

## ABSTRACT

GUISEWITE, LENA MARIE. Investigating Potential IPM Strategies in Swine Production: Efficacy of *Beauveria bassiana* Against Vinegar Flies (*Drosophila repleta*), and Understanding the Repellent Action of Geraniol Against House Flies (*Musca domestica*) and Stable Flies (*Stomoxys calcitrans*). (Under the direction of Dr. Wes Watson.)

House flies (*Musca domestica* L.), stable flies (*Stomoxys calcitrans* L.), and vinegar flies (*Drosophila repleta* Wollaston) are important economic and nuisance pests of livestock. Deep-bedded swine production systems offer challenges to traditional IPM strategies because the accumulation of manure provides a suitable habitat for fly development. Push-pull IPM strategies use repellents and attractants to manipulate pest populations. A push-pull approach for organic swine production may be applied by using both organically approved repellents and biological control agents. Geraniol, a plant-based monoterpene, exhibits repellency to mosquitoes, but no studies explore its efficacy against filth flies associated with livestock.

Insect pathogens contribute to the IPM strategy as one of many biological control options. *Beauveria bassiana* (Balsamo) Vuillemin has established pathogenicity against house flies and stable flies, however, little is known about the effects of this entomopathogenic fungus on vinegar flies. The main objectives for this project were 1) to understand the pathogenicity of a known entomopathogenic fungus, *B. bassiana*, against vinegar flies, and 2) to establish and characterize the repellency of geraniol against house flies and stable flies.

Adult house flies and vinegar flies were exposed to two strains (P89, and L90) of *B. bassiana* isolated from naturally infected house flies in a dust formulation in the lab ( $1 \times 10^8$  conidia per  $\text{cm}^2$ ) and in the field ( $1.076 \times 10^5$  c per  $\text{cm}^2$ ) on plywood surfaces. Mortality of

house flies was significantly higher ( $59.62\% \pm 8.3\%$ ) than vinegar flies ( $43.59\% \pm 8.4\%$ ), suggesting that vinegar flies are less susceptible to these isolates. However, the mean length of infection was not statistically different between house flies (10.39 days  $\pm$  1.25d) and vinegar flies (11.71 days  $\pm$  0.8d).  $LT_{50}$  values were shorter for house flies exposed to P89 (6.8) and L90 (7.5) than vinegar flies exposed to P89 (12.3) and L90 (10.2) in lab exposures. Lab exposures resulted in 85% mortality of house flies, whereas previous studies report 94.27% mortality across both strains. Infection rates for both species were greater in lab exposures ( $79.5\% \pm 3.6\%$ ) when compared to field exposures ( $23.69\% \pm 5.8\%$ ), likely due to differences in conidial dosage.

We evaluated geraniol at low concentrations (1-4%) for tactile and spatial repellency against house flies and stable flies. A treated filter paper bioassay was used to evaluate tactile repellency as indicated by fly specking frequency within a petri dish. Flies avoided contact with filter papers treated with concentrations as low as 2% geraniol suggesting a tactile repellency response for house flies ( $P$ -value= 0.0023), and 3% geraniol for stable flies ( $P$ -value= 0.0159). Dose dependent knockdown (KD) effects were noted 38 - 138 minutes during exposure in specking bioassays for both species, with 99.2% of house flies knocked down after exposure to 4% geraniol. Stable flies were more susceptible to the KD effects with 76.6% knocked down after exposure to 3% geraniol. Oviposition bioassays show geraniol was effective for stable flies at low concentrations (1-4%), and effective against house flies at only 4% geraniol (96.3% eggs on control,  $P$ -value= 0.0002). Comparative olfactometer tests evaluated the spatial activity of geraniol (1-4%) and swine manure (Positive control) to house flies. Results of these experiments do not support the hypothesis

that 1-4% geraniol is a spatial repellent for house flies (stable flies were not tested).

Our results demonstrate vinegar fly susceptibility to *B. bassiana*, and that mycoinsecticide treatments targeting pests can reduce vinegar fly populations that are present. In addition, tactile repellency of geraniol has the potential for use in livestock IPM strategies, especially those focusing on application to animal bedding or waste for oviposition prevention, or directly to animals for adult fly nuisance control.

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Investigating Potential IPM Strategies in Swine Production: Efficacy of *Beauveria bassiana*  
Against Vinegar Flies (*Drosophila repleta*), and Understanding the Repellent  
Action of Geraniol Against House Flies (*Musca domestica*) and  
Stable Flies (*Stomoxys calcitrans*)

by  
Lena Marie Guisewite

A thesis submitted to the Graduate Faculty of  
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APPROVED BY:

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Dr. Wes Watson  
Committee Chair

---

Dr. Coby Schal

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Dr. Hannah Burrack

## **DEDICATION**

I dedicate my thesis to my husband, Eric. The most patient, loving, supportive, and encouraging man I know. Thank you for always waiting.

## **BIOGRAPHY**

Lena Marie Guisewite grew up in Vista, California. She attended Las Positas Community College then transferred to the University of North Carolina at Pembroke where she completed her Bachelors of Science in Biology with a Concentration in Zoology in 2008. She then interned at the North Carolina Museum of Natural Sciences in the Naturalist Center under the direction of Dr. Colin Brammer. Lena began her Master's Degree at North Carolina State University in 2010 with Dr. Wes Watson.

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Many people have contributed to this adventure, to which I owe thanks. Particular gratitude is extended to my graduate committee: Dr. Wes Watson, Dr. Coby Schal, and Dr. Hannah Burrack. I am grateful to Dr. Watson for creating a lab where collaboration was not just encouraged, but expected. Thank you for your guidance and for stressing the importance of learning that there is always more to learn.

Steve Denning, our lab technician, deserves special praise not just for continually reminding me that anything can be built out of PVC, but he is truly an engineering magician. Without Steve many of my projects would have fallen apart, literally (and figuratively).

I sincerely thank Jonathan Cammack for his involvement in almost all of my experiments. From helping me collect flies in the field, to our tangential discussions on theoretical statistics I appreciate having a fellow lab mate to help me navigate the ropes.

Special thanks go to our summer student workers, Mindy Drewette, Colin Wong, and Chris Ackerman for their hard work in the field and lab collecting, counting, and identifying flies. So many hours of their work contributed to this body of research and it is much appreciated.

My family has been especially supportive throughout my schooling and I am glad for their continued encouragement. Thank you all for politely listening to my research ramblings.

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## CHAPTER 1

Guisewite: Introduction

L. M. Guisewite  
North Carolina State University  
Dept. of Entomology  
Campus Box 7626  
1106 Grinnells Laboratory  
Raleigh, NC 27695-7626  
Phone: (919) 515-1663  
Email: [lguisew@ncsu.edu](mailto:lguisew@ncsu.edu)

### **Introduction: IPM Strategies for Deep Bedded Swine Production**

L. M. Guisewite

North Carolina State University, Department of Entomology, 1106 Grinnells Laboratory,  
Raleigh, NC 27695-7626

**Abstract.** Integrated pest management approaches in livestock production use cultural, biological, mechanical, and chemical methods to manage target pests when applicable. Deep-bedded swine production systems offer challenges to traditional IPM strategies as the time between bedding replacement provides habitat for fly development. The push-pull strategy is a proposed option to managing flies in conventional as well as organic swine production systems. Push-pull uses repellents and attractants to manipulate the pest population. A push-pull strategy for organic livestock production may be applied by using a combination of organically approved repellents and biological control agents. Geraniol exhibits repellency to mosquitoes, but no studies explore its efficacy against filth flies associated with livestock. Our research indicates that organically approved geraniol may be an effective part of a push-pull strategy for fly control.

Insect pathogens contribute to the IPM strategy as one of many biological control options. *Beauveria bassiana* (Balsamo) Vuillemin has established pathogenicity against house flies, *Musca domestica* L., and stable flies, *Stomoxys calcitrans* L., and is able to be grown on artificial media for mass production. However, little is known about the effects of this entomopathogenic fungus on vinegar flies, *Drosophila repleta* Wollaston, one of the common flies in swine houses. The main objectives for this project were 1) to understand the pathogenicity of a known entomopathogenic fungus, *B. bassiana*, against the vinegar fly, and 2) to establish the repellency of geraniol against house flies and stable flies and characterize the repellency as tactile or spatial.

## Swine Production

North Carolina produces 13.9% of the nation's hogs, and is second only to Iowa in hog production (NCDA 2011). Averaging 9,500,000 head since 2007, cash receipts to hog producers in North Carolina exceeded \$2.2 billion in 2010, which is over 23% of the state's agricultural income (NCDA 2011). There has been a national increase in the number of hogs produced, yet a decrease in the number of farms raising pigs (USDA 2012). Swine in NC are distributed on 2,400 operations, each averaging about 4,130 animals per farm (NCDA 2007). The swine industry in NC and other southern states include both independent and contract growers. Within a vertically-integrated production system, individual farmers, under contract with companies (integrators), raise significant portions of market hogs. Of these, 61% house their hogs on less than 100 acres (NCDA 2011). Although NC ranks 2<sup>nd</sup> in the nation for swine production, organic swine farms are few, with only 16 certified organic operations as of 2008 (USDA ERS 2012).

These densely-packed, large-scale production swine systems have led to public health and environmental concerns. Lagoons provide a relatively inexpensive and simple means of treating swine by-products to reduce the nutrient content and biological activity prior to application to land (Andreadakis 1992). Lagoon-based swine production systems raise pigs indoors on concrete floors usually in pens sufficient to hold 6 to 8 animals. Accumulated wastes fall through the slotted concrete floor to a pit area below, and water flushes waste from the system into a nearby lagoon. Anaerobic bacteria in the lagoon break down the waste into liquid form that can be utilized as crop fertilizer (Hatfield et al. 1998). Recently, animal welfare advocates have urged the industry to increase space for pigs raised indoors and

eliminate the use of single animal crates (Bracke et al. 2002). In addition, lagoons are of environmental concern because of ammonia emissions (Aneja et al. 2000), odors (Schiffman et al. 2001), pathogens (Sobsey et al. 2001, Vanotti et al. 2005), and decreased water quality (Mallin 2000). In an attempt to decrease environmental impact, the North Carolina general assembly passed the Clean Water Responsibility and Environmentally Sound Policy Act of 1997, preventing any new construction of anaerobic lagoons. In 2007, the NC Senate made the ban on new lagoon operations permanent, increasing state and federal interest in waste removal alternatives.

Organic swine production has increased in the US with more than 10,000 meeting the standard as certified organic by 2008 (USDA ERS 2012). Organic production costs are driven by consumer demand for greater animal welfare and minimal environmental impact, typically by eliminating pesticides and antibiotics (Sundrum 2001). In order to fulfill consumer demands, pigs may need unique housing conditions. Current organic standards don't allow tail-docking or tooth-clipping, and require a minimum housing of 7.5 m<sup>2</sup> indoors and 5.0 m<sup>2</sup> outdoors on dry litter bedding for farrowing. Additionally, pigs must have access to pasture or be allowed to free-range a portion of everyday (Sundrum 2001).

Traditionally, when kept outdoors, pigs were penned in a pigsty or sturdy enclosure to prevent escape. Sanitation was a concern and the pigsty or pig pen was sometimes bedded with wood shavings or straw to keep the animals clean, reduce erosion, and provide firm footing (Thorton 1988). With sufficient land, the location of the pigsty may change year to year to help reduce nutrient loading. Pasture raised pig operations are few, limited by rising production output, consumer demand, and decreases in farm acreage (Hatfield et al. 1998).

The Center for Environmental Farming Systems in Goldsboro, NC constructed a deep-bedded dry pack management swine system that alleviates the environmental costs of traditional lagoon-style waste removal. These barns were populated with an antibiotic free swine herd with the goal of transitioning into organic swine production. Growing pigs on deep-bedded open floors allows pigs to move freely in a roofed building with open sides (Fig. 1). Providing bedding for pigs is considered a benefit for animal welfare (Bracke et al. 2002). As the pigs become accustomed to the housing, separate resting and dunging areas become established. Dunging areas are breeding sites for filth flies, specifically house flies, *Musca domestica* L., stable flies, *Stomoxys calcitrans* L., and vinegar flies, *Drosophila repleta* Wollaston. In addition to being a nuisance, these flies pose a biosecurity risk as they spread pathogens on or between farms (Ahmad et al. 2011). Inadequate waste management may compromise the health and well-being of the animals.

### **Pest Species Complex**

In a 2000 swine pest survey, NC swine producers scored the following pests as most common: rodents (67%), spiders (60%), flies (54%), and cockroaches (33%) (Stringham, unpublished data). Unfortunately, many alternative management strategies do not address insect pest issues adequately. Producers that have constructed swine barns that rely on a dry waste management system have observed increases in fly populations, due to accumulated manure and bedding.

Initial field monitoring (2010-2011) of insect species collected within a deep-bedded swine unit (Goldsboro, NC) yielded a diverse community. The main Dipteran species collected included house flies (*Musca domestica* L.), vinegar flies (*Drosophila repleta*

Wollaston), stable flies (*Stomoxys calcitrans* L.) and blow flies (Calliphoridae). Here, we focus on the biology of the most abundant pests in this specific swine system, and their impacts on livestock and people: house flies, stable flies and vinegar flies.

**House Fly Biology.** House flies are considered a synanthropic species, because they are well adapted to both domicile and farm, becoming one of the major pests of livestock. Adults are non-biting, and frequently land on humans and animals, animal feed, and other structural surfaces, contributing to their nuisance among rural residents (Miller 1993). Adults can live 2-3 weeks in the wild (West 1951), and up to 50 days in laboratory conditions (Rockstein 1957). Females are capable of laying between 120 and 150 eggs at one time, and will produce about six to eight egg masses in a lifetime (Skidmore 1985). Females deposit all of the eggs held within their ovaries in a single laying session, and store enough sperm from a single mating to fertilize all the eggs they produce in their lifetime (Moon 2009).

House flies feed and oviposit on decaying organic matter. Larvae develop in any decaying organic matter including manure, mixtures of straw and manure, spoiled feed and human food waste (Moon 2009). The entire house fly developmental cycle depends on temperature, where the fastest growth occurs at 27-32°C (Moon 2009). The minimum time from egg to adult is 7 days, but ranges between 10 and 21 days with three larval instars (Skidmore 1985).

It is well established that house flies can mechanically transmit pathogens that cause disease in humans including *Escherichia coli* (Foerster et al. 2007), *Helicobacter pylori*, (Grubel et al. 1997), and *Salmonella* (Mian et al. 2002). Similarly, house flies have been identified as potential mechanical vectors in the transfer of disease strictly between livestock

animals including Porcine Reproductive and Respiratory Syndrome Virus (PRRSv) which was identified from house fly homogenates (Otake et al. 2003). Perhaps most concerning is that house flies contribute to the dissemination of antibiotic-resistant bacteria (Ahmad et al. 2011). Recent studies have even shown conjugation transfer of the antibiotic resistant plasmid between bacteria within the house fly crop (Akhtar et al. 2009).

**Stable Fly Biology.** Stable flies are hematophagous, and almost strictly a livestock pest. Adults live up to 29 days in the wild, and both male and female stable flies feed on blood. Females utilize blood proteins to produce viable eggs, and can take the first blood meal as soon as 6 hours after eclosion (Skidmore 1985). Mating may occur as early as 24 hours after emergence, however, most oviposition begins after the 10<sup>th</sup> day. Eggs develop asynchronously, so feeding and oviposition are more distributed in time compared to house flies (Moon 2009). Stable flies produce only about 20 eggs in a typical batch (Skidmore 1985).

Like the house fly, female stable flies oviposit in decaying organic matter. Larvae have been found in decaying grass clippings, mixtures of straw, urine and manure, and aged spoiled hay (Schmidtman 1988, Broce et al. 2005). Larvae are saprophagous, but develop at a slower rate than house flies. Optimal developmental temperature is also 27-30°C for stable flies. Minimum time from egg to adult is 12 days, ranging from 15-30 days (Moon 2009).

Stable flies cause significant economic damage to the U.S. cattle industry with estimates of \$2.2 million per year of total damage (Taylor et al. 2012). Negative impacts of this pest are most evident in production losses including decreased weight gains (Campbell et al. 1987, Campbell et al. 2001), and decreased milk production by as much as 40% in dairy

cows (Bruce and Decker 1958). Stable flies have no economic impacts on swine, but deliver a painful bite and may contribute to overall animal welfare (Moon et al. 1987). Despite both male and female blood feeding, stable flies transmit few pathogens, and are considered chiefly an economic nuisance pest (Moon 2009).

**Vinegar Fly Biology.** There is very little information on the biology of *Drosophila repleta*. The life cycle of the vinegar fly consists of the egg, three instars, pupa, and adult stages. The developmental time at 25°C is approximately 15 days from egg to adult (Harrington 1993), and adults can live up to two months under lab conditions (Sohal 1970). Feeding behaviors of vinegar flies indicate a preference for decaying organic matter including fruit (Pipkin 1965, Parsons 1981) and fungi (Shorrocks and Wood 1973). Vinegar flies are also attracted to beer and wine (Malloch and McAtee 1924, Hottel et al. 2011), and are often pests in food processing plants (Zuska and Lastovka 1969). They have been noted in abundance in areas where manure is present (Sturtevant 1919). Due to their frequency in confined animal production facilities, the vinegar fly is considered a nuisance pest with the potential to mechanically transmit pathogens (Harrington and Axtell 1994).

### **Pest Management**

Insecticides have been the control method of choice in conventional livestock systems, but widespread resistance, limited availability of novel active ingredients, and regulatory cost makes this single strategy unrealistic. Beef, dairy and swine insecticides that are recommended for use against filth flies are frequently used as high rate premises treatments to target the pest habitat, and to avoid potential toxic reactions of animals.

Recommended premises treatments for North Carolina include bifenthrin, chlorpyrifos, cyfluthrin, dichlorovos, and fenvalerate, among others (Watson 2012).

House flies have developed resistance to most insecticides used to control them. House fly resistance to DDT was first reported in 1948, only 3 years after its introduction in the agricultural business (Lindquist and Wilson 1948). Many newer insecticides with different modes of action have become ineffective against house flies because of resistance. Permethrin (Scott and Georghiou 1985), methomyl (Price and Chapman 1987), imidacloprid (Wen and Scott 1997), cyromazine (Scott et al. 2000), and spinosad (Shono and Scott 2003) are all ineffective against resistant house flies. House flies can also develop cross-resistance to novel insecticides based on previous insecticide exposure (Liu and Yue 2000).

Stable flies are generally more susceptible to insecticides than house flies. Varying wild fly susceptibility to DDT was noted in 1956, and after only two generations of selection, stable fly  $LT_{50}$  values quadrupled (Somme 1958). However, accounts of stable fly resistance to recently developed insecticides are lacking. Despite the presumed susceptibility to insecticides, stable flies are still present at livestock systems that have been treated, and continue to cause significant economic damage (Taylor et al. 2012). It is likely that control failure is attributable to the necessity of prolonged chemical contact with the fly (Greene 1993). Premise-wide treatments in effort to contact and kill all pests contribute to widespread resistance (Georghiou and Taylor 1986). Additionally, it's impossible to ensure that chemical treatment will contact enough susceptible flies to force population levels below an economic threshold without inducing further resistance pressure. For these reasons there has been

growing interest within the pest control industry and livestock managers for sustainable alternatives to insecticides.

Integrated Pest management (IPM) was first defined as combining biological and chemical control, while monitoring economic thresholds to avoid reaching the economic injury level (the lowest population density that will cause economic damage) (Stern et al. 1959). Now IPM encompasses cultural and mechanical control strategies, and includes decision making based on ecological, economic, and social considerations to assess pest problems more comprehensively than chemical control strategies alone (Kogan 1998).

The push-pull management strategy further expands IPM to include semiochemicals, pheromones, repellents, and oviposition deterrents in combination to manipulate the target pest. Push-pull approaches may use a repellent (push) in combination with an attractive stimulus (pull) to manipulate and accumulate pest populations for elimination (Cook et al. 2007). Most work in push-pull strategies relies on manipulating insect behavior with visual or chemical cues. For example, trap crops are utilized to divert stem borer populations from maize and sorghum (pull) in combination with intercrops that repel the stem borers (push) (Kahn and Pickett 2004). Additionally, population abundance and distribution of the pea leaf weevil, *Sitona lineatus* L., for example, was altered with the combined use of a commercially available antifeedant, neem, and a synthetic aggregation pheromone, 4-methyl-3,5-heptanedione (Smart et al. 1994). Despite potential uses for push-pull in livestock systems, this strategy remains largely unexplored. One example resulted in increased tsetse fly trapping success when cattle were treated topically with a synthetic insect repellent (Hassanali et al. 2008).

Insecticides can be utilized in push-pull strategies, but when pests are concentrated to a smaller area, applications can be reduced or even eliminated (Cook et al. 2007). Push-pull implementations typically choose environmentally favorable methods including biological control agents, insect growth regulators, or botanical insecticides to control the target pest (Cook et al. 2007). The need for alternatives to traditional insecticides is magnified with current increases in organic dairy, beef, and hog farms due to rising consumer demands for organic products. Push-pull in organic systems may be approached by using organically approved repellents and biological control agents. Implementation of these strategies for organic production is not clear, particularly which biological control agents and repellents will be effective against filth flies.

**Biological Control.** Biological control agents for house and stable fly management have commonly included hymenopteran parasitoids from the genera *Muscidifurax*, *Spalangia*, and *Nasonia* (Morgan et al. 1975, Geden et al. 1992, Kaufman et al. 2001). Parasitoids are typically only a component of larger IPM programs because they are difficult to manipulate, and little is known about their population dynamics or what impacts management practices have on them (Petersen et al. 1990). House flies, for example, were effectively controlled in caged layer poultry installations when augmentative releases of *Spalangia endius* Walker were combined with cryomazine treatments in poultry feed applications (Morgan and Patterson 1990).

In addition, research efforts have included entomopathogenic fungi in filth fly IPM studies. *Entomophthora muscae* (Cohn) Fresenius, for example, causes mycosis in house flies and face flies (*Musca autumnalis* De Geer), but not in stable flies (Kramer and Steinkraus

1981). Epizootics of *E. muscae* have been successfully induced in the field by the release of infected adult house flies (Geden et al. 1993). *Metarhizium anisopliae* is also effective against house flies causing mortality within 5 days after exposure to a virulent strain (MaF52) isolated from Austria (Anderson et al. 2011).

The entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, has a wide host range with established pathogenicity against house flies and stable flies (Steinkraus et al. 1990, Watson et al. 1995). Unlike *E. muscae*, *B. bassiana* can be grown on artificial media, mass produced, and formulated for large scale applications (Kaufmann et al. 2005). The use of pathogens as microbial insecticides is an important stride in the development of pathogens as biological control agents (Van den Bosch et al. 1982). As a result, *B. bassiana* has been a successful biological control agent in livestock and poultry systems against house flies and the lesser mealworm, *Alphatobius diaperinus* (Panzer) (Watson et al. 1996, Geden and Steinkraus 2003, Kaufman et al. 2005). However, nothing is known of its activity against vinegar flies.

**Repellents.** Chemicals that are repellent cause insects to make oriented movements away from a source. Similarly, deterrents inhibit feeding or oviposition on otherwise attractive substrates (Dethier et al. 1960). Deet, *N, N*-Diethyl-3-methylbenzamide, was discovered in 1953 and remains the most important and effective repellent in use today (Roberts and Reigart 2004). Deet is also effective in repelling filth flies and biting flies from livestock, but it causes serious adverse effects in cattle and horses with symptoms including excessive salivation, sweating, and nasal discharge (Blume et al. 1971). Natural product repellents are often considered less harmful, and interest in plant-based insect repellents as an

alternative method of fly control has increased especially with added consumer demand of organic food products. Currently, the market for natural plant-based insect repellents is aimed at topically applied personal protection for humans against mosquitoes and other pests.

Common ingredients of natural insect repellents commercially available include geraniol (Repel® Natural, Bite Shield™, MosquitoSafe™), citronella (Natrapel®), lemon eucalyptus oil (Off! Botanicals®), Rosemary, lemongrass, or a combination of several ingredients (GonE!®, NeemAura®, SunSwat®, Bygone®). Many of these are essential oils, or monoterpenes, a constituent of essential oils (geraniol, citronellal). Geraniol is effective against mosquitoes in topical, candle, and diffuser formulations (Barnard and Xue 2004, Mueller et al. 2009). Citronella also exhibits repellency to mosquitoes, but it is less effective than geraniol (Mueller et al. 2008, Mueller et al. 2009).

Perimeter treatments using repellents to keep insects away from a resource have been largely unexplored. However, vetiver oil and its components, nootkatone and cedrene, were able to serve as a barrier and reduce subterranean termites that entered tunnels, and subsequently decreased wood feeding damage (Maistrello et al. 2001). Determination of topical or premises treatment of repellents relies on understanding of each species' response to specific compounds, and the physical properties of the repellent.

Volatile compounds are more likely to exhibit spatial activity against insects than compounds with low vapor pressure. The concept of spatial repellency is specifically defined as the vapor phase activity of a chemical that prevents an insect from reaching a target that is otherwise attractive (Bernier et al. 2007). Any spatial activity is directly dependent on the chemical's volatility and the sensitivity of the insect to the specific compound (Bernier et al.

2007). Spatially effective insect repellents provide more efficient control than traditional pesticides which require insect contact with the product (Greene 1993). Identification of a spatially active plant-based repellent can mitigate the effects of pervasive pesticide treatments which often lead to insect resistance (Georghiou and Taylor, 1986).

Repellents can exhibit a combination of spatial and tactile repellency, and it is not always necessary for vapor phase activity to elicit avoidance (Bernier et al. 2007). For example, sensitive chemoreception to a compound with low volatility may require an insect to come near or even contact the repellent to receive a dose above the repellency threshold concentration. Alternatively, plant based compounds with high volatility may act as fumigants and cause knockdown (KD) or mortality to insects in contact with the material (Isman 2000). Tactile repellency is advantageous as a secondary defense, with potential to induce KD or insecticidal effects. Understanding the function of the repellent, either tactile or spatial, against target species is important when establishing efficient and effective application methods.

### **Objectives**

The feasibility of using a biological control agent and a repellent to manage swine barn fly pests depends upon two factors 1) virulence of the pathogen for the target pest and 2) activity spectrum of the repellent. The objectives of this study were two-fold, 1) to understand the efficacy of a known mycoinsecticide, *B. bassiana*, against the vinegar fly and 2) to determine if geraniol, a monoterpene with known insect repellent properties, exhibits tactile or spatial repellency to house flies and stable flies. Experiments described in this thesis help address a broader purpose of understanding the feasibility of using push-pull

strategies for fly management in a dry swine waste management system.

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**Figure 1** Research swine unit at the Center for Environmental Farming Systems (CEFS) in Goldsboro, North Carolina. Five open-ended hoop barns (334.5 m<sup>2</sup> each) house sows (pictured), piglets, finishers, or boars which are allowed free range within the structure. The floor of the barns are constructed of solid concrete, and covered with loose wheat straw bedding to a depth of 50 to 60 cm. Manure is allowed to accumulate in the bedding, which is replaced weekly.

## CHAPTER 2

Guisewite: Susceptibility of *Drosophila*  
*repleta* to *Beauveria bassiana*

L. M. Guisewite  
North Carolina State University  
Dept. of Entomology  
Campus Box 7626  
1106 Grinnells Laboratory  
Raleigh, NC 27695-7626  
Phone: (919) 515-1663  
Email: [lguisew@ncsu.edu](mailto:lguisew@ncsu.edu)

### **Susceptibility of *Drosophila repleta* (Diptera: Drosophilidae) to Two Strains of *Beauveria bassiana* Isolated from House Flies (Diptera: Muscidae)**

L. M. Guisewite

North Carolina State University, Department of Entomology, 1106 Grinnells Laboratory,  
Raleigh, NC 27695-7626

**Abstract.** House flies (*Musca domestica* L.) and vinegar flies (*Drosophila repleta* Wollaston) are important economic and nuisance pests of livestock. Integrated pest management (IPM) strategies to control house flies are well researched and often include entomopathogens such as *Beauveria bassiana* (Balsamo) Vuillemin. The biological control potential of *B. bassiana* is well established against adult house flies, but the pathogenicity of this fungus to vinegar flies is unexplored. In this study we infected adult vinegar flies with two strains (P89, and L90) of *B. bassiana* isolated from naturally infected house flies. Adult house flies and vinegar flies were exposed to dust formulations of *B. bassiana* in the lab ( $1 \times 10^8$  conidia per  $\text{cm}^2$ ) and in the field ( $1.076 \times 10^5$  conidia per  $\text{cm}^2$ ) on plywood surfaces. Mortality of house flies was significantly higher ( $59.62\% \pm 8.3\%$ ) than vinegar flies ( $43.59\% \pm 8.4\%$ ), suggesting that vinegar flies are less susceptible to these isolates. However, the mean length of infection was not statistically different between house flies (10.39 days  $\pm$  1.25d) and vinegar flies (11.71 days  $\pm$  0.8d), yet the modes of infection duration were shorter for house flies (6 days) than vinegar flies (12 days). Lab exposures resulted in 85% mortality of house flies, whereas previous studies report 94% mortality across both strains. Infection rates for both species were greater in lab exposures ( $79.5\% \pm 3.6\%$ ) when compared to field exposures ( $23.69\% \pm 5.8\%$ ), likely due to differences in conidial dosage. Our results demonstrate vinegar fly susceptibility to P89 and L90 isolates, and that mycoinsecticide treatments targeting other pests (house flies) can also reduce vinegar fly populations.

**Keywords.** *Beauveria bassiana*, *Drosophila repleta*, biological control, entomopathogenic fungus, house fly

## Introduction

The vinegar fly, *Drosophila repleta* (Wollaston), was first described in 1858 (Wollaston M.A.F.L.S. 1858), and is characterized as a cosmopolitan species of Nearctic-Neotropical origin (Ashburner et al. 1981, Vilela 1983). The vinegar fly is more robust (2-3mm length) and typically darker than the common fruit fly, *Drosophila melanogaster* Meigen. A synanthropic species frequently found in confined animal production facilities, the vinegar fly is considered a nuisance pest (Harrington and Axtell 1994). Vinegar flies are often found in association with two primary insect pests of livestock and poultry, house flies (*Musca domestica* L.) and stable flies (*Stomoxys calcitrans* L.). Besides their annoyance to animals and people, house flies and stable flies are efficient mechanical vectors of disease (Graczyk et al. 2001, Foerster et al. 2007). Interestingly, information on vinegar flies is limited to nuisance concerns, and the potential for mechanical transmission of disease by this species is unknown.

Integrated pest management (IPM) strategies often include the use of biological control agents either in conjunction with, or in place of chemical insecticides. Biological control agents for house and stable fly management have commonly included hymenopteran parasitoids from the genera *Muscidifurax*, *Spalangia*, and *Nasonia* (Morgan et al. 1975, Geden et al. 1992, Kaufman et al. 2001). In addition, research efforts have included entomopathogenic fungi in filth fly IPM studies. *Entomophthora muscae* (Cohn) Fresenius, for example, causes mycosis in house flies and face flies (*Musca autumnalis* De Geer), but not in stable flies (Kramer and Steinkraus 1981). Epizootics of *E. muscae* have been successfully induced in the field by the release of infected adult house flies (Geden et al.

1993).

The entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, has a wide host range with established pathogenicity against house flies and stable flies (Steinkraus et al. 1990, Watson et al. 1995). Unlike *E. muscae*, *B. bassiana* can be grown on artificial media, mass produced, and formulated for large scale applications (Kaufman et al. 2005). As a result, *B. bassiana* has been a successful biological control agent in livestock and poultry systems against house flies and the lesser mealworm, *Alphatobius diaperinus* (Panzer) (Watson et al. 1996, Geden and Steinkraus 2003, Kaufman et al. 2005). Considered a generalist pathogen, when used as a biopesticide, *B. bassiana* can be expected to have an effect on non-target insects like the vinegar fly.

Two strains of *B. bassiana* originally isolated from wild house flies on a New York dairy (P89, L90) show virulence in laboratory tests (Steinkraus et al. 1990). The P89 strain induced mortality in 98.96% of house flies, while the L90 strain caused 89.58% mortality within 7 days (Watson et al. 1995). Strains of *B. bassiana* are typically most virulent to the original host species (Feng et al. 1994), but there have been noted exceptions (Feng and Johnson 1990). *B. bassiana* has induced 85% mortality of exposed *D. melanogaster* with variation in susceptibility observed across 6 genotypes (Tinsley et al. 2006). Still, no studies have examined the response of vinegar flies after exposure to *B. bassiana*. The objective of this study is to evaluate the susceptibility of vinegar flies to *B. bassiana* by examining vinegar fly mortality, mycosis and infection duration (time from initial exposure to death) in both a field and lab setting.

## Materials and Methods

**Fly Colonies & Rearing Procedures.** A vinegar fly colony was isolated from the Center for Environmental Farming Systems in Goldsboro, NC (CEFS) during May, 2010 (Fig. 3). Flies were positively identified as *Drosophila repleta* using the key in “The Drosophilidae of the Southwest” (Patterson 1943). Voucher specimens of both sexes for wild and lab reared flies were deposited at the North Carolina State University Insect Museum. Adults were maintained with water and a mixture of granulated sugar and milk powder. The oviposition substrate and larval rearing media consisted of a 1:10 ratio (by weight) of brewer’s yeast and plain instant mashed potato flakes, respectively. An equal volume of water supplemented with 1.056g/L of methyl paraben was added to the dry mixture as well as 15-20 granules of baker’s active dry yeast to yield the complete media mixture.

House flies were obtained from a colony originating in 1982 from a dairy farm near Cornell University, Ithaca, NY. Adults are maintained with water and a mixture of granulated sugar and milk powder. Larvae are reared on 250ml Milk Plus pellets (Cargill, Minneapolis, MN) 2L of wheat bran, and 1.5L of water.

**Fungal Preparation.** Two strains of *B. bassiana* (P89, L90) isolated from wild house flies (Steinkraus et al. 1990) were prepared for culturing. Dry conidia were added to 150ml of cooled sterile Bacto Saboraud Maltose Broth supplemented with 0.15g yeast extract (Anonymous 1984) and allowed to grow on a 100 rpm shaker at approximately 25°C for 5-9d. The liquid culture was poured over a sterile mixture of 240ml Moss’ Enriched Plain Fine Corn Meal (Buffaloe Milling Company, Inc, Kittrell, NC) and 700 ml of water on a half size (45.08 x 32.7cm) 19 gauge aluminum alloy sheet pan covered with aluminum foil. In a sterile

biological cabinet, the liquid culture was spread over the thin (approx. 0.6cm) cornmeal/water mixture with sterile plastic spreaders, covered with aluminum foil, and stored at 25° (Fig. 4). After 14d of growth, the sheet pans were then moved to a 25°C incubator for 7-9d with the aluminum foil covers removed to promote drying. The dried cornmeal and fungus mixture was processed to a fine power in a blender and preserved at -80°C until used (Fig. 5a and 5b).

**Lab Exposures.** *Beauveria bassiana* conidia were counted using a hemocytometer to calibrate a dose of  $1 \times 10^8$  per  $\text{cm}^2$  of treatment surface area. Conidia treatments were applied to a circular area (95mm diameter) immediately prior to fly exposure.

Plywood sheets ( $30.48\text{cm}^2$ ) were weathered outdoors for 2-11 weeks prior to bioassays to simulate natural barn conditions (Watson et al. 1995). Bioassays were conducted by placing 10 CO<sub>2</sub> anesthetized, mixed-sex, 5-9d old flies on the treated surface section) which were covered with an inverted petri dish bottom kept in place with transparent tape or cotton twine (Fig. 1). After a 3h exposure, flies were placed in clear plastic containers with a cotton sleeve and provisioned with water and sugar/dry milk mix. Each replicate consisted of two groups of vinegar flies (n= 10 ea., 20 total), with one group of house flies (n=10) exposed simultaneously as a positive control with a total of 4 replicates.

Dead flies were removed daily and the fly cadavers were placed on moistened filter paper inside of a petri dish to promote sporulation, indicative of patent infection, 2 to 3 days later (Steinkraus et al. 1990) (Figs. 2a and 2b). Water added to the filter paper was supplemented with 0.25g/L streptomycin to inhibit unwanted bacterial growth. Flies were examined under a dissecting microscope, and considered positive for *B. bassiana* infection

when white conidia emerged from the cadaver. A mounted slide prepared from an infected colony house fly exhibiting the distinct rachis pattern served as a positive specimen for all other fly cadavers with white conidial growth (Humber 1997).

**Field Exposures.** *Beauveria bassiana* was applied in 2 different swine barns once a week for 4 weeks during August 2011 at CEFS. The swine unit at CEFS consists of 5 open-ended hoop barns with solid concrete floors and cornstalk bedding. The center barn housing sows received the P89 strain, and the adjacent barn with finishing pigs received the L90 strain. Conidia were counted using a hemocytometer to calibrate a dose of  $1.076 \times 10^5$  conidia per  $\text{cm}^2$  (or  $1 \times 10^8$  c/ft.<sup>2</sup>). Dry material was volumetrically adjusted with bleached wheat flour so that each release had even coverage. This lower dose was necessary to cover the large area of the swine barns (3600 ft.<sup>2</sup>) with our limited supply of cultured material.

Dry material was spread in each barn using a backpack sprayer with a separate and simultaneous stream of water to promote adhesion on vertical surfaces. One plywood board was hung vertically at eye level in each barn before application and expected to have received an equal dose of conidia per  $\text{cm}^2$  as calculated for the entire barn. The boards were moved to an indoor horizontal surface after application for fly exposures with no more than 3 hours between the time of application and exposure. Each weekly replicate consisted of two groups of vinegar flies (n=10 ea.) and two groups of house flies (n=10 ea.) exposed simultaneously as a positive control with a total of 4 replicates.

To monitor wild fly infections, flies were collected 48h after each weekly field application using a sweep net. To avoid cross contamination, the sweep net was sprayed with 0.05% NaClO, then 70% ETOH and allowed to air dry between each barn's collections. Wild

flies were grouped per collection barn, and placed inside plastic containers with a cotton sleeve and provisioned with water and food for 7 days to allow *B. bassiana* pathogenesis to develop. After 7 days, wild collected flies were placed in a -21°C freezer. Fly cadavers were placed on moistened filter paper and monitored for patent infection as previously described.

**Calculations & Statistical Analyses.** All groups (n=10 ea.) of vinegar flies or house flies exposed to the same strain of *B. bassiana* within a replicate were considered individual subsamples. The proportion of flies infected was calculated by dividing the number of cadavers with patent infection by the total number of flies that were exposed within each subsample. The proportion that died due to infection were arcsine square root transformed to meet normality and equal variance assumptions. The number of days (duration) that infection lasted was included as a second response variable. The duration of infection was weighted by the number of flies that died that day and averaged per subsample. The modes of infection duration were calculated with replicates pooled, and frequencies generated from individual fly infection durations. Maximum likelihood analysis (PROC MIXED) was conducted with fly species, fungal strain, and application method (field vs. lab) as the main factors (SAS v. 9.1.3). Interactions between any of the 3 factors were not significant, and therefore were removed from the final statistical model.

LT<sub>50</sub> values were calculated only for lab exposures. Formulas were generated by simple linear regression (R v. 2.12.1) that compared probability units (probits) of the proportion of infected flies to the log (number of days after exposure).

## Results

Fly species, fungal strain, and application method all had a significant impact on mortality due to infection (henceforth referred to as “infection rate”) (Table 1). House fly infection rates were significantly higher than vinegar fly infection rates in both field and lab studies. In lab applications ( $1 \times 10^8$  per  $\text{cm}^2$ ) house fly infection rates averaged 86%, and vinegar flies averaged 74% infection (Table 1). Fewer flies died of mycosis in the field study for both house flies (34.24%) and vinegar flies (13.16%). There was no significant difference in mean infection duration between fly species (Table 1). However, vinegar fly  $\text{LT}_{50}$  values were higher in lab exposures, whereas house fly  $\text{LT}_{50}$  values were 6.8d when exposed to P89, and 7.5d when exposed to L90 (Fig. 7).

Application method of *B. bassiana* conidia had a significant impact on both infection rate and infection duration (Table 1, Table 2). The field applications resulted in lower infection rates, and the time from exposure to death was longer ( Table 1 and Table 2). House and vinegar fly infection rates were 55.82% higher when exposed to the lab application of  $1 \times 10^8$  conidia per  $\text{cm}^2$  placed directly to plywood boards when compared to field exposures at a rate of  $1.076 \times 10^5$  conidia per  $\text{cm}^2$  applied using a backpack sprayer (Table 1). Of the flies infected from the field exposures, infections lasted an average of 4.9 days longer when compared to the lab exposures (Table 2).

The L90 strain induced significantly higher infection rates (57.27%) compared to the P89 strain (45.95%) across both species (Table 1). There was no difference, however, in the mean duration of infection between P89 (10.50 days) and L90 (11.33 days, Table 1).

Wild flies collected after field applications had very low infection rates throughout

the 4 week experiment period (Table 3). The number of house flies collected was variable between barns and across weeks, and very few vinegar flies were captured overall. However, infections were recorded for both species in the barn treated with P89, and for house flies collected in the barn treated with L90. No vinegar flies were collected in the barn treated with L90 after *B. bassiana* applications; therefore infection rates were not determined. This is likely a result of low initial vinegar fly population levels since very few individuals were observed in barns prior to *B. bassiana* applications.

### **Discussion**

In our study, vinegar flies are susceptible to *B. bassiana*, but rates were significantly lower than those of the house fly (Table 1). Further investigation could expand any differences in infection across the two species not clearly identified in our results. For example, we found mean vinegar fly infection duration was not significantly different when compared to house flies (Table 2).

Certain factors may be responsible for differences in susceptibility. Cuticular protein composition variation across species is known to impact the conidial germination and germ tube development (Bidochka and Khachatourians 1992, Donatti et al. 2008). Delays in germ tube penetration of insect cuticle may explain the longer infection duration seen in vinegar flies. Additionally, differences of infection duration could be impacted by immune response variation. The *Drosophila* immune system responds aggressively through the Toll pathway against infections by microorganisms including fungi (Hoffman 2003). In part, this immune response is triggered by the release of different proteases of *B. bassiana* (Donatti et al. 2008). Furthermore, several genes have been identified that up-regulate after *B. bassiana* invasion of

larval silkworm, *Bombyx mori* L., tissue (Chengxiang et al. 2011). Examination of other *B. bassiana* host immune response differences could explain variation in fungal virulence across species.

Exposure of *B. bassiana* to *Drosophila melanogaster* ( $5.68 \times 10^5$  conidia per  $\text{cm}^2$  for 3 days) resulted in 85% mortality of *D. melanogaster* at 28 days postexposure, and infection durations ranging from 10 to 28 days (Tinsley et al. 2006). Additionally, 28% of flies in this study surviving beyond 30 days postexposure had *B. bassiana* colony forming units (CFUs) present internally (Tinsley et al. 2006). This suggests that long term ongoing infections are possible, and could contribute to potential resistance to this fungal pathogen.

Besides host immunity, factors influencing *B. bassiana* virulence could attribute to vinegar fly susceptibility. It is well established that recent in-vivo passages of *B. bassiana* through a specific host species has a positive impact on virulence to the same host species (Feng et al. 1994, Brownbridge et al. 2001, Song and Feng 2011). The P89 and L90 strains used here had only previously been passed through house flies. Conidia harvested from the vinegar fly cadavers in our study could be more virulent in subsequent exposures.

House fly infection rates in our study were less than observed in previous studies. In the lab exposures we had 85% mortality of house flies, whereas Watson et al. (1995) had 94.27% mortality across both strains. Also, infection duration of house flies in our study compared to Watson et al. (1995) took much longer. At 7 days post exposure we observed only 56.7% mortality (Fig. 6) compared to 94.27% in previous studies (Watson et al. 1995). It took 13 days after lab exposures for our house fly mortality to reach 85%. Our lower levels of house fly mortality could be attributed to differences in growth medium compared to

Watson et al. (1995), which is known to impact fungal virulence (Steinhaus 1949, Feng et al. 1994). Additionally, it is likely that despite host passages through our house fly colonies these fungal strains may have lost virulence since their original harvest in 1990. Vinegar fly susceptibility could be underestimated in this study because of low house fly infection rates.

Field exposures of *B. bassiana* resulted in less mortality and longer infection duration in both house and vinegar flies (Table 1 and Table 2). Vinegar fly infection decreased by 60.88%, and house fly infection decreased by 50.76% in field exposures when compared to the lab exposures. This is most likely a response to the lower conidial dose used for the field experiments. In a previous study, house flies exposed to  $1 \times 10^6$  conidia per  $\text{cm}^2$  had 22.8% mortality within 7 days (Watson et al. 1995). Our field exposure rate ( $1.076 \times 10^5$  conidia per  $\text{cm}^2$ ) induced only 8.4% mortality of house flies within 7 days, but 34.2% of house flies died from infection 38 days after exposure. With such extended mortalities after exposure, female flies would have opportunities to oviposit several times. House flies infected with *E. muscae*, with an average incubation period of 7 days, have shown decreased oviposition rates beginning 3 days post exposure (Mullens 1990). It is possible that physiological changes within female house flies exposed to *B. bassiana* are delayed in low-dose field exposures such that several gonotrophic cycles may continue to occur. High doses ( $1 \times 10^8$  c per  $\text{cm}^2$  at least) are necessary to induce field house fly epizootics that would adequately suppress population levels, because of the low infection rates and longer infection durations we observed.

Additionally, field application methods may have contributed to decreased infection rates and infection duration. The backpack sprayer application possibly resulted in

inconsistent coverage of material within each barn. Temperature, humidity, and precipitation are additional factors that may have confounded field infection rates. Conidial viability in the field decreases over time, with the first 4 days after application yielding the best infection rates (Watson et al. 1995). Previous studies also indicate that direct sunlight has more impact on efficacy than temperature alone, with direct sunlight decreasing *B. bassiana* viability to 1-2 days in the field (Dauost and Pereira 1986). Conidia in our field applications were applied inside of shaded hoop barns where direct sunlight is restricted. The high dust accumulation on walls and floors common in livestock systems, and the mixing of barn bedding from hog movement may have contributed to low wild (Table 3) and lab-reared fly infection rates (Table 1).

We observed a significant difference between infectivity of P89 and L90 strains of *B. bassiana* (Table 1). The L90 strain in our experiments induced infection in 84% of flies in the laboratory exposures while the P89 strain had an infection rate of 75% (Table 1). In contrast, previous studies found the P89 strain to be superior to L90 against house flies, and no strain differences in stable fly infectivity (Watson et al. 1995). The differences across strains in our studies are also evident in the field applications between L90 (30.64%) and P89 (16.76%) (Table 1). Factors in inoculum preparation could explain differences between the strains including the particle size of the blended powder, or moisture content. Again, it is likely these fungal strains have lost some virulence since their original harvest in 1990, even with several passages through host tissue.

It is clear that vinegar flies, a nuisance pest species, are susceptible to *B. bassiana*. Additional studies are needed to determine the specific population and individual

physiological effects that application of *B. bassiana* has on vinegar flies. Wide ranges in infection duration, and an overall lack of vinegar fly ecological, physiological, and biological information could result in unpredictable population fluctuations of adult vinegar flies if applied in the field. Our study has confirmed that application of *B. bassiana* targeting other pest species will impact any vinegar fly populations that are present. Cuticle composition, immune response, and the formulation of *B. bassiana* are all factors that could affect fungal virulence against vinegar flies. Future studies should concentrate on the physiological aspect of vinegar fly infection, with emphasis on strains that have previously passed through vinegar flies.

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**Table 1** Proportion of house flies and vinegar flies that died with patent infection (means  $\pm$  SE) exposed to P89 and L90 strains of *Beauveria bassiana* for both laboratory and field application methods

	Fly	Application	Strain	N	Mean $\pm$ SE
<i>P</i> -value:	0.014	<0.0001	0.0278		
	House	Lab	L90	4	0.93 $\pm$ 0.05
			P89	4	0.78 $\pm$ 0.085
		Field	L90	4	0.45 $\pm$ 0.15
			P89	4	0.24 $\pm$ 0.11
	Vinegar	Lab	L90	4	0.75 $\pm$ 0.083
			P89	4	0.73 $\pm$ 0.04
		Field	L90	4	0.16 $\pm$ 0.04
			P89	4	0.10 $\pm$ 0.10

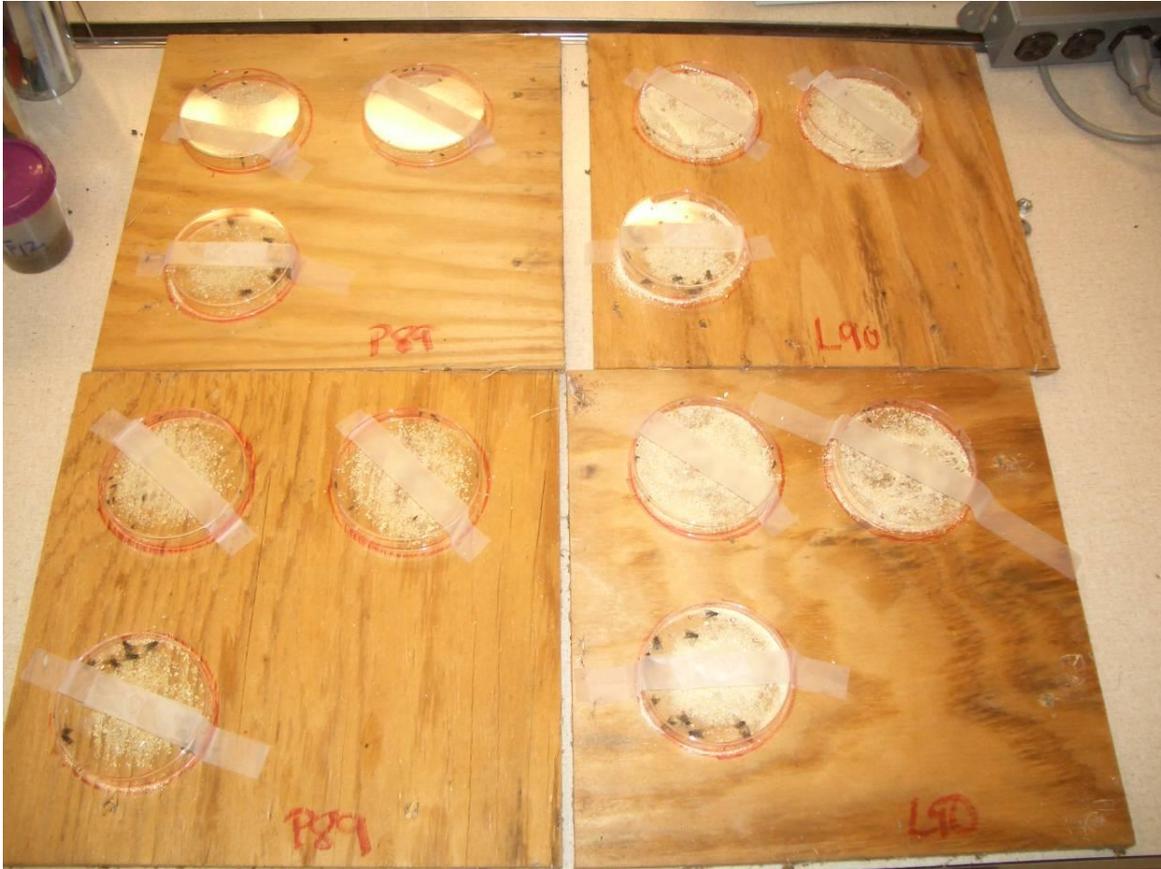
**Table 2** Duration of infection of house flies and vinegar flies that died with patent infection (days  $\pm$  SE) exposed to P89 and L90 strains of *Beauveria bassiana* in both laboratory and field application methods

	Fly	Application	Strain	N	Mean $\pm$ SE
<i>P</i> -value:	0.0853	0.0308	0.8669		
	House	Lab	L90	4	7.4 $\pm$ 0.05
			P89	4	7.37 $\pm$ 0.67
		Field	L90	4	14.74 $\pm$ 2.5
			P89	3	12.35 $\pm$ 3.8
	Vinegar	Lab	L90	4	10.06 $\pm$ 0.72
			P89	4	10.66 $\pm$ 0.73
		Field	L90	4	13.13 $\pm$ 1.79
			P89	1	16.87 <sup>a</sup>

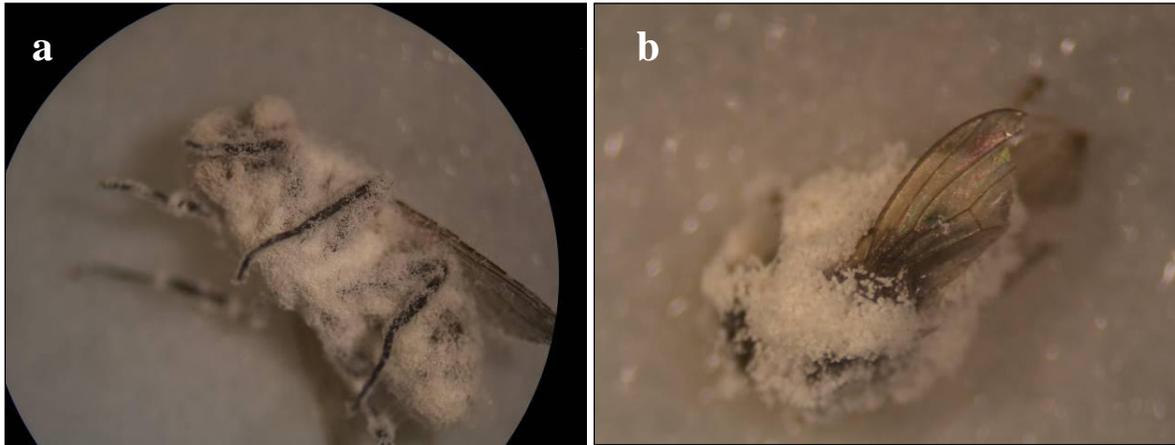
<sup>a</sup> Only one vinegar fly died from P89 *Beauveria bassiana* infection in field experiments; therefore, no standard error is calculated.

**Table 3** Infection rates of wild house flies and vinegar flies collected 48 hours after field application of *Beauveria bassiana*

Collection Date	Fly	Treatment	Collected	Infected
8/10/2011	House	P89	21	0
		L90	34	0
	Vinegar	P89	0	-
		L90	0	-
8/17/2011	House	P89	15	0
		L90	48	0
	Vinegar	P89	1	0
		L90	0	-
8/24/2011	House	P89	32	1
		L90	31	0
	Vinegar	P89	1	1
		L90	0	-
8/31/2011	House	P89	82	3
		L90	42	0
	Vinegar	P89	0	-
		L90	0	-



**Figure 2** Vinegar and house flies during exposure in the laboratory experiment ( $1 \times 10^8$  conidia per  $\text{cm}^2$ ) of P89 and L90 strains of *Beauveria bassiana*.



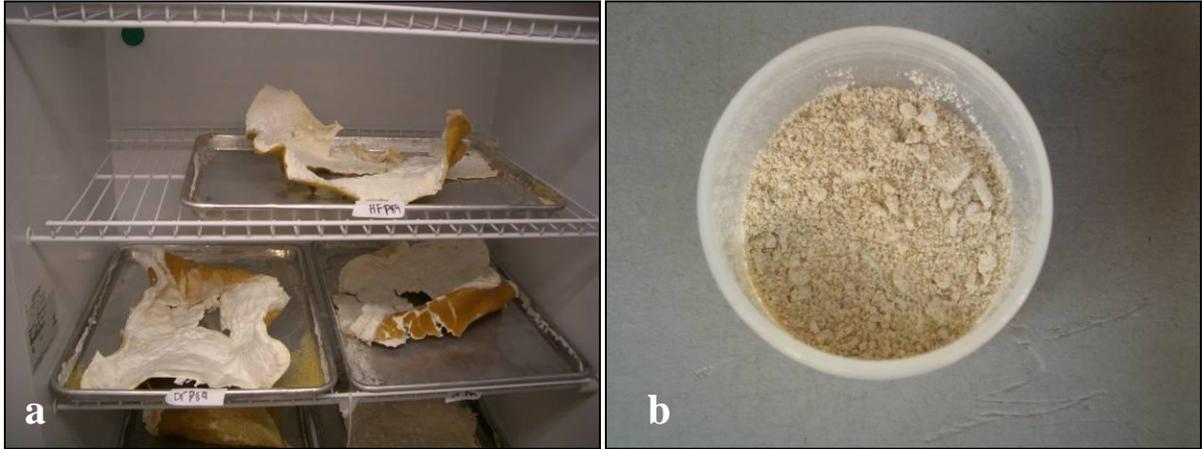
**Figure 3** House fly (a) and vinegar fly (b) cadavers with patent *Beauveria bassiana* infections.



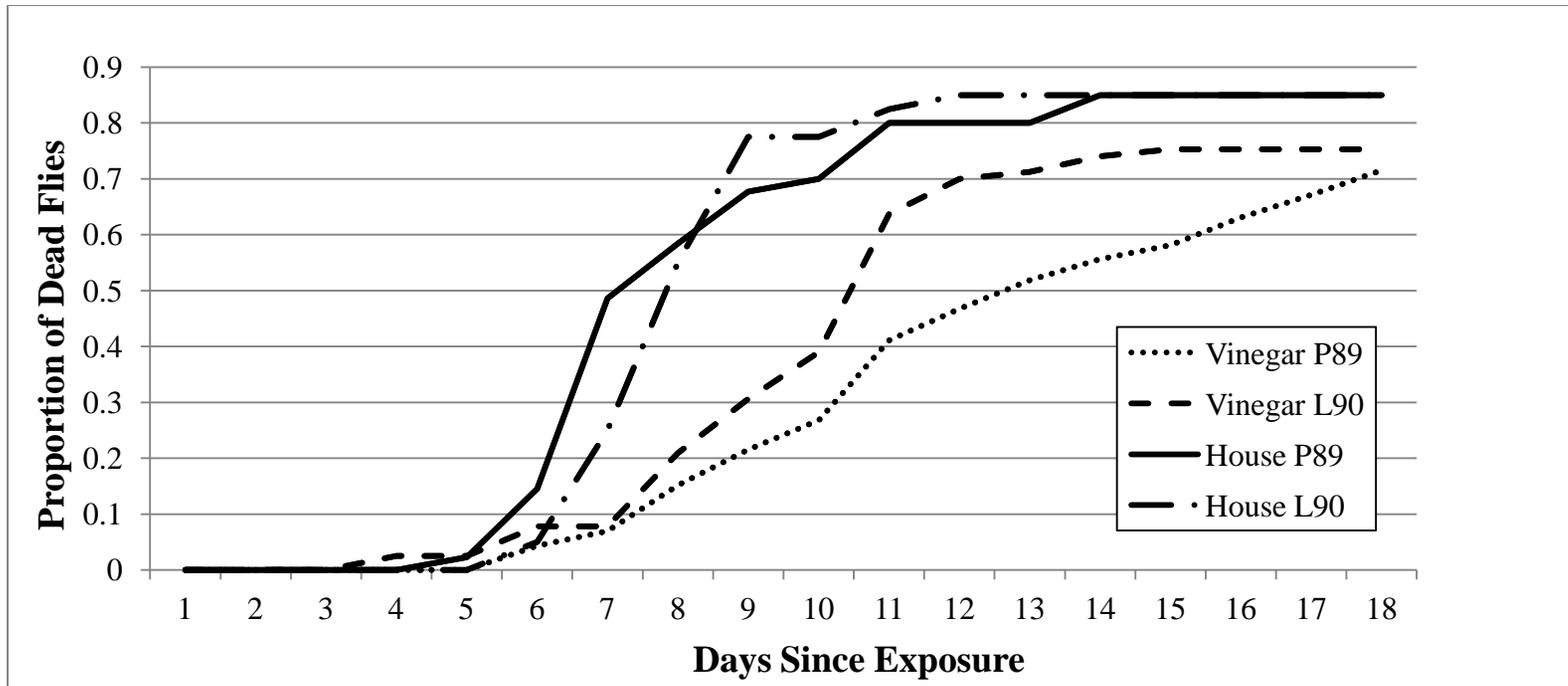
**Figure 4** Adult vinegar fly (*Drosophila repleta*) collected from Center of Environmental Farming Systems research swine unit Goldsboro, NC, May 2010.



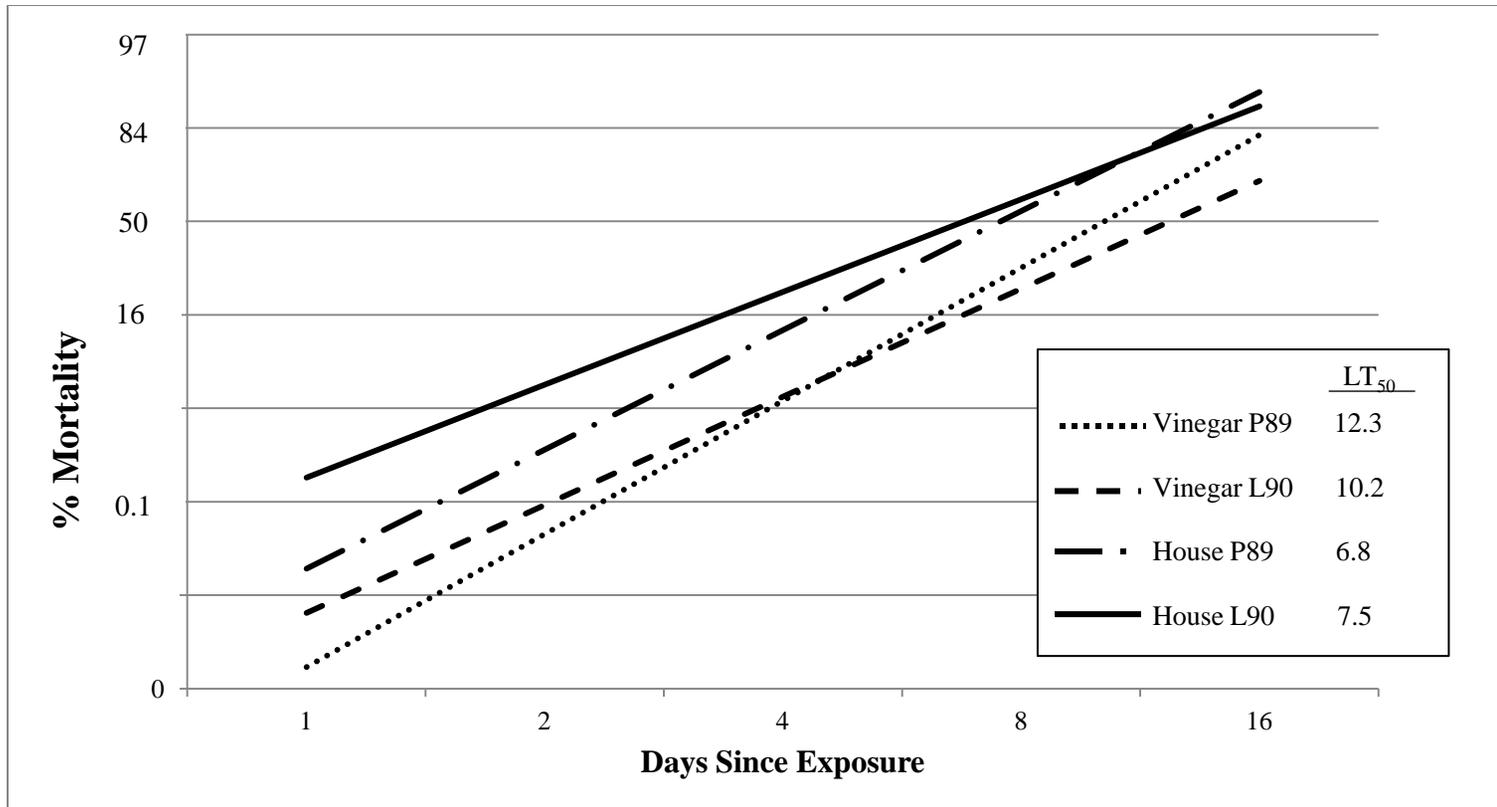
**Figure 5** Autoclaved corn meal and water mixture 2 days after liquid media *Beauveria bassiana* inoculation.



**Figure 6** a. *Beauveria bassiana* growth on cornmeal after 7 days of drying at room temperature. b. Final *Beauveria bassiana* inoculum blended into a fine powder.



**Figure 7** Time course of mortality (due to infection) of vinegar and house flies inoculated with the laboratory application method ( $1 \times 10^8$  conidia per  $\text{cm}^2$ ) of P89 and L90 strains of *Beauveria bassiana*.



**Figure 8** Probit analysis and LT<sub>50</sub> values of mortality (due to infection) over time of vinegar and house flies inoculated with the laboratory application method ( $1 \times 10^8$  conidia per cm<sup>2</sup>) of P89 and L90 strains of *Beauveria bassiana*.

## CHAPTER 3

Guisewite: Geraniol as a Fly (Diptera:  
Muscidae) Repellent

L. M. Guisewite  
North Carolina State University  
Dept. of Entomology  
Campus Box 7626  
1106 Grinnells Laboratory  
Raleigh, NC 27695-7626  
Phone: (919) 515-1663  
Email: [lguisew@ncsu.edu](mailto:lguisew@ncsu.edu)

### **Characterization and Efficacy of Geraniol as a Repellent against House Flies and Stable Flies (Diptera: Muscidae)**

L. M. Guisewite

North Carolina State University, Department of Entomology, 1106 Grinnells Laboratory,  
Raleigh, NC 27695-7626

**Abstract.** Geraniol is an active ingredient in many plant derived mosquito repellents for use on human skin, yet little is known about its efficacy against pests of livestock such as house flies, *Musca domestica* L., and stable flies, *Stomoxys calcitrans* L. Here we evaluated geraniol at low concentrations (1-4%) for tactile and spatial repellency against both species.

A treated filter paper bioassay was used to evaluate tactile repellency as indicated by fly specking frequency within a petri dish. Flies avoided contact with filter papers treated with concentrations as low as 2% geraniol suggesting a tactile repellency response for house flies ( $P$ -value= 0.0023), and 3% geraniol for stable flies ( $P$ -value= 0.0159). Dose dependent knockdown (KD) effects were noted 38 - 138 minutes during exposure in specking bioassays for both species, with 99.2% of house flies knocked down after exposure to 4% geraniol, and 61.7% knocked down after exposure to 3% geraniol. Stable flies were more susceptible to the KD effects with 76.6% knocked down after exposure to 3% geraniol. Oviposition deterrent bioassays show geraniol was effective for stable flies at low concentrations (1-4%), and effective against house flies at only 4% geraniol (96.3% eggs on control,  $P$ -value= 0.0002). Geraniol spatial repellency was tested using an olfactometer. Comparative tests evaluated the response of house flies to geraniol (1-4%) and swine manure, the positive control. Results of these experiments do not support the hypothesis that 1-4% geraniol is a spatial repellent for house flies (stable flies were not tested). Tactile repellency of geraniol has the potential for use in livestock IPM strategies, especially those focusing on application to animal bedding or waste for oviposition prevention, or application directly to animals for adult fly nuisance control.

**Keywords.** House fly, stable fly, geraniol, insect repellent, IPM

## Introduction

Filth flies associated with livestock pose serious health and economic problems. House flies can mechanically transmit pathogens that cause disease in humans including *Escherichia coli* (Foerster et al. 2007), and virulent enterococci (Ahmad et al. 2011) among others. Stable flies, *Stomoxys calcitrans* L. cause economic yield losses such as decreased weight gains in beef cattle (Campbell et al. 1987, Campbell et al. 2001), and decreased milk production in dairy cows (Bruce and Decker 1958). Widespread pesticide resistance in house fly populations (Scott et al. 2000) has led researchers and producers to examine alternatives to insecticides as a single use strategy. Deet, *N, N*-Diethyl-3-methylbenzamide, is an effective repellent for biting flies and ticks attacking humans (McCain and Leach 2007), but early studies suggest that deet causes acute toxicity in cattle and horses with symptoms including excessive salivation, sweating, and nasal discharge (Blume et al. 1971). Natural product repellents are often considered less aversive. Interest in plant-based insect repellents as an alternative method of fly control has increased with higher consumer demand of organic meat and dairy products. Currently, the market for natural plant-based insect repellents is aimed at personal protection, for application directly on human skin against biting mosquitoes and other pests.

Geraniol is a common plant-based active ingredient in commercially available natural insect repellents (Repel® Natural, United Industries; FASST Insect Repellent, FASST Products LLC; MosquitoSafe™, Naturale Ltd.). Geraniol is a biodegradable monoterpene, with low-toxicity to mammals, and it is approved for topical use on organically produced cattle by the Organic Materials Review Institute (OMRI). Topically applied to human

subjects, geraniol was effective against mosquitoes at a 25% concentration, but not as effective as a 15% formulation of Deet (Barnard and Xue 2004). A 5% candle formulation of Geraniol exhibited spatial repellency to 50% of female mosquitoes, and 97% of female mosquitoes in a 20g (100% geraniol) diffuser formulation. The spatial repellency to the 20g (100% geraniol) diffuser was reduced to 75% when tested outdoors (Mueller et al. 2009). Similarly, 100% of adult *Aedes albopictus* exposed to vapors of geraniol lose their host-seeking ability, and can take up to 24 hours to recover (Hao et al. 2008). Field studies also show geraniol provides 4h of protection against *Psorophora ferox* von Humbolt, *Aedes atlanticus* Skuse, and *Aedes mitchellae* Dyar (Qualls and Xue 2009).

Despite the evidence for tactile and spatial repellency of geraniol to mosquitoes, little is known about its efficacy against house flies and stable flies. Many farmers are concerned for the welfare of their animals and comment on the difficulty of controlling flies on the farm. Organic dairy farmers are particularly interested in natural product repellents to relieve livestock from fly pressures to meet the requirements of certification, and to limit pesticide use. As a result, investigations of plant-based repellents could provide effective fly management solutions.

Here we examine the tactile and spatial repellency of geraniol against house flies and stable flies in a series of two-choice bioassays. Specking, oviposition, and flight behaviors were monitored and quantified to characterize the repellency of geraniol against these filth flies.

## Materials and Methods

*Insects.* Flies were maintained on a 12:12 light/dark cycle at approximately 25°C. House flies were obtained from a colony established in 1982 from a dairy farm near Ithaca, NY. Adults were maintained with water and a mixture of granulated sugar and milk powder. Larvae were reared on a diet consisting of 250 ml of Milk Plus pellets (Cargill, Minneapolis, MN), 2L of wheat bran, and 1.5L of water.

Stable flies were isolated from a dairy farm in Hauge, FL in 1980. Larvae were reared on a diet of Milk Plus pellets (60 ml), 240 ml of vermiculite, 480 ml of wheat bran, and 450 ml of water. Adults were maintained on a 1:1 ratio of sodium citrated bovine blood and 10% sugar water solution.

**Tactile Repellency Bioassays.** We used house and stable fly specking patterns as an indirect measure of tactile repellency of geraniol. Specks are fecal or vomit spots deposited by flies on surfaces, which indicate the location of a fly at a moment in time (Axtell 1970). The testing arena was modified from tick repellent bioassays used by Bissinger et al. 2009. The test surfaces were comprised of white qualitative 90mm No. 1 filter paper semicircles (Whatman® PLC, Maidstone, UK) placed in 100mm diameter petri dish bottoms (BD Biosciences, Durham, NC), and attached with double-sided tape. Filter paper semicircles were dipped into either water-based geraniol (FASST Products LLC, Rockville Centre, NY, 30% proprietary formulation), or distilled water and allowed to air dry for 1 hour. Geraniol was diluted volumetrically with distilled water to produce 1, 2, 3, and 4% concentrations. Within each petri dish, 10 CO<sub>2</sub> anesthetized three to five day-old adult flies were placed on the filter paper seam separating the treated surfaces, the petri dish lid was closed, and the

anesthesia recovered flies were allowed to distribute themselves within the dish for three hours (Fig. 1). Fly distribution was determined by counting the number fecal and vomit spots on the treated and untreated filter paper surfaces.

The number of moribund flies was recorded every 15 minutes, beginning after flies recovered from CO<sub>2</sub> anesthesia. Flies were considered knocked down if movement had completely ceased, and there was no response to stimuli. Knocked down flies were removed at the end of the three hour exposure, placed in a container provisioned with food and water, and allowed to recover for 18-24 hours postexposure. The number of recovered flies (active and responding to stimuli) was recorded.

Repellency experiments were conducted in an evenly lit windowless laboratory with temperatures of at  $24 \pm 0.6^{\circ}\text{C}$  and  $45 \pm 13\%$  RH. Petri dishes were arranged on the bench top so that an equal number of petri dishes had treatment halves facing the right, left, top, and bottom to correct for sidedness.

*Calculation and Statistical Analysis.* Each of four replicates consisted of a new generation of flies tested across all concentrations of geraniol (stable flies were not tested at 4% geraniol). The number of specks on the treatment and control semicircles were averaged across three subsamples (petri dishes) within each replicate to reduce variation (subsample = 10 flies;  $N = 30$ ). To test the repellency of geraniol at each concentration, the proportion of specks on the control half were compared to the proportion of specks on the treatment half with two-sided paired t-tests for each fly species (PROC TTEST) (SAS Institute Inc. 2002-2005). To compare the efficacy of geraniol across concentrations, maximum likelihood analyses of the proportion of control specks were conducted for each fly species with

replicate as a random variable (PROC MIXED) (SAS Institute Inc. 2002-2005). Models with significant results ( $P \leq 0.05$ ) were compared by least squares means with a Tukey's adjustment (SAS Institute Inc. 2002-2005).

Fly knockdown (KD) was observed at some concentrations, therefore KD and recovery rates were calculated for house flies at 3% and 4% geraniol, and at 2% and 3% geraniol for stable flies. All KD values were calculated by pooling individual data points (n=120 at each concentration of geraniol for each fly species) unless otherwise noted.

**Oviposition Bioassays.** To evaluate geraniol as an oviposition deterrent, four 45cm<sup>3</sup> aluminum screen cages were evenly spaced inside a 8.95m x 4.62m x 2.46m (height) windowless room. The room was maintained at  $23 \pm 1^\circ\text{C}$  and  $37 \pm 13\%$  RH. Cages were placed one meter above the floor with an artificial light source above each. Two box fans were placed in opposite corners of the room to facilitate air flow in a clockwise direction. Each of the four cages was randomly assigned a concentration of geraniol for each of four replicates: 1, 2, 3, or 4% geraniol (FASST Products LLC, Rockville Centre, NY, 100% proprietary formulation) diluted with food-grade mineral oil (Nevastane® #15, by Total Lubricants, Paris, France).

Within each cage four 36.96 ml shot glasses containing the OSs (oviposition substrates) were placed in the corners (Fig. 2). OSs in opposite corners were treated with either 100µl of geraniol (treatment OSs), or 100µl of the mineral oil carrier (control OSs) pipetted directly to the OS surface.

Three to five days prior to experiments, house flies were provided evaporated milk as a protein source necessary for egg development. Stable flies were provided bovine blood

immediately after eclosion. Approximately 150 mixed-sex, 10-15 day-old flies were released into each cage and allowed to oviposit for six hours. A 10% sugar and water solution was provided as a carbohydrate and hydration source and placed in the center of each cage. An accurate count of the number of flies and sexes were determined after each replicate. Because of large egg deposits, many samples were counted volumetrically (house fly: 0.25ml= 2,492 eggs; stable fly: 0.2ml= 2,758 eggs) (Fig. 2).

*Oviposition Substrates.* Oviposition substrates (OSs) were composed of  $17 \pm 3$  grams of used house fly medium wrapped in black 100% cotton gauze (item #7649254, Jo-ann Fabrics and Craft Store®, LLC, Hudson, OH), twisted tightly into a ball shape, placed into 28.4 ml shot glasses, and moistened with 5ml of distilled water (Fig. 2b). Fiberglass window screen was secured over the top of shot glasses with rubber bands to prevent flies from getting coated with mineral oil and rendering them unable to fly. The top of the OSs touched the screen, and allowed the extended fly ovipositors to contact the OSs. The spent house fly medium was obtained from the house fly rearing process. The used medium was frozen at  $-20^{\circ}\text{C}$  until needed for these studies.

*Calculation and Statistical Analysis.* Each replicate consisted of a different generation of flies tested across all concentrations of geraniol. House fly and stable fly calculations and statistical analyses were performed separately. The proportion of eggs laid on the control and treatment OSs within each cage were calculated and arcsine square root transformed to meet equal variance and normality assumptions. Paired-t-tests (PROC TTEST) were conducted comparing transformed control and treatment values for each concentration of geraniol. Maximum likelihood analyses (PROC MIXED) with replicate as a random variable were also

conducted to compare across concentrations of geraniol. Models with significant results ( $P \leq 0.05$ ) were compared by least squares means with a Tukey's adjustment (SAS Institute Inc. 2002-2005).

**Olfactometer Bioassays.** A clear acrylic dual-choice Y-tube olfactometer with opaque PVC joints (25.5cm stem, 25.5cm arms, 90° angle, and 10.8 cm internal diameter) was used to evaluate the response of house flies to geraniol (stable flies were not tested in olfactometer bioassays) (Fig. 3). This diameter was selected to provide adequate space for insect flight, and to promote behavioral responses that would otherwise occur in open spaces. Each arm of the Y-tube was connected to one of two plastic vials, each containing either test or control substrates. Outside air was pulled into the olfactometer by a wet/dry vacuum connected to the base of the stem and set to 30% power with a voltage regulator. The vacuum produced airflow that was calibrated with the air flow meters attached to the arms at a rate of 2,310 ml/min, and at 4,620 ml/min through the stem where air streams coalesced. A smoke test was performed to confirm air movement and uniformity throughout the olfactometer. Air was exhausted through an outbound building duct which was not returned to the experiment room.

A microscope light (150 watt halogen bulb) was positioned over each distal end of the olfactometer arms. The olfactometer was placed in a dark room maintained at  $24.6^{\circ}\text{C} \pm 2.2^{\circ}\text{C}$ . Incoming outside air was equilibrated to inside temperature by passing air through 3.7 meters of 2.3cm diameter PVC tubing, then through 1.64m of 1cm diameter plastic tubing prior to entering the flow meters. Thermocouples were affixed inside each end of the arms, ensuring the internal olfactometer air was consistent with room temperature ( $24.7^{\circ}\text{C}$  in arms,

24.6°C in stem base). Relative humidity of internal air was measured between each experiment and averaged  $45.3\% \pm 11\%$ .

For each observation a single fly was placed in a screened holding chamber at the stem base. After a one minute acclimation period, a swivel gate was opened allowing the fly to enter the olfactometer. Once a fly passed the halfway mark (as indicated by rubber bands affixed around each arm) the location, treatment, and time were recorded. If a fly entered an arm without passing the halfway mark it was noted, but was only considered to have made a choice when it crossed the halfway mark. If the fly did not make a choice in three minutes, the fly was removed, and a new observation was initiated with a new fly.

Olfactometer choice comparisons were organized as follows: Empty/Empty (negative control); Pig manure/Pig manure (sidedness control); Pig manure/Empty (positive control); Geraniol/Carrier (repellency test); and Geraniol + manure/Manure + carrier (deterrence test). Repellency and deterrence tests were conducted using 1, 2, 3, and 4% geraniol. Between each observation the arms, PVC Y-joint, and PVC arm caps were washed with water and dish soap, then rinsed and dried with a cotton towel. The treatment and control arms were alternated between each observation.

*Test Substrates.* Geraniol (trans-3, 7-dimethyl-2, c-octadien-1-ol, 98 % technical grade, Sigma-Aldrich Co., LLC, St. Louis, MO) was volumetrically diluted with food-grade mineral oil (Nevastane® #15 by Total Lubricants, Paris, France) to obtain 1, 2, 3, and 4% concentrations. Pig manure was collected from sow pig pens at the NCSU Department of Animal Science Swine Educational Unit (Raleigh, NC). Manure was  $\leq 48$  hours old when used in olfactometer bioassays, and maintained in a 4°C refrigerator until used. For

repellency tests, 100µl of geraniol or mineral oil carrier was applied to a cotton ball with a pipetter. Deterrence tests consisted of 2g of pig manure with 100µl of geraniol or mineral oil applied directly to the manure surface with a pipetter. Test substrates were positioned on top of aluminum foil within the plastic vials that attached to the olfactometer arm caps. Test substrates were replaced with fresh material after every 10 observations.

*Calculation and Statistical Analysis.* Each of four replicates consisted of a different generation of flies tested across all concentrations of geraniol and choice comparisons. Experiment choice combinations within each replicate included five male and five female fly observations (N = 40-50 flies per choice comparison). To test the efficacy of geraniol in each experiment, the frequency of flies that chose the control side were compared to the frequency that chose the treatment side with a two-sided exact binomial test (PROC FREQ) (SAS Institute Inc. 2002-2005). To determine if the sex of the fly, or the presence of manure had an effect on response time, the amount of time (seconds) flies took to choose a side was compared across all experiments using a maximum likelihood analysis with replicate as a random variable (PROC MIXED) (SAS Institute Inc. 2002-2005). To determine whether the concentration of geraniol, or if the side chosen had an effect on fly response time, another maximum likelihood analysis was conducted across repellency and deterrence experiments (excluding control experiments) with replicate as a random variable (PROC MIXED) (SAS Institute Inc. 2002-2005).

## **Results**

**Tactile Repellency Bioassays.** Geraniol exhibited tactile repellency to both house and stable flies, though not uniformly across concentrations. House flies exposed to 2% and

3% geraniol left significantly more specks on the control semicircles when compared to treatment (Fig. 4). In contrast, there was no significant difference between control and treatment specks at 1% or 4% geraniol (Fig.4).

Stable flies avoided contact with semicircles treated with 3% geraniol (Fig. 5). Stable fly specking on semicircles for both 1% and 2% geraniol treatments were not significantly different from the controls. Interestingly, 3% geraniol significantly impacted specking behavior by both fly species, yet at this rate stable flies deposited only 55.4% of specks on the control side, while house flies deposited 84.4% (Figs. 4 and 5). The distribution of fly specks on untreated filter paper semicircles on both sides of the dish (controls) were not significantly different for house flies ( $P$ -value = 0.2288) or stable flies ( $P$ -value = 0.5166) (Figs. 4 and 5).

Knockdown (KD) activity was observed for both house and stable flies (Table 1). Only 3% and 4% geraniol caused a KD effect in house flies, whereas stable flies had KD at 2% and 3% concentrations. Stable flies appeared to be more susceptible to 3% geraniol KD effects than house flies. For stable flies, 76.7% of the flies were knocked down after 37.6 min. compared to house flies where 61.7% were knocked down after 113.9 min. The time to KD decreased as concentration increased for both fly species. Nearly all house flies appeared dead after approximately 65 minutes exposure to 4% geraniol (Table 1). As a result of these KD effects, the average number of house fly specks deposited on filter paper semicircles was less, 8.3 average specks at the 4% concentration, compared to 36.5 specks for the water/water control (Table 1).

Recovery rates were high for house flies exposed to 3% geraniol (96.5%), but only

63.8% recovered from exposure to 4% geraniol. Similarly, only 56.3% of stable flies recovered from exposure to 3% geraniol (Table 1). Unlike house flies, the average number of stable fly specks deposited on filter papers showed no trend as geraniol concentration increased (Table 1).

**Oviposition Bioassays.** Both fly species oviposited fewer eggs on OSs treated with geraniol at all concentrations relative to the mineral oil control (Figs. 6 and 7). There was great variation between house fly replicates, with almost all eggs being laid on a single substrate. For example, 4,073 eggs were laid on the treatment OSs within a single cage at 3% geraniol (vs. 41 eggs on control OSs in the same cage), and in a different replicate 7,979 eggs were laid on the control OSs (vs. 216 eggs on the treatment OSs). There were no significant differences in the number of house fly eggs deposited on treated and untreated substrates at 1, 2, and 3% geraniol concentrations. House flies avoided oviposition on OSs treated with 4% geraniol with 96.3% of the eggs deposited on the control media.

Stable flies deposited significantly fewer eggs on treatment OSs across all concentrations of geraniol, and had much less variation between and among replicates (Fig. 7). Similar to house flies, 4% geraniol resulted in the greatest difference between control (92.9%) and treatment OSs. However, there was no significant difference in stable fly oviposition avoidance between the different concentrations of geraniol (maximum likelihood analysis;  $F= 2.05$ ;  $df= 3,9$ ;  $P\text{-value}= 0.1773$ ). House flies averaged  $171 \pm 43$  flies released within each cage (93% female), and stable flies averaged  $88 \pm 29$  flies (61% female).

**Olfactometer Bioassays.** To validate the olfactometer as a study instrument, it was necessary to test the responses of flies to airstreams without the study material (geraniol).

Olfactometer control experiments (nothing/nothing; manure/manure) exhibited no difference in the number of flies that chose left or right olfactometer arms (Fig. 8). Positive control experiments resulted in significantly more flies choosing manure (64%) over the control. Few flies did not make a choice in the allotted 3 minutes (5.2%). Also, only 17 out of 494 observed flies selected a different path (reversed direction) before passing the half-way mark. Of those that reversed direction, 8 ultimately chose an arm treated with geraniol.

In olfactometer bioassays, no concentration of geraniol induced behaviors consistent with spatial repellency or deterrence for house flies (Fig. 8). All concentrations of geraniol in repellency tests (without manure) resulted in insignificant differences between control and treatment arms.

Similarly, in deterrence experiments (with manure) no concentration of geraniol resulted in significantly more flies choosing the control arm than the treatment arm (Fig 8). A statistically significant spatial action of geraniol on house fly behavior indicates a preference for 1% geraniol + manure (65%) over mineral oil + manure ( $P$ -value= 0.0357).

Flies took  $55 \pm 24$  seconds to choose an olfactometer arm. Interestingly, Females took significantly longer to choose a side than males (61.3 s and 44.9 s, respectively), and only in experiments with manure (Table 2). The sex of the fly had no effect on whether the treatment or control side was chosen ( $P$ -value = 0.8937,  $df = 1, 67$ ). Additionally, the presence of manure, the pathway chosen by the fly, and the concentration of geraniol tested had no effect on fly choice duration (Table 2).

## Discussion

These results clearly demonstrate geraniol is a tactile repellent against house and stable flies. Tactile repellency was measured indirectly by quantifying the number of fecal and regurgitation spots found on treated and untreated semicircles of filter paper. In these tactile repellency bioassays, house flies were significantly repelled at 2% and 3% concentrations, and stable flies were repelled by 3% geraniol (Figs. 4 and 5).

KD activity likely confounded these findings, leading to insignificant results at 4% geraniol. At this concentration, flies were knocked down on average in 86.7 minutes, less than half of the experiment duration (Table 1). As a result, the average number of specks deposited were greatly reduced at the 4% dose in comparison to other concentrations, due to KD activity (Table 1). It is difficult to determine the impact that KD had in stable fly experiments because the average number of specks per experiment did not decrease as geraniol concentration increased (Table 1). Despite having a faster KD onset than house flies at equivalent concentrations, stable flies still deposited enough specks to provide adequate evidence of repellency to geraniol (Table 1).

KD and lethal effects of geraniol have been documented with other arthropods including the ear mite, *Otodectes cynotis* Hering (Traina et al. 2005), with 100% mortality after a 1h exposure to 5% geraniol. Also, the food storage mite, *Tyrophagus putrescentiae* Schrank, had higher mortality rates when exposed to 1.95  $\mu\text{g}/\text{cm}^3$  of geraniol, compared to 11.27  $\mu\text{g}/\text{cm}^3$  of benzyl benzoate, a commercial acaricide (Jeon et al. 2009). However, no KD or lethal effects were observed with the Chagas disease vector, *Rhodnius prolixus* Stål, after a 9h exposure to 70  $\mu\text{g}/\text{cm}^2$  of geraniol (Sfara et al. 2009). Large differences in lethal

and KD activity of geraniol across arthropod taxa make the mode of action unclear. Neurophysiological effects of geraniol were found to inhibit spontaneous and stimulus-evoked impulses in the nerve cord of *Periplaneta americana* L. at concentrations above  $2.5 \times 10^{-4}$  M, and increased spontaneous firing at lower concentrations (Price and Berry 2006). Therefore, the pathway of geraniol does not mimic octopamine, as previously hypothesized for essential oils similar to geraniol (Enan 2001), since octopamine stimulates inactive neurons, and initiates only excitatory effects in *P. americana* (Price and Berry 2006).

Most importantly, KD activity revealed the volatile and fumigant nature of geraniol. With a high vapor pressure (0.02mm Hg), geraniol loses its dose at a higher rate (39% in 40 min) than other monoterpenes, such as citronellal (0.009mm Hg; 27% dose lost in 40 min) (Gilpin et al. 2009). Although the treatment semicircles air dried for 1 hr prior to bioassays, the geraniol remaining on filter papers was sufficient to cause KD and mortality in house and stable flies. Recovery rates were never less than 50% for either species, and for each exposure there were flies that never exhibited KD, even after a three hour exposure to 4% geraniol (Table 1). Knock down-like resistance may be at play in our results. House flies have developed knock down resistance (KDR) to pyrethroids and DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) through mutations on sodium channel gate coding genes, rendering them insensitive to effects on the nervous system (Knipple et al. 1994). KDR is distinguished from resistance due to up-regulation of metabolic detoxification genes. However, metabolic processing of geraniol may explain our high recovery rates. *Aedes aegypti* mosquitoes, for example, utilize P450 enzymes and glutathion-S-transferases to metabolize other essential oils including thymol, eugenol, pulegone, terpineol, and citronellal

(Waliwitiya et al. 2012). House and stable fly resistance, whether KDR-like or metabolic, to geraniol is a possibility based on these results, and should be further tested.

Results for oviposition bioassays were very different between fly species, except at 4% geraniol, which consistently resulted in high oviposition avoidance (Figs. 6 and 7). Stable flies showed strong oviposition avoidance at all concentrations of geraniol, and with much smaller standard error rates than house flies (Fig. 7). Significant oviposition deterrence was exhibited by house flies at 4% geraniol (Fig. 6).

Variation across lower concentrations may be due to semiochemicals associated with eggs laid on treatment OSs during the experiment, leading to further aggregative egg deposits on treatment substances. Female flies prefer to oviposit in OSs containing ovaries from mature females, and solvents, mainly tricosane and (Z)-9-tricosene, extracted from house fly ovaries are attractive to females (Jiang et al. 2002). Additionally, additive oviposition behavior is driven by *Klebsiella oxytoca* (Flügge) Lautrop, a bacteria found in house fly ovaries, that both triggers oviposition coordination, and serves as a negative feedback for oviposition once a threshold density is reached (Lam et al. 2007). It is unclear, however, if *K. oxytoca* serves as the bacterial source of tricosane or (Z)-9-tricosene. Strong behavior responses to these cues may explain large error rates with house flies. A single female able to overcome any repellent properties of geraniol would, in turn, increase the attractiveness of the OS, perhaps enough for other females to overcome the geraniol.

Stable flies have similar oviposition responses to semiochemicals associated with bacteria. *Citrobacter freundii* Werkman and Gillen, for example, was extracted from suitable OSs and elicited the greatest oviposition response from gravid stable fly females (Romero et

al. 2006). This may explain why females in our experiments oviposited on any OSs, but information is lacking on the cues elicited by bacteria directly in symbioses with female ovaries, or freshly laid eggs. Our results suggest that stable flies are not as influenced by odorants associated with egg masses, but by the fermenting nature of the OSs themselves, and the presence of geraniol on the substrate, which had the most impact on oviposition behavior. A similar oviposition study found comparable results with another plant based compound, catnip oil, which reduced stable fly oviposition by 98% (Zhu et al. 2012).

Despite the high volatility of geraniol (Gilpin et al. 2009) and the KD effects noted here, our olfactometer bioassays indicate that house flies are not spatially repelled by geraniol at concentrations less than 4%. No concentration of geraniol elicited avoidance in repellency tests (manure absent), or deterrence tests (manure present). Fly preference for 1% geraniol with manure present may yield a unique sensory blend attractive to house flies.

Despite efforts to account for any sidedness in olfactometer bioassays, minimal movements between observation set-ups were unavoidable. Differences in the angle of light penetration onto the distal portion of olfactometer stems may have produced results representative of insect responses to visual cues rather than olfaction (Hecht 1970, Roberts et al. 1992). Regardless, the volatile nature of geraniol was not effective in preventing oriented movements toward treatment arms despite potential subtle differences in light penetration angles.

Longer choice time of females in experiments with manure is perhaps attributed to the attractiveness of manure as both a food source and an oviposition substrate (Moon 2009). Making a more invested choice might require more time, though there are no studies that

explore this hypothesis directly, and requires further investigation. It is recognized, however, that females will use semiochemical cues to avoid oviposition on substrates with harmful microbes (Lam et al. 2010).

Geraniol clearly exhibits tactile repellency, and oviposition deterrence to both house flies and stable flies. Despite the volatile nature of geraniol, there is not enough evidence from these studies to suggest geraniol functions as a spatial repellent for house flies, probably due to house fly sensory insensitivity to volatilized geraniol (Bernier et al. 2007). Other insects, such as mosquitoes, are spatially repelled by geraniol both in lab (Barnard and Xue 2004, Hao et al. 2008, Mueller et al. 2008, Mueller et al. 2009) and field studies (Mueller et al. 2009, Qualls and Xue 2009). The Japanese beetle, *Popillia japonica* Newman, is attracted to the spatial vapors of geraniol (Ladd and McGovern 1980). These widely varied responses to geraniol imply geraniol has other potential uses as a repellent or baited trap.

Fly behavior may have been affected by geraniol formulation differences between experiments. Additives in proprietary formulations for tactile and oviposition experiments could have had an impact on fly behavior not exhibited in olfactometer experiments. Additionally, carrier solvents varied between tactile bioassays (water) and oviposition and olfactometer bioassays (mineral oil). Physical properties of solvents can impact geraniol volatility and rate of diffusion, potentially changing fly response.

There is potential for use of geraniol in IPM strategies, including push-pull management methods. Oviposition deterrence exhibited by both house and stable flies in our experiments suggests that animal bedding in hog barns or calf hutches, as well as urine saturated areas surrounding round hay bale feeders, where larval stable flies develop, may be

suitable sites for geraniol application (Moon 2009). Additionally, tactile repellency and KD effects of geraniol in our studies indicate that direct animal application may be effective for preventing stable fly biting, or house fly nuisance. Preventing stable fly landing may be sufficient to prevent stable fly probing. Use in livestock could have confounding variables including rainfall, amount to be applied per animal, and the frequency of application or type of application.

Overall the physiological mechanisms in house flies and stable flies that elicit avoidance behaviors to geraniol are unclear. Future studies should focus on better understanding the extent to which contact with geraniol is necessary to evoke avoidance behavior in filth flies, as well as utilizing these properties for field applications in livestock systems as part of IPM research programs.

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**Table 4** Knockdown (KD) activity of geraniol against house and stable flies after a 3 hour exposure, and fly recovery rates after 18-24 hours.

Fly	Geraniol (%)	Mean specks per assay $\pm$ SE	KD (%)	Mean time to KD (min) $\pm$ SE	Recovery (%)
House	1	39 $\pm$ 4.46	0	-	-
	2	30.83 $\pm$ 2.71	0	-	-
	3	27.41 $\pm$ 3.39	61.7	113.9 $\pm$ 14.5	96.5 <sup>b</sup>
	4	8.25 $\pm$ 1.53	99.2	65 $\pm$ 6.2 <sup>a</sup>	63.8
Stable	1	73 $\pm$ 12.95	0	-	-
	2	86.41 $\pm$ 18.09	16.7	137.7 $\pm$ 15.1	- <sup>c</sup>
	3	70.33 $\pm$ 10.09	76.7	37.6 $\pm$ 5.7	56.3 <sup>b</sup>

<sup>a</sup> Mean time to KD for house flies exposed to 4% geraniol calculated based on amount of time for the entire subsample of flies (n=10) to reach 100% KD. All other mean times were averaged across individual flies (n=120).

<sup>b</sup> Recovery for stable flies and house flies exposed to 3% geraniol calculated with data missing from one replicate (N=90).

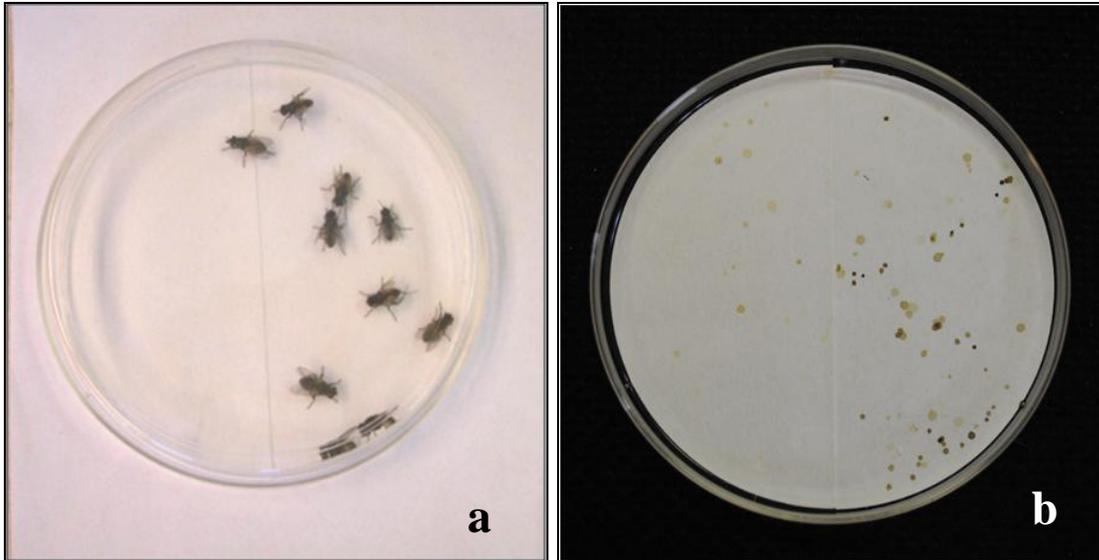
<sup>c</sup> No observation

**Table 5** Maximum likelihood analysis results and choice test duration (means  $\pm$  SE) of house flies in olfactometer bioassays. *N* represents the total number of observations for each statistic.

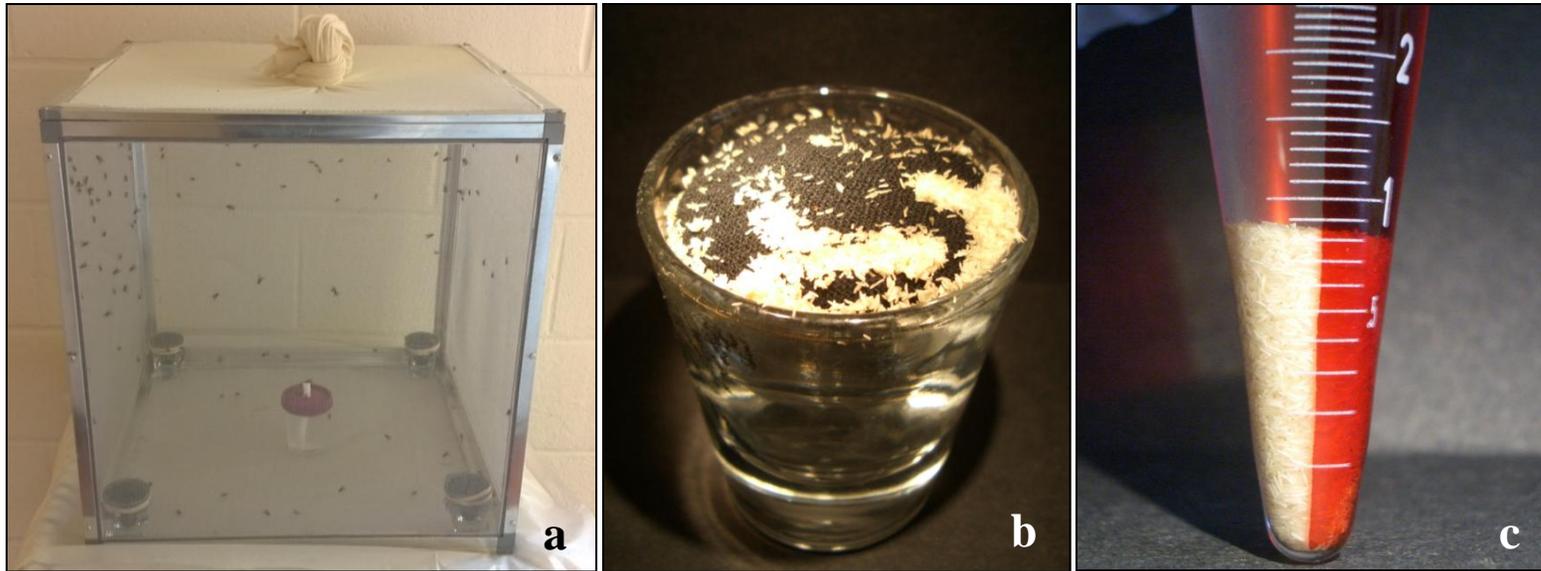
Factor	<i>N</i>	<i>P</i> -value	Levels	Seconds $\pm$ SE
Sex <sup>a</sup> (manure present)	286	0.0048	Male	44.9 $\pm$ 4.2
			Female	61.3 $\pm$ 4.4
Sex (no manure present)	201	0.2249	Male	50.9 $\pm$ 4.9
			Female	58.0 $\pm$ 5.1
Manure	494	0.5889	Present	53.1 $\pm$ 3.1
			Absent	54.4 $\pm$ 3.5
Side chosen <sup>b</sup>	355	0.1543	Control	47.1 $\pm$ 3.6
			Treatment	53.1 $\pm$ 3.7
Geraniol <sup>b</sup>	355	0.3923	1%	46.5 $\pm$ 4.9
			2%	56.8 $\pm$ 5.7
			3%	43.2 $\pm$ 4.9
			4%	54.9 $\pm$ 5.3

<sup>a</sup> Females took longer to choose a side when manure was present ( $F= 8.91$ ;  $df 1, 486$ )

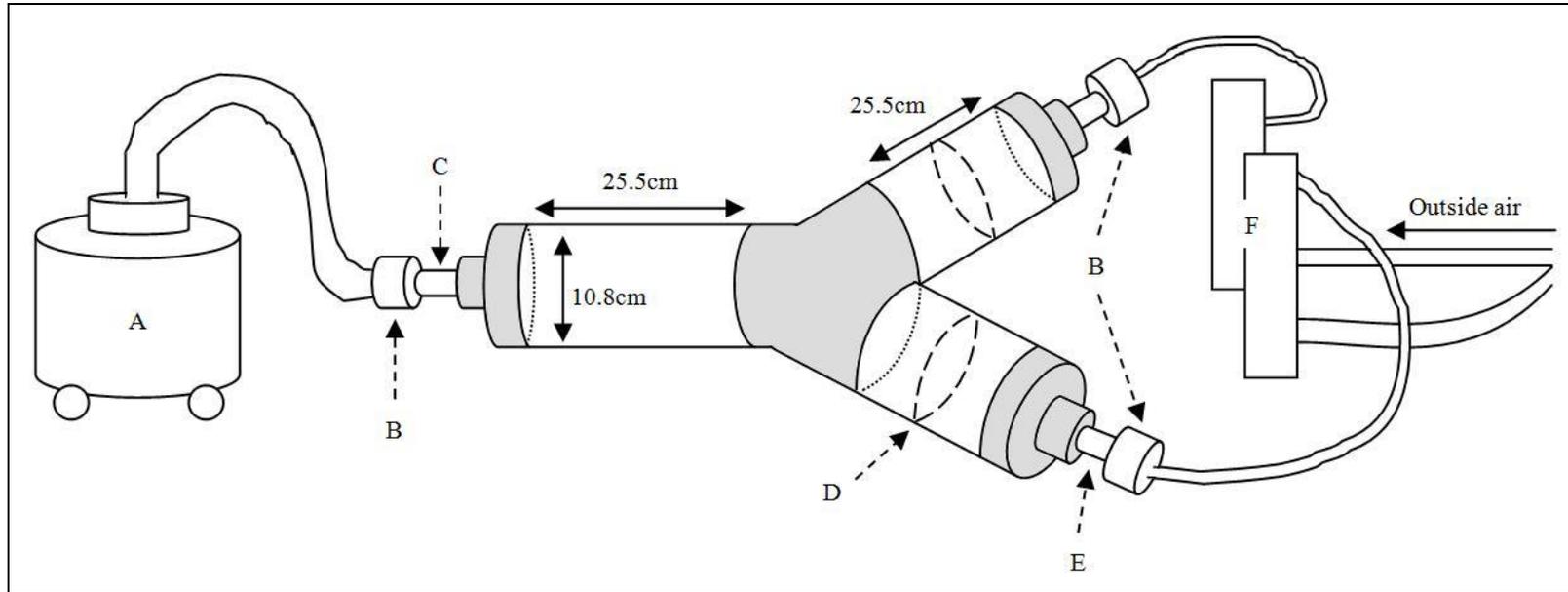
<sup>b</sup> Side chosen and Geraniol analyses only included observations where geraniol was present (no positive or negative control observations were included)



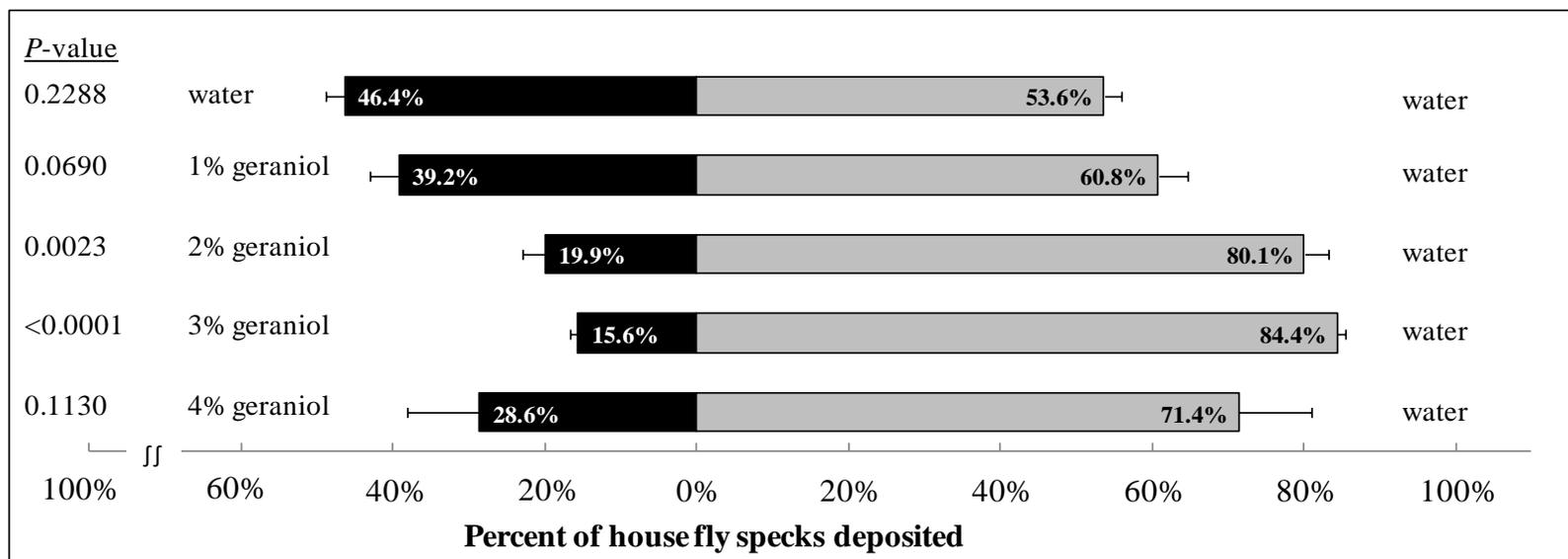
**Figure 9** a. House flies during three hour specking bioassay. Left filter paper half treated with 4% geraniol; Right half is distilled water control. b. Stable fly specks on filter paper halves after three hour specking bioassay. Left half treated with 2% geraniol; Right half is distilled water control.



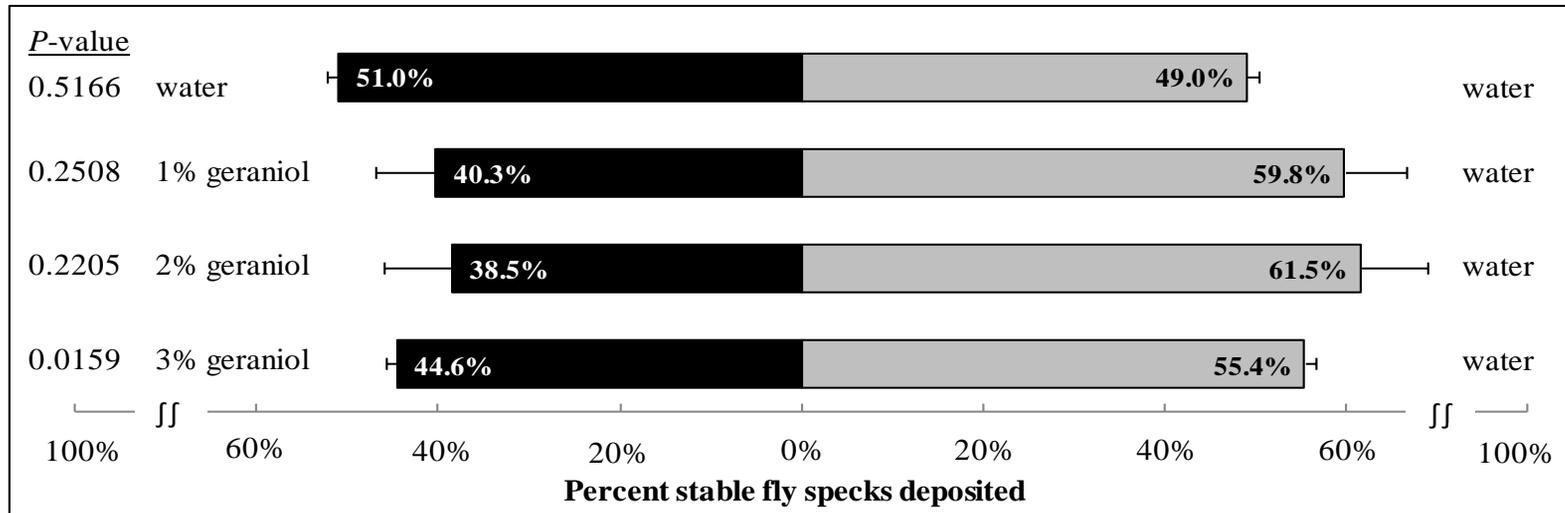
**Figure 10** a. Oviposition bioassay cage set-up. Oviposition substrates in corners (2 treatment, 2 control) and 10% sucrose solution wick in center. b. House fly eggs on control oviposition substrate after 6 hour oviposition bioassay. c. House fly eggs (8,473 eggs pictured) were quantified volumetrically after removal from oviposition substrate (shown in b).



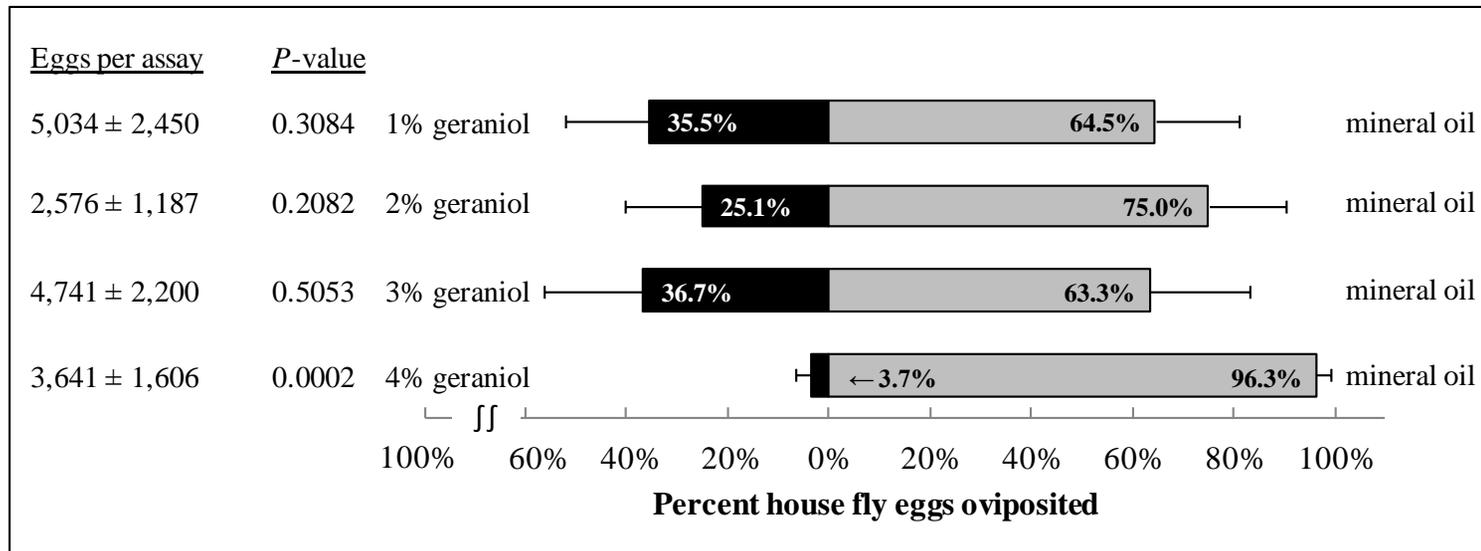
**Figure 11** Olfactometer schematic drawing (not to scale). A, wet/dry vacuum drawing in outside air; B, locations where thermocouples were affixed (stem, and both arms) for recording temperature; C, screened holding chamber for fly acclimation; D, half-way marks on each arm; E, test substrate holding chambers; F, flow meters set to 2,130 ml/min each. Shaded areas represent opaque PVC material. Internal diameter 10.8cm, length of exposed acrylic stem and arms: 25.5cm.



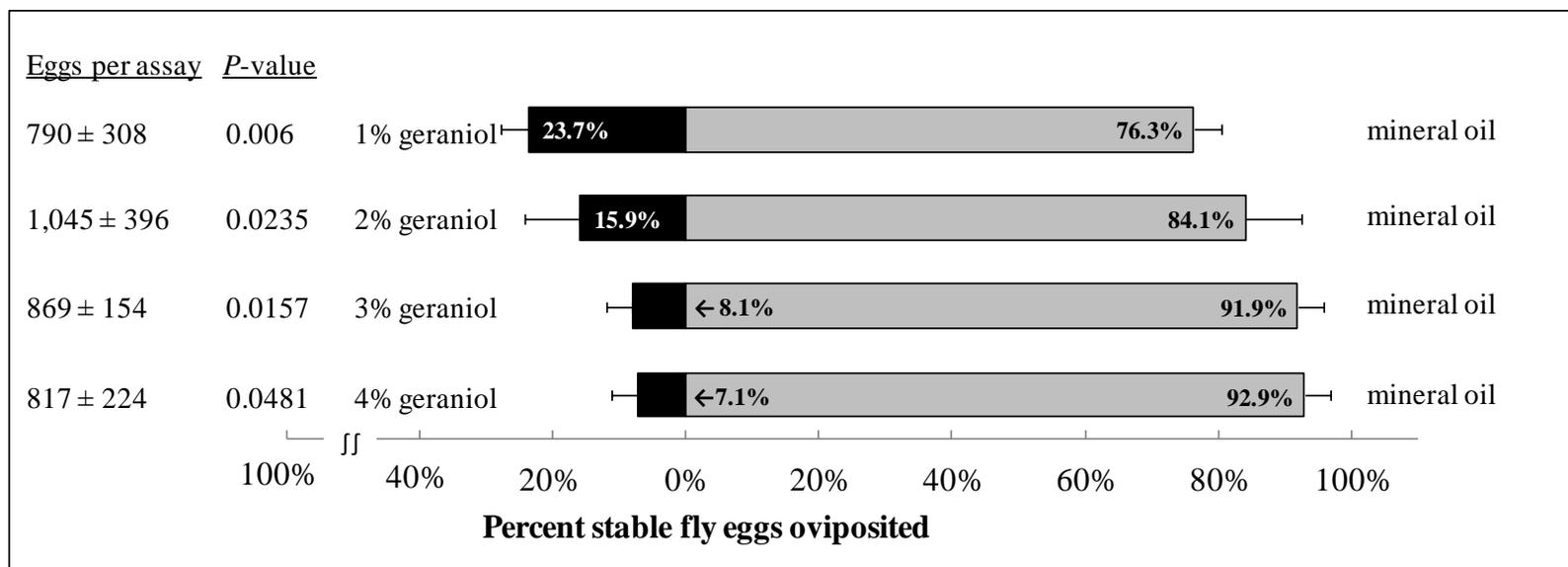
**Figure 12** Percent of specks deposited on treatment and control filter paper halves with  $\pm$  SE bars for house fly tactile repellency bioassays. Tactile bioassays conducted with 30% proprietary formulation of geraniol (FASST Products LLC, Rockville Centre, NY). *P*-values calculated with paired t-tests of proportions of specks on treatment and control halves.



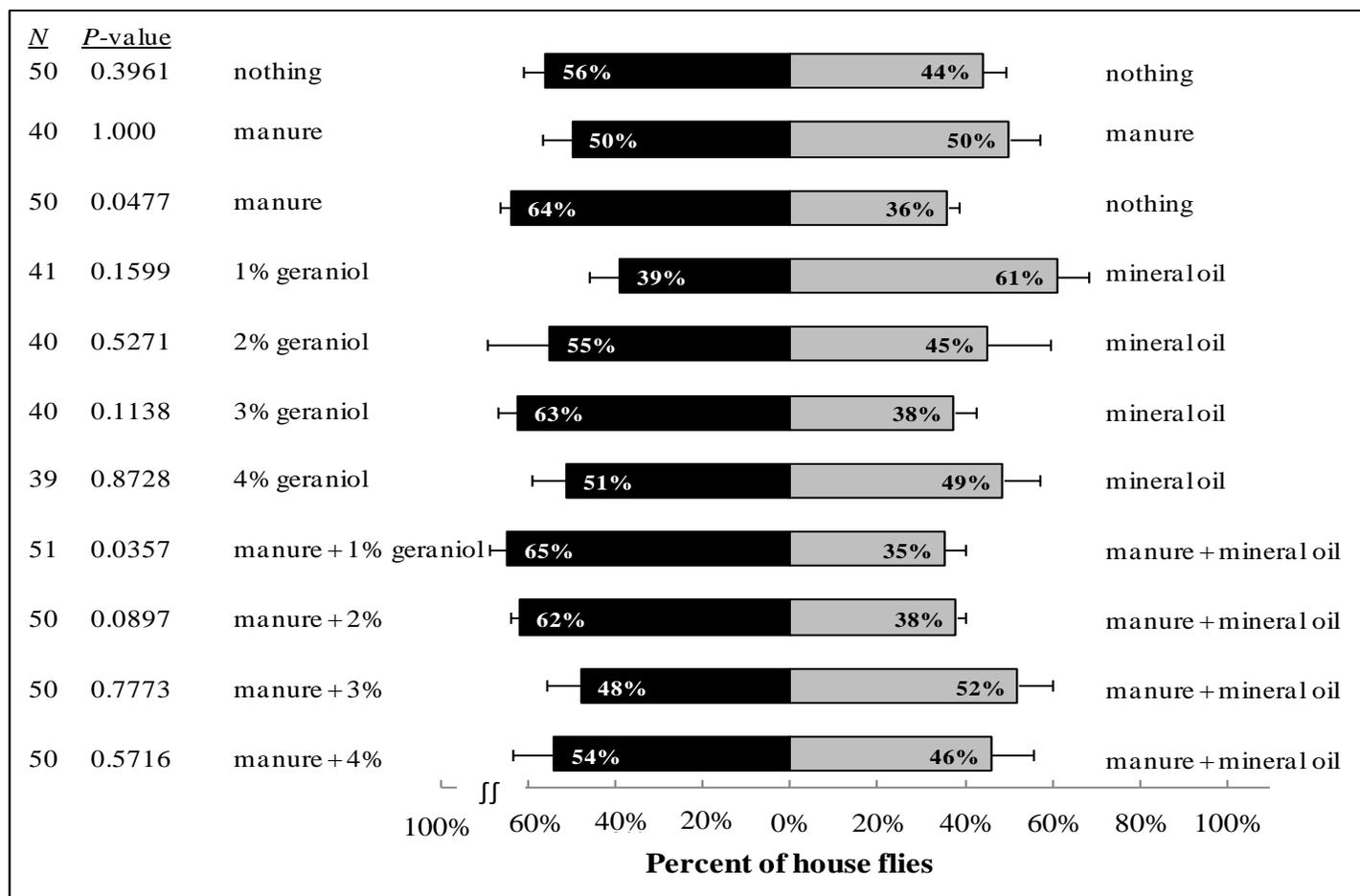
**Figure 13** Percent of specks deposited on treatment and control filter paper halves with  $\pm$  SE bars for stable fly tactile repellency bioassays. Tactile bioassays conducted with 30% proprietary formulation of geraniol (FASST Products LLC, Rockville Centre, NY). *P*-values calculated with paired t-tests of proportions of specks on treatment and control halves.



**Figure 14** Percent of eggs oviposited on treatment and control oviposition substrates (OSs) ± SE bars for house fly oviposition bioassays. Oviposition bioassays conducted with 100% proprietary formulation of geraniol (FASST Products LLC, Rockville Centre, NY). *P*-values calculated with paired t-tests of transformed proportions of eggs on treatment and control OSs, eggs per assay values were not used in any statistical analyses. Comparison between treatments were not significantly different ( $F= 1.02$ ,  $P$ -value= 0.41,  $df= 3,15$ ).



**Figure 15** Percent of eggs oviposited on treatment and control oviposition substrates (OSs) ± SE bars for stable fly oviposition bioassays. Oviposition bioassays conducted with 100% proprietary formulation of geraniol (FASST Products LLC, Rockville Centre, NY). *P*-values calculated with paired t-tests of transformed proportions of eggs on treatment and control OSs, eggs per assay values were not used in any statistical analyses. Comparison between treatments were not significantly different ( $F= 2.05$ ,  $P$ -value= 0.1773,  $df= 3,9$ ).



**Figure 16** Percent of house flies that chose treatment and control olfactometer arms  $\pm$  SE bars. Olfactometer bioassays conducted with technical grade geraniol (Sigma-Aldrich Co., LLC, St. Louis, MO). *P*-values calculated using exact binomial test.

## APPENDIX

## Appendix A

### **Effects of Geraniol and *Beauveria bassiana* Field Applications on House Fly Movement**

#### **(*Musca domestica*) in a Deep-Bedded Swine System**

Two marked house fly (*Musca domestica* L.) release and recapture experiments (summer 2011) were performed at a swine unit at the Center for Environmental Farming Systems (CEFS). The swine unit consists of 5 open hoop-style barns situated linearly (Fig. 1). Colony reared house flies were marked with luminous powders (Bioquip Products, Inc., Rancho Dominguez, CA) and released in barns weekly for four weeks (Fig. 2). Flies were caught in nithazene strips (Quik Strike® by StarBar, Schaumburg, IL) with plastic 2L buckets attached beneath. Recapture rates were recorded.

Simultaneous to fly releases, barns were treated with the insect repellent geraniol. Repellent treatments were applied twice weekly for 4 weeks at a rate of 4L per barn with a backpack sprayer. Geraniol was volumetrically diluted to a 6% concentration with mineral oil (Nevastane® #15, by Total Lubricants, Paris, France).

Two strains of *Beauveria bassiana* were applied once a week, concurrent to geraniol applications, for four weeks at a rate of  $1.076 \times 10^5$  conidia per  $\text{cm}^2$  with a backpack sprayer. Prior to each weekly application of *B. bassiana*, adult flies were collected from the each hoop barn with a sweep net (See Chapter 2, Table 3 for wild house fly infection rates and methodology).

## **Results**

Marked house flies tended to remain in the hoop barn where they were released (Table 1). Some flies moved to other barns, however the relative proportion of flies moving was small (Fig. 3). There was little indication that geraniol caused the flies to relocate.

**Table 6** Recapture rates of flies released into swine barns at the Center for Environmental Farming Systems (CEFS). Total flies represents the number of flies over four weeks that were recaptured within each barn. Percent recaptured represents the percent of flies that didn't leave the initial release barn.

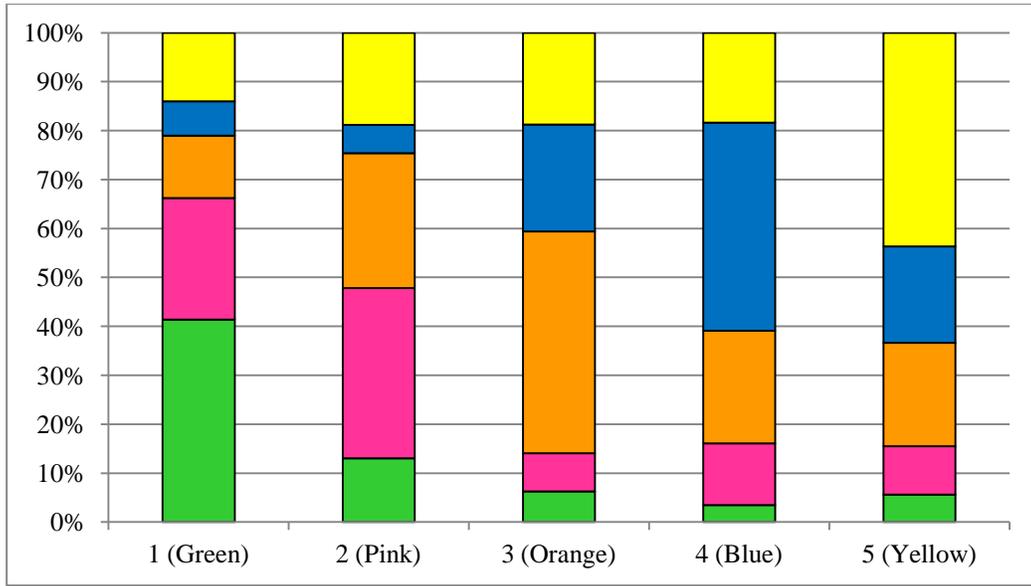
	Barn	Treatment	Pig Type	Total Flies Recaptured	% Recaptured in Release Barn
May 2011	1	Control	Sows	157	76.47%
	2	Geraniol	Sows	69	27.91%
	3	<i>Beauveria</i> P89	Sows	64	28.16%
	4	Geraniol	Finishers	87	46.25%
	5	Geraniol	Finishers	71	32.98%
August 2011	1	Control	Piglets	256	68.75%
	2	Geraniol	1 Farrowing	173	83.82%
	3	<i>B. bassiana</i> P89	Sows	186	73.66%
	4	<i>B. bassiana</i> L90	Finishers	138	59.42%
	5	Geraniol x2	Sows	116	83.62%



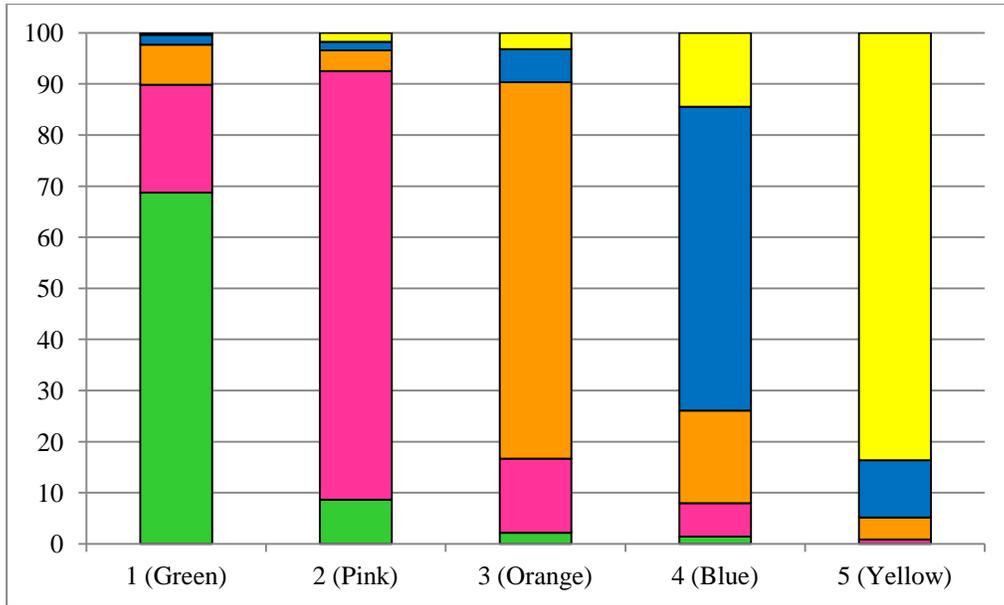
**Figure 17** Five hoop barns used in a mark and recapture study were numbered from left to right, barn numbers 1, 2, 3, 4, and 5.



**Figure 18** Colony reared flies were marked with luminous powders before they were released in each barn. Picture was taken with a UV black light, which was used to quickly identify marked flies within samples.



**Figure 19** Percent of flies captured within each barn during May 2011 field study. Colors represent original release barns (horizontal axis). For example, of the flies recaptured within barn 3, less than 10% were originally released in barn 1 (green).



**Figure 20** Percent of flies captured within each barn during August 2011 field study.

Colors represent original release barns (horizontal axis). For example, of the flies recaptured within barn 3, less than 5% were originally released in barn 1 (green).