The Effect of Nicotine on Carrion Feeding Insects with Considerations For Use Within Forensic Sciences

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“Crime is common. Logic is rare. Therefore it is upon the logic rather than upon the crime that you should dwell”

Sherlock Holmes. The Copper Beeches.

By Sir Arthur Conan Doyle

Abstract

The presence of invertebrates on decomposing animal matter has been used extensively by forensic entomologists to estimate time of death for over 100 years. The presence of toxins such as drugs and pesticides on carrion can affect the behaviour and life cycle of such invertebrates. The aim of this thesis was to examine the effects of nicotine upon the colonisation of carrion by invertebrates; nicotine was used because of its historical use as an insecticide and its ubiquitous use in society. The investigations aimed to examine these possible effects both in situ in field-based testing and *ex situ* in a controlled laboratory environment and to work towards an empirically testable correction factor for the estimation of *Postmortem* interval estimates in the presence of nicotine .

The field-based testing was done using *Sus domestica* (Linnaeus) carrion with a solution of nicotine injected into the cadaveric throat of the animal. The carrion was protected from feeding and removal by vertebrate scavengers. It was found that nicotine affected the time taken for Diptera to colonise the carrion as well as affecting the behaviour of feeding. Diptera larvae showed avoidance of the nicotine treated throat sites on the carrion, which is the normal site of oviposition. It was determined that the rove beetle *Creophilus maxillosus* (Linnaeus) was exclusively found on the higher dose nicotine carrion. The rare hoverfly *Rhingia rostrata* (Linnaeus) was discovered on the control animal; this is the first specimen reported in Nottinghamshire. The investigation also found the first record of the Soldierfly *Sargus bipunctatus* breeding in carrion; the late breeding period of this species and its significance to the forensic entomologist is considered. The experiments were conducted in the Autumn/Winter months and Spring/Summer months. Nicotine appeared to have a differing effect with the season as the autumnal fauna varied from that of the spring fauna. The presence of nicotine appeared to prevent the animal carcass from drying out, typified by mycophagus beetles in autumn and semi-liquid habitat breeding flies in the summer.

The laboratory based investigation examined the effects of nicotine upon the life cycle of *Calliphora vomitoria* including the effects upon oviposition, rate of development and survivablity. It was found that nicotine significantly affects rate of development of this forensically important fly.

This study has shown that a careful study of a single chemical compound and its interaction with carrion and entomology has profound effects upon the alteration of the normal activity of a range of forensically important invertebrates. It will assist in improving the evidential usefulness of entomology to the Forensic Science and Policing communities in criminal investigations.

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Chapter 1 Introduction

1.1 General introduction

On the 28th of June 1968 two thirteen year old fishermen were looking for small mammal carcasses in Bracknell woods, hoping that such carcases would be covered in maggots with which they could bait their hooks. After finding a mass of maggots their initial enthusiasm turned to horror as they discovered the maggots were not feeding upon a small mammal corpse, but a human body disposed of in a shallow grave. The investigating medical officer Keith Simpson determined that traditional methods were not suitable to determine time since death as the body appeared to be disarticulated. Under the working assumption that the maggots were that of a common bluebottle Dr Simpson attempted to estimate time of death by determining the age of the maggots. Dr Simpson's estimate called into question the story of a suspect who had admitted to owing the victim money, and had supposedly payed the deceased back on the very day that he was estimated to have been murdered. A subsequent trial found the suspect guilty of the murder (Wilkes, 2005). The use of insect evidence in murder cases, while novel, is not new. In fact the first recorded case of the use of insect evidence in the literature dates back to 13th century China in the "Hsi yuan lu" or "washing away of wrongs instruction to coroners." Hall's (2010) translation states that a coroner noted in a case of murder that the wounds on the victim had been made by a sickle. He ordered the possible suspects to lay their sickles down before him, and saw that out of seventy sickles, flies flocked to one in particular, that of a man known to have quarrelled with the victim over a loan of money. When the accused denied his guilt the coroner argued that the flies had singled out his sickle attracted by the smell of blood. The book goes on to state that when confronted with this evidence the accused promptly confessed his guilt.

1.2 The process of death and decomposition of animal remains

Decomposition of animal waste, both carrion and dung accounts for in excess of 95% of total community metabolism in terrestrial systems (Putman, 1983). By understanding the mechanisms of carrion decomposition, forensic practitioners are able to use biological indicators to estimate time of death in cases of suspicious death.

While death can be considered a process rather than an event, it is prudent to consider the moment of death as the second the heart stops beating. At this point the cells of the body are cut off from an oxygen supply and rapidly being to die individually. Within 3-7 minutes brain cells begin dying, whereas skin cells may still be alive up to 24 hours after a person is declared medically dead. The body no longer generates metabolic heat and begins to cool down to equilibrate with ambient temperatures. Between 20 minutes and 2 hours after death *livor mortis* or lividity is first observed, this is a reddish purple discoloration of the skin cause by the blood settling due to the lack of pulse and gravity, greenish discolouration of the body may be caused by metabolism of haemoglobin to sulphaemoglobin. Lividity becomes fixed between 10 and 12 hours after medically defined death, so discrepancies between expected lividity and observed lividity can suggest by a body has been moved *post mortem* (Gunn, 2006). *Rigor mortis* begins approximately 3-4 hours *post mortem* and is characterised by the stiffening of muscles and limbs, and is fully set in approximately 12 hours post mortem, characterised by total body rigidity. *Rigor mortis* is caused by an increase in the intracellular concentration of calcium ions in muscle cells from the sarcoplasmic reticulum *post mortem*. The rise in calcium concentration leads to the regulatory proteins troponin and tropomyosin to relocate, allowing actin and myosin to bind to each other. The binding of actin and myosin cannot be reversed as the the body is no longer producing the ATP required to separate the molecules, resulting in a prolonged stiffening of the body (Gunn, 2006) As the bodily chemicals required for muscle contraction break down *rigor mortis* passes and the body

relaxes usually after 36 hours. Like many elements of the decomposition process *rigor mortis* is affected by temperature with low temperatures prolonging the process, indeed at 4^{o}C total rigidity can be present for 16 days, and traces of *rigor mortis* may still be observed for up to 4 weeks (Gunn, 2006). Whittle and Ritchie (2004) note that notorious serial killer Doctor Harold Shipman used to turn- up the central heating in the houses of his victims, the authors suggested that this could have been to hasten the onset of *rigor mortis*.

The natural gut flora and enzymes, which are so effective in the breakdown of proteins during digestion, find the only proteins available for their own survival (after breaking down the contents of the digestive system) are the proteins that make up the gastrointestinal tract, whilst other gut bacteria such as *Clostridia* invade parts of the body and begin to break it down from the inside in a process known as autolysis (Gunn, 2006). Prescott *et al.* (1993) stated that as a result of the low pH (2-3) of the stomach less than 10 viable bacteria per ml of gastic fliud are contained within the stomach, however this may rise with pH. The small intestine contains few micro-organisms due to the steep pH increase from the stomach. The large intestine contains the largest population of microbial organisms in the human body with over 300 species with counts of approximately 10^{12} organisms per gram of faeces.

As the tissues of the body break down, the skin begins to discolour further and the respiratory gases of bacteria cause the abdomen to bloat, the tongue may protrude and the body begins to develop the characteristic odour of decay. This stage is normally reached in 4-6 days in the United Kingdom during the spring and summer months. However, the colder winter months would prolong this time, due to the reduced metabolism of the bacteria at lower temperatures (Gunn, 2006). The body itself and its bacteria are not the only agents of decay and decomposition; many different insects and other invertebrates specialise in recycling and returning to the environment the nutrients stored within a dead body. Odum (1967) notes that in decomposition micro-organisms can give off “environmental hormones” which can be

either inhibitory as with the antibiotic effect of penicillin producing fungi, or stimulatory. Odum (1967) further remarks that some micro-organisms combine vitamins and other growth substances to form "hormone-like substances". If it was not for decomposer organisms releasing organic matter back into the environment, production within the ecosystem would greatly decline. While it may be argued that bacteria and fungi are the most important decomposers, a wide range of different species are involved in recycling organic matter (Primack, 1998). Many of these are detritus or carrion feeding invertebrates, and when looking at average production efficiencies within an ecosystem, invertebrates have much higher production efficiency than birds or mammals as is shown in Table 1

Table 1.1: Average production efficiencies for various groups of animals (after Purves *et al.* 1998)

Group	***Production efficiency (%)**
Insect decomposers (detritivores)	47
Other invertebrate decomposers	36
Fish and social insects	10
Large mammals	3.1
Small mammals	1.5

*Production efficiency is defined as:

$$E = \frac{P}{(P + R)}$$

Where E is efficiency, P is net production, and R is respiration (Purves *et al.* 1998). Considering an animal carcass as a separate micro-ecosystem, the fundamental ecological principle of succession is observed during decomposition. Odum (1967) remarks that

succession is the process of community change, with a predictable sequence of community change beginning with pioneer stages and ending with a climax community in equilibrium with the local area. Chick (2010a) noted that flies or more specifically Calliphoridae, the common blue bottles and green bottles, are among the first insects to arrive at an unburied dead body in the UK, and act as the pioneer species of the carrion succession process.

Manlove (2010) states the rate at which these pioneer species decompose carrion is dependent upon the thermal environment. Knowledge of this successional pattern and how it is affected by environmental conditions is used by forensic practitioners to estimate the *post mortem* interval in cases of suspicious deaths, and it is the science of this process which is commonly referred to as Medicolegal Entomology.

1.3 Forensic Entomology

In the strictest sense Forensic Entomology is the application of insect science to legal matters (Smith, 1986). However most people assocciate the use of insects in matters of human decomposition as Forensic Entomology.

According to Gennard (2007) there are typically five stages of decomposition of a fresh organism (animal) that is deposited in a terrestrial environment:

- Stage 1: Fresh.

  Exists between the moment of death and the onset of bloating. In the U.K. this stage is associated with *Calliphora vicina* or *Calliphora vomitoria* ,*Lucilia* spp and possibly *Protophormia terraenovae* in early spring.

- Stage 2: Bloat.

  As previously stated the body's internal flora breaks down the internal tissue, the respiratory gases produced by these micro-organisms cause the abdomen

to swell, leading to the “Bloat corpse” appearance This stage is when predatory beetles such as Staphylinidae are attracted to the corpse.

- Stage 3: Active decay.

  The skin of the corpse starts to break up and slough off the body. As the abdomen is breached the respiratory gases of the gut flora escape and the body deflates. At the later stages of putrefaction fermentation occurs, which in turn generates butyric acids followed by caseic acids, Chick (2010a) notes that caseic fermentation occurs as the corpse begins to dry, and is recognisable by the odour of cheese the corpse exhibits. Gennard (2007) states this stage also includes ammoniacal fermentation, which Chick (2010) remarks is the point at which the sanious fluids (blood and other blood-tinged fluids) evaporate from the corpse. Gennard (2007) states that this stage typically includes colonisation of the corpse by beetles of the families Histeridae, Silphidae, such as *Nicrophorus humator*, and flies of the family Muscidae such as *Hyrdotaea capensis.*

- Stage 4: Post-decay

  Very little soft tissue remains, possibly small amounts of flesh and some of the intestines. Apart from this, all that remains is dried hide, cartilage, hair and bones. Normally this stage shows the dominance of Diptera upon the corpse gives way to waves of Coleoptera.

- Stage 5: Skeletonization

  All that remains is hair and bones, the final stage of decomposition for the purposes of forensic entomology. No obvious groups are associated with this stage although Nitidulidae may be found in some cases (Gennard, 2007), as may be Tenebrionidae (Smith, 1973) and Dermestidae (Peacock, 1993)

Smith (1973) considered there to be 8 waves of decomposition dividing the active-decay stage into the 3 fermentations (butyric, caseic and ammoniacal). Chick (2010a) used a modification of Smith's waves when discussing the faunal succession of Diptera; however it is noted that Chick's waves of decomposition were written with the Dipterist as a target audience, with passing reference to forensic science. Matuszewski *et al* (2010a) remarked that the traditional waves of decomposition fail to account for the continuous nature of decomposition; the boundaries between waves can be subjective to some investigators. Traditional wave models also fail to account for the mosaic nature of decomposition, wherein different parts of the body may be in different waves of decomposition (Matuszewski *et al*, 2010).

1.4 Case studies

Benecke (2001) states that the first scientific observations of arthropods as possible forensic indicators date back to the late 1880s when Reinhard and Hoffmann made observations during the exhumations of mass graves in Germany and France. Benecke (2001) also states that early case reports determined that humans had the same decomposition fauna as other mammals. Benecke also comments that early in the infancy of the field it was observed that the presence of invertebrates could cause damage to a body that could be mistaken for trauma or abuse. Case reports show that forensic entomology has a wide variety of uses in legal cases.

Vanin *et al* (2011) discovered the first recorded instance in Southern Europe of *Calliphora loewi* on a human corpse. The case from central Italy also recorded the presence of the Stratiomyid fly *Hermetia illucens* and the Clerid beetle *Necrobia rufipes*. The case was of particular importance as it provided data during Italy's cold season.

A review of cases in Switzerland between 1993 and 2008 found that in only 33 cases out of 160 were flesh flies (Diptera, Sarcophagidae) present upon a corpse (Cherix *et al.* 2012). The review states that in Switzerland, flesh flies colonise a corpse at much the same time as Calliphoridae, also the research discovered that *Sarcophaga argyrostoma* will colonise indoor corpses during the summer. However Chandler (1998) does not consider *S. argyrostoma* to be a British species. So this species' habits are of limited interest to the UK entomologist.

Benecke (1998) notes in one case that entomological evidence indicated a corpse in Germany was left outside for a considerable amount of time, before it was placed inside prior to discovery. This work also discusses how an alcoholic possibly died due to the presence of a fatal sepsis-causing bacterium, *Serratia marcescens,* which was discovered because the fly *Muscina stabulans* appeared out of sequence in the decomposition process. While most primary colonisers appeared as freshly laid eggs, or first instar larvae, *M. stabulans* appeared as pupae, so it was determined that the larvae must have fed upon the victim prior to death, a strange colouration of the pupa was interpreted as a culture of *S.marcescens.* Skidmore (1985) suggests that *M. stabulans* will consume dead or live tissue with equal preference.

In another case, Anderson (1999) remarked how forensic entomology was used to determine time of death of 3 adult black bears (*Ursus horribilis*) and 2 cubs which had been illegally killed and disembowelled, to obtain gall bladders for use in traditional Chinese medicine in Winnipeg, Canada. Yong (2013) stated that DNA extracted from carrion-frequenting flies can be used to map biodiversity of mammals in an inaccessible rainforest ecosystem, it is conceivable that such a methodology could be used to prove an insect was feeding upon a certain corpse, or be used to indentify a dead body. Mahat *et al* (2016) suggest that in cases of wildlife crime and animal abuse, evidence may be burnt to attempt to conceal a crime. Mahat

et al (2016) found that when rabbits were burned *post mortem* to the Crow-Glassman scale level 3 oviposition of both *Chrysomya megacephala* and *Chrysomya rufifacies* was delayed. Tomberlin and Sanford (2012) recommended caution in the use of forensic entomology in wildlife crime, suggesting that the differences between wild animals and humans need to be considered. Theoretically they posited that wild animals are more likely to have myiasis than humans, therefore may be carrying an infestation of primary colonisers for some time prior to being poached, suggesting that parasitic colonisation prior to death requires research in a forensic context.

Benecke *et al* (2004) observed that many of the Dipteran primary colonisers of cadavers are also facultative agents of myiasis (invasion of living tissues by Dipteran larvae) and as such can be theoretically used to determine how long an elderly person may have been neglected prior to death.Traditionally when primary colonisers are found on carrion their age must be accurately determined. This is based upon the point of development of the larvae compared with the environmental conditions (Byrd and Castner, 2001), with different species developing at different rates (Lefebvre and Pasquerault, 2003). Indeed, Ames and Turner (2003) showed that despite the poikilothermic nature of insects, cold tolerant species such as *Calliphora vicina* and *Calliphora vomitoria* do not have a minimum threshold for development in their northern range as previously thought. Determining the age of pupae is theoretically possible using molecular methods, since Ames *et al* (2006) showed that different genes were expressed more predominantly during the pupation of *C.vicina;* however further work is required in this area.

1.5 Factors affecting decompostion and insect development

The succession of invertebrates upon unburied corpses, in the Palaearctic ecozone is normally dominated by the blowflies (Diptera, Calliphoridae), house flies (Diptera, Muscidae) and

flesh flies (Diptera, Sarcophagidae), in the first 3 months. These are followed by Dermestid beetles (Coleoptera, Dermestidae) and pyralid moths (Lepidoptera, Pyralidae) approximately 3 months into the decomposition process, then by further Diptera and Coleoptera colonisation (Smith, 1973). However this process of succession may vary with location. Ortloff *et al* (2012) recorded the succession on pig carrion in semi-urban Chile during summer, noting that the carrion entered the dry stage of decomposition in only 11 days. Whereas Matuszewski *et al* (2008), investigating succession in forest habitats in Poland in autumn, observed higher decomposition rates in alder forests, than in pine-oak forests. When this investigation was expanded to examine the effect of seasonality of decomposition in Polish forests, it was found that during the spring months Alder forest active decay was driven by the larvae of *Necrodes littoralis* (in contrast to Calliphoridae in other forests). Decomposition was observed to proceed at a higher rate in summer, carrion in Alder forests again decomposing at a faster rate than other forests (Matuszewski *et al* 2010a). However it was later discovered that there was no significant difference between the compositions of the different forest faunas (Matuszewski *et al* 2010b). Matuszewski (2011) stated that in Poland the pre-appearance interval of *N.littoralis* was strongly inversely dependent upon the average ground temperature of the carrion deposition site. From this a number of methods of calculating pre-appearance interval were proposed; however it was stressed that the results were preliminary.

Kočárek (2003) explored the succession of Coleoptera on rat carcasses in varying ecosystems in the Czech Republic. 145 species from 22 families were recorded; the highest number of species was recorded during the decay stage, in forest ecosystems. Coleoptera were categorised into four groups, depending on carrion association and food specialisation. A study from Australia suggested that some Calliphoridae show a change in habitat preference with season, such as memebers of the genus *Chrysomya* will move from rural locations to urban locations over winter, (Kavazos and Wallman, 2012). The study also found that in

sample areas *Chrysomya* was most abundant in summer, whereas *Calliphora* was more abundant in winter. However Chandler (1998) does not record the genus *Chrysomya* in the UK. Whilst other Calliphorids may display similar behaviours, further work would be required for data to be of importance to the UK entomologist.

The most frequent primary colonisers in summer in the UK are blowflies from seven species of *Lucilia,* whereas in spring and autumn five species of the genus *Calliphora* are more likely to be seen. Blowflies of the genera *Phormia, Pollenia, Mellinda* and *Cynomya* may also be present on a corpse in the summer but these will appear at different waves in the decomposition (Dear, 1978).

Archer *et al* (2005) suggested that the artefacts such as pupal skins, left by carrion-frequenting insects during succession, are an indicator that a body has lain *in situ* for some time. This combined with a knowledge of insect ecology, for example that certain species are never observed within dwellings, or have a restricted geographical range, can provide information as to the possible movement of a body *post mortem*. This statement was echoed by Pohjoismäki *et al* (2010) who made a study of nine forensic cases in which bodies had been in an indoor environment for between five days and four months in Finland proving *Sarcophaga caerulescens* would colonise cadavers within a dwelling. This was also considered by Hwang and Turner (2005) who showed that some necrophagous Diptera are restricted to semi-natural habitats and therefore have limited distributions.

Apart from flies, other insects are important in the successional process of decomposition. Kulshrestha and Satpathy (2001) stated that in India, Dermestidae and Cleridae are the most common types of beetle found on human cadavers, although Smith (1986) notes that other families of beetle are present on carrion in Europe including the predatory Carabidae, Staphylinidae, Histeridae and terrestrial members of the Hydrophilidae. Zanetti, *et al* (2015)

state that dermestid beetles can alter *post mortem* wounds and disguise evidence within a month.

Leiodidae beetles are often present on cadavears, although their niche within the carrion ecosystem is not discussed by Smith. Cleridae, Dermestidae and Nitidulidae are associated with advanced decay, where they feed upon the flesh of a cadaver after the active dipterous decay. Other families are considered by Smith (1986), such as dung beetles and fungus feeding beetles. Dermestidae can in ideal conditions (i.e. high temperature and low humidity) out compete Diptera, and indeed accelerate the decomposition of a human corpse (Schroeder, *et al* 2002).

Many factors can affect the way in which invertebrates decompose carcases. As previously stated, temperature is a crucial factor in the development of invertebrates. Huntingdon *et al* (2007) showed that the metabolic heat generated by a mass of larval Diptera is significantly higher than the surrounding temperatures. In fact the metabolic heat generated by a larval mass is enough to overcome the effects of cold storage in a mortuary. Ireland and Turner (2006) showed that *Calliphora vomitoria* was prone to production of undersized maggots when incidences of larval crowding were high. This was observed on liver, muscle and brain tissue to varying extents. The effect of rainfall on decomposition is debated in the literature. A study by Archer (2004) in Victoria, Australia remarked that there was no statistical indication of the effect of rain on decomposition of neonatal remains, but anecdotally suggested that adult corpses will decompose at a faster ratewhen subjected to rainfall. Although rain may reduce the temperature surrounding a corpse, results in neonates suggested that rain increased the rate of decomposition, as the rainfall appears to aid break up of fleshy tissue and increases the rate of leeching of organic fluids. Archer also said that rainfall increased humidity and prevented the corpse from drying out. Conversely, based

upon data collected in Kelantan, Malaysia, Mahat *et al* (2009) suggested that dependant upon intensity, rain may delay oviposition on rabbit carcases for 1-2 days and extend the pupation of *Chrysomya* species by between 1-3 days.Thus it would appear that the effect of rainfall is varied dependent on the stage of decomposition. It should also be noted that the studies took place in separate areas of the world and thus local variation in climate may play a role.

Arnott and Turner (2008) showed that the post-feeding larval stage of *C.vicina* may be extended if the larvae cannot find a suitable pupation site and that this can introduce an error of up to two days in *post mortem* interval estimates. Manlove and Disney (2008) noted that in winter, the Phorid fly *Megaselia abdita* is more likely to present as a primary coloniser than the traditionally expected Calliphoridae, However this presents further problems in that Disney (1989) stated that females of the genus *Megaselia* are currently unidentifiable using traditional methods, and that *M. abdita* is restricted to Tyne and Wear and Midlothian, meaning that it is possibly of limited use to the forensic entomologist. In fact, the first case of *M. abdita* in a U.K. forensic investigation was only outlined by Disney and Manlove as recently as 2005. Zuha *et al* (2015) stated the Phoridae are often found in indoor crime scenes in Malaysia, particularly the species *Megaselia scalaris, Megaselia spiracularis* and *Dohrniphora cornuta.* Further points have been raised by Turchetto and Vanin (2004) who state that as most succession tables were compiled in the late 19th to early 20th centuries, they fail to take into account the effects of climate change or globalisation. To demonstrate this point Turchetto and Vanin (2004) illustrated how some forensically important species are spreading farther north. Nevertheless Chick *et al* (2008) argue that the majority of research is conducted in the U.S.A., which has a different fauna from Northern Europe and thus has limited value to a U.K. entomologist. This point was echoed by Martín-Vega (2011) who noted that European Piophilidae will colonise a cadaver much later in the successional process than was reported in literature based upon research from Hawaii.

Byrd and Castner (2001) stated that the ecosystem a cadaver is deposited in will have an effect upon how the body decays, A body disposed of in water, be it freshwater or marine, has a different fauna from that of a terrestrial ecosystem. A corpse deposited in a body of fresh water will undergo six stages of decomposition. These are:

1. Submerged fresh.
2. Early floating stage.
3. Floating decay.
4. Bloated deterioration.
5. Floating remains.
6. Sunken remains.

The corpse will initially sink, and will start to float during the bloat due to the build-up of gases. However the time taken for a corpse to float will depend on numerous factors including, but not limited to temperature, mass of the corpse and salinity of the water. During the floating stages a corpse will be accessible to numerous terrestrial insect species capable of flight which will colonise the corpse in a similar manner to decomposition on land. However after the abdomen is breached the gases dissipate and the corpse loses buoyancy and will sink, taking any terrestrial invertebrates with it (Byrd and Castner. 2001). The survival rates of submerged pupae and their parasitoids are variable. Survivability decreases as length of submergence increases. However the age of pupae prior to submergence is also an important factor; older pupae are more capable of surviving during submergence than younger pupae (Reigada *et al* 2011). However this study was conducted upon non U.K. species (Chandler, 1998) meaning that U.K. species might behave differently.

While it might appear obvious that placing a body in an aquatic ecosystem will have an effect upon how a corpse decomposes, a less drastic body disposal site might have an effect upon decomposition. For example Terrell-Nield and Macdonald (1997) stated that carrion

deposited in an environment such as a cave will decompose in a different manner depending upon how far into the cave the cadaver is, with carrion placed deep within a cave being more likely to be decomposed by fungi before primary insect colonisers can oviposit upon it.

Erzinçlioglu (1996) stated that soil coverage of 2-3 cm will be sufficient to prevent blowflies from ovipositing upon a cadaver. However the author also notes that the effects of soil structure need to be fully investigated to understand the impacts of burial upon arthropod succession. Smith (1973) wrote that the succession of arthropods on a buried cadaver contains 4 waves of succession over a period of 2 years, as opposed to a cadaver deposited above ground, which has 8 waves succession over a period of 3 years. Unfotunately this study fails to quote the depth of burial or soil type. Schotsman *et al* (2011) investigated the effect of lime, hydrated lime and quicklime upon the decomposition of pig cadavers buried in sandy loam soil in Belgium. The study demonstrated that while lime is often shown in television shows as a preferred method of body disposal, lime appears to delay the decomposition process in the first six months. The presence of the lime restricted the release of volatile organic compounds, which in turn resulted in reduced insect attraction. The effect of depth of burial of a cadaver upon larval dispersal of *Chrysomya albiceps* was studied by Gomes and Von Zuben (2005) who discovered a positive correlation between burial depth and distance dispersed by pre-pupal larvae, as well as a negative correlation between distance of dispersal and weight of pupae. Adams and Hall (2003) suggested that even the methods used to fix and preserve Dipteran larvae add a source of error into *post-mortem* interval estimates. The traditional hot water fixation method, increased length of larvae, and long term storage in 70% alcohol appeared to cause shrinkage. Furthermore time of deposition can affect the decomposition process. Brown *et al.* (2012) examined the methods used to preserve pupae of the Calliphoridae and determined that hot water killing followed by preservation in 80% alcohol or freezing at -20^0C was the optimal method.

Singh and Bharti (2001) focused upon the nocturnal oviposition of Calliphoridae. They state that while Calliphorid flies will lay eggs at night, the amount is greatly reduced. They noted that the ideal scenario would be where a body is deposited under bushes in which the flies are resting, as then the flies can crawl down to the cadaver. However it was maintained that Calliphoridae will fly during the night if they can detect carrion. Wooldridge *et al*. (2007) concurred with this work, stating that probability of oviposition resulting from nocturnal flight of *Calliphora vomitoria* and *Lucilia sericata* is relativity low. Further results suggested that *L.sericata* has a preference for oviposition at higher light intensities than *C.vomitoria.* This itself could suggest that *C.vomitoria* will have a preference for shaded oviposition sites, whereas *L.sericata* will have a preference for carrion in exposed sunlight. Charabidze *et al* (2015) stated that while it is commonly stated that blowflies will oviposit in wounds, on experiments using *Lucilia sericata* no oviposition directly in wounds was observed. Eggs laid in damp conditions show a increased survival rate (such as those laid around wounds), whereas those submerged in fluid appear to have a much lower survival (such as those laid in wounds).

Geography has an effect on decomposition; most of the current knowledge in forensic entomology comes from field based studies on human analogues, which are repeatedly sampled from during the course of decomposition. A study in Colorado U.S.A. investigated the effect of this repeated sampling using a total of 196 rat carcases (with a mass of between 153.2g and 176.7g) during the summer. A total of 38 arthropod taxa were collected from the carcases of which 18 were considered carrion feeders, the rest being categorised as incidental visitors. The results indicated that current sampling methods in carrion research have a negligible effect on the carrion community (De Jong and Hoback, 2006)

In carrion research, pigs are often used as a substitute for human cadavers. Haskell, *et al* (2002) investigated the suitability of porcine material as an analogue for human tissue in

entomological research. They determined that the carrion arthropods showed no significant preference as a whole for either porcine or human tissue, and also showed that 80% of insects were common to both types of carrion. Furthermore those taxa contributed 99.67% of the total catch, so it was determined to be an indication that only the rarest species do not colonise both humans and pigs. Additionally Erzinçlioglu (1996) noted that heart meat lacks vitamin A, which is required for vision in blowflies, and thus is not suitable for rearing purposes. However Clark *et al* (2006) concluded that different tissues of the body affect the growth rates of the Greenbottle *Lucilia sericata*. The study showed that porcine tissue increased growth as opposed to bovine tissue and that larvae left the food source thirty one hours quicker and grew by a length of 2mm when reared upon lung tissue as opposed to liver. Campobasso and Introna (2001) argued that if the entomologist doesn't understand forensic principles, or the forensic team undervalues entomology, then the value of entomological evidence will likewise be compromised.

1.6 Forensic entomotoxicology

Chick *et al* (2008) stated that if carrion is to be considered an ecosystem, then chemicals present in the body prior to death can be considered a form of pollution. Introna *et al* (2001) refer to the study of such toxins and their interactions with insects as **entomotoxicology**. This "cadaver pollution" can take the form of many different toxins, which in turn can have many effects on the carrion ecosystem. Commonly investigated cadaver pollution takes the form of abused drugs.

Entomotoxicology is generally split into two distinct branches, the first looks at the effect of chemicals on the development of insects feeding upon carrion, the second looks at the detection of toxins within insects feeding upon corpses.

According to Introna, *et al* (2001), cocaine increases the development rate of Sarcophagidae species. When reared on rabbit tissue administered with a fatal overdose of the drug, larval growth rate increased after seventy six hours post hatching and, overall the total development time was shortened. Carvalho *et al* (2012) remarked that cocaine makes *Chrysomya albiceps* and *Chrysoma putoria* develop at a faster rate, the difference being most observable after 12 hours of exposure to cocaine. It was also discovered that cocaine leads to reduced adult mortality, so it was determined that cocaine has a significant influence on stimulating larval growth. Introna *et al* (2001) also state that cocaine increases the size of maggots present on a corpse to larger than expected, or observed elsewhere on the corpse.This can create possible errors in a *post-mortem* interval estimate. Larger maggots were also observed by Chick *et al* (2008) in rats treated with common insecticides.

Bourel *et al* (1999) states that heroin increases the rate of growth in Sarcophagidae sufficiently to create inaccurate *post-mortem* intervals if uncorrected. They also explain that the related compound morphine causes the blowfly *Lucilia sericata* to develop at a slower rate than in uncontaminated carrion, when reared upon rabbit tissue treated with less than 50.0mg/h of morphine via ear perfusion of morphine chlorhydrate. Methamphetamine has been shown to accelerate the growth rate of *Parasarcophaga ruficornis* (Sarcophagidae) by between twenty four and sixty six hours, although past sixty hours the growth rate is slowed. The results showed that if unaccounted for, methamphetamine can alter *post-mortem* intervals in the larval stage by up to eighteen hours and by two full days if the specimens collected are in the pupal stage. It was also shown that larvae made it to their maximum length faster, and that this maximum size was smaller than in untreated larvae. Mullany *et al* (2014) investigated the effect of methamphetamine as well as its primary metabolite in humans, p-hydroxymethamphetamine on the development of *Calliphora stygia,* an Australian blowfly. Mullany *et al* (2014) discovered that the larval stages were significantly shortened, and the

size of all life stages was increased. The pupal stage took up to 78 hours longer to complete. According to Introna *et al* (2001) phencyclidine has been shown to reduce the length of the post feeding larval stage of *P.ruficornis,* although the pupal stage was shown to be increased in duration. Introna *et al* (2001) states the *P.ruficornis* larvae develop more rapidly between twenty four hours and one hundred and fourteen hours when reared on carrion treated with the party drug ecstasy. It was also noted that pupation took place at one hundred and ninety hours, with pupae having a maximum length 0.9mm less than control larvae. The synthetic opiate methadone was shown to affect the pupal mortality of *Lucilia sericata* significantly (P=0.02), with percentage emergence rising with methadone concentration.Slower development in *Lucilia sericata* was also observed. Emerged flies did not appear to have a distinct gender bias when exposed to methadone. It was also shown that although methadone can be extracted from pupal cases no linear relationship between the concentration of the corpse and the methadone obtained from the pupa could be obtained (Grosselin *et al,* 2011) The antidepressant amitriptyline, was shown to have no significant effect on the growth of *P.ruficornis* larvae to maximum size, although the post feeding stage of the larvae was increased to a significant level, so that an uncorrected *post mortem* interval estimate could have an error of approximately seventy-seven hours. George *et al* (2009) demonstrated that *Calliphora stygia* is not significantly affected by the presence of morphine in a corpse, and argued that *C.stygia* is a one of the most accurate carrion invertebrates with which to estimate *post-mortem* interval. This was further investigated by Parry *et al* (2011) who discovered that *Calliphora stygia* actively secretes morphine from the Malpighian tubules and suggested that the rate of secretion explains why *Calliphora stygia* appears to be unaffected by the presence of morphine. It was also shown that *Calliphora stygia* larvae metabolises morphine into a compound which is as yet uncategorised.

As previously stated, Chick *et al* (2008) have questioned the relevance of research conducted upon non-European faunas to the UK based researcher. Chandler (1998) does not list either *C.stygia* or *P.ruficornis* as British species, and in addition only one representative of the sub genus *Parasarcophaga* is listed as occurring in Britain. Furthermore Rognes (1991) does not list *C.stygia* as occurring in the Palaearctic, nor does Pape (1987) list *P.ruficornis* as a Palaearctic species within his catalogue. It could also be argued that the family Sarcophagidae as a whole is of limited interest to the British researcher, as Chick (2010a) placed the family in the second wave of decomposition after the Calliphoridae and Muscidae in Britain.

Zou *et al* (2013) investigated the effect of ketamine, an anaesthetic and commonly abused recreational drug on the development and pathology of *Lucilia sericata*. They discovered that while ketamine did not have an effect on the body length or weight of *Lucilia sericata*, the larvae developed at a faster rate. Zou *et al* (2013) also found that the growth of trophocytes in the fat bodies of the larvae could be caused by the presence of ketamine.

Entomotoxicology is also useful to the forensic toxicologist. Once again to consider toxins in the cadaver as pollution, the ecological principle of bioaccumulation may be observed, Purves *et al* (1998) note that toxins are ingested and concentrated as they move up food chains. Beyer *et al* (1980) provided the first technical note upon the extraction of drugs from insect larvae. The paper remarked how in a case involving a the suspicious death of a 22 year old female whose organs were too badly decomposed to serve as sample material for toxicological analysis, the larvae of *Cochliomyia macellaria* which had been feeding upon the corpse were tested, and it was determined that Phenobarbitol had been taken by the deceased prior to death. A further case report was communicated by Kintz *et al* (1990) who commented that in five cases where blood or urine was impossible to collect for toxicological analysis the authors were able to detect the presence of Triazolam, Oxazepam, Phenobarbitol,

Alimemazine and Clomipramine in fly larvae, albeit in smaller concentrations than would be expected from human tissues. Carvalho *et al,* (2001) investigated whether diazepam is detectable in the larvae of Brazilian *Chrysomya* species, and if the drug had any effect on the growth of the test species. It was determined that larvae developed at a more rapid rate between a period of 18 and 54 hours, whereas the duration of the pupal stage was significantly increased. Using gas chromatography-mass spectrometry, it was possible to detect diazepam in almost all of the test larvae. It has also been show that the psychostimulant methylphenidate can be extracted from *Lucilia sericata* feeding on mammalian brain tissue using Liquid-Liquid extraction followed by Liquid chromatography mass spectrometry. The authors expressed hope that related chemicals could be tested using such a method (Bushby *et al*, 2012). However Sadler *et al* (1997) in a study of the effects of barbiturates and analgesics on the larvae of blow flies, found that similar pharmaceutical compounds had different effects on the larvae suggesting that it is not possible to predict which chemicals will be detectable in necrophagous larvae based upon chemical structure,. Gosselin *et al* (2011) remarked although entomotoxicology has been in use since the 1980s providing evidence in actual criminal cases, there are several scientists who regard forensic entomotoxicology as little more than a laboratory curiosity at best. However it has been previously shown that in cases of advanced decay, insect larvae provide a more reliable toxicological sample than putrefied material, as drug concentration appears to be more stable in insects (Gosselin *et al*.2011). Karch (2008) concurs that in cases of advanced decay, insect larvae are a novel toxicological specimen, but also notes that larvae need to be preserved as soon as possible to prevent them from metabolising and bio-eliminating any drugs which they may have ingested. Further work is also required into the best selection sites of cadaver insects to be used as toxicological samples (Karch, 2008).

In a study concerned with the extraction of Amitriptyline and Nortriptyline from cadaver insects, Miller *et al* (1994) remarked that insects associated with mummified human remains may also bioaccumulate cadaver pollution. In the infancy of forensic entomotoxicology, research concentrated on the soft bodied larvae of primary colonisers. However as the ability to extract toxins from hair developed, similar attention was turned to the chitin rich remnants of Phoridae pupae and Dermestidae exuviae, as both hair and chitin are high in protein which must be digested by the invertebrates before toxins can be extracted. Miller *et al* (1994) also noted that both pupal cases and exuviae persist long after the insect which has produced it has migrated from the carrion. Bourel *et al (*2001) also showed that traces of morphine can be extracted from dried remains of adult *Lucilia sericata.* Although at a lower concentration than would be found in a corresponding pupal casing, it was noted that dried pupal cases and desiccated dead adult insects will persist for a long time in the environment, thus increasing their possible importance for a forensic investigation. Sadler *et al* (1995) noted that when *C.vicina* was fed on drug-laden tissue from 3 suicide victims involving the use of amitriptyline, temazepam and a combination of trazodone and trimipramine, the dipteran larvae were capable of metabolising the drugs. Levels of the drugs decreased during the post feeding stage of development, or if the larvae were moved mid-life cycle to drug free tissue. Sadler *et al* (1995) suggested that the forensic practitioner should collect larvae actively feeding on the corpse as a matter of preference if toxicological analysis was to be carried out using insect larvae, as they most certainly had ingested any toxins which may be in the corpse. Karampela *et al* (2015) developed a sufficiently sensitive and simple Liquid chromatography/ Mass spectrometry (LC/MS) protocol to detect THC and its primary metabolite THCA from Cannabis in the larvae of *Lucilia sericata*.

Oliveria *et al* (2014) attempted to utilise near infrared spectroscopy (NIRS) to detect and identify flunitrazepam in the larvae the blowfly of *Chrysomya megacephala.* The advantage

of NIRS over traditional toxicological methods such as GCMS and LCMS is that NIRS is non-destructive and portable.

1.7 Toxins other than drugs of abuse

Drugs of abuse are not the only cadaver pollutants that can affect the delicate carrion ecosystem. Charabidze *et al* (2009) suggest that various household products appear to affect the way in which *C.vicina* responds to the cadaver odour of carrion. In particular perfume, hydrochloric acid, citronella and paradichlorobenzene appeared to produce a significant repellent effect on female flies. Aubernon *et al* (2015) investigated the effects of chemical contamination of carrion, using bleach, caustic soda, patchouli perfume, mosquito repellent, insecticide and unleaded petrol. All of the treatments used appeared to increase the development times of *Lucilia sericata.*

Nuorteva and Nuorteva (1982) investigated the bioaccumulation of mercury in sarcosaprophagous insects in Finland; they found that carrion insects have a high tolerance for mercury and effective mechanisms for the elimination of inorganic mercury. Test flies had a high mercury concentration of up to 62.4ppm of their fresh weight, suggesting a high resistance, most likely due to evolutionary adaptation to the toxin. They argued that given the place of carrion insects in the food chain, a high tolerance to bioaccumulated toxins could have been an evolutionary selection factor, allowing carrion flies to feed upon mercury laden carrion such as fish predating birds. Since when non-carrion frequenting insectivorous beetles were fed the remains of the mercury laden flies, they showed symptoms of mercury poisoning Nuorteva and Nuorteva (1982) stated that this proves necrophagous insects have developed an evolutionary tolerance, in this case to mercury. Gunatatilake and Goff (1989) noted that during a legal case involving the suspected suicide by use of Malathion, a member of the organophosphate family of insecticides, it was argued that the presence of the

insecticide altered succession. This excluded certain taxa that would have been expected on at the successional stage of the carcase, such as Muscidae, Milichiidae and Fanniidae which would have been expected based up the level of decomposition. However it was suggested this may have also been due to possible accelerated development of the larvae in response to the toxin. Malathion was also detectable in the larvae of *Chrysomya* species feeding upon the corpse.

In a study from Kalantan, Malaysia it was suggested that the presence of Malathion on or within a corpse can delay oviposition by 1-3 days and may prolong the pupation period by 2-3 days by the species *Chrysomya megacephala* and *Chrysomya rufifacies.* It was suggested that Malathion is one of the most common poisons used as a suicide agent in Malaysia (Mahat, *et al*, 2009). However it is worth noting that *Chrysomya* is not a U.K. genus (Chandler, 1998) and the data from those case studies might not be applicable to U.K. fauna or climate.

In an investigation based in Nottingham U.K., Chick *et al* (2008) suggested that malathion inhibits initial colonisation of carrion and delays decomposition by approximately 6 days, but overall carrion treated with malathion decomposes at an accelerated rate. It is argued that this may be due to reduced competition on the carrion, leading to increased efficiency of decomposition by primary colonisers. Another explanation was offered by Erzinçlioglu (1996) who notes *L.sericata* develops at a slower rate on sterile tissue, suggesting that certain bacteria and yeasts appear to have a synergistic effect on larval growth. This would agree with Odum's (1967) remarks regarding "hormone-like substances" that increase the rate of decomposition. Erzinçlioglu (1996) also noted that Calliphoridae secrete antibiotic substances that reduce the growth of bacteria on carrion, whilst Cotter and Kilner (2010) commented that the burying beetle *Nicrophorus vespilloides* also secretes antibacterial chemicals to protect their young. With this in mind it is suspected that the initial delay in decomposition allows

for the bacteria to produce sufficient “hormone-like substances” to catalyse the decomposition when invertebrates eventually colonise the body. A final consideration is that bacteria break down proteins to liquids within the body by the release of enzymes from lysomes by the process of autolysis. Organs such as the pancreas, which is full of disgestive enymes, break down rapidly, producing gases such as hydrogen sulphide, methane and sulphur-containg organic molcules known as mercaptans (Gunn, 2006), so it is possible that this protein ‘soup’ is easier for larval blowflies to consume than fresh tissue, and as such a slight delay in oviposition creates a more palatable corpse.

With respect to other insecticides, Chick *et al.,* (2008) found that pyrethrum-based flea spray appears to reduce the rate of decomposition, and a fly spray containing a mixture of synthetic analogues of Pyrethrum and synergists, substances that increase the effect of other materials (Walker, 1999), appeared to inhibit decomposition for 6 days. This led to reduced weight loss rate of carrion. Chick *et al* (2008) also discovered that flies avoided areas treated with pesticides and changed their eating patterns in relation to said treated areas.

Oviposition behaviour was also affected by the presence of insecticides by Chick *et al* (2008) and the study maintains that on treated carcases the traditional oviposition sites of the natural orifices were avoided if treated with insecticide. Single egg laying was also observed on the test carrion as opposed to the more typical clumped egg laying seen on control carcases. The presence of *Abax parallelepipedus* on carrion, when it is referred to in literature as a scavenger on dead invertebrates could also be considered an indicator of toxins within a corpse, or could be due to decreased predator competition with less insecticide tolerant species

Biological indicators of pollution within ecosystems have been shown to be a reliable methodology by Woodiwiss (1964) who demonstrated how water quality in rivers can be determined by the fauna of the water course. Certain species are pollution tolerant, others

pollution intolerant, this being the basis of water quality checks upon the river Trent. Guglielmone *et al* (2001) studied the toxicity of the pyrethroids cypermethrin and diazinon on the Muscid fly *Haematobia irritans* also known as the horn fly. *H.irritans* is a semi-ectoparasite of cattle rather than a forensically important carrion breeding fly (Skidmore, 1985). It is rarely obtained by entomologists, as adults only leave horns of cattle which they cluster around, to oviposit upon dung (Fonseca, 1968) while it is still at body temperature (Skidmore, 1985). Guglielmone *et al* (2001) highlight a developing resistance to cypermethrin by *H.irritans* along its South American range and the idea of geographically dependent resistance could be of use to forensic entomologists in future research. The effect of pesticides on oviposition behaviour has also been observed in Grey tree frogs. Takahashi (2007) states that significant numbers of breeding pairs of these frogs avoid oviposition in spawning pools which have been treated with Glyphosate herbicides.

Chemical cues appear to be important to the cadaver fauna and Müller *et al* (2003) outline that burying beetles distinguish their mate from other burying beetles by the presence of cuticular chemicals obtained from the cadaver. It was shown that if a male's cuticle was washed with pentane, the previously tolerated male would be aggressively attacked by his mate. Fockink *et al* (2015) also found that males of the carrion beetles *Oxelytrum erythrurum* (Coleoptera: Silphidae) produced two volatile compounds, believed to be components of the pheromones used for sexual communication. However, Hanley *et al* (2009) argue that in field-based tests cuticular hydrocarbons had no significance on the trapping of the housefly *Musca domestica*, although it may react to other chemical stimuli. It was shown that in cases of heifer mastitis, bacteria responsible for the infection cause infected cattle to secrete volatile compounds which increase the attractiveness of the cattle to the Muscid fly, *Hydrotaea irritans* which acts as a vector of heifer mastitis (Thomas, *et al.*1985).

Dekeirsschieter *et al* (2009) proclaimed that the volatile organic compounds released from a corpse *post mortem* are the intermediate products of decomposition. They stated that as the corpse decomposes, the volatile organic compounds are broken down into smaller organic compounds so the pattern of volatiles changes as the corpse breaks down. No volatile organic compounds were detected during the fresh stage of decomposition; by the start of the bloat stage alcohols and sulphur based compounds are present. The active decay stage has the strongest olfactory signature and also contains the most compounds including phenol and a large number of organic acids such as butanoic acid. By the advanced decay stage the proportion of aldehydes has increased as the soft tissues have been removed. Given that each stage of decomposition has its own particular scent; it would appear that each species has its own set of chemical cues which attract them to the carrion. Von Hoermann (2012) showed that male *Dermestes maculatus* can differentiate different stages of decomposition and are attracted to carrion during the post bloat stage by the presence of benzyl butyrate in the odour bouquet. It was shown by further analysis that benzyl butyrate is present in the post bloat stage in higher concentrations that any other stage. However females appear to show no reaction to volatile organic compounds, so it was hypothesised that males act as pathfinders, and use a pheromone signal to attract females to a suitable breeding site (Von Hoermann *et al*, 2011). However the corpse itself is not the only thing that secretes chemicals. Staphylind beetles, as Dettner and Reissenweber (1991) reported, release defensive secretions. Dettner and Reissenweber (1991) investigated the chemistry of the subfamilies Omaliinae and Proteinae defensive secretions and stated that both subfamilies are traditionally considered to be closely related, based upon morphological characteristics and their saprophagous habits. Both subfamilies will feign death when maltreated, and discharge a secretion from an abdominal gland in reaction to any further mechanical stimulation. This discharge has a strong characteristic odour, which the beetle may distribute with its legs. The secretion has no

effect on members of the same species but appears to be used to frighten off predators. Max Barclay of the British Natural History Museum (Pers. Comm., 19/1/2010) remarked that when the staphylinid beetle *Creophilus maxillosus* has been on carrion, a dog will not approach the carrion, a behaviour which could have an effect on the use of cadaver dogs in criminal investigations. Dettner and Reissenweber (1991) found that the secretions of Omaliinae and Proteinae contained acids and aldehydes as active ingredients dissolved in esters, to improve penetration of the target organisms' integument. Furthermore Gnanasunderam *et al* (1981) found that the biochemical composition of some New Zealand staphylinid defensive secretions differs from those found in Australia. This has been observed in lab cultures and other invertebrates. It was suggested that staphylinid defensive secretions may change due to diet, or the beetles may evolve different compounds in different populations, due to environmental differences or differences in predators within differing ecosystems.

O'Hea *et al* (2010) suggest that species of *Aphodius* dung beetles are affected by the presence of the veterinary medicine Ivermectin in cow dung. The study states that the drug reduces the larval development rate and causes significant reductions to cohort size of the beetles. However there were no significant effects observed on the adults and this could be due to the differences between larval biology and adult biology as suggested by Chick *et al* (2008). Smith (1986) stated that many dung beetles including *Aphodius* construct tunnels under cadavers.

1.8 Nicotine and related compounds

Seckar *et al* (2008) stated that volatile nicotine is released into the environment during the production of cigarettes, and into the atmosphere when smoked, plus from waste water, as well as landfill, with tobacco dust being added to both fertilizer and compost. Secker *et al*

(2008) also suggested that environmental nicotine toxicity to both aquatic and terrestrial species is low. However it is noted that the authors work for the research and development department of R.J. Reynolds Tobacco Company, which many be construed as a conflict of interests.

Vickery and Vickery (1981) defined nicotine as an alkaloid and secondary plant metabolite. Carlile (2006) suggests that nicotine most likely evolved as a defensive mechanism by tobacco to prevent herbivory by insects. According to Shivastava and Saxena (2000) nicotine was first recommended as natural insecticide by Erasmus Darwin in 1763. Reay (1969) stated that it is the oldest of the botanical insecticides and while other alkaloids are present in the leaves of *Nicotiana* spp, none has enough merit to warrant extraction in favour of nicotine. Nicotine was expansively used as an insect control agent in the late nineteenth and early twentieth centuries in the forms of sprays and washes. In glass houses, where access can be restricted inside the enclosed structure, nicotine was burned in the form of shreds, which proved very effective (Carlile, 2006). Reay (1969) also maintained that while nicotine shows no herbicidal toxicity, it is an extremely potent toxin to mammals, with an LC_{50} of $55mg/kg^{-1}$ on rats. Roberts and Hutson (1999) stated that it is used to control insects including, but not limited to aphids, thrips and whitefly in a range of crops as well as glasshouse ornamentals.

Nicotine's mode of insecticidal action is primarily respiratory, however contact and stomach action is also observed (Roberts and Hutson, 1999). Carlile (2006) remarks that nicotine will rapidly penetrate the cuticle of the target organism, and can be used effectively as a contact insecticide; however this property will also affect warm blooded animals. Nicotine binds to nicotinic acetylcholine receptors at the synapses of insects, but is not broken down by the enzyme acetylcholinesterase. This leads to hyperexcitation followed by twitching and convulsions, and finally death of the insect (Carlile, 2006). Richardson and Gangolli (1994) noted that nicotine decomposes quickly under the influence of light and air, and has an EC_{50}

of 0.24mg 1^{-1} when tested upon *Daphnia magna.* As previously stated by Beketov and Liess (2007) Diptera may prove less toxicologically tolerant than other insect orders. Tomlin (2006) remarked that while nicotine is toxic to bees, it is also known to have a repellent effect upon them. This repellent effect is used by urban birds in Mexico who appear to be using smoked cigarette butts as part of their nesting material. It was suggested that the birds incorporated the cigarette butts to repel arthropod parasites. Use of plants which expel volatile compounds to repel parasites had been observed in wild birds previously. From this, a lab based study showed that smoked cigarette butts had a repellent effect on parasites, un-smoked butts did not (Suarez-Rodriguez *et al* 2013).

Nicotine has now mostly been replaced in agriculture due to its low persistence and hazards during use (Roberts and Hutson, 1999). During the mid-1990s neonicotinoids were introduced, which have a similar mode of action to nicotine, are systematic in plants and were seen as safer than organophosphates. Neonicotinoids also bind to the acetylcholine receptor in insects as does nicotine. However the non-target effect on vertebrates is much lower than nicotine as neonicotinoids are highly selective for insect receptors. The mechanism for this specificity is poorly understood but its low toxicity to vertebrates meant that synthetic pesticides in the neonicotinoid groups were seen to be one of the safest neurotoxic insecticides (Carlile, 2006).

As well as its use as a pesticide, nicotine is a widely used psychoactive drug, the use of which has been traced back as far as the Mayan culture of circa 600AD (Wonnacott *et al*, 1990).

Wonnacott *et al* (1990) outline the concentrations of typical nicotine contained within various tobacco products, and the amount typically consumed daily by a nicotine addict with 168mg of nicotine per 20 cigarettes daily. Nicotine has a high potency due to the normal method of delivery being smoking which allows for rapid access to brain neurons *via* the pulmonary vascular system. It has been suggested that nicotine can alter the excitability of neurons in the

central nervous system, both directly and modulatory, although further study is required (Deadwayler, *et al* 1993). Smoking has been linked to several types of illness, some of which are fatal and it is estimated that millions of people have died of illnesses related to smoking. However, nicotine has also been shown to be beneficial in the treatment or ulcerative colitis and Tourette's, as well as evidence to suggest that smokers are less likely to develop Parkinson's disease (Yildiz, 2004). In contrast, Allen *et al* (2015) link the use of electronic cigarettes and "pop corn lung" or servere bronchiolitis which can result in a servere loss of pulmonary funcution requiring a lung transplant.

Hafezi *et al* (2001) stated in a review of 100 illicit drug related deaths that 98% had also consumed nicotine prior to passing. Forty four percent also demonstrated yellowing of the finger tips, a sign of habitual smoking, 35% had symptoms of bronchial disease, 17% showed evidence of siderophages and 6% showed evidence of burns related to cigarette smoking, suggesting that in entomotoxicology cases involving illict drugs, nicotine is also likely to be present.Tracy (1984) suggests that nicotine acts as one of the most toxic and rapid poisons, and notes that "green tobacco sickness" has been observed in tobacco harvesters. McBribe *et al* (1998) stated that green tobacco sickness is a form of nicotine poisoning which affects those that come into direct contact with tobacco plants, such workers during harvesting and cultivation. The symptoms are similar to those presented by pesticide exposure, heat exhaustion or nicotine intoxication such as that experienced by novice smokers. McBride *et al* (1998) stated that green tobacco sickness is not typically associated with mortality or long term morbidity, and is typically seen as significant discomfort. Tracy (1984) also suggests that an adult human can be killed by an estimated dose of nicotine as low as 30mg (0.5mg/kg). However it is also stated that smokers demonstrate a marked tolerance to the alkaloid, so much so that there have been case reports of a game warden (and habitual smoker) who survived accidentally shooting himself with a tranquiliser dart containing over 3

times the upper estimated fatal dose (3.6mg/kg) of nicotine (Tracy, 1984). The literature also records a victim surviving a dose of 2g of nicotine by a confirmed smoker (Tracy, 1984).

Wexler (1998) outlined that while Nicotine is highly toxic, few deaths have been reported and also states that survival after ingesting doses of 4g have been reported. Tracy (1984) also maintains that tobacco appears to be less toxic to humans than its nicotine content would suggest, although contamination of infant formula with tobacco has proven fatal in the past.

It has been shown that children who die of sudden infant death syndrome (SIDS) tend to have higher concentrations of nicotine in their lungs compared to control children based upon biochemical assays. This higher concentration appears to be regardless of whether or not smoking is reported in the victim's house. This may be explained by exposure to environmental nicotine (McMartin *et al*, 2002). It has been suggested that infants affected by SIDS have either an environmentally acquired or latent defect that leaves them at risk of SIDS if exposed to nicotine, either actively in a smoking household or passively in the larger environment (Machaalani *et al* 2011). Nicotine has been discovered in the hair of infants that have been subjected to passive smoke in significantly higher levels than those of a control group and it has been suggested that hair sampling could be a non-invasive way of monitoring infant nicotine exposure (Pichini *et al* 1997). It is preferable to collect hair for analysis from the posterior vertex which is located at the back of the skull, where hair growth is relatively constant (Karch, 2008). Fenton (2005) suggests toxicological screening of hair is often impractical for forensic cases for several reasons, including low concentrations of toxin stored in hair, and controversy surrounding passive exposure verses active use of drugs. Karch (2008) argues that while the use of hair samples as toxicology specimens for live humans is controversial, this is far outweighed by its usefulness *post mortem*. Segments of the hair can be analysed to provide a temporal analysis of drug use or poisoning, including

periods of abstinence. Indeed Fenton (2005) states that drugs are persistent in segments of hair until it is cut.

Baselt (2004) remarked that virtually every member of society is exposed to nicotine in the air from smokers. It is also noted that 80%-100% of nicotine is presented to the upper respiratory trace by deep lung inhalation, and that it is present in a number of smoking cessation therapies. A report in the Daily Telegraph (2009) stated that a boy of fourteen collapsed with stomach pains and was hospitalised after chewing and swallowing 30 pieces of low strength nicotine gum in a one hour time frame. The boy survived consuming the equivalent of two days' worth of nicotine. Wexler (1998) stated that the symptoms of acute nicotine poisoning include nausea, vomiting and abdominal pain. Lethargy and coma may follow. Chronic use produces dependence, cessation can cause depression, hostility, anxiety and sleep disturbance. Chronic use may also lead to increased tolerance to nicotine (Wexler, 1998). Moriya and Hashimoto (2004) stated that suicidal smokers consumed more cigarettes than non-suicidal smokers prior to death, possibly due to lower serotonin function. Nishimura *et al* (2009) used the knowledge that suicidal smokers have raised nicotine levels along with other circumstantial evidence, such as a driving licence concealed in a sock, to determine that an adipocere body of a 42 year old man found in the sea off Japan had indeed committed suicide. As well as being an abused drug and accidental poison, nicotine has been implicated as a causative agent in suicides as in the above example. Chaturvedi *et al*, (1983) reported the use of the drug in a multi-chemical suicide, along with caffeine and Malathion in which a cocktail of the aforementioned toxins was ingested by a 21year old male found in a shower in North Dakota. Solarino *et al* (2010) outlined a case report in which the corpse of a 42 year old man was found in his garage and toxicological analysis failed to disclose any lethal agents other than a high nicotine level.

Corkey *et al* (2010) focused on a case report of two suicides in the United Kingdom in which nicotine was extracted from tobacco. The first case was that of a 19 year old male student in 2008. A document on the victim's personal computer was titled "death" and contained instructions from an internet site detailing the extraction of nicotine from tobacco to create a toxic drink. The second case involved a 32 year old male who was found lying face up on his bathroom floor, 400ml of a brown fluid was found in his stomach, mouth and airways. On his personal computer was found a suicide note which detailed that he felt he was incapable of earning money and that he had taken an overdose of nicotine. It was determined the deceased had used a website which quoted the anarchists' cookbook and a jar containing almost pure nicotine was found in the deceased's house. Moriya and Hashimoto (2005) relate the case report of a 35 year old male outpatient with psychiatric disorders in Japan who was found in a prone position in his home. Artefacts were found around the corpse including a cup of blue liquid and medicine package indicating a suicide. The victim was pronounced dead on arrival and white foam was noted around the mouth. At autopsy the stomach was found to contain shredded tobacco leaves suspended in 170g of greenish liquid. A toxicological screening revealed the presence of benzodiazepines, methomyl, nicotine, cotinine and triazolam in the stomach. The nicotine levels were found to be below the fatal blood levels but were higher that typical concentrations for a habitual smoker, and as methomyl and nicotine both cause respiratory paralysis it was theorised that the two compounds might have had a synergistic effect on each other. A 46 year old male was found dead in his bed in Germany. A suicide note was discovered next to his body, alongside two half empty bottles, one of which contained a black liquid with accompanying sediment. The body was covered with 25 transdermal patches each containing a 7mg dose of nicotine. No evidence of violence or external injuries was apparent on the corpse and analysis of the bottles found at the scene showed they contained tobacco and nicotine in the black sediment. Diphenhydramine and

zopiclone were found in the second bottle. A toxicological report showed the presence of nicotine, diphenhydramine, paracetamol and tramadol in the urine of the deceased. The diphenhydramine and paracetamol levels were in the therapeutic range, thus it was determined that tramadol and nicotine interacted to increase depression of the central nervous system leading to heart failure (Solarino *et al* 2010). However Karch (2008) stated that nicotine is only quantified in toxicological analysis when it is believed to be a causative agent in a suspicious death or if an abnormally large amount is detected in the preliminary screen process. Beketov and Liess (2007) suggested that the related neonicotinoid insecticide Thiacloprid causes delayed toxic effects to aquatic Diptera, even at low concentrations. However, they noted that the effect on fresh water crustaceans was more pronounced. It was also observed that dipteran larvae were highly sensitive to the insecticidal qualities of Thiacloprid. The European Food Safety Authority (EFSA, 2013a,b,c) compiled risk assessments that various neonicotinoid insecticides pose to European bees. It was found that neonicotinoids can be found in the nectar and pollen of plants, although the reports noted that data in other areas relating to non-target toxicology of neonicotinoids was lacking and further research was required. Neonicotinoids have previously been considered as a possible cause of Colony Collapse Disorder (CCD), a phenomena in which entire groups of bees appear to abandon their hives, Curiously the hives are not attacked by traditional honey robbing species such as wax moths, hive beetles or even other bees, even after the colony is abandoned (Benjamin and McCallum, 2008). Following the EFSA reports the European Union moved to tighten restrictions on neonicotinoids in Europe for two years, the decision divided both scientists and member nations. The restrictions included:

1. Prohibition of seeds pre-treated with neonicotinoids.
2. Ban on the use of neonicotinoids by amateurs.

However these restrictions do not apply to crops deemed non-attractive to bees, and winter crops (BBC, 2013, EFSA, 2013a,b,c).

Given the records in the literature of the common use of nicotine as a poison, an environmental hazard, and a substance of abuse which appears to have increased usage during times of stress, and given its insecticidal properties, it would be wise to investigate the effects it has as a form of cadaver pollution on carrion invertebrates. Thus it was hypothesised that the presence of nicotine within a corpse affects the behaviour and development of the carrion invertebrate fauna.

1.9 Aims Objectives and Outcomes

The overarching aim of this work was to investigate the effects of nicotine on the decomposition of animal carrion and to consider the possible implications such data would have upon forensic investigations.

To achieve this aim the following objectives were proposed:

1. A site was selected which was be viable for carrion placement in terms of security and ecological richness of carrion frequenting invertebrates. By use of baseline study this site was assessed prior to carrion placement.
2. Using porcine material treated with nicotine, the effect of nicotine on the carrion micro-ecosystem will be investigated in the field.
3. The field investigation will be repeated to account for differences in seasonal lifecycles of certain invertebrates.
4. The effect of nicotine on the life cycle of a named primary coloniser will be investigated under controlled laboratory conditions, including evaluating the fecundity and survivability of that species in relation to nicotine.

5. The lab-based data will be analysed to create a dose response correction that allows forensic practitioners to adjust *post-mortem* interval estimates in relation to nicotine.

Outcomes

The expected outcomes of this investigation are to:

1. Improve the accuracy of prediction of *post mortem* interval in forensic cases involving nicotine.
2. To add new knowledge to the field of applied entomotoxicology.
3. To offer new insight to the forensic and policing communities so they can fully evaluate entomological evidence.
4. To offer a predictive methodology from which other drugs and toxins can be considered in future investigations.

Chapter 2: Baseline study

2.1: Introduction and rationale

A two- part baseline study was carried out at several sites, with the aim of determining possible locations for field-based carrion investigations, and also to show the natural fauna of the sites prior to carrion placement. Suitability was defined by the following parameters where a site:

- Should contain both decomposer invertebrates, and non-decomposer invertebrates.
- Would not be visible to passers-by due to the sensitive nature of the experiment, or the possibility of vandalism.
- Would have to offer concealment of an investigator to passers-by, for the reasons considered in point 2.
- Should be low in vertebrate scavengers, or the effect of vertebrate scavengers would have to be minimised.

Four possible sites were identified on the Clifton campus of Nottingham Trent University (Figure 2.1)

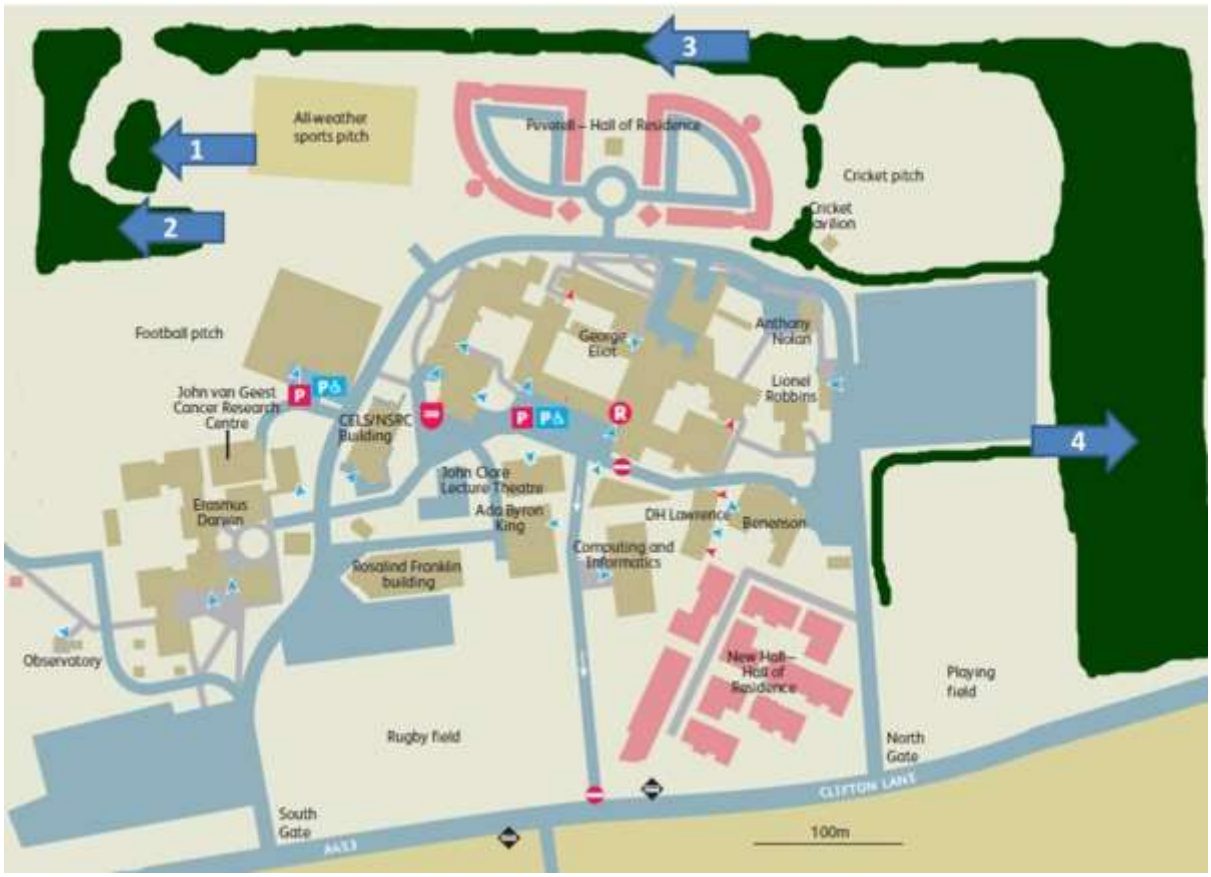


Figure 2.1: Modified campus map showing the relative positions of the sites selected for baseline studies.(Modified from https://www.ntu.ac.uk/map_files/4233.pdf)

Table 2.1 Ordinance Survey grid references of the Clifton Campus Baseline Sites.

Site 1	SK547353
Site 2	SK546353
Site 3	SK551345
Site 4	SK548354

2.2. Site descriptions:

Sites were selected based upon the parameters previously stated, regarding security and invertebrate fauna. Woodland offered the most natural privacy required for the placement of carrion, and as can be seen in Figure 2.1 Clifton campus contained a number of wooded areas which were deemed suitable for such study.

Site 1: SK547353

A small plantation of managed woodland in the grounds behind the Erasmus Darwin building. It was obscured from view on 3 sides by surrounding woodland, and by a hill on the fourth side. The site offered multiple entrances to a clearing where test equipment was placed and was dense enough to reduce visibility into and out of the woodland, until the 1st of February 2010 when the understory of the woodland was cut back (see autumn nicotine investigation for more details).

Site 2: SK546353

A large managed woodland site that made up the woods surrounding site one. It occupied the area between the Erasmus Darwin building and Site 1. It offered multiple concealed entrances as well as dense woodland that provided good cover combined with occasional clearings to place the carrion.

Site 3: SK551345

A dense woodland site situated between the student car park and the boundaries of the Clifton campus. Previously used by Chick *et al* (2008) for summer carrion research, the site offered two entrances, one of which is concealed by a second woodland, the other somewhat obscured by fencing. The site contained one clearing in which to place carrion.

Site 4: SK548354

A woodland site behind the Peverell halls of residence, which had a concealed entrance but offered less cover than sites 1-3, and only offered a small clearing.

2.3 Methodology

Phase 1 (Non-Carrion Studies)

The four sites illustrated above were passively sampled between the 1st of October 2008 and the 1st of March 2009 using un-baited pitfall traps in the manner described by Cooter (2006). A small plastic container, roughly the size of a vending machine drinks cup, containing glycol-based preservative was sunk into the ground with its top level with the ground, and a small roof added to prevent ingress of water. At each site 6 pit fall traps were placed. Samples collected from pitfalls were preserved in accordance with current entomological techniques (see curation) dependent upon order and family collected.

Phase 2 (Carrion Baseline)

Sites 1 and 2 were determined to be suitable for carrion placement in rotation (to allow for recovery of soil fauna and chemistry). Between the 2nd of March 2009 and the 1st of August 2009 the viability of the carrion fauna of these sites was tested using bones from a local butchers' and rat carrion (*Rattus norvegicus* Berkenhout). These were placed in anti-scavenger cages fashioned from steel mesh baskets in the style described by Chick (2008), who noted that the presence of vandals and/or vertebrate scavengers can affect, impede or even completely terminate the collection of invertebrates and data from carrion. These cages were surrounded by pitfall traps as in Phase 1 following Chick (2010a and 2010b). Webb (2012) argued that pitfall trapping misses some soil dwelling carrion species, since sieving

the soil under the carrion may collect approximately 17 species that pitfall trapping may miss including members of the Histeridae, Dermestidae and Staphylinidae , based upon tests carried out in Lincolnshire and Northamptonshire. However Webb (2012) also suggested that there is little difference between overall numbers collected between sieving and pitfall trapping, and pitfall trapping collects certain species that sieving fails to take into account. It was also noted that while pitfall trapping requires more preparation, it is a more pleasant way to collect specimens from carrion (Webb, 2012). This is because sieving requires moving the carrion directly into a sieve along with the surrounding leaf litter and soil which is a rather destructive method. Webb's (2012) objective was to investigate which method works best for total Coleoptera collected and not to study the successive waves of decomposition. Pitfall trapping by comparison uses a small container such as a plastic coffee cup, dug into the ground so the lip of the trap is level with the ground, and a small amount of ethylene glycol is added. A small roof is used to prevent the ingress of rain and small animals while allowing insects to fall into the trap (Cooter and Barclay, 2006). In comparison, carrion can be surrounded by pitfall traps making the technique non-destructive. It was determined that for this study, where the aim was to investigate long term succession on carrion, that the best sampling procedure would be the least invasive since sieving would require moving the carrion and disturbing the ground under the carrion Thus pit fall trapping was selected for use.

Carrion was either placed in the environment in a fresh state, or matured in zip-lock bags prior to exposure to the environment as suggested by Chick (2009) the aim being to allow putrefaction to occur in isolation from the primary carrion colonisers. Allowing for the carrion to effectively skip the first wave of insect carrion colonisers while still undergoing anaerobic microbial decomposition increases the opportunity for the full successional fauna expected of larger carrion to be collected with the smaller rat carcases.

2.3.1 Curation of specimens

Adequate details of basic curation methods can be found in Irwin (2010), Cooter and Barclay (2006), Gullen and Cranston (2004) and Betts and Laffoley (1986). As such, curation methods are only briefly outlined below.

Specimens were preserved in the manner recommended for the taxon in question.

Diptera: specimens were removed from the pitfall fluid and dried using acetone. This volatile solvent allows wet specimens to dry, removes any glycol residue from the specimen and prevents the delicate wings and hairs from sticking to each other or the body of the specimen, which facilities easier identification. Larger specimens were staged on micropins; smaller specimens pointed using a water based Gum Arabic adhesive.

Coleoptera: larger specimens were direct pined; medium specimens and larger Staphylinidae carded on Bristol board using a Gum Arabic paste. Small specimens were pointed on archival quality Bristol board. Specimens with pubescene (small hairs) were soaked and dried in acetone as per the Diptera.

Hymenoptera: larger specimens such as social wasps were staged on micropins and small specimens such as parasitic Braconidae were pointed.

Hemiptera: were pointed to archival card using a Gum Arabic-based mounting glue.

Acari: in early experiments these were slide mounted in Berlese fluid direct from 70% alcohol. However Chick (2010c) recommends mounting in Chick's modification of Dioni's mountant, and later specimens were mounted in this medium. As with all gum Arabic-based media, ringing with nail varnish was required to increase permanence of the slides.

Chick (2010c) states that traditional low refractive index mountants for mites use chloral hydrate as clearing agent in the mounting media. However chloral hydrate is difficult to obtain due to its illict use as "knock out drops", Chick's modification of Dioni's uses lactic acid as a clearing agent while maintaining a lower refractive index than traditional mountants like Canada Balsam required for the study of Acari.

Thus Chick's modification of Dioni's mountant was mixed using the following formula (Chick 2010c):

Powdered Gum Arabic 3g

Distilled water 10ml

Glycerine 5ml

Liquid Glucose 2.5ml

Lactic Acid 6ml

Antiseptic 1ml

The ingredients were combined and gently warmed overnight at 40^{o}C until the Gum had fully dissolved.

Identification of Specimens.

The collected specimens were identified using the literature listed in the Bibliography of Key Works (Appendix 1). Identification was aided using a stereo zoom microscope capable of magnifications between x7 and x80, or compound microscope capable of magnifications of between x40 and x400 depending upon size of specimen and preservation method used.

2.4: Results

During the course of the baseline study the specimens listed in Tables 2:1 to 2:5 were found. They were checked against the literature to see if they were traditionally considered to be associated with carrion. Where the literature on the group was unclear a "possible" result is recorded. The Discussion (section 2.5) elaborates on how the literature records important species.

Table 2.2: Baseline specimen log for site one (with and without carrion)

Order	Family	Species	Carrion association	Phase collected
Diptera	Cecidomyiidae	Unidentified species	No	1
	Tipulidae	Unidentified species	No	1
	Tipulidae	Unidentified species	No	1
	Anisopidae	*Sylvicola zetterstedti*	No	1
	Sciaridae	1 unidentified species	Limited (Smith, 1986)	1
	Mycetophilidae	Mycetopilinae spp	No	1
	Phoridae	*Megaselia* sp (female)	Yes	2
		Metopina oligoneura	Yes	2
	Sepsidae	*Nemopoda nitidula*	Yes	2
	Heleomyzidae	*Suillia flavifrons*	Yes	1
	Dryomyzidae	*Neuroctena anilis*	Yes	1 and 2
	Sphaeroceridae	*Leptocera fontinalis*	Yes	1
		Copromyza equia	Yes	1
		Copromyza stercoraria	Yes	1
	Muscidae	*Helina impucta*	No	1
		Phaonia subventa	Yes	2
		Thricops longipes	No	1
	Calliphoridae	*Lucilia caesar*	Yes	2
		Lucilia sericata	Yes	2
		Calliphora vicina	Yes	2
		Calliphora vomitoria	Yes	2
Coleoptera	Carabidae	*Carabus nemoralis*	Yes	1 and 2

		Calathus rotundicollis	Yes	1 and 2
		Nebria brevicollis	Yes	1 and 2
		Pterostichus madidus	Yes	1 and 2
		Leistus fulvibarbis	Yes	1
		Leistus rufomarginatus	Yes	1
		Abax parallelepipedus	Yes	1 and 2
		Trechus quadristriatus	Yes	1 and 2
	Leiodidae	*Nargus velox*	Yes	1 and 2
	Staphylinidae	*Ocypus olens*	Possible	1 and 2
		Philonthus decorus	Possible	1 and 2
		Tasgius globulifer	Possible	1 and 2
		Lesteva sicula	Possible	1 and 2
		Megalinus glabratus	Possible	1 and 2
		Othius punctulatus	Possible	1 and 2
Hymenoptera	Braconidae	*Alysia* spp	Yes	2
	Vespidae	*Vespula vulgaris*	Yes	1 and 2
Dermaptera	Forficulidae	*Forficula auricularia*	Possible	1
Oribatei (Acari)		Unidentified spp	No	1 and 2

Table 2.3: Baseline specimen log for site two. (with and without carrion)

Order	Family	Species	Carrion association	Phase Collected
Diptera	Lonchopteridae	*Lonchoptera furcata*	No	1
	Clusiidae	Unidentified spp	Yes	1
	Dryomyzidae	*Neuroctena anilis*	Yes	1 and 2
	Sepsidae	*Nemopoda nitidula*	Yes	2
	Calliphoridae	*Lucilia caesar*	Yes	2
		Lucilia sericata	Yes	2
		Calliphora vomitoria	Yes	2
		Calliphora vicina	Yes	2
Coleoptera	Carabidae	*Carabus nemoralis*	Yes	1 and 2
		Carabus violaceus	Yes	1 and 2
		Nebria brevicollis	Yes	1 and 2
		Trechus quadristriatus	Yes	1 and 2
		Pterostichus niger	Yes	1 and 2
	Hydrophilidae	*Megasternum obscurum*	Yes	2
	Leiodidae	*Nargus velox*	Yes	2
	Silphidae	*Nicrophorus humator*	Yes	2
		Nicrophorus vespilloides	Yes	2
	Histeridae	*Margarinotus ventralis*	Yes	2
	Staphylinidae	*Tinatus morion*	Possible	2
		Philonthus decorus	Possible	1 and 2
		Othius punctulatus	Possible	1 and 2
		Ocypus olens	Possible	1 and 2
		Tachinus spp	Possible	1 and 2
	Nitidulidae	*Glischrochilus*	*see description	2

		quadripunctatus	below	
	Curculionidae	*Apion uliseis*	No	1
		Adelognatha spp	No	1
Hymenoptera	Braconidae	*Alysia* spp	Yes	1 and 2
Heteroptera	Lygaeidae	*Kleidocerys resedae*	Possible	1
Dermaptera	Forficulidae	*Foricula auriculuria*	Possible	1
Lepidoptera	Tineidae	*Tineola bisselliella*	Yes	2
Oribatei (Acari)		Unidentified spp	No	1 and 2
Astigmata (Acari)		Unidentified spp	Yes (found on bones)	2
Mesostigmata (Acari)	Parasitidae	*Poecilochirus* spp	Yes, parasite of *Nicrophorus* spp	2

Table 2.4: Baseline specimen log for site three (without carrion)

Order	Family	Species	Carrion association
Diptera	Psychodidae	1 unidentified species	Yes
	Phoridae	*Metopina braueri*	Yes
	Dryomyzidae	*Neuroctena anilis*	Yes
	Muscidae	*Muscina prolapse*	Yes
	Calliphoridae	*Lucilia sericata*	Yes
		Calliphora vicina	Yes
Coleoptera	Carabidae	*Nebria brevicollis*	Yes
		Pterostichus madidus	Yes
		Abax parallelepipedus	Yes
	Staphylinidae	*Tasgius morsitans*	Possible
		Tinatus morion	Possible
		Ocypus olens	Possible
Hymenoptera	Braconidae	*Alysia* spp	Yes
Oribatei (Acari)		Unidentified spp	No

Table 2.5: Baseline specimen log for site four (without carrion)

Order	Family	Species	Carrion association
Diptera	Phoridae	*Metopina oligoneura*	Yes
	Muscidae	*Phaonia subventa*	Yes
		Helina impucta	No
Coleoptera	Carabidae	*Nebria brevicolis*	Yes
		Pterostichus madidus	Yes
	Staphylinidae	*Ocypus olens*	Possible
Heteroptera	Lygaeidae	*Kleidocerys resedae*	Possible
Oribatei (Acari)		Unidentified spp	No

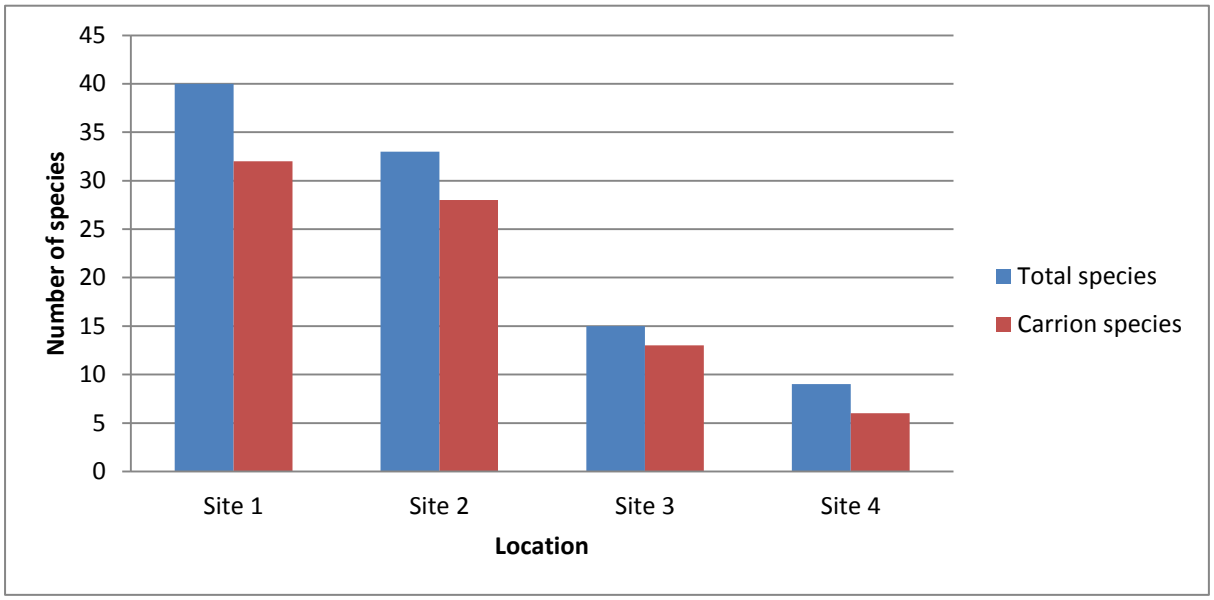


Figure 2.2- Comparative species numbers of the four potential sites during phase 1 and 2 showing total number of species and carrion associated species (Period of collection 1/10/2008- 1/8/2009)

Figure 2.2 shows that the greatest number of species was demonstrated by sites 1 and 2. with sites 3 and 4 showing less than half the species captured in pitfall traps. Figure 2.2 shows that when only carrion-associated species are considered the trend appears to be similar, with sites 1 and 2 having the greatest diversity of carrion-frequenting species with approximately twice as many as site 3 and between 5 and 6 times as many as the number of species collected from site 4.

Tables 2.4 and 2.5 show that carrion-frequenting invertebrates are common upon the Campus even in the absence of placed carrion. Calliphoridae, Muscidae, Carabidae and Staphylinidae appear to be the most species rich families of invertebrates collected from the sites, with a variety of Sphaeroceridae being present at site 1.

2.5 Discussion

The following points relate to families and species found that are carrion-associated, relating to the authority by which they are considered carrion frequenting and in terms of niches occupied within the carrion community.

2.5.1 Diptera: Sciaridae

The Sciaridae are known to occur on carrion (Smith, 1986) but are regarded as being of little forensic important in *post-mortem* interval determination. According to Freeman (1983) Sciaridae larvae will feed upon any decomposing organic material of animal or plant origin, including dung, dead adult Sciaridae, and will even become opportunistic predators of weakened larvae. Smith (1986) remarks that the generalist feeding behaviour of the sciarids leads to them appearing before the forensic entomologist in cases not related to *post mortem* interval investigations. Smith also notes that *Bradysia* species have been found feeding upon seized *Cannabis* and that while difficult due to the cosmopolitan distribution of stored products pests, it may be possible to determine the geographic origin of a *Cannabis* sample using the insects feeding upon it.

2.5.2 Phoridae

Phoridae were found at three of the four sites and while the family is known to breed in a wide variety of decomposing material, numerous genera are associated with decomposing vertebrate carrion. Indeed one member of the family, *Conicera tibialis,* has the common name of "the coffin fly" due to its being frequently encountered on exhumed corpses, although numerous species are known to have similar habits (Smith, 1986). *Metopina oligoneura*, was found at sites 1 and 4. Disney (1983) states the life history of the species is unknown but the species is widespread throughout the British Isles. *Metopina braueri* is considered not as

common as *M.oligonera* in the British Isles but its life history is equally unknown (Disney, 1983). However, Smith (1986) remarked that members of the genus *Metopina* are regularly found in vertebrate carrion in the U.K and Smith adds that the genus *Metopina* often occurs upon buried carrion.

A single female *Megaselia* species was recorded, but could not be identified because in the most current literature on the genus females are inadequately characterised and thus unidentifiable, according to Disney (1989). Smith (1989) states that the genus *Megaselia* comprises over 200 species, which have a wide range of larval habitats. Some *Megaselia* species are associated with general decomposition of organic material (Smith, 1989) and Smith (1986) remarks that some of the *Megaselia* are of forensic importance.

2.5.3 Psychodidae

A single specimen of Psychodidae was collected from site 3. Members of this family are commonly referred to as "moth flies", due to the characteristic hair on the wings of these small Diptera which give them a similar appearance to moths. Smith (1986) suggests that the moth flies are not regular parts of the faunal succession of a corpse, but have been frequently seen upon carrion. The moth flies require semi-liquid to liquid decomposing material to complete their life cycle, as was evident at site 3, which appeared to have poor drainage. Withers (1989) argued that psychodids are opportunistic colonisers of carrion, remarking that abattoir outwashes often harbour great numbers of species of psychodids normally associated with dung, suggesting that such species can tolerate high levels of organic matter and/or anaerobic conditions.

2.5.4 Heleomyzidae

Suillia flavifrons was found at site 1, Smith (1986) remarked that the larvae of this family develop in various forms of decaying organic material, including carrion, suggesting that

adults will arrive at a corpse within the first week, but maggots will not be observed for 3 weeks.

2.5.5. Sepsidae

The Sepsid *Nemopoda nitidula* was found at sites 1 and 2. Smith (1986) considers Sepsidae as a family of forensic importance, stating that larval sepsids will occur in a wide variety of decomposing organic materials. On human carrion Sepsidae are associated with the period between caseic and ammonical fermentation. Gennard (2007) states that dependent upon the location of a crime scene Sepsids may be present in swarms. *N. nitidula* is a very common member of the family often found upon carrion (Pont, 1979). It has a widespread distribution in the United Kingdom, often found in woodland, and described as shade loving. However it is also sometimes recorded in damp meadows (Pont, 1986). Smith (1986) remarked that on carrion *N.nitidula* adults were first observed upon the corpse after 12 days, with second and third instar larvae found 45 days *post mortem*; however,samples of adults have been observed within days of death. As the species is also associated with dung, Smith (1986) advocates examining the environment surrounding a corpse prior to using the presence of *N. nitidula* to determine *post mortem* interval. Pont (1986) remarked that *N.nitidula* is active in the UK between February and October.

2. 5. 6 Dryomyzidae

The Dryomyzid *Neuroctena anilis* was found at sites 1, 2 and 3. Oosterbroek (2006) remarks that this familyis frequently found in damp shaded woodland upon decaying material. Smith (1986) states *N. anilis* has a narrow temperature preference and is distinctly forest dwelling, suggesting that its presence upon a corpse can be used to indicate possible *post mortem* movement.

2.5.7. Sphaeroceridae

The Sphaerocerids *Leptocera fontinalis, Copromyza equia* and *Copromyza stercoraria* were found at site 1. Sphaeroceridae are small, usually dull brown-black stout flies approximately 0.7-5.5mm in

size. Members of the family are characterised by a swollen first tarsal segment of the hind leg and are found in wide variety of rotting organic materials (Ooesterbroek, 2006). Smith (1986) notes that sphaerocerids are often found upon human corpses but notes that little research had been done into their development rates. Chick (2010a) places the Sphaeroceridae in the fourth wave of Dipteran decomposition, which is contained within the 3rd wave of Gennard's (2007) forensic succession. *L. fontinalis* is a common U.K. species associated with numerous kinds of decomposing material including carrion (Pitkin, 1988). *C.equia* is most frequently recorded upon dung but has also be found on carrion, it is suggested to be most abundant in early summer (Pitkin 1988). *C. stercoraria* is occasionally found upon carrion and windows but is most common upon dung, rough grass and mouse runs (Pitkin, 1988). Its occasional presence indoors might be of importance to a forensic investigator. However further research into *C. stercorarias* development would be required. Smith (1986) argues that the presence of Sphaeroceridae upon a corpse might be due to dung upon or near the corpse. Ooesterbroek (2006) argues that sphaerocerid larvae feed upon microorganisms in decaying material rather that on the material itself.

2.5.8. Clussidae

An undetermined member of the Clusiidae was found at site 2. Dear (1978) comments that a single species of the family has been recorded on carrion during spring, but does not state which species it is. Ooesterbroek (2006) regards the Clusiidae as decomposers of wood, being often found in association with rotting wood and damp woodlands. Smith (1989) also remarks that while Clusiidae are often associated with rotting wood, the larval behaviour of the family is poorly understood.

2.5.9. Muscidae

The Muscid *Phaonia subventa* was collected at sites 1, 3 and 4. While not considered a fly of forensic importance by Gennard (2007), Smith (1986) or Gunn (2006), Skidmore (1985) remarks that *P.subventa* is often associated with carrion and is a highly adaptable species, and while primarily

associated with sap runs, *P.subventa* can be reared from most kinds of decomposing organic material. This is agreed by Gregor *et al* (2002) who list carrion as a larval habitat.

2.5.9 Calliphoridae

Calliphoridae of the genera *Calliphora* and *Lucilia* were collected from sites 1, 2 and 3. Chick (2010a) remarks that these species are among the first and second waves of dipteran colonisers of carrion. Gennard (2007) considers them among the most forensically important insects. Amat *et al* (2015) states that as well as their forensic importance, Calliphoridae are medically important as they can act as a vector of many pathogenic organisms.

2.5.10 Coleoptera: Carabidae

The Carabidae are predatory visitors to carrion, often considered to be too generalist in their habits to be of forensic importance (Smith, 1986). However Chick *et al* (2008) noted that *Abax parallelepipedus*' scavenging behaviour may be indicative of toxins acting as carrion pollution. The restrictive breeding behaviour of *Nebria brevicollis* may be of further importance to the forensic investigator (Chick *et al*, 2008). However, the nocturnal activity of their larvae means that in practice they are often not obvious as part of the carrion ecosystem (Gennard, 2007).

2.5.11 Leiodidae

The leiodid beetle *Nargus velox* was found in large numbers at sites 1 and 2. Harde (1984) considers *N.velox* to be primarily found in mammal burrows. Cooter and Barclay (2006) state *Nargus* is common in all decaying material including carrion, although Smith (1986) considers members of the related genus *Catops* to be forensically important, being frequently found under and on carrion. Duff (2012a) remarked that *Nargus velox* is common in woodlands in England and Wales often on carrion, leaf litter and moss.

2.5.12 Hydrophilidae

The hydrophilid *Megasternum obscurum,* one of the few terrestrial members of a predominantly aquatic family, was found during carrion testing in site 2. Smith (1986) states that *M. obscurum* has previously been collected from a human corpse in Surrey over 2 weeks after death and Skidmore (1991) remarks that it is often found in ungulate dung and decaying vegetable matter.

2.5.13 Histeridae

A histerid beetle identified as *Margarinotus ventralis* was recorded at site 2. Histerdae feed upon the larvae of flies on a corpse from the bloat stage through to the dry stage (Gennard, 2007). Halstead (1963) notes that *M. ventralis* is common throughout the British Isles in carrion and other rotting organic material. Also of note from the baseline studies is the presence of Histeridae and Hydrophilidae in the pitfall traps, as Webb (2012) stated that the only representatives found of these families during his study were collected using sieving, not pitfall trapping.

2.5.14 Nitidulidae

Glischrochilus quadripunctatus is noted by Wright (2009a) as being usually found associated with tree sap, an observation agreed by Harde (1984) who also stated that *G. quadripunctatus* is also found among the tunnels of wood boring beetles. Smith (1986) states that some of the beetles closely related to *G. quadripunctatus* are carrion-frequenters, and some are predaceous. However Gill (2005) reported that specimens of a non-UK member of the genus *Glischrochilus* were found on pig carrion placed above ground in rural Canada. When coupled with the evidence of multiple specimens on the rat carrion, it is possible that the

genus *Glischrochilus* is predatory and thus attracted to some of the other invertebrates found on the carrion, or to the presence of wood boring beetles.

2.5.15 Stapylinidae

The Staphylinidae or rove beetles are as a family considered to be associated with carrion (Smith, 1986). However Lott and Anderson (2011) noted that while most rove beetles are predatory (with the name rove coming from the Norse word *rov* meaning prey) the family has a diverse ecology, including herbivores, fungivores and parasitoids. Lott and Anderson (2011) also stated that many species have more specialised habitats than for example most of the Carabidae, although some are more generalist as predators of other invertebrates. The literature is patchy regarding the ecology of many of the Staphylinidae and as such their association with carrion has been marked as possible to reflect this. Nield (1976) remarks the *Ocypus olens*, is known to feed on carrion a trait that is ingrained in folklore and superstition giving rise to its common name, "the devils coach horse".

2.5.16 Hymenoptera: Braconidae

Numerous braconid wasps of the genus *Alysia* were collected from sites 1, 2 and 3. From the superfamily Ichneumonoidea, Braconidae are a family of parasitic wasps which tend to have a very narrow range of hosts (Shaw and Huddleston, 1991). The subfamily Alysiinae contains over 200 British species in 40 genera with all species acting as endoparasites of higher Diptera, with the tribe Alysiini preferring to attack Diptera species that breed in odorous and transient habitats such as carrion and dung (Shaw and Huddleston, 1991). Smith (1986) considers *Alysia manducator* the most likely braconid wasp to be encountered upon carrion in the British Isles. Erzinclioglu (1996) adds that the literature falsely considers *Alysia* to be a specific parasite of *Calliphora vicina* and not *Calliphora vomitoria*, but *Alysia* has

been observed parasitising both species in the wild. In laboratory tests. Smith (1986) adds that *A.manducator* will parasitise species of the genera *Calliphora, Lucilia, Phormia* and even *Chrysomya*.

2.5.17 Vespidae

Vespula vulgaris was collected from site 1. Known as the common wasp, this is probably the most well-known of the social wasps and nests can be formed in a variety of habitats, although underground nests appear to be the preference. Workers are fast and agile fliers able to take to flight in cloudy and windy conditions, and even able to fly in gentle rain (Zahradnik,1991). Common during summer and autumn, *V.vulgaris* is active until cold weather starts to bring its activity to an end as most of the workers die and the queens seek sites suitable for hibernation (Hickin, 1964). Spradbery (1973) stated that while predation upon other arthropods acts a major protein source for the nest, worker wasps have also been observed taking small amounts of meat and fish from butchers and fish mongers, as well as from chicken bones and even taking the eyes of a dead rat. Smith (1986) adds that on a human corpse *V.vulgaris* will appear early in the decomposition sequence taking bits of flesh, particular the eye from a corpse, but will most likely feed upon the eggs, larvae and adults of other insects frequenting the carrion including copulating pairs. This opportunistic feeding behaviour means that *V.vulgaris'* role in decomposition can vary and thus it can be considered either a scavenger or a predator. Smith (1986) also included *in addendum* that wasps can be a cause of death, remarking that single stings may kill a person with an allergy to the venom, and almost anyone can be killed if subjected to multiple stings, noting a case in which an infant was intentionally shut in a room full of wasps by "unnatural parents".

2.5.18 Lepidoptera: Tineidae

Tineola bisselliella is known as the common clothes moth due to its larvae traditionally being associated with damage to woollen clothes, However in nature it is associated with birds' nests and mammal burrows where it feeds upon animal remains (Novak, 1985). Smith (1986) remarks that *T.bisselliella* larvae will also feed upon dried skins, furs, feathers and leather, noting that while larvae can freely feed upon these animal remains often they will construct protective tubes from silk and feed from the safety of these tubes. The larval stage generally lasts between two and three months, but may last up to four years dependent upon the climate. Pupal cases are constructed from silk and fibres from the corpse. Smith (1986) places *T.bisselliella* in the seventh wave of decomposition, which is synonymous with Gennard's (2007) fourth wave of post decay.

2.5.19 Hemiptera: Lygaeidae

Kleidocerys resedae has been marked as a possible carrion associate, since although Southwood and Leston (2005) considered it to be found on alder or birch trees and McGavin (1993) considered the family Lygaeidae to be predominantly seed feeders, Smith (1986) noted that some of members of the family appear to be predaceous. At least one non-UK species of Lygaeidae (*Myodocha serripes*) has been observed actively feeding upon Calliphoridae larvae (Smith, 1986). It was not stated whether the non-UK species was typically an obligate predator or a facultative predator.

2.5.20 Dermaptera: Forficulidae

Forficula auricula is the common earwig, and listed as possibly associated with carrion. This is because although Smith (1986) does not consider the Dermaptera to be of forensic importance, Marshall and Haes (1988) note that *F. auricularia* like all earwigs is an

omnivorous scavenger. Chick *et al* (2008) previously stated that invertebrate scavengers on carrion may be of further importance to forensic entomotoxicology investigations, as if chemicals with insecticidal qualities lead to a number of dead insects around the carrion one would expect such specimens to attract scavengers.

2.5.21 Acari

The Acari or mites found at the sites were only partially identified due to the literature being very incomplete regarding the group, and because the higher classification varies from source to source. Gunn (2006) states that Acari are rarely used in forensic cases, due to their small size and the lack of expertise in identification. However the literature was followed as far as possible and inferences were drawn based upon the circumstances in which the specimens were collected. *Poecilochirus* spp was found at site two upon specimens of *Nicrophorus* which is a known host of the genus (Evans and Murphy, 1987). Mites associated with *Nicrophorus* are predatory upon blowfly eggs and it has been suggested that *Nicrophorus* are unable to produce offspring on carrion with more than 100 blowfly eggs. If the colony of *Nicrophorus* attempting to colonise the carrion have 30 or more mites upon them then the Diptera eggs are destroyed, but if the fly eggs hatch the mites and beetles are prevented from breeding on the carrion (Smith, 1986).

A number of Astigmata were found upon bones at site two during carrion testing but further identification proved problematic. Hughes (1976) lists four species typically associated with meat, bones, hides, dried fish and fishmeal, these being: *Lardoglyphus konoi, Lardoglyphus zacheri, Glycyphagus domesticus* and *Pyroglyphus africanus*.

Lardoglyphus konoi has been found in the U.K. in butcher's offal. In the rest of the world it is considered a serious pest of dried fish, and believed to be brought to animal remains by clinging to beetles of the genus *Dermestes* (Hughes. 1976)

Lardoglyphus zacheri is often transported on the larvae of *Dermestes maculatus* , and has been found on butcher's offal, bones, hides, sheep skins and on the outside of boxes containing casings (Hughes, 1976).

Glycyphagus domesticus is an opportunist, found on dried plant and animal remains, in association with human dwellings. It has been found in cheese, ham, dried calves' stomachs, birds' nests and on mouldy wallpaper. *G.domesticus* was also once discovered in the cold room of a pathology department, feeding upon the culture medium of cholera bacillus, most likely introduced as the cold room was occasionally used as a temporary food store (Hughes, 1976).

Pyroglyphus africanus has previously only been found in Suffolk, on fish meal imported from West Africa (Hughes, 1976), suggesting that it is rare in the U.K. Pimsler *et al* (2016) discovered *Myianoetus muscarum* (Acari: Histiostomatidae) on specimens of *Synthesiomyia nudiseta* (Diptera: Muscidae) found upon human remains. *M.muscarum* is an Astigmatid mite with a phoretic relationship with members of the Muscidae which they use to transport them to suitable carrion.

Oribatei were found at all sites, but are not considered forensically important by Gennard (2007), Smith (1986) or Byrd and Castner (2001). However Gunn (2006) states that Oribatei are more numerous in soil as the corpse starts to dry out. Dindal (1990) argues that Oribatei are slow moving particulate feeders upon fungi and plant material in the soil. This would suggest that the Oribatei are of incidental importance to the forensic scientist as they will be present prior to decomposition in the soil but most likely will leave the vicinity of the corpse during the decay process, to return as the soil chemistry and fauna returns to normal in the later stages. This information might be used to determine if a corpse has been moved, but is of little importance in terms of general sucession. As such the Oribatei are not considered forensically important for this investigation.

2.6 Conclusion and final site selection

It would appear that sites 1 and 2 are rich in invertebrates associated with carrion, from primary colonisers such as Calliphoridae, to carrion frequenting predators such as Staphylinidae and Carabidae as well as some families such as Pyschodidae which are considered understudied or of partial forensic importance. Consequently it was determined prior to the small carrion tests that sites 1 and 2 offered the best potential for carrion placement,

Site 4 was discounted early due to its low cover and close proximity to the halls of residence. The small clearing was also determined too small for larger carrion such as the porcine cadavers which were to be used in the main investigation. The initial baseline studies started prior to the undergraduate term, but once term started it was realised that there was a high footfall in the vicinity. When coupled with the low cover, it was determined that the site was unsuitable for carrion studies. The fact no carrion was placed at the site accounts for the low number of species collected from site 4.

Site 3 was discounted due to its close proximity to the student car park and to a fox's den and Chick *et al* (2008) commented that foxes readily removed carrion from the site within a week of placement. It was also noted that dependent upon the path of entrance, the investigator could be seen entering the site by students, reducing security. Finally, it was noticed that the student car park sits higher than the site and that during heavy rain this, combined with a heavy clay soil with poor drainage on site, meant the site was prone to flooding.

Sites 1 and 2 were seen as suitable for carrion studies based upon their species richness, site security and safety. Both had multiple entrances which reduced the chances of creating a "trodden in" path, and made entrance to the site less obvious. It was determined that Site 2 would be used for carrion baseline work, as this would keep site one "green", that is in a virgin state ready for the placement of porcine carrion. Given that the two sites were

approximately 20 metres apart the carrion fauna would be expected to be similar in both, especially since Erzinclioglu (1996) reported that blowflies can detect carrion from a distance of 6.4km. Site 1 was later used for the autumn/winter investigation until the understory of the woodland was cut back, reducing the effectiveness of the cover. Site 2 was then used for the spring/summer investigation. This had the bonus of giving the soil at the second site time to recover from any leachates from the small carrion baseline study. It was also noted that both sites one and two contained a number of coniferous trees in the understory. This was seen as an advantage, as it would offer greater cover in the winter months. In contrast it was noted that sites three and four had a lower density of coniferous trees than sites one and two, which in winter would lead to lower cover to obscure the experiments from the public.

Chapter 3: Autumn/winter pig carrion field tests.

3.1 Introduction

The baseline studies identified two suitable sites, in terms of security of site and richness of the pre-existing carrion fauna. Following the successful conclusion of these baseline studies it was determined that field-based testing on the possible effects of nicotine on the decomposition of cadavers could begin.

While it would have appeared the most rational carrion to have used would have be human cadavers, Gennard (2007) notes a few of the difficulties of using human remains for research, namely the Anatomy act of 1984 and the Human tissue act of 2004. Human carrion would also require additional site security, preferably a fenced compound which was unfeasible upon the campus. However as previously stated, Haskell et al (2002) considered there to be little difference between humans and pigs (*Sus scrofa domestica*) in terms of carrion fauna or decomposition patterns; this was echoed by Whitaker and Hall (2010). As *S.scrofa* decomposes in the same manner as humans and is easy to obtain, it was determined that pigs would be suitable for use in the investigation.

3.2 Methodology

Unwin and Corbet (1991) stated that the climate between the ground and a height of 1 metre varies from standardised meteorological measurements and refers to this area as a microclimate. As any carrion would be placed upon the ground it was deemed important that microclimate measurements needed to be collected on site; thus average temperature at ground level, soil temperature, humidity and rainfall were all recorded. Weather monitoring equipment (apart from the rain gauge) was placed in a modified anti scavenger cage (Figure 3.1). The cage was threaded with dyed jute in a similar manner to a sniper's ghillie suit to

camouflage the sensitive equipment in the undergrowth. The rain gauge was placed outside the cage as the presence of the camouflage might have affected the results.

Figure 3.1: The weather recording equipment in a modified anti-scavenger cage.

Three *S.scrofa* cadavers were obtained from an abattoir, each weighing approximately 15kg on the morning of dispatch. The cadavers were too large to be placed in the anti-scavenger cages mentioned by Chick (2008) and so were protected from vertebrate scavengers by the use of chicken wire (Figure 3.2). This was wrapped around the carrion and attached to the ground using tent pegs, as it was determined that a whole pig carcase would be too large for a fox to remove. For extra security the tent pegs were driven in at an acute angle making removal difficult. Each cadaver had four pitfall traps placed around it, filled with glycol based preservative, as in the baseline studies.

Figure 3.2: Pig carrion *in situ*, encased in chicken wire to prevent scavenging. Note pitfall traps underneath the square lids

The three pigs were treated as follows:

Pig 1 was left untreated as a control.

Pig 2 was injected with a dose of 8.4mg of aqueous nicotine. The nicotine was injected into the throat of the cadaver, this was to simulate the average amount of nicotine self-administered in a single cigarette the limitiations of which are discussed in chapter 6(based upon figures from Wonnacott *et al* 1990).

Pig 3 was injected with a dose of 168mg of aqueous nicotine. Wonnacott *et al* (1990) quote that figure as the average typical daily consumption of nicotine per 20 cigarettes by a smoker, this is also a possibly fatal dose for nicotine poisoning based upon the figures quoted by

Tracy (1984) although as previously outlined, in extreme circumstances a human can consume well over two grams of nicotine and survive.

The carrion was placed at site one (Grid reference: SK547353) on the 15th of September 2009 (Figure 3.3).

Figure 3.3: A view of the approach to Site 1.

In addition active trapping including aerial netting was used to collect further specimens not collected by passive means. Specimens were preserved in the manner recommended for the various taxonomic groups (c.f. baseline studies). Climate recording equipment was protected using an anti-scavenger cage much like the kind used to house the rat carrion; however this was covered with dyed jute strands and natural foliage to hide the equipment from view. Specimens actively collected were preserved for identification either by using an ethyl acetate charged kill jar as recommended by Cooter and Barclay (2006), by freezing, or by placing in 70% alcohol in the case of non-insect invertebrates. Soft bodied larvae were killed using

boiled water (Hot water kill/HWK) and preserved in 70% alcohol. Specimens were then curated using the methods listed in the baseline studies (section 2.3.1).

3.3 Results

3.3.1 Meteorological measurements

Using the onsite recording equipment, weather data was collected for one hundred days beginning on the 15th of September 2009 (see Figures 3.4-3.7). During this period a number of invertebrate specimens were collected and identified. (See tables 3.1-3.3).

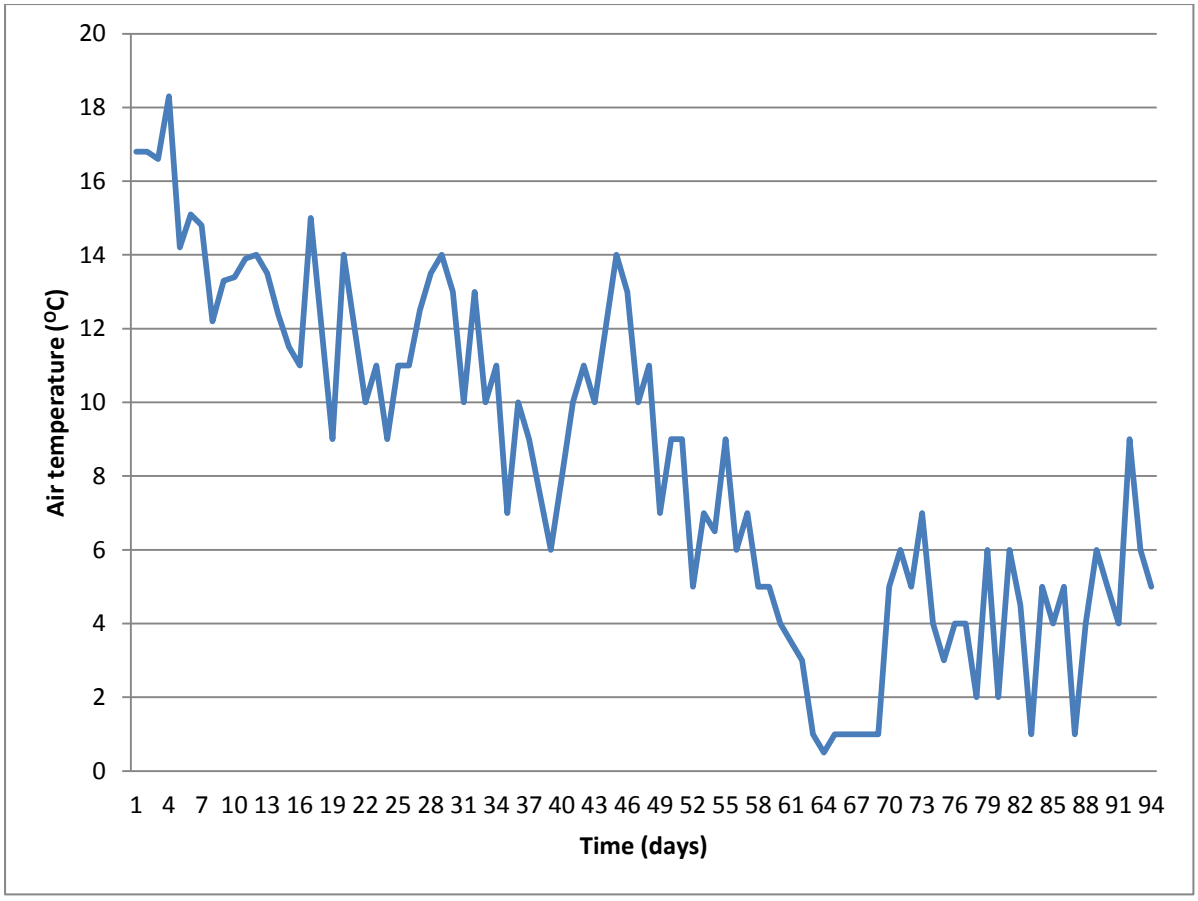


Figure 3.4: Mean air temperature (°C) of Site 1 during experiment 1.

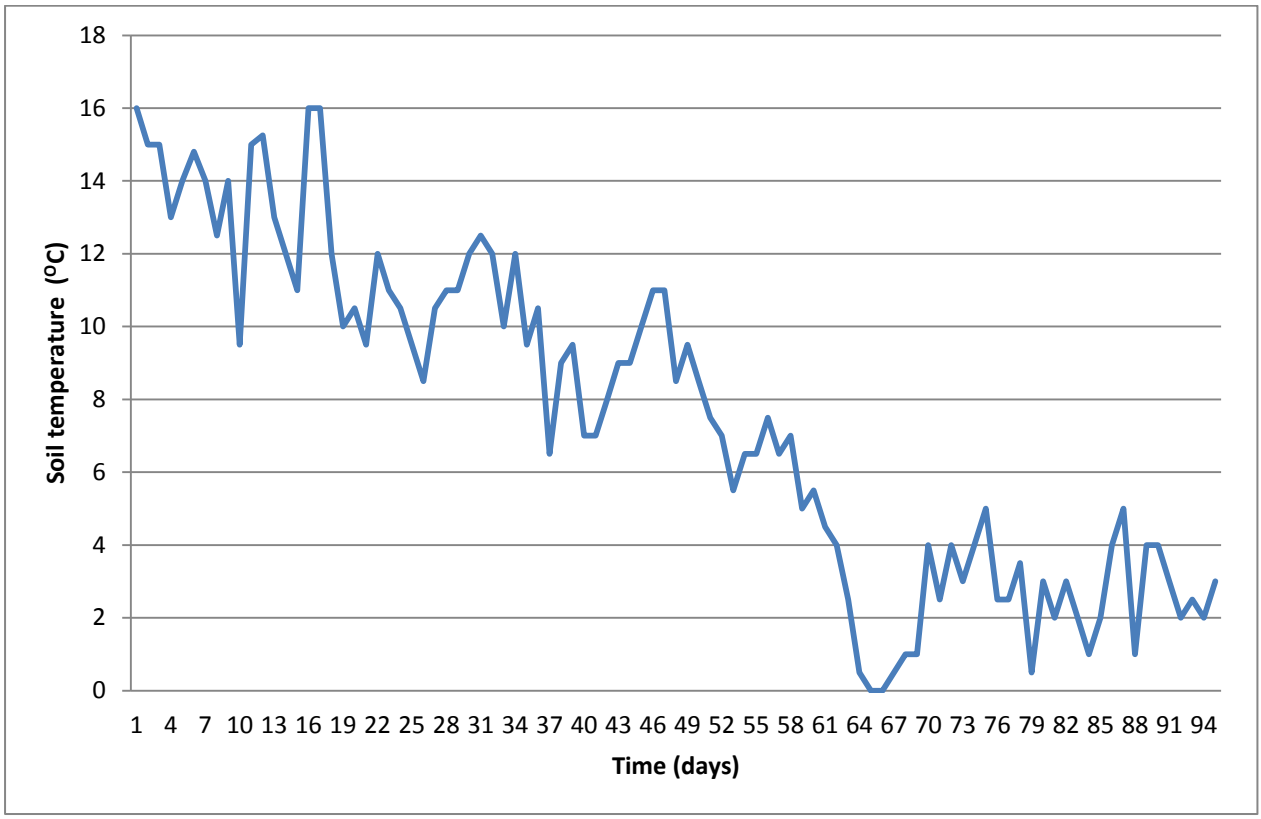


Figure 3.5: Soil temperature of Site 1 during the course of experiment 1.

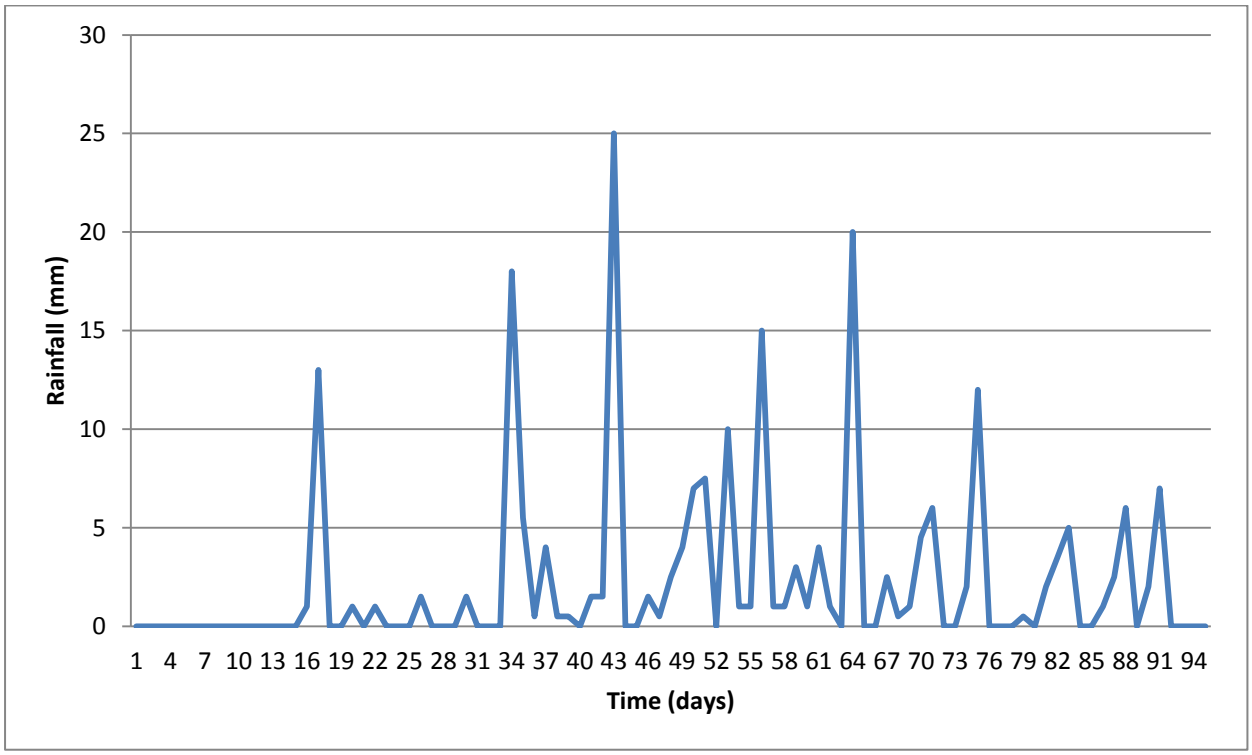


Figure 3.6: Rainfall during the course of experiment 1 at Site 1.

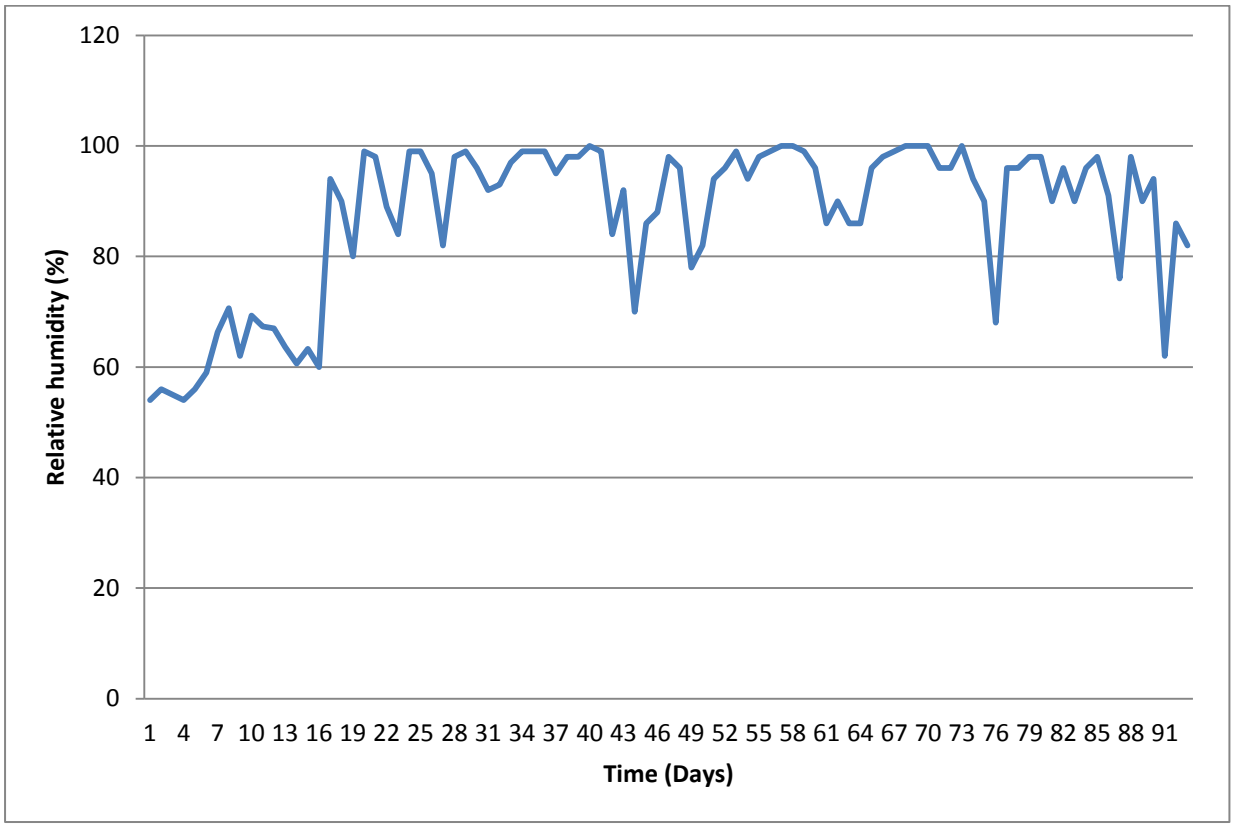


Figure 3.7: Relative humidity recorded from Site 1 during the course of experiment 1.

From Figures 3.4 -3.7 it would appear that average air temperature, whist fluctuating appeared to drop steadily for the first 40 days, with air temperature briefly increasing before dropping below the lower development threshold ($6^{O}C$) for the most cold tolerant Calliphoridae (Byrd and Castner, 2001) at day 60. After 70 days the temperature fluctuated around the lower development threshold. No rain was observed for the first 16 days; however, peaks of between 10mm and 25mm were sporadically observed during the course of the experiment. The humidity was between 55 and 70% for approximately 8 days, and then proceeded to fluctuate between 60% and 100% for the rest of the experiment.

Table 3.1: Specimen list for control pig carrion in the autumn and winter field test

Order	Family	Species
Diptera	Tipulidae	*Tipula staegeri*
	Stratiomyidae	*Sargus bipunctatus*
	Syrphidae	*Rhingia rostrata*
	Dryomyzidae	*Neuroctena anilis*
	Piophilidae	*Liopiophila varipes*
	Muscidae	*Phaonia subventa*
	Calliphoridae	*Calliphora vomitoria*
		Calliphora vicina
		Lucilia sericata
Coleoptera	Carabidae	*Carabus nemoralis*
		Carabus violaceus
		Abax parallelepipedus
		Nebria brevicollis
		Trechus quadristriatus
		Calathus rotundicollis
	Silphidae	*Nicrophorus humator*
	Ptiliidae	*Unidentified* spp.
	Staphylinidae	*Ocypus olens*
		Ontholestes tessellates
		Tachinus spp
		Tinotus morion
		aleocharinae spp 1
		Aleocharinae spp 2
		Aleocharinae spp 3
		Aleocharinae spp 4
		Aleocharinae spp 5
		Aleocharinae spp 6
	Scarabaeidae	*Aphodius prodromus*
	Lathridiidae	*Cartodere nodifer*
Hymenoptera	Braconidae	*Alysia spp*
	Pteromalidae	*Cerocephalinae spp*
	Vespidae	*Vespula vulgaris*
Dermaptera	Forficulidae	*Forficula auricularia*
Heteroptera	Lygaeidae	*Kleidocerys resedae*

Table 3.2: Specimen list for low dose pig carrion in the autumn and winter field test

Order	Family	Species
Diptera	Stratiomyidae	*Sargus bipunctatus*
	Dryomyzidae	*Neuroctena anilis*
	Muscidae	*Phaonia subventa*
	Calliphoridae	*Calliphora vomitoria*
		Calliphora vicina
		Lucilia sericata
Coleoptera	Carabidae	*Carabus nemoralis*
		Pterostichus madidus
		Abax parallelepipedus
		Nebria brevicollis
		Trechus quadristriatus
		Calathus rotundicollis
	Staphylinidae	*Ocypus olens*
		Philonthus decorus
		Tachinus spp 1
		Tachinus spp 2
		Micropeplus fulvus
	Silphidae	*Nicrophorus humator*
		Nicrophorus vespilloides
	Leiodidae	*Nargus velox*
	Lathridiidae	*Cartodere nodifer*
	Scarabaeidae	*Aphodius prodromus*
Hymenoptera	Braconidae	*Alysia* spp
	Pteromalidae	Cerocephalinae spp
Dermaptera	Forficulidae	*Forficula auricularia*
Heteroptera	Lygaeidae	*Kleidocerys resedae*
Pseudoscorpiones	Neobisiidae	*Neobisium carcinoides*

Table 3.3: Specimen list for high dose pig carrion in the autumn and winter field test

Order	Family	*Species*
Diptera	Tipulidae	*Tipula staegeri*
	Stratiomyidae	*Sargus bipunctatus*
	Phoridae	*Metopina oligeneura*
	Heleomyzidae	*Scolicentra brachyptera*
	Dryomyzidae	*Neuroctena anilis*
	Calliphoridae	*Calliphora vomitoria*
		Calliphora vicina
		Lucilia sericata
	Muscidae	*Phaonia subventa*
	Fanniidae	*Fannia fuscula*
		Fannia canicularis
Coleoptera	Carabidae	*Carabus violaceus*
		Carabus nemoralis
		Abax parallelepipedus
		Nebria brevicollis
		Pterostichus madidus
		Pterostichus melanarius
		Calathus rotundicollis
		Trechus quadristriatus
		Elaphrus riparius
	Staphylinidae	*Ocypus olens*
		Creophilus maxillosus
		Ontholestes tessellatus
		Tasgius morsitans
		Philonthus decorus
		Philonthus quisquilliarius
		Bisnius fimetarius
	Mycetophagidae	*Typhaea stercorea*
	Lathridiidae	*Stephostethus lardarius*
		Cartodere nodifer
	Scarabaeidae	*Aphodius contaminatus*
	Leiodidae	*Nargus velox*
	Nitidulidae	*Epuraea thoracica*
	Curculionidae	*Phyllobius argentatus*
	Coccinellidae	*Myrrha octodecimguttata*
Hymenoptera	Vespidae	*Vespula vulgaris*
	Braconidae	*Alysia* spp
	Pteromalidae	Cerocephalinae spp
Dermaptera	Forficulidae	*Forficula auricularia*
Pseudoscorpiones	Neobisiidae	*Neobisium carcinoides*

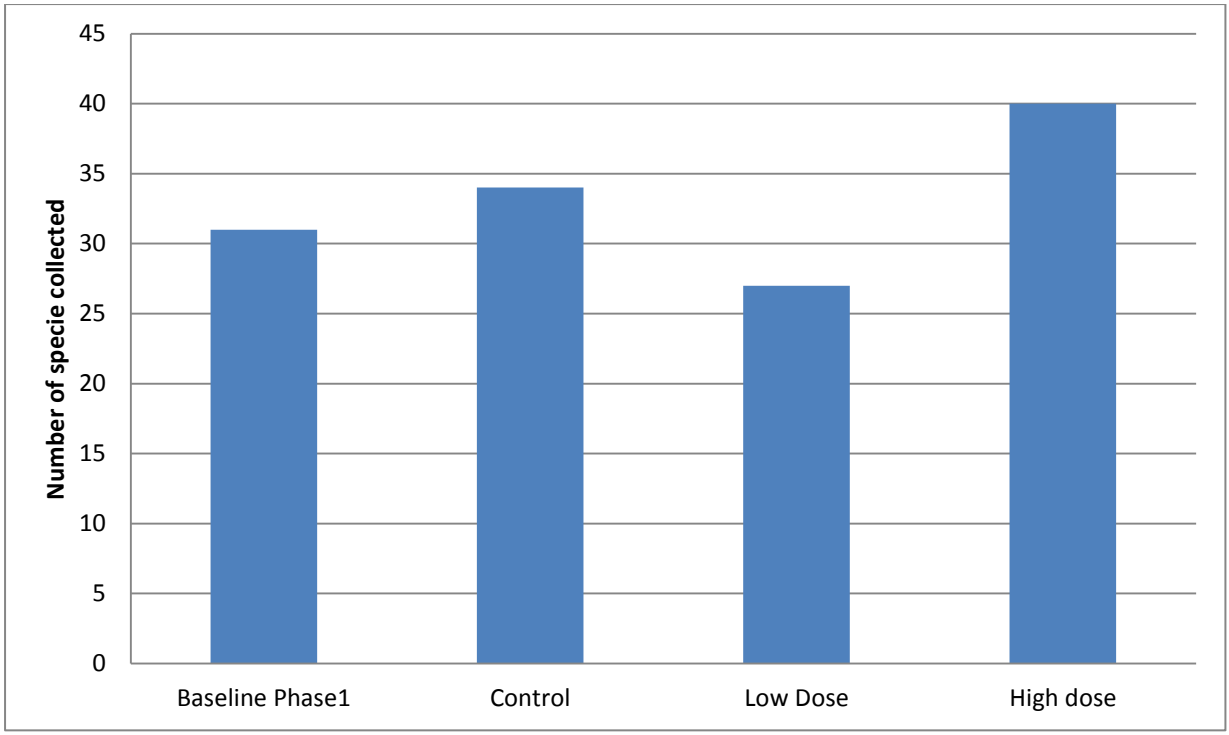


Figure 3.8: Comparison of number of species collected from each pig in relation to baseline studies.

From the above graph it would appear that high does nicotine contains approximately one third more species than were collected during the baseline study; with the control and low dose carrion having around plus five and minus five species in relation to the baseline studies respectively.

Invertebrate species lists collected from and around the various treatments were compared using a % similarity calculation based on presence/absence:

Where % similarity = (2J/(A+B)) x 100. J = number of joint occurrences, A = total species at the first site being compared and B = total species at the second site being compared. (See Table 3.4)

Table 3.4: a comparison of specimens collected by percentage similarity

	Baseline Phase1	Control	Low Dose	High dose
Total species	31	34	27	40
Similarity to baseline (%)	-	26.5%	40.7%	30.0%
Similarity to control (%)	-	-	74.1%	50.0%
Similarity to low dose (%)	-	-	-	52.5%

3.3.2 Observations on carrion

The carrion was placed *in situ* on the 15th of September 2009. Within twenty-four hours clusters of eggs were seen around the mouths of all three cadavers, with the largest number of eggs noted on the control, and the fewest on the high dose carrion. Social wasps (Vespidae) were observed around all carrion, and predatory ground beetles (Carabidae) were found in all pitfall traps. Within forty-eight hours the cadavers were attracting large numbers of *Calliphora* and *Lucilia* blowflies. Large predatory beetles of the families Carabidae and Staphylinidae were also observed on all carrion, as well as Vespidae (social wasps) and *Neuroctena anilis* (Diptera Dryomyzidae, a family of woodland flies attracted to rotting organic matter) on the control carrion. Bloating was evident after seventy-two hours. Eggs were laid singularly on the treated carrion, as opposed to the typical egg clumps on the control. After 7 days, a faint odour developed on the control which appeared to have had first instar maggots developing in the buccal cavity and ears; further eggs were laid on the head and neck and *Neuroctena anilis (*Diptera: Dryomyzidae*)* was present in the traps. The low and high dose pigs had eggs laid around the anal/genital area, and there were 1st instar larvae in the buccal cavity.

On the eighth day the head of the control animal was covered in eggs, however the

genital and anal regions were untouched; *Alysia sp* (Hymenoptera, Braconidae) was also observed on the control. Further egg laying was noted on the anal and genital regions of the low dose and high dose carrion. It was observed that the low dose carrion had higher fly activity than the control at that point; the high dose had higher fly activity than the control, but lower activity than the low dose cadaver. By the ninth day a larval mass was beginning to form in the buccal cavity of the control carrion with small amounts of oviposition in the anal/genital region, whereas the treated carrion appeared to have most activity in the area between the carrion and the ground. On the tenth day the larval mass on the control cadaver had moved to the throat. The genital and anal regions of the treated pigs showed signs of obvious larval feeding, also the high dose carrion appeared to attract coprophagous Diptera to its anal region. By day thirteen the flesh of the head of the control carrion had almost entirely been consumed. A larval mass was present in the chest region of the carrion between the forelegs. Large numbers of gravid Calliphoridae were present and further oviposition on the back of the corpse was observed. The low dose carrion also had a large amount of fly activity, a larval mass had formed below the site of injection, and Braconid wasps of the genus *Alysia* were observed. On the high dose carrion, larval feeding also appeared to avoid the site of injection and it was noted that the head of the cadaver was almost completely disarticulated. By the start of the second week of experimentation the head of the control had been completely devoured by larval feeding, with small Staphylinidae species present on the body. The low dose carrion had third instar larval feeding under the treatment area, whereas the high dose one appeared to have third instar larval masses feeding on the back of the neck, genitals and under the carrion.

On the 22nd day, female *Sargus bipunctatus* was first noted on the high dose cadaver,

which had a large anal/genital larval mass with a small larval mass on the head of the animal. The anal/genital and head larval masses of the low dose carrion were almost at the point of connecting with each other and *Nicrophorus vespillodes* was also present on the carrion. The control was starting to develop a smaller larval mass on the genital region at this point and the larval mass on the head had engulfed the shoulders. On the 24th day female *Sargus bipunctatus* were present on the control carrion, as was a feather wing beetle (Ptillidae). It was noted on the 25th day that more tissue had been removed from the treated carrion than the control carrion. By day 29 the low dose corpse could be described as having an odour like horse manure, with only bones and hide remaining, most larvae had migrated away.

Decomposition of the high dose pig appeared not as advanced as in the low dose one, yet was more advanced than the control, which had plenty of tissue remaining upon it. On the 31st day the control was surrounded by female *S.bipunctatus* and a specimen of *N.humator* was also present. The low dose cadaver was covered in numerous small Acalypterate flies. By day 32 the control appeared have Calliphorid larvae feeding on the abdomen. The legs were partially skinned. The low dose appeared to have a large number of migrating larvae in preparation for pupation with a few maggots remaining on the abdomen. The high dose only appeared to have larvae feeding on the abdomen. By day 36 the control pig showed signs of skin discoloration above the larval feeding. Both the low dose and high dose cadavers showed small signs of larval feeding; the skin of the treated pigs was leathery and yellow in places. On the 38th day heavy rain caused the remaining larvae to move to the top of all carrion. The pseudoscopion, *Neobisium carcinoides* was found on the low dose carrion on day 38 whereas the control was covered in large numbers of small/medium acalyptrate flies. On day 41 *N.carcinoides* was found on the high dose carrion along with acari. The

low dose pitfalls were also observed to contain acari, and a female specimen of the rare hoverfly *Rhingia rostrata* was found on the control carrion as were Sepsidae and some other small acalyptrate flies. Soldier fly larvae, most likely *S.bipunctatus* were found in the nutrient-rich leaf litter surrounding the control carrion on day 43. Evidence of digging around the control was noted on day 59, and by day 63 similar digging was noted around the low dose carrion. By this time numerous soldier fly larvae were noted on the control carrion. Digging around the high dose carrion by vertebrates was not observed until day 89. On the 90th day it appeared that a vertebrate had been digging for insects in the area around the control. On the 119th day it was noted that the bones of the control had been moved by rodents. The site was compromised by ground staff cutting back the dense foliage on the 1st of February 2010.

3.4 Discussion

From the results it would appear that in relation to the phase 1 baseline studies the presence of carrion does affect the invertebrates collected in a woodland ecosystem, the highest similarity to the baseline of invertebrates being found in the low dose carrion with 40.7 % similarity. This would be accounted for by the numbers of insects attracted specifically by the presence of the carrion. From the above data it would appear that nicotine has an effect on the behaviour of primary colonisers.

3.4.1 Diptera: Calliphoridae

As stated above on the treated carrion, Calliphoridae favoured oviposition on the genital and anal regions of the corpse rather than that of the eyes, ears and mouth as was favoured by Calliphoridae on the control carrion. A slight delay in oviposition was also noted on the treated carrion and oviposition behaviour was seen to change, in that single egg laying was

observed on the treated cadavers, whereas the more typical clumping of eggs was observed on the control pig. The single egg laying phenomenon was previously observed by Chick, *et al* (2008) in relation to oviposition on insecticide treated carrion, the study also stated that Calliphoridae avoided laying eggs in an area treated with an insecticide. Insecticides in or on the carrion appear to affect a behaviour that is so typical of the Calliphoridae that it forms the origin of the common name of the family. Gunn (2006) stated that the common name of the Calliphoridae (the Blowflies) is derived from the noun "blow" meaning a mass of eggs, and that meat, or carrion covered in these clumps, or masses is referred to as "flyblown". Greenberg and Kunich (2005) stated that the massing of eggs appears to be the result of many females ovipositing next to one another which was beneficial since it was known that Calliphoridae embryos have a limited ability to withstand desiccation. Greenberg and Kunich (2005) stated that this typical oviposition behaviour reduces the probability of desiccation, although *Calliphora* species have been known to lay eggs singularly in suboptimal conditions. Erzinclioglu (1996) stated that pheromones as well as smells appear to attract Calliphoridae to carrion. This, coupled with Müller *et al's* (2003) finding regarding detection of hydrocarbons by carrion insects, could suggest that the presence of toxins disrupts the hydrocarbon profile of a corpse.

It would appear that after an initial delay, the muscle tissue of the treated corpse was consumed at a faster rate. This appears to supplement the work of Chick *et al* (2008) who noticed a similar phenomenon with insecticide treated rat carrion, and attributed it to either reduced oviposition leading to increased efficiency, or possibly rain removing the toxins from the carrion. Given that in this investigation the toxin was applied by means of sub-dermal injection, removal by rain appears highly unlikely. As stated in the introduction other possibilities not considered by Chick *et al* (2008) include the likelihood of an increase in bacterial growth, coupled with the preference for the anal and genital regions as a site of

oviposition could have led to easier to access to the visera and thus an easier to consume corpse. Another theory is that the presence of increased "decomposition hormones" suggested by Odum (1967) in the treated pigs could have caused a higher consumption efficiency.

Figure 3.9: *Rhingia rostrata* specimen collected from site 1 control carrion.

3.4.2 Syrphidae

The presence of the hoverfly *Rhingia rostrata* (Figure 3.9) is noteworthy for a number of reasons. As stated by Chick (2010d) it was the first record of the species in Nottinghamshire. Stubbs and Falk (1996) noted that *R.rostrata* is prone to erratic occurrences, in two apparent broods, one in May-June and a second in August-September. However the abundance of *R.rostrata* fluctuates and it has been known to vanish from the records for many years. Shirt (1987) classified *R.rostrata* as vulnerable, however Stubbs (1996) stated that *R.rostrata* was

not as rare as previously thought, and noted that September is the best month to find it. According to Stubbs and Falk (1996) the larval biology of *R.rostrata* is unknown, and they suggest that carrion is a possible larval breeding medium. Rotheray (1993) remarked on the larval habits of *Rhingia campestris*, the closest British relative of *R.rostrata,* as occurring in cow dung. It was stated that the larvae coat themselves with fragments of the decomposing dung for camouflage, and a "patient and methodical" approach is required to discover them, suggesting that discovery of the larvae is destructive to the micro habitat. Falk (1991) noted that while *R.campestris*, uses cow dung as a larval food source, circumstantial evidence suggests this is not the case with *R.rostrata* and the finding of this species on carrion adds some support to the theory, although no larvae were found upon the carrion. Chick (2010d) noted that the size of carrion may be a possible reason for its scarcity, as the specimen was collected after the active feeding of the primary colonisers on medium sized carrion. Chick (2009) also stated that small carrion such as rats often are not able to sustain the later waves of coloniser due to the low amount of tissue. Also of interest is the collection site since Ball & Morris (2000) reported that *R.rostata* is often recorded in deciduous woodland, and the experimental site is managed woodland. However, Stubbs (1982) considered *R.rostrata* to be a relatively constant indicator of continuity in woodland habitats, as opposed to a general woodland species. Also as *R.rostrata* was only found on the control, this might suggest that its rarity might be in part due to its being carrion pollution intolerant. Finally the presence of *R.rostrata* on carrion counters the work of Smith (1986) who stated that the only hoverfly of potential forensic importance is *Eristalis tenax,* the adult of which is commonly called the Drone fly as the species is a honeybee mimic. The larvae of *E. tenax* are referred to as rat tailed maggots in reference to their telescopic breathing tube, which is an adaption to breathing in semi-liquid organic matter. Rotheray (1993) stated that the genus *Eristalis* is associated with wet decomposing organic matter, including vegetation in ponds. It is worth

noting that Smith (1986) considers that *E.tenax* is the lion–born "bee" of biblical reference, which forms the logo of the Lyles' sugar refiners and the quotation "out of the strong came forth sweetness" (Judges 14.14 Gideons bible)

Figure 3.10: Female *Sargus bipunctatus* collected from pig carrion at Site 1.

3.4.3 Stratiomyidae

The presence of *Sargus bipunctatus* (Figure 3.10) upon carrion is also noteworthy for a number of reasons. Firstly *S.bipunctatus* was first observed on the higher dose carrion and thus if the presence of nicotine is not accounted for then PMI estimates based upon the presence of this species in the succession of decomposition will add inaccuracies to legal investigations. Also while Smith (1986) noted the presence of the genus *Sargus* on dog carrion in the U.S.A., little consideration is given to individual species, or indeed species

from the United Kingdom. Dear (1979) does not report that *Sargus* is present on carrion, even when accounting for autumnal species. Stubbs and Drake (2001) note that *S.bipunctatus* is the largest member of the genus and shows strong sexual dimorphism, with females being more colourful than males Skidmore (1991). Stubbs and Drake (2001) also state that *S.bipunctatus* is the only UK member of the soldier flies to be found in autumn, being recorded as late as November although it is most abundant in September and can be found in July. The family Stratiomyidae to which *S.bipunctatus* belongs to is somewhat strange among the evolutionary lower flies, in that its larvae are neither parasitic nor predatory (Oosterbroek, 2006). The larvae of *Sargus* are reported to be terrestrial and have been recorded as mainly breeding in cow dung, but would also appear to be opportunists which will breed in a wide variety of decomposing organic matter, including rotting bracket fungus and compost heaps (Stubbs and Drake, 2001). Skidmore (1991) commented that *S.bipunctatus* is a common pasture species. Given the abundance of *S.bipunctatus* on the carrion and its late appearance in the year and the lack of information regarding it in the forensic literature, it would seem that it is an overlooked species that occupies a specific niche in the carrion community. Chick (2012) argued that given its abundance upon carrion, *S.bipunctatus* is of forensic importance, suggesting that given its limited and late breeding period its presence would be considered indicative of autumnal decomposition of carrion.

3.4.4 Fanniidae

The Fanniidae, *Fannia fuscula* and *Fannia canicularis* were both found exclusively upon the high nicotine carrion. *Fannia* is considered by Gennard (2007) to be part of the 3rd wave of decomposition, when the fats within the body have become rancid. Rozkosny *et al* (1997) remark that *F.canicularis* is a cosmopolitan species, the larvae develop in all kinds of rotting organic matter from plant material to dung. However, it has also been reared from wasp, bee

and bird nests, as well as various fungi and vertebrate carrion. It is remarked that *F.canicularis* is the most common cause of myiasis within the Fanniidae. Fonseca (1968) considered *F.canicularis* to be generally distributed and common almost all year round in Great Britain. Byrd and Castner (2001) state that the larvae of *F.canicularis* develop upon liquefying material and the larvae float upon this semiliquid environment. *F.canicularis* is often found upon corpses where the contents of the gut have been exposed.

F.fuscula is described as a Holarctic species, widespread throughout Europe but infrequently recorded by Rozkosny *et al* (1997).However, Fonseca (1968) considered it to be fairly common and generally distributed throughout Great Britain. Its primary habitats are described as vertebrate carrion and in the nests of social hymenoptera, although females are often attracted to meat and dung (Rozkosny *et al,* 1997). The presence of *Fannia* upon the high dose carrion might suggest that the remains are more likely to reach a semi liquid state in autumn in the presence of nicotine, and thus more likely to attract *Fannia*.

3.4.5 Coleoptera: Silphidae

Members of the genus *Nicrophorus* (Coleoptera, Silphidae) are often referred to as burying beetles. Joy (1933) remarked that these beetles gained their common name from their habit of burying small carrion, where small is taken to be around the size of a mouse. This is done between a breeding pair, which excavates the earth underneath the cadaver, gradually causing the dead animal to sink into the hole. The beetles then oviposit close to the buried carrion and the emerging larvae feed upon the carcase. Joy (1933) suggested that the burial of small carrion prevents desiccation, although Hall (1924) suggested the burying behaviour is used to reduce competition between the beetle larvae and other carrion decomposers. Smith (1986) stated that the burying beetles may also be found on human cadavers and noted a conflict in the literature regarding the feeding habits of *Nicrophorus,* adults either feeding on carrion

and/or dipterous larvae present on the corpse. Smith (1973) originally considered the genus *Nicrophorus* to be in the 5th wave of decomposition, on a corpse which had been exposed to the environment for approximately one year, but Smith (1986) amended the timing and considers the 5th wave to take place four months into the decomposition process. However it was also noted by Smith (1986) that there was a forensics case record in the UK, in which members of the Silphidae were found on a body which had been dead for seventeen days. The burying behaviour of the *Nicrophorus* is not mentioned in relation to larger carrion by Smith. Byrd and Castner (2001) noted that some American species make depressions in larger carrion to house groups of developing larvae. Byrd and Castner (2001) also suggest that the adults feed on maggots on carrion, whereas the larvae feed purely on decomposing animal flesh. However it is to be remembered that Chick *et al* (2008) stated that the American invertebrate fauna is distinctly different from that of the United Kingdom. *Nicrophorus humator* (Figure 3.11) is the largest member of the genus (Cooter and Barclay, 2006), Harde (1984) stated that *N.humator* is common across northern Europe. Its absence from the high dose but presence upon the low dose carrion could suggest that it is mildly tolerant to nicotine.

Figure 3.11: *Nicrophorus humator* dorsal view and head.

Wright (2009b) stated that *Nicrophorus vespillodes* (Figure 3.12) is also a common species of burying beetle.The fact it was only recorded on the low dose carrion could suggest that the initial delay in decomposition makes the carrion more attractive to *N.vespillodes* in a similar manner to the work of Chick (2009) who stated that if the colonisation of small carrion by Diptera is delayed then the relative attractiveness to carrion frequenting Coleoptera appears to be increased. Its absence from the high dose carrion would suggest the higher nicotine level in the high dose repelled *N.vespillodes.*

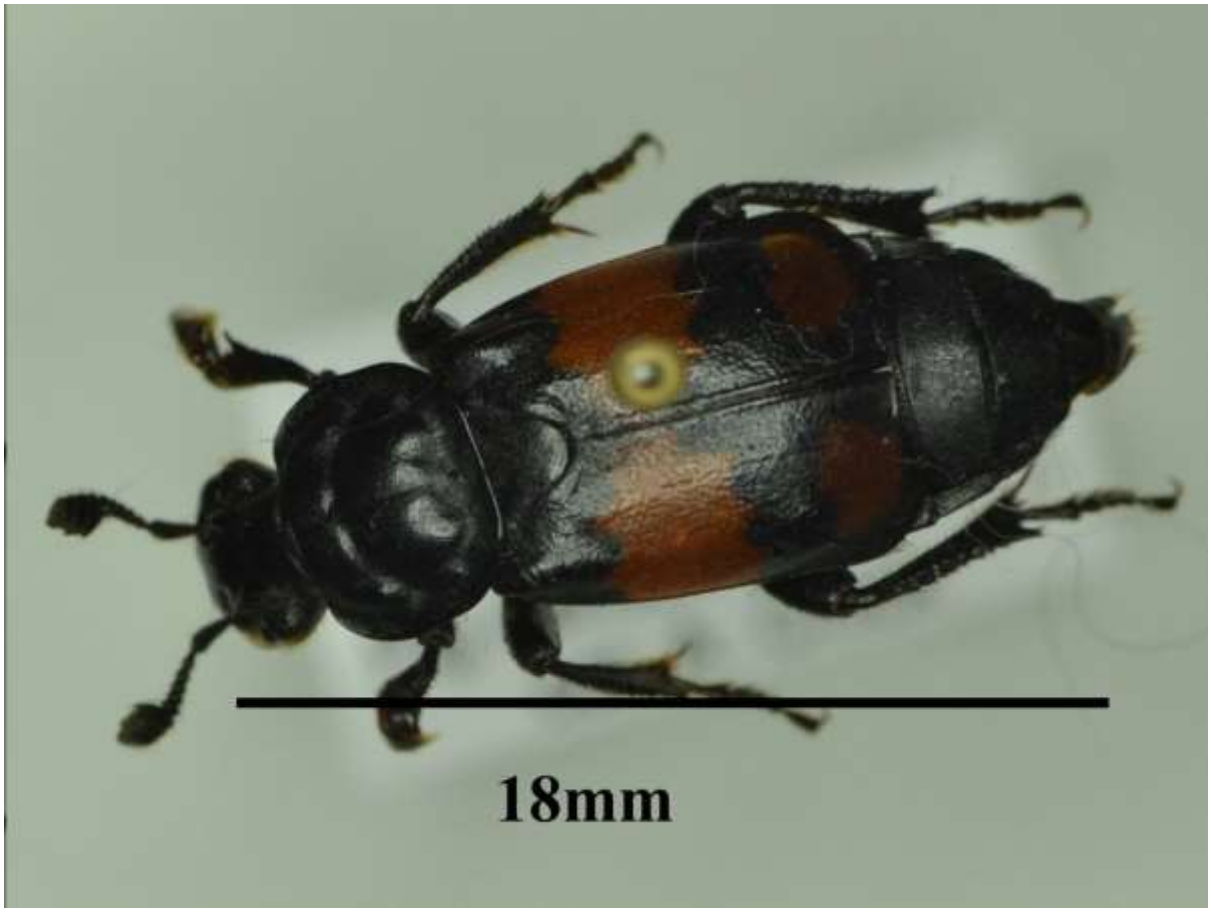


Figure 3.12: *Nicrophorus vespillodes* collected from low dose carrion.

3.4.6. Staphylinidae

Joy (1933) referred to *Creophilus maxillosus* (Figure 3.13), a single specimen of which was found on the high dose carrion, as a well-known carrion beetle, Harde (1984) stated that while *C.maxillosus* appears to have a preference for carrion, it will colonise other decomposing matter as well. Byrd and Castner (2001) referred to *C.maxillosus* by the common name of "the hairy rove beetle" and noted that the species is found throughout the eastern United States. It was also suggested that *C.maxillosus* may be found on carrion in the U.S.A. within hours of death, as well as in the advanced stages of decomposition, where both adults and larvae feed on maggots. Smith (1986) stated *C.maxillosus* was recorded in a forensic case in the U.K. on a body which had lain in the North Downs for 17 days in October 1969. *C.maxillosus* only being found on the treated carrion appears to contradict the

literature such as Joy (1976) who considered *C.maxillosus* to be a common carrion frequenter. However Lott and Anderson (2011) noted that *C.maxillosus* is prone to pupate during November which could offer an explanation for the lack of specimens.

Figure 3.13: *Creophilus maxillosus* collected from the high dose carrion

Ocypus olens (also known as the Devil's coach horse) (Figure 3.14) was found on all carrion suggesting that nicotine had little deterrent effect on it. Lott and Anderson (2011) noted that *O.olens* is the largest beetle in the Staphylinidae, and will eat a wide variety of foods, including large Carabid beetles, and earthworms. However *O.olens* apparently appears reluctant to eat live blowfly larvae due to problems relating to mandible purchase on the soft bodied larvae. It is also noted by Lott and Anderson (2011) that *O.olens* is found in a wide range of environments, albeit with a preference for soils with good drainage. *O.olens* is one of the most frequent specimens sent to museums for identification, particularly in autumn, when it breeds (Nield, 1976).

Figure 3.14: *Ocypus olens*.

Ontholestes tessellates was found upon the control and high nicotine carrion. The genus *Ontholestes* is found all over the world, apart from the Antarctic and the Australasian region, and in the U.K. it is represented by 2 species. *O.tessellates* has the wider distribution than the smaller *Ontholestes murinus*, being found throughout England, Wales, Scotland and the Isle of Man. *O. tessellates* feeds on fly and beetle larvae in dung and dung heaps, but is mainly found upon carrion which also has high concentrations of its prey of fly and beetle larvae (Lott and Anderson, 2011).

The high dose carrion also contained *Tasgius morsitans (=Staphylinus compressus).* Lott and Anderson (2011) remarked that *T.morsitans* can be found in a variety of environments on dry to damp soils and Chick *et al* (2008) previously discovered *T.morsitans* upon rat carrion treated with a commercial fly spray containing tetramethrin and d-phenothrin. This could suggest that *T.morsitans* is tolerant to insecticide pollution within a corpse. This could be due to a number of reasons such as insecticides acting as a chemical cue for *T.morsitans* (or its prey), *T.morsitans* being more tolerant of insecticides and thus able to out-compete intolerant species. Or it could be that *T.morsitans* is a scavenger, either in an obligate or facultative fashion, and thus is attached to dead insects which may be on the corpse. The Staphylinid

beetle *Bisnius(=Philonthus) fimetarius* was also found upon the high nicotine carrion exclusively. Lott and Anderson (2011) remark that *B.fimetarius* is common in litter piles, dung and rotting fungi, where it predates upon dense insect larvae populations, suggesting that carrion might be a possible habitat.

The Staphylinid fauna of the control carrion aside from the previously mentioned species was dominated by small species from the sub families Tachyporinae and Aleocharinae. Cooter and Barclay (2006) remark that both families present a number of identification problems due to their small size and reliance upon obscure identification characteristics; *Tinotus morion* was the only Aleocharinae species able to be fully identified to species level. Harde (1984) states *T.morion* is commonly considered a dung frequenting species common throughout Europe. Skidmore (1991) observed that the larvae of Aleocharinae, such as *T.morion,* act as internal parasites of Diptera upon dung, Smith (1986) remarked this parasitic behaviour might lead to such species being attached to Diptera upon carrion. Cooter and Barclay (2006) noted that Aleocharinae are most often found upon rotting vegetation, with carrion, dung and fungi being the other most common habitats. The Tachyporinae are found most commonly in moss, grass and leaf litter. Although the genus *Tachinus* is associated with carrion (Cooter and Barclay, 2006), identification beyond genus was not possible, due to a gap in the current literature. Harde (1984) state that *Tachinus* comprises 20 central European species, of which 14 are found in Britain, remarking that the genus is not only found in carrion, but also dung, decomposing plant material, sap runs and animal nests. The staphylind fauna of the low nicotine carrion also contained two *Tachinus* species, as well as *Micropeplus fulvus.* The sub-family Micropeplinae comprises four species in Great Britain (Lott and Anderson, 2011). Cooter and Barclay (2006) consider the Micropeplinae to be common in vegetable detritus. Harde (1984) states the sub-family is most common upon rotten and mouldy plant material. Tottenham (1954) remarked that *M.fulvus* is common throughout the U.K.

3.4.7 Scarabaeidae

Aphodius prodromus (Figure 3.15) was found on both the control and low dose carrion and *Aphodius contaminatus* (Figure 3.15) was found upon the high dose pig. Jessop (1986) remarks that the genus *Aphodius* is primarily a dung feeder, occasionally feeding upon decomposing plant material. Smith (1986) stated that members of the genus have previously been collected from pig carrion exposed to the elements de Almeida *et al* (2015) remarked that Aphodiinae have previously been found upon pig carrion in Brazil however the specimens were not identified beyond sub-family level. *A. prodromus* is most commonly found in various kinds of dung, but also occurs in flood refuse. It is a common species in England, Wales and Scotland which is most abundant in spring, but also found through the year (Jessop, 1986). *A.contaminates* generally feeds in dung, with a preference for horse dung. By contrast to *A. prodromus*, *A.contaminates* is most commonly encountered in the autumn months, (Jessop, 1986). Skidmore (1991) notes the adults are most common in the months of September and October in the U.K. The presence of *A.contaminates* like *Sargus bipunctatus* appears to be possibly characteristic of autumnal decomposition. Like with *S.bipunctatus* the presence of insects traditionally considered dung breeders might be due to organic leachates from the cadaver mixing with soil and/or leaf litter creating a form of synthetic pseudo-dung, although this is something that would require further investigation, possibly looking at the chemical composition of dung verses soil/leaf litter enriched with carrion fluids.

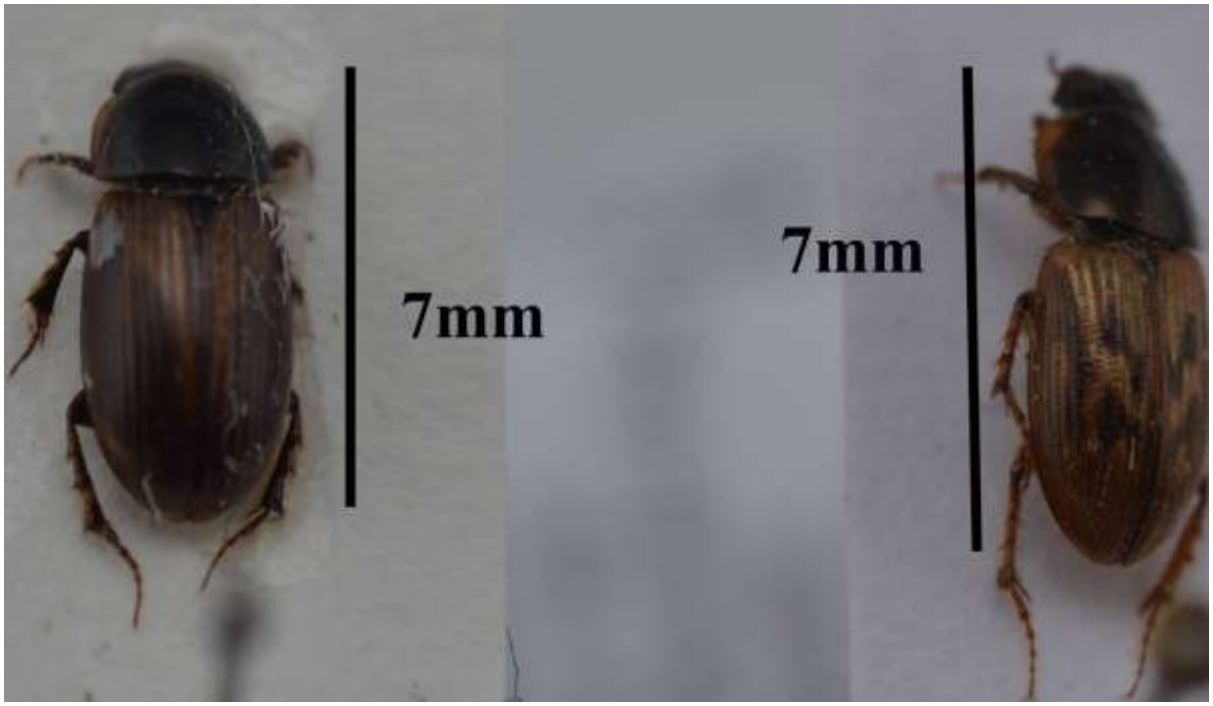


Figure 3.15: *Aphodius prodromus* (left) and *Aphodius contaminatus* (right)

3.4.8. Ptiliidae

A single unidentified specimen of the Ptiliidae (Figure 3.16) was found upon the control carcase. Ptiliidae are some of the smallest insects know to science, with none of the seventy British species having a length over 1mm (Cooter and Barclay, 2006). Smith (1986) remarks that that the Ptiliidae have been recorded from carrion, although species were most likely feeding upon fungal hyphae rather than upon the carrion itself. Terrell-Nield (Pers comm) stated that Ptillidae can be collected from kitchen waste compost. Cooter and Barclay (2006) remarked that due to the small size of the Ptilliidae special techniques are required for identification, dissection of the genitalia is required for critical determination, and the whole insect should be slide mounted in Euparal. Cooter and Barclay (2006) also remark that the most comprehensive current treatment of the family is written in German. Given that the Ptilliidae is not commonly considered forensically important and the small size of specimens, the presence or absence of Ptilliidae is considered inconclusive. While they might be highly intolerant of cadaver pollution such as nicotine, their absence could be explained simply by the difficulty normally experienced collecting Ptilliidae.

Figure 3.16: Ptilliidae specimen showing the characteristic "feather wings". 3rd part image removed

3.4.9. Lathridiidae

Lathridiidae were found on all carrion, *Cartodere (=Aridius) nodifer* (Figure3.17) being found on the control and treated carrion and *Stephostethus lardarius* (Figure 3.18) being found exclusively upon the high dose carrion. Like the Ptilliidae, Smith (1986) considered the Lathridiidae to be opportunistic fungal feeders as opposed to obligate carrion feeders. Harde (1984) remarks that the Lathridiidae feed upon the spores and mycelia of all kinds of fungi and as such are often found in association with damp decomposing material. Cooter and Barclay (2006) argue that the family are mostly detritus feeders found in association with a mouldy dark places. Although they occur in a wide range of habitats Cooter and Barclay (2006) stated that dung is a rare habitat for Lathridiids. *C.nodifer* is also known as "the common plaster beetle" (Cooter and Barclay 2006) and as its name suggests, it is cosmopolitan in distribution (Harde, 1984). Hinton (1945) observed that *C.nodifer* is found in association with mould. Records included mouldy plant material, wall paper and cheese among other substrates. *S. lardarius* has a more restricted range than *C.nodifer*, being common to only Europe and North America, and is slightly larger than *C.nodifer*. Previous records indicate that *S. lardarius* has been recorded from decaying vegetable matter and "a mouldy dried pig's bladder" (Hinton, 1945). The presence of *S. lardarius* upon the high nicotine cadaver is interesting, given the species is not as common as *C.nodifer*. Its absence from the other carrion could be due to the site supporting a smaller population, or it could be that high nicotine carrion supports more fungi than the lower dose and control carrion, thus reducing competition among the fungus feeders.

Figure 3.17: ***Cartodere nodifer. (3rd party material removed)***

3.4.10 Mycetophagidae

Typhaea stercorea (Figure 3.19) of the Mycetophagidae, was collected from the high dose carrion. Like the Ptiliidae and the Lathridiidae Smith (1986) considered the Mycetophagidae to most likely feed upon fungus growing upon the corpse rather than upon the corpse itself. Cooter and Barclay (2006) remark that none of the Mycetophagidae are host specific and will feed upon a variety of fungi. Harde (1984) considers *T.stercorea* a cosmopolitan species which can be found in mouldy plant refuse contained with buildings or in the wild.

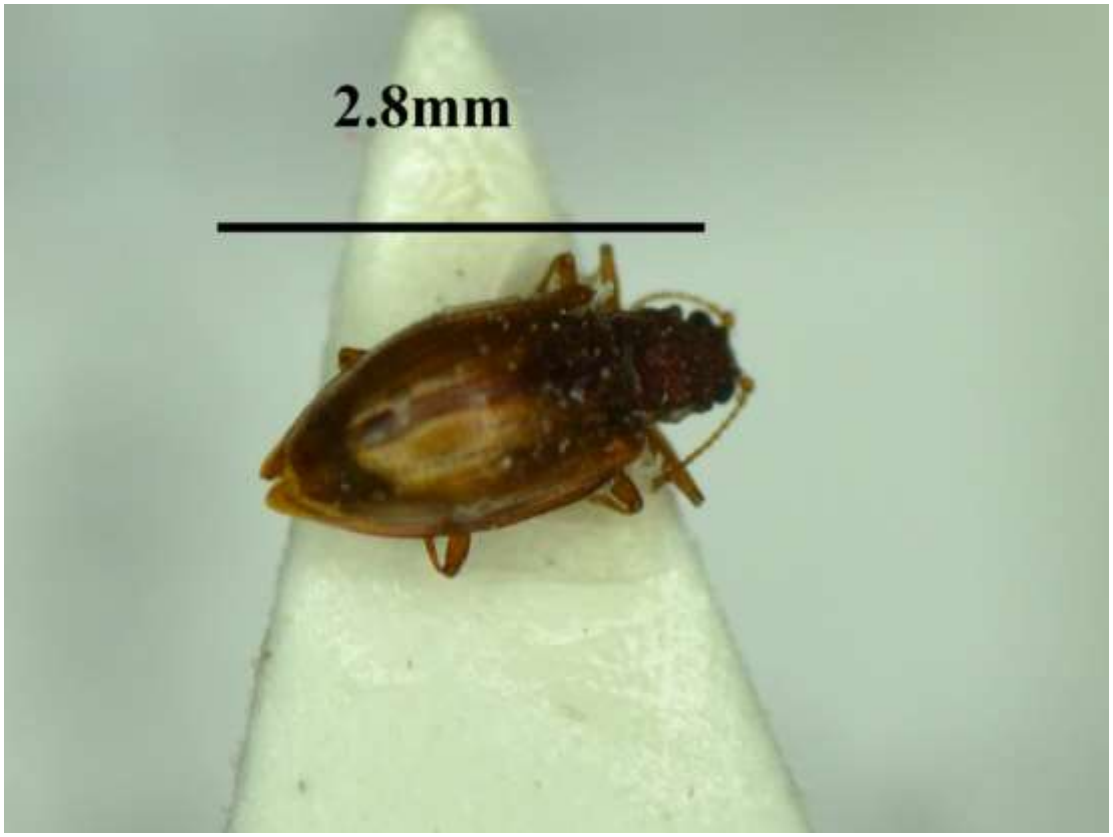


Figure 3.18: ***Stephostethus lardarius***

Figure 3.19: ***Typhaea stercorea***

The presence of these 3 families of fungus feeding beetles upon the carrion, particularly the high nicotine treatment, suggests that fungus plays an important role in the autumnal decomposition of corpses, the increased numbers of fungal feeders upon high nicotine dose carrion could suggest that more fungus is present upon this carrion than the other carrion, which in turn can support a larger sub-ecosystem of fungal feeders. This would require further study of the role of fungi in decomposition of animal remains. Gunn (2006) remarks that forensic mycology is a sadly under researched field and as such not much is known about the role of fungi in the decomposition process. Terrell-Nield and Macdonald (1997) report an analogous situation in caves. Decomposition in the deeper regions of the caves was seen to be primarily fungal, due to lower temperatures and exclusion of primary colonisers such as blowflies.

3.4.11. Nitidulidae

The Nitidulid beetle *Epuraea thoracica* was found upon the high dose carrion. Gennard (2007) remarks that of the Nitidulidae only the genera *Nitidula* and *Omosita* are known as carrion frequenters. Smith (1986) remarks the carrion-frequenting members of the family appear on a corpse during dry decay, often at the same time as Dermestidae, although the Nitidulidae have an apparent preference for moister flesh that the Dermestidae. Kirk-Spriggs (1996) states the *Epuraea* is the second largest genus in the Nitidulidae and that the biology of the genus is unknown in some instances and guessed at in others. A large number have been recorded in association with fungus. Cooter and Barclay (2006) note that some larvae the genus *Epuraea* have been observed to be predatory. Given the patchy literature upon the genus, the presence of *Epuraea* upon the high dose could either be as a fungus feeder as with the Mycetophagidae, the Ptiliidae and the Lathridiidae or it could be present as a predator of these mycophagus species. As previously, stated the presence of *Fannia* indicates that the high dose carrion is more likely to have been moister, or even semiliquid than the low dose and control. Consequently the liquid/moist carrion might be more prone to fungal colonisation, thus attracting fungal feeding Coleoptera. Also as previously stated, Smith (1986) remarked Nitidulidae appear to show a preference for moist flesh during the dry decay stage.

3.4.12. Pseudoscorpionida: Neobisiidae

The pseudoscorpion *Neobisium carcinoides* (Figure 3.20) was collected from both of the nicotine dosed cadavers. Legg and Jones (1988) remark that *N.carcinoides* is possibly Britain's most widespread and common pseudoscorpion. *N.carcinoides* is found in a variety of habitats including woodland and grassland, often in decaying vegetation and in soil. Olsen *et al* (2001) stated that pseudoscorpions are predatory invertebrates, which feed upon insect eggs and other small invertebrates such as mites and springtails. Given that some unidentified mites were found on the carrion at the same time as *N.carcinoides* it would suggest that the pseudoscorpions were upon the carrion as opportunistic predators. However as they were not noted upon the control carrion they may be attracted to invertebrates which in turn were attracted to the carrion because of the nicotine. As previously stated Acari identification is plagued by patchy and inconsistent literature meaning it wasn't possible to identify the mites upon the carrion.

Figure 3.20: ***Neobisium carcinoides.***

3.5 Conclusion

To conclude it would appear that the addition of carrion to the ecosystem changes the collected fauna, with only 40% species similarity beween the low dose carrion compared to the baseline studies, and other carrion showing lower similarities. The Presence of nicotine also has an effect upon the carrion fauna, with the control and low dose only sharing 3 quarters of species collected, and control and high dose only sharing half of the speices collected.

From the collected data it is possible to propose a provisional carrion biotic index for the effect of nicotine upon autumnal decomposition as illustrated in table 3.5, although this table should be considered provisional due to the lack of repeats.

Table 3.5: A provisional carrion biotic index of Autumnal decomposition in the presence of nicotine

Classification of Macro invertebrate	Indicative species
Strongly nicotine intolerant	**Diptera** Syrphidae *Rhingia rostrata* Piophilidae *Liopiophila varipes* **Coleoptera** Ptillidae Staphylinidae Aleocharinae *Tinotus morion*
Mildly intolerant	**Coleoptera** Silphidae *Nicrophorus humator* Scarabaeidae *Aphodius prodromus* **Hemiptera** Lygaeidae *Kleidocerys resedae*
Semi-Nicotine tolerant	**Coleoptera** Silphidae *Nicrophorus vespilliodes* Staphylinidae *Micropeplus fulvus*
Tolerant	**Coleoptera** Carabidae *Pterostichus madidus*

	Pseudoscorpiones Neobisiidae *Neobisium carcinoides*
Strongly Nicotine tolerant	**Diptera** Fanniidae *Fannia fuscula* *Fannia canicularis* Heleomyzidae *Scolicentra brachyptera* Phoridae *Metopina oligeneura* **Coleoptera** Staphylinidae *Tasgius morsitans* *Creophilus maxillosus* *Bisnius fimetarius* Carabidae *Pterostichius melanarius* *Elaphus riparius* Lathridiidae *Stephostethus lardarius* Mycetophagidae *Typhaea stercorea* Nitidulidae *Epuraea thoracica* Scarabidae *Aphodius contaminatus*
Key: Strongly nicotine intolerant = only found upon the control carrion Mildly intolerant = found upon both control and low dose carrion Semi-Nicotine tolerant = only found upon the low dose carrion Tolerant = found upon the low and high dose carrion Strongly tolerant = only found upon the high dose carrion	

The above table demonstrates that a number of species were exclusive to each treatment and as such may be considered to be indicative species of the presence of nicotine in an autumnal carrion ecosystem. As previously stated, the autumnal carrion ecosystem appears to include a number of fungal feeders. These species appear to be affected by the presence of nicotine more than some of the traditional primary colonisers such as Calliphoridae, which appear to not be totally excluded from nicotine treated carrion. Given the cosmopolitan distribution of the Calliphoridae, this would suggest that they have some level of evolutionary resistance to nicotine by previous exposure.

In conclusion it appeared that the nicotine treated pigs exhibited changes in the behaviour of primary colonisers, including a change in the preference of oviposition site. Single egg laying (which is not typical of blowflies) was observed in nicotine-treated carrion. Also observed was a change in the successional decomposition of the carrion. An example of this was that *Sargusbipunctatus* appeared earlier on treated carrion than on untreated carrion, also exclusion of species for differing pigs was observed. *R.rostrata* was only observed upon control carrion, suggesting it is pollution intolerant; however its "rare" status would suggest that the absence of *Rhingia rostrata* alone would not be enough to consider a corpse to be that of a smoker. *N.vespiloides* was only observed on the low doses of nicotine and not that of either the control or that of the high dose, suggesting that while it is susceptible to the higher doses of nicotine, the lower doses somehow make the carrion more attractive to it. *C.maxillosus* was only noted on the high dose carrion, suggesting that it has a high tolerance to nicotine. Its absence on the lower doses could be due to the change in the length of successional stages, or that the exclusion of species in the high dose carrion reduces competition in the niche that *C.maxillosus* occupies. It was also noted that *C.maxillosus* was approaching the end of its breeding year, and that both *S.bipunctatus* and *R.rostrata* were at the height of their abundance at the time of the experiment. If the experiment were to be repeated at a different point in the year a different faunal succession is likely to be observed, for example the presence of *S.bipunctatus* larvae on a corpse is highly indicative of a corpse being dead in the autumn months, whereas it is likely that neither *S.bipunctatus* nor *R.rostrata* would be present on a corpse that was found in March for example. With this in mind it was determined that the experiment should be re-run, with fresh pigs, in the early months of the year to account for species with a yearly brood.

Chapter 4: Spring/summer pig carrion field tests

4.1 Introduction

The autumn/winter experiments of Chapter 3 demonstrated that nicotine had an effect on both the time taken for a cadaver to decompose and upon the carrion fauna present on a corpse. It highlighted some species that are indicative of autumnal decomposition which are not traditionally considered to be of forensic importance. As previously stated, the fauna of decomposition is both seasonally and climatically dependent. Dear (1979) stated the families of flies associated with carrion differ with season; Cooter and Barclay (2006) noted that many species of Coleoptera appear early in the year. It is worth noting that they recommend the use of fish heads and viscera as a carrion bait as opposed to the whole carcase large mammals used in this investigation, which lead to a difference in decay. Chick (2009) observed that smaller carrion such as rat carcases decay too quickly in the summer for colonisation by Coleoptera such as Silphidae, which tend to arrive later in the decomposition sequence (Chick, 2010a) although predatory species of Coleoptera such as Staphylinidae and Carabidae may be present. Indeed the autumn investigation showed that fungal feeding invertebrates are found on carrion in the later months of the year, suggesting that the colder weather and reduced insect activity associated with autumn and winter leads to an increased mycological growth upon a corpse. It was deemed appropriate to repeat the investigation in the warmer spring and summer period.

Following the cutting-back of foliage at site one (Grid reference SK547353) by ground staff, it was determined that it was no longer suitable for use, as the site was exposed to the view of passers-by and no longer met the requirements of a site as outlined in Chapter 2. Consequently the spring and summer testing took place at site 2 (grid reference SK546353) (Figure 4.1). This change of site had the advantage that it had not had carrion recently placed in it, and as such the possibility of carrion leachates in the soil, which might affect results was

low and so the site could be considered “green”. This also eliminated risk of carryover of enhanced local fauna of sites previously exposed to carrion.

Figure 4.1: View of the entrance of site 2 visible from site 1 c.f. Figure 2.1 for location

4.2 Methodology

The methodology was unchanged from the autumn/winter investigation (section 3.2), so three pig cadavers (*Sus scrofa domestica*) of approximately 15kg were treated in the following manner:

Pig 1: was left untreated as a control.

Pig 2: was injected with a dose of 8.4mg of aqueous nicotine.

Pig 3: was injected with a dose of 168mg of aqueous nicotine.

Each corpse was protected from vertebrate scavengers using chicken wire above and below the carrion which was attached to the ground using tent pegs. Climate data was collected as before from a secure camouflaged cage.

Specimens were collected by both passive and active means, and were euthanized using ethylene glycol based preservative in passive trapping, and ethyl acetate, 70% alcohol or freezing in active trapping. Specimens were curated using the methods outlined in the baseline studies (section 2.3.1.)

4.3 Results

The microclimate weather data shown in Figures 4.2-4.5 was collected for 70 days from the 5th May 2010, and as with the autumn/winter experiment, a number of invertebrate specimens were collected during this period.

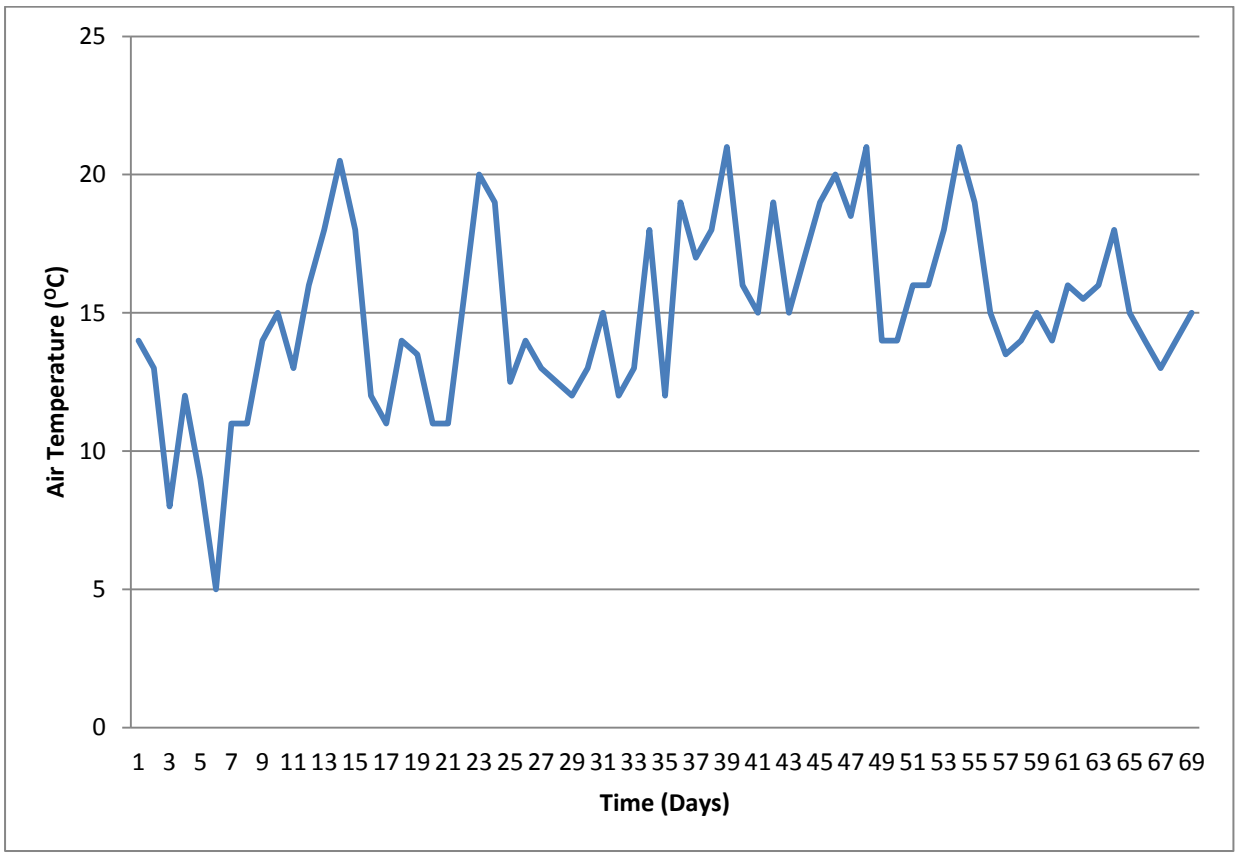


Figure 4.2: Mean air temperature at site two during experiment 2

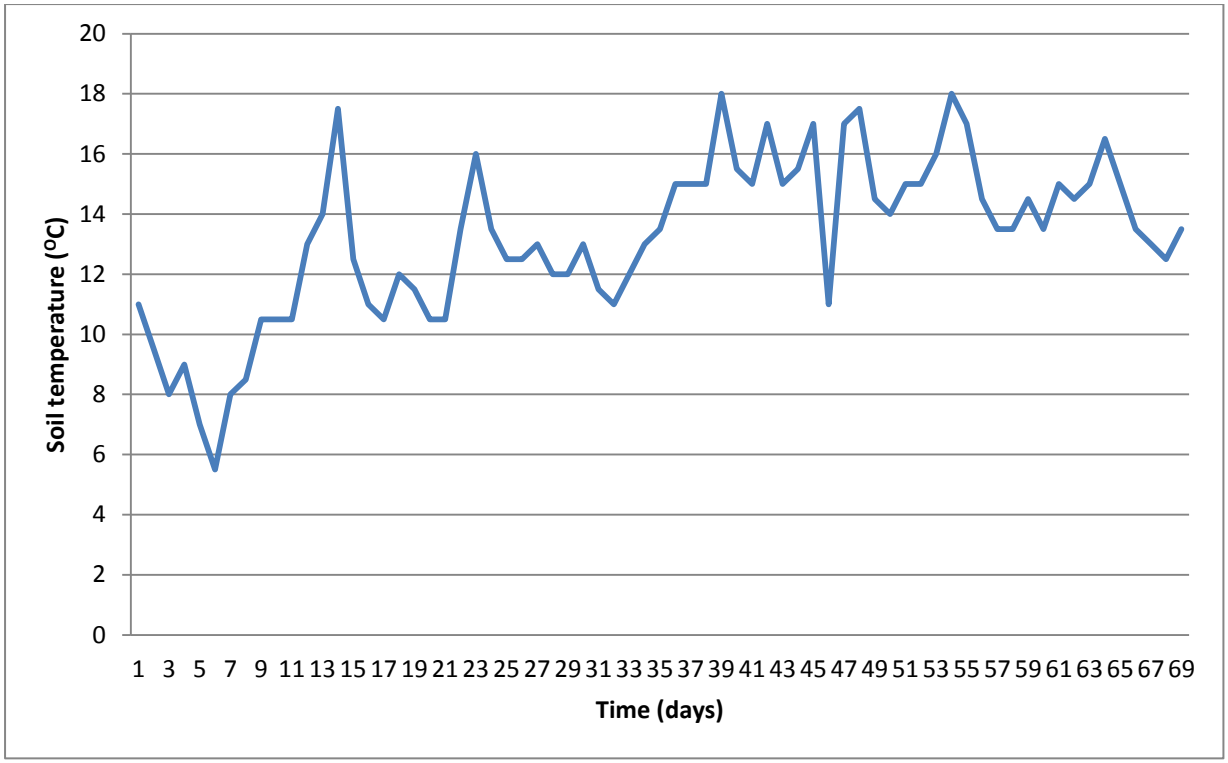


Figure 4.3: Soil temperature at site 2 during experiment 2

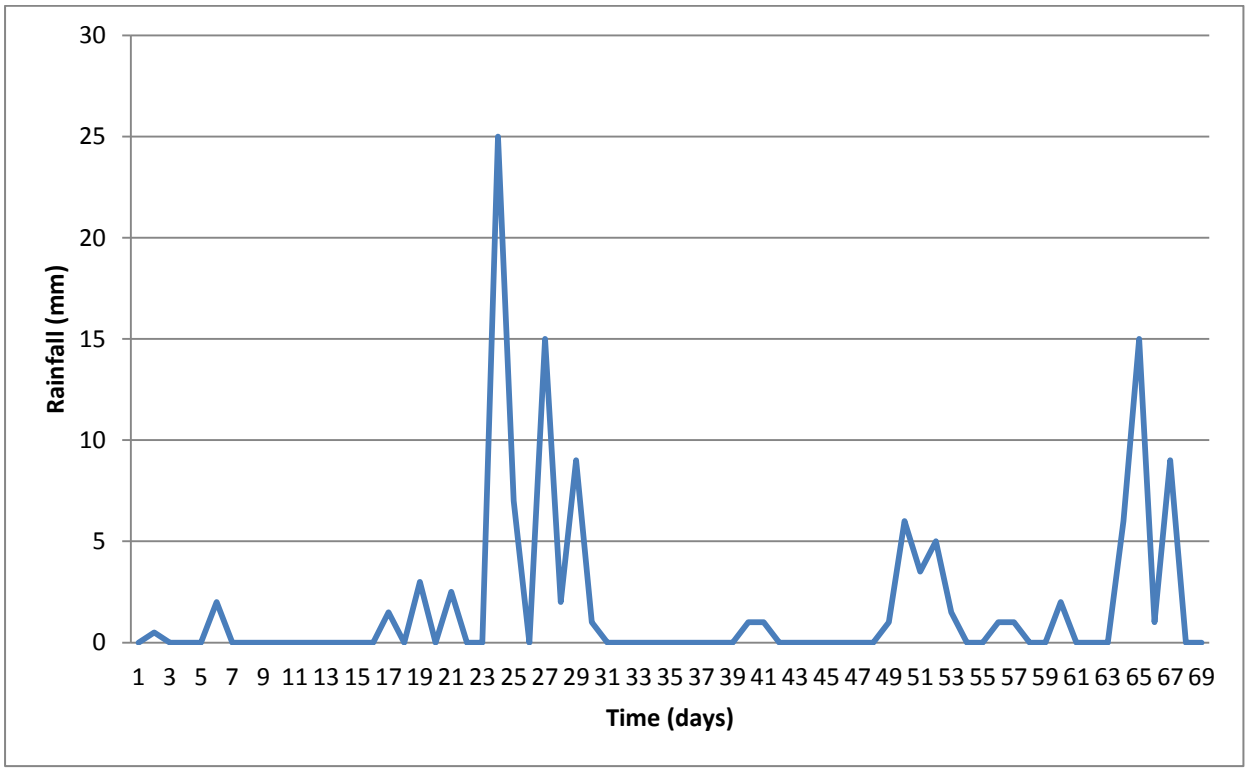


Figure 4.4: Average rainfall at site 2 during experiment 2

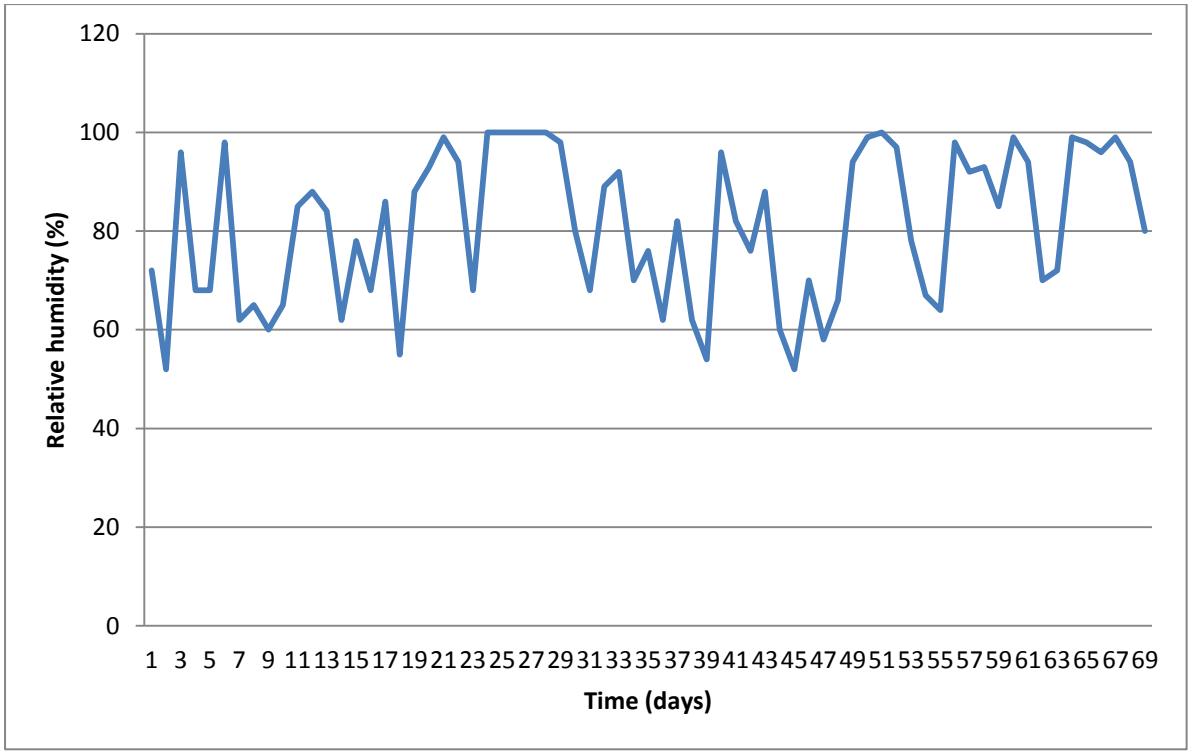


Figure 4.5: Relative humidity of site 2 during experiment 2

Figure 4.2 shows that during the first week of the experiment was the coldest.. Temperatures fluctuated by as much as 15^{O}C over the course of the investigation. The soil temperatures appear to follow the air temperatures closely. Overall the period of the investigation it was dry with a few periods of rain. The humidity varied by as much as 40% over the 70 day study period

Tables 4.1-4.3 shows the invertebrates collected.

Table 4.1: Specimen log for control carrion in spring/ summer

Order	Family	Species
Diptera	Phoridae	*Metopina oligoneura*
	Sphaeroceridae	undentified spp 1
		unidentified spp 2
	Piophilidae	undentified spp
	Sepsidae	undentified spp
	Calliphoridae	*Calliphora vomitoria*
		Calliphora vicina
		Lucilia sericata
Coleoptera	Carabidae	*Carabus nemoralis*
		Abax parallelepipidus
		Pterostichus madidus
	Silphidae	*Nicrophorus humator*
	Staphylinidae	*Philonthus decorus*
		Tachyporinae spp 1
		Tachyporinae spp 2
		Tachyporinae spp 3
	Curculionidae	unidentified spp 1
		unidentified spp 2
Hymenoptera	Braconidae	*Alysia* spp

Table 4.2:Specimen log for low dose nicotine treated carrion in spring/ summer

Order	Family	Species
Diptera	Sciaridae	undetermined spp 1
		unidentified spp 2
	Phoridae	*Metopina oligoneura*
		Megaselia spp
	Opomyyzidae	undetermined spp
	Sphaeroceridae	*Ischiolepta denticulata*
	Fanniidae	*Fannia lineata*
	Muscidae	*Phaonia subventa*
	Calliphoridae	*Calliphora vicina*
		Calliphora vomitoria
		Lucilia sericata
Coleoptera	Carabidae	*Carabus nemoralis*
		Pterostichus madidus
		Nebria brevicollis
	Staphylinidae	*Creophilus maxillosus*
		Philonthus decorus
		Aleocharinae spp 1
		Aleocharinae spp 2
		Tachinus spp 1
		Tachinus spp 2
		Tachinus spp 3
	Histeridae	*Maginarinotus cadaverinus*
Hymenoptera	Braconidae	*Alysia* spp
Dermaptera	Forficulidae	*Forficula auricularia*
Hemiptera	Aphidoidea	Undetermined spp 1
		Undetermined spp

Table 4.3: Specimen log for high dose nicotine treated carrion in spring /summer

Order	Family	Species
Diptera		
	Opomyyzidae	1 undetermined spp
	Lonchopteridae	*Lonchoptera fucata*
	Phoridae	*Anevrina unispinosa*
		Metopina oligoneura
	Sphaeroceridae	*Ischiolepta denticulata*
	Fanniidae	*Fannia lineata*
	Muscidae	*Graphomya maculata*
	Calliphoridae	*Calliphora vicina*
		Calliphora vomitoria
		Lucilia sericata
Coleoptera	Carabidae	*Carabus nemoralis*
		Carabus violaceus
		Abax parallelepipedus
		Nebria brevicollis
		Pterostichus niger
	Histeridae	*Maginarinotus cadaverinus*
	Silphidae	*Necrodes littoralis*
		Nicrophorus humator
	Staphylinidae	*Creophilus maxillosus*
		Philonthus decorus
		Bisnius fimetarius
		Aleocharinae spp 1
		Aleocharinae spp 2
		Aleocharinae spp 3
		Aleocharinae spp 4
		Aleocharinae spp 5
		Aleocharinae spp 6
		Tachinus spp 1
		Tachinus spp 2
		Tachinus spp 3
		Tachinus spp 4
		Tachinus spp 5
		Tachinus spp 6
		Tachinus spp 7
		Tachinus spp 8
		Tachinus spp 9
	Nitidulidae	*Omosita discoidea*
	Curculionidae	unidentified spp 1
		unidentified spp 2
Hymenoptera	Braconidae	*Alysia* spp
Dermaptera	Forficulidae	*Forficula auricularia*

Table 4.4: Comparision of the differing pig carrion in the spring /summer investigation

	Control	Low dose	High dose
Total species	19	25	41
Similarity to control (%)	-	28%	21%
Similarity to low dose (%)	-	-	37.5%

4.3.1 Observations

The carrion was treated and placed *in situ* on May 7th 2010 and attracted blowflies within a few minutes, the control appearing to draw the most attention. Within twenty four hours, the control cadaver appeared to have attracted beetles from the families Scarabaeidae *(Aphodus prodromus)*, and Staphylinidae *(Philonthus spp)*, and acalyptrate flies. The low dose corpse had attracted *Philonthus, Bibio marci* (Diptera: Bibionidae), *Nebria,* velvet mites and acalyptrate flies. The high dose had attracted *Philonthus, Bibio marci* and acalypterate flies including *Neuroctena anilis.* Within 48 hours oviposition by Calliphoridae was observed on the ears and nose of the control cadaver. Beetles of the genus *Carabus,* as well as representatives of the Tachyporinae (Coleoptera: Staphylinidae) were noted in the pitfalls. *Phaonia subventa* was observed on the low dose carrion, however no oviposition was seen. The high dose appeared to have some oviposition although in lower numbers than the control, and eggs were seen on the anal region and the mouth. Single egg laying was also observed. *Pterostichus madidus* was recorded upon the high dose carrion. By the fifth day, small egg clumps were noted on the treated pigs around the sites of the dispatch wounds, further egg laying was noted on the control pig. By the end of the first week the head of the control pig was covered in fly eggs and oviposition was noted on the ground around the head. By day eight Calliphoridae were noted on all carrion. The control had Calliphoridae on the head, whereas the high dose pig had Calliphoridae on the hind quarters. *Bibio marci* was found in the control pitfall. On the eleventh day, *Nicrophorus humator* was found upon the control cadaver and the high dose carrion had trombiculid mites in the pitfalls, and a large egg mass

upon the genital region. By the twelfth day *Aphodius* was observed on the low dose carrion. The first signs of 1st instar (blowfly) larvae were noted on the control and high dose pigs on the thirteenth day. When the carrion had lain *in situ* for two weeks, a large number of flies were observed including Calliphoridae, Sepsidae, Fanniidae and the Muscid *Phaonia subventa*. Braconid wasps of the genus *Alysia* were also present. The low dose carrion had oviposition on the rear legs and tail, as well as some first instar feeding on the eyes. Fly activity was low on the low dose, although *Calliphora*, *Lucilia* and *Phaonia subventa* were observed as well as members of the Fanniidae and some small acalyptrate flies. The high dose had *Lucilia*, *Calliphora*, sepsid and fanniid flies present as well as *Alysia* spp. Larval activity in the buccal caviety was also observed upon the carrion. On the fifteenth day *N. humator* was observed in the high dose pitfall trap. By day eighteen Histeridae were observed in pitfall traps of the high dose and the control. Another *N. humator* was found in the control pitfall traps. The flesh of the head on the control carrion was blackened due to decomposition, and small amounts of feeding upon the genital region were observed. The high dose cadaver showed signs of extensive bloating (Figures 4.6-4.7) and also had the smallest spread of feeding, although the maggots present upon it were much larger than those on the control carrion. The high dose carrion also showed the largest fly activity and diversity. The low dose carrion also had a low spread of feeding and larger maggots than were observed upon the control. On the nineteenth day the abdomen of the control corpse was breached by larval feeding. The larval mass on the low dose was noted to be producing a froth. On the twentieth day the low dose carrion was seen to have larvae surrounding the cadaver. The twenty first day saw the high dose carrion reach the stage of abdominal breach and marks were noted upon the carrion where it had pressed on the chicken wire which enclosed it. On the twenty second day the high dose carrion was fully deflated post larval breach, and had two larval masses, one on the head and one on the anal region. The low dose

cadaver had a large larval mass covering the abdomen, the feeding of which was so voracious that it produced an audible sound which had not been noted in any of the other experiments. The control had a larval mass feeding upon the anus, and numerous Histeridae feeding upon the Diptera larvae present on the remains. Active histerid feeding was noted on the high dose carrion on day twenty five. On the twenty seventh day it was apparent that the larval feeding had moved around the injection sites of the treated pigs, the same area was consumed on the control pigs. A strong smell of ammonia was observed at the site. On the twenty eighth day the first signs of migration were noted. The low dose pig showed some evidence of vertebrate scavenging on the twenty ninth day. By the thirty first day the control was showing the first signs of Skeletonisation, with the rib bones becoming visible *via* the site of the abdominal breach, and larval feeding had spread to the hind quarters.

Figure 4.6: Comparative bloating of the test carrion (Top: Control, Middle: Low dose, Bottom: High Dose).

Figure 4.7: Close up of the extensive bloating of the high dose carrion.

Creophilus maxillosus was observed upon the high dose cadaver and by the thirty second day the treated carrion had many of this species. A further *N.humator* specimen was seen on the control carrion on the thirty fifth day, and *Necrodes littoralis* was found upon the high dose carrion. By the thirty eighth day the control cadaver also appeared to have evidence of vertebrate scavenging. A fungal growth was observed upon the high dose carrion on the forty ninth day. By the sixty fifth day, the control had *N.humator* still upon it, whereas the low dose had Cardinal beetles upon its remains.

4.3.2 Comparative seasonal data

From Figures 4.8-4.11 below it would appear that while the temperatures of the spring/summer were lower in comparison to the autumn/winter investigation at the start of

the investigations, the temperatures dropped in the autumn/winter investigation and increased in the Spring/Summer investigation in line with expectations. The humidity and rain fall appeared to fluctuate in a similar manner but over different times.

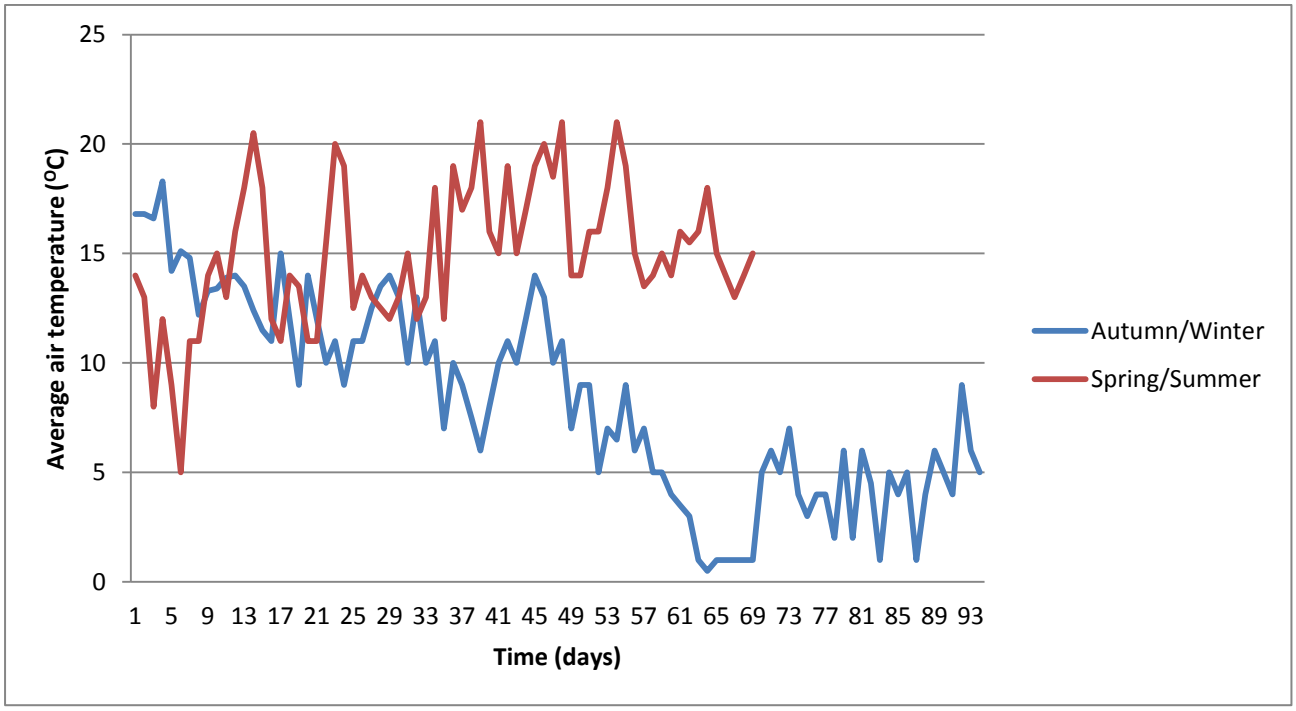


Figure 4.8: Comparission of average air temperature during experiments 1 and 2.

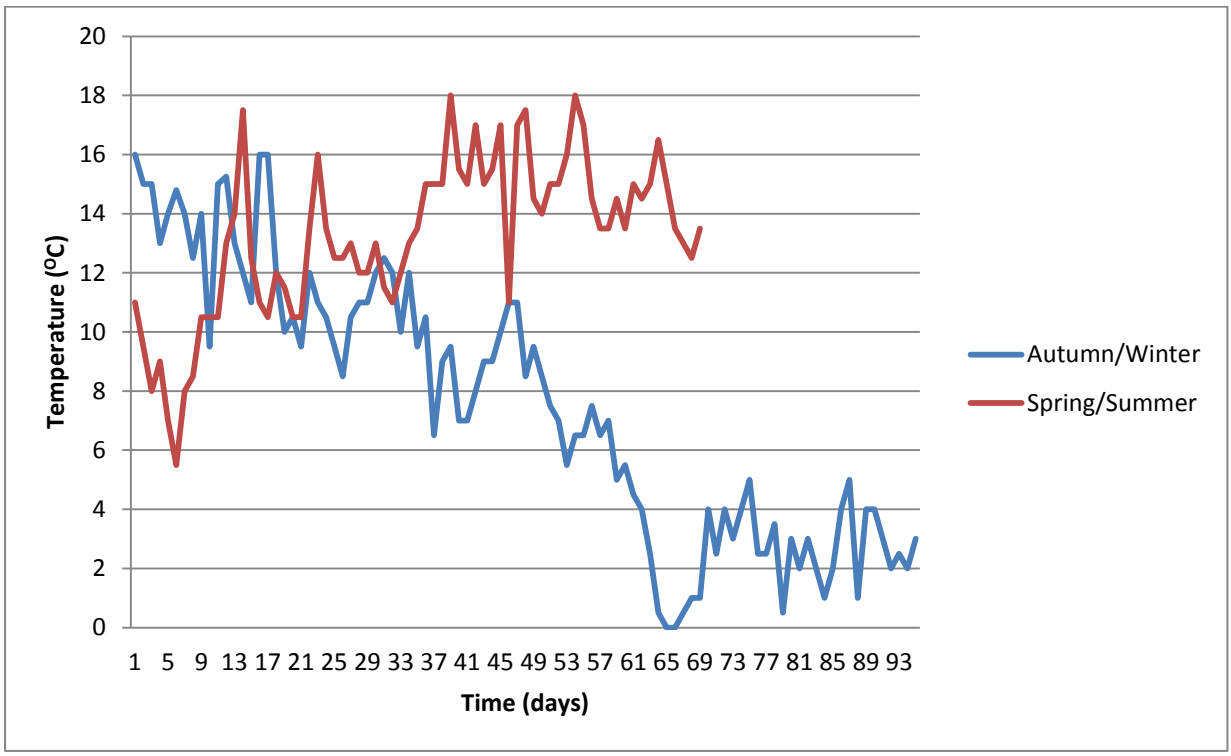


Figure 4.9: Comparission of soil temperatures during experiment 1 and 2.

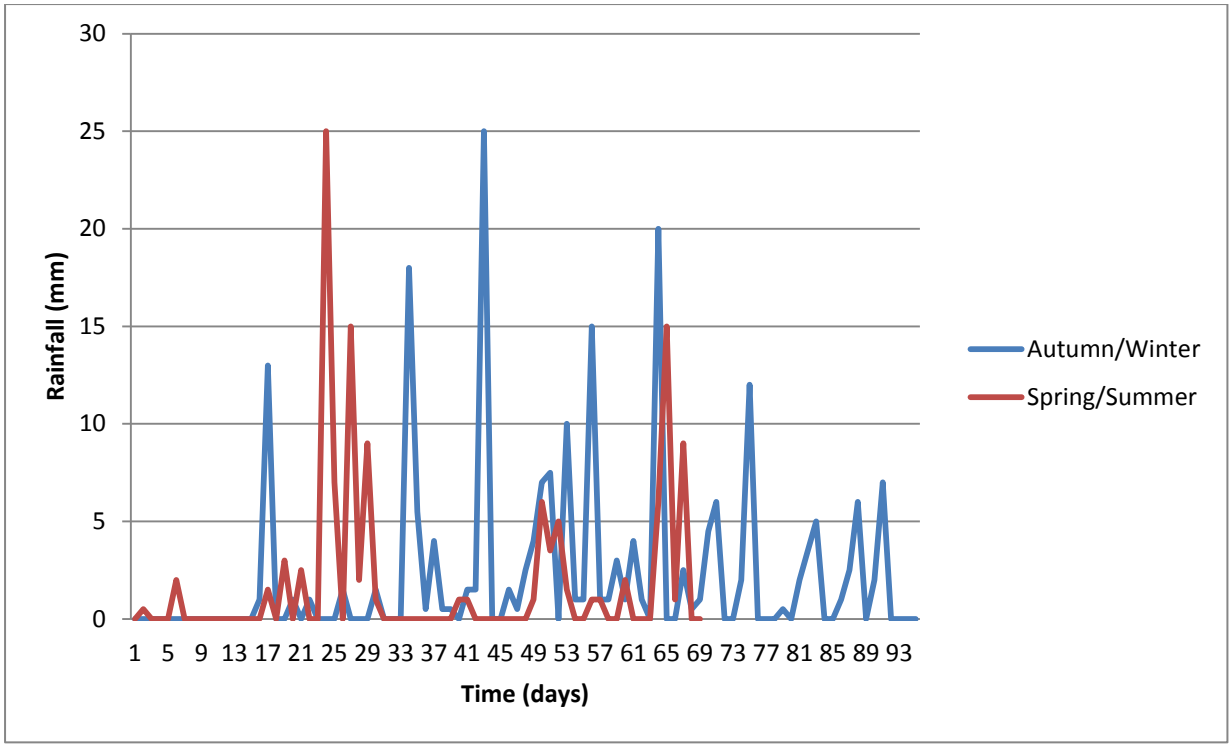


Figure 4.10: Comparision of rainfall during experiments 1 and 2.

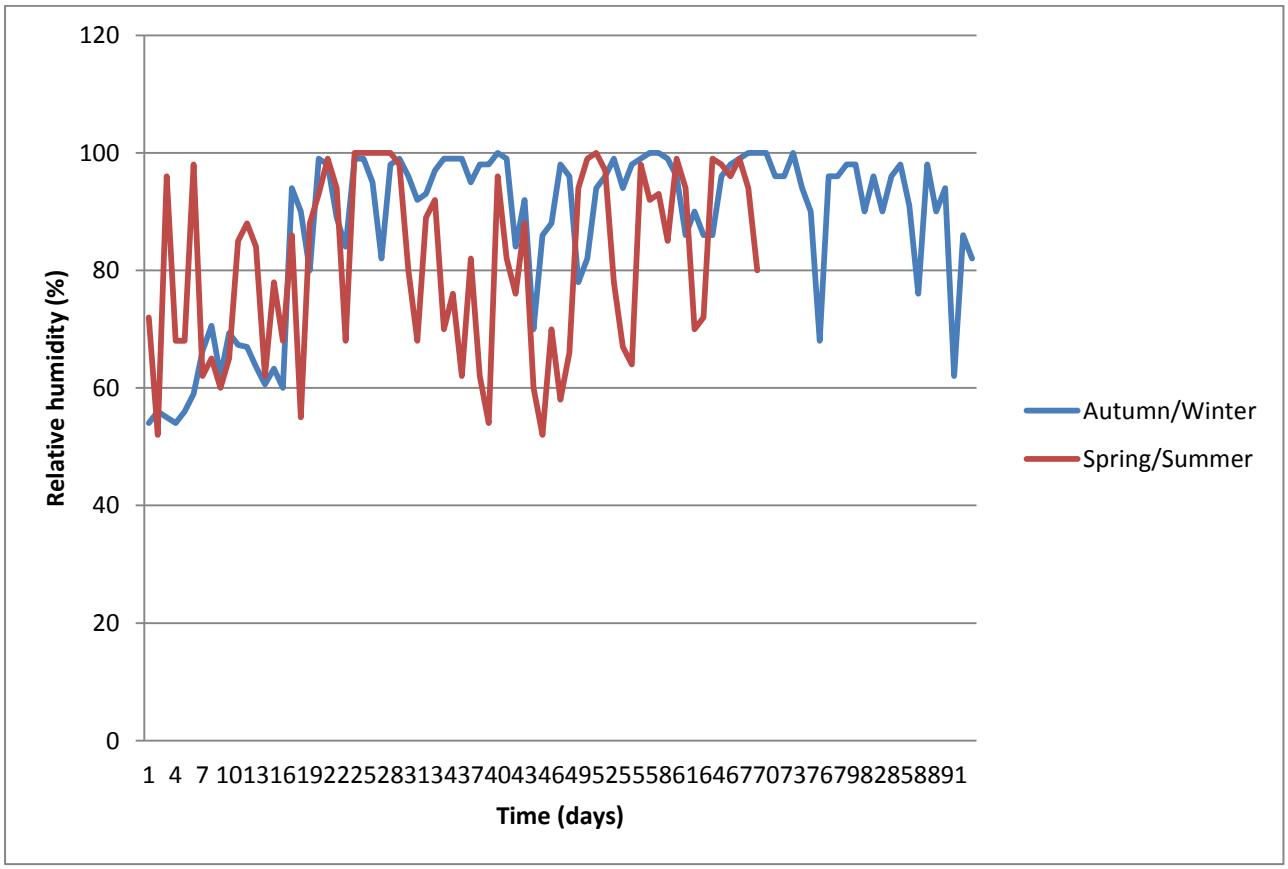


Figure 4.11: Comparision of relative humidity during experiments 1 and 2.

A similarity calculation for invertebrates was also carried out to compare the autumn/winter investigations with the spring/summer ones (Table 4.5)

Table 4.5:A comparison of the percentage of species from the autumn/winter investigation and the the spring/summer carrion

Percentage similarity between controls with season	Percentage similarity between low doses with season	Percentage similarity between high dose with season
24%	34%	28%

4.4: Discussion

From the above descriptions it would appear that as in the autumn investigation, nicotine affected both the primary colonisers and the carrion fauna as a whole. It seems that the way a nicotine treated corpse decomposes is markedly changed, with the initial delay in decomposition leading to an increase in decomposition rate in the later stages. As previously stated there maybe several reasons for this. Smith (1986) stated that larvae initially feed upon the liquid between muscle fibres, due to the acidity of tissue being above the optimum pH of the primary colonisers. During the course of autolysis and putrefaction, the pH of the intermuscular tissues is raised and this tissue is now attacked by necrophagus invertebrates (Smith, 1986). This is borne out by observations by Erzinclioglu (1996) who noted that carrion aged for 10 days can often be more attractive to blowflies than fresh carrion in laboratory based tests. Smith (1986) also listed the order in which various organs decompose, it was noted that the brain, bronchi and lungs decompose more slowly that the gastro-intestinal system and the circulatory system. Taking this into account it would appear that delayed oviposition around the anal and genital regions creates conditions for facilitating the increase in rate of decomposition observed in field based testing.

The level of bloating in the high dose pig prior to the active decay would appear to add new knowledge to the literature, which suggests that the behaviour of primary colonisers is

optimised by a small delay in decomposition, coupled with a change of oviposition site. By extrapolation it would suggest that knife wounds to the stomach region and a delay in decomposition might lead to even faster post oviposition decomposition. As previously stated primary colonisers will also oviposit on any wounds inflicted on the corpse.

The high level of bloating also suggests that the bacterial activity in the stomach is high and Gunn (2006) reported that during the onset of bloat, bacterial metabolism causes the corpse to give off various pungent gases which increase the attraction of primary colonisers to the corpse. The breakdown of tissues by bacteria changes the carrion at a chemical level. As previously stated, aged carrion and carrion with a higher pH appear to provide optimum conditions for blowfly feeding. It is therefore conceivable that bacteria in the corpse raise the pH. Gunn (2006) remarked that microbial decomposition begins in the intestines, yet the action of bacteria acidifies the corpse. It has also been previously noted that decomposition of the organs of the digestive system leads to the characteristic bloating of corpses. This is due to the digestive system having a distinct bacterial fauna that in life serves to break down proteins. This would appear to make the digestive system easier to digest by blowflies and will therefore increase the rate of decomposition after the initial delay in oviposition, which suggests this liquefaction of tissue is more important than pH level in the development of carrion frequenting blowflies. The high level of bloating also causes marks on the high dose carrion where the increase in size leads to the cadaver exerting a large amount of pressure on the mesh cage which protected it from scavengers. These marks did not fade after the bloating passed (Figure 4.12). Gunn (2006) noted that some *post mortem* injuries may be difficult to differentiate from *ante-mortem* injuries. It seems logical to consider that *post mortem* injuries caused by a combination of pressure on the corpse and a delay in decomposition could be confused with non-accidental injuries in a forensic environment.

Figure 4.12: Marking left on the flesh of the high nicotine treated carrion due to extensive bloating.

One important question that needs to be considered would be, if Calliphoridae require or prefer carrion that has laid *in situ* for a period of days, why are they the first to arrive and oviposit on carrion? In fact Smith (1986) noted that in nature Calliphoridae may breed in live tissue in some cases. This was also considered by Benecke *et al* (2004) who stated that blowflies may be indicators of neglect in the elderly. The simple answer could be due to the temporary nature of carrion and the genus *Nicrophorus* (Silphidae) will bury small carrion if they arrive first. Pape (1987) stated that the Dipteran family Sacrophagidae (the flesh flies) are also specialised to colonise smaller carrion. The flesh flies either larviposit (give birth to live offspring) or lay fully incubated eggs on the carrion. While this results in a reduced fecundity it gives an advantage in the exploitation of a transient food source. Smith (1986)

notes that Sarcophagidae will also fly in the rain and in such conditions will be the first arrivals at a corpse but may also arrive after the main blowfly sequence when carrion is older. If we consider carrion as a whole, small mammals and birds are the most likely to be encountered by carrion invertebrates, given that large livestock are used to feed the population, and humans and pets are mostly buried or cremated, Calliphoridae would most likely be out competed by *Nicrophorus* and Sarcophagidae, both of which show evolutionary adaptation to aid in the colonisation of smaller carrion and as all species named are found on human sized cadavers (Smith, 1986) it is possible this relationship is similar on larger carrion.

4.4.1 Coleoptera-Silphidae

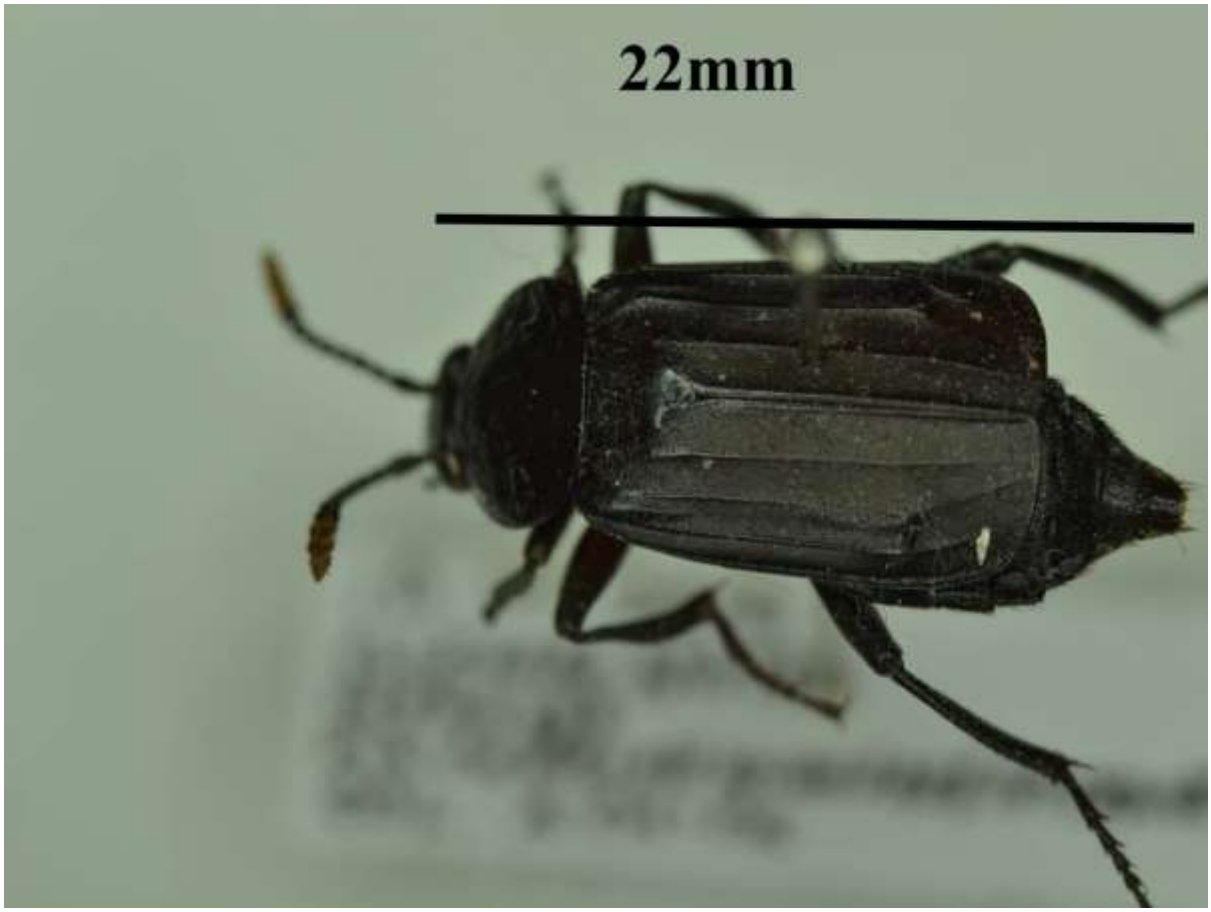


Figure 4.13: ***Necrodes littoralis***

Necrodes littoralis (Figure 4.13) was shown to be restricted to the high dose carrion. *N.littoralis* is stated by Harde (1984) to be declining in abundance in Europe, and to be associated with large cadavers. Harde (1984) also stated that *N.littoralis* is most likely to be found on or near the coasts of Great Britain. Wright (2009c) however disputed this and comments that the species is widespread throughout the British Isles. Smith (1986) recorded the presence of *N.littoralis* on a human body in the UK in October 17 days *post-mortem*, where the species was seen to be feeding upon Dipterous larvae. Smith (1986) suggested this was what most likely attracted *N.littoralis* to the corpse. The NBN Gateway (NBN, 2012) considered *N.littoralis* not to be a Nottinghamshire beetle, although there are records of it on the border of the county. However Sheila Wright (pers com) stated that *N.littoralis* was recorded previously in Nottinghamshire but the records had not been presented to the National Biodiversity Network gateway; Wright also considered *N.littoralis* an "under recorded" species in Nottinghamshire. Given that only a single specimen of *N.littoralis* was found on the high dose carrion this would suggest that *N.littoralis* is rare in Nottinghamshire as numerous specimens of *Nicrophorus* spp. were recorded over the course of the baseline and the autumn studies.

The lack of data regarding the distribution of *N.littoralis* on the National Biodiversity Network gateway is of concern for a forensically important insect. Given that distributional data is sometimes used to suggest if a body was moved, or indeed where a body was moved from, an absence of data could be misinterpreted as an absence of the species from a location. This could then lead to an erroneous conclusion that a body had been moved, when in fact the location in which the body was discovered was an understudied area. This would appear to be a similar sentiment to that of Jones (1980) who stated that his provisional atlas to the distribution of pseudoscorpions showed more the distribution of collectors that the

distribution of the animals themselves. Similar observations relate to other invertebrate groups.

4.4.2 Scarabaeidae

Aphodius prodromus was considered to be common throughout Britain, and it is most abundant in the spring months (Jessop 1986). Skidmore (1991) also commented that *A. prodromus* is common in late winter and spring. Harde (1984) noted that while this species prefers to lay its eggs in horse dung, it will lay in all kinds of rotting organic matter. Smith (1986) stated that 14 related species of *Aphodius* were recorded on above-ground carrion. As stated in the introduction (Section 1.7), life cycles and cohort sizes of members of the genus *Aphodus* were shown by O'Hea *et al* (2010) to be affected by the presence of Ivermectin in cow dung.

4.4.3 Nitidulidae

Omosita discoidea (Figure 4.14) is one of three species of the genus *Omosita* in Great Britain (Duff, 2012b). Harde (1984) stated that members of the genus *Omosita* are associated with carrion, bones and skin. Hinton (1945) expanded further upon this and reported that *Omosita* inhabits dry carcases, and old bones of carcases such as rabbits, dogs and calves. Smith (1986) noted that whilst Nitidulidae (the family of beetles to which the genus *Omosita* belongs) are often found in the company of Dermestidae during the dry stages of decomposition, Nitidulids appear to have a preference for moister skin than Dermestids. The presence of *Omosita discoidea* on the high dose and not on the other carrion could suggest that changes in decomposition created by the higher levels of nicotine lead to a decomposition stage with higher moisture content than expected. This could have been due to

a reduced “active decomposition stage”, meaning the corpse entered the “dry” stage before the moisture of the carrion had completely evaporated.

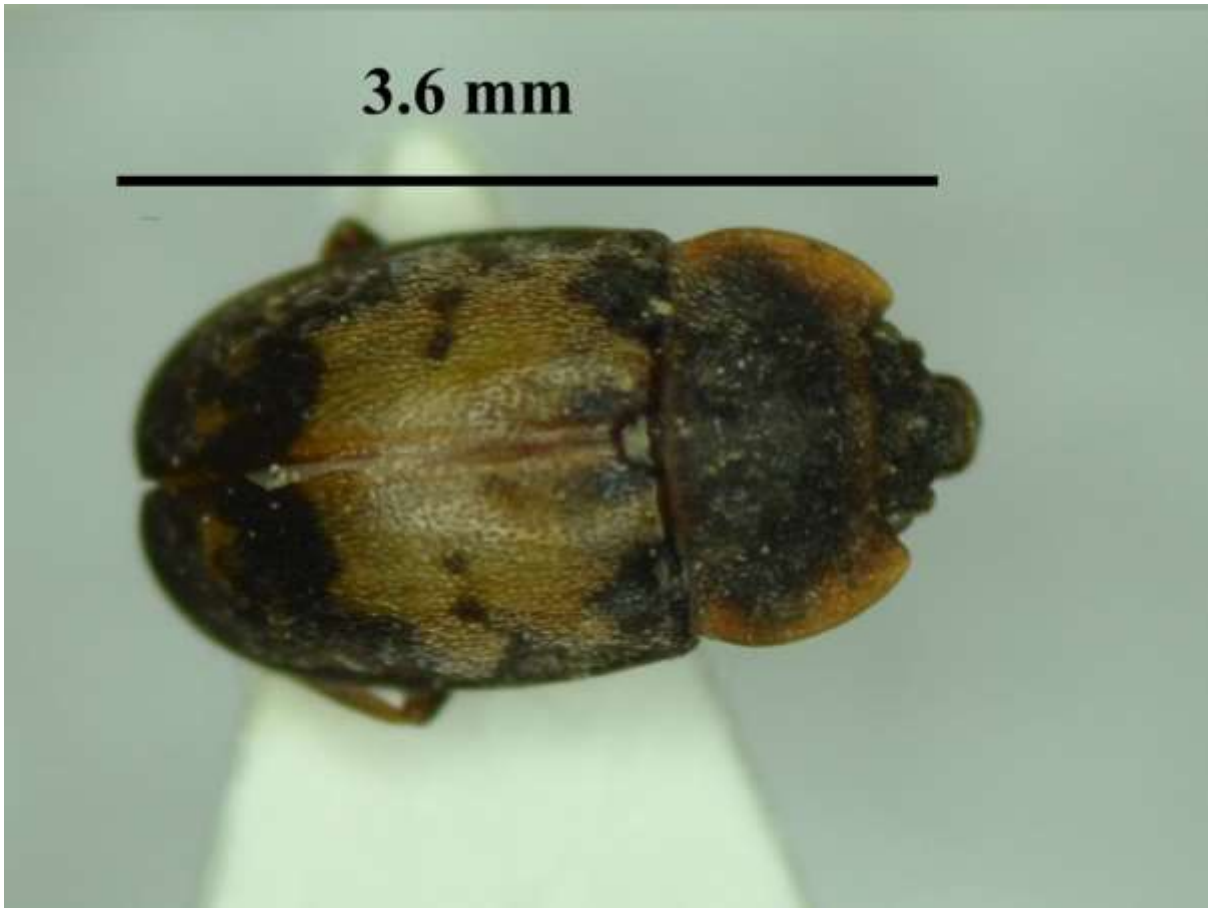


Figure 4.14: *Omosita discoidea*

4.4.4 Histeridae

Maginarinotus cadaverinus was found on both the low and high dose pig cadavers. Harde (1984) remarked that this species is one of the commonest European Histerids upon carrion, although it is mainly found on and in animal faeces. Harde also considers *M. cadaverinus* to be a predatory part of the carrion community and Gennard (2007) states that both the adults and the larvae of the Histeridae are common predators upon carrion. Barnard (2011) considers *M. cadaverinus* to be one of the most common Histerids in the British Isles. The absence of a common carrion species such as *M.cadaverinus* from the control carrion would

suggest that on the untreated carrion, Histerdae are outcompeted for their potential food source, and that upon the treated carrion this competition is reduced.

4.4.5 Diptera: Bibionidae

The presence of *Bibio marci* (Figure 4.15) is interesting; Smith (1986) did not consider the Bibionidae as a forensically important family. In fact Oosterbroek (2006) noted that the larvae feed on both decaying plant material as well as in some cases living roots and stems. Freeman and Lane (1985) stated that the common name of *B.marci* is the "Saint Marks fly" due to its limited active flight period, of around Saint Marks Day (25th April). However, numerous adults were observed around the carrion around the 5th of March. A possible explanation is offered in Smith's (1986) treatise of the Syrphidae, and the Apidae. The adults of non-carrion Syrphidae (hover-flies) are noted to visit carrion looking for moisture. Whereas the Apidae (honey and bumble bees) have been observed to suck-up the foul smelling juices from corpses, Oosterbroek (2006) notes that adults of the Bibionidae are known to visit flowers. Zahradnik (1998) said that bees visit flowers to obtain nectar as a form of food. It is conceivable that the "foul smelling juices" from carrion are chemically similar to that of nectar which would explain the presence of flower feeding invertebrates.

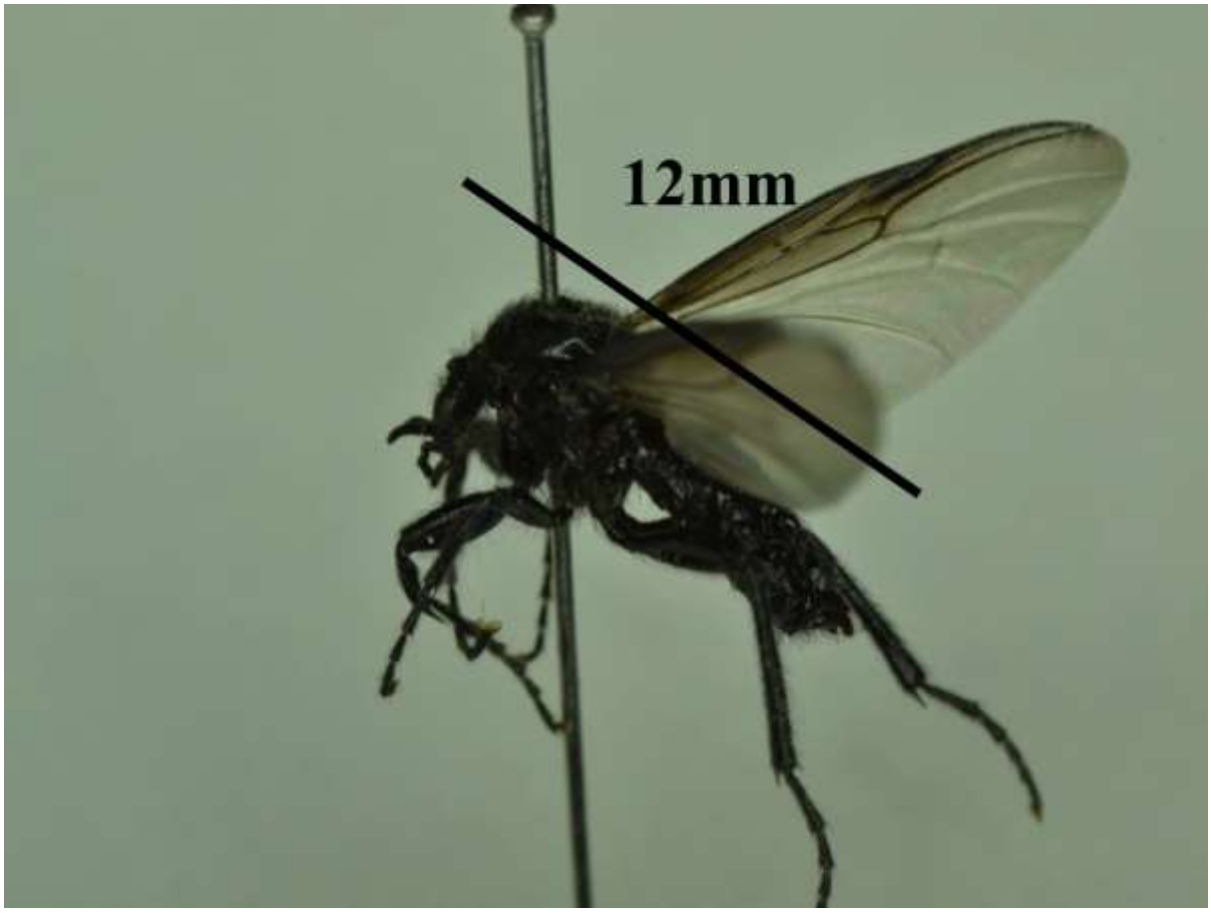


Figure 4.15: ***Bibio marci***

4.4.6 Sphaeroceridae

Ischiolepta denticulata was found upon the treated carrion as well as two specimens of Sphaeroceridae which were collected from the control carrion but in an unidentifiable state due to the pitfall preservative. Pitkin (1988) remarks that *I. denticulata,* which is widely distributed in the British Isles, is found upon carrion including frog and rabbit as well as on horse and cow dung and in decaying plant matter, Gennard (2007) stated that only a few members of the Sphaeroceridae are recorded from corpses, but from numerous stages of the decomposition process. They are generally attracted to ammonia, either from voiding of the bladder or by ammoniacal fermentation.

4.4.7 Phoridae

Metopina oligoneura was found upon all carrion in contrast to the autumn/winter investigation, when it was only found upon the high dose carrion. Another phorid, *Aneverina unispinosa*, which is widespread in England, occurred exclusively on the high dose carrion. Disney (1983) remarks that the larvae of *Aneverina* species are associated with small carrion, soil and mole's nests, however the natural history is poorly known,. A single female *Megaselia* was found on the low dose carrion, but, as previously stated by Disney (1989) females of the genus are impossible to identify: According to Bennard (2011) Disney (1989) is still the most comprehensive account of the family.

4.4.8 Muscidae

Phaonia subventa (Figure 4.16) was noted as being an early arrival at the low dose carrion. However it was found upon all carrion in the autumn/winter investigation. Neither Byrd and Castner (2001), Gennard (2007), Smith (1973) or Smith (1986) consider the genus *Phaonia* as forensically important. However Skidmore (1985) stated that *P.subventa* appears to be the most adaptable example of the genus in the Palaearctic region. In its larval stage *Phaonia* is described as an obligate carnivore (Skidmore, 1985). While the larval habitat preference is stated to be sap runs and rot holes in trees, all kinds of decomposing organic matter is a possible larval habitat. Gregor *et al* (2002) agree and note that carrion appears to be one of the larval habitats of *P.subventa.* Skidmore (1985) commented that the adults are often found on shade tree trunks, flowers, excrement and carrion. Fonseca (1968) observed that *P.subventa* is very common and generally distributed in the British Isles. Skidmore (1985) stated that the species overwinters as a larva pupating in late winter, with the first adults emerging in the wild in April. Fonseca (1968) reported that adults often emerge during the winter months indoors. Skidmore (1985) also made reference to adults being on the wing

during the winter months in Switzerland. It would appear that *P.subventa* is an under-reported carrion frequenter that could be of use to forensic entomologists. Given Fonseca's (1968) statement regarding the presence of early emergence indoors, it could have a niche as a useful coloniser of indoor corpses, but more research would be required to confirm this.

Figure 4.16: ***Phaonia subventa***

Graphomya maculata was found upon the high dose carrion. Although Fonseca (1968) describes it as common and widely distributed in the British Isles, Gennard (2007) Smith (1986) and Byrd and Castner (2001) do not consider the genus *Graphomya* to be forensically important. However Skidmore (1985) remarks that the larvae of *G.maculata,* although not a carrion specialist, have previously been recorded on carrion. Gregor *et al* (2002) maintain that *G.maculata* is a predacious larva that lives in liquid to semi-liquid substrates, a fact

supported by the presence of *Omosita discoidea* and *Fannia lineata,* both of which are typical of a more semi-liquid decomposition.

Skidmore (1985) remarks that *G.maculata* is a highly carnivorous larva, capable of overpowering and consuming other predatory larvae, yet is capable of surviving months without feeding if a suitable prey is unavailable. This trait of being able to survive long periods without feeding would suggest that *G.maculata* might not be suitable for PMI estimates even if laboratory data were available. Indeed Skidmore (1985) stated that under optimal conditions its life cycle may take over a year. The presence of predatory Muscidae is intriguing as the standard forensic literature (Gennard, 2007, Smith, 1986 and Byrd and Castner, 2001) only considers the carrion feeding members of the family. However Skidmore (1985) outlined the biology of the family on a worldwide basis and states that the larval life histories are very diverse, with numerous non-carrion feeders being associated with carrion as predators of carrion feeders.

4.4.9 Fanniidae

Fannia lineata was collected from both the nicotine treated pig corpses in the spring/summer investigation whereas, members of the Fanniidae were found exclusively upon the high dose carrion in the autumn/winter investigation. *F.lineata* is found throughout Europe in the nests of numerous birds, as well as rabbit burrows and vertebrate carrion (Rozkosny *et al.* 1997). Not considered to be a British species by Fonseca (1968) it was added to the British list by Pont (1983). The presence of this species exclusively upon treated carrion further suggests that that the carrion is more likely to attain a semi liquid state when infused with nicotine and thus more likely to attract *Fannia,* the larvae of which are adapted to be buoyant and thrive in semiliquid conditions.

Taking into consideration the above results, it is possible therefore to propose a provisional carrion biotic index for the effect of nicotine upon spring/summer decomposition as

illustrated in Table 4.6. As with table 3.5 due to the lack of replicates this table should be considered to be a provisional study.

Table 4.6: A provisional carrion biotic index of spring/summer decomposition in the presence of nicotine

Classification of Macroinvertebrate	Indicative species
Strongly nicotine intolerant	**Diptera** Piophilidae Sepsidae
Mildly intolerant carrion	**Coleoptera** Carabidae *Pterostichus madidus*
Semi-Nicotine tolerant	**Diptera** Sciaridae Phoridae *Megaselia* spp Muscidae *Phaonia subventa*
Tolerant	**Diptera** Sphaeroceridae *Ischiolepta denticula* Fanniidae *Fannia lineata* **Coleoptera** Carabidae *Nebria brevicolis* Staphylinidae *Creophilus maxillosus* Aleocharinae Histeridae *Maginarinatus cadaverinus* **Dermaptera** Forficulidae *Forficula auricularia*
Strongly tolerant	**Diptera** Lonchopteridae *Lonchoptera furcata* Phoridae *Anevrina unispinosa* Muscidae *Graphomya maculate* **Coleoptera** Carabidae *Pterostichus niger* Silphidae *Necrodes littoralis* Staphylinidae *Bisnius fimetarius* Nitidulidae *Omosita discoidea*
Key: Strongly nicotine intolerant = only found upon the control carrion	

Mildly intolerant = found upon both control and low dose carrion Semi-Nicotine tolerant = only found upon the low dose carrion Tolerant = found upon the low and high dose carrion Strongly tolerant = only found upon the high dose carrion

4.5 Conclusion

It would appear that the presence of nicotine leads to a greater amount of moisture in the carrion. The presence of Nitidulidae, Fanniidae and the Muscid specimens indicate a semi-liquid environment or higher moisture content. The increased bloating observed in the high dose nicotine would suggest that the bacterial decomposition in the corpse is either increased by the presence of nicotine or by the reduced early activity of invertebrates upon the carrion. This possible increased microbial decomposition allows the viscera to break down and liquefy, thus increasing the efficiency of primary colonisers and also changing that carrion fauna, with predacious larvae being attracted to the high dose carrion. The reduced primary colonisation appears to be caused by both a changed carrion fauna and a change in behaviour of the Calliphoridae, with delayed colonisation and a change in egg laying behaviours observed upon treated carrion in both autumn/winter and spring/summer. The single egg oviposition behaviour was previously observed by Greenberg and Kunich (2005) in response to unfavourable conditions and by Chick *et al* (2008) in relation to pesticides. While both the autumn experiment and the spring experiment showed interesting effects on the decomposition of carrion by invertebrates, in a legal case this data would create reasonable doubt that a PMI estimate based upon the carrion fauna of a smoker is accurate, but would not offer an accurate alternative for forensic practitioners. Therefore it was determined that a workable methodology to quantify the effect of nicotine dose upon the decomposition of a corpse would be of the most use to a forensic practitioner, and with this in mind a lab based element was designed as an extension of the research to quantify the effect of nicotine upon primary colonisers.

Chapter 5: The effect of nicotine on the life cycle and development of *Calliphora vomitoria*

5.1: Introduction

5.1.1 Rationale

Field testing demonstrated that nicotine had a number of effects upon the decomposition of carrion by invertebrates, including a delay in the initial colonisation of a corpse by Calliphoridae. Nicotine also affects the oviposition behaviour of the primary colonisers of carrion, leading to a preference for orifices in the hind quarters as opposed to the natural orifices of the face in relation to the site of injection. In normal circumstances the eggs of the primary colonisers are laid in clumps, but single eggs were observed upon nicotine treated corpses. After the initial delay in colonisation, decomposition appears to be accelerated, which could be due to the change of oviposition site allowing greater access to the digestive system of the corpse, which is reported to decompose at a faster rate than the respiratory system.

While the effect of nicotine on the rate of development of primary colonisers would be best studied in a laboratory environment under controlled conditions, such studies do not necessarily take into account the importance of the carrion as an ecosystem as a whole. For example, the carrion micro-ecosystem is a transient example of the ecological principle of succession. However, this is a complex system, with the surrounding community before carrion is placed being akin to a pseudo climax in the carrion ecosystem. Single species life cycle investigations, while providing further data regarding the possible effects of a toxin on a post mortem interval estimate based upon that species, do not provide any data on the

cumulative effect of a toxin on the wider ecosystem, or upon any of the later waves of decomposition succession. The presence of either *Rhingia rostata* or *Sargus bipunctatus* on the carrion could not have been factored into a laboratory test without the prior field test, as neither species had previously been recorded upon carrion, nor were they considered of forensic importance. Neither could the apparent preference of *C.maxillosus* for nicotine dosed pig carrion over control carrion. As previously stated the gut flora of the corpse has an effect upon the decomposition of carrion since it is a part of the decomposition ecosystem.

Thus it was determined that a laboratory based investigation into the effect of nicotine on a culture of primary colonising Calliphoridae must be used in conjunction with the field based approach to provide the maximum data for use in possible future forensic cases.

The aims of this section were as follows:

1. To determine a robust rearing protocol to act as a model for entomotoxicological studies.
2. To use the model to investigate the effect of nicotine upon the oviposition of a named species.
3. To investigate the effect of nicotine on the developmental rate of a named species.
4. To investigate the survivability of a named species in the presence of nicotine.

5.1.2 The use of primary invertebrate colonisers in medico legal forensic entomology.

Primary colonisers which feed upon the corpse may provide a link between the crime scene and the corpse, by comparing the geographical distribution and habitat preference of a species collected to the habitat in which the body has been found (Erzinclioglu, 1996). For example if a corpse is found in a southern urban environment, but has evidence of primary colonisers that are restricted to Scottish woodlands, it is possible to suggest a corpse has been moved *post mortem* from the primary crime scene or deposition site.

In the introduction (Section 1.3) it was shown that successional colonisation of the corpse by invertebrates follows a predictable pattern which can be used to estimate time since death. Erzincliogl u (1996) remarked that if the age of the primary colonising species of invertebrates is known, then it also is possible to infer the minimum amount of time for which a corpse has been decomposing. Since we know that blowfly maggots will only feed and develop upon decomposing carrion, if a corpse is found with maggots which are estimated to be 8 days old, the body must have been dead for at least 8 days.

However as stated by Gennard (2007) like other insects, the primary colonisers are poikilothermic and their metabolism and thus life cycle is dependent upon environmental temperature. This poses a problem for the forensic entomologist, if the life cycle of an insect is not a linear temporal response then how does one determine the age of a primary coloniser at a crime scene? The traditional method is to compare experimentally derived development data to crime scene temperatures to create a *post mortem* interval (P.M.I) estimate. Gunn (2006) suggests that if the experimental data is converted into an accumulation of thermal units over time, to produce Accumulated Degree Days (ADD) or Accumulated Degree Hours (ADH), then the estimate can be corrected to include variations in environmental temperature. Gennard (2007) provides formulae for calculation of ADD and ADH:

$$\text{Time}_{(\text{hours})} \times (\text{Temperature} - \text{base temperature}) = \text{ADH}$$

Or

$$\text{Time}_{(\text{days})} \times (\text{Temperature} - \text{base temperature}) = \text{ADD}$$

Here, temperature is the constant temperature of the experimental data, and Base Temperature is an experimentally derived lower development threshold, below which the growth and development of the species will be halted (Gennard, 2007).

ADD/ADH can be calculated for individual stages of the life cycle of various species of primary colonisers. For example Gunn (2006) gives the experimental data for *Calliphora vicina* at 25^0C as follows:

Egg Stage = 14.4 Hours

First Instar = 9.6 Hours

Second Instar = 24 Hours

Third Instar = 158.4 Hours

Gennard (2007) states *C.vicina* has a base temperature of 2.0^0C.

Therefore it is possible calculate the ADD/ADH of *C.vicina* for varying points in the life cycle.

If the forensic entomologist is presented with, for example, a third instar *C.vicina* the ADD can be estimated as follows:

14.4+9.6+24= 48 hours at 25^0C for *C.vicina* to reach the third instar

Therefore using:

$\text{Time}_{(\text{hours})}$ x (Temperature – base temperature) = ADH

48 hours x (25^0C - 2.0^0C) = 1104

Therefore the ADH for *C.vicina* is 1104

To determine the ADD for *C.vicina* 1104 is simply divided by 24 giving an ADD of 46.

The calculated ADD/ADH value can then be compared to how the temperature at a crime scene accumulates to determine minimum PMI where the experimental ADD/ADH is used as a development threshold (Gennard, 2007).

For example if the Crime scene spends 10 hours at 20^0C but then drops to 10^0C for 14 hours that would equal an ADH of 340 if this trend continued for 3 days the total ADH at the crime scene would be 1020, and a further 4 hours at 20^0C would bring the total ADH to 1100.

We know the experimentally determined ADH for *C.vicina* is 1104 to reach third instar. If third instar *C.vicina* larvae is found on this theoretical corpse in these conditions we can say that the body has been dead for *at least* 3 days and 4 hours.

Using the above methodology, the forensic entomologist can provide an estimate of minimum post mortem interval in the short term. However, as shown by Chick *et al* (2008) and the results reported in the current work (Chapters 3 and 4) that insecticides appear to affect the natural behaviour of primary colonisers, it is prudent to quantify this effect to allow forensic practitioners to calculate time since death to a greater degree of accuracy.

5.1.3 Selecting a test species (*Calliphora vomitoria*)

Calliphora vomitoria puparia were selected as a starting point for use in the following investigations. Oldroyd (1964) considered *C.vomitoria* to be one of the most common blowflies in Europe and North America. Smith (1986) stated that *C.vomitoria* along with the closely related *Calliphora vicina* are the most frequently encountered flies in European forensic entomology cases. Van Emden (1954) considered *C.vomitoria* to be common throughout the United Kingdom, and the Channel Isles. Busvine (1980) commented that *C.vomitoria* requires 4-5 days between pupal emergence and oviposition (at 24^0C), However, Erzincliogl u (1996) argued that blowflies will only oviposit between seven and ten days after the female has first consumed a protein meal. Rognes (1991) stated that in field conditions in Finland *C.vomitoria* has a developmental time of thirty eight days at approximately 15^0C and Byrd and Castner (2001) more accurately note that *C.vomitoria* required 737 accumulated degree days, or 17678 accumulated degree hours to develop from an egg to the point of emergence. Rognes (1991) also notes that *C.vomitoria* was recorded in cases of sheep strike in Norway, although it was said that *C.vomitoria* is most likely a secondary myiasis agent. Smith (1973) concurs that *C.vomitoria* most likely invades living tissue as a secondary

preference to carrion, however the presence of *C.vomitoria* in cases of myiasis means that any data relating to its development could be used in cases of neglect (as described by Benecke *et al*, 2004) of elderly smokers.

Like all species in the family Calliphoridae, the life cycle of *C.vomitoria* is composed of an egg, three larval instars, a pupal stage and the final adult stage (Erzincliogluo, 1996). Colyer and Hammond (1951) state that a female *C.vomitoria* will lay up to six hundred eggs, around natural orifices and Rognes (1991) noted that the eggs of the Calliphoridae are between 0.9 and 1.5mm in length, 0.3-0.4mm wide, and shining white. The pale larvae are of typical maggot shape with a truncated posterior end which tapers towards the anterior. The eggs hatch in one to two days (Erzincliogluo, 1996), and the first instar larvae are less than 2mm in length (Rognes, 1991). Erzincliogluo (1996) noted that this larval stage is susceptible to drowning or desiccation. Post hatching the larvae move to an area that provides the most suitable conditions for this most vulnerable stage of their life cycle. The first and second instars last little longer than twenty four hours each. Rognes (1991) reported that the second instar is between 2-9mm in length, with weaker mouth parts than the third instar. The third instar is stated to be between 9 and 22mm by Rognes (1991). Erzincliogluo (1996) reported that the third instar feeds voraciously for a period of 3-4 days although this varies with ambient temperature. After the larva is fully fed it empties its crop, migrates from the carrion and burrows after finding a suitable pupation site. In doing so, the larvae may cover a distance of over 30m of concrete (Erzincliogluo, 1996). On reaching a suitable site, the larvae burrow up to 3cm into the soil, at which point the cuticle contracts and hardens, forming the barrel-shaped puparium which protects the pupa (Erzincliogluo, 1996). When the adult has formed it inflates a blood-filled sack, the ptilinum, which will allow the insect to break through the tip of the puparium. The fly takes approximately 24 hours to harden and for its wings to fully expand. During this time the ptilinum is withdrawn into the head, never to be

used again, and the products of pupal metabolism are excreted through the anus (Erzinclioglu, 1996).

5.2 Methodology

5.2.1 Selecting a robust rearing protocol

The first requirement for testing was to determine how best to rear *C.vomitoria* in the laboratory. Rearing Calliphoridae specimens collected from a crime scene to adulthood for identification is covered in the standard forensic entomology literature (such as Byrd and Castner, 2001 and Smith 1986). However, establishing a laboratory colony was only briefly described in the texts. Erzinclioglu (1996) remarked that rearing cages can be fashioned from wire and knitted cotton material, whereas Gennard (2007) stated that an aquarium with a mesh lid, such as ladies tights with the feet removed, can be used as a rearing cage. Both authors recommend the use of sugar as a food source for adult flies; however, Erzinclioglu (1996) states that dry sugar lumps should be used, whereas Gennard (2007) remarked that a 50:50 mix of sugar and water should be used as pure dry sugar can lead to a reduction in size of insects. Liquids should either be in a wicked container (Gennard, 2007) or soaked into cotton wool in a dish (Erzinclioglu, 1996) to prevent the specimens from drowning (Gennard, 2007). Adults should be provided with a form of animal protein to allow the females to mature their eggs (Erzinclioglu, 1996); Singh and Moore (1985) suggest dried milk powder is suitable for this purpose although Erzinclioglu (1996) suggested liver may be used as a protein source but it must be changed often to prevent desiccation. Once the eggs have matured the females will be gravid, which is determined by an audible buzzing noise during flight (Erzinclioglu, 1996) and the colony can be provided with a medium such as liver for oviposition in a suitable container to reduce moisture lose (Gennard, 2007). After oviposition

this medium is placed on suitable material, such as sawdust or sand to encourage pupation in migrating larvae (Erzinclioglu, 1996).

After much experimentation the following rearing schedule was identified:

1) A rearing tank (Figure 5.1) was made by placing tights upon a tank as suggested by Gennard (2007). The tights allow for colony maintenance, such as addition of rearing media, without allowing escape of the flies.

2) The bottom of the tank was covered in a pupation medium of wood shavings (normally used as a substrate for small mammal husbandry).

3) A wicked specimen jar containing 50:50 sugar-water was added to the tank for adult nourishment.

4) A petri dish of powder milk was also added to provide protein for egg development.

5) 25 pupae were added to the tank and spread among the wood shavings. This was determined to be the optimum number of pupae for the size of tanks used; Gennard (2007) warned that overcrowding leads to damaged specimens' wings, which in turn can affect breeding.

6) After the adults emerged and gravid females were observed, rearing medium was added in a plastic drinks cup. The drinks cup acted to reduce the surface area of the medium exposed to air and thus to reduce desiccation.

7) After oviposition the medium plus eggs was removed to a separate tank.

Figure: 5.1 A Rearing cage for *C.vomitoria*

5.2.2 Choosing a suitable rearing medium for entomotoxiological studies

An artificial culture medium into which the nicotine could be added needed to be found or created. While Byrd and Castner (2001) recommended the use of organ tissue recovered from laboratory animals, Goff (2000) specified rabbits, to which the required toxin was administered prior to death, although this would raise a number of issues.

Firstly there is the matter of tissue type, since (as previously stated) members of the Calliphoridae will grow at different rates when fed on different animal tissues (Clark *et al* 2006). Clarks study investigated how *Lucilia sericata* developed on lung, liver, and heart tissues form both bovine and porcine orgins, both in a liquidised and solid state. It was found that lavae grew significantly faster on porcine material; also the study determined that larvae grew faster on heart and lung material than upon liver. Other researchers such as Haskell, *et al* (2002) stated porcine material behaves in a similar manner to human tissue, whereas nicotine dosed rabbit or rat liver could behave differently. Secondly there is the ethical concern of dosing a live animal with nicotine, prior to euthanasia and organ harvesting.

Numerous formulae for rearing media have been suggested by a variety of authors, each for a different requirement. Sherman and Trans (1995) required a sterile medium on which to grow therapeutic maggots. Sherman and Trans (1995) stated that beef liver and bacto agar media presented "equalled or exceeded" larvae grown on raw liver, suggesting that the media may not be useful for entomotoxicological studies.

It was determined that a porcine based culture medium which could be homogenised to contain a toxin would the most suitable compromise. Previously Erzinclioglu (1996) recommended the use of liver as a food source for Calliphoridae cultures; whereas Chick (2008) recommended the use of cat food as a rearing medium. Generally cat food is made of pieces of re-formed meat in either gravy or gelatine, with different animals used for different flavours.

Since porcine tissue is considered the closest to human tissue for the purposes of decomposition studies, if pureed porcine liver was homogenised with nicotine and reset using a thickener this might show promise as a possible culture medium. The most obvious thickening agent would be pork gelatine as this is of porcine origin. A number of possible formulae were proposed for investigation as follows:

Formula one was 3.5g gelatine, 25g ground pork liver and 20ml water

Formula two acted as a control with 7g of gelatine dissolved in 40ml of water

The gelatine was mixed with water and heated in a water bath at 40^0C until dissolved, and combined with the liver at room temperature and allowed to set.

Formula 2, was to be used as a pure gelatine control and a second pure liver control was also used in oviposition preference testing. The ratio of gelatine to water was based upon the ratios used in simple gelatine mountants for microscopy (Grey, 1973) to get the right consistency of setting agent, this was then weighed, and a simple 50:50 W/W solution of gelatine to liver was made.

The media were tested against 50g pure liver (Control 2) using the rearing methodology described in Section 5.2.1

A choice of rearing media, set in disposable cups was added to the cages to encourage oviposition. The medium was monitored for signs of egg laying, and replaced if it had dried out.

It was determined after testing that the liver and gelatine mixture behaved in a similar manner to pure liver, and was thus suitable for use in further experimentation.

5.2.3 Using the artificial med1ium for entomotoxicological studies.

With a robust rearing protocol and suitable medium for testing identified, it was important to use it to design a suitable method for rearing *C.vomitoria* in the presence of nicotine.

It was determined that an aqueous solution of nicotine could be used to dissolve the gelatine of the rearing medium, by using 20ml of aqueous nicotine solution being used to dissolve 3.5g of gelatine, to which 25g of pork liver was added.

What was then needed was to determine how much nicotine would be required in the small scale tests to be applicable as an analogue for the field testing described in Chapters 3 and 4, this was done as follows:

One batch of rearing media is equal to 45g

The test pigs weighed 15kg (15000g)

Therefore:

$$\frac{15000}{45} = 333.333$$

This gives a division factor to determine how much nicotine is required at a smaller scale for example:

High dose nicotine was 168mg, therefore:

$$\frac{168}{333.333} = 0.504\text{mg}$$

Therefore the high dose of 168mg used for a whole pig would be equivalent to a dose of 0.504mg in the small 45g rearing medium.

A stock solution of aqueous nicotine dissolved in 20ml distilled water was made thus:

For a 1 litre (1000ml) stock nicotine solution equal to the 168mg dose of a whole pig.

$$\frac{1000}{20} = 50$$

Therefore the 0.504mg dose multiplied by 50 gives 25.2mg of nicotine per litre as a stock solution.

Note that this stock solution created for a practical purpose does not relate to a standard measurement that can be applied to a crime scene or a toxicological screen.

The above figure of 0.504mg is per 45g of tissue and it would be better to determine dose as milligrams per kilogram which in the case of the high dose would be:

$$\frac{168}{15} = 11.2mg/kg$$

5.2.4 Investigating the effect of nicotine upon oviposition

As observed in sections 3.4.1 and 4.4 the initial oviposition behaviour of Calliphoridae changed in relation to the presence of nicotine both with respect to site selection and the practice of single egg laying. The following experiments attempt to quantify this effect by investigating the number of eggs laid in relation to specific concentrations of nicotine. To prevent impacting the survivability of the eggs, it was determined that this would be done in a non-invasive fashion by photographing the oviposition using a Nikon D3100 14.2 megapixel DSLR camera. This was fitted with a Tamron SP AF 90mm F/2,8 Di Macro lens capable of a reproduction ratio of 1:1 and a ring flash (Figure 5.2).

Figure 5.2 Camera set up used for photographing oviposition.

Photos of oviposition were then magnified upon a computer and examined on screen to count the number of eggs present in the frame.

5.2.5 Investigations into the effect of nicotine on larval development rates.

After a stable laboratory culture was established as stated in section 5.2.1 the next step was to investigate the effect of nicotine concentrations on the life cycle of *C.vomitoria.*

This was accomplished using the rearing medium described in section 5.2.2 dosed with nicotine as suggested in section 5.2.3. 25 *C.vomitoria* specimens were added, after oviposition had occurred and the resulting egg masses had been photographed for counting (Section 5.2.4 above) the colonised medium in its plastic cup was placed in a disposable 500ml cup with wood shavings at the bottom, similar to the main culture, to act as a rearing chamber. This was covered with muslin held in place using a rubber band (Figure 5.3) to allow air flow but to prevent escape of larvae or adult flies.

Figure 5.3 a Micro rearing pot for *C.vomitoria*

The micro rearing pots were kept in a Sanyo Fitotron environmental chamber at a constant temperature of 11^0C and a relative humidity of 50%, conditions at which *C.vomitoria* would be expected to be found (Smith, 1986) (Figure 5.4). The eggs were reared through to adulthood on varying concentrations of nicotine infused media. With a constant temperature it was possible to obtain experimental data similar to that used to create ADD calculations such as those shown in Section 5.1.2.

Figure 5.4 Sanyo Fitotron environmental chamber used for rearing *C.vomitoria*.

5.2.6 Pupal dissection

Gennard (2007) stated that the pupal stage of *C.vomitoria* at 12.5^{0}C lasts for 717.6 hours, longer than the sum of the previous life cycle stages, which last for a total of 614.4 hours. Given that high pupal mortality might affect PMI estimates it was determined that understanding when pupal mortality took place might provide further information in determining the PMI. As such a control group of pupae were reared in a plastic cup as described in section 5.2.5 and allowed to develop in the environmental chamber (Figure 5.4). At regular intervals a selection of pupae were removed from the culture, killed in hot water and preserved in 70% alcohol, as described in section 3.2. To facilitate dissection a number of micro-dissection tools were fabricated. Pantin (1969) describes how to fabricate micro-scalpel blades from a double edged razor blade, which was then mounted in a craft knife handle as suggested by Chick (2014). Cooter and Barclay (2006) describe how to fabricate micro dissection needles from insect pins mounted in wooden dowels.

A micro dissection dish was fabricated by melting histological embedding wax and pouring into a small petri dish in the manner suggested by Chick 2011 (Figure 5.5). The pupa for dissection was placed into a small pool of the paraffin wax melted using an unfolded paper clip mounted in a dowel as described by Chick (2014) which was heated until red hot. The melted wax was then left to set thus securing the specimen in place.

The specimen was then examined under a stereo zoom microscope at magnifications varying from 10-20x and dissection was carried out as follows:

1) The specimen was covered in glycerol to prevent desiccation during dissection as recommended by Chick (2011).

2) A shallow incision was made down the side of the dorsal surface of the pupae with a micro-scalpel, taking care not to damage the underlying specimen.

3) Two small incisions were made at the anterior and posterior ends of the specimen at either end of the primary incision.

4) The pupal skin was gently teased back using a mounted micro pin and pinned back using a fine pin, thus exposing the underlying specimen.

5) A photograph of the development stage was taken using an Olympus SP-320 7.1 mega pixel camera attached to the microscope.

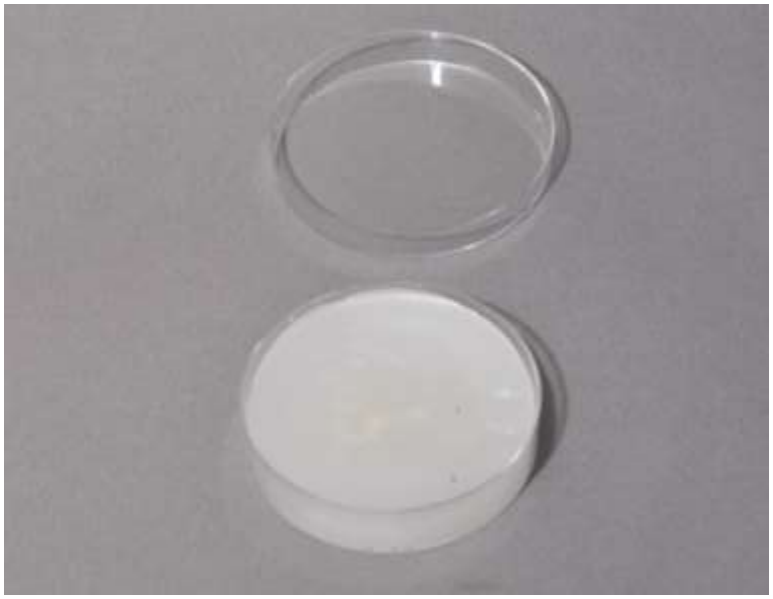


Figure 5.5 A Micro dissection dish

This protocol provided a baseline of pupal development under controlled conditions. The test pupae that had experienced mortality could then be preserved and dissected in a similar manner and compared with the control reared specimens to determine roughly how far into pupation the specimens had progressed.

5.2.7 Investigating survival rates of *C.vomitoria* pupae

After the investigations into development rates of *C.vomitoria* the cultures were counted and the percentage of emerged adults determined.

5.3 Results

The following results were obtained in the course of the investigations detailed in section 5.2. with the raw data presented in Appendix 2.

5.3.1 Effects of nicotine on oviposition on *C.vomitoria*

The average number of eggs laid in relation to the presence of nicotine is demonstrated in Figure 5.6.

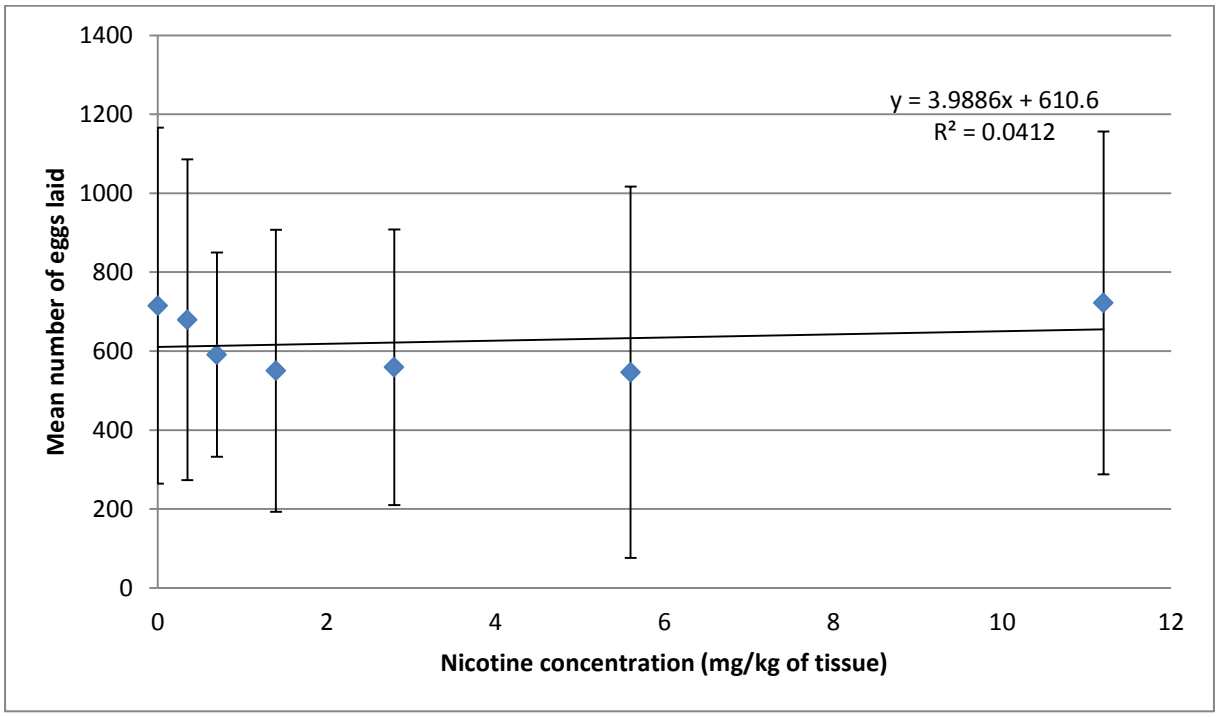


Figure 5.6. Average numbers of eggs laid in relation to concentration of nicotine +/- 1 standard deviation.

From the above it would appear that initially as the concentration of nicotine increases the number of eggs laid decreases, however at the highest dose of nicotine the number of eggs laid was shown to increase again.

The data was analysed using a one way ANOVA, the statistical analysis of the data suggests that the effect of nicotine concentration on the numbers of eggs laid is not significant (F= 0.258238, P=0.952705,)

Table 5.1 Summary statistics: Ovipostion data

Groups	*Count*	*Sum*	*Average*
Control	12	8580	715
0.35mg/kg	4	2717	679
0.7mg/kg	2	1182	591
1.4mg/kg	6	3298	550
2.8mg/kg	5	2795	559
5.6mg/kg	8	4371	546
11.2mg/kg	7	5053	722

5.3.2 Effect of nicotine on development of *C.vomitoria*

The following data was obtained during the investigation into the effect of nicotine on developmental rate of *C.vomitoria* from egg to adult emergence in Accumulated Degree Days.

The data was analysed as a one way ANOVA and the following results were obtained the effect of Nicotine on ADD of *C.vomitoria* is significant. (F= 56.77, P=<0.0001)

Table 5.2: Summary data: ADD of *Calliphora vomitoria* egg to emergence

Groups	*Count*	*Sum*	*Average*
Control	39	22888	587
0.35mg/kg	8	5680	710
0.7mg/kg	85	52336	616
1.4mg/kg	12	7392	616
2.8mg.kg	10	5840	584
11.2mg/kg	4	2400	600

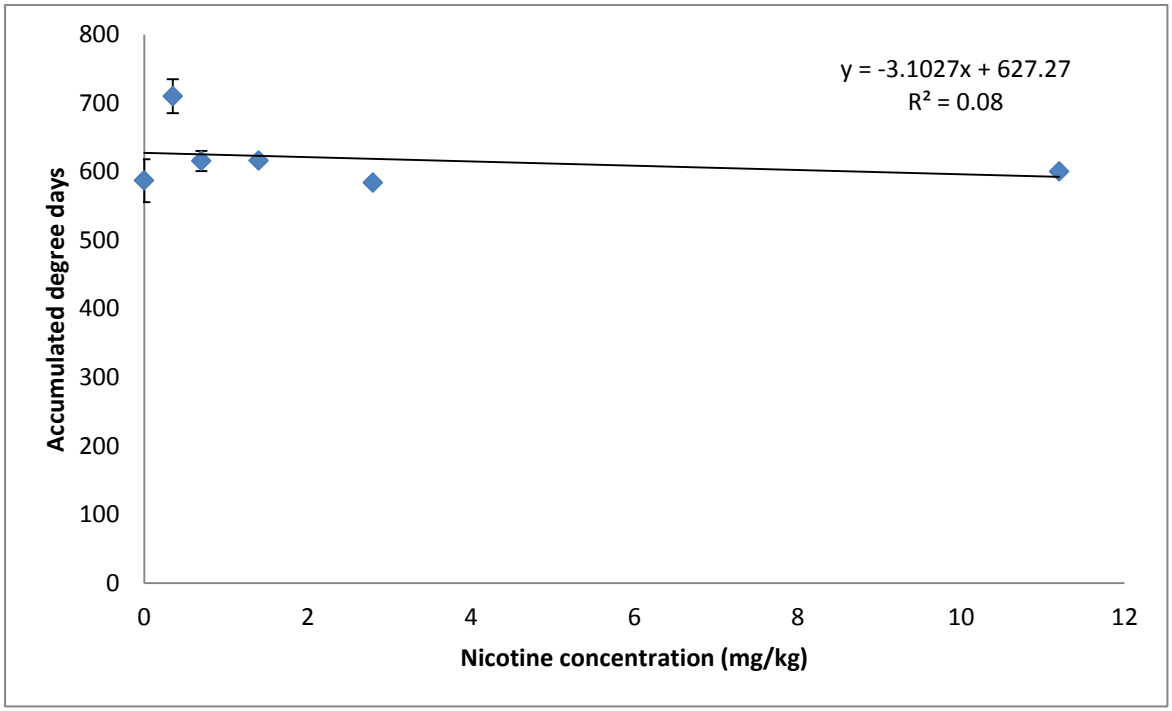
Table 5.3 Output of the Fisher LSD test for ADD from egg to emergence of *Calliphora vomitoria*

Grouping Information Using the Fisher LSD Method and 95% Confidence

Factor	N	Mean	Grouping		
0.35mg/kg	8	710.000000	A		
1.4mg/kg	12	616.000000		B	
0.7mg/kg	85	615.717647		B	
11.2mg/kg	4	600.000000		B	C
0	39	586.871795			C
2.8mg/kg	10	584.000000			C

Means that do not share a letter are significantly different.

Table 5.3 shows that 11.2mg and 2.8mg are not significantly different from the control and 0.35mg/kg is significantly different from all other treatments.



5.7 Average Accumulated Degree Days (ADD) for *C.vomitoria* to complete its life cycle in relation to the presence of nicotine calculated from an experimental temperature of 11^0C showing 1 standard deviation

ADD was calculated using following equation:

ADD= Time $_{(days)}$ × (temperature – base temperature) (Gennard, 2007)

As stated in section 5.2.5 the temperature of the investigation was 11^0C, and Gennard (2007) quoted the base temperature of *C.vomitoria* as 3.0^0C

The Equation of the line was calculated as:

y = -3.1027x + 627.27

Larvae reared at the highest concentrations of 5.6mg/kg and 11.2mg/kg of nicotine largely failed to emerge from the pupal stage.Wwith a total only 4 adults emerging (with an ADD of 600) from multiple runs of 11.2mg/kg and no emergences from 5.6mg/kg as such it was

determined that analysis and a similar graph required plotting taking in to account ADD to the point of pupation (Figure 5.13).

Table 5.4 Summary Data: ADD to pupation (*Calliphora vomitoria*)

Groups	*Count*	*Sum*	*Average*
Control	8	2312	289
0.35mg/kg	4	1440	360
0.7mg/kg	4	1408	352
1.4mg/kg	2	472	236
2.8mg/kg	6	1248	208
5.6mg/kg	2	688	344
11.2mg/kg	3	720	240

A one way ANOVA showed that the effect of nicotine upon the rate of growth of *C.vomitoria* to pupation is significant (F= 3.75 P== 0.01)

Table 5.5 Fishers LSD analysis of the effect of nicotine dose on ADD to pupation of *C.vomitoria*

Grouping Information Using the Fisher LSD Method and 95% Confidence

Factor	N	Mean	Grouping		
0.35	4	360	A		
0.7	4	352	A		
5.6	2	344	A	B	
control	8	289	A	B	
11.2	3	240		B	C
1.4	2	236		B	C
2.8	6	208			C

Means that do not share a letter are significantly different.

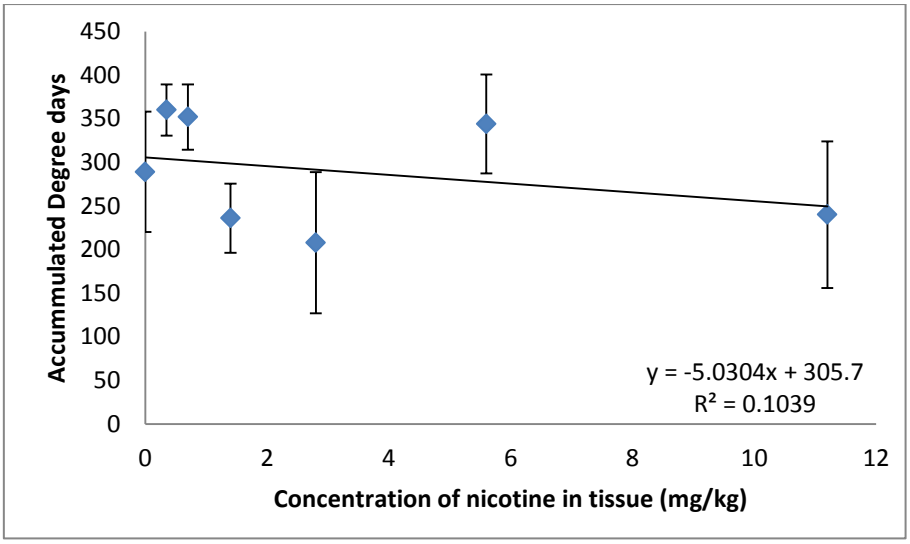


Figure 5.8 Average Accumulated Degree Days (ADD) for *C.vomitoria* to reach pupation in relation to the presence of nicotine calculated from an experimental temperature of 11^0C.

Figure 5.8 appears to show two distinct trends of ADD to pupation in relation to nicotine one in relation to low doses (Figure 5.9) and one in relation to higher dose (Figure 5.10)

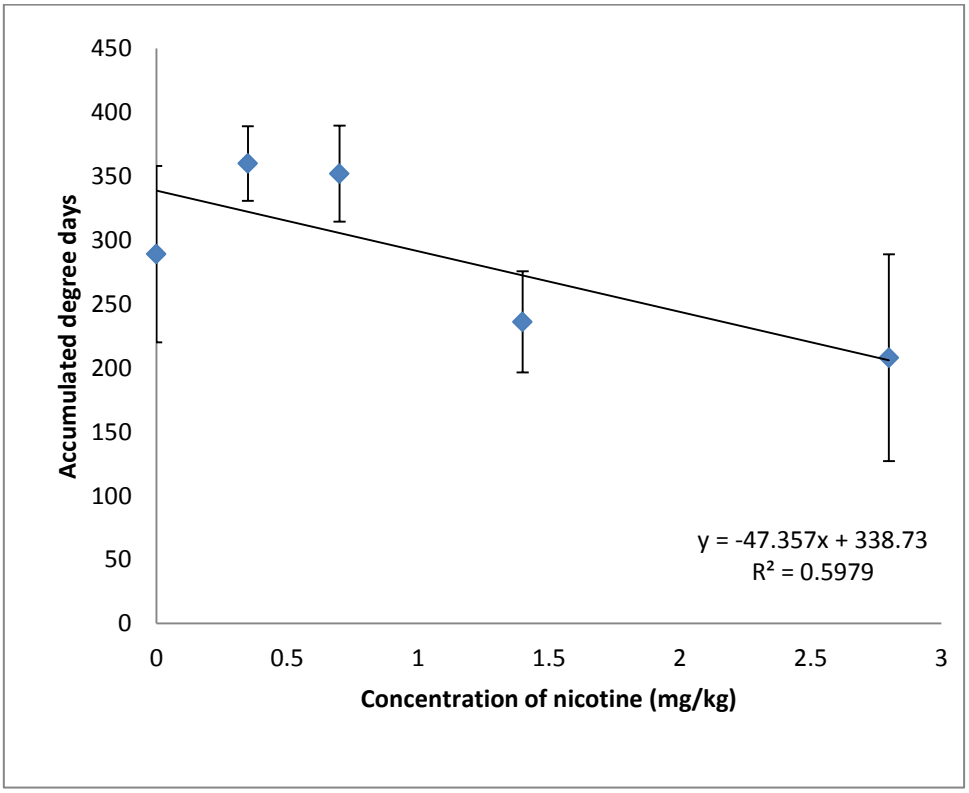


Figure 5.9 Average Accumulated Degree Days (ADD) for *C.vomitoria* to reach pupation in relation to the presence of low doses (up to 2.8mg/kg) of nicotine calculated from an experimental temperature of 11^0C showing 1 standard deviation.

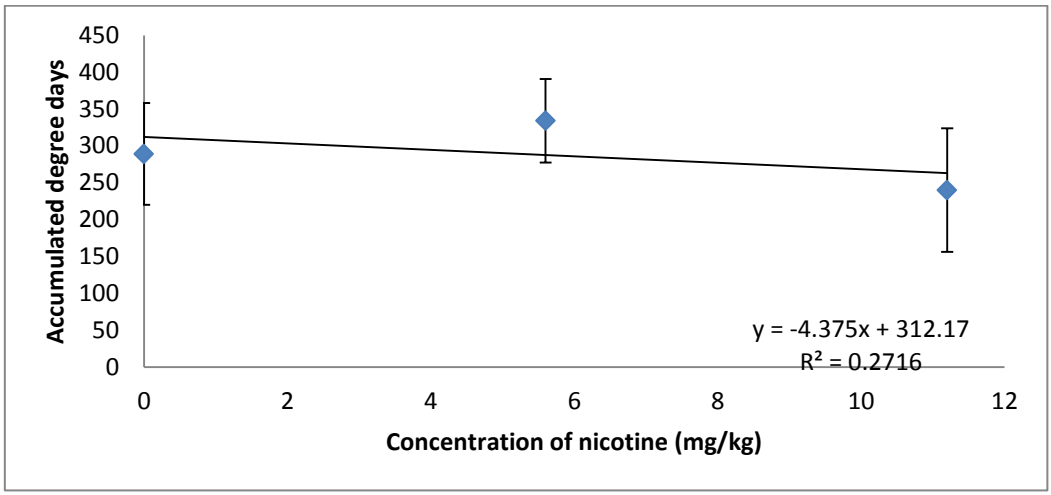


Figure 5.10 Average Accumulated Degree Days (ADD) for *C.vomitoria* to reach pupation cycle in relation to the presence of high doses of nicotine calculated from an experimental temperature of 11^0C.

When the length of pupal stage only is plotted against dose of nicotine the following graph is obtained (Figure 5.11):

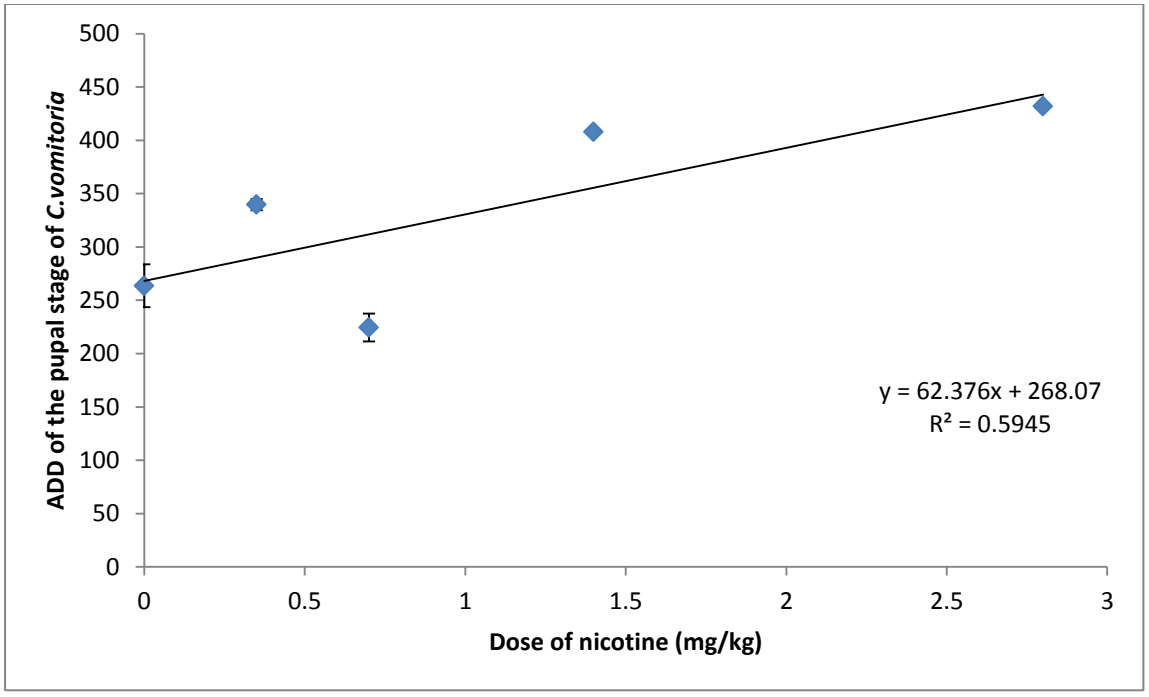


Figure 5.11 Average Accumulated Degree Days (ADD) for *C.vomitoria* spent in pupation in relation to the presence of low doses of nicotine calculated from an experimental temperature of 11^0C.

Table 5.6 Summary data: ADD of pupal duration (*Calliphora vomitoria*)

Groups	*Count*	*Average*
Control	39	264
0..35mg/kg	8	340
0.7mg/kg	85	224
1.4mg.kg	12	408
2.8mg/kg	10	432

One way ANOVA showed that there was a significant difference between the treatments of nicotine upon the ADD of pupal duration for *Calliphora vomitoria* (F=1809.11 P=<0.0001)

Table 5.7 Fishers LSD test results for ADD of *C.vomitoria* during pupation

Grouping Information Using the Fisher LSD Method and 95% Confidence

Factor	N	Mean	Grouping
2.8mg/kg	10	432.000000	A
1.4mg/kg	12	408.000000	B
0.35mg/kg	8	339.750000	C
control	39	263.589744	D
0.7mg/kg	85	224.470588	E

Means that do not share a letter are significantly different.

The above analysis shows a significant difference between all the test groups, and thus nicotine has a significant effect on pupal duration of *C.vomitoria*.

Given the failure of *C.vomitoria* to emerge from the pupal stage in the presence of high doses of nicotine it was deemed wise to further examine the unmerged pupal cases and the effect of nicotine on the survival rates of C.vomitoria

5.3.3 Pupal mortality and dissection of *C.vomitoria*

The pupae were dissected as described in section 5.2.6. And the following data was obtained.

5.3.3.1 Control dissections

Figure 5.12 Control pupa dissection at 8ADD.

Figure 5.13 Control pupa dissection at 16 ADD.

Figure 5.14 Control pupa dissection at 32 ADD.

Figure 5.15 Control dissection at 40 ADD; note the development of anatomical structures.

Figure 5.16 Control dissection at 56 ADD showing further development.

Figure 5.17 Control dissection at 80 ADD note the three body segments of an adult are visible.

Figure 5.18 Control dissection at 136 ADD.

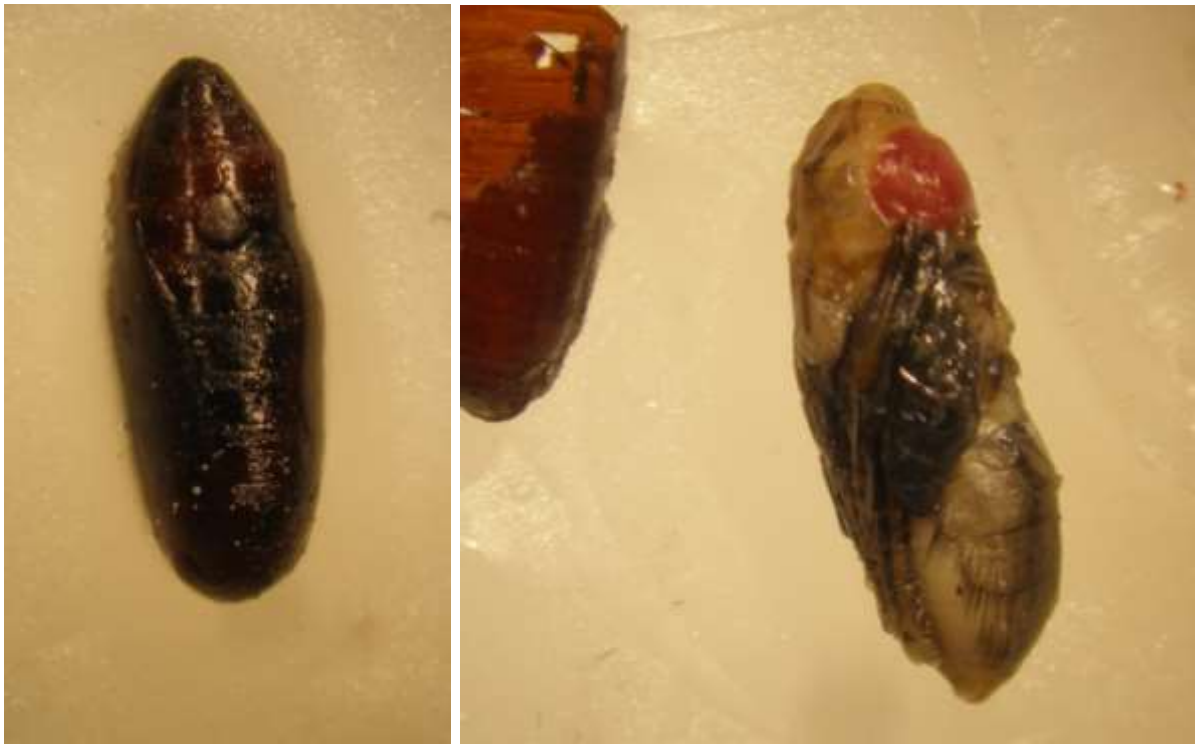


Figure 5.19 Control dissection at 232 ADD notice the fly almost fully formed.

5.3.3.2 Dissections of pupae exposed to nicotine

When the most advanced stages of the higher dose pupae were dissected the following was observed:

Figure 5.20 Dissected pupa exposed to 5.6mg/kg of nicotine. Note the fully developed fly, which has darkened beyond the colouration shown in Figure 5.19 and also the membrane encasing the fly

Figure 5.21. Dissected pupa exposed to 5.6mg/kg nicotine.

Figure 5.22 Dissected pupa exposed to 5.6mg/kg of nicotine.

Figure 5.23 Pupal dissection exposed to 11.2mg/kg of nicotine.

Figure 5.24 dissection of *C.vomitoria* pupa exposed to 11.2mg/kg of nicotine.

From the photos it would appear that the higher doses of nicotine caused the pupae to fail to emerge and not to develop into adults.

5.3.4 The effect of nicotine upon survival rates of C.vomitoria pupae

After counting the pupae and emerged adults the following data were obtained

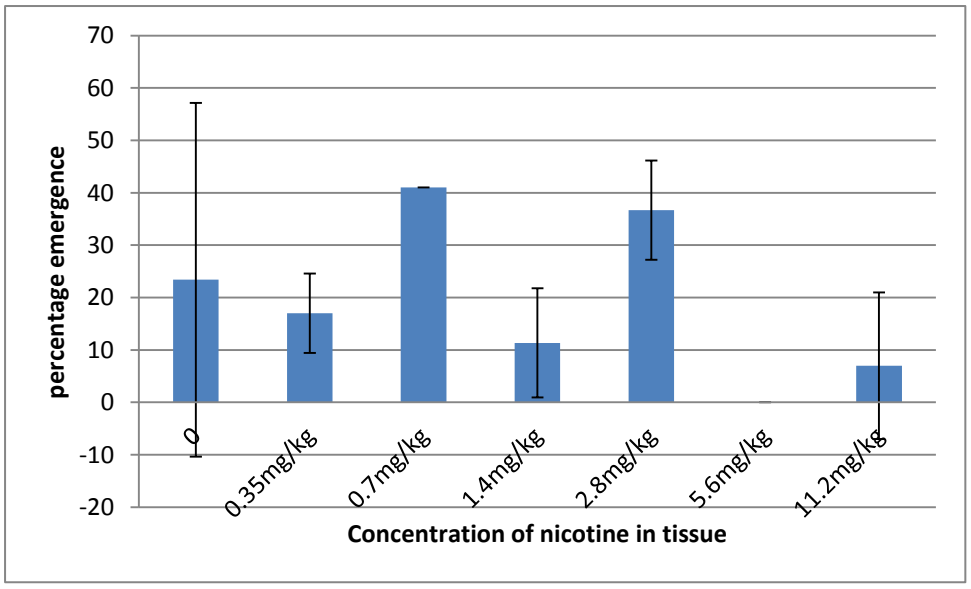


Figure 5.25 average adult emergence of *C.vomitoria* in relation to the presence of nicotine to 1 standard deviation

Figure 5.25 does not appear to show a defined trend in the relation between adult emergence and dose of nicotine, with 0.35mg/kg, 1.4mg/kg 5.6mg/kg and 11.2mg/kg showing a decline in percentage emergence from the control in relation to nicotine, but both 0.7mg/kg and 2.8mg/kg show and increase in emergence from the control. The data was analysed using a 1 way ANOVA and the following data were obtained.

Table 5.8 Summary data: Nicotine concentrationagainst percentage emergence

Groups	*Count*	*Sum*	*Average*
control	5	117	23
0.35mg/kg	3	51	17
0.7mg/kg	1	41	41
1.4mg/kg	3	34	11
2.8mg/kg	3	110	37
5.6mg/kg	3	0	0
11.2mg/kg	4	28	7

The results of the one way ANOVA -= shows there is no significant effect of nicotine upon emergence rates (F=1.30, P=0.31).

5.4 Discussion

From the above results it is possible to suggest the following inferences regarding the relationship between nicotine infused carrion and *C.vomitoria*.

5.4.1 The effect of nicotine upon the fecundity of *C.vomitoria*

Although the presence of nicotine does not seem to have a significant effect upon numbers of eggs laid, Figure 5.6 does show an initial trend of lowering egg numbers in relation to dose. However, this appears to increase at the highest dose investigated. The results of the ANOVA tests suggest this pattern is not significant and possibly the result of chance. Erzinclioglu (1996) stated that the average female calliphorid can lay over 600 eggs, so the variation of numbers of eggs could be due to a single sterile female and the standard deviations of the numbers of eggs would appear to support this hypothesis.

As previously shown in Chapters 3 and 4 the presence of nicotine has an effect upon the behaviour of egg laying with single eggs being observed in both the field and lab elements (Figure 5.26).However single egg laying was less pronounced, possibly due to the reduced surface area available for oviposition in the media cups, compared to the whole carrion used in the field tests.

Figure 5.26 Oviposition upon 11.2 mg/kg nicotine infused medium demonstrating single egg laying.

Given the smaller colonisable surface area of the medium and the reduced competition in comparison to the field study, it is probable that the single egg laying is in response to an environmental stress in the form of nicotine. As previously stated this single egg oviposition has previously been observed when flies are exposed to suboptimal conditions (Greenberg and Kunich, 2005) and the presence of insecticides (Chick *et al,* 2008). Given the insecticidal

properties of nicotine it is likely that the adult flies can detect the presence of the nicotine in carrion. However it would appear that the need to ensure a next generation of *C.vomitoria*, the transient nature of carrion and the unpredictability of carrion supply mean that outright avoidance of insecticide infused carrion is not an option to *C.vomitoria* and as such this atypical behaviour is observed. This is further evidenced in egg numbers, there was a fall in average eggs laid upon carrion however ANOVA suggested this effect was was not significant. , suggesting the flies were responding to the suboptimal condition of the carrion but the inherent uncertainty of carrion means that outright avoidance of toxin containing carrion is not an option for *C.vomitoria. C.vomitoria* exhibits many features typically considered that of a classical r-selected species.

Species can be classified as either r-selected or K-selected. An r-selected species is defined as having the potential to exhibit a high rate of population increase (Martin, 1999). They are often small organisms with short lifespans (Martin *et al,* 1996), which can rapidly colonise a new habitat in the early stages of succession by having an opportunistic nature (Allaby, 2004) as opposed to K-selected species which demonstrate low birth rates, with long developmental periods and high survivability, which is associated with a stable habitat (Allaby, 2004). Despite the remarks by Barnes *et al* (2000) that r-K selection has been largely superseded by life history theory which takes more into account that just growth characteristics, Reznick *et al* (2002) argue that while r-K selection fell out of vogue, the principles of r-K selection are still valid as theoretical ideals, and that the themes such as density dependent regulation, environmental fluctuations and availability of resources are still important in contemporary demographic theory.

Carrion is a transient resource which occupies a fixed space, and as such is irregular (Putman, 1983) providing an unstable and temporary ecosystem which favours colonisation by species with the ability to breed rapidly and colonise, with a wide dispersal range (i.e. r-selected).

With characteristics such as rapid development, high efficiency converting carrion into body mass, and development restricted to early decomposition with further feeding being used to stock pile nutrients required for pupation (Putman, 1983), Calliphoridae is considered to be an ideal r-selected family

Such front loaded development allows these flies to reduce later feeding if there are changes in the ecosystem or if there is less food available. This allows the larvae to maximise productivity and adult survival although resulting adults are likely to have a reduced fecundity (Putman, 1983). Given that *C.vomitoria* is adapted to survive in unfavourable and changing conditions it would seem logical that *C.vomitoria* would colonise suboptimal carrion such as nicotine infused or similar with almost as much vigour as that of optimal carrion given the inherent uncertainty of carrion size and supply.

The similar number of eggs upon nicotine treated carrion would appear to add to the work of Erzincliogl u (1996) who as stated in section 4.4 found that in a laboratory based setting Calliphoridae prefer to lay eggs upon carrion which has been aged for 10 days. This is counter to their behaviour in nature where researchers such as Chick (2010a) remark that Calliphoridae, when in competition with other insects, will colonise a corpse almost immediately after death or sometimes as Smith (1986) suggests, before death.

The colonisation behaviour of the Calliphoridae is an adaptation to changing environmental factors such as competition and carrion size, so suboptimal situations such as cadaver pollution by nicotine, which as shown in chapters 3 and 4 excludes certain competitive species, suggests that *C.vomitoria* has evolved to colonise suboptimal carrion due to the inherent instability and inconsistency of the carrion ecosystem.

5.4.2 The effect of nicotine upon pupal mortality and non-emergence of *C.vomitoria*

Following dissection of the non-emergedpupae exposed to nicotine infused tissue of doses of 5.6mg/kg and 11.2mg/kg, it would appear that the pupae failed to emerge, rather than failed to develop.

Typically the pupation of *C.vomitoria* is as follows (see Figures 5.12-5.19):

- Changes occur in the crop morphology of the larva. It finishes feeding, empties its gut and leaves the carcase at night, moving away from the carrion to find a suitable pupation site up to 30m away (Erincliogl u, 1996) at 22^0C. At 360 hours this prepupal stage in *C.vomitoria* is much longer than that of its close relative *C.vicina* (28 hours) increasing the overall life cycle from 18 days to 23 days (Smith, 1986).
- Having found a suitable pupation site the cuticle of the larvae contracts and begins to harden forming a brownish black puparium (Erzincliogl u, 1996) characteristic of higher flies such as the Calliphoridae which undergo true pupation (Rognes, 1991). The pupa is protected from the environment by both the puparium and an internal cuticle (Erzincliogl u, 1996). Smith (1989) states that while it is impossible see the development of the pupa of the Diptera which form puparia, upon emergence from the puparium the left over casing made from the skin of the third instar larvae can be used for identification in the absence of the emerged adult, as the puparium still has the morphological characteristics of the third instar larvae used for traditional identification, if keys exist.
- When the pupa has matured in the puparium the adult fly is ready to emerge from both the puparium and the pupal skin inside it, This is accomplished by *C.vomitoria* expanding and contracting a blood filled sac known as the ptilinum located upon the head of the pupa which forces the operculum (tip of the puparium) to split and detach from the rest of the puparium (Figure 5.26) (Erzincliogl u, 1996).

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Figure 5.27. The operculum in two parts detached from the puparium of *C.vomitoria.*

The adult fly (Figure 5.27) is now free of the puparium and makes its way towards light. The final stage of emergence is the withdrawal of the ptilinum, which is never used again, back in to the head (Erzinclioglu, 1996). As it is withdrawn a mark is left upon the adult fly known as the ptilinal suture, the presence of which is one of the ways of distinguishing between the lower Cyclorrhapha such as the Phoridae, and the Schizophora (higher Cyclorrhapha) such as Calliphoridae (Oostherbroek, 2006).

Figure 5.28 An adult female *C.vomitoria.*

Figure 5.25 shows average percentage emergence of *C.vomitoria* at different nicotine concentrations. Here there is no obviousthe relationship between adult emergence and dose of nicotine, with 0.35mg/kg, 1.4mg/kg 5.6mg/kg and 11.2mg/kg showing a decline in percentage emergence from the control in relation to nicotine, but both 0.7mg/kg and 2.8mg/kg showing an increase in emergence from the control. 5.6mg/kg had no emergence over the course of the investigation.

Figures 5.20-5.24 show the dissected pupae of *C.vomitoria* exposed to nicotine treated medium which failed to emerge from their puparium. What these figures show is that the pupae developed to the point of emergence, but for some reason did not inflate their ptilinum and force the operculum from the tip of the puparium, as evidenced by the shrivelled form of

the un-emerged pupa. Also visible is the intact pupal skin covering the pupa visible as a clear membranous substance .

Similar effects were observed by Chick *et al* (2008) who found that in the presence of Malathion *C.vicina* often failed to emerge from the puparium and in some cases adults which had emerged failed to harden and expand their wings.

There are a number of possible reasons why the adults may have failed to emerge. Erzincliogl (1996) stated that one of the adaptations of *C.vomitoria* and other species with the complete metamorphosis that allows them to be so successful is that the adult and larvae are completely different in terms of ecology, morphology and physiology, the bridging stage of this intense change being the pupation stage. As previously stated, pupation relies on nutrients gathered during the late larval stages, and since the larvae will often feed in unfavourable conditions it is possible that nicotine is fatally toxic to the adult flies but sub-lethal to the larvae.

Typically, in insects undergoing pupation, a few days prior to emergence a series of physiological and behavioural changes occur. Hormones such as ecdysis triggering hormone and eclosion hormone are released from neurosecretory cells in the larva. The build-up of these hormones aids the pupa in extricating itself from the larval cuticle (Gullan and Cranston, 2004). The build-up of eclosion hormone triggers the release of crustacean cardio active peptide (CCAP) from ventral nerve cells. CCAP causes a change in behaviour from pre-eclosion behaviour to eclosion behaviour, such as increased heart beat and the inflation of the ptilinum (Gullan and Cranston, 2004). The accumulation of eclosion hormone also stimulates the release of the post emergence neurohormones bursicon and cardiopeptides. The former causes the cuticle of the adult insect to harden after emergence after a brief period of elasticity and the latter stimulates the expansion of the wings (Gullan and Cranston, 2004). Looking at the dissected pupa, the cuticle appears to have hardened in the puparium

suggesting an absence of CCAP. Since Carlile (2006) states that nicotine binds to synapses in the central nervous system, and Shivastava and Saxena (2000) clarify nicotine attacks the synaptic ganglion in insects. It is possible that the presence of nicotine blocks the ventral nerve cells which detect a build-up of eclosion hormone and thus the presence of nicotine prevents the releasing CCAP, whilst the release of bursicon continues, leading to a fully developed adult being unable to emerge.

In adult insects a small amount of nicotine has been shown to increase insect activity, where as a large amount reduces activity and cause paralysis followed by death (Shivastava and Saxena, 2000) this could suggest why at lower doses *C.vomitoria* appears unaffected by nicotine in relation to pupal mortality.

As previously stated, 4 adults emerged from one test group of *C.vomitoria* exposed to 11.2mg/kg of nicotine treated media raising the average percentage emergence of 11.2mg/kg to 7% . A possible explanation of this could be insecticidal resistance. Carlile (2006) stated that the development of resistance is one of the major problems encountered by farmers with pesticides, akin to the issue of bacterial resistance to antibiotics in medicine. A single base change in DNA structure can lead to small changes in the binding site of insecticidal action, meaning that the pesticide does not bind to its biochemical target and the individual survives. Given that most pest species have highly efficient breeding methods, mutant variants are likely to appear in any normal population (Carlile, 2006). *C.vomitoria* being both an r-selected and common species with a preference for rural areas (Gennard, 2007) it is likely that it has been exposed to multiple insecticides and that resistance to nicotine is possible. The fact that at smaller doses *C.vomitoria* successfully emerged from pupation suggests that it has evolved a small level of resistance previously to nicotine. Table 5.8shows the output of the one way ANOVA of the percentage emergence of *C.vomitoria* and the result suggests that the presence of nicotine does not have a significant effect on the percentage of *C.vomitoria*

that emerge from their pupa, however the number of 0% emergences at higher doses suggests that this area requires further research possibly at even higher doses than those used in this investigation.

As Chick *et al* (2008) stated, traditionally specimens from a crime scene are reared through to adulthood to facilitate identification but if the specimens fail to emerge this may cause an issue with identification due to the lack of larval identification keys. This highlights that a suitable identification key for third instar larvae and puparia is required for the Calliphoridae of forensic importance.

5.4.3 Effect of nicotine upon Accumulated Degree Days (ADD) until pupation

Initially the ADD data was analysed as a whole, from egg to emergence of the adult *C.vomitoria*, using a one way ANOVA . A statistically significant P-value of 0.01 was found, showing that nicotine did indeed have an effect upon the development of *C.vomitoria* However, 11.2mg/kg and 2.8mg/kg were not significantly different from the control but 0.35mg/kg was significantly different from all other treatments this could be a form of hormesis, which Walker (1999) defines as the stimulating effect a normally toxic substance has at lower doses.

With the higher doses of nicotine showing an issue with emergence it was deemed wise to determine the accumulated degree days of *C.vomitoria* until the point of pupation so that if there was acase where PMI determination was based upon a smoker with a high dose of nicotine or a poisoning, then the PMI could be calculated.

The ANOVA test of the ADD to pupation showed a P-value of 0.01 that was also statistically significant.

Figures 5.8-5.10 show the change in ADD at varying concentrations of nicotine it would appear that in the presence of nicotine ADD initially increases in relation to small dose, and

the ADD starts to lower as the dose increases overall trend can be predicted with the following equation:

ADD to pupation (*C.vomitoria*) = -5.0304 x Dose of nicotine (mg/kg) + 305.7

This is derived using the equation of the line from Figure 5.9, however an R^2 of 0.1039 suggested the data points show a poor fit to the line.

When the higher doses are removed we get an equation:

ADD to pupation (*C.vomitoria*) = -47.357 x Dose of nicotine (mg/kg)* + 338.73

*to a maximum dose 6.3mg/kg nicotine

This was derived from the equation of the line for Figure 5.10 and gives an R^2 value of 0.5979 which shows a better correlation than the previous equation, although it fails to account for the higher doses.

When these are plotted independently the following equation is derived:

ADD to pupation (*C.vomitoria*) = -4.375 Dose of nicotine* + 312.17

*Where dose is above 6.3mg/kg nicotine

This equation was derived from Figure 5.9 and has a R^2 of 0.2716, having a better fit than that of equation 1 but a poorer correlation than equation 2. Given that the equation was drawn with just 3 data points, it would be wise to suggest equation one is the most accurate explanation of the relationship between ADD and nicotine concentration with the caveat that the relationship is not a simple linear one. Such a limitation would need to be noted in a witness statement if the equation was used to correct for ADD to pupation in the presence of

nicotine. Fishers LSD (Table 5.5) showed that the data fits into 3 different groups with some overlap between the groups, the higher doses showing the greatest difference from the control group.

5.4.4 The effect of nicotine upon the ADD to adult emergence of *C.vomitoria*

The raw results of the ADD investigations were tested using a one way ANOVA (Table 5.6) which showed that F is greater than F Crit and thus the effect of nicotine on ADD of *C.vomitoria* is significant. Fishers LSD (Table 5.7) test showed that there was a significant difference between each of the individual treatments, and suggests that nicotine has the most significant effect on the pupal stage of *C.vomitoria* as the specimen undergoes a number of physiological changes regulated by the central nervous system as previously stated.

The results were plotted in a dose response graph to attempt to create a predictive equation. Figure 5.11 shows the relationship between nicotine concentration in tissue and its effect upon the ADD of *C.vomitoria.* As previously stated, with the higher doses of nicotine most *C.vomitoria* fail to emerge from their puparia and as such ADD to emergence cannot be used. Figure 5.10 shows an increase in ADD in lower doses which drops as the dose of nicotine increases; and the following equation can be derived:

ADD to adult emergence (*C.vomitoria*) = -18.595 x dose of nicotine (mg/kg) + 642.04

This data has an R^2 of 0.1614 suggesting a low correlation of data points to the line of best fit. A much stronger relationship is observed in the equation of ADD for pupation. This further suggests that nicotine has the biggest effect upon the pupa stage of *C.vomitoria*

Figure 5.11 shows that nicotine causes an increase in the time spent in the pupal stage which can be predicted by the following equation:

ADD of pupation in response to nicotine (*C.vomitoria*) = 62.376 x Dose of nicotine + 268.7

With an R squared of 0.5945 there is a better fit of data than a straight ADD to emergence model would suggest. When considered in relation to the results of the pupa dissections (section 5.4.2), it would appear that this increased pupation occurs to a threshold at which the hormonal imbalance created by nicotine, causes non emergence in *C.vomitoria*.

5.4.5 Conclusion

From the above data it would appear that the relationship between nicotine and *C.vomitoria* is not a simple one, since nicotine affects different stages of the life cycle of *C.vomitoria* in diverse ways. The larval metabolism does appear to be reduced in low doses and increased in higher doses, the latter leading to a drop in ADD, indicating that in similar environmental conditions the larva will reach pupation in a shortened time, as evident for the predictive calculation:

ADD to pupation (*C.vomitoria*) = -47.357 x Dose of nicotine (mg/kg)* + 338.73
*to a maximum dose 6.3mg/kg nicotine

However once *C.vomitoria* reaches pupation, ADD increases in relation to nicotine concentration, meaning that in similar conditions the pupal stage will take longer, as shown by the calculation:

ADD of pupation in response to nicotine (*C.vomitoria*) = 62.376 x Dose of nicotine + 268.7

This appears similar to the effect of methamphetamine on the development of *Calliphora stygia* (Mullany *et al* 2014). This would suggest that to determine the ADD emergence of *C.vomitoria* from the corpse with nicotine in its tissue, two ADD calculations would be required. One to cover to the point of pupation and a second one from pupation to adult emergence, to accurately predict the *post mortem* interval. The use of correction factors from line equations and how R^2 values impact upon predictability is well known to the forensic entomologist. As Gennard (2007) states, line equations and R^2 values are used by the forensic entomologist to estimate historical crime scene temperatures by plotting crime scene temperature post discovery against values from a local weather station over the same time period. The equation of the line is used to estimate the temperature of the crime scene during the *post mortem* interval, based on corrected weather station temperatures.

Higher doses of nicotine seem to prevent *C.vomitoria* from emerging from the puparium. It is suggested that nicotine binds to the synapses of the central nervous system preventing ventral nerve cells from detecting a build-up of eclosion hormone, which in turn prevents the pupa from releasing CCAP. This appears to occur whilst bursicon is still released and thus a fully developed adult is unable to emerge from its puparium.

This work leads to some interesting questions, for example does nicotine have a similar effect on species related to *C.vomitoria* such as other Calliphoridae? Sadler *et al* (1997) remarked that it is not possible to predict how similar drug classes will affect necrophagus species, but if similar species react in different ways to the presence of a toxin, it is also conceivable that some common species show less reaction to common toxins than others. Resistance is always a possibility, and common species are more likely to be exposed or have survived exposure to a toxin than rare ones.

The possibility of resistant local populations was also suggested in this work and this leads to a number of possible questions, such as is it possible that local UK populations show more or

less of a resistant nature than that of those quoted in the literature? This would appear to add to the work of Chick *et al* (2008) who questioned the practicality of studies carried out in for example Hawaii by a U.K. entomologist.

Also highlighted, is the importance of larval identification. As previously shown by Chick *et al* (2008) the possibility of pupal mortality means that traditional identification of adult Calliphoridae from a crime scene may not always be possible. Erzincliogl (1996) provides partial keys to identification of third instar larvae, and by extension pupae, as the puparium is formed from the outer skin of the third instar larvae. However the key only covers some of the important species and identification the genera of *Lucilia* and *Calliphora* is incomplete.

In conclusion it would appear that the relationship between nicotine and *C.vomitoria* shows an increase in time spent in the pupal stage, to the point of fatality in higher doses of nicotine, most likely due to lack of CCAP release in the presence of nicotine. The relationship is not simple but is somewhat predictable, however limitations have been highlighted.

Chapter 6: General Discussion, Conclusions and Further Work

6.1 Introduction

The use of insect evidence to predict the *post mortem* interval (PMI) is well established in the literature, with the first use of insect evidence in a legal case being traced back to 13th century China. It has been shown that estimation of time since death can be based either upon the developmental stage of primary colonisers, or upon successional stage of the carrion community. However there are many factors both biotic and abiotic which can affect PMI estimation, including external temperature or the presence of toxins and it was apparent from the literature that these needed addressing to improve the quality of estimation (Introna *et al*, 2001, Chick *et al* 2008, Gosselin, *et al* 2011). The overarching aim of this investigation was therefore to examine the effect that one toxin (nicotine) had upon the decomposition of carrion and to consider how such an effect may impact upon forensic investigations. If the corpse is considered to be a micro ecosystem, then the presence of toxins within that ecosystem can be considered cadaver pollution, and as such may have an effect on the carrion fauna, both in terms of composition and development.

6.2 Field Investigations

A baseline study was used to select a site which was suitable for carrion placement, in terms of security and ecological richness of carrion frequenting invertebrates; this site was assessed prior to porcine carrion placement.

Once the viability of this site had been determined, the effect of nicotine on the carrion micro-ecosystem was investigated in the field using fresh whole piglets treated with nicotine. From this one of the primary colonisers on carrion at Nottingham Trent University, Clifton Campus, was determined to be *Calliphora vomitoria*.

To account for differences in seasonal occurrence of some invertebrates, the field investigation was carried out in both spring/summer and autumn/winter. Following the field investigations, the effect of nicotine on the life cycle of the primary coloniser (*C.vomitoria*) was investigated under controlled laboratory conditions. The fecundity and survivability of *C.vomitoria* was examined in relation to nicotine.

Finally, the laboratory based data was analysed to create a dose response correction that may allow forensic practitioners to adjust "*post-mortem* interval" estimates in relation to nicotine.

The expected outcomes of this investigation were:

- To add new knowledge to the field of applied entomotoxicology.
- To improve the accuracy of prediction of the *post mortem* interval in forensic cases involving nicotine.
- To offer new insight to the forensic and policing communities so that they can fully evaluate entomological evidence to assist criminal investigations and the courts.
- To offer a predictive methodology by which effects of other drugs and toxins on decomposition may be considered in future investigations.

The baseline studies identified a number of sites on the Campus which were rich in carrion-associated invertebrates including colonisers, predators and parasites of the carrion micro ecosystem. The presence of a fox den and issues with floral cover and potential disturbance lead to all but 2 areas being discounted as investigative sites for carrion. Foxes and other vertebrate scavengers, as noted by Chick *et al* (2008) have a destructive effect upon decomposing carrion, and a lack of suitable floral cover at some sites would have exposed the experimental animals and equipment to potential vandals. Both selected sites were used during the course of the field based investigations.

When nicotine treated porcine carrion was exposed to a woodland environment and left to decompose naturally during autumn and winter, a number of changes to the expected

behaviour of colonising invertebrates on untreated material were observed. Calliphoridae oviposited in an atypical fashion, with single eggs laid in contrast to the clumped eggs normally observed upon control carrion. It is reported in the literature that single egg laying has previously been witnessed under sub-optimal conditions (Greenberg and Kunich, 2005) such as on insecticide treated carrion (Chick, *et al*, 2008). It was also noted that on the nicotine treated carrion, primary colonisers showed a preference for the genital and anal regions as oviposition sites, in contrast to the eyes, ears, nose and mouth colonised on control carrion, suggesting avoidance of the areas of treatment, which were in the neck.

The nicotine dosed carrion also appeared to demonstrate an increase in mycological growth, which in turn lead to decomposition being typified by mycophagus Coleoptera such as *Stephostethus lardarius, Typhaea stercorea* and *Epuraea thoracica.*

Mourier and Winding (1977) state that fungus beetles are most common in damp conditions, suggesting an increase in the moisture content of nicotine containing carrion in contrast to the control, which did not attract mycophagus Coleoptera. A similar effect was observed by Terrell-Nield and Macdonald (1997) who reported that in a damp cave environment, if access to carrion by primary colonisers was prevented, in this case by placing it deep within the cave system, then decomposition was typified by the presence of fungus and mycophagus Coleoptera and Diptera. In the present work, in autumn and winter a change in the successional decomposition of the carrion was also noted. As an example, *Sargus bipunctatus* (Diptera: Stratiomyidae) appeared on treated carrion earlier than on untreated carrion, and exclusion of species was observed for different treatments of carrion. It was suggested the *Rhingia rostrata* (Diptera: Syrphidae) is toxin intolerant as it was only observed upon control carrion; however its status of "rare" would suggest that its absence alone would not be enough to consider a corpse to be that of a "smoker" given that only a single female specimen was collected. *R.rostrata* has previously been considered as a vulnerable species by Shirt

(1987). However, it has been argued that *R.rostrata* is not simply a rare species, it is an erratically occurring species. It has been known to alter in abundance to the point of appearing to vanish from a site, only to reappear many years later (Stubbs and Falk, 1996).

The presence of *R.rostrata* is of more general interest to the forensic scientist or entomologist than its particular relevance to nicotine treated carrion. Given that the life history of this species is unknown in the larval stage, and carrion is believed to be a possible breeding medium (Stubbs and Falk 1996), the scant information discovered adds anecdotal evidence to the theory but hardly proves *R.rostrata* is a carrion breeding fly.

As with the autumn/winter investigation, a marked change in Calliphorid oviposition site and behaviour was observed. The anal and genital regions once again appeared to be the site of preference for oviposition on the treated cadavers. Singular egg laying was once again observed in the primary colonisers of the treated carrion suggesting that nicotine creates sub-optimal conditions as suggested by Greenberg and Kunich (2005).

This observation is similar to other insecticides such as those reported by Chick *et al* (2008) who investigated the effect of pyrethium, pyrethium synergists and Malathion based insecticides on the decomposition of rat carrion, and observed single egg laying in the primary colonising Diptera. Also the presence of Nitidulidae, Fanniidae and Muscidae suggested a semi-liquid environment or higher moisture content, as with the mycophagus Coleoptera observed in the autumn winter investigation. This suggested that the presence of nicotine results in a lowered rate of desiccation in a corpse than in a non-nicotine treated corpse.

A marked extension to the bloat stage of decomposition was also observed in the high dose cadaver in the spring-summer investigation. This change would suggest that the effect of the nicotine is not limited to the site of injection and could be due to a number of reasons.

Firstly the change in the behaviour of the primary colonisers could create a domino effect with the chemical cues that attract the second wave of colonisers being altered. For instance if the primary colonisers take longer to breach the abdomen in the treated carrion, then the bloat stage would be increased, meaning that a greater percentage of tissue is digested by the gastrointestinal bacteria than would be normally expected. Chick (2009) showed how, by excluding the primary coloniser on small rodent carrion, a rat carcase can attract Coleoptera to a small corpse out of season, as well as those that would be expected on larger carrion during summer.

A second possibility would be *post-mortem* drug diffusion. Fuke *et al* (1996) showed that components of paint thinner when instilled into a corpse will diffuse within the body *post mortem*, for example more readily from the trachea than the gastric system. Levisky *et al* (2001) stated that *post-mortem* drug concentrations in adipose tissue are more stable, however the onset of lividity, may lead to differential distribution of toxins within a corpse. Moriya and Hashimoto (2001) stated in a case report that high levels diphenhydramine and dihydrocodine in the femoral venous blood of a sixteen year old deceased male were the result of *post mortem* diffusion of drugs from the bladder over the course of 9 days *post mortem*. Thus the literature suggests nicotine might be able to redistribute from within a corpse even in the absence of blood flow, however the accounts above also suggest that the site of application as well as the type of toxin applied has an effect upon how much *post mortem* diffusion is possible, if any (Levisky *et al,* 2001). It would appear that some diffusion is evident in the current study given that a change is observed throughout the decomposition process over the whole corpse.

When species indicative of nicotine are compared from the spring/summer and autumn/winter investigations it is possible to highlight a number of anomalous species, which

in isolation appear to be indicative of nicotine levels. However, when compared over both investigations these species appear to be more common than previously considered.

For example in the autumnal investigation, the scuttle fly *Metopina oligoneura* (Diptera: Phoridae) was only found on the high dose carrion and thus was considered indicative of nicotine within carrion. However in the spring/summer investigation it was found upon all of the carrion suggesting that it has no particular preference or intolerance to nicotine. As previously stated, *M. oligoneura* is common and widespread throughout the U.K. but the life history is poorly known (Disney, 1983) however the Genus *Metopina* has been associated particularly with buried carrion by previous authors (Smith, 1986).

The carabid beetle *Pterostchius madidus* appeared in the autumnal investigation to also favour nicotine treated cadavers, however in the spring/summer investigation it was shown also to be present upon the control corpse, suggesting that it has no preference to nicotine. Chick *et al* (2008) previously noted that in an investigation into various insecticides, various fly and flea treatments had no effect upon *P.madidus* which only appeared to be repelled from carrion by the organophosphate Malathion. *P.madidus* has a high level of autumn activity as this is when females prepare to over winter with their eggs, only females being present in the spring (Terrell-Nield, 1990) This could suggest that during the autumn when the population is at its greatest, nicotine infused carrion is colonised in preference, and in spring the female will colonise any carrion, given that egg development and nutrition is a priority after over wintering. Another possible explanation is that in the breeding season during autumn, the presence of one female specimen leads to the attraction of multiple male specimens for breeding and the apparent preference is the result of chance. However as the specimens collected were not sexed this cannot be answered with certainty.

In the autumnal investigation, the smaller species of Staphylinidae such as the Aleocharinae and the genus *Tachinus* appeared to be rather nicotine intolerant. However in the spring investigation *Tachinus* was common to all carrion and the Aleocharinae were exclusive to the treated carrion, suggesting little or no preference. With 456 species, the Aleocharinae have been described as the "Everest" of Coleoptera subfamilies, in that it is the biggest and most challenging group (Telfer, 2014), It is difficult to understand how they are attracted by nicotine, as with such a large number of species with varying habits and foodstuffs (Cooter and Barclay, 2006) and the problems of identification (Telfer, 20014) food preferences are hard to quantify. For example it could be that the species found in the spring investigation were mycophagus, whereas the autumn species were predatory, which would reinforce the other species found upon the carrion.

Nicrophorus humator (Coleoptera: Silphidae) in the autumnal investigation appeared to be mildly nicotine tolerant, however in the spring investigation it was found upon both the control and the high dose carrion. This would suggest that *N. humator* has no pollution intolerance and its use as an indicator species is limited. As with *P.madidus* the change in behaviour could be due to the time when carrion was placed in relation to the breeding cycle of the species. The genus *Nicrophorus* is known for its habit of burying small mammal carcases (around the size of a mouse or rat) leading to the common name of burying or sexton beetles. Byrd and Castner (2001) remarked that if a breeding pair is on larger carrion, such as a human, they will make a depression in the flesh to breed larvae. Another possibility is that the adults of *Nicrophorus* species feed upon maggots as well as carrion (Byrd and Castner, 2001) therefore it could be likely that the specimens on the control were searching for a suitable breeding site and the ones on the nicotine dosed carrion were searching for food for themselves. Putman (1983) states that the parents of Nicrophorus larvae bury the carrion and stay with the larvae to protect and care for their offspring. Given the degree of parental care

shown by *Nicrophorus*, carrion preference in relation to breeding makes sense, however further work on the species would be required.

6.3 Devising a biotic index

For convenience presented below are the biotic indices adjusted taking into account the reflections and comparisons discussed:

Table 6.1 A revised carrion biotic index for Autumnal decomposition in relation to nicotine

Classification of Macro invertebrate	Indicative species
Strongly nicotine intolerant	**Diptera** Piophilidae *Liopiophila varipes* **Coleoptera** Ptillidae Staphylinidae *Tinotus morion*
Mildly intolerant	**Coleoptera** Silphidae Scarabaeidae *Aphodius prodromus* **Hemiptera** Lygaeidae *Kleidocerys resedae*
Semi-Nicotine tolerant	**Coleoptera** Silphidae *Nicrophorus vespilliodes* Staphylinidae *Micropeplus fulvus*
Tolerant	**Pseudoscorpiones** Neobisiidae *Neobisium carcinoides*
Strongly tolerant	**Diptera** Fanniidae *Fannia fuscula* *Fannia canicularis* Heleomyzidae *Scolicentra brachyptera* **Coleoptera** Staphylinidae *Tasgius morsitans* *Creophilus maxillosus*

	Bisnius fimetarius Carabidae *Pterostichus melanarius* *Elaphus riparius* Lathridiidae *Stephostethus lardarius* Mycetophagidae *Typhaea stercorea* Nitidulidae *Epuraea thoracica* Scarabidae *Aphodius contaminates*
Key: Strongly nicotine intolerant = only found upon the control carrion Mildly intolerant = found upon both control and low dose carrion Semi-Nicotine tolerant = only found upon the low dose carrion Tolerant = found upon the low and high dose carrion Strongly tolerant = only found upon the high dose carrion	

Table 6.2: A revised carrion biotic index of spring/summer decomposition in the presence of nicotine

Classification of Macro invertebrate	Indicative species
Strongly nicotine intolerant	**Diptera** Piophilidae Sepsidae
Semi-Nicotine tolerant	**Diptera** Sciaridae Phoridae *Megaselia* spp Muscidae *Phaonia subventa*
Strongly tolerant	**Diptera** Sphaeroceridae *Ischiolepta denticula* Fanniidae *Fannia lineata* **Coleoptera** Carabidae *Nebria brevicolis* Staphylinidae *Creophilus maxillosus* Histeridae *Maginarinatus cadaverinus* **Dermaptera** Forficulidae *Forficula auricularia*

Strongly tolerant	**Diptera** Lonchopteridae *Lonchoptera furcata* Phoridae *Anevrina unispinosa* Muscidae *Graphomya maculate* **Coleoptera** Carabidae *Pterostichus niger* Silphidae *Necrodes littoralis* Staphylinidae *Bisnus fimetarius* Nitidulidae *Omosita discoidea*
Key: Strongly nicotine intolerant = only found upon the control carrion Mildly intolerant = found upon both control and low dose carrion Semi-Nicotine tolerant = only found upon the low dose carrion Tolerant = found upon the low and high dose carrion Strongly tolerant = only found upon the high dose carrion	

The above tables show that the Piophilidae (Diptera) appear to be the greatest indicators of the absence of nicotine. Chick (2010a) placed the Piophilidae in the 4th wave of decomposition during the onset of caseic fermentation, during which the corpse begins to dry out. Skowronek *et al* (2015) reported previously *Liopiophila varipes* feeding upon bone marrow cavities of deer in Poland. The absence of the Piophilidae in nicotine treated corpses supports the hypothesis that the presence of nicotine leads to a higher level of moisture retention in a corpse, and could suggest the Piophilidae don't have an a aversion to nicotine, but rather nicotine renders the corpses unsuitable for colonisation by the Piophilidae either due to the presence of nicotine, or the possible raised moisture level. Sphaeroceridae are also considered by Chick (2010a) to be part of the fourth wave. *Ischiolepta denticula* was determined to be mildly tolerant to nicotine. suggesting it is capable of withstanding a slightly higher moisture level in a corpse than can members of the Piophilidae. The only species of Sphaeroceridae are commonly recorded from corpses and are generally attracted to

ammonia, either from voiding of the bladder or by ammoniacal fermentation Gennard (2007). This could suggest a wetter corpse has a raised level of ammonia within it. As previously discussed, the nicotine tolerant species tend to be dominated by mycophagus Coleoptera, and semi-liquid frequenting flies as well as beetles such as *Omosita discoidea.* Smith (1986) previously stated that Nitidulids such as *Omosita* appear to have a preference for moister skin in carrion than do the Dermestidae.

These observations suggest that nicotine treated carrion retains moisture for a longer than does non-nicotine treated carrion, leading to an increase in mycological growth on the corpse and a consequent change in carrion fauna.

6.4 Laboratory experiments

The field based investigations highlighted a number of interesting behavioural and successional changes to the carrion ecosystem, so to further investigate and quantify the effect of nicotine, the specific effects on a primary coloniser were investigated. *Calliphora vomitoria* was selected as a suitable test species for single species lab investigations due to its common distribution and previously reported forensic relevance (Gennard 2007).

The first aim of the laboratory based element was to develop an artificial rearing medium for use in entomotoxicological investigations, using a mixture of porcine material and gelatine. The use of aqueous gelatine meant that test toxins could be homogenously added to the media prior to setting up an exposure to *C.vomitoria*. This allowed for quantitative investigations of the effect of nicotine on the development of *C.vomitoria* in a controlled environment. Subsequently, the effect of nicotine on total oviposition was investigated using *C.vomitoria* in the presence of rearing media dosed with nicotine. A one way ANOVA showed that the effect of nicotine on the numbers of eggs laid was not statistically significant. The apparent

fluctuations in egg numbers could have easily been natural variation given the number of eggs a single female can lay. Single egg laying was observed in the lab tests but not to the same extent as in the field testing, this was possibly due to the reduced surface area of carrion exposed to *C.vomitoria* in comparison to the whole carrion approach used in the field. Whole carrion, while larger, also is protected from desiccation by the presence of skin, while an attempt to replicate this effect in the laboratory by containing the media in a plastic cup thus only exposing a proportion to the air, may not have had the same effect as that of a whole carcase. The enclosed nature of a laboratory may also have had an effect on the behaviour of *C.vomitoria*.

A high level of pupal mortality was observed in the higher doses of nicotine treated carrion. Upon dissection of the un-emerged pupa it was determined that pupae developed to a pre-emergent imago, but the adult did not break the pupal casing to emerge from the puparium, possibly due to an absence of the crustacean cardio active peptide (CCAP) which triggers emergence behaviour (Gullan and Cranston, 2004) in *C.vomitoria* in unaffected pupae. Figure 5.25 showed that in general, the percentage emergence appeared to decrease in relation to higher doses of nicotine. However two anomalous results meant this trend was not clearly defined. The ANOVA results (P=0.31) suggested this trend was not significant, however further testing would be needed to determine if the two possible anomalous results have had an effect on the ANOVA.

One way ANOVA shows that nicotine has a significant effect on the accumulated degree days of *C.vomitoria* with a P= <0.0001.The exact relationship is not a simple one; the average development time of *C.vomitoria* at $11^{O}C$ in response to increasing nicotine concentration had a low R^2 of 0.1614. Fishers LSD test showed that there were 3 overlapping groups with differing levels of significance. When looking at the development of *C.vomitoria* at varying stages of its life cycle in relation to increasing nicotine concentration, the trend is

that nicotine reduces the time taken for *C.vomitoria* to reach pupation and then increases the time spent as a pupa. The data points have a larger R^2 than in the previous test with R^2=0.5979 for ADD from egg to pupation . R^2 for ADD for the duration of pupation in lower doses is 0.5945 both in lower doses of nicotine. Fishers LSD testing of the ADD from egg to pupation showed again that there were 3 groups of data with varying degrees of statistical significance. However when the ADD of pupation duration was analysed using Fishers LSD, each test was shown to be significantly different from each other, suggesting that nicotine has the greatest effect upon the pupal stage of *C.vomitoria*. This adds to the theory that the release of detection of CCAP is blocked by nicotine in *C.vomitoria.*

6.5Applications and considerations for forensic entomology

The forensic entomologist is primarily interested in how knowledge of insect ecology can be applied to legal and criminal matters. The most common way that insects impact upon forensic legal investigations is in the estimation of *post mortem* interval by use of their development and succession. Therefore any way in which the established order of insect colonisation or succession on a corpse is affected is of great interest to the forensic entomologist and the police.

As previously discussed there are several species that are indicative of seasonal decomposition in the presence of nicotine (tables 6.1 and 6.2) and this can be used by the forensic entomologist to suggest the presence of nicotine in the carrion ecosystem and request that a toxicological screening is required. Although Karch (2008) remarked that nicotine is not part of a normal toxicology screening, the concentration of nicotine would be useful information for the forensic entomologist to have at their disposal if the indicator species are

present. The effect of nicotine upon the calculation of *post mortem* Interval whilst not perfect can be somewhat taken into account using the equations derived in chapter 5:

ADD (egg to pupation *C.vomitoria*) = -47.357 x Dose of nicotine (mg/kg)* + 338.73

ADD (Pupation to emergence *C.vomitoria*) = 62.376 x Dose of nicotine (mg/kg) + 268.7ADD

*to a maximum dose 6.3mg/kg nicotine determined *via* laboratory culture

The non-emergence of *C.vomitoria* at higher doses reinforces the opinion of Gunatilake and Goff (1989) and Chick *et al* (2008) who observed a similar phenomenon in the presence of insecticidal compounds and stated that the absence of suitable identification methods for immature Diptera could pose problems for the forensic entomologist in cases involving cadaver pollution, given that the traditional methods of identification require rearing specimens to adulthood.

In conclusion, nicotine is a commonly abused toxin used both recreationally and as a method of poisoning in cases of suspicious deaths. The presence of nicotine in carrion affects the successional waves of decomposition differently depending upon season, with autumnal decomposition being typified by the presence of mycophagus species, and summer decomposition showing a preference for a semi liquid corpse. The behaviour of primary colonisers is shown to change, single egg laying observed with a marked change of oviposition site, in nature. In the laboratory it was shown that *C.vomitoria* shows a change in numbers of eggs laid and rate of development at different stages in its life cycle. While not perfect tentative predictive equations were proposed and limitations noted for the forensic entomologist to improve the estimation of *post mortem* interval.

6.6 Limitations of this study

The present study should be considered as a preliminary investigation into the effects of nicotine upon the decomposition of carrion. The field based study lacked concurrent replicates which make it difficult to draw definitive conclusions as to the effect of nicotine on decomposing carrion *in situ*. Ideally such a study would use at least 3 of each treatment in the same area Matuszewski *et al* (2010) used 4 replicates when they investigated the effects of season in Poland and remark that number of replicates was a limiting factor. Mahat *et al* (2016) also used 4 replicates when investigating the effect of burning on entomological decomposition. However previous provisional studies have used fewer replicates Zuha *et al* (2015) used only 1 repeat when investigating the effect of indoor decomposition in Malaysia. Oliveria-Costa *et al* (2013) didn't use a single replicate when investigating the effects of burning upon decomposition. Unfortunately space and resources were limiting factors in the current study meaning that the data was not as valid as could be hoped. This si not uncommon, for example Disney (2011) stated that underfunding is an issue in forensic science.

The swapping of sites that might have had an adverse effect upon the quality of the results between the autumn/winter investigation and the spring/summer was unavoidable, due to the reduction in cover by ground staff between investigations. While the sites were less than 50 metres from each other the baseline study did show a difference in invertebrate fauna. One would hope to reduce the number of variables when undertaking a scientific investigation and as such the lack of continuity at the site would have affected the results.

It is possible that the nicotine would not have been distributed throughout the carcases in the same manner as one would see in a real human corpse. The pigs were dosed with nicotine sub dermally post mortem, as per ethical approval. However Hukkanen *et al* (2005) state that

nicotine is distributed extensively throughout the body post absorption. They also remarked remarked that nicotine appears to have an affinity for the liver, kidneys, spleen and lungs in smokers with a low affinity for adipose tissue. However nicotine was often found in the brain, kidneys, blood and liver in case of poisoning by nicotine solution.

It was hypothesised that the presence of nicotine increases the presence of moisture/reduces lose of moisture in a corpse and therefore increases the instance of mycological growth, however this was not realised until the mycophagus specimens were indentified and as such the presence of fungi was not investigated or within the expertise of the investigator.

The lab based element was limited by the number of replicates. While each treatment was repeated at least once, cultures would occasionally spontaneously fail and die. Colleagues have reported similar deaths in other cultured in insects (Dave Gee, *Pers comm*). Unfortunately such failures are time and space consuming. The equations presented based upon the results of the lab based investigations have low R^2 values, and while their use in future forensic investigations might be limited due to the low correlations the method of using the equation of the line to correct ADD could be used in further investigations.

6.7 Scope for further work

The overarching aim of this investigation was to investigate what effects if any, the presence of nicotine had on the decomposition of carrion by the action of invertebrates, and how such effects can be factored in to estimations by the forensic entomologist.

The investigation was successful in that a number of changes were observed in how animal tissue decomposes in the presence of nicotine, both in a natural setting and a laboratory based setting. The changes included behavioural, faunal and developmental differences to the carrion fauna. The holistic approach adopted offered a better overview of the effect of

nicotine on decomposition than either element would have done if presented in isolation For example the laboratory investigation alone would not have indicated that autumn decomposition in the presence of nicotine leads to later waves of succession being dominated by mycophagus species. Also, the field investigation did not highlight the effect of the rate of *C.vomitoria* development, in response to nicotine.

A number of avenues of further work can be highlighted, the most obvious being to try and minimise the limitations stated above. A repeat of the field based experiments at a larger and more secure site (not requiring a switch of locations) with at least 4 replicates would be ideal. Another improvement for the field methodology would be to better mimic the distribution of nicotine in a corpse either as a poisoning, or as a therapeutic application.

It would be wise during any repeated field work to monitor moisture levels within the carrion, as well as to investigate the possible fungal growth upon the carrion.

As data used for PMI calculations will vary depending on the species present the laboratory based element could be repeated with other primary colonisers, for example common UK species such as *Calliphora vicina* or *Lucillia sericata* (Gennard, 2007). The orginal lab based investigation could be expanded to try and clarify the results with repeated tests.

Seasonal variation in *P.madidus* and *N.humator*, could also be investigated. Although Smith (1986) remarked that carabids are not considered forensically important despite their common status on corpses, in general their place in the carrion ecosystem appears poorly understood. Further work as to how the Carabidae fits into the carrion ecosystem throughout the year both in relation to the presence of nicotine and in general could provide interesting results. Since the work highlighted that the presence of the *P.madidus* on high dose carrion in autumn could have been due to the breeding season, a similar test concentrating on the ratios of sexes could be carried out to investigate this hypothesis.

N.humator was highlighted to be found on different carrion in both investigations. This could also be due to the time of year in relation to the breeding cycle of the species. A breeding methodology could be investigated to see if they show a preference in the laboratory, as could testing for a preference for nicotine containing maggots as a food source.

The hypothesis that nicotine leads to a corpse retaining moisture could be further investigated and the use of moisture probes could be used to log changes within the corpse and see how temperature relates to the faunistic succession on carrion.

There are a number of other compounds that should be investigated in place of nicotine. Although illegal drugs are the traditional area of entomotoxicology research, with the increasing number of deaths resulting from so called "legal highs" with unknown side effects being reported by the BBC (2014), a possible area of research would be the effect of such compounds on decomposition. Similarly, death by caffeine poisoning has recently been reported by CBS (2014) with a teenager in America. Even without fatal effects the presence of caffeine is possibly more ubiquitous than nicotine, and may also have an effect upon the determination of PMI.

A final avenue of investigation would be that since the start of this investigation the nature of recreational nicotine use has seen a change, with an increase in use of electronic cigarettes or e-cigerettes. The American Center for Disease Control (CDC) stated that nicotine poisoning due to e-cigerettes is increasing, with a high number of children under the age of 5 being involved in 51% of cases (WebMD, 2014). The e-cigerettes use a concentrated nicotine solution to provide the effect of nicotine, suggesting that poisoning *via* higher nicotine doses may become more common, although the CDC did note that a fatal case of accidental nicotine poisoning linked to e-cigerettes hasn't been seen yet, at least in humans (WebMD 2014). Offermann (2015) showed that e-cigerettes have passive effects similar to that of normal cigarettes, including the presence of nicotine in second hand exposure.

Nicotine is a ubiquitous toxin which is present in much of the population, and which is also known to have insecticidal effects. The present work has shown that the presence of nicotine within a corpse has a number of effects that are felt throughout the successional process of decomposition. These effects include a change in the natural behaviour of primary colonisers, as well as changes in secondary and tertiary colonisers. The effects vary throughout the year with autumn and winter showing an increase in the presence of mycophagus species. It was also shown that the Primary coloniser *Calliphora vomitoria* was shown to be significantly affected in terms of rate of development on nicotine infused tissue. A robust methodology that is transferable to other compounds and species has been suggested. The data goes some way towards a predictive methodology for use by forensic practitioners, and further avenues of research are highlighted.

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Appendix 2: Supplemental data of Laboratory testing of *Calliphora vomitoria*

Numbers of egg laid by *C.vomitoria* summary statistics

	Control	0.35mg/kg	0.7mg/kg	1.4mg/kg	2.8mg/kg	5.6mg/kg	11.2mg/kg
mean	715	679.25	591	549.6667	559	546.375	721.8571
Sd	450.8455	406.5221	258.8011	357.2801	349.1826	470.5777	434.28

Anova: Single Factor
(Microsoft Excel)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	12	8580	715	203261.6
0.35mg/kg	4	2717	679.25	165260.3
0.7mg/kg	2	1182	591	66978
1.4mg/kg	6	3298	549.6667	127649.1
2.8mg/kg	5	2795	559	121928.5
5.6mg/kg	8	4371	546.375	221443.4
11.2mg/kg	7	5053	721.8571	188599.1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	276647.9	6	46107.99	0.258238	0.952705	2.356179
Within Groups	660629	37	178548.5			
Total	688294	43				

Total Accumulated Degree Days (ADD) for the life cycle of *C.vomitoria*

Anova: Single Factor
(Microsoft Excel)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	39	22888	586.8718	968.3779
0.35mg/kg	8	5680	710	617.1429
0.7mg/kg	85	52336	615.7176	218.586
1.4mg/kg	12	7392	616	0
2.8mg.kg	10	5840	584	0
11.2mg/kg	4	2400	600	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	111078.4	5	22215.68	56.77214	4.6E-33	2.273686
Within Groups	59479.58	152	391.313			
Total	170558	157				
Total	170558	157				

ANOVA (one way) and Fishers LSD (Minitab)

Method

Null hypothesis	All means are equal
Alternative hypothesis	At least one mean is different
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	6	0, 0.35mg/kg, 0.7mg/kg, 1.4mg/kg, 2.8mg/kg, 11.2mg/kg

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	111078.392	22215.6784	56.77	<0.0001
Error	152	59479.583	391.3130		
Total	157	170557.975			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
19.7816340	65.13%	63.98%	62.94%

Means

Factor	N	Mean	StDev	95% CI
0	39	586.872	31.119	(580.614, 593.130)
0.35mg/kg	8	710.000	24.842	(696.182, 723.818)
0.7mg/kg	85	615.718	14.785	(611.479, 619.957)
1.4mg/kg	12	616	0	(604.717867, 627.282133)
2.8mg/kg	10	584	0	(571.641043, 596.358957)
11.2mg/kg	4	600	0	(580.458773, 619.541227)

Pooled StDev = 19.7816340

Grouping Information Using the Fisher LSD Method and 95% Confidence

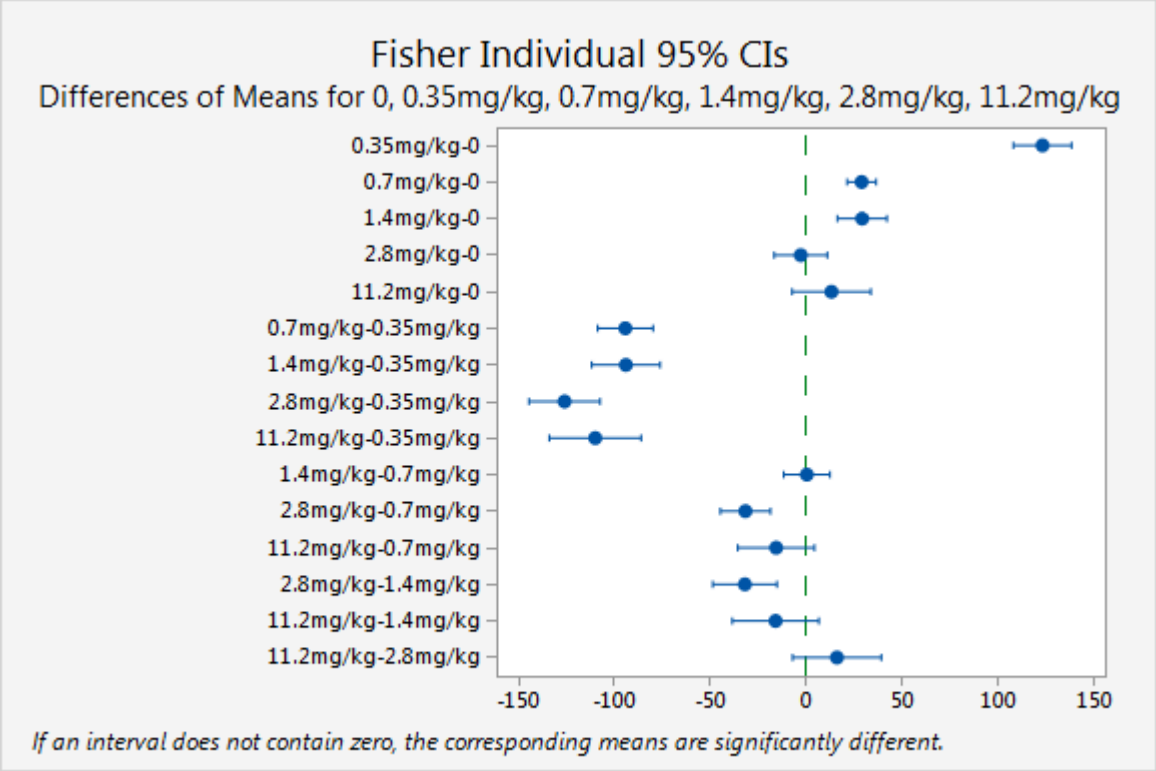
Factor	N	Mean	Grouping	
0.35mg/kg	8	710.000000	A	
1.4mg/kg	12	616.000000	B	
0.7mg/kg	85	615.717647	B	
11.2mg/kg	4	600.000000	B	C
0	39	586.871795		C
2.8mg/kg	10	584.000000		C

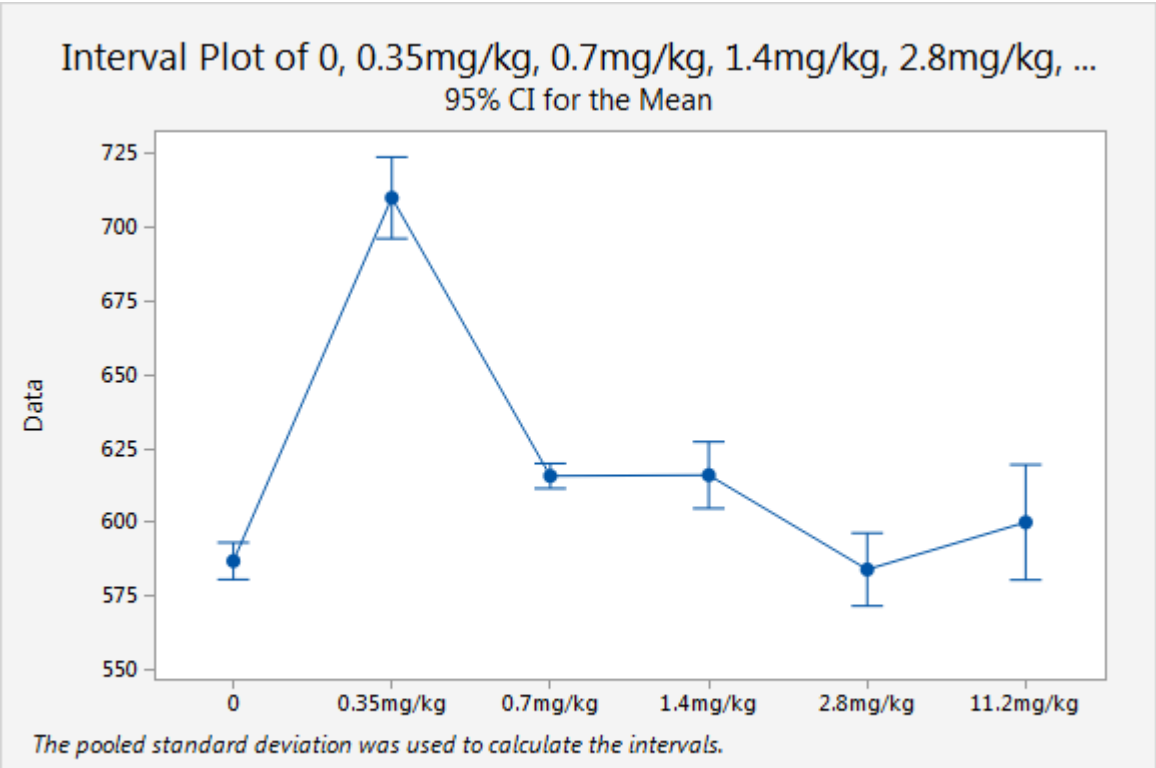
Means that do not share a letter are significantly different.

Fisher Individual Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.35mg/kg-0	123.128	7.678	(107.959, 138.297)	16.04	<0.0001
0.7mg/kg-0	28.846	3.826	(21.287, 36.405)	7.54	<0.0001
1.4mg/kg-0	29.128	6.530	(16.227, 42.030)	4.46	<0.0001
2.8mg/kg-0	-2.872	7.012	(-16.725, 10.981)	-0.41	0.6827
11.2mg/kg-0	13.13	10.39	(-7.39, 33.65)	1.26	0.2081
0.7mg/kg-0.35mg/kg	-94.282	7.316	(-108.736, -79.829)	-12.89	<0.0001
1.4mg/kg-0.35mg/kg	-94.000	9.029	(-111.839, -76.161)	-10.41	<0.0001
2.8mg/kg-0.35mg/kg	-126.000	9.383	(-144.538, -107.462)	-13.43	<0.0001
11.2mg/kg-0.35mg/kg	-110.00	12.11	(-133.93, -86.07)	-9.08	<0.0001
1.4mg/kg-0.7mg/kg	0.282	6.100	(-11.770, 12.335)	0.05	0.9631
2.8mg/kg-0.7mg/kg	-31.718	6.613	(-44.783, -18.652)	-4.80	<0.0001
11.2mg/kg-0.7mg/kg	-15.72	10.12	(-35.71, 4.28)	-1.55	0.1225
2.8mg/kg-1.4mg/kg	-32.000	8.470	(-48.734, -15.266)	-3.78	0.0002
11.2mg/kg-1.4mg/kg	-16.00	11.42	(-38.56, 6.56)	-1.40	0.1633
11.2mg/kg-2.8mg/kg	16.00	11.70	(-7.12, 39.12)	1.37	0.1736

Simultaneous confidence level = 63.89%


Fisher Individual 95% CIs
Differences of Means for 0, 0.35mg/kg, 0.7mg/kg, 1.4mg/kg, 2.8mg/kg, 11.2mg/kg
0.35mg/kg-0
0.7mg/kg-0
1.4mg/kg-0
2.8mg/kg-0
11.2mg/kg-0
0.7mg/kg-0.35mg/kg
1.4mg/kg-0.35mg/kg
2.8mg/kg-0.35mg/kg
11.2mg/kg-0.35mg/kg
1.4mg/kg-0.7mg/kg
2.8mg/kg-0.7mg/kg
11.2mg/kg-0.7mg/kg
2.8mg/kg-1.4mg/kg
11.2mg/kg-1.4mg/kg
11.2mg/kg-2.8mg/kg
-150
-100
-50
0
50
100
150
If an interval does not contain zero, the corresponding means are significantly different.


Interval Plot of 0, 0.35mg/kg, 0.7mg/kg, 1.4mg/kg, 2.8mg/kg, ...
95% CI for the Mean
Data
725
700
675
650
625
600
575
550
0
0.35mg/kg
0.7mg/kg
1.4mg/kg
2.8mg/kg
11.2mg/kg
The pooled standard deviation was used to calculate the intervals.

ANOVA(one way) ADD for *C.vomitoria* from egg to pupa

Anova: Single Factor

(Microsoft excel)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	8	2312	289	4762.286
0.35mg/kg	4	1440	360	853.3333
0.7mg/kg	4	1408	352	1408
1.4mg/kg	2	472	236	1568
2.8mg/kg	6	1248	208	6553.6
5.6mg/kg	2	688	344	3200
11.2mg/kg	3	720	240	7056

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	93978.76	6	15663.13	3.754999	0.010042	2.549061
Within Groups	91768	22	4171.273			
Total	185746.8	28				

ANOVA (one way) and Fishers LSD, ADD *C.vomitoria* from egg to pupa (Minitab)

Method

Null hypothesis	All means are equal
Alternative hypothesis	At least one mean is different
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	7	control, 0.35, 0.7, 1.4, 2.8, 5.6, 11.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	6	93978.759	15663.1264	3.75	0.0100
Error	22	91768.000	4171.2727		
Total	28	185746.759			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
64.5853910	50.60%	37.12%	17.30%

Means

Factor	N	Mean	StDev	95% CI
control	8	289.00	69.01	(241.64, 336.36)
0.35	4	360.00	29.21	(293.03, 426.97)
0.7	4	352.00	37.52	(285.03, 418.97)
1.4	2	236.00	39.60	(141.29, 330.71)
2.8	6	208.00	80.95	(153.32, 262.68)
5.6	2	344.00	56.57	(249.29, 438.71)
11.2	3	240.00	84.00	(162.67, 317.33)

Pooled StDev = 64.5853910

Grouping Information Using the Fisher LSD Method and 95% Confidence

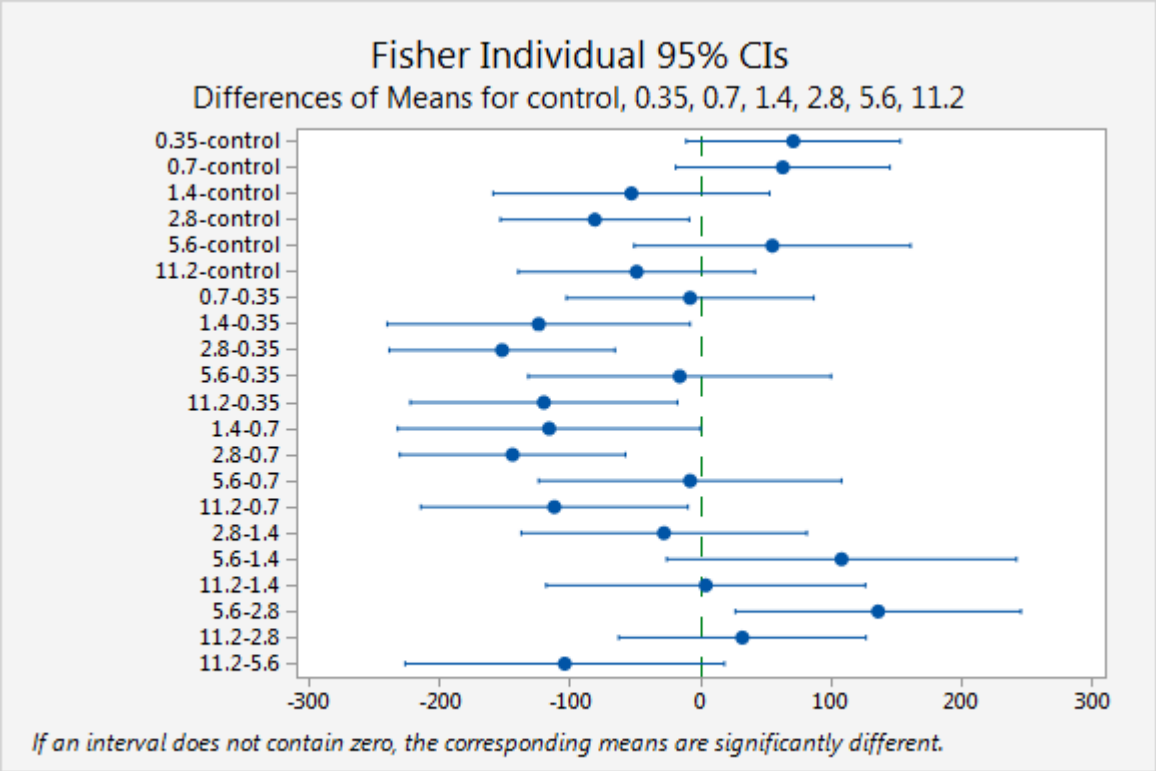
Factor	N	Mean	Grouping		
0.35	4	360	A		
0.7	4	352	A		
5.6	2	344	A	B	
control	8	289	A	B	
11.2	3	240		B	C
1.4	2	236		B	C
2.8	6	208			C

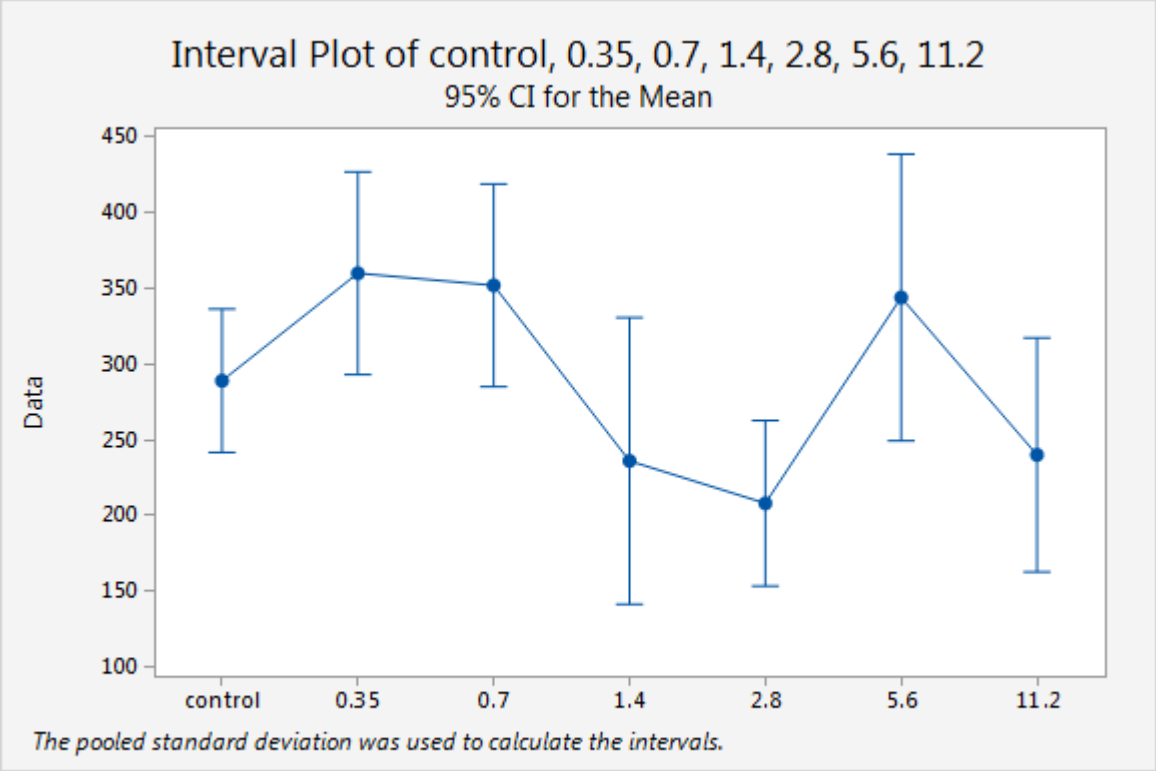
Means that do not share a letter are significantly different.

Fisher Individual Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.35-control	71.00	39.55	(-11.02, 153.02)	1.80	0.0864
0.7-control	63.00	39.55	(-19.02, 145.02)	1.59	0.1254
1.4-control	-53.00	51.06	(-158.89, 52.89)	-1.04	0.3105
2.8-control	-81.00	34.88	(-153.34, -8.66)	-2.32	0.0299
5.6-control	55.00	51.06	(-50.89, 160.89)	1.08	0.2931
11.2-control	-49.00	43.72	(-139.68, 41.68)	-1.12	0.2745
0.7-0.35	-8.00	45.67	(-102.71, 86.71)	-0.18	0.8625
1.4-0.35	-124.00	55.93	(-240.00, -8.00)	-2.22	0.0373
2.8-0.35	-152.00	41.69	(-238.46, -65.54)	-3.65	0.0014
5.6-0.35	-16.00	55.93	(-132.00, 100.00)	-0.29	0.7775
11.2-0.35	-120.00	49.33	(-222.30, -17.70)	-2.43	0.0236
1.4-0.7	-116.00	55.93	(-232.00, 0.00)	-2.07	0.0500
2.8-0.7	-144.00	41.69	(-230.46, -57.54)	-3.45	0.0023
5.6-0.7	-8.00	55.93	(-124.00, 108.00)	-0.14	0.8876
11.2-0.7	-112.00	49.33	(-214.30, -9.70)	-2.27	0.0333
2.8-1.4	-28.00	52.73	(-137.36, 81.36)	-0.53	0.6008
5.6-1.4	108.00	64.59	(-25.94, 241.94)	1.67	0.1086
11.2-1.4	4.00	58.96	(-118.27, 126.27)	0.07	0.9465
5.6-2.8	136.00	52.73	(26.64, 245.36)	2.58	0.0171
11.2-2.8	32.00	45.67	(-62.71, 126.71)	0.70	0.4908
11.2-5.6	-104.00	58.96	(-226.27, 18.27)	-1.76	0.0916

Simultaneous confidence level = 59.93%





One way ANOVA ADD (pupal duration of *C.vomitoria*)

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	8	2718	339.75	26.78571
Column 2	85	19080	224.4706	169.6807
Column 3	12	4896	408	0
Column 4	10	4320	432	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	706076.1	3	235358.7	1809.113	4.8E-94	2.686384
Within Groups	14440.68	111	130.0962			
Total	720516.7	114				

ANOVA (one way) and Fishers LSD, ADD *C.vomitoria* pupal duration (Minitab)

Method

Null hypothesis	All means are equal
Alternative hypothesis	At least one mean is different
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	5	control, 0.35mg/kg, 0.7mg/kg, 1.4mg/kg, 2.8mg/kg

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	707158.745	176789.686	880.34	<0.0001
Error	149	29922.112	200.820		
Total	153	737080.857			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
14.1710813	95.94%	95.83%	95.77%

Means

Factor	N	Mean	StDev	95% CI
control	39	263.590	20.184	(259.106, 268.074)
0.35mg/kg	8	339.750	5.175	(329.850, 349.650)
0.7mg/kg	85	224.471	13.026	(221.433, 227.508)
1.4mg/kg	12	408	0	(399.916449, 416.083551)
2.8mg/kg	10	432	0	(423.144913, 440.855087)

Pooled StDev = 14.1710813

Grouping Information Using the Fisher LSD Method and 95% Confidence

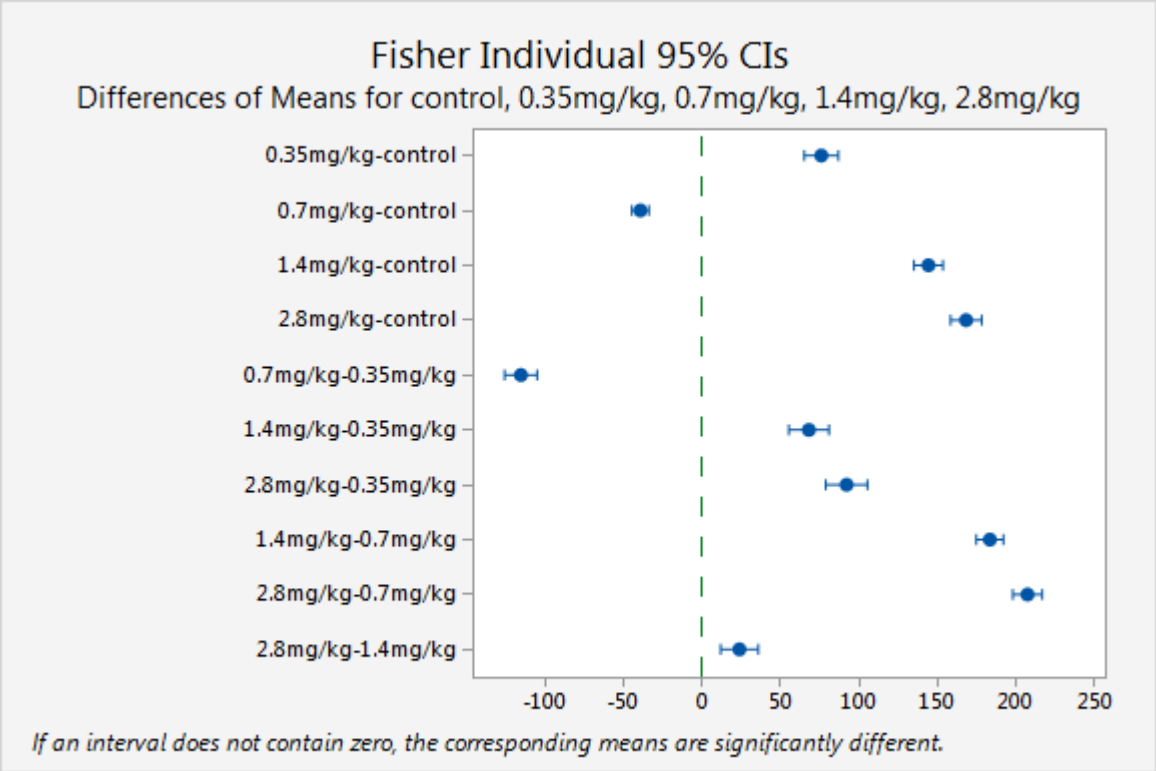
Factor	N	Mean	Grouping
2.8mg/kg	10	432.000000	A
1.4mg/kg	12	408.000000	B
0.35mg/kg	8	339.750000	C
control	39	263.589744	D
0.7mg/kg	85	224.470588	E

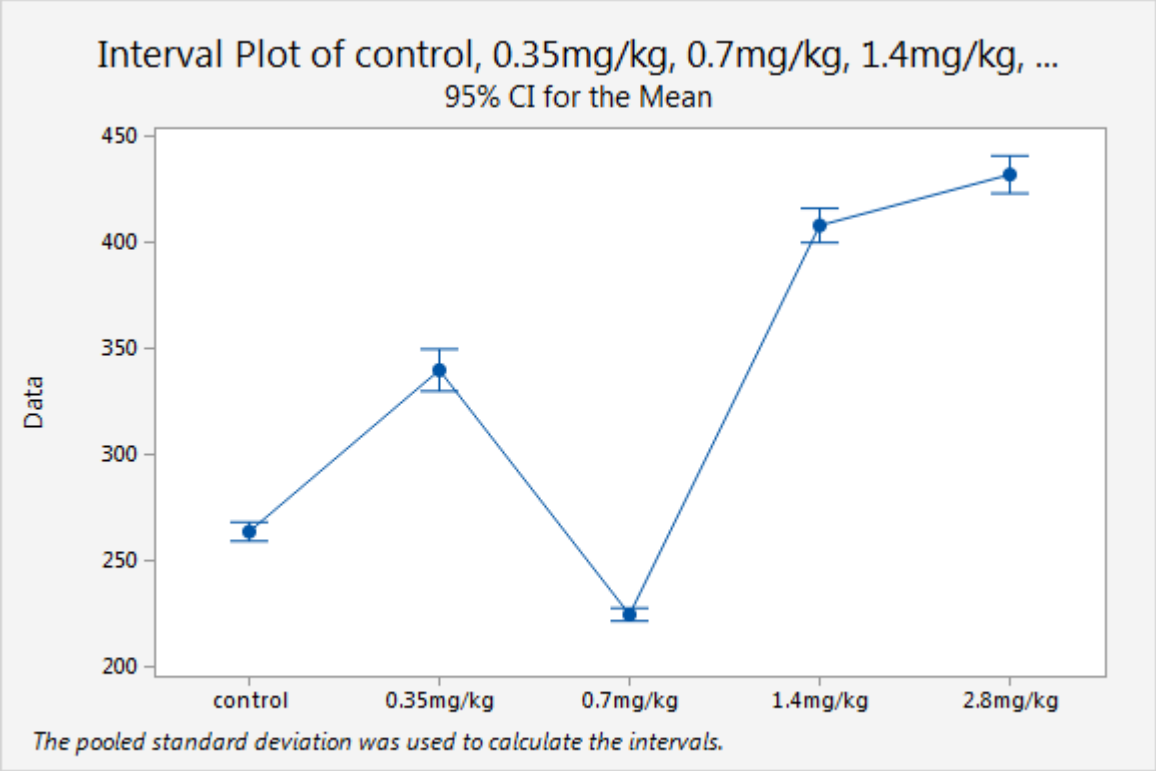
Means that do not share a letter are significantly different.

Fisher Individual Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.35mg/kg-control	76.160	5.500	(65.292, 87.029)	13.85	<0.0001
0.7mg/kg-control	-39.119	2.741	(-44.535, -33.703)	-14.27	<0.0001
1.4mg/kg-control	144.410	4.678	(135.166, 153.654)	30.87	<0.0001
2.8mg/kg-control	168.410	5.023	(158.485, 178.336)	33.53	<0.0001
0.7mg/kg-0.35mg/kg	-115.279	5.241	(-125.635, -104.924)	-22.00	<0.0001
1.4mg/kg-0.35mg/kg	68.250	6.468	(55.469, 81.031)	10.55	<0.0001
2.8mg/kg-0.35mg/kg	92.250	6.722	(78.967, 105.533)	13.72	<0.0001
1.4mg/kg-0.7mg/kg	183.529	4.370	(174.894, 192.165)	42.00	<0.0001
2.8mg/kg-0.7mg/kg	207.529	4.738	(198.168, 216.891)	43.81	<0.0001
2.8mg/kg-1.4mg/kg	24.000	6.068	(12.010, 35.990)	3.96	0.0001

Simultaneous confidence level = 71.72%





One way ANOVA of numbers of adult *C.vomitoria* emerged from pupa

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	5	117	23.4	1309.3
0.35mg/kg	3	51	17	57
0.7mg/kg	1	41	41	#DIV/0!
1.4mg/kg	3	34	11.33333	108.3333
2.8mg/kg	3	110	36.66667	89.33333
5.6mg/kg	3	0	0	0
11.2mg/kg	4	28	7	196

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3302.239	6	550.3732	1.303269	0.314385	2.790465
Within Groups	6334.533	15	422.3022			
Total	9636.773	21				

Appendix 3 Published work

Appendix 4

Abbreviations list

ADD Accumulated degree days

ADH Accumulated degree hours

HWK hot water kill

LC_{50} Lethal concentration required to kill 50% of a population

LD_{50} Lethal dose required to kill 50% of the population

PMI Post mortem interval