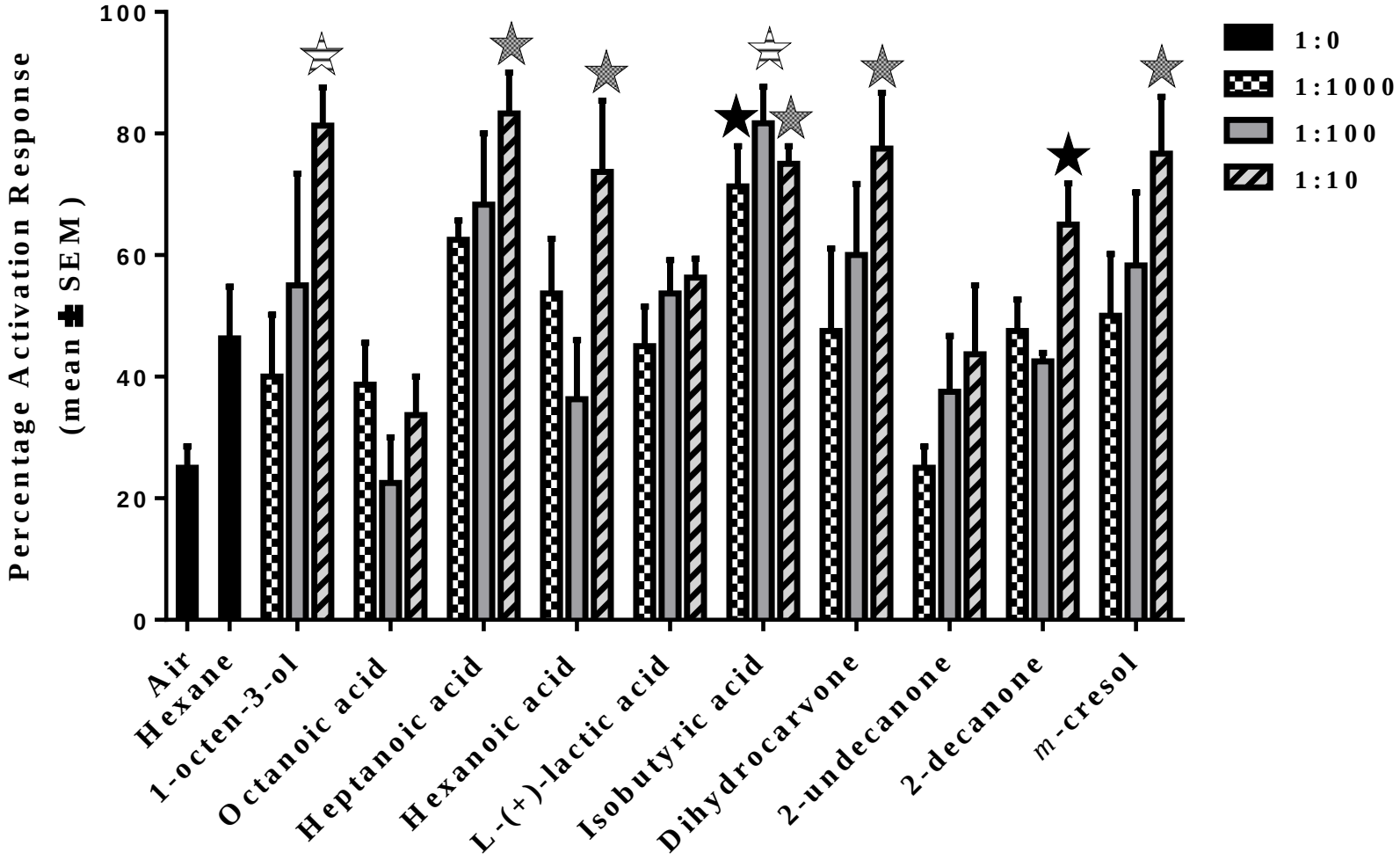

Figure 5.2. Activation responses of *S. vittatum*. Significance of the activation response of a test sample to the control of air alone is indicated by a black star ($P < 0.05$, one-way ANOVA), checkered star ($P < 0.005$, one-way ANOVA), or a star filled with horizontal lines ($P < 0.0005$, one-way ANOVA).

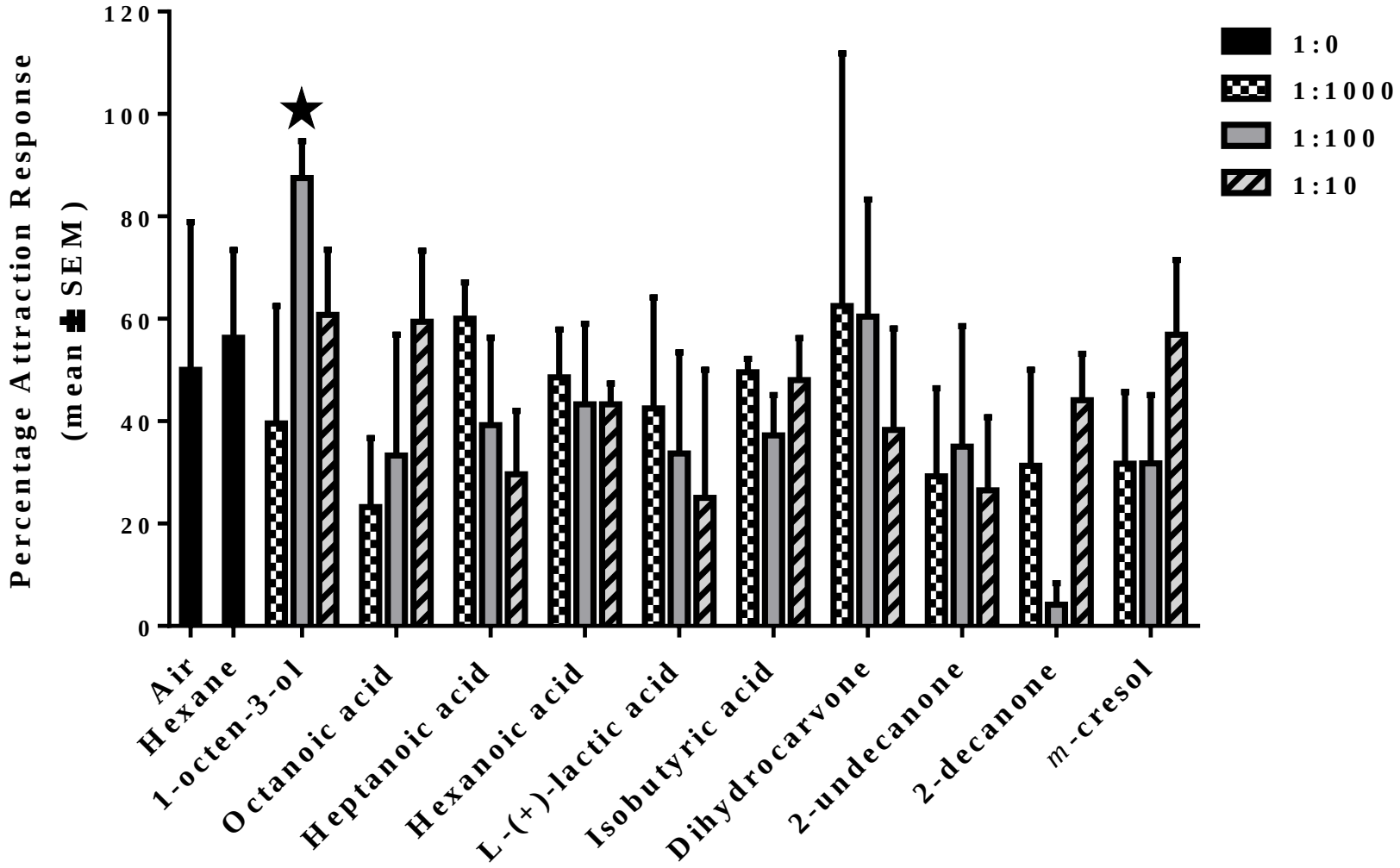

Figure 5.3. Attraction responses of *S. vittatum*. A black star above a bar indicates a significant ($P < 0.05$, paired t-test) attraction response to a test sample compared to its control.

CHAPTER 6

CONCLUSIONS

Objective A1- To determine the extent to which *Simulium vittatum* gravid females prefer to oviposit on a substrate that has fresh conspecific eggs.

Experiments in the binary choice chamber demonstrated the preference of gravid *S. vittatum* to oviposit on a substrate with fresh conspecific eggs rather than a substrate with no eggs when presented both treatments at the same time. The results from this study correlates with the findings from previous oviposition studies with other species of black flies (P. J. McCall et al., 1994; Rodriguez-Perez et al., 2003).

Objective A2- To determine and identify the factors responsible for *Simulium vittatum* gravid females' preference to oviposit on a substrate with fresh conspecific eggs.

Results from the oviposition attraction bioassays, in particular the oviposition attraction single chamber bioassay with the mesh screen covering the oviposition substrate, satisfied objective A2. In comparison to its respected control, a substrate with no eggs, significantly more flies were located on the substrate with eggs when the substrate was uncovered but not when it was covered. These results supported the hypothesis that gravid *S. vittatum* need to come into direct contact with the eggs or factors associated with the eggs to produce a response.

A more direct observation of the oviposition behavior of the flies when exposed to a stimulus was conducted with the petri dish bioassay. Induction of an oviposition response was observed when gravid *S. vittatum* females were exposed to a water or hexane extract of fresh eggs. Gas-chromatography coupled with mass spectrometry was used to identify compounds

from the active extracts. Two of the compounds, 1-pentadecene and 1-tridecene, identified from the active hexane extract elicited a significant oviposition response from the flies in comparison to a blank control. These results support the hypothesis that chemical factors on or associated with the surface of fresh *S. vittatum* eggs elicit a preferential behavioral response when gravid females are directly in contact with a substrate that has fresh eggs.

Objective B- To determine the extent to which a Y-tube olfactometer can be used to identify potential attractants to parous *Simulium vittatum* females.

A Y-tube olfactometer which was modified for our research was fabricated by the University of Georgia Instrument Shop, and was successfully used to demonstrate attractiveness of host-seeking *S. vittatum* females to volatile stimuli. Ten different test stimuli were identified previously from cattle and human odors and tested on host-seeking *S. vittatum*, and eight of the stimuli, CO_2, 1-octen-3-ol, heptanoic acid, hexanoic acid, isobutyric acid, dihydrocarvone, 2-decanone, and *m*-cresol, elicited an activation response. Only one stimulus, 1-octen-3-ol, elicited a significant attraction response. These results demonstrated the successful functionality of the Y-tube olfactometer in this study to assess the host-seeking behavior of *S. vittatum*.

Final Conclusions

Behavioral experiments combined with chemical analysis were able to verify and identify factors associated with eliciting an oviposition response from gravid *S. vittatum* females. These factors seemed to only elicit a significant response when the flies came into direct contact with them. These findings correlate with the hypothesis that communal oviposition of black flies are mediated chemicals emitted from fresh eggs.

In relation to the evolutionary process of black fly communal oviposition, the behavioral trait seems to be adaptive. The communal oviposition behavior has evolved in species that have

benefitted from several females depositing eggs on a single substrate which creates a mass of eggs. This mass of eggs could possibly prevent desiccation of the eggs during periods of low moisture in the environment. A higher number of eggs could also prevent a predator from eliminating all of single female's offspring. Although the previously stated benefits could increase survival of a female's offspring, costs such as oxygen deprivation and pathogen infection could reduce the number of viable offspring. An optimality modeled experiment could be conducted in a future study to assess if the benefits and costs of communal oviposition for black flies positively or negatively affect fitness of the species.

The Y-tube bioassay successfully identified attractive volatile compounds to host-seeking *S. vittatum* females. This allowed the Y-tube bioassay to be conducted on *Simulium ochraceum* and *Simulium damnosum* complexes, vectors of *Onchocerca volvulus*. Studies have continued with *S. ochraceum* and *S. damnosum* complexes in efforts to develop a lure for host-seeking flies in endemic areas of onchocerciasis.

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APPENDIX A

PRELIMINARY TRAP DEVELOPMENT FOR GRAVID *SIMULIUM TRIBULATUM* IN KINGSPORT, TN

Introduction

Trapping studies with black flies have focused primarily on attracting and capturing host-seeking females (Moore & Noblet 1974; Thompson 1976a, 1976b). Gravid flies have been the focus for trapping studies only a few times with limited success (Adler et al. 1983, Bellec 1976, Bellec et al. 1984). Different physiological states of a black fly can influence its behavior. For instance, gravid female black flies are more attracted to the colors yellow and green whereas host-seeking flies are more attracted to blue and black (Golini & Davies 1975, Moore & Noblet 1974).

This objective of this study was to improve previously developed oviposition traps, which would capture enough flies for onchocerciasis monitoring and be useful in vector control efforts.

Materials and Methods

Study sites. From June 9, 2011 to August 17, 2012 oviposition observations and gravid trap field trials with *Simulium tribulatum*, formerly *Simulium vittatum* IIIL-1, were conducted at two sites on the Big Sluice of the Holston River South Fork in Kingsport, TN, USA.

Site 1 (36º 31’54.36” N, 82º 33’52.34” W) consisted of a small grass island which was approximately 16.3 m long and 7.7 m wide. A picture from the east bank of site 1 is shown in figure A.1. Site 2 (36º 32’31.17” N, 82º 34’33.99” W), which was approximately 1578 m

downstream of site 1, was similar to site 1 in regard to its island of grass which was 30.3 m long and 10.5 m wide. The depth of the water was greater at site 2. A view from the West bank of site 2 is shown in figure A.2.

The Big Sluice diverges from the Holton River South Fork immediately below a Tennessee Valley Authority (TVA) controlled dam at the Fort Patrick Henry Reservoir. A small creek, Horse Creek, merges into the Big Sluice at approximately 400 m upstream of site 1.

Oviposition traps. An oviposition trap (Bellec plaque) with previous success of trapping gravid black flies was used initially. The trap was a 1-m^2 aluminum panel coated with an adhesive (Bellec 1976). The setup of the Bellec plaque was identical to that described in the original study (Figure A.3).

Non-floating oviposition traps consisted of a square or rectangular piece of plywood (UltraplyXL, Moreland Company, Sarasota, FL, USA) sealed with polyurethane (clear semi-gloss, Minwax Company, Upper Saddle River, NJ, USA). Industrial strength aluminum foil (Heavy Duty Foil, Handi-foil of America, Inc., Wheeling, IL, USA) was placed on top of the polyurethane treated plywood and secured with duct tape. Plastic strips, yellow (7.6-m wide), green (2.5-m wide), or a combination of the two, were then placed on top of the aluminum foil and adhered with duct tape. An adhesive, Tangle-Trap (The Tangle Foot Company, Grand Rapids, MI, USA) or a mixture of polyoxyethylene 20 (OmniPur, EMD Chemicals, Inc., Gibbstown, NJ, USA), which is similar to Tween 20, and alcohol, was applied only to the plastic strips. Experiments with the non-floating oviposition traps were conducted using two different size traps (1 m^2 and 0.5 m^2) (Figure A.4).

Floating oviposition traps, as shown in figure A.5, consisted of the same materials and set up as the non-floating oviposition traps but with the addition of enclosed 7.6-m wide internal

diameter polyvinyl chloride (PVC) pipes fixed on the bottom of the trap, which provided sufficient buoyancy for the trap to float in the river. A rope was tied to the front of the trap and to an anchored piece of rebar. Three different sizes of non-floating oviposition traps were used in this study: 1 m^2, 1-m wide x 0.5-m long, and 0.5 m^2. Foam was added between the PVC pipes of the 1-m^2 trap to ensure that the trap would float (Figure A.5).

Trip 1. Trip 1 was conducted from 0715 to 1030 on June 9, 2011. A total of 4 non-floating oviposition traps were used: two on the island of grass and two on the far bank opposite the road. A 1 m^2 with yellow tape and a 0.5 m^2 with green tape were used on the island of grass, and a 1 m^2 with green tape and a 0.5 m^2 with yellow tape was used on the far bank. Figure A.6 shows the designated trapping zones of site 1.

Trip 2. Trip 2 was conducted on June 30 and July 1, 2011. Non-floating and floating oviposition traps were used during this trip. On day 1, June 30, 2011, a total of 4 traps were used at site 1: a 1-m^2 floating trap with yellow tape at the end of the island of grass, 0.5-m^2 floating trap with yellow and green tape was located a few meters from the far bank, a 1-m^2 non-floating trap with yellow tape on the island of grass, and a 0.5-m^2 non-floating trap with yellow and green tape on the far bank. At site 2 on the island of grass, two (1 m^2 and 0.5 m^2) non-floating oviposition traps with yellow and green tape were used. All four oviposition traps were baited with octenol (Bioquip), which was applied to a piece of cotton gauze on the top of the trap. Traps were initially set up at 0815 and checked for collection at 1400. Figure A.7 shows the designated trapping zones of site 2. Site 2 was only used for trapping during day 1 of trip 2.

Day 2, July 1, 2011, a total of 2 traps were used: a 1-m^2 floating trap with yellow tape was located at the end of the island of grass and a 0.5-m^2 floating trap with yellow tape which

was a few feet from the far bank. Set up was at 0645 and collection was at 1030. No octenol was used during day 2.

Trip 3. Trip 3 was conducted on July 21-22, 2011. Day 1, July 21, 2011, a total of 6 traps were used: three 1-m^2 Bellec plaques and three 1-m^2 floating oviposition traps with yellow tape. One of each trap type was placed on the near bank, island of grass, and far bank. Tween 20 was used as the adhesive for both styles of traps. Set up was at 0700 and collection at 0845, 1100, 1500, and 1800. Tween 20 was reapplied at 1500.

Day 2, July 22, 2011, the same set up as day 1 was used but 1-m x 0.5-m traps were used for both styles. Set up was at 0655 and collection at 1015.

Trip 4. Trip 4 was conducted on July 28-29, 2011. On day 1, July 28, 2011, the same set up used for trip 3 was conducted with the exception of 0.5-m^2 traps being used and Tangle Foot was used for the floating oviposition traps and Tween 20 was used for the Bellec plaques. Set up was at 0645 and collection was at 1045, 1430, and 1800.

On day 2, July 29, 2011, only three 1-m^2 floating oviposition traps with yellow tape coated with Tangle foot were used at the locations: near bank, island of grass, and far bank (Figure A.6). Set up was at 0700 and collection was at 1030.

Trip 5. Trip 5 was conducted on August 17, 2012, and no traps were used during the trip as the purpose was only to observe oviposition behavior. Observations of the black fly population were only performed beginning at 0645 and ending at dusk.

Results

Trip 1. Oviposition behavior of *S. tribulatum* was observed in real-time at 0715 on trailing vegetation on the near side of the island of grass at site 1. Multiple flies were seen ovipositing on the same substrate that generated several masses of eggs on the substrate (Figure

A.8). Freshly deposited eggs were also found on vegetation at dusk, so some oviposition did occur in the evening hours. At site 2, eggs were found on trailing vegetation as at site 1, but no black flies or real-time oviposition was observed. Non-floating traps located adjacent to actively ovipositing black flies at site one resulted in zero black flies being captured.

Trip 2. Day 1 resulted in zero collected flies at sites 1 and 2. However, on day 2 at site 1, 22 females (16 gravid) were captured on the 1-m^2 floating oviposition trap with yellow tape at the end of the island of grass, and 31 females (25 gravid) were captured on the 0.5-m^2 floating oviposition trap with yellow tape located at the far bank. No other traps captured any flies. Most of the flies captured were at the distal end of the trap directly before it broke the surface of the water.

Trip 3. On day 1, only 1 female black fly was captured on each of the 1-m^2 floating oviposition traps with yellow tape on the far bank and behind the island of grass. No other flies were captured on the remaining traps.

On day 2, the 1 x 0.5-m floating oviposition traps with yellow tape at the far bank and behind the island of grass captured 6 and 11 females, respectively. The remaining traps failed to capture any flies.

Trip 4. Oviposition was observed at real-time and the primary period of activity was from 0645 until 1045. On day 1, the 0.5-m^2 floating oviposition traps with yellow tape at the far bank, behind the island of grass, and near bank captured 2, 17, and 7 females, respectively. One black fly was collected on the Bellec plaque at the near bank.

On day 2, only one black fly was collected on the 1-m^2 floating oviposition trap with yellow tape behind the island of grass.

Trip 5. Even though larvae and pupae were present, no eggs or adults were seen at the study site from 0645 to 0715. At 0950 several batches of freshly oviposited eggs were found on a couple of leaves which were trailing in the water (Figure A.9). No real-time oviposition was observed.

Discussion

Floating oviposition traps were more effective than the Bellec plaque and non-floating oviposition traps. Even though traps with yellow tape collected the most flies, a lack of replicates on days with black fly activity limited the conclusions from this study. Yellow and green have been documented to be the preferred color of black flies in choosing an oviposition substrate (Golini & Davies 1975).

The point at which an oviposition substrate breaks the water has been stated to be the preferred point on a substrate where North American black flies would oviposit (Adler et al. 2004). However, the Bellec plaque in a previous study collected thousands of black flies in the *Simulium damnosum* complex and was not in the water but on rocks on the bank of a river in Africa (Bellec 1976).

Oviposition was observed on clear and warm days, but days with an overcast or inclement weather resulted in no oviposition. Inconsistent weather conditions through our study made the behavior of the flies vary which made the comparison of traps and analyzing data difficult. More replicates are needed for this study to effectively decide which trap design and strategy would be the best for trapping the black fly population in Kingsport, TN.

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Tables and Figures

Figure A.1. Black fly study site 1 on the Big Sluice of the Holston River South Fork in Kingsport, TN.

Figure A.2. Black fly study site 2 on the Big Sluice of the Holston River South Fork in Kingsport, TN.

Figure A.3. The 1-m^2 Bellec plaque on the island of grass at site 1.

Figure A.4. Two non-floating oviposition traps, a 0.5-m^2 trap with green tape and a 1-m^2 trap with yellow tape, on the island of grass at site 1.

Figure A.5. (Left) 1-m^2 floating oviposition trap at the end of the island of grass at site 1. (Right) The underside of floating traps.

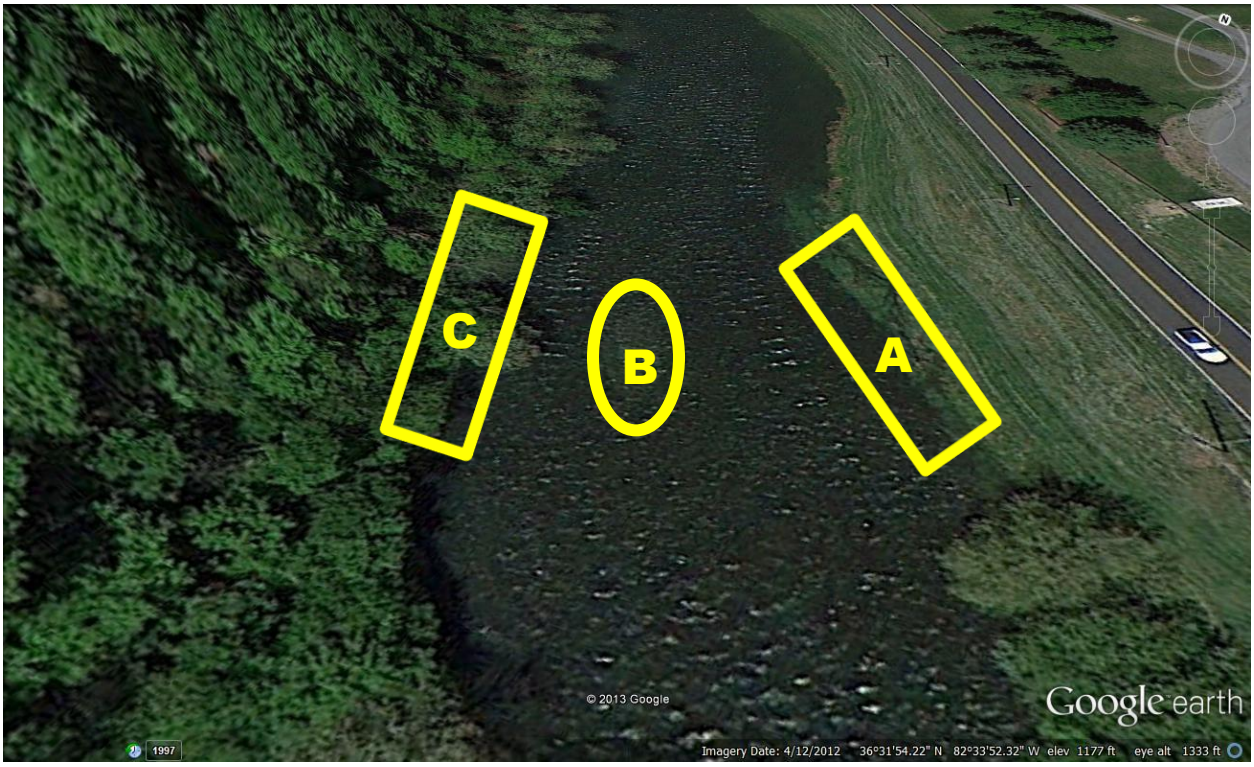

Figure A.6. An aerial picture of site 1 (Google Earth) with designated trapping zones: A) near bank, B) island of grass, and C) far bank.

Figure A.7. An aerial picture of site 2 (Google Earth) with designated trapping zones: A) island of grass.

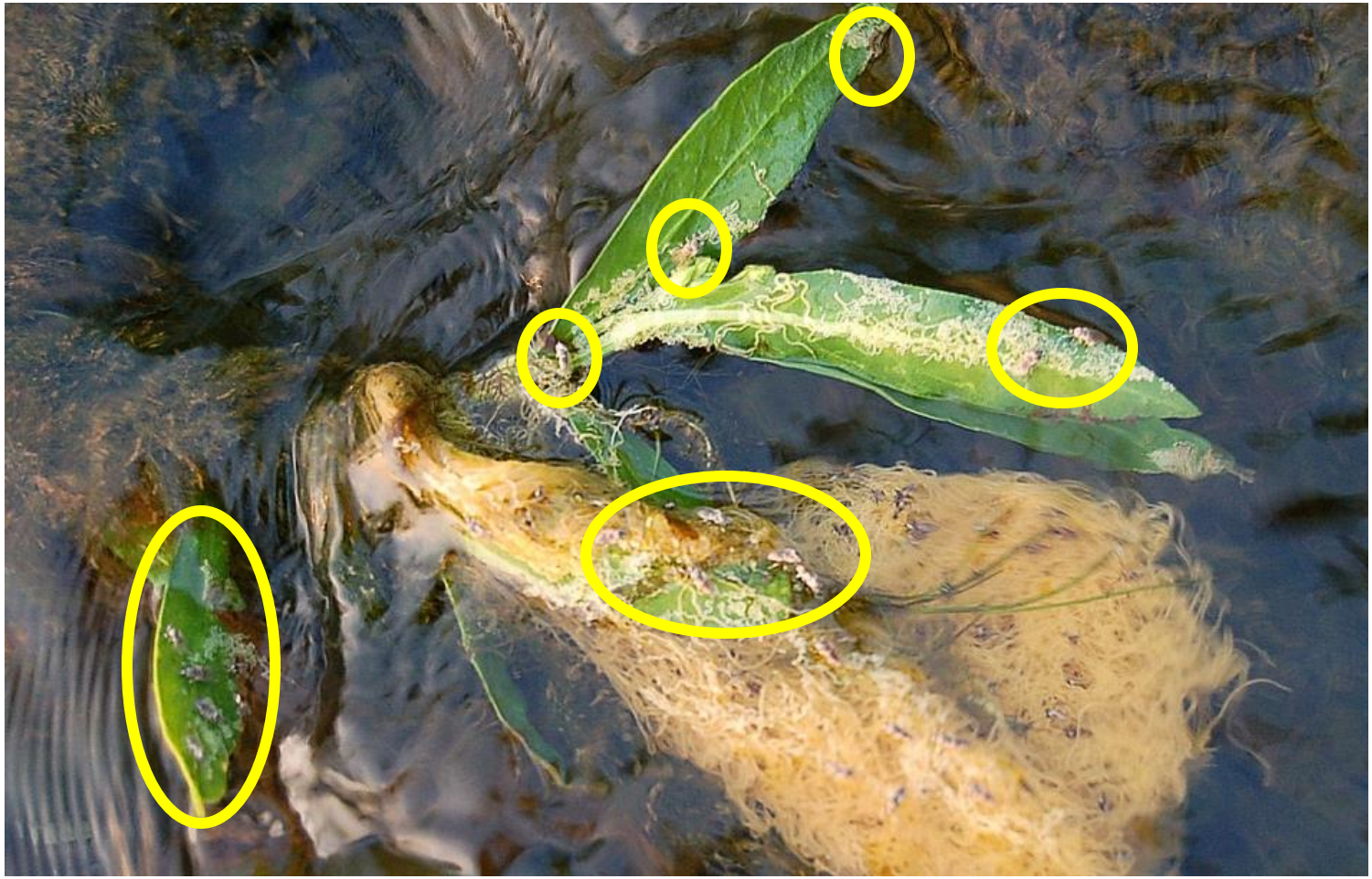

Figure A.8. Multiple black flies ovipositing (indicated by yellow circles) on a substrate at the east side of the island of grass of site 1.

Figure A.9. Freshly deposited eggs on the foreground and older black fly eggs on leaf behind the foreground leaf at site 1.