Defining the role of semiochemicals in host location and selection by UK *Culicoides* species biting midges

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I, James Iain Cook, confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The role of semiochemicals in host location and selection by UK *Culicoides* species biting midges is poorly understood. The spread of bluetongue virus (BTV) through northern Europe and its arrival in the UK in 2007, and the emergence and spread of Schmallenberg virus (SBV) in 2011, has focused attention on UK *Culicoides* spp. and their roles as arbovirus vectors. *Culicoides obsoletus* and *Culicoides pulicaris* species groups were implicated as vectors of BTV in the UK. Additionally, it has been reported that current trapping and surveillance tools drastically underestimated the presence of these species. As a result, there is a pressing need for a greater understanding of host location and selection by *C. obsoletus* and *C. pulicaris* species groups. Development of new equipment allowed for the successful collection of whole animal (sheep) air entrainment extracts. Significant attraction of wild *Culicoides* species was shown in the field to vented air from the whole animal entrainment box and in the laboratory using the extracts with *Culicoides nubeculosus.* Using gas chromatography-electroantennography (GC-EAG), 37 EAG-active peaks were identified from the sheep extracts, including 10 novel GC-EAG responses. Tentative identifications were made using GC-mass spectrometry (GC-MS) and 12 identifications were confirmed by peak enhancement co-injections. Behavioural activity was investigated in the laboratory resulting in significant attraction to some chemicals, including novel behavioural results. Chemicals were prepared in slow release formulations with release rates relevant to the natural host. Blends of chemicals were tested in the field, resulting in significant attraction of wild *Culicoides* spp. to a 3-chemical blend and 7-chemical blend in different trials. Refinement of these chemical blends may result in a slow release formulation that can be exploited to improve monitoring and control of vector *Culicoides* spp. Furthermore the novel identified chemicals may also play a role in host location of other haematophagous insects.

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Chapter 1: General introduction

Culicoides species biting midges (Diptera: Ceratopogonidae) are small haematophagous insects, typically 1-3 mm in length (reviewed by Mellor *et al*, 2000). Over 1500 *Culicoides* species are known and they have been found in most parts of the world except extreme polar regions, Patagonia, New Zealand and the Hawaiian Islands (Mellor *et al*, 2000). Forty eight *Culicoides* species have been recorded in the UK (Campbell and Pelham-Clinton, 1960, Boorman, 1986). Female *Culicoides* species feed on blood, which provides protein for the development of eggs, with one blood-meal usually required for each batch of eggs to mature (Mellor *et al*, 2000). However, approximately 38 species worldwide (for example, *Culicoides impunctatus* Goetghebuer, the Scottish biting midge) are autogenous, and therefore capable of producing the first batch of eggs without the need for a source of blood (Boorman and Goddard, 1970, Linley, 1983).

1.1 Biology of *Culicoides* species

The life cycle of *Culicoides* species includes the egg, four larval instars, pupa and imago or adult (Mellor *et al*, 2000) (Figure 1.1). *Culicoides* species generally breed in damp habitats with organic matter including swampy areas and pools of water with rotting vegetation (Kettle and Lawson, 1952). Only the females require blood for egg development. Egg batch sizes for *Culicoides* species can reach up to 450 eggs for *Culicoides circumscriptus* Kieffer while *C. impunctatus* in England has been found to lay 50 eggs on average (Boorman and Goddard, 1970, Service, 1968, Kettle, 1984). Development times are temperature dependent and higher temperatures tend to result in faster development. Veronesi *et al* (2009) found that the time from blood feeding *Culicoides imicola* Kieffer to the production of next generation adults was shorter at greater temperatures, up to 28 °C. While Allingham (1991) showed the optimum temperature range for survival of *Culicoides* brevitarsis Kieffer was 26 °C – 33 °C.

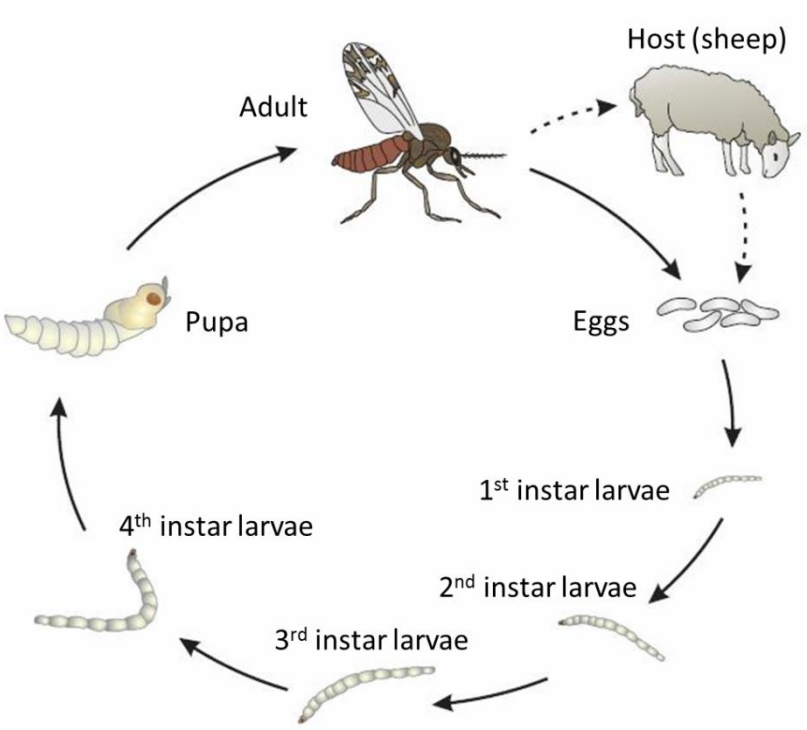


Figure 1.1: Illustrative life cycle of *Culicoides* species showing four stages: egg, larvae (4 instars), pupae and adult. Host location, and potential vectoring of pathogens occurs during the adult stage. Drawn by Lynda Castle, Rothamsted Research.

The *Culicoides* larvae emerge from the egg and pass through four instars, feeding on organic matter present in breeding sites. In the Palaearctic region, the development may arrest at the fourth larval instar and overwinter with pupation occurring the following spring (Kettle, 1984). In contrast, in warmer regions, larval development can take 4 – 5 days (Kettle, 1984). Similarly, the pupal stage often is brief, a few days, but temperature dependent, with low temperatures extending this development to a few weeks (Edwards, 1982).

Culicoides species adults tend to survive for 10 – 20 days, although individuals have been noted to survive for up to 92 days (Mellor *et al*, 2000, Goffredo *et al*, 2004). Most adult *Culicoides* species are crepuscular with peak activity occurring at dawn and dusk (Mellor *et al,* 2000, Sanders *et al*, 2012). Mating during swarming has been described for several *Culicoides* species including *Culicoides variipennis* Coquillett, *Culicoides obsoletus* Meigen and *Culicoides pulicaris* Linnaeus (Downes, 1955, Zimmerman *et al*, 1982).

1.2 Medical and veterinary importance

Culicoides species are mainly a nuisance to human beings due to irritation caused by their bites, as exemplified by *Culicoides impunctatus* in Scotland and *Culicoides furens* Poey in Florida and the Caribbean (Hendry and Godwin, 1988, Kettle, 1962, Linley and Davies, 1971). *Culicoides impunctatus* accounts for over 90 % of biting attacks in Scotland, and although it does not vector any pathogens that cause disease in the UK at present, it does cause significant losses to the economy through reduced tourism and outdoor activities (Hendry and Godwin, 1988). Oropouche virus which causes nausea, vomiting and fever, and is confined to the Caribbean and South America, is the only *Culicoides* species vectored pathogen of humans (Pinheiro *et al*, 1981). However, *Culicoides* species are vectors of veterinary importance, as reviewed by Mellor *et al* (2000). The saliva from *C. pulicaris* can cause an allergic dermatitis commonly called "sweet itch" in horses (Mellor and McCaig, 1974, Townley *et al*, 1984). Some *Culicoides* species, including *C. obsoletus*, *Culicoides nubeculosus* Meigen and *Culicoides parroti* Kieffer vector a filarial nematode, *Onchocerca cervicalis* Railliet and Henry, which affects horses (Mellor, 1973). *Culicoides* species also vector numerous livestock arboviruses, including those that cause bluetongue, African horse sickness, epizootic haemorrhagic disease, Akabane, bovine ephemeral fever, equine encephalosis and the Palyam viruses (Mellor *et al*, 2000). Of these arboviruses, bluetongue virus (BTV) and African horse sickness virus (AHSV) are classed as Office International des Epizooites (OIE) notifiable diseases, due to the international significance of the diseases they cause and their ability to spread rapidly (Mellor *et al*, 2000). Bluetongue virus and Schmallenberg virus are the most recent *Culicoides* species borne diseases of livestock to be recorded in the UK.

1.2.1 Bluetongue virus

Bluetongue virus is an Orbivirus, family Reoviridae, that infects domestic and wild ruminants and can have devastating effects on animal welfare and trade in some countries (Mellor *et al*, 2000, Wittmann and Baylis, 2000). The morbidity and mortality rate in susceptible flocks of sheep has been reported as greater than 70 % in some cases (Jennings and Mellor, 1988). Disruption to trade of animals and animal products

has been estimated to cause annual losses of $125 million in the United States during the 1990s (Tabachnick, 1996). Until recently, 24 different serotypes had been identified (Mellor *et al*, 2000, Purse *et al*, 2005). However, in 2008 a virus was detected in goats from Switzerland (Toggenburg virus – TOV) that has now been provisionally identified as a 25th BTV serotype (Hoffmann *et al*, 2008) and a 26th BTV serotype has been identified in Kuwait from a sample collected in 2010 (Maan *et al*, 2011). Bluetongue virus causes a disease with clinical signs including oedema, hyperaemia, pyrexia, coronitis, nasal discharge, excessive salivation and in severe cases death (Darpel *et al*, 2007). In susceptible sheep, such as the fine wool and mutton breeds common to Europe, morbidity and mortality may exceed 70 % (Mellor *et al*, 1983). The virus will replicate in all ruminants that have been investigated, although infection does not always manifest in a clinical disease (MacLachlan, 1994). Most transmission of BTV therefore occurs "silently" in disease-resistant host animals (Purse *et al*, 2005). Subclinically infected cattle form a “silent” reservoir, in most areas, developing prolonged viraemias that may last up to 100 days (Hourrigan and Klingspoorn, 1975). Bluetongue was first described in South Africa as malarial catarrhal fever of sheep in 1902 (Hutcheon, 1902). Cattle were not implicated as reservoir hosts for a further 30 years due to their less obvious clinical symptoms (Bekker *et al*, 1934). A decade later *Culicoides* species were found to be the likely vector of BTV (Du Toit, 1944). The name "bluetongue" came into use later, describing the cyanotic tongue which may occur in rare cases with severely affected sheep (MacLachlan, 2004).

The largest, longest and most costly series of BTV outbreaks in Europe began in the Mediterranean basin in 1998 (Mellor and Wittmann, 2002, Purse *et al*, 2005). Six strains of BTV (five serotypes: BTV-1, BTV-2, BTV-4, BTV-9 and BTV16) spread across 12 countries in southern Europe pushing 800 km further north than previously recorded (Purse *et al*, 2005, Saegerman *et al* , 2008). The northern expansion of *C. imicola* has been described as having a "baton effect", resulting in other Palaearctic *Culicoides* species being able to express vector competence for BTV through biting viraemic hosts (Mellor and Boorman, 1995). In August 2006, BTV was detected in northern Europe for the first time, where it rapidly spread across Belgium, France, Germany, Luxembourg and the Netherlands (Meiswinkel *et al*, 2008). This was a major event as the outbreak occurred 900 km further north than the latitudinal limits of previous European

incursions (Carpenter *et al*, 2009a). Although there is no overall total cost figure, it has been reported that a BTV-8 outbreak in the Netherlands in 2007 cost approximately $85 million, while a similar outbreak in France the same year was estimated to cost $1.4 billion (Tabachnick *et al*, 2008). The losses in the latter example were attributed to the inability to trade cattle on the international market. The trade in cattle is a very substantial industry in France (Tabachnick *et al*, 2008).

In 2006, another outbreak pushed BTV further north. The causal serotype was identified as BTV-8, a serotype that had not previously been found in Europe, which was shown to have a sub-Saharan origin (Maan, 2008). First detected in the Netherlands, BTV-8 spread to Belgium, Luxembourg, northern France and Germany (Mellor *et al*, 2009). The virus successfully over wintered and in September 2007, the first case of bluetongue (BTV-8) was identified in the UK at a farm in Suffolk (Szmaragd *et al*, 2010). As BTV-8 spread across England, approximately 150 premises were affected resulting in the implementation of restrictions on animal movement and a large scale vaccination programme during 2008 (Mellor *et al*, 2009, Szmaragd *et al*, 2010). Since the UK has the largest population of sheep in Europe (c. 34 million) the outbreak of BTV could have resulted in severe loses if not swiftly and effectively controlled (Carpenter *et al*, 2006). Following a successful vaccination campaign and cooler weather the last detected case of bluetongue in the UK was recorded in 2008 (post-import testing)(DEFRA, 2010). However, with bluetongue still circulating in areas of northern Europe, it remains a threat. Evidence suggests favourable winds can blow potentially infected midges more than 300km (Purse *et al*, 2005). Modelling of meteorological data has shown that weather conditions were suitable to carry air-borne infected *Culicoides* species from the Ostend area of Belgium to the east of England (Suffolk) around the 4 - 5th of August 2007, which suggests this was the entry route for BTV into the UK (Gloster *et al*, 2008). Additionally, during the northern Europe outbreak, temperatures reached a record high, providing ideal conditions for the replication and transmission of BTV-8 by a number of *Culicoides* species (Meiswinkel *et al*, 2008). During 2008, it was also noted that a different strain, BTV-1, expanded north from Spain reaching Brittany, in northern France (Wilson and Mellor, 2009).

Culicoides impunctatus is by far the most well studied of the midge species (Mordue (Luntz) and Mordue, 2003). *Culicoides impunctatus* can occur in high population densities in Scotland, often in close proximity to large numbers of sheep, thereby creating an ideal situation if it were to vector pathogens. However, it is unlikely to play a major role in BTV transmission in most of northern Europe (Carpenter *et al*, 2008a). Laboratory studies have shown that only a small proportion (< 1%) of *C. impunctatus* are capable of supporting the replication of BTV (Jennings and Mellor, 1988, Carpenter *et al*, 2006). Additionally, this species is found in the cooler northern regions of the continent and is generally not associated with areas of livestock-rearing (Boorman, 1986). Members of the *C. obsoletus* and *C. pulicaris* groups were found in catches at farms where *C. imicol*a was absent and their presence correlated with BTV outbreaks in Bulgaria and Italy (Torina *et al*, 2004, Purse *et al*, 2006). Previous studies have shown successful replication of BTV-4 and BTV-9 in wild caught *C. obsoletus* and *C. pulicaris* group midges following laboratory inoculation (Jennings and Mellor, 1988, Carpenter *et al*, 2006). Populations were shown to vary in their susceptibility to BTV-9 infection. However, the most susceptible populations were found to have infection rates that may exceed the levels noted for *C. imicola*, the primary vector associated with BTV outbreaks in southern Europe (Carpenter *et al*, 2006). Therefore, there is evidence that members of the *C. obsoletus* and *C. pulicaris* groups (Table 1.1) were the vectors of BTV in the UK in 2007 and will be the major vectors of BTV in the future in the UK and northern Europe (Carpenter *et al*, 2008a).

Table 1.1: Member species of the *Culicoides obsoletus* and *Culicoides pulicaris* groups, likely vectors of BTV in the UK.

Species Groups	
Culicoides obsoletus sensu lato	***Culicoides pulicaris sensu lato***
Culicoides obsoletus sensu stricto Meigen	*Culicoides pulicaris sensu stricto* Linnaeus
Culicoides dewulfi Goetghebuer	*Culicoides punctatus* Meigen
Culicoides chiopterus Meigen	
Culicoides scoticus Downes & Kettle	

1.2.2 Schmallenberg virus

In Autumn, 2011, a new disease of dairy cattle was reported in the North Rhine-Westphalia, Germany and in the Netherlands with clinical signs including fever,

diarrhoea and decreased milk production (Hoffman *et al*, 2012). Blood samples from a farm near the city of Schmallenberg (Germany) were analysed resulting in the detection of a novel orthobunyavirus in cattle (SBV) (Hoffman *et al* 2012). It was noted that members of the *Bunyaviridae*, widely distributed in Africa and Asia, are predominantly transmitted by *Culicoides* species and mosquitoes (Hoffman *et al* 2012). In early 2012 SBV was detected in the UK, in the south and east of England, following testing of deformed lambs. The virus subsequently spread and has affected over 278 farms to date (DEFRA 2013).

The outbreaks of BTV and the emergence of SBV have once again highlighted the need for better understanding of UK *Culicoides* species host location and selection.

1.2.3 African Horse Sickness Virus (AHSV)

In addition to the threat potentially posed by another UK bluetongue incursion, African horse sickness virus may also be a cause for concern, as it has been vectored by the same *Culicoides* species as BTV previously (*C. imicola*) (Mellor and Boorman, 1995). African horse sickness virus, also an Orbivirus like BTV, exists as nine antigenically distinct serotypes, causes an infectious, non-contagious disease of equids, and is prevalent in Africa, Asia, Australia, the Middle East, Europe and, Central and South America (Mellor, 1990, Mellor *et al*, 2000, Venter *et al*, 2000). African horse sickness is characterized by impaired respiratory and circulatory function and serious effusion and haemorrhage in various organs and tissues (Mellor *et al*, 2000). In susceptible populations, mortality rates often exceed 90% (Mellor, 1994). *Culicoides imicola* is considered the only field vector of AHSV, however, *Culicoides bolitinos* Meiswinkel has also been shown to be susceptible in the laboratory (Mellor, 1995, Venter *et al*, 2000).

1.3 Haematophagous insects and host location

Haematophagous insects require the ability to locate blood meals (from hosts) for energy and to gain the nutrients for egg production. Sutcliffe (1986), with reference to the black fly (Diptera: Simuliidae), described host location as any action or event in the

insects' adult life that allows it to respond to, or brings it into closer proximity to, a host. The process of host location involves a range of visual, physical and olfactory stimuli (Allan *et al*, 1987, Cork, 1996). Furthermore, host location is complicated by host mobility and their ability to defend themselves (Gibson and Torr, 1999). Investigations into the role of vision in *Culicoides* species host location are limited (Bhasin, 1996, Bishop *et al*, 2008). Bhasin (1996) provided the first quantitative record of visual responses by *Culicoides* species to targets. The study found that *C. impunctatus* were significantly more attracted to a two-dimensional solid black rectangular target compared with white or striped (black and white) targets. It was suggested that the contrast between the target and the background was an important factor in eliciting visual based orientation responses. Additionally, it was suggested that landings were inhibited by the reflective white surface which was not representative of a host (Bhasin, 1996). A more recent study in Australia baited a cattle shaped target with carbon dioxide (CO_2) and racemic 1-octen-3-ol and covered it in sticky traps to investigate attraction in *C. brevitarsis* (Bishop *et al*, 2008). Catches were greatest along the ridge line of the back of the cattle shaped target, suggesting that contrast with the background was important, similar to the results of Bhasin (1996). However, despite the potential role of visual contrast discrimination at short range, long range olfactory signals called semiochemicals (behaviour and physiology-modifying chemicals) are believed to be more important during host location (Cork, 1996, Takken and Knols, 1999, Mordue (Luntz) and Mordue, 2003).

Semiochemicals are chemicals involved in the transmission of a message between organisms (Law and Regnier, 1971). Pickett *et al* (1998) stated that an insect relies upon detection of semiochemicals to provide information about the physiological state and suitability of a host. Semiochemicals can be divided into pheromones (intraspecific) and allelochemicals (interspecific) based on their effects within or between species interactions (Norlund and Lewis, 1976). Allelochemicals can be further divided depending on the benefits for the emitting and receiving organisms resulting in, kairomones (beneficial to the receiver), allomones (beneficial to the emitter) and synomones (beneficial to both the emitter and receiver)(Norlund and Lewis, 1976). Additional divisions have been suggested based on the induced response of the receiving organism, such as foraging kairomone and aggregation kairomone,

however the understanding of the concept of a kairomone as beneficial to the receiving organism should be sufficient for this study (Ruther *et al*, 2002). Therefore, host location, via olfaction (kairomones), has potential to be exploited through the identification and development of host-derived semiochemicals slow release formulations that can be used in traps, or by the discovery of host-masking repellent semiochemicals (Cork, 1996, Mordue (Luntz) and Mordue, 2003, Pickett *et al*, 2010, Pickett *et al*, 2012).

Like most haematophagous insects, *Culicoides* species have a well-developed olfactory system and use their antenna and maxillary palps to detect semiochemicals (long and short range) as well as visual cues (short range) to gain information on location and suitability of a host (Gibson and Torr, 1999, Mordue (Luntz) and Mordue, 2003, Logan and Birkett, 2007). Additionally, evidence of abdominal olfactory receptors has recently been shown for *C. imicola* (Sollai *et al*, 2010). Semiochemicals, enter pores on the sensilla, present on the antennae and maxillary palps, and are transported across the lymph by odourant binding proteins to the olfactory neurone dendrites causing a change in membrane potential (McIver, 1982, Zweibel and Takken, 2004). The altered membrane potential allows an ion flux across it, creating a receptor potential or depolarisation of the receptor. It is this change in receptor potential that is measured in electroantennography (EAG) investigations. The magnitude of the receptor potential is related to the magnitude of the original stimulus. From the dendrite, the receptor potential to the cell body producing action potentials near the axon origin. The action potentials are rapid, lasting a few milliseconds and fire at different frequencies proportional to the magnitude of the receptor potential. Therefore, an odour molecule which is detected (e.g. a kairomone) is elicits electrical signals that can be processed by the central nervous system of the insect, resulting in a behavioural response (Hansson, 2002, Zhou, 2004, Zweibel and Takken, 2004).

In terms of behaviour, four distinct steps in the process of haematophagy in insects have been described: appetitive search; activation and orientation; attraction, and alightment and probing (Hamilton and Hurd, 2002). Gibson and Torr (1999) suggest two further scenarios for the first step in the haematophagy process of insects. Firstly, flight activation, whereby a stationary insect encounters host stimuli due to increased proximity of the host or when wind direction changes and carry volatile kairomones to

the insect. The second scenario, referred to as ranging behaviour, describes the detection of host stimuli by the insect during flight. In wind of variable direction, the insect flies upwind or downwind to increase the likelihood of encountering a host. In wind of a constant direction, flying across-wind increases the likelihood of detecting host stimuli (Knols and Meijerink, 1997, Gibson and Torr, 1999). Chemicals emanating from the body of a host are believed to form a continuous odour plume, with intermittent pockets of exhaled breath volatiles (Cardé, 1996, Geier *et al*, 1999). Upon detection of a host stimulus the insect is activated and host location behaviour is initiated (Hamilton and Hurd, 2002). Upwind flight and orientation involving odour-mediated anemotactic, optomotor anemotactic, klinokinetic and orthokinetic responses then occur (Gibson and Torr, 1999, Hamilton and Hurd, 2002). Attraction occurs as the insect moves towards the host. As the distance to the host decreases, other stimuli, such as visual cues, convective body heat or short range olfactory cues provide the insect with additional information, and may influence alightment. Following alightment selection of a feeding site is made and blood feeding begins (Hamilton and Hurd, 2002).

1.4 Olfactory stimuli

Due to their small size, and the difficulty in establishing laboratory colonies, investigations into olfactory cues for *Culicoides* species are rare compared to other Diptera. Host derived kairomones can be divided into three main categories: exhaled breath, skin emanations and urine (Takken, 1991).

Of the chemicals present in exhaled breath, carbon dioxide is considered an important kairomone for haematophagous insects acting as a universal activator/attractant (Gibson and Torr, 1999). Brown *et al* (1951) described CO_2 as an activator rather than an attractant. Activators prime insects for detection of other host volatiles, and often enhance responses (attraction) of the insect when presented with other stimuli (Brady *et al*, 1997, Gibson and Torr, 1999, Mordue (Luntz) and Mordue, 2003). Nelson (1965) first showed attraction of *Culicoides* species to CO_2 by collecting *Culicoides sonorensis* Wirth and Jones in a modified CO_2 baited mosquito trap. In the field, increased trap catches of *C. furens* and *Culicoides melleus* Coquillett were found with increasing CO_2

concentrations, in Georgia, USA (Kline *et al*, 1994). This same positive relationship between CO_2 concentration and behavioural response was reported for *C. impunctatus* in a wind tunnel (Bhasin, 2000a). Similarly, in an electrophysiological study on *C. furens*, increasing EAG responses were noted with increasing CO_2 concentration (Grant and Kline, 2003). The authors also noted that *C. furens* detected CO_2 at a lower concentration threshold than *Stegomyia (Aedes) aegypti* Linnaeus (Grant and Kline, 2003). Carbon dioxide was also shown to significantly increase trap catches of *Culicoides histrio* Johannsen and *Culicoides subimmaculatus* Lee and Reye compared with unbaited traps in Australia (Ritchie *et al*, 1994). In contrast, CO_2 baited traps did not significantly increase the trap catches of *C. obsoletus* during a study in California, USA (Mullens *et al*, 2005). Similarly, few *C. obsoletus* were caught in a CO_2 baited trap during a field trial in England, UK (Harrup *et al*, 2012). Of particular interest is a study by Mullens and Gerry (1998) where they compared trap catches of *C. sonorensis* in traps baited with CO_2 released at a similar rate to calves with catches direct from the calves. Their data showed that six times more *C. sonorensis* were caught on the calves suggesting that CO_2 alone does not reflect accurately the natural host.

Another well studied chemical component of exhaled breath is 1-octen-3-ol, identified from oxen breath (Vale and Hall, 1985). Y-tube olfactometer studies have shown that racemic 1-octen-3-ol, butanone, acetone and low doses of lactic acid significantly increase the relative attraction of *C. impunctatus* when compared with a solvent control (Bhasin *et al*, 2000a). Racemic 1-octen-3-ol and acetone were also found to significantly increase the relative attraction of *C. nubeculosus* in the Y-tube olfactometer (Bhasin *et al*, 2000a). Wind tunnel studies found racemic 1-octen-3-ol and acetone, presented in combination with CO_2, significantly increased the relative attraction of *C. impunctatus* compared with a solvent control (Bhasin *et al*, 2000b). Acetone has been identified in oxen breath and human breath (Krotoszynski *et al*, 1977, Vale and Hall, 1985). Sticky traps baited with racemic 1-octen-3-ol, acetone or a phenolic mixture were shown to significantly increase trap catches of *C. impunctatus* compared with an unbaited sticky trap (Bhasin *et al*, 2001). Phenols are commonly found in the urine of hosts (Sastry *et al*, 1980, Hassanali *et al*, 1986, Bursell *et al*, 1988).

Other studies with racemic 1-octen-3-ol have shown more variable results. In the USA, significantly greater numbers of *C. furens* were caught in an unlit Centers for Disease

Control (CDC) trap baited with racemic 1-octen-3-ol alone or in combination with CO_2 (200 ml min^{-1}) compared with CO_2 (200 ml min^{-1}) alone (Kline *et al*, 1994). Conversely, *Culicoides hollensis* Melander & Brues and *C. melleus* showed no significant difference in numbers caught for racemic 1-octen-3-ol in combination with CO_2 (200 ml min^{-1}), or a significant decrease in numbers caught for racemic 1-octen-3-ol alone, compared with CO_2 (200 ml min^{-1}) alone (Kline *et al*, 1994). In Australia, adding racemic 1-octen-3-ol and CO_2 (200 ml min^{-1}) in combination to encephalitis vector surveillance (EVS) traps significantly increased the catch of *Culicoides molestus* Skuse compared with CO_2 alone (Ritchie *et al*, 1994). Another Australian study used two-dimensional cattle shapes with sticky traps and showed that *C. brevitarsis* was in significantly greater numbers using racemic -1-octen-3-ol in combination with CO_2 (released from dry ice at 700 – 750 ml min^{-1}), but not on its own (Bishop *et al*, 2008). A summary of the known semiochemicals for *Culicoides* species has been provided (Table 1.2).

In addition to volatile chemicals being potentially released from the breath, skin and urine, it has also been shown that volatile chemicals can be released from bacterially infected myiatic lesions in sheep and these may need to be taken into consideration when studying whole animal odours (Khoga *et al*, 2002). Blowflies (*Lucilia cuprina* Wiedmann*, Lucilia sericata* Meigen) are attracted to the volatiles released by the bacteria present on the larvae that infest the myiatic lesions (Eisemann and Rice, 1987, Khoga *et al*, 2002).

Although semiochemicals have been identified for some *Culicoides* species in the past (Table 1.2), little is known about the host-location cues utilised by potential BTV, SBV and AHSV vectors in the UK (*C. obsoletus* and *C. pulicaris* groups).

Table 1.2: Reported semiochemicals that elicited behavioural activity in *Culicoides* species

Chemical stimulus	*Culicoides* spp.	Reference
(*E*)-2-Nonenal	*C. impunctatus*	Logan *et al*, 2009
6-Methyl-5-hepten-2-one	*C. impunctatus*	Logan *et al*, 2009
α-isomethyllionone	*C. impunctatus*	Logan *et al*, 2009
Acetone	*C. impunctatus, C. nubeculosus*	Bhasin *et al*, 2000a; Bhasin *et al* 2000b; Bhasin *et al*, 2001
Butanone	*C. impunctatus*	Bhasin *et al*, 2000a
Carbon dioxide	*C. impunctatus, C. furens*, *C. stellifer*, *C. mississippiensis, C. variipennis*	Kline *et al*, 1990; Bhasin, 1996; Grant & Kline, 2003; Kline *et al*, 1990; Kline *et al*, 1994; Takken & Kline, 1989; Mullens, 1995; Nelson, 1965; Weiser-Schimpf *et al*, 1991
Decanal	*C. impunctatus*	Logan et al, 2009
Geranylacetone	*C. impunctatus*	Logan et al, 2009
Isothiocyanates: allyl, butyl, phenyl, 2-phenyl	*C. impunctatus*	Blackwell *et al*, 1997
Lactic acid	*C. impunctatus*, *C. furens*	Bhasin *et al*, 2000a; Kline et al, 1990
Menthol	*C. impunctatus*	Logan et al, 2009
Methyl salicylate	*C. impunctatus*	Blackwell *et al*, 1997
Naphthalene	*C. impunctatus*	Logan et al, 2009
Phenols	*C. impunctatus*, *C. furens*	Bhasin *et al*, 2001; Kline *et al*, 1990
Racemic 1-octen-3-ol	*C. impunctatus, C. nubeculosus, C. furens, C. molestus, C. brevitarsis*	Bhasin *et al,* 2000a,b; Bhasin *et al*, 2001; Bishop *et al*, 2008; Blackwell *et al*, 1996; Logan, 2006; Kline et al, 1990; Kline *et al*, 1994; Takken & Kline, 1989; Ritchie *et al*, 1994

1.5 Differential attraction of haematophagous insects to hosts

Culicoides species can show specificity towards certain host species and host individuals. Blackwell *et al* (1995) demonstrated this concept for *C. impunctatus* based on enzyme linked immunosorbent assay (ELISA) analysis of bloodmeals, however, they did not relate this to host odours. *Culicoides nubeculosus* has been shown, using EAG, to respond differentially to animal solvent extracts (calf, deer, sheep, pony and water buffalo), while *C. impunctatus* respond differentially to the same animal extracts in a Y-tube olfactometer (Mands *et al*, 2004). It was also found that *C. impunctatus* and *C. pulicaris* showed differential attraction to a range of animal extracts when added to Mosquito Magnet® Pro traps in the field (Mands *et al*, 2004). Recently, differential attraction of *C. impunctatus* has also been shown to odours entrained from individual human beings (Logan e*t al*, 2009). Differential attraction has also been shown for horn fly loads in cattle herds (Jensen *et al*, 2004). Horn fly loads were determined for individual cattle and it was shown that moving specific cattle between different herds could alter the overall horn fly load of the herd. It was also determined that racemic 1-octen-3-ol and 6-methyl-5-hepten-2-one, isolated from cattle air entrainment extracts, and applied to heifers in slow-release formulations could reduce the fly load of individual animals (Birkett *et al*, 2004). As well as *Culicoides* species, differential attraction has also been shown many times for mosquito species (Schreck *et al*, 1990, Brady *et al*, 1997, Qiu *et al*, 2004, Logan *et al*, 2008). Interestingly, Logan *et al* (2008, 2009) also found a potential masking effect of 6-methyl-5-hepten-2-one, along with geranylacetone, on the attraction of *C. impunctatus* and *St. aegypti* to human beings.

Logan (2006, summarised in Logan *et al*, 2009) provided the first quantitative evidence that increases in certain naturally produced chemicals can mask and interfere with host location in *C. impunctatus*. However, this concept has not been investigated for other *Culicoides* species, and identification of specific chemicals that affect host selection for *C. obsoletus* and *C. pulicaris* groups could lead to improvements in the monitoring and control of these vectors of BTV and SBV.

1.6 Control and monitoring of *Culicoides* species

Options for *Culicoides* species control were reviewed by Carpenter *et al* (2008b) and included the use of larvicides, larval habitat destruction, adulticides, stabling of animals and the use attractant traps and repellents. The effective use of larvicides or larval habitat destruction is unlikely to be effective because of the limited knowledge of *Culicoides* species breeding sites. Similarly, mass use of insecticides (adulticides) is unlikely to be effective because of lack of knowledge of resting sites (Carpenter *et al*, 2008b). Stabling of animals may confer some protection against *Culicoides* species, however, it requires sufficient housing for the animals. Additionally, *Culicoides* species will enter farm buildings in search of a host. Therefore, semiochemical baited traps and repellents provide the most likely avenue of research for improved monitoring and control of *Culicoides* species.

Removal trapping has been investigated using traps baited with CO_2 and racemic 1-octen-3-ol in Florida, USA, with successful reductions in *Culicoides* species populations reported (Day *et al*, 2001, Logan *et al*, 2010). However, a separate trial, also in Florida, found inconsistent results when trying to reduce *Culicoides* species populations using traps baited with CO_2 and a mixture of racemic 1-octen-3-ol:3-propylphenol:4-methylphenol (Cilek and Hallmon, 2005).

A working group on vectors at the third OIE symposium on bluetongue recommended UV light downdraft suction type traps, namely the Onderstepoort Veterinary Institute (OVI) trap, as the "gold standard" monitoring tool for *Culicoides* species populations (Mellor *et al*, 2004). However, recently, the OVI light suction trap was shown to significantly underestimate numbers of *C. chiopterus* and *C. dewulfi* (part of the *C. obsoletus* group) compared with *Culicoides* species collected in a sheep baited drop trap (Carpenter *et al*, 2008a). The authors were not able to determine the underlying reason for this underestimation, however, they noted that activity levels of *C. chiopterus* were similar to other species collected (including *C. obsoletus*, *C. scoticus* and *C. dewulfi*). Conversely, *C. pulicaris* was found to comprise 5.2% of the light trap catches, despite none being found in the sheep baited drop trap (Carpenter *et al*, 2008a). In contrast, a study comparing five different types of light trap (Onderstepoort, Rieb, Pirbright, BG-Sentinel and mini-CDC) in South Africa found that the OVI trap

collected nearly twice as many *C. imicola* compared with the next best trap (Venter *et al,* 2009). Furthermore, preliminary trials directly comparing drop-trap catches on sheep with Mosquito Magnet® Pro™ and delta trap designs using semiochemicals (CO_2 and racemic 1-octen-3-ol) showed that the baited delta traps caught significantly lower numbers of *C. obsoletus* group midges. A similar study in the USA using CO_2-baited suction traps also found an underestimation of *C. obsoletus* when compared to insects obtained from the belly region of horses (Mullens *et al*, 2005). More recently a study in Spain showed an underestimation of numbers of *C. obsoletus* and *C. parroti* when comparing CO_2-baited traps or UV light traps with collections direct from sheep (Gerry *et al*, 2009). The CO_2-baited trap and the UV light trap caught two and 16 *C. obsoletus s. s.* respectively, compared with 313 directly aspirated from sheep (Gerry *et al*, 2009).

From the literature reviewed, it would seem that light alone, CO_2 alone or CO_2 in combination with racemic 1-octen-3-ol provides insufficient attractive stimuli for these species, most likely as the stimuli do not reflect accurately those encountered from a host. Other chemicals may be missing, that may attract *Culicoides* species if presented in the correct mixture and ratio. These limitations highlight the requirement for further research into attractants for improved monitoring of the midges that transmit BTV and AHSV to attempt to reduce the potential impact of the disease. A semiochemical-based monitoring and surveillance trapping system is likely to more accurately mimic a host, and therefore increase catches of host-seeking populations compared with current monitoring methods. Identification of suitable semiochemicals and optimization of such a surveillance method warrants urgent attention (Carpenter *et al*, 2008a).

1.7 Aims and objectives

The aim of this study was to investigate the role of volatile semiochemicals in host location and selection by UK *Culicoides* species biting midges. It was hypothesised that midges from the *C. obsoletus* and *C. pulicaris* groups were attracted to host species/breeds, as a consequence of qualitative and quantitative differences in semiochemical cues. Additionally, it was hypothesised that attraction was, in part, mediated by antennal perception of specific host/non-host cues or differing ratios of ubiquitous compounds.

1.7.1 Objectives

1. To investigate potential host volatile collection methods and collect sufficient volatile extracts for further investigation into the role of semiochemicals in *Culicoides* species host location (Chapter 2).

2. To demonstrate behavioural activity of the host volatiles in the field and laboratory using a Y-tube olfactometer with wild caught *Culicoides* species and laboratory-reared *C. nubeculosus* (Chapter 3).

3. To identify and quantify electrophysiologically-active compounds from host volatile extracts using coupled gas chromatography-electroanntenography (GC-EAG) and coupled gas chromatography-mass spectrometry (GC-MS) with wild caught *Culicoides* species and laboratory-reared *C. nubeculosus*. These chemicals are likely to play a role in host location by *Culicoides* species (Chapter 4).

4. To investigate behavioural responses of *Culicoides* species in the laboratory to confirmed EAG-active chemicals using a Y-tube olfactometer (Chapter 5).

5. To produce slow release formulations and determine release rates of semiochemicals which reflect those from natural hosts for identified EAG-active chemicals and investigate behavioural activity of *Culicoides* species in the field to individual chemicals and blends using CDC traps (Chapter 6).

The majority of work presented in this thesis was performed by the author. However, a small section of the work was carried out collaboratively with another PhD student, Andrew Hope, who was based at The Pirbright Institute. Any work presented here that was not done by the author is clearly described.

Chapter 2: Collection of host (sheep) volatiles – development of methods

2.1 Introduction

During field investigations of host attractiveness to *Culicoides* species it is possible to use the host itself (e.g. cattle, sheep, human) to measure biting rate or attraction of insects of interest. For example, direct aspiration of *Culicoides* species from sheep, or their collection via drop trapping have been used successfully in the past (Carpenter *et al*, 2008a, Gerry *et al*, 2009, Harrup *et al*, 2012, Viennet *et al*, 2011). However, these methods present visual and thermal cues in addition to volatile cues which means it is difficult to determine which cues are most important in host location. For investigations of volatiles it is preferable to remove visual and thermal cues to determine the true role of chemicals in host location. This has been attempted for studies on tsetse flies. Rayaisse *et al* (2010) placed hosts (pigs, cattle or humans) in Polyvinyl Chloride (PVC) tents and vented the air from the tent to a nearby (15 m away) trap, to investigate attraction of *Glossina* species tsetse flies. This potentially removed the effect of the visual stimuli by disassociating it from the volatile cues. Tchouassi *et al* (2012) baited traps with 19 g of fresh fleece (cut daily from the hosts) to investigate attraction of mosquito vectors of Rift Valley Fever (RVF). This method removed both the visual and thermal stimuli, however, it also potentially reduced the volatile stimuli, since only volatiles from the fleece were now present, and therefore may not represent accurately the host for the purposes of effectively eliciting attraction. All of these methods can be used to investigate attraction of insects to a host, however, without collection of the volatiles being released there is no way of further analysing those stimuli and discerning their components. Therefore, in order to study host-derived volatiles in the laboratory, it is necessary first to obtain a representative sample from the host.

A variety of techniques and methods have been reported in the literature, all with potential strengths and weaknesses. Two of the most regularly used techniques are solvent extraction and air entrainment. Solvent extraction involves washing or soaking the sample (host, host hair/fleece) in solvent thereby transferring chemicals from the host into the solvent extract for further analysis. Solvents may also be added to (ideally

inert) cloths or pads in order to wipe over areas of the host. This technique will transfer all the solvent soluble chemicals (volatile and non-volatile) from the host to the extract, which results in the presence of chemicals that do not necessarily play a role in odour-mediated host location by the insects in question. Despite this, the technique has been used successfully to demonstrate and rank attraction of *Culicoides* species to host hair solvent extracts in the field (Mands *et al*, 2004). From a range or hosts (calf, red deer, sheep, pony and water buffalo), 22 g of hair was soaked in hexane solvent (20 ml) for 7 days at 5 °C, then concentrated under a nitrogen flow by 50 %, yielding an extract of 2.2 g hair ml^{-1}. Addition of 1 ml of the extract(s) to Mosquito Magent® Pro™ traps resulted in a significant increase in mean trap catch of *Culicoides* species, compared with the control, for the water buffalo extract. However, no statistically significant differences were noted for the other host solvent extracts.

Another more common technique is air entrainment (often referred to as headspace collection), whereby volatile chemicals are collected onto absorbent polymers (e.g. Porapak™ Q or Tenax® TA) from a host source (Millar and Sims, 1998). In the laboratory this technique is often used for plants, insects or small animals, and it is likely that ratios of chemicals collected will be similar to those found in nature, since it is possible to keep the host alive during the entrainment and therefore the extract obtained should reflect the natural state of the host. It should be noted though that air entrainment provides a snapshot of the volatiles released over a given time period, and does not provide information on dynamic changes in volatile odour profiles. Unlike the solvent extraction method, with air entrainment only the volatile chemicals produced from the host are collected, therefore, contamination should be minimal (Agelopoulos *et al*, 1999). The technique requires a closed system so airflow can be controlled and air pulled out through the absorbent polymer. For small odour sources glass vessels or inert oven bags can be used (Stewart-Jones and Poppy, 2006), whereas for larger sources, such as humans, thermal survival bags have been successfully used (Logan, 2006). The human derived extract was subsequently shown to contain EAG-active chemicals for *C. impunctatus*, with behavioural responses shown to some of those chemicals in the laboratory (Logan, 2006, Logan *et al*, 2009). Air flow rates pumped into (charcoal filtered) and pulled out of the vessel are matched (or with oven/survival bags, the air flow rate in is set slightly higher) to avoid pulling potentially

contaminated air in from outside of the system. The duration of the entrainment can be altered to allow for sufficient collections of volatiles. There are some drawbacks to this method, primarily size restrictions on the odour source. While it is possible to place an amiable human in a survival bag for a period of time, animal hosts present a greater challenge. Birkett *et al* (2004) successfully collected volatiles from Holstein-Fresian heifers by suspending the absorbent polymer tube above the animal when it was in a stall. Despite an imperfect (open) system, sufficient volatiles were collected and differences discovered between the host and control entrainment. Air entrainment of larger animals from a PVC (or similar) tent, as used by Rayaisse *et al* (2010) when venting host volatiles, might present a possible solution. Such entrainment techniques have not been used previously with sheep. In fact, almost no research has taken place to investigate sheep related volatiles with relevance to host location of insects. Sheep-related volatile research has primarily focussed on the removal of undesired volatiles from sheep wool for the textile industry (Lisovac and Shooter, 2003). Therefore, further investigation of sheep derived volatiles may result in discovery of chemicals that could be exploited to improve surveillance and control trapping of *Culicoides* species.

The aim of this chapter was to investigate possible host (sheep) volatile collection techniques and to obtain suitable extracts for further analysis of behaviour responses in *Culicoides* species (Chapter 3 and 5), electrophysiological activity (Chapter 4) and development of slow release formulations to improve trapping of wild *Culicoides* species vectors (Chapter 6).

Objectives:

- To investigate potential host volatile collection methods including:
    - Solvent extraction of sheep fleece using two solvents (redistilled diethyl ether and redistilled hexane)
    - Air entrainment of sheep fleece
    - Air entrainment of whole sheep

- To collect sufficient volatile extracts for further investigation into the role of semiochemicals in *Culicoides* species host location

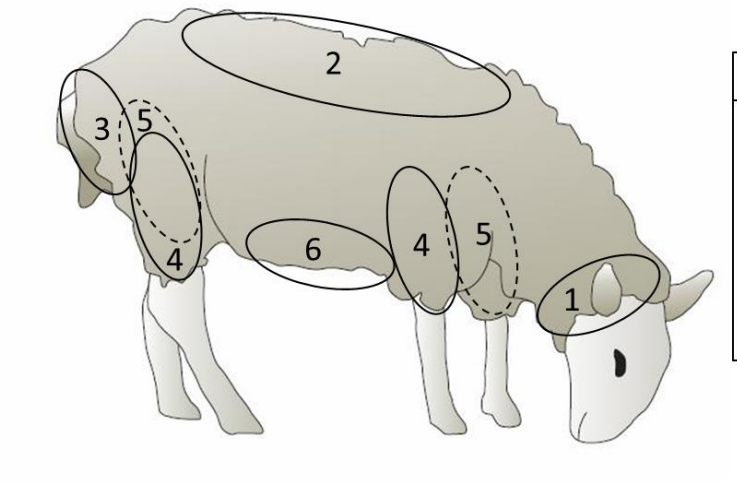
2.2 Materials and Methods

To determine the most appropriate volatile collection technique to provide relevant samples for further study, three different collection methods were investigated:

- solvent extraction of sheep fleece
- air entrainment of sheep fleece
- air entrainment of whole sheep

2.2.1 Sheep fleece

A whole sheep fleece (mature ewe, Texel/Charloais cross breed) was obtained from a local farm (Cross Farm, Harpenden, Hertfordshire, UK, 51°48’ 9.05’N; 0°19’ 58.5’W). The fleece was cut into sections, labelled, and sealed in seven oven bags (Sainsbury’s multi-purpose cooking bags, J Sainsbury plc, London, UK, approximately 250 g of fleece per bag) and stored at ambient room temperature (Figure 2.1).



ID	Samples
1	Neck
2	Back
3	Tail
4	Side/leg right
5	Side/leg left
6	Belly

Figure 2.1: Representation of a sheep showing sections of sheep fleece separated for experiments (250 g per bag).

2.2.2 Solvent extraction from sheep fleece

To determine the viability of extraction of chemicals from sheep fleece using solvents, two different solvents, redistilled diethyl ether (≥99.8 % pure, Sigma-Aldrich Co., St. Louis, MO, USA – redistilled at Rothamsted Research) and redistilled hexane (Petroleum ether fraction, 40 – 60 °C, Fisher Scientific Ltd., Leicestershire, UK – redistilled to collect hexane at Rothamsted Research, Hertofordshire, UK), were investigated. Sheep fleece (Bag ID: 4, side/leg right, Figure 2.1) was weighed (1 g) and placed in a glass vial (28.25 ml (S. Murray and Co. Ltd. Surrey, UK), to which one of the solvents (5 ml) was added. The experiment was replicated three times for each solvent. Controls consisting of solvent only were also set up. The vials were left for three hours then the solvent was recovered by pipette and filtered through a glass tube containing glass wool into a vial. A further 5 ml of redistilled diethyl ether or redistilled hexane solvent was passed through the glass wool and collected in a separate vial. The vials were stored at -20 °C prior to analysis.

2.2.3 Air entrainment from sheep fleece

Air entrainment was used to collect volatile chemicals from sheep fleece. The air entrainment kit consisted of pumps and flow meters which allowed control of a flow of air through the experimental equipment (Figure 2.2). Air was pumped via polytetrafluoroethylene (PTFE) tubing (1.5 mm internal diameter (ID) Alltech Associates, Camforth, Lancashire, UK), through an activated charcoal filter (BDH, 10-14 mesh, 50 g) to remove impurities and split by a Swagelok® T-junction (Swagelok®, Cleveland, Ohio, USA), before entering two 400 ml conical flasks. This was referred to as the "push" airflow and was set at 800 ml min^{-1}, resulting in 400 ml min^{-1} passing through each flask. A second pump was connected to two individual flow meters (both set at 400 ml min^{-1}) that "pulled" air via PTFE tubing (1.5 mm ID) from the conical flasks. Blank glass tubes (empty Porapak™ tubes) were connected to "pull" airflows and both airflows were turned on for two minutes to purge any contaminated air from the flasks. The blank glass tubes were then removed and the "pull" airflows were connected to Porapak™ Q polymer tubes (Porapak™ Q 50/80 mesh, 50 mg, Supelco analytical, Bellefonte, PA, USA) located in the top of the conical flasks. Sheep fleece (1

g, bag ID: 4, side/leg right, Section 2.2.1, Figure 2.1) was placed into the test conical flask. The experiments were run for three hours to match the duration of the solvent extraction from sheep fleece experiments. Following the experiment, the airflows were turned off and the Porapak™ Q polymer tubes recovered. The contents of the tubes were eluted using redistilled diethyl ether (750 µl) into pointed glass 1.5 ml Chromacol vials (Fisher Scientific UK Ltd. Leicestershire, UK) and stored at -20 °C prior to analysis. Three replicates of the experiment were completed. The glassware was cleaned using distilled water and detergent (Teepol multiple purpose detergent, Teepol (UK), Kent, UK), before being rinsed with acetone and distilled water. The PTFE tubing was rinsed with ethanol (99 % pure, Sigma-Aldrich Co., St. Louis, MO, USA) and distilled water. All equipment was baken in an oven at 180 °C for 2 hours. Charcoal filters were baked in an oven at 150 °C for two hours whilst attached to a flow of clean nitrogen gas from a cylinder (BOC Industrial Gases, UK).

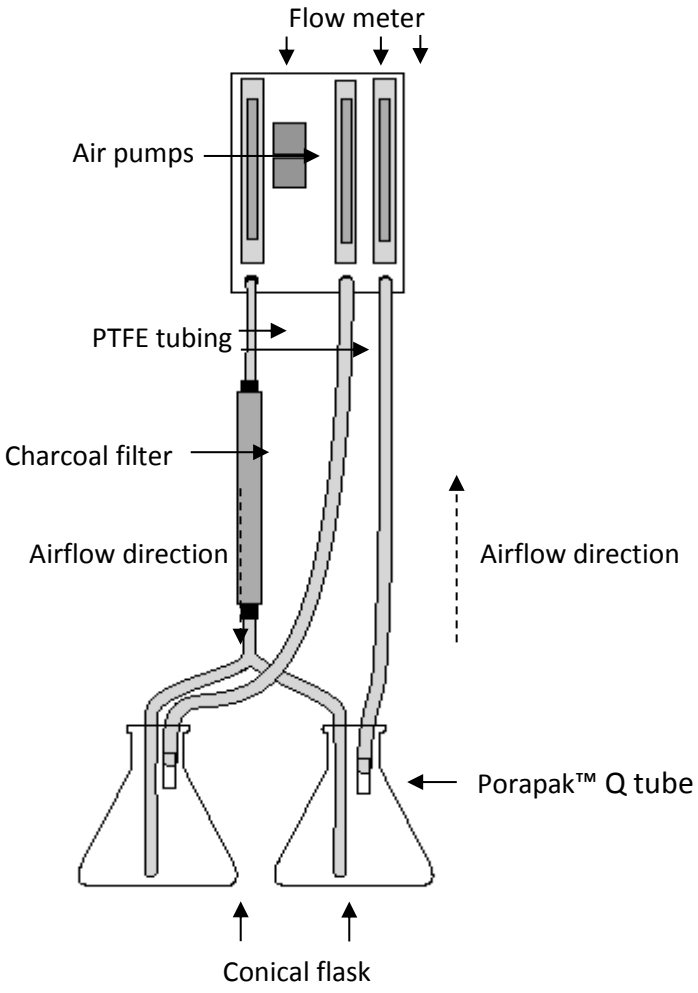


Figure 2.2: Air entrainment equipment used for the collection of volatiles from sheep fleece. Dashed lines show the direction of airflow.

2.2.4 Whole animal air entrainment

To collect volatile chemicals from whole animals a new piece of equipment was designed, developed and built (Rothamsted Research, UK), based on similar principles as laboratory air entrainment (Section 2.2.3), to allow for whole animal air entrainment (Figure 2.3).

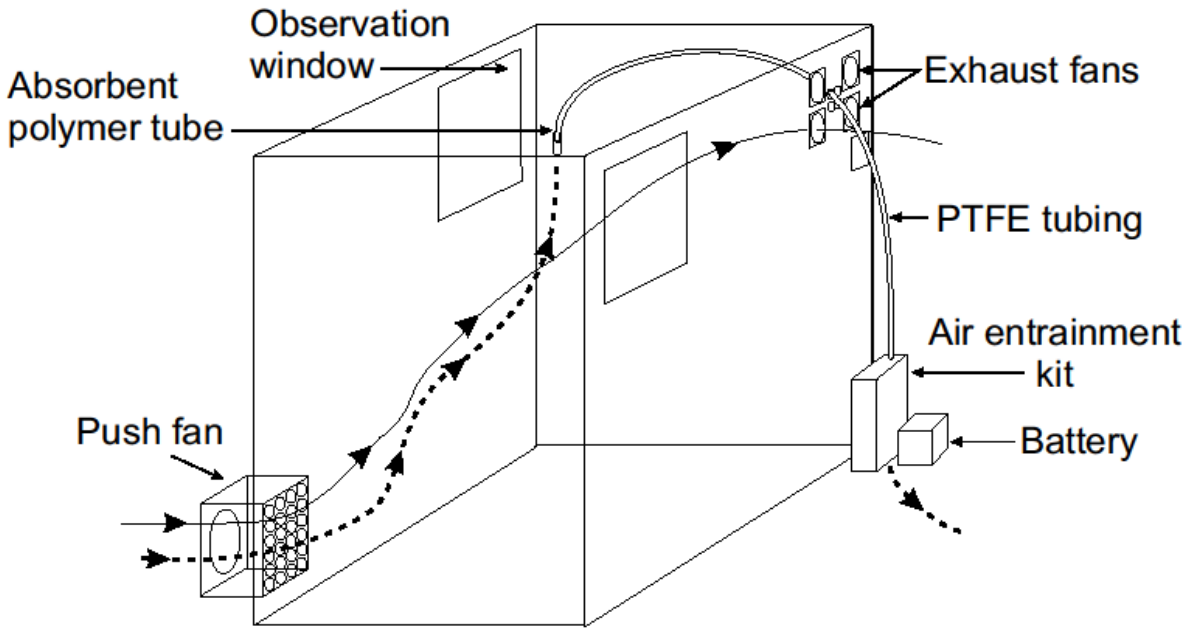


Figure 2.3: Whole animal air entrainment apparatus. The solid line with arrows shows the main airflow moving across the box from the push fan in the lower left to the exhaust fan in the upper right. The dashed line shows the airflow being sub-sampled, entering through the push fan, across the area where the animal(s) will be held and out through the adsorbent polymer tube connected, via PTFE tubing, to the "pull" pump of the entrainment kit.

A metal box measuring 1.82 m x 1.06 m x 1.64 m (length x width x height) was built consisting of a rolled-hollow-steel frame lined with 2 mm aluminium sheeting on the walls and ceiling, and a high strength five bar tread plate for the floor. A flat-pack design was created that allowed for easier transportation of the box from Rothamsted Research to appropriate field sites and for safer storage when not in use. The box was built to comply with animal housing and transport regulations (EU Regulation 1/2005 (Article 3 Annex I)). Two observation windows were located in the box, made from toughened Perspex, to allow for regular observations in order to maintain animal welfare. The doors at either end of the box (1.5 mm aluminium sheets) were designed

to slot into place and fasten with tensioned rubber clasps (RS Components Ltd, Corby, UK). Additionally, these rubber clasps allowed for the quick release and removal of the doors if required.

Air was pumped into the box by four 120 mm plastic computer fans (RS Components Ltd, Corby, UK) during the preliminary experiments through a coarse filter and a charcoal filter (296 mm x 296 mm x 20 mm Intersorb activated carbon panel, Vokes Air Group, Burnley, Lancashire, UK) to remove impurities. During the main volatile collection experiment the fan was changed to a mechanically throttled centrifugal "push" fan (single inlet, forward curved, 24 V, DC, ebm-papst UK Ltd, Chelmsford, Essex, UK) and flow rate through the carbon filter into the box was estimated at 73.5 $m^3 h^{-1}$. Air was exhausted out of the box (53.5 $m^3 h^{-1}$) via a 120 mm plastic computer fan (RS Components Ltd, Corby, UK) situated in the opposite far upper corner of the box, resulting in a positive pressure being maintained within the box and thereby preventing any non-charcoal filtered air from being drawn in. Situated near the exhaust fan were six ports formed using PTFE mat seals and Swagelok® fittings. These ports allowed for the attachment, via PTFE tubing, of air entrainment kits, situated outside the box, to Porapak™ Q or Tenax® TA (Tenax® TA 60/80 mesh, Supelco analytical, Bellafonte, PA, USA) adsorbent polymer tubes located inside the box. The entrainment kits drew air through each adsorbent polymer tube at 900 ml min^{-1}, taking a subsample of the total airflow from the whole animal air entrainment box. Following initial experiments, an additional larger pump (Cole-parmer, London, UK) was connected to two of the ports in the wall of the entrainment box and attached to two larger Porapak™ Q (250 mg) adsorbent polymer tubes. The pump drew air through the polymer tubes at approximately 5 l min^{-1} per tube, taking a subsample of the total airflow from the whole animal air entrainment box (1.15 %). All equipment was run from a leisure battery (12 V, sealed heavy duty, 85 amp hours, Alpha Batteries, Rochdale, UK). Batteries were swapped between control and test experiments and recharged overnight.

2.2.4.1 Collection of known volatile chemicals from slow release formulations

To determine if volatile chemicals could be collected on Porapak™ Q using the whole animal air entrainment box, slow release chemical formulations were produced that released at physiologically relevant levels based on previous experiments with cattle (personal communication: Dr. S. Y. Dewhirst). Chemicals were released individually by diffusion from either 1000 gauge polythene bag slow release formulations or 1500 gauge polythene bag slow release formulations. For 1000 gauge polythene bag formulations, 500 µl of chemical was added to cellulose sponge (3 mm thick, J Sainsbury plc, London) and heat-sealed into a 1000 gauge polyethylene tubing "bag" (A1 Packagings Ltd, London). For 1500 gauge polythene bag formulations, 500 µl of chemical was added to cellulose sponge and heat-sealed into a 500 gauge polyethylene tubing "bag". This slow release formulation bag was then heat-sealed into a 1000 gauge polyethylene tubing "bag". Slow release formulations were prepared for nonanal and decanal (Table 2.1).

Table 2.1: Details of chemical slow release formulation preparation for testing the whole animal air entrainment box

Chemical	Lure preparation
Nonanal	3 x 1000 gauge bag
Decanal	1 x 1500 gauge bag

Slow release formulations were suspended from a clamp stand in the centre of the whole animal air entrainment box during test experiments. Entrainments were carried out for 2 hours and 4 hours (non-concurrently) with Porapak™ Q tubes located near the fan and suspended centrally in the box. The box was cleaned thoroughly with 70 % ethanol after each set of replicates. These experiments were carried out at Rothamsted Research.

2.2.4.2 Collection of volatiles from whole animals (sheep) - Preliminary

The whole animal air entrainment box was dismantled and moved to a field site at a mixed dairy/sheep farm (near the former Institute of Animal Health (IAH) Compton

site, Berkshire, UK, 51˚30’ 25.09’N; -1˚16’ 30.12’W, Figure 2.4). It was erected in a field that had been used previously as an experimental site for trapping *Culicoides* species (Carpenter *et al*, 2008a). Control entrainments (empty box) lasting 2 hours and 4 hours concurrently, were conducted in the morning (08:00 – 12:00) and sheep entrainments (2 hours and 4 hours concurrently) were conducted in the afternoon (12:30 – 16:30). One Porapak™ Q and two Tenax® TA entrainment samples were collected for each time period.

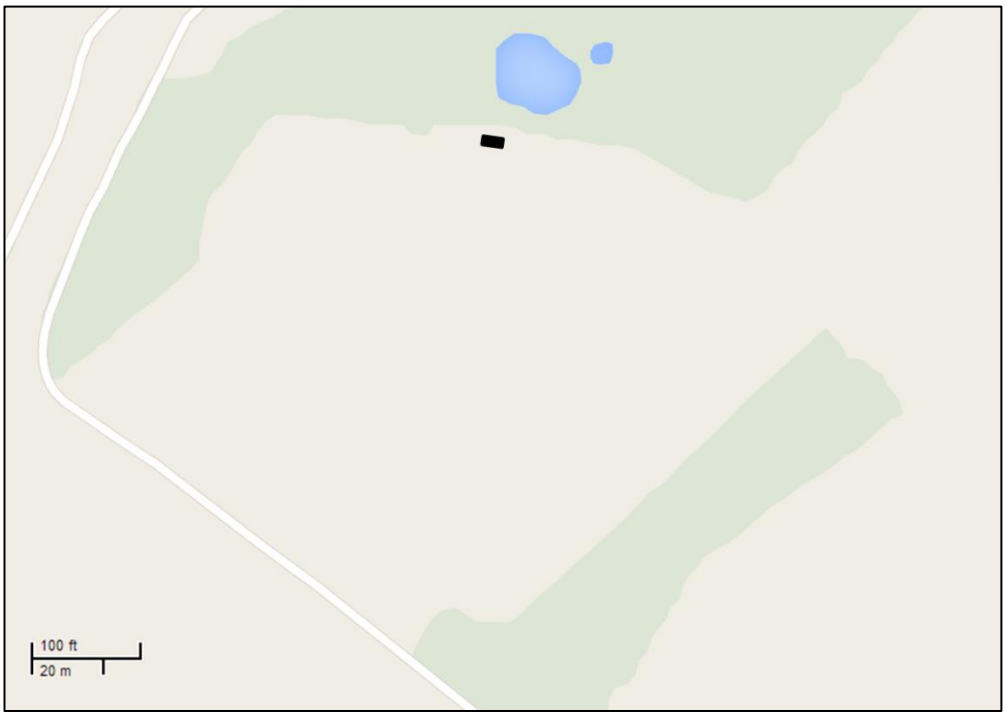


Figure 2.4: Map of the Compton field site used for preliminary collection of volatiles from sheep experiments and showing the position of the whole animal entrainment box (black rectangle).

Three adjoining fields contained six sheep (three female and three male) available for use in the experiments. During the control experiments the sheep were allowed to roam freely. Approximately 1 – 1.5 hours before the sheep entrainment experiments were due to begin the sheep were herded into a coral situated next to the whole animal entrainment box. Once the control experiment was completed and the absorbent polymer tubes had been replaced, two ewes were separated into the pen leading to the entrainment box. The sheep were selected so that each individual sheep was entrained, but as part of a pair, over the course of three replicates. The box door, and pen gate were opened and the sheep were ushered into the box. The door was

replaced and the entrainment kits were turned on to begin the sheep entrainment experiment. At the end of the experiment the entrainment kits were turned off and the door (opposite end to the pen) was opened releasing the sheep back into the field. The box was cleaned after each day by sweeping any dried or solid waste materials out of the box with a stiff bristled brush followed by thorough cleaning with water (mop and bucket). The box was then sprayed with 70 % ethanol and wiped down.

Temperature and humidity were monitored regularly during the experiment period and recorded via a data logger (TR-73U Thermo Recorder, T&D Corporation, Japan). Effects of stress on the animals can alter their odour profile and must therefore be minimised if possible. The previously mentioned regulations (EU Regulation 1/2005 (Article 3 Annex I) listed clear thresholds for temperature and relative humidity combinations which, if reached, indicated that the animal may suffer un-necessary stress (Table 2.2). At these combination points attempts to increase ventilation and decrease temperature were made. If such attempts were not immediately successful then the experiment was ended and the animals released.

Table 2.2: Limits of acceptable conditions inside the whole animal entrainment box based on animal housing/transport regulations (EU Regulation 1/2005 (Article 3 Annex I)).

Environmental condition thresholds	
Relative humidity (%)	Temperature (°C)
100	24
75	26
50	28
25	31
0	35

2.2.4.3 Collection of volatiles from whole animals (sheep) – main

Following initial field testing at the dairy/sheep farm site near Compton, the whole animal air entrainment box was dismantled and moved to a field site at a mixed cattle/sheep farm (Rushall Farm, near Bradfield, Berkshire, UK, 51°27’ 5.23”N; 1°9’ 28.44”W, Figure 2.5). The box was erected in a semi-sheltered area in the lower part of a field, and positioned to the right of a drop-trap and corral for sheep. Control entrainments (empty box) lasting 4 hours were conducted in the afternoon (approximately 12.45 – 16.45) and sheep entrainments were conducted in the evening

(approximately 18:00 – 22:00), designed to run for 3 hours before sunset and 1 hour after sunset. Two larger Porapak™ Q (250 mg) and two standard Tenax® TA (50 mg) entrainment samples were collected for each time period.

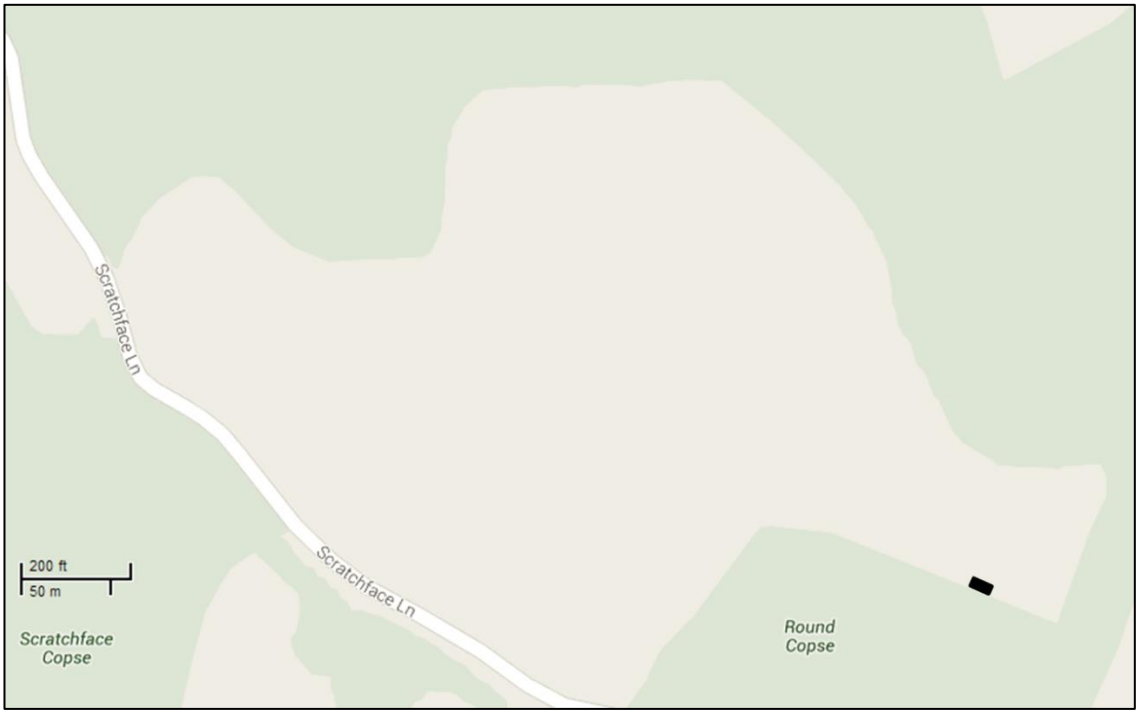


Figure 2.5: Map of the Rushall organic farm field site used for the main collection of volatiles from sheep experiments and showing the location of the whole animal entrainment box (black rectangle).

The field contained 12 female sheep (six “Pure” breed Hartline and six “Cross” breed Hartline/Suffolk) available for use in the experiments. The field also contained a herd of free roaming cattle (20 animals). The methodology proceeded as described above (Section 2.2.4.2) with a few exceptions. Three female sheep from one of the breeds were used for each test sheep entrainment and the breeds entrained were alternated daily. Both an entrainment kit and a larger pump were used in the experiments as described previously (Section 2.2.3). Air exhausted from the box was delivered to an unlit micro-CDC trap, running of a battery (6 V, 7 amp hours, Yuasa, Swindon, UK), via elephant trunking (ventilation duct, RS online, Northants, UK) to determine if the volatiles released within the box were attractive to *Culicoides* species (Chapter 3, Section 3.2.1). The box was cleaned following entrainments by scooping out any solid waste material using a dustpan and brush and disposing of it away from the entrainment area, prior to cleaning with water (mop and bucket). Charcoal panel filters

(Carbon C260 panel, 296 mm x 296 mm x 20 mm, Vokes Air, Burnley, Lancashire, UK) were replaced daily. Twenty replicates were completed, ten for each breed of sheep.

Temperature and humidity data were monitored as described above (Section 2.2.4.2).

2.2.5 Gas Chromatography and coupled GC-mass spectrometry

Sheep fleece solvent extraction samples and controls were analysed (1 µl, non-concentrated) using a high resolution Agilent Technologies, 6890N gas chromatography (GC) system fitted with a non-polar HP-1 (10 m, 0.53 mm internal diameter (ID), 2.65µm film thickness) column with a split/splitless injector, and a flame ionisation detector (FID). Hydrogen was the carrier gas. The following oven temperature programme was used: 30 °C hold for 2 min, then 10 °C min^{-1} to 250 °C, final hold for 55 min, total run time 79 min.

Air entrainment samples of sheep fleece and controls were concentrated to 50 µl under a gentle flow (10 ml min^{-1}) of high purity nitrogen (BOC Industrial Gases, UK). Samples were analysed (1 µl) using a Hewlett Packard, HP6890 GC system equipped with two GC columns. Column 1 comprised a non-polar HP-1 (50 m, 0.32 mm ID, 0.52 µm film thickness) column equipped with a cool-on-column (COC) injector and a non-polar fused silica pre column (1 m, 0.53 mm ID), whilst column 2 comprised a polar DB-WAX (30 m, 0.32 mm i.d., 0.5 µm film thickness) column with a COC injector and a non-polar fused silica (1 m, 0.53 mm ID) pre-column. Hydrogen was the carrier gas and both columns were fitted with FIDs. The following oven temperature programme was used: 30 °C hold for 0.5 min, then 5°C min^{-1} to 150 °C, hold for 0.1 min, then 10 °C min^{-1} to 230 °C, final hold for 35 min, total run time 67.7 min.

Whole animal air entrainment Porapak™ Q samples and controls were concentrated to 100 µl for preliminary field testing samples or 50 µl for main samples under a gentle flow (10 ml min^{-1}) of high purity nitrogen (BOC Industrial Gases, UK). Samples were injected (4 µl) and analysed using a Hewlett Packard, HP5890 Series II GC system fitted with a non-polar HP-1 (50 m, 0.32 mm ID, 0.52 µm film thickness) column with COC injector and a non-polar fused silica pre column (1 m, 0.53 mm ID) and a FID. Hydrogen was the carrier gas. The following oven temperature programme was used: 30 °C hold

for 1.0 min, then 5 °C min^{-1} to 150 °C hold for 0.1 min, then 10 °C min^{-1} to 230 °C, final hold for 27.0 min, total run time 60.1 min.

Whole animal air entrainment Tenax® TA samples and controls were analysed by thermal desorption using a Hewlett Packard, HP 6890 GC system and fitted with an Optic 400 programmable temperature vaporization (PTV) unit and a non-polar HP-1 (50 m, 0.32 mm ID, 0.52 µm film thickness) column with FID. Hydrogen was the carrier gas. The following temperature programme was used: 30 °C hold for 1.0 min, then 5 °C min^{-1} to 150 °C hold for 0.1 min, then 10 °C min^{-1} to 230 °C, final hold for 27.9 min, total run time 61 min. The temperature programme for the optic was: 30 °C start, then 16 °C sec^{-1} to 230 °C, hold for 40 min, total run time 40.1 min.

Coupled GC-MS analyses were carried out on the air entrainment of sheep fleece samples and the preliminary whole animal air entrainment samples to gain tentative identifications of chemicals present. Samples were separated using a HP-1 (50 m, 0.32 mm ID, 0.52 µm film thickness) capillary GC column directly coupled to a mass spectrometer (Mat 95 Thermo Finnigan) and attached to a COC injector and a FID. Helium was the carrier gas. Ionization was made by electron impact at 70 eV, 250 °C. The oven temperature programme was: 30 °C hold for 5 min, then 5 °C min^{-1} to 250 °C, final hold for 21 min, total run time 60 min.

Tentative identifications of EAG-active peaks were made by comparison to entries in the National Institute for Standards and Technology (NIST) 2005 database, before being reviewed by Dr. Mike Birkett (Rothamsted Research, UK).

To allow calculation of Retention Indices (RI) to facilitate mass spectrometry analysis, alkanes (100 ng μl^{-1}, C7 – C25) were run on the GC and analysed (under conditions used for each sample type). When required a larger alkanes series (C7 – C35) was analysed. The RI for a compound is a number that indicates its retention relative to adjacent alkanes and allows for comparison of data between different GC machines and columns (but maintaining the same column type e.g. HP-1). The RIs were compared to entries in a database (Rothamsted Research) of known compounds. An Excel Programme was used for fast and easy calculation RIs, and the formula is shown below (Equation 2.1):

$$RI = \left(\frac{100(\log rt(x) - \log rt(z-1))}{(\log rt(z+1) - \log rt(z-1))} \right) + 100(z-1)$$

Equation 2.1: rt = retention time, x = compound of interest, z+1 = alkane after the compound of interest, and z-1 = alkane before the compound of interest.

Where rt = Retention time, x = Compound of interest, z+1= alkane after the compound of interest, and z-1 = alkane before the compound of interest.

The total number of peaks present and the total peak area was compared between samples to estimate number and quantity of chemicals present.

2.3 Results

2.3.1 Solvent extraction from sheep fleece

Solvent extracts of sheep fleece were prepared and analysed using GC techniques. Some quantitative and qualitative differences in peaks were apparent depending on the type of solvent used (redistilled hexane or redistilled diethyl ether) (Figure 2.6 and 2.7).

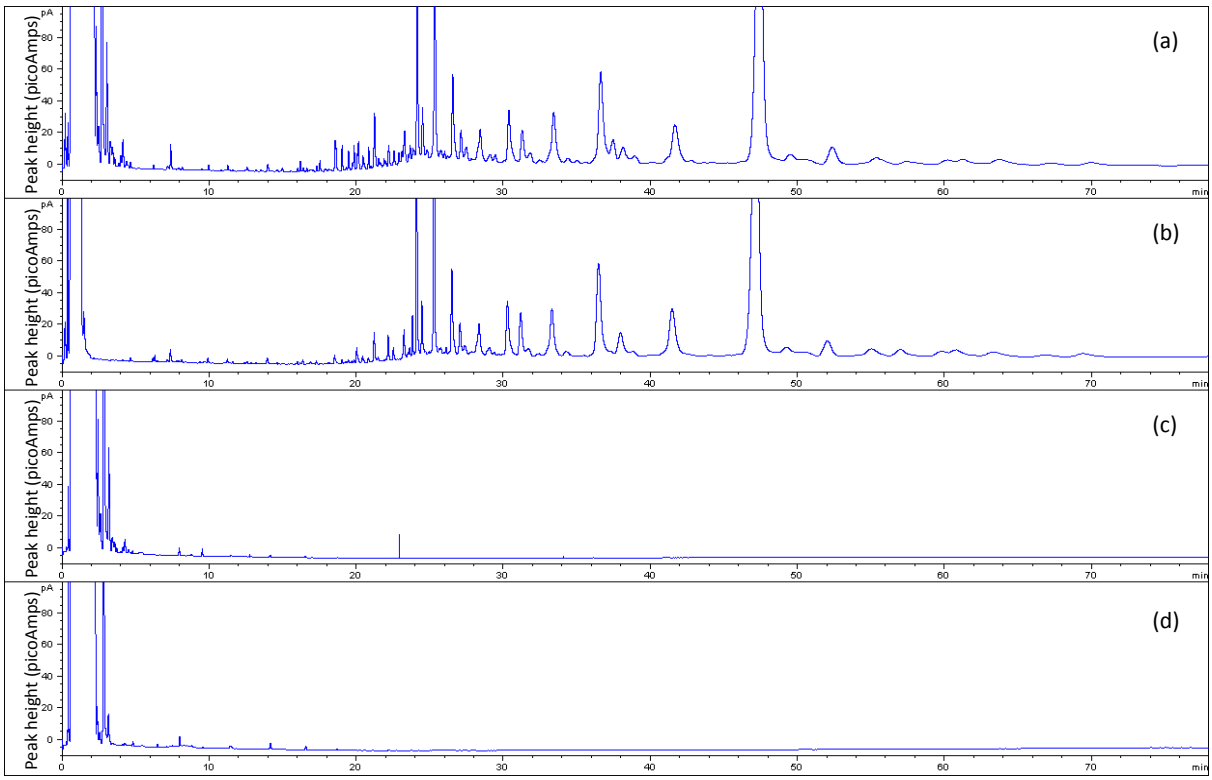


Figure 2.6: Typical gas chromatographs for sheep fleece solvent extraction (1 g of fleece in 5ml solvent, 28.25 ml vial), (a) hexane, (b) diethyl ether, (c) control (hexane only, no fleece) and (d) control (diethyl ether only, no fleece). GC trace produced on a HP-1 10 m column (Agilent Technologies, 6890N GC system).

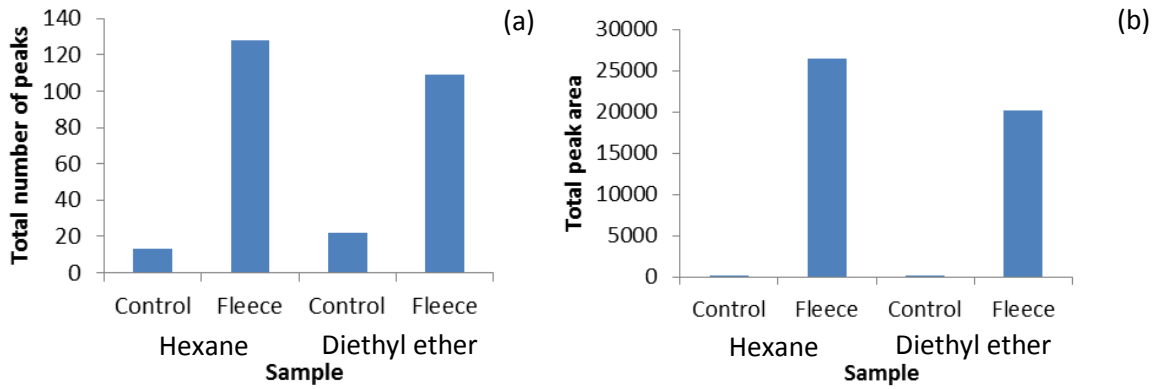


Figure 2.7: Total number of peaks (a) and total peak area (b) for solvent extraction from sheep fleece (n = 1).

Hexane solvent extraction (Figure 2.6a) contained 19 more peaks compared to the diethyl ether solvent extraction (Figure 2.6b, 2.7a) and also yielded a greater peak area (Figure 2.6b). Although both controls contained some peaks, the total peak area was around 1 % of that of the fleece samples (Figure 2.7b). A large peak was detected with a retention time of 47.5 min in samples from each of the solvent extraction of fleece experiments. Analysis showed that a 1 µl injection from a non-concentrated sample contained approximately 150 ng of this chemical.

2.3.2 Air entrainment from sheep fleece

Sheep fleece air entrainment samples were analysed by GC and GC-MS. Differences between the fleece and control entrainment samples were noted (Figure 2.8, 2.9) despite indication of large contamination at 15 min retention time (RT) and 30 min RT. Initial GC analysis using the HP-1 10 m column showed that the largest peak in the non-concentrated air entrainment sample was 0.342 ng. Therefore, the sample was concentrated to 50 µl and run on the HP-1 50 m to improve peak resolution (Figure 2.8 and 2.9).

Nearly twice as many peaks were detected in the fleece entrainment sample compared to the control with an increase in peak area also being noted (Figure 2.9a, b). Additionally, the sheep fleece air entrainment sample was analysed by coupled GC-

MS, which provided tentative peak identification of the most abundant peaks (Table 2.3).

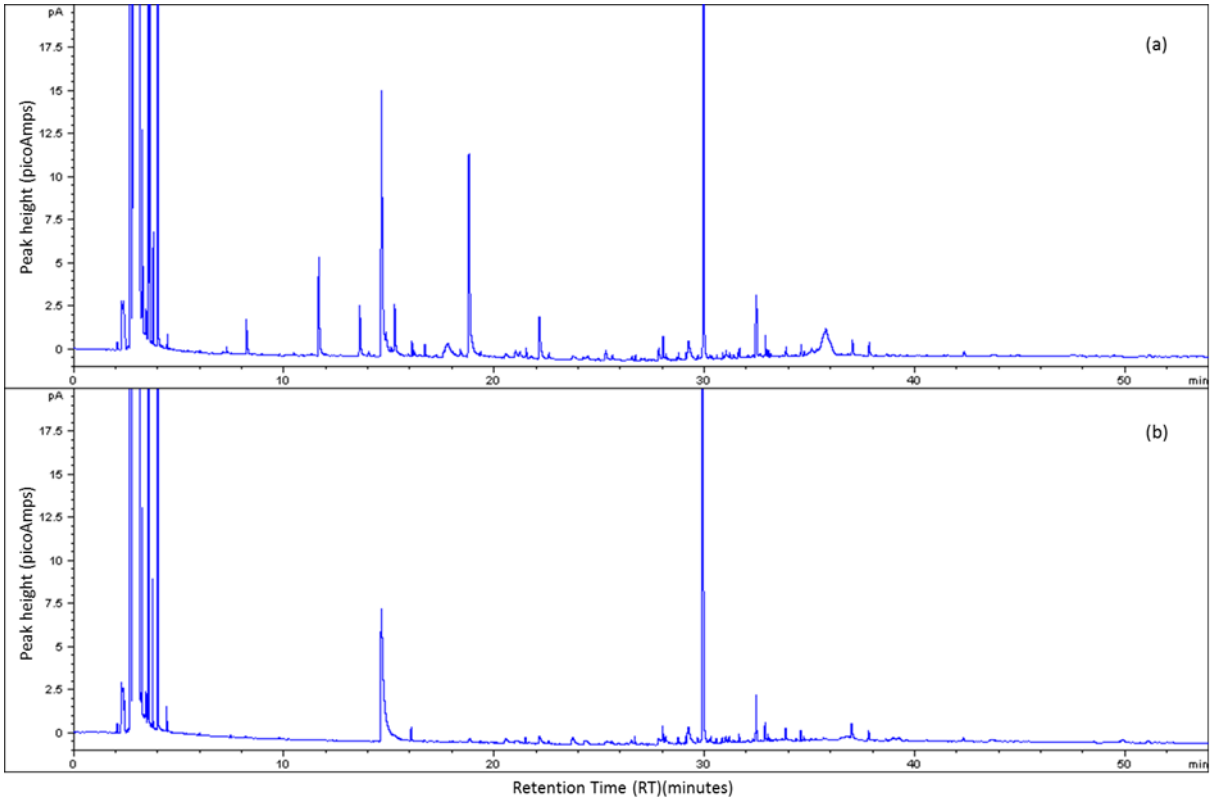


Figure 2.8: Typical gas chromatographs for air entrainment samples, (a) 1 g of fleece and (b) control. GC traces produced on a HP-1 50 m column (Hewlett Packard, HP6890 GC system). Tentative identification of chemicals compounds are detailed in Table 2.4.

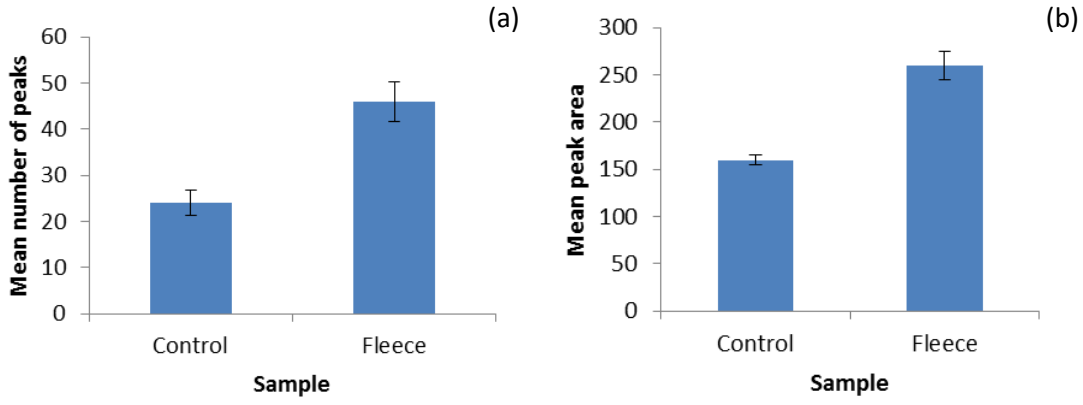


Figure 2.9: Mean number of peaks (a) and mean peak area (b) for air entrainment of sheep fleece experiment Porapak™ Q samples (n = 3, ± standard error of the mean (SEM)).

Table 2.3: Tentative identifications of compounds found in sheep fleece air entrainment (Figure 2.8) by coupled GC-MS.

RT (min)	Tentative chemical identification
10.11	2-Methylpropanoic acid
11.12	Hexanal
13.92	2-Methylbutanoic acid
15.39	Heptanal
17.6	6-Methyl-2-heptanone
18.72	6-Methyl-5-hepten-2-one
18.94	2-Octanone
19.15	Unknown *m*/*z* 41, 54, 67, 79, 80, 92, 106, 107 and 3-mercapto, 3-methyl butyl acetate
19.36	Octanal
20.86	Limonene
21.95	3-Methylphenol / 4-methylphenol
22.62	2-Nonanone
23.02	Nonanal
23.18	3,4-Dimethyl-2-hexanone
25.47	(*E*)-2-nonenal
25.98	2-Decanone
26.38	Decanal
29.55	Undecanal
32.48	Dodecanal
32.78	Tetradecane
35.34	Butylated hydroxytoluene
39.12	2,5-Bis(1,1-diemthylpropyl)-2,5-cyclohexadiene-1,4-dione
39.23	Decahydro-3-(3,3-dimethyl-2-oxobutenylideno)-quinoxalin-2-one
45.68	Hexadecanoic acid

2.3.3 Whole animal air entrainment

2.3.3.1 Collection of known volatile chemicals from slow release formulations

Testing of the whole animal air entrainment box was conducted at Rothamsted Research using known chemical lures (Table 2.4). Results showed successful recovery of nonanal and decanal in all instances except when located near the fan for 2 hours (11.4 % decrease in decanal) compared with the empty box control entrainments. The central location of the adsorbent polymer appeared more effective than the location directly next to the fan, recovering more of the test chemicals in two out of the three experiments.

Table 2.4: Percentage increase of chemicals recovered from slow release formulations compared to control runs.

	Percentage increase in chemicals collected on Porapak™ Q from lures (%)					
Location	Fan			Centre		
Duration	4hours		2hours	4 hours		2hours
Date	18/12/10	19/12/10	20/12/10	18/12/10	19/12/10	20/12/10
Nonanal	439.9	541.7	289.2	1564	277.3	934.7
Decanal	69.2	219.9	-11.4	901.7	37.5	235.3

2.3.3.2 Collection of volatiles from whole animals (sheep) - Preliminary

Preliminary tests with whole animals (sheep) were conducted at a mixed dairy/sheep farm near IAH Compton (Berkshire, UK) to assess the whole animal air entrainment methodology. The average temperature recorded inside the box during the second and third whole animal sheep entrainment replicate was notably higher than the first replicate and, conversely, the average relative humidity was lower during the second and third replicate compared to the first replicate (Figure 2.10a, b). All conditions remained within the guidelines (Table 2.2, Section 2.2.4.2)

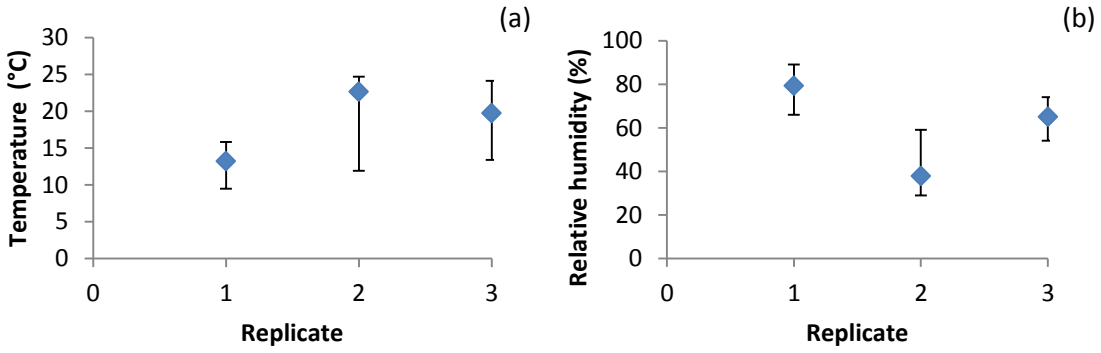


Figure 2.10: Average temperature (a) and relative humidity (b) during the course of each whole animal sheep entrainment replicate. The bars show the maximum and minimum values recorded during the entrainment period.

Collected Porapak™ Q samples were analysed by GC and GC-MS (Figure 2.11, Table 2.5). Differences between the sheep and control entrainment samples were noted (Figure 2.11, 2.12). In both the 2 hour and 4 hour sheep experiments, a greater mean number of peaks were recorded compared with the relevant control experiment (Figure 2.12a). The results also show an increase in the mean peak area found in the sheep experiments compared to the controls (Figure 2.12b). A 4 hour sheep

entrainment sample was analysed by GC-MS (Table 2.5). Some contamination was present from the solvent, notably the three large peaks around 12 min RT, and the six peaks between 14 min RT and 18 min RT (Figure 2.11c). Coupled GC-MS analysis provided tentative identifications of the most abundant peaks (Table, 2.5).

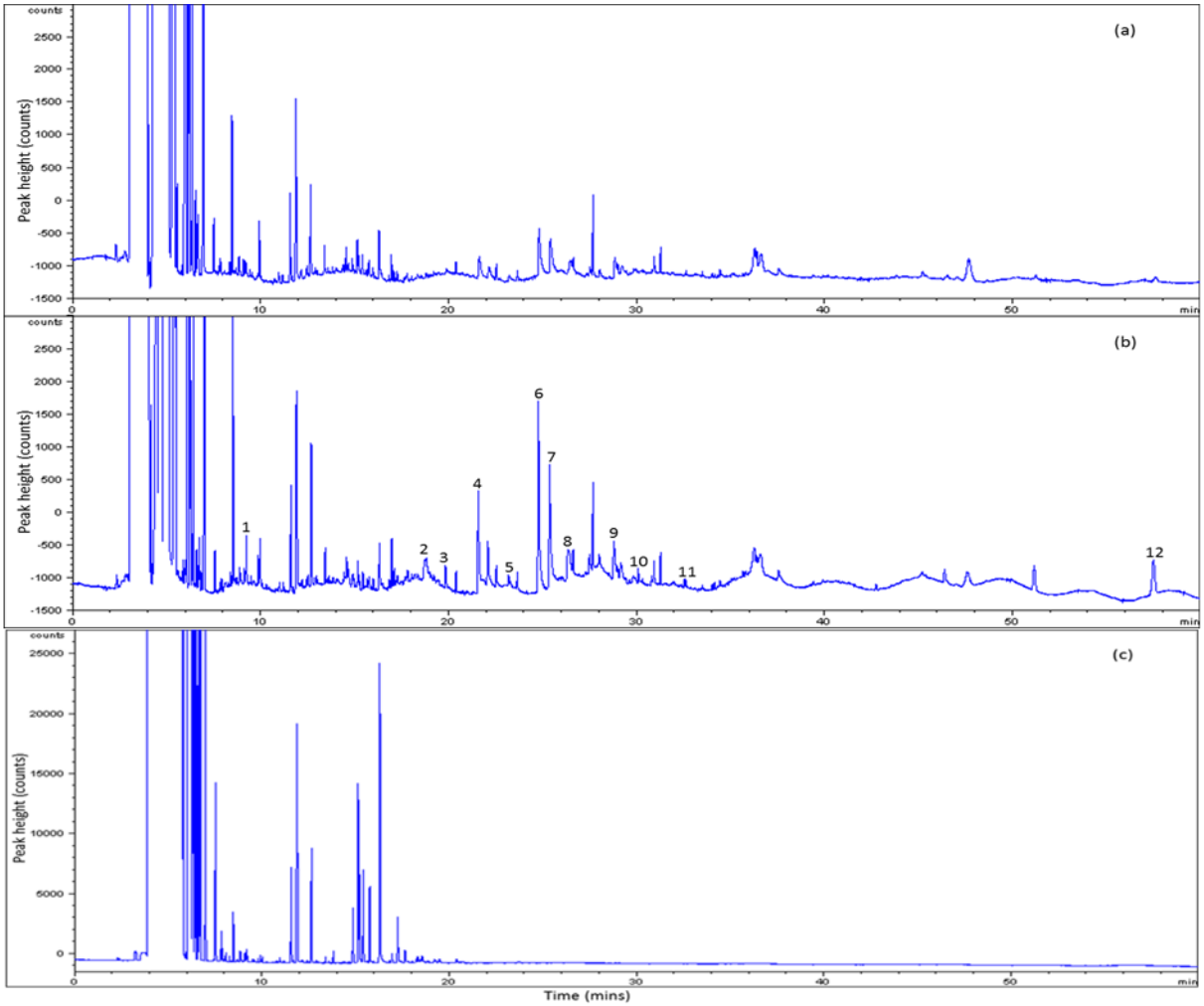


Figure 2.11: Typical gas chromatographs for whole animal sheep air entrainment Porapak™ Q samples, (a) 4 hour control, (b) 4 hour sheep and (c) redistilled hexane (4 µl) (contaminated). GC traces produced on an HP-1 50 m column (Hewlett Packard, HP5890 Series II, GC system). Numbered peaks show compounds tentatively identified by coupled GC-MS (Table 2.6).

Table 2.5: Tentative identifications of compounds found in whole sheep air entrainment (Figure 2.11) by coupled GC-MS.

Peak	RT (min)	Tentative Identification
1	11.48	Butanoic acid
2	21.88	3-Methylphenol / 4-methylphenol
3	23.07	Nonanal
4	24.78	3-Ethyl or 4-ethyl benzaldehyde
5	26.43	Decanal
6	28.01	2-Ethyl, 3-ethyl or 4-ethyl acetophenone
7	28.62	Indole
8	29.63	Tridecene (unknown double-bond position)/ 3,4-dimethylacetophenone
9	32.51	Dodecanal
10	34.27	Pentadecene (unknown double-bond position)
11	37.89	Tetradecanal
12	61.24	Pentacosane

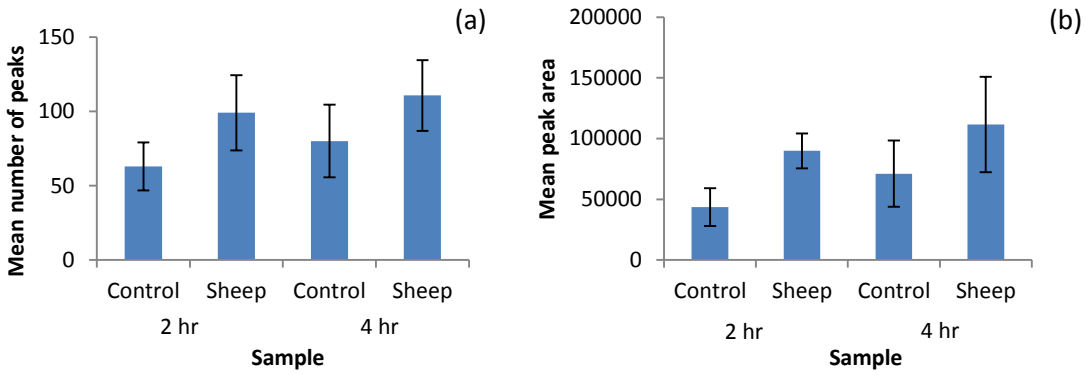


Figure 2.12: Mean number of peaks (a) and mean peak area (b) for whole animal air entrainment experiment Porapak™ Q samples (n = 3, ± standard error of the mean (SEM)).

Collected Tenax® TA samples were analysed by GC (Figure 2.13, 2.14). Differences were found between the test and control samples (Figure 2.13, 2.14). Additionally, 32 peaks of potential interest have been highlighted for further investigation by GC-MS. The results show an increase in the mean number of peaks and of the mean peak area for both the two hour and four hour sheep entrainments compared to the relevant controls, similar to the results from Porapak™ Q (Figure 2.11, 2.12). Additionally, the raw Tenax® TA data shows a trend of increasing number of peaks and peak area in the control and sheep replicates as the experiment progressed.

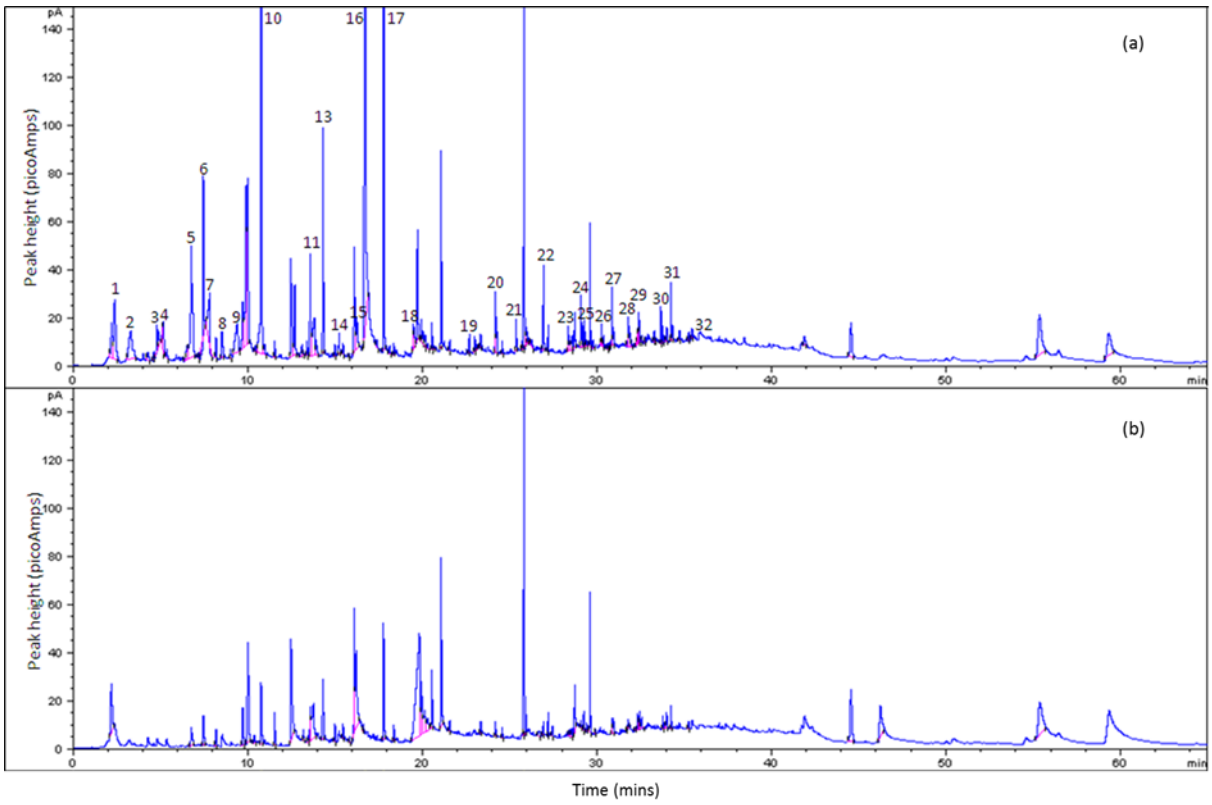


Figure 2.13: Gas chromatographs for whole animal air entrainment Tenax® TA samples, (a) 4 hour control and (b) 4 hour test (sheep). GC traces produced on an HP-1 50 m column (Hewlett Packard, HP6890, GC system). Numbered peaks show differences to be identified by coupled GC-MS (sample yet to be analysed).

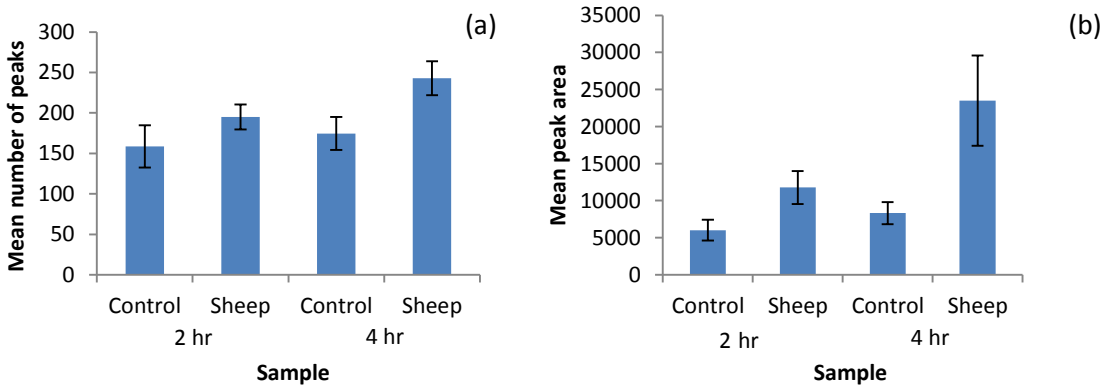


Figure 2.14: Mean number of peaks (a) and mean peak area (b) for whole animal air entrainment experiment Tenax® TA samples (n = 3, ± standard error of the mean (SEM)).

2.3.3.3 Collection of volatiles from whole animals (sheep) - Main

The main collection of volatiles using the whole animal air entrainment box was conducted at a mixed cattle/sheep farm (Rushall Farm, Berkshire, UK). The average

temperature and humidity recorded within the box remained within the acceptable limits as defined previously (Table 2.2, Section 2.2.4.2) with the exception of the first replicate evening where the temperature rose to a peak of 29 °C and resulted in the sheep being released from the box after 30 min (Figure 2.15). This replicate was repeated again at the end of the experiment. A drop in temperature and corresponding increase in humidity can also be seen during the 12th, 13th and 14th replicates.

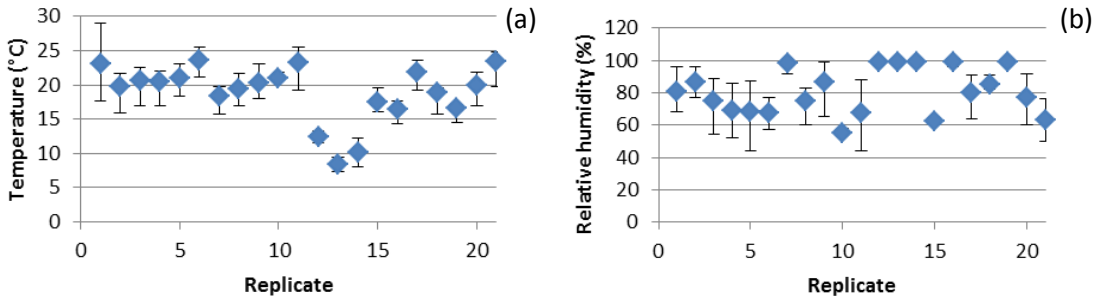


Figure 2.15: Average temperature (a) and relative humidity (b) during the course of each main whole animal sheep entrainment replicate. The bars show the maximum and minimum values recorded during the entrainment period.

Collected Porapak™ Q samples were individually analysed by GC (Figure 2.16). Differences between the sheep and control entrainment samples were noted (Figure 2.16, 2.17). A greater mean number of peaks and a larger mean peak area were found in the sheep experiments compared to the controls (Figure 2.17). The GC traces were checked visually for any atypical entrainment results. The samples were then combined to produce four bulk samples (pure breed control, pure breed sheep, cross breed control and cross breed sheep) for future work. When each individual entrainment sample was transferred to the bulk sample vial the original vial was also rinsed with 50 µl of redistilled diethyl ether, which was also transferred (thereby doubling the volume). After all the entrainments had been combined in the relevant bulk sample the bulk sample was concentrated down 50 % under a nitrogen flow to remove the diethyl ether added from rinsing.

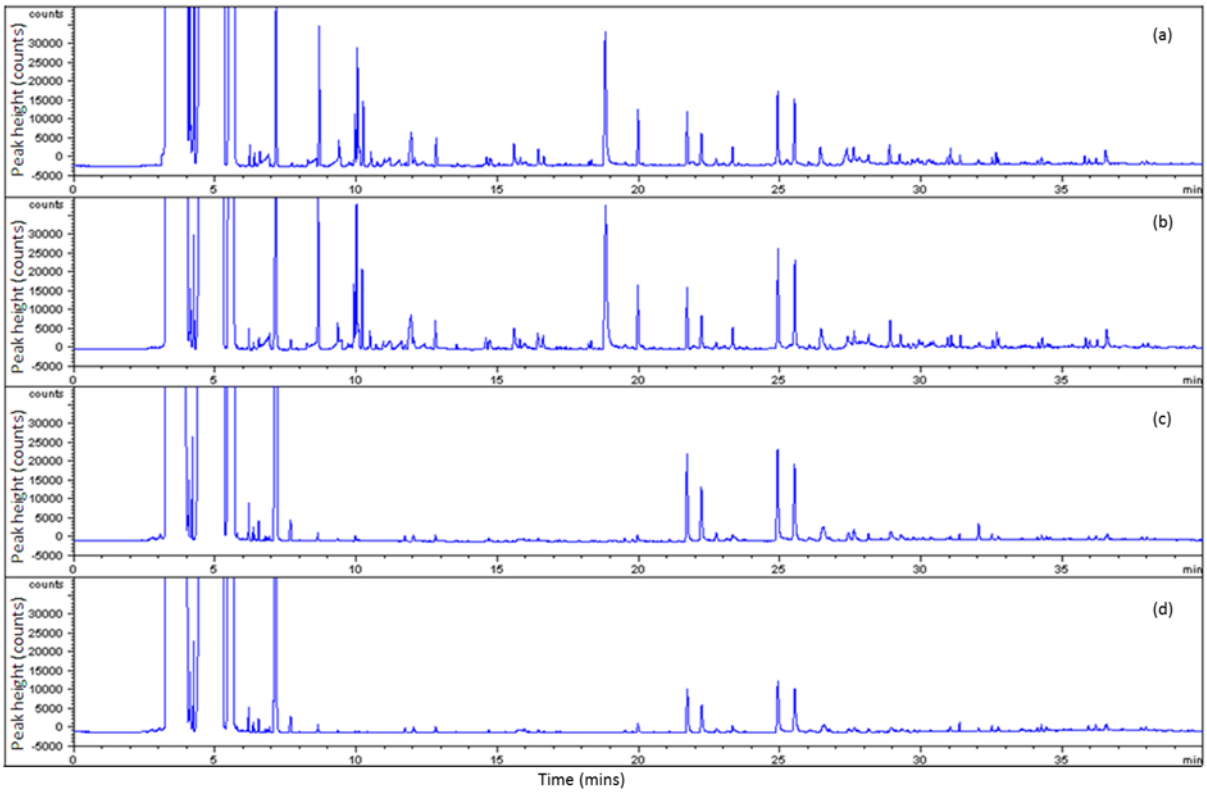


Figure 2.16: Gas chromatographs for whole animal sheep air entrainment larger Porapak™ Q samples, (a) 4 hour sheep tube 1 and (b) 4 hour sheep tube 2, (c) 4 hour control tube 1, (d) 4 hour control tube 2. GC traces produced on an HP-1 50 m column (Hewlett Packard, HP5890 Series II, GC system).

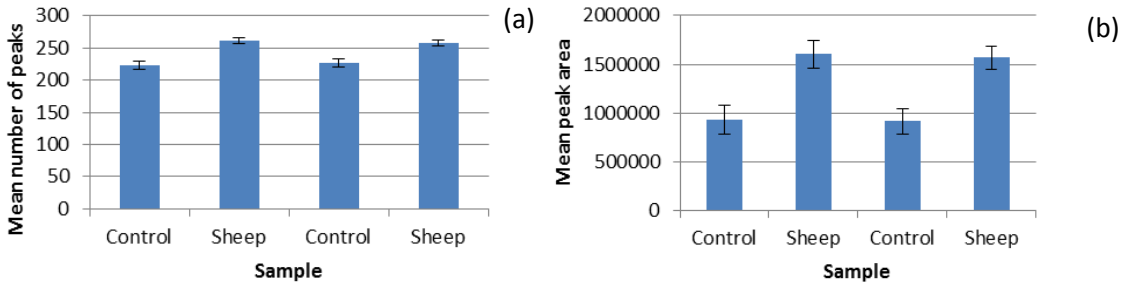


Figure 2.17: Mean number of peaks (a) and mean peak area (b) for main whole animal air entrainment experiment larger Porapak™ Q samples (n = 20, ± standard error of the mean (SEM)).

2.4 DISCUSSION

Identification of novel semiochemicals that attract *C. obsoletus and C. pulicaris* group midges may be exploited to develop improved monitoring and surveillance traps. An effective surveillance system would act as an early warning system and help protect or reduce the impact on livestock (and the wider financial impact), of future outbreaks of bluetongue (Carpenter *et al*, 2009b). The first step to achieving the identification of host-derived semiochemicals is the collection of appropriate volatile material. Three different methods were trialled in this chapter.

Chemicals were successfully extracted from the fleece of sheep directly with redistilled hexane and diethyl ether solvent, or by air entrainment using Porapak™ Q (Figure 2.6, 2.7, 2.8, 2.9). Analysis of solvent and air entrainment extracts revealed quantitative and qualitative differences between fleece samples and controls. The presence of the large peak (RT = 47.5 min, 150 ng μl^{-1}) meant that the solvent extract samples were not suitable for concentration (to increase the visibility/detection of the smaller peaks), as this would have resulted in deterioration of GC performance due to sample overloading. The injection of samples onto GC columns (HP-1 50 m) containing chemicals at concentrations greater than 100 ng μl^{-1} can damage and degrade the column and negatively affect the resolution of chemical peaks. It should be noted that this is not a contamination peak, as it was not found in the control samples. It is believed that this peak is part of the solvent-soluble fraction of suint (Stewart, 1962). Suint is the water-soluble material present in the greasy wool of sheep, comprised of organic acid salts, urea, and polypeptides (Truter, 1956). Separation techniques, such as vacuum distillation, should remove the large peak present in the solvent extraction samples and allow further investigation of the remaining, smaller and more volatile peaks (Pickett and Griffiths, 1980). Additionally, vacuum distillation provides more biological material compared to air entrainment which is mass-limiting. However, the solvent extracts will also contain more low volatile chemicals that are unlikely to be involved in longer range attraction of *Culicoides* species. The source of the contamination peaks in the air entrainment samples remains unclear, however, their presence highlights the care required when handling and cleaning the air entrainment equipment as well as the importance of ensuring the system is sealed. Glass conical flasks were used for the air entrainment, which are cleaned and re-used. A potential

solution to this would be the use of oven bags (J Sainsbury plc, London, UK) which are baked in the oven (180 °C for 2 hours) to clean, used for the entrainment, then disposed of, thereby removing the risk of contamination from imperfect cleaning.

Most of the literature regarding odours from sheep fleece concentrates on removal of undesirable volatile organic compounds (VOCs) in order to improve the odour of wool used in floor coverings and other indoor materials rather than the identification of VOCs involved in the attraction of insects (Lisovac and Shooter, 2003). Chemicals collected from the air entrainment of sheep fleece and tentatively identified as hexanal, heptanal, 2-octanone and 6-methyl-2-heptanone were previously reported from solid phase microextraction (SPME) of sheep wool and wool grease samples (Lisovac and Shooter, 2003). Phenol was also tentatively identified from analysis of the fleece solvent extraction in this study and, along with 3-methylphenol and 4-methylphenol, was shown to be a component of the skin secretions of oxen (Warnes, 1990). This suggests that relevant sheep-derived volatiles have been collected successfully. Bhasin *et al* (2000a) studied the EAG responses of *C. impunctatus* to several alkyphenols (including 3-methylphenol and 4-methylphenol) and found that they were more sensitive to the 3-substituted alkyphenols than the 4-substituted alky phenols. Previously, it had been shown that although the concentration of each individual component in a blend is important, the presence or absence of 3-methylphenol has a significant effect on the attractiveness of the blend to *C. impunctatus* (Bhasin, 1996). A recent study found that the addition of racemic 1-octen-3-ol and 4-methylphenol to OVI light suction traps in South Africa had no significant effect on the number of *Culicoides* species caught (Venter *et al*, 2011). However, it was noted that the release rates used were high. The authors used racemic 1-octen-3-ol at a release rate of 9.1 mg h^{-1}, which is 50 % higher than that used by Ritchie *et al* (1994) (6.05 mg h^{-1}) and 152 times higher than that used by Bhasin *et al* (2001) (0.06 mg h^{-1}). The authors note that the average concentration of 1-octen-3-ol found in natural ox odour is approximately 0.05 mg h^{-1}, or 182 times lower than their release rate (Venter *et al*, 2011). This highlights the importance of determining an appropriate release rate based on naturally occurring levels for any candidate chemicals prior to preparation of lures for field testing. Other chemicals tentatively identified from the air entrainment of sheep fleece experiments were 6-methyl-5-hepten-2-one, octanal, nonanal and

decanal, which have previously been shown to be EAG-active for *C. impunctatus* (Logan *et al*, 2009). Interestingly, these same chemicals also were found to be EAG-active for *St. aegypti* mosquitoes, occurring in greater amounts in entrainments from human being individuals deemed to be less attractive to the insects (Logan *et al*, 2008).

In summary, comparing the two approaches, air entrainment provides the best source of volatile chemicals and it would seem likely that longer distance attractants may be found in these samples. It is encouraging that semiochemicals have been tentatively identified from the entrainment samples that have been previously described as sheep-derived volatiles, and that are known to elicit behavioural and electrophysiological responses in *Culicoides* species midges. However, use of fleece samples, or even a whole fleece, still does not provide a true representation of a natural host as the starting point for investigation into *Culicoides* species host location. Therefore, the whole animal air entrainment box was conceived - a large scale air entrainment system that allowed collection of volatile from three sheep, thereby providing the most natural and representative extract for further research. Rather than just volatiles released from the fleece, the whole animal air entrainment box also allows collection of skin, breath, urine and faeces volatiles, plus any other source of volatiles that may be present on the animal (e.g. bacterially infected myiatic lesions)(Khoga *et al*, 2002).

The initial testing of the whole animal air entrainment box using known chemical lures at Rothamsted Research showed the successful collection of chemicals on Porapak™ Q after two and four hour entrainments, compared with control entrainments (Table 2.4). During these experiments some issues with the functionality of the box were encountered that resulted in the removal of the carbon filter (for the duration of the experiments) and the ordering of a stronger “push” fan and building of a new fan housing. The original array of four 120 mm computer fans did not generate sufficient force for the airflow to penetrate through the carbon panel filter, as a result much of the airflow generated by the fans was not making it into and through the whole animal entrainment box. This was likely to lead to negative air pressure inside the box, and increase the likelihood of contamination by pulling unfiltered air into the box. Additionally, it made calculation of the subsample of total airflow collected by the Porapak™ Q impossible. The temperature during these experiments rarely rose above

0 °C due to the experiments occurring over winter in preparation for the summer field season, resulting in lower than expected chemical release from the slow release formulations. The optimal release rate from the lures would have occurred at approximately 25 °C. Despite these issues the data suggested that a centrally suspended location for the absorbent polymer tubes would perform better than tubes located directly next to the exhaust fan array. This may have been due to turbulence in the flow of air near the exhaust fans affecting the collection of volatiles on the Porapak™ Q. The centrally suspended position for the Porapak™ Q tubes is also closer to the sheep and in keeping with previous successfully animal volatile entrainment studies (Birkett *et al*, 2004). Moreover, redesign of the fan and assessment of the new flow rates meant that only one exhaust fan would need to be running in order to maintain a positive pressure inside the box.

Collection of volatile chemicals from whole live animals in the field has been successful with differences in number of chemical peaks and total chemical constituents noted for both the Porapak™ Q and Tenax® TA samples at the 2 hour and 4 hour entrainment times during the preliminary experiments (Figure 2.11, 2.12, 2.13). These differences are more apparent with the Tenax® TA analysis because with thermal desorption the whole sample is analysed rather than a portion (Porapak™ Q). However, thermal desorption also destroys the whole sample, and is therefore not suitable for further investigation (i.e. determination of a behaviourally active sample using the Y-tube olfactometer, or electrophysiology). Some differences were present in the Porapak™ Q samples, including chemicals also tentatively identified from the laboratory entrainment of fleece, such as 3-methylphenol or 4-methylphenol, nonanal and decanal (Table 2.5). However, the amount of total and/or individual chemicals recovered was relatively low, making potential further work with Y-tube olfactometer bioassays difficult as concentration of the sample would reduce the volume to a point where insufficient quantities would be available. In contrast to laboratory air entrainment techniques with sheep fleece, where all the air from a given vessel is passed through the Porapak™ Q tube, only a subsample of the total airflow was collected during the whole animal entrainments in the field. Based on these results options were examined to increase the amount of volatiles available for analysis. Combining of individual entrainments to create a bulk sample would provide a larger

sample to work with, increasing concentration options. The decision was made to have multiple (two) Porapak™ Q tubes collecting volatile chemicals per replicate thereby doubling the quantity of chemicals collected. Other possible options were to increase the size of the Porapak™ Q tube, increase the airflow (stronger "pull" pump) and increase the mass of Porapak™ Q polymer present (Millar and Sims, 1998). As there is no indication that the Porapak™ Q was being saturated, based on the low quantities of collected chemicals, an increased airflow was investigated to allow for collection of a larger subsample of the total airflow (which may also require an increased diameter Porapak™ Q tube). There was also sufficient space in the box to introduce a third sheep during the whole animal air entrainments which would provide a further 50 % increase in volatile chemicals released. Additionally, during the preliminary whole animal entrainments, it was noted that the number of chemical peaks and peak area generally increased over the course of the replicates. This suggested that there was degradation in the carbon filter panel, resulting in impurities being drawn into the box, or that the current cleaning method was not sufficient at removing contaminants from the box. Therefore, new carbon filter panels were used daily during the main whole animal entrainment experiment and the box was cleaned thoroughly after each use.

For the main entrainment of whole sheep, alterations to the equipment and methodology were introduced. A larger pump was obtained that pulled a greater flow of air through the Porapak™ Q tube (5 l min^{-1}). Moreover, the pump could support multiple (two) Porapak™ Q tubes, both pulling at the increased flow rate. However, it was found during initial tests with the larger pump that breakthrough was a potential issue (saturation of the adsorbent polymer that results in volatiles not being bound as they pass over it). As a result, a larger bore Porapak™ Q polymer tube was developed and more absorbent polymer was added (250 mg) per tube (Millar and Sims, 1998). A third sheep was also added, as suggested above. The main experiment took place in June/July 2011 and the first evening's entrainment corresponded with the hottest day of the year. As a result the temperature in the box reached 29 °C and the combination with relative humidity breached the thresholds set out in Table 2.2 (Section 2.2.4.2). As a result the sheep were release from the box and the experiment abandoned for the night. The environmental conditions inside the box on all subsequent nights remained within the operating thresholds (Section 2.3.4.3, Figure 2.15). Clear differences could

be seen visually between the sheep entrainments and the control entrainments with the sheep entrainments collecting a greater number of peaks, and yielding a greater total peak area (estimated quantity of chemicals)(Section 2.3.4.3, Figure 2.16, 2.17). This provided a strong indication that the alterations had been successful and entrainment of sheep-derived volatiles had successfully occurred.

2.5 Conclusions

Initial investigations with solvent extraction and air entrainment of sheep fleece samples successfully collected sheep-derived volatiles. Following the decision to focus on air entrainment, a new piece of experimental equipment was designed and built at Rothamsted Research. Through iterative testing, the whole animal entrainment box was altered to improve the collections of volatiles from sheep. Using the whole animal entrainment box successful collection of sheep volatile samples allowed for bulk samples to be produced. These bulk samples were used to investigate behavioural responses of *Culicoides* species to the sheep extracts in the field and laboratory to confirm biological activity (Chapter 3). Electrophyisological responses of *Culicoides* species were investigated and active chemicals identified and quantified (Chapter 4). Behavioural responses of *Culicoides* species to identified chemicals were investigated (Chapter 5) and field trials of chemicals and blends with wild *Culicoides* species were conducted (Chapter 6).

Chapter 3: Behavioural responses of *Culicoides* species to sheep derived volatiles

3.1 Introduction

In the past, research into the host location of *Culicoides* species consisted primarily of incidental collections/observations during investigations into mosquito host location (Takken and Kline, 1989, Kline *et al*, 1990). Since then, a considerable amount of research has been conducted into the behavioural responses of *C. impunctatus* and *C. nubeculosus* (Blackwell *et al*, 1996, 1997, Bhasin, 1996, Bhasin *et al*, 2000a, 2001, Logan, 2006, Logan *et al*, 2009, 2010, Mands *et al*, 2004). However, little is known about other UK species, most notably the *C. obsoletus* and *C. pulicaris* species complexes implicated in the spread of BTV and SBV (Carpenter *et al*, 2008a, Veronesi *et al*, 2013).

There has been some research into *Culicoides* species associations with host animals in the field, especially since the spread of BTV into Northern Europe and the emergence of SBV (Carpenter *et al*, 2008a, Gerry *et al*, 2009, Kirkeby *et al*, 2013a, Viennet *et al*, 2011). Light traps are commonly used to investigate the attraction of *Culicoides* species to nearby potential hosts. An early investigation, around the Onderstepoort Veterinary Institute (OVI, Pretoria, South Africa), set up OVI traps close to areas housing different species of animals (including cattle, horses, sheep, chicken and pigs) and counted the number of *Culicoides* species caught (Venter *et al*, 1996). Several *Culicoides* species were caught, with *C. imicola* found to be the most prevalent. Bloodmeal analysis revealed greater numbers of *Culicoides* species had been feeding on sheep, horses and cattle respectively. Trapping took place in March and September 1988, and the later trapping period also revealed an increase in the numbers of *C. imicola* (and some other species) feeding on pigs compared with the March period (339 compared with 44, total *Culicoides* species) at these sites (Venter *et al*, 1996). In a similar study in Brandenburg, Germany, investigating trap catches (BG sentinel, UV light) at a farm site and analysing bloodmeals, it was shown that *C. obsoletus* and *C. pulicaris* group midges can feed on a variety of hosts with a preference for cattle even when other large vertebrates were kept in the near vicinity (Bartsch *et al*, 2009). Interestingly, none of the bloodmeals analysed tested positive for sheep, suggesting that, at least in the presence of larger livestock, sheep are not preferentially fed on by

Culicoides species (Bartsch, *et al*, 2009). Meanwhile, Foxi and Delrio (2010) used miniature blacklight traps (GZ International) to compare *Culicoides* species catches from a sheepfold with a pond habitat (containing reptiles, amphibians, and many aquatic birds). Their results show that *C. imicola* and *Culicoides newsteadi* Austen are mammalophilic, found in greater numbers at the sheepfold, while *Culicoides festivipennis* Kieffer, *Culicoides jumineri* Callot and Kremer and *Culicoides sahariensis* Kieffer are all ornithophilic, caught in greater numbers at the pond. Interestingly, and relevant to the above studies where traps were placed near animals, it has been shown that *Culicoides* species catches maybe 100% greater if within 200 m of hosts, compared with catches made at a greater distance (Kirkeby *et al*, 2013a).

Trapping direct with animals, either through drop trapping or direct aspiration, is probably the best way to clearly show the association between *Culicoides* species and hosts. Several studies have used these methods to compare catches with light and/or CO_2-baited traps (Carpenter *et al*, 2008a, Gerry *et al*, 2009, Viennet *et al*, 2011). All three of these studies (taking place in England, Spain and France, respectively) report an underestimation of the *C. obsoletus* group in light traps compared with drop trapping or direct aspiration, moreover, an over estimation of the *C. pulicaris* group was also found (Carpenter *et al*, 2008a). These results serve to highlight how current monitoring methods are not optimal by using traps and volatile chemical baits that do not reflect sufficiently the chemical signature of a host.

Also of note, Rayaisse *et al* (2010) used live animal hosts, cattle, humans or pigs in PVC tents and vented the air from tents to trap situated approximately 15 m away. They found significant increases in trap catches of *Glossina tacchinoides* Westwood (tsetse flies) to cattle volatiles, while no statistically significant differences were found for humans or pigs. This method represents another way of determining if a host is attractive to insects, while also removing other stimuli such as vision.

These studies all provide examples of how to establish if a host is attractive or not to *Culicoides* species and therefore worth further investigation to identify potential attractant (or repellent) chemicals. However, relatively few studies obtained and investigated host derived extracts in the field or laboratory, with fewer still analysing those extracts to determine active component chemicals. Tchouassi *et al* (2012) used

fresh fleece (approximately 19 g) taken from sheep to bait traps (cylinders with mesh tops) and found mixed results for attraction of mosquitoes in Kenya. Trap catches at one site showed a significant increase in mosquitoes caught when light, CO_2 and fleece were together compared with light and CO_2, although despite an increase in trap catch at the second site, the light, CO_2 and fleece combination did not show a statistically significant increase. This would seem to suggest that fleece on its own may not have been sufficiently representative of the volatile signature of the host (sheep). Fleece on its own will lack volatiles released from the skin and breath of the animal, as well as lacking volatiles present in urine or faeces (depending on where from the sheep the fleece is taken). This was hypothesized in Chapter 2, and was the main driving force behind development of the whole animal (sheep) entrainment box. Extracts of hosts have been investigated previously, based on solvent washings of 22 g of fresh hair/fleece (Mands *et al*, 2004). As with Touchassi *et al* (2012), the same potential problems remain, due to the complete volatile profile of the host animal not being represented purely by the fleece or fleece-based extract. This said, Mands *et al* (2004) was able to rank attractions of *C. impunctatus* based on trap (Mosquito Magnet® Pro™) catches from extract-baited traps. Water buffalo extracts increased trap catches by 262 % compared with an un-baited control, while sheep extracts were found to yield a 53 % reduction. Interestingly, dilutions of the extract were also prepared and tested in the laboratory using a Y-tube olfactometer. At the highest concentration tested, the sheep extract was found to significantly increase the relative attraction compared with the control (Mands *et al*, 2004).

The first study properly to identify and investigate the potentially active volatile chemicals from a host-derived extract was Logan (2006, summarized in Logan *et al*, 2008, 2009), where human-derived extracts were studied in relation to mosquitoes (*St. aegypti*) and biting midges (*C. impunctatus*). Following collection of a host-derived extract, the next step is to prove attraction of the associated insects in the laboratory, using a behavioural bioassay, thereby confirming that the extract does still maintain a behaviourally relevant effect, and allowing further investigation of the chemical components of the extract. The use of olfactometers which allow insects to crawl or make short flights anemotactically has become common as a first-step investigation of host-derived olfactory stimuli in the laboratory (Logan *et al*, 2010). This type of

olfactometer allows insects to move upwind towards a stimulus. When they reach the Y junction of the olfactometer they are forced to travel left or right, usually towards a stimulus (e.g. a volatile chemical) or control (no stimulus/solvent). The number of insects present in the stimulus arm, compared with the total in the stimulus plus the control arms, are determined to give a measurement of "attractiveness" referred to as relative attraction (Equation 3.1).

$$\text{Relative attraction} = \frac{\text{number of insects in the test arm}}{\text{number of insects in the test + control arms}}$$

Equation 3.1: Formula for the calculation of relative attraction in the Y-tube olfactometer

If the stimulus is attractive the results would show a significant difference from an expected 50/50 distribution in the treatment and control arms of the Y-tube. *Culicoides* species responses to a standard control (i.e. hexane solvent) in the Y-tube olfactometer tend to range between 45 – 55 %, close to the expected 50/50 distribution. A review of the history and development of the Y-tube olfactometer is included in Chapter 5 (Section 5.1). Although it is worth noting here that consistency has been shown in previous studies of 1-octen-3-ol between laboratory results, attracting *C. nubeculosus* and *C. impunctatus* (Bhasin *et al*, 2000a, Logan, 2006) and field results where 1-octen-3-ol statistically significantly increases trap catches of *C. impunctatus* (Bhasin *et al*, 2001, Harrup *et al*, 2012). This correlation shows that the Y-tube olfactometer is a suitable bioassay for the identification of volatile semiochemicals that are likely to elicit behavioural responses in a field setting.

In this study, a laboratory assay to determine whether volatile extracts collected from sheep (Chapter 2) were behaviourally active was used. The Y-tube was chosen as the most appropriate assay and was based on the design of Blackwell *et al* (1994). However, it was noted that improvements to this design could be made to remove unnecessary visual and olfactory stimuli which could lead to bias in Y-tube olfactometer. Orange rubber bungs were replaced at the ends of the Y-tube arms with sintered glass fittings and the horizontal entry port (that required insects to be "puffed" into the Y-tube by pipette) was replaced with a vertical port (that allowed insects to drop down into the Y-tube without additional stimuli).

The aim of this chapter was to determine whether volatiles from sheep in a semi-field setting are attractive to *Culicoides* species and to establish an effective Y-tube olfactometer, to investigate behavioural responses of laboratory reared and wild caught *Culicoides* species to volatile extracts collected from sheep. This would ensure that the volatile extracts, collected in Chapter 2, contain putative attractants for *Culicoides* species midges.

Objectives:

- To investigate behavioural responses of wild *Culicoides* species to air vented from a whole animal air entrainment box in the field.
- To test whether 1-octen-3-ol could be used as a positive control for further testing with *C. nubeculosus* and wild caught *Culicoides* species in the Y-tube olfactometer.
- To demonstrate behavioural activity of *C. nubeculosus* to whole sheep air entrainment extracts (collected in Chapter 2) in the laboratory.

3.2 Materials and methods

3.2.1 Field trapping using expelled air from whole sheep air entrainment box (wild *Culicoides* species) – with Andrew Hope (The Pirbright Institute)

Air exhausted from the whole sheep air entrainment box, containing 3 sheep, during extract collection (Chapter 2, Section 2.2.4.3), was delivered to an unlit micro-CDC trap, running off a battery (6 V, 7 amp hours, Yuasa, Swindon, UK), via elephant trunking (ventilation duct, RS online, Northants, UK) to determine if the volatiles from the sheep inside the box were attractive to *Culicoides* species (Figure 2.3, Chapter 2, Section 2.2.4). A negative control (blank micro-CDC trap with no extract and no light) and positive control (micro-CDC trap with UV light) were also set up each night. The vented air trap and the blank unlit trap were set up approximately 15 m apart, while the UV light trap was situated 100 m from these traps. Field caught midges were identified and data were analysed using a negative binomial model with temperature at sunset as a factor. This experiment was run in conjunction with Andrew Hope (PhD student, The Pirbright Institute) who was also running a drop trapping experiment with sheep at the same field site during this experiment (and the collection of volatiles from sheep experiment (Chapter 2) all of which ran concurrently. Andrew Hope had overall responsibility for setting up the CDC traps, collecting the trap catches, counting and identification of the insect catches and analysis of the data. I assisted with setting up the CDC traps and collecting the trap catches. The data analysis presented was completed by Andrew Hope. Due to weather restrictions (heavy rain), less replicates were conducted using the cross breed sheep compared to the pure breed sheep (Cross breed n = 6, Pure breed n = 9).

3.2.2 Insects used in laboratory experiments

3.2.2.1 *Culicoides nubeculosus*

Culicoides nubeculosus females were obtained from the laboratory colony at The Pirbright Institute (Pirbright, Surrey, UK), formerly the Institute for Animal Health, where they were reared according to the methods of Boorman (1974). The midges

were stored in an insectary at Rothamsted Research (Harpenden, Hertfordshire, UK), in small cardboard rearing pots in a polypropylene insect cage (BugDorm-1 insect cage, MegaView Science Co., Ltd, Taichung, Taiwan), at 27.0 ± 2 °C and 60-80% relative humidity (RH) on a 12:12 light:dark inverted cycle. Sucrose solution (~10 %) was provided on a small cotton wool pad as a food source.

3.2.2.2 Wild caught *Culicoides* species

Onderstepoort Veterinary Institute and CDC ultraviolet light, downdraft suction traps were used to collect wild *Culicoides* species. Traps were placed at locations around a local farm where sheep and horses were kept (Cross Farm, Harpenden, Hertfordshire, UK, 51°48' 9.05'N; 0°19' 58.5'W). The traps were set up nightly approximately 1 hour before sunset and allowed to run overnight. The OVI trap was run from a leisure battery (12 V, sealed heavy duty, 85 amp hours, Alpha Batteries, Rochdale, UK) and the CDC traps were run from smaller batteries (12 V, sealed rechargeable battery, 12 amp hours, PowerSonic, Essex, UK). Batteries were recharged during the day. To allow for the collection of live insects a damp cloth was placed in the collection bottle with a layer of filter paper placed on top. Due to the OVI traps producing greater downdraft suction, strips of newspaper were torn and crumpled, and placed on top of the filter paper to help reduce desiccation of the insects. Collected insects were recovered the following morning (approximately 08:00 hours) and taken to the insectary at Rothamsted Research where they were moved to small cardboard rearing pots by mouth pooter (fitted with a Hepa-Vent™ in-line filter device, Whatman Inc., NJ, USA). Rearing conditions were the same as described for *C. nubeculosus* (Section 3.1.1.1).

A sub-sample of collected insects was sent for analysis (Andrew Hope, PhD student, The Pirbright Institute, UK). All the *Culicoides* species present were members of the *C. obsoletus* or *C. pulicaris* complexes, and 90.3 % of the sample comprised female midges. Therefore, it was determined that the wild *Culicoides* species caught at the trapping site were relevant for the purposes of this study.

3.2.3 Extracts and chemicals

Whole sheep extracts ("Pure" Hartline breed extract and control and "Cross" Hartline/Suffolk breed extract and control) were obtained using the whole animal air entrainment box as described in Chapter 2.

Racemic 1-octen-ol (98 % pure, Avocado Research Chemicals Ltd, Heynsham, Lancs., UK) and (*R*)-(-)-1octen-3-ol (99 % pure, Bedoukian Research Inc., Danbury, CT, USA) were prepared at 100 mg ml^{-1} (100 µg $µl^{-1}$) concentrations in redistilled hexane (Petroleum ether fraction, 40 – 60 °C, Fisher Scientific Ltd., Leicestershire, UK – redistilled to collect hexane at Rothamsted Research, Hertofordshire, UK). Ten-fold serial dilutions were prepared down to 100 µg ml^{-1} (100 ng $µl^{-1}$). Due to the fact that 10 µl of the chemicals are used in the Y-tube olfactometer for each replicate this therefore gave a final dose range of 1 µg – 1 mg.

3.2.4 Y-tube olfactometer

A standard midge Y-tube olfactometer, comprising a Y-shaped glass tube (ID 15 mm, stem 160 mm, arms 140 mm, Figure 3.1) that allows insects to move towards volatiles/stimuli coming from the upwind end was used for behavioural bioassays, based on the design of Blackwell *et al* (1994). The Y-tube was located in a dark box, lined with white card, designed to remove any additional visual stimuli. The conditions in the bioassay room were maintained at 27.0 ± 2 °C and 60 – 80 % RH.

Air was pumped (diaphragm pump, 12 V, 0.5 amp, KNF Neuberger GmbH, Freiburg, Germany) via PTFE tubing (1/8" outside diameter (OD), 1.5 mm I.D., Omnifit, Cambridge, UK) through an activated charcoal filter (BDH, 10-14 mesh, 50 g) to remove impurities, then a conical flask containing distilled water (dH_2O) to provide humidity prior to being split, via a brass "T"-junction (Swagelok® London, Herts, UK), between the two arms of the olfactometer. Flow meters regulated the airflow in each arm at 150 ml min^{-1}. Extracts or chemicals (10 µl) in solvent (redistilled diethyl ether or redistilled hexane) were introduced onto filter paper (Whatman No. 1, 9 cm filter

papers, Whatman plc, UK, cut into strips 1 cm x 5 cm) by micropipette (10 µl Microcaps, Drummond Scientific, PA, USA) and placed in sintered glass expander/reducer couplings (Scientific Laboratory Supplies, Nottingham, UK, altered by Biochem Glass (Apparatus) Limited, glass blowers, Bletchley, UK). When sheep fleece was used, 400 ml conical flasks replaced the sintered glass couplings and airflow passed through the conical flasks via PTFE tubing. Groups of approximately 20 midges were collected and allowed to acclimatise in glass vials (4 ml flat bottom, screw cap, Chromacol, USA) for two hours in the dark (low-level red light). *Culicoides* species were inserted via the vertical port at the downwind end of the Y-tube apparatus (stem) and given three minutes to respond from when the lid sealed the dark box. In all bioassay experiments, relative attraction (the proportion of midges in the arm of the Y-tube containing the odour stimulus divided by the total number midges in both arms) was recorded. Treatments were randomised and for each replicate the arm containing the "test" treatment was selected at random.

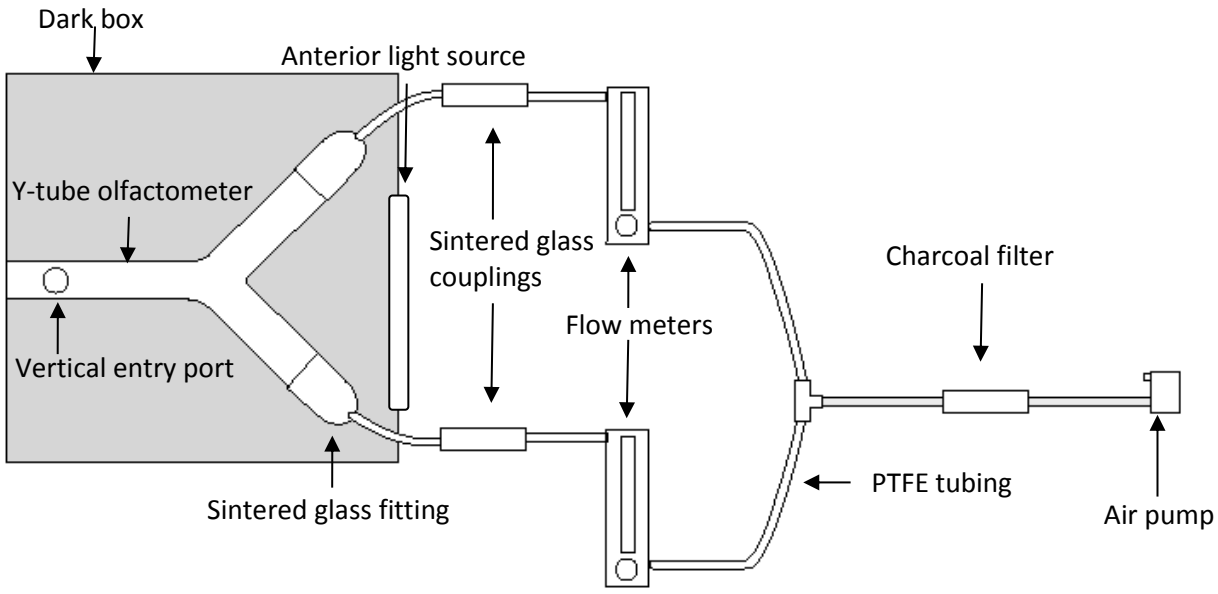


Figure 3.1: Y-tube olfactometer apparatus viewed from above. Airflow is from right to left. Sintered glass couplings (shown) are used when testing entrainment samples and chemicals.

All glassware was cleaned thoroughly using dH_2O and detergent (Teepol multiple purpose detergent, Teepol (UK), Kent, UK) then rinsed with acetone (99.8 % pure, Sigma-Aldrich Co., St. Louis, MO, USA) and distilled water, while the PTFE tubing was cleaned and rinsed with ethanol (99 % pure, Sigma-Aldrich Co., St. Louis, MO, USA) and distilled water. All equipment was baked for two hours at 180 °C in an oven. Charcoal

filters were cleaned weekly by baking in an oven (150 °C) for two hours whilst attached to a flow of clean nitrogen from a cylinder (BOC Industrial Gases, UK).

3.2.4.1 Positive control for *Culicoides* species

3.2.4.1.1 Laboratory reared *C. nubeculosus*

The behavioural response of laboratory reared *C. nubeculosus* to racemic 1-octen-3-ol, a known attractant to *Culicoides* species midges (Bhasin, 1996, Logan, 2006), was investigated to establish a positive control stimulus for use in future experiments, and to show that the functionality of the Y-tube olfactometer had not been impaired by the alterations. The equipment was set up as previously described (Section 3.2.4). A negative control consisting of redistilled hexane (10 µl) was tested along with 4 doses of racemic 1-octen-3-ol in hexane, 1 µg, 10 µg, 100 µg and 1 mg. All treatments were tested against redistilled hexane (10 µl). All experiments were conducted under red light with an anterior white light source. The experiment was replicated 12 times using a randomised complete block design.

3.2.4.1.2 Wild caught *Culicoides* species

The response of wild caught *Culicoides* species to the previously established positive control (racemic 1-octen-3-ol, 100 µg,) was investigated in the Y-tube olfactometer. No significant differences in relative attraction were noted (Generalised Linear Model (GLM), $P = 0.772$, n = 6, graph not shown).

Behavioural responses of wild caught *Culicoides* species to the enantiomer (*R*)-(-)-1-octen-3-ol were investigated in the Y-tube olfactometer. The same controls and dose range were used as for the positive control testing with *C. nubeculosus*. The experiment was replicated 12 times using a randomised complete block design (Section 3.3.1.2).

3.2.4.2 Response of *C. nubeculosus* in the absence of light

To determine if *C. nubeculosus* would respond in the Y-tube olfactometer without the necessity of an additional anterior light source stimulus (Blackwell *et al*, 1994, Bhasin, 1996, Mands *et al*, 2004) an experiment was carried out using the previously described equipment set up (Section 3.2.3). A negative control consisting of redistilled hexane (10 µl) on filter paper and a positive control consisting of racemic 1-octen-3-ol (100 µg) were tested. Treatments were tested under white light provided by two white fluorescent tubes and under red light provided by two fluorescent tubes surrounded by red acetate filters. As the lights were located directly above the box, the dark box was not sealed for this experiment. The experiment was replicated four times using a randomised complete block design.

3.2.4.3 Response of *C. nubeculosus* to volatiles from sheep fleece

Fleece from the neck, back and side/leg right (Table 2.1, Chapter 2, Section 2.2.1) areas of a sheep (Dorset Poll, mature ewe, approximately 85 kg) was weighed (0.1 g, 1 g and 10 g), providing nine "test" samples per replicate. In addition, a negative control consisting of a 400 ml conical flask containing glass wool (with an equivalent volume to 1 g of sheep fleece) and a positive control consisting of racemic 1-octen-3-ol (100 µg) were also tested. Racemic 1-octen-3-ol was presented on filter paper in glass sintered expander/reducing couplings, while the fleece was placed in 400 ml conical flasks as described previously (Section 3.2.3). Racemic 1-octen-3-ol was tested against hexane (10 µl), while the fleece samples were tested against equivalent volumes of glass wool. The experiment was replicated 16 times using a randomised block design.

3.2.4.4 Response of *C. nubeculosus* to whole sheep air entrainment extracts

For the investigation of behavioural activity of *C. nubeculosus* to sheep extracts a blank control (10 µl redistilled hexane) and a positive control (100 µg, racemic 1-octen-3-ol) were used. The extracts tested were 3 minute dose equivalents (of 1 sheep) of the "Pure" Hartline breed and "Cross" Hartline/Suffolk breed samples. Therefore, in the 3 minute duration in the Y-tube olfactometer, the insects were exposed to the

equivalent concentration of chemicals produced by 1 sheep during the 4 hour whole animal entrainment. Ten-fold and 100-fold dilutions of the 3 minute dose equivalent were also prepared. The extracts were tested against 3 minute dose equivalents (and dilutions) of the relevant control (empty box) entrainment. The experiment was replicated 12 times using a randomised block design.

3.2.5 Statistical analysis

A GLM (McCullagh and Nelder, 1989) with binomial distribution and logit link function was used to analyse the relative attraction to *C. nubeculosus* and wild caught *Culicoides* species to chemicals and the whole animal air entrainment sheep extract in the Y-tube olfactometer. Variation due to days (Day) was taken into account in the model as a blocking term. Also, time of day (Am_Pm) was accounted for when two or three replicates were run on one day. Time periods comprised: AM, 10:30 – 12:30; PM-1, 13:30 – 15:30 and PM-2, 15:30 – 17:30. A further possible explanatory variable, temperature (temp), and a factor (Arm_Trt) defined as the arm of the Y-tube olfactometer that the odour stimulus was delivered in were included to ascertain if they had a significant effect. Following these model terms the treatment effect was included (Trt). For the behavioural testing of the whole sheep entrainment extracts the treatment effect (Trt) was replaced with the term Controls/Sheep/TreatDil. This allowed more detailed analysis of the data, with the term Controls allowing for blocking by control type (i.e. separating the hexane control from the whole sheep entrainment controls), the term Sheep blocking by sheep breed and the term TreatDil effectively becoming the treatment factor showing the effects at the different dilutions of extract. The significance of all model terms was tested using an approximate F-test based on change in deviance. Particular treatments were compared to the control treatment using partial t-tests. The data, i.e. proportion (*p*) of midges in the arm of the Y-tube containing the odour stimulus divided by the total number midges in both arms, were analysed using GenStat (version 14.0). Model checking was carried out by considering residuals and evidence of over-dispersion (extra variation over and above that expected for a binomial distribution). Advice on experimental design and statistical analysis was provided by Dr. Stephen Powers (Biomathematics and Bioinformatics, Rothamsted Research, UK).

The GLM model for investigating behavioural response of *C. nubeculosus* to chemicals was:

$$y \sim Constant + Day + Day.AmPm + Arm\_Trt + Trt$$

where y was logit(*p*), Constant is an overall mean for the data on the logit scale, Day, AmPm and Arm_Trt are the blocking factors, Trt is the treatment factor, and the dot indicates the interaction between Day and AmPm.

The GLM model for investigating behavioural response of *C. nubeculosus* whole sheep entrainment extracts in the Y-tube olfactometer was:

$$y \sim Constant + Day + Arm\_Trt + (Controls/Sheep/TreatDil)$$

where y was logit(*p*), Constant is an overall mean for the data on the logit scale, Day and Arm_Trt are the blocking factors and Trt (treatment factor, seen above in a more simplified model) is replaced and split into Controls (blocking the hexane control and the whole animal sheep entrainment controls), Sheep (blocking by breed of sheep) and TreatDil (the treatment factor split by dilution).

Statistics on wild caught *C. obsoletus* to vented whole sheep entrainments were conducted by Andrew Hope (PhD Student, The Pirbright Institute) using a negative binomial model and taking into account total female *C. obsoletus* and the air temperature at sunset.

3.3 Results

3.3.1 Field trapping using expelled air from whole sheep air entrainment box (wild *Culicoides* species) – with Andrew Hope (The Pirbright Institute)

Venting of air from the whole animal entrainment box to a micro-CDC trap using elephant trunking resulted in significantly greater total catches when the sheep were present in the box, compared to the control (empty box, Table 3.1). Overall, a significant difference in the catches was found ($P < 0.05$)(Appendix 1). Both the vented air from the pure breed sheep ($P < 0.001$) and the cross breed sheep ($P < 0.05$) caught significantly more female *C. obsoletus* compared to the blank negative control (no extract, no light) trap. There was no significant difference noted between the catches from the two sheep breeds ($P = 0.24$). The CDC positive control (with light) caught significantly more than any other trap treatment ($P<0.001$).

Table 3.1: Total number of *Culicoides* species collected in the micro-CDC trap combined with vented air from the whole animal entrainment box. Pure breed n = 9, Cross breed n = 6.

Breed		Pure			Cross	
Sample	Control	Sheep	UV Light	Control	Sheep	UV Light
Total caught	6	108	1050	3	19	203

3.3.2 Determination of positive control for *Culicoides* species

3.3.2.1 Laboratory reared *C. nubeculosus*

Behavioural responses of *C. nubeculosus* to racemic 1-octen-3-ol concentrations were investigated in the Y-tube olfactometer (Figure 3.2). Of 1069 midges used, 735 midges responded by moving up the Y-tube into one of the arms (68.8 %). There was an overall significant difference between treatments ($F_{4,43} = 3.77$; $P = 0.01$, F-test from GLM model). The blank control showed 47.9 % relative attraction. A drop in relative attraction, compared to the blank control, was noted at the 1 µg dose, followed by an increase in relative attraction at 10 µg and a significant increase at 100 µg (63.1 %, $P = 0.005$, t-test following GLM model). At 1 mg a fall in relative attraction was observed to levels similar to that of the blank control.

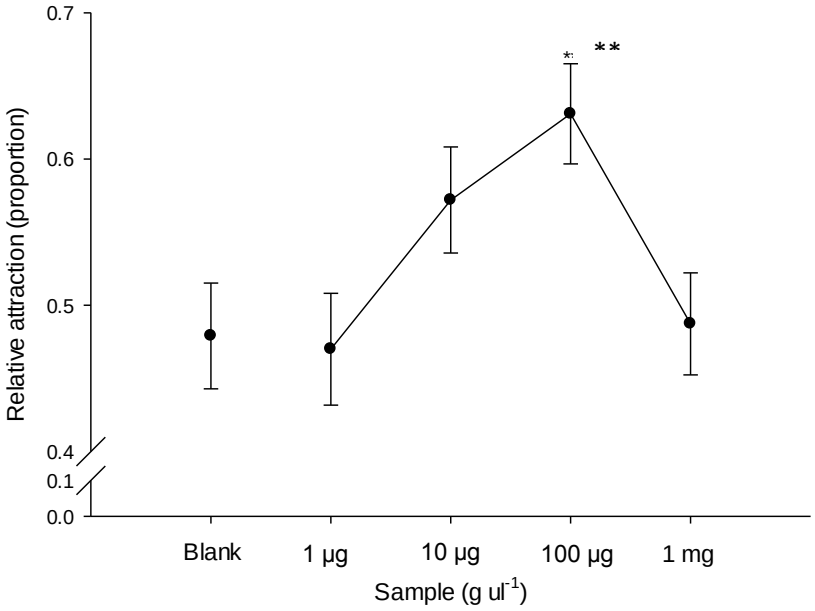


Figure 3.2: Relative attraction of *Culicoides nubeculosus* females to different doses of racemic 1-octen-3-ol and a blank control (hexane) in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared to the blank control. Results shown as mean relative attraction ± standard error. Relative attraction at 1 x 10^{-4} g μl^{-1} was 63.1 %. (GLM, $^{**}P < 0.01$, n = 12).

3.3.2.2 Wild caught *Culicoides* species

Behavioural responses of wild caught *Culicoides* species to the enantiomer (*R*)-(-)-1-octen-3-ol were investigated in the Y-tube olfactometer (Figure 3.3). There was an overall significant difference between treatments ($F_{4,35} = 17.71$; $P < 0.001$, F-test from GLM model, n = 10) and significant relative attraction, compared with the blank control, was found at 10µg (65.2 % ± 0.03 standard error (SE) $P = 0.008$, t-test within GLM model). The blank control showed 52.2 % (± 0.03 SE) relative attraction. There was no significant difference between the control and 1 µg or 100 µg doses of (*R*)-1-octen-3-ol. Relative attraction dropped significantly below the blank control at 1mg (28.0 % ± 0.03 SE, $P < 0.001$, t-test within GLM model).

For future Y-tube olfactometer behavioural work with wild-caught midges, (*R*)-(-)-1-octen-3-ol (10 µg) was used as the positive control.

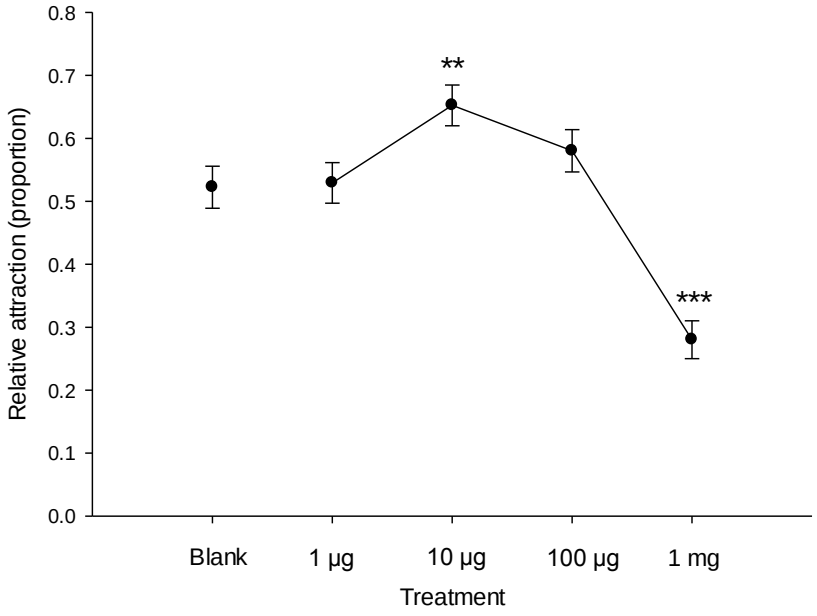


Figure 3.3: Relative attraction of wild *Culicoides* species to different doses of (*R*)-(-)-1-octen-3-ol and a blank control (hexane) in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared with the blank control. Results shown as mean relative attraction ± standard error. Relative attraction at 10 µg was 65.2 % (GLM, $^{**}P < 0.01$, n = 10) and at 1 mg was 28.0 % (GLM, $^{***}P < 0.001$, n = 10) compared with the blank control (hexane).

3.3.3 Response of *C. nubeculosus* in the absence of light

Behavioural responses of *C. nubeculosus* were investigated in the absence of an anterior light source. Out of 676 midges used only 12 in total moved into either arm of the Y-tube (1.7 %). Observations showed that the midges stayed in the release container, stayed in the Y-tube near the vertical entry point of the release chamber, or moved backwards down the stem of the Y-tube olfactometer. Furthermore, it was noted that under white light (from above), many of the midges attempted to crawl or fly vertically. As a result an anterior light source was kept for future experiments to stimulate upwind movement of the midges in the Y-tube olfactometer.

3.3.4 Response of *C. nubeculosus* to volatiles from sheep fleece

Behavioural responses of *C. nubeculosus* to different areas of sheep fleece tested at three different weights were investigated in a Y-tube olfactometer bioassay (Figure

3.4). A total of 3240 midges were used during the experiment and 2204 showed a response by moving into one of the arms of the Y-tube (68.0 %). As the temperature variate was not significant ($F_{1,146}$ = 0.71; *P* = 0.402, F-test from GLM model) it was excluded from the model. However, blocking terms for days, time of day (am/pm) and the arm of the Y-tube olfactometer that the treatment stimulus was delivered in were retained.

There was no overall significant difference between treatments ($F_{10,147}$ = 0.92; *P* = 0.513, F-test from GLM model). This appeared to be due to the high variation for the fleece data. Generally, further analysis would not be carried out following a negative F-test result, unless a pre-defined treatment was to be investigated, i.e. the positive control, racemic 1-octen-3-ol (100 µg). To check validity of the experiment the positive control was compared to the blank control and was found to show a significant increase in relative attraction (64.1 %, *P* = 0.038, t-test following GLM model). The blank control showed 49.9 % relative attraction and a trend of an increase in relative attraction was noted for Side 1 g (60.3 %).

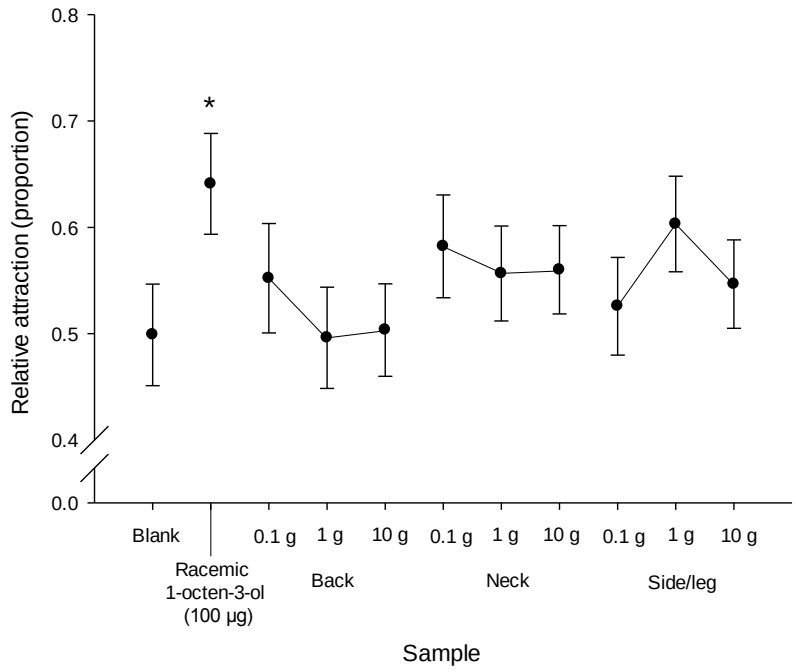


Figure 3.4: Relative attraction of *Culicoides nubeculosus* females to different areas and weights of sheep fleece and controls in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared to the blank control. Results shown as mean relative attraction ± standard error. Relative attraction for racemic 1-octen-3-ol was 64.1 %. (GLM, **P* < 0.05, n = 16).

3.3.5 Response of *C. nubeculosus* to whole sheep air entrainment extracts

A significant effect of treatment x dilution combinations was found ($F_{5,89}$ = 3.56; *P* = 0.006, F-test from GLM model) showing significant increases in relative attraction of *C. nubeculosus* at the 3 minute dose equivalent (61.7 % ± 0.04 SE, *P* < 0.05, based on Least Significant Differences (LSDs) predicted from the GLM model) and 100-fold dilution (53.0 % ± 0.04 SE, *P* < 0.05, based on LSDs predicted from the GLM model) for the combined (Pure + Cross breed) sheep extract data compared with the relevant combined control doses (Figure 3.5). Overall differences between the control extract dilutions and sheep extract dilutions were not significant ($F_{2,89}$ = 2.48; *P* = 0.090, F-test from GLM model) and there was no statistical differences between the sheep breed extracts ($F_{1,89}$ = 0.08; *P* = 0.777, F-test from GLM model, n = 8). The racemic 1-octen-3-ol (62.2 % ±0.05 SE *P* = 0.034, t-test within GLM model) showed significantly greater relative attraction of *C. nubeculosus* compared to the blank (hexane) control (44.6 % ± 0.06 SE).

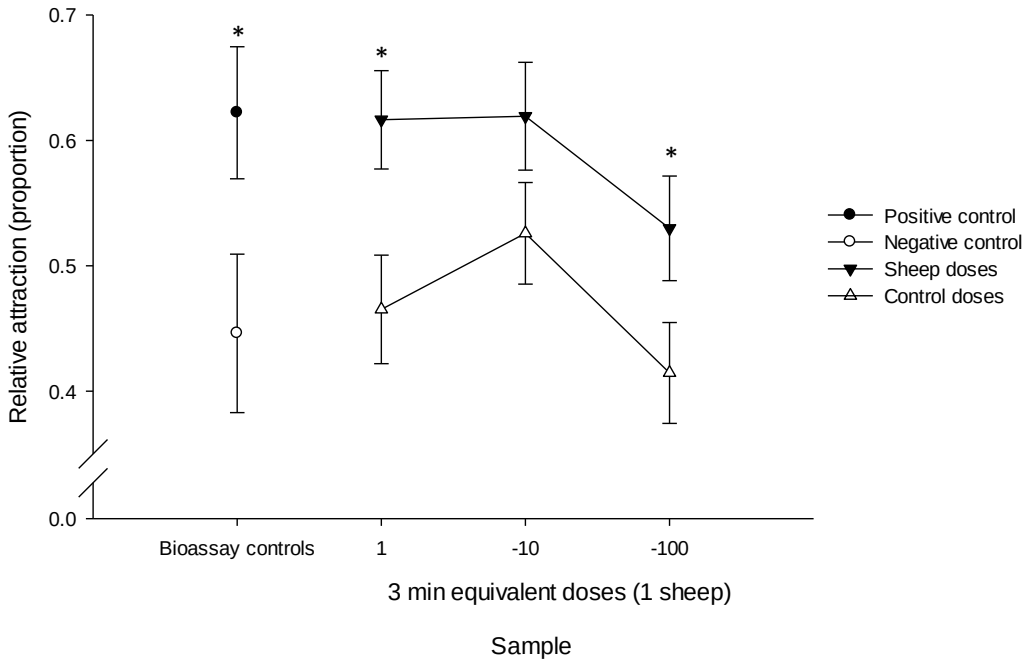


Figure 3.5: Relative attraction of *C. nubeculosus* to 3 minute equivalent sheep extract, 10-fold and 100-fold dilutions, a negative control (hexane) and a positive control racemic 1-octen-3-ol in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared to the relevant control. Results shown as mean relative attraction ± standard error. Relative attraction at the 3 minute dose equivalent sheep extract was 61.7 % and at the 100-fold dilution was 53.0 %. The racemic 1-octen-3-ol positive control was 62.2 % (GLM, **P* < 0.05, n = 8) and significantly different from the negative bioassay control.

3.4 Discussion

Having elected to develop the whole animal entrainment box for the purpose of collecting volatiles from sheep, the first priority was to determine whether the volatiles released by the sheep in the box were attractive to wild *Culicoides* species in the field. Trap catches of wild *Culicoides* species were significantly increased ($P < 0.001$, "Pure" breed, $P < 0.05$, "Cross" breed) by venting expelled air from the whole animal air entrainment equipment to an unlit micro-CDC trap, demonstrating that volatiles from the sheep attract *Culicoides* species. It should be noted, that the experiment used unlit CDC traps without the addition of CO_2, however, CO_2 would have been present in the vented air from the whole animal entrainment box due to its presence in exhaled sheep breath. Carbon dioxide comprises 4 % of exhalations from vertebrates (Cardé and Gibson, 2010). The average weight of mature Suffolk breed ewes is estimated at 84 kg (NSA, 2013). Information reported on breathing in sheep suggests a respiratory rate of 15 – 40 breaths min^{-1}, and a tidal volume of 4 – 9 ml kg^{-1} (Adams and McKinley, 1995). Taking the means for these figures yields 27.5 breaths min^{-1} and a tidal volume of 6.5 ml kg^{-1}. Therefore, total tidal volume (exhaled air) per breath for a mature ewe would be 546 ml of which 21.84 ml would be CO_2. At 27.5 breaths min^{-1} this equates to 600.6 ml min^{-1} of CO_2 per sheep, or potentially, 1801.8 ml min^{-1} CO_2 being present in the vented air from the whole animal air entrainment box (based on three sheep).

Previous investigations have compared the efficacy of CO_2 flow rates of 500 ml min^{-1} and 1000 ml min^{-1} in catching *Culicoides* species in England (Harrup, 2010). No significant difference between the two flow rates was found and it was noted that few *C. obsoletus* were caught. Bhasin *et al* (2001) reported a dose-response for CO_2 with increasing trap catches of female *C. impunctatus* at CO_2 flow rates from 600 ml min^{-1} to 2500 ml min^{-1}. The same dose response for CO_2 was recorded for *C. furens* and *C. melleus* in Georgia, USA, where increasing flow rates of CO_2 from 20 ml min^{-1} to 2000 ml min^{-1}, resulted in increasing trap catches (Kline *et al*, 1994). The Mosquito Magnet® Pro™ traps used in some trapping experiments of *Culicoides* species release CO_2 at approximately 500 ml min^{-1}, based on manufacturer's information, from the butane of butane or propane gas. Additionally, it is worth noting that this also provides a thermal cue (Harrup *et al*, 2012, Mands *et al*, 2004). These studies would suggest that the release rate of CO_2 estimated from the sheep in the experiment investigating vented

air from the whole animal entrainment box is comparable with previous experiments and within the dose-response ranges reported for other *Culicoides* species. Furthermore, a number of early investigations found attraction of *Culicoides* species using a CO_2 flow rate of 200 ml min^{-1} (Kline *et al*, 1990, Ritchie *et al*, 1994, Takken and Kline, 1989). The above studies all controlled release rates of CO_2 regulated from gas cylinders, however, other investigators have used dry ice as a CO_2 source (Gerry and Mullens, 1998, Mullens, 1995, Mullens *et al*, 2005, Weiser-Schimpf *et al*, 1991). As well as cooling the area around the trap as the dry ice sublimates, unstable release rates of CO_2 are produced, usually starting high and decreasing markedly over time. For example, Mullens (1995) found that the release rate of CO_2 during the first two hours was 1486 ml min^{-1}, dropping to 303 ml min^{-1} after 10 – 12 hours.

The estimated 600 ml min^{-1} release rate calculated for sheep in this study is comparable with flow rates used in previous investigations and even when accounting for a combined release rate of 1800 ml min^{-1}, vented from the whole animal entrainment box when three sheep were present, falls within the ranges previously shown to elicit a dose-response in *Culicoides* species (Kline *et al*, 1994, Mands *et al*, 2004). It should however, be noted that a number of studies reported low catches of *C. obsoletus* suggesting that CO_2 may not act as an important host cue for this species, at least on its own in the absence of other potential stimuli (semiochemical, thermal, visual)(Gerry, *et al*, 2009, Harrup, 2010, Mullens *et al*, 2005).

Additionally, a similar method of using vented/exhausted air from cattle or humans in a PVC-coated tent was utilised in Côte d'Ivoire and Burkina Faso investigated the effect of natural host odours on tsetse flies (Rayaisse *et al*, 2010). It was found that natural cattle odour increased mean trap (bi-conical design) catches per day by 5.1 times for *G. tachinoides* and 6.1 times for *Glossina palpalis gambiensis* Vanderplank compared with un-baited traps (Rayaisse *et al*, 2010). Cattle exhale much greater quantities of CO_2 with each breath; therefore, it is likely that each cow was releasing approximately 10 l min^{-1} of CO_2 during this experiment (Zöllner *et al*, 2004).

Gerry *et al* (2009) reviewed direct aspiration from hosts and drop trapping experiments in relation to *Culicoides* species and reported mean and max biting rates per minute (where possible). For *C. obsoletus* attacking sheep, mean biting rates

ranged from 0.9 attacks min^{-1} (Spain) to 20 attacks min^{-1} (New York). Carpenter *et al* (2008a) did not report a mean attack rate from their drop trapping experiment with sheep and *C. obsoletus*, but instead report a maximum attack rate of 17 attacks min^{-1}. The total number of *Culicoides* species caught through attraction to vented sheep volatiles was 127 (Section 3.3.1, Table 3.1), and therefore clearly underestimates these attack rates. It is clear that although *Culicoides* species (especially *C. obsoletus*) respond to volatiles from sheep in the absence of other stimuli (i.e. visual cues), this is typically at a lower response than is found by direct aspiration from sheep or with drop traps and implies that other stimuli may be necessary to improve attraction. However, in the meantime, successful attraction of wild *Culicoides* species in the field to host derived volatiles provided justification to further investigate the entrainment extracts in the laboratory using the Y-tube olfactometer and confirm the collection of behaviourally active extracts.

In laboratory experiments, preliminary studies demonstrated the necessity of the anterior light source as a co-stimulus in order to encourage activation and movement of the midges in the Y-tube olfactometer. This result corroborates previous work which found that only very low numbers of midges responded in the absence of an anterior light source (Blackwell et al, 1994). It is therefore worth noting that future discussion of attraction to chemicals shown by *Culicoides* species in the Y-tube (in this and later chapters) also includes light as a co-stimulus. Further optimisations of the Y-tube olfactometer successfully removed additional unnecessary stimuli. Following these alterations a dose response bioassay with racemic 1-octen-3-ol showed significant relative attraction (63.1 %) of *C. nubeculosus* at the 100 µg dose compared with the blank (redistilled hexane) control (Figure 3.2) in agreement with previous studies (Bhasin *et al*, 2000a). This suggests that the modified bioassay design was suitable for the behavioural experiments. Interestingly, a drop in relative attraction was noted at 1 mg, which is in contrast to a previous finding where a greater response by *C. nubeculosus* was observed in a Y-tube olfactometer at this dose compared with the 100 µg dose and a drop in attraction was not observed until the 10mg dose (Bhasin *et al*, 2000a). However, it is supported by previous work investigating the effects of racemic 1-octen-3-ol and enantiomeric mixtures of 1-octen-3-ol on *St. aegypti* and *Culex quinquefasciatus* Say mosquitoes (Cook *et al*, 2011). Drops in relative attraction

of mosquitoes to the chemicals in the Y-tube olfactometer were noted when a mixture contained the (*R*)-(-)-1-octen-3-ol enantiomer and the decreases in relative attraction were more pronounced for *Cx. quinquefasciatus,* dropping significantly below the level of the control and therefore implying a possible repellent effect (Cook *et al*, 2011). The level of response of *C. nubeculosus* to 1-octen-3-ol in the Y-tube olfactometer was also comparable to previous studies using *C. impunctatus* where significant responses were found at three concentrations (1×10^{-6} g μl^{-1}, 1×10^{-5} g μl^{-1} and 1×10^{-4} g μl^{-1} or 1 µg, 10 µg and 100 µg respectively) with relative attraction ranging between approximately 59 % to 68 % (Logan, 2006). Studies by Bhasin *et al* (2000a) and Blackwell *et al* (1997) have also looked at the response of *C. nubeculosus* and *C. impunctatus* in the Y-tube olfactometer, however, these studies report their results with relative attraction as the sum of differences between observed and expected results making direct comparison with Logan (2006) or the work presented here difficult. Following successful collection of wild *Culicoides* species during the field season it was discovered that they were not responding to the racemic 1-octen-3-ol positive control. The (*R*)-(-)-1-octen-3-ol enantiomer was trialled and found to elicit a significant increase in relative attraction of wild *Culicoides* species at a ten-fold lower sensitivity (10 µg) compared with *C. nubeculosus* response to racemic 1-octen-3-ol (100 µg). Furthermore, a significant drop in relative attraction of wild *Culicoides* species at the strongest does tested (1 mg) was observed, in line with the results of Cook *et al* (2011). Harrup (2010) investigated the traps baited with CO_2 and the enantiomers of 1-octen-3-ol, comparing catches with a standard OVI trap and drop trap catches with sheep. It was found that the (*R*)-(-)-1-octen-3-ol + CO_2-baited trap more accurately reflected the species composition found on the sheep (based on drop trapping) when compared to the OVI trap catch. Additionally, the semiochemical baited traps were shown to have collected all found member species of the *C. obesoletus* group however insufficient numbers of the *C. pulicaris* group were caught for statistical analysis (Harrup, 2010). This result, together with the findings reported in this chapter, suggest that (*R*)-(-)-1-octen-3-ol is more effective with wild *Culicoides* species. This may because (*R*)-1-octen-3-ol is the naturally occurring enantiomer which would also explain why it out performed the (*S*)-(+)-1-octen-3-ol enantiomer and the racemic blend (Kline *et al*, 2007, Syed and Leal, 2007).

Effective attraction of *C. nubeculosus* to whole sheep entrainment extracts in the laboratory, without the need for the addition of CO_2, suggests that CO_2 is not always necessary to elicit a response in *Culicoides* species. This is an interesting result, as when attraction was shown in the field, using air vented from the whole animal air entrainment box, CO_2 was present, released in the exhaled breath of the sheep (approximately 600 ml min^{-1} $sheep^{-1}$). However, although previous investigations have successfully collected *Culicoides* species in the field in the absence of CO_2, when presented in combination with a semiochemical (commonly racemic 1-octen-3-ol) it appears to act synergistically, significantly increasing trap catches. The addition of racemic 1-octen-3-ol (wick-in, low rate) to unlit CDC traps without the addition of CO_2 resulted in trap catches of 117.4 (± 34.4) *C. furens* per day (n = 16 days), and less than 1 *C. melleus* per day in Georgia, USA, compared with catches of 24447.3 (± 7377.2) *C. furens* per day and 98.0 (± 57.3) *C. melleus* per day (Kline *et al*, 1994). In two other experiments the trap catches for *C. furens* were 83.2 (± 32.9) and 22.7 (± 4.7) per day respectively, (n = 5 days) using racemic 1-octen-3-ol without the addition of CO_2 and 6290.2 (± 2090.5) and 1596.0 (± 490.4) with CO_2 (Kline *et al*, 1994). A different study, using delta traps (an unlit trap style) baited with racemic 1-octen-3-ol (0.06 mg hr^{-1}) found mean catches of *C. impunctatus* were greater at 19.5 (± 0.81) compared with an un-baited control at 11.7 (± 0.8) (n = 5) although there was no comparison with the addition CO_2 in this experiment (Bhasin *et al*, 2001). Harrup *et al* (2012) investigated the effect of CO_2 and racemic 1-octen-3-ol and the pure enantiomers of (*R*)-(-)-1-octen-3-ol and (*S*)-(+)-1octen-3-ol using unlit micro-CDC traps (1-octen-3-ol release rate was between 4.04 – 4.22 mg/h). The CO_2 + (*R*)-(-)-1-octen-3-ol trap caught significantly more *C. obsoletus* and *C. nubeculosus* compared with the CO_2 trap and significantly more *C. obsoletus* compared with the CO_2 + racemic 1-octen-3-ol trap. Interestingly in this experiment the numbers of *Culicoides* species caught with the volatile chemical + CO_2 baited unlit micro-CDC traps were atypically greater compared with two OVI UV light suctions traps (Harrup *et al*, 2012). Interestingly, a solvent extract of sheep fleece, tested in the field with the addition of CO_2 found a 53 % reduction in *C. impunctatus* caught compared with the standard baited control (CO_2 + racemic 1-octen-3-ol), however when the same extract was tested at a range of concentrations in the laboratory without the addition of CO_2, the greatest concentration showed a

significant increase in relative attraction of *C. impunctatus.* This implies that the extract may have performed better in the field without the addition of CO_2, or that CO_2 was in fact reducing the potential attraction of the extract.

The use of Y-tube olfactometers is common when investigating insect behaviour and responses to odours in the laboratory (Blackwell *et al*, 1997, Geier and Boeckh, 1999, Geier *et al*, 1999, Steib *et al*, 2001, Blackwell *et al*, 2004, Logan *et al*, 2009). A fuller critique of the Y-tube olfactometer as a bioassay is featured in Chapter 5. It is noted that, whilst laboratory models are useful (*C. nubeculosus*), it is preferable to work with the insect of interest, or better still field caught insects of interest (Logan *et al*, 2010). The differences in response of the laboratory reared *C. nubeculosus* and the wild caught *Culicoides* species, to 1-octen-3-ol, reported in this chapter serve to highlight that point. The dose of racemic 1-octen-3-ol (100 µg) found to significantly increase relative attraction in the Y-tube olfactometer for *C. nubeculosus* was ineffective with wild caught *Culicoides* species prompting the investigation and adoption of the natural (*R*)-(-)-1-octen-3-ol enantiomer (10 µg) as the positive control for wild *Culicoides* species. Clearly there are species specific differences in the response of *Culicoides* species to 1-octen-3-ol. While a positive result in the Y-tube olfactometer does not guarantee an effective attractant in the field, it is encouraging that there appears to be a degree of consistency in laboratory and field results when using 1-octen-3-ol to attract *C. impunctatus*, therefore implying that the Y-tube olfactometer is a suitable bioassay for the identification of potential behaviour eliciting semiochemicals (Bhasin *et al*, 2000a, 2001, Blackwell *et al*, 1996, Harrup *et al*, 2012, Logan, 2006, Mands *et al*, 2004). Any results obtained from the Y-tube olfactometer would need to be confirmed in field, however, as a bioassay it provides a relatively fast and easy way of screening potential attractants compared with the field (Logan *et al*, 2010).

It was found that the response of *C. nubeculosus* to sheep fleece samples in the Y-tube olfactometer was highly variable resulting in no statistically significant relative attraction being achieved. The greatest relative attraction was to 1 g fleece (side/leg right, Figure 3.4). The fact that the positive control produced a significant response suggests that the bioassay was functioning correctly and was not the cause of the non-response towards the fleece. It was feasible that fleece alone may not have provided all the necessary chemical signals to elicit attraction of *C. nubeculosus* in the Y-tube

olfactometer. Solvent washing of fleece, while good for collecting low volatile chemicals, may entirely miss volatile chemicals released from the skin of the animal. This was a major consideration in the design of the whole animal air entrainment equipment which allowed semiochemicals released from the fleece, breath, urine and faeces to be collected providing an extract, more representative of the natural host. Successful attraction to hexane extracts of animals has been shown previously, where the authors soaked 22 g of hair in 20 ml of hexane for 7 days at 5 °C (Mands *et al*, 2004). Using the extracts to bait Mosquito Magnet® Pro™ traps, they found that water buffalo provided a significant increase in total trap catch (+262 %) compared with the CO_2 + racemic 1-octen-3-ol control trap, and also caught significantly more *C. impunctatus* and *C. pulicaris* females. Interestingly, it was found that the sheep extract, along with deer, calf and pony, failed to significantly enhance the trap catches, with the sheep extract showing a 53 % reduction in total trap catch compared with the CO_2 + racemic 1-octen-3-ol control (Mands *et al*, 2004). However, it is noted that the release rate of racemic 1-octen-3-ol was quite high (6-8 mg/h) and may have produced a masking effect. As discussed previously, there is evidence that high doses of 1-octen-3-ol can result in a loss of attraction (Bhasin *et al*, 2000a, Cook *et al*, 2011). More recently, an experiment in East Africa investigated enhancing standard CDC light trap catches of mosquitoes using animal volatile baits from sheep (19 g of fresh hair from the belly and back areas, in a canister hung at the base of the CDC trap in the airflow) in combination with CO_2 or light (Tchouassi *et al*, 2012). It was found that traps with CO_2 (Light + CO_2 and Light + CO_2 + sheep hair) caught significantly more mosquitoes compared with the light only control trap. However, while increases in trap catches were noted when comparing Light + CO_2 + sheep hair with Light + CO_2 and also Light + sheep hair with Light only, these increases were not statistically significant, suggesting that CO_2 was responsible for the increased attraction rather than the sheep hair. (Tchouassi *et al*, 2012).

Significant relative attraction of *C. nubeculosus* was discovered for the combined whole sheep entrainment extracts at the 3 min dose equivalent and 100-fold dose equivalent level, proving behavioural attraction to the extract in the laboratory. No significant differences in relative attraction were recorded in the response of *C. nubeculosus* to the “Pure” Hartline breed extract, nor the “Cross” Hartline/Suffolk

breed extract at any of the doses tested. The lack of difference may not be surprising considering the two breeds involved were not distinct pure breeds. The previously mentioned investigation by Mands *et al* (2004) into responses of *Culicoides* species showed *C. nubeculosus* responded differentially to animal solvent extracts (calf, deer, sheep, pony and water buffalo) using EAG, while *C. impunctatus* responded differentially to the same animal extracts in a Y-tube olfactometer (Mands *et al*, 2004). It was also found that *C. impunctatus* and *C. pulicaris* showed differential attraction to the same range of animal extracts when added to Mosquito Magnet® Pro™ traps in the field (Mands *et al*, 2004). Recently, differential attraction of *C. impunctatus* has also been shown to odours entrained from individual human beings (Logan *et al*, 2009). Differential attraction has also been shown for horn fly loads in cattle herds (Birkett *et al*, 2004, Jensen *et al*, 2004). Horn fly loads were determined for individual cattle and it was shown that moving specific cattle between different herds could alter the overall horn fly load of the herd (Jensen *et al*, 2004). This intraspecific difference was not examined in this study as the design of the whole animal entrainment extract experiment did not allow for collection of volatiles from individual animals as three sheep were introduced into the box per replicate. This was to help reduce stress volatiles as sheep are social animals and can become distressed when isolated or separated from the flock (Price and Thos, 1980). Redesigning the whole animal entrainment box so that the walls were made from strong, relatively inert transparent Perspex (or similar) material, may allow entrainment of individual sheep without the associated stress caused by isolation. This would therefore allow an intraspecific study, similar to the one described above (Birkett *et al*, 2004, Jensen *et al*, 2004). A study of this nature may be able to further delineate the differential response of *Culicoides* species to sheep (within and between breeds).

In light of the significant attraction results with vented whole sheep volatiles in the field and the sheep-derived entrainment extracts in the laboratory (Section 3.3.5) compared with the non-significant laboratory results using sheep fleece alone (Section 3.3.4), it was decided further investigations would focus on the sheep-derived entrainment extracts. Additionally, as the whole sheep entrainment extracts should include all the volatiles obtained from laboratory entrainments of fleece (Chapter 2,

Section 2.2.3), in addition to volatiles from breath, urine and faeces, no further investigation of the laboratory fleece entrainments took place.

3.5 Conclusions

Volatiles from sheep have been shown to be behaviourally active in both the field (wild *C. obsoletus* complex species) and the laboratory (with *C. nubeculosus*). The sheep derived extract can now be studied further to identify electrophysiologically active chemicals (Chapter 4) and putative attractants/repellents (Chapter 5) for use in the field (Chapter 6).

The Y-tube olfactometer bioassay apparatus was successfully modified to investigate the behaviour of *C. nubeculosus* to volatiles. Statistically significant relative attraction responses of *C. nubeculosus* have been recorded to racemic 1-octen-3-ol (100 µg) and of wild caught *Culicoides* species to (*R*)-(-)-1-octen-3-ol (10 µg) providing positive controls for further experiments (Chapter 5).

Chapter 4: Identification of electrophysiologically active chemicals in whole sheep air entrainment extracts

4.1 Introduction

Culicoides species midges, like many other haematophagous insects, use olfaction, as well as heat, moisture and visual cues, to locate vertebrate hosts from a distance (Gibson and Torr, 1999). In chemical ecology studies, it is common practice to record electrophysiological responses from the antenna of insects, called EAG, to help in the identification of compounds of biological importance. This technique was first described by Schneider in 1957 and can be used for testing single compounds and/or whole odour extracts for determining olfactory sensitivity to the compounds.

Culicoides species possess olfactory sensilla on their antennae which play a role in host location. For example, a detailed study of the morphology of the antennae of *C. impunctatus* and *C. nubeculosus* demonstrated morphological differences in sensilla between midge species and suggested that these sensilla may play a role in the selection of different host species (Blackwell *et al*, 1992). Indeed, Mands *et al* (2004) used EAG to investigate the differential attraction of *C. nubeculosus* to solvent extracts from calf, sheep, deer, pony and water buffalo hair and showed differential EAG responses which may reflect host preferences. Subsequent studies have examined the electrophysiological response of *C. impunctatus* and/or *C. nubeculosus* to individual host-derived semiochemicals including 1-octen-3-ol, lactic acid, acetone, butanone and a range of phenolic compounds (Blackwell *et al*, 1996, 1997, Bhasin, *et al* 2000a). As well as studying electrophysiological responses from the insect antennae, the response of receptors on the maxillary palps (*Sensilla basiconica*) to CO_2 has been investigated for *C. furens*, *C. mississippiensis* and *C. stellifer* (Grant and Kline, 2003). Furthermore, the authors also tested the maxillary palps of *C. furens* for responses to previously proven EAG-active chemicals (for mosquitoes) including racemic 1-octen-3-ol and a 6-, 7- and 8-carbon branched ketone, however, no significant consistent responses were recorded (Grant and Kline, 2003). It is noted that the lack of electrophysiological response to these chemicals in the maxillary palps suggests that appropriate receptors are more likely to be found on the antennae, and this is supported by the results of Blackwell *et al* (1996, 1997) and Bhasin *et al* (2000a).

For a full investigation of natural host extracts EAG can be combined with gas chromatography (GC) to give a technique called coupled GC-EAG. Bearing in mind vertebrates can produce more than 300 volatile chemicals this technique allows for a rapid screening of many chemicals within a complex extract (Moorhouse *et al*, 1969). The employment of gas chromatography-electroantennography (GC-EAG) techniques to investigate host volatile extracts with *Culicoides* species was first done by Logan *et al*, (2009). In this study, they investigated the responses of *C. impunctatus* to human-derived air entrainment extracts. Fifteen EAG-active peaks were reported and 12 chemicals were identitified. These chemicals included benzaldehyde, 6-methyl-5-hepten-2-one, octanal, nonanal, decanal and geranylacetone. Additionally, three unknown chemicals were uniquely EAG-active for *C. impunctatus* compared with *St. Aegypti.* Although identified from a human derived extract by Logan (2006), octanal and nonanal have been shown to be present in air entrainments of 4-12 week old sheep wool during investigation of host-derived extracts with triatomine bugs (*Triatoma infestans* King) (Guerenstein and Guerin, 2001).

The aim of this chapter was to locate and identify electrophysiologically-active chemicals within the whole sheep entrainment extracts (obtained in Chapter 2).

Objectives:

- To locate electrophysiologically-active chemicals within the whole sheep entrainment extracts using GC-EAG.
- To tentatively identify the EAG-active chemicals using GC-MS.
- To confirm the identification of the EAG-active chemicals by peak enhancement.
- To quantify the confirmed EAG-active chemicals present in the whole sheep entrainment extracts.

4.2 Material and methods

4.2.1 Insects

The laboratory reared *C. nubeculosus* and wild caught *Culicoides* species used in GC-EAG experiments in this chapter were obtained or collected according to the methods detailed in Chapter 3 (Section 3.2.2).

4.2.2 Coupled gas chromatography-electroantennography (GC-EAG)

Coupled GC-EAG was used to investigate the presence of EAG-active chemicals for *Culicoides* species to the whole sheep entrainment extracts (Chapter 2, Section 2.2.4.3). Both the "Pure" Hartline breed sheep extract and the "Cross" Hartline/Suffolk breed sheep extract were tested using *C. impunctatus*, while the "Pure" breed extract was also tested using wild caught *Culicoides* species.

A Hewlett Packard, HP6890 gas chromatograph system equipped with a non-polar methylsiloxane HP-1 (30 m, 0.32 mm ID, 0.52 µm film thickness) column with a cool-on-column (COC) injector and an FID was used to analyse the combined whole sheep air entrainment extracts (3 µl injection). Helium was the carrier gas. The following oven temperature programme was used: 40 °C hold for 2 min, then 5 °C min^{-1} to 100 °C, hold for 0.1 min, then 10 °C min^{-1} to 250 °C, final hold for 35 min, total run time 54.1 min. The column was split with one end leading to the FID while the other end was inserted into the continuous airflow, described below, via a heated transfer line, so that the effluent passed over the insect head/antennae preparation. Signals were recorded/analysed using computer software (EAD 2000, Syntech®, The Netherlands).

Culicoides species were chilled on ice for approximately 1 minute then placed under a compound light microscope where the head, proboscis, maxillary palps and antennae tips were removed using a scalpel. Electrodes, made of silver/silver chloride (Ag/AgCl) wires (diameter, 0.37 mm, Harvard Apparatus, Edenbridge, UK), were inserted into glass pipettes made from borosilicate glass capillary tubes (OD 2.0 mm, ID 1.16 mm, Harvard Apparatus, Edenbridge, UK) filled with Ringers solution (7.55 g sodium chloride, 0.64 g potassium chloride, 0.22 g calcium chloride, 1.73 g magnesium

chloride, 0.86 g sodium bicarbonate and 0.61 g sodium orthophosphate l^{-1} in water, pH 7.4). The indifferent electrode was inserted into the back of the midge head and the distal ends of both antennae were inserted into the recording electrode. A continuous, charcoal filtered, humidified airflow (1 l min^{-1}) was passed over the insect head and antennae via a glass tube positioned 5 mm away. Five replicates (individual insects) were used for each set of GC-EAG runs.

Electrophysiological responses from GC-EAG analyses were examined by overlaying traces on a light box to identify EAG responses by visual inspection and match them to peaks on the GC trace. Three or more individuals (out of five) responding to the same peak on the GC trace was classed as a consistent EAG response and therefore the peak was considered to be EAG-active.

To allow for calculation of retention indices (RI) a solution of alkanes (C_7 – C_{25}, 100 ng μl^{-1}, 1 µl injection) in redistilled hexane was also analysed. The RI for a compound is a number that indicates its retention relative to adjacent alkanes and allows for comparison of data between different GC machines and columns (but maintaining the same column type i.e. HP-1). The RIs were compared to entries in a database (Rothamsted Research) of known compounds (Equation 4.1) (see Chapter 2, section 2.2.5, Equation 2.1).

4.2.3 Coupled gas chromatography-mass spectrometry (GC-MS)

Following GC-EAG, coupled gas chromatography-mass spectrometry analyses were carried out on the combined whole sheep ("Pure" and "Cross" breed) air entrainment extracts to gain tentative identifications for the EAG-active chemicals. Samples were separated using a HP-1 (50 m, 0.32 mm ID, 0.52 µm film thickness) capillary GC column directly coupled to a mass spectrometer (Mat 95 Thermo Finnigan) and attached to a COC injector and a FID. Helium was the carrier gas. Ionization was made by electron impact at 70 eV, 250 °C. The oven temperature programme was: 30 °C hold for 5 min, then 5°C min^{-1} to 250 °C, final hold for 21 min, total run time 60 min.

A solution of alkanes (C7 - C25, 100 ng μl^{-1}, 1 µl injection) was run on the GC-MS to allow for calculation of RIs and tentative identifications were made by comparison to entries in the National Institute for Standards and Technology (NIST) 2005 database. Tentative identifications of EAG-active peaks were made before being reviewed by Dr. Mike Birkett (Rothamsted Research, UK).

4.2.4 Peak enhancements

Peak enhancement was used to confirm the tentative chemical identifications found by GC-MS of the EAG-active peaks from the sheep extracts (Pickett, 1990). Where possible, commercially available chemicals were sourced and authentic chemical standards prepared (Table 4.1). Blends of these standards were then prepared with each component chemical present at 100 ng μl^{-1}. Blends of standards were used to reduce the volume of sheep entrainment extract required for the peak enhancement co-injections. Chemicals present in a blend were selected based on retention time to ensure they would resolve clearly and separately on the GC column. Each authentic chemical blend was injected onto the GC (HP-1 and DB-Wax columns). The RIs of the authentic chemical blends were compared to the combined sheep extract. If the location of the chemical standards matched, or were close to the EAG-active extract peaks, the approximate amount of tentatively identified chemical was calculated (Equation 4.1).

$$\text{Co} - \text{injection amount} = \frac{\text{Peak area of standard}}{\text{Peak area of EAG} - \text{active peak}} \text{ x Amount of standard } (\mu l)$$

Equation 4.1: Calculation of volume of chemical standard for co-injection with sheep entrainment extract.

The authentic chemical blend, adjusted to match the amount of each chemical in the sheep extract, was then co-injected with the combined whole sheep air entrainment extract (HP-1 and DB-Wax columns). The aim of the co-injection was to double the peak area while maintaining the peak width, thereby confirming the identity of the chemical.

Table 4.1: Source and purity of chemicals used for peak enhancement co-injections.

Chemical name	Supplier	Purity
2-Methylhexane	Sigma-Aldrich	99 %
Heptane	Sigma-Aldrich	99 %
Methylcyclohexane	Sigma-Aldrich	99 %
2-Methylpentanal	Sigma-Aldrich	98 %
Butanoic acid	Sigma-Aldrich	≥ 99 %
(*E*)-2-octene	Sigma-Aldrich	97 %
2-Methylbutanoic acid	Sigma-Aldrich	98 %
Styrene	Sigma-Aldrich	> 99 %
Heptanal	Sigma-Aldrich	95 %
3-Ethyltoluene	Sigma-Aldrich	99 %
Acetophenone	Sigma-Aldrich	99 %
4-Methylphenol	Sigma-Aldrich	99 %
4-Oxoisophorone	Sigma-Aldrich	≥ 98 %
Decanal	Sigma-Aldrich	99 %
1,3-Diacetylbenzene	Sigma-Aldrich	97 %
1,4-Diacetylbenzene	Sigma-Aldrich	96 %
Hexadecane	Sigma-Aldrich	> 99 %

4.2.5 Quantification of EAG-active identified chemicals

To quantify EAG-active compounds from the whole animal air entrainment sheep extract, a multiple point external standard method was used. Authentic chemical standards were prepared at four concentrations (100 ng μl^{-1}, 50 ng μl^{-1}, 10 ng μl^{-1} and 1 ng μl^{-1}) and each was injected three times onto a GC (1 µl, HP-1, method as described in section 2.1.5). An auto-sampler was used for the injections as this minimized the error introduced by manual injection. The peak areas from these injections were calculated and used to produce calibration curves. From these curves the mean peak area of the EAG-active chemicals could be calculated. Average areas for individual peaks were calculated from each replicate of the raw GC data and adjusted using the calibration curves. The natural release rates of the chemicals were then calculated based on three sheep (mg day^{-1}).

4.2.6 Statistical analysis

The extracts obtained from whole sheep air entrainment (Chapter 2) were analysed based on the chemicals successfully confirmed by peak enhancement. Canonical variates analysis (CVA) was used (Krzanowski, 2000). This multivariate technique analyses the data from all 12 of the confirmed chemicals together. Using the four treatments ("Pure" breed sheep, "Cross" breed sheep and the two sets of controls) the analysis attempts to maximise the ratio of the between treatments variation to the within treatments variation, thereby performing a discrimination between the treatments. In the analysis the fewest number of Canonical variates (CVs) (linear combinations of the chemicals) are retained that take up the most variation in the data and therefore make the most discrimination. The magnitudes of the CV loadings (on the chemicals) are inspected for each CV to determine which chemicals are important in the discrimination in each retained dimension. The data are then visualised in the new dimensions by plotting the CV scores for each sample. The mean CV scores in each dimension for each treatment combination are also plotted. Assuming a Normal distribution for the data on the natural logarithm ($\log_e$) scale with a small adjustment (0.005) to account for zero observations, 95 % confidence circles are placed around the CV means for the treatments. The radius of these circles is $\sqrt{\chi^2_{2,0.05}} / \sqrt{n}$, where *n* is the replication and $\chi^2_{2,0.05}$ = 5.99, is the upper 5 % point of a chi-squared distribution on 2 degrees of freedom. Non-overlapping confidence circles give evidence of significant differences between treatments at the 5 % level of significance.

Following CVA, analysis of variance (ANOVA) was applied to the confirmed chemicals data, with the two sets of controls combined, based on the CVA results. Variance due to statistical blocks was removed. When there was an overall significant difference between treatments ($P < 0.05$, F-test), appropriate treatments were compared using the least significant difference (LSD) value at the 5 % level of significance. Therefore, a difference in a given pair of means greater than the corresponding LSD value was significant ($P < 0.05$, LSD).

The GenStat® (2011, 14th edition, © VSN International, Hemel Hempstead, UK) statistical system was used for the analysis.

4.3 Results

4.3.1 Coupled GC-EAG with *Culicoides* species

Visual inspection of wild caught *Culicoides* species GC-EAG traces to whole sheep ("Pure" breed) air entrainment extract resulted in 37 EAG-active peaks (threshold > or = 3/5 responses)(Figure 4.1). GC-EAG results with laboratory reared *C. nubeculosus* were compared with the results from wild *Culicoides* species (Figure 4.2 and 4.3).

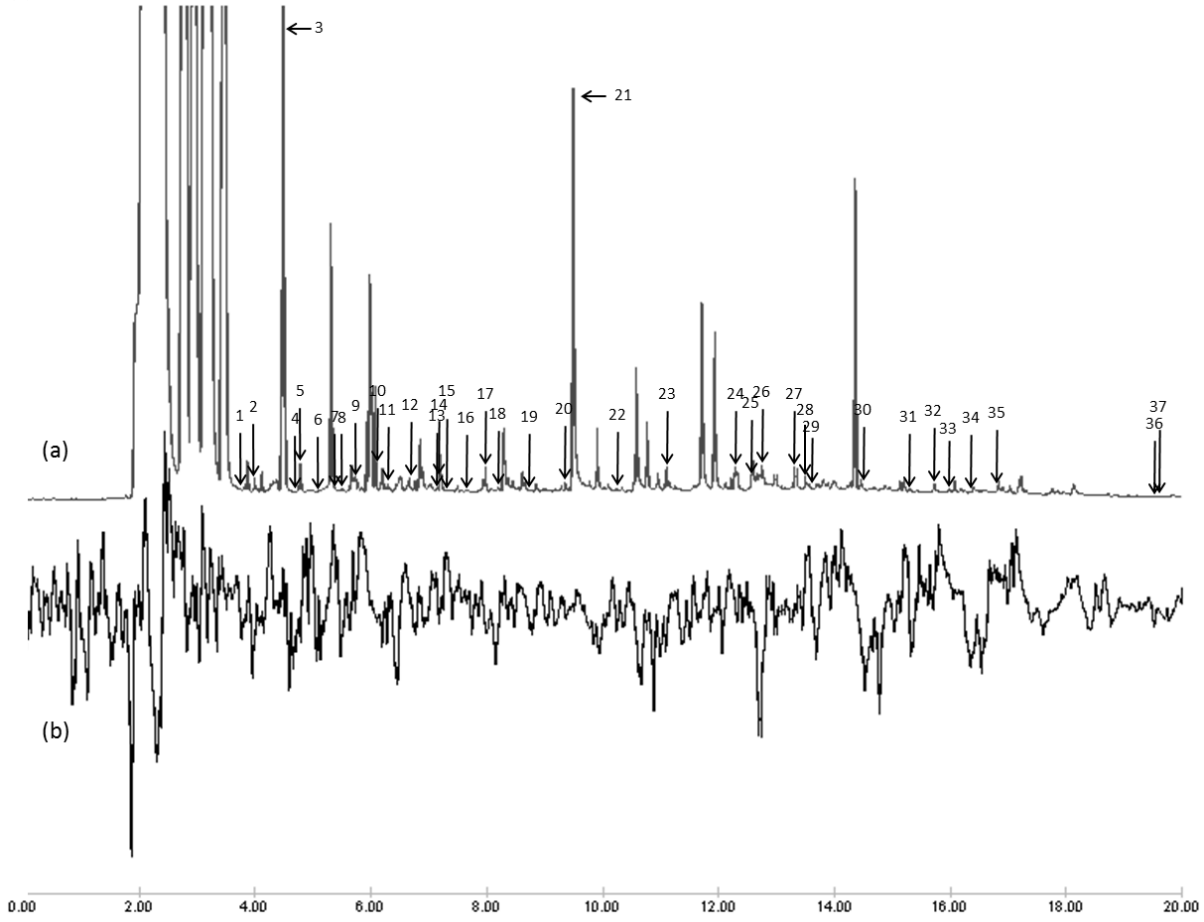


Figure 4.1: Typical coupled GC-EAG trace showing position of EAG-active peaks identified by visual inspection of responses of wild caught *Culicoides* species and *Culicoides nubeculosus* to whole sheep ("Pure" breed) air entrainment extract(threshold > or = 3/5 responses), (a) = GC trace, (b) = EAG trace.

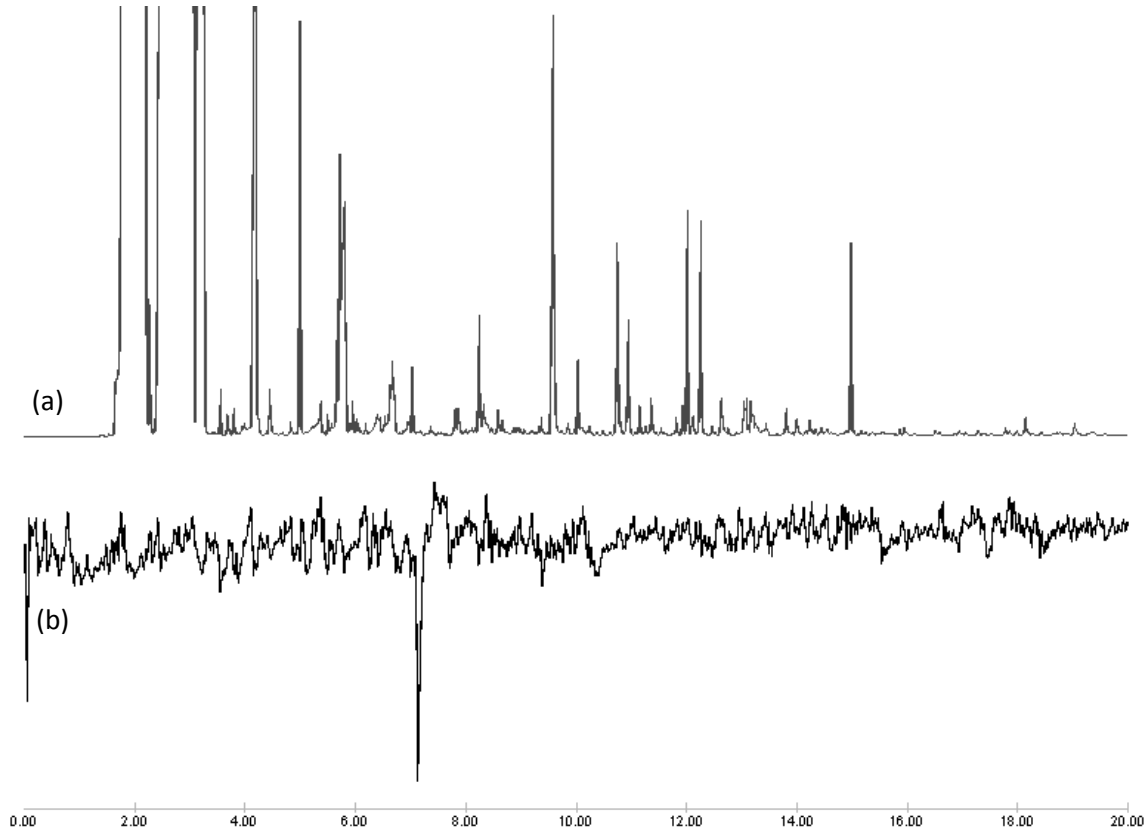


Figure 4.2: Typical coupled GC-EAG trace showing responses of *Culicoides nubeculosus* to whole sheep ("Pure" breed) air entrainment extract (a) = GC trace, (b) = EAG trace.

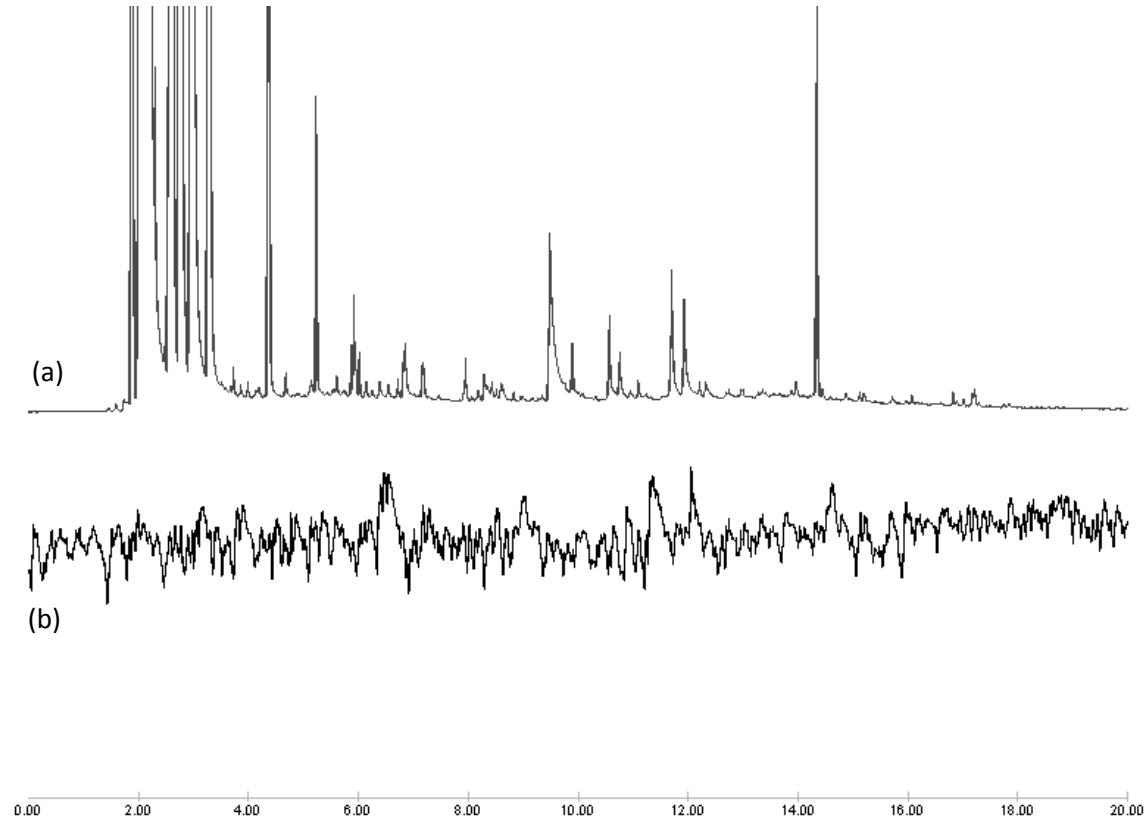


Figure 4.3: Typical coupled GC-EAG trace showing responses of *Culicoides nubeculosus* to whole sheep ("Cross" breed) air entrainment extract (a) = GC trace, (b) = EAG trace.

4.3.2 Identifications by GC-MS and peak enhancement

Tentative identifications were made, where possible, for the 37 EAG-active peaks (Table 4.2, Figure 4.4, 4.5 and 4.6). Thirteen of the peaks could not be tentatively identified. A further 7 were not commercially available readily.

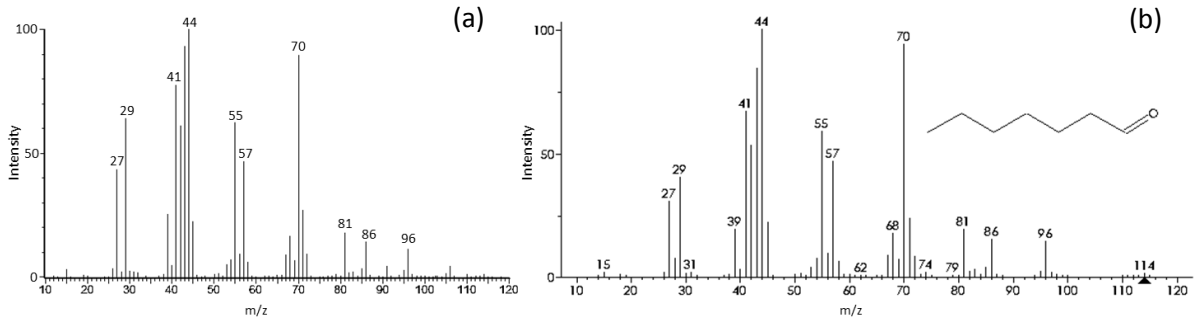


Figure 4.4: Typical fragmentograms from the GC-MS analysis (a) and the NIST database (b) used for comparison leading to tentative identification of the GC-EAG-active chemical peaks.

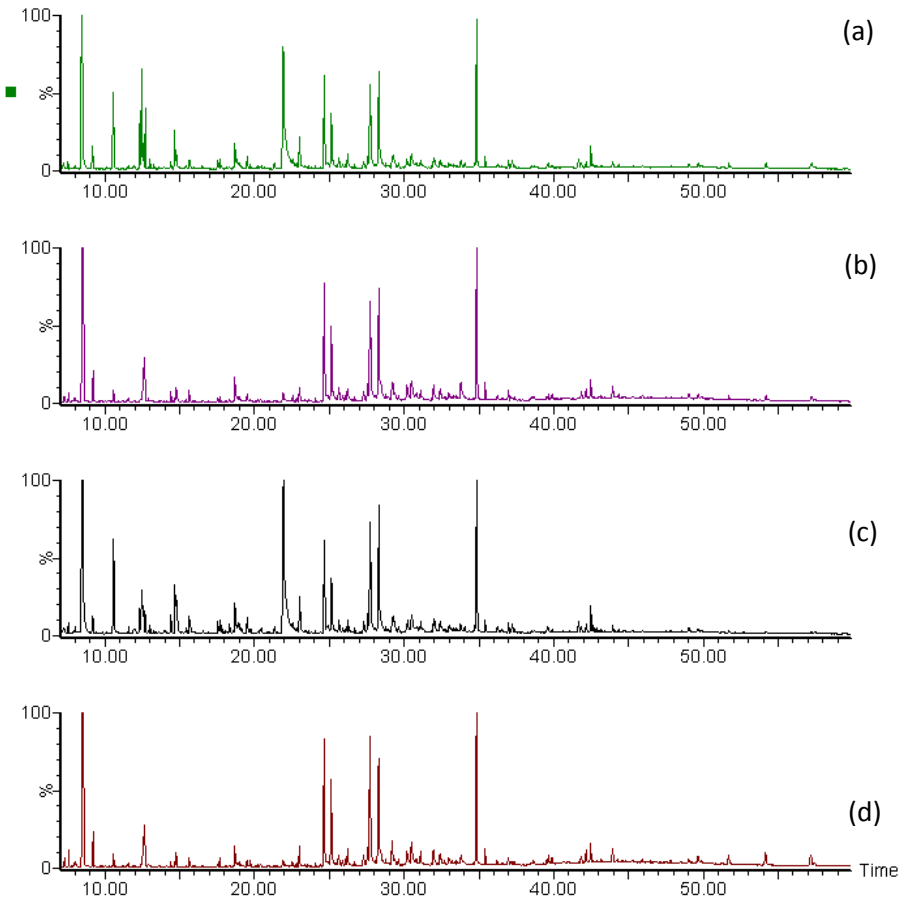


Figure 4.5: Total ion count (TIC) traces from coupled GC-MS for whole sheep air entrainment. Pure breed sheep entrainment combined (a), pure breed control entrainment combined (b), cross breed sheep entrainment combined (c) and cross breed control entrainment combined (d).

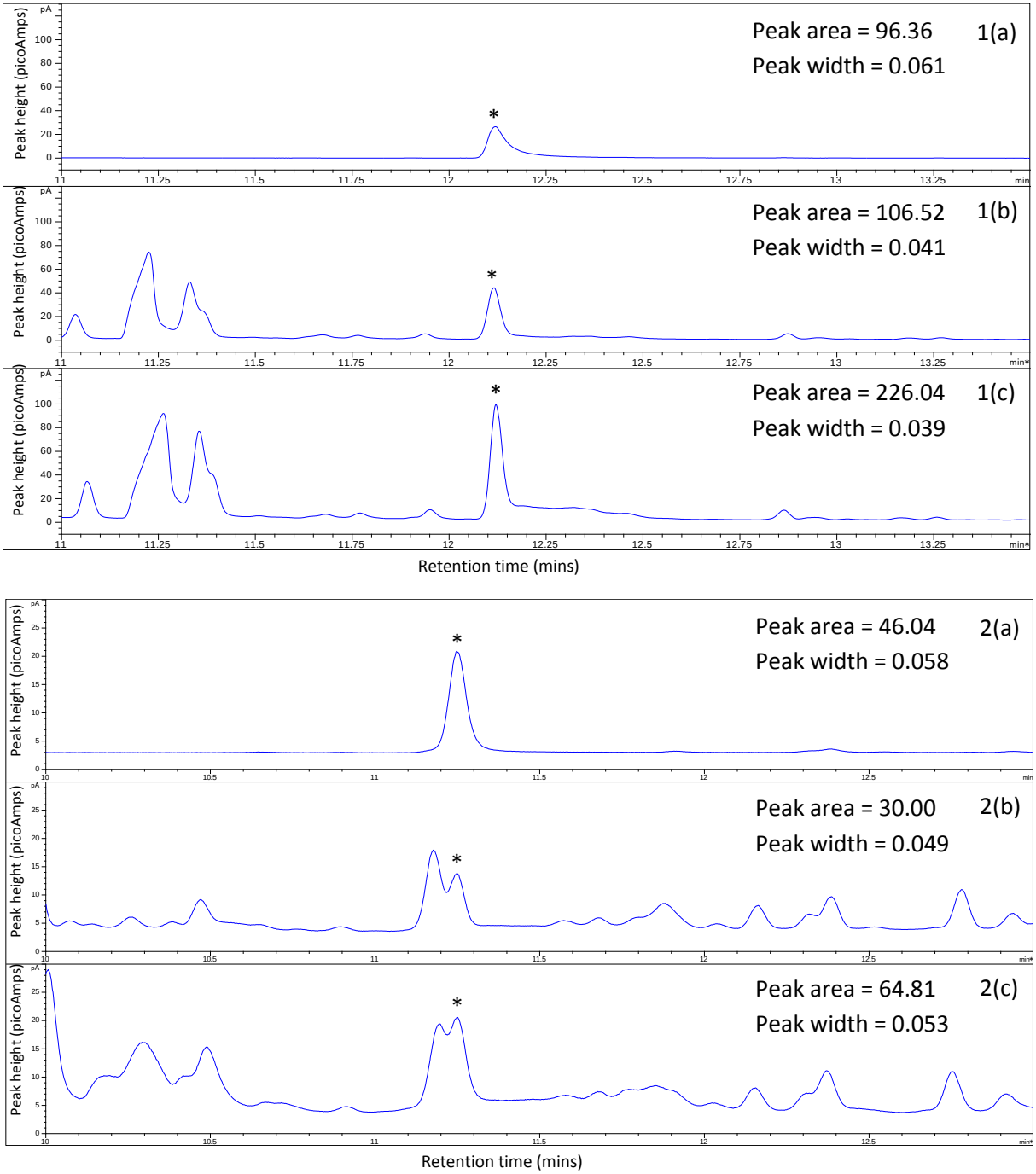


Figure 4.6: Typical peak enhancement gas chromtaographs for HP-1 (1) and DB-Wax (2) columns showing (a) heptanal standard, (b) whole animal air entrainment sheep extract and (c) peak enhancement by co-injection. Peak area and width are shown.

Of the 17 EAG-active peaks available for confirmation, 11 were confirmed by peak enhancement co-injections with the relevant chemical standard (Figure 4.6). One likely chemical identity was assumed (1,3-diacetylbenzene) based on the retention time and RI of the chemical standard compared with the tentatively identified peak in the sheep

entrainment extracts and the successful confirmation of 1,4-diacetylbenzene (Table 4.2). Hereafter, 1,3-diacetylbenzene is referred to as confirmed, for simplicity, when discussing the group of 12 chemicals collectively.

4.3.3 Quantification of EAG-active identified chemicals

Following tentative identification of EAG-active chemical peaks by GC-MS and peak enhancement co-injection confirmations, quantification of the confirmed chemicals was carried out. Determination of the gradient in the equation of the line allowed for the calculation of the amount of chemical (ng) present (y) based on the area of the chemical peak on the GC (x) (Figure 4.7, Table 4.2).

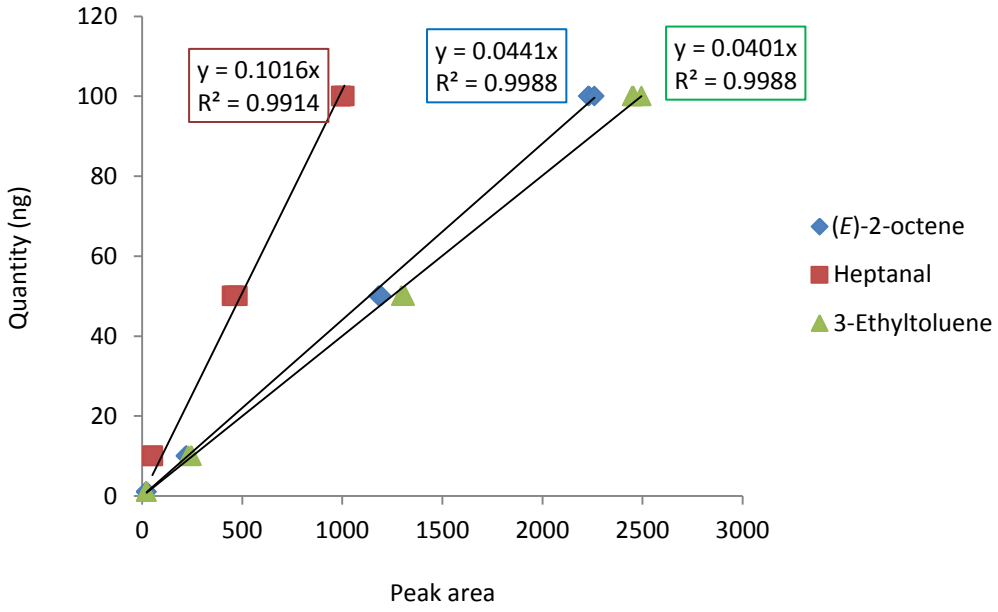


Figure 4.7: Typical external standards response curve for quantification of EAG-active confirmed chemicals. The equation of the line and regression coefficient is shown for each chemical.

Table 4.2: Tentative identification of electroantennography (EAG)-active compounds by gas chromatography-mass spectrometry (GC-MS). GC-EAG retention times (RT) and retention index (RI) values are provided. Peak numbers correspond with figure 4.1 (Section 4.3.1). Confirmed chemicals are shown and quantification is provided for "Pure" and "Cross" breed sheep entrainment extracts. Average areas for peaks were calculated from the raw GC data and adjusted using the response curves created during GC-EAG quantification (section 4.3.3).

Identification					Quantification (natural release rate of 3 sheep, mg day^{-1})			
Peak >3/5 EAG	EAG RT	EAG RI	GC-MS ID	Confirmation by co-injection	Pure		Cross	
					Sheep	Control	Sheep	Control
1	3.74	#N/A	No ID	No	-	-	-	-
2	3.96	#N/A	2-Methylhexane	No	-	-	-	-
3	4.49	704	Heptane	Confirmed	10.748	16.427	10.952	14.058
4	4.69	719	No ID (m/z 69,84)	No	-	-	-	-
5	4.77	725	Methylcyclohexane	No	-	-	-	-
6	5.08	745	No ID	No	-	-	-	-
7	5.36	763	3-Methyl-2-butenal	Not readily available	-	-	-	-
8	5.49	771	2-Methylpentanal	No	-	-	-	-
9	5.72	785	Butyric acid	No	-	-	-	-
10	6.1	808	(*E*)-2-Octene	Confirmed	0.298	0.000	0.117	0.000
11	6.29	822	1,3-Octadiene	Not readily available	-	-	-	-
12	6.66	848	2-Methylbutanoic acid	No	-	-	-	-
13	7.15	880	Styrene	Confirmed	0.010	0.006	0.017	0.003
14	7.21	883	Heptanal	Confirmed	0.327	0.132	0.445	0.129
15	7.31	890	No ID (m/z 43,57,72,84,110,125)	No	-	-	-	-
16	7.63	912	No ID (m/z 43,57,72,101)	No	-	-	-	-
17	7.97	938	6-Methyl-2-heptanone	Not readily available	-	-	-	-
18	8.19	954	3-Ethyl toluene	Confirmed	0.010	0.009	0.033	0.007
19	8.72	991	No ID	No	-	-	-	-
20	9.35	1042	Acetophenone	Confirmed	0.036	0.000	0.045	0.000
21	9.49	1053	4-Methylphenol	Confirmed	3.782	0.141	4.353	0.097
22	10.24	1114	4-Oxoisophorone	Confirmed	0.010	0.000	0.004	0.002
23	11.1	1188	Decanal	Confirmed	0.161	0.140	0.164	0.181
24	12.31	1297	2-Ethylacetophenone	Not readily available	-	-	-	-
25	12.55	1321	No ID	No	-	-	-	-
26	12.73	1340	allyl 4-ethylbenzoate	Not readily available	-	-	-	-
27	13.31	1396	1,3-Diacetylbenzene	Likely	0.199	0.171	0.207	0.184
28	13.48	1414	1,4-Diacetylbenzene	Confirmed	0.097	0.098	0.101	0.101
29	13.6	1427	No ID	No	-	-	-	-
30	14.49	1521	2,5-di-isopentylthiophene	Not readily available	-	-	-	-
31	15.28	1608	Hexadecane	Confirmed	0.027	0.002	0.002	0.002
32	15.7	1658	No ID (m/z 193,250)	No	-	-	-	-
33	15.96	1689	Pentadecanal	Not readily available	-	-	-	-
34	16.33	1733	No ID (m/z 41,53,133,159)	No	-	-	-	-
35	16.79	1789	No ID (m/z 73,218)	No	-	-	-	-
36	19.51	2073	No ID	No	-	-	-	-
37	19.61	2082	No ID	No	-	-	-	-

4.3.4 Statistical analysis

Initial CVA was performed on four treatments: “Pure” breed sheep, “Pure” breed associated controls, “Cross” breed sheep and “Cross” breed associated controls (Figure 4.8). The 95 % confidence circles for the two control treatments given the confirmed chemicals can be clearly seen to overlap, giving evidence of no significant difference between those treatments. The data for the two sets of controls was therefore combined and the CVA was run again (Figure 4.9).

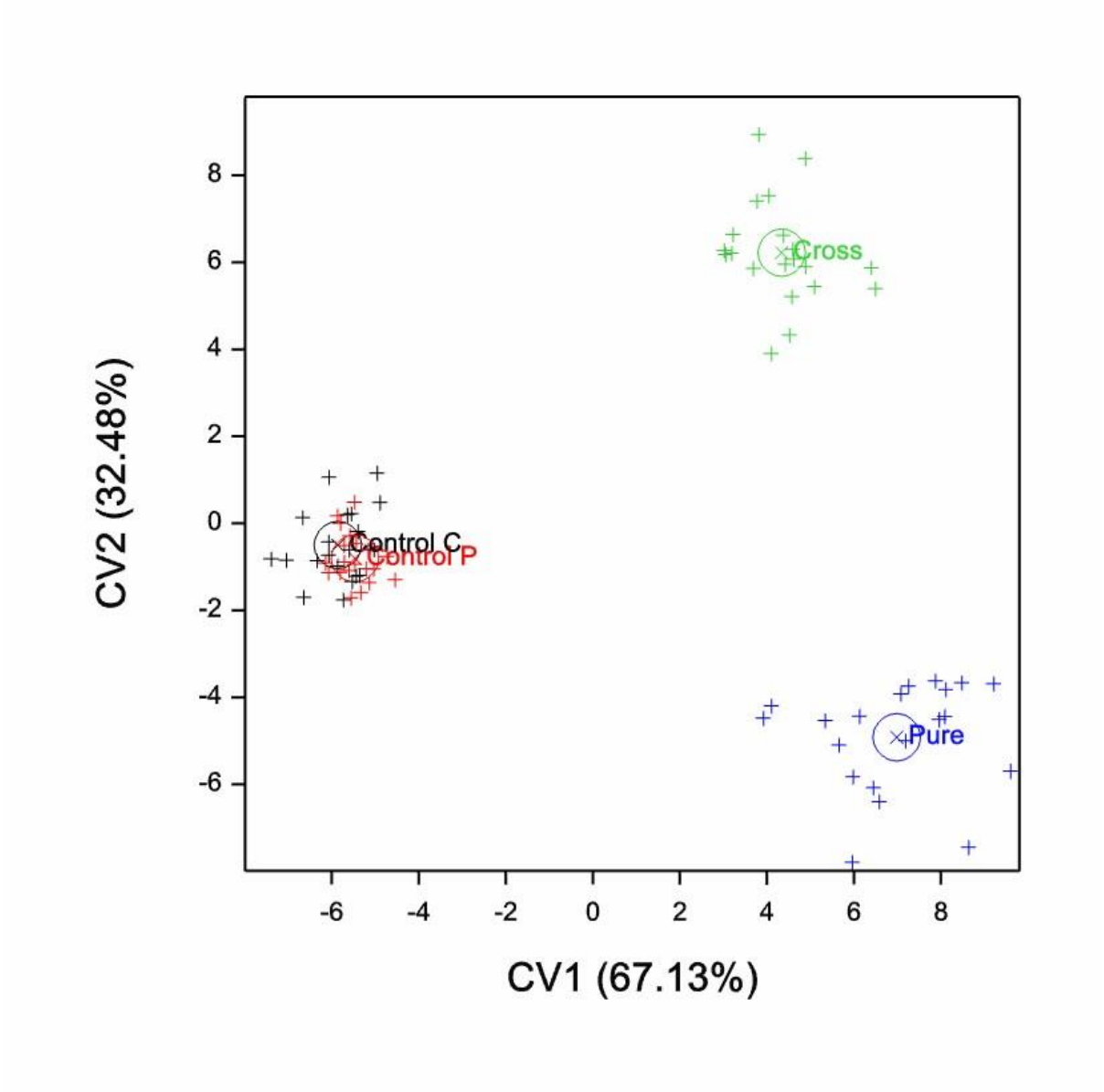


Figure 4.8: Canonical variate analysis plot based on the amounts of each of the 12 confirmed EAG-active chemicals identified from the sheep entrainment extracts in the four treatments: “Pure” breed sheep, “Pure” breed associated controls, “Cross” breed sheep and “Cross” breed associated controls.

The combining of the controls allowed all the variation (CV1, 67.26 %, CV2, 32.74 %) to be accounted for within the two dimensions visualised in the CVA plot (Figure 4.9). Discrimination between the treatments can clearly be seen with the CV1 dimension discriminating between the controls and the sheep breed treatments and the CV2 dimension discriminating between the “Pure” and “Cross” sheep breeds.

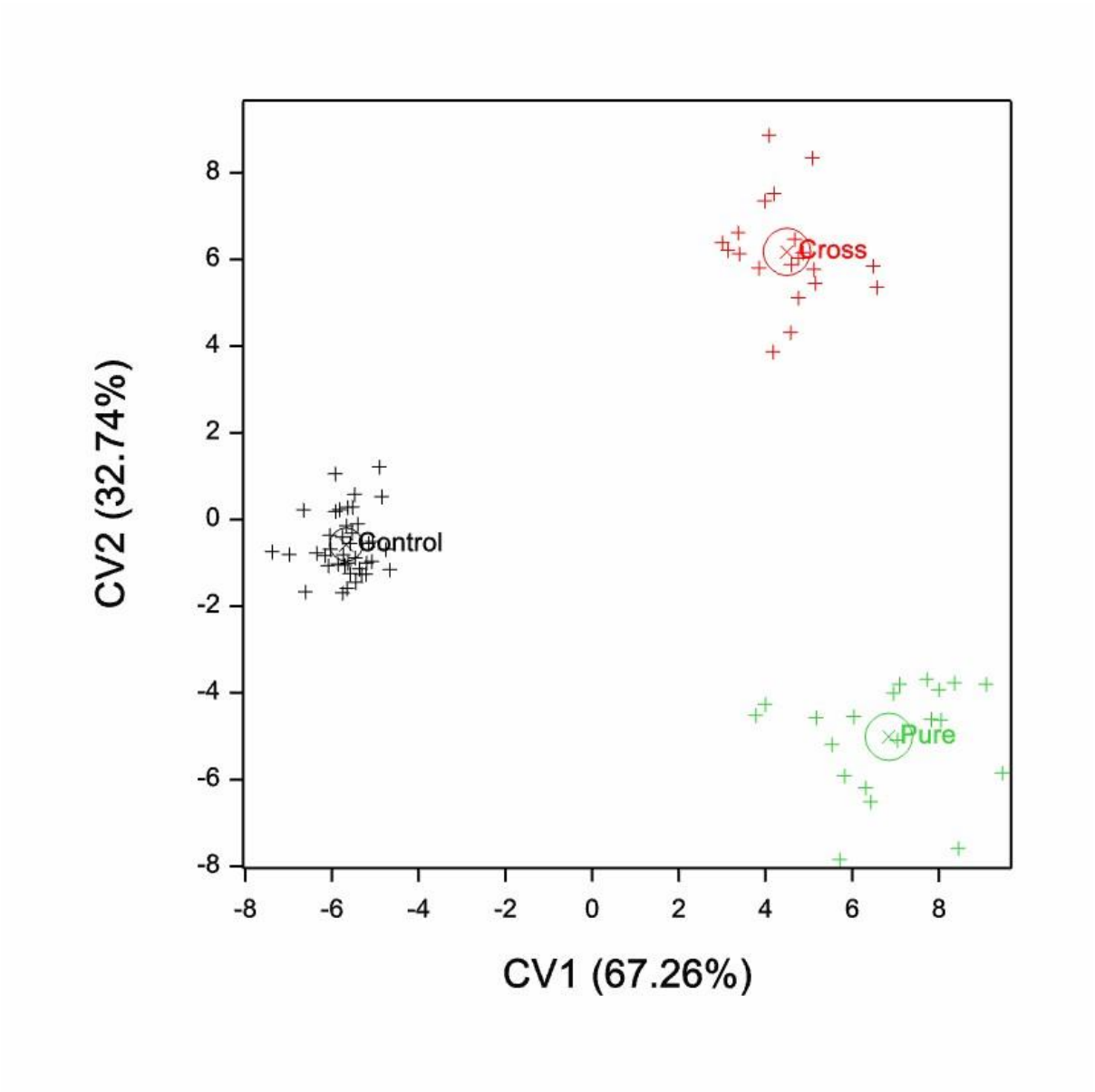


Figure 4.9: Canonical variate analysis plot based on the amounts of each of the 12 confirmed EAG-active chemicals identified from the sheep entrainment extracts in the three treatments: “Pure” breed sheep, “Cross” breed sheep and control.

The loadings (Table 4.3) on the chemicals show that (*E*)-2-octene (1.469), heptanal (2.191), acetophenone (1.107) and hexadecane (1.173) have the most influence when

discriminating on the CV1 dimension (between controls and sheep). Whereas, heptanal (2.315), acetophenone (2.849) and hexadecane (-6.293) have the most influence when discriminating on the CV2 dimension (between the sheep breeds).

Table 4.3: Loadings (latent vectors) from CVA for confirmed EAG-active chemicals.

Chemical name	**CV1 (67.26 %)**	**CV2 (32.74 %)**
Heptane	0.282	0.06
(*E*)-2-Octene	1.469	-0.563
Styrene	-0.567	-0.519
Heptanal	2.191	2.315
3-Ethyltoluene	-0.512	-0.729
Acetophenone	1.107	2.849
4-Methylphenol	0.434	0.205
4-Oxoisophorone	-0.273	-1.106
Decanal	-0.87	-0.654
1,3-Diacetylbenzene	-0.196	1.394
1,4-Diacetylbenzene	-0.653	0.316
Hexadecane	1.173	-6.293

Since the two sets of controls were found to show no significant differences in the CVA analysis they were combined for the ANOVA of individual chemicals (Table 4.4).

Table 4.4: Collated ANOVA data showing the relevant F-statistics, *P*-values, treatment means (transformed), back transformed data means (BT)(mg day^{-1} for three sheep) and LSDs for each of the 12 confirmed EAG-active chemicals identified from the sheep entrainment extracts.

			Control		Cross		Pure			
Chemical	**$F_{2,28}$**	***P*-value**	**mean**	**mean (BT)**	**mean**	**mean (BT)**	**mean**	**mean (BT)**	**LSD (5%)[1]**	**LSD (5%)[2]**
Heptane	3.97	0.03	2.45	11.583	1.92	6.816	2.05	7.763	0.421	0.486
(*E*)-2-Octene*	306.43	<0.001	-5.298	0.000	-2.256	0.100	-1.432	0.234	0.3551	0.41
Styrene	15.51	<0.001	-4.796	0.003	-3.958	0.014	-4.288	0.009	0.3208	0.371
Heptanal	51.77	<0.001	-2.115	0.116	-0.927	0.391	-1.175	0.304	0.2659	0.307
3-Ethyltoluene*	4.9	0.015	-4.454	0.007	-3.85	0.016	-4.353	0.008	0.4017	0.464
Acetophenone	230.48	<0.001	-5.283	0.000	-3.094	0.040	-3.353	0.030	0.2416	0.279
4-Methylphenol	103.41	<0.001	-2.79	0.056	1.282	3.599	1.161	3.188	0.6999	0.808
4-Oxoisophorone*	36.71	<0.001	-5.156	0.001	-4.906	0.002	-4.212	0.010	0.2262	0.261
Decanal	0.43	0.653	-1.956	0.136	-1.859	0.151	-1.86	0.151	0.2619	0.302
1,3-Diacetylbenzene	3.32	0.051	-1.811	0.158	-1.621	0.193	-1.651	0.187	0.1716	0.198
1,4-Diacetylbenzene	0.26	0.772	-2.431	0.083	-2.357	0.090	-2.394	0.086	0.215	0.248
Hexadecane*	349.21	<0.001	-5.026	0.002	-4.963	0.002	-3.515	0.025	0.1226	0.142

LSD (5 %)[1]: Comparing control to sheep breeds
LSD (5 %)[2]: Comparing between sheep breeds
* Significant difference between sheep breeds based on LSD (5 %)[2]

There were significant differences between the treatments (pure, cross and control, $P < 0.05$, F-test) for all the chemicals tested with the exception of decanal ($P = 0.653$), 1,3-diacetylbenzene ($P = 0.051$) and 1,4-diacetylbenzene ($P = 0.772$). Using the LSDs at $P < 0.05$ for significance, the ANOVA revealed differences between the sheep extracts (both "Pure" and "Cross") compared with the control for (*E*)-2-octene, styrene, heptanal, acetophenone, 4-methylphenol and 4-oxoisophorone (Table 4.4). For 3-ethyltoluene and 1,3-diacetylbenzene the "cross" breed extract was found to be significantly different compared with the control, however, no significant difference was found between the "Pure" breed extract and control. Conversely, with regard to hexadecane, the "Pure" breed extract showed a significant difference compared with the control, while no significance was found between the "Cross" breed extract and control. Interestingly, heptane showed a decrease in the mean amount of chemical present in the sheep extracts compared with the control which was significant for the "Cross" breed extract. No significant differences were found between either sheep breed extract and the control for decanal and 1,4-diacetylbenzene. Additionally, significant differences were found between the two sheep breed extracts for (*E*)-2-octene, 3-ethyltoluene, 4-oxoisophorone and hexadecane.

4.4 Discussion

Coupled GC-EAG was used successfully to investigate electrophysiological responses to the whole sheep entrainment extracts (obtained in Chapter 2). Thirty seven GC-EAG-active peaks were identified by GC-EAG using wild caught *Culicoides* species to the whole sheep ("Pure" breed) air entrainment extract (Figure 4.1) while responses of laboratory reared *C. nubeculosus* to both the "Pure" and "Cross" breed sheep entrainment extracts gave above threshold (>3/5 reps) GC-EAG responses to twelve of those thirty-seven peaks, or to twenty-one chemical peaks including below threshold responses. *Culicoides nubeculosus* only gave GC-EAG responses to six of the chemical peaks for both the "Pure" and "Cross" breed samples (styrene, 3-ethyltoluene, 4-methylphenol, 4-oxoisophorone, peak 25 (no tentative identification) and 1,4-diacetylbenzene). Furthermore, only two of these chemicals, 4-methylphenol and the unknown chemical (peak 25, Table 4.2, Section 4.3.2), elicited above threshold (>3/5 reps) responses by *C. nubeculosus* for both breeds. Overall, less above threshold GC-EAG responses were noted for the "Cross" breed (5) compared to the "Pure" breed (9) for *C. nubeculosus*. It was not possible to acquire GC-EAG data for wild *Culicoides* species with the "Cross" breed extract, therefore it is not known if a similar difference in overall responses would have been found for the wild caught midges. These results clearly show differences in the detection/electrophysiological response of wild caught *Culicoides* species compared with the laboratory reared *C. nubeculosus*. This may reflect differences in host preference between these *Culicoides* species. Alternatively, it may suggest a reduction or alteration of host seeking behaviour in *C. nubeculosus* due genetic drift from successive generations in colony, where no host seeking has been required. Genetic drift in sand fly colonies has been reported previously (Mukhopadhyay *et al*, 1997). This is an important consideration when *C. nubeculosus* is being used as a laboratory model species for testing of potential behaviour inducing chemicals and may be worth further investigation in a separate study. Tentative identifications of the EAG-active compounds were then obtained by GC-MS analysis (Table 4.2, Figure 4.4 and 4.5, Section 4.3.2). It was not possible to obtain viable tentative identifications for some peaks, often due to the GC-EAG response of the insect correlating with a flat GC baseline most likely because of the amount of chemical present in the sheep extract being below the detectable threshold of the GC.

Moreover, some tentatively identified chemicals were not commercially available making confirmation and subsequent use in the laboratory or field unlikely. Taking these factors into account, seventeen tentatively identified compounds remained. Successful peak enhancement co-injections, on two columns of different polarity (HP1 and DB-Wax) confirmed the identification of 12 chemicals (including one likely identification). These were: heptane, (*E*)-2-octene, styrene, heptanal, 3-ethyltoluene, acetophenone, 4-methylphenol, 4-oxoisophorone, decanal, 1,3-diacetylbenzene, 1,4-diacetylbenzene and hexadecane. *Culicoides nubeculosus* gave above threshold GC-EAG responses to 8 of these identified chemicals ((*E*)-2-octene, styrene, 3-ethyltoluene, 4-methylphenol, 4-oxoisophorone and 1,4-diacetylbenzene) from the "Pure" extract and three (heptane, acetophenone and 4-methylphenol) from the "Cross" extract.

It is believed that this is the first time that electrophysiological responses by *Culicoides* species have been reported for the following chemicals: heptane, (E)-2-octene, styrene, heptanal, 3-ethyltoluene, acetophenone, 4-oxoisophorone, 1,3-diacetylbenzene, 1,4-diacetylbenzene and hexadecane. Moreover this appears to be the first time (E)-2-octene, 3-ethyltoluene and 1,3-diacetylbenzene have been reported as electrophysiologically active for any insect species. Therefore, each of these chemicals may elicit behavioural responses in *Culicoides* species, resulting in the potential for new attractants and/or repellents to be discovered following suitable laboratory and field studies.

Chemicals present in the sheep extract, which were found in significantly greater quantities compared with the control extracts (Table 4.2, Section 4.3.2) (and therefore present in greater quantities than any potential residual contamination) are most likely to be host-derived volatiles released by the sheep ((*E*)-2-octene, styrene, heptanal, 3-ethyltoluene, acetophenone, 4-methylphenol, 4-oxoisophorone, 1,3-diacetylbenzene and hexadecane). Chemicals not significantly different from the control extracts are most likely to be contaminants that have been released by components of the box, have built up over time due to imperfect cleaning, entered the box via the pump, through the charcoal filter, or were Porapak™ Q contaminants (decanal and 1,4-diacetylbenzene) (Sturaro *et al*, 1992). An unexpected finding was the significant reduction in the quantity of heptane present in the sheep extracts compared with the

controls. Despite re-distillation of the hexane solvent used to elute the entrainment extracts, it is known that some heptane will be present in the solvent, however, the level of heptane would be expected to be consistent across the samples and would therefore not account for the differences in the amount of heptane present in the sheep and control entrainments. It is hypothesised that the lanolin rich sheep fleece may have absorbed heptane thus reducing the amount recovered from the Porapak Q™ polymer, or heptane is being inhaled and metabolised by the sheep.

Chemicals shown to be significantly different between the breeds of sheep are of interest as they may act as cues that help distinguish between the hosts for *Culicoides* species accounting for differential attraction between breeds ((*E*)-2-octene, 3-ethyltoluene, 4-oxoisophorone and hexadecane). Differential attraction has been shown interspecifically between host animal species (hair solvent extracts) for *C. impunctatus* (Mands *et al*, 2004) and intraspecifically with humans for *C. impunctatus*, (Logan, 2006, Logan *et al*, 2009). Differential attraction has also been shown within a herd of Holstein-Friesian heifers for the cattle fly *Haematobia irritans* Linnaeus (Birkett *et al*, 2004). However, studies have not properly investigated differential attraction of sheep breeds to *Culicoides* species or differential attraction within a breed/flock. Having said this, previous behavioural results with the extracts in the field, and the laboratory (Chapter 3, Section 3.3.4) found no significant differences in responses to the two breeds of sheep, despite the discovery of significant differences in the quantities of some chemicals between the breeds. This may suggest that these chemicals may not be important for differential attraction, but may form part of a core set of ubiquitous sheep volatile cues which indicate the presence of a host and attract *Culicoides* species, regardless of their amount, therefore the greater importance is the presence or absence of these chemicals in a blend, as described by Logan and Birkett (2007). This would also imply that the chemicals which were different between the breeds may play a role in differential attraction of *Culicoides* species.

Canonical variate analysis (Figure 4.9, CV2) showed that heptanal, acetophenone and hexadecane had the most influence for discriminating between the sheep breeds, while ANOVA revealed that (*E*)-2-octene, 3-ethyltoluene, 4-oxoisophorone and hexadecane were significantly different between the sheep breed extracts. The three chemicals shown by the CVA to discriminate between the two sheep breeds were also

shown to discriminate between control (Figure 4.9, CV1) and the sheep breeds, with the addition of (*E*)-2-octene. It is not surprising to see (*E*)-2-octene and acetophenone discriminating between the control and sheep breeds as they were only present in the sheep breed extracts and not detected in the control samples. Canonical variate analysis is designed to find the maximum possible differences with which to distinguish treatments and can be affected by the precision of the measurements (personal communication, Dr. Stephen Powers) as for heptanal and acetophenone in the current data. The back transformed means for the hexadecane (Table 4.2, Section 4.3.2) show over a ten-fold difference between the sheep breeds and it is therefore clear how the CVA determined it to be important for making such distinctions. The differences in the back transformed means for heptanal and acetophenone are less pronounced, suggesting the raw data for these chemicals was quite precise, thus enabling the CVA to identify them as discriminators between the breeds. However, it seems likely the overall variation present in the ANOVA meant that the differences between means of heptanal and acetophenone for the two sheep breeds were not great enough to be found significant. It is also worth noting that while ANOVA showed statistically significant differences between the sheep breeds for four of the EAG-active confirmed chemicals, no significant difference was found for the other eight confirmed EAG-active chemicals and no statistically significant difference was observed in the laboratory or the field when the breeds were tested with *C. nubeculosus* and wild *Culicoides* species respectively (Chapter 3).

Previous GC-EAG investigations on human derived entrainment extracts with *St. aegypti* mosquitoes and *C. impunctatus* midges reported some EAG-active chemicals also found during this study (Logan, 2006). It is worth noting that Logan (2006, summarized in Logan *et* al, 2009) is the only other reported investigation that used GC-EAG with *Culicoides* species. Decanal, reported here as EAG-active for wild caught *Culicoides* species, was reported as EAG-active for both *St. aegypti* and *C. impunctatus* while hexadecane was only reported as EAG-active for *St. aegypti* with no response noted for *C. impunctatus* (Logan, 2006). Interestingly, hexadecane, present in the sheep derived entrainment extracts, has been shown here to be EAG-active for wild caught *Culicoides* species, although no response was discovered for laboratory reared *C. nubeculosus*. Furthermore, Logan (2006) reported GC-EAG responses by *C.*

impunctatus to an unknown chemical with an RI (HP1) of 807. In the work presented here (*E*)-2-octene, confirmed by peak enhancement, showed GC-EAG responses with wild *Culicoides* species and *C. nubeculosus* (with the "Pure" Hartline breed extract) and the RI (HP1) was determined to be 808. It is possible that the unknown chemical reported by Logan (2006) could be (*E*)-2-octene identified in this study. The potential for the unknown chemical to be (*E*)-2-octene is further supported by the presence of 2-octene in the list of GC-MS tentative identifications derived from human skin emanations (Bernier *et al*, 2000). This evidence shows that (*E*)-2-octene is a volatile chemical produced by both humans and sheep which elicits a GC-EAG response in wild *Culicoides* species and laboratory reared *C. nubeculosus*. The work presented here is the first instance of the confirmed chemical, (E)-2-octene, being reported as EAG-active for insects, and the data shows GC-EAG responses to the chemical for wild *Culicoides* species (mainly *C. obsoletus*), *C. nubeculosus* and *C. impunctatus* (based on Logan, 2006). The recording of GC-EAG responses from three (potentially) different *Culicoides* species suggests that (*E*)-2-octene may have a role in host location and discrimination by these insects. Further investigation in the laboratory (Chapter 5) and the field (Chapter 6) will determine if (*E*)-2-octene is behaviourally active and reveal whether it may have potential as a semiochemical-bait to improve surveillance of, or assist with localized control of, *Culicoides* species populations. No GC-EAG response was reported for *St. aegypti* mosquitoes, suggesting that the chemical does not play a role in mosquito host location (Logan, 2006). Therefore, it may also be a *Culicoides* species specific host signal, unlike the more ubiquitous semiochemicals such as 1-octen-3-ol that act on a variety of haematophagous insect species (as reviewed by Gibson and Torr, 1999, Logan and Birkett, 2007). However, further laboratory and/or field-based investigations with mosquitoes, tsetse flies and other species would be required to confirm whether (*E*)-2-octene elicits behavioural responses in these species.

Previous studies support the role of some of the identified chemicals as potential host location cues. Heptane and heptanal have been reported in the headspace of sheep wool grease samples by SPME collections, while 4-methylphenol has been shown to be a component of skin secretions from oxen (Lisovac and Shooter, 2003, Warnes, 1990). Using EAG, rather than GC-EAG, Bhasin *et al* (2000a) studied the responses of *C.*

impunctatus to several alky phenols (including 3-methylphenol and 4-methylphenol) and found that they were more sensitive to the 3-substituted alkyphenols than the 4-substituted alkyphenols. Also, investigations of skin washes of *C. impunctatus* hosts has shown that the presence or absence of 3-methylphenol in a blend had a significant effect on the level of attraction of that blend (Bhasin, 1996). This is an interesting result, as contrary to what may have been expected, based on these findings, none of the electrophysiologically active peaks reported here were 3-derivate alkylphenols. Instead, as stated above, 4-methylphenol was found to be EAG-active for wild caught *Culicoides* species and *C. nubeculosus*. However, Bhasin *et al* (2000a), went on to report that behavioural investigations of these alkylphenols in the Y-tube olfactometer with *C. impunctatus*, did not suggest the same increased sensitivity with for the 3-derivative alkylphenols as seen with the EAG responses. Additionally, GC-MS tentative identifications for chemicals derived from cow, pig, human and monitor lizard entrainments were found to contain some chemicals also reported in this study: heptanal, 3-ethyltoluene, 4-methylphenol, decanal and hexadecane (personal communication, Dr. S. Y. Dewhirst). Furthermore, GC-MS tentative identifications based analysis of human skin emanations found heptane, 2-octene, styrene, heptanal, decanal and hexadecane as potential component chemicals (Bernier *et al*, 2000). Although only tentative identifications were made in these studies, this information still suggests that these chemicals are hosts derived and therefore may elicit behavioural responses in *Culicoides* species. They also show that the collection and identification methods utilised in this investigation are sound, as they are producing results comparable to previous findings while also discovering novel EAG-active chemicals for *Culicoides* species (and wider insect species).

Electrophysiological activity and behavioural attraction has been shown for the oriental fruit fly, *Bactocera dorsalis* Hendel, to heptane, identified from air entrainments of Alphonso mango (*Mangifera indica* Linnaeus) (Jayanthi *et al*, 2012). Guédot *et al* (2008) showed that 4-oxoisophorone, detected from flowers of the Butterfly Bush (*Buddleja davidii*) elicited GC-EAG antennal responses in two pest moth species, cabbage loopers (*Trichoplusia ni* Hübner) and alfalfa loopers (*Autographa californica* Speyer). In a different study, volatile entrainments of *Salix caprea* and *Salix atrocinerea* plants were tested with *Andrena vaga* Panzer bees and GC-EAG responses

recorded to a compound tentatively identified as 4-oxoisophorone (or benzyl nitrile)(Dotterl *et al*, 2005). While these examples show insect responses to plant-derived volatiles rather than animal-derived volatiles, they nevertheless provide evidence that heptane and 4-oxoisophorone can elicit electrophysiological and behavioural responses in different insect species. It is also interesting that 4-oxoisophorone appears as a plant-derived volatile in the literature, whereas here it is believed to be sheep-derived, found in greater quantities in the sheep extracts than the controls, and also shown to be present in significantly different quantities between the two breeds of sheep (Table 4.2, Section 4.3.2).

Acetophenone, 1,3-diacetylbenzene and 1,4-diacetylbenzene have been shown to be Porapak™ Q (ethylvinylbenzene-divinylbenzene co-polymer) contaminants, produced from the breakdown of the co-polymer during heating (Sturaro *et al*, 1992). However, these chemicals are shown here to be EAG-active for wild caught *Culicoides* species to the "Pure" breed extract, eliciting responses in more than three out of five replicates. Furthermore *C. nubeculosus* elicited an EAG response to acetophenone in the "Cross" breed sheep extract (>3/5 reps) and an EAG response to 1,4-diacetylbenzene in the "Pure" breed sheep extract (>3/5 reps). An EAG response from *C. nubeculosus* to 1,4-diacetylbenzene was noted in the "Cross" breed sheep extract (<3/5 reps), however, was below the cut-off level to be classed as a consistent response. No EAG response was noted for *C. nubeculosus* to 1,3-diacetylbenzene in either of the sheep breed extracts. Similarly, styrene (vinylbenzene) has been reported as a potential artefact of Porapak™ Q (Lewis and Williams, 1980). However, styrene was shown here to be EAG-active with wild *Culicoides* species and *C. nubeculosus* to the "Pure" breed extract, while *C. nubeculosus* showed a below cut-off level response (< 3/5 reps) to styrene in the "Cross" breed extract. As a result, some caution should be taken inferring roles for these chemicals in host location until the EAG-activity is backed up with proof of attraction or repellency of *Culicoides* species in the laboratory or field. These studies suggest that styrene, acetophenone, 1,3-diacetylbenzene and 1,4-diacetylbenzene may not be host-derived. However, previously identification as potential contaminants does not preclude these chemicals from being produced through biological pathways in hosts and therefore still being viable host-derived semiochemicals. Investigating behavioural responses of *Culicoides* species regardless of the origin of these volatiles is

worthwhile in light of the positive GC-EAG responses (often recorded for two different *Culicoides* species).

Based on the statistical analysis, and the mean amount of chemicals present calculated from the sheep entrainment extracts (Table 4.2, Section 4.3.2), decanal, 1,3-diacetylbenzene and 1,4-diacetylbenzene appear to be true contaminants, showing no significant differences between the breeds, nor between either breed and the control extract (data combined following initial CVA). As 1,3-diacetylbenzene and 1,4-diacetylbenzene have been previously identified in the literature as possible breakdown contaminants of Porapak™ Q, it seems likely that this is their source (Sturaro *et al*, 1992). To facilitate the desired collection airflow rate through the Porapak™ Q tubes, large bore tubes (glass tubing, 5.5 mm ID, rather than 3.5 mm ID, Fisher Scientific UK Ltd, Leicestershire, UK) with a greater amount of Porapak™ Q (250 mg rather than 50 mg) were used. The standard cleaning protocols were followed when preparing the Porapak™ Q tubes, however, it is possible that due to the increase in size, or the increase in polymer amount, that this cleaning process was imperfect. Decanal, however, does not appear to have been reported as a Porapak™ Q contaminant previously. Instead it is a known volatile from humans (Bernier *et al*, 2000, Logan, 2006,) and EAG-active for *C. impunctatus* (Logan, 2006). Additionally, it has been shown to have repellent effect with *C. impunctatus* (Logan *et al*, 2009). This data would seem to suggest that decanal is a sheep volatile, however, this does not explain its presence in the control extracts, especially at the same amounts as found in the sheep extracts. Regardless of the source, 1,3-diacetylbenzene and decanal were both found to be EAG-active for *Culicoides* species in this investigation. As behavioural effects of decanal have previously been shown (Logan *et al*, 2009), 1,3-diacetylbenzene may elicit a behavioural response for *Culicoides* species individually or as a component of a blend.

Despite being previously reported as a potential Porapak™ Q contaminant (Sturaro *et al*, 1992), acetophenone was only found in the sheep extracts, with no trace being found in the control extracts (as was the case with (*E*)-2-octene) (Table 4.2, Section 4.3.2). This proves that acetophenone is a sheep derived volatile and further, due to only being found in the sheep extracts, potentially one of the most important of the 12 EAG-active chemicals identified (along with (*E*)-2-octene). Moreover, it highlights the

fact that chemicals reported as potential breakdown contaminants of Porapak™ Q, may also be produced biologically in hosts.

4.5 Conclusions

Gas chromatography-electroantennography was successfully used with *C. nubeculosus* and wild caught *Culicoides* species to investigate the behaviourally active sheep entrainment extracts. Thirty-seven EAG-active peaks were tentatively identified by GC-MS and twelve have been confirmed using peak enhancement co-injections. The confirmations included ten chemicals that are reported here for the first time as EAG-active for *Culicoides* species including three ((*E*)-2-octene, 3-ethyltoluene and 1,3-diacetylbenzene) that have not been reported as EAG-active for any insect species previously. The confirmed chemicals were quantified and statistical analysis revealed significant differences between the sheep breed extracts for some of these chemicals. Investigations into behavioural activity of the chemicals can now take place in the laboratory (Chapter 5) and the field (Chapter 6).

Chapter 5: Behavioural responses of *Culicoides* species to EAG-active identified chemicals

5.1 Introduction

The first laboratory-based study using an olfactometer with *Culicoides* species was carried out in 1994 by Blackwell *et al*. This relatively recent appearance of the Y-tube for this insect may be due to the difficulties in rearing *Culicoides* species in the laboratory (Veronesi *et al*, 2009, Logan *et al*, 2010b). Since then, the Y-tube olfactometer has been used to successfully investigate behavioural responses of *Culicoides* species to a variety of stimuli. Attraction of parous, un-fed, female *C. impunctatus* to live, parous, un-fed females has been reported, demonstrating the presence of a midge pheromone (Blackwell *et al*, 1994). Racemic 1-octen-3-ol was combined with live, parous, un-fed females or hexane extract equivalents and a significant increase in attraction of *C. impunctatus* to the 10 µg combined dose was found (Blackwell *et al*, 1994). It was noted that at greater concentrations, racemic 1-octen-3-ol elicited a repellent effect on *C. impunctatus.* Strictly speaking, the definition of a repellent is a substance that dissuades an insect (or other pest) from approaching or alighting on a host, and repels them, driving them away from the host. Due to the design of the Y-tube olfactometer, in the stem insects are moving towards the "repellent" source as the approach the Y-junction before moving out of the airflow into the other arm of the Y-tube. In a true repellent response it might insects would be expected to remain at the far (downwind) end of the stem. As such, the Y-tube is not suited to determining if a chemical is repellent, and such results would be better termed to show a reduction in attraction with possible repellents effects that would need to be confirmed in a different experiment. Further investigation with racemic 1-octen-3-ol showed statistically significant increases in relative attraction of *C. impunctatus* in the Y-tube olfactometer to doses reported as 1×10^3 µg and 1×10^4 µg over a concentration range of 10-1×10^4 µg (Blackwell *et al*, 1997). In addition to attractants the Y-tube olfactometer has also been used to suggest possible repellent effects of chemical stimuli, by reduction in attraction levels compared with an expected 50/50 response. Phenol, 3-n-propylphenol, 3-methylphenol and 4-methylphenol were all shown to exhibit a significant reducing in expected attraction of

C. impunctatus at varying concentrations, while 1-octen-3-ol, butanone and acetone all showed significant increases in relative attraction of *C. impunctatus* (Bhasin *et al*, 2000). Interestingly, lactic acid showed significant attraction of *C. impunctatus* at very low concentration; however, significant reduction in attraction was noted at high concentrations. Furthermore, behavioural responses of *C. nubeculosus* to racemic 1-octen-3-ol and acetone were investigated, producing classic bell-shaped dose response curves with significant increases in relative attraction reported for both chemicals (Bhasin *et al*, 2000). Reductions in relative attraction of *C. impunctatus* to neem oil when compared with ethanol (95 %) have also been reported (Blackwell *et al* 2004). Logan (2006) showed significant increases in relative attraction of *C. impunctatus* to human air entrainment extracts, as well as racemic 1-octen-3-ol, 6-methyl-5-hepten-2-one and (*E*)-2-nonenal while also reporting significant decreases in relative attraction of *C. impunctatus* (potential repellent effects) for linalool, menthol, naphthalene, decanal, geranylacetone and α-isomethylionone.

The development of the olfactometer, or olfactory meter, dates back to Barrows (1907) who investigated reactions of the pomace fly (*Drosphila ampelophila* Loew) to odorous substances. The first appearance of a Y-tube olfactometer appears to be an adaption of Barrows (1907) technique for use with the potato beetle (*Leptinotarsa decemlineata* Say) to investigate attractants and repellents (McIndoo, 1926). An early olfactometer for haematophagous insect (mosquito) research was developed based on a design by Wieting-Hoskins (1939) for investigating responses of house flies (*Musca domesticus* Linnaeus) (Willis, 1947). The design was further altered for the investigation of *St. aegypti* after the Willis design was found to be too restrictive for those mosquitoes (Brown *et al*, 1951). One of the most recent designs was produced by Geier and Boeckh (1999) and was designed to investigate *St. aegypti* responses to host-derived volatiles using human hands, extracts of human skin residues, L-(+)-lactic acid and CO_2. Designs similar to Geier and Boeckh (1999) have proved successful in investigating responses of mosquitoes to host volatiles (Geier *et al*, 1999, Logan, 2006, Logan *et al* 2008, Cook *et al*, 2011).

Although the Y-tube has been used successfully, there are several potential issues that should be considered. Insects may not respond in the same way to odour stimuli when walking compared to flying (Kennedy, 1977) as the diameter of the Y-tube does not

allow for free flight, but rather short hops or walking. There is also the potential for odour streams not to mix properly, resulting in steep odour gradients where they interface which may affect responses (Kennedy, 1977). Despite these caveats associated with using a Y-tube olfactometer in the laboratory, there is evidence that a Y-tube olfactometer is an appropriate tool as described in Chapter 3.

Objective:

- To investigate behavioural responses of *Culicoides* species in the laboratory to confirmed EAG-active chemicals using a Y-tube olfactometer.

5.2 Materials and Methods

5.2.1 Insects

Laboratory reared *C. nubeculosus* and wild caught *Culicoides* species used in Y-tube olfactometer bioassays in this chapter were obtained or collected according to the methods detailed in Chapter 3 (Section 3.2.1).

5.2.2 Chemical standards

The source and purity of all chemicals used in this chapter are detailed in Chapter 4 (Section 4.2.4, Table 4.1). Chemicals were prepared in redistilled hexane at 100 mg ml^{-1} and serial diluted in 10-fold dilutions down to 1 µg $µl^{-1}$. As 10 µl of chemical dilution are loaded onto the filter paper in the Y-tube olfactometer for each replicate this gave a final dose range of 10 ng – 100 µg for investigation with wild caught *Culicoides* species.

For investigating 4-methylphenol and 4-oxoisophorone, with *C. nubeculosus*, a 10 pg – 1 µg dose range was used, while for (*E*)-2-octene and *C. nubeculosus* a 10 pg – 100 µg dose range was used.

5.2.3 Y-tube olfactometer

The Y-tube olfactometer was used as a laboratory bioassay to investigate the behavioural responses of laboratory reared *C. nubeculosus* and wild caught *Culicoides* species to GC-EAG-active chemicals with confirmed identification from Chapter 4. Insects are able to move upwind towards volatile chemicals released into the airflow coming down one of the arms of the Y-tube olfactometer (with a solvent released into the other arm of the Y-tube as a control). As the insects move up the stem of the Y-tube olfactometer the reach the apex of the Y-junction and move into one of the arms of the olfactometer. Relative attraction can be counted as the number of insects in the "test" arm, divided by the total number of insects in both the "test" and control arms.

The methods for use of the Y-tube olfactometer are described in full in Chapter 3 (Section 3.2.3). No alterations to those methods were made.

5.2.4 Statistical analysis

Full details of the statistical analyses used are described in Chapter 3 (Section 3.2.5). The GLM model: used for behavioural investigations of *Culicoides* species with the Y-tube olfactometer in this chapter was

$$y \sim \text{Constant} + \text{Day} + \text{Day.AmPm} + \text{Arm\_Trt} + \text{Trt}$$

where y was logit(*p*), Constant is an overall mean for the data on the logit scale, Day, AmPm and Arm_Trt are the blocking factors, Trt is the treatment factor, and the dot indicates the interaction between Day and AmPm.

5.3 Results

5.3.1 Responses of *C. nubeculosus* to EAG-active identified chemicals

5.3.1.1 Behavioural responses of *C. nubeculosus* to 4-methylphenol

The behavioural responses of laboratory reared *C. nubeculosus* to the confirmed chemical 4-methylphenol were investigated in the Y-tube olfactometer (Figure 5.1). An overall significant difference between treatments was found ($F_{7,48}$ = 3.24, P < 0.05, F-test from GLM model, n = 8) with a significant decrease in relative attraction, compared with the blank control, at 1µg (39.6 % ± 0.04 S. E., P = 0.028, t-test within GLM model). The blank control showed 53.9 % (± 0.04 SE) relative attraction. The racemic 1-octen-3-ol positive control showed a significant increase in relative attraction, at 66.5 % (± 0.04 SE) (P = 0.047, t-test within GLM model). No significant difference in relative attraction was seen at any of the other doses tested.

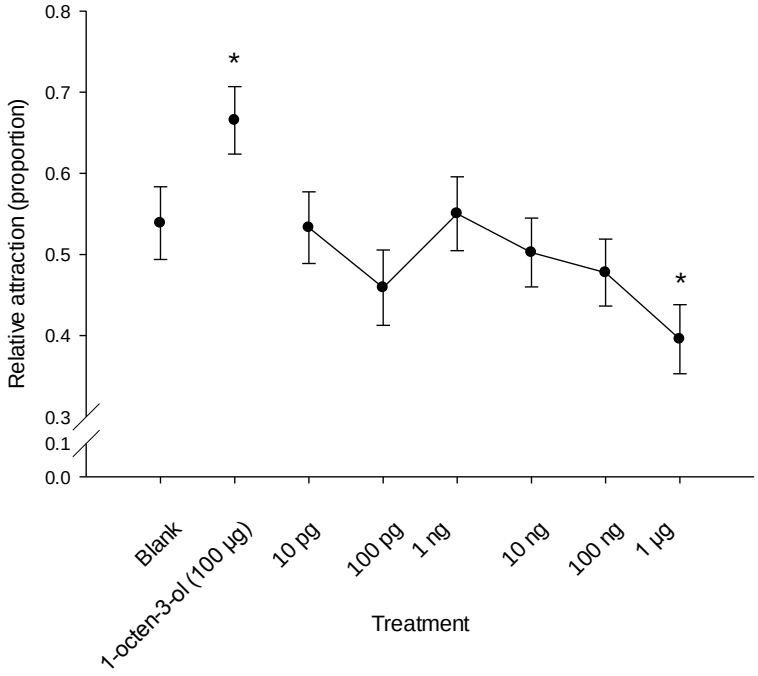


Figure 5.1: Relative attraction of laboratory reared *C. nubeculosus* to different doses of 4-methylphenol, a blank control (hexane) and a positive control racemic 1-octen-3-ol in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared with the blank control. Results shown as mean relative attraction ± standard error. Relative attraction at 1µg of 4-methylphenol was 39.6 % and the racemic 1-octen-3-ol positive control was 66.5 % (GLM, *P < 0.05, n = 8).

5.3.1.2 Behavioural responses of *C. nubeculosus* to 4-oxoisophorone

The behavioural responses of laboratory reared *C. nubeculosus* to the confirmed chemical 4-oxoisophorone were investigated in the Y-tube olfactometer (Figure 5.2). An overall significant difference between treatments was found ($F_{7,61}$ = 2.46, P < 0.05, F-test from GLM model, n = 10). No significant differences in relative attraction were found for the dose range of 4-oxoiophorone investigated, although a large decrease in relative attraction was observed at 10 pg 44.5 % (± 0.05 SE). The blank control showed 54.8 % (± 0.05 SE) relative attraction. The racemic 1-octen-3-ol positive control showed a significant increase in relative attraction, at 66.7 % (± 0.04 SE) (P = 0.048, t-test within GLM model).

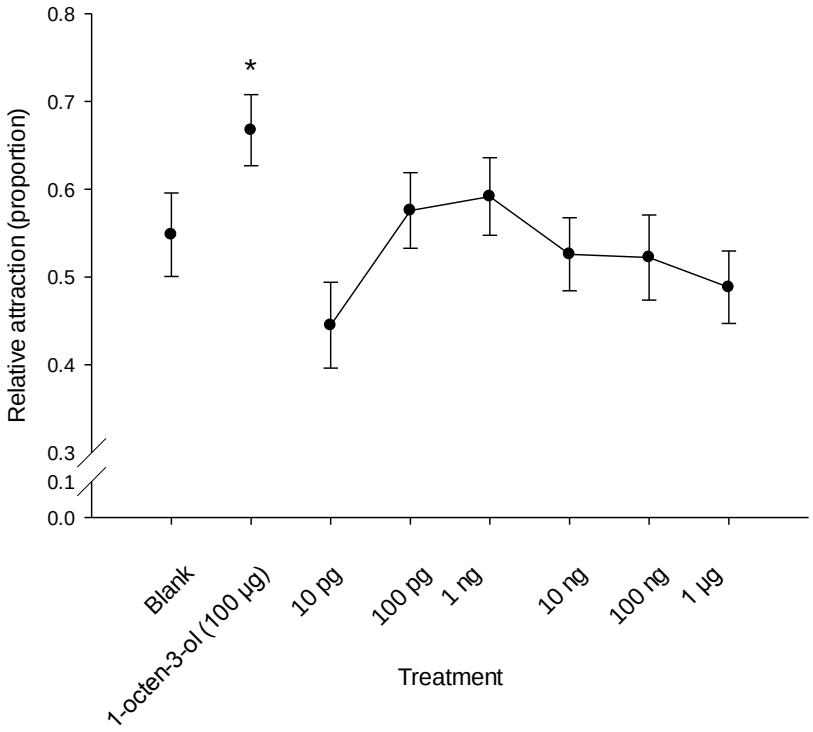


Figure 5.2: Relative attraction of laboratory reared *C. nubeculosus* to different doses of 4-oxoisophorone, a blank control (hexane) and a positive control racemic 1-octen-3-ol in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared with the blank control. Results shown as mean relative attraction ± standard error. Relative attraction to the racemic 1-octen-3-ol positive control was 66.7 % (GLM, *P < 0.05, n = 10).

5.3.1.3 Behavioural responses of *C. nubeculosus* to (E)-2-octene

The behavioural responses of laboratory reared *C. nubeculosus* to the confirmed chemical (*E*)-2-octene were investigated in the Y-tube olfactometer (Figure 5.3). No overall significant difference between treatments was found ($F_{9,80}$ = 1.30, *P* = 0.248, F-test from GLM model, n = 10). No significant differences in relative attraction were found for the dose range of (*E*)-2-octene investigated, although all doses showed a greater response than the blank control at 45.6 % (± 0.05 SE) relative attraction. The greatest increase in relative attraction was observed at the 100 pg dose, 58.5 % (± 0.05 SE). The racemic 1-octen-3-ol positive control showed a significant increase in relative attraction, at 64.4 % (± 0.04 SE) (*P* = 0.007, t-test within GLM model).

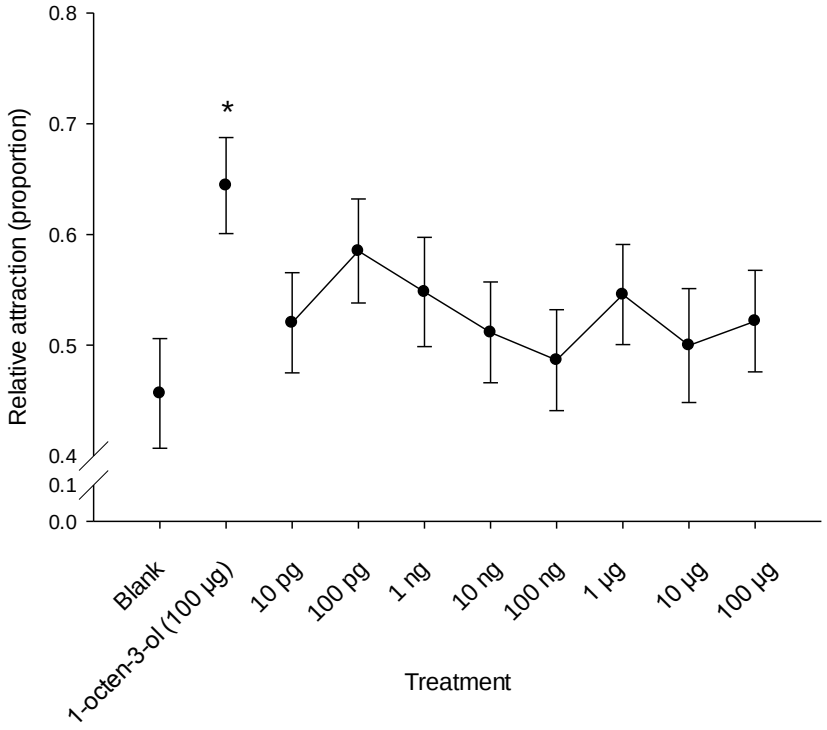


Figure 5.3: Relative attraction of laboratory reared *C. nubeculosus* to different doses of (*E*)-2-octene, a blank control (hexane) and a positive control racemic 1-octen-3-ol in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared with the blank control. Results shown as mean relative attraction ± standard error. Relative attraction to the racemic 1-octen-3-ol positive control was 64.4 % (GLM, **P* < 0.05, n = 10).

5.3.2 Responses of wild caught *Culicoides* species to EAG-active identified chemicals

5.3.2.1 Behavioural response of wild caught *Culicoides* species to (*E*)-2-Octene

Behavioural response of wild caught *Culicoides* species (*E*)-2-octene were investigated (Figure 5.4). An overall significant difference between treatments was found ($F_{6,65}$ = 1.89, $P < 0.05$, F-test from GLM model, n = 12) with a significant increase in relative attraction, compared with the blank control, at 1µg (60.6 % (± 0.03 S. E., $P = 0.027$, t-test within GLM model). The blank control showed 50.1 % (± 0.03 SE) relative attraction. The (*R*)-(-)-1-octen-3-ol positive control showed a significant increase in relative attraction, at 59.7 % (± 0.03 SE) ($P = 0.045$, t-test within GLM model). No significant difference in relative attraction was seen at 10 ng, 100 ng, 10 µg nor 100 µg of (*E*)-2-octene.

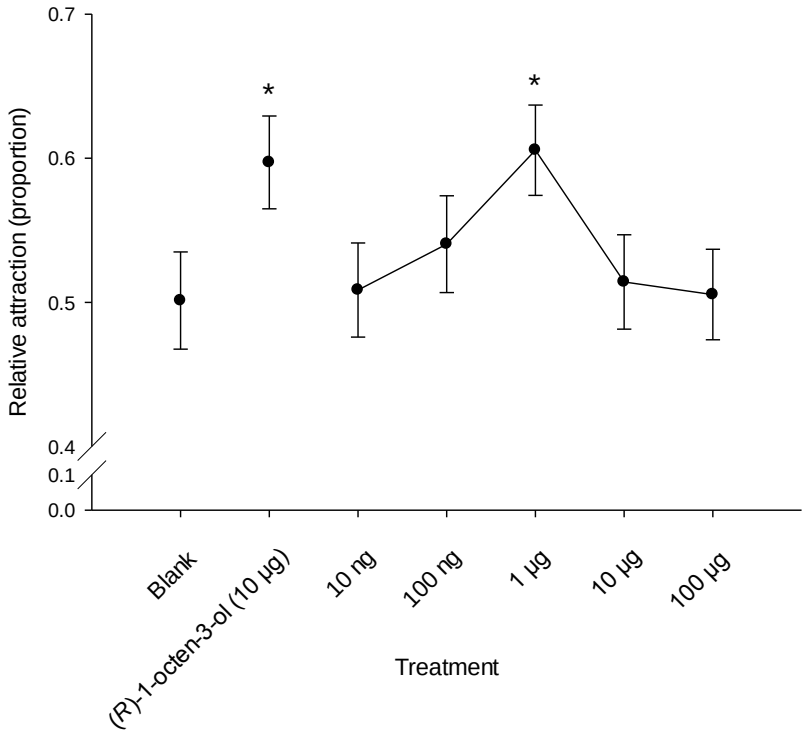


Figure 5.4: Relative attraction of wild *Culicoides* species to different doses of (*E*)-2-octene, a blank control (hexane) and a positive control (*R*)-(-)-1-octen-3-ol in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared with the blank control. Results shown as mean relative attraction ± standard error. Relative attraction at 1 µg of (*E*)-2-octene was 60.6 % and the (*R*)-(-)-1-octen-3-ol positive control was 59.7 % (GLM, *$P < 0.05$, n = 12).

5.3.2.2 Behavioural response of wild caught *Culicoides* species to 3-ethyltoluene

The behavioural response of wild caught *Culicoides* species to 3-ethyltoluene was investigated (Figure 5.5). An overall significant difference between treatments was found ($F_{6,65}$ = 2.27, P < 0.05, F-test from GLM model, n = 12) and significant increases in relative attraction, compared with the blank control, were discovered at 1 µg (55.8 % ± 0.03 S. E., P = 0.040, t-test within GLM model) and 100 µg of 3-ethyltoluene (58.5 % ± 0.03 SE, P = 0.008, t-test within GLM model). The blank control showed 47.0 % (±0.04 SE) relative attraction and the (*R*)-(-)-1-octen-3-ol positive control showed a significant increase in relative attraction (60.3 % ± 0.03 SE P = 0.002, t-test within GLM model) compared with the blank control. No significant difference in relative attraction as seen at 10 ng, 100 ng nor 10 µg of 3-ethyltoluene.

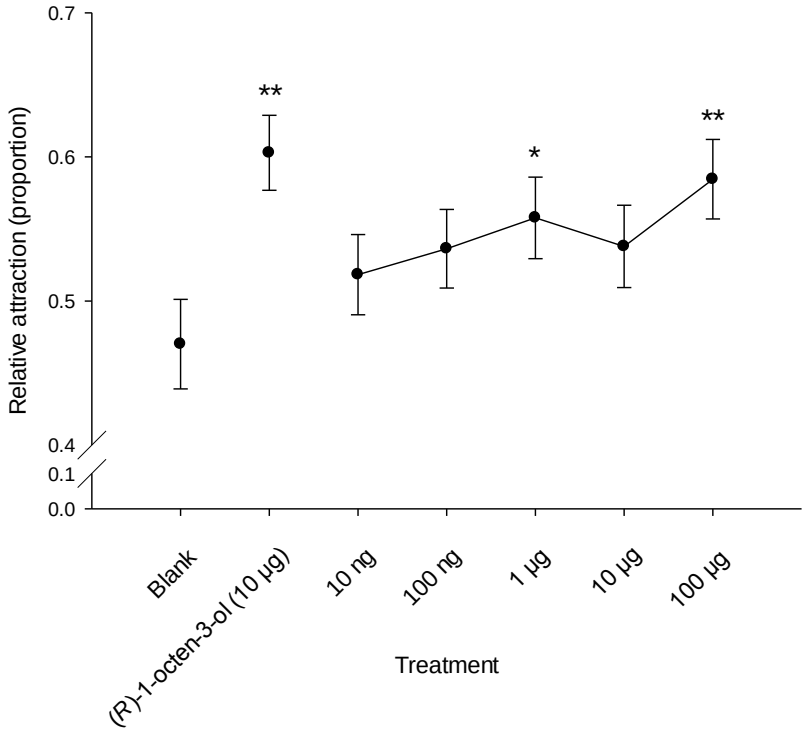


Figure 5.5: Relative attraction of wild *Culicoides* species to different doses of 3-ethyltoluene, a blank control (hexane) and a positive control (*R*)-(-)-1-octen-3-ol in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared with the blank control. Results shown as mean relative attraction ± standard error. Relative attraction at 1 µg of 3-ethyltoluene was 55.8 % (GLM, *P < 0.05, n = 12) and at 100 µg was 58.5 % (GLM, **P < 0.01, n = 12). The (*R*)-(-)-1-octen-3-ol positive control was 60.3 % (GLM, **P < 0.01, n = 12).

5.3.2.3 Behavioural response of wild caught *Culicoides* species to heptanal

An overall significant difference between treatments was found ($F_{6,65}$ = 4.24, *P* = 0.001, F-test from GLM model, n = 12) with a significant increase in relative attraction, compared with the blank control, at 1µg (60.1 % ± 0.04 S. E., *P* = 0.03, t-test within GLM model, Figure 5.6). The blank control showed 48.3 % (± 0.04 SE) relative attraction. The (*R*)-(-)-1-octen-3-ol positive control showed a significant increase in relative attraction compared with the blank control 63.0 % (± 0.03 SE) (*P* = 0.004, t-test within GLM model). No significant difference in relative attraction was seen at 10 ng, 100 ng, 10 µg nor 100 µg of heptanal.

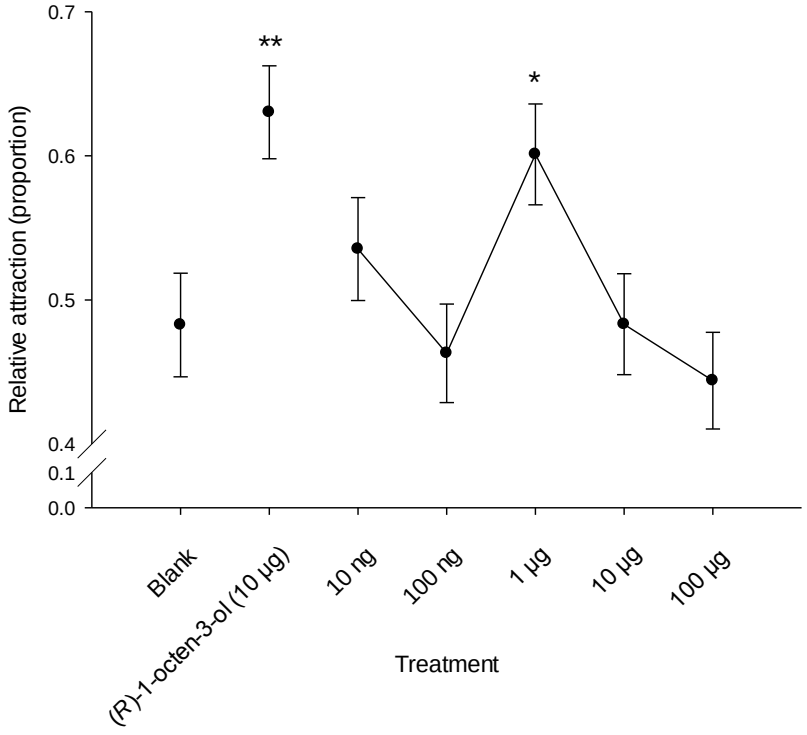


Figure 5.6: Relative attraction of wild *Culicoides* species to different doses of heptanal, a blank control (hexane) and a positive control (*R*)-(-)-1-octen-3-ol in a Y-tube olfactometer bioassay. Relative attraction is the proportion of midges that flew upwind and were counted in the "test" arm of the Y-tube olfactometer. Asterisks show statistically significant differences in response compared with the blank control. Results shown as mean relative attraction ± standard error. Relative attraction at 1 µg of heptanal was 60.1 % (GLM, *P < 0.05, n = 12) and the (*R*)-(-)-1-octen-3-ol positive control was 63.0 % (GLM, **P < 0.01, n = 12).

5.4 Discussion

Behavioural investigations for some of the EAG-active confirmed chemicals, identified in Chapter 4, using the Y-tube olfactometer (as described in Chapter 3) with laboratory reared and wild caught *Culicoides* species have shown behavioural responses to (*E*)-2-octene, 3-ethyltoluene, heptanal and 4-methylphenol. Significant increases in relative attraction of wild caught *Culicoides* species compared with the hexane control were recorded for (*E*)-2-octene, 3-ethyltoluene and heptanal. It is believed that this is the first time behavioural responses to (*E*)-2-octene, 3-ethyltoluene and heptanal have been reported for *Culicoides* species. Furthermore, it is believed to be the first time behavioural responses have been reported for (*E*)-2-octene and 3-ethyltoluene for any insect species, thereby making them novel attractants.

Initial investigations into behavioural responses of *Culicoides* species to the confirmed electrophysiologically active chemicals began with the laboratory reared model species, *C. nubeculosus*. This was due to lack of access to wild caught *Culicoides* species as it was winter. Wild caught *Culicoides* species were used preferentially when available.

Trans-2-octene was found to be a novel EAG-active chemical for wild *Culicoides* species and *C. nubeculosus* ("Pure" breed sheep extract) (Chapter 4, Section 4.3). This chemical was shown to be sheep-derived as it was only found in the sheep extracts with no trace recorded for the control extracts. A statistically significant difference were also found between the two sheep breeds for quantities of (*E*)-2-octene present in the extracts with the "pure" breed" showing almost 2.5 times as much as the "Cross" breed ($P < 0.05$, LSDs from ANOVA, Chapter 4, Section 4.3.4, Table 4.4). Behavioural dose response investigations with the Y-tube olfactometer found no significant difference in relative attraction of *C. nubeculosus* to (*E*)-2-octene compared with the hexane control. In contrast, a statistically significant increase in relative attraction of wild caught *Culicoides* species was discovered at the 1 µg dose (60.6 % ± 0.03 SE, GLM, partial t-test, $P < 0.05$) compared with the hexane control (50.1 % ± 0.03 SE). This is the first reported instance of behavioural activity (attraction) to (*E*)-2-octene for *Culicoides* species and for any insect species.

Culicoides nubeculosus is one of the few species successfully reared in the laboratory and as a result it has been used as a lab model for *Culicoides* species behaviour in this study and in previous studies (Boorman, 1974, Bhasin *et al*, 2000a, Logan *et al*, 2010). The fact that no response to (*E*)-2-octene was achieved using *C. nubeculosus*, in contrast to the significant increase in response noted for wild caught *Culicoides* species, raises questions and concerns about its usefulness as this species is a model for studying host seeking behavioural responses. Differences in the response between *C. nubeculosus* and wild caught *Culicoides* species were also noted for 1-octen-3-ol. Wild caught *Culicoides* species were found not to respond to racemic 1-octen-3-ol (100 µg), which was the positive control established using *C. nubeculosus* (Chapter 3, Section 3.3.2.1, Figure 3.2). As a result, the enantiomer (*R*)-(-)-1-octen-3-ol was investigated and found to statistically significantly increase the relative attraction of wild caught *Culicoides* species at a dose of 10 µg (Chapter 3, Section 3.3.2.2, Figure 3.3). It had been intended to use laboratory reared *C. nubeculosus* as a model species to test EAG-active confirmed chemicals in the Y-tube olfactometer over winter, highlighting chemicals for further investigation in the field during summer (with wild *Culicoides* species). As a result, wild *Culicoides* species were used preferentially, when available, for studying potential behavioural attraction of other confirmed EAG-active chemicals. Evidence exists, for sand flies, showing that in laboratory colonies selection for "laboratory-adapted" insects and genetic bottlenecks may result in fixation of rare alleles, thereby potentially altering normal responses (Mukhopadhyay *et al*, 1997). This is reiterated in reference to mosquitoes by Benedict *et al* (2009) who were trying to colonise and mass rear mosquitoes for use in sterile insect technique (SIT) programmes. They noted that they are in fact not trying to rear "natural" mosquitoes, but instead select for desirable traits. The potential selection of traits over time may lead to a reduction in the host seeking behaviour of laboratory reared insects, especially where there is no host seeking necessary for obtaining of food sources (sugar and blood). It may be interesting to compare the behavioural responses of wild caught *C. nubeculosus* with those of the laboratory colony *C. nubeculosus*, and if differences are found then further investigate the genetic basis of this. However, at present this is outside the scope of the current investigation. Therefore, it was deemed better to use wild caught *Culicoides* species where possible, a preference noted in Logan *et al* (2010).

Another EAG-active confirmed chemical shown to be a novel behaviourally active attractant, was 3-ethyltoluene, significantly increasing relative attraction of wild caught *Culicoides* species at a dose of 1 µg (55.8 % ± 0.03 SE, $P = 0.040$) and 100 µg (58.5 % ± 0.03 SE, $P = 0.008$) compared with the hexane control (47.0 % ± 0.04 SE). Significant differences in the quantities of 3-ethyltoluene were found between the two sheep breed extracts (the "Cross" breed showed twice as much as the "Pure" breed), and between the "Cross" breed extract and the control extract ($P < 0.05$, LSDs from ANOVA, Chapter 4, Section 4.3.4, Table 4.4). There are no previous reports of behavioural activity for any insect species in the literature. The only reference for 3-ethyltoluene related to insects is for its potential use as a solvent additive for "knockdown" pesticides in a patent from French pharmaceutical company Roussel-Uclaf (1991). In addition to the significant increase in relative attraction at the 1 µg dose, comparable with dose reported for (*E*)-2-octene, 3-ethyltoluene also showed an increase in relative attraction at the 100 µg dose. The trend shown in the dose response data (Section 5.3.2.2, Figure 5.5) implies an increase in relative attraction with increasing dose, therefore suggesting that higher doses of 3-ethyltoluene may provide further statistically significant increases in relative attraction of wild caught *Culicoides* species.

Novel behavioural activity of wild caught *Culicoides* species in response to heptanal has also been shown for the first time in the laboratory (section 5.3.2.3, Figure 5.6). The quantities of heptanal present in the both sheep entrainment extracts were significantly different from the control extracts ($P < 0.05$, LSDs from ANOVA, Chapter 4, Section 4.3.4, Table 4.4), although no statistically significant differences in quantities of heptanal were found between the two sheep breed extracts. Heptanal was shown to be behaviourally active in the Y-tube olfactometer, significantly increasing relative attraction of wild caught *Culicoides* species (60.1 %, ± 0.04 SE, $P = 0.03$) at a 1 µg dose compared with the hexane control. The response reported here, at a 1 µg dose, is the same as was found for (*E*)-2-octene and 3-ethyltoluene with the wild caught *Culicoides* species. Responses at the 1 µg dose seem consistent for wild caught *Culicoides* species based on these results, and it is interesting that this is a ten-fold lower dose than was discovered for the (*R*)-(-)-1-octen-3-ol positive control (10 µg, Chapter 3, Section 3.3.2.2, Figure 3.3). Previous studies have shown mixed results when investigating the

efficacy of heptanal using mosquitoes. Puri *et al* (2006) found heptanal to be attractive, significantly increasing the response of *Cx. quinquefasciatus* mosquitoes, compared with the solvent control, in a Y-tube olfactometer at doses of 0.1 µg, 1 µg and 10 µg. In contrast, no significant attraction to heptanal was discovered for *St. aegypti* when tested in a dual port olfactometer (Bernier *et al*, 2002). This contrasting result from the two mosquito species was also evident in the GC-EAG result for *Culicoides* species where it was shown to be EAG-active in the "Pure" breed extract with wild caught *Culicoides* species but not with *C. nubeculosus*. Heptanal, shown here to be a sheep-derived volatile, was previously reported to be present in SPME of sheep wool and wool grease samples (Lisovac and Shooter, 2003). Heptanal has also been referred to in previous studies as a plant volatile and has been shown to affect the detection of pheromones in male moths, *Agrotis ipsilon* Hufnagel (Chaffiol *et al*, 2012).

Using laboratory reared *C. nubeculosus*, 4-methylphenol was shown to elicit a behavioural response in the Y-tube olfactometer (Section 5.3.1.1, Figure 5.1). A statistically significant decrease in relative attraction was noted at the 1 µg dose (39.6 % ± 0.04 SE, P = 0.028, partial t-test within GLM model) compared with the hexane control (53.9 % ± 0.04 SE). The dose range used with *C. nubeculosus* was lower than that used with wild caught *Culicoides* species, mainly due to increased time pressure being present in the latter case as the field season approached. The data showed a decreasing trend with a reduction in relative attraction as the dose increased. Therefore, it may be likely that doses greater than 1 µg may reduce further the relative attraction of *C. nubeculosus*. Quantities of 4-methylphenol were significantly greater in both sheep breed entrainment extracts compared to the control extracts, confirming it as a sheep-derived volatile ($P < 0.05$, LSDs from ANOVA, Chapter 4, Section 4.3.4, Table 4.4). In a separate study, *C. nubeculosus* were shown here to exhibit a significant decrease in relative attraction, compared with the hexane control, at the 1 µg dose in the Y-tube olfactometer, while no significant differences in relative attraction were found for the other doses tested (10 pg – 100 ng). This result is supported by previous research into the behavioural responses of *C. impunctatus* to several phenolic compounds (phenol, 3-n-propylphenol, 3-mehtylphenol and 4methylphenol) using the Y-tube olfactometer (Bhasin *et al*, 2000a). Phenol was found to significantly increase relative attraction of *C. impunctatus* at a 10^{-10} g dose compared with the expected

response based on the solvent control, however, significant decreases in relative attraction were noted at the 10^{-6} g, 10^{-4} g and 10^{-3} g doses. Only reductions in relative attraction of *C. impunctatus* were found with 3-n-propylphenol and 3-methylphenol, with significant reductions recorded at the 10^{-5} g and 10^{-3} g doses (3-n-propylphenol) and 10^{-8} g, 10^{-7} g and 10^{-6} g doses (3-methylphenol). A non-significant increase in relative attraction of *C. impunctatus* compared with the control was reported for 4-methylphenol at the 10^{-10} g and 10^{-8} g doses while significant reductions in relative attraction were found at 10^{-7} g and 10^{3} g doses (Bhasin *et al*, 2000a). The results presented here and in Bhasin *et al* (2000a) suggest that the phenolic chemicals are most likely to elicit no behavioural response, or a decrease in relative attraction at higher doses with *Culicoides* species. Although repellency cannot strictly be shown in the Y-tube olfactometer, a significant decrease in relative attraction would indicate that these chemicals may be worth investigating further using suitable repellency tests in the laboratory or field. Identification of effective repellents could be useful in protecting both animals and humans against *Culicoides* species bites. However, the increase in relative attraction of *C. impunctatus* at the lowest dose tested (10^{-10} g) to phenol (significant) and 4-methylphenol (non-significant) may suggest a role as a potential attractant (Bhasin *et al*, 2000a). Both Hassanali *et al* (1986) and Bursell *et al* (1988) tentatively identified (GC-MS) 4-methylphenol as the major component of buffalo urine, which had been shown previously to be an effective attractant of tsetse flies in the field by other studies. Additionally, 4-methylphenol has been shown to be a component of skin secretions from oxen and has been tentatively identified as a component chemical in the air entrainment extracts of cows from Burkina Faso (personal communication, Dr. Sarah Dewhirst, Warnes, 1990). With regard to tsetse flies in Côte d'Ivoire and Burkina Faso, blends incorporating 4-methylphenol were found to be about twice as effective at attracting *Glossina* species as those including 3-methlyphenol (Rayaisse *et al*, 2010). Based on the results of this study, and previous findings from the literature, it would appear that 4-methylphenol does not form part of the core set of chemicals (as described by Logan and Birkett, 2007) that discriminate a host for *Culicoides* species.

Also investigated in the Y-tube olfactometer with *C. nubeculosus* was the confirmed EAG-active chemical 4-oxoisophorone, however, no statistically significant behavioural

responses were found (Section 5.3.1.2, Figure 5.2). Analysis of the quantities of 4-oxoisophorone present in the entrainment extracts revealed almost no trace in the control extract (0.001 mg day^{-1} for 3 sheep, Chapter 4, Section 4.3.4, Table 4.4) and significantly greater quantities found in both sheep breed extracts ($P < 0.05$, LSDs from ANOVA, Chapter 4, Section 4.3.4, Table 4.4) suggesting it was a sheep-derived volatile. Additionally, significant differences in quantities of this chemical were found between the two sheep breeds, with a 5-fold increase in the "Pure" breed. There appears to be no previous references in the literature for behavioural responses of insects to 4-oxoisophorone, however, there is evidence of EAG-activity from several insects (full details in Chapter 4, Section 4.4). It is interesting to find this chemical identified from host sheep as it has primarily been described as an irregular terpenoid plant volatile (Knudsen and Gershenzon, 2006). *Culicoides* species may also use this volatile, along with heptanal, as chemical cues when locating plant sugars to feed on (an area of work which has received less attention due to most volatile based research for haematophagous insects focussing on host location and host volatiles). If behavioural activity can be shown to this potential host (and plant) volatile in the laboratory or the field it would represent a novel chemical attractant/repellent for *Culicoides* species (and insects of other species) and could be useful for monitoring or control strategies.

Logan *et al* (2009) investigated the behavioural responses of *C. impunctatus* to decanal using a Y-tube olfactometer and found significant decreases in relative attraction compared with the control at doses of 100 µg and 10 µg while no significant difference in relative attraction was discovered at doses of 1 µg and 100 ng. Therefore both decanal and 4-methylphenol have been shown to significantly reduce relative attraction of *C. impunctatus*. This is something that should be considered when producing blends of chemicals to test in the field (Chapter 6). Behavioural responses of *St. aegypti* mosquitoes to decanal were also investigated in the Y-tube olfactometer covering a decadic dose range of 100 µg to 10 ng (Logan *et al*, 2008). Decanal was presented in addition to a standard hand and a significant increase in relative attraction, compared with the control, was found at all doses except the highest dose (100 µg) for *St. aegypti* (Logan *et al*, 2008). However, when compared with the standard hand on its own, decanal plus the standard hand resulted in a significant decrease in relative attraction at 1 µg, 10 µg and 100 µg doses, suggesting a masking

effect on the standard hand attractant (Logan *et* al, 2008). Interestingly, the statistical analysis based on the mean amount of the 12 confirmed chemicals present in the sheep breed extracts (Chapter 4, Section 4.3.4, Table 4.4) revealed that there was no significant difference in the amount of decanal present in either sheep breed compared with the control, nor between the sheep breeds. This would imply that decanal was in fact a contaminant (as discussed in Chapter 4, Section 4.4). Therefore, although detected in the sheep extracts and EAG-active for wild caught *Culicoides* species (no responses were noted with *C. nubeculosus*), decanal may not be a primary host location cue for *Culicoides* species, hence the lack of attraction reported for *C. impunctatus* (Logan *et al*, 2009). For non-haematophagous insects, decanal, detected in plant entrainments of *Cinnamomum camphora* elicited EAG-activity in the common bluebottle, *Graphium sarpedon nipponum* Fruhstorfer (Li *et al*, 2010). Additionally, in a touch bioassay, a significant increase in touches of a decanal treated twig was reported compared with the untreated control twig (Li *et al*, 2010).

Of the 12 EAG-active confirmed chemicals, heptane was the only one to show a significant decrease in mean quantity in the sheep breed extracts compared with the control extract (Chapter 4, Section 4.3.4, Table 4.4). The reason for this decrease in chemical quantity in the sheep entrainment extracts remains unclear. In addition to reporting EAG-activity to heptane, present in entrainments of Alphonso mangoes, with the oriental fruit fly (*B. dorsalis*), Jayanthi *et al* (2012) also noted behavioural attraction in the Y-tube olfactometer.

For the remaining EAG-active identified compounds reported in Chapter 4 (Section 4.3.4, Table 4.4), there is no reference in the literature to behavioural responses by any insect species to styrene, 1,3-diacetylbenzene, 1,4-diacetylbenzene or hexadecane, which suggests they may be novel attractants or repellents for *Culicoides* species if behavioural activity can be shown in future investigations. However, based on the analysis of quantities of 1,4-diacetylbenzene present in the entrainment extracts, it appeared to be a Porapak™ Q breakdown contaminant (Sturaro *et al*, 1992). Hexadecane was one of the four chemicals (along with (*E*)-2-octene, 3-ethyltoluene and 4-oxoisophorone) that were found to be statistically different between the sheep breeds based on quantities of chemicals in the entrainment extracts with twelve times as much hexadecane present in the “Pure” breed extract

compared with the “Cross” breed extract (Chapter 4, Section 4.3.4, Table 4.4). As discussed in Chapter 4 (Section 4.4) all of these chemicals may have a role in the differential attraction of *Culicoides* species to sheep, or may be part of a core set of chemicals that define a host (sheep) volatile cue, which would explain the lack of behavioural difference found to the two sheep breeds in the field and laboratory (Chapter 3).

Acetophenone was the only EAG-active confirmed chemical, other than (*E*)-2-octene, to show no trace in the control extract, thereby confirming it was a sheep-derived volatile (Chapter 4, Section 4.3.4, Table 4.4). It has been shown to significantly reduce the attraction of the western pine beetle (*Dendroctonos brevicomis* Leconte) to its aggregation pheromone (Erbilgin *et al*, 2008). However, in contrast, it has also been shown to be a component of the oviposition pheromone of the gregarious desert locust (*Schistocera gregaria* Forskal), inducing gravid females to oviposit in behavioural bioassays (Rai, *et al*, 1997). Due to a late emergence (approximately 5-6 weeks late) of wild *Culicoides* species in the final summer (2013), it was not possible to investigate the behavioural responses of acetophenone using, wild caught *Culicoides* species, in the Y-tube olfactometer. Based on the previous studies reporting behavioural effects, it remains unclear whether acetophenone may act as an attractant or repellent with wild *Culicoides* species. It is therefore suggested that the potential behavioural responses of this chemical are investigated as a priority in any future work derived from this thesis.

5.5 Conclusions

Novel behavioural attraction of wild caught *Culicoides* species has been successfully shown in the laboratory using a Y-tube olfactometer for the confirmed EAG-active chemicals: (*E*)-2-octene, 3-ethyltoluene and heptanal. Additionally, this is the first time behavioural responses have been reported for any insect species for (*E*)-2-octene and 3-ethyltoluene. Of the remaining EAG-active chemicals, further novel behavioural responses may be discovered subject to investigation in the laboratory. However, there was not time to achieve this within this study. Acetophenone is highlighted as a potentially behaviourally active host-derived chemical as it was only found in the

sheep derived entrainment extracts. Further investigations with these chemicals and other host-seeking insects, such as mosquitoes and tsetse flies, may reveal them to be wider insect attractants (or repellents).

These results provide evidence for further research of the confirmed EAG-active chemicals in the field (Chapter 6).

Chapter 6: Field trials of EAG-active identified chemicals and blends

6.1 Introduction

In the first recorded field based investigations involving *Culicoides* species, incidental collections of *Culicoides* species were found in studies focussed on mosquitoes and mosquito host-related cues (Takken and Kline, 1989, Kline *et al* 1990). For example, using CDC traps, Takken and Kline (1989) investigated the effects of CO_2 (200 and 1000 ml min^{-1}) and racemic 1-octen-3-ol (1.57 – 2.26 mg h^{-1}) on collections of mosquitoes at two sites in Florida, USA. They found that in addition to mosquitoes and some other insect species, the traps caught *C. furens* and *C. mississippiensis*. At the Snake Bight site collection of *Culicoides* species with the CO_2 (200 ml min^{-1}), racemic 1-octen-3-ol and CO_2 (1000 ml min^{-1}) treatments traps were similar (3.8 ± 4.1, 5.7 ± 2.8 and 6.0 ± 3.0 *Culicoides* species caught per trap day^{-1}, respectively) while the CO_2 (200 ml min^{-1}) + racemic 1-octen-3-ol treatment trap caught significantly more *Culicoides* species (272.2 ± 152.1 caught per trap day^{-1}). Similarly, at the Suwannee site catches using the CO_2 (200 ml min^{-1}) and racemic 1-octen-3-ol treatment traps were not significantly different from each other, with catches elevated for a greater concentration of CO_2 (1000 ml min^{-1}) treatment traps and significantly increased catches for the CO_2 (200 ml min^{-1}) + racemic 1-octen-3-ol treatment traps (Takken and Kline, 1989). The study shows early evidence of synergism between CO_2 and racemic 1-octen-3-ol for *Culicoides* species trap catches. In the same area of Florida, Kline *et al* (1990) investigated the potential of butanone, CO_2 (200 ml min^{-1}), honey extract, 1-octen-3-ol (3 mg h^{-1}), (L)-lactic acid and phenols as mosquito attractants. Carbon dioxide and (L)-lactic acid on their own caught similar low numbers of *C. furens*, however, racemic 1-octen-3-ol and phenol (each individually) attracted larger numbers. Racemic 1-octen-3-ol + phenol, and racemic 1-octen-3-ol + CO_2 increased trap catches by 100-fold compared with CO_2 alone (Kline et al, 1990).

Following these discoveries *Culicoides* species specific studies began investigating common attractants found to be effective for mosquitoes or tsetse flies. In South-eastern Queensland, Australia, Ritchie *et al* (1994) investigated the response of *Culicoides* species to CO_2 (200 ml min^{-1}), racemic 1-octen-3-ol (6.05 mg h^{-1}) and light using encephalitis vector surveillance (EVS) traps. The study found the CO_2 + light, or

CO_2 + racemic-1-octen-3-ol tended to increase trap catches of *Culicoides* species compared with CO_2 or racemic 1-octen-3-ol alone. The authors suggest that the synergistic effect between CO_2 and racemic 1-octen-3-ol could be utilised to improve surveillance of disease vectors in relation to BTV (Ritchie *et al*, 1994). In the UK (Ormsary, Scotland) Blackwell *et al* (1994) investigated attraction of *C. impunctatus* to Delta traps baited with live female midges. Increases in trap catches baited with freshly squashed female *C. impunctatus*, or hexane extracts of live females provided evidence of midge produced volatile attractant/pheromone (Blackwell *et al*, 1994). At the same field site the response of *C. impunctatus* to Delta traps baited with racemic 1-octen-3-ol (0.11 mg h^{-1}) was investigated with consistently greater numbers of *C. impunctatus* caught in the test traps compared with the control (Blackwell *et al*, 1996). Later, Bhasin *et al* (2001) used that site to investigate responses of *C. impunctatus* to racemic 1-octen-3-ol (0.06 mg h^{-1}), acetone, butanone and a variety of phenolic compounds were investigated using Delta traps. Racemic 1-octen-3-ol, acetone and a mix of 6 phenolic compounds were all shown significantly to increase trap catches compared with an unbaited control. When tested in combination with CO_2 (200 ml min^{-1}), synergism was also reported for racemic 1-octen-3-ol, acetone and cow urine (a known tsetse fly attractant) compared with CO_2 alone. However, combinations of CO_2 + racemic 1-octen-3-ol/acetone + cow urine produced the largest increase in trap catches (Bhasin *et al*, 2001). Additionally, the authors reported a significant dose-dependent increase in trap catches covering a range of CO_2 release rates (0.2 – 2.5 l min^{-1})(Bhasin *et al*, 2001). Also, as discussed in Chapter 3, Mands *et al* (2004) investigated *Culicoides* species trap enhancement using hexane extracts of animal hair samples with water buffalo causing the greatest increase in trap catch (+262 %) while the sheep extract caused a reduction (-53 %) in trap catch.

Commercial traps have been developed for trapping of mosquitoes and *Culicoides* species (e.g. Mosquito Magnet® range), as well as traps marketed specifically for *Culicoides* species trapping (e.g. Midgeater) which often use the addition of a slow release formulation of racemic 1-octen-3-ol. However, these traps are not commonly used for surveillance purposes. Previous studies have not investigated host derived volatiles, relevant to *Culicoides* species, with a view of attempting to mimic the natural host. Furthermore, research has shown that traps for catching *Culicoides* species,

including the recommended OVI light downdraft suction trap underestimate *Culicoides* species population numbers and biting rates on animals (Carpenter *et al*, 2008a, Gerry *et al*, 2009, Harrup *et al* , 2012, Mellor *et al*, 2004). Current surveillance traps tend to use light suction traps on their own, or in combination with CO_2, which does not reflect accurately the volatile profile of a host.

Recently, following the spread of BTV into Northern Europe, the 2007 UK outbreak, and the emergence of SBV, there has been increased focus on improving vector surveillance traps and ensuring their catches are representative of the circulating *Culicoides* species populations. In a study comparing the efficacy of light trapping (OVI UV black light) with live animal drop trapping in the UK (Compton, England), Carpenter *et al* (2008a) found that light trapping did not provide an accurate reflection of the *Culicoides* species biting sheep. *Culicoides chiopterus* was the second most abundant species present on sheep, yet only accounted for <1 % of the total light trap catch. Conversely, *C. pulicaris* and *C. punctatus* were caught in relatively high number in the light trap, but completely absent from drop trap catches. Similarly, in Spain, Gerry *et al* (2009) found that CO_2 baited traps and CDC black light traps underestimated the numbers of *C. obsoletus* and *C. parroti* when compared with direct aspirations of *Culicoides* species from live sheep. A comparison of animal-baited traps with UV light suction traps for collecting *Culicodes* species in France was conducted by Viennet *et al* (2011).The authors found that UV light suction traps did not estimate accurately biting populations of *Culicoides* species. Drop trapping caught the greatest abundance of *Culicoides* species while the use of sticky traps did reflect accurately biting rates, however, only direct aspiration exclusively caught host seeking females. These results highlight the need for host derived semiochemical slow release formulations that improve trap catches and accurately reflect the circulating *Culicoides* species populations.

The aim of this chapter was to investigate behavioural activity of identified EAG-active chemicals and blends in a field setting using slow release formulations and CDC style traps.

Objectives:

- To produce slow release formulations and determine release rates which match the natural host (sheep) for identified EAG-active chemicals for use in the field.
- To investigate behavioural activity of individual chemicals and blends with wild *Culicoides* species in the field using CDC traps.

6.2 Materials and methods

6.2.1 Location

Field trials were conducted at Rushall organic farm (Bradfield, Berkshire, UK, 51° 27' 7.64" N, 1° 9' 38.48" W) in the summer of 2012 and 2013 in a field known as "Annie's Field" (Figure 6.1). This is the same field site used for the main collection of sheep volatiles using the whole sheep air entrainment box as described previously (Chapter 2, Section 2.2.4.2). It should be noted that during the collection of sheep volatiles the field contained a mixture of sheep (Hartline and Hartline Suffolk cross breeds) and cattle. During the summer 2012 field trial the field contained only sheep, whereas during the summer 2013 field trial the field contained a mixture of sheep and cattle again (as well as two donkeys for one night).

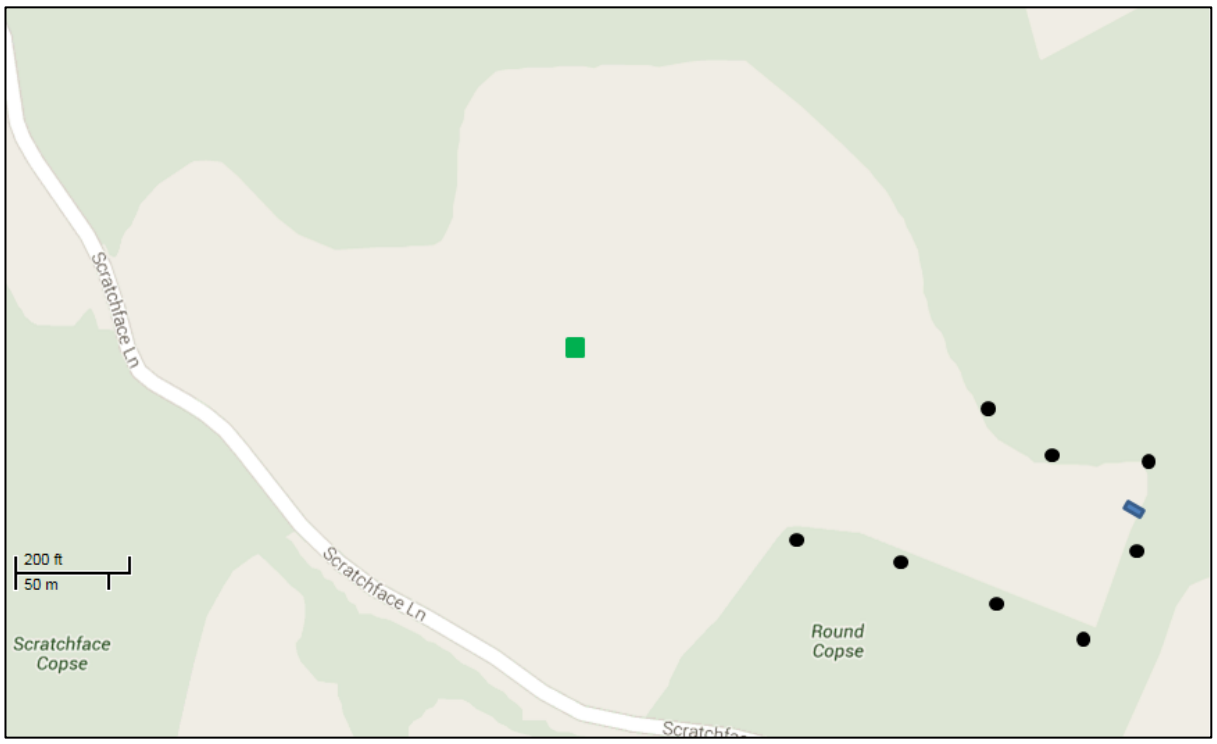


Figure 6.1: Field trial site map for Rushall organic farm. Black circles show trap positions, blue rectangle = water trough, green square = weather station.

6.2.2 Release rates of confirmed EAG-active chemicals from slow release formulations

EAG-active chemicals (identified in Chapter 4) were formulated using different slow release techniques achieve the required release rates (measured by daily weight loss) based on the natural release rates of chemicals from three sheep per day (Chapter 4, Section, 4.3.2, Table 4.2). The methods used were:

(1) Small polyvial: 400 µl of chemical was sealed in a 0.5 ml polythene vial (Just Plastics Ltd, Norfolk, UK).
(2) Large polyvial: 500 µl of chemical was added to a thick cellulose sponge (10 mm thick, J Sainsbury plc, London, UK) and sealed in a 2.5 ml polythene vial (Just Plastics Ltd, Norfolk, UK).
(3) 500 gauge bag: 500 µl of chemical was added to a thin cellulose sponge (3 mm thick, J Sainsbury plc, London, UK) and heat-sealed in a 500 gauge polythene tubing bag (A1 Packagings Ltd, London, UK).
(4) 1000 gauge bag: 500 µl of chemical was added to a thin cellulose sponge (3 mm thick, J Sainsbury plc, London, UK) and heat-sealed in a 1000 gauge polythene tubing bag (A1 Packagings Ltd, London, UK).
(5) 1500 gauge bag: 500 µl of chemical was added to a thin cellulose sponge (3 mm thick, J Sainsbury plc, London, UK) and heat-sealed in a 500 gauge polythene tubing bag, which in turn was heat-sealed in a 1000 gauge polythene tubing bag (A1 Packagings Ltd, London, UK).

Four replicates for each slow release formulation type were prepared for each identified chemical. Slow release formulations were placed in a wind tunnel maintained at 20 °C with a flow rate of 0.4 m sec^{-1}. The slow release formulations were weighed three times every 24 hours and average weight loss calculated.

When release rates that matched that of the whole sheep entrainment were achieved, slow release formulations were prepared in advance, sealed in a heat-sealed foil pouches and stored at -20 °C until delivered to Andrew Hope for the summer 2012 field trial. For the summer 2013 field trial, slow release formulations were prepared as needed and either taken directly to the field on the day of preparation, or placed in the wind tunnel described above until the optimum day(s) of release (Table 6.4).

6.2.3 Field trial design

6.2.3.1 Field trial, summer 2012 – Andrew Hope (The Pirbright Institute)

A field trial was designed in conjunction with Andrew Hope (PhD student, The Pirbright Institue) to investigate the identified EAG-active chemicals from the whole sheep entrainment extracts. The randomised Latin square design consisted of eight treatments (traps) with an 8 night rotation and three replicates, resulting in a total of 24 trapping nights. A new randomisation was made at the start of each 8 night block (replicate), and the traps were moved to new positions each night. The traps were all CDC style traps and included:

(1) Blank + no CO_2 (negative control)

(2) CO_2 (negative control)

(3) UV light (positive control)

(4) (*R*)-(-)-1-octen-3-ol + CO_2

(5) (*E*)-2-Octene + CO_2

(6) 3-Ethyltoluene + CO_2

(7) Heptanal + CO_2

(8) 3-Chemical blend: (*E*)-2-octene + 3-ethyltoluene and heptanal + CO_2

All chemicals were released from individual slow release formulations. For the three-chemical blend treatment, each chemical was released from an individual slow release formulation attached to the trap in a close group. The field trial was run by Andrew Hope and insects were collected and identified by him at The Pirbright Insititue (Pirbright, UK).

Carbon dioxide (22 kg, BOC Industrial Gases, UK) was delivered from a cylinder, located near the trap and resting upright against a tree (to reduce additional visual stimuli) fitted with a two stage CO_2 regulator (BOC Industrial Gases, UK) and connected using Tygon tubing (Saint-Gobain, Performance Plastics, Poestenkill, NJ, USA), via a flow meter (Key Instruments, Trevose, PA, USA), to the underside of the CDC trap (J. W. Hock, Gainesville, USA) where the tubing was fixed in place with duct tape. (*R*)-(-)-1-Octen-3-ol was released from a glass vial (1 ml Chromacol vial with 20 mm exposed wick) at a rate of approximately 4.1 mg h^{-1} (98.4 mg day^{-1}) as described by Harrup *et al* (2012). Slow release formulations were grouped together using string and stuck to the

underside of the CDC trap, near the CO_2 tubing (when appropriate) using duct tape. The UV trap was powered by a 12V 7Ah battery (Yuasa, Swindon, UK) while the unlit CDC traps were powered by 6V 12 Ah batteries (Yuasa, Swindon, UK). All other CDC traps had the lights turned off.

The traps were turned on 1 hour before sunset and run until three hours after sunset (4 hour trapping period). Insects attracted to the traps were caught by downdraft fan and blown into a plastic container (that screwed into the trap) containing water and a drop of detergent. At the end of each night, when the traps (and CO_2 cylinders) were turned off, the batteries and plastic containers were collected and taken back to The Pirbright Institute (2012) or Rothamsted Research (2013). The trap catches were transferred into 70 % ethanol for storage and later identification. The batteries were recharged each night using a 12 V 10 amp hours battery charger.

6.2.3.2 Field trial summer 2013

A field trial was designed similar to that used in summer 2012. The randomised Latin square design consisted of 8 treatments (traps) and 8 positions, with an 8 night block and 3 replicates, resulting in a total of 24 trapping nights. A new Latin square randomisation was made at the start of each 8 night block (replicate), and the treatments were moved randomly to new positions each night rather than rotated (as had occurred in the summer 2012 field trial). The traps were all CDC style traps and included:

(1) Blank + no CO_2 (negative control)

(2) CO_2 (negative control)

(3) UV light (positive control)

(4) (*R*)-(-)-1-octen-3-ol + CO_2

(5) 3-Chemical blend + CO_2

(6) 7-Chemical blend + CO_2

(7) 10-Chemical blend + CO_2

(8) 12-Chemical blend + CO_2

All chemicals were released from individual slow release formulations (Table 6.1). The 3-chemical blend contained the same chemicals as were used in the 2012 field trial.

All other details are the same as in section 6.2.3.1.

Table 6.1: Chemicals present in the slow release formulation blends used during the summer 2013 field trial. ✓ = chemical included in the blend.

Chemical	Chemicals present in each blend			
	3-Chemical blend	7-Chemical blend	10-Chemical blend	12-Chemical blend
Heptane	-	-	✓	✓
(*E*)-2-Octene	✓	✓	✓	✓
Styrene	-	✓	✓	✓
Heptanal	✓	✓	✓	✓
3-Ethyltoluene	✓	✓	✓	✓
Acetophenone	-	✓	✓	✓
4-Methylphenol	-	-	-	✓
4-Oxoisophorone	-	✓	✓	✓
Decanal	-	-	-	✓
1,3-Diacetylbenzene	-	-	✓	✓
1,4-Diacetylbenzene	-	-	✓	✓
Hexadecane	-	✓	✓	✓

6.2.4 Statistical analysis

6.2.4.1 Field trial summer 2012 – Andrew Hope (The Pirbright Institute)

A negative binomial model was used to analyse the trap count data from the summer 2012 field trial. Total numbers of wild *Culicoides* species caught for each trap treatment were compared, along with covariates relating to the variation in wind direction and the air temperature at sunset. Due to zero counts the blank trap and 3-ethyltoluene trap data were omitted from the final analysis model.

6.2.4.2 Field trial summer 2013

A GLM (as described in Chapter 3, Section 3.2.5) with a Poisson distribution and a logarithmic transformation was used to analyse the trap count data from the summer 2013 field trial. Variation due to due to the position of the trap at the field site was accounted for using the term “Position”, while variation over the duration of the field

trial was accounted for using the term “Time”. The 3 x 8 night design of the field trial was included using the term “Block” and the slow release formulation treatments were investigated using the term “Treat”. The four covariates; air temperature (°C), relative humidity (%), wind direction (degrees) and wind speed (m s^{-1}) were investigated for statistical significance ($P < 0.05$, F-tests) in the model when added in after the design terms using a forward selection process. The procedure added in the most significant covariate first and then checked the other covariates in a step-wise manner.

The GLM model for investigating behavioural response of wild *Culicoides* species to slow release formulation treatments on CDC traps was then found to be:

y ~ Constant + Block + Block.Position + Relative humidity + Air temperature + Wind direction + Time + Treat + Time.Treat

where y was log(count), Constant was an overall mean for the data on the log scale, Block and Position were blocking factors, Relative humidity, Air temperature , Wind direction and Time were variables and Treat was the treatment factor. The dot indicates the interaction between Block and Position, and the interaction between Treat and Time.

The initial analysis highlighted that while the UV CDC light trap (positive control) was proven to catch a greater number of wild *Culicoides* species compared with the CO_2-baited CDC trap (negative control), the UV trap data also accounted for a high proportion of the variance in the data, potentially swamping the signal from the other treatments over time. As a result the UV trap data was removed from the model and the data were re-analysed.

Following a further forward selection process the GLM model was then found to be:

y ~ Constant + Block + Block.Position + Wind speed + Air temperature + Time + Treat + Time.Treat

where y was log(count), Constant was an overall mean for the data on the log scale, Block and Position were blocking factors, Air temperature, Wind speed and Time were variables and Treat was the treatment factor. The dot indicates the interaction between Block and Position, and the interaction between Treat and Time.

Predictions, with standard errors, were made at relevant levels of factors and values of variables included in the model.

The GenStat® (2011, 14th edition, © VSN International, Hemel Hempstead, UK) statistical system was used for the analysis.

6.3 Results

6.3.1 Release rates of confirmed EAG-active chemicals from slow release formulations

Five different types of slow release formulation were prepared for each of the twelve EAG-active confirmed compounds. Periods of stable release (measured by daily weight loss) were determined (Table 6.2).

Table 6.2: Release rates of each slow release formulation type for three of the twelve EAG-active confirmed compounds SE is the standard error, and "Days of stable release" shows the active period counted from slow release formulation preparation.

Chemical	Slow release formulation type	Release rate	SE	Days of stable release
(*E*)-2-Octene	500 gauge bag	0.39	0.04	5-8
	1000 gauge bag	-	-	-
	1500 gauge bag	2.97	0.65	3-4
	Small polyvial	17.75	0.10	3-13
	Large polyvial	40.77	0.92	3-7
		6.57	0.46	10-13
Heptanal	500 gauge bag	33.47	1.84	1-4
		3.03	0.17	12-18
	1000 gauge bag	3.40	0.35	14-18
	1500 gauge bag	5.28	0.67	12-18
	Small polyvial	0.47	0.08	2-4
		1.27	0.01	5-22
	Large polyvial	0.42	0.08	2-3
		1.72	0.10	4-6
		2.63	0.08	5-22
3-Ethyltoluene	500 gauge bag	0.16	0.02	3-4
	1000 gauge bag	-	-	-
	1500 gauge bag	0.35	0.04	6-7
	Small polyvial	19.05	0.14	3-11
	Large polyvial	38.12	0.87	3-7

The natural release rate of the twelve EAG-active confirmed chemicals, based on the whole animal air entrainment extract (three sheep, "Pure" breed), was determined (Table 6.3). Average areas for these peaks were calculated from the raw GC data and adjusted using the response curves created during GC-EAG quantification (Chapter 4,

Section 4.3.3). It was not possible to match the natural release rate, from three sheep, for all 12 confirmed chemicals with the slow release formulations that had been prepared. However, a closer match at a release rate 10 times higher than natural was possible (Table 6.4). As a result, slow release formulations at ten times the natural release rate were chosen for use in field trials. It is common for chemicals to be released at greater than "natural" rates during field trials and often leads to increased traps catches, for example the release rate of 1-octen-3-ol from ox breath is 0.05 mg h^{-1}, yet it has been shown to significantly increase trap catches of *Culicoides* species as (*R*)-(-)-1-octen-3-ol at a release rate of 4.1 mg h^{-1} (82 times greater than natural)(Vale and Hall, 1985, Harrup *et al*, 2012).

Table 6.3: Natural release rate of chemicals from three sheep ("Pure" breed, mg day^{-1}). SE = standard error.

	(mg day^{-1})	
Chemical	**Mean**	**SE**
Heptane	10.748	1.798
(*E*)-2-Octene	0.298	0.052
Styrene	0.010	0.002
Heptanal	0.327	0.024
3-Ethyltoluene	0.009	0.003
Acetophenone	0.036	0.006
4-Methylphenol	3.782	0.523
4-Oxoisophorone	0.010	0.001
Decanal	0.161	0.009
1,3-Diacetylbenzene	0.199	0.012
1,4-Diacetylbenzene	0.097	0.011
Hexadecane	0.027	0.003

6.3.2 Field trial summer 2012 – Andrew Hope (The Pirbright Institute)

The summer 2012 field trial using slow release formulations prepared from chemicals identified in this study, was run by Andrew Hope (PhD student, The Pirbright Institute)(Table 6.5). The 3-chemical blend trap ((*E*)-2-octene, heptanal and 3-ethyltoluene) caught significantly more wild midges (29, $P < 0.05$) than the CO_2 (negative control), (*E*)-2-octene and heptanal-baited traps. No significant difference in trap catch was found between the 3-chemical blend baited trap and the (*R*)-(-)-1-octen-3-ol baited trap. The positive control UV light trap caught the significantly more midges than any of the other trap treatments (5601, $P < 0.001$).

Table 6.4: Matching of available slow release formulation release rates to approximately 10x natural release rate of three sheep ("Pure" breed). Optimal available release rate and desired release rate of chemicals shown, S.E. = standard error. SP – small polyvial, LP – large polyvial, 500g – 500 gauge bag, 1500g – 1500 gauge bag.

		(mg day^{-1})					
Chemical	**Slow release formulation**	**Release rate**	**SE**	**Days to use**	**Number**	**Total release rate**	**Desired release rate**
Heptane	SP	32.23	0.21	2-7	3	96.68	107.48
(*E*)-2-Octene	1500g	2.97	0.60	3-4	1	2.97	2.98
Styrene	500g	0.13	0.01	3-4	1	0.13	0.10
Heptanal	SP	1.28	0.02	5-19	3	3.84	3.27
3-Ethyltoluene	500g	0.16	0.02	3-4	1	0.16	0.09
Acetophenone	SP	0.35	0.09	1	1	0.35	0.36
4-Methylphenol	500g	3.77	0.03	1-10	10	37.72	37.82
4-Oxoisophorone	SP	0.10	0.09	3	1	0.10	0.10
Decanal	LP	1.52	0.06	6-8	1	1.52	1.61
1,3-Diacetylbenzene	500g	1.00	0.03	1-7	2	2.01	1.99
1,4-Diacetylbenzene	500g	0.81	0.02	1-2	1	0.81	0.97
Hexadecane	SP	0.24	0.06	5-6	1	0.24	0.27

Table 6.5: *Culicoides* species collected during summer 2012 field trial with EAG-active confirmed compounds. Data separated into *Culicoides obsoletus* and *Culicoides pulicaris* complexes and by sex.

Treatment	**Total**	***C. obsoletus s.l.***		***C. pulicaris s.l.***	
		Female	**Male**	**Female**	**Male**
Blank	0	0	0	0	0
CO2	1	1	0	0	0
UV light	5601	4957	60	560	24
(*R*)-(-)-1-Octen-3-ol	63	63	0	0	0
(*E*)-2-Octene	1	1	0	0	0
3-Ethyltoluene	0	0	0	0	0
Heptanal	1	1	0	0	0
3-Chemical blend	29	29	0	0	0

6.3.3 Field trial summer 2013

The summer 2013 field trial was successfully completed and trap collections were counted based on total *Culicoides* species (due to time constraints)(Table 6.6).

Table 6.6: Total *Culicoides* species collected during field trial with EAG-active confirmed compounds.

Treatment	Total *Culicoides* species
Blank	1
CO2	5
UV	822
(*R*)-1-octen-3-ol	160
3-Chemical Blend	1
7-Chemical Blend	17
10-Chemical Blend	1
12-Chemical Blend	3

Statistical analysis showed a significant time by treatment interaction ($F_{6,129}$ = 9.40, P < 0.001, F-test from GLM model), with both those main effects also significant (P < 0.001, F-test). There was a positive effect of wind speed ($F_{1,129}$ = 184.71, P < 0.001) and air temperature ($F_{1,129}$ = 89.38, P < 0.001) with regression coefficients of 1.499 (SE 0.207) and 0.2546 (SE 0.063) respectively. Therefore, trap catches increased with increases in both wind speed and air temperature. There were no significant interactions between these two variables and treatment. The positive control UV trap caught the most *Culicoides* species (822), catching significantly more compared with each of the other trap treatments (P < 0.001, partial t-test, from GLM model). The UV data was removed prior to final analysis. The positive chemical control, (*R*)-(-)-1-octen-3-ol (160), caught significantly more *Culicoides* species (P < 0.001, partial t-test) than each of the other treatments. The 7-chemical blend baited trap (17) caught significantly more *Culicoides* species (P < 0.001) compared with the CO_2 control. The remaining chemical blend baited traps and the blank trap were not significantly different in total *Culicoides* species caught when compared with the CO_2 control. The predicted counts were calculated at the mean wind speed (1.51 m s^{-1}) and air temperature (21.84 °C) for each of the experimental days and visualised as a line graph (Figure 6.2). Trap catches (counts) for both (*R*)-(-)-1-octen-3-ol and the 7-chemical blend can be seen to decrease over the duration of the experiment, with the 7-chemical blend showing a more rapid decrease. The catches for the other treatments (blank, CO_2, 3-chemical blend, 10-chemical blend and 12-chemical blend) were low or zero and therefore no significant interaction between time and these treatments was established.

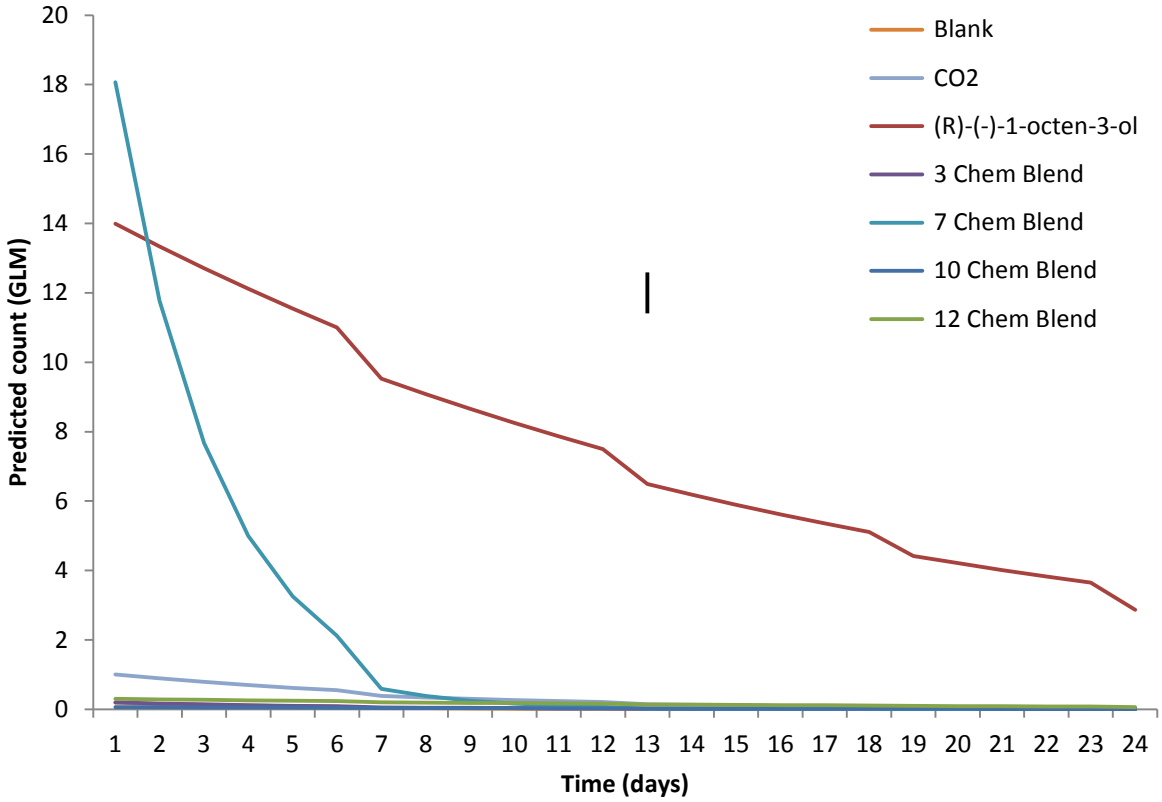


Figure 6.2: Interaction of Time and Treatment as highlighted in the GLM analysis of the summer 2013 trap count data. All treatments except the blank control were presented with the addition of 500 ml min^{-1} CO_2. The black bar in the centre of the graph represents the mean standard error for the data.

6.4 Discussion

6.4.1 Summer 2012 field trial (run by Andrew Hope, The Pirbright Institute)

A range of slow release formulations were prepared resulting in suitable release rates of confirmed EAG-active compounds, based on the calculated release rates of the chemicals from three sheep per day. It was not possible to obtain an optimal (natural) release rate for 3-ethyltoluene and as a result it was decided to use release rates at ten times the natural level for the field trial. It may have been possible to slow the release rate of 3-ethyltoluene using microencapsulation or paraffin oil/emulsifying wax mixtures with the slow release formulation, however, this was not investigated. Wax formulation for delivery of volatile chemicals has been used previously to investigate repellents for *C. impunctatus* in Scotland (Logan *et al*, 2009). Analysis of the total trap catch data showed that the individual chemical slow release formulations did not significantly increase the trap catch of *Culicoides* species compared with the CO_2-baited control trap. However, the 3-chemical blend (containing (*E*)-2-octene, heptanal and 3-ethyltoluene) caught significantly more ($P < 0.05$) *Culicoides* species than the blank or CO_2-baited control traps and showed an increased effect compared with the catches from the individual chemical-baited traps. This suggests that the blend provided a semiochemical cue that was closer to that of a natural host. The UV, positive control, trap caught the most *Culicoides* species (5601), and was significantly more attractive compared with the CO_2-baited control trap ($P < 0.001$). The total trap catch for the semiochemical positive control, (*R*)-(-)-1-octen-3-ol (63), was not significantly different from the 3-chemical blend (29). This is mostly likely due to high variation in the nightly catch data, and the fact that one night accounted for 54 of the *Culicoides* species caught in the (*R*)-(-)-1-octen-3-ol-baited trap. Interestingly, the data also showed that the (*R*)-(-)-1-octen-3-ol and the 3-chemical blend treatment trap were more specific in the species they caught, collecting only female members of the *C. obsoletus* species complex whereas the UV trap caught male *C. obsoletus* as well as both sexes of the *C. pulicaris* species complex. This difference in trap catch composition suggests differences in the way the members of the two species complexes potentially host seek. A similar disparity in the host seeking behaviour of these two species complexes was shown in an investigation comparing drop trapping

and light trapping of *Culicoides* species (Carpenter *et al*, 2008a). The data showed that while *C. obsoletus* group members were caught in both light traps and drop traps, *C. pulicaris* group members were only caught in the light traps, with none caught on sheep by the drop trapping (Carpenter *et al,* 2008a). Additionally, within the *C. obsoletus* group members, the data showed that *C. chiopterus* was underestimated in light trap catches compared to number found on sheep during drop trapping (Carpenter *et al*, 2008a). Such differences in host seeking behaviour would need to be taken into account to allow for improved monitoring of *Culicoides* species population or attempts at localized control strategies.

Following the summer 2012 field trial and the success of the 3-chemical blend compared with the individual chemicals, and given the finite time period of this study, it was decided to focus on blends for the 2013 field trial.

6.4.2 Summer 2013 field trial

During the summer 2012 field trial the treatment traps were rotated round the trap positions. This resulted in the same traps being adjacent to any given trap within each of the 8 night replicate blocks (a new Latin square randomisation was used for the start of each 8 night replicate block). As this was not a fully randomised design, during the 2013 field trial the design was rectified with treatment traps moving randomly between position each night rather than being rotated round the positions.

Initial analysis of the total *Culicoides* species trap catch data found that the positive control UV trap caught the most *Culicoides* species (822) and significantly more than the CO_2 control. This was in keeping with the summer 2012 field trial results. However, it was found that the variation in the UV data was masking potential effects from the other treatments, and therefore the UV data was removed and the remaining data re-analysed. This second analysis revealed significant increases in trap *Culicoides* species trap catches for the (*R*)-(-)-1-octen-3-ol positive chemical control ($P < 0.001$) and the 7-chemical blend baited trap ($P < 0.001$). Interestingly, despite catch numbers generally being lower during the 2013 field trial, the (*R*)-(-)-1-octen-3-ol trap caught more (160)

than in 2012 (63). Additionally, the 3-cheimcal blend baited trap only caught one *Culicoides* species in 2013 and was not significantly different from the CO_2 control.

The early July to mid-august trapping period for 2013 appears to have occurred during a drop in overall population numbers as highlighted in the GLM analysis of the data by the interaction between Time and Treatments (Figure 6.2, Section 6.3.3). This drop in numbers was also noted independently by other researchers around mid to late July for 2013 (personal communication, Andrew Hope, The Pirbright Institute). Additionally, an investigation using Rothamsted suction traps (RSTs), at twelve sites across England, to determine the influence of seasonal and meteorological variables on the flight activity of *Culicoides* species noted that most sites showed a greater abundance of *Culicoides* species during spring (April/May) and autumn (September/October), with all showing a reduction in abundance during June (Sanders *et al*, 2011). The summer 2012 field trial was run later (late august to late September) and avoided this drop in population during late July/early-mid august as evidenced by a 6-fold increase in total wild *Culicoides* species caught over the duration of the experiment compared with summer 2013. However, it should be noted that 3842 of the *Culicoides* species caught during the summer 2012 field trial, accounting for over 67.4 % of the total catch, were collected on one night (15/09/2012).

The statistical analysis from the summer 2013 field trial found a positive regression coefficient (1.499) for wind speed showing that as the wind speed increased the catches in the treatment traps increased. This may be due to increases in wind speed allowing for a greater dispersion of the volatile chemicals emitted from the slow release formulations in the treatment traps, and therefore creating a greater catchment area from which to attract *Culicoides* species to the trap. A study investigating the spatial abundance and clustering of *C. obsoletus* and *C. pulicaris* group midges also found a positive impact of wind speed on their data (Kirkeby *et al*, 2013a). The authors suggested that if the wind speed was weak, *Culicoides* species may find it difficult to determine the direction of hosts from the volatile signals and therefore be “reluctant to waste energy on flying”. Therefore, within the ranges of wind speed they recorded, higher wind speed resulted in a greater abundance of *Culicoides* species (Kirkeby *et al*, 2013a). This is true up to a point, as eventually wind speed would increase to levels that are prohibitive to *Culicoides* species flight and

therefore trap catches would decrease. Sanders *et al* (2012), in contrast, found an inverse relationship between wind speed and *Culicoides* species catches during a truck trapping (large net fitted to the top of a vehicle) experiment investigating diel activity and noted that few *Culicoides* species were caught at wind speeds above 4 m s^{-1}. However, the wind speeds recorded during that study ranged from 0 – 6.0 m s^{-1} (Sanders *et al*, 2012). Similarly, a different investigation into trapping of *Culicoides* species, using the OVI design UV down draft light trap, found an increase in zero trap counts when wind speeds were greater than 3.0 m s^{-1} (data ranged from 0 – 5.6 m s^{-1})(Carpenter *et al*, 2008a). During the field trials reported here wind speed peaked at 3.066 m s^{-1} (based on weather station data at sunset). The lower wind speeds recorded here may account for the positive relationship between wind speed and trap catch noted in the summer 2013 field trial data analysis, as the negative effects of higher wind speeds were not present in the data set.

A positive regression coefficient was also found for air temperature (0.2546) showing that as the air temperature increased, trap catches increased. This follows a similar pattern to that described above for wind speed, whereby, above a certain level the temperature would be prohibitive to *Culicoides* species flight and therefore trap catches would decrease. It has been shown previously that relative humidity and air temperature effect the biting rate of *Culicoides* species with optimal variables reported at 60 % relative humidity and 20.2 °C optimal air temperature, based on light and drop trapping of *Culicoides* species during summer (June/July) (Carpenter *et* al, 2008a). It seems reasonable that biting rate and host location are linked, as the insects must first locate a host in order to be able to bite it. Kirkeby *et al* (2013a) reported a positive impact of air temperature on their spatial abundance and clustering data for *C. obsoletus* and *C. pulicaris* group midges, noting an optimum temperature just below 16°C. The trial took place in Denmark between June and August (Kirkeby *et al*, 2013a). With regard to the UK, a reduction in the activity of *Culicoides* species was recorded at temperatures above 21 °C during a truck trapping experiment (Sanders *et al*, 2012). Data collected by the weather station, located in the same field as the summer 2012/2013 field trials, shows that during the 2012 field trial, air temperature at sunset averaged 13.92 °C, however, during the 2013 field trial, air temperature at sunset was greater, averaging 21.8 °C over the course of the experiment. The greater temperature

noted during the summer 2013 field trial may, in part, account for the lower overall catch of *Culicoides* species together with the drop in population shown over time (Figure 6.2, Section 6.3.3).

Rigot and Gilbert (2012) showed a significant spatial interaction between OVI black light traps placed 50 m apart, compared with 100 m or 200 m, for catches of *C. obsoletus* group midges. However, no spatial interaction was noted for other *Culicoides* species. Their data suggests that female *Culicoides* (across all species) had a range of attraction of 29.6 m (26.3 m – 31.9 m, 95% confidence interval). This implies that OVI black light traps need to be at least 64 m apart to avoid interacting with each other. Similarly, Kirkeby *et al* (2013b) investigated the interactions of CDC black light traps with respect to *Culicoides* species and found that they had an attraction range of 15.25 m (12.7 m – 18.3 m). From this data, a distance of at least 36.6 m would be advised when using CDC traps to avoid a trap based interaction. The distances between the CDC traps for both the field trials were approx. 50 m, therefore, no light based trap interactions should have taken place. However, it is unclear whether this interaction, shown for the both the CDC black light trap and the OVI black light trap also effects un-lit chemical-baited CDC traps. If the CDC UV light trap interaction distance was greater than reported by Kirkeby *et al* (2013b), then it is possible that it was competing with those traps and preferentially drawing *Culicoides* species to the UV trap, thereby reducing the trap catches for the adjacent un-lit chemical-baited traps. Moreover, if the un-lit CDC chemical-baited traps were also to show a similar interaction, at approximately 50 m distances apart, then the (*R*)-(-)-1-octen-3-ol (and potentially the 7-chemical blend) could have competed with adjacent traps, reducing their potential catches of *Culicoides* species. This may partially explain why the 3-cheimcal blend treatment, despite showing significant attraction during the summer 2012 field trial (Section 6.3.2), was not significantly attractive for *Culicoides* species (one caught) during the 2013 field trial (Section 6.3.3). The UV trap, (*R*)-(-)-1-octen-3-ol trap and 7-cheimcal blend trap treatments (shown by the data to be the preferential treatments, Table 6.6, Section 6.3.3) potentially interacted with, and out competed, adjacent treatment traps. However, any potential effects in the data from distance interactions should be lower for the summer 2013 field trial as the treatments were moved to new positions randomly each night rather than being rotated, as was they

were during the summer 2012 field trial. Further studies to investigate the spatial interaction of chemical-baited traps with reference to *Culicoides* species are clearly needed to determine and quantify such effects. In the meantime, it would seem advisable for traps to be placed at distances greater than 64 m apart to avoid potential interaction and competition between traps during field trials.

The field trials took place in an area of a large field. As a result the animals (sheep and cattle) present in the field were able to move away from the area where the traps were. However, there was a water trough present within the trapping area, so the cattle regularly entered this area in order to drink. It has been shown that traps placed near (<100 m) to host animals increased catch abundance by 50 % - 100 %, compared with traps placed 300 m – 400 m away (Kirkeby *et al*, 2013a). The length of the field used for the field trials was approximately 588 m at its longest section. Therefore, if the sheep and cattle were to move to the far end of the field (away for the area with the traps), as they often did, it is possible that a significant drop in *Culicoides* species numbers in the trapping area may occur, due to the insects following the volatile cues from the animals, thus reducing potential trap catches. Future trapping experiments may benefit from selecting a field, or restricted area of a field, whereby the animals cannot move as far as 300 m from the traps. However, as mentioned above, such a field site/area would still need to allow sufficient distance between the treatment traps to avoid potential interactions.

A further consideration is highlighted by a study investigating local scale spatial abundance and clustering of *Culicoides* species in Denmark (Kirkeby *et al*, 2013a). Using a grid of fifty mini CDC UV light traps spaced 50 m apart the authors sampled the *Culicoides* species populations over a 7 week period and analysed the data using conditional autoregressive (CAR) models. Their analysis found a dynamic pattern of clusters of *Culicoides* species moving around the field each night, not accounted for by other variables (Kirkeby *et al*, 2013a). Additionally, these clusters showed a mean abundance of *C. obsoletus* 0.62 – 10.82 times greater than the rest of the field, with *C. pulicaris* recorded mean abundances of 1.75 – 4.16 times greater. The authors note that such random clustering effects will potentially create a lot of noise in field trial data, and suggest the best way to combat this is with large scale studies involving many traps and locations (Kirkeby *et al*, 2013a). Interestingly, Sanders *et al* (2012)

reported occasional large numbers of *Culicoides* species with the truck trap and attributed this to clustering of males. Both the field trials reported in this chapter (especially summer 2012) featured a number of nights of greater catches, which may be attributable to clusters of *Culicoides* species moving randomly round the field site. Together with trap interaction distances and proximity to animal hosts, these are all factors to consider when designing future field trials.

(*R*)-(-)-1-Octen-3-ol was not detected in the sheep extract samples and therefore natural release rates from sheep could not be determined. Similarly, Logan (2006) failed to find 1-octen-3-ol as an EAG-active component of human-derived entrainment extracts when tested with *C. impunctatus*. Instead, (*R*)-(-)-1-octen-3-ol was released from a neat chemical source with an approximately 100 mg day^{-1} release rate, which represented a 30 - 1000 fold increase in release rate based on the chemicals present in the 7-chemical blend. As (*R*)-(-)-1-octen-3-ol caught the most *Culicoides* species out of the chemical treatment traps, using different slow release formulations to increase the release rate to 100 times natural or 1000 times natural may increase the trap catches for the chemical-baited traps. Additionally, as sheep are flock animals it seems reasonable to assume that release rates of chemicals from the flock will be significantly greater than released in the current field experiments (based on the release rate from 3 sheep). Two previous field studies on *C. impunctatus* (Ormsary, Argyll, Scotland) which have seen significant increases in trap catches using racemic 1-octen-3-ol baited traps compared with controls, have used release rates of 0.11 mg day^{-1} and 0.06 mg h^{-1} (1.44 mg day^{-1})(Blackwell *et al*, 1996, Bhasin *et al*, 2001). Both of these investigations used Delta traps placed 5 m apart. As described above, if chemical-baited traps were to interact in a similar way to OVI black light traps, then it is highly likely that there would have been interaction/competition between treatment traps in these investigations. Moreover, given the greater abundance of midges within *C. impunctatus* populations in Scotland compared to *Culicoides* species populations in England, the mean trap catches for racemic 1-octen-3-ol baited traps in those investigations, 22.7 $\pm$ 1.8 and 19.5 $\pm$ 0.81 respectively, do not seem particularly large. Using 1-octen-3-ol (in different enantiomeric ratios) with Mosquito Magnet® Pro™ traps (50 m apart) to catch *C. impunctatus* (Ormsary, Argyll Scotland), significant

increases in trap collections were recorded for ratios greater than *R*:*S* 19:81 compared with the CO_2 control (CO_2 released at approximately 500 ml min^{-1}) with mean trap catch peaking at 944.7 for the *R*:*S* 79:21 enantiomer ratio (Harrup *et al*, 2012). The difference in traps catches, for the same *Culicoides* species, at the same location, albeit in different years, suggests that the type of trap used also plays an important role in determining *Culicoides* species trap catches. The Mosquito Magnet® Pro™ trap emits heat as well as CO_2, both of which are lacking in the Delta trap design. These additional cues may help provide a better representation of host stimuli. Furthermore, both Blackwell *et al* (1996) and Bhasin *et al* (2001) used racemic 1-octen-3-ol. Recently, a study in England (Compton, Berkshire) used unlit CDC chemical baited CDC traps to compare catches of *Culicoides* species using 1-octen-3-ol enantiomers ((*S*)-(+)-1octen-3-ol, racemic (50:50) 1-octen-3-ol and (*R*)-(-)-1-octen-3-ol) with the addition of CO_2 (500 ml min^{-1})(Harrup *et al*, 2012). The authors found that (*R*)-(-)-1-octen-3-ol enhanced trap catches of livestock associated *Culicoides* species compared with the other treatments (Harrup *et al*, 2012). These data suggest that future studies should consider using the (*R*)-(-)-1-octen-3-ol enantiomer rather than the more commonly used racemic 1-octen-3-ol. This was highlighted previously (Chapter 3, Section 3.3.1) where the laboratory reared *C. nubeculosus* responded to racemic 1-octen-3-ol (100 µg) in the Y-tube olfactometer, however, wild caught *Culicoides* species did not. The wild caught *Culicoides* species were subsequently found to respond to (*R*)-(-)-1-octen-3-ol (10 µg).

Carbon dioxide was released at 500 ml min^{-1}, based on previous studies, and estimates of CO_2 composition of exhaled sheep breath (4 % above background levels) as described previously (Chapter 3, Section 3.6)(Harrup *et al*, 2012, Cardé and Gibson, 2010). The carbon dioxide baited trap caught no *Culicoides* species, during summer 2012 field trial and only five *Culicoides* species during the summer 2013 field trial. Based on these results, it would appear that CO_2 is not an effective attractant for wild *Culicoides* species in Southern England. Harrup *et al* (2012) caught relative few *Culicoides* species (311 in 80 samples) in a CO_2-baited unlit CDC trap with comparing the efficacy of 1-octen-3-ol enantiomers in Southern England. However, 285 of those midges caught were *C. nubeculosus* with *C. obsoletus* only accounting for 22 out of the total catch (Harrup *et al*, 2012). Similarly, while investigating biting rates of *Culicoides*

species in North-eastern Spain, Gerry *et al* (2009) caught 41 *Culicoides* species in a CO_2 (1kg dry ice) baited trap over 8 nights, of which only two were *C. obsoletus*, 38 were *Culicoides parroti* and one was *Culicoides circumscriptus*. For comparison, the UV trap caught 16 *C. obsoletus* while mechanical aspiration from a sheep resulted in a catch of 313 *C. obsoletus* (Gerry *et al*, 2009). This large discrepancy in *C. obsoletus* catch between the UV trap and direct aspiration from sheep also serves to highlight the issue of underestimation of potential disease vectors when using standard UV light based trapping techniques for monitoring *Culicoides* species populations.

6.5 Conclusions

Chemicals identified from whole sheep entrainments have been shown to be effective at significantly increasing trap catches of wild *Culicoides* species when presented as slow release formulation blends in unlit CDC traps. Initial attraction to the 3-chemical blend (summer 2012) and subsequent attraction to the 7-chemical blend (summer 2013) shows the potential for these chemicals to be used to enhance trap catches. Both these chemical blends contain novel chemicals never reported for *Culicoides* species previously. Further refinement of composition and release rate may lead to a suitable blend that can enhance trap catches to improve monitoring of *Culicoides* species populations as an early warning system for the presence of potential BTV/SBV vectors.

Chapter 7: General Discussion

The aim of this study was to investigate the semiochemical basis of host location in UK *Culicoides* species biting midges. Previous studies have used samples of sheep fleece or solvent extracts of sheep fleece to attract mosquitoes and *Culicoides* species to traps, or have used whole live animals in tents and vented the air to traps as a volatile bait (Mands *et al*, 2004, Rayaisse *et al*, 2010, Tchouassi *et al*, 2012). One study investigated the response of *C. impunctatus* to air entrainment extracts of humans (Logan, 2006, summarized in Logan *et al*, 2009). Following initial investigations it was decided that air entrainment would provide the best source of host (sheep) derived volatiles, and that whole animal air entrainment would most accurately reflect a natural host because of the inclusion of breath, skin, fleece, urine and faeces volatiles rather than a limited selection of these sources.

A new piece of experimental equipment, the whole animal entrainment box, was designed and developed, in-house at Rothamsted Research, to enable the collection of volatiles from whole live animals (sheep). Sheep odour vented from this box was shown to contain behaviourally active host volatiles from sheep, as it significantly increased the attraction of wild *Culicoides* species to a CDC trap. After volatiles were collected they were tested in the Y-tube olfactometer in the laboratory to confirm behavioural activity. Statistically significant behavioural responses (increases in relative attraction) compared with a solvent control were observed in *C. nubeculosus* in response to the combined sheep breed extracts. This is the first study to entrain volatiles emitted from a whole sheep and to show behavioural responses of wild *Culicoides* species to vented whole animal volatiles in the field and behavioural responses of *C. nubeculosus* to whole sheep air entrainment extracts in the laboratory. Further analysis by GC-EAG and GC-MS revealed twelve electrophysiologically active chemicals based on the response of wild caught *Culicoides* species (mostly *C. obsoletus* group) to the “Pure” breed extract. These chemicals were: heptane, (*E*)-2-octene, styrene, heptanal, 3-ethyltoluene, acetophenone, 4-methylphenol, 4-oxoisophorone, decanal, 1,3-diacetylbenzene, 1,4-diacetylbenzene and hexadecane. Ten of these chemicals represent novel reporting of EAG-activity for *Culicoides* species and two (decanal and 4-methylphenol) have been reported previously. This also appears to be

the first time (*E*)-2-octene, 3-ethyltoluene and 1,3-diacetylbenzene have been reported as EAG-active for any insect species.

Laboratory investigation using the wild caught *Culicoides* species in the Y-tube olfactometer revealed statistically significant increases in relative attraction compared with the redistilled hexane control for (*E*)-2-octene, 3-ethyltoluene and heptanal. This is the first time behavioural attraction of *Culicoides* species to these chemicals has been reported. It is also the first time behavioural attraction has been shown to (*E*)-2-octene and 3-ethyltoluene for any insect species.

7.1 Implications for monitoring and control

Improved attractants for use in semiochemicals baited traps, resulting in enhanced catches, and providing a more accurate representation of *Culicoides* species populations, would be valuable in both monitoring and control attempts of vector *Culicoides* species. Current traps are known to underestimate *C. obsoletus* and *C. pulicaris* group populations (Carpenter *et al*, 2008a, Gerry *et al*, 2009). Better understanding of *Culicoides* species population sizes and locations would allow reactive measures to take place to protect animals from vectored pathogens as population densities increased. Additionally, in the event of an outbreak of BTV or SBV, this knowledge would allow accurate restriction zones to be put in place more quickly and thereby reduce the potential impact of such an outbreak.

Reduction of *Culicoides* species populations by removal trapping in localized areas has been investigated previously, (Logan *et al*, 2010, Day *et al*, 2001, Cilek and Hallmon, 2005). Florida, USA, is similar to areas of the Highlands, Scotland, in that *Culicoides* species populations have an impact on tourism through nuisance biting of humans (Linley and Davies, 1971, Hendry and Godwin, 1988). A line barrier of CDC traps, baited with CO_2 and racemic 1-octen-3-ol reduced mosquito and *Culicoides* species populations on an island resort in Southwest Florida (Logan *et al*, 2010). Similarly, at two coastal areas in South Florida, and on an island in the Bahamas, CO_2 and racemic 1-octen-3-ol baited insecticide treated fabric targets were found to significantly reduce populations of *C. furens* (Day *et al*, 2001). Another study investigated the reduction of

Culicoides populations around individual homes using Mosquito Magnet® Pro™ or ABC Pro traps in north-western Florida (Cilek and Hallmon, 2005). Inconsistent results were found, with population reduction ranging from 2.3 % - 70.6 % and the authors suggested that one trap per backyard was insufficient to effectively reduce the *Culicoides* species population. It is noted that with the ABC Pro trap a bait of CO_2 (500 ml min^{-1}) and a mixture of racemic 1-octen-3-ol:3-n-propylphenol:4-methylphenol (4:1:8) with an overall release rate of 5.39 ($\pm$ 0.54) mg h^{-1} was used (Cilek and Hallmon, 2005). Results presented here showed that the addition of 4-methylphenol and decanal to a blend of chemicals did not alter the level of attraction to baited CDC traps with no significant difference in attraction being discovered compared with the CO_2 control (Chapter 6, Section 6.3.3). Therefore, 4-methylphenol may have contributed to the inconsistent results noted by Cilek and Hallmon (2005). These studies highlight the potential of localised reduction and control of *Culicoides* species populations with improved baits for insect traps. Small farm holdings where animals roam in a defined area may be able to deploy traps around the perimeter that can remove *Culicoides* species each night and suppress the population to reduce vector potential. This effect may be further enhanced by utilising a push/pull trapping strategy, where by chemicals that mask host volatiles, or repellents, are used to "push" insects away from an area while attractant baited traps "pull" the insects towards them, removing them (Cook *et al*, 2007). As research into *Culicoides* species host location improves such push/pull strategies may be optimised to provide localized control of a grazing area. Recently, 6-methyl-5-hepten-2-one and geranylacetone were shown to be effective repellents against *C. impunctatus*, and therefore have potential for use in the "push" part of such a strategy (Logan *et al*, 2009). In addition to common attractants such as racemic or (*R*)-(-)-1-octen-3-ol, this study has discovered novel attractants for use with the pull side of the system ((*E*)-2-octene, 3-ethyltoluene and heptanal) and further investigation of GC-EAG-active identified chemicals may yield more behaviourally active results with *Culicoides* species that could improve either the "push" or "pull" part of this strategy.

7.2 Coincidence detection and *Culicoides* species host location

In addition to the meteorological variables and potential trap interactions mentioned discussed in Chapter 6, another important concept that may affect the trap catches of host seeking *Culicoides* species is coincidence detection. The behavioural indication of coincidence detection was first identified for the moth, *Manduca sexta* Linnaeus, when it was noted that males would not fly unless two specific pheromone components were presented simultaneously (Tumlinson *et al*, 1989). The physiological basis for this effect was confirmed by Christensen and Hildebrand (1997) who showed that the neuronal response in *M. sexta* was optimal when they received simultaneous inputs. In a review of host location of phytophagous insects, Bruce *et al* (2005) described coincidence detection suggesting that chemicals in a blend released from a single source, i.e. a host, will be encountered by the insect at the same time, resulting in the relevant olfactory receptor neurons being triggered simultaneously. Whereas, the same chemical components of the blend may exist in the environment, released by a variety of non-hosts, however, because they originate from different sources they are encountered by the insect at different times, and as such the olfactory receptor neurons fire at different times. Therefore, the same chemicals can signal a host or non-host. Therefore, the individual chemicals ((*E*)-2-octene, heptanal and 3-ehtyltoluene) may have been effective in the Y-tube olfactometer in the laboratory due to a lack of alternative choices – i.e. the options were test chemical or hexane (blank). However, in the field environment, in the presence of a range of volatile semiochemical signals from hosts (sheep and cattle) and the chemical blend baited trap, the individual chemicals were not effective as they were did not sufficiently represent a host volatile signal, resulting in the *Culicoides* species being preferentially attracted by the other trap/host options available. This theory may also help explain why, in the summer 2013 field trial, the 3-chemical blend (effective in summer 2012) caught only one *Culicoides* species while the 7-chemical blend baited trap was found to significantly attract and catch *Culicoides* species compared with the CO_2 control trap. The 7-chemical blend may have better represented the volatile signature that defines a host for *Culicoides* species and therefore the insects were preferentially attracted to the trap baited with that blend. The 7-chemical blend contained all of the GC-EAG-active identified chemicals highlighted as important based on the means of the chemicals

present in the extracts of the pure and cross breeds compared to their relevant controls (with 4-methylphenol removed as it was a known repellent) (Chapter 4, Section 4.3.4, Table 4.4). Thus the 7-chemical blend contained all of the chemicals present in the 3-chem blend plus additional chemicals the data suggested were important. The 10-chemical blend had the addition of heptane (found to be reduced in the sheep extracts compared with the controls) and 1,3- and 1,4-diacetylbenzene (two potential Porapak™ Q contaminants), while the 12-cheimcal blend also contained two known repellents (decanal and 4-methylphenol). The additional chemicals present in the 10-chemical and 12-chemical blends may represent non-host cues, hence the lower, non-significant, trap catches. Bearing this theory of coincidence detection in mind, it may be possible to further enhance trap catches by improving the delivery methods of the slow release chemical formulations so that they are emitted as a premixed blend from a single point source. This was attempted at the start of the summer 2013 field trial by placing the slow release chemical formulations into a polypropylene box with brass tube fittings (Swagelok®, Cleveland, Ohio, USA) screwed into opposite sides, thus allowing the CO_2 flow from the CO_2 cylinder to be passed through the box via Tygon tubing (Saint-Gobain, Performance Plastics, Poestenkill, NJ, USA). However, the design of the container for the slow release formulations was not air-tight and the flow rate of CO_2 through the container could not be maintained at 500 ml min^{-1} due to CO_2 escaping from the lid and around the Swagelok® fittings. Therefore, successful routing of CO_2 through an air-tight container, allowing for the volatile chemicals to be released from a single point source near the CO_2 delivery tube may further enhance the trap catches for the chemical based treatments.

7.3 Further work

7.3.1 Confirm the identity of other EAG-active chemicals

Twelve of the 37 EAG-active chemicals were confirmed by peak enhancement co-injection (11 confirmed chemicals and one likely identification). No clear tentative identification was available following GC-MS analysis for thirteen of the EAG-active peaks. Five EAG-active peaks have tentative identifications (2-methylhexane, methylcyclohexane, 2-methylpentanal, butyric (butanoic) acid, 2-methylbutanoic acid)

that were not confirmed during this investigation because of the sheep entrainment extracts being used up before successful peak enhancement confirmations were obtained. These five chemicals are worth investigating in the laboratory and/or field in the future to determine if there is any behavioural activity with *Culicoides* species. Additionally, there were seven EAG-active chemicals tentatively identified using GC-MS analysis that were not commercially available and therefore not investigated further in the study (3-methyl-2-butenal, 1,3-octadiene, 6-methyl-2-heptanone, 2-ethylacetophenone, allyl 4-ethylbenzoate, 2,5-diisopentylthiophene and pentadecanal). Of these tentative identifications, 6-methyl-2-heptanone has previously been reported as a tentative identification in air entrainment analysis of sheep wool grease samples using solid phase microextraction (SPME) suggesting it may be a sheep-derived volatile (Lisovac and Shooter, 2003). Lewis and Williams (1980) have previously reported 2-ethylacetophenone may be a contaminant from unconditioned Porpak™ Q tubes upon heating suggesting that it may not be relevant to *Culicoides* species host location. It may be possible to synthesise these chemicals to allow for behavioural investigations to take place in the laboratory with *Culicoides* species.

7.3.2 Investigate behavioural responses of remaining confirmed EAG-active chemicals

Due to time and seasonal constraints, a focus on using wild *Culicoides* species and ensuring investigation of potential chemical blends in the field, studies on the behavioural activity of identified EAG-active chemicals currently is incomplete. Further investigation into the behavioural responses of wild *Culicoides* species in the laboratory using the Y-tube olfactometer with these chemicals may yield more potential attractants or putative repellents. The priority of any future investigation would be acetophenone. Although previously reported as a potential Porapak™ Q contaminant, analysis in Chapter 4 (Section 4.3.4, Table 4.4) found that it was only present in the sheep-derived entrainment samples with no trace present in the control samples (Sturaro, *et al*, 1992). Therefore, this would suggest that, like (*E*)-2-octene which was also only found in the sheep-derived entrainment samples, it may prove to be behaviourally active for wild *Culicoides* species and potentially one of the “core”

chemicals determining the host cue used by *Culicoides* species as described by Logan and Birkett (2007). Along with (*E*)-2-octene and 3-ethyltoluene, 4-oxoisophorone and hexadecane were found to be present in statistically significant different mean quantities in the sheep breed extracts compared with the control extract, and between the two sheep breed extracts (Chapter 4, Section 4.3.4, Table 4.4). Behavioural attraction was shown for (*E*)-2-octene and 3-ethyltoluene. Therefore, investigation of behavioural responses of wild caught *Culicoides* species in the Y-tube olfactometer with these chemicals may provide further attractants. 4-oxoisophorone was investigated in the Y-tube olfactometer with *C. nubeculosus* and no statistically significant increases in relative attraction were noted (Chapter 5, Section 5.3.1.2, figure 5.2) However, *C. nubeculosus* was also found to not respond to (*E*)-2-octene, despite responses being recorded with wild *Culicoides* species (Chapter 5, Section 5.3.1.3, Figure 5.3). As no significant difference in behavioural attraction of *Culicoides* species to the breeds or breed extracts were found, it may be that 4-oxoisophorone and hexadecane also form part of a "core" sheep cue for *Culicoides* species rather than being involved in the differential attraction of *Culicoides* species to individual sheep or breeds (Logan and Birkett, 2007). Similar to 4-oxoisophorone, 4-methylphenol was investigated with *C. nubeculosus*, and found to show a statistically significant reduction in relative attraction compared with the hexane control, however, it was not investigated with wild *Culicoides* species. It would therefore be interesting to determine if this reduction in relative attraction also occurs with wild *Culicoides* species. While attractants are of interest to improve trapping and therefore monitoring of *Culicoides* species populations, it may be possible to integrate attractants and repellents into a localized control or protection strategy. The remaining chemical which showed a statistically significant difference in to the mean quantity present in the sheep extracts compared with the control extracts, was styrene (chapter 4, Section 4.3.4, Table 4.4). Styrene, like acetophenone, has been previously reported as a potential Porpak™ Q contaminant, although analysis in this study suggests it is an EAG-active sheep-derived volatile (Lewis and Williams, 1980). Therefore, further investigation of behavioural responses of *Culicoides* species to styrene is advised.

It would be interesting to determine if the differences in behavioural response of the laboratory reared *C. nubeculosus* compared with the wild caught *Culicoides* species

exist for the other identified EAG-active chemicals, as was found with (*E*)-2-octene (Chapter 5, Section 5.3.1.3, Figure 5.3 and Section 5.3.2.1, Figure 5.4). It is unclear at present whether such differences in behavioural response are due to different host preference between the *Culicoides* species or whether the laboratory reared *C. nubeculosus* are not host seeking in a comparable way to wild *C. nubeculosus,* or other wild *Culicoides* species, due to genetic drift within the colony (Mukhopadhyay *et al*, 1997). As mentioned previously in chapter 5, this may present an interesting and useful study, especially since laboratory reared *C. nubeculosus* are a common model species for behavioural investigations (Bhasin *et al*, 2000a, Mands *et al*, 2004).

The previous investigation into GC-EAG responses of *C. impunctatus* to host (human)-derived volatiles reported 15 EAG-active chemicals, of which only one, decanal (or two if the unknown chemical with an RI of 807 is (*E*)-2-octene, reported here with an RI of 808), was also found in the list of GC-EAG active chemicals identified from sheep extracts with wild *Culicoides* species (Logan, 2006).

Further field trials to modify or refine the chemical blends would be of value. Increasing the release rates of the chemicals in the blend by 100 fold or 1000 fold may represent more accurately a flock of sheep and therefore increase trap catches. It may also be worth investigating the effectiveness of the 7-chemical blend in the absence of additional CO_2, as from this study, and others, there appears to be evidence that *C. obsoletus* group do not respond well to CO_2 in the field (Gerry *et al*, 2009, Harrup *et al*, 2012). Incorporating additional, identified chemicals from further research may improve the efficacy of blends, or determining the behavioural effects of currently identified chemicals may reveal potential repellents which could be removed from the current 7-chemical blend. It may also be possible to reduce this blend down to fewer component chemicals while still maintaining a useful effect in the field. A simpler blend would be more straightforward to produce and therefore increase the likelihood of its use in monitoring or localized control strategies.

7.3.3 Improvements to the whole animal entrainment box

These results demonstrate the success of the whole animal entrainment box as a method for the collection of behaviourally relevant host volatiles from large animals. During these investigations, possible alterations to the whole animal entrainment box have been noticed that would further improve its usefulness as a volatile collection tool. While the whole animal entrainment box was designed for transport and storage, the individual parts are still heavy and require two people to move them. It may be possible to change the materials used to make the box, thereby maintaining the strength and rigidity in the build structure, but saving on weight. Material changes were mentioned in Chapter 3 (Section 3.4) when discussing the potential for an intraspecific study of sheep. Transparent, high strength Perspex (or equivalent) sides for the box were suggested, to allow individual sheep to see the rest of the flock and therefore reduce stress/distress in the animals. The 2 mm aluminium sheet walls contribute less to the total weight of the box than the rolled hollow steel tube frame to which they are welded. Therefore, the supporting frame would also need to be re-engineered. The 5-bar tread plate floor also constitutes a large portion of the total weight of the box, although alterations to the rolled hollow steel tube frame would reduce this weight. The converse option, rather than reduce the overall weight of the equipment, would be to build it onto a wheeled base, creating a trailer which houses the flat packed box for transport and storage, however, reduces carrying distances of component when the structure is erected. This option also provides the ability to move the box around field locations once built without the need to dismantle and re-build each time, which is a time consuming task and one that requires at least two people. The design of the box (i.e. the size and space inside) is sufficient to allow the investigation of volatiles from animals larger than sheep, such as calves, that would also be relevant to *Culicoides* species host volatile research due to the fact that calves/cattle are often bitten preferentially to sheep (Bartsch *et al*, 2009).

Another factor that could be altered and improved in the whole animal entrainment box would be the airflow. The airflow used in the collection of host volatile experiments was greater than necessary. An airflow rate was calculated that would support three hyperventilating sheep for 4 hours. The maximum breathing rate of a sheep is 350 breaths min^{-1} compared to an average breathing rate of 15 - 40 breaths

min $^{-1}$ (Adams and McKinley, 1995). However, the experiment would have been stopped if the sheep had become distressed and hyperventilated, therefore, it was not necessary to have the airflow so high. Decreasing the main airflow through the box would increase the sub sample percentage collected by the Porapak™ Q tubes and thereby allow for collection of more host volatiles (as it was not believed that the Porapak™ Q tube had reached saturation point). However, there is a limiting factor on the alteration of the main airflow because of a threshold flow rate below which the airflow does not penetrate through the carbon panel filter. This was noticed during the preliminary experiments with the whole animal entrainment box where the array of 4 x 120 mm computer fans were found not to be strong enough and the air was felt escaping from the side of the fan housing and back out through the fans. The new, more powerful, fan did not experience this problem. Therefore, to reduce the airflow rate penetration threshold a different design of carbon filter may be required.

7.3.4 Contamination of Porapak™ Q tubes

It was noticed during analysis of the sheep volatile entrainment samples that some of the peaks (including EAG-active peaks) were potential Porapak™ Q contaminants: styrene, acetophenone, 1,3-diacetylbenzene and 1,4-diacetylbenzene (Lewis and Williams, 1980, Sturaro *et al*, 1992). An increase in the likelihood of potential contaminants is attributed to changes in the design of the Porapak™ Q tubes. The diameter of the glass tube was increased and the quantity of Porapak™ Q used per tube was increased from 50 mg to 250 mg. Refinement of cleaning methods for these larger Porpak™ Q tubes is required to remove or reduce the level of contaminants present. However, previous reporting as a potential contaminant does not preclude these chemicals from being host-derived and potentially behaviourally active, as seen for acetophenone which was EAG-active and shown to be only present in the sheep-derived entrainment extracts (Chapter 4, Section 4.3.4, Table 4.4). Therefore, chemicals found to be GC-EAG-active for *Culicoides* species are worth investigating further for behavioural effects regardless of whether they may be host-derived or a potential contaminant.

6.4 Conclusions

This study is the first to entrain whole sheep volatiles. New equipment was designed and developed at Rothamsted Research to facilitate the collection of volatile chemicals emitted from whole animals (sheep), allowing for the simultaneous collection of volatiles from breath, skin, fleece, urine and faeces. Air vented from the box, containing emitted volatiles from three sheep was shown to significantly increase attraction of wild *Culicoides* species to unlit CDC traps compared with a blank control. Whole sheep air entrainment extracts, eluted from Porapak™ Q polymer tubes were subsequently shown to significantly increase the relative attraction of *C. nubeculosus* (for combined breeds) in the Y-tube olfactometer in the laboratory compared with the redistilled hexane control.

The behaviourally active extracts were analysed by GC-EAG using wild caught *Culicoides* species and *C. nubeculosus* with 37 GC-EAG-active peaks discovered based on the responses of wild *Culicoides* species to the "Pure" Hartline breed extract. Using GC-MS analysis, tentative identifications of these active peaks were obtained and the identity of twelve of the peaks was confirmed by peak enhancement co-injections. These 12 peaks were: heptane, (*E*)-2-octene, styrene, heptanal, 3-ethyltoluene, acetophenone, 4-methylphenol, 4-oxoisophorone, decanal, 1,3-diacetylbenzene, 1,4-diacetylbenzene and hexadecane. Of these chemicals, ten represent novel identifications of EAG-activity for *Culicoides* species: heptane, (*E*)-2-octene, styrene, heptanal, 3-ethyltoluene, acetophenone, 4-oxoisophorone, 1,3-diacetylbenzene, 1,4-diacetylbenzene and hexadecane. This is also the first time (*E*)-2-octene, 3-ethyltoluene and 1,3-diacetylbenzene have been reported as EAG-active for any insect species.

Behavioural responses of *C. nubeculosus* and/or wild caught *Culicoides* species to *a* selection of confirmed GC-EAG-active chemicals were investigated in the laboratory using a Y-tube olfactometer. Significant increases in relative attraction, compared with a redistilled hexane control were found for (*E*)-2-octene, 3-ethyltoluene and heptanal. This is the first time behavioural attraction has been reported for *Culicoides* species to these chemicals and the first time behavioural activity has been reported to (*E*)-2-octene and 3-ethyltoluene for any insect species.

Over two summer seasons chemical traps were baited with confirmed GC-EAG-active and behaviourally active chemicals and blends (with the addition of CO_2) to investigate attraction of wild *Culicoides* species in the field. During the first field trapping season the three-chemical blend baited trap (containing (*E*)-2-octene, 3-ethyltoluene and heptanal) caught significantly more wild *Culicoides* species compared with the CO_2 control and trap baited with the individual chemicals. In the second field trapping season four different chemical blends were investigated, with the seven-chemical blend catching significantly more wild *Culicoides* species compared with the CO_2 control. In the second field season the seven-chemical blend also caught significantly more wild *Culicoides* species than the three-chemical blend. The seven-chemical blend contained: (*E*)-2-octene, styrene, heptanal, 3-ethyltoluene, acetophenone, 4-oxoisophorone and hexadecane. These are the chemicals shown by statistical analysis to be present in greater mean quantities in the sheep extracts compared with the control extracts, and therefore most likely to have a role in host location of *Culicoides* species

This study is one of the few investigations into host location of UK *Culicoides* species biting midges known to vector BTV and SBV, namely the *C. obsoletus* and *C. pulicaris* groups. These results may help improve monitoring and trapping of UK *Culicoides* species vector populations and provides a basis for further investigation of GC-EAG-active and behaviourally active chemicals.

Appendices

Appendix 1 – Field trapping using expelled air from whole sheep air entrainment box (wild *Culicoides* species) – statistical analysis (Andrew hope, The Pirbright Institute).

Appendix 1.1: Regression co-efficients included in the Generalised Linear Model to describe collections of total *Culicoides* species and *Culicoides obsoletus* group females.

Parameter	Total *Culicoides*	*C. obsoletus* group females
Intercept	0.998	0.619
Light Trap 1	3.88***	4.118***
Light Trap 2	2.107**	2.354***
Unbaited Trap	-1.634*	-1.66*
Pure breed	1.381*	1.621*

Appendix 1.2: Tukey's analysis of differences between traps, estimates for total *Culicoides* species Gemeralised Linear Model are in the upper diagonal, estimates for *Culicoides obsoletus* group females Generalised Linear Model are in the lower diagonal, estimates are treatments on the top line relative to treatments on the left. (*=p<0.05, **=p<0.01, ***=p<0.001)

Treatment	Light Trap 1	Light Trap 2	Pure	Cross	Un-baited
Light Trap 1	-	-1.773*	-2.499***	-3.880***	-5.515***
Light Trap 2	1.764*	-	-0.726	-2.107*	-3.742***
Pure	2.496***	0.732	-	-1.381	-3.015***
Cross	4.118***	2.354**	1.622	-	-1.634
Un-baited	5.779***	4.014***	3.282***	1.660	-

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