MICROBIAL VOLATILE ORGANIC COMPOUNDS ASSOCIATED WITH DECOMPOSITION MEDIATE FORAGING BEHAVIOR OF THE RED IMPORTED FIRE ANT (*SOLENOPSIS INVICTA* BUREN) (HYMENOPTERA: FORMICIDAE)

A Thesis

by

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ABSTRACT

Examining the chemicals involved in interkingdom interactions (e.g., microbe-insect) is useful for understanding the mechanisms governing insect behavior. Recent studies have shown that blow flies (Diptera: Calliphoridae) involved in carrion decomposition are attracted to concentrations of volatile chemical signals emitted by swarming bacterial strains such as *Proteus mirabilis* (Enterobacteriales: Enterobacteriaceae). This research presents field and laboratory responses of the red imported fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae) (RIFA) to baits with VOCs associated with *P. mirabilis* to determine dose dependent responses that may be useful for understanding interkingdom interactions and potential applications in forensic entomology and urban pest control.

Field trials took place in two environments in College Station, TX, USA: an agricultural enclosure (rural) and a manicured lawn (urban). Responses to baits treated with one of four compounds diluted to one of two different concentrations were site specific. In the urban environment, indole (IND) at 5.0 µg concentration displayed the highest RIFA attraction to baits at 15% overall; 34% more than the control. Dimethyl disulfide (DMDS) at 0.005 µg concentration displayed the least attraction at 6%; 45% less than the control. In the rural environment, phenylacetic acid (PAA) at 0.1 µg concentration and dimethyl disulfide at 0.25 µg concentration displayed the highest attraction of RIFA response to baits with 17.7% and 17.3% overall attraction; 148% and 142% more than the control bait, respectively. Isobutylamine (IBA) at 0.01 µg concentration displayed the least attraction with 3.5% overall attraction to bait; 50% less attractive than the control.

Laboratory choice assays were conducted to validate results from fieldwork. Following three trials, RIFA attraction to a compound concentration compared to controls of either plain bait or bait with acetone were variable for RIFA attracted to a bait and amount of bait removed. However, IND 0.05 µg and DMDS at both high and low concentrations were the most attractive of the compounds compared to controls.

Like other insects, RIFA respond differently to compounds depending on concentration and environment. Microbial communities may have a significant impact on motivating generalist species to select one resource over another, leading to better pest management strategies.

DEDICATION

I dedicate this thesis to my parents, John and Barbara. You taught me to listen to my instincts, take chances, and always love what I do. To my grandparents for their support and level- headedness. To my sister Katie, who inspires me every day.

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NOMENCLATURE

TSC	Time since colonization
PMI	Post mortem interval
AIs	Autoinducers
QS	Quorum sensing
VOCs	Volatile organic compounds
DMDS	Dimethyl disulfide
DMS	Dimethyl sulfide
IBA	Isobutylamine
IND	Indole
PAA	Phenylacetic acid
CTRL	Control
ORs	Odorant receptors
IPM	Integrated Pest Management
SEM	Standard Error Margin
GC/MS	Gas chromatography mass spectometry

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I. 1. Foraging and Information Transfer

The survival of an organism depends on its ability to successfully acquire and use energy. The theoretical basis for how animals exploit essential nutrients in their environments for functional use known as optimal foraging theory. Described by MacArthur and Pianka (1966), the theory was the first to mathematically illustrate how time spent searching for food versus the net energy gained from capturing prey is correlated with natural selection; optimal foraging behavior, which maximizes net energy, reflects the overall fitness of the organism(s). Furthermore, this successful behavior is plastic in that it changes with varying environmental resources and their distribution; in environments where resources are more widely spaced, predators have a broader diet range than those whose preferred food is plentiful and close-range (Macarthur and Pianka, 1966; Hernández et al., 2016).

The eusocial red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae) (RIFA) serves as a model example for investigations of optimal foraging theory. A mature colony may defend an average territorial area of $100m^2$ in pastureland (Eldridge, 2003; Tschinkel, 2006), and foragers of the worker caste utilize underground tunnels throughout the territory, emerging to the surface near foraging areas that are rarely more than a meter from any given foraging tunnel opening, reducing aboveground exposure time. Ink-marking experiments (Tshinkel, 2006) have shown that only 10% of foragers are typically found in the central mound. RIFA further optimizes food capture

through the reliance on scout ants to locate food sources and provide a signal using trail pheromone (a secretion of α-farnesenes), whereby the majority of foragers waiting in tunnel branches will collect before the trail pheromone wears off. However, if a resource is of particularly high quality, a scout may use a series of other, nonpheromonal, signals such as food display or body waggling to recruit more foragers (Tshinkel, 2006). In experiments by Cassill (2000; 2003), scouts used a total of six nonpheromonal recruitment behaviors (the maximum) to recruit foragers to a food source of 27% sugar water; the highest sugar concentration tested.

Even armed with an arsenal of advanced sensory input, a sole forager is vulnerable to predation. Group foraging strategy increases the likelihood of prey-capture success. A shift in behavior from individual driven foraging to group foraging provides numerous advantages; predation risk decreases (Pullman, 1973; Powell 1974; Lazarus, 1979), feeding time increases as individuals' vigilance towards predators decreases (Caraco, 1979; Sullivan, 1984), and groups can make better and swifter estimates of a foraging patch's resources through cohesive learning behavior whether or not each individual shares the same information about patch quality (Valone, 1989). Moreover, coordinated group behaviors in RIFA ensure that energy expenditure is divided amongst individual foragers while increasing the likelihood of capture success. For RIFA, this is required to feed hundreds of thousands of colony members.

RIFA colonies vary greatly in terms of the number of queens found per mound. They may form monogyne colonies with a single reproductive queen, or polygyne communities that support multiple queens; anywhere from several to several hundred, who

all lay eggs (Glancey et al., 1973; Lofgren and Williams, 1984; Vargo and Fletcher, 1987). The type of community structure is determined by a queen's social chromosome, made up of 527 genes, that is detectible by the worker caste through cuticular pheromones (Wang et al., 2013). The number of queens is highly variable and polygyne nests may contain hundreds of them. Fletcher et al (1980) found that polygyne queens were less physogsatric than monogyne, though 87.2% of queens in polygyne nests brought to the lab produced between one and 75 eggs in five hours. Polygyne mounds produce less brood per queen on average than those of monogyne colonies (Fletcher et al. 1980; Greenberg et al. 1985; Vargo and Fletcher, 1989; Vander Meer and Morel, 2007), but sustain larger mounds and greater brood output overall (Vander Meer et al., 1992; Macom and Porter, 1996). To provide adequate nutrition for these colonies, and most importantly the queens, individual foragers of the sterile worker caste must have a system of within-group information transfer to efficiently collect and transfer solid food, which is digested by the brood and transferred throughout colony members through trophallaxis. In general, information transfer is accomplished by the use and interpretation of volatile organic compounds (VOCs) as signals or cues (Wilson, 1962). A disruption in the way ants communicate may hinder foraging and, therefore, colony success.

'Social information' is an umbrella term that refers to the gathering of data, or interpreting cues from others to improve individual fitness. Encompassed under this general term are private and public information (Blanchet, 2010) which are evolved responses organisms use to learn about environmental quality. The work of Valone (1989; 2007) describes private information as "knowledge" not shared with surrounding

organisms, and public information as a form of indirect social information; instead of successful individuals directly displaying information about a given resource for others to use (i.e., a honey bee waggle dance) (*Apis mellifera* Linneaus) (Hymenoptera: Apidae), individuals instead indirectly "eavesdrop" on the actions or decision-making of others and mimic their behaviors if it will yield fitness increasing results. Examples of public information include female zebra finches (*Taeniopygia guttata castanoti*) (Passeriformes: Estreldidae) copying mate-choice preferences of their conspecifics (Kniel et al., 2015), and parasitoids homing in on information in the form of VOC molecules (also called green leaf volatiles or secondary metabolites) when *Manduca sexta* L. (Lepidoptera: Sphingidae) larval feeding (i.e. disruption of leaf cell structure) triggers a volatile conversion in the leaves of tobacco plants *Nicotiana attenuate* (Solanales: Solanaceae) (Allman et al., 2013; Halitschke et al., 2008).

Social insects serve as an appropriate model for exploring the concept of public information. Their behavior is easily manipulated, genomic data is plentiful, and their basic assessments in deciding whether to copy others in social learning situations are similar to those of vertebrates. Gruter and Leadbeater (2014) found that a variety of insects will use social learning (in the form of copying) to enhance their fitness if exploration is costly, if they are "dissatisfied" from a personal result, if the majority is behaving a certain way, or if others have displayed success in some task.

From a practical standpoint, insect reproductive rates are generally high (relative to the higher organisms) and multiple individuals are easily attainable for breeding purposes and maintaining a colony in a laboratory setting. Macom and Porter (1996)

determined that the highest population density of RIFA individuals in a polygyne field site, per square hectare, was 123.4 million. With this many individuals sustained by a high turnover rate of worker ants, a colony's system of coordination and information exchange is an ideal model for studying within-colony information transfer and interpretation.

Ants, and eusocial Hymenoptera in general, owe much of their evolutionary success to a diverse array of chemical cue interpretation (Zhou et al., 2015). They exhibit pheromones for trail marking, aggregation, formation of territorial boundaries, alarm, and sexual stages (Hölldobler, 1978), and cuticular hydrocarbons are known to assist in nestmate recognition (Torres et al., 2007), sexual mimicry (Cremer et al., 2002), and determining oogenesis potential between queens and workers (Liebig et al., 2000), among other functions. RIFA have approximately 333 odorant receptors (ORs) (Zhou et al., 2015); heteromeric ligand-gated ion channels with odor-gated currents of differing ion permeabilities (Sato et al., 2008). In contrast, another eusocial insect, the honey bee, has approximately 164 ORs (Robertson and Wanner, 2006), while the mosquito *Anopheles gambie* (Diptera: Culicidae) has 79 (Carey et al., 2010). *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) have 62 ORs (Jafari and Alenius, 2015), and the blow fly *Calliphora stygia* (Fabricius) (Diptera: Calliphoridae) has 50 (Leitch et al. 2015). Ants are highly successful in their ability to maintain complex societies and large territories by perceiving and interpreting chemical compounds emitted by each other as well as their surrounding environment (Sharma et al., 2015).

I. 2. The Red Imported Fire Ant

Since its introduction to Mobile, Alabama, USA in the 1930s, the red imported fire ant has become a major pest of the western, southern, and eastern USA (Vinson, 1997; Tshinkel, 2006; Neff, 2011) and is expected to expand its range with higher climatic temperatures (Morrison et al., 2005). Native to the flood plains of northern Argentina (Tschinkel 2006; Caldera et al. 2008), this species was introduced to the Mobile Bay in Alabama most likely through cargo shipments of potted plants and soil (Tchinkel, 2006). These ants soon began to outcompete populations of native and non-native ants including another invasive *Solenopsis* species that was already established; *Solenopsis richteri* Buren (Hymenoptera: Formicidae). It has been estimated that RIFA cost the United States approximately $5 billion annually in household and institutional costs and nearly $1 billion in agricultural losses (USDA; Lard et al. 2002; Lard et al. 2006).

Fire ants are a health hazard to humans and their pets, livestock, and crops. Their stings often result in painful then itchy pustule-like bumps (Caro et al., 1957; Apperson and Adams, 1983) and can cause allergic reactions (Lockey, 1974; Hoffman, 1988) that, if severe, can lead to hospital stay due to decreased blood pressure, allergic symptoms, or anaphylactic shock (Haddad and Larson, 2015). RIFA are therefore of medical importance as they pose risk to humans, particularly so for patients in establishments like hospitals and nursing homes by vectoring bacteria such as *P. mirablis* (Chadee and Le Maitre, 1990), and anaphylaxis due to venom allergy has been known to cause human death (Lofgren, 1986; Rhoades et al., 1989; DeShazo et al., 2004).

The deleterious effects of RIFA introduction continue as RIFA are also known to displace native ants through resource competition and predation, (Porter and Savignano, 1990; Morrison and Porter 2003; Calixto et al., 2007; Calcaterra et al., 2008; Cumberland et al., 2012). In agriculture, *S. invicta* have been shown to reduce soybean yield by 0.22 to 0.64 hectoliters per hectare due to crop collecting issues and inability to reach all useable crops (Lofgren and Adams, 1981; Apperson and Powell, 1983), interference with combine operations, aggregations around root systems of plants, feeding on crops like citrus, corn, okra, and cucumber (Jetter et al., 2002), and threatening other arthropod species (Neff et al., 2011). While it is true and well cited that RIFA survive well in disturbed or simple habitats, they can also be successful in complex and conserved habitats (Calcaterra et al. 2008).

I. 3. Bacteria and Quorum Sensing

As insects evolve interpretive communicative cues within their species, this process is further complicated by a world of interspecific relationships, specifically, with microorganisms. Microbes are anything but static in terms of impact on host health and behavior. Some bacteria may be symbiotic; many of these beneficial bacteria are seen in fourth instar RIFA larval midguts (i.e. *Lactococcus garvieae* (Lactobacillales: Streptococcaceae), *Staphylococcus saprophyticus* (Bacillales: Staphylococcaceae), *Enterococcus avium* (Lactobacillales: Enterococcaceae)) and in hemolymph (*Bacillus* (Bacillales: Bacillaceae) species not yet determined to species level using gyrA and SG850 genes) (Gunawan, 2008). As mentioned above, these larvae act as the “stomach” of the

colony by digesting the solid foods that will be fed back to the colony as liquid material (Tschinkel, 1988; Peloquin and Greenberg, 2003).

Ezenwa (2012) described how different strains of bacteria, whether symbiotic or pathogenic, affect an organism's behavior. The author cites Verhulst (2011), which determined that under both laboratory and semi-field trials, mosquitoes *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) are attracted to volatiles emitted from bacteria on human skin ($p = 0.69$ for the no-odor bait traps versus $p \leq 0.001$ with baits of incubated skin microbiota). The communication that occurs between bacterial cells to emit these volatile compounds may be a result of quorum sensing swarming behavior of the bacteria. Zhang et al. (2015) used *Aedes aegypti aegypti* Linnaeus (Diptera: Culicidae) and determined that *Ae. aegypti* was attracted to blood-feeder devices inoculated with wildtype *Staphylococcus epidermis* (Bacillales: Staphylococcaceae) 2.6 times (or 74%) more than an agr-strain of *S. epidermis* with the agr gene knocked out ($p < 0.0001$); preventing bacteria from quorum sensing (swarming) capabilities.

When some bacterial species reach a threshold population (i.e., quorum) in response to extracellular signaling molecules called autoinducers (AIs) in the environment, they are able to unify this signaling into one massive communicative unit. This communication is quorum sensing (QS). Since congregates of certain bacteria are sensitive to fluctuations in their population densities, at certain thresholds they release proteins such as "autoinducer" molecules (Fuqua et al., 1994) or volatile organic compounds (VOCs), capable of regulating an animal's gene expression and downstream behavior (Miller and Bassler, 2001). For example, the gram-negative bacteria *Proteus*

mirabilis Hauser (Gamma Proteobacteria: Enterobacteriales) carries a homologue of LuxS, a gene and signal molecule generating enzyme required for an autoinducer molecule called AI-2 to synthesize and express swarming quorum sensing behavior as seen in laboratory agar plating experiments (Schneider et al., 2002). Miller and Bassler (2001) state that in the bacterium *Vibrio harveyi* (Vibrionales: Vibrionaceae), the AI-1 quorum sensing system is used for intraspecific cell-cell communication and the AI-2 quorum sensing circuit for interspecific cell-cell communication, later confirmed by Winzer et al. (2003) and Pereira et al. (2013). At the same time bacteria are releasing these autoinducing molecules to perform a host of regulating processes (i.e., biofilm formation, motility, antibiotic resistance, expression of proteins and peptides) (Pereira et al., 2013), the cells may also emit swarming-capable VOCs readily perceived by other organisms to induce an attraction or repellency response.

I. 4. Relationship of *Proteus mirabilis* with Arthropods

Ma et al. (2012) and Tomberlin et al. (2012) used *Lucilia sericata* (Diptera: Calliphoridae) to confirm that the metabolites lactic acid, phenol, NaOH, KOH and ammonia produced by *P. mirabilis*, known to be fly attractants, could restore the reduced swarming behavior in one-third of mutant cells, and that an organism's nutrition, sex, and gravidity play critical factors in how well they respond to those cues. Archie and Theis (2011), although not using the terms "quorum sensing," explain a variety of examples of bacterial-mammal interactions including human recognition by bacteria associated with sweat, animal scent-marking, and bird plumage color augmented by symbiotic bacteria; providing many outlets to understand if quorum sensing plays a role. Hughes and

Sperandino (2008), among their many examples, describe plant interactions with quorum sensing bacteria in that some plants and algae are able to mimic the autoinducer AHL to confuse potential pathogenic bacteria and prevent attack. Ezenwa et al. (2012) describes how insects and vertebrates and bacteria interact with behavior bidirectionally; animals can manipulate their bacterial microbiomes while bacteria are able to influence animal behavior.

Preliminary results in our lab show that differing densities of the same bacteria may produce different behavioral outcomes in RIFA. Specifically, that *P. mirabilis* elicits an attractive response by RIFA at lower concentrations but begins to repel the ants when concentrations near 10^9 colony forming units (CFU), depending on what bait substrate is used (Dr. Elida Espinoza, personal communication). At specific bacterial concentration thresholds in which the swarming behavior was observed, four VOCs were emitted that are known to impact the behavior of necrophagous insects; indole, dimethyl disulfide, isobutylamine, and phenylethyl alcohol. These compounds have indicated potential roles in interkingdom (*sensu lato*, microorganisms exchange hormonal communication with eukaryotes) interactions between bacteria and *L. sericata* through differential regulation when compared to non-swarming *P. mirabilis* mutants (Tomberlin et al., 2012). As potential QS signaling molecules, these compounds are likely to influence the behavior of other arthropod species depending on factors such as sex, gravidity, age, nutritional status, and environment (Liu et al., 2016; Dekeirsschieter et al., 2013).

I. 5. Insects Respond to Bacterial Related Volatile Organic Compounds

Insect-microbe interactions in which insects use, or are manipulated by, bacterial chemical stimuli to make decisions about feeding, oviposition, and host-seeking have been well documented (Davis et al. 2013). The four compounds that are mentioned above are products of the essential amino acids tryptophan, methionine, valine, and phenylalanine; important cadaveric VOCs with the capability of influencing necrophagous insect behavior (Dekeirsschieter et al., 2009).

Indole is produced by bacteria through the degradation of tryptophan, an amino acid considered rare in the environment compared to other essential amino acids (Hrazdina and Jensen, 1992) and one that is costly to synthesize (Yanofsky et al., 1991). For this reason, it is a valuable resource to many organisms. For over 80 known bacterial species that produce indole, the compound is known to assist in spore formation, drug resistance, virulence, plasmid stability, and biofilm formation (Lee and Lee, 2010). This VOC is an aromatic heterocyclic organic and nitrogen containing compound. Indole acts as an extracellular signaling molecule for higher organisms (Bansal et al., 2009) and is a fly attractant (Liu et al., 2016), commonly used in fly traps in combination with other chemicals (Urech et al., 2004). Molecules of IND, when combined with sulfur-containing molecules such as DMDS, create the distinct smell of dung and decomposition (Jürgens et al., 2013).

DMDS is a well-known bacterial VOC containing sulfur (Stotzky et al., 1976; Tomita et al., 1987), and is the degradation product of the essential amino acids methionine and cysteine (Jürgens et al., 2013) by various bacterial species including *Proteus*

(Hayward et al., 1977; Tomita et al., 1987). This compound, and derivations of the compound, is attractive to a host of organisms. Copepods; a class of small aquatic crustaceans (Calanoida: Maxillopoda), forage for phytoplankton prey using underwater chemoreception and can detect plumes of dimethyl sulfide (DMS) given off by the algae (Steinke et al., 2006). The mosquito *Ae. aegypti* is attracted to DMDS individually or in blends with lactic acid and acetone (Bernier et al. 2003; Allan et al. 2006). The compound is also utilized by carrion mimicking plants like the dead-horse arum (*Helicodiceros muscivorus*) to attract flies (Stensmyr et al., 2002).

Phenylacetic acid (PAA) is produced by many bacteria, including *P. mirabilis*, as an antifungal/antibacterial agent (Kim et al., 2007). It is a catabolite of the essential amino acid phenylalanine and it is also the oxidation product of phenethylamine. Phenethylamine can also be biosynthesized from phenylalanine though decarboxylation. The ubiquity of phenylacetic acid in vegetal tissues may be linked with its production by plant-associated microorganisms (Kim et al. 2007). Besides the characteristics of antibiotic agent and its association with the common bacteria *Proteus* isolated from *L. sericata*, phenylacetic acid also has similarity in structure as well as sharing the same decomposing pathway with phenylethyl alcohol (Weatherston and Percy, 1976).

Isobutylamine is formed through decarboxylation by the amino acid valine (Richardson, 1966). The reaction of the amine has been studied in many bacteria including *Proteus* spp. (Gale, 1941; Proom and Woiwod, 1951; Ekladius et al. 1957).

I. 6. RIFA Interacts with QS bacteria

Historically, studies investigating ant/bacteria associations have focused on the symbiotic interactions between the ant species and its natural bacterial biota (Lee et al., 2008; Medina, 2011; Woodhams and Brucker, 2013). Studies have also linked ants with quorum sensing in foraging ecology in the sense that the ants will reach a "quorum" capacity before making decisions whether to exploit a new food resource. That is, a representative number of foragers need to "agree," by reaching a "quorum," that the resource is worthwhile before complete exploitation by the rest of the colony (Pratt et al., 2002; Cronin, 2014; Franks et al., 2015). This study is the first to link microbial quorum sensing associated with RIFA, with RIFA using public information to eavesdrop on the signals given off by the gram-negative bacteria *P. mirabilis*.

The aim of this study is to use volatile organic compounds given off by *P. mirabilis* (See: Tomberlin et al., 2012), at varying but deliberate concentrations that have been previously tested by the Texas A&M Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S.) to develop and test novel RIFA bait attractants and/or repellants. Volatile compounds associated with this particular strain were used because *P. mirabilis* is known for swarming behavior and a QS gene has been found with the bacteria (Schneider, 2002; Stankowska, 2012). The strain has been used successfully with blow fly studies (Ma et al., 2012; Tomberlin et al., 2012) and the strain has previously been isolated from RIFA (Chadee and Le Maitre, 1990; Hon Yu Lee, 2007).

Using bacteria to manage RIFA population densities is not uncommon. A commonly used granular fire ant pesticide contains the active ingredient Abamectin,

derived from fermentation by the soil bacterium *Streptomyces avermitilis* Goodfellow (Actinobacteria: Actinomycetales) (Neff et al., 2011). Abamectin is an insecticide, acaricide, and nematicide with high levels of toxicity if swallowed or inhaled (PubChem Chemistry Database: Avermectin B1A). The junction between bacterial quorum sensing and this invasive ant species requires scientific inquiry as behavioral manipulation using quorum sensing bacteria may prove to be a more successful, cost effective alternative to pest control than current insecticides or may serve as one implementation measure for IPM.

I. 7. Objectives and Hypotheses

Objective 1: Determine the rate and level of attraction of RIFA to QS related compounds in the field

H_0: There are no observable rates or levels of attraction of RIFA to QS related compounds

Relevance of Objective 1: Porter and Tshinkel (1987) found that RIFA, at a soil depth of 2 cm, will forage at temperatures between 15°C and 43°C, with optimal foraging occurring between 22°C and 36°C. We seek to know if there is a similar pattern or Gaussian distribution in the foraging rate of RIFA to known bacterial VOCs related to QS. More specifically, by quantifying the number of RIFA to baits over time and weighing the bait taken away at the end of experimentation, I aim to determine how long these VOCs last in the environment and patterns of attraction over time.

Objective 2: Determine if there exists a dose dependent response of RIFA to QS related compounds in the field

H_0: There are no observable dose dependent responses of RIFA to QS related compounds

Relevance of Objective 2: These four compounds are found in nature but have proven to elicit different responses based on compound concentration and other factors. Liu et al. (2016) found differences in gravidity and sex played a role in *L. sericata* attraction to these compounds but it is presently unknown how all female eusocial and generalist organisms like RIFA interpret and behave in the presence of similar compounds and concentrations. This objective will be assessed through a series of field and laboratory trials analyzing recruitment to baits using VOC concentrations that represent what may be encountered in the environment.

Objective 3: Validate fieldwork by conducting choice assay trials in the laboratory

H_0: There are no observable differences of RIFA to compounds in the field versus laboratory environment.

Relevance of Objective 2: Laboratory trials are necessary for validation measures, to determine what variables, if any, may contribute to differences in RIFA behavior response in a given environment.

CHAPTER II

RESEARCH, RESULTS, AND DISCUSSION

II. 1. Introduction

Public information (*sensu lato*, 'information about the quality of a patch that can be obtained by observing the foraging success of other individuals in that patch') (Valone, 1989) exchanged between quorum sensing bacteria and eukaryotes are fundamental products of evolutionary adaptation. Research has begun to elucidate which mechanisms contribute to bacterial swarming capability and the interspecific and intraspecific actions occurring during such events, but there remains much to know about how far reaching this bacterial capability extends into influencing eukaryotic behavior.

Recent calculations suggest that the number of bacterial cells in an average human body rival human cells slightly more 1:1 (Sender et al, 2016). In general, we recognize that bacterial cells are ubiquitous in the environment and that bacteria that can communicate via quorum sensing (the coordination of gene expression after reaching a threshold population (i.e., quorum) in response to extracellular signaling molecules called auto inducers (AIs)) are able to unify this signaling into a communicative unit with impressive implications. Through this language, quorum sensing bacteria can perform a host of regulating processes (i.e., biofilm formation, motility, antibiotic resistance, expression of proteins and peptides) that can impact other organisms' behavior. For example, Ezenwa et al (2012) explained that bacteria impact a variety of eukaryotic behavior; from predator- prey interactions to feeding and habitat preferences. Furthermore, a single microbial species may be responsible for the behavior, or it may be

caused by a variety of microbial species associate with a host. Overall, microbes may influence the host via the host's microbiome, or may extend their influence outward to other organisms via the environmental macrobiome.

Ecologically, ephemeral carrion resources serve as significant examples of spatial environments in which bacteria colonize in large numbers and attract a variety of species. Insects are often the first colonizers to sense volatile organic compounds (VOCs) related to decomposition, as carrion resources are nutrient rich and therefore competitive.

This study examines the behavior effects of the VOCs indole, dimethyl disulfide, isobutylamine, and phenylacetic acid, in their pure form, on foraging behavior of the red imported fire ant (RIFA) *Solenopsis invicta* Buren (Hymenoptera: Formicidae). In the past, these compounds have been isolated from *P. mirabilis*, which has been found on RIFA (Chaddee and Le Maitre, 1990; Hon Yu Lee, 2007) and have biological relevance to carrion decomposition (Tomberlin et al., 2012); resources of which ants are known to take advantage (Houston, 1987; Clark and Blom et al., 1991; Wells and Greenberg, 1994; Campobasso et al., 2009; Reinert and McCoy, 2010). Concentrations for these compounds were modeled from Liu et al. (2016) in their dosage form, and were prepared by dilution in acetone and stored for the study duration.

RIFA serves as a model organism in this study as it is both an expanding pest species across the world and uses carrion as a food resource. As social insects, RIFA rely on a high number of olfactory receptors to navigate the nest and to communicate with other colony members. For this reason, many studies have focused on trail laying

pheromones and defense chemicals. However, growing literature has shown that insects such as blow flies rely on VOCs associated with microbial decomposition for egg laying and mating, and it is presently unknown whether successful pest species such as RIFA rely on such cues; choosing one food resource over another based on molecules emitted by microbes. The objective of this study is to expose RIFA foragers to common VOCs related to bacterial decomposition, to understand how RIFA behavior is altered so that we may better understand the ecological dynamics at work in fields such as forensic entomology and pest management.

II. 2. Methods

II. 2. 1. Field Experiments

II. 2. 1. 1. Experiment Sites

Two field sites containing polygyne RIFA colonies in Brazos County, Texas, USA were selected for this study; one manicured (urban) and one agricultural (rural) site. The urban site contained mostly Bermudagrass (*Cynodon dactylon*) (Poales: Poaceae) and patches of Bahiagrass (***Paspalum notatum***) (Poales: Poaceae) and other native grasses (Steven Canon, Texas A&M Hildebrand Equine Complex, personal communication), was mown weekly, and was sometimes used for social events (i.e. cross country track meets). The rural site, which was typically grazed by four or five cows, contained a heterogeneous mixture of thick native grasses and plants; annual ryegrass (*Lolium multiflorum* Lam.) (Poales: Poaceae), Chinese tallow (*Triadica sebifera* L.) (Malpighiales: Euphorbiaceae), dallisgrass (*Paspalum dilatatum*) (Poales: Poaceae), dewberry (*Rubus* species) (Rosales: Rosaceae), Texas thistle (*Cirsium texanum*) (Asterales: Asteraceae), honey mesquite

(*Prosopis glandulosa*) (Fabales: Fabaceae), Pennsylvania pellitory (*Parietaria pensylvanica*) (Rosales: Urticaceae), western wheatgrass (*Pascopyrum smithii*) (Poales: Poaceae), Texas nightshade (*Solanum triquetrum*) (Solanales: Solanaceae) and silverleaf nightshade (*Solanum elaeagnifolium*) (Solanales: Solanaceae) (Alex Homesley, USDA-NRCS, personal communication). Sites are approximately 5.2 km apart. Temperature and humidity were logged at each trial using weather station data located less than 1 km from the sites.

II. 2. 1. 2. Experiment Design

Field sites were divided into 30.5 m by 30.5 m plots (Martin et al., 1998; Calixto and Harris, 2010) and the coordinates of the center point of each plot was recorded using Google Maps™. Overall dimensions of the plots were 91.5 m x 122 m (Figure 1). RIFA densities were characterized in both field sites prior to initiating this research. Two methods were employed; lures to attract RIFA foragers, and quantification of RIFA mounds within plots. These were repeated at the conclusion of experiments to document any changes in RIFA densities over the research period.

For assessing RIFA with food lures, slices of hot dog (approximately 2.54 cm diameter, 0.6 cm cylindrical height, 3.0 g) (Bar S Franks, Bar S Foods, Phoenix, AZ) were used as bait and were placed on the ground within the predetermined grid system at each field site (Bestelmeyer et al., 2000). One hot dog slice was placed in the center point of each plot, pinned to the ground with a vinyl ground marking flag, and three more slices were placed in the same manner every 3 m off the center point in each cardinal direction. The number of RIFA foragers at each bait was recorded after one hour had elapsed.

Mound densities were determined by marking the plot center points with a metal stake and anchoring a rope or measuring tape, carrying it to the edge of the plot (approximately 15.25 m) and walking the 360 degrees throughout the plot, counting the number of active mounds. This method ensures no mounds are counted twice once the beginning point has been reached again (Morrison and Porter, 2005). A mound was considered active if, when prodded with a walking stick or flag, RIFA workers emerged from the mound. Plots used for experimentation contained an average of 17 RIFA mounds.

II. 2. 1. 3. Compound Dilution and Testing as Bait

Nine treatments were tested; with two chemical concentrations per compound and the control a dry granular bait. Concentrations were chosen based on previous studies testing volatiles associated with carrion feeding insects (Liu et al. 2016; Dekeirsschieter et al. 2013).

For these experiments, each compound was diluted with acetone to an amount per 10 µL, as 10 µL were applied to the granular baits. IND (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥ 99.0%) was diluted to doses of 5.0 µg and 0.05 µg. DMDS (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥ 99.0%) was prepared at doses 0.005 µg and 0.25 µg. PAA (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥ 99.0%) was prepared to doses of 0.10 µg and 10.0 µg. IBA (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥ 99.0%) was prepared to doses of 0.01 µg and 1.00 µg.

Of the four compounds tested, only indole has been shown to be a QS capable compound (Lee et al, 2015; Zhang et al., 2015). There is much research to be done in the

realm of what compounds from QS capable bacteria account for QS capabilities. Therefore, these compounds were chosen also for their likelihood of affecting the behavior of RIFA through the species' naturally occurring *P. mirabilis*, with RIFA being driven to some extent by its naturally occurring bacteria to favor differing concentrations of DMDS, PAA, and IBA.

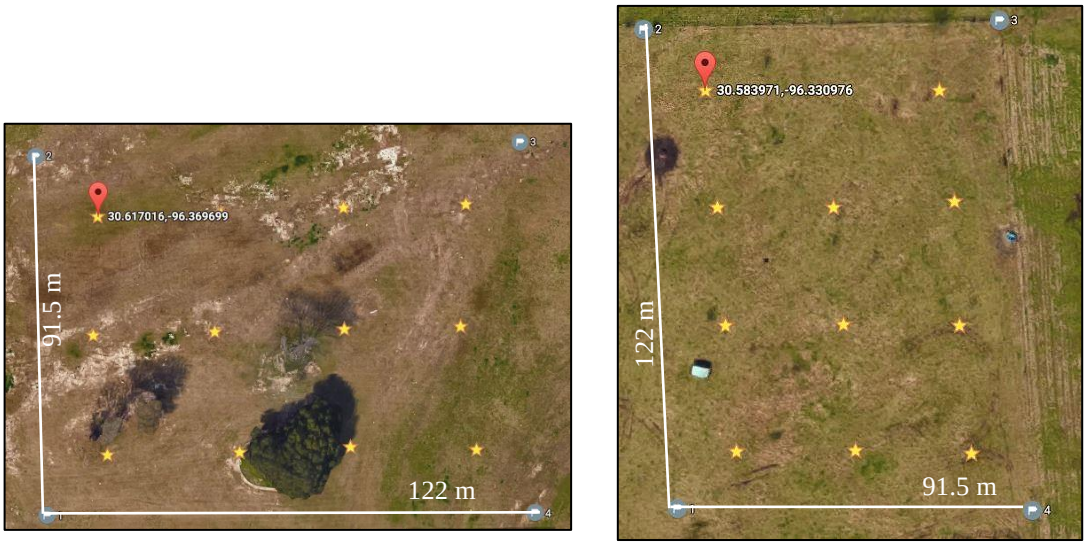

Figure 1: Overhead view of urban (left) and rural (right) field sights in College Station, Brazos Valley, Texas. Fields were divided into 30.5 m by 30.5 m plots for mound and bait counts, with yellow stars representing plot center points and blue flags marking the corners.
Reprinted from (Google, 2017).

II. 2. 1. 4. Treatments

Compounds were placed on a standard ant bait (Cook et al. 2010); an agar-based diet with a 1:1 protein (whey protein and calcium caseinate) to carbohydrate (sucrose) ratio. The prepared bait was dried overnight at 50°C to ensure removal of any water weight, ground with a KitchenAid® grinder attachment, and sieved through U.S. standard sieve (size #18; 1.00 mm), (Neff et al. 2011). Prior to each trial, 2 g allotments were placed

in 90 Dart® Conex Complements® condiment cups (59 mL). Each was assigned as treatment or control and cup lids were labeled with a mm scale, compound and concentration, and date (Figure 2). Five replicates were made for each treatment (n = 9), with 45 total cups deployed at each field site (Figure 3). For each treatment, 10 µL (as used in Liu et al., 2016) of the assigned compound and concentration was pipetted onto the granular bait particles and allowed to set for 5 minutes, capped with a lid, and immediately transported to the field sites.

Figure 2: Example of a 2 g, size #18, dry bait and 1.5 cm scale label; "DMDS 0.25 µg, Jun 30 16". Ten µL of compound concentration (unless a control) were added to the bait granules. Once in field, baits were poured onto the lid tops, below the label, for picture documentation. Number "24" represents specific cup location in field.

II. 2. 1. 5. Field Procedure

Trials began between 0800 and 1000 h. Cup locations were flagged and recorded with a handheld Garmin eTrex® 10 data logger or a cellular Android™ phone connected

to Google Maps™ labeled coordinates. Photographs were taken of each treated bait beginning at time point "0" with the removal of the first cup lid, and every 15 minutes for two hours using a Canon® EOS 50D and Canon® EOS 70D. For each digital photograph, the number of RIFA present at the bait was recorded. Concluding each trial, cups were returned to the laboratory and the remaining bait was quantified. Ants present in the cups were freeze-killed and preserved in 90-95% alcohol as voucher specimens. Six trials were conducted at each location between 24 June and 15 September 2016.

II. 2. 1. 6. Statistics

RIFA data were analyzed with an analysis of variance (ANOVA) followed by a Tukey HSD test ($P < 0.05$) (Tukey, 1949) (JMP® Pro 12). The statistical models tested used factors of trial, time, and treatment as predictors of RIFA response, with initial analyses also including location and replicate as predictors.

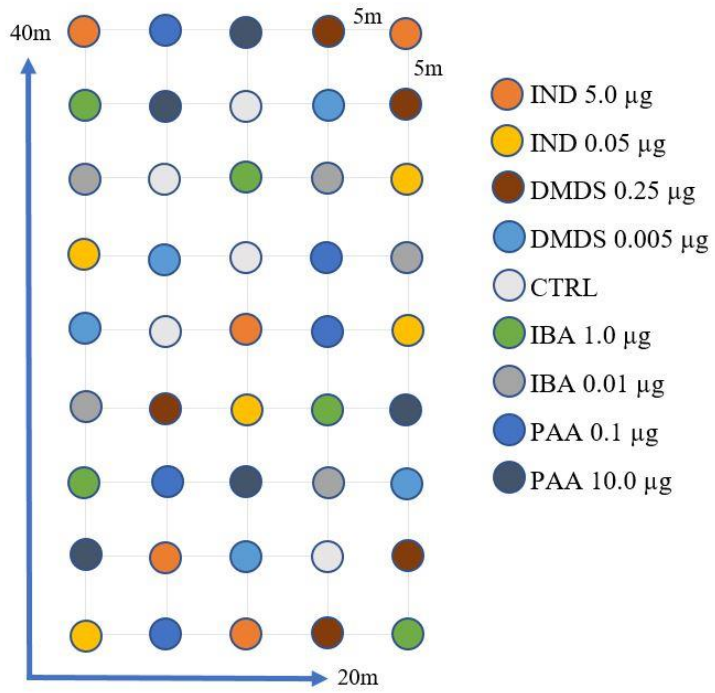

Figure 3: Example of an experimental field plot layout for rural and urban field trials in College Station, Brazos County, Texas. 45 cup lids in total; 9 total treatments including control, with 5 replications per treatment. All baits were 5m apart and labeled with a number corresponding to compound, concentration, date, and location in field (#1-45). Replicates and location in field were randomized prior to each trial.

II. 2. 2. Laboratory Experiments

Laboratory colonies of RIFA were collected in Brazos County, Texas, USA between February and April of 2017. Queens, workers, and brood gathered in the field were transferred to subsequent laboratory colonies via methods described in Banks *et al.*, (1981). Colonies were fed a 1:3 mixture of honey and water, apple slices, and mealworms *ad libitum*. With each trial, new colonies were collected from the field and utilized to avoid behavioral bias due to prolonged time spent in artificial settings. Colonies were kept and choice assay experiments were run in a temperature controlled room at the Rollins Urban and Structural Entomology Facility at Texas A&M University at a constant temperature

of 25.5ºC ± 1.2 ºC and relative humidity of 40.0% ± 1.0%, with a photo period of 8:16 (L:D) h.

II. 2. 2. 1. Experiment Design

Compound dilutions used in laboratory experiments were prepared using methods previously described. Preliminary data indicated starving RIFA foragers for 48 h prior to experimentation yielded best results. For each experiment, 105 RIFA foragers were starved in 150 mm plastic petri dishes (Thermo Fisher Scientific) containing a 75 mm test tube (VWR®), which held 3 mL of water and was plugged with a cotton ball, leaving room for RIFA to enter the tube. One hundred foragers were used for experimentation, with 5 extra included in case of any deaths over the 48 h starvation period. One hour prior to initiating an experiment (at 47 h), 20 brood (including ≥ 5 fourth instar larvae) were taken from their colonies and, for consistency, added to the 100 RIFA from their same colonies. This gave the RIFA foragers an hour to move the brood into the water tube to keep them moist, which made transferring the water tube and the 120 total RIFA (100 foragers and 20 brood) into the choice assay much easier, since most congregated inside the tube. Fourth instar larvae act as the stomach of the colony by digesting solid food which is then fed to the colony members in liquid form through trophallaxis (Tshinkel, 2006). RIFA foragers are more likely to initiate foraging behavior of granular bait if these larvae are present (Dr. Elida Espinoza, personal communication).

At 48 h, the 120 RIFA (foragers and brood) were removed from the petri dish and placed in the arena (described below). Experiments were performed in blocks. One block consisted of examining the response of RIFA to a negative control (bait alone), and

positive control (bait with 10µL acetone), versus bait treated with 10 µL of the low dose of a compound; IND 0.05 µg, DMDS 0.005 µg, IBA 0.1 µg, or PAA 1.0 µg. The second block examined the response of RIFA to these same treatments but with the high dose of a compound; IND 5.0 µg, DMDS 0.25 µg, IBA 1.0 µg, or PAA 10.0 µg. All experiments were replicated three times over two months.

The choice assay set-up consisted of one "nest" chamber; an airtight plastic cylinder (10 cm diameter, Pioneer Plastics Inc®, Dixon, KY, USA) attached to three similar "choice" chambers, drilled once using a 9.13 mm drill bit, and connected by 15 cm polyethylene tubing. Each choice chamber contained a 50 mm petri dish top (Thermo Fisher Scientific) with 2 g of bait; with choice chambers containing either a bait with 10 µL compound and associated concentration, a negative control of dry bait, or a positive control bait with 10 µL acetone (Figure 4). Five minutes before the start of the experiment, 10 µL of the compounds and positive controls were placed on the baits to allow the acetone to volatilize. Doses were blocked with low doses being examined concurrently. High doses were examined independent of low doses. After each trial was run, the set-up and tubing was cleaned with 70% ethanol and allowed to dry.

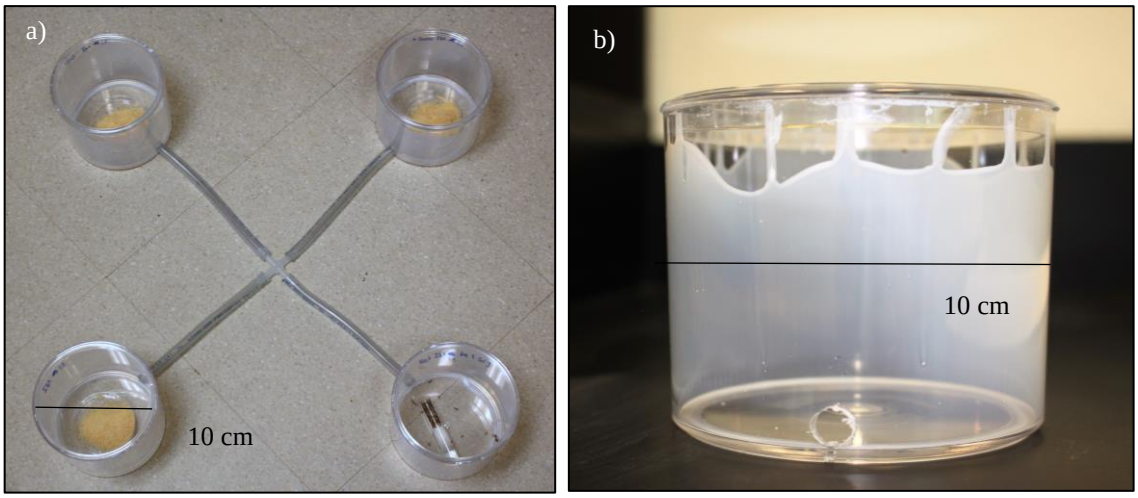

Figure 4: Laboratory design. a) Nest chamber (bottom right) houses 100 starved RIFA foragers, 20 brood, and one water test tube containing 3 mL water blocked with a cotton ball, with room for RIFA to enter. Foragers were given 2 hours to "choose" which randomized treatment (bait with compound, positive control bait with acetone, or plain bait negative control) to collect bait from and bring back to the nest for the larvae. Pictures were taken at time point "0" and every 15 minutes to document RIFA accumulation on baits, and bait weighed following experiments for determining bait removal. b) Chambers were made from 10 cm diameter plastic containers, coated with Fluon® to prevent RIFA from climbing, and drilled with a 23/64" drill bit for attaching plastic tubing.

II. 2. 2. 2. Statistics. RIFA response data were analyzed with an analysis of variance (ANOVA) followed by a Tukey HSD test ($P < 0.05$) (Tukey, 1949). The statistical models tested used factors of trial, time, and treatment as predictors of RIFA response. In treatment and grams of bait removed.

II. 3. Results

II. 3. 1. Field Experiments

II. 3. 1. 1. RIFA Assessment and Response at Urban and Rural Sites

Based on preliminary mound counts at the field sites, RIFA populations were not significantly different ($P = 0.9657$) across urban and rural sites (Figure 5). Preliminary RIFA recruitment to food lures (Figure 6) was also not significantly ($P = 0.3735$) different across the urban and rural sites. Total RIFA recruited to baits across all six trials in urban

and rural sites were not significantly (P = 0.6305) different as well (Figure 7 and Figure 8).

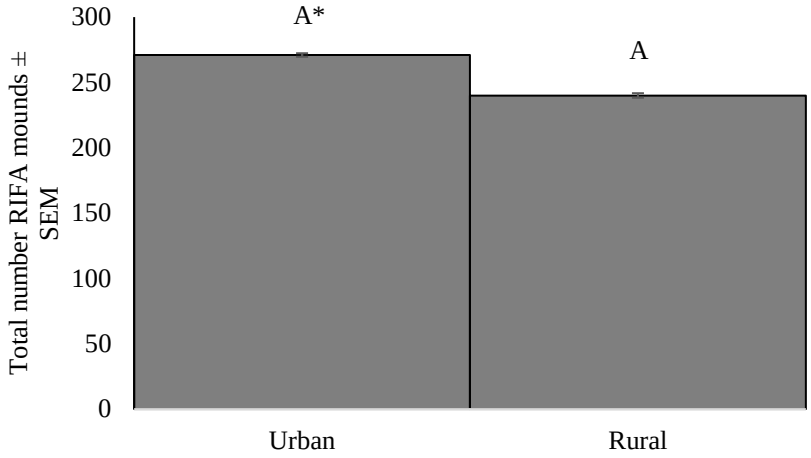

Figure 5: Total RIFA mound counts from urban and rural sites prior to and following experimentation; June 24, 2016- September 15, 2016. Urban mounds, N = 271. Rural mounds, N = 240. *Different letters indicate significant (P < 0.05) difference in RIFA response.

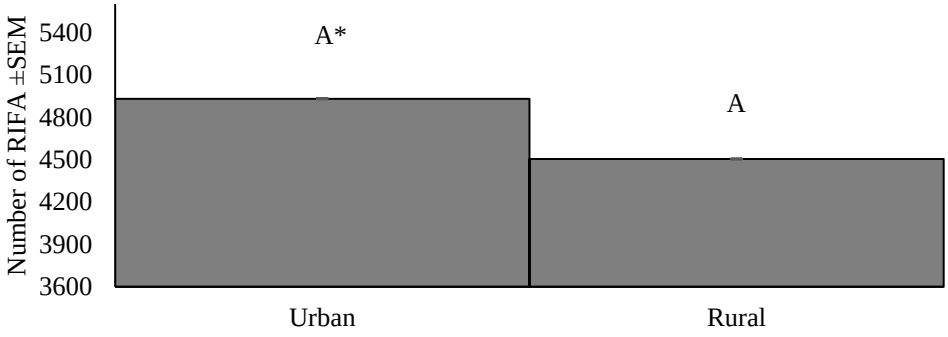

Figure 6: Total number RIFA observed during preliminary hot dog counts at both urban and rural field sites, gathered the morning of June 20, 2016. Urban RIFA, N = 4,930. Rural RIFA, N = 4,504. *Different letters indicate significant (P < 0.05) difference in RIFA response.

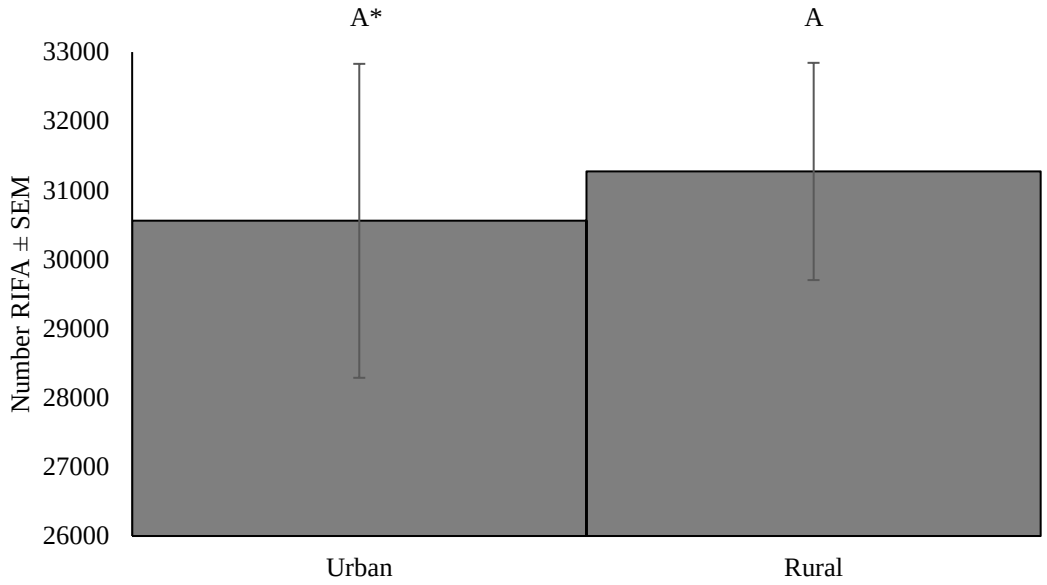

Figure 7: Total number of RIFA attracted to baits from all six trials and separated by site. Urban RIFA, N = 30,559. Rural RIFA, N = 31,273. *Different letters indicate significant ($P < 0.05$) difference in RIFA response.

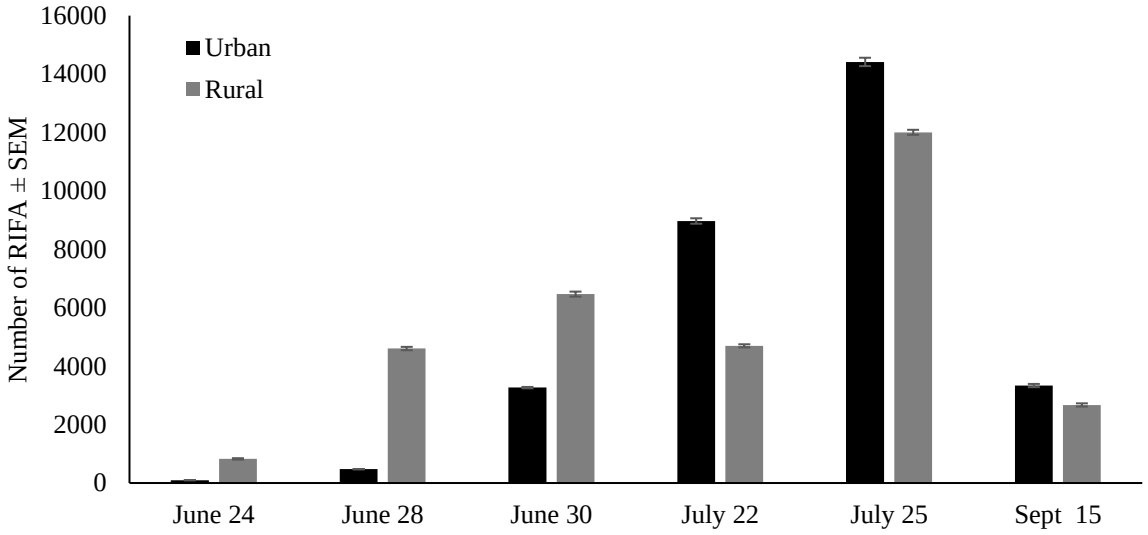

Figure 8: Total number of RIFA counted on baits and separated by all six trials in both urban and rural field locations; June 24, 2016 through September 15, 2016. No significant ($P > 0.05$) difference determine in response between sites.

II. 3. 1. 2. Overall RIFA Response to Treatments

Analysis (ANOVA presented in Appendix 1) of total RIFA attracted to all treatments from all six trials based on location (urban and rural), replicate (N = 5), time (15 minute intervals), treatment (compound concentrations and controls), and trial (N = 6) showed significance (df = 1079, 3240; F = 4.022; $P < 0.0001$). A five-way interaction between trial, treatment, time, location, and replicate was determined (df = 160, 3240; F = 1.393; $P < 0.001$). However, treatment was not significant (df = 8, 3240; F = 1.565; $P = 0.130$).

II. 3. 1. 3. RIFA Response in Urban Environment

Analysis (ANOVA presented in Appendix 2) of total RIFA numbers attracted to all treatments from all six urban trials based on replicate, time, treatment, and trial was significant (df = 107, 431; F = 5.73; $P < 0.0001$) (Figure 9).

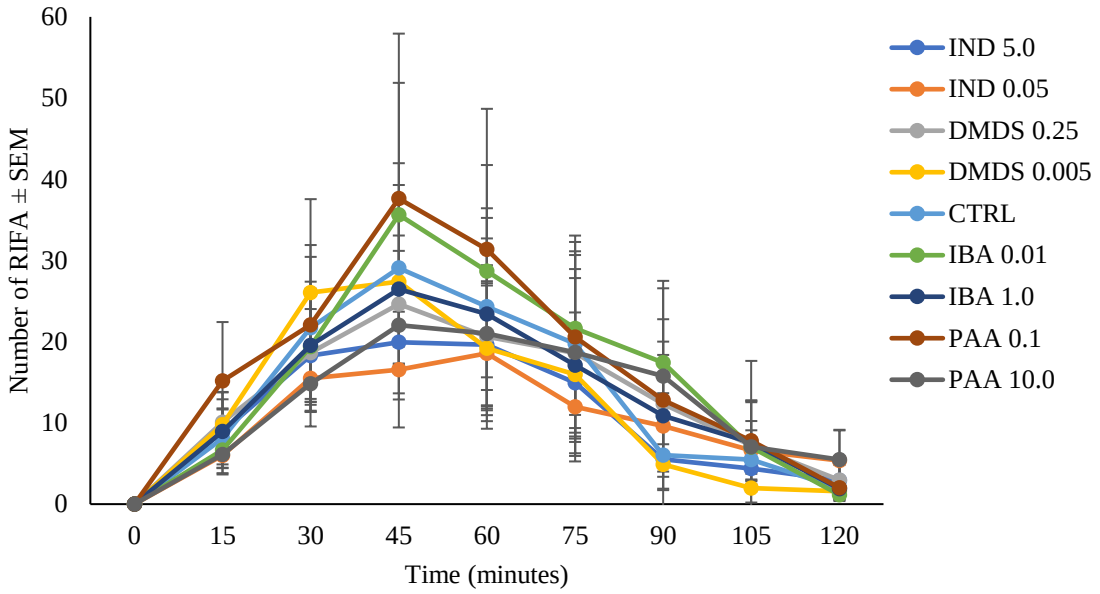

Figure 9: Average urban field site RIFA activity over time using mean RIFA totals, and including data from all trials. No significant ($P > 0.05$) difference determine in response over time between treatments.

Time and trial significantly interacted (df = 5, 324; F = 4.98; $P < 0.0001$). Treatment was not significant (df = 8, 324; F = 1.71; $P = 0.0946$). Analyses of trial indicate trials from June 30th and September 15th were not significantly different ($P > 0.05$) from one another and represented greatest level of similar RIFA responses to baits (Figure 5). Therefore, remaining analyses conducted on RIFA responses were restricted to these two trials. Remaining trials from June 24th and June 28th grouped; however, most observations (82.1%) were zero and thus considered uninformative as the model was not significant ($P > 0.05$) (Figure 10).

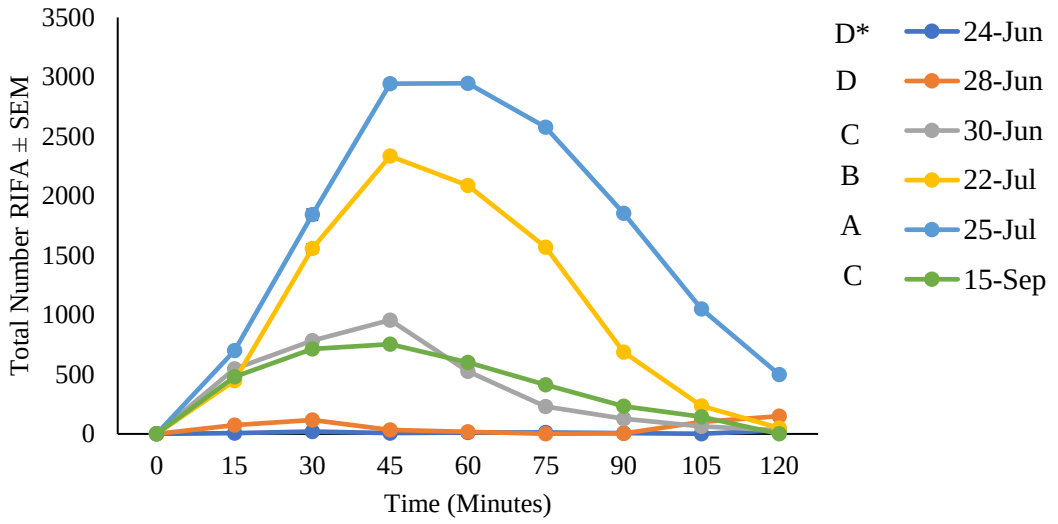

Figure 10: Total RIFA accumulation to baits over time in the urban environment, separated by trials 1-6. *Different letters indicate significant difference in RIFA response. No significant ($P > 0.05$) difference determine in response over time between treatments.

Analysis (ANOVA presented in Appendix 3) of total RIFA numbers responding to treatments during urban trials from June 30th and September 15th based on treatment and time was significant (df = 17, 71; F = 7.73; $P < 0.0001$). No significant interactions were determined. Time (df = 1, 54; F = 100.73; $P < 0.0001$) and treatment (df = 8, 54; F = 2.44; $P = 0.0250$) were significant. For the treatments, IND 5.0 µg and DMDS 0.005 µg were significantly different from each other as well as the remaining treatments. Indole 5.0 µg displayed the greatest (15%) attraction for RIFA, while DMDS 0.005 µg served as the least attractive (6.1%) for RIFA recruitment. Remaining treatments, including control, accounted for 78.9% of RIFA response, with the control bait responsible for 11.2% of RIFA recruitment. Furthermore, IND 5.0 µg attracted 34.1% more RIFA than the control bait, while IND 0.05 µg was 24.3% less attractive. Similar results were determined for

DMDS with the high dose attracting 23.9% more RIFA, while the low dose was 45.6% less attracted than the control bait. Both doses of IBA were marginally more attractive (11-16%), while PAA was less attractive (8-15%), than the control bait (Figure 11).

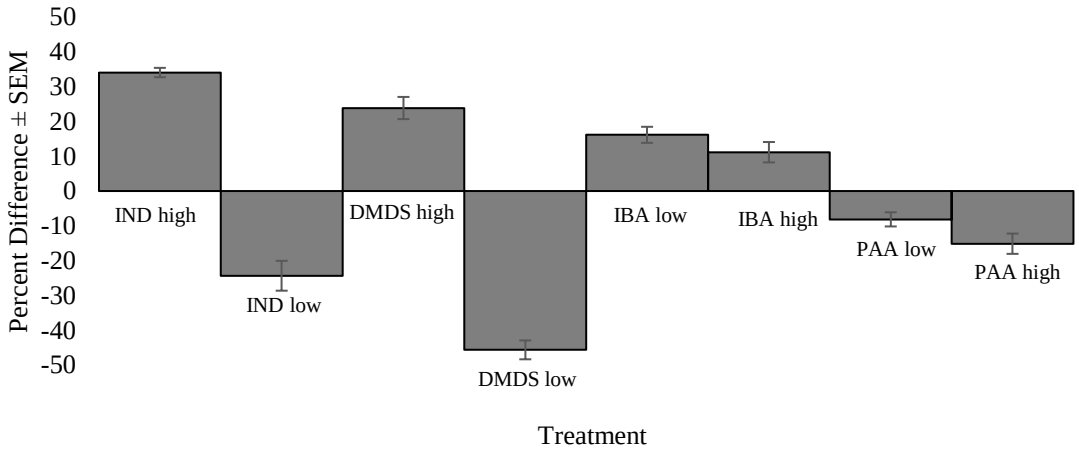

Figure 11: Percent difference of total RIFA attraction to each treatment compared to the control (0) from urban trials 3 and 6. Compounds are measured in µg. No significant ($P > 0.05$) difference determine in response between treatments.

II. 3. 1. 4. RIFA Response in Rural Environment

Analysis (ANOVA presented in Appendix 4) of RIFA response from all six rural trials based on replicate, time, treatment, and trial was significant (df = 107, 431; F = 3.16; $P < 0.0001$) (Figure 12).

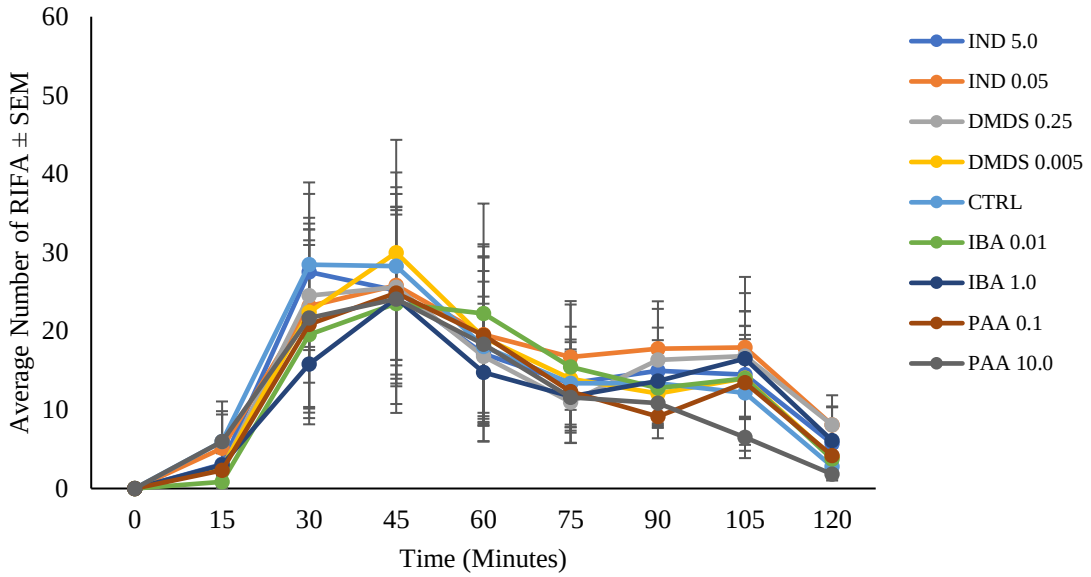

Figure 12: Average rural field site RIFA activity over time using mean RIFA totals, and including data from all trials. No significant ($P > 0.05$) difference determine in response over time between treatments.

A significant (df = 5, 324; F = 21.88; P < 0.0001) interaction between trial and time was determined. However, a trial effect was observed (Appendix 4), and analyses based on grouping were not significant ($P > 0.05$) or had an interaction effect (ANOVA presented in Appendix 5), except for rural trials 1 and 6 (ANOVA presented in Appendix 6) (Figure 13). Though these two trials had significantly different ($P = 0.0005$) RIFA recruitment numbers, they provide a starting point for comparing the differences of RIFA behavior possibly affected by environmental differences (Figure 14).

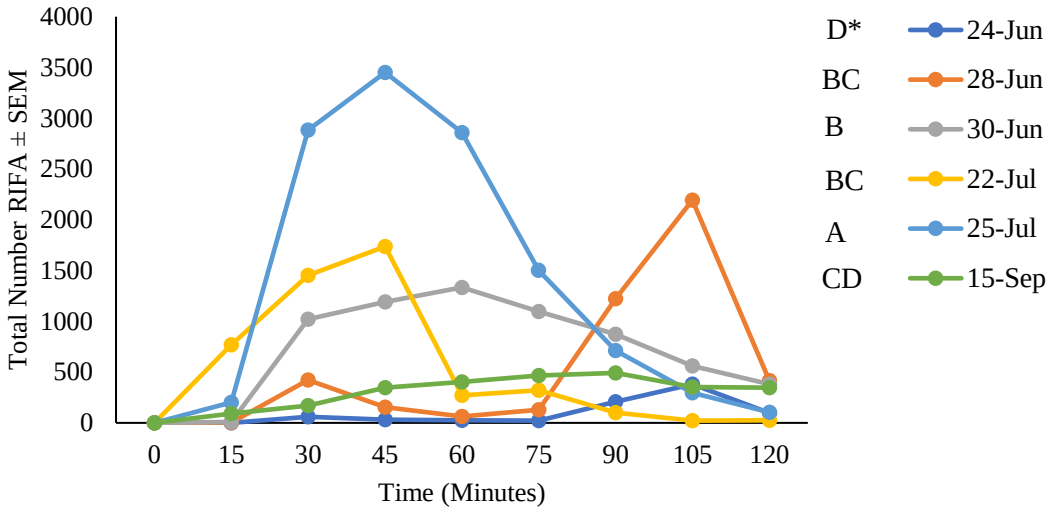

Figure 13: Total RIFA accumulation to baits over time in the rural environment, separated by trials 1-6. *Different letters indicate significant difference in RIFA response.

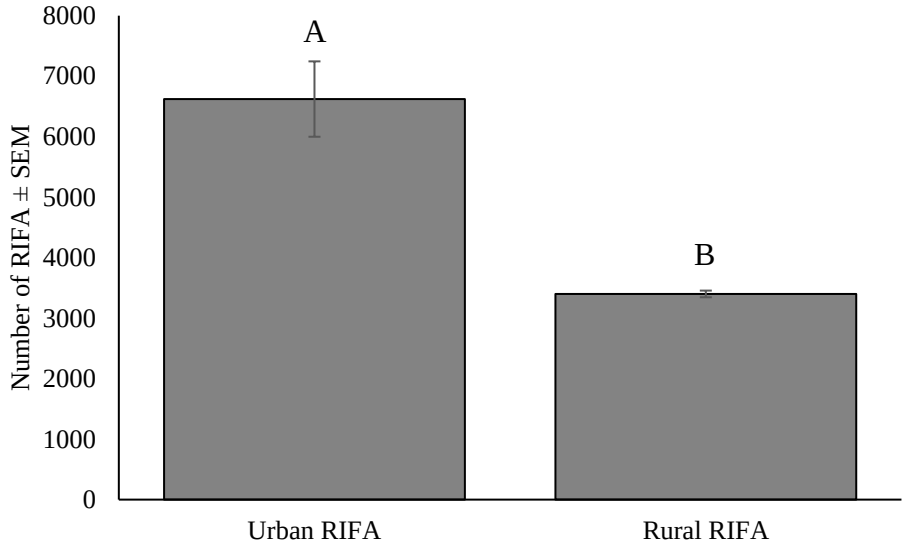

Figure 14. Total number of RIFA attracted to all baits from urban trials 3 and 6 (June 30th and September 15th) and rural trials 1 and 6 (June 24th and September 15th). *Different letters indicate significant ($P < 0.05$) difference in RIFA response.

Analysis (ANOVA presented in Appendix 6) of RIFA response during rural trials 1 and 6 based on treatment and time was significant (df = 17, 71; F = 5.856; $P < 0.0001$).

No significant interactions were determined. Time (df = 1, 54; F = 40.412; $P < 0.0001$) and treatment (df = 8, 54; F = 6.168; $P < 0.0001$) were significant. For the treatments, DMDS 0.25 µg and IBA 0.01 µg were significantly different from each other as well as the remaining treatments. PAA 0.1 µg (17.7%) and DMDS 0.25 (17.3%) µg displayed the greatest attraction for RIFA, while IBA 0.01 µg served as the least attractive (3.5%) for RIFA recruitment. Remaining treatments, including control, accounted for 61.6% of RIFA response, with the control bait responsible for 7.1% of RIFA recruitment. For percent difference in rural RIFA attraction to baits treated with compounds compared to control baits, the treatment PAA 0.1 µg attracted 148.3% more RIFA than the control bait, while the higher dose was 3.7% more attractive. Similar results were determined for IBA, with the high dose 94.2% more attractive than control bait and lower dose 50.4% less attractive. Finally, high dose DMDS attracted 142.1% more RIFA than the control bait, while the low dose was 88% more attractive, and 62.8% of RIFA were more attractive to the high indole dose, and 16.1% of RIFA were more attracted to the low indole dose than to control (Figure 15).

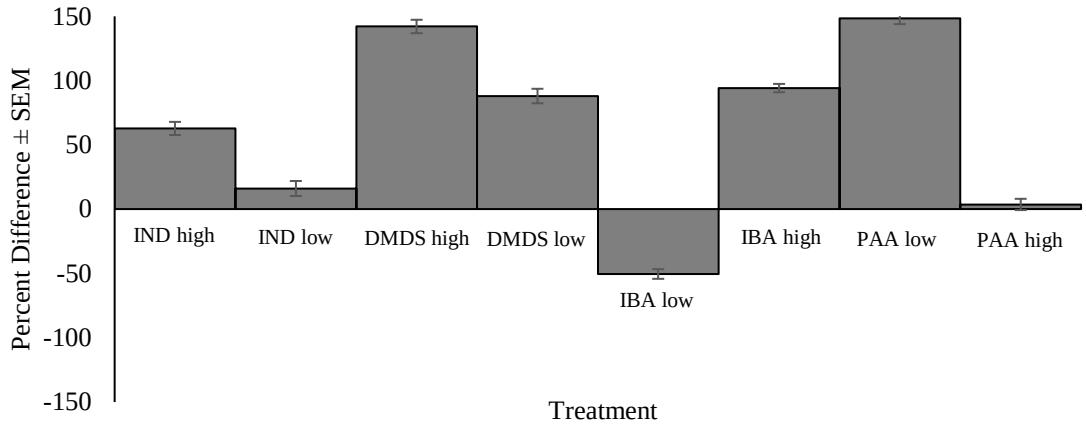

Figure 15: Percent difference of RIFA attraction to treated baits compared to the control (0) from rural trials 1 and 6. Compounds are measured in µg. No significant ($P > 0.05$) difference determine in response between treatments.

II. 3. 2. Laboratory Experiments

II. 3. 2. 1. Overall RIFA Response to Treatments

Analysis (ANOVA presented in Appendix 8a) of RIFA response from high dose trials based on time, treatment, and trial was significant (df = 47, 287; F = 4.39; $P < 0.0001$). Treatment was significant ($P < 0.0001$), however the interaction between trial, treatment, and time was not ($P = 0.0993$).

Analysis (ANOVA presented in Appendix 8b) of RIFA response from low dose trials based on time, treatment, and trial was significant (df = 47, 287; F = 6.88; $P < 0.0001$). Trial ($P <.0001$), treatment ($P <.0001$), and time ($P = 0.0040$) were significant, while the interaction term based on trial, treatment, and time was not significant ($P = 0.6600$).

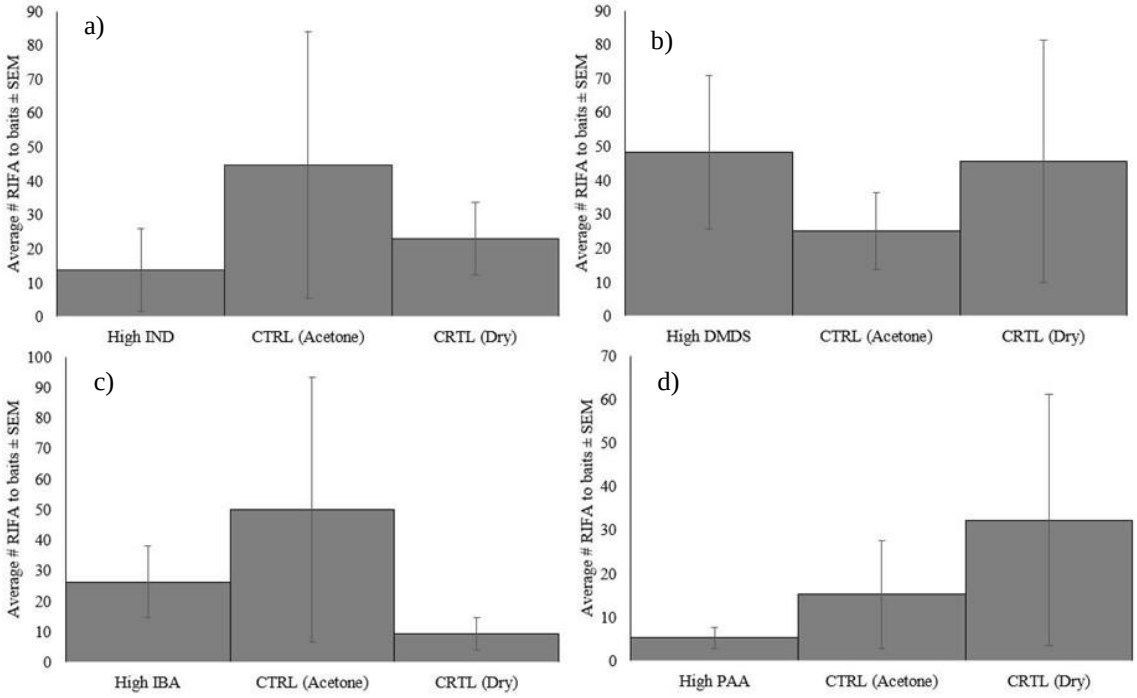

Figure 16: Average total number of RIFA to laboratory baits in high concentration assay experiments over three trial periods. Experimental conditions; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h. a) Indole (5.0 µg) treatments not significant, $P = 0.6742$. b) Dimethyl disulfide (0.25 µg) treatments not significant, $P = 0.7822$. c) Isobutylamine (1.0 µg) treatments not significant, $P = 0.5662$. d) Phenylacetic acid (10.0 µg) treatments not significant, $P = 0.5966$.

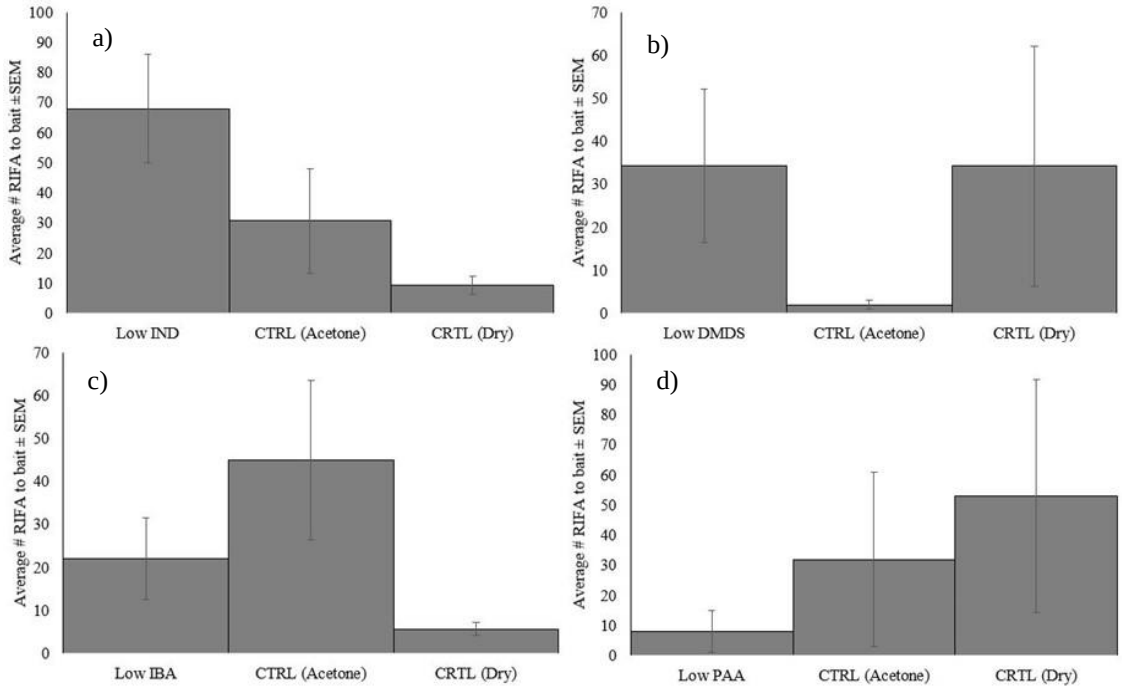

Figure 17: Average total number of RIFA to laboratory baits in low concentration assay experiments over three trial periods. Experimental conditions; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h. a) Indole (0.05 µg) treatments not significant, $P = 0.0723$. b) Dimethyl disulfide (0.005 µg) treatments not significant, $P = 0.4368$. c) Isobutylamine (0.1 µg) treatments not significant, $P = 0.1480$ d) Phenylacetic acid (0.1 µg) treatments not significant, $P = 0.5600$.

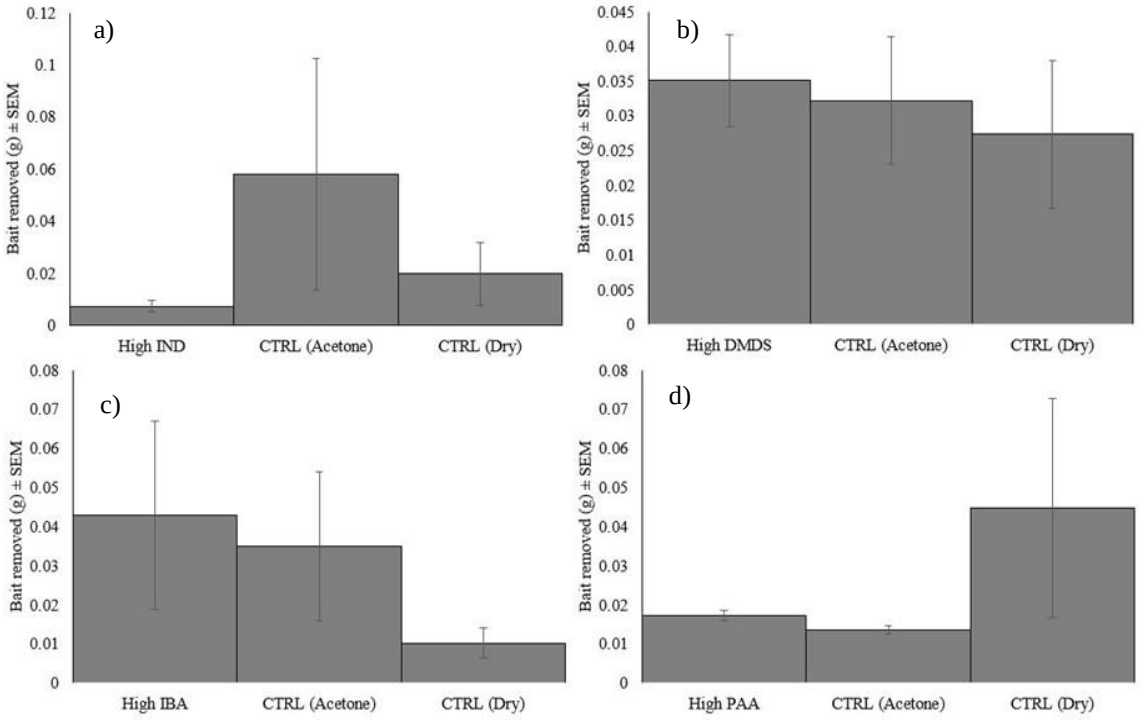

Figure 18. Average amount of bait removed by RIFA from trials 1-3 of high concentration laboratory choice assays transported from original 2g baits to nest chamber. Experimental conditions; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h. a) IND 5.0 µg treatments, $P = 0.4252$. b) DMDS 0.25 µg treatments, $P = 0.8320$. c) IBA 1.0 µg treatments, $P = 0.4520$. d) PAA 10.0 µg treatments, $P = 0.3924$.

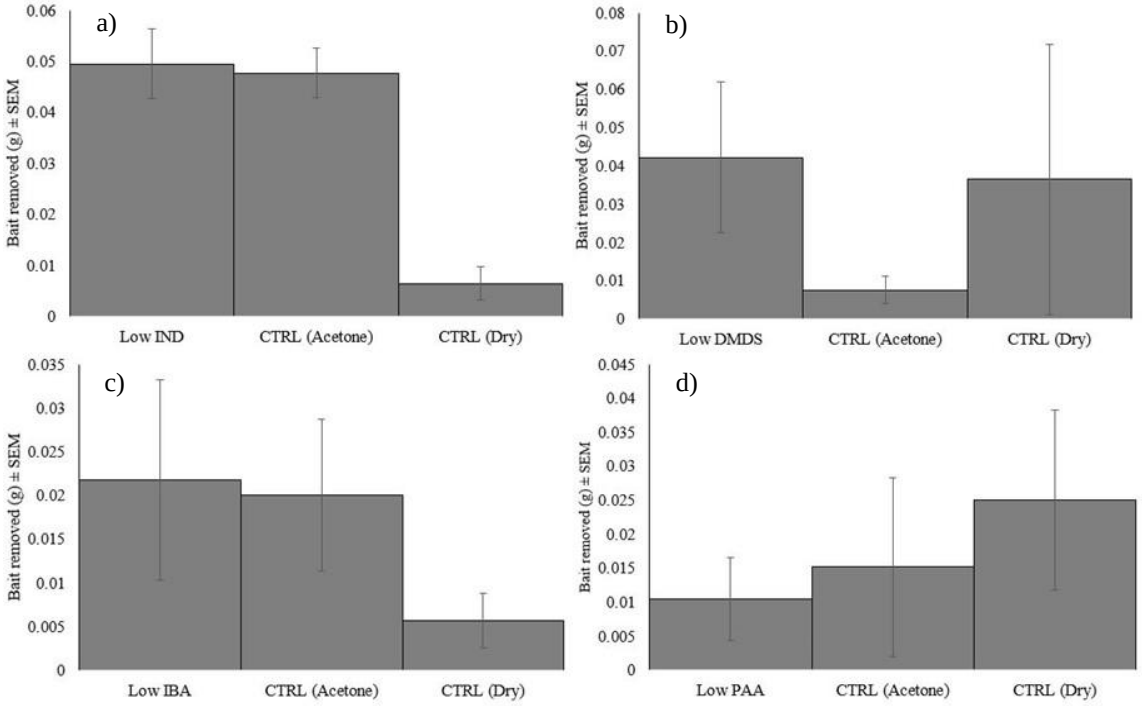

Figure 19. Average amount of bait removed by RIFA from trials 1-3 of low concentration laboratory choice assays transported from original 2g baits to nest chamber. Experimental conditions; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h. a) IND 0.05 µg treatments, $P = 0.0017$. b) DMDS 0.005 µg treatments, $P = 0.5654$. c) IBA 0.01 µg treatments, $P = 0.3986$. d) PAA 1.0 µg treatments, $P = 0.6686$. Letters that differ above indole assay indicate significant difference in treatment ($P < 0.05$).

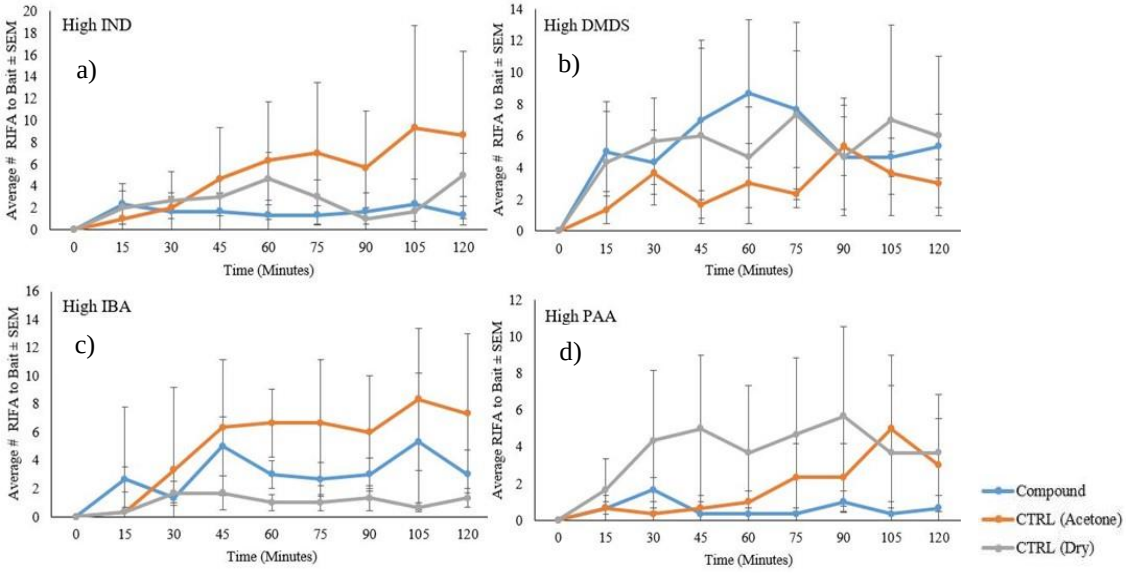

Figure 20: Average number of RIFA on baits from trials 1-3 of high concentration laboratory choice assays. Experimental conditions; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%, 8:16 L:D h. No significant ($P > 0.05$) difference determine in response between treatments.

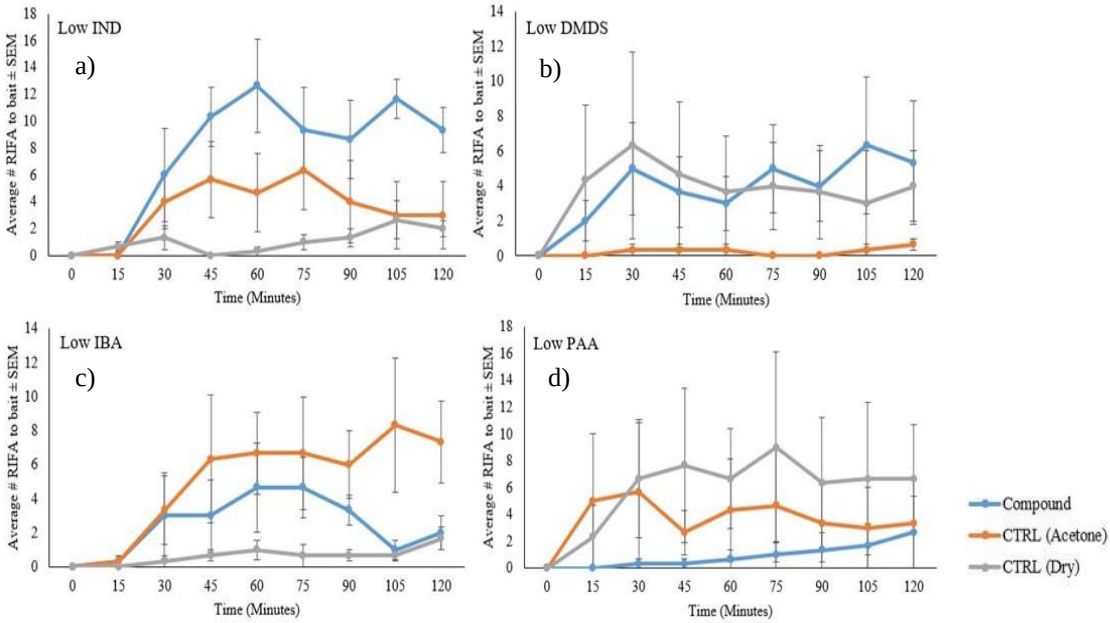

Figure 21: Average number of RIFA on baits from trials 1-3 of low concentration laboratory choice assays. Experimental conditions; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%, 8:16 L:D h. No significant ($P > 0.05$) difference determine in response between treatments.

II. 3. 2. 2. Average Total RIFA to High Dose Baits

II. 3. 2. 2. 1. IND 5.0 µg

Analysis (presented in Appendix 9a) of total RIFA attracted to bait treated with IND 5.0 µg, 10µL acetone, or not at all, was not significant ($P = 0.6742$) (Figure 16a). Analysis (presented in Appendix 10a) of total bait removed from the treatments was not significant ($P = 0.4252$) either (Figure 18a). However, analysis (presented in Appendix 11a) of RIFA attracted to baits over time was significant ($P < 0.0001$) (Figure 20a). A two-way interaction between trial and time was determined (df = 2, 18; F = 13.512; $P = 0.0003$). The control and bait treated with the high dose of IND has a similarly low level of RIFA response over time (approximately 50% less than bait treated with acetone).

II. 3. 2. 2. 2. DMDS 0.25 µg

Analysis (presented in Appendix 9b) of total RIFA attracted to bait treated with DMDS 0.25 µg, 10µL acetone, or not at all, was not significant ($P = 0.7822$) (Figure 16b). Analysis (presented in Appendix 10b) of total bait removed from the treatments was not significant ($P = 0.8320$) either (Figure 18b). Analysis (presented in Appendix 11b) of RIFA attracted to baits over time was significant ($P < 0.0001$) (Figure 20b) for treatment (df = 2, 18; F = 27.4763; $P = 0.0007$) but not time (df = 1, 18; F = 10.8930; $P = 0.2701$), and had an interaction effect that was not significant (df = 2, 18; F = 5.8217; $P = 0.5576$).

II. 3. 2. 2. 3. IBA 1.0 µg

Analysis (presented in Appendix 9c) of total RIFA attracted to bait treated with IBA 1.0 µg, 10µL acetone, or not at all, was not significant ($P = 0.5662$) (Figure 16c). Analysis (presented in Appendix 10c) of total bait removed from the treatments was not

significant (P = 0.4520) either (Figure 18c). Analysis (presented in Appendix 11c) of RIFA attracted to baits over time was significant (P < 0.0001) (Figure 20c). A two-way interaction between trial and time was determined (df = 2, 18; F = 5.8217; P = 0.0112).

II. 3. 2. 2. 4. PAA 10.0 µg

Analysis (presented in Appendix 9d) of total RIFA attracted to bait treated with PAA 10.0 µg, 10µL acetone, or not at all, was not significant (P = 0.5966) (Figure 16d). Analysis (presented in Appendix 10d) of total bait removed from the treatments was not significant (P = 0.3924) either (Figure 18d). Analysis (presented in Appendix 11d) of RIFA attracted to baits over time was significant (P < 0.0001) (Figure 20d). A two-way interaction between trial and time was determined (df = 2, 18; F = 4.6038; P = 0.0243).

II. 3. 2. 3. Average Total RIFA to Low Dose Baits

II. 3. 2. 3. 1. IND 0.05 µg

Analysis (presented in Appendix 9e) of total RIFA attracted to bait treated with IND 0.05 µg, 10µL acetone, or not at all, was not significant (P = 0.0723) (Figure 17a). Analysis (presented in Appendix 10e) of total bait removed from the treatments displayed a significant model (df = 2, 8; F = 21.9232; P = 0.0017) and treatment was significant (P = 0.0017) (Figure 19a). Analysis (presented in Appendix 11e) of RIFA attracted to baits over time was significant (df = 5, 23; F = 9.6340; P = 0.0002) (Figure 21a), with significance in treatment (df = 2, 18; F = 19.9725; P < 0.0001) and time (df = 1, 18; F = 5.0951; P = 0.0374). A two-way interaction between trial and time was not determined (df = 2, 18; F = 1.6652; P = 0.2186).

II. 3. 2. 3. 2. DMDS 0.005 µg

Analysis (presented in Appendix 9f) of total RIFA attracted to bait treated with DMDS 0.005 µg, 10µL acetone, or not at all, was not significant (P = 0.4368) (Figure 17b). Analysis (presented in Appendix 10f) of total bait removed from the treatments was not significant (P = 0.5654) either (Figure 19b). Analysis (presented in Appendix 11f) of RIFA attracted to baits over time was significant (P < 0.0001) (Figure 21b). A two-way interaction between trial and time was determined (df = 2, 18; F = 6.9316; P = 0.0059), although time was not significant (df = 1, 18; F = 0.6641; P = 0.4258).

II. 3. 2. 3. 3. IBA 0.01 µg

Analysis (presented in Appendix 9g) of total RIFA attracted to bait treated with IBA 0.01 µg, 10µL acetone, or not at all, was not significant (P = 0.1480) (Figure 17c). Analysis (presented in Appendix 10g) of total bait removed from the treatments was not significant (P = 0.3986) either (Figure 19c). Analysis (presented in Appendix 11g) of RIFA attracted to baits over time was significant (P < 0.0001) (Figure 21c). A two-way interaction between trial and time was determined (df = 2, 18; F = 4.7563; P = 0.0220).

II. 3. 2. 3. 4. PAA 0.1 µg

Analysis (presented in Appendix 9h) of total RIFA attracted to bait treated with PAA 0.1 µg, 10µL acetone, or not at all, was not significant (P = 0.5600) (Figure 17d). Analysis (presented in Appendix 10h) of total bait removed from the treatments was not significant (P = 0.6686) either (Figure 19d). Analysis (presented in Appendix 11h) of RIFA attracted to baits over time was significant (P < 0.0001) (Figure 21d). A two-way interaction between trial and time was determined (df = 2, 18; F = 22.8904; P = 0.0001).

II. 4. Discussion

RIFA responses to baits was partly regulated by compound and concentration placed on the bait. However, RIFA responses to various compounds at different concentrations varied between urban and rural trials. Furthermore, the responses across trials within a given location differed. Of the six trials conducted in both field sites, only two trials produced significant results. In the case of the urban site, IND 5.0 µg attracted significantly (15%) more RIFA than the control bait. In contrast, DMDS 0.005 µg attracted significantly fewer ants (45%) than the control bait. In the case of the rural site, PAA 0.1 µg and DMDS 0.25 µg attracted similarly high amounts of RIFA and were approximately 145% more attractive compared to the control.

To investigate RIFA responses to these compounds and associated concentrations in more detail, three laboratory trials were conducted. However, in these trials, RIFA response was restricted to a specific compound and concentration versus positive and negative control diets containing the solvent or the diet alone (three-way choice). RIFA response was most interesting with regards to two compounds, IND and DMDS. Based on results generated, the low dose of IND was attractive (63.1% of attraction compared to the negative control at 8.6% and positive control at 28.3%) while the high dose did not elicit a RIFA response. In contrast, the high dose of DMDS elicited an attractive response over time and making up 40.6% total recruitment with the positive control making up 38.4% and the negative control 21% recruitment. The low dose of DMDS did not show significance over time, and both the negative control and compound treated baits elicited the same number of RIFA, both making up 48.6% of RIFA response. Of the models for

total average RIFA to baits, no models were significant. These results demonstrate RIFA respond differently to volatiles associated with bacteria common in their environment. Furthermore, these compounds hold promise for developing novel baits for RIFA control; however, additional research is still needed to optimize these compounds for use as attractants. As previously mentioned, results varied across sites and trials. Furthermore, based on these results, it might be possible to develop methods for attracting RIFA to specific locations without using a bait; for example, incorporating VOC concentrations into different substrates to dissuade or persuade RIFA foraging.

Many factors may be influencing RIFA preference for one bait over another across sites (i.e., urban versus rural). It is well-known that insects perform differently under differing environmental conditions. An exemplary case of this is the desert locust *Schistocerca gregaria* Forsk (Orthoptera: Acrididae). In times of nutritional resource scarcity, the typically solitary locusts aggregate on the available food sources, triggering their serotonin levels, which induce a gregarious behavioral form (Ansley et al., 2009). It is also known that insects may show preferences for certain host plants or habitats over others, if given the choice. For example, Davis (2008) found that the differences observed in female *Drosophila melanogaster* breeding site preferences were positively correlated with their natal habitats; that is, flies spent the shortest amount of time searching for a breeding site if it resembled their natal habitat.

New polygyne RIFA queens are known to develop their colonies closer to their natal nests than monogyne queens (Tshinkel, 2006). It is possible that the differing RIFA foraging preferences for compound doses in the urban and rural environments may be

correlated with the resources in that specific environment. In the urban field, Bermudagrass, Bahiagrass, and native grasses are the dominant plant species. RIFA in this environment were most attracted to IND at the higher dose and were least attracted to DMDS at the lowest dose. As mentioned, indole is a common attractant in varying doses for many insects including mosquitoes (Diptera: Culicidae) and blow flies (Diptera: Calliphoridae), and was found to be the best attractant for laboratory RIFA trials. This compound is also a derivative of tryptophan, one of the rarer essential amino acids. In a more homogenous environment, indole may serve as a ubiquitous attractant molecule for insect foragers.

The rural landscape is dominated by a heterogeneous variety of plants and native grasses and, as it were, cow dung. DMDS may be more attractive in this environment because there are high amounts of DMDS in manure, which was common on the landscape and was observed to be a nesting site used by RIFA (observed when performing preliminary mound counts). PAA was another high attractant in this area of higher plant variety. Plants contain auxins; powerful growth hormones that are affected by insect herbivory (Erb et al., 2012). PAA is one of these auxins and is found in many crop plants, several times more abundant than 3-indoleacetic acid (Wightman and Lighty, 1982). It has been found that herbivores induce jasmonic acid levels to fluctuate, which may affect a plant's auxin homeostasis and further causing plants to modulate their auxin levels (Ding et al., 2008; Pauwels et al., 2009; Erb et al., 2012). Insect parasitoid detection to host volatiles is a well-studied topic in the literature. As a generalist predator with heightened

ORs compared to other insect species, it would be interesting to test if RIFA play a role in detecting herbivory through similar chemical means.

Regarding other insects' roles in the mediation of RIFA preference to baits, competition could vary across sites and thus impact RIFA response. In the case of the behavior changing locusts, it was found that those in the gregarious form, opposed to their solitary counterparts, had larger brains to cope with the onset of higher competition and heightened sociality (Ott and Rogers, 2010). This raises the question of what RIFA's genes or physiological make-up is different between groups of different environments. In the urban environment, the odorous house ant, *Tapinoma sessile* Say (Hymenoptera: Formicidae), were sometimes found on baits either with RIFA or by themselves. In the rural environment, this ant was rarely observed but grasshoppers and spiders frequented the baits. It is less likely that there would be intraspecific competition between members of different colonies because polygynous (opposed to monogynous) RIFA do not tend to fight for territory (Tschinkel, 2006). However, it is possible that competing species could have impacted RIFA accumulation. Furthermore, what we determine to be differences in VOC preference may also be impacted by abiotic factors. One factor may be the ability of the ants to access the baits. In conducting field trials, if the grass impeded the cup from lying flat on the soil, it was pushed aside so that the cup lid with bait could sit on the soil. However, the ease of access to these baits is unknown.

Both field sites were not irrigated, and rainfall experienced at both sites most likely was similar, given geographic proximity (5.2 km). Further, soil type is unknown in both environments. It is possible that factors such as consistent manure patches in the rural field

and frequent mowing in the urban field could aid in differences of soil type. Cattle manure is known to increase pH of acidic soils (Whalen et al., 2000) and increase phosphorous content (Sharpley et al., 2004). Kitchen et al. (2009) found that mowing of grass altered soil carbon and nitrogen content over time. Also, thicker grasses in the rural site may have allowed for more shade for foragers than the more open design of the urban site; affecting ease of access to baits on days with more sun exposure. Finally, season is an abiotic factor that has also been linked to changes in fire ant behavior, and animal behavior in general. For example, Cook et al. (2011) found that there is a higher need for protein and lesser need for carbohydrates in the summer months than in the fall months, likely due to larval development needs.

Liu et al. (2016) found that sex, gravidity, and nutritional status had a significant impact on flies to doses of the compounds tested here. All RIFA foragers are female, are the eldest members of their colonies, and by definition of their caste are fed less nutritious food than the reproductive caste members, who require additional nutritional resources for flight and reproduction. This raises the question if perhaps age or nutritional status of the workers would impact individual preferences to baits. For instance, do newly promoted foragers react differently to baits treated with decomposition related VOCs than those who are elder and more "practiced" at the job? In a choice experiment using all four compounds, how might RIFA "decide" which amino acid derivative is most needed by the colony (i.e. providing needed nutrition)? From these experiments, indole consistently showed attractive properties and is a rare amino acid in the environment; known to attract many insects.

There is still much work to do in determining dose specific behavioral changes of insects to VOCs related to decomposition. For example, Frederickx et al. (2012) did not observe any behavioral attraction of *L. sericata* to doses of indole or phenol at 100 µg and 0.05 µg. Also, Dekeirsschieter et al. (2009) found no cadaveric VOCs were detected during the fresh decomposition stage. This is important as we tend to see blow flies and fire ants within minutes after death, though most studies that look at decomposition-related VOCs focus on those associated with later decomposition stages such as bloat and post-bloat; times when VOC concentrations are at their peak.

Statheropoulos et al. (2005) found variation in compounds emitted by decomposing human bodies thought to have died around the same time. Cadaver volatiles were measured by nmol/L, with DMDS being one of the most prominent compound concentrations detected; 7.27 nmol/L for one cadaver and 19.51 nmol/L for the other.

Although molecular in concentration, variation in compound concentrations can have a significant impact on insect behavior. For example, it has been shown that ants presented a choice of a trail with a higher and lower dose of trail pheromone will choose the path with the higher concentration (Hangartner, 1969). Further, in studying trail laying of the Argentine ant (*Linepithema humile* Mayr) (Hymenoptera: Formicidae), Choe et al., (2012) estimated that the rate of (Z)-9-hexadecenal could not exceed 0.3 pg/cm, while von Thienen et al. (2014) found that, in France, the same species could lay trails that were much stronger at 18.5 pg/cm. Future work should seek to better quantify and standardize volatile concentrations in the environment that are relevant to insect attraction. Finally, future work should explore more doses likely to occur in fresh decomposition scenarios.

Additional research with these compounds is still needed to develop an acceptable bait for RIFA. At present, we do not know enough about what concentrations of VOCs, or what bouquets of VOCs, ants respond in seeking food resources (Youngsteadt et al., 2010). This paper shows that by using varying doses of decomposition related VOC concentrations, RIFA are interpreting these chemical doses differently and are altering their behavior accordingly. Future work should test combinations of these compounds likely to occur in nature, and determine VOC dissipation rate over time by locating an appropriate carrier.

These results are exciting as these compounds that are known to be produced by *P. mirabilis* and other bacteria common in the environment, and have been found associated with RIFA (Chadee and Le Maitre, 1990; Medina, 2011), have not been studied as prospective tools for RIFA management. Furthermore, I show that RIFA responds differently to VOC concentrations in terms of attraction to baits, despite being a generalist pest, and that environment plays a role in attraction to a food resource. These data could lead to the development of novel baits for suppressing RIFA activity within a given area.

CHAPTER III

CONCLUSION

The current study examined dose dependent olfactory response of the red imported fire ant *S. invicta* Buren to VOCs related to decomposition. A better understanding of RIFA foraging strategy can be directly applied to pest management, and impacts behavioral ecology regarding what it means to be labeled a generalist species. Furthermore, the VOCs tested are byproducts of bacteria, which play a pivotal role in attracting or repelling an insect to a potential resource. Previous studies of *L. sericata* determined an interkingdom relationship to *P. mirabilis*-emitted compounds compared to a non-swarming mutant, and further that there is a VOC concentration-dependent effect on fly behavior based on gravidity, sex, and nutritional status.

Ants have highly evolved social systems explained by the high number of olfactory receptors they possess. To our knowledge, this is the first study to link these same concepts to an urban, generalist, and eusocial organism. Our findings suggest that, like other insects that utilize carrion resources for food, ants may be biologically programmed to utilize the same resources based off similar VOC cues emitted by quorum sensing capable bacteria. Here, it is shown that RIFA exposed to low concentrations of VOCs related to decomposition behave differently based on compound, concentration, and environment. In field trials, RIFA in the manicured and more homogenous urban environment were more attracted to the higher dose of indole and were most repelled by the low dose of DMDS. RIFA in the more heterogeneous rural environment were mostly attracted to low amounts of PAA and higher amounts of DMDS. In laboratory trials, variability of RIFA

to baits was high but indole in both the high and low doses elicited the most attractive responses overall. These behavioral changes may be mediated by resources available in those environments.

There is still much to know about how insect behavior is mediated by bacteria, and what other factors, such as environment, are at play. There is much research on perceived pheromones by ants related to trail laying and reproduction, however, we are limited in our knowledge about how VOCs emitted from aspects of the outside environment are sensed and interpreted. Furthermore, volatiles are often not solitary in the environment but form a bouquet, which in many cases is necessary to provoke a certain organismal response. While this historically is known to be true for herbivore specialists, it is lesser known for generalist insects such as those that utilize carrion resources. This report provides preliminary data for future work in this realm. However, it is also important to note that although the compounds tested were used in their true form, baits used in field and laboratory trials containing egg and whey protein will naturally contain the amino acids tryptophan, methionine/cysteine, valine and phenylalanine. At present, it is unknown what kinds of VOCs could have been detected by RIFA from the plain control bait. Related to this, current trends tend to dilute potential behavior-mediating compounds to see if lower doses elicit the same or different organismal responses. Future research should attempt to better standardize this method so that concentrations used reflect GC/MS concentrations of decomposition related to forensic casework, for example.

My research has provided a foundation for these inquiries, and has demonstrated that the mechanisms RIFA employ for food retrieval goes beyond seasonality or

carbohydrate to protein ratios, but extends into complex relationships with the microbial community. Future research should closely examine these relationships between the microbial community, environment, and RIFA olfactory perception before conducting field or laboratory experiments meant to tease apart these factors.

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APPENDIX

Appendix 1. ANOVA results of RIFA recruitment in both urban and rural fields showing effects of trial, treatment, time, replicate, and location on average RIFA recruitment for all trials (1-6). Significance ($P < 0.05$)

Source	Df	SumSq	F Ratio	Prob. >F
Model	1079	1597377.6	4.0215	**<.0001**
Error	3240	1192742.1		
Total	4319	2790119.7		
Trial	5	562343.57	305.5133	**<.0001**
Treatment	8	4609.14	1.5651	0.1299
Time	1	41905.96	113.8346	**<.0001**
Replicate	4	12102.18	8.2187	**<.0001**
Location	1	110.21	0.2994	0.5843
Trial*Treatment	40	44195.08	3.0013	**<.0001**
Trial*Time	5	120227.72	65.3180	**<.0001**
Trial*Treatment*Time	40	21607.87	1.4674	**0.0295**
Trial*Location	5	72315.54	39.2880	**<.0001**
Treatment*Location	8	11580.39	3.9322	**.0001**
Time*Location	1	4924.28	13.3765	**0.0003**
Trial*Rep	20	33001.79	4.4824	**<.0001**
Time*Rep	4	1705.72	1.1584	0.3272
Location*Rep	4	1131.24	0.7682	0.5458
Trial*Treatment*Time*Location*Rep	160	82054.68	1.3931	**0.0010**

Appendix 2. ANOVA results showing urban field site effects of trial, treatment, and time on average RIFA recruitment for all trials (1-6). Significance ($P < 0.05$)

Source	Df	SumSq	F Ratio	Prob. >F
Model	107	115702.42	5.7252	**<.0001**
Error	324	61194.06		
Total	431	176896.47		
Trial	5	84927.057	89.9315	**<.0001**
Treatment	8	2586.832	1.7120	0.0946
Time	1	7490.572	39.6598	**<.0001**
Trial*Treatment	40	9255.769	1.2251	0.9583
Trial*Time	5	4700.346	4.9773	**<.0001**
Treatment*Time	8	900.862	0.5962	0.4245
Trial*Treatment*Time	40	5840.977	0.7731	0.9860
Tukey HSD	Level		Least Sq Mean	
	5	A	40.041667	
	4	B	24.526389	
	6	C	9.269444	
	3	C	9.247222	
	2	D	1.452778	
	1	D	0.278472	

Appendix 3. ANOVA showing urban field site effects of treatment and time on average RIFA recruitment for trials 3 and 6. Significance ($P < 0.05$). N = 10

Source	Df	SumSq	F Ratio	Prob. >F
Model	17	2657.0882	7.7333	**<.0001**
Error	54	1091.4006		
Total	71	3748.4888		
Treatment	8	394.0675	2.4372	**0.0250**
Time	1	2035.8930	100.7313	**<.0001**
Treatment*Time	8	227.1276	1.4047	0.2157
Tukey HSD	Level		Least Sq Mean	
	IND 5.0	A	12.437500	
	DMDS 0.25	A B	11.787500	
	IBA 0.01	A B	10.712500	
	IBA 1.0	A B	10.125000	
	CTRL	A B	9.275000	
	PAA 10.0	A B	7.950000	
	IND 0.05	A B	7.025000	
	PAA 0.1	A B	7.000000	
	DMDS 0.005	B	4.950000	

Appendix 4. ANOVA results showing rural field site effects of trial, treatment, and time on average RIFA recruitment for all trials (1-6). Significance ($P < 0.05$)

Source	Df	SumSq	F Ratio	Prob. >F
Model	107	81522.02	3.1597	**<.0001**
Error	324	78125.11		
Total	431	159647.13		
Trial	5	41233.278	34.2005	**<.0001**
Treatment	8	641.307	0.3325	0.9532
Time	1	1790.267	7.4246	**0.0068**
Trial*Treatment	40	8285.586	0.8590	0.7141
Trial*Time	5	26377.016	21.8781	**<.0001**
Treatment*Time	8	758.471	0.3932	0.9240
Trial*Treatment*Time	40	2436.095	0.2526	1.0000
Tukey HSD	Level		Least Sq Mean	
	5	A	33.350000	
	3	B	17.963889	
	4	B C	12.983333	
	2	B C	12.854861	
	6	C D	7.336111	
	1	D	2.291667	

Appendix 5. ANOVA showing rural field site effects of treatment and time on average RIFA recruitment for trials 2 and 4. Significance ($P < 0.05$). N = 10

Source	Df	SumSq	F Ratio	Prob. >F
Model	17	22811.456	13.7217	**<.0001**
Error	54	5280.680		
Total	71	28092.137		
Treatment	8	18267.698	23.3506	**<.0001**
Time	1	280.206	2.8654	0.0963
Treatment*Time	8	4263.553	5.4499	**<.0001**
Tukey HSD	Level		Least Sq Mean	
	IND 0.05	A	62.050000	
	CTRL	B	16.087500	
	IND 5.0	B	15.950000	
	IBA 1.0	B	13.287500	
	DMDS 0.25	B	12.837500	
	DMDS 0.005	B	11.650000	
	PAA 10.0	B	11.075000	
	IBA 0.01	B	8.562500	
	PAA 0.1	B	7.512500	

Appendix 6. ANOVA showing rural field site effects of treatment and time on average RIFA recruitment for trials 1 and 6. Significance ($P < 0.05$). N = 10

Source	Df	SumSq	F Ratio	Prob. >F
Model	17	610.10818	5.8557	**<.0001**
Error	54	330.95802		
Total	71	941.06620		
Treatment	8	302.42360	6.1680	**<.0001**
Time	1	247.68191	40.4124	**<.0001**
Treatment*Time	8	60.00268	1.2238	0.3032
Tukey HSD	Level		Least Sq Mean	
	DMDS 0.25	A	8.2250	
	PAA 0.1	A B	7.0475	
	IBA 1.0	A B C	6.1250	
	DMDS 0.005	A B C	5.6875	
	IND 5.0	A B C D	4.9250	
	IND 0.05	B C D	3.5125	
	PAA 10.0	B C D	3.1375	
	CTRL	C D	3.0250	
	IBA 0.01	D	1.5000	

Appendix 7. Laboratory RIFA recruitment without treatment concentration blocks. Significance ($P < 0.05$).

Source	Df	SumSq	F Ratio	Prob. >F
Model	95	7296.397	5.3345	**<.0001**
Error	480	6910.837		
Total	575	14207.234		
Trial	1	283.5938		**<.0001**
Treatment	23	2726.1094		**<.0001**
Time	1	137.7878		**0.0021**
Trial*Treatment	23	3323.6562		**<.0001**
Trial*Time	1	16.9767		0.2781
Treatment*Time	23	405.3333		0.2176
Trial*Treatment*Time	23	402.9400		0.2238

Tukey HSD	Level							Least Sq Mean
	IND 0.05	A						8.5000
	PAA 1.0 CTRL –	A	B					6.5000
	IBA 1.0 CTRL +	A	B					6.2500
	DMDS 0.25	A	B	C				5.9166
	DMDS 0.25 CTRL –	A	B	C	D			5.7083
	IBA 0.01 CTRL +	A	B	C	D			5.6250
	IND 5.0 CTRL +	A	B	C	D			5.5833
	DMDS 0.005		B	C	D	E		4.2916
	DMDS 0.005 CTRL -		B	C	D	E		4.2083
	PAA 10.0 CTRL –		B	C	D	E		4.0416
	PAA 1.0 CTRL +		B	C	D	E		4.0000
	IND 0.005 CTRL +		B	C	D	E		3.8333
	IBA 1.0		B	C	D	E		3.2500
	DMDS 0.25 CTRL +		B	C	D	E		3.0000
	IND 5.0 CTRL –		B	C	D	E		2.8750
	IBA 0.01		B	C	D	E		2.7500
	PAA 10.0 CTRL +			C	D	E		1.9166
	IND 5.0				D	E	F	1.7083
	IND 0.05 CTRL –					E	F	1.1666
	IBA 1.0 CTRL –					E	F	1.1250
	PAA 0.1					E	F	1.0000
	IBA 0.01 CTRL –					E	F	0.7083
	PAA 10.0					E	F	0.6666
	DMDS 0.005 CTRL						F	0.2500

Appendix 8a. Laboratory RIFA recruitment to high dose treatments. Significance ($P < 0.05$).

Source	Df	SumSq	F Ratio	Prob. >F
Model	47	3552.4568	4.3907	**<.0001**
Error	240	4131.5397		
Total	287	7683.9965		
Trial	1	14.0833	0.8181	0.3666
Treatment	11	1032.0382	5.4501	**<.0001**
Time	1	44.8811	2.6071	0.1077
Trial*Treatment	11	1960.0417	10.3508	**<.0001**
Trial*Time	1	5.5804	0.3242	0.5696
Treatment*Time	11	192.8590	1.0185	0.4305
Trial*Treatment*Time	11	302.9732	1.6000	0.0993

Tukey HSD	Level		Least Sq Mean
	IBA 1.0 CTRL +	A	6.2500
	DMDS 0.25	A	5.9166
	DMDS 0.25 CTRL –	A B	5.7083
	IND 5.0 CTRL +	A B C	5.5833
	PAA 10.0 CTRL –	A B C D	4.0416
	IBA 1.0	A B C D	3.2500
	DMDS 0.25 CTRL +	A B C D	3.0000
	IND 5.0 CTRL –	A B C D	2.8750
	PAA 10.0 CTRL +	B C D	1.9166
	IND 5.0	C D	1.7083
	IBA 1.0 CTRL -	D	1.1250
	PAA 10.0	D	0.6666

Appendix 8b. Laboratory RIFA recruitment to low dose treatments. Significance ($P < 0.05$).

Source	Df	SumSq	F Ratio	Prob. >F
Model	47	3743.3135	6.8776	**<.0001**
Error	240	2779.2976		
Total	287	6522.6111		
Trial	1	402.5208	34.7588	**<.0001**
Treatment	11	1693.4444	13.2940	**<.0001**
Time	1	98.0324	8.4654	**0.0040**
Trial*Treatment	11	1230.6042	9.6606	**<.0001**
Trial*Time	1	12.0040	1.0366	0.3096
Treatment*Time	11	207.3485	1.6277	0.0916
Trial*Treatment*Time	11	99.3591	0.7800	0.6600
Tukey HSD	Level		Least Sq Mean	
	IND 0.05	A	8.5000	
	PAA 1.0 CTRL –	A B	6.5000	
	IBA 0.01 CTRL +	A B C	5.6250	
	DMDS 0.005	B C D	4.2916	
	DMDS 0.005 CTRL –	B C D E	4.2083	
	PAA 1.0 CTRL +	B C D E	4.0000	
	IND 0.005 CTRL +	B C D E F	3.8333	
	IBA 0.01	C D E F G	2.7500	
	IND 0.05 CTRL –	D E F G	1.1666	
	PAA 0.1	E F G	1.0000	
	IBA 0.01 CTRL –	F G	0.7083	
	DMDS 0.005 CTRL +	G	0.2500	

Appendix 9a. Indole high dose (5.0 µg acetone) choice assay analysis for total RIFA accumulation to baits based on trials 1-3. Significance ($P < 0.05$).

Source	**DF**	**Sum of Squares**	**F Ratio**	**P Value**
Model	2	1517.556	0.4213	0.6742
Error	6	10807.333		
Total	8	12324.889		
Treatment	2	1517.556	0.4213	0.6742

Appendix 9b. Dimethyl disulfide high dose (0.25 µg acetone) choice assay analysis for total RIFA accumulation to baits based on trials 1-3. Significance ($P < 0.05$).

Source	**DF**	**Sum of Squares**	**F Ratio**	**P Value**
Model	2	978.667	0.2560	0.7822
Error	6	11467.333		
Total	8	12446.000		
Treatment	2	978.667	0.2560	0.7822

Appendix 9c. Isobutylamine high dose (1.0 µg acetone) choice assay analysis for total RIFA accumulation to baits based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	2541.5556	0.6263	0.5662
Error	6	12174.667		
Total	8	14716.222		
Treatment	2	2541.5556	0.6263	0.5662

Appendix 9d. Phenylacetic acid high dose (10.0 µg acetone) choice assay analysis for total RIFA accumulation to baits based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	1118.0000	0.5637	0.5966
Error	6	5950.0000		
Total	8	7068.0000		
Treatment	2	1118.0000	0.5637	0.5966

Appendix 9e. Indole low dose (0.05 µg acetone) choice assay analysis for total RIFA accumulation to baits based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	5290.6667	4.1997	0.0723
Error	6	3779.3333		
Total	8	9070.0000		
Treatment	2	5290.6667	4.1997	0.0723

Appendix 9f. Dimethyl disulfide low dose (0.005 µg acetone) choice assay analysis for total RIFA accumulation to baits based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	2090.8889	0.9540	0.4368
Error	6	6575.3333		
Total	8	8666.2222		
Treatment	2	2090.8889	0.9540	0.4368

Appendix 9g. Isobutylamine low dose (0.01 µg acetone) choice assay analysis for total RIFA accumulation to baits based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	2342.8889	2.6718	0.1480
Error	6	2630.6667		
Total	8	4973.5556		
Treatment	2	2342.8889	2.6718	0.1480

Appendix 9h. Phenylacetic acid low dose (0.1 µg acetone) choice assay analysis for total RIFA accumulation to baits based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	3042.000	0.6396	0.5600
Error	6	14268.000		
Total	8	17310.000		
Treatment	2	3042.000	0.6396	0.5600

Appendix 10a. Bait removal from indole high dose (5.0 µg acetone) laboratory choice assay experiment; based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	0.00420206	0.9894	0.4252
Error	6	0.01274089		
Total	8	0.01694296		
Treatment	2	0.00420206	0.9894	0.4252

Appendix 10b. Bait removal from dimethyl disulfide high dose (0.25 µg acetone) laboratory choice assay experiment; based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	0.00009171	0.1896	0.8320
Error	6	0.00145079		
Total	8	0.00154250		
Treatment	2	0.00009171	0.1896	0.8320

Appendix 10c. Bait removal from isobutylamine high dose (1.0 µg acetone) laboratory choice assay experiment; based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	0.00173709	0.9090	0.4520
Error	6	0.00573291		
Total	8	0.00747000		
Treatment	2	0.00173709	0.9090	0.4520

Appendix 10d. Bait removal from phenylacetic acid high dose (10.0 µg acetone) laboratory choice assay experiment; based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	0.00174338	1.0979	0.3924
Error	6	0.00476398		
Total	8	0.00650736		
Treatment	2	0.00174338	1.0979	0.3924

Appendix 10e. Bait removal from indole low dose (0.05 µg acetone) laboratory choice assay experiment; based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	0.00356355	21.9232	**0.0017**
Error	6	0.00048764		
Total	8	0.00405119		
Treatment	2	0.00356355	21.9232	**0.0017**

Appendix 10f. Bait removal from dimethyl disulfide low dose (0.005 µg acetone) laboratory choice assay experiment; based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	0.00207024	0.6280	0.5654
Error	6	0.00989015		
Total	8	0.01196039		
Treatment	2	0.00207024	0.6280	0.5654

Appendix 10g. Bait removal from isobutylamine low dose (0.01 µg acetone) laboratory choice assay experiment; based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	0.00046659	1.0763	0.3986
Error	6	0.00130055		
Total	8	0.00176714		
Treatment	2	0.00046659	1.0763	0.3986

Appendix 10h. Bait removal from phenylacetic acid low dose (0.1 µg acetone) laboratory choice assay experiment; based on trials 1-3. Significance ($P < 0.05$).

Source	DF	Sum of Squares	F Ratio	P Value
Model	2	0.00033163	0.4309	0.6686
Error	6	0.00230880		
Total	8	0.00264043		
Treatment	2	0.00033163	0.4309	0.6686

Appendix 11a. Average RIFA to baits treated with either indole at the high dose (5.0 µg acetone), acetone, or nothing (control) during a laboratory choice assay; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h .

Source	DF	Sum of Squares	F Ratio	P Value
Model	5	116.32011	18.6156	**<.0001***
Error	18	22.49471		
Total	23	138.81481		
Treatment	2	63.231481	25.2985	**<.0001**
Time	1	19.315697	15.4562	**0.0010**
Treatment*Time	2	33.772928	13.5123	**0.0003**

*Significance set at $P < 0.05$

Appendix 11b. Average RIFA to baits treated with either dimethyl disulfide at the high dose (0.25 µg acetone), acetone, or nothing (control) during a laboratory choice assay; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h .

Source	DF	Sum of Squares	F Ratio	P Value
Model	5	47.057540	5.0136	**<.0001***
Error	18	33.789683		
Total	23	80.847222		
Treatment	2	42.361111	11.2830	**0.0007**
Time	1	2.430556	1.2948	0.2701
Treatment*Time	2	2.265873	0.6035	0.5576

*Significance set at $P < 0.05$

Appendix 11c. Average RIFA to baits treated with either isobutylamine at the high dose (1.0 µg acetone), acetone, or nothing (control) during a laboratory choice assay; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h .

Source	DF	Sum of Squares	F Ratio	P Value
Model	5	114.32962	15.4978	**<.0001***
Error	18	26.55771		
Total	23	140.88733		
Treatment	2	81.078750	27.4763	**<.0001**
Time	1	16.071786	10.8930	**0.0040**
Treatment*Time	2	17.179083	5.8217	**0.0112**

*Significance set at $P < 0.05$

Appendix 11d. Average RIFA to baits treated with either phenylacetic acid at the high dose (10.0 µg acetone), acetone, or nothing (control) during a laboratory choice assay; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h .

Source	DF	Sum of Squares	F Ratio	P Value
Model	5	60.419974	14.2015	**<.0001***
Error	18	15.316138		
Total	23	75.736111		
Treatment	2	46.583333	27.3731	**<.0001**
Time	1	6.001984	7.0537	**0.0161**
Treatment*Time	2	7.834656	4.6038	**0.0243**

*Significance set at $P < 0.05$

Appendix 11e. Average RIFA to baits treated with either indole at the low dose (0.05 µg acetone), acetone, or nothing (control) during a laboratory choice assay; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h .

Source	DF	Sum of Squares	F Ratio	P Value
Model	5	268.20971	9.6340	**0.0002***
Error	18	94.65563		
Total	23	362.86534		
Treatment	2	222.41303	19.9725	**<.0001**
Time	1	28.36967	5.0951	**0.0374**
Treatment*Time	2	18.54371	1.6652	0.2186

*Significance set at $P < 0.05$

Appendix 11f. Average RIFA to baits treated with either dimethyl disulfide at the low dose (0.005 µg acetone), acetone, or nothing (control) during a laboratory choice assay; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h .

Source	DF	Sum of Squares	F Ratio	P Value
Model	5	94.70622	29.4614	**<.0001***
Error	18	11.57251		
Total	23	106.27872		
Treatment	2	85.366389	66.3899	**<.0001**
Time	1	0.426944	0.6641	0.4258
Treatment*Time	2	8.912885	6.9316	**0.0059**

*Significance set at $P < 0.05$

Appendix 11g. Average RIFA to baits treated with either isobutylamine at the low dose (0.01 µg acetone), acetone, or nothing (control) during a laboratory choice assay; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h .

Source	DF	Sum of Squares	F Ratio	P Value
Model	5	130.37935	14.4477	**<.0001***
Error	18	32.48723		
Total	23	162.86658		
Treatment	2	97.608991	27.0408	**<.0001**
Time	1	15.601698	8.6443	**0.0088**
Treatment*Time	2	17.168661	4.7563	**0.0220**

*Significance set at $P < 0.05$

Appendix 11h. Average RIFA to baits treated with either phenylacetic acid at the low dose (1.0 µg acetone), acetone, or nothing (control) during a laboratory choice assay; temperature = 25.5°C ± 1.2°C, humidity = 40.0% ± 1%. 8:16 L:D h .

Source	DF	Sum of Squares	F Ratio	P Value
Model	5	79.741648	33.4715	**<.0001***
Error	18	8.576541		
Total	23	88.318189		
Treatment	2	48.664965	51.0678	**<.0001**
Time	1	9.263325	19.4414	**0.0003**
Treatment*Time	2	21.813358	22.8904	**0.0001**

*Significance set at $P < 0.05$