Response of *Stomoxys calcitrans* and *Musca domestica* (Diptera: Muscidae) to Volatile Organic Compound Profiles from Ontario, Canada Dairy Farms

by

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The above committee determined that the thesis is acceptable in form and content and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate during an oral examination. A signed copy of the Certificate of Approval is available from the School of Graduate and Postdoctoral Studies.

Abstract

Stomoxys calcitrans are significant pests of dairy and beef cattle livestock facilities. Their seasonal abundance causes damage to the industry in the United States. While similar damage has been documented in Canada, the majority of the research explores population dynamics of stable flies with respect to seasonal and temporal conditions. Exploring potential semiochemical targets that impact host-parasite interactions, at livestock facilities, can give insight in creating novel integrated pest management (IPM) approaches. We have sampled Volatile Organic Compounds (VOCs) at dairy farms in Southern Ontario, to determine important components of volatile profiles at such sites. Further, we have used Gas-Chromatography-Electroantennography (GC-EAG) to identify VOCs presenting electrophysiological activity in *S. calcitrans*, for further exploration of their significance in behavioural modification. The data collected illustrates that VOC profiles taken at livestock facilities are subject to change over the summer months, and has produced novel targets for IPM programs.

Keywords: *Stomoxys Calcitrans*; VOCs; semiochemicals; electroantennography; chemical ecology

Author's Declaration

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Statement of Contributions

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication. I have used standard referencing practices to acknowledge ideas, research techniques, or other materials that belong to others. Furthermore, I hereby certify that I am the sole source of the creative works and/or inventive knowledge described in this thesis.

Dedicated to my mother, who taught me the value of perseverance even when I could not distinguish it from stubbornness. To my grandmother, who gave me the gift of patience, while I learned I sometimes had to wait. And to my uncle Cameron, who was excited to celebrate each of my progresses until the end.

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List of Abbreviations

ANOVA	Analysis of Variance
BAME	Butanoic Acid Methyl Ester
DMDS	Dimethyl Disulfide
EAG	Electroantennography
GC-EAG	Gas Chromatography-Electroantennography
GC-MS	Gas Chromatography-Mass Spectrometry
HAEE	Hexanoic Acid Ethyl Ester
HAME	Hexanoic Acid Methyl Ester
IDAC	Intelligent Data Acquisition Controller
IPA	Isopropyl Alcohol
IPM	Integrated Pest Management
OR	Olfactory Receptor
ORN	Olfactory Receptor Neuron
SSR	Single Sensillum Recording
VOC	Volatile Organic Compound

Chapter 1. Introduction

The stable fly, *Stomoxys calcitrans* Linnaeus (Diptera: Muscidae), is an important pest of both humans and livestock, which causes both medical and economic concerns globally. As a synanthropic species, the stable fly is distributed world wide, but is closely associated with stables and dairies, where they exploit resources allowing them to feed and propagate (LaBrecque, Meifert et al. 1972, Broce and Haas 1999, Talley, Broce et al. 2009). The stable fly is a haematophageous species, which requires blood meals to complete its life cycle. As a result, it feeds on domesticated animals, such as cows, horses, sheep, dogs, and chickens (Yeruham and Braverman 1995, Avancini and Silveira 2000, Koehler and Kaufman 2006, Pitzer, Kaufman et al. 2011). In Canada, and the majority of North America, preferential feeding occurs on dairy and beef cattle (Pitzer, Kaufman et al. 2011). The painful bite causes decreased milk and beef production in cows (Campbell, Berry et al. 1987, Campbell, Skoda et al. 2001). Moreover, bites can transmit infections that cause veterinary disease in the livestock (Turell and Knudson 1987, Baldacchino, Muenworn et al. 2012, Kahana-Sutin, Klement et al. 2017). The damage due to stable flies is estimated to cost up to 2.2 billion dollars annually (Taylor, Moon et al. 2012).

The housefly, *Musca domestica* Linnaeus (Diptera: Muscidae), a related pest, is similarly distributed and is a vector of infectious disease, such as shigellosis, anthrax, and cholera in human and in animals, (Keiding, World Health Organization. Division of Vector et al. 1986, Cohen, Green et al. 1991, Yap, Kalpana et al. 2008, Fasanella, Scasciamacchia et al. 2010). Like the stable fly, house flies decrease dairy productivity, in these facilities, and can also reduce egg production at poultry farms. For house flies,

the approximate cost of insecticides on poultry farms alone is $1.6 million dollars each year (Crespo, Lecuona et al. 1998). Insecticides pose risks to other species, including humans, and development of resistance is already occurring in both the stable fly and housefly (Devonshire 1975, Williamson, Denholm et al. 1993, Walsh, Dolden et al. 2001, Khan, Shad et al. 2013). Therefore, the development of novel control methods to reduce damage due to these pest is greatly needed.

1.1 Stable fly morphology and life cycle

Stable flies vary in length ranging from 5.5-9 mm—close in size to the related *M. domestica*. They can be differentiated however from house flies, by their grey colouring, checkered dark spots on the abdomen, and 4 distinctive dark stripes running vertically down the thorax. Unlike the house fly they have a non-retractable proboscis, which is specialized for piercing, tearing, and digesting blood from their hosts. Venation of the wings also differs, curving toward the r4 and r5th veins, not bending like those in the house fly.

S. calcitrans are sexually dimorphic; the female flies having compound eyes placed farther apart on the frons than those of the males. Depending on environmental factors, gravid females can lay up to 1000 eggs in their life time, either singly or in bunches of 20-200 eggs per batch (Foil, Hogsette et al. 1994). Eggs are small white ovids, measuring about 1mm in length by 0.3 mm in width. The eggs hatch in 1-4 days depending on environmental factors, such as temperature and humidity, and female retention of the eggs (Lysyk 1998). Larvae appear similar to those of *M. domestica*, about 5-12 mm long, yellow/white cylindrical maggots, with 2 triangular shaped spiracles on rear end separated by 1 ½ width of the spiracles. Once hatched the larvae will seek moist

areas to feed and grow, often digging further down into the site of oviposition. They pass through 3 instar stages becoming larger with the subsequent molt. This stage is largely variable and can last 11-30 days (Lysyk 1998), depending heavily on the temperature, quality and abundance of nutrients in the substrate, and presence of a live microbial community (Wienhold and Taylor 2012, Albuquerque and Zurek 2014). Prior to pupating, 3rd instar larvae will discontinue to feed and contract to form the puparium (Rochon, Baker et al. 2011)). The pupae are approximately 4-7mm in length, and begin a light reddish brown on the exterior, tanning as the age of pupae increases. Posterior spiracles on puparia are black with three 's' shaped yellow slits and sclerotized. This stage lasts from 6-10 days, depending on the environment, as with the larvae. Poor food sources, or below freezing temperatures can result in hibernation for 90-120 days, until conditions improve (Lysyk 1998). The average life cycle from egg to adult takes approximately 3-4 weeks to complete.

1.2 Feeding

While stable flies will feed lightly on manure and spilt feed, in addition to nectar feeding from several plants, blood meals are required for nutritional and reproductive aspects of the stable fly life cycle (Müller, Hogsette et al. 2012). Bovid species appear to be the preferred host in the US, UK and Canada, reports have noted stable flies can subsist on blood meals from horse, chicken and pig species as well. Equine blood seems to be the secondary preference in the west; and although *S. calcitrans* show reduced fecundity when fed on horse blood meals, larval development increases when oviposition occurs in horse manure rather than cattle manure. A study performed by J.B. Pitzer et al. (2011), used blood meal analysis to determine stable flies fed on significantly more on cows

(70%), then horse (26.3%), humans (10.3%) and finally dogs (1.7%). This occurred even when stable flies were collected near to horse stables.

Reproductive females must have at least 3 blood meals before depositing her eggs, and fewer than this results in eggs maturing only to the first stage of development. Accordingly, males who do not feed on blood show decreased or no sperm transfer (Jones, Milne et al. 1992, Tangtrakulwanich 2012) . This creates a demand for a source of blood that is available and consistent during their greatest periods of seasonal activity (May—September) (Taylor, Berkebile et al. 2007). *S. calcitrans* are strong fliers, with the ability to travel up to 3.2 km from their breeding site, to take a blood meal (Pitzer, Kaufman et al. 2011). Stable flies display daytime feeding, and most activity occurs in a diurnal pattern between 10 AM – and 4 PM. The flies can take from 1-5 minutes to feed, however defensive behaviour on part of the host may dislodge the flies, in which case they may feed several times on the same or many hosts (Schofield and Torr 2002, Gerry 2007, Tangtrakulwanich 2012) .

1.3 Host orientation

Stable flies locate hosts through a complex combination of olfactory, and visual cues, both of which are modulated by physical cues from the environment, such as temperature, humidity, background odour, and time of day. Host orientation of haematophagous flies can be broken down into 3 behaviours: activation, orientation or ranging, and visual identification of the food source (Gibson and Torr 1999). Activation and ranging of host seeking flies, both include olfactory components.

1.3.1 Activation

Activation is an increase in flight activity and behavioural shift towards seeking responses in mature flies. In haematophagous flies, activation largely occurs in response to an abundance of carbon dioxide—a cue indicative of host metabolism, given off in large quantities in the host's breath. Activation to carbon dioxide, acetone, and human breath increase landing responses in stable flies (Warnes and Finlayson 1985). Human skin and breath volatiles also activate stable flies and increase their orientation and ranging (Alzogaray and Carlson 2000). It is important to note that studies in mosquitoes indicate that carbon dioxide alone, while causing activation and some host orientation is not enough to keep promoting upwind flight at long ranges (Hoel, Kline et al. 2007). Specifically the source of the carbon dioxide is not found, but rather followed, until a host odour arises in the plume. A similar response is seen in *Stomoxys spp*. Alzogaray and Carlson (2000), demonstrated that flies were significantly activated by low doses of carbon dioxide (0.0001ml s^{-1}), but only showed significant orientation at any but high doses of carbon dioxide (0.001-0.01 ml s^{-1}). An individual cow of 500 kg can emit between 33.3 ml s^{-1} and 46.6ml ml s^{-1} of carbon dioxide during the day, which greatly exceeds the highest amount tested in this study (Torr, Mangwiro et al. 2007).

1.3.2 Orientation

Stable flies show a demonstrable preference in host species and some individuals in a species will gain more biting attacks than others (Warnes and Finlayson 1987, Torr, Mangwiro et al. 2006). It has been suggested that increased avoidance behaviour displayed by the host has some effect on decreasing *S. calcitrans* biting preferences. However, it is clear that odour and attractant discrimination also play a role in species

preference, and host individualization. For example, while *S. calcitrans* show some activation to human breath and skin volatiles, their behavioural response to compounds known to be attractant to mosquitos—whose primary host is humans— can differ significantly. Similarly, while studying *Glossina spp*. (Gikonyo, Hassanali et al. 2003), demonstrated a difference in skin odour profiles between waterbuck and the non-preferred hosts—ox and buffalo. Studies using traps baited with various host odours, have given insight into kairomone mediated anemotaxis. Vale (1985) showed that using ox odour to bait traps increased the catch of *Stomoxys calcitrans*, while filtering the air reduced trap catches. More recent studies have used GC-MS to directly identify compounds in rumen digestia (Jeanbourquin and Guerin 2007) and cattle manure slurries (Tangtrakulwanich, Chen et al. 2011, Tangtrakulwanich, Albuquerque et al. 2015). In olfactometer and field studies, key compounds were found to be attractive both singly and in mixtures and included, indole, cresol, 4-ethyl phenol, and octanol. Notably, mixtures of the compounds tended to be more attractive than single compounds. Torr (1990), also demonstrated the additivity of attractive compounds such as acetone, carbon dioxide and octenol, can increase trap catches in the field. Comparably, Hieu et al. (2013) showed that there are detractive effects when adding repellent compounds to attractive ones. Adding another layer, studies have determined that not all host odours arise from the host; kairomones can also be present in VOCs given off by symbiotic or parasitic species living on the host—such as the skin microflora (Braks, Anderson et al. 1999). Odours originating from the bacteria *Peptococcus indolicus*, the causative agent of mastitis infections, attract *Hydrotaea irritans* (Diptera: Muscidae) to cows (Thomas, Schomaker et al. 1985). Additionally, phenolic compounds associated with buffalo urine,

attracts members of *Glossindae spp.* and can come from *Aerococcus viridans* (Okech and Hassanali 1990). In stable flies, fecal coliforms are higher when flies are most prevalent, and oviposition is more likely to occur in manure that has a bacterial presence, over those that have been sterilized (Romero, Broce et al. 2006). Taken together the results indicate the complexity of host odours in the natural environment, and the behavioural effects exhibited by flies towards them.

1.3.3 Visual Identification

Stable flies exhibit visual sensitivities in both the UV and visual range of the spectra, with peaks specifically at 360 nm, 450-550nm, and a plateau at 625 nm (Agee and Patterson 1983). Studies demonstrate that flies are in fact phototaxic to blue wavelengths, and trap catches can be improved using phthalogen blue cloth in Nzi traps (Allan, Day et al. 1987). Colours of traps are known to alter the efficacy of trap catches, as well as reflectance of light off the trap surface (Cilek 2003, Beresford and Sutcliffe 2006). A study by Beresford and Sutcliffe (2006), showed that white Coroplast cards traps are more effective than Alsynite traps, especially when paired with direct adhesives such as tangle trap, rather than adhesive sheets. Shape and orientation of traps also have an effect on effectiveness of catches and this can vary between individual species. For example, catches of *Stomoxys spp.* decrease in response to shape, with horizontal rectangular shapes being the most likely to trap flies, whereas circular traps were more effective for tsetse flies (Gibson and Torr 1999). Still, unlike tsetse flies, stable flies are more likely to arrive at an odour source regardless of the presence of a visual target, which underscores the importance of effective baits for such traps.

1.4 Pestilence

S. calcitrans are serious pests of mammals; including cows, horses, and humans. As a result of their painful bite, stable flies are one of the most damaging arthropod species, causing up 2.2 billion dollars in economic damage each year in the American agricultural industry (Taylor, Moon et al. 2012) and up to 26.8 million in Canada (Colautti, Bailey et al. 2006). They are a key cause of loss in beef cattle weight gains, in addition to decreased production of milk in dairy cattle (Campbell, Berry et al. 1987, Campbell, Skoda et al. 2001). *Stomoxys calcitrans,* belong to the family Muscidae, also known as the 'filth flies', and pose a threat to humans as the potential carriers of harmful infectious bacteria such as *Bacillus anthracis* (Turell and Knudson 1987) and African Trypanosomiasis (D'Amico, Gouteux et al. 1996) .

Stable flies have been previously associated with dairy and cattle farms, where the livestock are typically kept in enclosed stables. Over the past 2 decades however, they are becoming a growing concern in open pastures, due to the increased prevalence of round hay bale feeding sites (Talley, Broce et al. 2009). Reproduction of the stable fly is dependent on many factors such as oviposition site, temperature for larval development, pH, and moisture of hay-manure layers. *S. calcitrans* preferentially deposit eggs into moist and decomposing organic material, which makes these feeding sites ideal as the feed becomes combined with accumulating manure. Generally, the flies are found feeding on the lower flanks of the animals, including the lower legs (Gerry 2007). Irritation of the cattle by the flies causes stomping, 'tail flicking', and in extreme infestation, cattle will form protective groups in order to relieve themselves from the bites (Mullens, Lii et al. 2006). This behaviour means cows are not feeding, and also contributes to heat and

contact damage. Wieman, Campbell et al. (1992) showed stable flies are the cause of up to 28% of the weight lost by cattle, due to biting and energy needed by cattle to evade them. The rest of the damage is due to bunch of the cows and the heat stressed caused by this. In addition, consistent biting of cows can lead to dermatitis and the thinning of skin and hair of calves (Yeruham and Braverman 1995). Bite wounds on the cows act as oviposition sites for other flies, leading to myasis of the affected area (Foil, Hogsette et al. 1994). Extreme swarming and repetitive biting of the flies can also cause blood loss and anemia of the cows (Foil, Hogsette et al. 1994, Baldacchino, Muenworn et al. 2012). Dairy cattle exhibit similar fly repelling behaviour, and milk production can be decreased by 1.49 kg/ day (Kaufman 2002). It has been shown that up to 5 flies per leg is sufficient to cause serious economic damage, with a minimum of 2.5 flies listed as the economic injury level (Campbell, Skoda et al. 2001). In addition to decreased beef cattle and milk production, the damage to cow and horse hides caused by *Stomoxys spp.* puncturing the skin, is also a relevant economic concern (Gerry 2007). During the spring and summer months when stable flies are prevalent, milk and buttermilk production is depressed, while in farms with good pest control in effect, production is increased (Kaufman 2002).

1.5 Integrated Pest Management

Integrated Pest Management (IPM) is the globally accepted policy put in place to consciously reduce the risks of crop loss, due to arthropod and other pests. Increasing reliance on chemical pesticides has caused pronounced effects on agro-ecosystems, and are potentially damaging to human health. Thus, IPM can also be understood as the set of

protocols put in place to manage and decrease use of potentially harmful synthetic pesticides (Ehler 2006, Pimentel and Peshin 2014). Generally, IPM is a process that uses a range of techniques to suppress pests below agricultural economic injury level in an efficient, but environmentally conscious and economic way (Ehler 2006). Central to the use of IPM is identifying and monitoring pest populations, followed by implementing appropriate controls to keep pests below the acceptable thresholds.

IPM, as the name implies, makes use of several different types of preventative, and active pest management controls, which are categorized based on the technique of pest reduction; these control methods include: cultural, mechanical, biological, behavioural, and chemical controls (Norris and Kogan 2003). Control methods are best used in conjunction with each other to mitigate the pitfalls in any individual method.

1.5.1 Cultural Control

The cultural management technique places emphasis on altering the way a crop is grown in order to make it less susceptible to the pest or maximize its suitability for natural predators of the pest. Cultural management targets the prevention of transportation of new pest populations into the area. This can occur through active transport, where the pest physically moves into the area of protection, or passive transport including wind dispersal or human activity (Norris and Kogan 2003). Sanitation plays a large role as a method of prevention, which is an important reason why crop/ food removal is advised where they present ideal conditions for eggs or immatures to be harboured (Anthony 2005). Also key, is the control of alternate hosts at nearby risk areas, which can harbour pests, and upregulate their presence and density elsewhere. It is notable that many

cultural practices are geared specifically to plant insect-interactions and do not apply to animal/host interactions (Norris and Kogan 2003). Ultimately, the goal is to reduce the level of impact below economic injury level.

1.5.2 Mechanical Control

The methods which result in the killing or exhaustion of pests, through the alteration of their typical environment. Mechanical methods work primarily through the direct modification of the pests' optimal environment, adding physical barriers, and using physical removal. Environmental modification methods include those which seek to increase or decrease temperatures, access to water, and light. An example might be the complete desiccation of removed manure and crop waste. Physical removal methods work by actively seeking and killing pests by: trapping, tillage, drowning, burning and shooting.

1.5.3 Biological Control

Biological control of pests is centred on the principle that organisms within an ecosystem or agrosystem, interact with each other in trophic hierarchy that modulates feeding interactions. The pests that feed on the crop of importance then, will also have natural enemies or predators, which can be exploited for population control (Norris and Kogan 2003). Classical biological control is the principle of finding these natural enemies, native or exotic to the area, and exposing them to the pest population (Foil, Hogsette et al. 1994, Hogsette 1999). Beneficial organisms—endemic natural enemies of the pest of concern—may already be present in proportions insufficient to provide full control of the pest, but

exotic agents need to be investigated for their safety in a new environment. Biological invasions are closely associated human activities whether direct or indirect. In many cases bioagents are screened for their success in an environment, and can easily invade, disrupting the native habitat (Alpert 2006). An example is the ladybug *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), which has been introduced to North America for biological control strategies of *Aphidoidea* species. Having a highly non-selective diet, which can include the larvae and pupae of other Coccinellids in addition to other non-target species, *H. axyridis* poses direct competition to endogenous insects. Yet, although it is an avid feeder, it is often ineffective for control due to its long life cycle relative to pest species, and propensity to disperse.

Biological control programs exploit interactions between the beneficial organism and the pest, therefore many subtleties must be considered. Parasitoid wasps are at the forefront of biological pest control programs for house flies and stable flies, especially *Spalangia cameroni* Perkins, *Spalangia endius* Walker, and *Muscidifurax raptor* Girault & Sanders (Hymenoptera: Pteromalidae) (Petersen and Meyer 1985, Floate and Skovgård 2004, Skovgård 2004, Skovgård and Nachman 2004). Adult wasps oviposit eggs in pupal cases of house and stable flies, where the wasp larvae will continue to develop until maturity. Because of its complex life cycle there exists a lack of mass rearing protocols, rendering it difficult to grow in labs (Foil, Hogsette et al. 1994). Compared to typical larvicidal controls, implementing biological control with these species is expensive, and there is little agreement about the correct ratio of parasitoids needed to effectively control dipteran pests (Petersen and Meyer 1985, Axtell 1986, Skovgård 2004). There are also

inconsistencies regarding target specificity and extent of the effect on house flies and stable flies. A study by Skovgård and Nachman (2004), showed that house flies are readily targeted and knocked down by control programs using *S. cameroni*, while stable flies are not. Yet other studies, showed that *S. cameroni* is more likely to parasitize *S. calcitrans* pupae (Petersen and Meyer 1983).

Integration of biological control methods including these wasps also require careful consideration. *S. cameroni* and *M. raptor*, due to their predation on dipteran larvae, live in similar environments of manure and decomposing feedstock. Since the wasp larvae have longer development times than their dipteran hosts, cultural controls that include removing manure decrease the total population of the adult parasitoids (Axtell 1986). In addition, both species are more susceptible to insecticides and larvicides than *S. calcitrans* and *M. domestica* (Crespo, Lecuona et al. 1998). Applying larvicides to potential incubation sites kills immature stages of wasps as well as pests, but dipterans recover quickly owing to their relatively short life cycle (Crespo, Lecuona et al. 1998). The program should also account for the lag occurring between the introduction of the agent, and its population growth and dispersal. Due to the length of development, wasps should be released prior to increased *M. domestica* and S. *calcitrans* population growth in mid-summer (Crespo, Lecuona et al. 1998, Skovgård 2004). Even with population growth, dissemination of wasps can occur on large dairies, such that necessary quantity of parasitoids needed to decrease flies were low. Also, biological control can be sensitive to many parameters such as temperature, and competition of species to resources. For instance, many species of the parasitoid wasps display low or no survivability of Canadian winter months, and use would therefore be limited to one season without

reapplication (Floate and Skovgård 2004). While some protocols have shown success, further investigation is needed understand the intricacies of their implementation.

1.5.4 Behavioural controls

Behavioural controls are the set of controls which use the pests natural responses to a signal or stimulus, to control or monitor pest populations. Within the scope of insects this mostly applies to visual and olfactory based tactics; however, more broadly behavioural control looks at how pests use their senses to exploit and modify the behaviour harmful to the crop. This can include the use of semiochemicals for monitoring pest populations, pheromones for the interruption of mating and reproduction (Norris and Kogan 2003). Behavioural approaches can in some cases be more effective than pesticides, as in the case of protecting orchards from apple maggot flies *Rhagoletis pomonella* Walsh (Diptera: Tephritidae) by the use of odorant butyl hexanoate (Prokopy, Wright et al. 2001). This is especially true when trapping and killing methods can be combined with the use of attractants or pheromones as a bait (Chapman, Knapp et al. 1998, Mihok, Carlson et al. 2007). Uses of plant volatiles or other repellent odorants, can also contribute to control (Schreck and Kline 1983, Müller, Junnila et al. 2009). J.J. Zhu et al. (2010) found that catnip oil could provide effective spatial repellence of stable flies on dairy farms, though effectiveness was reduced three hours after application. This being due to the volatility of the repellents.

One benefit of behavioural controls is their potential for target specificity. In one study, traps baited with trianary blend of sex pheromones of the dogwood borer, *Synanthedon scitula* Harris (Lepidoptera: Sesiidae), was able to increased catch specificity to 97%. In houseflies, one pheromone—9-tricosene—has been identified and

exploited for commercial use; but attracts only male flies (Chapman, Knapp et al. 1998). Similarly, cuticular hydrocarbons in female stable flies, attract males and stimulate mating (Uebel, Sonnet et al. 1975). Studies investigating Stomoxyine sugar-feeding on fruit and flower targets, demonstrated variance in attraction of *S. calcitrans*, *S. niger bilineatus*, and *S. sitiens* to certain targets. For instance, of five fruit tested, the three species were all significantly attracted to Camel's Foot fruit *Piliostigma reticulatum*, but only 2 species, *S. niger bilineatus* and *S. Sitiens*, were attracted to Tamarind pods from *Tamarindus indica* (Müller, Hogsette et al. 2012). Results like these ones can indicate that with more insight into mechanisms surrounding attraction and preference, behavioural controls can be a good candidate for pest control programs.

1.5.5 Chemical Control

Chemical control is the direct application of chemicals to an area to prevent, incapacitate or kill pests to mitigate damage done to the crop or livestock. There are various types of chemical controls, which target a variety of organisms, and stages of their respective life cycles. In some cases, pesticides can be easier and cheaper to apply than the other pest control tactics and they have the benefit of rapidly knocking down pests where resistance hasn't been developed. Despite this, they can frequently cause harm to the ecosystem, especially when applied incorrectly.

Together cultural, mechanical, and biological controls are sought to lessen the impact of chemical controls on the environment and on the financial burden of farmers. In accordance with these practices, regular removal of feed and manure are advised to

cattle and dairy farms, but this can be time consuming and costly. It is estimated that 226 million dollars are wasted yearly on cattle feed due to this preventative practice (Taylor, Moon et al. 2012). Many farms also employ the use of Alsynite fiberglass sheet sticky traps, which were shown to provide only minor control in infestation, due to saturation of the adhesive surface area (Gilles, David et al. 2007). Other common traps exploit phthalogen blue cloth which is known to stimulate the flight of stable flies, but are less efficient in control, catching only older populations of flies (Taylor and Berkebile 2006, Gilles, David et al. 2007). Natural parasites of stable flies and horns flies are also commercially available, however are less effective in open spaces, and are less applicable to range cattle and dairy farms (Lysyk, Kalischuk-Tymensen et al. 2010).

Similar to cultural controls, there are many ways to implement chemical controls around farms and stables, which include space sprays, residual sprays, larvicides, chemical tags, and whole animal sprays. Whole animal sprays which constitute one of the more widely used methods, present a few problems. First, stable flies mainly feed on the lower legs of the animal, which is the area where the spray is frequently subject to removal as the animal walks through wet grass, or standing water (Taylor and Berkebile 2006). Secondly, as flies do not spend more time on the cattle than it takes to feed (1-5 minutes), they may bite several animals, or several times before the insecticide is able to take action (Schofield and Torr 2002). Additionally, there is a limited amount of these chemical sprays which are approved on all animals, especially those which are lactating, due to suspected toxicity (Gerry 2007). The primary distribution of insecticide by insecticide impregnated ear tags is on the face and back of the animal, however self-grooming and other interactions help spread the insecticide over the body of the animal.

Previous studies have shown that fenvalerate and permethrin tags are helpful in dissuading other haematophagous flies and filth flies, such as horn and face flies from feeding (Haufe 1982). This area of permethrin distribution is outside of the characteristic bite pattern of stable flies however, and the use of insecticide impregnated tags is insufficient to aid in cattle relief from *S. calcitrans*. The usage of space sprays (synergized permethrins for example), is effective for the rapid knockdown of stable flies, but can only be used at specific times within the season and at intervals during the day when flies are known to congregate and be inactive. Space sprays have low residual effects, lasting up to two days, and need to be reapplied frequently to gain control (Kaufman 2002, Rutz et al. 1994). Comparatively, residual sprays used where stable flies are likely to land, are rapidly gaining resistant populations in *S. calcitrans* (Gerry 2007). Control of stable fly population, is also attempted by the application of larvicides either directly to manure, or by administration to the animal to be excreted in a controlled method. Larvicides can employ the use of biological controls, wherein bacteria known to cause harm to the pest is applied to manure, or where the application of chemical compounds to the manure causes the same result (Miller and Chamberlain 1989, Lysyk 1998, Hogsette 1999, Lysyk, Kalischuk-Tymensen et al. 2010). While larvicide treatments show promise, they may cause harm to other commensal species of insects (Gerry 2007).

1.6 Insect sensory system

Chemoreception, the organism's ability to perceive and interpret chemical cues in their environment either in the form of gustation or olfaction may be one of the oldest senses evolved. This is because the mechanisms between taste and scent are similar; however,

olfactory organs typically receive gaseous chemosensory cues (Zacharuk 1980). In the insect, the main organs of olfaction are the antenna—paired symmetrical appendages found on the head—and to a lesser extent the maxillary palps. Flies of the family Muscidae have an aristate antennae, morphologically composed of 3 segments: the scape, the pedicel, and the flagellum which is attached dorso-laterally to a feather-like arista (Sukontason, Sukontason et al. 2004). Both the antenna and maxillary palps bear sensilla, which are sensory hairs housing olfactory receptor neurons. Each of the antennal segments have sensilla, though the flagellum segment has more both in number and classification (Vosshall and Stocker 2007).

Sensilla structure and shape varies across and even within insect families, and there is not consensus on both morphological identification and function of sensillum types. Nevertheless, in Muscidae, classification seems to align with presence of 3 distinct sensilla: trichoid, basiconic, and coeloconic (Albuquerque and Zurek 2014). While all three types have been described as having olfactory function, some studies have suggested due to the absence of cuticular pores, coeloconic sensilla do not serve a chemoreceptor role but instead function as mechanoreceptors (Tangtrakulwanich, Chen et al. 2011). Regardless, trichoid, basiconic and coelconic sensilla have all been described in Muscid flies, including *Stomoxys calcitrans* and *Musca domestica*. Two more types, the clavate, and the sensory pit, have been described in *Stomoxys calcitrans*. While basiconic sensilla have been reported as the most abundant type of sensillum in Calliphoridae spp., including *Chrsyoma megacephala*, *Chrysoma ruffifaces*, *Chrysomya nigripes* and *Lucillia cuprina*, and Muscid species including *Musca domestica*, trichoid sensilla are most abundant in male and female stable flies, with basiconic sensilla being secondary

(Sukontason, Sukontason et al. 2004). Trichoid sensilla in stable flies exhibiting sexual dimorphism with the amount of medium trichoid sensilla being more numerous in males of the species (Tangtrakulwanich, Chen et al. 2011). This may account for difference in olfactory response between female and male stable flies, and support the proposal of pheromone signalling and communication in the species (Muhammed et al, 1975). Sensory pits are less abundant in Muscids, with stable flies having only 2 versus 3 or more in other species (Lewis, 1971). This is thought to be due to an increased reliance on visual cues over odour in these species, which corroborates findings that *Stomoxys calcitrans* uses both odour and visual cues, in host seeking.

Regardless of the structure each sensillum is a sensory hair, containing pores through which chemical stimulants can travel, and contains up to four olfactory receptor neurons (ORNs); the dendrites of which, extend to the surface of the sensillum (Figure 1.1). The basal portion of the ORNs extend into the lymph of the sensillum and conserved olfactory receptors (OR) in the membrane (Couto, Alenius et al. 2005, Vosshall and Stocker 2007). The basal portions integrate into the olfactory glomerulus combining with like receptors, and many glomeruli integrate at the antennal lobe to signal to higher brain centres (Vosshall and Stocker 2007).

When an odour is received in a column of air, odour binding proteins in the sensillum lymph bind odorants and transmit it to membrane bound olfactory receptors on the olfactory receptor neurons (ORNs) (Sánchez-Gracia, Vieira et al. 2009, Olafson 2013). This process is aided by a co-receptor Orco, whose dimerization results in the production of a ligand gated ion channel. The receptors function similarly to g-protein coupled receptors and their activation signals for the firing of action potentials—electrical

signals that can be detected and recorded by an electroantennographic device (Vosshall and Stocker 2007).

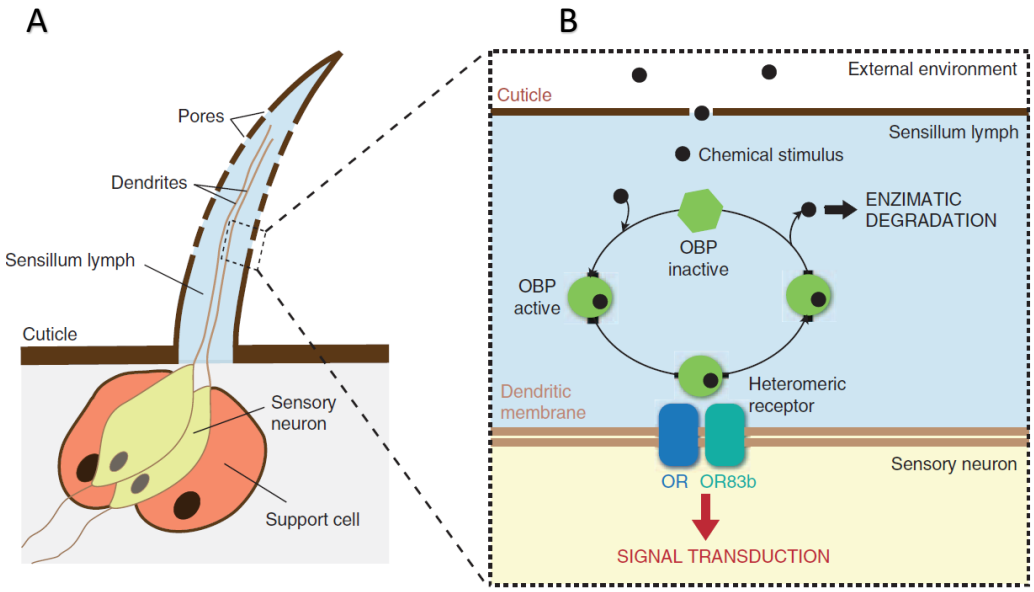

Figure 1.1. A) schematic of the insect olfactory sensillum structure from Sánchez-Gracia, Vieira et al. (2009). B) sensillum house binding proteins in the sensillum lymph, which aid in the transport of odorants to receptors located in olfactory receptor neurons. Binding of the chemostimulant leads to transduction and integration of the signal at higher brain centres.

Action potentials in the brain are the biological basis of integrating information from the periphery, with reaction and modifying responses from the central nervous system (CNS). Information-carrying scents that are received at the antenna need to be integrated in the CNS of the fly before any modification of behaviour occurs. In electrophysiological experiments, it is the action potential that can be detected for measurement and further study.

1.7 Semiochemicals

Semiochemicals are a group of information carrying molecules that induce behavioural changes in the organism that receives them (Kline 2007). Often these chemicals are volatile organic compounds (VOCs)—compounds with high vapour pressures that are typically associated with scents or types of gaseous emissions—and are released by most organisms including plants and humans (Filipy, Rumburg et al. 2006, Betancourt, Krebs et al. 2013, Stadler 2013, Chaudhury, Zhu et al. 2014, Zito, Dötterl et al. 2015). They are divided into two groups: those that evoke a response within a species are pheromones, while those that evoke a response across different species are termed allelochemicals. The intraspecific pheromones typically prompt behaviours that are physiological in nature; they either prime insects to undertake physiological changes leading to behavioural changes, or cause immediate responses—typically involved in mating or reproduction (Alaux and Robinson, 2007). Allelochemicals are different in that they provoke behavioural changes across different species. The allelochemicals are further classified, by the object which emits the chemical, and who benefits from the emission. As described in section 1.1 flies are able to detect host odours, which they may use to navigate towards a food source. Certain chemicals in these odours could be therefore be described as kairomones for the species, as they are emitted by the host, and they benefit the receiver—in this case the haematophageous fly. In general, these compounds can also be classified by the specific behaviour or taxes they induce in the species that receives them. For example, a chemical that cause the orientation of an insect towards the emission is an attractant, and in the reverse, one that causes the orientation of an insect away from the emission is a repellent (Hedin, Maxwell et al. 1974).

Because of their ability to affect the behaviour of organisms there are the newer target of pest management approaches with a few benefits. As they are received and targeted only by sensory systems, they are less harmful than pesticides which often target vital pathways of pests causing death, and resistance is less likely, as is toxicity in the host (Norris and Kogan 2003). Semiochemicals are ubiquitous in biological systems; for example, many repellents arise from plants as natural defence mechanisms. Still, the synthetic versions produce the same behavioural responses in the organisms as they would if derived from the natural source; a trait which is useful to exploit, as it means compounds can be mass-produced for behavioural control. Plant essential oils can be more repellent than diethyltoluamide (DEET) an insecticide and active ingredient in common insect repellents, especially in stable flies (Zhu, Wienhold et al. 2013). Several studies done on livestock facilities, show promise for the repellency of various essential oils.

As an argument for the use of attractants, baits for traps can be easily be produced in concentrations above natural levels, for species who show augmented anemotaxic responses to higher concentrations of host kairomones, such as is seen in Glossina. Moreover, since semiochemical profiles vary across species, baited traps, and push-pull systems can be produced to have higher specificity decreasing harmful effects in the ecosystem. In mosquitos, it has been shown that attractiveness to human hosts differs not due to repellents secreted by hosts, but rather an attractance to certain human odours and VOCs emanating from the skin microflora (Braks, Anderson et al. 1999) . Regardless of attractance or repellency it is clear that insects are extremely sensitive to the presence of

semiochemicals in their environment. Garnering insight into the mechanisms by which this occurs, will be beneficial in the modification of insect behaviour.

1.8 Objectives

Stable flies are present during the summer in large amounts at dairy and cattle farm operations, and their presence overwhelms the cows decreasing the overall yield of the crop. While efforts have been made to understand how volatile organic compounds play a role in attracting stable flies to these facilities, previous studies have demonstrated differential behavioural responses by location and a host of other environmental factors. Therefore, the overall aim of the project was to characterize profiles of VOCs across dairy farm and to understand the effect they have on the presence of stable flies at these facilities, along with the nuisance fly *Musca domestica*. It was also the goal, to study whether the composition of these VOC profiles change during the presence of stable fly activity and to determine the effect this contributes to stable fly presence. This was achieved by:

1. Identifying compounds that were consistent in the profiles of dairy farming facilities across southern Ontario. The aim was to characterize the occurrence of the compounds at the farms, elucidate whether the profiles were affected by external environmental factors, and in turn study how this affected the seasonal abundance of *S. calcitrans* and *M. domestica*. Accordingly, air samples collected from several dairy farms in Ontario, aided in the identification of reoccurring compounds, by comparative analysis of the rumen volatile profiles.
2. Identifying key compounds in the volatile profiles that elicited a physiological response with the use of electroantennography. Electroantennography was further

used to characterize and quantify the physiological effect of sampled volatile organic compounds and their synthetic analogues on *S. calcitrans* and *M. domestica*.

3. Isolating active compounds known to produce antennal stimulation, dose response curves were created in order to understand how the concentration of the VOCs affect the physiological responses. Through these experiments, the quantification of response thresholds and receptor saturation levels was possible, allowing for the determination of a range of physiological activity. With the resulting information, GC-EAG studies could be used to aid in identifying the importance of each of the selected compounds in a biologically relevant context.

Chapter 2. Investigation of VOC Profiles at Dairy Facilities and Effect on *Stomoxys calcitrans* and *Musca Domestica*

2.1 Introduction

Stable flies are obligate haematophageous insects that cause significant damage to livestock on dairy and beef cattle farms in Canada (Colautti, Bailey et al. 2006).The combination of consistent access to hosts as well as moist and decaying feed for oviposition makes livestock facilities the perfect environment for flies to complete their life cycle (Wienhold and Taylor 2012). Not only can the flies cause damage to cows through painful bites, they can also cause injurious veterinary disease, which include shigellosis, trypanosomiasis, and lumpy skin disease (Baldacchino, Muenworn et al. 2012, Kahana-Sutin, Klement et al. 2017).

A closely related fly species, *Musca domestica* L. (Diptera: Muscidae), is also found in abundance on dairy farms. While they do not bite, these flies are also capable of causing disease to both human and livestock. Associated with the manure in which they oviposit, they act as mechanical vectors for the spread of dangerous diseases like anthrax, salmonellosis, and shigellosis, making them a risk for causing serious harm to cows and humans (Cohen, Green et al. 1991, Levine and Levine 1991, Ugbogu, Nwachukwu et al. 2006). Moreover, studies have demonstrated *M. domestica* resistance to organophosphate and carbamate pesticides, as well as those in the pyrethroid class (Devonshire 1975, Williamson, Denholm et al. 1993, Walsh, Dolden et al. 2001, Khan, Shad et al. 2013). Like stable flies, house flies have well developed olfactory systems. Housefly antennae and sensilla are morphologically similar to stable flies and other Muscid flies (Sukontason, Sukontason et al. 2004, Smallegange, Kelling et al. 2008). Thus, their sensitivity to olfactory cues allow them to search for food, (Chapman, Knapp et al. 1998)

mates, and oviposition sites (Jiang, Lei et al. 2002, Tang, Zhang et al. 2016). For example, house flies have been shown to display avoidance to chicken feces inoculated with fungi, potentially in response to fungus emitted semiochemicals, dimethyl trisulfide, and 2-phenylethanol (Lam, Tsang et al. 2010). This speaks to the potential of VOCs to significantly impact behavioral decisions made by dipteran species.

Volatile organic compounds associated with cattle, can come from a variety of sources, excretions, such as manure and urine; gaseous emanations, such as the breath; skin secretions; and other metabolic functions (Birkett, Agelopoulos et al. 2004, Hobbs, Webb et al. 2004, Filipy, Rumburg et al. 2006, Sun, Trabue et al. 2008). Crops, feed, and bedding, in addition to waste management methods such as flushing, make dairy farm facilities a major source of odor that can be detected from downwind of the site (National Research Council 2003). One cow weighing 500kg can produce CO_2 at a rate of $2.0Lmin^{1}$ in the morning, and $2.8Lmin^{-1}$, in the afternoon (Torr, Mangwiro et al. 2007). With dairies of larger herd sizes, the accumulation of VOCs could easily draw attraction of insects whose life cycles are dependent on the sources from which they come. Thus, understanding the volatile profiles that arise from such sites can lead to important insights about semiochemical mediated attraction in pest fly species.

This knowledge is valuable as traps are still a popular method of insect control. Alongside pesticides, baited traps are more effective for knock down of pest fly populations than those without any attractant (Torr 1990, Mihok, Carlson et al. 2007). Appropriate attractants can also lead to the specificity of the insects being trapped, hence beneficial insects remain unaffected (Leskey, Bergh et al. 2006, Kline 2007). Repellents can also be found at such sites by noting which areas differ in the level of flies, or by

identification of a host that attracts more parasites than others. For example Warnes and Finlayson (1985) noted that cows producing less CO_2 attracted significantly less flies, and controlling for CO_2 release reduced significant differences in attractiveness between cows. Some individuals also produce repellents at higher rates than others, such as 2-methoxyphenol and aliphatic carboxylic acids (Torr, Mangwiro et al. 1996). In the field compounds 2-methoxyphenol, pentanoic acid, reduced catches at traps baited with ox odours. Pentanoic acid was also able to reduce tsetse fly feeding on ox. Together this means analysis of dairy farm profiles can lead to finding a variety of VOCs from natural sources which cause behavioral effects in flies. Volatile organic compounds with such effects can be used for push-pull strategies to protect livestock. Push pull systems target innate insect behaviours, by exploiting the insects' reactions to semiochemicals. In these systems, repellent volatiles can be employed to deter or 'push' insects away from livestock hosts or other protected crops. Meanwhile, attractive compounds can be added to traps or targets, to further draw or 'pull' insects away for removal from these sites (Hassanali, Herren et al. 2008). Haematophageous arthropods apply, these approaches in site selection for feeding on hosts. The red-legged tick, *Rhipicephalus evertsi* preferentially feeds on anal regions of bovids. In field and lab studies they show attraction to odorants from anal regions, and are repelled by odors from other sites of the cow, directly resulting in migration away from these sites (Wanzala, Sika et al. 2004). Push pull systems employing catnip oil have been developed with early signs of success for *A. aegepti* mosquitos, but require optimization for use in the field (Obermayr, Ruther et al. 2015). These studies provide evidence that push-pull systems can be adapted for other haematophageous insects, to minimize the burden on their respective hosts.

The objectives of this research were to characterize volatile profiles of dairy farm facilities in southern Ontario, in order to understand how the range of VOCs changed as a function of time and region at each site. During the first sampling season at Ponsonby Research Station, the species *M. domestica* was found consistently alongside *S. calcitrans*. Therefore, we sought to explore the effect of *M. domestica* on *S. calcitrans* populations, and observe interactions between the fly species, VOCs, and environmental patterns. In turn, the profiles were used to identify VOCs with putative activity towards *S. calcitrans* and *M. domestica* species. This information would help to give insight if the presence of certain VOCs are consistent with the presence of Stomoxyine flies, and whether variations and fluctuations affect this presence on dairy farm facilities.

2.2 Methods

2.2.1 Fly rearing

Stomoxys calcitrans

S. calcitrans pupae were obtained from the University of Florida, as gracious gifts from Dr. Philip Kaufman. *Stomoxys calcitrans* were a permethrin susceptible strain; however, wild type species were obtained during sampling at the farm to increase the genetic variance of the colony. The flies were held at 25 ± 0.5 ˚C and enclosed in a mesh cage measuring 30cm x 25cm x 25cm (L x W x H) (Figure 2.1). The mesh cages were held in a fly rearing enclosure, located at beside an east facing window to maintain a natural light dark cycle. During the winter months, however, a timed lamp was added to compensate for the diminished daylight. This enclosure was created for the purpose of preventing the

escape of the biting flies into the lab and surrounding area. The enclosure was a ceiling to floor, 4 vinyl-walled structured, fitted with a mesh ceiling to allow suitable air flow to the cages. The area can house up to 6 cages, which are placed on mounted wooden shelves—3 per side. It also housed a heater to maintain a more constant temperature, and a humidifier to overcome the desiccating effects of the heater.

The stable flies had access to citrated bovine blood, delivered on a soaked cotton gauze, and changed daily. The flies were also given access to 30% sucrose-water solution, ad libitum. After approximately 4-5 blood meals (around 5 to 7 days after eclosure), the flies begin to deposit eggs in their blood cup. At this point, an oviposition cup was added to the cage to allow for egg laying. The oviposition cup was constructed out of a 40 mL cup filled with a black nylon material wrapped around cotton balls (Figure 2.1). The cotton balls were previously moistened with water that was used to rinse the eggs from blood-meal cups, into beaker for future separation into the rearing media. Water from the egg rinsings, helped to promote deposition onto the oviposition cup, rather than blood cups.

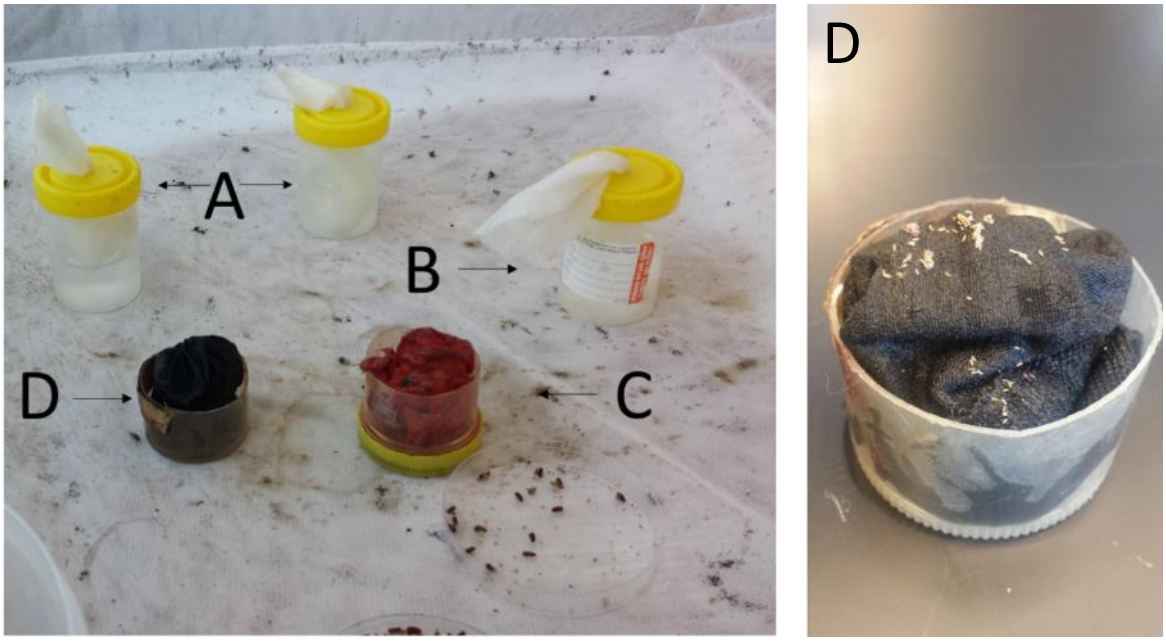

Figure 2.1. *Stomoxys calcitrans* colony housing. Flies were contained within a cotton mesh netting (30cm x 30cm x 30cm). *Stomoxys calcitrans* were given access to water (A) and a 30% sucrose solution ad libitum (B). Fresh citrated bovine blood (C) was added to the cage daily. (D) When eggs began to appear in blood cups, an oviposition cup was introduced to the cage.

During the egg laying period, eggs were rinsed from the oviposition cups and added to a mixture containing 50% woodchips (hamster bedding), 35% wheat bran, and 15% fish meal, then moistened with water. The rearing mixture was incubated in a Caron Refrigerated Incubator (Caron Products & Services, Inc, Marietta, OH, USA) at 25 ˚C ± 0.1˚C, set to mimic the optimal environmental conditions for larval development as prescribed by (Lysyk 1998). Newly eclosed flies were sexed and approximately 1/4th of the female flies were separated and placed in a new cage (30cm x 30 cm x 30 cm) and fed 30% sucrose solution. Flies were kept here for 1-10 days during electroantennography studies.

Musca domestica

Flies were enclosed in a cotton mesh cage (30cm x 25cm x 25cm), and held at 22 ± 0.5˚C. House flies were provided with water, honey, sugar, and milk powder, ad libitum (Figure 2.2). The milk powder was added as a protein supplement to enrich their diet, and fecundity (Shipp and Osborn 1967). After allowing the flies to equilibrate the flies were able to oviposit in a rearing mixture containing 50% woodchips, 35% wheat bran, and 15% fish meal, then moistened with water. The bowl containing the mixture was left in the cage for up to 2 hours, at which point the batch contained enough egg clusters to rear.

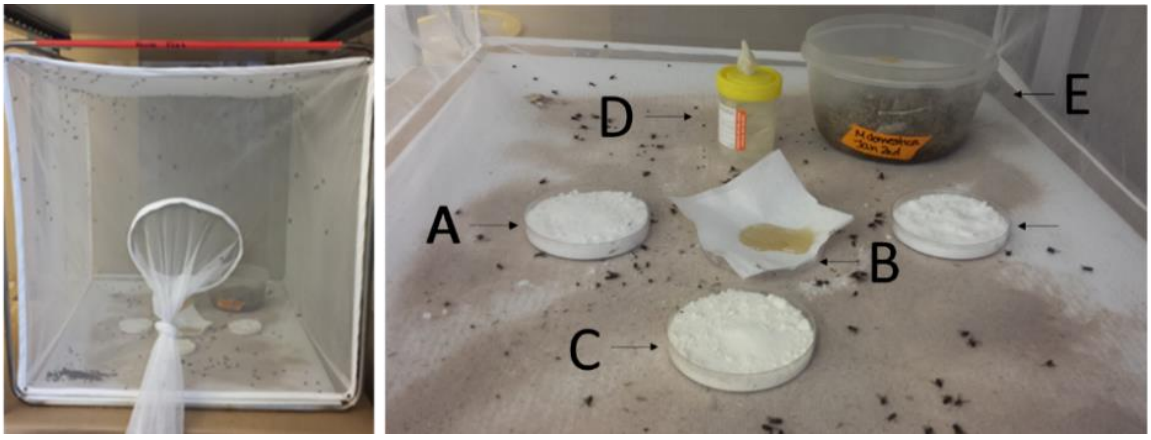

Figure 2.1. Cage housing *Musca domestica* Fly colony. (Left) Flies are contained within a cotton mesh netting (30cm x 30cm x 30cm). (Right) *M. domestica* have access to sugar (A), honey (B), milk powder (C) and water (D) ad libitum. A bowl containing a moistened mixture of 50% woodchips, 35% wheat bran, and 15% fish meal (E), is added to the cage to allow for the oviposition of eggs.

The bowl was then removed, and covered with a fine black mesh to allow oxygen circulation throughout the mixture. The mixture was incubated at 25 ˚C ± 0.1˚C (Lysyk 1998) and the larvae were checked daily for development. Approximately 3/4th of the eclosed flies were added directly to the mesh cage, while the others were sexed. Virgin female flies were then added to another cotton mesh cage (30cm x 30cm x 30cm) and had

access to sugar and water. The unmated female flies were kept 1-10 days for electroantennography studies.

2.2.2 Research Trial Dairy Farm Facilities

Ponsonby Research Station (2014)

The first sampling trial was executed at the University of Guelph, Guelph-Elora Ontario, Canada. The research site, Ponsonby Livestock Research Station (Ponsonby), is located at (43°37'38.4"N 80°20'31.0"W). Ponsonby is primarily characterized by grassy and crop fields.

Figure 2.3. The Ponsonby Dairy Centre facilities. (Left) Exterior of one of the animal rearing facilities managed Ponsonby Dairy Centre. (Right) Interior of the wing where sampling occurred. Cows were held in tie stalls, with straw bedding, and had access to food and water ad libitum.

The farm houses 150 mature cows and 160 replacement heifers, and consists of 120 tie-stalls and 40 free-stalls, in addition to a common parlor, and physiology and maternity wings (Figure. 2.3). Cows were fed twice daily, but had access to the feed and water ad libitum over the duration of the day. Cows were milked twice daily, 12 hours apart, at 6:30 AM and 6:30 PM.

Sampling on the Ponsonby Livestock Research Station was held over the period of June-September 2013. Samples and traps were collected on at least 3 sample days each month with the exception of August 2013, until the sample period had concluded. The termination of sampling on September 28, 2013, coincided with a decrease of stable fly population on the farm, and was supported by previous research trials observing population dynamics of *S. calcitrans* on dairy and cattle farms (Beresford and Sutcliffe 2009).

Elora Dairy Research Centre (2015)

In 2015, Ponsonby Research Facility was closed by the University of Guelph, to introduce a new research facility named the Elora Research Station—Dairy Facility (EDF). During the construction of the EDF, the previous herd stationed at Ponsonby, was relocated to another research facility operated by the University of Guelph—the Elora Dairy Research Centre (Elora). Elora was located less than 7 km North-East of Ponsonby Research Facility, (43°38'21.4"N, 80°23'59.2"W). The facility at Elora, was similar in construction to Ponsonby, and had a maternity wing, physiology wings, a common parlour, and both free and tie stalls. Approximately 300 dairy cows, from the Ponsonby herd were relocated to this facility, and therefore sampling in 2015 was continued at Elora.

A notable difference between EDRC and the Ponsonby and Shadyway farms, was the closed structure of the barn. There were fewer windows and the doors were opened less frequently, despite this however; it remained very well-ventilated with fans. Because of this factor, there were significantly less house flies and stable flies present throughout the barn even though they were present outside (Figure 2.4).

Figure 2.4. Elora Dairy Research Centre. (Left) The outside of the barn housing the dairy cows. (Right) Interior of the physiology wing, housing approximately 30 of the facilities cows.

Sampling at Elora took place weekly from May to mid-September of 2015. The end date (August 23, 2015) was picked to coincide with a decrease in fly presence on the farm, and the moving of livestock to the new EDRC facility.

Shadyway Farm (2015)

Shadyway Farm is a family operated business run by Warren and Lorne Gibbs, and their sons in Sunderland Ontario. This farm was the secondary sample site, for the 2015 summer trial, and was located at (44° 13' 56.9742”N, 79° 1' 28.473”W). The surrounding area was characterized by a multitude of other cattle farms and crop fields, and Shadyway Farm itself produces corn, wheat, and soybean crops during the season. The herd was approximately 120 cows, housed in tie stalls and free stalls, though dry cows also had access to a large field during the day (Figure 2.5).

Figure 2.5. Shadyway Farm. (Left) Outside view of the main barn housing the dairy herd. (Right) Cows in this part of the barn are held in tie stalls. Cows have straw bedding, and have access to food and water ad libitum. A row behind the stalls has a flushing system to remove cattle slurries.

Cows had access to feed and water ad libitum over the duration of the day. Cows were milked twice daily, 12 hours apart, at 4:30 AM and 4:30 PM. Much like Ponsonby, the open structure of the barn permitted good air circulation throughout farm, simultaneously allowing flies to access the interior of the housing. To combat this, the barn was sprayed, during the summer with Dragnet FT Emulsifiable Concentrate Insecticide (FMC Corporation)—a pyrethroid class insecticide containing a concentration of 384 g/L Permethrin. Spray dates were July 21st, and August 10th, 2015. Sampling at Shadyway, began in June and took place weekly until mid-September, when insect presence declined. Sampling concluded on September 15, 2015.

2.2.3 Sample Collection

Air Entrainment

Volatile organic compounds were sampled using two different single sorbent tubes, Tenax TA and Porapak Q (Markes International Inc.). These sorbents were chosen as they are each capable of sampling a wide variety of mid to high volatility compounds, with boiling point ranges of 100˚C-400˚C and 50-200˚C, respectively. Unlike Tenax,

Porapak does not have the heat capacity to withstand thermal desorption, and desorption of compounds is primarily completed through solvent elution. Therefore, overlap in compound detection of Tenax (C_2 –C_{12}) and Porapak (C_4—C_{12}) allows for the compensation of solvent peak masked analytes during the GC-MS analysis of Porapak samples, while also giving the maximum range for examination of volatile profiles. A total of 101 Tenax and 29 Porapak samples were taken during the study.

All samples were taken using a LeMotte air sampling pump (GENEQ Inc.) with adjustable flow rate settings, to draw air onto the sorbent tubes (Figure 2.6). Due to the dilution of VOCs with solvent with the Porapak sorbent, the samples must be concentrated prior to elution; therefore, all samples were acquired over a duration of 4 to 8 hours at 1.5 LPM. Tenax tubes were sampled to a maximum of 15 minutes for a final volume of <5L of air to avoid breakthrough of analytes of interest; the majority of the tubes being run for 5 minutes at 1.0 LPM (Litres per Minute), or 10 minutes at 0.5LPM.

Since stable flies preferentially bite the legs of their host, volatile profiles were sought from this area of the animal. Cows were chosen at random, and the sorbent tubes attached to pump were placed no greater than 50cm away from the leg of the host. In addition, tubes were placed above a height of 30-50cms to avoid dust and volatiles emitted from the bedding of the tie stall. To discern whether there was a shift in volatile profiles over the duration of the day, a minimum of 3 Tenax samples were taken, 2-3 hours apart, over the duration of the sample day.

Figure 2.6. LeMotte Air pump is used with Tenax sorbent tube at 1.5 LPM for 5 minutes for VOC sample collection.

Traps

Multiple traps were put in place over the course of the sampling period, to observe how the stable fly population differed over time. Traps were composed out of white paperboard measuring 8” x 11” (L x H), coated with the Tangle-trap Sticky Coating adhesive (approximately 1/16”), purchased from Natural Insect Control (Stevensville, ON, Canada). Three traps were mounted at the beginning of the sample day, immediately after the start of Porapak sample collection (Fig. 2.7). Traps were placed in locations which would illustrate the movement and behaviour of the flies within the barn during normal activity, but barred bias and interference of the traps with each other. Trap placement regularly included: one trap located near the entrance of the barn; one trap located mid-barn, near animal host or oviposition site; one trap located in a sun exposed resting location.

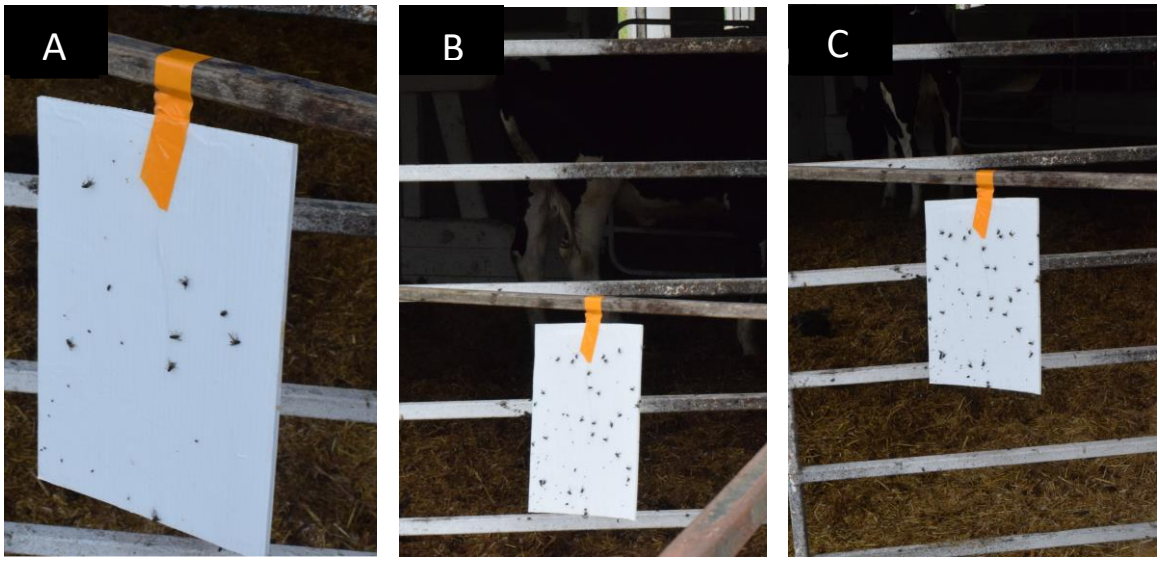

Figure 2.7. Traps are put in place at the beginning of air sampling, and removed after the collection of the last sample. Traps are monitored throughout the day—A) Early morning B) Noon and C) Late Afternoon—last sample collection. This is to assess trap buildup, or collection of debris.

Traps were placed at least 100 cm above any vegetation, and low enough to be in regular flight paths. Over the course of the day, they were then monitored, for fullness and/or debris build-up. Pictures of the traps are given for times of the day at which point the Tenax sample collection occurred—typically early morning, noon, and mid-late afternoon. Traps were removed at the end of the sample day after the completion of sample collection and counted. Unknown fly catches were removed from traps stored in a sample jar for later identification. Traps were wrapped with clear plastic wrap and stored in a freezer.

2.2.4 Sample Analysis

Thermal Desorption

Thermal desorption is a technique that has a range of applications in VOC collection and analysis, in forensic, food, and environmental investigation (Torr, Mangwiro et al. 1996, Borusiewicz, Zadora et al. 2004, Filipy, Rumburg et al. 2006). Samples desorbed using thermal desorption have the benefit of not being diluted by solvent, making it valuable for low concentration analytes, in addition to removing the risk of solvent masked peaks. All collected Tenax samples were run via thermal desorption gas-chromatography mass-spectrometry (TD-GC-MS), using a Markes International, Unity 2 Thermal Desorption Unit. Unity series 2 offers a two-step thermal desorption process, during which compounds from the sorbent tube are released, concentrated, and refocused onto the gas-chromatograph for analysis. The two step thermal desorption program, is not only the most effective way to deliver all the compounds onto the GC column but also prevents loss of sample, as was the previous case with thermal desorption. With this method, sample can actually be recollected onto a clean sorbent tube for additional analysis or method calibration. During the primary desorption step analytes are heated and released from the sample tube, then pre-concentrated onto a focusing trap held at low temperatures to condense the analytes refocusing or recollection. The secondary desorption releases the analytes transferring them from the cold trap to be injected directly onto the HP5 GC column (Agilent) via heated transferline connecting the instruments.

The exact method is as follows: a pre-purge step lasting one minute, flowing high purity (99.999%) Nitrogen through the sample tube to remove any debris, or water condensation. The sample tube was then heated to 300˚C and held for 15 minutes.

Following this step the cold trap was held at a temperature of -20˚C for 1 minutes and heated for 4 minutes at a temperature of 300˚C. The transfer line was held at a temperature of 200˚C at a flow 5.0 of using high purity (99.999%) Helium gas for injection of sample onto the column. After the first samples were found to overload the column and inhibit identification of compounds by mass spectrometry, a double split was added to the method, where in $1/7^{th}$ of the sample is split during primary desorption and another $1/6^{th}$ was split during injection onto the column for a final split of $1/42^{th}$. Prior to injection, an internal standard consisting of 50 µg bromobenzene was added to the sample. Bromobenzene was chosen as an internal standard as it separates well from other analytes, therefore not masking any relevant peaks, and is not found readily at significant concentrations in ambient air.

Before use of the sample tubes, all tubes were conditioned for 30-60 minutes. Tenax tubes were heated to a temperature of 335˚C, and held for 45-60 minutes. Clean tubes were tested using the GC method for sample analysis prior to use, to verify the suitability of the TD conditioning method.

Porapak Sample Analysis

Before any sampling all Porapak tubes were conditioned with thermal desorption at lower temperatures to avoid damaging the sorbent packing. Porapak tubes had a condition method as followed. Tubes were leak tested, purged for 1 minute, and heated to 220˚C for 30 minutes. After sampling, volatiles adsorbed to Porapak tubes are eluted through the use of an appropriate solvent, capable of liquid extraction of similar organic compounds. Organic solvent, diethyl ether, was chosen based on its ability to efficiently recover VOC compounds (Wada and Shibamoto 1997, Linskens, Adams et al. 2012). Additionally,

diethyl ether does not provoke large electrophysiological responses in Muscid flies; therefore, elution of the compound off the gas chromatograph column would not desensitize flies to further compounds. The samples were desorbed, by drawing 2 mL of ether through the sorbent tube, and collecting the eluent in screw cap vials (Agilent Technologies, USA), which were then wrapped with Parafilm and stored at -20˚C. Samples had to be manually injected onto the GC-MS, with a syringe. Per analysis, 2 µl were injected onto the column, with 1 µl of bromobenzene standard (50 µg/µl).

Gas Chromatography-Mass Spectrometry

Tenax and Porapak samples were analyzed using a Varian 240 gas chromatograph in tandem with a Varian 450 EI mass spectrometer detection unit. The gas chromatograph was equipped with an HP5 capillary column (30 m x 1.25 x 0.3m OD), purchased from Agilent Technology. The samples were run with a 46 minute thermal ramp method: a 4 minute hold at 35˚C, a 3 ˚C/min ramp to 80 degrees, a 10˚C/min to 120, and finally a 40˚C/min to 240˚C, which was held for 20 minutes.

Gas Chromatography-Electroantennography

Virgin female flies were anaesthetised in a sample container placed in ice. In the meantime, preparation of electrodes and software was conducted. In preparation for EAG trials, borosilicate capillary tubes (0.8mm OD) were pulled to create the glass electrodes. The glass electrodes were filled with non-lactated Ringer's saline solution. The ringer's solution prevents dehydration of the excised tissue and provides an isotonic environment with the cations responsible for signal propagation within olfactory neurons across the antennae (Miller 1992). A small amount of electrically conductive gel (Spectra 360 Electrode Gel, Parker Inc.) was applied to the antenna. This helped to improve the

contact between the hydrophobic antennae, and solutions which in turn helped to eliminate noise which can obscure the signal (Syntech 2015). The electrode was mounted onto electrode holder housing a silver wire. Each probe —different and indifferent—was secured on an electrode holder and held by micromanipulators (Harvard Apparatus Canada) to facilitate movement of the antennal preparation.

While incapacitated, the flies' heads were excised with a scalpel and pierced through the foramen of the fly head with one of the prepared glass electrodes. The electrode was then remounted onto the electrode holder; this probe is the indifferent (non-recording) electrode. In some studies, the 3rd antennal segment is excised and mounted between the two electrodes, however due to the size of the segment in the dipteran flies, this can be hard to manipulate, and can cause extraneous noise in the preparation. A similarly prepared and filled electrode is mounted onto the different (recording) electrode. The recording electrode is connected to the Intelligent Data Acquisition Controller (IDAC-2) (Syntech), which is responsible for the amplification, and integration of the EAG, to Syntech's Autospike software (Version 3.9). The fly head was brought into proximity with the recording electrode, allowing one of the antennae to rest within an appropriately sized opening thereby allowing contact with the ringer solution and electrode gel (Figure 2.8 D). The contact of the antennal tip to the ringer solution is extremely important, due to the sensitivity of the instrument; therefore, the hollow in the glass electrode is minimized to an inner diameter just larger than that of the fly antennae, in order to avoid extraneous noise. Further, the EAG set-up itself is held within a Faraday Cage (Harvard Apparatus Canada), to reduce electromagnetic noise caused by nearby instruments and electronic devices, housed on a TMC 63-series High Performance Lab

Table. The whole preparation sits in front of a charcoal humidified air stream through which the GC column transferline is run (Figure 2.8). The charcoal humidified air provides an inert and regulated background, which does not interfere with the test compounds, and acts as control for non-olfactory receptors (mechanoreceptors, hygroreceptors, etc.) present on the surface of the antenna (Keil 1997, Myrick and Baker 2018).

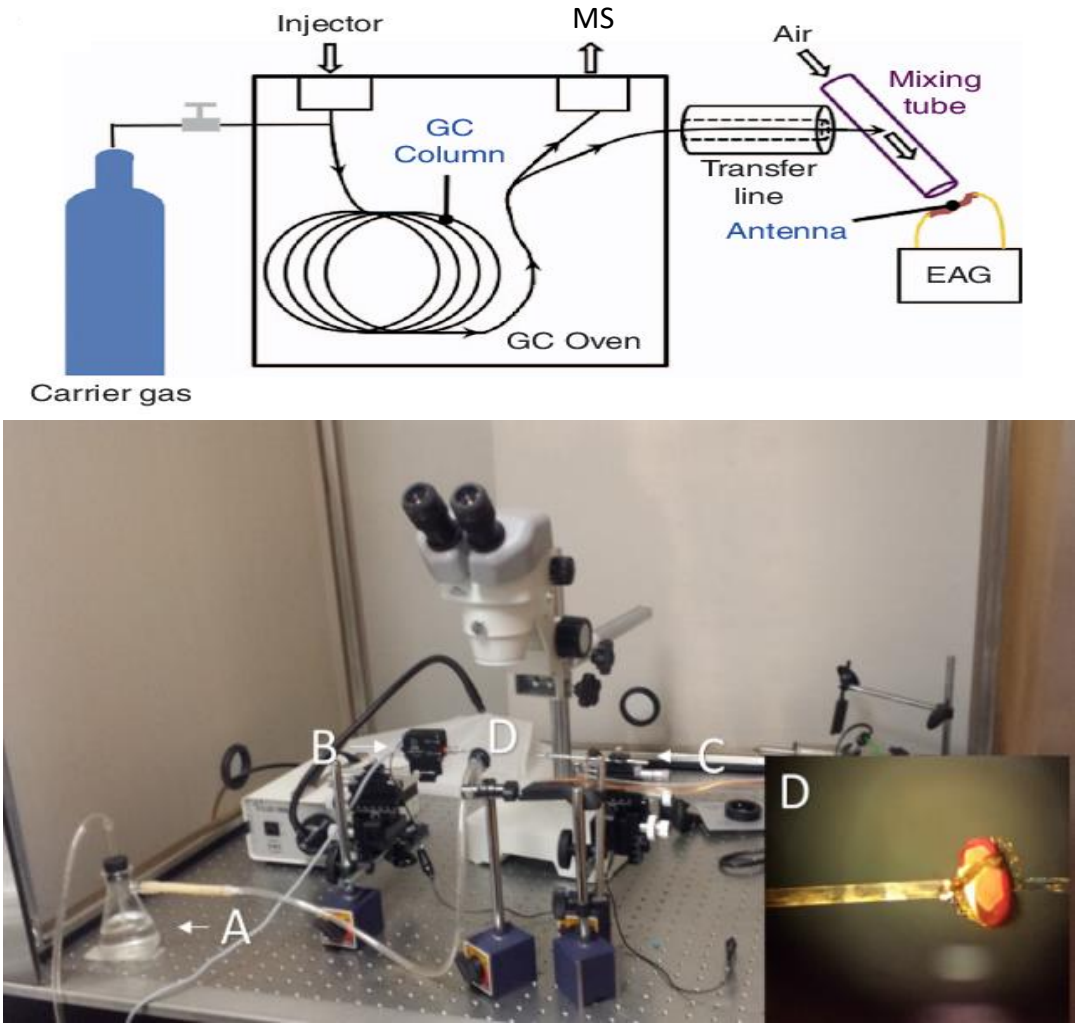

Figure 2.8. Schematic of GC-EAG system adapted from Zhou, Akbar et al. (2017) and electroantennography installation. (A) Humidified water stream leading to incised head preparation. (B) The recording electrode is attached to the Intelligent Data Acquisition Controller (not pictured). Both it and the (C) indifferent electrode are held on micromanipulators which can move forward and back in 3 axes. The indifferent electrode pierces (D) The excised fly head through the foramen, which is brought into contact with the recording electrode, allowing its antenna to come into contact with ringer solution in the glass electrode. (E) GC-MS transferline can be directed onto the preparation for GC-EAG analysis.

GC-MS was run with the same conditions as mentioned in the previous section. The capillary column was fitted with a Y-splitter, which allowed half of the sample to be transferred to the MS for analysis of eluted compounds. Simultaneously, the other half of the sample, was projected onto the EAG preparation, through a transferline that could be inserted into the humidified airstream. Active compounds triggered voltage changes across the fly antennae, as they eluted off the compound. The resulting depolarizations were recorded by the probe and integrated with Autospike software, during the duration of the run. Autospike software was used to measure the magnitude and length of each depolarization, and gave information about the time at which the stimulus was delivered (Figure 2.9).

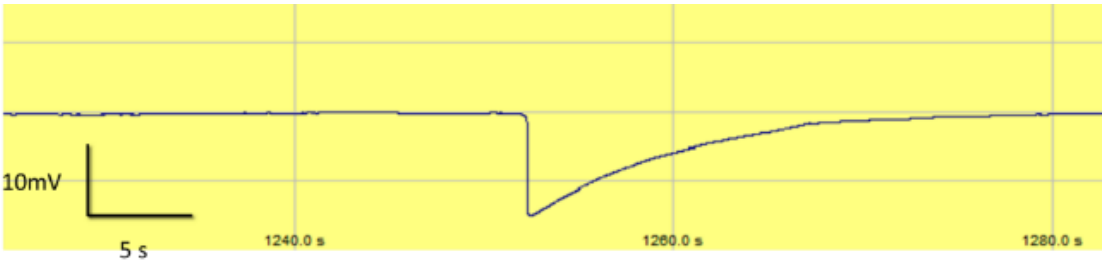

Figure 2.9. Example of a depolarization (14.2 mV) in response to 1-methyl-4-(1-methylethyl) benzene (p-cymene) in female stable fly head. Porapak sample taken on July 9th, at Shadyway Farm.

2.2.5 Statistical Analysis

Statistical analysis was performed using R software V. 3.5.1. Prior to statistical analysis, data was tested for normality and heteroscedasticity, using the Shapiro-Wilks, and Levene's tests respectively. The appropriate parametric or non-parametric tests were performed, p-values for each test are stated below. Pairwise post hoc tests were performed for all significant tests. For Kruskal-Wallis test, Dunn's Multiple Comparison test with Bonferroni adjustments were used. For Chi-Squared (χ^2) analysis, McNemar's

post-hocs were performed using the package Fifer. Assumption testing, ANOVA or Kruskal-Wallis, and post-hocs were performed using the Stats or Car packages. Analysis of Covariance (ANCOVA) model was performed using SPSS. ANCOVA was used to determine the effects of VOCs of interest on fly abundance for *Stomoxys calcitrans* and *Musca domestica*. Covariants tested were temperature, humidity and abundance of the other fly species. Model simplification was then performed using backward elimination of non-significant terms, and compared to previous models. Terms that did not significantly decrease the power of the model were dropped, all others were retained in the model.

2.3 Results

The spring-summer months (May—September) is when *Stomoxys calcitrans* species was most prevalent on farms and, therefore, displaying the largest amount of biting activity (Beresford and Sutcliffe 2010, Beresford and Sutcliffe 2012). Trap data and air entrainment to produce the sample sets occurred over the course of two seasons: season 1 (2014), being conducted at Ponsonby Dairy Farm Facility; and season 2 (2015) held simultaneously at EDRC, and Shadyway Farm. A minimum of 3 sampling days were held for each month during the seasons; however, due to a low abundance of flies in June of the 2nd sample season (2015), sampling was instead held biweekly during this month, at both Elora Dairy Research Centre and Shadyway. Further, the structure of EDRC farm presented a physical barrier to flies in the area of sampling, and reliable trap data could not be obtained. Trapping at this site was discontinued, while air samples were still collected.

Fly trap data was obtained at Ponsonby and Shadyway farms a total of 1520 flies were caught, on 63 traps. Overall trap catches of stable flies between Ponsonby and Shadyway were not significantly different (Kruskal Wallis; P= .14), shown in Figure 2.10. Therefore, data was pooled between the trials to test whether there were changes in fly presence between the months of the trial. Further analysis showed there was an interaction between month and site, with September catches at Shadyway being significantly higher than those in all months at Ponsonby. It was determined that fly catches did vary between months (Kruskal Wallis; $P < .001$), with significant increases in flies caught between June-July ($P <$ .001), June-August (P = .0012) and June-September (P = .0042).

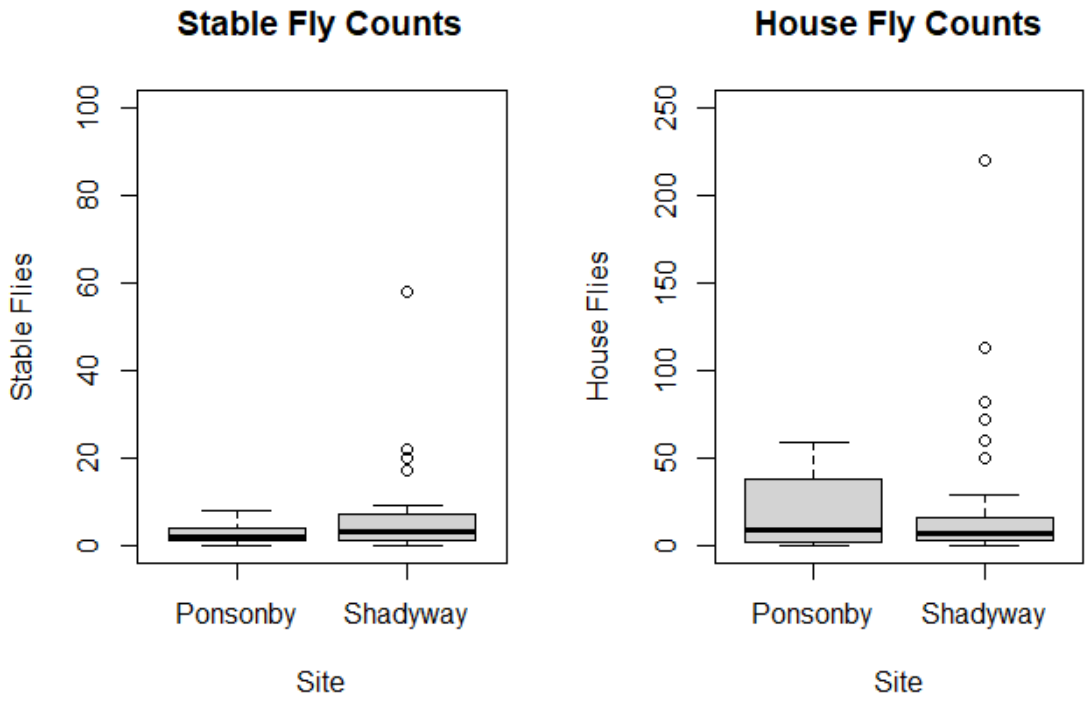

Figure 2.10. Box and whisker plot of the stable fly and house fly catches in the Ponsonby (2013), and Shadyway (2015) trials. The solid horizontal line within the box indicates the median value, and the lower boundary and upper boundary of the box indicates the 25th and 75th percentiles, respectively. The whiskers are indicative of the maximum and minimum values, open circles represent outliers.

Consideration of the sites individually resulted in similar trends. At Ponsonby, flies catches in each month differed significantly (P = .011) and post hoc tests demonstrated that stable fly counts were significantly higher between June-July (P = .0025). At Shadyway, significant increases in stable fly catches were found between June-August (P = .029) and June-September (P = .0018) (Figure 2.11).

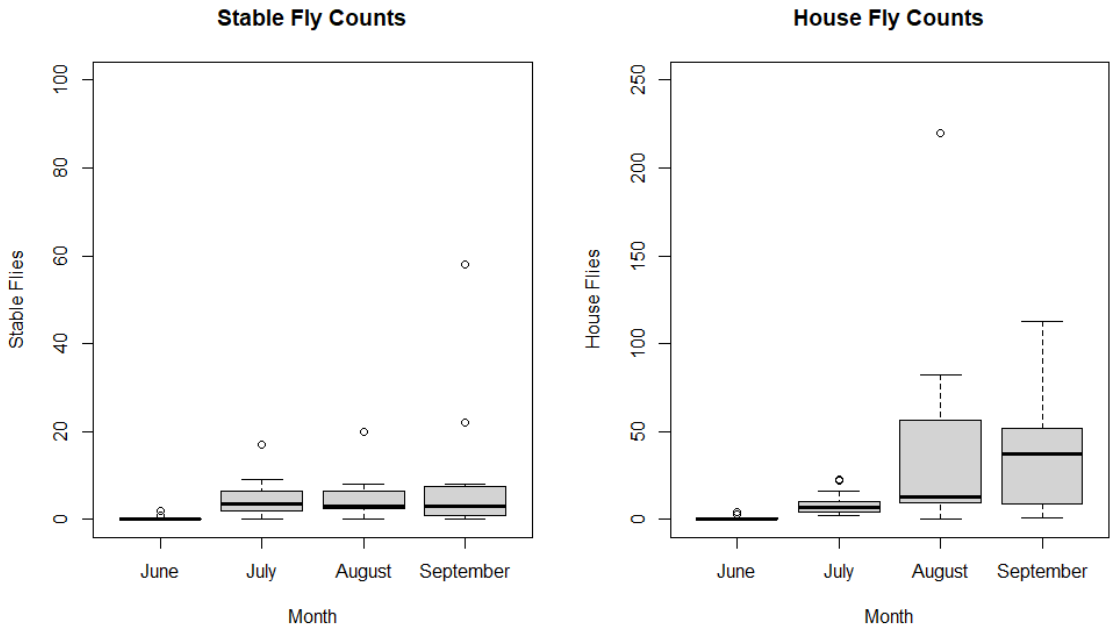

Figure 2.11. Box and whisker plot displaying pooled fly catches at both sites across the summer months. House fly catches were frequently in higher abundance than stable flies. Open circles represent outliers.

Housefly counts grew over of the summer as well, with significantly larger catches between June-July (P = .0017), June-August (P < .001) and June-September (P < .001). Similarly, there was no significant difference between fly catches between the sites (P = .92). At Ponsonby trap catches differed (P = .020) with an increase between June-September (P = .0066) while at Shadyway, catches were different between both June-August (P < .001) and June-September (P = .0039). Catches are displayed by

month for both species below in Figure 2.12. It was determined that at each site house flies were caught in a larger abundance than stable flies (Welsh's T.Test; $P < .001$).

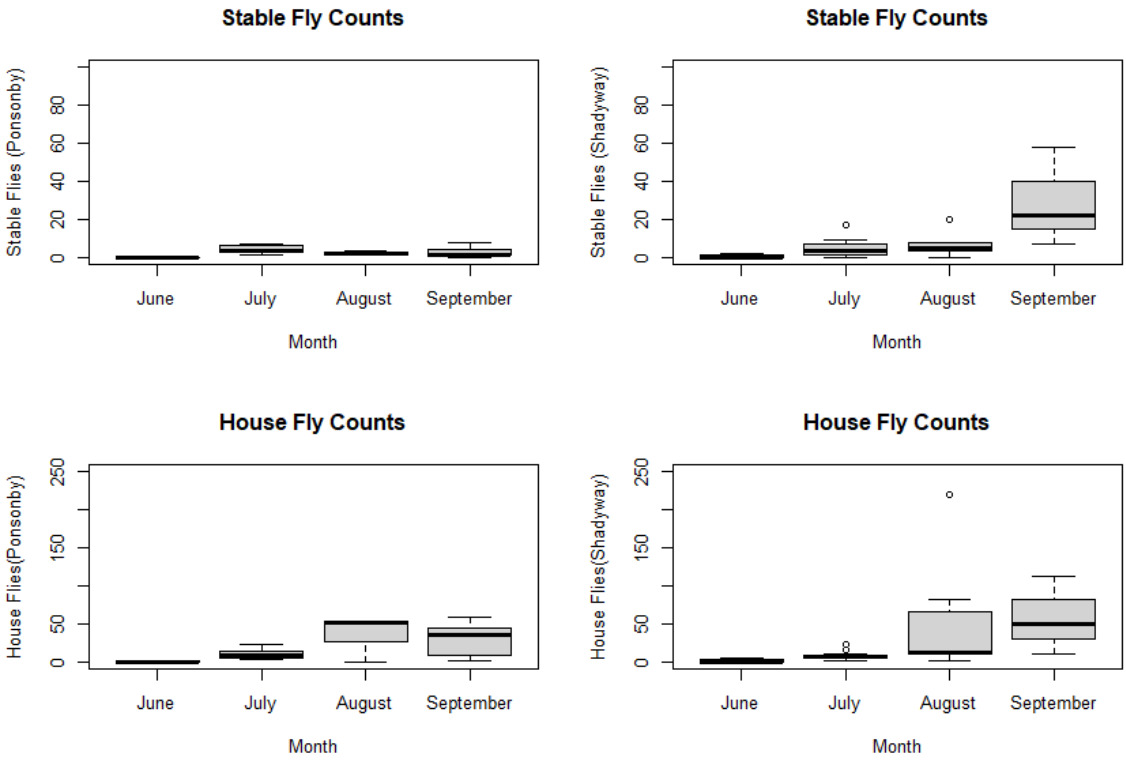

Figure 2.12. Trap catches of *Stomoxys calcitrans* and *Musca domestica* by month at each farm. Catches of *S. calcitrans* in September at Shadyway are significantly higher than all other catches, at either Ponsonby or Shadyway.

Stable fly and house fly catches were each plotted with respect to temperature and humidity (Figure 2.13). Catches appeared to increase in response to temperature, and therefore linearity of the relationship was tested. Regression analysis showed a significant relationship of stable flies to temperature ($P = .018$) however the correlation was low ($R^2 = 0.272$) and could not explain all of the variance in the data. Stable fly survival is increased at median temperatures and humidity, therefore a quadratic model was also tested but none were significant. Neither linear nor quadratic regressions could

be fit with respect to stable flies and humidity; however, with regard to both stable flies and house flies, abundances seemed to follow a normal distribution with higher catches at a relative humidity between 60-75% relative humidity. Similarly, housefly counts were largest between 19-25°C; however, stable fly counts seemed to increase beyond this point. By month, catches were maximized in July and August, which is when average daily temperatures were highest, therefore, temperature could not be excluded as an important factor in fly abundance at farms.

Stable flies are known to be attracted to volatiles that are suggestive of host metabolism, present good oviposition sites, and are repelled by a variety of plant compounds. It has been suggested that functional groups of compounds may play a role in stable fly behavior, therefore, compound species were analyzed for important functional groups compound in VOCs. In addition, samples were used to determine a list of compounds found at each site that might cause such behavioural responses in stable flies. A total of 101 Tenax samples were analysed to identify VOC profiles. Tenax and Porapak samples were analysed by GC-MS and used to determine a list of VOCs present at each site. Compounds were identified according to forward and reverse retention time alignment (>800) and cross-reference of mass spectra with the NIST Mass Spectral Database. Standards were selected and analyzed with identical programs to confirm the identity of each compound. While comparing VOC profiles across different facilities' environments, temperature and trap data were taken at the trial sites, to support the data's significance. The resulting compound distributions of the samples were considered as a whole and as a unique function of individual sites for further comparison. Taken together the compounds were analysed for important functional groups common in VOCs.

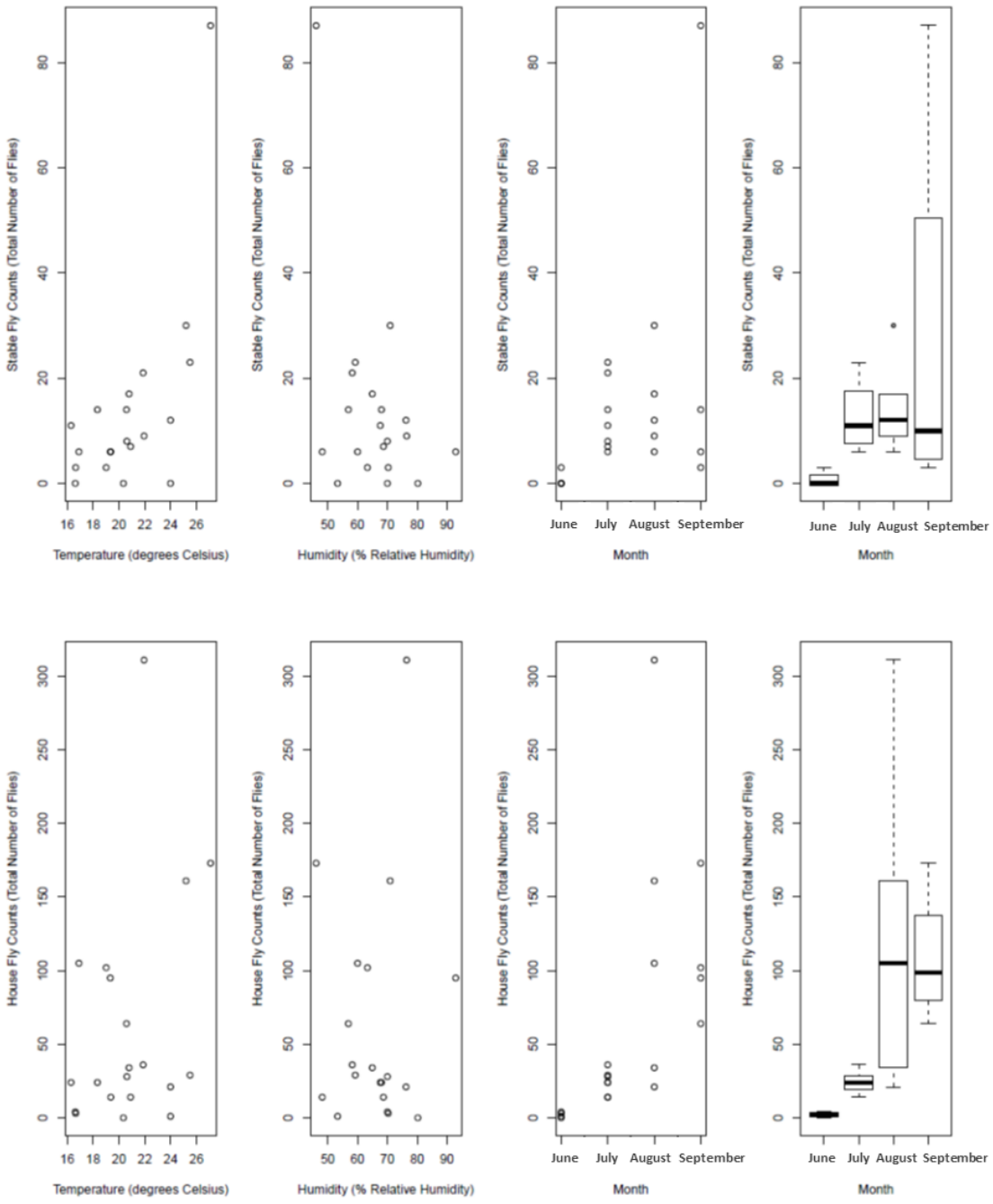

Figure 2.13. *Stomoxys calcitrans* and *Musca domestica* abundance, with respect to Temperature, Humidity, and Month. Traps were placed at several sites around the farm and traps were assessed for the number of each fly species per day. Average temperature and humidity was recorded with data loggers placed at the facilities during the day.

Compound species separated primarily into the following classes: aromatic (benzene derivatives), alcohols, aldehydes, volatile fatty acids, and hydrocarbons. Hydrocarbons were primarily characterized by short to medium hydrocarbons with either no functional groups or ones outside those previously listed.

Collectively, the pooled data from all the sites suggested the VOCs were dominated by hydrocarbons compounds (44.8%), followed by aromatic (21.8%) and fatty acid (14.1%) compounds. Terpenes (7.7%), aldehydes (6.5%), and alcohols (5.2%) were recovered with similar abundances, and when looking at the sites separately these compounds separately this generally held true (Figure 2.14). Representation of compounds at each of the sites were fairly similar, with Shadyway and Elora farms being the most similar in terms of distribution. Ponsonby seemed to have a slightly larger incidence of aromatic, and fatty acid compounds than the other sites, with a lower frequency of the hydrocarbons. At all sites but Ponsonby, the predominantly recovered volatiles were hydrocarbons. After this, the largest group of compounds were the benzene derived species at each of the sites; the group was the most prevalent at Ponsonby. The aromatic compounds were present in 25.13% of all of the samples taken. Of these, benzene, toluene, and ethylbenzene were frequent. At Ponsonby and Shadyway alcohols were found in the least abundance, however this was inverted at Elora, where terpenes were the least abundant followed by alcohols. Shadyway, distribution of fatty acids, aldehydes and terpenes were no different. Similarly, at Ponsonby distributions of terpenes and aldehydes were the same. In general, the aromatic groups were more abundant than those of the alcohols, aldehydes and terpene groups, and likewise so were the aliphatic compounds over the alcohols, aldehydes, and terpene groups.

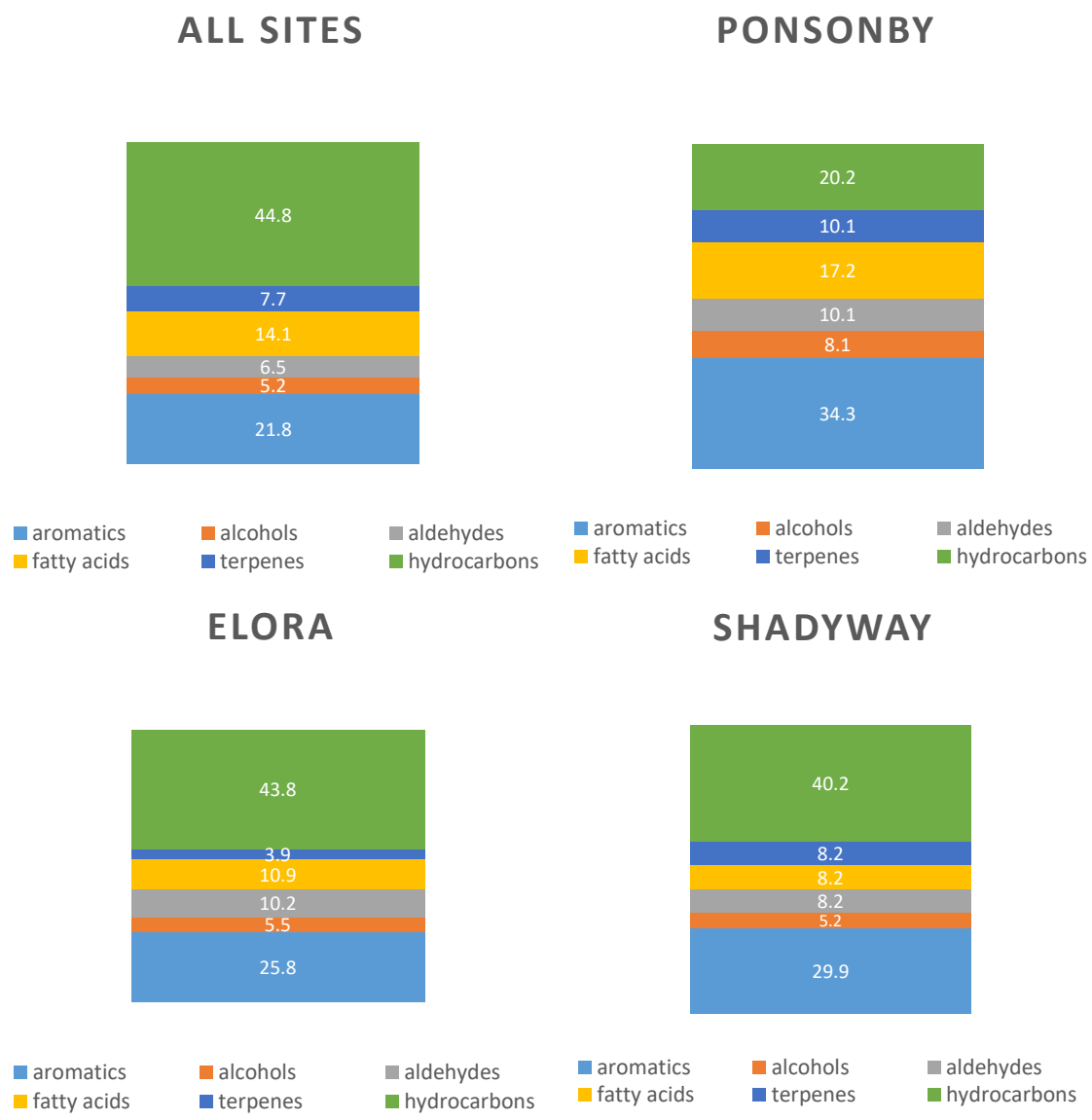

Figure 2.14. Distribution of functional groups in recovered Volatile Organic Compounds. Air samples from each site were analyzed by Gas Chromatography-Mass spectroscopy, and Volatile Organic Compounds were assessed for functional group. For individual sites, the percentage for each group was calculated out of the total number of sample obtained at that site. For the distribution of all sites, recovered VOCs were pooled and calculated out of the total 101 samples.

Due to the magnitude of compounds that were present amongst the samples, it was necessary to pick a few targets that could be studied with respect to fly activity. During analysis it was observed that some VOCs recurred frequently in the air samples. In order to see whether these compounds varied by farm, the 10 most abundant compounds were observed at each site (Table 2.1). The presence of the VOCs were similar across the sites, however their abundances sometimes changed; for example toluene was present at all sites, but was recovered in almost 90% of samples taken at Ponsonby but only 60% of those at Elora. 2-nonen-1-ol was present at all sites, and was the 3rd most abundant compound recovered overall, but was recovered in only 21% of samples at Elora versus 60% of Ponsonby air samples. Recurrent volatiles are shown in Table 2.1 below. Compounds that were common to all 3 sites were 2-nonen-1-ol, dimethyl disulfide (DMDS), heptanal, isopropyl myristate, and toluene.

Porapak samples taken at the farm were also analyzed by GC-EAG. This method was used to test for putative activity of the VOCs towards both *Stomoxys calcitrans* and *Musca domestica*. For each sample taken during the entrainment, 10 flies were tested for EAG responses. The percentage of flies which responded to an individual compound was calculated, for all samples in which that compound appeared. Those compounds which elicited a response greater than 40% of the time are presented below, in Table 2.2.

Table 2.1. List of Volatile Organic Compounds found at each dairy farm facility, and percent occurrence in sample Tenax tubes.

Compound	Site			
	Ponsonby (%)	Elora (%)	Shadyway (%)	All Sites (%)
Alcohols				
2-Decen-1-ol	30.43	9.62	--	13.86
2-Ethyl-1-hexanol	26.09	--	--	--
2-Nonen-1-ol	60.87	21.15	38.46	42.56
Cyclodecanol	39.13	21.15	--	24.75
Aldehydes				
Pentanal	--	15.38	19.23	12.87
Hexanal	--	13.46	19.23	13.86
Heptanal	34.78	34.62	30.77	33.66
Dodecanal	26.09	--	--	38.61
Aromatics				
Toluene	86.96	59.60	69.20	68.30
Phenol	21.74	--	--	--
Fatty Acids				
Isopropyl myristate	52.17	50.00	76.92	58.42
Hexadecanoic acid methyl ester	--	17.31	15.38	14.85
Eicosanol	--	--	19.23	--
Terpenes				
α-pinene	26.09	--	--	--
Isopulegol	--	--	11.54	--
Limonene	--	--	11.54	--
Sulfides				
Dimethyl Disulfide	21.74	26.92	19.23	23.76

Interestingly, many compounds that demonstrated some activity were similar between the flies, though not necessarily to the same extent. An example is butanoic acid methyl ester, which elicited a response in 80% of the house flies, but only demonstrated 50% in the stable flies. In contrast, the compound DMDS demonstrated a large response of 78% in stable flies, while in the houseflies the response was 50%. Some compounds, such as propionic acid, elicited similar activities in both flies.

Commonalities between the recurrent VOCs at the facilities, and those which most frequently provoked a response, were explored. Of the compounds that demonstrated some response in the stable flies, seven compounds appeared frequently at one or all of the sites. These were: DMDS, hexanal, limonene, phenol, toluene, 2-nonen-1-ol, and α-pinene. Five compounds to which the housefly were responsive appeared at the farms, and these were: DMDS, toluene, 2-nonen-1-ol, phenol, and α-pinene. Three of these compounds— DMDS, toluene, and 2-nonen-1-ol—appeared at high frequencies at all three sites.

Because these volatiles appeared frequently and elicited responses in the flies, it was questioned whether their abundances fluctuated over the course of the summer, and whether this could be correlated with stable fly activity. Tenax samples were taken at all sites, and multiple times over the course of the day, and therefore compounds were studied to determine whether changes in their presence occurred on a site, month, and time dependant basis. Of the compounds that demonstrated consistent responses in the flies and were frequently present at the farms, 15 were chosen for further exploration.

Table 2.2. Electroantennogram responses of *Stomoxys calcitrans* and *Musca domestica* in response to Volatile Organic Compounds found in the samples of dairy farm profiles. Gas-Chromatography-Electroantennography was used to analyze Porapak samples taken from 3 dairy facilities in Southern Ontario. For each sample, a minimum of 5 replicates were performed with each species. The percentage was the pooled result of all responses to the VOC for each time it appeared in the samples.

Compound Activities over 40%			
VOCs isolated from dairy farm	*Stomoxys calcitrans* % Response to VOCs	VOCs isolated from dairy farm	*Musca domestica* % Response to VOCs
Dimethyl disulfide	78.3	Butanoic acid methyl ester	80.0
1-Methylethyl benzene	70.0	2-Pentanone	72.4
3-Methyl-1-butanol	66.7	Phorone	66.7
Pentanoic acid	63.8	Propionic acid	66.7
2-Pentanone	55.6	p-Cresol	58.2
2-Butanol	54.6	Pentanoic acid	57.1
Hexanal	53.8	Dimethyl disulfide	50.1
Phenol	53.4	α-pinene	49.1
2-Nonen-1-ol	51.9	Phenol	45.9
p-Cresol	51.2	Benzaldehyde	44.4
Isopropyl alcohol	51.0	Toluene	44.2
Butanoic acid methyl ester	50.0	diptone	43.3
Camphene	50.0	4-Hydroxy-4-methyl-2-pentanone	41.4
Ethanedioic acid	50.0	2-Nonen-1-ol	40.4
o-Xylene	48.8	2,3-Dimethyl-1-pentanol	40.0
α-pinene	48.2	2-Methyl-butanoic acid methyl ester	40.0
3-Ethyl phenol	47.0	4-Fluoro-3-trifluoromethylbenzoic acid phenyl ester	40.0
2,2-Dimethoxy butane	45.6		
Hexanoic acid ethyl ester	44.4		
Toluene	44.0		
diptone	43.0		
o-Cymene	40.9		
Butanoic acid propyl ester	40.0		
Limonene	40.0		

These compounds were DMDS, dodecanal, heptanal, hexanal, hexanoic acid ethyl ester, isopropyl alcohol, isopropyl myristate, limonene, pentanal, phenol, toluene, α-pinene, 2-decen-1-ol, 2-nonen-1-ol, 4-carene. Three other compounds—hexadecanoic acid methyl ester, pentanoic acid, and diptone (this name was given an the original compound; the identity of which cannot be stated here)—were also chosen for examination, however their detection while relatively frequent in Porapak samples, did not occur in Tenax samples. Likewise while the compounds pentanal, dodecanal, and isopropyl myristate, were abundant in Tenax samples their presence was below the limit of quantitation in Porapak samples and could not be further examined for their effect on stable flies.

Chi square analysis was used to determine whether the presence or absence of the 15 compounds changed across the sites. It was determined 10 compounds varied significantly between the sites (Table 2.3). McNeymar's chi square post hoc was conducted to determine the site-wise differences. Compounds mainly varied between Ponsonby and the other sites, with Elora and Shadyway being the most similar. This is in accordance with the fact that of the compounds tested, those present at Elora were also present at Shadyway, or were absent at both sites.

Eight compounds demonstrated significant differences between the months (Table 2.4). On average the compounds varied the most often between June and later months—primarily July and August. This corresponded with the increase in fly abundance, found in the trap data. Finally the variation in compounds, time dependently during the sample day was tested Table 2.5).

Table 2.3. Site-wise differences of 15 volatiles of interest at three dairy facilities in Ontario. Absence or presence of each volatile was tested for significant differences with Pearson's chi square analysis, and McNeymar's post-hoc was used to test pairwise differences between each site. P-values are listed in the table below.

Site	***Compound***	X^2	***Mcneymar's Chi Squared Post-hoc***		
		p-value	Ponsonby -Elora	Ponsonby - Shadyway	Shadyway - Elora
	Dimethyl Disulfide	0.76			
	Dodecanol	0.0051**	0.0066	0.028	
	Heptanal	0.77			
	Hexanal	0.17			
	Hexadecanoic acid Methyl Ester	0.37			
	Hexanoic acid ethyl ester	<.001**	0.0065	0.038	
	Isopropyl Alcohol	<.001**	<.001	0.017	
	Isopropyl Myristate	0.095*			0.058
	Limonene	0.76			
	Pentanal	0.012**	0.0086	0.014	
	Phenol	<.001**	0.0024	0.037	
	Toluene	0.14			
	α- pinene	<.001**	0.0032	0.062	
	2-decen-1-ol	0.0064**	0.034	0.0045	
	2-nonen-1-ol	0.0073**	0.013	0.0083	
	4-Carene	0.017**	0.037		

**significant difference at $P < .05$
* significant difference at $P < .10$

The sampling period was divided into 3 time groups: early morning (7-10:30AM), mid-morning (10:30AM-1PM), and afternoon (1-4PM). Here, only a few compounds, DMDS, α-pinene, isopropyl myristate and hexadecanoic acid methyl ester varied. Changes in compounds typically varied the most between mid-morning and the other time points.

Table 2.4. Differences of 15 compounds of interest between trial months at three dairy facilities in Ontario. Porapak samples were separated by the month in which they were taken, and the presence of each volatile was assessed. Absence or presence of each volatile was tested for significant differences with Pearson's chi square analysis, and McNeymar's post-hoc was used to test pairwise differences between each site. P-values are listed in the table below.

Month	***Compound***	***X^2***	***McNeymar's Chi Squared Post-Hoc***									
		p-value	May June	May July	May Aug	May Sept	June July	June Aug	June Sept	July Aug	July Sept	Aug Sept
	Dimethyl Disulfide	0.78										
	Dodecanol	0.0014 **	0.024				<.001	0.0088				
	Heptanal	<.001 **		0.041			<.001	0.0019	0.0098			0.042
	Hexanal	0.20										
	Hexadecanoic acid Methyl Ester	0.032 **					0.0379				0.060	
	Hexanoic acid ethyl ester	0.45										
	Isopropyl Alcohol	0.23										
	Isopropyl Myristate	<.001 **	<.001	0.0094	0.011	0.010						
	Limonene	0.59										
	Pentanal	0.0081 **					0.0024	0.088				
	Phenol	0.40										
	Toluene	0.010 **	0.096				0.0083	0.0096				
	α-pinene	0.16										
	2-decen-1-ol	0.0069 **					0.0033	0.032			0.088	
	2-nonen-1-ol	0.0044 **					0.088	0.068			0.016	0.057
	4-carene	0.16										

**significant difference at $P < .05$
* significant difference at $P < .10$

Table 2.5. Differences between 15 VOCs of interest over a daily time course. Porapak samples were divided based on the time of day sampled, and assessed for presence of each compound. Pearson's chi square and McNeymar's chi squared post hoc were used to discover difference in abundance of each compound over the day.

Time of Day	***Compound***	***X^2***	***McNeymar's Chi Squared Post-Hoc***		
		p-value	Early Morning - Midmorning	Early Morning-Afternoon	Mid morning-Afternoon
	Dimethyl Disulfide	0.083*		0.082	
	Dodecanol	0.59			
	Heptanal	0.79			
	Hexanal	0.87			
	Hexadecanoic acid Methyl Ester	0.083*			0.051
	Hexanoic acid ethyl ester	0.83			
	Isopropyl Alcohol	0.34			
	Isopropyl Myristate	0.034**	0.071		0.044
	Limonene	0.99			
	Pentanal	0.34			
	Phenol	0.12			
	Toluene	0.56			
	α-pinene	0.044**	0.071		0.044
	2-decen-1-ol	0.80			
	2-nonen-1-ol	0.83			
	4-Carene	0.67			

**significant difference at $P < .05$
* significant difference at $P < .10$

Stable fly abundance was investigated as a function of the fifteen volatiles selected. In general stable fly abundance increased with respect to the presence of the volatiles. Volatiles such as toluene, HAME, and diptone expressed large differences in the median stable fly abundance dependent on the presence of the compound (Figure 2.15).

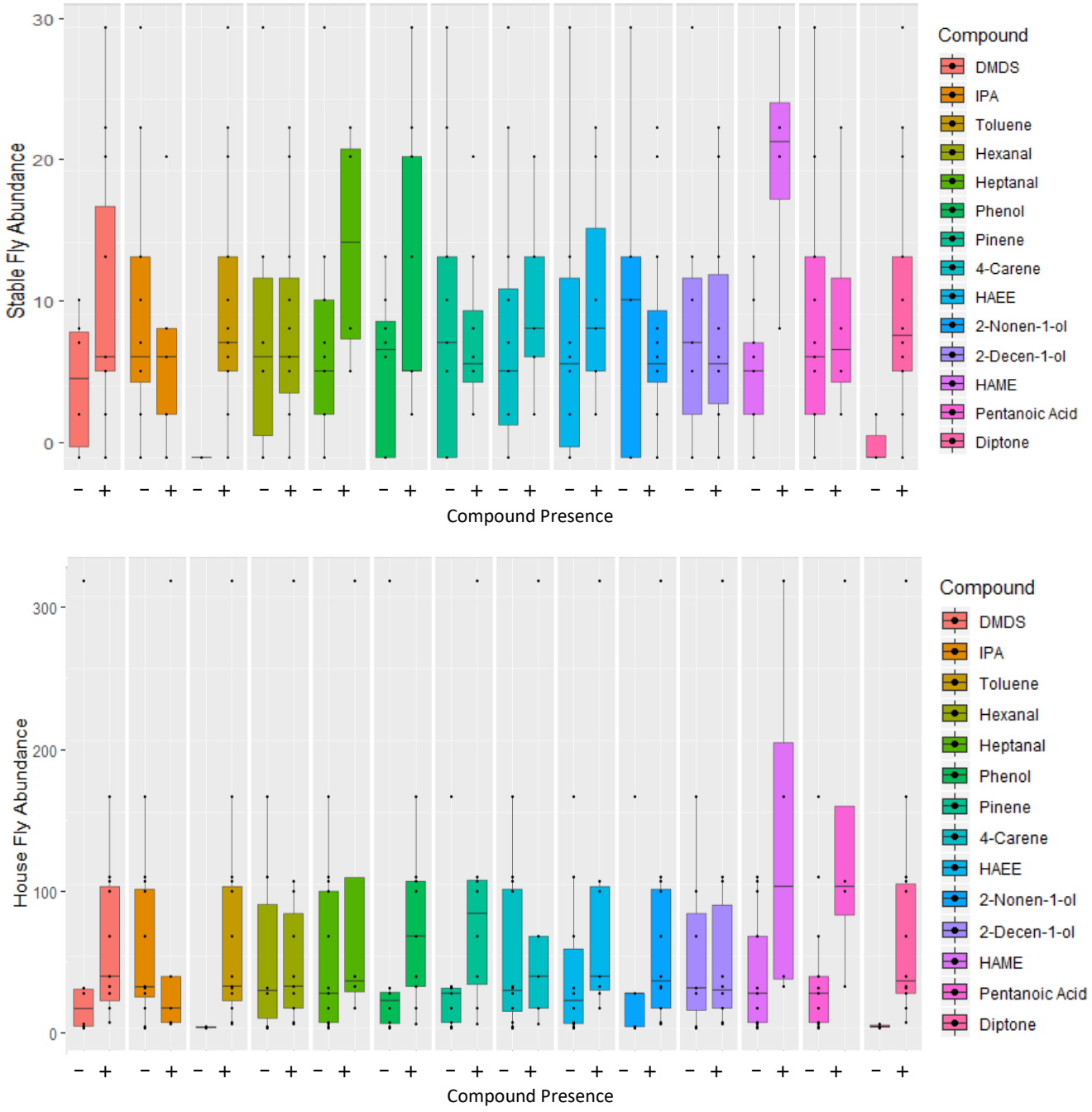

Figure 2.15. Box and whisker plots representing effect of compound presence on stable fly and house fly abundance. + and – denote presence and absence respectively. HAEE represents hexanoic acid ethyl ester and HAME represents hexadecanoic acid methyl ester. Horizontal solid line represents median value. Black circles are outliers represent fly counts associated with the day sample was taken.

Table 2.6. Analysis of Covariance table of the final model of *Stomoxys calcitrans* abundance. The non-significant terms were dropped from the model and the new models were compared with the null model. Terms were retained when their removal caused worsening of the model.

Variable	***P-Value***
Corrected Model	<.001
Intercept	<.001
Dimethyl Disulfide	<.001
Isopropyl Alcohol	<.001
Toluene	<.001
Heptanal	.001
Phenol	<.001
4-carene	.001
Hexanoic Acid Ethyl Ester	<.001
2-nonen-1-ol	.001
2-decen-1-ol	.003
Hexadecanoic Acid Methyl Ester	<.001
Pentanoic Acid	<.001
Diptone	<.001
α-pinene	.050

ANCOVA was used to test if VOCs of interest had affected the abundance of either the stable flies or house flies (Table 2.6). Of the tested compounds, hexanal was found to be insignificant and therefore improved the model by its removal. Because temperature and humidity affect fly life history, in addition to the emission of VOCs, these factors were tested as covariants. House fly abundance was also tested as a covariant, as house flies compete with stable flies for similar resources, such as

oviposition sites. However, none of these variables had significant effects on stable fly abundance, and therefore they did not appear in the final model below.

For the house flies, the same variables were tested, with temperature, humidity, and stable flies being examined as covariants. The compounds that had no significant effect on the house fly abundance were hexanal, 2-decen-1-ol, α-pinene, and 3-carene. Likewise, temperature and humidity were not found to be significant to the model. While stable fly counts did not significantly affect the houseflies, this variable did account for some variance in the data. Therefore the variable remained in the final model (Table 2.7). Results suggested that many compounds tested did have effects on the stable flies, while factors such as temperature, and humidity did not appear to have any positive effect in either fly. Similarly hexanal was the only compound which presented no significant effect in either fly species. In the housefly model, there were fewer which compounds induced significant effects, compared to the stable flies.

Table 2.7. Analysis of Covariance table of the final model for *Musca domestica* abundance. The non-significant terms were dropped from the model and the new models were compared with the null model. Non-significant terms were retained when their removal caused worsening of the model.

Variable	***P-Value***
Corrected Model	.001
Intercept	.138
Stable Fly Abundance	.099
Temperature	.275
Dimethyl Disulfide	<.001
Isopropyl Alcohol	.001
Toluene	.005
Heptanal	.002
Phenol	<.001
Hexanoic Acid Ethyl Ester	<.001
2-nonen-1-ol	<.001
Hexadecanoic Acid Methyl Ester	.013
Pentanoic acid	.001
Diptone	<.001

2.4 Discussion

Dairy farms are the site of a complex mixture of volatile organic compounds, which help stable flies locate hosts and find nutrients. One goal of this research project was to identify reoccurring VOCs present at dairy farm and discover what impact that these would have on stable fly species. At the farms, many compounds were recovered from the air samples. It was hypothesized that the VOCs which occurred most frequently

amongst the sites might represent important semiochemical cues to the nuisance flies, and therefore explain their presence at the farms. Therefore, it was examined whether these compounds had electrophysiological activity. It was further tested whether those compounds that were active, presented a significant increase or decrease on the abundance of pest flies at the farms during the summer months. This was the case, as at least eight of the compounds found most frequently at the sites were electrophysiologically active in stable flies and five compounds elicited responses in houseflies. Notably, while there remained much overlap in VOC species between the two sorbents used in the study, there were a few discrepancies in recovery of some VOCs of interests. An example, is isopropyl myristate, which was found frequently in the Tenax tubes, while recovery of the same species was rare in Porapak samples. Some of these compounds might have elicited responses had they been present for analysis in GC-EAG studies. Additionally, compounds which elicited responses in a high percentage of flies, such as pentanoic acid and diptone, were also found quite frequently amongst the sites, but not to the extent as those listed in Table 1.

In VOCs that were recovered and were found to significantly affect stable fly or houseflies, it was questioned whether similar characteristics existed amongst them, either in structure, source, or promotion of behavioral activity. VOCs at farms come from two main sources, the first being compounds from livestock metabolism and waste (Filipy, Rumburg et al. 2006, Sun, Pan et al. 2008, Sun, Trabue et al. 2008); the second source, emissions from plant compounds, either from livestock feed, bedding, or nearby plant crops (Sunesson, Gullberg et al. 2001, Alanis, Sorenson et al. 2008). Volatile compounds from either source can be further categorized based on their functional groups, which

tend to differ by source. Volatiles derived from animal sources, contain 6 major groups including volatile fatty acids (VFA), volatile amines, ammonia, phenol and indole derivatives, and sulfurous compounds (Tripathi, Upadhyay et al. 2009). Plants, especially those of more aromatic varieties, can produce compounds which interrupt and alter the physiological functions and behaviors of insect species. The properties of these compounds are what make essential oils a growing interest in integrated pest management (Tripathi, Upadhyay et al. 2009, Verdeguer, Blázquez et al. 2009, Hieu, Jung et al. 2013, Zhu, Wienhold et al. 2013). Components of active plant compounds can be divided into four classes including terpenes, benzene derivatives, hydrocarbons, and other miscellaneous compounds, such as alcohols and ketones (Ngoh, Choo et al. 1998). Activity of compounds in insects might be related to the presence and position of functional group, rather than volatility. Further, functional group analysis of VOCs might lead to further insight about insect behaviors and physiological traits. For instance, it has been reported that moth pollinated plants have decreased terpenoid compounds but instead plants emit high amounts of benzenoids (Dudareva, Klempien et al. 2013). In the cockroach *Periplaneta Americana L. (*Blattodea: Blattidae*)*, aromatic compounds were more repellent and toxic than terpenes, and additions of methoxy groups to these compounds mediated higher lethality in the insects (Ngoh, Choo et al. 1998).

As a part of analysis of all VOCs recovered during air entrainment separated into six major groups based on compound functionality. These groups included: hydrocarbons, benzene derivatives, volatile fatty acids, terpenes, alcohols, and aldehydes. It was interesting to see how the distribution of these groups varied amongst the sites that were studied, as well as how they compared to those reported elsewhere. Thirteen

compounds were determined to have a significant impact on stable flies, these were: toluene, phenol, α-pinene, 4-carene, pentanoic acid, hexanoic acid ethyl ether, hexadecanoic acid methyl ester, heptanal, isopropyl alcohol, 2-nonen-1-ol, 2-decen-1-ol, dimethyl disulfide, and diptone. In houseflies, ten compounds were found to significantly affect the abundances, and these were: toluene, phenol, pentanoic acid, hexanoic acid ethyl ether, hexadecanoic acid methyl ester, heptanal, isopropyl alcohol, 2-nonen-1-ol, dimethyl disulfide, and diptone. These compounds were related closely to those that recurred on the farm, and could be described mainly by four of the six groups; terpenes, aldehydes, alcohols (especially fatty alcohols), and benzene derivative compounds. Of the four groups several have prevalence in both plant and livestock VOC odors.

Benzene derivative compounds and sulfurous compounds, such as dimethyl disulfide tend to be resultant from the metabolism of amino acids and proteinaceous foods in the diet. Volatiles of this type are increased by anaerobic fermentation of livestock urine, and manure. 3-methyl phenol (m-cresol) and 4-methyl phenol (p-cresol) for example, are byproducts of tyrosine digestion, and are present in urine and cow manure slurries (Hobbs, Webb et al. 2004). Cresol causes attraction in stable flies and is a precursor to pesticides such as Fenthion. Phenol, cresols, 3-ethyl phenols, all found active during this study, were also recovered in oxen host odors attractive to tsetse and stable flies (Torr, Mangwiro et al. 2006). In the same study, propylphenols were also recovered; these were present in our samples, but did not show high activity, like the other phenolics. In houseflies, p-cresol and 2-phenylethanol, were found in attractive vinegar baits, but oddly it was male house flies who responded most strongly to p-cresol (vinegar baits). Here, p-cresol was active in nearly 60% of female houseflies tested by

GC-EAG, but perhaps this number could have been larger at increased concentrations, or tested with both sexes. In compounds that were tested by GC-EAG analysis, 1-methylethyl benzene, and toluene, o-xylene were amongst those to frequently produce responses. Benzaldehyde, which produced responses in 44.4% of houseflies, was also noted to be active in human and rumen odours, towards mosquitoes and tsetse flies respectively (Harraca, Syed et al. 2009, Tauxe, MacWilliam et al. 2013) . Ngoh, Choo et al. (1998) demonstrated that in *P. americana*, benzene derivatives are more toxic than terpenes. This cytotoxicity, could be why fewer benzene derivative compounds were found to elicit responses at high frequencies.

Alcohols and fatty alcohols were recovered frequently at the farms. 2-nonen-ol, which was recovered at all sites, was also found in the compounds to which either fly species responded. 2-nonen-1-ol, along with 2-decen-1-ol, exhibited a significant impact on stable fly abundance. These two fatty alcohols are also emitted by the carnivorous plant *Sarracenia purpurea*. As this plant traps flies as prey, this gives a good indication that odorants associated with it might also have attractive properties. Studies performed at dairy facilities elsewhere have reported high emissions (Filipy, Rumburg et al. 2006, Sun, Trabue et al. 2008). Ethanol did not occur in the same abundances in VOC samples recovered during the present study. However of the compounds with EAG activity, many shorter chained alcohols, such as isopropyl alcohol, 2-butanol, and 3-methyl-1-butanol promoted frequent responses. 3-methyl-1-butanol is an attractant for female *Tephritidae spp*., and is associated with bacterial fermentation (Epsky, Heath et al. 1998). Jeanbourquin and Guerin (2007), also found numerous alcohol compounds, from rumen digestia that activated *S. calcitrans* when tested by GC-EAG. Amongst them were, oct-1-

en-3-ol, (Z)-hex-3-en-1-ol, and heptan-1-ol. They reported the compound 2-ethylhexan-1-ol, but noted no significant EAG activity to the compound. This is in accordance with the findings here; 2-ethylhexan-1-ol, was recovered frequenly at Ponsonby, but displayed no activity in either fly. A related group, volatile fatty acids (VFAs) and their methyl esters, such as propionic acid and butanoic acid methyl ester also produced many responses in either of the fly species. Emissions mainly originate from animal feeds, and secondarily by bacterial degradation of wastes (Alanis, Sorenson et al. 2008). Further study of VFA emissions by this group found that after acetic acid,the compounds propionic acid, butanoic acid, and hexanoic acid had the highest emission rates. In this study pentanoic acid occurred below the limit of detection in all samples. Conversely, while acetic acid was not recovered at high abundance in this study, propionic acid, and pentanoic acid were recovered and were both responsible for a high percentage of responses in both flies. Yet, some differences in compounds may be due to the variances in cows sampled. At all dairies in the present study sampling occurred near lactating cows. Lactating and dry cows show a trend differing emission rates of VOCS. For example, emissions of propionic acid and 2-methyl phenol are higher in lactating cows, while valeric acid emissions are lower (Sun, Trabue et al. 2008). Regardless, volatile fatty acids represent a large component of dairy cow emissions, specifically those associated with aged wastes and skin odors, and have been demonstrated to show repellent activity in other haematophageous species, such as mosquitos. A study of human sweat volatiles attractive to mosquitos, found alimphatic carboxylic acids (C_1-C_{18}), were the main acid components of human sweat (Cork and Park 1996). They additionally noticed C_1-C_5 acids elicited larger EAG responses than those with longer carbon chain lengths. This appears to be

true in the present study, as larger VFAs did not tend to elicit responses in over 40% of the flies. In addition, in house flies, propionic acids gained more responses than did pentanoic acid.

The compound diptone was identified as EAG active in both *S. calcitrans* and *M. domestica*. This compound has been recovered as an odorant at other sites (Filipy, Rumburg, Mount, Westberg, & Lamb, 2006). With respect to electrophysiological activity however, mentions in the literature exist only to the beetle *Kepher bonelli* (MacLeay), (Coleoptera: Scarabaeidae), where it is constituent of sex attractant secretions (Burger, Petersen et al. 2008). No activity in either *S. calcitrans* or *M. domestica* have been described and no dose response trials were performed in these species. diptone is also likely to be a plant-derived compound, as it 3-hexen-1-ol, and methylated substituents of the compound, have been recovered in plant essential oils and volatile profiles (Zeringue and McCormick 1989, Verdeguer, Blázquez et al. 2009)

There was significant recovery of monoterpenes from VOC samples, including α-pinene, limonene, and isopulegol. Due to of the presence of plant crops at each site, in addition to livestock feed and bedding, plant compounds were expected. Several other terpenoid compounds were present amongst the sites and appeared within the compounds eliciting some stable of house fly electrophysiological activity. Camphene, α-pinene and o-cymene, for example, all gave some response, upwards of 40%. Phorone elicited a response in 66% of houseflies tested. Carene, and α-pinene were also significantly related to stable fly abundance but had no effect on the houseflies. In fact, of the compounds that were excluded from housefly abundance model, many were plant related. A possible

explanation for this, is the reliance of stable flies on plant nectars to acquire energy for host location between blood meals (Jones, Milne et al. 1992, Taylor and Berkebile 2014).

In a study on airborne chemical compounds at dairies, in Sweden, Sunesson, Gullberg et al. (2001) reported α-pinene, and 3-carene amongst those found in the highest levels, and related their abundance to the presence of sawdust on the floor. Several others have noted this pattern. In Nebraska, Miller Miller and Varel (2001), reported volatile fatty acids and alcohols from cattle slurries. In California, two studies confirmed emissions of VFAs as major components of farm odors. Additionally, the presence of alcohol, and phenolic components from dairy cows was confirmed (Sun, Trabue et al. 2008). Amine groups and nitrogenous groups were not found with high frequency in this study, as was noted by two other groups (Sunesson, Gullberg et al. 2001, Rabaud, Ebeler et al. 2003). The distribution of volatile functional groups found during this project aligns with those presently reported, as do the compounds that were found in high abundance during the study.

Of volatiles that demonstrated significant effects on the stable fly abundance, several also fluctuated on a monthly basis. Regarding these compounds, fluctuations often occurred in months, where the presence of the stable flies also increased significantly. Figure 2.11 shows increases of stable flies between June-July, June-August. During this same time, all compounds varied with the exception of isopropyl myristate. IPM was below the limit of detection in Porapak samples, thereby leaving no way to test its relationship to the flies. Hexanal, was not found to significantly vary by month, and accordingly did not affect changes in fly abundance. Conversely, other VOCs such as dimethyl disulfide, and phenol, affected abundances but did not vary by month. Dimethyl

disulfide is a noted attractant of stable flies, however. Phenol, DMDS and a related compound dimethyl sulfide, have been associated with farms and most specifically with livestock urine and manure, thus explaining their lack of variation between the months.

Interestingly, while DMDS did not vary by month, its presence increased during the day, between the early morning and afternoon. In a study by Filipy, Rumburg et al. (2006), dimethyl sulfide DMS was found in high concentration in stall areas, while DMDS was found in areas characterized by high volumes of cattle manure slurries. In these areas, DMDS has similarly high concentration ranges and gave emissions from the slurries at similar rates. IPM and HAME are VOCs that would similarly be associated with livestock manure (Miller and Varel 2001). Aged manures were demonstrated to emit higher amounts of VFAs than fresh manure, due to bacterial fermentation over time. Further, compounds whose presence relies on fermentation processes, can also be affected by temperature and humidity. Ethanol which depends on the fermentation process, is more dependent on temperature on DMS (Filipy, Rumburg et al. 2006). Emission rates of volatiles associated with manure also increase monotonically over time period, increasing with the addition of fresh manure, and decrease when the fermentable sugars present in the manure have been entirely consumed. Thus, differences in volatile profiles over a daily time course, might be related to the fermentation and accumulation of VOCs associated with cattle slurries.

Both stable flies and house flies are nuisance pests at dairy farms in southern Ontario, and their respective presences can cause substantial damages to livestock and economic losses to farmers. It is consequently important to understand in greater detail, the population dynamics of the two fly species, and the factors causing their sustained

presence at such sites. It has reported previously that fly catches can not only vary by site, but also within different areas of the same site, depending on the species and sex of fly being observed. A group in Thailand, (Phasuk, Prabaripai et al. 2013), has demonstrated site-wise difference between stable fly species, so it would not be unexpected to find differences. Yet, fly abundance did not vary significantly between the farms, studied in this analysis. Of the three sample sites, Ponsonby and Shadyway were the most similar, in terms of structure, layout and herd size. These farms however differed in location, as Shadyway was situated greater than 2.5 hours North-East of Ponsonby and Elora. Since stable fly abundances at the sites were similar, it was reasoned that the volatile profiles of the two sites may be similar, yet analysis of the volatiles showed several differences.

Chi squared analysis of chosen compounds, indicated the differences mainly occurred between Ponsonby and other sites. When looking at the distribution of the recurrent compounds (Table 2.1), only 5 of the VOCs appeared at both Shadyway and Ponsonby. These compounds were 2-nonen-1-ol, DMDS, heptanal, IPM, and toluene. Of the five compounds which appeared both at Ponsonby and Shadyway none but 2-nonen1-ol significantly differed in frequency from each other (Table 2.3). Significant differences mainly arose from compounds that occurred only at one site. Elora shared more compound similarities with either farm than they presented with each other, but ultimately was most similar to Shadyway, rather than Ponsonby. Still there were commonalities in the VOC profiles of Elora and its sister farm Ponsonby, and many more than Ponsonby shared with Shadyway. This is perhaps appropriate as the two farms, both managed by the University of Guelph, were located less than 15 minutes away from each other. As such, they tended to experience more similar weather conditions. While the

compounds present between Ponsonby and Elora were similar, it tended to be their frequency in the samples that varied.

Farms had similar distributions of the recovered function groups as described earlier, yet again the biggest differences between farms again occurred again between Ponsonby and Shadyway, with Ponsonby having twice as many instances of VFAs than Shadyway, and twice fewer hydrocarbons (Figure 2.13). Altogether, this data suggests that Ponsonby samples were the most dissimilar of the three sites, but particularly varied from Shadyway. Therefore, similarities in fly abundance cannot be fully explained by a similarity in volatile profiles between Shadyway and Ponsonby. It is likely that VOC profiles were not varied enough to reflect large changes.

Considering fly trap data was discontinued at Elora, due to the amount of insect exclusion at the sample site, further observations here may have resulted in larger differences. Of the compounds assessed by ANCOVA, thirteen compounds were significant to stable fly abundance, but only five of these compounds had significant differences between Ponsonby and Shadyway. In houseflies, this number was reduced as compounds 2-decen-1-ol, and α-pinene, which did vary between the sites were not found to have a significant effect on housefly abundance. Larger variations in more of these compounds might contribute to greater differences in both fly species at the farm. In addition, experimental conditions such as trap location, and height, as well as environmental factors such as precipitation, wind speed and weather conditions can also play a role in stable fly presence at farm (Beresford and Sutcliffe 2009, Showler and Osbrink 2015). Taken together, these factors might help to explain why no significant differences were seen between the farms.

Outside of VOC variables, stable fly abundance was not found to differ significantly with respect to temperature or humidity. Yet, temperature and humidity varied significantly by month and time of day, with temperature rising over the course of the day, while humidity fell. An explanation for this is the fact that while temperature and humidity varied significantly, the range during the sampling season was insufficient to be detrimental to flies. Over the trials, variation in temperature varied 14 degrees, from 14.0°C to 27.7°C. The higher end of this range overlaps with the optimum survival rates of the stable flies. The difference in temperature range decreases when looking at the average daily temperature which ranges just over 9°C (16.29°C -25.50°C). The average daily temperature was the one used to determine the differences in fly abundances as traps were considered on a per day basis. It is likely that the small difference in temperature plays a role in our observations as temperature has been an important factor in other models (Semelbauer, Mangova et al. 2018). In this study, temperature was weakly correlated with stable fly abundance (Spearman's rho, ρ=0.349) as well as house fly abundance (ρ =0.428), but no important association was found for humidity. This was at odds with (Phasuk, Prabaripai et al. 2013), who found at least a weak correlation between relative humidity and stable fly activity, as well as light intensities, but like in the present study found no significant correlation for temperature.

Housefly populations at the sites of study were larger than those of the stable flies. An earlier study in Florida, reported lower averages of stable flies at several different locations, including dairies and horse stables (LaBrecque, Meifert et al. 1972). Broce and Haas (1999) reported colonization of aged manure, by both fly species on dairy feedlots, and found larval populations of stable flies were lower than those of house flies.

Houseflies were also in higher abundance at poultry farms in Brazil (Avancini and Silveira 2000). A study in Alberta, Canada, came to an opposite conclusion, finding houseflies to be present in far lower quantities that stable flies at all sites examined (Lysyk 1993). Given the proximity of the research locations, it was surprising to see such variability in stable fly and house fly densities. Lysyk (1993) was also able to correlate fluctuations in stable fly and house fly abundance to weather conditions such as temperature. It was noted though, that house fly abundance relied less on environmental factors, that did stable flies, and to an extent that mirrored what was seen in these studies. Most studies were in agreement that population of stable flies increased after June and had at least one peak in population in late august or early fall. Lysyk (1993) showed that in Alberta stable fly populations were small in May and did not begin to increase until June or later. Data was obtained over three years, and in the first two years, populations increased steadily through June and July. In two years, either a single population peak was observed in September or, two peaks in fly abundance was observed in late august and September. In 1990, peaks in fly populations as late as October were recorded. In Ontario, some agreement occurs, in that May populations of both fly species were very small. During this time cooler temperatures dominated, as well as precipitation. Fly populations increase throughout late June and into July. At Ponsonby, fly catches were low by September, while peaks in populations were seen as late as September while at sampling at Shadyway in 2015. Still, subsequent trips to this site revealed low populations of stable flies and provided evidence that termination of sampling could occur. In southern Ontario, peaks in populations of stable flies were observed until late September, before decreasing (Beresford and Sutcliffe 2010). Broce and Haas (1999),

observed larvae present in cattle manures decrease by late august, consistent with the fly populations. Similarly, in the Labrecque study, populations of both flies decreased by late august (LaBrecque, Meifert et al. 1972). Like Lysyk (1993), other authors have reported patterns of bimodal stable fly activity, but these occur predominantly in areas with warmer climates. The threshold for immature development is 12.5°C (Lysyk 1998). The grand mean temperature in October 2015, was 8.303°C, which below this threshold, and the minimum temperature decreased to -4.5°C. Holding the assumption temperature is related to fly abundance, it is likely larval and immature development will be severely decreased, if not completely inhibited. However as the maximum temperature reached up to 21.7°C, perhaps in years with warmer fall seasons such bimodal population spikes would be seen.

Stable fly populations at dairy farms, are impacted by a range of interrelated factors. This study was able to provide evidence that VOCs can significantly affect the abundance of stable flies. While weak correlations environmental variables like temperature were observed, no significant effect or model could be drawn from the current data set. Instead, significant increases in fly abundance that were month and time dependent, were demonstrated. A closer look at covariant factors would be useful in future analysis. Recurrent compound recovered in the study showed overlap with those that provoked electrophysiological responses in flies. Electrophysiological responses however, tell us little about the importance of these compounds on fly behavior, and therefore demonstrates the importance of behavioral tests and dose response analysis.

Chapter 3. Dose Response Study on Selected Active Compounds

3.1 Introduction

Activation and anemotaxis in stable flies occur in response to host VOCs. At low doses, carbon dioxide can elicit activation of host orientation in stable flies, but only at high doses will orientation begin (Warnes and Finlayson 1985, Schofield and Brady 1997, Alzogaray and Carlson 2000). This behavior illustrates the importance of VOC concentration and VOC concentration gradients in host-parasite interactions of haematophageous flies. Numerous studies have shown that increasing host odour concentrations at baited traps have increased fly catches (Torr 1990, Alzogaray and Carlson 2000, Mihok, Carlson et al. 2007). Likewise, filtering the odour results in decreased fly catches (Vale and Hall 1985, Schofield and Brady 1997). In addition, the effects of individual components in host odors can produce additive behavioural effects. As seen in, a study by Hieu, Jung et al. (2013), two attractive compounds together will increase the amount of catches seen with only one compound and so on. Similarly, mixing an attractive compound with a repellent one, will decrease the catches found with the attractant alone. This provides evidences that behavioural responses can be overcome or manipulated by understanding insect response patterns to odorants in their environment.

In identifying compounds that can augment the efficacy of traps, synthetic analogues have been evaluated for their use (Hoel, Kline et al. 2007, Okumu, Killeen et al. 2010, Hieu, Jung et al. 2013). Synthetic compounds when tested individually, tend to show different behavioural properties than the natural compositions from which they are derived (Berger and Estes 1987, Torr 1990, Gibson and Torr 1999). One cause for the

difference seems to be that responses from insects are dependant on specific ratios of volatile mixtures. For instance, behavioural bioassays in oriental food moths *Grapholita molesta* Busck (Lepidoptera: Tortricidae), determined that altering natural peach odours, by varying the component benzonitrile, had no effect on normal bioactivity within a range of 2 orders of magnitude (Najar-Rodriguez, Galizia et al. 2010). Outside of this range however, moths could no longer distinguish between the synthetic mixture and the blank solvent. Another study performed with grapevine moths *Lobesia botrana* Denis & Schiffermüller (Lepidoptera: Tortricidae), found that moths were attracted to a 3 volatile mixture found in the same ratio as emitted by grapes. A mixture of the same components were found unattractive by moths, however, when tested in the ratio released in the headspace of apples (Tasin, Bäckman et al. 2006). Another factor stems from the fact pure compounds and their mixtures lack the authenticity of the natural odours, due to the absence of context and missing components. It has been shown for example, that *Aedes aegypti* (Diptera: Cilicidae) mosquitoes do not show attractive responses to lactic acid in the absence of carbon dioxide released from the host's breath (Hoel, Kline et al. 2007).

Electroantennography studies have been used to investigate the activity of VOCs and their mixtures. Electroantennography (EAG) is a method developed by Schneider (1957) which can be used to determine the electrophysiological response of insects to semiochemicals of interest. The tool is powerful as a bioassay to understand how sensory information in perceived by olfactory systems. The method measures changes in voltage across the antennae caused by the depolarization in olfactory neurons responding to chemical stimuli. When a VOC is bound to an olfactory receptor, the heterodimerization of OR and olfactory receptor coreceptor (ORCO) allows for pore formation, resulting in

an influx of positively charged calcium ions entering the neuron (Neuhaus, Gisselmann et al. 2005). The decreased negative charge, sensitizes nearby ion channels, and causes a cascade across the membrane, thereby depolarizing the cell. This triggers an action potential, which is responsible for cell to cell communication to nearby postsynaptic neurons, via the release of acetylcholine (Guo and Smith 2017). In EAG, the change in voltage due to the influx of ions into the cell is measured, by placing electrodes, in the head and/or antennal segments of the insect. Whereas in single sensillum recordings (SSR), the change due to 1-4 olfactory neurons can be measured, the combination of depolarized cells across the antenna are measured in EAG (Syntech 2004, Vosshall and Stocker 2007). The technique has been used within the Muscidae family, specifically *Stomoxys calcitrans* and *Musca domestica*, to classify the electrophysiological responses to semiochemicals of interest. Electroantennogram recordings have also been used in *Stomoxys calcitrans* to study host interactions. Notably, electroantennography has been used very successfully with tsetse flies (*Glossina spp.*) an important hematophagous pest and known vector of African trypanosomiasis contributing to live stock damage in sub-Saharan Africa (Gibson and Torr 1999). Because of their damage to bovine and suide species, identification and analysis of attractants have been sought after to enhance the catching ability of traps to knock down disease in the population. EAG has been used in the identification of many attractants and repellents of hematophagous flies such as carbon dioxide, octen-3-ol, phenols and active aldehydes (Torr 1990, Van der Goes van Naters, Den Otter et al. 1998). Due to the similarities between *Glossina* and *Stomoxine* feeding behaviour, risk of disease transfer, and breadth of economic damage both in America and sub-Saharan Africa, attractants and repellents of *Stomoxine* flies have been

pursued—often starting with compounds previously known to attract *Glossina spp.* (Gibson and Torr 1999, Schofield and Torr 2002). Both electroantennography and single cell recordings have been useful in identifying active compounds, especially when paired with wind tunnel or field studies. Coupled gas chromatography-EAG in particular has been able to relate electrophysiological response directly to compounds found in host breath, host degradation products, microbiota (Romero, Broce et al. 2006), and plant volatiles (Wibe 2004, Hieu, Jung et al. 2013) . Because action potentials are an 'all or none' response to these compounds, and insects are both sensitive and selective to sensory stimulation, EAG is applicable for the detection of any compound that is capable of triggering a response in insects. This translates to a signal for stimulants for which the concentration is as low as a femtogram (Jeanbourquin and Guerin 2007, Poole 2012) and illustrates differential reactions to chemical enantiomers (Mustaparta, Angst et al. 1979, Mustaparta, Angst et al. 1980, Wojtasek, Hansson et al. 1998). Therefore, each compound evoking a response in the insect brain can be identified and characterized.

While EAG can help to identify putative attractants and repellents, dose response is useful for examining the extent of the activity. Such experiments can determine the dose dependent responses of single and combined VOCs of interests (Hieu, Jung et al. 2013). Dose response experiments measure the relationship of physiological response to a given chemical stimulus, or drug, at varying concentrations. Within the context of chemical ecology, dose response can be used with electroantennography (EAG) or single sensillum recording (SSR) to measure the response (the depolarization across the fly antennae) to a VOC of interest (Schofield, Cork et al. 1995, Jeanbourquin and Guerin 2007, Hieu, Jung et al. 2013). These experiments are particularly useful because they can

be used to generate information on key dynamics related to olfaction. These studies have been applied in a pharmacological context with the purpose of identifying neurological receptors and target sites of action of specific drugs (Dethier, Browne et al. 1960, Brownlee and Johnson 1965). For example, studies of physiological behaviours displayed by *Drosophila* in response to cocaine, have to lead to the identification of previously unknown targets in humans (Lease and Hirsh 2005). Using dose response-electrophysiology, Sakurai, Nakagawa et al. (2004), have located male specific olfactory receptors in *Bombyx mori* (Lepidoptera: Bombycidae), giving evidence of sexual dimorphism in olfactory responses. Accordingly, differences in male and female EAG responses to the same compounds have been documented in a variety of species including beetles, moths and flies (Otter, Tchicaya et al. 1991, Blight, Pickett et al. 1995).

The dose response relationship is useful since it can be used to generate information on the threshold at which a given compound can be perceived by an insect, and demonstrate the range of concentrations at which physiological responses to host odours occur. Typical experiments plot the magnitude of the EAG response of the insect as a function of the VOC concentration, as dose-response curves. Dose response curves allow for the visualization of the minimum concentration of a VOC that will provoke a response, as well as the concentration above which the magnitude of the EAG response will no longer increase. This describes the range at which saturation of olfactory receptors (ORs) occurs, and can help in the estimation of how many receptors exist in a species for a given VOC (Wright, Carlton et al. 2009). Functionally, this describes the point above which increasing the concentration of a VOC will not increase any behavioral response associated with it. Dose response can also inform as to whether

multiple VOCs share similar binding sites (Schofield, Cork et al. 1995, Hoskovec, Hovorka et al. 1996, Oka, Omura et al. 2004). For example, Hoskovec, Hovorka et al. (1996) determined neuron sensitivities to the moth pheromone Z-8-dodecenyl acetate showed reduced but similar responses to substituted analogues. In another study, *Aedes* and *Anopheles* mosquitoes, electrophysiology experiments performed while selectively inhibiting the CO_2 sensitive olfactory neuron, noted decreased activation responses to other compounds present in host odors, with noticeable changes in dose response curves post inhibition (Tauxe, MacWilliam et al. 2013). These findings underscore the relationship and chemical similarities of compounds which share binding sites. Additionally, they illustrate the ability of certain compounds to hamper the ability of insects to detect important cues in their environment.

Dose response trials can therefore help us to visualize the effect of many important factors associated with olfactory and gustatory responses. Experiments by Gillary (1966), monitored blowfly sodium chloride receptors and their response with respect to concentration, pH, and age. The study demonstrated that sensitivity of flies increased with age, decreased at extremes of pH, and noted dose dependent responses varied in reproducibility and adaptation, based on the sequence the doses were tested. Specifically, when concentrations were tested with increasing magnitudes, data was more reproducible. Stimulations with several concentrations tested repeatedly, resulted in the opposite—higher adaptation in receptors and lower reproducibility. This effect was reiterated in stable flies by Schofield, Cork et al. (1995) who described increases in recovery time required for olfactory receptors, with dose and increasing chain length of active compounds. Further, EAG dose response series performed in the absence and

presence of background odours also describe adaptation of olfactory receptors in house flies (Kelling, Ialenti et al. 2002). As such, dose response studies with EAG can provide detail on how fast adaptation to the compounds can occur, as well as how quickly it can be reversed; which is beneficial for understanding the behavioral and electrophysiological response to olfactory cues.

Therefore, generation of dose response curves using EAG was used to confirm the selected compounds isolated from dairy cow farms were semiochemicals in *Stomoxys calcitrans* and *Musca domestica.* Further characterization of responses in both species, toward active compounds was necessary, especially with respect to threshold and saturation levels, as well as sensitivity.

3.2Methods

3.2.1 Insect Rearing for Dose Response Trials

Stomoxys calcitrans

Female stable flies aged 3-10 days old were selected for the dose response experiments. Females were chosen for their slightly increased sensitivity over male stable flies. While female and male flies generally display activity to the same compounds, sensitivity and intensity of response may sometimes vary. For example, in EAG studies, female stable flies show greater magnitude of response than males for a variety of compounds, such as attractant 1-oceten-3-ol and C2-C8 primary alcohols. In a study by Kelling, Biancaniello et al. (2003), housefly responses were similar between sexes, but in the only variation noted, female houseflies were more sensitive to acetic acid. It is interesting that there is no major sexual dimorphism in most sensillum types, but there is evidence that females

have more medium trichoid sensilla present on their antennae (Tangtrakulwanich, Chen et al. 2011). Combined with different gene expression between males and females tsetse flies (Nyanjom, Tare et al. 2018), this may support evidence that female flies are more sensitive to stimuli in their environment.

Additionally, observations in EAG studies also show interaction between age, sex and hunger in sensitivity to response. In stable flies starved up to 50 hours, significant increases in response were observed relative to those starved 20-23 hours after eclosure (Warnes and Finlayson 1986). Similar intensification of response to lactic acid in host odors has been observed in the *A. aegypti* mosquitoes, as a function of lactic acid receptor development (Davis 1984). Females are inactive in the first 24 hours after emergence, while sensitivity of the receptors increase until the 3rd day, at which time active host seeking behavior commences. Houseflies, who are capable of detecting host odours immediately after emergence also demonstrate increased sensitivity with age (Kelling, Biancaniello et al. 2003). In addition to heightened responses, reproducibility is important in EAG studies. It was demonstrated that in Glossina spp. (Diptera: Glossinidae) stability of fly responses to stimuli are relatively even during the time period between 3 and 10 days. While, on the 1st day after emergence, fly EAG responses are at their highest, the magnitude of the responses decrease significantly over the next few days. After 3 days old, flies still display heighted responses to stimuli, with no more significant decreases until after 10 days (Otter, Tchicaya et al. 1991). Therefore, starved female stable flies between 3-10 days were selected for EAG studies, due to the increased responses after 48 hours, and the stability of response after 3 days as seen in other haematophageous dipterans. Female stable flies were separated as they emerged and housed in mesh cages

at 22°C at 66% RH. Blood meals were not given, however they had access to 15% sucrose solution and water, which increases energy stores and elongates lifespan in absence of blood (Jones, Milne et al. 1992, Taylor and Berkebile 2014).

Musca domestica

Dose response experiments with female house flies were also conducted within 3-10 day after eclosure. Not only were female houseflies, more sensitive to acetic acid, but mature flies (4-10) days, were found to be more sensitive than newly emerged flies (under 24 hours), to compounds amyl acetate and limonene (Kelling, Biancaniello et al. 2002). Therefore, in order to keep consistency between trials, and to gain increased responses in female house flies, EAG studies were also completed between 3-10 days with these flies. The female flies were kept separately from the main colony and separated at emergence. Like the stable flies they were given access to 15% sucrose solution and water ad libitum. Blood-meals are unnecessary for mating or oviposition in house flies, but protein is still needed to promote fecundity. Milk powder is used as a protein supplement in the housefly diet, and therefore this was withheld from the female house flies used for dose response.

3.2.2 Compound Choice, Confirmation, and Preparation

The Porapak samples collected from the three farm trials, were used to run GC-EAG trials and observe potentially active compounds-those that elicited a depolarization from the fly as they eluted from the compounds. VOCs, which elicited a response greater than 50% of the times that they appeared in samples, were considered electrophysiologically active. Compounds that had the most reoccurrence in the samples, the most

electrophysiological activity, and were previously demonstrated to be present in host odours were chosen as candidates for dose response. The test compounds chosen were Butanoic Acid-Methyl Ester (BAME), dimethyl disulfide, isopropyl alcohol, pentanoic acid, propionic acid, and diptone.

Once selected, each compound was confirmed by GC-MS analysis with temperature programs as described in Chapter 2. Porapak samples had previously been run on a Varian 240 gas chromatograph with an HP-5ms column. Dairy farm samples which contained the compound of interest were spiked with chemical standard solutions diluted to 100 µg/µl using diethyl ether. Previously observed peak areas increased in response to the added standard solution, thereby serving as confirmation of the compound.

Compound diptone was synthesized by Jeffrey Regier (PhD candidate, Bolshan lab group, University of Ontario Institute of Technology), having been detected in multiple samples, and consistently generating responses in both flies. The compound had not been referenced as a chemostimulant in Dipterans, however has been referred to as a secretion by male Khepri beetles (Mucignat-Caretta 2014).This compound was also confirmed by GC-MS co-injection, and analyzed for impurities.

Initially, dilutions of each of the samples were prepared in diethyl ether at 100 µg/µl, 10 µg/µl, 1 µg/µl, 1.0x10-1 µg/µl, 1.0x10-2 µg/µl, 1.0x10-3 µg/µl, and 1.0x10-4 µg/µl. However, higher concentrations (400 µg/µl and 200 µg/µl) were added to determine at which point saturation of the antennae occurred. Diethyl ether was used as a negative control since it was used as the solvent for the compounds. Acetone was used as a solvent for the compound diptone, and therefore was used as negative control for this

compound. As a positive control, methyl salicylate (2 x10-3 µg/µl) was chosen. Methyl salicylate elicits a consistent response in both stable flies and house flies, however as a plant compound which it is unlikely to cause any activating behavior in flies looking for a blood meal. Other studies have shown that a range of insects respond reliably to this compound, in both EAG and SSR experiments (Wadhams, Blight et al. 1994, Blight, Pickett et al. 1995, Birkett, Agelopoulos et al. 2004, Xu, Cai et al. 2015). As such, methyl salicylate was ideal and set a threshold for a perceivable positive response in the other compounds. The negative control stimuli were delivered first at the beginning of the recording, in the order: air, diethyl ether, methyl salicylate positive control, finally followed by the test compounds. This order ensures we are able to measure the baseline responses of the flies to the negative controls without interference of the positive control.

3.2.3 Electroantennography

Dose response experiments were conducted using virgin female flies. Flies were anaesthetized on ice and antennal preparations were carried out as described in section 2.2.4. Due to the extreme sensitivity of the flies to smell, all dose response compounds were prepared in a separate room adjacent to the faraday cage housing part of the EAG system. Test solution was applied to Fisherbrand Q2 fine porosity filter paper, (Fisher Scientific, Pittsburgh, PA) measuring approximately (4mm x 40mm). Halved Pyrex borosilicate glass capillaries (0.8-1.1 x100mm) were used to apply the solution, and the filter paper was then allowed to dry for 15 seconds. The filter paper was transferred into a 15cm (5 ¾ inch) borosilicate Pasteur pipettes (Fisher Scientific) and the top opening was held closed to allow the volatiles to equilibrate for another 10 seconds. The Pasteur pipette was then attached to the charcoal filtered humidified air source and air was

delivered on to the fly head preparation. The preparation of the next sample allowed the antenna to return to baseline, leaving 2 minutes between each test. Time was left between the sample, to minimize adaption of the fly to stimulus, and to avoid the occurrence of baseline drift.

3.2.4 Method Development for Application of Stimuli

In order to create dose response curves it was necessary to ensure there was both no bias in the samples as well as no interference between compounds tested. Two different methods were tested here to optimize the response and reproducibility of the experiments.

The first method, the dose response was performed such that one fly received a stimulus by all 8 of the compounds at one concentration. In essence, each fly would receive stimulus by BAME, followed by DMDS, then IPA, etc. at a dose of 0.0001 µg/µl. The experiment would then repeated at differing doses, with at least 5 replicates. Multiple doses were never performed on the same fly preparation as the length of time required to test each compound, and allow for the baseline to return to normal, would result in suboptimal responses. Once each run was performed a new fly preparation would be tested. A large variation was observed in fly responses with this methods, even between the control compounds, such that runs of the same doses were hardly comparable. Also, as it has been noted, odorants exhibit antagonism amongst each other and can diminish the observed EAG responses of other compounds being tested (Oka, Omura et al. 2004). Within this study, this was particularly observed at higher doses of the tested compounds. For example, following mid to large doses of dimethyl disulfide or propionic acid, responses to the test compounds were increasingly small and the recovery time for the antennal preparation became long if it did ever recover.

Alternatively, flies were tested for one compound at the full range of the dose. For example, BAME would be tested on one fly preparation 0.0001 µg/µl-400 µg/µl. All test compounds were delivered in increasing concentrations only, once a compound of higher concentration was started, there were no tests on compounds of lower concentrations. This was done in order to avoid losing the sensitivity of the fly to the lower concentrations. The result was an increased similarity between the responses across the tested fly heads and greater consistency in responses within the dose across the entire range. As such the second protocol was chosen to generate the dose response curves.

3.2.5 Statistical Analysis

The individual antennal response varies, according to many factors such as: age, sex, time of day and so on. In order to account for individual differences, responses were normalized using the following procedure. All ether control responses were subtracted from the test stimuli responses as in the method outlined by Beck, Light et al. (2012). In the case of diptone, which was prepared in acetone, acetone was subtracted from the test compound response. The resulting responses were then normalized, with the highest response, in each run being designated as 100%. The means were taken from 5 replicate experiements, and the corresponding data was fit to dose response curves using GraphPad Prism (Version 6.00, GraphPad Software, La Jolla California USA). Statistical analysis for the dose-response relationships were performed using R software (R Development Core Team, 2010). In R, the mean relative response was tested for significant differences between each dose, using ANOVA and Tukey's HSD post hoc tests. Compact Letter Display (CLD) of pairwise comparisons are included for visualization of statistical

differences at each dose. Mean responses to the positive control methyl salicylate are displayed on the models.

3.3 Results

3.3.1 Stable flies

Butanoic Acid Methyl Ester

Stable flies were tested for their response to Butanoic Acid Methyl Ester. The response data was fit to a 4-parameter logistic (sigmoidal) curve (Figure 3.1). The fitted hill slope for the observed stable fly response was 0.3 and the EC_{50} of the model is 0.6 µg/µl. Stable flies gave a maximal response to the 100 µg/µl dose with a mean response of 95.8%. The doses 10, 200, and 400 µg/µl were not significantly different from the 100 µg/µl dose nor each other. The minimal response was given at 0.0001 µg/µl, wherein the mean response was 6.3%. The first dose that elicits a response larger than that of the positive control is 0.1 µg/µl with a mean response of 35.1%.

Dimethyl Disulfide

The relative responses elicited by the compound DMDS in the stable flies fit a 4 parameter logistic model with a hill slope of 0.5 (Figure 3.1). The EC_{50} of the model is 3.8 µg/µl. The relative response of the stable flies peaked at 200 µg/µl, which in all replicates gave the largest response. The minimum response was given by 0.0001 µg/µl, and had a mean response of 2.6% of the maximum response in stable flies.

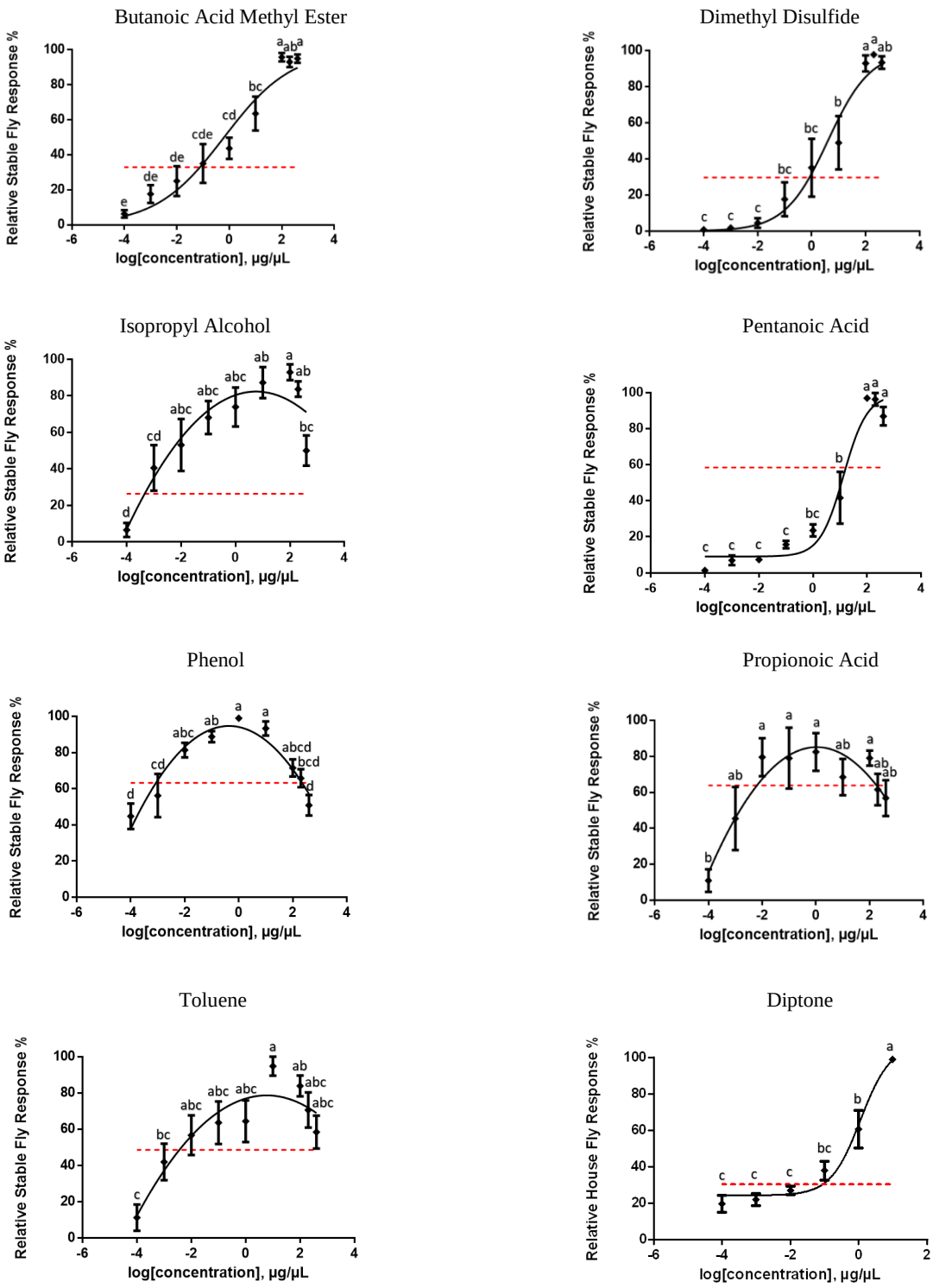

Figure 3.1. Dose response curves of normalized responses to *Stomoxys calcitrans*. Mean responses (n=5) are presented at each concentration; concentration is displayed on a logarithmic scale. Red dashed line indicates positive control methyl salicylate. Error bars represent standard error of the mean. The mean response of the dose, were compared with ANOVA and pairwise comparisons were performed with Tukey's HSD. Statistical differences are demonstrated using Compact Letter Display (CLD), means between stimuli with the same letter are not significantly different.

Therefore, the lowest dose elicited almost no response from the stable flies to DMDS and the following two doses gave similarly low responses (3.6% and 6.1%). The first dose that elicits a significantly different response from this concentration is 10 µg/µl, giving 49.1% of the maximal response, however the 1 µg/µl dose is the first to provoke a response larger than that of the positive control.

Isopropyl Alcohol

IPA was first fitted to a 4 parameter logistic curve as with the rest of the compounds. However, this curve fit poorly especially with respect to the highest doses (100-400 µg/µl). The data was therefore fit to a second order polynomial curve (U-curve) giving an R-value of 0.6583 (Figure 3.1). As a predictive model, the non-monotonic shape of the curve fit the data more appropriately. Due to the biphasic nature of the curve, EC_{50} values are not estimated in the model, as there are at least two doses at which relative response can be 50%. The primary EC_{50} value (the first dose that would stimulate a response of 50%) was approximated for this and future biphasic models, based on the line equation given for the curve. The estimated EC_{50} value for IPA was 0.004. At the 400 µg/µl the mean response dropped down to 50-57% of the maximal response and the smallest dose 0.0001 µg/µl, gave a mean response of 6.6% of the maximum dose (100 µg/µl). Relative to the other compounds, IPA provokes larger relative responses at low dosages, with the 0.001 µg/µl dose being the first to have a magnitude above the positive control.

Pentanoic Acid

Stable fly responses to pentanoic acid were fit to 3 parameter logistic curve and show a clear dose dependent response (Figure 3.1). For the curve given, there is hillslope of 1. When compared to other sigmoidal curves found in stable flies, this represents a relatively steeper change in stable fly responses between 1- 100 µg/µl. The EC_{50} for the data is 13.46 µg/µl and the dose giving the maximum response was 100 µg/µl with a mean response of 97.0%. A minimum response of 1.4% is given to the smallest dose at 0.0001 µg/µl. There is a quite a low response, through the lower concentrations until the 1-10 µg/µl dose at which point the responses are significantly higher. Notably the first dose to rise above the positive control is 100 µg/µl, which is large relative to the other compounds tested in stable flies.

Phenol

The observed responses for Phenol were fit to a second order polynomial curve (R= 0.6485) similarly to IPA (Figure 3.1). While there is a dose dependent response, the relationship seems to also be non-monotonic, with the responses having decreased at high values, to as low as 50.9% of the maximum response. The primary EC_{50} of the curve is 0.0003 µg/µl. The mean maximum response of 99.0% was given at a dose of 1 µg/µl., and accordingly median doses 0.01-10 µg/µl elicited the highest responses in the data set. The dose 0.01 µg/µl is the first concentration to exceed the positive control with a response of 81.4%. The minimum dose tested exhibited a mean response of 44.8%, the largest of all of the compounds tested in the stable flies. This illustrates that small doses of the compound can still provoke a stimulatory response in stable flies, perhaps even below those tested here.

Propionic Acid

The relative stable fly responses were fit to a second order polynomial curve (R=0.4366), as no fit could be determined for a sigmoidal dose response curve (Figure 3.1). As with previous biphasic curves, the primary EC_{50} was extrapolated using the line of the curve, and determined to be 0.0003 µg/µl. There was large variation in the final doses 100-400 µg/µl, with 2 responses dropping below that of the control at 200 and 400 µg/µl. The maximum response is given at a relatively small dose of 1 µg/µl, however there was no significant difference expressed between doses 0.01, 0.1, and 1 µg/µl. The minimum concentration at 0.0001 µg/µl, is the only dose at which the responses are significantly different from the others. As such, it seemed possible that this curve represented the top of a typical sigmoid function, and that at the chosen concentrations, stable flies exhibit extreme sensitivity. Yet, at the minimum dose, the mean response is 11.0612%, leaving little room for smaller doses to minimize the model.

Toluene

Toluene was fitted to a second order polynomial curve, with an R^2 of 0.5101 (Figure 3.1). The maximum response 94.8% is given at a dose of 10 µg/µl, and the minimum value at the 0.0001 µg/µl dose for a mean response of 11.3%. At 400 µg/µl the response drops down to 58.5% of the maximum dose. The bulk of the doses are not significantly different from each other, the exceptions being significant differences between the maximum and the minimum doses at 1 µg/µl, and 0.0001 µg/µl. All doses above 0.001 µg/µl give responses larger than that of the positive control.

Diptone

The responses for diptone were fit to a 3 parameter logistic model and demonstrates dose dependence (Figure 3.1). The EC_{50} of the curve is 1.1 µg/µl with a hillslope of 1. The maximum response of the doses tested was given at 10 µg/µl, which was the highest concentration available for this compound. The minimum response given is 19.7% of the maximum, at 0.0001 µg/µl indicating that there could be some reaction to diptone even below this concentration. Accordingly, the model predicts an increase of responses beyond the largest dose tested. Doses above 0.1 µg/µl provoke responses larger than the positive control.

3.3.2 House flies

Butanoic Acid Methyl Ester

Observed responses for house flies were fit on a 4 parameter logistic curve with a hill slope of 0.3 (Figure 3.2). The EC_{50} associated with the model is 0.2 µg/µl. The maximum response of 95.3% was given at 200 µg/µl, and the minimum at 0.0001 µg/µl had a mean of 16.0%. Doses above 0.1 µg/µl provoke larger responses than that of the positive control, although responses at 0.01 µg/µl fall upon this magnitude. There is a steady, nearly linear increase in response with dose, indicating there might be further response at higher concentrations than those tested here.

Dimethyl Disulfide

Responses from house flies to the compound DMDS were fitted to the standard 3-parameter logistic curve with a hillslope of 1 (Figure 3.2). The EC_{50} for the model is 20.2 µg/µl. The minimum response at 0.0001 µg/µl is 10.1%. There is a dose dependent

relationship although, until the 10 µg/µl dose, all of the relative responses are quite low. Accordingly, the 10 µg/µl concentration is the first to fall above the positive control. Doses 100, 200, and 400 are significantly larger than all previous doses, and there is a sharp increase in response between 10-100 µg/µl from a relative response of 35.9% to 97.8%. The dose at which the maximum response was given was 100 µg/µl. In the following doses, there is a small but non-significant decrease in housefly response.

Isopropyl Alcohol

The observed responses for house flies were fit to a 3 parameter logistic curve with an EC_{50} of 20.4 µg/µl (Figure 3.2). At 400 µg/µl there is a decrease in response, but this is not significantly different from either the 100 µg/µl or 200 µg/µl doses. These are the only concentrations eliciting responses larger than that of the positive control. The minimum response is given at 0.0001 µg/µl, however doses between 0.0001-10 µg/µl all give relatively low responses, with no significantly different groups among them. Between doses 10 µg/µl and 100 µg/µl there is almost a 3-fold increase in response, suggesting response by houseflies to isopropyl alcohol occurs only when it is present in high concentrations.

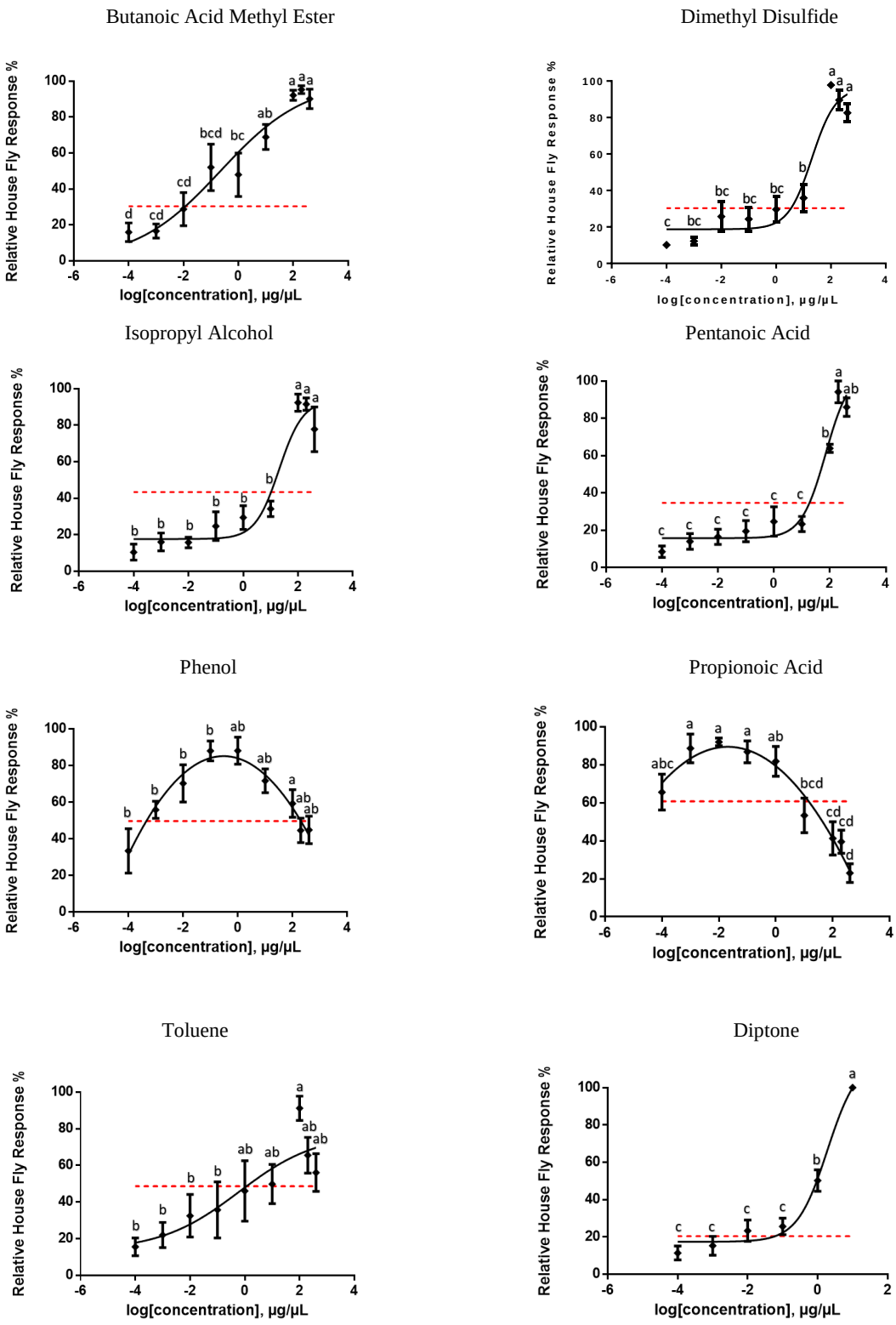

Figure 3.2. Dose response curves of normalized responses to synthetic compounds in *Musca domestica*. Mean responses are plotted at each dose tested. Red dashed line represents mean response to positive control, methyl salicylate. Significant differences are represented with Compact Letter Display (CLD); dosages which have the same letter are not significantly different from each other.

Pentanoic Acid

Housefly responses to pentanoic acid fit a 3 parameter logistic curve with an EC_{50} of 70.8 µg/µl, which was high relative to the other models (Figure 3.2). The maximum response occurs at 200 µg/µl, while the minimum value is 8.5%. Doses 0.0001-10 µg/µl are not significantly different from each other and do not exceed 25% of the maximum response. While the first dose to provoke a larger response than the positive control is 100 µg/µl, it is considerably larger in magnitude than the control. The curve has a standard hillslope of 1, reflective of a sharp increase in response between 10-200 µg/µl from 23.3% to 94.1%.

Phenol

Housefly responses to Phenol were fit to a second order polynomial curve similar to the stable flies (Figure 3.2). The minimum response (mean 33.4%) is given at dose at 0.0001, yet the dose at 400 µg/µl comes close to this range with 44.9%. The curve is essentially symmetrical with median values eliciting the highest responses, and no significant difference between the highest and lowest concentrations. The maximum response occurs at 1 µg/µl, with a mean response of 88.1%. This response value is low in comparison to the other models, but deviance in the two neighboring doses may account for this. In the range of 0.001-100 µg/µl, all responses fall above the control. The remaining doses do not provoke responses much lower than this, and suggests that concentrations below 0.0001 µg/µl could still yield some reaction in the flies.

Propionic Acid

Propionic acid was fit to a second order polynomial curve with an R^2 of 0.7031 (Figure 3.2). All doses below 10 µg/µl, cause responses greater than the positive control. The maximum response occurs at dose 0.01 µg/µl. At the smallest dose, the mean response of the house flies was 65.6%, which is the largest response at this concentration in any of the compounds tested. Such a response suggests, extreme sensitivity to this compound in the house flies, extending into the picogram range. Conversely, responses are significantly decreased at high concentrations. The smallest response to this compound occurred at largest dose tested (400 µg/µl), with a mean of 23.0%; such a trend might indicate at greater dosages, response could be completely inhibited.

Toluene

Housefly data for the compound Toluene were fit to a 4 parameter sigmoidal curve with a hillslope of 0.3025 (Figure 3.2). The model shows a large amount of linearity, with the doses 100 µg/µl and 400 µg/µl being the only deviating responses. The maximum response (91.2%) occurs at 100 µg/µl, and is significantly different from the 3 smallest concentrations. There are no significant differences between the rest of the concentrations tested, therefore dose dependence if any is minimal. The minimum value occurs at 0.0001 µg/µl with a mean response of 15.5%. Throughout the mid-range doses, there is considerable deviation in response, potentially accounting for the lack of differences between the doses. The given EC_{50} value of the curve is 0.6 µg/µl.

Diptone

Observed housefly responses to diptone were fitted to a 3 parameter logistic model (Figure 3.2). The EC_{50} of the curve is 1.9 µg/µl with a hillslope of 1. The maximum response of the doses tested was given at 10 µg/µl, which was the highest tested, however it is likely that there are increased responses at higher doses. The minimum response given is 11.4% of the maximum, at 0.0001 µg/µl. With the exception of the first two concentrations tested, all responses are greater than the positive control. There is an increase in response by nearly 5 times in the range of 0.1-10 µg/µl.

3.3.3 Comparison of Dose Dependent Responses between Species

Compounds that were tested for dose response fit two different types of curves; either the typical sigmoidal model, or biphasic 2^{nd} order polynomial curve. Compounds that exhibited the U-shaped curves were isopropyl alcohol, phenol, propionic acid, and toluene.. The shape of the curves for a given compound were often but not necessarily conserved across the fly species. For example responses towards phenol and propionic acid were fit to 2^{nd} order polynomial curves in both the stable flies and the house flies, yet for IPA and toluene, while stable flies fit a 2^{nd} order polynomial curve, house flies were fit to a sigmoidal model. Flies were most sensitive to compounds that elicited responses modelled by the 2^{nd} order polynomial curves. Of all the compounds, these ubiquitously elicited the highest responses at small doses, and appropriately had the lowest EC_{50} values, ranging between 0.0001-0.005 µg/µl. Generally, stable flies had lower EC_{50} values than house flies in response to the same compound; there were also more incidences of U-shaped curves in stable flies however. Of the compounds stable flies were most sensitive to phenol (EC_{50}: 0.0003 µg/µl), and then propionic acid (EC_{50}:

0.0003 µg/µl). In the house flies, highest sensitivity was towards propionic acid, in which the EC_{50} value occurred below the lowest dose tested, and then phenol (EC_{50}: 0.0005 µg/µl). In the compounds following the typical dose response curves, both stable flies and house flies were most sensitive towards BAME (EC_{50}: 0.6 µg/µl and 0.2 µg/µl, respectively) then diptone (EC_{50}: 1.1 µg/µl and 1.9 µg/µl). Similarly, both stable flies and house flies appeared to be the least sensitive to pentanoic acid of all the compounds tested here (EC_{50}: 13.5 µg/µl and 70.8 µg/µl).

Overall, the bulk of the lowest responses occur at the dose 0.0001 µg/µl, which was the smallest dose tested. The only exception to this occurred when housefly responses were tested towards propionic acid, in which the smallest response was given in response to the 400 µg/µl dose. The concentration eliciting the maximum response was more varied, generally ranging anywhere from 1-200 µg/µl in stable flies and as low as 0.01-200 µg/µl in house flies. A heatmap was used to compare the difference in the magnitude of responses between the two fly species. Housefly responses were subtracted from those of stable flies, and are illustrated in Figure 3.3. To further visualize differences in dose response curves, the fitted models of stable fly responses were overlaid with the housefly responses to the same compound in Figure 3.4

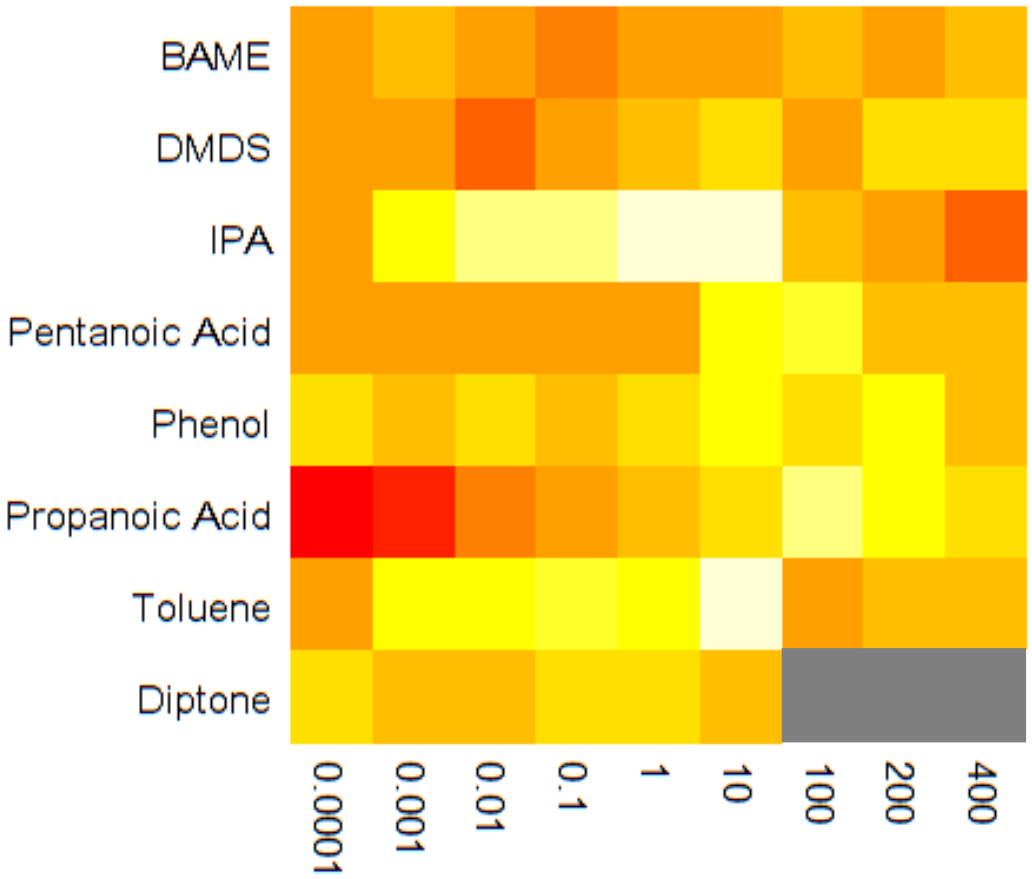

Figure 3.3. Heatmap of differential responses towards tested compounds at all dose levels (µg/µl). Displayed responses are relative to stable flies (house flies subtracted). Data is unscaled within rows (tested compounds). Absent data for diptone at doses 100, 200, and 400 ug/ul (dark grey). Large differences and negative differences are represented dark red squares, white-yellow squares representative of large positive differences.

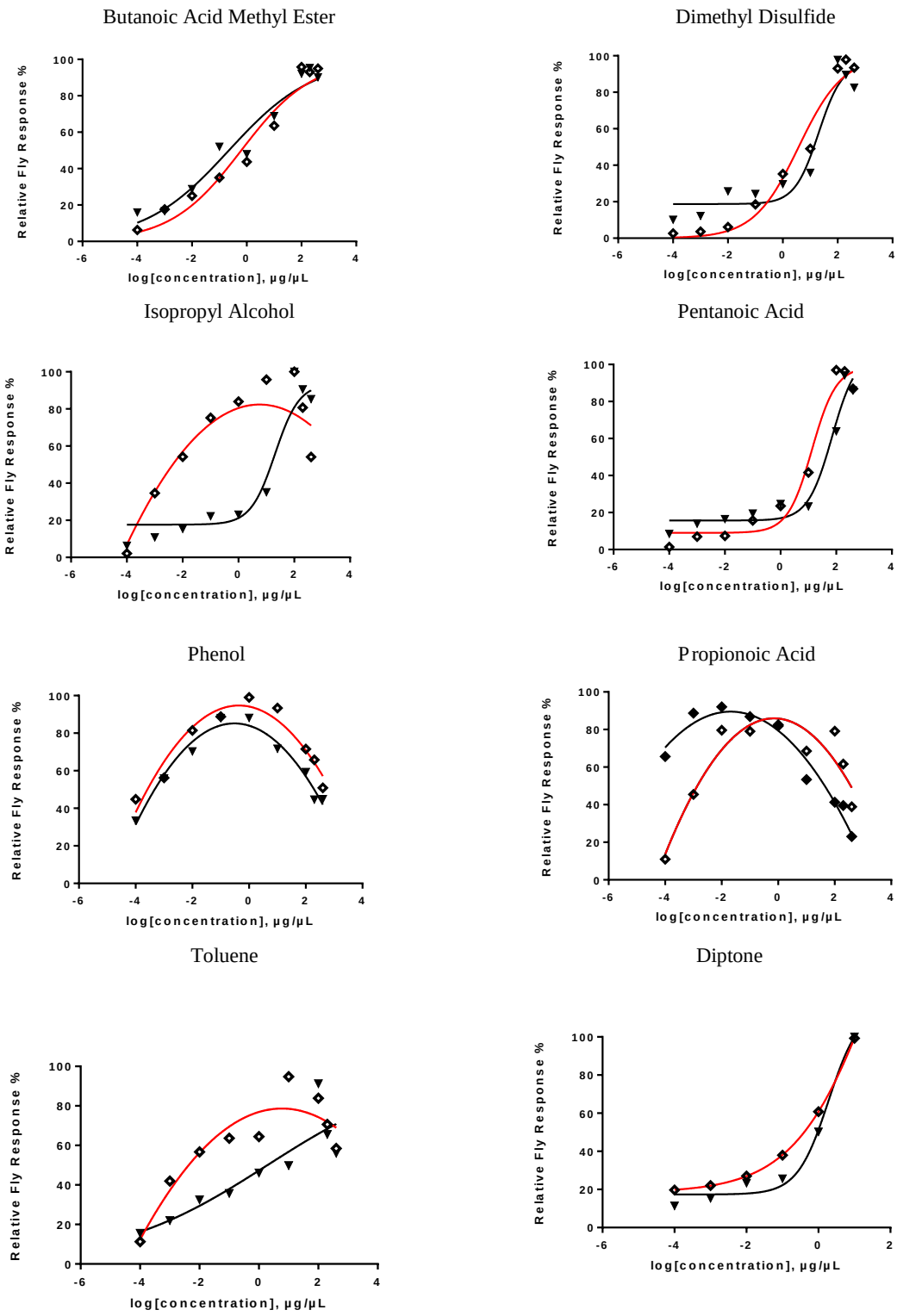

Figure 3.4. Dose response models for *Stomoxys calcitrans* were overlaid with *Musca domestica* models to the same compounds. Open diamonds and black triangles represent mean responses at each dose for *Stomoxys calcitrans* and *Musca domestica* respectively. For SEM, see Figure 3.1 and 3.2. Red solid line is the fitted model *Stomoxys calcitrans* and the black like is the fitted model for *Musca domestica.*

Butanoic acid Methyl Ester

Butanoic acid methyl ester was fit to the same curve in both stable flies and house flies. Neither curve had a particularly high slope and both were fairly linearized. The stable fly curve had the higher slope representing the greater change in response relative to concentration, however this did not represent a significant difference between curves (*P*= .86). The stable fly model also had the higher EC_{50} value, but no significant difference was determined (*P* = .86). The larger response at the minimum value in stable flies; is indicative of a lower threshold value overall, but this value could not be determined here. In both species, the first response to produce a response greater than the magnitude of the positive response was 0.1 µg/µl, though in the house flies, the response at 0.01 µg/µl falls just under this value. While stable fly responses peaked at 100 µg/µl, those of the stable flies peaked at 200 µg/µl. Across the dose range, the response values are similar with an exception to the 0.1 µg/µl concentration. There is a trend of the housefly responses being greater than those of the stable flies, until the higher doses (Figure 3.3).

Dimethyl Disulfide

Both fly species were fit to sigmoid curves for the compound dimethyl disulfide, however while housefly responses could be modelled to the standard curve, stable flies were better fitted towards a 4 parameter logistic function, which allowed for variance in the slope. As such, the stable fly model had a function with a hillslope of 0.5—almost half as steep as the standard 1 demonstrated by the housefly model. This is consistent with the large increase in response from 10 -100 µg/µl in the house flies, while there is a more gradual increase seen with the stable fly curve. The stable fly curve gives evidence that the

approximate threshold value towards DMDS is near 0.0001 µg/µl. Stable fly responses peaked at 200 µg/µl with a mean of 100%, while in the house flies the maximum responses occurred earlier at 100 µg/µl, but with a mean response at 97.8%. There was also a large rightward shift in the housefly curve relative to that of the stable fly above 1 µg/µl, signifying reduced sensitivity of the house flies to DMDS at higher values. Accordingly, at all doses below 1 µg/µl stable fly responses are smaller than those of the house flies (Figure 3.3). Also, there is a relatively large EC_{50} OF 20.2 µg/µl in the house flies over the 3.785 µg/µl in stable flies.

Isopropyl Alcohol

The curves for IPA were interesting due to the large difference in the models. House flies were fit to a standard 3-parameter model, while stable fly responses were fit to a biphasic 2^{nd} order polynomial curve. In the stable flies, the 0.0001 µg/µl gave a low response of 6.6% therefore the full range of response was likely observed, unlike with some of the other biphasic curves. Following the trend of the other biphasic curves however, the primary EC_{50} value of the stable fly curve was 0.004 µg/µl. The house flies however were found to have an EC_{50} of 20.4 µg/µl, suggesting that stable flies are more sensitive to the compound. This is demonstrated in Figure 3.3 above, where it can be seen stable fly responses are consistently greater below 100 µg/µl—the inflection point of the curve. Both minimum and maximum response values at 0.0001 µg/µl and 100 µg/µl are similar in magnitude; with respect to the house flies, the largest increase in response happens between 1-100 µg/µl, whereas rapid change in response is present from the onset in the stable fly data.

Pentanoic Acid

Pentanoic acid stimulated the highest EC_{50} values of all the compounds tested in both the house flies and stable flies. The stable flies have a higher response threshold to pentanoic acid, than do the house flies. At 0.0001 µg/µl, the response in the stable flies was 1.4% compared to 8.5% in the house flies, and at lower doses, the compound evokes greater responses, but this reverses through the higher doses. Data from both flies fit a standard 3-parameter logistic curve, and a hillslope of 1 in both curves indicated equally increased responses with respect to concentration after 1 µg/µl. The main difference between the models is a rightward shift of the house flies relative to the stable flies, with the house flies reaching a maximum value at 200 µg/µl rather than at 100 µg/µl as occurred in the stable flies (Figure 3.4). While the peak occurred later, the mean value was lower than was seen in the stable flies (94.1% vs 97.0%). Since the curves could be fitted to the same model, a comparison of curve fits could be generated in Graphpad, and the log EC_{50} of the two curves were tested for significant differences using an F-test. Overall, house flies showed less sensitivity (P =.0013) to pentanoic acid with an EC_{50} of 70.8 µg/µl—more than 5 times larger than that of the stable flies.

Phenol

The biphasic 2nd order polynomial curve was conserved in both fly species in response to phenol. Both curves are similar in that responses were maximized at 1 µg/µl, had reduced responses at the 400 µg/µl dose, and potentially converge at values below the tested range. Primary EC_{50} values of the both curves were 0.0003 µg/µl in stable flies and 0.0005 µg/µl in house flies. This is in accordance with the relatively high responses triggered at the minimum dose tested; in both species, these were the highest values

obtained of all the compounds at the 0.0001 µg/µl dose. At all values, phenol provokes a larger response in stable flies than in house flies. At the inflection point of the curve, stable fly responses towards phenol are consistently larger than housefly (Figure 3.4). Further, responses do not decrease in magnitude as rapidly as seen in the housefly model. This could indicate that stable flies are better able to overcome saturation of ORNs for this compound.

Propionic acid

Data for both stable flies and house flies were fit to biphasic 2nd order polynomial curve, yet in the range tested the models appear to be mirrored versions of each other. Stable fly responses are small at low doses and relatively high at larger ones, whereas in the house flies, responses are extremely high at low doses and small at large ones (Figure 3.3). There is a rightward shift of the stable fly curve relative to the house flies. Moreover, at the 0.0001 µg/µl the response mean given by the house flies was 65.6% and 11.0% in stable flies, making propionic acid both more potent against the house flies. Notably, in the house flies the highest dose elicits a response that is significantly smaller than at the lowest dose. In both species, propionic acid was the only compound that elicited such weak responses at the 400 µg/µl concentration. Similarly, it was the only compound in which the maximum response occurred at such small doses (0.01 µg/µl). As was seen with phenol, housefly responses tended to diminish more quickly after the maximum response was achieved, than did the stable flies' responses (Figure 3.4). The mean maximum response (79.6%) towards propionic acid in the stable flies was relatively low however, and was coupled with a large response variance within many of the doses. As

such these were, the lowest of the maximum responses observed and might account for some of the difference.

Toluene

Responses to toluene were variable in both of the fly species tested. Stable flies responses were fit to a 2nd order polynomial curve, while the model used for the house flies was a 4 parameter logistic function, though the resulting model is almost completely linear. The housefly curve is shifted rightward, relative to the stable fly curve (Figure 3.1 and 3.2). In both curves, there are few significant difference between the majorities of the doses tested, giving little evidence of dose dependence. This could account for the large variation in response, which was present at all doses in the range tested. In most of the doses tested, stable fly responses were much greater than those of the house flies.

Diptone

Both fly species were fit to 3-parameter logistic models. 10 µg/µl was the highest dose tested for this compound and both species had maximum values converging at this dose, signifying there could be higher responses at larger doses. The rightward shift of the housefly curve as well as lower responses at smaller doses relative to the stable fly data suggests that house flies are not as sensitive to this compound as are the stable flies (Figure 3.4). Yet, models share the same slope, and while the EC_{50} for the stable flies are lower, this value is not significant (P = .31).

3.4 Discussion

All of the compounds tested here demonstrated dose dependent effects in both fly species and all compounds tested resulted in at least 3 doses that elicited responses larger than that of the positive control. This study confirms the electrophysiological activity of a few compounds recovered from dairy farms which can be candidates for behavioural testing.

In general dose response curves are expected to follow a sigmoidal shape with linearity between 20-80% of the measured response on a logarithmic-dose scale, corresponding with exponential increase in the measured values. Sigmoidal curves were in fact observed for the compounds butanoic acid methyl ester, dimethyl disulfide, pentanoic acid, diptone, as well as isopropyl alcohol and toluene in house flies. However, in a few cases dose dependent responses exhibited biphasic curves (also called non-monotonic curves) as with phenol, propionic acid, isopropyl acid, and toluene. Therefore this study provides further evidence that dose dependent electrophysiological responses can exhibit hormesis. Within a toxicological context, hormesis has been defined as a dose response relationship in which there is a stimulatory response at low doses and an inhibitory response at high doses, resulting in a U-shaped or an inverse U-shaped curve, over the typical sigmodal or J-shaped model (Calabrese and Baldwin 2001). Since its primary observations in the late 1800s the phenomenon been associated with a large amount of concern; primarily because of the inability to determine whether the response was a result of direct, indirect, or injurious stimulation (Calabrese and Baldwin 2001, Wang 2013) . Generally, there is a consensus that hormesis arises in an evolutionary context as an adaptation to stressors in the environment and as a biological imperative to restore homeostatic conditions. Regardless it occurs within many cytoprotective

pathways and can be considered in receptor-agonist systems such as the one presented here as a direct stimulation (Calabrese and Baldwin 2001, Kim, Lee et al. 2018).

Hormetic responses in insects have been observed in a toxicological contexts, but most are measured with factors concerning behavioural and physiological aspects, wherein pesticides cause off target effects altering mating, fecundity, or life history parameters (Nascarella, Stoffolano et al. 2003, Cutler 2013, Wang, Guo et al. 2014). For example it has been shown that at low levels the pesticide Dieldrin promoted increased fecundity and weight in *M. domestica* (Georghiou, March et al. 1963). Other examples show that pesticides can alter responses of fly species to sex pheromones in a beneficial way, by changing the response threshold in olfactory neurons (Rabhi, Deisig et al. 2016). Still within the literature there remains almost no examples of hormetic response to VOCs in electrophysiology studies. With respect to EAG identification of active VOCs in *Stomoxys calcitrans*, there is one study which demonstrated dose dependent response with hormetic effects, however the authors did not comment on the occurrence (Jelvez Serra, Goulart et al. 2017). In the same study, the biphasic dose response curve was given in response to phenol, displaying diminished responses at high concentration; this was in accord with the findings presented here. This study is the first to demonstrate this type of response in *Musca domestica* and gives evidence to further compounds eliciting such a response in *Stomoxys calcitrans*.

As it has been noted previously, flies showed high sensitivity to compounds that provoked the biphasic curves. In these curves, responses were decreased at high doses by up to 69.0301% of the maximum value. Of the compounds that exhibited this response in the flies, there are no overt similarities that explain this property. One consideration is

that phenol, toluene, and IPA all present mild to severe neurotoxicity in humans and animals. This is in accord with the suggestion that hormesis occurs as an overcompensation towards injurious chemical stressors in the fly's environment (Calabrese and Baldwin 2001). Yet, propionic acid has not been shown to have neurotoxic effects. In fact, propionic acid is a short chain fatty acid, prevalent in rumen secretions, and excretions, such as in sweat and urine (Jeanbourquin and Guerin 2007, Torr, Mangwiro et al. 2007). In *Aedes* and *Culex* mosquitoes it has been shown to repel oviposition when present in high concentrations, in the substrate. It has also been involved in host location. Interestingly, a study concerned with mapping drosophilid odorant responses in OSNs using computational approaches, found that propionic acid elicited a response across at least 63 OSNs (Munch and Galizia 2016). It was shown that propionic acid produced mid to high kurtosis, suggesting that it produced a large and definitive response in at least one OSN.

One of five compounds that showed the broadest ensemble response was 4-methylphenol, a phenolic derivative that provoked a response across less OSNs, but with a relatively large affinity across all of them. A similar study which investigated putative ligands for drosophila odorant receptors, also demonstrated the OSNs responding to phenolic derivatives increased spikes above the activation threshold, especially halogenated phenols such as 2-bromophenol (Boyle, McInally et al. 2013). 4-methylphenol and 3-methylphenol stimulated responses just under the activation threshold; phenol to a lesser extent also activated the receptor. In the same study, methyl propionate, bypassed the activator threshold for one OSN by 5 times, and also elicited responses across many compounds. Thus a possible explanation for the hormetic

response seen here, could be related to the compounds' high affinity towards many odorant receptors, such that large responses overwhelm olfactory mechanisms at high doses. Toluene and IPA were not mentioned in either study, however differences in food and oviposition preferences between drosophilids might account for their absence in the studies.

There is a notable difference between the fly species in response to IPA and toluene, both of which only produced biphasic responses in the stable flies. A study by (Schofield, Cork et al. 1995) showed recovery responses to components of rumen host odours. While investigating the recovery response of primary aliphatic alcohols, it was show that recovered period decreased with a decrease in alcohol chain length. While IPA is not a primary alcohol, such results suggest that the size or complexity of the molecule might increase the recovery rate. In the same study, toluene nor phenol where observed, however responses to 3-methylphenol, 4-methylphenol, and naphthalene showed high recovery time in stable flies. Similarly, recovery rate increased with increased doses. Mechanistically, this is reasonable as at the saturation of ORNs there will be a decreased response as a lack of binding sites are available to odorants. If increased dosages present with an increased recovery time, this may account for decreased responses at high concentrations of the compounds tested here.

If differences in curves can occur as a function of saturation and low recovery response, the desensitization of ORNS might help to further explain differences both between the compounds and between the species. Desensitization at the ORN level is thought to be an adaptation mechanism occurring both directly in the ORN as well as downstream to coordinate signal transmission to higher olfactory centres (Guo and Smith

2017). Some inhibition by downstream GABAnergenic local neurons might play a role in delaying signalling for the purpose of generating a better odorant profile (Vosshall, Wong et al. 2000, Vosshall and Stocker 2007). Intrinsic desensitization occurs in response to odorant binding which triggers calcium ion mediated dephosphorylation of the ORCO receptor and concurrent desensitization, thereby providing a direct effect on the sensitivity of the receptors (Guo and Smith 2017).

Many genes as well as sensilla structure and distribution are well conserved amongst Dipterans, yet altered responses to odorants, is well documented in between the sexes and between species (Otter, Tchicaya et al. 1991, Kelling, Ialenti et al. 2002, Vosshall and Stocker 2007, Smallegange, Kelling et al. 2008, Olafson 2013). At least within *Stomoxys calcitrans* sexual dimorphism cannot be attributed to differences in the number of sensilla (Tangtrakulwanich, Chen et al. 2011, Tangtrakulwanich 2012). However studies have established that differential gene expression within ORNs can account for variation in response to VOCs (Nyanjom, Tare et al. 2018). All together this suggests differential expression is targeted towards different needs such as mate detection, oviposition, and nutritional preferences. This could explain differences in response, sensitivity and recovery response rate to VOCs.

In terms of sensitivity, there was some accord with previous results as to which species were most sensitive to a given compound. The comparison of the dose response curves allows for the identification of factors such as potency, efficacy and maximum response—all of which can help to provide information on how VOCs affect flies and the ORN level. This can help to determine sensitivity at all levels or at a given dose. When identifying putative test compounds via GC-EAG, it became apparent that a species of

flies would respond more readily that others for a given compound. In accordance, the percentages of total responses by each species to given compound during GC-EAG was taken (Table 1). In the compounds that were tested by dose response, that the fly species that were described to have the greater sensitivity by dose response, were also observed to have a greater percentage of response to the compounds in the Porapak samples. For example, stable flies generated a response to 63% of the all the pentanoic acid peaks present in the Porapak samples, while house flies responded approximately 57% of the time. Comparison of the models and EC_{50} confirmed that house flies showed lesser sensitivity to the compound. Moreover, the curve reflects GC-EAG results with the consideration that concentration plays a vital role and a lower percentage of response might be due to samples containing low concentrations of the volatile recovered.

Corresponding to concentration and gene expression in odorant receptors as a factor in differential response patterns, is the role the compound plays in an ecological context. While electroantennography cannot predict the behavioural response designated by an active compound, differences in the response patterns may help us to infer which compounds are more or less important to the species. Responses between *S. calcitrans* and *M. domestica* for example were found to be significantly different, with house flies being less sensitive to the compound. Pentanoic acid (or valeric acid) is a short chain fatty acid, that is formed as a fermentation product, and is a component of decomposing protein sources. Similar to propionic acid, its occurrence is well known in animal secretions and excretions, such as in sweat and urines. It is attractive to haematophageous species such as mosquitoes, though conversely carboxylic acids are repellent in Tsetse flies. With its prevalence amongst host odours, and association with wastes, it seems

counterintuitive that house flies should show decreased responses to it, and that there should be such a large difference between the sensitivity of the flies. It has been shown that this compound induces oviposition by *Stomoxys calcitran*s, so perhaps increased sensitivity correlates to its use as a predictor of suitable oviposition substrates. Another possible explanation could be that tested separately, house flies are less sensitive to pentanoic acid, than would be if tested alongside an associated mixture, such as sweat or urine. It is well known that components of mixtures tested singly often do not produce behavioural traits that are associated with the full mixture. In such a case, other components would aid in relaying a more meaningful message to the fly.

Cases such as these demonstrate the biological differences present even in cohabitating species. They also show a further need for behavioural tests alongside EAG dose response to clarify VOCs role in behavioural responses, such as host seeking and oviposition preference.

Chapter 4. Conclusions and Future Considerations

4.1 Gas Chromatography-Electroantennography and Dose Response Studies

During the course of the study, volatile profiles were characterized throughout Spring and Summer from three farms in Ontario. The VOCs spanned a wide range of components associated with plant and ruminant host odors. Frequently recovered volatiles could be further categorized by chemical functionality. Aldehydes, alcohols, aromatics, terpenes, volatile fatty acids and their derivatives, emerged as frequently recovered groups. Alcohol, aldehydes, and fatty acid groups emanated from skin secretions such as sweat or

other gaseous emissions. Unlike other studies, ethanol was only recovered in low abundances, and instead the most frequently isolated alcohols were the long-chained unsaturated groups found in sweat. However, there were other short, unsaturated alcohols found in recovered in the samples, and this is in accordance with other findings. These types of alcohols along with ethanol, result from fermentation of manure and feed by bacterial processes. This highlights the importance of bacterial originating VOCs and their role in insect orientation towards hosts. Other compounds which were correlated with bacterial fermentation were aromatic and sulfide groups. These VOCs arose from proteinaceous wastes originating in urine and manure and were found at each site with some of the highest abundances. Aromatic compounds are important components in a variety of mammalian odours and might represent an important target to further investigate attractants which can be used as trap baits.

Through the use of GC-EAG, compounds with electrophysiological activity could be identified directly from samples, with relatively high throughput. Many of the VOCs frequently recovered at each site displayed activity in *S. calcitrans* and *M. domestica*. Of groups demonstrated to have the highest electrophysiological activity, aromatic groups (especially phenolics), alcohols, and volatile fatty acids were well represented. Many of compounds in these groups have appeared as attractants in haematophageous flies and other Dipterans. In a similar manner, the active volatiles for *S. calcitrans* and *M. domestica* overlapped frequently. This can be attributed to the shared surroundings they navigate, with special consideration to oviposition sites and host preference. As the two species have a mutual need to lay eggs in a moist and nutrient rich environment, it is appropriate they share a mutual ability to detect volatiles associated with such an

environment. Differences in behavioral requirements, can therefore contribute to the variations in EAG active compounds, and fly responses to them. For instance, a subtle difference noted was the more frequent responses of stable flies to terpenoid compounds relative to house flies. While stable flies demonstrated activity towards camphene, α-pinene, o-cymene and limonene, houseflies only responded to phorone and α-pinene. Despite *S. calcitrans'* need for blood meals, this species also relies on plant and fruit nectars as an energy source; therefore, sensitivity to these VOCs may be born out of a need to supplement the diet. Other compounds, such as DMDS and butanoic acid methyl ester (BAME), were active in both species, but varied in the percentage of flies that responded to them. In dose response trials, it was determined that overall sensitivity to these compounds differed between *S. calcitrans* and *M. domestica*. Instances like these, are useful for understanding the necessity and prioritization of certain odorants by insects in a biological context. However, a restraint on electrophysiology experiments, is the inability to predict behavioural modifications, if any, that active VOCs are capable of producing in insects. Behavioural bioassays are needed to determine the significance of active compounds and their roles in host orientation, mating, and oviposition.

During the course of GC-EAG analysis, eight compounds were selected for dose response to further explore the range of activity they present in *S. calcitrans* and *M. domestica*. Most strikingly, two types of dose response curves were observed, a typical sigmoidal curve, and a biphasic or U-shaped curve. Biphasic curves occurred in response to four compounds: isopropyl alcohol, phenol, propionic acid, and toluene. These curves were also observed in both species, though not necessarily to the same volatiles. Isopropyl alcohol and toluene, generated these curves only in *S. calcitrans*, while

propionic acid and phenol were biphasic in both species. These results suggest that hormesis can occur in insects in response to stimulation by volatile organic compounds. Hormetic responses are thought to occur in response to overstimulation by harmful compounds. Accordingly, each compound which produced this response in the flies, presented some neurotoxicity in animals and humans at high doses. Further, of the VOCs tested, *S. calcitrans* and *M. domestica* were the most sensitive to these compounds. In some cases, threshold concentrations could not be determined, as concentrations as low as 10^{-4} resulted in relative responses above 30%. Additionally, since the curves were non-monotonic, EC_{50} values could only be estimated based on the fitted model, but because models were quadratic two such values would exist. Further, extrapolating these values is inadvisable, due to uncertainty in the progression of the curve beyond the experimental doses. The significance of a second EC_{50} value is unclear, and would require bioassays to describe changes in behaviour at important inflection points in each model. It is also important to understand the role receptor saturation, and recovery time might play in the occurrence of these curves.

Sigmoidal models, were more easily compared with each other, and gave valuable insight into the sensitivity of each species to VOCs. In most cases, threshold values could be determined, and existed at the lowest concentrations. Likewise, saturation was detected for most sigmoidal curves between 100-200 µg/µl. The compound diptone, was the only compound in which saturation was likely not determined. In all flies, 100% relative response to diptone was reached at 10 µg/µl, without variation, which suggests that increased responses would be obtained at higher doses. At the lowest doses of this compound, responses remained above 10% indicating flies could have sensitivity below

this point as well. Dose response curves for diptone have not yet been described in *S. calcitrans* or *M. domestica*. Therefore, further exploration of the importance of this compound is necessary.

4.2 Characterization of Dairy Farm Profiles and Analysis of Fly Abundance

VOCs were evaluated for changes in their presence during the active season in *S. calcitrans,* and several were found to vary by time of day, month, and across the sampled sites. VOCs that varied by time of day tended to be associated with cattle feed and wastes, and changed significantly between early morning and other time points. Accumulation of waste increases throughout the day as cattle become more active, and as the moisture and temperature increases, so does subsequent bacterial degradation. These changes may be responsible for changes in volatile profiles over the course of many hours. Of these compounds, DMDS and α-pinene were found to have a significant effect on stable fly presence. In houseflies, only DMDS had an effect on housefly presence. In fact, many monoterpenoid compounds were found to have no significant effect on housefly abundance, which underscores housefly preference for volatiles associated with cattle slurries.

Eight compounds, dodecanol, heptanal, hexadecanoic acid methyl ester, isopropyl myristate, pentanal, toluene, 2-decen-1-ol, and 2-nonen-1-ol, varied between the months. Seven of these VOCs differed between early and late summer months, namely between June-July, and June-August; only isopropyl myristate changed between May and later months. These months correspond to peaks in stable fly abundance at dairy farm

facilities, and were often found to significantly affect the abundance of both flies. These VOCs were largely comprised of aromatic compounds, and fatty acid derivatives (fatty alcohols, and fatty acid methyl esters), which are associated with rumen metabolism and wastes. While waste produced by ruminants remains consistent throughout the months, the increased temperature during the later summer months likely increases the metabolic rate of the cattle, and increases the emissions of VOCs arising from bacterial fermentation. Additionally, higher temperatures can increase the kinetic energy required for the longer chained fatty acids to volatilize. Despite the association of temperature and humidity on VOCs, and the life history of flies, this study was unable to significantly relate either to stable fly or housefly abundance at dairies. It is likely, the variation in either factor was too small during the courses of the summer for these differences to be notable. One limitation of this study, is that sampling did not occur in early spring months, nor late into the fall. Expanding the length of sampling would increase the range temperatures, and environmental conditions experienced by the flies, thereby intensifying any differences these factors control. For instance, the bimodal spike in stable fly populations noted by other groups may have been more clearly observed, as these have been documented as late as October. This study focused on the VOCs sampled inside the facility, and closest to the hosts. While temperature and humidity were tracked closely, the samples were all taken indoors, and therefore this study, did not take into account other weather conditions, such as: rainfall, light conditions, and wind speed. Thus, it is unclear what effects variables might have on VOCs and insect interactions with them.

Abundances of *S. calcitrans* and *M. domestica* did not vary significantly between farms, though there occurred variation in VOC profiles at each site. It was expected that

farms with similar location and practices, would share more similarities in VOC profiles, but this was not the case. Ponsonby and Elora, which were more closely located, shared less of the frequently recovered volatiles than did Ponsonby and Shadyway. In fact, these two farms also had more significant differences in the 15 compounds tested, although these differences came mainly from the percent of recovery, and not from absence of common compounds, as was the case with Ponsonby and Shadyway. Regardless, it was expected that large differences in volatile profiles, may result in differences between fly abundances. At least between Ponsonby and Shadyway this could not be observed. It is possible that VOC profiles did not express large enough variations to see changes in fly populations. This seems most probable, as volatiles associated with host odors and dairy facilities, are frequently reproduced with high fidelity between studies. Still, because reliable trap data could not be obtained for Elora, it is difficult to properly compare the effect location and site-wise differences may have on fly abundance.

4.3 Future Considerations

Insects navigate through a complex environment of chemostimulants, which are affected by many intrinsic and extrinsic factors. To better understand behavioral modification through the use of attractants and repellents, it is necessary to understand the relationship between semiochemicals, and the environments in which they are found. Examination of a wider range of environmental factors, such as temperature, humidity, wind speed, and rainfall, will give a more thorough understanding of how VOCs are perceived by pests.

It was determined in this study, VOC profiles at dairy facilities differed by time and location. In addition, VOCs that affect fly abundance were also determined. However, more information is needed on the critical differences necessary to

significantly alter population dynamics. Supplementary studies should be geared toward understanding the underlying causes for changes in volatile profiles, and what shifts result in increased presences of nuisance flies. Optimization of insect trapping, and identification of potential biases, would increase statistical power needed for monitoring population dynamics.

Putative activity of VOCs can be determined through GC-EAG studies, but behavioral bioassays are at all times critical to understanding the response of *S. calcitrans* to compounds of interest. Identification of hormetic curves in an olfactory context are offered here, but their impact on dipteran behavior remains uncertain. Future studies should be dedicated to understanding, the relationship between biphasic curves, sensitivity, and receptor saturation levels. It is important to know whether these curves are reversible, or whether they remain even with longer recovery times between stimulation.

This body of research was largely exploratory and should be considered an early step in examining the importance VOC modulation on the seasonal abundance of *S. calcitrans* and *M. domestica*. The suggestions here will hopefully contribute to novel insights for identifying active compounds for their targeted use in integrated pest management approaches.

Chapter 5. References

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