

# **Olfactory basis of host-recognition in the black bean aphid, *Aphis fabae***

by

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## **DECLARATION**

I hereby declare that I have composed this thesis and that it has not been accepted in any previous application for a degree. The work of this thesis is a record of my own work and any collaborative work has been specifically acknowledged.

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## ABSTRACT

Behavioural and electrophysiological responses of winged virginoparous *Aphis fabae* to volatile compounds of faba bean, *Vicia faba*, were studied and semiochemicals used in host location identified. In olfactometer bioassays, aphids responded positively to *V. faba* volatiles from an intact plant. This response also occurred when volatiles from an air entrainment sample of a *V. faba* plant were tested. Coupled gas chromatography-electroantennography revealed the presence of 16 electrophysiologically active volatile compounds in the air entrainment sample and 15 of these were identified as (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 1-hexanol, benzaldehyde, 6-methyl-5-hepten-2-one, octanal, (*Z*)-3-hexen-1-yl acetate, (*R*)-linalool, methyl salicylate, decanal, undecanal, (*E*)-caryophyllene, (*E*)- $\beta$ -farnesene, (*S*)-germacrene D, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. A synthetic blend consisting of all identified compounds in the same concentration and ratio as in the air entrainment sample elicited a similar behavioural response from the aphids as the air entrainment sample. Each compound was tested for behavioural activity individually at the same concentration as in the air entrainment sample and subsequently over a range of different doses. It was found that the response to the complete blend was not due to a response to a single compound. Dose response experiments also revealed ten of the compounds elicited negative behavioural responses from aphids. Further behavioural experiments revealed that these responses were context-specific and behavioural activity of individual compounds was different when they were presented alongside other compounds in the blend.

It was hypothesised that a blend of host volatiles in a species-specific ratio may be used by *A. fabae* to recognise its host. To determine whether or not ratios of volatiles could provide a reliable cue to host seeking aphids, intra-specific and diurnal variation of ratios of volatiles emitted were investigated. Although considerable variation in ratios was observed the quantities of some pairs of compounds were positively correlated, indicating a degree of consistency in the ratios.

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## ABBREVIATIONS

|        |                                                          |
|--------|----------------------------------------------------------|
| ANOVA  | Analysis of variance                                     |
| EAG    | Electroantennography                                     |
| GLV    | Green-leaf volatile                                      |
| GC     | Gas chromatograph                                        |
| MS     | Mass spectrometer                                        |
| ORN    | Olfactory receptor neurone                               |
| RI     | Retention index                                          |
| S.E.   | Standard error                                           |
| S.E.D. | Standard error of the difference                         |
| SSR    | Single sensillum recording                               |
| TMTT   | ( <i>E,E</i> )-4,8,12-Trimethyl-1,3,7,11-tridecatetraene |

## GLOSSARY

|                            |                                                                                                     |
|----------------------------|-----------------------------------------------------------------------------------------------------|
| <b>Alate</b>               | Winged aphid morph                                                                                  |
| <b>Allelochemical</b>      | Semiochemical mediating inter-specific interactions                                                 |
| <b>Allomone</b>            | An allelochemical which benefits the emitter of the signal but not the receiver                     |
| <b>Apneumone</b>           | A semiochemical emitted by non-living material which benefits the receiver                          |
| <b>Apterous</b>            | Wingless aphid morph                                                                                |
| <b>Attraction</b>          | Oriented movement towards a stimulus                                                                |
| <b>Arrestment</b>          | Cessation or reduction of movement upon encounter of stimulus                                       |
| <b>Deterrent</b>           | Inhibits a behaviour                                                                                |
| <b>Enantiomer</b>          | One of two molecules which are mirror images of each other                                          |
| <b>Fundatrix</b>           | Parthenogenetic female aphid developing from fertilised egg                                         |
| <b>Gynoparae</b>           | Parthenogenetic female aphid that migrates to and colonises winter host and produces sexual females |
| <b>Host</b>                | Plant on which an insect feeds and develops                                                         |
| <b>Kairomone</b>           | An allelochemical which benefits the receiver of the signal but not the emitter                     |
| <b>Locomotor stimulant</b> | A stimulus which causes an insect to move or speed up                                               |
| <b>Olfactometer</b>        | Equipment used to record behavioural responses of insects to semiochemicals                         |
| <b>Oviparae</b>            | Sexual female aphids                                                                                |

|                      |                                                                                    |
|----------------------|------------------------------------------------------------------------------------|
| <b>Pheromone</b>     | A semiochemical mediating intra-specific interactions                              |
| <b>Repulsion</b>     | Oriented movement away from a stimulus                                             |
| <b>Semiochemical</b> | Volatile chemicals that act as signals between organisms                           |
| <b>Stimulant</b>     | Initiates a behaviour                                                              |
| <b>Synonome</b>      | An allelochemical which benefits the emitter of the signal <i>and</i> the receiver |
| <b>Virginoparae</b>  | Parthenogenetic female aphid that reproduces on the summer host                    |

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# CHAPTER 1. INTRODUCTION

Amongst other senses aphids use smell to recognise their host plants. Volatile compounds are detected by receptor neurones on the insects' antennae and processed in the central nervous system. Insects then make behavioural responses appropriate to the odorant molecules detected. If the volatile compounds originate from a host plant, insects may make oriented movements upwind towards the source of the odour. If volatile compounds originate from a non-host plant, insects may move away from the odour source. An understanding of which volatile compounds elicit which kind of behavioural response in an insect can be useful in integrated pest control where behaviourally active volatile compounds can be used to manipulate the behaviours of pest insects so as to protect crops.

The black bean aphid, *Aphis fabae* Scop. (Hemiptera:Aphididae), is an economically important pest of faba bean, *Vicia faba* (Fabales:Fabaceae). Aphids cause damage directly through phloem feeding, resulting in significant impairment of plant growth and yield (Pruter & Zebitz 1991; Parker & Biddle 1998; Shannag & Ababneh 2007) and can also cause indirect damage by acting as vectors for plant viruses (El-Amri 1999; Neeraj et al. 1999). Due to parthenogenetic reproduction and viviparity, aphids have short generation times and therefore high rates of reproduction meaning that even a small initial infestation can quickly lead to large populations and significant damage to the plant (El-Amri 1999). Effective management of aphid pests is essential in modern agriculture. Traditionally, pesticides have been effectively employed for this but research into alternative pest control strategies is warranted due to increasing instances of pesticide resistance by insects (Jutsum et al. 1998) and increasing concerns over environmental and economic costs of such practices (Pretty et al. 2000; Pimentel 2005; Carvalho 2006). Semiochemicals are chemicals that act as signals between organisms but have no direct effect on the physiology of the receiving organism. Semiochemicals can be used by phytophagous insects to locate and recognise a suitable host plant. *Aphis fabae* is known to use volatile chemical cues to locate its host, *V. faba* (Nottingham et al. 1991) and an understanding of the mechanisms behind olfactory recognition of its host could potentially lead to the development of novel means of pest control. The purpose of this introductory chapter is to give an outline of how this might be achieved by first discussing the general biology and life cycle of *A. fabae*, then discussing

key concepts in chemical ecology, a summary of the literature on aphid chemical ecology and finally how chemical ecology may be applied in the context of pest control.

## 1.1. Semiochemicals

Semiochemicals are volatile chemicals that act as signals between organisms (Howse et al. 1998). They are classified according to whether they act between individuals of the same or different species and by whether they benefit or detriment the receiver or emitter of the signal. Figure 1.1 shows the different classes of semiochemicals.

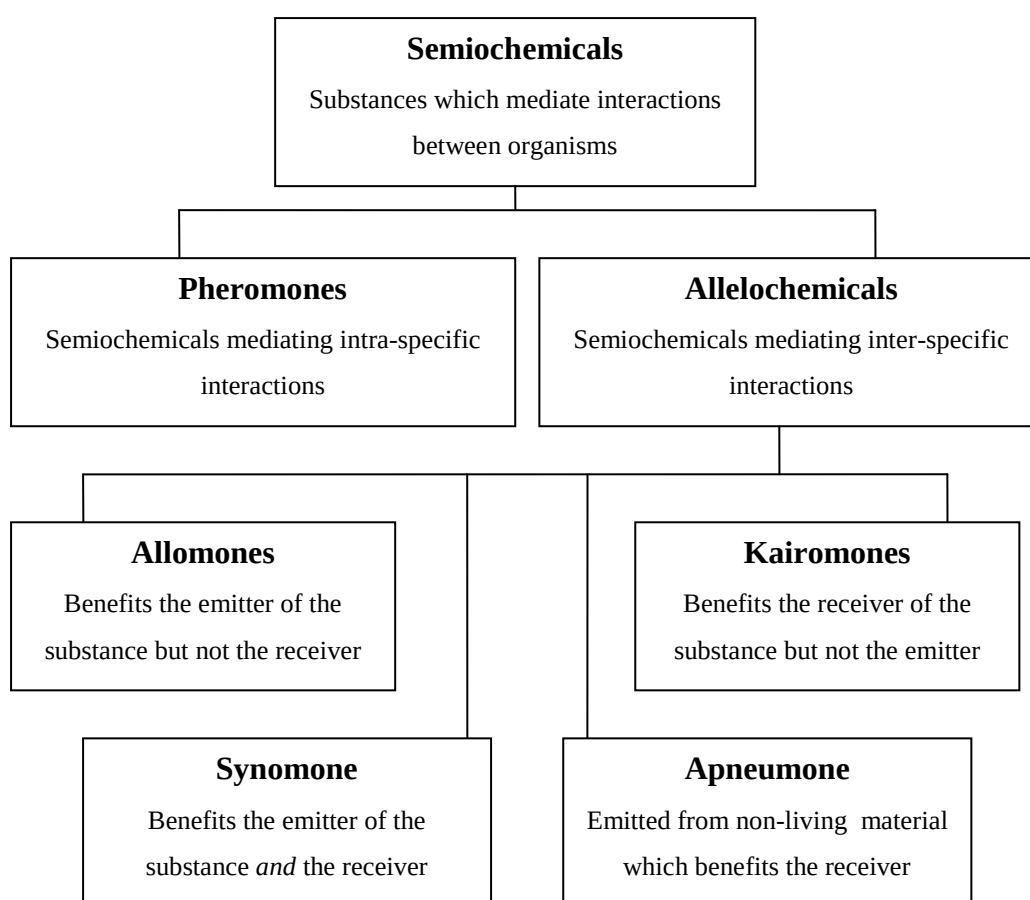


**Figure 1.1. Classification of semiochemicals.**

Individual volatile compounds can belong to more than one of the above classes. For example, (*E*)- $\beta$ -farnesene is emitted by aphids under attack from parasitoids and causes nearby aphids to disperse (Nault et al. 1973). In this sense it is a pheromone because it is a form of communication between individuals of the same species. However, as the same

compound serves the same function in a number of a different aphid species, it can also be said to be an allelochemical.

Plant volatile compounds may be released continuously, released upon damage of plant tissue or may only be released after induction, for example upon feeding by phytophagous insects. Volatile induction may result from tissue damage caused by the mouthparts of chewing insects and numerous studies have shown how mechanical damage to plant tissue alters the volatile compounds emitted (Saijo & Takeo 1975; Agelopoulos et al. 1999b; Moretti et al. 2002). Additionally, oral secretions from herbivorous insects can induce systemic release of volatile compounds by interacting with chemicals in the plant tissue (Mattiacci et al. 1995; Alborn et al. 1997; Halitschke et al. 2001). These are called elicitors.

## **1.2. Insect olfaction**

Insects have evolved sophisticated mechanisms for detecting volatile compounds. The insect olfactory apparatus comprises receptor neurones on the antennae, the antennal nerve, the antennal lobe, the mushroom bodies and the antennal protocerebrum (Lemon & Getz 1999; Mustaparta 2002; Couto et al. 2005). Olfactory receptor neurones which respond to volatile compounds are housed in sensilla arranged on the surface of the antennae and may also be present on the maxillary palps in some species (Shambaugh et al. 1978; Hansson et al. 1986; Bland 1991; Baker & Chandrapatya 1992; Ljungberg & Hallberg 1992; Skilbeck & Anderson 1996). Numerous structural types of sensilla exist. Among the most common structures are hair-like sensilla (trichoid) (Aljunid & Anderson 1983; Hansson et al. 1986), sunken peg-like structures (coeloconic) (Bromley et al. 1979b; Baker & Chandrapatya 1992) and flattened plate-like structures (placoid) (Bromley et al. 1979b; Bland 1991). In aphids, sensilla are arranged in clusters called rhinaria which house a number of receptor neurones (Bromley et al. 1979b, 1980; Park & Hardie 2004). These have been characterised as primary and secondary rhinaria. All aphid morphs possess proximal and distal primary rhinaria which are found on the fifth and sixth antennal segments (Bromley et al. 1979b, 1980), respectively, and respond primarily to plant volatiles (Park & Hardie 2002). Electroantennogram studies of the pea aphid, *Acyrtosiphon pisum*, have suggested that the two types of primary rhinaria are specialised to detect different volatile compounds, with the proximal responding more strongly to aldehydes and the distal responding more strongly to alcohols. The secondary

rhinaria are associated with the sex pheromone components nepetalactol and nepetalactone (Park & Hardie 2002) and distribution varies between morphs with sexual males possessing the most (Bromley et al. 1979b; Hardie et al. 1994b).

Figure 1.2 shows a cross section of an aphid placoid sensillum (Bromley et al. 1979b). The cuticle is perforated with numerous small pores. Odorant molecules enter the lumen of the sensilla through these pores and come into contact with odourant binding proteins (OBPs) (Vogt & Riddiford 1981; Field et al. 2000; Pelosi et al. 2006). OBPs are thought to act as transport molecules which carry the odorant to the membrane-bound receptors on the dendrites of the olfactory receptor neurones (ORNs). There are OBPs which are specific to pheromones (PBPs) and also OBPs that bind to other semiochemicals (GOBPs) and these both appear to have some selectivity (Pelosi et al. 2006). The interaction of the membrane-bound receptors leads to an intracellular cascade reaction which results in depolarisation of the cell membrane. This depolarisation opens voltage-gated channels resulting in the generation of action potentials (Krieger & Breer 1999).

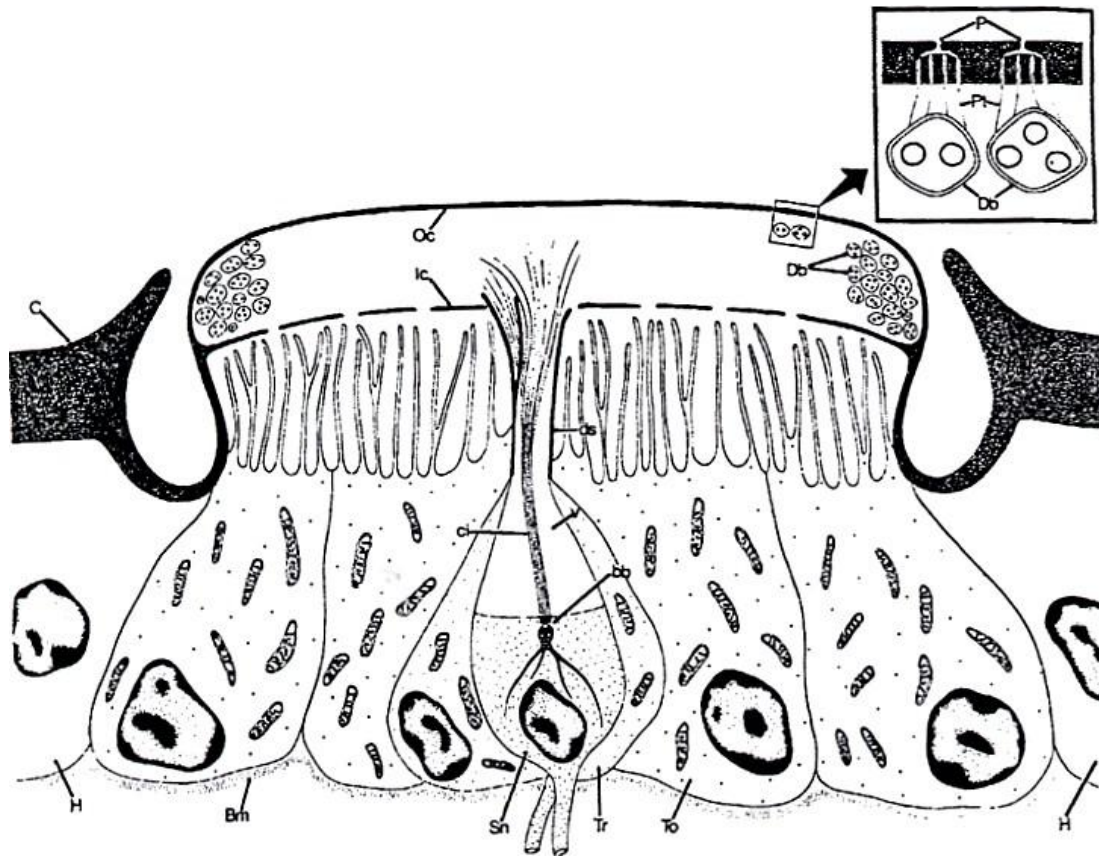


Figure 1.2. Diagrammatic representation of a placoid sensillum of an aphid. (Bromley et al. 1979a). Bb: Basal body apparatus, Bm: Basal lamina, C: cuticle, Db: Dendritic branch, ds: dendritic sheath, H: Hypodermal cell, Ic: Inner cuticle, Oc: Outer cuticle, P: Pore, Pt: Pore tubules, Sn: Sensory neurone, To: Tormogen cell, Tr: Trichogen cell, v: vacuole.

Each sensillum usually contains one or a small number of distinct ORNs (Bromley et al. 1979b; Ljungberg & Hallberg 1992; Blight et al. 1995; Cosse et al. 1998; Stensmyr et al. 2003). The dendrites of the ORNs are enclosed in a dendritic sheath which ends at the base of the structure and the dendrites may branch in the lumen just beneath the perforated surface of the sensillum (Bromley et al. 1979b, 1980; Lopes et al. 2002). The dendrites of different ORNs bear receptors for different volatile compounds. Some receptors detect pheromones and are usually highly specific whereas other receptors respond to other volatile compounds, such as those originating from the insects' host. Originally the receptors responding to plant volatile compounds were thought to be generalist receptors, responding to a wide range of plant volatile compounds but recent evidence suggests they may be highly specific, exhibiting only weak responses to other volatile compounds (Anderson et al. 1995; Blight et al. 1995; Hansson et al. 1999; Stensmyr et al. 2003). It has been found that ORNs sometimes

occur in clusters, the same ORNs consistently co-located in the same sensilla (Al Abassi et al. 2000; Stensmyr et al. 2003). Olfactory receptor neurones responding to pheromones and plant volatile compounds are often located separately in specialised sensilla but in some species they can be located together in the same sensillum (Anderson et al. 1995).

From the cell bodies of the ORNs, axons project directly, via the antennal nerve, into the antennal lobe within the central nervous system where they branch extensively (Bernays & Chapman 1994). The antennal lobe is divided into a number of functional units called glomeruli. Each glomerulus receives input from one or a small number of ORN classes and communication between glomeruli is facilitated by local interneurons (Ng et al. 2002). On presentation of odorants, activity can be seen in the corresponding glomerulus (Rodrigues 1988; Distler et al. 1998; Carlsson et al. 2002). The antennal lobe is divided into two regions (Mustaparta 2002). The macro-glomerula complex (MGC) receives input from ORNs responding to pheromones while the main area of the antennal lobe receives input from ORNs responding to plant volatile compounds. Christensen and Hildebrand (2002) suggested that the MGC is not a separate region of the insect brain but merely an extension of the spatial arrangement of glomeruli where ORNs responding to similar odorants project to adjacent glomeruli. Studies have shown that as the concentration of an odorant is increased, excitation of the glomerulus receiving input from the ORN class responding to the particular odorant spreads to adjacent glomeruli (Carlsson & Hansson 2003). This is perhaps because these adjacent glomeruli receive input from ORNs which exhibit secondary responses to the odorant, only becoming activated at higher concentrations (Carlsson & Hansson 2003). In this way it is possible to encode odorant concentration as a spatial pattern of activity in the antennal lobe. Presentation of blends of volatile compounds can cause an additive effect in patterns of glomerula excitation (Joerges et al. 1997; Rappert et al. 1998) but inhibition through local interneurons can also result in patterns of activity which cannot be explained by a simple additive effect (Joerges et al. 1997). Patterns of excitation across the antennal lobe can therefore be used to encode information about odorant quantity and quality.

Projection neurones then transmit information from the antennal lobe to the mushroom bodies, which are associated with learning and memory in some insect species (Heisenberg 2003). Conflicting studies have suggested that these projection neurones can be more or less broadly tuned than ORNs and that the temporal responses of these projection neurones can also differ from those of the ORNs (Ng et al. 2002; Wilson et al. 2004). This suggests that

some integration of odorant information occurs in the antennal lobe. The lateral protocerebrum receives projection neurones from the mushroom bodies as well as from the antennal lobe (Mustaparta 2002). Descending premotor neurones extend from this region to make contact with motor neurones in other parts of the central nervous system to initiate odour-induced motor responses (e.g. proboscis extension reflex during sucrose stimulation in some species) (Kloppenburg et al. 1997; Mishima & Kanzaki 1999).

### **1.3. Plant volatile compounds**

Numerous studies have succeeded in identifying a wide range of plant volatile compounds. Many of these are ubiquitous as they are found in a wide range of unrelated plant species. In contrast, others are specific to certain plant families. Some of the more well-known classes of volatiles are discussed below.

#### **1.3.1. The fatty acid derivatives**

The fatty acid derivatives, or ‘green leaf volatiles’ (GLVs), comprise a number of C-6 alcohols, aldehydes and their esters and are found ubiquitously throughout the plant kingdom, produced in all green plant tissue (Visser & Ave 1978; Hatanaka et al. 1987). They are formed from linolenic and linoleic acids through the lipoxygenase pathway. Lipases catalyse the breakdown of fatty acid esters into linolenic and linoleic acid (Galliard 1971; Hatanaka et al. 1976b). Lipoxygenase converts linoleic acid into hydroperoxylinoleic acid (Matthew & Galliard 1978; Croft et al. 1993) and hydroperoxide lyase catalyses the breakdown of this product into hexanal which is then converted to hexanol by alcohol dehydrogenase (Kajiwarra et al. 1977). Linolenic acid is converted to hydroperoxylinolenic acid by a lipoxygenase (Matthew & Galliard 1978; Croft et al. 1993). Hydroperoxide lyase catalyses the breakdown of this compound into (Z)-3-hexenal which is then converted enzymatically into the C6 products (*E*)-3-hexenal, (Z)-3-hexen-1-ol, (*E*)-3-hexenol, (*E*)-2-hexenal and (*E*)-2-hexenol by alcohol dehydrogenase and isomerization enzymes (Hatanaka et al. 1976b; Croft et al. 1993). An acyltransferase catalyzes the formation of (Z)-3-hexenyl acetate from (Z)-3-hexen-1-ol and acetyl CoA (D'Auria et al. 2002).

Increased emission of GLVs is often associated with injury to plant tissue (Saijo & Takeo 1975; Agelopoulos et al. 1999b; Moretti et al. 2002; Myung et al. 2006). Physical wounding

leads to loss of cell integrity and the release/activation of enzymes (Bostock & Stermer 1989). Lipases attack membrane lipids giving rise to free fatty acids which are converted to GLVs. Antifeedant properties of GLVs have been found for some species of insect (Hildebrand et al. 1993; Faccoli et al. 2005) as well as antifungal properties (Vaughn & Gardner 1993; Brown et al. 1995) and exposure of some plants to GLVs can result in the induction of plant defences (Farag et al. 2005; Kost & Heil 2006).

### **1.3.2. The phenylpropanoids**

The phenylpropanoids are also found ubiquitously throughout the plant kingdom and consist of an aromatic ring with a three-carbon side chain. Phenylpropanoids are derived from L-phenylalanine which is converted to (*E*)-cinnamic acid by L-phenylalanine ammonia lyase. This product is converted into a variety of hydroxycinnamic acids, aldehydes and alcohols by a series of hydroxylation and methylation reactions (Dudareva et al. 2006).

Phenylpropanoids are often associated with plant defence. A pertinent example is methyl salicylate, which is typically emitted by plants that have been physically damaged by herbivory or after exposure to herbivore-induced volatile compounds (Dicke et al. 1999; Rodriguez-Saona et al. 2001; Van Den Boom et al. 2004). Methyl salicylate acts as a repellent to a number of herbivorous insects (Hardie et al. 1994a; Markovic et al. 1996; Jayasekara et al. 2005) and has been found to attract predators and parasitoids of some herbivores (James & Grasswitz 2005; Zhu & Park 2005; James 2006). It is often found at elevated levels in plants under attack from insects (Van Poecke et al. 2001; Zhu & Park 2005; John et al. 2006) and exposure of plants to methyl salicylate can result in the induction of plant defences (Shulaev et al. 1997; Deng et al. 2004; Hountondji et al. 2006).

### **1.3.3. The isoprenoids**

The isoprenoid group of organic compounds are formally derived from the C<sub>5</sub> hydrocarbon isoprene. Isoprene is the most abundant plant volatile compound in nature, accounting for around half of all biological hydrocarbon emission (Loreto & Sharkey 1993; Mlot 1995). Isoprenoids are classified according to how many isoprene molecules they are made from. Monoterpenoids consist of two isoprene molecules and sesquiterpenoids consist of three. The biosynthesis of monoterpenoids in plants occurs via the methylerythritol phosphate (MEP) pathway in the chloroplasts of plants and the biosynthesis of sesquiterpenoids occurs via the

mevalonate pathway in the cytosol. Both biosynthetic pathways result in the formation of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Rohmer et al. 1993; Rohmer 1999) which, through a series of reactions, are converted into geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate from which other isoprenoids are derived. Monoterpenoids and sesquiterpenoids tend to have high volatility. They are extremely widespread in the plant kingdom and often function as semiochemicals in insect-insect communication and insect host location (Bowers et al. 1972; Rocca et al. 1992; Burger et al. 1993).

#### **1.3.4. The isothiocyanates**

The fatty-acid derivatives, phenylpropanoids and isoprenoids are widely distributed throughout the plant kingdom (Knudsen et al. 1993). However, other classes of plant volatile compounds are restricted to certain taxonomic groups. The isothiocyanates represent such a class and warrants special attention because of widespread responses to these compounds in a number of insect species and their implication in host location and non-host avoidance (Nottingham et al. 1991; Kostal 1992b; Blight et al. 1995; Visser & Yan 1995; Evans & Allen-Williams 1998; Mewis et al. 2002; Renwick et al. 2006). Isothiocyanates are produced by hydrolysis of glucosinolates (Cole 1976), which are found almost exclusively in the order Brassicales. These are compartmentalised in the plant tissue (Matile 1980) and only released after mechanical damage, for example by insect chewing. During this process myrosinases come into contact with the glucosinolates and catalyse their breakdown into aglycones (Bjorkman & Lonerda 1973; Fahey et al. 2001), which rearrange spontaneously into the isothiocyanate and other catabolites. Isothiocyanates are toxic to some species (Borek et al. 1997; Li et al. 2000; Park et al. 2006) which suggests they may be used in plant defence. While they may be used as non-host cues by many species of insect (Nottingham et al. 1991; Isaacs et al. 1993; Hardie et al. 1994b), insects specialising on brassicaceous plants often use these compounds as host cues, displaying attraction in laboratory and field studies (Bartlett et al. 1993; Blight et al. 1995; Smart et al. 1997; Blight & Smart 1999; Barker et al. 2006; Renwick et al. 2006).

## **1.4. Host-recognition by phytophagous insects**

Recognising a host plant is important to allow insects to find suitable feeding sites and also to find suitable oviposition sites (Jallow et al. 1999; Liu et al. 2006) and rendezvous sites for mating (Linn et al. 2003; Ginzel & Hanks 2005). At a distance, host location can involve the use of visual and olfactory cues. The main visual organ in adult insects is the compound eye, which consists of a series of closely packed light-sensing units called ommatidia (Doring & Chittka 2007; Borst 2009). Light enters these visual units through a transparent cuticle covering the surface of the eye and is then focussed as it passes through a lens within the ommatidium. Beneath this lens is a receptor system which consists of a number of pigment-containing cells which respond to different wavelengths of light and convey information to the central nervous system. The ability of insects to respond to visual cues is well-documented and some insects have demonstrated strong abilities to use visual cues in host-plant discrimination (Harris et al. 1993; Bernays et al. 2000; Hirota & Kato 2004).

Successful host recognition using plant volatile compounds relies on an insect being able to recognise characteristic features of its host-plant's odour. The 'token stimulus' hypothesis originally put forward by Fraenkel (1959) suggested that insects respond to volatile compounds which are specific to their host plants. A pertinent example of this is insects specialising on brassicaceous plants. The presence of isothiocyanates in an odour plume presents a relatively accurate means of recognising a plant from this family. There are many examples of brassica-specialising insects which exhibit behavioural responses towards isothiocyanates (Nottingham et al. 1991; Pivnick et al. 1991; Kostal 1992a; Bartlet et al. 1993; Blight & Smart 1999; Mewis et al. 2002; Reddy et al. 2002; Renwick et al. 2006). There are also a number of other examples of host-specific plant volatile compounds eliciting behavioural responses in insects and some of these are listed in Table 1.1.

**Table 1.1. A list of examples of insects which respond behaviourally to host-specific volatile compounds (Pierce et al. 1978; Guerin et al. 1983; Judd & Borden 1989; Krasnoff & Dussourd 1989; Bjostad & Hibbard 1992; Knight & Light 2001).**

| Insect                                                                                                               | Hosts         | Host-specific compound                     |
|----------------------------------------------------------------------------------------------------------------------|---------------|--------------------------------------------|
| Arctiid moths; <i>Cisseps fulvicollis</i> ,<br><i>Ctenucha virginica</i> and <i>Halysidota</i><br><i>tessellaris</i> | Asteraceae    | (S)-hydroxydanaidal<br>(R)-hydroxydanaidal |
| Corn rootworm larvae, <i>Diabrotica</i><br><i>virgifera</i>                                                          | Cucurbitaceae | 6-methoxy-2-benzoxazolinone                |
| Onion fly, <i>Delia antiqua</i>                                                                                      | Alliceae      | dipropyl disulphide                        |
| Codling moth, <i>Cydia pomonella</i>                                                                                 | Rosaceae      | ethyl (2E,4Z)-2,4-decadienoate             |
| Carrot fly, <i>Psila rosae</i>                                                                                       | Apiceae       | (E)-asarone                                |

In the absence of host-specific volatile compounds insects must somehow identify their host plants using more generally distributed plant volatiles and it is thought by some that this scenario applies to the majority of host-seeking insects (Bruce et al. 2005b). One possible way this could be achieved is for insects to recognise species-specific blends of volatile compounds. Although many of the volatiles emitted by a particular host plant may be found ubiquitously throughout the plant kingdom, it may be that the host is the only plant species to produce a particular blend of them. Host recognition could therefore be achieved by responding behaviourally only when a combination of compounds in a blend are detected, the absence of particular volatiles indicative of a non-host. The orange wheat blossom midge, *Sitodiplosis mosellana*, was found to respond behaviourally to a three-component blend of acetophenone, (Z)-3-hexenyl acetate and 3-carene present in a ratio representative of that found in the volatile blend emitted by wheat panicles (cv. “Lynx”) (Birkett et al. 2004). No attraction was observed to any of these three compounds when presented individually at quantities equivalent to that present in the three-component blend. The only study to date which has demonstrated that aphids respond preferentially to blends of host volatiles rather than individual compounds was conducted by Ngumbi et al., (2007). It was found that the peach potato aphid, *Myzus persicae*, was arrested by the odour of potato leaf roll virus-

infected potato plants. When individual compounds isolated from the headspace of infected plants were tested for behavioural activity, only one compound,  $\beta$ -pinene, was a mild arrestant. However, aphids were arrested far more strongly by the complete blend.

Tasin et al. (2007) showed that the behavioural responses of the grapevine moth, *Lobesia botrana*, to grape odour was mediated by a blend of grape volatile compounds. Upwind flight could not be initiated by a single compound but moths responded to a number of different blends composed of different grape volatiles. This suggested that there was redundancy in the behaviourally active blend and that no single species-characteristic combination of volatile compounds is necessary to elicit upwind flight in this species of moth. Similar conclusions were made by Cha et al. (2008), who found that (*Z*)-3-hexen-1-yl acetate and methyl salicylate were essential for eliciting upwind flight in the Grape berry moth (*Paralobesia viteana*) in the presence of certain host volatiles, but were not essential host cues in the presence of other host volatiles. The behavioural importance of these volatiles was therefore context specific. These two studies suggest that where blends of ubiquitous plant volatiles are used in host location, host-seeking insects may exhibit some plasticity in the blends they use to recognise their hosts, possibly to allow for natural intraspecific variation in the volatile compounds emitted by their host plants (Tasin et al. 2007).

Visser (1986) proposed that host location using blends of ubiquitous volatile compounds may be achieved by recognising species-characteristic ratios of volatile compounds. This would involve insects responding to a blend of volatile compounds only when they are present in ratios corresponding to those emitted by the host plant. The first evidence of this came from studies of the Colorado potato beetle, *Leptinotarsa decemlineata*, which was shown to respond behaviourally to the odour of potato plants (Visser & Ave 1978). No response was detected, however, when the potato odour was mixed with additional quantities of (*E*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, (*Z*)-2-hexen-1-ol or (*E*)-2-hexenal. Since these volatiles were found to be already present in the headspace of the potato odour it was concluded that the addition of these same volatile compounds distorted the natural ratios detected by the beetles and this was responsible for the switching off of the behavioural response. Later work found that this masking effect could be achieved by the mixing of potato odour with the odour of wild tomato (*Lycopersicon hirsutum* f. *glabratum* C. H. Mull) or cabbage (*Brassica oleracea* L. var. *gemmifera* DC.) (Thiery & Visser 1986). Since no repellency was observed to the odours of wild tomato or cabbage when presented alone, it was concluded that the masking

effect observed when these were mixed with the potato odour must be due to the effect of volatile compounds emitted by the tomato and cabbage plants distorting the ratios of volatile compounds from the potato plants (Visser & Ave 1978). More recently, synthetic blends based on the volatile compounds emitted by potato plants have been found to elicit behavioural responses from the Colorado potato beetle and this work highlighted the significance of volatile-ratios in eliciting a behavioural response (Dickens 2000; Martel et al. 2005a, b). A number of other species have also been found to respond to blends of plant volatiles with evidence suggesting the behavioural importance of ratios (Robacker et al. 1992; Roseland et al. 1992; De Moraes et al. 1998; Zhang et al. 1999; Ayasse et al. 2000; Wright et al. 2002; Natale et al. 2003; Nojima et al. 2003a; Nojima et al. 2003b; Tooker & Hanks 2004; Linn et al. 2005).

Other than providing information on species, ratios of volatile compounds can provide useful information about the condition of the plant they originate from. The quantities of some volatile compounds emitted by plants can vary depending on factors such as time of day, season and physiological status of the plant. Temporal variation in volatile emission has been observed in a variety of plant species. Time of day can have a strong influence on quantities of certain volatile compounds released, due to the circadian rhythms of plants and changes in light availability influencing pathways responsible for production of volatile compounds (Loughrin et al. 1993; Loughrin et al. 1994; Loughrin et al. 1997; De Moraes et al. 2001; Karl et al. 2002; Graus et al. 2004; Sellegri et al. 2005; Chamberlain et al. 2006). Seasonal variation in emission of volatile compounds has also been observed (Hatanaka et al. 1976a; Mita et al. 2002; Bai et al. 2006; Holzinger et al. 2006) and host-seeking insects that are more active at certain times of the day may therefore evolve to respond preferentially to blends of host volatiles associated with that particular time of day. Other studies have shown that volatile compound emission can reflect stage of development (Engel et al. 1988; Agelopoulos et al. 2000; Dudareva et al. 2000; Shiojiri & Karban 2006). Engel *et al.* (1988) found that the ratios of green leaf volatile compounds emitted from the pulp of nectarines changed significantly as the fruit ripened. It was suggested that this could provide the Mediterranean fruit fly, *Ceratitis capitata*, which feeds on nectarines, with a means of avoiding unsuitable hosts such as under-ripe or damaged fruit (Light et al. 1988). A similar situation can be found with the apple maggot which is only attracted to the odour of apples which have not yet reached maturity (Fein et al. 1982). The proportions of green leaf volatile compounds emitted

by apples were shown to vary at different stages of maturity and this was suggested as the means by which apple maggot flies are able to discriminate apples which are not yet mature. A number of environmental factors have also been found to influence emission of volatile compounds which could reflect the physiological status of a potential host plant. These include temperature, humidity, light quality, nitrogen availability, and humidity (Johnson et al. 1999; Gouinguene & Turlings 2002; Schmelz et al. 2003; Vallat et al. 2005; Ferreyra et al. 2006).

Mechanical damage caused by herbivory can cause an increase in the release of GLVs (Saijo & Takeo 1975; Agelopoulos et al. 1999b; Moretti et al. 2002). An increase in the ratio of GLVs to other volatile compounds not influenced to the same extent by mechanical damage could provide a reliable indicator of the physical condition of the host. It has also been demonstrated that different species of insect can cause species-characteristic alterations to the proportions of volatile compounds released by a plant (De Moraes et al. 1998). This could provide insects with a way of identifying feeding sites that are already under heavy attack, allowing insects either to avoid competition or to join forces with conspecifics in an attempt to overcome plant defences (Werner 1972; Lanier 1983).

## **1.5. Identification of semiochemicals**

Identification of plant semiochemicals involves a series of experimental steps. The first is to confirm the behavioural response of an insect to a natural odour source. The second step is to collect the volatile compounds from the natural source. Gas chromatography (GC) coupled with electrophysiology is then used to pinpoint electrophysiologically active volatile compounds for identification by mass spectrometry and GC analysis. Finally, the behavioural responses of the insects to the identified volatile compounds are compared to the response to the natural sample to confirm identification. Each of these steps is discussed in more detail below.

### **1.5.1. Behaviour**

The first step in identification of semiochemicals is to determine the behavioural response of an insect to the natural odour source. Behavioural responses are often classified as simply attraction or repulsion. Such simplistic terminology can lead to confusion, however, as the

two terms are used to cover a range of different types of behaviour. Dethier (1960) attempted to clarify the definitions of behavioural responses and this is summarised in Table 1.2.

**Table 1.2. Definitions of behavioural responses (Dethier et al. 1960).**

| Type of behaviour   | Description                                                           |
|---------------------|-----------------------------------------------------------------------|
| Arrestant           | Causes an insect to stop moving or move at a slower speed             |
| Locomotor stimulant | Causes an insect to start or speed up movement                        |
| Attractant          | Causes an insect to make oriented movements towards an odour source   |
| Repellent           | Causes an insect to make oriented movements away from an odour source |
| Stimulant           | Initiates or drives a behaviour                                       |
| Deterrent           | Inhibits a behaviour                                                  |

Distinguishing between these different types of behaviour is not just a matter of semantics; different terms describe very different behaviours which may impact on the potential application of a semiochemical in pest control. For example, if a new semiochemical was sought to increase the catch of an insect sticky trap used for monitoring pest populations, an attractant would be far more effective than an arrestant as an attractant would lure an insect from a distance whereas an arrestant would only elicit a behavioural response once the insect has already arrived at the trap.

The behavioural activity of volatile compounds can be studied using olfactometers. These usually involve a container in which an insect is placed which is sealed except for a number of channels through which air carrying test odours is drawn. Insects inside the olfactometer are able to move around and ‘choose’ an odour source. Examples of such olfactometers are the Y-tube (Thorpe & Jones 1937; Dwumfour 1992; Girling et al. 2006), the linear track (Sakuma & Fukami 1985; Nottingham et al. 1991; Cook et al. 2002), the four-arm

olfactometer (Pettersson 1970; Birkett et al. 2004), and the locomotion compensator (Kramer 1976; Visser & Taanman 1987; Dewhirst 2007) and each of these has been used to study aphid behaviour (Pettersson 1970; Visser & Taanman 1987; Nottingham et al. 1991; Girling et al. 2006; Dewhirst 2007). In the Y-tube and linear track olfactometers, the insect walks towards a junction where two different odour streams meet and then ‘chooses’ between them by making an oriented movement in the direction of either odour stream. In this way, the Y-tube and linear track measure attraction and repulsion as defined by Dethier (1960) as insects must make an oriented movement in the direction of an odour source for a behaviour to be scored. In the four-arm olfactometer, the insect is enclosed in an arena divided into 4 regions with each region exposed to a different odour source. Time spent in each region is then recorded to determine preference of the insect to different odour sources. The four-arm olfactometer does not, therefore, distinguish between attraction and arrestment or between repulsion and locomotor stimulation as only time spent is recorded and not how the insect has moved in relation to the odour source. This can, however, be an advantage when it is unknown what type of behavioural response an odour source will elicit. The four-arm olfactometer also has the advantage of allowing insects to make multiple choices which is desirable if initial behavioural responses are simply escape responses due to the stress of being placed into an olfactometer. In a locomotion compensator, insects are placed on a sphere over which an odour source is blown. The sphere is rotated to compensate for the insects’ movement so that it always remains on the top of the sphere. The rotation of the sphere is then recorded and used to calculate direction of movement, speed of movement and rate of turning of the insect, allowing for the recording of several different types of behaviour.

When selecting an olfactometer for use in behavioural bioassays, it is important to be aware of the types of behaviour each olfactometer is capable of measuring. For example, the Y-tube and linear track olfactometers are only capable of detecting attraction or repulsion. The four-arm olfactometer records time spent in each region and therefore makes no distinction between attraction and arrestment or between repulsion and locomotion stimulation but can be initially useful in establishing a behavioural response to a semiochemical where the nature of the response is unknown. The locomotion compensator is capable of recording a range of behavioural responses and so can potentially be very useful in describing an insect’s behaviour. However, this method was found to be difficult to use with aphids due to the build

up of static electricity caused by the rotation of the sphere and difficulties introducing the odour source over the surface of the sphere (Dewhirst 2007).

### **1.5.2. Collection of volatile compounds**

Headspace sampling can be used to collect volatile compounds from living odour sources such as intact plants. Static headspace sampling involves allowing the sample to come into equilibrium with its vapours, which are then collected either with a simple syringe or by using a more sophisticated extraction technique such as solid-phase micro-extraction (SPME) which uses a polymer-coated fused silica fiber to extract volatile compounds from a sample (Agelopoulos & Pickett 1998; Lagalante & Montgomery 2003; Scascighini et al. 2005). Dynamic headspace analysis, commonly referred to as air entrainment (Agelopoulos et al. 1999b; Birkett et al. 2004), involves drawing clean air over the odour source (e.g. a plant) and then through an adsorbent polymer that collects the volatile compounds. Volatile compounds are then removed either by thermal desorption or by eluting with a solvent. Thermal desorption is useful for analysing small quantities of volatile compounds, for example if the air entrainment is done over a short period of time, as all captured volatiles are released simultaneously providing a highly concentrated sample for analysis. The drawback of thermal desorption, however, is that the whole sample is used during one analysis and thus it cannot be used again, for example in behavioural bioassays. Elution with a solvent, on the other hand, provides a sample from which aliquots can be repeatedly drawn to allow multiple chemical analyses and repeated behavioural and electrophysiological experiments with the same sample. Comparison of sampling techniques has revealed that different methods result in the differential isolation of volatile compounds such that ratios of some volatile compounds may appear different depending on the method used, with dynamic headspace sampling generally result in the best preservation of ratios of compounds (Agelopoulos & Pickett 1998; Faldt et al. 2000; Vercammen et al. 2000; Wanakhachornkrai & Lertsiri 2003). Differential adsorption can also be caused by some compounds breaking through the filter without being trapped, by compounds becoming adsorbed onto the surface of the material used to contain the odour source during entrainment, and due to differential affinities of the adsorbent material used. (Stewart-Jones & Poppy 2006).

As well as dynamic and static headspace sampling, more aggressive methods of volatile collection can also be employed. Steam distillation involves lowering the boiling points of

compounds within a sample by adding steam so that they evaporate at temperatures below which significant deterioration of these compounds would normally occur (Konstantopoulou et al. 2004; Agostini et al. 2006; Brophy et al. 2006). The compounds can then be collected as they evaporate. The breakdown of plant tissue by the steam can be a problem, however, as damage to plant material can cause an increase in production of certain volatile compounds (Saijo & Takeo 1975; Agelopoulos et al. 1999b; Moretti et al. 2002). This may result in qualitative and quantitative differences in the volatile compounds collected. A less destructive method is the use of vacuum distillation which uses a vacuum, rather than steam, to lower the boiling points of the volatile compounds being emitted by the plant and collecting them in solution (Pickett & Stephenson 1980; Schuh & Schieberle 2005; Tomova et al. 2005). An advantage of both these methods is that they allow large quantities of plant volatile compounds to be collected in a relatively short space of time. However, there is a risk that they may be unlikely to provide accurate information on quantities and ratios of compounds emitted naturally by the plant.

### **1.5.3. Electrophysiology**

Plants can emit hundreds of different volatile compounds, making the task of identifying each compound and testing for behavioural activity impractical. Electroantennography is a technique used to determine whether or not an insect possesses olfactory receptor neurones (ORNs) capable of detecting particular volatile compounds. By determining which volatile compounds within a sample are electrophysiologically active to an insect, it is possible to significantly reduce the number of compounds which need to be identified. Electroantennography involves attaching an electrode to either end of an antenna, making it possible to record changes in potential caused by depolarisations of ORNs brought on by the binding of volatile compounds. The technique was first developed in 1957 (Schneider 1957) and was later refined to allow recordings to be made from individual olfactory sensilla (Boeckh 1962). Electrophysiological techniques have since been combined with the powerful separation capability provided by GC to determine the electrophysiological responses of an insect to each compound as it exits the GC column (Moorhouse et al. 1969; Wadhams 1982, 1990).

Separation of volatile compounds involves injecting a sample onto a GC column housed in an oven. As the oven heats up the different volatile compounds within the sample move through

the column at different speeds due to differences in boiling points. Separation is aided by the stationary phase, which is a material lining the inside of the column which interacts differently with different compounds travelling through by formation of hydrogen bonds, dipole-dipole interactions and Van der Waals forces. Volatile compounds therefore separate and exit the GC column according to boiling point and interaction with the stationary phase (Flanagan 1993). In coupled GC-EAG, the GC column terminates in an air stream being blown over an insect antenna. In this way, electrophysiological responses can be recorded to each volatile compound as it leaves the GC column. Coupled GC-EAG analyses thus allow individual peaks on a GC trace to be labelled as electrophysiologically active. These compounds are then prioritised for identification of chemical structure (Wadhams 1990).

#### **1.5.4. Identification of volatile compounds**

Identification of volatile compounds can be achieved using mass spectrometry and comparison of GC retention times with those of authentic standards. Electron impact mass spectrometry works by bombarding a sample with electrons causing the molecules to lose electrons and to fragment into ions (Watson & Sparkman 2007). A high voltage is then used to accelerate the ions through a magnetic field which deflects them onto a detector. The angle of deflection is dependent on the mass:charge ratio ( $m/z$ ) of the ions. As each molecule is broken into several ions this provides characteristic mass spectra giving the fragmentation pattern for different compounds. These spectra can give the molecular weight for the molecular ion and provide a characteristic ‘fingerprint’ of a compound. This can be compared with spectra of known compounds from a database to provide a tentative identification or be used, from mass spectrometry theory, to predict a tentative structure. In coupled GC-MS, the GC column terminates in the ionization source of the mass spectrometer, allowing spectra to be obtained for each of compound as it leaves the GC column (Watson & Sparkman 2007). This allows spectra to be taken for GC peaks identified as electrophysiologically active during coupled GC-EAG.

Comparison of mass spectra only provides a tentative identification of a compound. Identification needs to be confirmed using GC analysis by comparing retention times of the compound with authentic standards on two GC columns possessing stationary phases of different polarities (Pickett 1990). Retention time of a compound is a product of boiling point and interaction with the stationary phase of the GC column. Use of 2 GC columns of different

polarities therefore ensures that compounds with similar boiling points can be distinguished from one another as they will pass through columns of different polarities at different speeds. For compounds that exist as different enantiomers (mirror images of the same molecule), an additional step in the identification process is required as mass spectrometry and conventional GC analysis cannot be used to distinguish between different enantiomers. This is because different enantiomers possess identical boiling points and have the same polarities and hence interact with the stationary phase of a GC column in the same way. Chiral GC analysis employs a GC column with a stationary phase which interacts differently with different enantiomers, resulting in different retention times for each (Taylor 1993). By comparing retention times of the compound to be identified with authentic standards of each enantiomer on such a column, stereochemistry can be determined.

To quantify compounds within a sample, a GC fitted with a flame ionization detector (FID) may be used. The magnitude of the FID response is proportional to the number of carbon atoms reaching the detector (Flanagan 1993). Known concentrations of a compound can be used to plot a calibration curve of FID response against quantity of a compound. Since the FID responds differently to different chemical structures, separate calibration curves are usually constructed for each compound that needs to be identified if accurate determination of quantities is required (Bartle 1993). This can be very time consuming, however, so simpler, more convenient methods of quantification may be employed if only estimates of quantities are required. A single known concentration of the compound to be quantified can be injected onto a GC column and the area of its FID peak used to estimate quantities of the same compound within a sample by dividing the peak area of the known standard by its quantity and multiplying by the peak area of the compound to be quantified. When a large number of compounds need to be quantified, the peak area from a single known standard or the average peak area of several known standards (e.g. from an alkane series) may be used to estimate the quantities of all compounds in this way. This results in lower accuracy as it does not take into account differential FID responses to different compounds but has the advantage of being far less time consuming when a large number of compounds need to be quantified.

## 1.6. General biology and life cycle of *Aphis fabae*

*Aphis fabae* has a complex, host-alternating life cycle (Figure 1.3) (Powell & Hardie 2001). It over-winters as diapausing eggs on its primary host, spindle, *Euonymus europaeus*, before foundress females (fundatrices) hatch in the spring, giving rise to several parthenogenetic generations of viviparous females. These eventually give birth to winged spring migrants (virginoparae) who go on to colonise a variety of secondary host plants including bean (*V. faba*), sugar beet (*Beta vulgaris*) and a variety of other species (Cammell 1981; Fernandez-Quintanilla et al. 2002). Once a secondary host plant has been located and accepted by a spring migrant, the aphid gives birth to wingless females (apterous virginoparae) which undergo several generations of parthenogenetic viviparous reproduction. High population densities on the secondary host plant stimulate the production of winged virginoparae (Lees 1966; Sutherland 1969; Müller et al. 2001) which move to other plants to start new colonies. Although winged virginoparae are known to fly to new hosts there is evidence to suggest that walking also plays a role in aphid dispersal over short distances (Hardie 1980; Hodgson 1991; Mann et al. 1995).

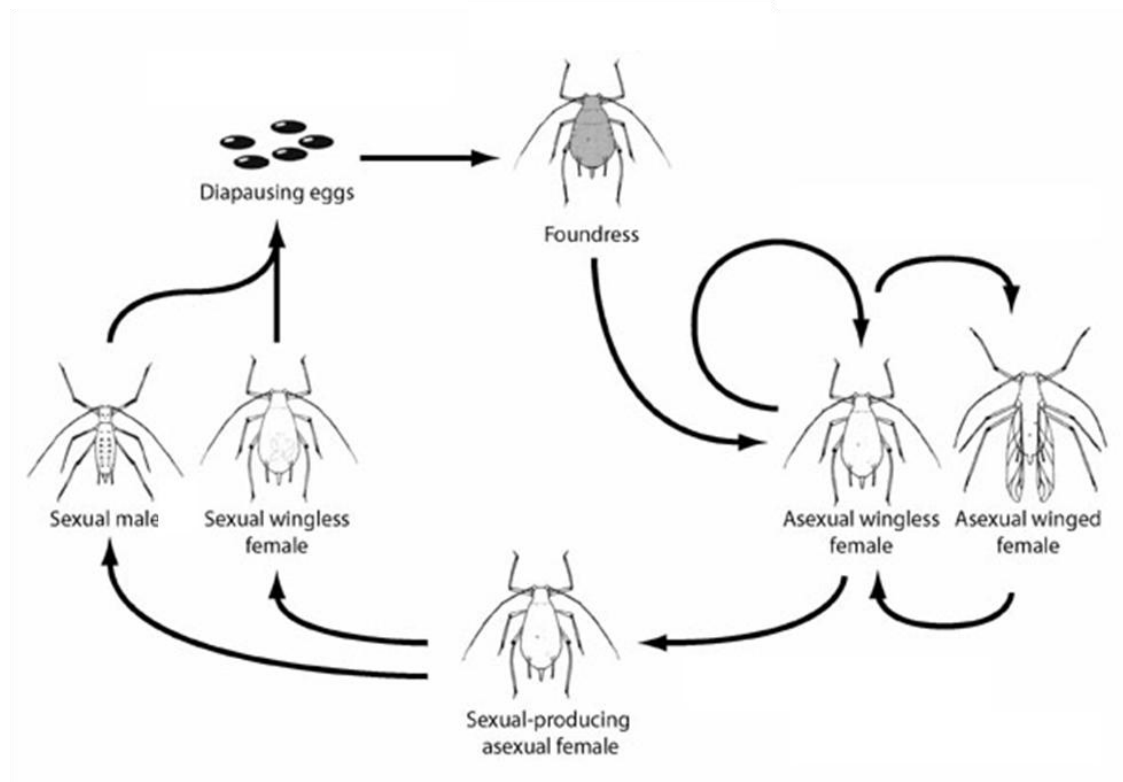


Figure 1.3. Aphid life cycle (adapted from Blackman 1974).

Despite *A. fabae* having been observed on a wide range of secondary hosts, evidence exists for a number of host-races rather than it being a truly polyphagous species (Thieme 1987). Douglas (1997) found that twenty nine different clones of *A. fabae* developed preferentially on either *V. faba* or *Tropaeolum majus* but none of them developed well on both hosts, suggesting that different clones are adapted to different summer host plants. Similar conclusions have been drawn from other studies (Mackenzie 1996; Tosh et al. 2004) lending further weight to the hypothesis that *A. fabae* exists as a number of host-races, each possessing a narrow host range.

Shorter days signal the beginning of autumn during which winged female autumn migrants (gynoparae) are produced. These return to the primary host and give birth to the wingless sexual females (oviparae). Shortly afterwards winged males are produced on the secondary host that fly to the primary host to mate with the females. Females subsequently lay the eggs that over-winter. As well as possessing a sexual phase, it has also been shown that *A. fabae* can remain on some of its summer hosts indefinitely if winter temperatures and abundance of the secondary host during winter months allow continuous development (Cammell 1981).

## **1.7. Aphid chemical ecology**

Aphid chemical ecology has received some previous attention which has been reviewed in Dawson et al., (1990), Pickett et al., (1992) and Pickett and Glinwood (2008). Some of the key topics are discussed in the following sections.

### **1.7.1. Host and non-host recognition**

Olfaction is known to play a prominent role in aphid host location (Pickett et al. 1992; Pickett & Glinwood 2008) and behavioural responses of aphids to host volatiles have been demonstrated in a wide range of species (Pospisil 1972; Pettersson 1973; Chapman et al. 1981; Visser & Taanman 1987; Pickett et al. 1992; Nottingham & Hardie 1993; Pettersson et al. 1994; Jimenez-Martinez et al. 2004; Vargas et al. 2005; Gish & Inbar 2006). In *A. fabae*, early evidence showed that gynoparae responded to the odour of leaves of its winter host, spindle (*Euonymus europaeus*) (Jones 1944). Subsequently it was demonstrated that both winged and wingless virginoparae *A. fabae* move towards the odour of excised *V. faba* (var.

Sutton dwarf) leaves in a linear track olfactometer (Nottingham et al. 1991). The volatile compounds used in host location, however, were not identified.

Comparatively more is known about the behavioural responses of *A. fabae* to non-host volatiles than to host volatiles. *Aphis fabae* possesses ORNs capable of detecting Brassica-associated isothiocyanates (Nottingham et al. 1991; Visser et al. 1996) and it was found that *A. fabae* is repelled by these compounds in laboratory and field bioassays (Isaacs et al. 1993). This suggested that *A. fabae* used these non-host cues to avoid settling on non-host Brassicaceous plants. A similar example was found in (1R,5S)-myrtenal, a volatile compound associated with gymnosperms that has not been identified in the headspace of *V. faba*. It elicited electrophysiological responses from *A. fabae* and masked the attractive odour of *V. faba* plants, as well as being repellent when presented on its own (Hardie et al. 1994a).

Non-host cues have also been found to play a role in settling responses of *A. fabae*, in the form of epicuticular waxes (Powell et al. 1999). Aphids were found to readily initiate stylet penetration on *V. faba* plants but not on non-host oat plants. When aphids were transferred to glass that had been coated with plant epicuticular waxes, stylet penetration was not significantly greater when waxes from bean leaf were applied but were significantly reduced when waxes from oat leaves were applied. This suggested that a non-host contact cue rather than a host cue was responsible for mediating stylet penetration. It was suggested that 1-hexacosanol, found on the surface of oat leaves but not *V. faba* leaves, was responsible for inhibition of stylet penetration.

### **1.7.2. Aphid sex pheromone**

The first evidence for the existence of the aphid sex pheromone came from Pettersson (1970) who demonstrated that male aphids were attracted to the odour of oviparous females. Since then, the sex pheromone has been found to comprise two compounds for most aphids species, nepetalactone and nepetalactol with different species employing different enantiomers of the two compounds (Dawson et al. 1989; Pickett & Glinwood 2008). Different aphid species also produced different ratios of the sex pheromone components and males responded preferentially to ratios associated with conspecific females (Hardie et al. 1990; Lilley & Hardie 1996; Stewart-Jones et al. 2007). More recently, a third component of the aphid sex pheromone released by the rosy apple aphid, *Dysaphis plantaginea*, was identified as

(1S,2R,3S)-dolichodial (Dewhirst et al. 2008). The behavioural response of male aphids to sex pheromones was increased in the presence of host volatiles for some species of aphid. The bird cherry-oat aphid, *Rhopalosiphum padi*, and the hop aphid, *Phorodon humuli*, were caught in greater numbers in trap catches when host-plant volatiles benzaldehyde and methyl salicylate were combined with sex pheromones compared to traps baited with sex pheromones alone (Pope et al. 2007). This suggested that, as well as using species-specific enantiomers and ratios of sex pheromone components to recognise conspecifics, males are also able to use host-plant cues to ensure they are able to locate females of the correct species.

### 1.7.3. Aphid alarm pheromone

When disturbed by a predator or parasitoid, aphids secrete cornicle droplets containing the monoterpenoid (*E*)- $\beta$ -farnesene (Bowers et al. 1972; Nault et al. 1973; Pickett & Griffiths 1980). (*E*)- $\beta$ -Farnesene causes nearby aphids to disperse to escape the threat that triggered the secretion of this compound (Montgomery & Nault 1977). It was suggested that other aphids, upon detection of the alarm pheromone, would then secrete the same compound themselves to facilitate rapid spread of the signal. Recent attempts to demonstrate this behaviour experimentally, however, have shown that this is not the case (Verheggen et al. 2008). The threshold for initiating a dispersal response varies between different species of aphid. Montgomery and Nault (1977) showed the dose required to disperse 50 % of aphids varied from less than 0.02 ng for *Schizaphis graminum* to over 100 ng for *Aphis illinoisensis*. *Aphis fabae* also only responded at relatively high doses, requiring nearly 100 ng of (*E*)- $\beta$ -farnesene before 50 % dispersal was observed.

For some species, activity of the alarm pheromone may be synergised with plant volatiles. The mustard aphid, *Lipaphis erysimi*, responds more strongly when the alarm pheromone is presented along with Chinese cabbage volatiles (Dawson et al. 1987). The same response was also observed when the Chinese cabbage volatiles were replaced with the host volatile 3-butenyl isothiocyanate. Similar results were obtained for *A. fabae*, when it was found that aphids were alarmed more strongly when (*E*)- $\beta$ -farnesene was presented alongside bean volatiles, but the volatiles responsible were not identified. Other plant volatiles may inhibit the behavioural response to the alarm pheromone. As well as being secreted by aphids, (*E*)- $\beta$ -farnesene is also a common plant volatile (Knudsen et al. 1993) and so aphids must be able to

discriminate between host- and aphid originating (*E*)- $\beta$ -farnesene. This is achieved by using certain plant volatiles as alarm pheromone inhibitors. The first evidence of this came from studies with *Myzus persicae*, which showed response to the alarm pheromone was inhibited by the presence of the plant compound, (*E*)-caryophyllene (Dawson et al. 1986). More recently, it has been shown that the alarm response of *A. fabae* can be inhibited by the presence of linalool (Bruce *et al.*, personal communication), a volatile compound emitted by its summer host *V. faba* (Colazza et al. 2004; Pareja et al. 2009).

## **1.8. The use of semiochemicals in pest control**

The use of semiochemicals in pest control is receiving increased attention due to increasing instances of pesticide resistance by insects (Jutsum et al. 1998) and increasing concerns over environmental and economic costs of such practices (Pretty et al. 2000; Pimentel 2005; Carvalho 2006), as well as recent changes in pesticide legislation (e.g. EU directive 91/414). Aphids can be particularly difficult to control with insecticides. Due to congregation on the underside of leaves and within the furls of young leaves, aphids are difficult to target with spraying meaning that systemic pesticides are usually required and these often suffer from a lack of specificity. The use of such broad-spectrum pesticides can be disadvantageous as they may cause reductions in populations of natural enemies (Cork et al. 2005), which would normally help suppress pest populations. Semiochemicals, on the other hand, are usually species-specific and so the use of semiochemicals in control of aphid pests may prove useful when combined with other practices in integrated pest management (IPM). Some of the major ways in which semiochemicals have been used in pest control are discussed below.

### **1.8.1. Monitoring pest populations**

Incorporation of attractive semiochemicals into traps used for monitoring insect pests can increase the efficiency of traps, helping farmers to predict outbreaks and make informed decisions about the application of pesticides. An example of such a practice is that used to monitor the orange wheat blossom midge, *Sitodiplosis mosellana*, a serious pest of wheat in the Northern hemisphere. Infestations of this pest vary from year to year, making it difficult for farmers to predict when to apply pesticides. The sex pheromone of the orange wheat blossom midge was identified as (2*S*,7*S*)-nonadiyl dibutyrate, (Gries et al. 2000) and has

since been incorporated into a commercial monitoring trap (Phero Tech Inc., Delta, Canada). Field trials showed that the trap gave a reliable indication of peak midge emergence and showed a significant correlation between maximum trap catch and crop infestation levels (Bruce et al. 2007). The incorporation of host-volatiles into monitoring traps for the orange wheat blossom midge has also been discussed (Birkett et al. 2004). This would overcome the problem associated with pheromone-baited traps which usually only attract males and only during certain times of the year. Kairomones, however, are often not as attractive or species specific as pheromones and so the effectiveness of a kairomone-baited trap may be limited.

Aphid populations can be effectively monitored with suction traps (Cammell et al. 1989; Perez et al. 2007) but these are only available in a small number of locations. The use of pheromone- or kairomone-baited monitoring traps could also potentially aid farmers in predicting outbreaks. There is evidence to suggest that some species of aphid may produce and respond to aggregation pheromones (Pettersson et al. 1998) and this could possibly be integrated into a monitoring trap. The use of sex pheromones and attractive plant kairomones may also be effectively incorporated into such traps. An advantage of semiochemical-baited traps over non-discriminating methods of monitoring such as the use of suction traps is that semiochemicals are often specific to the insect pest, allowing more efficient monitoring of the pest species without catching non-target insects.

### **1.8.2. Mating disruption**

Male insects generally locate conspecific females by following a pheromone plume released by the female. Synthesis of the pheromone artificially and release of it in large quantities in the field has been found to successfully disrupt mating in some species and it is believed this is achieved by camouflaging the signal emitted by females and providing competing sources for males, reducing their searching efficiency (Carde & Minks 1995). Gaston et al., (1967) were the first to successfully demonstrate the potential application of this technique when they succeeded in disrupting mating of the cabbage looper moth (*Trichoplusia ni*) by evaporating its pheromone from steel planchettes mounted on stakes. Since then the technique has been used to disrupt mating in a number of other insect pest species (Hegazi et al. 2007; Alfaro et al. 2009; Gambon et al. 2009) and represents an example of successful pest control using semiochemicals.

### 1.8.3. Mass trapping

As well as being used for monitoring pest populations, semiochemical-baited traps can also be used to directly reduce pest populations through mass trapping. This has achieved considerable success in controlling certain pest species where highly attractive semiochemicals have been identified (El-Sayed et al. 2006). A pertinent example of mass trapping is that used to control the bark beetle. It was already known that deliberately felled trees could be used as “trap trees” to concentrate populations into one area for easy removal. Following the discovery of the aggregation pheromone of the western pine beetle (Silverstein et al. 1968), attempts at large scale trapping using mechanical traps began and met with mixed results. Initial trials in 1970 in California indicated that mass trapping significantly reduced tree mortality caused by bark beetles but were contrasted by subsequent trials which showed little reduction in tree mortality (El-Sayed et al. 2006). Norway saw the largest scale attempts at controlling bark beetle populations by mass trapping when in 1979 approx. 600,000 pheromone-baited traps were deployed in forests suffering minor outbreaks (El-Sayed et al. 2006). Damage to trees was reduced but the success of the programme is debated, however, as no control plots were used to compare its effectiveness. Mass trapping has also been employed on a much smaller scale. A successful programme was developed for commercial sawmills when it was found that sticky traps baited with 6-methyl-5-hepten-2-ol were effective at reducing damage to stored wood by the Ambrosia beetle, *Gnathotrichus sulcatus* (McLean & Borden 1979). A similar technique is ‘lure and kill’ where an attractive agent, such as a pheromone, is used to lure insects in large numbers (El-Sayed et al. 2009). As the name suggests, insects are then killed rather than trapped and this is typically achieved using insecticides. As the attractive lure concentrates the insects in one place, far less insecticide is required than if it were applied to the crop and so there are economical advantages of such a practice over conventional insecticide usage.

The success of either of these techniques relies on an attractive lure that can draw sufficient numbers of pest insects to significantly reduce the damage they cause to the crop. Due to parthenogenetic reproduction and viviparity, aphids have short generation times and therefore high rates of reproduction meaning that even a small initial infestation can quickly lead to large populations and significant damage to the crop (El-Amri 1999). Any mass trapping or

lure and kill programme developed for aphids is therefore likely to meet with difficulty as if even a small number of aphids evade the lure, this could be enough to lead to significant crop damage.

#### **1.8.4. Transgenic approaches**

The aphid alarm pheromone, (*E*)- $\beta$ -farnesene, is secreted by aphids under attack from predators or parasitoids and causes nearby aphids to disperse (Nault et al. 1973; Montgomery & Nault 1977). (*E*)- $\beta$ -Farnesene is also produced in relatively smaller quantities by some plant species but aphids use other plant volatiles as alarm pheromone inhibitors to avoid being dispersed by this volatile when released by plants (Dawson et al. 1986). Recently, it was shown that high levels of expression in *Arabidopsis thaliana* plants of an (*E*)- $\beta$ -farnesene synthase gene cloned from *Mentha piperita*, resulted in the increased emission of (*E*)- $\beta$ -farnesene which was sufficiently high to elicit an alarm response from the aphid *Myzus persicae* and an arrestant response from its parasitoid *Diaeretiella rapae* (Beale et al. 2006). Work is currently underway to develop transgenic wheat which emits high levels of (*E*)- $\beta$ -farnesene capable of alarming aphids which attempt to settle on the wheat (Pickett et al., personal communication) and, if successful, would be the first example of a practical means of pest control combining transgenic approaches with chemical ecology. In theory, it should be possible to upregulate genes or incorporate new genes involved in the production of other semiochemicals repellent to aphids into crop plants such as *V. faba* as a way of deterring aphids from settling on crops.

#### **1.8.5. Stimulo-deterrent diversionary strategies**

Stimulo-deterrent diversionary strategies (SDDS) or “push-pull” involve using a repellent within the crop and an attractive lure outside the crop to divert pests. The method was first developed by Miller and Cowles (1990) for the control of onion fly, *Delia antiqua*, and has since been used to great effect in other systems, significantly reducing yield loss due to agricultural pests. Semiochemicals used to recruit natural enemies of pests may also be incorporated into such a system to further reduce pest populations. A recent example of a successful push-pull programme is that used to control the stem borer, a pest of maize in Africa (Khan & Pickett 2004). Stem borers are Lepidoptera whose larvae infest maize crops at all stages of development, from seedling to maturity. There are several species which infest

maize, with *Chilo partellus* and *Busseola fusca* being of particular significance in Eastern Africa. It was found that stemborers were preferentially attracted to Napier grass (*Pennisetum purpureum*) and Sudan grass (*Sorghum vulgare*) over maize (Khan & Pickett 2004). Stemborers did not, however, survive well on Napier grass and showed higher rates of parasitisation on Sudan grass, suggesting the plant was recruiting parasitoids of the stemborers. This made both grasses excellent candidates for trap crops which could be used to divert the pest. It was also found that intercropping the maize with *Desmodium uncinatum* protected crops further by emitting volatiles that repelled stemborers (Khan & Pickett 2004). In this way an attractive trap crop planted around the main crop and a repellent crop intercropped with the main crop have been successfully used to divert stemborers and significantly reduce damage caused by these pests (Khan et al. 2008).

Push-pull has also been explored as a way of controlling pests of *V. faba* (Smart et al. 1994). It was found that application of neem oil to *V. faba* crops significantly reduced infestation by the pea and bean weevil, *Sitona lineatus*, and that application of the aggregation pheromone, 4-methyl-3,5-heptanedione significantly increased numbers of weevils. Combining these two semiochemicals into a push-pull system could, therefore, theoretically be used to protect *V. faba* crops from this pest. A similar system may also be possible for controlling *A. fabae* by combining repellent semiochemicals applied to a crop and attractive semiochemicals used to lure aphids away from the crop. Special care must be taken, however, when using semiochemicals in such systems as some compounds can have opposite effects on different pests. For example, *cis*-jasmone is a plant volatile that repels the peach-potato aphid, *Myzus persicae*, which is a pest of brassicaceous crops. The same compound, however, is attractive to the specialist aphid *Lipaphis erysimi* and this could potentially limit its potential use in IPM (Bruce et al. 2008). The success of any semiochemical-based approach to IPM therefore requires a complete understanding of the ecology of the insects associated with the crop.

A number of compounds which repel *A. fabae* and deter them from settling on *V. faba* have already been identified (Nottingham et al. 1991; Isaacs et al. 1993; Nottingham & Hardie 1993; Hardie et al. 1994a; Storer et al. 1996) and the aphid sex pheromone has also been identified (Dawson et al. 1989; Dewhurst et al. 2008). In contrast, comparatively little is known about volatile compounds used in host recognition by this species of aphid. Investigating the mechanisms of olfactory host recognition in *A. fabae* would therefore provide a valuable contribution to an understanding of the chemical ecology of this insect

pest and may ultimately help lead to the development of novel semiochemical-based means of pest control.

## 1.9. Aims and objectives

The aim of this research was to determine how *A. fabae* uses volatile chemical cues to recognise its host plant, *V. faba*. To achieve this aim, the following objectives were envisaged:

- Devise a suitable behavioural bioassay to determine behavioural responses of *A. fabae* to host volatiles. It was hypothesised that the four-arm olfactometer would prove to be suitable for recording aphid responses to host volatiles. (Chapter 2)
- Determine behavioural responses of *A. fabae* to host-plant odour. It was hypothesised that *A. fabae* would respond positively to the odour of its host plant by spending more time in the treated region of the olfactometer than controls. (Chapter 2)
- Use air entrainment to collect volatile compounds from *V. faba* plants. (Chapter 2)
- Use coupled GC-EAG to determine electrophysiological responses of *A. fabae* to collected *V. faba* volatile compounds. (Chapter 3)
- Identify electrophysiologically active volatile compounds using coupled GC-MS and GC analysis. It was hypothesised that all identified compounds would be general plant volatiles and not taxonomically characteristic of *V. faba*. (Chapter 4)
- Determine behavioural responses of *A. fabae* to identified compounds and to determine whether or not host recognition is mediated by a single compound or a blend of host volatiles. It was hypothesised that host recognition is not mediated by a single compound and that the complete blend would be preferred over individual compounds alone. (Chapter 5)
- To identify any host-characteristic volatile cue by determining intraspecific and diurnal variation in emission of volatile compounds by *V. faba*. It was hypothesised that while there would be variation in the quantities of volatiles produced there would be some consistency in the ratios and this could theoretically provide a species-characteristic cue for host-seeking *A. fabae*. (Chapter 6)

# CHAPTER 2. BEHAVIOURAL RESPONSES OF *Aphis fabae* TO *Vicia faba* VOLATILE COMPOUNDS

## 2.1. Introduction

Olfaction plays a prominent role in aphid host location (Pickett et al. 1992; Pickett & Glinwood 2008). Early work into host location in *A. fabae* showed that gynoparae responded to the odour of leaves of the winter host, spindle (*Euonymus europaeus*) (Jones 1944). More recently it was demonstrated that both winged and wingless virginoparae *A. fabae* move towards the odour of excised *V. faba* (var. Sutton dwarf) leaves in a linear track olfactometer (Nottingham et al. 1991). The use of olfaction in host location has now been demonstrated in a wide range of aphid species (Pospisil 1972; Pettersson 1973; Visser & Taanman 1987; Pickett et al. 1992; Nottingham & Hardie 1993; Pettersson et al. 1994; Jimenez-Martinez et al. 2004; Vargas et al. 2005; Gish & Inbar 2006) but the volatile compounds used in host location by *A. fabae* have not yet been identified. One of the aims of this project was to identify *V. faba* volatile compounds used in host location by *A. fabae*. The first step was to select an appropriate behavioural bioassay to record the behavioural responses of *A. fabae* to the odour of its host plant. This chapter also deals with the first steps in identifying *V. faba* volatile compounds used in host location by *A. fabae*. These were the isolation of *V. faba* volatile compounds by headspace sampling and the use of olfactometer bioassays to determine the behavioural responses of *A. fabae* to the collected volatiles.

### 2.1.1. Aims and objectives

The aims of the experiments described in this chapter were to identify a suitable bioassay for testing behavioural responses of aphids, to collect volatile compounds from the headspace of intact *V. faba* plants and to determine the behavioural responses of alate virginoparae *A. fabae* to these volatile compounds. To achieve these aims the following objectives were envisaged:

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Note: a refereed paper relating to the work in this Chapter has been published (Webster et al., 2008a).

### **Determine suitability of four-arm olfactometer for measuring behavioural responses of *Aphis fabae* to host volatiles**

The four-arm olfactometer has not previously been used to record behavioural responses of *A. fabae* to host volatiles. To determine the suitability of this olfactometer for the present study, behavioural responses of aphids to excised *V. faba* leaves and mechanically damaged leaves as tested by Nottingham *et al.*, (1991) using the linear track olfactometer were determined. It was hypothesised that responses will be similar to those described by Nottingham *et al.*, (1991). That is, aphids will spend significantly more time in the treated region of the olfactometer compared to controls when excised leaves are used as an odour source but not when mechanically damaged leaves are used as an odour source.

### **Collect volatile compounds from the headspace of intact *Vicia faba* plants**

In order to identify volatile compounds used in host location by *A. fabae*, the volatile compounds emitted by *V. faba* must first be collected. Dynamic headspace analysis was employed as this method avoids causing damage to the plant, which could alter the ratios of volatile compounds collected. Porapak Q was used to collect volatiles as it meant a solution containing isolated compounds could be obtained at the end of the entrainment that could be used in behavioural bioassays and future electrophysiology studies and gas chromatography and mass spectrometry analyses.

### **Determine behavioural response of winged virginoparae *Aphis fabae* to the odour of intact *Vicia faba* plants and to the *Vicia faba* air entrainment sample**

The volatiles emitted by intact *V. faba* plants may differ from those emitted by excised leaves so it was important to determine whether aphids also respond behaviourally to intact plants. This was achieved by using a four-arm olfactometer to monitor the aphids' preferences. To determine whether or not volatile compounds used in host location were successfully collected from the headspace of *V. faba* plants, behavioural responses of *A. fabae* to the air entrainment sample were determined using the four-arm olfactometer. It was hypothesised that aphids would respond behaviourally both to the odour of intact *V. faba* plants and to the *V. faba* air entrainment sample.

## 2.2. Materials and methods

### 2.2.1. Insects

The Kennedy and Booth clone of *A. fabae* (Kennedy & Booth 1950) was reared on tick beans (*Vicia faba* var. *minor*) in a Perspex rearing cabinet under controlled conditions (22±1 °C, LD 16:8 h). Winged aphids were induced by allowing the colony to become crowded. Prior to behavioural bioassays, aphids were starved for 24 h in Petri dishes with moistened filter paper to prevent dehydration. Aphids were then left in the bioassay room for at least 2 h to acclimatise prior to experiments.

### 2.2.2. Plants

All plants used were glasshouse-grown in rooms maintained at 20 °C. Faba bean plants (*Vicia faba* cv. Sutton dwarf) were grown individually in 7.5 cm pots in Rothamsted prescription mix compost (Petersfield Products, Leics, UK) and removed from the glasshouse for use in experiments prior to flowering, when the plants were 3 weeks old before the stage of inflorescence emergence.

### 2.2.3. Air entrainment of intact *Vicia faba* plants

Prior to entrainments, all glassware was washed with Teepol detergent (Herts. County Supplies, Herts., UK), rinsed with acetone and distilled water and baked overnight at 160 °C. Three-week-old *V. faba* plants at the vegetative growth stage were enclosed individually in glass vessels (190 mm high x 100 mm wide), open at the bottom and closed at the top except for an inlet and outlet port. The bottom was closed without pressure around the plant stem using two semicircular aluminium plates with a hole in the centre to accommodate the stem. Charcoal filtered air was pumped in at 400 ml min<sup>-1</sup> and drawn out at 300 ml min<sup>-1</sup> through a Porapak Q adsorbent tube (Alltech Associates Inc., Carnforth, Lancashire, 50 mg Porapak Q in 5-mm diameter glass tube) which was used to collect the volatile compounds. Prior to use, Porapak tubes were eluted with 2 ml of redistilled diethyl ether and conditioned at 132 °C for 2 h. The difference in flow rates created a positive pressure to ensure that unfiltered air did not enter the system, removing the need for an air-tight seal around the stem. All connections were made with 1.6 mm i.d. PTFE tubing (Alltech Associates Inc., Lancashire, UK) with

swage lock brass ferules and fittings (North London Valve Co., London, UK) and sealed with PTFE tape (Gibbs & Dandy Ltd., Luton, UK). Entrainments lasted 7 days, after which the Porapak tubes were eluted with 0.5 ml of redistilled diethyl ether, providing an air entrainment sample that contained the collected volatile compounds. The air entrainment samples from entrainments of 4 different plants were combined to give a bulk air entrainment sample for use in subsequent experiments.

#### **2.2.4. Behavioural bioassays using four-arm olfactometer**

A Perspex four-arm olfactometer (Pettersson 1970) was used to determine behavioural responses of *A. fabae* winged virginoparae to host volatiles. Prior to each experiment all glassware was washed with Teepol detergent, rinsed with acetone and distilled water and baked in an oven overnight at 160 °C. Perspex components were washed with Teepol solution and then rinsed with 80 % ethanol solution and distilled water and left to air dry. The olfactometer was fitted with a filter-paper base (Whatman, Maidstone, UK) to provide purchase for the walking insect and was illuminated from above by diffuse uniform lighting from two 18W/35 white fluorescent light bulbs screened with grease-proof paper. The lamps were fitted with solid state electronic ballast causing the lamp flickering rate (26000 Hz) to be above the insects sensitivity level (Shields 1989). It was surrounded by black paper to remove any external visual stimuli and an extractor fan was used to prevent accumulation of volatile compounds in the room. For bioassays with plants, 2-l glass vessels were connected to each of the inlets of the olfactometer by 1.6 mm i.d. PTFE tubing (Alltech Associates Inc., Lancashire, UK) (Figure 2.1). For all other odour sources tested, the glass vessels were replaced with glass arms (900 mm in length with 200 mm i.d. with connecting tubes 500 mm in length with 3 mm i.d.) (Figure 2.2).

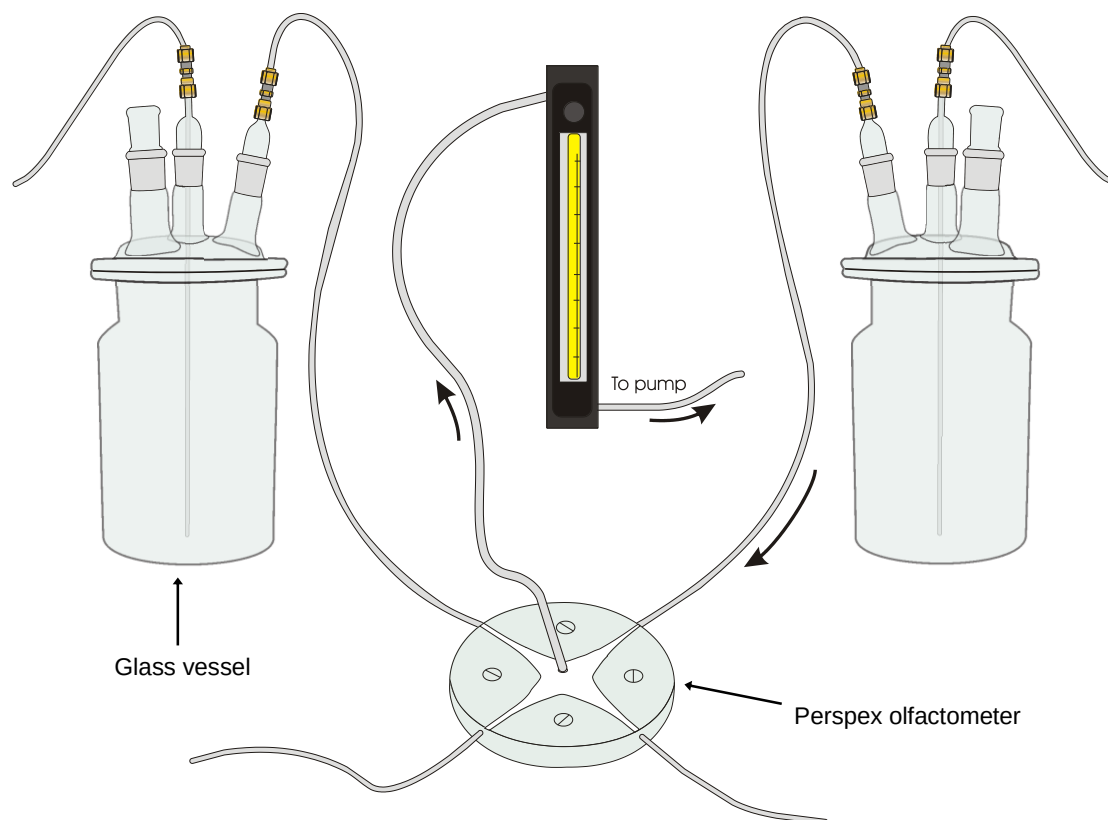


**Figure 2.1. Four-arm olfactometer (120 mm diameter) with 2-l glass vessels to contain odour sources. Large arrows indicate direction of airflow.**

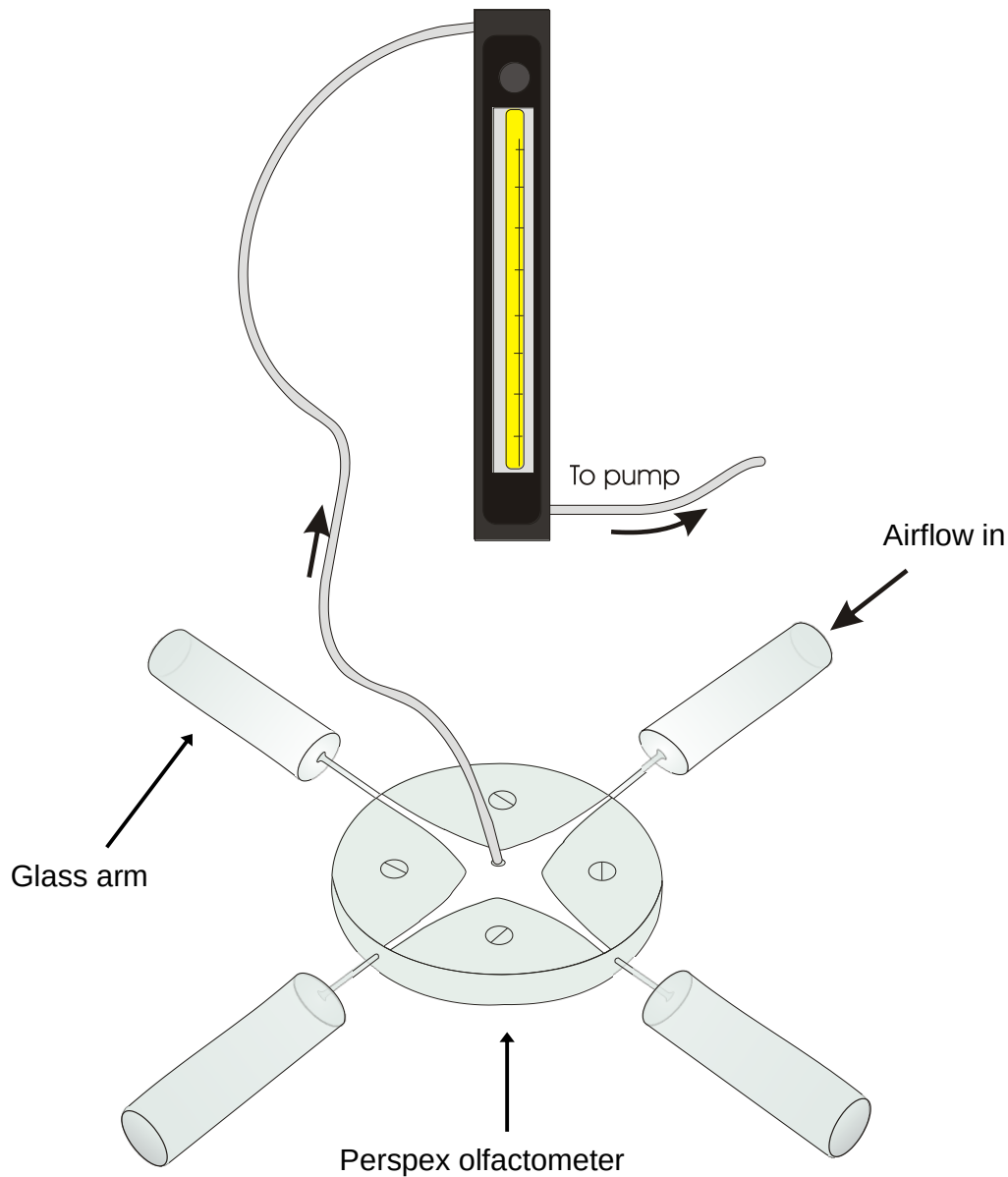


**Figure 2.2.** Four-arm olfactometer (120 mm diameter) with glass arms (900 mm x 200 mm i.d. with 500 mm x 3 mm i.d. connecting arms) to contain odour sources. Large arrows indicate direction of airflow.

### Experimental procedure

A single aphid was introduced through a hole in the top of the olfactometer using a fine paintbrush. Air was then drawn through the central hole at a rate of  $400 \text{ ml min}^{-1}$  to a 2-l glass vessel used to buffer the air flow before being subsequently exhausted from the room. Each aphid was given 2 min to acclimatise in the olfactometer after which the experiment was run for 16 min. The olfactometer was rotated 90 degrees every 2 min to control for any

directional bias in the room. When excised *V. faba* leaves, mechanically damaged *V. faba* leaves, and *V. faba* plants were used as odour sources, the olfactometer was divided into five regions. Regions 1 to 4 corresponded to each of the four glass vessels/arms and region 5 the central region (Figure 2.3). After preliminary observations indicated that host volatiles may act as an arrestant, it was decided to modify the experimental design to more effectively record arrestant behaviours. Consequently, the central region was incorporated into each of the 4 other regions (Figure 2.3) and this method of recording responses was used when the odour of intact plants and the *V. faba* air entrainment sample were used as odour sources. Time spent in each region was recorded using Olfa software (F. Nazzi, Udine, Italy). If an aphid remained motionless for 2 min continuously it was considered inactive and the replicate was rejected. Twelve replicates were carried out for each odour source tested.

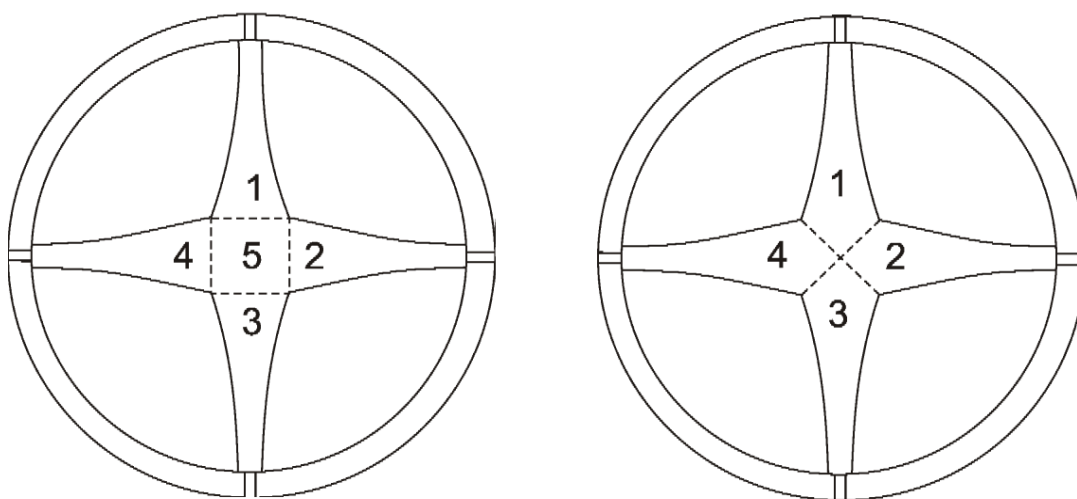


**Figure 2.3. Division of regions in four-arm olfactometer.**

### **List of experiments**

The odour source tested, number of replicates and division of regions in the four-arm olfactometer are summarised in Table 2.1.

**Behavioural response to clean air.** To determine if there was any underlying bias in the olfactometer, time spent in each region of the olfactometer when clean air was used as the odour source in all four arms was tested. This experiment was carried out twice, the first with the olfactometer divided into five regions, the second time divided into 4 regions. Twelve replicates were completed for each.

**Behavioural response to excised *Vicia faba* leaves.** Using a scalpel, 2 g ( $\pm$  0.2 g) of leaves were removed from a three-week old *V. faba* plant and gently placed into one of the glass arms. A triangle of filter paper moistened with distilled water was placed into each of the other three arms to create equivalent humidity. The olfactometer was divided into five regions. Twelve replicates were completed.

**Behavioural response to mechanically damaged *Vicia faba* leaves.** Using a scalpel, 2 g ( $\pm$  0.2 g) of leaves were removed from a three-week old *V. faba* plant. A Pasteur pipette was then used to pierce the leaves approx. 30 times and the damaged leaves placed inside one of the glass arms. A triangle of filter paper moistened with distilled water was placed into each of the other three arms to create equivalent humidity. The olfactometer was divided into five regions. Twelve replicates were completed.

**Behavioural response to intact *Vicia faba* plant.** A single three-week-old *V. faba* plant enclosed in a 2 l glass vessel was used as the odour source. Filter paper saturated with distilled water to create equivalent humidity was placed into each of the other three glass vessels and used as controls. A fresh plant was used for each replicate. Observations made when testing responses to excised leaves suggested that host volatiles may act as an arrestant. For this reason it was decided to incorporate the central region into each of the other four regions (Figure 2.3). To compare the effectiveness of dividing the olfactometer into four regions instead of five, time spent in each region using both division methods was recorded during each experiment. Twelve replicates were completed.

**Behavioural response to *Vicia faba* air entrainment sample.** Ten  $\mu$ l of the *V. faba* air entrainment sample was added to a triangle of filter paper (approx. 50 mm<sup>2</sup> area). Thirty seconds was allowed for the solvent to evaporate and then placed into one of the glass arms. A similar triangle of filter paper which had ten  $\mu$ l ether added was placed in each of the other glass arms and used as controls. The olfactometer was divided into four regions (see Figure b). Twelve replicates were completed.

**Table 2.1. List of four-arm olfactometer experiments.**

| Odour source                                | Control                 | n  | No. olfactometer regions |
|---------------------------------------------|-------------------------|----|--------------------------|
| clean air                                   | clean air               | 12 | 5                        |
| clean air                                   | clean air               | 12 | 4                        |
| 2 g intact <i>V. faba</i> leaves            | moistened filter paper  | 12 | 5                        |
| 2 g damaged <i>V. faba</i> leaves           | moistened filter paper  | 12 | 5                        |
| intact <i>V. faba</i> plant                 | moistened filter paper  | 12 | 5                        |
| intact <i>V. faba</i> plant                 | moistened filter paper  | 12 | 4                        |
| 10 µl <i>V. faba</i> air entrainment sample | 10 µl redistilled ether | 12 | 4                        |

### Statistical analysis

Time spent in the treated region was converted to a percentage of total time spent in regions 1 to 4 and transformed using a logit transformation. The transformed data were found to be normally distributed and then compared to a test mean of -1.099 (logit transformation of 25%) using a one sample *t*-test (Genstat v. 10) to test the hypothesis that aphids spent more than 25% of their time in the treated region.

To compare the time spent in each of the four regions when clean air was used as an odour source, the transformed data were compared using a one-way ANOVA test (Genstat v. 10).

## 2.3. Results

Results of each experiment are described below. The vast majority of aphids used in experiments were active and did not need to be removed from the analysis as non-responders.

### 2.3.1. Behavioural response to clean air

When clean air was used as the test odour, aphids showed no preference for any of the regions of the olfactometer when divided into five ( $P=0.391$ ) (Figure 2.4) or four regions ( $P=0.942$ ) (Figure 2.5).

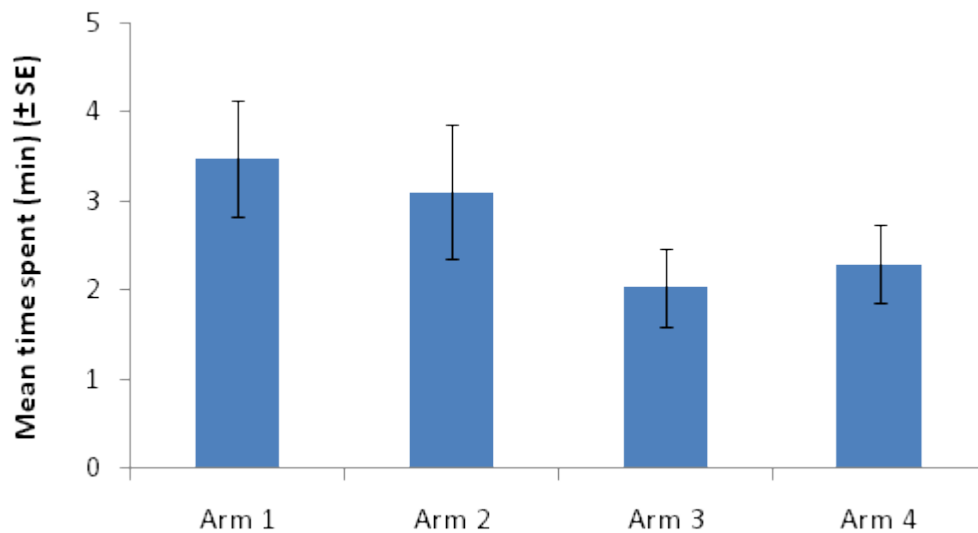


Figure 2.4. Mean time spent in each arm of the olfactometer when clean air was used as the odour source with olfactometer divided into five regions (n=12).

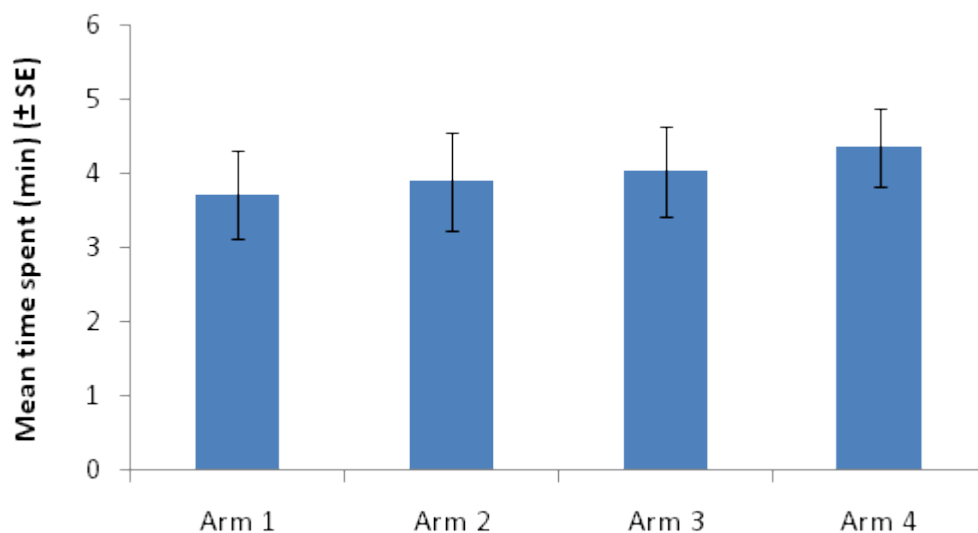


Figure 2.5. Mean time spent in each arm of the olfactometer when clean air was used as the odour source with olfactometer divided into four regions (n=12).

### 2.3.2. Behavioural responses to intact and mechanically damaged excised *Vicia faba* leaves

When 2 g excised *V. faba* leaves were used as the test odour, aphids spent significantly more than 25% of their time in the treated region of the olfactometer ( $P=0.003$ ) but not when leaves which had been mechanically damaged were used as the test odour ( $P=0.198$ ) (Figure 2.6).

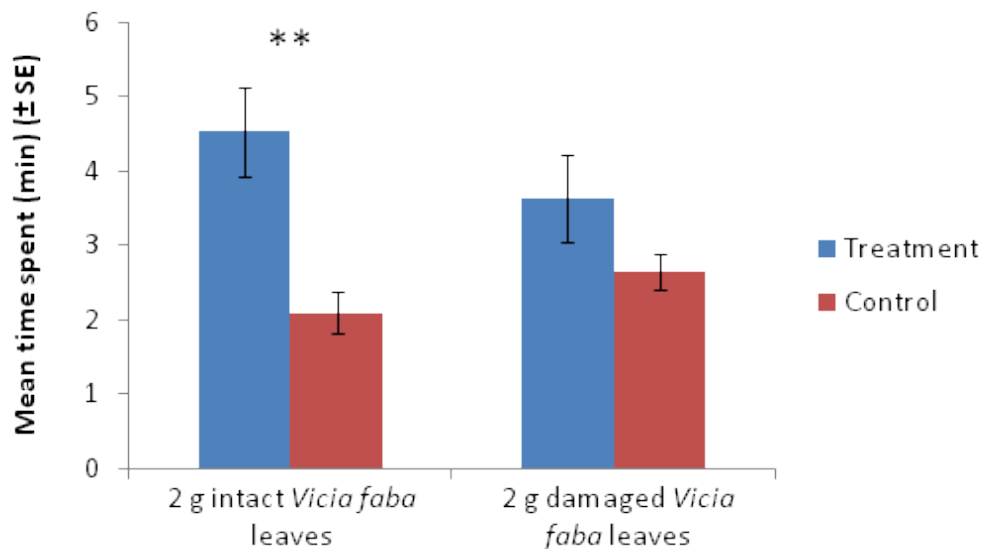


Figure 2.6. Behavioural response of winged virginoparae *Aphis fabae* to 2 g intact and mechanically damaged *Vicia faba* leaves with olfactometer divided into five regions ( $n=12$ ). \*\* Significantly different  $P<0.01$ .

### 2.3.3. Behavioural responses to intact *Vicia faba* plants and *Vicia faba* air entrainment sample

When an intact *V. faba* plant was used as the test odour, aphids spent significantly more than 25 % of the time in the treated region of the olfactometer both when the olfactometer was divided into five regions ( $P=0.004$ ) and four regions ( $P<0.001$ ) (Figure 2.7), and also when the bulk air entrainment sample was used as the test odour ( $P=0.048$ ) (Figure 2.8).

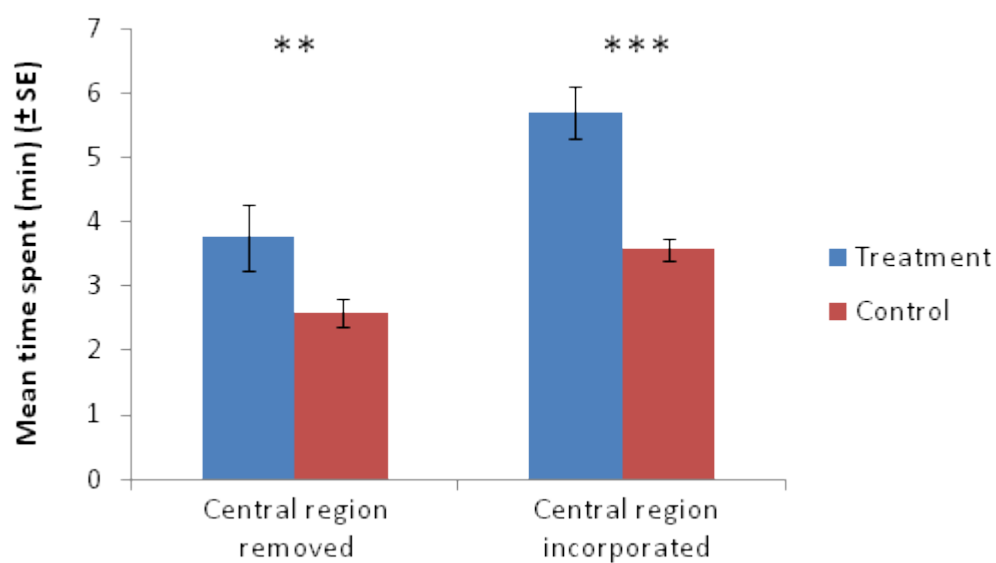


Figure 2.7. Behavioural response of winged virginoparae *Aphis fabae* to *Vicia faba* plant with central region of olfactometer divided into four and five regions ( $n=12$ ). \*\* Significantly different  $P<0.01$ . \*\*\* Significantly different  $P<0.001$ .

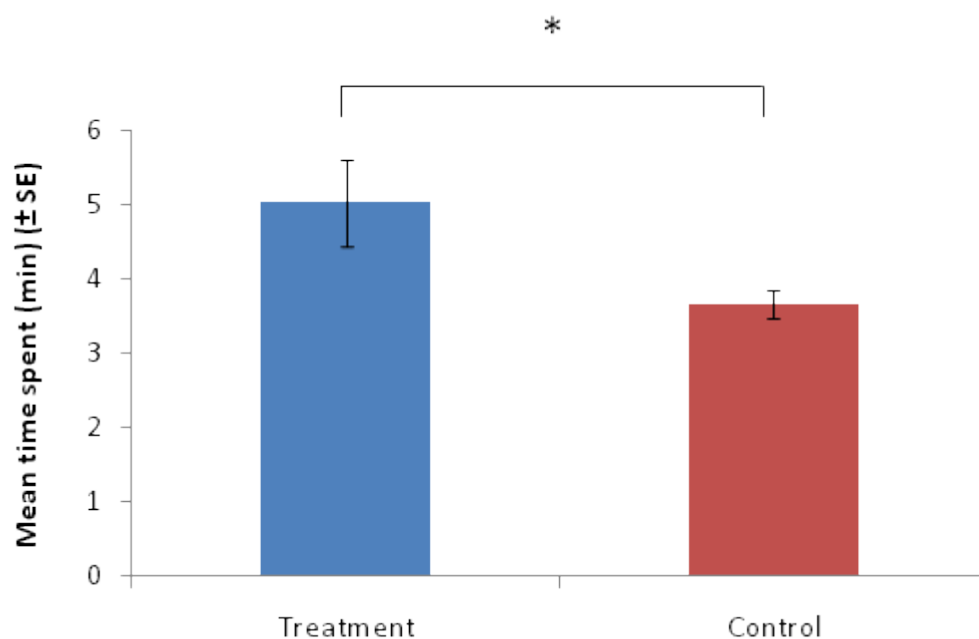


Figure 2.8. Behavioural response of winged virginoparae *Aphis fabae* to *Vicia faba* air entrainment sample with olfactometer divided into four regions ( $n=12$ ). \* Significantly different  $P<0.05$ .

## 2.4. Discussion

### 2.4.1. Behavioural responses of *Aphis fabae* to clean air

In olfactometer bioassays, aphids did not show any preference for any region of the olfactometer when clean air was used as the test odour. This demonstrated that the aphids showed no underlying preference for any region of the olfactometer. Testing the behavioural responses of aphids to clean air can be used to determine if there is a fundamental bias in the design of the olfactometer which causes aphids to spend more time in one region than others. However, as a new olfactometer is used in each experiment this method will not pick up on bias present in individual olfactometers; for example, a region of an olfactometer contaminated with a behaviourally active volatile compound. This potential problem can be eliminated by thorough cleaning of olfactometers prior to experiments and sufficient replication with a clean olfactometer used for each replicate.

As well as problems with the olfactometers, sources of bias may also exist in the bioassay room. For example, a behavioural response due to a visual response to an imbalance in the lighting in the room may override responses to test compounds. This potential problem was accounted for by surrounding the olfactometer with black paper to remove any external visual stimuli. Another possible source of bias would be a contaminating odour in the room which could influence the behaviour of the aphids. The room used in the present study was dedicated to behavioural bioassays and did not contain any plant material other than that in the treated arm of the olfactometer. Use of an extractor fan to prevent build up of contaminating volatile compounds also helped minimise this problem. Finally, adjusting to the new environment of the room in which bioassays were conducted prior to experiments could affect the aphids' behavioural responses. For example, behavioural responses to the change in environment could override responses to plant volatiles. To minimise this risk, aphids were left to acclimatise in the bioassay room for at least 2 hours prior to bioassays and were also allowed 2 min to acclimatise before any recordings were made once they had been placed in the olfactometer. Table 2.2 summarises these potential sources of bias and the measures taken to minimise their effects.

**Table 2.2. Potential sources of bias in olfactometer experiments and actions taken to avoid or minimise their effects.**

| Source of bias                                        | Actions taken to avoid or minimise bias                                                                                                                      |
|-------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Asymmetric design of olfactometer                     | Bioassays using clean air were carried out to determine if there was any underlying preference for any region of olfactometer                                |
| Chemical contamination of olfactometers               | Olfactometers were cleaned thoroughly prior to use<br>Replication of experiments using new olfactometers each time                                           |
| Contaminating odour in bioassay room                  | Extractor fan was used to prevent build up of volatile compounds<br>Room was dedicated to behavioural bioassays and did not contain any other plant material |
| Light imbalance in the bioassay room                  | Olfactometer was surrounded with black paper to minimise light imbalance<br>Grease-proof paper was used to diffuse light source above olfactometer           |
| Any other directional source of bias in bioassay room | The olfactometers were rotated every 2 min during bioassays                                                                                                  |
| Stress caused to aphids due to change in environment  | Aphids were given at least 2 h to acclimatise to the bioassay room and 2 min to acclimatise to the olfactometer prior to recording behaviour                 |

### **2.4.2. Behavioural responses of *Aphis fabae* to intact and mechanically damaged *Vicia faba* leaves**

Aphids spent significantly more than 25 % of their time in the treated region of the olfactometer when intact *V. faba* leaves were used as the test odour but this response was lost when the leaves were mechanically damaged by punching holes in them with a Pasteur pipette. This is consistent with previous work which showed that aphids moved towards the odour of excised *V. faba* leaves in a linear track olfactometer but failed to respond in the same way when the leaves were mechanically damaged (Nottingham et al. 1991). The purpose of repeating this experiment here was to determine if the four-arm olfactometer could be successfully used to determine behavioural responses of *A. fabae* to host volatiles and would yield similar results to the previous study. As the results are similar to those obtained by Nottingham *et al.*, (1991), this suggests that the four-arm olfactometer is suitable for behavioural bioassays carried out in this project and so the decision was made to continue working with this olfactometer.

### **2.4.3. Behavioural responses of *Aphis fabae* to *Vicia faba* plant odour**

Behavioural responses of *A. fabae* to *V. faba* leaves may be different from the behavioural response to an intact plant. The minor damage caused to the leaf when it is cut from the plant may result in qualitative and quantitative differences in volatile emissions and it is already known that significant differences in volatile emission are observed between intact and mechanically damaged *V. faba* plants (Agelopoulos et al. 1999b). For this reason, it was deemed necessary to determine behavioural responses of the aphids to intact plants.

Preliminary observations of aphids responding to the odour of *V. faba* leaves suggested that host volatiles may act as an arrestant, as the aphids appeared to pause upon entering a region where the odour stream would be encountered. Other species of aphid are also known to be arrested by host volatiles (Eigenbrode et al. 2002) and so an experimental design which is capable of recording arrestment behaviour would be a useful tool in determining behavioural responses to host volatiles. Traditionally, the four-arm olfactometer has been divided into five regions: four regions corresponding to each of the treatment arms and a fifth central region which is usually removed from statistical analysis (Pettersson 1970; Vet et al. 1983). First encounter of the odour plume by a walking aphid would normally occur in the region of the

central area closest to the treated arm. This means that any initial arrestment behaviour upon encounter of the odour plume would normally happen in the central region. For this reason, it was decided to incorporate the central region into the four other regions so that any initial arrestment behaviour upon encounter of the odour plume would count towards proportion of time spent in the treated region. When an intact *V. faba* plant was used as an odour source, recordings were made using both methods of olfactometer division simultaneously to compare the effect the different methods have on the results obtained. Aphids spent significantly more than 25% of their time in the treated region of the olfactometer when both methods were employed. This was marginally more significant when the olfactometer was divided into four regions ( $P < 0.001$ ) than when it was divided into five regions ( $P = 0.004$ ) but the difference is small and both methods yielded significant results. The reason for this small difference may be that both methods are so highly effective at recording responses to *V. faba* volatiles that differences in effectiveness are negligible. It may also be that the behavioural response of the aphids to host odour is partly due to an attraction response (i.e. oriented movement towards the odour source) rather than just an arrestant response, meaning that the new division of regions makes less of a difference to the proportion of time spent in the treated region. The possibility remains, however, that for other olfactory stimuli the effectiveness of the two methods could differ more significantly. A highly arrestant volatile compound could induce cessation of movement in the central region of the olfactometer, resulting in no recorded behavioural response. For this reason, it was decided to divide the olfactometer into 4 regions for all future behavioural experiments.

#### **2.4.4. Isolation of volatile compounds from *Vicia faba* plants**

When the air entrainment sample was used as the test odour, aphids spent significantly more than 25 % of their time in the treated region of the olfactometer. This suggested that volatile compounds mediating the behavioural response to host odour were successfully collected and were present in the air entrainment sample. Time spent in the treated region when the air entrainment sample was used as the test odour was slightly less than time spent when a *V. faba* plant was used as the test odour (Figures 2.7 and 2.8) but these times cannot easily be compared. Not only were the bioassays carried out on different days, but it is difficult to accurately quantify the volatile compounds reaching the aphids from the intact plant and so differences in behavioural responses may be the result of different concentrations of volatiles. Differences in humidity between the two experiments also make comparisons difficult. When

plants were used as the odour source, increased humidity from the plants and moistened filter paper used in controls may have affected the behavioural response to host odour. Previous work has found levels of humidity can influence behaviour of *A. fabae* (Jones 1944) as well as other insect species (Pospisil 1972; Green & Pitman 2003; Martínez 2008).

Air entrainment is a useful method for collecting volatile compounds but it is important to be aware that the air entrainment sample obtained at the end may possess qualitative differences to the headspace of the plant entrained. During air entrainment, volatile compounds are collected by adsorption onto a polymer. The quantity of each volatile compound isolated is a product of the quantity emitted by the plant and the effectiveness of the air entrainment method and polymer at collecting each volatile compound. Some compounds may be susceptible to adhering to the sides of the entrainment vessel resulting in less of these compounds being collected by the adsorbent filter, reducing their relative quantity in the air entrainment sample at the end of the entrainment period (Stewart-Jones & Poppy 2006). Some volatiles may also have a greater tendency than others to ‘break through’ the polymer without being adsorbed (Raguso & Pellmyr 1998; Stewart-Jones & Poppy 2006). This means that the ratios of volatile compounds in the air entrainment sample may not accurately reflect those in the headspace of the plant. Furthermore, the quantities and ratios of volatile compounds released from filter paper impregnated with the air entrainment sample may differ substantially from those present in the air entrainment sample itself. Differences in volatilities of different compounds mean they will be released from the filter paper at different rates. This will result in ratios of volatiles altering over time with compounds of high-volatility more likely to dominate immediately after adding the sample to the filter paper compared to volatiles of lower volatility. As the aphids responded behaviourally to the air entrainment sample, any differences between volatiles released from filter paper impregnated with the air entrainment sample and volatiles emitted from the headspace of a *V. faba* plant were insufficient for the behavioural response to host odour to be lost. Despite the problems associated with dynamic headspace sampling, it is still preferable to other methods of collecting volatile compounds from plants as it does not necessitate causing damage to the plant which would result in large changes in quantity and quality of volatile compounds collected (Saijo & Takeo 1975; Agelopoulos et al. 1999b).

### 2.4.5. Conclusions

The work carried out here showed that winged virginoparous *A. fabae* responded behaviourally to the odour of *V. faba* plants and that the four-arm olfactometer is an effective means of recording such behavioural responses. Incorporating the central region of the olfactometer into the four outer regions will allow more accurate recordings to be made of arrestment behaviour and may improve the effectiveness of this olfactometer at recording response to host volatiles. Air entrainment was used to isolate volatile compounds from the headspace of *V. faba* plants and the behavioural response of *A. fabae* to the air entrainment sample demonstrated that volatile compounds essential for host location were successfully collected. The next chapter reports the use of coupled gas chromatography-electroantennography to identify the presence of any electrophysiologically active volatile compounds in the *V. faba* air entrainment sample as the first step in identifying volatile compounds used in host location.

# **CHAPTER 3. USE OF COUPLED GAS CHROMATOGRAPHY- ELECTROANTENNOGRAPHY TO IDENTIFY *VICIA FABA* VOLATILE COMPOUNDS ELECTROPHYSIOLOGICALLY ACTIVE TO *APHIS FABAE***

## **3.1. Introduction**

In the previous chapter it was established that winged virginoparous *A. fabae* respond behaviourally to the odour of their host plant, *V. faba*, and also to an air entrainment sample of the plant by spending more time in the treated region of a four-arm olfactometer than in controls. The behavioural response of the aphids towards host odour may be the result of a response to a single volatile compound or to a blend. Plants can emit hundreds of different volatile compounds and the task of identifying each compound and testing for behavioural activity would be impractical. Using coupled GC-EAG, it is possible to reduce the number of compounds which need to be identified by pinpointing those which are electrophysiologically active. The work carried out in this chapter represents the first steps in identifying behaviourally relevant volatile compounds within the *V. faba* air entrainment sample by identifying those which are electrophysiologically active to winged virginoparous *A. fabae*.

### **3.1.1. Aims and objectives**

The aim of this chapter was to determine the presence of electrophysiologically active volatile compounds in the headspace of *V. faba* plants. To achieve this aim, coupled GC-EAG was used to separate volatile compounds isolated from the headspace of *V. faba* plants and identify electrophysiologically active peaks. Retention indices for these peaks were

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Note: a refereed paper relating to the work in this Chapter has been published (Webster et al., 2008a).

calculated and used to aid identification of electrophysiologically active FID peaks in the subsequent chapter.

## **3.2. Materials and Methods**

### **3.2.1. Antennal preparation**

Winged virginoparous *A. fabae* were collected from the walls of the Perspex rearing cabinet and stored in Petri dishes prior to use. Antennal preparations were set up by separating the head from the body using a scalpel and cutting the tips of the antennae to ensure a good contact with the electrodes. Two Ag-AgCl borosilicate glass micro electrodes (2 mm o.d. X 1.16 mm i.d. with an inner filament) filled with Ringer's solution (7.55 g l<sup>-1</sup> sodium chloride, 0.64 g l<sup>-1</sup> potassium chloride, 0.22 g l<sup>-1</sup> calcium chloride, 1.73 g l<sup>-1</sup> magnesium chloride, 0.86 g l<sup>-1</sup> sodium bicarbonate, 0.61 g l<sup>-1</sup> sodium orthophosphate) as in Maddrell (1969) but without glucose were used for electroantennogram recordings. The head was placed into the tip of the indifferent electrode and the tips of the antennae were positioned into the end of the recording electrode.

### **3.2.2. Electroantennography**

A continuous humidified, charcoal filtered air stream was blown over the antennal preparation at a rate of 1 l min<sup>-1</sup> through a glass tube (8 mm i.d.). The outlet of the glass tube was positioned 5 mm from the antennal preparation. Test compounds were introduced via a hole in the side of the glass tube using an 'odour cartridge' which consisted of 10 µl of the test compound applied to a strip of filter paper placed in a Pasteur pipette. The stimulus delivery device (Syntech Stimulus Air Controller CS-05) generated a 2-s 'puff' of air through the odour cartridge which joined the main air stream being blown continuously over the antennal preparation. The Syntech Stimulus Air Controller ensured a continuous flow rate of air over the insect, compensating for the increased air flow through the odour cartridge during stimulus delivery. Electrophysiological responses were recorded using specialised software (EAD version 2.3; Syntech, the Netherlands).

### 3.2.3. Antennal preparation longevity test

To determine if the antennal preparations lasted sufficiently long to conduct coupled GC-EAG recordings that lasted 30 min, the response to a standard stimulus was tested on preparations at 5 min intervals to determine consistency of electrophysiological responses over time. The standard test stimulus used was (1*R*,5*S*)-myrtenal, a compound previously demonstrated to be electrophysiologically active to *A. fabae* (Hardie et al. 1994a), and was tested at a dose of  $10^{-5}$  g. Clean air (empty odour cartridge) was used as a control. The test stimulus and control were tested one after the other 10 min after excision of the aphid head and then tested every subsequent 5 min until 60 min had passed. The order of standard stimulus and control was randomised for each 5 min test slot and 1 min was allowed between stimulations to allow for antennal recovery. Odour cartridges were prepared 1 min prior to stimulation, with a fresh odour cartridge used each time to ensure test stimulus was at the same concentration for each stimulation. The experiment was replicated 6 times using a fresh aphid each time. Responses were calibrated by dividing by the amplitude of a 100  $\mu$ v calibration pulse. Responses to the test compound at each time period were compared to the responses to controls at corresponding time periods using a paired t-test (Genstat).

### 3.2.4. Coupled gas chromatography-electroantennography

Figure 3.1 shows a coupled GC-EAG system. The capillary column effluent from the gas chromatograph is split between the flame ionization detector (FID) and a transfer line which leads to a purified air stream which passes over the insect antenna. A humidified, charcoal filtered air stream ( $1 \text{ l min}^{-1}$ ) flowed continuously over the insect preparation through a glass tube positioned 5 mm from the antenna. It had a hole in the side through which the column effluent was introduced. The splitter was constructed from glass-lined stainless steel tubing and deactivated fused silica tubing.

Volatile compounds were separated by gas chromatography. To provide sufficient quantities of compounds for successful GC resolution, the bulk air entrainment sample was concentrated down by placing it in a glass vial and directing a constant stream of charcoal-filtered nitrogen over the surface. Using this technique, the air entrainment sample was concentrated down approximately 5-fold. After GC analysis, the sample was reconstituted with redistilled diethyl ether to its original concentration. One  $\mu$ l of the concentrated air

entrainment sample was injected onto a nonpolar column (HP-1, 50 m× 0.32 mm i.d.× 0.52 µm film thickness) in an HP5890 GC equipped with a cool on-column injector and a flame ionization detector (FID). The oven temperature was maintained at 30 °C for 2 min and then programmed at 5 °C min<sup>-1</sup> to 100 °C and then at 10 °C min<sup>-1</sup> to 250 °C. Hydrogen was used as the carrier gas.

Simultaneous recordings of the EAG and FID responses were obtained with specialised software (Electro Antenna Detection version 2.3, Syntech, Netherlands). A total of six coupled runs were completed. Traces were then printed onto white paper and compared by placing them over one another on a light box. Only FID peaks which corresponded to an EAG peak in 3 or more replicates were considered electrophysiologically active.

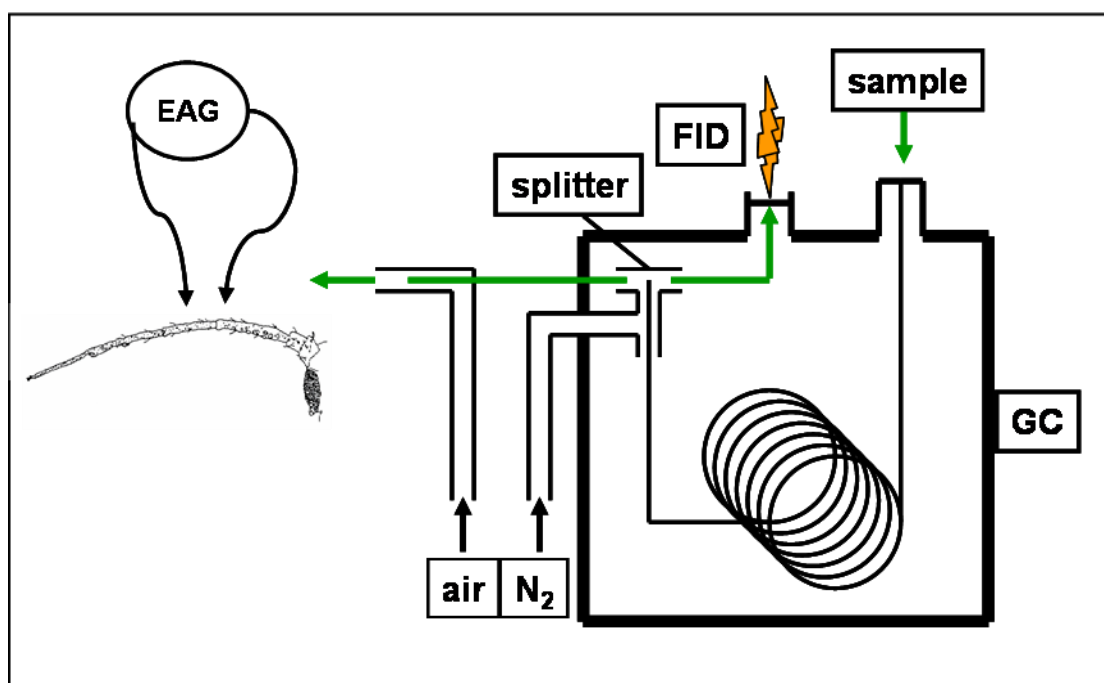


Figure 3.1. Coupled gas chromatography-electroantennography showing GC (gas chromatograph) fitted with splitter directing sample between FID (flame ionization detector) and antennal preparation connected to EAG (electroantennogram) apparatus. Based on drawing by C. Woodcock.

### 3.2.5. Retention indices of electrophysiologically active FID peaks

To calculate retention indices of electrophysiologically active FID peaks, an alkane series consisting of C8 to C25 alkanes each at 100 ng in redistilled hexane was injected onto the

same GC column used in coupled GC-EAG with the same temperature gradient. Retention times of each of the alkanes was recorded and used to calculate retention indices of electrophysiologically active FID peaks using the following equation (Bartle 1993):

$$RI = 100n + 100 \frac{\log RT_{(\text{unknown})} - \log RT_{(n)}}{\log RT_{(N)} - \log RT_{(n)}}$$

where:

RI = retention index

$RT_{(\text{unknown})}$  = retention time of peak to be identified

$RT_{(N)}$  = retention time of alkane to elute immediately *after* peak to be identified

$RT_{(n)}$  = retention time of alkane to elute immediately *before* peak to be identified

n = number of carbon atoms in alkane eluting immediately *before* peak to be identified

### 3.3. Results

#### 3.3.1. Antennal preparation longevity test

Electrophysiological responses to the standard test stimulus were significantly greater than controls at each time period tested up to 60 min. This showed that antennal preparations were receptive and had a longevity of at least 60 min. Mean EAG responses to  $10^{-5}$  g (1R,5S)-myrtenal compared to controls are shown Figure 3.2. The magnitude of the response declined but the key feature was that there was a significantly higher response to the test stimulus than to the control stimulus at all time intervals tested.

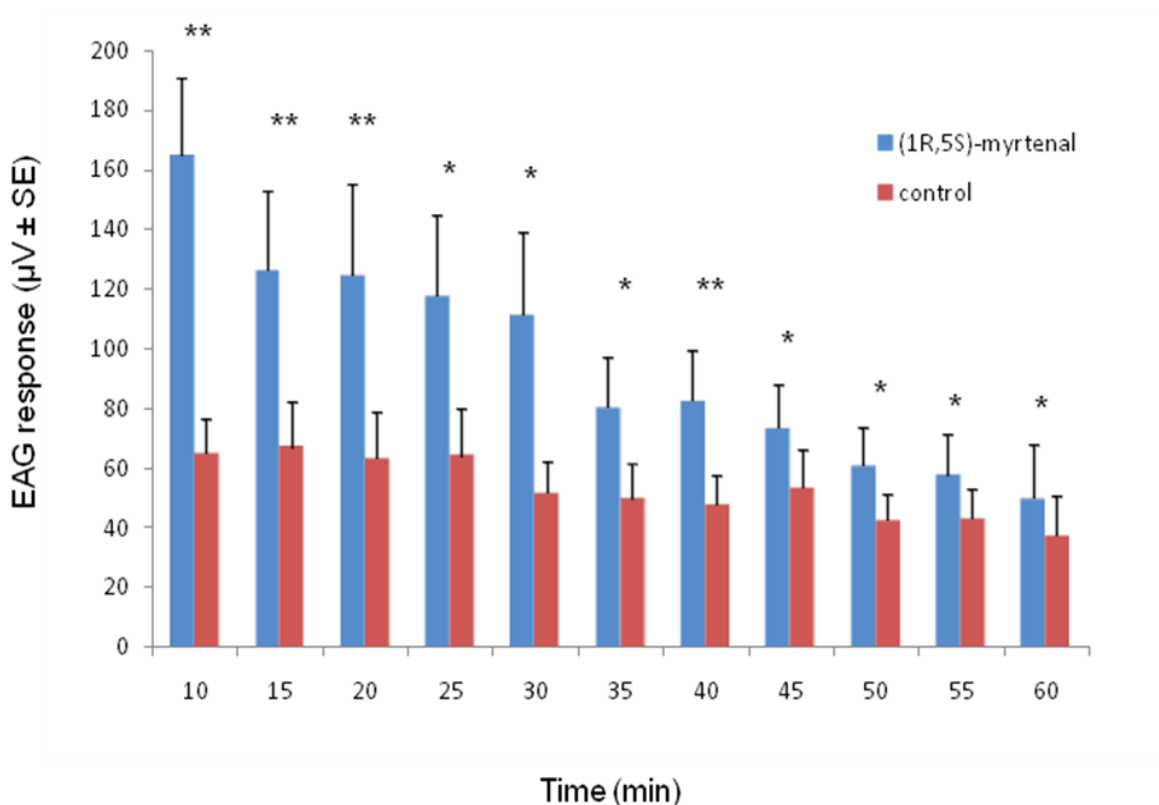


Figure 3.2. Mean EAG responses of winged virginoparae *Aphis fabae* to  $10^{-5}$  g (1R,5S)-myrtenal compared to clean air controls. \* Treatment significantly different from control ( $P < 0.05$ ). \*\* Treatment significantly different from control ( $P < 0.01$ ).

### 3.3.2. Coupled GC-EAG

Coupled GC-EAG revealed 16 electrophysiologically active FID peaks in the air entrainment sample. Each trace is shown in Figure 3.3 with corresponding electrophysiological and FID peaks labelled for each trace. The retention times of C8-C25 alkanes are listed in Table 3.1. Retention times and indices of electrophysiologically active compounds are listed in Table 3.2.

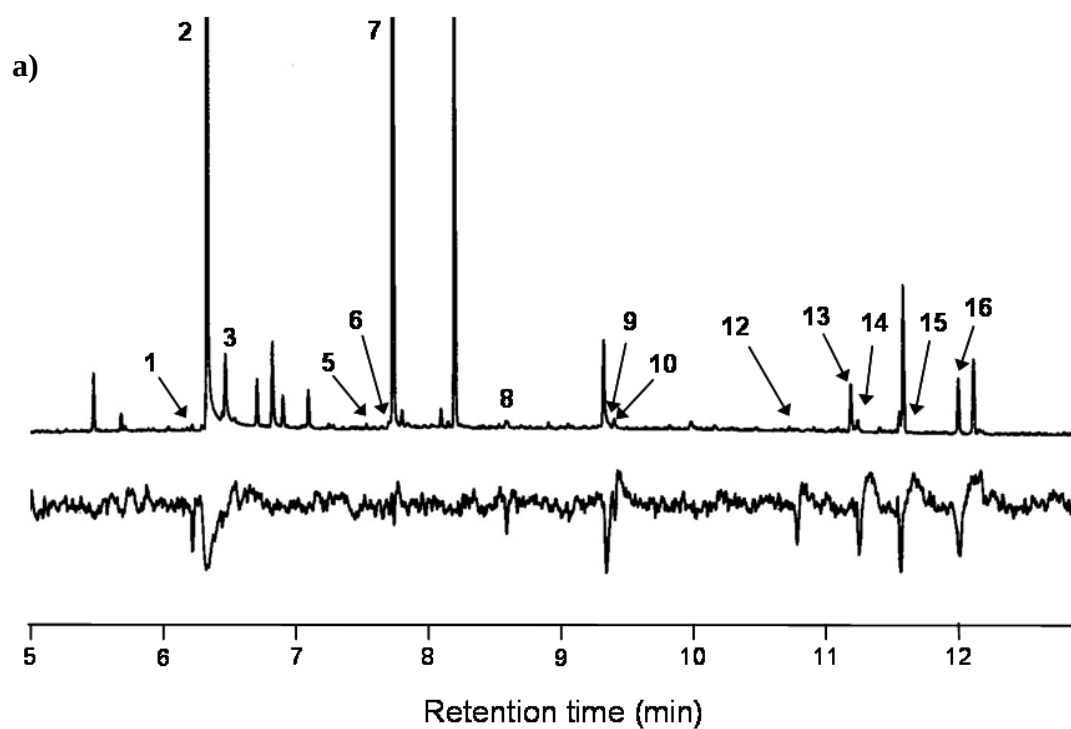
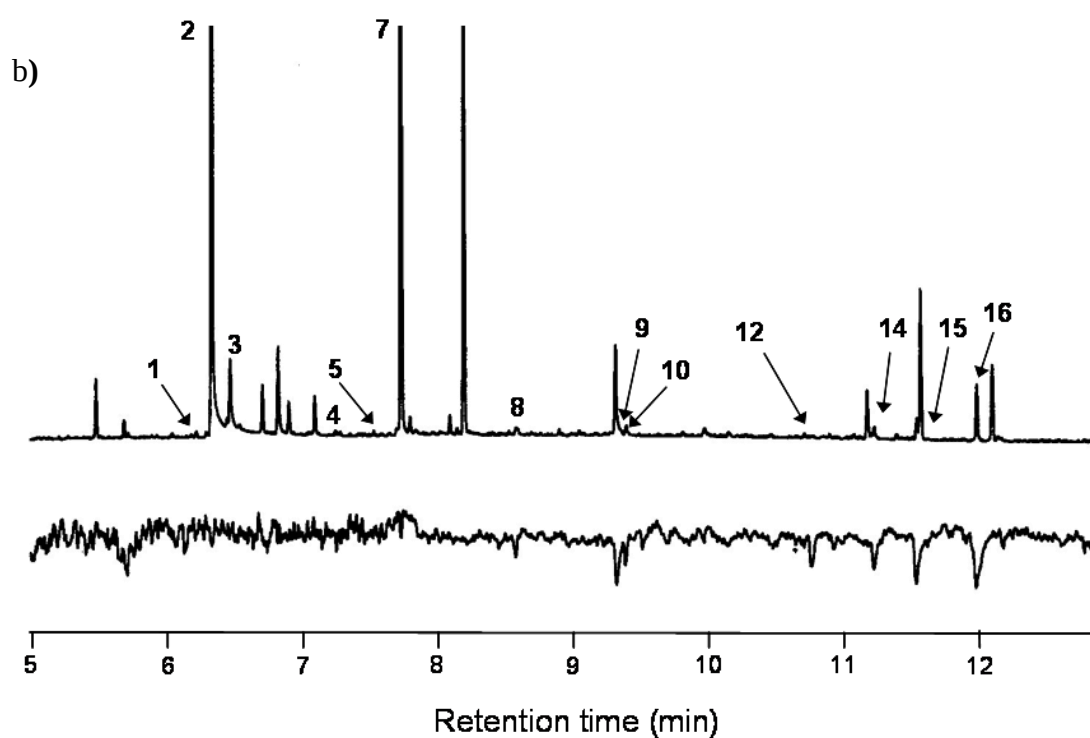


Figure 3.3. Coupled GC-EAG traces showing FID peaks corresponding to peaks of electrophysiological activity. Six replicates were performed (a-f).

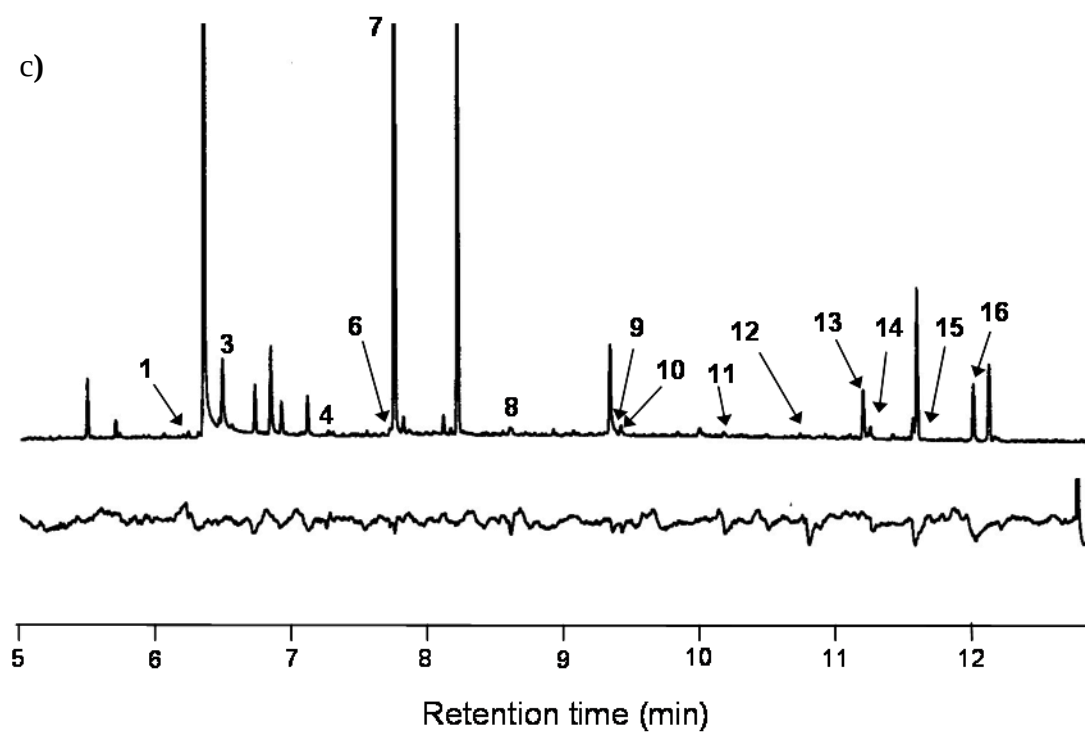
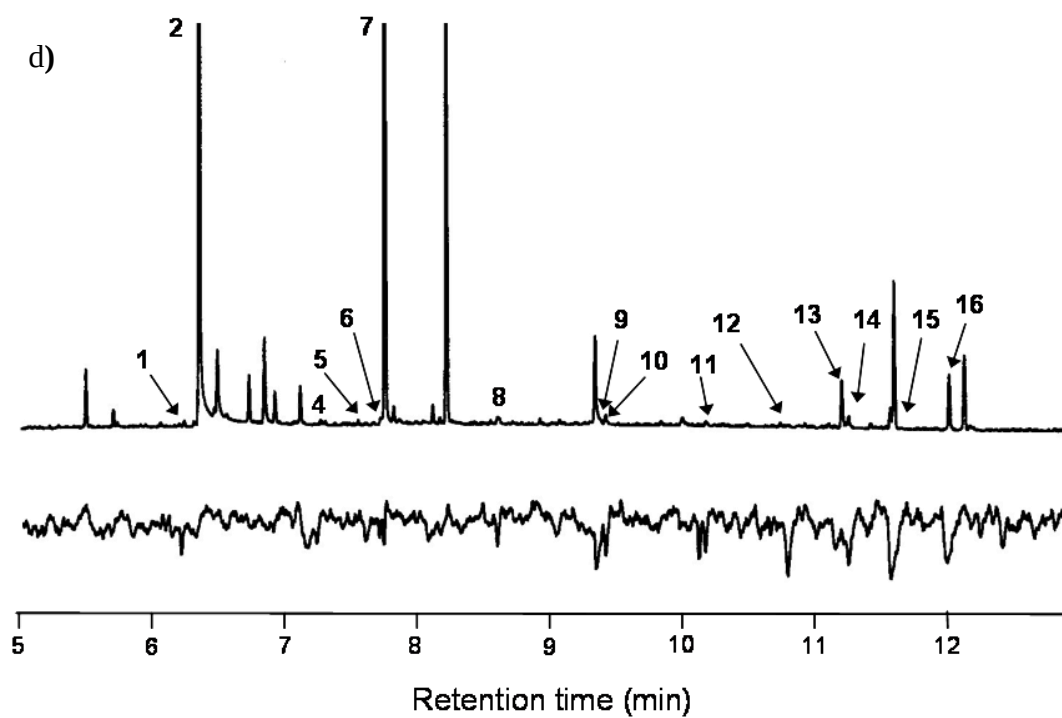


Figure 3.3 continued. Coupled GC-EAG traces showing FID peaks corresponding to peaks of electrophysiological activity. Six replicates were performed (a-f).

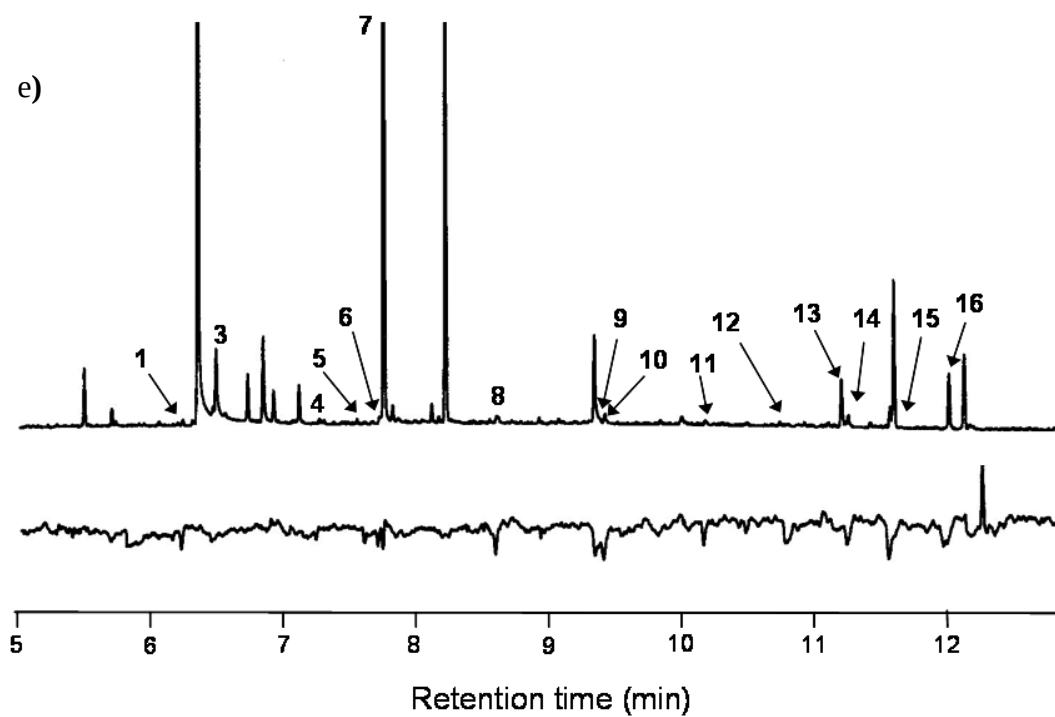
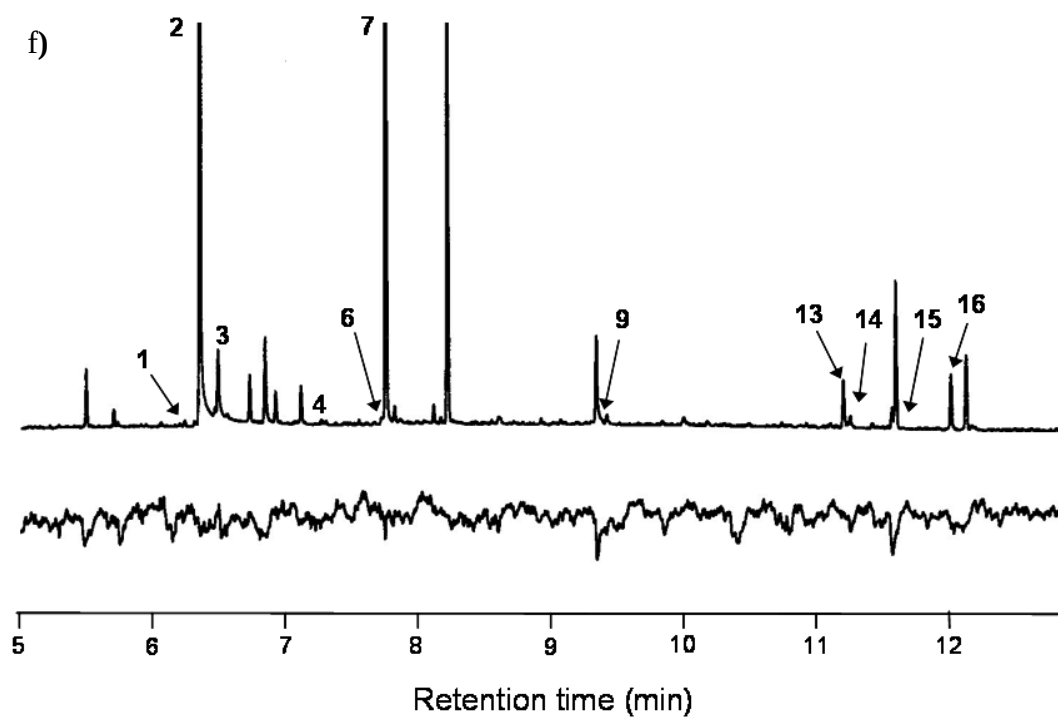


Figure 3.3 continued. Coupled GC-EAG traces showing FID peaks corresponding to peaks of electrophysiological activity. Six replicates were performed (a-f).

**Table 3.1. Retention times of alkanes C8-C25.**

| Alkane no.           | C8   | C9   | C10  | C11   | C12   | C13   | C14   | C15   | C16   | C17   |
|----------------------|------|------|------|-------|-------|-------|-------|-------|-------|-------|
| Retention time (min) | 6.49 | 7.94 | 9.30 | 10.55 | 11.72 | 12.81 | 13.84 | 14.81 | 15.72 | 16.59 |

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| Alkane no.           | C18   | C19   | C20   | C21   | C22   | C23   | C24   | C25   |
|----------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Retention time (min) | 17.47 | 18.42 | 19.49 | 20.75 | 22.29 | 24.20 | 16.61 | 29.71 |

**Table 3.2. Retention times and indices of electrophysiologically active FID peaks eluting on a non-polar column (HP1, 50 m× 0.32 mm i.d.× 0.52 µm film thickness).**

| Peak no. | Retention time (min) | Retention index |
|----------|----------------------|-----------------|
| 1        | 6.84                 | 826             |
| 2        | 7.09                 | 844             |
| 3        | 7.27                 | 856             |
| 4        | 8.40                 | 936             |
| 5        | 8.82                 | 966             |
| 6        | 9.10                 | 984             |
| 7        | 9.15                 | 990             |
| 8        | 10.41                | 1089            |
| 9        | 11.56                | 1182            |
| 10       | 11.63                | 1193            |
| 11       | 12.50                | 1272            |
| 12       | 13.71                | 1388            |
| 13       | 14.28                | 1446            |
| 14       | 14.36                | 1454            |
| 15       | 14.89                | 1509            |
| 16       | 15.55                | 1582            |

## 3.4. Discussion

### 3.4.1. Antennal preparation longevity test

Previous work has suggested that aphid antennal preparations which involve the excision of the head may be short-lived (Hardie et al. 1994b; Park & Hardie 1998), making them unsuitable for electrophysiological techniques conducted over an extended period of time such as coupled GC-EAG. Successful use of coupled GC-EAG requires an antennal preparation that will remain responsive for long enough to cover the period of time during which all plant volatiles within a sample pass through the GC column. The antennal preparations in this study continued to give statistically significant electrophysiological responses to the test stimulus of  $10^{-5}$  g (1*R*,5*S*)-myrtenal up to 60 min after excision of the aphid head. No more recordings were made after 60 min had passed so it is possible that antennal preparations may have continued to give significant electrophysiological responses even beyond this time. Although significant responses were observed up to 60 min the magnitude of the response had notably diminished. EAG responses after 60 min were on average 30% of the magnitude of EAG responses after 10 min, indicating that some gradual degradation of the antennal preparation had occurred.

Time taken from excision of the aphid head to the start of the coupled GC-EAG run was typically 5-10 min. When the alkane series was run on the GC, the alkane *n*-pentacosane (C<sub>25</sub>), with a boiling point of 254 °C, passed through the column in just under 30 min (Table 3.1Table ). As antennal preparations remained responsive for up to 60 min this indicates that they could be successfully used with coupled GC-EAG to identify electrophysiologically active volatile compounds with retention indices of up to at least 2500 (the retention index of *n*-pentacosane), even taking into account the 5-10 min required to set up the preparation before the coupled run begins. The majority of identified plant volatiles possess retention indices lower than this value (Knudsen et al. 1993), showing that the method of antennal preparation used here allows sufficient time for most plant volatile compounds to pass through the GC column.

### 3.4.2. Coupled GC-EAG

Coupled GC-EAG revealed 16 volatile compounds in the air entrainment sample which were electrophysiologically active to *A. fabae*. Only compounds which corresponded to peaks of electrophysiological activity in at least 3 out of 6 traces were considered to be electrophysiologically active rather than the product of background noise. Only 1 compound, peak 11, corresponded to a peak of electrophysiological activity in the minimum number of 3 traces. Two more compounds, peaks 2 and 5, corresponded to peaks of electrophysiological activity on 4 out of 6 traces and 7 more compounds - peaks 3, 4, 6, 8, 10, 12, and 13 – were present on 5 traces. Only six of the compounds showed electrophysiological activity in all 6 traces; peaks 1, 7, 9, 14, 15, and 16 and these compounds generally corresponded to electrophysiological peaks with the largest amplitudes.

The fact that electrophysiological activity of some compounds was not detected on all traces raises the possibility that some electrophysiologically active compounds may have been missed by not showing up on at least 3 traces. An electrophysiological response is thought to be the sum of changes in potential of many receptors and the amplitude of the response decreases as the concentration of the electrophysiologically active volatile compound decreases (Schneider 1957; Wadhams 1990). For a response to be successfully identified the amplitude of the response must be greater than the random fluctuations in potential across the antennae ('background noise'); that is, it must possess a sufficiently large 'signal to noise ratio'. At lower concentrations, the amplitude of the response may be too small to be distinguished from the background noise and may be missed. However, volatile compounds are known to elicit physiological and behavioural responses in insects at extremely low concentrations. For example, the female-emitted sex pheromone of the silk moth, *Bombyx mori*, elicits a behavioural response from male moths when just 85 ORNs per antenna intercept one molecule each per second (Kaissling & Preisner 1970) and 5 molecules reaching the antenna per second is enough to trigger a change in heartbeat frequency (Angioy et al. 2003). It is therefore possible that a volatile compound within the air entrainment sample could be behaviourally or physiologically active at concentrations too low to induce a detectable electrophysiological response. On the other hand, it is also possible that some of the highlighted compounds may be false positives; that is, compounds which are not electrophysiologically active to the aphids but appear to correspond to peaks of electrophysiological activity which are in fact merely the result of 'background noise'. This

possibility was minimised by only accepting electrophysiologically active peaks which occurred in at least 3 out of 6 traces so the occurrence of a false positive in these results is unlikely. Furthermore, electrophysiological activity was confirmed by tests with synthetic standards once the compounds had been identified (see Chapter 4).

Some of the electrophysiologically active compounds corresponded to very small FID peaks (see Figure 3.3) indicating very high sensitivity of the insect antenna to these compounds. In particular, peak 12 corresponded to a virtually flat region of the FID trace despite the fact that this showed detectable electrophysiological activity in 5 out of 6 traces. The magnitude of an electrophysiological response is thought to correlate with the number of olfactory cells responding to the compound (Schneider 1957) but it does not necessarily reflect the magnitude of the behavioural response that the compound may induce. This fact is demonstrated by the physiological and behavioural responses of the silk moth to the female sex pheromone after stimulation of only a few olfactory receptor neurones (Kaissling & Preisner 1970; Kaissling 1971; Angioy et al. 2003). It is therefore difficult to speculate on the behavioural relevance of the differences in magnitudes of different peaks.

### **3.4.3. Conclusions**

Coupled GC-EAG was successfully used to pinpoint 16 volatile compounds within the *V. faba* air entrainment sample which are electrophysiologically active to winged virginoparous *A. fabae*. The retention indices calculated for each compound provided the first step in identification of these volatile compounds. The next step is to identify the electrophysiologically active volatile compounds using GC analysis and mass spectrometry, and to confirm their electrophysiological activity using electroantennography, which is discussed in Chapter 4.

# CHAPTER 4. IDENTIFICATION OF *Vicia faba* VOLATILE COMPOUNDS ELECTROPHYSIOLOGICALLY ACTIVE TO *Aphis fabae*

## 4.1. Introduction

Previous studies have demonstrated that *A. fabae* moves towards the odour of its host plant, *V. faba* (Alikhan 1960; Nottingham et al. 1991) but the semiochemicals responsible for eliciting the behavioural response were not identified. In Chapter 3, coupled GC-EAG showed 16 volatile compounds in a *V. faba* air entrainment sample that were electrophysiologically active to *A. fabae*. The aim of this chapter was to identify those compounds using GC-analysis and coupled gas chromatography-mass spectrometry (GC-MS), to quantify the identified compounds and to confirm their electrophysiological activity to *A. fabae*.

### 4.1.1. Volatile compounds emitted by *Vicia faba*

Some information on the volatile compounds emitted by *V. faba* is already available due to the work of Sutton *et al.* (1992) and Griffiths *et al.*, (1999). Their studies characterised some of the more abundant volatile compounds present in the headspace of *V. faba* flowers. Other research investigating volatile compounds used as semiochemicals by herbivorous insects other than *A. fabae* and their parasitoids has yielded further information on the volatile compounds found in the headspace of *V. faba* plants (Blight et al. 1984; Du et al. 1998; Colazza et al. 2004; Mendgen et al. 2006; Pareja et al. 2009). The volatile compounds identified in these studies are listed in Table 4.1. The majority of these compounds are terpenoids, phenylpropanoids and GLVs, classes of compounds which are found universally throughout the plant kingdom.

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Note: a refereed paper relating to the work in this Chapter has been published (Webster et al., 2008a).

**Table 4.1. List of volatile compounds known to be emitted by *Vicia faba* (Blight et al. 1984; Sutton et al. 1992; Du et al. 1998; Agelopoulos et al. 1999b; Griffiths et al. 1999; Colazza et al. 2004; Mendgen et al. 2006; Pareja et al. 2009).**

| Class                | Compound                                                                                                                                                                                                                                                                        |
|----------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Monoterpenoids       | Geraniol, geranyl acetate, limonene, linalool, <i>p</i> -menthatrienenerol, 6-methyl-5-hepten-2-one, $\beta$ -myrcene, neryl acetate, ( <i>Z</i> )- $\beta$ -ocimene, ( <i>E</i> )- $\beta$ -ocimene, $\alpha$ -pinene, <i>p</i> -propenyl anisole, sabinene, $\alpha$ -thujene |
| Sesquiterpenoids     | ( <i>E</i> )-Caryophyllene, ( <i>E,E</i> )- $\alpha$ -farnesene, farnesyl acetate, germacrene D, $\alpha$ -humulene,                                                                                                                                                            |
| homoterpenoids       | (3 <i>E</i> )-4,8-Dimethyl-1,3,7-nonatriene, ( <i>E,E</i> )-4,8,12-trimethyl-1,3,7,11-tridecatetraene                                                                                                                                                                           |
| Phenylpropanoids     | <i>p</i> -Allyl anisole, benzaldehyde, benzyl alcohol, cinnamyl acetate, cinnamyl alcohol, eugenol, <i>O</i> -methyl eugenol, methyl isoeugenol, methyl salicylate,                                                                                                             |
| Green leaf volatiles | Hexanal, 1-hexanol, ( <i>E</i> )-2-hexenal, ( <i>Z</i> )-3-hexenal, ( <i>Z</i> )-3-hexenol, ( <i>Z</i> )-3-hexen-yl acetate, 4-oxo-( <i>E</i> )-2-hexenal                                                                                                                       |
| Other                | Benzothiazole, decanal, decane, indole, nonanal, nonane, undecane, methyl jasmonate, 2-phenoxyethanol                                                                                                                                                                           |

#### **4.1.2. Electrophysiological responses of *Aphis fabae* to plant volatile compounds**

The identification of so many ubiquitous plant volatiles from *V. faba* plants lends weight to the hypothesis that pests of *V. faba* must rely on blends of non-specific plant volatiles rather than species-specific volatiles to locate their host. However, most of the previous studies focussed on compounds which were present in the greatest quantities of the headspace of *V. faba* plants, rather than focusing on volatile compounds electrophysiologically active to

individual insect pests. Compounds relevant to insect behaviour are not necessarily the most abundant and electrophysiological activity is a more important criterion.

Considerable work has already been done on the electrophysiological responses of *A. fabae* to plant volatile compounds (Nottingham et al. 1991; Hardie et al. 1994b; Visser et al. 1996; Park & Hardie 2004). Table 4.2 shows a list of volatile compounds known to be electrophysiologicaly active to *A. fabae*, many of which have been previously identified in the headspace of *V. faba* plants (Table 4.1). Most are ubiquitous plant volatiles, found in a diverse range of plant species and not restricted to the hosts of *A. fabae* (Knudsen et al. 1993). This lends weight to the hypothesis that *A. fabae* uses blends of common plant volatiles rather than a single species-specific volatile compound to recognise its host. It is important to note that the list of electrophysiologicaly active volatiles in Table 4.2 is not comprehensive as only a limited number of volatiles have been tested in each of the studies referenced. Electrophysiological responses of *A. fabae* to volatiles collected from *V. faba* plants have not been previously recorded. The possibility therefore remains that *V. faba* emits a unique volatile compound used by host-seeking *A. fabae* that has not yet been identified.

**Table 4.2. Plant volatile compounds electrophysiologically active to *Aphis fabae* (Hardie et al. 1994a; Visser et al. 1996; Park & Hardie 2004).**

| <b>Class</b>         | <b>Compounds</b>                                                                                                                                                                                                       |
|----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Monoterpenoids       | (1 <i>R</i> ,5 <i>S</i> )-Myrtenal, (1 <i>S</i> )- $\alpha$ -pinene, (1 <i>S</i> )- $\beta$ -pinene, (S)-carvone, (R)-carvone, $\alpha$ -terpineol, linalool, geraniol, nerol, citronellal, (+)-citronellol, sabinene. |
| Sesquiterpenoids     | ( <i>E</i> )-Caryophyllene, ( <i>E,E</i> )- $\alpha$ -farnesene, ( <i>E</i> )- $\beta$ -farnesene.                                                                                                                     |
| Phenylpropanoids     | Methyl salicylate, benzaldehyde, 2-methoxybenzaldehyde, 3-methoxybenzaldehyde, 4-methoxybenzaldehyde, 2-hydroxybenzaldehyde.                                                                                           |
| Green leaf volatiles | ( <i>E</i> )-2-Hexenal, ( <i>E</i> )-2-hexenol, ( <i>Z</i> )-3-hexenol, ( <i>Z</i> )-3-hexenyl acetate, 1-hexanol, hexanal, ( <i>E</i> )-2-heptenal, 2-hexanone, 2-hepanone.                                           |
| Other                | Butyl isothiocyanate, allyl isothiocyanate, 3-butenyl isothiocyanate, 4-pentenyl isothiocyanate, hexanonitrile, heptanonitrile.                                                                                        |

#### **4.1.3. Aims and objectives**

The aim of this chapter was to identify the chemical structure of the electrophysiologically active compounds occurring in the *V. faba* air entrainment sample, to quantify the compounds and confirm their electrophysiological activity to *A. fabae*. To achieve these aims, the following objectives were envisaged:

#### **Coupled gas chromatography-mass spectrometry (GC-MS) to provide tentative identifications of electrophysiologically active volatile compounds**

Coupled GC-MS was used to obtain mass spectra for FID peaks that were associated with electrophysiological activity (Chapter 3). These spectra were then compared to a database of known spectra to make tentative identifications.

### **Gas chromatography analysis to confirm tentative identifications**

Gas chromatography analysis was used to compare retention times of compounds tentatively identified by GC-MS with those of authentic standards of each compound. Chiral GC analysis using a  $\beta$ -cyclodextrin chiral capillary column was used to determine the stereochemistry of enantiomeric compounds by comparing retention times with authentic standards of each enantiomer.

### **Electroantennograms to confirm electrophysiological activity of identified compounds**

Electrophysiological responses of *A. fabae* to identified compounds using electroantennography was used to confirm the identities of compounds.

### **Quantification of identified compounds**

To determine the quantities of each compound, calibration curves were constructed of FID peak area against quantity of authentic standards of known concentrations for each identified compound.

## **4.2. Materials and methods**

### **4.2.1. Insects**

The Kennedy and Booth clone of *A. fabae* (Kennedy & Booth 1950) was reared on tick beans (*Vicia faba* var. *minor*) in a Perspex rearing cabinet under controlled conditions ( $22 \pm 1$  °C, LD 16:8 hr). Winged aphids were induced by allowing the colony to become crowded. Aphids were collected from the walls of the rearing cabinet on the morning of the experiments and stored in Petri dishes.

### **4.2.2. Chemicals**

Authentic chemical standards used were (*E*)-2-hexenal, benzaldehyde, 6-methyl-5-hepten-2-one, decanal, 1-hexanol (all 99% purity, Sigma Aldrich Inc., St. Louis, Missouri, USA), (1*R*,5*S*)-myrtenal (98% purity, Sigma Aldrich Inc.) undecanal (97% purity, Sigma Aldrich Inc.), (*R*)-linalool, (*S*)-linalool (both 98% purity, Sigma Aldrich Inc.), (*Z*)-3-hexen-1-yl acetate (99% purity, Avocado Research Chemicals Ltd.) octanal, methyl salicylate, (*Z*)-3-

hexen-1-ol, (all 98% purity, Avocado Research Chemicals Ltd., Lancs., UK), and (*E*)-caryophyllene (85% purity, Pfaltz & Bauer inc., Stamford, Connecticut, USA). (*E*)- $\beta$ -Farnesene (98% purity) was synthesized from (*E*)-farnesyl chloride by Dr. Mike Birkett at Rothamsted Research (Kang et al. 1987). (*S*)-Germacrene D and (*R*)-germacrene D (both 98% purity) were obtained by incubation of farnesyl pyrophosphate with purified, expressed (*R*) or (*S*)-germacrene-D synthase and subsequent hexane extraction and purification through a short column of silica gel (BDH, 40-63  $\mu\text{m}$ )/ $\text{MgSO}_4$  (10:1) by Dr. Ian Prosser at Rothamsted Research (Prosser et al. 2004). (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT) was synthesised from (*E,E*)-farnesol by oxidation to its aldehyde followed by Wittig methylenation performed by Dr. Samuel Dufour at Rothamsted Research (Leopold 1990).

#### 4.2.3. Coupled Gas Chromatography-Mass Spectrometry

Tentative identification of electrophysiologically active peaks was achieved by coupled gas chromatography-mass spectrometry (conducted with the assistance of Dr. Claudia Birkemeyer at Rothamsted Research). A 1  $\mu\text{l}$  aliquot of the air entrainment sample was injected onto an HP1 capillary GC column (50 m x 0.32 mm i.d. methylsilicone fitted with an on-column injector) directly coupled to a mass spectrometer (Thermo-Finnigan, MAT95). Ionization was achieved by electron impact at 70 eV at 250  $^{\circ}\text{C}$ . The GC oven temperature was maintained at 30  $^{\circ}\text{C}$  for 5 minutes and then programmed at 5  $^{\circ}\text{C min}^{-1}$  to 250  $^{\circ}\text{C}$ . Spectra were analysed using Qual Browser v. 1.3 (Thermo Finnigan, Herts, UK). Tentative identifications were made by comparison of spectra with those of authentic standards in a database (NIST 2005). Compounds with similar spectra which also possessed retention indices similar to those of electrophysiologically active compounds calculated in Table 3.2 were taken as tentative identifications.

#### 4.2.4. Gas chromatography analysis

##### Peak enhancement

The air entrainment sample was analysed on a Hewlett-Packard 6890 GC equipped with a cool on-column injector, a FID, a non-polar HP-1 bonded phase fused silica capillary column (50 m x 0.32 mm i.d., film thickness 0.52  $\mu\text{m}$ ) and a polar DB-WAX column (30 m x 0.32 mm i.d., film thickness 0.82  $\mu\text{m}$ ). The oven temperature was maintained at 30  $^{\circ}\text{C}$  for 1 min and then programmed at 5  $^{\circ}\text{C min}^{-1}$  to 150  $^{\circ}\text{C}$  and held for 0.1 min, then 10  $^{\circ}\text{C min}^{-1}$  to 230

°C. A 1 µl aliquot portion of the air entrainment sample was injected onto the HP1 column and the areas of the peaks to be identified were calculated using GC Chemstation software (Agilent Technologies, Santa Clara, CA). A 1 µl aliquot portion of alkane series consisting of C8 to C25 alkanes each at 100 ng µl<sup>-1</sup> in redistilled hexane was injected onto the HP-1 column and the mean areas of the peaks corresponding to the alkanes were calculated. Estimates of the quantities of the compounds to be identified in 1 µl of the air entrainment sample were then calculated from the areas of the alkane peaks using the following equation:

$$\text{Estimated quantity (ng)} = \frac{\text{Mean } n\text{-alkane peak area}}{100} \times \text{area of peak to be identified}$$

Authentic standards of each compound tentatively identified by GC-MS were then made up in redistilled hexane at concentrations similar to those estimated for each compound. To determine if compounds in the air entrainment sample co-eluted with the authentic standards both were co-injected by taking up 1 µl of each in the same syringe and injecting them together onto both the HP1 and DB-WAX columns. Co-elution on both columns, indicated by an increase in peak height without an increase in width, was taken as confirmation of identity.

### **Chiral gas chromatography analysis**

To determine the stereochemistry of germacrene D and linalool, authentic standards of both *S* and *R* enantiomers for both compounds were made up in redistilled hexane at concentrations similar to those of each compound in the air entrainment sample as estimated using the peak areas of the *n*-alkane series. Both enantiomers were then co-injected with the air entrainment sample on an HP5890 GC (Agilent Technologies, UK) equipped with a cool on-column injector, a FID, and fitted with a β-cyclodextrin chiral capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). The GC oven was maintained at 40 °C for 1 min and then raised by 5 °C min<sup>-1</sup> to 150 °C where it was held for 30 min. The carrier gas was hydrogen. Co-elution with either enantiomer indicated by an increase in FID peak height without an increase in width was taken as confirmation of the presence of that enantiomer.

## Quantification of identified compounds

To determine the quantities of identified compounds, authentic standards were made up of each compound in redistilled hexane at 3 different concentrations around the quantities estimated using peak areas of *n*-alkanes. Each authentic standard was injected 3 times onto the HP1 column of a Hewlett-Packard 6890 GC equipped with a cool on-column injector and FID. The oven temperature was maintained at 30 °C for 1 min and then programmed at 5 °C min<sup>-1</sup> to 150 °C and held for 0.1 min, then 10 °C min<sup>-1</sup> to 230 °C. A mean peak area for each quantity of each compound was calculated using GC Chemstation software (Agilent Technologies, Santa Clara, CA). A linear line of best fit was plotted using Microsoft Excel (2002) and this was used to calculate a quantity for a given peak area for each compound. Due to limited quantities of available germacrene D, authentic standards for quantification were not made for this compound. Instead, the calibration curve for (*E*)- $\beta$ -farnesene was used as both compounds are sesquiterpenes and so FID would respond similarly to both.

### 4.2.5. Electroantennography

Antennal preparation and stimulus delivery system was as described in section 3.2.1 and 3.2.2. Test compounds were randomly assigned to five different groups and arranged within each group in a randomised block design consisting of 8 replicates. Each compound was tested at a dose of 10  $\mu$ g in 10  $\mu$ l redistilled hexane applied to a strip of filter paper at the start of the experiment and contained in a Pasteur pipette odour cartridge. One minute was left between stimulations to allow antennal recovery. At the start and end of each replicate a standard stimulus of (1*R*,5*S*)-myrtenal and a control consisting of 10  $\mu$ l hexane applied to a strip of filter paper were tested. Electrophysiological responses to each test compound, standard stimulus and hexane control were corrected by dividing by the amplitude of 0.1 mV. Responses to (1*R*,5*S*)-myrtenal were compared with responses to hexane at the start and end of the experiment to ensure that preparations were functional throughout the experiment. Responses to each test compound were compared to the average of the responses to the two hexane stimulations for each replicate. Compounds which failed to elicit a statistically significant electrophysiological response at a dose of 10  $\mu$ g were subsequently tested at a dose of 100  $\mu$ g. All comparisons were made using a paired *t*-test (Genstat v. 10).

## 4.3. Results

### 4.3.1. GC-MS and peak enhancement

Fifteen out of 16 electrophysiologically active compounds were tentatively identified using coupled GC-MS as (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 1-hexanol, benzaldehyde, 6-methyl-5-hepten-2-one, octanal, (*Z*)-3-hexen-1-yl acetate, (*R*)-linalool, methyl salicylate, decanal, undecanal, (*E*)-caryophyllene, (*E*)- $\beta$ -farnesene, (*S*)-germacrene D, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). Mass spectra for each compound are shown in figures 4.1 to 4.15. Compound 12 could not be identified due to the absence of any visible FID peak meaning a mass spectrum could not be obtained. The identities of all 15 remaining compounds were confirmed by peak enhancement on both HP1 and DB-WAX columns (Table 4.3). Figure 4.16 shows an example of peak enhancement used to confirm identity of a compound.

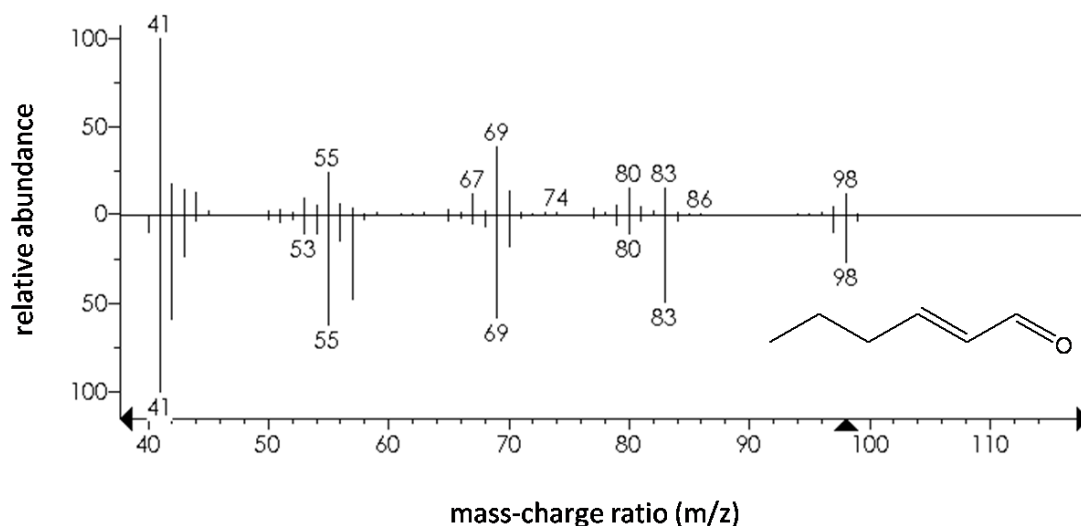


Figure 4.1. Mass spectra of peak 1 (top) and best library match, (*E*)-2-hexenal (bottom).

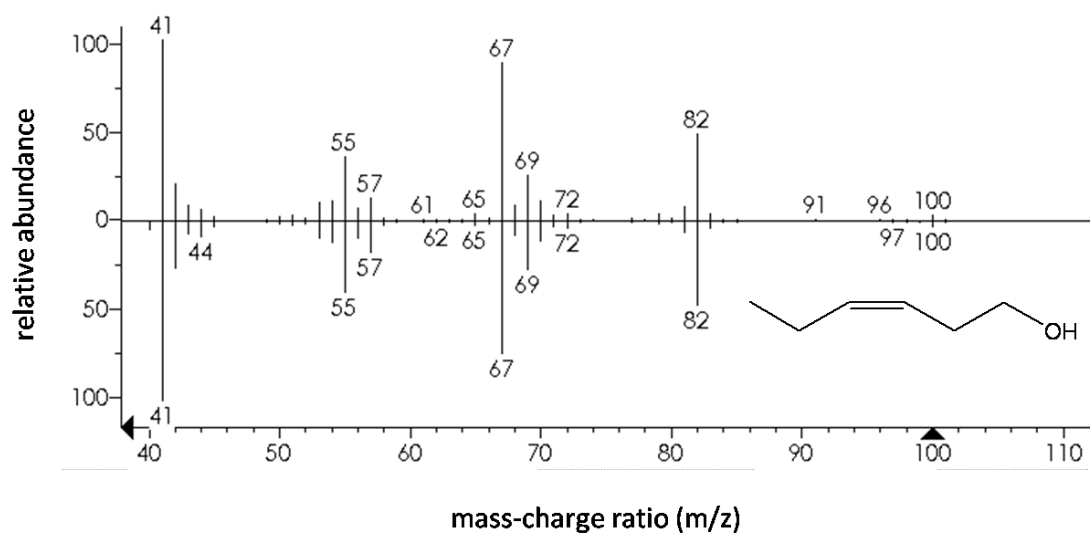


Figure 4.2. Mass spectra of peak 2 (top) and best library match, (Z)-3-hexen-1-ol (bottom).

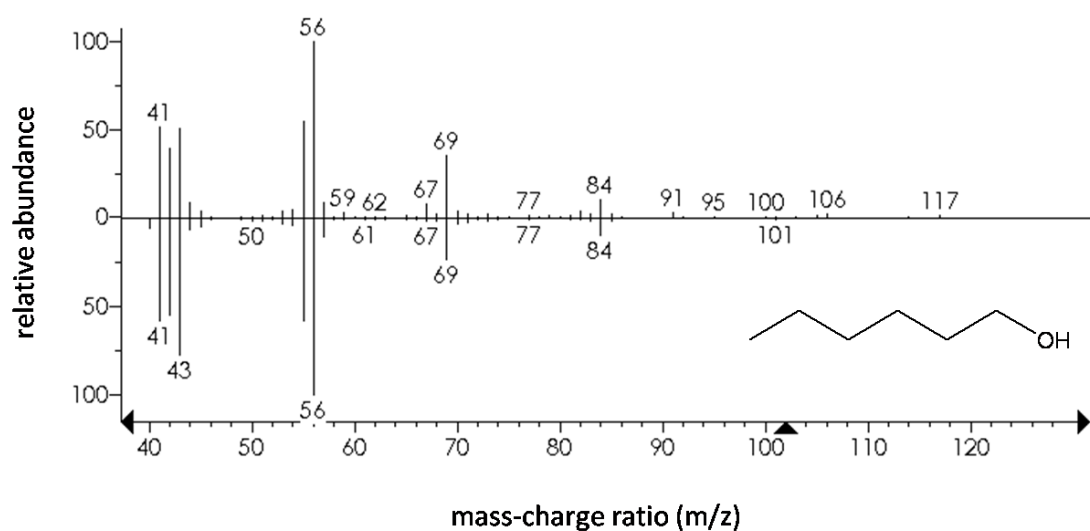


Figure 4.3. Mass spectra of peak 3 (top) and best library match, 1-hexanol (bottom).

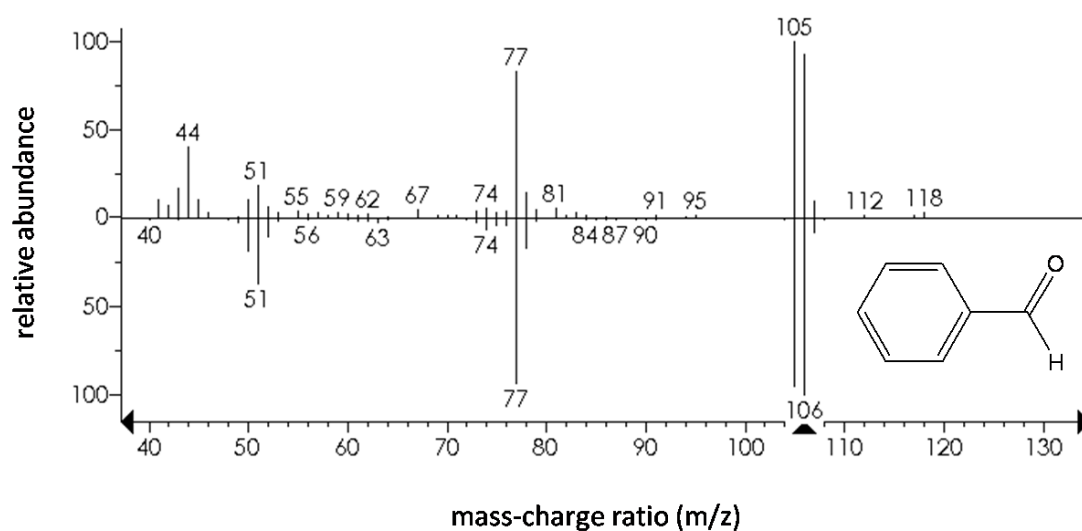


Figure 4.4. Mass spectra of peak 4 (top) and best library match, benzaldehyde (bottom).

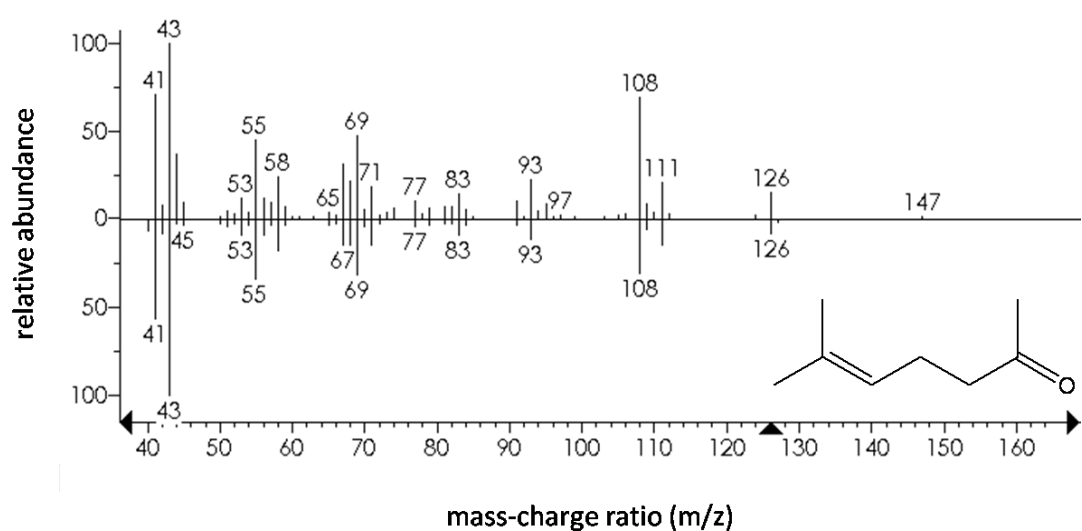


Figure 4.5. Mass spectra of peak 5 (top) and best library match, 6-methyl-5-hepten-2-one (bottom).

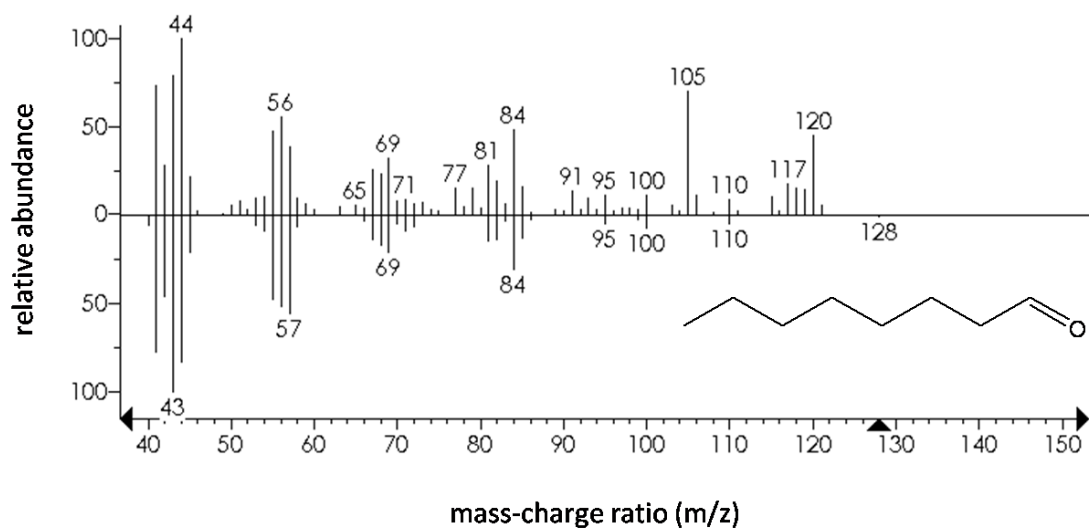


Figure 4.6. Mass spectra of peak 6 (top) and best library match, octanal (bottom).

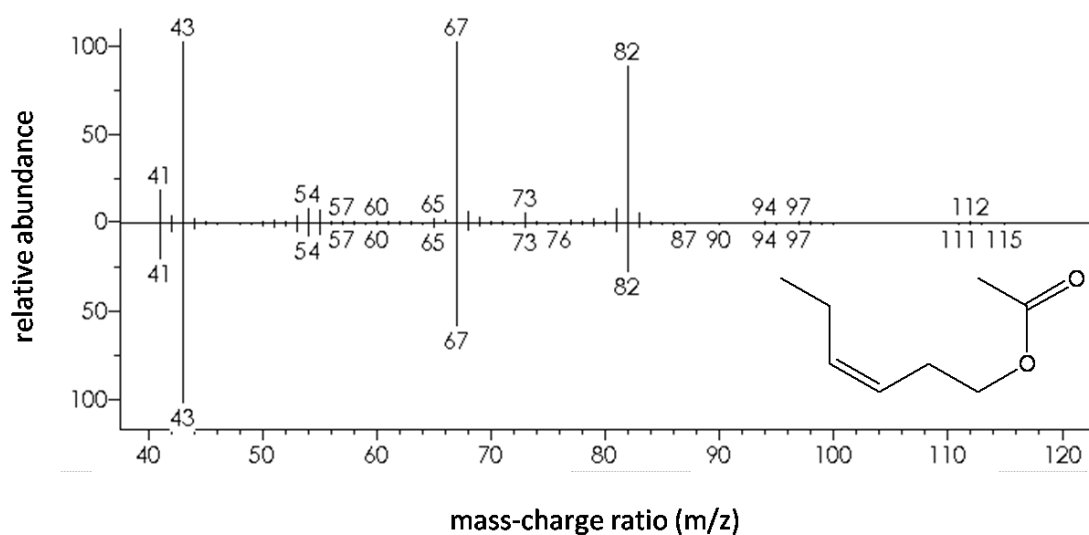


Figure 4.7. Mass spectra of peak 7 (top) and best library match, (Z)-3-hexen-1-yl acetate (bottom).

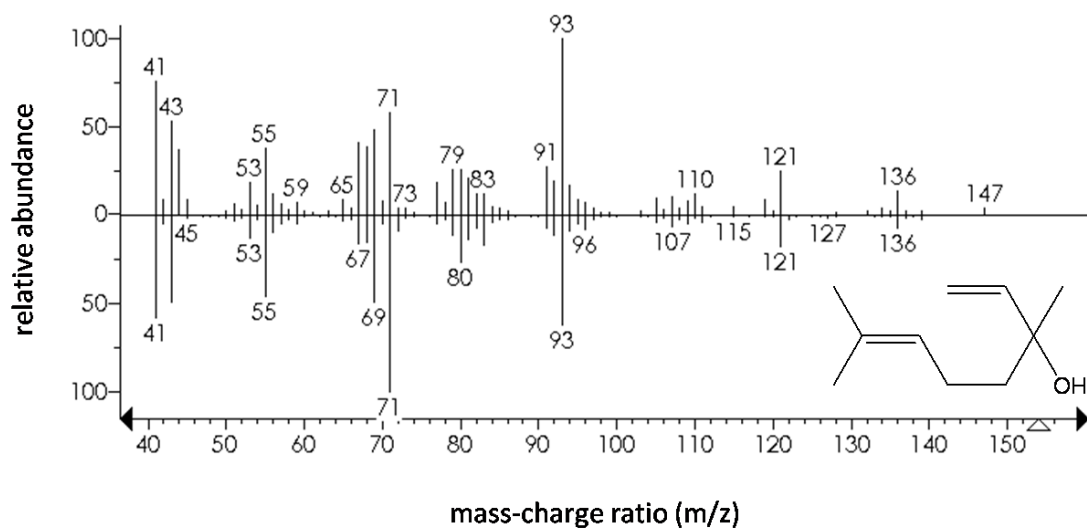


Figure 4.8. Mass spectra of peak 8 (top) and best library match, linalool (bottom).

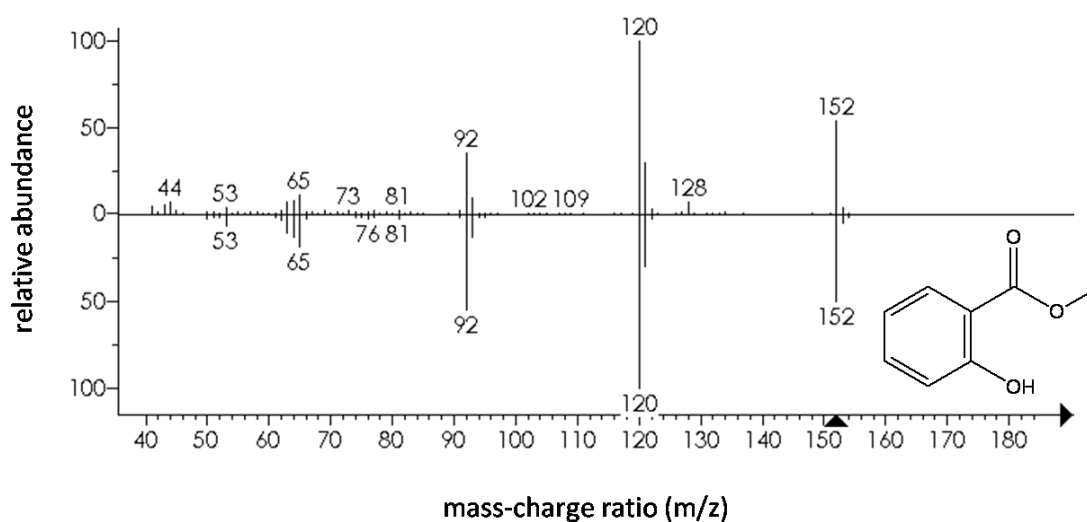


Figure 4.9. Mass spectra of peak 9 (top) and best library match, methyl salicylate (bottom).

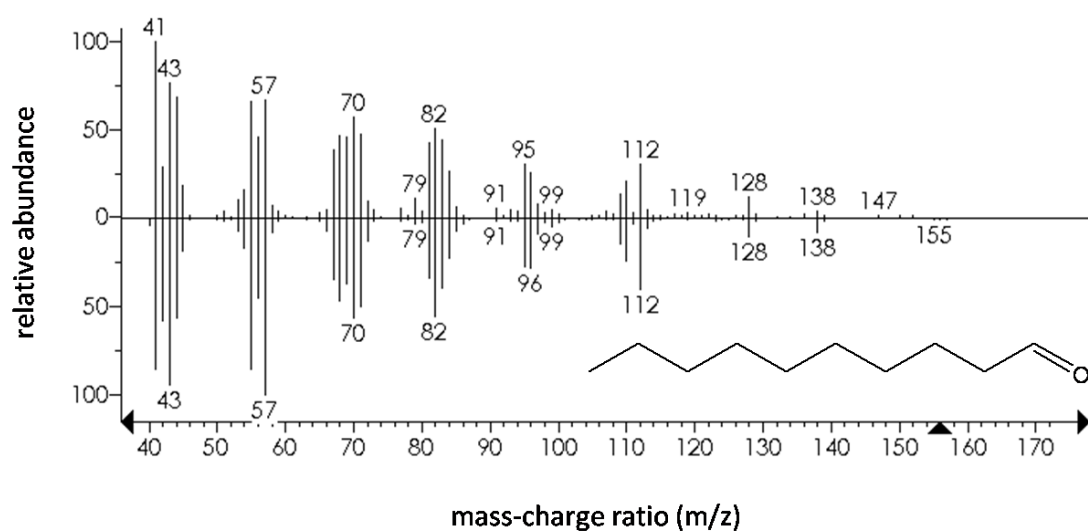


Figure 4.10. Mass spectra of peak 10 (top) and best library match, decanal (bottom).

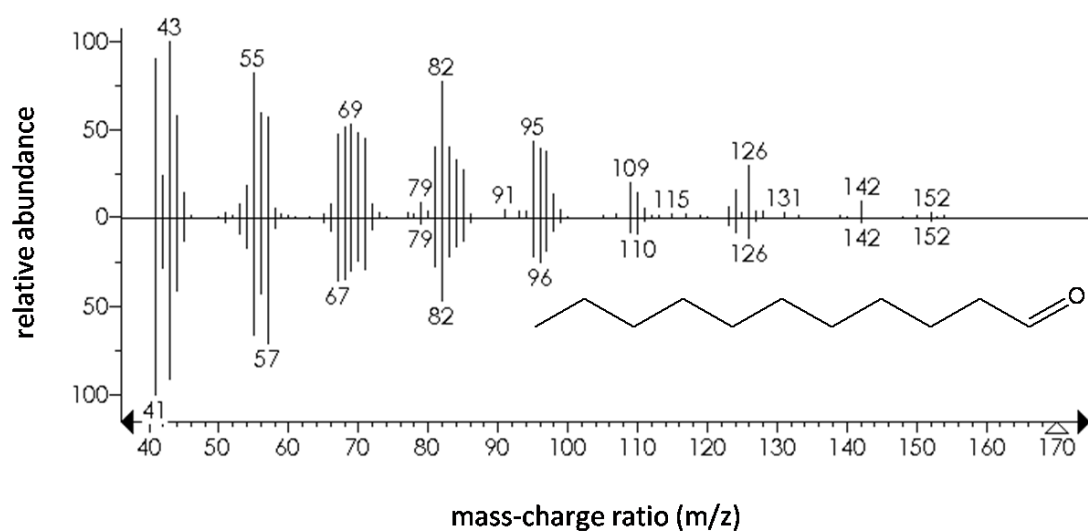


Figure 4.11. Mass spectra of peak 11 (top) and best library match, undecanal (bottom).

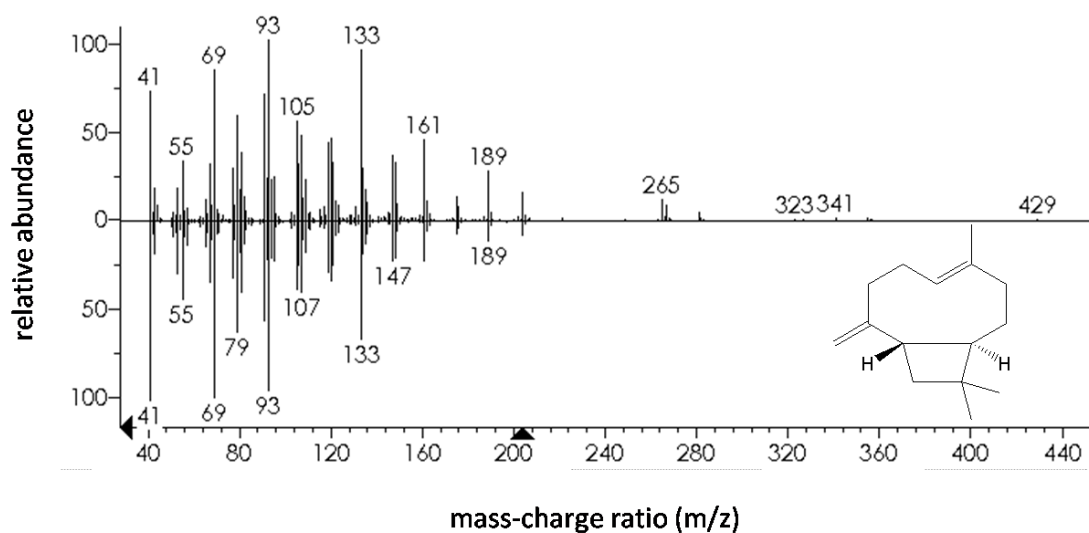


Figure 4.12. Mass spectra of peak 13 (top) and best library match, *(E)*-caryophyllene (bottom).

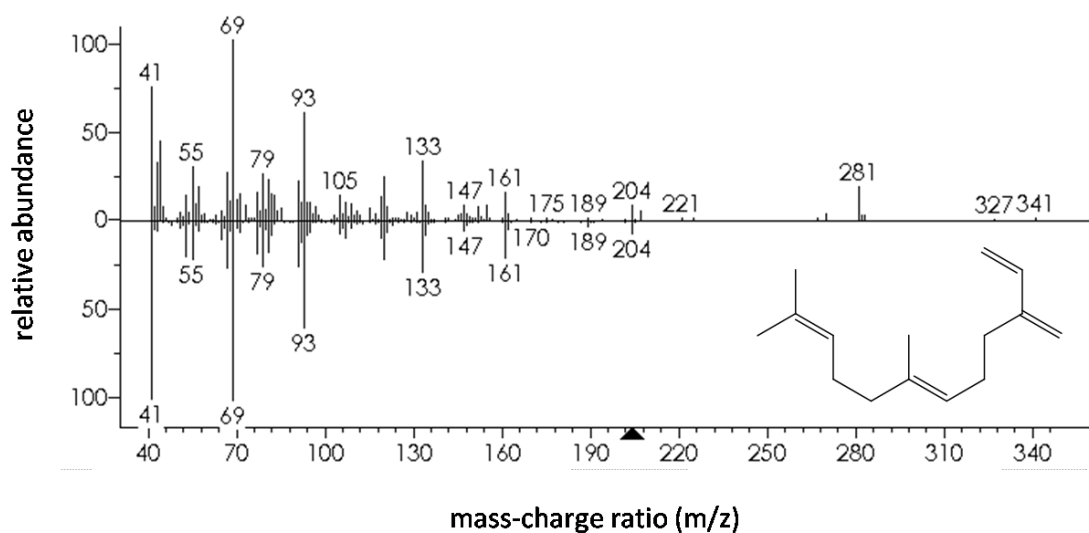


Figure 4.13. Mass spectra of peak 14 (top) and best library match, *(E)*- $\beta$ -farnesene (bottom).

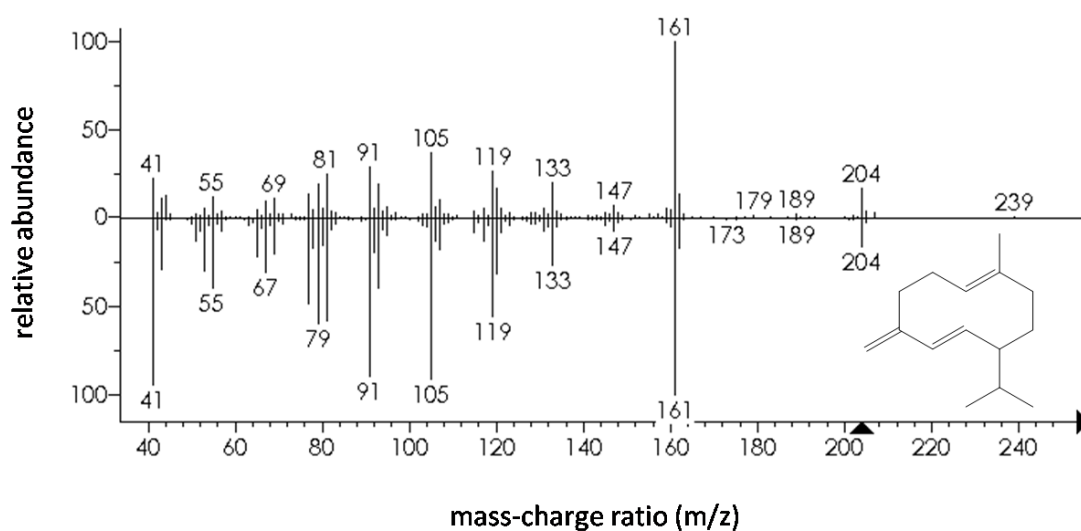


Figure 4.14. Mass spectra of peak 15 (top) and best library match, germacrene D (bottom).

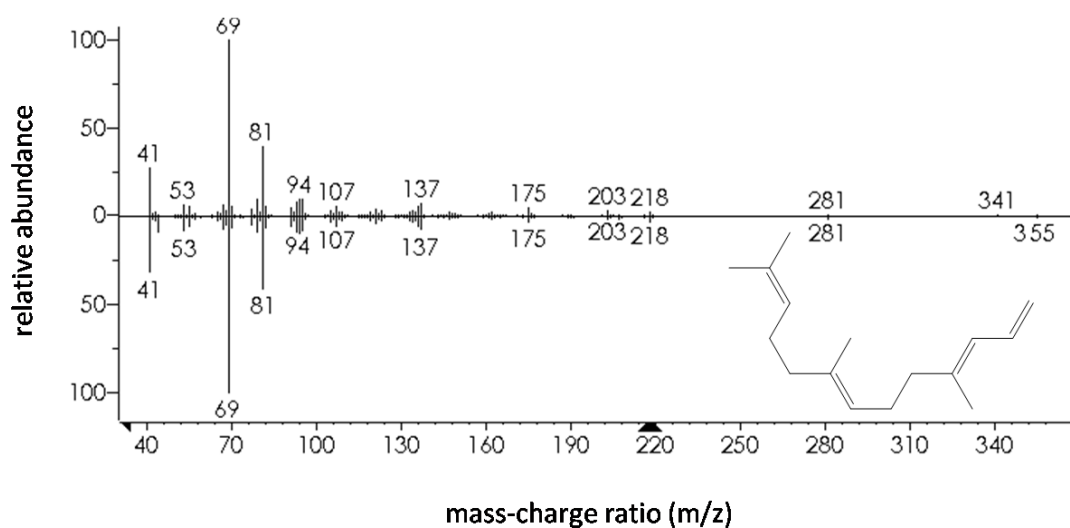


Figure 4.15. Mass spectra of peak 16 (top) and best library match, (E,E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (bottom).

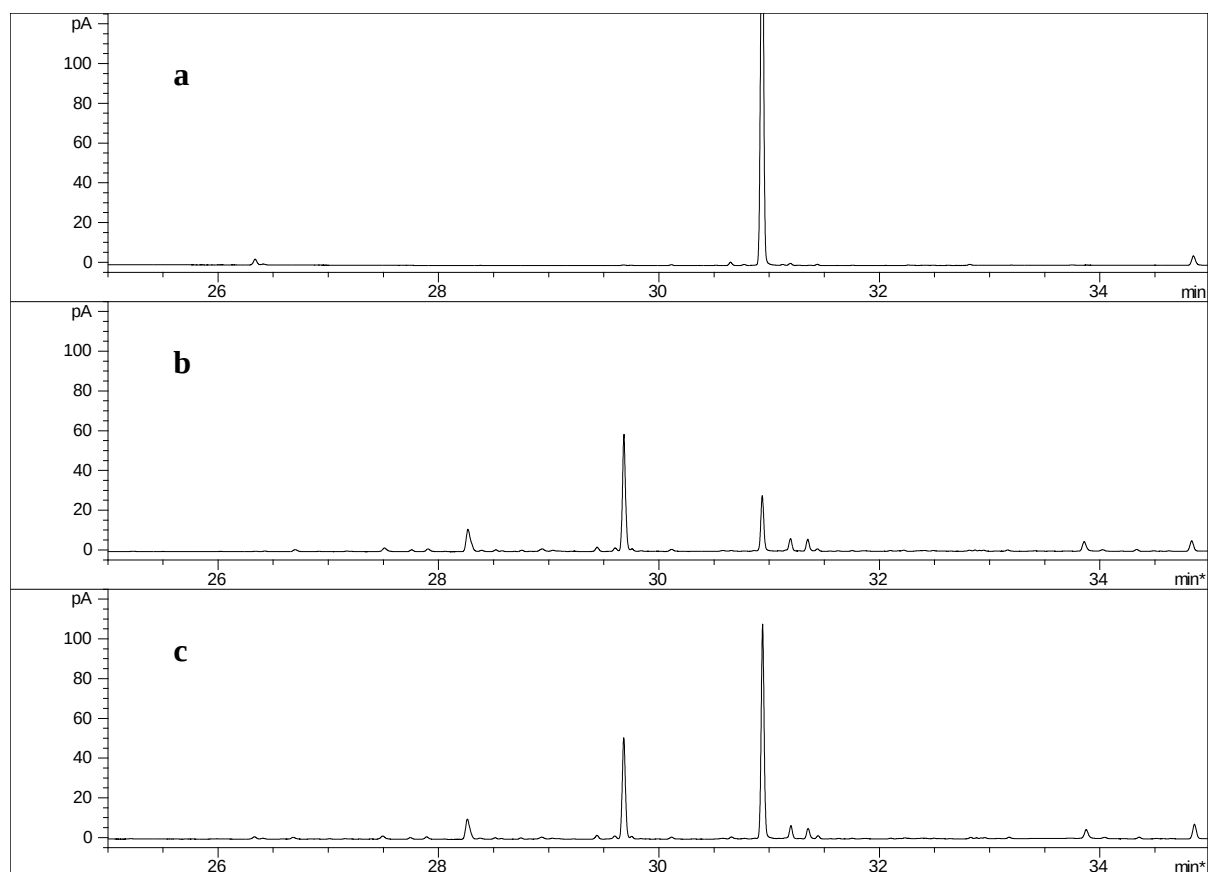


**Figure 4.16.** Example of peak enhancement used to confirm presence of (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT) in *V. faba* air entrainment sample. a) GC FID trace of authentic standard of TMTT. b) GC FID trace of *V. faba* air entrainment sample. c) GC FID trace of co-injection of air entrainment sample with authentic standard of TMTT showing increase in peak height relative to nearby peaks without an increase in width.

**Table 4.3. Retention indices and identities of *Vicia faba* volatile compounds electrophysiologically active to *A. fabae*.**

| Peak no. | RI (HP1) | RI (DB-WAX) | Compound name                                            |
|----------|----------|-------------|----------------------------------------------------------|
| 1        | 826      | 1218        | ( <i>E</i> )-2-hexenal                                   |
| 2        | 844      | 1351        | ( <i>Z</i> )-3-hexen-1-ol                                |
| 3        | 856      | 1377        | 1-hexanol                                                |
| 4        | 936      | 1528        | benzaldehyde                                             |
| 5        | 966      | 1328        | 6-methyl-5-hepten-2-one                                  |
| 6        | 984      | 1285        | octanal                                                  |
| 7        | 990      | 1317        | ( <i>Z</i> )-3-hexen-1-yl acetate                        |
| 8        | 1089     | 1545        | ( <i>R</i> )-linalool                                    |
| 9        | 1182     | 1785        | methyl salicylate                                        |
| 10       | 1193     | 1498        | decanal                                                  |
| 11       | 1272     | 1629        | undecanal                                                |
| 12       | 1388     | -           | unknown                                                  |
| 13       | 1446     | 1596        | ( <i>E</i> )-caryophyllene                               |
| 14       | 1454     | 1666        | ( <i>E</i> )- $\beta$ -farnesene                         |
| 15       | 1509     | 1706        | ( <i>S</i> )-germacrene D                                |
| 16       | 1582     | 1804        | ( <i>E,E</i> )-4,8,12-Trimethyl-1,3,7,11-tridecatetraene |

#### 4.3.2. Quantification

Figure 4.17 shows an example of a calibration curve used to calculate quantity from FID peak area. Quantities and ratios of all identified compounds in 10  $\mu$ l of the air entrainment sample are listed in Table 4.4.

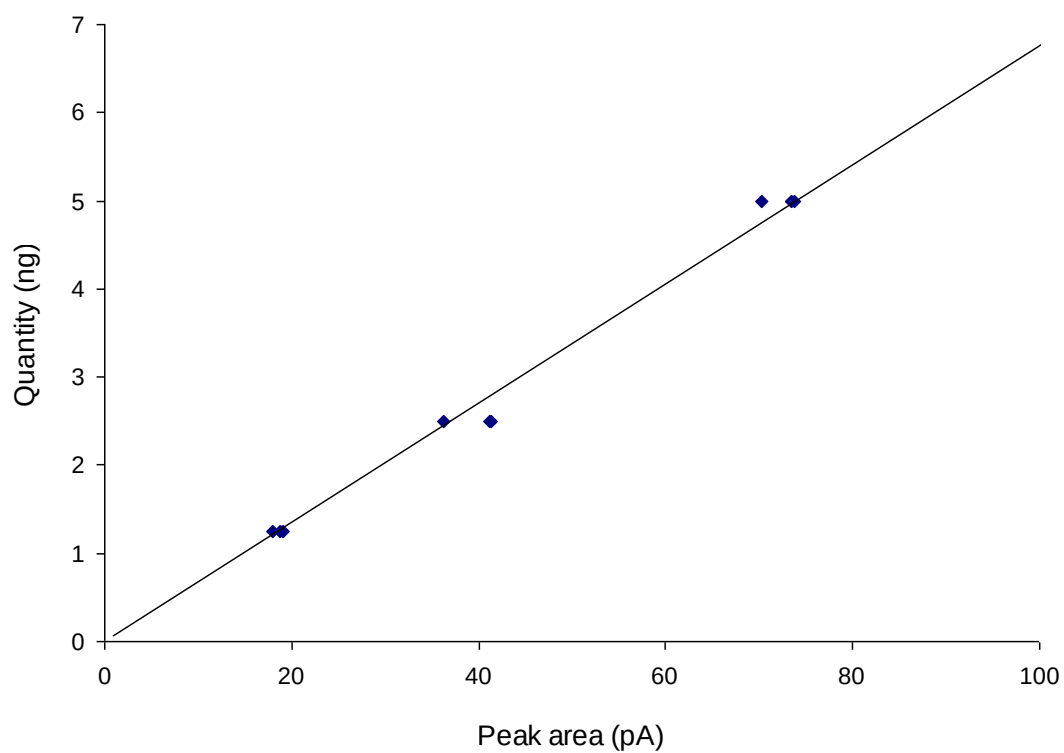


**Figure 4.17. Example of a calibration curve showing FID peak area against quantity (ng) with line of best fit used to quantify amount of *(E,E)*-4,8,12-Trimethyl-1,3,7,11-tridecatetraene in air entrainment sample**

**Table 4.4. Quantities and ratios of all identified compounds in 10 µl of *V. faba* air entrainment sample.**

| Compound no. | Compound name                     | Quantity in 10 µl of <i>Vicia faba</i> air entrainment sample (ng) | Ratio  |
|--------------|-----------------------------------|--------------------------------------------------------------------|--------|
| 1            | ( <i>E</i> )-2-hexenal            | 0.36                                                               | 0.42   |
| 2            | ( <i>Z</i> )-3-hexen-1-ol         | 85.80                                                              | 100.00 |
| 3            | 1-hexanol                         | 14.20                                                              | 16.55  |
| 4            | benzaldehyde                      | 0.04                                                               | 0.05   |
| 5            | 6-methyl-5-hepten-2-one           | 0.58                                                               | 0.68   |
| 6            | octanal                           | 0.34                                                               | 0.40   |
| 7            | ( <i>Z</i> )-3-hexen-1-yl acetate | 42.00                                                              | 48.95  |
| 8            | ( <i>R</i> )-linalool             | 1.02                                                               | 1.19   |
| 9            | methyl salicylate                 | 0.70                                                               | 0.82   |
| 10           | decanal                           | 0.76                                                               | 0.89   |
| 11           | undecanal                         | 0.52                                                               | 0.61   |
| 12           | unknown                           | -                                                                  | -      |
| 13           | ( <i>E</i> )-caryophyllene        | 4.60                                                               | 5.36   |
| 14           | ( <i>E</i> )- $\beta$ -farnesene  | 0.44                                                               | 0.51   |
| 15           | ( <i>S</i> )-germacrene D         | 0.36                                                               | 0.42   |
| 16           | TMTT                              | 4.96                                                               | 5.78   |

#### **4.3.3. Electrophysiological responses of aphids to identified compounds**

All compounds elicited statistically significant electrophysiological responses when tested at 10 µg with the exception of (*Z*)-3-hexen-1-ol, 6-methyl-5-hepten-2-one and benzaldehyde. These three compounds did, however, yield a statistically significant electrophysiological response when tested at 100 µg (Table 4.5).

**Table 4.5. Corrected EAG responses of winged virginoparae *Aphis fabae* to compounds identified in headspace of *Vicia faba*.**

| Compound name                                   | Corrected EAG response<br>( $\pm$ SE) [- $\mu$ V] at 10 $\mu$ g<br>dose | Corrected EAG response<br>( $\pm$ SE) [- $\mu$ V] at 100 $\mu$ g<br>dose |
|-------------------------------------------------|-------------------------------------------------------------------------|--------------------------------------------------------------------------|
| (E)-2-hexenal                                   | 56 ( $\pm$ 7.0)**                                                       | NT                                                                       |
| (Z)-3-hexen-1-ol                                | 7 ( $\pm$ 3.9)                                                          | 50 ( $\pm$ 5.3)**                                                        |
| 1-hexanol                                       | 48 ( $\pm$ 3.7)***                                                      | NT                                                                       |
| benzaldehyde                                    | -2 ( $\pm$ 6.4)                                                         | 36 ( $\pm$ 6.4)**                                                        |
| 6-methyl-5-hepten-2-one                         | -25 ( $\pm$ 6.2)                                                        | 59 ( $\pm$ 7.3)*                                                         |
| octanal                                         | 85 ( $\pm$ 5.3)***                                                      | NT                                                                       |
| (Z)-3-hexen-1-yl acetate                        | 22 ( $\pm$ 6.4)*                                                        | NT                                                                       |
| (R)-linalool                                    | 43 ( $\pm$ 4.8)**                                                       | NT                                                                       |
| methyl salicylate                               | 36 ( $\pm$ 4.7)**                                                       | NT                                                                       |
| decanal                                         | 82 ( $\pm$ 7.6)**                                                       | NT                                                                       |
| undecanal                                       | 60 ( $\pm$ 6.9)***                                                      | NT                                                                       |
| (E)-caryophyllene                               | 110 ( $\pm$ 5.5)***                                                     | NT                                                                       |
| (E)- $\beta$ -farnesene                         | 36 ( $\pm$ 6.1)*                                                        | NT                                                                       |
| (S)-germacrene D                                | 111 ( $\pm$ 8.0)**                                                      | NT                                                                       |
| (E,E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene | 75 ( $\pm$ 7.6)**                                                       | NT                                                                       |

*Note. Compounds which elicited a significant EAG response at the dose of 10  $\mu$ g were not tested (NT) at the dose of 100  $\mu$ g. EAG responses were corrected by subtracting the response to the hexane solvent control. \* Significantly different from control ( $P < 0.05$ ). \*\* Significantly different from control ( $P < 0.01$ ). \*\*\* Significantly different from control ( $P < 0.001$ ).*

## 4.4. Discussion

### 4.4.1. Identification of *V. faba* volatile compounds

Using coupled GC-MS, 15 out of 16 electrophysiologically active volatile compounds yielded mass spectra that matched closely with spectra of known compounds from a database. The tentative identities of each compound were successfully confirmed by comparison of their retention times with authentic standards on GC columns of two different polarities. The mass spectrum of octanal contained two dominant ions with  $m/z$  ratios of 105 and 120 which were not present in the mass spectrum of an authentic standard of octanal. The presence of octanal in the air entrainment sample was confirmed by peak enhancement on two GC columns of different polarities and its electrophysiological activity confirmed by electroantennography. These additional ions are therefore most likely impurities rather than an indication of a mismatched mass spectrum, most likely the result of another compound within the air entrainment sample co-eluting with octanal on the GC column used in coupled GC-MS.

Twelve of the compounds reported here have been identified previously in the headspace of undamaged *V. faba* plants, namely (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 1-hexanol, benzaldehyde, 6-methyl-5-hepten-2-one, (*Z*)-3-hexen-1-yl acetate, (*R*)-linalool, decanal, methyl salicylate, (*E*)-caryophyllene, (*S*)-germacrene D and TMTT (Blight et al. 1984; Griffiths et al. 1999; Colazza et al. 2004; Pareja et al. 2009). For the remaining three compounds; octanal, undecanal, and (*E*)- $\beta$ -farnesene; this study represents the first time these compounds have been identified in the headspace of *V. faba* plants. All 15 identified compounds are ubiquitous plants volatiles, found in a diverse range of families and not restricted to *V. faba* (Knudsen et al. 1993). This lends support to the hypothesis that *A. fabae* uses a blend of general plant volatiles to recognise its host rather than a single host-specific volatile compound. One compound, number 12 with a retention index of 1388 on an HP1 column, could not be identified due to its presence in very small quantities making it difficult to obtain a mass spectrum. It is possible that this compound could be specific to *V. faba* and olfactory host recognition mediated by this compound alone. This possibility will be discussed in Chapter 5.

The relatively high quantity of TMTT is an unusual trait of *V. faba* not common to other plant species. Although this compound is emitted by a wide variety of plant species it is usually

only released in response to herbivory (e.g. Pare & Tumlinson 1997; Buttery et al. 2000; Blackmer et al. 2004; Williams et al. 2005) whereas *V. faba* emits this compound constitutively. There is evidence to suggest that this compound may play an ecological role in some species, emitted upon infestation by herbivorous insects and attracting natural enemies of the herbivores to the infested plant (e.g. De Boer et al. 2004). It has also been found to act as a repellent for the aphid *Myzus persicae* (Bruce et al., unpublished). The constitutive emission of this compound may, therefore, be an adaptive trait to deter herbivores and recruit their natural enemies.

It was surprising to find methyl salicylate and (*E*)- $\beta$ -farnesene within an attractive/arrestive blend as these compounds have been reported to repel *A. fabae* in previous studies. Hardie et al., (1994a) observed that methyl salicylate caused *A. fabae* to make oriented movements away from the odour source in a linear-track olfactometer and also masked the attractive odour of *V. faba* leaves. The presence of methyl salicylate in the air entrainment sample therefore raises the question of why the aphids spent more time in the region of the olfactometer corresponding to the air entrainment sample despite the presence of this compound (section 2.2.1). The behavioural response observed by Hardie et al., (1994a) only occurred when methyl salicylate was present at release rates at and above 50  $\mu\text{g h}^{-1}$ . The relatively small quantity of methyl salicylate in the air entrainment sample (0.7 ng) is therefore likely below the threshold for which a masking effect would normally be observed, hence the aphids responded to the air-entrainment sample despite the presence of this compound. (*E*)- $\beta$ -Farnesene is used by *A. fabae* as an alarm pheromone and released when under attack from predators or parasitoids and causing nearby aphids to disperse (Nault et al. 1973; Pickett et al. 1992; Bruce et al. 2005a). This compound was present in relatively small quantities in the air entrainment sample, at 0.44 ng in 10  $\mu\text{l}$  of the air entrainment sample. *Aphis fabae* responds weakly to its alarm pheromone compared to other aphid species. Montgomery and Nault (1977) found that an average dose of 100 ng was required to disperse 50% of *A. fabae*, much higher than that present in 10  $\mu\text{l}$  of the air entrainment sample. This could possibly explain why aphids still responded positively to the air entrainment sample despite the presence of this compound. (*E*)- $\beta$ -Farnesene is also a widely occurring plant volatile (Knudsen et al. 1993) and aphids distinguish between plant- and aphid-originating (*E*)- $\beta$ -farnesene by the presence of certain other plant volatiles which inhibit the behavioural response to the alarm pheromone (Dawson et al. 1984). *Aphis fabae* uses linalool as an alarm

pheromone inhibitor (Bruce *et al.*, unpublished) which could also help explain why the aphids responded behaviourally towards the air entrainment sample despite the presence of (*E*)- $\beta$ -farnesene.

The quantities of compounds identified here were similar to those identified by Colazza *et al.*, (2004) and Pareja *et al.*, (2009) who both found relatively high quantities of (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl acetate in the headspace of undamaged *V. faba* plants. Smaller quantities of (*E*)-caryophyllene and TMTT in approximately equal quantities were also found which are also in agreement with the findings of the present study. Several compounds known to be electrophysiologically active to *A. fabae* were identified in the previous studies but not identified in the current study; namely hexanal and (*E,E*)- $\alpha$ -farnesene. As both of these compounds are electrophysiologically active to *A. fabae* (Visser *et al.* 1996) they should have been detected by coupled GC-EAG if they had been present in the air entrainment sample in sufficient quantities to elicit detectable electrophysiological responses. Differences in volatiles detected in different studies could be due to natural variation in volatile emissions between different plants used in each study. Large intra-specific variation in volatile emission has already been demonstrated in a number of different plant species (e.g. Degen *et al.* 2004; Lou *et al.* 2006). This idea is discussed in more detail in Chapter 6. Also, a wide variety of factors are known to influence emission of volatile compounds. These include stage of development of plants (Engel *et al.* 1988; Agelopoulos *et al.* 2000; Dudareva *et al.* 2000; Shiojiri & Karban 2006), time of day (Loughrin *et al.* 1993; Graus *et al.* 2004; Chamberlain *et al.* 2006) and a variety of environmental factors such as temperature, humidity, light availability and nitrogen availability (Johnson *et al.* 1999; Gouinguene & Turlings 2002; Schmelz *et al.* 2003; Vallat *et al.* 2005; Ferreyra *et al.* 2006). Any of these factors could vary between studies and potentially explain minor differences in volatiles collected. The work of Griffiths *et al.*, (1999) and Sutton *et al.*, (1992) also reported different volatiles than those identified in the present study. These earlier studies, however, collected volatiles from *V. faba* flowers rather than from plants prior to the flowering stage which is a likely explanation for any differences between these studies and the present one.

#### **4.4.2. Electrophysiological responses of *A. fabae* to identified compounds**

Statistically significant electrophysiological responses were obtained for all identified compounds at a dose of 10  $\mu$ g, with the exception of (*Z*)-3-hexen-1-ol, benzaldehyde and 6-

methyl-5-hepten-2-one, which only induced significant electrophysiological responses when tested at the higher dose of 100  $\mu\text{g}$ . This confirmed the identities of each compound tentatively identified by GC-MS and GC analysis. (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 1-hexanol, benzaldehyde, (*Z*)-3-hexen-1-yl acetate, linalool, methyl salicylate, (*E*)-caryophyllene and (*E*)- $\beta$ -farnesene have all previously been shown to be electrophysiologically active to *A. fabae* (Visser et al. 1996). However, this study represents the first time that 6-methyl-5-hepten-2-one, octanal, decanal, undecanal, (*S*)-germacrene D and TMTT have been shown to elicit electrophysiological responses in winged virginoparous *A. fabae*.

The magnitude of the electrophysiological responses varied widely between compounds. Construction of dose response curves of electrophysiological responses to each compound would have provided additional information but this was beyond the scope of the current study, the aim of which was merely to confirm electrophysiological activity of identified compounds. The amplitude of an electroantennogram response is believed to be proportional to the number of olfactory receptor neurones responding to a volatile compound (Schneider 1957; Wadhams 1990) so differences in response could be due to differences in numbers of receptors specific to each volatile compound present on the aphids' antennae. Test compounds were applied to filter paper at a dose of 10 or 100  $\mu\text{g}$ . Differences in molecular weight between test compounds means that the same weights of different compounds could contain different numbers of molecules. For example, (*E*)-caryophyllene has a molecular mass of 204.36  $\text{g mol}^{-1}$  whereas (*E*)-2-hexenal has a molecular mass of 98.14  $\text{g mol}^{-1}$ . This means that there are approximately twice the number of molecules of (*E*)-2-hexenal as there are (*E*)-caryophyllene in 10  $\mu\text{g}$  of each compound. Differences in molecular weight therefore mean that the aphids are exposed to different doses of different compounds. Differences in vapour pressures also affect the actual dose the aphids receive. Compounds with higher vapour pressures are more volatile and will evaporate from the filter paper at a faster rate resulting in more molecules reaching the aphid antenna during the 2 second stimulation than test compounds with lower vapour pressures. This makes comparison of electrophysiological responses to different compounds difficult. Steps could be taken to control for differences in molecular weight and volatility so that aphids are exposed to the same number of molecules for each compound tested. However, the magnitude of an electrophysiological response does not necessarily reflect the behavioural activity of a compound and so further characterisation

of the electrophysiological activity of each compound was beyond the scope of the present study. The aim of the present study was simply to confirm the electrophysiological activity of each compound before behavioural activity is tested in subsequent experiments and so the methods employed here were sufficient.

#### **4.4.3. Conclusions**

Fifteen out of 16 electrophysiologically active volatile compounds have been identified by GC-MS and GC analysis and their electrophysiological activity confirmed using electroantennography. The next step was to determine the behavioural responses of *A. fabae* to these compounds and this is discussed in Chapter 5.

Identification and quantification of volatile compounds was made based on a bulked air entrainment sample consisting of entrainments of four plants. The relative quantities of the different volatile compounds emitted by *V. faba* plants in the field may vary considerably from plant-to-plant due to genetic, developmental and environmental differences between individual plants. It is possible that the quantities and ratios of the compounds reported here represent only a part of the range of different quantities and ratios that are naturally emitted by *V. faba*. The extent of this range of ratios may be important to host-seeking aphids as it is believed that ratios may provide a means of host-discrimination for herbivorous insects (Visser 1986; Bruce et al. 2005b). Determination of this plant-to-plant variation would contribute considerably to an understanding of how volatile compounds are used in host location by *A. fabae* and this will be addressed in Chapter 6.

# **CHAPTER 5. BEHAVIOURAL RESPONSES OF *Aphis fabae* TO VOLATILE COMPOUNDS IDENTIFIED IN THE HEADSPACE OF *Vicia faba* PLANTS**

## **5.1. Introduction**

In the previous chapter, fifteen electrophysiologically active volatile compounds were identified from the headspace of *V. faba* plants. To confirm that they are used by *A. fabae* in host location their behavioural activity must first be determined. This can be achieved by bioassay of aphid behavioural responses in an olfactometer bioassay which is the subject of this chapter. The ultimate test is to formulate a synthetic blend comprising all identified compounds at the same concentrations and ratios as in the air entrainment sample and comparing the behavioural response of the aphids to this blend to the response to the air entrainment sample. If aphids show no difference in preference for the synthetic blend and the air entrainment sample then this will provide strong evidence that all behaviourally relevant compounds have been identified and quantified correctly.

Visser (1986) suggested that host location using volatile compounds could be achieved in two possible ways. The first hypothesis was that host-seeking insects respond to taxonomically characteristic volatile compounds which are specific to their hosts and not found in unrelated plant species. There are a number of examples in the literature supporting this hypothesis (Pierce et al. 1978; Guerin et al. 1983; Judd & Borden 1989; Krasnoff & Dussourd 1989; Bjostad & Hibbard 1992; Knight & Light 2001). The second hypothesis was that host-seeking insects use ubiquitous volatile compounds not specific to their host, but which are emitted in species-characteristic ratios. There are several examples in the literature suggesting that insects respond optimally to host volatiles when they are in a particular ratio (Visser & Ave 1978; Robacker et al. 1992; Ngumbi et al. 2007), lending weight to this hypothesis. Due to the ubiquity of the electrophysiologically active volatile compounds identified in the *V.*

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Note: a refereed paper relating to the work in this Chapter has been published (Webster et al., 2008b).

*faba* air entrainment sample (Knudsen et al. 1993), it is more likely that the second hypothesis applies to *A. fabae* as a single volatile compound would not constitute a host-specific cue. By comparing behavioural responses of the aphids to each volatile compound presented alone to the response to the whole air entrainment sample, it can be tested whether or not a single host volatile compound can explain the response to the whole headspace sample.

Another possibility is that *A. fabae* relies on non-host avoidance as well as host-recognition. In this scenario, *A. fabae* may respond to general plant volatiles, perhaps a single volatile emitted by its host, and be repelled or deterred by volatiles originating from non-host plants. It has been shown that *A. fabae* moves away from isothiocyanates in an olfactometer, associated with non-host brassicaceous plants (Nottingham et al. 1991; Isaacs et al. 1993; Storer et al. 1996; Fahey et al. 2001). It has also been shown that, upon alighting on a non-host oat plant, *A. fabae* is able to use non-host cues in the form of epicuticular lipids to avoid settling on a non-host plant (Powell et al. 1999). The use of ubiquitous plant volatiles as host-cues has been discussed previously (Visser 1986; Bruce et al. 2005b) but their potential use as non-host cues has received relatively little attention. If a species of plant reliably and consistently produces several general plant volatile compounds, then the presence of one of those volatiles in the absence of all the others would constitute a reliable non-host cue. As *A. fabae* has already been shown to possess the ability to use non-host cues to avoid non-host plants (Nottingham et al. 1991; Isaacs et al. 1993; Hardie et al. 1994a), this hypothesis merits further investigation. An investigation of behavioural responses to individual volatile compounds alone and in the context of other *V. faba* volatiles would provide a valuable starting point to testing this hypothesis.

### **5.1.1. Aims and objectives**

The aim of this chapter was to determine the behavioural responses of *A. fabae* to volatile compounds identified from the *V. faba* air entrainment sample in Chapter 4. To achieve this aim, the following objectives were envisaged:

### **Determine the behavioural responses of *Aphis fabae* to a synthetic blend based on *Vicia faba* air entrainment sample**

The behavioural responses of winged virginoparous *A. fabae* to a synthetic blend comprising all 15 identified volatile compounds at the same concentration and ratio as in the *V. faba* air entrainment sample was determined using a four-arm olfactometer. To test whether or not the synthetic blend elicited the same behavioural response as the air entrainment sample, a choice test was conducted where aphids were offered the choice of both odour sources in the same olfactometer. It was hypothesised that neither odour source would be preferred over the other.

### **Determine the behavioural response of *Aphis fabae* to each volatile compound at the same concentration as in the air entrainment sample**

Behavioural responses of winged virginoparous *A. fabae* to each volatile compound at the same concentration as in the air entrainment sample were determined using a four-arm olfactometer. It was hypothesised that aphids would spend more or less time in the treated region of the olfactometer than controls. Any compound(s) which caused the aphids to spend significantly more time in the treated region of the olfactometer than controls were tested again using a choice-test, where aphids were offered the choice between the single compound and the synthetic blend in the same olfactometer. It was hypothesised that one or more volatile compounds would cause the aphids to spend more time in the treated region of the olfactometer but that aphids would prefer the complete blend over the individual compound(s).

### **Determine behavioural response of *Aphis fabae* to each volatile compound at a range of different doses**

To gain further insight into the behavioural responses of aphids to each compound, dose response experiments were conducted. Compounds which caused aphids to spend more time in the treated region of the olfactometer at any dose were combined into a blend. Behavioural responses to this blend were compared to the response to the complete blend. It was hypothesised that the aphids would prefer the complete blend over the reduced blend.

## **Determine context-dependent behavioural responses of *Aphis fabae* to volatile compounds**

Compounds which caused aphids to spend less time in the treated region of the olfactometer when tested at the same concentration as in the air entrainment sample were combined into a blend and tested for behavioural activity using a four-arm olfactometer. It was hypothesised that aphids would spend less time in the treated region of the olfactometer than controls. A second blend, consisting of all compounds in the air entrainment sample except those causing aphids to spend less time in the treated region of the olfactometer was offered to aphids alongside the full blend in a choice test. This was to determine if the behavioural effect of the individual compounds was the same when in the context of the blend. It was hypothesised that aphids would prefer the reduced blend.

## **5.2. Materials and Methods**

### **5.2.1. Insects**

The Kennedy and Booth clone of *A. fabae* (Kennedy & Booth 1950) was reared on tick beans (*Vicia faba* var. *minor*) in a Perspex rearing cabinet under controlled conditions (22±1 °C, LD 16:8 h). Winged aphids were induced by allowing the colony to become crowded. Prior to behavioural bioassays, aphids were starved for 24 h in Petri dishes with moistened filter paper to prevent dehydration. Aphids were then left in the bioassay room for at least 2 h to acclimatise prior to experiments.

### **5.2.2. Chemicals**

Chemicals used were (*E*)-2-hexenal, benzaldehyde, 6-methyl-5-hepten-2-one, decanal, 1-hexanol (all 99% purity, Sigma Aldrich Inc., St. Louis, Missouri, USA), (1*R*,5*S*)-myrtenal (98% purity, Sigma Aldrich Inc.) undecanal (97% purity, Sigma Aldrich Inc.), (*R*)-linalool, (*S*)-linalool (both 98% purity, Sigma Aldrich Inc.), (*Z*)-3-hexen-1-yl acetate (99% purity, Avocado Research Chemicals Ltd.) octanal, methyl salicylate, (*Z*)-3-hexen-1-ol, (all 98% purity, Avocado Research Chemicals Ltd., Lancs., UK), and (*E*)-caryophyllene (85% purity, Pfaltz & Bauer inc., Stamford, Connecticut, USA). (*E*)- $\beta$ -Farnesene (98% purity) was synthesized from (*E*)-farnesyl chloride by Dr. Mike Birkett at Rothamsted Research (Kang et

al. 1987). (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT) was synthesised from (*E,E*)-farnesol by oxidation to its aldehyde followed by Wittig methylenation performed by Dr. Samuel Dufour at Rothamsted Research. (Leopold 1990). (*S*)-Germacrene D was extracted from ylang ylang oil (Boots, Nottingham, UK). Approx. 1g of the oil was mixed with an equal amount of redistilled hexane and run through a 25 cm by 2 cm i.d. column filled with florisil (60-100 US mesh, Fisons scientific apparatus, Loughborough, Leics, UK) and hexane. Collected fractions were run through a column (i.d. 1 cm) filled with silica gel (particle size 0.035 – 0.07 mm, 220-440 mesh, Fluka, Dorset, UK) and hexane, resulting in a 85% purity of (*S*)-germacrene D in hexane.

### 5.2.3. Test stimuli

A complete synthetic blend was made up in redistilled hexane comprising all identified volatile compounds at the same concentration and ratio as in the air entrainment sample as listed in Chapter 4 (Table 4.4).

Single compound solutions were also made up of each compound in redistilled hexane at the same concentration as in the air entrainment sample (Chapter 4, Table 4.4) and also at 0.01 ng  $\mu\text{l}^{-1}$ , 0.1 ng  $\mu\text{l}^{-1}$ , 1 ng  $\mu\text{l}^{-1}$ , 10 ng  $\mu\text{l}^{-1}$ , and 100 ng  $\mu\text{l}^{-1}$  for dose response experiments. A two-component blend consisting of (*Z*)-3-hexen-1-ol and 1-hexanol at the same concentration and ratio as in the air entrainment was also made up. Additionally, a three component blend consisting of 0.34 ng  $\mu\text{l}^{-1}$  octanal, 1.02 ng  $\mu\text{l}^{-1}$  (*R*)-linalool and 0.36 ng  $\mu\text{l}^{-1}$  (*S*)-germacrene D, and also a reduced-component blend consisting of all the compounds at the same concentration and ratio as in the air entrainment sample except octanal, (*R*)-linalool and (*S*)-germacrene D, both in redistilled hexane.

### 5.2.4. Behavioural bioassays

A four-arm olfactometer was used to determine behavioural responses of aphids to volatile compounds as described in Chapter 2 (section 2.2.4) with the olfactometer divided into 4 regions for each experiment.

For choice tests where two different odour sources were tested, each odour source was randomly assigned to one of the glass arms for each replicate and the other two odour sources

were used as controls. Time spent in each region of the olfactometer was then recorded for 16 min.

### **List of experiments**

All experiments are described below and summarised in Table 5.1. In all experiments test compounds and controls were added to triangles of filter paper (approx. 50 mm<sup>2</sup> area) and allowed 30 s for the solvent to evaporate before being placed into one of the glass arms.

**Behavioural response to complete synthetic blend.** Ten µl of the complete synthetic blend comprising all 15 identified electrophysiologically active volatile compounds at the same concentration and ratio as in the air entrainment sample (see Chapter 4 Table 4.4) was tested in one of the glass arms. Ten µl hexane was placed in each of the other glass arms and used as controls. Twelve replicates were carried out.

**Choice test: complete synthetic blend vs. air entrainment sample.** Ten µl of the complete synthetic blend was added to a triangle of filter paper and ten µl of the air entrainment sample added to a second triangle of filter paper. As the two odour sources were dissolved in different solvents, ten µl of ether or hexane was also added to the filter paper containing the complete synthetic blend and air entrainment sample, respectively. Ten µl of ether and ten µl hexane were added to each of two pieces of filter paper and used as controls. Twenty-four replicates were carried out.

**Behavioural response to individual compounds.** A one µl solution of each test compound at the same concentration as in the air entrainment sample was applied to a triangle of filter paper. One µl hexane was applied to three pieces of filter paper as controls. Test compounds were arranged in a randomised block design consisting of twelve replicates. To ensure aphids were responsive on the days of testing, ten µl of the complete synthetic blend was also tested using ten µl of hexane as controls.

**Dose response behavioural experiments to individual compounds.** One µl solutions of each test compound at doses of 0.01 ng, 0.1 ng, 1ng, 10 ng, and 100 ng was applied to a triangle of filter paper and one µl hexane applied to triangles of filter paper used as controls. Test compounds were arranged in a randomised block design consisting of twelve replicates.

**Choice test: (Z)-3-hexen-1-ol vs. complete synthetic blend.** Ten  $\mu\text{l}$  of the complete synthetic blend was applied to a triangle of filter paper and ten  $\mu\text{l}$  containing 85.8 ng (Z)-3-hexen-1-ol was applied to another filter paper triangle. Ten  $\mu\text{l}$  redistilled hexane was applied to two pieces of filter paper and used as controls. Eighteen replicates were carried out.

**Behavioural response to two-component blend.** One  $\mu\text{l}$  of a two-component blend consisting of 85.8 ng (Z)-3-hexen-1-ol and 14.2 ng 1-hexanol was applied to a triangle of filter paper and one  $\mu\text{l}$  hexane applied to triangles of filter paper used as controls. Twelve replicates were completed. A choice test was then performed with one arm containing filter paper which had the two-component blend applied and another containing filter paper with the complete synthetic blend applied. Eighteen replicates were carried out.

**Behavioural response to three-component blend.** One  $\mu\text{l}$  of a three-component blend consisting of 0.34 ng octanal, 1.02 ng (R)-linalool, and 0.36 ng (S)-germacrene D was applied to a triangle of filter paper and one  $\mu\text{l}$  hexane applied to triangles of filter paper used as controls. To ensure aphids were responsive on the days of testing, 0.34 ng octanal was also tested. The two were arranged in a randomised block design consisting of twelve replicates.

**Choice test: reduced blend vs. complete synthetic blend.** One  $\mu\text{l}$  of a blend consisting of all compounds in the complete synthetic blend except octanal, (R)-linalool, and (S)-germacrene D plus 9  $\mu\text{l}$  hexane was applied to a triangle of filter paper. Ten  $\mu\text{l}$  of the complete synthetic blend was applied to another triangle of filter paper and ten  $\mu\text{l}$  hexane applied to triangles of filter paper used as controls. Eighteen replicates were carried out.

### **Statistical analysis**

Responses to single compounds were analysed as described in Chapter 2 (section 2.2.3). To determine if more time was spent in either of the treated regions when a choice test was conducted, the difference between times spent in each was calculated. The data were found to be normally distributed and then compared to a test mean of zero using a one sample t-test. To determine if the combined time spent in the two treated arms was greater than time spent in the control arms, the summed time spent in the controls was subtracted from summed time spent in treated arms and compared to a test mean of zero using a one-sample t-test.

For test odours which it was predicted that either more or less time would be spent in the treated region of the olfactometer compared to controls (for example, compounds which were already known to elicit a particular behavioural response), a one-sided t-test was performed. For test odours for which it could not be predicted whether aphids would spend either more or less time in treated region of the olfactometer compared to controls, a two-sided t-test was performed. In experiment 3, for the majority of compounds it could not be predicted whether individual compounds would cause aphids to spend more or less time in treated region of olfactometer than controls so a two-sided test was carried out for each. The exceptions were methyl salicylate and (*E*)- $\beta$ -farnesene which were already known to cause aphids to avoid the treated region of the olfactometer (Montgomery & Nault 1977; Hardie et al. 1994a). To allow fair comparison with the synthetic blend, for which a one-sided t-test was appropriate, a one-sided test was also carried out for all compounds tested in experiment 3. In experiment 4, compounds which had been shown to be behaviourally active in experiment 3 or previously in the literature (Montgomery & Nault 1977; Hardie et al. 1994a) were tested using a one-sided t-test. All other compounds were tested using a two-sided t-test.

**Table 5.1. List of behavioural experiments including compounds and doses.**

| Experiment no. | Test odour                                          | Dose (ng)             |
|----------------|-----------------------------------------------------|-----------------------|
| 1              | complete synthetic blend                            | *                     |
| 2              | complete synthetic blend vs. air entrainment sample | *                     |
| 3              | complete synthetic blend                            | *                     |
|                | ( <i>E</i> )-2-hexenal                              | 0.36                  |
|                | ( <i>Z</i> )-3-hexen-1-ol                           | 85.80                 |
|                | 1-hexanol                                           | 14.20                 |
|                | benzaldehyde                                        | 0.04                  |
|                | 6-methyl-5-hepten-2-one                             | 0.58                  |
|                | octanal                                             | 0.34                  |
|                | ( <i>Z</i> )-3-hexen-1-yl acetate                   | 42.00                 |
|                | ( <i>R</i> )-linalool                               | 1.02                  |
|                | methyl salicylate                                   | 0.70                  |
|                | decanal                                             | 0.76                  |
|                | undecanal                                           | 0.52                  |
|                | ( <i>E</i> )-caryophyllene                          | 4.60                  |
|                | ( <i>E</i> )- $\beta$ -farnesene                    | 0.44                  |
|                | ( <i>S</i> )-germacrene D                           | 0.36                  |
|                | TMTT                                                | 4.96                  |
| 4              | ( <i>E</i> )-2-hexenal                              | 100, 10, 1, 0.1, 0.01 |
|                | ( <i>Z</i> )-3-hexen-1-ol                           | 100, 10, 1, 0.1, 0.01 |
|                | 1-hexanol                                           | 100, 10, 1, 0.1, 0.01 |
|                | benzaldehyde                                        | 100, 10, 1, 0.1, 0.01 |
|                | 6-methyl-5-hepten-2-one                             | 100, 10, 1, 0.1, 0.01 |
|                | octanal                                             | 100, 10, 1, 0.1, 0.01 |
|                | ( <i>Z</i> )-3-hexen-1-yl acetate                   | 100, 10, 1, 0.1, 0.01 |
|                | ( <i>R</i> )-linalool                               | 100, 10, 1, 0.1, 0.01 |
|                | methyl salicylate                                   | 100, 10, 1, 0.1, 0.01 |
|                | decanal                                             | 100, 10, 1, 0.1, 0.01 |

|   |                                                                                                                 |                       |
|---|-----------------------------------------------------------------------------------------------------------------|-----------------------|
|   | undecanal                                                                                                       | 100, 10, 1, 0.1, 0.01 |
|   | ( <i>E</i> )-caryophyllene                                                                                      | 100, 10, 1, 0.1, 0.01 |
|   | ( <i>E</i> )- $\beta$ -farnesene                                                                                | 100, 10, 1, 0.1, 0.01 |
|   | ( <i>S</i> )-germacrene D                                                                                       | 100, 10, 1, 0.1, 0.01 |
|   | TMTT                                                                                                            | 100, 10, 1, 0.1, 0.01 |
| 5 | ( <i>Z</i> )-3-hexen-1-ol vs.<br>complete synthetic blend                                                       | 85.80<br>*            |
| 6 | ( <i>Z</i> )-3-hexen-1-ol & 1-hexanol<br>complete synthetic blend vs. ( <i>Z</i> )-3-<br>hexen-1-ol & 1-hexanol | *<br>*                |
| 7 | octanal<br>octanal, ( <i>R</i> )-linalool and ( <i>S</i> )-<br>germacrene D                                     | 0.34<br>*             |
| 8 | reduced-component blend vs.<br>complete synthetic blend                                                         | *                     |

\* Complete synthetic blend was comprised of compounds at same dose as listed in Chapter 4 (Table 4.4).

## 5.3. Results

Results of each experiment are described below. The vast majority of aphids were active and did not need to be removed from the analysis for all experiments.

### 5.3.1. Behavioural responses to complete synthetic blend

Aphids spent significantly more time in the treated region of the olfactometer when the complete synthetic blend was used as the test odour ( $P=0.018$ ). This is shown in Figure 5.1 alongside responses to *V. faba* plant and air entrainment sample from Chapter 2 for comparison. When the synthetic blend and air entrainment samples were tested in a choice test, the total time spent in the two treatment olfactometer arms was significantly greater than

the total time spent in the two control arms ( $P=0.012$ ) but the aphids did not show any preference for either of the two treatments ( $P=0.844$ ), demonstrating that the synthetic blend had similar activity to the natural sample (Figure 5.2).

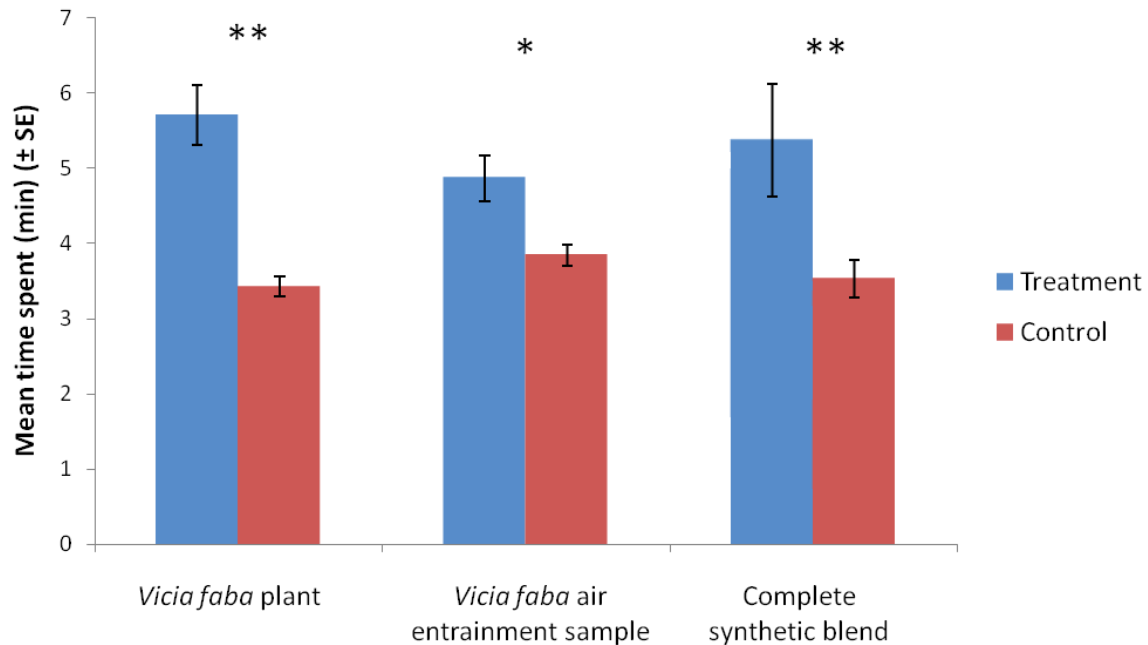


Figure 5.1. Behavioural response of winged virginoparae *Aphis fabae* to complete 15-component synthetic blend ( $n=12$ ). Data is shown alongside behavioural response to *V. faba* plant and *V. faba* air entrainment sample from Chapter 2 for comparison. \* Significantly different ( $P<0.05$ ). \*\* Significantly different ( $P<0.01$ ).

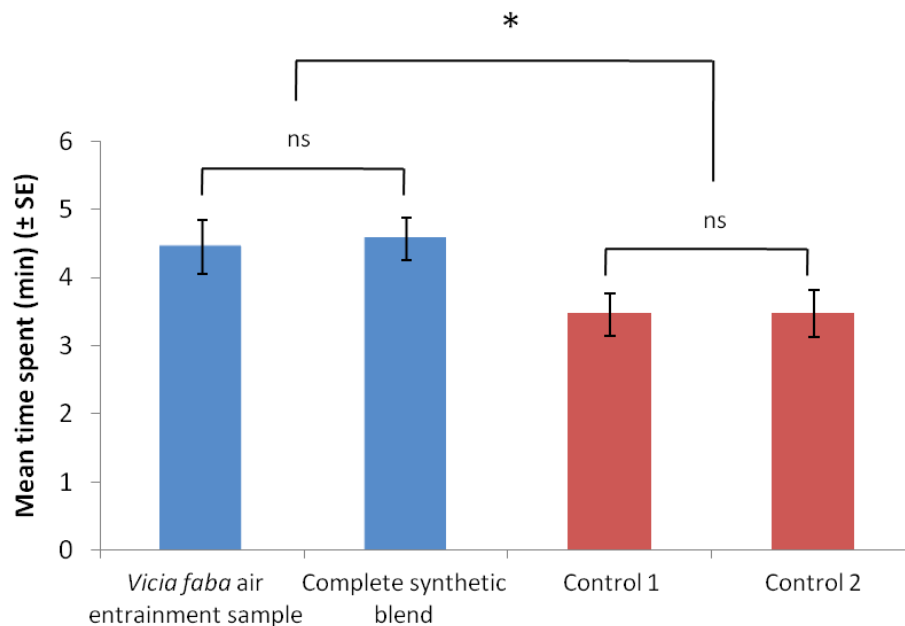


Figure 5.2. Behavioural response of winged virginoparae *Aphis fabae* to *Vicia faba* air entrainment sample and complete 15-component synthetic blend when both odour sources present together in the same olfactometer ( $n=24$ ). \* Significantly different ( $P<0.05$ ).

### 5.3.2. Behavioural responses to individual compounds at same concentration as in air entrainment sample

Figure 5.3 shows time spent in the treated region of olfactometer compared to controls when each compound was tested individually at the same concentration as in the air entrainment sample. Time spent in the treated region was significantly greater than 25 % of 16 min when the complete synthetic blend was tested ( $P=0.032$ ), indicating that aphids were responsive to odours during the experiments. However, no single compound caused aphids to spend significantly more time in treated region of olfactometer than control. However, when a one-sided test was performed on the data, this suggested that aphids spent significantly more than 25 % of their time in the treated region when 85.8 ng (*Z*)-3-hexen-1-ol was used as the test odour ( $P=0.047$ ) (Figure 5.3). When aphids were offered the choice of 85.8 ng (*Z*)-3-hexen-1-ol and the complete synthetic blend in a choice test, however, the aphids preferred the complete synthetic blend over this compound alone ( $P=0.006$ ) (Figure 5.4).

Surprisingly, three of the compounds when tested individually and at the same concentration as in the air entrainment sample caused the aphids to spend significantly less than 25 % of their time in the treated region of the olfactometer. These were octanal ( $P=0.018$ ), (*R*)-linalool ( $P=0.012$ ), and (*S*)-germacrene D ( $P=0.034$ ) (Figure 5.3).

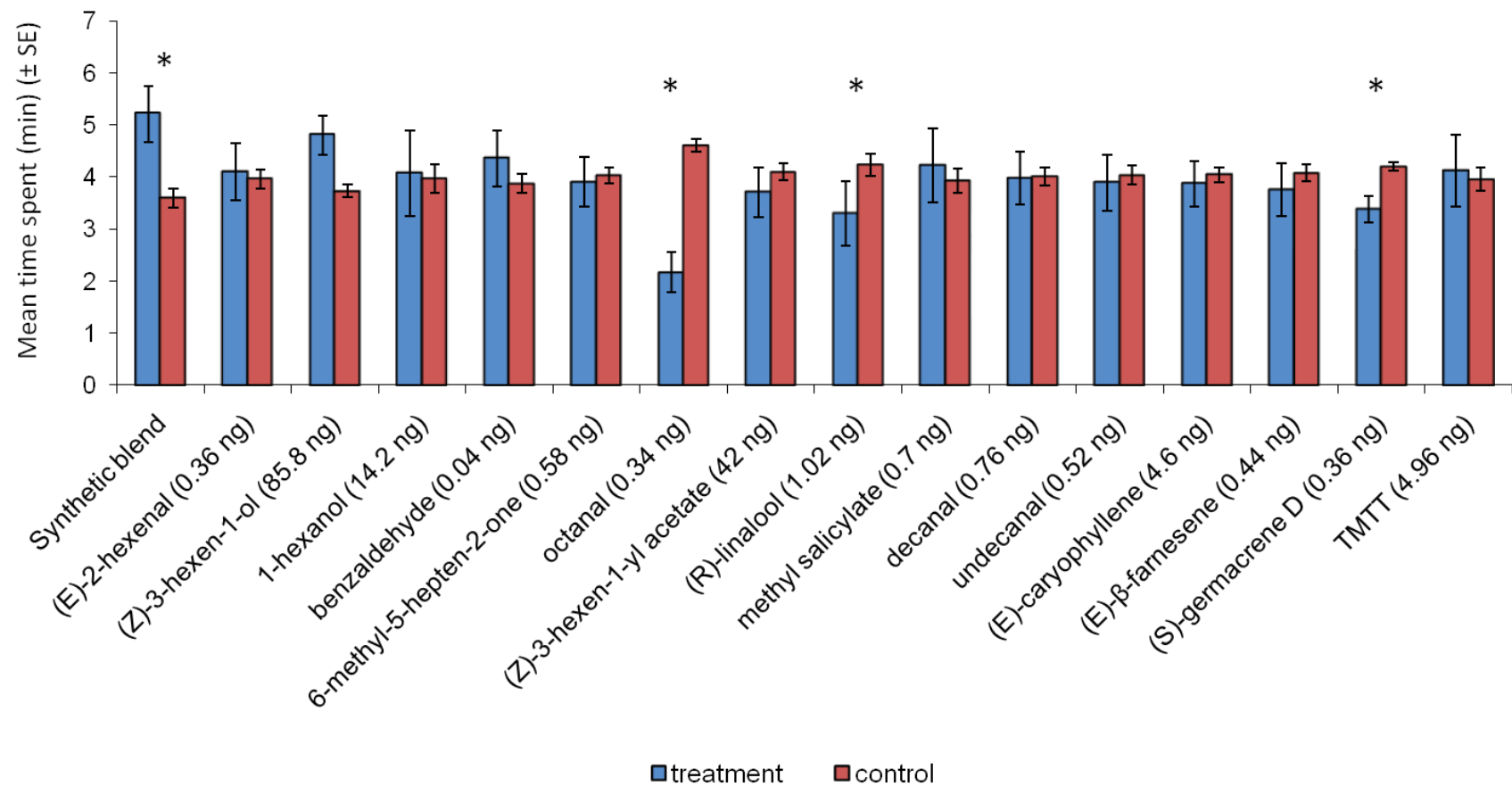


Figure 5.3. Behavioral response of winged virginoparae *Aphis fabae* to individual compounds at same concentration as determined in air entrainment sample ( $n=9-12$ ). \* Significant ( $P<0.05$ ).

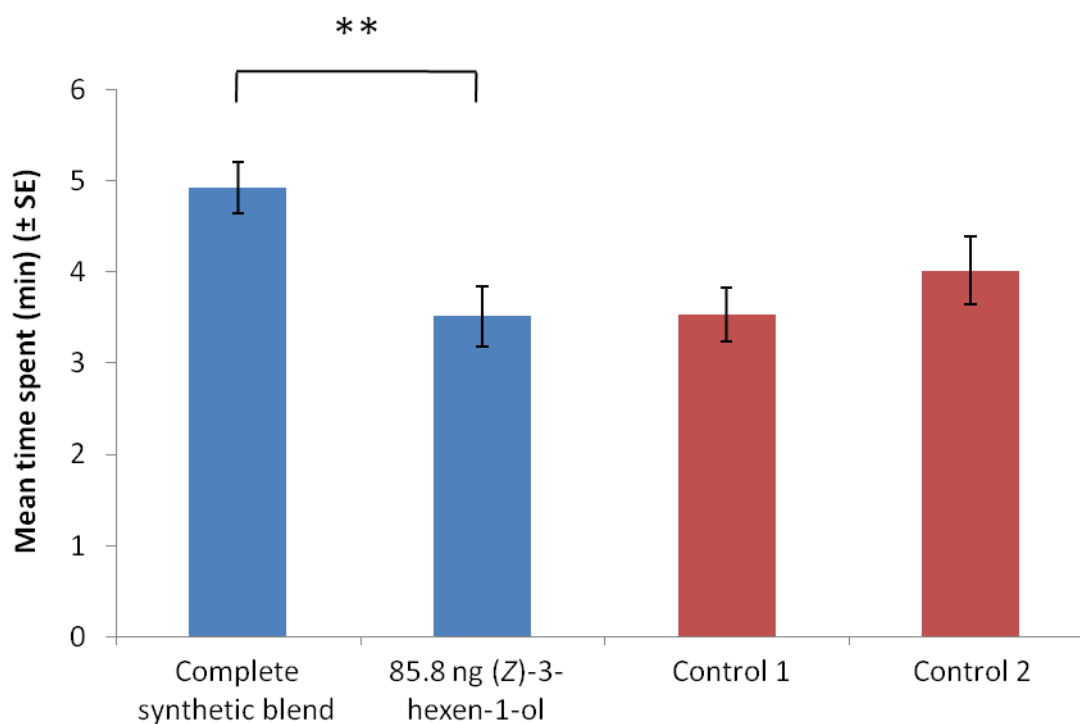


Figure 5.4. Behavioural responses of winged virginoparae *Aphis fabae* to synthetic blend and 85.8 ng (Z)-3-hexen-1-ol when both odour sources present together in the same olfactometer ( $n=18$ ). \*\*Significantly different ( $P < 0.01$ ).

### 5.3.3. Dose response experiments

The behavioural responses of aphids to each compound at each dose tested are shown in Figures 5.5 to 5.19.

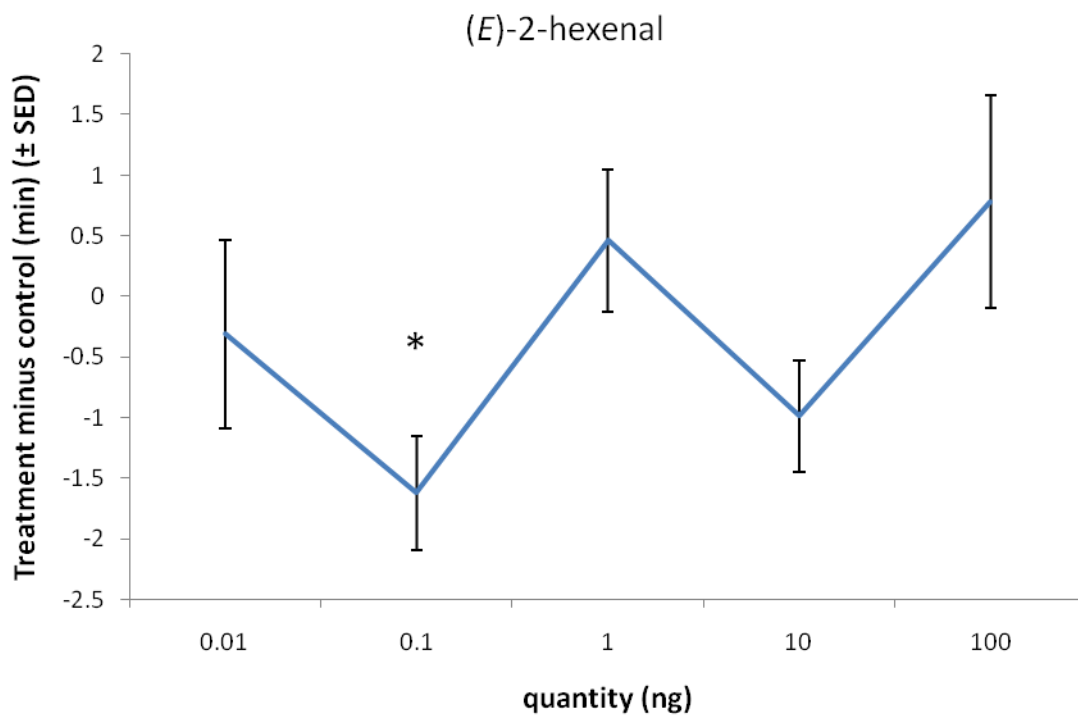


Figure 5.5. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when (E)-2-hexenal was used as odour source ( $n=9-12$ ). \* Significant ( $P<0.05$ ).

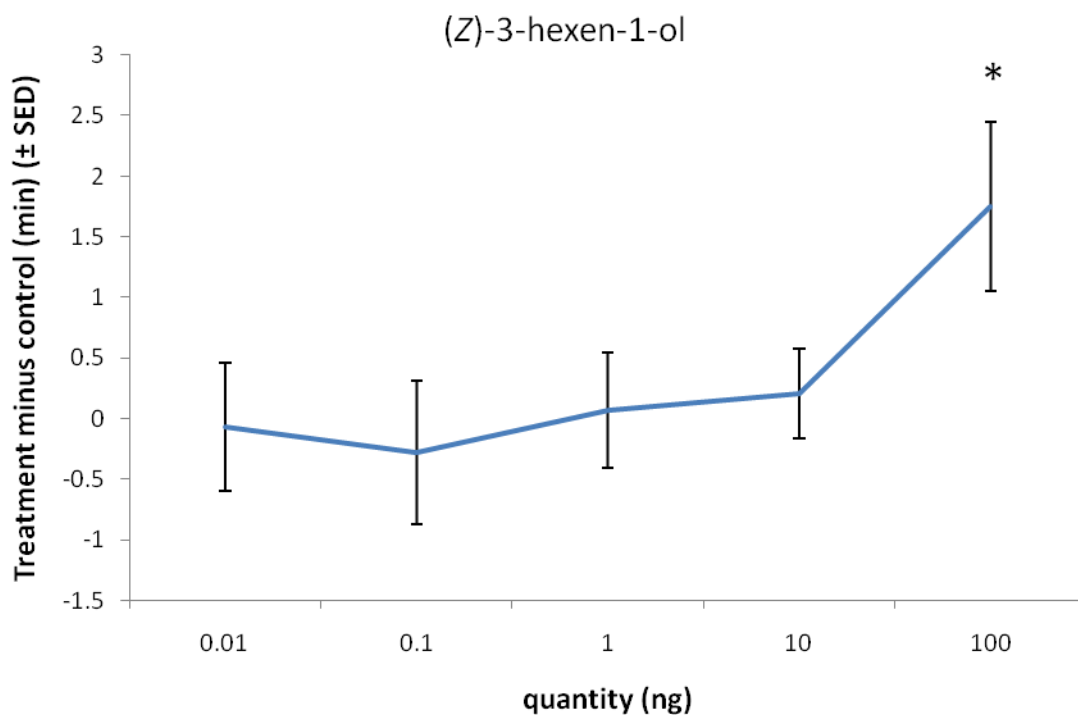


Figure 5.6. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when (Z)-3-hexen-1-ol was used as odour source ( $n=8-12$ ). \* Significant ( $P<0.05$ ).

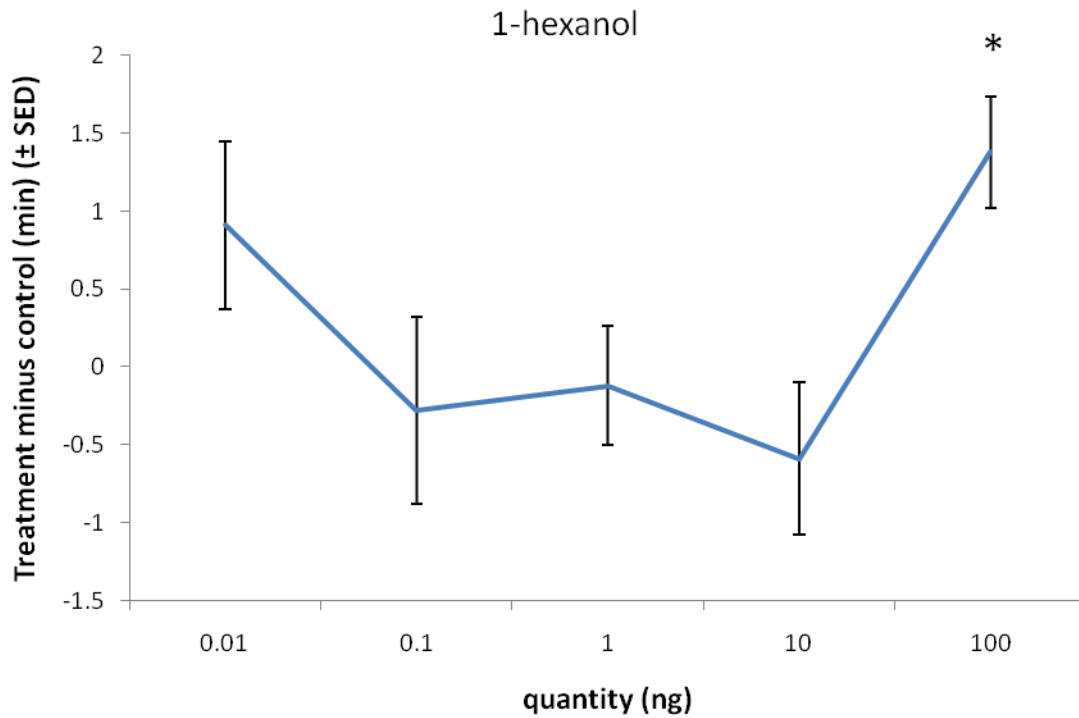


Figure 5.7. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when 1-hexanol was used as odour source ( $n=9-10$ ). \* Significant ( $P<0.05$ ).

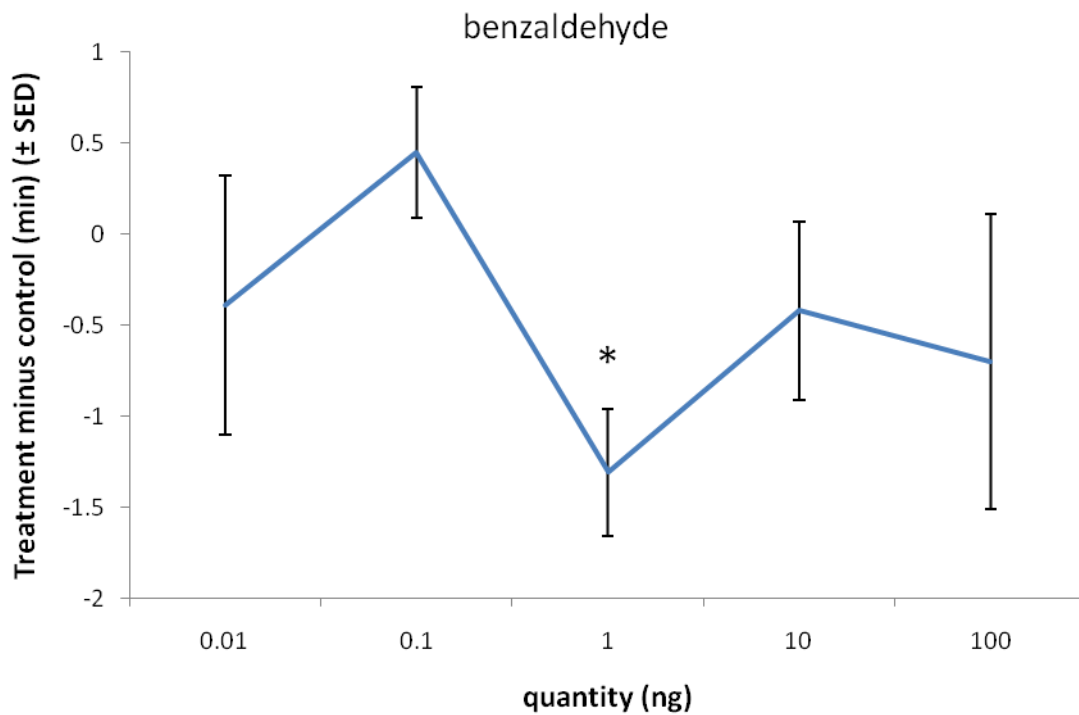


Figure 5.8. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when benzaldehyde was used as odour source ( $n=10-12$ ). \* Significant ( $P<0.05$ ).

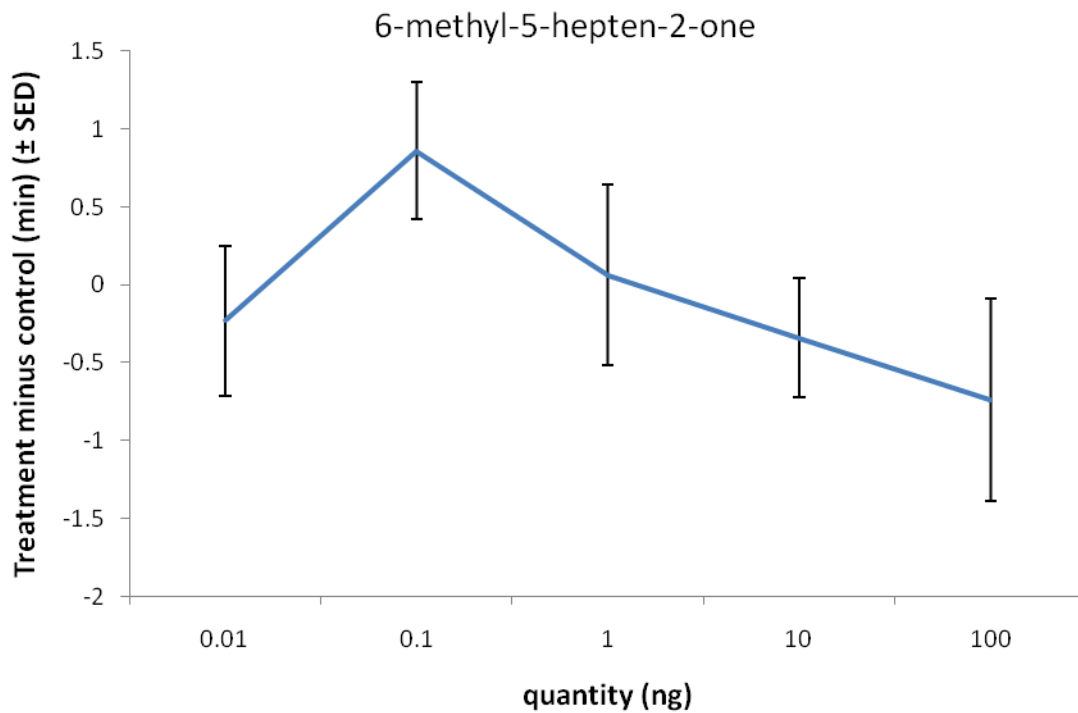


Figure 5.9. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when 6-methyl-5-hepten-2-one was used as odour source ( $n=9-11$ ).

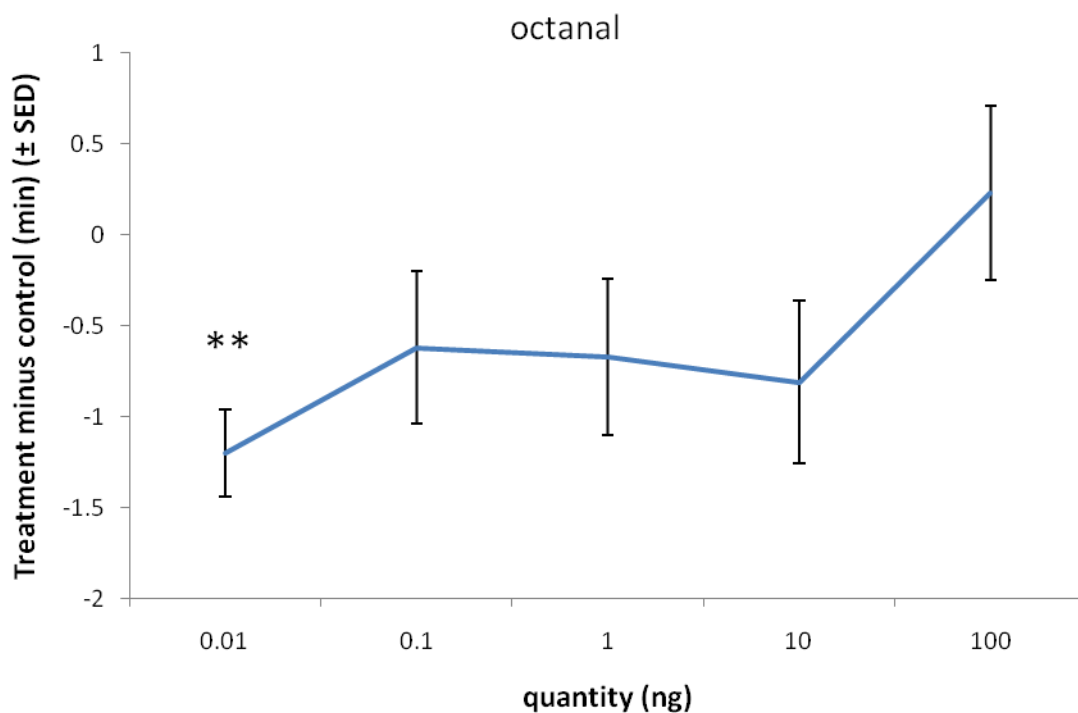


Figure 5.10. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when octanal was used as odour source ( $n=12$ ). \*\* Significant ( $P<0.01$ ).

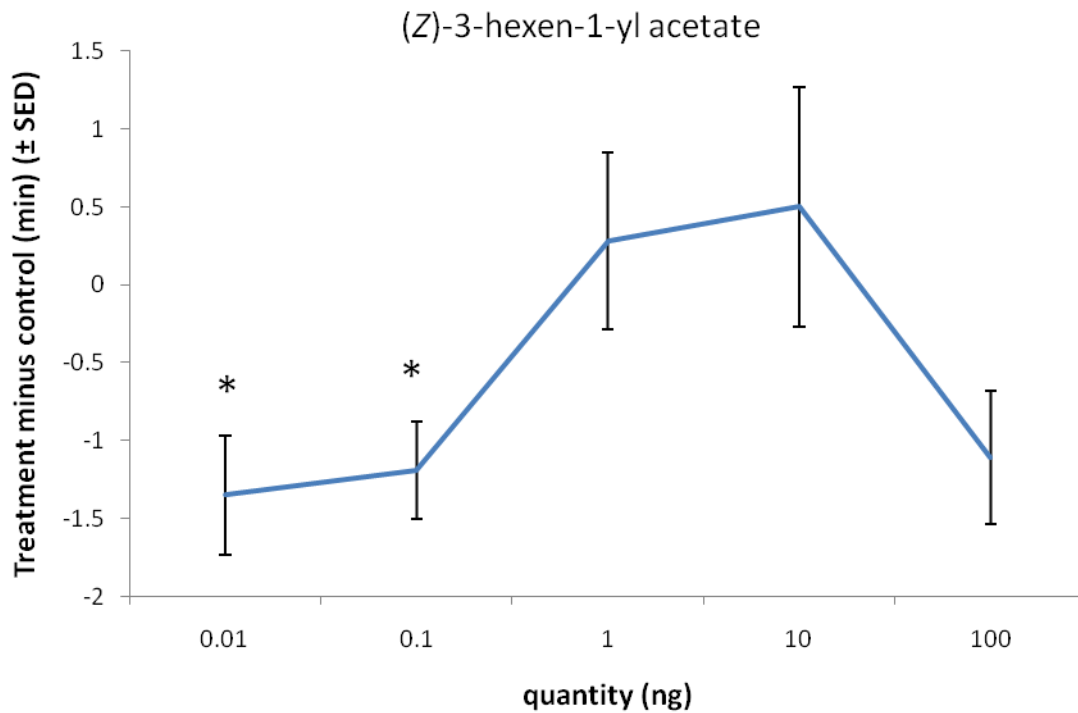


Figure 5.11. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when (Z)-3-hexen-1-yl acetate was used as odour source ( $n=9-12$ ). \* Significant ( $P<0.05$ ).

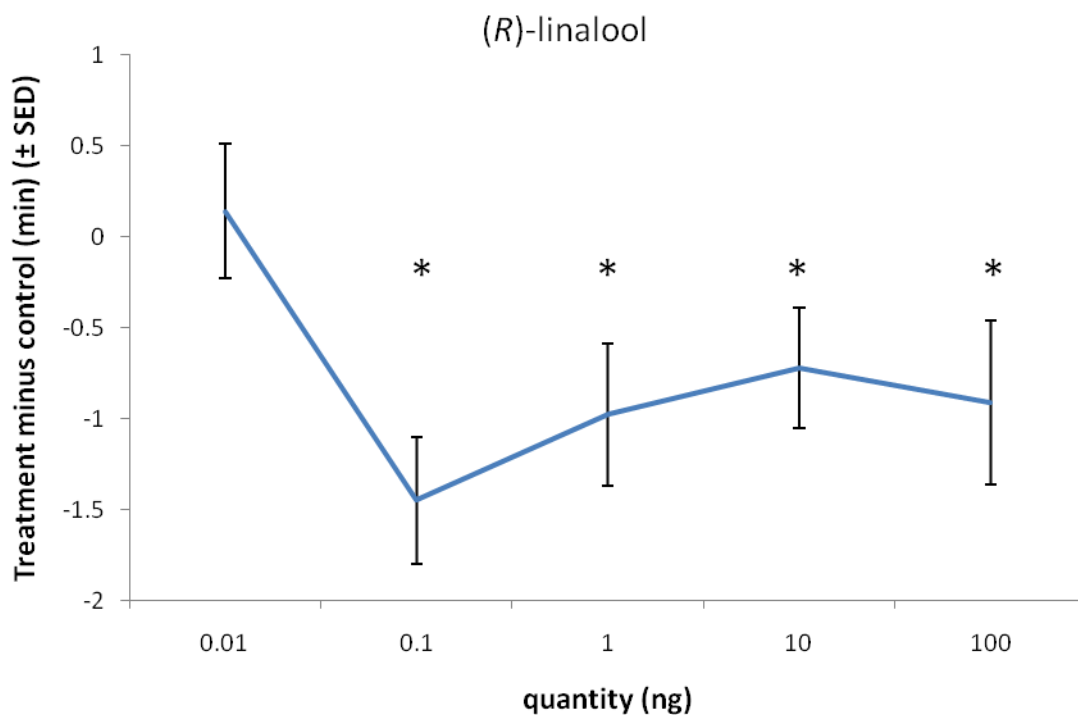


Figure 5.12. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when (R)-linalool was used as odour source ( $n=11-12$ ). \* Significant ( $P<0.05$ ).

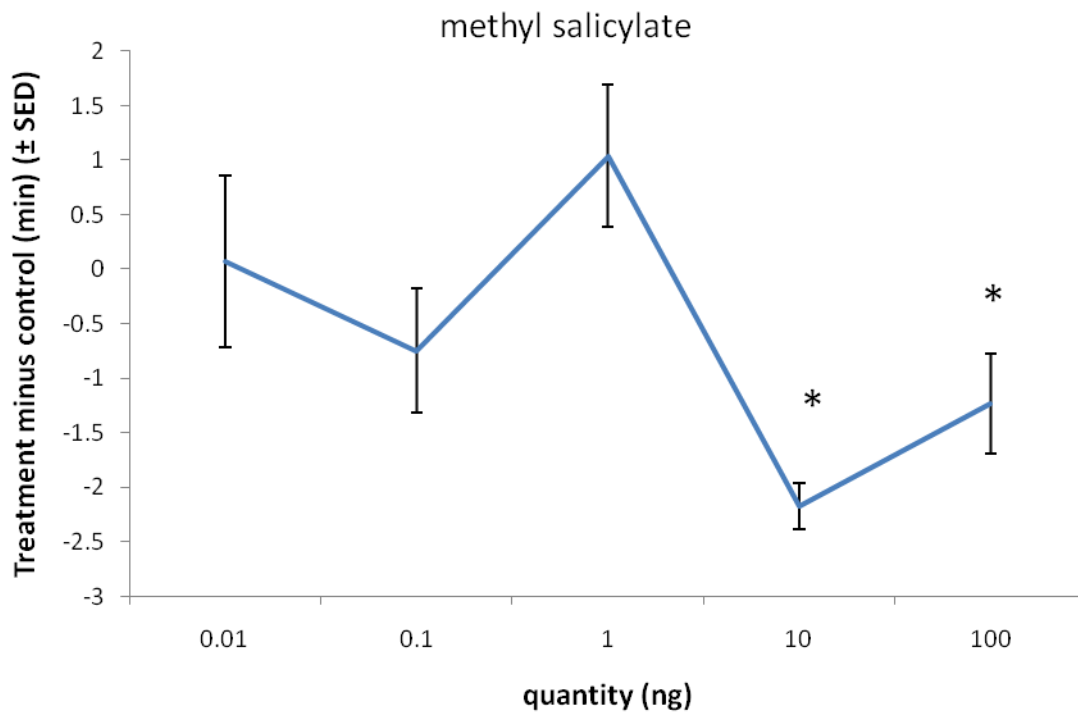


Figure 5.13. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when methyl salicylate was used as odour source ( $n=8-11$ ). \* Significant ( $P<0.05$ ).

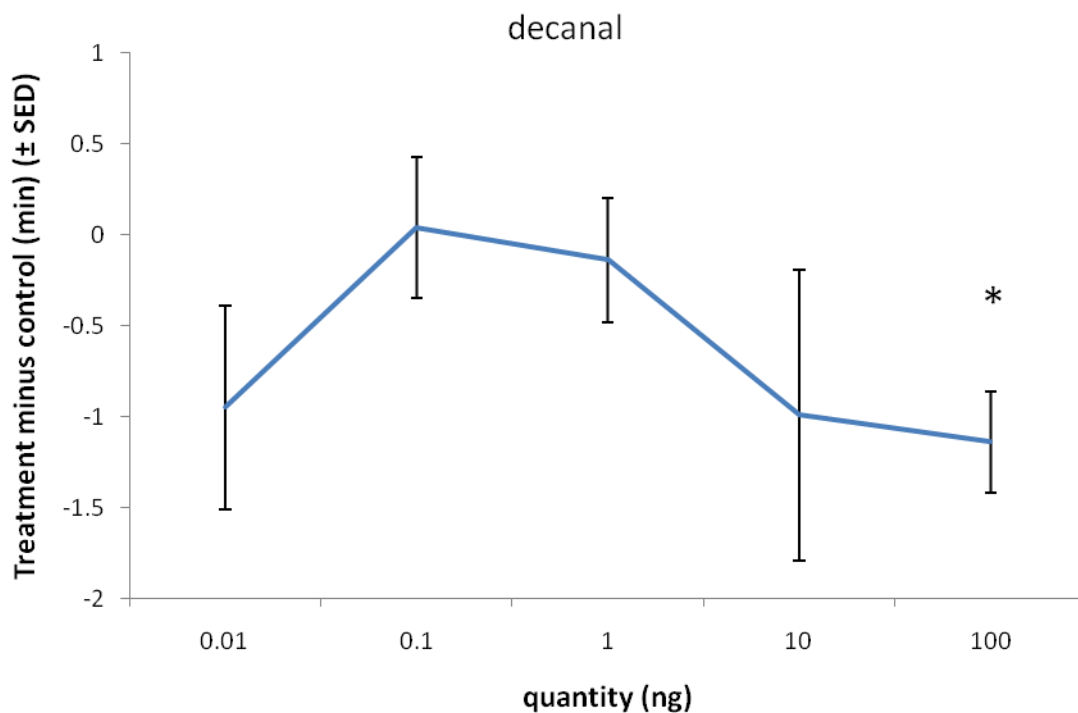


Figure 5.14. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when decanal was used as odour source ( $n=10-12$ ). \* Significant ( $P<0.05$ ).

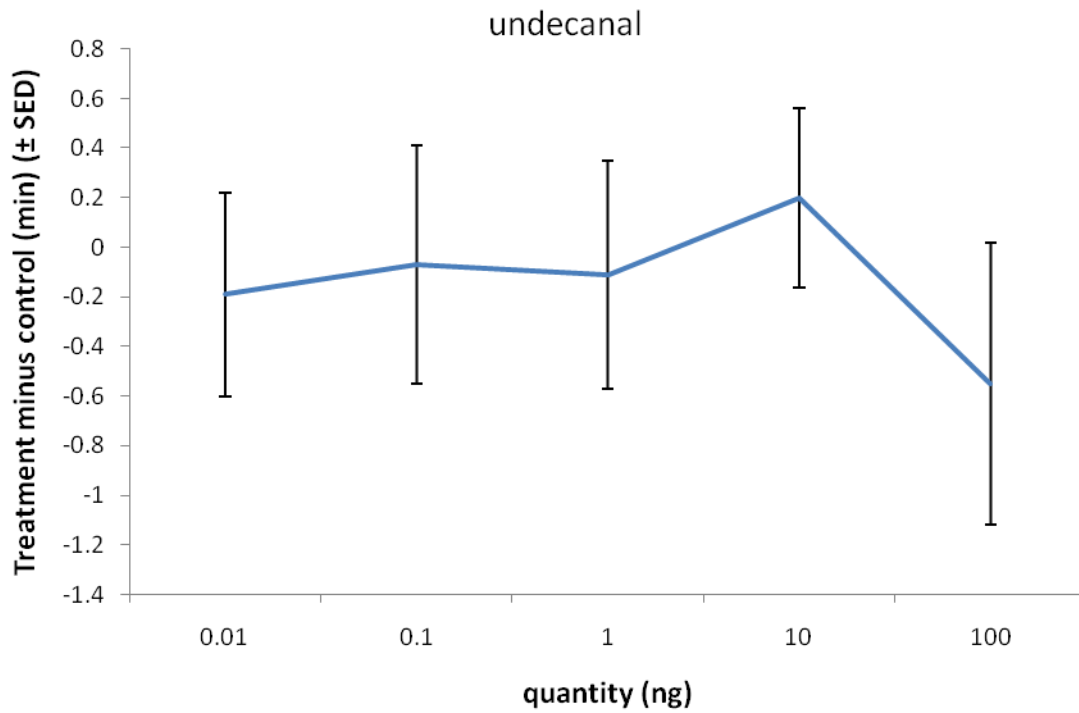


Figure 5.15. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when undecanal was used as odour source ( $n=9-11$ ).

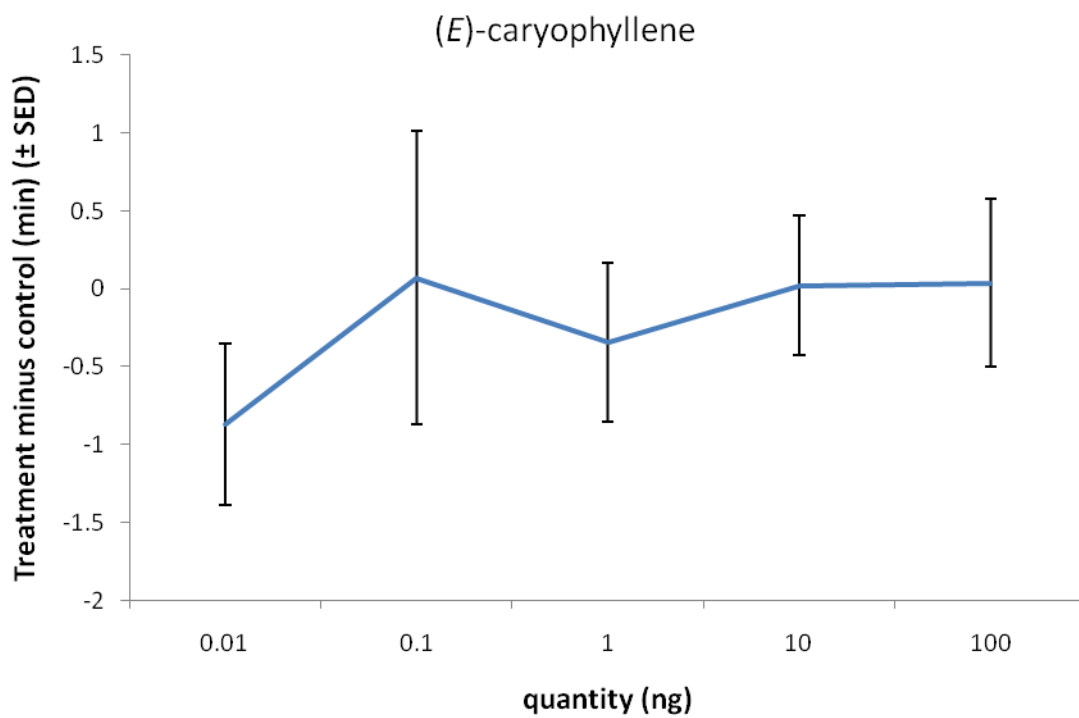


Figure 5.16. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when (E)-caryophyllene was used as odour source ( $n=9-12$ ).

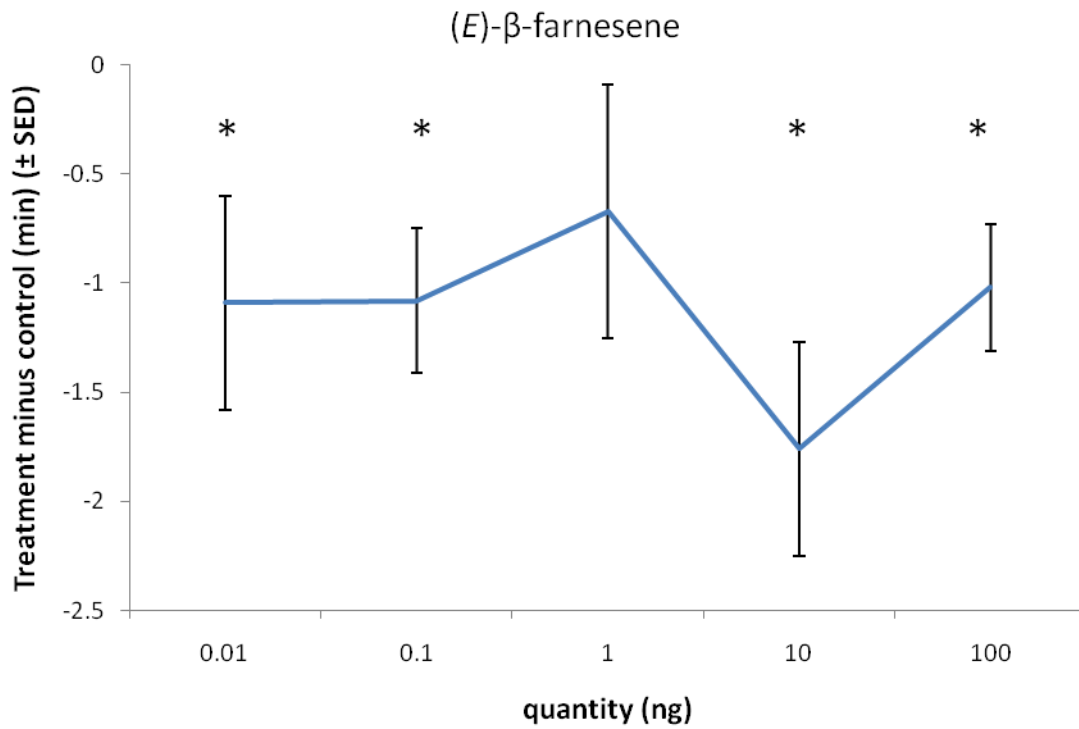


Figure 5.17. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when (*E*)- $\beta$ -farnesene was used as odour source ( $n=8-12$ ). \* Significant ( $P<0.05$ ).

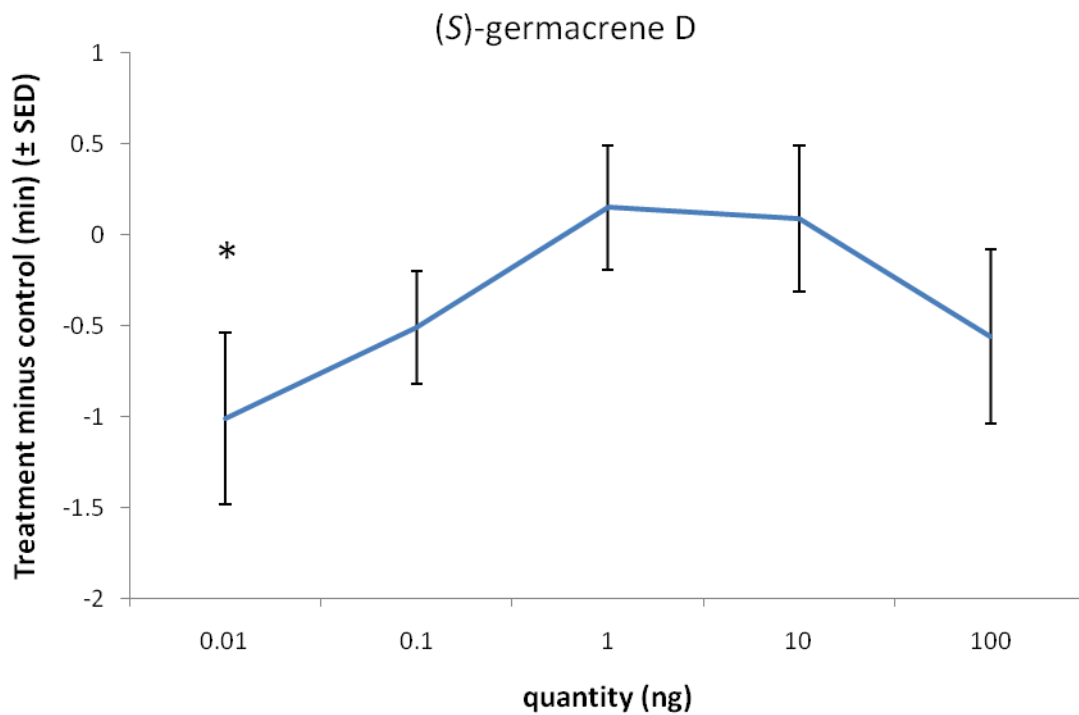


Figure 5.18. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when (*S*)-germacrene D was used as odour source ( $n=11-12$ ). \* Significant ( $P<0.05$ ).

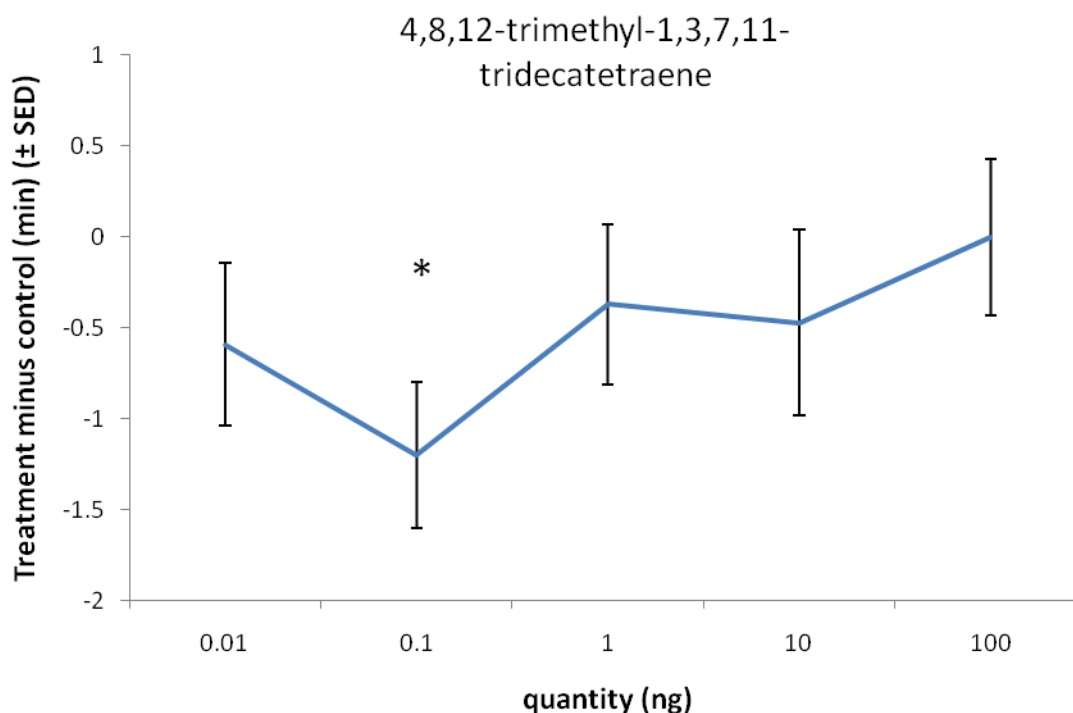


Figure 5.19. Difference between mean time spent by winged virginoparae *Aphis fabae* in treated arm compared to control when (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene was used as odour source ( $n=10-12$ ). \* Significant ( $P<0.05$ ).

(Z)-3-hexen-1-ol and 1-hexanol caused aphids to spend significantly more than 25 % of their time in the treated region of the olfactometer, both at a dose of 100 ng. Consequently, these were combined to make a two-component blend consisting of  $85.8 \text{ ng } \mu\text{l}^{-1}$  (Z)-3-hexen-1-ol and  $14.2 \text{ ng } \mu\text{l}^{-1}$  1-hexanol. Aphids spent significantly more time in the treated region of the olfactometer when this blend was used as the test odour ( $P=0.019$ ) (Figure 5.20). When aphids were offered a choice between this blend and the complete synthetic blend, aphids spent more time in the region corresponding to the complete blend compared to the two-component blend and this difference was significant ( $P=0.05$ ) (Figure 5.21).

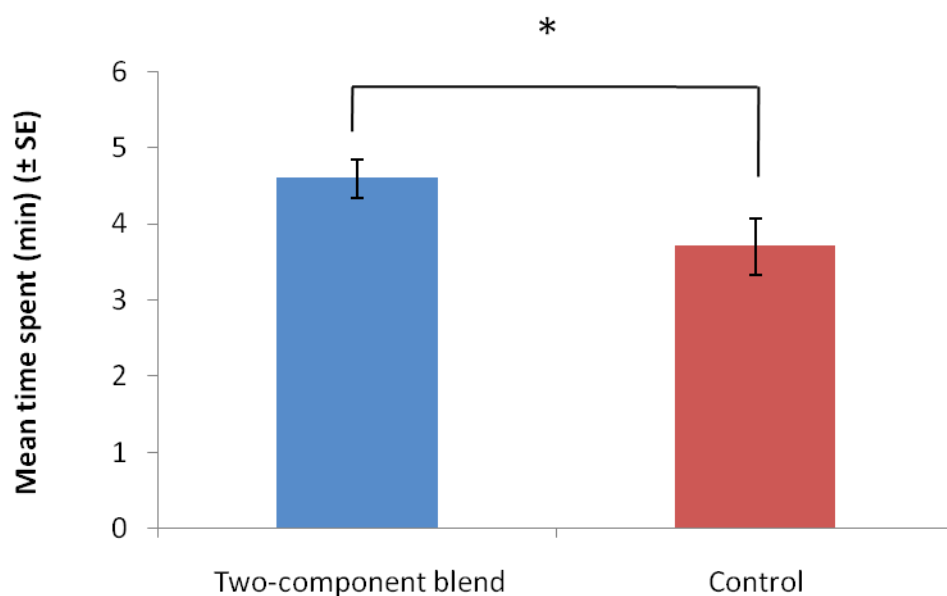


Figure 5.20. Behavioural response of winged virginoparae *Aphis fabae* to a two component blend consisting of 85.8 ng (Z)-3-hexen-1-ol and 14.2 ng 1-hexanol compared to control ( $n=12$ ). \* Significantly different ( $P<0.05$ ).

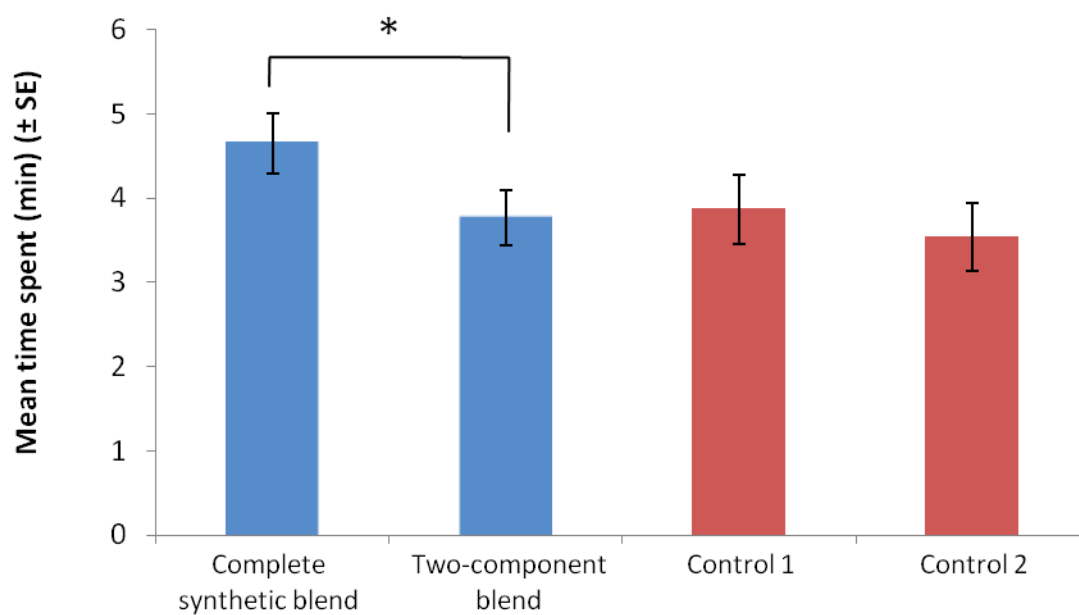


Figure 5.21. Behavioural response of winged virginoparae *Aphis fabae* to complete synthetic blend and two-component blend consisting of 85.8 ng (Z)-3-hexen-1-ol and 14.2 ng 1-hexanol when both odour sources offered together in same olfactometer ( $n=18$ ). \* Significantly different ( $P=0.05$ ).

#### 5.3.4. Context-dependent behavioural responses to octanal, (*R*)-linalool, and (*S*)-germacrene D

A three component blend consisting of octanal, (*R*)-linalool and (*S*)-germacrene D at the same concentration and ratio as in the air entrainment sample did not cause aphids to spend less than 25 % of their time in the treated region of the olfactometer ( $P=0.931$ ). However, 0.34 ng octanal tested on the same day did ( $P=0.019$ ) (Figure 5.22).

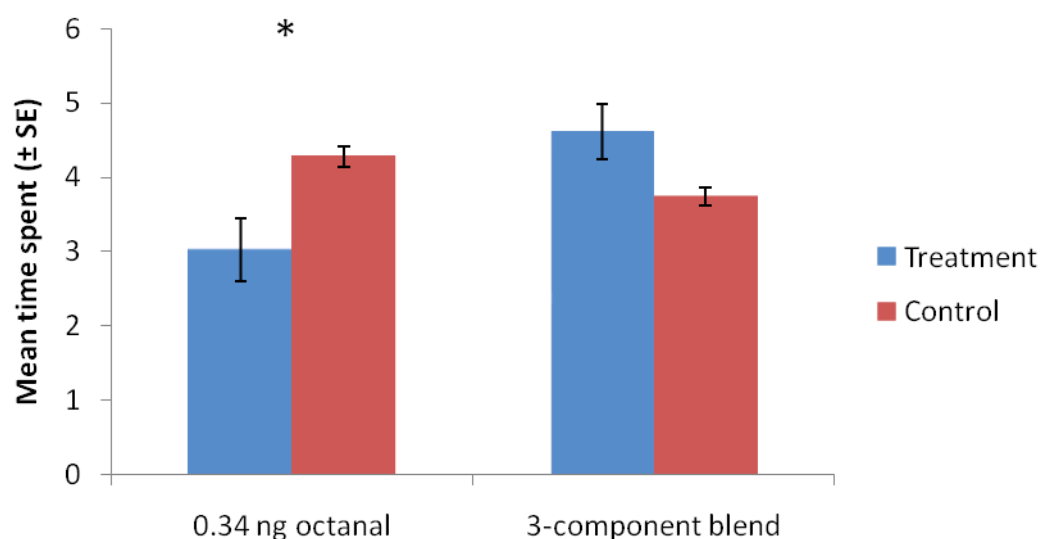


Figure 5.22. Behavioural response of winged virginoparae *Aphis fabae* to 0.34 ng octanal and a 3-component blend consisting of 0.34 ng octanal, 1.02 ng (*R*)-linalool, and 0.36 ng (*S*)-germacrene D ( $n=12$ ). \* Significantly different ( $P<0.05$ ).

When a new blend was constructed with all the original components of the complete synthetic blend except octanal, (*R*)-linalool and (*S*)-germacrene D, the aphids did not show any preference for this reduced blend over the complete synthetic blend ( $P=0.976$ ) but did spend more time in the two treated arms combined than the control arms ( $P=0.047$ ) (Figure 5.23).

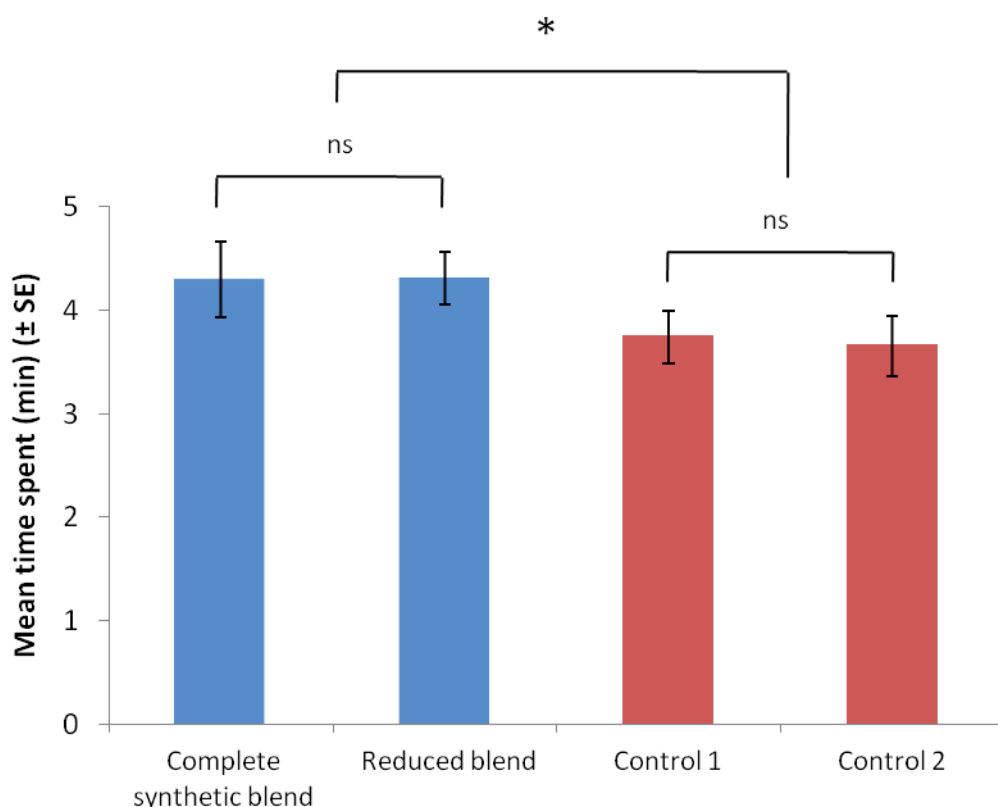


Figure 5.23. Behavioural responses of winged virginoparae *Aphis fabae* to complete synthetic blend and reduced blend consisting of all compounds except octanal, (*R*)-linalool and (*S*)-germacrene D when both presented together in same olfactometer ( $n=18$ ). \* Significantly different ( $P<0.05$ ).

## 5.4. Discussion

### 5.4.1. Behavioural responses to complete synthetic blend

When the complete 15-component synthetic blend comprising all identified compounds from the air entrainment sample was used as the test odour in a four-arm olfactometer aphids spent significantly more than 25% of their time in the treated region of the olfactometer. The amount of time spent in the treated region was similar to that when the air entrainment sample and *V. faba* plant were used as test odours in Chapter 2 (Figure 5.1), suggesting that all volatile compounds essential for host location were present and in the correct quantities in the complete synthetic blend. This was supported by a choice test, which demonstrated that aphids showed no preference for the air entrainment sample over the complete synthetic blend (Figure 5.2).

In Chapter 4, one electrophysiologically active volatile compound could not be identified and so was not included in the complete synthetic blend. As the aphids showed no preference for the air entrainment sample over the synthetic blend, it is unlikely that this unidentified compound is essential for host location in *A. fabae*.

#### **5.4.2. Behavioural responses to individual compounds at same concentration as in air entrainment sample**

A behavioural response to a blend of 15 volatile compounds does not necessarily mean that all 15 are essential for host location. There are many examples in the literature demonstrating host location can be mediated by only a small fraction of the electrophysiologically active volatiles identified in the headspace of an insect's host plant, the other compounds being redundant (e.g. Blight et al. 1997; Nojima et al. 2003a; Birkett et al. 2004; Tasin et al. 2007). Other examples have shown that host location can be mediated by a single compound alone (e.g. Guerin et al. 1983; Hern & Dorn 2004), although such cases usually involve taxonomically specific volatile compounds that are not found in unrelated plant species (Bruce et al. 2005b). As all of the compounds identified in Chapter 4 are ubiquitous plant volatiles and not specific to *V. faba*, it was hypothesised that no single volatile compound would fully account for the behavioural response to the synthetic blend. This hypothesis was tested by determining behavioural responses to each compound individually at the same concentration as in the air entrainment sample.

Aphids did not spend significantly more than 25% of their time in the treated region of the olfactometer for any single compound when tested at the same concentration as in the air entrainment sample. Aphids did, however, spend significantly more time in the treated region when the complete synthetic blend was tested as an odour source on the same days as the individual compounds (Figure 5.3), demonstrating that aphids were responsive on the days of the experiments. As it was not known how aphids would respond to individual compounds, a two-sided t-test was appropriate to test behavioural responses to individual compounds. The complete synthetic blend was already known to cause aphids to spend more time in the treated region (Figure 5.1) and so a one-sided t-test was appropriate to test responses to the complete blend, giving a *P* value of 0.032. Had this been a two-sided test, the response would not have been significant to the 0.05 significance level. In light of this fact, all test compounds were also compared using a one-sided t-test, to allow for accurate comparison of

behavioural responses. Any conclusions made from this test were regarded as purely tentative and merely used to put compounds forward as candidates for choice tests where responses would be compared directly with the response to the synthetic blend in the same olfactometer. Using this kind of test, (Z)-3-hexen-1-ol elicited a significant behavioural response when tested alone at a dose of 85.8 ng ( $P=0.047$ ). However, when a choice test was carried out, aphids preferred the complete synthetic blend over this compound alone (Figure 5.4), indicating that host location in *A. fabae* is not mediated by this single compound alone.

(Z)-3-Hexen-1-ol, like all of the electrophysiologically active volatiles identified in Chapter 4, is an ubiquitous plant volatile, emitted by a diverse range of plant species and not specific to *V. faba* (Knudsen et al. 1993). It is therefore not surprising that host location is not mediated by this compound or any of the other compounds alone as they would not constitute a host-specific volatile cue. Visser (1986) suggested that insects can recognise their hosts using ubiquitous volatiles by recognising host-characteristic ratios of volatile compounds. In light of the results of the present study, this hypothesis seems more likely than *A. fabae* uses a single volatile compound to locate its host.

(Z)-3-Hexen-1-ol has been reported previously in the headspace of undamaged *V. faba* plants (Colazza et al. 2004; Pareja et al. 2009) but has been shown to be produced in greater quantities following mechanical damage to the plant (Agelopoulos et al. 1999b). As *A. fabae* does not respond behaviourally to the odour of damaged *V. faba* leaves (see Chapter 2) (Nottingham et al. 1991), it is surprising that this compound, associated with mechanical damage, should cause the aphids to spend more time in the treated region of the olfactometer. It is possible that this volatile compound reflects the physiological status of the plant in some other way in addition to reflecting mechanical damage so that high levels of this compound indicate a particularly suitable host. *Aphis fabae* occurs on its host in very large numbers (Hodgson & Godfray 1999; Bruce, personal communication) causing significant stress to the plant. This volatile may be associated with such stress and used by *A. fabae* to locate hosts whose defences are being overwhelmed by conspecifics. This could only be determined by entraining plants with different levels of infestation to determine the quantity of (Z)-3-hexen-1-ol produced and seeing if this correlates with behavioural responses to the same plants. This is beyond the scope of the present study but would provide an interesting starting point for future work into olfactory cues used to indicate host suitability.

### 5.4.3. Dose response experiments

Dose response experiments showed that 12 out of 15 compounds were behaviourally active at at least one of the doses tested. The surprising finding was that these single compounds mostly elicited negative behavioural responses (i.e. they caused aphids to spend less time in the treated region of the olfactometer). The doses used were chosen because they corresponded roughly to the range of quantities present in 10  $\mu$ l of the air entrainment sample used in behavioural bioassays and so are physiologically relevant. Several of the compounds were behaviourally active at the lowest dose tested of 0.01 ng. These were octanal, (*Z*)-3-hexen-1-yl acetate, (*E*)- $\beta$ -farnesene, and (*S*)-germacrene D. To the author's knowledge, these represent the lowest doses for which any plant volatile has been shown to elicit significant behavioural responses from a species of aphid. The quantities of the test compounds applied to the filter paper triangles used in experiments do not equate to the quantities the aphids are exposed to, which are actually even lower. It is difficult to estimate actual quantities encountered by the aphids because of differences in molecular weight and volatility of different compounds. Concentrations may also vary throughout the duration of the bioassay as the sample gradually evaporates.

Only two compounds when presented alone caused the aphids to spend more than 25% of their time in the treated region of the olfactometer. These were 1-hexanol and (*Z*)-3-hexen-1-ol, both of which only elicited a significant response at a dose of 100 ng. The 14.2 ng of 1-hexanol tested in experiment 3 (Figure 5.3) and present in the air entrainment sample and synthetic blend was too low a dose to elicit a significant behavioural response with a 5 % significance threshold. It was hypothesised that by combining this compound with (*Z*)-3-hexen-1-ol, the summed response to the two compounds may elicit a behavioural response similar to that elicited by the complete synthetic blend. In this way, the response to the blend could simply be the sum of the responses to two of its compounds. To test this hypothesis, the two compounds were combined in a two-component blend and tested for behavioural activity. A significant behavioural response to the blend was observed (Figure 5.20) but when offered a choice the aphids preferred the complete synthetic blend over the two-component blend and this preference was significant (Figure 5.21). Host location may be partly mediated by these two compounds but it is clear that other compounds must also play a role. As (*Z*)-3-hexen-1-ol and 1-hexanol were the only compounds to elicit a positive behavioural response at any dose tested, the response to the blend is unlikely to be simply the sum of responses to

individual compounds. A more complex synergistic effect is more likely and investigating whether or not this is the case could form an interesting basis for future work.

Methyl salicylate has previously been shown to cause aphids to move away from its source in a linear track olfactometer (Hardie et al. 1994a), supporting the results obtained in the present study, where it elicited a negative behavioural response at doses of 10 ng and 100 ng. It has been suggested that this compound is associated with plant defence, produced in elevated quantities when the plant is under attack from herbivores (Hardie et al. 1994a), and used by aphids to avoid over-exploited hosts. However, it is also produced by undamaged *V. faba* plants constitutively in small quantities (Chapter 4) (Pareja et al. 2009). This could explain why aphids only respond negatively to this compound at the relatively high doses of 10 ng and 100 ng and not at the lower doses which may correspond to levels emitted by healthy, undamaged plants which are not infested with herbivores. (*E*)- $\beta$ -farnesene is the aphid alarm pheromone and causes aphids to disperse (Montgomery & Nault 1977; Pickett et al. 1992). The present study supports this as (*E*)- $\beta$ -farnesene caused aphids to spend significantly less than 25 % of their time in the treated region of the olfactometer at all doses tested with the exception of the intermediate dose of 1 ng, although the time spent in the treated region of the olfactometer was less than 25 % at this dose. A similar lack of a behavioural response was also observed at a dose of 0.44 ng in experiment 3. This could suggest that around these concentrations (*E*)- $\beta$ -farnesene is not behaviourally active to *A. fabae*, perhaps because this is approximately the dose at which it is emitted by plants. Combined with the use of alarm pheromone inhibitor volatiles, this could help prevent *A. fabae* from being alarmed by volatiles from its host plant.

(*E*)-2-Hexenal, benzaldehyde, octanal, (*R*)-linalool, decanal, (*S*)-germacrene D and TMTT also all caused the aphids to spend significantly less than 25% of their time in the treated region of the olfactometer at one or more doses tested. This is the first time such responses to these compounds have been observed in *A. fabae*, though all have been found to be behaviourally active to other species of aphid (Quiroz & Niemeyer 1998; Bruce et al. 2005a; Guo & Liu 2005; Pope et al. 2007; Halbert et al. 2009). The fact that so many compounds caused *A. fabae* to spend less than 25% of their time in the treated region of the olfactometer suggests that *A. fabae* may use general plant volatiles as non-host cues. Each of these volatile compounds would normally be encountered in the context of the complete blend if the aphid were exposed to volatiles from a *V. faba* plant and thus the blend is of crucial importance.

The presence of some of these plant volatiles in the absence of the others would indicate the volatiles did not originate from the host plant and would therefore constitute a non-host cue. This could explain why the aphids responded the way they did. To fully test this hypothesis, however, it would need to be confirmed that non-host plants do not produce all of these same volatile compounds so that the presence of an individual or a number of these compounds in the absence of all the others is a *reliable* non-host cue. This would be a time consuming task and is beyond the resources of the present study. However, the findings here offer a valuable starting point for future work aiming to investigate this idea further.

The question remains as to why the aphids generally only respond to these volatiles when they are present at low doses. Only decanal and (*R*)-linalool elicited negative behavioural responses at the highest dose tested of 100 ng, whereas (*E*)-2-hexenal, benzaldehyde, octanal, (*Z*)-3-hexen-1-yl acetate, (*S*)-germacrene D and TMTT only elicited significant behavioural responses at lower doses. This suggests that these compounds may have different ecological meanings at different doses. Volatiles are often released at higher rates when a plant is subject to herbivory or under physiological stress. (*E*)-2-hexenal and (*Z*)-3-hexen-1-yl acetate are both green leaf volatiles and are often associated with mechanical damage to plants, when they are released in greater quantities (Saijo & Takeo 1975; Agelopoulos et al. 1999b). Benzaldehyde, germacrene D and TMTT have all been shown to be produced in elevated quantities by some plant species which are subject to herbivory (Rose & Tumlinson 2004; Johné et al. 2006; Li et al. 2006). It is currently unknown whether *V. faba* produces these compounds in elevated quantities when subject to herbivory or physiological stress, or when produced at high levels reflect some other physiological condition of the plant, but if so it could explain the different behavioural responses to these compounds at elevated levels. At low levels, these compounds could be used as non-host cues whereas at high levels they may be used to provide information on the physiological status of the host.

#### **5.4.4. Context-dependent behavioural responses to volatile compounds**

The hypothesis that general plant volatiles may be used as non-host cues unless presented together in the correct blend is further supported by experiments involving octanal, (*R*)-linalool, and (*S*)-germacrene D. When these three compounds were tested individually at the same concentration as in the air entrainment sample aphids spent significantly less than 25 % of their time in the treated region of the olfactometer (Figure 5.3). However, when these three

compounds were combined into a three-component blend aphids actually spent more time in the treated region of the olfactometer, but this was not significant (Figure 5.22). This finding was supported by the fact that when a new blend was made up comprising all compounds from the complete synthetic blend except these three, aphids showed no preference for the reduced blend over the complete blend (Figure 5.23). This suggests that the response to individual compounds is different when in the context of the blend. Individually, these three compounds are avoided by *A. fabae* whereas together they are not. This lends further weight to the hypothesis that *A. fabae* uses these ubiquitous plant volatiles as non-host cues when encountered in the absence of other *V. faba* volatiles.

### 5.4.5. Conclusions

The work carried out here has made considerable progress towards understanding how *A. fabae* uses olfaction to recognise its host, *V. faba*. Aphids did not show any preference for the air entrainment sample over the complete synthetic blend, suggesting that all volatile compounds used in host location have been successfully identified and quantified. Host location could not be explained as a behavioural response to any single compound presented individually. Only (Z)-3-hexen-1-ol elicited a similar behavioural response to the complete synthetic blend when tested alone at the same concentration as in the air entrainment sample. However, the blend was preferred over this compound when both were offered together in an olfactometer choice test. Dose response experiments suggested that 1-hexanol may also contribute to the positive behavioural response to the complete synthetic blend as it elicited a positive behavioural response at a dose of 100 ng. A two-component blend consisting of (Z)-3-hexen-1-ol and 1-hexanol was found to cause aphids to spend significantly more than 25 % of their time in the treated region of the olfactometer. However, in a choice test the complete synthetic blend was preferred over this two-component blend. This suggests that host-location is not mediated by these two compounds alone.

Some individual compounds elicited responses that suggested they were repellents. The context in which volatiles were perceived was of crucial importance because a totally different response was observed to complex blends of host volatiles. This suggests that odour quality is an emergent property dependent on particular combinations of volatiles rather than a simple summation of responses to individual components. This perhaps is a general principle that could apply more widely to the animal kingdom. While the use of general plant

volatiles has been implicated in host-location (Visser 1986; Bruce et al. 2005b), their use in non-host avoidance has received relatively little attention in the literature (Bruce et al. 2005b). This hypothesis is compatible with the hypothesis that *A. fabae* responds to ratios of host volatiles and together could allow *A. fabae* to accurately recognise its host and avoid non-hosts.

Future work aiming to test these hypotheses would look further at how *A. fabae* responds to compounds within the context of different blends and how it responds to different ratios of volatile compounds. When attempting to identify the mechanism of host recognition and non-host avoidance, two facts must be determined. The first is that the cue in question is *characteristic* of the host. This is a relatively straightforward task and can be determined by entraining a large number of plants and characterising the volatile profiles of each to determine if there is a consistent, species-characteristic cue. The second task is to determine if the cue is *specific* to the host. It is worth remembering that *A. fabae* is capable of developing on a range of different host plants (Cammell 1981; Fernandez-Quintanilla et al. 2002). *Aphis fabae* may use a blend associated with all its potential hosts. Alternatively it may employ different blends to recognise different hosts or a small number of different blends that are each characteristic of a small number of its potential hosts. Determining whether the host blend is restricted to *V. faba* or to the range of hosts exploited by *A. fabae* would be a difficult and time consuming task as it requires the entrainment of a large number of host and non-host plants. This is beyond the resources of the present study. A review of the literature would not be sufficient as studies of volatiles emitted by plants usually only attempt to identify a small number of volatiles which are of relevance to the study, for example those that are electrophysiologically active to a particular insect pest and so not all volatiles may be reported. Valuable information can still be obtained by addressing the first question; that is, are volatiles produced consistently. Do volatiles vary from plant to plant and at different times of day and are they produced in species-characteristic ratios? These questions will be addressed in Chapter 6 which looks at the plant-to-plant and diurnal variation in volatile compounds emitted by *V. faba*.

# CHAPTER 6. INTRA-SPECIFIC AND DIURNAL VARIATION IN VOLATILE EMISSIONS OF *Vicia faba*

## 6.1. Introduction

In Chapter 4, fifteen volatile compounds were identified from the headspace of undamaged *V. faba* plants which were electrophysiologically active to *A. fabae*. None of the identified volatile compounds were specific to *V. faba*, instead being produced by a wide range of plant species. In Chapter 5 it was shown that no single volatile compound could fully account for the behavioural response to the complete synthetic blend. It was hypothesised that *A. fabae* may recognise its host using species-characteristic ratios of ubiquitous plant volatiles as suggested by Visser (1986) rather than using taxonomically characteristic volatile compounds. To determine whether this hypothesis would be feasible, it must first be determined whether or not ratios of volatiles emitted by *V. faba* constitute a species-characteristic cue. If ratios vary widely from plant to plant, it is unlikely that ratios of volatiles could play a role in host location. If, however, there is a degree of consistency then this would suggest it is possible for *A. fabae* to recognise its host in this way

Volatile compound emission by plants can reflect stage of development (Engel et al. 1988; Agelopoulos et al. 2000; Dudareva et al. 2000; Shiojiri & Karban 2006), season (Hatanaka et al. 1976a; Mita et al. 2002; Bai et al. 2006; Holzinger et al. 2006) as well as environmental conditions such as temperature, humidity, light quality, nitrogen availability, and humidity (Johnson et al. 1999; Gouinguene & Turlings 2002; Schmelz et al. 2003; Vallat et al. 2005; Ferreyra et al. 2006). Volatile blends emitted by plants may also show large intra-specific variation due to natural genetic variation between individuals. For example, Degen *et al.*, (2004) found that 31 maize inbred lines varied dramatically in the total quantity of volatiles emitted and also showed qualitative differences in volatile blends as well as differences in volatiles induced by herbivory. Several other studies have yielded similar results, showing that genetic variation accounts for large variability in volatile blends emitted by plants (Takabayashi et al. 1991; Loughrin et al. 1995; Lou et al. 2006; Hare 2007; Delphia et al.

2009). Variation in volatile emission has also been found in a single cultivar of *V. faba* (cv. Hangdown Grunkernig) (Pareja et al. 2009), though consistency in ratios of volatiles was not investigated. Some studies have suggested that the ratios of volatiles may be consistent from plant to plant in some plant species. Agelopoulos *et al.*, (2000) entrained a number of potato plants and found that the ratios of major compounds did not vary much from plant to plant. Evidence has been found to suggest that the Colorado potato beetle, a pest of this plant species, may use ratios of volatiles to recognise its host (Visser & Ave 1978) and the findings of Agelopoulos *et al.* (2000) lend weight to this hypothesis. A lack of statistical analysis to support their findings, however, means the conclusions of this study must be considered tentative.

As well as plant-to-plant variation, host-seeking insects are also confronted with diurnal variation in the volatile blends emitted by their host plants. Several studies have shown that the quantities of volatiles emitted by plants vary according to time of day and some have found significant qualitative differences, with different ratios of volatiles being emitted at different times of day (Loughrin et al. 1991; Loughrin et al. 1994; Agelopoulos et al. 2000; Casado et al. 2006; Chamberlain et al. 2006). Some insect species are more responsive to plant odours and exhibit different behaviours at different times of the day (Lazzari et al. 2004). For example, the potato aphid (*Macrosiphum euphorbiae*) responds more strongly to the odour of its host plant during daylight than at night (Narayandas & Alyokhin 2006). Diurnal variation in behaviour has also been shown for the damson-hop aphid, *Phorodon humuli* when it was found that the median period of flight activity was from 2 h after sunrise until 30 min before sunset (Campbell & Muir 2005). In *A. fabae*, peak flight activity occurs during mid-morning and early afternoon and flight typically lasts for up to two hours before alighting occurs (Cammell 1981). Behavioural responses of host-seeking aphids may therefore be ‘tuned’ to the volatile profile associated with their host at the time of day in which they are most likely to alight on a potential host plant. When attempting to determine the optimum blend which an insect uses to locate its host it may therefore be important to consider the diurnal variation in volatile blends emitted.

In most of the above studies concerned with intra-specific and diurnal variation in volatile emission by plants, the volatiles chosen for study were those which occurred in the greatest quantities in the headspace of the plants being studied. In Chapter 3 it was shown that not all of the compounds in the air entrainment sample which were electrophysiologically active to

*A. fabae* were those which were present in the greatest quantities. Indeed, some of the electrophysiologically active volatile compounds were collected in relatively small amounts compared to other compounds which did not show electrophysiological activity. When attempting to determine intra-specific and diurnal variation of ratios of plant volatiles in the context of host location, it is therefore important to consider those which are electrophysiologically active to the host-seeking insect in question. The work carried out in the previous chapters has therefore enabled selection of volatiles relevant to host location by *A. fabae* with which to study intra-specific and diurnal variation in emission.

### **6.1.1. Aims and objectives**

The aim of this chapter was to determine the intra-specific and diurnal variation of *V. faba* volatile compounds electrophysiologically active to *A. fabae*. To achieve this aim, the day was divided into four, six-hour periods (04.00 h to 10.00 h, 10.00 h to 16.00 h, 16.00 h to 22.00 h, and 22.00 h to 04.00 h) and air entrainments of 16 plants were carried out over these periods of time. Quantities of each of the 15 volatile compounds at each time of day for each plant were determined and compared. It was hypothesised that the quantities of some compounds would correlate and therefore collected in consistent ratios.

## **6.2. Materials & methods**

### **6.2.1. Plants**

All plants used were glasshouse-grown in rooms maintained at 20 °C. No other plants except those used in experiments were grown in the glasshouse room. Faba bean plants (*Vicia faba* cv. Sutton dwarf) were grown individually in 7.5 cm pots in Rothamsted prescription mix compost (Petersfield Products, Leics, UK) and used in experiments when the plants were three weeks old before the stage of inflorescence emergence.

### **6.2.2. Air entrainment**

In Chapter 2, volatiles were collected from plants enclosed in glass vessels. However, for the experiments in this chapter, a large number of replicates were required which meant that the use of glass vessels was not practical. The use of PET (polyethylene terephthalate) oven bags

provides a cheap, disposable alternative to glass vessels that is appropriate for dynamic headspace sampling of intact plants (Stewart-Jones & Poppy 2006) and has been used in a number of studies (Turlings et al. 1998; Dufa et al. 2004; Huber et al. 2005). This method also has the advantage of making it easier to avoid causing damage to the plant during entrainment which can sometimes occur when placing a glass vessel over a plant and can result in qualitative and quantitative changes in blends emitted by the plant (Agelopoulos et al. 1999b). To minimise damage caused to plants during entrainments, and to facilitate replication, PET oven bags were used for all entrainments of plants in this chapter.

To avoid causing damage to the plant when transporting them, plants were entrained in the same glasshouse they were grown in. The pot was covered in aluminium foil which had been previously rinsed with acetone and baked in an oven maintained at 150 °C for 2 hours. It was arranged around the pot so that only a minimal gap around the stem of the plant was exposed. A PET (polyethylene terephthalate) oven bag (250 mm x 380 mm, poly-lina®, Telford, UK), which had been baked in an oven maintained at 150 °C for 2 hours, was carefully placed over the plant and sealed around the pot using rubber bands. One of the top corners of the bag was cut off with scissors so as to accommodate a glass tube (80 mm x 5 mm OD), which contained Tennax TA (60/80 mesh, 0.05 g, Supelco Inc., Bellefonte, PA) adsorbent polymer plugged with glass wool at both ends. The Tennax tube was attached to a brass fitting with a PTFE septa that connected to PTFE tubing connected to a pump. The PET oven bag was sealed around the end of the Tennax tube using wire and wrapped in PTFE tape to create an air-tight seal. It was held in place by a clamp supporting the brass fitting. PTFE tubing connected to a pump was inserted under the bag through the rubber bands and charcoal-filtered air was pumped in at a rate of 500 ml min<sup>-1</sup>. Air was drawn through the Tennax tube at a rate of 400 ml min<sup>-1</sup>. The difference in pressure was to ensure air from the glasshouse did not enter the PET bag. As the rubber bands around the base of the pot did not form an air-tight seal there was no build up of positive pressure during the entrainment period. Charcoal-filtered air was pumped into the system for four hours before the Tennax tube was inserted and entrainments began to ensure any contaminating odours introduced while setting up the experiment were flushed from the system. As the method of entrainment included the pot and compost in the system, on each day a pot of the same compost the plants were grown in (Petersfield Products, Leics, UK) covered in aluminium foil with a small gap in the foil (equivalent to the gap around the stem of entrained plants) was entrained as a control.

Plants were entrained in groups of four on four different days during May and June 2008. New plants were entrained on different days. Entrainments began at 16.00 h and continued until 16.00 h the following day. Every six hours the Tennax tube was removed for analysis and replaced with a new one. In this way, entrainments were made over four six-hour periods: evening (16.00 h to 22.00 h), night (22.00 h to 04.00 h), morning (04.00 h to 10.00 h), and midday (10.00 h to 16.00 h). Time periods were chosen so that sunrise occurred shortly after the start of the morning period and sunset occurred towards the end of the evening period. Dates of entrainments and times of sunrise and sunset are listed in Table 6.1. No artificial lighting was used during entrainments. Light intensity (photosynthetically active radiation, PAR) was recorded at plant height throughout the entrainment using a Sunfleck Ceptometer (Decagon, Pullman, WA). Temperature was recorded using a TR-73U Thermo Recorder (T&D, Matsumoto, Japan).

**Table 6.1. Dates of entrainments with times of sunset and sunrise.**

| Group | Dates           | Sunset  | Sunrise |
|-------|-----------------|---------|---------|
| 1     | 13-14 May 2008  | 20.43 h | 05.09 h |
| 2     | 27-28 May 2008  | 21.02 h | 04.52 h |
| 3     | 05-06 June 2008 | 21.12 h | 04.45 h |
| 4     | 08-09 June 2008 | 21.15 h | 04.44 h |

After entrainments Tennax tubes were sealed in glass ampoules under a stream of purified nitrogen and stored in a fridge maintained at 4 °C until they were analysed by GC.

### **6.2.3. Gas chromatography analysis**

Separation of volatiles was carried out on a nonpolar (HP-1, 50m x 0.32 mm ID x 0.52 µm film thickness) capillary column using an HP6890 GC (Agilent Technologies, UK) fitted with a flame ionization detector. Volatiles on the Tennax tube were transferred to the GC column by thermal desorption using an Optic 2 programmed temperature vaporization inlet (Anatune, UK), set for rapid heating from 30 °C to 220 °C in 12 sec. The oven temperature was maintained at 30 °C for 1 min and then programmed at 5 °C min<sup>-1</sup> to 150 °C and held for 0.1 min, then 10 °C min<sup>-1</sup> to 230 °C. Compounds were identified by comparison of GC

retention times with authentic standards of electrophysiologically active compounds identified in Chapter 4 (Table 4.4).

As a different GC was used to that in Chapter 4, new calibration curves were constructed. This was done using the same methodology as described in Chapter 4 (section 4.2.4). Quantities of individual compounds were calculated by comparing peak areas with calibration curves.

#### **6.2.4. Statistical analysis**

Quantities of compounds (ng) were log transformed ( $\ln[x+0.1]$ ) to normalise the distribution. To determine if more volatiles were collected from plants than controls, quantities of individual compounds from plants at different time periods were compared with controls using two-way ANOVA with blocking. Treatment factors were plant/control and time of day. Blocking structure was entrainment group (1-4). To determine if ratios of volatiles were consistent from plant to plant, all zero values were first removed and a correlation matrix constructed for quantities of all compounds collected from plants in greater quantities than controls for each time period. As a large number of correlations were calculated, a lower significance level of  $P=0.02$  was used, corresponding to a false discovery rate of 10%. All statistical tests were performed using Genstat v.11.

### **6.3. Results**

#### **6.3.1. Variation in light intensity and temperature during entrainments**

Figure 6.1 shows variation in light intensity throughout each group of entrainments. Available PAR showed a similar variation throughout the day for each group of entrainments, with the greatest available PAR to be found during the evening (16.00 h – 22.00 h) and midday (10.00 h – 16.00 h) time periods. Table 6.2 shows mean, maximum, and minimum temperatures in glasshouse during each group of entrainments. Temperatures were similar for each group of entrainments, with the highest temperatures recorded during evening and midday entrainments.

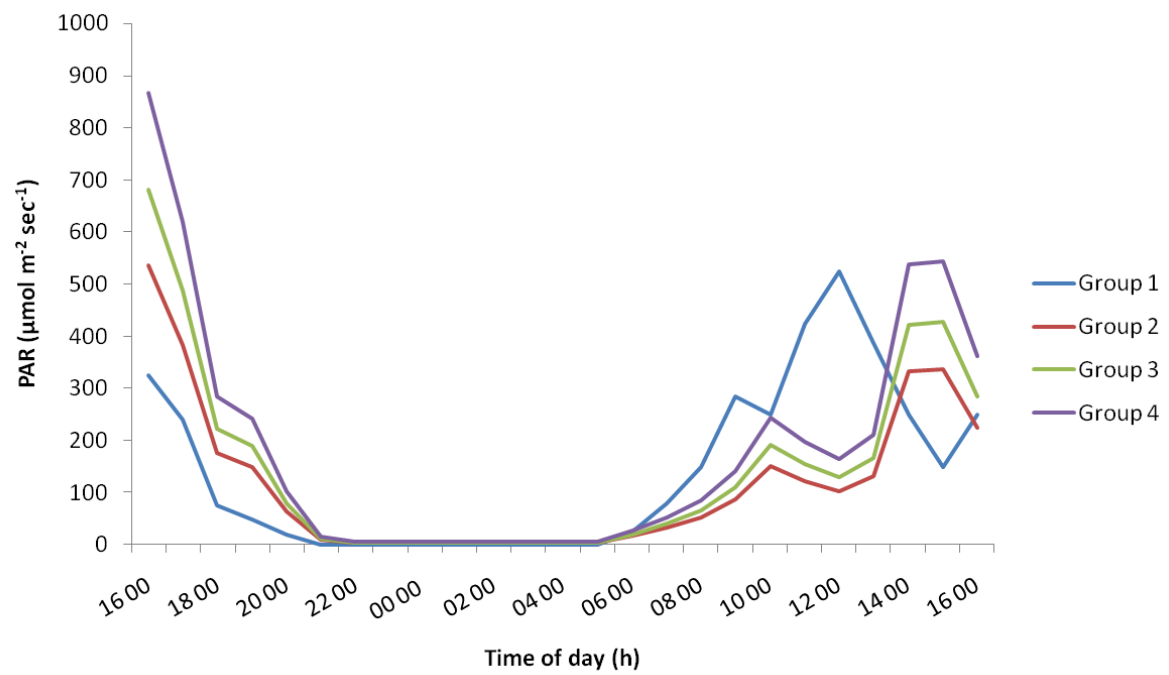


**Figure 6.1.** Variation in available photosynthetically active radiation at bench height in the greenhouse during each group of entrainments.

**Table 6.2. Mean, minimum and maximum temperatures during air entrainments.**

| Group | Time period (h) | mean temp. (°C) | min temp. (°C) | max temp. (°C) |
|-------|-----------------|-----------------|----------------|----------------|
| 1     | 16.00 – 22.00   | 23.4            | 21.0           | 25.0           |
|       | 22.00 – 04.00   | 18.9            | 17.5           | 21.0           |
|       | 04.00 – 10.00   | 16.7            | 16.0           | 17.5           |
|       | 10.00 – 16.00   | 25.9            | 16.5           | 32.5           |
| 2     | 16.00 – 22.00   | 20.7            | 19.5           | 22.5           |
|       | 22.00 – 04.00   | 19.4            | 19.5           | 20.0           |
|       | 04.00 – 10.00   | 20.0            | 19.5           | 22.0           |
|       | 10.00 – 16.00   | 21.3            | 20.5           | 24.0           |
| 3     | 16.00 – 22.00   | 22.8            | 20.5           | 25.0           |
|       | 22.00 – 04.00   | 20.3            | 19.5           | 21.0           |
|       | 04.00 – 10.00   | 21.3            | 20.0           | 22.5           |
|       | 10.00 – 16.00   | 21.5            | 20.0           | 23.0           |
| 4     | 16.00 – 22.00   | 24.8            | 21.5           | 28.0           |
|       | 22.00 – 04.00   | 20.8            | 19.5           | 22.0           |
|       | 04.00 – 10.00   | 23.0            | 20.0           | 26.0           |
|       | 10.00 – 16.00   | 27.8            | 26.0           | 29.5           |

### 6.3.2. Quantities and ratios of collected volatile compounds

Table 6.3 shows quantities of volatile compounds compared to controls for the total 24 h of the entrainment period. Data from three entrainments are missing from the analysis due to technical problems with the GC. These were an evening entrainment of one of the plants from group 3, a night-time entrainment of one of the plants in group 4, and a midday entrainment of another of the plants in group 4. One replicate, the evening entrainment of control in group 4 was removed as an outlier. Ten of the volatile compounds were collected from plants in significantly greater quantities than controls. These were (*Z*)-3-hexen-1-ol, 1-hexanol, 6-methyl-5-hepten-2-one, (*Z*)-3-hexen-1-yl acetate, (*R*)-linalool, methyl salicylate, (*E*)-caryophyllene, (*E*)- $\beta$ -farnesene, (*S*)-germacrene D and TMTT (Table 6.2).

**Table 6.3. Mean quantities of volatile compounds emitted by plants compared to controls.**

| Compound                                                     | Mean quantity collected<br>from plant (ng) ( $\pm$ SE) | Mean quantity collected<br>from control (ng) ( $\pm$ SE) | <i>P</i> value |
|--------------------------------------------------------------|--------------------------------------------------------|----------------------------------------------------------|----------------|
| ( <i>E</i> )-2-hexenal                                       | 5.38 ( $\pm$ 1.08)                                     | 3.92 ( $\pm$ 1.87)                                       | 0.589          |
| ( <i>Z</i> )-3-hexen-1-ol                                    | 9.74 ( $\pm$ 4.57)                                     | 0.11 ( $\pm$ 0.08)                                       | < 0.001        |
| 1-hexanol                                                    | 0.48 ( $\pm$ 0.17)                                     | 0.07 ( $\pm$ 0.03)                                       | 0.002          |
| benzaldehyde                                                 | 5.81 ( $\pm$ 0.51)                                     | 6.99 ( $\pm$ 1.53)                                       | 0.589          |
| 6-methyl-5-hepten-2-one                                      | 3.35 ( $\pm$ 0.39)                                     | 1.61 ( $\pm$ 0.47)                                       | <0.001         |
| octanal                                                      | 4.71 ( $\pm$ 0.49)                                     | 4.49 ( $\pm$ 1.06)                                       | 0.298          |
| ( <i>Z</i> )-3-hexen-1-yl acetate                            | 7.72 ( $\pm$ 2.34)                                     | 0.00                                                     | < 0.001        |
| ( <i>R</i> )-linalool                                        | 0.72 ( $\pm$ 0.14)                                     | 0.31 ( $\pm$ 0.19)                                       | 0.014          |
| methyl salicylate                                            | 2.62 ( $\pm$ 0.32)                                     | 0.16 ( $\pm$ 0.15)                                       | < 0.001        |
| decanal                                                      | 12.88 ( $\pm$ 1.28)                                    | 16.62 ( $\pm$ 3.29)                                      | 0.220          |
| undecanal                                                    | 1.56 ( $\pm$ 0.16)                                     | 1.99 ( $\pm$ 0.38)                                       | 0.140          |
| ( <i>E</i> )-caryophyllene                                   | 37.31 ( $\pm$ 6.39)                                    | 0.60 ( $\pm$ 0.23)                                       | < 0.001        |
| ( <i>E</i> )- $\beta$ -farnesene                             | 0.73 ( $\pm$ 0.11)                                     | 0.00                                                     | < 0.001        |
| ( <i>S</i> )-germacrene D                                    | 1.33 ( $\pm$ 0.20)                                     | 0.02 ( $\pm$ 0.01)                                       | < 0.001        |
| ( <i>E,E</i> )-4,8,12-trimethyl-<br>1,3,7,11-tridecatetraene | 19.17 ( $\pm$ 2.15)                                    | 0.00                                                     | < 0.001        |

Mean quantities of these compounds at each time period are shown in Figure 6.2. Quantities of compound emitted by each individual plant at different time periods are shown in Figures 6.3 to 6.6. The green leaf volatiles, (*Z*)-3-hexen-1-ol, 1-hexanol and (*Z*)-3-hexen-1-yl acetate, were produced in greatest quantities during morning and midday entrainments but showed large variation in quantities collected during these time periods. In particular, the large quantities of green leaf volatiles collected during morning entrainments appear to be largely the result of one plant skewing the mean. The isoprenoids, 6-methyl-5-hepten-2-one, (*E*)-caryophyllene, (*S*)-germacrene D, (*E*)- $\beta$ -farnesene, and TMTT tended to be collected in greater quantities during evening and midday entrainments with TMTT also collected in relatively large quantities during night entrainments. Some of these compounds showed less diurnal variation than others. Methyl salicylate was collected in a similar pattern to the

isoprenoids. (*R*)-linalool was an exception and was only collected during morning and midday entrainments. 6-Methyl-5-hepten-2-one, (*S*)-germacrene D, and (*E*)- $\beta$ -farnesene showed relatively little diurnal variation whereas (*E*)-caryophyllene and TMTT showed varied widely in quantities collected at different times of day. These two compounds also displayed amongst the largest plant-to-plant variation.

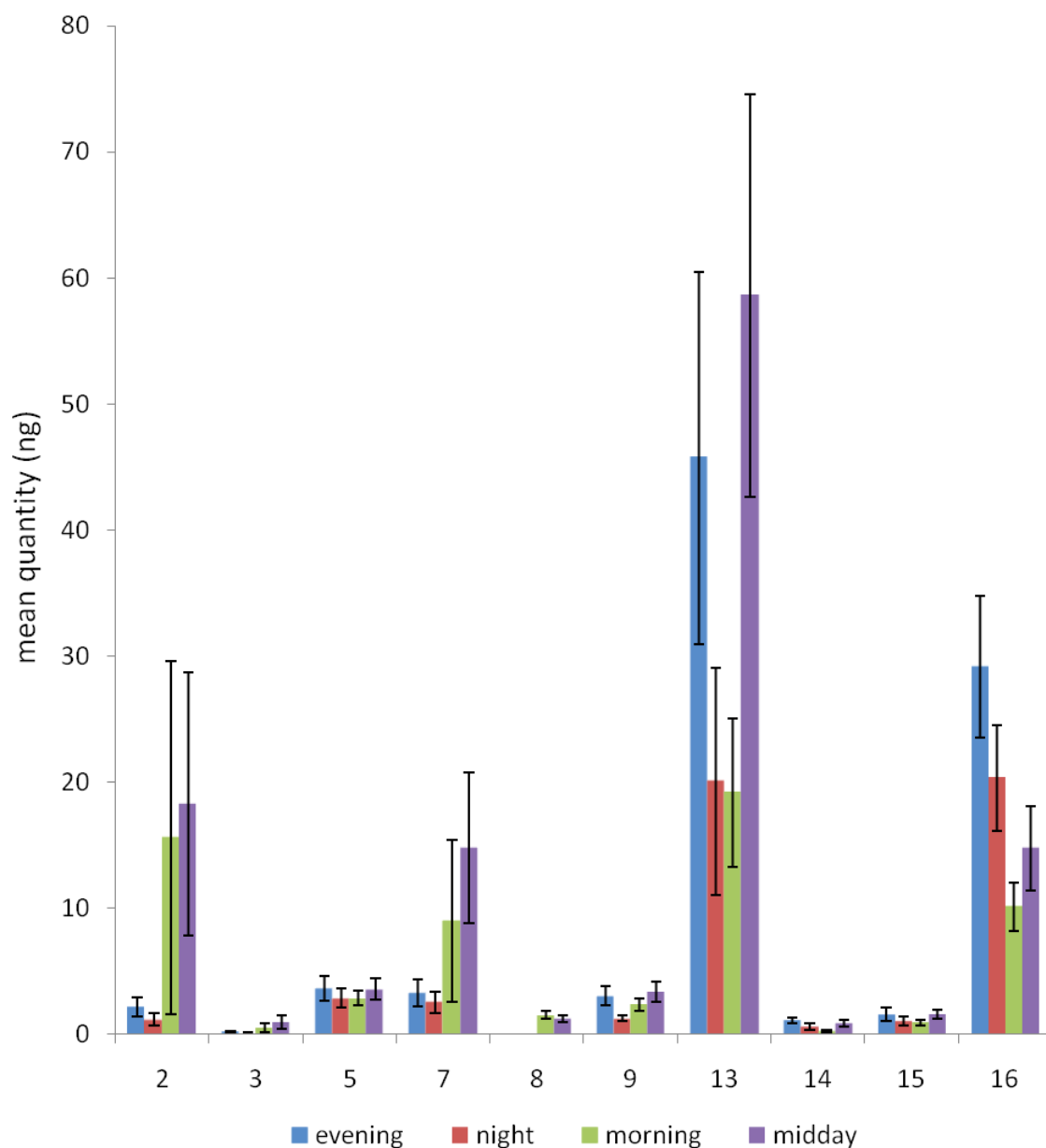


**Figure 6.2.** Mean quantities of compounds collected at different time periods ( $\pm$  SE). Time periods: evening, 16.00 h – 22.00 h; night, 22.00 h – 04.00 h; morning, 04.00 h – 10.00 h; midday, 10.00 h – 16.00 h. Compound numbers: 2. (Z)-3-hexen-1-ol; 3. 1-hexanol; 5. 6-methyl-5-hepten-2-one; 7. (Z)-3-hexen-1-yl acetate; 8. (R)-linalool; 9. methyl salicylate; 13. (E)-caryophyllene; 14. (E)- $\beta$ -farnesene; 15. (S)-germacrene D; 16. (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

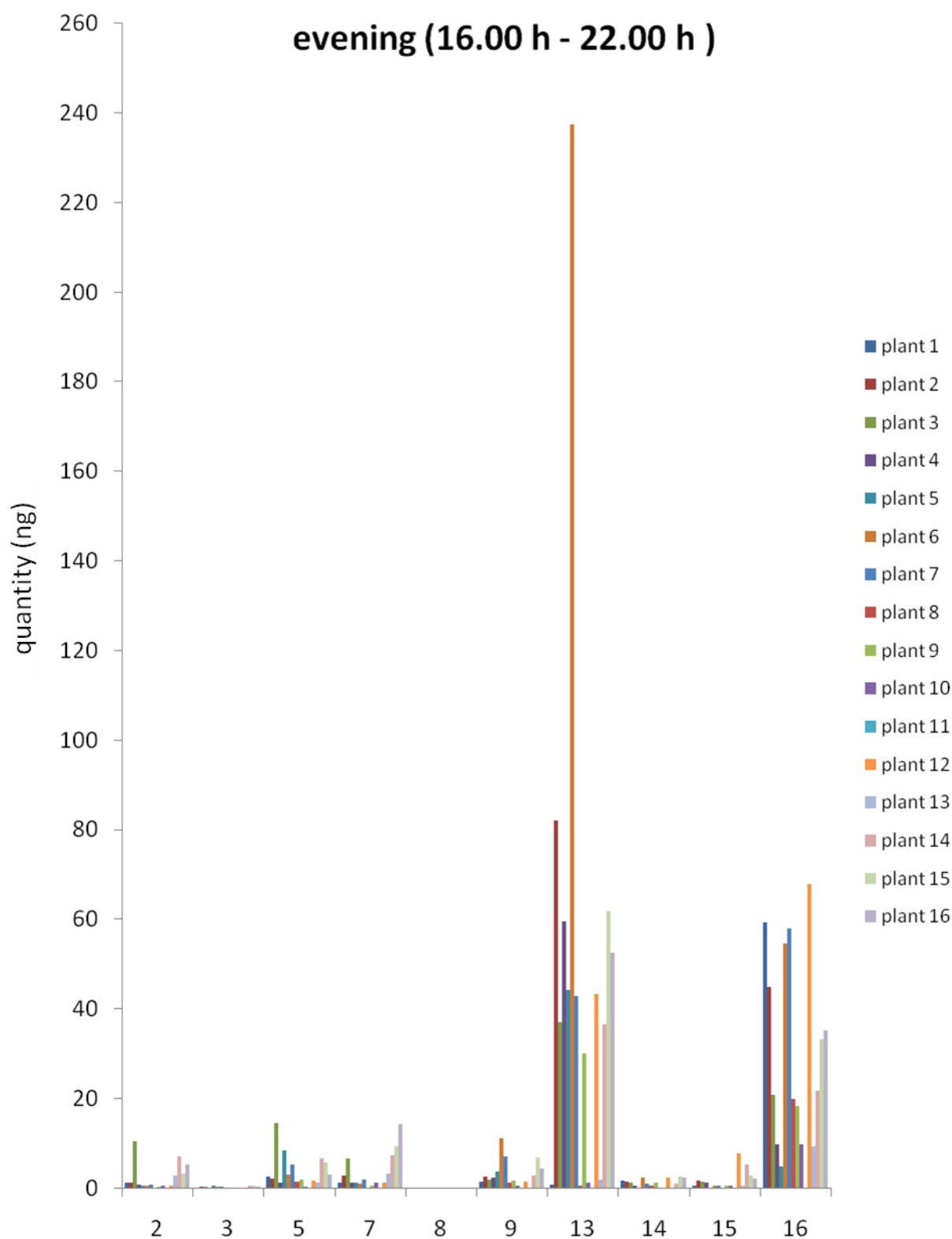


**Figure 6.3.** Range of quantities of compounds emitted during evening (16.00 h – 22.00 h) entrainments. Each colour corresponds to a different plant (plant 11 missing). Compound numbers: 2. (*Z*)-3-hexen-1-ol; 3. 1-hexanol; 5. 6-methyl-5-hepten-2-one; 7. (*Z*)-3-hexen-1-yl acetate; 8. (*R*)-linalool; 9. methyl salicylate; 13. (*E*)-caryophyllene; 14. (*E*)- $\beta$ -farnesene; 15. (*S*)-germacrene D; 16. (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

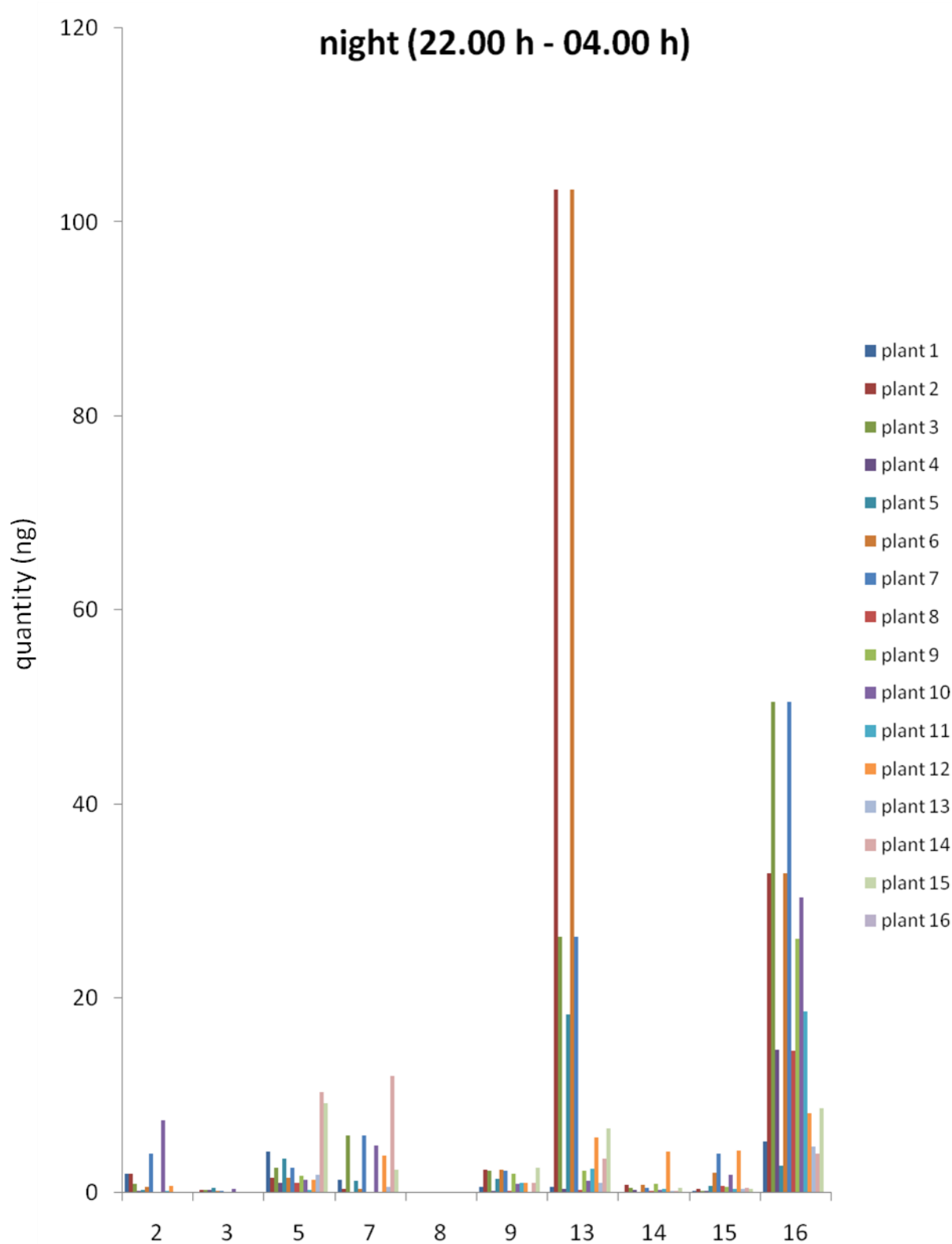


**Figure 6.4.** Range of quantities of compounds emitted during night time (22.00 h – 04.00 h) entrainments. Each colour corresponds to a different plant (plant 16 missing). Compound numbers: 2. (*Z*)-3-hexen-1-ol; 3. 1-hexanol; 5. 6-methyl-5-hepten-2-one; 7. (*Z*)-3-hexen-1-yl acetate; 8. (*R*)-linalool; 9. methyl salicylate; 13. (*E*)-caryophyllene; 14. (*E*)- $\beta$ -farnesene; 15. (*S*)-germacrene D; 16. (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

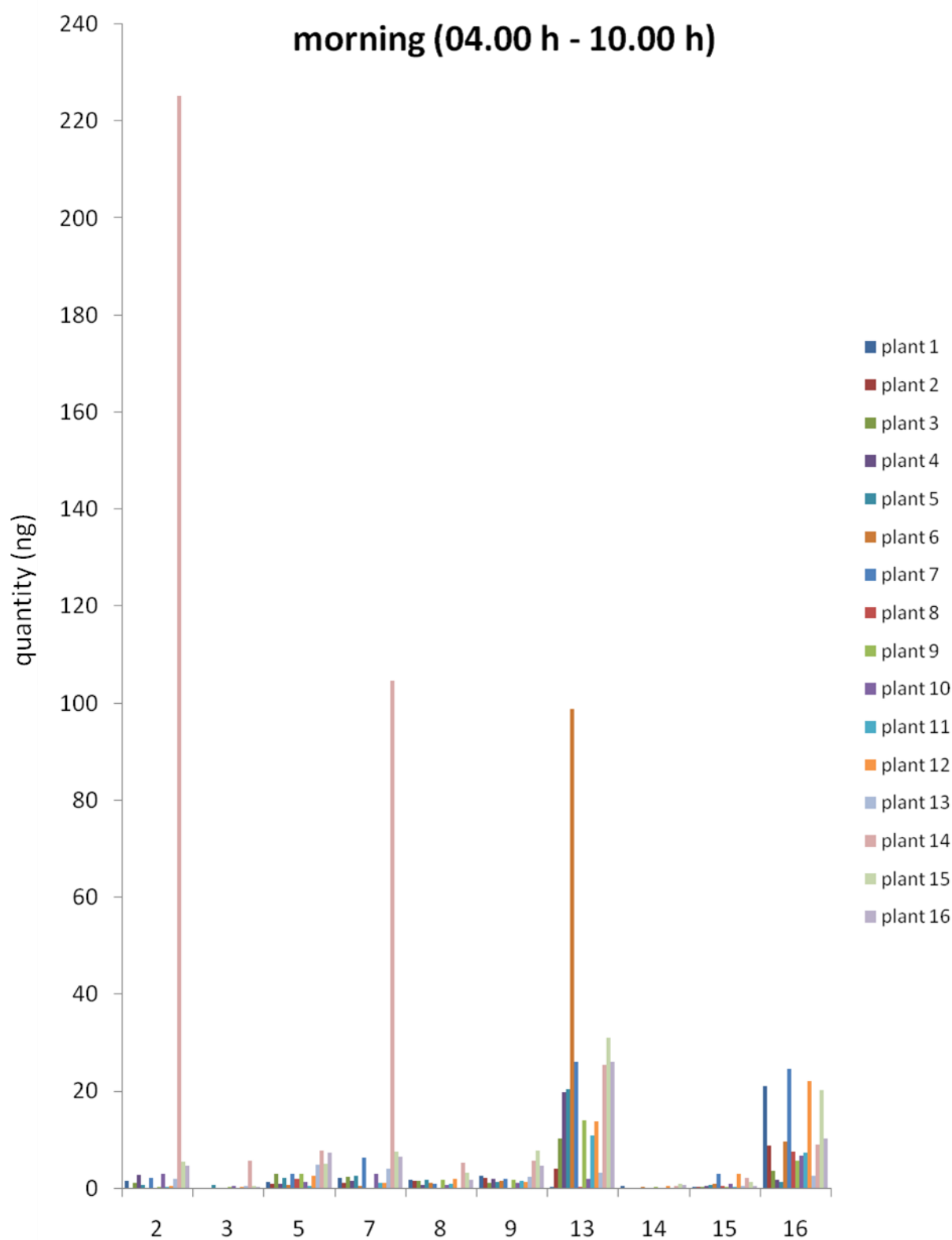


**Figure 6.5.** Range of quantities of compounds emitted during morning (04.00 h – 10.00 h) entrainments. Each colour corresponds to a different plant. Compound numbers: 2. (Z)-3-hexen-1-ol; 3. 1-hexanol; 5. 6-methyl-5-hepten-2-one; 7. (Z)-3-hexen-1-yl acetate; 8. (R)-linalool; 9. methyl salicylate; 13. (E)-caryophyllene; 14. (E)- $\beta$ -farnesene; 15. (S)-germacrene D; 16. (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

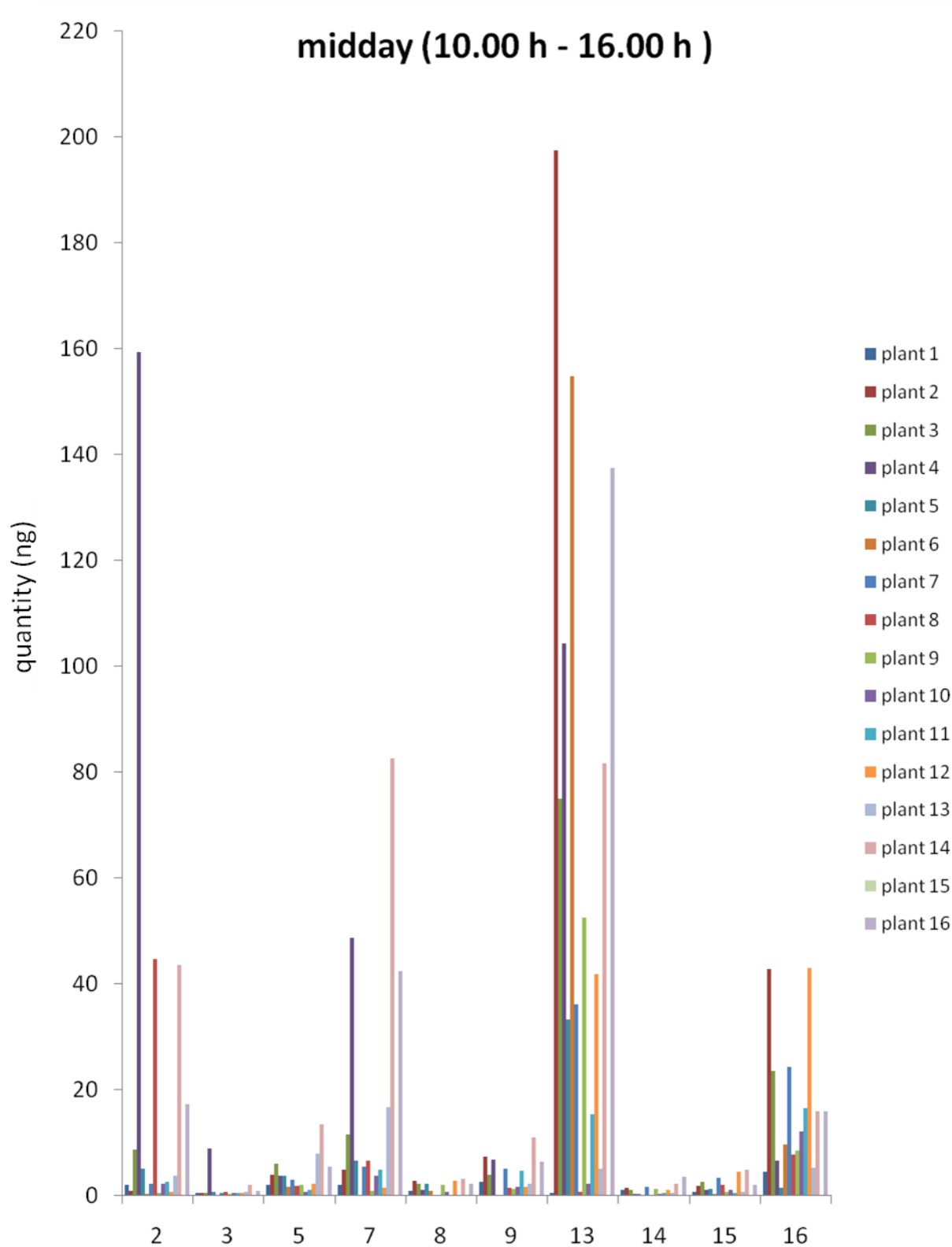


**Figure 6.6.** Range of quantities of compounds emitted during midday (10.00 h – 16.00 h) entrainments. Each colour corresponds to a different plant (plant 15 missing). Compound numbers: 2. (*Z*)-3-hexen-1-ol; 3. 1-hexanol; 5. 6-methyl-5-hepten-2-one; 7. (*Z*)-3-hexen-1-yl acetate; 8. (*R*)-linalool; 9. methyl salicylate; 13. (*E*)-caryophyllene; 14. (*E*)- $\beta$ -farnesene; 15. (*S*)-germacrene D; 16. (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

To determine if ratios of volatiles were consistent from plant to plant for those compounds produced in greater quantities by plants compared to controls, correlations between pairs of compounds at all different time periods were calculated. Two values were removed as outliers when calculating correlations. These were the values of compound 3 (1-hexanol) for one plant in group 1 and one in group 4. Correlations are summarised in Table 6.4 and all pairs of compounds that showed significant correlations are presented in Figure 6.7. Correlations varied at different times of day. The most correlations were found during the morning and midday entrainments with 9 and 7 significant correlations, respectively. Five significant correlations were found during morning entrainments and only three found during night entrainments. (*Z*)-3-Hexen-1-yl acetate showed the greatest numbers of correlations with other compounds, accounting for 11 out of 24 of the significant correlations found. Methyl salicylate correlated with eight other compounds and (*Z*)-3-hexen-1-ol and 6-methyl-5-hepten-2-one correlated with six each. 1-Hexanol and (*R*)-linalool correlated with 4 other compounds each, while (*E*)-caryophyllene only correlated with two other compounds. (*E*)- $\beta$ -Farnese, (*S*)-germacrene D and TMTT accounted for the smallest number of correlations, each only correlating with one other compound.

**Table 6.4.** Table of correlation coefficients for compounds collected during entrainments of *Vicia faba* at a) evening (16.00 h – 22.00 h), b) night time (22.00 h – 04.00 h), c) morning (04.00 h – 10.00 h), and d) midday (10.00 h – 16.00 h). Compound numbers: 2. (Z)-3-hexen-1-ol; 3. 1-hexanol; 5. 6-methyl-5-hepten-2-one; 7. (Z)-3-hexen-1-yl acetate; 8. (R)-linalool; 9. methyl salicylate; 13. (E)-caryophyllene; 14. (E)- $\beta$ -farnesene; 15. (S)-germacrene D; 16. (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Significant correlations are highlighted in bold type. \* Significant ( $P < 0.02$ ). \*\* Significant ( $P < 0.01$ ). \*\*\* Significant ( $P < 0.001$ ).

a) Evening entrainments (16.00 h – 22.00 h)

|    |                 |                |       |       |   |                |       |                 |       |       |
|----|-----------------|----------------|-------|-------|---|----------------|-------|-----------------|-------|-------|
| 2  | 1.000           |                |       |       |   |                |       |                 |       |       |
| 3  | 0.459           | 1.000          |       |       |   |                |       |                 |       |       |
| 5  | 0.483           | <b>0.716**</b> | 1.000 |       |   |                |       |                 |       |       |
| 7  | <b>0.897***</b> | 0.504          | 0.442 | 1.000 |   |                |       |                 |       |       |
| 8  | -               | -              | -     | -     | - |                |       |                 |       |       |
| 9  | 0.157           | <b>0.775**</b> | 0.541 | 0.359 | - | 1.000          |       |                 |       |       |
| 13 | 0.171           | 0.461          | 0.457 | 0.417 | - | <b>0.705**</b> | 1.000 |                 |       |       |
| 14 | 0.117           | 0.392          | 0.550 | 0.258 | - | 0.572          | 0.579 | 1.000           |       |       |
| 15 | 0.449           | 0.203          | 0.273 | 0.508 | - | -0.104         | 0.305 | 0.353           | 1.000 | 0.182 |
| 16 | 0.065           | -0.066         | 0.145 | 0.119 | - | 0.320          | 0.230 | <b>0.817***</b> | 0.182 | 1.000 |
|    | 2               | 3              | 5     | 7     | 8 | 9              | 13    | 14              | 15    | 16    |

b) Night entrainments (22.00 h – 04.00 h)

|    |                |        |                |        |   |                 |       |       |       |       |
|----|----------------|--------|----------------|--------|---|-----------------|-------|-------|-------|-------|
| 2  | 1.000          |        |                |        |   |                 |       |       |       |       |
| 3  | -0.020         | 1.000  |                |        |   |                 |       |       |       |       |
| 5  | 0.410          | 0.079  | 1.000          |        |   |                 |       |       |       |       |
| 7  | <b>0.746**</b> | -0.036 | <b>0.642**</b> | 1.000  |   |                 |       |       |       |       |
| 8  | -              | -      | -              | -      | - |                 |       |       |       |       |
| 9  | 0.489          | -0.763 | 0.316          | 0.399  | - | 1.000           |       |       |       |       |
| 13 | 0.261          | -0.543 | 0.165          | 0.317  | - | <b>0.836***</b> | 1.000 |       |       |       |
| 14 | 0.100          | -0.566 | -0.146         | 0.077  | - | 0.359           | 0.450 | 1.000 |       |       |
| 15 | 0.326          | -0.220 | -0.100         | 0.323  | - | 0.217           | 0.305 | 0.514 | 1.000 |       |
| 16 | 0.403          | -0.713 | -0.428         | -0.098 | - | 0.312           | 0.378 | 0.243 | 0.218 | 1.000 |
|    | 2              | 3      | 5              | 7      | 8 | 9               | 13    | 14    | 15    | 16    |

c) Morning entrainments (04.00 h – 10.00 h)

|    |                 |        |                 |                |               |               |        |       |       |       |
|----|-----------------|--------|-----------------|----------------|---------------|---------------|--------|-------|-------|-------|
| 2  | 1.000           |        |                 |                |               |               |        |       |       |       |
| 3  | 0.415           | 1.000  |                 |                |               |               |        |       |       |       |
| 5  | 0.595           | 0.295  | 1.000           |                |               |               |        |       |       |       |
| 7  | <b>0.950***</b> | 0.348  | <b>0.780***</b> | 1.000          |               |               |        |       |       |       |
| 8  | 0.573           | 0.430  | 0.529           | <b>0.681**</b> | 1.000         |               |        |       |       |       |
| 9  | <b>0.697**</b>  | 0.268  | <b>0.598*</b>   | <b>0.687**</b> | <b>0.656*</b> | 1.000         |        |       |       |       |
| 13 | 0.014           | -0.125 | 0.186           | 0.117          | 0.449         | 0.158         | 1.000  |       |       |       |
| 14 | 0.305           | 0.280  | 0.650           | 0.307          | <b>0.713*</b> | <b>0.758*</b> | 0.443  | 1.000 |       |       |
| 15 | 0.349           | -0.231 | 0.393           | 0.431          | 0.236         | 0.228         | 0.365  | 0.268 | 1.000 |       |
| 16 | 0.111           | -0.480 | 0.126           | 0.121          | 0.195         | 0.385         | -0.055 | 0.544 | 0.503 | 1.000 |
|    | 2               | 3      | 5               | 7              | 8             | 9             | 13     | 14    | 15    | 16    |

d) Midday entrainments (10.00 h – 16.00 h)

|    |                 |                 |                |                |               |       |       |       |       |       |
|----|-----------------|-----------------|----------------|----------------|---------------|-------|-------|-------|-------|-------|
| 2  | 1.000           |                 |                |                |               |       |       |       |       |       |
| 3  | <b>0.862***</b> | 1.000           |                |                |               |       |       |       |       |       |
| 5  | 0.413           | 0.407           | 1.000          |                |               |       |       |       |       |       |
| 7  | <b>0.847***</b> | <b>0.916***</b> | <b>0.637**</b> | 1.000          |               |       |       |       |       |       |
| 8  | 0.068           | 0.268           | <b>0.700*</b>  | 0.310          | 1.000         |       |       |       |       |       |
| 9  | 0.422           | 0.330           | 0.576          | <b>0.713**</b> | 0.362         | 1.000 |       |       |       |       |
| 13 | -0.058          | -0.203          | 0.417          | 0.121          | 0.644         | 0.602 | 1.000 |       |       |       |
| 14 | -0.058          | 0.069           | 0.396          | 0.142          | 0.559         | 0.372 | 0.414 | 1.000 |       |       |
| 15 | 0.304           | 0.274           | 0.350          | 0.403          | <b>0.699*</b> | 0.254 | 0.269 | 0.542 | 1.000 |       |
| 16 | -0.222          | -0.191          | 0.010          | -0.024         | 0.388         | 0.295 | 0.388 | 0.574 | 0.516 | 1.000 |
|    | 2               | 3               | 5              | 7              | 8             | 9     | 13    | 14    | 15    | 16    |

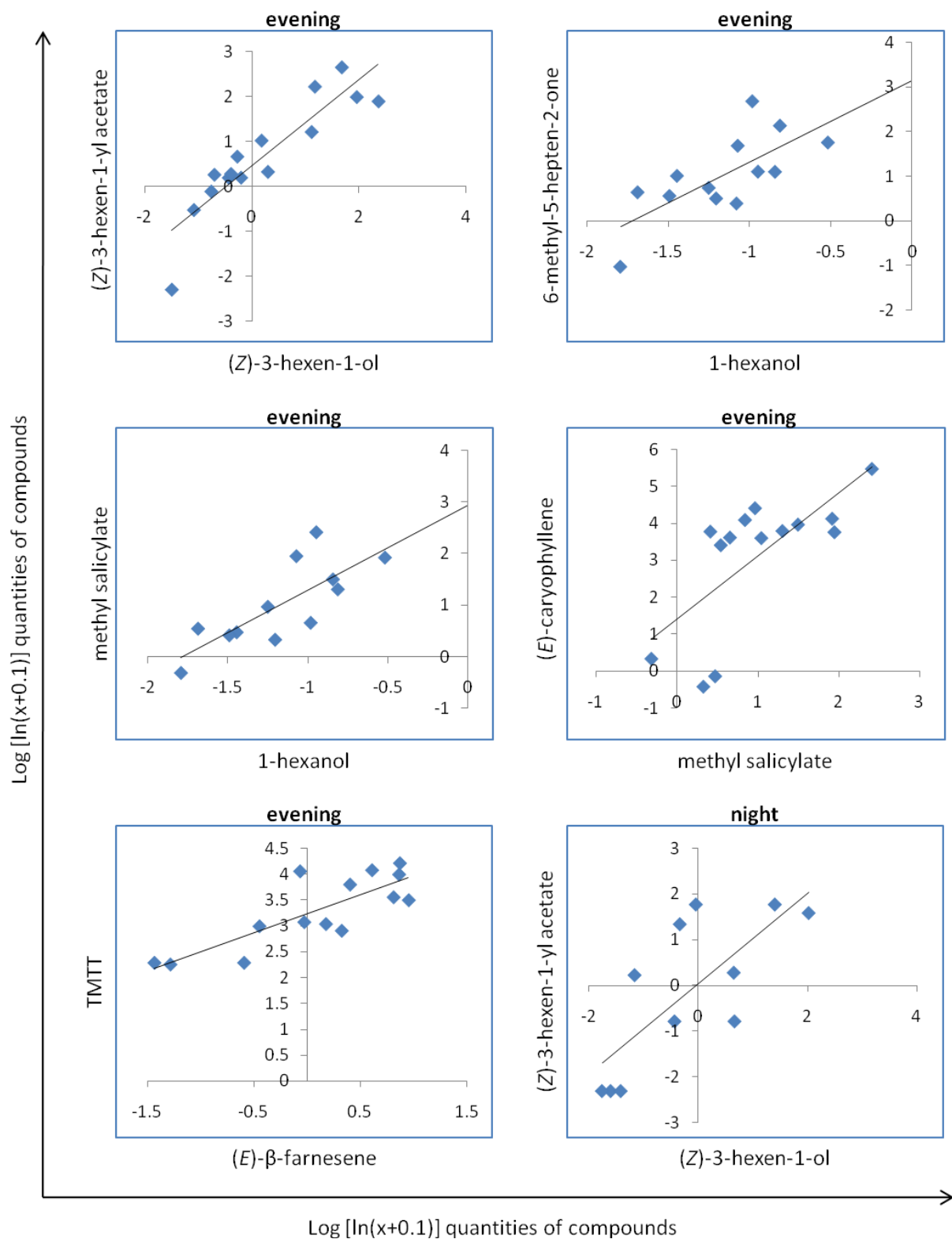


**Figure 6.7.** Correlations between quantities of compounds for entrainments at different time periods: evening (16.00 h – 22.00 h), night (22.00 h – 04.00 h), morning (04.00 h – 10.00 h), and midday (10.00 h – 16.00 h).

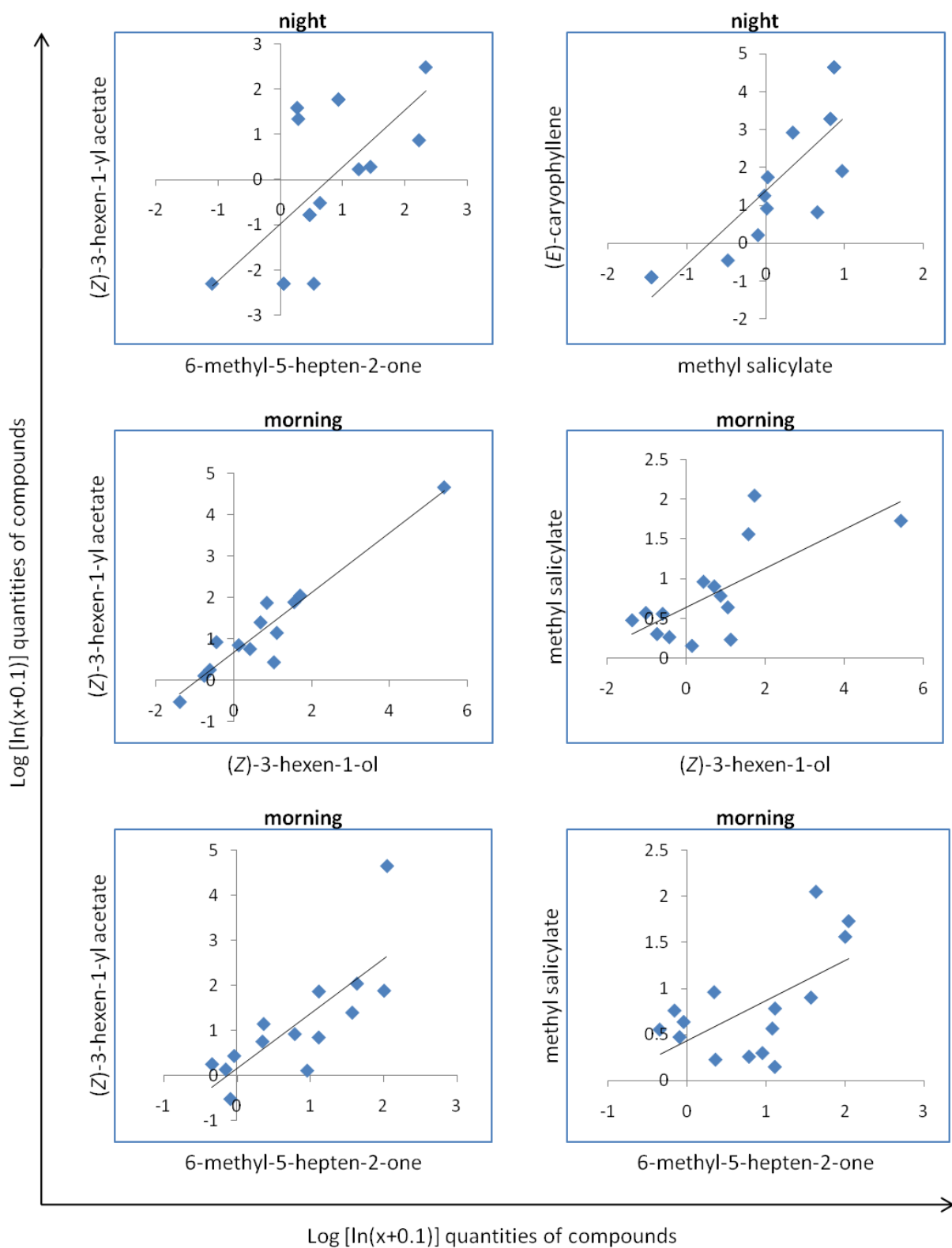


Figure 6.7 continued. Correlations between quantities of compounds for entrainments at different time periods: evening (16.00 h – 22.00 h), night (22.00 h – 04.00 h), morning (04.00 h – 10.00 h), and midday (10.00 h – 16.00 h).

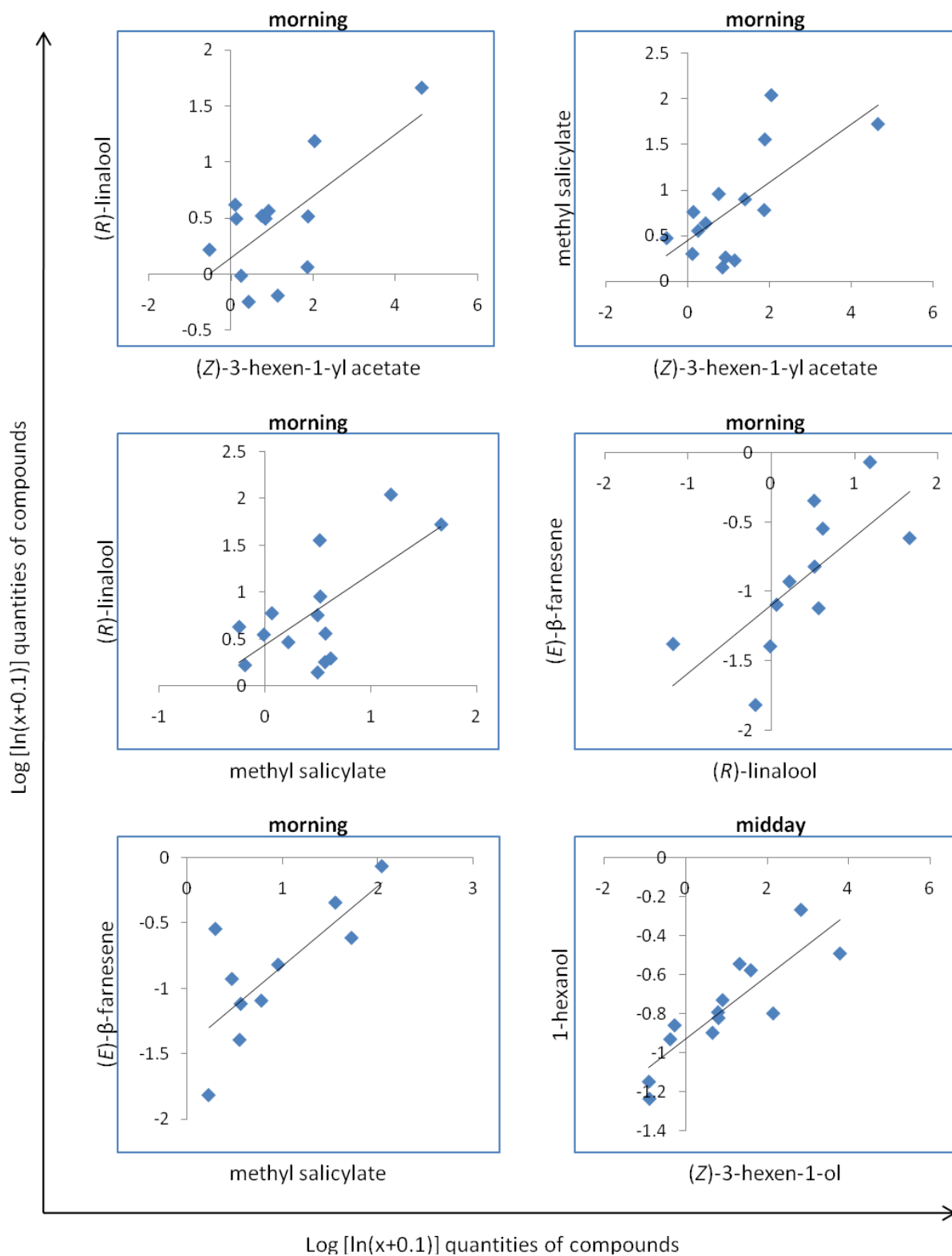


Figure 6.7 continued. Correlations between quantities of compounds for entrainments at different time periods: evening (16.00 h – 22.00 h), night (22.00 h – 04.00 h), morning (04.00 h – 10.00 h), and midday (10.00 h – 16.00 h).

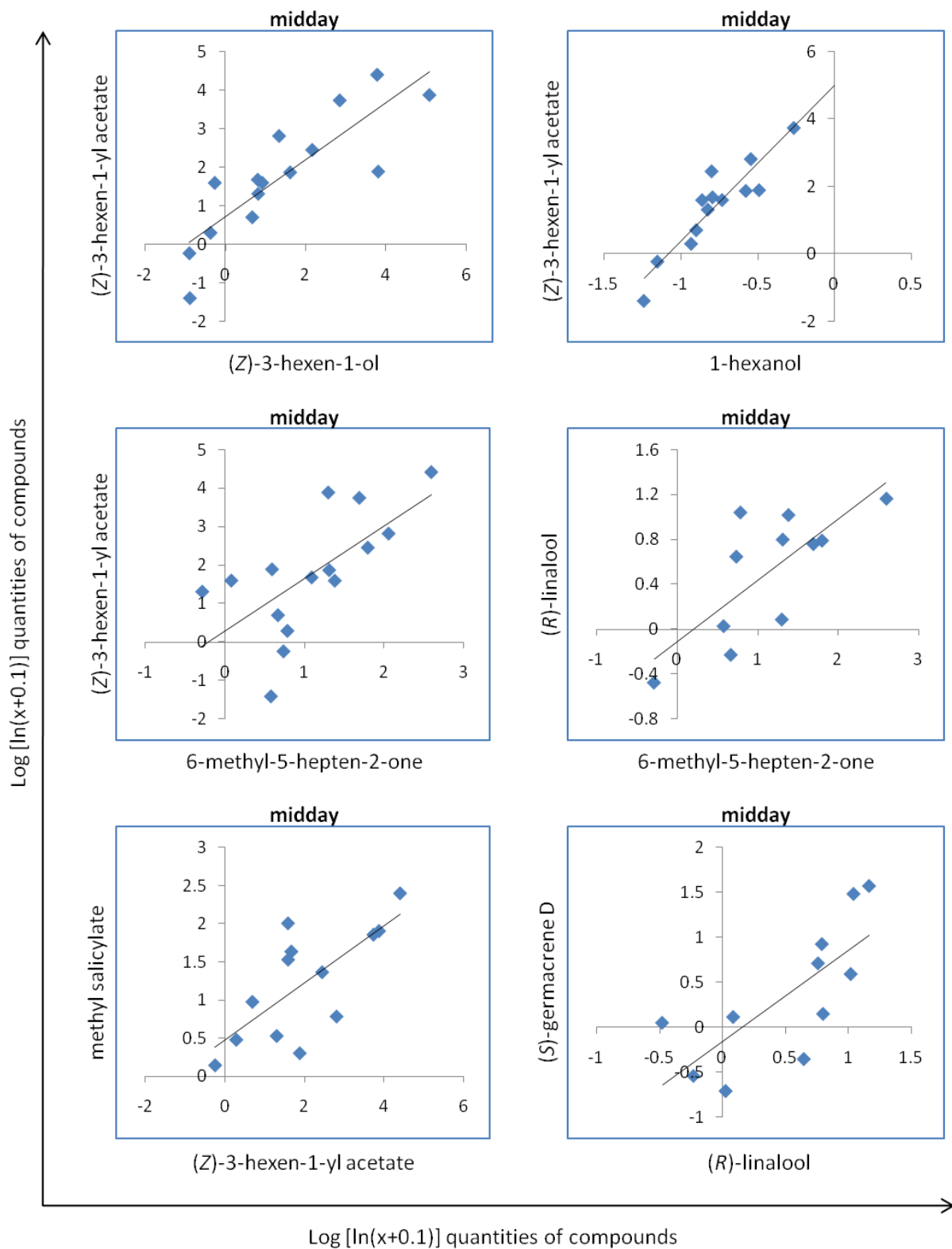


Figure 6.7 continued. Correlations between quantities of compounds for entrainments at different time periods: evening (16.00 h – 22.00 h), night (22.00 h – 04.00 h), morning (04.00 h – 10.00 h), and midday (10.00 h – 16.00 h).

## 6.4. Discussion

### 6.4.1. Comparison of volatiles collected from plants and controls

Ten compounds were collected in significantly greater quantities from plants than pots of compost used as controls. These were (Z)-3-hexen-1-ol, 1-hexanol, 6-methyl-5-hepten-2-one, (Z)-3-hexen-1-yl acetate, methyl salicylate, (R)-linalool, (E)-caryophyllene, (E)- $\beta$ -farnesene, (S)-germacrene D, and TMTT. The remaining five compounds; (E)-2-hexenal, benzaldehyde, octanal, decanal and undecanal, were not collected in significantly greater quantities from plants than controls. It is therefore possible that the plants did not produce these five volatile compounds. If this is the case then microbes in the compost may be responsible for the production of these volatiles rather than the plants (Kai et al. 2009). However, (E)-2-hexenal, benzaldehyde, and decanal have all been reported previously in the headspace of *V. faba* plants so it is likely that these three at least were produced by the plants (Sutton et al. 1992; Griffiths et al. 1999; Colazza et al. 2004; Mendgen et al. 2006; Pareja et al. 2009). Another possibility is that the five compounds were produced by the plants but not in quantities detectably higher than that given off by the compost in the control entrainments. These five compounds are among those collected in the smallest quantities in the air entrainments performed in Chapter 2 so this is a plausible explanation. This could be determined by further entrainments of plants using more stringent methodology to minimise contamination from the compost compounds. If these volatiles are not produced by the plants then removing them from the complete synthetic blend would be a logical starting point for any future work into the volatile compounds used by *A. fabae* in host location.

In Chapter 2, air entrainments were made by enclosing the plants in a glass vessel and securing aluminium plates around the base of the stem. A positive pressure was used to ensure that volatiles from the glasshouse and compost did not enter the system. In this chapter, due to the larger number of entrainments that needed to be performed, a different method was employed which involved covering the compost of the plant pot in aluminium foil and enclosing the plant and pot in a PET oven bag. As it was possible that volatiles from the compost could enter the system using this method, pots containing just compost covered in aluminium foil save for a small gap were entrained as controls. Many studies involving air entrainment of plants do not mention the use of pots of compost as controls (e.g. Du et al.

1998; Nojima et al. 2003b; Birkett et al. 2004; Chamberlain et al. 2006; Pareja et al. 2009) whereas others do (De Moraes et al. 1998; Quiroz & Niemeyer 1998). The present study suggests that entrainment of control pots is worthwhile as detectable levels of some electrophysiologically active volatiles were collected from these controls.

#### **6.4.2. Variation in quantities of *V. faba* volatile compounds**

In Chapter 5, (Z)-3-hexen-1-ol and 1-hexanol were the only compounds to elicit positive behavioural responses from *A. fabae* in olfactometer bioassays but did not fully account for the behavioural response to the complete synthetic blend. Both these volatiles showed large plant-to-plant variation in emission and were produced in negligible quantities by some plants at certain time periods. This could partly explain why these two compounds are not used solely by *A. fabae* to recognise its host as their presence does not appear to offer a reliable and consistent host cue at any time period tested. All other volatile compounds were also collected in variable quantities, possibly explaining why no single compound is used on its own by *A. fabae* to recognise its host.

Several studies have demonstrated that there can be large diurnal variation in volatile emission by plants (Loughrin et al. 1993; Dudareva et al. 2000; Karl et al. 2002; Graus et al. 2004; Chamberlain et al. 2006) and each of the ten volatile compounds in the present study showed clear diurnal variation in emission by *V. faba*. Compounds of the same structural class tended to show similar diurnal patterns in emission, probably due to shared biosynthetic pathways involved in their production. The terpenoids, 6-methyl-5-hepten-2-one, (*R*)-linalool, (*E*)-caryophyllene, (*E*)- $\beta$ -farnesene, (*S*)-germacrene D, and TMTT, as well as the phenylpropanoid methyl salicylate all showed similar diurnal patterns in emission. These were generally collected in the greatest quantities during midday and evening. TMTT was also collected in relatively large quantities during night entrainments. (*R*)-linalool was another exception, as this compound was only collected during morning and midday entrainments. The green leaf volatiles (Z)-3-hexen-1-ol, 1-hexanol, and (Z)-3-hexen-1-yl acetate were collected in greater quantities during morning and midday entrainments and in smaller quantities during evening and night entrainments. Green leaf volatiles are produced by the enzymatic breakdown of free fatty acids in the plasma membrane. They tend to be emitted in greater quantities following mechanical damage to plant tissue (Saijo & Takeo 1975; Agelopoulos et al. 1999b) but Chamberlain *et al.*, (2006) found that green leaf volatiles

were also emitted in greater quantities by *Pennisetum purpureum* and *Hyparrhenia tamba* when these plants first entered the scotophase. In contrast to these findings, the present study has shown that the green leaf volatiles are produced by *V. faba* in the smallest quantities during the time period encapsulating the onset of the scotophase (evening entrainments) and found greater quantities collected during the onset of the photophase (morning) and during the photophase (midday). Large plant-to-plant variation in quantities of volatiles collected was observed during both these time periods, however. The differences between the present study and the findings of Chamberlain et al., (2006) could be due to different methodologies, as the previous study used artificial lighting which led to a more dramatic change from light to dark than that in the present study. Differences are most likely due to the different plant species used in the different studies, however.

The present study divided the day into four six-hour time periods. More information could be gained by entraining plants over even shorter time periods to get a clearer idea of the variation in emission over the course of the day. Such a task would be difficult as the quantities of some volatiles are already close to the threshold for detection, but this could perhaps form the basis of future work once there have been technological advances in the field allowing easier detection of smaller quantities.

#### **6.4.3. Variation in ratios of *Vicia faba* volatile compounds**

Correlations between the ten compounds collected in significantly greater quantities than controls were performed for each of the time periods: evening (16.00 h to 22.00 h), night (22.00 h to 04.00 h), morning (04.00 h to 10.00 h), and midday (10.00 h to 16.00 h). In total, 24 significant correlations between quantities of pairs of compounds were found (Table 6.4, Figure 6.4). This suggests that *V. faba* emits some volatile compounds in species characteristic ratios. These could, in theory, be used by host-seeking aphids to recognise their host (Visser 1986; Bruce et al. 2005b). Unlike previous work attempting to determine the consistency of ratios of plant volatiles in the headspace of plants (Agelopoulos et al. 2000; Degen et al. 2004), the present study focused on volatile compounds electrophysiologically active to the insect in question rather than focusing on those which were collected in the greatest quantities. This therefore provides stronger evidence to support the hypothesis that insects may be able to recognise their hosts using ratios of volatile compounds. This hypothesis cannot be confirmed, however, without determining behavioural responses of

aphids to different volatile blends with components at different ratios. If aphids respond preferentially to blends whose ratios are within the natural range determined in this chapter, then it could be concluded that ratios of volatiles are indeed used by *A. fabae* to recognise its host.

Diurnal variation in the ratios of volatiles collected was also found. Indeed, only one pair of compounds was found to be correlated at all four time periods. This was the correlation between (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate. Quantities of (Z)-3-hexen-1-yl acetate correlated with 6-methyl-5-hepten-2-one during night, morning and midday entrainments. Methyl salicylate correlated with (E)-caryophyllene during evening and night entrainments and with (Z)-3-hexen-1-yl acetate during morning and midday entrainments. All other correlations between quantities of pairs of compounds were only significant during one of the four time periods. Diurnal variation in ratios of volatiles may not necessarily make the task of host-location more difficult for aphids, however. Aphids are more active at certain times of the day (Cammell 1981; Campbell & Muir 2005; Narayandas & Alyokhin 2006) and so may be tuned to ratios associated with the time of day in which they are more active. *Aphis fabae* shows peak flight activity during mid-morning and early afternoon and flight typically lasts for up to 2 hours after which alighting occurs (Cammell 1981). These periods of alighting would correspond most closely to the midday and evening entrainments. It would be interesting to see if aphid responses are tuned more closely to blends associated with these times of day and could form an interesting basis for future work.

(R)-Linalool inhibits the alarm response of *A. fabae* to (E)- $\beta$ -farnesene (Bruce, personal communication), released by aphids under attack and used to alert to nearby aphids to the presence of predators or parasitoids (Nault et al. 1973). The correlation of this compound with (E)- $\beta$ -farnesene from entrainments carried out at midday could explain why aphids use this volatile as an alarm pheromone inhibitor, as the quantity it is emitted in is proportional to the quantity of (E)- $\beta$ -farnesene being emitted by the plant. In this way, (R)-linalool may constitute a reliable alarm pheromone inhibitor emitted by the plant at this time of day. The lack of (R)-linalool emitted at other times of day is surprising, however, and begs the question as to how *A. fabae* avoids an alarm response at other times of the day. Investigations into the alarm responses of *A. fabae* at different times of day in light of these findings would be interesting.

Several of the correlated pairs of compounds are derived from the same biosynthetic pathways and this may partly explain correlations between quantities of certain pairs of compounds. (*Z*)-3-hexen-1-ol, 1-hexanol and (*Z*)-3-hexen-1-yl acetate all correlate with one another at various time periods. Each of these compounds is derived from the lipoxygenase pathway (Dudareva et al. 2006) which may explain why they tend to be produced in similar quantities. Similarly, the correlation between (*R*)-linalool and 6-methyl-5-hepten-2-one may be due to similar biosynthetic pathways as all these compounds are monoterpenoids and derived via the methylerythritol phosphate (MEP) pathway. Shared biosynthetic pathways could also explain the correlation between the two sesquiterpenes (*E*)-caryophyllene and (*S*)-germacrene D. The precursors of monoterpenoids, sesquiterpenoids and homoterpenoids are derived via different biosynthetic pathways but some cross-talk between the pathways has been observed (Dudareva et al. 2006). Correlations between quantities of (*R*)-linalool and (*E*)- $\beta$ -farnesene, and between (*R*)-linalool and (*S*)-germacrene D may therefore be in part a result of this cross-talk.

Where correlations between pairs of compounds can be explained as the result of common biosynthetic pathways, the ratios of the volatiles may be less likely to constitute a host-specific cue as the same biosynthetic pathways are common to many different plant species. There are, however, several examples of significant correlations between all three classes of compounds that do not share common biosynthetic pathways, and these may be more likely to constitute ratios specific to *V. faba*. Methyl salicylate is a phenylpropanoid and correlates with the terpenoids (*E*)-caryophyllene, 6-methyl-5-hepten-2-one, (*R*)-linalool and (*E*)- $\beta$ -farnesene during various time periods. Methyl salicylate also correlates with the green leaf volatiles 1-hexanol, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl acetate during various time periods. This could be result of a common physiological cause of increased emission of these volatiles. Production of green leaf volatiles is associated with plant stress in *V. faba* (Agelopoulos et al. 1999b), as is the production of methyl salicylate (Hardie et al. 1994a). Although plants were entrained under conditions designed to minimise stress it is possible that minor stress was endured by some of the plants which could explain the correlations between these compounds. Correlations were also observed between the green leaf volatiles 1-hexanol, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl-acetate and the terpenoid 6-methyl-5-hepten-2-one, and between (*Z*)-3-hexen-1-yl-acetate and (*R*)-linalool. As these are examples

of correlations between different classes of compounds they could represent ratios which are specific to *V. faba*.

Entrainments carried out in this chapter were only performed on plants of a single cultivar. It is likely that larger degrees of variation in volatile emission would be found if entrainments were made of more than one cultivar as the larger degree of genetic variability would probably influence the outcome of the experiments. This could form the basis of future work. In particular, the entrainment of inbred lines in a similar vein to other studies (Degen et al. 2004) could yield useful information on genetic variability in volatile emissions of *V. faba*.

#### **6.4.4. Conclusions**

Large variability in quantities of volatile compounds electrophysiologically active to *A. fabae* has been found but some consistency in the ratios of some of these volatiles was also found. Many of the pairs of compounds whose quantities are correlated are compounds that share common biosynthetic pathways which may explain why they are produced in consistent ratios. If this is the case then such correlations may not be specific to *V. faba* as many different plant species share the same biosynthetic pathways. Other pairs of significantly correlated compounds, however, are not derived from the same biosynthetic pathways and these may be more likely to be specific to *V. faba*. Whether or not consistent ratios of compounds are specific to *V. faba* is a question that can only be answered by determining the ratios of the same volatiles emitted by non-host plants. A comparative study with several non-related plant species would therefore form an interesting basis for future work. If ratios of volatile compounds found to be consistent from plant-to-plant in this study are found to be unique to *V. faba*, then this would lend weight to the hypothesis that *A. fabae* uses ratios of host volatiles to discriminate its host from non-host plants. If, however, these ratios and blend compositions are found to be common throughout the plant kingdom then this would cast doubt on this hypothesis.

## CHAPTER 7. GENERAL DISCUSSION

The main aim of this research project was to investigate the olfactory basis of host recognition in *A. fabae*. This involved identifying relevant semiochemicals of its major summer host, *V. faba*, and determining behavioural responses to identified semiochemicals. A positive behavioural response to the odour of a *V. faba* plant was shown and also to an air entrainment sample comprising volatiles collected from the headspace of *V. faba* plants. It was then shown that a blend of 15 electrophysiologically active volatile compounds, in the same quantities and ratios as collected from *V. faba* headspace, elicited a similar positive behavioural response. Behavioural responses to individual compounds and to blends was also demonstrated and provided further insight into the role of volatile chemical cues in host recognition by *A. fabae*. Surprisingly, some of the identified compounds elicited negative behavioural responses when presented alone. Finally, plant-to-plant and diurnal variation in emission of the 15 electrophysiologically active volatile compounds from *V. faba* was measured in order to gain an understanding of whether or not there is any consistent host-characteristic volatile cue that may be used by host-seeking *A. fabae*. In this chapter these key findings are discussed as well as future directions that this work may take and how these might be used in integrated pest control strategies for the control of *A. fabae* populations.

### 7.1. Identification of *Vicia faba* volatile compounds

In Chapter 3, coupled GC-EAG revealed the presence of 16 electrophysiologically active volatile compounds in the *V. faba* air entrainment sample (Chapter 3, Figure 3.3). Using coupled GC-MS and comparison of GC retention times with those of authentic standards, 15 of the volatile compounds were identified as (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 1-hexanol, benzaldehyde, 6-methyl-5-hepten-2-one, octanal, (*Z*)-3-hexen-1-yl acetate, (*R*)-linalool, methyl salicylate, decanal, undecanal, (*E*)-caryophyllene, (*E*)- $\beta$ -farnesene, (*S*)-germacrene D, and TMTT (Chapter 4, Table 4.3). These findings are in agreement with previous research into the volatile compounds emitted by *V. faba* as twelve of the compounds have been identified previously in the headspace of undamaged *V. faba* plants; (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 1-hexanol, benzaldehyde, 6-methyl-5-hepten-2-one, (*Z*)-3-hexen-1-yl acetate, (*R*)-linalool, decanal, methyl salicylate, (*E*)-caryophyllene, (*S*)-germacrene D and TMTT

(Blight et al. 1984; Griffiths et al. 1999; Colazza et al. 2004; Pareja et al. 2009). This study also represents the first time that 6-methyl-5-hepten-2-one, octanal, decanal, undecanal, (S)-germacrene D and TMTT have been shown to elicit electrophysiological responses in winged virginoparous *A. fabae*.

Although fifteen compounds were successfully identified, one compound could not be identified due its presence in extremely low quantities which meant that a mass spectrum could not be obtained. This compound elicited a relatively large electrophysiological response considering it was present in such low quantities and identification of this compound may be a worthwhile pursuit for future research. While it is sometimes possible to use mass spectrometry to identify compounds present in extremely low quantities (Pickett 1990), identification could be made easier by employing a different method of volatile collection so that larger quantities of the compound could be collected. Vacuum distillation is an example of such a technique that allows for collection of large quantities of volatiles from plants (Pickett & Stephenson 1980). If used to collect volatiles from *V. faba* plants, the identity of this unknown compound may be revealed and its role, if it has one, in host location in *A. fabae* could then be determined with behavioural bioassays

It is possible that other volatile compounds for which *A. fabae* possesses specific ORNS were also not identified. Electroantennography works by detecting changes in potential across the antennae caused by depolarisation of ORNs when they bind to odourant molecules. The magnitude of the response is thought to be proportional to the number of receptor neurones responding (Schneider 1957; Wadhams 1990). If aphids possess only a few receptor neurones associated with a particular volatile compound, an electrophysiological response may not be detected as the amplitude of the response may be too small. It is also possible that if some compounds were only collected from *V. faba* in small quantities, they may not have elicited a detectable electrophysiological response even though the aphid could detect the presence of the compound. Single sensillum recording (SSR) is an electrophysiological technique which provides greater sensitivity than EAG (Wadhams 1982) and could facilitate the identification of compounds present in small quantities. Coupled GC-SSR could be used to determine if there are any other volatile compounds within the air entrainment sample which *A. fabae* possesses ORNs capable of detecting that were missed using coupled GC-EAG. However, since the 15-component synthetic blend elicited a similar behavioural response to the air entrainment sample (Chapter 5, Figure 5.1), and aphids showed no preference between them

(Figure 5.2), it is unlikely that any compounds not identified in the present study are essential for host recognition.

## 7.2. Host recognition using blends or single compounds

The token stimulus hypothesis, originally put forward by Fraenkel (1959), suggested that host-seeking insects recognise their host plants using volatile compounds which are restricted to their hosts. There are a number of examples in the literature of insects responding to taxonomically characteristic host volatile compounds, including aphids (Dilawari & Atwal 1989; Nottingham et al. 1991). Where host-specific volatile compounds are not present, an alternative hypothesis was provided by Visser (1986). This suggested that host-seeking insects could recognise their hosts by recognising host-specific ratios of general plant volatiles. One of the aims of this project was to determine if either hypothesis applies to *A. fabae*.

All 15 electrophysiologically active volatile compounds identified in the headspace of *V. faba* are found ubiquitously throughout the plant kingdom and are not restricted to this species of plant (Knudsen et al. 1993). It is possible that the compound which could not be identified in Chapter 4 is specific to *V. faba* and could be used as a host-specific cue by host-seeking insects. It is also possible that other volatile compounds in the air entrainment sample which did not elicit a detectable electrophysiological response (either because they were not electrophysiologically active or because they were present in too low concentrations) are also specific to *V. faba*. However, the olfactometer choice test between the synthetic blend and air entrainment sample in Chapter 5 (Figure 5.2) showed aphids had no preference for the air entrainment sample over the synthetic blend. This suggests that all volatile compounds essential for host recognition by *A. fabae* were identified and present in the synthetic blend. Even if any unidentified compounds are specific to *V. faba*, they are unlikely to be essential for host recognition by *A. fabae*. It can therefore be concluded that the token stimulus hypothesis probably does not apply to *A. fabae* responding to *V. faba* odour.

Despite the absence of a host-specific volatile compound from the synthetic blend, it was still possible that host location could be mediated by a single compound alone. This would probably not, however, allow *A. fabae* to discriminate host from non-host; instead it would merely enable the recognition of the odour of plants in general. When each volatile

compound was tested individually for behavioural activity at the same concentration as in the air entrainment sample, only (Z)-3-hexen-1-ol elicited a similar response to the synthetic blend (Chapter 5, Figure 5.3). However, a choice test showed that aphids preferred the complete 15-component blend over this compound alone (Figure 5.4). This suggests that host recognition in *A. fabae* is not mediated by this single compound alone and must instead be mediated by a blend. The present study describes the second such example of a species of aphid responding preferentially to a blend than to an individual host volatile. The first example came from Ngumbi et al., (2007) who found that the peach potato aphid *Myzus persicae* was arrested more strongly by blends than by individual host volatiles.

When a behavioural response is elicited by a blend, the response may be due to the summated response to the individual components or due to a more complex synergistic effect. If several of the compounds within the blend had weak behavioural activity to *A. fabae* on their own (and perhaps too low to elicit a detectable behavioural response), they may add up to a strong behavioural response when presented together in the complete blend. Dose response experiments can help to determine whether or not this may be the case as a compound which is weakly behaviourally active at one dose may elicit a more easily detectable response at a slightly higher or lower dose. Dose response experiments showed that only (Z)-3-hexen-1-ol and 1-hexanol elicited significant positive behavioural responses, both at a dose of 100 ng (Chapter 5, Figure 5.6 and Figure 5.7). When these two compounds were combined into a two-component blend they elicited a similar behavioural response to the synthetic blend (Figure 5.20) but aphids preferred the synthetic blend in a choice test (Figure 5.21). As no other compounds elicited a positive behavioural response at any dose tested, it is unlikely that the response to the blend is simply due to the sum of responses to individual compounds. The response to the blend is therefore most likely due to a synergistic effect.

### **7.3. Context-dependent behavioural responses of *Aphis fabae* to host volatile compounds**

Three of the compounds, octanal, (R)-linalool and (S)-germacrene D, caused aphids to spend less than 25 % of their time in the treated region of the olfactometer when tested at the same concentration as in the air entrainment sample (Chapter 5, Figure 5.3). This suggested that these compounds may function as repellents to *A. fabae*. When these three compounds were

combined into a three-component blend, however, they did not elicit any significant behavioural response from the aphids (Figure 5.22). This showed that the behavioural response to individual compounds is context-specific and is further evidence that the response to a blend is not simply the sum of responses to individual components. This was further confirmed when the three compounds were removed from the complete 15-component blend and aphids showed no preference for the reduced 12-component blend over the complete 15-component blend (Figure 5.23).

Seven other compounds elicited similar negative behavioural responses when tested in dose response experiments. These were (*E*)-2-hexenal, benzaldehyde, (*Z*)-3-hexen-1-yl acetate, methyl salicylate, decanal, (*E*)- $\beta$ -farnesene, and TMTT, all of which caused aphids to spend less than 25 % of their time in the treated region of the olfactometer at one or more concentrations tested (Chapter 5, Figures 5.5, 5.8, 5.11, 5.13, 5.14, 5.17, 5.18, and 5.19). The presence of so many compounds which elicited a negative behavioural response within a blend that was attractive/arrestant was surprising. In total, ten out of the fifteen compounds comprising the complete blend which elicited a positive behavioural response elicited a negative response when tested alone while only two elicited a positive response. This strongly suggests that the way in which the aphids perceive a complex odour blend is very much dependent on emergent properties of the blend rather than a simple summation of responses to component compounds. This may reflect a mechanism of non-host recognition that has received relatively little attention in the literature but which could have important implications for our understanding of animal olfaction in general. If a single host volatile compound is encountered in the absence of the other host volatiles, it could not have originated from the host. Instead, it must have originated from a non-host plant. In this way, volatile compounds which are also produced by the host may be used by *A. fabae* as non-host cues. By recognising volatiles as originating from a non-host plant, aphids could minimise their risk of settling on the wrong plant species by actively avoiding these volatiles, thereby increasing their foraging efficiency. *Aphis fabae* is already known to use volatile compounds specific to non-host plants to avoid settling on non-hosts (Nottingham et al. 1991; Isaacs et al. 1993; Nottingham & Hardie 1993; Hardie et al. 1994a; Storer et al. 1996) and the present study suggests that *A. fabae* may also use ubiquitous plant volatiles as non-host cues. This hypothesis could be further tested by determining behavioural responses to various blends consisting of various combinations of the ten volatiles which elicit negative behavioural

responses to determine if the behavioural effect of individual volatiles is inhibited by the presence of other volatiles. Some work in this area was attempted here with the three-component blend consisting of octanal, (*R*)-linalool, and (*S*)-germacrene D. An emergent property of perception of the three compounds as a blend caused a response different to the responses to component compounds. This represents a striking example of volatile compounds possessing context-dependent behavioural activity. Each of these three volatile compounds would normally be encountered in the context of the complete blend if the aphids were exposed to volatiles from a *V. faba* plant but encountered individually could not have originated from a *V. faba* plant and so could, in theory, represent a non-host cue.

While the present study focused on aphids, huge similarities in the mechanisms of olfaction across phyla mean that these findings may also be relevant to our understanding of animal olfaction in general (Hildebrand & Shepherd 1997; Eisthen 2004). The ability to use the same individual volatile compounds to recognise two different biological entities could help explain how animals are capable of using olfaction to discriminate such a wide range of different odour blends while possessing olfactory receptor neurones specific to a restricted range of volatile compounds. Awareness of context-dependent volatile-recognition could even have implications beyond animal olfaction. In recent years attempts have been made to develop mechanical devices for the detection of volatile compounds (Wilson & Baietto 2009). These have been used, to varying effectiveness, for the detection of volatiles associated with cancer in humans (Kateb et al. 2009; Wang et al. 2009), for assessment of food quality (Ayudhaya et al. 2009; Campagnoli et al. 2009; Rubino et al. 2009), and even for the detection of volatiles associated with explosives in the prevention of terrorist activities (Senesac & Thundat 2008). By recognising the context in which volatile compounds are detected, the range of odour blends such devices are capable of discriminating could be increased substantially without the need to increase the range of individual structural classes of volatile compounds that can be detected.

Awareness of the context-dependent behavioural activity of volatile compounds could also have implications for the use of semiochemicals in integrated pest management (IPM) strategies. Volatile compounds are often described as simply ‘repellent’ or ‘attractive’. Such descriptions may often be inaccurate due to oversimplification as the behavioural activity of a volatile compound may depend upon the ecological context in which it is perceived, as highlighted by the present study. The use of semiochemicals in IPM has received increasing

attention over the years (Agelopoulos et al. 1999a; Rodriguez-Saona & Stelinski 2009) and awareness of the complex behavioural activity of semiochemicals is essential for the success of any such practice. The context-dependent behavioural activity of certain semiochemicals could mean that different behavioural activity is observed when transferred from the clean-air conditions of the laboratory to the odour-rich environment of the field where other volatile compounds present in the atmosphere may alter the behavioural activity of the semiochemicals intended for use in pest management (Knudsen et al. 2008). Future applied studies should therefore consider this aspect when developing IPM strategies using semiochemicals and could significantly increase the chances of such practices being successful.

The response to the reduced blend consisting of all compounds except octanal, (*R*)-linalool, and (*S*)-germacrene D (Chapter 5, Figure 5.23) also suggested that not all compounds within the blend may be essential for host location. This is in agreement with other studies which have shown that insects often respond behaviourally to a blend of just a few of the volatile compounds identified in the headspace of their hosts (Birkett et al. 2004; Tasin et al. 2007; Cha et al. 2008). It may be possible to reduce the blend further. This would be useful if these host volatiles were ever used as an attractive lure used to monitor populations or as part of a push-pull system as a reduced blend would be cheaper to manufacture for large-scale field trials. Reducing the blend could be achieved through subtraction bioassays, where compounds are systematically removed from the blend until the behavioural response is lost in order to determine which components are essential and which are redundant. It is possible that different compounds may possess different levels of ‘behavioural importance’ within the blend. Previous work has shown that, for some insect species, some compounds can be removed and replaced with others without impacting significantly on insect behavioural responses whereas other compounds are essential, their removal resulting in a switching off of the insects’ behaviour (Tasin et al. 2007). The same could be true for *A. fabae* responding to host volatiles. Some of the volatiles may be removed or substituted without affecting behavioural responses whereas the removal of others may result in aphids failing to respond behaviourally to the blend at all. Investigation of the behavioural hierarchy of *V. faba* volatile compounds would therefore be an interesting focus for future work into host location in *A. fabae*.

## 7.4. Intra-specific and diurnal variability in emission of volatiles

As a starting point to testing the hypothesis that *A. fabae* uses host-characteristic ratios of volatiles to recognise its host, intra-specific and diurnal variation in ratios of volatiles emitted by *V. faba* was determined. Large variability in quantities of volatiles was observed between plants and between different time periods but some consistency in ratios of volatiles was observed with some compounds (Chapter 6, Figures 6.3 to 6.8). These findings are similar to those of previous work on potato plants which showed intra-specific and diurnal consistency in ratios of volatiles emitted (Agelopoulos et al. 2000). The difference between this and the present study is that the present study focused on volatiles electrophysiologically active to one of the herbivores of the plant whereas the previous study only focussed on those volatiles produced in the greatest quantities. The present study suggests that *A. fabae* may be able to use ratios of volatiles to recognise its host as there is a consistent signal in the form of ratios available.

The next logical step would be to follow this up with behavioural studies. The results gained in Chapter 6 show which volatile compounds are produced in consistent ratios and what the natural ranges of these ratios are. The next step would be to construct blends comprising *V. faba* volatiles at different ratios and testing them for behavioural activity in olfactometer bioassays. Some blends would contain compounds which are at ratios within the natural range of those emitted by the plants whereas others would be at ratios beyond that natural range, and therefore not representative of a *V. faba* plant. The hypothesis is that aphids will prefer blends whose ratios most closely match those that are representative of an intact *V. faba* plant and respond to blends that are not representative as though they were non-host plants. The ratios of some compounds may be behaviourally more important than others. It is already known that the presence of certain individual compounds within a blend may be more important in eliciting a behavioural response than others (Tasin et al. 2007) and the same may be true for ratios of volatiles. Altering the ratios of certain pairs of compounds may have a stronger effect on behaviour than altering the ratios of other compounds. It is expected that the ratios that are behaviourally more important will be those that showed intra-specific consistency as these are the most likely to constitute reliable host cues rather than the ratios of volatiles which did not show any consistency.

Correlations between quantities of compounds could in some cases be explained as the compounds sharing common biosynthetic pathways. In these cases, the ratios may not be specific to *V. faba* as the same biosynthetic pathways are common to many different plant species. Quantities of other pairs of compounds which did not share common biosynthetic pathways were also correlated and these may be more specific to *V. faba*. This cannot be confirmed, however, without the entrainment of sufficient non-host plants. This could be a difficult task as the number of potential non-host plants is vast and a review of the literature would not be sufficient as studies usually only report volatiles that are either produced in the greatest quantities or are electrophysiologically active to a particular insect being studied. Valuable information could still be obtained, however, by entrainment studies of some representative non-host plants.

It would be interesting to see if all the potential host plants of *A. fabae* emit the same volatile compounds in the same ratios as *V. faba*. *Aphis fabae* may have evolved to respond to a particular volatile blend which is representative of its full range of potential hosts or may respond to different cues associated with each of its potential hosts. *Aphis fabae* is believed to exist as a number of host races, each race specialised on a restricted range of summer host plant species. Previous work has shown that specialisation on a particular host can result in fitness trade-offs, restricting the ability to develop on other hosts (Thieme 1987; Mackenzie 1996; Douglas 1997). Behavioural preference for the optimal host would therefore provide a selective advantage to *A. fabae* and this has been found to be the case for some host races (Gorur et al. 2007). Studies have also shown that different host races can adapt to developing on novel hosts over time and modify their preferences accordingly (Gorur et al. 2005, 2007). Determining whether or not preference for these different hosts is mediated by species-specific blends or ratios of ubiquitous volatiles would provide an interesting starting point in determining whether or not aphids employ ratios of volatiles to locate suitable hosts and avoid those which are less suitable. This could also form an interesting basis for future work.

It is important to remember that plants entrained in Chapter 6 were glasshouse grown in the same compost and under constant environmental conditions and all at the same stage of development. In the field, aphids are faced with a much greater diversity as plants will be subject to different environmental conditions, be at different stages of development and may be subject to herbivory. All of these factors may have an effect on quantities and ratios of volatiles emitted (Engel et al. 1988; Johnson et al. 1999; Agelopoulos et al. 2000; Dudareva

et al. 2000; Gouinguene & Turlings 2002; Colazza et al. 2004; Shiojiri & Karban 2006). Entrainments of plants under field conditions would therefore provide valuable information on the diversity of volatile blends with which host-seeking *A. fabae* are faced. It would also be interesting to entrain plants under controlled conditions to determine the effects of various environmental factors on volatile emission and how aphids respond to these different blends. It is possible that ratios of different groups of volatiles represent different things to host-seeking insects. For example, the ratios of one group of compounds may be used to provide information on the identity of the plant whereas the ratios of another group may be used to gain information on the physiological status of the host. In this way the host volatiles may be divided into 'behavioural units', with different sub-blends conveying different information to insects. By understanding the ecological meaning of any such subunits it would be possible to gain much greater insight into the host location mechanisms employed by *A. fabae*.

Strong diurnal variation in ratios of volatiles was observed. This may not necessarily make the task of host-location more difficult for host-seeking aphids, however, as they tend to be more active at certain times of day. *Aphis fabae* shows peak flight activity during mid-morning and early afternoon and flight typically lasts for up to 2 h after which alighting occurs (Cammell 1981). These periods of alighting would correspond most closely to the midday and evening time periods used in the present study and aphids may respond preferentially to odour blends associated with these particular times of day. The initiation of this behaviour could occur in two possible ways. Firstly, it is possible that aphids will respond to a particular blend at any time of the day and the reason for their midday activity is that this is the only time the stimulatory blend is emitted by its host. Conversely, it is possible that aphids' behavioural responses are affected by their own circadian rhythm and aphids are only responsive to plant volatiles during a certain period of the day. Most behavioural experiments carried out during this project were conducted during the midday time period and so little information was gained to determine which of these hypotheses applies to *A. fabae*. By testing behavioural responses of aphids at different times of the day to each of the blends associated with different times of day, it would be possible to see if behaviour is driven by the circadian rhythm of the aphids or by the plants' diurnal variation in volatile emission, or by a combination of both. This would provide valuable insight into how host-seeking behaviour is initiated in *A. fabae* and would therefore be a worthwhile pursuit for future work.

## 7.5. The use of host-cues in integrated pest management

Before host volatiles can be incorporated into pest control strategies, it is necessary to gain further understanding of the type of behaviour that the host volatiles elicit. For example, an attractant would be far more useful in a monitoring trap or mass trapping system than an arrestant. Bioassays using olfactometers designed to record specific behaviours such as the linear-track olfactometer (Sakuma & Fukami 1985) or locomotion compensator (Dewhirst 2007) would be useful to this end and so a starting point for future work could be to use such methods to provide a more detailed description of the behavioural responses of *A. fabae* to identified host volatiles.

Attractive chemical cues could potentially be used in integrated pest management for monitoring aphid populations (Birkett et al. 2004), mass-trapping (El-Sayed et al. 2006), or as part of a ‘push-pull’ system or stimulo-deterrent diversionary strategy (Khan & Pickett 2004; Cook et al. 2007; Hassanali et al. 2008). Pheromones are usually selected for use in integrated pest management because they tend to elicit stronger behavioural responses than plant volatiles and so tend to be more effective at luring insects. A downside to pheromones is that they do not attract all insects at all times of the year. In *A. fabae*, only the males produced in the Autumn respond to the sex pheromone (Cammell 1981). Lures employing host-plant volatiles would therefore have the advantage of attracting more insects at the right time of year but may not compete for the insects’ attention over the crop. A more detailed understanding of olfactory host location could help improve the usefulness of plant volatiles in integrated pest management. The present study showed that different volatile blends were emitted at different times of the day. The behavioural responses of *A. fabae* may therefore be tuned to the odour of *V. faba* at a particular time of day. If a lure could be constructed to produce that optimum blend continuously, the lure may be more attractive than the crop at certain times of the day. Volatile blends can provide information on the physiological status and stage of development of the host (Engel et al. 1988; Agelopoulos et al. 2000; Dudareva et al. 2000; Shiojiri & Karban 2006). It may be possible to identify the ratios associated with an optimum host and incorporate these into an attractive lure which would be more attractive to host-seeking aphids than the main crop. Conversely, it may also be possible to use volatile blends associated with less-preferred *V. faba* plants to protect crops. As well as being a result of time of day, different individual *V. faba* plants may be more or less acceptable to *A. fabae*

due to being at different stages of development or due to differences in physiological status. These differences in acceptability may be reflected in the volatile blends emitted by the plants. By assessing olfactory preference for different *V. faba* plants it may be possible to identify which blends are associated with plants that are least preferred by *A. fabae*. Genetic modification or selective breeding could then be used to create *V. faba* plants which emit volatile blends making them naturally unattractive to aphid pests, potentially reducing levels of infestation and damage to crops.

Ten of the identified compounds were found to elicit negative behavioural responses from *A. fabae*. These compounds did not, however, deter aphids when in the context of other *V. faba* volatiles. It is therefore difficult to imagine how these volatiles could be used as repellents in the field to protect crops. It is possible that at higher concentrations they may deter aphids from settling on plants but this could only be determined with more laboratory and field experiments. It would also be interesting to see if these volatiles have similar behavioural effects on other pests of *V. faba*, to determine if any could be used as broad spectrum repellents against a range of *V. faba* pests.

All behavioural experiments carried out in this project were with the *A. fabae* clone originally isolated by Kennedy and Booth (1950). This clone was selected because much of the work on the behaviour and electrophysiology of this species has been conducted on individuals of this clone. Inter-clonal variation in host-plant preference in *A. fabae* (Thieme 1987; Mackenzie 1996; Douglas 1997) means that any conclusions drawn from the present study may only apply to this particular clone. Several clones are capable of developing on *V. faba* (Douglas 1997; Gorur et al. 2007) but may nevertheless employ different olfactory cues to recognise the same host. It is also possible that *V. faba* plants at different stages of development or in different physical condition may possess physiologies which are more or less acceptable to different clones. If this were the case then different clones may respond preferentially to different blends emitted by *V. faba* associated with these different physiological conditions. It would be useful to compare the behavioural and electrophysiological responses of other clones with that used in the present study to determine if all clones respond in the same way to the same volatile compounds. Such research would be important for understanding the potential applications of these semiochemicals in IPM. Should each clone respond differently to different *V. faba* volatiles then the use of any of these semiochemicals in future IPM strategies would be severely limited as they may be ineffective against all but a few clones.

## 7.6. Conclusions

Volatile compounds emitted by *V. faba* which are electrophysiologically active to *A. fabae* were identified. None of the identified compounds were specific to *V. faba* and olfactometer bioassays showed that the response to the complete blend could not be explained by a response to a single compound. Several of the compounds elicited negative behavioural responses from the aphids when presented alone but not when in the context of the blend. It was hypothesised that *A. fabae* recognises its host using species-characteristic blends of volatile compounds. Entrainments of *V. faba* plants showed that there is some consistency in ratios of certain volatile compounds, meaning that ratios could feasibly provide a host-characteristic cue. Olfactometer bioassays also suggested that aphids may use individual volatile compounds as non-host cues when not found in the context of other *V. faba* volatiles. There are several directions that could be taken for future work. The preliminary evidence of context-dependent behavioural activity of volatile compounds could lead to some interesting future studies on this method of odour-recognition. Such studies could greatly enhance our understanding of animal olfaction and perhaps even enhance the success of future IPM strategies by helping us to understand how the behavioural activity of semiochemicals may be affected by the presence of other volatiles in the field. Another direction for future work would be to determine behavioural responses of *A. fabae* to blends with components at different ratios both within and beyond the natural range of ratios emitted by *V. faba*. This would make it possible to determine whether or not *A. fabae* uses ratios of volatiles to recognise its host. Further work could also look at how ratios of volatiles reflect the physiological condition of a potential host plant and whether or not *A. fabae* can use ratios to identify optimum host plants in this way. Diurnal variation in ratios may be the factor which initiates host-seeking behaviour in *A. fabae* and by determining behavioural responses to blends associated with different times of day, this hypothesis could be tested. Finally, by testing the behavioural responses of other *A. fabae* clones to the semiochemicals identified in the present study it should be possible to determine whether the conclusions drawn here apply to the species as a whole or just to this single clone. This could have serious implications for the potential application of the findings of this study in future IPM strategies. As well as adding to our understanding of aphid host location, several new questions have been raised as a result of this research project. Answering these questions in future studies would greatly

enhance our understanding of how *A. fabae* uses volatile compounds to recognise its host and may eventually lead to new methods of controlling this insect pest.

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