The Role of Chemicals in the Location of Host Plants by Midge Pests of UK Fruit Crops

Helen Sarah Thomas

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DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not currently submitted for any degree other than that of Master of Philosophy (M.Phil) of the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise stated.

Name……
(Student)

Name……..
(First Supervisor)

Name…….
(Second Supervisor)

ABSTRACT

Gall midges (Diptera: Cecidomyiidae) are important pests of many horticultural crops. They are difficult to control by conventional means as the larvae develop in galls surrounded by plant tissue. Gravid females need to locate suitable host plant material on which to lay their eggs and there is good evidence that host-produced odour cues are involved. Gall midge pheromones are now available commercially; these attract males to allow monitoring in the field. However there are currently no lures available to attract females. This work aims to develop lures, for up to three gall midge species, which attract gravid females and can be used to monitor females in the field. Three species of gall midges were investigated: the raspberry cane midge, *Resseliella theobaldi* (Barnes), the blackcurrant leaf midge, *Dasineura tetensi* (Rübsaamen), and the apple leaf midge, *Dasineura mali* (Kiefer). Field studies showed gravid raspberry cane midge females are attracted to split canes for oviposition. Chemicals were identified using solid phase microextraction (SPME) which were present only or in much larger quantities after canes split. A lure based on these chemicals was tested in the field with different trap types but no significant attraction was seen to the lures of either male or female raspberry cane midges. Blackcurrant leaf midge females lay their eggs on blackcurrant shoots. A suite of volatiles was identified using SPME which were produced by blackcurrant shoots. Bioassay work was carried out in a four-way olfactometer and a wind tunnel to assess female attraction to shoot material but no attraction was seen in either experiment. Solutions containing natural volatile extracts were obtained using entrainment onto Porapak resin. The effect of these chemicals on female blackcurrant leaf midge antennae was assessed using electroantennography (EAG) but there were not enough responses to conclude that any compound was EAG-active. Apple leaf midges lay their eggs on apple leaf shoots. A suite of chemicals was identified which are produced by apple shoots. EAG runs were carried out on female midges with both natural and synthetic solutions but numbers of responses were low and it was not possible to conclude that any of the compounds were definitively EAG active.

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Chapter 1
INTRODUCTION

1.1. OVERVIEW

Species of gall midge (Diptera: Cecidomyiidae) are important pests of many horticultural crops and often very difficult to control by conventional means as the feeding larvae are protected by galls. Scientists at The Natural Resources Institute (NRI) and East Malling Research (EMR) have made considerable progress in identification of female sex pheromones in this group of insects (Hall et al., 2012), and these are now in use for monitoring populations of several pest species. However, the female-produced sex pheromones attract only males. Attractants for the females, particularly mated females, would potentially be far more valuable for both monitoring and control of the pests. There is good evidence in several species of midge that mated females are attracted to their host plants for oviposition by specific odours from the plants. Although this has been known for over 40 years in some cases, the chemicals responsible for this attraction have not yet been identified.

This project aimed to identify the chemicals responsible for attraction of mated female midges to oviposition sites on their host crop for up to three species which are important pests of soft fruit and tree crops in the UK and where such attraction has been demonstrated previously. These are the raspberry cane midge, *Resseliella theobaldi* (Barnes), the blackcurrant leaf midge, *Dasineura tetensi*, (Rübsaamen) and the apple leaf midge, *Dasineura mali* (Kieffer). The results will also advance our knowledge of the remarkable ability of insects to find their host plants in terms of whether a few key chemicals are involved or whether they use specific blends of several more ubiquitous chemicals.

1.2. GENERAL GALL MIDGE BIOLOGY

Gall midges are small Diptera belonging to the family Cecidomyiidae. The family is divided into three subfamilies: Lestremiinae, Poricondylinae and Cecidomyiinae. They may be phytophagous, fungivorous or zoophagous (Skuhravá et al., 1984). All phytophagous gall midges are found in the subfamily Cecidomyiinae.

In the case of most phytophagous gall midges, adult females lay their eggs into or onto plant tissue and the larvae feed, stimulating cell growth leading to the formation of plant tissue structures around them known as galls (Rohfritsch and Shorthouse, 1982). A gall can be defined as 'an abnormal growth produced by a plant under the influence of another organism,' (Redfern et al., 2011). A minority of phytophagous gall midges do not cause the production of galls, for example the raspberry cane midge, *R. theobaldi*, which attacks raspberry canes, *Rubus idaeus* (Linnaeus), resulting in dark patches on the cane but no gall structure. . Similarly the larvae of *Resseliella oculiperda* (Rübsaamen) feed at grafting sites on roses, *Rosa* (Linnaeus)., and fruit trees, causing damage but not abnormal growth (Skuhravá et al., 1984).

The galling habit is thought to be an adaptation in response to dry conditions Price et al., 1987, Yukawa and Rohfritsch, 2005) with the galls protecting larvae from desiccation as well as improving the nutrition obtained by the feeding insect (Price et al., 1987). The ancestral feeding mode of Cecidomyiidae was probably fungivory, and there has been a transition to gall feeding with some species which have ambrosia galls, containing fungi, possibly representing an intermediate stage (Skuhravá et al., 1984, Roskam, 2005,).

Typically the egg stage lasts 4-8 days, and the larvae then feed for a few weeks, 2-3 weeks in the case of *R. theobaldi* and apple leaf midge, *D. mali* (Alford, 2007). The larvae then spin cocoons and pupate and for many species the larvae pupate in the soil (Buczacki and Harris, 1998). The adults emerge from the cocoons and live from one to several days and may disperse over long distances. It is not common for adult midges to feed although some take water from dew drops (Yukawa and Rohfritsch, 2005).

Many parasitoids which prey on gall midge have been identified; for example the primary natural enemies of *D.mali* are an egg-larval parasitoid, *Platygaster demades* (Walker), and anthocorid *(Anthocoridae)* and mirid *(Miridae*) bugs which feed on the eggs and larvae in galls (Cross et al., 1999, Cross and Hall, 2009).

In the case of some midges, such as *R. theobaldi*, it is not the physical damage by the midge which causes problems. Female midges lay their eggs in natural splits in raspberry canes and the larvae feed causing limited damage (Alford, 2007).This damage however leaves the canes susceptible to a fungal complex known as 'midge blight'. This fungal complex, which is made up of *Leptosphaeria coniothyrioum* (Fuckel), and *Didymella applanata* (Niessl) Sacc, can kill young canes and reduce the fruiting potential the following year (Alford, 2007).

Research has been undertaken by several groups to identify midge sex pheromones and the chemical ecology of Cecidomyiid midges was reviewed by Hall et al. (2012).

1.3. RASPBERRIES AND THE RASPBERRY CANE MIDGE

1.3.1 General information about raspberries

Raspberries, *Rubus idaeus* (Linnaeus), are an important soft fruit crop used for a variety of products including desserts, preserves, juices and cosmetics as well as being consumed fresh. They contain high levels of antioxidants (Liu et al., 2002, Szajdek and Borowska, 2008) and good levels of vitamin C (25 mg/100 g - Bender and Bender, 1986) making them a valuable addition to the diet. According to the FAO, approximately 495,718 MT of raspberries were produced by the world's top 10 producers in 2011. Of the top ten producers, seven are located in Europe (or at least partially, for example Azerbaijan), accounting for around 413,017 MT in 2011 (FAOSTAT, 2011c) (Figure 1.1). European production mainly occurs in Northern regions although interest in southern Europe is growing. In the UK much of the fruit produced goes into the fresh fruit market but in more central areas, such as Poland, the majority of fruit is processed (The James Hutton Institute, 2011).

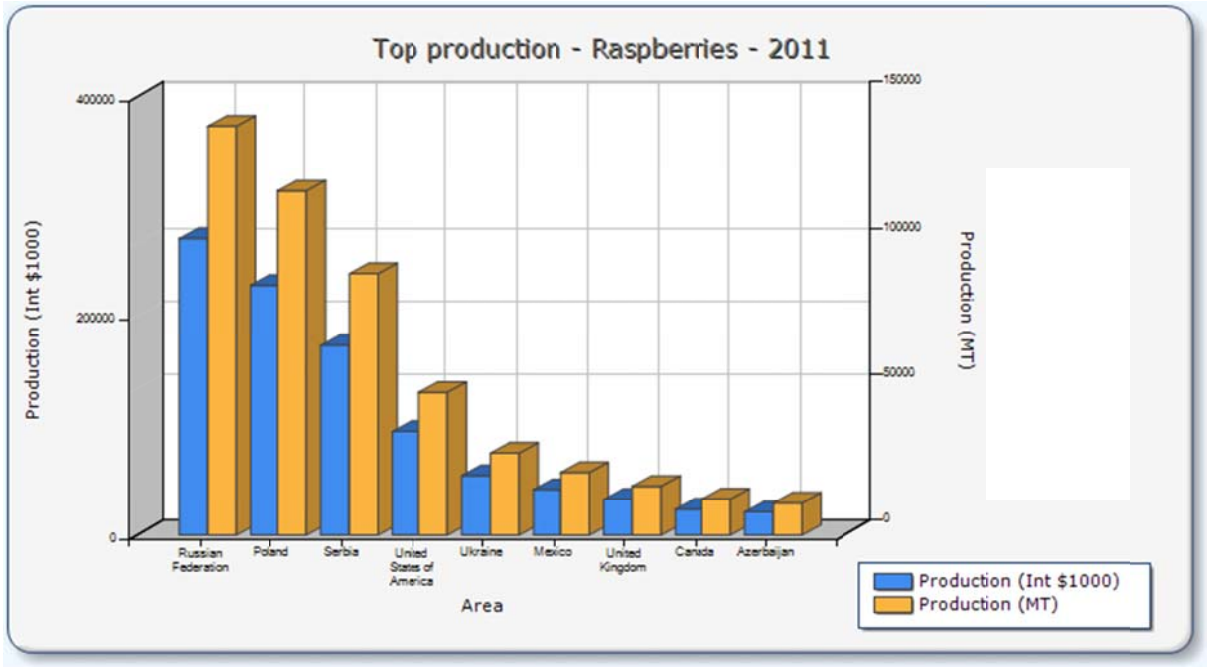

Figure 1.1. FAO Raspberry Production Values for 2011 both by Weight and Value (FAOSTAT, 2011c)

Traditional varieties of raspberry are summer fruiting with fruit harvested from the one-year-old floricanes which then die back and are removed at the end of the fruiting season (Bowling, 2000). The production of fruit in autumn occurs in some wild varieties. The first financially viable autumn fruiting variety was developed at East Malling Research Station (Now East Malling Research) and combined autumn fruit production with good berry yields (Felicidad Fernandes (EMR) pers. comm.). Autumn fruiting varieties grow fruit on the upper nodes of the primocanes (i.e. canes which are new that year) late in the season. Some very early fruits can also be harvested from the lower nodes the following spring in some varieties (Bowling, 2000,The James Hutton Institute, 2012).

There is a range of pests which attack raspberry plants reducing productivity and lifespan. These include large raspberry aphid, *Amphorophora idaei* Börner, (Jennings et al., 2003), raspberry clearwing moth, *Pennisetia hylaeiformis* Laspeyres, raspberry beetle, *Byturus tomentosus* De Geer, raspberry jewel beetle, *Agrilus aurichalceus* Redtenbacher and the raspberry leaf and bud mite, *Phyllocoptes gracilis* Nalepa (Alford, 2007). One of the key pests is the raspberry cane midge, *R. theobaldi*, on which the following section focuses.

1.3.2 The raspberry cane midge

The raspberry cane midge, *R. theobaldi,* is a common pest in the UK and Europe. The adult midge has a dark red body, long legs and measures 1.2-2.1 mm in length. The first-generation adults emerge from cocoons in the soil in early May, although this may be delayed or brought forward depending on temperature, and mate. Virgin females display characteristic calling behaviour extending the terminal segments of their ovipositor and releasing pheromone to attract males (Figure 1.2). The females lay eggs in natural splits in the raspberry primocanes (Figure 1.3) when they reach 20-30 cm in height (Alford, 2007). The eggs hatch approximately one week after they are laid. The larvae feed under the bark of the primocane for up to a month (Buczacki and Harris, 1998) passing through three instars, which range from colourless to pinky-yellow (Alford, 2007) (Figure 1.4). Once mature, the larvae fall to the ground close to the base of the canes; enter the soil and spin a cocoon (Figure 1.5) in which they pupate for two to three weeks after which the second generation of adults emerges. The cycle continues with the second generation mating and laying eggs. When the third generation hatches in September they feed to maturity before pupating in cocoons in the soil over winter (Alford, 2007).

The cane is damaged by the larvae as they feed causing the plant tissue to become black or brown (Alford, 2007) (Figure 1.4) and the bark to peel away from the cane (Buczacki and Harris, 1998). This damage leaves the plant susceptible to fungal infection by raspberry cane blight, *L. coniothyrium,* and raspberry spur blight, *D. applanta*. Collectively these fungal infections are called midge blight and can lead to the death of young canes and thus a reduction in fruit production the following year (Alford, 2007). Canes which split easily such as Malling Jewel and Malling Enterprise are particularly susceptible to midge blight (Buczacki and Harris, 1998). Some varieties which do not readily split are seen not to suffer from midge infestation and midge blight (Jennings et al., 2003).

Figure 1.2 Adult virgin female *Resseliella theobaldi* calling with ovipositor extended.

Figure 1.3 Adult female *Ressiella theobaldi* laying eggs in a fresh split in a raspberry, *Rubus idaeus,* cane.

Figure 1.4 Immature raspberry cane midge (*Resseliella theobaldi*) larvae feeding in a split in a raspberry cane. Feeding leads to the formation of dark patches on the cane.

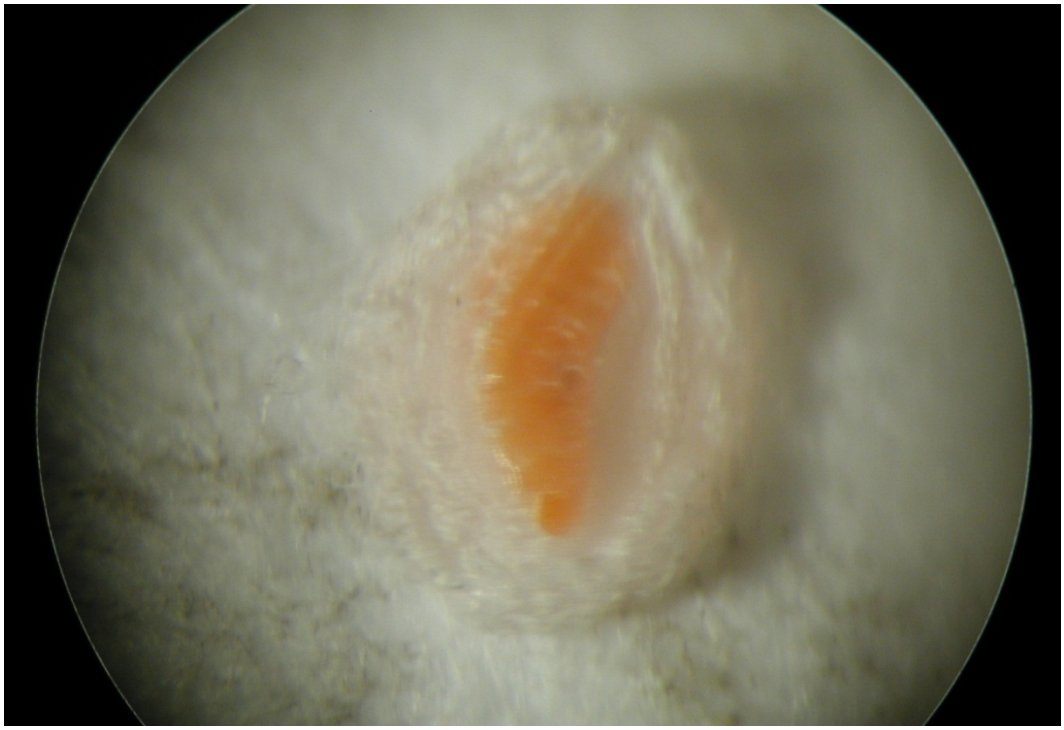

Figure 1.5 Mature *Resseliella theobaldi* larva in cocoon in the early stages of pupation.

Cultural practices can be used to reduce the chances of midge infestation such as treating early spring primocanes of vigorous cultivars with herbicide, leading to their replacement with less split canes at the time when the first generation midges are laying (Jennings et al., 2003). The main treatment used to control the raspberry cane midge is to spray canes with a broad spectrum insecticide in the first week of May and then again two weeks later (Buczacki and Harris, 1998). This is most effective if combined with some kind of monitoring system which can give an idea of whether emergence has occurred and the numbers of midges in the field. A model has been created (Gordon et al., 1989,

Buczacki and Harris, 1998) which uses mean daily 10 cm soil temperatures to predict the first date of *R. theobaldi* oviposition in the field. It was found that 339°C days above the base of 4°C were needed for first oviposition although this was affected by the direction of the slope on which the raspberry plantation was located.

Hall et al., (2009) have identified the sex pheromone of the raspberry cane midge as (*S*)-2-acetoxy-5-undecanone. Traps baited with the synthetic pheromone can be used in traps to assess populations in the field (Cross et al., 2008). Synthetic lures are available from Agralan, (Swindon, SN6 6QR, UK)

1.3.3 Host plant volatiles

Females of *R. theobaldi* lay their eggs in splits in the primocanes (new season growth). The splits may arise from wind rubbing, human activities such as pruning, and night frosts. Vétek et al. (2006) found that plants where canes split and bark peeled easily had higher larval infestation rates than those where canes split less easily.

In laboratory bioassays, Nijveldt (1963) observed that when presented with undamaged canes females did nothing, but when presented with damaged canes they immediately laid their eggs in the wounds. Females did not lay eggs in older dry wounds suggesting that sap played a role in the selection of oviposition sites.

To investigate the role of sap, damaged willow twigs were put into a cage with female midges and there was no oviposition. The twigs were then sprayed with raspberry sap and the females immediately laid eggs in the wounds (Nijveldt, 1963). The eggs did hatch but the larvae died soon after. Observations in the field found females were rapidly attracted to artificial splits made in canes. Females landed close to the damaged area and searched the surface with their antennae until they found the wounds and then laid their eggs underneath the epidermis (Nijveldt, 1963).

Solid-phase microextraction (SPME) was used to identify six volatiles produced by intact stems and twelve from split canes (Hall et al., 2011). Four of these chemicals elicited EAG responses in the female midges, three of which were produced only when the cane was split. It could be suggested that it is these volatiles which females are using to locate the split canes. Field tests were carried out in England and Hungary, of a synthetically produced blend of volatiles presented in the same proportions as found from the split canes. This gave inconclusive results with few females caught at any site; and at some sites significantly more and at some significantly fewer males caught than in un-baited controls. Hall et al. (unpublished) also carried out a trial with bamboo canes coated in synthetic cane volatiles. No females were found caught on the sticks. Males were caught but it varied as to whether numbers were significantly different to those caught on un-baited canes.

Female raspberry cane midges will lay their eggs on blackberry, rose and to a lesser extent apple and hawthorn as well as raspberry (Pitcher, 1955). Larvae were only found on raspberry and to a small extent rose, but this finding does suggest that these other plants may also be giving off some visual or olfactory cues prompting oviposition.

1.4. BLACKCURRANTS AND THE BLACKCURRANT LEAF MIDGE

1.4.1 General information about blackcurrants

Blackcurrants, *Ribes nigrum* Linnaeus, are a small but economically important crop. They are a member of the *Ribes* genus which also includes redcurrants, *Ribes rubrum* Linnaeus, whitecurrants (also *R. rubrum*) and gooseberries, *Ribes uva-crispa* Linnaeus. Europe is the main producer of currant fruits with the twelve largest producers all being European countries or countries at least part in Europe such as Turkey. The Russian federation is the leading currant growing country with the UK ranking the world's fifth largest producer yielding 12,060 MT in 2011 (FAOSTAT, 2011b)

Blackcurrants are high in antioxidants (Moyer et al., 2002, Brennan, 2008) including vitamin C (Johns and Stevenson, 1979, Szajdek and Borowska, 2008). Levels of vitamin C vary between cultivars from 130-200 mg/100 ml juice although cultivars developed at SCRI (now the James Hutton Institute) have reached over 350 mg/100 ml (

Brennan, 2008). A study in Finland looking at the amount of vitamin C in fresh berries found 137 ± 16.6 mg/100 g fresh weight compared to 51 ± 3.2 mg/100 g fresh weight for oranges, *Citrus x sinensis* (Linnaeus) Osbeck (Hägg et al., 1995). Most of the blackcurrants produced in Europe are used in juice production (Brennan, 2008). Most large scale blackcurrant production in the UK and other countries is now completely mechanised (Bowling, 2000).

The genus *Ribes* is found growing wild in the northern hemisphere. Blackcurrant foliage has a distinctive scent which is produced by yellow glands located on the underside of leaves (Figure 1.5) (Johns and Stevenson, 1979).

Figure 1.5. Yellow glands visible on the underside of a blackcurrant, *Ribes nigrum* leaf as seen under a microscope.

Blackcurrant bushes are cultivated from cuttings. From the third year onwards fruit is produced on the shoots of the previous year. The bush produces its largest yields between the ages of four and eight with the average bush fruiting up to the age of twelve. Flowering occurs in April and May with the resultant berries harvested in July and August. From October to March the bushes are dormant. Per acre of bushes, three to five tons of fruit are harvested each year (GlaxoSmithKline, 2010). A single bush can yield 2-3 kg once mature (Bowling, 2000).

There is a range of pests which affect blackcurrant plants including blackcurrant gall mite, *Cecidophyopsis ribis* Westwood, which can act as a vector for blackcurrant reversion virus (Jennings et al., 2003, Brennan, 2008), currant/sowthistle aphid, *Hyperomyzus lactucae* Linnaeus, blackcurrant sawfly, *Nematus olfaciens* Benson, winter moth, *Operophtera brumata* Linnaeus, and

also the pest examined here, the blackcurrant leaf curling midge, *Dasineura tetensi* (Mitchell et al. 2011)

1.4.2 The blackcurrant leaf curling midge, *D. tetensi*

The blackcurrant leaf curling midges lay their eggs on young opening leaves. The eggs hatch after a week and the larvae feed causing the leaves to remain folded and twisted (Figure 1.6), and eventually turn black. If the green galls are untwisted, white-orange larvae can be seen with an average of 4-5 larvae in each infested leaflet (Figure 1.7). Young affected plants may become misshapen and the leaf distortion can mask other problems such as viruses and rogue cultivars (Buczacki and Harris, 1998, Alford, 2007).

Figure 1.6 – Twisted blackcurrant, *Ribes nigrum,* leaf galls containing feeding *D. tetensi* larvae.

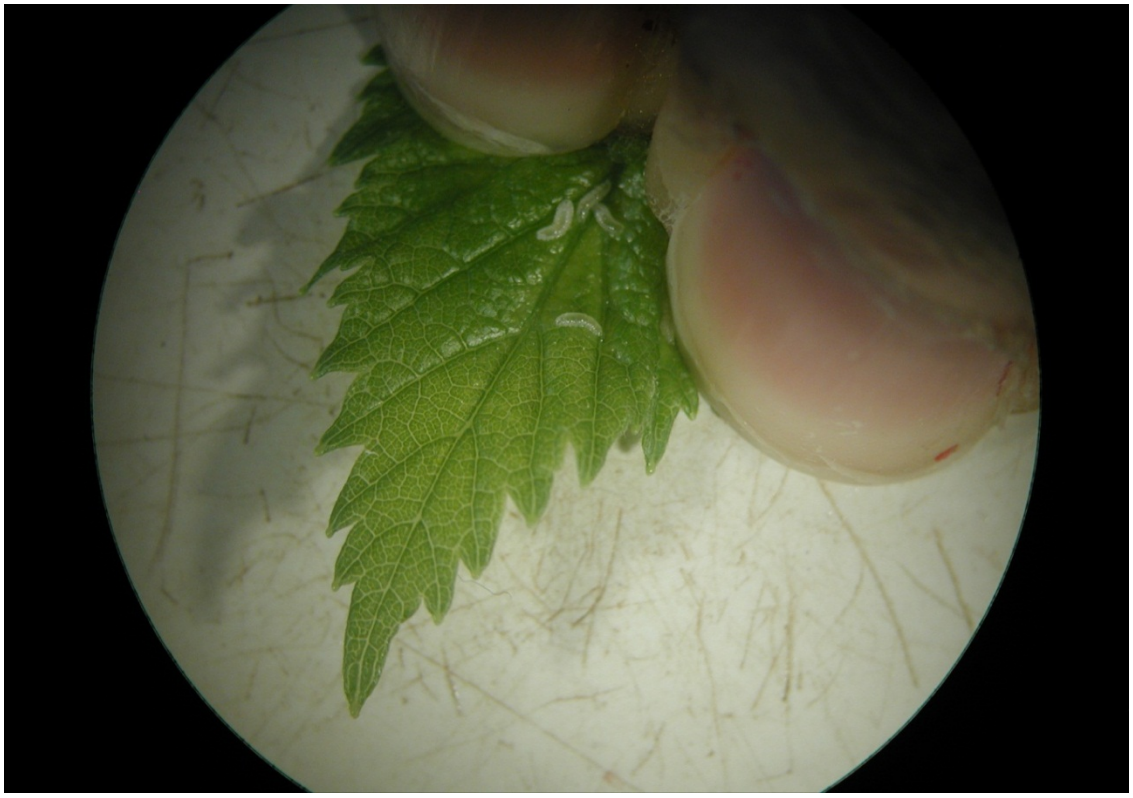

Figure 1.7. Young *D. tetensi* larvae in an unrolled blackcurrant, *Ribes nigrum,* leaf gall.

Figure 1.8. Adult virgin female *D. tetensi* displaying characteristic calling behaviour.

Adults emerge from the cocoons in the soil where they overwintered from April and mate, with the females then laying eggs. The larvae feed in the galls for 10-14 days before falling to the soil and spinning cocoons in which they remain for two weeks before the next generation emerges. In the UK there are three main generations per year but there may be a fourth on cultivars producing new growth in late summer. The final generation overwinters in cocoons in the soil.

Adult blackcurrant leaf midges are 1-2 mm long with a brown-yellow body (Figure 1.8). The abdomen is paler with dark cross bands above. The larvae are up to 2.5 mm long and white, becoming orange as they mature. The adults fly when weather conditions are calm but remain under the shelter of blackcurrant bushes when it is windy (Buczacki and Harris, 1998, Alford, 2007).

Emergence from cocoons usually occurs between 7 and 8 am in the lab and mating occurs between 10 am and 12.00 pm, which is the time when the midges are most active (Crook and Mordue, 1999). The exact date of the spring emergence of adult midges from the soil is dependent on the air temperatures in the area directly around the soil where the midges have been pupating (Cross and Crook, 1999).

Synthetic pyrethroid insecticides sprayed to control blackcurrant gall mites have also kept blackcurrant leaf curling midge populations under control. However, these chemicals and other organophosphate insecticides are now being withdrawn from sale due to health and environmental concerns (Jennings et al., 2003).The organophosphates Lindane and Fenitrothion, which were both used for the control of midges in horticultural crops were banned in the European Union in 2000 (Byrne, 2000)) and 2007 (Kyprianou, 2007) respectively. These bans coupled with increasing consumer pressure to reduce residues on fruits and worries about midge pesticide resistance mean that more work into the control of midges with reduced use of pesticides is needed (C Bird, Agrii, personal communication, 2013).

It has been shown that mated but not virgin females respond to blackcurrant leaf volatiles (Crook and Mordue, 1999). Insects were introduced to a four-way olfactometer and allowed to choose where to go with one arm connected to a flask containing blackcurrant foliage and the other three acting as controls. Virgin

females and males showed no response to the foliage but mated females were positively attracted. Antennae of males and females were studied under scanning and transmission electron microscopes. It was found that females had more of a stalked type of sensilla, called circumfilla, which are unique to gall midges and whose structure suggests they may be important in olfaction.

Some blackcurrant varieties show partial resistance to midge attack with little or no larval development, for example Ben Connan (Buczacki and Harris, 1998). Volatiles from leaves of susceptible and resistant cultivars were analysed using gas-chromatography linked to mass spectrometry (Griffiths et al., 1999). It was found that the chemical make-up of the odour plumes produced by two susceptible and one resistant variety were identical with only small quantitative differences between the three plants. There were no clear trends with one volatile present in higher concentrations in resistant vs. susceptible plants or vice versa.

Crook et al. (2001), investigated midge oviposition on blackcurrant plants of both resistant and susceptible cultivars. Single mated females, which were less than twenty four hours old, were introduced to shoots of either Ben Alder (susceptible) or Ben Connan (midge resistant). The shoots were examined for eggs twenty four hours later under a microscope. The females showed no preference between the two cultivars and also showed no discrimination between volatiles from the two varieties in a four-way olfactometer. Larvae which were reared on Ben Connan plants were significantly smaller than those reared on Ben Alder and in field experiments Ben Connan showed significantly less leaf curl than Ben Alder and another susceptible variety, Baldwin.

Hellqvist (2001) studied larvae which could survive on resistant cultivars (virulent). These larvae develop on both resistant and susceptible blackcurrant varieties although development on the resistant varieties was found to be initially slower. It was also found that although avirulent larvae did not develop alone on the resistant varieties they would when virulent larvae were also present. An intermediate midge type, which could develop on gall resistant plants but at a lower rate to the virulent type, was also discovered. It was concluded that there must be no cost to being virulent as larvae of this type were also found in areas where only susceptible varieties were present.

1.5. APPLE AND THE APPLE LEAF CURLING MIDGE

1.5.1 General information about apples

Apples, *Malus domestica* Borkh, are an important crop in several regions of the world including North America, India, Russia, Poland and Iran. By far the highest producer is China (Figure 1.9).

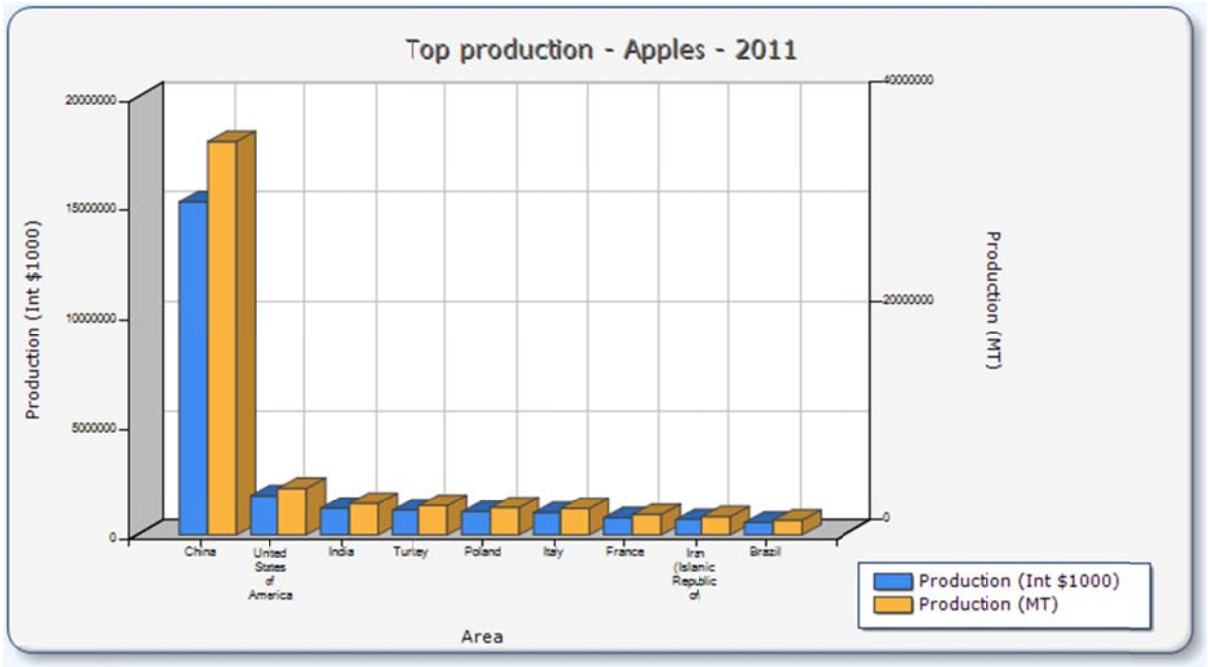

Figure 1.9. Apple Production in 2011 - FAOSTAT, (2011a)

Once planted, apple trees take several years (between three and ten) before they produce their first fruit with an average tree producing fruit after five years. Trees produce their largest harvests between ten and thirty years old after which fruiting ceases. Apple trees can live for between thirty five and one hundred years (AppleTreeHill, 2000).

Each year an apple tree passes through a range of stages from flowering in spring to fruit maturity in autumn (Apple Tree Hill 2000).

There is a variety of pests which affect apple orchards including apple capsid, *Plesiocoris rugicollis* Fallén, apple sucker, *Psylla mali* Schmidberger, a range of

aphids (family *Aphididae*), codling moth, *Cydia pomonella* Linnaeus, apple sawfly, *Hoplocampa testudinea* Klug, and apple leaf curling midge, *Dasineura mali* (Alford, 2007).

1.5.2 The apple leaf curling midge, *D. mali*

The apple leaf curling midge, *D. mali*, is found throughout Europe, Argentina, New Zealand and North America (Cross and Hall, 2009). Adult midges are 1.5 to 2.5 mm long and are dark brown. Adult females have a red abdomen. Larvae are around 3 mm long and are white in early life becoming red to orange later (Alford, 2007) (Figure 1.10). Pupae overwinter, with the first generation of adults emerging in spring. Adults mate and eggs are laid in between the hairs of unfurled young leaves (Cross and Hall, 2009) and the larvae hatch after three to five days. After 2-3 weeks of larval feeding the mature larvae spin cocoons and fall to the ground with adults emerging two weeks later (Alford, 2007). There are three to four generations per year with the first occurring around blossom time. In pheromone trap experiments in the UK (Kent) and New Zealand (Cross et al., 2009) it was found that there were regional differences in generation number; with 2-3 generations per year in the UK compared to 5 in New Zealand.

Infested leaves become tightly rolled and slightly thickened due to larval feeding (Redfern et al., 2011) (Figure 1.11), forming galls which makes control of the midge with insecticide difficult as they are protected (Cross and Hall, 2009). Midge damage stunts tree growth making it a serious problem for young nursery trees (Alford, 2007). Another problem for growers is that midge cocoons may become lodged in the surface of fruits restricting export to countries where midges are not present.

Figure 1.10. Mature *Dasineura mali* larva.

Figure 1.11. Rolled apple, *Malus domestica*, leaf containing feeding *Dasineura mali* larvae.

The main natural enemies of *D. mali* are the egg and larval parasitoid, *Platygaster demades* Walker, (Cross and Jay, 2001, Cross et al., 1999) and anthocorid (family *Anthocoridae*) and mirid bugs (family *Miridae*) which predate on the eggs and larvae in the leaf galls (Cross and Hall, 2009). This biological control is not, however, enough to deal with serious midge infestations.

Field work has been done with delta traps baited with synthetic pheromone. (Cross and Hall, 2009). It was found that there was an optimum pheromone septa load of 3 µg, and delta trap height of 0.5 m. It was also found that peak male numbers occurred at 9 am, with the number of ovipositing females peaking later at 12 pm. Research by Cross et al. (2009) using the pheromone baited delta traps found that there was a relationship between the number of male midges caught in traps for a particular generation and number of galls formed subsequently for that generation; with each male caught corresponding to 137 galls per hectare. It was also found that generational peak numbers were earlier the closer to the orchard the trap was. In addition Cross et al. (2009) also found that the life cycle of midges was considerably lengthened during dry periods as rain is needed for the larvae to exit the leaves for pupation.

As well as population monitoring, pheromone traps can be used to attract males and then kill them. Suckling et al. (2007) tested both delta traps and Lynfield type traps consisting of a plastic container with four entrances and vegetable oil at the bottom acting as a killing material. There were far fewer males found in areas where Lynfield traps were used, as assessed using delta traps, suggesting they may act as a good control method.

1.5.3 Host plant volatiles

Female *D.mali* need to locate young apple leaves on which to lay their eggs. Galanihe and Harris (1997) investigated the responses of mated female midges to host and non-host foliage in a wind tunnel. When released downwind of apple and pear, *Pyrus communis* Linaeus, foliage, females were more likely to fly upwind to the apple. Landings on the apple were mostly concentrated on the buds and young leaves, the area in which eggs are normally laid. When the foliage was

obscured form view, females still showed a preference for apple, evidence that odour cues are involved in host selection. This was further corroborated by the orientation responses displayed by females exposed to a dichloromethane extract of apple volatiles. Pear leaves were found to be more attractive than clean air suggesting that there may be some attractive volatiles present in the foliage of both plants. To see if visual cues played a role, foliage models were used in some tests. It was found that the presence of these models reduced the number of females taking flight in both the presence and absence of apple odours. This suggests that the models were probably recognised as alien by the midge and this disrupted their behaviour.

1.6. THE IMPORTANCE OF OLFACTORY CUES

Insects, like other animals, need to locate important resources such as food and mates for reproduction. Resource location relies on a number of cues including visual, auditory, mechanical and olfactory. In the case of phytophagous insects, finding plant hosts on which to feed and oviposit is very important. In some cases, target host plants are not visible due to other plant cover and in this case olfactory cues are particularly important (Bruce et al., 2005).

Olfactory cues are airborne chemical cues which are received by specialised cells or groups of cells called sensilla (Bruce et al., 2005), generally in the antennae. There are a range of different sensillum structures including hair like, pore plates and pit pegs. Several sensilla types are usually present on the antennae of a single insect and there is great diversity of sensilla even within members of the same species (Steinbrecht, 2008).

1.6.1 Semiochemicals

Semiochemicals are signalling chemicals involved in the interaction between organisms, including between those of the same species. Semiochemicals can be divided into four types.

1) *Pheromones* are semiochemicals acting intraspecifically (within species) and are involved in mate location, aggregation and territory marking, for example (Bruce et al., 2005).

2) *Allomones* are interspecific (between species) chemicals which are detrimental to the receiver (Collins, 2013) for example the toxic monoterpenes and hydrocarbons squirted by soldier termites at attackers (Evans and Schmidt, 1990).

3) *Synomones* are interspecific chemicals which benefit both the emitter and the receiver, for example plant volatiles which attract insect pollinators (Meyer, 2006).

4) *Kairomones* are interspecific chemicals which are involved in interactions which are beneficial to the receiver but disadvantageous to the emitter (Bruce et al., 2005). This applies to the chemicals (odours) produced by plants which allow them to be located by insects which will subsequently feed on and damage them.

1.7. THEORIES OF HOST RECOGNITION

There are two distinct theories which have been proposed to explain how phytophagous insects detect and identify host plants.

1) Insects respond to one or two volatile chemicals which are specific to their host plant. In the review by Bruce et al. (2005), it is noted that there are few examples of insects detecting host-specific chemicals in the literature. Most of these involve the detection of brassica host plants. Brassicas are the only plant family to produce isothiocyanate volatiles and it is these unique volatiles which insects use to detect them.

2) Insects respond to a specific blend of a range of chemicals. The chemicals may be ubiquitous to many plant species but the exact blend is host specific (Visser, 1986). Bruce et al. (2005) found much more evidence for this in the literature. Under this theory it is the ratio of different chemicals which is likely to be

important with the same chemicals acting as attractants and repellents when combined in different blends. Webster et al. (2010) found that when the compounds present in a blend which attracts the black bean aphid, *Aphis fabae* Scopoli, were presented alone ten of them were repellent. When these compounds were combined in a blend at their most repellent concentrations they produced an attractant effect.

1.8. IDENTIFICATION OF HOST PLANT VOLATILES INFLUENCING INSECT BEHAVIOUR

Vicia faba (Linnaeus) volatiles have been collected on Porapak-Q cartidges for use in tests with the black bean aphid, *Aphis fabae* (Webster et al., 2008). Electroantennography (EAG) tests were carried out on excised aphid heads in conjunction with gas chromatography (GC) to identify chemicals within the bean volatile sample which the aphid could detect. Further identification was achieved by comparing GC-mass spectrometry (MS) results for the volatile sample with known standards. Tests with the aphid in a four way, perspex olfactometer found that they spent significantly more time in the areas connected to glass arms containing both natural bean material and synthetic blends of chemicals identified from the GC-EAG. The volatile chemicals which the aphid responded to included alcohols, aldehydes and acetates (Webster et al., 2008).

Another key group of pests for whom host volatiles are important are the moths (Lepidoptera) which cause losses to a wide variety of agricultural crops. Work on the sphinx moth, *Manduca sexta* (Linnaeus) (Fraser et al., 2003), used gas chromatography linked to EAG, and found 23 active compounds in volatiles collected from the headspace around tomato plants, *Solanum lycopersicum* (Linnaeus). Fifteen of these were identified using GC-MS. Collection was achieved by placing the plants in a glass tank, with the volatiles collected on Porapak-Q. Volatiles were extracted from the polymer using dichloromethane. Wind tunnel experiments showed that female moths were attracted to a synthetic blend of eight compounds identified as active by the EAG.

Female moths of *Lobesia botrana* (Denis and Schiffermuller), lay their eggs on flower buds and berries of grape plants, *Vitis vinifera* (Linnaeus). Once hatched, the larvae feed causing damage (Cooper et al., 2010). GC-EAG has been used to identify active volatiles produced by grape berries and inflorescences and create a lure which attracted females in both lab and field bioassays proving that this moth species uses odours to select oviposition sites (Anfora et al., 2009).

Plant odours are also used by parasitoids of phytophgous insects to locate their prey. Research by Onagbola and Fadamiro (2011) investigated attraction of the parasitoid, *Pteromalus cerealellae* (Boucek), to the cowpea seed beetle, *Callosobruchus maculatus* (Fabricius). Investigations using EAG and bioassays with both Y-tube and four-way olfactometers showed that *P. cerealellae* was strongly attracted to the odour of the cowpeas, in some cases more so than to the actual cowpea seed beetle prey. In this interaction the cowpea odour is acting in a way which is beneficial to the plant by attracting parasitoids which will kill the insects which are feeding on it. In this case the plant volatile is acting as a synomone benefiting both the producer and the receiver.

A gall midge example is that of the brassica pod midge (*Dasineura brassicae* (Winnertz)) and its parasitoids *Platygaster subuliformis* (Kieffer) and *Omphale clypealis* (Thomson) (Murchie et al. 1997). *D. brassicae* is a summer pest of brassica crops including oilseed rape (*Brassica Napus* (Linneaus)). The brassica family is characterised by the presence of glucosinolate compounds in plant tissues. When the plant is injured these compounds are broken down by enzymes to isothiocyanate volatiles and other compounds. Traps baited with two different isothiocyanates were tested in the field. It was found that both male and female midges were significantly attracted to traps baited with allyl isothiocyanate baited traps as were female *O. clypealis* which lay their eggs in larvae of the midges. Significant attraction was seen by both males and female of another midge parasitioud *P. subuliformis* to 2-phenylethyl iso-thiocyanate. In this example the midges are using the volatiles to locate their host plant and the parasitoids to locate sites which are likely to have midge larvae in which to lay their eggs.

1.9. RESEARCH TO IDENTIFY HOST PLANT VOLATILES: MIDGE RELATED EXAMPLES

The above examples illustrate some of the techniques used in host plant volatile identification and insect-plant interactions. Work detailed in this project focuses on small Diptera from the family Cecidomyiidae.

Birkett et al. (2004) investigated the volatiles produced by the Lynx cultivar of wheat, *Triticum aestivum* (Linnaeus), and their effect on the orange wheat blossom midge, *Sitodiplosis mosellana* (Géhin) which lays its eggs on wheat ears. They collected volatiles from intact wheat ears at the time of emergence using air entrainment. Olfactometer bioassays showed that both the entrainment samples and freshly cut wheat samples attracted female midges. Gas chromatography linked to electroantenography (GC-EAG) experiments showed that there were six components which female midges detected. These were identified using GC linked to a mass spectrometer as acetophenone, (*Z*)-3-hexenyl acetate, 3-carene, 2-tridecanone, 2-ethyl-1-hexanol and 1-octen-3-ol. None of the chemicals was attractive alone at the concentrations found in the air entrainment sample, but a blend of the six components at the concentrations observed naturally (3.4 ng/µl (*Z*)-3-hexenyl acetate, 7.4 ng/µl 3-carene, 1.3 ng/µl acetophenone, 1.7 ng/µl octen-3-ol, 1.8 ng/µl 2-ethyl-1-hexanol, and 607 ng/µl 2-tridecanone) was, as was a blend of acetophenone, (*Z*)-3-hexenyl acetate and 3-carene found in the same natural concentrations. The paper concludes that blends of all six compounds or acetophenone, (*Z*)-3-hexenyl acetate and 3-carene attracted female midges and could be used to monitor midge populations in the field.

Research with brassica pod midge, *D. brassicae*, allowed the midge to choose between the normal preferred host rape, *B. napus* , and less preferred brown mustard, *Brassica juncea* (Linnaeus) (Åhman, 1985). Female midges were more likely to land on rape, although once landed the size of the egg batch laid was the same as for brown mustard. It was also noted that more batches were laid on the rape. Åhman (1985) concluded that the females were using visual and/or odour cues to locate host plants and make decisions before landing. Murchie et al. (1997) found in field trials that both male and female *D. brassicae* were caught in significantly higher numbers in traps baited with allyl isothiocyanate compared to

unbaited controls providing evidence that this midge uses odour cues to locate host plants.

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In South Africa, the midge *Dasinura dielsi* (Rübsaamen) has been introduced as a biological control agent to control *Acacia cyclops* (Cunningham. ex Don), an invasive tree species from Australia (Kotze et al., 2010). The midge lays its eggs in the acacia flowers preventing seed set (Adair, 2005). Headspace volatile samples were collected from various flowering stages (Kotze et al., 2010). These were analysed using GC-MS and it was found that there were four characteristic chemicals of the open flowers found only in this stage and the bud stage (when the insect is physically unable to oviposit). It was suggested that it is these characteristic chemicals which the midge uses to identify its host at the appropriate stage. A high concentration of (*Z*)-3-hexen-1-ol was found in the green buds, which is known to deter oviposition in *Heliothis virescens* (Fabricius) moths (Pichersky and Gershenzon, 2002) and may function in a similar way for the midge.

1.10. AIMS AND OBJECTIVES

Female midges of all three species, *R. theobaldi, D. tetensi* and *D. mali*, are attracted to their host plants, raspberry, blackcurrant and apple, at particular stages of development or in a particular condition. It has been suggested that all three use olfactory cues to find appropriate hosts on which to lay their eggs. In the case of *D. mali* and *D. tetensi* attraction to the host material has been demonstrated but no chemical attractants found. In the case of the *R. theobaldi,* chemicals detected by the antennae have been found but the results of field work were inconclusive. This project sought to build on this body of work and develop lures for all three species which mimicked the female host plant in its attractive condition. These lures can be used to monitor females in the field and possibly as part of attract-and-kill strategies.

Specific objectives were as follows, for one or more of the three target species:

a) To develop methods for collecting volatiles from appropriate host plant material.

b) To use electroantennography (EAG) to detect components of the host plant volatile collections which are detected by female midges and hence are candidate attractants
c) To develop laboratory and/or field bioassays to evaluate the attractiveness of plant volatiles and synthetic chemicals to female midges.
d) To develop lures with attractive blends of synthetic compounds.

Chapter 2
GENERAL MATERIALS AND METHODS

2.1. INTRODUCTION

There are various materials and methods used more than once in different chapters of this thesis. For clarity and to avoid repetition they are detailed here in this general chapter.

2.2. VOLATILE COLLECTION METHODS

2.2.1 Solid phase microextraction (SPME)

Solid phase microextraction allows the rapid collection of volatiles produced by plant material. A cylindrical cage (8 cm high x 5 cm diameter) was made from wire which was placed around the plant tissue to be sampled (cane or shoot etc.). The bottom was cut off a 1l capacity oven bag (poly(ethyleneterephthalate), Sainsbury's PLC) and this was put over the cage and secured above and below with ties. A hole was made in the bag with a mounted needle and volatiles sampled with a solid-phase microextraction (SPME) fibre (blue polysiloxane/polydivinyl benzene; Supelco) pushed through the hole (Fig. 2.1). The fibre was then exposed for 15 minutes. A blank SPME fibre was analysed as a control. Once collection was complete the fibre was inserted into the injector of a gas chromatograph (GC) linked to a mass spectrometer (MS) to desorb and analyses the trapped volatile compounds.

Figure 2.1.Collection of volatiles from a split raspberry cane, *Rubus idaeus*, using an exposed solid phase microextraction (SPME) fibre

2.2.2 Entrainment

Entrainment is another method of volatile collection. While not as rapid as SPME it does allow a solution to be made which can be used for multiple experiments.

Volatiles were collected using filters made from Pasteur pipettes (4 mm i.d.) containing Porapak Q (200 mg 50/80 mesh; Waters Associates,).

The material under investigation, for example a shoot tip, was enclosed in a 1l oven bag (Sainsbury's PLC). The neck of the bag was secured around the plant stem with a piece of wire. The portable entrainment kit (made by Barry Pye, 4 Whitehall Cottages, SG4 8JS) was used which consists of the set up shown in Figure 2.2. The set up allows collection of volatiles from two volatile sources at once.

The lines coming from the push and pull were made of Teflon tubing (2 mm diameter and 2 m long) and for each sample one push tube was inserted in to the bag through the neck and secured with the wire. The push pump was set to just over 420 ml/min into each bag so that charcoal filtered air was pushed into the

bag and it inflated. A collection cartridge (4 mm i.d) containing Porapak-Q was connected to the pull tube using a connector (Figure 2.3 A). The corner of the bag was cut off the inflated oven bag and the end of the cartridge inserted into the bag and secured with wire (Figure 2.3 B). A clamp stand was used to hold the cartridge at an appropriate height. The pull pump was turned on and set to 400 ml/min. Collections were carried out for various lengths of time depending on situation (see individual chapters). The slight difference in the rate of air entering and leaving the bag ensured that the bag remained inflated with a small amount of air always leaving the bag to prevent impurities from the atmosphere entering the bag. Volatiles were desorbed by eluting the cartridge with 1ml of dichloromethane (Pesticide Residue Grade) to make a solution which could be injected into the GC.

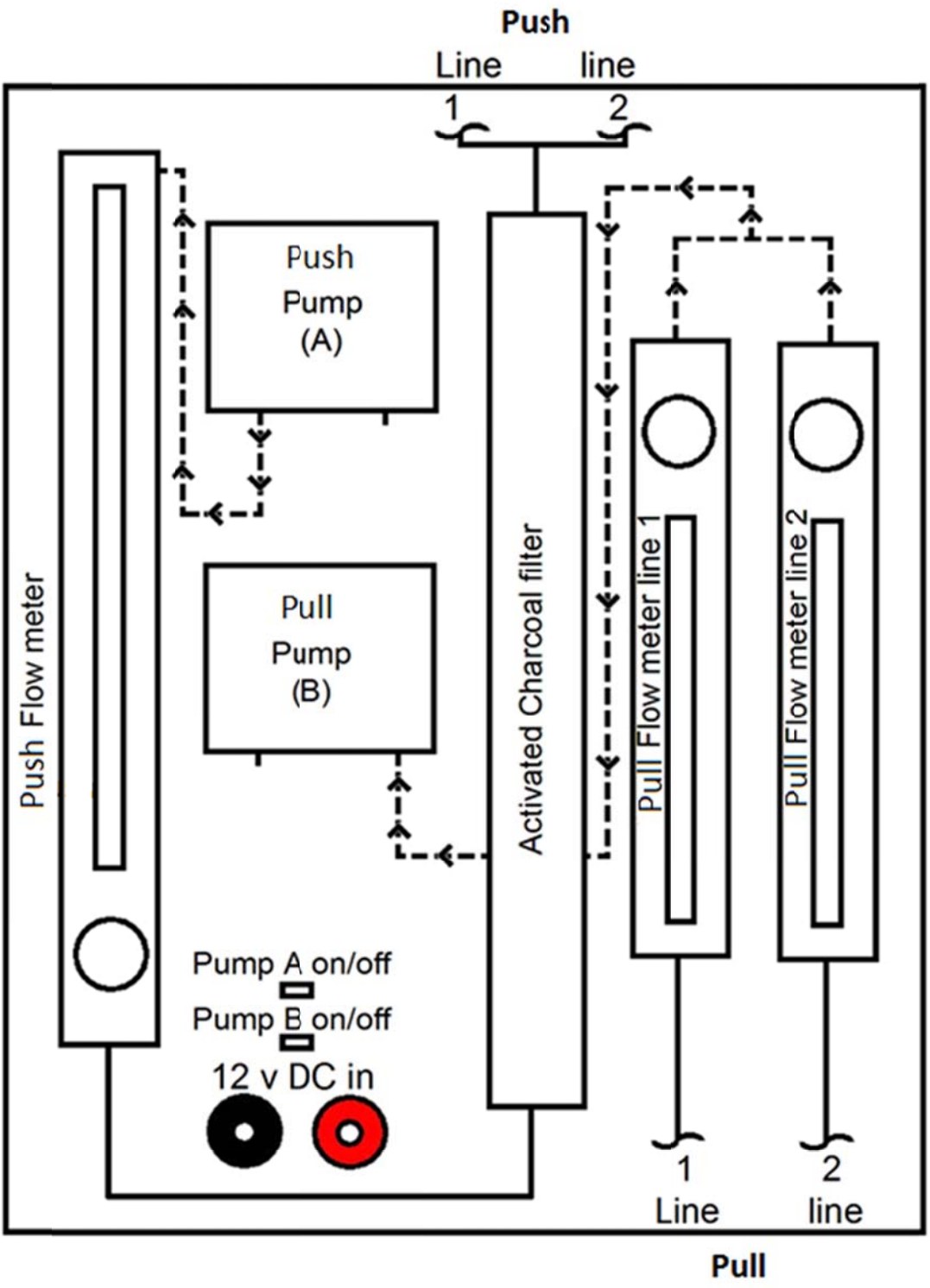


Figure 2.2 Diagram of the portable entrainment kit (courtesy of A Harris, EMR)

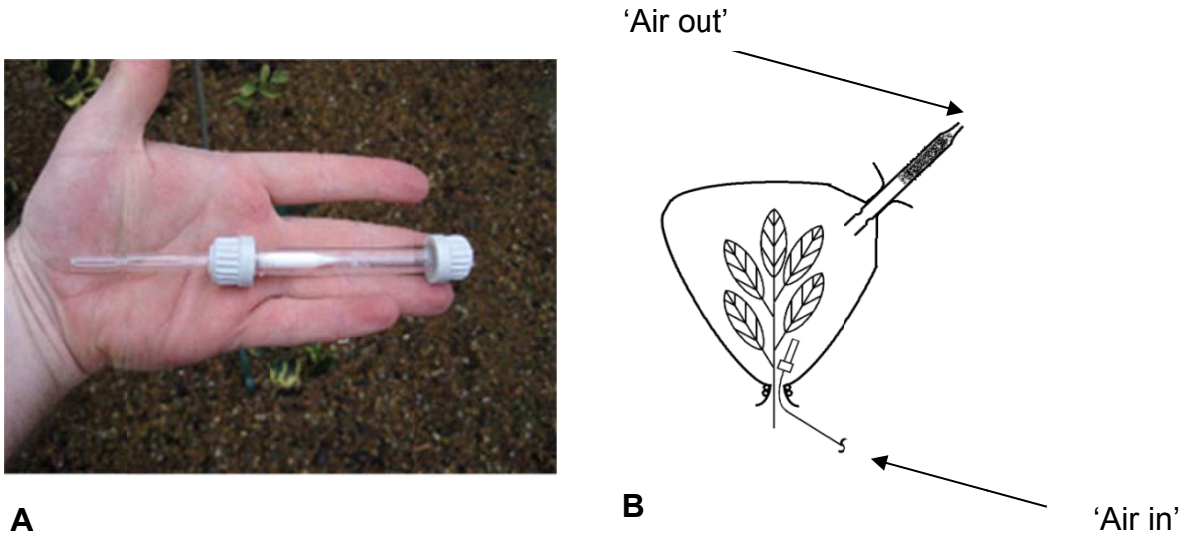


Figure 2.3 A. Push fit connector with the Porapak Q filter inserted. **B.** Diagram of the plant entrainment set up with Porapak Q filter inserted and arrows showing airflow (both courtesy of A Harris, EMR).

2.3. ANALYSIS OF COLLECTIONS OF VOLATILES

2.3.1 Gas Chromatography linked to mass spectrometry (GC-MS)

Gas chromatography linked to mass spectrometry (GC-MS) was used to analyse the volatile collections made using both the SPME and entrainment methods. For SPME the fibre was injected directly into the GC, whereas in the case of entrainment the solution of volatiles was injected. For SPME there was no solvent delay but with entrainment samples a solvent delay of 4 minutes was used to avoid solvent saturation of the system.

GC-MS Analyses on Agilent instrument

Analyses were carried out on a HP6890 GC coupled to a HP 5973 Mass Selective Detector (Agilent, Stockport, Cheshire, UK). The GC was fitted with a fused silica capillary column (30 m x 0.25 mm i.d. x 0.125 µ film thickness) coated with non-polar DB5 (Agilent). Injection was splitless (220ºC) with helium as the carrier gas (1 ml/min). The oven temperature was programmed at 50ºC for 2 min then at 6ºC/min to 240ºC. *n*-Alkane standards were run each day and retention times of compounds of interest converted to Kovats Indices (KI).

GC-MS Analyses on Varian instrument

The GC–MS set up used consisted of a gas chromatograph (Varian CP 3800) coupled to a mass spectrometer (Varian Saturn 2200 ion trap). The GC had fused capillary columns (30 m x 0.25 mm i.d.) coated with polar (Supelcowax-10, Supelco, USA) and non-polar (30 m x 0.25 mm i.d.; VF5, Varian) phases. Both polar and non-polar analyses were carried out (see individual chapters for details).

Injection was splitless (220ºC) with helium as the carrier gas (1 ml/min). The oven temperature was programmed at 40ºC for 2 min then at 10ºC/min to 240ºC. *n*-Alkane standards were run each day and retention times of compounds of interest converted to Kovats Indices (KI).

Kovats Indices

The Kovats retention index is a method used in Gas Chromatography to turn retention times (which vary with GC system) into constants which are system-independent. The calculation makes use of the retention times of hydrocarbon standards which are injected into the same GC being used for analysis. The calculation used for temperature programmed chromatography (used here) can be seen in Figure 2.4.

$$I = 100 \times \left(n + (N-n) \frac{RT\,(\text{unknown}) - RT\,(n)}{RT\,(N) - RT\,(n)} \right)$$

Figure 2.4 The equation used to calculate a Kovats Retention Index using gas chromatograph (GC) retention times (I = Kovats Index; n = number of carbon atoms in the smaller n-alkane; N = number of carbon atoms in the larger n-alkane; RT = Retention Time on the GC).

Compound identification

Data were captured and processed using Chemstation Software (Agilent) or MS Workstation v6.8 (Varian). Compounds were identified by comparison of their mass spectra with those in the NIST Mass-Spectral library and of their retention

indices with those in the Pherobase (El-Sayed, 2012) and Flavornet (Acree, T and Arn, H, 2004) internet databases. If there was good matching between the Kovats Index found and those on the databases then it was concluded that the compound had been correctly identified.

2.3.2 Gas Chromatography linked to electroantennographic recording (GC-EAG)

The effect of components of volatile collections on the antennae of midges was assessed using a GC-EAG set up following a similar method to Amarawardana (2009).

The GC used was an HP6890 (Agilent Technologies) with a flame ionisation detector (FID) and fused silica capillary columns (30 m x 0.32 mm x 0.25 µm film thickness). The columns were coated with polar (Supelcowax-10, Supelco, USA) and non-polar (SPB-1, Supelco, USA) phases. The column ends were connected to a push-fit-Y connector, the outlet of which was connected to a second Y-connector. This was connected to two identical lengths of deactivated silica capillary column, one leading to the FID and the other to the antennal preparation area (Figure 2.5).

Two microliters of the volatile solution was injected into a the GC running the following program : 2 minutes at 50°C followed by an increase of 10°C/min to 250°C and then held for 5 minutes. Injection was onto the polar column for all except the blackcurrant SPME EAG, and splitless at 220°C and helium was used as the carrier gas (2.4 ml/min).

Two separate delivery systems were used for delivering volatiles to the antennal preparation. Volatiles were either delivered in a constant stream of humidified air (200 ml/min) or in puffs. The puff system increased the concentration of compounds delivered to the antenna. Under this system volatiles were collected in a reservoir for 17 seconds and released over the preparation for 3 seconds with air (200 ml/min), this was repeated throughout the run. During the 17 second collection period humidified clean air was passed over the preparation to prevent drying.

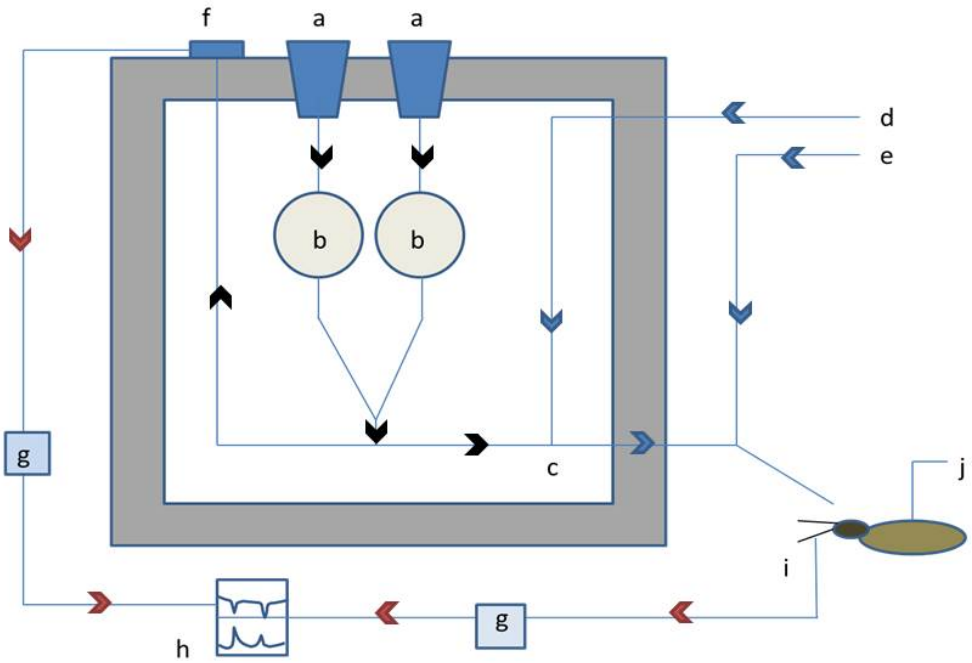


Figure 2.5. Diagram of the GC-EAG set up ((a) split/splitless injectors; (b) columns; (c) GC column outlet to EAG preparation; (d) inlet for pulsed 3 second air (when pulsed method not used then this was constant all the time and humidified); (e) inlet for constant 17 sec humidified air flow (not used when pulsed flow not present); (f) GC column outlet to the flame ionisation detector (FID); (g) amplifiers; (h) computer I recording FID and EAG data; (i) recording electrode; (j) reference electrode). Direction of airflow (blue), electrical signals (red) and chemical compounds (black) can be seen.

Antennal responses were recorded using a portable recording unit (INR-2, Syntech, The Netherlands) which consisted of electrode holders and an amplifier. Silver electrodes were placed in the electrode holders.

The insect was first sedated with carbon dioxide and the wings and legs removed so that their movement could not interfere with the electrical signal. The body was placed into a freshly pulled capillary tube filled with 0.1M KCL solution with 1% polyvinylpyrrolidine (PVP) (to reduce evaporation) and mounted on the reference

silver electrode of the EAG system. One of the antennae was put into an electrolyte filled capillary tube mounted on the recording silver electrode (Figure 2.6).

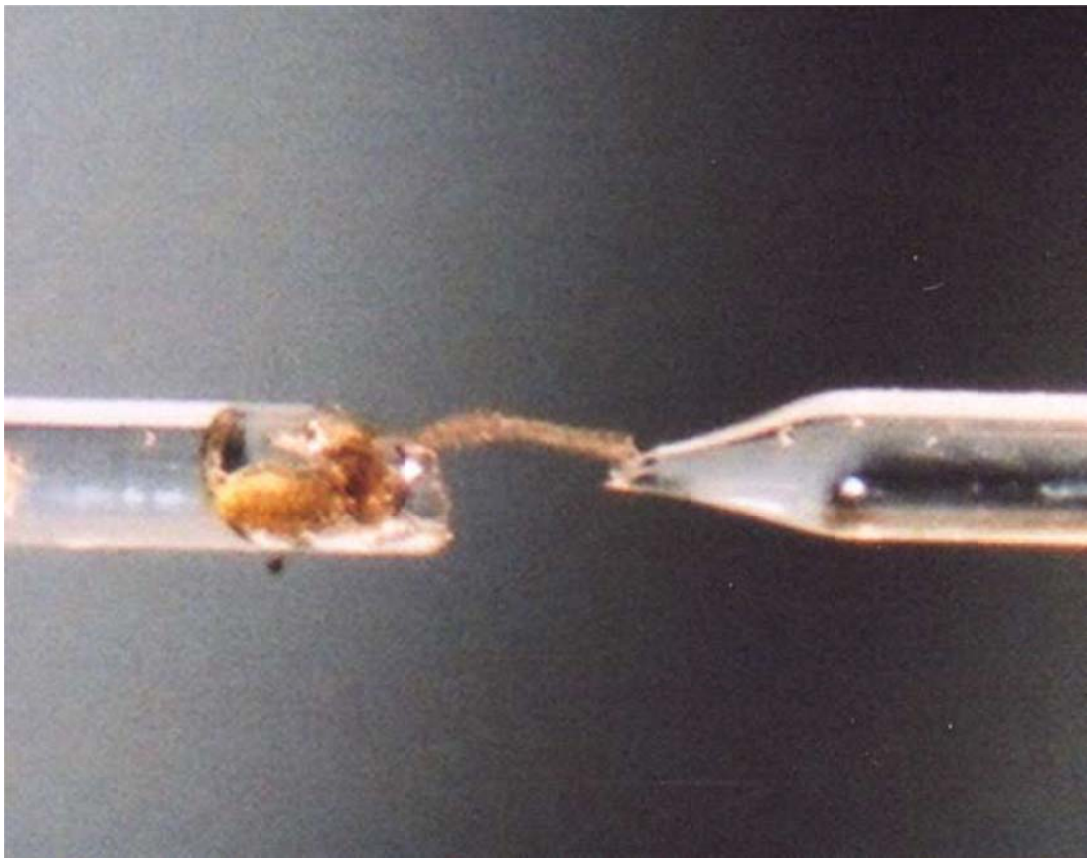


Figure 2.6 An example of a midge (in this case male *Resseliella theobaldi*) with body inserted into the reference electrode and antennal tip into the recording electrode.

The signals were captured and analysed using EZChrom software (Agilent).

n-Alkane standards were also injected into the GC-EAG system at the beginning of each day to allow the calculation of Kovats Retention Indices for the peaks on the GC. The Kovats Indices could be compared with those identified for peaks on the GC-MS and in databases such as the Pherobase (El Sayed 2012).

Chapter 3
IDENTIFICATION OF VOLATILES FROM RASPBERRY CANES

3.1. INTRODUCTION

The first gall midge species to be focussed on was the raspberry cane midge, *Resseliella theobaldi* Barnes. Females of this species lay their eggs in split raspberry canes, *Rubus idaeus* (Linnaeus). Investigation into the volatiles produced by split and intact canes was carried out by Hall et al. (2011) and it was found that six volatiles were produced by intact canes and 12 by split canes. Four of these chemicals were found to give electroantennographic (EAG) responses with three of the four being found only in split canes. Field trapping tests with blends of these EAG active chemicals were carried out, but results were inconclusive

This chapter focuses on volatile collection work and follows the method of Hall et al. (2011) to study the differences in the volatile profiles of canes of two raspberry varieties before and after they are split. Once these chemicals have been identified further work can be undertaken using EAG, behavioural bioassays and field testing to identify attractive chemicals and blends.

3.1.1 Selection of method for volatile collection

There are several systems available for the capture of volatiles emitted by plants. These include entrainment were the plant is enclosed in a vessel and air is pumped in over the plant material and sucked out through an adsorbent material. The volatiles are then eluted by passing a solvent through the adsorbent. This method required a collection time of several hours to build up a sufficient quantity of volatiles but produced a solution which can be used for further experiments.

Volatiles can also be captured onto a solid phase microextraction (SPME) fibre. The fibre is exposed near the plant material and volatiles are rapidly adsorbed onto the fibre's surface. The fibre can then be directly injected into a Gas Chromatographe (GC) for analysis. This method does not give a solution for

further analysis but does give a measure of the chemicals present rapidly (Prosen and Zupančič-Kralj, 1999). SPME was chosen as an appropriate method for the collection of the short-lived burst of volatiles produced when canes split, with a collection time of fifteen minutes

3.2. MATERIALS AND METHODS

3.2.1 Plants

Raspberry plants of the varieties Octavia and Glen Ample were purchased in summer 2011. Some were left out on the sandbeds at EMR over the winter while others were transferred in November 2011 to smaller 2-litre pots and put into heated glass houses. Those plants which overwintered under glass produced primocanes early in spring 2012 allowing volatile collection to begin.

3.2.2 Collection of Volatiles

Plants with fresh green primocane were selected and taken to the laboratory at NRI. Solid phase microextraction collections were made using the method detailed in Chapter 2. Collections were made from the intact stem and immediately after making a slit in the epidermis (3 cm) with a razor blade.

3.2.3 Analysis

Once the collection was complete the fibre was inserted into the injector of the Agilent GC-MS with non-polar column as detailed in Chapter 2. A table of the common plant volatiles and their structures can be found in the appendix at the end of the thesis.

3.3. RESULTS

3.3.1 Volatiles from raspberry primocanes

Collections were made from nine plants before and after artificial splitting, including plants from both the varieties Glen Ample and Octavia. Chemicals identified are listed in Table 3.1.

To identify differences between the cane before and after splitting it was possible to plot the two GC traces against each other and compare the peaks which are present in the two traces at the same time (Figure 3.1). In this example from Octavia, six compounds were observed to be greatly enhanced in the volatiles collected after splitting - linalool, citronellal, myrtenal, citronellol, neral and geranial.

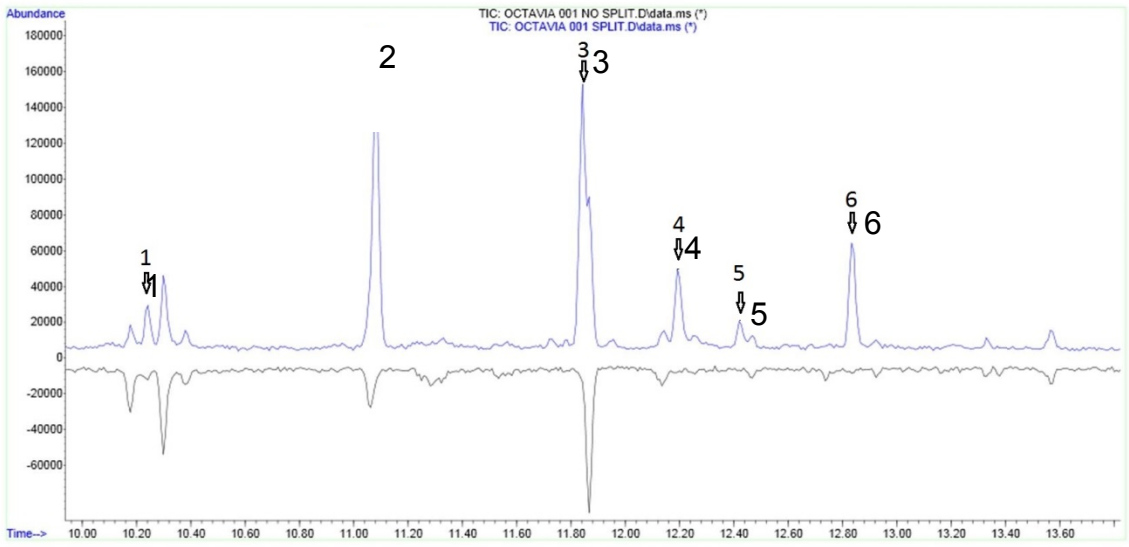
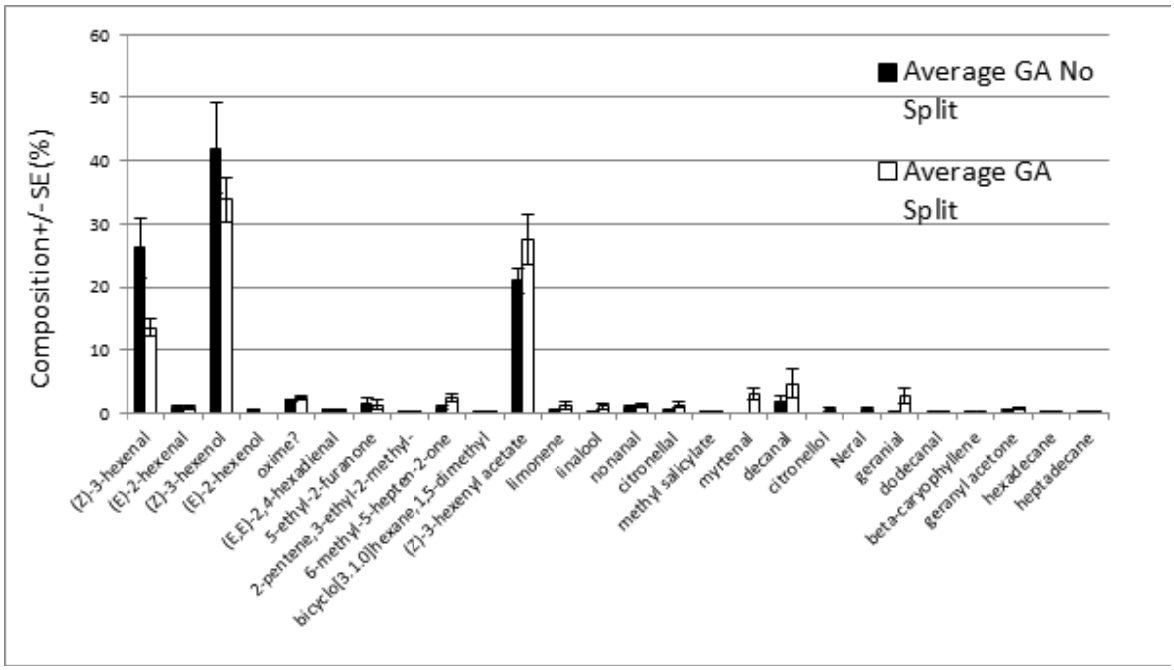


Figure 3.1. GC-MS traces for SPME collections from a single Octavia raspberry plant primocane before (bottom) and after (top) a split was made (1 linalool, 2 citronellal, 3 myrtenal, 4 citronellol, 5 neral, 6 geranial).

Table 3.1. Chemicals identified in solid phase microextraction (SPME) collections made from intact and split raspberry canes. RT = retention time in minutes; KI = Kovats Indicex; Pherobase = Kovats Indices from Pherobase (El-Sayed, 2012)

RT (min)	KI	Compound	Pherobase
4.95	784	(*Z*)-3-hexenal	784
5.94	844	(*E*)-2-hexenal	845
6.02	848	(*Z*)-3-hexenol	857
6.18	858	(*E*)-2-hexenol	861
6.78	895	oxime?	?
6.96	905	(*E,E*)-2,4-hexadienal	909
7.80	954	5-ethyl-2-furanone	984
8.04	968	2-pentene,3-ethyl-2-methyl-	?
8.34	986	6-methyl-5-hepten-2-one	985
8.44	992	bicyclo[3.1.0]hexane,1,5-dimethyl	?
8.68	1006	(*Z*)-3-hexenyl acetate	1007
9.16	1036	limonene	1036
10.24	1104	linalool	1107
10.30	1108	nonanal	1108
11.08	1156	citronellal	1153
11.79	1201	methyl salicylate	1206
11.85	1205	myrtenal	1193
11.87	1206	decanal	1204
12.19	1229	citronellol	1228
12.40	1235	neral	1235
12.83	1275	geranial	1270
14.71	1411	dodecanal	1413
15.06	1440	beta-caryophyllene	1428
15.29	1458	geranyl acetone	1453
17.05	1600	hexadecane	1600
18.18	1701	heptadecane	1700



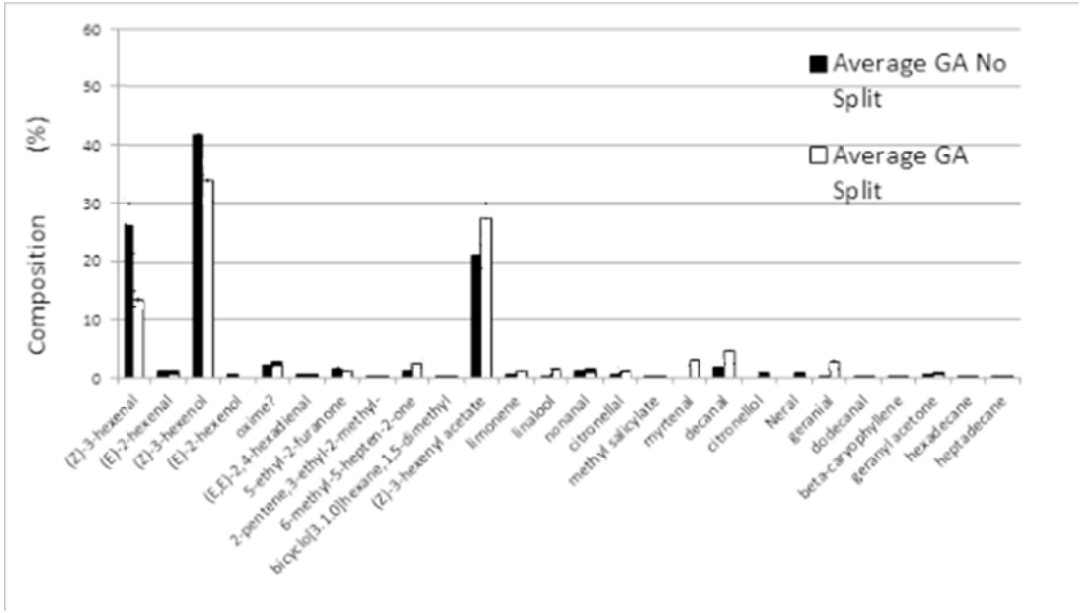


Figure 3.2. Comparison of volatiles produced before and after an artificial split was made in a Glen Ample raspberry, *Rubus ideaus,* cane in terms of percentage of each chemical in the total volatile emission (*N* = 5).

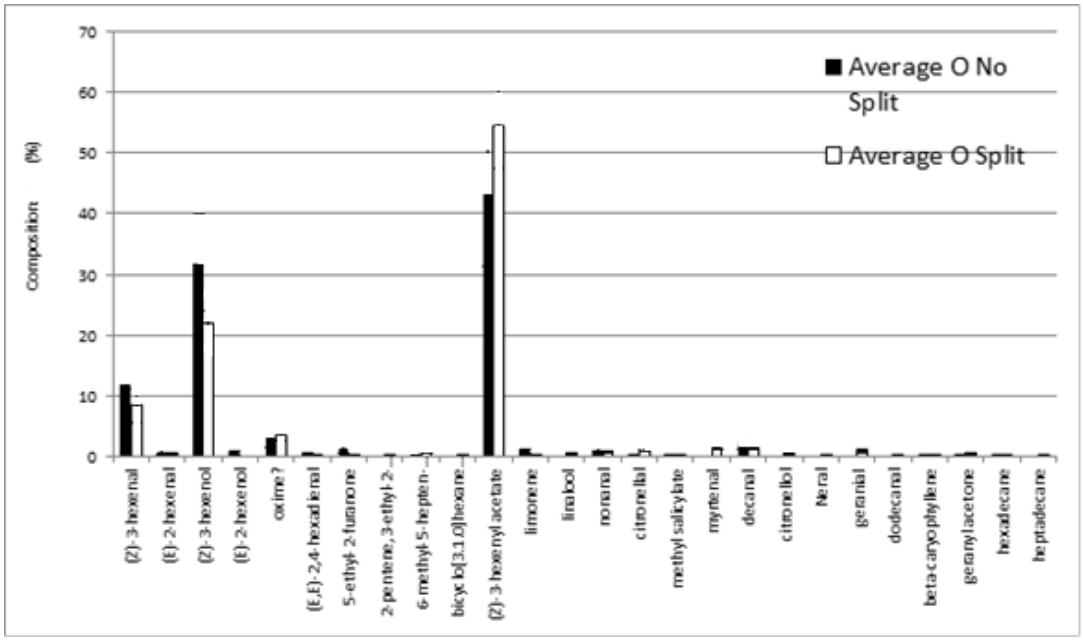


Figure 3.3 Comparison of volatiles produced before and after an artificial split was made in an Octavia raspberry, *Rubus ideaus,* cane in terms of percentage of each chemical in the total volatile emission (*N* = 4; cf. Figure 3.1).

Peak areas are measured in abundance (a measure of the total ions present in the mass spectrometer at a particular moment), this gives a measure of the amount of the different compounds in the sample. These values were converted to percentages of the total blend and also quantified relative to the peak area of an internal standard, decyl acetate. The average percentage composition of the volatiles, before and after splitting are shown for Glen Ample (Figure 3.2) and Octavia (Figure 3.3).

The major components in these collections were the “green leaf volatiles”, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate. However, in both varieties six compounds were either present only after splitting of the stem or their relative proportions were greatly increased – linalool, citronellal, myrtenal, citronellol, neral and geranial. Interestingly, 6-methyl-5-hepten-2-one and geranyl acetone, compounds often emitted by plants after wounding, were present both before and

after splitting.

These patterns can be seen more clearly when the three most abundant chemicals (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate are removed from the graphs (Figures 3.4 and 3.5).

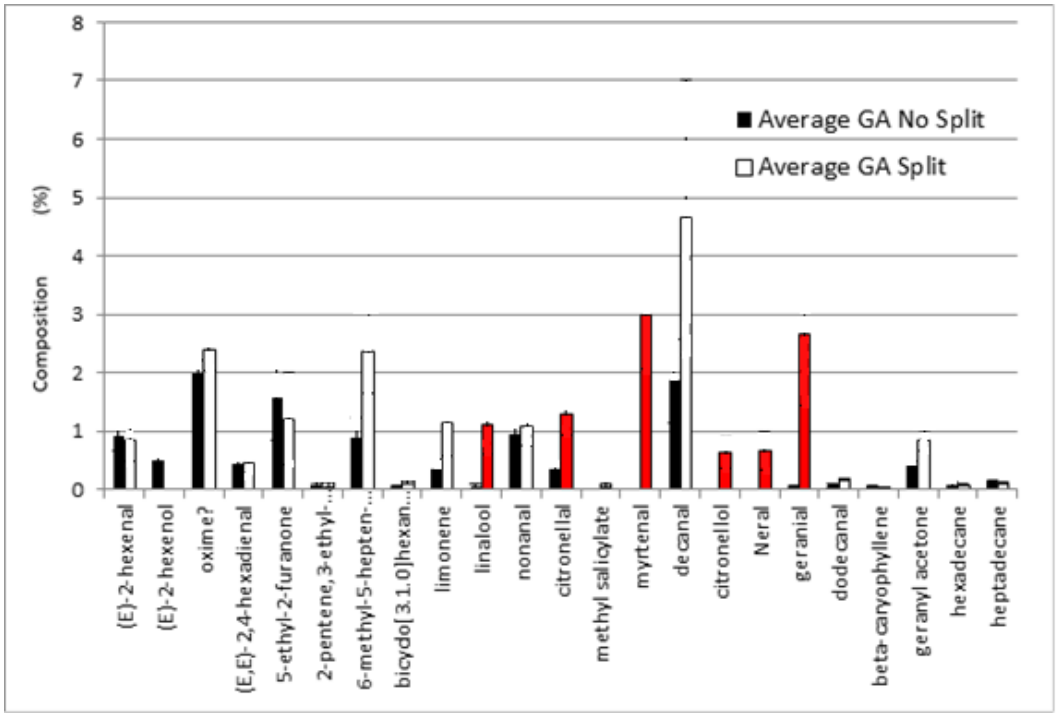


Figure 3.4 - Comparison of volatiles produced before and after an artificial split was made in a Glen Ample raspberry (*Rubus idaeus*) cane in terms of percentage of each chemical in the total volatile emission (N = 5) with the three largest peaks removed. The bars highlighted in red are those compounds present only or in higher quantities after splitting.

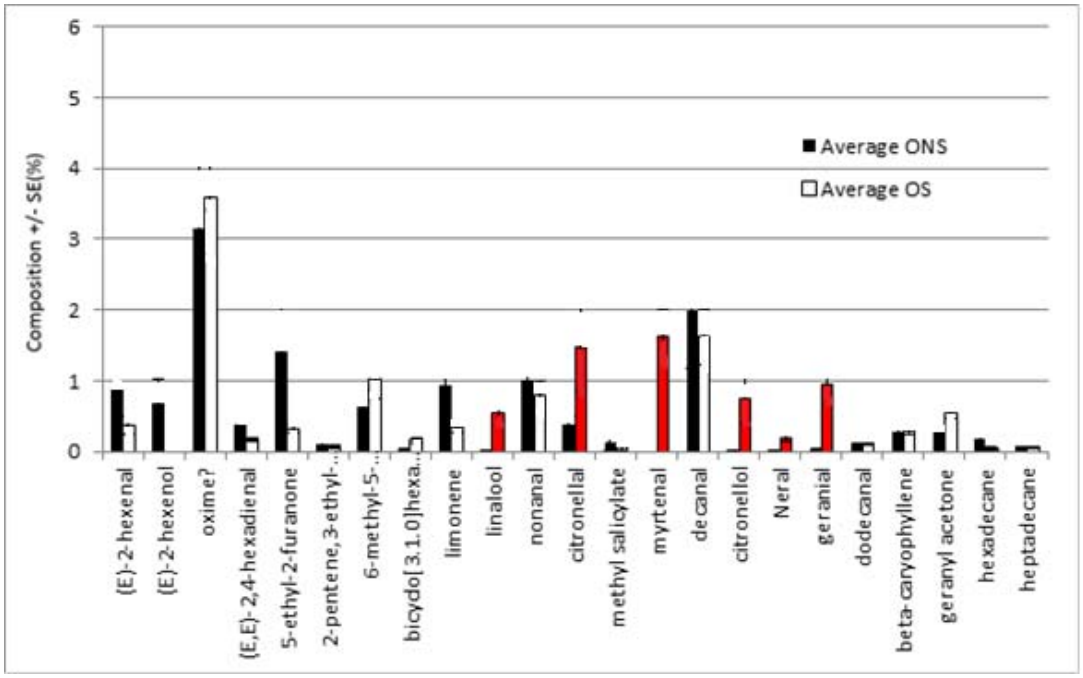


Figure 3.5- Comparison of volatiles produced before and after an artificial split was made in an Octavia raspberry (*Rubus idaeus*) cane in terms of percentage of each chemical in the total volatile emission (N = 4; cf. Fig. 3.1) with the three largest peaks removed. The bars highlighted in red are those chemicals present only or in higher quantities after the canes were split.

To assess for significant differences between the amount of chemicals released by split and non-split canes the data was transformed using a log (abundance+1) transform and subjected to an Analysis of Variance. It was found that for both varieties the presence of a split gave a significant difference in chemical profile with a probability below 0.01. Due to the large number of possible combinations it was not possible to carry out an ad-hoc test to compare compounds before and after splitting. However the graphs produced using the log abundance transform give a good indication of which chemicals increased after splitting with those pairs of bars in which the error bars do not overlap indicating significant differences (figures 3.6 and 3.7).

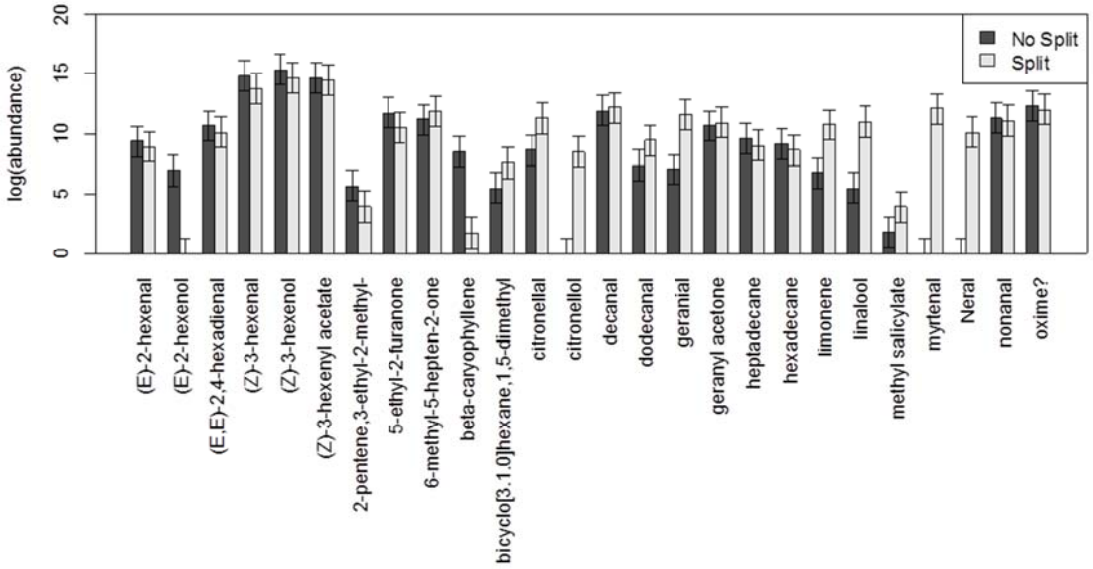


Figure 3.6 – Graph of average log abundance for the different compounds collected before and after splitting in Glen Ample (n=5). Compounds with significant increases after splitting are citronellal, citronellol, geranial, limonene, linalool, myrtenal and neral. It is interesting to note that using the log transform limonene now shows an increase after splitting.

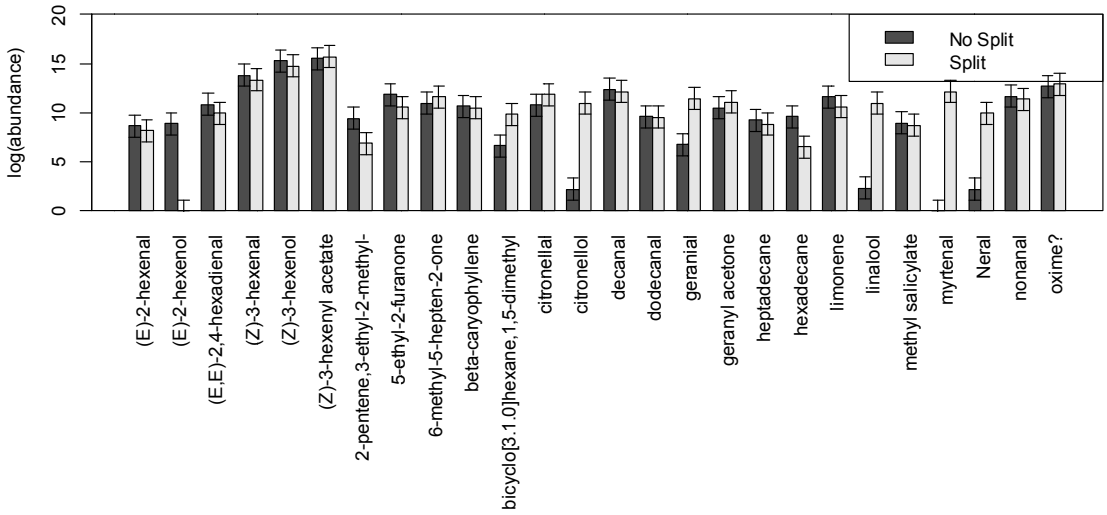


Figure 3.7. Graph of average log abundance for the different compounds collected before and after splitting Octavia (n=4). Compounds with significant increases after splitting are bicyclo(3.1.0) hexane,1,5,dimethyl, citronellol, geranial, linalool, myrtenal and neral.

3.3.2 Cluster analysis of volatile data

In order to determine if there were statistically significant differences between the species and intact and split canes the data was organised and subjected to a cluster analysis in the R statistical package. The cluster analysis looks at traits (in this case the amount of the different compounds present in each sample) and groups samples into groups accordingly. Figure 3.6 shows the cluster analysis output graphically.

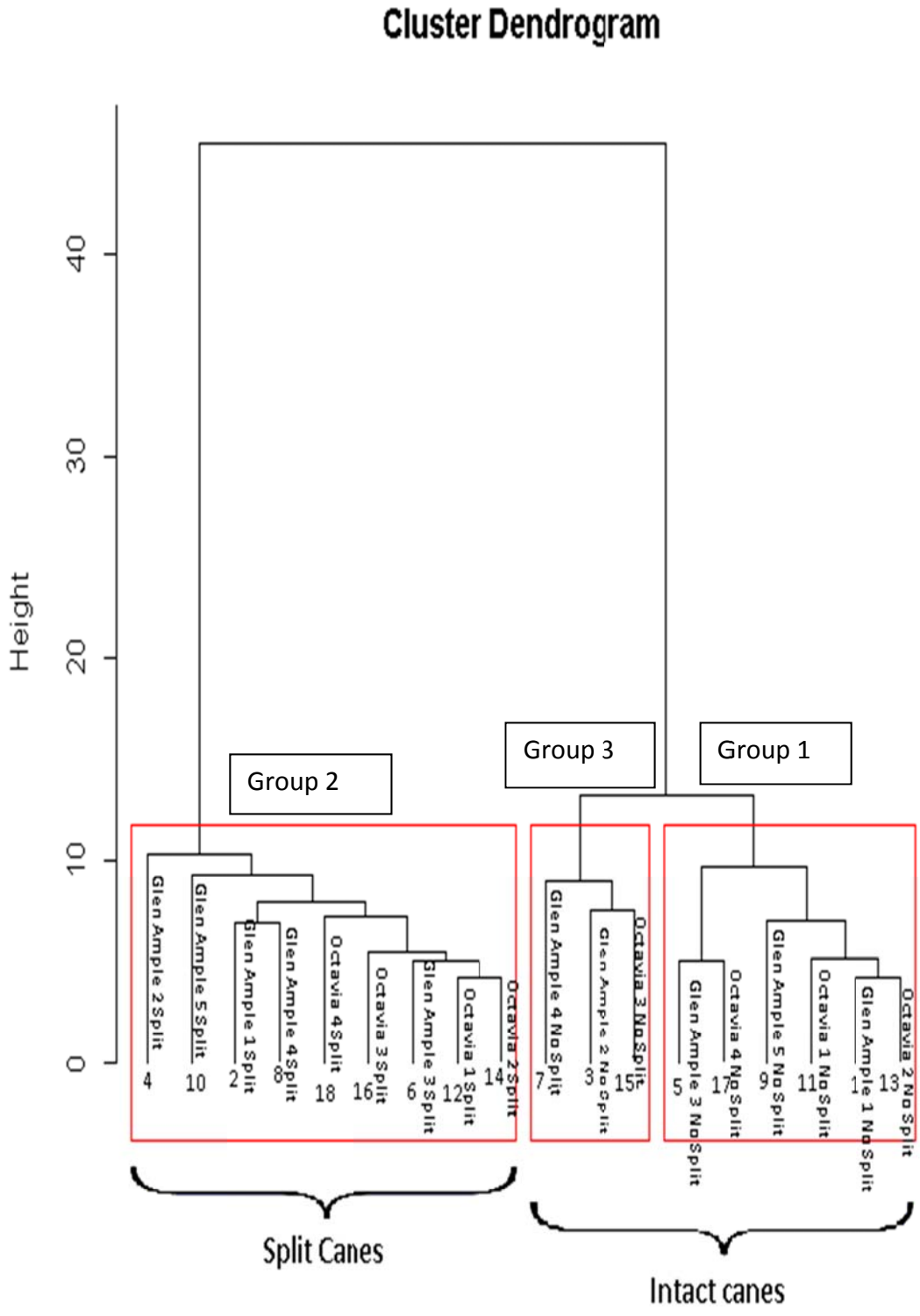


Figure 3.6. Graphical representation of the cluster analysis results for the different volatile collections obtained using the R statistical package. The split and intact canes were clearly split into separate groups. The height is a measure of how far the criterion of the model need to be relaxed for the cluster members to be in the same group. The dendrogram was cut to give a sensible number of clusters.

The analysis grouped the data into three groups. There is a clear division between group 2 which includes all the split canes and groups 1 and 3 which contain all the intact canes. Groups 1 and 3 both contain a mix of the two raspberry varieties. Differences in volatiles between the three groups can be seen in the graphs produced as part of the cluster analysis output (Figure 3.7).

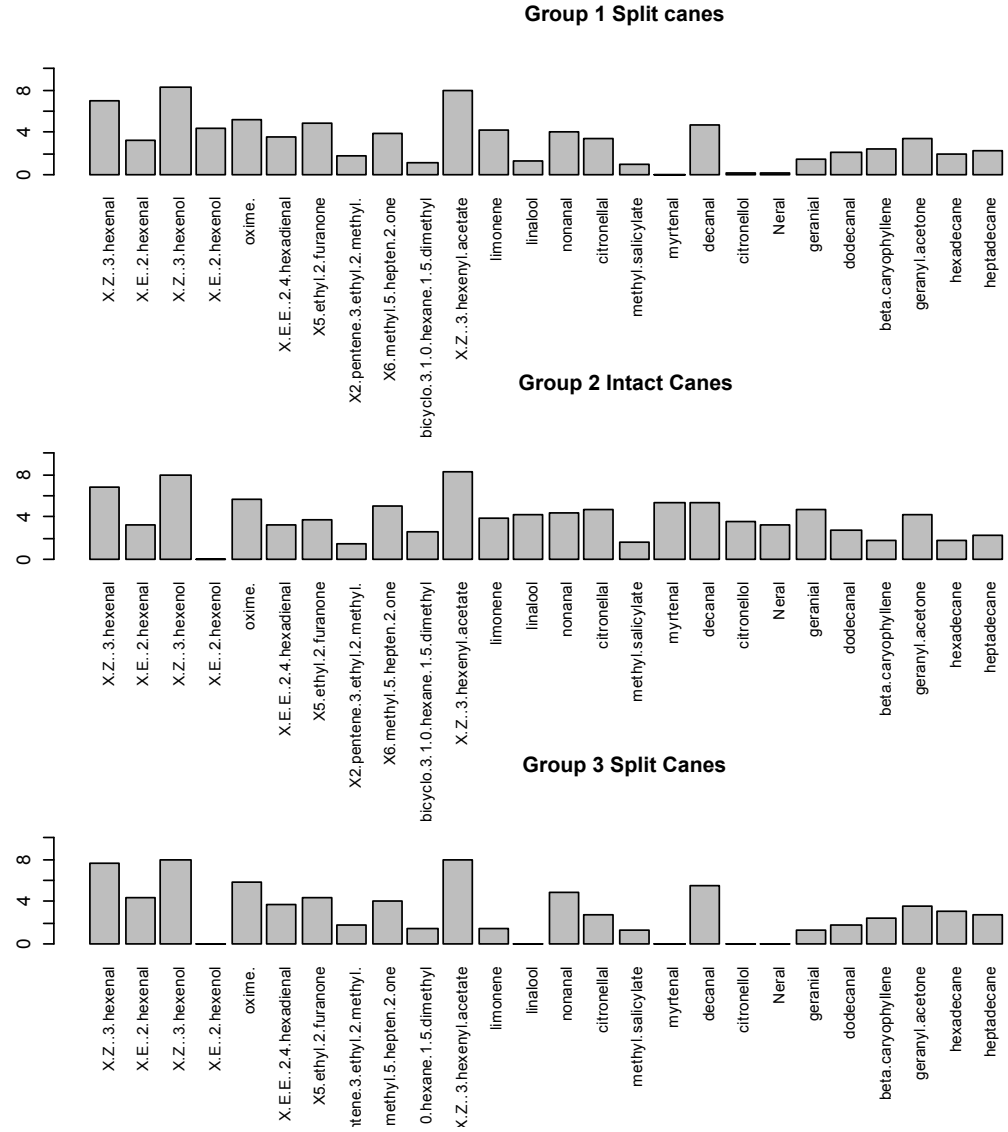


Fig 3.7 Graphs produced by R statistical package showing the differences in amounts and presence of chemicals between the 3 groups of volatile samples identified using the cluster analysis. The units give a measure of average volatile compositions and are on a log scale.

From the graphs in Figure 3.7 linalool, myrtenal, citronellol, neral and geranial are the main chemicals which have higher amounts in Group 2 compared to the other

two groups. This backs up the findings when the plants before and after splitting were compared in Figures 2.6 and 2.7.

To confirm that the difference in grouping was statistically significant a Fisher test was carried out in R on the relationship between membership of Group 2 and presence of a split. The test yielded a *P* value of less than 0.001, which is significant meaning that the results are not random and there is a relationship between membership of Group 2 and weather the cane is split.

A table of chemicals present when a clean SPME fibre was run was made. Most of the chemicals were present in very small amounts during the raspberry volatile collection and were not affected by presence of splits. These chemicals were eliminated from further analysis and are not present in Table 3.1. The only exception to this is was the peak identified as oxime (identified on the NIST library as “Oxime-, methoxy-phenyl-”). This compound is not found on the Pherobase (El-Sayed, 2012) and is not known to be plant produced.

3.4. DISCUSSION AND CONCLUSIONS

3.4.1 Volatiles from raspberry primocanes

SPME was found to be a convenient means to sample the rapid burst of chemicals produced when raspberry primocanes are split. Sampling before and after splitting showed that in both Octavia and Glen Ample varieties six compounds were either present only after splitting of the stem or their relative proportions were greatly increased – linalool, myrtenal, citronellol, neral and geranial. These chemical differences before and after splitting were great enough to lead to a clear separation of split and non-split canes which was statistically significant at the 0.05 level. The six compounds were also reported by Hall et al. (2011) for the Glen Moy variety. Interestingly, 6-methyl-5-hepten-2-one and geranyl acetone, compounds often emitted by plants after wounding, were present both before and after splitting.

The inclusion of a blank control allowed chemicals coming from the volatile sampling apparatus to be excluded from analysis. This included the oxime

compound which was previously included as it was present in significant amounts and seemed to be slightly higher after the canes were split. This slight increase is still unexplained but the fact that it is not known as a plant volatile and is present even when plants are not, suggests it may not be as important as the other plant chemicals identified.Most of the compounds identified here as produced by split canes are characteristic plant damage volatiles. These same volatiles are seen to be produced by a range of different plants suggesting that a common set of biosynthetic pathways is found in a wide variety of plant families (Paré and Tumlinson, 1999). These volatiles are often released in response to herbivore damage and can be divided into the terpenes such as linalool and the green leaf volatiles such as hexenyl acetate. These volatiles can play a role in plant defence by repelling herbivores; for example the bird cherry-oat aphid (*Rhopalosiphum padi* (L.)) repellent odours released by wheat (Triticum spp. (L.)) seedlings with high aphid infestations (Quiroz et al., 1997). In addition volatiles can also attract herbivore predators and parasites. Green leaf volatiles can help prime the defences of surrounding conspecifics to herbivore attack (Arimura et al., 2009). In some cases C6 aldehyde green leaf volatiles are responsible for resistance to fungal pathogens; for example they were found to mediate the resistance of transgenic *Arabidopsis thaliana* ((L.) Heynh) to the pathogen *Botrytis cinerea* ((De Bary) Whetzel) (Shiojiri et al, 2006). The volatiles do, however, in many cases act in a way which is detrimental to the plant by attracting herbivores and insects looking to lay eggs such as the raspberry cane midge.

Chapter 4
FIELD OBSERVATIONS AND TRAPPING EXPERIMENTS ON RASPBERRY CANE MIDGE

4.1. INTRODUCTION

In Chapter 3 a suite of volatiles produced only or in larger quantities after raspberry canes were split was identified. These chemicals were also identified along with others by Hall et al. (2011) and an artificial blend created which was tested in the field for attraction of raspberry cane midge, *Resseliella theobaldi* Barnes, in the UK and Hungary with inconclusive results. In this Chapter a field experiment was carried out using the same blend used in the field work of Hall et al. (2011) which included most of the chemicals identified in Chapter 3. A range of trap types (some novel) were used and both natural split canes and empty traps used as controls The wider range of trap types and the inclusion of a positive (cane) control were not present in the work of Hall et al. (2011) and are thus novel research.

Although following on from the work of Chapter 3 in this thesis, the work in this chapter was in fact carried out in summer 2011

4.2. MATERIALS AND METHODS

4.2.1 Initial field observations

Once *R. theobaldi* began to emerge in April 2011 preliminary observations were carried out in plantations at Mockbeggar Farm, Higham, and East Malling Research Station, both in Kent. Red delta traps (Agralan, Swindon, SN6 6QR, UK) baited with synthetic sex pheromone lures were hung in an open ended polytunnel at Mockbeggar (raspberry variety Tulameen) and in plots of variety Glen Ample (Figure 4.1), at EMR. The sticky bases were monitored twice a week and once the numbers in the field were more than 10 midges on the sticky base, artificial split observations were begun.

Splits (approx 10 cm long) were made with a mounted needle in growing primocane and observed to see if female *R. theobaldi* were attracted. In some of the later observations a pooter was used to collect the midges visiting the splits, these midges were taken in pots to the lab and examined under a microscope to confirm the species and the sex. Each observation period lasted 30 minutes.

Figure 4.1. Raspberry (*Rubus Idaeus*) field (WE 195) at EMR where field observations were carried out.

4.2.2 Trapping experiment

Location

The experimental work was carried out in cv. Maravilla potted primocane raspberries at Court Lodge Farm, Kenward Road, Yalding, Kent. The site is farmed by Clockhouse Farms Ltd, Linton, owner Robert Pascall. The total study

area measured 85 m x 228.8 m and includes a total of 30 rows of raspberry cane under polytunel (Figure 4.2).

Figure 4.2. Aerial photograph of Court Lodge Farm, Yalding, Kent with study area (polytunnels of Maravilla raspberry, *Rubus idaeus,* marked in red).

Treatments

Four different trap designs (Figure 4.3) were compared.

1. White delta trap (20 cm x 27 cm x 11 cm high) with sticky base (18.5 cm x 18.5 cm). (Agralan, Swindon, SN6 6QR, UK)
2. Horizontal white water trap constructed from a plant saucer (24 cm diameter x 4 cm deep) with an upright section of plastic pipe (2 cm diameter, 5 cm high) glued in the centre to support the cane sections.
3. Horizontal sticky trap consisting of a piece of red corrugated plastic (20 cm x 20 cm) with a sticky base (18.5 cm x18.5 cm) clipped to the upper surface using bulldog clips. A nail was pushed through from the underside for mounting the cane.
4. Vertical sticky trap made from a piece of corrugated plastic (19 cm x 11 cm) with aperture (2.5 cm x 12 cm) cut into the centre. A sticky base was cut into four strips and one strip attached with bulldog clips to either side of the aperture on both the front and back of the trap. A loop of wire was pushed down through the top of the trap to make legs which could be pushed into the soil to support the trap. Nails were pushed up through the bottom and down through the top of the trap to hold the cane sections.

The size of the catching surface was the same in the vertical, horizontal and delta traps (18.5 cm x 18.5 cm sticky base) and the water trap size was chosen to be as close to these dimensions as possible.

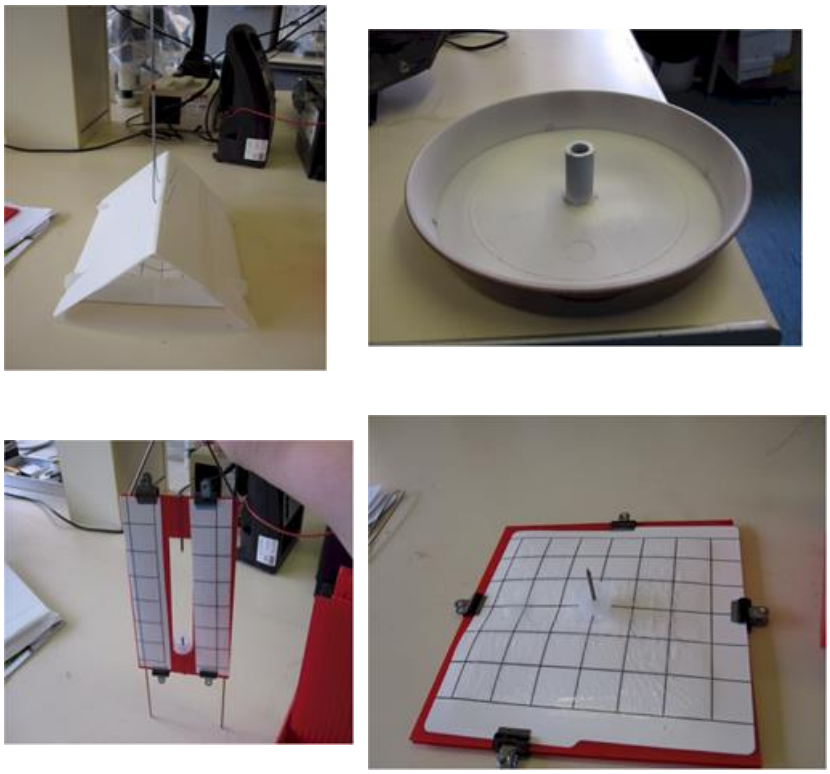


Figure 4.3. The trap designs (clockwise from top): white delta trap, water trap, horizontal sticky trap, vertical sticky trap.

Traps were un-baited or baited with one of two different lures. The “natural” lure was an excised cane section (12 cm) with a split (10 cm long) made in the epidermis using a razor blade. The “synthetic” lure was that developed by Hall et al. (2011) containing a blend of 9 compounds in a sealed polyethylene sachet (5 cm x 5 cm x 120 µ thick) and 4 compounds in a polyethylene vial (22 mm x 8 mm x 1 mm thick) (Table 1). The vial was placed inside the sachet. The lures were secured in the centre of the traps.

Table 4.1 Components of the synthetic lure used in the field experiment – there were equal amounts of each compound and 100µl of each mix in the sachet and vial. The compounds highlighted in bold were found in higher quantities or were only present when canes were split in the SPME work in Chapter 3.

Vial	**Sachet**
(*Z*)-3-Hexenyl acetate	6-Methyl-5-hepten-2-ol
6-Methyl-5-hepten-2-one	Decanal
(*Z*)-3-Hexenol	**Linalool**
Benzyl alcohol	**Myrtenal**
	Citral (50% **Neral**, 50% **Geranial**)
	Citronellol
	Myrtenol
	Geraniol
	Geranyl acetone

The synthetic lures were suspended in the delta and vertical traps using a paperclip. A section of old dried out cane was used as a support for the synthetic sachets in the horizontal sticky and water traps. The synthetic volatile sachet was attached to the top of the dry cane using a nail. Sections of primocane with the base cut at an angle of 45° were wedged into the white delta traps and secured with a nail (Figure 4.4).

Figure 4.4. Sachet suspended on dry cane in water trap (left) and raspberry primocane wedged in delta trap (right).

4.2.3 Experimental design

A randomized complete block design was used with four blocks. The plots were a single trap, and each block ran the full length of the study area (228.8 m or 104 metal poly tunnel hoops). Block one was located in the third row, block two in row eleven, block three in row nineteen and block four in row twenty seven. The traps were laid out along the row at a distance of eight hoops (18 m) apart. Rows were 2.8m apart.

In addition two red delta traps baited with pheromone lures and located near to but not in the study area were checked daily to gauge the male raspberry cane midge population. Also splits were made in 10 canes close to each of the pheromone traps. These splits were marked with coloured tape and checked for eggs the day after being made, although this was abandoned after two days as no eggs were found.

Each day the excised cane sections were changed and water in the water traps topped up. Counts of the numbers of *R. theobaldi* males and females were made every three days. Sticky card inserts and trap water were changed after each count had been made. Sticky card inserts were stored in small pizza boxes until the number of midges and other insects had been counted.

The insects caught in the traps were sorted into three groups: male *R. theobaldi*, female *R. theobaldi* and by-catch which included all insects which were not *R. theobaldi*, including midges of other species.

The data were analyzed using ANOVA in Genstat (VSN International) with trap design and presence/absence of lure or cane section as treatment factors.

During the study two data loggers were put out in the centre of the study area inside a Stevenson screen. This allowed the daily temperature and humidity to be taken into account when analysing the data.

4.3. RESULTS

4.3.1 Initial field observations

During the field observations at the farm and research station in Kent it was seen that *R. theobaldi* were attracted to the splits within 20 min (Table 4.2). Once the midges arrived they walked the length of the split before inserting their ovipositors under the primocane skin, presumably to lay eggs, before flying off.

Table 4.2. Summary of data collected from observing arrival of *Resseliella theobaldi* at artificial splits. Observations = the number of 30 minute observation periods carried out. *R. theobaldi* seen = the total number of sightings of raspberry cane midge visiting artificially split canes in each plot. Females = the total number of females caught in a pooter and identified and Males = the total number of males caught and identified. These last numbers are lower than the total number of midge seen as not all the midges observed were caught and it was difficult to judge sex without the use of a microscope.

		R. theobaldi		
Location	Observations	Seen	Females	Males
Mockbeggar Farm	8	6	0	1
EMR – Plot WE195	18	8	3	0

The observational work led to the baiting of a red delta trap in the Mockbeggar Farm polytunnel with a section of excised split cane. This was left for seven days after which time 94 midges had been caught, all of which were male *R. theobaldi*. This finding suggests that males are also attracted to splits in primocane, possibly looking to find females.

These initial observations also led to investigation into different types of trap which could catch insects visiting a split, living cane. A delta trap with an aperture cut in the top and bottom to allow the cane to pass through was tried, but it was found that the delta trap was too heavy for the cane to support. A trap was also created using the central section of a 2-litre drinks bottle with sticky paper attached to the inside surface. This trap did not catch many insects at all so was abandoned.

4.3.2 Trapping experiment

Table 4.3 shows the mean number of males, females and by-catch caught in each of the two weeks of the experiment. Combined mean catches of males and by-catch for the two weeks are shown in Figures 4.5 and 4.6.

Table 4.3. Mean numbers of male and female *Resseliella theobaldi* and by-catch insects caught in four trap designs (D delta trap; W water trap; H horizontal sticky trap; V vertical sticky trap) with three lures over two successive weeks. In week one there were a total of 1,341 males, 0 females and 2,093 by-catch insects caught over all the traps. In week two there were a total of 1,414 males, 16 females and 5,915 by-catch insects caught over all the traps.

	Week 1				**Week 2**			
Lure	**D**	**W**	**H**	**V**	**D**	**W**	**H**	**V**
Females								
Synthetic	0.0	0.0	0.0	0.0	0.75	0.00	0.00	0.00
Cane	0.0	0.0	0.0	0.0	1.00	0.00	0.00	0.25
No Bait	0.0	0.0	0.0	0.0	0.75	0.25	0.50	0.50
Males								
Synthetic	45.3	0.0	32.2	41.5	30.0	0.2	18.7	77.8
Cane	13.3	0.0	35.3	57.5	14.2	0.5	12.7	87.8
No Bait	18.5	0.0	78.4	46.8	32.5	0.0	16.2	62.8
By-catch								
Synthetic	68.5	5.0	105.2	48.8	275.0	35.5	164.8	168.5
Cane	22.5	5.7	72.1	70.0	22.0	84.8	161.5	173.5
No Bait	16.2	3.7	73.1	70.8	31.0	48.8	158.2	155.2

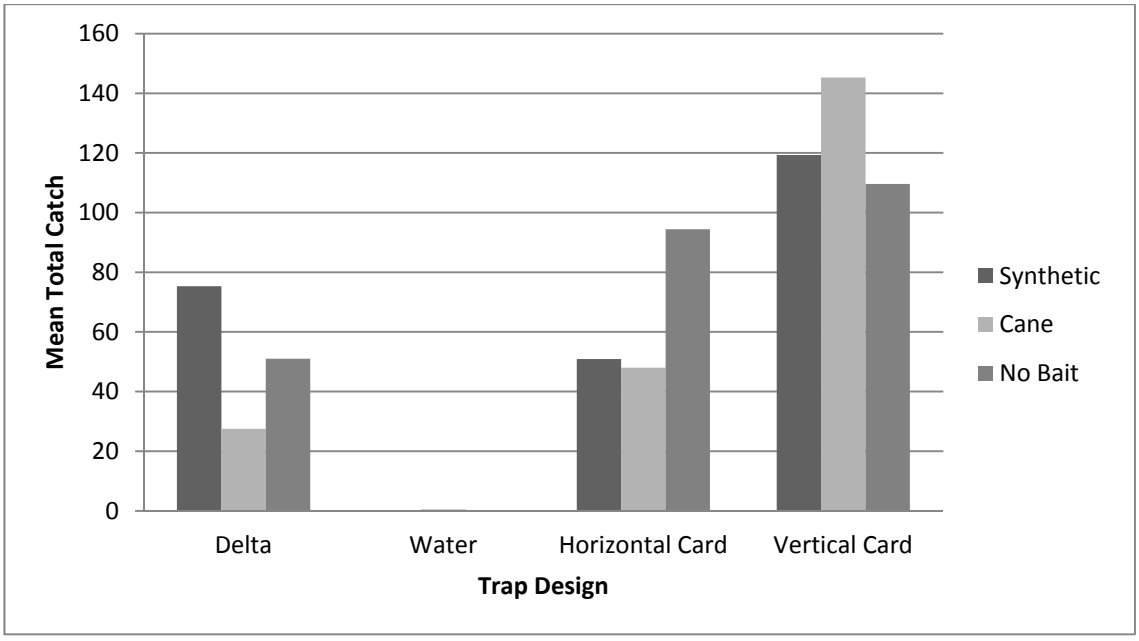


Figure 4.5. Mean catches of male *Resseliella theobaldi* in different trap designs baited with synthetic lures or a split raspberry cane (Mean catches over 2 weeks summed, 8-22 July 2011).

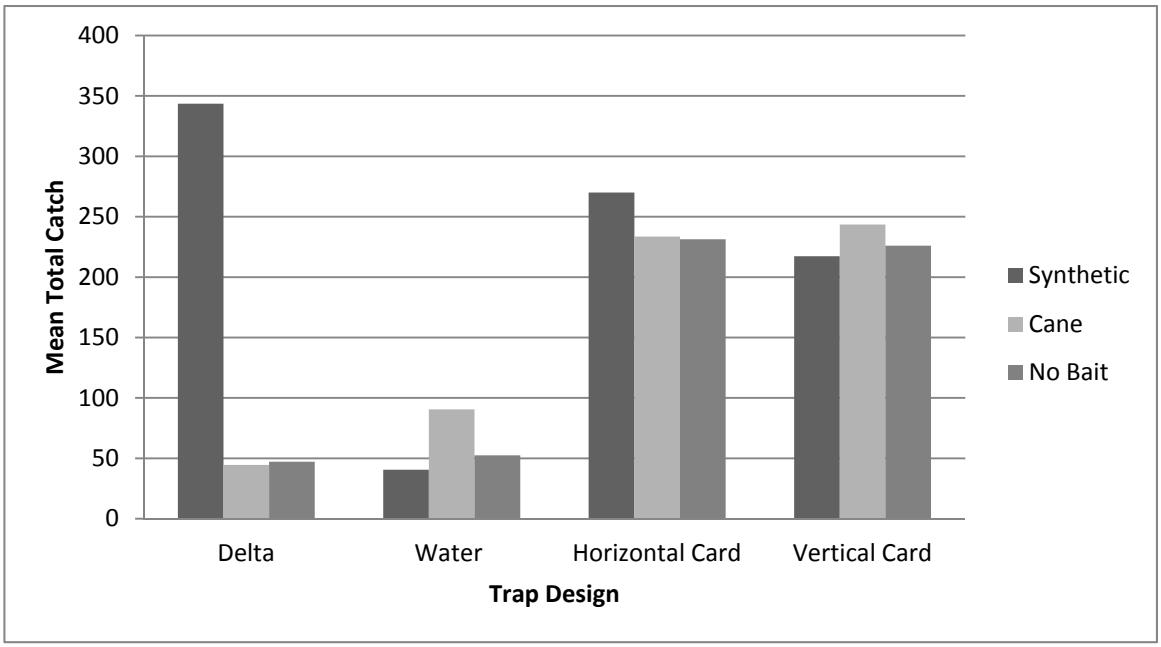


Figure 4.6. Mean by-catch (all insects which are not *Resseliella theobaldi* caught in traps in different trap designs baited with synthetic lures or a split raspberry cane summed over 2 weeks (8-22 July 2011).

An analysis of deviance was carried out based on a negative binomial generalised linear model to look for relationships within the data. The analysis yielded the

following *P*-Values (Table 4.4) indicating the significance of trap and lure type on the number of males, females and by-catch over the two week period.

Table 4.4. *P*-Values after analysis of devience was carried out based on a negative binomial generalised linear model on trap catch data (males and females are *Resseliella theobaldi* and by-catch are all other insects).

Variable	Males	Females	By-catch
Lure	0.416	0.249	0.017
Trap	<0.001	0.045	<0.001
Lure and trap	0.097	0.446	<0.001

From Table 4.3 it can be seen that the numbers of female *R. theobaldi* caught were low with all trap and lure combinations. There was a slightly significant effect of trap type on the number of females caught. However, as the numbers of females caught were so small more field work needs to be done.

In both weeks the numbers of males caught were lower in the water traps than in all the other traps (Table 4.3) giving a significant trap effect in the analysis (Table 4.4). The numbers of males in the other traps varied with bait and week, but generally the vertical traps seem to catch most male *R. theobaldi* (Figure 4.5).

In the case of the by-catch the water traps again have lower catch numbers (Table 4.3), giving a significant effect in the ANOVA in both weeks (Table 4.4). The catches with the other trap types are extremely variable. The effect of trap and lure combined was significant for the by-catch . The by-catch in the delta traps baited with the synthetic lure was much higher than with the other lures (Figure 4.6).

There did seem to be more males and by-catch caught in the second week of the trial (table 4.3) although this was not investigated as it was outside the remit of the study.

The dataloggers showed that temperature and humidity fluctuated on a daily basis but was on a day-to-day basis fairly constant during the study duration (Figure 4.7).

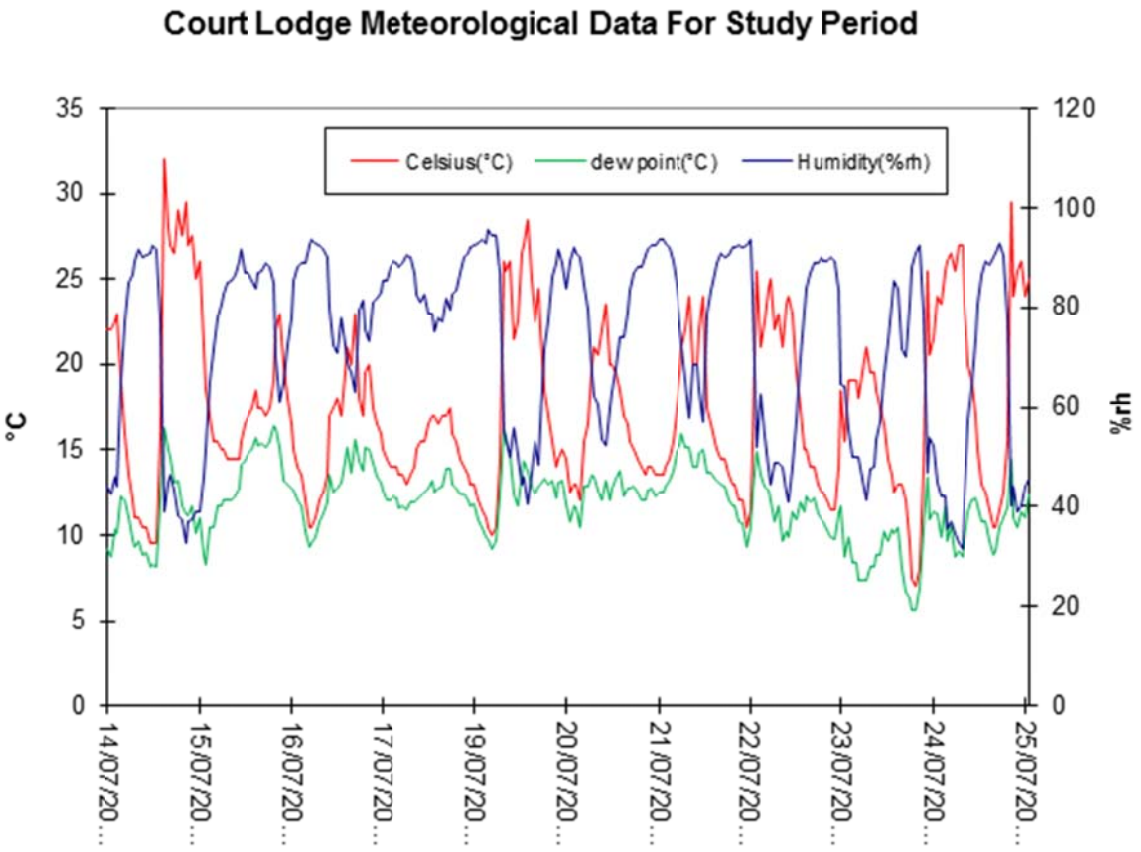


Figure 4.7. Meteorological data taken in the polytunnels at Court Lodge Farm, Yalding, Kent, during the study.

4.4. DISCUSSION AND CONCLUSIONS

The initial observations suggested that females of *R. theobaldi* are attracted to split primocanes which fits with the observations of Nijveldt (1963). However, this was not evident in the trapping experiment. It is possible that excising the cane from the plant reduced its attractiveness or that females are simply put off by the appearance of the traps as they are using more than simply olfactory cues.

Similarly the synthetic blend did not attract midges of either sex. Hall et al. (2011) reported some attraction, but results were very variable and inconsistent.

It is not known why there were so few females caught. Attempts were initially made to look for eggs in the field but could not be seen using available magnification. It must be assumed that females were present as several generations were witnessed at the study site in 2011.

The by-catch insect group included other Diptera, Hymenoptera and Lepidoptera. These by-catch insects did show some attraction to the synthetic blend and this may be due to the fact that blend components appear in their host plants also. It would be useful, if similar work is carried out, to identify species of by-catch and see how these are affected by synthetic volatiles.

At this stage it would be valuable to develop a bioassay to demonstrate that the female midges are attracted to split canes. This could then be used to test the attractive properties of synthetic chemicals.

Chapter 5
IDENTIFICATION OF VOLATILES FROM BLACKCURRANT SHOOTS, BIOASSAY AND WIND TUNNEL WORK

5.1. INTRODUCTION

Due to the cool, wet summer of 2012 in south eastern England, a population of the raspberry cane midge, *Resseliella theobaldi* (Barnes), could not be located for lab and field work. As a result work was moved to another midge species, the blackcurrant leaf midge, *Dasineura tetensi* (Rübsaamen), which lays its eggs in new blackcurrant, *Ribes nigrum* (Linnaeus), shoot foliage causing it to become twisted . This midge was found on site in a polytunnel at EMR.

.

The work in this chapter sought to identify the volatiles produced by blackcurrant shoots which may be attractive to female *D. tetensi.* Bioassays were also developed to assess attraction to natural material with a view to testing the attractiveness of synthetic compounds.

The Pettersson olfactometer bioassay was chosen as this method had successfully been used by Birket et. al (2004) to investigate the volatiles produced by wheat, *Triticum aestivum* (Linnaeus), which attract the orange wheat blossom midge, *Sitodoplosis mosellana* (Géhin). A wind tunnel bioassay was successfully used by Galanihe and Harris (1997) to demonstrate that mated female apple leaf midges, *Dasineura mali* (Kiefer), are attracted to foliage of their host plant, *Malus domestica* Borkh, even when visual stimuli were obscured.

5.2. MATERIALS AND METHODS

5.2.1 Plant material

All the plant foliage used in this chapter was of the variety Ben Connan. Young plants were obtained from a nursery supplier and potted up in compost. They were stored on the sand beds at EMR and watered regularly. Sampling was carried out when shoots and young leaves were present on the plant. In the case of the wind tunnel and olfactometer work the plants were cut back three weeks before being used so that new growth was present.

5.2.2 Insect Material

Twisted shoots were collected from the polytunnel at EMR which contained a mix of blackcurrant varieties

Insects for Pettersson olfactometer

For the olfactometer work plant material was stored in ventilated Perspex boxes (22.5cm x 11.5cm x 7.5cm) with a piece of damp blue tissue. The boxes were checked every few days and mature orange larvae removed and potted up individually in 1.5ml Autoanalyzer (AA) cups (Sarstedt, Leicester, UK) with a piece of damp filter paper. The AA cups were checked regularly and adults removed and sexed.

The majority of insects tested were virgin although attempts were made to obtain mated females. To do this a newly emerged male and female were put into a Perspex screw top jar (12 cm high with a 6 cm radius) together and left for two hours. After this period they were separated into their individual AA cups again. After thirty minutes the female was observed to see if she was displaying calling behaviour, extending her ovipositor. If she was not calling it was deemed that she had mated and could be used in olfactometer work. This followed the method of Galanihe and Harris (1997). During experiments this method did not prove reliable with females which did not call after thirty minutes proceeding to call once in the olfactometer.

Insects for wind tunnel

Collected shoot tips were put into a nylon rearing cage (0.5 m x 0.5 m) with damp blue tissue paper. The cage was located in a controlled temperature room set at 22°C with 18 hours light and 6 hours dark each day. The cage was checked every 2-4 days and water added to keep the leaves and larvae from desiccating. After a few days it was noticed that the cage was drying out quickly and as a result clear plastic bags were draped over the cage to reduce evaporation.

Once adults had begun to emerge the cage was checked daily and adults removed to individual plastic pots (3 cm tall and 4.5 cm in diameter). Crook and Mordue (1999) observed that midges emerged between 7 and 8 am and that mating occurred between 10 and 12 am. As a result collections of adults were carried out between 11 am and 1 pm to allow time for the midges to emerge and mate. It was hoped that this method of natural emergence and mating in the cage would work better than the mating method detailed in the olfactometer method above.

Only mated females were used in the wind tunnel. Mating status was difficult to determine but females were viewed as mated if males were present in the rearing cage when the females were collected and if no calling was observed after a 30 minute acclimatisation period in their individual pots. This acclimatisation period was carried out in the room where the wind tunnel was located. Any females seen to be calling were not released in the wind tunnel.

5.2.3 Volatile sampling and analysis

Using the method detailed in Chapter 2, volatiles were collected from new shoots on blackcurrant plants. Shoots are the site where mated female midges lay their eggs. Figure 5.1 shows the collection of volatiles. This work was carried out in Spring 2012.

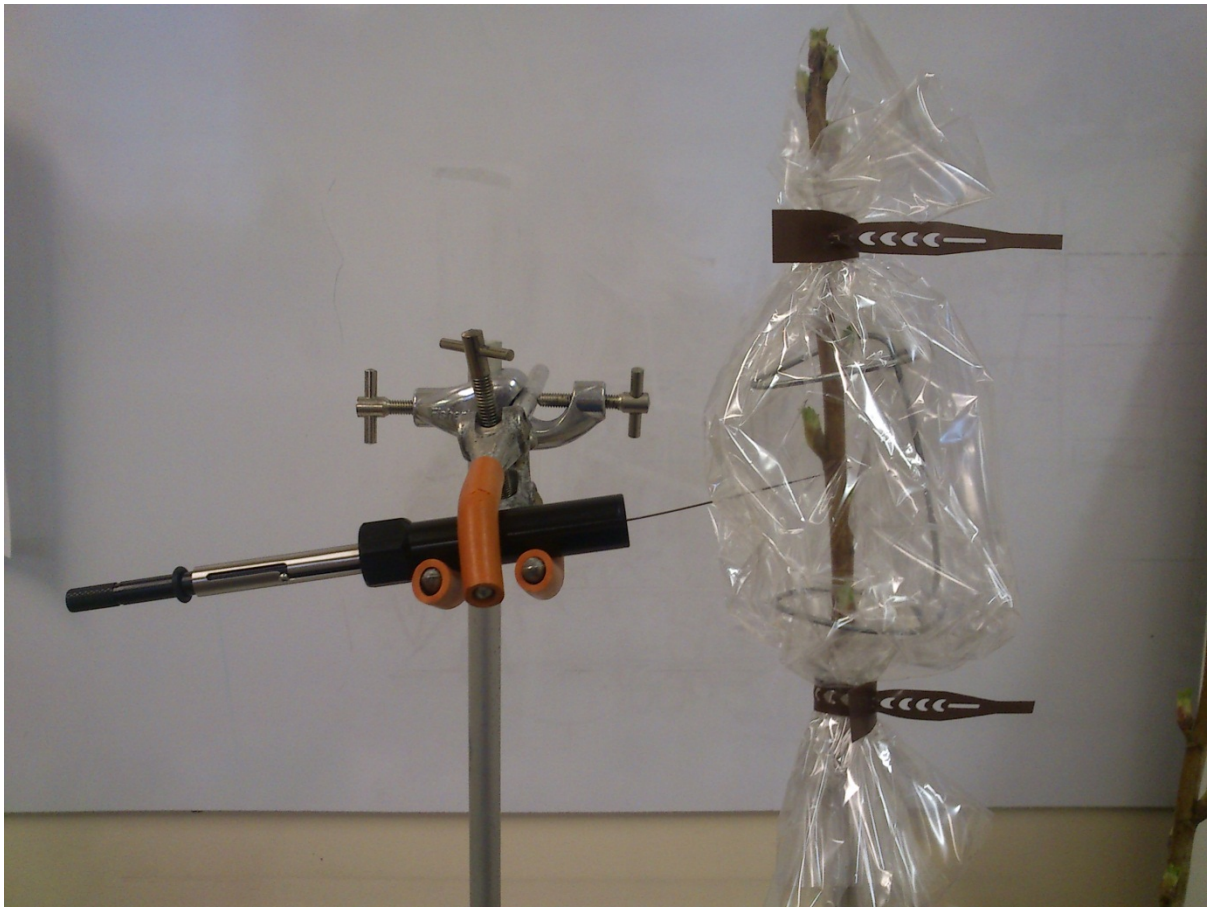


Figure 5.1 – SPME collection of volatiles from a young Ben Connan blackcurrant bud.

Collections were analysed using the gas chromatography linked to mass spectrometry using the method detailed in Chapter 2. A table of common volatile compounds with chemical structures can be found in the Appendix.

5.2.4 Pettersson olfactometer bioassay

This work was carried out in summer 2012.

Perspex olfactometers were obtained from Rothamstead (Birkett et al., 2004) (Figure 5.2). These consisted of circular top and bottom sections and a central section with a cut–out, 4-pointed star arena space. The total arena diameter was 12 cm with distance from one tip of the star to the opposite being 10 cm. The

three sections screwed together with plastic screws and nuts but could be taken apart for cleaning. At the tip of each of the points was a hole (5 mm) through which a glass arm section could be inserted. The glass arms had one wide (20 mm) end where plant material or filter papers could be placed and then a step down to a much narrower (5 mm) end which fitted into the hole in the arena.

There was also a hole (5 mm diameter) in the centre of the top plate to allow insects to be put in and a pump attached to draw air in through the glass arms and out through the top (22V, 50Hz WISA model 110). In initial trial runs it was noted that the midges could fly up through the top opening and become stuck in the pipe leading to the pump. To solve this problem a pipe connecter with one side covered in a square of net curtain secured with PTFE tape, was connected to the end of the pipe entering the hole in the top of the arena. This prevented insects entering the pipe and the PTFE tape gave a good tight fit.

Small squares of muslin were put over the ends of the arms to prevent insects escaping and make a tight fit. Muslin was used as, although less robust than net curtain, it was disposable and clean (important as the air flowed through it to reach the insect). These squares of muslin were replaced after each run.

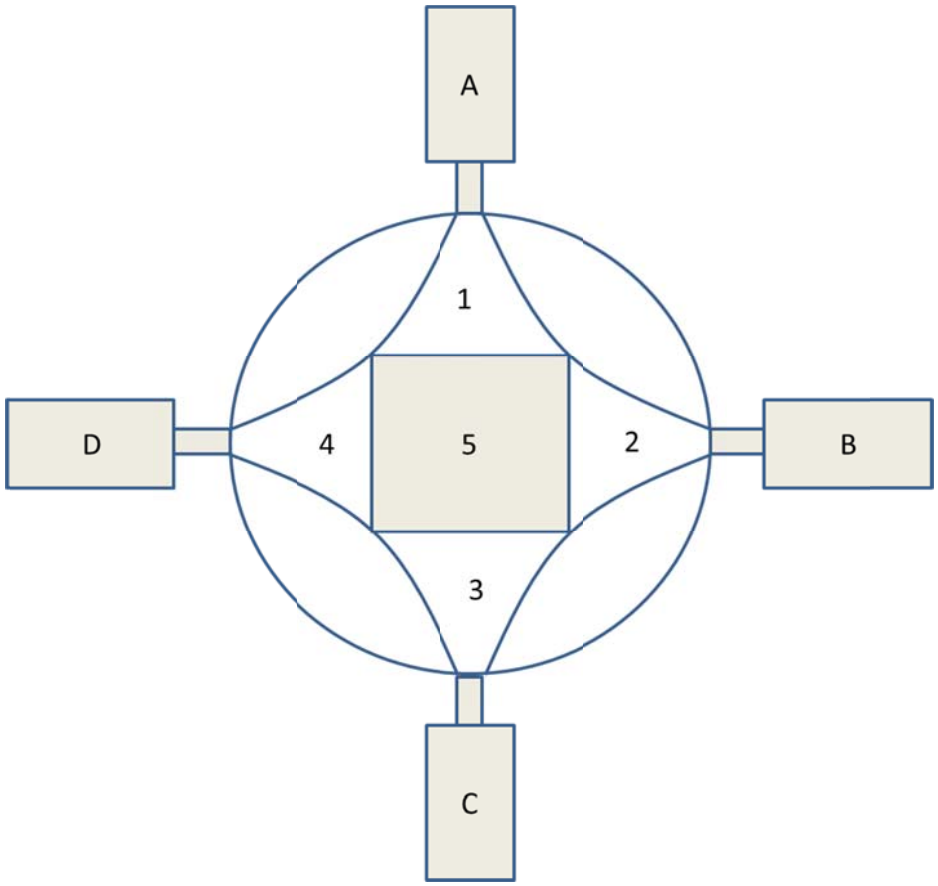


Figure 5.2. Diagram of the Petersson olfactometer (numbers represent zones of the arena and letters represent the different treatment arms).

To avoid odour contamination affecting the results a clean arena was used for each insect. To clean the arena the screws were unscrewed and each of the three sections rinsed with distilled water and then 70% ethanol (made up with distilled water) three times. The top and bottom plates were left to dry upright on a clean incubator shelf over some blue paper. The central sections could be placed on the shelf horizontally as the edge protected the central section from contamination. The glass arms were washed in the same way each time the treatment in the arm was changed. All equipment was left to air dry (preferably overnight).

The olfactometer was set up in dark room at EMR. The olfactometer and pump were placed onto the bench. A flow-meter was connected between the tube joining the top of the arena and the pump to measure the airflow in the system. For each run the airflow was set to 400 cm^3/min meaning 100 cm^3/min of air was being drawn in through each of the arms. The pump was moved to a different

table as early observations indicated insects moved to the arm nearest the pump regardless of bait. The set up was lit using a light box which contained a 22W fluorescent lamp which shone out through a sheet of opaque plastic giving a diffuse light. The light box was suspended approximately 30 cm above the bench.

The arena was divided into five sections (Figure 5.2), noted on a white piece of paper underneath the set up – the central section, section 5 into which the midges first entered the arena through the hole; and sections 1-4 which each corresponded to a glass arm. For tests using plant material a shoot tip was placed into one of the arms with the other three left as blank controls. The insect was observed for 15 minutes and the time spent and entries into each area recorded using the computer program OLFA (Boorland International, California, USA). After each run the arms were moved through 90° to eliminate any directional bias (i.e. arm 1 moved to the arm 2 position etc.).

The arena with pump attached was tested using smoke from a bee smoker to visualise the air entering the arena (Figure 5.3). Although only faintly seen in the camera image, when the smoker was placed close to one of the side openings the smoke could be seen being sucked in. Clear boundaries could be seen between the four quarters of the arena with very little mixing of airflow.

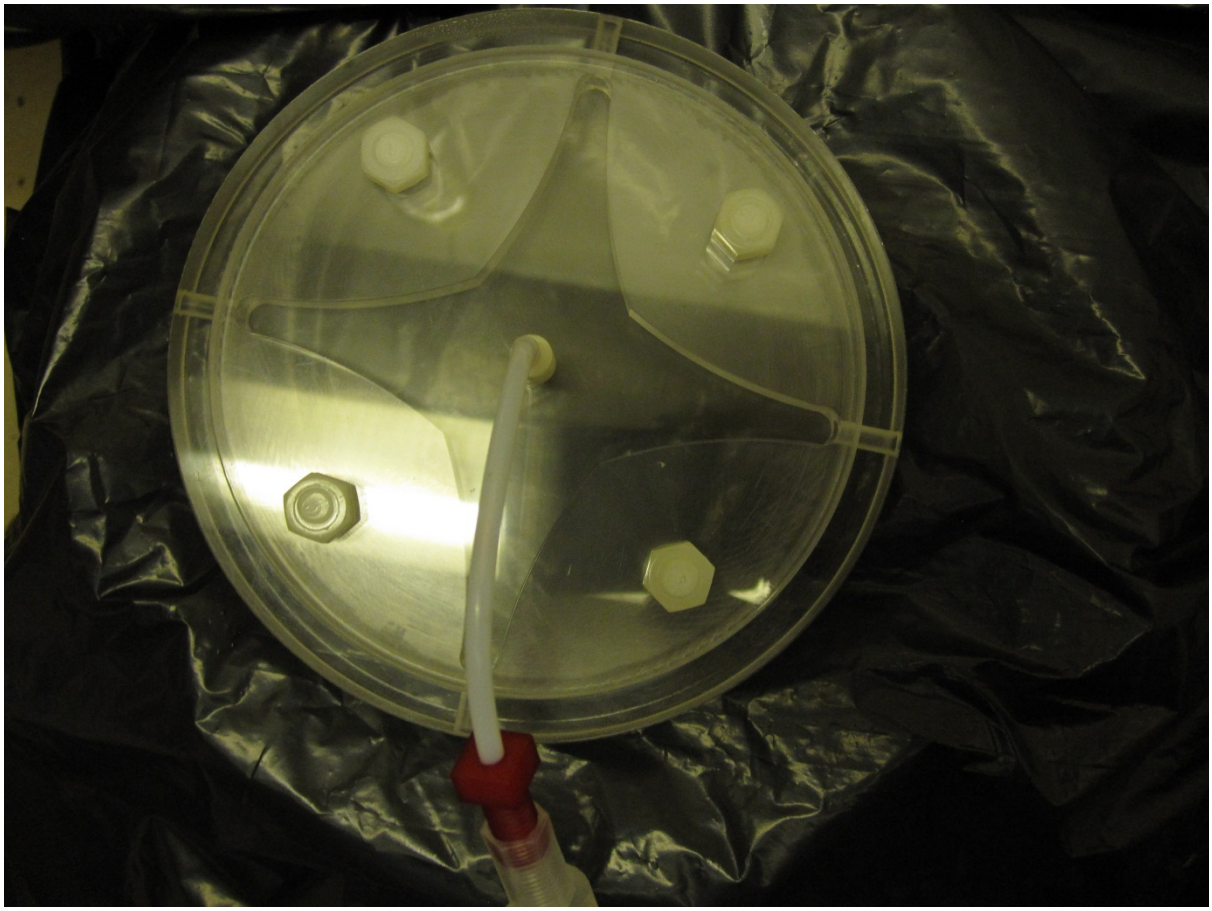


Figure 5.3. Arena with pump attached and smoke used to visualise air flow. The smoker was located near the leftmost opening and smoke can faintly be seen entering the arena.

5.2.5 Wind tunnel bioassay

This experiment was carried out during a week long period of blackcurrant leaf midge emergence in July 2013.

The windtunnel was located in a ventilated room at the Natural Resources Institute (Figure 5.4). The air was drawn in from outside the building by a fan through a charcoal filter (50 cm x 50 cm) – passing across the whole area of the air inlet. Vents present near the wall could be opened and closed to allow/restrict airflow. The air passed through the two upwind gauzes and through the main body of the tunnel. The air was drawn through the downwind gauze and out of the room through an air vent at the downwind end of the room.

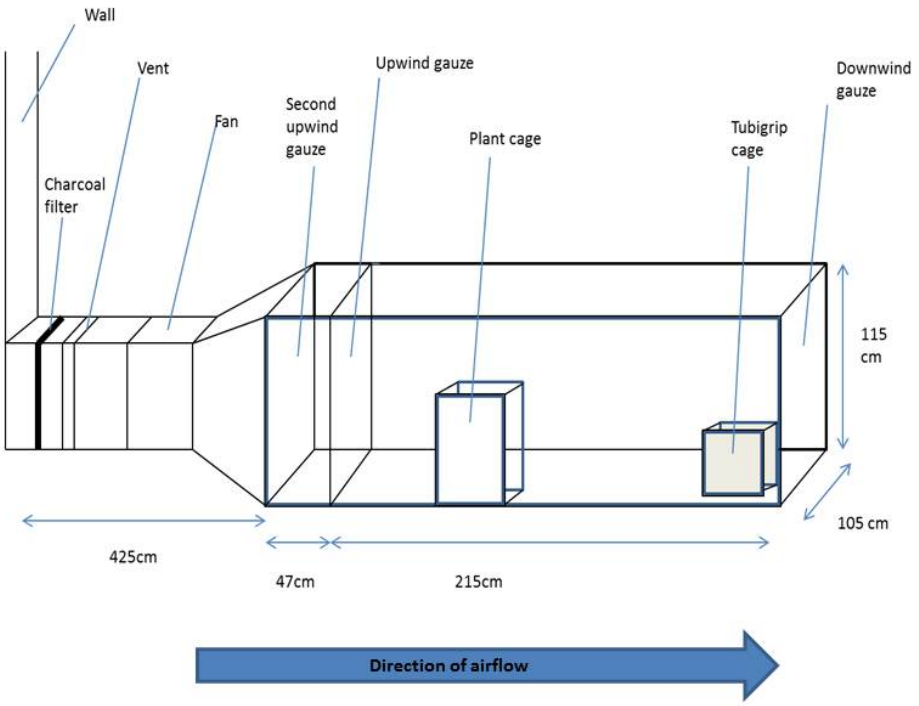


Figure 5.4. Diagram of the wind tunnel

The main body of the tunnel was constructed from opaque white acrylic with a clear Perspex roof. This allowed light through while limiting visual distractions. The tunnel was lit by an array of fluorescent tubes on the lab roof. On the floor of the tunnel were scattered 15 black acrylic shapes (10 cm x 10 cm) to aid insect navigation. All the gauzes were made from fine white netting which could not be easily seen through; but which allowed a uniform passage of air. The upwind end of the tunnel had a small chamber partitioned off to allow material to be placed out of site but permit volatiles to pass into the tunnel (this section was not used in my experimental work).

Also present in the body of the tunnel was the plant cage in which the whole blackcurrant plant was placed (Figure 5.5).

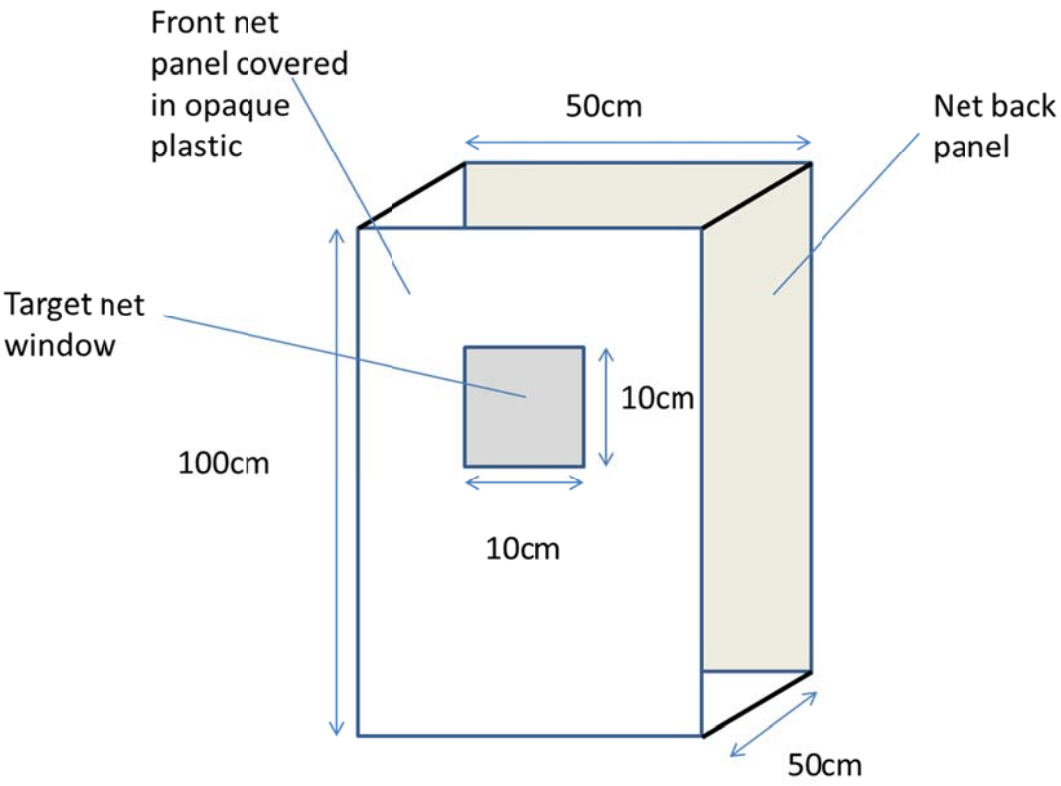


Figure 5.5. Diagram of the plant cage

The cage frame was constructed from fibre glass poles connected with plastic corners. A cover which fitted over the frame was made. The top and two sides were made from opaque plastic sheet and the front (upwind) and back (downwind) sides from white netting. The plastic sheet and netting were heat sealed together. The base of the cage was left open to allow the cage to be placed over the blackcurrant plant. A sheet of opaque plastic with a small (10 cm x 10 cm) window cut in was placed over the upwind side of the cage and secured with bulldog clips. This gave a clear downwind target through which blackcurrant volatiles could pass and onto which the blackcurrant leaf midges could land.

To observe behaviour over a period of several hours a high resolution analogue video camera was used (SHC-735p; Samsung, Korea) fitted with a 1/3' infrared corrected, C mount varifocal lens (f:1.0) with auto-iris. The camera signal was sent to a digital video recorder (SRD-470D, Samsung, Korea).

The camera was mounted on a tripod outside the downwind end of the tunnel. The lens of the camera was pushed through a hole in the downwind gauze. The camera was focused on the window in the plant cage so that landings and behaviour could be recorded.

The individual pots containing mated female midges were placed on a tubigrip cage (30 x 30 x 30 cm). This put the midges on a level with the window in the plant cage. The pots were angled so that the tops pointed upwind. The lids were removed to release the midges and recording begun. The number of mated females released in each run varied from 11 to 23.

The set up was left for a period of between three and five hours. The footage was then watched back and midge flights, landings and behaviours in the field of view noted.

5.3. RESULTS

5.3.1 Volatiles emitted by young blackcurrant shoots

A typical trace for GC-MS analyses of volatiles collected from blackcurrant shoots is shown in Figure 5.6 and a list of chemicals identified is shown in Table 5.1. The main components were the monoterpenes 3-carene and α-terpinolene with smaller amounts of *Z*- and *E*-β-ocimene.

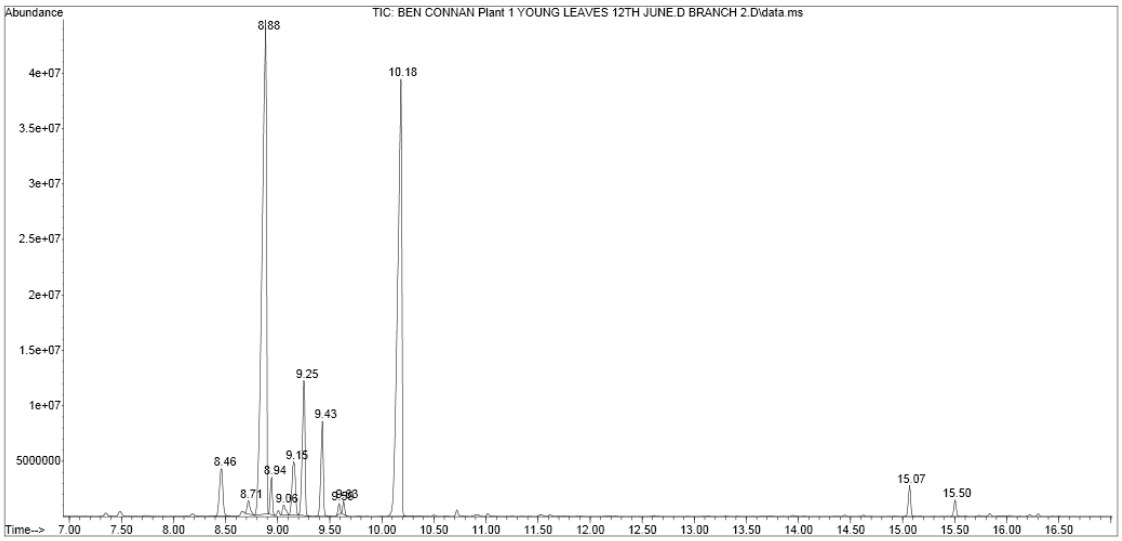


Figure 5.6. GC-MS Analysis of SPME sample from shoot of Ben Connan

blackcurrant plant (non-polar DB5 column; 3-carene at 8.88 min, α-terpinolene at 10.18 min).

Table 5.1. Chemicals identified in SPME samples taken from new leaves of Ben Conan blackcurrant plants (RT - retention time on non-polar DB 5 column (mins), KI - Kovats Indices, Pherobase - KI from the Pherobase and Relative Quantity - gives an indication of the size of the peaks * being smallest and ***** largest. .

RT (min)	KI	Compound	Pherobase	Relative Quantity
7.34	927	α-thujene	923	*
7.49	936	α-pinene	939	*
8.46	993	myrcene	991	***
8.71	1008	α-phellandrene	1005	**
8.88	1019	3-carene	1011	*****
8.94	1023	α-terpinene	1018	***
9.01	1027	*o*-cymene	1020	*
9.06	1030	*p*-cymene	1026	**
9.25	1042	*E*-β-ocimene	1040	****
9.43	1053	*Z*-β-ocimene	1050	****
9.59	1063	γ-terpinene	1062	**
9.63	1066	β-terpinene	1071	**
10.18	1100	α-terpinolene	1088	*****
10.72	1134	dimethyloctatriene(?)	?	*
15.07	1436	β-caryophyllene	1418	***
15.51	1467	α-caryophyllene	1454	**

During analysis of the blackcurrant traces the results of the blank SPME run were consulted and those chemicals present in the empty SPME system excluded from further analysis.

5.3.2 Pettersson olfactometer bioassay

Results of bioassays with the Pettersson olfactometer are shown in Table 5.2.

All insects survived for the whole run except the mated female on the 11 September which was dead on entry to the olfactometer. As a result the experiment was abandoned shortly after beginning.

Unfortunately due to the erratic emergence and low numbers of insects, the actual date of emergence could not be accurately determined.

Due to the low number of observations, statistical analysis could not be carried out although from Table 5.2 the midges do not seem to be attracted to the excised shoots. As the initial results did not look promising the focus of work was moved to the wind tunnel in the hope of seeing female attraction to blackcurrant shoot material.

Table 5.2. Time spent in each arm of Pettersson olfactometer by adult blackcurrant leaf midge, *Dasineura tetensi*, in experiments carried out between the 21 August and 24 September 2012 with baited arm in bold (experiments between 16.00 and 18.00 hours; temperature 20-23°C and 48-61% relative humidity; bait was a fresh blackcurrant shoot with end sealed with PTFE tape). One mated female died shortly after entry (7th in table below).

Insect		Bait	Time in arm (min)				
Sex	**Mated Status**	**Arm**	**1**	**2**	**3**	**4**	**5 (centre)**
Female	Virgin	2	0	**0**	0	0	15.00
Female	Virgin	3	0	0	**0**	1.40	13.60
Female	Virgin	3	0	0	**0.09**	13.51	1.40
Female	Virgin	2	12.95	**0**	0	1.75	0.33
Female	Virgin	1	**2.95**	0	2.26	9.05	0.71
Female	Mated	4	0	0	0	**0**	15.00
Female	Mated	4	0	0	0	**0**	0.12
Male	Virgin	3	1.78	0	**0**	11.68	1.53
Male	Mated	1	**1.06**	0	0	13.85	0.07
Male	Mated	4	0	0	0	**0**	15.02

5.3.3 Wind tunnel bioassay

In the windtunnel bioassay, adult blackcurrant midges, *D. tetensi,* were observed passing through the recording field and landing on the window of the plant cage (Table 5.3). However, no ovipositing behaviour was ever seen.

Table 5.3. Blackcurrant leaf midges, *Dasineura tetensi,* seen passing through the camera viewing field in the wind tunnel and landing on the cage

Date	No. Females	Time of Recording	Time midge seen flying across viewing field	Time of midge landing in viewing field
15.7.2013	20	13.03-18.01	15.14 and 16.10.	None
16.7.2013	14	14.29-17.31	No midges seen	None
17.7.2013	11	12.59-16.10	13.00, 13.01, 13.02, 13.03 and 15.06	None
19.7.2013	23	12.10-17.00	12.56, 12.58, 13.05	12.15, 15.50, 16.10, 16.35, 16.49.

After this five day emergence period the number of adults emerging dropped dramatically so the experiment could not continue. Midges were only seen to be attracted to the window on one of the days and no ovipositing behaviour was seen.

5.4. DISCUSSION

5.4.1 Collection and analysis of volatiles

A method was developed using SPME which proved effective for the collection of volatiles from blackcurrant shoot tips. A suite of chemicals was identified which was produced by shoot tips, the main components being the monoterpenes 3-carene and α-terpinolene with smaller amounts of *Z*- and *E*-β-ocimene.

5.4.2 Olfactometer and Wind tunnel

Both bioassay methods were chosen as they had been used successfully with other insects. The same perspex olfactometers were used by Birket et al. (2004) to successfully show the attraction of orange wheat blossom midges to host plant volatile blends. The wind tunnel set up at NRI was successfully used by Hawkes et al. (2012) to investigate the behaviour of the mosquito *Anopheles gambiae sensu stricto* (Giles).

Attraction to blackcurrant shoots was not seen in either the olfactometer or wind tunnel experiments. These results contrast with the work of Crook and Mordue (1999) who found that mated but not virgin females were attracted to blackcurrant shoot volatiles in an olfactometer. There are many possibilities for this lack of attraction. One key problem was ensuring the mating status of the females. Crook and Mordue (1999) put males and females together and observed them for mating. This was tried in some preliminary work (not detailed here) and it was found that pairs were not often seen to mate and observations often lasted for many hours. This method was deemed too labour intensive and time consuming. For the olfactometer work pairs were put together for a two hour period unobserved and then females were observed for thirty minutes to see if they were calling. A similar method was used for the wind tunnel but the midges were taken from a mixed rearing cage and observed rather than reared virgin and deliberately paired. These methods may still not have resulted in mated females and hence the lack of responses.

Another possibility is that the environment of the experiments may have been too unnatural. Laboratory bioassays are by definition carried out in the laboratory where conditions such as humidity and lighting will not be the same as in the field. In both the olfactometer and windtunnel, humidity was not controlled and this may have affected the insects' health and behaviour. Another environmental factor in the windtunnel especially was lack of shelter. In the field the insects spend most of their time within the sheltered plant canopy surrounded by leaves. This is very different to the open wind tunnel environment used here.

The lighting in the wind tunnel was provided by fluorescent tubing mounted on the lab ceiling and a fluorescent light box was used in the olfactometer work.

Fluorescent tube lights flicker with the mains alternating current at a rate of 50 cycles per second in the UK. The human eye can only distinguish flickers of 16 Hz or below (the flicker threshold). Rapid moving diurnal flies such as blackcurrant leaf midges have much higher flicker thresholds than humans. The fusion frequency of house flies has been estimated electrophysiologically as 265 Hz (Mathews and Mathews, 2010). If the frequency of *D. tetensi* is anything above 50HZ then the insects would perceive the light as flickering on and off and this may have led to their lack of response in the wind tunnel and olfactometer experiments.It was concluded that these behavioural experiments were not successful and that further work was needed. However due to the sporadic availability of adults throughout the year it was decided that the limited number of insects could be best used in another way. Electroantennogram technology allows the compounds present in volatile collections which insects can detect to be determined. It was proposed that this technique could be used to show if any of the blackcurrant shoot volatile compounds were detected by mated females and to see if there were differences in detection between mated females, virgins and males. The chemicals which were responded to by the antennae could then be focused on in further work.

Chapter 6
ELECTROANTENNOGRAM STUDIES ON BLACKCURRANT LEAF MIDGE

6.1. INTRODUCTION

Olfactometer and wind tunnel studies in Chapter 5 failed to show any evidence for attraction of blackcurrant leaf midge, *Dasineura tetensi,* to host-plant volatiles. Electroantennography (EAG) is an alternative bioassay approach which allows identification of chemicals which stimulate an insect's antenna; these components can then be focused on in behavioural work to see whether they elicit behavioural responses.

EAG has been used successfully in the identification of pheromones for several midge species (Amarawardana, 2009), and this approach was investigated to determine if *D. tetensi* adults can detect the components of the volatiles from blackcurrant shoots.

6.2. MATERIALS AND METHODS

A preliminary EAG investigation was carried out using volatiles collected from blackcurrant shoots by SPME, as was done for GC-MS analysis of the volatiles in Chapter 5. Further work was carried out with volatiles from blackcurrant shoots collected on Porapak Q as this made it possible to carry out replicate analyses on the same sample.

6.2.1 Insect material

For EAG analyses of plant volatiles collected by SPME the same insect collection and rearing method was used as in 5.2.2. for the olfactometer insects. The AA cups were checked every few days and then daily once adults had begun to emerge. The adults were sexed and taken to the EAG lab.

For EAG analyses of volatiles collected on Porapak Q the same method was used as in 5.2.2 for insects for the wind tunnel.Rolled shoot tips were collected from a mixed variety polytunnel at EMR and picked from an infested field at Burrs Hill Farm in Brenchley, Kent (owner Ian Overy). Once emergence had begun the cage was checked daily after 11 am and adults removed into individual plastic pots and taken to the EAG laboratory. The mating status of the insects was tentatively assigned based on whether both males and females were found in the cage on that day. If the cage had both sexes then the midges were noted as mated; and if only one sex was present, virgin.

The insects were prepared for GC-EAG analyses using the methods detailed in Chapter 2.

6.2.2 Collection of volatiles

Volatiles were collected from young Ben Connan shoots following the SPME method detailed in Chapter 2 and injected straight into the GC-EAG. The fibre was left in the GC injector for 10 minutes to ensure all volatiles had left the fibre.

Both polar and non-polar GC columns were used, although in subsequent work only the polar column was used as it gave better separation of compounds.

Volatiles were also collected from young blackcurrant shoots on Porapak Q using the method described in Chapter 2. This gave a solution which could be used for multiple GC-EAG runs. Volatiles were collected from single shoot tips of potted plants of the variety Ben Connan which had been cut back and left for two weeks to encourage new growth. Collections lasted for 1 hour.

The collections of volatiles were analysed by GC-MS on the polar column in the Varian instrument as detailed in Chapter 2. The same solutions were then analysed by GC-EAG on the polar GC column as described in Chapter 2. A table of common volatile compounds with chemical structures can be found in the Appendix.

6.2.3 Gas Chromatography linked to electroantennographic recording (GC-EAG)

The effect of components of the blackcurrant volatile collection on the antennae of the midges was assessed using the GC-EAG method detailed in Chapter 2. The polar GC column was used for all GC-EAG runs.

In the main experiment initially the volatiles were delivered to the antenna in a constant stream of nitrogen but in later runs the puff system was used to concentrate the volatiles and increase the chances of obtaining responses.

6.3. RESULTS

6.3.1 GC-EAG Analyses of volatiles collected by SPME

A table was created showing which times each insect was seen to respond. From this table males and those preparations which did not respond at all were removed. The table was then split into those runs which were polar and those which were non polar. From the twelve runs, only 4 were responding females.

There was only one response time at which a response was seen more than once, at 12.3 minutes. This did correspond to a peak on the GC at the same time. This response was seen twice on the non-polar runs.

To investigate the identity of the active peak, the GC traces in this experiment were compared with those obtained in analyses of volatiles collected from blackcurrant shoots by SPME in Chapter 5. This was done as different GCs running different programs were used in both experiments and accurate HC

standard values were not available for the GC-EAG at this time and therefore Kovats Indices could not be calculated. A corresponding peak was detected at 15.5 min and this was identified as α-caryophyllene. However the number of insects was low and the peak was only responded to twice thus it was concluded that more EAG work would be needed. It was also the case that virgin rather mated females were used and this project mainly focusses on mated females.

The time which taking an SPME collection for each EAG run adds led this method to be abandoned in favour of Porapak Q entrainment as this allows a solution to be made which can be used for many runs. Rearing the insects in AA cups worked but only produced virgins, e.g Hall et al. (2009), and involved a great time commitment potting up the individual larvae. Thus the rearing cage method was developed which was simple and gave the possibility of obtaining mated females.

6.3.2 GC-EAG analyses of volatiles collected on Porapak Q

GC-MS analysis

Results of GC-MS analysis of collection of volatiles from blackcurrant shoots are shown in Table 6.1. Compounds were identified by the results of searching the GC-MS library and comparison of retention indices with those on the Pherobase (El Sayed, 2012) on similar GC phases.

Table 6.1. GC-MS analyses on a polar GC column of volatiles collected from Ben Connan blackurrant shoots collected on Porapak Q and relative quantities (***** high, * low)

Retention Time (min)	Identification	Kovats Index	Pherobase KI	Relative Quantity
5.66	3-carene	1138	1148[1]	*****
5.87	myrcene	1154	1168[2]	*
6.35	limonene	1189	1212[2]	*
6.51	β-phellandrene	1200	1241[2]	*
6.86	(*E*)-β-ocimene	1226	1242[1]	***
7.10	(*Z*)-β-ocimene	1243	1245[2]	***
7.55	α-terpinolene	1276	1297[2]	*****
8.01	(*Z*)-3-hexenyl acetate	1310	1308[2]	**
9.00	(*Z*)-3-hexen-1-ol	1375	1378[2]	*
11.75	β-Caryophyllene	1598	1608[2]	**
13.06	germacrene D	1708	1705[1]	*
14.37	Hexanoic acid	1820	1863[2]	*

[1] HP Wax phase; [2] DB Wax phase

For those compounds which have been identified and found on the Pherobase there is good matching of Kovats suggesting that the peaks were correctly identified.

Comparison of GC-MS and GC-EAG peaks

Comparison of GC-MS and GC-EAG allowed the peaks on the GC-EAG to be identified. (Table 6.2)

Table 6.2. Analysis of volatiles collected from blackcurrant shoots on Porapak Q on GC-EAG equipment using polar GC column (2013).

Retention Time (min)	Identification	Kovats Index	Pherobase KI	Relative Quantity
5.41	3-carene	1142	1148[1]	*****
5.59	myrcene	1154	1168[2]	*
6.10	limonene	1193	1212[2]	*
6.21	β-phellandrene	1201	1241[2]	*
6.55	(*E*)-β-ocimene	1227	1242[1]	***
6.78	(*Z*)-β-ocimene	1243	1245[2]	***
7.22	α-terpinolene	1276	1297[2]	*****
7.67	(*Z*)-3-hexenyl acetate	1310	1308[2]	**
8.70	(*Z*)-3-hexen-1-ol	1387	1378[2]	*
11.30	β-Caryophyllene	1598	1608[2]	**

[1] HP Wax phase; [2] DB Wax phase

GC-EAG results

Examples of GC-EAG traces are shown in Figures 6.1 and 6.2.

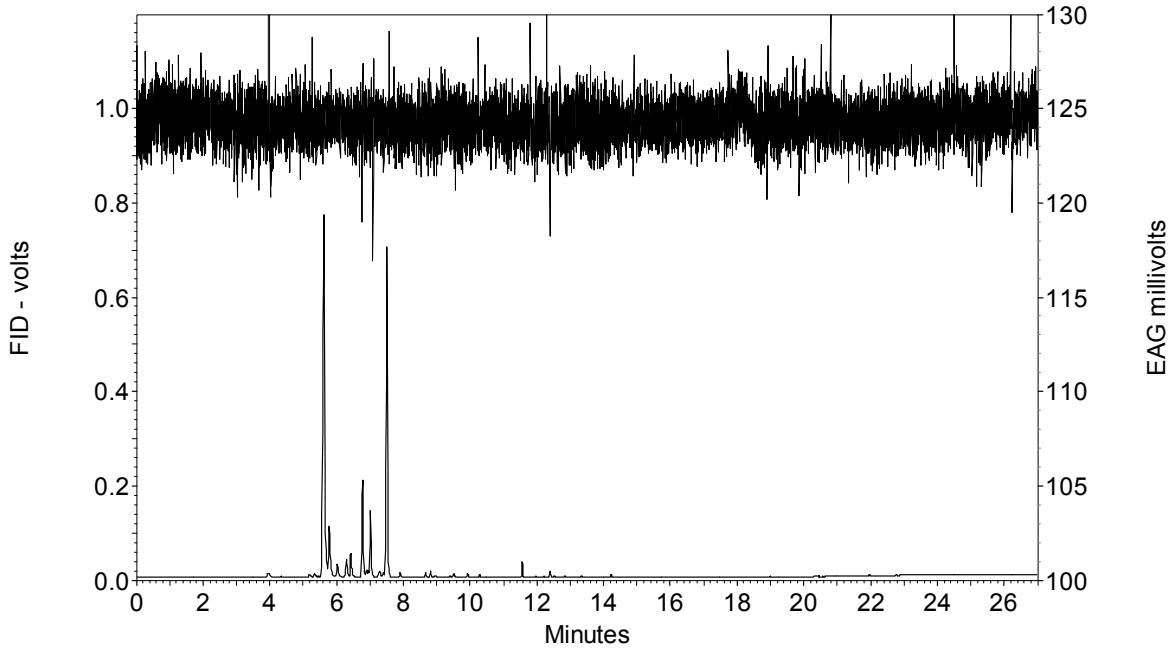


Figure 6.1. GC-EAG trace for female *Dasineura tetensi* with SPME sample from blackcurrant shoot volatile solution. Upper trace is EAG and lower trace is FID.

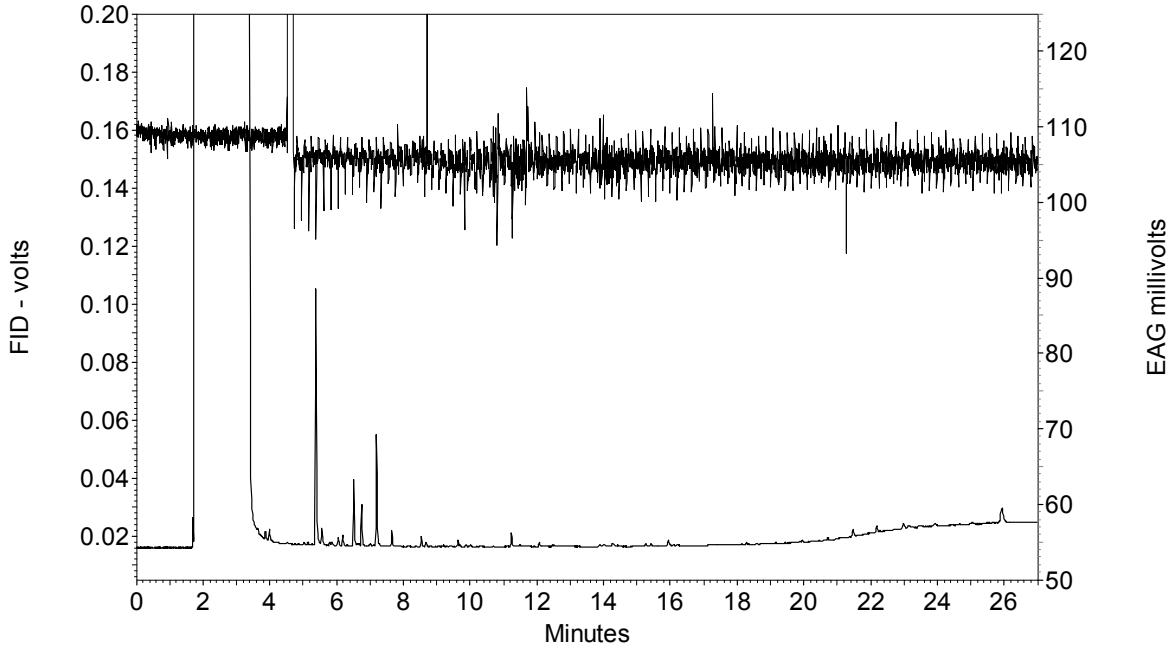


Figure 6.2. GC-EAG trace for female *Dasineura tetensi* with Porapak sample from blackcurrant shoot. Volatiles were delivered in puffs of humidified air. Upper trace is EAG and lower trace is FID.

The first step in the EAG analysis was to identify and note any midge responses and the times at which they occurred. The number of times each particular response was seen was also noted. At this stage all runs were removed which were replicates of the same insect – this is because these were pseudo- rather than real replicates. Runs were also removed which were of male midges or which showed no depolarisations. Responses were also removed if they only occurred once. Of 26 runs performed for which a connection was established on 12 traces EAG responses could be seen. There were 12 times at which EAG responses were seen more than once (Table 6.3).

Table 6.3. EAG responses from female *Dasineura tetensi* in GC-EAG analyses which were seen more than once.

RT EAG response (min)	No. responses	RT GC peak (min)	Kovats Index	Compound
4.9	2	4.86	1154	3-carene
5.0	2	5.05	1169	No identity
5.2	2	5.17	1179	No identity
6.8	3	6.78	1271	α-terpinolene
7.2	3	7.20	1293	Ethyl-acetopheonone
7.9	2	7.89	1330	No identity
9.0	2	9.07	1392	No Identity
9.4	2	9.43	1411	Acetamide,2,2,2-trifluro-N-methyl-
9.9	2	9.90	1435	No identity
21.5	2	21.48	2091	Siloxy
24.4	2	nd		
25.4	2	nd		

Two of the responses (24.4 min and 25.4 min) did not correspond to any peaks on the GC connected to the EAG set up; these responses can thus be discounted.

Both ethyl-acetophenone and the siloxy compound are probably contaminants from the Porapak-Q and do not occur in the blackcurrant volatile blend and these responses can thus be discounted.

This leaves five responses to compounds which produced GC peaks but which remain unknown. One of these peaks was seen on the GC-MS run but was not identified from the fragmentation pattern and the other four were seen as peaks on the GC connected to the EAG but not on the GC-MS. Three known compounds were also seen to elicit EAG responses and these were 3-carene, α-terpinolene and 2,2,2-trifluro-N-methylacetamide.

6.4. DISCUSSION AND CONCLUSIONS

Initial GC-EAG work using SPME as the volatile collection method was carried out as SPME had proved a convenient way to collect volatiles in previous chapters. The method, however, was time consuming and each collection was not exactly the same as the one before leading to a lack of reproducibility. The preliminary work also only used virgin females when it is the mated females which lay eggs on shoot tips. Attempts were made to remedy these problems in the main experiment.

In the main experiment entrainment samples were collected from a blackcurrant shoot tip onto Porapak Q, eluted into solution and some of the components were identified using the GC-MS. This solution was then used in GC-EAG work with female midges.

Over half of the EAG runs carried out failed with no responses seen to any of the chemicals (12/26 runs). On other days an electrical connection across the midge could not be established and as a result several experimental days yielded no data at all. It was found that only ten GC peaks elicited more than one EAG depolarisation result and two of these were contaminants from the Porapak-Q system. Of the peaks responded to none were responded to more than three times and of these several remain unidentified. Overall there were not enough responses to conclude that any EAG active components were present in the blackcurrant volatile blend. As a result further work is needed.

It is possible that the solution of volatiles was not concentrated enough and further work could test preparations with a collection carried out over a longer period of time or which had been concentrated under a stream of nitrogen. Further work could also include the testing of synthetic versions of the chemicals identified as present in the blackcurrant volatile profile with the insect EAG preparation as the chemicals will be at higher concentrations and may cause responses.

It is of interest that a caryophyllene peak was seen in the entrainment solution but this was not responded to by the midges.

Chapter 7
IDENTIFICATION OF VOLATILES PRODUCED BY YOUNG APPLE LEAVES

7.1. INTRODUCTION

The final midge species examined was the apple leaf midge, *Dasineura mali* Kiefer. Mated females lay their eggs on young apple, *Malus domestica* Borkh, shoots and larval feeding causes the leaves to form characteristic twisted leaf galls. Galanihe and Harris (1997) found that mated female *D.mali* would fly upwind in a wind tunnel to young apple leaves even if the leaves were not visible; they suggested that the volatiles the leaves were producing were attracting the females. This chapter focuses on the collection of volatiles from apple shoots using the two methods, entrainment and SPME, employed in previous chapters.

Studies have already been carried out into the volatiles produced by apple foliage (Anfora et al., 2005; Bengtsson et al., 2001) but no full analysis of apple volatiles has been carried out as part of this project. Work here was a comparison with work previously done but in a different (UK) climate with different apple cultivars.

This work was carried out in December 2013 a year after the apple EAG work detailed in Chapter 8.

7.2. MATERIALS AND METHODS

7.2.1 Plant material

In December 2013 leafy apple trees which were cultivars based on the Israeli rootstock Hashabi and English variety Falstaff were sourced from a glasshouse open to the air on site at EMR. These plants were part of a breeding experiment by Feli Fernandez, . The glasshouse was open to the air (the door was open) but the roof provided shelter.

The entrainment was carried out on plants in the glass house but plants were taken to the lab at NRI for SPME collections.

7.2.2 Collection of volatiles

Collection of volatiles on Porapak Q

Collections were made using the portable entrainment kit set up in the glasshouse following the method detailed in Chapter 2 (Figure 7.1). The kit was run off a battery (Halfords lead acid battery HB063 with 360 Amp start up power). Two plants were entrained at the same time (both Hashabi varieties). Collections were carried out for 48 hrs from 4 pm on the 11 December to 4 pm on the 13 December 2013.

Figure 7.1. Porapak Q entrainment of an apple, *Malus domestica*, shoot in the glasshouse at East Malling Research.

Porapak Q collection filters were eluted with dichloromethane (1 ml). The solutions were injected (1 µl) into the GC-MS for analysis.

Collection of volatiles by SPME

Collections were carried out in December 2013 as the final piece of practical work for this project.

Four trees were brought over to NRI for sampling. The trees sampled were three Hashabi and one Falstaff.

Falstaff is an English apple variety developed at East Malling Research. The other cultivars are based on the Israeli rootstock Hashabi.

The plants were brought over from EMR and collections were carried out in the lab at NRI following the SPME method detailed in Chapter 2.

Young apple leaves were enclosed in a 1l oven bag (Sainsbury's PLC) which was secured around the stem using a plastic tie (Figure 7.2).

Initially volatiles were collected from young growth on each plant in turn. Unfortunately due to the time of year (December) only small amounts of new growth could be seen and even this may have become dormant. To ensure that the leaves tested as shoots were still juvenile and not yet mature, clearly mature leaves were also sampled and the profiles compared.

Figure 7.2. Solid phase microextraction (SPME) collection from an apple, *Malus domestica,* shoot in the lab at NRI.

7.2.3 GC-MS analysis

Volatiles were analysed by GC-MS on the Varian instrument with polar GC column and splitless injection using the method detailed in Chapter 2. A table of common volatile compounds with chemical structures can be found in the Appendix.

7.3. RESULTS

7.3.1 Volatiles collected using entrainment onto Porapak-Q

An example of a GC-MS trace is shown in Figure 7.3. The peaks are listed in Table 7.1.

Only one of the compounds had good matching with the Pherobase; 4-ethylbenzaldehyde which had a peak at 13.22 min.

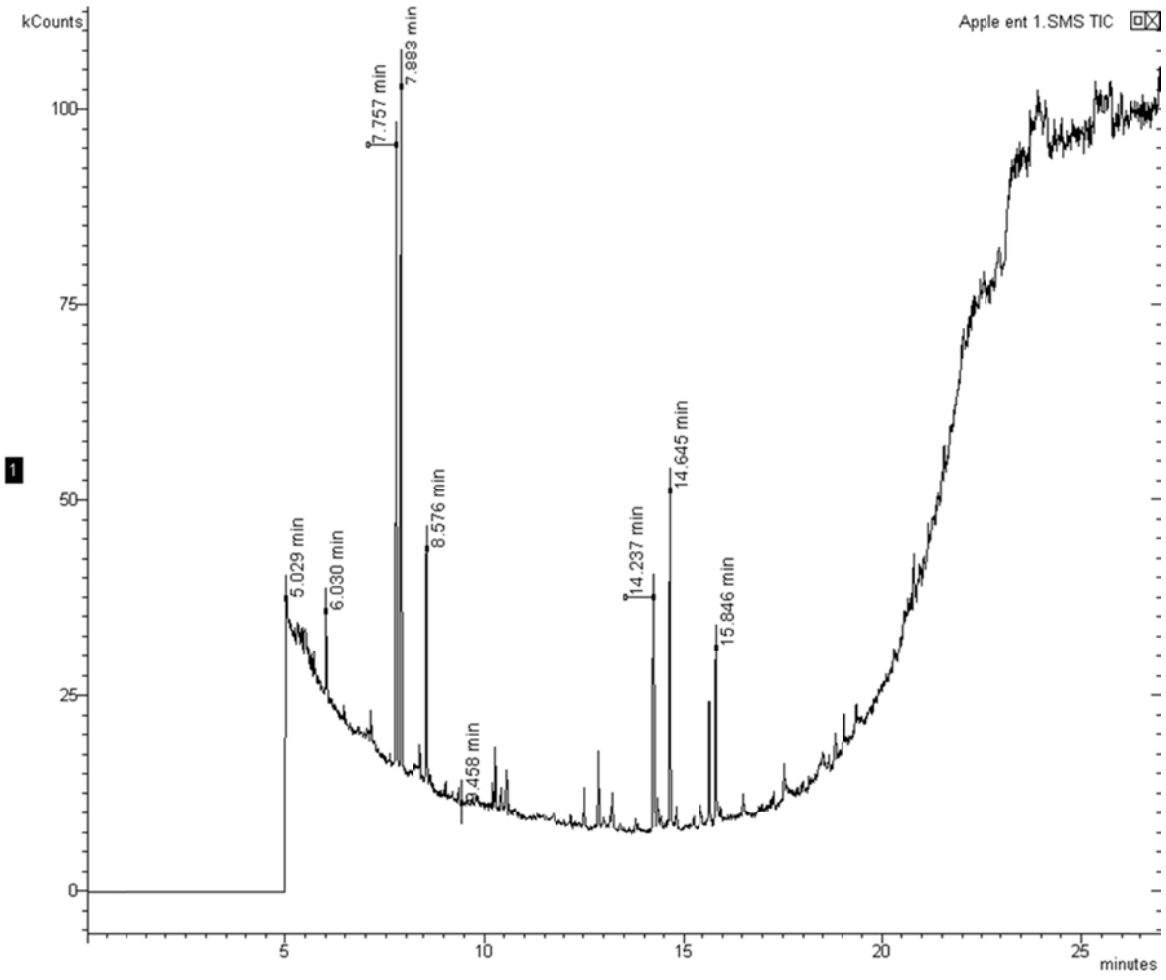


Figure 7.3. Example of a GC trace of the volatiles collected using entrainment from an apple shoot.

Table 7.1. Compounds identified in significant quantities in apple volatiles collected on Porapak Q and analysed by GC/MS (RT = retention time in minutes, Pherobase = Kovats Index for the named compound on the Pherobase (El Sayed, 2012); asterisks in Sol 1 and Sol 2 indicated whether the peak was present in entrainment solutions 1 and 2; Relative quantity = a measure of the relative quantity present in the sample from * to *****).

RT (min)	Compound	Kovats Index	Pherobase	Sol 1	Sol 2	Relative quantity
6.03	Siloxane	1168	na	*	*	**
7.77	Benzene 1,4-diethyl-	1294	na	*	*	*****
8.59	2-Pentene,4-hydroxy-4-methyl	1354	na	*	*	****
10.27	1-Hexene,3,5-dimethyl-	1483	na	*		**
12.86	Ethylbenzaldehyde,	1699	1753	*	*	**
14.23	Benzoic acid, 4-ethyl, 4-cyanophyenyl ester	1822	na	*	*	****
15.68	4-(1-hydroxyethyl) benzaldehyde	1962	na	*	*	***

7.3.2 Volatiles collected using SPME

An example of a GC trace of volatiles collected from an apple shoot by SPME is shown in Figure 7.4.

A table was created of all peaks seen on the GC and the identities based on comparison of the fragmentation patterns to those in the NIST library. The blank SPME run in Chapter 2 was consulted and known SPME fibre contaminants removed from the table and further analysis.

The number of times each peak was seen was noted, as was the peak's presence in shoots and mature foliage. The variety each peak was present in was also noted. This allowed relationships to be identified (Table 7.2).

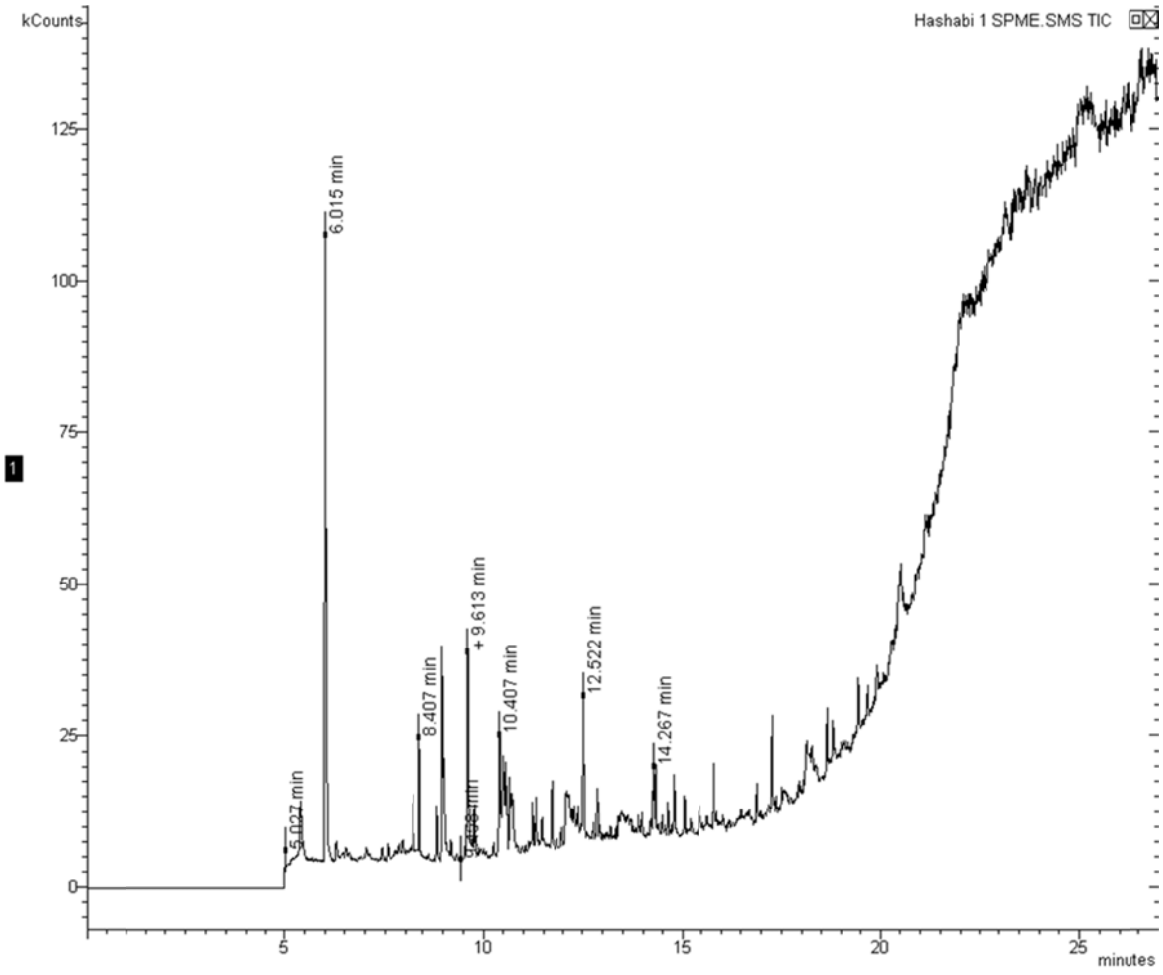


Figure 7.4. Example of a GC-MS trace of the volatiles collected using SPME from an apple shoot, *Malus domestica,* analysed on polar GC column.

Table 7.2. GC/MS Analyses of volatiles from apple shoots collected by SPME and analysed on polar GC column showing how often each peak was seen, whether it was present in shoots and or young foliage and presence in the different apple varieties, and a measure of relative quantity present (* low, ***** high).

RT (min)	Compound	No. times observed	Presence shoots/mature	Presence Hashabi/Falstaff	Relative Quantity
5.42	?	3	S, M	H	**
6.55	(*E*)-2-Hexenal	2	S, M	H, F	*
7.05	(*E*)-β-ocimene	1	S	H	*
7.87	tridecane	2	M	H, F	*
8.85	(*Z*)-3-Hexen-1-ol	3	S, M	H	**
9.03	Nonanal	5	S, M	H, F	****
9.15	(*E*)-2-hexenol	1	S	H	*
9.60	Undecanol	1	M	F	****
9.61	Acetic acid	Always	S, M	H, F	**
10.41	?	Always	S, M	H, F	***
10.51	?	Always	S, M	H, F	***
11.23	?	Always	S, M	H, F	**
11.32	?	Always	S, M	H, F	**

Table 7.2 (cont). GC/MS Analyses of volatiles from apple shoots collected by SPME showing how often each peak was seen, whether it was present in shoots and or young foliage and presence in the different apple varieties, and a measure of relative quantity present (* low, ***** high).

RT (min)	Compound	No. times observed	Presence shoots/mature	Presence Hashabi/Falstaff	Relative Quantity
13.35	(*E,E*)-α-Farnesene	3	S, M	H, F	*
13.59	Methyl salicylate	2	S	H, F	*
14.33	1-Methylethyl dodecanoate	1	S	H	**
14.78	Benzyl alcohol	2	S	H	**
18.81	2-Ethylhexyl salicylate	2	S	H	**
19.46	1-Eicosanol	1	S	H	**

From Table 7.3 it seems that there are differences between the two varieties, although the number of samples was not great enough to carry out statistical analyses and determine whether these differences are significant. Table 7.3 also shows that a suite of peaks were only present in the shoots and this confirms that the leaves tested as shoots were not the same as mature leaves and thus likely to be still growing.

7.4. DISCUSSION

7.4.1 Collection of volatiles on Porapak-Q

Many of the compounds identified from the MS were not on the Pherobase or Flavornet sites which suggests they may not be natural plant volatiles. These chemicals also did not match with those found by Anfora et al. (2005) or Bengtsson et al. (2001). This was only a very small study but it was not taken further. Due to the poor results, focus was shifted to collection of volatiles using SPME fibres.

7.4.2 Collection of volatiles by SPME

Solid phase microextraction was found to be a convenient way of sampling apple volatiles. A suite of volatiles was identified which are produced by immature and mature apple foliage. Those chemicals which showed good Kovats Indices matching to the Pherobase and were concluded to be correctly identified were compared to tables of volatiles found in apple collections in the literature. Table 7.4 shows the findings of this chapter along with the work of Anfora et al. (2005) and Bengtsson et al (2001) studying immature and mature apple foliage respectively. The volatiles identified in this work such as acetic acid and (*E,E*)-alpha-farnesene agree with the literature.

Table 7.4. Table of compounds conclusively identified as present in mature and juvenile apple leaves in this Chapter compared to those identified in the work of Anfora et al. (2005) and Bengtsson et al. (2001) on immature and mature apple foliage respectively.

			This Study	
Compound	**Anfora**	**Bengtsson**	**immature**	**mature**
Acids				
Acetic acid	*		*	*
Hydrocarbons				
Pentadecane	*			
Hexadecane	*			
Tridecane			*	*
4,8-Dimethyl-1,3,E7-nonatriene		*		
Alcohols				
Hexanol	*			
(*Z*)-3-Hexen-1-ol	*	*	*	*
2-Ethyl-hexanol	*			
Octanol	*			
1-Octen-3-ol	*			
Aldehydes				
(*E*)-2-Hexenal	*		*	*
Octanal	*			
Nonanal	*			
Ketones				
6-Methyl-5-hepten-2-one	*			
Esters				
(*Z*)-3-Hexenyl-acetate	*	*		
Hexyl acetate		*		
Geranyl-acetate	*			

Table 7.4 (cont). Table of compounds conclusively identified as present in mature and juvenile apple leaves in this Chapter compared to those identified in the work of Anfora et al. (2005) and Bengtsson et al. (2001) on immature and mature apple foliage respectively.

			This Study	
Compound	**Anfora**	**Bengtsson**	**immature**	**mature**
Aromatic Compounds				
Ethyl benzoate	*			
Benzyl alcohol			*	
Methyl salicylate			*	
Monoterpenes				
β - pinene	*			
S-limonene	*			
Δ3-carene	*			
β - linalool	*	*		
β - ocimene	*	*	*	
α - terpineol	*			
Sesquiterpenes				
β–caryophyllene	*	*		
(*E*,*E*)-α-farnesene	*	*	*	*
(*E*)-beta-farnesene		*		
(*Z*,*E*)-alpha-farnesene		*		
Germacrene D		*		

These findings are promising but more sampling is needed to conclude if there are differences between varieties and different ages of foliage. This was not possible here as work was carried out in December 2013 and the trees sampled were the only ones available showing any sign of young leaves.

There is quite a difference between the findings in this chapter and those of Bengtsson et al., (2001) and Anfora et al. (2005). This may be related to differences in cultivar or in climate or due to the fact that the trees may have been moving towards dormancy as it was late in the growing season. This work led on to the investigation of which compounds present in the apple volatile profile which

D.mali can detect and the use of the EAG technique already used for the blackcurrant leaf curling midge.

Chapter 8
EAG STUDIES ON APPLE LEAF MIDGE

8.1. INTRODUCTION

As mentioned in Chapter one, mated female *Dasineura mali* lay eggs on young apple foliage. Volatiles produced by immature apple (*Malus domesticus* Borkh) foliage were identified in Chapter 7 and the following work sought to identify which of these volatiles female midges could detect. Once identified these volatiles could then be used in behavioural work to determine if they are attractive and a blend developed to attract females in the field.

In early 2013 a supply of apple leaf midge, *Dasineura mali* Kieffer, larvae was obtained from New Zealand and used for EAG studies In Autumn 2013 work was continued using midges collected from a mixed orchard at East Malling Research.

8.2. MATERIALS AND METHODS

8.2.1 Plant material

Early 2013

As this work was to be carried out during winter when new young foliage was not available in the field, another source had to be found for volatile collections. In November 2012 rootstocks of the variety MM106 were taken from the cold store at EMR and potted up in compost. The plants were then placed in a glass house compartment with lights used as needed to provide 16 hour day conditions with lights coming on when the external light levels fell below 15 K Lux. Temperatures in the glass house did vary with time of day and year but there was an average temperature of 15°C at midnight and 22.5°C at midday for the year from November 2012 to November 2013. The plants were left until young shoots had been produced.

Autumn 2013

Volatiles were collected from apple trees which were located in a polytunnel at EMR. The plants were part of a breeding experiment run by Feli Fernanadez. As they had been kept under cover these plants still had some new growth from which shoot volatiles could be collected.

8.2.2 Volatile collection

Early 2013

Collections of volatiles on Porapak-Q were carried out using the method detailed in Chapter 2 except that a 1 l oven bag (Sainsbury's PLC) was put around the apple shoots alone without a cage. Six collections were made for periods of 24 – 72 hr.

The collections were analysed by GC-MS, with Kovats Indices calculated and compared to those in the Pheroase (El Sayed, 2012) to provide peak identities as detailed in Chapter 2.

During this analysis it was found that two of the solutions had the greatest quantity of volatiles and as one of these was selected for use in GC-EAG analyses.

Autumn 2013

Volatiles were collected on Porapak-Q from two plants from the polytunnel – the varieties are unknown but were both on M9 rootstocks and had the codes E611-281 and E699-4. Collections were carried out using the portable entrainment kit in situ in the polytunnel. The same method was used as detailed as above. Both plants were sampled at the same time for 4 hours.

Due to the low number of insects, EAG work was begun before the solutions had been analysed using the GC-MS.

Later in the autumn the solutions (1µl) were injected into the GC-MS. Analysis was carried out on any peaks using the MS patterns in conjunction with the NIST library as detailed in chapter 2. A table of common volatile compounds with chemical structures can be found in the appendix.

8.2.3 Insects

Early 2013

Foliage infested with apple leaf midge larvae was sent from New Zealand by Peter Shaw (Plant and Food Research, Motueka, New Zealand). This foliage was put into a nylon rearing cage (0.3 m x 0.3 m) with damp tissue. The cage was covered with a clear plastic bag to reduce drying in the cage. The cage was located in a climate controlled room set at 22°C with 18 hr light and 6 hr dark each day, in the NRI insectaries. The cage was checked every 2-4 days and water was added to keep the larvae from desiccating.

Once the larvae started to emerge, the cage was checked daily and adults removed, sexed and taken for EAG. In the end mating status was not assigned as it was difficult to determine and there were only a limited number of females available.

Autumn 2013

Infested foliage was collected from a mixed variety orchard onsite at East Malling Research and a polytunnel of experimental breeding varieties. The foliage was put into a nylon rearing cage (0.3 m x 0.3 m) with damp tissue. The cage was checked regularly and water added to the cage to prevent larval desiccation. Once emergence had begun the cage was checked daily and adults were removed and sexed.

Apple leaf midges emerge between 6 and 10 am and mate shortly after (Galanihe and Harris, 1997). Insects were only collected after 11 am to allow time for the insects to emerge and mate. Initially all adults which had emerged were removed to individual plastic pots when the cage was checked each day, but it was found

that insects did not survive well in the individual pots and connections could only be established when they had just been removed from the cage. As a result the midges were removed as needed. Mating status were not assigned as it was difficult to determine.

8.2.4 GC-EAG analyses

Early 2013

The effect of the apple volatiles on the antennae of the apple leaf midges was investigated using the GC-EAG set up described in Chapter 2. Two microliters of apple volatile solution was injected into the GC. The EAG delivery system used in this work gave a continuous stream of volatiles in humidified air, rather than the puff system used in some parts of the blackcurrant midge work.

GC peaks which were responded to only once were discounted from further analysis. Kovats Indices were used to match peaks responded to those identified on the GC-MS. The patterns of the GC traces from the GC-MS and GC-EAG were also compared so that peaks could be identified based on the MS.

Autumn 2013

The same methods were used as earlier in the year. The only difference was that the volatiles were delivered to the antennal preparation using the puff system with volatiles delivered in three second dry puffs separated by 17 seconds of humidified clean air. This system should concentrate the volatiles, leading to greater responses. During the course of the GC-EAG runs the solution was concentrated under a stream of nitrogen to twice its initial concentration (half the original volume). Then later in the experiment the solution was concentrated by further halving the volume making it four times as concentrated as the initial solution.

8.2.5 Use of synthetic solutions in autumn 2013

After problems with the natural entrainments it was decided that EAG should be carried out using synthetic versions of volatiles found in apple entrainments in the literature (Anfora et al 2005 and Bengtsson et al 2001) and the findings of Chapter 7. The Kovats Index for each compound was identified using the Pherobase (El Sayed, 2012) or, if Kovats were not found on the Pherobase, Flavornet (Acree and Arn, 2004) was consulted. The compounds were then allocated to one of six blends with care taken to include a spread of retention indices so that no compounds with similar retention times were included in the same blend as these would be hard to distinguish between during analyses. Table 8.1. shows compounds including retention indices and to which blend (A-F) they were assigned.

Solutions contained 10 ng/µl concentration of each compound in hexane. Each day females were collected which had emerged over the last 24 hr. Females were collected at 11am, this would have allowed time for mating to occur although it was difficult to determine the mating status of the females so this data was omitted from further analysis. For each EAG run 2µl of solution was injected and the same temperature programme as used as with the natural collections. Females were run with each of the solutions in turn although none survived a full six runs.

Table 8.1 Volatiles identified in apple samples (based on Table 7.3) and by Anfora et al. (2005) in immature leaves and Bengtsson et al. (2001) in mature foliage with Kovats Indices and which blend they were in ((Z,E)-alpha-farnesene was not included in any of the synthetic blends as it was only present in the mature sample in the Bengtsson et al. (2001) paper).

			This Study		Pherobase	
Compound	**Anfora**	**Bengtsson**	**immature foliage**	**mature foliage**	**KI (DB 5)**	**Blend**
Acids						
Acetic acid	*		*	*	600	A
Hydrocarbons						
Pentadecane	*				1500	A
Hexadecane	*				1600	A
4,8-Dimethyl-1,3,E7-nonatriene		*			1302 (DB Wax)	F
Alcohols						
Hexanol	*				851	B
(Z)-3-Hexen-1-ol	*	*	*	*	857	D
2-Ethyl-hexanol	*				1032	A
Octanol	*				1072	B
1-Octen-3-ol	*				978	B

Table 8.1 (cont) Volatiles identified in apple samples (based on Table 7.3) and by Anfora et al. (2005) in immature leaves and Bengtsson et al. (2001) in mature foliage with Kovats Indices and which blend they were in ((Z,E)-alpha-farnesene was not included in any of the synthetic blends as it was only present in the mature sample in the Bengtsson et al. (2001) paper).

			This Study		Pherobase	
Compound	**Anfora**	**Bengtsson**	**immature foliage**	**mature foliage**	**KI (DB 5)**	**Blend**
Aldehydes						
(*E*)-2-Hexenal	*		*	*	854	C
Octanal	*				1004	C
Nonanal	*				1104	C
Ketones						
6-Methyl-5-hepten-2-one	*				985	D
Esters						
(Z)-3-Hexenyl-acetate	*	*			1007	E
Hexyl acetate		*			1008	A
Geranyl-acetate	*				1383	B

Table 8.1 (cont) Volatiles identified in apple samples (based on Table 7.3) and by Anfora et al. (2005) in immature leaves and Bengtsson et al. (2001) in mature foliage with Kovats Indices and which blend they were in ((Z,E)-alpha-farnesene was not included in any of the synthetic blends as it was only present in the mature sample in the Bengtsson et al. (2001) paper).

Compound	Anfora	Bengtsson	This Study		Pherobase KI (DB 5)	Blend
			immature foliage	mature foliage		
Aromatic Compounds						
Ethyl-benzoate	*				1170	B
Benzyl alcohol		*			1039	D
Methyl salicylate		*			1191	C
Monoterpenes						
β-pinene	*				980	F
S-limonene	*				1036	E
Δ3-carene	*				1009	F
β-linalool	*	*			1098	D
β-ocimene	*	*	*		1050	F
α-terpineol	*				1189	E

Table 8.1 (cont) Volatiles identified in apple samples (based on Table 7.3) and by Anfora et al. (2005) in immature leaves and Bengtsson et al. (2001) in mature foliage with Kovats Indices and which blend they were in ((Z,E)-alpha-farnesene was not included in any of the synthetic blends as it was only present in the mature sample in the Bengtsson et al. (2001) paper).

			This Study		Pherobase	
Compound	**Anfora**	**Bengtsson**	**immature foliage**	**mature foliage**	**KI (DB 5)**	**Blend**
Sesquiterpenes						
β–Caryophyllene	*	*			1428	C
(*E*,*E*)-α-Farnesene	*	*	*	*	1508	B
(*E*)-β-Farnesene		*			1458	F
(*Z*,*E*)-α-Farnesene		*				
Germacrene D		*			1480	E

8.3. RESULTS

8.3.1 Early 2013

Examples of GC-EAG traces are shown in Figures 8.1 and 8.2.

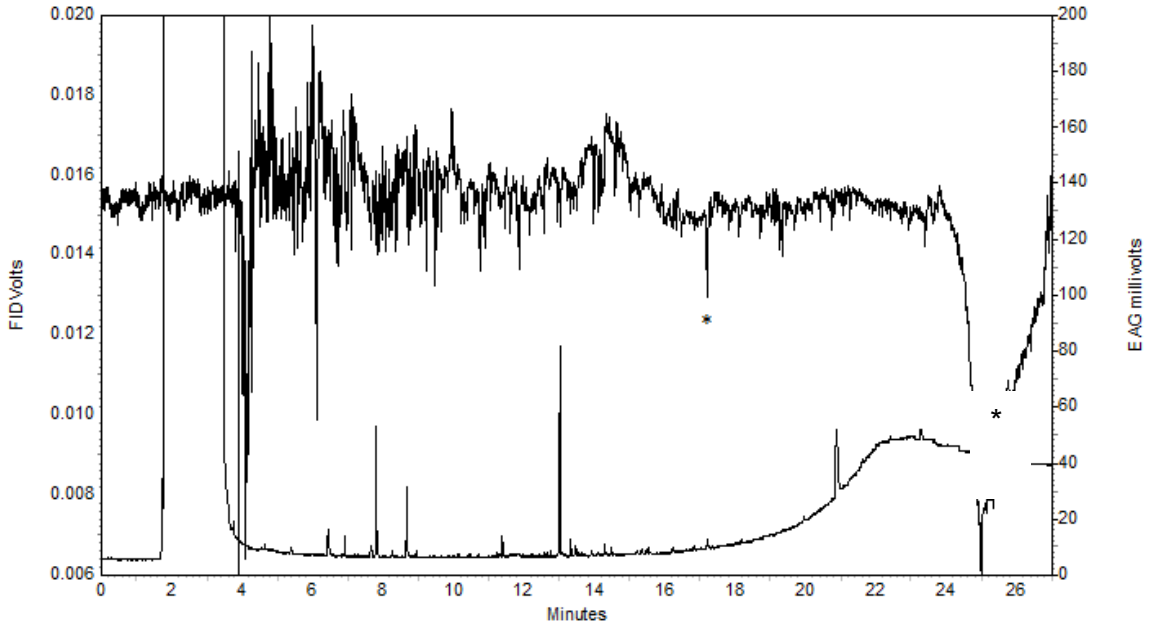


Figure 8.1 An example of an GC-EAG trace with female *Dasineura mali* and sample of apple volatiles collected on Porapak-Q (EAG response at 17.20 minutes to a small peak on the GC later identified as a probable siloxane contaminant – marked with an asterisk). The top trace is the EAG and the bottom trace the FID.

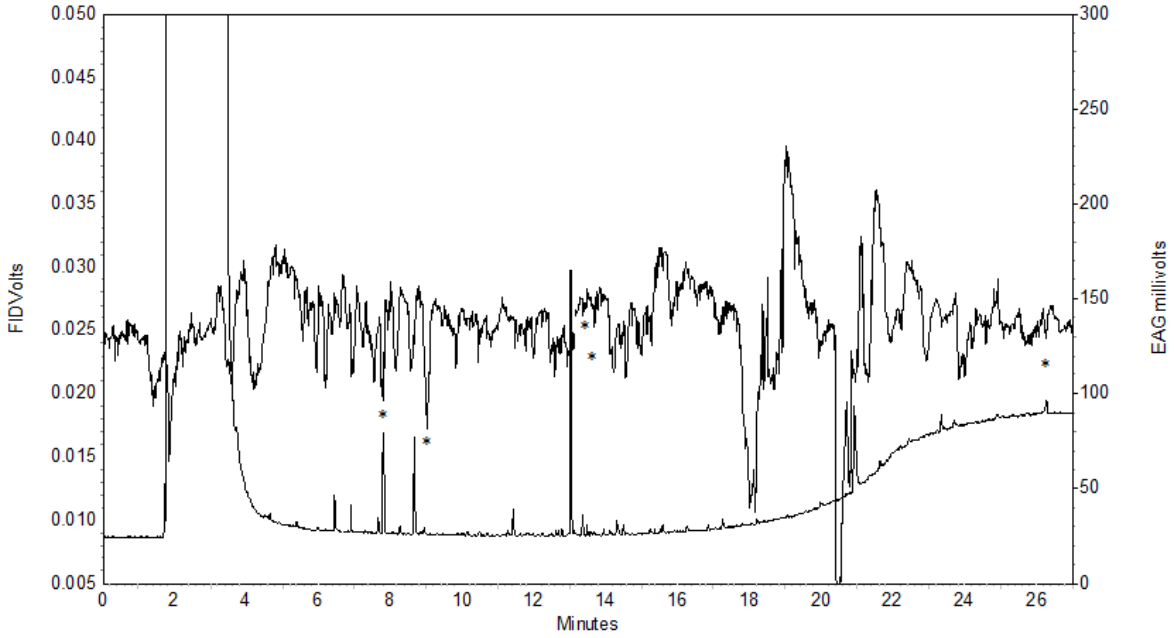


Fig 8.2 Example of GC-EAG trace of female *Dasineura mali* showing EAG responses to GC peaks at 6.91 (3-carene), 7.79 (Cyclohexane, 2-ethyl-1-1-dimethyl-3-methylene), 8.98 (unknown), 13.36 (methyl salicylate), 13.49 1-cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl)- and 26.27 (unknown) in an apple entrainment sample – responses marked with asterisks. The top trace shows the EAG and the bottom trace the FID.

Peaks on the GC which were responded to by more than one EAG preparation were identified using Kovats Indices and MS data (Table 8.3).

Table 8.3. Identities of peaks in apple entrainment sample responded to more than once by female *Dasineura mali* in GC-EAG analyses on polar GC column as determined by comparison with Kovats Indices in GC-MS analyses (RT=retention time, relative quantities * low, ***** high).

RT (min)	Kovats Index	No. responses	Compound	Relative Quantities
3.73	1002	2	(in solvent peak)	*
6.42	1217	3	(*E*)-2-Hexenal	***
6.91	1254	3	3-Carene	***
7.66	1309	3	(Z)-3-Hexen-1-ol acetate	*
7.81	1320	2	Cyclohexane, 2-ethyl-1-1-dimethyl-3-methylene	***
8.68	1385	2	(*Z*)-3-hexen-1-ol	**
8.96	1407	3	Unknown	*
11.41	1608	4	Caryopyllene	**
13.02	1752	2	α-Farnesene	*****
13.33	1779	2	Methyl salicylate	**
13.5	1795	3	1-cyclohexene-1-carboxaldehyde,4-(1-methylethenyl)-	*
14.3	1873	3	2-Methyl-propanoic acid	*
15.25	1967	2	Siloxane	*
16.81	2130	2	Unknown	*
17.2	2172	5	Siloxane	*
22.49	2788	2	Unknown	*
26.2	3222	2	Unknown	*

Some of the peaks were identified as siloxane compounds from the Porapak-Q or GC column or septum and are thus contaminants not present in the apple volatile profile. Some of the peaks were not identified on the MS.

To check the identity of the peaks identified as responded to on the GC-EAG available synthetic compounds were injected into the GC-MS. The solutions were all made up at a concentration of 10 ng/µl. Hydrocarbon standards were also run to allow Kovats Indices to be calculated for the synthetics.

The Kovats Indices for peaks identified as caryophllene, (*E,E)*-α farnesene and methyl salicylate show good matching to the Kovats Indices of the synthetic compounds indicating that these peaks were identified correctly. It was not possible to calculate the Kovats Indices for 3-carene as the hydrocarbon standards below C12 (RT 6.646) came through during the solvent delay.

Of the 26 runs carried out 20 showed at least one depolarisation.

8.3.2 Autumn 2013

GC-EAG Analyses of collection of apple volatiles on Porapak-Q

A total of twelve runs were carried out using a solution of apple volatiles collected over 4 hr on Porapak-Q. Of these runs only 3 females showed any EAG responses. The responses are shown Table 8.5.

Table 8.5. Responses of female *Dasineura mali* in GC-EAG analyses of volatiles from apple collected on Porapak-Q.

	Magnitude of response (mV)							
Noise level (mV)	**5.0 min**	**6.1 min**	**6.8 min**	**8.1 min**	**9.4 min**	**17.4 min**	**18.4 min**	**25.6 min**
3.8			10.0					
0.15		0.25				0.21		
0.7	1.7			2.4	1.4		1.1	1.3

The GC output on the GC-EAG set up showed no significant peaks in the one sample used. This was further corroborated by the fact that no real peaks were found in the sample when it was injected into the GC-MS. This was probably due to the sort collection time and this was not pursued further.

EAG with synthetic blends

In GC-EAG analyses of synthetic compounds, depolarisation responses were only seen in four runs, two with solution C and one with solutions B and D respectively. The results are shown in Table 8.6. There were a total of 18 runs – 6 with solution B, 4 with solution C, 4 with solution D,2 with solution E and 2 with solution F. No runs were carried out with solution A due to lack of time.

Only two of the EAG responses lined up with peaks on the GC. These were the responses at 11.0 min and 15.1 min seen for the first run of solution C (highlighted in bold). These were to caryophyllene (at 11.0 minutes) and caryophyllene oxide (at 15.1 min), which was not intentionally included in the blend and only gave a small peak on the GC. As these only occurred once there is not enough evidence to draw any clear conclusions at the present time.

Table 8.6. Responses or female *Dasineura mali* to solutions of synthetic compounds (responses in bold correspond to GC peaks).

Solution	Noise Level (mV)	Magnitude of response (mV)														
		7.5 min	**11 min**	**11.6 min**	**13.9 min**	**14.1 min**	**14.2 min**	**14.3 min**	**15.1 min**	**16 min**	**18.6 min**	**19.2 min**	**19.6 min**	**20.8 min**	**21.8 min**	**22.9 min**
C	0.4		**0.6**						**0.8**							1.8
B	0.6									1.0						
C	0.9				1.1	4.5	2.0	1.6								
D	1.8											2.0			2.0	

8.4. DISCUSSION

The initial GC-EAG work in early 2013 suggested that female apple leaf midges were able to detect a subset of chemicals within the apple shoot volatile profile. Some of the volatiles identified as responded to were also identified as present in apple volatile collections in one or both the studies by Anfora et al. (2005) and Bengtsson et al. (2001). These were (*E*)-2-hexenal, 3-carene, (*Z*)-3-hexen-1-ol, caryopyllene, α-farnesene and methyl salicylate. Some of the compounds responded to were contaminants from the Porapak-Q collection system (siloxane compounds) and these were discounted from further investigation. Other compounds were tentatively identified from the MS and seen to be responded to but were not found in other apple literature. The findings were promising, but as each peak was only responded to in a small number of the total runs it was still possible that the responses may not have been significant. The most responded to peak was the siloxane peak at 17.2 minutes and this was only responded to 5 times out of 26 runs.

As these results were inconclusive more GC-EAG analyses were carried out in autumn 2013. However, the volatile collection used did not contain significant quantities of volatiles. This was most likely due to the short collection time.

Attention was moved to the testing of synthetic chemicals. However, in only very few runs were EAG responses seen.

One of the key problems with this GC-EAG work is the fact that many preparations failed during runs as the insect died or the antennae became disconnected from the electrode. Of the runs in which signal was established only a few showed responses to the compounds injected into the GC. This may be because apple leaf midge females are not able to detect compounds in the collections of apple volatiles made. However, this disagrees with the findings of Galanihe and Harris (1997) who showed that mated female apple leaf midges would fly upwind to apple foliage even when it was obscured suggesting volatiles were involved. It is also possible that the insects were not in good condition. In early 2013 the insects used had travelled through the post from New Zealand. The work later in the year used the last generations of insects which would naturally have been approaching diapause.

Another problem was assessing the mating status of the females. Using a cage increased the chances of mating happening, as males and females emerged naturally together, but it was hard to tell if the females were mated. Attempts were initially made to observe females for calling but this was time-consuming with the numbers of midges low and adults dying within a few hours of collection from the cage. Attempts to determine mating status were therefore abandoned. Galanihe and Harris (1997) found that mated females were attracted to apple foliage in the wind tunnel but they did not test the responses of virgin females. Crook and Mordue (1999) found that mated but not virgin female *D. tetensii* were attracted to their host plant, blackcurrant, in olfactometer studies and this may have been due to the fact that virgins could not detect the host plant volatiles. It is possible that virgin female apple leaf midges cannot detect apple volatile compounds as they do not need to be attracted to apple trees for egg laying. If the majority of insect tested in this chapter were not mated then this might explain the lack of antennal responses to apple volatiles.

In future work females could be dissected under a microscope after being used for EAG runs to look for sperm in the spermathecae – this would definitively prove their mating status so that only data for mated females is included in analysis.

Overall the early 2013 work suggests the possibility that female apple leaf midges can detect certain volatiles but much more EAG work is needed to prove this conclusively. Behavioural work is also needed to determine what effect these volatiles have on the insect behaviour in the lab and field.

Chapter 9
GENERAL DISCUSSION

This research set out to identify the volatiles produced by host plants which attract gall midges of three different species which cause problems in horticultural crops in the UK with a view to creating a lure to attract mated females. The midges in question were the raspberry cane midge, *Resseliella theobaldi* Barnes, the blackcurrant leaf curling midge, *Dasineura tetensi* Rübsaamen, and the apple leaf midge, *D. mali* Kieffer.

9.1. IDENTIFICATION OF HOST PLANT VOLATILES

For each of the midge species the first step was to identify the volatiles produced by host plant material at the appropriate developmental stage and in the appropriate condition for female midge oviposition. These volatiles formed the basis of further work on behavioural and physiological activity.

Solid phase microactraction (SPME) was found to be a convenient way of capturing volatiles rapidly which was especially important for the sampling of the burst of volatiles emitted by split raspberry, *Rubus idaeus* Linneaus, canes, the site of egg laying for female *R. theobaldi*, as it is rapid (Prosen and Zupančič-Kralj, 1999). The volatile collections were analysed using gas chromatography linked to mass spectrometry (GC-MS) which allowed the identification of compounds from both their MS fragmentation patterns and their retention times through the use of Kovats Indices. This allowed compounds to be reliably identified.

These collections fulfilled objective (a) as detailed in Chapter 1 of this thesis.

9.1.1 Raspberry cane volatiles

A suite of volatiles was identified which were present only or in much larger amounts after canes split. These chemicals were: linalool, myrtenal, citronellol, neral and geranial. These along with (*Z*)-3-hexenol, 6-methyl-5-hepten-2-ol, methyl salicylate, myrtenol, benzyl alcohol, nerol and geraniol were identified as chemicals produced when raspberry canes are split by Hall et al. (2011).

Interestingly (*Z*)-3-hexenol, 6-methyl-5-hepten-2-ol and methyl salicylate were identified in the SPME collections. In the case of (*Z*)-3-hexenol it was actually seen to decrease after splitting. The amount of 6-methyl-5-hepten-2-ol did increase after splitting but this increase was not as great as for the other compounds listed above and methyl salicylate was only present in very small quantities both before and after splitting.

Myrtenal, geranial and neral are the products of the oxidation of myrtenol, nerol and geraniol and it is possible that these alcohols were present when the canes split but quickly oxidised to their respective aldehyde forms which were observed.

Benzyl alcohol was identified by Hall et al. (2011) in split cane collections but was not found in this study.

Other than the work of Hall et al (2011) a comparison of split and intact cane volatiles has not been carried out in the literature.

9.1.2 Blackcurrant shoot volatiles

A range of compounds was identified which were produced by young blackcurrant shoot tips. The main components were the monooterpene hydrocarbons 3-carene and α-terpinolene, with smaller amounts of (*Z*)- and (*E*)-β-ocimene.

Although shoot volatiles were used in the behavioural work of Crook and Mordue (1999) with female *D.tetensi,* this is the first time shoot headspace volatiles have been identified systematically.

9.1.3 Apple shoot volatiles

A suite of compounds was identified which were produced by immature apple foliage. The compounds showed good matches with Kovats Indices on the Pherobase and were thus concluded to have been identified correctly. Several of these compounds were also found in the study of shoot volatiles by Anfora et al. (2005) (Table 9.1).

Table 9.1. Compounds identified as produced by apple shoots in this study and in a paper by Anfora et al. (2005).

Compounds	**Present in Anfora et al. (2005)**
(*E*)-2-Hexenal	Yes
(*E*)-β-Ocimene	Yes
6-Methyl-5-hepten-2-one	No
(*Z*)-3-Hexen-1-ol	Yes
Acetic Acid	Yes
(*E,E*)-α-Farnesene	Yes
Methyl Salicylate	No
Benzyl Alcohol	No

9.2. GAS CHROMATOGRAPHY LINKED TO ELECTROANTENNOGRAPHY (GC-EAG) ANALYSES

Use of gas chromatography linked to electroantenography (GC-EAG) allows the identification of compounds which the insect can detect. It was used during the identification of female attractive host volatiles produced by wheat, *Triticum aestivum* Linnaeus, which attract female orange wheat blossom midge, *Sitodiplosis mosellana* Géhin (Birkett et al. (2004)). EAG was also used successfully by Amarawardana (2009) to identify the female-produced pheromones of several gall midge species.

Entrainment was found to be the best method of volatile collection for EAG as it allows the creation of a solution which can be used in multiple runs and can also be concentrated.

EAG would have been carried out for all three midge species but was not carried out for *R. theobaldi* as a significant population could not be found after 2011.

This work fulfils the objective (b) as detailed in Chapter 1.

9.2.1 GC-EAG studies with *Dasineura tetensi*

In GC-EAG analyses of collections of volatiles from blackcurrant shoots with a female *D. tetensi* EAG preparation, only ten peaks on the GC had more than one EAG response. Two of these were contaminants from the Porapak-Q collection system and were thus excluded from further analysis. None of the compounds was responded to more than three times; and several remain unidentified. Overall there is not enough reliable data to draw conclusions regarding which compounds the females can detect.

During this work there was no reliable method of obtaining mated females. In this case females were assumed to be mated if males were present in the cage on the day of collection. It is possible that changes in the antennal receptors occur when the females mate and this makes them receptive to the shoot volatiles. If this is the case and the females tested here were virgin then it would explain the lack of responses. Crook and Mordue (1999) found that in an olfactometer female *D. tetensi* were attracted to blackcurrant foliage when mated but not when virgin. Antennal changes following physiological events have been seen on mosquitoes. Qiu et al (2006) saw both up and down regulation of olfactory receptor neurones in female *Anopheles gambiae* (Giles) following a blood meal leading to suppression of host seeking.

9.2.2 GC-EAG studies with *Dasineura mali*

The work in early 2013 suggested that female apple leaf midges respond to a subset of the natural volatiles produced by apple shoots. Some of these volatiles matched those found in apple entrainments by Anfora et al. (2005). These were: (*E*)-2-hexenal, 3-carene, (*Z*)-3-hexen-1-ol, caryophyllene, (*E,E*)-α-farnesene and methyl salicylate. Some of the compounds responded to were contaminants from the Porapak-Q entrainment system and were thus excluded from analysis. As well as the identification of volatiles, Anfora et al. (2005) also carried out some EAG work with *D.mali* using synthetic volatiles identified as produced by apple foliage. Mated females responded significantly to 1-hexanol, (*Z*)-3-hexen-1-ol, α-terpineol and 6-methyl-5-hepten-2-one. This agrees with my findings that females respond to (*Z*)-3-hexen-1-ol.

However in the study in early 2013 only a small number of responses were seen to each of the peaks and as a result further work was undertaken in autumn 2013. However, the volatile solution used contained negligible amounts of volatiles, probably due to the short collection time used.

Only a few EAG runs were carried out with synthetic volatiles as not many insects were available. No significant responses were found to any of the synthetic compounds tested.

There were many failed runs throughout all the EAG work with problems achieving a good connection. Possibilities for lack of responses include the fact that females may not have been mated and the fact that the midges may not have been in good condition. In early 2013 they had travelled though the post from New Zealand and in autumn 2013 they were at the end of their season.

There are also conflicting results in the literature. In behavioural work Anfora et al. (2005) found that of the four compounds identified as EAG-active only one produced a behavioural response, (*Z*)-3-hexen-1-ol, and this was found to repel females. Galanihe and Harris (1997) found mated females to be attracted to apple volatiles in the wind tunnel. It is possible that midges may be using a blend of volatiles in a specific ratio to locate their host and this is why the single volatiles used by Anfora et al. (2005) were not attractive.

9.3. LABORATORY BIOASSAY WORK

GC-EAG allowed the identification of compounds which the midge antennae can detect but it does not tell us if these compounds lead to a behavioural response or the nature of any behavioural response. To find out these things bioassay work is needed.

Two bioassays were selected based on their successful use with midges in the literature. Small olfactometers based on the work of Pettersson (1970) and Vet et al. (1983) were used successfully with *D. tetensi* by Crook and Mordue (1999) to show mated but not virgin females were attracted to blackcurrant shoots. These olfactometers were also used by Birkett et al. (2004) to show attraction of female *S. mosellana* to wheat volatiles.

Attraction was also investigated using a wind tunnel. This was selected as it had successfully been used by Galanihe and Harris (1997) to demonstrate attraction of female *D.mali* to apple volatiles.

The bioassays were used only on *D.tetensi* as this was the most abundant of the three midges during the period of research.

This partly fulfils objective (c) as detailed in Chapter 1 although both bioassays were not very successful and only *D. tetensi* was tested.

9.3.1 Wind tunnel and olfactometer work with *Dasineura tetensi*

In neither the wind tunnel nor the olfactometer was attraction seen to host plant material. These results are not what was expected and disagree with the work of Crook and Mordue (1999) who found that mated female *D. tetensi* were attracted to plant material in an olfactometer bioassay. However Crook and Mordue (1999) also found that virgin females were not attracted and this may explain my results. Crook and Mordue (1999) put pairs of midges together and observed for mating, this was found here to be too time consuming for a single experimenter to undertake as individual pairs had to be observed for many hours. So far a satisfactory method to provide mated females reliably has not been found and

even females not seen to be calling during the observation period then often called once in the windtunnel/olfactometer. It is thus likely that many or even all midges used were virgin, hence the lack of attraction.

Another explanation stems from the fact that fluorescent lights were used to illuminate both the wind tunnel and the olfactometer. These lights flicker at 50 Hz in the UK and although not distinguished by the human eye this flickering would be obvious to the midges and may disrupt their natural behaviour.

9.4. FIELD BIOASSAY

Field bioassays offer a chance to test female attraction under natural conditions and are the best way to test lures. As a lure had already been developed by Hall et al. (2011) a field experiment was developed to assess its attractiveness with a view to using this method with other lures developed throughout the project.

This also partly fulfils objectives (c) and (d) as detailed in Chapter 1 although the lure tested was not found to be attractive to female *R. theobaldi*.

9.4.1 Field bioassay with *R. theobaldi*

Lures based on the work of Hall et al. (2011) and the work in Chapter 3 were field tested on a farm in Kent. The aim was to see if artificial forms of the volatiles produced when canes split were attractive to female midges in the field. A range of trap types was also tested.

Numbers of females were low throughout the field experiment and no significant differences were seen between the number of females caught in baited and un-baited traps. There were also no significant differences in the number of females caught in the different trap types.

Hall et al. (2011) found that there was some attraction of midges in the field using baits with the same composition as used here. As in this study low numbers of females were caught, but significantly more males were caught in traps baited

with the synthetic blend than in un-baited traps in some trials. This was not seen in this project with neither male nor female catch numbers affected by the presence of the lure.

In the field traps baited with excised cane sections representing the natural split cane situation and acting as a positive control, significant numbers of midges were not caught relative to catches in unabaited traps. It is possible that the cut canes produced a different volatile blend to those on the living plant and they dried out quickly. If repeating this study it might be better to use living canes as the control.

This field trial may not have worked as the blend of chemicals used in the lures was not correct or simply because the female midges were put off by the appearance of the traps.

9.5. FURTHER WORK

9.5.1 General further work

One key problem identified during this work was the difficulty of obtaining mated female midges. It is important that a method is identified before further work is carried out. One possibility, although time consuming, would be to put plant material into a rearing cage and observe each day once emergence has begun for a period of hours during peak emergence, removing females as they are seen to be mated.

For all species it would be useful to carry out EAG with synthetic versions of the compounds identified in the SPME work as different concentrations can thus be tested. Blends of synthetics can also be tested in the olfactometer and wind tunnel with different components and ratios to identify the most attractive lure blend.

Problems were encountered with the EAG in terms of getting a connection and a stable base line. This problem was not observed with other insects being worked on (by other scientists) at these times, and is presumably due to the delicate nature of the midges and their possible poor condition.

Once blends have been identified which are attractive in the laboratory, synthetic lures can be developed and tested in the field using a similar method to that detailed in Chapter 4.

9.5.2 Specific further work by species

R. theobaldi

EAG and bioassay work still needs to be carried out to identify compounds which mated females can detect and test behavioural responses. These results can be used to develop a lure which can be field tested for attraction on natural populations.

D. tetensi

Although EAG and bioassay work has been carried out, a clear set of compounds eliciting antennal responses and attraction has not been identified. This work should be repeated once a reliable method of obtaining mated females has been identified. Repetition of bioassay work would also be valuable with females which have definitely been mated.

D. mali

Although some compounds which were responded to a small number of times on the EAG were identified it would be useful to repeat this work with a larger number of females which are definitely mated. It would also be of value to test natural and synthetic volatiles in the olfactometer and wind tunnel bioassays.

9.6. FINAL CONCLUSIONS

Although much of the work in this project did not run to plan and results were not as expected a number of different techniques were used and a number of methods developed.

Referring back to the objectives listed in Chapter 1:

(a) Compounds produced by all three host plants at the development stage at which female midges lay eggs were identified.

(b) A GC-EAG method was developed for use with midges based on the work of Amarawardana (2009).This method was used on *D. mali* and *D. tetensi* but no definitive EAG-active compounds were found. The method involved the placing of the insect body in a pulled capillary tube filled with electrolyte (acting as the reference electrode) and the tip of the antenna into another capillary tube (acting as the recording electrode). It was found that with midges it was best to use insects as soon as they had been removed from the rearing cage and that desiccation is a major cause of mortality.

(c) Windtunnel and olfactometer bioassays were developed in the laboratory to test attraction to natural and synthetic material. *D.tetensi* were tested using natural plant volatiles but clear attraction was not seen in either bioassay.

(d) Field tests were carried out with natural split canes and synthetic lures based on the volatiles produced by split raspberry canes. Female *R. theobaldi* were not significantly attracted to the lures relative to unbaited traps.

(e) Lures which are attractive to the female gall midges have not yet been developed.

Novel aspects of this research can be seen summarised below:

- This is the first time volatile collections from split raspberry canes have been compared for two varieties, one autumn and the other spring fruiting. Variety was not found to have a significant difference on volatile profile.
- This is the first time EAG has been carried out on *D. mali* and *D. tetensi* to look for EAG active compounds in mated females.
- Novel trap types were used in the field to try and catch female *R. theobaldi* using synthetic host volatile baits.
- It is the first time a wind tunnel of the specification detailed in this project has been used for any of the three midge species.

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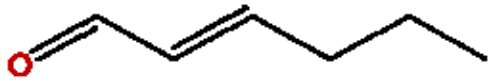
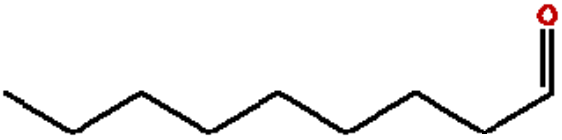
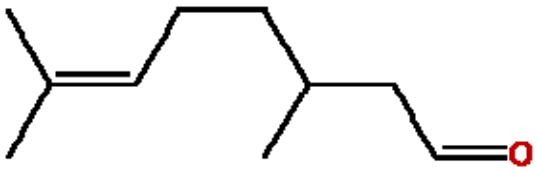
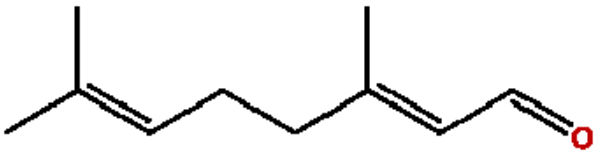
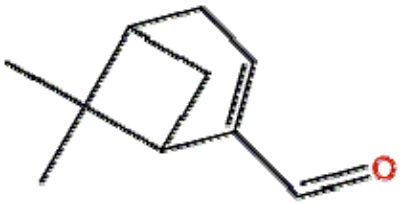
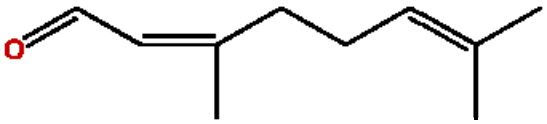
APPENDIX

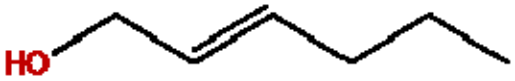
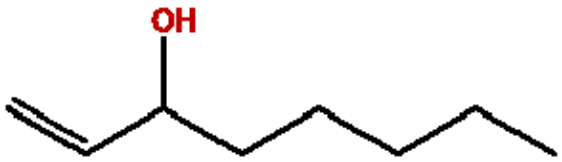
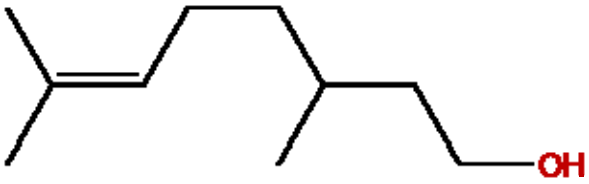
Table. (A-J) Common plant volatile compounds mentioned in this thesis with common names, chemical names and structures taken from the Pherobase (El Sayed 2012).

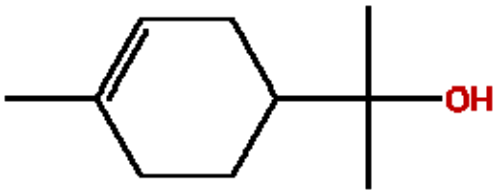
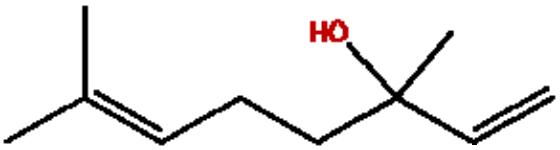
A) Aldehydes

Common Name	**Full name**	**Chemical Structure**
(*E*)-2-Hexenal	(*E*)-2-Hexenal	
Decanal	Decanal	
Dodecanal	Dodecanal	
Nonanal	Nonanal	

Common Name	Full name	Chemical Structure
Octanal	Octanal	
Citronellal	3,7-Dimethyl-6-octenal	
Geranial	(*E*)-3,7-Dimethyl-2,6-octadienal	
Myrtenal	6,6-Dimethylbicyclo[3.1.1]hept-2-ene-2-carbaldehyde	
Neral	(*Z*)-3,7-Dimethyl-2,6-octadienal	

B) Alcohols

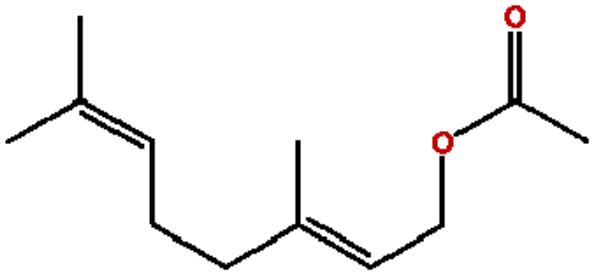
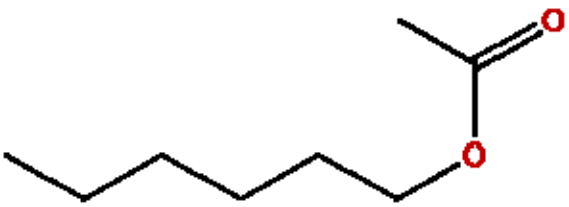
Common Name	Full name	Chemical Structure
(*E*)-2-hexen-1-ol	(*E*)-2-hexen-1-ol	
(*Z*)-3-hexen-1-ol	(*Z*)-3-hexen-1-ol	
1-octen-3-ol	1-octen-3-ol	
Citronellol	3,7-Dimethyl-6-octen-1-ol	

Common Name	Full name	Chemical Structure
α – terpineol	2-(4-Methylcyclohex-3-enyl)-propan-2-ol	
Linalool	2,6-Dimethyl-2,7-octadien-6-ol	

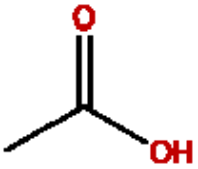
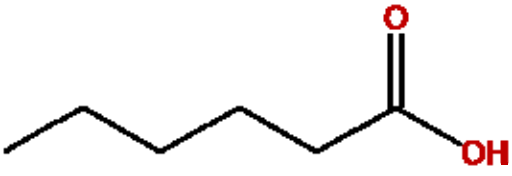
C) Alkanes

Common Name	**Full name**	**Chemical Structure**
Heptadecane	Heptadecane	
Hexadecane	Hexadecane	
Tridecane	Tridecane	
Pentadecane	Pentadecane	

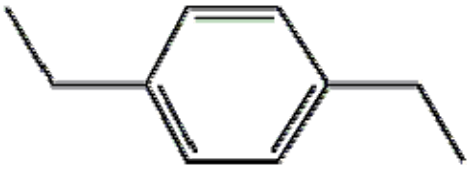
D) Acetates

Common Name	Full name	Chemical Structure
Geranyl-acetate	(E)-3,7-Dimethyl-2,6-octadienyl acetate	
Hexyl acetate	Hexyl acetate	

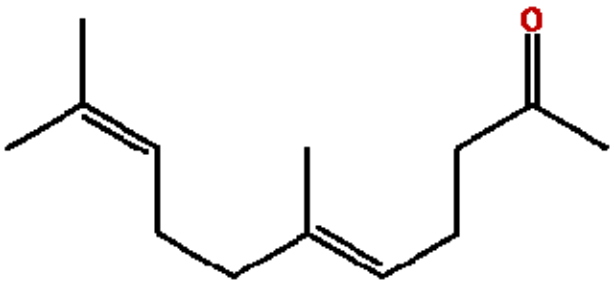
E) Acids

Common Name	Full name	Chemical Structure
Acetic acid	Acetic Acid	
Caproic Acid	Hexanoic Acid	

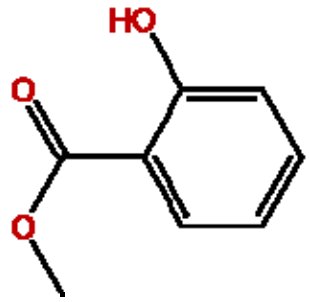
F) Aromatic compounds

Common Name	Full name	Chemical Structure
1,4-Diethylbenzene	1,4-Diethylbenzene	

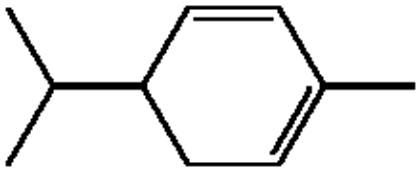
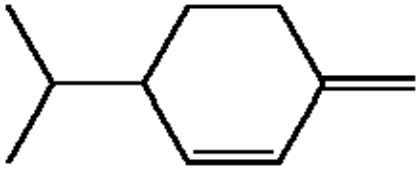
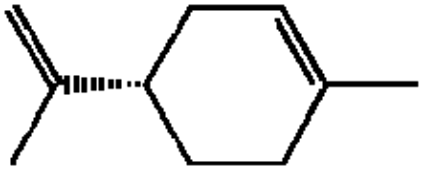
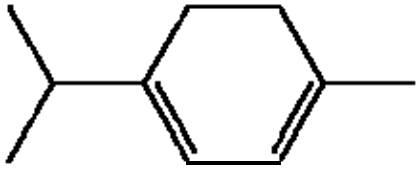
G) Ketones

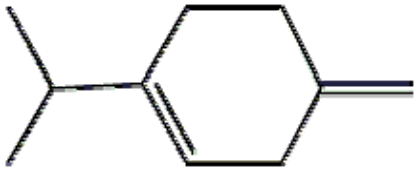
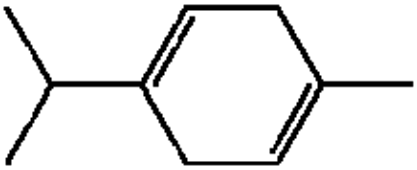
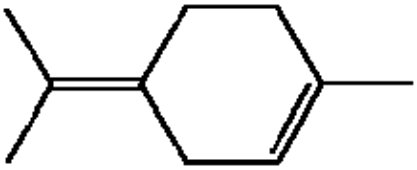
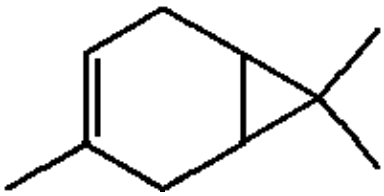
Common Name	Full name	Chemical Structure
Geranyl acetone	(*E*)-6,10-Dimethyl-5,9-undecadien-2-one	

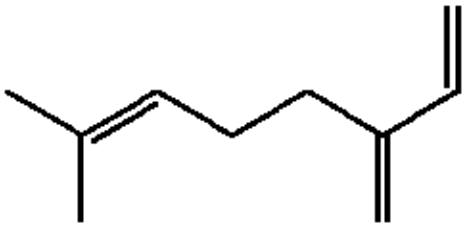
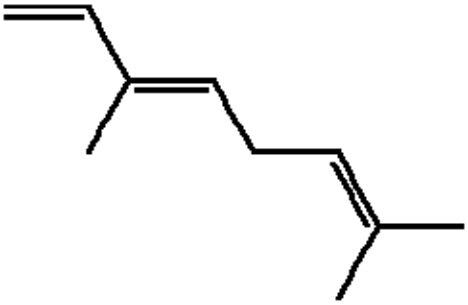
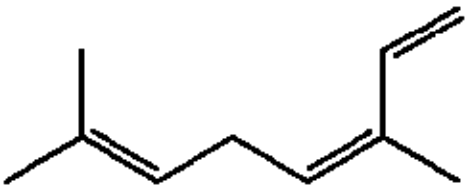
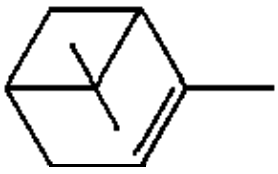
H) Esters

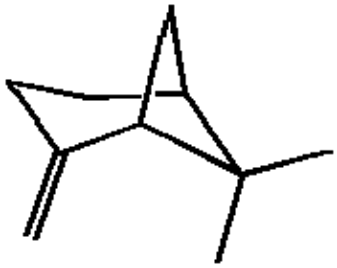
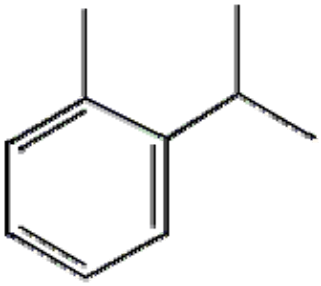
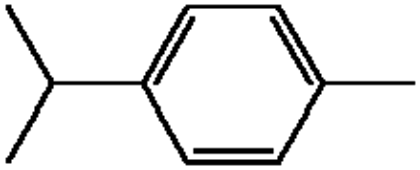
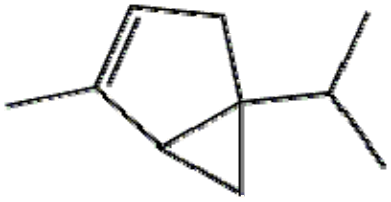
Common Name	Full name	Chemical Structure
Methyl Salicylate	Methyl Salicylate	

I) Monoterpenes

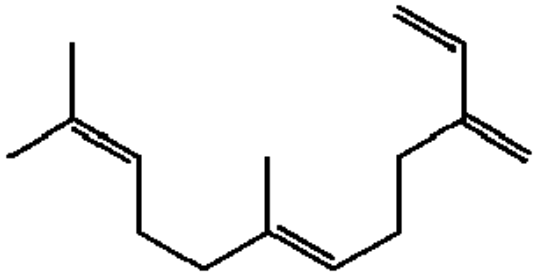
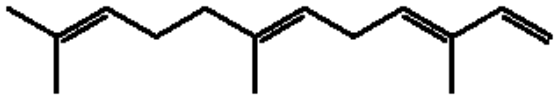
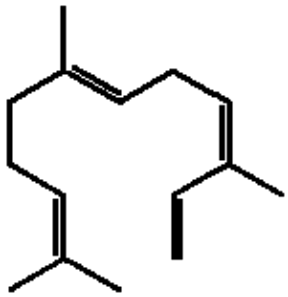
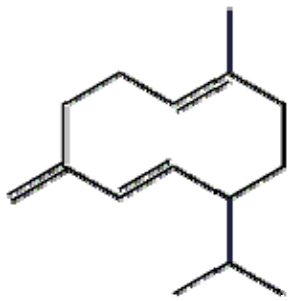
Common Name	Full name	Chemical Structure
α-phellandrene	5-Isopropyl-2-methylcyclohexa-1,3-diene	
β-phellandrene	4-Isopropyl-1-methylene-2-cyclohexene	
S-limonene	(S)-1-Methyl-4-(1-methylethenyl)-cyclohexene	
α-terpinene	1-Isopropyl-4-methylcyclohexa-1,3-diene	

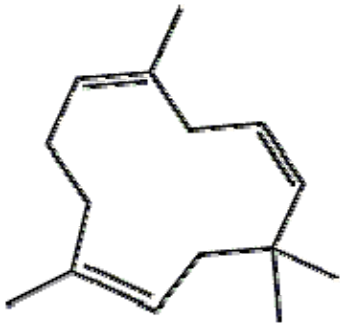
Common Name	Full name	Chemical Structure
β-terpinene	1-Isopropyl-4-methylene-1-cyclohexene	
γ-terpinene	1-Isopropyl-4-methyl-cyclohexa-1,4-diene	
α-terpinolene	1-Methyl-4-(1-methylethylidene)-cyclohexene	
3-Carene	3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene	

Common Name	Full name	Chemical Structure
Myrcene	7-Methyl-3-methylene-1,6-octadiene	
(*E*)-β-ocimene	(*E*)-3,7-Dimethyl-1,3,6-octatriene	
(*Z*)-β-ocimene	(*Z*)-3,7-Dimethyl-1,3,6-octatriene	
α-pinene	2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	

Common Name	Full name	Chemical Structure
β - pinene	6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane	
o-cymene	1-Methyl-2-(1-methylethyl)-benzene	
p-cymene	1-Methyl-4-(1-methylethyl)-benzene	
α-thujene	5-Isopropyl-2-methylbicyclo[3.1.0]hex-2-ene	

J) Sesquiterpenes

Common Name	**Full name**	**Chemical Structure**
(*E*)-β-farnesene	(*E*)-7,11-Dimethyl-3-methylene-1,6,10-dodecatriene	
(*E,E*)-α-farnesene	(*E,E*)-3,7,11-Trimethyl-1,3,6,10-dodecatetraene	
(*Z,E*)-α-farnesene	(*Z,E*)-3,7,11-Trimethyl-1,3,6,10-dodecatetraene	
Germacrene D	(*E,E*)-1-Methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene	

Common Name	Full name	Chemical Structure
α-caryophyllene	(*E,E,E*)-2,6,6,9-Tetramethyl-1,4,8-cycloundecatriene	
β-caryophyllene	1R-(1R,4E,9S)-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene