UNIVERSITY OF DERBY

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

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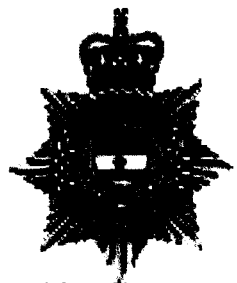

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Abstract

Research was undertaken to explore the succession of insects on a decomposing body and to identify the semiochemicals responsible in initially attracting blowflies, specifically *Calliphora vomitoria*, to the body.

Five adult pigs were used for studies of insect succession and decomposition. Different species of insects are attracted to a decomposing body at different stages of decomposition. This was evident throughout all the experiments with blowflies *C. vicina* and *C. vomitoria* being the first to arrive on the experimental pigs and colonise the body. Muscidae and *Lucilia* sp. closely followed. Silphidae and Staphylinidae beetles, adults and immature stages, were present early during decomposition; however, during the last stage of decomposition Cleridae, Histeridae, and Dermestidae beetles were much more numerous. Warmer temperatures presented increased insect activity and rate of tissue removal, while rain triggered a mass larval migration from the body. Maggot masses greatly elevated the temperature inside a corpse. While the presence of adults of a single species on the body could not alone be used to identify a specific point in insect succession, their presence with a particular combination of other insect species, their abundance, and that of their offspring all helped define a succession pattern.

Combined gas chromatography (GC) and electroantennogram (EAG) experiments identified four volatile chemicals recovered from the decomposing pigs which elicited a receptor response from *C. vomitoria*: dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, and a compound of unknown structure (KI 881-885). Dose response experiments demonstrated that dimethyl trisulfide and dimethyl tetrasulfide elicited the greatest receptor responses. A positive correlation was found between the concentration of these compounds and the number of flies on the pig carcass. The chemical composition and concentration of the compounds associated with decay, in particular the EAG-active compounds, varied between the different stages of decomposition. None of the EAG-active compounds caught a significantly greater number of blowflies than unbaited traps when water trap and sticky trap experiments were conducted. The positive control, a mixture of sodium sulfide and liver, also failed to capture significantly more blowflies than the controls. While fewer than expected blowflies were captured during these experiments, a greater insight into suitable trapping methods and bait composition was gained.

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CHAPTER 1 – INTRODUCTION

1.1 INSECTS AND CRIMINAL INVESTIGATIONS

Decomposition is a natural process and allows nutrients to be re-absorbed and recycled. This can be a very slow process by bacterial action alone; however, insects significantly accelerate this progression thus playing an important role within our ecosystem (Haskell *et al.*, 1997). The insect and other arthropods associated with decomposing bodies exhibit predictable behaviours allowing for reliable conclusions to be made about a particular corpse.

Forensic Entomology has been described as the study of insects and other arthropods associated with legal issues (Keh, 1985; Haskell *et al.*, 1997; Hall, 2001; Gennard, 2007). Insects can be found in an infinite amount of locations opening a wide range of applications for forensic entomology, from a simple food contamination case to a serious homicide investigation.

When suspicion is cast upon a death, insects associated with decomposing bodies become important in the formation of a criminal investigation. Insects can be used to detect the presence of drugs in a body, manner or cause of death, and find an association of a suspect with the crime scene (Catts & Goff, 1992; Gennard, 2007). Movement of a body after death can also be detected. As insects have a relatively defined diversity that exists in specific geographical areas, insect species found on a corpse that do not correspond with species normally found in the area can be a good indicator that the body in question was moved from one area to another (LeBlanc, 1998; Wolff *et al.*, 2001). However, forensic entomology is primarily used to determine time since death, also called the post-mortem interval (PMI) (Haskell *et al.*, 1997; Amendt *et al.*, 2007a).

Traditional methods of determining the PMI, such as the rate of body cooling, can only be used up to the first 72 hours after death as the medical information then becomes unreliable (Dirkmaat, 1997; Gennard, 2007). After this time, other methods of determining PMI must be employed. Thus, entomology still remains the most reliable

method of assessing the time period since death (Kashyap & Pillay, 1989; Buchan & Anderson, 2001; Williams *et al.*, 2001; Vass *et al.*, 2002).

Insects visiting the decomposing carcass can be divided into different groups according to their ecological role on the body (Easton & Smith, 1970). Catts & Goff (1992) list four categories of arthropods found on decomposing bodies:

- Necrophagous insects or scavengers – These feed on the carcass itself. This group is formed primarily by blowfly larvae (Calliphoridae), other Dipteran larvae (e.g. Sarcophagidae, Muscidae), as well as both immature and adult stages of Coleoptera (Silphidae and Dermestidae) which feed on necrotic tissue and liquid during their development.
- Predators and Parasites – Predators feed on the insects present on the decomposing body, most often feeding on Dipteran larvae. These predators include such Coleoptera as Staphylinidae, Histeridae, Silphidae, and Cleridae. Mites (Families: Macrochelids, Parasitids, Parholaspids, and Uropodids) and spiders (Family Lycosidae) which predate on insects, nematodes, and other mites on the carcass also belong to this group. Hymenoptera, such as the parasitic Brachonid wasp, are often present on the corpse to parasitize Dipteran larvae.
- Omnivorous insects – These feed on both the corpse and the associated fauna. Such behaviour can be seen of Hymenoptera (Family Vespidae) and Coleoptera (Families Silphidae and Staphylinidae). Some Dipteran larvae which are necrophagous early in their larval development may become predaceous during their later larval instar, such as the Muscid, *Hydrotaea* sp.
- Incidentals – This includes arthropods that use the body as an extension of their normal habitat. Some are on the body seeking shelter or have simply arrived accidentally. These include springtails (Order Collembola), some spiders (Order Aranae), centipedes (Class Chilopoda), woodlice (Order Isopoda), and certain mites (Families: Acaridae, Lardoglyphidae, and Winterschmidtiidae).

1.1.1 Blowflies

Flies of the family Calliphoridae (Order Diptera), called blowflies, are among the most common of all insects and can be found in many diverse environments (Erzinçlioğlu, 1996). While location and food source may vary, the majority of blowflies develop in decomposing organic matter, including carrion (Smith, 1986).

The two most significant groups in relation to their associations with humans are bluebottles (*Calliphora* sp.) and greenbottles (*Lucilia* sp.) flies:

- The bluebottles are distinguished by their shiny blue abdomen and their large size. These blowflies are robust and can be present on a decomposing carcass during shady or sunny conditions and have been know to frequent bodies outdoors and indoors during both warm and cool temperatures (Erzinçlioğlu, 1996; Smith, 1986; Anderson, 2001; Campobasso *et al.*, 2001).
- The greenbottles are recognised by their metallic green abdomen. These particular blowflies are generally smaller in size and prefer sunlight and warm weather. They are often present on bodies found indoors and are much less likely to be active in cool weather (Erzinçlioğlu, 1996; Smith, 1986; Anderson, 2001; Campobasso *et al.*, 2001).

Blowflies exhibit behaviours which can have a severe economic impact on the animal farming industry. Slaughter-houses, for example, have always been potential breeding grounds for blowflies, of which the most numerous species include: *Calliphora vomitoria*, *C. vicina*, *Lucilia caesar*, *L. illustris*, *L. sericata*, and *Protophormia terraenovae*. These are most often attracted to refuse of animal slaughter including inedible guts and offal (Green, 1951). This not only gives the appearance of unsanitary conditions, but can also potentially cause the spread of diseases and extend to the downgrading of meat which has been previously graded fit for the consumer. The large quantities of migrating larvae also bring forward complaints by the local community as increased sheep strike in neighbouring farms has been linked to the unusually high population of potentially parasitic blowflies, such as *Lucilia sericata,* around slaughter houses (Green, 1951).

While many blowflies lay eggs on dead carcasses, others will also lay eggs on living tissue (Fisher *et al.*, 1998). This adaptation possibly evolved due to increased

competition on decomposing bodies. A common form of myiasis is sheep-strike. *Lucilia sericata* is the most problematic species associated with sheep strike in England and Wales (Ashworth & Wall, 1994). The adult flies lay eggs on wool soiled with urine or faeces where the larvae develop and cause considerable distress, if not death, to the animal (Smith, 1973). This is a major problem in the United Kingdom resulting in high costs associated with the prevention of infestations, treatment, and loss of livestock each year (Ashworth & Wall, 1994; Fisher *et al.*, 1998). *C. vicina* and *C. vomitoria* have not yet been linked with sheep-strike; however, cases of wound myiasis in humans have occasionally been reported (Smith, 1973).

Despite their obvious disadvantages, blowflies have proved themselves invaluable in certain areas of life. Along with other arthropods, they play an important role in decomposition and nutrient recycling; they are the largest contributors of tissue removal on a carcass (Haskell *et al.*, 1997). Blowflies also hold the greatest importance when determining the post-mortem interval (Erzinçlioğlu, 1996; Smith, 1986). They are often the first to arrive on a human corpse and, therefore, can give the most valuable information concerning time since death and other valuable information about the body (discussed in Section 1.1.2).

Life Cycle

Blowflies, such as *Calliphora vomitoria*, go through a relatively rapid and predictable life cycle. Most of their life cycle is spent on a decomposing body. Female flies lay their eggs on a decomposing carcass, usually near moist areas so that the eggs do not desiccate. The eggs are white or pale yellow in colour and measure approximately 1.5mm in length (Figure 1.1). Within 12-24 hours at room temperature, the larva hatches, leaves the egg case, and begins to feed on the body fluids. The larva will moult its outer layer twice resulting in three distinct stages, forming the 1st instar, 2nd instar, and 3rd instar larva (Figure 1.2); each stage feeding more voraciously than the next. Finally once the larva has sufficiently fed it will empty its gut and leave the carcass to seek shelter by burrowing into the soil. The outer cuticle of the larva hardens to form a hard brown hard shell, called the puparium (Figure 1.3). The pupa can remain in this stage for several weeks depending on the temperature. The greatest metamorphosis takes place during this stage and once the development is complete, the adult fly emerges (Erzinçlioğlu, 1996).

Figure 1.1: *Calliphora vomitoria* – eggs

Figure 1.2: *Calliphora vomitoria* – 3rd instar larvae

Figure 1.3: *Calliphora vomitoria* - puparia containing pupae

1.1.2 Establishing the Post-Mortem Interval

Time since death is established using two methods, larval development and insect succession (Haskell *et al.*, 1997; Amendt *et al.*, 2007a):

Larval Age Determination

During the early post-mortem period, time since death is determined by assessing the development stage of the oldest immature specimen present on the body. Blowflies are often the first to arrive on a body; they can arrive and oviposit within minutes or hours of death (Catts & Goff, 1992; Anderson, 2001). These immature stages are vital in determining the PMI. The larvae must be identified to the correct species before any further assessments can take place, as rates of development vary between species. The larvae are measured and/or weighed, and the stage of development must be determined. Temperature has a great influence on the rate at which the larvae will develop; therefore, the temperature and environmental conditions to which the immature stages were exposed must be estimated. The final age determination is made by comparing the stage of development of the larvae and the environmental conditions with known growth rate data for that particular species (Amendt *et al.*, 2007a). This is a determination of when the first eggs were laid on the body and not the actual time of death. This is why a minimum estimation is given.

Arthropod Succession

As the first immature insects leave the body and complete their life cycle, successional patterns become the primary method of PMI estimation. Certain arthropods can be linked to a well-established decomposition stage, thus, allowing for an estimation of PMI (Campobasso *et al.*, 2001). This estimation is rarely based on one species alone, but rather the entire composition of the arthropod community, associating it with a known successional pattern, the environment, and the stage of decomposition (Amendt *et al*, 2007a). As the arthropod fauna varies with geographical location and the changing seasons, it is mandatory to be familiarised with the successional pattern relating to the location of interest (Catts & Goff, 1992; Haskell *et al.*, 1997; Amendt *et al.*, 2007a).

For both methods of time since death determination, a shorter PMI will allow for a narrower estimated time range. Therefore, the longer the time period since death, the less precise the PMI estimation will be (Haskell *et al.*, 1997; Dix & Graham, 2000). However, the season in which the person died can also be determined by insect artefacts, such as empty fly puparia, left behind on the decomposed body (Haskell *et al.*, 1997).

While insects play a great role in decomposition, decay of a body is also influenced by weather, temperature, scavengers, location, and the circumstances of death (Haskell *et al.*, 1997; LeBlanc & Strongman, 2002; Anderson, 2001). Weather directly affects ambient temperature and insects associated with the body. Weather can change the temperature of a corpse and even delay the colonisation of the body. Rain cools the temperature of a body found outdoors. Heavy rain will delay access to the corpse and even dislodge eggs that have been recently laid. For example, the greenbottle blowflies, *Lucilia* sp., are much less likely to come to a corpse during overcast conditions (Haskell *et al.*, 1997; Erzincliogly, 1996).

Temperature has a direct influence on the decomposition process and insect activity of the corpse. Low temperatures can slow the decay of organic matter, while warm temperatures increase the number and type of carrion insects found on and around the body (Campobasso *et al.*, 2001). Mass aggregation of Diptera larvae on a body can greatly increase the micro-environmental temperature. Anderson and VanLaerhoven (1996) recorded maggot mass temperatures of up to 20°C above the body or ambient temperature, thereby increasing the rate of insect activity.

Decomposition is not uniform; the breakdown of tissue can progress faster in some areas than others and is largely affected by insect activity. Flies lay their eggs in moist areas in the natural orifices of the body such as the eyes, nostrils, and mouth (Smith, 1986). Areas of injury are suitable oviposition sites and therefore decomposition will occur more rapidly in those areas (Catts & Goff, 1992). For example, a larval mass in the palm of a hand often indicates the site of defensive wounds seen in stabbing cases. A similar aggregation of insect activity in the chest can indicate gunshot wounds, blunt force trauma, or stabbing. Nude human remains with a large collection of larvae in the pelvic area may indicate sexual violence (Haskell *et al.*, 1997).

Decomposition is also affected by the environment in which the body has been placed. For example, buried bodies and those found in water will attract different species of insects and will decompose at a slower rate than those exposed to direct sunlight. Although buried remains are still colonised by insects, the sequence of colonisation and the time elapsed before the first insects reach the body is greatly altered (Anderson & VanLaerhoven, 1996). Phorid flies are the insects most commonly found on buried bodies (Easton & Smith, 1970; Anderson, 2001). An even greater difference in fauna is seen in bodies found in water (Anderson, 2001). The body of water (pool, lake, stream, ocean) in which a body is found has a great affect on the aquatic life present on the corpse (Anderson, 2001; Merritt & Wallace, 2001). Most of the research concerning decomposition of a body under water has been focussed in the freshwater environment. While scavenging fish and crustacean will feed on the body, aquatic insects which can be found feeding on submerged corpses include caddisflies (Order Trichoptera), springtails, and heptageniid mayflies (Order Ephemeroptera) (Easton & Smith, 1970; Merritt & Wallace, 2001; Hobischak & Anderson, 2002).

In an attempt to disguise the remains, some bodies may be concealed or wrapped. Those placed in large freezers or in the well sealed boot of a car will deny insects' access to the body. Those loosely wrapped will often not present any difficulty for insect access. However, other bodies tightly wrapped in sheets or plastic wrapping often restrict access to the insects until scavengers, such as foxes or rodents, create an opening and an opportunity for insects to colonise the body (Anderson, 2001).

Decomposition and insect behaviour are dependent on numerous variables; none can be ignored and all must be understood. Therefore, all of the environmental information available needs to be gathered by the forensic entomologist. As well, in-depth knowledge about the life cycle, behaviours, and geographical fauna of the insects found on the body needs to be acquired. Often, the questions asked of a forensic entomologist are very important to the process of a criminal investigation and can greatly affect its direction. Thus, a clear understanding of the above will only serve to help the entomologist's investigation. The use of arthropods in criminal investigations is becoming increasingly important as their role in determining time since death is becoming widely recognised.

1.2 INSECT ANTENNA – THE SENSORY ORGAN

The capacity to sense and respond to the chemicals present in the environment exists in nearly all living creatures; however, this ability is particularly important in insects (Vickers, 2000). Insect olfactory organs involved in the response to volatile chemicals are located primarily on the antennae (Borror *et al.*, 1989). Insects use chemical signals to navigate through their environment. The chemicals in their environment are used to find a mate, a food source, and an oviposition site (Cragg & Cole, 1956; Borror *et al.*, 1989; Castner, 2001). They must be able to quickly process the information within an odour plume as this will affect their entire behavioural response.

Insect antennae are covered with a very large number of sensillae (Castner, 2001; Shields, 2001). These sensillae house the olfactory receptor cells (ORC) which detect environmental status, any change in this, and transmit the information to the central nervous system (Shields & Hildebrand, 2001). Odour molecules reach the olfactory sensillum, which is lined with the ORC, and then make their way to the underlying sensory neurons. It is here that the chemical signal is converted into an electrical one which is then sent to the brain of the insect (Shields & Hildebrand, 2001).

Insects not only detect the presence of odours but also the chemical gradient, giving them vital information about the location of an odour source (Vickers, 2000). They detect volatile chemicals that indicate host suitability and also the presence of potential competitors (Shields & Hildebrand, 2001).

The morphology and position of chemosensory appendages, such as the antenna, may help determine its importance and efficiency in capturing chemical cues. For example, most insects possess long, movable antennae which provide greater capacity to detect volatiles without require the insect to re-position its body frequently to detect an odour (Vickers, 2000). These evolutionary features indicate that chemical cues play an important role in insect behaviour and survival.

1.3 THE DECOMPOSITION PROCESS

Decomposition is said to commence almost immediately after death (Vass *et al.* 1992; Dix & Graham, 2000). The body can go through several phases and rates of decomposition, which are highly dependent on weather, temperature, humidity, and the environment in which the body is left, *i.e.* indoors, outdoors, buried, under water, wrapped, etc. (Clark *et al.*, 1997; Dix & Graham, 2000).

Immediately after death, the cells start to die and enzymes begin to digest the cells from the inside out, a process called autolysis or self-digestion. This action causes the cells to rupture and release nutrient-rich fluid (Dix & Graham, 2000; Vass, 2001). Tissues containing more digestive enzymes, such as the liver, will be digested at a faster rate than those containing fewer enzymes. Putrefaction occurs as the bacteria, already present in the large intestine, begin to destroy the soft tissues resulting in the production of liquids and various gasses (hydrogen sulfide, carbon dioxide, methane, ammonia, sulphur dioxide, and hydrogen) (Vass, 2001). These bacteria gain access to the vascular system and spread throughout the body. There is often a green discolouration associated with these changes (Williams *et al.*, 2001). As the blood begins to break down within the blood vessels and the skin loses pigmentation, the dark stained blood vessels can be observed through the skin producing an effect called marbling. The outer layers of skin begin to slip off the body while fluid under the slipping skin form blisters. Trapped gasses cause the body to become 'bloated'. The body swells, primarily within the abdomen, and decomposed blood or faecal matter may be 'purged' from the lungs, airways, or rectum.

Once the trapped gasses have escaped, a much more active stage of decomposition can be observed. Volatile compounds derived from the decomposition of materials, such as proteins and fats, are subsequently produced. These include indole, 3-methylindole (skatole), putrescine, cadaverine, phenols together with carboxylic acids and glycols (Vass *et al.*, 2002). The greatest physical changes to the cadaver occur at this time. The organs degrade and become unrecognisable forming a grey exudate within and beneath the body. Following this active decomposition the body may become skeletonised leaving behind just dry leathery skin and bones, depending on the environment in which the body has been resting (Clark *et al.*, 1997; Dix & Graham, 2000).

Throughout these physical changes different volatile gasses are released from the body. The chemical compounds released will vary in composition and concentration as decomposition progresses (Vass *et al.*, 1992). While Vass has studied the compounds produced during the decomposition of a buried body, the specific volatiles in relation to the attraction of carrion insects to the decomposing unburied corpse have not been examined.

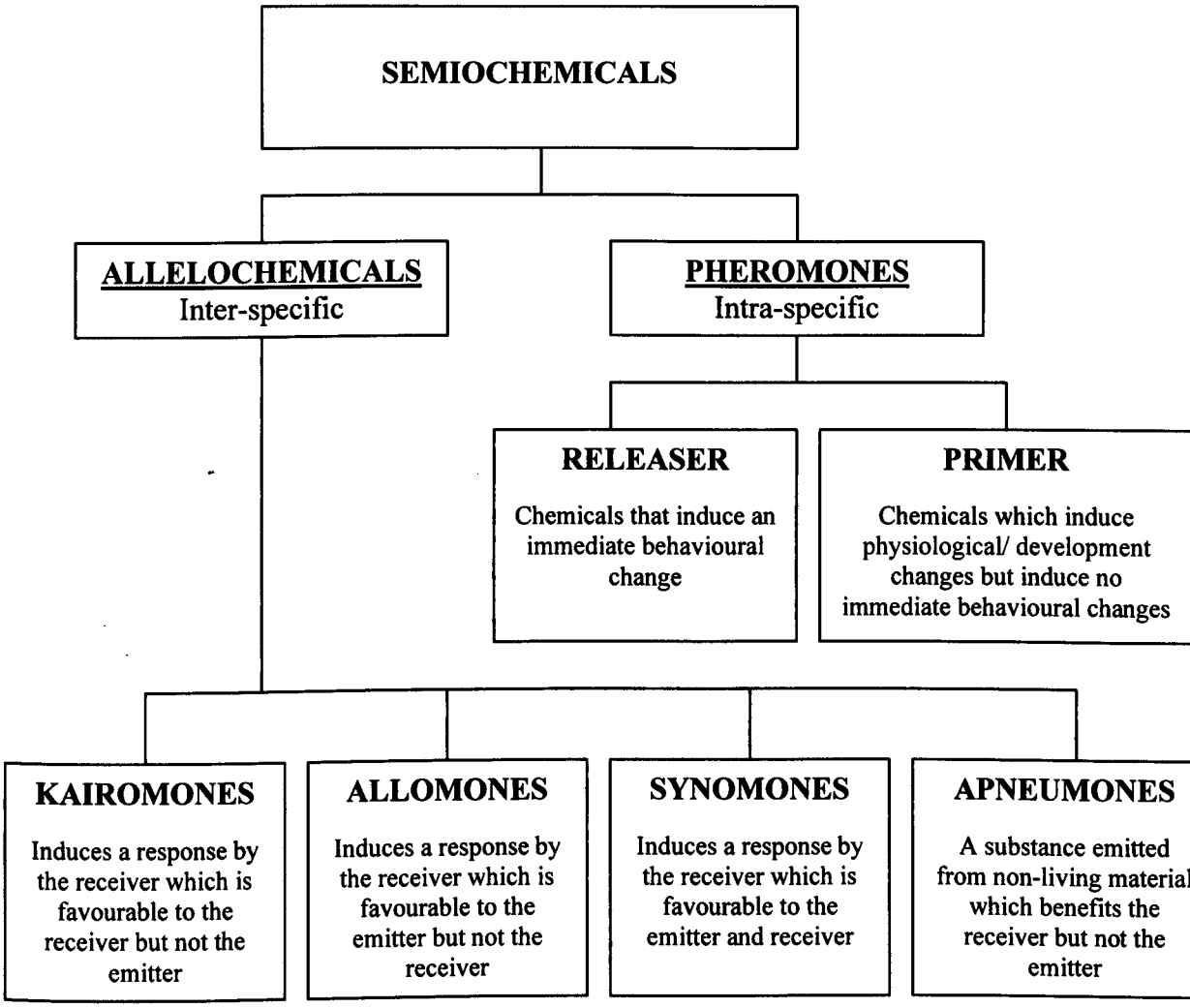
1.4 SEMIOCHEMICALS - THE STUDY OF ODOURS

Carrion odour releases a wide range of chemicals. Volatile molecules appear within four minutes of death but human olfactory perception is too insensitive to detect such short-term degradation. As discussed in section 1.2, blowflies have evolved a mechanism to detect matter, such as a decomposing corpse, in order to survive. They detect the decomposing body by olfactory stimuli which mediate a behaviour mechanism to lead them to the food source (Pickett *et al.*, 1998). Chemical compounds emitted from the decomposing body provide the location and physiological information about the body from which the volatile compounds are emitted (Angelopoulos *et al.*, 1999).

Behaviour-modifying compounds are called semiochemicals. These are chemical compounds that convey information between organisms (Nordlund & Lewis, 1976; Blight, 1990). Semiochemical is derived from the Greek word *semeion*, which means 'sign' or 'signal' (Angelopoulos *et al.*, 1999).

The categories of semiochemicals described by Nordlund & Lewis (1976) are used in this thesis (Figure 1.4). Chemical agents are classified by categories according to the effect on the receiver or emitter. Semiochemicals are divided into two groups, pheromones and allelochemicals. Pheromones are those chemicals which causes interactions between individuals of the same species (intra-specific), while allelochemicals create interactions between different species (inter-specific) (Angelopoulos *et al.*, 1999). However, no function is assigned to a single chemical as a particular compound may evoke different responses from different organisms (Isaacs, 1994).

Table 1.4: Classification of the different categories of semiochemicals (Norlund & Lewis, 1976; Howse *et al.*, 1998)



Those semiochemicals emitted from decomposing bodies are classified as 'apneumones'. These are chemicals which are emitted by non-living material, such as a corpse, and evoke a behavioural reaction from the receiver. The word apneumone was derived from the Greek word *ā-pneum*, meaning breathless or lifeless. This category was originally described by Nordlund & Lewis (1976).

Semiochemicals convey specific information concerning the location of the emitter, the suitability of host, and its physiological state (Pickett *et al.*, 1998). A behavioural response can be triggered with only very small quantities of chemicals (Cork *et al.*, 1990). Odour emitted by the emitter is often perceived by the receiver beyond its

visual range (Gikonyo *et al.*, 2003). Semiochemicals are volatile in nature and airborne, which means that they can be detected from long distances and perceived by a number of other organisms of the same or different species (Angelopoulos & Pickett, 1998; Selby, 2003). The response generated will be different depending on the source emitting the odour and the insect receiving this information (Picket *et al.*, 1992).

Insects have high specificity to individual compounds. This sensitivity is to single molecular structures or a specific group of molecules, rather than a human's general response to a whole series of structural types (Pickett *et al.*, 1998). To properly understand the blowfly's role in decomposition, and therefore forensic entomology, it is important to differentiate between the behaviourally active compounds and those that do not generate any behavioural response (Park & Cork, 1999). Air entrainments are capable of isolating volatile chemicals emitted by a body. Electrophysiological and behavioural assays single-out biologically and ecologically relevant natural extracts and single semiochemicals (Logan, 2005). Following the isolation of the semiochemicals, gas chromatography (GC) and GC-mass spectrometry (GC-MS) techniques are used to accurately identify the compounds. Finally, behavioural assays can determine the role of the isolated semiochemicals in the biology of the insect (e.g., repellent, attractant).

1.5 JUSTIFICATION OF AIMS

In-depth knowledge of the blowfly's life cycle and behaviour has always been important for matters concerning myiasis or forensic entomology; however, very little is know about the chemical stimuli which directly influence the actions of these insects. As of yet, there has never been a direct study of carrion insects in relation to the odours emitted by a decomposing body.

As electrophysiology and analytical chemistry techniques continue to be developed, it is possible to learn more about theories which were simply assumed in the past. Semiochemicals have many possible applications in the present economy. They can potentially be used as a controlling agent to modify insect behaviour (Cork *et al.*, 1990). Successful attempts have already been made in the United Kingdom (Ashworth

& Wall, 1994) and Australia (Muirhead-Thomson, 1991) using chemical lures to reduce the number of sheep-strike on livestock.

The entomological fauna currently active in the Derbyshire region has been subject to only a few research papers and no carrion insect data is currently available. Clearly, a better understanding of insects associated with decomposing bodies in this region is needed before studies concerning behaviour modifying chemicals can be undertaken.

The combined understanding of carrion entomo-fauna and the effect of the chemical-releasing stimuli present many possibilities. For example, knowing precisely when a female blowfly is attracted to a corpse and understanding other behaviours initiated by the semiochemicals would help to extract infinitely more information about the blowfly as well as the assessment of an ever more accurate minimum PMI estimate.

1.5.1 Aims and Objectives

Chapter2 – Insect succession field studies using pigs

The aim of this chapter was to determine the entomo-fauna associated with decomposing pig carcasses in the Derbyshire region in order to become familiarised with these insects and to gain a better understanding of their interactions with the body and other arthropods. Therefore, the following work will be carried out:

- Collection of insects, comprising various stages of development, in order to identify the different species attracted to and inhabiting the carcass.
- Make observations to establish whether ambient temperature, other arthropods, and different weather conditions, such as sunshine, rainfall, and wind, could be linked to different behaviours exhibited by the insects.
- Record species composition on the body.

Chapter 3 – Analysis of semiochemicals and insect receptor responses

The aim of this chapter was to determine which compounds, associated with the decomposing body, elicited an electrophysiological response from the blowfly species, *Calliphora vomitoria*. The following will be necessary:

- Determine the most suitable method of collecting volatiles from pig carcasses during all stages of decomposition.

- Using combined gas chromatography (GC) and electroantennogram (EAG) experiments to isolate compounds which elicit an electrophysiological response from *C. vomitoria* and to confirm the tentatively identified volatile chemicals.
- Another important objective of this chapter is to determine whether the chemicals associated with the decomposing pig, particularly the EAG-active compounds, fluctuate between the different stages of decomposition in order to determine whether an association can made between the compounds and state of decay.

Chapter 4 – Behavioural responses of blowflies to trap experiments incorporating the previously identified electrophysiological-active compounds

The aim of this chapter is to combine the findings of the previous examinations to determine the possible behaviour initiated by the semiochemicals in order to further explain the link between certain decomposition volatiles and blowflies commonly found on corpses.

This will be accomplished by conducting bioassay field experiments using water and sticky traps as trapping devices and the EAG-active compounds as bait.

CHAPTER 2 – INSECT SUCCESSION FIELD STUDIES USING PIGS

2.1 INTRODUCTION

Death and the events that occur thereafter have always been intriguing and wondrous to man. Early historical collections uncover jewellery of carrion insects; old scriptures of "worms" on cadavers; paintings and carvings depicting decomposition (Benecke, 2001). In *The Iliad* (Book XIX), written by the Greek poet Homer in approximately 800 BCE, Achilles expresses worry that flies might get into the wounds of his dead friend Patroklis showing that flies' link to death was recognised (Greenberg & Kunich, 2005).

The first documented cases of murder being solved using flies were published in 1247 by Sung Tźu, Chinese lawyer and death investigator, in the book *The Washing Away of Wrongs*. One case in particular describes how a sickle, which had attracted many flies due to invisible traces of blood, led to a confession from the murderer (Greenberg & Kunich, 2005; Benecke, 2001). It can only be speculated if Sung Tźu realised the impact entomology would have on today's world and, more specifically, the legal system.

Flies are often the first to arrive at the scene of a murder, and therefore very important in determining time since death. In-depth knowledge of their life cycle, habitat, and behaviours can help to determine the time since death and countless other details about the crime (Greenberg & Kunich, 2005, Gennard, 2007). These living witnesses may differ in many respects from other insects; however, each particular species leads a very predictable life cycle. This "predictability" needs to be understood before any worthwhile legal investigation can take place.

Since the early references to carrion flies, their notoriety has grown and, as well as their Latin binomial titles, they can be referred to as Blowflies. More recently, research has been conducted to expand our knowledge of decomposition and carrion insects. Megnin (1894) originally listed eight insect "waves" that appeared on exposed decomposing human bodies, producing the first detailed list of insect succession. This list was revised by Johnson & Villeneuve (1897) who showed that different

geographical locations give varying results. While in theory these "waves" would depict a simple and clear-cut answer to the time-line of an investigation; the reality is that the relationship between insects, as living organisms, and their environment, including carrion, is much more complex. Many factors influence what species will reach the carcass and at what time in the decomposition. Location will have a significant influence on the entomofauna: Carcasses in Australia, for example, will not contain all of the same insect species as bodies decomposing in the United Kingdom. *Calliphora dubia* (Diptera: Calliphoridae), the primary colonizer of carrion during the summer months in south-western Australia (Dadour *et al.*, 2001) has not been found on bodies in the United Kingdom; It is rather *Calliphora vicina* and *C. vomitoria* that are the primary colonizer of bodies in the UK during the summer months (Davies, 1999; also observed by author). Even within a country entomofauna can differ due to altitude, temperature, and concealment (Erzinçlioğlu, 1983). Fortunately there are some species that make relatively predictable appearances on the carcass and leave evidence of their presence in the form of offspring, as in the blowflies, or faeces (frass), such as the Dermestid beetle. It is therefore important that many geographical areas be researched in order to accumulate the relevant information.

Studies to increase our knowledge of carrion insects have been conducted on many animals such as mice (Davies, 1990), guinea pigs (Bornemissza, 1957), foetal pigs (Payne, 1965; LeBlanc & Strongman, 2002; Archer, 2004), and sheep (Davies, 1990). While these have allowed us to gather valuable information, the direct comparison to human cadavers cannot so easily be made as size and the presence of fur or wool can alter decomposition (Anderson, 1997b). Smaller carcasses are attractive to burying beetle (examples: *Nicrophorus defodiens*); however, these are rarely found on larger cadavers (Scott, 1998). The blowfly *Calliphora vomitoria*, in some cases, has been noted to completely avoid smaller carcasses (Greenberg and Tantawi, 1993). Decomposition can also be affected by carcass size as smaller carcasses cannot sustain large larval masses, also know as maggot masses, which are known to increase the rate of tissue removal and therefore rate of decomposition due to their feeding activity (James *et al.*, 2002; Rodriguez & Bass, 1985). It has been found that, next to human cadavers, pigs are the most reliable models of human decomposition (Dadour *et al.*, 2001). The domestic pig (*Sus scrofa*) is most often used in research as it is omnivorous, with a digestion (including digestive enzymes) similar to that of humans,

and it is relatively hairless with skin so similar to ours that it can even be used in human skin grafts (Anderson, 1997b). It is also possible to obtain pigs of similar weight to that of an average human torso, about 35-50Kg. All of the reasons above contribute to the increasing popularity of this animal as a primary research subject.

In Britain, very few research studies have been published on the behaviour of arthropods found on decomposing pigs (Turner & Wiltshire, 1999). For a period of two years, three succession experiments, using pigs, were conducted in Ripley, Derbyshire, England. The purpose of these experiments were to:

- Establish a possible link between the number of Calliphorid individuals on a body compared to the ambient temperature.
- To investigate the number of species of insects attracted to pig carcasses and their behaviour when in the presence of other arthropods and when subjected to varying weather conditions such as sunshine, rainfall, and wind.
- To establish whether a faunal succession actually did appear on decomposing carcasses in this geographical region.
- To determine the relative numbers of *Calliphora* individuals, and other forensically important insects, attracted to the carcasses in relation to the stage of decomposition.
- To provide carrion insect fauna information for the Derbyshire Constabulary and aid other forensic entomologists during their investigations.

2.2 METHODS

2.2.1 Experiment 1 – Summer 2002

This first experiment was carried out in the spring and summer of 2002 from the 20th of May to the 16th of October. Observations described below were recorded in order to determine the behaviour of carrion insects and the effects of weather.

Research Site

A site that was secure and away from people was needed to comply with Health and Safety regulations. Therefore, experiments were conducted at The Police Headquarters in Ripley, Derbyshire (Figure 2.1). The land in this area is in possession of the police,

all of which is kept very secure. An open area near the helicopter pad and the skid-pan was chosen as the particular site to conduct the experiments. This site was completely surrounded by a high fence keeping the public and many animals away from the area. Police officers and mechanics working for the Constabulary had access to this site but worked several hundred metres away from the chosen location. The dead pigs were placed 20 to 70 metres north-west of the skid-pan. This did not pose a problem as the skid-pan vehicles did not leave the paved (slippery) surface and officers had no need to come to the far end of the skid-pan. The site had a flat grassy surface with trees, such as birch trees (*Betula* sp.), at the far end. Behind the trees was a small trench containing vegetation such as grasses, low shrubs, and ferns. Beyond the fence, to the east, was a privately owned field accommodating a few cows and a horse more than 250 metres away from where the dead pigs were placed and the experiments were conducted. To the north-west of the experimental site was an open field, owned by the Constabulary, reaching a row of trees. This open field was at times used on rare occasions to train police dogs. Many rabbits, as well as some foxes, were present in all fields.

Figure 2.1: Photo of the research site at the Derbyshire Constabulary, looking north.

Placement of Pig (set-up)

A 45 Kg pig (male) was acquired from Broomfield Hall. It was killed in a humane condition causing the least amount of blood loss. The pig was killed with one shot to the top of the head using a 22 calibre rifle by farmer Michael Cookson, assisted by the farm's veterinarian at 0900 hours on the 20th of May 2002. Almost immediately the corpse was wrapped in a bag and brought to Police Headquarters using an unmarked police van.

At the research site, a 1.5 metre by 1.0 metre area was prepared for the placement of the pig. To meet with Health and Safety requirements, a metal grating was placed under the pig and a wire cage over the pig to keep scavenging animals from reaching the carcass. Grass and topsoil were carefully removed with a shovel. A thick wire mesh, with a 2 cm square size, was placed in the hole. The grass and topsoil was then re-laid on top of the wire mesh. This was done so that the pig did not lie directly on the wire mesh.

The pig was laid on its right side on the prepared area. The removable wire cage, lined with 1cm square wire, was placed over the pig (Figure 2.2). This first day of the experiment was described as Day 0.

Figure 2.2: Experimental set-up. Pig carcass in protective cage.

Data Collection

Visits to the experimental site were made daily, until the Dry stage of decay (described in Section 2.2.3) when the visits were reduced to 3 to 2 times per week. A minimum of four hours was spent at the site each day. The following observations and recordings were collected during each visit:

Visual Observations

Observations of such behaviours as feeding, mating, oviposition, parasitism, and predation were noted. Fly activity and the number of fly species present on the pig were observed and new egg masses were also recorded. A record was made of the physical state of the pig to determine the state of decomposition (Section 3.2.3). Counts of flies present on the body were made during each visit, within 30 minutes of arrival to the site. Modifications were made in later studies (Section 2.2.3) with one pig to improve the quantitative nature of data collection.

Insect collection

One to four adult flies and beetles representing each different species seen on the body that day were collected, killed using ether, and taken to the laboratory for identification.

Temperature

Temperature recordings were taken two to three times during the course of the day using temperature probes. Measurements were taken from the sub-soil (5cm below), the soil surface, a height level with the pig but not over it (25cm), at 1 metre, at 2 metres, just over the pig, the skin surface, inside the pig (about 5cm below the skin surface), on top of the exudates, and just below the surface of the exudate (4cm below). The weather conditions were also noted. An example of the data collection sheets developed for this research can be seen in Appendix 1. Weather data, including temperature and total rainfall, was also obtained from the weather station (Campbell Scientific's PC208W data logger) at the University of Derby in Derbyshire.

Insect Rearing

A portion of the first eggs found on the body were collected and taken to the laboratory provided by the Derbyshire Constabulary. These were reared in 700mL plastic containers holding 200mL of vermiculite and a piece of pork liver. The eggs or newly emerged larvae were placed on the liver and allowed to develop into adults. Throughout the experiment dipteran larvae and certain coleopteran immature stages

were collected from the decomposing pig or the liquid exudate associated with the pig in order to be reared; as adult insects can be much simpler to identify. All insects were reared at room temperature, which was normally between 18-21°C. For every collection of immature insects, a small portion was preserved by placing the sample in just boiled water for 30 seconds and then storing them in 80% ethanol (Adams & Hall, 2003). The results of these were combined with the list of arthropods collected.

Insect Identification

Insects were grouped by day of capture on the pig carcass. These were then identified with the aid of a Nikon stereomicroscope. Insects were identified using the keys described by Borror (1989); D'Assis Fonseca (1968); Erzinçlioğlu (1996); Joy (1932); Pont (1979); Rognes (1991); and Smith (1986). To confirm the identification of certain species, trips to the Natural History Museum in London and University of Oxford were made for use of their vast reference collections. Zoe Adams and Nigel Wyatt of the Natural History Museum helped with the identifications of the Calliphoridae. Maxwell Barkley, also of the Natural History Museum, was vital in the identifications of the Coleoptera. Dr. Adrian Pont and Dr. John Ismay of the Natural History Museum of the University of Oxford taught the techniques used to properly identify Muscids and Sepsids and confirmed many of the identifications.

End of Experiment/ Soil Recovery

On the 16th of October 2002, Day 140 of this experiment, the remains were removed and examined. Any living insects and insect remains, such as empty pupal cases, were recovered. The soil underneath the remains (depth of 10cm) was divided into 16 squares of similar size and collected and taken to the laboratory where each portion of soil was searched. The soil and the remains of the pig were placed in yellow Biohazard bags and collected by the Derbyshire Constabulary for disposal in an incinerator.

Note: Placing the wire mesh under the topsoil, used in Experiment 1, created a trench (8cm depth x 10cm width) around the pig, between the caging and the undisturbed soil. This trench was unintentional but was a result of the wet weather and loosely packed soil at the perimeter of the disturbed area. It was felt that this trench created a disturbance in a research experiment that was meant to mimic real life; therefore, the method was changed. In subsequent experiments the pigs were laid directly on the wire

mesh. The wire mesh underneath the pig did not seem to affect the decomposition, growth of plants, access of the arthropods to the pig, or the soil fauna underneath the grating.

2.2.2 Experiment 2 – Autumn 2002

On the 4th of October 2002, another 45 Kg pig was placed at the research site to observe decomposition and insect behaviour in this cooler season. Insect collections and observations were carried out until the 4th of November 2002, for a total of 28 collection days. An air entrainment experiment, described in Chapter 3, was run simultaneously.

Placement of pig

Due to a misunderstanding in the manner of death, the pig (female) was euthanized by the visiting veterinarian at Broomfield Hall at 0700 hours. The pig was immediately wrapped in plastic and brought to the research site. A location 30 metres away from the first pig was chosen (Figure 2.3). At 1215 hours the pig was placed on its right side directly onto a thick wire mesh and a removable cage lid was placed over the pig as described in section 2.2.1.

Data Collection

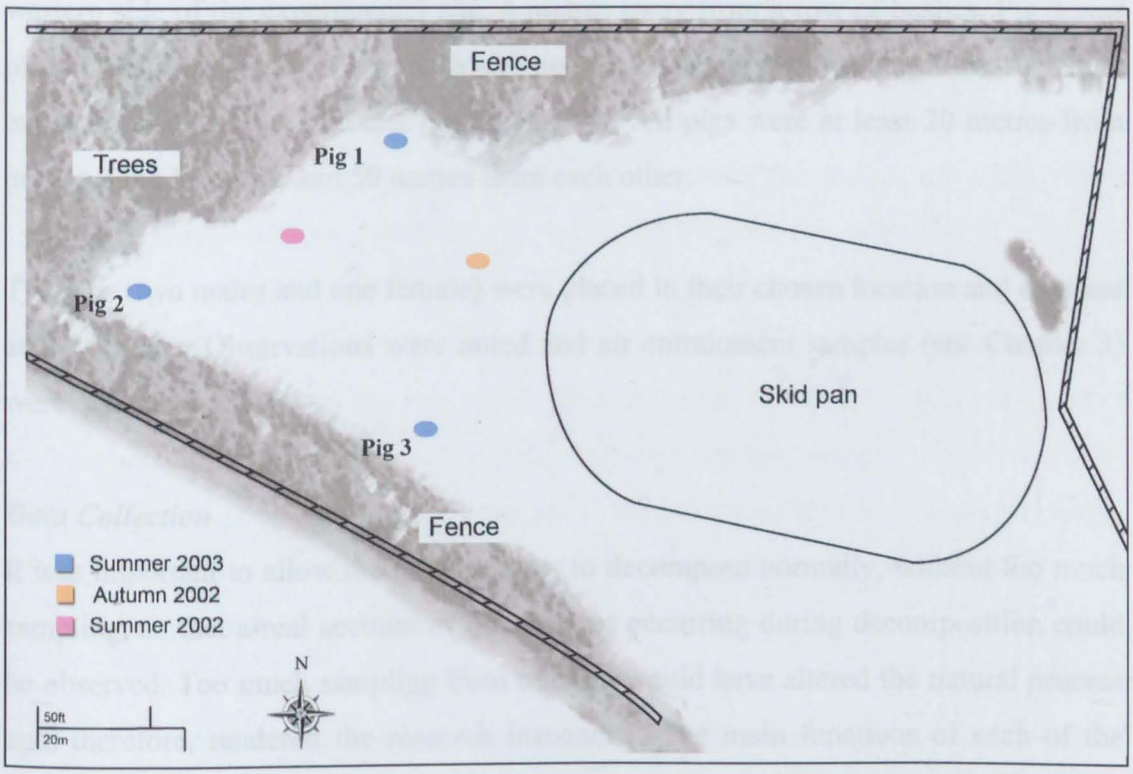
Collections and observations were conducted as explained in section 2.2.1. While the temperature measurements were collected similarly to the previous experiment, a HOBO Pro Data Logger (ONSET Computer, Massachusetts, USA – more details are provided in Appendix 2), containing a sensor and an external probe capable of recording temperatures, was also employed. The data logger itself was placed at soil level while the probe was inserted into the rectum of the pig carcass where temperature recordings were made hourly.

The temperature recordings were compared to temperature data collected with weather station (Campbell Scientifics PC208W data logger) at the University of Derby.

The insect identifications were done as described in section 2.2.1. On the 1st of December 2002 the remains of the pig were collected and incinerated. The soil underneath the pig was not recovered after this experiment as the soil was frozen and

difficult to recover, as well; other experiments were already underway in Hertfordshire.

Figure 2.3: Research site with location of pigs indicated by the coloured circles.



Created by Alexander R.H. Tully

2.2.3 Experiment 3 – Summer 2003

The final pig experiment was conducted in the summer of 2003 between the 5th of July and the 9th of October. While good results were obtained with the previous two experiments a few changes were made to even further improve the research experiment; these are listed below. Collections and observations were made daily (with the exception of three days) until the Dry stage. Once the decomposition had reached this later stage the visits were reduced to four times per week and later to twice a week.

Placement of Pigs & Research Site

For this experiment three pigs were used each with a specific function. The pigs were killed at 1400 hours by farmer David Deville with one shot to the top of the head using a 22 calibre rifle. These were immediately wrapped in heavy plastic sheets and delivered to the research site in a Derbyshire Constabulary transport van.

The pigs were labelled Pig 1, Pig 2, and Pig 3. Pig 1 was placed near the top end of the site, 20 metres away from where the pig had been positioned during the first experiment (Figures 2.1 & 2.3) - The remains of the pigs used in the first two experiments had been completely removed the previous year. Pig 2 was places at the western side of the experimental site, 4 metres away from a row of bushes. Pig 3 was placed south of Pig 2. All were positioned in a manner that would allow for equal exposure to direct sunlight and partial shading. All pigs were at least 20 metres from previous pig locations and 50 metres from each other.

The pigs (two males and one female) were placed in their chosen location and exposed at 1710 hours. Observations were noted and air entrainment samples (see Chapter 3) were collected.

Data Collection

It was important to allow the pig carcasses to decompose normally, without too much sampling, so that a real account of the changes occurring during decomposition could be observed. Too much sampling from one pig would have altered the natural process and, therefore, rendered the research inaccurate. The main functions of each of the three pigs were considered very important yet possibly damaging to the experiment if combined on one pig. Therefore, the sampling was divided in the following manner:

Pig 1 was essentially used to collect arthropods associated with the body (as described in section 2.2.1) and to take temperature measurements of occurrences such as larval masses and sudden changes in decomposition of an area. An estimate of the amount of insects arriving on the body was noted by taking a count of the number of insects present on the pig. Counts were taken in the morning and in the afternoon during a period of 40 seconds each time. Most of the photography documenting the progression of the decomposition was made from this pig.

Pig 2 was used primarily to conduct air entrainments explained in Chapter 3.

Pig 3 was reserved for the collection of temperature data in, on, and around the carcass. Two HOBO Pro Data Loggers containing a sensor and a probe and one

HOBO 4-Channel External Data Logger (ONSET Computer, Massachusetts, USA) with 4 external channels (probes) were combined to take the temperature recordings (Figures 2.4 & 2.5). The data logger containing the four probes was placed inside the cage with the pig to avoid damage by scavenging animals. The following eight temperature measurements were taken hourly: 2 metres and 1 metres above soil level, pig height (0.35 metres), 5cm below the soil, surface of the skin of the pig, inside the mouth (15-20 cm), inside the rectum (15-20 cm), and underneath the pig (placed under the abdomen). Temperature recordings were collected until Day 103, the 16th of September 2003.

While each pig had a specific purpose, temperature recordings, insect collections, and observations were noted for all three pigs in order to confirm that these were decomposing in a similar manner. Individual recordings for each pig were noted daily until the Dry stage of decay, where the visits were reduced to 3 to 2 times per week. Particular attention was spent on determining the state of decomposition in which each pig had reached (described below).

Three pitfall traps were placed into the ground for eight days prior to the start of the experiment to determine the normal arthropod fauna of the area. All 250mL clear plastic cups were filled with 175mL of water mixed with a detergent solution and inserted in a hole made in the soil. The top of the cup was level with the soil surface (Anderson, 1996). The pitfall traps and arthropods were collected before the pigs were brought to the site. At the start of the experiment a pitfall trap was placed 15cm from the abdomen of each pig carcass. One of the original pitfall traps, 100 metres away from the pigs, was left in place to collect arthropod samples not associated with the decomposing carcasses. The arthropods in the detergent solution were checked daily and collected weekly. The insects were preserved in 75% ethanol and a fresh solution of water and dish detergent was added to each cup once emptied.

Insect identification was achieved as described in section 2.2.1

Figure 2.4: HOBO Pro Data Loggers positioned at 2 metres and 1 metres, 0.35 metres, and 10cm inside the soil.

Figure 2.5: Pig 3 with HOBO 4-Channel External Data Logger. The four probes were located: on the surface of the skin of the pig, inside the mouth, inside the rectum, and underneath the pig.

Stages of Decomposition

Five stages of decomposition, originally described by Payne (1965) and Anderson & VanLaerhoven (1996), were observed during the above experiments. The stages were previously applied by the author, in research conducted using still-born piglets in Nova Scotia, Canada (LeBlanc and Strongman, 2002), and were found to describe the condition of the decomposing carcass accurately. Therefore, the author felt confident in applying the same decomposition stages to the current research.

The descriptions were based on physical condition, odour, and at times varying insect activity (Payne, 1965). These stages included:

Fresh – This stage commences immediately after death and continues until the body is bloated. Chemical breakdown occurs during this stage; however, few morphological changes are observed. There is no obvious odour to humans.

Bloated – An accumulation of gasses from the activity of anaerobic bacteria produce a swollen, bloated appearance. There is an obvious odour present.

Active Decay – This stage is recognisable by the deflation of the carcass due to the gases escaping from the body. There is a very strong putrid odour.

Advanced Decay – During this stage, a large amount of the flesh has been removed; however, there is still some moist tissue present. The odour is less obtrusive than in the previous stage, but it is still quite noticeable.

Dry – At this stage the carcass has been reduced to bones, cartilage, and dry skin. Only a slight odour is present.

While there are recognisable stages in the physical decomposition of the carcass (Anderson & VanLaerhoven, 1996), there is often no clearly defined beginning or end to a decomposition stage, especially during the later stages, making the decision of stage change somewhat subjective (Bornemissza, 1957; Early & Goff, 1986; Shoenly & Reed, 1987; Archer, 2004; Grassberger & Frank, 2004). That is why this author was the only one to make comments or determine stage changes in all the experiments conducted to keep consistency in the experiments each year.

Payne (1965) includes a sixth stage called "remains stage" where carrion-feeding and carrion-visiting insects are no longer present on the remains. This stage was not included in these studies, as it was never observed in the time frame designated to this research.

2.2.4 Statistical Analysis

A possible link between the number of flies (*Calliphora*, *Lucilia*, *Protophormia terraenovae*, *Hydrotaea* – formerly *Ophyra*, other Muscidae, Piophilidae, and Sepsidae) on the decomposing carcass and the ambient temperature was explored using Pearson's correlation. The analysis was done using statistical software SPSS with the data collections made during Experiment 3 – Summer 2003.

2.3 RESULTS

Arthropod succession and key observations regarding insect behaviour during the different stages of decomposition were noted for all three experiments. Within each stage of decomposition the physical appearance of the pig, the weather conditions, and the insect activity are described.

Counts of adult insects present on the carcass were made during the visits to the experimental site. The counts are referred to in the following manner: "few", meaning 1-5 flies counted; "some", referring to 6-19; "many", meaning 20-40; and "numerous", for those instances where the count exceeded 40 flies.

2.3.1 Experiment 1 – Summer 2002

From the 20th of May to the 16th of October 2002 the following decomposition stages and other observations were made:

Fresh Stage – (Days 0 to 3)

The carcass was exposed on the afternoon of this first day of research (Day 0). Muscids and *Calliphora* species (Calliphoridae), also known as blue-bottles, immediately came to the pig but stayed on the body for only a few minutes as it was beginning to rain. It rained heavily on the evening of Day 0; therefore, no remaining flies were seen until the next morning.

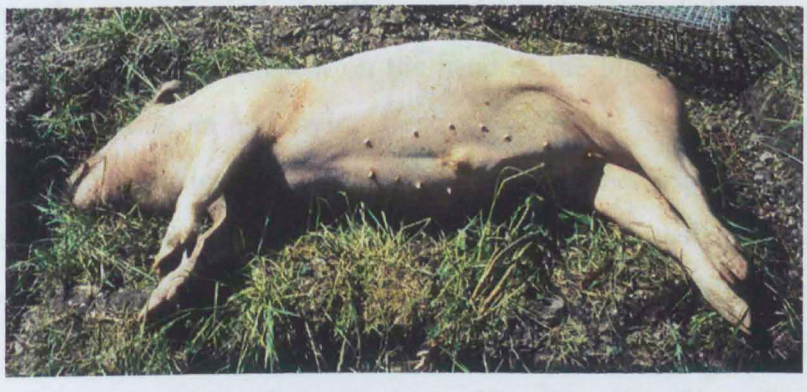
Appearance of Pig

During this stage there was no great change in the appearance of the pig (Figure 2.6). The skin seemed to be losing its pigmentation and blood was being purged from of the nose, which was attracting many flies looking to feed, mate, and lay eggs. A slight odour was becoming noticeable by Day 3.

Weather

The weather was variable with a constantly changing climate of rain, wind, and sun. Occasional rain would wash away unprotected eggs or those that were not firmly attached. The temperatures recorded are discussed in greater detail in Temperature and Rainfall, Section 2.3.4.

Figure 2.6: Experiment 1 (Summer 2002), Day 1 – Fresh stage.



Insect Activity

Early in the morning of Day 1, some (6-19 flies) blue-bottles (*Calliphora* sp.) were actively laying eggs in the nostrils, mouth, and on the top of the head where the pig was shot (Figure 2.7). The eggs were collected and reared; all adults that emerged were of *Calliphora vicina*. *Lucilia* species (Calliphoridae), commonly called green-bottles, were also quickly attracted to the decomposing corpse and were seen laying eggs by late morning of Day 1.

Figure 2.7: Experiment 1 (Summer 2002), Day 1 – Fresh stage. Showing the clump of eggs and flies on the top of the head where the bullet wound was located.

During cloudy periods the *Calliphora* sp. outnumbered any other fly species present; however, during the sunny periods *Lucilia* sp. quickly dominated the carcass. Many (20-40 flies) Calliphorids were present on the head of the pig all trying to lay eggs, which can be described as an egg-laying frenzy. *Lucilia* sp. laid eggs together in a common area (in folds of skin under chin), while the *Calliphora* sp. were laying eggs on the nose, mouth, and the top of the head. However, eventually *Lucilia* sp. also began to lay eggs on top of the head along with the *Calliphora* sp. Muscids were also present but in fewer numbers than the Calliphorids. Some were seen trying to lay eggs near the head but were pushed away by the larger blowflies. Few Sarcophagidae were seen on and near the pig but were not observed laying larvae. Piophilidae began to appear on Day 3.

By late morning of Day 2 first instar Calliphoridae larvae were observed and second instar larvae were noted on Day 3. There was a mass attraction of adults to the carcass where many Calliphoridae and Muscidae came to lay eggs. The frenzied egg laying around the head and face continued throughout this stage. Few Piophilidae were seen mating on and around the pig carcass by the afternoon of Day 2. Two spiders, Lycosidae, were present on the carcass but were only observed attacking flies (especially green-bottles) rather than approaching the eggs. Parasitic Brachonid wasps were already present on Day 3 and observed parasitizing the Calliphoridae larvae. At this stage, insect activity appeared to be confined to the head region. More details on the arthropods present can be found in Table 2.1.

Bloated Stage – (Days 4 to 7)

Appearance of Pig

By Day 4 the pig carcass was very noticeably bloated. The mouth was now open and blood and other fluids were flowing out of the nose. Many areas of the carcass had taken on a marbled appearance and the veins and arteries had become a dark brown-greyish colour (Figure 2.8). The skin on the facial area was becoming darker every day. Faecal material was being pushed out of the rectum. The odour was now much stronger than in the previous stage.

Table 2.1: Arthropods observed on the pig carcass during Experiment 1 – Summer 2002.

Order	Family	Species	Fresh Day 0-3	Bloated Day 4-7	Active Decay Day 8-22	Advanced Decay Day 23-28	Dry Day 29-
Diptera	Anthomyiidae	Not identified further	A				AL
	Calliphoridae	*Calliphora vicina*	AL	AL	AL	AL	A
		Calliphora vomitoria	AL	AL	AL	AL	A
		Lucilia caesar	AL	AL	AL	AL	
		Lucilia sericata	AL	AL	AL	AL	A
		Protophormia terranovae		A	AL	AL	AL
	Muscidae	*Graphomya maculata*	A	A			
		Hydrotaea dentipes	A	A	A	A	AL
		Hydrotaea similis			A		
		Hydrotaea irritans			A		AL
		Hydrotaea meteorica	A				
		H. ignava (Ophyra leucostoma)			AL	AL	AL
		H. capensis (O. capensis)					AL
		H. aenescens (O. aenescens)			AL	AL	AL
		Limnophora sp.	A				
		Morellia hortorum		A	A		
		Muscina assimilis	A	A			
		Mesembrina meridiana	A				
		Stomoxys calcitrans	A				
	Fanniidae	*Fannia lustrator*		A			
		Fannia maricata			A		
		Fannia scalaris			A		
		Fannia sp.		A			AL
	Phoridae	*Megaselia sp.*					AL
	Piophilidae	*Piophila foveolata*	A	A	A	A	AL
	Psychodidae	Not identified further					A
	Sarcophagidae	*Sarcophaga sp.*	A	A	A		A
	Sepsidae	*Nemopoda nitidula*					AL
		Meroplius minutus					AL
		Sepsis cynipsea					AL
	Stratiomyidae	Not identified further					AL
	Syrphidae	*Eristalis tenax*					AL
Homoptera	Not identified						A
Hymenoptera	Braconidae	Not identified further	A	A	A		
	Ichneumonidae	Small black wasp, unidentified					A
	Formicidae	Not identified further			A	A	A
	Vespidae	Yellow jacket wasp					A

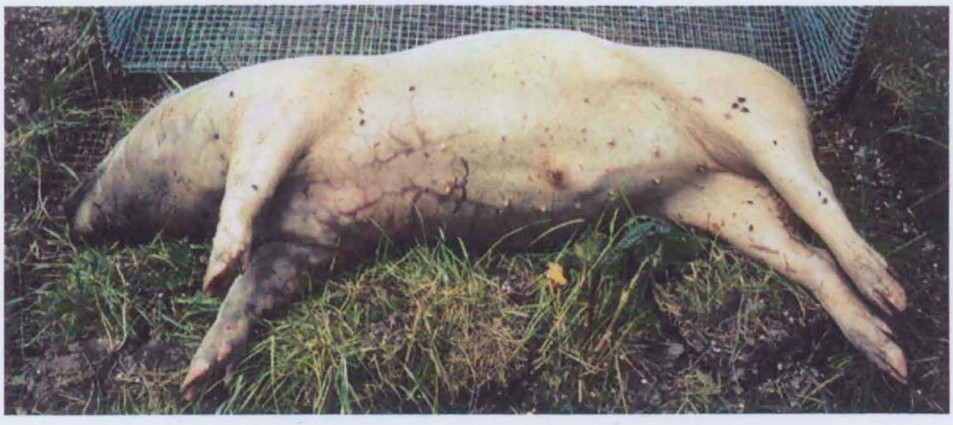
A = adults, L = larvae

Table 2.1: Arthropods observed on the pig carcass during Experiment 1 – Summer 2002, Continued.

Order	Family	Species	Fresh Day 0-3	Bloated Day 4-7	Active Decay Day 8-22	Advanced Decay Day 23-28	Dry Day 29-
Coleoptera	Cleridae	*Necrobia rufipes*					A
		Necrobia violacea					A
	Histeridae	*Margarinotus cadaverinus*					AL
		Margarinotus carbonarius					A
	Hydrophilidae	*Cercyon sp.*					A
	Silphidae	*Necrodes littoralis*			AL	AL	AL
		Nicrophorous humator			A		
		Thanatophilus sinuatus			A		
	Staphylinidae	*Aleochara curtula*	A		A		
		Creophilus maxillosus			A		A
		Philonthus politus					A
Spider	Not identified		A	A			
Acari	Mites					A	A
Collembola	Not identified						AL

A = adults, L = larvae

Figure 2.8: Experiment 1 (Summer 2002), Day 5 – Bloated stage.



Weather

Throughout most of this stage there was either rain or the sky was overcast. The cloudy conditions did not prevent flies (*Calliphora*, Muscidae, and Piophilidae) from coming to the corpse; however, it did possibly contribute to the fewer numbers of *Lucilia* sp. present on the carcass. During short periods of rain, the flies would take shelter underneath the pig and surrounding vegetation. The adults would quickly reappear once the rain had ceased. Staphylinidae beetles were observed on three occasions during this stage.

Insect Activity

On Day 4 scattered Muscidae eggs and a few first instar larvae started to appear at the posterior end of the carcass, near the rectum. A larger amount of Calliphorid larvae were present in the mouth than previously described. Sarcophagidae adults were seen during this stage, however, only very few (1-5). *Protophormia terraenovae* (Calliphoridae) were now present on the carcass. Brachonid wasps were more numerous during this stage than the previous one, reaching counts of up to 8 adults. They were observed attacking the Calliphorid larvae crawling on the skin of the carcass, more often choosing the larger ones. These parasites were seen attacking larvae, which were congregated into masses; however, once the maggot masses became larger (approx. 7 x 8cm) the Brachonid wasps moved to single larvae away from the mass. Calliphorids continued to lay eggs during this stage but no longer did so near the head, except for one clump of eggs inside the ear. Possibly due to a repellent effect from the mass, flies were most commonly laying their eggs near the rectum or along the back and legs of the pig carcass closest to the ground as the larval mass grew larger in the mouth and head.

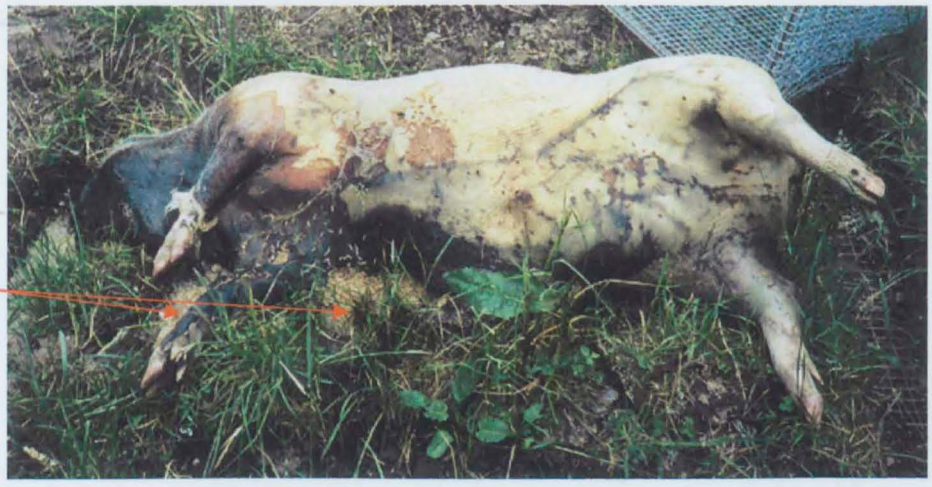
Active Decay Stage – (Days 8 to 22)

Appearance of Pig

The stage of active decay was initiated by the escape of gases from the carcass. The mouth, snout, and eye regions had already experienced a substantial amount of insect activity by this time and were beginning to turn black and the mouth and jaw region was becoming disarticulated. By the end of this stage the dry black skin in the facial area had extended to the head and neck region. The rest of the carcass, however, still retained much of its flesh but had a more significant marbled appearance. As tissues

were breaking down, the first layer of skin was slipping off the legs (Figure 2.9). The abdomen of the decomposing pig was beginning to lose its shape. It gave a slightly sunken appearance as decomposition of the internal organs progressed and feeding of insects increased. This made the carcass appear to be turning onto its back as opposed to lying on its side, as originally positioned. As the insects continued to feed on the carcass, holes were created in the abdomen near the rectum causing the intestines to be exposed. By Day 18, a white fungus had begun growing on the dry remains of the pig. The odour was now quite strong and putrid. This stage resulted in, by far, the most unpleasant odours to the human sense.

Figure 2.9: Experiment 1 (Summer 2002), Day 18 – Advanced Decay stage.



Weather

The active decay stage endured varying temperatures. Some days were sunny and warm with temperatures upwards of 23°C, while other days were cloudy with rain (more details in Section 2.3.4).

Insect Activity

Numerous (41+) adult *Calliphora* sp., Muscids, *Lucilia* sp. and some (6-19) mating Piophilids were regularly observed on the carcass. The numbers of mating Piophilids increased during this stage compared to the previous stages. Few Scathophagidae were present but were not observed laying larvae. Brachonid wasps were still present but they did not approach the flies, rather, they stayed on the area of the carcass where there was less fly activity. Beetles, such as Cleridae, Staphylinidae, and Silphidae,

were being noted more often during this stage than in the previous. Silphid beetle larvae (*Necrodes littoralis*) were beginning to appear in great numbers (many, 20-40) as the active decay stage progressed and were observed to feed on decaying flesh and to be predacious on dipteran larvae. By Day 12 the number of Calliphorid and Muscid flies was so great (numerous 41+) that all that could be heard was the buzzing of the wings. *Protophormia terraenovae* and Muscidae (*Hydrotaea* sp., synonymous with *Ophyra* sp.) were present during this stage. *Hydrotaea* sp. flies were numerous on the posterior half of the carcass after Day 16. By the end of this stage adult Piophilidae were very numerous on the carcass.

This stage revealed certain interesting behaviours: During sunny periods numerous flies, such as *Calliphora* sp., *Lucilia* sp., Muscidae, and at times Piophilidae, came to the body to lay eggs, while the wet periods initiated mass exodus of post-feeding larvae from the carcass. On the days with light rain, the wet skin of the carcass provided a slippery surface on which the larvae could travel. In fact, the larvae seemed to be following each other in several lines on the wet skin indicating that there could be a contact chemical to inform the larvae where others have passed.

On the first day of this stage larger second instar larvae had completely covered the mouth and clumps of larvae were falling onto the ground as the larvae on the outer edge of the maggot mass were being pushed out due to the constant motion of the larvae and the increasing size of the mass. Meanwhile Calliphorid and Muscid flies were laying numerous eggs near the rectum, lower abdomen and legs. As decomposition progressed, the carcass experienced waves of larvae all over the body resulting in an almost constant mixture of 1^{st}, 2^{nd}, and 3^{rd} instar larvae due to the continual egg laying by the flies. During this stage mass larval dispersal periods were observed. The first mass exodus of post-feeding larvae, previously feeding on the head region, was observed on the morning of Day 16 resulting in a noticeable void in the head region and the skull becoming exposed around the nose and jaw. Other significant mass exoduses were observed on Day 20 and Day 22 from larvae previously feeding all over the carcass. Rain seemed to trigger the mass exodus of the larvae. These post-feeding larvae were found, the same morning, over 2 metres away not yet burrowed in the soil. Toward the end of this stage fewer clumps of eggs were being noted while larvae of all stages of development were consuming the carcass.

Local birds, Chaffinch (*Frigilla coelebs*) being the most common in this case, were regularly coming near the carcass to collect migrating larvae. Even those already in the ground were picked out by the birds. In fact, the birds were so good at locating the larvae that they were, at times, more reliable than random soil searches for locating post-feeding larvae.

This stage resulted in the most impressive damage to the carcass due to feeding larvae. Continuous egg laying, resulting in a growing number of larvae, and mass exodus of third instar larvae from the carcass resulted in very significant changes almost on a daily basis. As the tissue from the head was almost entirely consumed, new maggot masses were forming at the posterior end of the carcass. As the posterior end was becoming consumed new interest was being given to the abdomen of the carcass. Changes to the carcass and in insect activity were often drastic. Certain days the carcass was covered by hundreds of flies laying eggs while the next day only few flies would be noted, often due to rain. Also, maggot masses were often covering certain regions of the carcass but, in some instances, after a mass exodus only smaller larvae feeding inside the body cavity would remain giving the appearance of little insect activity. This resulted in very significant changes to the appearance and odour of the carcass leading into the next stage.

Advanced Decay Stage – (Days 23 to 28)

Appearance of Pig

Important factors determining this stage of decay were the state of the carcass (amount of tissue remaining), the number of flies attracted to the carcass, and the amount of larvae present on the carcass. The skin around the abdomen and legs was beginning to blacken leaving only a few portions still creamy brown. Due to decomposition and insect activity all organs and other tissues had liquefied resulting in a grey exudate under and around the carcass. The strong putrid odour was not as overpowering. There was less rain in this stage than in the previous and the body was drying quickly.

Weather

During this stage of decomposition the sky was overcast with only brief period of sunshine. No heavy periods of rain were recorded.

Insect Activity

Fly activity was beginning to change; Calliphoridae were no longer as attracted to the carcass. While the *Calliphora* sp., *Lucilia* sp., and Muscidae were present during this stage, they were on the body in much fewer numbers (1-19) and were only seen laying eggs on one day during this entire stage. The Piophilidae were present (many, 20-40) throughout this stage and were seen laying eggs on most days. Toward the end of this stage Calliphoridae adults were observed landing on surrounding vegetation but not on the carcass. There was also a noticeable decrease in the number of dipterous larvae and a considerable increase (numerous, 41+) in Silphidae beetle larvae, *Necrodes littoralis*. Fewer Calliphoridae larvae were seen; however, Muscidae and *P. terraenovae* larvae were observed migrating away from the carcass. An important factor to note is that there were no longer any large maggot masses and the carcass had been taken over by a large amount of Silphidae beetle larvae. It is possible, through the effect of competition and predation, that the beetle larvae may have contributed to a decrease of dipterous larvae.

Dry Stage – (Days 29 to 149)

Appearance of the Pig

The skin was dry and "leathery"; much fewer flies observed in the previous stages were attracted to the carcass. Early in this stage, due to the beetle larvae infestation described in the previous stage, the dry skin of the carcass was eventually completely covered with faeces and cast beetle skin giving it a rough, black appearance (Figure 2.10). After a heavy rainfall the carcass was cleaned of this excrement and regained a pinkish-grey appearance (Figure 2.11). The sun would eventually bleach the leathery skin. While the carcass consisted almost only of bone covered with dry skin, there was still some exudate present. Throughout this stage the exudate dried slightly and the skin retracted to expose more bones. Rain would temporarily re-hydrate the skin, amplifying the slight odour present around the carcass. In this stage the odour was significantly reduced and gained a somewhat sour quality. By Day 73 the odour had changed from sour to having a distinct leathery and peppery quality to it.

Weather

During this lengthy stage of decomposition the weather was variable with days of bright sunshine and others with heavy rainfalls.

Figure 2.10: Experiment 1 (Summer 2002), Day 30 – Dry stage, before rain.

Figure 2.11: Experiment 1 (Summer 2002), Day 46 – Dry stage, after rain.

Insect Activity

Flies such as the Calliphoridae and few Muscidae were still found on the carcass yet their behaviour was quite different to that seen in the earlier stages of decomposition. These would only spend a few minutes or seconds on the carcass and were not seen ovipositing at any time. The entomofauna had changed drastically and gave way to numerous flies such as Piophilidae, Sepsidae, as well as many beetles. At the beginning of this stage Piophilidae outnumbered any other fly species and were often observed mating. By Day 58 numerous Sepsidae were observed on the carcass and remained a normal presence on the carcass for the remainder of this research, far outnumbering Piophilidae on most days late in decomposition. The hoverfly, *Eristalis tenax* (Syrphidae), appeared to the carcass early in this stage and was seen laying eggs on several occasions. Some Stratiomyidae were seen on the carcass on Day 60. A few

Psychodidae flies appeared on the carcass on Day 83 and remained until the end of the research. Newly emerged adult flies of Muscidae, especially *Ophyra* sp., were appearing by Day 64. Tiny Ichnumonid wasps were seen three times late in this stage of decomposition flying 1.5 metres above the pig in a swarm in what resembled a grey cloud.

Beetles such as Staphylinidae (*Creophilus maxillosus*), Cleridae, Histeridae, Silphidae (*Nicrophorous humator*) were becoming more numerous or being observed much more frequently. Beetle frass was observed on the pig on Day 40; subsequently Dermestidae adults and larvae were observed on the carcass. With the beetles came numerous mites of several different species, both found on the beetles and the pig. Silphid beetle larvae were still present on the first three days of this stage but they were also seen leaving the carcass and burrowing under stones and clumps of soil. These were seen leaving until there were almost none left on the carcass. Some were found under stones up to 2 metres away from the carcass by the end of this decomposition stage and into the Dry stage. The first newly emerged adult Silphidae (*Necrodes littoralis*) were observed on Day 66.

During this stage there was no longer the regular presence of large numbers of Calliphoridae larvae. Only small numbers of Muscidae larvae were noted early on in this stage. The considerably smaller Piophilidae and Sepsidae larvae were now common on the carcass. Piophilidae larvae were first noted very early during this stage. Weather conditions, such as sunshine and rainfall, had a great affect on the behaviour of the insects on the carcass. When the sun was shining a noticeably larger group of flies gathered on the carcass. It was the rain, however, that caused the most interesting reactions. On warm days the larvae would be observed in the moist exudate only. However, as it started to rain the 3rd instar larvae of the Piophilidae would begin to emerge from the exudate and "jump" or "skip" off the carcass. Some were observed "jumping" in an area 80cm away from the carcass. The rain triggered an almost immediate reaction. Within two minutes of the rain falling, hiding Piophilidae larvae were observed exiting the exudate and immediately "jumping" in what seemed like random directions. Whether the larvae's behaviour was a direct reaction to falling water or increased humidity still needs to be investigated. One must wonder if the moisture facilitated movement or whether the disturbance created a defensive

response. Sepsidae larvae also emerged from the exudate when the skin was moist but did not exhibit any "jumping" behaviour. On Day 76 Stratiomyidae and Sepsidae larvae were observed on the carcass; no rain had fallen. Wolf spiders, Lycosidae, were seen attacking flies, most often Muscidae, and carrying eggs away from the carcass. Throughout this stage birds such as Magpies and Chaffinches were often observed 0.5m to 2m from the carcass looking for migrated larvae.

2.3.2 Experiment 2 – Autumn 2002

The short autumn experiment was primarily conducted to test air entrainment techniques described in Chapter 3. However, it proved to be also informative from an entomological point of view.

The experiment was conducted from the 4th of October 2002 and continued until the 11th of November 2002. The following observations were made:

Fresh Stage – (Days 0 to 3)

The pig carcass was exposed at 1215 hours on a partly sunny afternoon and almost immediately attracted many *Calliphora* and *Lucilia* species. Muscidae and Piophilidae were also present on this day but in fewer number. Three Sarcophagidae were also observed on the carcass. At this time the carcass was 23°C. Within two hours of exposure eggs had already been laid in the ear, which were later established to be *C. vomitoria*. In the following hours many more eggs would be laid in the facial area.

The probe inserted into the rectum was lost by Day 2. An animal, possibly a fox, chewed through the probe connected to the HOBO Pro Data Logger, which was located outside the cage, and was taken away. The probe was never recovered.

Appearance of Pig

On Day 1 the skin of the carcass was taking a dark blue marbled appearance. There was no strong odour and all the insect activity seemed confined to the head region.

Weather

This stage of decomposition experienced a mixture of cloudy and sunny periods. More details are provided in Section 2.3.4.

Insect Activity

This stage noted the presence of some (6-19 flies per species) *Calliphora* sp.; *Lucilia* sp.; Muscidae, including *Ophyra* sp.; Piophilidae; few (1-5 flies) Sarcophagidae; few parasitic Brachonid wasps; and even a yellow jacket (Vespidae) wasp attacking a *Lucilia* sp. Many more eggs were laid on the head, nose, ears, and mouth. More details on the arthropods present can be found in Table 2.2.

Bloated Stage – (Days 4 to 8)

Appearance of Pig

This stage was recognisable due to the very bloated appearance of the pig carcass and pockets of blood, or blisters, forming under the first layers of skin. The odour was stronger and the gases were still mostly contained within the body.

Weather

For the following days the weather was cool and cloudy; therefore, no large numbers of flies were noted. Periods of rain were also recorded.

Insect Activity

Calliphora sp., *Lucilia* sp. appeared on the pig in groups of four up to ten during this stage. *Lucilia* sp., however, was not seen on Days 7 and 8 due to the frequent periods of rain. Muscidae, Piophilidae, Sarcophagidae, parasitic Brachonid wasps, and yellow jacket wasps were present only one to three at a time on the body for each family. Second instar larvae were observed feeding around the mouth. Eggs were noted for the first time near the rectum on Day 6.

Table 2.2: Arthropods observed on the pig carcass during Experiment 2 – Autumn 2002.

Order	Family	Species	Fresh Day 0-3	Bloated Day 4-8	Act. Decay Day 9-38
Diptera	Calliphoridae	*Calliphora vicina*	AL	AL	AL
		Calliphora vomitoria	AL	AL	AL
		Lucilia caesar	AL	AL	AL
		Lucilia sericata	AL	AL	
	Heleomizidae	*Neoleria inscripta*			A
	Muscidae	*Graphomya maculata*			A
		Hydrotaea dentipes	A		A
		Hydrotaea ignava (Ophyra leucostoma)	A		AL
		Scatophaga stercoraria	A	A	A
	Phoridae	*Megaselia sp.*			A
	Piophilidae	*Piophila foveolata*	A	A	A
	Sarcophagidae	Not identified further	A		A
	Tipulidae	Not identified further			AL
Hymenoptera	Braconidae	Not identified further	A	A	A
	Vespidae	Yellow jacket wasp	A	A	A
Coleoptera	Cleridae	*Necrobia rufipes*			A
		Necrobia violacea			A
	Nitidulidae	*Omosita colon*			A
		Nitidula sp.			A
	Staphylinidae	*Ocypus olens* - Devil's Coach-horse			A
		Ontholestes sp.	A		
		Philonthus politus			A

A = adults, L = larvae

Active Decay Stage – (Days 9 to 38)

Appearance of Pig

The active decay stage began as a large amount of larvae were observed feeding on the body, mainly in the mouth; the rectum, and underneath the carcass. Most of the gasses had escaped and a very strong putrid odour was present.

Decomposition progressed much slower than in the previous experiment. While similar characteristics and insect behaviour indicated that the carcass was in the active stage of decay, the cool weather and slowing rate of insect activity did not physically alter the carcass sufficiently to reach the next stage.

Weather

Throughout this stage the ambient temperature was becoming considerably cooler. There were periods of heavy rain and high winds. A maximum temperature of 16°C and a minimum of -3°C were recorded.

Insect Activity

Very few flies were seen on the carcass early is this stage due to the rainy and windy conditions. However, by Day 14, rain and winds had ceased and considerable (many, 41+ in total) fly activity ensued for the next two days. An almost daily change from cool, wet, and windy weather to slightly milder weather caused a drastic change in adult insect activity. The insects noted were *Calliphora* sp., *Lucilia* sp., Muscidae, parasitic Brachonid wasp, Sarcophagidae, Piophilidae, Heleomizidae, Tipulidae, and Phoridae. While immature stages indicated that beetles had been present, there was very little other evidence of them on the carcass. No adult beetles were recorded during the observations made and no species of beetle larvae were recorded in great numbers (some, 6-19).

During this stage the dipteran larvae were quite active, causing considerable tissue damage to the carcass. Third instar larvae were first noted on Day 10 of the experiment. For the remainder of the research period the weather stayed cool, often below 13°C. The carcass, however, often reached temperatures of 19°C or above as the larvae developed and formed small masses. Fewer larvae were seen on the skin of the carcass, as the weather was getting cooler. However, larvae could be seen crawling on the skin after rain. A similar "larval wandering" behaviour to that described in the previous experiment was occasionally observed: the larvae were moving in a straight line on the skin of the carcass. On the morning of Day 20 these were observed moving from the mouth to the anus, east to west, away from the sun. On Day 18 more eggs were laid on the carcass; though, following a spell of cold weather, did not appear to survive as none hatched. Toward the end of this experiment, Day 26, Calliphoridae and Muscidae larvae were observed leaving the carcass. By Day 38 many third instar larvae were observed on the carcass, as well as a few eggs. It was not determined whether or not these eggs survived.

Due to the cold weather slowing the progression of insect activity and decomposition, as well as prior engagements at Rothamsted Research, the experiment was terminated on Day 38 (11 November 2002) never having reached the Advanced Decay stage.

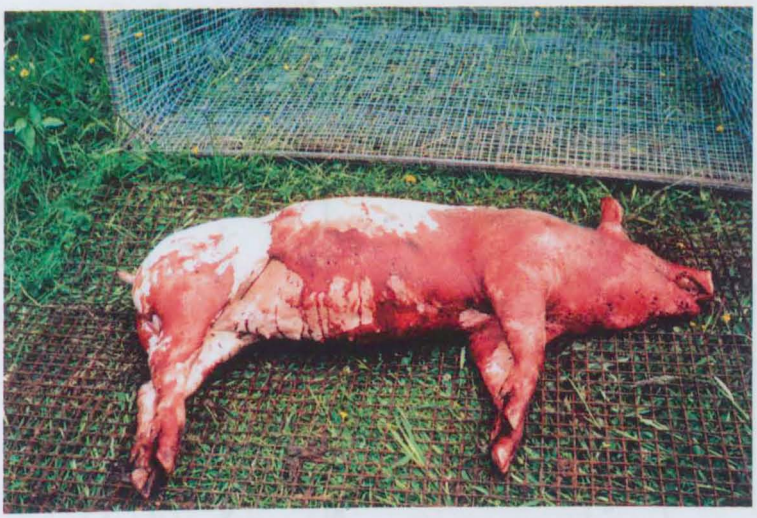
2.3.3 Experiment 3 – Summer 2003

The following observations were made of Pig 1, described in Section 2.2.3, during the course of the experiment conducted from the 5th of June 2003 to the 9th of October 2003:

Fresh Stage – (Days 0 to 3)

The pig, which had been killed using a 22-calibre weapon, was exposed on the 5th of June 2003 at 1710 hours. No odour, other than a those associated with a farm, was detected. Flies, such as *Calliphora* sp. and Muscids, were immediately attracted to the carcass possibly due to the blood covering the body (Figure 2.12).

Figure 2.12: Experiment 3 (Summer 2003), Day 0 – Fresh stage.



Appearance of Pig

For the remainder of the fresh stage the carcass kept a relatively unchanged appearance, except for the gases inside the body pushing blood out of the nose.

Weather

The weather was sunny for most of this period. More weather detail can be found in Section 2.3.4, Temperature and Rainfall.

Insect Activity

On the morning of Day 1, the first eggs were observed on the carcass. These were located in the nose and were later found to be that of *Calliphora vicina*. Insects such as many (20-40 flies) *Calliphora* sp. and *Lucilia* sp., as well as some (6-19 flies) Muscidae (including *Ophyra sp.*), Piophilidae, *P. terraenovae*, Heleomizidae, parasitic Brachonid wasps, and Staphylinidae beetles (*Aleochara curtula*) were attracted to the carcass during this stage. During the sunny periods of Days 2 and 3, with temperatures reaching above 30°C, *Lucilia* sp. outnumbered any other fly species on the carcass. The occasional spider was also spotted on the carcass. Little larval activity was observed during this stage. More details on the adult insects present can be found in Figure 2.13 and Table 2.3.

Figure 2.13: Counts of adult insects on the decomposing pig taken from Pig 1 throughout Experiment 3 – Summer 2003.

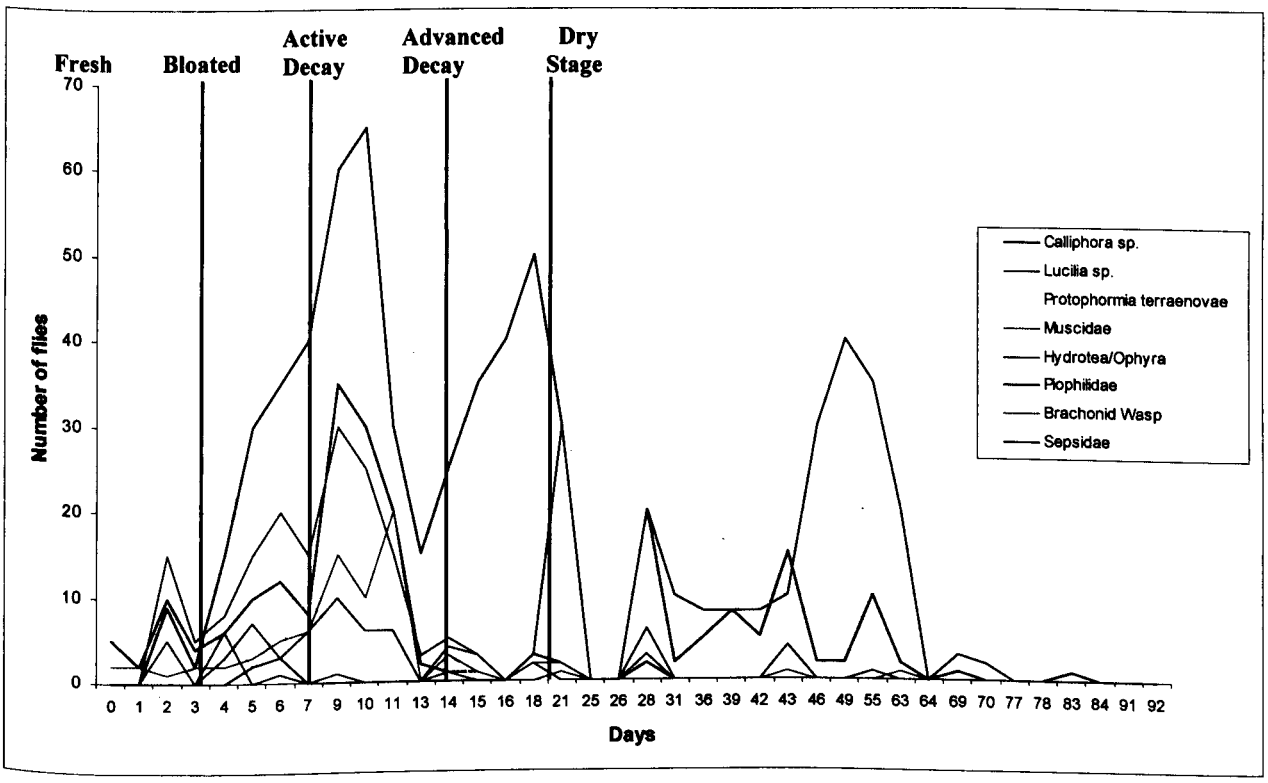


Table 2.3: Arthropods observed on the pig carcasses during Experiment 3 – Summer 2003.

Order	Family	Species	Fresh Day 0-3	Bloated Day 4-7	Active Decay Day 8-14	Advanced Decay Day 15-18	Dry Day 19-
Diptera	Calliphoridae	*Calliphora vicina*	AL	AL	AL	AL	A
		Calliphora vomitoria	AL	AL	AL	AL	
		Lucilia illustris	A				
		Lucilia caesar	AL	AL	AL	AL	AL
		Lucilia sericata	AL	AL	AL	AL	AL
		Protophormia terranovae		AL	AL	AL	
	Empididae	Not identified further		A		A	
	Heleomizidae	*Neoleria inscripta*	A				
	Muscidae	*Hydrotaea dentipes*		A	A		
		Hydrotaea similis		AL	AL		
		Hydrotaea cinerea			AL		
		H. ignava (Ophyra leucostoma)		A	AL	AL	AL
		Muscina pabulorum		A			
	Fannidae	*Fannia tuberculata*		A			
		Fannia sp.			A		
	Phoridae	*Megaselia sp.*					A
	Piophilidae	*Piophila foveolata*	A	A	A	A	AL
	Psychodidae	Not identified further					A
	Sarcophagidae	*Sarcophaga sp.*		A	A		A
	Scatopsidae	Not identified further					A
	Sepsidae	*Nemopoda nitidula*	A	A			AL
		Meroplius minutus					AL
		Sepsis cynipsea	A			A	AL
	Stratiomyidae	Not identified further			AL	AL	AL
	Syrphidae	*Eristalis tenax*					AL
Hymenoptera	Braconidae	Not identified further	A	A		A	
	Ichneumonidae	Small black wasp, unidentified				A	A
	Formicidae	Not identified further					A
Homoptera	Cercopidae	Not identified further					A
		Not identified further		A			A
Coleoptera	Cantharidae	Not identified further		A			
	Cleridae	*Necrobia rufipes*			A	A	A
		Necrobia violacea			A	A	A
	Dermestidae	*Dermestes murinus*					AL
	Elateridae	*Agriotes obscurus*		A			
	Histeridae	*Margarinotus cadaverinus*					AL
		Margarinotus carbonarius					AL
	Nitidulidae	*Omosita colon*					A
	Silphidae	*Necrodes littoralis*		A	AL	AL	AL
		Nicrophorous humator		A			

A = adults, L = larvae

Table 2.3: Arthropods observed on the pig carcasses during Experiment 3 – Summer 2003, Continued.

Order	Family	Species	Fresh Day 0-3	Bloated Day 4-7	Active Decay Day 8-14	Advanced Decay Day 15-18	Dry Day 19-
Coleoptera	Staphylinidae	*Aleochara curtula*	A	A			
		Anotylus rugosus					A
		Creophilus maxillosus			A		
		Gyrohypnus sp.					A
		Philonthus cephalotes					A
		Philonthus politus					A
		Platystethus arenarius					A
		Rugilus orbiculatus					A
Spider			A	A		A	A
Acari	Mites					A	A
Collembola	Isotomidae	Not identified further					AL
	Hypogastruridae	Not identified further					AL

A = adults, L = larvae

Bloated Stage – (Days 4 to 7)

Appearance of Pig

The carcass had a very bloated appearance and the skin was becoming marbled. Much of the flesh on the face was being removed due to insects feeding (Figure 2.14). A strong odour was beginning to escape from the carcass.

Figure 2.14: Experiment 3 (Summer 2003), Day 7 – Bloated stage.

Weather

The weather was sunny for this entire stage of decomposition.

Insect Activity

An increase in fly activity was observed during this stage as more insects were coming to the body to mate, feed, and oviposit. Present during the bloated stage were the same species as listed in the previous stage with the addition of few (1-5 flies) Sarcophagidae and Empididae, as well as beetles Cantharidae, Staphylinidae, and Silphidae. Unexpectedly, Sepsids were observed on the carcass on three occasions during this stage. New eggs were regularly being observed on the face and head region due to the continua egg laying; *Calliphora* and *Lucilia* species were the flies primarily seen laying eggs on the this region. Larval masses, comprising of mainly second instar Calliphorid and Muscid larvae, were forming in the mouth and on the head of the carcass. Towards the end of this stage, small larvae were observed near the rectum and large third instar larvae were feeding on tissues on the head and face. Spiders were seen feeding on adult *Lucilia* sp and a Staphylinid beetle was feeding on an adult Piophilid.

Active Decay Stage – (Days 8 to 14)

Appearance of Pig

This stage was marked with the obvious deflation of the carcass. As decomposition and insect feeding continued, the carcass became more flattened. Areas that were subjected to heavy insect feeding, such as the face and head region, were drying and becoming black (Figure 2.15). The first layer of skin was separating from the body and sliding from its original location. A white fungus was noted on the carcass by Day 11. The very strong putrid odour was overwhelming. Insect activity was at its greatest during the active decay stage.

Weather

Warm weather persisted throughout most of this stage with sunshine and very little rain. Temperatures of above 20°C were often recorded.

Figure 2.15: Experiment 3 (Summer 2003), Day 13 – Active Decay stage.

Insect Activity

On Days 9 & 10 hundreds of flies were present on and around the carcass; *Calliphora* sp., *Lucilia* sp., and Piophilidae outnumbered all other species on the carcass by at least 3 times. Many of these were observed ovipositing. Sepsidae were no longer present on the carcass at this point. Calliphoridae larvae (later identified as *C. vicina*) were migrating away from the carcass on Days 8 & 9, during the first observed mass exodus. Maggot masses engulfed the carcass including a large range of size and species. A "popping" sound could be heard coming from these maggot masses. The highest maggot mass temperature was measured at 37.6°C, which was an elevation of 17°C above the ambient temperature. Larvae on the head were reared and found to be *C. vomitoria*. New eggs, some which were identified as *C. vicina*, *C. vomitoria*, and Stratiomyidae, could be found on various locations on the carcass. Signs of Silphidae beetle larvae, such as cast skins and faeces, were observed on the carcass; however, none had been captured in the pitfall traps.

Advanced Decay Stage – (Days 15 to 19)

Appearance of Pig

Entering the advanced decay stage a significant amount of the flesh had been removed and the carcass looked flattened (Figure 2,16). The anterior end of the carcass was dry; the bones in the head were disarticulated, while the posterior end still retained some tissue. Exudate surrounded the carcass, which still emitted a strong putrid odour.

Weather

The weather consisted of mostly sunny periods with some clouds.

Figure 2.16: Experiment 3 (Summer 2003), Day 18 – Advanced Decay stage.

Insect Activity

The diversity of species observed in this stage was similar to that of the previous stage; however, the amount of adult insects had decreased considerably. Calliphoridae and Muscidae were only present in small numbers (few, 1-5 per species). Piophilidae, on the other hand, remained quite numerous (at times 41+ flies). Beetles, such as Cleridae, as well as mites were becoming more common. Sepsids began to reappear on the final day of this stage. Throughout this stage, small groups of Calliphoridae and Muscidae larvae were observed in migration away from the carcass. Most larvae were confined to the posterior end of the carcass due to the limited availability of tissue and moisture at the anterior end. No large maggot masses were observed on the carcass. Silphid beetle larvae were finally collected from the body during this stage, even though evidence of these had been apparent for several days.

Dry Stage – (Days 20 to 126)

Appearance of Pig

The carcass appeared very dry (Figure 2.17). Very little tissue remained and only exudate below the carcass was moist. As this stage progressed the dry skin became bleached and exposed more of the bones beneath it. The white fungus on the skin,

originally noted on Day 11, was slowly spreading. The tall grass started to spread over the carcass toward the end of this stage. There was very little odour present.

Figure 2.17: Experiment 3 (Summer 2003), Day 49 – Dry stage.

Weather

As this stage was considerably longer than the others, varying weather was recorded from sunny days to heavy rainfalls.

Insect Activity

Fly activity was greatly reduced. Calliphoridae and Muscidae, which arrived one or two at a time, only remained on the carcass for one to five seconds. None were observed mating, feeding, or laying eggs. Piophilidae were still present in larger numbers (many, 20-40) but by Day 31 had dropped in abundance (0-15 flies). Sepsidae were present in greater numbers during this later stage. While Piophilidae adults were not numerous, the three species of Sepsidae (*Nemopoda nitidula, Meroplius minutes, Sepsis cynipsea*) were observed mating and laying eggs in greater magnitude (10-40 flies). The number of Sepsidae remained constant until Day 69. Other insects, such as Psychodidae, Eristalis, Homoptera, and small Ichnumanid wasps were observed on the carcass during this stage, but only one to five were seen per visit. As the vegetation started to grow over the carcass, flies were more commonly being found on the vegetation itself. Beetles were detected during this stage. Some Cleridae were observed almost daily, while Dermestids (adults and larvae) and Histeridae were only

seen during a few visits. Pitfall traps gave a better indication of the diversity of beetles present on or near the carcass than visual observations could offer (Table 2.4).

While the carcass appeared dry, "popping" sounds could be heard from the exudate under the carcass, indicating that there was ongoing insect activity. Piophilidae larvae were noted as early as Day 21. Similarly to the previous experiments, the larvae could be found on the skin after rainfall. However, during heavy rainfalls many would drown in the small puddles formed in depressions in the skin where water would collect. On Day 42 and again on Day 50 large amounts of migrating Piophilidae larvae were collected in the pitfall traps immediately after rainy periods. Third instar Sepsidae larvae were first noted on Day 55 and remained in view until Day 69. It was observed that on Day 76 there was a period of heavy rain yet no larvae were recovered from the pitfall traps.

Silphidae beetle larvae (*Necrodes littoralis*) were present early in this stage. On Day 31, many beetle larvae were collected in the pitfall traps indicating that they had begun to leave the carcass; however, they did not disappear entirely as some were still present in the exudate. Increasing amount of spiders, many Lycosidae, and mites were being seen on the carcass. The flies collected towards the end of this stage were often covered with many mites. Ants were also being observed during this stage.

For a visual comparison of insect colonisation for different decay stages between Summer 2002 and Summer 2003 see Appendix 3.

Pitfall Traps

Pitfall traps used in summer 2003 proved helpful before, to give a baseline, and during the experiment. Initially, adult *Lucilia* sp., Muscidae, and Carabid beetles were noted in the pitfall trap near (15cm) the pig. However, by Day 9 the traps also collected migrating larvae. While some arthropods, such as ants, Carabid beetles, Curculionid beetles, some Staphylinid beetles, Spiders, and Isopods, were found before the pig carcasses were brought to the research site (as well as near and far away from the carcasses) the pitfall traps near the carcasses showed that there was a drastic change in arthropod fauna when a decomposing animal was present. The pitfall traps placed before the pigs were introduced to the site and away from the pigs recovered fewer

species. These did not trap any adult or immature Calliphoridae, Piophilidae, Sepsidae, or Silphidae which were all present on the pig carcass and recovered from the pitfall traps near the pig. Table 2.4a and Table 2.4b list the arthropods collected in the pitfall traps.

2.3.4 Temperature and Rainfall

Along with the HOBO data loggers used at the experimental site, weather information was also provided by the weather station (PC208W) located at the University of Derby.

Experiment 1, Summer 2002, was conducted during a very wet period. An average of 91.0mm of rain was measured per month during Experiment 1, compared with 45.7mm of rain per month during Experiment 3, Summer 2003 (Table 2.5, Figure 2.18). The average ambient temperature was also 2°C cooler in Experiment 1 than for Experiment 3. Experiment 2, Autumn 2002, reached cooler temperatures and more rain was recorded than in the other two experiments.

With the help of temperature probes, the temperature inside the pig in relation to the ambient temperature could be monitored closely. Eight different temperature recordings were taken, using HOBO Pro Data Loggers, every hour for Experiment 3 – Summer 2003. Appendix 4 shows the temperature changes throughout the experiment.

Table 2.4a: Arthropods present in pitfall traps 100 metres away from the pig carcass before and during Experiment 3 – Summer 2003.

Order	Family	Species	100m away from pig				
			day –8*	day -6	Day 4	Day 11	Day 16
Diptera	Chloropidae	Not identified further	A				
	Heleomyzidae	*Neoleria inscripta*		A			
	Muscidae	*Helina sp*	A				
		Hydrotaea sp.	A				
	Psychodidae	Not identified further	A				
	Scathophagidae	Not identified further			A		
	Tipulidae	Not identified further	A		A		
Homoptera	Aphididae	Not identified further	A				
Hymenoptera	Formicidae	Not identified further	A	A			
	Sphecidae			A			
Coleoptera	Carabidae	Not identified further	A		A	A	A
	Curculionidae	Not identified further		A			
		poss. *Rugilus orbiculatus*	A				
	Staphylinidae	Not identified further		AL	AL		
Araneae		Spiders	A	A	A		
Acari	Acariformes	Mites species 2	A				
Collombola		*Springtails*	AL				
Phylum Annelida		Earthworms			A		
Isipoda	Not identified		AL	AL			
Class Chilopoda		Centipedes			A		

A = adults, L = larvae * Day –8 indicates 8 days before the commencement of the experiment.

Table 2.4b: *Arthropods collected in pitfall traps near (15cm) the pig carcass during Experiment 3 – Summer 2003.*

Order	Family	Species	Day 9	Day 11	Day 12	Day 25	Day 36	Day 39	Day 42	Day 49	Day 50	Day 55	Day 63	Day 69	Day 77	Day 83
Diptera	Calliphoridae	*Calliphora vomitoria*		A												
		Calliphora sp.	L			L					L					
		Lucilia caesar	A													
		Lucilia sp.			L											
	Muscidae	Not identified further	L	L	A											A
	Phoridae	*Megaselia sp.*	A										A		A	
	Piophilidae	*Piophila foveolata*					L		L	L	L		L			
	Sepsidae	*Nemopoda nitidula*					A	A	A							
		Meroplius minutus					A	A	A							
		Sepsis cynipsea				A	A						L	A	A	
	Spheaeroceridae						A	A						A		
	Tipulidae	Not identified further							A							
Hemiptera							A		A				A			A
Homoptera	Not identified		A					A								A
Hymenoptera	Braconidae	Not identified further						A								
	Ichneumonidae	Small black wasp, unidentified	A				A	A						A	A	
	Formicidae	Not identified further	A	A	A								A	A	A	A
	Platygasteridae							A								

A = adults, L = larvae

Table 2.4b: *Arthropods present in pitfall traps near (15cm) the pig carcass during Experiment 3 – Summer 2003, Continued.*

Order	Family	Species	Day 9	Day 11	Day 12	Day 25	Day 36	Day 39	Day 42	Day 49	Day 50	Day 55	Day 63	Day 69	Day 77	Day 83
Coleoptera	Carabidae	Not identified further	A		A		A	A	A	A			A	A	A	A
	Cleridae	*Necrobia sp.*					A									
	Curculionidae	Not identified further					A	A					A		A	
	Histeridae	*Margarinotus sp.*	A				A	A	A				A	A	A	A
	Nitidulidae	Not identified further	A					A								
	Silphidae	*Necrodes littoralis*				L	L			A	A	A				
		Nicrophorous humator		A												
	Staphylinidae	*Aleochara curtula*					A									
		Philonthus politus				A					A				A	
		poss. *Rugilus orbiculatus*						A	A				A			A
	Staphylinidae	Not identified further			A			L	L	A	A			A		L
Araneae		Spiders	A	A			A	A	A	A		A	A	A	A	A
Acari	Acariformes	Mites species 1					A	A	A				A	A	A	A
Collombola		*Springtails*					AL	AL	AL							AL
Phylum Annelida		Earthworms										A				
		cocoon												C	C	C
Phylum Mollusca	Order Stylommatophora	Snails - class Gastropoda										A				
Phylum Mollusca	Order Stylommatophora	Slugs						A		A	A	A				
Class Chilopoda		Centipedes					A									

A = adults, L = larvae, C = cocoon

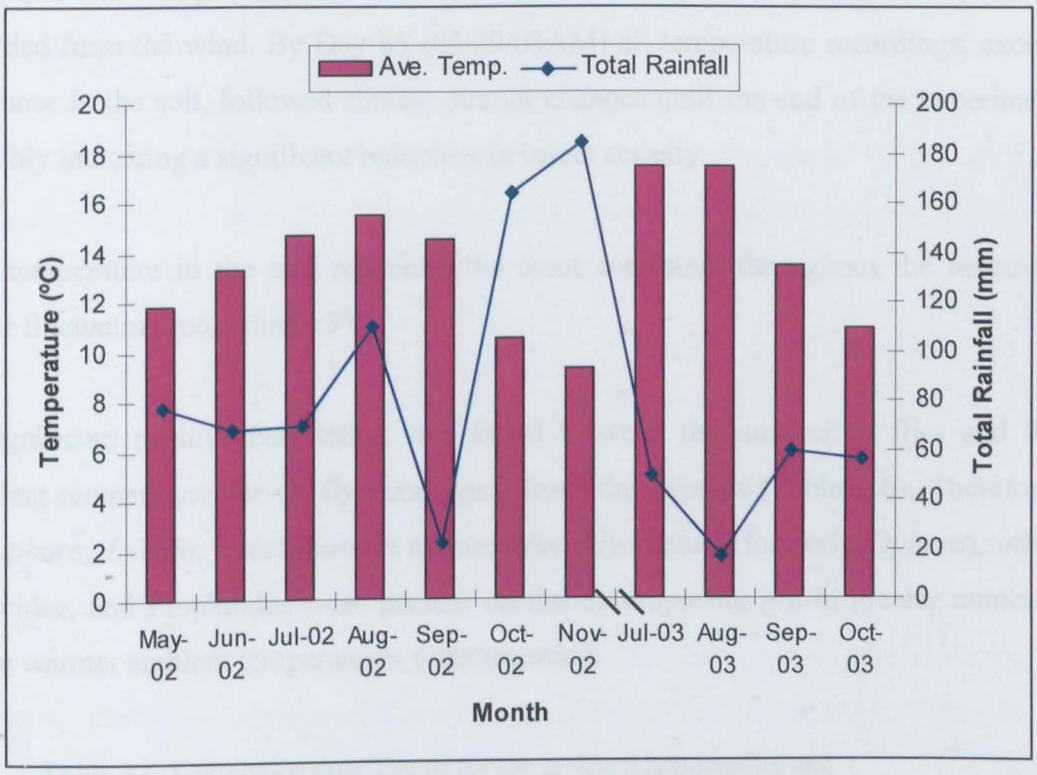
The recordings revealed that the ambient temperatures taken at 2 metres, 1 metre, and 0.35 metres were similar and fluctuated in the same diurnal manner. The surface of the skin of the pig carcass followed the general trend of the ambient temperatures but always 1-3°C warmer, especially during the daytime. During the very sunny days the difference between the surface of the skin and the ambient temperatures became greater, at times up to 9°C warmer.

On Day 4 (06/09/03AM), as maggot masses began to form in the mouth and head area of the carcass, the temperature in the mouth rose above all other temperature recordings to 19.9°C and remained warmer than the other recordings until Day 14 (06/19/03AM). By Day 14 the area was losing tissues and bones were being exposed. The temperature in the mouth began to decrease and followed temperature recordings similar to that recorded from the surface of the skin. While they remained similar, the mouth temperature recordings were 0.5°C to 2.5°C cooler than the skin surface temperatures, possibly due to the dry skin of the face still partially shielding the probe from direct sunlight.

Table 2.5: Average temperature (°C) and average rainfall (mm) data recorded

	Exp 1 Summer 2002	Exp 2 Autumn 2002	Exp 3 Summer 2003
Ave. Temp.	12.8	9.9	14.8
Ave. Rainfall	91.0	143.7	45.7

Figure 2.18: Monthly temperature recording (°C) and total rainfall measurements (mm) during the experimental months.



Until Day 10 (06/15/03PM), the temperature probes under the abdomen, inside the rectum, and in the soil were not subjected to as large temperature changes as the probes exposed to the air and the elements; therefore, these temperatures remained fairly consistent. As insect activity was moving to the tail end of the carcass, on Day 11 (06/16/03PM) the temperatures in the rectum started to increase above the ambient temperature and remained above the other temperature recordings, with the exception of mouth and under the pig, until Day 19 (06/24/03PM) when larvae were observed to have migrated from the body. As in the rectum, the temperature beneath the carcass rose as the maggot masses spread throughout the body. By Day 20 (06/25/03AM), as the large maggot masses disappeared, the temperature in the rectum and under the carcass fell and began to follow closely the temperatures of the skin and mouth. By Day 32 (07/07/03AM), when little tissue and few larvae remained on the carcass, the abdomen and rectum exhibited temperatures closer to ambient temperatures. As the probe under the pig carcass was shielded from the wind, it demonstrated temperatures slightly warmer than ambient temperatures. As of Day 60 (05/07/03AM), the temperatures underneath the carcass and in the rectum continued to closely follow the trends of the ambient temperature; however, they did not experience the highs and

lows seen in the ambient recordings. Instead they remained relatively stable across the 24 hours. This was possibly due to the fact that these temperature probes were partially shielded from the wind. By Day 85 (08/29/03AM) all temperature recordings, except for those in the soil, followed similar diurnal changes until the end of the experiment possibly indicating a significant reduction in insect activity.

The temperature in the soil remained the most consistent throughout the research; never fluctuating more than ±5°C.

A significant positive correlation was found between the number of flies and the ambient temperature for all fly taxa, apart from the Sepsids (Table 2.6). Therefore, *Calliphora*, *Lucilia*, *Protophormia terraenovae*, *Hydrotaea* (formerly *Ophyra*), other Muscidae, and Piophilidae were present on the decomposing pig in greater numbers when warmer ambient temperatures were recorded.

Table 2.6: Correlation coefficients for the ambient temperature and numbers of flies on the decomposing pig, n = 32.

	Pearson Correlation (r)
Temperature	1
Calliphora	.433(*)
Lucilia	.460(**)
Protophormia terraenovae	.401(*)
Hydrotaea* – formerly *Ophyra	.478(**)
Other Muscidae	.387(*)
Piophilidae	.353(*)
Sepsidae	-.157

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Maggot Mass Temperatures

Maggot mass temperatures were recorded when possible and compared to the other temperatures taken on or around the carcass at the same time. It was found that maggot masses could measure up to 17°C above the ambient temperature during summer and autumn experiments; however, the larvae would still maintain a temperature below their lethal limit of over 35°C (Donovan *et al.*, 2006; Greenberg & Tantawi, 1993; Reiter, 1984; Wall *et al.*, 2001), except for two occasions where the larval mass

temperature rose to 37.5 °C and 38 °C. This behaviour of maintaining a constant temperature was also exhibited when the surface of the skin was heated by the sun to temperatures well above 40°C. Maggot masses did not reach these high temperatures. The exudate showed a similar pattern. This was often warmer than the ambient temperature but yet much cooler than the crust formed just above it. The average maggot mass temperature for the summer experiments was 31.5 and 18.6 °C in the autumn experiment, compared to the average ambient temperatures of 13.8 and 9.9 °C in the same experiments.

2.3.5 Exploration of the Soil Post Decomposition

Arthropod artefacts recovered at the end of Experiments 1 and 3 (Summer 2002 & 2003) are listed in Table 2.7. Both experiments resulted in similar findings. As expected, arthropods not associated with carrion had begun to reappear in the soil and many empty pupal cases were recovered. Immature and adult stages of insects known to appear later in decomposition, such as Sepsidae and Piophilidae, were present in the soil; however, surprisingly, immature stages of Calliphoridae were still found in the soil even at this late stage of decay.

Table 2.7: Arthropods present in soil under the pig carcasses at the end of both summer experiments.

Order	Family	Species	Summer 2002	Summer 2003
Empty pupal cases				
Diptera	Calliphoridae	*Calliphora vicina*	X	
		Calliphora sp.	X	X
		Lucilia sp.		X
	Muscidae	*sp.*	X	X
	Piophilidae	*Piophila foveolata*	X	X
	Sepsidae	*sp.*	X	X
	Stratiomyidae	*sp.*		X
	Syrphidae	*Eristalis tenax*	X	X
Coleoptera	Silphidae	*Necrodes littoralis* - cast larvae skins	X	X
Pupae				
Diptera	Calliphoridae	*Calliphora sp.*		X
		Lucilia sp.		X
	Muscidae	*Hydrotaea ignava (Ophyra leucostoma)*	X	X
		Hydrotaea capensis (Ophyra capensis)	X	X
		sp.		X
	Piophilidae	*Piophila foveolata*	X	X
	Sepsidae	*Nemopoda nitidula*	X	X
		sp.	X	X
	Stratiomyidae	*sp.*	X	X
	Syrphidae	*Eristalis tenax*	X	X
Coleoptera	Histeridae	*Margarinotus cadaverinus*		X
	Silphidae		X	X
Larvae				
Diptera	Calliphoridae	*Calliphora sp.*	X	
		Lucilia sp.	X	
		Protophormia terranovae	X	
	Muscidae	*sp.*	X	X
	Piophilidae	*Piophila foveolata*	X	
	Sepsidae	*sp.*	X	X
	Stratiomyidae	*sp.*		X
	Syrphidae	*Eristalis tenax*		X
Coleoptera	Histeridae	*Margarinotus sp.*		X
	Silphidae			X
	Staphylinidae		X	X

Table 2.7: Arthropods present in soil under the pig carcasses at the end of both summer experiments, Continued.

Order	Family	Species	Summer 2002	Summer 2003
Adults				
Diptera	Mycetophilidae			X
	Sepsidae	*Nemopoda nitidula*		X
Homoptera	Aphididae	Not identified further		X
Coleoptera	Histeridae	*Margarinotus cadaverinus*		X
		Margarinotus carbonarius		X
	Nitidulidae	*Omosita colon*		X
	Staphylinidae	*Anotylus rugosus*	X	X
		Gyrohypnus sp.	X	X
		Philonthus cephalotes		X
		Philonthus politus	X	
		Platystethus arenarius		X
		Rugilus orbiculatus		X
Araneae		Spider	X	X
Acari	Acariformes	Mites	X	X
Collombola		*Springtails*	X	X
Phylum Annelida		Earthworms	X	X
Phylum Mollusca	Order Stylommatophora	Snails - class Gastropoda	X	X
Phylum Mollusca	Order Stylommatophora	Slugs	X	X
Isipoda	Not identifies further		X	X
Class Diplopoda		Millipedes		X
Class Chilopoda		Centipedes		X
Other			X	X

2.4 DISCUSSION

Insects were observed feeding on the carcasses during the entire duration of the three experiments stressing that insects have a great role to play in decomposition. Insects are valuable evidence types to a Forensic Entomologist; however, as living organisms their behaviour must be studied closely to better understand their presence on a corpse.

For both summer experiments *C. vicina* was the first species to lay eggs; followed by *C. vomitoria* and *Lucilia* sp. *C. vomitoria* was the first to appear during the autumn experiment. Some research has shown that *C. vicina* is primarily an urban species and *C. vomitoria* a rural species; however, both species can be found in either location (Smith, 1986; Erzinçlioğlu, 1996; LeBlanc & Strongman, 2002). *C. vomitoria* has been described as a 'cold-adapted fly' (Greenberg & Tantawi, 1993) which might explain its early arrival during the autumn experiment. These results do not indicate whether the pig carcasses were in an urban or rural setting. In reality the site was in a rural location with a small city-like community (compound of Derbyshire Constabulary) built next to it. The author does not feel that the arrival of the first insect species could have been used as an indication of the type of location used as, in the case of the summer experiments, *C. vomitoria* also laid eggs and arrived on the carcass in great numbers. Many factors could have caused this outcome. One could speculate that *C. vicina* females can detect odours of decay at lower concentrations or they could have merely been there by chance. More research would have to be done before making any reliable conclusions. *Lucilia caesar* and *Lucilia illustris* appeared within the first two days of decomposition. It was found during this research, and stated by other authors (Erzinçlioğlu, 1983; Smith, 1986; etc.), that *Lucilia* sp. was most prevalent during sunny periods.

Muscidae were present throughout the entire duration of all three experiments but never in an abundance matching that of the Calliphoridae. A great diversity of Muscidae appeared on the carcass with *Hydrotaea dentipes* being collected the most amount of times during all three experiments. Experiment 1 (Summer 2002) showed *Stomoxys calcitrans* early during decomposition. This species was only captured once during all three experiments and was later determined to be accidental on the carcass as it is a blood feeder; not a carrion feeder (D'Assis Fonseca, 1968). Adult Muscids *Hydrotaea ignava* (formerly *Ophyra leucostoma*) and *Hydrotaea capensis* (formerly

Ophyra capensis) were commonly found on the carcass after the Fresh stage. Many of their larvae and pupae were recovered during the Dry stage of decomposition. These are thought to be predaceous on the larvae of other fly species (Smith, 1986; Lefebvre & Pasquerault, 2004). *Hydrotaea aenescens* (formerly *Ophyra aenescens*), a New World species, was regularly observed on the carcass during Experiment 1 (Summer 2002). This was the first published record for this species in the United Kingdom (Pont *et al.*, 2007). Lefebvre & Pasquerault (2004) suggest that *H. capensis* and *H. aenescens* are forensically significant species in France; however, the present study found that *H. ignava* was the primary *Hydrotaea* species present during decomposition in the Derbyshire region.

Piophilidae had almost a constant presence on the carcasses as adults, however, was not observed mating until the Active Decay stage. Megnin (1894) and Erzinçlioğlu (1983) list that adult Piophilids have a later presence on a carcass, while Anderson & VanLaerhoven (1996) agree with the early arrival of this species observed in the present study.

In the third experiment (Summer 2003) adult Sepsids were observed on the carcass during the Fresh, Bloated, and Dry stages. In the Summer 2002 experiment, these were not observed until the Dry stage. Erzinçlioğlu (1983) found that Sepsids could appear on a corpse merely a few hours after death but does not indicate whether these were mating or laying eggs. Immature Sepsids were not observed migrating nor were they collected in the pitfall traps more than once during the experiments. This would agree with Pont (1979) and my soil findings (Table 2.7) that these tend not to migrate away from the carcass to pupate; rather they pupate beneath it.

Psychodidae were only observed as adults towards the end of Experiments 1 and 3. Payne (1968) and Smith (1986) both noted the adults slightly earlier than this author. Smith (1986) states that Psychodidae larvae are found in semi-liquid habitats such as excrement or wet and decaying organic matter. The exudate would have been an ideal environment for these larvae; however, this author did not observe any or rear them from the soil samples at the end of the experiment.

Beetles were normally observed after the fresh stage with their abundance and diversity building as decomposition progressed. One exception to this was a Staphylinid, *Aleochara curtula*, which was only captured on the carcass in the first three stages of decomposition. This species has been documented as a parasite of Diptera pupae (Chapman & Sankey, 1955; Linssen, 1959; Payne & King, 1970; Smith, 1986). Chapman & Sankey (1955) reported this species as being the most common of all beetles on rabbit carcasses during studies conducted in July, 1951. They observed that *A. curtula* appeared on the third day after death, peaked on the seventh day, and then their abundance fell rapidly. While the days vary slightly, this finding agrees closely with the observations made by this author, suggesting that *A. curtula* arrived on the corpse soon after dipteran larvae were present. None were observed, however, during Experiment 2 (Autumn 2002), possibly due to seasonal effect.

Silphidae larvae, *Necrodes littoralis*, were observed feeding on the decomposing tissue and Dipteran larvae. These beetles were possibly one of the factors in the reduction of dipterous larvae on the pig carcasses during the advanced decay stage. Payne and King (1970) noted in their research that Silphidae larvae were carrion feeders and not predacious, which was quite different to what was found in this study.

Cleridae, Histeridae, and Dermestidae were all recovered from the carcasses once the skin was dry. Cleridae are described feeding on carrion (Gennard, 2007), as well as predators that will occupy dry carrion and other decomposing animal matter (Harde, 1984), which fits the findings of this research. Dermestidae feed on a large selection of dried animal matter (Smith, 1986), which would agree with the late collections of this group of beetles. Histeridae, however, are described by Payne & King (1970) as predators present during the bloated, decay, and early parts of the dry stage. This was not the case in this research; however, Payne & King (1970) also go on to explain that Histerids stay hidden beneath the carcass during daylight and become active at night. This could provide an explanation why these were not observed prior to the Dry stage.

Hydrophilidae, *Cercyon* sp., were only found during the Dry stage of Experiment 1 (Summer 2002). Smith (1986) reports that the terrestrial species can be associated with mud, dung, or vegetable matter, where the larvae will occur in moist situations. He also explains that Hydrophilidae recovered from a fox corpse were possibly predacious

on the smaller dipteran larvae present. These findings would agree with the situation present on the carcass at the time the Hydrophilid beetles were present: Experiment 1 (Summer 2002) was conducted in a relatively wet summer, which contributed to a significant amount of exudate beneath the pig, where mainly Piophilidae and Sepsidae larvae were present on the carcass and exudate.

Carabid beetles are predacious and can be found on carcasses due to the concentrated food supply (Payne & King, 1970). The only Carabidae collected throughout the experiments were in the pitfall traps (Experiment 3) away (100m) and near the body (Table 2.4a&b). However, none were collected directly from the pig carcasses. Gennard (2007) describes that these beetles are nocturnal, possibly explaining the fact that none were recorded on the pig.

Some species of Hymenoptera were present; however, these were only observed attacking flies rather than feeding on the carrion itself. Only ants occasionally left signs of having fed on the skin of the carcass (Payne & Mason, 1971; Castner *et al.*, 1997).

An interesting phenomenon was observed with the maggot mass temperatures inside the body. While it is common knowledge that maggot masses greatly elevate the temperature inside a corpse (Campobasso *et.al.*, 2001; Grassberger & Frank, 2004), it was surprising to find that the larvae actually kept their habitat at a relatively constant temperature. These seemed to regulate the temperature inside the body to an optimal development temperature. At times, the maggot mass temperature was often up to 17°C above the ambient temperature due to their aggregation and constant movement. When the weather was warm and the sun was shining on the skin of the pig carcass, temperatures the pig's skin was sometimes up to 48°C; well above the lethal temperature of a maggot. However, when the probe was inserted into the body, the maggot mass temperatures were below this lethal limit of approximately 35 °C. It is the movement of the larvae that generates heat, but it is also this movement that possibly kept the maggot mass temperatures below 35 °C. It was observed that there was a constant cycle of the larvae on the inside of the mass being pushed to the outside, while the larvae on the outside made their way inside. This constant cycle could have contributed to cooling the mass as the warmer larvae left the centre and the cooler

larvae entered the mass. There is also the possibility that the mass was less compact or a bit more spread out during the warm days allowing the heat to dissipate; however, no larval mass size measurements were routinely taken during these experiments. Further investigation into this activity would help to explain their cooling process.

Pitfall traps were helpful in collecting post-feeding third instar larvae migrating away from the carcass. Days where larvae were witnessed leaving the carcass to pupate, larvae were also collected from the pitfall traps. Larvae were also collected from the pitfall traps when none were witnessed leaving the carcass. Soil samples were also collected to determine days of migration from the carcass; however, larvae were not always found in the soil samples. Therefore, it was demonstrated that some groups of larvae migrating away from the carcass would have been missed entirely without the use of pitfall traps.

The varying weather conditions were shown to be a great indicator of decomposition and insect activity. A positive correlation was found between the number of flies on the body and the ambient temperature. Rain and sunshine both initiated different behaviours from insects on and around the carcass. Sunny periods revealed larger numbers of flies on the carcass while light rain initiated larvae migration away from the pig. Rainfall possibly had an important effect on decomposition as Grassberger & Frank (2004) found that heavy rainfall reduced the internal temperature, which retarded larval development and thus slowing down biomass removal. In this study, rain did lower the temperature inside the pig but not as much as the external temperature recordings, nor did the inside of the pig exhibit the temperature fluctuations recorded of the ambient temperature. During the later stages of decomposition, rain was found to re-hydrate the carcass and the surrounding exudate, leading to more insect activity therefore prolonging decomposition; which is a view also shared by Archer (2004). The amount of rain and cooler temperatures in Summer 2002 may have contributed to the reduced rate of decomposition. Experiment3, Summer 2003, experienced warmer temperatures, much less rain, and a faster decomposition rate than seen in Experiment 1.

There were important differences, such as species composition and abundance, observed between the autumn and summer experiments, suggesting that season has a

great effect on decomposition. Archer (2004) agrees with this statement but warns that the extent of seasonal differences depends greatly on the year. This author feels that seasons are loosely used to describe the time of year; however, they do not always give a good indication of the actual weather and ambient temperature.

The temperatures recorded by the data loggers corresponded well with the changes observed during the experiments. With temperature probes placed in several locations inside and outside the carcass, it was possible to track immature insect movement and development throughout the carcass in the earlier stages of development.

Megnin (1894) originally listed "waves", or successions, of insects associated with the decomposing human body. While it is true that certain insect species are attracted to the carcass at a particular time, this research did not produce such specific insect successions due to the amount of overlap. While no insect succession was found that would provide an unambiguous estimate of minimum time since death, many species did prove to be more active during specific stages of decomposition and certain Calliphoridae, such as *C. vicina*, *C. vomitoria*, and *Lucilia* sp, were predictably the first species to lay eggs on the carcass. It is not only their presence that is important but also the species composition of insects (Erzinçlioğlu, 1983), their abundance and that of their offspring which must be considered. While Calliphoridae (and at times Muscidae) were the first insects to appear on the carcass and were still collected during the final stage of decomposition; their abundance, however, was significantly reduced in the later stages. Adult Piophilidae were seen very early on during all three experiments, but the larvae were not apparent until later in the Dry stage of decomposition. Adult Silphidae beetles, *Necrodes littoralis*, were present early during the Bloated stage; however, it was their larvae that were the most apparent and had the most significant impact on the carcass during the Advance Decay stage. Adult Sepsidae were briefly observed on the carcass during the Fresh stage but it was only during the Dry stage that adults and immature Sepsids were abundant on the carcass. Adult Cleridae were present during the Active Decay stage but was observed in much greater numbers during the Dry stage. All these families were present on the carcass throughout most of the decomposition, yet exhibited behaviours which lead to the assumption that certain stages or days in the decomposition were favourable to others.

While findings made during these experiments were similar to those published by other authors conducting similar experiments, there were enough differences to stress the fact that studies in a particular region of interest are very important to better appreciate insect species and behaviour in specific geographical locations. Waves or specific days of insect activity given by authors such as Megnin (1894) and Erzinçlioğlu (1983), were often very different from results found by this author. However, stages of decomposition proved to be a much better description of insect activity on the carcass, with many commonalities shared with this author's experiments. I therefore feel that invaluable information was collected during this research to aid the Derbyshire Constabulary in their effort to further their knowledge in the field of forensic entomology.

Many factors mentioned earlier, such as temperature, weather conditions, and stage of decay, do affect the amount of arthropods on a corpse as well as their behaviour. Another important factor to consider is semiochemicals. Odour plays an important role in initiating insect behaviour (Pickett *et al.*, 1998). The information gathered during this chapter's investigations was applied in the next chapter, in conjunction with chemical analysis and electrophysiological tests, to determine whether olfactory stimuli had an effect on the attraction of certain insects to the corpses.

2.5 SUMMARY

Carrion insects have become important evidence types in criminal investigations as, with the proper knowledge and understanding, a large amount of information concerning the scene and the decomposing body can be extrapolated. While entomology is currently the best method of determining time since death (Buchan & Anderson, 2001; Vass *et al.*, 2002), changing geographical locations present different entomo-fauna and varying insect development and behaviours. In the United Kingdom, few succession experiments have been conducted. In fact, only one of these has been conducted using pig carcasses, which most closely resembles the human body (Turner & Wiltshire, 1999). Therefore, at the request of the Derbyshire Constabulary, studies were undertaken to gather carrion entomo-fauna data in the Derbyshire region of England.

The major aims of this investigation were to record the different species of Calliphora and other arthropods present on the decomposing carcass and determine whether a clear insect succession could be identified. It was also important to determine if a link could be establish between the number and the species of insects on the carcass with other arthropods, the varying weather conditions, temperature, and the different stages of decomposition.

Three experiments were conducted at the Derbyshire Constabulary Headquarters. Dead 45Kg pigs were placed in a field in order to study decomposition, the insects and the effects stated above. Two experiments were conducted in the summer months while one more was carried out in the autumn. Many insect counts and collections were made. Weather data and temperature recordings were also collected.

Calliphora vicina and *C. vomitoria* were found to arrive within minutes of exposing the body and were the first to colonise the pig carcasses. *Lucilia caesar* and *L. illustris* also arrived very soon after placing the body and were most common during sunny periods. Muscidae were present throughout the duration of the experiments, however, in much fewer number than Calliphoridae. Silphidae and Staphylinidae beetles, adults and immature stages, were present early during decomposition; however, during the last stage of decomposition Cleridae, Histeridae, and Dermestidae beetles were much more numerous. Temperature and weather conditions such as sunshine, rainfall, and wind had a great effect on decomposition and larval behaviour. Cool temperatures reduced the rate of insect activity and decomposition while rain triggered mass larval migration from the body during the early stages of decomposition. Larval (maggot) masses greatly elevated the temperature of that area, often up to 17°C above the ambient temperature. However, the larval masses seemed to maintain an optimal development temperature by keeping the mass below 35°C. Insect succession on the decomposing pigs could only be discerned when species composition of insects, their abundance and that of their offspring were taken into account.

In conclusion this research established important carrion entomo-fauna data for the Derbyshire region, but also revealed new insight into behaviours, such as those exhibited by the larval masses and migrating larvae, never before studied in depth in the field of forensic entomology.

CHAPTER 3 – ANALYSIS OF SEMIOCHEMICALS AND INSECT RECEPTOR RESPONSES

3.1 INTRODUCTION

In forensic entomology research, it is general practice to record insect succession and compare this to the state of decomposition of the body through visual observations, as demonstrated in the previous chapter (Payne, 1965; Smith, 1986; Anderson & VanLaerhoven, 1996). This chapter introduces an additional factor; the identification of olfactory stimuli which could be associated with insect succession.

Human decomposition is said to begin approximately four minutes after death initiated by the self-digestion of cells, called autolysis (Vass *et al.*, 2002). There are no visual or odour effects obvious to humans at this time; however, it is believed that insects are able to detect the decomposition immediately (Anderson, 2001). In central England blowflies are most often the first insects to oviposit on a carcass, therefore, it is important to determine which factors initially attract these insects to the body. Decomposing bodies go through constant change and emit hundreds of chemicals (Vass *et al.*, 2002). Determining which compounds, called semiochemicals, elicit an electrophysiological response in insects was the objective of this chapter.

As described in Chapter 1, semiochemicals play a considerable role in mediating insect behaviour (Birkett *et al.*, 2004; Pickett *et al.*, 1998). Identifying these specific compounds and investigating the responses they elicit could provide a better understanding of the insects and, in some cases, allow manipulation of their behaviour. The period between death and the arrival of the first ovipositing blowflies is of great interest to the forensic entomologist, yet not fully understood. Therefore, the identification of semiochemicals present which attract the blowfly, *Calliphora vomitoria*, to a decomposing body could potentially allow the calculation of a more accurate post mortem interval (PMI), as the semiochemicals could indicate when *C. vomitoria* was initially attracted to the carcass, providing a more accurate period of interval between death.

The majority of insect semiochemicals are volatile, making gas chromatography (GC) the ideal technique for both separation and quantification of the compounds present in the extracts (collected volatiles) (Cork, 1999). Gas chromatography allows for the separation, quantification, and subsequent identification of compounds within complex mixtures of volatile chemicals. However, additional techniques are required to discriminate between the biologically active and inactive compounds in the chromatogram (Wadhams, 1990).

Electroantennogram (EAG) recordings were originally utilised by Schneider in 1957. Using microelectrodes, he found that it was possible to record a depolarization of the affected sensillae on the antenna stimulated by a volatile compound. This is an effective means of initially identifying semiochemicals because EAG responses are recorded without the influence of environmental or neurological factors which could affect behavioural responses (Cork *et al.*, 1990). To take advantage of both techniques, a coupled GC-EAG system was employed for this research as it combines the separating ability of GC with the sensitivity and selectivity of the EAG, making it possible to isolate olfactory stimuli in a complex mixture (Wadhams, 1990). This provides a more focused consequent investigation, looking only at the biologically relevant compounds.

Odour studies, primarily bioassay experiments, have shown that *C. vomitoria* respond to substances such as liver (Wooldridge *et al.*, 2007) and synthetic dimethyl trisulfide (Nilsen *et al.*, 1996). However, no previous studies have explored the role of electrophysiology nor have the specific compounds associated with dead bodies, which elicit a response from *C. vomitoria,* been fully investigated and identified. Studies, using GC-EAG, have concluded that the housefly, *Musca domestica,* is attracted to specific compounds found in pig manure (Cossé & Baker, 1996). Such investigations into decomposition and the blowfly *C. vomitoria* would provide valuable information which could possibly be used in the field of forensic entomology.

An electroantennogram (EAG) preparation, which uses the structure that bears most of the olfactory sensory neurons, i.e. the antenna, coupled with high-resolution capillary column gas chromatography (GC) provides a powerful tool for locating the active components in behaviourally active samples (Pickett *et al*, 1992). Using this tool it was

hoped to solve many of the uncertainties regarding insect olfactory attractions and hopefully result in a clearer understanding of forensic entomology.

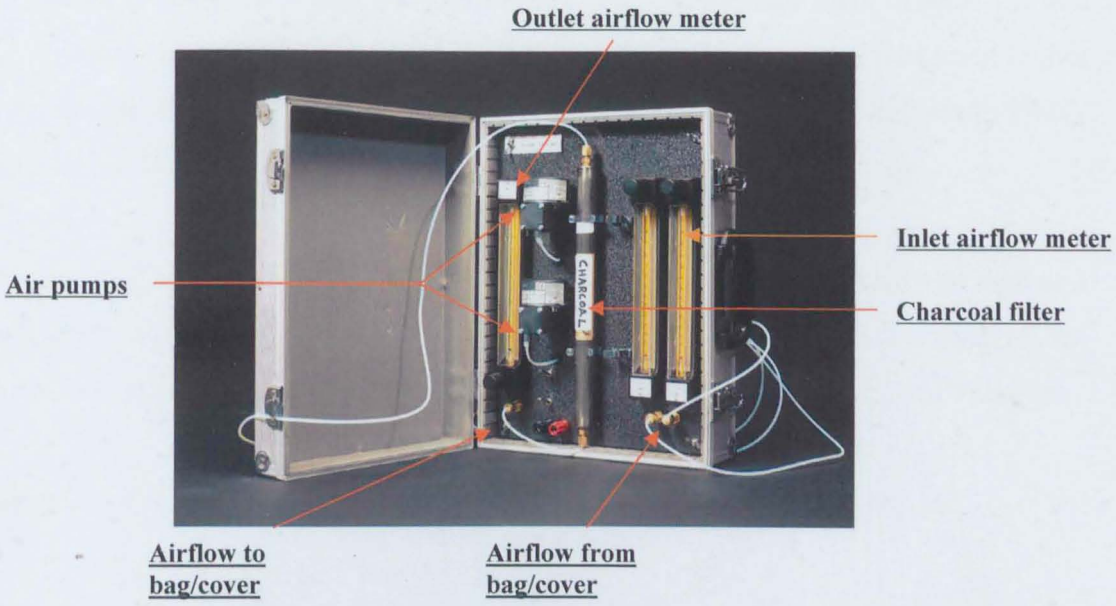
The focus of this investigation was to collect volatiles from pig carcasses during all stages of decomposition and determine specifically which semiochemicals elicited an EAG response from *C. vomitoria*. Developing the method for volatile collection, attaining reliable and consistent EAG results for *C. vomitoria*, and confirming the identity of possible semiochemicals were all essential components of this chapter.

3.2 METHODS

3.2.1 Air Entrainment

The air entrainment apparatus used to collect volatiles from decomposing pigs was made by Research and Development Engineer, Barry Pye, at Rothamsted Research, Hertfordshire, U.K. Each apparatus is made to suit the specific needs of the experiment, whether it be for the collection of volatile chemicals from plants, insects, or animals. The air entrainment apparatus normally works on a 'push' and 'pull' principal (positive air pressure system; Agelopoulos *et al.*, 1999) (Figure 3.1), but the 'push' was later dropped (Sections 3.2.3) as it was not necessary in this case. A stream of charcoal filtered air is 'pushed' by air pump into the area or chamber sealed for odour collection (positive airflow – mL/min). This ensures that no unfiltered air is drawn into the collection apparatus and that a vacuum is not created. The 'pull' system draws air from the headspace through an appropriate collection tube containing a polymer (description below) which collects the volatile chemicals within the chamber. For the following experiments, the apparatus was powered by a 12-volt car battery in order to operate it in the field.

Figure 3.1: Air entrainment kit.



3.2.2 Semiochemical Volatile Collection Tubes

Porapak Tubes

The odour collection tubes were prepared by me in the Rothamsted Research analytical chemistry laboratory. They were made from 5 mm glass tubing shaped to measure 8cm in length with a slightly pointed tip. Glass wool was inserted and pushed toward the pointed end of the tubes. 50mg of adsorbent Porapak Q (50-80 mesh, Waters Assoc. Ins, U.S.A.), a cross-linked polymer resin which traps volatiles, was added into the tube followed by more glass wool (Figure 3.2). The tubes were then held vertically and washed with 1mL of re-distilled diethyl ether, placed in an oven heated to 138°C, with a stream of nitrogen passing through them for approximately 12 hours. Each was then sealed inside a sterilised 10 mm glass ampoule and carefully stored in padded containers until required.

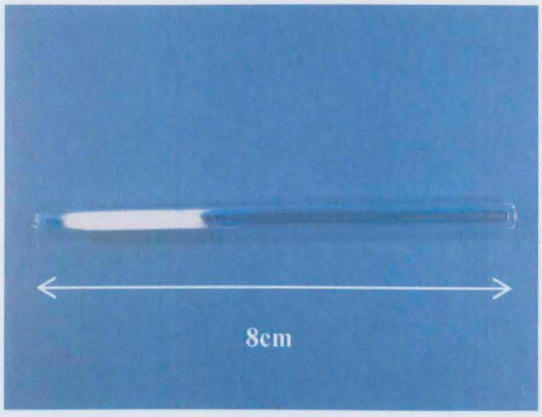
Tenax Tubes

The tubes used above were not adequate for use in thermals desorption; therefore, glass tubes (8cm long; 5mm ID) were purchased from ATAS (Cambridge, UK). These tubes did not have a pointed tip but instead had a slight constriction 1cm from the lower end. These were prepared (filled and washed) in a similar fashion to the Porapak tubes; however, Tenax TA

(Supelco) resin was used instead of Porapak Q and tubes were placed in a 210°C oven for 12 hours with nitrogen passing through them. Tenax TA is a porous polymer resin based on 2.6-diphenylene oxide and can withstand higher temperatures than the Porapak Q polymer resin (Agelopoulos & Pickett, 1998). It has been specifically designed for the trapping of volatiles in air.

For every batch of Porapak or Tenax tubes sterilised and sealed one tube was kept as a blank control to be run in the GC at a later date to confirm the success of the sterilisation technique. When tested, all blank tubes were found to be free of volatiles.

Figure 3.2: Porapak tube used for the collection of volatiles during air entrainment.



3.2.3 Air Entrainment of Pig

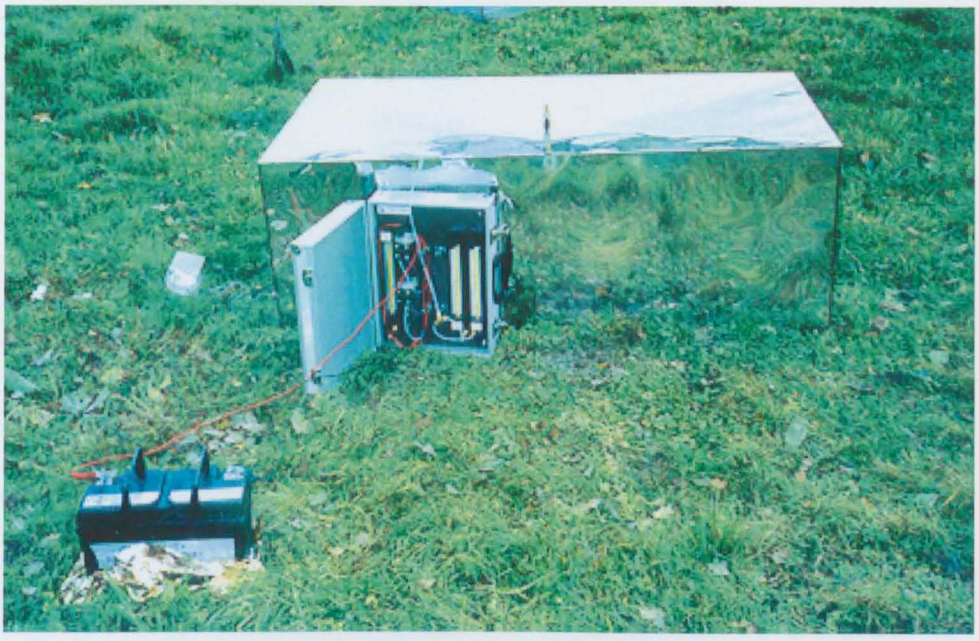
It was important to determine the best means in which to proceed with air entrainment experiments. Therefore, trials were conducted to test the methods and equipment required. During initial trials at Rothamsted, a thermal blanket, similar to that used by Logan (2005), was laid outdoors over shaped stainless steel wires positioned over an area matching the size of a 50 Kg pig in a way that would prevent the thermal blanket from touching the pig, if one had been in place (Figure 3.3). Sponges (2x3cm) permeated with 1µL of 3-methylindole (skatole) were placed under the wires. The thermal blanket was held down by long and slender sand bags to minimize the intake of air from outside the blanket. Porapak tubes were connected to the thermal blanket using Swagelok brass connectors. These were connected to air pumps and flowmeters using PTFE tubing. The stream of charcoal filtered air flowing inside the thermal

blanket also prevented its collapse inwards. The air entrainment was run for 1 hour and then the extracts were analysed using gas chromatography.

Figure 3.3: Original equipment used for the collection of volatiles showing the air entrainment kit, car battery, and the thermal blanket.

An experiment, using the same equipment as above, was conducted at the Derbyshire Constabulary Headquarters over the first decomposing pig used for research (Chapter 2). Air entrainments were conducted for 1hr, 2hrs, and 4hrs. The extracts were analysed using gas chromatography. While the thermal blanket allowed for the simple addition of new openings for extra sample tubes, it proved to be too fragile and much too difficult to work with, especially in windy conditions. The sharp stones on the soil caused scratches, thinning, and eventually holes in the blanket, while the wind caused the blanket to blow onto the wet skin of the pig and often prevented proper positioning of the thermal blanket. To resolve this problem, a rectangular 1.5m × 1.0m × 1.0m cover made entirely of stainless steel was constructed for the specific purpose of odour collection. Stainless steel was used as it would not absorb the volatiles from the decomposing pig and it is resistant to corrosion (Safra & Aguilar-Cauz, 2007). A Swagelok brass connector was fitted on one side of the box for the air inlet and another connector was placed on the top of the box for the air outlet (Figure 3.4).

Figure 3.4: Equipment used for the collection of volatiles showing the air entrainment kit, car battery, and stainless steel cover.



The shiny surface of the stainless steel cover shown above is reflecting light.

After three test trials, the decision was made to stop airflow into the stainless steel lid as it was too little to adequately circulate the air under the cover. Instead all of the power was re-diverted to the 'pull' system to extract a greater volume of air. As the cover was solid there was little chance of collapse. The weight and the sharp edges of the stainless steel cover caused the cover to dig into the soil such that only little air entered the cover from the outside environment; however, not to the point of creating a vacuum. No part of the cover came into contact with the pig. This cover was only over the pig during air entrainments. The remainder of the time the pig was covered with a wire cage described in Chapter 2. The cover was washed, when required (usually once per week), to remove any plant and soil debris with a detergent solution in water, and then rinsed with 95% ethanol which was allowed to evaporate.

During the trials, the air entrainment machine operated with an air flow near 800 mL/min for the 'push' system and almost 1000 mL/min for the 'pull' system. However after entirely re-diverting the 'push' flow, the 'pull' flow was 1400 mL/min.

3.2.4 Test Experiment

A female 45kg pig was killed by lethal injection administered by a local veterinarian and placed in the experimental field at Derbyshire Constabulary on the 4th of October 2002 for the purposes of semiochemical study. This experiment was conducted 40 metres away from the first pig, which only consisted of skin and bones at this time (Chapter 2). Odour collections were made daily until the 4th of November 2002. Temperature recordings and insect samples were also taken each day (method defined in Chapter 2).

Control entrainments of the grass were carried out by placing the stainless steel cover over a patch of grass 150 metres away from the pig, to eliminate volatiles emanating from the grass rather than the pig. The control samples were taken just before the pig was brought to the site (Day 0) and again halfway through the experiment (Day 19, 23rd of October 2002).

The daily odour collections were taken using Porapak tubes. Each sampling time was two hours and the liquid sample resulting from the elution of the Porapak with redistilled diethyl ether (extract) was analysed using GC (further described in Section 3.2.9). There was a concern that certain volatile compounds had a similar retention time to that of diethyl ether, resulting in some of the volatile peaks being concealed under the large solvent peak in the GC traces. Therefore, a new method (described below) of collection was introduced to the experiment to either complement or replace the Porapak method. It was also found that the GC peaks were quite small indicating that the concentration of the volatiles was very low. The entrainment time, when using Porapak tubes, would have to be extended.

On the 11th of November 2002, collections were made using Tenax tubes with sampling times of 10 minutes, 20 minutes, and 30 minutes, including a control sample of the surrounding grass performed as described above. Each sample was sealed in a sterilised 10 mm glass ampoule immediately after the collection of volatiles. The tubes were analysed by GC using thermal desorption (described in Section 3.2.9).

It was decided that a combination of the two techniques (i.e. Porapak and Tenax) was required. The Tenax tubes were used during the day as they required shorter

entrainment time and the Porapak tubes were used in the evening to avoid the hot sun shining directly on the metal cover, essentially turning the container into an oven. Tenax allowed for shorter air entrainments; however, as the samples were completely destroyed during thermal desorption, Porapak was also used as the volatiles were collected in solvent and could be used for multiple assays, including coupled GC-EAG experiments described in Section 3.2.10. The entrainments using Tenax tubes were run for 30 minutes each and the entrainments using Porapak tubes were conducted overnight (minimum of 12 hours).

3.2.5 Experiment, Summer 2003

In the summer of 2003, three pigs were obtained to carry out an experiment as above but with some adjustments. One pig was assigned for insect collections, one for temperature data, and another specifically for air entrainment experiments. This was done so that each pig was disturbed as little as possible. Further details of the complete experiment are listed in Chapter 2, Section 2.2. The pig assigned for air entrainment experiments, pig 2, was placed four metres away from a row of tall bushes and received approximately the same amount of sunlight and shade as the other two pigs. Both Porapak and Tenax tubes were used in this experiment. It was found in the Test Experiment that 2 hours was not enough time to collect a sample using Porapak as the sample had to be concentrated considerably, leaving very little sample available for future work. Because the sample had to be taken over several hours, the air entrainments were done in the evening as the weather was cooler and this decreased the chance of a sharp rise in temperature under the cover.

Tenax samples only required 30 minutes and therefore, could be taken during daytime hours. As the entire sample was used for analysis by thermal desorption GC, two consecutive samples were taken for most sampling days, one for normal GC analysis using a flame ionisation detector (FID) and the other for GC-mass spectrometry (MS).

When air entrainments were not being conducted, the stainless steel cover was removed from the decomposing pig and replaced with a cage cover similar to pigs 1 and 3 to protect it from scavengers.

Air entrainment collections were carried out at regular intervals (explained below) throughout the experiment extending from the 5th of June 2003 to the 4th of September 2003. On the first day of the experiment, Day 0, control samples were taken of the grass before the pigs were brought to the site; one using a Tenax tube (30min entrainment) and another using a Porapak tube (4hr entrainment). Immediately after the pig assigned for air entrainment was taken out of the body-bag, visual observations were made, temperature measurements were taken, and two air entrainments were done using Tenax tubes. The pig had been dead, but kept inside the body-bag, for 4 hours at this point. A Porapak sample was taken overnight.

Tenax Samples

As explained in Chapter 2, five decomposition stages were observed: Fresh, Bloating, Active Decay, Advanced Decay, and Dry. During the first four stages of decomposition (the first 17 days), Tenax samples were taken daily as decomposition progressed quickly. Once in the Dry stage, samples were then taken 2 to 3 times per week for the remainder of the experiment.

Porapak Samples

Porapak samples were taken twice a week during the first four stages. When the Dry stage was reached, samples were taken once a week.

3.2.6 Supplementary Experiment

While conducting the experiment of summer 2003, a quick trial was performed to see if future air entrainment studies were warranted. The results of the whole body of experiments in this thesis are hoped to be used in murder investigations in the future. Therefore, a quick trial was done, solely for the interest of the author, to see if experiments could eventually be adapted for human bodies. On the 20th of June 2003, Day 15 of the experiment, three additional air entrainment collections were made using Tenax tubes. As a stainless steel cage would not be practical or helpful in the preservation of trace evidence at a scene of crime, a simpler method of collection of volatiles had to be explored. For this trial, each tube was held by hand 10cm over the selected area and the entrainments were performed for 15 minutes. One air entrainment collection was done over the head area, the next was done over the hind area of the pig

(tail), and the final was a control (grass) taken 50 metres away from the pig upwind of the research site.

3.2.7 Electrophysiology

Microelectrodes were prepared from 1 mm glass capillaries using an electrode-puller, to give a tip diameter of < 0.1 mm, and then cut to the required length. The microelectrodes were slipped onto Ag/AgCl coated silver wires and filled with insect ringer solution (refer to Appendix 5 for composition) to maintain electrical contact between the insect and the electrode. Female flies, *Calliphora vomitoria* (further details of flies in Section 3.2.11), were briefly anaesthetised by collecting each in a separate specimen tube which was then placed on ice. Once anesthetised, the head of a fly was excised from the thorax using a micro-scalpel and mounted on the indifferent electrode so that the tip of the electrode reached the base of the antennae. The tip of the antenna was placed against the recording electrode (described below Section 3.2.7.1). The preparation was held in a continuous, charcoal-filtered, humidified air stream (1 L/min) delivered from a Teflon tube. During stimulation, the tip of a Pasteur pipette, containing the odour sample, was inserted into the Teflon tube through a small hole, and a 2-second puff of air (600 mL/min) was delivered to the pipette and into the Teflon tube with the air stream flowing continuously over the preparation. Signals from the antennae were amplified (x10 000) (Syntech®, The Netherlands) and analysed using a customised software package (AutoSpike, Syntech).

The Pasteur pipettes were prepared by applying a solution of the test compound onto a strip of filter paper and allowing the solvent to evaporate for 30 seconds. The filter paper strip was then placed inside the Pasteur pipettes. This method is explained further in Section 3.2.12 – *Dose Response Test.*

3.2.7.1 Development of Electrophysiology Method

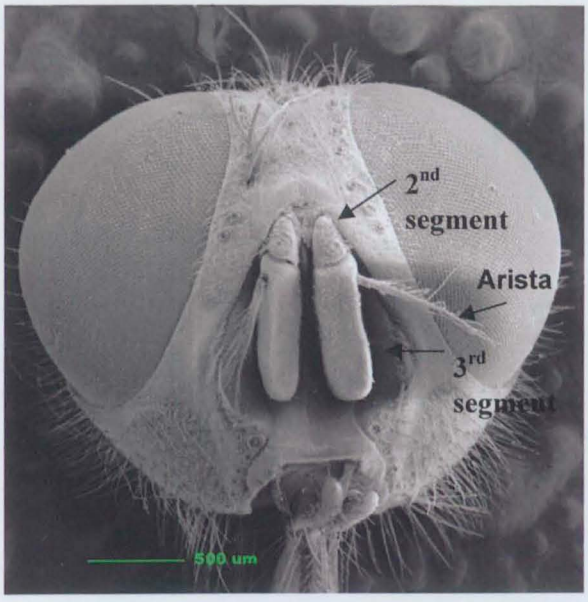
To conduct electroantennogram studies the best mounting and electrode position for the fly, *Calliphora vomitoria,* had to be found. Individual test stimuli suspected to elicit a response from blowflies was used to test the mounting technique. These stimuli were heptanone, linalool, and 3-methylindole (skatole). Solvent (hexane) alone was used as the control. A different fly was used for each experiment.

The following trials were conducted:

1. One antenna was removed from the fly's head and the base was placed into the indifferent electrode. The tip of the arista (Figure 3.5) was cut and connected to the recording electrode.

   Results: The antenna was difficult to manipulate. The results were very poor; there was little background noise but the responses to the stimuli were small and indiscriminate. The response appeared to be from the mechanoreceptors, which respond to mechanical stimuli such as the puff of air, rather than the actual compound.

Figure 3.5: Scanning electron microscope (SEM) image of Muscidae.



Provided by Images of Nature

2. One antenna was cut off and its base joined to the indifferent electrode. The tip of the third antennal segment, rather than the arista, was joined to the recording electrode. The tip of the recording electrode was cut to a wider size so that the tip of the antenna could rest onto the electrode without being pierced.

   Results: The antenna by itself was more complicated to manipulate. The background noise was low once the preparation was finally connected. The peaks obtained when a stimulus was introduced were not as "clean" as the others. For example they dipped down quickly but then would go up above the base line. The peaks were generally small.

3. Both of the antennae were removed still joined at the base. This base was placed on the tip of the indifferent electrode and the third antennal segment of one of the antennae was joined to the recording electrode.

   Results: Peaks were produced when the antenna was exposed to stimuli and much less background noise was exhibited. However, the preparation was only good for 30 minutes or less, which was much too short to conduct all experiments required.

4. The fly was anaesthetised; the legs were then cut off, the wings taped to its abdomen and the whole fly taped onto a stage. The indifferent electrode was placed into the eye of the fly and the recording electrode was joined to the tip of the third segment of the antenna.

   Results: The method was too complicated and did not yield any useful results.

5. The head of the fly was excised and mounted onto the indifferent electrode with the tip of the electrode positioned at the base of the antennae. The tip of the arista was cut and connected to the recording electrode.

   Results: The flies reacted with strong responses to the test compounds but a much smaller response to the solvent. However, the cut arista created background noise. This reduced slowly as time went by. The preparation was good for at least 1.5 hour.

6. The head of the fly was excised and mounted onto the indifferent electrode so that the tip of the electrode touched the base of the antennae, while the tip of the third antennal segment was joined to the recording electrode. A small amount of Spectra 360 electrode gel (Parker Laboratories Inc., Orange, NJ, U.S.A.) was placed on the tip of the third antennal segment to reduce background noise and to allow stable electrical contact (Costantini *et al.*, 2001). The proboscis was cut off to reduce any movement in the head.

   Results: The gel helped to reduce the background noise and produced a straighter baseline. The responses were very strong for the compounds and weaker for the solvent alone. The preparation was good for almost 2.5 hours with a slight decrease in reaction as time went by.

7. The same procedure as number 6; however no electrode gel was used.

   Results: Results were similar to number 6; however, it took more time for the background noise to subside.

Method number 6 was chosen as the most reliable to conduct the electroantennogram studies as it was the simplest to set up and gave the best results.

3.2.8 Gas Chromatography- Mass Spectrometry (GC-MS)

Tenax and Porapak tube extracts were analysed using GC-MS to identify EAG-active compounds. A Hewlett-Packard 5890 GC was connected to a VG Autospec mass spectrometer (Fisions, Manchester, U.K.). Ionization was by electron impact at 70 eV, 230°C, and the GC, using the HP-1 column, was maintained at 30°C for 5 min then programmed to heat up at 5°C min^{-1} to 180°C (Birkett, 1999). Analyses were made by Senior Research Scientist in the Chemical Ecology Group at Rothamsted Research, Dr. Mike Birkett.

3.2.9 Analysis by Gas Chromatography

Internal Standard

In order to quantify compounds within the volatile profile of an extract, an internal standard was run with each Porapak extract. An alkane series, incorporating $C_8 - C_{17}$, was run in order to obtain the retention times of each. The internal standard should elute relatively close to the peaks of interest, yet not overlap with any of the compounds (Selby, 2003). Undecane (C_{11}) was decided to be the most suitable internal standard.

Quantification of EAG-active chemicals from Porapak samples:

Concentration (ng/μL) = $((PA/ ET)/SPA))*(1/Inj)$

PA – Peak area
ET – Total entrainment time to collect sample
SPA – Internal standard (undecane) peak area
Inj – Amount of sample injected

The retention index, more specifically the Kovats indices (KI), of each peak was calculated for comparing the gas chromatographic retention time of different peaks and to aid in the identification of the compounds of interest. This calculation makes it possible to compare peaks from different machine (i.e. GC, GC-MS, & GC-EAG):

$$KI_X = 100n + 100 \frac{(LogRt_X - LogRt_{Cn})}{(LogRt_{Cn+1} - LogRt_{Cn})}$$

where,

Rt_X	the retention time of compound "X"
Rt_{Cn} & Rt_{Cn+1}	retention times of the reference n-alkane hydrocarbons eluting immediately before and after chemical compound "X"
n	carbon number of the reference n-alkane hydrocarbon eluting immediately before chemical compound "X"

Analysis of Extracts

Each of the Porapak tubes used during the experiment was washed with 700 µL of re-distilled diethyl ether to elute the volatile compounds. The volatiles were retrieved through solvent desorption. This was done by positioning the tube vertically, injecting re-distilled diethyl ether with a syringe at the top of the tube, and collecting the extract as it dripped into an ampoule (approximately 500 µL is collected while 200 µL remains inside the tube). The ampoules were then stored at –20°C. The liquid samples were analysed using Agilent 6890 GC equipped with an on-column injector and a flame ionization detector (FID). The GC was fitted with a non-polar HP-1 bonded phase fused silica capillary column (50 m x 0.32 mm i.d., film thickness 0.52µm). The carrier gas was hydrogen. The oven was maintained at 30°C for 1 minute after injection then programmed at 5°C min^{-1} to 150°C, followed by a second ramp at 10°C min^{-1} to 230°C.

A sample of the re-distilled diethyl ether used to elute the volatiles was kept in similar conditions as all the extracts and was tested regularly to confirm that no contaminants were in the ether. None were found.

The samples collected using Tenax tubes were analysed directly by thermal desorption on an Agilent-6890 model gas chromatograph fitted with an Optic 2 programmed temperature vaporization (PTV) injector (ATAS) and FID. Desorption inside the PTV unit was performed with a rapid temperature ramp starting at 30°C during introduction of the sample and reaching 220°C in 12 sec. The carrier gas was hydrogen. The column was non-polar (HP-1, 50 m x 0.32 mm i.d., 0.52 µm film thickness). Five Tenax tubes, corresponding with the stages of decomposition, were selected for analysis by GC-MS.

Confirmation of Identifications

Co-injections were performed using polar (DB-WAX) and non-polar (HP1) columns to confirm tentative identifications made by GC-MS. Extracts and authentic standards were co-injected in quantities that approximately doubled the area under the peak of the compound of interest. A positive identification of a compound was considered to have been obtained when peak enhancement was achieved on the two GC-columns of differing polarity.

GC analysis on a polar column was carried out using a Hewlett-Packard 6890 GC fitted with a polar DB-WAX capillary column (30m x 320μm i.d., film thickness 0.82μm), a cool on injector and an FID. The carrier gas was hydrogen. The GC oven was maintained at 30°C for 1 minute after injection then programmed at 5°C min^{-1} to 150°C, then 10°C min^{-1} to 240 °C.

3.2.10 Coupled Gas Chromatography – Electroantennogram (GC-EAG)

A coupled GC-electrophysiology system was used, in which the cooled (using nitrogen gas) effluent from the GC capillary column was delivered simultaneously to the antennal preparation and the GC detector. Separation of the volatile sample was achieved on an AI 93 GC (AI, Cambridge, U.K.) equipped with a cold-on-column injector and a FID. The column (HP-1, 30 m x 0.53 mm i.d., 0.5 μm film thickness) was maintained at 40°C for 2 min and then programmed to rise at 10°C min^{-1} to 250°C. The carrier gas was hydrogen. The output from the EAG amplifier and the FID were monitored simultaneously on a chart recorder. In total, eight trials were conducted using *Calliphora vomitoria* antennae and the extracts collected from pig 2 during Summer 2003 (Figure 3.6).

Figure 3.6: System design used for coupled gas chromatography and electroantennogram experiments

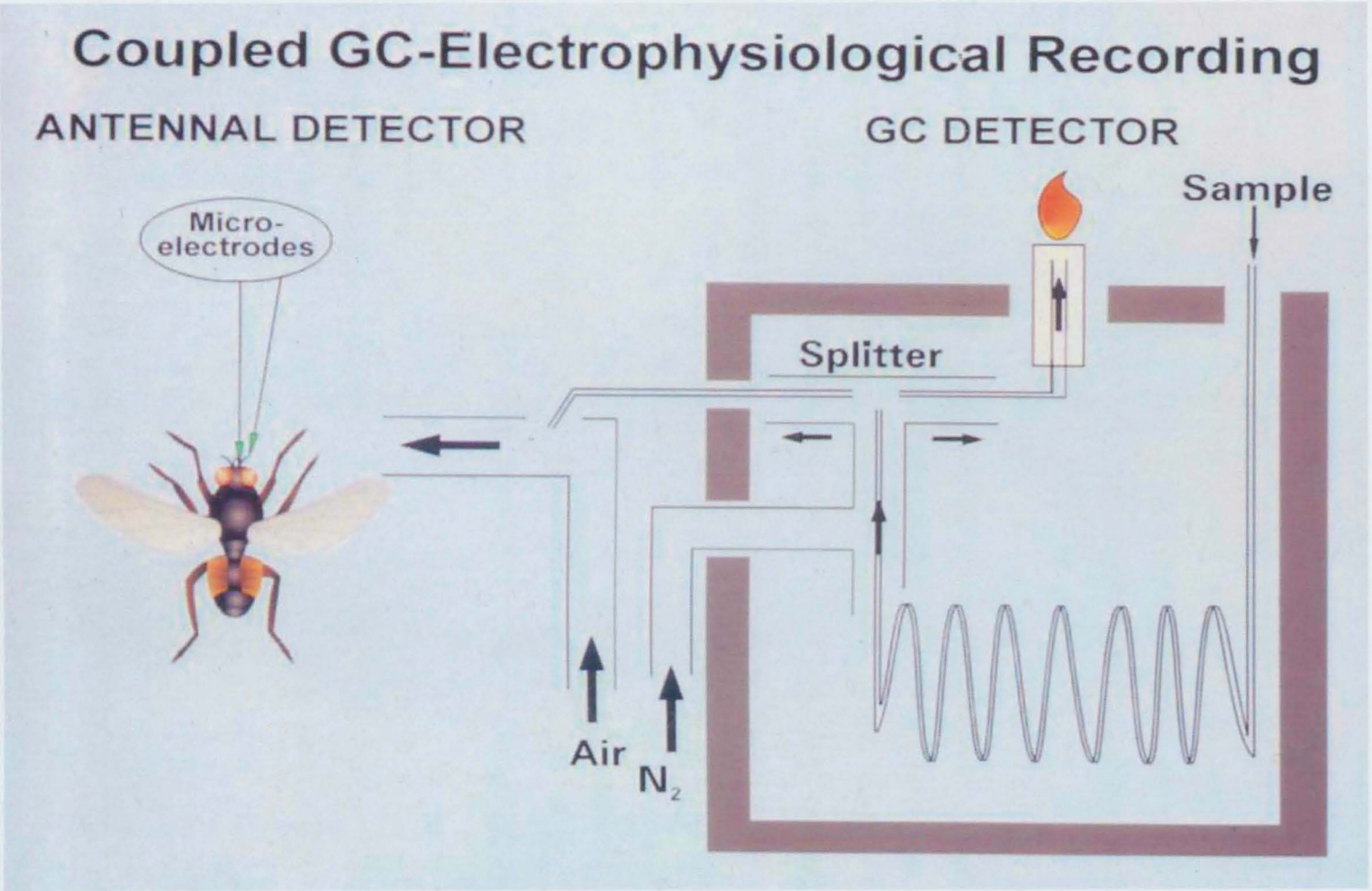


Image provided by Rothamsted Research

3.2.11 Flies

Calliphora vomitoria

Larvae were obtained at a local angling shop and reared in cages measuring 40cm x 35cm x 35cm. Females were separated from males within one day of emergence of the adults. These were fed water, sugar, and casein powder. The cages for the flies were kept in an entomology laboratory near a window and were therefore subjected to natural night. Female flies, between 1 and 4 days old, were used for the GC-EAG experiments because it is females which lay eggs, thereby initiating larval infestations of cadavers. Therefore, females are the most important sex in relation to forensic entomology investigations.

The experiments were conducted during daytime hours, from 8h30 to 15h30, only. After this time the responses to EAG-active compounds were significantly decreased (personal observation). Flies which had been kept in natural daylight and those which had been kept under artificial lighting (light 10hrs : dark 14hrs) all reacted in this manner. Hall (1980) reported that increased responsiveness (extension of the proboscis) from *Protophormia terraenovae*, was recorded twice (early morning and late afternoon) during photophase. Also, Minnich (1931) found that *C. vomitoria* responded to much lower concentrations of sugar mixtures during the first few trials of the day; however, as already expressed by Hall (1980), no mention was made of the exact time of the tests nor whether exhaustion played a role in the decreased response.

3.2.12 Dose Response

Dose response experiments, using EAG, were conducted to determine the threshold concentration for an insect response with selected active compounds and to confirm the electrophysiological activity.

Preparation of compounds

Test compounds were prepared in decreasing concentrations using hexane or diethyl ether as the diluting solvents. The test compounds, or treatments, consisted of all four electrophysiologically active compounds, propyl butyrate (tentative identification, EAG-active peak later referred to as KI 881-885), dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide, as well as 3-methylindole (skatole) and indole, which appeared in the samples collected from the pig. The last two compounds, 3-

methylindole and indole, were used as standards as it was known that they were EAG-active compounds of *M. domestica* (Cossé & Baker, 1996). Both solvents, diethyl ether and hexane, were used as negative controls as both were employed in the preparation of the dilutions. Hexane is the common solvent used as it is stable; however, due to some concerns of solubility with compounds such as dimethyl disulfide and dimethyl trisulfide, ether was used for those dilutions.

All tests were conducted by using 10μL of each treatment resulting in the concentrations listed below. Propyl butyrate and dimethyl disulfide were tested at concentrations of 10^{-7}g/10μL (10^{-8}g/μL, 10 ng/μL), 10^{-6}g/10μL (10^{-7}g/μL, 100 ng/μL), and 10^{-5}g/10μL (10^{-6}g /μL, 1000 ng/μL). All others were tested at concentrations from 10^{-9}g/10μL (10^{-10}g /μL, 0.1 ng/μL) to 10^{-5}g/10μL (or -10 to -6 in log scale).

EAG responses can vary with time; they can also be affected by temperature, airflow rate, juxtaposition of the air tube to the antenna, and previous responses (Roelofs, 1984). As each fly used in these experiments was exposed to compounds over a period of time, it was important to normalise the response amplitudes to a particular standard so that a percent value could be obtained. The following calculation was used to normalise the data: (100/value for standard) × value for compound, which was carried-out within the customised software package provided by Syntech (The Netherlands). Methyl salicylate was used as the standard (positive control) as it elicits a detectable EAG response in many dipterous species (Birkett *et al.*, 2004) and is chemically unrelated to the compounds tested in these experiments (Bhasin *et al.*, 2000). The concentration was kept constant at 10^{-7}g/10μL and was administered at 10 minute intervals. A dose response test for 2-heptanone was done at a later date using methyl salicylate and hexane as controls.

Dose Response Tests

Female flies, *Calliphora vomitoria*, were mounted as described in sections 3.2.7.1.

A solution of the test compound was applied onto a clean strip of filter paper (4mm x 40mm) using a capillary. The solvent was allowed to evaporate for 30 seconds. The filter paper was then carefully placed inside a Pasteur pipette and held with the thumb placed over the larger opening to allow the volatiles to collect inside the pipette for a further 30 seconds. The compound was then administered to the fly with a puff of air

as described in section 3.2.7. The next sample was prepared, allowing three minutes between each test.

Lower concentrations of the test compounds were administered first to avoid desensitising the fly with a stronger concentration. The same fly was used for up to two different concentrations. The fly was never exposed to the same concentration of a semiochemical more than once, with the exception of the positive control. Any response measuring a peak below the peak of the negative control was considered to be "non-responsive". Any peaks measuring a response similar or above the positive control was considered "responsive". As the experiment led into the more concentrated solutions, more time was allowed for the background noise (baseline) to stabilise between each sample. Results were normalised with respect to the average response from the positive control, methyl salicylate, to give a percentage value.
Each concentration was tested five times with a total of 12 EAG preparations (flies).

3.2.13 Statistical Analysis

3.2.13.1 Dose Response Analysis

EAG responses were compared statistically, as described below, to test differences between the treatments and to compare them with the positive and negative controls. With the help of Dr. Salvador A. Gezan the tests below were performed.

The data set encompassed a total of 5 experiments on which 9 treatments at different concentrations were compared. There were a total of 5 replicates for each treatment at different concentrations. In the tables, the different concentrations are listed as -10, -9, -8, -7, and -6 (in log-scale). The response variable (RESP) corresponds to the ratio of the measurement over the reference treatment: methyl salicylate (MS). No transformation was required in this response.

The analysis consisted in fitting simultaneously a linear regression for each treatment based in the following linear model:

$$y_{ijkl} = \mu + SET_i + TRT_j + CONC_k + TRT.CONC_{jk} + e_{ijkl}$$

where,

y_{ijkl}	response for a particular observation
μ	intercept
SET_i	random effect of experiment or set (block)
TRT_j	fixed effect of treatment
$CONC_k$	general concentration slope (covariate)
$TRT.CONC_{jk}$	specific concentration slope for each treatment
e_{ijkl}	random noise

This model was fitted using REML procedure as implemented in GenStat 8.0. A quadratic model was fitted and tested but it was not significant, hence, a simple linear regression model was preferred.

The main objective of this analysis was to compare treatments across different concentration levels. In a first stage, pairs of selected regression lines were compared. A significant result in this first stage was indicated when these lines had statistically significantly different intercepts and slopes, and it was expected that they would differ in most of the concentration levels. Therefore, for the second stage on those significant comparisons, the means at all concentration levels were individually compared. All the comparisons were obtained using the software SAS v.8. In all cases a significance level of α (p-value) = 0.05 was considered. Due to the large number of comparisons performed in the second stage, a Bonferroni correction was applied in order to avoid a large number of false positives. Therefore, significance levels corresponded to α^* = 0.000855 for a total of 60 comparisons.

3.2.13.2 Analysis of the GC-EAG Active compounds

Analyses were conducted using the four EAG-active compounds found in the extracts: propyl butyrate, dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide. Some analyses also included indole and 3-methylindole, as they had also been included in the dose response experiments. All statistical tests were performed using GenStat 8.0.

3.2.13.2.1 Multivariate Analysis

Due to the large number of data and different groups involved, multivariate analysis was conducted to reduce the complexity of the data by identifying which combinations of variables best summarise the compounds in the extracts.

Principal Component Analysis (PCA)

Principal component analysis (PCA) was carried-out due to the many variables being examined. PCA allows the number of variables in a multivariate data set to be reduced, while still retaining as much of the variation present as possible in the data set (Smith, 2002). This reduces the dimensionality of the data and identifies which combinations of variables best explain the largest amount of variation in the multivariate data set (Fowler-*et al.*, 1998). Principle components (PC) are created to explain the variation in the data set using scores with graphical representation.

The factor loadings show which variables contribute to the greatest differences between individuals. Loadings closest to 1 or -1 have a greater effect on the principal component, while those closest to 0 contribute very little to the value of that PC.

PCA generates a graphical display of the output by determining the best line to pass through the long axis of the grouping of the data, forming the first principal component (PC1). The positioning of the line explains the most variation between the variables, in this case chemicals. The next PC makes a line, which goes through the grouping of points at a right angle to the first axis, to generate a second PC and is designed to be as different from the first as possible (Dytham, 2003). Therefore, the first axis determines the most variations between the variables, the second explains the next most variations, and this continues in diminishing order. The maximum number of components is the same as the number of variables in the data set (Dytham, 2003); however it is hoped that the first few PCs will jointly explain a reasonably large proportion of the variations in the original sample (Fowler *et al.*, 1998).

PCA transforms a set of correlated variables to a new set of uncorrelated variables, making it particularly suited to data set with variables that have a higher correlation between themselves as very few principal components will be required to account for a large portion of the variance in the variables. Low correlation would lead to too many

of the PCs being required to determine the most variation in the data (Chatfield & Collins, 1980).

Canonical Variate Analysis (CVA)

Since five stages of decomposition were identified when collecting the volatiles and insects from the decomposing pigs (Chapter 2), it was important to also analyse the data (chemicals) while grouped into these different stages. Therefore, canonical variates analysis (CVA) was explored.

The test, while very similar to PCA, calculates the loadings of the variates that will maximise the differences between *groups* rather than the chemicals overall, as in the case with PCA. CVA produces loading values, which allow for the identification of those variables that are the most different between groups relative to the within group variability and discards those that are the same (Dytham, 2003). Hence, CVA minimises the spread within a group and maximise the differences between groups.

In this case, the analysis compared the five decomposition stages observed throughout the experiment and looked for variability in the concentrations of the four semiochemicals (in some cases six compounds) maximising differences between the group.

The scores of the first two canonical variates (CV) were plotted on a graph showing the differences between groups. Each group mean was plotted on the graph and encircled by a 95% confidence interval assuming multivariate Normality for the data. Overlapping of the circles from the group indicated that there was no significant difference between these groups at the $P = 0.05$ level.

This was only conducted with the Tenax samples as Porapak samples did not offer enough days per decomposition stage/group for this statistical test.

3.2.13.2.2 Univariate Analysis

One-Way ANOVA

A one-way ANOVA was used to compare the mean amount of the volatiles collected from the decomposing pig during the five decomposition stages. LSDs were used to locate the significant differences with a 95% and a 99% confidence interval.

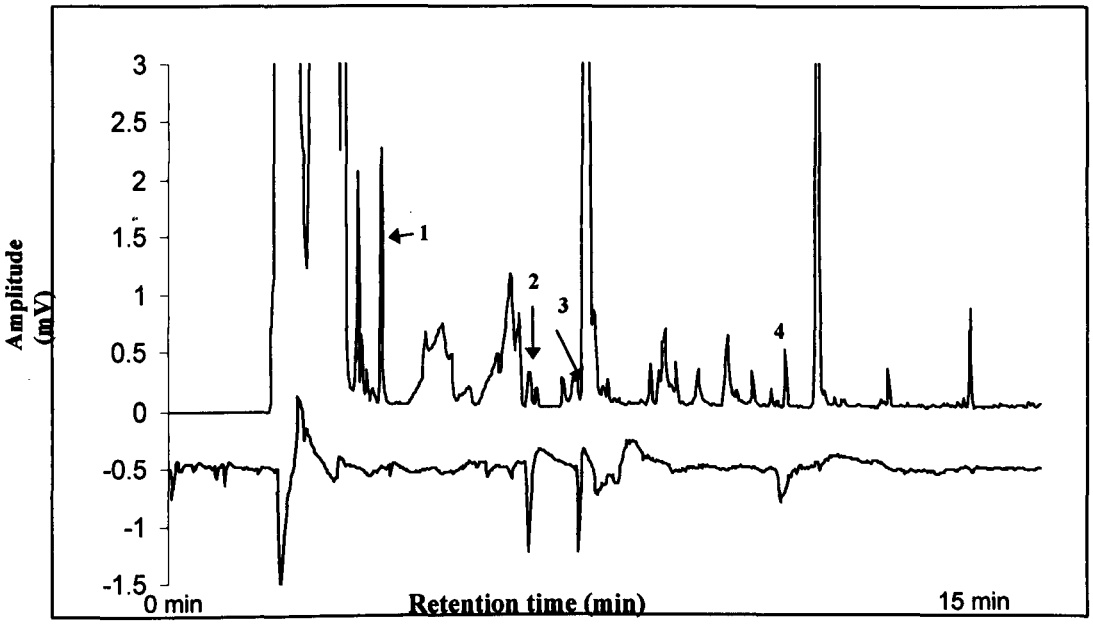
Correlation Analysis

The compounds found to be EAG-active, as well as indole and 3-methylindole, were located on the GC traces obtained from the decomposing pig volatiles using KI values. The concentration of these compounds were calculated and compared to the number of bluebottle flies (*Calliphora* sp.) present on the body near the time that the air entrainments were conducted. For this Pearson's correlation coefficient was performed, using statistical software SPSS, to see if there was any link between high concentration of these compounds and the number of bluebottle flies present on the pig during the different days of decomposition. The numbers of flies on the pig were calculated for Chapter 2 and the method is explained in Section 2.3 of that chapter.

3.3 RESULTS

Each fly tested in GC-EAG experiments reacted to specific peaks (active peaks) showing that the decomposing pig volatiles contained specific compounds of interest which could be detected by the fly's antennae (Figure 3.7).

Figure 3.7: Coupled GC-EAG trace. The upper trace shows the chromatogram of volatiles from the pig carcass extract at day 21 of the experiment. The lower trace shows the corresponding EAG response of *Calliphora vomitoria* antenna.



The numbered peaks represent: 1) dimethyl disulfide, 2) KI 881-885, 3) dimethyl trisulfide, 4) dimethyl tetrasulfide.

3.3.1 Experiment 1 – Test Experiment

The samples collected during this experiment were part of a test exercise to perfect the essential techniques.

Analysis of Extracts

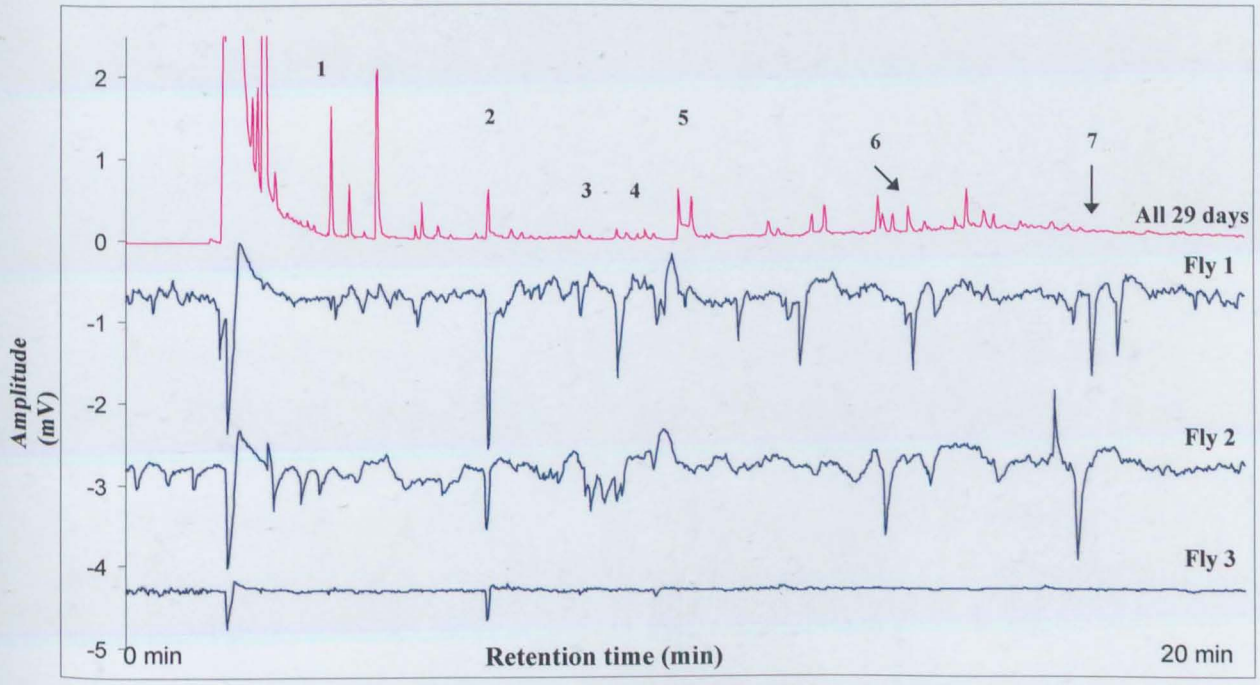
A total of 29 Porapak samples were collected during air entrainments of the decomposing pig. The extracts were run on the GC. The gas chromatograms obtained showed that there were numerous volatiles collected; however, the peaks obtained through GC analysis were very small. Therefore, each Porapak sample was concentrated using a nitrogen air stream to evaporate the solvent (diethyl ether) until only 50 to 100 μL of solvent remained. The subsequent gas chromatograms (traces) gave larger and clearer peaks mapping the changes throughout the decomposition. This

reduction in solvent was noted when calculating the concentration of each semiochemical.

Electrophysiology

The concentrated liquid samples were used in coupled GC-EAG experiments. Each produced a trace displaying the chromatographic separation of compounds and the electrophysiological responses. Using a syringe, 2μL of a sample was injected onto the GC column. The GC-peaks were small; the responses from the flies were minimal and difficult to interpret. In order to obtain a stronger response, 25μL of each of the 29 concentrated samples were combined to make one single sample and then further concentrated. The combination of all 29 extracts meant that this particular sample could not be used for comparison between different days; however, it was important at this time to determine if GC-EAG experiments could be successful. A larger amount of the combined sample, 4μL, was injected onto the GC column. The resulting EAG responses were stronger than with the individual day sample alone (Figure 3.8).

Figure 3.8: Traces of coupled GC-EAG trials with *Calliphora vomitoria*. The sample injected into the GC is a mixture containing all 29 extracts collected from the decomposing pig (pink). Corresponding EAG response are plotted in blue. The peaks measurements (amplitude) are in millivolts (mV).



Compounds were tentatively identified using GC-MS by Dr. Mike Birkett.
1) dimethyl disulfide, 2) dimethyl trisulfide, 3) decanal/ 2-phenylethanol (mixture), 4) ethyl phenol (2, 3, or 4), 5) 4-Isopropyl phenol (or benzyl alcohol), 6) unidentified, 7) unidentified (very small peak)

Six consistently active peaks were then analysed using coupled GC-MS to tentatively identify the six EAG-active compounds. These were dimethyl disulfide, dimethyl trisulfide, decanal/2-phenylethanol (mixture), ethylphenol, dodecane, 4-isopropyl-phenol and benzyl alcohol. Other peaks were present but did not elicit an EAG response and so they were not identified.

Solvent

Diethyl ether as an eluting solvent for Porapak tubes proved to be a problem. The ether solvent peaks, obtained through GC, covered potentially important semiochemicals normally found early on the GC traces.

Solvents of varying molecular weights, such as pentane, hexane, and heptane, were run on the GC (non-polar column) to determine their retention time (Table 3.1). However, none were found to elute significantly earlier or later than diethyl ether; therefore, all future experiments were continued using the diethyl ether. As Tenax tubes did not require solvent for the elution of volatiles, these were used in conjunction with the Porapak tubes to locate any compounds with short retention times.

Table 3.1 Retention times for different solvent peaks

Solvent	**Retention Time** (minutes)
Diethyl Ether	3 – 6.2
Hexane	3 – 5
Pentane	3 - 5
Heptane	3 - 8

Tenax

Air entrainments of the pig were made using Tenax tubes. The entrainment times were 10mins, 20mins, and 30mins. The GC traces showed that the 10 minutes entrainment did not provide clear results with some peaks hidden in the noise of the baseline; 20 and 30 minutes entrainments, however, provided clear GC traces with many large and well defined peaks. The longer entrainment of 30 minutes was chosen as the ideal entrainment time.

Summary of Technical Trials

In conclusion it was determined that coupled GC-EAG was possible using pig volatiles and the blowfly, *Calliphora vomitoria*. Air entrainments for a period of 2 hours, when using Porapak tubes, was an insufficient amount of time to collect the amount of semiochemicals required for these experiments. Concentrating the extract using nitrogen was not an option for the next experiment for fear of losing the more volatile semiochemicals, as well, a larger amount of extract would allow for additional experiments, if required. Therefore, the air entrainment time was extended to a minimum of 12 hours. As the time required for Porapak air entrainments limited when and how often the entrainments could be conducted (as discussed in section 3.2.4) it was concluded that using a combination of Porapak and Tenax tubes would give the desired amount of information required for the next experiment.

3.3.2 Experiment 2 - Odour Experiment, Summer 2003

3.3.2.1 GC-EAG & GC-MS

Biologically Active Compounds from the Decomposing Pig

A *Calliphora vomitoria* preparation was exposed to the effluent from the GC of the volatile mixture from the decomposing pig in order to determine which semiochemicals were electrophysiologically active. Flies were tested using extracts from days 4, 14, and 21, which corresponded with three different stages of decomposition (Bloated, Active Decay, Advanced Decay), to determine whether the flies responded to different semiochemicals at varying stages in decomposition.

Six experiments (two for each collection day tested) were carried out using GC-EAG. *C. vomitoria* responded to four compounds which were tentatively identified as dimethyl disulfide, propyl butyrate (EAG-active peak later referred to as KI 881-885), dimethyl trisulfide, and dimethyl tetrasulfide. Dimethyl trisulfide, dimethyl tetrasulfide and propyl butyrate, were consistently the most active throughout the experiments (Table 3.2). Dimethyl trisulfide was present in each test and all EAG reactions were strong. Dimethyl tetrasulfide and propyl butyrate elicited a response in all but one of the GC-EAG experiments; dimethyl tetrasulfide elicited strong reactions each time. Dimethyl disulfide was EAG-active in only one of the experiments; however, as it was consistently active during the 2002 experiments it was considered an important semiochemical. Four unidentified compounds appeared EAG-active in only one of the

experiments; therefore, their identifications were not pursued any further. The four EAG-active compounds were detected on all three days (4, 14, & 21) except for propyl butyrate, which was not found on day 21. *Calliphora vomitoria*, however, reacted to a compound present on day 21, suggesting that the wrong compound has been identified. This is further discussed in Section 3.3.2.2.

Table 3.2 Summary of coupled GC-EAG activity found in extracts.

Trial no.	Dimethyl disulfide	KI 881-883	Dimethyl trisulfide	Dimethyl tetrasulfide
a – Day 4	√	√	√	√
b – Day 4		√	√	√
c – Day 14		√	√	√
d – Day 14			√	√
e – Day 21		√	√	√
f – Day 21		√	√	

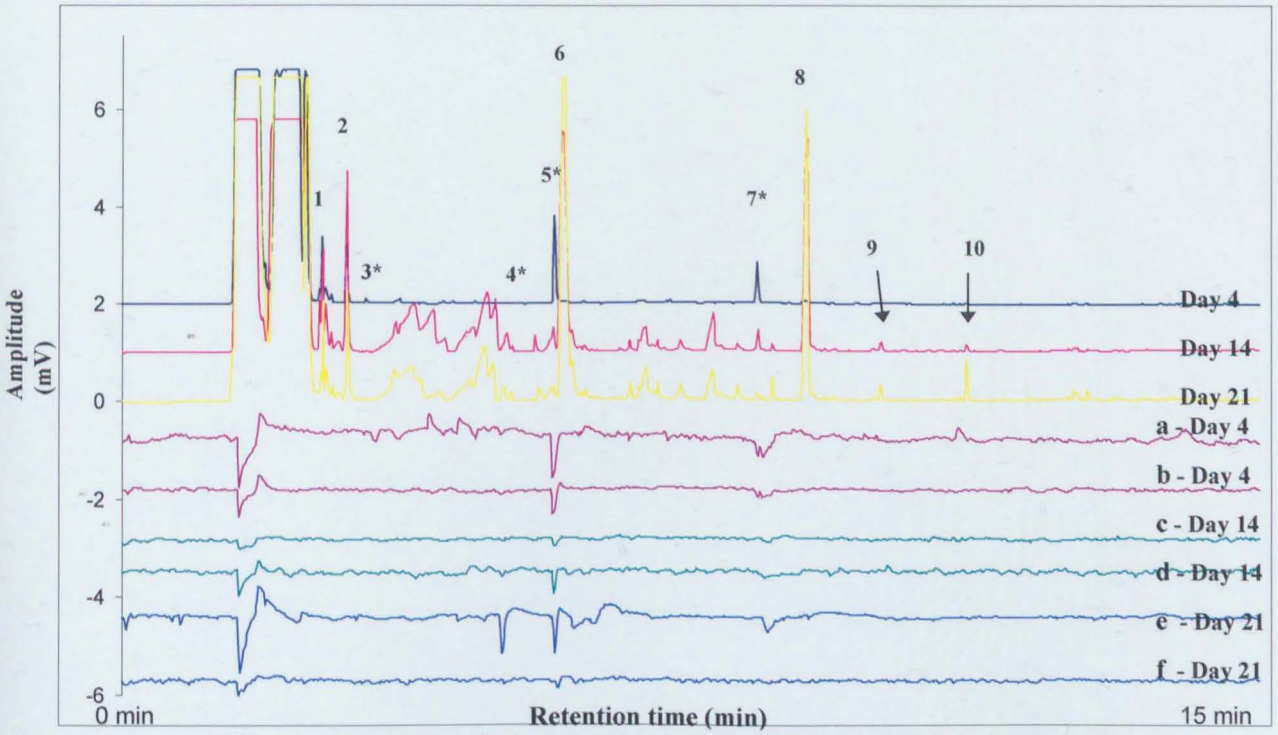
√ indicates a response from *C. vomitoria* during the GC-EAG experiment.

There were variations in the chemical composition of the volatile samples used in the GC-EAG experiments, days 4, 14, and 21 (Figure 3.9). Certain compounds, represented by peaks 1 and 2, remained constant throughout the experiments, while phenol, indole, tetradecane, and pentadecane (peaks 6, 8, 9, and 10), appeared in larger amounts only later during decomposition and increased in concentration as decomposition progressed. Electrophysiologically active compounds dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide (peaks 3, 5, and 7), were present early in decomposition; however, decreased considerably in concentration as decomposition progressed. Propyl butyrate (peak 4), was present throughout with higher peaks during Advance Decay. Concentrations are listed in Appendix 6.

C. vomitoria did not react to new compounds as decomposition progressed; nor was there a significant decrease in electrophysiological response to the compounds even though the concentrations were much less later on in decomposition. As demonstrated in Figure 3.10, chemicals such as phenol and indole (peaks 6 & 8) increased in concentration after the Fresh stage, while tetradecane and pentadecane (peaks 9 & 10) increased in concentration only in the Dry stage of decomposition. In the case of phenol and indole, their concentrations dropped significantly after Day 42. However,

as can be seen in Figure 3.9, these compounds, even in elevated concentrations, did not stimulate a response from *C. vomitoria*.

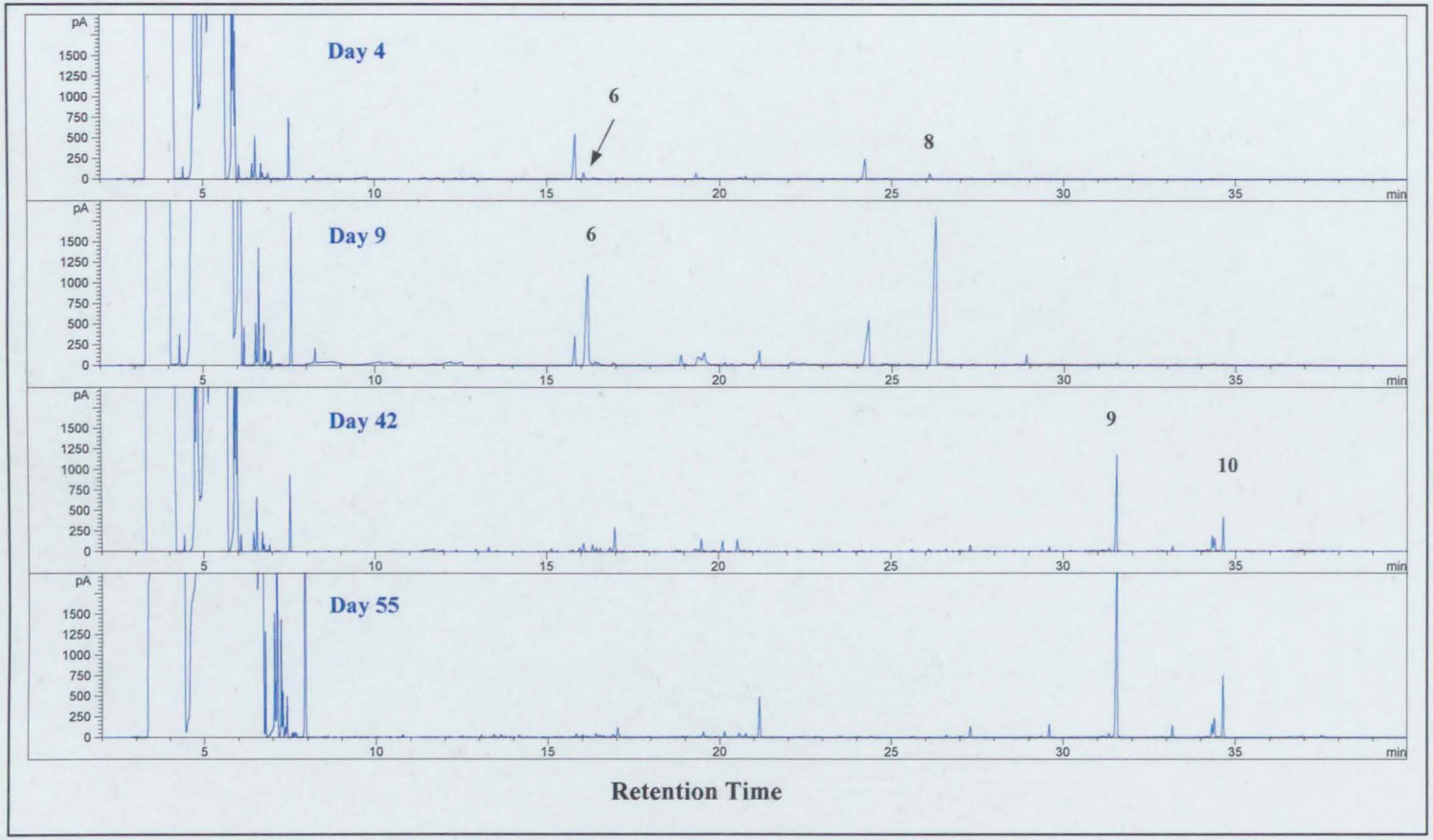
Figure 3.9: Traces of coupled GC-EAG trials of with *C. vomitoria*, all trials combined. The extracts were of decomposing pig volatiles collected on Days 4, 14, & 21. The corresponding EAG (a to f) response are plotted below.



Compounds were tentatively identified using GC-MS by Dr. Mike Birkett. The four EAG-active compounds are denoted by the *.
1) unidentified, 2) unidentified, 3) dimethyl disulfide*, 4) KI 881-885*, 5) dimethyl trisulfide*, 6) phenol, 7) dimethyl tetrasulfide*, 8) Indole, 9) tetradecane, 10) pentadecane.

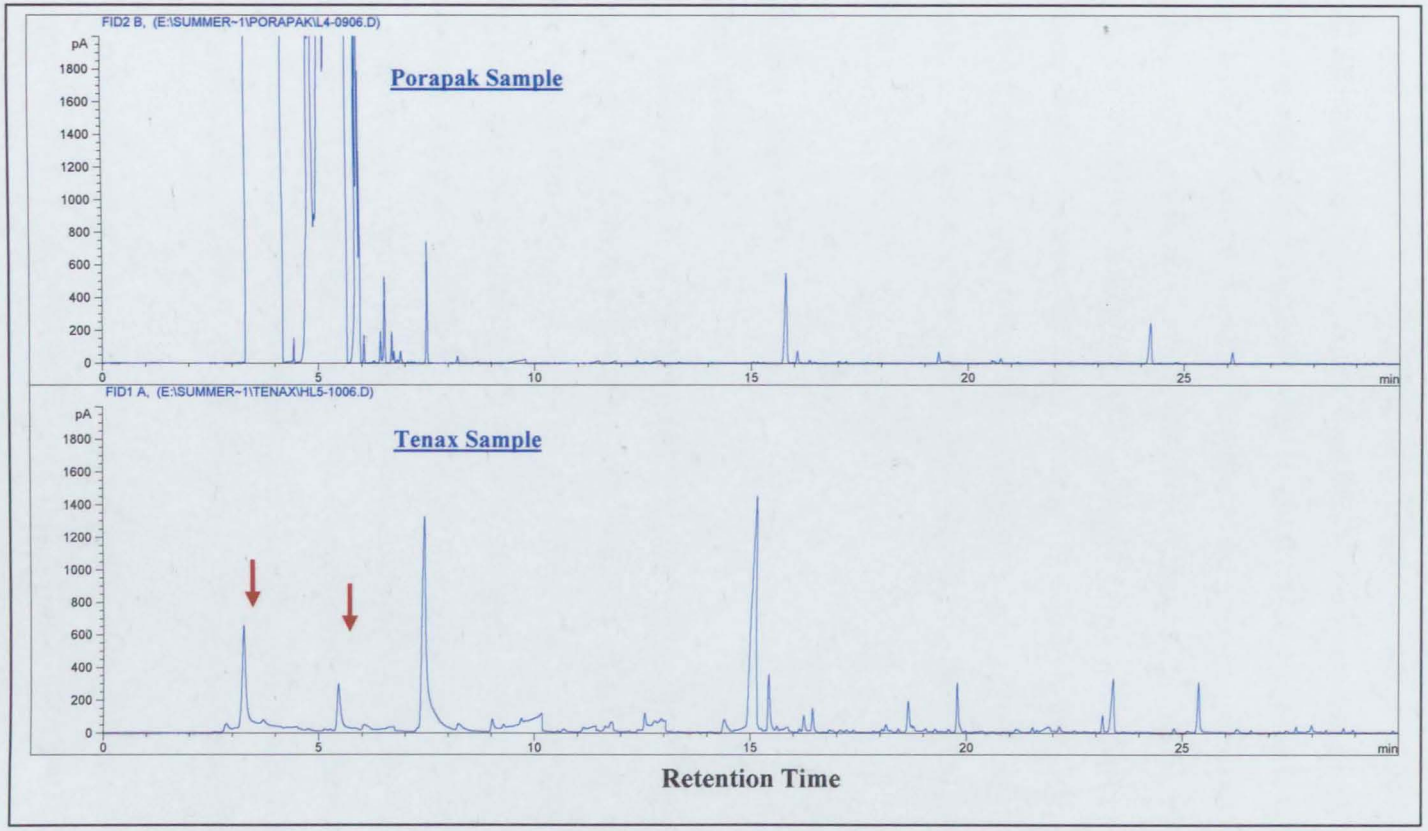
In all GC-EAG experiments, the flies reacted to one or more elements of the chemical mix (including the solvent) appearing early on the chromatogram. Dose response experiments showed that the reaction to solvent alone was low (section 3.3.3), indicating that semiochemicals, with a very small molecular weight, were also present in the mixture. As coupled GC-EAG experiments could not be done using Tenax tubes, the EAG activity of these chemicals could not be confirmed. GC alone indicated that some chemicals were present (Figure 3.11); however no positive identification was successfully made when using GC-MS.

Figure 3.10: Gas chromatograms from Porapak extracts of days 4, 9, 42, and 55. The peaks measurements are shown in peak height (pA).



Numbered peaks: 6) phenol, 8) Indole, 9) tetradecane, 10) pentadecane. The numbered peaks correspond to peaks numbered in Figure 3.8.

Figure 3.11: Gas chromatograms of Porapak and Tenax samples. The arrows point to compounds potentially hidden by the diethyl ether peaks in the Porapak samples. The peaks measurements are shown in peak height (pA).



3.3.2.2 Semiochemical Identification

Confirmation of Identifications

Mass spectroscopy tentatively identified the four main peaks of interest as propyl butyrate, dimethyl trisulfide, dimethyl tetrasulfide, and dimethyl disulfide. Co-injections on polar and non-polar columns confirmed the identification of dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide. The identification of propyl butyrate, however, proved to be much more difficult to confirm. Finally the peak found to have KI 881-885 was re-evaluated using GC-MS and was tentatively identified as 2-heptanone and not propyl butyrate. Again, polar column and non-polar column co-injections failed to confirm the identification of 2-heptanone as that of the compound that elicited a reaction from *C.* vomitoria during coupled GC-EAG experiments. The primary reason for the difficulty in identifying this particular EAG-active compound was because many compounds were present near each other, all with a similar retention time, making isolation of the EAG-active compound very difficult. As no confirmation could be made, this peak is referred to as KI 881-885 for the remainder of this chapter.

3.3.3 Statistical Analysis

3.3.3.1 Dose Response

The dose response curves, along with standard error, were plotted (Figure 3.12). Test compounds dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide, as well as 3-methylindole and indole all obtained reactions from *C. vomitoria* near or above that of the standard, methyl salicylate. Diethyl ether and hexane (controls) consistently generated responses near the 50% region. Propyl butyrate peaks were near those of the negative controls; even with the highest concentration administered in this test, the response to propyl butyrate by *C. vomitoria* did not increase. In later experiments, 2-hepatanone (see Section 3.3.2.2) was found to have no significant difference from the negative controls and induced reactions similar to those of propyl butyrate.

Statistical analysis demonstrated that when fitting the linear model, the results showed that all components were significant by treatment and at different concentration levels and that there was no effect of *SET* (random effect of experiment or grouping) (Table 3.3).

From the regression comparisons, it was clear that the treatments indole, 3-methylindole, dimethyl trisulfide, and dimethyl tetrasulfide were significantly different, due to the strong responses, to any of the positive or negative controls (methyl salicylate, diethyl ether, and hexane). What was also revealed is that the controls showed no significant differences among each other (Table 3.4).

None of the individual comparisons were significant at the concentration level of -10 (0.1 ng/μL), and all were significantly different at -6 (1000 ng/μL) with the exception of indole vs. methyl salicylate, which was not significant at any level (Table 3.5). It is interesting to note that at a concentration of -8 (10 ng/μL) most of the relevant differences were detected, indicating that higher concentrations are not required to produce a differential response.

Figure 3.12: Dose response curves. Responses were normalised, making the positive control 100%. Positive control was methyl salicylate and the negative controls were hexane and diethyl ether. Standard error is represented by the vertical bars.

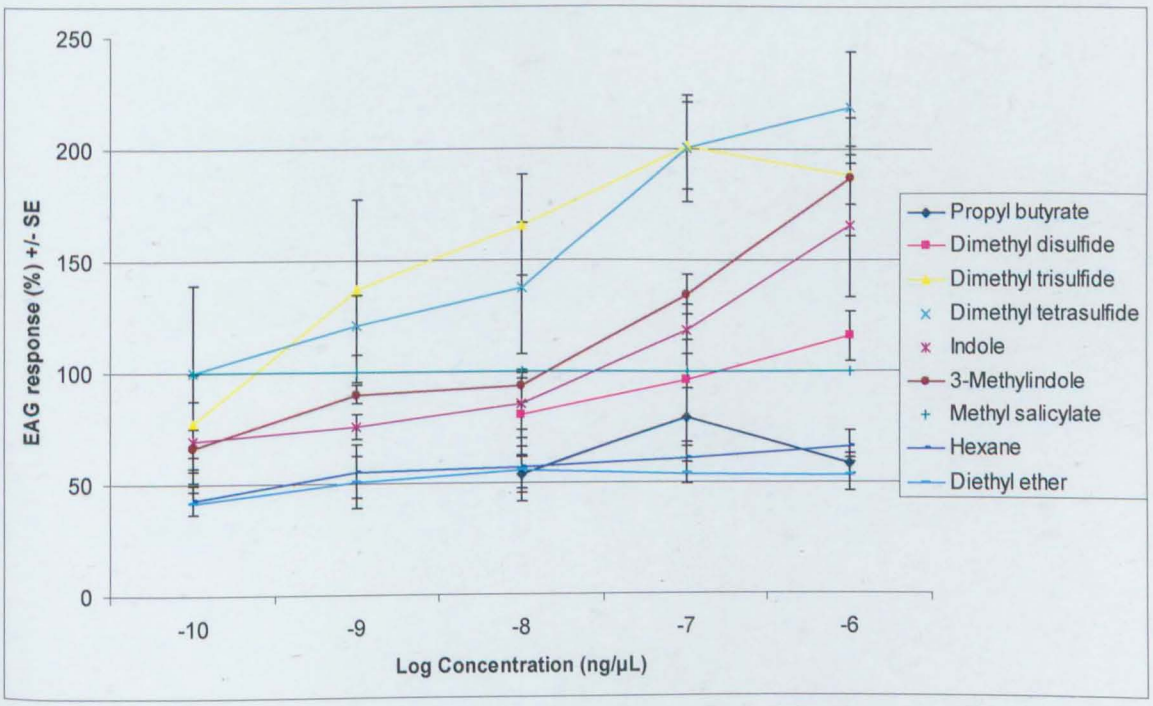


Table 3.3: Summary of results from fitting the mixed linear model for each treatment used in dose response experiments.

	sqWalk
VARIANCE COMPONENT	**Estimate**
SET	0.0
Residual	929.4
EFFECT	**p-value**
TRT	< 0.001
CONC	< 0.001
TRT.CONC	< 0.001

SET = random effect of experiment or set (block)
TRT = fixed effect of treatment
CONC = general concentration slope (covariate)
TRT.CONC = specific concentration slope for each treatment

Table 3.4: Contrasts comparing intercepts and slopes of the regression lines among pairs of treatments tested in dose response experiments. Results (p-values) showing significant differences between two treatments are listed in red.

Treatment	**Diethyl ether**	**Hexane**	**Methyl salicylate**
Dimethyl disulfide	0.0541	0.1097	0.1156
Indole	**0.0017**	**0.0080**	**0.0089**
Propyl butyrate	0.9305	0.8080	0.7879
3-Methylindole	**< 0.0001**	**< 0.0001**	**< 0.0001**
Dimethyl tetrasulfide	**< 0.0001**	**< 0.0001**	**< 0.0001**
Dimethyl trisulfide	**< 0.0001**	**< 0.0001**	**< 0.0001**
Diethyl ether	-	0.6104	0.5825
Hexane	0.6104	-	0.9678

Significance level of $\alpha = 0.05$

Table 3.5: Contrasts comparing the estimated means of different concentration levels for significant regression lines (listed in Table 3.4). Results (p-values) showing significant differences are listed in red.

Treatment	Least concentrated -10	-9	-8	-7	Most concentrated -6
IN vs ET	0.2825	0.0041	**< 0.0001**	**< 0.0001**	**< 0.0001**
SK vs ET	0.4559	**0.0006**	**< 0.0001**	**< 0.0001**	**< 0.0001**
TE vs ET	0.1091	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
TR vs ET	0.0147	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
IN vs HE	0.2909	0.0089	**< 0.0001**	**< 0.0001**	**< 0.0001**
SK vs HE	0.4673	0.0014	**< 0.0001**	**< 0.0001**	**< 0.0001**
TE vs HE	0.1131	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
TR vs HE	0.0155	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
IN vs MS	0.0123	0.0517	0.6938	0.2060	0.0458
SK vs MS	0.0045	0.1774	0.0985	**< 0.0001**	**< 0.0001**
TE vs MS	0.0599	0.3805	**< 0.0001**	**< 0.0001**	**< 0.0001**
TR vs MS	0.2693	0.1579	**< 0.0001**	**< 0.0001**	**< 0.0001**

-10, -9, -8, -7, & -6 in log scale. ET = diethyl ether, HE = hexane, MS = methyl salicylate, IN = indole, SK = 3-methylindole (skatole), TE = dimethyl tetrasulfide, TR = dimethyl trisulfide. Significance level of $\alpha = 0.000855$

3.3.3.2 Multivariate Analysis for the GC-EAG Active compounds

As the dose response experiments recorded significant responses by *C. vomitoria* to both indole and 3-methylindole, these were included in the following calculations. However, as the GC-EAG experiments did not identify these as semiochemicals, the same tests were also conducted without indole and 3-methylindole to see if there were any differences in the results.

There were a total of 16 days sampled using Porapak tubes and 23 days sampled using Tenax tubes, plus control samples. Certain important Tenax samples (*e.g.* day 1) were lost in the GC and the GC-MS due to equipment failure.

Principal Component Analysis

Porapak

Including Indole and 3-Methylindole

Principal component analysis was used to distinguish between the days of study during decomposition of the dead pig. The first two principal components (PC) accounted for 99.12% (PC1 = 97.36%, PC2 = 1.97%) of the variance in the data. Two groups of outliers could be observed (Figure 3.13). Days 14 (Adv. Decay), 21 & 25 (Dry) were near each other while days 9 & 12 (Act Decay) were away from all other groups,

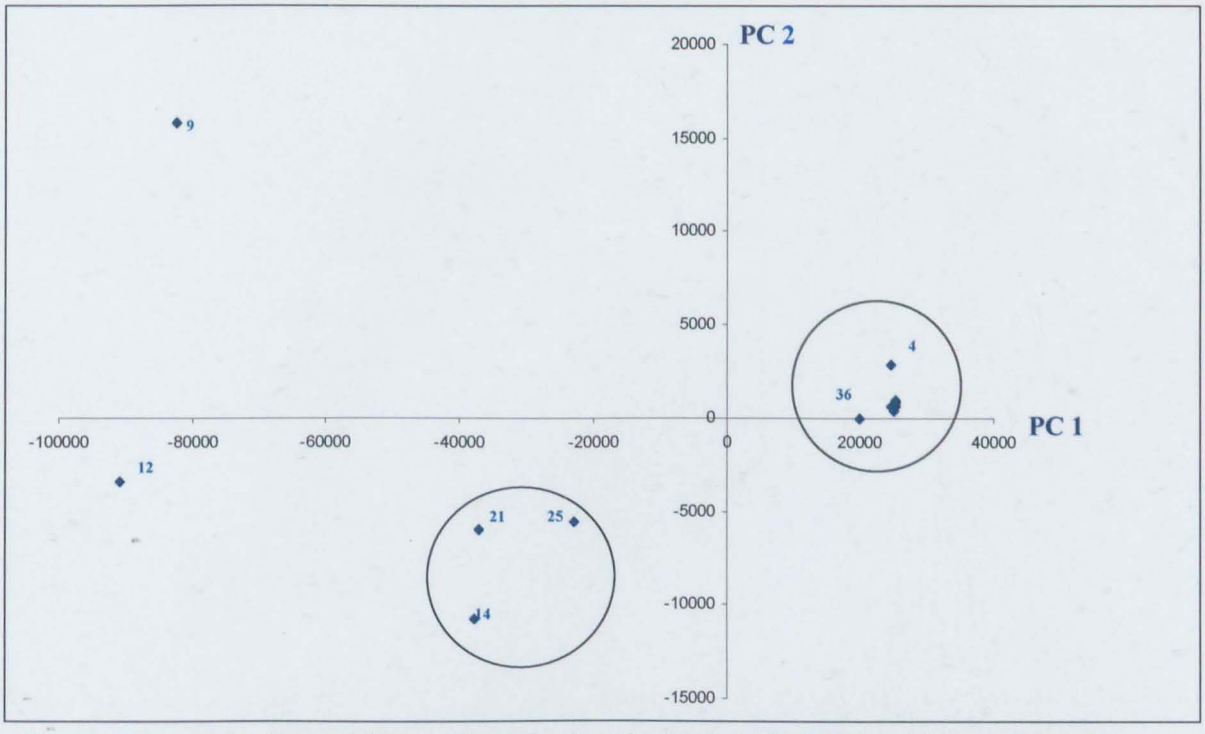
although not very close together. All other days (decomposition stages) were grouped together. Indole and dimethyl tetrasulfide were the two chemicals that most affected PC1. Dimethyl tetrasulfide and KI 881-885 influenced PC2. All latent vectors, or loadings, can be found in Appendix 7.

Not Including Indole and 3-Methylindole

The first two principal components accounted for 95.37% (PC1 = 76.71%, PC2 = 18.66%) of the variance in the data. In this case, only days 9 & 12 (Act Decay) and 14 (Adv Decay) were outliers; none were close together (Figure 3.14). All other days were closely grouped together. PC1 was most heavily influenced by dimethyl tetrasulfide and dimethyl trisulfide. KI 881-885 most affected PC2. These results would suggest that indole and, to a lesser extent, 3-methylindole had an effect on results in the previous test, particularly on day 21 and day 25.

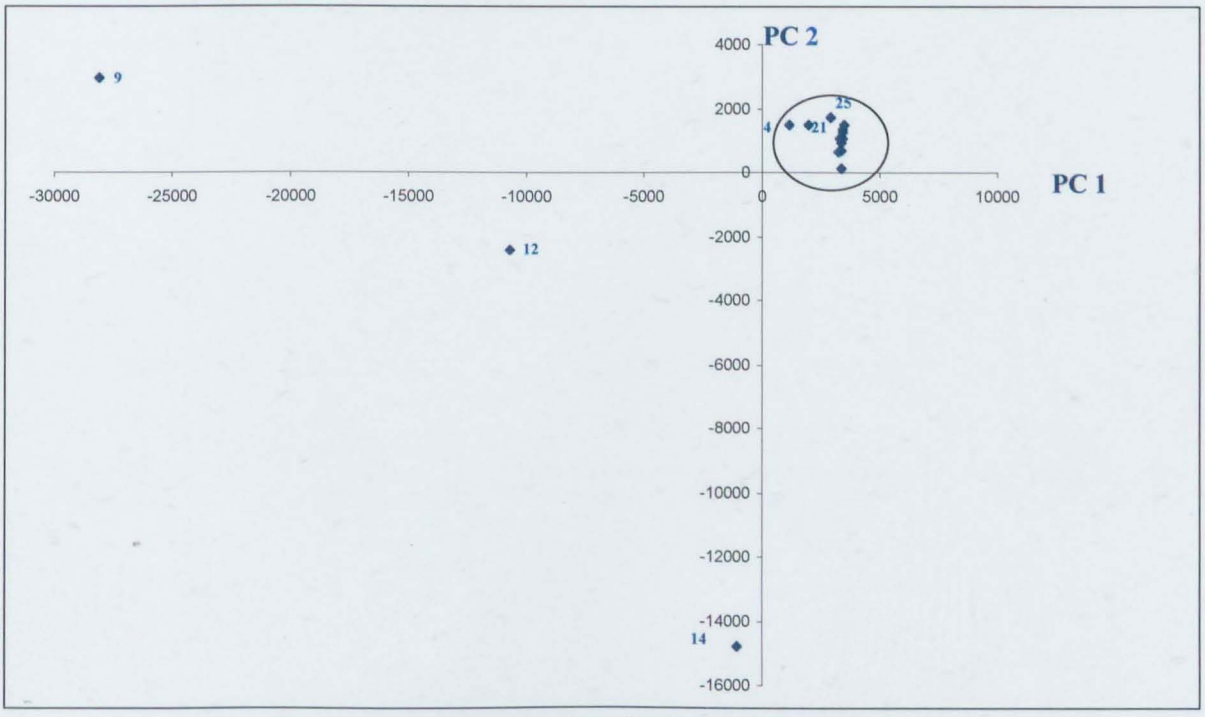
The concentrations of indole and skatole were elevated at days 21 & 25 which would explain their disappearance in the group of outliers in the second test done without the influence of these chemicals. Indole concentrations peaked from day 9 to day 14, dimethyl tetrasulfide and dimethyl trisulfide peaked on day 9.

Figure 3.13: Ordination plot of the principal component scores based on the concentration of EAG-active chemicals, including indole and 3-methylindole, collected with Porapak tubes.



The numbers near the diamond shapes refer to the day of collection. The horizontal (x) axis represents principal component 1 (PC1), while the vertical (y) axis represents principal component 2 (PC2). The two principal components explain 99.12% of variance in the data, which in this case is primarily influenced by indole and dimethyl tetrasulfide.

Figure 3.14: Ordination plot of the principal component scores based on the concentration of EAG-active chemicals, not including indole and 3-methylindole, collected with Porapak tubes.



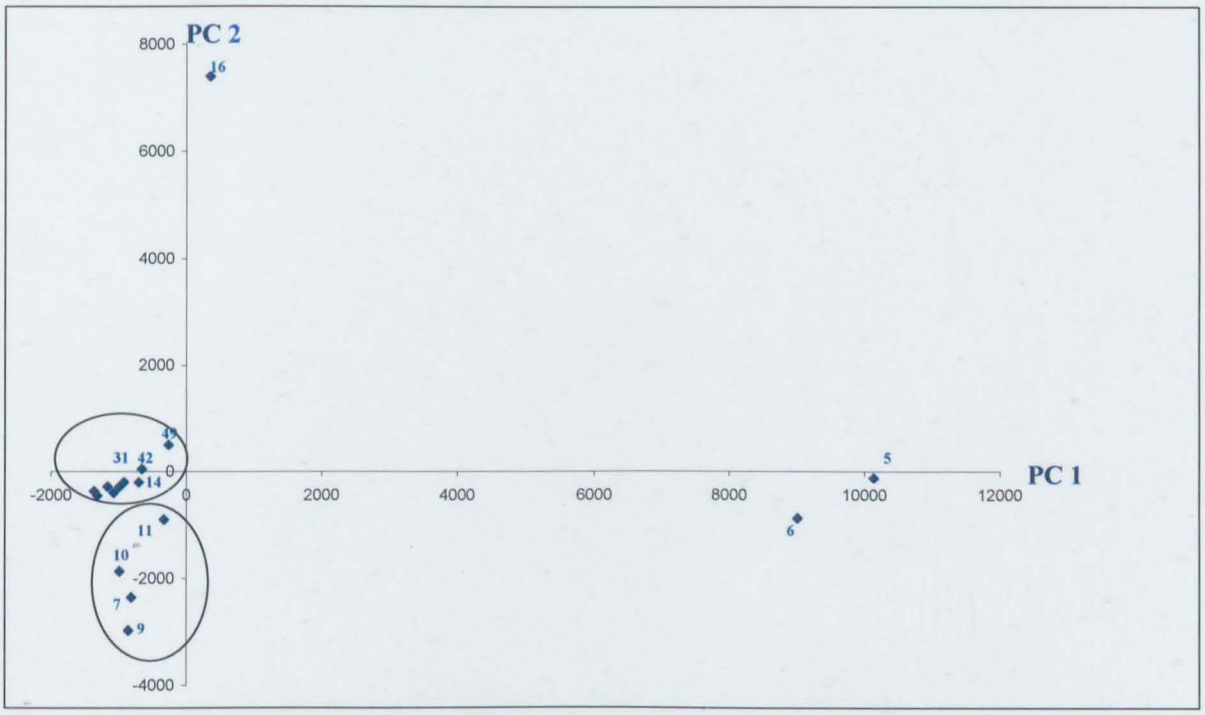
The numbers near the diamond shapes refer to the day of collection. The horizontal (x) axis represents principal component 1 (PC1), while the vertical (y) axis represents principal component 2 (PC2). The two principal components explain 95.37% of variance in the data, which in this case is primarily influenced by dimethyl tetrasulfide and dimethyl trisulfide.

Tenax

Including Indole and 3-Methylindole

The first two principal components accounted for 82.53% (PC1 = 59.07%, PC2 = 23.46%) of the variance in the data. There were three outliers; Days 5 & 6 (Bloated), and 16 (Adv decay) (Figure 3.15). Days 5 & 6 were near each other. All other days of decomposition were close together; however, these could be separated into two groups: Days 7, 9, 10, 11 (Act decay) and all other days 0, 2, 3, 4, 13, 14, 18, 25, 28, 31, 42, 49, 55, 69, 77, 91, which encompass Fresh, some Bloated, one Act Decays, Adv Decay and Dry stages. Days 31, 42, 49, while still in the second group, were slightly aside from the group possibly due to the higher concentrations of KI 881-885 on those days. In this case dimethyl trisulfide and, to a lesser extent, dimethyl tetrasulfide were the two compounds most affecting the graph at PC1, while KI 881-885 and dimethyl tetrasulfide most influenced PC2.

Figure 3.15: Ordination plot of the principal component scores based on the concentration of EAG-active chemicals, including indole and 3-methylindole, collected with Tenax tubes.



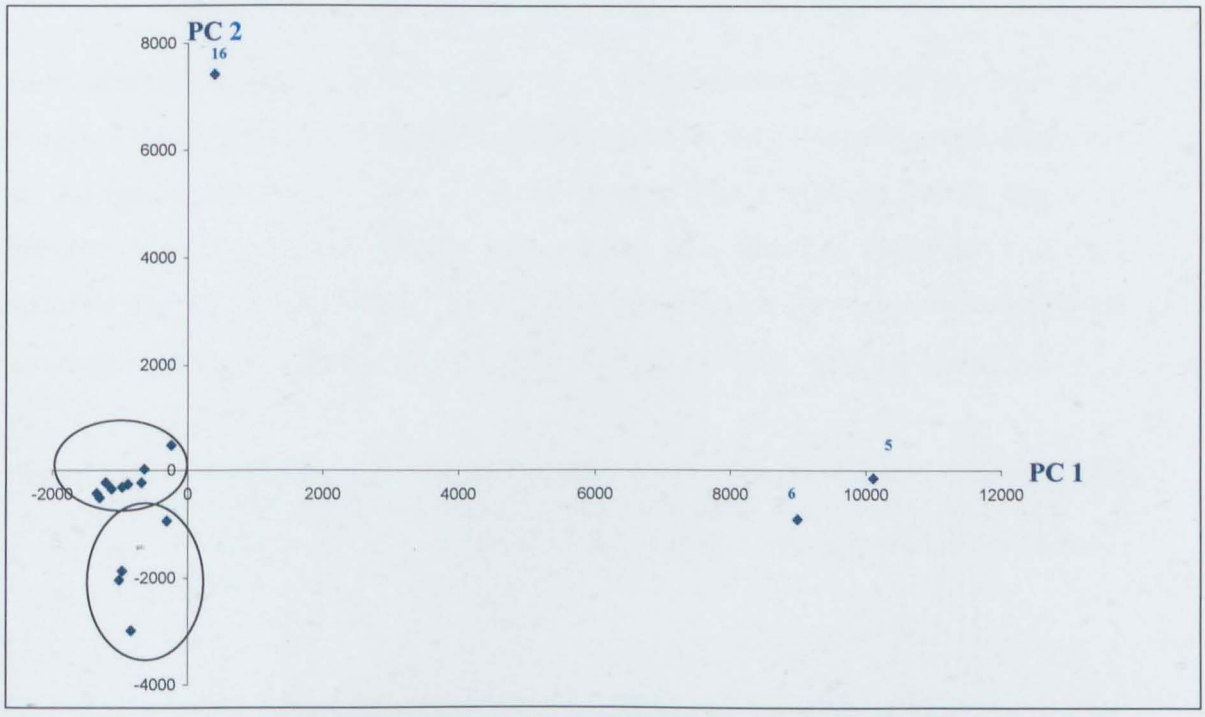
The numbers near the diamond shapes refer to the day of collection. The horizontal (x) axis represents principal component 1 (PC1), while the vertical (y) axis represents principal component 2 (PC2). The two principal components explain 82.53% of variance in the data, which in this case is primarily influenced by dimethyl trisulfide and dimethyl tetrasulfide.

Not Including Indole and 3-Methylindole

The first two principal components jointly accounted for 85.66% (PC1 = 61.39%, PC2 = 24.27%) of the variance in the data. There was little change in the results obtained without indole and 3-methylindole, indicating that these chemicals were not determining factors in the final graph (Figure 3.16).

With the Tenax samples, the data was slightly less predictable. Days of sampling from groups, such as Active Decay, were close to each other even though they appeared to be different in the Porapak collections. This may be due to the unexplained lack of dimethyl trisulfide in the Tenax samples (missing concentration data for dimethyl trisulfide) during days 7 & 9.

Figure 3.16: Ordination plot of the principal component scores based on the concentration of EAG-active chemicals, not including indole and 3-methylindole, collected with Tenax tubes.



The numbers near the diamond shapes refer to the day of collection. The horizontal (x) axis represents principal component 1 (PC1), while the vertical (y) axis represents principal component 2 (PC2). The two principal components explain 85.66% of variance in the data, which in this case is primarily influenced by dimethyl trisulfide and dimethyl tetrasulfide.

Canonical Variate Analysis

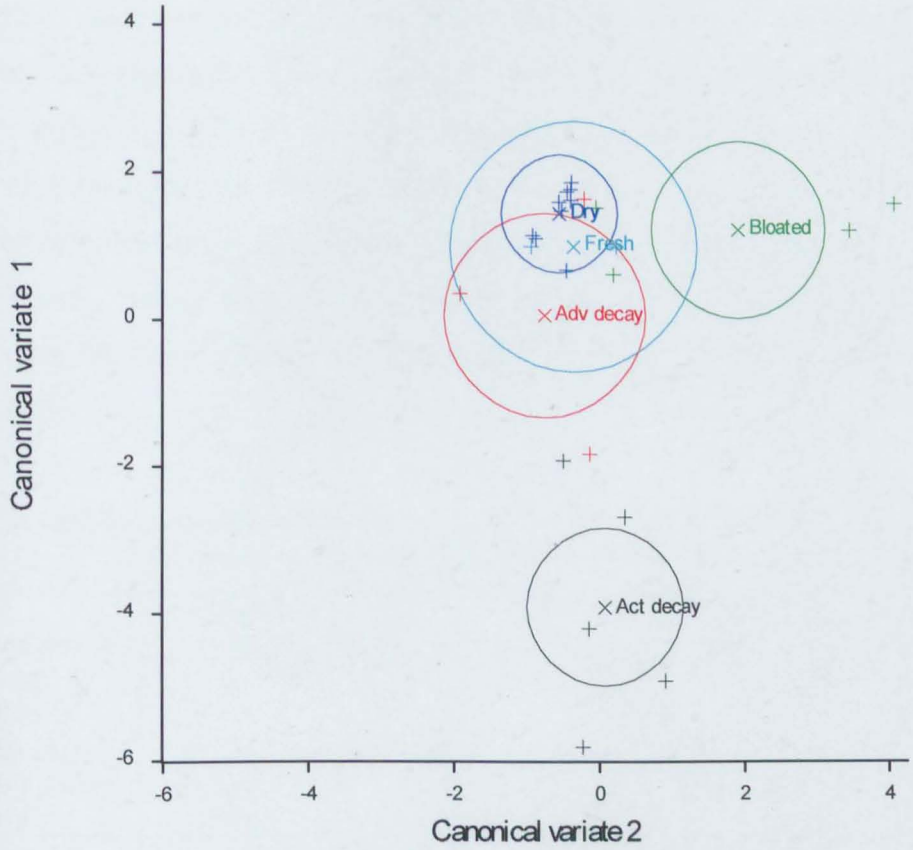
To better show distinction between the five decomposition stages, canonical variate analyses were conducted to analyse these groups.

The five decomposition stages/groups were compared using the semiochemicals identified plus indole and 3-methylindole. The first two principal components accounted for 89.69% (PC1 = 75.49%, PC2 = 14.20%) of the variation in the data. Those circles, not overlapping, were considered to be significantly different ($P < 0.05$) (Figure 3.17). The Fresh stage was found to only be significantly different to the Active Decay stage. The Bloated stage was found to be significantly different to all but the Fresh stage. Active Decay stage was significantly different to all other stages. The Advanced Decay stage was significantly different to the Bloated stage and Active Decays stage. The Dry stage was significantly different to the Bloated and Active Decays stage. The loadings indicated that 3-methylindole and dimethyl tetrasulfide

heavily influenced canonical variate 1 (CV1), while 3-methylindole and dimethyl disulfide affected CV2. The loadings are listed in Appendix 7.

Another test was conducted without indole or 3-methylindole, leading to very similar results to the previous test. While the groups moved slightly along the graph, there was no change in the overlap of any of the groups. The chemicals having the most influence on CV1 were dimethyl tetrasulfide and dimethyl disulfide. Dimethyl disulfide and KI 881-885 mostly influenced CV2. The first two principal components accounted for 89.39% (PC1 = 75.18%, PC2 = 14.21%) of the variation in the data.

Figure 3.17: Ordination plot of the canonical-variate scores based on the concentration of EAG-active chemicals within the five stages of decomposition, Fresh (light blue), Bloated (green), Active Decay (black), Advanced Decay (red), and Dry (dark blue).



The circles represent 95% confidence intervals. Those circles which are overlapping indicate that there is no significant difference between these groups at the $P = 0.05$ level.

3.3.3.3 Univariate Analysis for the GC-EAG Active compounds

One-way ANOVA was employed to find the difference between the means of the concentration values of the 5 decomposition groups. Logarithmic transformation was performed on the means as the data was not normally distributed. Dimethyl trisulfide, dimethyl tetrasulfide, and 3-methylindole were all found to be significantly different in the separate stages of decay (Table 3.6, Figure 3.18, & Table 3.7). Of these three compounds the comparisons of means were calculated (Table 3.8) indicating that for dimethyl trisulfide, the most important differences were due to the significant differences between the Bloated stage & the Fresh and Bloated stage & Active Decay stages. An anomaly with the collection of dimethyl trisulfide during two days in the Active Decay stage is discussed further in Section 3.4. As for dimethyl tetrasulfide the most important differences, or significant differences to the 1% level, were between the Active Decay stage and the following three stages: Fresh, Advanced Decay and Dry. There was no need to conduct another test without indole and 3-methylindole as all the chemicals were analysed separately, independently of each other. The correlation coefficients for the mean amounts of the chemicals showed that the Fresh vs. Dry stages were the most similar as they showed the highest correlation coefficient, closely followed by the Fresh vs. Active Decay and Bloated vs. Dry stages (Table 3.9). Those showing the lowest correlation coefficient, and therefore the most difference, were the Active Decay vs. Advanced Decay and the Bloated vs. Advanced Decay stages.

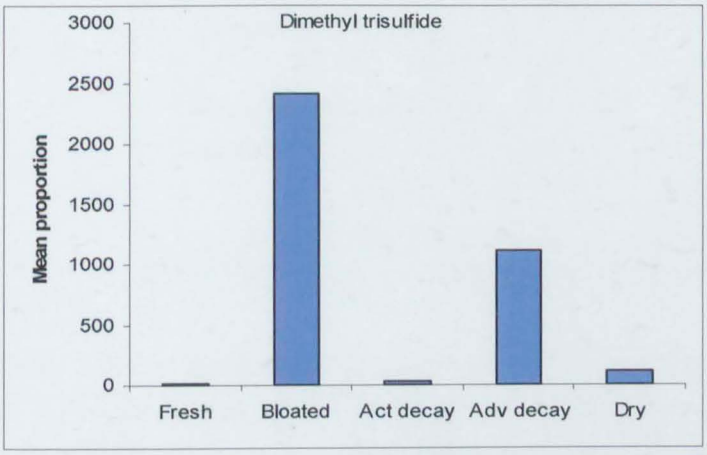
Table 3.6: Logarithm transformation of the mean values of the compounds (ng) collected from the decomposing pig compared to the different stages of decomposition.

Log means	Dimethyl disulfide	KI 881-885	Dimethyl trisulfide	Dimethyl tetrasulfide	Indole	3-Methylindole
Fresh	5.3975	5.4529	3.0217	3.2661	3.9356	4.2183
Bloated	4.7841	5.9017	7.7900	5.9284	5.6409	3.8910
Act decay	5.2270	5.7741	3.2432	7.9935	3.5398	4.8337
Adv decay	5.9043	7.6668	7.0126	3.4680	3.3662	2.9147
Dry	3.3904	6.5476	4.7413	3.4526	4.5433	2.4289
LSD 5 %	2.946	1.677	2.981	2.589	4.024	2.213
LSD 1 %	4.036	2.298	4.084	3.548	5.514	3.032

Values highlighted in red are significantly different at 5%.

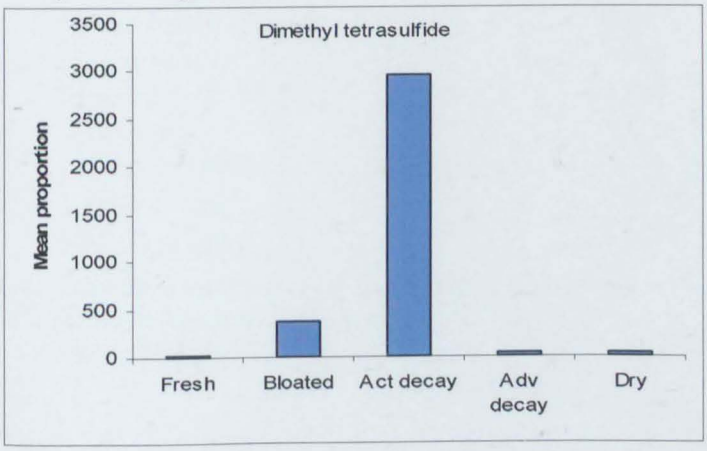
Figure 3.18: Mean proportions of the three chemicals that are significantly different from each other within each stage of decomposition.

a) Dimethyl trisulfide



The low level of dimethyl trisulfide in the Active Decay stage is discussed in Section 3.4.

b) Dimethyl tetrasulfide



c) 3-Methylindole

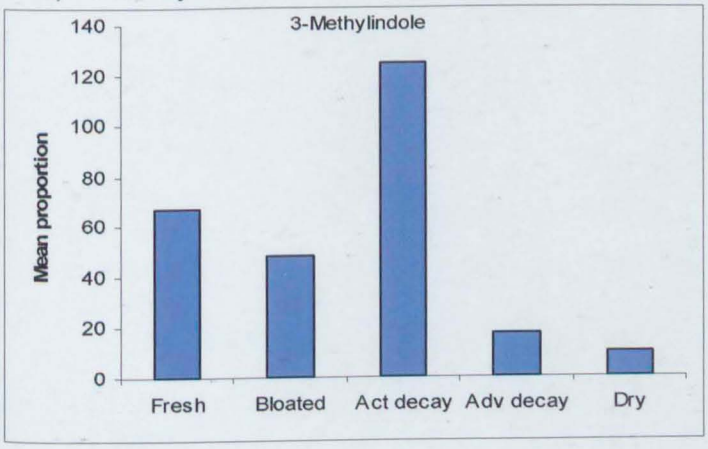


Table 3.7: Comparison of the mean values of the compounds collected from the decomposing pig between the different stages of decomposition.

	F-value	P-value
Dimethyl disulfide	1.67	0.200
KI 881-885	2.30	0.098
Dimethyl trisulfide	5.08	0.006
Dimethyl tetrasulfide	8.29	<0.001
Indole	0.56	0.693
3-Methylindole	3.00	0.047

Values highlighted in red are significantly different at 5%.

Table 3.8: Comparison of means between the different stages of decomposition.

Decomposition stages	Dimethyl trisulfide	Dimethyl tetrasulfide	3-Methylindole
Fresh vs Blt	-4.7683	-2.6623	0.3274
Fresh vs Act D	-0.2214	-4.7274	-0.6154
Fresh vs Adv D	-3.9908	-0.2019	1.3036
Fresh vs Dry	-1.7196	-0.1865	1.7895
Blt vs Act D	4.5468	-2.0651	-0.9427
Blt vs Adv D	0.7774	2.4604	0.9763
Blt vs Dry	3.0487	2.4758	1.4621
Act D vs Adv D	-3.7694	4.5255	1.9190
Act D vs Dry	-1.4981	4.5409	2.4048
Adv D vs Dry	2.2713	0.0154	0.4859

Blue indicates where significantly different at 5%
Green indicates where significantly different at 1%
Blt = Bloated, Act D = Active Decay, Adv D = Advanced Decay.

Table 3.9: Correlation coefficient for the mean amounts of chemicals in the Tenax data.

a) With indole and 3-methylindole

Fresh	1				
Bloated	0.67	1			
Act decay	0.61	0.64	1		
Adv decay	0.80	0.51	0.49	1	
Dry	0.84	0.80	0.59	0.64	1
	Fresh	**Bloated**	**Act decay**	**Adv decay**	**Dry**

Values closest to 1 are more correlated than values furthest from 1.

b) Without indole and 3-methylindole

Fresh	1				
Bloated	0.66	1			
Act decay	0.75	0.59	1		
Adv decay	0.70	0.41	0.50	1	
Dry	0.89	0.74	0.68	0.59	1
	Fresh	**Bloated**	**Act decay**	**Adv decay**	**Dry**

Values closest to 1 are more correlated than values furthest from 1.

3.3.4 Chemicals vs. Flies

Porapak samples were found to have significant positive correlations between the daily levels of dimethyl trisulfide, dimethyl tetrasulfide, indole, and 3-methylindole and the numbers of bluebottles (*Calliphora* sp.) found each day on the decomposing pig (Table 3.10a). However, Tenax samples did not show such results. Only 3-methylindole showed a significant positive correlation with *Calliphora* sp. on the pig (Table 3.10b).

Table 3.10: Correlation coefficients for the concentrations of chemicals and numbers of bluebottles, *Calliphora* sp., on the decomposing pig.

a) Using Porapak concentrations, n = 16.

	Pearson Correlation (r)
Calliphora	1
Dimethyl disulfide	.121
KI 881-885	-.028
Dimethyl trisulfide	.964(**)
Dimethyl tetrasulfide	.987(**)
Indole	.757(**)
3-Methylindole	.558(*)

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

b) Using Tenax concentrations, n = 23.

	Pearson Correlation (r)
Calliphora	1
Dimethyl disulfide	-.034
KI 881-885	.357
Dimethyl trisulfide	-.083
Dimethyl tetrasulfide	-.268
Indole	.132
3-Methylindole	.880(**)

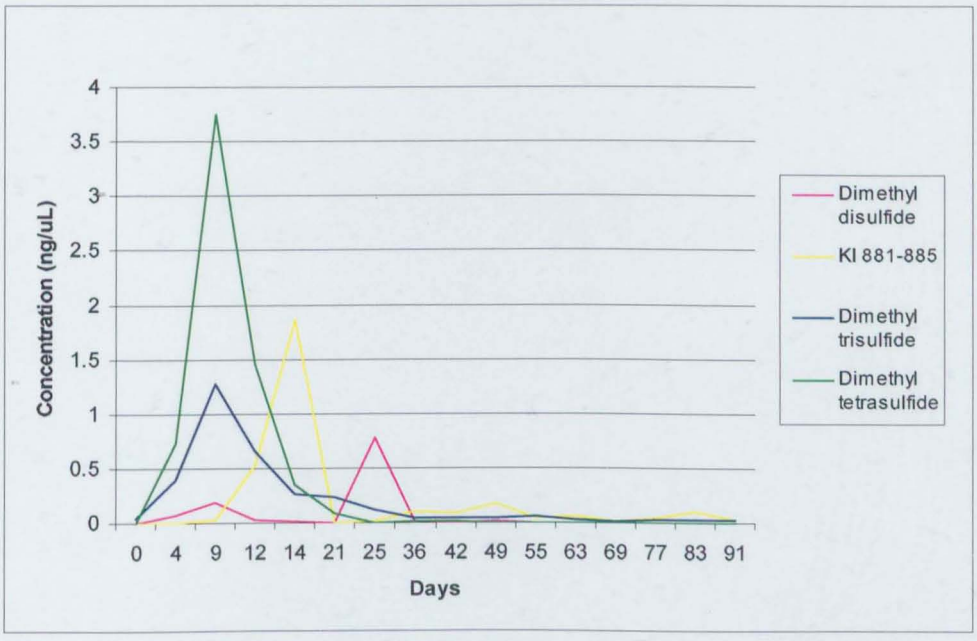
** Correlation is significant at the 0.01 level (2-tailed).

Concentration of the EAG-active compounds from Tenax and Porapak samples were plotted against the days of the experiment (Figures 3.19 & 3.20). The peaks in the Porapak samples indicate the concentration of dimethyl trisulfide and dimethyl tetrasulfide were at their highest on Day 9 – Active Decay. Dimethyl tetrasulfide, from the Tenax samples, also peaked around Day 9. Dimethyl trisulfide, as stated above, was not present during these sample collections (Days 7 & 9) but the peak was present on days 5 & 6 – Bloated stage. When the presence of flies was plotted against days

bluebottles, greenbottles, and Piophilidae numbers all peaked on day 9. There is no statistical data to support correlation between numbers of the other species of flies and the chemicals. GC-EAG experiments would preferably be performed in order to identify the EAG-active compounds for specific species of flies.

Figure 3.19: Concentration of the chemicals collected using Porapak tubes during decomposition vs. the flies counted on the decomposing pig on the same dates as volatile samples were collected.

a) Concentration of the EAG-active compounds



b) Number of flies on the decomposing pig

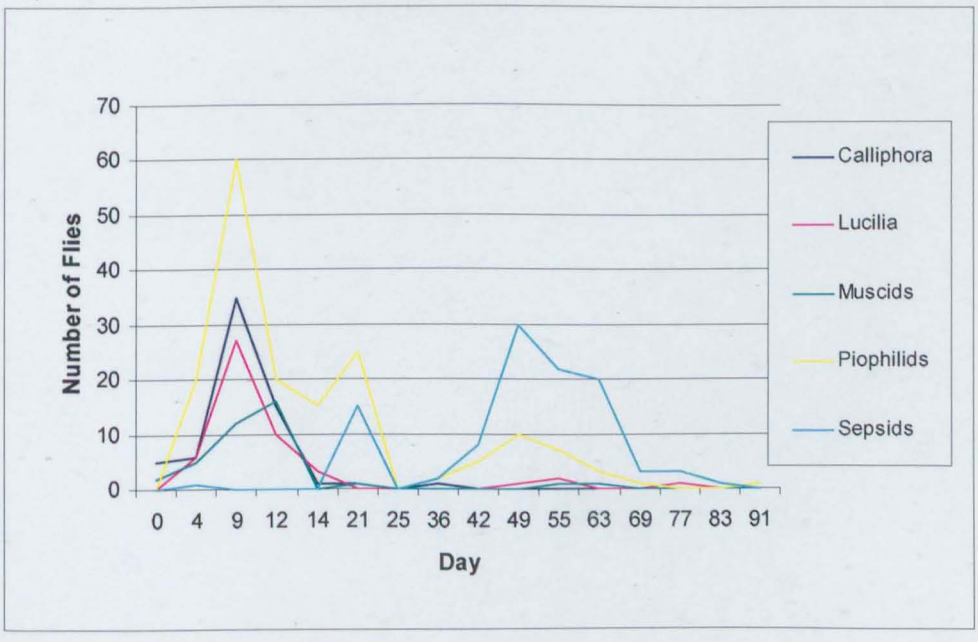
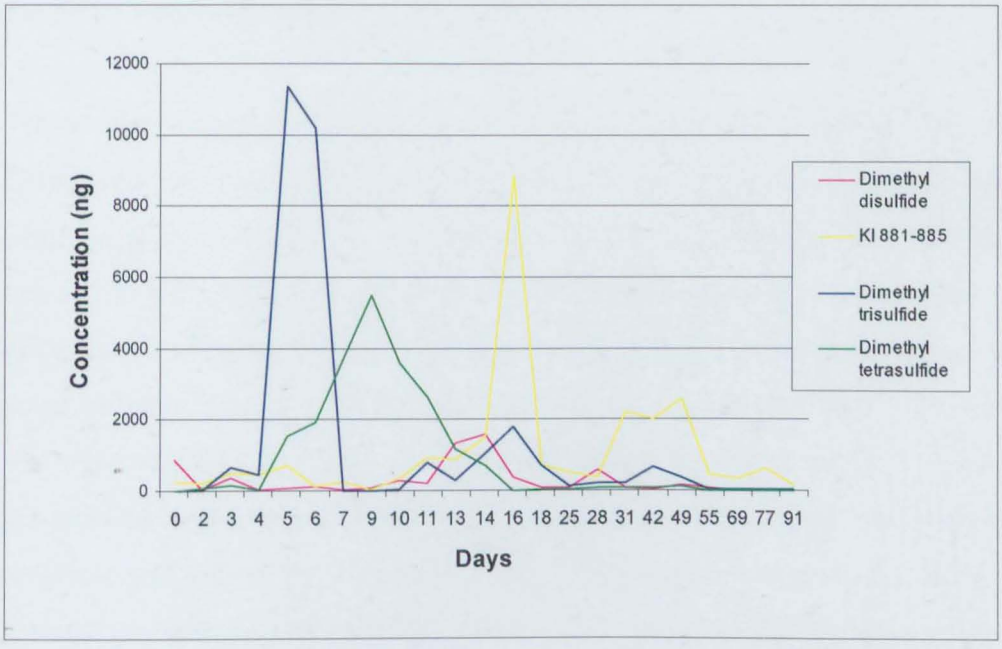
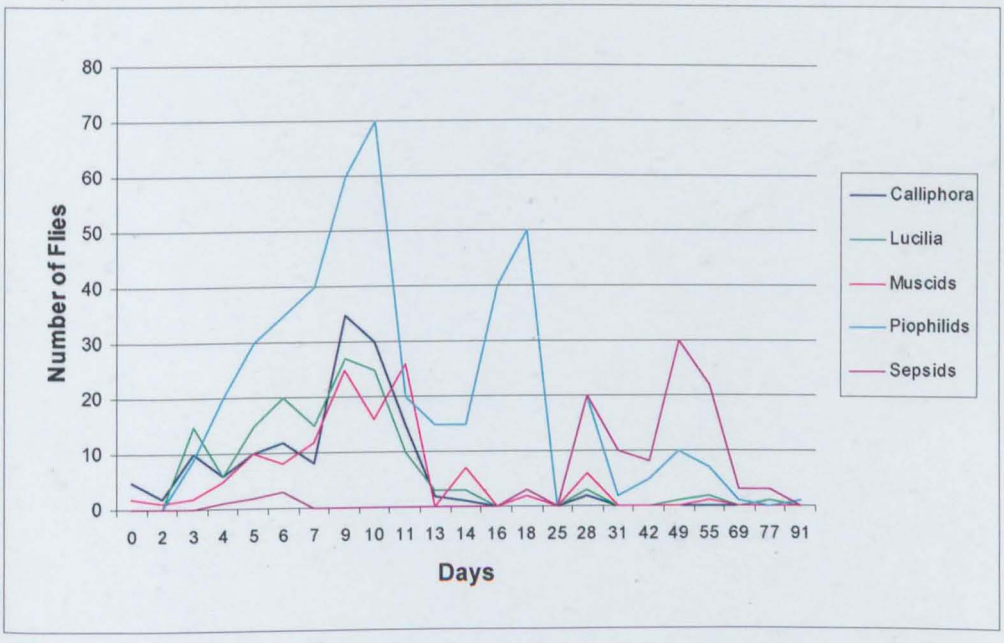


Figure 3.20: Concentration of the chemicals collected using Tenax tubes during decomposition vs. the flies counted on the decomposing pig on the same dates as volatile samples were collected.

a) Concentration of the EAG-active compounds



b) Number of flies on the decomposing pig

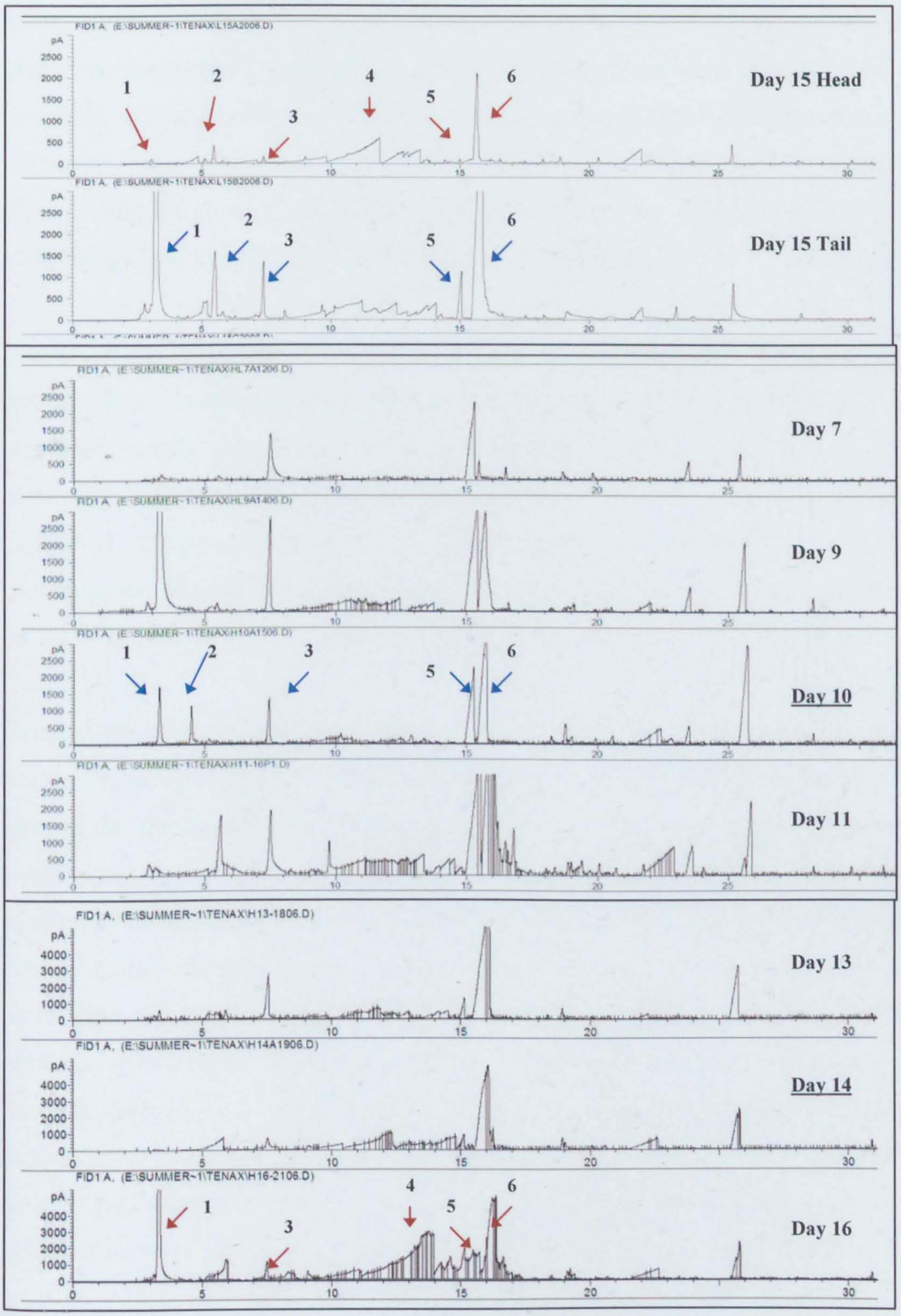


3.3.5 Supplementary Experiment

On Day 15 of the experiment (20th June 2003), a short trial was conducted to determine if volatiles could still be collected at a crime scene in a non-disruptive way, as space can be an issue and evidence is critical.

Tenax tubes successfully recovered volatiles from the head and the tail of the pig. These were analysed using gas chromatography and two different gas chromatograms emerged (Figure 3.21). Visual observations showed that the head of the pig was reduced to a dry state where the skin was darkened, the bones were exposed, and little or no larvae were present. The peaks present on the chromatogram closely resembled those of the Tenax air entrainments done on Pig 2 on Day 14 (19th June 2003). This was represented by the height ratio between peaks 2 (unknown) and 4 (unknown), and the reduced height of peak 5 (dimethyl trisulfide) and the larger peak 6 (phenol). The posterior end of the pig, however, was not as dry and contained a mass of larvae feeding on the moist tissues. The gas chromatogram resembled that of Day 10 (15th June 2003). This was represented by the difference in height ratio between peaks 1 and 2, the greater heights of peaks 3 (dimethyl disulfide).

Figure 3.21: Supplementary experiment: head vs. tail. Chromatogram of Tenax samples of decomposing pig volatiles. Corresponding EAG response are plotted in brown and blue. The peaks measurements are shown in peak height (pA).



1) unidentified, 2) unidentified, 3) dimethyl disulfide, 4) unidentified, 5) dimethyl trisulfide, 6) phenol.

3.4 DISCUSSION

The changes implemented after the test experiment, such as to the collection medium used and the air entrainment time, proved to be helpful as each sample examined had sufficient amounts of volatiles for analysis. Tenax tubes were less practical than originally hoped as they could not be used in coupled GC-EAG experiments and important tubes were destroyed due to problems with the analytical equipment. Porapak and Tenax tubes were both found to be useful and complimentary; however, each method had benefits and disadvantages, as discussed below.

Tenax TA can withstand much higher temperatures than Porapak Q, which means a sample can be analysed through thermals desorption removing the need for solvent. As the entire sample is analysed only a short entrainment time is required, causing less disruption to the organism being entrained and providing information on the release profile of compounds (Figure 3.11 – Tenax sample) (Angelopoulos *et al.*, 1999). However, the feature that makes Tenax TA so useful is also its biggest disadvantage - analysis through thermal desorption. A Tenax sample can only be used once.

Porapak samples, on the other hand, are collected with a solvent and can be used many times for several types of analysis. This includes: quantification of the chemicals present in the sample by injecting a standard, co-injections for the purpose of confirming the identity of a compound, analysis using different GC columns, electrophysiology and bioassay experiments, replication of tests, and finally the sample can be sealed and stored in a freezer for later use (Angelopoulos & Pickett, 1998). Disadvantages of using Porapak Q include its lower thermal stability making it unsuitable for thermal desorption (Raguso & Pellmyr, 1998). As only a few micro-litres of sample are analysed out of the entire liquid sample, much longer entrainments are required to collect more volatiles. Finally, as seen throughout this chapter, the solvent peaks can mask compounds with shorter retention times (Angelopoulos *et al.*, 1999).

Both sampling methods are beneficial because used together, the thermal desorption sample can provide information on the compounds with short retention times, while the solvent desorption sample can be used for identification and validation of the thermal desorption sample (Angelopoulos & Pickett, 1998). Therefore it seems that

using both methods is important in revealing the most information on decomposition processes and should be continued in future experiments.

The four semiochemicals identified, dimethyl disulfide, KI 881-885, dimethyl trisulfide, and dimethyl tetrasulfide, were present throughout most of the experiment; however, the concentration of these chemicals changed greatly, and in a statistically significant way, during different stages of decomposition. Throughout decomposition dimethyl disulfide was recovered in the lowest concentrations of all four EAG-active compounds, which could explain the inconsistencies in the fly's reaction to this chemical during GC-EAG experiments. It would seem that the concentrations may have affected the presence of *Calliphora* sp. on the corpse, as higher concentrations of dimethyl. trisulfide, dimethyl tetrasulfide, indole, and 3-methylindole resulted in a much more substantial number of flies, in the case of Porapak samples. However, one cannot exclude the possibility that other factors, such as temperature, could have also had an influence on the volatiles and the number of flies present on the corpse.

Even though *C. vomitoria* responded to peak KI 881-885 during GC-EAG trials, dose response experiments showed that the two compounds tentatively identified at this peak, propyl butyrate and 2-heptanone, had no significant effect on *C. vomitoria.* When observing the dose response curves in Figure 3.12, it can be seen that the response to dimethyl disulfide appears clearly greater than that to methyl salicylate, the positive control, at log concentration of -6. However, as these results were found not to be significantly different to the negative controls (diethyl ether and hexane) dimethyl disulfide could not be confirmed as causing a significant response from *C. vomitoria.* Propyl butyrate, on the other hand, was at the same level, and at times, below those of the negative controls which would indicate that there is clearly no significant response to this chemical by *C. vomitoria.* It would seem that the correct compound was identified. It should be noted that several compounds were co-eluting at the same retention time causing confusion at the EAG-active peak (KI 881-885).

When analysed statistically, dimethyl trisulfide and dimethyl tetrasulfide had the most significant comparison of means (to the 5% and 1% levels). Throughout all the principal component statistical tests these two compounds were responsible for explaining the greatest amount of variation. As these two chemicals peaked in the

Active Decay stage, it is not surprising that this stage stood apart from the other days or stages of decomposition. It might suggest that dimethyl trisulfide and dimethyl tetrasulfide have the strongest effect in attracting the blowfly *C. vomitoria* to a decomposing carcass as the Active Decay stage was also the one showing the most insect activity.

It is interesting to note that statistical evaluation revealed that the Fresh and Dry stages were similar to each other. In physical appearance these stages were very different; however, the chemical compounds showed otherwise, although this is a somewhat artificial result due to the focus being simply on the four EAG-active compounds discussed below. In fact, the stages Fresh, Advanced Decay, and Dry, scored relatively close to each other when the chemical concentrations present at each stage were analysed. As these were all lower in sulphur-containing compounds, the results were not surprising. In this study, only the EAG-active compounds were tested. However, were calculations to be re-done to include all the chemical compounds recovered from the decomposing pig carcass (EAG and non-EAG active compounds for *C. vomitoria*) the later occurring compounds would be expected to have an affect on the results such that the Fresh and Dry stage would not be grouped together as many new peaks appear later in decomposition.

Three days in particular, days 5 & 6 (Bloated), and 16 (Advanced Decay), were found to be outliers due to the sudden high concentrations of dimethyl trisulfide recovered from the decomposing pig using Tenax tubes. These days also link with higher than average temperatures recorded in the mouth of the decomposing pig and later the rectum (Chapter 2, Section 3.4). Tenax samples yielded no dimethyl trisulfide on days 7 & 9. This must have been due to a problem with the collection method as Porapak samples prove that high levels of dimethyl trisulfide were present on those days.

The EAG-active compounds recovered from the decomposing pig can be identified in other sources described below and have been found to evoke antennal responses in other dipterans. Dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide are just some of the chemicals that have been isolated from raw chicken (Senter *et al.*, 1999; Miller & Horn, 2001), ox faeces (Ding *et al.*, 1998), and swine breeding areas (Ranau & Steinhart, 2005). These three sulphur compounds, as well as 3-methylindole

and indole were also identified in pig manure and found to be attractants for the housefly, *Musca domestica*, during GC-EAG and wind tunnel experiments (Cossé & Baker, 1996). These findings indicate that *M. domestica* and *C. vomitoria* have some similarities in their responses to semiochemicals.

Lucilia sericata, most often associated with sheep strike in England and Wales, is said to be attracted to sulphur-based chemicals, including dimethyl disulfide, released during the breakdown of cystine in the wool keratin (Ashworth & Wall, 1994). When Park & Cork (1999) investigated electrophysiological responses of receptor neurons on the antennae of female *L. cuprina*, a serious sheep pest in Australia, they found that there was a response to dimethyl disulfide. Dimethyl disulfide has been identified in fleece volatiles, pig excreta, and is part of the formulation of the chemical attractant, Swormlure-4 (SL-4). This synthetic attractant is used to control populations of the New World Screwworm, *Cochliomyia hominivorax*, and other parasitic flies against infestation of livestock. (Mackley & Brown, 1984).

Morris *et al,*. (1998) also recovered dimethyl disulfide from liver, liver + sodium sulfide mixtures, 7-day old sheep gut mucus, and *Proteus mirabilis*, *Serratia marcesens*, *Pseudomonas aeruginosa* & *Citrobacter freundii* bacterial cultures. However, they found that bioassay experiments using dimethyl disulfide did not elicit a response from *L. cuprina*. While *L. cuprina* was not responsive to dimethyl disulfide, it was very responsive to dimethyl sulfide - also found in liver, liver + sodium sulfide mixtures, sheep faeces, and *Pseudomonas aeruginosa* and *Citrobacter freundii* bacterial cultures. Dimethyl sulfide was not identified during this chapter's experiments, although this could be due to the dimethyl sulfide peak being located under the diethyl ether peak, as it has a shorter retention time than dimethyl disulfide. Further testing will have to be conducted.

Propyl butyrate is most often a volatile of vegetation such as the ripe fruit of *Spondia mombin* (Cruz-Lopez *et al.*, 2006). It has been described as having a sweet smell (Mosciano, 2006) but has not yet been linked to decomposing animals. As *C. vomitoria* is not normally attracted to plants, the poor dose response results were not surprising.

Birkett *et al.* (2004) found that, during electroantennogram experiments, *Haematobia irritans*, *Hydrotaea irritans*, and *Stomoxys calcitrans* responded to 2-heptanone, while *Musca autumnalis* and *Wohlfahrtia magnifica* responded to indole and 3-methylindole collected from the vapour mixture above cattle, termed the headspace, and urine (48 hrs old).

Du & Millar (1999) recorded antennal responses from *Culex quinquefasciatus* and *C. tarsalis* (Diptera: Culicidae) to indole and 3-methylindole, among other compounds, collected from the headspace of fermented infusions of Bermuda grass, during GC-EAG experiments. These compounds elicited an antennal response from *C. vomitoria*, but only in the dose response experiments; the GC-EAG trials failed to identify these as semiochemicals.

Indole and 3-methylindole, along with many other compounds (Chapter 1), are produced during the further decomposition of protein and fat shortly after the gases escape the body (Vass, 2002). The period, described by Vass (2002), in which indole and 3-methylindole become prevalent during decomposition seems to agree with the findings of this research, as the concentration of these compounds were at their highest during the Bloated and Active Decay stage.

Indole was present in high concentrations on certain days of decomposition; however, it did not elicit a response from *C. vomitoria* in the coupled GC-EAG study. When a strong response to one compound is exhibited, this can cause super-saturation of receptors and it then takes longer for them to recover, so creating a delay before they can respond to the next EAG-active compound (Burguiere *et al.*, 2001). In this case, *C. vomitoria* responded strongly to dimethyl tetrasulfide, which is released from the GC column just before indole. The strong response to dimethyl tetrasulfide and potential super-saturation is one possible reason for the lack of response to indole, however, this scenario seems unlikely as there was a gap of 1 minute and 20 seconds between the two compounds. The potential for indole to stimulate an EAG response from *C. vomitoria* should be studied in dose-response trials with the single compound.

3-Methylindole was present in very low concentrations, lower than that of any of the EAG-active compounds (see averages - Appendix 6). Dose response experiments

concluded that *C. vomitoria* only responded to 3-methylindole, in a significant manner at 10 ng/μL which is well above the maximum concentration of 0.13 ng/μL recovered from the Porapak samples. Therefore the response noted in the dose-response experiments might have no natural significance. Receptors have specifically evolved to respond to particular compounds that have some behavioural significance, e.g., semiochemicals, and respond to these at low levels (Bruce *et al.*, 2005). It is possible that 3-methylindole is not one of these EAG-active compounds and therefore requires larger concentrations for a reaction from *C. vomitoria*. It is also possible that the high stimulus concentrations, used during dose response experiments, may have caused a general response, since at high levels specialised receptors may display some response to other compounds (Pickett *et al.*, 1992).

Birkett *et al.* (2004) have found that at times when hosts are not attractive to pests, there are elevated levels of certain semiochemicals which may act as active repellents or more passively by 'masking' attractants, thereby reducing the sensitivity of flies to these attractants. This could be relevant to decomposing bodies. The combination of the semiochemicals with later occurring compounds could have also had an effect on the presence of insects on the pig. The drop in sulphur compounds along with a rise in many other compounds could work together to indicate to *C. vomitoria*, and other flies, that the body is no longer suitable for feeding or oviposition. Volatiles, that are not present early in decomposition, could develop and become a repellent to the early colonizers of the corpse or 'mask' the attractant semiochemicals. Similarly, late colonizers could be repelled by early decomposition volatiles or by those produced by the immature stages (larvae) of the early colonizers to help avoid competition. However, the alternative is that the flies are simply not attracted to the odours present on a body at a particular time.

The detection of semiochemicals mediates the behaviour of insects. For example, the Mediterranean flower dead-horse arum, *Helicodiceros muscivorus* (Araceae: Aroideae) lures blowflies to act as pollinators by emitting dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, and dimethyl trisulfide derivatives (confirmed through GC-EAG experiments), which are also found in decomposing meat. The flies are thus enticed and trapped into a floral chamber that surrounds the female florets (Stensmyr *et al.*, 2002). Other factors such as the appearance of the plant - the flower is said to resemble

the anal area of a dead mammal - and the pseudo-thermogenic properties of the plant are also key factors in the fly's attraction to the plant (Stensmyr *et al.*, 2003). Circadian activity may also play a role in fly attraction as the odours are only emitted between sunrise and noon. GC-EAG tests also showed that female *C. vicina*, *L. caesar*, and other Calliphoridae species were attracted to these compounds and were not able to discriminate between decomposing meat and the flower through olfaction alone (Stensmyr *et al.*, 2002).

The data also suggests that the concentrations of the semiochemicals may have had a strong influence on the number of flies around the decomposing corpses. However, during GC-EAG experiments very low concentrations elicited a response from *C. vomitoria* in amplitudes often as high as those samples with stronger concentrations, indicating that in GC-EAG experiments the presence of the semiochemicals was at times more important than the concentration. In nature, higher concentrations of chemicals allow flies to detect the volatiles of a decomposing corpse from further away. Therefore, outside of the laboratory experiment setting, concentration plays an important role in attracting flies from a larger area resulting in greater numbers on flies on the carcass.

As seen in Chapter 2, Sepsidae were observed in large numbers on the decomposing bodies while in the Dry stage of decay. These results were consistent during each the spring/summer experiments. GC-EAG trials using these later occurring flies would be very interesting and possibly valuable when considering post mortem interval.

General observations of decomposing carcasses show that, depending on the circumstances of death and method of disposal of the remains, different areas of the body may decompose at different rates due to the insect activity (Smith, 1986; Haskell *et al.*, 1997). The supplementary experiment potentially supports these observations as the concentration levels of volatiles emitted from the head and posterior end of the pig differed. More research will have to be done to fully evaluate this technique for application at crime scenes, both for research and for case work. A direct comparison to the air entrainments done using the stainless steel cover is difficult at the moment as they take the complete suite of volatiles from the whole cadaver instead of from just one area of the body.

While the issue of whether or not blowflies oviposit on a body at night is still highly debated, a recent study by Wooldridge, *et al.*, (2007) found that *C. vomitoria* was less accurate at finding a food source (liver) in the darkness. Though it might suggest that these blowflies do not oviposit at night, this particular research based its finding on light and dark rather than time of day (all experiment were conducted in the early afternoon – information provided by author R. Wall). Spencer (2002), who conducted nocturnal oviposition studies in the southwest of England, did not find any evidence of oviposition during any of her nine trials. Studies conducted in Central Europe reported similar findings (Amendt *et al.*, 2007b). Reckel *et al.*, (2006) suggest that the time of year and cooler temperatures possibly contribute to the lack of oviposition at night. Although, in this body of work, no experiments were conducted to determine whether *C. vomitoria* would oviposit at night, it was evident that GC-EAG experiments would only yield results if conducted before 15:30 hours. All trials were performed during the winter months; however, *C. vomitoria* were kept in an insectary and were not subjected to the cool winter weather.

Coupled GC-EAG assays measure only the amplitudes of antennal responses; they will not reveal the behaviour which a specific volatile will have on a particular insect species (Du & Millar, 1999). Therefore further tests, such as behavioural bioassays, are required to understand how *C. vomitoria* arrives on a carcass so early in decomposition.

Ultimately, this research is hoped to be expanded to human decomposition volatiles. The use of insects and other arthropods is the most reliable method of determining the post mortem interval (PMI) to date (Vass *et al.*, 2002; Buchan & Anderson, 2001). However, this determination is often highly dependent on when the first flies initially arrived on the body to lay their eggs. With a stronger knowledge of the volatiles from decomposing human bodies and a better understanding of the behaviour of carrion insects to these particular volatiles, this author believe a more accurate PMI could be calculated.

3.5 SUMMARY

Blowflies are attracted to a carcass by olfactory stimulus. However, this association between the blowfly and decomposing body has never been explored. Determination of time since death is greatly dependent on the oldest immature stages of blowfly on the carcass. Therefore, research was undertaken to find the many factors which influence the behaviour of insects on the decomposing body and to identify the semiochemicals responsible in initially luring blowflies, specifically *Calliphora vomitoria*, to the body.

The main focus of this investigation was to collect volatiles from pig carcasses during all stages of decomposition and determine specifically which semiochemicals elicited an electrophysiological response from *C. vomitoria*. It was also essential to determine the best means by which to collect the volatile chemicals associated with a decomposing body, finding the most reliable method to conduct electroantennogram (EAG) experiments, and confirm the identity of possible semiochemicals.

Volatile chemicals were collected from a decomposing pig during all stages of decomposition and analysed using gas chromatography (GC) with mass spectrometry (MS). Combined GC-EAG experiments, using the volatile chemical samples and *C. vomitoria*, were conducted in order to isolate the EAG-active compounds. Dose response experiments were performed to determine the threshold concentration of these tentatively identified compounds for *C. vomitoria* with selected active compounds and to confirm their electrophysiological activity.

Four compounds were found to be EAG-active: dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, and a compound of unknown structure (KI 881-885). Dose response experiments confirmed that dimethyl trisulfide and dimethyl tetrasulfide had the greatest effect on *C. vomitoria*, which was evident when the concentration of these semiochemicals was compared to the number of flies on the body at that time. As decomposition progressed, the concentrations of the EAG-active compounds fluctuated, with dimethyl trisulfide and dimethyl tetrasulfide peaking during the Active Decay stage. The Active Decay stage also recorded the greatest number of *Calliphora*.

Certain volatile compounds, such as phenol, indole, tetradecane, and pentadecane, occurring later in decomposition were recorded in high concentrations. However, despite the high concentrations, the flies did not respond to these indicating that *C. vomitoria* is very selective in the chemicals to which it will respond.

GC-EAG experiments could only be conducted between the daytime hours of 8h30 and 15h30, as response from *C. vomitoria* decreased considerably after this time. This phenomenon was not explored further at the time; however, a possible diurnal rhythm is suspected.

In conclusion, this research explored aspects of blowflies never before evaluated. Four semiochemicals were identified and I believe that an understanding of their influence on *C. vomitoria* should be further explored in order to extrapolate useful information to benefit the fields of forensic entomology and chemical ecology.

CHAPTER 4 – BEHAVIOURAL RESPONSE OF BLOWFLIES TO TRAP EXPERIMENTS INCORPORATING THE PREVIOUSLY IDENTIFIED ELECTROPHYSIOLOGICAL-ACTIVE COMPOUNDS

4.1 INTRODUCTION

Semiochemicals and insects are often studied in laboratory conditions where the environment and many other factors can be controlled. While these yield important details, they explain very little about the insects' behaviour in their natural environment. As explored in the previous chapter, insects rely heavily on odours; blowflies are no exception to this. There are many techniques to conduct behavioural experiments in regards to semiochemicals, these include Y-tube and wind-tunnel trials (Logan, 2005); however, field trapping experiments have been widely encouraged (Muirhead-Thomson, 1991). These studies are important to research examining the ecology and field behaviour of insects; they help study or control insect populations in the most natural environment possible (Hall, 1995). Adequate replication can help mitigate the effects of uncontrolled factors such as site, time, and weather. While it is very difficult to control factors affecting insects in the field, these natural conditions will provide valuable information that would not be possible in a controlled laboratory setting.

Insects have evolved specific behaviours to survive. Carrion insects, for example, have developed different seasonal life-cycle development times, variations in habitat, and preferences in carcass size, species, and decomposition stage due to the high levels of interspecific competition (Fisher *et al.*, 1998). Some, like the blowfly *Lucilia sericata* (Diptera: Calliphoridae), are able to develop on live sheep and other warm-blooded vertebrates as well as in carrion (Fisher *et al.*, 1998).

Insects have also developed different behavioural mechanisms to locate a food source, and sites for mating or oviposition. Visual cues such as size, colour, and shape can be important to certain insect species (Muirhead-Thomson, 1991).The oriental fruit moths, *Grapholita molesta* (Lepidoptera: Tortricidae), were found to be unable to remain orientated when placed in a visually diminished "blank" environment containing an attractant odour, implying that a visual cue is vital for site location

(Vickers, 2000). One study revealed that the blackfly, *Simulium arcticum* (Diptera: Simuliidae), relied heavily on visual cues to locate a host at close range and, therefore, responded not only to the CO_2 being released by the living host (Sutcliffe *et al.*, 1991), as had been originally assumed.

For many insects, a visual cue is important for the final location of a food source; however, most require an olfactory cue to initiate movement towards the source. Semiochemicals play an important role in the activation and flight orientation towards a food source, mating, or oviposition site (Wall and Fisher 2001). The blowfly, *L. sericata*, was shown to land closer to an odour source when accompanied by a visual cue, therefore showing that it combined the effects of the visual and chemical cues to reach its desired location (Wall and Fisher 2001). Certain insects do not always require such obvious visual cues and rely almost completely on olfactory information (Vickers, 2000). Another study using blackfly, this time *Simulium damnosum* (Diptera: Simuliidae), showed that when the response of the fly was tested using human baited traps, it was discovered that the visible bait, rendered odourless inside a plastic enclosure was completely non-attractive, showing that these blackflies rely more heavily on the odour attractant (Muirhead-Thomson, 1991).

Different behaviours will be observed from flies, at the same odour source, depending on their sex and, in the case of females, level of oocyte maturation (Wall & Fisher, 2001). For example, previtellogenic *L. sericata* (Diptera: Calliphoridae) females are attracted to a dead animal for its protein meal; later, changes in behaviour will occur as the ovaries mature and these will become attracted to a dead carcass, regardless of the protein meal, to mate or lay eggs (Wall & Fisher, 2001; Ashworth & Wall, 1995; Hays & Wall, 1999).

The importance of many different factors such as visual cues, olfactory cues, and sex vary within the insect communities. It cannot be assumed that the behaviour mechanisms of related species will be identical, even when in the same family or apparently similar habitats. Over time, each organism has evolved its own specific behaviour (Vickers, 2000). Scientists are continually developing traps in the light of new knowledge about biology and behaviour of pest species. Traps may have varying functions. They may be used for monitoring population densities. Others, especially in

the case of biting flies which have a significant economic impact, are employed for population suppression (Mands *et al.*, 2004). Traps can be designed to be highly selective or general.

Trap design presents a wide array of choice from the very simple, like the sticky trap, to the complex, such as the 'West Australia' blowfly trap described below. These designs can be used to test visual factors such as colour, shape, size, or motion as well as chemical lures.

Water traps, using a detergent and water mixture in a bowl to capture visiting flies, are simple and inexpensive. Odour or colour bait is used to lure the insects, which inadvertently land on the water and sink. These traps can be used to test such factors as visual and odour cues (Smart *et al.*, 1997). Others such as sticky traps use sheets or cards covered in an adhesive to trap landing flies (Fisher *et al.*, 1998). Sticky traps are ideal in research as they only require the flies to land on the adhesive surface where they become fixed (Hall, 1995). No complicated trap entry and containment mechanism is required such as in the 'West Australia' blowfly trap which uses a series of chambers, including an ant exclusion device, to trap and hold blowflies. This trap requires considerable maintenance and can be awkward to operate (Muirhead-Thomson, 1991; Vogt *et al.*, 1985).

There are traps made to resemble animals, called 'silhouette' traps, to attract a particular pest of interest. Cow silhouettes used in blackfly, *Simulium luggeri* and *S. arcticum*, studies appeared to suggest that the surface area and size of the openings were more important than shape (Fredeen, 1961). The 'silhouette' traps have more recently been combined with odour baits to create a much more successful trap. These found that *Simulium* were more attracted to the "tail" region of the silhouette; however, ultimately the odour bait location determines the pattern of fly distribution (Muirhead-Thomson, 1991).

Some traps use live bait such as living humans or animals. These not only provide the natural odour but they also offer the visual cue and, depending on the specific style of trap and bait, can also offer motion as attractants to lure the desired insect species (Muirhead-Thomson, 1991). Other traps are designed by accident. Horse-flies,

Tabanus sp. (Diptera: Tabanidae), were found to be attracted to a weather balloon in Manitoba, Canada. From this came about the successful trapping method called the 'Manitoba Trap'. The trap design uses the principal that horse flies are attracted to the contrast between the light and dark colours of the canopy and the moving sphere – a dark animal walking through an open pasture or a trail (Stringham, 2001). Thermal stimuli created from the heat absorbed by the black sphere may also contribute to this traps' success (Muirhead-Thomson, 1991).

It has been widely accepted that blowflies rely heavily on odour attractants to locate decomposing bodies (Archer & Elgar, 2003). While no other research has examined volatiles collected from a decomposing body, some researchers have tested the attraction of blowflies, such as *Lucilia sericata*, to sodium sulphate and liver mixture as well as aged liver with success (Freney, 1937; Hall, 1995; Smith & Wall, 1998; Fisher *et al.*, 1998; Hall *et al.*, 2003; Wooldridge *et al.*, 2007).

In the previous chapters, succession experiments showed that blowflies, such as *Calliphora vomitoria*, *C. vicina*, *L. sericata*, and *L. caesar* (Diptera: Calliphoridae), were often the first to arrive on a decomposing carcass and were, during the early stages of decomposition, the most numerous on the body. Electrophysiology experiments, using *C. vomitoria* and volatiles from a decomposing pig, tentatively identified compounds that elicited a reaction from *C. vomitoria*. These EAG-active compounds were: dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, propyl butyrate, and 2-heptanone. The aims of this chapter were to further explore the findings discovered in the previous chapters by conducting Bioassay experiments using the EAG-active compounds in order to determine if these chemicals, detected physiologically by blowflies, will attract them, repel them, or have no behavioural impact.

4.2 METHODS

Many possible methods of testing were considered, including experiments conducted in wind-tunnels, as used by such authors as Wall & Fisher (2001) and Cossé & Baker (1996). However, these were discounted as none of the wind-tunnels at the research location were equipped to take air directly to the outside making them unsuitable for

this particular research. It should also be noted that Cossé & Baker (1996) had difficulty in getting houseflies to respond to sulphur compounds during wind-tunnel experiments, ultimately influencing my decision not to conduct wind-tunnel experiments. Bioassay experiments conducted in the field, in the blowflies' own environment, were chosen as the most suitable for these trials.

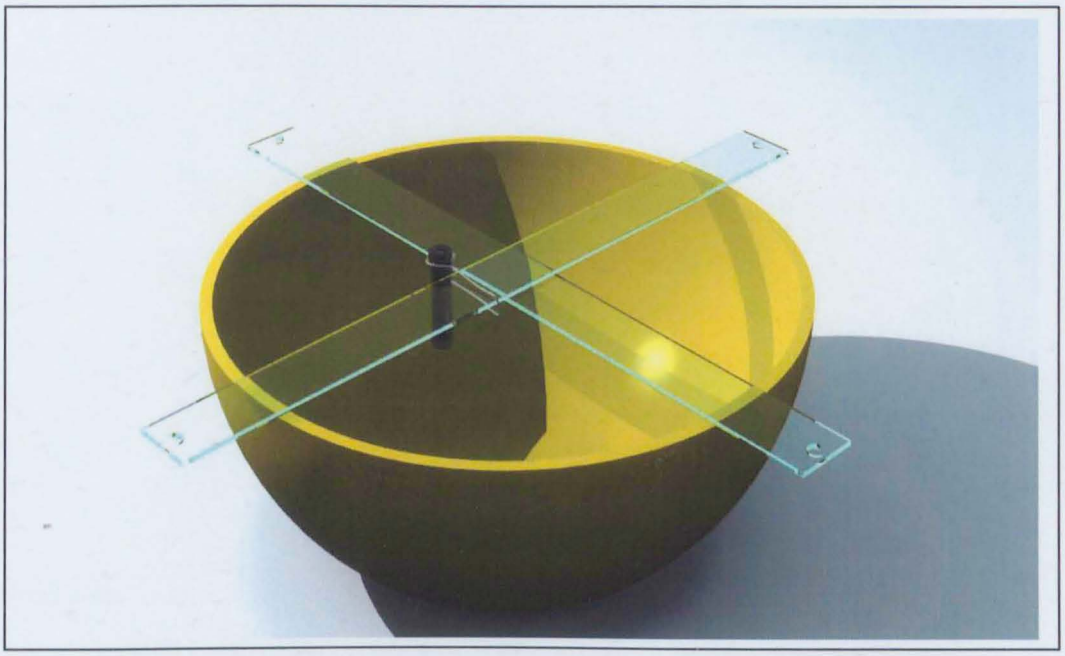
4.2.1 Field Trial 1 - Water Traps

Water trap experiments were carried out between the 23rd of April 2004 and 2nd of June 2004.

Traps

Water traps were chosen as they were inexpensive and easy to manage. Each trap consisted of a canary yellow plastic bowl (Just Plastics Ltd.; 21cm diam. × 9cm) filled with a detergent solution (0.5% in water) up to 10mm from the edge of the bowl (Smart *et al.*, 1997; Muirhead-Thomson, 1991). Yellow bowls were chosen for these experiments as blowfly, *L. sericata*, showed a preference to yellow traps during field studies conducted by Wall *et al.* (1992). To keep the bowl in place two strips of clear plastic, one of which had a hole in each end, were joined together to form a cross and placed on top of the bowl. Plastic bands were tied to the holes in the "cross bar" (Smart & Blight, 1997). Tent pegs were driven into the ground and the elastics were slipped over these to keep the bowl in place (Figure 4.1). The bait (odour sample) was held in position by a thin wire in the middle of the cross and was hung just over the water.

Figure 4.1: Visual representation of the water trap used in experiments conducted from the 23rd of April 2004 to the 2nd of June 2004. Included are the yellow bowl, plastic cross bar, and the darkened Choromacol vial containing the bait.



Created by Alexander R.H. Tully

The traps were placed in a randomised block (Latin Square) design (Smart *et al.*, 1997) as this configuration allows for many replicates to be laid at the same time and to account for the effects of factors present in the experimental area without having to move the traps (Dytham, 1999). There were five rows and five columns; these were positioned in a manner where only one replicate and each treatment occurred only once in every row or column (Figure 4.2). There was 10m spacing between each trap. One side of the field used for the experiments was near a wooded area with coniferous and non-coniferous trees (15m away); but the randomised experiment design, with ANOVA, took into account any edge effects that the site might have introduced.

Figure 4.2: Latin square design of water traps in Appletree Field.

Rows (a-e)

Columns (1-5)	a	b	c	d	e
1	1C	2E	3D	4B	5A
2	6E	7B	8A	9D	10C
3	11D	12A	13E	14C	15B
4	16B	17D	18C	19A	20E
5	21A	22C	23B	24E	25D

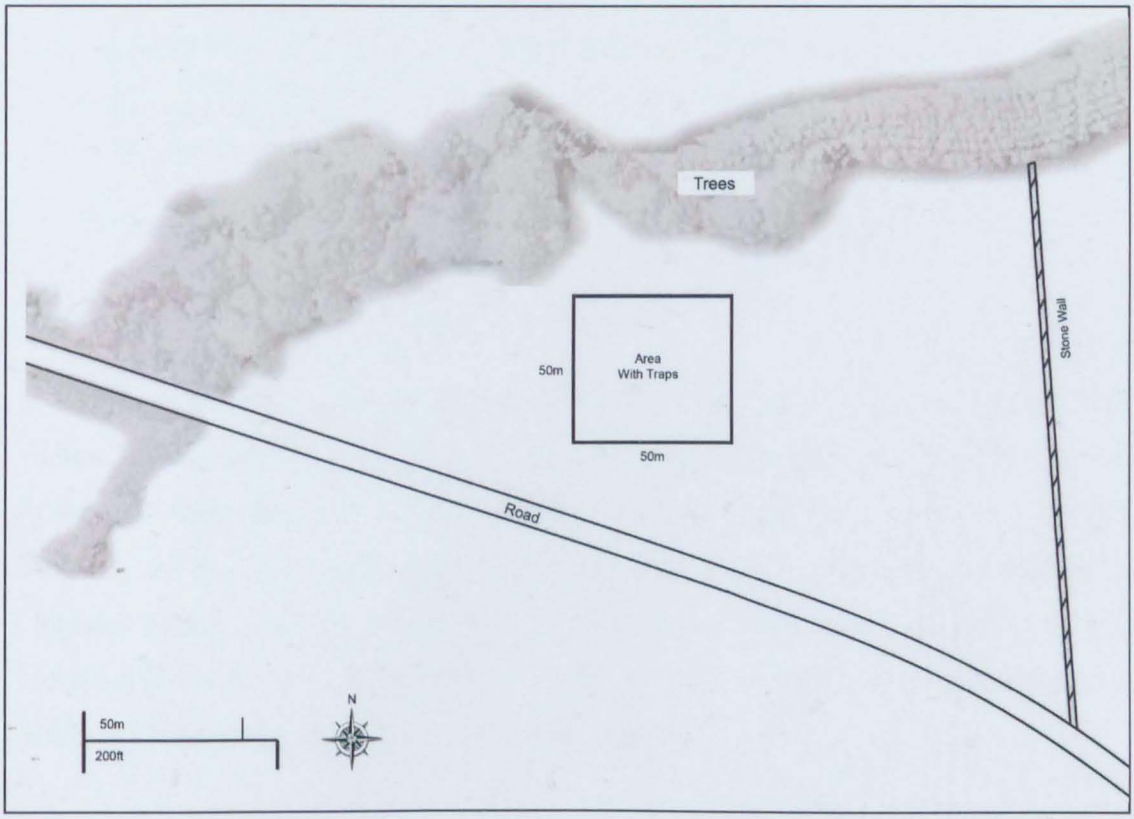
*A total of 25 water traps.

Treatment:
A – Propyl butyrate
B – Dimethyl disulfide
C – Dimethyl trisulfide
D – Dimethyl tetrasulfide
E – Control – empty vial

Research Site

Appletree Field in Scout Farm, Rothamsted Research, Harpenden, Hertfordshire, UK. This field was adjacent to the road B5183. There were two horses 600-700 metres away, in a field with gates denying the horses access to the research site. The research field was a 'set-aside', or an untouched area of land, containing a diverse habitat including bushes along the road, wheat, grass, and weeds, with clay-like soil and stones. There was a small wooded area on the NW side of the research area. Column 1 was facing the NW side of the field and Column 5 was towards the SE side of the field. The minor road, B5183, was 200m away from Row 'a' (Figure 4.3).

Figure 4.3: Appletree Field in Scout Farm, Rothamsted Research, Harpenden, England. Research site where water trap experiments were conducted.



Created by Alexander R.H. Tully

Bait

Darkened 800µL Choromacol vials were used with 1.1mm diameter hole drilled into the lid so that the chemical compound could be released by diffusion. Each vial contained 100mg of compound (Table 4.1). The compounds were chosen 'neat' as when mixed with solvent there was a worry that the solvent would be released (diffuse) first and would not give an accurate representation of the release rate of the compound in question. The compounds tested were propyl butyrate, dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, and a negative control (empty vial).

Table 4.1: Volume of test compound (bait) used for release rate experiments and bioassays made up to 100mg.

Compound	Density (g/mL)	Volume (µL)
2-Heptanone	0.82	122
Propyl butyrate	0.87	115
Dimethyl disulfide	1.06	94
Dimethyl trisulfide	1.19	84
Dimethyl tetrasulfide	1.30	77

Conversion was done by dividing the desired weight (100mg) by the density.

A single water trap was later placed in Little Hoos Field (described below) with sodium sulfide and liver bait (also described below) as a positive control. The trap was in the field for a duration of six days in the month of October (from the 1st to the 6th). This test did not lead to the capture of many blowflies; however, it was used as an indicator of the efficiency of the trap, as the bait had been used successfully to trap blowflies in the past (Ashworth & Wall, 1994; Hall, 1995; Fisher *et al.*, 1998; Smith & Wall, 1998; Wall & Fisher, 2001; Hall *et al.*, 2003).

Six experiments were conducted with varying lengths of time to determine the effect of bait exposure period on catch of blowflies (Table 4.2). At the end of each experiment the insects captured (explained below) were collected and the bait was replenished for the next experiment.

Table 4.2: Duration of each water trap experiment conducted in Appletree Field.

Trial	Date	Duration
1	2304/04 - 26/04/04	3 days
2	26/04/04 - 29/04/04	3 days
3	29/04/04 - 05/05/04	6 days
4	05/05/04 - 11/05/04	6 days
5	1105/04 - 17/05/04	8 days
6	17/05/04 - 02/06/04	16 days

Collections

The insects were collected by pouring the detergent solution and water through a sieve made from a $10cm^2$ piece of muslin. The insects and the muslin were then preserved in a vial containing 95% ethanol and 5% glycerol.

Insects were examined under a Nikon stereomicroscope and identified using the keys of Borror (1989); D'Assis Fonseca (1968), Erzinçlioğlu (1996), Joy (1932), Pont (1979), Rognes (1991), Smith (1986), and Kloet, (1978).

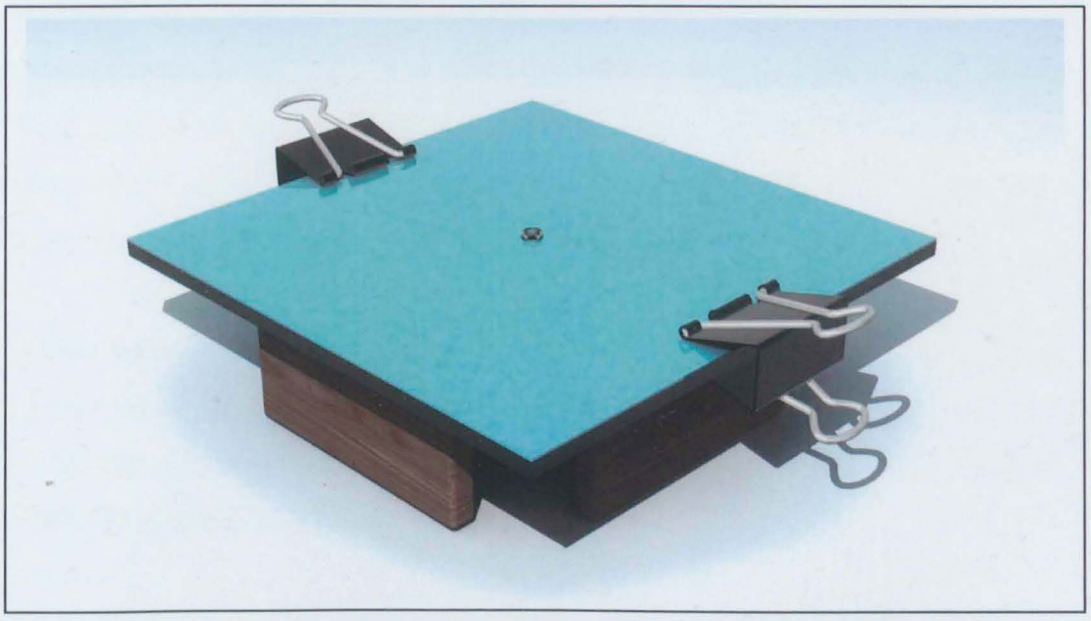
4.2.2 Field Trial 2 - Sticky Traps

The above water trap techniques did not provide clear results; therefore, a second method was employed for the bioassay studies. This experiment was conducted from the 1st to the 7th of October 2004.

Traps

Blocks of wood measuring 5cm in height were glued to the bottom of a $20cm^2$ plastic (plexi glass) sheet to lift the trap off the ground, and together forming the base of the trap (Figure 4.4). The horizontal plastic square had a 1cm hole drilled in the centre to place the bait flush with the sticky card (Muirhead-Thomson, 1991). Two holes at the edge of the plastic square were made where elastic bands were placed and fastened to tent pegs. Sticky cards were used to form the 'trapping' mechanism of the sticky traps. The sticky cards were placed onto the horizontal plastic square and kept in place with bull clips. Blue sticky cards were chosen as yellow cards attracted too many non-target arthropods. Two cards (Oecos Ltd., 10 × 20cm, coated with Oecotak adhesive) were clipped onto the flat plastic square of each trap. In both experiments the traps were kept close to the ground to simulate the height of a carcass.

Figure 4.4: Visual representation of the sticky trap used in experiments conducted from the 1st to the 7th of October 2004. Included are the trap base, the blue sticky cards, bull clips, and the top of the darkened Choromacol vial containing the bait (centre of trap).



Created by Alexander R.H. Tully

Research Site

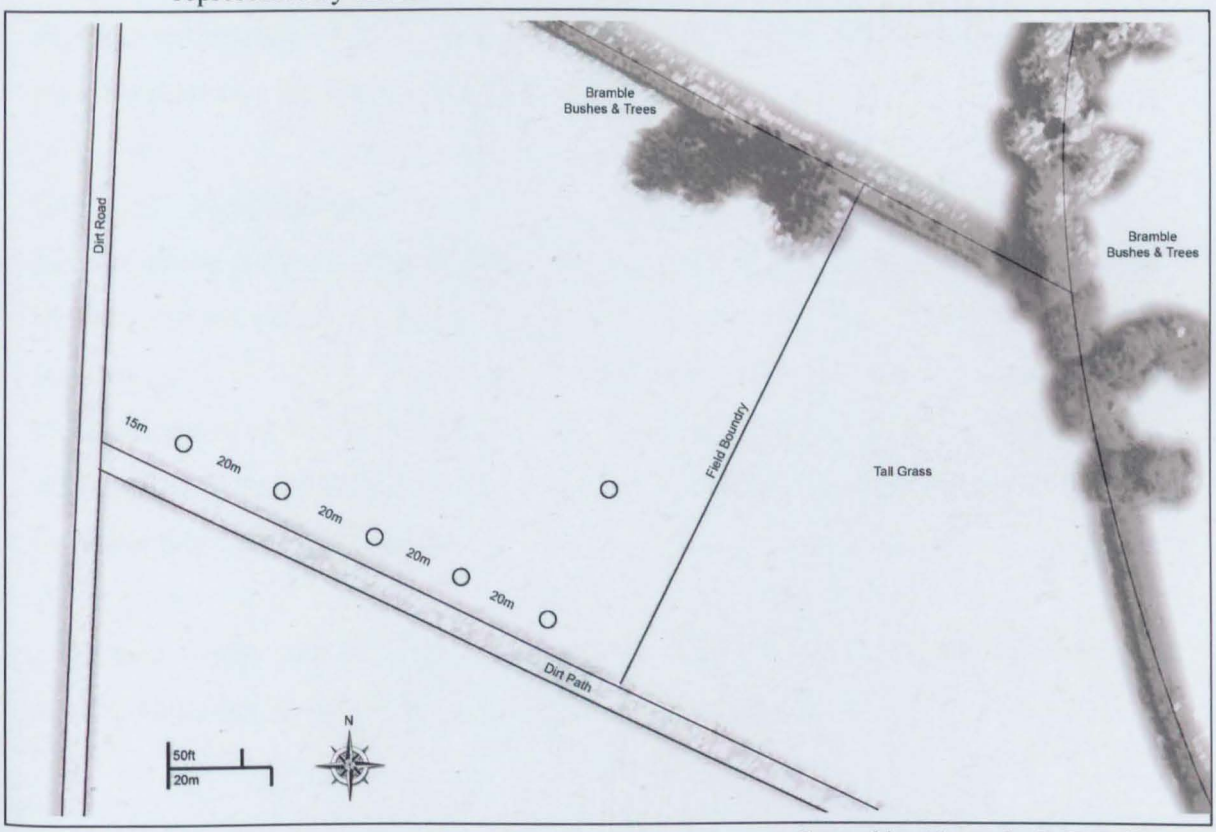
Little Hoos Field in Scout Farm, Rothamsted Research. Harpenden, was one of several agricultural fields used to study plant growth. At the centre of these fields was a single lane, gravel road to allow access to the site. At the far-east end of the chosen location was a footpath which was separated from the fields by large, thick bramble bushes. The traps were placed on the west side of the field where predominantly short grass (planted 2 months earlier) was present. Taller grass took over the eastern half of the field. The first of six traps was placed 15m away from the gravel road (SW corner of the chosen location) and the other four traps were positioned in a straight line leading SE away from the road (Figure 4.5). The fifth trap (second last) was 20m away from the tall grass; the final trap was positioned at a right angle from the previous one forming an L shape design.

Bait

The chemical compounds used as bait were dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, sodium sulfide with liver as the positive control, and a negative control (empty vial). Propyl butyrate, used in 4.2.1, was replaced with 2-heptanone as the gas chromatogram peak previously thought to be propyl butyrate (tentative) was later identified as 2-heptanone (tentative) (Chapter 3). 100mg of the chemical compounds were placed in darkened 800µL Choromacol Vials with 1.1mm hole drilled into the lid, exactly as in the previous experiment (Table 4.1).

The sodium sulfide and liver mixture was created by placing 25g of ground pig liver into a graduated cylinder then adding a 10% solution of sodium sulfide with the liver to the 30ml line (Ashworth & Wall, 1994; Hall, 1995; Fisher *et al.*, 1998; Smith & Wall, 1998; Wall & Fisher, 2001).

Figure 4.5: Little Hoos Field in Scout Farm, Rothamsted Research, Harpenden, England. Research site where sticky trap experiments were conducted. Sticky traps are represented by circles.



Created by Alexander R.H. Tully

Collection

Every day at 1500 hours the sticky cards were collected from each trap and placed in separate clear plastic bags. To minimize damage to the flies captured, the sticky cards were set back to back (sticky side out) and then placed into the clear plastic bag. The bag was positioned so that there were no creases over the sticky surface. Identifications could be made without removing the sticky cards from the bag; small incisions in the bag directly over the insects were done when required.

Each day the old odour baits were removed and replaced with a new one. Each trap and bait was rotated by one position every day of the experiment to minimise the effect of positional bias (Fisher *et al.*, 1998). New sticky cards were placed on each trap. The sticky cards collected were returned to the lab and placed in the freezer until examination was possible. Fly identifications were done as in the previous experiment.

4.2.3 Release Rates

The release rate of each chemical compound was calculated through controlled experiments. One group of vials were kept in a fume cupboard and the other group was placed outdoors at the first experimental site (Appletree Field).

Control – Fume Cupboard

The vials were prepared and weighed at the same time as the experimental vials, using the same measurements (Table 4.1). The Choromacol vials were fitted with lids each containing a 1.1mm hole, as in the field experiments. They were then placed in a fume cupboard reserved for this experiment only. The temperature in the fume cupboard remained at approximately 19°C. The vials were weighed throughout the duration of the water trap field experiments.

2-Heptanone was tested at a later date (October 2004) in the same fume cupboard set at 19°C and using the same balance as in the above experiment.

Experimental – in the Field

Vials prepared at the same time as the ones in the control group were placed at Appletree Farm, 15m west of water trap 1C. These were mounted on a pole at 80cm. The vials were returned to the laboratory and weighed on each insect collection day.

As the experiment progressed, it was becoming clear that water was collecting in certain vials during periods of rain. Other matter such as small flies and debris were also appearing in the vials during windy periods. Therefore, empty vials (as those above) were left at Appletree Farm to determine how much rain-water, insects, or debris would be collected and ultimately block the small opening in the lid which could affect the release rate of the test compound. The vials were placed in the field on the 6th of May 2004 and collected on the 7th of June 2004; these were weighed on four separate occasions. All vials were weighed using the same balance to avoid as many variables as possible.

4.2.4 Statistical Analyses

The catches of blowflies were subjected to one-way analyses of variance (ANOVA) to test the effect of the experimental treatment and position of the traps. The possible links between the effects of weather on fly catch were compared by correlation (Pearson's). Chi-square was used to determine differences between the male and female blowflies captured in the traps.

4.3 RESULTS

4.3.1 Field Trial 1 – Water traps

Six field trials of varying duration (Table 4.2) were conducted during the spring season of 2004. The total duration extended from the 23rd of April to the 2nd of June.

The counts were restricted to the four blowflies most commonly found on the decomposing pigs discussed in Chapter 2, *C. vicina*, *C. vomitoria*, *L. caesar*, and *L. sericata*. *Melinda sp.* (Diptera: Calliphoridae) was captured on three occasions.

Individual trials collected very few blowflies, the collections ranging from 3 to 62 blowflies per trial (Table 4.3). The blowflies comprised just a small minority of the

total insects collected. Other insects captured in the water traps are listed in Table 4.4. Diptera represented the greater number of families present in the bowls; however, Hymenoptera (primarily Apidae and Vespoidea) and Coleoptera (mainly Curculionidae and Nitidulidae) made up the majority of the insects captured. These were possibly attracted to the yellow colour of the bowl, a typical response of phytophagous insects (Smart *et al.*, 1997). Due to the lack of blowflies collected, data for the trials was combined in the analysis.

Table 4.3: Total number of blowflies collected in the water trap experiments conducted in Appletree Field.

Trial	Date	*C.vicina*	*C.vomitoria*	*L.caesar*	*L.sericata*	Total flies
1	2304/04 - 26/04/04	0	0	3	1	4
2	26/04/04 - 29/04/04	2	0	2	0	4
3	29/04/04 - 05/05/04	28	2	1	2	34
4	05/05/04 - 11/05/04	44	4	10	4	62
5	1105/04 - 17/05/04	8	3	4	4	19
6	17/05/04 - 02/06/04	2	0	1	0	3
		85	9	21	11	126

Table 4.4: Arthropods collected from the water traps

Order	Family
Coleoptera	Carabidae
	Coccinelidae
	Curculionidae
	Elateridae
	Nitidulidae
	Staphilinidae
Collembola	unknown
Diptera	Anthomyiidae
	Bibionidae
	Calliphoridae
	Dryomyzidae
	Heleomyzidae
	Muscidae
	Phoridae
	Tachinidae
Hemiptera	unknown
Homoptera	unknown
Hymenoptera	Apidae
	super-family Chalcidoidea
	Formicidae
	Ichneumonidae
	Platygasteridae
	Sphecoidea
	sub-order Symphyta
	Tenthredinidae
	Vespoidea

None of the chemical compounds tested was found to have a significant effect on the capture of blowflies (all species combined) (Table 4.5 & 'Total' in Table 4.6). None of the four blowfly species observed exhibited a preference to any of the chemical compounds tested (Table 4.5). There was no statistical difference between catches in the negative control and in the baited traps.

Table 4.5: Total number of blowflies collected per treatment during the water trap experiments.

Treatment	*C. vicina*	*C. vomitoria*	*L. caesar*	*L. sericata*	Total
Propyl butyrate	14	3	1	0	18
Dimethyl disulfide	6	2	5	2	15
Dimethyl trisulfide	21	1	6	6	34
Dimethyl tetrasulfide	22	2	3	0	27
Negative Control	22	1	6	3	32
Total per species	85	9	21	11	126

For two species of blowflies there was a statistically significant positional bias in Column 1, regardless of the bait (Table 4.7a). Traps in Column 1, on the NW edge of the plot near the trees (Figure 4.1), contained at least five times more blowflies than any other column. *C. vicina* and *L. caesar* were caught significantly more in traps of Column 1. However, it is important to note that they were present in the largest numbers and it was not possible to make such claims with *C. vomitoria* and *L. sericata* due to their small capture numbers ($P>0.10$). The traps collected four times more *C. vicina* than any other blowfly species (Table 4.2). There was no positional preference within the rows (Table 4.7b).

A slightly higher number of female than male blowflies were caught in the water traps, but this difference was not statistically significant (Table 4.8, $X^2 = 3.8$, d.f. = 1, $P = 0.284$).

The results from the water trap consisting of the positive control (liver and sodium sulfide mixture) were not included in the above calculations because they did not form a part of the Latin Square; however, for reference, only two *C. vicina* were recovered from this positive control over a period of six days.

Table 4.6: Results of one-way analysis of variance comparing blowfly species recovered from the water traps to the treatment used as bait.

Species	Treatment	n	Mean*	Std. Error	df	*F*	*p*
					Between Groups		
C. vicina	1	5	2.80	1.83	4	.375	.824
	2	5	1.20	1.20			
	3	5	4.20	1.59			
	4	5	4.40	3.41			
	5	5	4.40	2.68			
	Total	25	3.40	.97			
C. vomitoria	1	5	.60	.24	4	.304	.872
	2	5	.40	.40			
	3	5	.20	.20			
	4	5	.40	.40			
	5	5	.20	.20			
	Total	25	.36	.13			
L. caesar	1	5	.20	.20	4	.452	.770
	2	5	1.00	.77			
	3	5	1.20	.73			
	4	5	.60	.60			
	5	5	1.20	.73			
	Total	25	.84	.27			
L. sericata	1	5	.00	.00	4	1.170	.354
	2	5	.40	.24			
	3	5	1.20	.80			
	4	5	.00	.00			
	5	5	.60	.60			
	Total	25	.44	.21			
TOTAL combined data for all four species	1	5	3.60	2.14	4	.284	.885
	2	5	3.00	1.90			
	3	5	6.80	2.18			
	4	5	5.40	4.41			
	5	5	6.40	4.17			
	Total	25	5.04	1.32			

Treatments: 1 = propyl butyrate, 2 = dimethyl disulfide, 3 = dimethyl trisulfide, 4 = dimethyl trisulfide, 5 = empty vial (negative control).
*** "Mean" refers to the mean number of individuals per trap for all trial combined.**

Table 4.7: Results of one-way analysis of variance comparing blowfly species recovered to the position (columns & rows) of the water traps. Values highlighted in red are significantly different at 5%.

a) Columns

Species	Column	n	Mean*	Std. Error	df	*F*	*p*
					Between Groups		
C. vicina	1	5	11.80	2.11	4	19.058	<.001
	2	5	1.20	.49			
	3	5	1.60	.93			
	4	5	.60	.24			
	5	5	1.80	.49			
	Total	25	3.40	.97			
C. vomitoria	1	5	.80	.37	4	1.778	.173
	2	5	.00	.00			
	3	5	.60	.40			
	4	5	.00	.00			
	5	5	.40	.24			
	Total	25	.36	.13			
L. caesar	1	5	3.20	.58	4	17.680	<.001
	2	5	.40	.24			
	3	5	.40	.24			
	4	5	.00	.00			
	5	5	.20	.20			
	Total	25	.84	.27			
L. sericata	1	5	.60	.60	4	.450	.771
	2	5	.20	.20			
	3	5	.60	.40			
	4	5	.00	.00			
	5	5	.80	.80			
	Total	25	.44	.21			
TOTAL combined data for all four species	1	5	16.40	2.77	4	18.799	<.001
	2	5	1.80	.58			
	3	5	3.20	1.11			
	4	5	.60	.24			
	5	5	3.20	1.32			
	Total	25	5.04	1.32			

*** "Mean" refers to the mean number of individuals per trap for all trial combined. Columns are listed in Figure 4.2.**

b) Rows

Species	Row	n	Mean*	Std. Error	df	*F*	*p*
					Between Groups		
C. vicina	1	5	2.80	1.83	4	.065	.992
	2	5	3.40	2.93			
	3	5	4.40	3.41			
	4	5	3.20	1.10			
	5	5	3.20	1.77			
	Total	25	3.40	.97			
C. vomitoria	1	5	.20	.20	4	.810	.534
	2	5	.60	.24			
	3	5	.40	.40			
	4	5	.00	.00			
	5	5	.60	.40			
	Total	25	.36	.13			
L. caesar	1	5	1.00	.77	4	.063	.992
	2	5	1.00	.77			
	3	5	.80	.58			
	4	5	.80	.80			
	5	5	.60	.24			
	Total	25	.84	.27			
L. sericata	1	5	.00	.00	4	2.605	.067
	2	5	1.60	.81			
	3	5	.00	.00			
	4	5	.40	.40			
	5	5	.20	.20			
	Total	25	.44	.21			
TOTAL combined data for all four species	1	5	4.00	2.55	4	.108	.978
	2	5	6.60	4.34			
	3	5	5.60	4.38			
	4	5	4.40	1.78			
	5	5	4.60	1.94			
	Total	25	5.04	1.32			

*** "Mean" refers to the mean number of individuals per trap for all trial combined. Rows are listed in Figure 4.2.**

Table 4.8: Number of females and males blowflies collected in both the water trap and sticky trap experiments.

Water Traps	*C. vicina*	*C.vomitoria*	*L. caesar*	*L. sericata*	Total blowflies
Females	49	7	13	5	74
Male	36	2	8	6	52
Total male & female	**85**	**9**	**21**	**11**	**126**
Sticky traps	*C. vicina*	*C.vomitoria*	*L. caesar*	*L. sericata*	Total blowflies
Females	7	4	8	0	19
Male	3	1	0	1	5
Total male & female	**10**	**5**	**8**	**1**	**24**

4.3.2 Field Trial 2 – Sticky Traps

One field trial was conducted from the 1st to the 6th of October 2004. Only *C. vicina*, *C. vomitoria*, *L. caesar*, and *L. sericata* were recorded.

As with the water trap experiment performed in the spring, very few blowflies were collected (Table 4.9). The most abundant species of blowflies captured was *C. vicina*; however, all captures were low. The blue cards did not attract many other insects or arthropods; mostly Muscidae were collected.

Table 4.9: Total number of blowflies collected on the sticky traps experiments conducted in Little Hoos Field.

Treatment	*C. vicina*	*C. vomitoria*	*L. caesar*	*L. sericata*	Total
2-Heptanone	1	0	2	0	3
Dimethyl disulfide	1	0	1	0	2
Dimethyl trisulfide	4	1	2	0	7
Dimethyl tetrasulfide	0	0	1	0	1
Empty vial	2	0	1	0	3
Sodium sulfide + liver	2	4	1	1	8
Total per species	10	5	8	1	24

None of the chemical compounds tested had a significant effect on the capture of blowflies (Table 4.10). A positional bias was found; Trap number 6, positioned at the SE end of the field at a right angle (Figure 4.5), captured at least four times the amount of flies than any of the other traps, regardless of the treatment (Table 4.11). Traps at position number 4 did not capture any blowflies throughout the experiment.

Female blowflies were more prevalent than male blowflies in this particular experiment by at least three times (Table 4.8). A chi-squared analysis confirmed that there was an association between the catches of blowflies and the sex of the flies recovered from the sticky traps ($X^2 = 8.167$, d.f. = 3, $P < 0.05$).

4.3.3 Weather

Meteorological information was provided by the Rothamsted Research weather station. The amount of average rainfall, sunlight, wind run, and temperature to which the traps were exposed for each experiment are listed in Table 4.12.

Daily maximum and minimum temperatures were recorded during the water trap and sticky trap field experiments (Figure 4.6). The maximum temperature throughout the spring water trap experiments was 22.2°C while the minimum was 3.8°C. The maximum temperature recorded during the autumn sticky trap experiments was 16.2°C and the minimum was 5.2°C. During both experiments the weather was often cool with only short periods of sunshine and there was little shelter from the strong winds, especially in Little Hoos Field.

It was found that there were no significant correlations between the total number of blowfly individuals of all four (*C. vicina, C. vomitoria, L. caesar, L. sericata*) species combined and either mean rainfall ($r = -0.38$, $P = 0.80$, $n = 6$), mean sunshine ($r = -0.63$, $p = 0.18$, $n = 6$), mean wind run ($r = 0.74$, $p = 0.89$, $n = 6$) or mean temperature during each sampling period ($r = -0.32$, $p = 0.54$, $n = 6$).

Table 4.10: Results of one-way analysis of variance comparing blowfly species recovered from the sticky traps to the treatment used as bait.

Species	Treatment	n	Mean*	Std. Error	df	*F*	*p*
					Between Groups		
C. vicina	A	6	.17	.17	5	.528	.753
	B	6	.17	.17			
	C	6	.67	.67			
	D	6	.00	.00			
	E	6	.33	.21			
	F	6	.33	.21			
	Total	36	.28	.12			
C. vomitoria	A	6	.00	.00	5	.906	.490
	B	6	.00	.00			
	C	6	.17	.17			
	D	6	.00	.00			
	E	6	.00	.00			
	F	6	.67	.67			
	Total	36	.14	.11			
L. caesar	A	6	.33	.21	5	.222	.950
	B	6	.17	.17			
	C	6	.33	.21			
	D	6	.17	.17			
	E	6	.17	.17			
	F	6	.17	.17			
	Total	36	.22	.07			
L. sericata	A	6	.00	.00	5	1.000	.435
	B	6	.00	.00			
	C	6	.00	.00			
	D	6	.00	.00			
	E	6	.00	.00			
	F	6	.17	.17			
	Total	36	.03	.028			
TOTAL combined data for all four species	A	6	.50	.34	5	.517	.761
	B	6	.33	.21			
	C	6	1.17	.98			
	D	6	.17	.17			
	E	6	.50	.34			
	F	6	1.33	1.15			
	Total	36	.67	.26			

Treatments: A = 2-heptanone, B = dimethyl disulfide, C = dimethyl trisulfide, D = dimethyl trisulfide, E = sodium sulfide + liver (positive control), F = empty vial (negative control).

*** "Mean" refers to the mean number of individuals per trap for all trial combined.**

Table 4.11: Results of one-way analysis of variance comparing blowfly species recovered to the position of the sticky traps. Values highlighted in red are significantly different at 5%.

Species	Position	n	Mean*	Std. Error	df	*F*	*p*
					Between Groups		
C. vicina	1	6	.00	.00	5	2.872	.031
	2	6	.00	.00			
	3	6	.17	.17			
	4	6	.00	.00			
	5	6	.33	.21			
	6	6	1.17	.60			
	Total	36	.28	.12			
C. vomitoria	1	6	.00	.00	5	1.623	.184
	2	6	.00	.00			
	3	6	.00	.00			
	4	6	.00	.00			
	5	6	.00	.00			
	6	6	.83	.65			
	Total	36	.14	.11			
L. caesar	1	6	.17	.17	5	1.000	.435
	2	6	.17	.17			
	3	6	.17	.17			
	4	6	.00	.00			
	5	6	.33	.21			
	6	6	.50	.22			
	Total	36	.22	.07			
L. sericata	1	6	.00	.00	5	1.000	.435
	2	6	.00	.00			
	3	6	.00	.00			
	4	6	.00	.00			
	5	6	.00	.00			
	6	6	.17	.17			
	Total	36	.03	.028			
TOTAL combined data for all four species	1	6	.17	.17	5	3.391	.015
	2	6	.17	.17			
	3	6	.33	.21			
	4	6	.00	.00			
	5	6	.67	.33			
	6	6	2.67	1.26			
	Total	36	.67	.26			

*** "Mean" refers to the mean number of individuals per trap for all trial combined.**

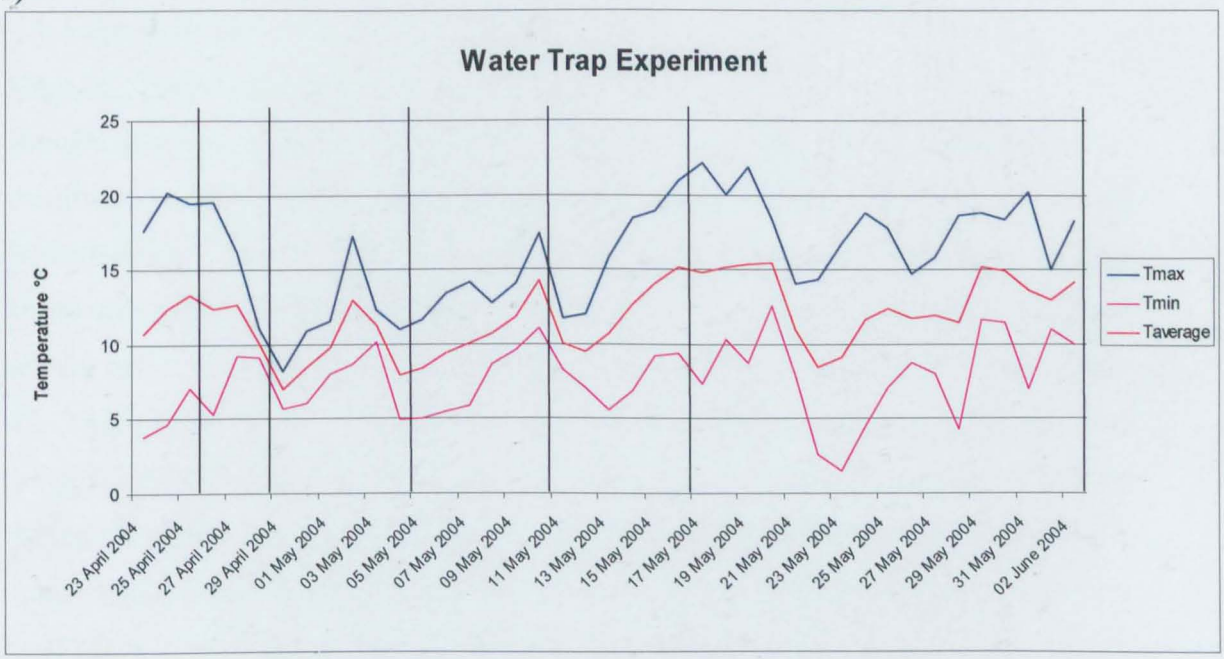
Table 4.12: Weather data - average daily calculations for each experiment.

Water Traps Date	Duration	Rain (mm)	Sun (hr)	Wind run (km)*	Temp (°C)	Total No. blowflies
23/04/04 - 26/04/04	3 days	0.0	11.4	110	12.23	4
26/04/04 - 29/04/04	3 days	5.1	4.3	328	9.92	4
29/04/04 - 05/05/04	6 days	6.1	3.6	204	9.81	34
05/05/04 - 11/05/04	6 days	0.5	1.5	216	11.07	62
11/05/04 - 17/05/04	6 days	0.0	9.7	134	12.84	19
17/05/04 - 02/06/04	16 days	3.2	5.7	172	12.81	3
Sticky Traps Date	Duration	Rain (mm)	Sun (hr)	Wind (km)	Temp (°C)	Total No. blowflies
01/10/04 - 06/10/04	6 days	4.1	4.0	258	11.58	24

Weather data provided by Rothamsted Research.
* "Wind run" refers to the total distance that air (wind) has travelled past the measuring point during a 24hr period.

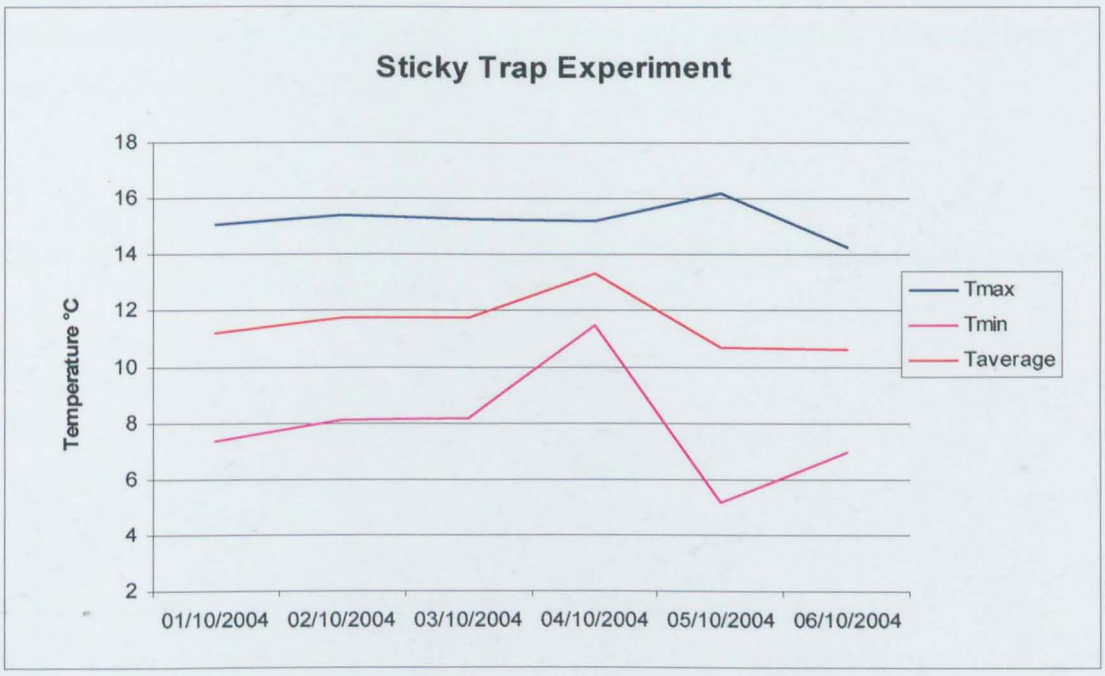
Figure 4.6: Daily minimum and maximum temperatures for the water trap (a) and sticky trap (b) experiments.

a)



T = temperature. Vertical lines indicate the dates that insects were collected and the baits were replenished.

b)



T = temperature.

4.3.4 Bait Release Rates

Control - Fume Cupboard:

Release rates are shown in Figures 4.7. The weight of the vial containing dimethyl disulfide decreased consistently and appeared empty on day 6 (28/04/04) of this experiment yet there was still a strong odour present. As the most volatile of the five compounds tested, dimethyl disulfide was found, not surprisingly, to have the highest release rate (16.58mg/day). The vial containing propyl butyrate appeared empty on day 27 (19/05/04) of this experiment. Dimethyl tetrasulfide was the heaviest (molecular weight) of the compounds tested and the only one to exhibit an increase in weight during the period of exposure. One of these increases was recorded on day 6, when the fume cupboard was off for 3-5 hours during a power failure at the laboratory. Moisture could have been absorbed during this time.

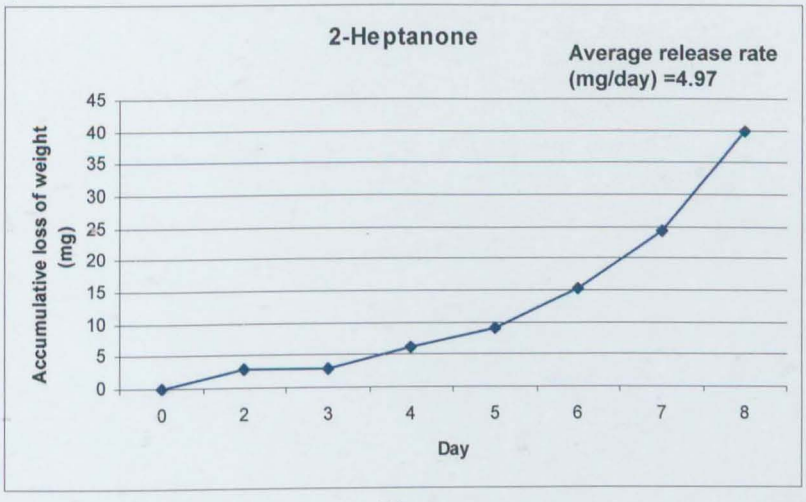
Experimental – in the Field:

Water and debris were found inside the Choromacol vials and on the lids, therefore, the release rates could not be determined. A dead 1mm fly (unidentifiable) was found inside the vial containing propyl butyrate. The empty vial placed in the field was found to collect water (increased weight) at times of rain and lost some of this water through evaporation (loss of weight) when the weather was warmer and the sun was out. As the

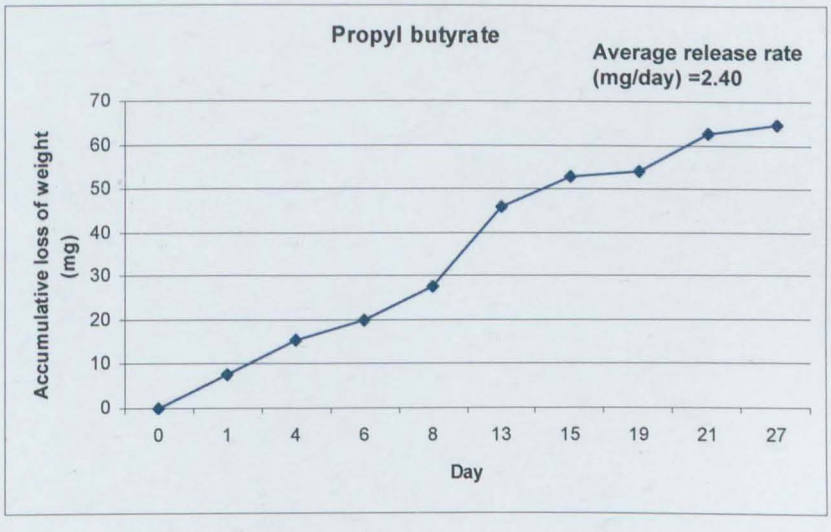
vials used in the traps were changed regularly, these were not as affected by rain and debris and therefore, the compounds were likely to be successfully diffusing from the vials.

Figure 4.7: Release rates of compounds in 800µL Choromacol vials with a 1.1mm hole in the lid. Tests were carried out in a fume cupboard.

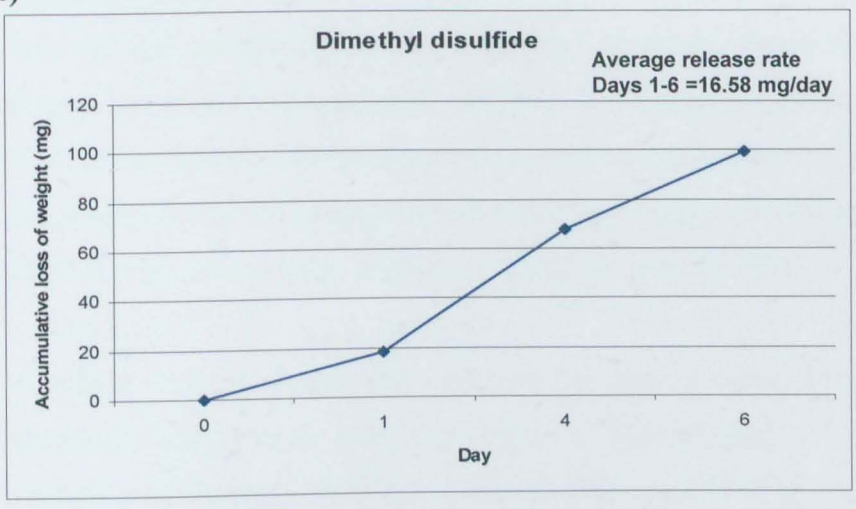
a)



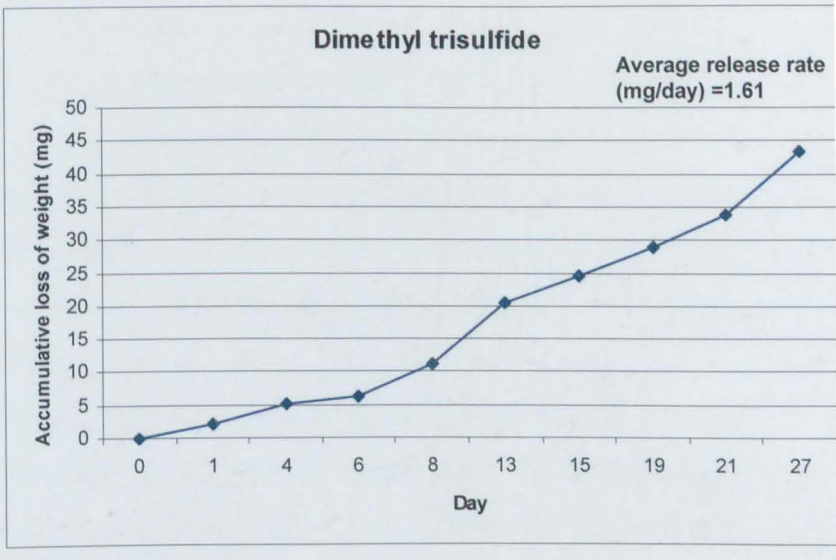
b)



c)


Dimethyl disulfide
Average release rate
Days 1-6 =16.58 mg/day
Accumulative loss of weight (mg)
120
100
80
60
40
20
0
0
1
4
6
Day

d)


Dimethyl trisulfide
Average release rate
(mg/day) =1.61
Accumulative loss of weight (mg)
50
45
40
35
30
25
20
15
10
5
0
0
1
4
6
8
13
15
19
21
27
Day

e)


Dimethyl tetrasulfide
Average release rate
(mg/day) =0.33
Accumulative loss of weight (mg)
10
8
6
4
2
0
-2
0
1
4
6
8
13
15
19
21
27
Day

4.4 DISCUSSION

None of the compounds tested – dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, propyl butyrate, and 2-heptanone – caught a significantly greater number of blowflies than the unbaited control traps. This could have been as a result of single compounds being used instead of mixtures, the trapping technique, the concentration of the compounds, the trapping site, or a combination of these.

Nilssen *et al.* (1996) found that, at the receptor level, the reindeer oestrids, *Hypoderma (Oedemagena) tarandi* and *Cephenemyia trompe* (Diptera: Oestridae), reacted positively to dimethyl trisulfide; however, during flight trap experiments these were only caught in small numbers. To the contrary, *Hydrotaea anxia* (Diptera: Muscidae), *Protophormia terraenovae*, *C. vomitoria*, *C. uralensis* and *C. loewi* (Diptera: Calliphoridae) were all captured in significant numbers during their field experiments. Similarly to the reindeer oestrids, *C. vomitoria* showed an EAG response to dimethyl trisulfide but were not caught in large numbers during this chapter's water or sticky trap experiments. However, as Nilssen *et al.* (1996) did report larger catches of *C. vomitoria*, it is possible that the difference lies with the trapping methods.

Dimethyl disulfide is an important compound of the chemical attractant Swormlure-4, developed to more efficiently trap the New World screwworm fly, *Cochliomyia hominivorax* (Diptera: Calliphoridae) (Mackley & Brown, 1984). When dimethyl disulfide was used alone in the water trap and sticky trap field experiments, however, no strong attraction was noted to the traps.

Cossé & Baker (1996) conducted wind tunnel behavioural assays with the housefly, *Musca domestica*, using EAG-active compounds (including dimethyl tetrasulfide and dimethyl trisulfide) resulting in no significant difference in the number of individuals attracted between the single compounds and the control (water). When combined together, however, the results were quite different; these blends attracted *M. domestica* in a similar manner as pig manure, a highly attractive bait. Ashworth & Wall (1994) found that both dimethyl disulfide and ethanethiol were attractants for *L. sericata* when mixed with each other or with hydrogen sulfide; however, neither was effective alone.

While a number of research experiments concluded that blends were more efficient in attracting certain Diptera (Cossé & Baker, 1996; Du & Millar, 1999; Muirhead-Thomson, 1991), some studies found that *M. domestica*, during field experiments, were attracted to single compounds, such as indole, 3-methyllindole, and butanoic acid (Brown *et al.*, 1961; Frishman & Matthysee, 1966; Mulla *et al.*, 1977). Smart & Blight (1997), when testing the affect of oilseed rape odours on *Ceutorhynchus assimilis* (Coleoptera: Curculionidae), found that three of the seven single compounds (selected from EAG studies: 3-butenyl, 4-pentenyl, and 2-phenylethyl isothiocyanate) were capable of luring these beetles to the traps. The complexity of preparing insect baits based on EAG studies is demonstrated by the responses of the New World screwworm fly, *Cochliomyia hominivorax*, to various blends of synthetic odour bait (Cork and Hall, 2007).

In some cases, the concentration of the chemical baits used was an important factor in the response of flies to a particular trap. Du & Millar (1999) found that, in high concentrations (10µg/litre), compounds present in Bermuda grass became repellents to *Culex quinquefasciatus* and *C. tarsis* (Diptera: Culicidae). When an excess of 0.5mg h^{-1} octenol was added to ox odour, Muirhead-Thomson (1991), recorded that tsetse flies, *Glossina sp.* (Diptera: Glossinidae), were strongly attracted to the mixture, yet when the dose was increased to 500mg h^{-1} it became a strong repellent. However, the opposite effect was found when using a combination of ox, other cattle, sheep, goats, donkey, and buffalo urine as bait. This did not have a repellent effect; tsetse fly catches consistently increased as the concentrations of these natural host odours also increased.

Bacterial decomposition in animal proteins results in the formation of hydrogen sulfide. Similarly, during the breakdown of sodium sulfide in water, hydrogen sulfide gas is emitted forming a strong attractant for blowflies (Fisher *et al.*, 1998). For this reason a water diluted sodium sulfide and liver mixture is often used to attract *Lucilia sericata*. Ashworth & Wall (1994), Hall (1995), and Wall & Fisher (2001) have reported success while using this combination; in fact, Fisher *et al.* (1998) found that fresh liver combined with sodium sulfide acted similarly to aged liver. From this data the control bait, used in the field experiments conducted in 2004, should have yielded much larger catches of blowflies, in particular *L. sericata*. This does put in question the suitability of the traps and possibly the site.

In field trapping experiments, *L. sericata* has been recorded as preferring traps of a yellow colour by Wall *et al.* (1992) and those of a black colour by Hall *et al.* (1995). Visual cues are used to select a final landing site; therefore, in many species this plays an important role (Wall & Fisher 2001; Muirhead-Thomson, 1991). The yellow bowl attracted many insects not of interest to this research, such as Hymenoptera and Coleoptera. For this reason the blue cards were used in the sticky trap experiments as much fewer non-target species were attracted to this colour.

The blowflies tested in these experiments rely heavily on odour cues, especially at long range (Ashworth & Wall, 1994; Archer & Elgar, 2003). Spivak *et al.* (1991) suggest that flies which arrive to a resource (host, food source, etc.) earlier than other colonizers rely mainly on olfactory stimuli emitting from this resource, whereas the orientation of later arrivals may depend on the chemical cues initially and then on visual information (i.e. a cluster of feeding flies) to locate a resource more precisely (Spivak *et al.*, 1991). As blowflies generally attend the body in great numbers, forming large groups of flies and larval masses, the odours released and visual cues present may ultimately alert other blowflies to the carrion. The visual information may take place with the later colonising carrion insects on a decomposing body; however, the blowflies first to arrive on a carcass clearly do not rely on the presence of other carrion insects. The small numbers of flies captured in these studies likely would not have offered these cues.

Column 1 of the water trap experiments attracted more blowflies possibly because it was closest to the line of trees and offered protection from the wind. The bias for position 6, in the sticky trap experiment, was perhaps due to its closer proximity to the tall grass and bramble bushes where shelter is provided for the blowflies. Even though there was a positional bias, this did not have an influence on the results of the treatments because of the randomised layout and rotation of the traps.

Design is vital to any experiment. Du & Millar (1999) demonstrated this by showing that two experimental designs, using the same odour cues, presented very different results when calculating the attraction of *C. quinquefasciatus* and *C. tarsalis* to compounds found in Bermuda grass. Therefore, the species of fly that is targeted will influence the type of trap required.

Advantages and disadvantages of the water and sticky traps used for this study were noted. Both trap types were very inexpensive and simple to construct. The arthropods collected from the water traps were easier to identify because they were not covered in glue as those collected on the sticky cards. Those trapped on the sticky cards were left *in situ* and frozen. Certain features were difficult to reach and damage to the insect during the identifications was common, even with the use of a solvent. By far the biggest disadvantage of all, discovered in the water traps, was due to the large collections of non-target and potentially beneficial insects, such as certain Coleoptera and Hymenoptera.

During both experiments, female blowflies were caught more often than male blowflies, although this difference was only statistically significant during the water trap experiment. It has not yet been determined if the difference between male and female blowfly captures between the water trap and sticky trap experiments was seasonal or due to the different types of traps. It is possible that the autumn experiment attracted females perhaps looking to oviposit as, this late in the season, the immature offspring could over-winter as pupae (Rognes, 1991; Pitts & Wall, 2005). It has been well documented that the level of oocyte maturation of a female fly will affect the rate at which they land on a target (Campan, 1977; Muirhead-Thomson, 1991; Wall & Fisher, 2001). It is also possible that there are higher overall fly populations in the autumn and that female flies live longer than male flies. Further research on the effect of the bait used in these experiments and the time of year would provide more insight into the differences in female and male fly catches.

Environmental variables did not influence the number of blowflies captured during these trap experiments. The daily average temperatures (9.81-12.84°C) were within the activity threshold of the blowflies (Faucherre *et al.*, 1999), therefore, should not have restricted access to the traps on any day of the research, even though average hours of sunlight per day was often low. While wind speed factors exceeding $2.5ms^{-1}$ depressed catches of *L. cuprina* (Muirhead-Thomson, 1991), this does not seem to have been the case with these water trap and sticky trap experiments.

Sticky trap experiments conducted by Fisher *et al.* (1998) trapped over 200 female *L. sericata* over July and August of 1994, and an even greater number in 1997. The

catches made were considerably greater than the catches recovered in these experiments (24 blowflies). Differences in these experiments and those conducted by Fisher *et al.* (1998) include differing seasons and field location, as well trap design. Fisher *et al.* (1998) used larger sized sticky traps (41cm × 41cm), compared to the sticky cards used in these experiments (20cm × 20cm).

It is clear that the techniques used here need to be re-evaluated. A new trap could be designed, though it also seems appropriate to test combinations of the EAG-active compounds; in combinations and concentrations resembling the different stages of pig decomposition. Although, as the positive control was itself not successful, it does cast doubt on the efficiency of the traps to catch and retain flies attracted to the bait. The size of the darkened 800µL Choromacol vials with a 1.1mm hole drilled into the lid may not have allowed enough volatiles out to attract a large number of blowflies from longer distances; a larger bait container and bigger opening should be used in future studies.

Other possibilities of research include Y-tube experiments (Logan, 2005). A wind tunnel of adequate size to conduct tests on blowflies and to allow for flight track analysis, would be a reasonable next step as Cossé & Baker (1996) did have a degree of success with this technique when conducting experiments on the house fly, *M. domestica.*

In these experiments, it was not possible to calculate how many blowflies were attracted to the vicinity of baited traps, only how many actually landed on the water or sticky surface. New trapping methods could be explored, such as baited electrified field nets, which would leave little chance for the flies to escape capture (Torr & Hall, 1992; Green *et al.*, 1993; Hall *et al.*, 1995).

Trapping experiments have been conducted on *L. cuprina*, *L. sericata*, *C. hominivorax*, and other blowflies presenting health, and economic implications (Mackley & Brown, 1984; Muirhead-Thomson, 1991; Fisher *et al.*, 1998; Smith & Wall, 1998). *Calliphora vicina* and *C. vomitoria* are not regarded as great pests; therefore, there is considerably less information available on trapping preferences. As the field of Forensic Entomology grows, more information regarding these previously overlooked blowflies

will be required. Baited trap designs and techniques are endless; I look forward to discovering a suitable method of trapping these blowflies and testing semiochemicals to their full capacity.

4.5 SUMMARY

Field trapping is a popular method of conducting Bioassay experiments. However, while this is popular with many economically important insect species, such as *Ceutorhynchus assimilis* (Coleoptera: Curculionidae), *Anopheles gambiae* (Diptera: Culicidae), *Simulium arcticum* (Diptera: Simuliidae), *Lucilia sericata*, very little testing has been done to further understand the chemical attractants of certain blowflies such aš *C. vicina, C. vomitoria* in their natural environment.

The previous chapters demonstrated that blowflies, such as *Calliphora vomitoria*, *C. vicina*, *L. sericata*, and *L. caesar* (Diptera: Calliphoridae), were attracted to a decomposing pig early in decomposition. Volatiles collected from a decomposing pig combined with electrophysiology studies tentatively identified EAG-active compounds (dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, propyl butyrate, and 2-heptanone) which may attract the blowflies, specifically *C. vomitoria*, to the decomposing corpse. Therefore, the aim of this chapter was to further understand the findings by testing blowflies in the field to determine their attraction to the EAG-active compounds.

Water trap experiments were conducted in the spring of 2004 using yellow bowls placed in a randomised (Latin Square) design. Each trap contained one of the EAG-active compounds as bait, including an empty vial as a negative control. Six separate experiments had catches totalling only 126 blowflies. A large number of non-target insects, such as Hymenoptera and Coleoptera, were captured.

A sticky trap experiment was conducted in the autumn of 2004. Blue adhesive cards were used to avoid capturing many non-target insects. The traps were moved by one position each day to minimise the effects of a positional bias. Along with the EAG-active compounds and empty vial, a mixture of liver and sodium sulfide was used as positive control in the sticky trap experiment. This sodium sulfide and liver mixture

was also applied with a water trap in the autumn to test the efficacy of the trap. A total of 24 blowflies were captured during this the sticky trap experiment.

None of the chemical compounds tested, in either trap study, was found to have a significant effect on the capture of blowflies whether by individual species or combined. Not even the positive control, which is known to attract and capture *L. sericata* in large numbers, successfully captured a significantly greater amount of blowflies than the EAG-active compounds or the negative control. During both trap experiments there was a positional bias to those traps closest to the trees or tall grass. However, this did not affect the results due to the randomised layout of the traps. *C. vicina* was more prevalent in the water trap catches, while female blowflies were captured significantly more often than males during the sticky trap experiments. No correlation was found between the number of blowflies captured and meteorological parameters such as the rain, sunshine, wind speed, and daily temperature.

In conclusion, it is clear that a new trapping method needs to be designed in order to better capture blowflies. While the blowfly captures were still low, the blue sticky traps were preferred by this author because they attracted very few non-target insects. Blends of the EAG-active compounds also need to be explored in the future.

CHAPTER 5 – SUMMARY AND CONCLUSIONS

The chief aims of this study were to identify the insects associated with decomposition, whether the presence of these was linked to the physical state and/or the volatiles released from the decomposing pig carcass, and to determine which chemicals initially lure the adult Dipterans, specifically *Calliphora vomitoria* (Diptera: Calliphoridae), to the carcass. Particular emphasis was placed on the isolation and identification of those chemical compounds associated with decomposition, triggering an electrophysiological response from *C. vomitoria* as such links between the decomposing body and blowflies had never been examined.

Given that this research was novel and many of the studies had never been performed, many different aspects concerning decomposition, insect succession, volatile collection, electrophysiology technique, and behavioural experiments had to be explored in order to proceed with the investigation. The full progression of decomposition of the carrion was observed, the insects on the carcass were noted as well as their behaviour once on the body, volatiles were collected and utilised to detect the physiological response of *C. vomitoria* to semiochemicals. Those that elicited a response were identified, and finally bioassay experiments were undertaken to give a complete picture of the effects of decomposition on carrion insects in order to develop methods for further research and, potentially, the use of insect's natural behaviour to our benefit in the future.

Insect Succession on Decomposing Pigs

A total of five pig carcasses were exposed for the purpose of conducting decomposition and insect succession studies. In each case, Muscidae and Calliphoridae adults arrived at the carcass immediately after the plastic covering was removed from the body (Chapter 2). Within hours eggs were laid in the nostrils, mouth, and on top of the head at the wound site where the pigs were shot. Successful rearing of the first eggs laid revealed that *Calliphora vicina* (Diptera: Calliphoridae) were the first flies to lay eggs on the carcasses in both summer experiments while *C. vomitoria* were the first to lay eggs during the autumn experiment. *Calliphora sp.* and *Lucilia sp.* often

laid eggs in clumps in the same location while Muscids oviposited individual eggs away from the Calliphorid egg masses.

Adult and immature insects on the carcass were monitored to determine whether "waves" or successions of insects could be confirmed. This presence of "waves" on a decomposing body was originally described by Megnin (1894), who produced the first detailed list of insect succession. While no clear "waves", or more specifically distinct blocks with the presence of only certain species of insects, were noted, it was found that certain insects did dominate during specific stages of decomposition. Many of the insect families could be found, at least in small numbers, during all stages of decomposition. In fact, factors such as the insect species composition, their abundance, the presence of the adult and immature stages, and their behaviour on the food source all needed to be considered when describing the succession of insects. It is important to note that the insect succession found in the Derbyshire region of England will not necessarily agree with similar research undertaken elsewhere, even within the English Midlands. Succession data is very specific to geographical location and temperature (season), which can further be affected by other meteorological parameters (Johnson & Villeneuve, 1897; Erzinçlioğlu, 1983; Archer, 2004). Therefore, it is important to undertake studies as close to the site of a suspicious death as possible to be able to interpret the insect succession on the body.

The temperature inside the carcass was consistently higher than the ambient temperature, especially on cooler days, due to insect activity and shelter from the wind and rain by the remaining leathery skin (Chapter 2, Section 3.4). Larval masses maintained near optimal temperature for their development by remaining in constant motion; the larvae inside the mass would move out while the larvae at the edge of the mass would move deeper into the mass. This activity was seen both on warm and cool days. On cool days the constant motion served to warm the larval mass, while during warm days the cooler larvae on the outside of the mass moved inside while the warmer larvae moved out for cooling.

Rain had a great effect on the behaviour of flies and the larvae. During periods of rain, adult flies would not visit the carcass; however, they would slowly reappear once the rain had ceased. Larvae, on the other hand, would emerge from inside the body and

glide in straight lines across the wet skin. Third instar larvae would make mass exodus from the carcass to find a suitable site to pupate. This particular behaviour may have come about since rain offers more suitable conditions for the larvae to migrate, *i.e.* the larvae are less likely to dehydrate, it is easier to burrow into the soil, and they are less likely to be predated by birds. However, heavy rain dislodged clumps of eggs from the carcass and lowered the temperature of the body. Such considerable changes in the behaviour of insects are important to note as larval migration and temperature of the body were largely dictated by weather conditions such as rainfall and sunshine.

The stages of decomposition described in Chapter 2 (Fresh, Bloated, Active Decay, Advanced Decay, and Dry) were all easily identifiable to the trained eye; however, the relative duration of each stage differed greatly in all three experiments due the varying weather conditions and resulting temperature. Very different weather conditions, such were experienced in the two summer experiments (Experiment 1, 2002 & Experiment 3, 2003). The pigs exposed in summer 2003 decomposed and progressed through the stages of decomposition much faster than the pig exposed in the summer 2002, due to warmer ambient temperature and fewer days of rainfall. Experiment 2, conducted in the autumn of 2002, saw the coolest temperatures of all and the pig exposed then spent the longest time in the Active Decay stage. The progression of the decomposition was not followed past this third stage.

In Experiment 1, Silphid beetle larvae had an effect on the number of fly larvae present on the carcass. At their peak, the number of *Necrodes littoralis* (Coleoptera: Silphidae) larvae and their mass feeding on other larvae greatly reduced the number of Dipteran larvae (Chapter 2, Section 3.1) on the carcass. This amount of predation could have had an affect on decomposition by reducing the rate of tissue removal by Dipteran larvae.

Electrophysiology studies using *Calliphora vomitoria*

Odour samples were collected from the decomposing pig, using Porapak and Tenax tubes, in order to identify the volatiles which elicited a receptor response from *C. vomitoria* during electrophysiology (EAG) studies described in Chapter 3. Coupled gas chromatography (GC) and EAG experiments revealed that *C. vomitoria* reacted most strongly and repeatedly to four compounds in the volatile mixtures. These four EAG-

active compounds, or semiochemicals, were identified as dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, and KI 881-885 (tentatively identified as propyl butyrate or 2-heptanone). These appeared early on in decomposition with elevated concentrations detected during the Bloated and Active Decay stages, and then greatly diminishing during the later stages of decomposition.

During the later stages of decomposition, including Advanced Decay and Dry stages, other compounds such as phenol, indole, tetradecane, and pentadecane, became more prominent while the concentrations of the four EAG-active compounds decreased significantly. Even though the later occurring compounds were present in high concentrations, none triggered a response from *C. vomitoria* during GC-EAG experiments, demonstrating that this species only responds to specific compounds.

While the five stages of decomposition were determined largely on visual characteristics – physical conditions such as colour of the skin and the amount of flesh remaining on the body – it was found that the chemical composition and concentration of the volatiles associated with the decomposing pig changed in a way that closely followed these decomposition stages (Chapter 3, Section 3.3). Differences in the chemicals present, in specific the four EAG-active compounds, and the different concentrations varied between the different stages of decomposition, suggesting that many of the physical changes to the carcass have an important effect on the chemicals produced surrounding the carcass and consequently an effect on the attraction of insects to the carcass.

The Active Decay stage differed in chemical composition and concentration from all other decomposition stages with the increased occurrence of dimethyl tetrasulfide and dimethyl trisulfide having the greatest influence on this result. Visual observations of the pigs supported these findings in that: 1) there were a much greater number of Calliphoridae, both adult and immature, on the carcass at this stage and 2) the greatest physical changes to the pigs were noted during this time.

Dose response experiments using EAG, for the purpose of determining the response threshold of *C. vomitoria*, revealed that dimethyl trisulfide and dimethyl tetrasulfide were significantly different from the negative controls, diethyl ether and hexane. These

sulfide compounds have been previously identified in raw chicken (Senter *et al.*, 1999; Miller & Horn, 2001), ox faeces (Ding *et al.*, 1998), and pig manure (Cossé & Baker, 1996). These, along with dimethyl disulfide, indole, and 3-methylindole have also been found to evoke a physiological and behavioural response from other Dipterans such as *Musca domestica* (Cossé & Baker, 1996).

Within the volatile samples collected using Tenax tubes, three days were found to exhibit a sudden high concentration of dimethyl trisulfide: days 5&6 (Bloated) and day 16 (Active Decay stage). These days of high concentration were concurrent with higher temperature recordings in the mouth and rectum of the decomposing pig. According to Vass *et al.* (2004), the production of sulphur compounds is dependent on temperature. Therefore, it could be assumed that the sulphur concentrations are elevated during warmer days or during times of high larval activity in the carcass, thereby attracting more *C. vomitoria* adults. It is also possible that the levels of sulphur compounds are more elevated during the daytime, rather than the cooler evening hours.

Both Porapak Q and Tenax TA polymer resin were used for the collection of decomposing pig volatiles. Porapak collections provided a liquid sample that could be used for confirming the identity of a compound through co-injections in different GC columns, quantification of the compounds in the sample, conducting electrophysiology experiments, and could also be stored for use at a later (Angelopoulos & Pickett, 1998). Tenax TA was analysed through thermal desorption, eliminating the use of a solvent, and only required a short entrainment time. Both methods, however, presented clear problems possibly critical to the research. The solvent present in the Porapak samples covered many peaks with a similar retention time, masking important compounds in the sample. The longer entrainment time, required of Porapak samples, potentially caused more disruption with the organism being entrained. Tenax samples, however, could only be used once, eliminating the possibility of quantification or confirmatory identifications, nor could the samples be used for GC-EAG experiments. Employed together, however, these two different sampling methods offered the data required in the experiment and led to a thorough investigation. Therefore, the findings made by this research strongly indicate that both methods should be used in future studies.

Field Trapping Studies

Electrophysiology studies are not a determinant of behaviour but simply indicate whether or not there is a receptor response from a particular insect to a single chemical within a group of compounds (Du & Millar, 1999). Field trapping experiments were therefore conducted to determine the behaviour of blowflies to the individual EAG-active compounds identified during GC-EAG experiments (dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, propyl butyrate, and 2-heptanone). Two trapping methods were selected to carry out the field experiments, water traps and sticky traps (Chapter 4, Section 2).

Water trap experiments were conducted in the spring and consisted of a yellow bowl, a detergent solution to trap the blowflies, and the bait. A total of six experiments were performed; however, the blowfly catches were very low (Chapter 4, Section 3.1). The blowflies made-up only the minority of insects caught in the water traps. Many non-target and beneficial insects were captured in the water traps; Hymenoptera (primarily Apidae and Vespoidea) and Coleoptera (mostly Curculionidae and Nitidulidae) made up the majority of the insects captured. These non-target insects were attracted to the yellow colour of the bowl (Blight *et al.*, 1992; Greggers & Menzel, 1993), a typical response of phytophagous insects (Smart *et al.*, 1997).

In the autumn, a sticky trap experiment was conducted in order to capture blowflies attracted to the bait. Blue sticky cards were used to avoid large captures of non-target insects. While they succeeded in not capturing many non-target insects, these also failed to capture a large number of blowflies (Chapter 4, Section 3.2).

Neither of the trapping methods succeeded in collecting a large amount of blowflies and none of the compounds tested caught a significantly greater number of blowflies than the unbaited traps. The traps holding the mixture of sodium sulfide and pig liver (positive control) also captured very few blowflies. This combination of liver and sodium sulfide mixture has been successfully used by other authors to trap blowfly *Lucilia sericata* (Diptera: Calliphoridae) in large numbers (Ashworth & Wall, 1994; Hall, 1995; Hall *et al.*, 2003; and Wall & Fisher, 2001). This information suggests that the trap designs employed in the present study were ineffective or that the blowfly populations at the chosen site were below statistically acceptable levels for this study.

While some trials indicate that single compounds can lure insects (Brown *et al.*, 1961; Frishman & Matthysee, 1966; Mulla *et al.*, 1977; Smart & Blight 1997), other studies report that a blend of compounds is more successful at luring insects (Ashworth & Wall, 1994; Cossé & Baker, 1996; Du & Millar, 1999). Though the experiments did not capture many blowflies, a lot was learned from the trials. In addition to newly designed traps, future studies will explore the use of blends of semiochemicals matching the concentrations of those samples recovered from the pig at different stages of decomposition.

Conclusion and Final Thoughts

In conclusion, within this body of research, it was discovered that decomposing pig carcasses do emit volatiles that trigger an electrophysiological response from *C. vomitoria*, supporting the initial theory that blowflies are attracted to a decomposing body by odour (Ashworth & Wall, 1994; Nilsen *et al.*, 1996; Castner, 2001: Archer & Elgar, 2003). As decomposition progresses and the physical state of the pig changes, so do the volatiles. The volatiles associated with the corpse will change in composition and in concentration, almost certainly affecting the number and species of carrion insects on the body. The succession of insects is not demonstrated by non-overlapping groups of insects but rather a combination of factors leads to a more ill-defined but very evident change in species composition on the corpse. Temperature and rain have a great affect on the rate of development and behaviour of larvae, which stresses the need for consideration of effects of weather when considering the behaviour and development of Dipteran larval infestation; a view shared by Higley & Haskell (2001) and Wardhaugh *et al.*, (2007). *C. vomitoria* reacts to compounds present early in decomposition and not to those increasing in concentration during the later stages of decay. Insects appearing during the later stages of decay, such as Sepsidae, might not exhibit an electrophysiological response to dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, or KI 881-885, but instead respond to the later occurring compounds such as phenol, indole, tetradecane, and pentadecane. Further research would clarify this matter.

The field of Forensic Entomology is ever growing and expanding. As it is used more often in criminal cases (Benecke, 2001), further details will need to be understood about the semiochemicals which attract carrion insects to decomposing bodies in order

to help determine exactly when the first flies were attracted to the corpse. Identifying the exact semiochemicals or blends of these that initially attract blowflies, specifically gravid females, to the decomposing corpse and identifying exactly when these compounds are emitted from the body will need to be established. Sulphur compounds, which are released early during decomposition (Vass *et al.*, 2004) and were found to trigger an electrophysiological response from *C. vomitoria*, would be the focus of worthwhile research.

In an effort to develop new techniques which will locate concealed bodies, Smedts (2004) found that pigs and humans share chemical markers used to detect buried bodies; this includes dimethyl disulfide. Such similarities between pigs and humans is encouraging, as the new discoveries made during this doctoral research could hopefully lead to examinations on humans and the development of new more accurate techniques for determining the post mortem interval.

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APPENDIX 1: Experimental Sheets used when making observations and recordings.

FORENSIC ENTOMOLOGY EXPERIMENT NO.3

Time: _____ **Initials:** _____ **Date:** _____ **Day:**_____

Weather: ☐ Raining ☐ Sunny
☐ Cloudy ☐ Hot Sun
☐ Partially Cloudy ☐ Other: _____

Estimated State of Decomposition:

☐ Fresh: No strong smell and few changes to body

☐ Bloated: Bloated appearance, marbled skin, many flies, beginning to smell

☐ Active Decay: Deflation of body, flesh still present, still wet, skin starting to go black, very strong smell

☐ Advanced Decay: Much of the flesh has been removed

☐ Dry: Bones, cartilage and some skin, very little odour, small flies and beetles

Notes:

_____

_____

_____

Temperatures (°C): Time: _____ Weather: _____
Sub-soil _____ Soil surface_____ Level of pig _____ Over pig _____
1.0 metres _____ 2.0 metres _____ Pig skin_____ Inside pig_____
Maggot masses _____ Other: _____
Exudate: surface_____ just below (4cm)_____

Insects observed (Preferably in order of most to least common): Time: _____

Pig:_____

_____

_____

_____

_____

_____

_____

Notes:

See back of page for extra notes

Air Entrainment:

1) Date:_____ **Day:**_____ **Tenax®** or **Porapak®**

Start:_____ **End:** _____ **Pull (mL/min):** _____

Notes:_____

2) Date:_____ **Day:**_____ **Tenax®** or **Porapak®**

Start:_____ **End:** _____ **Pull (mL/min):** _____

Notes:_____

APPENDIX 2

ONSET Computer Corporation, Bourne, Massachusetts, USA

Software: BoxCar Pro.

HOBO Pro Temp/External Temp Data Logger

Part # H8-031-08

Two-channel logger with internal temperature sensor and external soil/water temperature probe on 2-meter (6-foot) cable.

Key Specifications

Internal Temperature Measurement Range: -30° to 50°C (-22° to 122°F)

- External Temperature Measurement Range: -40° to 100°C (-40° to 212°F)
- Temperature Accuracy: ±0.2° at 21°C (±0.33° at 70°F)

HOBO H8 4 External Channel Data Logger

Part # H08-006-04

Four-channel logger accepts a wide range of external sensors and cables for Temp, CO2, AC Current, 4-20mA, and DC Voltage.

Key Specifications

32K readings

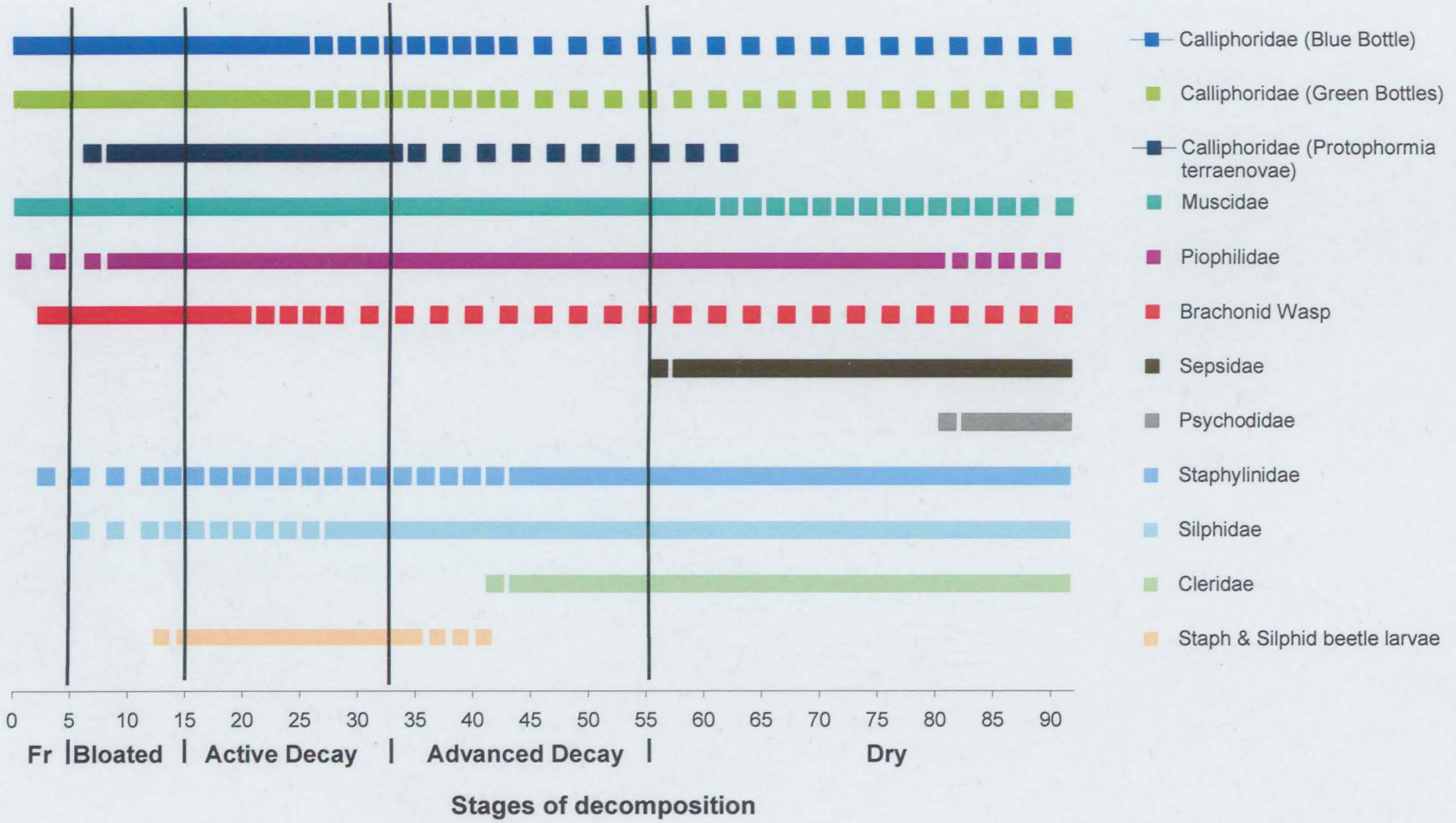
- External Input Channel Measurement Range: 0-2.5 DC Volts (See External Sensors)
- External Input Channel Accuracy: ±10 mV ±3% of reading

APPENDIX 3:

A list of the adult insects (with the exception of Staphylinid and Silphid larvae) observed during the succession experiments; however, this does not give an indication the specific abundance. The solid lines indicate consistent presence of a particular group of insects while the broken lines indicate an occasional presence.

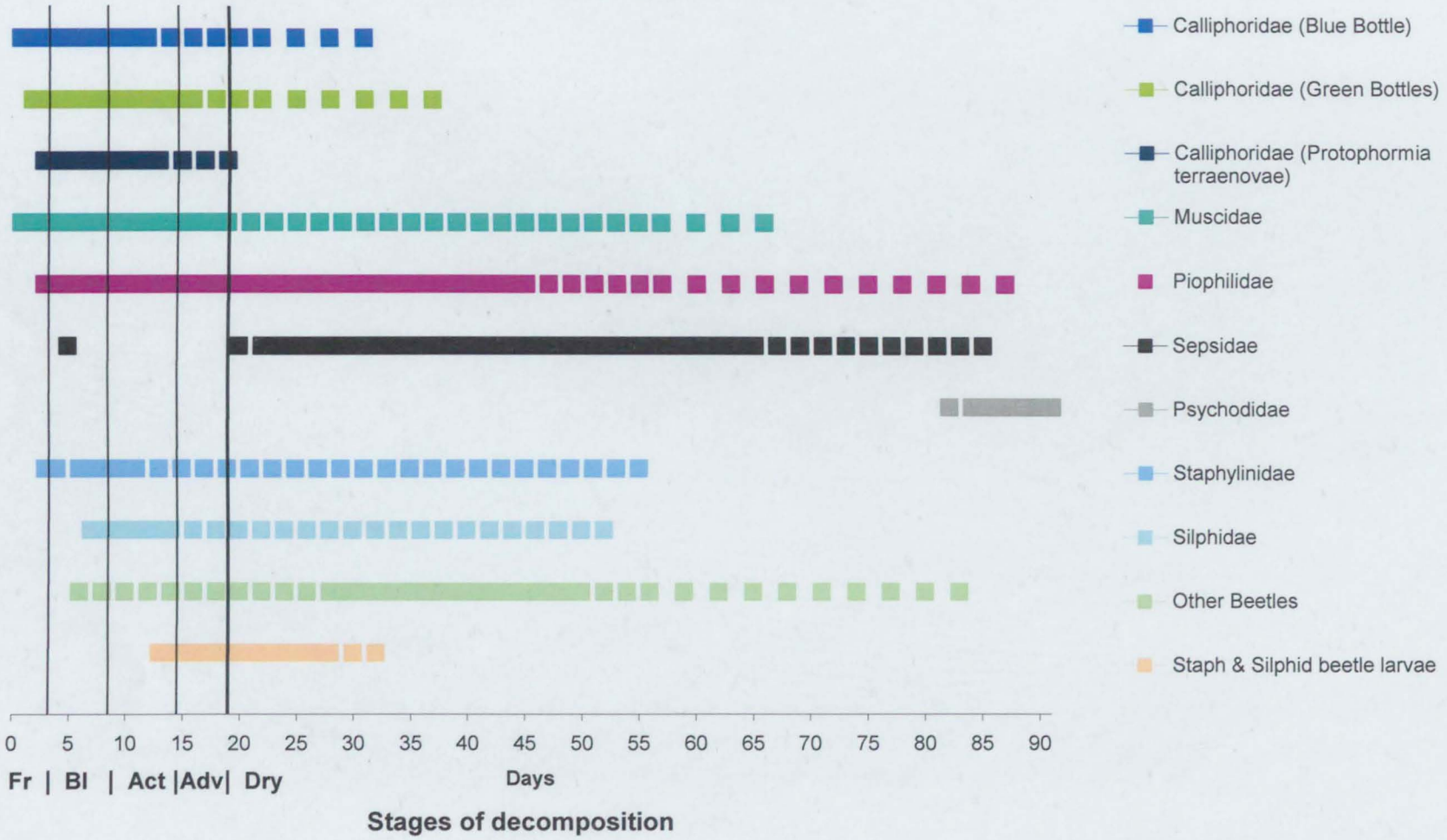
See next page

Experiment 1 – Summer 2002



Fr = Fresh stage

Experiment 3 – Summer 2003

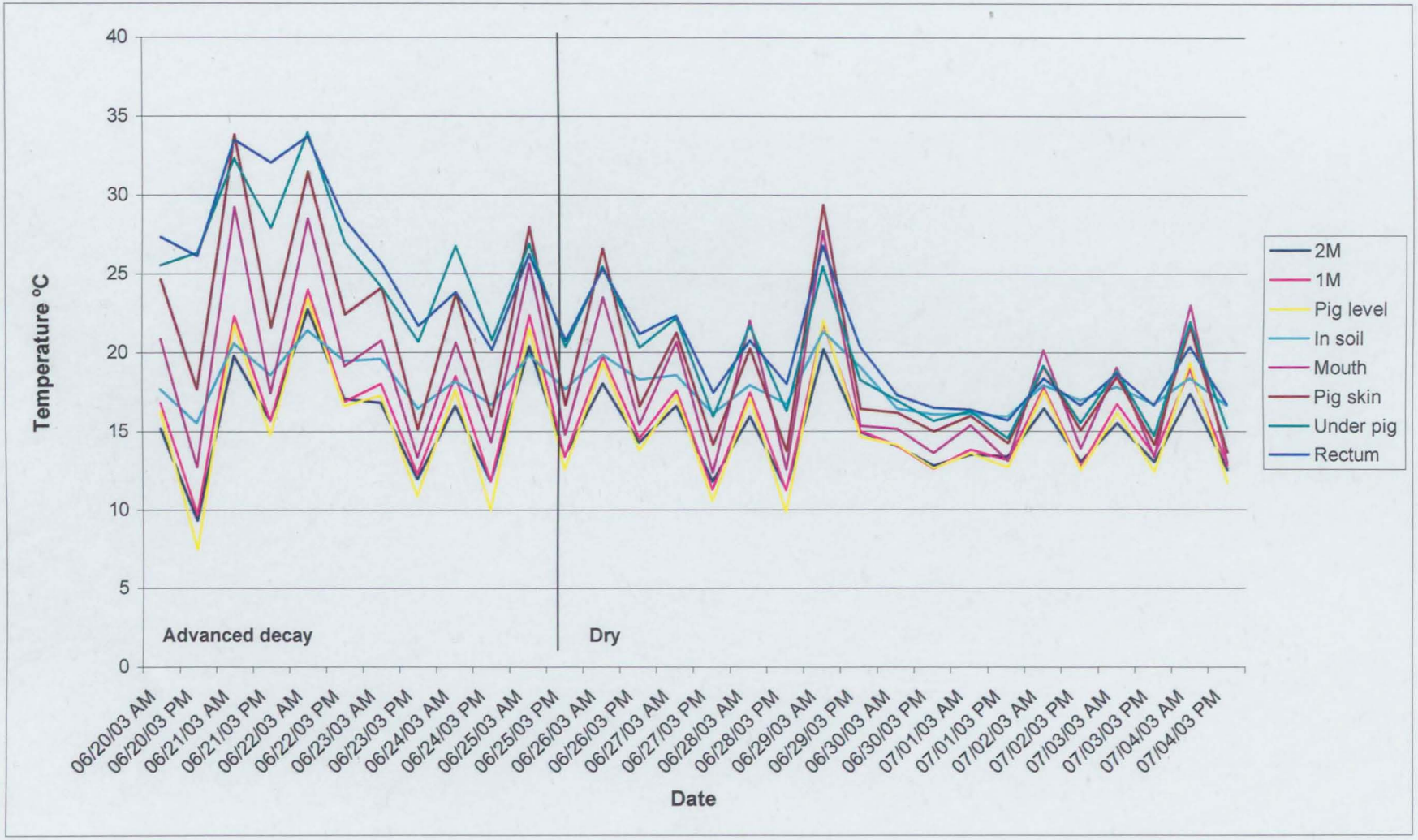


Fr = Fresh, Bl = Bloated, Act = Active Decay, Adv = Advanced Decay stage

APPENDIX 4: Temperature (°C) data for Experiment 3 – Summer 2003. Measured using HOBO Pro Data Loggers.

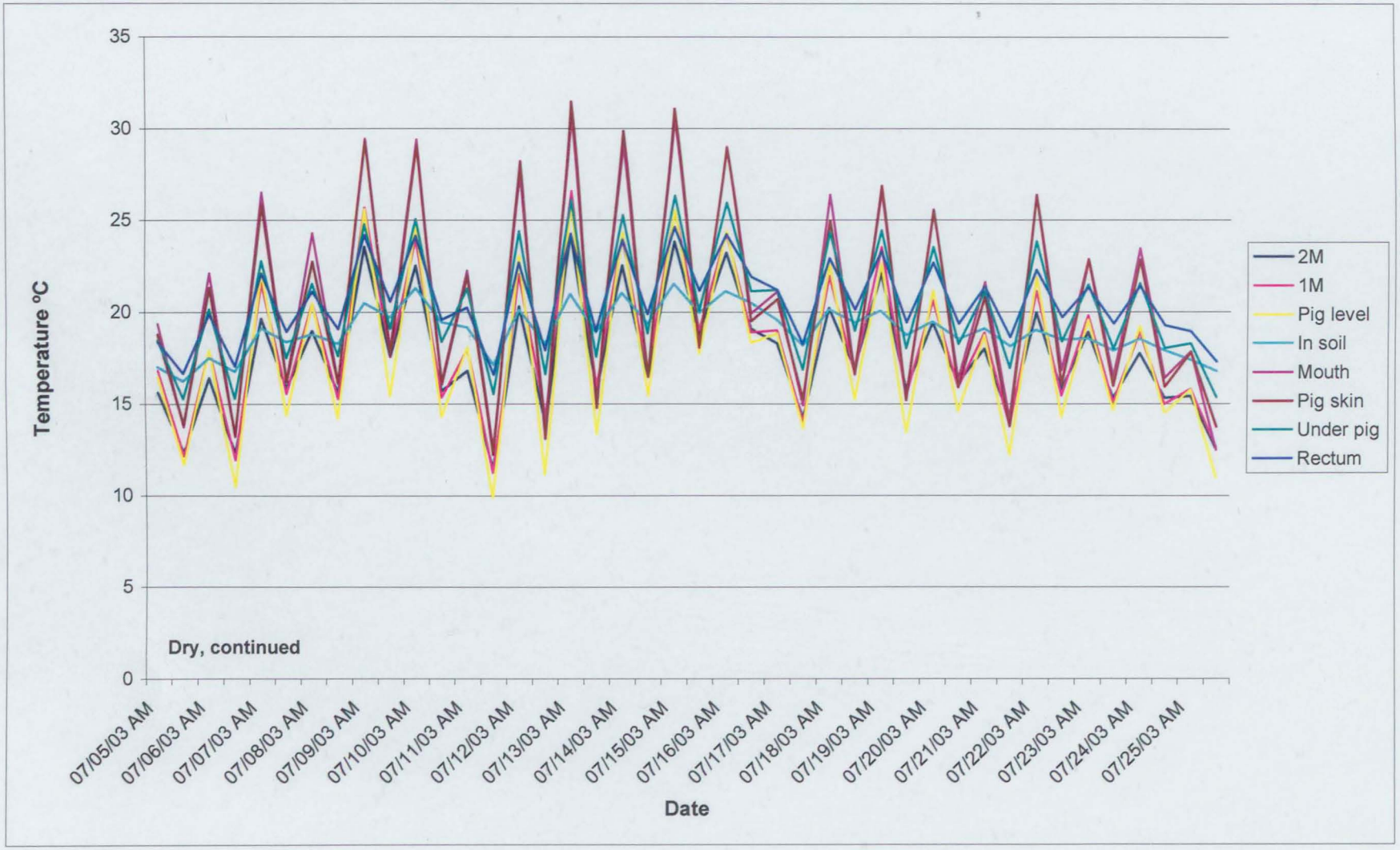
Date is listed in month/day/year

Temperature data for Experiment 3 – Summer 2003, Continued.

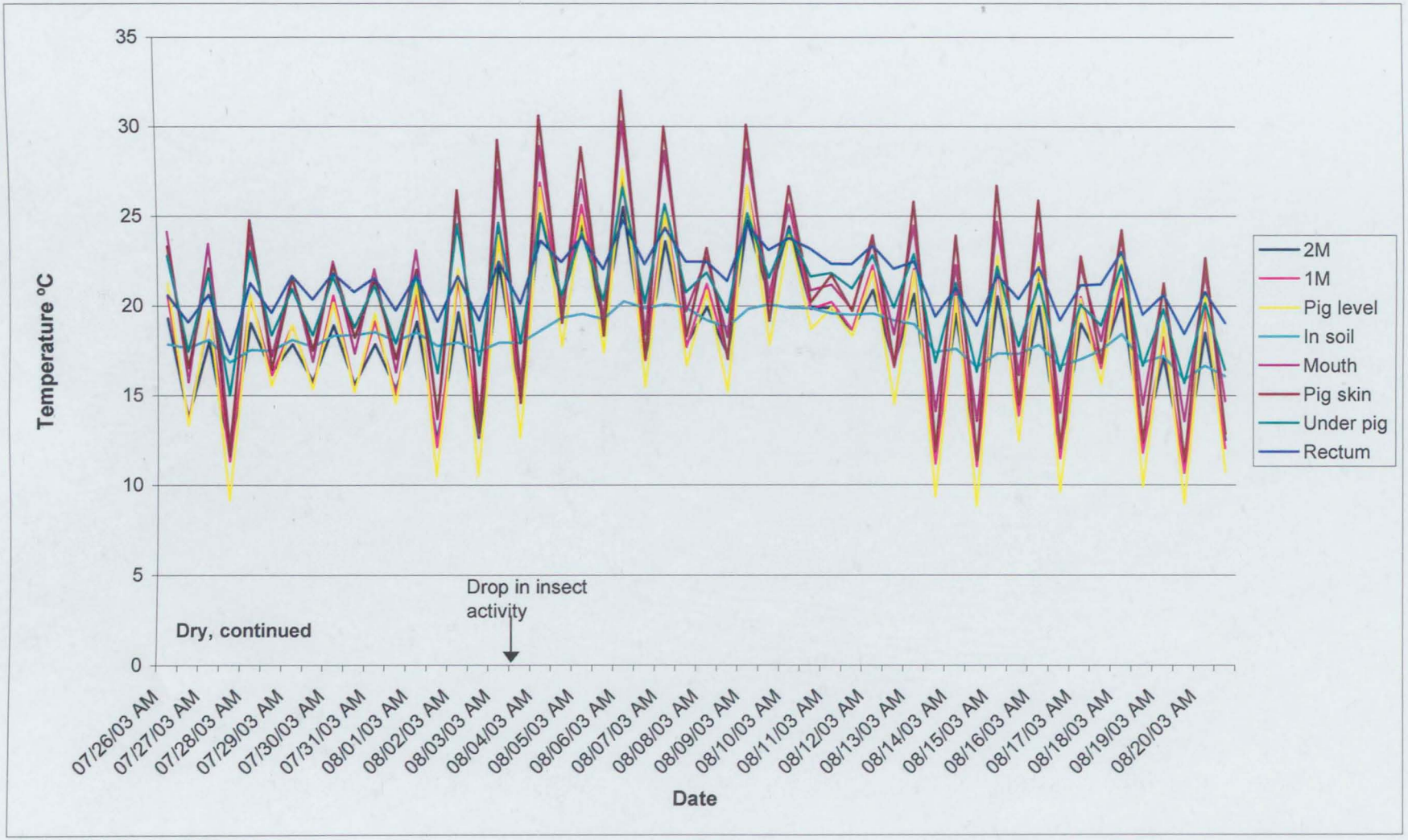


Date is listed in month/day/year

Temperature data for Experiment 3 – Summer 2003, Continued.

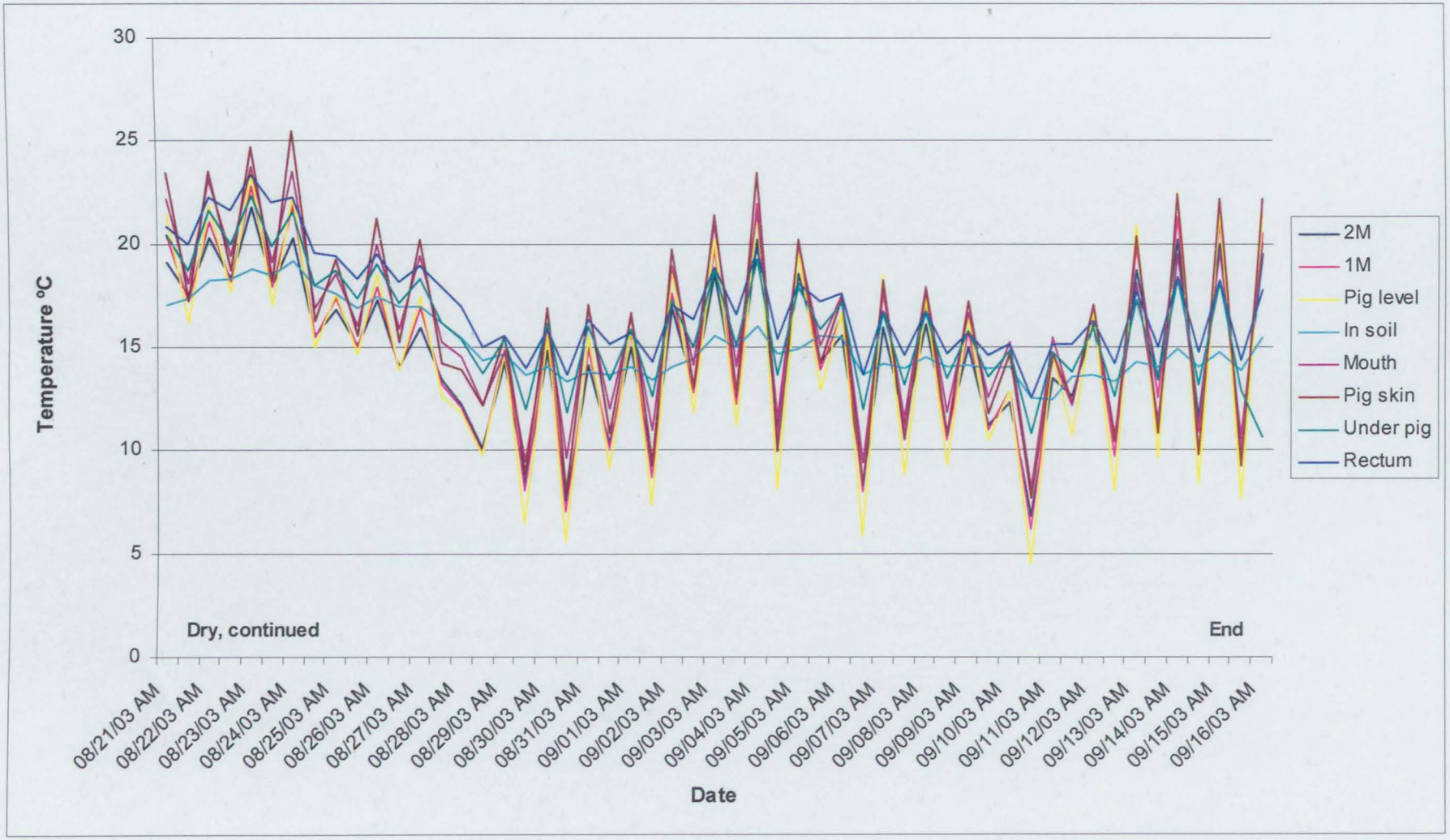


Temperature data for Experiment 3 – Summer 2003, Continued.



Date is listed in month/day/year

Temperature data for Experiment 3 – Summer 2003, Continued.



Date is listed in month/day/year

APPENDIX 5

Composition of Insect Ringer Solution (in 1 litre)

Sodium chloride	7.55g
Potassium chloride	0.64g
Calcium chloride (dehydrate)	0.22g
Magnesium chloride	1.73g
Sodium bicarbonate	0.86g
Sodium orthophosphate	0.61g

APPENDIX 6

Concentrations

Concentrations of chemicals (ng/μL) collected during air entrainments using Porapak tubes. Experiment 2 – summer 2003

EAG-active compound + indole & 3-methylindole	Day 0	Day 4	Day 9	Day 12	Day 14	Day 21	Day 25	Day 36	Day 42	Day 49	Day 55	Day 63	Day 69	Day 77	Day 83	Day 91
Dimethyl disulfide	0.0038	0.0679	0.1921	0.036	0.0137	0.0059	0.7752	0.0087	0.0044	0.0118	0	0.0031	0	0	0	0
KI 881-885	0	0.0046	0.0232	0.52	1.8678	0	0.0213	0.0969	0.086	0.1797	0.0409	0.0624	0.0168	0.0501	0.0964	0.0268
Dimethyl trisulfide	0.0425	0.3907	1.2686	0.661	0.2702	0.2302	0.1207	0.0455	0.0459	0.0381	0.0589	0.0274	0.0141	0.0251	0.0297	0.0329
Dimethyl tetrasulfide	0	0.7265	3.7456	1.457	0.3526	0.0867	0	0.0075	0.0086	0	0	0.0054	0	0	0	0
Indole	0	0.1525	13.025	13.13	7.1239	6.3122	4.8703	0.6394	0.072	0.0426	0.0177	0.0138	0.0058	0.019	0	0.0082
3-Methylindole	0	0.0057	0.1059	0.133	0.0883	0.0933	0.0801	0.0496	0.0264	0	0	0.0034	0	0	0	0

Concentrations of chemicals (ng) collected during air entrainments using Tenax tubes. Experiment 2 – summer 2003

EAG-active compound + indole & 3-methylindole	Day 0	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 10	Day 11	Day 13	Day 14	Day 16	Day 18
Dimethyl disulfide	875.62	54.638	397.83	43.99	81.682	136.89	21.426	103.73	312.75	223.42	1353.9	1573.5	370.49	83.242
KI 881-885	260.42	207.45	505.5	447.6	716.82	108.6	254.32	45.697	351.95	917.05	892.8	1503.5	8865.3	729.73
Dimethyl trisulfide	12.816	29.497	655.6	446.8	11364	10200	0	0	47.661	796.32	283.2	1061.6	1822	705.95
Dimethyl tetrasulfide	8.3938	72.126	160.06	39.97	1560.8	1929.4	3717.4	5511.7	3591	2648.9	1167	704.38	0	45.775
Indole	82.056	30.554	153.06	35.6	1146.5	972.49	3441.7	0	0	97.722	141.96	157.71	0	152.18
3-Methylindole	37.252	119.6	134.22	49.83	60.751	12.537	115.09	34.577	279.07	138.68	193.05	261.46	0	22.906

EAG-active compound + indole & 3-methylindole	Day 25	Day 28	Day 31	Day 42	Day 49	Day 55	Day 69	Day 77	Day 91
Dimethyl disulfide	71.361	600.18	83.319	29.45	164.98	68.682	12.831	0	0
KI 881-885	489.79	383.46	2236.6	2033	2619.3	469.36	355.47	634.28	162.14
Dimethyl trisulfide	124.57	214.82	227.39	676.6	382.99	35.237	35.802	48.817	30.822
Dimethyl tetrasulfide	22.441	92.13	83.861	76.08	122.6	13.243	13.91	9.7995	6.7249
Indole	1136.8	1138.8	1405.5	30.17	111.3	33.982	14.067	10.287	14.091
3-Methylindole	8.004	19.559	13.17	9.148	86.187	20.703	7.3023	6.4524	0

Average concentrations of chemicals collected throughout Experiment 2 – Summer 2003 from day 0 to day 91, as listed above.

EAG-active compound + indole & 3-methylindole	Porapak (ng/µL)	Tenax (ng)
Dimethyl disulfide	0.0702	289.74
KI 881-885	0.1933	1095.2
Dimethyl trisulfide	0.2063	1269.7
Dimethyl tetrasulfide	0.3994	939.03
Indole	2.8394	448.11
3-Methylindole	0.0366	70.85

Porapak: n = 16, Tenax: n = 23

APPENDIX 7

Loadings

Note: Loadings in each box are in order from highest weighting to lowest weighting and are also listed in absolute value.

Principal Component Analysis

A. Loadings from PCA of all EAG-active compounds, plus indole and 3-methylindole, collected using Porapak tubes.
The first two principal components (PC) accounted for 99.12% of the variance in the data - PC1 = 97.36% and PC2 = 1.97%.

	PC1	Abs. Value
Indole	-0.98637	0.98637
Dimethyl tetrasulfide	-0.14915	0.14915
Dimethyl trisulfide	-0.05661	0.05661
KI 881-885	-0.03688	0.03688
Dimethyl disulfide	-0.01304	0.01304
3-Methylindole	-0.00967	0.00967

	PC2	Abs. Value
Dimethyl tetrasulfide	0.87001	0.87001
KI 881-885	-0.41257	0.41257
Dimethyl trisulfide	0.23215	0.23215
Indole	-0.12867	0.12867
Dimethyl disulfide	-0.04529	0.04529
3-Methylindole	-0.01899	0.01899

B. Loadings from PCA of all EAG-active compounds, without indole and 3-methylindole, collected using Porapak tubes.
The first two principal components (PC) accounted for 95.37% of the variance in the data - PC1 = 76.71% and PC2 = 18.66%.

	PC1	Abs. Value
Dimethyl tetrasulfide	-0.94656	0.94656
Dimethyl trisulfide	-0.31788	0.31788
KI 881-885	-0.05004	0.05004
Dimethyl disulfide	-0.02162	0.02162

	PC2	Abs. Value
KI 881-885	-0.99577	0.99577
Dimethyl disulfide	0.06456	0.06456
Dimethyl tetrasulfide	0.05996	0.05996
Dimethyl trisulfide	-0.02619	0.02619

C. Loadings from PCA of all EAG-active compounds, plus indole and 3-methylindole, collected using Tenax tubes.
The first two principal components (PC) accounted for 82.53% of the variance in the data - PC1 = 59.07% and PC2 = 23.46%.

	PC1	Abs. Value
Dimethyl trisulfide	0.9936	0.9936
Dimethyl tetrasulfide	0.09271	0.09271
Indole	0.06203	0.06203
Dimethyl disulphide	-0.01369	0.01369
KI 881-885	-0.01108	0.01108
3-Methylindole	-0.00254	0.00254

	PC2	Abs. Value
KI 881-885	0.89246	0.89246
Dimethyl tetrasulfide	-0.43627	0.43627
Indole	-0.09707	0.09707
Dimethyl trisulfide	0.05697	0.05697
Dimethyl disulphide	0.01994	0.01994
3-Methylindole	-0.01099	0.01099

D. Loadings from PCA of all EAG-active compounds, without indole and 3-methylindole, collected using Tenax tubes.
The first two principal components (PC) accounted for 85.66% of the variance in the data - PC1 = 61.39% and PC2 = 24.27%.

	PC1	Abs. Value
Dimethyl trisulfide	0.99581	0.99581
Dimethyl tetrasulfide	0.09006	0.09006
Dimethyl disulfide	-0.01338	0.01338
KI 881-885	-0.00882	0.00882

	PC2	Abs. Value
KI 881-885	0.90166	0.90166
Dimethyl tetrasulfide	-0.42945	0.42945
Dimethyl trisulfide	0.04708	0.04708
Dimethyl disulfide	0.01915	0.01915

Canonical Variate Analysis

E. Loadings from CVA of all EAG-active compounds, plus indole and 3-methylindole, collected using Tenax tubes.
The first two principal components (PC) accounted for 89.69% of the variance in the data - PC1 = 75.49% and PC2 = 14.20%.

	CV1	Abs. Value
3-Methylindole	-0.006072	0.006072
Dimethyl tetrasulfide	-0.001341	0.001341
Dimethyl disulfide	-0.000704	0.000704
KI 881-885	-0.000189	0.000189
Dimethyl trisulfide	0.000218	0.000218
Indole	-0.000096	0.000096

	CV2	Abs. Value
3-Methylindole	0.005610	0.005610
Dimethyl disulfide	-0.000828	0.000828
Dimethyl trisulfide	0.000388	0.000388
KI 881-885	-0.000220	0.000220
Indole	-0.000110	0.000110
Dimethyl tetrasulfide	0.000007	0.000007

F. Loadings from CVA of all EAG-active compounds, without indole and 3-methylindole, collected using Tenax tubes.
The first two principal components (PC) accounted for 89.39% of the variance in the data - PC1 = 75.18% and PC2 = 14.21%.

	CV1	Abs. Value
Dimethyl tetrasulfide	-0.0014042	0.0014042
Dimethyl disulfide	-0.0012913	0.0012913
Dimethyl trisulfide	0.0002566	0.0002566
KI 881-885	-0.0001582	0.0001582

	CV2	Abs. Value
Dimethyl disulfide	0.0004309	0.0004309
KI 881-885	0.0003313	0.0003313
Dimethyl trisulfide	-0.0003158	0.0003158
Dimethyl tetrasulfide	-0.0001731	0.0001731

APPENDIX 8:

Publication

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