Electrophysiological responses of *Chrysomya rufifacies* (Diptera: Calliphoridae) to active volatile organic compounds released by human and pig decomposition

By

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Abstract

Forensic entomology is an important discipline which utilizes the developmental and behavioural patterns of insects which colonize decomposing tissue in a medicolegal context, most commonly to determine the post-mortem interval (PMI). *Chryosmya rufifacies* (Macquart) (Diptera: Calliphoridae) is a secondary colonizer of human decomposition in North America and its predatory behaviour can affect successional data, and therefore alter PMI estimations. Determining the specific volatile organic compounds which induce a response in *C. rufifacies* could mitigate the effects of this predatory species by providing empirical indications of the behaviourally active compounds released by decomposition. The specific compounds which cause a response in this species were isolated and identified via GC-MS, electroantennography (EAG) and GC-EAG. Electrophysiologically active volatile organic compounds (VOCs) derived from human and pig decomposition were analysed and compared, indicating that pigs are an acceptable human analogue. Six EAG-active compounds were identified via coupled GC-EAG of the VOC samples; BAME, DMDS, DMTS, ethanol, indole and phenol. Dose response testing was conducted, confirming DMTS and BAME as EAG-active compounds. Ethanol was determined to not be EAG-active in *C. rufifacies*.

Keywords: *Chrysomya rufifacies*, VOCs, decomposition, electroantennography, human analogue, GC-EAG, dose response

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BAME	Butanoic acid, methyl ester
CASC	Continuous Airflow Stimulus Controller
DMDS	Dimethyl disulfide
DMTS	Dimethyl trisulfide
EAG	Electroantennography
GC-MS	Gas chromatograph-mass spectrometer
IDAC	Intelligent Data Acquisition Controller
PCA	Principal Component Analysis
PMI	Post-Mortem Interval
ORN	Olfactory Receptor Neuron
VOC	Volatile Organic Compound
FID	Flame Ionization Detector

CHAPTER 1: Introduction

1.1 Forensic Entomology: History

Forensic entomology is the study of insects and other arthropods in the context of a medico-legal investigation (Hall, 2001; Amendt et al, 2004). It is an important discipline that makes use of the ubiquitous nature of insects, their discrete and predictable life cycles and development, their affinity to create habitats in and feed from decomposing organic matter (such as a human cadaver), and the predictable patterns with which different species of insects will colonize said matter (Amendt et al., 2004). Forensic entomology is one of the earliest recorded sciences to be used in the aid of a criminal investigation. A treatise by Sung Tz'u, named "The Washing Away of Wrongs" from the 13th century (translation by McKnight, 1981) discusses the use of insects to investigate a crime in the 13th century. A murder in a small village that was determined to have been committed using a sickle. The investigators ordered every person who owned a similar blade bring it into public and lay them on the ground. The perpetrator of the murder had cleaned the weapon, but not well enough to erase all traces of blood and tissues (Amendt et al., 2004). During the examination of the weapons, flies began to swarm the murder weapon, which the murderer felt was a divine indictment and subsequently confessed (Anderson, 2009). This account shows one of the oldest uses of forensic entomology on record (Amendt et al., 2004).

Despite these early reports, forensic entomology did not emerge as published scientific research until the late 1800s with the work of Jean Pierre Mégnin (Anderson, 2009). Mégnin was the first to record the observation of different successional waves of insects arriving at and colonizing, by laying eggs and thus introducing offspring to, decomposing tissue in a predictable pattern. Two Canadian scientists named Wyatt Johnston and Geoffrey Villeneuve were inspired by the work of Mégnin and furthered his research in 1897, in a climate similar to that of France

where Mégnin's work was completed. Johnston and Villeneuve were some of the first researchers to use the predictable successional patterns to help determine the length of time in which a person had been deceased (Benecke, 2001). Despite this early Canadian work in forensic entomology, this discipline was not commonly used in North America in the following several decades. Forensic entomologists have only been routinely asked to give their expertise in medico-legal investigations in the past thirty years or so, even though it was common practice in Europe for the majority of the 20th century (Anderson, 2009). Modern forensic entomology can have several applications to medico-legal investigations, as insects can begin to colonize decomposing tissue in a matter of minutes (Archer & Elgar, 2003).

1.2 Blow Fly Development

Fly species have discrete life cycle stages including eggs, larvae, pupae and adults (Figure 1).

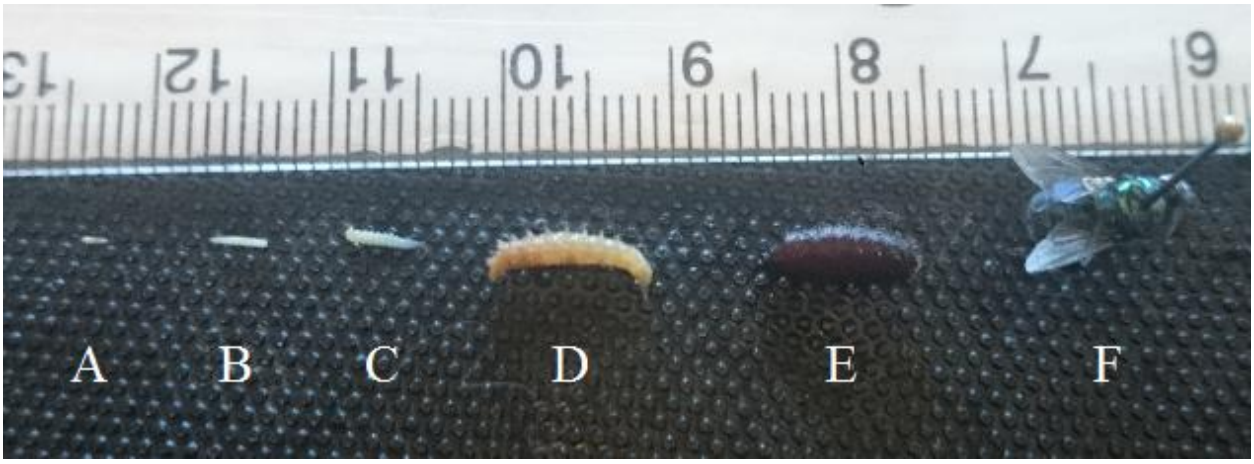

Figure 1: Life cycle stages of *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae), represented by preserved individuals. (A) egg (B) 1st instar (C) 2nd instar (D) 3rd instar (E) pupa and (F) adult fly. © S. Kolodij

The amount of time a species spends in each stage of their life cycle is heavily dependent upon temperature as well as species. A higher temperature results in a shorter growth cycle, as blowflies are cold blooded and, within species-specific limits, grow more quickly in warmer environments. Once the first instars emerge from the eggs, they require a liquid food source as they do not possess mouth parts that are capable of rending solid food. The larvae will molt

twice, into second and third instar larvae, and will develop a more voracious appetite with each developmental stage, until they complete the third instar stage and prepare for pupation (Amendt et al., 2011). Blowfly species only consume dead tissue during their larval stages, in order to build up energy and protein stores to prepare for pupation. The period of time between active feeding and pupation is called post-feeding. Once larvae have entered the post-feeding stage of their development, they leave their food source by burrowing into the ground, or behind objects such as refrigerators or sofas in an indoor environment. This is done in order to better guard the larvae from predators as they begin the non-motile stage of development and can no longer escape predation. The larval bodies begin to shorten and harden until the pupa inside is covered in a hard outer layer, called the puparium. When the flies have completed metamorphosis, the adults emerge from the puparia, leaving pupal cases behind as evidence of their presence. Once these pupal casings are located, it is an indication that the primary colonizers have completed their life cycle (Amendt et al., 2007).

Natural selection has acted strongly in favour of those female flies that are able to find a high quality food source for their offspring, leading to the efficiency with which gravid (pregnant or egg-carrying) females are able to locate wounds and open orifices in which to oviposit (Archer & Elgar, 2003). Blowfly females have to be efficient in locating mucosal membranes and blood, as upon emerging from the egg, fly larvae do not have strong enough mouthparts to be able to rend and consume solid flesh and therefore require a liquid source of protein such as mucus in the mouth, nose and eye areas or blood from wounds (Amendt et al., 2004). Adult female flies that have not yet mated or become gravid also require a protein meal in order to begin vitellogenesis (the formation of oocytes), and are also therefore strongly attracted to decomposition even prior to mating (Hayes, et al., 1999). Once adult females have secured a

protein meal, their responses to decomposition are markedly diminished until such a time as they become gravid and need to locate a food source for their offspring, wherein responses to carrion once again increase and drop off again post-oviposition (Hayes et al., 1999). Males do not show as strong of an attraction to protein sources as females do, but are still attracted by and will occur on decomposing tissue for the purposes of finding a mate (Spradberry, 1979).

1.3 Olfaction

Chemoreception in adult flies is necessary for the detection of food sources, the determination of suitable oviposition sites, and the location of mates, and is therefore extremely important for the survival of individual flies (Sukontason, et al., 2005). Blowflies receive chemical signals through olfactory receptor neurons (ORNs), which are primarily located in the third antennal segment (Vosshall & Stocker, 2007). It is through these highly sensitive and specialized chemosensory receptors that densely populate the antennal surface that blowflies and other forensically important species collect sensory information that mediates rapid location and colonization of decomposing tissue (Sukontason, et al., 2005).

Dipteran antennae are comprised of three different sections; the scape, which is the smallest relative segment and closest to the head; the pedicel, which is approximately twice the size of the scape; and the funiculus (also known as the third antennal segment) which is approximately three times larger than the other two components combined and contains the majority of the chemosensory receptors. The arista is a projection from the third antennal segment which is filamentous, thin, long relative to the other antennal components and attached to the funiculus laterally (Figure 2) (Sukontason, et al., 2007). The funiculus is covered in sensilla, which have a cuticular component which appear as tiny hairs and function to contain and protect the ORNs housed within from damage. The ORNs exist within the cuticle, with up to

four neurons per hair, wherein the sensory dendrite of an ORN extends into the shaft of the cuticle. The entire sensory unit is called a sensilla (Sukantason et. al, 2004). The individual sensilla are electrically insulated from each other via the secretion of sensillum lymph from support cells which surround the ORNs (Vosshall & Stocker, 2007). In blowflies, there are variations in the type of sensilla located on different antennal segments; the scape features only trichoid sensilla; the pedicel is populated by trichoid sensilla as well as styloconic sensilla; and the flagellum contains basiconic sensilla, coeloconic sensilla and sensory pits (Sukantason et. al, 2004). This variation in the location and clustering of sensilla on the antennae is important to note, as literature has shown differing responses in insect species to increasing concentrations of compounds (Wright & Smith, 2004). That is, some chemosensory organs will only respond to lower concentrations of stimuli, whereas others will only respond to higher concentrations, and it has been hypothesized that this leads to the activation of different combinations of receptor types depending upon the concentration of the compound or compounds of interest (Wright & Smith, 2004). It was discovered by Dietrich Schneider in 1957, that due to the dense population of sensilla and ORNs on the third antennal segment, when a stimulus is directly applied to the antenna, there is a measurable voltage fluctuation that occurs between the tip and the base of the funiculus, which forms the basis of electroantennographical investigation (Roelofs, 1984).

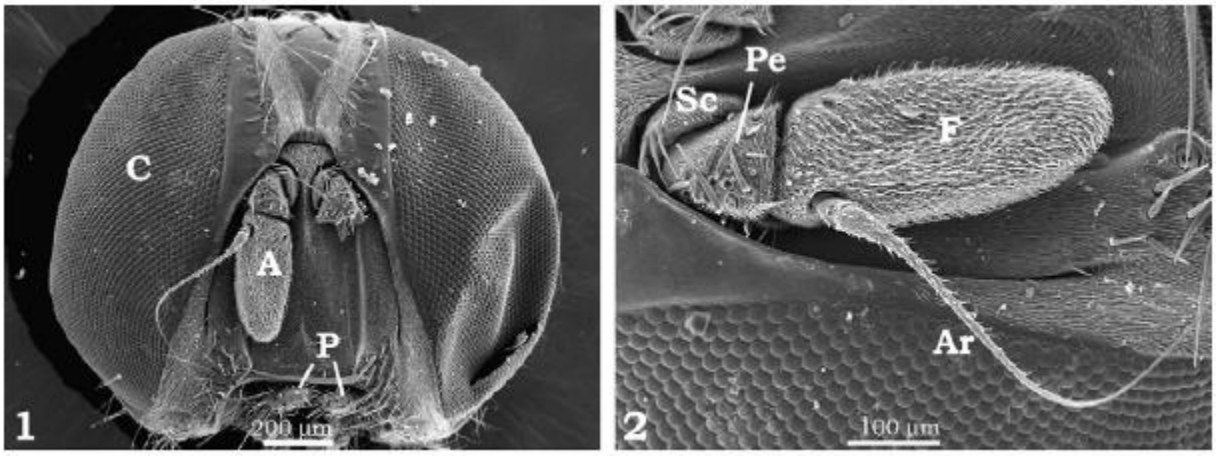

Figure 2: Scanning electron micrographs from Sukontason, et al., 2007. 1) Head of *Hydrotaea (= Ophyra) chalcogaster* featuring compound eyes (c), antenna (a) and palps (p). 2) Antenna of *H. chalcogaster* showing scrape (Sc), pedicel (Pe), funiculus (F) and arista (Ar).

1.4 Decomposition

Gravid female blowflies will utilize olfactory signals to locate appropriate food sources for their necrophageous offspring, commonly leading to the colonization of decomposing mammals and other decomposing organic matter (Amendt et al., 2007). Decomposition occurs in five stages; fresh, bloat, active decay, advanced decay and dry/remains (Payne, 1965). Originally the dry and remains stages were classified as different stages but have since been combined due to a lack of obvious differentiation of these stages (Anderson & VanLaerhoven 1996). Fresh decomposition occurs immediately following death and involves chemical changes within the body that result in rigor mortis, livor mortis and breakdown of the structural integrity of the cells. Decomposition is first driven by the chemically induced autolysis of cells which is induced by the absence of oxygen (anoxia) within cells. Anoxia results in a decline in pH, which in turn causes the disgorgement of hydrolytic cellular enzymes which denature the cellular membrane and results in apoptosis (Gill-King, 1997). The compounds released during this stage attract primary colonizers, even though there are few visual cues to indicate that decomposition is occurring (Anderson & VanLaerhoven 1996). Next the body will enter the bloat phase of decomposition,

where the anaerobic environment created by autolytic cell processes allows for the rapid growth of anaerobic microbial communities in the body. The microbiota of the bowel will begin to degrade and metabolize the lipids, proteins and carbohydrates in the body tissues, producing gaseous waste such as carbon dioxide and hydrogen sulfide (Gill-King, 1997). These processes cause gases to build up in the abdominal cavity and leads to a distended or bloated appearance of the decomposing body which characterizes this phase. Microbial activity also produces the detectable odour changes associated with putrefaction, and therefore the release of volatile organic compounds into the surrounding area, which are hypothesized to attract necrophagous insects to colonize the decomposing tissues (Gill-King, 1997). As the tissues are consumed by maggots and other necrophagous organisms, the body enters into the active decay stage, where liquefied tissues exit the body and contribute to the loss of mass caused by insect consumption. Insect activity decreases significantly during the next stage, advanced decay, because the majority of the nutritious tissues have been consumed or degraded by this point in time. The last stage of decomposition is the Dry/remains stage where only the inedible and dry components of the body such as bones and cartilage remain, and necrophagous insect activity has all but ceased as there is no longer any food source for the growth of larvae (Micozzi, 1991; Bass, 1997; Janaway et al., 2009). Insect activity on carrion tissue has a significant effect on the rate of decomposition of that tissue, as a majority of the remains can be removed from the initial site within 6 days post mortem when insects are allowed to colonize, whereas tissue from which insects have been excluded can retain its form for several months (Payne, 1965).

Temperature is an influential factor in the rate of decomposition of organic matter, wherein higher temperatures induce a faster rate of decay and lower temperatures can slow or stop decomposition. Higher temperatures will induce more rapid growth of the anaerobic

bacteria which facilitate putrefaction, as well as increasing the growth and consumption rate of necrophagous organisms such as blow flies. Due to the temperature fluctuations that can occur across seasons, geographical areas, and years, accumulated degree days (ADD) are commonly used to more accurately compare decomposition across varying environments and study conditions. ADD is a summation of the average daily temperature, in degrees Celsius, across the days that decomposition has occurred (Vass, et al., 1992; Vass, et al., 2002). ADD is presented as a score for comparison wherein, for example, one subject could take approximately 5 days to reach an ADD score of 75 if the average daily temperature is 15˚C, while another subject would take 15 days to achieve the same score at an average daily temperature of 5˚C. This was of expressing the amount of thermal energy available in the environment allows for a more reliable discussion of the similarities and differences between different types of decaying organic matter, as well as the VOCs produced thereby.

1.5 Volatile Organic Compounds

As decomposition of organic matter proceeds, volatile organic compounds are produced and released into the surrounding environment via the degradation of the basic components of bodily tissues. These VOCs are detected by the chemosensory organs of blowflies which will then colonize the decomposing tissue, as it is an optimal food source for developing larvae and essential to the offspring's survival. Blow flies locate these protein at very low concentrations sources primarily via olfaction (Archer & Elgar, 2003).

The VOC profile released during decomposition can change in composition over the course of the different stages of decomposition. This is because different components of the body will decompose at different rates, and the rate depends on temperature and other environmental factors (Archer & Elgar, 2003). For example, early stage decomposition VOCs mainly consist of

phosphorous compounds due to the breaking down of the proteinaceous structures in the body. When the proteins have all been degraded, the chemical profile of the VOCs will alter reflecting this change (Archer & Elgar, 2003). It is therefore important to study the composition of the VOCs released during the different decomposition stages, as studying only the VOCs from a single time point will not yield a proper understanding of the full range of compounds which might be produced, and therefore which compounds might attract insects to decomposition.

Much of the literature on blowfly response to VOCs makes use of pig carcass (*Sus scrofa*) VOCs, such as presented in Frederickx, et al. (2012). Pigs are a convenient replacement for human decomposition due to the physical and physiological similarities between pigs and humans. Pigs have similarly hairless skin and share a similar fat distribution, which allows for insects to behave closely to how they would with a human carcass. Further, pigs and humans share similar gut flora and similar average abdominal lengths, which means that the stages of decomposition in pigs follow a similar pattern to human decay (Borel, 2013). Due to the similarities of human and pig decomposition, pig VOCs are seen as an acceptable analog to stimulate necrophagous insect behaviour. However, it is important to explore the differences between human and pig decomposition empirically, in order to determine how accurately pig decomposition reflects human decomposition. This is especially important given the malleable nature of successional data and insect behaviour. Based on the shifting chemical profiles of VOCs and the predictable patterns of succession shown in the behaviour of insects colonizing decomposing tissue at different times, it stands to reason that different insects will be attracted to different chemical profiles in the VOCs released by decomposition (Archer & Elgar, 2003). These VOCs can be collected using air sampling techniques such as sorbent tubes in conjunction with air pumps to collect the air surrounding the decomposing tissue (LeBlanc & Logan, 2010).

1.6 The Post-Mortem Interval: Insect Aging and Succession

The phenomenon of blowflies detecting decomposition via olfactory reception of VOCs is what leads to the location and colonization of decomposing tissue. Since colonization can occur within minutes of the cessation of cellular function, the most important use of forensic entomology is the determination of time since death (Amendt et al., 2011). Forensic entomology is currently the only accurate method to determine the amount of time since death once 72 hours have elapsed (Amendt, et al., 2011). Before this time, a pathologist will use biological indicators of time of death such as rigor mortis and livor mortis (Amendt, et al., 2011). Forensic entomologists most commonly look for the presence and life cycle stages of necrophagous insects (insects that consume dead flesh) on a corpse, in the context of a medico-legal investigation, in order to determine how long a cadaver has been deceased. This is referred to as the post-mortem interval or PMI (Amendt et al., 2007). Determining an accurate PMI is important to investigative work for several reasons, such as assisting to determine the validity of the alibi of a suspect, as the date and time of a murder will drastically change the story of the suspect as to their whereabouts. The PMI can also corroborate or completely discredit witnesses, by having direct forensic evidence to determine the last time a victim could have been seen alive. Forensic entomology is also used in cases of insurance coverage, and whether or not the deceased person was still covered under their policy at the time of their death and how much an insurance company will pay the surviving family, if that is the case. Further, an accurate PMI estimation can give peace of mind to the families of missing persons, especially if the person was missing for a long period of time before their body is discovered. Knowing as much as possible about the events and timing leading up to the death of a loved one can provide untold comfort to grieving families (Anderson, 2009).

The PMI is determined using a number of factors, including an identification of the species present, and the determination of the life stage of the insects on and around the body.

Samples must be taken from all areas of the body that has been colonized, as different species could have colonized different orifices or wounds at different times. The individual insects representing the oldest life stage present on the body are considered the primary colonizers, as those individuals will have been deposited, as eggs or larvae, on the body first. It is important to know the species in question as different species have different timeframes within which they complete each stage of their life cycle (Amendt et al., 2007). These developmental timelines are heavily reliant upon temperature, which will determine and can help predict the developmental rate of insect species, within the individual species' own developmental pattern. Due to the immense effect that temperature has on the growth rates of most insects, meteorological data is collected for the location of the body in order to compare that, and the life stage, to current knowledge of developmental rates of the insect in question (Amendt, et al., 2011). Once the primary colonizer has been identified to the species level, the life cycle stage of the specific insects found at the scene needs to be determined. Estimation of the minimum amount of time that tissue has been colonized can be estimated with this information, and therefore PMI can also be estimated.

Once the full life cycle of a primary colonizing species has been completed, however, other techniques must be utilized to estimate the PMI. Flies that have reached the adult stage cannot be accurately attributed to the body, as there is no evidence to indicate whether that adult developed on the body; was attracted to the body as an adult; or arrived there by chance. Therefore, the only indication that the primary colonizing species has reached adulthood is the presence of pupal casings in the area around the body, which are the hard outer casings that the pupae inhabit while completing the transformation to adulthood. If this stage has been reached, a forensic entomologist must also use successional data to make an estimation of the PMI.

Succession refers to the order in which different species of insect colonize or are attracted to a corpse (Schoenly et al. 1996). These data are comprised of the predictable pattern of insects that colonize decomposing tissue in succession in different geographic regions and in different conditions. Decomposing bodies undergo rapid changes that provide significantly different growth environments for insects as time and decomposition progresses. This shifting environment causes different insects to be attracted to decomposition at different stages, and it has been hypothesized that it is due to the olfactory cues released by the tissues and fluids associated with decomposition (Amendt et al., 2004). Species can be considered primary or secondary colonizers depending on when they arrive to lay eggs on decomposing tissue. These successional waves were documented and studied in forensic entomology as early as the late 1800s. However, these patterns are affected by many environmental factors such as climate, weather, urban development and geographical features, and can therefore be difficult to predict without consistent study of these patterns. Different species can change their roles depending on the geographic region, the insect community composition and the various aforementioned environmental factors. For example, *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) is a primary colonizer in Thailand, but a secondary colonizer in Texas (Sukontason et al., 2005 & Tomberlin et al., 2006). Further, the species *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) has been confirmed as a primary colonizer in a study performed in the Yukon territories, whereas this species has not previously shown to be a dominant presence in decomposition studies, much less a primary colonizer anywhere else in North America (Bygarski & LeBlanc, 2013). The main reason for a difference in colonization behaviour across geographical areas alongside insect community composition is temperature and climate. As in the case of *C. rufifacies*, Thailand is more consistently warmer and humid than Texas, which

makes Texas a less than ideal environment for the development of a foreign species such as *C. rufifacies,* which originated in the Australasian region of the world, and thrives in high heat and humidity.

Reliable successional data for a specific geographical region, and its local weather and climate, are needed to predict the timing of when certain insects will occur at a crime scene. Based on this, and the age determination of the secondary colonizers found on the body, an estimate of the PMI can be reached. Due to the possible alterations that can occur in these patterns due to climate change or invasive species, it is exceedingly important to have continual study of what affects successional patterns of colonization. Regular updates to successional data are needed in order to maintain accurate information, so as not to produce an inaccurate PMI estimation as, unfortunately, successional data is less accurate than the aging of primary colonizers when trying to determine the PMI (Amendt et al. 2007).

It is important to recognize that these methods provide an estimate of the minimum PMI. because even though insects can colonize a body within minutes, this does not always happen as insects may not have access to a body immediately (Amendt et al., 2004). Such cases would include a body submerged in water, buried or wrapped in something airtight. Colonization of a body can also be delayed by meteorological events such as high winds and heavy rainfall, which would also affect the PMI estimation. This is a further reason why it is important for forensic investigators to collect meteorological data from the scene at which evidence was recovered for several days before and after the entomological evidence was collected.

1.7 *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae)

Chrysomya rufifacies is a forensically important blowfly species of the family Calliphoridae (Rosati & VanLaerhoven, 2007). The term "forensically important" indicates that it is a necrophagous species that is known to colonize decomposing tissue and may provide information that canbe used to calculate PMI. *C. rufifacies* is a tropical species that usually inhabits warm climates, such as the southern United States. This species originated in the Australasian region of the world and migrated into North America by way of introductions in Texas beginning in the 1980s (Byrd et al., 1996; Rosati & VanLaerhoven, 2007). Its territory has been steadily expanding northward, to the point where it was reported to exist in Southern Ontario in recent years (Rosati, & VanLaerhoven, 2007). Its territory is expected to expand even further north due to climbing temperatures attributed to climate change. It is often referred to as "the hairy maggot blowfly" due to the appearance of the larvae. During the third larval stage of *C. rufifacies*, fleshy tubercles occur on the surface of the larvae, giving it the appearance of having "fur" (Castner et al., 1996) (Figure 3).

Figure 3: 3rd instar *Chrysomya rufifacies* larvae showing indicative fleshy tubercles. © S. Kolodij

C. rufifacies is responsible for many cases of myiasis (infestation of a live mammal by fly larvae) including sheep-strike, in Australia especially (Morris, 2005). Sheep-strike can cause major economic distress in agricultural sectors by decimating livestock populations and siphoning time and resources in order to eradicate the infestation (Morris, 2005). While not immediately applicable to Canada at this point in time, it could become a larger issue as *C. rufifacies* moves farther and more permanently into Canada.

C. rufifacies is a tropical species with a lower threshold of activity and development at approximately 12˚C, optimal growth at 29.3 ˚C and a maximum growth temperature of 31.5˚C (O'Flynn, 1983; Baumgartner, 1993). Therefore, this species remains primarily active in the warm summer months in Canada (Cammack et al., 2010). This species undergoes a full life cycle of development from egg to adult from between 190 to 598 hours (O'Flynn, 1983; Byrd et al., 1996). The timing is dependent upon the temperature, with higher temperatures corresponding with shorter development times, as is common in most insect species. Once the female lays eggs on a food source, the first instar larvae eclose after approximately 26 hours at optimal growth temperatures. *C. rufifacies* undergoes three instar stages of larval development before reaching pre-pupal stage and moving away from the food source after approximately 60 hours at optimal growth temperatures. This post-feeding stage lasts 36 hours at optimal temperatures as the larval body shortens in preparation for the outer cuticlar layers to harden into a puparium. The pupal stage follows then, and continues for approximately 72 hours at optimal temperatures before the adults emerge. The adults will then mate between 3 to 7 days after emerging from the puparium, and the females will lay eggs on a suitable food source at approximately 5 days post-mating (O'Flynn, 1983; Byrd et al., 1996).

C. rufifacies is reported as being a secondary colonizer during human decomposition in North America, as it is not often the first insect to colonize decomposing tissue in this geographic area (Cammack & Nelder 2010). In scientific literature from Thailand, this species is often reported as a primary colonizer, which stresses how different climates and ecological conditions can affect entomological data (Sukontason, et al., 2005). Normally, information about secondary colonizers is used in combination with the information collected about primary colonizing species in order to accurately determine the PMI as they do not represent the oldest individuals at the scene of decomposition, but can provide useful information via succession patterns (Amendt et al., 2007; Cammack & Nelder, 2010). This often means that *C. rufifacies* is not considered as vital in forensic investigations as a common primary colonizer such as *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) would be. However, this secondary colonizer is a facultative predator in its larval stage, and has the ability to completely eradicate the older populations of primary colonizers that might be competing for the same food source, via a shortening of the larval developmental cycle and utilization of their characteristic shiny tubercles to immobilize prey larvae (Shiao & Yeh, 2008).

The appearance and emergence of *C. rufifacies* larvae can be problematic for primary colonizing insects, due to the predatory nature of this species. This trait is important in forensic investigations due to the possibility that this predatory behaviour could drastically alter the succession data from a specific corpse, especially as *C. rufifacies* acts as a secondary colonizer. If a primary species is completely cleared from the area through predation, it could render the estimation of PMI completely incorrect by hours or possibly days (Cammack & Nelder, 2010). This is extremely problematic to a medico-legal investigation.

1.8 Rationale

The VOCs which induce an electrophysiological response in *Chrysomya rufifacies* have as yet not been studied, identified or quantified. The timing of the first occurrence of this species attending decomposition is not fully understood or accurately predictable, as they have an expected fitness advantage over other species by their behaviour as a secondary colonizer as well as a predator, but have unexpectedly been shown to occur almost simultaneously with primary colonizers such as *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) (Mohr & Tomberlin, 2014). Due to the conflicting behaviour of this species, and the potentially devastating effects of its predation on other species, more empirical study of the exact compounds which might attract *C. rufifacies* needs to be completed in order to more accurately predict the precise times when this species is likely to colonize decomposing matter.

The data I collect on the olfactory stimulating compounds of this forensically important species could help to produce more accurate PMI estimations by showing the chemical profiles to which *C. rufifacies* is likely respond, that could lead to this species colonizing human decomposition, even as a secondary presence. This could be a first step to allow forensic experts to have the tools to determine an accurate time since death, even if predation of primary colonizers occurs.

1.9 Purpose

The objectives of this research are three-fold.

1. To isolate the active compounds present in the VOC profile of human and pig decomposition which cause a response in the forensically important blowfly species, *Chrysomya rufifacies* via the identification of the major compounds present in the VOCs utilizing GC-MS techniques.

2. To compare the VOC profiles of human decomposition to pig decomposition to determine the efficacy of using pigs as an analogue for human subjects. The pig subjects were included as a comparison to previous literature published on the subject of decomposition VOCs, as most literature has to do with pigs rather than humans (Bygarski, 2013; Frederickx, et al., 2012; Payne, 1965; Anderson & VanLaerhoven, 1996). This is the case because pig subjects are much more readily available for experimentation. As such, this study of human decomposition is a rare and exciting opportunity.
3. To identify isolated active compounds and confirm their identity as EAG-active candidates, as well as determine the lower and upper detection thresholds of each compound in relation to *C. rufifacies* through dose response experimentation.

CHAPTER 2: Electroantennography & Gas-Chromatography-Electroantennography Investigation to Determine Active VOC Candidates

2.1 Introduction

Insect species respond uniquely to VOCs released during decomposition. A measurement of the response of one species to a particular compound will often not be applicable to another species. It is therefore important to measure the response of a species of interest to various compounds in order to determine how that specific species responds, as the response indicated in previous studies is often not directly applicable. It is also important to note that due to physiological and behavioural differences that occur between different species of insects, compounds which are behaviourally active in one species may not be behaviourally active in another. For example, Ranger et. al, showed in 2014 that ethanol was a behaviourally active compound in honey bees, whereas Chaudhurty et al, also in 2014, utilized ethanol as a negative control due to its lack of effect on the behaviour of *Lucilia sericata.*

2.1.1 Electroantennography

An empirical recording of the responses induced by VOCs can be taken using a technique known as Electroantennography (EAG). EAG equipment measures the average voltage fluctuation which occurs across all sensilla which populate the surface of an antenna of an insect of study, as caused by exposure to an olfactory stimulus. The phenomenon of a measurable potential difference between the tip (distal or recording end) and base (proximal or indifferent end) of the antenna of an insect during stimulation was discovered by Dietrich Schneider in 1957 (Roelofs, 1984). This voltage difference on the antenna has been hypothesized to be due to the membrane depolarization that occurs when an ORN is stimulated (Roelofs, 1984). Stimulatory chemicals enter through pores which populate the cuticular surfaces of the sensilla that populate the antenna. These molecules are then transported to the dendritic membranes inside the peg or

hair generating a receptor potential along the dendrites which, if strong enough, will, in turn, stimulate the ORNs to produce action potentials (Logan et al., 2007). The voltage change in the dendritic membranes is what created the EAG response and the amplitude of the negative spike correlates with the frequency of the nerve impulses induced by the stimulus (Roelofs, 1984). The depolarizations are recorded by positioning a live antenna (i.e. recently resected from the insect) between two glass capillary electrodes which are then attached to an electrical amplifier and a recording device (Roelofs, 1984). Syntech, a company currently based in Germany, specialises in the manufacture of original laboratory equipment, such as that used in EAG experimentation. Their focus as a company is on the production of equipment that is used in the study of insect behaviourals and electrophysiological responses induced by chemical stimuli. They are currently the only manufacturer of such specialised electroantennography equipment.

The output of the recordings produced by the EAG is represented graphically, showing the response as a depolarization wave, the amplitude of which is dependent on several factors. Female blow flies often produce higher amplitude responses than males to decomposition VOCs, and more highly concentrated stimuli will also show higher responses in the insects. However, there is a saturation point that is reached when the concentration of a biologically active compound (i.e. a compound that causes a response) becomes too high for the antenna to register a response (Wright & Smith, 2004; Syntech, 2004).

When a compound is shown to consistently induce a depolarization across the antenna, that compound is considered to be "EAG-active" for that species (Cork, 1996; Gikonyo, 2003). EAG-active compounds are considered to be likely to induce a behavioural response in the species of interest. However, the precise behavioural effects of an EAG-active compound cannot be determined or characterized solely based on the incidences of depolarizations across the

olfactory cell membranes. Further testing, such as wind tunnel bioassays which measure and characterize flight patterns of live insects to determine attraction or repulsion in the presence of these chemicals, is required to elucidate their behavioural qualities. It is also important to note that due to physiological and behavioural differences that occur between different species of insects, compounds which are EAG-active in one species may not be EAG-active in another.

2.1.2 GC-MS and Coupled GC-EAG Testing

Gas chromatography (GC) is an efficient and sensitive way of separating out the components of a VOC profile. A sample is injected into the GC sample inlet/injection port, where it is then volatilized and transferred to the column in order to be separated into component parts. The column is a glass capillary tube which is coated internally with a microscopic layer that acts as a "solid phase", or "stationary phase", as this coating does not travel through the column. A non-reactive gas, such as helium, is utilized as the "gas phase" or "mobile phase" which carries the volatilized sample through the capillary, over the stationary phase. As the sample moves through the column, some compounds in the sample will adsorb (adhere) to the column walls at varying strengths, which then affects the rate at which those molecules will travel through the column (Pavia et al., 2006). This separates the sample molecules based on their retention times in the column, as the adsorbance strength is based on the chemical properties of the compounds being separated.

After the sample is separated in the GC column, the sample can be split or entirely sent to a detection apparatus such as a Mass Spectrometer (MS). A mass spectrometer takes the newly separated components from the GC and bombards the molecules with electrons. The electron flow causes the molecules to fragment into smaller components, as charged ions. These ions are processed through an electric or magnetic field, which has the effect of separating the ions based

on their mass-to-charge ratios. An electron multiplier detects the charged particles and records the information in the form of a mass spectrum. A mass spectrum is a graphical representation of the ion signal against the mass-to-charge ratio. These spectra can then be compared to previously collected data regarding the known fragmentation patterns of various compounds, thereby allowing the components of the test sample to be identified (Sparkman, 2000). The data is represented graphically as a comparison between the detector response and the retention time, showing peaks of varying sizes that represent the amount of a compound present in the sample. When a peak is selected, the ion spectrum of that peak as produced by the MS is shown and can be used to elucidate the identity of the compound producing that peak.

When this technique is coupled with electroantennographical detection, the sample is split as it exits the GC column, so that some of the sample is sent to the MS, and some is sent to the EAG. With this technique, it is possible to get a real-time recording of the insect's EAG response to different compounds as they are separated from a complex mixture in a complex mixture (Syntech, 2004). Since the split column carries half the sample through to the stimulus tube component of the EAG apparatus, the stimuli are applied to the antenna at the same time as the components are being recorded by the MS. The data produced by both the MS and EAG are represented as a function of the time at which each component was ejected from the GC column and can therefore be directly compared.

By determining which specific components of the sample VOCs coincide with a depolarization EAG response in the fly subject, it can be concluded which of those components are EAG active compounds (Syntech, 2004). This information is collected by determining if any of the times of depolarization occur in concert with the retention times and spectra produced through the GC-MS data. If they EAG and MS responses coincide, that is a possible indication

that that specific compound responsible for both is EAG-active for that species, and can be considered a candidate for affecting the insect's behaviour (Frederickx et al., 2012).

These techniques of GC-MS analysis and coupled GC-EAG testing were utilized in order to address the previously discussed objectives of isolating the EAG- active compounds present in the VOC profile of human and pig decomposition and to determine the efficacy of using pigs as an analogue for human subjects.

2.2 METHODS

2.2.1 Sample Collection

Samples of volatiles generated by human and pig decomposition were collected by Dr. Hélène LeBlanc in trials conducted in 2011 and 2012. The collection took place at the Forensic Anthropology Center at Texas State (FACTS), in San Marcos Texas, United Stated of America. Two of the three trials performed were utilized in this research, from May 2012 and November 2012, with each trial consisting of two human subjects and one porcine subject. All of the human subjects at FACTS are provided on a completely voluntary basis via the United States Universal Anatomical Gift Act (UAGA) (Stefanuto et al., 2015).

The donors for the May 2012 trial were; a 59 year old Caucasian male (herein referred to as H3) whose cause of death (COD) was anoxic encephalopathy; and a 90 year old Caucasian female (herein referred to as H4) whose COD was respiratory failure. The donors for the November 2012 trial were; a 67 year old Hispanic female (herein referred to as H5) whose COD was metastatic breast cancer; and a 57 year old Caucasian male (herein referred to as H6) whose COD was pneumonia and lung cancer. In each trial, one human cadaver was exposed to insect activity and the other was excluded from insect activity and all cadavers were unclothed. All

human, pig and control sites were separated by a minimum of 10 metres, within an open, grassy field. Control sites were ensured to be devoid of decomposing tissue. (Stadler, 2013).

The human remains decomposition trials were conducted over the course of 6 experimental days, with VOC samples taken daily. Volatile sampling was conducted with the use of a 100 cm x 70 cm x 40 cm stainless steel sampling hood placed over the pig experimental site, a sampling hood measuring 120 cm x 76 cm x 76 cm over the human experimental site for a period of 30 minutes prior to collection in order to allow accumulation of VOCs. A control site devoid of decomposing organic matter was also studied during each trial (Stadler, 2013). Collection occurred via the attachment of a sorbent tube to a sampling port on the top of the hood, which was attached to a LaMotte model BD (Chestertown, Maryland, USA) constant flow airsampling pump for 2 hours at a flow rate of 2 litres per minute (Stefanuto et al., 2015). Table 1 shows the summary of the samples utilized in this research and their source. Control samples were only collected on Day 0.

Table 1: Summary of sample sources utilized in this research. Samples which were not accessible or utilized in this research are indicated with N/A.

Subject	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Human 3	✓	✓	✓	✓	N/A	✓
Human 4	✓	✓	✓	✓	N/A	✓
Pig 2	N/A	N/A	N/A	N/A	N/A	✓
Human 5	✓	✓	✓	✓	✓	✓
Human 6	✓	✓	✓	✓	✓	✓
Pig 3	N/A	✓	✓	✓	✓	✓

The human remains and pig samples were collected using Porapak and Tenax TA sorbent tubes. The sorbent tubes contain porous polymers such as Tenax TA or Porapak Q, which adsorb the compounds without altering them in any way, keeping the samples to be tested at a later time

(McDermott, 2004). The most often used compound is Tenax, which is a polymer resin with high thermal resistance. This means that samples collected using Tenax can be desorbed using Thermal Desorption (TD), where the entire sample is heated and put through the analytical equipment at the same time, instead of breaking up the components or diluting them (McDermott, 2004). It takes less than one hour to collect samples adsorbed by Tenax polymer resin as the sample does not need to be highly concentrated in order to achieve detectable levels of compounds. A drawback to this polymer is that the samples can only be analyzed once, due to sample degradation caused by high thermal stress via TD (McDermott, 2004).

Porapak sampling takes a longer amount of time to collect, several hours as opposed to less than an hour in Tenax samples. Due to this long sampling time, the samples collected are highly concentrated. Porapak samples are not analyzed using TD methods, but are analyzed through solvent desorption. This means that the sample separates from the sorbent material by moving with the mobile solvent instead of remaining associated with the solid phase. This solvent-desorption approach allows for multiple analyses as the solvents involved do not have to be associated with the sorbent materials, which means the solvent-sample solution can be stored and used in subsequent trials (McDermott, 2004). The Porapak samples were kept in a cooled diethyl ether solvent that is highly volatile. The samples need to be regularly checked to ensure that the solvent levels are not too low, and topped up with minute amounts of diethyl ether so as to not lose the samples to evaporation, or alter the concentration of the samples.

2.2.2 Maintenance and rearing of test subjects

A corhort of several dozen *Chrysomya rufifacies* pupae were received from the laboratory of Dr. Jeffery Tomberlin at Texas A&M University, which were sent via airmail as either post-feeding third instars or early pupa, and formed the research colony. Four different cohorts were received

in total. Since this species already occurs in Canada, (Rosati & VanLaerhoven, 2007) there are no concerns regarding the invasiveness of this species. Once the pupae emerged as adults (eclosed), the adults were transferred to a mesh cage (60cm x 60cm x 75cm), supported on an aluminum frame, hereby referred to as a single unit as the colony. The colony was initially kept at room temperature, next to a window so as to achieve a natural light/dark cycle. However, it was determined that this environment was not ideal for the optimal lifespan or fecundity of *C. rufifacies* due to an observed low oviposition rate. Therefore, the colony was transferred to a vinyl walled, 6-shelved, stacked enclosure (Figure 4).

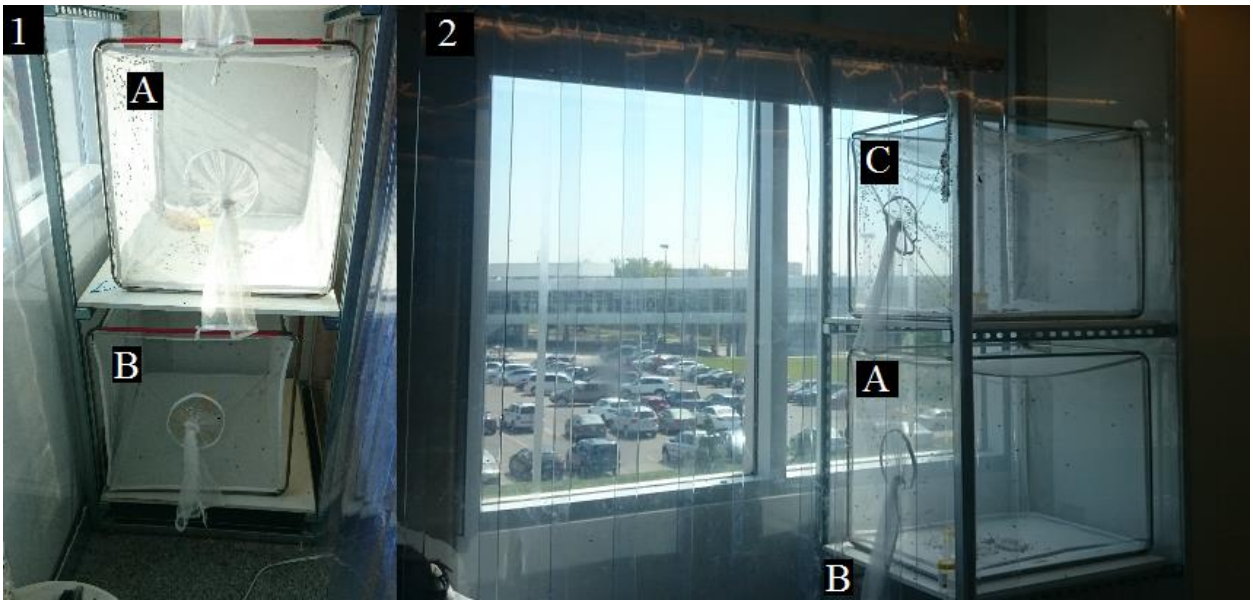

Figure 4: LeBlanc Entomology Lab Fly Enclosure. 1) Internal view of enclosure showing mating colony of *Chrysomya rufifacies* (a) and female isolation colony (b). 2) External view of enclosure showing mating (a) and female isolation (b) of *C. rufifacies* and resident colony of *Lucilia sericata* (c).

The enclosure included six shelves, three stacked on top of each other on the left and right sides of the enclosure. Each shelf contained one aluminum framed cage, to a total of six possible separate colonies co-existing within the enclosure. The structure included three clear vinyl walls, and an opening in the fourth wall that acted as an entrance to the enclosure which consisted of several strips of overlapping vinyl. The top of the enclosure is a layer of mesh

netting in order to allow a proper flow of oxygen into and out of the enclosure while preventing flies that have escaped the cages from travelling outside of the enclosure. The back wall of the enclosure is against an east-facing window, which allowed for a natural light/dark cycle.

In order to optimize the environment for the *C. rufifacies* adults, the colony was placed on the lowest shelf on the right hand side of the enclosure with a small space heater installed approximately 1 metre from the front of the colony. This increased the ambient temperature in the colony to an average of 29˚C and a relative humidity of 38%, as recorded by a HOBO Pro V2 data logger (©Onset). Once the temperature was increased, there was also an almost immediate increase in fecundity and instances of oviposition in *C. rufifacies*. In order to mitigate any drying effects from the installation of an artificial heat source, a humidifier was also installed approximately 1 metre from the front of the colony.

C. rufifacies adults were provided with clean drinking water from a potable drinking source, from a closed, clean, urine collection jar. The lid of the jar was perforated in order to allow a paper wick to passively transfer the water through capillary action to the outside of the jar. The closed jar and wick system is necessary to mitigate the risk of the adult flies falling into the water and being unable to escape before drowning. Granulated white sugar in an open petri dish was provided as a source of food, while powdered skim milk in another petri dish was provided as a continual protein source (Figure 5).

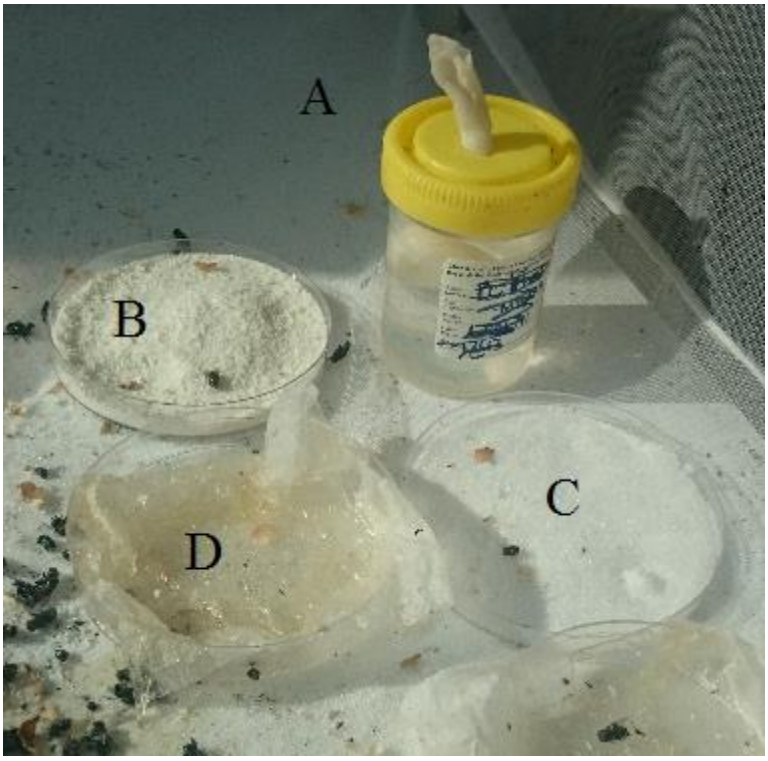

Figure 5: Nutritional sources for *C. rufifacies* colonies, showing water source in jar-and-wick system (a), protein source from skim milk powder (b), secondary carbohydrate source from granulated white sugar (c), and preferred nutrition and carbohydrate source from food-grade honey (d)

On the advice of Dr. Meaghan Pimsler from Dr. Jeffery Tomberlin's lab, these food sources were provided primarily as a secondary source of sustenance. The recommended food source was food-grade honey, which was initially presented on a lint-free laboratory wipe (Kimtech) in an open petri dish. However, as the adults aged and lost peak physical performance, they began to become stuck in the viscous honey and caused an unacceptable level of casualties. The optimal presentation of honey was determined to be between single layers of a lint-free laboratory wipe in an open petri dish that was sprayed with a small amount of water in order to decrease the viscosity of the honey and increase the ease with which the adults could consume it (Figure 5d). This proved to decrease unnecessary deaths of adult flies while still providing adequate nutrition.

Further, *C. rufifacies* showed increased longevity and overall health when provided with a more robust protein source than provided by the skim milk powder. Blood was collected from the drippings of commercially available food-grade bovine or porcine liver. The liver source was dependent upon local availability. Lint-free laboratory wipes were used to collect the blood drippings from empty packages of liver, and frozen until required. Approximately every two days, a blood-laden tissue was thawed by exposure to approximately 2 mL of boiling water in a small open petri dish. This had the dual effect of bringing the blood to a consumable temperature, while also diluting and dissociating the blood from the physical substrate. The amount of water was selected to be enough to properly moisten the wipe without flooding the petri dish and possibly causing adult casualties.

In order to maintain the population of the colony, it was important to collect and rear a new cohort of *C. rufifacies* on a regular basis. To do so, a rearing container with fresh liver was placed into the colony as needed. A rearing container consists of a clean plastic kitchen container, filled approximately half-full with pet-grade aspen or pine wood chips, which are moistened with potable water from a spray bottle. Fresh liver was added to the rearing container in a hand folded, open, aluminum foil packet that contained the food source for the duration of the larval growth period. A small amount of moistened liver was placed in the colony overnight in order to induce oviposition. The rearing container was removed from the colony when a sufficient amount of eggs were collected, usually at approximately 3 hours after sunrise. This was done in order to most efficiently collect eggs because the oviposition tended to occur in the few hours before and after sunrise, and desiccation can occur if the eggs are collected any later.

Once the eggs were established on the food source and the rearing container was collected, fresh liver was added to the aluminum packet and moistened. The food source needed

to be moist in order to provide a liquid protein source for the larvae. After approximately 26 hours, the first instar larvae emerge and begin consuming the food source. Approximately 30g of liver was added every morning at approximately the same time of day for the sake of consistency. If there were an unusually large number of larvae in a particular cohort, additional liver was provided as needed at mid-day or at sunset. The rearing container was covered with a fine, thin cheesecloth and secured with an elastic band around the rim of the container.

The rearing container was kept inside of a Caron Environmental Chamber (Model 6022) for the duration of larval and pupal development. The environmental chamber was kept at the optimal growth temperature for *C. rufifacies*, at 29.3˚C (O'Flynn, 1983; Baumgartner, 1993). Larval development lasted for approximately 2.5 days at this temperature, from first instar to third instar. The third instar larvae were the most voracious, and were the most likely to require additional liver. Once *C. rufifacies* entered the post-feeding stage of development, as evidenced by larvae abandoning the food source instead of consuming it, the aluminum packet was removed from the rearing container once any lingering individuals were relocated to the woodchips. The rearing container was kept moist during the pupation stage of growth so as to not risk desiccation of the pupae.

Once adults began to emerge from the puparia, female adults were separated from the males and placed into a separate cage. This was done via temporary anesthesia of the adults, which employed the use of pure Nitrogen gas inside of a closed container until adult movement ceased. Anaesthesia was performed in this fashion because the physiology of *C. rufifacies* as a tropical species did not allow for safe anesthesia through a cold shock as is possible in other species such as *Lucilia sericata*. Once the adults were immobile, they were quickly sorted by sex based on sexually dimorphic physical characteristics, specifically eye shape and placement.

Males and a small number of females were returned to the main cage, while the majority of females were placed into the aforementioned separate cage. This was done because only unmated females that had never received a blood meal were to be used for EAG testing. The females used for testing were between the ages of 2 to 15 days old.

2.2.3 Electroantennography testing

All coupled GC-EAG testing was completed with the use of a Varian Saturn 2000 MS and Varian 3800 GC, with an HP-1 column. Coupled GC-EAG testing is often performed with the use of a Flame Ionization Detector (FID), wherein the FID identifies the concentration of a compound within a sample via combustion (Syntech, 2004). This option was replaced with the use of mass spectrometry. The quantification of compound concentration was calculated via the use of an internal standard applied to the column simultaneously with the test samples. The selected internal standard was bromobenzene, which was chosen because it was unlikely to co-elute with EAG-active compounds, does not occur as a by-product of decomposition and is not readily available in the atmosphere (Stadler, 2013).

In order to perform coupled GC-EAG testing, a Varian Saturn 2000 GC/MS was altered to accommodate for the inclusion of the EAG equipment. The column output of the GC was diverted with the use of a press-fit glass Y-tube (©Agilent Technologies), which allowed for some of the sample to travel to the MS while the remainder exited the GC oven and was delivered to the EAG equipment. The EAG was housed in an adjoining room and, as a result approximately 2 metres of extra column were required for the sample to be directly applied to the EAG preparation from the GC oven. As gas chromatography functions based on how quickly molecules travel through the length of the column, several calculations were performed to ensure that there was as little discrepancy as possible between the retention time reported by the MS,

and the time at which the sample eluted from the column and was applied to the EAG preparation. Due to the sensitivity of the equipment involved and the complicated nature of splitting a column in the fashion that was necessary for this research, an expert from Agilent Technologies was contracted to perform the installation and alterations. Figures 29 and 30, located in Appendix A, show the experimental equipment set-up that was utilized for all electroantennography trials.

The first step of preparing an antenna for EAG testing was to decapitate a fly anesthetised with nitrogen. Once the head was excised from the body, a glass capillary filled with Ringer's solution was inserted into the fly brain via the neck foramen where the body was removed. Ringer's solution provides an isotonic environment to allow the electrical conductivity of the brain to function after it is separated from its blood source thus ensuring that the electrical impulses flow in the most natural and applicable way possible, via effective electrically indifferent contact (Hille, 1984). The capillary can also be inserted through the eye of the subject, but it was determined to be most efficient to complete the preparation as previously described. The capillary was then slid over the silver wire in the grounding (or indifferent) electrode holder. The positioning gears on the indifferent staging head were then adjusted to manipulate the subject's head into viewing position underneath the stereomicroscope. Once the head was in focus under the microscope, an entomological pinning needle was used to re-position the antennae away from the front of the head, in order to more easily establish a connection with the recording electrode. The pinning needle was also used to apply a minute amount of electrode gel to the very end of the third segment of the antenna. Spectra 360 electrode gel (Syntech) and had the effect of reducing background noise during recording. Once the test antenna was in position, the recording electrode was brought into view using the

micromanipulators on the recording electrode staging head. A recording capillary, which measured the voltage fluctuation which occurred across the third antennal segment, was carefully positioned until the end of the third antennal segment of the subject was submerged in Ringer's solution within the tip of the glass capillary tube. This was then considered a finished preparation (Figure 6).

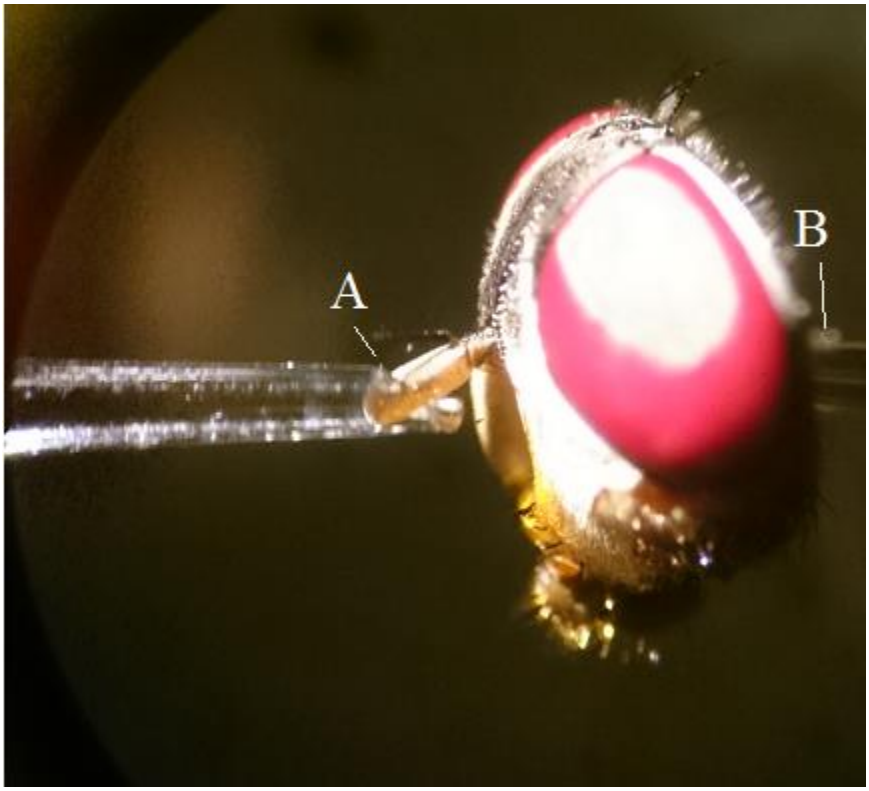

Figure 6: Female *Chrysomya rufifacies* antennal preparation for electroantennography, showing third antennal segment captured in recording electrode, in contact with Ringer's solution (a) and indifferent electrode capillary entering via decapitation wound (b). © S. Kolodij

A magnetic clamp stand installed directly in front of the viewing area underneath the stereomicroscope was used to support the stimulus application equipment. The stimulus was applied to the subject via a Continuous Airflow Stimulus Controller (CASC) (©Syntech). This machine created a continuous flow of air at a variable rate depending on the sensitivity of the antennal preparation through a plastic tube, which was attached to a solid glass tube mounted in front of the electrodes on the magnetic clamp, directed towards the preparation. The end of the

stimulus tube was positioned 1 to 2 cm away from the preparation to ensure a direct application of the stimuli without causing confounding responses based on physical response to air flow instead of electrical responses to the test compounds. The continuous airflow was also humidified before passing through the glass stimulus tube, by being routed through deionized water inside a Büchner (or side-arm) flask before reaching the glass stimulus application tube. The stimuli were applied to the antenna of the subject via air puffed over a dosed filter paper which was injected into a continuous airflow applied to the subject, which is important when exposing the subject to stimuli because otherwise it would be unclear if any responses recorded were due to the chemical stimulus or the physical stimulus of discontinuous airflow. This application is referred to as Direct EAG testing (Syntech, 2004).

An opening was created in the side of the stimulus tube to allow for the end of the GC column to be inserted. This allowed for stimuli to be applied directly into the continuous airflow and to the subject in real time as the compounds were being separated and released by the column. This procedure is referred to as coupled GC-EAG testing, as the GC equipment separates the compounds from a mixed sample in order to apply the compounds to the EAG equipment recording responses at the same time as the MS is determining the identity of the compounds present in the sample. Electrophysiological responses were recorded through an IDAC-2 data acquisition system and Autospike software (V3.9, © Syntech NL).

After the mixed VOC sample was applied to the column, EAG recording was started within 5 seconds. The sample was delivered to the Saturn 2000 GC-MS through an HP-1 column with a flow rate of 1 mL/minute and to the EAG at a flow rate of 2.5 mL/minute, as these were determined to be the rates that would compensate for the difference between retention time and stimulus delivery resulting from the distance of the EAG from the GC.. The GC-MS

methodology is such that the MS does not begin recording or analysing the samples until 3 minutes and 30 seconds after the sample has been applied to the column. This design was done because high levels of the diethyl ether solvent elute beginning at 1m 45s and spanning almost 2 minutes of retention time, until approximately 3m 20s. It was indicated by a technician from Agilent technologies that this extreme peak might damage the sensitive detecting components in the Saturn 2000 MS. The initiation time of 3 minutes and 30 seconds was chosen as the earliest timepoint that the full solvent peak was shown to have eluted, through a similar methodology performed on a Varian 240 GC-MS from Agilent technologies.

2.2.4 Depolarization Analysis

In order to determine which compounds in human decomposition were electrophysiologically active in *C. rufifacies*, 138 separate GC-EAG coupled experimental trials were conducted, using 30 different samples. The data was recorded and analysed using Autospike (© Syntech) software, wherein any visually distinct peaks were considered to be a response. The depolarizations were measured using the measurement tool option within Autospike wherein the amplitude and duration of each negative spike was collected via the use of two perpendicular vectors, the end points of which could be shifted along the graphical representation of the voltage response recorded in the subject (Figure 7).

Figure 7: Sample EAG output in Autospike (V3.9, ©Syntech) showing measurement tool

Possibly active compounds were identified based on the number of trials in which a depolarization occurred, and the consistency at which a depolarization re-occurred at each timepoint, counted from the initial timepoint +5 seconds. The reason for the positive addition of 5 seconds is that during testing, the GC-MS equipment and EAG equipment were housed in different rooms, and 5 seconds was the maximum amount of time between the initiation of the GC-MS methodology and the initiation of the EAG recording. The EAG recording was never initiated prior to the initiation of the GC-MS protocol, and therefore the added counts were only included in a forward direction, not the reverse.

2.2.5 Data analyses

Samples were grouped from each of the experimental subjects (H3, H4, H5, H6, P2 and P3) were analysed and compared using principal component analysis (PCA) and hierarchical clustering of the variance-covariance of the percent of total samples which contained each of the identified EAG-active candidate compounds via Past 3.10 Software (© Ø Hammer 1999-2015). The comparison was made based on the inclusion or exclusion of each of the six EAG-active

candidates within each of the sample groups, and also within each day a sample was collected from that experimental subject.

2.3 Results

2.3.1 Peak Analysis Results

A total of 138 separate trials were run, which produced a cumulative 798 different peak values. The majority of peaks did not occur more than once or twice at most timepoints, leading to a wide range of peak timepoints which did not occur with any consistency across trials. Sixteen different time points were identified as showing consistent peaks, wherein there was an evident depolarization across the antennae in at least 5 trials (Table 2).

Table 2: Time points with possibly active peaks as a percentage of the total number of trials.

Time (min:sec)	Number of Trials with Peak	Percentage of Total Trials with Peak
0:05	7	5.1%
1:15	7	5.1%
1:45	102	73.9%
3:05	6	4.4%
4:45	8	5.8%
5:30	9	6.5%
7:50	8	5.8%
10:05	9	6.5%
11:45	5	3.6%
15:10	18	13.0%
16:05	4	2.9%
24:50	9	6.5%
25:15	6	4.4%
27:30	7	5.1%
28:10	6	4.4%

Peaks at timepoint 1:45 were determined to be artifacts of the solvent peak, which possibly co-eluted with select highly volatile compounds, but a clear separation was not achievable with the use of Porapak samples. In order to determine whether *C. rufifacies* was

responding to the solvent or to possibly hidden co-eluting compounds, several solvent tests were performed wherein a dose of pure solvent was applied to an EAG preparation. These control trials showed that it was, in fact, the solvent front to which the flies were responding, as there was no test sample applied to those trials but a consistent response occurred at the expected solvent peak timepoint in 87% of tests performed. This phenomenon is hypothesized to occur due to the extremely high level of solvent that elutes at that time, as it is several orders of magnitude higher than the low concentration of compounds that the flies are able to detect in other circumstances.

Timepoints consistently found to have peaks were identified via comparison to GC-MS chromatograms which were recorded at the same time through a Saturn 2000 MS. Due to the minor discrepancy between the initiation times of the two different procedures, compound identity at this stage was approached via identification of any and all significant chromatogram peaks around the salient timepoints, in each of the 30 different samples, using the NIST08 and nist_ri libraries (©Agilent Technologies). Figure 8 shows a sample chromatogram for the most consistent timepoint of 15 minutes and 10 seconds.

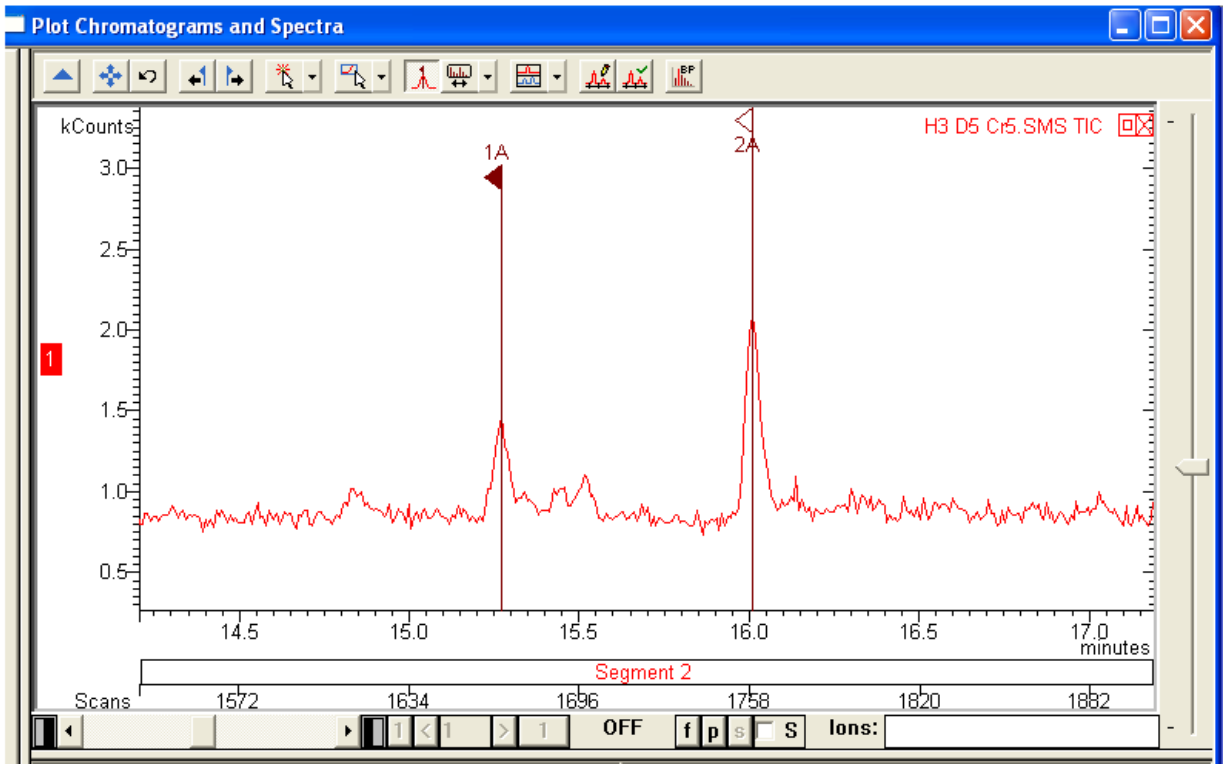

Figure 8: Sample chromatogram showing DMTS (1A) and Phenol (2A) peaks in Sample H3 D5

Based on the retention times most closely associated with the previously collected EAG timepoints, 6 different compounds were identified as being electroantennographically active, while it was not possible to associate the 11 other possible timepoints with eluted compounds with confidence. The 6 EAG-active compounds were butanoic acid methyl ester (BAME), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), ethanol, indole and phenol. Because it is known that different compounds are released from decaying tissue at different rates and different stages of decomposition, the sample groups were analysed with respect to whether or not each of the 6 identified compounds were found within the samples. Table 10 in Appendix B shows which compounds were present or absent from each of the samples, and shows that not all compounds of interest occurred in all samples taken. To account for this, Table 3 shows the trials which produced a response in *C. rufifacies* as a percentage of the number of trials performed for each sample which contained the compound of interest.

Table 3: EAG active timepoints with unconfirmed identities

Time (min:sec)	Number of Trials with Peak	Percentage of Trials with Peak out of Total Compound Trials	Identity
4:45	8	7.7%	BAME
5:30	9	36.0%	DMDS
7:50	8	5.8%	Ethanol
15:10	18	94.7%	DMTS
16:05	4	20.0%	Phenol
24:50	9	81.8%	Indole

2.3.2 Comparison of Human and Pig Data

Twenty-five pig sample trials were performed yielding a total of 3 different timepoints that were considered to consistently show depolarizations, as opposed to 16 timepoints that induced depolarizations in the human samples. The solvent peak timepoint occurred in both the human and pig samples, as did the depolarization at timepoint 15 minutes and 10 seconds, which was identified to be DMTS. A unique peak, identified as 2,2-dimethoxybutane, occurred in the pig samples at 5 minutes and 45 seconds.

In order to explore the relatedness of pig decomposition to human decomposition VOCs, principal component analysis (PCA) of the variance between sample groups was performed (Figure 9). The groups were delineated by source such that Human 3 (H3), Human 4 (H4), Human 5 (H5), Human 6 (H6), Pig 2 (P2) and Pig 3 (P3) were each considered a separate sample group. Each sample group was compared based on the number of samples from that sample group (H3, H4, H5, H6, P2, P3) which contained each of the six compounds of interest. Those scores were then converted to a percentage of the number of total samples collected for that sample group.

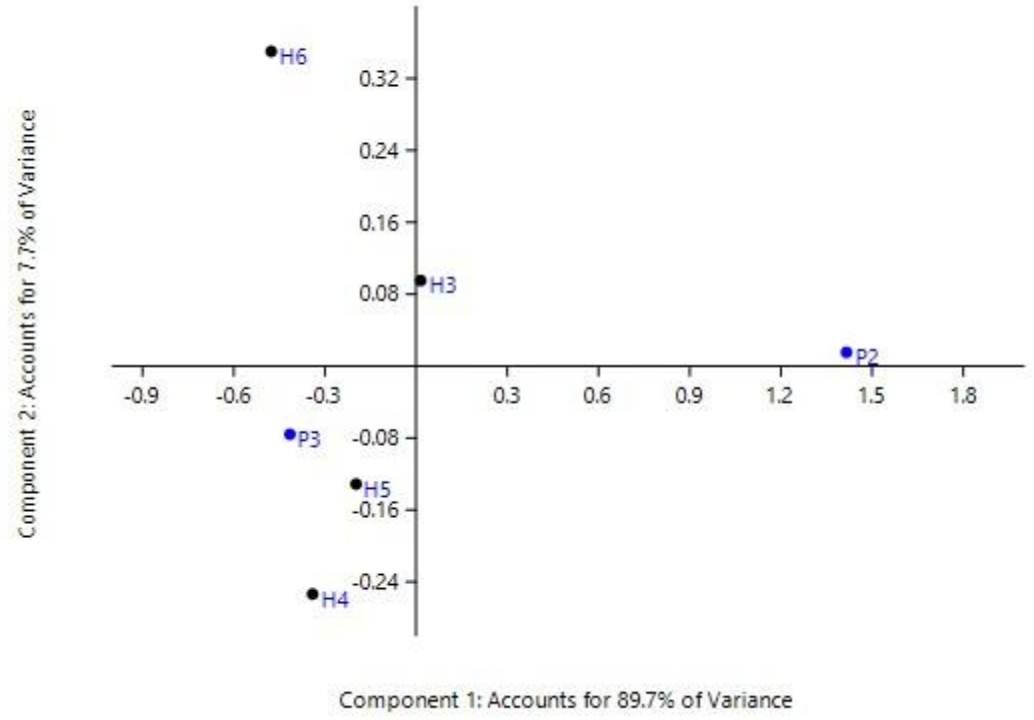

Figure 9: Scatterplot of Principal Component Analysis (PCA) of variance amongst sample groups in terms of inclusion of 6 different identified compounds: BAME, DMDS, DMTS, ethanol (EtOH), indole and phenol. PCA shows the least variance between human samples, which cluster close to the midline of PC1. Pig 2 shows the most variance against the other groups, while Human 6 shows the largest vertical variance which only accounts for approximately 8% of the total variance. Green biplot lines indicate the variance across compound groups, the length of which indicates strength of the variance, wherein a longer line denotes stronger variance.

The scatterplot of the PCA shows that the first principal component (the x-axis) accounts for approximately 90% of the variation between sample groups. The blue and black dots indicate the sample groups, wherein black represents the human samples (H3, H4, H5 and H6) and blue represents the pig samples (P2 and P3). Two of the human samples (H4 and H5) cluster in the bottom-left quadrant, with H6 occurring in the top-left quadrant and H3 occurring on the midline between the two top quadrants. P3 clusters closely with the human samples, whereas P2 is an obvious outlier. The close clustering of these points as opposed to their distance from the P2

sample group indicates a lower variance among the these groups than the variance that exists between P2 and the remaining sample groups. The P2 sample group shows the most variance as opposed to all other groups sampled. To more clearly display which sample groups associate most closely together, a hierarchical clustering of the sample groups was also performed, based on the percentages of samples which contained each of the 6 compounds (Figure 10).

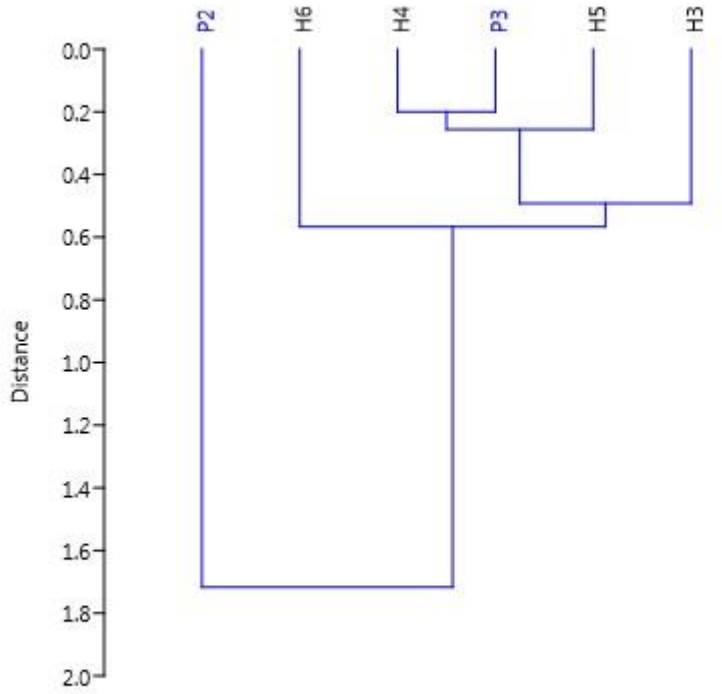

Figure 10: Heirarchical clustering of sample groups, using a Euclidian similarity index and unweighted pair-group average (UPGMA), presented as a dendrogram.

The hierarchical clustering (Figure 10) shows groupings or associations between each of the sample groups, wherein hierarchical clustering shows groupings or associations between variables. The hierarchical clustering shows that Human 4 and Pig 3 are more closely associated with each other than with any other groups; that Human 5 is more closely associated with Human

4 and Pig 3 equally; that Human 3 is more closely associated with Human 4, Pig 3 and Human 5 equally; Human 6 is more closely related to all other groups than it is to Pig 2; and that Pig 2 is the shows the least association to the rest of the sample groups.

The dendrogram shows that P3 clusters within the human sample group, showing a close association with H4, as opposed to P2 which shows a clear separation from all other groups. This is likely to have occurred due to the larger sample pool available in the Pig 3 sample group, which had a total of 5 different samples within the group which were collected over 5 days, as opposed to the sample pool for Pig 2, which only had one sample collected from the fifth day of sample collection. Considering that different VOCs are likely to occur in some days and not others as decomposition progresses, comparing data from a sample group which only contains one sample from the last day of sample collection is unlikely to closely follow the more robust data collected over the course of 4-5 days, as is the case in the 5 other sample groups. In order to explore this hypothesis, a second PCA and hierarchical cluster were performed to determine which sampling days would show the closest relationships. Figure 11 shows the PCA scatterplot of the data after it was sorted into categories based on the experimental day that sample was collected, regardless of the source of the sample.

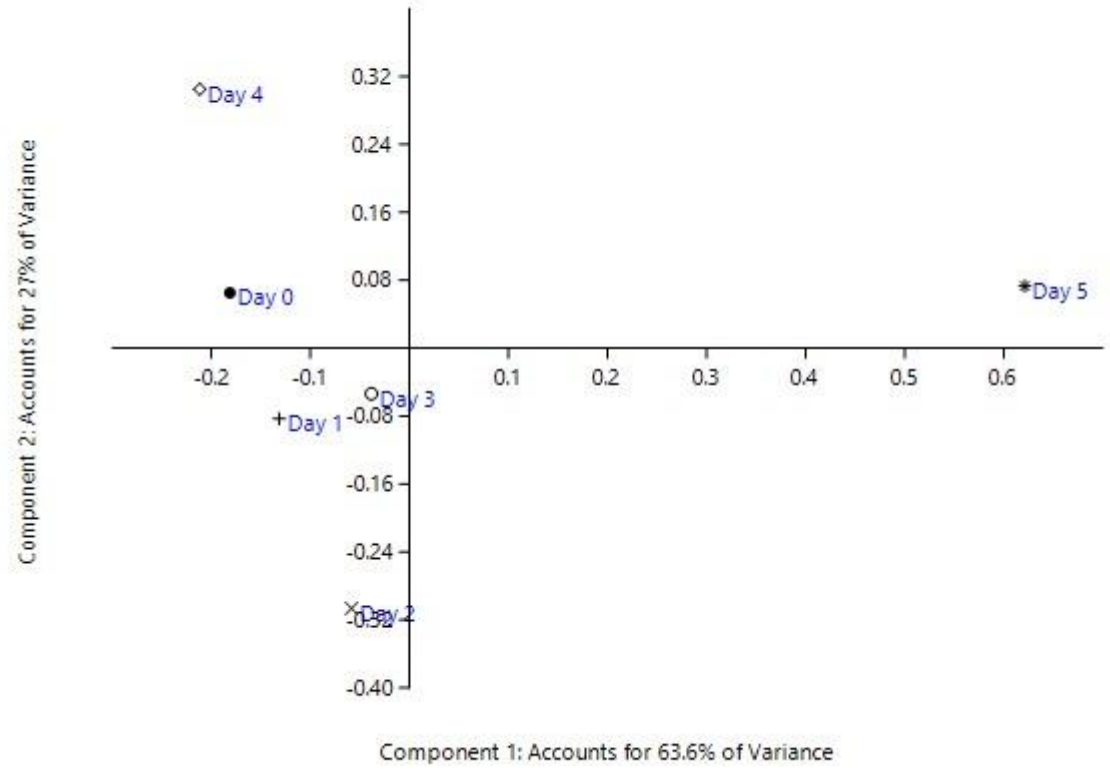

Figure 11: Principal component analysis of variance amongst sampling days in terms of inclusion of 6 different identified compounds: BAME, DMDS, DMTS, ethanol (EtOH), indole and phenol collected over 5 days of decomposition, wherein approximately 64% of the variance is accounted for by the x-axis. PCA shows clustering of the earlier days (D0, D1, D2, D3, D4) in the left half of the PCA while the last day (D5) occurs on the right side of the PCA. Green biplot lines indicate the variance across compound groups, the length of which indicates strength of the variance, wherein a longer line denotes stronger variance.

The variance that exists between days wherein collected samples contain each of the test compounds supports the postulation that a sample taken solely from Day 5 would not be able to fully represent the variance in sample composition which occurs over the full span of decomposition. Since approximately 64% of the variance between samples is accounted for across principal component 1 (PC1), the positions of the data points representing each separate day show more information by their arrangement in horizontal proximity to each other. This is as opposed to their position along PC2, which accounts for approximately 27% of the variance between the sampling day groups. Each day is represented by a different shape, and variance

amongst which compounds occur within each day is indicated by the green biplot lines, wherein the angle of the line indicates which days that compound is most closely associated with. DMTS, DMDS and phenol showed a bias towards clustering with the last day of decomposition, while ethanol showed no obvious bias and indole showed a weak trend towards Day 5. BAME was the only compound to show clustering trends towards the earlier days of decomposition in this research, and specifically showed a bias towards Day 4.

To clarify which days showed the closest associations, a hierarchical clustering of the sample days was also performed based on the percentages of trials which showed a depolarization at the timepoints of interest within each sampling day (Figure 12).

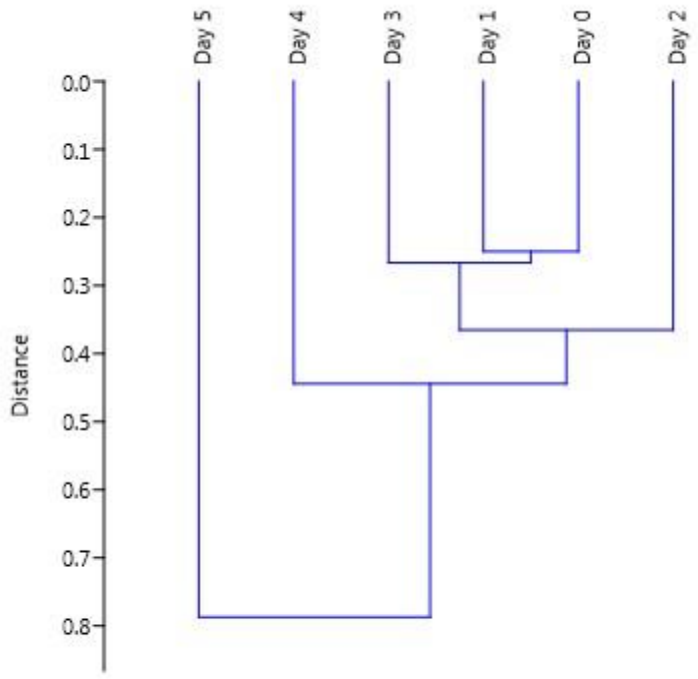

Figure 12: Heirarchical clustering of sampling days, using a Euclidian similarity index and unweighted pair-group average (UPGMA), presented as a dendrogram.

The hierarchical clustering of sampling days shows similar results to the PCA displayed in Figure 11, which is that the first 5 days cluster together as opposed to Day 5. Days 1 and 0 are more associated with each other than they are to Day 3, which is equally associated with both earlier days. Day 2 is more closely associated with Day 3, Day 0 and Day 1 than it is to Day 4 more closely associated with all other groups than it is with Day 5, but shows no preferential association with any of the early days it clusters with. Day 5 is an outgroup as compared to all other sampling days. The separation of Day 5 from the rest of the sampling days shows that there is a recognizable difference across days in terms of which compounds are released by human decomposition. This supports the argument that the extreme variation shown in the P2 trials as opposed to all other sampling groups in Figures 9 and 10 could possibly be explained by the lack of a full array of sampling days for the P2 sample group.

2.4 Discussion

2.4.1 Compound Identification

Six compounds qualified for further study based on the consistency with which they were able to produce an EAG response. These compounds were BAME, DMDS, DMTS, ethanol, indole and phenol. Due to the variable nature of decomposition VOC profiles these compounds were not identified in all samples, nor were they expected to be (LeBlanc, 2008). It was therefore hypothesized that a depolarization caused by these compounds would not occur with a high frequency relative to the total number of trials conducted. The random depolarizations can occur because several external factors such as airflow over the EAG preparation or post-decapitation antennal movement have the possibility of causing a response during EAG testing, which would cause depolarizations to occur at random timepoints that do not correlate to a compound eluting from the GC-MS column. It is therefore important to identify EAG-active candidate compounds based on repetitive responses at the same timepoint, rather than as a percentage of the total

number of peaks collected over all trials. The 6 identified compounds showed consistency above these random depolarizations. Therefore, responses were scored based on the percentage of depolarizations that occurred only within those samples which contained the compound of interest.

Four of the five identified compounds (DMDS, DMTS, ethanol and indole) have been shown in literature to cause consistent EAG-active responses in forensically important flies (Frederickx et al., 2012; Birkett et al., 2013; Ranger et al., 2014). BAME has as yet not been shown to be EAG-active in any forensically important species, and the results presented here, as well as in the dose response trials discussed in Chapter 3. This represents the first report of BAME as a potentially EAG-active, and behaviourally important compound for *C. rufifacies* or any other dipteran species. This compound was included because previous studies of its base compound with no ester group, butanoic acid, have produced significant EAG responses in forensically important blowflies (Frederickx et al., 2012; Dekeirsschieter et al., 2013). Based on the literature, the five other compounds were considered to be the best candidates for dose response testing, alongside BAME. The inclusion of ethanol occurred, despite low depolarization consistency due to previously performed EAG studies which showed ethanol as an EAG active compound (Ranger, et al., 2014). This result was not repeated in this research, despite ethanol occurring in more samples than all other EAG-active compounds for *C. rufifacies*. This could indicate that ethanol is unlikely to be EAG-active, which further expands the understanding of which compounds are important or unimportant in the olfactory response of *C. rufifacies.*

2.4.2 Pig Sample Comparison

The principal component analyses, which compared the instances of compound occurrence as a percentage of the total number of samples analysed, suggest that when samples are collected over a range of days, there is close association between pig samples and human samples that

does not occur when only one sampling day is included. This is likely to have occurred due to the recognized phenomenon of different VOCs being released at different times during decomposition (LeBlanc, 2008). It is likely that if a full range of sampling days was represented in the P2 sampling group, that that group would more closely associate with the human groups, as does the P3 sample group. This hypothesis is supported by the PCA of the variance of responses across the decomposition timeline of different sampling days, which showed that the early days cluster together in a group separate from the later sampling days. Since there is a large variance of EAG responses in Day 5 as opposed to Days 0-4, it follows that P2 would behave as an outgroup in the analysis considering the source sample was solely from Day 5, whereas the other sample groups had a range of samples collected across the 5 days of sampling that occurred in both the May and November 2012 trials.

This data supports the use of *Sus scrofa* decomposition as an analogue for human decomposition because when there is a robust sample of the full range of decomposition VOCs, the response percentage is similar for the two groups. This result is consistent with larger scale comparisons which have shown the VOC profiles of pig decomposition followed the same trends as human decomposition VOCs (Stefanuto et al., 2015). The largest difference between the human and pig samples is the occurence of 2-2-dimethoxybutane in the pig samples which does not occur as strongly or as often in the human samples; however, this is likely an artifact of plant disturbance at the site of VOC collection, as the compound is a component of fruit extracts and other plant materials (Kalt & Cock, 2014).

In order to confirm this preliminary exploration of the similarities between human decomposition VOCs and pig decomposition VOCs, the number of pig samples and trials should be more closely matched to the human decomposition samples and trials. This will show more

robust trends across both decomposition VOC profiles and help to expand upon the results presented here. Further, a full examination of the observed differences in the VOC profiles and instances of a response between samples collected on different days post mortem should be conducted in order to properly determine the similarity of human and pig decomposition, in terms of not only composition but of rate at which different compounds will occur in each VOC profile. This would most clearly reveal any significant differences which would affect the efficacy of pig decomposition as a human analogue.

CHAPTER 3: CONFIRMATION OF COMPOUND IDENTITIES AND DOSE RESPONSE

3.1 Introduction

It is important to perform dose response trials due to the nature of olfactory reception, wherein some receptor cells will only respond to certain compounds at higher concentrations, but will also only respond to others at low concentrations (Wright & Smith, 2004). Alternately, compounds can cause an altered response in an adult fly at differing concentrations, and it has been hypothesized that this phenomenon is due to the activation of different combinations of receptor types caused by a variation in concentration of the compound of interest (Wright & Smith, 2004). The saturation point, as well as the lowest effective dose, varies between different compounds as well as across different species (Frederickx, et al., 2012).

Electroantennographical testing measures the voltage fluctuation across an antenna in response to a chemical stimulus, and therefore is a measurement of whether there is a sensory response or not, and the strength of the response if one occurs. The voltage differential does not give an indication of the nature of the possible behavioural response induced via olfactory stimulation. The behavioural response induced by an EAG-active compound could be positive, negative or neutral, in that the insect could show attraction to the stimulus, repulsion, or no behavioural response at all. Further testing once a compound is indicated to be EAG-active needs to be conducted. In order to determine whether a compound is attractive or repulsive to a species, bioassays such as wind-tunnel experiments must be conducted (Frederickx, et al., 2012). To most efficiently conduct bioassay experiments, the concentration of the bait compounds needs to be optimized to the level at which the compounds will be most detectable by the species of interest, and therefore allow the best representation of the effect of those compounds on insect behaviour. This optimization occurs via dose response experimentation.

A dosage response curve is a graphical representation of the relationship between the concentration of a chemical stimulus and the response of the subject to that concentration (Frederickx et al., 2012). The response curve is often plotted with concentration along the x-axis with logarithmic, step-wise increases, and the level of response plotted along the y-axis. Ideally, the curve will follow a sigmoidal pattern (Figure 13); that is, dosage increases at the low end will have an effect small enough that the slope of the curve will initially be close to zero until an effective dose level is reached, at which point the slope of the curve will sharply increase until a saturation point is reached. Past this point, the change in response that occurs with further dosage increases will diminish, lowering the slope of the curve close to zero once again (Altshuler, 1981). The saturation point occurs in sensory dose response testing because individual ORNs on the antennae of the insect may become inhibited via over-stimulation and can no longer register an increase in the concentration of the stimulus (Wright & Smith, 2004).

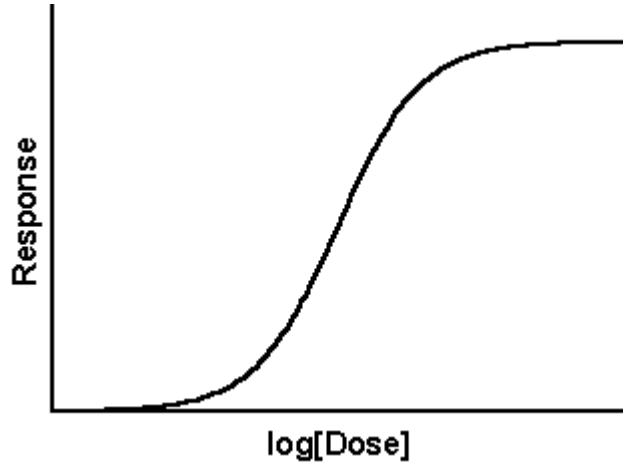

Figure 13: Illustration of expected dose response sigmoidal curve shape, from curvefit.com**.**

Therefore, in order to determine the true activity of EAG-active compounds, several different doses need to be applied to the test species in order to determine the lower and upper thresholds of response for each electrophysiologically active compound of interest. This technique was utilized to determine the lower and upper detection thresholds of each confirmed EAG-active compound in relation to *C. rufifacies*.

3.1.2 Dose Response Compounds

3.1.2.1 Butanoic acid methyl ester (BAME)

Butanoic acid, also commonly referred to as butyric acid, is a short chain fatty acid. This compound is a component of human vomit and is also found in milk, butter, and parmesan cheese. Butanoic acid has a strong odour which produces the characteristic smell associated with human vomit, as well as the indicative scent of rancid butter (Wishart, et al., 2007). It occurs as a product of anaerobic fermentation in the human colon and as body odour and is present in animal fats, in the form of an ester. Butanoic acid has been previously shown to be a consistent component of decompositional VOCs (Statheropoulos, et al., 2005**;** Dekeirsschieter, et al., 2009).

The esterification of butanoic acid occurs due to the saponification of fatty acids during the decomposition process (Statheropoulos, et al., 2005)**.** The methyl ester of butanoic acid, also referred to as methyl butyrate and herein referred to as BAME, is a low molecular weight ester which has a sweet, fruity odour and as such is often used in food flavouring and perfumes (Wishart, et al., 2007). Further, it has been studied as a possible alternative biodiesel fuel source, but was determined to be unsuitable for such applications (Gail et al., 2007). The chemical structure of BAME is shown in Figure 14.

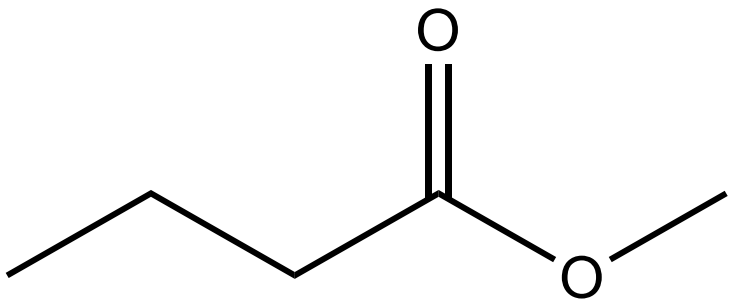

Figure 14: Chemical structure of BAME produced in ChemDraw 2015 © 2015 PerkinElmer Inc.

3.1.2.2 Dimethyl disulphide (DMDS)

Dimethyl disulphide, referred to as DMDS, is a previously studied decomposition-associated volatile organic compound, which has been shown to be electrophysiologically active in some species (Statheropoulos, et al., 2005; LeBlanc, 2008; Frederickx, et al., 2012)**.** The scent of DMDS is described as unpleasant and characteristic of fetid meat, due to the sulphurous components of the compound (Stensmyr, et al., 2002). It occurs naturally in several plants such as garlic, onions and cabbage and as such is accepted as a food flavouring additive, according to the Occupational Safety & Administration under the United States Department of Labor. The chemical formula of DMDS is CH_3SSCH_3 and its chemical structure is shown in Figure 15.

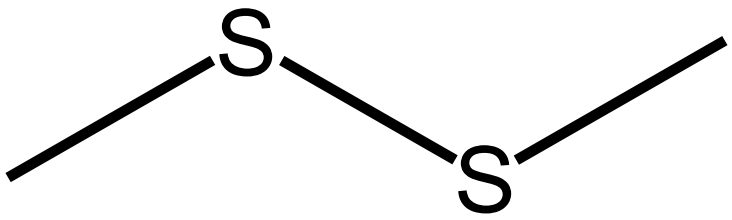

Figure 15: Chemical structure of DMDS produced in ChemDraw 2015 © 2015 PerkinElmer Inc.

3.1.2.3 Dimethyl trisulphide

Dimethyl trisulphide, herein referred to as DMTS, is an accepted decomposition-associated VOC, similar to DMDS in composition and occurrence (Statheropoulos, et al., 2005; LeBlanc, 2008). It can be produced as a by-product of bacterial decomposition, and is specifically associated with human decomposition. This compound is also found in several different plant

species (Stensmyr, et al., 2002). DMTS has a strong, unpleasant odour that is a component of the scent of fungating cancerous wound and human feces and is detectable at levels as low as 1 part per trillion (Shirasu, et al., 2005). Several studies have been conducted to investigate the components of the volatile organic compounds which are associated with decomposition and their effects on the insects which colonize decomposition, and DMTS has been shown to be an important component which acts as an attractant to forensically important blowfly species (Frederickx, et al., 2012; Zito et al., 2014). The chemical structure of DMTS is shown in Figure 16.

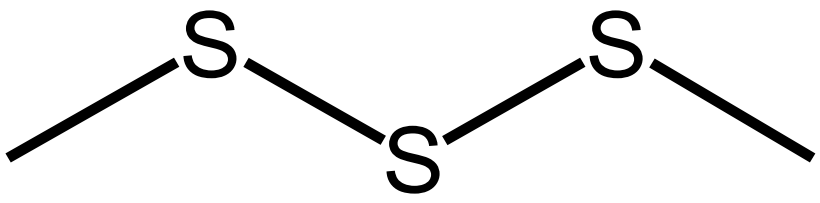

Figure 16: Chemical structure of DMTS produced in ChemDraw 2015 © 2015 PerkinElmer Inc.

3.1.2.4 Ethanol

Ethanol is a ubiquitous and highly studied alcohol that has many applications to human society in general and entomological studies specifically. It is a neurotoxic psychoactive drug, and is used recreationally across the majority of the planet legally (Brust, 2010). It is often used as a solvent to dissolve non-water soluble compounds, and has been studied and utilized as an alternative fuel source to gasoline (Sanchez & Cordona, 2008). Some studies have used ethanol as a possible electrophysiologically-active compound in terms of behavioural response in forensically important insect species (Ranger, et al., 2014)**,** while some other studies have used it as a negative control or inert solvent for the same purpose (Chaudhury et al., 2014). The full structural formula of ethanol is shown in Figure 17.

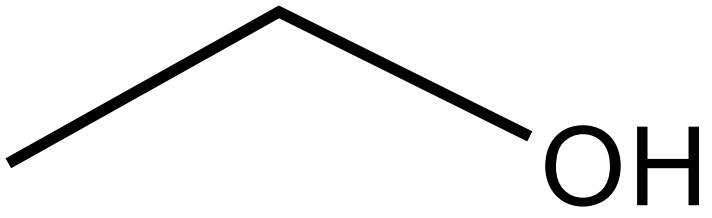

Figure 17: Chemical structure of ethanol produced in ChemDraw 2015 © 2015 PerkinElmer Inc.

3.1.2.5 Indole

Indole is an organic compound comprised of a benzene ring and a nitrogen-containing pyrrole ring. It is produced by bacteria via the degradation of the amino acid tryptophan and produces a malodourous fecal scent (Nelson et al., 2005; Statheropoulos, et al., 2005)**.** Indole has been previously shown to be a compound produced by both human and animal decomposition and has been utilized to study the olfactory response of some forensically important blowfly species (Dekeirsschieter, et al., 2013; Frederickx, et al., 2012**).** The heterocyclic chemical structure of indole is shown in Figure 18.

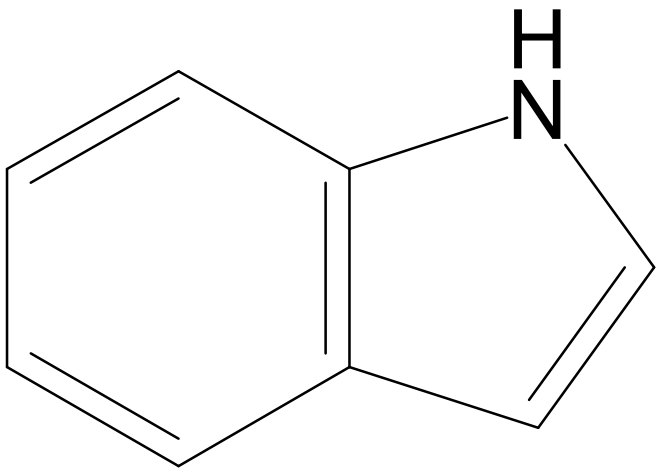

Figure 18: Chemical structure of indole produced in ChemDraw 2015 © 2015 PerkinElmer Inc.

3.1.2.6 Phenol

Phenol can also be referred to a carbolic acid, and is comprised of a phenyl group and a hydroxyl group. It is most commonly produced from petroleum and acts as the precursor to many compounds which can used in commercial products such as nylon, cosmetics, detergents and herbicides (Weber, et al., 2005). Phenol is soluble in water and mildly acidic and has the potential to cause chemical burns. It has been previously reported to be a decomposition-associated volatile organic compound, and has also been shown to be an EAG-active compound in some insect species (Dekeirsschieter, et al., 2013; Frederickx, et al., 2012)**.** The chemical structure of phenol is shown in Figure 19.

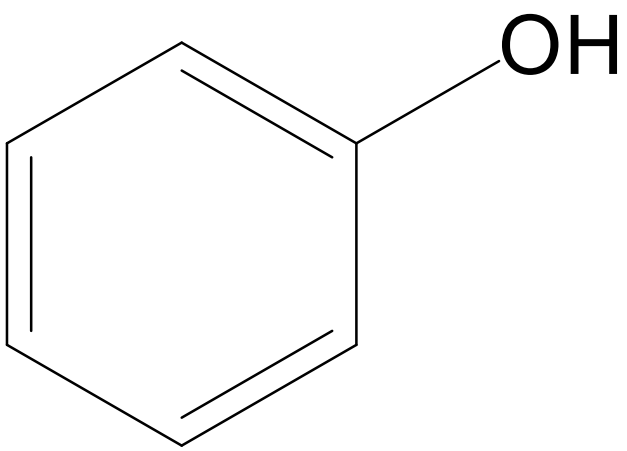

Figure 19: Chemical structure of phenol ChemDraw 2015 © 2015 PerkinElmer Inc.

3.2 Methods

3.2.1 Confirmation of Compounds

Initial analysis of the samples was conducted via the use of a Varian 240 GC-MS (©Agilent Technologies) through an HP-1 column, wherein each sample was investigated for any major peaks. Coupled GC-EAG testing was performed utilizing a Saturn 2000 GC-MS (©Agilent

Technologies), and therefore confirmation of the compound identities via the use of standard solutions of each compound was required. Based on the data collected from the coupled GC-EAG testing, six different compounds were considered to be possibly active. Those six compounds were BAME, DMDS, DMTS, ethanol, indole, and phenol. These compounds were identified based on the proximity of their retention times to the time at which a depolarization occurred in the EAG subject, if that depolarization occurred consistently across several trials. In order to confirm this information, further GC-MS testing was required.

Stock solutions at a concentration of 2 ppm of the 6 possibly active compounds were applied to the column in combination with a sample containing each of the compounds, in order to determine if the peaks from the known stock solution would co-elute with the sample peaks identified previously. This would indicate a shared mass spectrum and therefore a shared identity. A human VOC sample containing the compound of interest was used to positively identify the 6 compounds, and the sample chosen varied depending on the compound as not all samples contained all compounds of interest simultaneously.

The stock solutions were diluted to 2 ppm of solute in diethyl ether. The chromatograms showed a positive identity via co-elution and shared spectral data for all 6 of the compounds of interest: BAME, DMDS, DMTS, ethanol, indole, and phenol. These compounds were considered to be active compounds and were utilized in dosage response testing.

3.2.2 Dose response trials

The active compounds determined via GC-EAG and confirmation testing required further trials in order to determine the upper and lower levels of concentration that would produce an electrophysiological response in *C. rufifacies.* To do this, dose response trials were performed at concentrations of 2M, 0.2M, 0.02M, 0.002M and 0.0002M of BAME, DMDS, DMTS, ethanol,

indole, and phenol, with the addition of concentration of 1M and 4M for BAME, to a total of 32 different test solutions. The concentration levels were chosen based on the previous dose response work of Wright and Smith (2004). Dose response trials were conducted with a sample size of five or more at each dose concentration for each of the candidate compounds.

Dose response trials were performed without the coupled use of the GC-MS. The GC column was removed from the stimulus tube and a Pasteur Pipette was inserted in its place. A section of filter paper with no alterations was first inserted into the pipette and the pipette was attached to the end of a secondary air output on the Continuous Airflow Stimulus Controller (CASC), called the pulse flow. The pulse flow is controlled by a foot pedal, which activates a 2 second pulse of air to travel through the secondary tube once depressed (Figure 20).

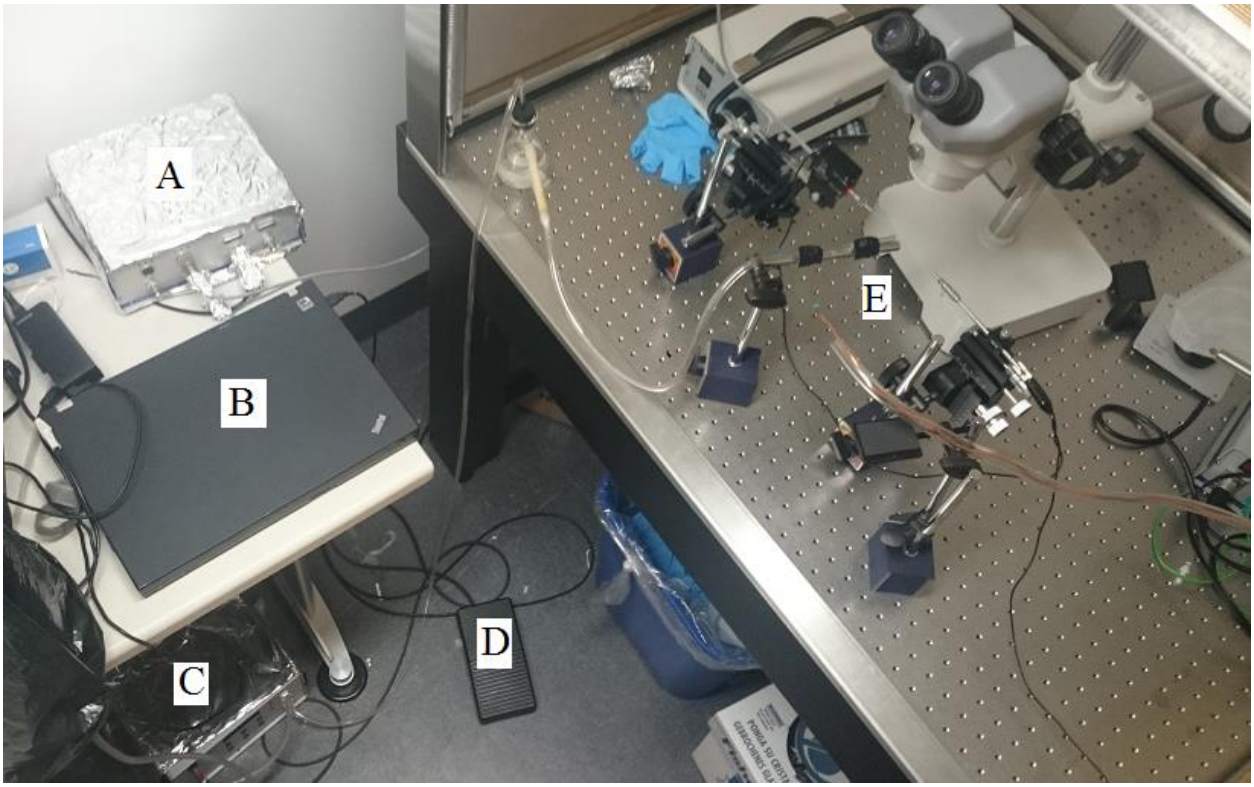

Figure 20: IDAC-2 (A); Recording computer (B); Continuous Airflow Stimulus Controller (C); and foot pedal (D), the foot pedal initiates flow from air stimulus controller through pulse flow tube; EAG preparation area, including stimulus glass tube mounted on magnetic clam stand (E).

The pipette was inserted into the side of the stimulus application tube and air was puffed through the pipette into the continuous airflow that is directed at the subject, applying the stimulus. A plain filter paper was used first as a control, followed by new filter paper that was dosed with pure solvent, once the filter paper was fully dried. Filter paper was dosed via the application of a minute amount of solution through dabbing of an uncontaminated pipette tip or capillary tube. The paper was allowed to dry completely before insertion. Next, a dose of 0.2 M concentration of methyl salicylate was applied as a positive control in order to normalize the response. Methyl salicylate was chosen based on previous electroantennographical studies, as its application shows a consistent response across many different insect species (Birkett, 2004; Blight et al., 1995; Chamberlain, et al., 2001; Wadhams et al., 1994).

Once control trials were performed, dosage testing was performed via application of test compounds in alphabetical order and increasing concentration. A full dose application was performed as follows: methyl salicylate, DMDS, DMTS, ethanol, indole and then phenol. Butanoic acid, methyl ester dose response testing was conducted separately, following the same control procedures. Control and test compounds were applied to the EAG preparation with at least a 1 minute rest period occurring between each stimulus application. The lowest concentrations were administered first as application of higher doses could show artificially diminished responses in the flies once the lower doses were re-administered. Therefore, the 0.0002M dose of all compounds was administered in the above order, followed by the 0.002M concentration, continuing through in this pattern until the highest concentration of 2M was reached. However, after approximately 20 minutes elapsed and the strength of the response of the subject would soon begin to diminish as the excised components lost moisture. When this point was reached the highest concentration of each compound was applied, even if it was not

the next concentration level in the established order. The reason the highest concentrations was separated was due to the possibility that the highest concentration could saturate the olfactory receptors of the subject early into the testing period, and prevent any functional data from being recorded following that application. A total of 54 different adult female flies were utilized, in order to reach 5 separate trials for each compound at each dose.

3.2.3 Data analyses

Data was analysed using SigmaPlot (SigmaPlot 12.5 ©Systat Software Inc.). Two-tailed t-test were performed to analyse variance of test compound induced responses to the negative controls of air and solvent. When normality and equal variance assumptions required to perform t-testss were not achieved, a Mann-Whitney Rank Sum Test was used instead. Data was normalized to the positive control prior to analysis, and presented as a percentage of the methyl salicylate positive control.

3.3 Results

3.3.1 Confirmation of Compounds

In order to control for the possible discrepancy between timepoints and the retention times of the possibly active compounds, further GC-MS trials were conducted using 2 ppm standard solutions for BAME, DMDS, DMTS, ethanol, indole and phenol. Figure 21 shows a sample chromatogram showing the identified DMTS peak occurring at the same retention time that DMTS was identified to exist within the human decomposition samples. This was considered to be a confirmation that DMTS was present in the samples at the retention time identified, and therefore it was approved to be utilized in dose response testing in the next phase. Each of the other four compounds were shown to elute at the hypothesized times with BAME eluting at 4 minutes, 45 seconds; DMDS eluting at 5 minutes, 30 seconds; ethanol eluting at 7 minutes and 50 second; indole eluting at 24 minutes, 50 seconds; and phenol eluting at 16 minutes and 5

seconds. Thus, these 5 compounds were considered to be EAG active compounds, and the upper and lower dosage thresholds needed to be determined.

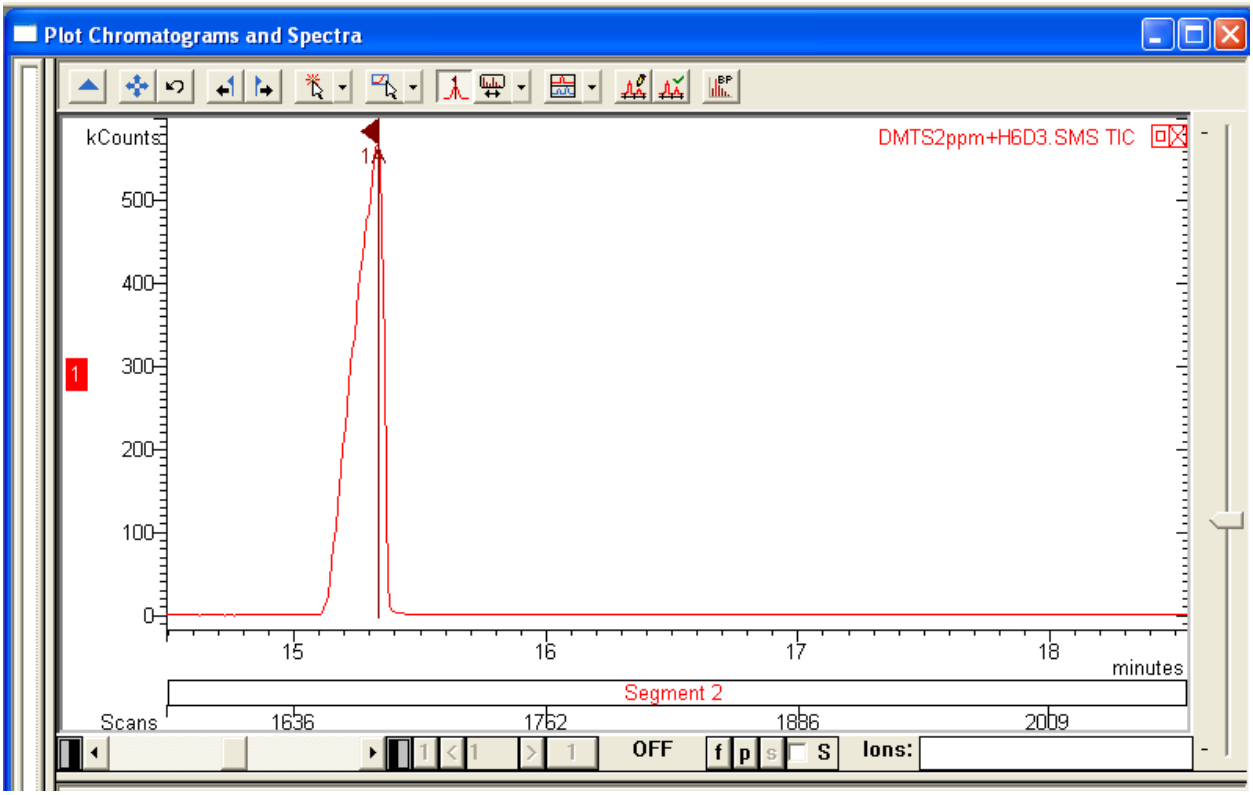

Figure 21: Sample chromatogram showing strong DMTS peak in H6 D3 sample spiked with 2 ppm solution of DMTS in diethyl ether

3.3.2 Dose Response

Dose response trials were quantified using AutoSpike software's measurement tool, which measures the amplitude and duration of electrical depolarizations that differ from the baseline. Each depolarization was measured to its most extreme value. The data was compiled and normalized to the most closely associated methyl salicylate induced depolarization via dividing the amplitude of the stimulus response to the most closely associated methyl salicylate response, as this compound was used as a positive control test. The normalized data was separated into 5 different doses, within 6 different compounds.

The response to each dose of the compounds was compared to the negative controls using a two-tailed t-test with SigmaPlot software, unless the data did not pass a Normality test or an Equal Variance test, and was therefore subject to a Mann-Whitney Rank Sum Test. This is because t-tests function under the assumption of normally distributed data, and if the data fails to pass a normality test such as the equal variance test, another statistical examination was suggested and utilized to better represent the data.

3.3.2.1 Butanoic acid, methyl ester

Table 4 shows the results of the two-tailed t-tests and Mann-Whitney analyses performed on the electrophysiological responses produced by *C. rufifacies* when exposed to different dose concentrations of butanoic acid, methyl ester (BAME). Significant differences from the negative controls were observed at all concentrations higher than the original median of 0.02M. Due to observations made during dose response testing, extra concentrations were tested for this compound at 1M and 4M, in order to test for a specific saturation point for this compound.

Table 4: Statistical t-tests of dose response to BAME at each dosage level as compared to negative controls. P-values for dosages that produced EAG responses that are significantly different from the negative control are bolded. Doses which failed normality assumptions and were therefore subjected to Mann-Whitney Rank Sum test are indicated by MW.

Dose Concentration (M)	P-value	Test
0.0002	0.31	MW
0.002	0.605	t
0.02	0.690	MW
0.2	**0.015**	**MW**
1	**0.008**	**MW**
2	**<0.001**	**MW**
4	**0.008**	**MW**

Due to the occurrence of a significantly different response from the negative controls at the penultimate and highest concentrations, BAME was considered to be an EAG-active

compound. Figure 22 shows the relationship between dose concentration and average electrophysiological response of *C. rufifacies* to BAME. The EAG response did not change significantly at the 3 lowest concentrations of 0.0002M, 0.002M and 0.02M, though it did at higher concentrations. Further, an increased response with increased dose was observed in the original concentrations tested as well as the additional concentrations of 1M and 4M. A saturation point was not determined, as the response continued to increase in the highest doses as compared to the other lower doses.

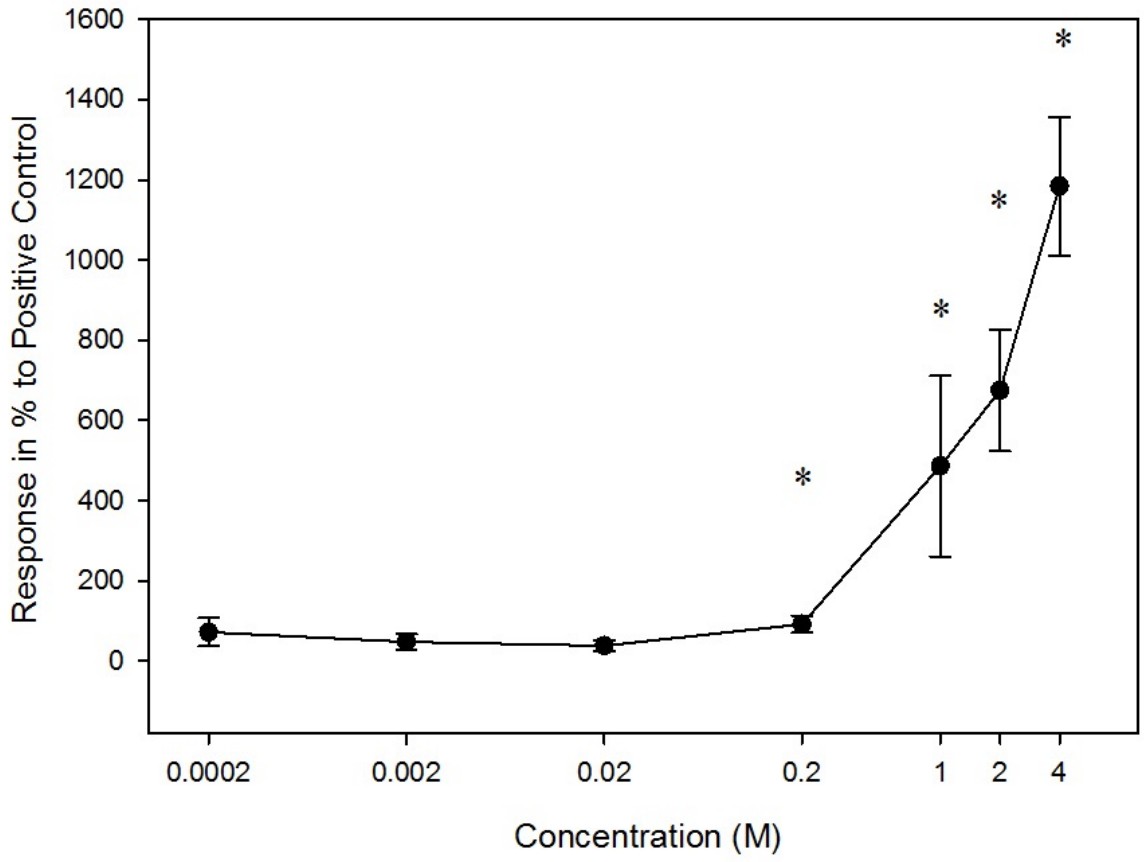

Figure 22: Average dose responses in µV of *C. rufifacies* to BAME at concentrations of 0.0002M, 0.002M, 0.02M, 0.2M, 1M, 2M and 4M, including standard error and normalized to positive control. Doses that produced significantly greater responses than the negative controls have been indicated with an asterisks (*).

3.3.2.2 DMDS

Table 5 shows the results of the two-tailed t-tests and Mann-Whitney analyses performed on the electrophysiological responses produced by *C. rufifacies* when exposed to different dose concentrations of DMDS. Significant differences from the negative controls were not observed at any concentration.

Table 5: Statistical t-tests of dose response to DMDS at each dosage level as compared to negative controls. Doses which failed normality assumptions and were therefore subjected to Mann-Whitney Rank Sum Tests are indicated by MW.

Dose Concentration (M)	P-value	Test
0.0002	0.486	MW
0.002	0.486	MW
0.02	0.199	t
0.2	0.165	MW
2	0.097	MW

Figure 23 shows the relationship between dose concentration and average electrophysiological response of *C. rufifacies* to DMDS. The overall trend observed in the dose response trials was that DMDS showed a generally linear trend of increasing strength of depolarization response in *C. rufifacies* when exposed to increased concentrations. Trend data was normalized as a percentage of the positive control.

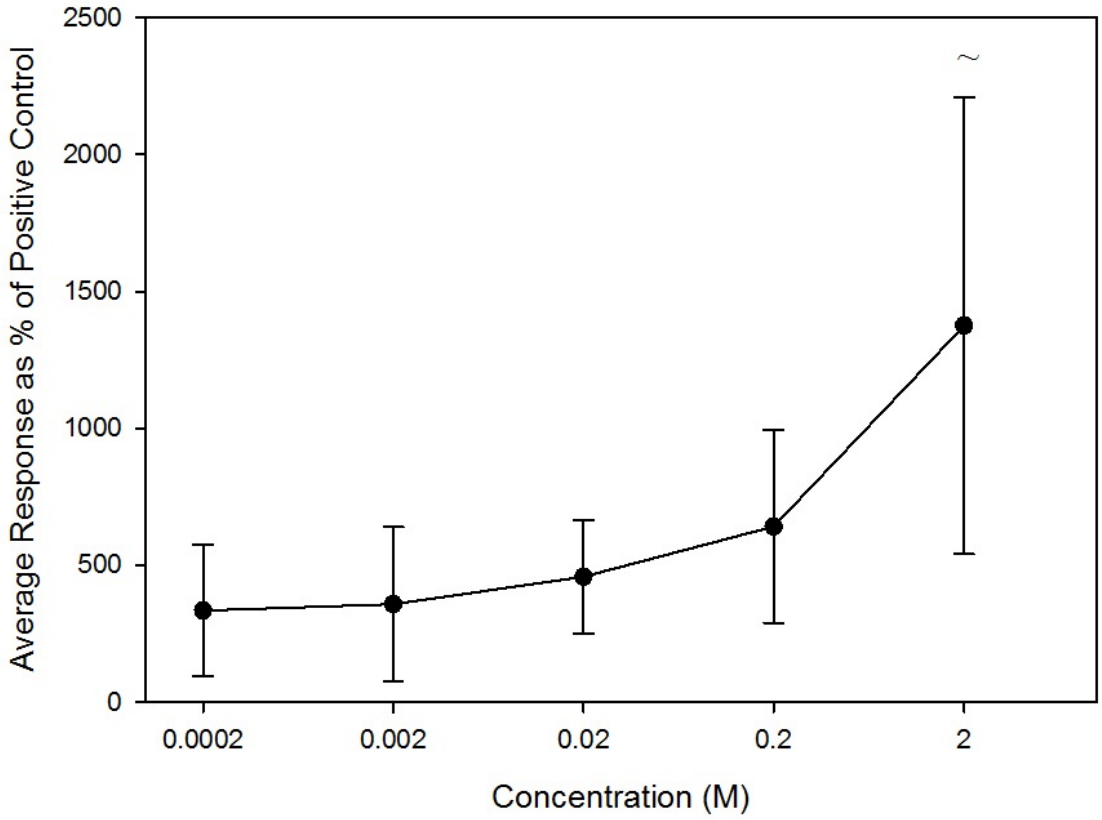

Figure 23: Average dose responses in µV of *C. rufifacies* to DMDS at concentrations of 0.0002M, 0.002M, 0.02M, 0.2M and 2M, including standard error, and normalized as a percentage of the positive control. No significant differences from the negative controls were observed, p = < 0.1 occurred at the highest concentration and is denoted by (~).

3.3.2.3 DMTS

Table 6 shows the results of the two-tailed t-tests performed on the electrophysiological responses produced by *C. rufifacies* when exposed to different dose concentrations of DMTS. The penultimate concentration (0.2 M) and the highest concentration (2 M) showed significantly different responses to DMTS in *C. rufifacies* as opposed to the negative controls (p=0.038, and p=0.038 respectively, with α=0.05). Significance was not observed across the other concentration levels of 0.0002 M, 0.002 M, and 0.02 M. However, the median concentration of 0.02 M showed near significance at a p value of 0.065, and when the normality and equal

variance assumptions were ignored, and a t-test was applied instead of the Mann-Whitney analysis, significance was observed at p=0.034.

Table 6: Statistical t-tests of dose response to DMTS at each dosage level as compared to negative controls. P-values for dosages that produced EAG responses that are significantly different from the negative control are bolded. Doses which failed normality assumptions and were therefore subjected to Mann-Whitney Rank Sum Test are indicated by MW.

Dose Concentration (M)	P-Value	Test
0.0002	0.149	t
0.002	0.114	MW
0.02	0.065	MW
0.2	**0.038**	**MW**
2	**0.038**	**MW**

Due to the occurrence of a significantly different response from the negative controls at the penultimate and highest concentrations, DMTS was considered to be an EAG-active compound and showed the highest values for responses out of all 6 compound groups. Figure 24 shows the relationship between dose concentration and average electrophysiological response of *C. rufifacies* to DMTS. The trend of the average EAG response to the different concentrations shows an increased response with increased strength of the dose up to the median concentration of 0.2 M, at which point the average response to increasing concentration began to decline.

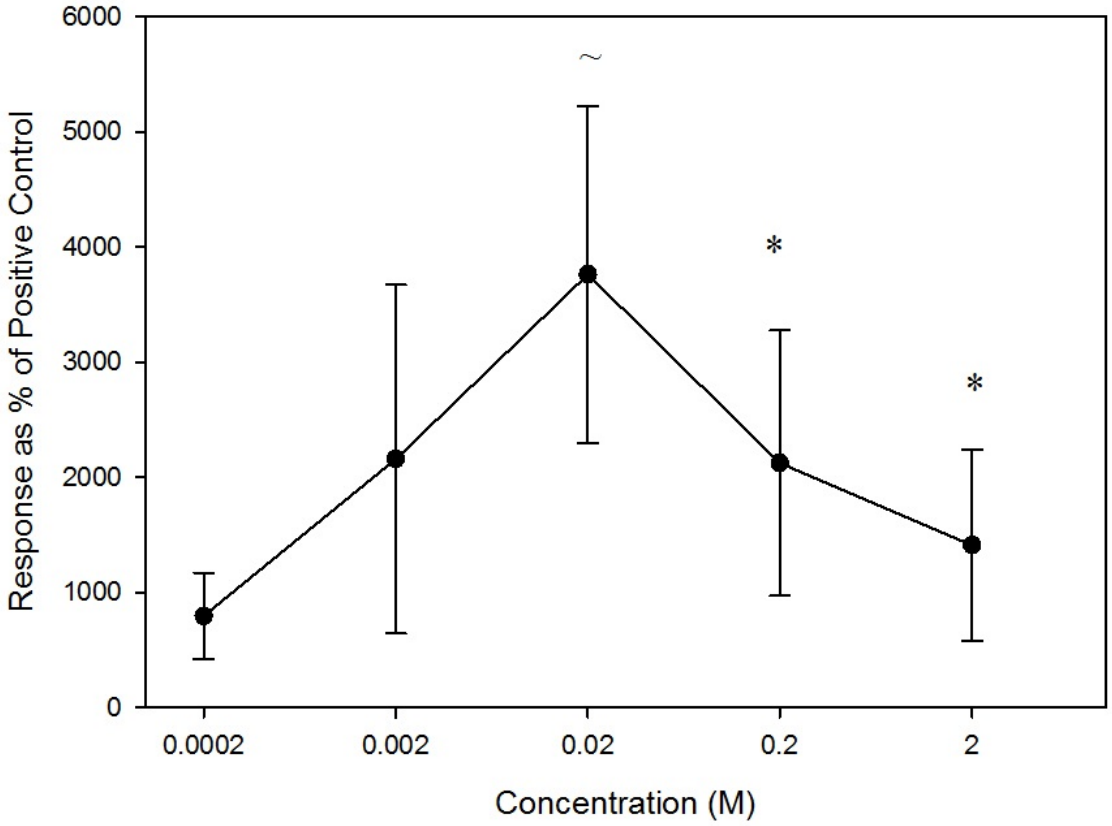

Figure 24: Average dose responses in µV of *C. rufifacies* to DMTS at concentrations of 0.0002M, 0.002M, 0.02M, 0.2M and 2M, including standard error and normalized to positive control. Doses which showed a significant difference as opposed to the negative controls have been indicated with an asterisks (*), and near significance is indicated with a (~).

Due to the peak response occurring at the median concentration, further testing of 3 lower concentrations of DMTS was performed, at concentrations of $2x10^{-5}$M, $2x10^{-6}$M, and $2x10^{-7}$M. The sample size was not robust enough to be included in statistical analyses in terms of significance; however, the trends are presented in Figure 25. When the lower doses are included, it can be seen that DMTS follows the expected sigmoidal trend line wherein the response increases with increasing concentrations until an olfactory saturation point is reached and the response thereby declines with increasing concentration.

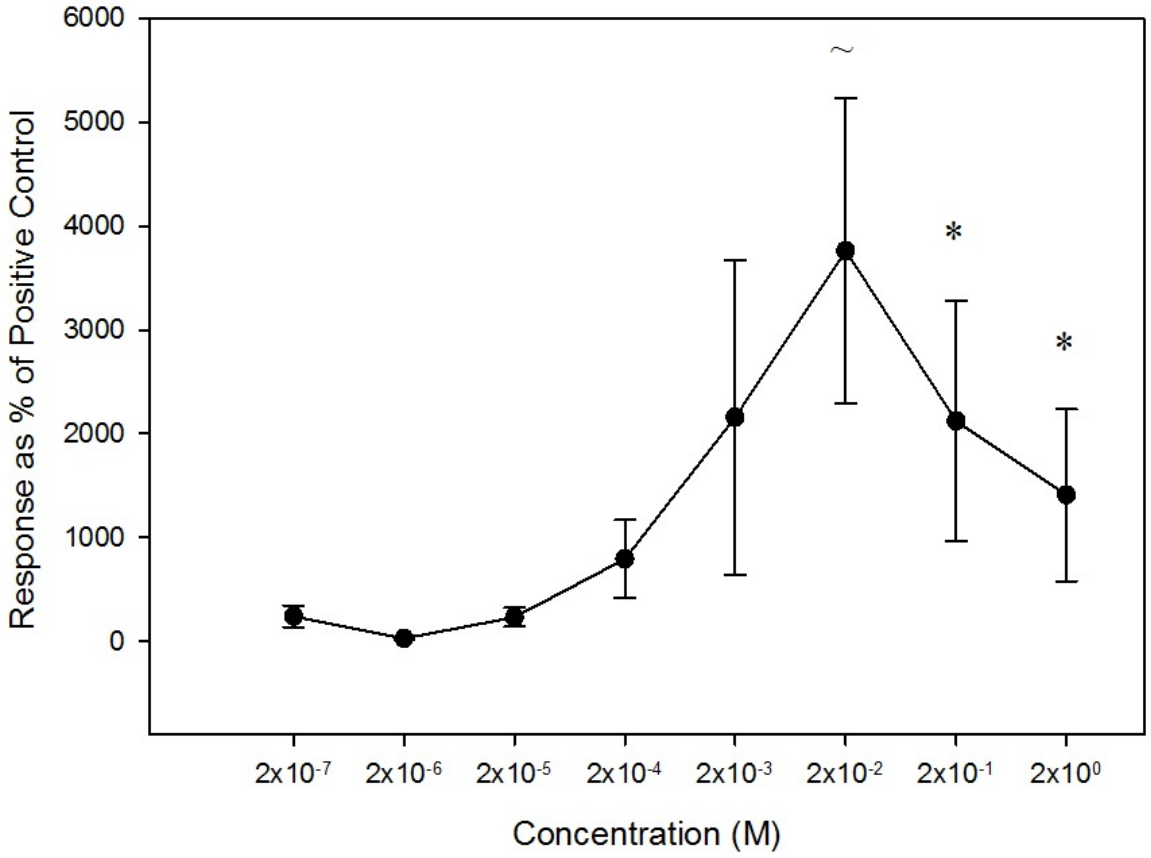

Figure 25: Average dose responses in µV of *C. rufifacies* to DMTS at concentrations of 0.0000002M, 0.000002M, 0.00002M, 0.0002M, 0.002M, 0.02M, 0.2M and 2M, including standard error and normalized to positive control. Doses which showed a significant difference as opposed to the negative controls have been indicated with an asterisks (*), and near significance is indicated with a (~).

3.3.2.4 Ethanol

Table 7 shows the results of the two-tailed t-tests performed on the electrophysiological responses produced by *C. rufifacies* when exposed to different dose concentrations of ethanol. Significant differences from the negative controls were not observed at any concentration.

Table 7: Statistical t-tests of dose response to ethanol at each dosage level as compared to negative controls. Doses which failed normality assumptions and were therefore subjected to Mann-Whitney Rank Sum Tests are indicated by MW.

Dose Concentration (M)	P-value	Power
0.0002	0.501	t
0.002	1.000	MW
0.02	0.214	t
0.2	0.710	MW
2	0.620	MW

As the response was not significantly different from the negative controls, ethanol was considered to not be an EAG-active compound. However, ethanol did not show an overall trend that was similar to the trends produced by DMTS in terms of the average response level to different doses presented (Figure 26). The strongest response was recorded at the median concentration of 0.02M, after which the response to the two highest concentrations diminished to similar levels to the two lowest concentrations.

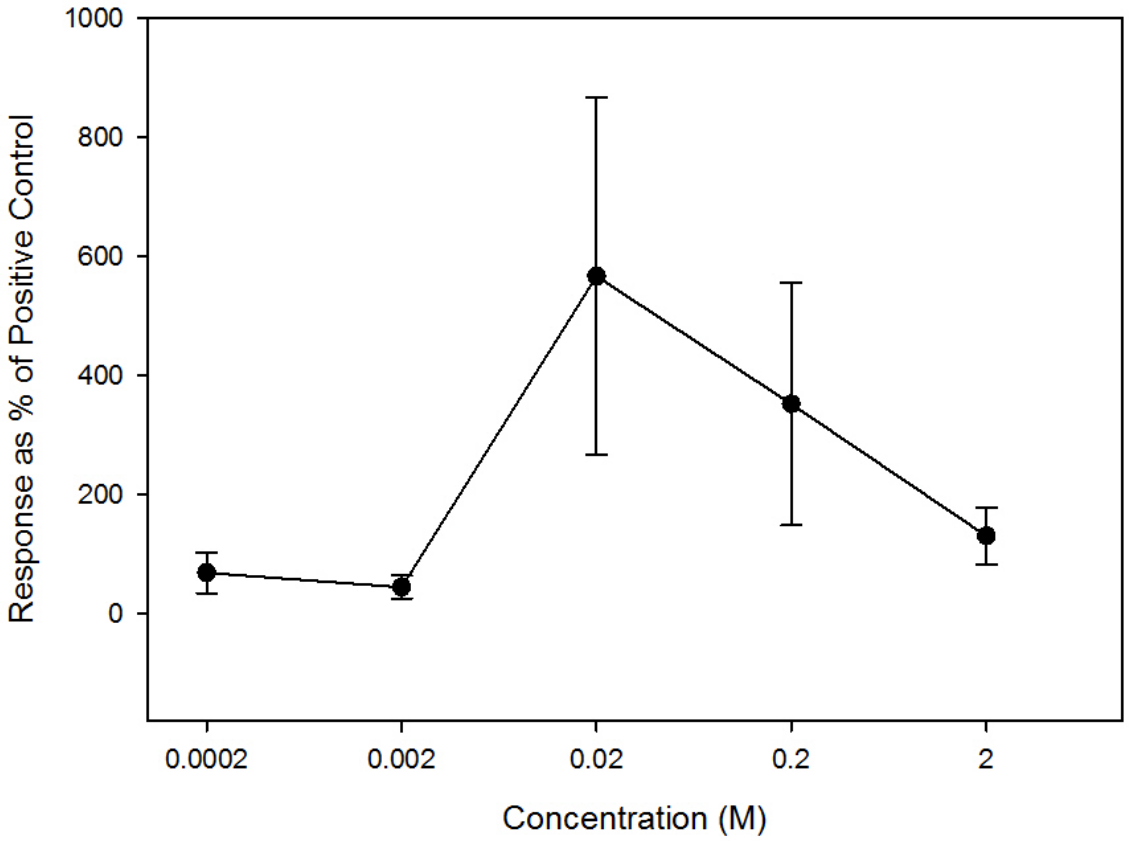

Figure 26: Average dose responses in µV of *C. rufifacies* to Ethanol at concentrations of 0.0002M, 0.002M, 0.02M, 0.2M and 2M, including standard error and normalized to positive control.

3.3.2.5 Indole

Table 8 shows the results of the two-tailed t-tests performed on the electrophysiological responses produced by *C. rufifacies* when exposed to different dose concentrations of indole. Significance was not observed at any concentration, though near significance was observed at the median concentration of 0.02M, at $p = 0.065$.

Table 8: Statistical t-tests of dose response to indole at each dosage level as compared to negative controls. Doses which failed normality assumptions and were therefore subjected to Mann-Whitney Rank Sum Tests are indicated by MW.

Dose Concentration (M)	P-value	Test
0.0002	0.867	t
0.002	0.686	MW
0.02	0.065	MW
0.2	0.456	MW
2	0.71	MW

Due to the lack of statistical significance between the different doses of indole, the line plot of the trend of the average responses shows the clearest depiction of the relationship between the dose responses (Figure 27). Indole showed a general trend to increasing strength of response correlating to increase in concentration of the compound, with the peak response level occurring at the median concentration of 0.02M. Similarly to DMTS and ethanol, the response diminished for the two highest concentrations, indicating a likely saturation point at the median concentration.

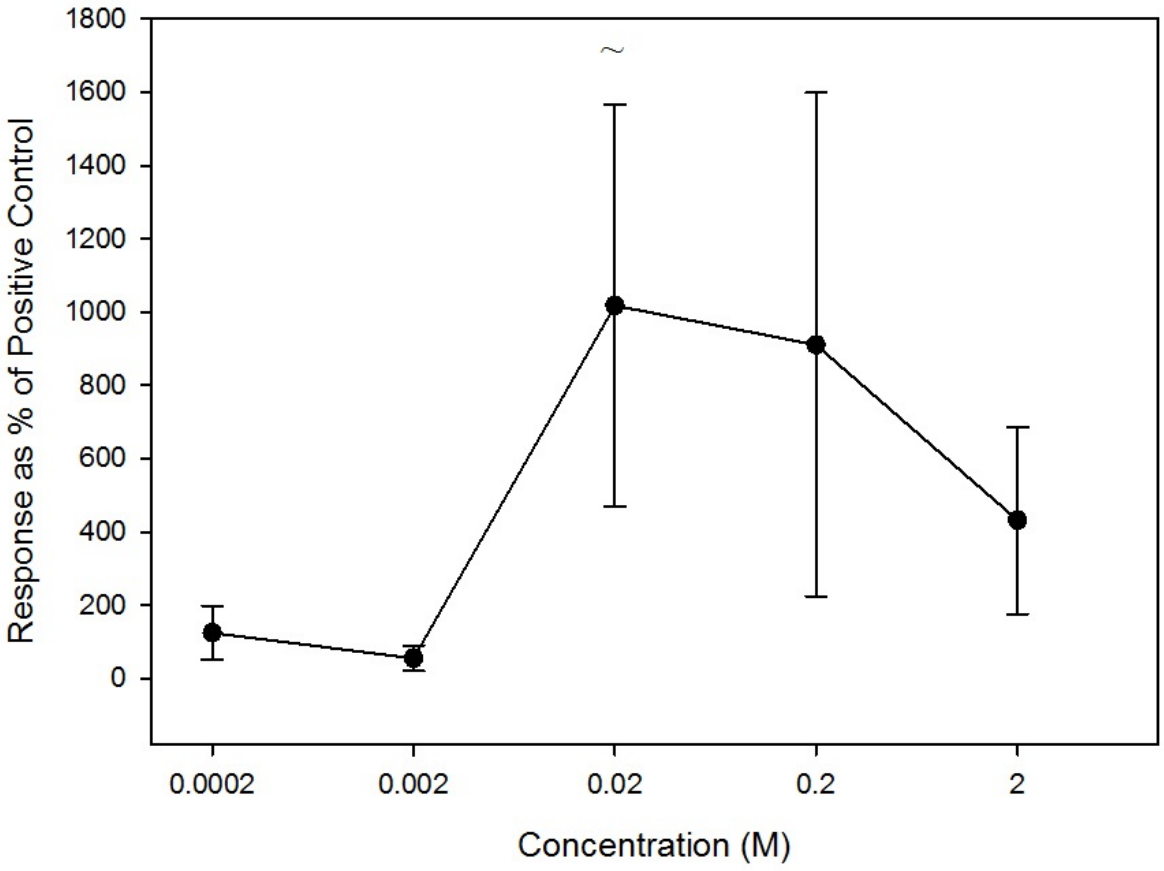

Figure 27: Average dose responses in µV of *C. rufifacies* to Indole at concentrations of 0.0002M, 0.002M, 0.02M, 0.2M and 2M, including standard error and normalized to positive control. No significant differences from the negative controls were observed. Near significance is denoted with a (~).

3.3.2.6 Phenol

Table 9 shows the results of the two-tailed t-tests performed on the electrophysiological responses produced by *C. rufifacies* when exposed to different dose concentrations of phenol. No true significance was observed at any dose concentration.

Table 9: Statistical t-tests of dose response to phenol at each dosage level as compared to negative controls. Doses which failed normality assumptions and were therefore subjected to Mann-Whitney Rank Sum Tests are indicated by MW.

Dose Concentration (M)	P-value	Power
0.0002	0.36	t
0.002	1	MW
0.02	0.699	MW
0.2	1	MW
2	0.71	MW

The trend of the average response to each dose concentration of phenol shows an overall weak response as compared to the positive control (Figure 28). It also follows a trend towards increased responses to increased concentrations, wherein the large variation in response to the penultimate concentration at 0.2 M caused a deviation from this trend. This is somewhat different from the saturation response seen in DMTS and indole, as the decrease shown in the highest concentration is likely an artifact of deviation error rather than saturation.

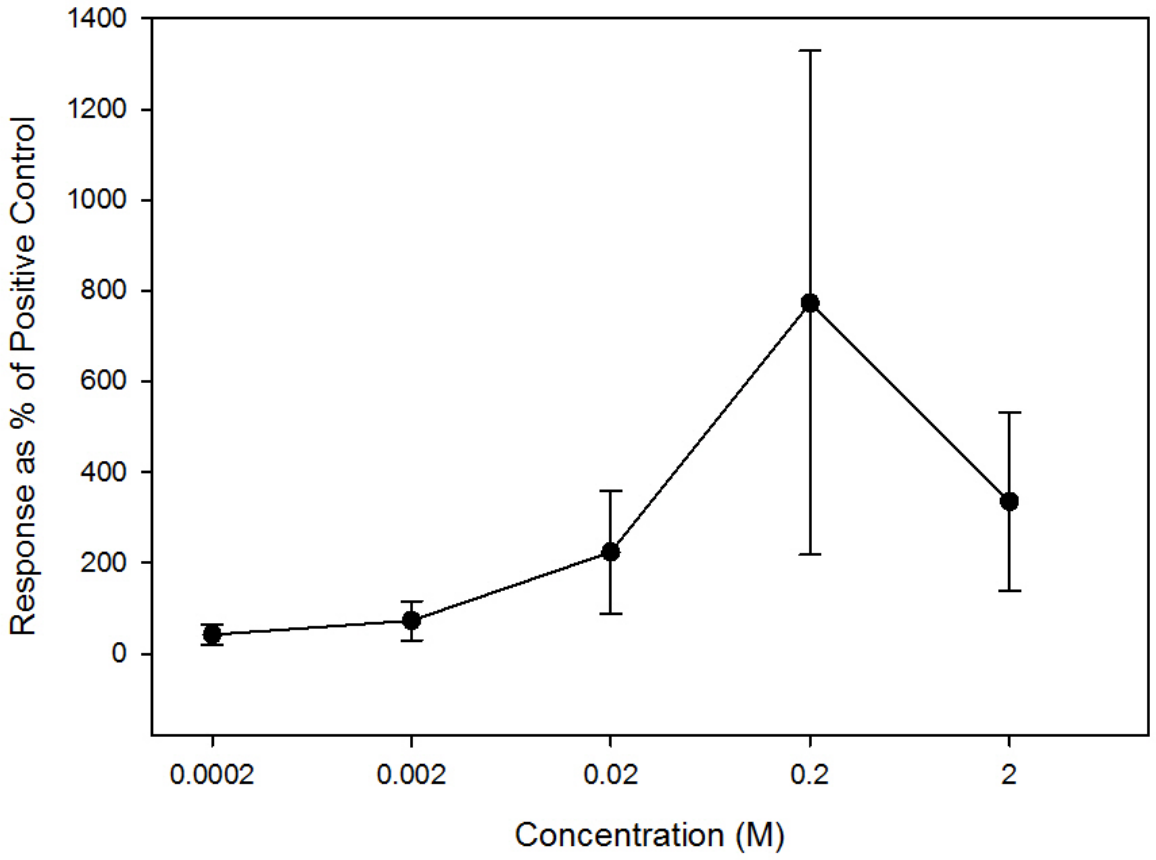

Figure 28: Dose responses in µV of *C. rufifacies* to Phenol at concentrations of 0.0002M, 0.002M, 0.02M, 0.2M and 2M, including standard error and normalized to positive control. No significant differences from the negative controls were observed.

3.4 Discussion

3.4.1 Interpretation of Dose responses

Two out of the six compounds identified as EAG-active candidates from human decomposition, were confirmed because they produced a significantly stronger EAG responses than controls at least one dose. These compounds were BAME and DMTS. EAG-activity has been shown previously for DMTS in forensically important blowfly species, such as *Lucilia sericata* (Zito, et al., 2014), but this is the first time it has been shown for *Chrysomya rufifacies* for this compound and the first time BAME has been shown to be EAG-active for any forensically-important species.

Dimethyl trisulfide showed a stronger average response at all dosage levels than any of the other four compounds. This result is consistent with a recent study that showed DMTS as a more significant and attractive component of decomposition VOCs compared to other compounds such as DMDS in terms of its ability to produce a response in adult flies (Zito, et al., 2014). Therefore, this compound should be studied more extensively in decomposition ecology, especially considering the fact that it occurs in both human and pig decomposition samples and causes a consistent response in different species of interest.

BAME as an EAG-active compound in *C. rufifacies* is a novel result, as this compound not been previously studied as an electrophysiologically important compound. Despite a lack of literature to indicate the inclusion of BAME, the choice to examine it as opposed to butanoic acid was due to the consistent identification via the NIST library of BAME in a large number of samples, whereas butanoic acid was not identified. Butanoic acid has been previously studied to determine its EAG-activity in some forensically important species, and found to be electrophysiologically active (Frederickx, et al., 2012; Dekeirsschieter et al., 2013). However, the methyl ester of butanoic acid was not examined in these studies. Due to the significant responses of *C. rufifacies* to BAME occurring only at high concentrations, further testing of butanoic acid, as compared to BAME, should be conducted in order to determine if the esterification of this compound in animal fat and during the saponification in decomposing remains causes an inhibition of the response in *C. rufifacies* that is only overcome at high concentrations of the compound.

The compounds which did not elicit statistically significant EAG responses were dimethyl disulfide, ethanol, indole and phenol, and were not confirmed as EAG-active compounds despite each compound (aside from ethanol) consistently eliciting an

electrophysiological response in *Chrysomya rufifacies* during the GC-EAG coupled experiments performed in this study. This result is at odds with previous studies in which DMDS has been shown to cause very strong and significant EAG responses in forensically important species (Frederickx, et al., 2012; Dekeirsschieter et al., 2013). However, this result is consistent with a study from 2014 which investigated the effects of DMDS and DMTS on the behaviour of 5 forensically important species, which showed no clear electroantennographical response to DMDS but a very strong response to DMTS (Zito et al., 2014). Some studies have shown phenol to be a behaviourally active compound on some species (Frederickx, et al., 2012; Dekeirsschieter et al., 2013) whereas others have shown that phenol failed to elicit behavioural responses (Birkett, 2004).

Ethanol did not elicit consistent activity in the GC-EAG coupled experiments to the same degree as the other studied compounds, but was included in dose testing due to previous literature which showed responses to ethanol in other species (Ranger, et al., 2014). This could be attributed to the abundance of ethanol which occurred in all collected samples. Figure 11 shows the principal component analysis of the associations that exist between sampling days in terms of the responses of *C. rufifacies* to the 6 different test compounds. The green biplot lines indicate the variance of the occurrence of each compound, wherein the angle of the line indicates which days that compound is most closely associated with. DMDS, DMTS and phenol showed a bias towards clustering with the last day of sampling, while indole trended only slightly towards the latest sampling day, and BAME showed a bias towards the earlier sampling days. Ethanol was the only compound to show no clustering trends towards any days of decomposition in this research. More extensive testing on the variation of VOC profile composition across days needs to be conducted in order to test this but this preliminary assessment could account for the lack of

EAG response in *C. rufifacies* to ethanol. This is because *C. rufifacies* behaves as a secondary colonizer in the geographic region in which the samples were collected and the olfactory senses of a secondary colonizer are hypothesized to be specialized to respond to compounds which are released during later stages of decomposition, such as DMTS which only occurred in the Day 5 samples analysed in this research. The continuous presence of ethanol would likely therefore cause it to be an ineffective signal to *C. rufifacies* that a suitable food source is nearby. This absence of EAG-active characteristics of ethanol is supported by literature which uses ethanol as negative control (Chaudhury et al., 2014).

This variance in responses to the same compound across different studies and different species is indicative of the necessity to study the specific responses of forensically important insect species within the geographical areas in which they impact forensic investigations. For example, the study performed by Frederickx et al. in 2012 showed a very strong response in *Lucilia sericata* to DMDS, whereas Zito et al. in 2014 showed the opposite wherein they did not detect a significant response in the same species to DMDS. Further studies which examine the effects of compounds within decomposition VOC profiles on multiple species of interest should be performed in order to determine if the lack of significant responses to compounds which were hypothesized to be EAG-active is due to species-level differences in olfactory behaviour before DMDS, indole or phenol can be definitively dismissed as being physiologically active in *C. rufifacies.*

In terms of the trends, all of the compound groups showed a generally increasing trend wherein strength of the response tended to increase with increased concentration. This follows the trends shown in previous studies, which often report a linear increase in response which occurs with increasing log concentration of compounds (Wright & Smith, 2004). The trends

presented in this study did not show consistency in terms of this increase however, which could be an artifact of the low sample size. This could be rectified by the performance of extra dose response trials. However, this trend could also be due to the concentrations themselves, in that it has been previously shown that compounds can exhibit a nonlinear response at differing concentrations due to the activation of different combinations of receptor types at these different concentrations (Wright & Smith, 2004). Lower doses are suggested as the doses chosen for this study were based on dose response trials performed using honey bees as the species of interest, and not forensically important blowflies. In order to investigate this possibility, the concentration of the 5 test compounds were calculated as compared to an internal standard of 160 ng of bromobenzene which the samples were spiked with prior to elution from the sorbent tubes. The highest average concentration of any compound of interest was indole with 0.00012 M, while each of other compound were either $1/10^{th}$ or $1/100^{th}$ as concentrated as indole. Considering the levels of the compounds that occurred in the samples, which showed a response during the coupled GC-EAG testing, it would follow that the concentration levels utilized in dose response testing presented in this research could possibly have been above the saturation concentration. In order to account for this possibility, further dose response testing was performed at three 10-fold dilution steps lower than the concentrations presented in Section 3.2, in order to identify if the doses follow a linear trend at lower thresholds. However, the majority of trials across all compounds did not show any evidence of a response at these low concentration levels. This could be due to the concentration or due to electrical background noise recorded by the highly sensitive EAG equipment which could mask lower voltage depolarizations. Ultimately, only 3 of the DMTS trials showed any response in *C. rufifacies* to the extremely low dose concentrations, and were therefore not robust enough to include in the overall statistical analysis for DMTS

activity. The trends were included to illustrate that further testing of these lower concentrations should be completed, if the methodology were able to be optimized to reduce background noise while testing less strongly responsive compounds at a level to which statistical power can be assured.

CHAPTER 4: Conclusions and Recommendations

4.1 GC-EAG

From the results obtained from the coupled GC-EAG trials conducted, 5 electroantennographically-active candidate compounds were identified based on consistent depolarizations during the trials. The identities of these compounds, and their inclusion within the human and pig samples collected were subsequently confirmed based on the retention times of a standard solution of each compound, and the spectral data produced by mass spectrometry. These 5 compounds were butanoic acid methyl ester (BAME), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), indole and phenol. They were identified as EAG-active due to the consistency with which they produced an electrical response as recorded across the antenna of an adult female *Chysomya rufifacies.* A sixth compound, ethanol, was included in in dose response trials despite having low consistency in its induction of a depolarization as compared to the number of samples which contained ethanol, due to its reported EAG-active status in previous studies (Ranger, et al., 2014). Ethanol elicited weak responses in the dose response trials which were not significantly different than the negative controls. Ethanol was ultimately determined to not possess EAG-active qualities in *C. rufifacies* based on its lack of consistency in GC-EAG coupled testing, and a lack of significant results in dose response trials. This is a novel result as compared to some studies (Ranger, et al., 2014), but follows the treatment of ethanol in other studies as a negative control compound (Chaudhury et al., 2014). Ethanol was detected in all samples, and therefore further testing should be done to determine more precisely when ethanol is released by human decomposition. *C. rufifacies* is less likely to respond to compounds which occur continuously during decomposition, as compared to compounds which occur more consistently in later days of decomposition, such as DMTS. Other compounds such as DMDS, phenol and indole elicited a higher incidence of response despite a lack of significant difference

from the control in the dose response testing, so those compounds cannot be definitively stated to be biologically inactive until further testing is completed.

4.2 Pig as Human Analogue

The comparison of human decomposition VOCs and pig decomposition VOCs showed that one pig subject (P3) was very similar to human samples in terms of fractions that elicited responses in *C. rufifacies* whereas samples from the other pig subject (P2) were very different from the human samples in this regard. However, samples from P2 were not taken on all days and therefore represented only the last day of decomposition VOCs. This shows the variance which can exist in VOC profiles from different days post mortem, and that data from one day of sampling is not necessarily comparable to the full spectrum of profiles collected across 5 sampling days. This is bolstered by the way in which P3 clustered closely with the human samples, Human 4 specifically. It is possible that if the full range of samples were available for P2, it would also cluster closely with the human samples. Further testing of pig derived VOCs should be conducted in order to more fully investigate this hypothesis.

4.3 Dose Response

The dose response trials performed showed a significant difference in response to test compounds as compared to the negative control for at least one dosage of both DMTS and BAME. This result, along with the general increase in strength of the response to increasing concentration solidified the finding of DMTS as an EAG-active compound in *C. rufifacies*, and a likely saturation threshold was determined via the decrease in response to the highest dose. BAME showed a similar trend in terms of the average response to increasing doses, but an upper threshold was not determined, and the response was not significant until the median dose was applied. Further testing should be conducted in order to determine the saturation threshold for

BAME, and comparison testing should be performed between BAME and butanoic acid to determine if esterification of this acid decreases or increases the response in *C. rufifacies*.

The dose response results for DMDS, ethanol, indole and phenol show a lack of significant difference from the negative controls, as well as a generally weak average response to the different concentrations when compared to the response generated by the significant values for DMTS and BAME. Indole, phenol and DMDS cannot be fully dismissed as possibly EAG-active compounds due to the consistency with which those compounds elicited a response in the GC-EAG coupled testing conducted with the samples which contained those compounds, and further trials should be run to increase the statistical power until such a time as a lack of significance cannot be attributed to low power. Ethanol, however, was concluded to be electrophysiologically inactive in *C. rufifacies* due to low consistency, and lack of statistically significant differences in dose response.

4.4 Limitations

The most significant limitation to this study was the extreme sensitivity of the olfactory organs in the species of interest. Adult female flies when attempting to locate a blood meal, a mate, or an oviposition substrate, are capable of detecting minute traces of compounds from several metres away within minutes after cell death begins. Due to this, their olfactory system is highly sensitive to even very small stimulations, and electrophysiological recordings taken from antennae can produce results that are not due to applied or controlled stimuli. Procedures were put in place during testing to minimize contamination of the air surrounding the subject prior to exposure to each compound of interest, but in some cases simply opening the odour compound containers resulted in the odours being detectable to people in an adjacent room. This kind of contamination can decrease the response in an EAG test subject through sensory adaptation and/or habituation.

Further, this can affect the response to the negative control stimulations if airborne compounds are present when they are applied. This source of contamination rendered an early round of dose response testing that was originally thought to have shown an ethanol responses invalid because traces of DMTS were present in the stimulus applicator. The contamination effect was rectified by increasing the distance between the EAG equipment and the station where test chemicals were applied to the filter paper, and through the systematic retraction and removal of the end portion of the stimulus applicator so as to prevent any trace of previous compounds within the applicator. Unfortunately the original data set could not be utilized to bolster the statistical power of the results presented in this research.

Another limitation is the use of solvent-based samples, due to the large solvent peak that occurs in the chromatogram which could possibly mask some highly volatile compounds which might be EAG-active. In order to investigate that possibility, samples collected with Tenax sorbent tubes which can be thermally desorbed instead of eluted via solvent could be performed. However, samples collected in that fashion are highly delicate and can only be utilized for one analysis, which does not allow for the replication required of electoantennographical investigation. Further, samples collected in this fashion would have to be analysed using a different GC-MS than the equipment utilized for the coupled GC-EAG testing, and would therefore not be directly comparable to the data collected by the EAG.

4.5 Recommendations

Based on the results obtained in this study, the following recommendations are proposed:

1. In order to fully determine the applicability of pig decomposition as an analogue for human decomposition, further pig VOC collection and investigation should be conducted and compared to the GC-EAG results presented here, to locate any

possible differences that cannot be attributed to plant or other site-specific artifacts not directly related to decomposition.

2. Further dose response testing should be conducted in order to increase the statistical power, definitively identify any non-significant results and more clearly show response trends.

3. Dose response testing should be conducted with a range of concentrations more closely associated to the concentration of the compounds within the collected samples, to more closely follow the conditions which would attract the species of interest to decomposing tissue in its natural environment. The EAG methodology should be optimized to diminish any electrical background noise that could mask any electrical responses to these lower concentrations.

4. Butanoic acid should be investigated via dose response testing and compared to the results obtained in the BAME trials in order to determine the effect of esterification on the EAG-activity of this acid.
5. Since only isolated compounds were studied in this research, direct EAG and dose response testing should be conducted utilizing combinations of the EAG-active candidate compounds, to more accurately reflect the environmental conditions of natural decomposition.
6. Bioassay experimentation should be conducted utilizing identified EAG-active compounds in order to determine the nature of the response induced in *C. rufifacies*.

CHAPTER 5: References

Altshuler, B (1981). Modeling of dose-response relationships. *Environmental Health Perspectives* 42: 23–7.

Amendt, J., Krettek, R., & Zehner, R. (2004). Forensic entomology. *Naturwissenschaften* 91(2): 51-65.

Amendt, J., Campobasso, C. P., Gaudry, E., Reiter, C., LeBlanc, H. N., & Hall, M. J. R. (2007). Best practice in forensic entomology—standards and guidelines. *International Journal of Legal Medicine* 121(2): 90-104.

Amendt, J., Richards, C. S., Campobasso, C. P., Zehner, R. & Hall, J. R. (2011) Forensic Entomology: applications and limitations. *Forensic Sci Med Pathol*, 7: 379–392.

Anderson, G. S. (2009) Forensic Entomology. In James, S.H., Nordby, J. J. & Bell, S. (Eds.). *Forensic Science: An Introduction to Scientific and Investigative Techniques* (3rd ed.) (pp. 137-164). Boca Raton, FL: CRC Press.

Anderson, G. S., & VanLaerhoven, S. L. (1996). Initial studies on insect succession on carrion in southwestern British Colombia. *Journal of Forensic Sciences* 41(4): 617-625.

Archer, M. S., & Elgar, M. A. (2003). Effects of decomposition on carcass attendance in a guild of carrion-breeding flies. *Medical & Veterinary Entomology*, *17*(3), 263. doi:10.1046/j.1365-2915.2003.00430.x

Bass, W. M. (1997) "Outdoor decomposition rates in Tennessee." In WD Haglund and MH Sorg eds. *Forensic Taphonomy: The Postmortem Fate of Human Remains*. London: CRC Press, pp. 181–186.

Baumgartner, D. L. (1993). Review of *Chrysomya rufifacies* (Diptera: Calliphoridae). *J. Med. Entomol.* 30:338–352.

Benecke, M. (2001). A brief history of forensic entomology. *Forensic Science International* 120(1–2): 2-14.

Birkett, M.A., Angelopoulos, N., Jensen, K.-M.V., Jespersen, J.B., Pickett, J.A., Prijs, H.J., Thomas, G., Trapman, J.J., Wadhams, L.J., & Woodcock, C.M. (2004). The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting cattle flies. *Medical and Veterinary Entomology.* 18:313-322.

Blight, M. M., Pickett, J. A., Ryan, J., Wadhams, L .J., & Woodcock, C. M. (1995). Recognition of oilseed rape volatiles by pollen beetles, *Meligethes spp*.: electrophysiological and chemical studies. *Proceedings, GCIRC 9th international rapeseed congress* 3:1043–1045.

Borel, B. (2013). "What Pig Carcasses Could Teach Coroners About Human Death." *Popular Science*. Retrieved on February 13, 2014, from http://www.popsci.com/science/article/2013-06/burial-sea.

Brust, J. C. M. *(2010).* Ethanol and Cognition: Indirect Effects, Neurotoxicity and Neuroprotection: A Review. *International Journal of Environmental Research and Public Health* ***7*** *(4): 1540–57.*

Bygarski, K., & LeBlanc, H. N. (2013). Decomposition and Arthropod Succession in Whitehorse, Yukon Territory, Canada. *J Forensic Sci*, March 2013, Vol. 58, No. 2. doi: 10.1111/1556-4029.12032

Byrd, J. H. & Butler, J.F. (1996). Effects of temperature on *Chrysomya rufifacies* (Diptera: Calliphoridae) development. Journal of Medical Entomology 34: 353-358.

Cammack, J. A., & Nelder, M. P. (2010). Cool-weather activity of the forensically important hairy maggot blow fly Chrysomya rufifacies (Macquart) (Diptera: Calliphoridae) on carrion in Upstate South Carolina, United States. *Forensic Science International* 195(1–3): 139-142.

Castner, J. L., Byrd, J. H., & Butler, J. F. (1996). Forensic Insect Field Identification Cards. Forensic Sciences Foundation, American Academy of Forensic Sciences. Colorado Springs, CO.

Chamberlain, K., Guerrieri, E., Pennacchio, F., Pettersson, J., Pickett, J. A., Poppy, G. M., Powell, W., Wadhams, L. J., & Woodcock, C. M. (2001). Can aphid-induced plant signals be transmitted aerially and through the rhizosphere? *Biochemical Systematics and Ecology* 29(10):1063 - 1074

Chaudhury, M. F., Zhu, J. J., Sagel, A., Chen, H. & Skoda, S. R. (2014). Volatiles from Waste Larval Rearing Media Attract Gravid Screwworm Flies (Diptera: Calliphoridae) to Oviposit. *Journal of Medical Entomology* 51(3): 591 – 595.

Cork, A., & Park, K.C. (1996). Identification of electrophysiologically-active compounds for the malaria mosquito, Anopheles gambiae, in human sweat extracts. *Med Vet Entomol*. 10(3): 269-76.

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.

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.

Gaïl, S., Thomson, M. J., Sarathy, S. M., Syed, S. A., Dagaut, P., Diévart, P., Marchese, A. J., & Dryer, F.L. (2007). A wide-ranging kinetic modeling study of methyl butanoate combustion. *Proceedings of the Combustion Institute* 31: 305.

Gikonyo, N.K., Hassanali, A., Njagi, P. G. N., & Saini, R. K. (2003) Responses of *Glossina morsitans* morsitans to blends of electroantennographically active compounds in the odors of its preferred (buffalo and ox) and non-preferred (waterbuck) hosts. *J Chem Ecol* 29(10): 2331–2345.

Gill-King, H. (1997) Chemical and ultrastructural aspects of decomposition.

In: Haglund, W. D., & Sorg, M. H. (eds.) *Forensic Taphonomy: the Postmortem Fate of Human Remains.* CRC Press, Boca Raton, Fla, pp. 93–108.

Hall RD (2001) Perceptions and status of forensic entomology.

In: Byrd J. H., & Castner J. L. (eds) *Forensic entomology: the utility of arthropods in legal investigations*. CRC, Boca Raton, Fla., pp 1–15.

Hayes, E.J., Wall, R., & Smith, K.E. (1999) Mortality rate, reproductive output, and trap response bias in populations of the blowfly *Lucilia sericata*. *Ecological Entomology*, 24, 300–307.

Hille, Bertil (1984). *Ionic Channels of Excitable Membranes*. Sunderland, Mass. 01375: Sinauer Associates, Inc.

Janaway, R.C., Percival, S.L., & Wilson, A.S. (2009). "Decomposition of Human Remains". In Percival, S.L. *Microbiology and Aging*. Springer Science + Business. pp. 13–334.

Kalt, F. R., & Cock, I. E. (2014) Gas chromatography-mass spectroscopy analysis of bioactive petalostigma extracts: Toxicity, antibacterial and antiviral activities. *Pharmacognosy magazine* 10(37): S37-S49.

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. In Current concepts in forensic entomology. (pp. 205-221). Springer Netherlands.

Lee, S., & Kruse, J. (2008). "Biopotential Electrode Sensors in ECG/EEG/EMG Systems". Analog Devices, Inc.

Logan, J. G., & Birkett, M. A. (2007). Semiochemicals for biting fly control: their identification and exploitation. *Pest Manag Sci* 63:647–657.

McDermott, H. J. (2004). *Air Monitoring for Toxic Exposures* (2nd ed.) (pp. 173-173). Hoboken, New Jersey: Wiley-Interscience.

McKnight, B. E. (1981). *The Washing Away of Wrongs: Forensic Medicine in Thirteenth Century China by Sung T'zu*,

Micozzi M.S. (1991). *Postmortem Changes in Human and Animal Remains: A Systematic Approach*. Springfield, IL: Charles C. Thomas.

Morris, M. C. (2005). Tests on a new bait for flies (diptera: Calliphoridae) causing cutaneous myiasis (flystrike) in sheep. *New Zealand Journal of Agricultural Research, 48*(1), 151-156.

Nelson, D. L., & Cox, M. M. (2005), *Principles of Biochemistry* (4th ed.), New York: W. H. Freeman.

O'Flynn, M.A. (1983). The succession and rate of development of blowflies in carrion in southern Queensland and the application of these data to forensic entomology. *Journal of the Australian Entomological Society* 22: 137–148.

Pavia, D. L., Lampman, G. M., Kritz, G. S., & Engel, R. G. (2006). *Introduction to Organic Laboratory Techniques (4th Ed.)*. Thomson Brooks/Cole. pp. 797–817.

Payne, J. A. (1965). A Summer Carrion Study of the Baby *Pig Sus Scrofa* Linnaeus. *Ecology* 46(5): 592–602.

Ranger, C. M., Gorzlancyk, A. M., Addesso, K. M., Oliver, J. B., Reding M. E., Schultz, P. B. & Held, D. W. (2014). Conophthorin enhances the electroantennogram and field behavioural response of Xylosandrus germanus (Coleoptera: Curculionidae) to ethanol. *Agricultural and Forest Entomology*, 16, 327–334.

Roelofs, W.L. (1984). Electroantennogram Assay: Rapid and Convenient Screening Procedures for Pheromones.
In: Hummel, H. E. & Miller, T. A. (Eds.) *Techniques in Pheromone Research* (pp. 131-160). Springer Verlag.

Rosati, J. Y., & VanLaerhoven, S. L. (2007). New record of *Chrysomya rufifacies* (diptera: Calliphoridae) in Canada: Predicted range expansion and potential effects on native species. *Canadian Entomologist*, 139(5): 670-677.

Sanchez, O. J. & Cardona, C.A (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology* 99(13): 5270-5295.

Shiao, S. F., & Yeh, T. C. (2008). Larval Competition of *Chrysomya megacephala* and *Chrysomya rufifacies* (Diptera: Calliphoridae): Behavior and Ecological Studies of Two Blow Fly Species of Forensic Significance. *Journal of Medical Entomology* 45(4): 785-799.

Shirasu, M., Nagai, S., Hayashi, R., Ochiai, A., & Touhara, K. (2009). Dimethyl trisulfide as a characteristic odor associated with fungating cancer wounds. *Bioscience, Biotechnology, and Biochemistry* 73 (9): 2117–20.

Schoenly, K., Goff, M. L., Wells, J. D., & Lord, W. D. (1996). Quantifying statistical uncertainty in succession-based entomological estimates of the postmortem interval in death scene investigations: a simulation study. *Am Entomol* 42:106–112.

Sparkman, O. D. (2000). *Mass spectrometry desk reference*. Pittsburgh: Global View Pub. ISBN 0-9660813-2-3.

Spradberry, J.P. (1979) The reproductive status of *Chrysomya* species (Diptera: Calliphoridae) attracted to liver-baited blowfly traps in Papua New Guinea. *Journal of the Australian Entomological Society* 18: 57–61.

Stadler, S. (2013). Analysis of the Volatile Organic Compounds Produced by the Decomposition of Pig Carcasses and Human Remains. The University of Ontario Institute of Technology for the Degree of Doctor of Philosophy.

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

Stensmyr, M. C., *Urru,* I., *Collu,* I., *Celander,* M., *Hansson,* B. S., & *Angioy*, A. M. (2002). *Rotting s*mell of dead-horse arum florets. *Nature 420 (6916): 625–626*.

Sukontason K., Methanitikorn R., Chaiwong, T., Kurahashi, H., Vogtsberger, R. C., & Sukontason, K. L. (2007) Sensilla of the antenna and palp of *Hydrotaea chalcogaster* (Diptera: Muscidae). *Micron,* 38: 218–223.

Sukontason, K. L., Narongchai, P., Sripakdee, D., Boonchu, N., Chaiwong, T., Ngern-Klun, R., Piangjai, S., & Sukontason, K. (2005). First Report of Human Myiasis Caused by *Chrysomya megacephala* and *Chrysomya rufifacies* (Diptera: Calliphoridae) in Thailand, and Its Implication in Forensic Entomology. *Journal of Medical Entomology* 42(4): 702-704.

SYNTECH (2004) Eletroantennography: A practical introduction. Hilversum, The Netherlands.

Tomberlin, J. K., Albert, A. M., Byrd, J. H. & Hall, D. W. (2006). Interdisciplinary Workshop Yields New Entomological Data for Forensic Sciences: Chrysomya rufifacies (Diptera: Calliphoridae) Established in North Carolina. *Journal of Medical Entomology* 43(6): 1287-1288.

Vass, A. A., Bass, W. M., Wolt, J. D., Foss, J. E. & Ammons, J. T. (1992). Time since death determinations of human cadavers using soil solution. *Journal of Forensic Sciences* 37(5): 1236-1253.

Vass, A. A., Barshick, S. A., Sega, G., Caton, J., Skeen, J. T., Love, J. C. and 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.

Vosshall, L. B., & Stocker, R. F. (2007). Molecular architecture of smell and taste in Drosophila. *Annual Revue Neuroscience*, 30: 505-533.

Wadhams, L. J., Blight, M. M., Kerguelen, V., Le Métayer, M., Marion-Poll, F., Masson, C., Pham-Delègue, M. H., & Woodcock, C. M. (1994). Discrimination of oilseed rape volatiles by honey bee: Novel combined gas chromatographic-electrophysiological behavioral assay. *Journal of chemical ecology* 20(12): 3221 – 3231.

Weber, M., Weber, M., & Kleine-Boymann, M. (2004). "Ullmann's Encyclopedia of Industrial *Chemistry*". Wiley-VCH, Weinheim.

Wishart D. S., Tzur, D., Knox C, et al., (2007) HMDB: the Human Metabolome Database. Nucleic Acids Res. Jan;35(Database issue):D521-6. 17202168

Wright, G. A., & Smith, B. H. (2004). Different thresholds for detection and discrimination of odors in the honeybee (*Apis mellifera*). *Chem. Senses* 29: 127-135.

Zito, P., Sajeva, M., Raspi, A., & Dötterl, S. (2014). Dimethyl disulfide and dimethyl trisulfide: so similar yet so different in evoking biological responses in saprophilous flies. *Chemoecology* (2014) 24:261–267.

APPENDIX A: Establishment of EAG Equipment

Before any trials could be performed on the test subjects, the electroantennography (EAG) equipment (Figure 29) needed to be established and optimized. In order to properly establish the equipment, manuals and reference material provided by Syntech were used as the main resource and guide to proper functionality.

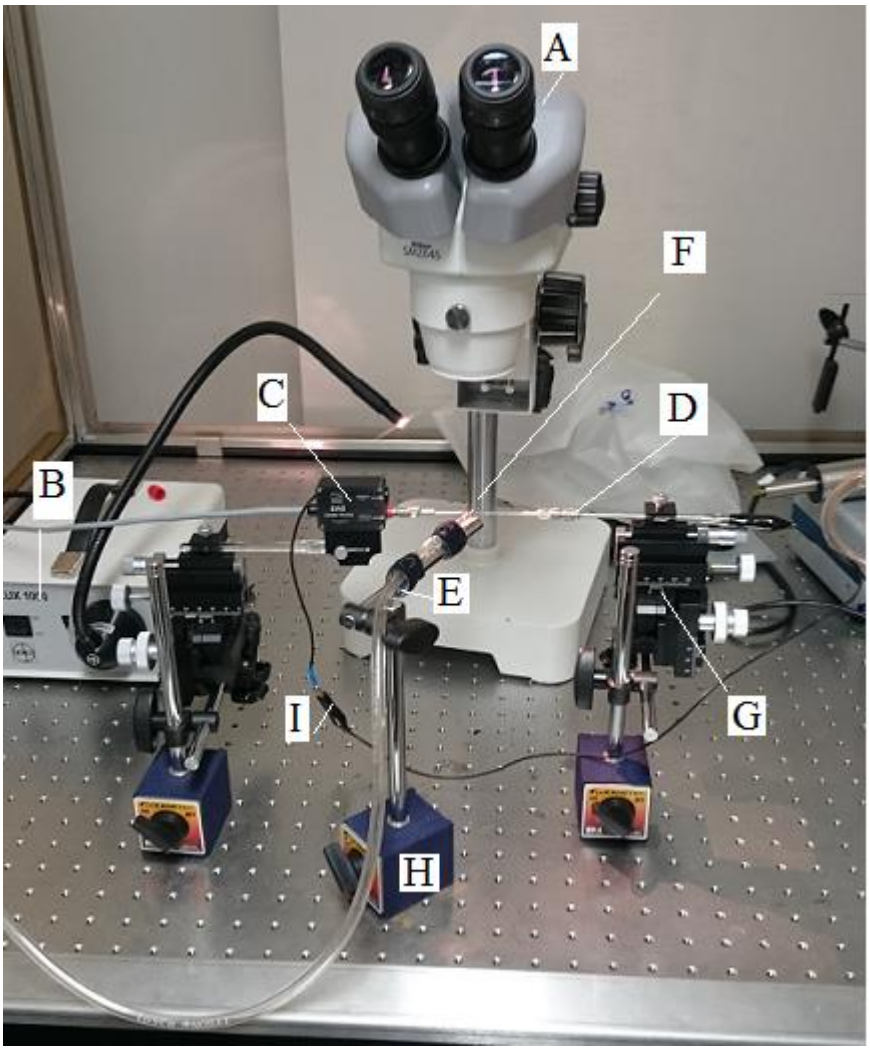

Figure 29: Electroantennography equipment including stereomicroscope (a), external light source (b), recording electrode (c), indifferent electrode (d), stimulus and continuous airflow application tube (e), fly subject mounted on electrodes (f), mobile staging heads (g), magnetic clamp stands (h), and grounding wire with alligator clamps (i)

Firstly, a stereo microscope and external light source were placed inside of a Type II Faraday cage (TMC). The Faraday cage was necessary to mitigate any confounding electrical

interference from the surrounding environment. A TMC 63-series High Performance Lab Table was installed in order to protect from possible noise contamination caused by physical interference such as vibrations. Staging heads (Harvard Apparatus Canada) were attached to strongly magnetized clamp stands, wherein the magnet could be turned off if repositioning the staging heads became necessary. The staging heads were comprised of an electrode holder and 3 gears which can be manipulated in turn in order to precisely position the electrodes in 3 dimensions for testing purposes.

The staging head which holds the recording electrode is attached via a wire to a 2-channel USB acquisition controller, known as the Intelligent Data Acquisition Controller (IDAC-2) (©Syntech). The IDAC-2 collects and amplifies the electrical signal reported by the recording electrode, and relays the signal to the recording software. The IDAC-2 has two possible input channels, and relays the information through a USB cord, which also functions as its power source.

The recording electrode (also referred to as the different electrode) is one of two types of electrodes used in this equipment design (Figure 30). This electrode is comprised of a silver wire that runs through a metal brace, which is secured into the staging head via metal threading similar to a standard screw. The silver wire must make contact with the metal base on the inside of the electrode holder in order for a response to be recorded. The metal brace has a circular opening which allows for a glass capillary tube filled with Ringer's solution to be slid over the silver wire, so that the silver wire is fully encased in the isotonic solution.

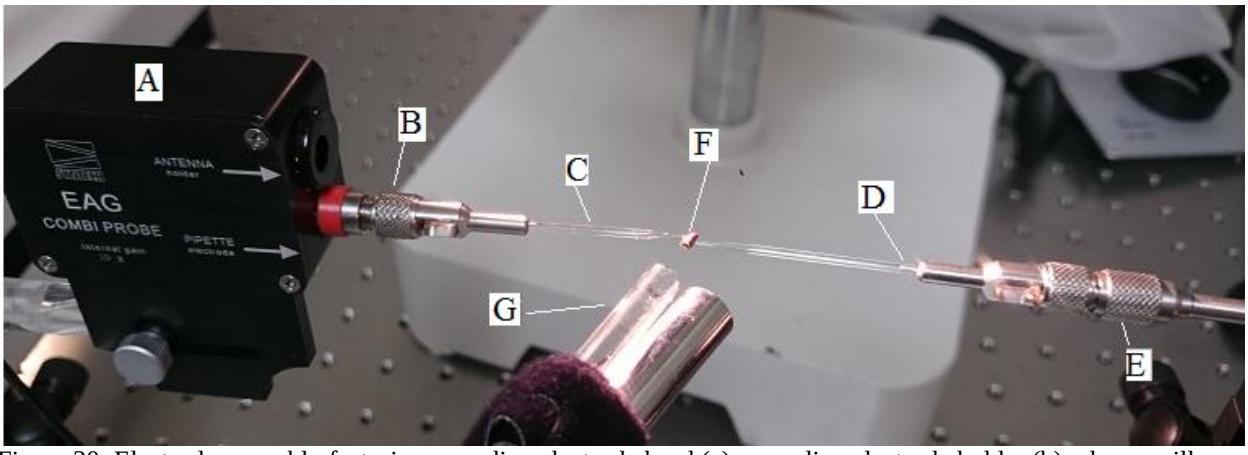

Figure 30: Electrode assembly featuring recording electrode head (a), recording electrode holder (b), glass capillary tube containing Ringer's solution (c), silver electrode wire (d), grounding electrode holder (e), excised *C. rufifacies* head (f), and stimulus/air application tube (g).

The glass capillary tubes are 1.5 mm in diameter and approximately 10 cm in length before being pulled. The EAG procedure requires a fine glass point in order to mount the test preparation, and the capillary tubes therefore need to be altered. The tubes were heated over a low-level blue flame on a Bunsen burner. The glass was twirled by hand at the apex of the flame until the glass began to soften. The tube was then removed from the heat and a pulling pressure was applied to both ends of the tube as evenly as possible. This allowed for the tubes to be narrowed to a very thin diameter before cooling, at which point the tubes were broken in the middle and two functional capillary tubes were created. The capillary tube that was used to cover the silver wire of the recording electrode needed to be of wide enough diameter at the tip that the end of the third antennal segment of the fly subject could be cradled in the glass and partially submerged in Ringer's solution. However, if the tip of the recording capillary was wide enough that the antennal tip did not fully plug the opening, this was considered too wide, as it allowed for an increase of electrical background noise to be recorded.

The indifferent (or grounding) electrode was positioned to mirror the recording electrode. The indifferent staging head was comprised of an electrode holder and positioning gears, as well as a 3 pronged, y-shaped connecting wire which had an attached metal alligator clip at each of

the 3 ends of the wire. One alligator clip was mounted on the electrode holder, with one clip on the grounding wire of the recording electrode and one clip attached to the Faraday cage. This completes the circuit via grounding the system, and allows for proper recording. The indifferent electrode included a silver wire within a metal brace, similar to the recording electrode. A glass capillary tube filled with Ringer's was also placed over the indifferent silver wire, but the indifferent capillary tube had a much narrower tip. This tip only needed to be wide enough to allow contact between the Ringer's solution and the inside of the test subject's brain.

Given that EAG measures minute changes in the electrical gradient across an antenna, the equipment is very sensitive and delicate. Further, the rarity of this equipment has the effect of limiting the resources available to assist in establishment and maintenance. Due to this, several extra measures were required prior to the use of the EAG equipment, as the initial configuration based solely on the provided manuals and instructional materials did not produce a functional or recordable signal.

In order to create a reliable signal to be properly recorded by the IDAC, completing the electrical circuit was necessary. Several different iterations of wire placement patterning were tested in order to properly connect and ground the indifferent electrode until a functional configuration as found. A functional grounding circuit was created via the use of a 3-pronged, y-shaped alligator clip wire, wherein the metal of the alligator clips connects the various components. One clip is attached to the end of the metal electrode holder which houses the indifferent electrode. A second clip is clamped to the Faraday cage, which acts to ground the electrode and circuit. The last clip is attached to the recording electrode via a specialized grounding ring that is housed within the staging head, which completes the circuit (Figure 31).

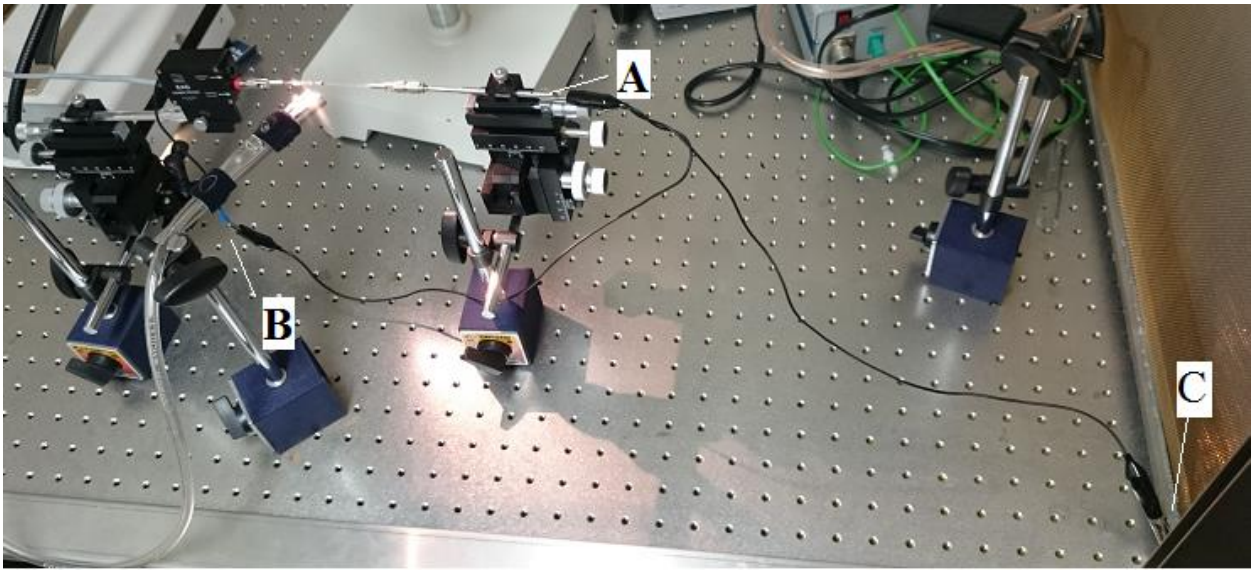

Figure 31: Grounding wire configuration, showing 3 alligator clamps attached to indifferent electrode (a), recording electrode (b) and Faraday cage (c).

In order for the electrodes to record a current, the silver wire of both electrodes first needed to be chlorided. When the silver wire is coated in a layer of silver chloride, it functions to convert, through contact, the ion current within the subject into an electron current that can be amplified and recorded by the IDAC (Lee & Krusse, 2008). In order to create chlorided silver electrodes, the silver wire purchased through Syntech was exposed to household brand bleach for several minutes until a layer of silver chloride was created along the surface of the electrode. As silver chloride is white in colour, the establishment of a coating was recognized by the distinct colour change of the electrode from shiny silver to dull white.

The configuration of the continuous airflow from the stimulus controller needed to be altered in order to account for the addition of humidified air, once the necessity became apparent. The rubber tubing that carries the air from the stimulus controller was severed and redirected into a 200 mL Büchner flask, which was filled with approximately 100 mL of deionised water. A glass insert delivered the air directly into the water, and the connection was considered complete when bubbles formed once the stimulus controller was activated. The air, once forced through

the water, was humidified and released through the side-arm of the Büchner flask. The continuous airflow line was attached to this side-arm, which then delivered the humidified air to the subject mounted upon the electrodes.

The inclusion of humidified air exacerbated the development of rust within the electrode holders, and at the grounding connections. This had the effect of dulling and eventually causing complete cessation of any electrical signal being detected by the IDAC. Once this concern was identified, maintenance measures were put in place to regularly identify and remove rust from any areas on the equipment.

APPENDIX B: Compounds Occurring within Samples

Table 10: Sample list showing which test compounds occurred within each of the collected samples

	BAME	DMDS	DMTS	Ethanol	Indole	Phenol
Cont D0-1				✓		
Cont D0-2				✓		
H3 D0	✓			✓		
H3 D1	✓			✓		
H3 D2				✓		
H3 D3		✓		✓		
H3 D5		✓	✓	✓	✓	✓
H4 D0				✓		
H4 D1				✓		
H4 D2	✓			✓		
H4 D3				✓		
H4 D5	✓			✓		
H5 D0	✓			✓		
H5 D1				✓		
H5 D2				✓		
H5 D3				✓		
H5 D4	✓			✓		
H5 D5	✓	✓	✓	✓		
H6 D0	✓			✓		
H6 D1	✓			✓		
H6 D2	✓			✓		✓
H6 D3	✓			✓		
H6 D4	✓			✓		
H6 D5	✓			✓		
P2 D5		✓	✓	✓		✓
P3 D1	✓			✓	✓	
P3 D2				✓		
P3 D3	✓			✓		
P3 D4	✓			✓		
P3 D5	✓			✓		