NECROPHILOUS INSECT ATTRACTION TO CADAVERIC VOLATILE ORGANIC COMPOUNDS

by

Kelie Yoho

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STATEMENT OF COMMITTEE APPROVAL

Dr. Trevor Stamper, Chair
Department of Entomology
Dr. Matthew Ginzel
Department of Entomology
Dr. Jeffrey Holland
Department of Entomology

Approved by:
Dr. Linda Mason
Head of the Graduate Program

To my grandpa, Chuck
for always believing I could
and making me believe too

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ABSTRACT

Author: Yoho, Kelie, C . MS
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Committee Chair: Trevor Stamper

Though it is well established that insects are attracted to decomposing vertebrates, little is known about the mechanism of attraction. Olfaction is thought to be the primary sense involved in resource location by necrophilous insects, and several studies have attempted to identify cadaveric-produced volatile organic compounds (VOCs) that contribute to insect attraction. This previous research has been completed almost exclusively in lab settings, with no field studies to verify these findings.

The goal of my thesis was to test necrophilous insect attraction to nine cadaveric VOCs (1-butanol, 1-pentanol, *p*-xylene, *o*-xylene, ethanol, cyclohexanone, acetamide, dimethyl disulfide, and dimethyl trisulfide) in a field setting. Pitfall traps at six research locations were baited with the chemicals for one week. Specimens collected from the traps were identified and species assemblages were compared to those of a pig carcass. Replicates were conducted in fall 2018 and spring 2019.

Two chemicals were identified as important attractants for a variety of necrophilous insects: dimethyl disulfide and dimethyl trisulfide. These chemicals were especially attractive to *Phormia regina*, a blow fly (Diptera: Calliphoridae) found worldwide. VOC insect communities did not include all of the insects found on carcasses, possibly indicating the necessity of chemical blends for effective attraction. This study is only the second study to test necrophilous insect attraction in a field setting.

CHAPTER 1. THESIS INTRODUCTION

1.1 Background

Forensic entomology is a field that has been growing steadily in recent years. While forensic entomology may refer to urban, stored product, or medico-legal entomology, the latter is the most common application of the field (Catts and Goff 1992). Medico-legal entomology is the use of insects in criminal investigations, often homicide or abuse cases. Perhaps the most important piece of information in such cases is the length of time that someone has been dead or abused/neglected. This period of time between death and discovery is known as the postmortem interval (PMI). Necrophilous insects, particularly blow flies (Diptera: Calliphoridae) can colonize decomposing tissue within minutes (Anderson 2001), making them useful for estimating PMI: the age of the insects on the body should be less than or equal to the PMI. Therefore, if a forensic entomologist is able to determine the age of the insects, they will be able to estimate a PMI.

In addition to aging insects, faunal succession patterns can also be useful for PMI estimations. Insects colonize remains in sequential waves that depend on a number of factors, such as location, season, cadaver size, and light availability (Amendt *et al.* 2010). Therefore, the set of species found on a cadaver, or conversely the species that are *not* present on a cadaver, can give an indication of PMI. Succession models track the presence of species across the decomposition process and have become the primary method of PMI estimation once immature insects have left the body (LeBlanc 2008).

1.2 Limitations

Current methods for age determination in insects rely heavily on species-specific development models (Catts and Goff 1992). These models provide the range of times that an individual of a particular species will spend in a given life stage, at a given temperature (or, occasionally, temperature cycle). Unfortunately, interspecific variation across geographic regions changes these models, so no single model will be representative of the entire species (Catts and Goff 1992). Additionally, accurate and reliable taxonomic identification of dipteran species often requires that specimens be reared to adulthood, which can be time consuming and costly.

Succession is highly dependent on location and time, as species assemblages can vary greatly across geographic locations and seasons. Additionally, little is known about what triggers different species to colonize at particular times, though it is likely heavily dependent on olfaction (LeBlanc and Logan 2010). A corpse releases a variety of chemicals during the decomposition process, many of which volatilize. These volatile organic chemicals (VOCs), are thought to be the main attractants of necrophilous insects (LeBlanc and Logan 2010).

1.3 Finding Solutions Using Chemical Ecology

Interdisciplinary work is essential for the growth of any field. In Chapter 2, I explore the research that has been completed to date regarding chemical ecology in a forensic entomological context. Current research is improving species identifications and larval aging using cuticular hydrocarbon profiles and identifying cadaveric VOCs that act as insect attractants. In Chapter 3, I present my own cadaveric VOC attractant study. This field study tested nine cadaveric VOCs, as selected from a literature review, for necrophilous insect attraction. Pitfall traps were laid across six field sites and baited with the selected chemicals. Working under the hypothesis that cadaveric VOCs are responsible for insect attraction, I predicted that all of the chemicals used would attract forensically important insects. The results from this study provide insight into important insect attractants, which can be used to improve PMI estimates and body recovery techniques.

1.4 References

Amendt, J., Zehner, R., Johnson, D. G., & Wells, J. (2010). Future Trends in Forensic Entomology. *Current Concepts in Forensic Entomology*, 353-368. doi:10.1007/978-1-4020-9684-6_16

Anderson, G. S. (2001). Insect succession on carrion and its relationship to determining time of death. In JH Byrd and JL Castner (Eds) *Forensic entomology: the utility of arthropods in legal investigations* (143-175). Boca Raton: CRC Press.

Catts, E. P., & Goff, M. L. (1992). Forensic Entomology In Criminal Investigations. *Annual Review of Entomology, 37,* 253-272. doi:10.1146/annurev.en.37.010192.001345

LeBlanc, H. N. (2008). Olfactory stimuli associated with the different stages of vertebrate decomposition and their role in the attraction of the blowfly Calliphora vomitoria (Diptera: Calliphoridae) to carcasses. The University of Derby for the Degree of Doctor of Philosophy.

LeBlanc, H. N., & Logan, J. G. (2010). Exploiting Insect Olfaction in Forensic Entomology. *Current Concepts in Forensic Entomology*, 205-221. doi:10.1007/978-1-4020-9684-6_11

CHAPTER 2. CHEMICAL ECOLOGY AND FORENSIC ENTOMOLOGY- A REVIEW

In the past decade or so, interest in the intersection of forensic entomology and chemical ecology has been on the rise. Chemical ecology integrates chemistry and biology to better understand how organisms interact with their environments, and may it offer solutions to questions that currently plague forensic entomologists. The majority of chemical ecology/forensic entomology research published to date focuses on one of two main themes: cuticular hydrocarbons or cadaveric VOC attractants. Cuticular hydrocarbon studies typically examine the use of cuticular hydrocarbons for taxonomic identification or for age determination of specimens, while attractant studies seek to identify the compounds released by a decomposing corpse that act to draw insects.

2.1 Cuticular Hydrocarbons

2.1.1 Introduction

Insect epicuticle is a thin, waxy layer made of nonpolar compounds that covers the cuticle and performs many functions for the insect, including waterproofing, deterrence of predation, mimicry and camouflage, reflections of radiation, and chemical signaling (Gullan and Cranston 2014). Hydrocarbons are among the most prominent components of the epicuticle and assist in its two primary functions (Drijfhout 2010): prevention of desiccation and microorganismal/toxinal penetration and chemical signaling. Hydrocarbons are critical in species and gender recognition, nestmate recognition, task specific cues, dominance and fertility cues, and chemical mimicry, and can act as pheromones as well (Howard and Blomquist 2005). Hydrocarbons are found in the cuticles of all insect life stages, though their profiles vary over time and across castes and species (Drijfhout 2010). The variability in cuticular hydrocarbons, both across species (Braga *et al.* 2013) and within life stages (Roux *et al*. 2008), makes hydrocarbon analysis a promising tool for forensic entomologists, particularly for species identification and specimen age assessment.

2.1.2 Collection

Cuticular hydrocarbons can be collected using liquid extraction or solid-phase microextraction (SPME). The liquid extraction method requires whole insects to be submerged in

an organic solvent, such as hexane, for 10-20 minutes. (Drijfhout 2010). While insects may be submerged individually (e.g. Byrne *et al.* 1995; Ye *et al.* 2007; Moore *et al.* 2017a), it is not uncommon to pool insects of the same age and species together to ensure a higher concentration of hydrocarbons is extracted (e.g. Braga *et al*. 2013; Butcher *et al*. 2013; Moore *et al*. 2016). The collected extract is often dried—such as under a stream of nitrogen (Zhu *et al*. 2007; Braga *et al*. 2013), a vacuum (Ye *et al*. 2007), or in the air (Roux *et al.* 2008; Moore *et al*. 2014). The extract can be analyzed directly or re-dissolved in a smaller volume of solvent to increase its concentration (Pechal *et al.* 2014). The extract may also be passed through a silica gel chromatography column to remove polar compound contaminants (Moore *et al*. 2012; Braga *et al.* 2013).

The other method, SPME (Pawliszyn 1999), uses fibers coated in an absorbent that are rubbed directly on the insect cuticle to collect hydrocarbons. Absorbents vary by thermal stability, selectivity, and polarity, and are chosen based on the needs of the experiment (Mani 1999). Commercially available fiber coatings include polydimethylsiloxane (PDMS), polyacrylate, PMDS/divinylbenzene (DVB), PDMS/carboxen, carbowax/DVB, and carbowax/templated resin (TPR; Mani 1999). The SPME fiber assemblies are rubbed against the insect's cuticle for thirty seconds and may then be loaded directly into a gas chromatography injection port (Ginzel *et al.* 2003; Chen *et al*. 2017). SPME has the added benefit of being nondestructive—it can even be performed on live insects (Drijfhout 2010; Chen *et al*. 2017).

2.1.3 Chemical Analysis

Chemical analysis of cuticular hydrocarbons is carried out using gas chromatography coupled with mass spectrometry (GC-MS; Gohlke 1959). This method is ideal given that many cuticular hydrocarbons are volatile (Drijfhout 2010). GC separates compounds based on volatility and attraction to the column coating (i.e. their polarity). For cuticular hydrocarbons, this generally means that heavy, long hydrocarbons will volatilize last. GC analysis can be carried out in either split or splitless mode, depending on the concentration of the solution. GC columns cannot effectively separate analytes in concentrated solutions, so split mode allows only a portion of the injected solution into the column to prevent overloading (Drijfhout 2010). In cuticular hydrocarbon studies, GC is often carried out in splitless mode (e.g. Zhu *et al.* 2006; Ye *et al.* 2007; Braga *et al.* 2013), indicating relatively dilute solutions. Several studies (Zhu *et al*. 2006; Roux *et al*. 2008; Xu *et al.* 2014) mention coupling GC analysis with a flame ionization detector (FID) in addition to

the MS analysis. FID is a nonselective detector that can provide valuable information about the concentration of the analyte but offers no insight into the compound's identity (Drijfhout 2010). On the other hand, MS can lead to a positive identification when experimental mass spectra are compared to known literature or mass spectra libraries, such as those of the National Institute of Standard and Technology, NIST (Drijfhout 2010).

For GC-MS analysis to be successful, solutes must separate into distinct, unique peaks. Incrementally increasing the temperature of column allows for solutes to separate based on their boiling points. For cuticular hydrocarbons, the oven is generally set to 50 – 60°C initially, and programmed to rise 25°C/minute until a final temperature of around 200°C is reached (e.g. Roux *et al.* 2008; Moore *et. al* 2014; Pechal *et al*. 2014). At this higher temperature, hydrocarbons volatilize more quickly, so the incremental temperature increase is reduced significantly, to about 3°C/minute (e.g. Zhu *et al.* 2006; Ye *et al.* 2007; Moore *et al.* 2014). The majority of compounds are thought to have eluted between 260 – 320°C, at which time GC is considered complete. Some studies (e.g. Braga *et. al* 2013; Barbosa *et al.* 2017; Paula *et al.* 2017) chose to set the initial temperature of the oven higher, to 150°C, with an incremental increase of 3 – 5°C until a final temperature of 320°C was reached. This second method eliminates the need to set a new temperature increase. There is currently no indication that one method is superior to the other.

Additionally, direct analysis in real time mass spectrometry (DART-MS) can be used for hydrocarbon analysis (Musah *et al.* 2015). In a forensic context, DART-MS is most frequently used to identify drugs, explosives, toxic chemicals, inks and dyes, and illegally trafficked plant and animal species (Pavlovich *et al*. 2016), though Musah *et al*. (2015) demonstrated its potential for identifying insects of forensic interest as well. DART-MS uses electronically excited inert gas to ionize molecules in a sample, which can then be read with a mass spectrometer (Pavlovich *et al.* 2016). This technique does not make use of chromatographic separation technique, so it does not require any one specific sample preparation, i.e. it may be used on solids or liquids (Chernetsova *et al.* 2011). Avoiding chromatography prior to mass analysis also allows for rapid results—mass spectra output is almost instantaneous (Chernetsova *et al.* 2011). The spectra received are often complex and difficult to analyze, however (Pavlovich *et al.* 2016). DART-MS is a relatively new technology, and though promising for use in forensic entomology, further development in this particular field is needed for it to be used in investigations (Pavlovich *et al.* 2016).

2.1.4 Chemotaxonomy

Most current methods of insect species identification rely on morphological keys (e.g. Whitworth 2006) or DNA barcoding (e.g. DeBry *et al.* 2013), but these methods pose some problems. Morphological keys can be subjective and easily misinterpreted, and are not useful for some life stages, such as with eggs or some larvae (Drijfhout 2010). Many require that collected specimens be reared to adulthood as well, which may result in high mortality. DNA barcoding can be very useful for the identification of eggs and larvae, but can also be time consuming and DNA extraction from empty puparia is extremely difficult (Drijfhout 2010).

Cuticular hydrocarbon profiles are both species-specific and heritable (Kather and Martin 2012), making them good candidates for taxonomic identification. It is possible to use these profiles in conjunction with traditional methods to increase confidence in a species decision (Ye *et al.* 2007). Cuticular hydrocarbons may also be one of the earliest indicators of speciation (Seppa *et al.* 2001). Many insects use cuticular hydrocarbons as sex pheromones (Ginzel *et al.* 2003; Howard and Blomquist 2005; Nojima *et al.* 2007), demonstrating their importance for species survival. Even small changes in cuticular hydrocarbon profiles can lead to reproductive isolation through homogamy (Stennet and Etges 1997) and, ultimately, speciation (Roelofs *et al.* 2002). Seppä *et al.* (2011) showed that short term isolation events can lead to large changes in cuticular hydrocarbon profiles, causing reproductive isolation between sister populations before genetic markers can indicate a speciation event.

The potential to use cuticular hydrocarbon profiles to delimit cryptic species is perhaps the most important chemotaxonomical benefit of cuticular hydrocarbons. It is notoriously difficult to identify forensically important flies in their larval and pupal stages (Ye *et al.* 2007; Paula *et al.* 2017); even more difficult if only the pupal exuviae remain (Braga 2013). In cases where morphological features fail to delimit species, cuticular hydrocarbons can be used as a taxonomic tool (Drijfhout 2010). Many studies have been completed on Diptera of forensic interest, at every life stage, with the goal of identifying species (Table 1). Invariably, these studies have shown that cuticular hydrocarbon profiles are unique to each species. Differences between profiles rely primarily on the presence/absence of certain compounds (Kather and Martin 2012), making chemotaxonomic identification relatively simple and objective compared to traditional morphological analysis (though see *1.1.6 Limitations and Problems*).

2.1.5 Age Estimations

Arguably the most promising use of cuticular hydrocarbons in forensic entomology is for age determination of necrophilous insects. Age determination of the insects colonizing a body can provide one of the most accurate indicators of the minimum post-mortem interval (mPMI; LeBlanc and Logan 2010; Sharma *et al.* 2015). Age is often determined using temperature- and species-specific development models (Sharma *et al.* 2015). With this datum, an entomologist can provide a range of approximate ages for a particular larva. This method is quite effective, though it does present some issues. First, development models are often created at one steady temperature (e.g. Grassberger and Reiter 2002; Weidner *et al.* 2014), though developing insects are unlikely to experience such conditions in a field setting. Second, temperature, while important, is not the only variable that affects insect growth (type and amount of food, moisture, photoperiod, toxins, and interactions with other organisms also affect growth; Gullan and Crantston 2014). Third, estimated age ranges can be quite large, reducing the utility of the information (Moore *et al.* 2013). Lastly, age determination is extremely difficult for certain life stages, such as post feeding 3rd instars (Zhu *et al.* 2006) and pupal exuviae (Moore *et al.* 2017b).

Insect cuticular hydrocarbon profiles change over time as an individual's environment, and thus requirements for survival, changes. For example, blow fly larvae (Diptera: Calliphoridae) spend the majority of their time in moist, decaying tissues, while adults live in more arid environments. Adults, therefore, have a greater need for long chain, saturated hydrocarbons to prevent desiccation, and corresponding increases in such hydrocarbons have been observed as a fly ages (Moore *et al.* 2013). Additionally, short chain hydrocarbons are found more abundantly in young larvae than in later life stages, which could be linked to observable aggregation behaviors (Zhu *et al.* 2006). Several studies have used these changes to mathematically model larval age (e.g. Zhu *et al*. 2006; Xu *et al.* 2014), while others have used more visual methods, such as heat maps (e.g. Pechal *et al.* 2014), principal component analysis (e.g. Moore *et al.* 2016), and discriminant analysis (e.g. Roux *et al*. 2008), to demonstrate unique profiles within and between life stages.

The use of cuticular hydrocarbons for larval age determination has produced tremendous results—larvae have been successfully assessed to within a day's precision (Roux *et al.* 2008; Butcher *et al.* 2013). The contracted range of mPMI estimates is obviously desirable for casework, but cuticular hydrocarbon analysis comes with another perk—some life stages have increased utility. Historically, adult flies collected at a scene have rarely been used for mPMI estimation,

they are more frequently used for species confirmation of immature stages (Pechal *et al.* 2014). One reason for this limited use of adults is the difficulty of separating adults that arrive at a scene to feed, mate, and lay eggs from freshly eclosed adults that developed at the scene. Gravid female blow flies are primarily attracted to carrion as an oviposition site (Mohr and Tomberlin 2015). These females require a protein meal for reproductive development but may not begin protein feeding immediately post-eclosion (Mackerras 1933). Identification of freshly emerged adult females may be useful then, as a newly eclosed female will be more likely to have developed on a body than other adults in the area. Several studies (Pechal *et al.* 2014; Braga *et al.* 2016; Moore *et al.* 2017a) have shown that adult blow flies continue to demonstrate changes in their cuticular hydrocarbon profiles as they age, with the first day post-eclosion being the most distinct. These studies, along with other techniques in development (e.g. Mohr and Tomberlin 2015) could contribute to the usefulness of adult flies in PMI estimation.

Pupal *exuviae* have also limited demonstrated utility, as morphological aging is not possible, and even species identification can be difficult given the destructive mechanism of eclosion (Braga *et al.* 2013). Cuticular hydrocarbon analysis could be the new standard for aging these previously underutilized life stages. Pupal *exuviae* have demonstrated such hydrocarbon changes as well (Zhu *et al.* 2007; Moore *et al.* 2017b; Zhu *et al.* 2017), though the changes are much smaller. Pupal *exuviae* are very hardy and can remain intact for years after eclosion. In cases where corpses are left undiscovered for long periods of time, *exuviae* may be the only entomological evidence remaining at a scene, and thus can be crucial for mPMI estimation.

Although not currently discussed in the literature, determining mPMI through cuticular hydrocarbons could also allow for statistical analysis of forensic entomological data. Wells and LaMotte (2017) discuss the need for experimental designs that allow for the calculation of confidence intervals. Without such intervals, validation of an mPMI estimate is extremely difficult. Age determination of insects with cuticular hydrocarbon analysis relies not only on chromatogram peak presence/absence (qualitative data), but also on peak size (quantitative data). The area under peaks is used for confidence interval calculations when compared to library standards. Cuticular hydrocarbon profiles do not rely exclusively on age, however—they may also be influenced by environmental factors. Little work has been done regarding the influence of such factors, but a model that takes into account covariates may be necessary for such calculations to be made.

2.1.6 Limitations and Problems

Cuticular hydrocarbon analysis has several potential shortcomings. Overexposure of the insect to the solvent during liquid extraction can lead to contamination (Drijfhout 2010). Internal polar compounds, such as fatty acids, and endocrine gland secretions may also be extracted (Drijfhout 2010; Chen *et al.* 2017). Purification of the extract using a silica gel column or a TLC plate was not widespread in previous studies, and may increase the occurrence of co-elution or "dirty" extracts (discussed below; Drijhout 2010). This issue can be avoided by using SPME instead of a liquid extraction, as the two techniques have been shown to have qualitatively similar results (Chen *et al.* 2017). SPME does not offer quantitative results, however, limiting the usefulness of this technique for age determination. Furthermore, SPME cannot be used for further testing, such as behavioral assays, after GC analysis is complete because there is no additional extract remains. SPME fibers can also be coated with many different absorbents, and there currently is no standard SPME fiber assembly to use in cuticular hydrocarbon analysis (Chen *et al.* 2017).

Though useful for hydrocarbon analysis, GC-MS does not guarantee complete separation of compounds. Compounds with similar retention times—such as two alkenes with identical chain lengths but varying double bond positions—may elute simultaneously, causing peaks that are mixtures of more than one compound (Drijfhout 2010). Co-eluted peaks hinder identification of the compounds, which may be essential for chemotaxonomic identification. Additionally, particularly long carbon chains require high temperatures to boil, which may not be reached in standard GC columns (Akino 2006). High temperature columns are capable of detecting these long chain hydrocarbons but are significantly more expensive than standard columns (Kather and Martin 2012).

The success of chemotaxonomy is dependent on comprehensive standard libraries. While the use of cuticular hydrocarbons as semiochemicals makes them useful for species identification, it also means that they vary over the life of the insect (Roux *et al.* 2008). Mature insects produce hydrocarbons that are used for mate location and selection, while immatures of the same species are less likely to produce the same chemicals (e.g. Roux *et al.* 2008). This also means that males and females of the same species are likely to produce varying hydrocarbon profiles (e.g. Byrne *et al.* 1995). Chemical libraries must contain standards for various life stages and both sexes to ensure

proper identification of the species. For this same reason, age determination also depends on comprehensive library standards.

While the use of cuticular hydrocarbons has tremendous potential as a tool for aging specimens, profiles are not dependent exclusively on species or age. Environmental influences have some impact on profile development, though the exact role of abiotic factors has not been conclusively identified (Pechal *et al.* 2014). Furthermore, most of the studies that have been completed have used insects reared in a laboratory setting (e.g. Byrne *et al*. 1995; Ye *et al.* 2007; Butcher *et al* 2013), not in the field (e.g. Barbosa *et al.* 2017). This affects especially the use of pupal *exuviae*, as their cuticular hydrocarbon profiles have been shown to change significantly (albeit predictably) with weather (Zhu *et al.* 2017). More work is needed into how weathering affects these profiles for pupal *exuviae* aging to be useful in a field setting.

2.2 Insect Attraction to Cadaveric VOCs

2.2.1 Introduction

It is well known that insects are attracted to rotting meat, but what actually draws them is somewhat of a mystery. Studies suggest that olfactory cues and visual cues may work in tandem to help an insect locate a resource (Vickers 2000), with olfaction being the most important sense in this endeavor (LeBlanc and Logan 2010). Insects have extremely sensitive olfaction, and can respond to as little as one molecule of a chemical (LeBlanc and Logan 2010). This sensitivity allows necrophilous insects to detect a corpse long before the human nose can, producing interest in exploiting this sense. Identification of cadaveric produced VOC attractants is the first step in harnessing insect olfaction for forensic purposes. Studies that focus on pinpointing these attractants typically focus on identification of cadaveric VOCs, insect olfactory neuron responses to VOCs, and/or insect behavioral responses to VOCs.

2.2.2 Collection and Identification of Cadaveric VOCs

Cadaveric VOCs must be identified before attractant studies can take place. To date, hundreds of cadaveric VOCs have been identified (LeBlanc and Logan 2010) from pig and human decomposition studies. These compounds are produced primarily by microbes as they metabolize bodily tissues (Paczkowski and Shütz 2011; though see *2.2.4 Limitations and Problems* below).

As the body progresses through decomposition microbes digest different biological compounds, forming different VOCs and thus, a unique odor bouquet.

Identification of cadaveric VOCs begins with their collection. Often studies rely on domestic pig (*Sus scrofa*) carcasses rather than human cadavers. Pig carcasses are often used as analogs for humans because they have similar body hair coverage, internal organs, muscle structure, gut fauna, etc., and are easier and cheaper to obtain, with fewer ethical restrictions (Statheropoulos *et al*. 2011). Regardless of whether a pig or a human is used, collection techniques remain the same. Headspace, the air above a given sample, may be sampled either passively or dynamically. Passive collection relies on adherence to a sorbent, such as a SPME fiber assembly (e.g. Hoffman *et al*. 2009). The sorbent remains exposed to the odor bouquet for a given time period, which can vary greatly from study to study: Kalinová *et al*. (2009) report 15-minute exposure times and Hoffman *et al*. (2009) used 20- and 40-minute exposure times, whereas Dekeirsschieter *et al*. (2009) collected for a full week. Vass (2012) sampled passively without a sorbent by heating samples in vials and collecting the headspace with a glass syringe. Passive sampling is cheap and requires little specialized equipment, but is less effective at collection than dynamic sampling.

Dynamic sampling, which uses air pumps to actively pull the headspace sample into a sample tube, is more commonly used in cadaveric VOC studies. Dynamic sampling is particularly effective for volatile collection, because more volatile containing air is exposed to the sorbent. This technique requires the volatile producing sample to be placed in an enclosed container, such as a body bag (e.g. Statheropoulos *et al*. 2007; Statheropoulos *et al*. 2011), a stainless-steel hood (e.g. Armstrong *et al*. 2016), or a zip lock plastic bag (e.g. Brasseur *et al*. 2012). Air is pumped into the enclosed space through a charcoal filter to prevent a vacuum from forming. At the same time, air is pulled out of the space and into a collection tube containing a polymer to capture the VOCs (LeBlanc 2008). There is no agreed upon length of time for which to run dynamic sampling, and it seems to rely partially on the state of decomposition (as decomposition progresses, the concentration of volatiles increases) and the polymer in the collection tube. LeBlanc (2008) used two different polymers, Tenax and Porapak. Tenax samples were collected over a 30-minute period, while Porapak samples required 12 hours of sampling.

In both passive and dynamic sampling techniques, blank samples must also be taken. VOCs are produced by every living and many nonliving things, and so are ubiquitous in the environment

(von Hoermann 2013). By taking blank samples, a researcher is able to identify volatiles attributable to nearby plants, animals, ambient odors, or equipment. Blank samples are taken using the same techniques as the other samples, but in the absence of the carcass. For example, if samples are taken from a carcass in an oven bag using a dynamic sampling technique, blank samples are taken from an empty oven bag using the sample dynamic sampling technique (e.g. von Hoermann 2013).

After collection, volatile samples are typically analyzed using GC-MS (see *2.1.3 Chemical Analysis* for more on GC-MS). Headspace samples require either thermal or liquid desorption before analysis. Thermal desorption utilizes heat to re-volatilize the samples from the polymer sorbent (e.g. Statheropoulos *et al*. 2005; Dekeirsschieter *et al*. 2009). In liquid desorption, a solvent, such as diethyl ether (e.g. Dekeirsschieter *et al*. 2012) or a pentane/acetone solution (e.g. von Hoermann *et al*. 2016) is used.

Not every study uses GC-MS for analysis, however. Given the wide range of compounds produced by decomposing vertebrates, it is not surprising that there is a lot of variability in the properties of those compounds as well. As discussed before, traditional GC-MS relies on sample abosrbance to the column coating, which is largely dependent on polarity. Cadaveric VOCs vary in their polarities (Stefanuto *et al*. 2015), so a single column would not attract every chemical in a sample, causing an increase in coelution. Some studies (e.g. Kalinavá *et al*. 2009; Brasseur *et al*. 2012; Dekiersschieter *et al*. 2012; Armstrong *et al*. 2015; Stefanuto *et al*. 2015) have employed two-dimensional gas chromatography (GCxGC; Liu and Phillips 1991) as a solution to this problem. GCxGC uses two columns, typically one with opposing affinities and different lengths, to increase peak capacity and decrease coelution (Shields *et al*. 2015). As peaks elute from the first column, they are injected into the second column. Single peaks may be broken down into multiple peaks with this technique (Shields *et al*. 2015). Compounds typically elute very quickly from the second column, and standard MS techniques are not fast enough to pick up individual signals. Instead, GCxGC is often coupled with time of flight mass spectrometry (TOFMS; Pareige *et al*. 2016). TOFMS takes into account the time it takes a particle to reach the detector and produces results much more quickly than standard MS. GCxGC-TOMF is an extremely sensitive technique that has tremendous potential in many forensic fields (Shields *et al*. 2015).

2.2.3 Electrophysiological Responses

Many attraction studies begin by identifying the compounds that stimulate the insect nervous system. Given the vast number of VOCs produced by a given carcass the preferred method of analysis is gas chromatography–electroantennographic detection (GC-EAD; Cork *et al*. 1990). Electroantennograms (EAGs) record insect nervous system responses as the insect is exposed to an odor plume. EAGs may be performed on whole insects or portions (the head or antennae); in studies where either whole insects or heads are used, immobilization is required (Cork *et al*. 1990). Microelectrodes are inserted into the antennae, which record voltage changes in the olfactory neurons as an air stream containing the desired volatile or volatile blend flow over the antennae (LeBlanc and Logan 2010). Voltage changes indicate that the chemical or chemical blend is electrophysiologically active in the insect.

Many cadaveric VOC attractant studies use GC-EAD to identify potential insect attractants (e.g. LeBlanc 2008; von Hoermann *et al*. 2011; von Hoermann *et al*. 2016). GC identifies components of complex odor blends while the EAG concurrently records which of those same compounds are electrophysiologically active. In these studies, a headspace sample is collected from the vertebrate host (see section 1.2.2 of these chapter for more details on this technique). The sample is injected into a GC and subsequently splits—half of the sample flows to the detector for identification, while the other half simultaneously passes over the antennae of the insect (LeBlanc and Logan 2010). A chromatogram is produced that contains information about both the retention time of the compounds and the voltage change, if any, of the olfactory neurons (LeBlanc and Logan 2010). Electrophysiologically active chemicals may then be selected for further use in behavioral assays.

In some studies, compounds may be chosen without the aid of GC-EAD. In these cases, potential volatile attractants are often selected based on established literature or prior personal experience (e.g. Kalinová *et al*. 2009; Frederickx *et al*. 2012; Dekeirsschieter 2013; Zhu *et al*. 2013; Liu *et al*. 2016; Yan *et al*. 2018). Because the compounds are defined, the need for GC analysis is negated. Frequently in these cases the compounds are diluted to several different concentrations, so standard EAG is still used to determine electrophysiological activity at each concentration before bioassays are completed.

2.2.4 Behavioral Responses

While EAG studies are useful in identifying potential attractants, they do not provide any information about how the insect will respond in the presence of a compound. Behavioral bioassays are often used in tandem with EAG studies to fill this knowledge gap. Often these bioassays are performed in a laboratory setting to account for factors such as insect age, temperature, humidity, and light (Liu *et al*. 2016). Laboratory based bioassays typically present an insect with two options—the chemical or not the chemical—and the insect's choice is taken as an indication of attraction, repulsion, or neither.

Y-tube olfactometer studies are among the most commonly utilized bioassays for attractant research (e.g. Kalinová *et al*. 2009; Dekeirsschieter 2013; Liu *et al*. 2016). Generally, a Y-tube olfactometer contains two glass or Teflon® chambers, also called arenas, connected into a Y shape with glass arms. The chemical (or chemical blend) of interest is applied to filter paper and placed into one of the arenas, while an untreated piece of filter paper (control) is added to the other arena. A pump is attached to both arenas to facilitate air flow. The insect is introduced into the tube that makes up the base of the Y. Frequently these insects are starved, because starved specimens are more likely to seek out a potential food source. The insect is allowed to roam the Y-tube for a specified amount of time (this varies greatly between studies), either until the time expires or the insect enters one of the two possible arenas. At this point the insect's choice, or lack thereof, is recorded and the insect is removed from the olfactometer. The Y-tube is then cleaned with ethanol or dichloromethane and dried before the next insect is introduced.

A variety of other bioassays are used in attractant studies as well. In a similar two-choice bioassay, Yan *et al*. (2018) placed two plastic funnel bottle traps in a mesh cage to measure responses by gravid and nongravid *Lucilia cuprina* (Wiedemann). One bottle was baited with a cadaveric chemical applied to filter paper, the other was a negative control. Martin and Verheggen (2018) used a multiple-choice bioassay to examine *Lucilia sericata*'s (Meigen) ability to differentiate between carcasses with and without conspecific infestation. They split a large Petri dish into four quarters – each quarter contained a piece of filter paper impregnated with a different chemical blend. The dish was covered with a piece of tulle to aid in air flow and insect containment as the insect chose one of the four quarters. The attraction of *Nicrophorus vespilloides* (Herbst) to various odor bouquets was examined using a Kramer sphere—a free rotating ball attached to an optical mouse (von Hoermann *et al*. 2013). An insect's protonum is glued to a bar located directly

above the sphere. This prevents the insect from leaving the sphere, but still allows for movement of the legs and abdomen. An introduced odor stimulus flows onto the insect, which may then begin to walk. The optical mouse tracks the movement of the ball, and thus the insect, as the insect walks across it.

Baker and Cardé (1984) separate behavioral bioassays into a few general categories: assays with or without airflow, and with or without displacement of the insect monitored. A wide variety of behavioral bioassays have been developed following these basic guidelines (e.g. Miller and Roelofs 1978; Sommonds *et al.* 1990), but many have not been used for cadaveric VOC studies. The prevalence of current techniques, particularly Y-tube studies, is likely due to cost and ease. Other techniques are complex and require specialized equipment, and so are unlikely to become widespread in this relatively small area of study.

2.2.5 Limitations and Problems

One of the major setbacks for attractant studies is the wide variation in cadaveric VOCs found between bodies, likely due to different speeds of decomposition (Statheropoulos *et al*. 2005) and body composition. VOC profiles can be influenced by weather, temperature, humidity, environment, and other factors (LeBlanc and Logan 2010), and the role of these abiotic factors in volatile production has not been studied in depth. Additionally, as previously stated pig carcasses are often used in place of human cadavers, yet Cablk *et al*. (2012) found that porcine remains share very few human-specific VOCs, and their profiles contain additional volatiles not present in human remains (though see Stefanuto *et al*. 2015). Though the overlap of these profiles may be important to explore, few studies seem to take into account the inherent differences between human and pig remains. The volatiles identified from porcine carcasses that are selected for behavioral assays may not be applicable to human cases if those chemicals are pig-exclusive.

Likewise, electrophysiological studies using EAG or GC-EAD are useful for understanding neuronal responses of insects to specific compounds or compound blends. They do not, however, provide any information regarding behavior (LeBlanc and Logan 2010). A voltage change does not indicate attraction—it merely suggests that the compound might be in perceived by the insect. The chemical could be attractive, repulsive, or only important as part of a blend or at a certain concentration. This also becomes a limitation in behavioral assays. It has been shown (e.g. Liu *et al*. 2016) that at different concentrations the *same* chemical may be attractive, repulsive,

or neutral to a given species. Attraction may also depend on the sex and reproductive state (gravid vs. nongravid) of the individual tested (e.g. Liu *et al*. 2016; Yan *et al*. 2018). This high variation makes pinpointing important compounds difficult.

Lastly, many cadaveric VOC studies have been conducted in relatively sterile environments, i.e. in the absence of insects (Dekeirsschieter *et al*. 2009). Though this makes identification of human produced volatiles easier, it is also unrealistic in a field setting—rarely is a corpse insect free. These studies ignore the impact that insects may have on the odor profile of a corpse. In fact, the author is unaware of any studies that have identified and/or tested larval fly produced VOCs. This is a huge knowledge gap, as certain insects have been shown to prefer carcasses with conspecific larvae present (e.g. Martin and Verheggen 2018). It is also conceivable that insects would show an aversion to carcasses inhabited by certain allospecific larvae, such as *Chrysomya rufificies* (Macquart) or *Creophilus maxillosus* (Linnaeus), that are predatory on other larvae. These carcass preferences could potentially impact succession, and thus PMI estimates.

2.3 Conclusions

Though still in its infancy, the application of chemical ecology has tremendous promise for the field of forensic entomology. Cuticular hydrocarbons can be used to identify cryptic species without the need for in-depth morphological knowledge. They are also capable of determining an insect's age to within a range much smaller than the most popular methods currently used. Cadaveric VOC and attractant studies show potential for new methods of PMI estimation through the development of a chemical fingerprint. These studies often begin with collecting cadaveric VOCs, either with a passive, which requires less equipment, or dynamic, which requires more equipment but is also more effective, technique. Collection often followed by GC-EAD to identify electrophysiologically active compounds, and behavioral assays, especially Y-tube studies, to determine behavioral responses. Vass *et al*. (2002) have suggested that with the use of chemical fingerprints, the accuracy of the PMI estimation is dependent only on an investigator's access to accurate temperature data. The data from these studies may also be used for body recovery, either to train cadaver dogs or else to develop portable detection systems (Hoffman *et al*. 2009). This research may also prove to be useful for the medical and veterinary entomological community, as certain species of necrophilous insects, such as *Lucilia sericata* and *Cochliomyia hominivorax* (Coquerel), are also pests of humans and/or livestock. Lures have already been developed for these

species using cadaveric VOC attractants (LeBlanc and Logan 2010). Identification of repellents may be useful in a similar way. In Chapter 3, I apply the concepts of attractant studies to field studies, to provide an environment that more closely resembles the conditions experienced in a case. Hopefully, the success of these studies will spark further interdisciplinary work to improve the utility of insects in legal investigations.

Table 1 : Studies that examined cuticular hydrocarbon profiles of forensically important Diptera. Species names are listed as presented in the literature cited.

Species	Life Stage(s)	Study Focus	Authors
Aldrichina grahami	Pupal *exuviae*	Chemotaxonomy	Ye *et al*. 2007
	Larvae (3-8 days)	Age determination	Xu *et al*. 2014
Calliphora vicina	All	Intraspecies variation	Roux *et al*. 2008
	Larvae (1 day)	Chemotaxonomy	Moore *et al*. 2014
	M/F adults (≤ 20 days)	Age determination	Bernhardt *et al*. 2017
	Larvae	Age determination	Moore *et al*. 2016
	Pupal *exuviae*	Age determination	Moore *et al*. 2017b
	Adults (≤ 30 days)	Age determination	Moore *et al*. 2017a
Calliphora vomitoria	All	Intraspecies variation	Roux *et al*. 2008
	Larvae (1 day)	Chemotaxonomy	Moore *et al*. 2014
	Larvae	Age determination	Moore *et al*. 2016
	Adults (≤ 30 days)	Age determination	Moore *et al*. 2017a
Chrysomya rufifacies	Larvae (2.5-8 days)	Age determination	Zhu *et al*. 2006
	Pupal *exuviae*	Chemotaxonomy	Ye *et al*. 2007
	F adults (≤ 30 days)	Age determination	Pechal *et al*. 2014
	Pupal *exuviae*	Chemotaxonomy	Musah *et al*. 2015
	Pupal *exuviae*	Weathering effects	Zhu *et al*. 2017
Chrysomya putoria	M/F adults (≤ 5 days)	Age determination	Braga *et al*. 2016
Chrysomya megacephala	Pupal *exuviae*	Chemotaxonomy	Ye *et al*. 2007
	Pupal *exuviae*	Age determination	Zhu *et al*. 2007
	All	Intraspecies variation	Paula *et al.* 2017
Cochliomya macellaria	F adults (≤ 30 days)	Age determination	Pechal *et al*. 2014
	Pupal *exuviae*	Chemotaxonomy	Musah *et al*. 2015
	M/F adults	Chemotaxonomy	Barbosa *et al*. 2017
Hemilucilia segmentaria	M/F adults	Chemotaxonomy	Barbosa *et al*. 2017
Protophormia terraenovae	All	Intraspecies variation	Roux *et al*. 2008
Lucilia cuprina	Pupal *exuviae*	Chemotaxonomy	Musah *et al*. 2015
	M/F adults	Chemotaxonomy	Barbosa *et al*. 2017
Lucilia sericata	Pupal *exuviae*	Chemotaxonomy	Ye *et al*. 2007
	Larvae (≤ 9 days)	Age determination	Butcher *et al*. 2013
	Larvae (≤ 10 days)	Age determination	Moore *et al*. 2013
	Larvae (1 day)	Chemotaxonomy	Moore *et al*. 2014
	Pupal *exuviae*	Chemotaxonomy	Musah *et al*. 2015
	M/F adults (≤ 20 days)	Age determination	Bernhardt *et al*. 2017
	Pupal *exuviae*	Age determination	Moore *et al*. 2017b
	Adults (≤ 30 days)	Age determination	Moore *et al*. 2017a
Phormia regina	M/F adults (7 days)	Intraspecies and sex variation	Byrne *et al*. 1995
Boettcherisca peregrine	Pupal *exuviae*	Chemotaxonomy	Ye *et al*. 2007
Parasarcopaga crassipalpis	Pupal *exuviae*	Chemotaxonomy	Ye *et al* 2007
Peckia chrysostoma	Pupal *exuviae*	Chemotaxonomy	Braga *et al*. 2013
Peckia intermutans	Pupal *exuviae*	Chemotaxonomy	Braga *et al*. 2013
Sarcodexia lambens	Pupal *exuviae*	Chemotaxonomy	Braga *et al*. 2013
Sarcho ruficornis	Pupal *exuviae*	Chemotaxonomy	Braga *et al*. 2013
Musca domestica	Pupal *exuviae*	Chemotaxonomy	Musah *et al*. 2015

Table 2 : Studies that examined behavioral responses of forensically important insects to cadaveric VOCs.

Species	Tests Run	Chemicals Tested	Behavioral Response	Authors
Calliphora vomitoria	GC-EAG, field trapping	2-heptanone	No significant response	LeBlanc 2008
		propyl butyrate	No significant response	
		dimethyl disulfide	No significant response	
		dimethyl trisulfide	No significant response	
		dimethyl tetrasulfide	No significant response	
Lucilia cuprina	EAG, two-choice bioassays	dimethyl trisulfide	Attractive (F)	Yan *et al*. 2018
		butyric acid	Attractive (F)	
		1-octen-3-ol	No significant response	
		indole	No significant response	
Lucilia sericata	Electroantennography, Y-tube bioassays	putrescine	Repulsive (F)	Frederickx *et al*. 2012
		cadaverine	No significant response	
		1-butanol	Attractive (M & F)	
		butanoic acid	No significant response	
		indole	No significant response	
		dimethyl disulfide	Attractive (F)	
		phenol	No significant response	
	Y-tube bioassays	dimethyl disulfide	Dose dependent responses	Liu *et al*. 2016
		indole	Dose dependent responses	
		isobutylamine	Dose dependent responses	
		phenylacetic acid	Attractive (F)	
Nicrophorus vespilloides	EAG, Y-tube bioassays	dimethyl sulfide	Attractive (M & F)	Kalinová *et al*. 2009
		dimethyl disulfide	No significant response	
		dimethyl trisulfide	Attractive (M & F)	
Nicrophorus vespillo	GC-EAG	dimethyl sulfide	Attractive (M & F)	Kalinova *et al*. 2009
		dimethyl disulfide	Attractive (M & F)	
		dimethyl trisulfide	Attractive (M & F)	
Thanatophilus sinuatus	Electroantennography, Y-tube bioassays	1-butanol	No significant response	Dekeirsschieter *et al*. 2013
		n-butanoic acid	No significant response	
		dimethyl disulfide	Attractive (M & F)	
		phenol	No significant response	
		p-cresol	Attractive (M)	
		Indole	No significant response	
		Cadaverine	No significant response	
		putrescine	Repellent (M)	

2.5 References

Akino, T. (2006). Cuticular hydrocarbons of Formica truncorum (Hymenoptera : Formicidae): Description of new very long chained hydrocarbon components. *Applied Entomology and Zoology, 41*(4), 667-677. doi:10.1303/aez.2006.667

Armstrong, P., Nizio, K. D., Perrault, K. A., & Forbes, S. L. (2016). Establishing the volatile profile of pig carcasses as analogues for human decomposition during the early postmortem period. *Heliyon, 2*(2). doi:10.1016/j.heliyon.2016.e00070

Baker, T.C., Cardé, R.T. (1984) Techniques for Behavioral Bioassays. In Hummel H.E., Miller T.A. (eds) *Techniques in Pheromone Research*. Springer Series in Experimental Entomology. New York: Springer.

Barbosa, R. R., Braga, M. V., Blomquist, G. J., & Queiroz, M. M. D. (2017). Cuticular hydrocarbon profiles as a chemotaxonomic tool for three blowfly species (Diptera: Calliphoridae) of forensic interest. *Journal of Natural History, 51*(25-26), 1491-1498. doi:10.1080/00222933.2017.1337944

Bernhardt, V., Pogoda, W., Verhoff, M. A., Toennes, S. W., & Amendt, J. (2017). Estimating the age of the adult stages of the blow flies Lucilia sericata and Calliphora vicina (Diptera: Calliphoridae) by means of the cuticular hydrocarbon n-pentacosane. *Science & Justice, 57*(5), 361-365. doi:10.1016/j.scijus.2017.04.007

Braga, M. V., Pinto, Z. T., Queiroz, M. M. D., & Blomquist, G. J. (2016). Effect of age on cuticular hydrocarbon profiles in adult Chrysomya putoria (Diptera: Calliphoridae). *Forensic Science International, 259*, E37-E47. doi:10.1016/j.forsciint.2015.11.006

Braga, M. V., Pinto, Z. T., Queiroz, M. M. D., Matsumoto, N., & Blomquist, G. J. (2013). Cuticular hydrocarbons as a tool for the identification of insect species: Puparial cases from Sarcophagidae. *Acta Tropica, 128*(3), 479-485. doi:10.1016/j.actatropica.2013.07.014

Brasseur, C., Dekeirsschieter, J., Schotsmans, E. M. J., de Koning, S., Wilson, A. S., Haubruge, E., & Focant, J. F. (2012). Comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry for the forensic study of cadaveric volatile organic compounds released in soil by buried decaying pig carcasses. *Journal of Chromatography A, 1255*, 163-170. doi:10.1016/j.chroma.2012.03.048

Butcher, J. B., Moore, H. E., Day, C. R., Adam, C. D., & Drijfhout, F. P. (2013). Artificial neural network analysis of hydrocarbon profiles for the ageing of Lucilia sericata for post mortem interval estimation. *Forensic Science International, 232*(1-3), 25-31. doi:10.1016/j.forsciint.2013.06.018

Byrne, A. L., Camann, M. A., Cyr, T. L., Catts, E. P., & Espelie, K. E. (1995). Forensic Implications Of Biochemical Differences Among Geographic Populations Of The Black Blow Fly, Phormia-regina (Meigen). *Journal of Forensic Sciences, 40*(3), 372-377.

Cablk, M. E., Szelagowski, E. E., & Sagebiel, J. C. (2012). Characterization of the volatile organic compounds present in the headspace of decomposing animal remains, and compared with human remains. *Forensic Science International, 220*(1-3), 118-125. doi:10.1016/j.forsciint.2012.02.007

Chen, N., Bai, Y., Fan, Y. L., & Liu, T. X. (2017). Solid-phase microextraction-based cuticular hydrocarbon profiling for intraspecific delimitation in Acyrthosiphon pisum. *Plos One, 12*(8). doi:10.1371/journal.pone.0184243

Cork, A, Beevor ,PS, Gough, AJE, & Hall, DR (1990). Gas chromatography linked to electroantennography: A versatile technique for identifying insect semiochemicals. In AR McCaffery and ID Wilson (Eds) *Chromatography and isolation of insect hormones and pheromones,* 271-279. New York: Springer.

DeBry, R. W., Timm, A., Wong, E. S., Stamper, T., Cookman, C., & Dahlem, G. A. (2013). DNA-Based Identification of Forensically Important Lucilia (Diptera: Calliphoridae) in the Continental United States. *Journal of Forensic Sciences, 58*(1), 73-78. doi:10.1111/j.1556-4029.2012.02176.x

Dekeirsschieter, J., Frederickx, C., Lognay, G., Brostaux, Y., Verheggen, F. J., & Haubruge, E. (2013). Electrophysiological and Behavioral Responses of Thanatophilus sinuatus Fabricius (Coleoptera: Silphidae) to Selected Cadaveric Volatile Organic Compounds. *Journal of Forensic Sciences, 58*(4), 917-923. doi:10.1111/1556-4029.12123

Dekeirsschieter, J., Verheggen, F. J., Gohy, M., Hubrecht, F., Bourguignon, L., Lognay, G., & Haubruge, E. (2009). Cadaveric volatile organic compounds released by decaying pig carcasses (Sus domesticus L.) in different biotopes. *Forensic Science International, 189*(1-3), 46-53. doi:10.1016/j.forsciint.2009.03.034

Drijfhout, F. P. (2010). Cuticular Hydrocarbons: A New Tool in Forensic Entomology? *Current Concepts in Forensic Entomology*, 179-203. doi:10.1007/978-1-4020-9684-6_10

Frederickx, C., Dekeirsschieter, J., Verheggen, F. J., & Haubruge, E. (2012). Responses of Lucilia sericata Meigen (Diptera: Calliphoridae) to Cadaveric Volatile Organic Compounds. *Journal of Forensic Sciences, 57*(2), 386-390. doi:10.1111/j.1556-4029.2011.02010.x

Ginzel, M. D., Millar, J. G., & Hanks, L. M. (2003). (Z)-9-Pentacosene - contact sex pheromone of the locust borer, Megacyllene robiniae. *Chemoecology, 13*(3), 135-141. doi:10.1007/s00049-003-0239-z

Gohlke, R. S. (1959). Time-Of-Flight Mass Spectrometry and Gas-Liquid Partition Chromatography. *Analytical Chemistry, 31*(4), 535-541. doi:10.1021/ac50164a024

Grassberger, M., & Reiter, C. (2002). Effect of temperature on development of the forensically important holarctic blow fly Protophormia terraenovae (Robineau-Desvoidy) (Diptera : Calliphoridae). *Forensic Science International, 128*(3), 177-182. doi:10.1016/s0379-0738(02)00199-8

Gullan, P. J. & Cranston, P. S. (2014). *The insects: An outline of entomology*. Chichester, West Sussex: Wiley Blackwell.

Hoffman, E. M., Curran, A. M., Dulgerian, N., Stockham, R. A., & Eckenrode, B. A. (2009). Characterization of the volatile organic compounds present in the headspace of decomposing human remains. *Forensic Science International, 186*(1-3), 6-13. doi:10.1016/j.forsciint.2008.12.022

Howard, R. W., & Blomquist, G. J. (2005). Ecological, behavioral, andbiochemical aspects of insect hydrocarbons. *Annual Review of Entomology, 50*, 371-393. doi:10.1146/annurev.ento.50.071803.130359

Kalinova, B., Podskalska, H., Ruzicka, J., & Hoskovec, M. (2009). Irresistible bouquet of death-how are burying beetles (Coleoptera: Silphidae: Nicrophorus) attracted by carcasses. *Naturwissenschaften, 96*(8), 889-899. doi:10.1007/s00114-009-0545-6

Kather, R., & Martin, S. J. (2012). Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. *Physiological Entomology, 37*(1), 25-32. doi:10.1111/j.1365-3032.2011.00826.x

LeBlanc, H. N. (2008). Olfactory stimuli associated with the different stages of vertebrate decomposition and their role in the attraction of the blowfly Calliphora vomitoria (Diptera: Calliphoridae) to carcasses. The University of Derby for the Degree of Doctor of Philosophy.

LeBlanc, H. N., & Logan, J. G. (2010). Exploiting Insect Olfaction in Forensic Entomology. *Current Concepts in Forensic Entomology*, 205-221. doi:10.1007/978-1-4020-9684-6_11

Liu, W. Q., Longnecker, M., Tarone, A. M., & Tomberlin, J. K. (2016). Responses of Lucilia sericata (Diptera: Calliphoridae) to compounds from microbial decomposition of larval resources. *Animal Behaviour, 115*, 217-225. doi:10.1016/j.anbehav.2016.03.022

Mani, V (1999). Properties of Commercial SPME Coatings. In J. Pawliszyn (Ed), *Applications of solid phase microextraction,* 57-72. Cambridge: Royal Society of Chemistry.

Martin, C., & Verheggen, F. (2018). Behavioural response of Lucilia sericata to a decaying body infested by necrophagous insects. *Physiological Entomology, 43*(3), 188-195. doi:10.1111/phen.12244

Miller, J. R., & Roelofs, W. L. (1978). Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *Journal of Chemical Ecology*, *4*(2), 187-198.

Mohr, R. M., & Tomberlin, J. K. (2015). Development and validation of a new technique for estimating a minimum postmortem interval using adult blow fly (Diptera: Calliphoridae) carcass attendance. *International Journal of Legal Medicine, 129*(4), 851-859. doi:10.1007/s00414-014-1094-x

Moore, H. E., Adam, C. D., & Drijfhout, F. P. (2013). Potential Use of Hydrocarbons for Aging Lucilia sericata Blowfly Larvae to Establish the Postmortem Interval. *Journal of Forensic Sciences, 58*(2), 404-412. doi:10.1111/1556-4029.12016

Moore, H. E., Adam, C. D., & Drijfhout, F. P. (2014). Identifying 1st instar larvae for three forensically important blowfly species using "fingerprint" cuticular hydrocarbon analysis. *Forensic Science International, 240*, 48-53. doi:10.1016/j.forsciint.2014.04.002

Moore, H. E., Butcher, J. B., Adam, C. D., Day, C. R., & Drijfhout, F. P. (2016). Age estimation of Calliphora (Diptera: Calliphoridae) larvae using cuticular hydrocarbon analysis and Artificial Neural Networks. *Forensic Science International, 268*, 81-91. doi:10.1016/j.forsciint.2016.09.012

Moore, H. E., Butcher, J. B., Day, C. R., & Drijfhout, F. P. (2017). Adult fly age estimations using cuticular hydrocarbons and Artificial Neural Networks in forensically important Calliphoridae species. *Forensic Science International, 280*, 233-244. doi:10.1016/j.forsciint.2017.10.001

Moore, H. E., Pechal, J. L., Benbow, M. E., & Drijfhout, F. P. (2017). The potential use of cuticular hydrocarbons and multivariate analysis to age empty puparial cases of Calliphora vicina and Lucilia sericata. *Scientific Reports, 7*. doi:10.1038/s41598-017-01667-7

Musah, R. A., Espinoza, E. O., Cody, R. B., Lesiak, A. D., Christensen, E. D., Moore, H. E., . . . Drijfhout, F. P. (2015). A High Throughput Ambient Mass Spectrometric Approach to Species Identification and Classification from Chemical Fingerprint Signatures. *Scientific Reports, 5*. doi:10.1038/srep11520

Nojima, S., Shimomura, K., Honda, H., Yamamoto, I., & Ohsawa, K. (2007). Contact sex pheromone components of the cowpea weevil, Callosobruchus maculatus. *Journal of Chemical Ecology, 33*(5), 923-933. doi:10.1007/s10886-007-9266-5

Paczkowski, S., Maibaum, F., Paczkowska, M., & Schutz, S. (2012). Decaying Mouse Volatiles Perceived by Calliphora vicina Rob.-Desv. *Journal of Forensic Sciences, 57*(6), 1497-1506. doi:10.1111/j.1556-4029.2012.02245.x

Paczkowski, S., & Schutz, S. (2011). Post-mortem volatiles of vertebrate tissue. *Applied Microbiology and Biotechnology, 91*(4), 917-935. doi:10.1007/s00253-011-3417-x

Pareige, C., Lefebvre-Ulrikson, W., Vurpillot, F., & Sauvage, X. (2016). Time-of-Flight mass spectrometry and composition measurements. In *Atom Probe Tomography* (123-154). Academic Press.

Paula, M. C., Antonialli, W. F., Mendonca, A., Michelutti, K. B., Eulalio, A., Cardoso, C. A. L., . . . von Zuben, C. J. (2017). Chemotaxonomic Profile and Intraspecific Variation in the Blow Fly of Forensic Interest Chrysomya megacephala (Diptera: Calliphoridae). *Journal of Medical Entomology, 54*(1), 14-23. doi:10.1093/jme/tjw142

Pavlovich, M. J., Musselman, B., & Hall, A. B. (2018). Direct analysis in real time-mass spectrometry (DART-MS) in forensic and security applications. *Mass Spectrometry Reviews, 37*(2), 171-187. doi:10.1002/mas.21509

Pawliszyn, J (1999). *Applications of solid phase microextraction*. Cambridge: Royal Society of Chemistry.

Pechal, J. L., Moore, H., Drijfhout, F., & Benbow, M. E. (2014). Hydrocarbon profiles throughout adult Calliphoridae aging: A promising tool for forensic entomology. *Forensic Science International, 245*, 65-71. doi:10.1016/j.forsciint.2014.10.019

Roelofs, W. L., Liu, W. T., Hao, G. X., Jiao, H. M., Rooney, A. P., & Linn, C. E. (2002). Evolution of moth sex pheromones via ancestral genes. *Proceedings of the National Academy of Sciences of the United States of America, 99*(21), 13621-13626. doi:10.1073/pnas.152445399

Roux, O., Gers, C., & Legal, L. (2008). Ontogenetic study of three Calliphoridae of forensic importance through cuticular hydrocarbon analysis. *Medical and Veterinary Entomology, 22*(4), 309-317. doi:10.1111/j.1365-2915.2008.00752.x

Seppa, P., Helantera, H., Trontti, K., Punttila, P., Chernenko, A., Martin, S. J., & Sundstrom, L. (2011). The many ways to delimit species: hairs, genes and surface chemistry. *Myrmecological News, 15*, 31-41.

Sharma, R, Garg, RK, & Gaur, JR (2015). Various methods for the estimation of post mortem interval from Calliphoridae: A review. *Egyptian Journal of Forensic Sciences*, *5,* 1-12.

Shields, WJ, Saba, T, Boehm, PD, & Pietari, J (2015). Cogeners: A Forensics Analysis. In B. Murphy and R. Morrison (Eds), *Introduction to Environmental Forensics, 3rd ed* (347-393). Oxford: Academic Press.

Simmonds, M. S. J., Blaney, W. M., & Fellows, L. E. (1990). Behavioral and electrophysiological study of antifeedant mechanisms associated with polyhydroxy alkaloids. *Journal of Chemical Ecology*, *16*(11), 3167-3196.

Statheropoulos, M., Agapiou, A., Spiliopouiou, C., Pallis, G. C., & Sianos, E. (2007). Environmental aspects of VOCs evolved in the early stages of human decomposition. *Science of the Total Environment, 385*(1-3), 221-227. doi:10.1016/j.scitotenv.2007.07.003

Statheropoulos, M., Agapiou, A., Zorba, E., Mikedi, K., Karma, S., Pallis, G. C., . . . Spiliopoulou, C. (2011). Combined chemical and optical methods for monitoring the early decay stages of surrogate human models. *Forensic Science International, 210*(1-3), 154-163. doi:10.1016/j.forsciint.2011.02.023

Statheropoulos, M., Spiliopouiou, C., & Agapiou, A. (2005). A study of volatile organic compounds evolved from the decaying human body. *Forensic Science International, 153*(2-3), 147-155. doi:10.1016/j.forsciint.2004.08.015

Stefanuto, P. H., Perrault, K. A., Stadler, S., Pesesse, R., LeBlanc, H. N., Forbes, S. L., & Focant, J. F. (2015). GC× GC–TOFMS and supervised multivariate approaches to study human cadaveric decomposition olfactive signatures. *Analytical and bioanalytical chemistry*, *407*(16), 4767-4778.

Stennett, M. D., & Etges, W. J. (1997). Premating isolation is determined by larval rearing substrates in cactophilic Drosophila mojavensis. III. Epicuticular hydrocarbon variation is determined by use of different host plants in Drosophila mojavensis and Drosophila arizonae. *Journal of Chemical Ecology, 23*(12), 2803-2824. doi:10.1023/a:1022519228346

Vass, A. A. (2012). Odor mortis. *Forensic Science International, 222*(1-3), 234-241. doi:10.1016/j.forsciint.2012.06.006

Vass, A. A., Barshick, S. A., Sega, G., Caton, J., Skeen, J. T., Love, J. C., & Synstelien, J. A. (2002). Decomposition chemistry of human remains: A new methodology for determining the postmortem interval. *Journal of Forensic Sciences, 47*(3), 542-553.

Vickers, N. J. (2000). Mechanisms of animal navigation in odor plumes. *Biological Bulletin, 198*(2), 203-212. doi:10.2307/1542524

von Hoermann, C., Ruther, J., & Ayasse, M. (2016). Volatile Organic Compounds of Decaying Piglet Cadavers Perceived by Nicrophorus vespilloides. *Journal of Chemical Ecology, 42*(8), 756-767. doi:10.1007/s10886-016-0719-6

von Hoermann, C., Ruther, J., Reibe, S., Madea, B., & Ayasse, M. (2011). The importance of carcass volatiles as attractants for the hide beetle Dermestes maculatus (De Geer). *Forensic Science International, 212*(1-3), 173-179. doi:10.1016/j.forsciint.2011.06.009

von Hoermann, C., Steiger, S., Muller, J. K., & Ayasse, M. (2013). Too Fresh Is Unattractive! The Attraction of Newly Emerged Nicrophorus vespilloides Females to Odour Bouquets of Large Cadavers at Various Stages of Decomposition. *Plos One, 8*(3), 11. doi:10.1371/journal.pone.0058524

Weidner, L. M., Tomberlin, J. K., & Hamilton, G. C. (2014). Development of Lucilia Coeruleiviridis (Diptera: Calliphoridae) In New Jersey, USA. *Florida Entomologist, 97*(2), 849-851. doi:10.1653/024.097.0277

Wells, J., & LaMotte, L. (2017). The role of a PMI-prediction model in evaluating forensic entomology experimental design, the importance of covariates, and the utility of response variables for estimating time since death. *Insects*, *8*(2), 47.

Whitworth, T. (2006). Keys to the genera and species of blow flies (Diptera : Calliphoridae) of America North of Mexico. *Proceedings of the Entomological Society of Washington, 108*(3), 689-725.

Xu, H., Ye, G. Y., Xu, Y., Hu, C., & Zhu, G. H. (2014). Age-dependent changes in cuticular hydrocarbons of larvae in Aldrichina grahami (Aldrich) (Diptera: Calliphoridae). *Forensic Science International, 242*, 236-241. doi:10.1016/j.forsciint.2014.07.003

Yan, G. J., Liu, S. M., Schlink, A. C., Flematti, G. R., Brodie, B. S., Bohman, B., . . . Martini, G. B. (2018). Behavior and Electrophysiological Response of Gravid and Non-Gravid Lucilia cuprina (Diptera: Calliphoridae) to Carrion-Associated Compounds. *Journal of Economic Entomology, 111*(4), 1958-1965. doi:10.1093/jee/toy115

Ye, G. Y., Li, K., Zhu, J. Y., Zhu, G. H., & Hu, C. (2007). Cuticular hydrocarbon composition in pupal exuviae for taxonomic differentiation of six necrophagous flies. *Journal of Medical Entomology, 44*(3), 450-456. doi:10.1603/0022-2585(2007)44[450:chcipe]2.0.co;2

Zhu, G. H., Jia, Z. J., Yu, X. J., Wu, K. S., Chen, L. S., Lv, J. Y., & Benbow, M. E. (2017). Predictable weathering of puparial hydrocarbons of necrophagous flies for determining the postmortem interval: a field experiment using Chrysomya rufifacies. *International Journal of Legal Medicine, 131*(3), 885-894. doi:10.1007/s00414-016-1507-0

Zhu, G. H., Xu, X. H., Yu, X. J., Zhang, Y., & Wang, J. R. (2007). Puparial case hydrocarbons of Chrysomya megacephala as an indicator of the postmortem interval. *Forensic Science International, 169*(1), 1-5. doi:10.1016/j.forsciint.2006.06.078

Zhu, G. H., Ye, G. Y., Hu, C., Xu, X. H., & Li, K. (2006). Development changes of cuticular hydrocarbons in Chrysomya rufifacies larvae: potential for determining larval age. *Medical and Veterinary Entomology, 20*(4), 438-444. doi:10.1111/j.1365-2915.2006.00651.x

Zhu, J. W. J., Chaudhury, M. F., Tangtrakulwanich, K., & Skoda, S. R. (2013). Identification of Oviposition Attractants of the Secondary Screwworm, Cochliomyia macellaria (F.) Released from Rotten Chicken Liver. *Journal of Chemical Ecology, 39*(11-12), 1407-1414. doi:10.1007/s10886-013-0359-z

CHAPTER 3. NECROPHILOUS INSECT RESPONSES TO SELECTED CADAVERIC VOCS

3.1 Introduction

Vertebrate decomposition is a complex process that is influenced by a number of factors, both biotic and abiotic. Among the biotic influencers are bacteria, fungi, protozoa, and insects (Campobasso *et al.* 2001). Insect colonizers of carrion can be quite conspicuous, with dipteran larvae often blanketing the carcass in enormous masses while Coleoptera, Hymenoptera, Lepidoptera, and insects of other orders are also frequently present (Byrd and Castner 2001). Carrion is a nutrient rich food source, but is also ephemeral and scarce—organisms ultilizing such a resource must act quickly. As such, necrophilous insects are to the volatile organic compounds (VOCs) produced by decomposing animals (LeBlanc and Logan 2010). The nature of the response (attractive or antagonistic) may depend on chemical concentration and gradient (e.g. Frederickx *et al.* 2012; Liu *et al.* 2016), which, in the case of cadaveric VOCs specifically, provides information about the stage of decomposition and the location of the resource (LeBlanc and Logan 2010).

Necrophilous insects are particularly attracted to carcasses that meet their unique nutritional needs. For instance, burying beetles (*Nicrophorus spp.*) are carrion feeding insects that specialize on small carcasses, which they bury in the ground and subsequently raise their young upon (Kalinová *et al.* 2009). In some species, females with well-developed ovaries show a preference for small carcasses, such as mice, that are easy to bury, whereas recently emerged females prefer larger carcasses for ovary development, perhaps because of reduced competition (Wilson and Knollenberg 1984). Furthermore, older females prefer fresh carcasses, which provide more food for their young, while younger females are more attracted to older, more decomposed carcasses, which offer more immediate nutrition (von Hoermann *et al.* 2013). For these sorts of preferences to develop, an insect must be able to distinguish carcass quality.

One reason that such distinctions are possible is that decomposing vertebrates produce unique, odor profiles that change over time—a newly deceased animal smells different than a heavily decomposed one. Putrefaction begins immediately following death as cells autolyze and are digested by bacteria and fungi (Campobasso *et al.* 2001). Destruction of the soft tissues in these ways produces the liquids and gases that make up the odor of death (LeBlanc and Logan 2010). Each type of soft tissue is made up of a unique combination of biological molecules, and thus

break down into unique blends of VOCs. These tissues also decompose at different rates—liver tissue, for example, which contains a lot of digestive enzymes, decomposes faster than many other types of soft tissue (LeBlanc and Logan 2010). The odor profile of a body is therefore dependent on which tissues have been broken down, which is in turn dependent on how long a body has been dead. Recent research has suggested that odor production is also heavily dependent on the microbial community found in and on the decomposing vertebrate (Davis *et al.* 2013). VOCs are produced as byproducts of microbial digestion. At the onset of decomposition, microorganisms inhabiting a body have access to an abundance of nutrients. This availability of resources allows the microbes to prioritize primary metabolic pathways, which prioritize growth and reproduction (Davis *et al.* 2013). As decomposition progresses the resource dwindles, causing many microbial species to activate lower priority pathways. These secondary pathways may utilize alternative substances for energy or enhance survival strategies in other ways. Each pathway produces a unique blend of chemical byproducts, and the odor profile of the corpse changes as the microbial flora utilize different pathways over time. Vass *et al.* (2002) created models to estimate this postmortem interval, or PMI, based on the VOCs produced by a body. The accuracy of these models is limited only by the accessibility of temperature data, which plays a significant role in decomposition rate (Campobasso *et al.* 2001).

PMI estimations, or more specifically minimum PMI or mPMI estimations (Villet *et al.* 2010), are one of the most important roles of forensic entomologists. Often this is accomplished by collecting larval samples at the scene and using species- and temperature-specific development models to determine larval age (Sharma *et al.* 2015). Certain insects, such as blow flies (Diptera: Calliphoridae), can arrive at a body and deposit eggs within minutes (Anderson 2001a), making the age of the larvae representative of the mPMI. This method assumes that the larvae collected were the oldest larvae at the scene, which may not always hold true (Tarone and Sanford 2017). Forensic entomologists may choose to combine this method with additional ones, such as succession analysis, to improve their mPMI estimates. Because insects are attracted by specific odor bouquets produced at specific points in the decomposition process, they tend to arrive in a predictable pattern, called succession. Species assemblages vary at different points in the decomposition process, and the presence or absence of a particular species may contribute information for mPMI estimation (Amendt *et al.* 2010). In cases of advanced decomposition and

skeletal remains, few, if any, larvae are left at the scene, making succession analysis an even more important tool for forensic entomologists.

There is currently interest in improving PMI estimates by combining succession with VOC-based PMI models. Identification of the cadaveric VOCs responsible for insect attraction is essential for this integration. Researchers typically collect VOCs at various stages of the decomposition process, determine the electrophysiologically active compounds using gas chromatography–electroantennographic detection, and then test the behavioral responses of these compounds using one or more behavioral assays (see Chapter 2 for more details). Frequently these studies are completed in a laboratory setting to control for certain conditions that might affect behavior, such as insect age, source population, temperature, humidity, and lighting (Liu *et al.* 2016). These studies, however, fail to recreate these conditions in a field setting. In fact, to the author's knowledge, only one study has examined insect responses to cadaveric VOCs in a field setting (LeBlanc 2008). Field studies are beneficial in that they offer a more realistic method of exposure and can provide information for many species at once, rather than a single species as is frequently encountered in attractant studies.

The goal of this study was to test the attraction of forensically important insects to nine selected volatile organic compounds: ethanol, *o*-xylene, *p*-xylene, 1-pentanol, cyclohexanone, acetamide, dimethyl disulfide, dimethyl trisulfide, and 1-butanol. I hypothesized that cadaver-produced VOCs are responsible for insect attraction, and would be able to attract forensically important insects even in the absence of a corpse. I predicted that all of the chemicals would show significant attraction to forensically important insects. Experiments were carried out in a field setting to collect more useful data about a variety of necrophilous insect species.

3.2 Materials and Methods

3.2.1 Field Sites

Three Purdue university owned properties were selected for use in this project: Purdue Wildlife Area, Lugar Farm, and Martell Forest (Figure 1). These sites were selected as per the advice of the property manager, based on their similar landscapes and proximity to one another. Two field sites within each property were selected based on availability and accessibility: Purdue Wildlife Area 1 (40°27'33" N, 87°03'33" W; Figure 2), Purdue Wildlife Area 2 (40°27'10" N,

87°03’24” W; Figure 2), Lugar Farm 1(40°25’47” N, 86°57’28” W; Figure 3), Lugar Farm 2 (40°25’48” N, 86°57’07” W; Figure 3); Martell Forest 1 (40°25’55” N, 87°02’15” W, Figure 4) and Martell Forest 2 (40°25’55” N, 87°01’54” W; Figure 4). The number of total field sites was limited by manpower availability. Pitfall traps were placed in each of the six field sites along the edge of a path or unpaved road next to a forested area, for ease of collection. Holes for traps were dug at least 10.5 meters apart.

3.2.2 Pitfall traps

Each field site held 18 no kill pitfall traps—one trap per chemical bait and one negative control per chemical bait (Figure 5). No kill traps were used to avoid any potential attractive effects the preservative may have had on insects.

Empty two-liter soda bottles were triple rinsed with DI water and allowed to air-dry. Once dry, an incision was made around the bottle, approximately 20.3 cm from the base of the bottle. The top of the bottle was then inverted to form a funnel. A small, 5 mL plastic bag containing a clean cotton roll was strung onto a length of fishing line (4.5 kg Omniflex, W.C. Bradley/Zebco Holdings, Inc.). The fishing line was strung through two holes about 4 cm from the top rim of the funnel. The funnel was then placed into the base of the bottle and the ends of the fishing line were drawn tight to reduce slack (Figure 6). The completed traps were placed into previously dug holes at the six selected field sites (Figure 7).

The location of each pitfall trap was denoted with a pink marker flag. The flags were labeled with the name of the test chemical or “control”; negative control traps were placed between each baited trap in a line. Each baited trap had one associated negative control. The order of the baited traps along the line was chosen using a random number generator (Figure 5). Once the order of the baits was determined, it was held constant throughout the trial. The order at each site was repeated from the fall to the spring trial.

3.2.3 Cadaveric Chemical Baits

Chemicals were chosen from a literature review based on the following criteria: 1) chemical was identified in multiple studies (Table 3), 2) chemical is produced by both human and porcine remains, 3) chemicals chosen encompass the entire decomposition process (Table 3), 4)

availability, and 5) cost. Several chemicals that were initially chosen were not used because they were not commercially available and/or they were too expensive to purchase in large quantities.

Nine carrion produced VOCs were ultimately tested for insect attraction: ethanol (100% pure, Decon Laboratories, PA, USA), *o*-xylene (99% pure, Alfa Aesar, MA, USA), *p*-xylene (99% pure, Alfa Aesar, Haverhill, MA), cyclohexanone (99.8% pure, Acros Organics, Thermo Fisher Scientific, NJ, USA), 1-pentanol (99+% pure, Alfa Aesar, MA, USA), 1-butanol (99.9% pure, Thermo Fisher Scientific, NJ, USA), acetamide (99% pure, Acros Organics, Thermo Fisher Scientific, NJ, USA), dimethyl disulfide (99% pure, Alfa Aesar, MA, USA), and dimethyl trisulfide (98% pure, OR, USA). All chemicals were received as liquids and applied in pure form, with the exception of acetamide, which was received as a solid. A 177 mg/mL aqueous acetamide solution was made for application. This concentration was chosen because it was the highest concentration possible given the mass of acetamide received and the volume of liquid needed for the trials.

Six drops (approximately 0.5mL) from a 7.7mL disposable pipette (Cat. No. 13-711-7m, Fisher Scientific) of the appropriate chemical was applied to the cotton roll of a pitfall trap, and the bag left unsealed. Chemicals were reapplied every 24 hours. At the time of each application, the funnel of the pitfall trap was removed, and all insects inside the trap were collected—by hand or with an insect net—and stored in a freezer at -20° C for later analysis. This experiment was initially carried out in October 2018 (October 21, 2018 – October 25, 2018) and repeated in May 2019 (May 13, 2019 – May 20, 2019).

I was unable to collect from all six field sites at one time during the October 2018 trial due to time restrictions. Instead, the sites were split into AM/PM collection times, with one site from each Purdue owned research area serviced in the morning, and one site from each research area serviced in the fall. In the May 2019 trial, all traps were serviced in the afternoon.

3.2.4 Positive Controls

Pig carcasses were used as positive controls to determine what forensically important insects were present in the surrounding area during each field trial. All insects collected from the baited traps were compared to insects found on pig carcasses at the Entomology Field Operations Building (EFOB; 40°25’41” N, 86°56’54” W). The positive control for the October 2018 trial was a set of three adult pig carcasses laid out in October 2017. Despite being a year apart, the data set

were considered comparable because of similar average temperatures and precipitation between the two years at the time of collection. Collections were made from these carcasses twice daily, with larval, flying, and crawling insect specimens collected. Larval specimens were reared to adulthood on beef liver for identification. All specimens were pinned and stored for later identification. The pig carcasses were left in the field from October until early March, but only specimens collected from the carcasses in October were included for comparison. This was done to ensure that the species present would be representative of those that may be found at the same time the traps were in the field.

The positive control for the May 2019 was a previously frozen stillborn piglet carcass. The piglet was laid out on the same day that the traps were initially baited, and collected from once daily. Flying and crawling insects were collected, but no larval collections were made. This decision was made primarily due to time constraints for rearing. All specimens were stored in a freezer at -20° C for later identification. Collections were made for 10 days, until the piglet skeletonized. This was done to ensure that all of the volatiles used as baits, which encompass the entirety of the decomposition process, would be represented by the carcass.

3.2.5 Insect Identification

All insects were identified using a stereomicroscope (Leica M80) based on morphological identification keys. All insects were identified to family (McAlpine 1981; Borror *et al.* 1989). Insects belonging to families of forensic significance were further identified to genus or species when possible (Huckett and Vockeroth 1987; Evans 2014, Whitworth unpublished). No Myriapoda or Chelicerata were identified past subphylum.

3.2.6 Statistical Analysis

All analyses were conducted using R 3.6.0 (R Development Core Team 2019). Species abundances were compiled for each trap across the sampling period. Species that were collected only once were omitted from analyses. Each site was treated as a replicate for analysis, and thus all sites were analyzed together. Data from the spring trial were square root transformed to meet assumptions of homogeneity of beta dispersions. Species assemblages from chemically baited traps were compared to their negative controls with Permutational Multivariate Analysis of Variance (Anderson 2001b) with 999 permutations, using the ADONIS function in the “vegan”

package (Oksanen *et al.* 2019) and Bray-Curtis dissimilarity. Any baited trap communities that were found to be significantly different from their negative controls were then compared to one another using Bray-Curtis dissimilarity and nonmetric multidimensional scaling (NMDS; Kruskal 1964).

3.2.7 Chemical Release Rates

The release rate of each chemical was measured in controlled experiments under a fume hood. Cotton rolls were placed into small plastic bags and weighed. Six drops (approximately 0.5mL) from a 7.7mL disposable pipette (Cat. No. 13-711-7m, Fisher Scientific) of a chemical was applied to the cotton roll and weighed again. This was repeated for all chemicals. The bags were hung in a fume hood and weighed once more after 24 hours. This procedure was conducted three times for each chemical, with the exception of dimethyl trisulfide, which was only tested once due to limited availability. For the second and third trials, 30 drops (approximately 2.5mL) were added to the cotton rolls.

3.3 Results

3.3.1 Chemical Release Rates

The mean release rates and standard errors are reported in Table 4. Many of the rates exhibited high standard errors, indicating a lot of variation in the sample distribution, likely because of the change in methodology from the first to the subsequent trials. The change was deemed necessary because several of the chemicals (dimethyl disulfide and *p*-xylene) volatilized completely during the first trial, so an accurate representation of the release rate may not have been recorded. Despite the possibility that some chemicals may have completely volatilized prior to reapplication, all of the baited pitfall traps (with the exception of acetamide) still exuded a strong scent at the time of reapplication.

3.3.2 Fall 2018

A total of 39 different arthropod taxa were collected and identified across both the pitfall traps and positive control during the fall study (Table 5). The following specimens collected from pitfall traps were determined to be of potential forensic importance: *Creophilus maxillosus* (Linnaeus), *Necrophila americana* (Linnaeus), *Nicrophorus sayi* (Laporte), *Nicrophorus*

tomentosus (Weber)*,* Staphylindae, *Muscina sp.,* and Phoridae. *Creophilus maxillosus, Necrophila americana,* Staphylindae, and Phoridae specimens were also found on the positive control carcass. Each trap demonstrated homogeneity of beta dispersion when compared with its negative control, fulfilling the assumptions of ADONIS without the need for transforming the data. Analysis with ADONIS revealed that no baited trap was significantly different from its negative control at p = 0.05 (though dimethyl trisulfide was closest at p = 0.058).

Despite a lack of statistical significance, certain trends are evident in the data. Specifically, all of the specimens deemed to be forensically important were collected from dimethyl trisulfide baited pitfall traps, with the exception of *Nicrophorus sayi and Nicrophorus tomentosus,* which were collected from dimethyl disulfide baited traps (Table 6). It may also be noted that many of the forensically important species collected from the positive control were not represented in the pitfall trap collections, most notably all of the Calliphoridae species.

3.3.3 Spring 2019

A total of 27 different arthropod taxa were collected and identified across both the pitfall traps and positive control during the spring study (Table 7). The following specimens collected from pitfall traps were determined to be of potential forensic importance: *Creophilus maxillosus, Euspilotus assimilis* (Paykull)*, Necrophila americana, Oiceoptoma noveboracense* (Forster)*,* Staphylinidae*, Trox sp., Cochliomyia macellaria* (Fabricus)*,* and *Phormia regina* (Meigen)*. Euspilotus assimilis, Oiceoptoma noveboracense,* Staphylinidae*, Cochliomyia macellaria,* and *Phormia regina* were also found on the positive control. Analysis with ADONIS revealed that the species assemblages from six chemically baited traps were significantly different from their negative controls: dimethyl trisulfide (p = 0.004), dimethyl disulfide (p = 0.011), ethanol (p =0.031), cyclohexanone (p = 0.05), acetamide (p = 0.036) and *p*-xylene (p = 0.045).

The species communities from these six chemicals were further compared with NMDS (stress = 0.204; Figure 8), which provides a visual representation of the differences between the groups. The closer together the groups are in the plot, the more similar they are. The following taxa were included in the comparison: Chelicerata, Myriapoda, Gryllidae, Carabidae, Formicidae, Staphylinidae, Scarabaeidae, *Trox sp., Pherbellia sp., Phaonia spp., Euspilotus assimilis, Phormia regina, Creophilus maxillosus, Necrophila americana, Oiceoptoma noveboracense,* and *Cochliomyia macellaria*. The acetamide, ethanol, cyclohexanone, and *p*-

xylene communities demonstrated a lot of overlap, whereas the dimethyl disulfide, dimethyl trisulfide, and positive control communities were separate and distinct. This indicates that dimethyl disulfide and dimethyl trisulfide attracted unique species when compared to the other four chemicals.

One possible driving force for the differences observed in the NMDS is the attraction of forensically important insects. Although acetamide, ethanol, cyclohexanone, and *p*-xylene were different from their negative controls, no forensically important insects were captured with these baits. A second NMDS was run using only dimethyl disulfide and dimethyl trisulfide, as all of the forensically important insects collected during the study came from these traps. Only the forensically important groups previously mentioned were included in these tests. The NMDS (stress = 0.044; Figure 9) reiterates that the two communities are distinct.

3.4 Discussion

In the fall 2018 trial, the treatments were non-significant, and there was limited overlap between the baited traps and the positive control. This is not necessarily indicative of the selected chemicals not being attractive, however. The fall study was not conducted for a full week due to severe weather, which limited the number of specimens collected, and thus how different from the negative controls the baited traps were. Additionally, because the majority of the specimens collected from the positive control were larvae, the positive control collections were not necessarily indicative of the adult specimens that were visiting the carcass at that time. Larval development can take days to weeks depending on temperature, so it is possible that the larvae hatched from eggs that were laid long before the collections were made. No larvae were collected in any pitfall traps at any time, so if adult calliphorids were in diapause or otherwise inactive at the time of the experiment, there would be no representation of those species in the traps. During this study the bags containing the dimethyl trisulfide baited cotton rolls were also frequently scavenged from the traps, so the traps may not have remained baited for a full 24 hours.

In the spring 2019 trial, the species assemblages from six chemicals were found to be significantly different from their negative controls: dimethyl trisulfide, dimethyl disulfide, ethanol, acetamide, cyclohexanone, and *p*-xylene. Of the six, only two attracted forensically important insects—dimethyl trisfulfide and dimethyl disulfide. Dimethyl trisulfide was particularly attractive to *Phormia regina*—thousands of specimens were collected from these traps (Table 8). Although

sex of each fly was not recorded, it was noted that the majority, if not all, were female. Many were also gravid, and on several occasions, eggs coated the bottoms of the pitfall traps, though they never hatched and no larvae were found in the traps. There is not currently any published literature that examines the attraction of *Phormia regina* to cadaveric organic compounds, but these personal observations indicate that sex and reproductive status may be factors in attraction for this species, as has been noted for other species as well (e.g. Liu *et al.* 2016; Mohr and Tomberlin 2015). Dimethyl disulfide also attracted *Phormia regina*, but in far fewer quantities, typically only one or two over a 24-hour period.

There were several species collected from the positive control that were not represented in the baited traps. These differences may be due to the inherent differences in collection—the baited traps were left out for 24 hours, and all insects attracted in that time were collected, whereas the positive control was collected from only once per day by hand, with forceps and an insect net. If certain species were not present at the time of collection, they would not have been collected. Several species that have been well documented at carcasses, such as *Creophilus maxillosus* and *Necrophila americana*, were not collected from the positive control but were collected from the baited pitfall traps, lending credence to this hypothesis. There were also species that were present on the pig carcass that were not collected in the pitfall traps, such as *Lucilia coeruleiviridis* (Macquart) and *Lucilia sericata* (Meigen). In a previous study (Liu *et al.* 2016), dimethyl disulfide was shown to be attractive to *Lucilia sericata*. This study seems to contradict that, but Liu *et al.* (2016) found attraction only at specific concentrations (0.25 μg for nongravid females and 0.005 μg for males). This field study used pure dimethyl disulfide, without dilution, which has not previously been tested for attraction with this species. Both in this study and in previous studies (see Table 2 in Chapter 2), dimethyl trisulfide and dimethyl disulfide have been shown to be important attractants to a variety of species.

Species communities in the baited traps were very different from fall to spring. Notably, fall traps saw no blow fly activity and reduced numbers of forensically important beetles, whereas spring traps saw large numbers of both flies and beetles. Many species begin to enter diapause as the days grow shorter in the fall, so these differences are not surprising. The species at the positive controls, however, showed more overlap between seasons. As previously mentioned, many of the specimens identified from the fall study were collected as larvae from the pig carcasses. These larvae may have been laid as eggs days to weeks before collection. Adults of the species may have

already died or entered diapause at the time of these collections, which could explain why no blow flies were found in the baited traps in the fall, despite record of them on the carcasses. Adults are very active in the spring, however, and thus would be attracted to the traps. At no time in either fall or spring were larvae found in the pitfall traps.

I encountered several setbacks during the course of the two trials. The fall trial was delayed because the chemicals did not arrive by the expected start date, which corresponded to a large drop in average temperature, more precipitation, and fewer insects than when the trial was originally scheduled to begin.

I also experienced bait thefts during the fall trial: on several occasions the dimethyl disulfide and dimethyl trisulfide baited cotton rolls had been scavenged from the pitfall traps. As previously stated, this may have led to the traps not being baited for a full 24 hours, but it could also be indicative of vertebrate attraction to these chemicals. No scavenging was observed during the spring trial, so the attraction to these chemicals may be stronger as winter approaches and resources dwindle. During both the fall and spring trials, heavy thunderstorms flooded the traps on multiple occasions. The storms may have also affected insect abundance as insects are unlikely to be mobile during such weather. Bait concentrations may have also been affected by dilution, which could further affect attraction. Despite the extreme weather, forensically important insects were still attracted daily, so the effect of weather was not considered to be strong enough to warrant rescheduling the trials.

In addition to environmental problems, there may have been some problems with experimental design as well. Space limitations required that traps be placed relatively close to one other (10.5m between each trap, 21m between each chemically baited trap; Figure 5), which could have resulted in overlap of odor plumes. Additionally, the geography of the field sites did not allow for the traps to be placed in a straight line. The curvature of the paths may have also affected resulted in overlap of odor plumes. These possible effects were also deemed insignificant, as no forensically important insects were caught in any negative control trap at any time, despite being positioned between baited traps.

This and other attractant studies (see Chapter 2) support the hypothesis that cadaveric VOCs are responsible for insect attraction. Not every VOC tested was shown to be attractive, however. Only two of the chemical baits captured any forensically important insects: dimethyl disulfide and dimethyl trisulfide. The other chemicals were not attractive on their own in a pure

state, but may be attractive at different concentrations or in blends with other compounds. It is also possible that these chemicals are not unique enough to decomposition to trigger behavioral responses. This study could not utilize GC-EAD before the behavioral assay because many species were being targeted at once. Including this step would provide insight into whether or not these chemicals are perceived by forensically important insects. It is also important to note that not every forensically important insect species shown to be present in the area was represented in the traps. This was expected, as each chemical represents a "static" point in the decomposition process – no chemical used has been shown to be present throughout decomposition. The positive control, however, was allowed to decompose fully, and faunal succession brought about a wider variety of forensically important insects. Additionally, no chemical blends were tested, which are likely very important for insect resource location. Furthermore, larvae were present on the positive control carcass, which may influence resource location via pheromone and VOC production.

The information gleaned from this study can be used to improve body recovery techniques and mPMI estimates. Portable detection systems based on insect olfaction could be used to locate decomposing bodies (Hoffman *et al.* 2009). These devices would detect the same chemicals responsible for triggering insect behavioral responses. In addition, the chemicals attractive to necrophilous insects identified in this and similar studies may be used to help train canine recovery teams. Cadaver dogs are often trained using small pieces of flesh or bones, which may not be representative of all of the odors a body might produce during decomposition (Hoffman *et al.* 2009). Pinpointing the VOCs throughout the decomposition process that allow necrophilous insects to selectively forage provides a scent profile that encompasses the entirety of decomposition. Trainers can use these chemicals to train the dogs to recognize a body at any stage of the decomposition process.

Tomberlin *et al.* (2011) describes the need to include the pre-colonization interval, the period of time after death but before insect colonization, in mPMI estimates. The pre-colonization interval is especially important in cases where bodies were not immediately exposed to the environment—such as when a body is frozen, left in a trunk, or buried—but is frequently left out of mPMI estimates. This exclusion is largely due to the difficulty of measuring the duration of the phase. As I have discussed previously in this chapter, cadaveric odor profiles change over the course of the decomposition process, and as they change different insect colonizers are attracted to the corpse. Given that microbial produced VOC profiles vary over time, knowing what chemical

cues attract primary colonizers, and when those cues are emitted, offers a way to measure the precolonization interval (Tomberlin *et al*. 2011).

To make this research practically applicable in the field, more work needs to be done. More field validation studies need to be completed to assess how insects react to certain cues in a natural setting. These studies should make an effort to use chemical concentrations that were shown to be attractive in laboratory settings as well, as opposed to using pure compounds that may not have the same effect. The effect of hetero- and conspecific colonization should also be examined. Larval produced VOCs might prove to be important cues for adults as well as certain predatory insects, such as *Creophilus maxillosus*. Larval presence may also affect cadaveric VOC profiles, which could affect colonization patterns and timing. More research into how odor profiles are influenced by microbial communities, biotopes, and taphonomic factors would also be beneficial.

Figure 1 : Locations of every Purdue owned research area used for the study

Figure 2 : Locations of the sites within Lugar Farm

Figure 3 : Locations of the sites within Purdue Wildlife Area

Figure 4 : Locations of the sites within Martell Forest

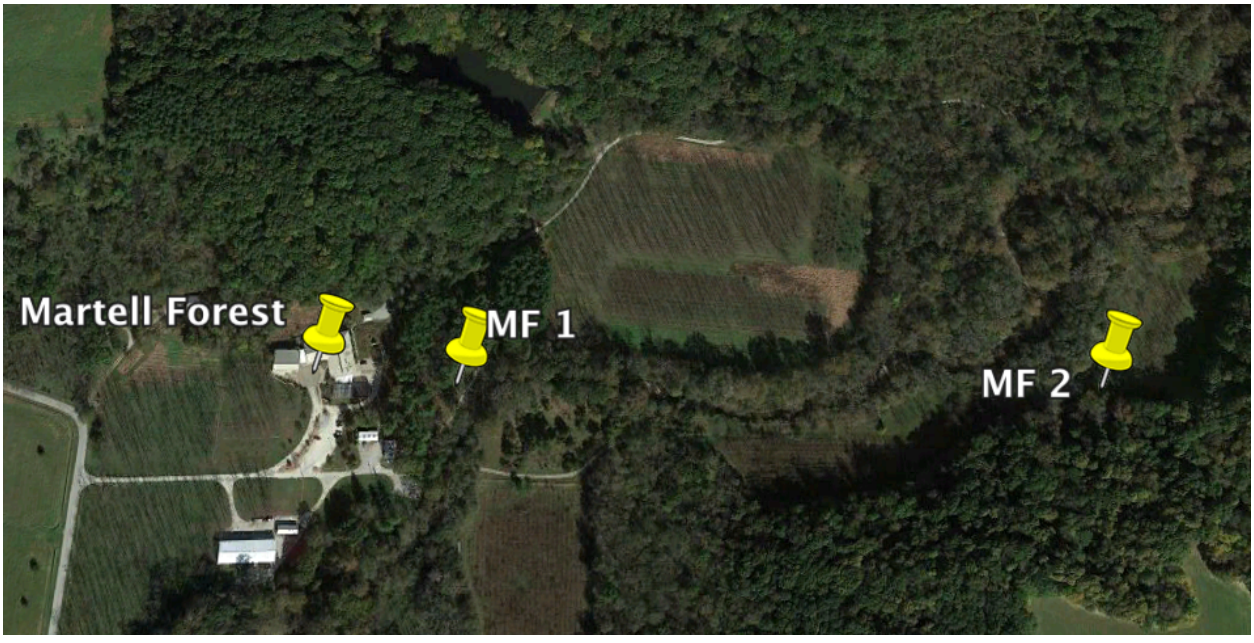


Figure 5: Pitfall trap organization at each field site. Traps were approximately 10.5m apart.

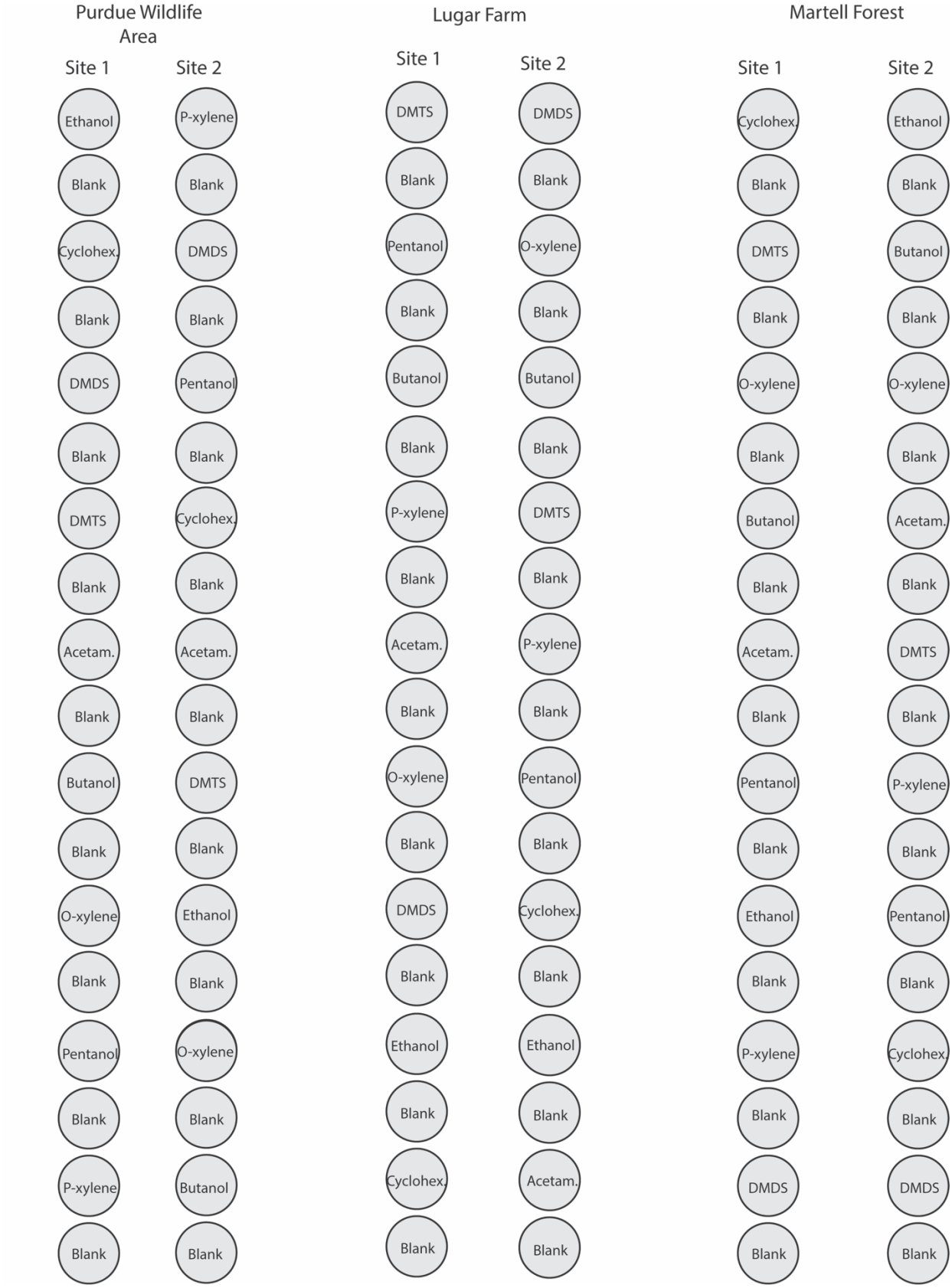


Figure 6 : Drawing of the pitfall trap design.

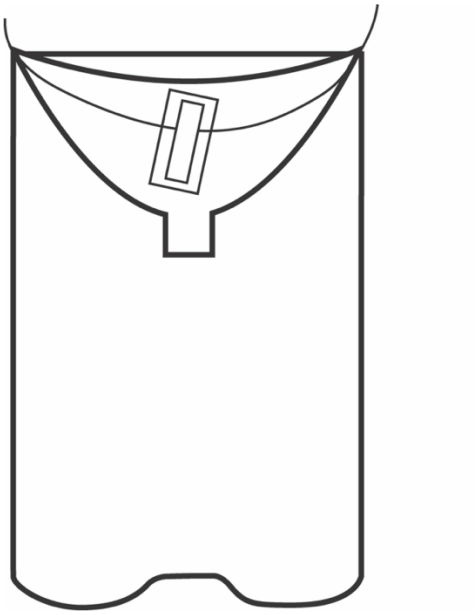


Figure 7 : Pitfall trap in place

Table 3: Chemical baits, the stage of decomposition each is found in, and some studies in which they have been identified

Chemical	Stage of Decomposition*	Literature References
ethanol	Fresh, bloat, post bloat	Statheropoulos *et al*. 2005; Dekeirsschieter *et al*. 2012; Armstrong *et al.* 2016
o-xylene	Fresh, bloat	Statheropoulos *et al*. 2007; Dekeirsschieter *et al*. 2012; Vass *et al.* 2012
p-xylene	Post bloat	Statheropoulos *et al*. 2005; Hoffman *et al.* 2009; Vass *et al.* 2012
cyclohexanone	Fresh, bloat, post bloat	Hoffman *et al.* 2009; DeGreef and Furton 2011; Statheropoulos *et al*. 2011
1-pentanol	Post bloat	Vass *et al.* 2008; Dekeirsschieter *et al*. 2009; Armstrong *et al.* 2016
1-butanol	Bloat, post bloat	Statheropoulos *et al*. 2005; Boumba *et al.* 2008*;* Dekeirsschieter *et al*. 2009
acetamide	Post bloat	Dekeirsschieter *et al*. 2009; Dekeirsschieter *et al*. 2012
dimethyl disulfide	Post bloat	Kalinova *et al.* 2009; Brasseur *et al. 2012;* Armstrong *et al.* 2016
dimethyl trisulfide	Bloat, post bloat	Kalinova *et al.* 2009; DeGreef and Furton 2011; Armstrong *et al.* 2016

*As reported by Dekeirsschieter *et al*. 2012

Table 4 : Chemical release rates

Chemical	Mean Release Rate (mg/day)	Standard Error
ethanol	445.73	180.76
o-xylene	721.27	262.59
p-xylene	675.47	221.75
cyclohexanone	67.10	11.56
1-pentanol	34.47	23.18
1-butanol	43.13	7.36
acetamide	27.4	8.07
dimethyl disulfide	764.00	267.93
dimethyl trisulfide	198.40	N/A

Table 5 : Specimens collected in fall 2018

Order or Subphylum	Family or Species (Pitfall Traps)	Family or Species (Positive Control)
Chelicerata	unknown	none
Coleoptera	Carabidae *Ceratoma trifurcata* *Creophilus maxillosus* *Necrophila americana* *Nicrophorus sayi* *Nicrophorus tomentosus* Scarabaeidae Staphylinidae *Tachyporus elegans* Tenebrionidae	*Creophilus maxillosus* *Geotrupes blackburnii* *Necrophila americana* *Nicrophorus orbicollis* *Nicrophorus sp.* Staphylinidae
Dermaptera	unknown	none
Diptera	*Apotropina sp.* Culicidae. *Muscina sp.* *Pherbellia sp.* Phoridae Tipulidae	*Calliphora vomitoria* *Chrysomya rufifacies* *Cochliomyia macellaria* *Lucilia coeruleiviridis* *Lucilia illustris* *Lucilia sericata* *Lucilia silvarum* *Lucilia spp.* Muscidae Phoridae *Phormia regina*
Hemiptera	Pentatomidae unknown	unknown
Hymenoptera	Formicidae unknown	none
Lepidoptera	unknown (larvae)	none
Myriapoda	unknown	none
Orthoptera	Gryllidae unknown	unknown

Table 6: Forensically important specimens captured per trap in Fall 2018

Bait	Insect	Total Captured	Mean ± Standard Error
Dimethyl disulfide	*Nicrophorus tomentosus*	2	0.33 ± 0.33
	Nicrophorus sayi	2	0.33 ± 0.33
Dimethyl trisulfide	*Mucina sp.*	33	5.5 ± 3.47
	Creophilus maxillosus	6	1 ± 0.68
	Necrophila americana	2	0.33 ± 0.33
	Staphylinidae	1	0.16 ±0.16

Table 7 : Specimens collected in spring 2019

Order or Subphylum	Family or Species (Pitfall Traps)	Family or Species (Positive Control)
Chelicerata	unknown	none
Coleoptera	Carabidae *Ceratoma trifurcata* *Copris fricator* *Creophilus maxillosus* *Euspilotus assimilis* *Necrophila americana* *Oiceoptoma noveboracense* *Onthophagus hecate* *Phyllophaga sp.* Scarabaeidae Staphylinidae *Trox sp.*	*Euspilotus assimilis* *Oiceoptoma noveboracense* *Onthophagus hecate* Silphidae (larvae) Staphylinidae
Diptera	*Cochliomyia macellaria* *Phaonia spp.* *Pherbellia sp.* *Phormia regina*	*Cochliomyia macellaria* *Lucilia coeruleiviridis* *Lucilia sericata* *Phaonia spp.* *Phormia regina*
Hymenoptera	Formicidae unknown	Formicidae unknown
Lepidoptera	unknown (larvae)	none
Myriapoda	unknown	none
Orthoptera	Gryllidae unknown	none

Table 8: Forensically important specimens captured per trap in Spring 2019

Bait	Insect	Total Captured	Mean ± Standard Error
Dimethyl disulfide	*Phormia regina*	21	3.5 ± 2.78
	Creophilus maxillosus	1	0.16 ± 0.16
	Trox sp.	30	5 ± 3.06
	Staphylinidae	1	0.16 ± 0.16
Dimethyl trisulfide	*Phormia regina*	5573	928.8 ± 588.2
	Cochliomyia macellaria	7	1.16 ± 0.98
	Creophilus maxillosus	67	11.12 ± 5.99
	Oiceoptoma noveboracense	7	1.16 ± 0.83
	Necrophila americana	6	1 ± 0.68
	Euspilotus assimilis	10	1.67 ± 0.84
	Trox sp.	3	0.5 ± 0.34
	Staphylinidae	11	1.83 ± 1.17

Figure 8 : NMDS plot depicting the six chemicals deemed different from their negative controls. Points represent field sites.

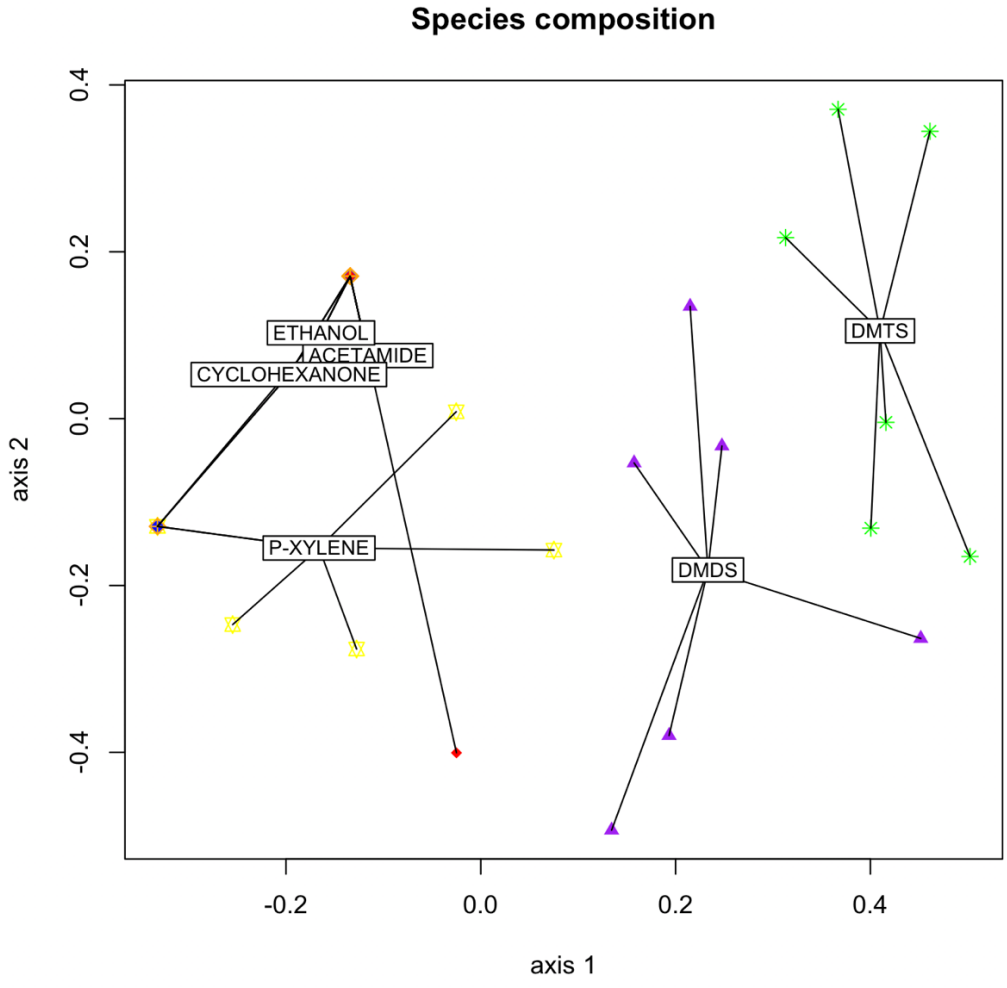
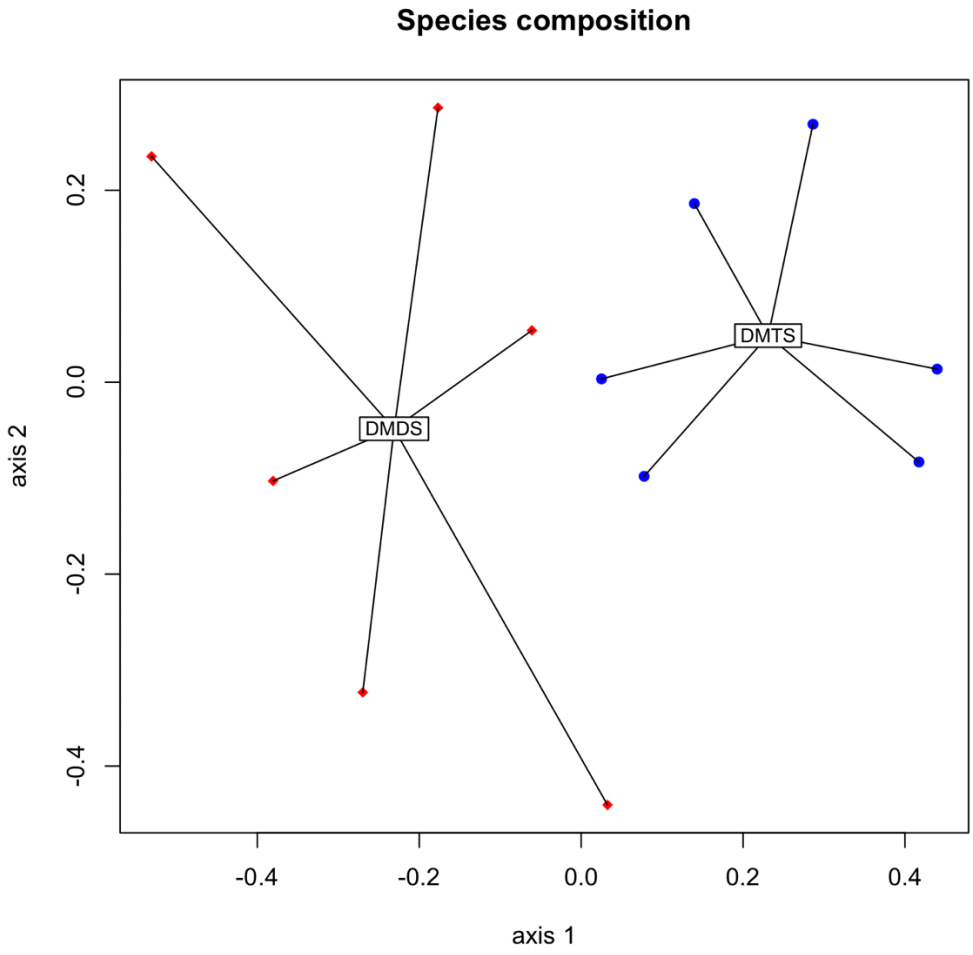


Figure 9: NMDS plot depicting the two chemicals that attracted forensically important insects. Points represent field sites.



3.5 References

Amendt, J., Zehner, R., Johnson, D. G., & Wells, J. (2010). Future Trends in Forensic Entomology. *Current Concepts in Forensic Entomology*, 353-368. doi:10.1007/978-1-4020-9684-6_16

Anderson, G. S. (2001a). Insect succession on carrion and its relationship to determining time of death. In JH Byrd and JL Castner (Eds) *Forensic entomology: the utility of arthropods in legal investigations* (143-175). Boca Raton: CRC Press.

Anderson, M. J. (2001b). A new method for non-parametric multivariate analysis of variance. *Austral Ecology, 26*(1), 32-46. doi:10.1111/j.1442-9993.2001.01070.pp.x

Armstrong, P., Nizio, K. D., Perrault, K. A., & Forbes, S. L. (2016). Establishing the volatile profile of pig carcasses as analogues for human decomposition during the early postmortem period. *Heliyon, 2*(2). doi:10.1016/j.heliyon.2016.e00070

Borror, D. J., Triplehorn, C.A., & Johnson, N. F. (1989). *An introduction to the study of insects*. Philadelphia: Saunders College.

Boumba, V. A., Ziavrou, K. S., & Vougiouklakis, T. (2008). Biochemical pathways generating post-mortem volatile compounds co-detected during forensic ethanol analyses. *Forensic Science International, 174*(2-3), 133-151. doi:10.1016/j.forsciint.2007.03.018

Brasseur, C., Dekeirsschieter, J., Schotsmans, E. M. J., de Koning, S., Wilson, A. S., Haubruge, E., & Focant, J. F. (2012). Comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry for the forensic study of cadaveric volatile organic compounds released in soil by buried decaying pig carcasses. *Journal of Chromatography A, 1255*, 163-170. doi:10.1016/j.chroma.2012.03.048

Byrd, J.H. & Castner, J.L. (2001). Insects of Forensic Importance. In JH Byrd and JL Castner (Eds) *Forensic entomology: the utility of arthropods in legal investigations* (143-175). Boca Raton: CRC Press.

Campobasso, C. P., Di Vella, G., & Introna, F. (2001). Factors affecting decomposition and Diptera colonization. *Forensic Science International, 120*(1-2), 18-27. doi:10.1016/s0379-0738(01)00411-x

Davis, T. S., Crippen, T. L., Hofstetter, R. W., & Tomberlin, J. K. (2013). Microbial Volatile Emissions as Insect Semiochemicals. *Journal of Chemical Ecology, 39*(7), 840-859. doi:10.1007/s10886-013-0306-z

DeGreeff, L. E., & Furton, K. G. (2011). Collection and identification of human remains volatiles by non-contact, dynamic airflow sampling and SPME-GC/MS using various sorbent materials. *Analytical and Bioanalytical Chemistry, 401*(4), 1295-1307. doi:10.1007/s00216-011-5167-0

Dekeirsschieter, J., Stefanuto, P. H., Brasseur, C., Haubruge, E., & Focant, J. F. (2012). Enhanced Characterization of the Smell of Death by Comprehensive Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry (GCxGC-TOFMS). *Plos One, 7*(6). doi:10.1371/journal.pone.0039005

Dekeirsschieter, J., Verheggen, F. J., Gohy, M., Hubrecht, F., Bourguignon, L., Lognay, G., & Haubruge, E. (2009). Cadaveric volatile organic compounds released by decaying pig carcasses (Sus domesticus L.) in different biotopes. *Forensic Science International, 189*(1-3), 46-53. doi:10.1016/j.forsciint.2009.03.034

Evans, A. V. (2014). *Beetles of Eastern North America*. Princeton: Princeton University Press.

Frederickx, C., Dekeirsschieter, J., Verheggen, F. J., & Haubruge, E. (2012). Responses of Lucilia sericata Meigen (Diptera: Calliphoridae) to Cadaveric Volatile Organic Compounds*. *Journal of Forensic Sciences, 57*(2), 386-390. doi:10.1111/j.1556-4029.2011.02010.x

Hoffman, E. M., Curran, A. M., Dulgerian, N., Stockham, R. A., & Eckenrode, B. A. (2009). Characterization of the volatile organic compounds present in the headspace of decomposing human remains. *Forensic Science International, 186*(1-3), 6-13. doi:10.1016/j.forsciint.2008.12.022

Huckett, H.C. and Vockeroth, J.R. (1981). Muscidae. In JF McAlpine (Ed) *Manual of Diptera Vol. 2* (1115-1132). Ottawa: Research Branch, Agriculture Canada.

Kalinova, B., Podskalska, H., Ruzicka, J., & Hoskovec, M. (2009). Irresistible bouquet of death-how are burying beetles (Coleoptera: Silphidae: Nicrophorus) attracted by carcasses. *Naturwissenschaften, 96*(8), 889-899. doi:10.1007/s00114-009-0545-6

Kruskal, J. B. (1964). Multidimensional-scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika, 29*(1), 1-27. doi:10.1007/bf02289565

LeBlanc, H. N. (2008). Olfactory stimuli associated with the different stages of vertebrate decomposition and their role in the attraction of the blowfly Calliphora vomitoria (Diptera: Calliphoridae) to carcasses. The University of Derby for the Degree of Doctor of Philosophy.

LeBlanc, H. N., & Logan, J. G. (2010). Exploiting Insect Olfaction in Forensic Entomology. *Current Concepts in Forensic Entomology*, 205-221. doi:10.1007/978-1-4020-9684-6_11

Liu, W. Q., Longnecker, M., Tarone, A. M., & Tomberlin, J. K. (2016). Responses of Lucilia sericata (Diptera: Calliphoridae) to compounds from microbial decomposition of larval resources. *Animal Behaviour, 115*, 217-225. doi:10.1016/j.anbehav.2016.

McAlpine, J. F. (1981). Key to Families – Adults. In JF McAlpine (Ed) *Manual of Nearctic Diptera Vol. 1* (89-105). Ottawa: Research Branch, Agriculture Canada.

Mohr, R. M., & Tomberlin, J. K. (2015). Development and validation of a new technique for 03.022 estimating a minimum postmortem interval using adult blow fly (Diptera: Calliphoridae) carcass attendance. *International Journal of Legal Medicine, 129*(4), 851-859. doi:10.1007/s00414-014-1094-x

Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R. B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., & Wagner, H. (2019). vegan: Community Ecology Package. R package version 2.5-5. https://CRAN.R-project.org/package=vegan

R Development Core Team (2019). R: A language and environment for statistical computing. R 243 Foundation for statistical Computing, Vienna, Austria. Retrieved from http://www.R-project.org

Sharma, R, Garg, RK, & Gaur, JR (2015). Various methods for the estimation of post mortem interval from Calliphoridae: A review. *Egyptian Journal of Forensic Sciences*, *5,* 1-12.

Statheropoulos, M., Spiliopouiou, C., & Agapiou, A. (2005). A study of volatile organic compounds evolved from the decaying human body. *Forensic Science International, 153*(2-3), 147-155. doi:10.1016/j.forsciint.2004.08.015

Statheropoulos, M., Agapiou, A., Spiliopouiou, C., Pallis, G. C., & Sianos, E. (2007). Environmental aspects of VOCs evolved in the early stages of human decomposition. *Science of the Total Environment, 385*(1-3), 221-227. doi:10.1016/j.scitotenv.2007.07.003

Statheropoulos, M., Agapiou, A., Zorba, E., Mikedi, K., Karma, S., Pallis, G. C., .Spiliopoulou, C. (2011). Combined chemical and optical methods for monitoring the early decay stages of surrogate human models. *Forensic Science International, 210*(1-3), 154-163. doi:10.1016/j.forsciint.2011.02.023

Vass, A. A., Barshick, S. A., Sega, G., Caton, J., Skeen, J. T., Love, J. C., & Synstelien, J. A. (2002). Decomposition chemistry of human remains: A new methodology for determining the postmortem interval. *Journal of Forensic Sciences, 47*(3), 542-553.

Vass, A. A., Smith, R. R., Thompson, C. V., Burnett, M. N., Dulgerian, N., & Eckenrode, B. A. (2008). Odor analysis of decomposing buried human remains. *Journal of Forensic Sciences, 53*(2), 384-391. doi:10.1111/j.1556-4029.2008.00680.x

Vass, A. A. (2012). Odor mortis. *Forensic Science International, 222*(1-3), 234-241. doi:10.1016/j.forsciint.2012.06.006

Villet, M. H., Richards, C. S., & Midgley, J. M. (2010). Contemporary Precision, Bias and Accuracy of Minimum Post-Mortem Intervals Estimated Using Development of Carrion-Feeding Insects. *Current Concepts in Forensic Entomology*, 109-137. doi:10.1007/978-1-4020-9684-6_7

von Hoermann, C., Steiger, S., Muller, J. K., & Ayasse, M. (2013). Too Fresh Is Unattractive! The Attraction of Newly Emerged Nicrophorus vespilloides Females to Odour Bouquets of Large Cadavers at Various Stages of Decomposition. *Plos One, 8*(3), 11. doi:10.1371/journal.pone.0058524

Wilson, D. S., & Knollenberg, W. G. (1984). Food discrimination and ovarian development in burying beetles (Coleoptera-Silphidae-Nicrophorus). *Annals of the Entomological Society of America, 77*(2), 165-170. doi:10.1093/aesa/77.2.165

Whitworth, T. (unpublished). Blow flies of North America: keys to the subfamilies, genera, and selected species of Calliphoridae with emphasis on species of forensic importance.