Floral derived compounds as attractants for agricultural pests in the family Noctuidae

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A thesis submitted in partial fulfilment of requirements of the

University of Greenwich for the degree of Doctor of Philosophy

December 2014

DECLARATION

I certify that this work has not been accepted in substance for any degree, and

it is not currently being submitted for any degree other than that of Doctor of

Philosophy being studied at the University of Greenwich. I also declare that this

work is the result of my own investigations except where otherwise identified by

references and that I have not plagiarised the work of others.

Student's signature_____Date_____

Supervisor's signature_____Date_____

Acknowledgements

I am hugely thankful to my supervisors, Professors David Hall, John Colvin, and Alan Cork, without whom this thesis would not have been possible. A special note of thanks to David, whose proof-reading, excellent suggestions, and regular emails to say "just get it finished", finally came to fruition – it finally is finished. Thanks David.

I thank Alan for setting me on the interesting path of floral semiochemicals and for his support during the early stages of my research.

I am extremely grateful to the University of Greenwich for funding the research, and to the Natural Resources Institute for being flexible with regard to me studying and working for them at the same time. This would not have been possible without the support of Mark Parnell, Heather McAvoy-Marshall, John Orchard, and Natalie Morley. It was a real pleasure working with you and all of the staff and students at the NRI.

A special thanks goes to David Grzywacz and Aliyu Aminu for their help with the insect colonies, and to Dudley Farman and Paul Douglas for practical scientific (and non-scientific) advice - sometimes given whether I wanted it or not but always useful. I would like to thank Simon Springate whose wit kept me just about sane whilst in the throes of writing and Stephen Young who taught me to somehow enjoy statistical analysis.

The field trials in Argentina were made possible, not to mention hugely enjoyable, by Enrique Lobos and his team who provided such excellent help and advice as well as their time.

Abstract

Many species of moths within the family Noctuidae are significant agricultural pests. Specific floral volatiles are attractive to both male and female Noctuidae and may be used to as attractants in crop protection. For the first time the following research compares two types of floral volatile blends - those that mimic natural floral odours and those that are artifical odour blends ('super-blends'). In wind tunnel bioassays and field trials in two diverse geographic locations (Argentina and the United Kingdom) a range of noctuid moth species that are considered crop pests were found to be attracted to both types of the floral odour blends. However, a 'super-blend' containing phenylacetaldehyde, salicylaldehyde, methyl 2-methoxybenzoate, linalool, and limonene (in a 10 : 4 : 2 : 2 : 1 ratio) was found to be the most effective general attractant across the following species: *Helicoverpa armigera* and *gelotopoeon*, *Heliothis zea, Spodoptera frugiperda*, and *Autographa gamma* suggesting that these compounds are universal cues to this family of moths when searching for flowers.

Further behavioural bioassays found that the physiological state of the insect had an important effect on its behavioural response to the floral odour super-blend. Bioassays carried out on *H. armigera* revealed that gravid insects were significantly less likely compared to virgin insects to make contact with an odour blend baited lure. In addition, insects provided with sucrose solution were significantly less likely to make contact with the odour source compared to starved insects or insects only provided with water. This is the first time this affect has been seen in this species and may have important implications for using these types of floral odours for crop protection.

Investigations into the most effective trap for capturing Noctuidae found that a homemade bucket and water trap or funnel and sleeve traps were significantly more effective than UniTraps or sticky traps. During the field trials large numbers of non-target insects were also captured, including beneficial insects and pest species. By using green coloured traps captures of beneficial hymenoptera (Syrphidae and Apoidea) were significantly reduced. Taken together, the current findings provide insights into how Noctuid moths interact with host odour cues and how they may be used in developing pest management techniques.

Contents

List of abbreviations

AIC = Akaike information criterion

AL = antennal lobe

ap = after pupation

AVI = Audio Video Interleaved

CRD = Chemicals Regulation Directorate

FV = floral volatile

GLV = green leaf volatile

i.d. = internal diameter

IR = infra red light

KI = Kovat's Indices

LIS = linalool synthase

Lm = limonene

Ln = linalool

M2M = methyl 2-methoxybenzoate

OBP = odorant binding protein

ORN = olfactory receptor neuron

PAA = phenylacetaldehyde

RI = retention indices

RPA = relative peak area from a gas-chromatograph signal

RPH = EAG response relative to peak area

Sa = salicylaldehyde

SAG = sex accessory gland

sEAG = standardised EAG value

UoG = University of Greenwich floral odour blend (comprising phenylacetaldehyde, salicylaldehyde, methyl 2-methoxybenzoate, linalool, and limonene)

VOC = volatile organic compound

CHAPTER 1 - INTRODUCTION

Plants and insects have been sharing terrestrial land since the Devonian age (*ca.* 400 million years ago) (Metcalf and Metcalf, 1992a). From this time the two groups have been engaged in a constant battle to survive and reproduce, leading to the continuous need for species to better utilise resouces around them and to defend themselves from other organisms and the environment. In some instances this has led to a mutualistic coevolution (e.g. pollinators and angiosperms, predatory insects and plants under attack from herbivores) and in others an antangonistic relationship has developed (e.g. herbivores and plants) (Ehrlich and Raven, 1964; Schoonhoven *et al.*, 2005a; Farré-Armengol *et al.*, 2013). The coevolution between plants and insects is evident in the close connection between the floral VOCs (volatile organic compounds) produced by plants and the insect's ability to detect and behaviourally respond to those compounds. Angiosperms have evolved a system of reproduction that relies on the transfer of their genetic material via other species (pollinators). In order to achieve this reproduction they recruit insects by providing food (nectar). Flower constancy and insect associative learning increases the chance that an insect will carry the plant's genetic material to a conspecific (Schoonhoven *et al.*, 2005c). Further evidence for coevolution between plants and insects comes from the large crossover in VOCs produced by the two groups. Schiestl (2010) showed that of the 71 common floral volatiles 87 % of these were also produced by insects and used in insect-insect communication (i.e. as a pheromone or allomone - see section 1.1.1 for definitions) with a correlation in volatile production between angiosperms and pollinators but not between gymnosperms and pollinators. This suggests adaptive evolution either from plants utilising VOCs to recruit insects by producing the compounds insects themselves use to communicate, or vice versa, i.e. insects

evolving to use plant compounds in their own communication, or more likely a combination of the two.

Plants and insects have the ability to evolve quickly and this has led to one of greatest risks currently facing agriculture: the development of resistance to pesticides. In an incredibly short period of time (chemical pesticides have been deployed since the 1940s) and at an exponential rate weeds have developed resistance to many herbicide modes of action (Prather *et al.*, 2000) and insects have developed resistance to almost every insecticide mode of action (Hardy, 2014). For this reason it is imperative that research is carried out into alternative pest control strategies.

The notion that an insect may be controlled by altering its behaviour in such a way that it is removed or kept out of an area where it is not wanted or encouraged into an area where it is wanted is a captivating one. It allows us to encourage beneficial insects into crops and attract pests away from crops, reducing the need for spraying pesticides directly onto crop plants (further benefiting predators and parasitoids of pests, as well as pollinating insects) and reducing pesticide residues. It is not surprising therefore that much research has been and still is underway to find semiochemicals that will fulfil these roles. Many types of semiochemical are in use or under investigation for use as crop protection tools. One of the most commonly used group of semiochemicals being sex pheromones which are generally used either for pest population monitoring or mating disruption (Witzgall *et al.*, 2010).

Although sex pheromones fit some of the requisites of a suitable semiochemical type for crop protection (i.e. highly attractive at relatively long distances, species-specific) they suffer from an integral problem in that they only attract one sex which in Lepidoptera is the male. This problem is exacerbated because it is the larvae that

cause the loss of yield, and a larva's location is determined by the female parent. Therefore, what is required is an attractant that is also attractive to females. As female Noctuids do not respond behaviourally to sex pheromones, this leaves plant-emitted gustatory semiochemicals and oviposition semiochemicals, or perhaps a combination of the two. The aim of this study is therefore to identify highly attractive floral odours for noctuid moths and identify whether these blends are attractive because they stimulate foraging for food or oviposition sites, the former being more likely to be attractive to females and males and latter being more attractive to gravid females.

1.1 CHEMICAL COMMUNICATION IN INSECTS

1.1.1 Semiochemicals

It may be said that insects live in a chemical world. This is succinctly stating that insects use chemical cues to a large extent for many of their behaviours. Searching for food, mates, oviposition sites, and social communication all require information received and/or delivered in the form of chemicals. It is therefore not surprising that insects have evolved a highly sensitive array of organs, cells and behaviours for the detection and use of chemicals.

For Lepidoptera searching for food or an oviposition site, these chemical information packets come in the form of volatile chemicals (Shields and Hildebrand, 2001) which are sensed by the insect's olfactory system. For oviposition the primary cue is olfactory (Ramaswamy, 1988). Chemical information in the form of solid or dissolved chemicals sensed by the gustatory system require much higher concentration of these chemicals to elicit a response (Schneider, 1969). This project is concerned with the effect of volatile organic compounds (VOCs) emitted from plants and their corresponding detection and behaviour elicited in insects, specifically those from the

family Noctuidae. It is hoped that the work presented here will lead towards an attractant that may be used in the management of noctuid agricultural pests.

1.1.1.1 <u>Semiochemicals and insect behaviour</u>

Semiochemicals are chemicals that transmit information with the ability to modify the behaviour of the receiver. They may benefit the sender (allomones), disadvantage the sender (kairomones) or benefit both (synomones). Dethier (1960) categorised semiochemicals by the behaviour they elicit in the receiver (see Table 1.1 for a description of terms).

Table 1.1. Semiochemicals categorised by the behavioural response they elicit in the receiver. reproduced from Dethier *et al.* (1960).

Attractant	A chemical that causes insects to make orientated movements to locate the source
Repellent	A chemical that causes insects to make orientated movements away from the source
Arrestant	A chemical that may slow the linear progression of an insect by reducing actual speed of locomotion or by increasing turning rate.
Feeding or oviposition stimulant	A chemical that elicits feeding or oviposition in insects ('feeding stimulant' is synonymous with 'phagostimulant')
Feeding or oviposition deterrent	A chemical that inhibits feeding or oviposition when present in a place where insects would, in its absence, feed or oviposit

Semiochemicals are primarily received in the gaseous state having volatilised from the source and are carried by air currents and by diffusion to the receiver (see section 1.1.1.2 for further details of VOC's movement through air). In order to make use of these olfactory cues, insects have evolved complex behaviours with which they can locate potential mates, food sources, or oviposition sites.

From a stationary, grounded position a moth may be stimulated to take flight by non-directional (kineses) or directional cues (taxes). Non-directional cues may be internal (idiothetic e.g. reduced level of carbohydrate in the haemolymph), or external (alleothetic e.g. temperature or humidity) (Hardie *et al.*, 2001). For behaviours relating to location of a resource by moths the initial cue to take flight is likely to be an olfactory cue. However, even if the olfactory stimulus is combined with information on the direction of air movement, the turbulent nature of the wind makes it unlikely that the moth can use the air direction information to ascertain the direction of the odour source. Therefore, for a moth to locate the odour source actively the initial stimulus must be followed by taxis (Figure 1.1), i.e. cues that provide information on the direction of the odour source relative to the position of the moth. The moth achieves this directional information by employing pre-programmed behaviour patterns that aid the moth in maintaining contact with the odour plume as much as possible, whilst also reducing the distance between the odour source and the moth. These behaviour patterns are known as 'casting' and 'zigzagging' and aid the moth in locating and relocating and hence following an odour plume (see Figure 1.2) (David and Kennedy, 1987; Cardé and Willis, 2008). Casting occurs when the moth has lost the olfactory stimuli (or perhaps contacted an incorrect stimulus rather than the one it is tracking). The behaviour is defined by lateral movement, relative to its previous trajectory, with little longitudinal movement and larger turns (David and Kennedy, 1987; Hardie *et al.*, 2001; Schoonhoven *et al.*, 2005b). The aim of this behaviour is to

maximise the chance of re-contacting the lost odour plume. Zigzagging occurs while the insect is in contact with the odour plume, and is defined by lateral movement combined with longitudinal movement such that the insect travels towards the odour source with short sharp turns (David and Kennedy, 1987). The odour plume is not a continuous region of odour molecules, but rather patches of space containing odour molecules (olfactory stimulating) interspersed with air that contains no relevant odour molecules (non-stimulating). Moths attempting to locate an odour source respond to the frequency of stimulating to non-stimulating air (Gibson and Torr, 1999). The amplitude of the zigzag may be determined by the length of time between olfactory stimuli, i.e. the 'patchiness' of the plume.

The casting behaviour also occurs when a moth enters an odour cloud (evenly dispersed continuous odour molecules), presumably because continuous or over stimulation of the olfactory system conveys as little information as no olfactory stimulus (Cardé and Willis, 2008).
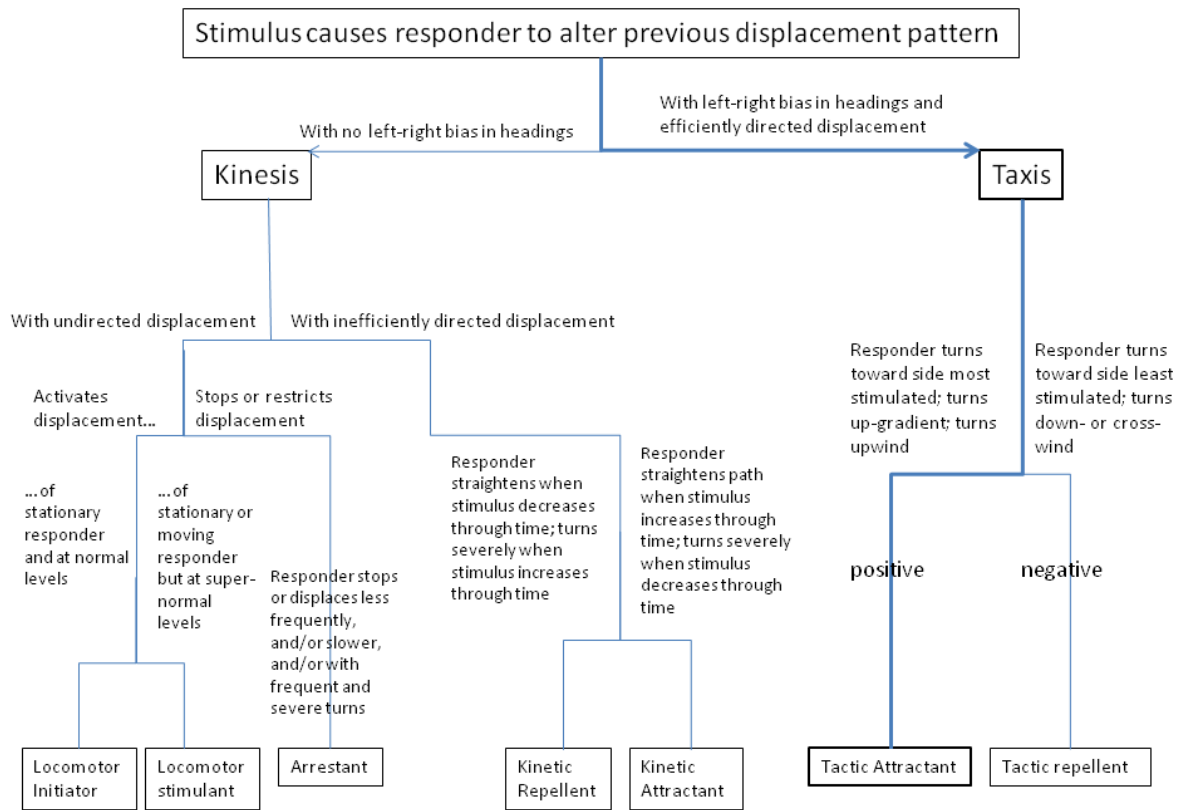
Figure 1.1: Behaviours that define types of semiochemicals. The bold lines indicate the desired effect of a floral lure. Reproduced and modified from Miller *et al.* (2009).
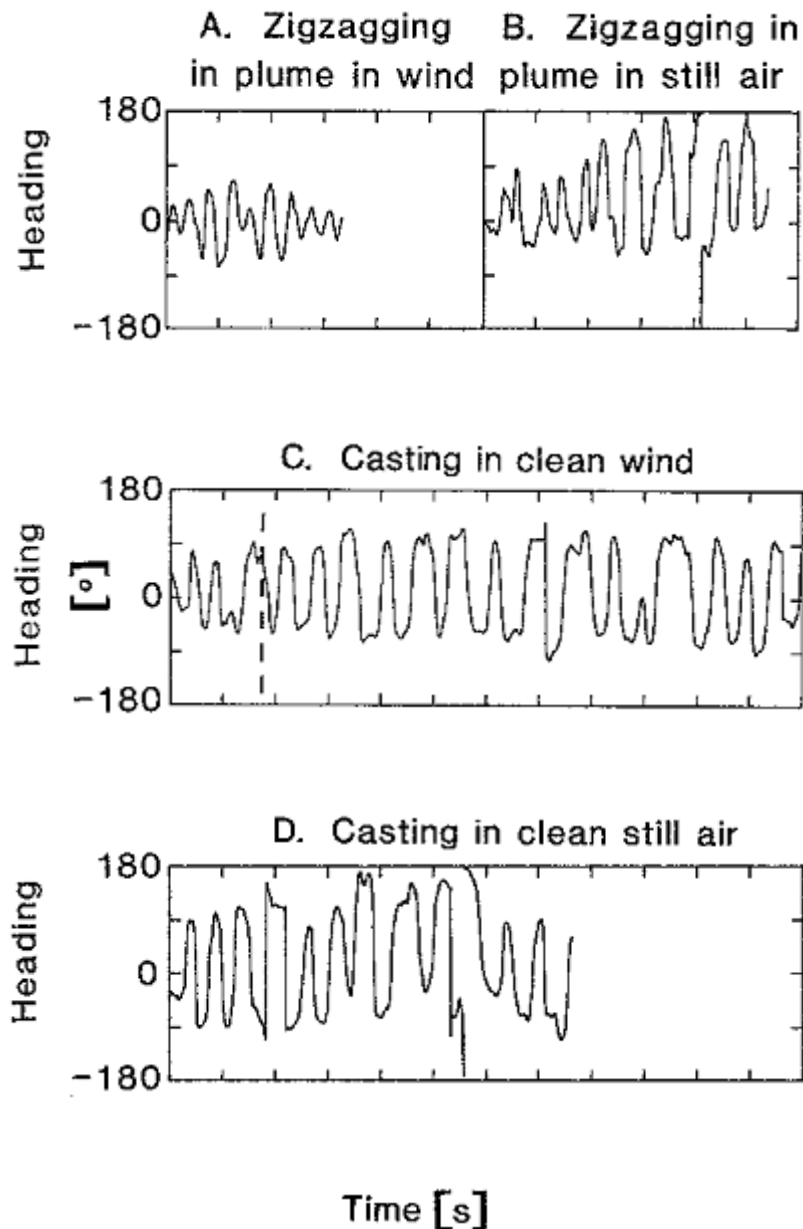
Figure 1.2: Time series of heading from a male Gypsy moth (*Lymantria dispar*) flying in a wind tunnel. Where present, the plume is the sex pheromone. The vertical dashed line in C. indicates where the odour plume was stopped. Taken from David and Kennedy (1987).

1.1.1.2 Structure of Odour Plumes

Murlis (1986) defined an odour plume as the structure of the trail of odour molecules as they travel from their source, carried by wind. The structure of an odour plume depends on the scale that is being visualised, for as resolution increases so does the complexity of the odour plume structure. Early models (low resolution) used to describe plume structure applied a Gaussian distribution for concentration over time

(with diffusion coefficients as constants defined by the atmospheric conditions) and distance from the source applied as a power. This 'time-average' model of plume structure creates a simple expanding structure of continuous odour molecules with decreasing concentration downwind of the source and from the central plume line (i.e. longitudinally and laterally) (Murlis *et al.*, 1992). This model was expanded upon to incorporate the meandering feature of odour plumes (Gifford, 1959; Murlis *et al.*, 1992). This development showed that at a fixed downwind position, an insect will come into contact with large differences in odour concentration as the plume meanders into and out of the insect's space. At any given point along the plume length, a cross-section 'slice' of the odour plume will show an evenly dispersed concentration gradient in all directions from the centre (or plume line) of that slice. It is this centre line that meanders, giving rise to the changing concentration the insect experiences in its fixed position.

Further increases in the resolution of the odour plume, using an ion sensor, have found that within the meandering odour plume there are small-scale fluctuations made by odorous regions interspersed with pockets of odourless regions (Murlis and Jones, 1981; Cardé and Willis, 2008). These are relative to the small-scale air turbulence and the size of the odour source. An odour source that is smaller than the smallest air turbulence will have a greater number of odourless pockets than a larger source (Murlis *et al.*, 1992). This is due to the way odour molecules behave immediately after evaporation from a source. The initial movement after evaporation is governed by Brownian motion which dilutes molecules evenly. Once the odour molecules reach a volume equal to the size of the smallest air turbulences (eddies), their movement becomes governed by these eddies which will mix pockets of air in with the odour molecules. The size of the eddies is defined by the Kolmogoroff scale, which in the atmosphere is "very approximately a centimetre" but only a fraction of a

millimetre in a wind tunnel (Murlis, 1986). This forms the plume into what has been described as turbulent filaments of odour interspersed with non-odorous air, with the odour concentration within filaments near the source and far from the source being extremely similar (Murlis, 1986). Therefore, until insects come extremely close to the source, the odour concentration cannot be used to estimate distance from that source (Murlis and Jones, 1981).

Thus an odour plume's shape is a function of the resolution at which it is measured, the environment in which it is present, and the size of the odour source.

Odours of interest to flying insects may originate from three general source sizes: intraspecific pheromone (characterised by a point-source, possibly intermittent in some species), floral odours (characterised by numerous point-sources) (see section 1.2.1), and green leaf plant odours (characterised by a wide-area source).

1.1.1.3 <u>Pheromones</u>

Semiochemicals that are used for communication within a species are known as pheromones. These intraspecific chemicals may be used to signal various types of information within a species' population. For example, a sexually mature female may advertise her presence to males using sex pheromone, or an aphid suffering predation may emit alarm pheromone to signal the danger to its conspecifics triggering them to move away from the area (Pickett *et al.*, 1992). In Lepidoptera sex pheromones are volatile and generally emitted by the female. The requirement of volatility means that the compounds generally have a molecular weight of between 80 - 300 daltons and 5 - 20 carbon atoms (Cork, 2004). In some lepidopteran species males also emit short-range sex pheromone from their pencil-hairs on their abdomen which increases the chances of copulation with an accepting female once in close proximity (Nishida *et al.*, 1982). In contrast the female produced sex pheromone is

extremely long-range and can stimulate upwind flight in males several hundreds of meters away and perhaps further (Wall and Perry, 1987). Short-range and long-range may be defined by the method of odour particle movement, short-range being by diffusion, long-range being carried on air currents. One of the characteristics of pheromones (and in particular for sex pheromones) is the requirement for an accurate ratio and composition of the volatiles that comprise the pheromone. This ratio is maintained as the pheromone odour packets move away from the emitter; the mechanism for this is described in section 1.1.1.2. Pheromone specificity is evident in the closely related Noctuids of the Plusiinae species: *A. gamma*, *Trichoplusia ni*, and *Chrysodeixis chalcites*. As is common in other Plusiinae, the pheromone of all three species contains (*Z*)-7-dodecenyl acetate (Z7-12Ac) as the major component, but each species also contains minor components. The presence of one of the minor components: Z5-12Ac, Z9-14Ac, or Z7-14Ac from the other species' pheromone significantly inhibited the behavioural responses of male *A. gamma* to its normal pheromone (Mazor and Dunkelblum, 1992).

1.1.1.4 <u>Kairomones</u>

Kairomone is the term given to semiochemicals that benefit the receiver but not the emitter. Examples of these types of compounds include plant compounds used by herbivores to locate their host plants. Compounds emanating from plants that are insect kairomones generally have a maximum molecular weight of 250 and originate from osmophores, glandular trichromes of leaves and stems, ducted oil cavities or glands, and oil cells of leaves and fruits. Odour production and release is related to air temperature and utilises diffusion and exudation from plants tissues to the external environment (Metcalf and Metcalf, 1992a). One of the most powerful plant-insect interactions involving kairomones can be seen in fruit flies within the family

Dacinae. Many species are highly attracted to the plant-emitted compounds methyl eugenol or cuelure (and raspberry ketone, which cuelure readily breaks down to) (e.g. Vargas *et al.*, 2000; Keng-Hong and Nishida, 2005). The level of attraction and distance can rival that of sex-pheromones in Lepidoptera and similarly attracts male Dacus flies to their host plants and conspecifics. Subsequently feeding and mating occurs and the gravid females lay their eggs into the fruit of the host plant (for a review see Metcalf and Metcalf, 1992b and the references therein).

Whether a compound acts as a kairomone or otherwise is not necessarily as clear cut as it might seem. The effect of a kairomone on the receiver depends on a variety of factors including the accompanying compounds, concentration and ratio of the compounds, background odour, and the physiological state of the receiver (described in more detail in sections 1.1.2 and 1.1.3). For example, identified host volatiles of the black bean aphid, *Aphis fabae* (Scopoli) (Aphididae), were found to act as repellents when presented individually (at behaviourally relevant concentrations). However, when these repellent compounds were combined this blend was attractive to the aphid (Webster *et al.*, 2010). This highlights that interpretation of VOCs by insects is dependant not only on the chemicals received but also the context of the chemicals.

1.1.1.5 <u>Synomones</u>

Communication via semiochemicals is not always one-sided and mutually beneficial interactions also occur. The floral compounds emitted by flowers to offer nectar and pollen in return for pollination are the most well known example of these semiochemicals, termed synomones. Floral scents are incredibly diverse with over 1700 compounds from 990 taxa identified so far (Knudsen *et al.*, 2006) and the mutal benefit to the plant (pollination) and to insect (food) is clear. However, there are other

more subtle examples of mutual semiochemical communication which are evident in certain types of plant defence mechanisms. During oviposition the elm leaf beetle, *Xanthogaleruca luteola,* mechanically damages elm leaves and also secretes an "egg glue" that fixes its eggs in place. The combination of mechanical damage and oviduct secretion was found to initiate systemic release of volatiles by the plant that were attractive to the egg parasitoid *Oomyzus gallerucae* (Meiners and Hilker, 2000). In a similar plant-insect-carnivore interaction involving the pine sawfly, *Diprion pini,* pine needles from *Pinus sylvestris*, and the egg parasitoid *Chrysonotomyia ruforum,* it was found that this mechanism of plant defence probably utilises jasmonic acid (Hilker *et al.*, 2002). The responses of plants to oviposition can vary depending on the species that placed the eggs. For example, oviposition from a generalist moth (*Mamestra brassicae*) onto wild crucifer (*Brassica nigra*) plants caused different changes in VOC emissions compared to the plants being infested by a specialist moth (*Pieris brassicae*) (Fatouros *et al.*, 2012). However, although parasitoids attracted to these volatile emissions could discriminate between infested and clean plants they could not discriminate between plants with either *M. brassiae* or *P. brassicae* eggs even though for one of the species of parasitoid *M. brassicae* was an unacceptable host.

Volicitin (N-(17-hydroxylinolenoyl)-L-glutamine) identified from the regurgitate of recently fed *Spodoptera exigua* (beet armyworm) was found to trigger an indirect plant defence in *Zea mays* (Alborn *et al.*, 1997). The effect of volicitin and mechanical damage on maize plants induced the plants to released specific volatiles including: (*Z*)-3-hexenyl acetate, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, α-*trans*-bergamotene, (*E*)-β-farnesene, (*E*)-nerolidol, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. These compounds and others had been previously found to be released by maize seedlings under attack from *S. exigua* larvae. Experienced (i.e.

had previously successfully oviposited) larval parasitoids, *Cotesia marginiventris* (Braconidae), learned to use these induced plant volatiles to locate their prey (Turlings *et al.*, 1990). This demonstrates magnificantly how plants use their secondary metabolites as synomones to enlist the help of herbivore predators.

These types of plant defence mechanisms that have a mutual benefit for the plant and the parasitoid or predator are reviewed by Takabayashi and Dicke (1996) and (with a more molecular theme) by Fürstenberg-Hägg *et al.* (2013) .

1.1.1.6 <u>Allomones</u>

In constrast to kairomones, allomones benefit the emitter but not the receiver. The classic example of allomones are plant defence compounds that protect plants from herbivory.

One of the most potent and studied plant derived anti-feedants is azadirachtin (commonly known as 'neem') produced by *Azadirachta indica* (Meliaceae), the neem tree. The chemical is present in various parts of the tree but is highest in the seed kernel and has several main effects on insects that ingest it, i.e. it stops the insect from feeding and it causes physiological effects such as disruption and inhibition of growth, and death (Schoonhoven *et al.*, 2005d and the references therein).

More subtle plant defence compounds have been identified that influence the host selection of ovipositing *Manduca quinquemaculata* (Sphingidae). In *Nicotiana attenuata* (Solanaceae) the release of specific plant volatiles increased in response to herbivory by several insects including *M. quinquemaculata*; these volatiles included methyl salicylate, *cis*-α-bergamotene, linalool, and other green leaf volatiles (Kessler and Baldwin, 2001). When a dispenser releasing racemic-linalool was applied to the base of *N. attenuata* the plants received significantly fewer (2.4 fold

reduction) eggs from ovipositing *M. quinquemaculata*. A similar (but less pronounced) effect was seen with the application of the known plant defence primer methyl jasmonate. The greatest effect was seen by the presence of *M. quinquemaculata* larvae. The authors conclude that after herbivory the plant increased releases of specific volatiles to deter further oviposition (Kessler and Baldwin, 2001).

1.1.2 Perception of Semiochemicals

1.1.2.1 Antennal Development

Dethier (1986) stated that *"chemoreception, the capacity of a cell to react with foreign molecules without transporting them into itself for metabolic purposes, is one of the earliest hallmarks of life"*. Selective permeability in the membranes of the earliest of organisms or proteinoid structures was the birth of chemoreception. From this ability to selectively allow entrance into the cell of the external chemicals evolved the processes that became 'olfaction' and 'taste'. The membrane structures permitting specific external chemicals to enter the cell led to the development of other membrane structures that were capable of binding and reacting with external chemicals, thus starting the process of chemoreception (Dethier, 1986).

By linking motility and chemoreception these early pioneers of life developed chemokinesis (and chemotaxis). This important leap allowed organisms to develop behaviours common to all modern motile species: attraction and repellency (as defined by Dethier (1960), Table 1.1).

In Lepidoptera the main olfactory organs are the antenna. For holometabolans, even at the larval stage they have become innervated and thus join the two chemoreceptor organs to the deutocerebrum. However, they are little more than stumps made of

three segments, and contain only three basiconic sensilla used in detection of VOCs (Hansson, 1995; Juma *et al.*, 2008). These larval antennae will form the imaginal disks (or imaginal primordium) that develop into the antennal structures seen in adult Lepidoptera. The development of the adult antenna during pupation in lepidopteran *Manduca sexta* (L.) (Sphingidae) can be seen within the first 2 h. after pupation (a.p.) and the annuli are visible. Prior to pupation the antennal primordia cells are pulled into position by the shedding of the larval cuticle and hydrostatic pressure continues this process. Before the third day a.p. the developing antennae remain inverted with the 'to be external' scale producing epithelium facing inwards and the sensory epithelium facing outwards, whilst the antennal epithelium cells remains attached to the cuticle. After approximately 72 h. the antennal epithelium detaches from the cuticle (apolysis) which allows it to develop the adult antenna and sensilla. It is thought that some or all of these processes may be regulated by the hormone group of ecdysteroids (Franco *et al.*, 2007). For structurally simple antennae such as those seen in the Noctuids of interest in this project, both the pupal and adult antenna are approximately tubular and therefore little morphological changes are required (Keil, 1999).

1.1.2.2 <u>Antennal Structure</u>

The insect antenna is the primary organ used to sense molecules in the gaseous phase. In Lepidoptera the antenna are annulated and are divided into three sections (Chapman, 1983; Koh *et al.*, 1995; Hansson, 1999):

1. *Scapus* – the segment at the base of the antenna. It is attached to the head by the antennifer with a ball and socket joint. It is therefore able to move in all directions. The levator and depressor muscles connect the anterior tentorial

arms in the neck to the scape and are partly responsible for antennal movement.

2. *Pedicellus* – is attached to the scapus with an elastic membrane. Flexor and extensor muscles connecting the scape to the pedicel are responsible for the rest of the antennal movement. The pedicellus also contains Johnston's organ which is involved in mechanoreception.

3. *Flagellum* – is the longest part of the antenna and contains most of the sensilla. The flagellum is sub-divided into annuli which are not true segments.

(i) Sensilla

The adult lepidopteran antennae may contain thousands (and in some cases tens of thousands) of sensilla. The sensilla are the cuticular structures used for allowing the olfactory receptor neurons (ORN) access to odour molecules. They are usually located on the ventral side of the antenna while the dorsal side is covered in scales.

The sensilla can be divided into groups defined by their form and function. However, all types share a basic structure: one or more ORN lie slightly below the base of the sensillum (see Figure 1.3). The ORN's outer dendrite arborise into the body of the sensillum (Hansson, 1995). These branches of the outer dendrite are surrounded in dendritic fluid. The dendritic fluid bridges the lymph space between the dendrite and the cuticle wall of the sensillum. In olfactory sensilla the cuticle wall is multi-pored. There are various ancillary cells positioned around the ORN which are first involved with the development of the sensillum, and a.p. provide the ionic chemicals in the sensillum lymph required for the sensillum to function. These ancillary cells are the: thecogen, tormogen, and trichogen (Hansson, 1995). The inner sensillum lymph is contained within the thecogen, which at the cuticular end terminates in the fine

cuticular sheath. The outer sensillum lymph is contained within the trichogen; the dendrites protrude into this liquid filled space (Hallberg *et al.*, 1999).
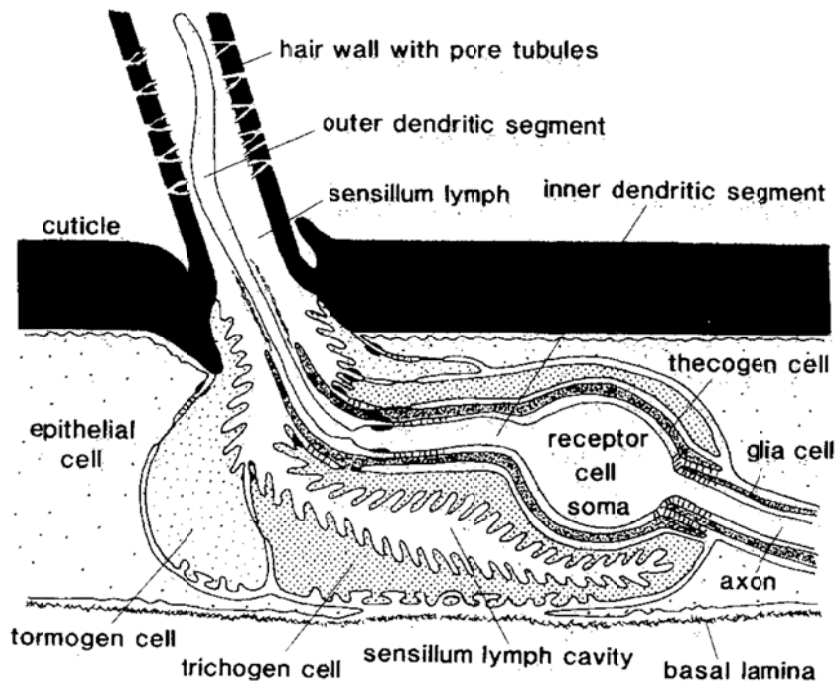
Figure 1.3: Schematic drawing of a lepidopteran olfactory sensillum. (Redrawn after an original by Keil (1999).

(ii) Sensilla trichodea and basiconica

Sensilla known to be involved in plant odour detection are the trichodeum, basiconicum, and possibly the auricillica. The s. trichodea (or trichoidea) are long and thin (hair-like) with lengths of 30 – 300 µm. They are known to be used for pheromone and odour detection. The s. basiconica are shorter and fatter and also involved in pheromone and plant odour detection. The pores present in the cuticle of both of these sensilla allow odour molecules to enter the sensillum lymph (Hansson, 1995).

1.1.2.3 Olfaction in adult Lepidoptera

As previously indicated odour molecules enter a sensillum via the pores in the cuticle and follow the route described below (Figure 1.4).

Odour molecule contacts sensillum cuticle

↓

Odour molecule enters the pores of the cuticle (possibly aided by the lipid surface of the pores)

↓

At the base of the pores the odour molecules comes into contact with the dendritic fluid in the lymph space

↓

Odour molecule is bound with Odour Binding Proteins (OBPs) (pheromones are bound by Pheromone Binding Proteins (PBPs))

↓

The bound complex allows the odour molecule to dissolve in the dendritic fluid and travel by diffusion to the branched tendrils of the dendrite membrane

↓

The OBP-odour molecule complex binds to the olfactory receptors (OR) on the dendrite's membrane

↓

Binding of the complex results in opening of ion channels in the dendrite membrane

↓

Leading to an influx of ions (Ca^{2+}) which depolarises the dendrite

↓

Once the depolarising threshold has been reached an action potential is initiated

↓

The neuron transmits the action potential along the axon to the glomeruli in the antennal lobe (AL)

Figure 1.4: Route taken of an odour stimulus starting from external odour molecules to termination of nerve pulse in the antennal lobe.

The action potential is generated by the ORN and delivered to the antennal lobe (AL) via the axon. Within the AL there is a degree of sexual dimorphism. For males ORNs

receptive to pheromones terminate in the region known as the macroglomerular complex (MCG) which is a large region containing a number of glomeruli specific to their sex (Lei and Vickers, 2008). The glomeruli within the MCG are named the cumulus, toroid 1, and toroid 2 (Shields and Hildebrand, 2001). Female *Manduca sexta* have a female specific region of glomeruli homologous to the males' MCG. This region contains two large glomeruli (the 'lateral large glomeruli' and the 'medial large glomeruli'), and a third smaller glomeruli (the 'small female glomeruli') (Rospars and Hildebrand, 2000). This female specific region of three glomeruli may play an important role in host-plant recognition as stimulation of the antenna with linalool (a plant emitted monoterpene) showed excitation of the neurons feeding into the Large Female Glomeruli (LFG) (King *et al.*, 2000; Shields and Hildebrand, 2001). This LFG region was also found (in *H. virescens*) to be the terminal point for sensilla responsive to intra-specific pheromone compounds (Hillier *et al.*, 2006). The ability of females to recognise their own species' sex pheromone may allow conspecifics to avoid intraspecies competition.

1.1.2.4 Stimulus load and olfactory response

Insects are staggeringly sensitive to odour molecules. Work measuring cardio responses to odours in *S. littoralis* found that male insects were capable of detecting as few as 6 molecules of their sex pheromone contacting their antenna in 1 s, and females could detect approximately 10 molecules of certain plant volatiles (Angioy *et al.*, 2003). Studies have shown that the concentration of chemicals presented to a lepidopteran antenna (or odour receptor at the smaller scale) influences the size of the neuron response that enters the insect's antennal lobe (Hartlieb *et al.*, 1997; Angioy *et al.*, 2003; Fraser *et al.*, 2003). Further, extremely high concentrations can change an odour receptor's specificity causing a neurological response to a chemical

the receptor would not respond to at a lower concentration (Hartlieb *et al.*, 1997). Moreover, changes in concentration (of a single chemical) have been shown to alter the principle activated glomeruli in the antennal lobe, meaning that as concentration changes, so does the pattern of activated glomeruli and presumably therefore the insect's perception of the odour (Carlsson and Hansson, 2003). This may explain why at high concentration compounds can become behaviourally repellent when at lower concentrations they were attractive – the odour is perceived differently by the insect at the high concentrations. Once initial processing has occurred in the antennal lobe the olfactory data continues on to the mushroom bodies, and lateral protocerebrum (Mustaparta, 2002). The mushroom bodies are associated with learning, whilst the lateral protocerebrum connects with motor neurons (Mustaparta, 2002 and the references therein). Electroantennogram (EAG) responses to volatile chemicals are often found to exhibit an '*S*'-shaped curve (on a log scale x-axis, see section 5.3.2.3) i.e. response increases in relation to concentration up to a plateaux but then sharply falls. Likewise, behavioural response to attractants has been found to follow a similar trend; as dose increases attraction increases until the dose becomes too high and the insect is no longer attracted or even repelled.

1.1.2.5 <u>Odour-identification by insects</u>

As this thesis is focused on floral volatiles, sex pheromone odour identification will not be discussed in great detail other than to say there is obviously much overlap in the processes involved in insects identifying the two types of compounds. Receptors to plant volatiles and sex pheromones are found on both sexes in lepidoptera, although it has been found that males are usually more sensitive to sex pheromones whereas females are more sensitive to some plant volatiles (Angioy *et al.*, 2003). Behavioural responses to pheromones tend to require the correct compounds in the

correct ratio (Mazor and Dunkelblum, 1992). This can also be the case for plant volatiles when they are being used by the insect for host location (Visser and Avé, 1978). Or the presence of a single specific plant volatile can be enough to indicate the presence of the host and trigger a behavioural response (Rojas, 1999a). However, if adult lepidoptera are searching for a food resource such as nectar the need for odour blends to be in precise ratios will be much less. The evolutionary pressure is much less, and therefore the need for precision is reduced. A moth that flies to a flower that turns out to be a poor nectar resource is unlikely to affect its offspring's chances of survival – it can learn from its mistakes and fly to the next flower. Therefore in terms of nectar feeding moths, the likelihood is that its innate responses will cause it to fly to wide range of floral odours, but learning may play an important role in its future responses to floral odours. For a floral attractant the key to success may be to identify volatile compounds that stimulate the greatest level of innate responses in the target organism. Research on *Manduca sexta* by Riffell *et al.* (2013) has shown that although the moth learns new odour-food associations from its experiences of nectar resouces, these do not override its innate responses to floral volatiles. The authors conclude that the olfactory system has two parallel channels: one for innate responses, and one for learned experiences. It should also be noted that the many of the plant odours that stimulated innate responses in the moths were from the family Solanaceae, the host plants of this species. It is possible therefore that the 'innate olfactory channel' the authors propose is more connected to host plant identification and the association with nectar is a by-product of the plant-herbivore relationship. It would be interesting and useful to know if there was any difference in innate behaviour between males and females, however, the authors do not state whether their work was carried out with one or both of the sexes.

(i) Mechanisms for odour identification

Although the methods insects use to identify one odour from another are still being studied, much progress has made since the discovery by Jean-Henri Casimir Fabre that the male Great Peacock moth, *Saturnia pyri* (Denis & Schiffermüller) (Saturniidae), uses olfactory cues to locate mature females (Fabre, 1911). Fabre noted that males were able to locate females through an odour fog of other volatiles including naphthalene and lavender oil. The ability of insects to do this is quite incredible and has stimulated much research into the matter.

'Odour-identification' falls into two sections, first detection of molecules that the insect comes into contact with, and subsequent transmission of this information to the brain via the olfactory neurons as described in section 1.1.2.2, 'Olfaction in adult Lepidoptera'. The second part involves the interpretation of this information, i.e. perception of odour.

The detection of volatiles is carried out on the peripheral olfactory organs –primarily the antennae. As soon as odour molecules contact pores on the sensilla of the insect's antennae, the information is passed into the insect's antennal lobe. The mechanics and layout of the insect peripheral olfactory system (antennae) allows them to distinguish odours spatially and temporally, i.e. upon contact with an array of odours they are capable of distinguishing which volatiles originated from the same source compared to other sources and the background odour (Bruce *et al.*, 2005; Bruce and Pickett, 2011; Martin *et al.*, 2011; Szyszka and Stierle, 2014). This is acheived firstly because not all ORNs are the same (Hildebrand, 1996). Some are finely tuned to certain volatiles (e.g. pheromones or specific host volatiles), some respond to groups of similar molecules (e.g. grouped by the metabolic pathway the volatile is derived from, or their chemical structure), whereas some respond to dissimilar molecules, and some are general responding to a wider selection of

volatiles (Rostelien *et al.*, 2005; Hillier *et al.*, 2006). In addition, the quantity of a particular volatile also affects whether an ORN may respond or not (further detail in section 1.1.2.4). Secondly, by grouping certain ORNs within sensilla (Hansson, 1995) on the antenna it allows the insect to detect which packets of odours (see section 1.1.1.2 for details on odour packets and odour plumes) arrive at exactly the same instance (with millisecond precision (Hansson, 2002)), thus providing the insect's antennal lobe with information on which volatiles are from one odour source compared to another or from the background odour. Finally, the location of the insect's olfactory organs (antennae) mean that the information is constantly being refreshed as it moves, unlike in mammalian odour detection which relies on the breathing cycle (Szyszka and Stierle, 2014).

Once odour molecules make contact with the antennae the information is passed into the antennal lobe (AL). The AL is compartmentalised into glomeruli (Hansson, 2002), each being stimulated by specific groups of ORNs, i.e. there are glomeruli for specific types of odour (see section 1.1.2.3). However, there is also a complex network of neurons between the glomeruli, which excite or inhibit other glomeruli. In this way single odours and odour mixes create unique patterns of stimulation within the AL. This segregation of odour objects means that once processed by the AL an odour blend from one source is distinguishable from an odour blend from another source even if contains the same volatiles but in a different ratio. This information from the AL is then passed to the mushroom bodies or Kenyon cells, which are known to be involved in learning (Szyszka and Stierle, 2014). Research on *A. mellifera carnica* (honey bees) has found that prior exposure to a compound effects the insect's neurological response to a binary mixture containing that compound (Locatelli *et al.*, 2013). After exposure to compound A, the insects subsequently perceived a mixture

containing A+X to be more like compound X, i.e. the insect was able to increasingly differentiate A from A+X after it had experienced A.

To conclude, this demonstrates that insects are capable of differentiating complex mixtures of odours from one another as well as from background odour. This in turn highlights the importance of odour blend composition and delivery. Two volatiles emanating from separate (but extremely close) sources will be perceived differently by an insect compared to the same two volatiles combined in a blend (Bruce *et al.*, 2005). Furthermore, odour blends containing similar compounds but in different ratios can also be differentiated by insects (Bruce and Pickett, 2011) and can therefore lead to different behaviours (Visser and Avé, 1978). The capacity for insect learning means that the results seen in laboratories using odour naïve insects may not translate to similar results in the field, and similarly insects with prior experience to different odours may exhibit different behaviours.

An insect's behavioural response to host plant volatiles also depends on its physiological state. The same blend of plant volatiles may trigger one response in a moth looking for oviposition sites, and another response in a moth looking for nectar.

1.1.3 Changes in behaviour towards odours due to physiological state

Noctuid moths respond differently to stimuli depending on changes in their environment (alleothetic cues) and changes in their physiological state (idiothetic cues). This has been termed behavioural plasticity and is required in order for the organism to maintain homeostasis. Behavioural changes can be either learnt or innate.

Learnt changes or 'experienced-induced plasticity' occur in response to prior experiences and many studies on insects have been carried out in this area

particularly using Pavlovian-esque techniques with odours and rewards on bees and moths (for example Cunningham *et al.*, 1999; Carlsson and Hansson, 2006; Wright and Schiestl, 2009; Matsumoto *et al.*, 2012). It is clear that some, if not all, insects are highly capable of learning to associate specific odours with a food resource and that this may play a crucial role in the insect's resource foraging choices. However, innate behaviours may over-ride learnt behaviours when both cues are available (Riffell *et al.*, 2008b). Not only are insects capable of learning to associate specific odour cues with a food resource, the efficiency of memory development can be modulated by chemicals within that food resource. For example, a bee's ability to remember an association between an odour stimulus and synthetic nectar increased in duration if the nectar contained caffeine (as found in the nectar of coffee and citrus trees). Caffeine was shown to cause an increase in the excitability of Kenyon cells (associated with memory formation) which may allow for these cells to have a greater propensity to fire (than without caffeine) leading to greater memory formation (Wright *et al.*, 2013).

Within the laboratory, the work carried out in this thesis will relate to innate behaviours as all of the moths tested will only be used once and will be odour naïve (i.e. have had minimal, if any, experience of floral odours). Moths caught during field trials will have experienced many odours present in their natural surroundings and therefore will not be odour naïve and may have developed associations between nectar and certain plant volatiles. This may have important implications on the results of the field trials.

The major resources that an insect requires are: food (and water), a mate(s), and a suitable ovipositional site. With regard to floral volatiles the most likely resource that these are odours mimic is carbohydrate and water (as nectar). However, it is

possible that these cues also are used to locate ovipositional sites by some noctuid species. It has been shown that *Helicoverpa* species oviposit more in flowering crops than pre- or post-flowering (Firempong and Zalucki, 1989; Fitt, 1989 and the references therein; Jallow *et al.*, 1999; Sequeira *et al.*, 2001). Similarly, the common blue butterfly *Polyommatus icarus* (Lycaenidae) has been found to select ovipostional sites based on prevalence of flowers on *Lotus corniculatus* (Fabaceae), its preferred host. Study of the insect's ovipositional behaviour showed that the insect "almost always" fed upon nectar prior to ovipositing (Janz *et al.*, 2005) suggesting that there maybe an advantage to the insect by combining oviposition and nectar finding at the same resource. Conversely, in *Spodoptera littoralis* there is a distinct change in behaviour pre- and post-mating that has been demonstrated with flowering *Syringa vulgaris* (L.) (Oleaceae) (common lilac) and one of the moths' host plants, *Gossypium hirsutum* (L.) (Malvaceae) (cotton). Virgin females exhibited a strong preference for the flowering plants over the host plant, whereas mated females flew towards and landed on the host plant rather than the flowering plant (Saveer *et al.*, 2012), leading us to conclude that once mated the insect is no longer interested in nectar and is only searching for a site on which to oviposit. However, it should be noted that as *S. littoralis* is polyphagous the results seen with cotton and flowering lilac may not be universal across the insect's other host plants, and may differ from a oligophagous moth such as *H. armigera*.

The distinction between ovipositional cues and floral/nectar cues may not always be clear-cut, and will depend on the particular plant-insect interaction. The evidence cited above suggests that in the case of *Helicoverpa*, mated females may well be strongly attracted to odour cues relating to flowers.

1.1.3.1 <u>Mating-induced behaviour changes</u>

(i) Males

Mating results in significant changes in insect physiology and behaviour in both sexes. In Noctuidae (with the possible exception of the Agaristinae (Poole, unpublished)) males transfer their reproductive material to the female within a protective sheath called a spermatophore containing nutrients and other factors which improve the female's fecundity, stimulating oogensis (Jin and Gong, 2001), and inhibit further pheromone production by the female (Hanin *et al.*, 2011 and the references therein). The male's protein reserves within its sex accessory gland (SAG) also become depleted post mating (Duportets *et al.*, 1998). Once mated, *Agrotis ipsilon* males have been shown to switch off their behaviour directed to finding a mate (i.e. they become behaviourally unresponsive to sex pheromones). This may, in part, be explained by significant changes in the response of AL neurons which display a reduction in sensitivity towards sex-pheromone post-mating (Gadenne *et al.*, 2001; Anton *et al.*, 2007; Barrozo *et al.*, 2011). The behavioural change lasts for approximately 24 h with males exhibiting normal sex pheromone searching flight behaviour the following night, and presumably neurons within the antennal lobe return to normal sensitivity towards sex-pheromone.

Barrozo *et al.* (2011) also found that when stimulated with the plant volatile heptanal, activity within a mated insect's AL was greater in comparison to that in virgin insects. The authors conclude that once an *A. ipsilon* male mates there is a decrease in AL activity towards the sex-pheromone in conjunction with a slight increase in AL activity towards a plant volatile which will aid the insect in locating potential food sources over females with which it is unable to mate with for 24 h. Similar results were seen in unmated and mated *S. littoralis* males, in that prior to mating males flew towards host plants, flowers, and female calling *S. littoralis*. Post mating the males were significantly less attracted to females and host volatiles but remained attracted to the

flowers (Kromann *et al.,* 2015). In addition, EAG and antennal lobe recordings suggested that there was modulation of the moth's olfactory system post mating, but this modulation only occurred in the AL areas related to detection of pheromone and host plant volatiles.

(ii) Females

Once mated a female moth's primary aim is to locate a suitable site on which to oviposit. A switch in behaviour has been clearly demonstrated by Saveer *et al.* (2012) who also made significant progress in elucidating some of the mechanisms behind this switch. The authors found that mated female *S. littoralis* altered their behaviour towards either a nectar rich flowering plant, or the leaves of a host plant suitable for oviposition (Figure 1.5). The mated female *S. littoralis* exhibited significantly different EAG responses to a range of plant and floral volatiles compared to virgin moths. As well as changes in the peripheral sensory apparatus (antennae) of female *S. littoralis* mating was found to trigger modulation of the antennal lobe networks where the mated insects exhibited a down-regulation of the glomeruli associated with floral odours and an up-regulation of glomeruli associated with cotton leaf odours.

Part of the package that males transfer to females during copulation includes proteins and peptides (produced in the male's SAG). One of these, identified in *Drosophila melanogaster*, is the *Drm* sex-peptide which enters the female's haemolymph from its reproductive tract (Pilpel *et al.*, 2008). This sex-peptide is involved in suppression of female sex pheromone production (Nagalakshmi *et al.,* 2007) and subsequently influences the female's nervous system (Häsemeyer *et al.*, 2009). It is possible that these male derived sex-peptides are involved in the changes to the female's olfactory system and behaviour seen in *S. littoralis*.
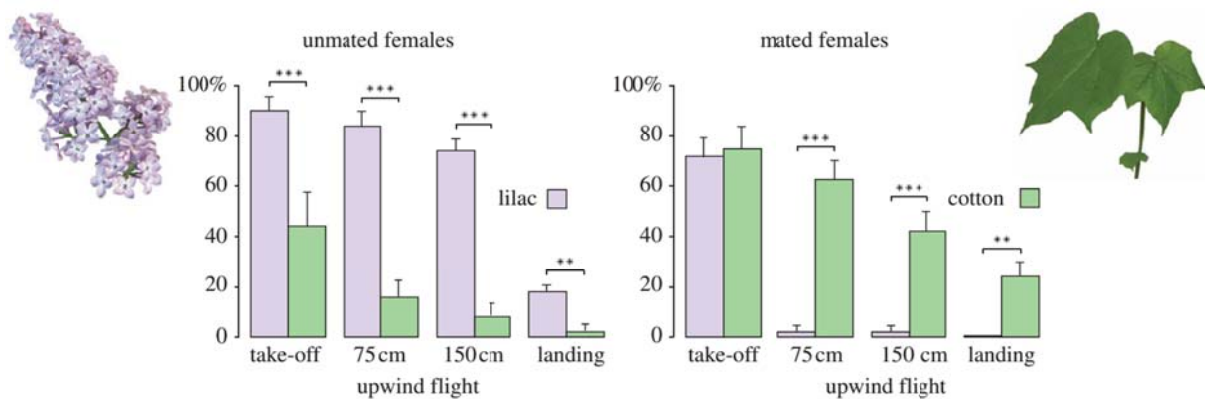
Figure 1.5: Attraction of unmated (a) and mated (b) *Spodoptera littoralis* females in a wind tunnel, toward lilac flowers (*Syringa vulgaris*) and cotton foliage (*Gossypium hirsutum*) (n = 50; mean ± s.e.m.). Differences between unmated and mated females for all steps of the behavioural sequence are significant at **$P < 0.001$, and ***$P < 0.0001$, according to Fisher's Exact Probability test. Taken from Saveer *et al.* (2012).

1.1.3.2 Feeding

Adult Noctuidae can regularly be seen to visit flowers from which they imbibe nectar. This rich source of carbohydrates also contains amino acids, secondary plant compounds, proteins, and lipids, (Nicolson and Thornburg, 2007 and the references therein) is an excellent energy source for adult Lepidoptera, increasing female pheromone production, fecundity, life span, and offspring fittness (Song *et al.*, 2007; Wackers *et al.*, 2007; Foster, 2009; Foster and Johnson, 2010). Adult *A. ipsilon* are known to seek water or sucrose solution immediately after eclosion, and if provided sucrose solution their consumption of it was greatest during the first three days of adult life and declined over time, whereas those provided with water only increased their consumption over time (Binder, 1996) suggesting water alone does not satisfy the moths' requirements. For female virgin *Heliothis virescens* hemolymph trehalose concentration (HTC) was significantly lower in moths that had been provided with water only compared to moths allowed to drink from 10% sucrose solution. Starvation of sucrose for 4 days or more reduces HTC to levels that significantly

reduce pheromone production (Foster and Johnson, 2010); mating also significantly reduces HTC (Foster, 2009), but HTC was quickly restored to normal levels if starved or mated insects were given 10% sucrose solution. This demonstrates the benefits and importance of feeding on nectar to females prior to mating and after mating.

With regard to behaviour towards odours and satiated/starved status there has been little research so far on floral odour and noctuid moths. However, starved *Trichoplusia ni* (Hübner) (Noctuidae) female moths were more attracted to the male sex pheromone than those fed with sucrose solution (Landolt *et al.*, 1996). Male sex pheromone for lepidopteran species is uncommon, and may be linked to signifying a host plant or food resource to females. Landolt *et al.* (1996) suggest that their findings corroborate with this theory and that the starved females (unlike the satiatied females) were more attracted to the male sex pheromone because it may be triggering a food foraging response.

In other insect species starvation has been shown to affect behaviour, for example in female western flower thrips, *Frankliniella occidentalis* (Pergande) (Thripidae), a greater percentage (77%) of individuals starved for 24 h chose the host-odour containing arm of a Y-tube olfactometer compared to satiated insects (58.7%) (Davidson *et al.*, 2006). In the triatomine, *Rhodnius prolixus* (Stål), starved insects orientated towards all tested volatiles associated with food, whereas satiated insects orientated away from these volatiles (except for α-pinene). In addition, the starved *R. prolixus* preferred clean air over aggregation pheromone (Reisenman *et al.*, 2013). This demonstrates the importance of the biological meaning of the odour cue and its relevance to the insect's physiological state, i.e. for starved insects food odours are important but in some cases avoiding competition for resources with their conspecifics is more important.

Much progress has been made in discovering the biochemical and molecular mechanisms involved in modulating behavioural plasticity in invertebrates (reviewed by Sengupta, 2013), and a complex mixture of hormones, peptides, and metabolites is known to inform the brain of the current physiological status of the organism. However, the peripheral olfactory sensory apparatus (antennae) in *S. littoralis* is not affected by the insect's fed status (Martel *et al.*, 2009).

1.2 FLORAL VOLATILES

The flowering parts of a plant emit a wide array of volatiles that make up the floral odour of that plant. Currently, in excess of 1700 compounds have been identified, with the most common occurring in more than 50% of families examined so far (Table 1.2). These common compounds are a selection of monoterpenes (limonene, (*E*)-β-ocimene, myrcene, linalool, α- and β-pinene) and benzenoids (benzaldehyde, methyl salicylate, benzyl alcohol, and 2-phenylethanol) (Knudsen *et al.*, 2006). The main chemical categories that floral volatiles fit into are the terpenoids, fatty acids, and phenylpropanoids (including the benzenoids); within these groups compounds with a wide range of functional groups are found, e.g. hydrocarbons, alcohols, aldehydes, ketones, acids and esters. Although the floral bouquet from a specific plant species may contain tens or hundreds of compounds, most are found in trace quantities and the odour may be dominated by only a few main components. However, insects are often sensitive to minor components and may use these as well as major components to identify resources.

Table 1.2: Percentage of families of seed plants that produce some of the most common components of floral volatiles. Taken and modified from Knudsen *et al.* (2006).

Compound	Percentage present
Limonene	71
(*E*)-Ocimene	71
Myrcene	70
Linalool	70
α-Pinene	67
Benzaldehyde	64
β-Pinene	59
Methyl salicylate (Methyl 2-hydroxybenzoate)	57
Benzyl alcohol	56
2-Phenolethanol	54
Caryophyllene	52
6-Methyl-5-hepten-2-one	52
Phenylacetaldehyde	32

Aside from the reproductive parts of the plant, other parts both above and below ground also emit volatiles into the atmosphere and rhizosphere. As this project is primarily concerned with floral volatiles this review will concentrate on the production and release of compounds from the inflorescence part of the plant.

1.2.1 Origin of Floral Volatiles

As Dudareva and Pichersky (2000) point out, identifying exactly where on a flower volatiles are emitted from is more complicated than it may seem. Staining techniques (e.g. chemical staining (Stern *et al.*, 1986) or using bio-molecular techniques (Dudareva *et al.*, 1996; Rohrbeck *et al.*, 2006) may identify where non-volatilised compounds or their precursors are stored, but this indirect method does not actually measure airborne compounds. Headspace entrainment does provide information on compounds released from a plant, but to infer exactly where this originates is tricky

and many studies have relied on volatiles from excised plants organs which presents problems of its own due to changes in plant chemistry in response to the mechanical damage. Potential issues aside, the quantity of the enzyme, linalool synthase (LIS), involved in the final steps of linalool production was found to correlate positively with linalool emissions from floral organs (Pichersky *et al.*, 1994), suggesting that in situ visualization of compounds involved in the biosynthetic pathways of a particular volatile may provide good evidence of the source of that volatile.

Floral emissions primarily emanate from the petals (Pichersky *et al.*, 1994; Raguso and Pichersky, 1995; Rohrbeck *et al.*, 2006), but other floral organs (e.g. pistils and sepals) of some plant species also emit volatiles (Pichersky *et al.*, 1994; Mactavish and Menary, 1997). Within the petals volatile release is not uniform with some areas producing significantly more VOCs than other areas (Effmert *et al.*, 2005; Rohrbeck *et al.*, 2006). Rohrbeck *et al.* (2006) found that floral emissions across the petals varied in *Nicotiana suaveolens* and *Stephanotis floribunda*. The petal rim of *N. suaveolens* emitted twice as much methyl benzoate than the petal centre, where as in *S floribunda* the floral signature contained benzyl alcohol, (*E*)-β-ocimene, β-linalool, methyl benzoate, α-farnesene (plus one unidentified compound) which were all found to be released from the petal rim and centre but not from tube of the flower. The authors hypothesised that a greater release of VOCs at more peripheral areas of the petal allows for better distribution of the floral scent and thus improves attraction and guidance of pollinators. In contrast however, in the flower of *Mirabilis jalapa*, Effmert *et al.* (2005) found that (*E*)-β-ocimene release was predominantly from the central area of the petals, with less emanating from the edges of the petal, and very little being emitted from the lower areas and tube. Whether from the petal edge or centre, or from other areas of the flower, floral volatiles can be considered to be emitted from multiple point sources.

Research into the floral scent of the flower *C. breweri* found that VOCs were emitted from stamens, pistils, petals, and sepals (Pichersky *et al.,* 1994). In addition, emission levels for each flower organ approximately corresponded quantitatively and qualitatively with the total floral scent, and enzyme (LIS) activity was only found in flower parts that emitted floral VOCs suggesting that synthesis and emission occur at the same site and translocation of scent compounds is not active.

1.2.2 Floral and other plant volatiles as kairomones for Noctuids

Plants that are insect pollinated advertise their flowers by vision and scent. Crepuscular moths (e.g. certain hawkmoths, and the noctuid *A. gamma* used in this study) may use both visual and olfactory cues but are more stimulated by odour (Plepys *et al.*, 2002a; Theobald *et al.*, 2010; Bisch-Knaden *et al.*, 2012). For night flying moths (such as *Helicoverpa* species and many other noctuids) the most influential cue is odour (Balkenius *et al.*, 2006).

Numerous studies have investigated the effect of floral volatiles on the behaviour of noctuid moths, either in field trials (Table 1.3) or in wind tunnel bioassays (Table 1.4). Phenylacetaldehyde (PAA) is the most commonly tested floral volatile and was often tested alone or as the major component of odour blends. In nearly all cases the compounds or blends of compounds were found to be attractive; only two field studies found that PAA combined with pheromone caught significantly fewer male moths than the pheromone alone (Burgio and Maini, 1995; Meagher, 2001b), and one wind tunnel bioassay (Deng *et al.*, 2004) found some compounds elicited fewer landings than pheromone or PAA alone. The compounds that reduced the number of moth landings in the Deng *et al.* (2004) wind tunnel study were methyl salicylate, eugenol, 2-phenylethanol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, and (*Z*)-3-hexanol.

Many of the field studies included data on the sex of the caught insects and it was often found that more females were caught than males.

Several compounds stand out as being highly attractive to noctuids either in combination with PAA or alone. The addition of β-myrcene to phenylacetaldehyde was found to greatly increase catches of many noctuid species, and the binary combination of phenylacetaldehyde plus limonene, benzyl acetate, *cis*-jasmone, or linalool also increased catches of several noctuid species (Meagher and Landolt, 2008). The authors acknowledge that without further testing they cannot tell whether the compounds added to phenylacetaldehyde, are attractive themselves, or whether there is some synergistic effect occurring. However, previous work has found synergistic effects with β-myrcene as alone it was only weakly attractive to *Autographa californica* (Plusiinae) (Speyer) (alfalfa looper) and *Trichoplusia ni* (Plusiinae) (Hübner), but the combination of phenylacetaldehyde with β-myrcene attracted many more insects that the sum of the two compounds individually (Landolt *et al.*, 2006).

Using wind tunnel bioassays, lilac aldehyde isomers from the Lesser Butterfly Orchid, *Platanthera bifolia*, were identified as the primary attractive compounds for *A. gamma* (Plepys *et al.*, 2002b) and also in White Campion, *Silene latifolia*, for *H. bicruris* (Dötterl *et al.*, 2006). Unfortunately the authors were not able to identify whether attraction was limited to specific isomers or a selection of them. In the case of *A. gamma*, the moths were found to be just as attracted to lilac aldehyde as a synthetic blend of six compounds mimicking the odour from flowers of *Platanthera bifolia* L. (Rich.) which had been previously identified as highly attractive to *A. gamma*.

Other compounds that were commonly tested and found to be highly attractive to noctuid moths include: benzaldehyde, methyl salicylate, methyl 2-methoxybenzoate,

and 2-phenylethanol. Specifically in relation to *Helicoverpa* species compounds that resulted in increased catches or positive movement in wind tunnel bioassays include: PAA, benzaldehyde, salicylaldehyde, benzyl alcohol, 2-phenylethanol, α-pinene, cineol, limonene, (*Z*)-(3)-hexenyl salicylate, α-bulnesene, and myrcene. Similarly for *Autographa* species compounds include: phenylacetaldehyde, lilac aldehyde, *cis*-jasmone, myrcene, and benzyl acetate.

Table 1.3: Compounds that have been tested in field trials to assess their affect on trap catches of (primarily) noctuid moths. The list is ordered by the number of compounds in the blend.

Compound(s)	Lepidoptera	Host plant	Trap catches (increased/ decreased)	Reference
Phenylacetaldehyde	*Autographa gamma* (Noctuidae)	Polyphagous	increased	1
Phenylacetaldehyde	male *Ostrinia nubilalis* (Pyralidae)	Polyphagous	decreased	1
Phenylacetaldehyde	female *Ostrinia nubilalis* (Pyralidae)	Polyphagous	increased	1
Phenylacetaldehyde	*Autographa califonica* (Noctuidae)	Polyphagous	increased	2
Benzyl acetate	*Autographa califonica* (Noctuidae)	Polyphagous	increased	2
Phenylacetaldehyde	male *Spodoptera frugiperda* (Noctuidae)	Grasses	decreased	11
Phenylacetaldehyde	*Anticarsia gemmatalis* (Noctuidae)	Legumes	increased	4
Phenylacetaldehyde	*Feltia subterranea* (Noctuidae)	Polyphagous	increased	4
Phenylacetaldehyde	*Argyrogramma verruca* (Noctuidae)	Polyphagous	increased	4
Phenylacetaldehyde	*Helicoverpa zea* (Noctuidae)	Polyphagous	increased	4
Phenylacetaldehyde	*Spodoptera sp.* (Noctuidae)	Polyphagous	increased	4
Myrcene	*Helicoverpa zea* (Noctuidae)	Polyphagous	increased	5

Compound(s)	Lepidoptera	Host plant	Trap catches (increased/ decreased)	Reference
Phenylacetaldehyde	*Argyrogramma verruca* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde	*Autographa gamma* (Noctuidae)	Polyphagous	increased	9
Phenylacetaldehyde	*Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	9
Phenylacetaldehyde	*Macdunnoughia confusa* (Noctuidae)	Polyphagous	increased	9
Phenylacetaldehyde	*Euclidia glyphica* (Noctuidae)	Trifolium	increased	9
Phenylacetaldehyde	*Autographa califonica* (Noctuidae)	Polyphagous	increased	10
Phenylacetaldehyde	*Trichoplusia ni* (Noctuidae)	Polyphagous	increased	10
Phenylacetaldehyde; myrcene	*Trichoplusia ni* (Noctuidae)	Polyphagous	increased	10
Phenylacetaldehyde; *cis*-jasmone;	*Autographa califonica* (Noctuidae)	Polyphagous	increased	2
Phenylacetaldehyde; benzyl acetate	*Chrysodeixis includens* (Noctuidae)	Polyphagous	increased	4
Phenylacetaldehyde; *cis*-jasmone	*Argyrogramma verruca* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; myrcene	*Argyrogramma verruca* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; myrcene	*Autographa califonica* (Noctuidae)	Polyphagous	increased	10
Phenylacetaldehyde; methyl salicylate	*Argyrogramma verruca* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; linalool	*Mocis sp.*(*disseverans + latipes*) (Noctuidae)	grasses and legumes	increased	5

Compound(s)	Lepidoptera	Host plant	Trap catches (increased/ decreased)	Reference
Phenylacetaldehyde; methyl-2 methoxybenzoate	*Mocis sp.*(*disseverans + latipes*) (Noctuidae)	grasses and legumes	increased	5
Phenylacetaldehyde; linalool	*Anticarsia gemmatalis* (Noctuidae)	Legumes	increased	5
Phenylacetaldehyde; myrcene	*Anticarsia gemmatalis* (Noctuidae)	Legumes	increased	5
Phenylacetaldehyde; limonene	*Anticarsia gemmatalis* (Noctuidae)	Legumes	increased	5
Phenylacetaldehyde; myrcene	*Heliothis virescens* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; *cis*-jasmone	*Heliothis virescens* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; methyl-2 methoxybenzoate	*Heliothis virescens* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; myrcene	*Helicoverpa zea* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; myrcene	Diaphania hyalinata (Pyralidae)	Curcurbits	increased	5
Phenylacetaldehyde; *cis*-jasmone;	*Chrysodeixis includens* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; myrcene	*Chrysodeixis includens* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; benzyl acetate	*Chrysodeixis includens* (Noctuidae)	Polyphagous	increased	5
Phenylacetaldehyde; limonene	*Chrysodeixis includens* (Noctuidae)	Polyphagous	increased	5
Benzyl acetate; myrcene	*Autographa califonica* (Noctuidae)	Polyphagous	increased	10

Compound(s)	Lepidoptera	Host plant	Trap catches (increased/ decreased)	Reference
Phenylacetaldehyde; (±) - linalool	*Anticarsia gemmatalis* (Noctuidae)	Legumes	increased	6
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Agrotis segetum* (Noctuidae)	Polyphagous	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Agrotis bigramma* (Noctuidae)	Poaceae	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Agrotis exclamationis* (Noctuidae)	Polyphagous	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Xestia c-nigrum* (Noctuidae)	Polyphagous	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Apatele rumicis* (Noctuidae)		increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Amphipyra pyramidea* (Noctuidae)	Oak	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Dypterygia scabriuscula* (Noctuidae)	Rumex and Polygonum	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Hadula trifolii* (Noctuidae)	Polyphagous	increased	9

Compound(s)	Lepidoptera	Host plant	Trap catches (increased/ decreased)	Reference
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Euxoa aquilina* (Noctuidae)	Poaceae	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Mamestra brassicae* (Noctuidae)	Brassicae + polyphagous	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Mamestra oleracea* (Noctuidae)	Polyphagous	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Mamestra suasa* (Noctuidae)	Polyphagous	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Mythimna sp.* (Noctuidae)	Grasses	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Noctua pronuba* (Noctuidae)	Polyphagous	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Trachea atriplicis* (Noctuidae)	Rumex and Polygonum	increased	9
Isoamyl alcohol (3-methylbutanol); acetic acid; isobutanol (2-methylpropanol)	*Euclidia glyphica* (Noctuidae)	Trifolium	increased	9
Phenylacetaldehyde; Benzaldehyde; salicylaldehyde; benzyl alcohol	*Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	3

Compound(s)	Lepidoptera	Host plant	Trap catches (increased/ decreased)	Reference
Phenylacetaldehyde; Benzaldehyde; salicylaldehyde; benzyl alcohol; 2-phenylethanol	*Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	3
Phenylacetaldehyde; α-pinene; cineol; limonene; (*Z*)-(3)-hexenyl salicylate	*Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	7
Phenylacetaldehyde; 2-phenylethanol; methyl salicylate; methyl-2 methoxybenzoate; benzaldehyde; benzyl alcohol	Thysanoplusia orichalcea (Noctuidae)	Polyphagous	increased	8

References: 1, (Burgio and Maini, 1995); 2, (Landolt *et al.*, 2001); 3, (Li *et al.*, 2005); 4, (Meagher, 2002); 5, (Meagher and Landolt, 2008); 6, (Meagher and Landolt, 2010); 7, (Gregg *et al.*, 2010); 8, (Stringer *et al.*, 2008); 9, (Toth *et al.*, 2010); 10, (Landolt *et al.*, 2006); 11, (Meagher, 2001b)

Table 1.4: Compounds that have been found in influence the flight behaviour of noctuid moths in wind tunnels. The list is ordered by the number of compounds combined.

Compound(s)	Lepidoptera	Host plant	Upwind flight (increased/ decreased)	Reference
(-)-germacrene-D	*Heliothis virescens* (Noctuidae)	Polyphagous	increased	10
Phenylacetaldehyde	*Helicoverpa armigera* (Noctuidae) male	Polyphagous	increased	12
Methyl salicylate	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	decreased	12
Benzaldehyde	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	12
Eugenol	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	decreased	12
2-phenylethanol	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	decreased	12
(*Z*)-3-hexenyl acetate	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	decreased	12
(*E*)-2-Hexenal	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	decreased	12
(*Z*)-3-hexenol	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	decreased	12
(*Z*)-6-nonenol	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	12
Salicylaldehyde	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	12
Linalool	male *Helicoverpa armigera* (Noctuidae)	Polyphagous	decreased	12

Compound(s)	Lepidoptera	Host plant	Upwind flight (increased/ decreased)	Reference
Phenylacetaldehyde	male *Spodoptera frugiperda* (Noctuidae)	Grasses	increased	18
Phenylacetaldehyde	*Hadena bicruris* (Noctuidae)	Caryophyllaceae	increased	13
Lilac aldehyde isomers	*Hadena bicruris* (Noctuidae)	Caryophyllaceae	increased	13
Lilac aldehyde isomers	*Autographa gamma* (Noctuidae)	Polyphagous	increased	15
Borneol; α-pinene; citronellol; caryophyllene oxide	*Spodoptera littoralis* (Noctuidae)	Polyphagous	increased	11
Phenylacetaldehyde; 2-phenylethanol; benzaldehyde; benzyl alcohol	*Trichoplusia ni* (Noctuidae)	Polyphagous	increased	14
Lilac aldehyde isomers; methyl benzoate; benzyl benzoate; benzyl salicylate; methyl salicylate; cinnamyl alcohol	*Autographa gamma* (Noctuidae)	Polyphagous	increased	15
β-caryophyllene; α-humulene; β-guaiene; α-muurolene; γ-muurolene; α-bulnesene	*Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	16
Phenylacetaldehyde; benzaldehyde; (*S*)-(-)-limonene; (*R,S*)-(+-)-linalool; (*E*)-myroxide; (*Z*)-β-ocimene; (*R*)-(-)-piperitone	*Helicoverpa armigera* (Noctuidae)	Polyphagous	increased	17

References: 10, (Mozuraitis *et al.*, 2002); 11, (Salama *et al.*, 1984); 12, (Deng *et al.*, 2004); 13, (Dötterl *et al.*, 2006); 14, (Haynes *et al.*, 1991); 15, (Plepys *et al.*, 2002b); 16, (Hartlieb and Rembold, 1996); 17, (Bruce and Cork, 2001); 18, (Meagher and Mitchell, 1998).

1.2.3 Use of Floral Volatiles in Management of Insect Pests

1.2.3.1 <u>Attractants</u>

The idea of using floral volatiles to control insect pest behaviour and protect crops has been around since the 1960s when developments in technology allowed scientists to record the sensitivity of insect antennae to specific plant volatiles (Schneider, 1957; Moorhouse *et al.*, 1969). However, since then the number of products based on floral volatile remains small. As more and more pesticides are withdrawn from the market due to concerns over ecological damage, novel methods of protecting our crops are required. Utilising the innate behaviours of insects towards compounds found naturally in the environment has inherent benefits in terms of environmental safety and reduced chances of development of resistance. However, other hurdles must be overcome. The first and most obvious hurdle is identifying the correct chemical(s) to stimulate the desired behaviour in the target insect. If more than one compound is present in the odour it must then be optimised to ensure that the chemicals are in the most effective ratio (as discussed in section 1.1.1.1, 'Odour-identification by insects').

There are already a few examples of commercial products in use or being trialled that utilise plant volatiles to act as an attractant to control crop pests. In Australia researchers identified a blend of floral and leaf volatiles that is extremely attractive to the two major lepidopteran pests in cotton, *H. armigera* and *H. punctigera*. (Anonymous, 2004; Del Socorro *et al.*, 2010a; Del Socorro *et al.*, 2010b; Gregg *et al.*, 2010). The attractant is sold under the name 'Magnet', produced by Ag Biotech, Australia, and the odour blend is comprised of compounds that were identified from host and non-host plants of *H. armigera*.

The reason for the use of non-host plant odours is that although larval development may not succeed on these plants, the adults may still use them as a nectar resource and therefore be attracted to the floral scent. The authors state that they are not investigating the basis of attraction (i.e. for oviposition or for nectar foraging) but only the level of attraction and whether there was any sex bias. The final odour blend in Magnet is a combination of volatiles from different plant species which is a novel idea as most research in this area aims to mimic a single preferential host plant of the pest, rather than create a "super blend" as coined by Del Socorro *et al.* (2010a). Perhaps because the target pest is highly polyphagous in the larval and adult phase a 'super blend' is more suitable opposed to trying to mimic a single plant species. As Plepys et al (2002a) note with regard to another generalist flower forager, *A gamma*, the insect probably "relies on a number of chemical compounds or blends for the attraction to flowers". There is no single 'key compound' that attracts these types of polyphagous nectar foragers which allows them to maximise their foraging range. Therefore if an odour blend can be devised that stimulates the maximum innate food foraging response, it should be an excellent bait for generalist nectar feeders such as the noctuid moths.

Other floral blends have been devised for use as crop protection tools, for example, a floral blend for use in an 'attract and kill' system to control white-spotted flower chafers, *Protaetia brevitarsis* (Cetoniidae), in maize has been trialled in China (Chen and Li, 2011). The composition of the odour blend was derived from searching scientific literature relating to plant compounds that have been found to be attractive to Cetoniidae, and testing these compounds in an olfactometer with *P. brevitarsis*. Eight compounds were found to give significant positive behavioural responses in the olfactometer and were

subsequently tested in field trials. The chemicals were formulated into dispensers and used as baits for sacrificial trap plants within the maize crop. All of the plots with baits suffered significantly less damage from *P. brevitarsis* than plots without baited sacrificial plants. Within the plots the sacrificial maize plants suffered significantly greater damage than unbaited plants. The authors conclude that the two most effective compounds from the field trial, reported as propenol and benzyl carbinol, could be used as the basis of an 'attract and kill' control method for *P. brevitarsis*. These two compounds are presumably better known as 2-propenol (allyl alcohol) and 2-phenylethanol, respectively.

Plants that are being attacked by herbivorous insects are known to produce specific volatiles (e.g. McCall *et al.*, 1994; Loughrin *et al.*, 1995), synomones, which recruit parasitoids and insect predators to the plant to dispatch the plant's attackers (Turlings *et al.*, 1990). James and Price (2004) investigated using the plant volatile methyl salicylate (present in many floral bouquets and also known to be a herbivory induced plant compound) as an attractant for beneficial insects in a grape vineyard in Prosser, Washington stage, USA. The authors placed dispensers of methyl salicylate in the vineyard and recorded the populations of beneficial insects in the crop to control grape vine pests. The presence of methyl salicylate dispensers was found to have a significant effect on the prevalence of some pest predator species (e.g. *Stethorus punctum picipes* (Coccinellidae) and *Orius tristicolor* (Anthocoridae)) and parasitic wasps with an increase in numbers of nearly four times for some species compared to untreated areas. The authors conclude that application of methyl salicylate may be a useful method of ensuring beneficial insect populations are maintained throughout the crop cycle providing control of many insect pests. Similar work covering a wider range of crops and plant volatiles was carried out by Simpson

et al. (2011) and the results were equally encouraging with many of the tested volatiles significantly increasing populations of beneficial insects.

1.2.3.2 <u>Repellents</u>

Much of the research into the application of plant volatiles in crop protection is aimed at using them as attractants in an 'attract and kill' system, but other areas of application are also being developed. For example, research into plant defence mechanisms and plant-plant communication has highlighted the possibility of using specific volatile compounds to prime plants to make them respond faster to herbivore attack or stimulate the plant to produce secondary metabolites that repel herbivorous insects. This has been demonstrated in cotton plants, *Gossypium hirsutum* (L.) (Malvaceae), which were primed with the floral volatile *cis*-jasmone causing the plants to produce volatiles that the cotton aphid, *Aphis gossypii* (Glover) (Aphididae), find repellent ((Z)-3-hexenyl acetate, (E)-4,8-dimethyl-1,3,7-nonatriene, methyl salicylate, and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) (Hegde *et al.*, 2000).

Genetic modification of plants in conjunction with knowledge of floral volatiles and chemical ecology has opened up other potentially useful avenues for crop protection to make plants more resistant to pests. For example the work currently being carried out by Rothamsted Research is conducting field trials with wheat plants that have had a (E)-β-farnesene synthase gene inserted into their genome causing the plants to expresses (E)-β-farnesene in much greater quantities than non-transformed wheat (Beale *et al.*, 2006; Rothamsted Research, 2013). (E)-β-farnesene is commonly found in the floral odours of plants, but some aphid species also use this VOC as an alarm pheromone triggering them to move away from the area when they sense the compound.

As the compound is commonly found in plant odours, the insect only responds to significantly larger quantities of (E)-β-farnesene than that normally emitted by plants and at a level above other common plant volatiles (e.g. β-caryophyllene, and (-)-germacrene D), therefore the GM wheat needs to produce (E)-β-farnesene in 'above background' levels (Beale et $al.$, 2006). Other research in this area has investigated the benefit of inserting genes for other volatile compounds to stimulate the searching behaviour of parasitoids or other beneficial insects (Kos et $al.$, 2009 and the references therein).

1.3 NOCTUIDS AS AGRICULTURAL PESTS

Several species within the family Noctuidae are significant agricultural pests around the world. Notable genera include *Helicoverpa* species; *Spodoptera* species, *Heliothis* species, *Agrotis* species, *Autographa* species) and the species *Mamestra brassicae*.

Three noctuid species were used in this research: *Autographa gamma*, *Helicoverpa armigera*, and *Helicoverpa gelotopoeon*. The reason for selecting these geographically diverse species was that the aim of this research was to identify a blend of floral compounds that is a general attractant to Noctuids. Therefore, if a blend is found which is attractive to these diverse noctuid species it is hoped that it will also be attractive to other noctuid species. Although the blend may be generally attractive to Noctuids it may also be optimised for specific species by the addition of certain volatiles to the blend.

1.3.1 Autographa gamma

Figure 1.6: *Autographa gamma* (L.) (Noctuidae). Left, mounted (unknown, 2007); right, *A. gamma* found inside a trap during a field trial.

A. gamma is a crepuscular moth with a distinctive 'Y' shaped silver/white mark on its forewings (Figure 1.6) (Waring *et al.*, 2009). It is an agricultural pest in Europe, North Africa, and Asia, but also poses a high risk to North America (Venette *et al.*, 2003). It is polyphagous and a pest in the following crops: brassica, legumes, potato, beets, and others. Females are highly fecund and are capable of two to five generations per year in its native ranges across Europe to North Africa, China and Japan (Carter, 1984; Venette *et al.*, 2003; Chapman *et al.*, 2012). In the UK the species does not generally survive the winter months (Carter, 1984). Thus the initial UK generation migrates from further south (North Africa and the Middle East) during the spring, and it is thought to do the reverse journey emigrating from the UK in late Summer/Autumn (Chapman *et al.*, 2012).

1.3.2 Helicoverpa armigera
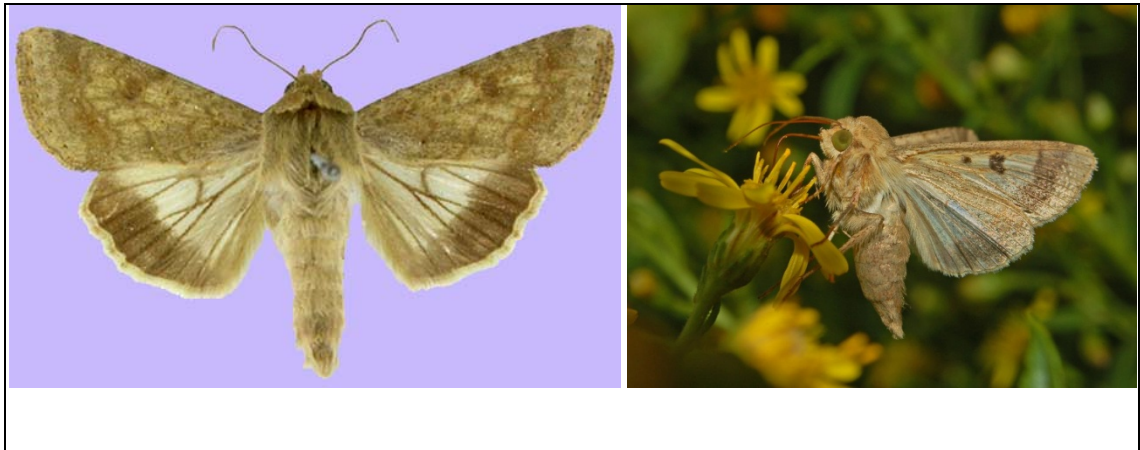
Figure 1.7: *Helicoverpa armigera* (Hübner) (Noctuidae). Left, mounted (Anonymous, 2007); right, feeding on nectar from a flower (Balocchi, 2008)

H. armigera is one of the most economically-damaging agricultural pests in the world. It causes an estimated $5 billion in yield losses *per annum* despite the use of pesticides costing $1 billion *per annum* (Sharma, 2005). The insect is polyphagous, highly fecund, capable of migrating extremely long distances, and is found across the globe (Fitt, 1989; Anonymous, 2006; Lammers and MacLeod, 2007). It is found on cotton, tobacco, maize, tomato, chickpea, pigeonpea, sorghum, soybean, oilseed rape, groundnuts, sunflower and safflower (Fitt, 1989 and the references therein). The larval stages of the moth feed on the reproductive and fruiting structures of its host plant, which has a direct effect on crop yields and is particularly damaging for farmers in high value cash crops (e.g. cotton and soybean). Its wide distribution and economic impact have resulted in the species being one of the most targeted organisms for insecticidal control. This chemical pressure has led to the species developing resistance to many common pesticide groups, including pyrethroids, endosulfan, and *Cry* proteins used in Bt genetically modified crops (Armes *et al.*, 1996; Downes and Mahon, 2012). Climate change is allowing the species to expand its range raising the risk of it causing serious problems in Northern Europe (FAO, 2008) and it has been recorded in relatively large numbers during

some years in the UK, e.g. over 11,700 in 2006 and distributed all over Britain (Waring *et al.*, 2009).

1.3.3 Helicoverpa gelotopoeon

Figure 1.8: *Helicoverpa gelotopoeon* (Dyar) (Noctuidae). Left, mounted; right, *H. gelotopoeon* photographed during field trials in Argentina.

H. gelotopoeon is restricted to South America and is common in Argentina, Chile, and Uruguay (Mitter *et al.*, 1993). It is a pest of cotton, maize, tomato, soybean, pea, sunflower, onion, flax, and tobacco (Specht *et al.*, 2004). In conjunction with *H. virescens*, it is a major pest of cotton requiring several insecticide applications per season (Cork and Lobos, 2003 and the references therein).

1.4 AIMS AND OBJECTIVES

The aim of this work was to identify blends of floral compounds which attract both male and female Noctuid Lepidoptera for use in the field as crop protection tools.

The objectives were:

- identify a blend of chemicals attractive to both sexes by assessing the responses of male and female noctuids to floral mimic blends and artificial odour blends in wind tunnel bioassays and field trials;

- optimise the blend in terms of its attractiveness to Noctuid moths. This will be achieved by measuring moths' EAG responses to selected floral compounds to identify potential chemicals to add to the previously identified floral odour blend which will then be assessed in field trials;

- improve capture rates of Noctuid moths by assessing various trap types baited with the floral blend in field trials;

- investigate how physiological status affects the moth's behaviour towards the floral blend in wind tunnel bioassays;

- assess captures of non-target insects in the floral baited traps, and investigate whether addition of specific floral chemicals or modification of trap colour may reduce captures of non-targets in field trials.

The null hypotheses of this thesis were that floral odour blends are equally attractive to male and female Noctuids, and the physiological status of the moth does not influence its behaviour towards that odour.

To identify a general attractant for Noctuids three species from different geographical locations were used. Initially research was focused on *Autographa gamma* (L.) (Noctuidae) as it is found locally and is an extremely common crop pest in the UK. In 2009 an opportunity arose to carry out field-work in Argentina. Local crop pest species were trapped and one of these, *Helicoverpa gelotopoeon* (Dyar) (Noctuidae), was imported back to the UK to continue laboratory-based work on the species. Due to continual problems with rearing field-collected insects in the laboratory, a pathogen-free colony of *Helicoverpa*

armigera (Hübner) (Noctuidae) was procured from Ag. Biotech Pty Ltd, Australia in February 2011.

CHAPTER 2 - GENERAL MATERIALS AND METHODS

2.1 INTRODUCTION

Throughout the behavioural and electrophysiological experiments a supply of live insects was required. Three species of insect were reared using similar methods, the details of which are described in this Chapter.

Techniques that were common throughout the electrophysiology experiments are also described in this chapter. Some further detail on electrophysiology methodology is reported in the chapters in which electroantennography was used.

The basic wind tunnel design and specifications are described in detail within this Chapter. Further details on the methodologies used to carry out the bioassays are in the relevant chapters involving the wind tunnel.

2.2 INSECT REARING

The method used for rearing the insects followed that described by Armes *et al.* (1992), with some modifications.

2.2.1 Autographa gamma

To initiate the *A. gamma* colony, gravid female moths were collected from the field (Intercrop Ltd. Deal, Kent, UK) during July 2008 using Unitraps baited with floral odours. The larvae were initially fed on fresh, field collected dandelion, *Taraxacum officinale* (F. H. Wigg) (Asteraceae) as this plant was found to be the most successful for rearing *A. gamma* at 20°C (Honek *et al.*, 2002). For

ensuing generations insects were reared on a modified semi-synthetic diet developed for the tobacco hornworm, *Manduca sexta* (L.) (Hoffman, 1966). The diet was modified with the addition of 50 g of freeze dried ground *T. officinale* to 2.75 L of synthetic diet. Additional field collected insects were added to the colony the following year to maintain genetic diversity.

After multiple generations, unidentified problems caused a decline in the survival rate of larvae to the pupal stage. Larvae were found to reach the final instar but failed to pupate, became black, and died. In addition, there was an increase in the percentage of adults emerging with deformed wings and a shortened lifespan. Eventually the survival rate of larvae decreased to less than 10%. Therefore insects from a known clean colony were procured from Tim Carty, Centre for Ecology and Hydrology (CEH), Oxford. Although the old colony was destroyed and the rearing laboratory and tools sterilised, after several generations the new colony exhibited the same symptoms.

A. gamma was reared at 20°C with 16:8 (L:D) photoperiod as described by Hill and Gatehouse (1992).

2.2.2 Helicoverpa gelotopoeon

The *H. gelotopoeon* colony was started from moths collected in soybean fields in the Santiago Del Estero district, (at 28°01'29"S, 64°14'01"W) Argentina in 2009. Eggs were transported to the Natural Resources Institute UK and upon emergence the neonate larva were reared on Hoffman's semi-synthetic diet (1966). The insects were imported to the UK under DEFRA licence PHL 176C/6528 - Licence to Import, Move and Keep Prohibited Invertebrates.

In the absence of peer reviewed information on rearing *H. gelotopoeon,* the method described for *H. armigera* was used. Hence, the colony was reared at 25-28°C with 16:8 (L:D) photoperiod which provided suitable conditions for a successful colony (Armes *et al.*, 1992). Varying the temperature by one or two degrees allowed some control over the development rate of the insects making preparations for the timing of experiments easier.

After many generations this colony started showing similar symptoms to those seen in the previous *A. gamma* colony. New pupae were sent from Argentina, but within 6 months these also exhibited the same symptoms and the colony collapsed.

2.2.3 Helicoverpa armigera

H. armigera eggs were received from Ag. Biotech Pty Ltd, Australia in February 2011. The insects were imported to the UK under DEFRA licence PHL 176C/6528 - Licence to Import, Move and Keep Prohibited Invertebrates. The insects were reared using protocols outlined in The Laboratory Culture and Development of *Helicoverpa armigera* (Armes *et al.*, 1992) at 26°C with a photoperiod of 14 : 10 h (light : dark). Relative humidity was not actively controlled but was at approximately 50 %RH.

Initially the colony appeared healthy, but after several months these insects also suffered from very low survival rates with very similar symptoms to the previous colonies. After ruling out the possibility of adverse environmental conditions, or diet, the cause was eventually suspected to be an unidentified viral pathogen (see section 2.2.9).

2.2.4 Pupae

Once the larvae had pupated they were removed from the larval pots (*Helicoverpa* spp. usually from under/inside the diet block; *Autographa gamma* from cocoons attached to the lid of the pot). Pupae were washed in 1 % sodium hypochlorite and then in deionised water. If required, the pupae were sexed according to the markings on the final segment on the abdominal tip (Figure 2.1).
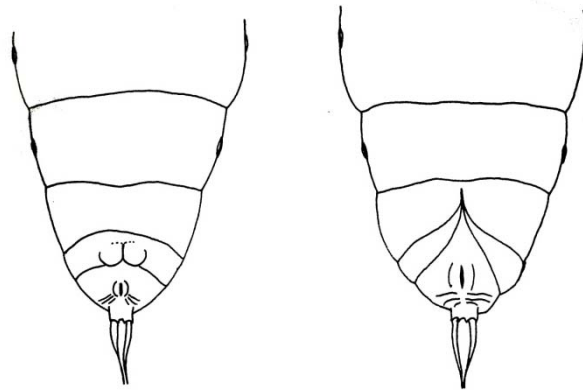
Figure 2.1: Ventral view of male (left) and female (right) *Helicoverpa armigera* pupae to show sex determination. Modified from Armes *et al.* (1992).

Once washed (and sexed if required), pupa were placed onto a bed of vermiculite in a 9 oz. disposable pot and placed into an emergence cage.

For *Autographa gamma*, emergence cages were wire-framed (40 x 40 x 40 cm) covered with black terylene mesh with tissue paper placed on the floor of the cage. Water and/or 10 % sucrose solution was provided once the adults started to eclose. Up to 40 pupae were placed in these cages.

For *Helicoverpa* spp. pupae were placed into cylindrical acrylic plastic cages (40 cm height, 20 cm diameter) with metalic mesh lids and floor. Water and/or 10 % sucrose solution was provided once the adults started to emerge. Tissue paper was placed on the floor, roof and on the inside of the tube for the adults

to climb up onto during wing fanning and drying after they had emerged. Up to 20 pupae were placed in these cages.

After every generation all the cages, equipment and bench surfaces were washed in 1 % Virkon®.

2.2.5 Adults

Adults eclosed in the emergence cages. If the insects were to be used for experiments males and females were kept in separate cages. Insects used for the next generation were placed in mixed-sex cages with 10 % sucrose solution and a substrate on which to oviposit. *A. gamma* and *H. armigera* were provided with fine, soft tissue paper (nappy liner), and *H. gelotopoeon* were provided with cotton plant leaves. The cotton leaves were removed from the plant and the petiole placed into a pot with water and a cotton wool bung to stop insects falling in. The presence of cotton leaves greatly increased the number of fertile eggs laid by *H. gelotopoeon* and may indicate that maing in this species is strongly influenced by the presence of host volatiles

2.2.6 Eggs

Eggs were collected from the cages on their respective substrates. The sheets or leaves were placed in 9 oz. pots with a cube of diet (c. 1.5 cm^2). Larvae emerged within 3 - 4 days. This could be extended by placing the pots into the fridge for up to 1 week and allowed the emergence of the next generation to be spread over a few days which was useful when conducting experiments.

2.2.7 Larvae

Larvae were removed from the 9 oz. pots within 1 week of hatching (1st or 2nd instar) and placed individually into small plastic pots (45 mL). Each pot contained a cube of diet (c. 1.5 cm^2), a single larva, and a strip of tissue paper (c. 1 x 4 cm) over the pot and held in place by the lid. A cut was made into the lid to improve airflow into and out of the pot and to reduce instances of high humidity leading to fungal growth. The small pots were places onto trays holding 72 pots each. The trays were labelled with the date prepared, the date the neonates emerged. In addition, the date the first larva pupated, and the number of successful pupae was recorded for each tray.

2.2.8 Diets

The *Helicoverpa* spp. were fed on a modified Hoffman's semi-synthetic diet (1966). *A. gamma* was fed on this diet with the addition of 50 g of freeze dried ground dandelion, *Taraxacum officinale* (F. H. Wigg) (Asteraceae).

2.2.9 Colony collapse

As mentioned above all of the three noctuid species cultured suffered from colony decline and the eventual collapse of the colony due to reduced fecundity and low survival rates of larvae. The most prominent symptom was failure of the larvae to pupate after the final instar (Figure 2.2), often leading to losses in excess of 50% and up to 90%.

Figure 2.2: pupa and infected larva of *Helicoverpa armigera*. Both are the same age and reared in the same conditions but the right plate shows the typical symptoms of the larvae that failed to pupate.

The percentage of larvae that exhibited this symptom generally increased in ensuing generations. Some larvae also appeared to be lethargic in their later instars compared to their siblings. The fecundity of the adults decreased as the colony progressed; this was particularly evident for the *H. gelotopoeon* species which initially produced high numbers of fertile eggs, in a similar quantity to *H. armigera*, but later individual females were only ovipositing c. 50-100 eggs of which half or less were fertile. Providing fresh cotton leaves as a substrate for oviposition initially appeared to increase egg laying, but the eventual result was the same with some females only producing c. 10 neonates from c. 20 eggs. In addition, successive generations suffered from increasing numbers of malformed pupae (particularly in *A. gamma*), and pupae failing to emerge (in all species).

Although all equipment and materials were sterilised using Virkon® between each generation, and the floors and walls of the laboratory were sterilised before bringing in a new colony of insects, similar symptoms eventually occurred in the three species of noctuid being reared in that laboratory. Insects

brought into the laboratory from a known clean colony also exhibited the same symptoms within a few generations even though the previous infected insects had all been removed and the laboratory decontaminated.

The observation that the phenomena seemed to occur in all colonies and indeed "spread" to newly established cultures derived from cultures known to be clean could be consistent with either an environmental cause or an infectious agent. The slow appearance of the condition and the chronic nature of the decline rule out a number of well known Lepidopteran pathogens such as the nucleopolyhedroviruses whose presence typically involves rapid epidemic pathology.

There were no signs of any fungal infection in any of the insects examined. The symptoms were consistent with infection from protozoan pathogens such as the microsporidia. However examination of wet smears of larvae from a number of the species on several occaisions when mortality was prevalent with phase contrast microscope showed no evidence of presence of the distinctive spores associated with infection by protozoa known to infect Lepidoptera such as *Variamorpha* spp, *Nosema* spp. etc., let alone the heavy infestations commonly seen in cultures in decline due to these parasites (Undeen and Vavra, 1997). In addition, the insects were found to have a healthy amount of body fat (Figure 2.3) which is not consistent with microsporidian infection (Solter *et al.*, 2012).
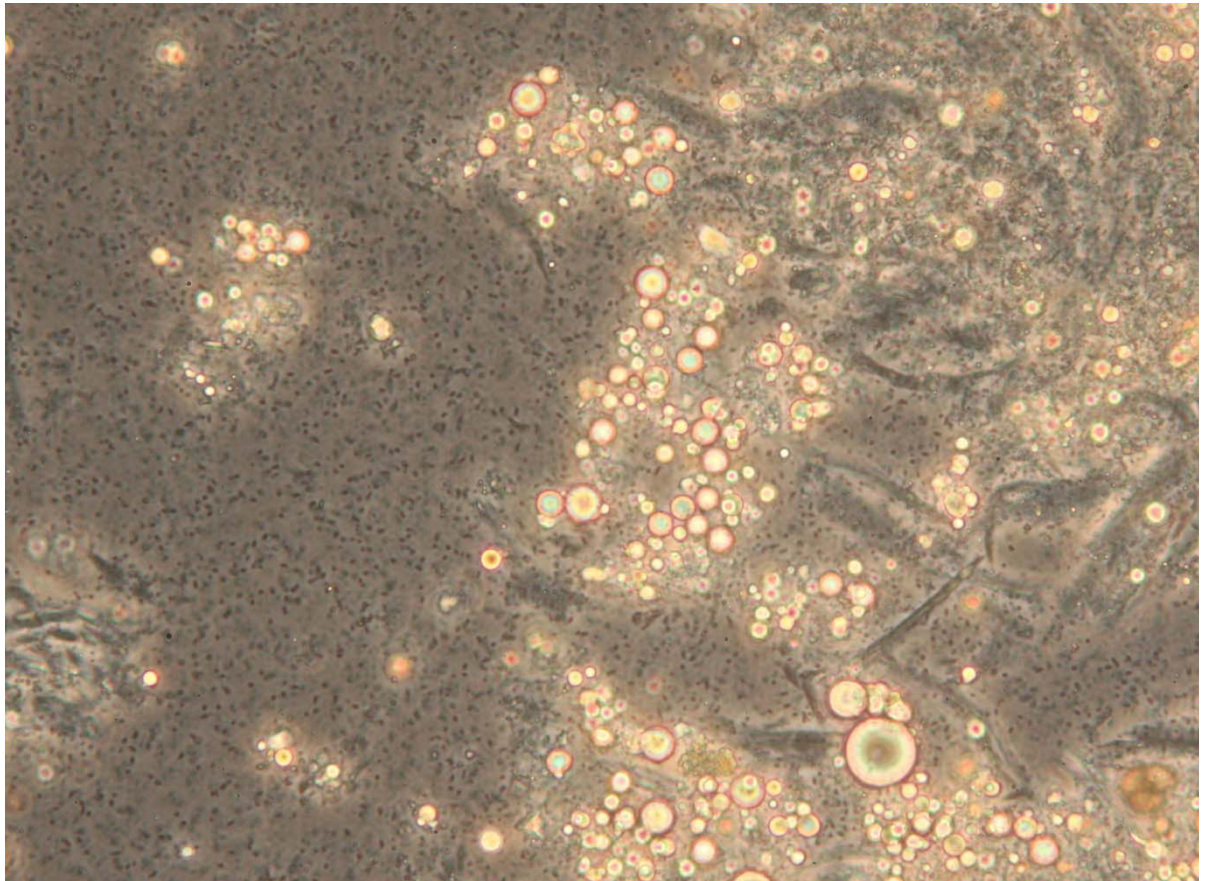
Figure 2.3: light microscope, 40 x magnification. Body tissue from Helicoverpa gelotopoeon larva showing a large number of black oval bodies and fatty deposits.

Phase contrast examination of wet smears showed no sign of the infectious particles characteristic of some other viral pathogens associated with colony decline in Lepidoptera such as Granulovirus or Cypovirus. It remains possible that the cause may have been due to infection by other non-occluded viruses that are known to infect Lepidoptera whose particles not visible under light microscopy such as Tetravirus or picornavirus. Identification of both of these requires complex purification protocols and electron microscopy (Christian and Scotti, 1998; Gordon and Hanzlik, 1998) and so was not followed up due to the time and resources needed.

In summary while the symptoms and epidemiology of the colony declines were consistent with a number of known Lepidopteran chronic pathogens it was not possible to identify any specific pathogen as the cause.

2.3 TEST CHEMICALS

The following table (Table 2.1) summarises the compounds used within this thesis. The chemicals are grouped by biosynthetic origin and then alphabetically. Kovat's Indices (KI) values are shown. However it should be noted that these are not true KI values as they were not obtained under isothermal conditions, but rather the same oven settings as used during the GC-EAG work (Bernier *et al.*, 1998). The KI values shown here are calculated from the peaks of a known standard solution containing *n*-alkanes (C6, 8, 10, 12, 14, 16, 18, and 20). This standard solution was run at the beginning of the GC-EAG work to ensure the GC and method were as working as expected. KI was calculated using the following formula:

$$KI = \left[\frac{Tr(unknown) - Tr(n)}{Tr(N) - Tr(n)}\right] \times (100 \times Z) + (100 \times n)$$

Figure 2.2: equation used to calculate Kovat's Indices for chemicals used in this thesis. *K* = Kovat's Indices; *Tr* = retention time; *unknown* = the test chemical; *n* = the smaller hydrocarbon; *N* = the larger hydrocarbon; *Z* = the difference in carbons between the smaller and larger hydrocarbons.

Table 2.1: Chemicals used in this thesis with CAS number, purity, molecular weight and Kovat's Indices (KI) measured on an Agilent model 6850 with a DB-Wax polar column (30m x 0.32mm x 0.25μm) against *n*-alkane standards; all chemicals were obtained from SigmAldrich (Gillingham, Dorset, UK).

Common Name	CAS No.	Purity	MW	KI
Benzenoids				
anisyl alcohol	105-13-5	≥98%	138	2252
Benzaldehyde	100-52-7	≥99%	106	1503
benzyl acetate	140-11-4	≥99%	150	1711
benzyl alcohol	100-51-6	≥99.9%	108	1857
benzyl benzoate	120-51-4	≥99%	212	
benzyl salicylate	118-58-1	≥98%	228	2719
butyl salicylate	2052-14-4	≥99%	194	1975
methyl 2-methoxybenzoate	606-45-1	≥99%	166	2071
methyl benzoate	93-58-3	≥99%	136	1602
Phenylpropanoids				
2-phenylethanol	60-12-8	≥99%	122	1893
3-hydroxybenzaldehyde	100-83-4	≥97%	122	1656
4-hydroxybenzaldehyde	123-08-0	≥97%	122	2850
cinnamyl alcohol	104-54-1	≥98%	134	2258
Eugenol	97-53-0	≥99%	164	2142
hydrocinnamaldehyde	104-53-0	≥99%	134	1756
Phenylacetaldehyde	122-78-1	≥95%	120	1641
methyl salicylate	119-36-8	≥99%	152	1424
Salicylaldehyde	90-02-8	≥98%	122	1656
Nitrogen containing compounds				
Indole	120-72-9	≥99%	117	2403
methyl anthranilate	134-20-3	≥98%	151	2204
Monoterpenoids				
(-)-linalool	126-91-0	≥97%	154	1541
(+)-(3)-carene	498-15-7	≥99%	136	1166
(±)-linalool	78-70-6	≥97%	154	1550
(±)-(α)-pinene	80-56-8	≥98%	136	1108

β-myrcene	123-35-3	≥90%	136	1174
Camphene	79-92-5	≥95%	136	1125
1,8-cineol	470-82-6	≥99%	154	1206
Geraniol	106-24-1	≥98%	154	1840
lilac aldehyde *		**	168	1145, 1153, 1167
(S)-(-)-limonene	5989-54-8	≥95%	136	1197
(R)-(+)-limonene	5989-27-5	≥97%	136	1197
Gree Leaf Volatiles (GLVs)				
(Z)-3-Hexen-1-ol	928-96-1	≥98%	100	1384

* lilac aldehyde synthesised by Dr. Paul Douglas. ** GC analysis showed three peaks giving KIs of 1145 (23%), 1153 (50%), and 1167 (27%).

2.4 ELECTROPHYSIOLOGY

Electroantennography (EAG) measures changes in potential across an insect antenna when receptors on the antenna are stimulated (Schneider, 1957). The method was originally developed to measure the responses of antennal receptors to insect sex pheromones, but has since been used for a wide range of insect semiochemicals, including host-plant volatiles (e.g. Raguso *et al.*, 1996; Rojas, 1999a; Bruce and Cork, 2001; Plepys *et al.*, 2002a).

In this thesis, three different methods were used to deliver the odour stimulus to the antenna preparation. The reason for this was to improve the clarity of the results and speed up the process of carrying out experiments and collecting EAG data. The first method (A) used a Pasteur pipette to direct a small jet of air carrying the stimulus chemical onto the insect's antenna. Although this provided good responses to the test chemicals, the insect's mechanoreceptors also responded to the changes in air movement caused by the jet of air exiting the Pasteur pipette. To overcome this method B was used in which the air from the Pasteur pipette was injected into a continuous air stream directed at the insect's antenna. The volume of air directed at the insect's antenna was maintained at a constant rate thus reducing the neurological noise in the EAG recordings created by changes in air movement. Both methods A and B rely on the volatilisation of the chemical stimuli from filter paper inside the Pasteur pipette into the air stream moving through the Pasteur pipette. Therefore, the concentration of the test chemical in the air coming into contact with the insect will vary due to differences in volatility of the chemicals under investigation. Method C aimed to address this problem by using gas chromatography (GC) linked to the EAG recording. A known quantity of the test chemical was injected

into the GC from which all of the injected molecules were delivered in a vaporised state to the antennal preparation, thus allowing the actual amount of the test chemical stimulating the antennal receptors to be accurately quantified.

2.4.1 Insect preparation

Moths were placed into 45 mL holding pots and anaesthetised using CO_2. They were subsequently transferred to a groove carved into a block of modelling-clay with the insect's ventral side facing upwards. A thin strip of modelling-clay was used to hold the insect's body in place so that only the ventral and topside of the head and antenna were exposed (see Figure 2.3). The insects generally recovered from the CO_2 within 30 s. Each antenna was spread out from the head in a V-shape and held in place by staples (0.2 mm diameter and 5-7 mm length cut from copper wire) in such a way that the ventral sides of the antenna were exposed. Extreme care was taken not to push the staples in too far to avoid undue pressure and damage to the antennae. Usually as the insect recovered from the CO_2 it would spread out its antennae making pinning them down easier.

2.4.2 Electroantennography preparation

Microelectrodes were made using an electrode puller to stretch borosilicate glass capillaries (1.5 mm O.D. and 0.86 mm I.D.). The glass capillary was heated at its midpoint and pulled at both ends stretching the glass out and forming two fine-tipped points (c. 30 mm long) which were then filled with 0.1 M KCl electrolyte solution containing 1% (w/v) polyvinylpyrrolidone (PVP). To make the microelectrode these fine-tipped glass capillaries were then placed into the electrode holder containing a fine silver wire, which acts as the

connection between the recording equipment and the haemolymph of the insect.

The microelectrodes were attached to silver electrodes held in micromanipulators via clamps on a portable EAG device developed by Syntech (INR-02; Syntech, Hilversum, The Netherlands). Using the micromanipulators the fine tip of the reference electrode was inserted through the antennal cuticle in the pedicel so as to connect the electrode with the haemolymph. The circuit was completed by snipping off the last two or three segments at the distal end of the antenna and inserting the antenna into the recording electrode as shown in Figure 2.3.

Using this method, the insect remained intact and alive. Thus the preparation could be used for many hours with little loss of neurological response. It was found that once removed from the EAG apparatus the insect would recover and its life span did not appear to be reduced.
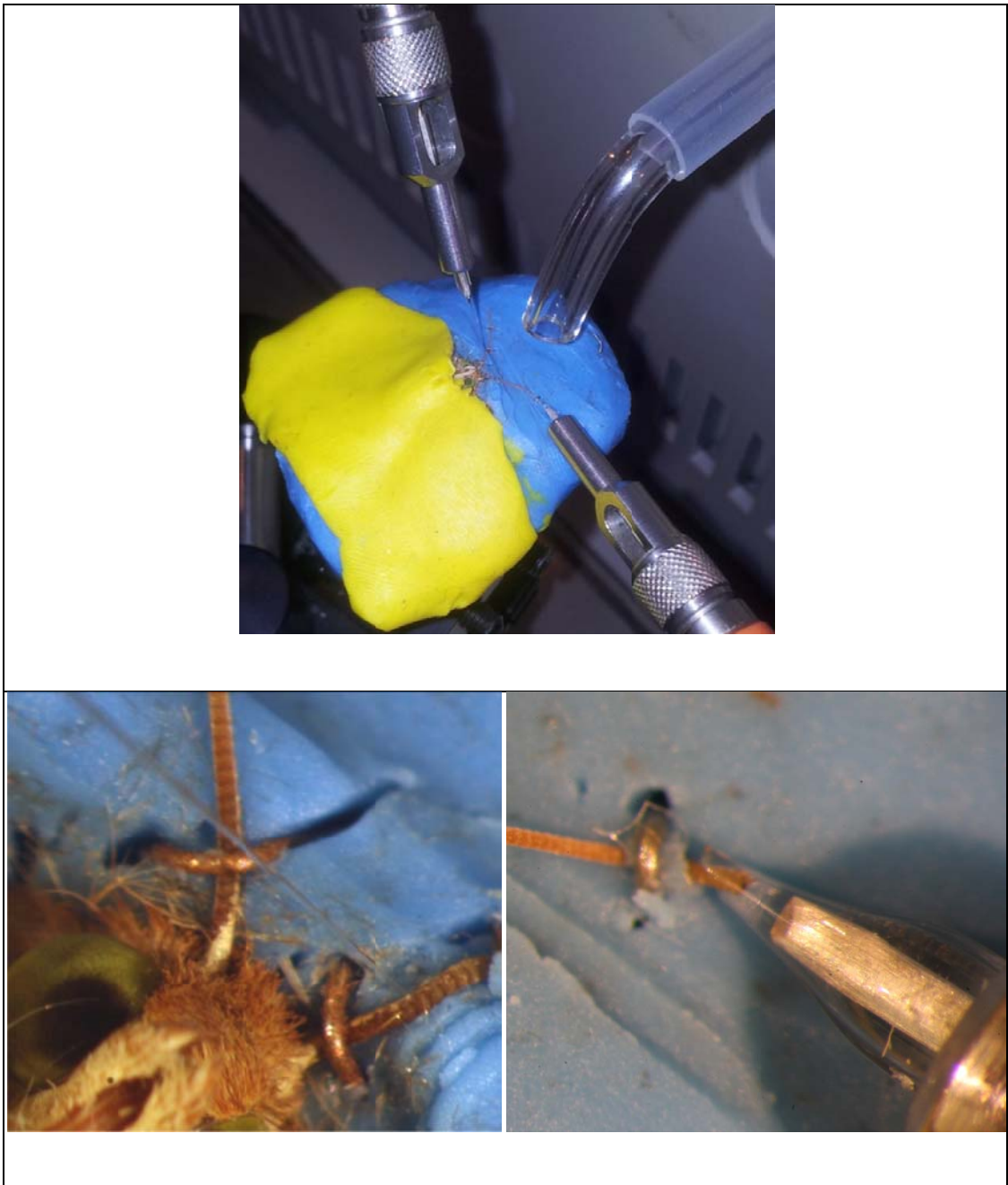
Figure 2.3: GC-EAG preparation of *H. armigera*. Top image: overview showing the insect held in plasticine, with the reference electrode on the left, recording electrode on the right, and GC effluent coming from the right directed down towards the antenna; lower-left image: zoomed in image of the reference electrode coming from the top-left of the image and entering the proximal end of the antenna; lower-right image: zoomed in image of the distal end of the antenna inserted into the recording electrode.

2.4.3 Presenting the chemical stimuli to the EAG preparation

Three methods were used to present the chemical stimuli under investigation.

2.4.3.1 <u>Method A</u>

The volatile test chemical was deposited on a strip of filter paper (20 x 5 mm) which was inserted into a Pasteur pipette. The wide end of the Pasteur pipette was attached to plastic piping connected to a charcoal filter, followed by an air pump. The air pump was set to provide air at 350 mL min^{-1} for 3 s, and controlled by a foot-peddle. The fine end of the Pasteur pipette was aimed at the mid-section of the EAG preparation and held 1 cm away from it. Pressing the foot peddle triggered the air pump to pass a fixed volume of air containing the test chemical over the insect's antenna (Figure 2.4).
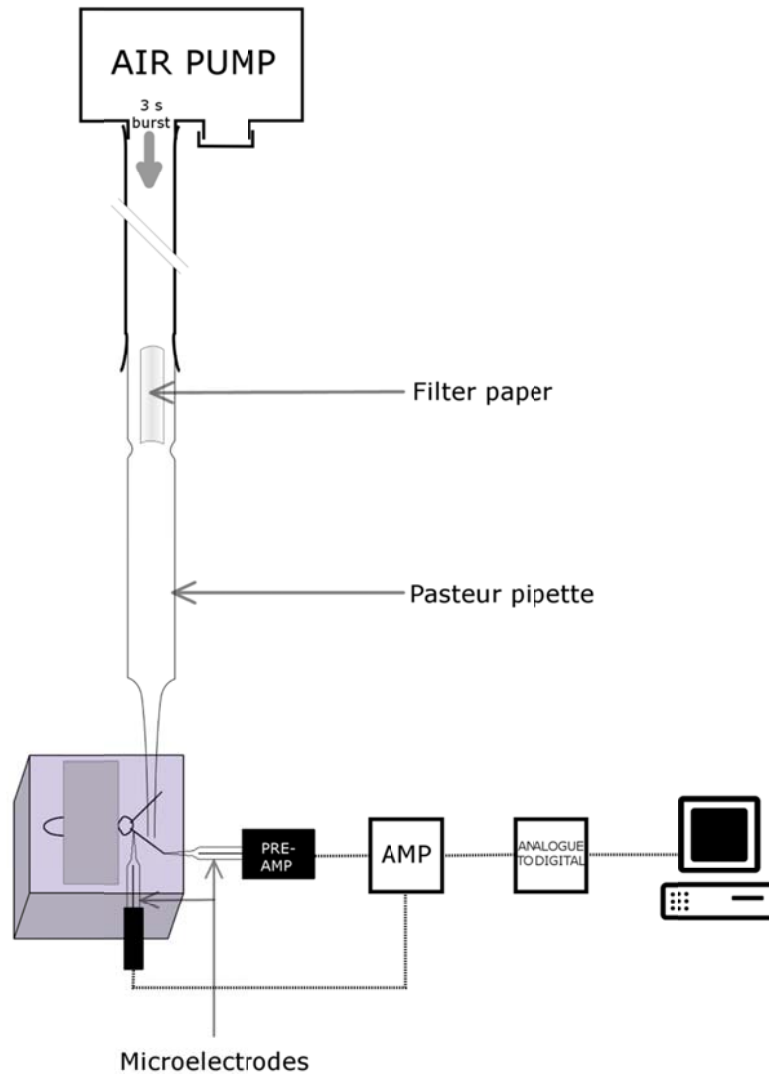
Figure 2.4: Method A. Chemical stimuli is blown directly onto the insect's antenna. Not drawn to scale.

2.4.3.2 <u>Method B</u>

A similar same setup was used as for method A, with one alteration. The effluent from the Pasteur pipette containing the odour stimulus was injected into a constant odour stream passing over the insect's antenna. A glass tube (I.D. 1 cm, length 10 cm) was positioned 1 cm from the midpoint of the insect's antenna. The tube had a hole at the midpoint, into which the tip of a Pasteur pipette could be inserted. The glass tube and Pasteur pipette were connected to an air pump via charcoal filters and supplied a stream of air at 1150 mL min^{-1} and 350 mL min^{-1} respectively. As the 3 s burst of air came through the Pasteur

pipette and into the glass tube, the airflow into the glass tube was reduced by an equal amount, thus the rate of air leaving the delivery tube and reaching the antennae remained constant (Figure 2.5).
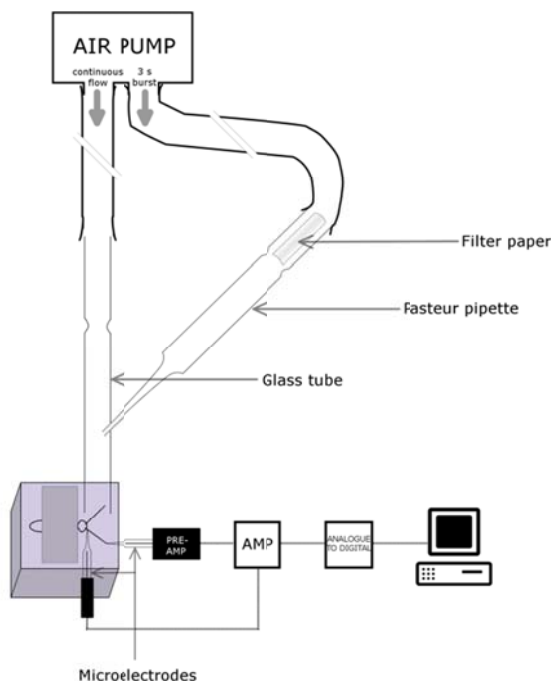
Figure 2.5: Method B. The chemical stimulus is blown from the filter paper into a continual airstream directed at the insect's antenna. The volume of air entering the continuous airflow reduced at the same rate that was being injected into it via the Pasteur pipette, thus maintaining a constant airflow onto the insect's antenna. Not drawn to scale.

2.4.3.3 <u>Method C</u>

The third method utilised a GC (Agilent, HP6890) to deliver the test chemical over the antenna. The test stimuli was injected into the polar column (Wax10; 30 m × 0.32 mm i.d. 0.25 μm film thickness; Supelco) of the GC. The carrier gas was helium (2.4 mL min^{-1}), and injection was splitless (220 °C). After exiting the GC's column the volatiles were split (1:1 ratio) with a push-fit Y-piece between the flame ionisation detector (FID) (at 250 °C) and a silanized glass T-piece in the column oven leading to the EAG preparation (Figure 2.6) as described by (Cork *et al.,* 1990). The effluent directed towards the antenna was mixed with a

stream of charcoal-filtered, humidified air with a combined outflow rate of 400 mL min^{-1}. The exit pipe was positioned approximately 10 mm from the insect's antenna.
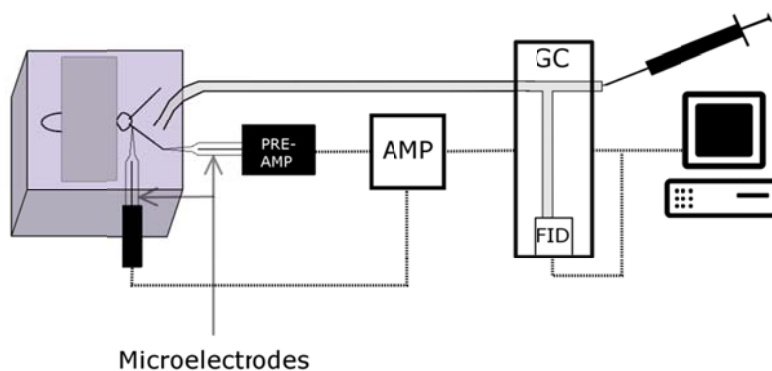
Figure 2.6: Method C. The chemical stimuli was injected into the GC and the resulting column effluent is split (1:1) to the FID and EAG preparation. Key: GC, gas chromatograph; FID, flame ionisation detector. Not drawn to scale.

2.4.4 Recording the EAG signal and delivery protocol

2.4.4.1 Data-collection for stand-alone EAG preparation (Methods A and B)

This method was used for EAG methods A and B. The recording electrode was connected to a 10x pre-amplifier (Syntech) and the signal was fed to the high impedance amplifier (Syntech, AC/DC Amplifier UN-06). This analogue signal was passed into a digital interface (Nelson 400) converting it to a digital signal for processing and storage using EZ Chrom Elite (version 3.3.2, Agilent) on a PC.

To test the antennae with the samples a similar technique was used as described by Cork *et al.* (1990).

(i) Test protocol

To test an odour the following protocol was used:

1. A standard was presented to the antenna
2. A hexane blank was presented
3. The test odour was presented
4. The standard was presented again
5. The hexane blank was presented again.

There was 60 s between each part.

EAG peak height was calculated manually from the EZ Chrom output and converted to a value relative to the standard (2.5 mmol of phenylacetaldehyde). This standardised EAG value (sEAG) was calculated with the following formula:

$$\frac{(O_1 - B_1)}{(S_1 - B_1)} = \text{sEAG}$$

O_1 = first odour peak height, S_1 = first standard peak height, B_1 = first blank peak height, sEAG = standardised value used in analysis.

2.4.4.2 Data collection for Method C (GC-EAG preparation)

This was used for EAG method C. The recording electrode was connected to a 10 x pre-amplifier (Syntech) and the signal was fed through a high impedance amplifier (INR-02; Syntech, The Netherlands) and on to the GC. Both the signals from the GC's FID and the EAG amplifier were recorded digitally via a PC running EZ Chrom Elite (v3.0; Scientific Software Inc., Pleasanton, CA, USA).

(i) Test protocol

2 µL of a test solution was injected into the polar column of the gas chromatograph (Agilent, Classic 6890). As the effluent from the GC's column

was split 1:1, half of the material injected would pass over the insect's antenna and the other half would pass through the FID.

The insect's antenna was placed under the EAG air flow from the GC after the solvent had exited the GC (approximately 4 min after the sample was injected). Each insect was tested with one or more groups of compounds at multiple doses. The *(Z)*-3-hexen-1-ol internal standard was used to test for degradation of the antenna's response over time. All EAG responses were calculated relative to the response to *(Z)*-3-hexen-1-ol for that particular run. A single run lasted 22 min and although the moths remained useable for $\geq$8 h they were generally discarded after 4 h.

(ii) Data extraction and analysis
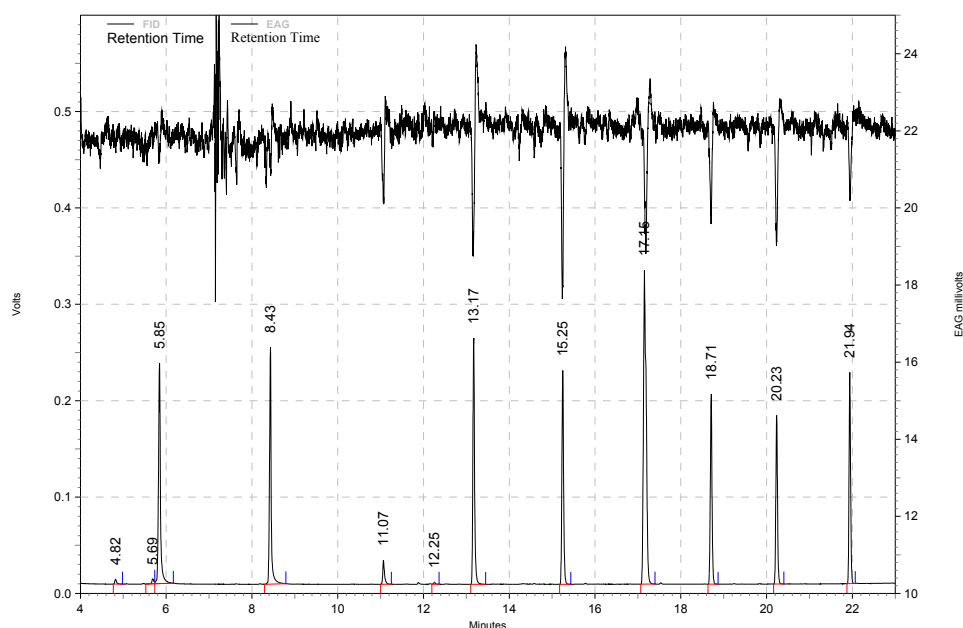
Figure 2.7: Typical data from a single linked GC-EAG analysis using test solution group A at 495 µmol tested on the antenna of *Helicoverpa gelotopoeon*. Upper line is the EAG response, the lower line the FID response. The FID y-axis is on the left, the EAG y-axis on the right side. The compound with a retention time of 11.07 min. is the internal standard. Note that EAG responses elicited negative peaks followed by smaller positive peaks.

Due to the large volume of data collected (several thousand EAG responses) in these experiments, an automatic method of calculating EAG peak size was developed. To do this, the output from the FID and EAG were exported into a spreadsheet (Microsoft Office Excel 2007). A baseline for the EAG data was calculated by averaging all the values 30 s each side of a single moment. The size of each EAG peak was calculated by subtracting the lowest point in the trough from the baseline to give a positive number (in millivolts) for each EAG peak (see Figure 2.8). As there was often noise present in the EAG data (EAG recording electrodes also picked up vibrations in the preparation, biological functions in the insect, and other external disruptions) not all peaks in the EAG data were due to the insect's olfactory responses. It was therefore necessary define at which point on the EAG trace corresponded to the chemicals exiting the GC. This was done by adding a formula that searched along the FID trace for moments when the FID value was above a certain threshold. The threshold was manually set depending on the quantity of material exiting the GC, e.g. for a 5 µmol run the threshold was set very low, and for a 495 µmol run the threshold was set very high. This allowed the FID peaks to be identified by the spreadsheet and defined at which points on the EAG trace differences between troughs and the baseline should be calculated.
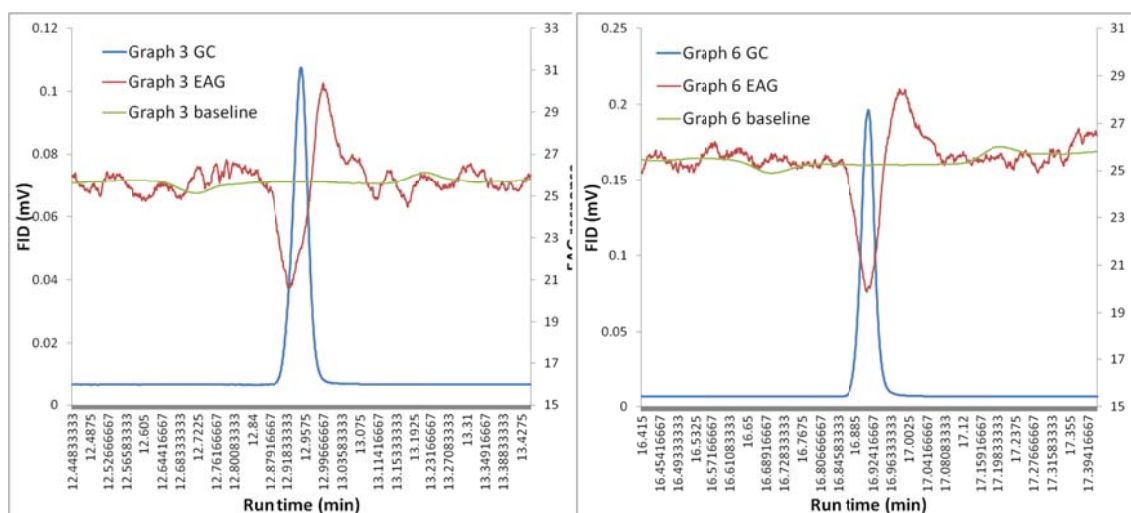
Figure 2.8: Representative GC-EAG traces after importing into the Excel spreadsheet used to automatically calculate EAG responses. The blue line shows the FID trace, the red line shows the EAG trace, and the green line is a baseline calculated from the EAG values 30 s before and after that instance. The spreadsheet calculates the difference between the trough in the EAG trace and the baseline during the period that the FID shows a peak.

Several hundred EAG peaks were also calculated manually which allowed comparison between the automated spreadsheet method and the manual method (see next section for statistical analysis comparing manually and automatically collected data).

All statistical analyses were carried out on EAG peak height values relative to the internal standard (50 µmol of (Z)-3-hexen-1-ol) (standardised peak height - sEAG).

(iii) Comparing automatically calculated values against manually calculated values

To test the accuracy of the Excel spreadsheet a selection of EAG response peaks were calculated manually and automatically (using the Excel spreadsheet). The means of the two groups were compared using a Wilcoxon Rank Sum test and no significant differences were found ($P > 0.05$). However,

there was a significant difference in the variance between automatically and manually collected data ($P < 0.05$) with the former having more variance than the latter. The increased variance may impact the statistical analysis of the EAG experiments making making it more difficult to identify significantly different treatments.

2.5 BEHAVIOURAL BIOASSAYS

Behavioural bioassays were carried out in a wind tunnel (described below). Initially the insect's behaviour was recorded manually, but for later experiments a video camera, PC, and tracking software were added which allowed the insects' movement to be tracked digitally providing increased acuity.

2.5.1 Wind tunnel

The wind tunnel was a 'closed circuit, closed jet' similar to that described by (Vogel, 1969). The unit had an upper and a lower section, the upper containing the flight area (1.7 m long x 0.6 m high x 0.6 m wide) (Figure 2.9). The flight area was defined by four Perspex panels. One side-panel had two Perspex doors (0.2 m x 0.2 m) cut into it. The panels (except the bottom panel) were held in place with chrome plated steel frames and twist-clips that allow for easy and fast removal of the panels. The bottom panel rests on the base of the upper section and can be easily removed for cleaning and maintenance.

The upper and lower sections of the unit were connected with galvanized steel ducting that contains closable vents which allowed external air to be brought into the unit if required. At each end of the wind tunnel section were two filter plates to remove large and small particles (grade G4 to EN779) which stopped any moths from entering the lower sections and also assisted in providing a

more uniform air flow through the wind tunnel by providing a pressure difference between each side of the filter plate.

The lower section contained an 'Extra Duty' filter (Airclean Ltd.) consisting of 6 'grade 50', activated carbon filter cells rated as being able to adsorb 55% of carbon tetrachloride in 0.1 s, with a surface area of 1100-1200 m^2/g. The filter contained 25 kg of carbon and was positioned after the fan unit. There was space in the lower section for another filter block of equal or less capacity in front of the fan should it be required. The fan used was a direct driven DIDW centrifugal fan (Imofa, (UK) Ltd., model DD-12-9-9). The motor was enclosed to ensure it cannot contaminate the air stream, and was an F class with overheat protection suitable for temperatures up to 50°C. Anti-vibration motor mountings were incorporated to reduce the risk of transferring vibration to the upper section. The fan was capable of supplying 0.78 m^3/s of air with a motor speed of 900 rpm.
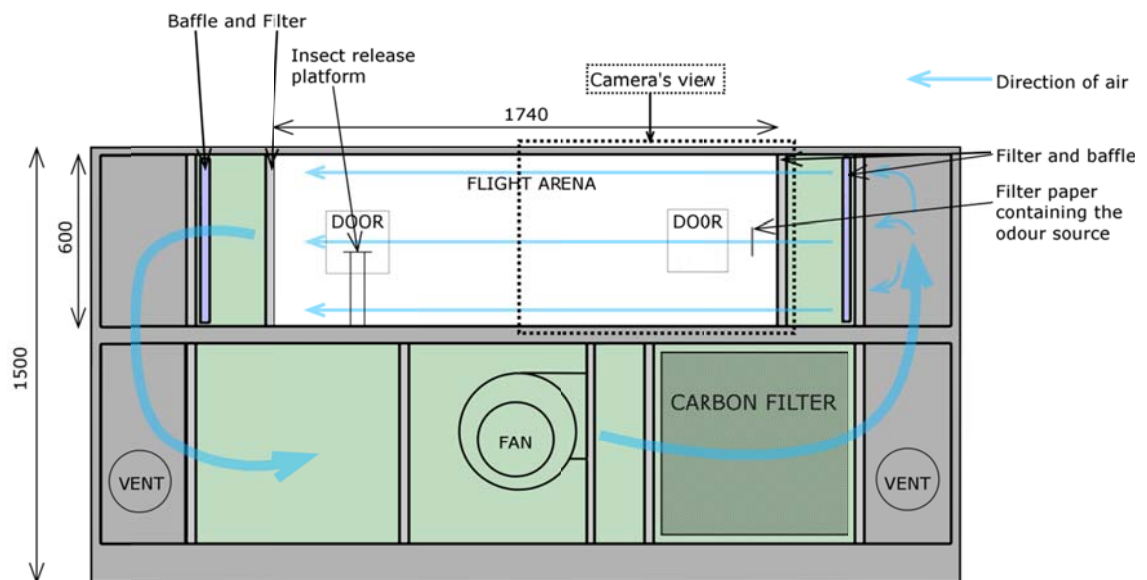
Figure 2.9: Schematic of the wind tunnel used for the experiments.

To allow recording of the insects' flight, a digital camera (Sony video camera with a swivel lens and VISCA port, unknown model) was positioned

approximately 1.5 m from the flight arena, pointing at the upwind section of the arena to record a side view as indicated by the dotted lines in Figure 2.9. As the experiments were carried out in low lighting (using a 15 W lamp covered with a red filter), infra red lighting was positioned behind the wind tunnel arena facing the camera (Figure 2.11). The IR light was provided by a bespoke IR LED array. The LED array was made from 96 infrared LEDs (T-1¾ LED 940nm 60° HIRL5040, RS-components), wired onto a mirrored Perspex board (570 x 870 mm), connected to a 12V 1.5 amp power supply. Immediately infront of the LED array were paper sheets used to diffuse the light and provide a uniform background for the tracking software (Figure 2.10).

Figure 2.10: Wind tunnel used in behaviour bioassays. Air movement travels right to left (example odour source filter paper can be seen in right hand photograph).

Figure 2.11: Video camera's view of the wind tunnel in the dark with the IR LEDs on. The white disc on the right hand side is the filter paper. Air flow is right to left.

2.5.2 Assessment of air speed

Air speeds were assessed using a hot wire anemometer and a cup anemometer. The fan was connected to a control box with a variable dial (0 – 10). The air speed results can be seen in Figure 2.12. The hot-wire anemometer provided a much more accurate reading than the cup anemometer at low speeds but it was only able to measure air speeds up to 1.8 m/s. Therefore a cup anemometer was used to measure the speeds for the upper dial positions. It should be noted that the results of the cup anemometer were similar to those of the hot-wire anemometer for dial positions 5 and 6, but it was not accurate at lower air speeds.

Measurements were taken at three locations along the wind tunnel: upwind, middle, and downwind. At each of these locations three measurements were taken at different heights from the floor of the flight arena: bottom, middle, and top. These data were pooled as there was so little variation between the locations.

The results show a steady increase in wind speed from 0.5 m/s at dial position 0 to 2.6 m/s at dial position 10. Variation of wind speed at the positions measured was minimal (shown by the small error bars for the data points measured using the hot wire anemometer). Measurements taken using the cup anemometer were more variable. The low amount of variation indicates that the wind tunnel provides a highly unified airflow. Tests using smoke trails of titanium tetrachloride showed that the airflow was also highly laminar.
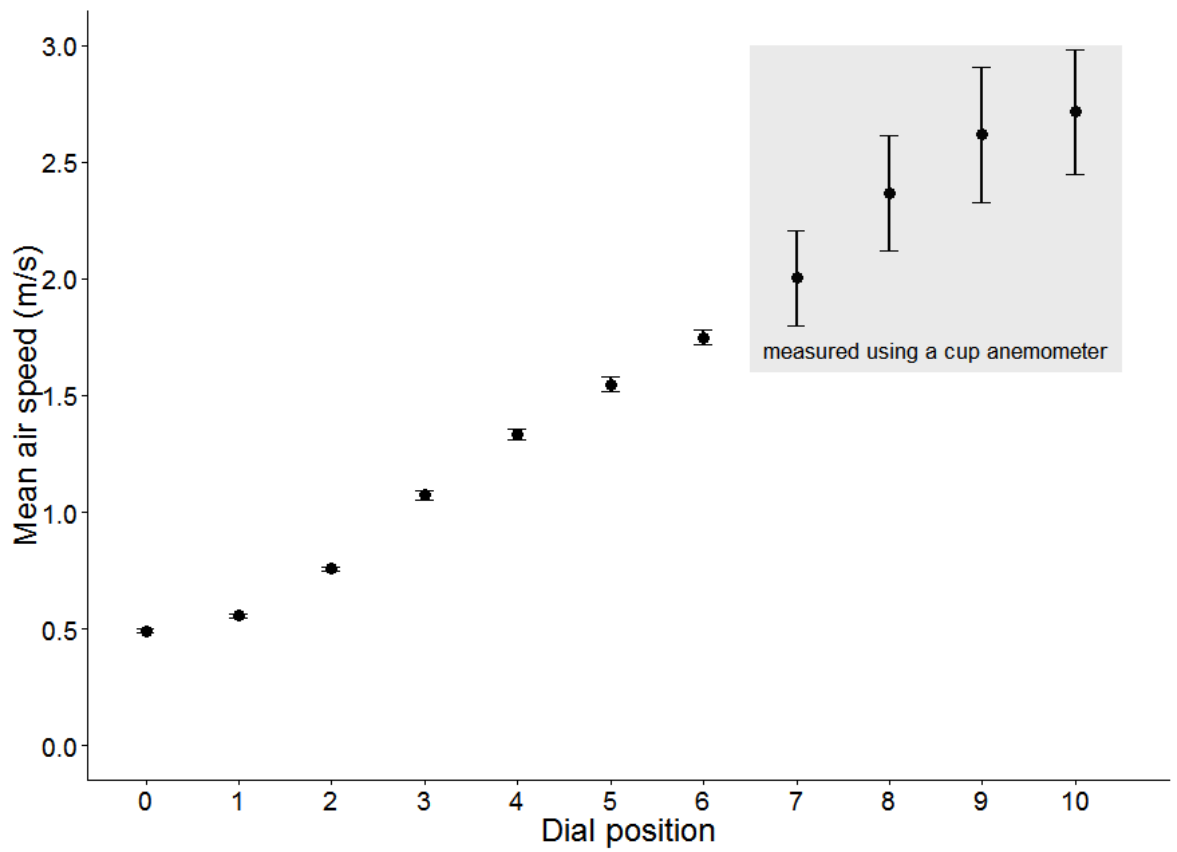
Figure 2.12: air speed (m/s) measured at several different points within the wind tunnel at different positions of the control dial. Dial positions 0 – 6 were measured with a hot-wire anemometer, and positions 7 – 10 were measured with a cup anemometer.

2.5.3 Presentation of odours in the wind tunnel

In order to test odours in the wind tunnel a suitable method had to be devised to present the odours. Several delivery methods were trialled: (1) pipette with cotton wool as described by Cunningham *et al.* (2004); (2) the plastic vials and sachets used in field trials, (3) a filter paper with freshly pipetted solutions. The pipette and cotton wool method did not stimulate any behavioural response in any of the insects tested. Using titanium tetrachloride to visualise the odour plume coming from the pipette showed the plume to be thin and uniform with little dispersion. It is possible that the plume's shape made it unlikely that the insects would come into contact with the odours. The plastic vials and sachets used in the field trials were found to release too much odour and the whole laboratory filled with the odours' scent especially whilst changing treatments between runs. The filter paper method was found to be quick, simple and effective. Some additional investigations were carried out to assess how much odour should be placed onto the filter paper (Figure 2.13 and Figure 2.14) and to test whether the insects were responding to the visual stimuli of the paper as well as the olfactory stimulus (data not shown). In both sets of experiments each odour source point was made from one aliquot of solution containing 10 µL of the University of Greenwich blend (UoG blend, containing phenylacetaldehyde, salicylaldehyde, methyl 2-methoxybenzoate, linalool, and limonene in a 10 : 4 : 2 : 2 : 1 ratio) dissolved in hexane at the reported concentration. Where multiple odour sources points were applied to a filter paper, each aliquot was placed 20 mm apart around the centre of the filter paper.

The results in Figure 2.13 show that the number of odour source points to place on a filter paper in the wind tunnel in order to achieve an optimal behavioural

response from *A. gamma* was four point sources. With four odour source points on the fitler paper 90% of the moths contacted the odour source. A Fisher's Exact test found a significant difference between the number of moths that contacted or did not contact the odour source for the three treatments (Fisher's Exact test, $P < 0.001$, N = 10, 20, 20 for treatments with 1, 4, or 9 odour source points respectively).
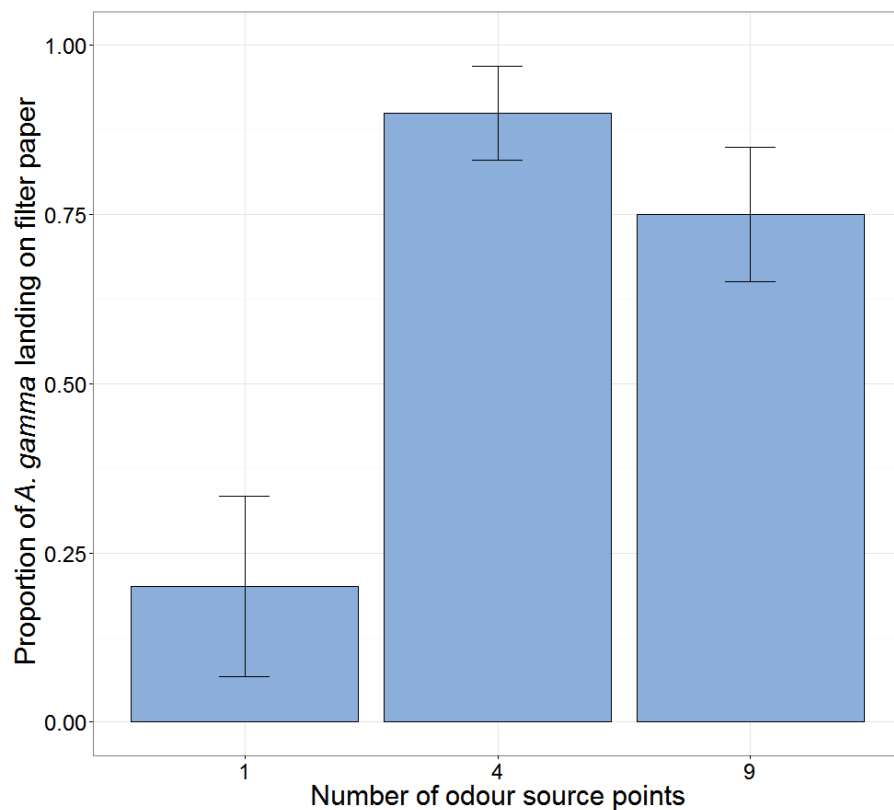
Figure 2.13: The proportion of *A. gamma* moths that landed on filter paper treated with a floral attractant. The filter papers contained 1, 4, or 9 aliquots of the odour solution (10 µL of the UoG blend dissolved in hexane). A significant difference between treatments was found (Fisher's Exact test, $P < 0.001$, N = 10, 20, 20 for treatments with 1, 4, or 9 odour source points respectively). All moths were 7 - 9 days old, and starved for 24 h prior to testing.

The results in Figure 2.14 show that there was no significant difference between the concentrations tested and the proportion of *A. gamma* that contacted an odour source containing four or nine aliquots of the test solution.
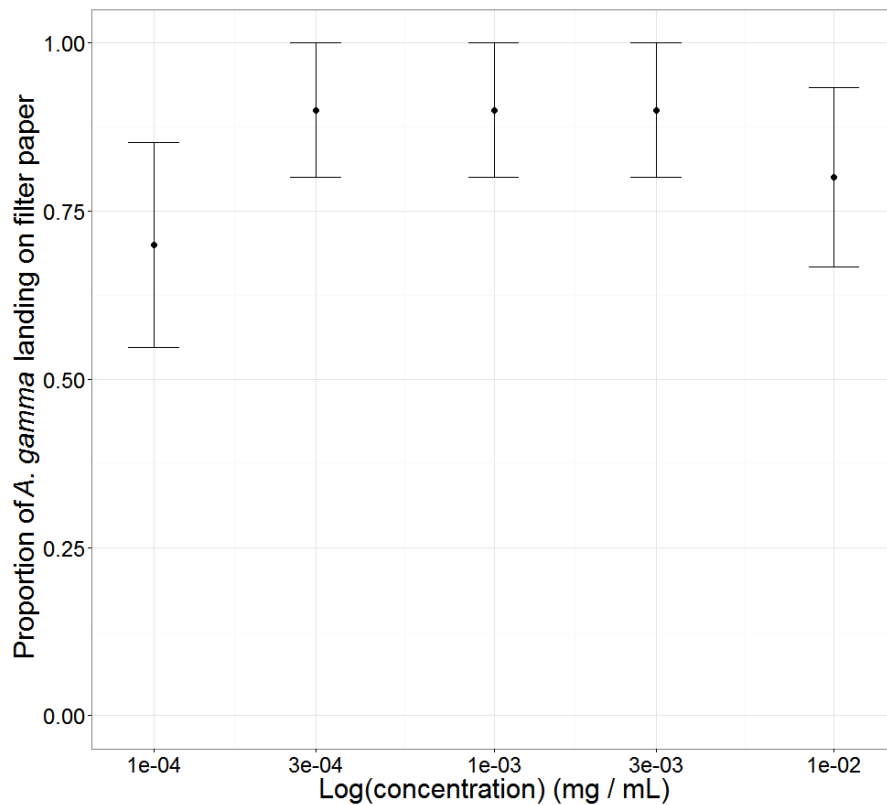
Figure 2.14: The proportion of *A. gamma* moths that landed on filter paper treated with a floral attractant at different concentrations. The papers contained either 4 or 9 aliquots of the odour solution. No significant difference was found between treatments (Fisher's Exact test *P* > 0.05, N = 10 for all treatments. All moths were 7 - 9 days old, and starved for 24 h prior to testing.

2.5.4 Statistical analysis

Statistical analysis and data plotting was carried out using R (ver. 3.03) for Windows with R-Studio (ver. 0.98). Additional R packages installed include: denstrip (ver. 1.5.3), ggplot2 (ver. 0.9.3.1), grid (3.0.3), lattice (ver. 0.20), Lme4 (ver. 1.1), Lmertest (ver. 2.0), MASS (ver. 7.3), Multcomp (ver. 1.3), reshape2 (ver. 1.22), survival (ver. 2.37).

2.6 DISCUSSION

2.6.1 Insect rearing

The importance of good hygiene and using colonies known to be free of disease is evident from the spread of disease through all of the colonies reared during

this work. If disease occurs within the colony it is extremely difficult to control and remove it. Attempts to completely clean out and sterilise the rearing laboratory followed by re-establishment of the insect colony did not succeed in keeping disease out of the new colonies.

Aside from the difficulties of running experiments when disease is affecting the availability of insects with which to carry out the work, certain pathogens that affect Noctuids are known to affect their behaviour (e.g. Vasconcelos *et al.*, 1996; Georgievska *et al.*, 2010). This has obvious implications on the behaviour work conducted during this project as it is possible that disease load may have influenced the behaviour of the insects being tested. Furthermore, it is conceivable that the health of the insects may have influenced the antennal responses seen in the electroantennography (EAG) work. Successful EAG requires a good connection between the insect's haemolymph and the electrodes. Insects in poor health may have reduced electrolytes in their haemolymph or be dehydrated which could negatively impact the EAG responses. In addition, it is conceivable that an insect's olfactory system may not function correctly if infected with pathogens. However, all of the insects used in the EAG work did not exhibit visible signs of disease and there were no indications that the EAG responses were impeded in any way.

2.6.2 Electrophysiology

Three methods were used to carry out electrophysiology experiments and each had its advantages and disadvantages. Methods A and B both had the advantage that a GC was not required which simplifies the amount of equipment needed. This also allows the order of the chemicals under investigation to be randomised making the statistical analysis more robust. In

addition, method B produced cleaner signals (i.e. a better noise : signal ratio) compared to method A. Both methods suffered from several disadvantages, the main being that quantification of the chemical stimuli is extremely difficult so that carrying out accurate dose-response experiments would be difficult. Methods A and B also required an operator to be present all the time the experiment was being carried out. This inevitably created additional noise in the EAG signal.

Method C overcame some of the disadvantages of the first methods, by utilising a GC to dispense the test chemicals. This allowed for quantification of the material and automation of the chemical delivery (so that the operator could leave the room whilst the experiment was going, reducing disturbance within the room and therefore reducing noise in the EAG signal). However, it did have the disadvantage of the chemical delivery being dependant on the retention time of each compound, thus not allowing for randomisation of the order the chemicals were delivered. There was also a limit on the quantity of the chemicals that could safely be injected into the GC (to avoid overloading the column).

The Excel spreadsheet used to calculate EAG responses automatically in Method C greatly sped up the data collection process (see 2.4.4.2). Although the variance was significantly greater for the automatically collected data compared to the manually collected data there was no significant difference between the two (auto and manually collected) EAG data sets. To reduce the chances of erroneous auto-calculated values the data was visually checked (using scatterplots) to identify any values that seemed unusually high or low compared to the other values. This highlighted a few miscalculations usually caused by the baseline being out of position due to large jumps in EAG recording electrode (probably due to insect movement, outside influences, or

building vibrations). Overall the spreadsheet was a huge time saver and if more time or funds had been available its basic premise could be used to design and program a simple piece of bespoke software that should handle the calculations required more efficiently. The processor used to carry out the calculations in this thesis was an Intel E6550 CPU (Core: 2.33 GHz, FSB: 1333 MHz) which, due to the extremely large number of calculations required, was a little slow to work out the EAG response values in Excel (c. 15 s to refresh the spreadsheet).

The spreadsheet was also designed to create line charts for each GC peak so that the user could see a zoomed in version of the GC-EAG data and check that the EAG response lined up correctly with GC peak and the calculated baseline was in the correct place (see Figure 2.8 for examples of these charts).

2.6.3 Behavioural bioassays

2.6.3.1 Assessment of airspeed

For all experiments an air speed of 0.5 - 0.6 m/s was used. This is similar to the air speed used in previous behavioural experiments for Lepidoptera (e.g. Tingle *et al.*, 1989; Bruce and Cork, 2001; Mechaber *et al.*, 2002; Fraser *et al.*, 2003).

The airflow within the wind tunnel was relatively uniform with little difference in air speed across the latitudinal and longitudinal sections of the flight arena (top, middle, and bottom, and also in front, middle, and back). The baffles at each end of the arena even out the flow in the latitudinal plane by providing a slight positive pressure on the windward side of the baffle and negative pressure on the leeward side; likewise, the equal pull and push of air at the respective ends of the arena give it a very even flow in the longitudinal plane.

2.6.3.2 <u>Presenting odours in the wind tunnel</u>

Filter paper treated with at least 4 evenly spaced (20 mm apart) aliquots of odour dissolved in hexane at a concentration of between 0.0003 - 0.003 mg / mL elicited the highest behavioural responses. Testing the plume shape with titanium chloride showed a larger more dispersing plume (compared to that seen using the pipette and cotton wool method). This was probably due to the larger number of odour source points and the increase air turbulence created around the filter paper. The larger surface area from which the odour emanated was also more representative of how floral volatiles are released from flowers as discussed in section 1.2.1. No moths landed on untreated filter paper and therefore we may assume that their responses were due to olfactory stimulation only and not visual.

CHAPTER 3 - INITIAL ASSESSMENT OF THE ATTRACTIVENESS OF FLORAL BLENDS IN THE UK AND ARGENTINA

3.1 INTRODUCTION

Many floral odour blends have been previously researched for their attractiveness to noctuid species (for a review see section 1.2.2). In this chapter some of those blends that may be particularly attractive to the target species in this thesis were assessed in field trials and in wind tunnel bioassays to identify which blend is most attractive to *A. gamma* and *Helicoverpa* species. Previous research has focused on odour blends that mimic host plants or the preferred flowers of the target species. The research in this chapter investigates these types of mimic odour blends but also 'super-blends', which are an artificial combination of VOCs known to act as attractants for the target species, i.e. do not mimic a single plant species.

For several of the blends tested this is the first time they have been assessed in these geographical locations (UK and Argentina) with the associated insect species. In addition, this the first time these blends have been compared to one another.

Bruce (2000) identified two odour blends that were designed to attract the noctuid *H. armigera*, one based on the floral odours of sweetpea, *Lathyrus odoratus* (L.) (Fabaceae) and the other on marigold, *Tagetes erecta* (L.) (Asteraceae). However, during field testing of these blends in Israel in chickpea and cotton fields, a significant number of *A. gamma* were also caught in the baited traps. The UK field sites used in the current study were known (after

consultation with the farm managers) to have regular and large numbers of *A. gamma* present, and therefore were an ideal location to assess how the species responded to the two blends identified by Bruce (2000).

An odour blend was tested which was being developed by Professor Alan Cork as a general noctuid attractant (Cork, 2011). This odour blend was the result of a systematic series of field trials in Bangladesh and India investigating five floral volatiles blended in a series of ratios to identify which ratio is the most potent attractant. The two main components of Cork's (2011) odour blend (from hereon called the University of Greenwich blend or UoG blend) are phenylacetaldehyde and salicylaldehyde, with three minor components: methyl-2-methoxybenzoate, linalool, and limonene. The ratio for these compounds is approximately 50:20:10:10:10 (phenylacetaldehyde : salicylaldehyde : methyl-2-methoxybenzoate : linalool : limonene) (Cork, 2011). All of these compounds have been found to be highly attractive for Noctuids (see Table 1.3 and Table 1.4), but linalool has also been shown to be repellent to *H. armigera* males in search of sex pheromone (Deng *et al.*, 2004).

In later field trials, two other super-blends were tested. The commercial product traded under the name "Magnet" contains phenylacetaldehyde, limonene, α-pinene, anisyl alcohol, butyl salicylate and cineole (see section 1.2.3.1 for further details). A blend that mimics leaves of the Lombardy Poplar, *Populus nigra* (L.) (Salicaceae), identified by Li *et al.* (2005) was also tested as it was found by the authors to be highly attractive to *H. armigera* and other noctuids in China. Although the authors based their odour blends on the wilted leaves of *P. nigra*, the compounds they identified as being attractive to *H. armigera* are

common constituents of floral odours, i.e. phenylacetaldehyde, salicylaldehyde, benzaldehyde, benzyl alcohol and 2-phenylethanol (Knudsen *et al.*, 2006).

3.2 MATERIALS AND METHODS

3.2.1 Lures

3.2.1.1 <u>Field trials</u>

Lures for field trials were made of the components shown in Table 3.1. The compounds were pipetted onto a cellulose acetate cigarette filter (14 x 6 mm, Swan, Republic Technologies Ltd., UK) in polyethylene sachets (5 cm x 5 cm, Transatlantic Plastics, Southampton, UK). The sachets were heat sealed and stored at -18°C until used.

3.2.1.2 <u>Wind tunnel bioassay</u>

To make the lures, the constituent chemicals of the odour blends were dissolved in pesticide grade hexane at 0.001 mg/mL. For details of the contents of each treatment see Table 3.1. Subsequently 4 aliquots (10 µL in each aliquot) of an odour blend dissolved in hexane was pipetted onto the central area of a filter paper 20 mm apart (Figure 3.1). The solvent was allowed to evaporate for a few seconds before being placed in the wind tunnel. The total quantity of the odour blend applied to the filter paper was 0.04 µg.
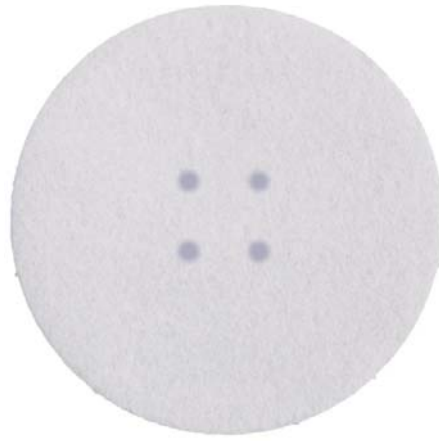
Figure 3.1: Odour lure used in wind tunnel bioassay with approximate locations of the odour source points on a 60 mm diameter filter paper (Whatman no. 9).

Table 3.1: Composition of blends used in field trial 1, 2, 3, and wind tunnel bioassay. Compounds are measured in µL unless otherwise stated.

Blend name	Total vol (uL)	Phenylacetaldehyde	Salicylaldehyde	Methyl 2-methoxybenzoate	(±)-Linalool	(S)-(-)-Limonene	Diacetone	Benzaldehyde	Benzyl alcohol	Phenylethyl alcohol	Benzyl Benzoate	Cinnamyl alcohol	Alpha-pinen	anisyl alcohol	Butyl salicylate	Cineol	4-Hydroxybenzaldehyde
Odour blends used in field trial 1																	
PAA	150	150															
PAA + linalool	180	150			30												
Sweetpea	600	150			150		150		150								
Marigold	165	150			5	5		5									
UoG	255	150	30	30	30	15											
Odour blends used in the wind tunnel bioassay (values are in percentages)																	
Magnet	100%	24.3				5							15.2	13.9	27.8	13.6	
P. nigra	100%	3.4	46.1					5.3	24.2	20.9							
UoG	100%	54	22	9.5	9.5	5											
Binary	100%	70	30														
Hexane	100%																

Blend name	Total vol (uL)	Phenylacetaldehyde	Salicylaldehyde	Methyl 2-methoxybenzoate	(±)-Linalool	(S)-(–)-Limonene	Diacetone	Benzaldehyde	Benzyl alcohol	Phenylethyl alcohol	Benzyl Benzoate	Cinnamyl alcohol	Alpha-pinen	anisyl alcohol	Butyl salicylate	Cineol	4-Hydroxybenzaldehyde
Odour blends used in field trial 2																	
Magnet	300	73				15							45	42	84	41	
P. nigra	300	10	138					16	73	63							
UoG	300	178	35	35	35	17											
Binary	300	210	90														
G9	300	150	30	30	30	15					15	15					15
Control	0																
Odour blends used in field trial 3																	
Magnet	250	41.7				41.7							41.7	41.7	41.7	41.7	
P. nigra	250	8.55	115				13.3	60.5	52								
UoG	250	135	55	23.8	23.8	12.5											
Binary	250	176	74														

3.2.1.3 <u>Sex Pheromones</u>

For field trial 1 *A. gamma* pheromone lures and VARL+ traps were supplied by Csalomon®, Plant Protection Institute, Hungary.

The pheromone traps used in field trial 2 were UniTraps baited with *A. gamma* pheromone lures. The lures were rubber septa (Z124389, SigmaAldrich, Gillingham, UK) impregnated with 0.1 mg of (*Z*)-7-dodecenyl acetate (Z7-12Ac) and (*Z*)-7-dodecen-1-ol (Z7-12OH) in a 10:1 ratio (Mazor and Dunkelblum, 1992).

In field trial 3 the *H. gelotopoeon* pheromone traps were bucket traps baited with rubber septa impregnated with 1 mg total of a 1:1 mixture of hexadecanal (16:Ald) and (*Z*)-9-hexadecenal (Z9-16:Ald) and an equal amount of BHT as antioxidant in hexane (Cork and Lobos, 2003). The *H. zea* pheromone traps were bucket traps baited with rubber septa impregnated with 1 mg of (*Z*)-11-hexadecenal (Z11-16Ald), (*Z*)-9-hexadecenal (Z9-16Ald), (*Z*)-7-hexadecenal (Z7-16Ald) and hexadecanal (16Ald) in a 90 : 1.4 : 1.2 : 7 ratio and an equal amount of BHT as antioxidant in hexane (Mistrot Pope *et al.*, 1984).

3.2.2 Traps

For trials 1 and 2 Unitraps (AgriSense, Treforest, UK) were used. These are funnel and bucket traps commonly used for trapping Lepidoptera. A photograph and further details can be seen in Figure 8.1.

For field trial 3 bucket traps were used. These are a simple homemade trap containing water and a thin layer of oil (to stop water evaporation) as shown in Figure 8.1.

The VARL+ traps used to monitor the male *A. gamma* population in field trial 1 were also funnel and bucket traps of similar proportions but with a larger bucket and made of semi-transparent plastic. An example can be seen in Figure 3.2.

Figure 3.2: A VARL+ trap baited with sex pheromone used to monitor *A. gamma* populations during field trial 1

3.2.3 Trial design for field trial 1 - assessing the performance of floral blends (UK)

The trial was run at a field site, known as 'Shacklinge Holden' (51°14'1.79"N, 1°21'26.06"E), and is owned by Intercrop Ltd. based in Deal, Kent. It was chosen because it contained a large area of lettuce (*Lactuca sp.*) and spinach (*Spinacia oleracea*) at various growing stages, and was known to suffer from lepidopteran damage especially by the noctuid *Autographa gamma* and the plutellid *Plutella xylostella*. The trial was run between 05/06/2008 and 24/07/2008. The traps were checked once per week. However, from 12/06/2008 to the 17/06/2008 no moths were caught in any traps and therefore

the data were omitted from the statistical analyses. After checking the traps they were randomly re-ordered (within their block).

Unitraps ™ (AgriSense) were placed in four blocks (of two rows) each block containing all five treatments in a randomised order (Figure 3.3). Traps were tied to wooden stakes so that the top of the trap was 50 cm above the ground and approximately 30 - 40 cm above the crop.

Three blends were tested: the four-component sweetpea and marigold blends reported by Bruce (2000) and the five-component UoG blend (Table 3.1). In addition, two simpler odours were included in the trial, one containing only phenylacetaldehyde and the other containing phenylacetaldehyde combined with linalool, both known to be highly attractive to many lepidoptera especially those in the family Noctuidae. These were included to assess if there was any synergy with phenylacetaldehyde and linalool, as suggested by Meagher and Landolt (2008; 2010).

Two VARL+ traps baited with *A. gamma* pheromone were positioned 100 m from the floral baited UniTraps. The pheromone traps were also positioned 100 m apart at either side of the field.

Figure 3.3: Approximate locations for traps in trial 1 at Shacklinge Holden, Deal, Kent. There were four blocks of five traps. The blocks were 15 m apart, and the traps within the blocks were 10 m apart. Aerial photo taken from Google Earth (2008) and modified.

3.2.4 Wind tunnel bioassay to assess behaviour of the attractiveness of four previously identified floral blends.

For a description of the wind tunnel see section 2.5. Insects were reared as described in section 2.2. All insects used were virgin, odour naive, between 2 - 8 days old, and had not been fed. The experiment was run between 0 - 4 h into scotophase, and a 15 W incandescent lamp covered with a red filter provided some illumination. The lamp was positioned facing away from the flight arena such that the illumination provided was indirect and did not influence the moth's behaviour. A moth selected at random was tested for activity by touching the moth's antenna with a cotton bud soaked in 10% sucrose solution. Those that did not extend their proboscis were not used. The moth was then placed onto the release platform in the wind tunnel. Lures were prepared as described above. A stop watch was used to measure the run time (5 min) and the insect's behaviour was recorded manually. The experiment was run with *A. gamma* and *H. gelotopoeon* moths of both sexes.

Using a similar method to that used by Plepys *et al.* (2002a) the moths' behaviour was recorded and categorised into the following six levels:

1. Moth takes flight from the release platform

2. Moth crosses the halfway point of the wind tunnel (80 cm from release point)

3. Moth flies within 10 cm of the lure

4. Moth contacts but does not land upon the lure

5. Moth lands on the lure but does not stay

6. Moth lands and remains on the lure for at least 3 s.

Behaviour levels were recorded sequentially, e.g. a moth that did not take flight but walked across the halfway point was not counted as reaching level 2.

Treatments tested in the wind tunnel bioassay were the Magnet, *P. nigra* and UoG blends plus a two component blend which contained the main components of UoG and Magnet blends (phenylacetaldehyde) and of the *P. nigra* blend (salicylaldehyde) in approximately a 2.3:1 ratio (Table 3.1).

3.2.5 Trial design for field trial 2 - assessing the performance of floral blends (UK)

The trial was conducted at the site known as 'Park Field' (51°13'12.84"N 1°20'56.90"E) at Intercrop Ltd, Deal, Kent (Figure 3.4). The trial was positioned in an area of grassland adjacent to fields of salad crops (spinach, lettuce, and coriander) that suffered from Lepidopteran damage (predominately *A. gamma* and *P. xylostella*).

At the time of the trial the crops were at all stages from seedlings to mature plants ready for harvest. The trial was run from 30/07/2010 to 24/08/2010 and

the traps checked every three to five days a total of six times during the trial. After checking the traps they were repositioned within their blocks. The lures were renewed on the 16/08/2010. Unitraps™ (AgriSense) were placed in six blocks each block containing all six treatments in a randomised order. Traps were tied to bamboo stakes so that the top of the trap was 30 cm above the ground and approximately 0 - 20 cm above the foliage. The trial was conducted in a latin-square with six treatments, six replicates, and checked six times.

The six treatments included an unbaited control and the Magnet, *P. nigra* and UoG blends evaluated in the wind tunnel bioassay (Table 3.1). In addition another version of the UoG blend was tested which contained three additional VOCs, cinnamyl alcohol, benzyl benzoate, and 4-hydroxy benzoate. The three additional VOCs were added to the basic UoG blend as part of experiments described in the next chapter to increase the effectiveness of the UoG blend and is referred to as treatment number 'UoG+BB/CA +4Hb' in trial 5 (see Table 6.1), but shortened to 'G9' for this chapter. The final treatment was the two-component blend of phenylacetaldehyde and salicylaldehyde in a 2.3:1 ratio which had been tested in the wind tunnel bioassay (Table 3.1).

Two pheromone traps (UniTraps baited with *A. gamma* pheromone) were positioned 100 m away from the floral baited traps - one to the south-west and one to the north-east.

Figure 3.4: Approximate locations for traps in trial 2 at Park Field, Deal, Kent. There were six blocks of six traps. The blocks were 20 m apart, and the traps within the blocks were 10 m apart. Aerial photo taken from Google Earth (2008) and modified.

3.2.6 Trial design for field trial 3 - assessing the performance of floral blends (Argentina)

The trail was carried out at an agricultural research station run by The National Institute of Agricultural Technology (INTA) in the Santiago Del Estero district in Argentina (at 28°01'29"S, 64°14'01"W). Two cropping areas were used, one containing maize at the silking stage, the other cotton at the flowering stage.

At each of the two crop sites the bucket traps were positioned in three blocks, each block containing the four treatments. All traps were checked and repositioned within their blocks every week for four weeks.

The four treatments were the Magnet, *P. nigra* and UoG blends with the binary blend of phenylacetaldehyde and salicylaldehyde tested previously in the UK (Table 3.1).

Three bucket traps baited with *H. gelotopoeon* pheromone were positioned 30 m from the floral traps around the cotton field. Three bucket traps baited with *H. zea* pheromone were positioned 30 m from the floral traps around the maize field.

The trials were set up with the assistance of Dr. Enrique Lobos and two of his Masters students, German Sarria and Andres Wintten. After I had left Argentina, the students continued to check the traps and sent the data to me.

Figure 3.5: Approximate trap locations for field trial 3. Cotton was growing at the south-western site, and maize at the north-eastern site. Each site contained three blocks (replicates) of the four treatments with the traps being positioned approximately 10 m apart. Pheromone traps were placed 30m from each sites (*H. gelotopoeon* at the cotton site and *H. zea* at the maize site) to monitor local populations. Aerial photo taken from Google Earth (2008) and modified.

3.3 RESULTS

3.3.1 Field trial 1

In field trial 1 overall catches were low, but significant differences were found between the treatments (Figure 3.6) and *post-hoc* analysis showed that the

UoG blend (Table 3.1) caught significantly more *A. gamma* moths than phenylacetaldehyde alone and the synthetic sweatpea blend but not the phenylacetaldehyde + linalool blend or the marigold blend.

The pheromone VARL+ traps caught a mean of 5.75 male *A. gamma* (±se = 0.89).
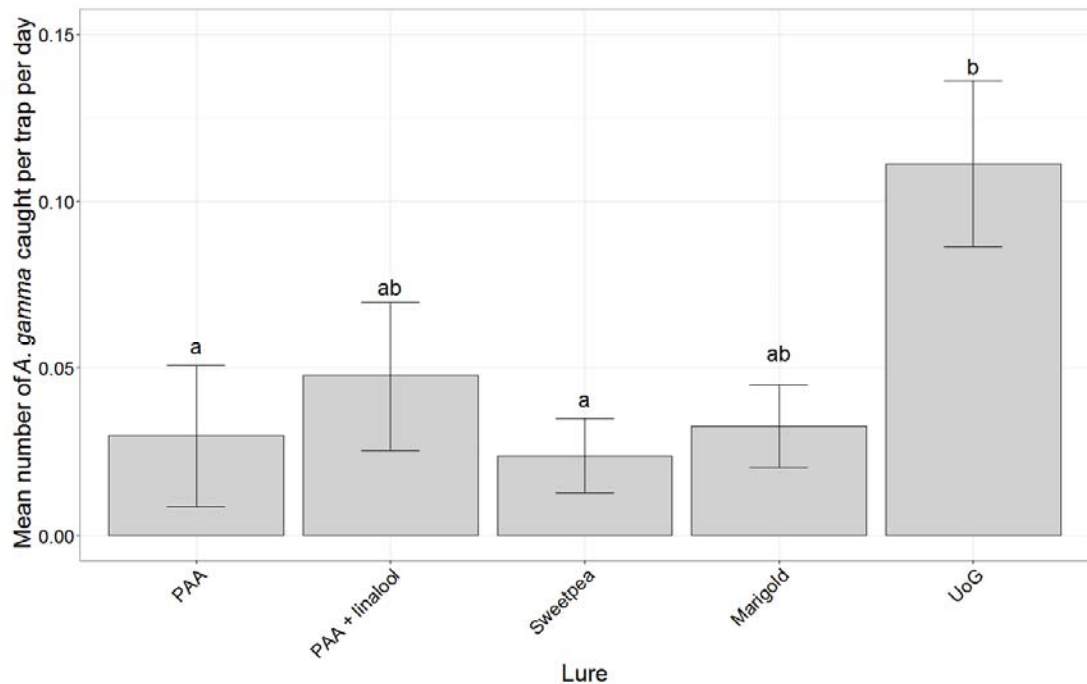
Figure 3.6: Mean number of *Autographa gamma* caught per trap per day baited with blends of floral volatiles. Error bars indicate standard error of the means. Data analysed using a GLM with a quasipoisson distribution; N = 24, χ^2 = 2.14, d.f. = 4, *P* < 0.05; letters denote significant differences (*P* < 0.05) between treatments, tested by Tukey's. Pheromone traps 100 m from the floral baited traps caught a mean of 0.88 (±se 0.15) male *A. gamma* per trap per day.

3.3.2 Wind tunnel bioassay

In the wind tunnel bioassay, behavioural responses to four different blends and a hexane control were compared (Table 3.1) with both *H. gelotopoeon* and *A. gamma* moths. With all treatments, including the hexane control, the number of moths that took flight was high (> 75%). The results at behaviour levels 1 and 2 show that all treatments elicited upwind flight causing over 50% of the *H.*

geloptopoeon moths tested for each treatment to cross the halfway point of the wind tunnel (Figure 3.7). However, there was a significant difference in the number of *H. gelotopoeon* that came to within 10 cm of the odour source for the floral treatments compared to the hexane control. For the UoG blend significantly more *H. gelotopoeon* moths were stimulated to land on the odour source compared to *P. nigra* blend, binary blend, and hexane control.

The responses of both species were similar with the UoG blend achieving the highest number of landings (behaviour levels 5 and 6 Figure 3.8 and Figure 3.9), and the hexane control treatment not stimulating the moths to come within 10 cm of the odour source.
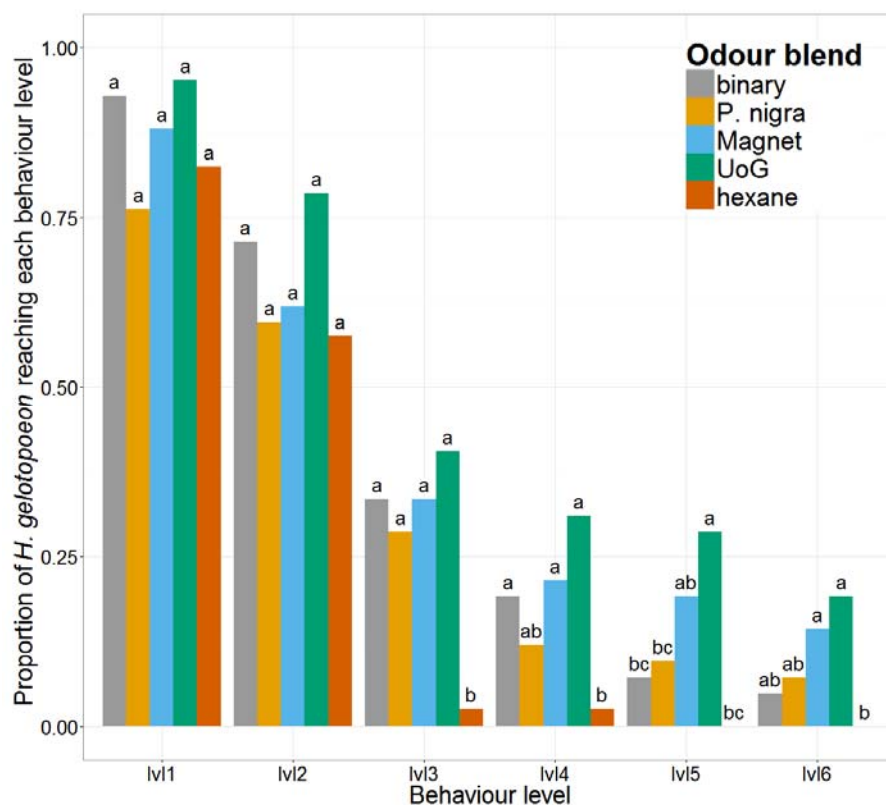
Figure 3.7: The proportion of *Helicoverpa geloptopoeon* males and females (data pooled) that reached each behaviour level in response to odour attractants in a wind tunnel. Bars with the same letters are not significantly different (chi-square test, $P < 0.05$, N = 42 for all treatments except hexane N = 40). Comparisons were made only within each behaviour level. Lvl 1 - insect takes flight, lvl 2 - flies to halfway, lvl 3 - comes within 10 cm of odour source, lvl 4 - contacts odour source, lvl 5 - lands on odour source, lvl 6 - lands on odour source and stays for at least 3 seconds.

Figure 3.8: The proportion of *Autographa gamma* males and females (data pooled) that reached each behaviour level in response to odour attractants in a wind tunnel. No significant differences were found (chi-square test, *P* > 0.05, N = 12 for all treatments except 'binary' N = 11, and hexane N = 10). Comparisons were made only within each behaviour level. Lvl 1 - insect takes flight, lvl 2 - flies to halfway, lvl 3 - comes within 10 cm of odour source, lvl 4 - contacts odour source, lvl 5 - lands on odour source.

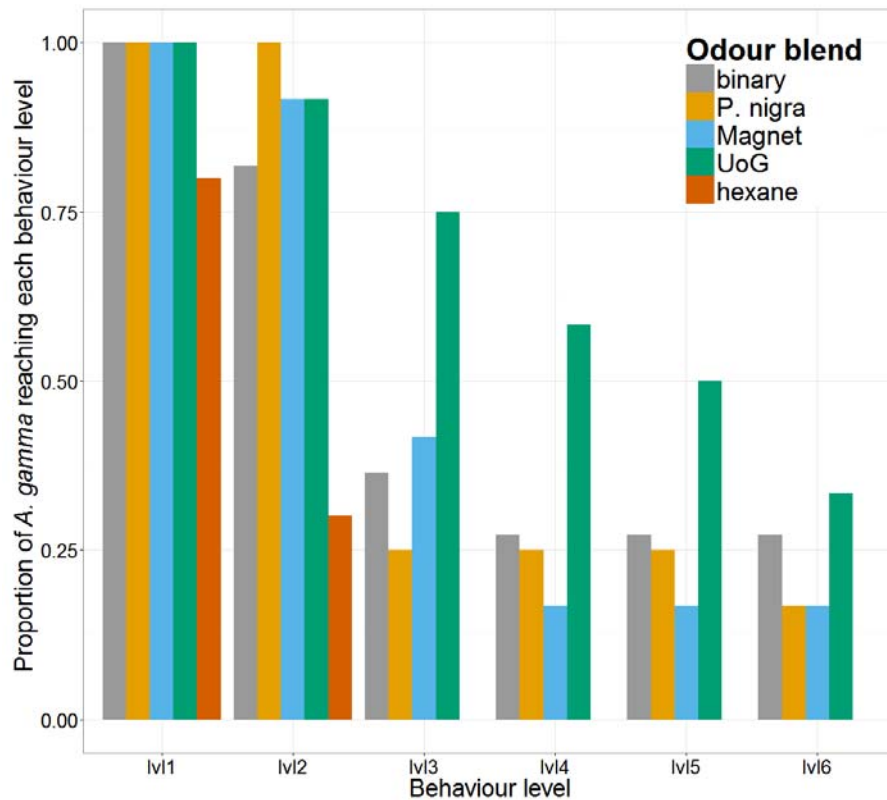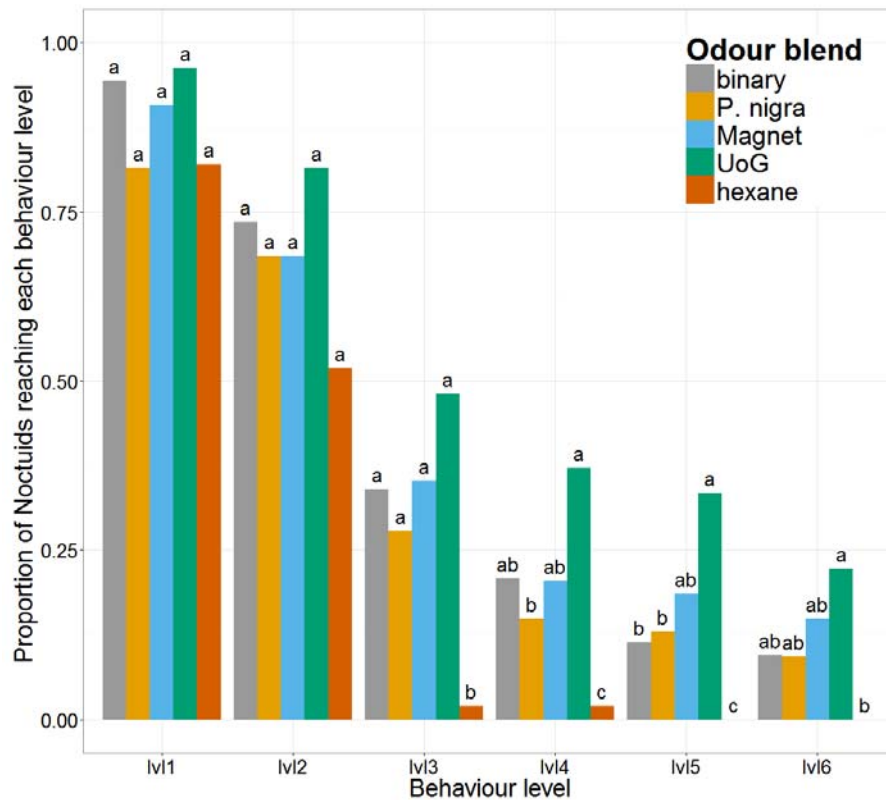Figure 3.9: The proportion of *Helicoverpa geloptopoeon* and *Autographa gamma* males and females (data pooled) that reached each behaviour level in response to odour attractants in a wind tunnel. Bars with the same letters are not significantly different (chi-square test, $P < 0.05$, N = 54 for all treatments except 'binary' N = 53, and hexane N = 50). Comparisons were made only within each behaviour level. Lvl 1 - insect takes flight, lvl 2 - flies to halfway, lvl 3 - comes within 10 cm of odour source, lvl 4 - contacts odour source, lvl 5 - lands on odour source.

3.3.3 Field trial 2

In field trial 2, all six treatments caught noctuids but the blank control only caught one. The majority of noctuids caught were *A. gamma*. Between the odour treatments, the *P. nigra* blend caught significantly fewer moths than the other odours (Figure 3.10). All of the 'super-blends' performed similarly but the UoG blend caught slightly more moths than the Magnet and G9 blends (not significantly different). Only the Magnet and *P. nigra* blends caught more males than females (not significantly different).

The pheromone traps in this field trial only caught 1 *A. gamma* moth during the whole trial.
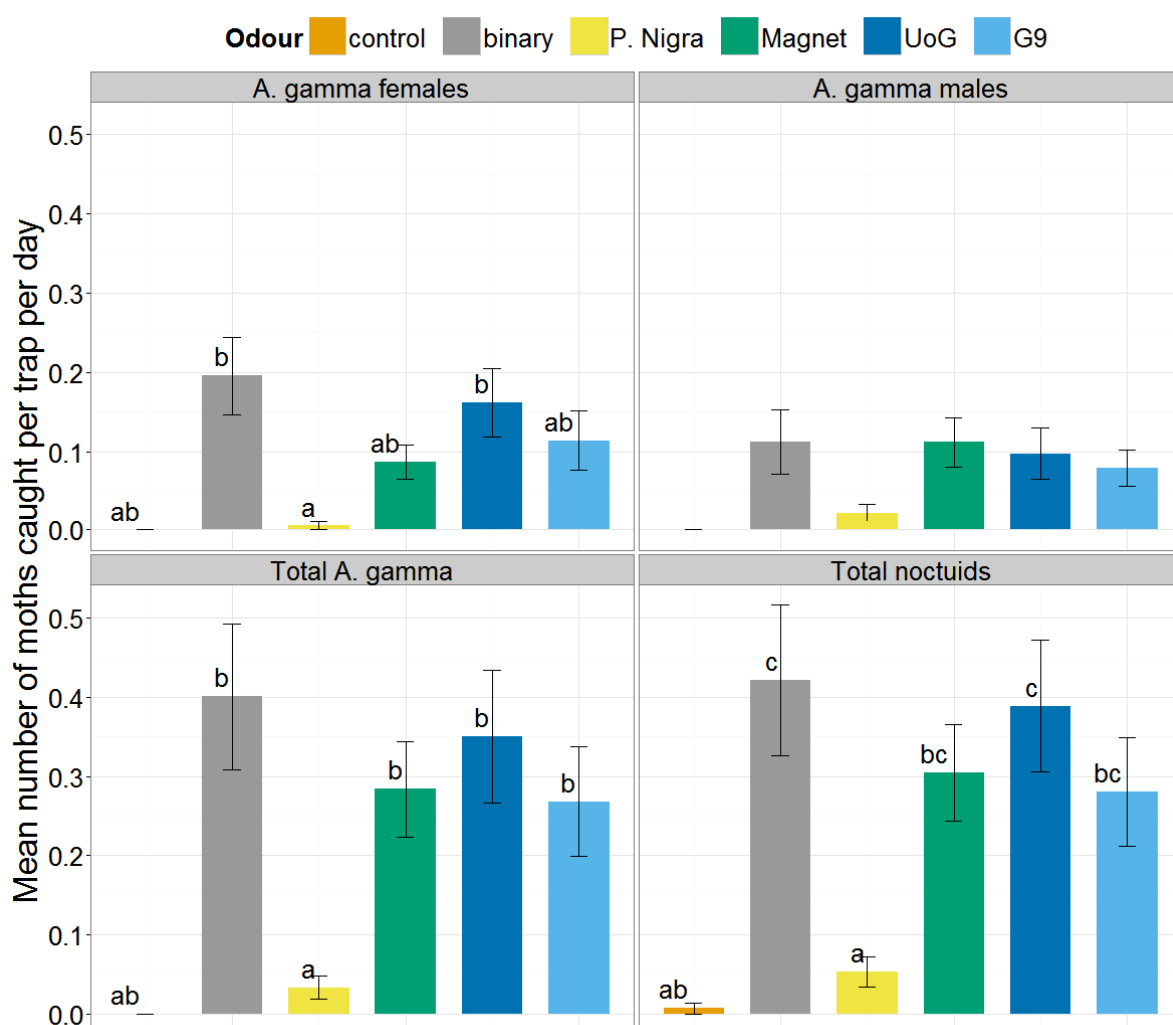
Figure 3.10: The mean number of moths caught per trap per day in a UK field trial (trial 2) in traps baited with floral odours (see Table 3.1 for components of odour blends). Data analysed using a GLM with a poisson distribution (overdispersion was detected and corrected for using a quasi-GLM), N = 36, X^2 = 16.6 (*Autographa gamma* females), 8.5 (*A. gamma* males), 32.3 (total *A. gamma*), 30.3 (total noctuids), d.f. = 5, *P* < 0.001 for all groups. Error bars show ±se; bars with the same letters within each chart are not significantly different tested by Tukey's test.

3.3.4 Field trial 3

In field trial 3 carried out in Argentina, three species of noctuids were caught:

Heliothis zea, Helicoverpa gelotopoeon and *Spodoptera frugiperda*. All of the

odour blends caught noctuids of both sexes, but only the *P. nigra* blend caught a higher number of male *Helicoverpa* species than females. All the other blends caught more females (not significantly different) (Table 3.2). The UoG blend caught significantly more Noctuids that the Magnet and binary blend but not the *P. nigra* blend (Figure 3.11). Significantly more *H. gelotopoeon* were caught in traps baited with the *P. nigra* blend than the binary blend (Figure 3.11).

Table 3.2: Mean number of Noctuids species caught per trap per day at two field sites (one corn, one cotton) at the National Institute of Agricultural Technology (INTA) in the Santiago Del Estero district in Argentina.

Corn

Treatment	*H. zea* males	*H. zea* females	*H. zea* total	*H. gelotopoeon* males	*H. gelotopoeon* females	*H. gelotopoeon* total	*S. frugiperda* males	*S. frugiperda* females	*S. frugiperda* total	Total noctuids
Magnet	0.09	0.13	0.22	0.00	0.00	0.00	0.08	0.12	0.20	0.49
P. nigra	0.22	0.13	0.35	0.00	0.00	0.00	0.12	0.21	0.33	0.72
UoG	0.17	0.21	0.38	0.00	0.00	0.00	0.15	0.21	0.36	0.81
Binary	0.10	0.15	0.25	0.00	0.00	0.00	0.14	0.11	0.25	0.54
H. zea pheromone	2.46									

Cotton

Treatment	*H. zea* males	*H. zea* females	*H. zea* total	*H. gelotopoeon* males	*H. gelotopoeon* females	*H. gelotopoeon* total	*S. frugiperda* males	*S. frugiperda* females	*S. frugiperda* total	Total noctuids
Magnet	0.00	0.00	0.00	0.09	0.07	0.17	0.08	0.09	0.17	0.37
P. nigra	0.00	0.00	0.00	0.18	0.08	0.26	0.05	0.09	0.14	0.46
UoG	0.00	0.00	0.00	0.13	0.10	0.23	0.06	0.16	0.22	0.50
Binary	0.00	0.00	0.00	0.06	0.06	0.12	0.09	0.11	0.20	0.33
H. gelotopoeon pheromone				3.44						
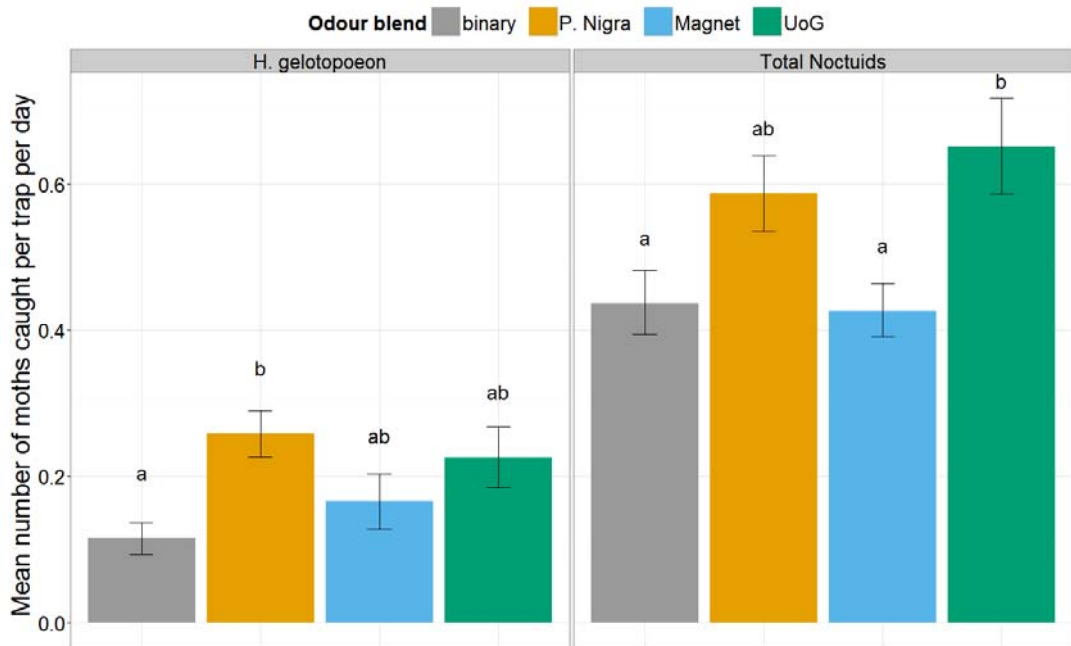
Figure 3.11: Mean number of *H. gelotopoeon* and total Noctuid moths caught per trap per day in traps baited with floral odour blends in fields in Argentina. Data analysed by GLM with poisson distribution (a quasi-GLM was used). Error bars show ±se, bars that do not share the same letters within each chart are significantly different. Left chart, N = 12, X^2 = 0.79, d.f. = 3, $P < 0.05$; right chart, N = 24, X^2 = 1.7, d.f. = 3, $P < 0.01$.

3.4 DISCUSSION

In this chapter, blends of floral volatiles previously reported to be attractive to Noctuids were evaluated in a wind tunnel bioassay in the laboratory and in three trapping trials in the field. Two of the latter were carried out in the UK, where the main noctuid species was *A. gamma*, and one was in Argentina where the target species were *Helicoverpa sp.*, but also caught *Spodoptera sp.* This is the first time that these blends have been compared to one another and the tested in these diverse geographical locations. The purpose of these experiments was to compare floral odour blends that had been reported as

being highly attractive to Noctuidae, and assess these blends for the their attractiveness for a range of noctuid species. Significant differences in the numbers of noctuid species were found between the blends tested.

3.4.1 Field trial 1

In this trial, catches of *A. gamma* were compared in traps baited with three blends having phenylacetaldehyde as a major component, with phenyl acetaldehyde alone and phenylacetaldehyde with linalool. The three blends were the four-component sweetpea and marigold blends of Bruce *et al.* (2000) and the five-component UoG blend.

The UoG blend baited traps caught most *A. gamma* moths, significantly more than the sweetpea blend and the phenylacetaldehyde only blend and more than twice the catches with the marigold blend and the phenylacetaldehyde with linalool, but not significantly more. All of the treatments contained the same quantity of phenylacetaldehyde so any differences in the numbers of moths caught were due to the additional compounds present in the odour. In order to maintain an equal quantity of phenylacetaldehyde between treatments the total volume of the treatments varied (Table 3.1). Testing with linear regression (data not shown) found that there was no correlation in volume of attractant and the number of moths caught. Therefore, we may conclude that the additional compounds in each odour blend acted in some additive or synergistic way to increase the catch rate. In which case, the addition of salicylaldehyde, linalool, limonene, and methyl 2-methoxybenzoate to phenylacetaldehyde significantly increases the number of *A. gamma* caught per day per trap compared to phenylacetaldehyde alone. Previous work by Landolt and Smithhisler (2005) found that individually both linalool and methyl-2-methoxybenzoate were

compounds that had a positive synergistic effect on the attractiveness of phenylacetaldehyde for the cabbage looper, *Trichoplusia ni* (Hübner) (Noctuidae) (within the same subfamily, *Plusiinae*, as *Autographa gamma* and *californica*). However, when linalool and methyl-2-methoxybenzoate were added to phenylacetaldehyde together they did not have an additive effect. In the present study linalool alone added to phenylacetaldehyde increased catches (not significant), but when methyl-2-methoxybenzoate (with limonene and salicylaldehyde) were added as the UoG blend, catches increased further (significant). Without further testing it is not possible to know for certain how the individual components of the UoG blend effect the number of *A. gamma* caught in baited traps. However, the evidence from this trial suggests that phenylacetaldehyde alone, or phenylacetaldehyde with linalool (5 : 1 ratio) is not as behaviourally stimulating to *A. gamma* as those two compounds with the addition of salicyladehyde, methyl 2-methoxybenzoate, and limonene. As the Marigold blend also contained linalool and limonene albeit in much lower quantities compared to the UoG blend, we may conclude that salicylaldehyde and methyl 2-methoxybenzoate are important in stimulating *A. gamma* moths to fly to the odour source.

The synthetic sweetpea blend contained phenylacetaldehyde, linalool, benzyl alcohol and diacetone (in a 1:1:1:1 ratio) but although these compounds are all thought to be attractive either individually or in combination the catches were significantly lower than for the UoG blend. Benzyl alcohol has been shown to be attractive to other noctuid species alone or in conjunction with other compounds, e.g. alone and in conjunction with phenylacetaldehyde, limonene and linalool for *Helicoverpa armigera* (Gregg *et al.*, 2010), and in conjunction with phenylacetaldehyde, benzaldehyde, and 2-phenylethanol for *T. ni* (Haynes

et al., 1991), but in both cases benzyl alcohol was a much lower percentage of the total blend compared to other compounds. It is possible that the ratio of compounds in the sweetpea blend, in particular the high percentage of benzyl alcohol or linalool, did not stimulate the desired behaviour and entice the moths into the traps. The UoG blend contained a much lower percentage of linalool and was significantly more attractive.

Catch rates overall were lower than hoped, and compared to pheromone traps the best floral blend caught five times fewer *A. gamma* (data not shown).

3.4.2 Wind tunnel bioassay

Due to problems rearing insects fewer replications of the bioassay were completed with *A. gamma* than had been planned making statistical analysis with this species difficult.

For *H. gelotopoeon* (and pooling the data for two Noctuid species) the experiment found a high percentage of the insects responding (in excess of 75% taking flight) and that whether the insects took flight or not (behaviour level 1) and flew up wind to the halfway point of the tunnel (level 2) was independent of the presence of a floral odour source (Figure 3.7 and Figure 3.9) as there no significant difference between the floral treatments and the blank control for behaviour level 1. However, further upwind flight to the vicinity of the filter paper (level 3) was only achieved in the presence of an odour blend. The stimulation to land on the odour source (levels 5 - 6) was found to be dependent on the blend, with significantly more noctuids (Figure 3.9) landing on the UoG odour source compared to the binary and *P. nigra* blends, as well as the hexane control. The difference in composition of the binary blend and the UoG blend is the addition of the three minor components: linalool, methyl-2-

methyoxybenzoate, and limonene. The quantity of odour was uniform for all the treatments, therefore the behavioural difference found between the UoG and binary blends is the consequence of the three minor components. This is in agreement with a previous field study comparing a binary blend of phenylacetaldehyde and salicylaldehyde with the same UoG blend used in the current study (Cork, 2011). This also fits with the results from the field trial 3 which shows a significant difference in catches of noctuids between the binary and UoG blend, although not with catch data for *H. gelotopoeon* (Figure 3.11).

3.4.3 Field trials 2 and 3

Field trials 2 and 3 compared the same blends (the 'G9' blend is absent in field trial 3) in the UK and in Argentina. However, there were some minor differences in the blends used in the two trials. In trial 3 the compounds making up the Magnet blend were combined in a 1:1:1:1 ratio rather than the actual ratio used by Ag Biotech. This was due to an error when the lures were being made which was not realised until after trials were started in Argentina. In addition, the total volume of VOCs for the lures was 250 mL for Argentina, rather than 300 mL as used in the UK trial.

With the exception of the G9 blend used in field trial 2, the other blends have all been published previously and their suitability as field attractants for noctuid agricultural pests has been considered. However, these blends had not yet been compared to one another nor have they been tested in diverse geographic locations as they have in the current study. The results from the UK and Argentinean field trials (trials 2 and 3) showed that all of the blends were attractive to a variety of noctuid species, including *Autographa gamma*, *Helicoverpa gelotopoeon*, *Helicoverpa zea*, and *Spodoptera frugiperda*, all of

which are important agricultural pests. This confirmed that these types of floral odour blends are general attractants which appeal to multiple species and both sexes.

The G9 blend was the result of field trials conducted in the UK to try and improve the effectiveness of the UoG blend for attracting *A. gamma*. From the results in trial 2 it did not achieve this aim, and although not statistically significantly so, the G9 blend caught fewer *A. gamma* than the UoG blend.

3.4.4 General discussion

The floral odours that mimiced natural blends performed poorly compared to the super-blends. This is seen in trial 1 (section 3.3.1) with the sweetpea and marigold blends compared to the UoG blend, and the *P. nigra* blend in the wind tunnel (section 3.3.2) and and field trial 2 (3.3.3). However, in contrast to the results seen in the wind tunnel with *H. gelotopoeon* the *P. nigra* blend performed well in field trial 3 catching more *H. gelotopoeon* than the other blends tested (although not significantly different from the Magnet or UoG blend).

In the wind tunnel bioassays odour naïve insects were used. Therefore the behaviours seen may be considered innate responses to the floral odours presented. In the Introduction (section 1.1.2.5) I state that the key to a successful floral attractant may be to identify an odour blend that stimulates the greatest level of innate responses. Of the blends tested the UoG blend stimulated the greatest number of innate responses in the wind tunnel bioassays for both species tested. For *H. gelotopoeon* this odour blend stimulated more than 25% of the moths to landed on the odour source within the 5 min bioassay; for *A. gamma* 50% of the moths landed. Riffell *et al.* (2013)

showed that a moth's innate responses remain after it had learnt to associate other floral odours with nectar. In addition it has been demonstrated that innate responses to a particular floral odour can be reinforced by experiencing that odour in conjunction with nectar (or sucrose substitute) (Cunningham *et al.*, 2004). Moths in the field may well have had experience of some of the floral odours used in the test odour blends as many are extremely common floral volatiles, particularly limonene and linalool (Knudsen *et al.*, 2006). The presence of both of these compounds in the UoG blend may explain why it attracted the highest number of noctuids in the Argentina field trials.

Traps baited with the UoG blend caught the highest numbers of *Spodoptera frugipera* and *Helicoverpa zea* and all noctuids combined (significantly different from the binary and Magnet blends) in trial 3 and *A. gamma* in trial 1; filter papers loaded with the UoG blend stimulated more moths to land on the odour source than the other blends tested for both *A. gamma* and *H. gelotopoeon* in the wind tunnel. Throughout these investigations the UoG blend either caught the highest number of the noctuid species or was not significantly different from the blend that did, and therefore may be considered the most effective general attractant for noctuids of those blends tested.

CHAPTER 4 - WIND TUNNEL BIOASSAY OF THE UOG FLORAL BLEND – EFFECT OF PHYSIOLOGICAL STATE OF MOTHS

4.1 INTRODUCTION

The behaviour of insects is determined by many factors, both exo- and endogenous. As in all organisms, the reactions of insects to a specific stimuli change in response to their current situation/status. Endogenous factors generally relate to the physiological state of the insect, and those that may cause behavioural changes to a stimulus include: development stage, nutritional state, age, and mated status. The ability of an insect to change its behaviour towards a stimulus as a result of its physiological state allows the insect to prioritise its resource finding so that the insect locates what is most pertinent to it at that moment. For excellent reviews on this matter see the review papers by Barton Browne (1993) and Anton *et al.* (2007). The work within this chapter relates to two physiological states that affect the behaviour of insects: nutritional and mated status, in conjunction with the strength of the odour stimulus they receive.

The use of floral attractants as a crop protection tool has great potential, primarily because volatile chemicals have been identified that are attractive to both male and (more importantly) female crop pests (for example Haynes *et al.*, 1991; Hartlieb and Rembold, 1996; Plepys *et al.*, 2002b; Hern and Dorn, 2004; Del Socorro *et al.*, 2010a). The success of odour lures (for crop protection) based on floral volatiles relies on them being able to attract female insects before they have oviposited in the crop. Numerous studies have found that mated female *H. armigera* are more attracted to flowering host plants then non-

flowering host plants (Parsons, 1940; Firempong and Zalucki, 1989; Riley *et al.*, 1992; Sequeira *et al.*, 2001). Liu *et al.* (2010) also found a highly significant preference for adult *H. armigera* to oviposit on flowering host plants (tobacco and sunflower) compared to host plants with either their flowers covered or absent. In addition, nectar feeding was shown to increase fecundity eight-fold due to improved egg maturation. In oviposition bioassays on *Nicotiana tobacum* McCallum *et al.* (2011) noted that gravid *H. armigera* laid 64(±6)% of their eggs on inflorescences compared to 36(±6)% on leaves. As well as being attracted to flowers for nectar feeding prior to and during oviposion (Riley *et al.*, 1992), *Helicoverpa spp.* are also attracted to flowers to feed from nectar as immature virgins (Beerwinkle *et al.*, 1993). Therefore a lure that mimics floral scent should be highly attractive to both virgin and mated females. In addition, it is hypothesised that a sated moth (i.e. the insect has fed on an energy providing solution, in this case 10 % w/v sucrose solution) will be less likely to complete the behaviour sequence resulting in locating/contacting the odour source.

Female *Spodoptera littoralis* have been shown to exhibit a behavioural switch post-mating so that they change from being more likely to fly towards lilac flowers prior to mating and green-leaf odours of host-plants (cotton) post-mating (Saveer *et al.*, 2012). This behavioural switch was found to relate to the insects' olfactory system which activated or deactivated specific glomeruli associated with green leaf volatiles (GLVs) or floral volatiles in response to mating. This suggests that Lepidoptera behaviour towards plant volatiles is highly dependent on the physiological status of the insect, and mating may reduce the chances of the insect flying towards floral odours.

The following research investigated the flight behaviour of *H. armigera* towards an odour blend under different nutritional conditions: starved (no food, no water), hydrated (water *ad libitum*) and sated (10 % sucrose solution *ad libitum*); different reproductive states: gravid or virgin; and three odour loadings (concentration of the odour blend used to make the lures).

4.2 MATERIALS AND METHODS

4.2.1 Lures

All chemicals tested were purchased from Sigma Aldrich (percentage purity in parentheses): (±)-linalool (≥ 97%) (Ln), (S)-(-)-limonene (≥ 99%) (Lm), methyl-2-methoxybenzoate (≥ 99%) (M2M), phenylacetaldehyde (≥ 90%) (PAA), salicylaldehyde (≥ 98% ≤ 1% phenol) (Sa). The chemicals were dissolved in hexane at three concentrations: 0.01, 0.1, 1.0 mg/ml.

Each lure was made immediately prior to use in order to minimise loss of the plant volatiles due to evaporation before the run had started. 10 µl of the odour solution was pipetted onto a 6 cm filterpaper (Grade 1) four times approximately 2 cm apart to give a total of 40 µl of solution on a single lure (as described in section 2.5.3). Odour loadings were: 40 µg, 4 µg, and 0.4 µg. See Table 4.1 for quantities of plant volatiles at each loading.

Table 4.1. Ratio of compounds that comprise the UoG blend and quantities at each odour loading used. PAA = phenylacetaldehyde, Sa = salicylaldehyde, M2M = methyl 2-methoxybenzoate, Ln = linalool, Lm = limonene.

	Chemical				
	PAA	Sa	M2M	Ln	Lm
Percentage of blend					
	54	22	9.5	9.5	5
Odour loading					
40 ug	21.6	8.8	3.8	3.8	2
4 ug	2.16	0.88	0.38	0.38	0.2
0.4 ug	0.216	0.088	0.038	0.038	0.02

4.2.2 Feeding solutions

Helicoverpa armigera moths were either fed on tap water, 10% w/v sucrose solution, or nothing. Feeding wicks were prepared using a 45 ml plastic container. A hole was cut (approx. 10 x 5 mm) into the lid of the container and a wick of absorbent cotton inserted through the hole (see Figure 4.1).

Figure 4.1: a water / sucrose solution dispenser, made from a 45 ml plastic pot with a hole (c. 10 x 5 mm) cut out of the lid and piece of absorbent cotton wool inserted to act as a wick. Moths would land on the pot and insert their proboscis into the cotton wool to feed.

4.2.3 Physiological state of insects

4.2.3.1 <u>Feeding conditions</u>

H. armigera were transferred from the rearing cages (described in section 2.2) to 1 L Kilner jars 0 - 2 days after eclosion. The Kilner jars contained a 6 cm diam. filter paper on the floor of the jar, a feeding pot if required, and a metal wire-mesh lid. Each Kilner jar contained a maximum of 5 insects of the same sex. Both males and females were tested.

4.2.3.2 <u>Odour loading conditions</u>

Insects were transferred from the rearing cages (described in section 2.2) to 1 L Kilner jars 0 - 2 days after eclosion. The Kilner jars contained a 6 cm diam. filter paper on the floor of the jar, a feeding pot containing water, and a metal wire-mesh lid. Each Kilner jar contained a maximum of 5 insects of the same sex. Both males and females were tested.

4.2.3.3 <u>Mated status conditions</u>

Insects were transferred from the rearing cages (described in section 2.2) to 1 L Kilner jars 1 - 3 days after eclosion. The extra day for insects used in the mated status experiments was found to improve the likelihood of mating. The Kilner jars contained a 6 cm diam. filter paper on the floor of the jar, a feeding solution of either water or 10% sucrose solution, and a metal wire-mesh lid. Each Kilner jar contained a maximum of 5 insects: 2 females were kept with 3 males. Only females were tested in the wind tunnel. After the bioassay females were kept separately and checked every 24 h for oviposition of fertile eggs.

4.2.3.4 <u>General conditions</u>

The insects were kept in the Kilner jars for 48 h at the same environmental conditions as the wind tunnel bioassay room, which was 26 °C with a photoperiod of 14 : 10 h (light : dark). Relative humidity was not actively controlled but was at approximately 50 %RH.

4.2.3.5 <u>Comparison of males and females</u>

For comparing the responses of males and females, only the data from virgin insects was used. These insects were used in either the odour loading experiment or the feeding experiment.

4.2.4 Experimental protocol

The insects were brought to the wind tunnel laboratory 5 – 10 min prior to the experiment start time. The experiment started at the beginning of scotophase and lasted up to 3 h, each run taking approximately 7 – 10 min including preparation time. The actual time of each run was 5 min in order to allow enough time to test a reasonable number of insects per day. Constant room lighting was provided by a 15 W incandescent lamp covered with a red filter. To provide additional lighting during the time taken to prepare the odour lures an additional 11 W fluorescent lamp covered with a red filter was also used.

At the start of each run an odour lure was prepared and immediately hung in the wind tunnel. An insect was randomly selected and placed onto the release platform. Once the wind tunnel access panels were closed the experiment was started and run for 5 min. The insect's behaviour was recorded digitally via the camera (Sony video camera with a swivel lens and VISCA port, unknown model) and computer running MS Windows XP with a video capture card (Euresys Picolo Pro 2, PCI video capture card).

Insects used in the mated status experiment were replaced back into the same (labelled) plastic pots they were in before the experiment. At the end of the experiment, all pots were checked to see if the females had oviposited. The pots were checked again every 24 h for 6 days. The number of days between the experiment and oviposition and larval emergence was recorded. If fertile eggs were present in the pot within 24 h of the experiment, that female was counted as being gravid at the time of the experiment.

It was found to be not possible to test unfed mated insects as they invariably died before the experiment was complete.

4.2.5 Digital tracking and analysis

Each replicate was recorded via the camera and computer and compressed to an Audio Video Interleaved (AVI) file format with the XVID codec using VirtualDub (version 1.9.9). The video files were analysed using EthoVision XT (ver. 7.1) tracking software. Each flight track was manually checked for tracking errors and corrected where necessary. EthoVision was then used to calculate the time taken for the insect to:

- cross the halfway point of the wind tunnel

- contact the odour source

- count the total time spend moving and stationary (within the tracking arena)

The camera was set up to only record insects that entered the upwind half of the wind tunnel. Therefore all of the data calculated by EthoVision pertains only to insects that crossed that halfway point. Insects that did not cross this point

may have moved from their starting position but were simply counted as 0 cm moved by EthoVision.

The calculated data from EthoVision was exported and analysed using R.

4.2.5.1 <u>Time spent moving/stationary</u>

The amount of time the insect spent moving or stationary was calculated. Only insects that entered the tracking area were counted, i.e. those that crossed the halfway point of the wind tunnel. The data was analysed using a GLM with guassian distribution and a quasi GLM was fitted to compensate for overdispersion with Tukey's pairwise comparisons where appropriate.

4.2.5.2 <u>Proportion of insects that contacted the odour source</u>

The proportion of insects that contacted the odour source for each treatment was calculated and analysed using a GLM with a binomial distribution.

4.2.5.3 <u>Latency to contacting the odour source</u>

'Survival' statistics was used to demonstrate how the factors investigated (sex, prior feeding, odour loading, and mated status) affect the time taken for insects to either contact or not contact the lure. In all the related figures the y-axis is reversed and indicates the proportion of the insects tested that made contact with the odour source; the x-axis shows the number of seconds taken for each insect to fly from the halfway point of the wind tunnel to the odour source. The marks on the lines indicate 'events' which were when an insect failed to contact the odour source within the time given for the experiment (310 s). Therefore curves that rise sharply mean that a greater proportion of the insects tested contacted the odour quickly after making upwind flight; and the position of the

marks along the curves show the length of time the insect took between making upwind flight and the end of the run without making contact with odour source. For each factor tested the variables were tested for significant differences using the log-rank test from the *G-rho* family of tests which are used for comparing groups in survival data (Harrington and Fleming, 1982; Therneau, 2014).

4.2.5.4 <u>Release rate of the blend in the wind tunnel</u>

The release rate of the odour blend was estimated by placing strips of filter paper (30 x 10 mm; Whatman No. 9 see above) loaded with 40 µl of the odour blend into the wind tunnel for between 0 and 310 s. The concentration of the odour blend was 0.1 mg/mL dissolved in hexane. 40 µL of this solution was pipetted onto a strip of filter paper and placed into the wind tunnel in the same location as the odour lures were placed during the behavioural bioassay. The wind tunnel was set to provide an air speed of 0.5 m/s.

After being in the wind tunnel for the required length of time the strips were removed and placed directly into a 5 mL vial containing 4 mL of hexane. The 4 mL of hexane also contained an internal standard (decyl acetate, C10:Ac at 0.001 mg/mL).

There were five replicates for each treatment, which was the length of time the strip of filter paper spent in the wind tunnel as described in Table 4.2.

After the contents of the filter papers were eluted into 4 mL of hexane, 2 µL of this solution was injected into a GC (Agilent, 6850 with a DB-Wax polar column) via an autosampler (Agilent, 7693). Peak area was recorded and normalised with the internal standard.

Table 4.2: the length of time that strips of filter paper containing 40 µL of the UoG odour blend at one of three concentrations was kept in the wind tunnel before being placed into a 5 mL vial containing 4 mL of hexane. There were three replicates of each concentration for each time period. The wind tunnel was set to run at 0.5 m/s.

Total time (s)	Time in wind tunnel (WT) (s)	Description
0	0	Pipetted onto the filter paper and transferred immediately to the 5 mL vial.
10	0	Pipetted onto the filter paper, left for 10 s and then transferred to the 5 mL vial.
40	30	Pipetted onto the filter paper, transferred to the WT, then placed into a 5 mL vial.
70	60	Pipetted onto the filter paper, transferred to the WT, then placed into a 5 mL vial.
100	90	Pipetted onto the filter paper, transferred to the WT, then placed into a 5 mL vial.
130	120	Pipetted onto the filter paper, transferred to the WT, then placed into a 5 mL vial.
190	180	Pipetted onto the filter paper, transferred to the WT, then placed into a 5 mL vial.
250	240	Pipetted onto the filter paper, transferred to the WT, then placed into a 5 mL vial.
310	300	Pipetted onto the filter paper, transferred to the WT, then placed into a 5 mL vial.

4.3 RESULTS

4.3.1 Release rate

The peak areas of the UoG blend compounds were calculated relative to the internal standard peak area to give relative peak area (RPA). The means of the UoG blend compounds were plotted against the total time since pipetting (i.e. 10 s longer than time spent in the wind tunnel) and presented in Figure 4.2. The results show that during the first 40 s (30 s of which was in the wind tunnel) a considerable amount of the odour compounds was lost (between 2 to 11 fold losses). After the first 40 s the release rate plateaued apart from for linalool which continued a modest decline. However, post 40 s the ratio of the compounds was also different with the quantity of remaining salicylaldehyde

dropping to similar levels as limonene, whereas methyl 2-methoxybenzoate maintained relatively high quantities.
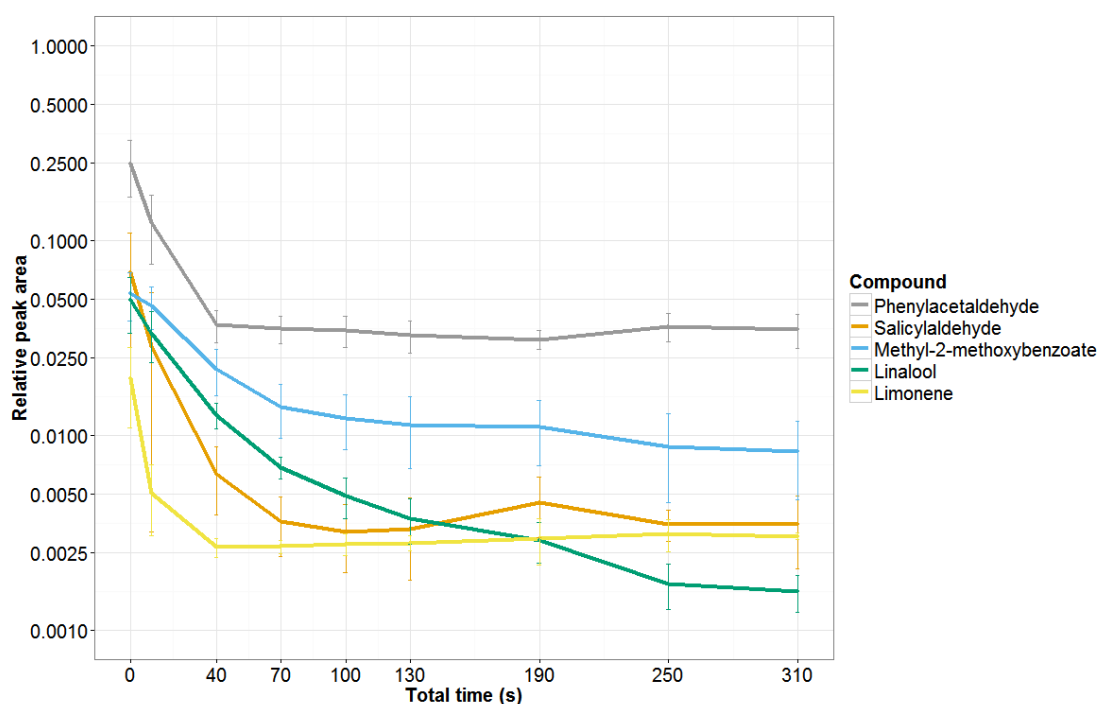

Figure 4.2: the relative peak area (log scale) of the compounds in the UoG blend, used to give an estimation of the quantity remaining of each compound on a filter paper in a wind tunnel over 310 s. The filter paper had had 40 µL of the UoG blend at 0.1 mg/mL pipetted onto it providing approximately 4 µg of the odour blend. At each time interval the remaining quantity of the compounds was estimated by calculating the peak area from a GC trace relative to a known quantity of an internal standard.

Assuming that at the initial time the total quantity of the UoG blend on the filter paper was 4 µg with the quantities of the individual as described in Table 4.1, then an approximation of the total quantities of each compound at each time point can be calculated and consequently the losses (Table 4.3).

Table 4.3: approximate quantities remaining on the lure and quantities lost from the initial time of the individual components of the UoG blend during 310 s from being pipetted onto filter paper and placed into a wind tunnel. The starting concentration was 0.1 mg/mL of the UoG blend and 40 μL of this was pipetted onto the filter paper, giving a total starting quantity of approximately 4 μg. Rates of loss are calculated by measuring the remaining quantity of the compounds on the filter paper after being eluted into hexane with an internal standard and quantified by measuring the peak area for each compound by GC. All values are approximate as it assumes that peak area of one compound is directly relative to the peak area of another compound.

	Time (s)								
	0	10	40	70	100	130	190	250	310
Compound	Approximate quantities remaining (μg)								
Limonene	0.200	0.052	0.027	0.028	0.028	0.029	0.030	0.032	0.031
Linalool	0.380	0.259	0.098	0.053	0.038	0.029	0.022	0.013	0.012
Methyl-2-methoxybenzoate	0.380	0.330	0.156	0.100	0.088	0.080	0.079	0.062	0.059
Phenylacetaldehyde	2.160	1.079	0.322	0.309	0.303	0.284	0.271	0.316	0.305
Salicylaldehyde	0.880	0.369	0.081	0.047	0.041	0.043	0.058	0.045	0.045
Total (μg)	**4**	**2.088**	**0.685**	**0.536**	**0.498**	**0.465**	**0.461**	**0.468**	**0.452**

	Approximate quanties lost since 0 s (μg)								
Limonene	0.000	0.148	0.173	0.172	0.172	0.171	0.170	0.168	0.169
Linalool	0.000	0.121	0.282	0.327	0.342	0.351	0.358	0.367	0.368
Methyl-2-methoxybenzoate	0.000	0.050	0.224	0.280	0.292	0.300	0.301	0.318	0.321
Phenylacetaldehyde	0.000	1.081	1.838	1.851	1.857	1.876	1.889	1.844	1.855
Salicylaldehyde	0.000	0.511	0.799	0.833	0.839	0.837	0.822	0.835	0.835
Total (μg)	**0**	**1.912**	**3.315**	**3.464**	**3.502**	**3.535**	**3.539**	**3.532**	**3.548**

4.3.2 Example tracks

Using an infra-red camera with an infra-red LED light array to backlight the arena allowed the digital recording of the movement of insects in the wind tunnel. The digital video files were analysed by EthoVision in order to trace and quantify that movement. An example of the insect tracks movement can be seen in Figure 4.3. There were considerable differences in the movement insects undertook, with some spending relatively little time moving around the arena and some taking much longer.
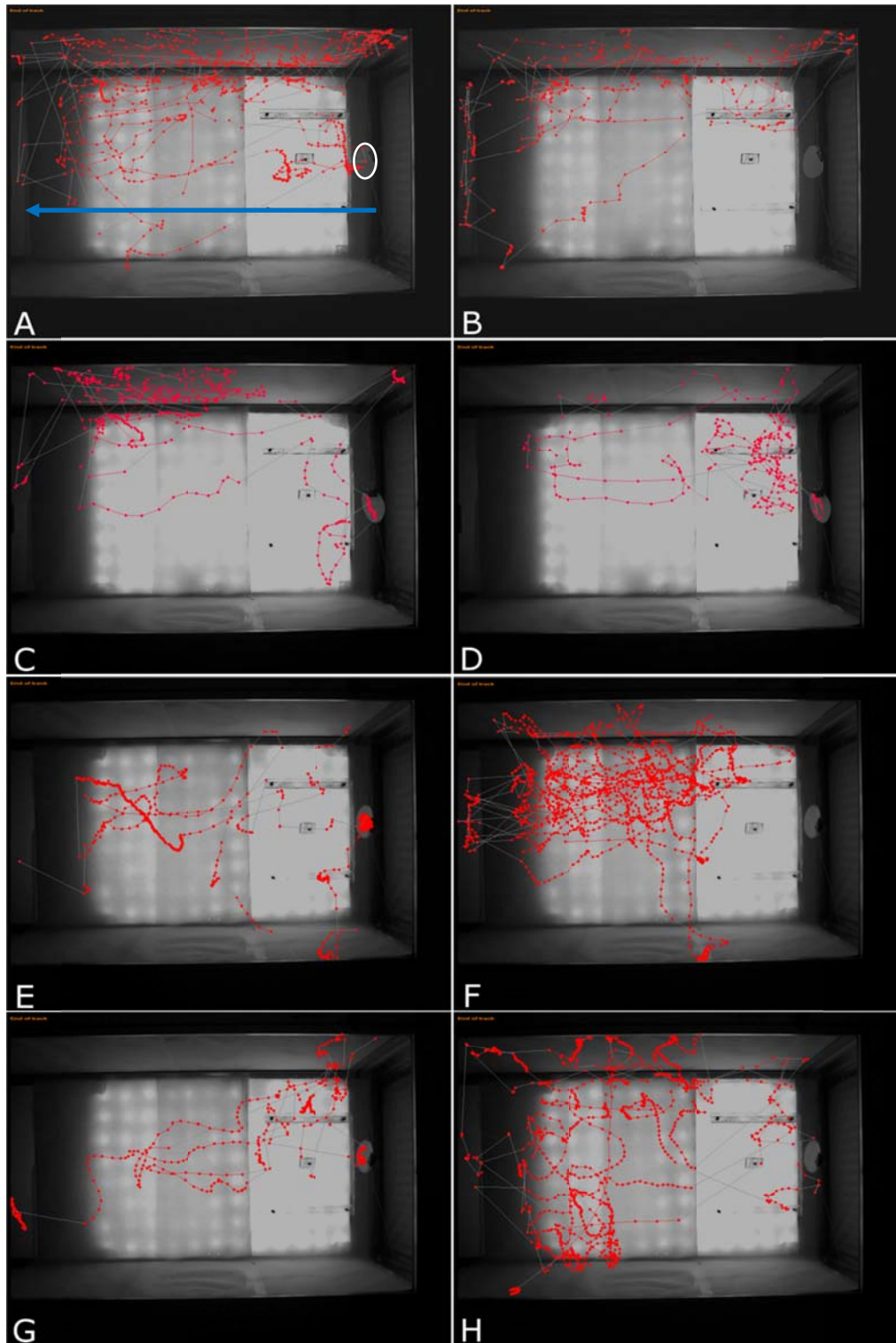
Figure 4.3: Example flight tracks of *Helicoverpa armigera* created by EthoVision. The backlight is infra red and therefore only visible to the camera. The red lines and dots show the insect's movement over the course of the 310 s run. The grey lines indicate that the tracking software has interpolated the track. The halfway point is approximately where the lit area starts; the odour source is the filter paper hanging on a wire at the upwind (right) end of the arena (highlighted in plate A); the direction of airflow is shown by the blue arrow in plate A.

Figure 4.3 (continued): (A) A female, virgin, unfed, odour loading of 40 µg, contacted the odour source 2.88 s after crossing the halfway point. (B) A female, virgin, fed with sucrose solution, odour loading of 40 µg, did not contact the odour source. (C) A male, virgin, fed with water, odour loading of 4 µg, contacted the odour source 12.72 s after crossing the halfway point. (D) A male, virgin, fed with sucrose solution, odour loading of 0.4 µg, contacted the odour source 32.48 s after crossing the halfway point. (E) A female, virgin, fed with water, odour loading of 4 µg, contacted the odour source 38.96 s after crossing the halfway point. (F) A female, gravid, fed with sucrose solution, odour loading of 4 µg, did not contact the odour source. (G) A female, virgin, fed with water, odour loading 4 µg, contacted the odour source 6.2 s after crossing the halfway point. (H) A female, gravid, fed with water, odour loading of 4 µg, hovered around the odour source but did not contact it.

4.3.3 ANOVA table of treatments and the proportion of *H. armigera* that contacted the odour source

The physiological status treatments (nutritional status and mated status), sex and the amount of odour placed onto the lure were analysed by GLM (with a binomial distribution) to identify if the factors had a significant effect on the proportion of H. armigera insects to contact a lure baited with the UoG blend. Both prior feeding experience and odour load was found to have a significant effect (Table 4.4) and mated status was also found to have a significant effect (Table 4.5).

Table 4.4: Factors affecting the proportion of *H. armigera* moths that contacted the odour source compared to those that did not make contact. Analysed by GLM (binomial distribution), with the reduction in deviance of the factors in the model tested using chi-squared.

	Df	Deviance	Residual Df	Residual deviance	P
NULL			49	83.73	
Fed	2	18.93	47	64.80	< 0.001
Odour load	2	8.80	45	56.00	< 0.05
Sex	1	2.70	44	53.34	> 0.05
Fed:odour load	4	4.14	40	49.19	> 0.05
Fed:sex	2	0.13	38	49.10	> 0.05
Odour load:sex	2	2.46	36	46.60	> 0.05
Fed:odour load:sex	4	5.13	32	41.48	> 0.05

Table 4.5: The effect of the mated status of *H. armigera* females on the proportion of insects that made contact with an odour source. Analysed by GLM (binomial distribution), with the reduction in deviance of the factors in the model tested using chi-square analysis.

	Df	Deviance	Residual Df	Residual deviance	P
NULL			21	37.05	
Mating status	1	11.09	20	25.96	< 0.001
Fed	1	10.47	19	15.49	< 0.01
Mating status:fed	1	0.45	18	15.04	> 0.05

4.3.4 Insect sex

No significant differences were found in the time moths spent moving or were stationary between either of the sexes (time moving: N = 111 (female), N = 65 (male), DF_{sex} = 1, F = 0.27, *P* > 0.05; time stationary: GLM, N = 111 (female), N = 65 (male), DF_{sex} = 1, F = 0.46, *P* > 0.05).

No significant difference was found for the proportion of moths that contacted the odour sources between the sexes (Table 4.4) (GLM with binomial distribution, X^2 = 2.24, d.f. = 1, N = 91 (males), 140 (females), *P* > 0.05).

No significant difference was found between the sexes for the 'survival curve' depicting the time the insects took to make contact with the odour source (Figure 4.4).
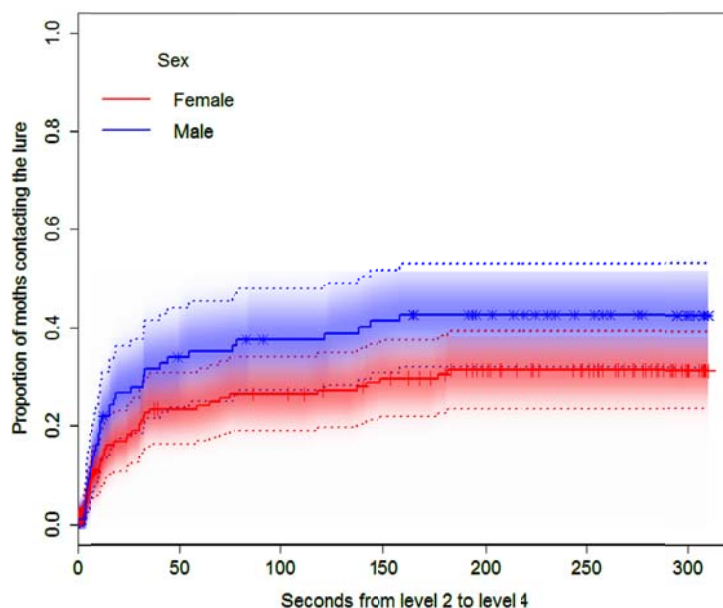
Figure 4.4: The proportion of male and female *Helicoverpa armigera* moths responding to an odour source and the time taken (from the halfway point of the wind tunnel) to contact that odour source. The cross-marks indicate moths that did not contact the odour source in the time given. Dotted lines and shading indicate 95% confidence intervals. Data analysed by a log-rank test N = 136 (females), 86 (males), X^2 = 3, df = 1, P > 0.05.

4.3.5 Odour loading

No significant differences were found between the three odour loadings tested for the amount of time insects spent moving or stationary. For the three odour loadings: time spent moving, GLM, N = 59 (0.4 µg), N = 53 (4 µg), 64 (40 µg), DF_{odour} = 1, F = 0.72, P > 0.05; time spent stationary, GLM N = 59 (0.4 µg), N = 53 (4 µg), 64 (40 µg), DF_{odour} = 1, F = 0.36, P > 0.05).

A significant difference was found between the three odour loadings and the proportion of insects that contacted the odour source (Table 4.4). A significantly greater proportion of moths contacted the odour source with a higher loading (40 µg) compared to the lowest loading (0.4 µg) (Figure 4.5).
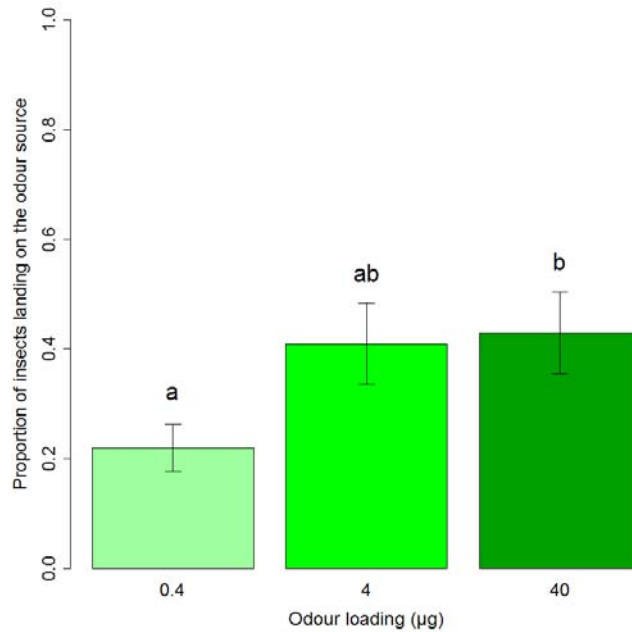
Figure 4.5: The proportion of *Helicoverpa armigera* moths contacting an odour source containing difference odour loadings of the UoG blend in a wind tunnel bioassay. Data analysed using a GLM with binomial distribution, N = 84 (0.4 µg), 73 (4 µg), 74 (40 µg), $X^2_{(2, 48)}$ = 5.06, *P* < 0.01.

A significant difference was found in the 'survival curves' for odour loading (X^2 = 6.9, df = 2, *P* < 0.05) (Figure 4.6). At the highest odour loading, the curve is initially steeper than for the other loadings indicating that more moths contacted the odour source in a relatively short space of time for a loading of 40 µg compared to the other two loadings.
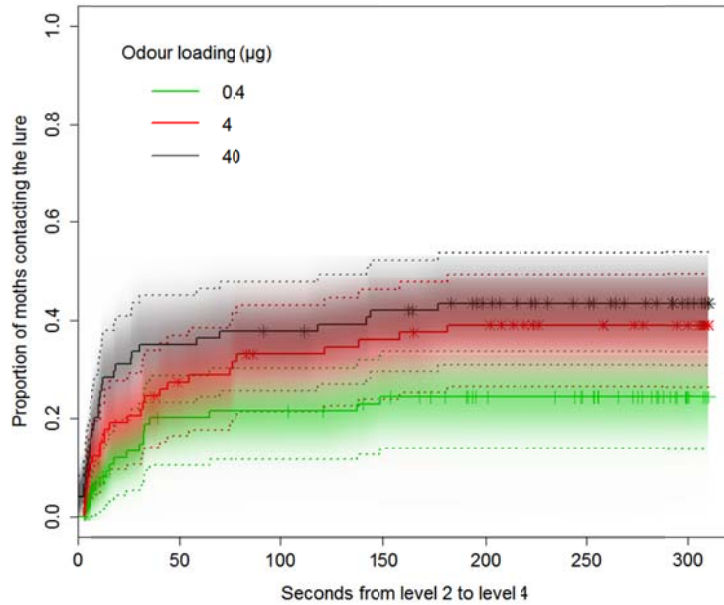
Figure 4.6: The proportion of *Helicoverpa armigera* moths responding to an odour source with different odour loadings and the time taken (from the halfway point of the wind tunnel) to contact that odour source. The cross-marks indicate moths that did not contact the odour source in the time given. Dotted lines and shading indicate 95% confidence intervals. N = 75 (0.4 µg), 73 (4 µg), 74 (40 µg), X^2 = 6.9, df = 2, *P* < 0.05.

4.3.6 Prior feeding

A significant difference was found in the time moths spent moving (Figure 4.7) or were stationary (Figure 4.8) for the three feeding regimes. Moths that had been able to feed on sucrose solution spent significantly less time moving than moths that were either not fed or were only provided with water, and vice versa for the amount of time spent stationary.
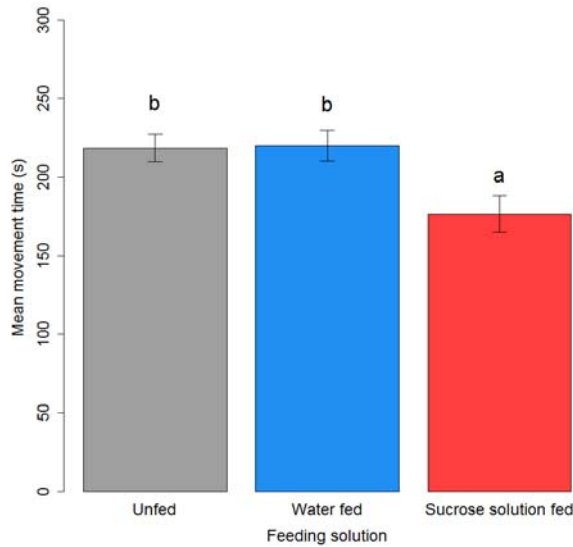
Figure 4.7: Mean time (s) that moths that crossed the halfway point of the wind tunnel spent moving. GLM with a quasi distribution, N = 64 (unfed), N = 60 (water fed), N = 52 (sucrose fed), $F_{(2, 173)} = 5.57$, $P < 0.01$.
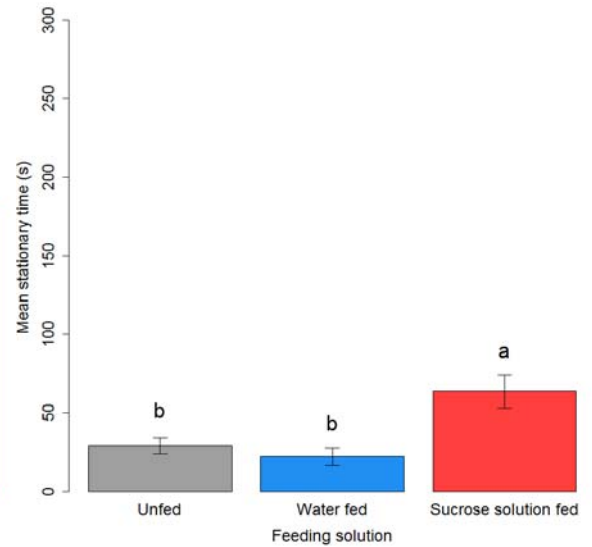
Figure 4.8: Mean time (s) that moths that crossed the halfway point of the wind tunnel spent stationary. GLM with a quasi distribution, N = 64 (unfed), N = 60 (water fed), N = 52 (sucrose fed), $F_{(2, 173)} = 9.12$, $P < 0.001$.

A significantly lower proportion of moths that were allowed to feed on 10 % sucrose solution contacted the odour source compared to moths that were starved or fed on water (Figure 4.9).
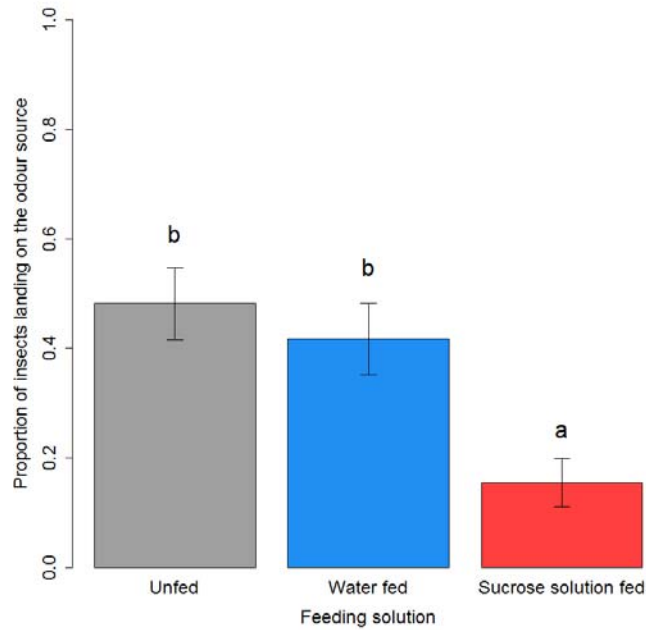
Figure 4.9: The proportion of *Helicovera armigera* moths in a wind tunnel bioassay that contacted an odour source containing the UoG blend after exposure to different feeding solutions. Data analysed using a GLM with binomial distribution, N = 76 (unfed), 74 (water fed), 81 (sucrose solution), $X^2_{(2,47)}$ = 18.93, *P* < 0.001.

Significant differences were found between the treatments for the 'survival curves' for the tests on prior feeding (X^2 = 15, df = 2, *P* < 0.001) (Figure 4.10), and mated status (X^2 = 11.3, df = 1, *P* < 0.001) (Figure 4.12). Moths that were unfed or provided only with water exhibited similar shaped curves.
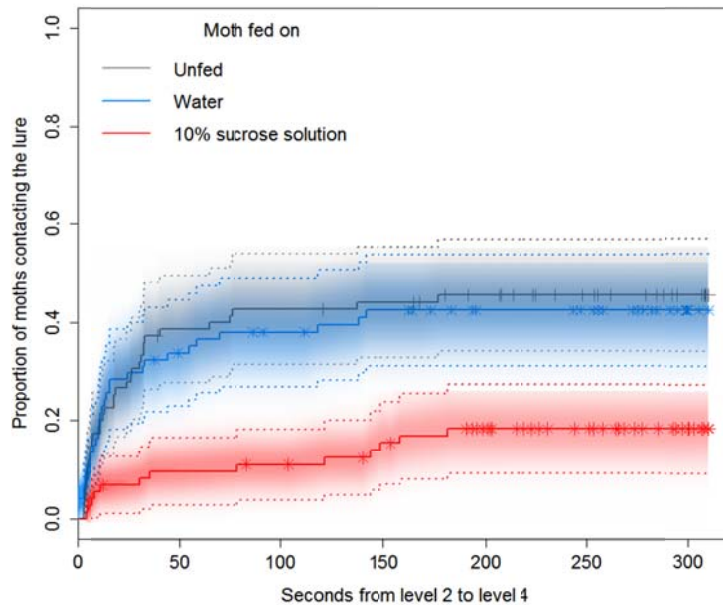
Figure 4.10: The proportion of *H. armigera* moths given different regimes responding to an odour source and the time taken (from the halfway point of the wind tunnel) to contact that odour source. The cross-marks indicate moths that did not contact the odour source in the time given. Dotted lines and shading indicate 95% confidence intervals. N = 75 (unfed), 74 (water fed), 73 (sucrose solution fed), X^2 = 15, df = 2, P < 0.001.

4.3.7 Mated status

No significant differences were found between the mated status of the moths for: the time spent moving, GLM, N = 53 (non-gravid), N = 32 (gravid), $DF_{mated\ status}$ = 1, F = 1.30, P > 0.05; the time spent stationary, GLM, N = 53 (non-gravid), N = 32 (gravid), $DF_{mated\ status}$ = 1, F = 0.10, P > 0.05).

The mated status of female *H. armigera* had a significant effect on the proportion of insects that contacted the odour source (Table 4.5 and Figure 4.11). The proportion of insects that were gravid was significantly less than for insects that were not gravid. The result was the same regardless of what the insects had been able to feed on and the lowest proportion of insects that contacted the odour source were those that had both fed on sucrose solution and had mated.
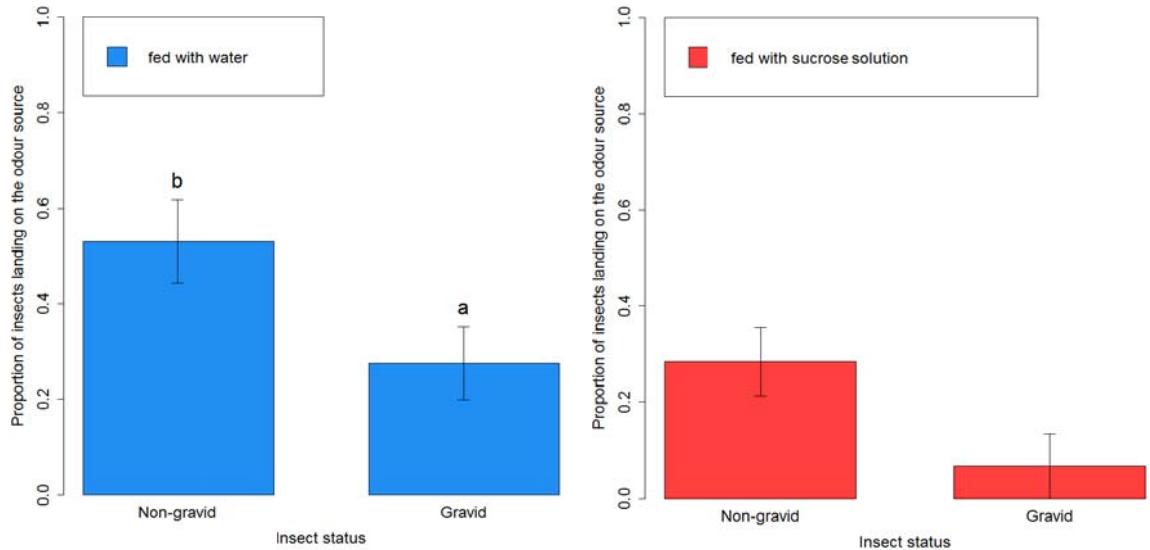
Figure 4.11: The proportion of water fed and sucrose solution fed, gravid or non-gravid *H. armigera* females that contacted the UoG odour source. Data analysed using a GLM with a binomial distribution, N = 50 (non-gravid, water fed), 33 (gravid, water fed); N = 21 (non-gravid, sucrose fed), 19 (gravid, sucrose fed); for water fed insects, $X^2_{(1,14)}$ = 6.67, P < 0.01.

Significant differences were found between the treatments for the 'survival curves' for the tests on mated status (X^2 = 11.3, df = 1, P < 0.001) (Figure 4.12).

Figure 4.12: The proportion of gravid or non-gravid *Helicoverpa armigera* moths responding to an odour source in a wind tunnel bioassay and the time taken (from the halfway point of the wind tunnel) to contact that odour source. The cross-marks indicate moths that did not contact the odour source in the time given. Dotted lines and shading indicate 95% confidence intervals. N = 68 (non-gravid), 52 (gravid), X^2 = 11.3, df = 1, P < 0.001.
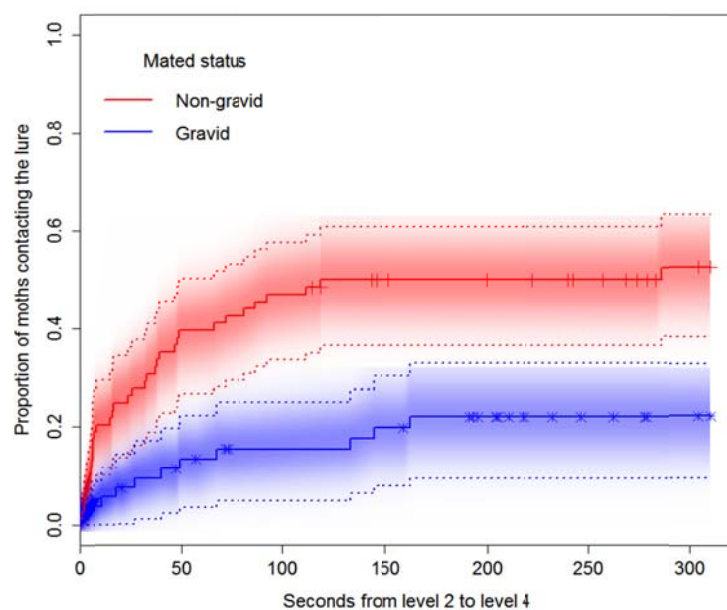
4.4 DISCUSSION

4.4.1 Release rate

The results of the release rate experiment showed that a large proportion of the odours was lost from the lure within the first 0 - 40 s from being pipetted onto the filter paper. Table 4.3 shows the approximate quantities lost for each compound, and if the quantity at 10 s is compared to the quantity at 310 s we can see that the loss for each compound during these 300 s is: PAA = 0.77, Sa = 0.32, M2M = 0.27, Ln = 0.25, Lm = 0.02 (μg). The extremely high loss of limonene within the first 10 s meant that little was released during the remainder of the run. Apart from limonene, the initial ratio was similar to the final ratio, and the release rate was approximately 2.6 ng / s for PAA, 1.1 ng/ s for Sa, 0.9 ng / s for M2M, 0.8 ng / s for Ln, and 0.07 ng / s for Lm. If this is scaled up this be a release rate of 0.22 mg / day for PAA, or 0.48 mg / day for the UoG blend as a whole.

4.4.2 Effect of sex

As was found in the other studies carried out during this thesis, there was no significant difference in behaviour that could be attributed to the sex of the insect. This confirms the hypothesis that the synthetic floral attractant used in these studies is equally attractive to both sexes.

4.4.3 Insect movement

The behaviour a moth displays in order to locate an odour source is described as a sequence: (i) taking flight, (ii) upwind movement, (iii) flying to within a few centimetres of the odour source, (iv) contacting the odour source, (v) landing on and probing the odour source (e.g. Rojas *et al.*, 2000; Plepys *et al.*, 2002a; Saveer *et al.*, 2012). For the analysis of the time moths spent moving or stationary, only moths that made upwind movement and crossed the halfway point of the wind tunnel (i.e. travelled to within c. 900 mm of the odour source) were counted. Therefore, any statistical differences found are for moths that have already displayed behaviour indicative of attempting to locate the odour source.

In all treatments insects spent considerably more time in motion than stationary; insects were found to spend approximately 200 s in motion and less than 50 s stationary (out of a total of 310 s). The remainder of the time was spent outside of the tracking arena. The differences between in motion or stationary for moths under different treatments were all non-significant except for the prior feeding test. Insects were found to be in motion for significantly less time and stationary for significantly more time if they had been fed sucrose solution compared to those fed with water or nothing. A possible hypothesis for explaining the difference in movement (or lack of) can be attributed to a behaviour switch, whereby insects that are sated are less likely to utilise their energy in the search of a resource they do not currently require. As they had recently fed on a source of sugar they do not need to expel further energy in the pursuit of locating more 'nectar'.

In these experiments the method used for tracking the insect's movement (2D tracking from a side view) did not allow for the recording of certain typical

behaviours thought to be used by moths to locate an odour source. Specifically, 'casting' and 'zigzagging' were not recorded as the direction the moth would travel to exhibit these behaviours would be in the same plane as the camera's line of sight. Although these movements were not recorded nor analysed the author notes that many moths did not display these types of behaviours and instead flew directly to the odour source within a few seconds of what appeared to be the insect locking on to the odour plume. As shown by the results of the 'latency to contact data' the majority of moths that did contact the odour source did so within the first few seconds. The likely explanation for the highly direct flight pattern is that the air movement in the wind tunnel was highly laminar with little formation of vortices. The straight and relatively uniform odour plume may have facilitated the moths in locating the odour source quickly. In addition, the experimental protocol used was a 'no-choice' experiment. Thus the chances of the insect losing the odour plume or becoming distracted by non-target scent was minimal. The lack of other odours and straight and relatively uniform odour plume would presumably make it easy for the insects to track it meaning the casting or zigzagging was not necessary.

4.4.4 Proportion of insects that contacted the odour source

4.4.4.1 Odour loading

"... up to a point, the accuracy of orientation improves with stimulus intensity." (Bell, 1991)

A significant difference was found between the three odour loading levels tested here. The highest odour load (40 µg) had a significantly greater proportion of insects contacting it than the lowest odour load (0.4 µg). The 40 µg load received double the proportion of contacts compared to the lowest odour

loading (0.4 µg). This has important implications for the use of this floral attractant in the field in terms of the release rate, lifespan, and formulation. The results seen here suggest that the quantity of odour being released affects the number of insects that will be attracted to the lure. The release rate of the volatile chemicals from certain types of lure (e.g. rubber septa) have a very large release rate initially and then decrease quickly over time. Both the high initial release and latter low release rates could negatively affect insect captures. This effect may be negated by using alternative formulations or lures, e.g. plastic sachets, which have a more uniform release rate and were therefore used in the field work in chapters 3, 6 and 7.

The effect of odour concentration on attraction has been investigated in many plant odour-insect interactions (e.g. Plepys *et al.*, 2002b; Fraser *et al.*, 2003; Dötterl *et al.*, 2006) and the results seen here agree with those before them such that higher doses increases the proportion of insects that respond to the odour lure. There is clear evidence that odour concentrations can be differentiated to several orders of magnitude by insects (for a review see Carlsson and Hansson, 2006 and the references therein) and play an important role in odour discrimination. The ability of an insect to discriminate between odours may be reduced at extremely low or high concentrations. When investigating the behavioural response of odour-conditioned honeybees it was found the bees were less able to discriminate between the odour they were conditioned with and a test odour if both odours were presented at a low dose, as the dose increased discriminated also increased (Smith *et al.*, 2006). At the lowest and highest doses tested, Plepys *et al.* (2002b) found that a lower proportion *A. gamma* moths responded in a wind tunnel than at the other moderate doses tested. Within the moderate doses, the authors found that

slightly higher doses gave a greater response than lower doses. This suggests there is an optimum odour dose to initiate a positive behavioural response in insects, and if the antennae receives odours at concentrations outside of its preferred range the chances of stimulating flight or searching behaviour are reduced. From the results seen in this experiment it would suggest that the doses tested were within the insects' preferred range as behavioural responses were good at all doses. It would be useful to test higher doses to ascertain whether the proportion of insects stimulated to land on the lure could be increased.

The reasons for these behavioural changes in response to varying odour loadings is likely due to the insect's ability to discriminate between olfactory stimuli at extreme concentrations. At very high doses ORNs have been shown to become less odour specific and will respond to chemicals that would not stimulate them at lower doses (Hartlieb *et al.*, 1997). This may initially increase behavioural responses until a stimulation threshold is reached where the insect's olfactory system is overloaded causing the insect to no long be attracted to the odour. This over stimulation of the ORNs by high odour concentrations is known to alter how the odours are processed in the antennal lobe causing different (adjacent) glomeruli to be activated than the usual glomeruli for that particular odour (Carlsson and Hansson, 2003). In addition, at very low odour concentrations the insect may be able to detect the presence of odour molecules but not identify them (Wright *et al.*, 2005).

Moving out of the laboratory, field trials carried out by Meagher and Landolt (2010) found that the release rate of binary lures (containing phenylacetaldhyde and linalool) did not significantly affect the captures of the velvetbean moth,

Anticarsia gemmatalis. More importantly their results indicated an inverse relationship between release rate and moth captures. Higher release rates (estimated to be in the order of 4.9 mg / d for PAA at the highest release rate) caught fewer moths than the lower release rates. Due to the conditions and methods used the authors were not able to accurately calculate the release rate of their lures in the field. Other studies have estimated the release rate of their lures at 11.8 mg / d (Meagher, 2001a), and 4 mg / d for linalool (Landolt *et al.*, 2001). In the current wind tunnel bioassays the release rate excluding the time taken to position the lure (i.e. the first 10 s) to the end of the run (300 s) was extrapolated and estimated to be equivalent to 0.48 mg / d from an intital loading of 4 µg (see section 4.4.1). This would obviously be much lower for the initial loading of 0.4 µg, and much higher for the initial loading of 40 µg. However, even at the lowest dose insects responded positively towards the odour lure, and at the highest odour loading the insects did not exhibit behaviour synonymous with a repellent. If the behaviour response curve follows a typical sigmoid shape then it is possible that loadings of 400 or 4000 µg would result in the moths being repelled. Unfortunately the type of wind tunnel used here (circulating air with a carbon filter) makes testing very high odour loadings impractical.

4.4.4.2 Prior feeding

The effect of the feeding solutions (or absence of) was highly significant in terms of the proportion of insects that contacted the odour source. There was no significant difference between the moths that were fed only with water or not fed at all, but both of these treatments were significantly different from the moths that were provided with sucrose solution. A significantly lower proportion

of moths that were able to feed on sucrose solution contacted the odour source compared to the moths that were only able to feed on water or were not fed at all. This strongly supports the hypothesis that the synthetic floral odour blend used in this study is a 'feeding attractant' because moths that were sated with sucrose solution were less likely to contact the odour source (Figure 4.9), and took longer to do so (Figure 4.10) than moths that were only provided with water or nothing at all. Moths that were starved exhibited a slightly higher propensity for contacting the odour source compared to moths that were water fed (non-significant).

In this experiment it was not known when the insects tested had last fed or even if they had fed at all. It is likely that at least some of the insects tested that were in the 'fed on sucrose solution' group had not fed for some time particularly those that were tested towards the end of the replicate (up to three hours into scotophase). It is conceivable that this experimental design may have caused the difference in response between the treatments to be reduced. Regardless, it is clear that sated insects are not as attracted to this particular blend of floral volatiles compared to insects that were only fed on water or on nothing at all. This is of particular importance when considering using this odour blend as a crop protection tool in environments that have a large source of nectar, e.g. during the flowering stages of the crop, multicropping environments that contain large numbers of flowering plants, or areas with a high floral biodiversity providing the insect with a large number of nectar resources. Conversely, this may mean that the most successful area for using this odour blend would be an area with minimal nectar resources, e.g. large mono-crop areas with a crop in the pre- or post-flowering stage. The timing, relative to the crop cycle, of

implementing a crop protection system using this (or perhaps other floral odours) could strongly influence its success.

4.4.4.3 Mated status

Mating status had a significant effect on the proportion of insects that contacted the odour source. Gravid insects (i.e. those that oviposited fertile eggs within 24 h of being tested in the wind tunnel) were significantly less likely to contact the odour source than virgin moths. The effect was similar to that of the moths that were fed on water or sucrose and the lowest proportion of contacts overall were made by gravid, sucrose fed moths. Previous research has found that mating alters the behavioural responses to plant volatiles in insects causing mated females to become more likely to fly towards host plant odour sources (Rojas, 1999b; Mechaber *et al.*, 2002; reviewed by Anton *et al.*, 2007; Saveer *et al.*, 2012).

Nectar feeding (or feeding on sucrose solution) has been shown to increase fecundity and larval performance in *H armigera* (Song *et al.*, 2007; Wackers *et al.*, 2007 and the references therein). The results seen in the current experiment are a little surprising as ovipositing *H. armigera* are known to be more attracted to flowering host plants than non-flowering host plants (Firempong and Zalucki, 1989; Sequeira *et al.*, 2001; Wackers *et al.*, 2007 and the references therein) and also feed on nectar between ovipositing. Tingle and Mitchell (1992) found that significantly more mated *H. virescens* landed on lures baited with cotton flower extracts than virgin females; most of these insects then proceeded to probe the dispensers with their antennae, proboscis, and/or ovipositor. The effect of mating in *A. gamma* was found to alter the priority of important stimuli between the sound of predatory bats and the smell of flowers,

causing mated females to take less heed of the sound of bats compared to virgin females when searching for the source of floral odours (Skals *et al.*, 2003). In field tests of synthetic lures (containing phenylacetaldehyde, benzyl acetate, and benzaldehyde, Meagher (2002)) found that the majority of the female noctuid moths (mostly *Pseudoplusia includes*) trapped were mated (c. 93%). In the current study, exactly why the proportion of mated females contacting the odour source was significantly lower than for non-mated females is not clear. However, it is possible that the reason lies in the origins of the chemicals that make up the blend. All five chemicals in the blend are putative floral volatiles (Knudsen *et al.*, 2006), and it is likely that mated females are attracted to odours that combine floral <u>and</u> green leaf chemicals, as these compounds would indicate the ideal oviposition host for the gravid female, whereas perhaps an odour that only indicates the presence of flowers does not provide quite the correct stimulus for a mated female looking to oviposit. Indeed, Saveer *et al.* (Saveer *et al.*, 2012) found that mated female *S. littoralis* were significantly more attracted to the green leaves of cotton plants than the nectar-rich flowers of *S. vulgaris*. It is possible that the presence of specific floral compounds at certain quantities may effect the behaviour of gravid insects, for example, transgenic *Nicotiana tabacum* (tobacco) (L.) (Solanaceae), which produced increased quantities of (*S*)-linalool relative to non-transgenic tobacco was tested for oviposition preference by gravid *H. armigera*. The moths laid significantly more eggs on the wild-type compared to the transgenic plants suggesting that the gravid insects find odour profiles containing larger than normal quantities of (*S*)-linalool less suitable for oviposition (McCallum *et al.*, 2011). The presence of linalool in the UoG blend and its relatively high release

rate (Figure 4.2 and Table 4.3) may have negatively affected the likelihood of the gravid insects making upwind flight.

The behavioural change towards the UoG floral odour blend in gravid insects is clear, but quite how this behavioural switch may come about is not yet entirely understood. Rajapakse *et al.* (2006) found that the EAG responses in mated and non-mated *H. armigera* to the common host plant, pigeon pea, were not significantly different. The chemicals tested in their study included two that are in the UoG blend (limonene and linalool). Electroantennagraphy measures the neurological response of the odour receptor neurons in the insect's antennae. The results of the Rajapakse *et al.* (2006) study suggests that the change in response between mated and non-mated takes place further up the neurological pathway rather than in the antennae. Indeed, using calcium imaging, Saveer *et al.* (2012) found differences in the antennal lobes of mated and non-mated *S. littoralis* in the insects' response to the odour from a host plant (*Gossypium hirsutum*) and a nectar-rich flowering plant (*Syringa vulgaris*). Thus, the processing of the volatile chemical data within the antennal lobe is the first point in the insect's sensory and processing mechanism which leads to a behavioural change. It is currently unknown how the differences seen in the antennal lobe modulate the behavioural response.

4.4.5 Latency to contact

Significant differences were found between the treatments for the variables 'prior feeding' (Figure 4.10), 'odour load' (Figure 4.6), and 'mated status' (Figure 4.12), and but not 'sex' (Figure 4.10).

The most interesting result from these analyses is the shape of the curves. Curves that rise sharply and plateau out early indicate that the majority of

insects that contacted the odour source did so very quickly. The marks on the lines denote situations where an insect has failed to contact the odour source within the time given. Curves that have many markers towards the end of the experiment indicate the insect crossed the halfway point early on, but then failed to contact the odour source. For example, in the prior feeding experiment, the curve for the unfed insects rose very sharply with the majority of insects contacting the odour source within 75 s of crossing the halfway point of the wind tunnel. In contrast, the curve for the insects that fed on sucrose solution rose very slowly implying that the insects took a long time to locate the odour source even after they had made upwind flight.

In the odour loading experiment, the highest loading (40 µg) curve was the steepest, rising much quicker than the middle odour load (of 4 µg) for the first 30 seconds. In the mated status experiment, the curve for the unmated insects rose much more quickly than for the gravid insects.

Overall, the results show that where the insects were more likely to contact the odour source, they also made this contact quicker than in treatments where the proportion of insects making contact was lower. This is likely, in part, due to the design of the experiment – a 'no choice' experiment. The insect had only to decide to attempt to locate the odour source or to ignore it. If it chose to locate it, this posed no problem as the air movement in the wind tunnel was continuous and linear, the odour source was stationary, and there were no competing odours or stimuli to distract the insect or disrupt the odour stream.

4.4.6 Conclusion

 In terms of using this blend of chemicals as a crop protection tool in an 'attract-and-kill' system or as a lure in a trap, the observations that mated and fed

moths were the least likely to be attracted to the lure are important. This suggests that the odour blend is a feeding attractant rather than an ovipostion attractant, and should cause a reduction in the numbers of virgin and unfed moths from the field, but may have less of an impact on the numbers of mated and/or fed moths in the field. Therefore, to maximise the effectiveness of the odour blend it should be used when there are more virgin moths compared to mated, as well at times when there are few natural nectar resources, or when moths are likely to feed. Noctuid behaviour that relates to this includes the following: female Noctuid moths do not normally mate for the first 2 - 3 days post-eclosion, do not usually mate prior to migration, will feed on the first evening after eclosion, and may feed prior to and during bouts of oviposition. Consequently, the odour lures should be in place before the arrival or emergence of populations of the target moth species, in order to capture the arrival of virgin and unfed moths.

The proportion of unfed, virgin insects that contacted the odour source within the 5 min given was relatively high (c. 50 %). Assuming that as time increases this proportion will increase the odour blend may be usable as a crop protection tool. Moreover, it is possible that with the addition of specific plant volatiles that *H. armigera* (and other noctuid pests) utilise to identify and locate ovipositional sites, then the blend could become increasingly attractive to mated females. For example Jallow *et al.* (1999), found a blend of terpenes (containing: trans-β-caryophyllene, β-bisabolol, α-pinene, myrcene, β-pinene, and α-humulene) attracted significantly more eggs from gravid *H. armigera* than a blank control. Rajapakse *et al.* (2006) found that small quantities of the GLV (*Z*)-3-hexenyl 2-methylbutyrate was present only in the odour profile of their preferred host pigeon pea, and this compound elicited high EAG responses. It is possible this

and other GLVs are oviposition stimulants which could be added to improve the attraction of gravid moths to the UoG blend. (Saveer *et al.*, 2012) showed that mated *S. littoralis* females were more attracted to cotton leaves than the flowers of *S. vulgaris*. The compounds identified in the cotton leaves' headspace that elicited an EAG response in *S. littoralis* included benzaldehyde and β-myrcene, both of which were tested as additions to the UoG blend to improve its attractiveness in the field trials in Chapter 6.

The topic of oviposition odour cues in Noctuid moths is relatively un-researched and would be an interesting avenue to follow especially when combined with the research within this thesis.

As the results of this chapter show that the physiological status of the moth affects its behaviour towards the UoG floral odour blend, electrophysiological work was carried out in Chapter 5 to see if the basis for the changes in behaviour could be attributed to changes in sensitivity within the antenna. It would also have been useful to record the mated status (or count the presence of spermatophores) of noctuids caught in the odour baited traps in the field trials in Chapter 6; unfortunately, due to time constraints this was not possible.

CHAPTER 5 - ELECTROPHYSIOLOGICAL RESPONSES OF NOCTUIDS TO FLORAL COMPOUNDS

5.1 INTRODUCTION

The aim of this chapter was to test a suite of candidate floral volatiles on the antennae of *Autographa gamma* and *Helicoverpa gelotopoeon*. The results from this chapter would then form the basis for selecting compounds to test in the field in the following chapter to try to improve the attractiveness of the UoG odour blend. This is the first time the South American Heliothine species has been tested with floral volatiles in this manner.

The method used here for optimising the UoG blend is similar to the methods used in the research that culminated in the production of the Magnet blend (Del Socorro *et al.*, 2010b; Gregg *et al.*, 2010). Gregg *et al.* (2010) selected compounds that elicited EAG responses in *H. armigera* and tested these floral and leaf volatiles in a series of odour blends in a novel olfactometer in order to identify the most effective odour blend for attracting *H. armigera* and other Helicoverpa species.

Initially a review of literature related to floral volatiles and noctuids was conducted to identify any compounds that may be attractive. A summary of this is presented in Table 1.3 and Table 1.4. Once a list of candidate compounds was identified they were tested on the antennae of *A. gamma* and *H. gelotopoeon* using the EAG methods as described in section 2.4. Although sensitivity to compounds does not necessarily predict behaviour, EAG

techniques allow researchers to identify compounds that an insect's antennae are particularly sensitive to and which may elicit a behavioural response.

In Chapter 4 significant behavioural differences towards the UoG blend were found between *H. armigera* females that were either virgin or gravid. Previous research has also found differences in behaviour to plant odours between mated and virgin female Lepidoptera (e.g. Tingle and Mitchell, 1992; Rojas, 1999b; Mechaber *et al.*, 2002; Skals *et al.*, 2003; Saveer *et al.*, 2012). However, it is not yet clear where in the olfactory system changes due to mating occurs – in the periphery (antennae), in the antennal lobe, or both. Some research has found no difference in antennal response to odours between mated and virgin insects (Rajapakse *et al.*, 2006), whereas other research has found a difference in EAG responses between the two physiological states (Li *et al.*, 2005; Martel *et al.*, 2009), and Barrozo *et al.* (2011) found that peripheral perception (single-cell antennal recordings) to the plant odour heptanal did not change post-mating, whereas within the antennal lobe, changes were apparent.

5.2 MATERIALS AND METHODS

5.2.1 Insect material

Autographa gamma, *Helicoverpa gelotopoeon,* and *Helicoverpa armigera* were reared as described in Section 2.2. EAG studies were carried out on both sexes. The insects were provided with 10% w/v sucrose solution and were 0 - 6 days old. Insects that were used in the mating experiments were prepared as described in Chapter 4 (section 4.2.3.3) and were 2 - 4 days old.

5.2.2 Electroantennogram (EAG) studies

Three different methods were used to carry out the EAG work in this chapter as described in Section 2.4. In methods A and B the test material was delivered to the EAG preparation by blowing air over the compound impregnated onto filter paper and onto the preparation. In method C compounds were delivered to the EAG preparation in fully volatilised form through a gas chromatograph.

5.2.2.1 <u>Testing candidate attractants by EAG Methods A and B</u>

Initial EAG experiments to test individual chemicals at a range of doses were carried out using EAG methods A and B (section 2.4.3) with *A. gamma* as the test insect. Chemicals were dissolved in hexane (pesticide grade) to the required concentration (Table 5.1). To prepare the Pasteur pipettes containing the test compounds for EAG methods A and B, 10 µL of a solution was pipetted onto a strip of filter paper (5 x 20 mm, Whatman No. 9) and inserted into a Pasteur pipette using forceps. Samples were made immediately prior to being used. The samples were tested in a random order (by blind selection) except for the 10 mg/mL dose which was tested last to avoid the problem of over saturating the antenna causing erroneous EAG responses for subsequent tests. Complete enzymatic degradation of volatile compounds within the antennae may take several minutes after saturation (Kaissling, 2001; Kaissling, 2009).

Initially a dose-response test was carried out with phenylacetaldehyde and methyl salicylate using Method A (Table 5.1). Subsequently, EAG responses to 10 candidate attractants were compared at a single dose using Method B (Table 5.1).

For the dose response tests the data was transformed (log(sEAG + 0.1) and analysed by linear regression using R. Plots show non-transformed data with the treatment concentration on a log-scale (Figure 5.2 and Figure 5.1).

For the candidate attractants the data was normalised against a standard of 10 µL of phenylacetaldehyde at 2.5 mM. This standard was a different solution of phenylacetaldehyde than the one used as a test compound. After being normalised the data was analysed by ANOVA using R to identify differences between the doses and compounds tested.

Table 5.1: list of quantities of compounds tested on the antennae of *A. gamma* using two different EAG methods

Compound	Concentration	Quantity applied to filter paper (µg)	EAG method used
Dose-response test			
Phenylacetaldehyde	0.1 (mg / mL)	1	A
Phenylacetaldehyde	0.3 (mg / mL)	3	A
Phenylacetaldehyde	1 (mg / mL)	10	A
Phenylacetaldehyde	3 (mg / mL)	30	A
Phenylacetaldehyde	10 (mg / mL)	100	A
Methyl salicylate	0.1 (mg / mL)	1	A
Methyl salicylate	0.3 (mg / mL)	3	A
Methyl salicylate	1 (mg / mL)	10	A
Methyl salicylate	3 (mg / mL)	30	A
Methyl salicylate	10 (mg / mL)	100	A
Candidate attractants			
(±)-linalool	2.5 (mM)	3.9	B
2-phenylethanol	2.5 (mM)	3.05	B
Benzaldehyde	2.5 (mM)	2.65	B
Benzyl alcohol	2.5 (mM)	2.7	B
Cinnamyl alcohol	2.5 (mM)	3.35	B
(S)-(-)-limonene	2.5 (mM)	3.4	B
Methyl salicylate	2.5 (mM)	3.8	B
Methyl-2-methoxybenzoate	2.5 (mM)	4.15	B
Phenylacetaldehyde	2.5 (mM)	3	B
Salicylaldehyde	2.5 (mM)	3.05	B

5.2.2.2 <u>Testing candidate attractants by EAG Method C</u>

EAG method C was used (Section 2.4.3.3) to test the EAG response of *H. gelotopoeon* to a range of candidate floral attractant compounds (Table 5.2) at a range of doses.

The retention index (RI) for each compound was calculated with respect to *n*-hydrocarbon standards. The RI's were used to group compounds into two groups such that within a group the RI of each compound differed by at least 100 which would mean at least a 1 min gap between compounds exiting the GC under the temperature-programmed GC conditions used. The GC oven was set to 40 °C for 4 min, then 10 °C / min to 230 °C.

Compounds within the same group were dissolved together in hexane (Fisher, pesticide grade) at one of seven concentrations: 1, 5, 10, 50, 100, 250, 495 µM (Table 5.2). In addition, all test solutions contained 50 µM of (*Z*)-3-hexen-1-ol (RI 1384) as an internal standard. The EAG peaks of the test compounds was normalised against the internal standard to give a 'standardised EAG value' (sEAG). To check the responses of males and females to the internal standard, the EAG responses of the insects to (*Z*)-3-hexen-1-ol was calculated relative to the GC peak area to give 'relative peak height' (RPH). A T-test was carried out to identify any sigificant difference between the sexes and their responses to the internal standard.

In total 40 moths were tested, 21 females and 19 males. As each moth was tested with more than one compound (i.e. psuedoreplication) an error term was added to the model to account for this. The sEAG data was analysed by mixed effect linear model to identify which factors: sex, compound, or dose, may be significant. Following this, the data was subsetted by dose and mixed effect linear models were used to analyse the sEAG responses for the effects of compound and sex within each dose. In addition, t-tests were applied to the dose-subsets to identify specific differences between the sexes for each compound.

The variables sex and compound were pooled and the data visually analysed to identify the shape of the overall sEAG response against dose on a log scale.

The linear portion of the response curve (between 5 and 250 µM) for each compound was analysed by mixed effect linear model.

Table 5.2: list of chemicals tested on the antennae of *Helicoverpa gelotopoeon* with retention indices and concentrations used. Quantities in ng/µL are amounts present in an air stream flowing over the antenna. Retention indices were calculated with respect to *n*-hydrocarbon standards on polar DBWax column. The compounds were split into two groups (A and B) so that compounds within the same group did not exit from the GC at the same time.

Compound name	Retention Indices	Group	1 µM	5 µM	10 µM	50 µM	100 µM	250 µM	495 µM
			ng / µL for µM specified						
β-Myrcene	1174	A	0.136	0.68	1.4	7	14	34	67
Benzaldehyde	1503	A	0.106	0.53	1.1	5	11	27	52
Methyl benzoate	1602	A	0.136	0.68	1.4	7	14	34	67
Benzyl alcohol	1857	A	0.108	0.54	1.1	5	11	27	53
Butyl salicylate	1975	A	0.194	0.97	1.9	10	19	49	96
Eugenol	2142	A	0.164	0.82	1.6	8	16	41	81
Phenylacetaldehyde	1641	A	0.12	0.6	1.2	6	12	30	59
Camphene	1125	B	0.136	0.68	1.4	7	14	34	67
Cineol	1206	B	0.154	0.77	1.5	8	15	39	76
(-)-Linalool	1541	B	0.154	0.77	1.5	8	15	39	76
Benzyl acetate	1711	B	0.15	0.75	1.5	8	15	38	74
2-Phenylethanol	1893	B	0.122	0.61	1.2	6	12	31	60
Methyl 2-methoxybenzoate	2046	B	0.166	0.83	1.7	8	17	42	82
Methyl anthranilate	2204	B	0.151	0.755	1.5	8	15	38	75
Indole	2403	B	0.117	0.585	1.2	6	12	29	58

5.2.2.3 <u>Testing components of the UoG blend by EAG Method C</u>

EAG Method C (Section 2.4.3.3) was used to measure the EAG responses of virgin and gravid female *H. armigera* to the components of the UoG blend at six different concentrations. The chemicals were dissolved in hexane (Fisher, pesticide grade) and diluted to one of six concentrations: 5, 10, 50, 100, 250, 500 µM (Table 5.3). In addition, all test solutions contained 50 µM of (*Z*)-3-hexen-1-ol as an internal standard.

In total 17 insects were tested, 8 virgin and 9 gravid. Each insect was tested with a series of test compounds at different doses. The pseudo-replication was accounted for during statistical analysis by adding the individual moths' identification as an error term in the GLM model.

The GC oven was set to 60 °C for 2 min, then programmed at 10 °C / min to 190 °C.

Table 5.3: list of chemicals in the UoG floral odour blend (plus an internal standard) and concentrations tested on the antennae of virgin and gravid female *H. armigera*. Quantities in ng/µL are amounts present in the air stream flowing over the antenna. The internal standard ((Z)-3-hexen-1-ol) was fixed at 50 µM for all samples. Retention indicies indicate the order the test compounds exited the GC.

Compound name	Retention Indices	5 µM	10 µM	50 µM	100 µM	250 µM	500 µM
		ng / µL for µM specified					
S-(-)-limonene	1199	0.68	1.4	7	14	34	68
Linalool	1550	0.77	1.5	8	15	39	77
Phenylacetaldehyde	1641	0.6	1.2	6	12	30	60
Salicylaldehyde	1679	0.61	1.2	6	12	31	61
Methyl 2-methoxybenzoate	2071	0.83	1.7	8	17	42	83
(Z)-3-hexen-1-ol	1384	5	5	5	5	5	5

The peak height data from the FID and EAG signals was collected using EZChrom and then exported to a MS Excel spreadsheet containing formulae to

extract the peak heights of the EAG responses as described in Section 2.4.4.2.

The EAG peak heights were normalised to the internal standard (5 ng of (*Z*)-3-hexen-1-ol)) to give standardised EAG values (sEAG).

The responses of virgin and gravid female *H. armigera* to the internal standard ((Z)-3-hexen-1-ol) were analysed by linear mixed-effect model with moth ID number as error term to account for the same moth being tested several times (psuedoreplication). EAG response was calculated relative to the peak area of (*Z*)-3-hexen-1-ol on the GC trace giving RPH (relative peak height).

5.3 RESULTS

5.3.1 EAG responses of *A. gamma* to floral volatiles (Methods A and B)

5.3.1.1 <u>Dose-response test</u>

The effects of dose on the standardised EAG response (sEAG) of *A. gamma* to phenylacetaldehyde and methyl salicylate are shown in Figure 5.1 and Figure 5.2 respectively. No significant difference in sEAG response was found between the sexes nor between the two compounds (data not shown). A significant log linear relationship between dose and sEAG response was found (R^2 for phenylacetaldehyde = 0.21, and for methyl salicylate = 0.38, $P < 0.001$).
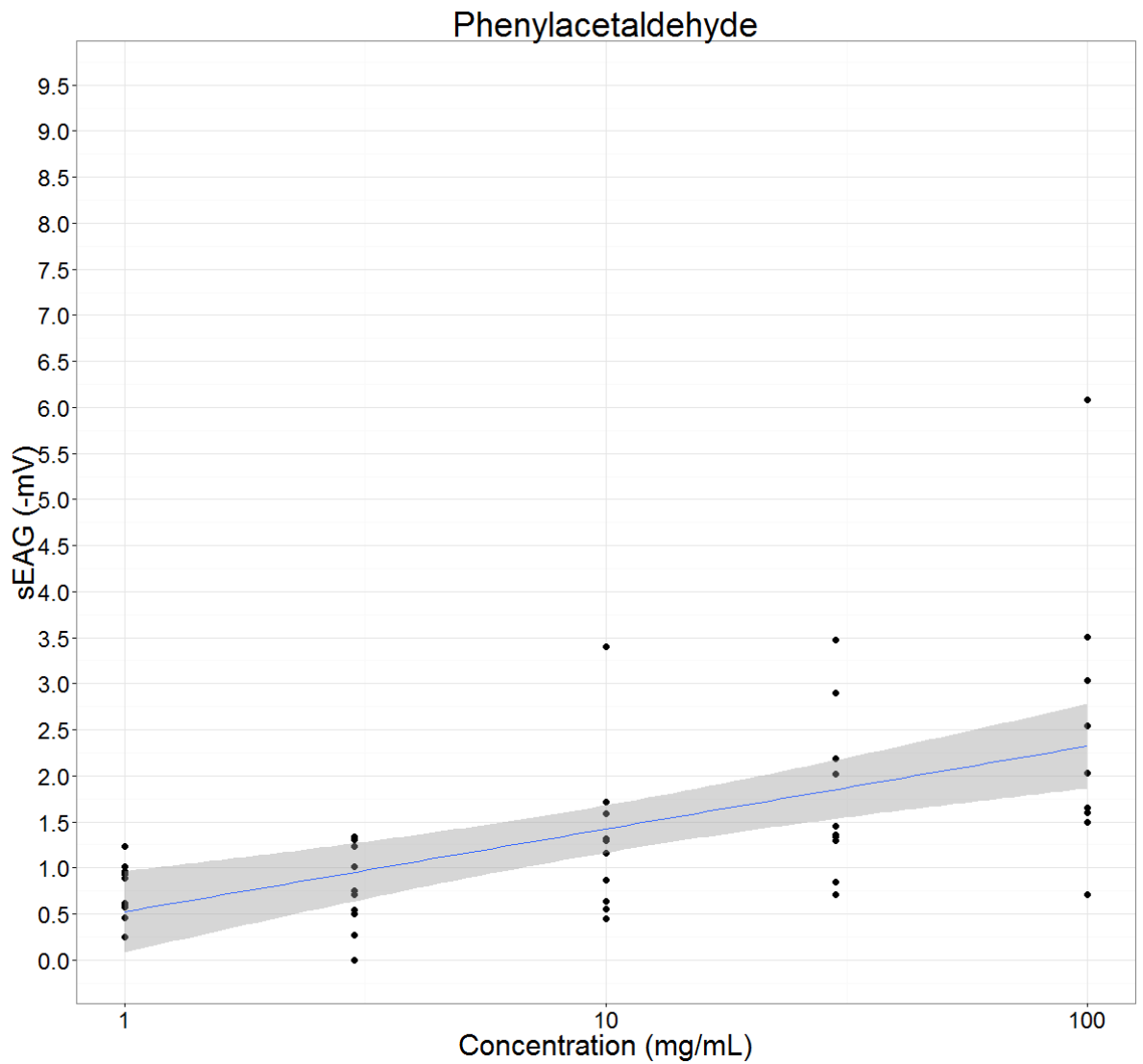
Figure 5.1: Scatterplot of the sEAG responses of *A. gamma* to a range of doses of phenylacetaldehyde. Linear regression line and 95% confidence intervals shown. $R^2 = 0.21$, *P* < 0.001.
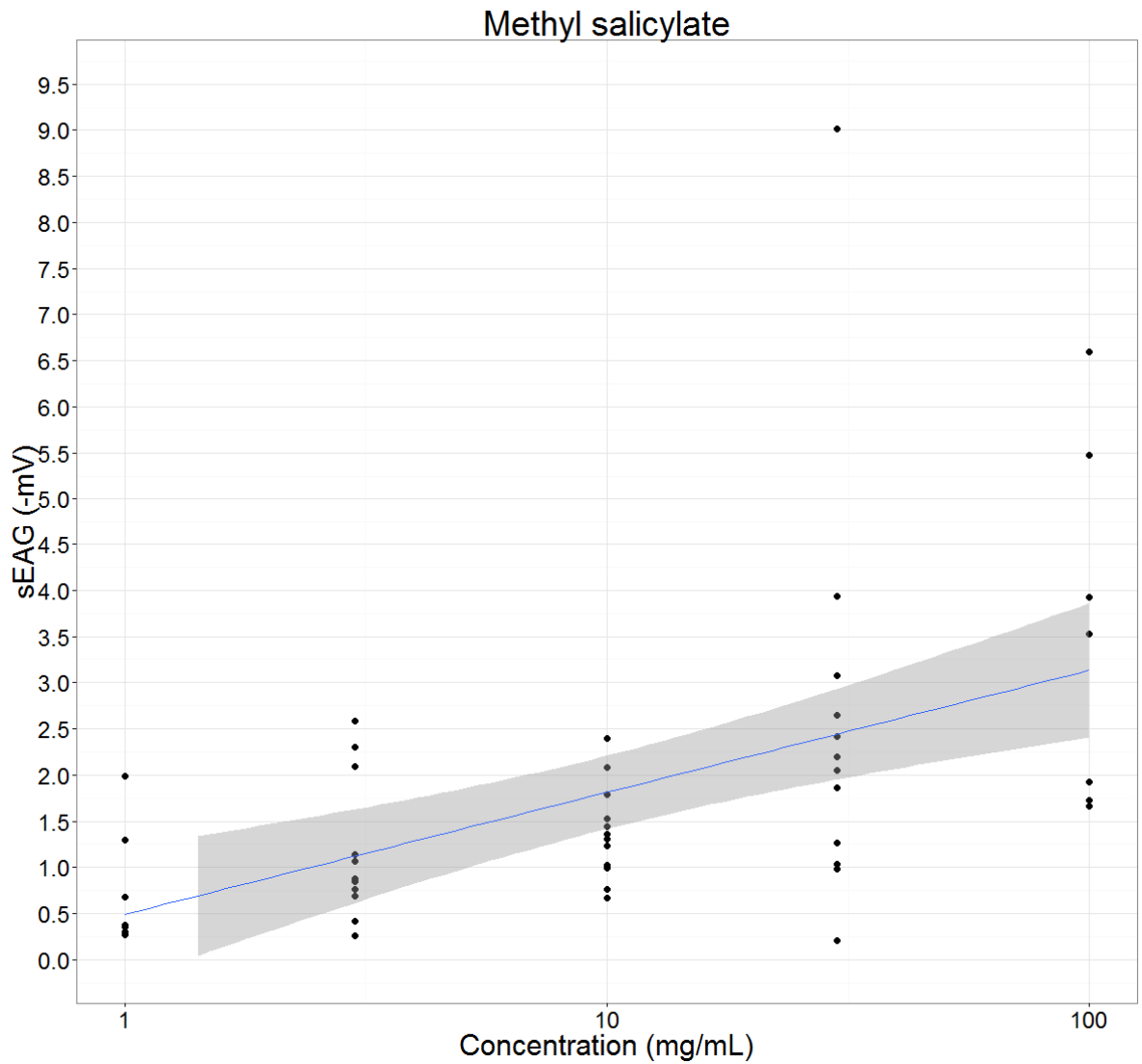
Figure 5.2: Scatterplot of the sEAG responses of *A. gamma* to a range of doses of methyl salicylate. Linear regression line and 95% confidence intervals shown. $R^2 = 0.38$, *P* < 0.001.

5.3.1.2 EAG Responses to candidate attractants

The standardised EAG values (sEAG) of *A. gamma* to candidate attractants delivered by Method B are shown in Figure 46.
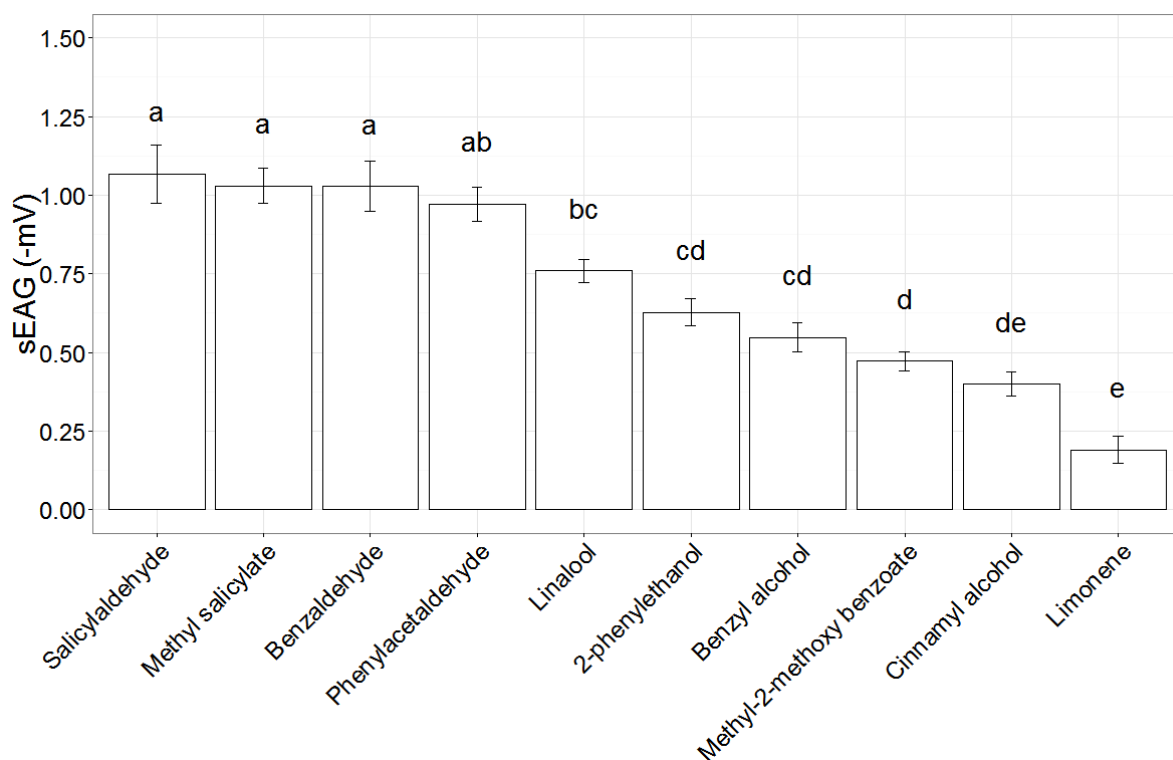
Figure 5.3: Mean sEAG responses of *A. gamma* to ten floral volatiles at 2.5 mM. EAG response is relative to a standard (10 µL of phenylacetaldehyde at 2.5 mM). Salicylaldehyde (N=11), methyl salicylate (N=12), benzaldehyde (N=11), phenylacetaldehyde (N=11), linalool (N=11), 2-phenylethanol (N=11), benzyl alcohol (N=10), methyl-2-methoxy benzoate (N=9), cinnamyl alcohol (N=11), limonene (N=10). Letters denote significant differences (data analysed by ANOVA with TukeyHSD, *P* < 0.001). Error bars show SE mean.

All of the compounds elicited EAG responses from male and female *A. gamma* (Figure 5.3). Significant differences were found between the compounds tested (ANOVA, df = 9, *F* = 29.42, *P* < 0.001) but not between the sexes (ANOVA, df. = 1, *F* = 0.82, *P* > 0.05). Salicylaldehyde, methyl salicylate, benzaldehyde, and phenylacetaldehyde elicited the highest responses, and cinnamyl alcohol and limonene the lowest responses.

The sEAG responses were plotted against predicted vapour pressure of the compounds tested (Figure 5.4) and a quadratic regression applied, giving an R^2 of 0.51, and *P* < 0.01.
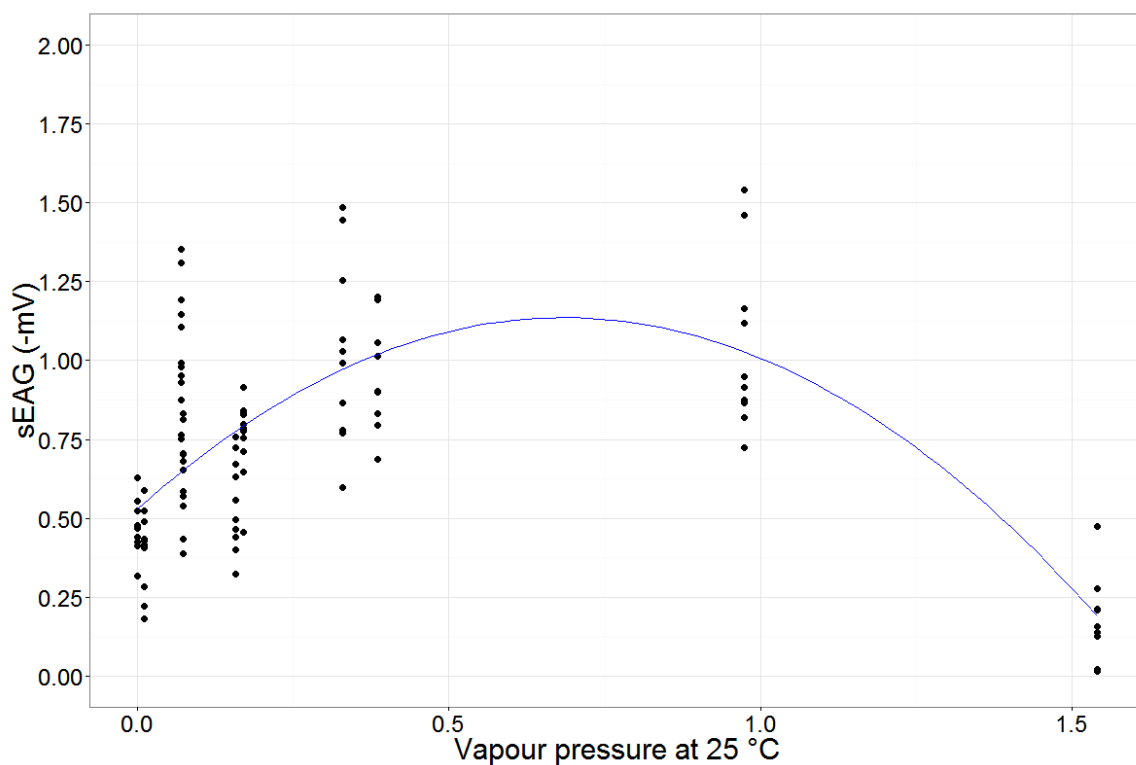
Figure 5.4: sEAG responses of *A. gamma* to a range of compounds with difference vapour pressures. Analysed by quadratic regression (y = 0.53 + 1.77x - 1.29x2, R^2 = 0.51, d.f. = 105, F = 14.41, P < 0.01).

The sEAG responses of *A. gamma* and the vapour pressure of the compounds tested in the experiment investigating ten floral volatiles do not show a linear correlation (R^2 = 0.03, *P* > 0.05), but do show a non-linear, quadratic relationship (y = 0.53 + 1.77x - 1.29x^2, R^2 = 0.51, d.f. = 105, *F* = 14.41, *P* < 0.01).

5.3.2 EAG responses of *H. gelotopoeon* to floral volatiles (EAG Method C)

5.3.2.1 The responses of males and females towards the internal standard

No significant difference was found between the responses of males and females to the internal standard of (*Z*)-3-hexenol (data analysed using a two

sample t-test: t = -0.54, d.f. = 224, *P* > 0.05; mean for females = 1.04 relative peak height (RPH), mean for males = 1.1 RPH) (Figure 5.5).
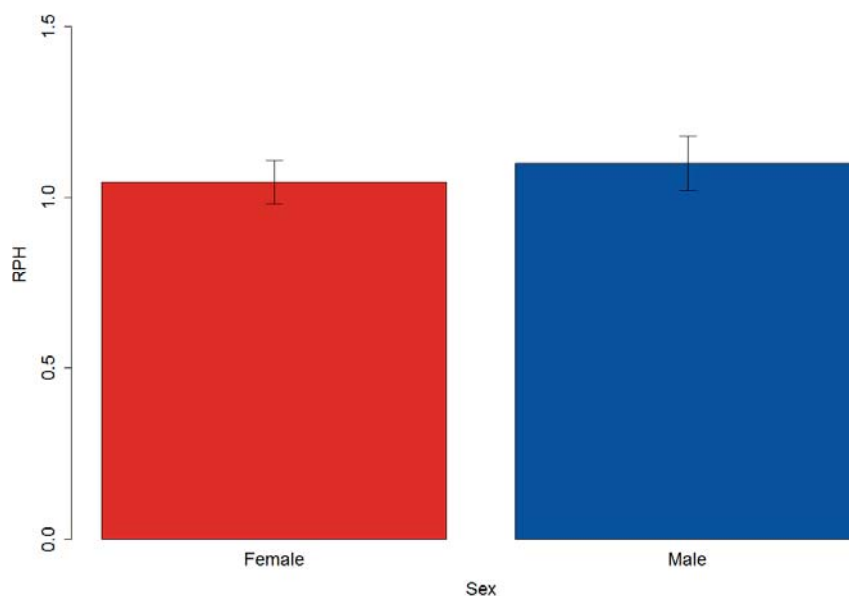

Figure 5.5: Relative peak heights of female and male *H. gelotopoeon* to the internal standard with ±SE mean. RPH is the EAG peak height relative to the peak area of the internal standard (5 ng of (*Z*)-3-hexen-1-ol). No significant difference between the variables (two sample t-test: t = -0.54, d.f. = 224, *P* > 0.05).

5.3.2.2 <u>Comparison of male and female sEAG responses to compounds</u>

Analysis of the data by mixed effect linear model found that the 3-way interaction including compound:sex:log(dose) was a significant factor in sEAG response (N = 35, $F_{30,4}$ = 51.37, *P* < 0.001). However, 'sex' alone was not a significant factor, and the model with the lowest AIC (Akaike information criterion) had only compound, log(dose), and the interaction compound:log(dose) as significant factors.

Further analysis on the effect of sex was carried out by separating the data by dose. At each dose the data were analysed by linear mixed effect model. For

almost every dose sex was found not have a significant effect on the sEAG (Figure 5.6), but the compound tested did have a significant effect for every dose ($P < 0.0001$, d.f. = 14) (Figure 5.7). Pairwise comparisons between the compounds at each dose were carried out using the Multcomp package available for R (Hothorn *et al.*, 2008).
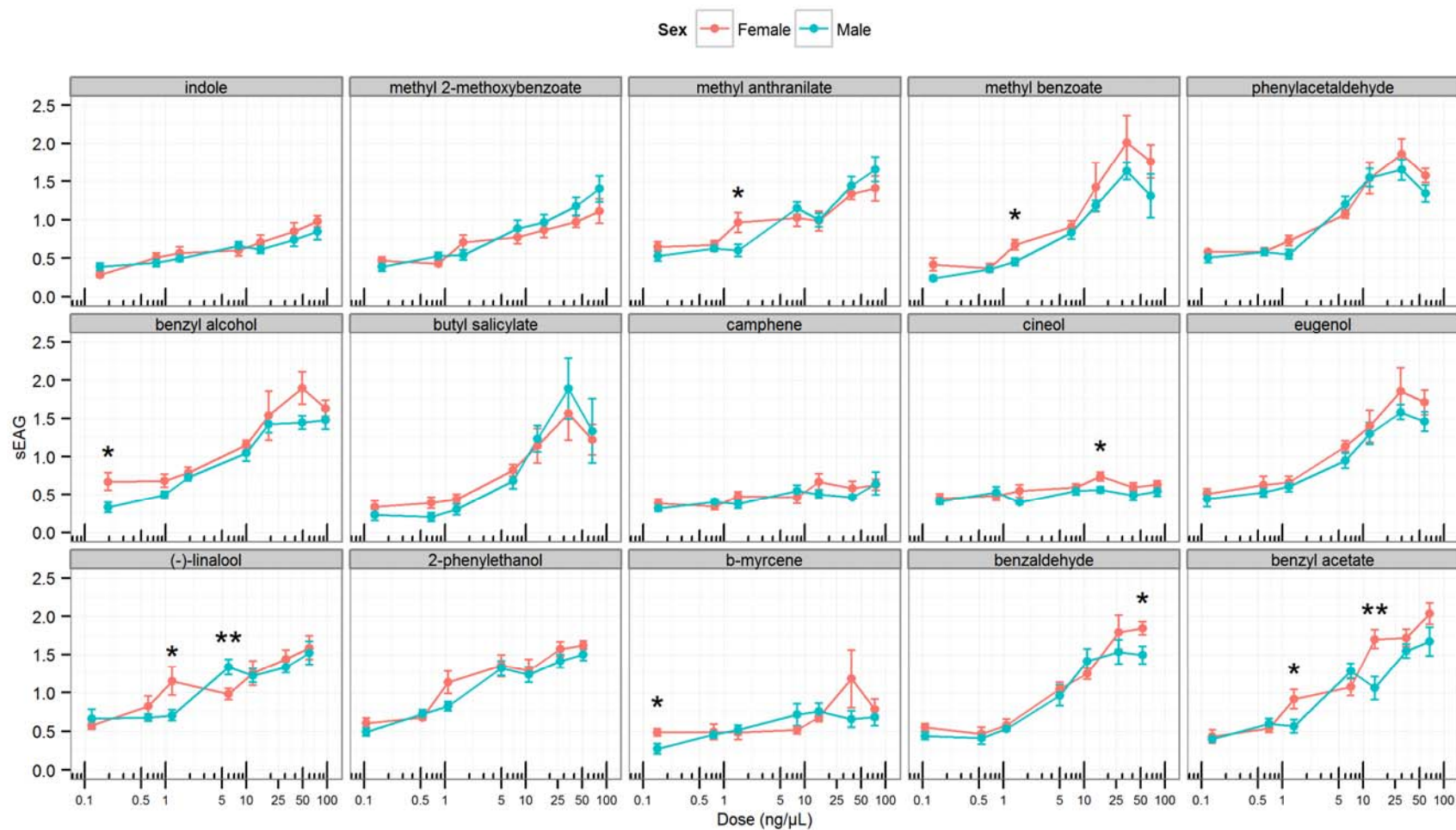
Figure 5.6: sEAG responses relative to an internal standard of male and female *Helicoverpa gelotopoeon* in response to doses of test compounds. Significant differences between males and females indicated by asterisks (t-test, * = $P < 0.05$, ** = $P < 0.001$). Error bars indicate standard error; N = 5 to 12. The insect's antenna received 1 µL of each test solution.

Carrying out t-tests comparing the responses of males and females for each compound at each dose revealed some significant differences as shown in Figure 5.6. The significant results of these t-tests are presented in Table 5.4.

Table 5.4: summary of t-tests showing significant differences between mean sEAG responses of male and female *Helicoverpa gelotopoeon* for compounds at specific concentrations.

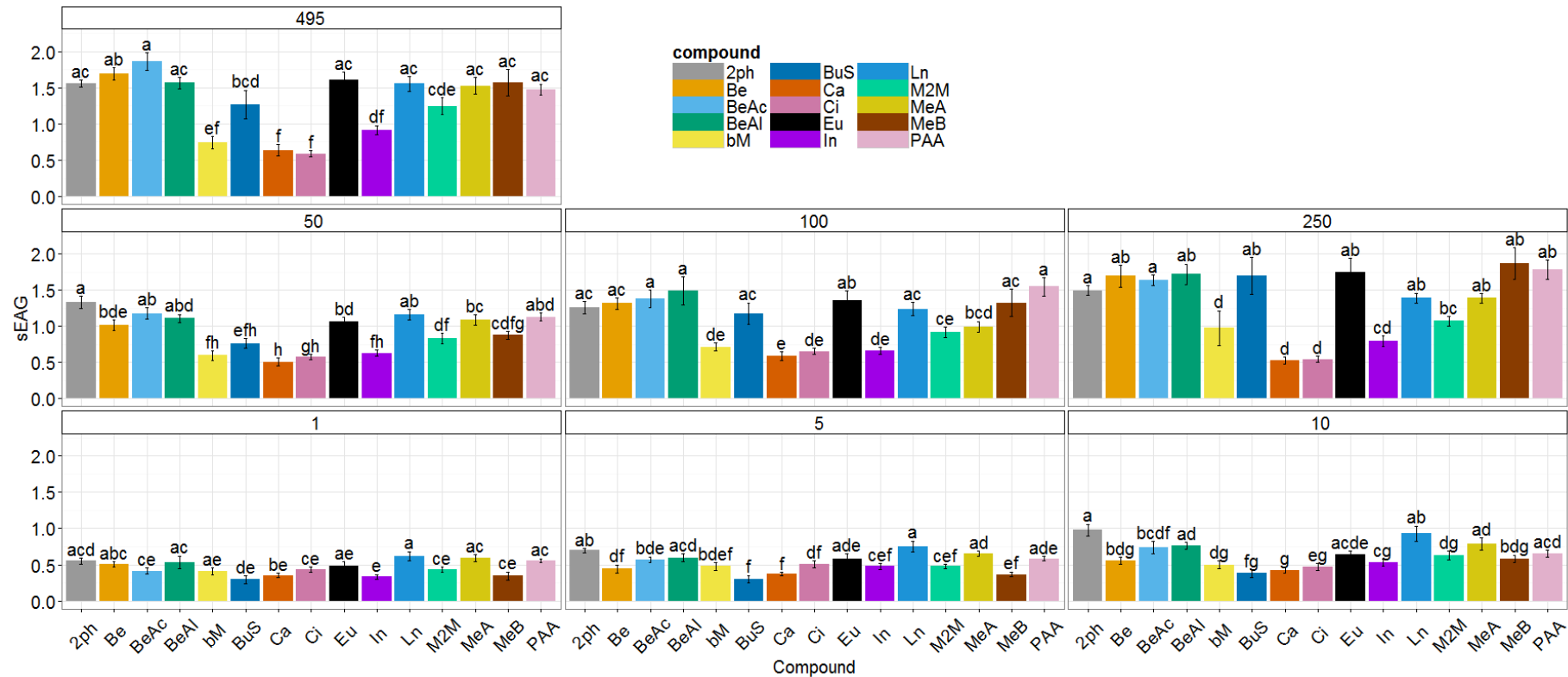| Compound (concentration)	sEAG (female)	sEAG (male)	T	df	$P <$
(-)-linalool (10 µM)	1.149	0.706	2.26	11.64	0.05 *
(-)-linalool (50 µM)	0.982	1.333	-2.87	20.62	0.01 **
benzaldehyde (495 µM)	1.843	1.488	2.51	8.03	0.05 *
benzyl acetate (10 µM)	0.914	0.564	2.29	16.07	0.05 *
benzyl acetate (100 µM)	1.699	1.058	3.34	17.31	0.01 **
benzyl alcohol (1 µM)	0.672	0.331	2.59	11.80	0.05 *
β-myrcene (1 µM)	0.484	0.271	2.67	7.78	0.05 *
cineol (100 µM)	0.740	0.566	2.51	16.01	0.05 *
methyl anthranilate (10 µM)	0.964	0.600	2.39	14.79	0.05 *
methyl benzoate (10 µM)	0.675	0.455	2.65	11.79	0.05 *

Figure 5.7: Mean sEAG from *Helicoverpa gelotopoeon* (males and females pooled) for each compound at each dose with standard errors. Chart titles show concentration (μM). Letter codes indicate significant differences between compounds within columns (linear mixed-effect model, df = 14, residual df = 20, *P* < 0.001 for all doses, followed by Tukey's pairwise comparison). Compound key: 2ph = 2-phenylethanol, Be = benzaldehyde, BeAc = benzyl acetate, BeAl = benzyl alcohol, bM = β-myrcene, BuS = Butyl salicylate, Ca = Camphene, Ci = Cineol, Eu = Eugenol, In = Indole, Ln = (-)-linalool, M2M = Methyl 2-methoxybenzoate, MeA = Methyl anthranilate, MeB = Methyl benzoate, PAA = Phenylacetaldehyde.

5.3.2.3 Pooling the data for all compounds and both sexes

Pooling all data across all of the compounds tested and comparing the sEAG responses to the dose (µM) on a log scale gave a sigmoid shaped curve (Figure 5.8).
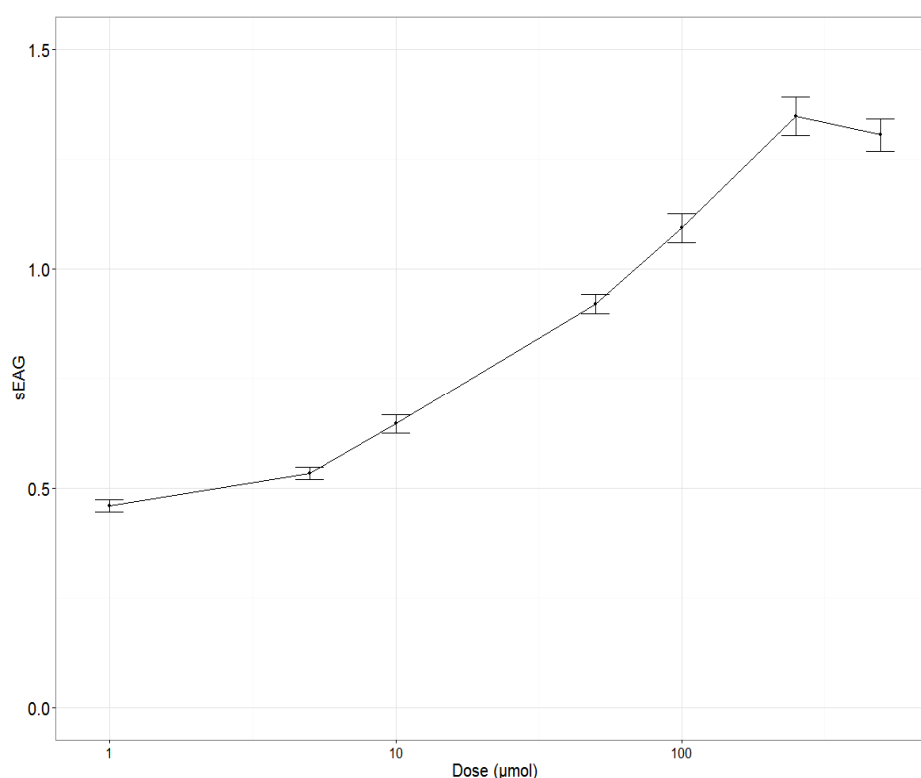

Figure 5.8: sEAG responses to all doses for all moths tested with all compounds. Error bars show standard error, N = 220 to 311 for each dose.

5.3.2.4 Analysis of the linear portion of the dose response

Overall the data showed that the sEAG response was a sigmoid curve (Figure 5.8) using a log scale on the abscissa. Between the doses of 5 - 250 µM the response was relatively linear, therefore to investigate the effect of dose, compound and sex on sEAG a mixed effect linear model was used between these doses. The analysis found that sex was not a significant factor, but that dose (logged), compound, and the interaction between compound and dose (logged) was significant (Table 5.5).

Table 5.5: analysis of variance table of the effect of compound and dose on the sEAG of *H. gelotopoeon* tested with a variety of floral volatiles at concentrations between 5 - 250 μM.

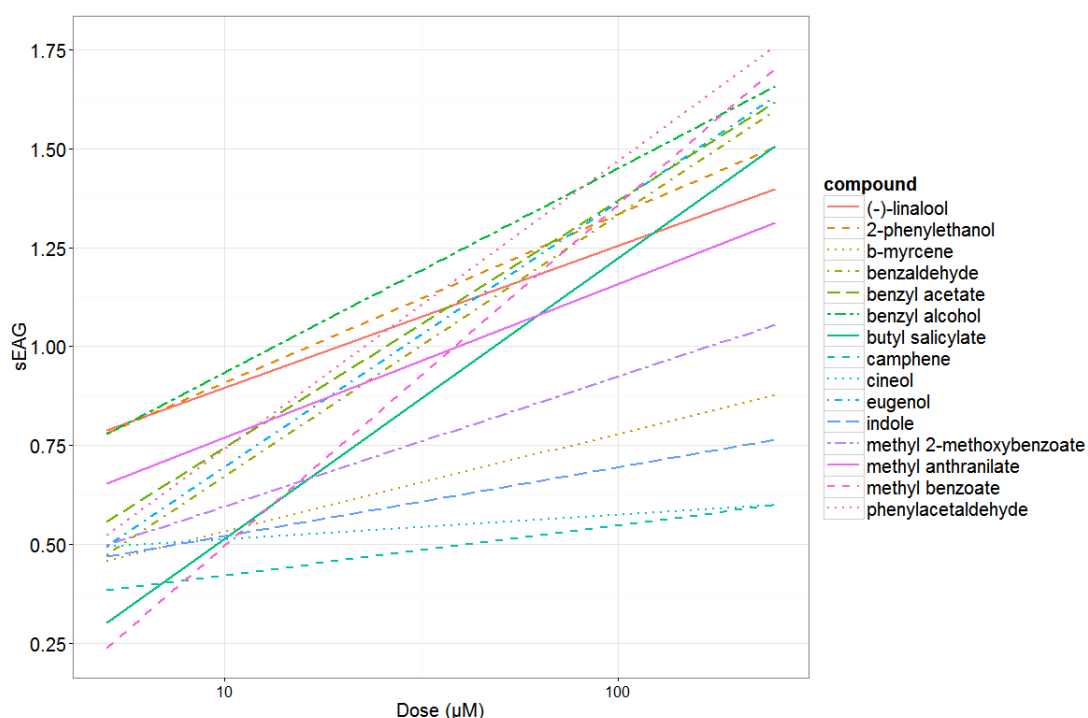|	Df	Sum of squares	Mean square	F	P
Compound	14	70.3	5.0	42.3	< 0.001
Log(conc.)	1	103.8	103.8	875.0	< 0.001
Compound:log(conc.)	14	31.3	2.2	18.9	< 0.001
Residual	2				

Figure 5.9: Slopes of sEAG responses of *Helicoverpa gelotopoeon* to candidate attractants plotted against (log) dose between 5 and 250 μM. Linear model analysis found that compound, log(dose), and the interaction compound:log(dose) were significant factors (*P* < 0.001).

The graph in Figure 5.9 highlights several types of compounds:

(i) Compounds that elicit a relatively low response to low doses and high doses:

cineol, indol, camphene, and β-myrcene.

(ii) Compounds that elicit relatively low responses to low doses and relatively

high responses to high doses (i.e. steep slopes):

methyl benzoate, and butyl salicylate.

(iii) Compound that elicit relatively high response to low doses but moderate response to high doses:

methyl 2-methoxybenzoate

(iv) Compounds that elicit relatively high responses to low and high doses:

benzaldehyde, eugenol, phenylacetaldehyde, benzyl acetate, methyl anthranilate, (-)-linalool, 2-phenylethanol, and benzyl alcohol.

5.3.3 EAG responses of gravid and virgin female *H. armigera* to the UoG blend of chemicals (Method C)

5.3.3.1 <u>Responses to the internal standard</u>

The responses of virgin and gravid female *H. armigera* to the internal standard ((Z)-3-hexen-1-ol) were analysed by linear mixed-effect model with moth ID number as a random effect to account for the same moth being tested several times. The mean relative peak height (RPH) for males and females is shown in Figure 5.10. The mean RPH to (Z)-3-hexen-1-ol was slightly lower for non-gravid moths than for gravid moths but this difference was not significant.
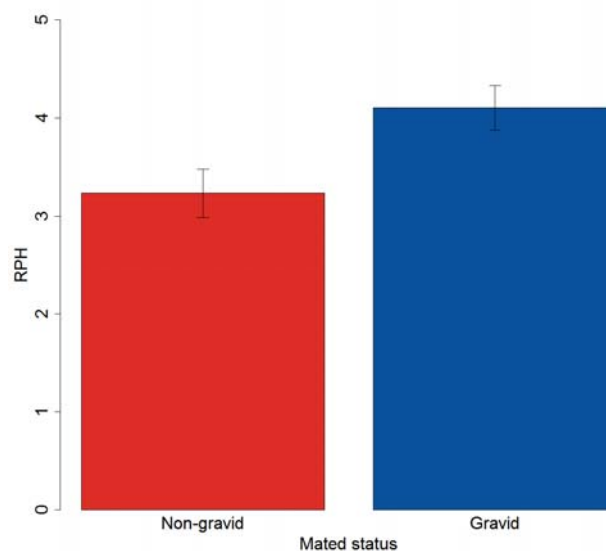
Figure 5.10: Mean sEAG responses to the internal standard (Z)-3-hexen-1-ol (at 50 μM) relative to the peak area of the (Z)-3-hexen-1-ol measured by the GC (RPH) for gravid and non-gravid female *Helicoverpa armigera*. Error bars indicate standard error. No significant difference was found between the means. The data was analysed using a linear mixed-effect model (to account for repeated measures); N = 53 (non-gravid) 55 (gravid), $F_{(1,16)}$ = 1.2, P > 0.05.

5.3.3.2 Responses to the test compounds

(i) t-tests

The insects' responses to the test compounds at each dose was analysed by two sampled t-tests (Figure 5.11). For all compounds (except methyl 2-methoxybenzoate) the responses of non-gravid moths were higher than for gravid moths, but only in two cases out of 30 was this difference significant: phenylacetaldehyde at 500 μM, and (-)-R-limonene at 500 μM); in one instance the sEAG responses for gravid moths was significantly higher than for non-gravid moths (P < 0.05 for salicylaldehyde at 5 μM).
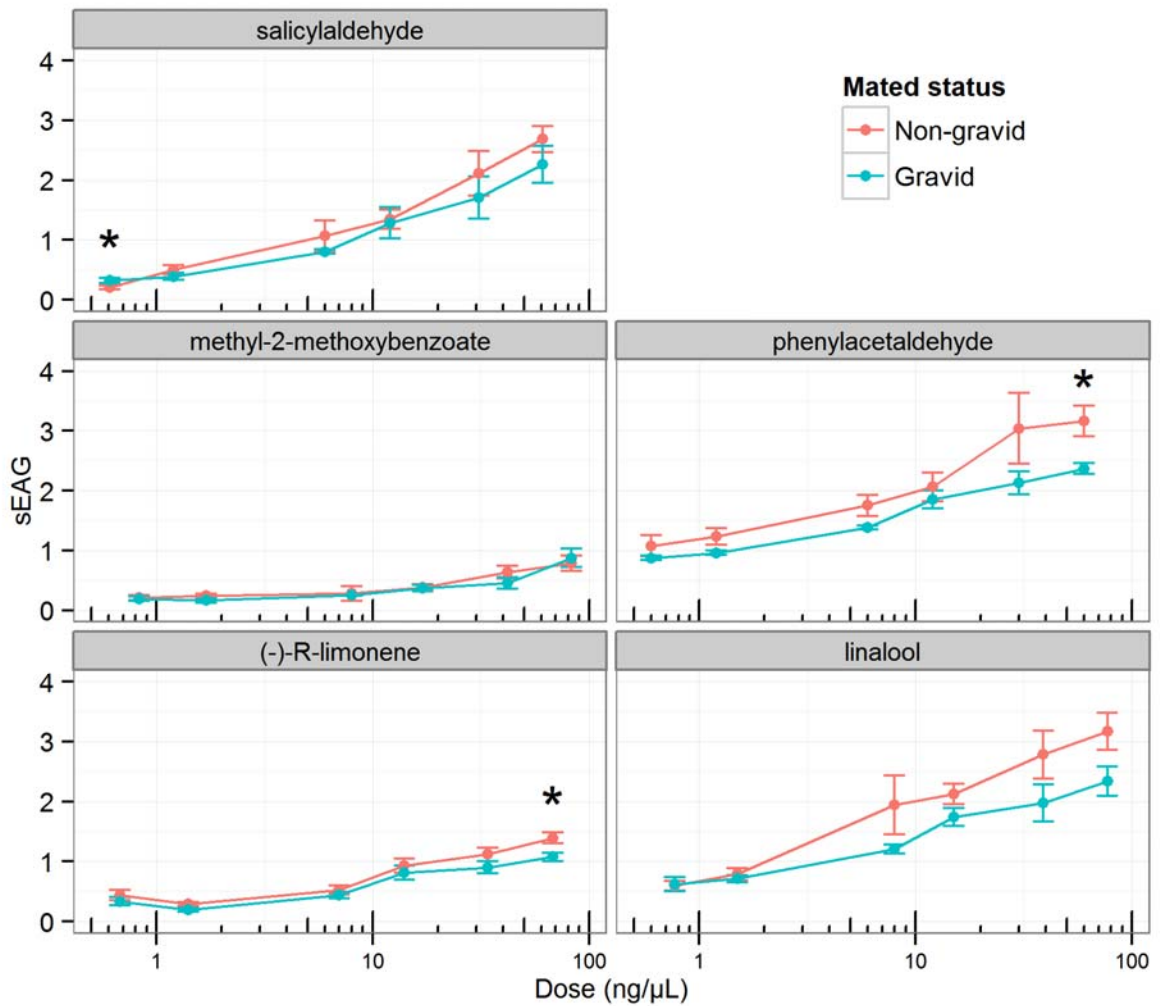
Figure 5.11: Mean sEAG of gravid and non-gravid (virgin) female *H. armigera* to the five compounds in the UoG blend at a range of doses. Asterisks indicate significant differences between the treatments (P < 0.05). Error bars show standard error.

(ii) Mixed effect linear model

The sEAG data was normalised by log transformation (+0.01 to remove zeros) and tested against the factors. Analysis found that 'log dose' and 'compound' had a significant effect on the 'log sEAG', but 'mated-status' did not. Further analysis was carried out at each dose and 'compound' was found to have a significant effect on the sEAG ($P < 0.001$) and followed up with pairwise comparisons (TukeysHSD) (Table 5.6).

Table 5.6: Mean sEAG of *H. armigera* to compounds at each dose with standard errors. Letter subscript codes denote significant differences between the compounds within each dose.

Compound	5 µmol		10 µmol		50 µmol		100 µmol		250 µmol		500 µmol	
	Mean sEAG	± SE mean	Mean sEAG	± SE mean	Mean sEAG	± SE mean	Mean sEAG	± SE mean	Mean sEAG	± SE mean	Mean sEAG	± SE mean
Phenylacetaldehyde	0.98a	0.09	1.1a	0.08	1.57a	0.1	1.94a	0.13	2.56a	0.31	2.74a	0.16
Linalool	0.61b	0.07	0.75b	0.06	1.58a	0.26	1.91a	0.12	2.36ab	0.26	2.73a	0.22
Salicylaldehyde	0.26c	0.03	0.45c	0.05	0.94b	0.13	1.31b	0.16	1.90b	0.25	2.46a	0.19
(-)-R-limonene	0.39c	0.06	0.24d	0.03	0.48c	0.04	0.86c	0.08	1.00c	0.08	1.23b	0.07
methyl-2-methoxybenzoate	0.2c	0.03	0.2d	0.03	0.27c	0.06	0.38d	0.04	0.54c	0.07	0.83c	0.1

From the linear model intercepts and slopes were calculated for each compound and plotted against sEAG (Figure 5.12).
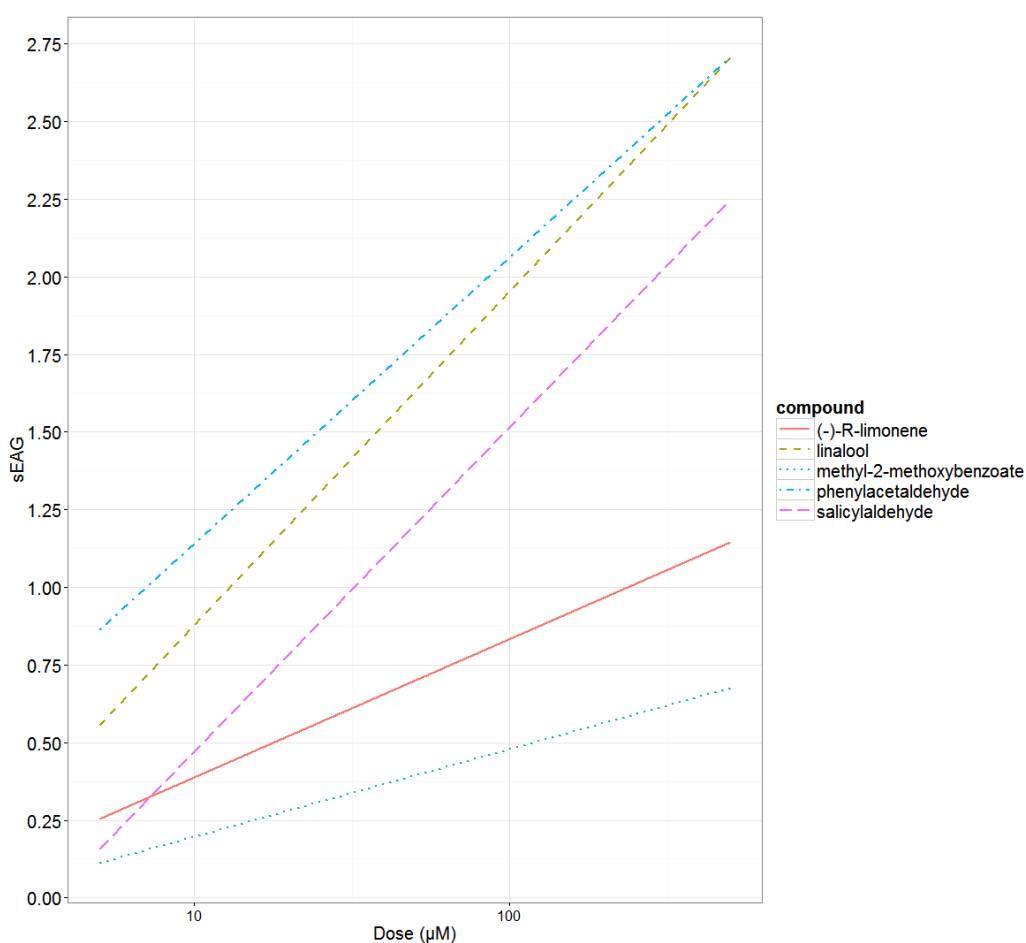

Figure 5.12: Slopes of sEAG responses of *Helicoverpa armigera* to five components of UoG blend plotted against (log) dose between 5 and 250 μM. Linear model analysis found that compound, log(dose), and the interaction compound:log(dose) were significant factors ($P < 0.001$).

The greatest sEAG responses were to phenylacetaldehyde, salicylaldehyde, and linalool at the highest does. The weakest EAG responses were to methyl 2-methoxybenzoate, R-limonene, and salicyaldehyde at the lowest does. Salicylaldehyde gave the steepest slope and methyl 2-methoxybenzoate gave the shallowest slope. The slopes can be grouped into two groups: limonene and methyl 2-methoxybenzoate in one group, and salicylaldehyde, linalool, and

phenylacetaldehyde in the other group. The former group characterised by low responses overall and the latter group characterised by high sEAG responses overall.

5.4 DISCUSSION

The EAG responses of three noctuid species to a selection of floral volatiles were assessed in order to identify differences between the sexes, dose of the compound, and mated status of the insect. Three different odour delivery systems were used to address the problems of the previous system as discussed in section 2.6.2.

5.4.1 EAG responses of *A. gamma* to floral volatiles

A. gamma responded to all of the chemicals tested. The factors that were found to have a significant effect on the sEAG responses were the chemical tested and the dose of either phenylacetaldehyde and methyl salicylate (page 164). No significant differences were found between the sexes. This is consistent with electroantennography work carried out by Plepys *et al.* (2002a) who tested the antennae of *A. gamma* to a range of plant compounds identified from *Cirsium arvense* (L.) (Asteraceae), *Platanthera bifolia* (L.) (Orchidaceae), *Saponaria officinalis* (L.) (Caryophyllaceae), *Centaurea scabiosa* (L.) (Asteraceae), *Trifolium pratense* (L.) (Fabaceae), and *Nepeta faasseni* (Bergmans) (Lamiaceae), of which some were tested in the current experiment including: benzaldehyde, benzyl alcohol, 2-phenylethanol, cinnamyl alcohol, methyl salicylate, (±)-linalool, and methyl-2-methoxybenzoate. Plepys *et al.* found no significant differences between male and female *A. gamma* antennal responses, but significant differences between compounds tested.

With the exception of linalool, all the compounds tested contain aromatic rings. Those that also contained an aldehyde group elicited higher responses than the alcohol and methyl containing compounds at the dose tested. In particular, benzaldehyde elicited a significantly greater EAG response than benzyl alcohol, and methyl salicylate elicited a significantly greater response than methyl 2-methoxybenzoate. Between benzaldehyde and benzyl alcohol the only structural difference is the functional group at the end of the carbon chain; between methyl salicylate and methyl-2-methoxybenzoate the structural difference is that the phenolic group is replaced with a methoxy group.

Exactly how structural variation may cause differences in EAG response is unknown. Previous work has also discussed the importance of specific chemical groups and structure on EAG response (e.g. Burguiere *et al.*, 2001; Fraser *et al.*, 2003) and in both cases surmised that chemical groups and carbon chain length do play an important role in olfactory stimulation irrespective of other factors such as volatility.

The method used (Method A, see page 72) for this experiment relied on the test compounds volatilising from a filter paper. The quantity of each compound that reached the insects' antenna (or dose) would be influenced by the volatility of each compound. For this reason sEAG response was also compared to vapour pressure of the compounds at 25 °C (Figure 5.4). The size of an EAG response may be related to the strength of the stimuli (Venard and Pichon, 1981; Venard and Pichon, 1984; Hartlieb and Rembold, 1996; Riffell *et al.*, 2008a). Maekawa *et al.* (1999), found a strong correlation (R^2 = 0.9) between the volatility of compounds and EAG response when investigating the responses of the soybean beetle, *Anomala rufocuprea* (Motschulsky) (Scarabaeidae), and

surmised that this correlation was related to the number of molecules hitting generalist olfactory neurons, which in turn was related to the volatility of the compounds they were testing. Standard relative vapour pressures have been suggested as a means for estimating (or correcting) the amount of a compound that has volatilised from a substrate when carrying out EAG experiments (Bengtsson *et al.*, 1990).

Using the methods in these experiments (methods A and B) the number of molecules of the test chemical on the filter paper was controlled (e.g. 10 μL at 2.5 mM), the air flow was the same throughout, exposure time was kept as constant as possible, but the vapour pressure varied between the compounds. These different vapour pressures will influence the dose of the stimulus that the antenna receives and may have an effect on the sEAG response. The results from this experiment (Figure 5.4) show that volatility did not have a linear correlation with the mean EAG responses but there was evidence of a non-linear, quadratic correlation. It is possible that compounds with low volatility produce small EAG responses because there are fewer molecules contacting the insect's antenna; as volatility increases, so does the number of molecules contacting the antenna and consequently the EAG response increases - up to a maximum. After this point the high volatility of the compound causes so much to be lost before the experiment has taken place very few molecules remain to stimulate the antenna and a very small EAG response is seen. For example, the compound with the highest vapour pressure was limonene, which elicited relatively low sEAG responses. Using this EAG method B allows for some of the test chemical to evaporate from the filter paper before it is placed into the Pasteur pipette. It is therefore possible that the low sEAG response was due to a reduced dose as limonene molecules may have been lost to the environment

before they were blown over the insects' antennae. Using a GC as a delivery mechanism in EAG method C with *H. gelotopoeon* found no quadratic correlation of EAG response to vapour pressure, and only a very weakly linear correlation (R^2 = 0.20, *P* < 0.001) (data not shown). As later experiments showed that dose is a significant predictor in EAG response, and dose <u>may</u> be impacted by vapour pressure when using EAG methods A and B, all subsequent EAG experiments were carried out using method C which should minimise the influence of volatility on the dose of the chemicals the antennae receive.

5.4.1.1 <u>Selecting compounds for testing in the field</u>

Large EAG responses to specific compounds indicate that the insect's antenna has a high number of ORNs tuned to that compound. This suggests the detection of the compound is important to the insect, and consequently the compound may give rise to a behavioural response. Pheromone compounds elicit extrememly large EAG responses in male noctuids and give rise to strong behavioural responses. Floral compounds such as phenylacetaldehyde have shown similar (but less pronounced) effects in male and female noctuids. However, previous research has also demonstrated the opposite. For example, the antennae of the cabbage moth, *M. brassicae*, was shown to produce large EAG responses to 4-pentenyl isothiocyanate and benzyl isothiocyanate but in wind tunnel bioassays these compounds triggered almost zero response, whereas allyl isothiocyanate evoked relatively low EAG responses but triggered over 30% of female *M. brassicae* to respond in upwind flight (Rojas, 1999a). With this is in mind, potentially any compound that the insect can detect may trigger a behavioural response. All of the compounds tested elicited EAG

responses in *A. gamma*, and therefore were tested in field trials (in Chapter 6) to try to improve the attractiveness of the UoG blend to this and other noctuid species.

5.4.2 EAG responses of *H. gelotopoeon* to floral volatiles

5.4.2.1 Responses of males and females to the internal standard

If male and female *H. gelotopoeon* had responded differently towards the internal standard this would skew all of the subsequent sEAG results. Therefore the responses of males and females to the internal standard was tested statistically and no significant was found. The internal standard used was (*Z*)-3-hexen-1-ol. The results reported by Li *et al.* (2005) showed that the mean EAG responses of virgin male and female *H. armigera* to (*Z*)-3-hexen-1-ol was 0.81 (±0.32 SE) and 1.76 (±0.32 SE) respectively. It is therefore perhaps surprising that the similar species *H. gelotopoeon* exhibited no such significant difference in the responses of males and females to this compound.

5.4.2.2 Responses of male and female *H. gelotopoeon* to floral volatiles

Some significant differences were found between the sEAG responses of male and female *H. gelotopoeon* (Figure 5.6). For all of these differences the responses of females were significantly larger than for males (except the dose of 8 ng of (-)-linalool in which males showed a larger response). Previous studies have found that the antennae of female Lepidoptera produce larger responses to floral volatiles than males, for example Rojas (1999a) found that the antennae of female *M. brassicae* elicited a significantly greater EAG response to a range of doses to the floral volatile caryophyllene, but not to other floral and green leaf volatiles. Significantly larger EAG responses were also

found for female *H. virescens*, compared to males, towards a range of doses of several floral volatiles (benzaldehyde, benzyl alcohol, 2-phenylethanol, phenylacetaldehyde, and phenylacetylene) (Hillier *et al.*, 2006). The results seen in the current experiment corroborate those of Raguso *et al.* (1996) who also found some large differences between male and female *Hyles lineata* (Fabricius) (Sphingidae) antennae to specific floral volatiles (females responses were usually higher) but did not find that sex was a significant factor to EAG response in their statistical analyses. The authors proposed three theories as to how female's antennae may be more sensitive: (1) sexual diamorphism in the populations of ORNs; (2) differences in olfactory physiology; (3) altered sensitivity due to prior experience before the insects were captured. In the current study, the third theory may be ruled out as the insects used were not exposed to floral odours until the experiment started. Theories 1 and 2 may explain the differences seen between the EAG responses of males and females to floral odours. Sexual diamorphism in the populations of ORNs on Lepidopteran antennae has been clearly demonstrated many times in studies of the EAG responses of males and females to their species' sex-pheromone. The antennae of males contain distinct sensilla trichodea tuned to sex-pheromone. Whereas the female antenna does not contain these specialised type of sensilla trichodea (Franco *et al.*, 2007; Gu *et al.*, 2009), and as host plant detection is more important to females they have greater sensitivity to these odours. Differences in the olfactory physiology has also been found and is discussed in more detail in the Section 1.1.2.2 - Olfaction in adult Lepidoptera.

5.4.2.3 <u>Pooling the data for all compounds and sex</u>

Pooling all the compounds and sex together showed an sEAG response to the doses tested with a characteristic sigmoid shaped curve (with dose on a log-scale) (Figure 5.8). Many of the individual compounds also resulted in a sigmoid-shaped sEAG response (Figure 5.6). This indicates that the antennae were reaching saturation point for those compounds - as the stimulus reaches a certain threshold the EAG responses plateaux due to saturation of the ORNs. The concentrations at which the insect is most able to discriminate odours are found within the linear portion of the sigmoid curve (Schoonhoven *et al.*, 2005b) whereas at extremely low concentrations the ability of insects to differentiate between odours may decrease (Wright *et al.*, 2005) and at very high concentrations ORNs that would not normally be triggered for that particular compound may do so (Hartlieb *et al.*, 1997; Smith *et al.*, 2006). Therefore analyses were carried out for the compounds between the concentraions of 5 to 250 µmol.

5.4.2.4 Analysis of the linear portion of the dose range

The *H. gelotopoeon* antennae responded to all 15 of the floral volatiles (Figure 5.7 and Figure 5.9). The lowest responses were to camphene, cineol, indole, and β-myrcene, which is consistent with previous research on the EAG responses of noctuids to these compounds (Burguiere *et al.*, 2001; Birkett *et al.*, 2006). However, Rojas (1999a) found that cineol elicited moderate EAG responses (similar to that of linalool) in *Mamestra brassicae* (L.) (Noctuidae) and incited a behavioural response (upwind flight and landing on the odour source) in mated females in a wind tunnel.

The responses to methyl benzoate and butyl salicylate showed a steep slope (Figure 5.9) and very high values at 250 µmol (Figure 5.7). Specific RNs for

methyl benzoate have been found in *H. armigera* (Rostelien *et al.*, 2005), moderate EAG responses were seen in *Hyles lineata* (Fabricius) (Sphingidae) (Raguso *et al.*, 1996), and also in *Hadena bicruris* (Hufnagel) (Noctuidae) (Dötterl, 2004). Interestingly, Raguso *et al.* (1996) also investigated the effect of the *ortho*-R group on the EAG response and found no significant differences between methyl benzoate (R = H) and methyl anthranilate (R - NH_2) or methyl 2-methoxybenzoate (R = OCH_3), but a much greater (and statistically significant) EAG response to methyl salicylate (R = OH).

Methyl 2-methoxybenzoate elicited a moderate EAG response at low and high doses (a steady rising slope). Compared to the structurally similar compounds of methyl benzoate and methyl anthranilate, methyl 2-methoxybenzoate elicited similar or slightly higher responses at the low doses, but the peak response at the higher doses was much lower than those to the other two compounds. Even though it was found to elicit relatively low EAG responses in *Helicoverpa spp.* in this research and that of others (Deng *et al.*, 2004), it has been shown to elicit moderate responses in *H. lineata* males, and slightly higher in *H. lineata* females (Raguso *et al.*, 1996).

The compounds that elicited the largest EAG responses were benzaldehyde, eugenol, phenylacetaldehyde, benzyl acetate, methyl anthranilate, (-)-linalool, 2-phenylethanol, and benzyl alcohol. The largest sEAG values were in response to benzyl acetate (at 495 μmol) and methyl benzoate (at 250 μmol) (Figure 5.7). Benzyl acetate has been shown to elicit moderately high EAG responses from other Heliothinae spp. (*H. zea* and *H. virescens*) relative to many other volatile plant compounds (Park *et al.*, 2002). Many of the compounds that elicited high sEAG responses did so at the penultimate highest

dose tested (250 µmol), and the response then decreased slightly for the 450 µmol dose, suggesting that for these compounds the antennae reached saturation point. The quantity of the compounds exiting the GC and passing over the antennae at the highest dose was between 52 and 90 ng (Table 5.2) over approximately 10 s (or between 5.2 and 9 ng / s). This highlights the importance of using doses that are within the range of naturally occurring odour plumes when conducting behaviour or electroantennography experiments as doses that are unnaturally high may trigger abnormal responses.

This experiment was originally designed to test another 15 floral volatiles but unfortunately this became unfeasible due to the colony of *H. gelotopoeon* collapsing several times. The other compounds to be tested included: (+)-linalool, salicyaldehyde and limonene (i.e. the other compounds in the UoG blend), and compounds that would have allowed some analysis into the effect of changing the –R group(s) around the aromatic ring (e.g. methyl salicylate in conjunction with methyl benzoate and methyl anthranilate), or extending the carbon-chain attached to the aromatic ring (e.g. hydrocinnamaldehyde in conjunction with benzaldehyde, and phenylacetaldehyde). In addition, all of the compounds tested on *A. gamma* were intended to be tested on *H. gelotopoeon* to identify any similarities.

5.4.3 EAG responses of *A. gamma*, *H. gelotopoeon*, and *H. armigera*

The three different Noctuid species tested within this chapter exhibited similar responses to the test compounds in some instances. Notably phenylacetaldehyde elicited high responses in all three species; in addition linalool also exhibited high or moderately high responses in all three species. The EAG responses elicited by methyl 2-methoxybenzoate were consistently

low in all three species. Salicylaldehyde triggered high responses in both *H. armigera* and *A. gamma*. These similarities could indicate that for some floral compounds there is an inter-species trend in olfactory responses for these polyphagous Noctuids. Indeed Rostelien *et al.* (2005) found that two Heliothine Noctuids (*Heliothis virescens* and *Helicoverpa armigera*) had similar olfactory systems in terms of neuron specificity toward odours and the co-location of these ORNs within sensilla. Both species had a high number of neurons that were tuned to linalool. Birkett *et al.* (2006) also found similarities between *Busseola fusca* (Fuller) (Noctuidae) and *Chilo partelus* (Swinhoe) (Crambidae) and their EAG responses to linalool, which was identified as one of the key compounds the insects used to locate their hosts (Khan *et al.*, 2000).

Between *H. gelotopoeon* and *A. gamma* several compounds stand out as eliciting high EAG responses in both species, namely: benzaldehyde, phenylacetaldehyde, linalool, and 2-phenylethanol. All of these compounds except phenylacetaldehyde are found in more than 50% of the families of seed plants (Knudsen *et al.*, 2006). The high degree of olfactory sensitivity of the two Noctuids towards these ubiquitousness floral odours will likely assist these generalist nectar feeders in locating this food resource from a wide range of angiosperms.

5.4.4 EAG responses of gravid and non-gravid *H. armigera*

All of the gravid or non-gravid *H. armigera* tested responded to all of the test chemicals at all the doses and the internal standard. This supports the findings from previous experiments in this chapter suggesting that there are similarities in olfactory sensitivity to floral odours between Noctuid species. The results indicate that the insects' antennae are highly sensitive to phenylacetaldehyde at

all doses and (relative to the other compounds tested here) less sensitive to methyl 2-methoxybenzoate.

The mated status of the insects was not found to have a significant effect on the EAG responses to the compounds tested. This result is in agreement with that found by (Rajapakse *et al.*, 2006) in which no differences in EAG responses of mated and virgin *H. armigera* between a range of compounds identified from pigeon pea, including limonene and linalool. Saveer *et al.* (2012) investigated the effects of mated status on the EAG responses of *S. littoralis* and found that there were no significant differences for phenylacetaldehyde but a difference (P < 0.05) for (R)-(-)-linalool at 100 μg (mated insects showed a greater EAG response than virgin insects). Most of the compounds Saveer *et al.* tested were not found to elicit a significant difference at a range of doses, except for nonanal and benzyl methyl ether in which mated females elicited greater responses and for (S)-(+)-linalool in which virgin females elicited greater EAG responses.

Li *et al.* (2005) found large differences in the EAG responses of mated and virgin female *H. armigera* to the aromatic compounds: phenylacetaldehyde, salicylaldehyde, and benzaldehyde. However, the authors did not test for significance and the number of replicates was not stated, so conclusions on the effect of mating cannot be made.

In the current study, although there was a difference in the means of the internal standard ((Z)-3-hexen-1-ol) this was not found to be significant (Figure 5.10). It is important to note the slight difference between the means as it has implications for the rest of the results. As the mean response to (Z)-3-hexen-1-ol was slightly higher for gravid moths than for non-gravid moths, the responses to the test compounds (which were calculated relative to an internal standard of

(*Z*)-3-hexen-1-ol) for gravid moths will be slightly depressed compared to responses for non-gravid moths.

It is interesting that in this research the EAG responses to the internal standard was slightly higher for gravid insects compared to non-gravid moths. Sun *et al.* (2012) showed that this GLV is electrophysiologically active and (within a blend) behaviourally active to mated *H. assulta*. The importance of GLVs in host-location for gravid female *H. armigera* has not yet been elucidated, but it would seem possible that gravid females make a switch in their odour-receptor neuro-physiological system to improve their ability to locate suitable oviposition sites rather than locate energy sources such as those provided the nectar within flowers, similar that seen in the work of Saveer *et al.* (2012). Therefore, on the basis of the results seen here it would be useful to test a range of both GLVs and floral odours on the odour sensory system of gravid and non-gravid *H. armigera* to see if a similar switch occurs to that seen in *S. littoralis* demonstrated by Saveer *et al.* (2012).

5.4.5 Conclusion

The antennae of the three species of Noctuid moths responded to the floral volatiles tested in a similar fashion, both in terms of responding to all the floral chemicals tested and their responses being dependent on the dose of the chemical. Both *H. gelotopoeon* and *H. armigera*, analysis showed that the interaction of chemical and dose was a significant factor in determining the EAG response. The increased number of compounds tested at different doses with *H. gelotopoeon*, demonstrated significant differences in the sensitivity between the compounds. Of particular note, were those compounds that produced relatively low responses across all the doses tested: cineol, camphene, indole,

and β-myrcene; whilst others gave low responses initially but rose steeply: butyl salicylate and methyl benzoate; and other compounds elicited relatively large responses to both high and low doses: e.g. phenylacetaldehyde, linalool, and benzyl alcohol.

Although overall, analysis found that the sex of the insect was not a significant factor in determining sEAG response, some small significant differences were found between the responses of males and females of *H. gelotopoeon* at certain doses for some chemicals. Particularly for benzyl alcohol and β-myrcene at low doses, benzyl acetate at mid doses, and benzaldehyde at high doses. For all of these compounds the sEAG responses of females was higher than for males. This may indicate that the sensitivity to floral compounds at very low or very high doses is higher for females than for males.

Whether *H. armigera* females were gravid or not had little discernible effect on the EAG responses. The mean RPH (relative peak height) of gravid and non-gravid females to the internal standard was different 3.2 (non-gravid) to 4 (gravid), but this was not significantly different. However, it may have influenced the sEAG values which were calculated relative to the internal standard.

It had been the intention to test another 15 compounds on the antennae of *H. gelotopoeon* moths, followed by behavioural bioassays with both *H. gelotopoeon* and *A. gamma* to try to assess and improve the attractiveness of the UoG blend for those species. Unfortunately this was not possible due to problems rearing the insects. Although limited by the work not being completed, some conclusions may be drawn by the results in this chapter. Within the compounds that make up the UoG blend, several were found to elicit relatively high EAG responses across one or more of the species tested. Furthermore,

the relative EAG responses for these five compounds across the Noctuid species was consistent. Phenylacetaldehyde and linalool, elicited high EAG responses for all three Noctuid species tested, and salicylaldehyde in *A. gamma* and *H. armigera* (the compound was not tested on *H. gelotopoeon*, although it was planned to do so). By contrast, limonene and methyl 2-methoxybenzoate elicited relatively low responses across the three Noctuid species. Other compounds that elicited relatively high-moderate EAG responses included methyl salicylate in *A. gamma*, 2-phenylethanol, benzyl alcohol, and benzaldehyde in both *A. gamma* and *H. gelotopoeon*. The sensitivity of the Noctuid species to these compounds may suggest that these compounds are behaviourally important. Therefore these compounds were tested in field trials in Chapter 6 by adding them indivually as a minor component to the UoG blend in order to assess whether they had an effect on the number of Noctuids caught.

CHAPTER 6 - FIELD TRIALS TO IMPROVE ATTRACTIVENESS OF FLORAL BLENDS TO NOCTUIDS

6.1 INTRODUCTION

There is very little published work assessing the trapping of *Autographa gamma* by floral baited traps in the field. Aside from three studies that found the species was caught in traps baited with phenylacetaldehyde (Burgio and Maini, 1995; Toth *et al.*, 2010; Landolt *et al.*, 2013) there has been no other published work that I have found. The work here aims to address that knowledge gap by assessing the captures of *A. gamma* and other Noctuidae species in trap baited with a wide range of floral volatiles.

This chapter aims to improve the general Noctuid attractant found to be effective in Chapter 3 (the UoG blend) by the addition of other floral compounds. Floral compounds were selected from the results to the EAG work in Chapter 5, and also compounds identified from the literature.

The results from Chapter 5 suggested that the antennae of noctuid moths are sensitive to a range of floral volatiles but the size of the EAG responses across a dose range varies between compounds. However, the antennal sensitivity or amplitude of the EAG response is not necessarily an indication of behavioural activity, or may induce negative taxis rather than positive (Ômura *et al.*, 2000; Dötterl *et al.*, 2006). Consequently behavioural tests must be carried out to ascertain the effect of the candidate attractive floral odours.

All of the odour blends tested in Chapter 3 were found to be attractive to several noctuid species. However, overall the UoG blend proved to be the most

consistently attractive. Therefore, this blend was used as a basis from which to build a more attractive odour blend for the noctuids *A. gamma* and *H. gelotopoeon*. Candidate compounds to add to the UoG blend were identified in Chapter 5 and electrophysiology work was carried out to identify those that the species were most sensitive to.

The field trials were carried out in the UK and therefore the predominant target noctuid species was *A. gamma*. This species has previously been shown to be attracted to floral compounds found in the flowers of *Cirsium arvense* (thistle), *Platanthera bifolia* (lesser butterfly-orchid), and *Saponaria officinalis* (common soapwort) (Plepys *et al.*, 2002a). There is a degree of crossover in the floral odour profile of these three plant species and several compounds are present in more than one of their odour plumes: benzaldehyde, benzyl alcohol, 2-phenyl ethanol, methyl benzoate, methyl salicylate, and benzyl benzoate. In Chapter 5 several of these compounds were found to elicit high EAG responses in *A. gamma* and *H. geletopoeon*. Therefore these floral compounds were tested in the field in conjunction with the UoG blend at an equal percentage as the other minor components of the UoG blend with the aim of increasing captures of Noctuid moths. The reason for adding the test compounds at this percentage was to mainting the integrity of the UoG blend whilst trying to find compounds that increased attractiveness for certain species.

As the *A. gamma* antennae were found to respond to all of the compounds in the EAG experiments, all of the remaining compounds were also tested in the field, for example cinnamyl alcohol elicited moderate EAG responses in (Figure 5.3). In addition, searching the literature (see Table 1.3 for a summary of literature on field trials testing floral volatiles and Noctuid moths) highlighted

compounds that were commonly found to be attractive to noctuid moths, for example β-mrycene, lilac aldehyde (especially for *A. gamma*), and α-pinene. Finally, two compounds, 3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde were tested because of their structural similarity to salicylaldehyde - salicylaldehyde having the phenol group in the ortho- position, 3-hydroxybenzaldehyde having the phenol group in the meta- position, and 4-hydroxybenzaldehyde in the para- position. Previous research has found that EAG responses to structurally similar compounds are not significantly different (Raguso *et al.*, 1996) and that they can stimulate the same ORNs (albeit to a lesser degree) (Bengtsson *et al.*, 1990). The purpose of including these structurally similar compounds was to see if their presence in the blend would increase moth captures.

Two field trials (field trial 4 and 5) were carried out to assess the effect of adding specific floral compounds to an already proven attractive blend of floral odours - the UoG blend - and the effect they may have on UK Noctuids (predominantly *A. gamma*). The aim of field trial 6 was to assess the affect of removing individual components from the UoG odour blend completely. This was deemed necessary because the blend was originally devised in Bangladesh and India to target local species crop pests, primarily *H. armigera*. Although the UoG blend was tested in the UK in the initial study in Chapter 3 and found to be highly attractive to *A. gamma*, it was not developed to target this species.

6.2 MATERIALS AND METHODS

6.2.1 Lures

Lures were made of the components shown in Table 6.1. The compounds were pipetted onto a cellulose acetate cigarette filter (14 x 6 mm, Swan, Republic

Technologies Ltd., UK) in polyethylene sachets (5 cm x 5 cm, Transatlantic Plastics, Southampton, UK). The sachets were heat sealed and stored at -18°C until used.

Table 6.1: Composition of lures used in field trials in Chapter 6. Quantities are in µL.

Treatment Name	Total volume (µl)	Phenylacetaldehyde (µl)	Linalool (µl)	(-)-Limonene (µl)	Methyl 2-methoxvbenzoate (µl)	Salicylaldehyde (µl)	Methyl salicylate (µl)	Beta-myrcene (µl)	Methyl benzoate (µl)	Benzyl benzoate (µl)	Benzyl salicylate (µl)	Cinnamyl alcohol (µl)	Benzaldehyde (µl)	Benzyl alcohol (µl)	2-phenylethanol (µl)	α-pinene (µl)	Cineole (µl)	3-hydroxybenzaldehyde (µl)	4-hydroxybenzaldehyde (µl)	Lilac aldehydes (µl)
Odour blends used in field trial 4																				
UoG	255	150	30	15	30	30														
UoG+MSa	255	150	26	13	26	26	13													
UoG+bM	255	150	26	13	26	26		13												
UoG+MBe	255	150	26	13	26	26			13											
UoG+BeBe	255	150	26	13	26	26				13										
UoG+BeSa	255	150	26	13	26	26					13									
UoG+CiAl	255	150	26	13	26	26						13								
Odour blends used in field trial 5																				
UoG	255	150	30	15	30	30														
UoG+BB/CA	300	158	32	16	32	32				15		15								
UoG+BB/CA +Be	300	150	30	15	30	30				15		15	15							
UoG+BB/CA +BA	300	150	30	15	30	30				15		15		15						
UoG+BB/CA +2Ph	300	150	30	15	30	30				15		15			15					
UoG+BB/CA +aP	300	150	30	15	30	30				15		15				15				
UoG+BB/CA +Ci	300	150	30	15	30	30				15		15					15			

Treatment Name	Total volume (µl)	Phenylacetaldehyde (µl)	Linalool (µl)	(-)-Limonene (µl)	Methyl 2-methoxybenzoate (µl)	Salicylaldehyde (µl)	Methyl salicylate (µl)	Beta-myrcene (µl)	Methyl benzoate (µl)	Benzyl benzoate (µl)	Benzyl salicylate (µl)	Cinnamyl alcohol (µl)	Benzaldehyde (µl)	Benzyl alcohol (µl)	2-phenylethanol (µl)	α-pinene (µl)	Cineole (µl)	3-hydroxybenzaldehyde (µl)	4-hydroxybenzaldehyde (µl)	Lilac aldehydes (µl)
UoG+BB/CA +3Hb	300	150	30	15	30	30				15	15							15		
UoG+BB/CA +4Hb	300	150	30	15	30	30				15	15								15	
UoG+BB/CA +LA *	300	150	30	15	30	30				15	15									15

* Lilac aldehyde was not commercially available. A small quantity was provided by Dr. P Douglas allowing 3 lures to be made containing lilac aldehyde.

Odour blends used in field trial 6

Treatment Name	Total volume (µl)	Phenylacetaldehyde (µl)	Linalool (µl)	(-)-Limonene (µl)	Methyl 2-methoxybenzoate (µl)	Salicylaldehyde (µl)
UoG	255	150	30	15	30	30
UoG-PAA	105		30	15	30	30
UoG-Ln	225	150		15	30	30
UoG-Lm	240	150	30		30	30
UoG-M2M	225	150	30	15		30
UoG-Sa	225	150	30	15	30	
Control						

6.2.2 Traps

In all of the field trials in this chapter Unitrap (Agrisense, Treforest, UK) traps were used (Figure 8.1).

6.3 TRIAL DESIGN

6.3.1 Field trail 4

The trial was conducted at the site known as 'Park Field' (51°13'12.84"N 1°20'56.90"E) at Intercrop Ltd, Deal, Kent (Figure 6.1). The trial was positioned in an area of grassland adjacent to fields of salad crops (spinach, lettuce, and coriander) that suffered from lepidopteran damage (predominately *A. gamma* and *P. xylostella*). This area was next to the site used in field trial 2 (Section 3.2.5).

The trial contained eight treatments (including a blank control) (Table 6.1) replicated four times in blocks. Treatments within the blocks were positioned randomly and were 10 m apart. There was 15 m between the block rows (Figure 6.1). Unitraps were tied to bamboo stakes so that the top of the trap was 30 cm above the ground and 0 - 20 cm above the foliage.

At the time of the trial the crops were at various growth stages from seedlings to mature plants ready for harvest. The trial was run from 18/07/08 to 22/08/08 and the traps checked one or two times per week and a total of seven times during that period. After checking the traps they were repositioned within their blocks.

Figure 6.1: Approximate locations for traps in trial 4 at Park Field, Deal, Kent. There were four blocks of eight traps. The blocks were 15 m apart, and the traps within the blocks were 10 m apart. Aerial photo taken from Google Earth (2008) and modified.

6.3.2 Field trial 5

The trial was carried out at two sites (approximately 1 km apart) at Gosmere Farm. One site was a pea field (*Pisum sativum*) (L.) (Fabaceae) adjacent to wheat field and a line of Poplar trees (51°16'54.36"N 0°53'36.34"E) (Figure 6.2) and the other site was a field of *Echium vulgare* (L.) (Boraginaceae) adjacent to marigold, wheat fields, and woodland, with oilseed rape nearby (51°16'19.90"N 0°54'9.14"E) (Figure 6.3).

The trial contained 10 treatments (Table 6.1) with 9 replicates in blocks (there were only 3 replicates of the treatment containing lilac aldehyde). Five replicates were at the pea field site and 4 replicates were at the *Echium* site. UniTraps were positioned 10 m apart, 1 m from the crop edge, and 10 to 30 cm above the field margin foliage. The traps were checked 9 times between

02/06/09 and 28/07/09 and treatments were repositioned within their block after being checked.

A single pheromone trap was placed approximately 100 m from the field site at each of the two locations.

Figure 6.2: Field site one at Gosmere farm. Approximate locations of traps for field trial 5 are shown. Peas were being grown in the field to the west of the traps, whilst the field north of the traps was a wheat field. The field to the east was grass and traps followed a line of Poplar trees. Traps were positioned 10 m apart and less than 1 m from the edge of the crop. Aerial photo taken from Google Earth (2008) and modified.

Figure 6.3: Field site two at Gosmere farm. Approximate locations of traps for field trial 5 are shown. The field contained *Echium vulgare*. To the west was a field of marigolds and to the north oilseed rape. Traps were positioned 10 m apart and less than 1 m from the edge of the crop. Aerial photo taken from Google Earth (2008) and modified.

6.3.3 Field trial 6

The trial was carried out at the same site as field trial 2, known as 'Park field'

(51°13'12.84"N   1°20'56.90"E) at Intercrop Ltd, Deal, Kent (Figure 3.4) and

carried out in a similar fashion. Traps were placed on 17/07/2008 and checked

at least once per week seven times until 22/08/2008. After checking the traps were re-positioned in their blocks.

There were four blocks of replicates of each of the seven treatments (Table 6.1). The traps were hung from bamboo stakes so that the top of the trap was 0 - 30 cm above the foliage.

A pheromone trap was placed 100 m from the field site and checked at the same time as the field trial traps.

6.4 RESULTS

6.4.1 Field trial 4

The addition of minor components to the UoG blend was found to significantly influence the number of *A. gamma* caught and the total number of noctuids caught. The addition of benzyl benzoate (UoG+BeBe) or cinnamyl alcohol (UoG+CiAl) to the UoG blend resulted in significantly more noctuids being caught in traps compared to the standard UoG blend (Figure 6.4).
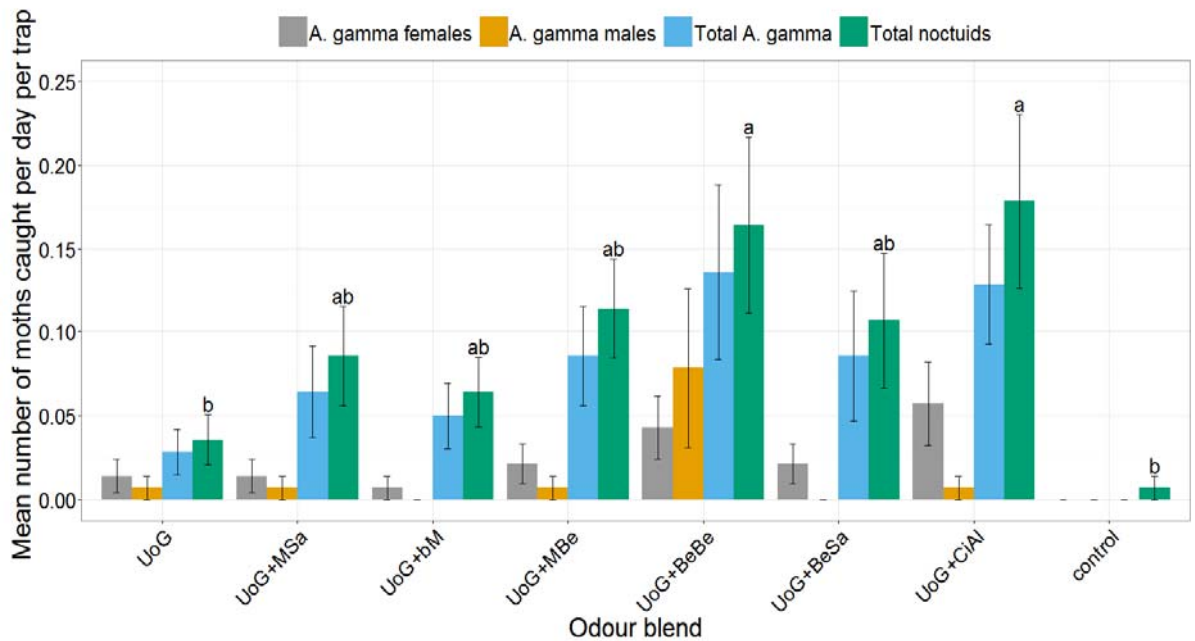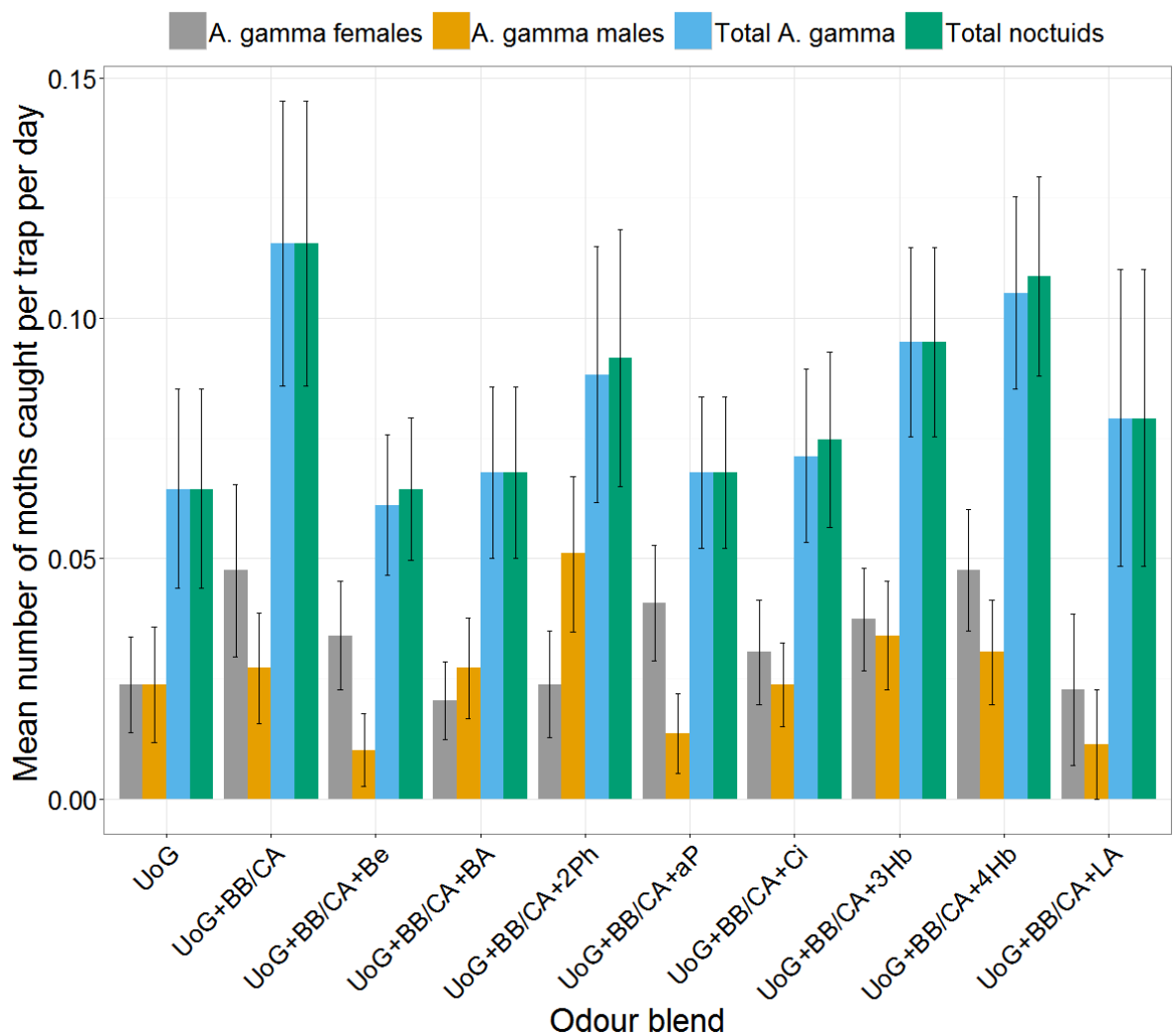
Figure 6.4: Mean number of *A. gamma* and noctuid moths caught per trap per day in traps baited with the UoG blend with a series of additional compounds located in a fallow field site adjacent to fields containing salad crops. The presence of additional compounds to the UoG blend was found to have a significant effect on the number of moths caught for all groups. Data analysed by GLM with a negative binomial distribution, N = 28, d.f. = 7, *A. gamma* females, $P < 0.05$; *A gamma* males, $P < 0.001$; total *A. gamma*, $P < 0.001$; total noctuids, $X^2 = 31.25$, $P < 0.001$). Error bars show ±se, bars of the same colour that do not share the same letters are significantly different (pairwise comparisons, $P < 0.05$).

6.4.2 Field trial 5

Catch rates were low and no significant differences were found between the odour blends for any groups of moths.

The trial was carried out at two field sites, one growing peas, the other *Echium vulgare* plants. A significant difference was found in the numbers of noctuids caught at the two field sites, with significantly more noctuids caught at the pea field (mean of 0.14, SE of ±0.01) than the *Echium* field (mean of 0.03, SE of ±0.01) (DF = 1, $F = 110.58$, $P < 0.001$). Within each field site the odour blends did not have a significant effect on the catches of *A. gamma* or total Noctuids.

The blend that caught the most *A. gamma* and noctuids contained the UoG blend with the added compounds identified from field trial 4 (benzyl benzoate and cinnamyl alcohol), but this was not significantly different from any other blend. Most blends caught more females than males with the exception of blends containing 2-phenylethanol and benzyl alcohol which caught more males, although none of these differences were significant ($P > 0.05$).

Pheromone traps caught a mean of 0.23 male *A. gamma* at the *Echium* field site, and a mean of 2.4 (per trap per day) at the field pea site.

Figure 6.5: Mean number of *A. gamma* and noctuid moths caught per trap per day in traps baited with the UoG blend alone, the UoG blend combined with compounds previously found to significantly improve catches in field trial 4 (benzyl benzoate + cinnamyl alcohol), and this blend combined with additional compounds identified during EAG work. The additional chemicals added to the odour blend were not found to have a significant effect on the number of moths caught. The data was analysed by GLM with negative binomial distribution, d.f. = 9, *P* > 0.05). Error bars show ±se; N = 90 for all treatments except for UoG+BB/CA+LA (the addition of lilac aldehyde) where N = 27.

6.4.3 Field trial 6

Overall catches were very low. The odour blends tested were not found to be significantly different from a blank control with regards to the mean number of *A. gamma* males, females, total, and Noctuids caught (Figure 6.6). Likewise, odour blend had no significant affect on the ratio of male to female *A. gamma* caught.

The pheromone trap caught a mean of 12 male *A. gamma* during the course of the field trial.
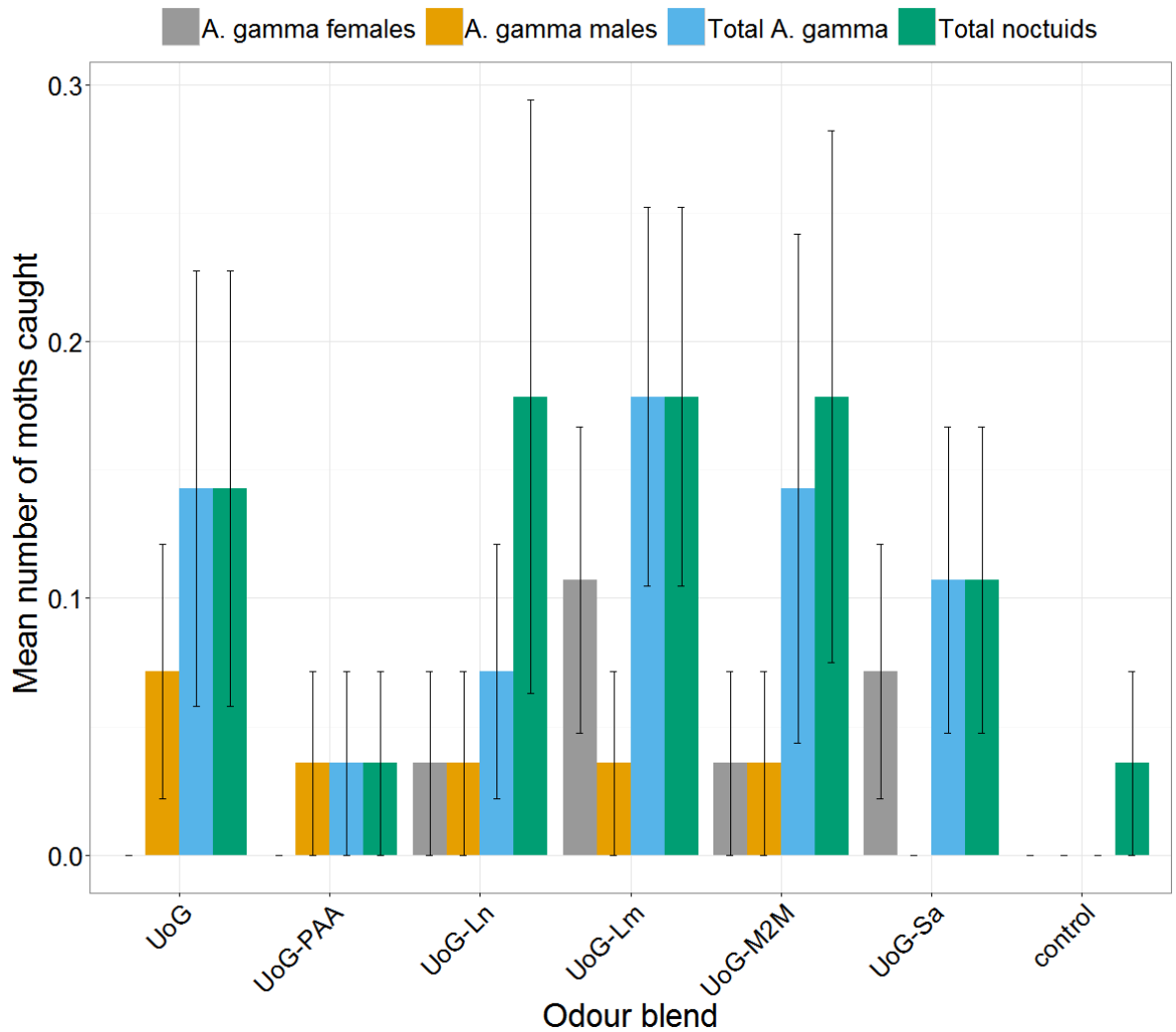
Figure 6.6: Mean number of *A. gamma* and noctuid moths caught in traps baited with the UoG blend, the UoG blend with one component absent, and a blank control. Odour blend was found not to be a significant predictor for number of moths caught for all groups. The data was analysed by a GLM with a poisson distribution, d.f. = 6, *P* > 0.05. Error bars show ±se; N = 28.

6.5 DISCUSSION

6.5.1 Field trials 4 and 5

As discussed in Chapter 3, the UoG blend performed the most consistently in

attracting several species of noctuid moths. It was therefore used as the basis

from which to test other compounds that were identified as potentially attractive

to Noctuids by searching published literature and by conducting

electroantennography (Chapter 5). In field trial 4 the addition of certain floral volatiles to the UoG blend significantly increased the catches of Noctuids (the vast majority of which were *A. gamma*). By adding a small quantity (the same amount as that of the minor components) to the UoG blend, both benzyl benzoate and cinnamyl alcohol significantly increased catches compared to the UoG blend alone. The EAG responses of *A. gamma* to cinnamyl alcohol were relatively low compared to some of the other volatiles tested (eliciting approximately half the response of the highest EAG responses) (Figure 5.3, Chapter 5). Plepys *et al.* (2002a) showed that the antennae of *A. gamma* are sensitive to both benzyl benzoate and cinnamyl alcohol. Whilst the former was moderately behaviourally stimulating the latter was not as no insects made contact with the cinnamyl alcohol odour sources (Plepys *et al.*, 2002b). The reason why a compound may be non-stimulating on its own but may increase attraction when mixed with other odours is that certain compounds have been shown to provide a synergistic effect on insects behaviour when mixed in the correct ratios; even if these compounds are non-stimulating or even repellent when alone, once added to other compounds they may induce positive taxis in the receiver (for a detailed review see Bruce *et al.*, 2005; and Bruce and Pickett, 2011). Other floral volatiles have been shown to have a very limited level of attraction when tested alone, but have a positive synergistic effect when in combination with other volatiles. For example, β-myrcene alone caught very few *T. ni* moths, but when combined with either phenylacetaldehyde or benzyl acetate, it significantly increased the catches of those compounds - far more than the sum of those compounds when presented individually (Landolt and Smithhisler, 2005).

The addition of the other compounds tested in field trial 4 also increased captures of Noctuids compared to the UoG blend alone, but not significantly so. Compounds that elicited strong EAG responses in *A. gamma* comparative to other compounds that are behaviourally stimulating were not found to significantly increase trap catches. For example, methyl salicylate and benzaldehyde both elicited EAG responses similar to that of phenylacetaldehyde and salicylaldehyde, but did not significantly increase trap catches (tested in field trial 4 and 5 respectively). Previous work has found methyl salicylate to be only mildly attractive to *A. gamma* (Plepys *et al.*, 2002b), whilst benzaldehyde has been identified as attractive for several species of noctuid: *H. armigera* (Bruce and Cork, 2001; Deng *et al.*, 2004; Li *et al.*, 2005), *T. orichalcea* (Stringer *et al.*, 2008), and *T. ni* (Haynes *et al.*, 1991). However, in field trial 5 the addition of benzaldehyde to the UoG blend+benzyl benzoate+cinnamyl alcohol reduced trap catches (not significantly).

It was surprising to find that the addition of lilac aldehyde (as an isomeric mix) did not increase catches of *A. gamma*. The results of Plepys *et al.* (2002b) showed that lilac aldehyde was highly attractive to *A. gamma*, significantly more than any other single compound tested and equal to a synthetic mimic of the odour blend of *P. bifolia* flowers, which itself was known to be highly attractive to this moth. An explanation for the result in the current work is possible the isomeric mix of lilac aldehydes used in this research were not sufficiently behaviourally stimulating to *A. gamma*, and perhaps the insect responds to only some or one of the isomers of lilac aldehyde. In addition, the reduced number of replications containing lilac aldehyde (due to difficulties in procuring it) in conjunction with the poor overall catch rates may have caused erroneous or unclear results.

No significant differences were found between the ratios of males and females caught with the different odour blends tested in the field. However, it should be noted that for almost all blends more females were caught than males, with the exception of the blends containing benzyl alcohol and 2-phenylethanol. These two compounds are structurally very similar and differ only in the length of the carbon-chain arm linking the benzene ring to the alcohol and its possible these structures may have some behavioural effect specifically for males, but further investigations are needed to explore this. Previous work with floral volatiles has shown some compounds trigger slightly different levels of attraction between male and female *H. armigera* (e.g. Gregg *et al.*, 2010).

Although no significant differences were found between the odour blends tested in field trial 5, the UoG blend with benzyl benzoate and cinnamyl alcohol caught the highest mean number of moths (Figure 6.5). Therefore, the overall results of field trials 4 and 5 indicate that the UoG blend with the additional components: benzyl benzoate and cinnamyl alcohol, is the most effective floral blend of those tested for capturing *A. gamma*. The lack of significant differences between the blends tested in field trial 5 suggests that the standard UoG blend is already a good attractant for these moths and additional components have only a small affect if any at all. Further analysis should be carried out to test whether it is purely the presence and absence of specific compounds or whether different ratios of these compounds that impact the level of attraction.

The investigation on the effect of structurally similar compounds, salicylaldehyde, 3-hydroxybenzaldehyde, and 4-hydroxybenzaldehyde perhaps would have been more informative if salicylaldehyde in the UoG blend had been

replaced by each of the structurally similar compounds rather than added as a minor component.

6.5.2 Field trial 6

The UoG blend was developed in Bangladesh and India to target local noctuid crops pests (primarily *H. armigera*). The results in Chapter 3 show the blend to be highly attractive to other Noctuid species including *A. gamma*, *H. gelotopeon*, *H. zea*, and *S. frugiperda*. The 'binary' blend from Chapter 3 contained the two major components of the UoG blend, and was found to be significantly less attractive than the full UoG blend. However, no further testing was carried out on the minor components of the blend. Field trial 6 attempted to address this by removing individual components from the blend and assessing the mean captures of Noctuids in the UK. Statistical analysis of field trial 6 found no significant differences between the treatments (including the blank control). This was probably due to the very low capture rates during that trial. Unfortunately there was no time to repeat the trial. It had been the intention to do the same type of experiment in the wind tunnel with *A. gamma* and *H. gelotopoeon*, however, that was not possible due to problems rearing insects in the laboratory.

Some of the UK field trials suffered from low catch rates making statistical analysis difficult. Reports of the *A. gamma* affecting crops was low for the years 2009 through to 2012 (ADAS UK Ltd, 2010; ADAS UK Ltd, 2011; ADAS UK Ltd, 2013) and it is therefore likely that for those years the wild populations of this crop pest were low.

CHAPTER 7 - ATTRACTION OF NON-TARGET INSECTS TO FLORAL BLENDS IN FIELD TRAPPING TESTS

7.1 INTRODUCTION

Throughout all of the field trials large numbers of non-target species were caught in the traps. Depending on the non-target species trapped this may be an advantage or disadvantage. For conventional chemical insecticides, the killing of non-target organisms is one of their main disadvantages and has resulted in immense ecological damage (for a detailed review, see Devine and Furlong, 2007 and the references therein). It is therefore vital that any crop protection technology is properly assessed for its affects (direct and indirect) on non-target organisms, whether this be non-target pests, rare species, pollinators, or any others. Categorising insect species into groups by their status with regards to farmers and ecologists, i.e. crop pest, beneficial insect, pollinator, rare species, key indicator species, etc., may be a useful way to analyse the insects caught. Due to the time required to correctly record and identify insects down to species level it was not possible to classify all of the insects trapped in these field trials, but classification to family level was possible for all insects and genus or species level for many. Although there is large diversity within many families, it is hoped that this basic level of taxonomic classification will provide some insight into the types of insects that the floral odours tested in the field are attracting outside their intended targets.

The odour blends tested throughout the field trials included common floral volatiles identified in the odour profiles of many plant genera and many are not only emitted from the reproductive areas of the plants, but also the green leaf

areas. It is likely that these non-specific chemicals would be attractive to a wide array of insects (e.g. James, 2005; James and Grasswitz, 2005). The targeted insects (Noctuid moths) were expected to be attracted to the traps because they are searching for a nectar resource and it is highly likely that other insect groups will be attracted to the traps for the same reasons. However, the 'meaning' inferred by an insect from a plant volatile is dependent on many things (e.g. species or trophic level of the receiver, physiological state of the receiver, other volatiles within the odour plume, ratio of those other volatiles, etc) and can alter due to changing context (Vet and Dicke, 1992). Benzaldehyde is commonly found in flower odour plumes (Plepys *et al.*, 2002a; Knudsen and Gershenzon, 2006) and known to be attractive to herbivorous insects (Bruce and Cork, 2001) and to stimulate proboscis extension in nectar feeding insects (Eby *et al.*, 2013), yet it is also a compound utilised by carnivorous insects (e.g. *Chrysopa sp.*, *Aphidius sp.*, and *Coccinella sp.*) to locate their prey (aphids feeding on tea shoots) (Han and Zongmao, 2002). Thus not only insects that are commonly thought of as flower visitors (pollinators and herbivorous insects) will be trapped in the floral odour baited traps but also insects from the third trophic or fourth level that may be searching for their prey.

Some floral volatiles have previously been found to be repellent to certain insect species, for example the floral volatile methyl salicylate for aphids (Hardie *et al.*, 1994; Losel *et al.*, 1996), bees (Henning *et al.*, 1992; Sahebzadeh *et al.*, 2009), and Lepidoptera (Gregg *et al.*, 2010). During field trials 4 and 5 it was hoped that a floral volatile(s) might be identified that did not negatively affect the attractiveness for the target species but was repellent to beneficial insects such as bees.

The traps used in the trials were UniTrap (Figure 8.1) which have a green top, yellow funnel, and white base. Some insect species are known to utilise colour and shape to identify nectar resources. The honey bee, *Apis mellifera* (L.) (Apidae), has been shown to have an innate colour preference for blue and green (Giurfa *et al.*, 1995) and is attracted to shapes with radial patterns (reminiscent of a 'classical' flower's petals) (Lehrer *et al.*, 1995). *Bombus impatiens* (Cresson) (Apidae) has a preference for blue and yellow colours and also radial patterns, but is capable of learning from its experiences (Simonds and Plowright, 2004). Other insect groups, such as species of Syrphidae, have been shown to have a preference to yellow coloured flowers (Campbell *et al.*, 2010). Given the yellow and green colours of the trap in conjunction with the floral odour baits, it was expected that large numbers of non-target insects would be caught in the traps.

The aims of this chapter were to assess which non-target species were attracted to the floral blends; to identify which of these non-target species were beneficial or crop pests; to assess whether the addition of small quantities of specific floral compounds to the UoG blend increased or decreased non-target captures; and to attempt to address the problem of capturing non-target beneficial insects by changing the colour of the traps used. As the target Noctuid species are crepuscular or nocturnal they were not expected to be heavily influenced by trap colour.

7.2 MATERIALS AND METHODS

7.2.1 Field trials 2, 3, 4, and 5

For field trials 2, 3, 4, and 5 the lures and methodologies used were described in detail in Chapters 3 and 6.

7.2.2 Field trial 7 – the effect of trap colour on non-target insects

7.2.2.1 <u>Lures</u>

The lures contained the UoG blend as shown in Table 7.1. The compounds were pipetted onto a cellulose acetate cigarette filter (14 x 6 mm, 14 x 6 mm, Swan, Republic Technologies Ltd., UK) in polyethylene sachets (5 cm x 5 cm, Transatlantic Plastics, Southampton, UK). The sachets were heat sealed and stored at -18°C until used.

Table 7.1: Composition of the odour blend used in field trial 7. Quantities are in µL.

Treatment Name	total volume (µl)	Phenylacetaldehyde (µl)	Linalool (µl)	(-)-Limonene (µl)	Methyl 2-methoxybenzoate (µl)	Salicylaldehyde (µl)
UoG	255	150	30	15	30	30

7.2.2.2 <u>Trap colours</u>

Unitraps (Agrisense, Treforest, UK, Figure 8.1), originally with a green lid, yellow funnel, and white bucket were coloured blue, white, red, yellow, green using 'Plasti-kote Project Paint' Gloss (Table 7.2). The relative reflectance of the traps was measured using an AvaSpec-2048 with an AvaLight-DH-S-BAL light from Avantes Ltd. Colours are measured relative to a $BaSO_4$ white standard. Three to four samples of each colour were measured and means of these results are presented in Figure 7.1.

To colour the traps the exterior of each trap was completely covered, allowed to dry for 24 h then sprayed again and left for another 24 h. They were then left

outside in the sun for a further 24 h to reduce the influence of any odours given off by the product. To the human eye the colours looked even and uniform with no evidence of the original colour showing through. After 24 h outside there was no noticeable odour from the paint detectable by the human nose.

Table 7.2: Colours of traps used in trial

Treatment colour	'Plasti-kote' color and product code
Blue	Blue (1134 Royal Blue)
White	White (1109 White RAL 9010)
Red	Red (1120 Bright Red)
Yellow	Yellow (1115 Yellow)
Green	Green (1127 April Green)
Control trap	Green lid, yellow funnel, white base
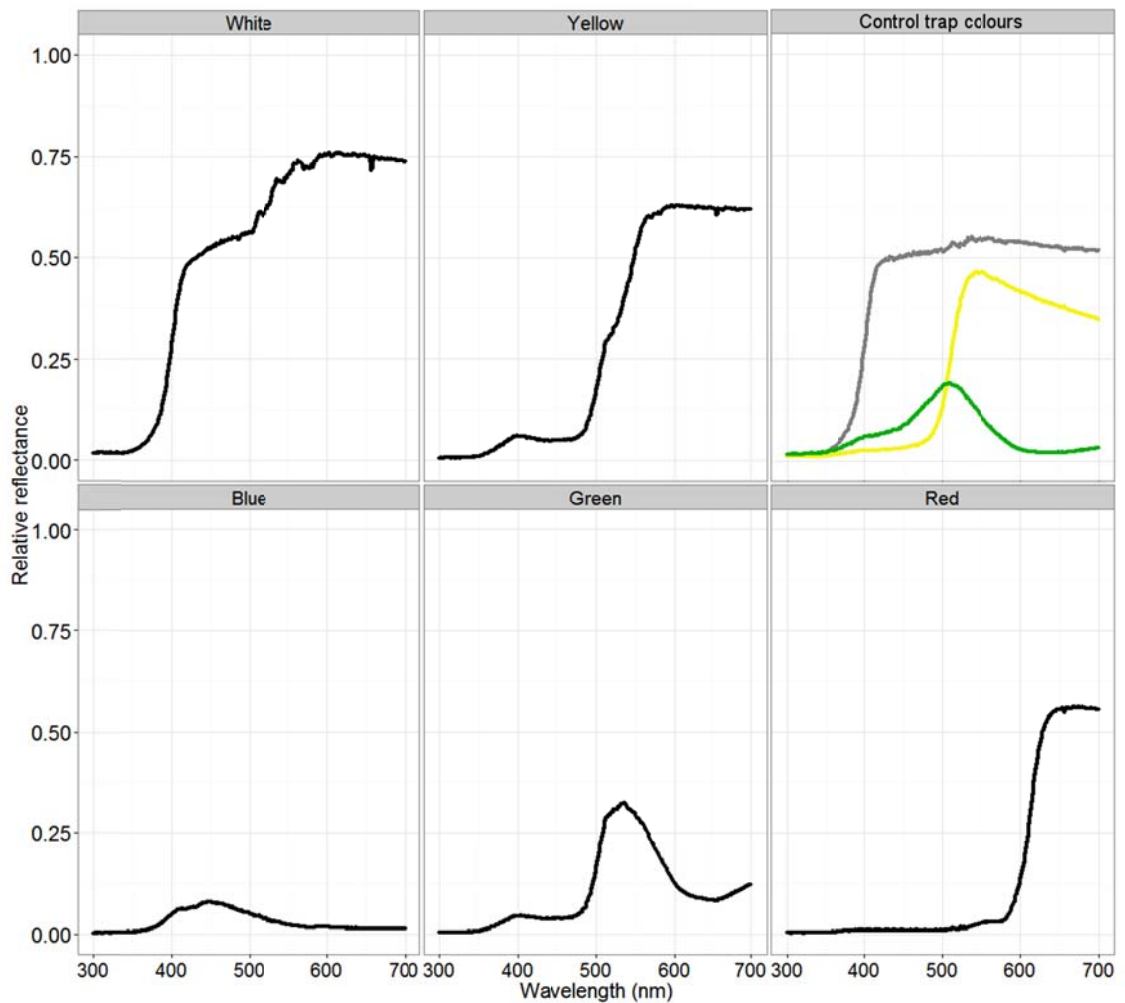
Figure 7.1: Relative reflectance of the traps sprayed with 'Plasti-kote'. The 'control trap colours' shows the relative reflectance of each of the three components of an unsprayed trap: the green line shows reflectance of the green lid, the yellow line shows the yellow funnel, and the grey line shows the white base. See Figure 8.1 for a photograph of a UniTrap.

7.2.2.3 Trial design

The trial was carried out at the same *Echium vulgare* field used for field trial 5

(51°16'19.90"N 0°54'9.14"E) (Figure 7.2).

Figure 7.2: Field site two at Gosmere farm. Approximate locations of traps for field trial 7 are shown. The field contained *Echium vulgare*. To the west was a field of marigolds and to the north oilseed rape. Traps were positioned 10 m apart and less than 1 m from the edge of the crop. Aerial photo taken from Google Earth (2008) and modified.

The trial contained four replicates of six treatments (Table 7.2). The traps were baited with a floral attractant lure (Table 7.1). A block of six traps consisted of one replicate. The treatments within each block were randomly distributed; once the traps had been checked they were repositioned within that block. The traps were checked once per week, seven times in total, between 08/08/2009 and 08/09/2009.

7.3 RESULTS

7.3.1 Field trial 2

The largest number of insects of any group that was caught in the traps was that of insects within the order Syrphidae (hoverflies) with a mean of +9 insects per odour baited trap which was significantly different from the mean number of Syrphidae caught in unbaited control traps (Figure 7.3). Other insect groups that were caught in significantly greater numbers in odour baited traps (regardless of the specific odour blend) compared to unbaited traps include the genus Meligethes (pollen beetles) (Figure 7.3) and the species *Forficula auricularia* (L.) (Forficulidae) (Earwigs) (Figure 7.4).

For other insect groups some odours resulted in a greater mean catch compared to the other odours and the control. For insects in the order Muscidae (flies), the *P. nigra* odour blend was not significantly more attractive than the unbaited control, but other blends were. The Magnet, UoG and G9 odour blends caught significantly more Muscidae than the *P. nigra* blend. Similarly, the UoG and G9 blends caught significantly more insects in the group Oedemeridae than the *P. nigra* blend. In addition, for this insect group the binary, *P. nigra* and Magnet blends were not significantly different from the unbaited control (Figure 7.4).

In the final set of results (Figure 7.5), although for the mean captures of individual bee groups (Apis, Bombus, Lasioglossum) no significant differences were found between the treatments, pooling the bee data ("Total.Bees", Figure 7.5) did result in significant differences such that all of the odour blends apart from the *P. nigra* blend caught significantly more bees than the unbaited control traps. In addition, for the insect order Miridae, significantly more insects were

found in traps baited with the binary or G9 odour blends compared to unbaited traps.

For comparison, in field trial 2, the number of total *A. gamma* caught was 0.4 per day per trap (Chapter 3, Figure 3.10), therefore more moths were caught compared to bees (0.2 bees per trap per day) (Figure 7.5), but a much greater number of Syrphidae were caught (9 per trap per day) (Figure 7.3).
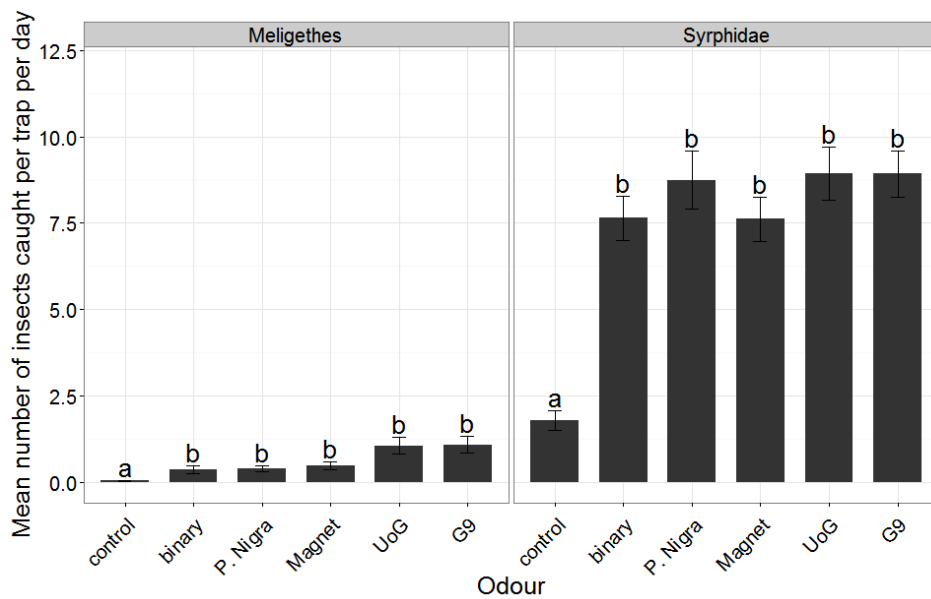

Figure 7.3: Mean number of insects per trap per day in the groups Meligethes and Syrphidae caught in traps baited with floral odour blends from field trial 2. Error bars show ±se mean, bars with the same letters are not significantly different within each chart. Data analysed by GLM with a negative binomial distribution and Tukey's pairwise comparisons, for Meligethes N = 36, $X^2_{(5, 210)}$ = 51.76, P < 0.001; for Syrphidae N = 36, $X^2_{(5,210)}$ = 147.88, P < 0.001.
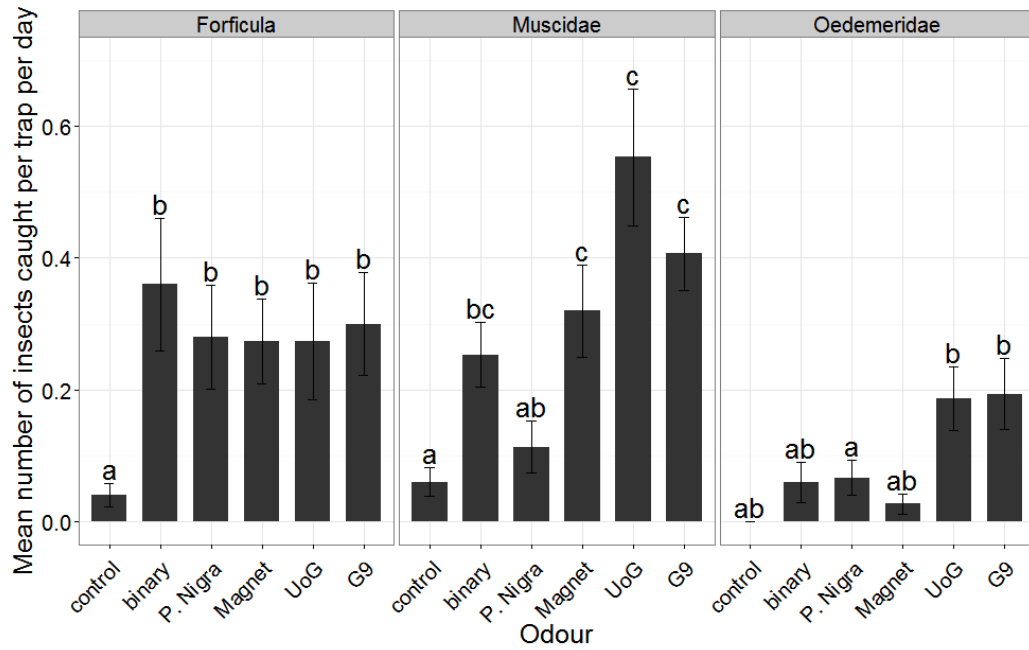
Figure 7.4: Mean number of insects per trap per day in the groups Forficula (auricularia), Muscidae, and Oedemeridae caught in traps baited with floral odour blends from field trial 2. Error bars show ±se mean, bars with the same letters are not significantly different within each chart. Data analysed by GLM with negative bionomial distribution and Tukey's pairwise comparisons, for Forficula N = 36, $X^2_{(5,210)}$ = 20.89, P < 0.001; for Muscidae N = 36, $X^2_{(5,210)}$ = 57.09, P < 0.001; for Oedemeridae N = 36, $X^2_{(5,210)}$ = 41.70, P < 0.001.
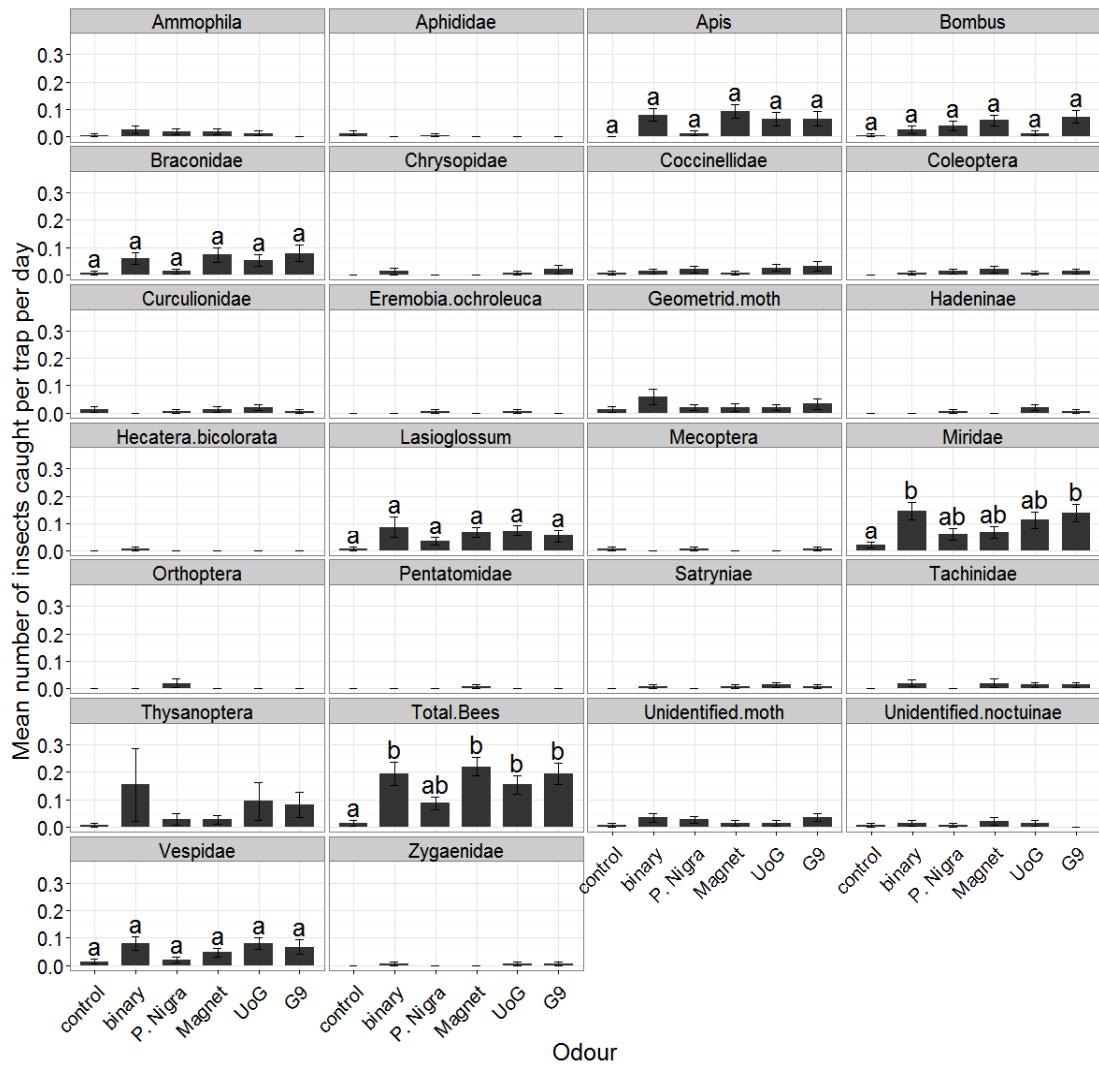
Figure 7.5: Mean number of insects per trap per day in the groups Ammophila, Aphididae, Apis, Bombus, Braconidae, Chrysopidae, Coccinellidae, Coleoptera, Curculionidae, *Eremobia ochroleuca* (Noctuidae), Geometrids, Hadeninae, *Hecatera bicolorata* (Noctuidae), Lasioglossum, Mercoptera, Miridae, Orthoptera, Pentatomidae, Satryniae, Tachinidae, Thysanoptera, total bees, Vespidae, and Zygaenidae caught in traps baited with floral odour blends from field trial 2. Error bars show ±se mean, bars with the same letters are not significantly different within each chart. Data analysed by GLM with negative binomial distributions, followed by Tukey's pairwise comparisons where appropriate. For all groups, N = 36, df = 5; for Apis, X^2 = 26.60, P < 0.001; for total bees X^2 = 42.05, P < 0.001; for Miridae X^2 = 22.09, P < 0.001; for Bombus X^2 = 14.87, P < 0.05; for Braconidae X^2 = 14.38, P < 0.05; for Lasioglossum X^2 = 13.64, P < 0.05; and for Vespidae X^2 = 14.24, P < 0.05.

For insect groups that may be considered as crop pests (Aphididae, Curculionidae, Miridae, Meligethes, Oedemeridae, Thysanoptera) the UoG and G9 odour blends caught significantly more insects than the *P. nigra* and Magnet blends, but not the binary blend. All of the odour blend baited traps caught significantly more crop pests than the unbaited control traps (Figure 7.6).

For insect groups that may be considered as agriculturally beneficial no significant differences were found between the odour blends, although they all caught significantly more insects than the unbaited control traps (Figure 7.7). The vast majority of insects that made up this group were those of the order Syrphidae.
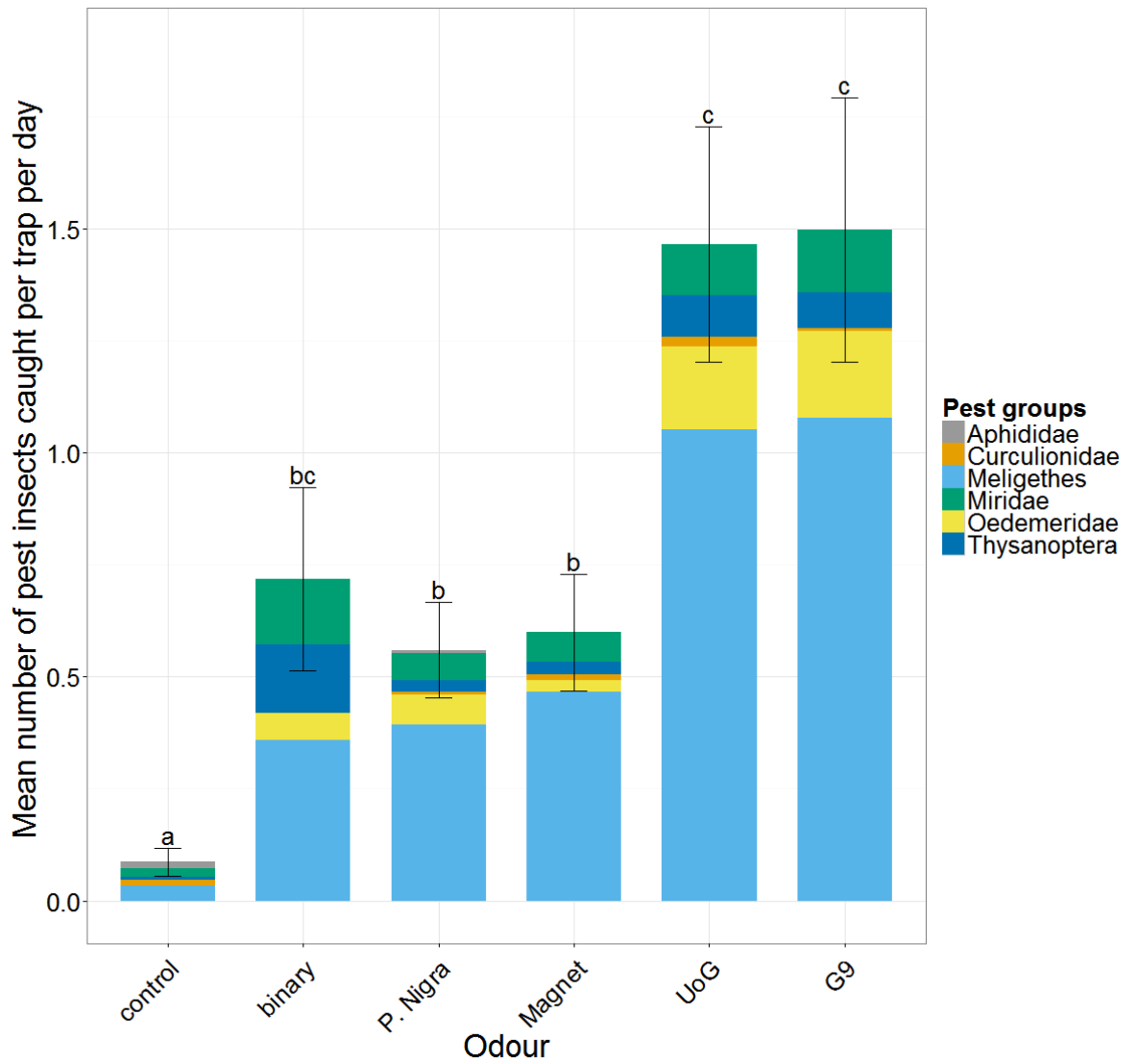
Figure 7.6: Mean number of insects considered as crop pests caught per trap per day in traps baited with floral odour blends from field trial 2. For statistical analysis pest groups were pooled. Error bars show ±se mean, bars with the same letters are not significantly different. Data analysed using a GLM with negative binomial distribution and Tukey's pairwise comparisons, N = 36, $X^2_{(5,210)}$ = 69.42, P < 0.001).
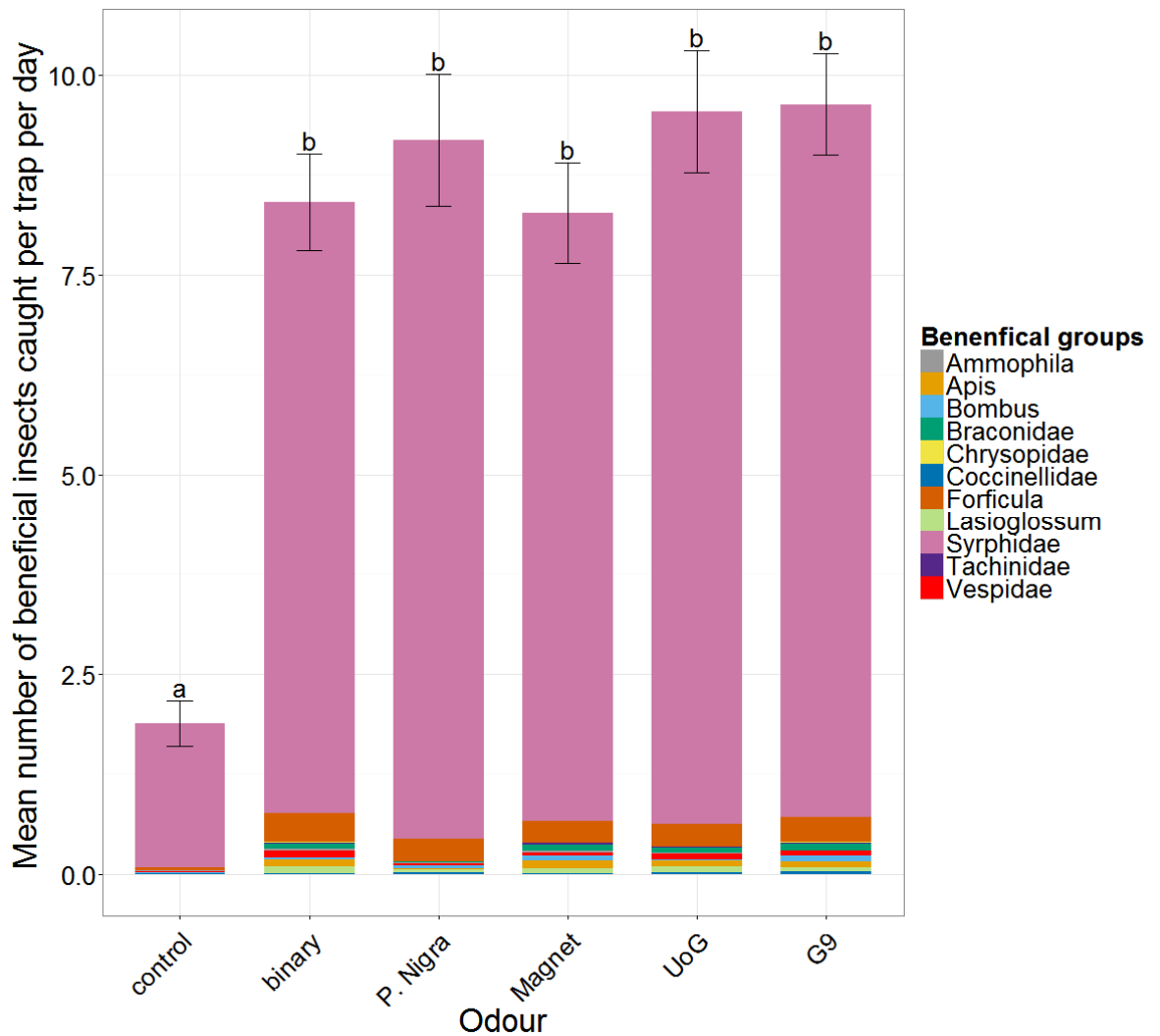
Figure 7.7: Mean number of insects considered as beneficial insects caught per trap per day in traps baited with floral odour blends from field trial 2. For statistical analysis beneficial groups were pooled. Error bars show ±se mean, bars with the same letters are not significantly. Data analysed using a GLM with negative binomial distribution and Tukey's pairwise comparisons, N = 36, $X^2_{(5,210)}$ = 188.84, P < 0.001).

7.3.2 Field trial 3

No significant differences were found between catches with any of the odour blends for any of the insect groups (Figure 7.8). Insects within the orders Apoidea and Coleoptera were caught in significantly larger numbers than the other insect groups (data analysed by GLM with a negative binomial distribution and Tukey's pairwise comparison, N = 96, $X^2_{(7,760)}$ = 101.47, P < 0.001). Neither the terms 'date' nor 'crop' (N.B. data was collected from two field sites, one

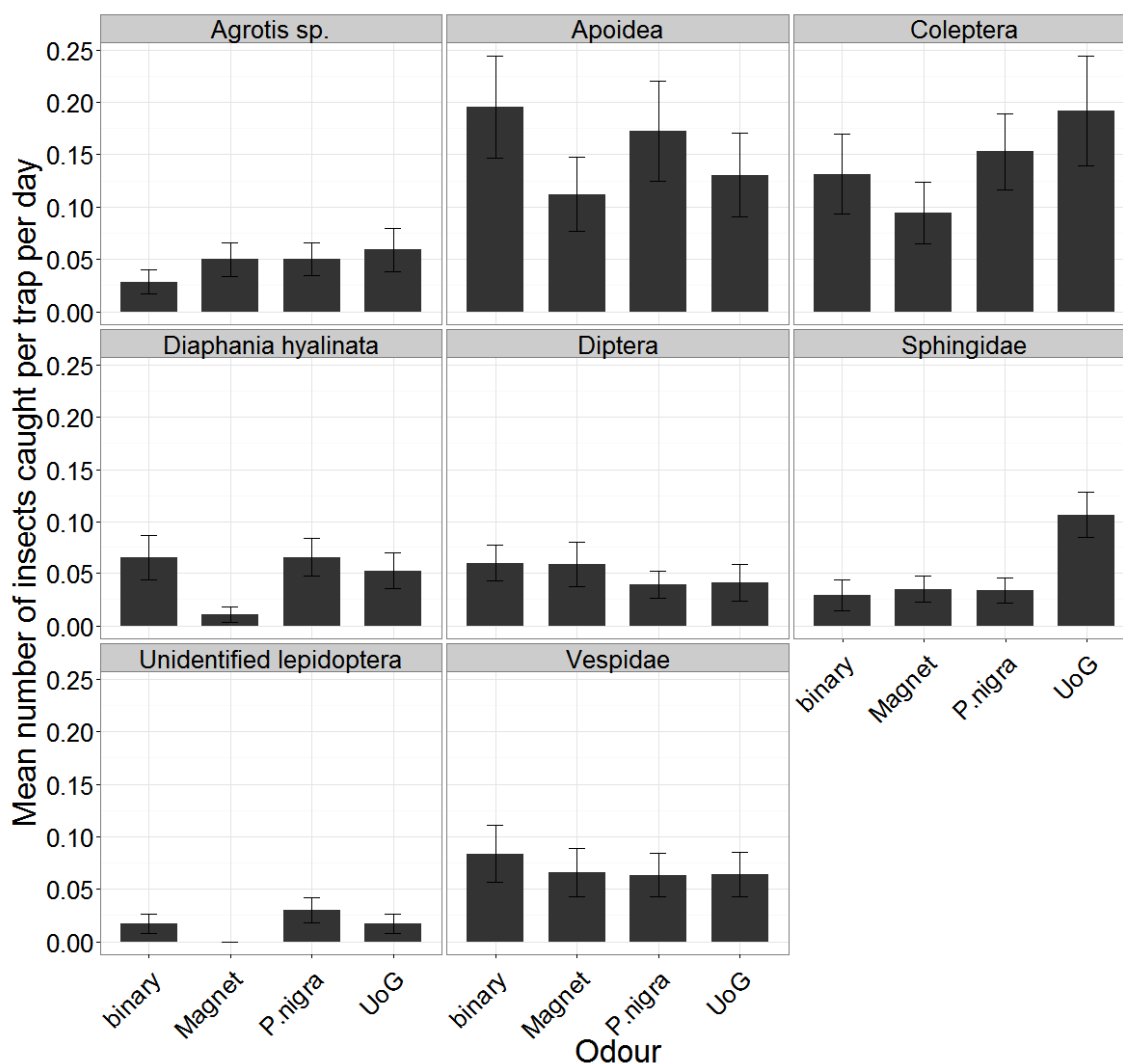growing cotton the other maize) were found to have significant effects on the species caught in the traps.

Figure 7.8: Mean number of insect groups caught per trap per day in traps baited with floral odour blends in field trial 3. Error bars show ±se mean. Data analysed by GLM with negative binomial distribution; for the insect group 'Sphingidae' the factor 'treatment' was found to be significant, N = 24, $X^2_{(3,92)}$ = 11.32, *P* < 0.05, but Tukey's pairwise comparisons found no significant differences between the treatments. No other significant differences were found between the odour blends for the other insect groups.

7.3.3 Field trial 4

The numbers of non-target insects caught in traps during field trial 4 were significantly affected by the odour treatment and the date. All of the odour

baited lures caught significantly more of the insect groups Apoidea and Syrphidae than the unbaited control traps (Figure 7.9). The addition of methyl salicylate, β-myrcene, or benzyl salicylate to the UoG blend resulted in a significant increase in the mean catches of insects within the Meligethes genus; the addition of methy salicylate, β-myrcene, methyl benzoate or benzyl benzoate resulted in a significant increase in the mean catches of insects in the Tachinidae group.
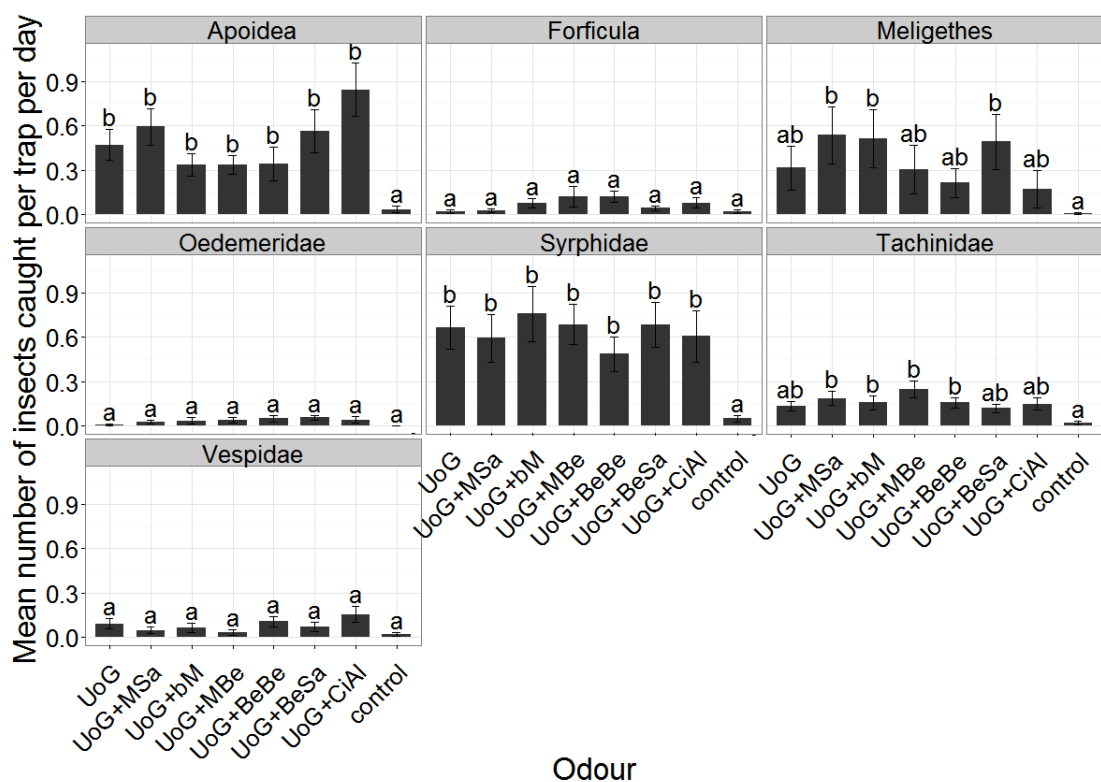

Figure 7.9: Mean number of insect groups caught in traps baited with floral odour blends in field trial 4. Error bars show ±se mean, within each chart bars that share the same letter are not significantly different. The data was tested by GLM with negative binomial distribution and Tukey's pairwise comparisons. For Apoidea $X^2_{(7,216)}$ = 57.65, P < 0.001; for Syrphidae $X^2_{(7,216)}$ = 46.61, P < 0.001; and for Tachinidae $X^2_{(7,216)}$ = 25.64, P < 0.001; for Forficula $X^2_{(7,216)}$ = 17.36, P < 0.05; for Meligethes $X^2_{(7,216)}$ = 14.95, P < 0.05; for Oedemeridae $X^2_{(7,216)}$ = 16.00, P < 0.05; and for Vespidae $X^2_{(7,216)}$ = 14.22, P < 0.05.
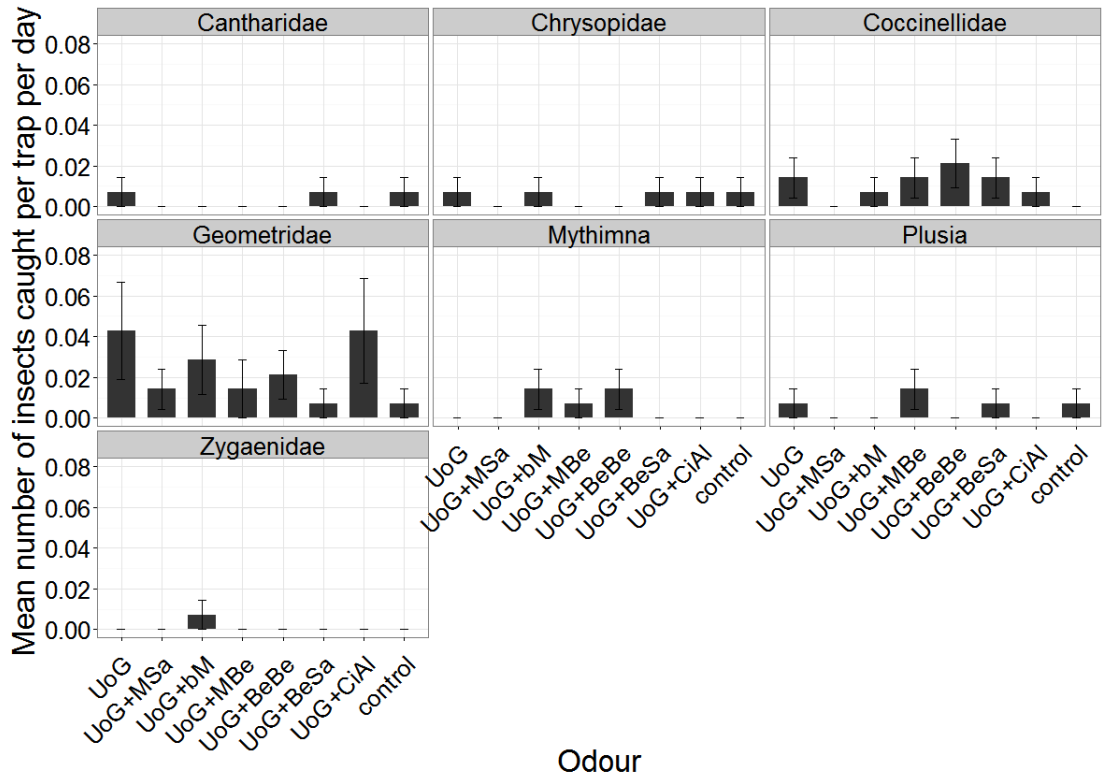
Figure 7.10: Mean number of insect groups caught in traps baited with floral odour blends in field trial 4. Error bars show ±se mean, no significant differences were found between treatments for each of the insect groupds. Data was tested by GLM with negative binomial distribution.
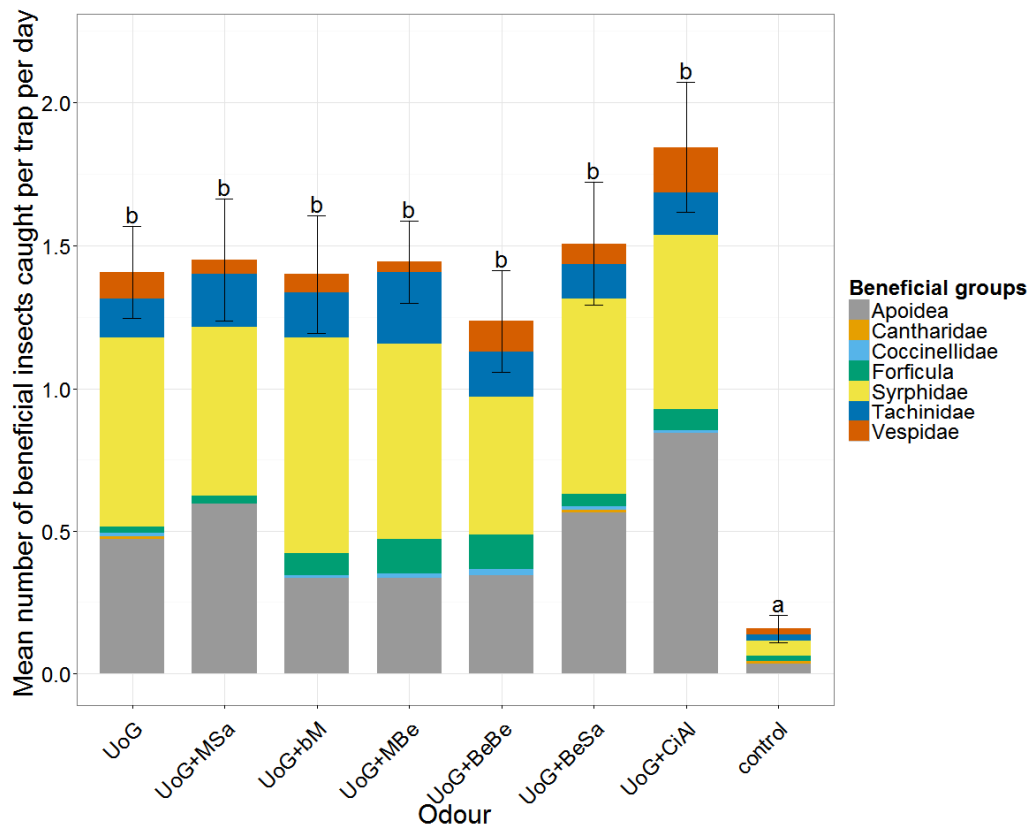
Figure 7.11: Mean number of insects considered as beneficial caught per trap per day in traps baited with floral odour blends from field trial 4. For statistical analysis insect groups were pooled. Error bars show ±se mean, bars with the same letters are not significantly different. Data analysed by GLM with negative binomial distribution followed by Tukey's pairwise comparisons. N = 28, $X^2_{(7,216)}$ = 119.09, $P < 0.001$).
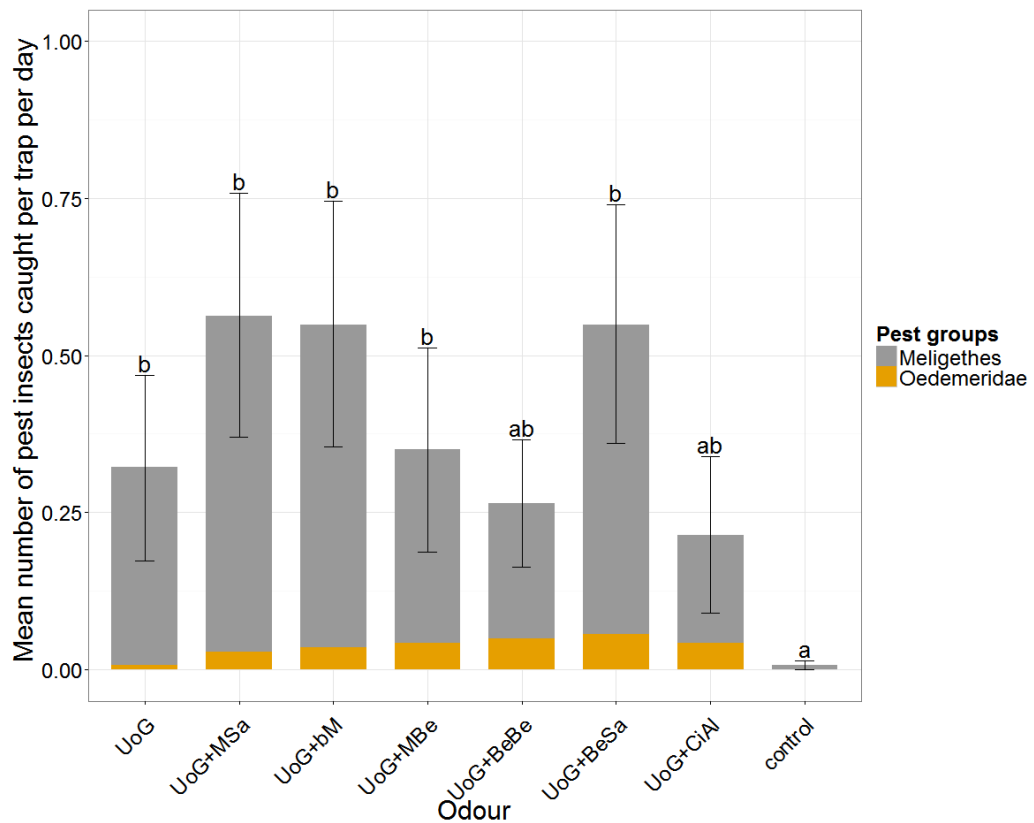
Figure 7.12: Mean number of insects considered as pests caught per trap per day in traps baited with floral odour blends from field trial 4. For statistical analysis insect groups were pooled. Error bars show ±se mean, bars with the same letters are not significantly different. Data analysed by GLM with negative binomial distribution followed by Tukey's pairwise comparisons. N = 28, $X^2_{(7,216)}$ = 22.64, $P < 0.01$).

For the insects in the groups Apoidea, Meligethes, and Syrphidae (i.e. insects caught in the largest numbers relative to the other groups) the numbers of insects caught declined over the course of the four week trial (Figure 7.13). Other insect groups (Cantharidae, Chrysopidae, Coccinellidae, Dermaptera, Oedemeridae, Tachinidae, and Vespidae) showed low initial catch rates which rose during the four week trial (Figure 7.14).
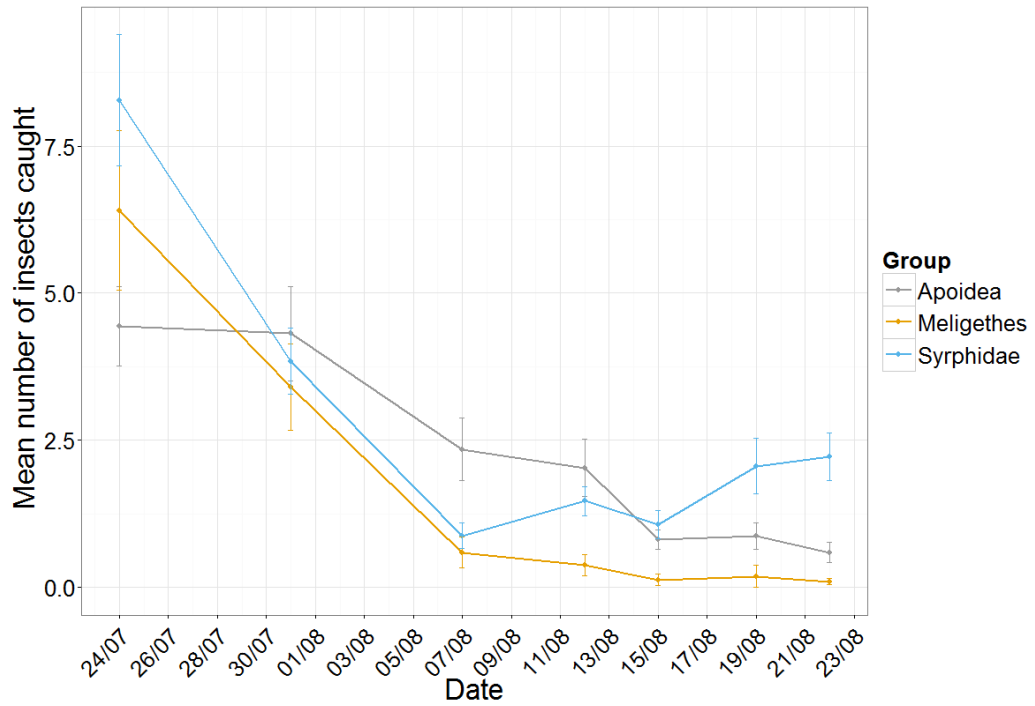
Figure 7.13: Mean number of insect groups caught in traps baited with floral odour blends over the course of field trial 4. Error bars show ±se mean.
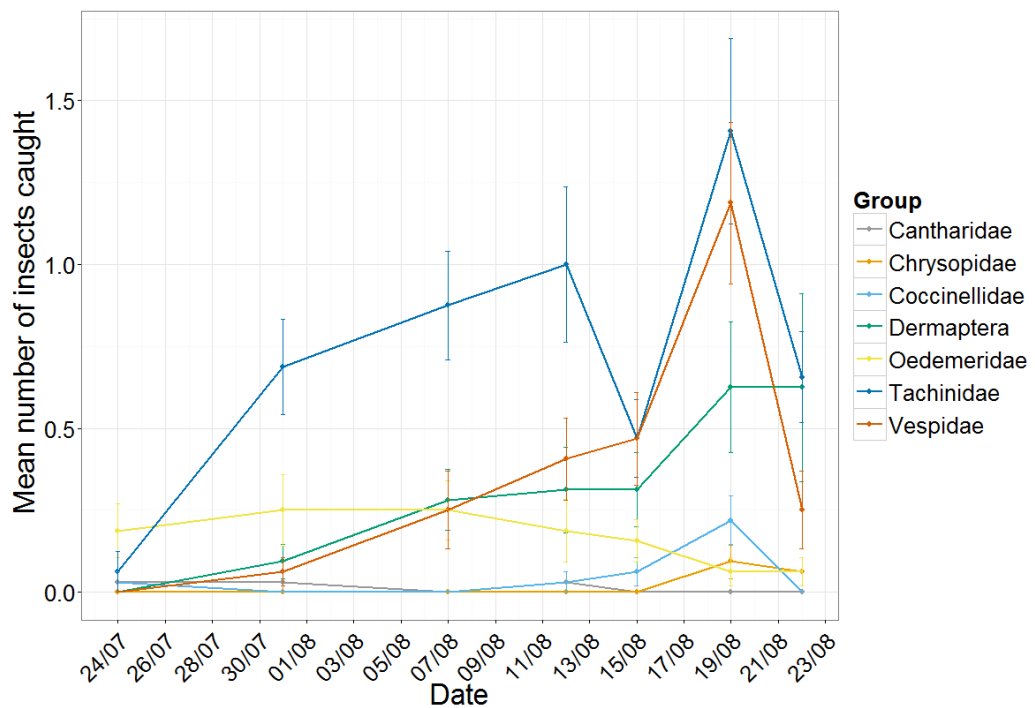

Figure 7.14: Mean number of insect groups caught in traps baited with floral odour blends over the course of field trial 4. Error bars show ±se mean.

7.3.4 Field trial 5

The numbers of non-target species insects caught in traps during field trial 5 were not significantly affected by the odour lure. However, the date was found to be a significant variable as was the field location for some insect groups.

Insects in the groups Meligethes and Syrphidae followed similar patterns of rising and falling during the field trial. Other insect groups (Empididae and Cantharidae) were not caught in traps until 16/07 peaking on the 20/07 and dropping on 25/07, whereas catches of *Apis mellifera* rose on this date. The groups Chrysopidae and Oedemeridae maintained a steady low catch rate dropping off towards the end of the trial.
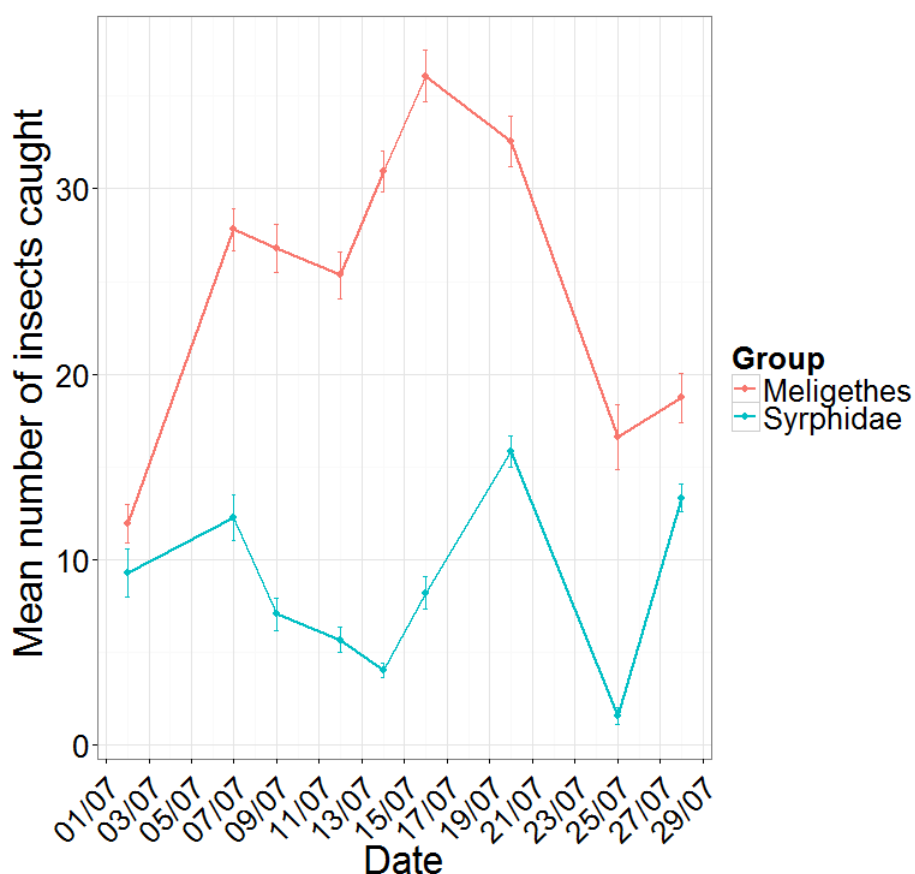

Figure 7.15: Mean number of insect groups caught in traps baited with floral odour blends over the course of field trial 5. Error bars show ±se mean.
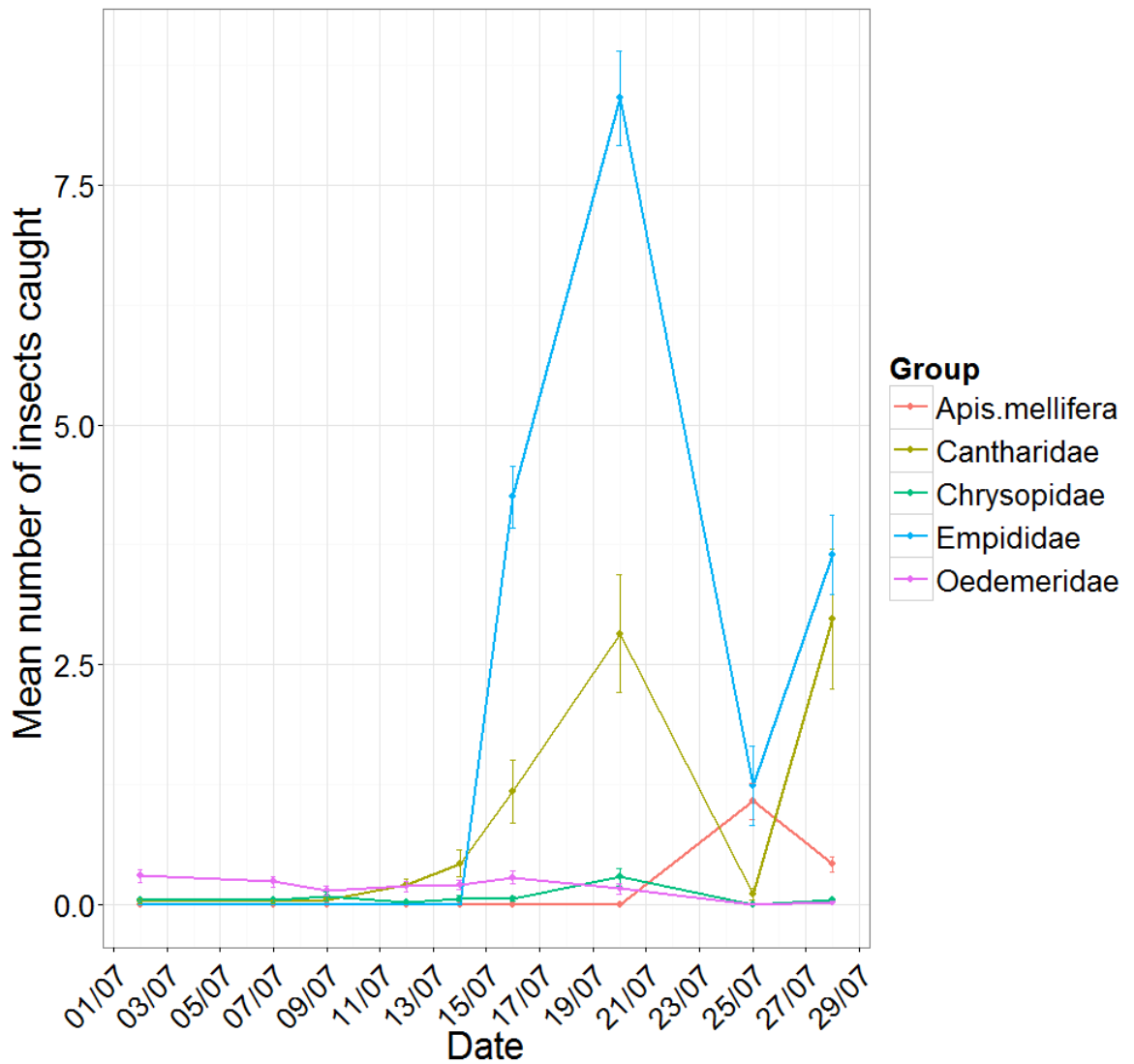
Figure 7.16: Mean number of insect groups caught in traps baited with floral odour blends over the course of field trial 5. Error bars show ±se mean.

The majority of insect groups were caught in significantly greater numbers in the traps positioned around the *Echium vulgare* crop (Figure 7.17, Figure 7.18, and Figure 7.19). The exception to this was those insects within the Syrphidae group and the Chrysopidae which were caught in significantly greater numbers in the field containing field peas (Figure 7.17 and Figure 7.19).
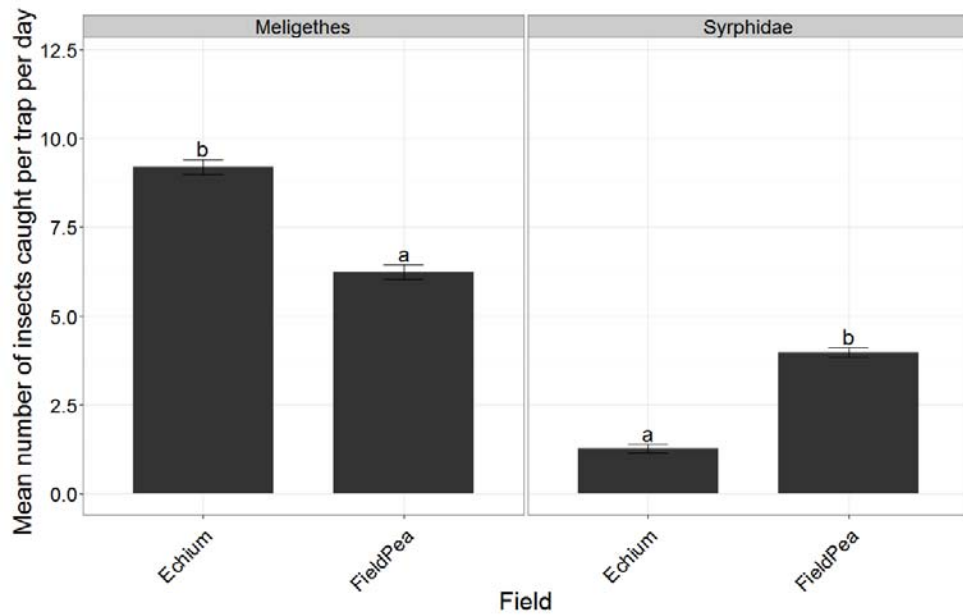
Figure 7.17: Mean number of insect groups caught per trap per day in traps baited with floral odour blends at the two field sites used in field trial 5. Error bars show ±se mean, within each chart bars that share the same letter are not significantly different. Data analysed by test by GLM with a quasipoisson distribution and Tukey's pairwise comparisons. For Meligethes N = 411 and 407 (Echium and Field pea, respectively), $X^2_{(1, 816)}$ = 763, P < 0.001; for Syrphidae N = 411 and 407 (Echium and Field pea, respectively), $X^2_{(1, 816)}$ = 1971.3, P < 0.001.

Figure 7.18: Mean number of insect groups caught per trap per day in traps baited with floral odour blends at the two field sites used in field trial 5. Error bars show ±se mean, within each chart bars that share the same letter are not significantly different. Data tested using GLM with negative binomial distribution and Tukey's pairwise comparisons. N = 411 and 407 (Echium and Field pea, respectively) for all insect groups. For Bombus $X^2_{(1,816)}$ = 15.65, P < 0.001; for Cantharidae $X^2_{(1,816)}$ = 127.68, P < 0.001; and for Oedemeridae $X^2_{(1,816)}$ = 24.87, P < 0.001; for Apoidea $X^2_{(1,816)}$ = 7.12, P < 0.01.
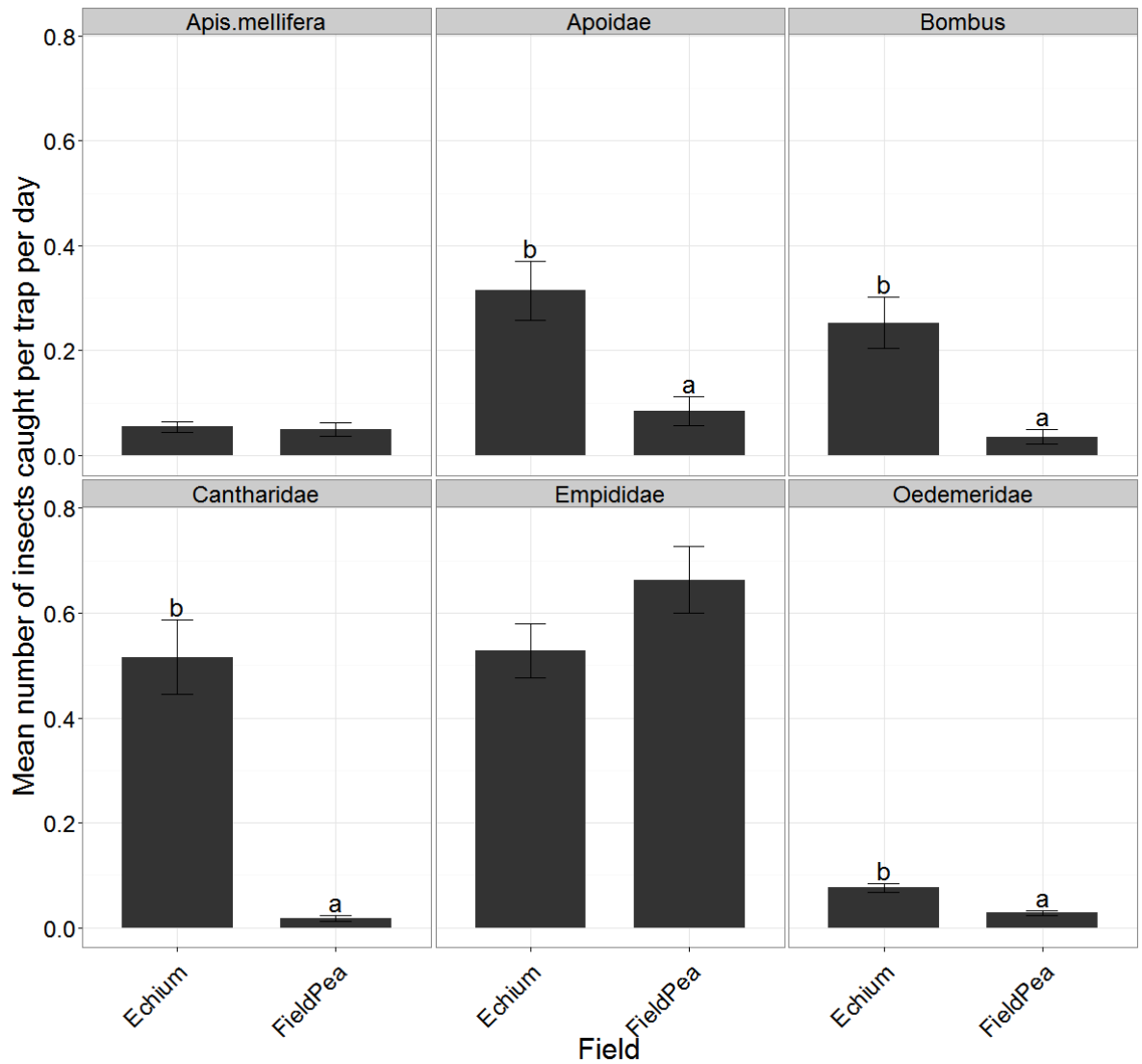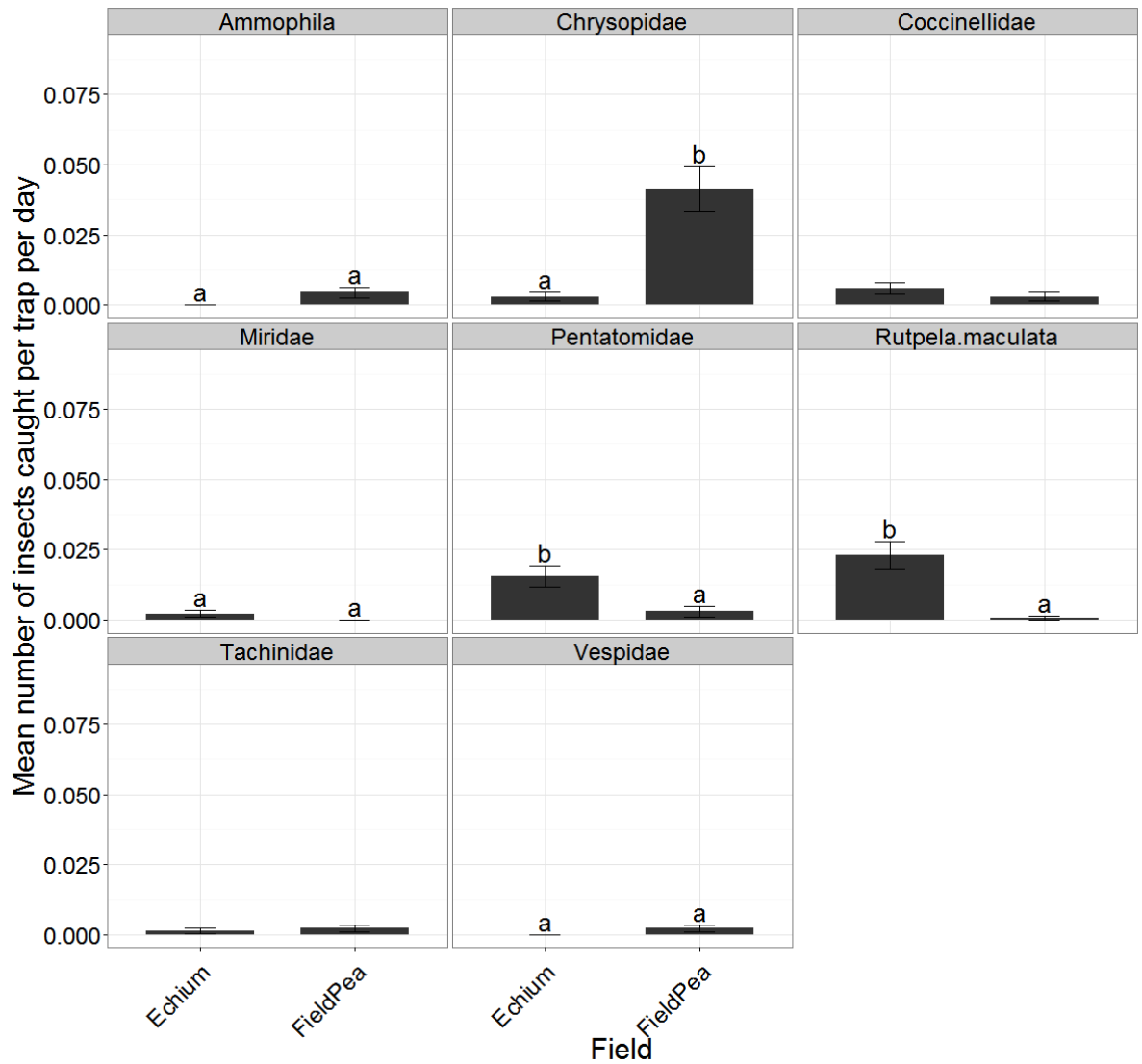
Figure 7.19: Mean number of insect groups caught per trap per day in traps baited with floral odour blends at the two field sites used in field trial 5. Error bars show ±se mean, within each chart bars that share the same letter are not significantly different. Data analysed by GLM with negative binomial distribution and Tukey's pairwise comparisons. N = 411 and 407 (Echium and Field pea, respectively) for all insect groups. For Chrysopidae $X^2_{(1,816)}$ = 38.96, P < 0.001; for Rutpela maculata $X^2_{(1,816)}$ = 31.17, P < 0.001; for Ammophila $X^2_{(1,816)}$ = 8.38, P < 0.01; and for Pentatomidae $X^2_{(1,816)}$ = 10.25, P < 0.01; for Miridae $X^2_{(1,816)}$ = 4.13, P < 0.05; and for Vespidae $X^2_{(1,816)}$ = 4.19, P < 0.05.

7.3.5 Field trial 7

The colour of the traps baited with the UoG odour blend significantly affected

the catches of non-target insects. For the bees species caught (*Apis mellifera*,

Bombus hortorum, *B. lapidarius*, *B. lucorum/B terrestris/B. ruderatus*, *B.*

pascuorum, and *Lasioglossum leucozonium*) highly significant differences were

found for the colour of the trap and the mean catches. The Bombus species: *B. lucorum, B. terrestris,* and *B. ruderatus* can be difficult to tell apart accurately in field collected specimens due to hair loss and discolouration, and therefore were grouped. *A. mellifera* and *L. leucozonium* were caught in significantly larger numbers in control and white traps compared to yellow, red, and green traps (the control traps remained in their original colours of a white base, yellow funnel, and green lid). Of the genus *Bombus* the majority of species were either *lucorum*, *terrestris*, or *ruderatus* which were caught significantly more in blue and white traps compared to green traps. As it is difficult to separate these three *Bombus* species it was thought sensible to group them, but the likelihood is that they were primarily *B. terrestris* which is more common than the others. Pooling the bee data showed that green traps caught significantly fewer bees than the other traps (Figure 7.20).
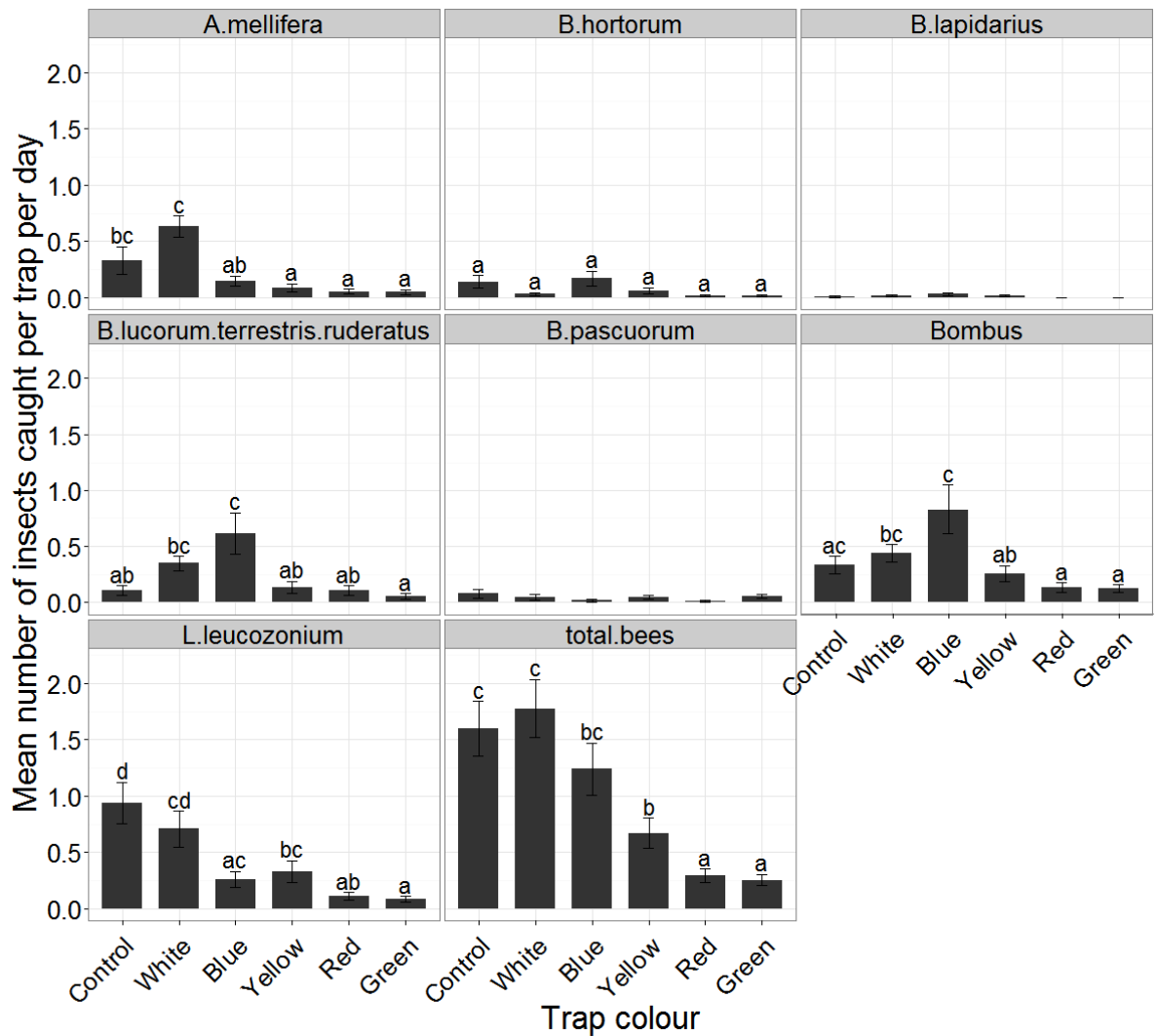
Figure 7.20: Mean number of insect caught per trap per day in different coloured traps baited with the UoG odour blend in field trial 7. Error bars show ±se mean, within each chart bars that share the same letter are not significantly different. The data was analysed by GLM with negative binomial distributions, followed by Tukey's pairwise comparisons. For all insect groups N = 26. For Total bees $X^2_{(5,150)}$ = 98.72, P < 0.001; for *L. leucozonium* $X^2_{(5,150)}$ = 59.25, P < 0.001; for *B. hortorum* $X^2_{(5,150)}$ = 22.35, P < 0.001; for *B.lucorum/terrestris/ruderatus* $X^2_{(5,150)}$ = 41.10, P < 0.001; for *B.*, and for *A. mellifera* $X^2_{(5,150)}$ = 66.43, P < 0.001; for *B. lapidarius* and *pascuorum* P > 0.05.

For the other insect groups significant differences were found for the Syrphidae which were caught in significantly greater numbers in the control traps compared to the blue, red and green coloured traps. In addition the blue coloured traps caught significantly fewer Vespidae than the control traps. For the other insect groups, although 'trap colour' was found to be a significant

factor for some groups, no differences were found after Tukey's pairwise comparisons (Figure 7.21).
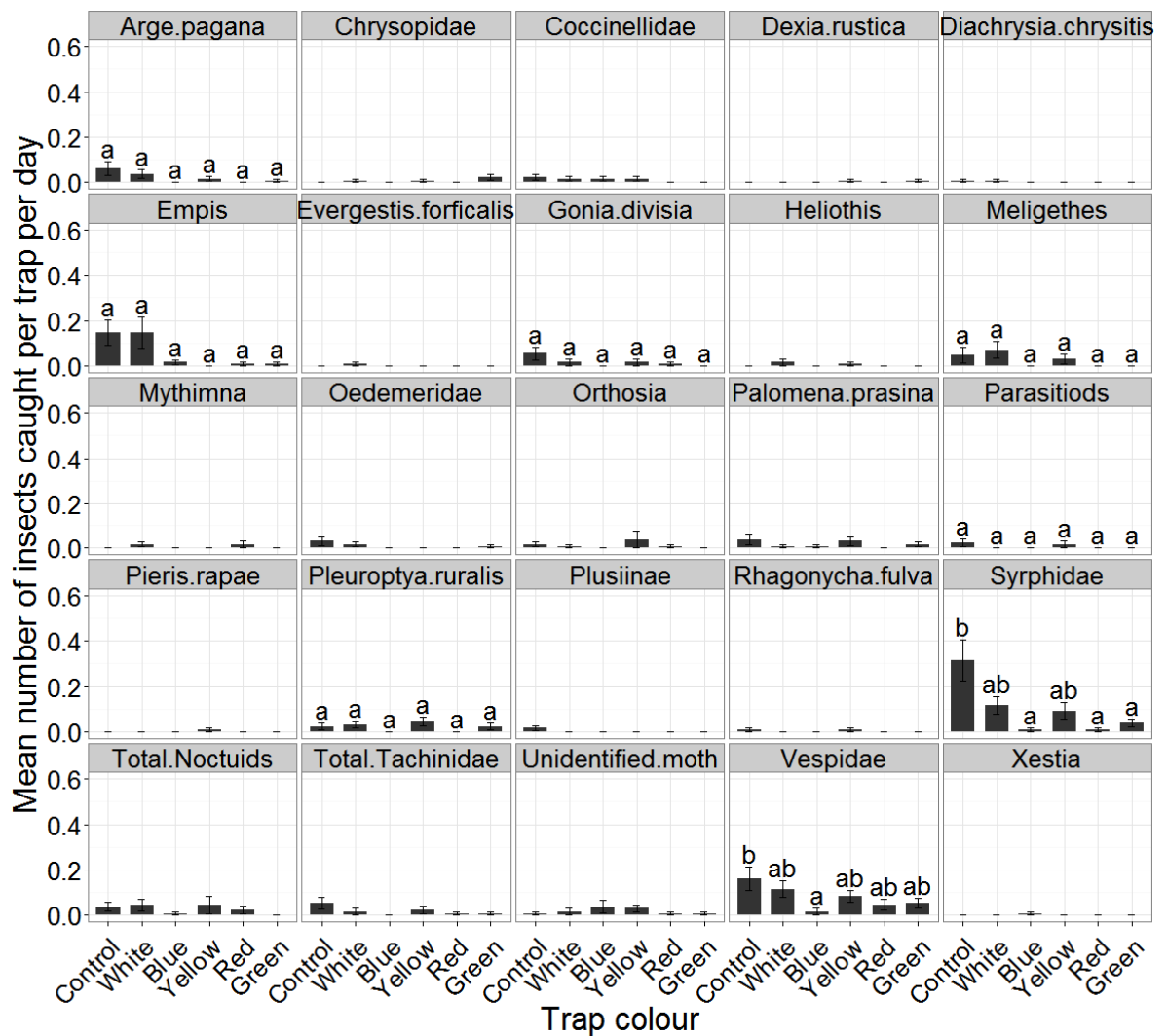
Figure 7.21: Mean number of insect caught per trap per day in different coloured traps baited with the UoG odour blend in field trial 7. Error bars show ±se mean, within each chart bars that share the same letter are not significantly different. The data was analysed by GLM with negative binomial distribution followed by Tukey's pairwise comparisons where appropriate. For all insect groups N = 26, df = 5. For Meligethes X^2 = 28.34, P < 0.001; for Syrphidae X^2 = 41.79, P < 0.001; and for Empis X^2 = 34.59, P < 0.001; for *Arge pagana* X^2 = 16.04, P < 0.01; for Vespidae X^2 = 16.42, P < 0.01; for Parasitoids X^2 = 11.19, P < 0.05.

7.4 DISCUSSION

During the UK field trials the insect groups caught in the largest numbers were those in the family Syrphidae (hoverflies), the genus Meligethes and the family

Apoidae (bees). Of the genus Meligethes it is most likely that all of the insects caught were of the species *Meligethes aeneus*, a pollen beetle commonly found in the UK and a pest of oilseed rape (which was growing near to the *Echium vulgare* field in trial 5). Other insect groups caught in notable numbers included those in the family Muscidae, the genus Forficula (earwigs almost certainly of the species *F. auricularia*), and the family Oedemeridae (pollen-feeding beetles or 'false blister beetles').

7.4.1 Syrphidae

Hoverflies were caught in traps at all of the field trials. In Argentina Diptera were not identified beyond Order due to time constraints, but it was reported by the students collecting the field data that hoverflies made up a large proportion of the Diptera caught there. Some species of hoverfly species include some important pollinators and their larvae are predators of numerous sap-sucking crop pests (e.g. aphids, thrips, etc), and so the high numbers of hoverflies present in the floral baited traps may be considered a significant disadvantage to using these compounds in a crop protection strategy.

It is know that the antennae of Syrphids are sensitive to floral volatiles. For example, *Episyrphus balteatus* (De Geer) (Syrphidae) can detect several floral compounds including phenylacetaldehyde, methyl salicylate, linalool, and 2-phenylethanol (Primante and Dotterl, 2010). The results from the current study clearly demonstrate that hoverflies are attracted to floral volatiles by the significantly greater numbers that were caught in odour baited traps compared to the unbaited control traps (Figure 7.3 and Figure 7.9). However, no differences were found between the odour blends tested which may suggest that as generalists they are not attracted to specific floral compounds but a wide

range of plant compounds. It is also possible that because the insect group was only identified down to the Family level, potential differences in responses to the floral blends between Syrphid species were not identified.

Previous research has found that hoverflies are attracted to floral compounds, specifically: methyl salicylate (James and Price, 2004), 2-phenylethanol (Zhu and Park, 2005). However, neither of these significantly increased catches when added to the UoG blend in field trial 4 and 5. Perhaps this is because the proportion of these compounds in the blends was low, or perhaps the added compounds are not any more attractive than the other compounds present in the UoG blend. Some species within the Family Syrphidae have been reported to not respond behaviourally to several of the floral compounds used during these field studies (benzaldehyde, linalool, and limonene) (for a review see Kaplan, 2012). Indeed, although the binary blend (phenylacetaldehyde and salicyaldehyde) had a lower mean catch of Syrphids than the UoG blend (which is similar to the binary blend with the addition of linalool, limonene, and methyl 2-methoxybenzoate) the difference was not found to be significant (Figure 7.3), suggesting that linalool, limonene (and methyl 2-methoxybenzoate) are not particularly behaviourally stimulating for Syrphids, but phenylacetaldehyde and/or salicylaldehyde are. Likewise the addition of benzaldehyde to the UoG+benzyl benzoate+cinnamyl alcohol (UoG+BB/CA) blend did not significantly affect the mean catches of Syrphidae (section 7.3.4).

Although significantly fewer hoverflies were caught in the unbaited control traps a substantial number were still found in these traps, which suggests that the insects are not only using olfactory cues to locate their resources but also visual cues and it is likely that the colour of the traps was attractive to these day-flying

insects. Syrphids have previously been shown to have a preference for yellow coloured flowers over white, but were attracted to both colours (Campbell *et al.*, 2010). In field trial 7, the control traps caught significantly more Syrphids than the blue, red, and green coloured traps, but not more than the white and yellow traps. As the control traps were predominately white and yellow, this suggests that this family of insects do have a propensity towards white and yellow colours as demonstrated in the work by Campbell *et al.* (2010). However, although the insects do utilise visual cues their primary sensory apparatus is likely to be their olfactory organs as the addition of odour baits to the traps significantly increased Syrphid captures. Further testing by removing any visual stimulus and providing only olfactory cues may confirm this but is out of the scope of this research.

7.4.2 Meligethes

Pollen beetles (*Meligethes aeneus*) were trapped in substantial numbers in almost all of the UK-based field trials (field trials 2, 4, and 5) with the exception of field trial 7 (testing trap colour) where it was caught in much lower numbers. Pollen beetles are considered one of the most important agricultural pests of oilseed rape (Alford, 2000) damaging the buds and flowers and therefore an effective odour bait could prove useful for crop protection. Previous work on the olfaction of pollen beetles has found that they respond electrophysiologically and behaviourally to a wide range floral and green leaf volatiles, including the aromatic isothiocyanates relating to their preferred hosts, the Brassicaceae (Smart and Blight, 2000 and the references therein; Cook *et al.*, 2002). Floral volatiles found to stimulate significant attractive behavioural responses in *M. aeneus*, included phenylacetaldehyde, 2-phenylethanol, indole, and (*E*)-4,8-

dimethyl-1,3,7-nonatriene (DMNT) (Smart and Blight, 2000), whilst (±)-linalool and (±)-linalyl acetate were shown to repel the insect (Mauchline *et al.*, 2008). All of the odour blends tested in field trial 2 caught significantly more pollen beetles than the odour blank control traps. This may be attributed to the binary, Magnet, UoG, and G9 blends having a large proportion of phenylacetaldehyde whilst the *P. nigra* blend had a much smaller proportion of phenylacetaldehyde but also contained 2-phenylethanol (phenylethyl alcohol). In field trial 4, the addition of either methyl salicylate, β-myrcene, or benzyl salicylate appeared to increase the attraction of the UoG odour blend to *M. aeneus*. Methyl salicylate has previously shown to be attractive to *M. aeneus* (Smart and Blight, 2000 and the references therein). β-Myrcene is known to be a major component of oilseed rape flower and bud headspace (Jönsson *et al.*, 2005) and the odour of whole flowers have been shown to be attractive to the beetles (Cook *et al.*, 2002). The other main volatiles emitted from oilseed rape flowers include benzaldehyde, methyl benzoate, limonene, and phenylacetaldehyde, the latter two being present in UoG and G9 blends might also explain their slightly higher mean captures compared to the other odour blends.

Although trap colour was found to have a significant effect on the mean captures of Meligethes in field trial 7, pairwise testing did not identify any significant differences between the trap colours tested (Figure 7.21). *M. aeneus* are known to be attracted to yellow and white objects (Blight and Smart, 1999 and the references therein) and the slightly higher mean capture of Meligethes in the control, white, and yellow traps (Figure 7.21) is in agreement with the results of these previous researchers.

The absence of an odour bait significantly reduced the captures of Meligethes indicating the importance of olfactory over visual cues for these insects. Indeed, Ruther and Thiemann (1997) showed that *M. aeneus* locates its host plant before the typical yellow colour has developed further confirming that olfactory cues are more important to this insect than visual.

7.4.3 Apoidea

Within the individual bee families no significant differences were identified between the odour treatments. However, by pooling the bee data (termed "Apoidea" and "Total.Bees" in the figures) it was found that the odour blends caught significantly more bees than the unbaited control traps (Figure 7.9) except for the *P. nigra* odour blend (Figure 7.5). It is not surprising that bees were found to be attractive to the floral odour blends as they are known to use olfactory cues to locate nectar resources (Wenner *et al.*, 1969). The lower mean captures of bees in the *P. nigra* baited traps may be due to the blend containing only a very small proportion of phenylacetaldehyde and a large proportion of salicylaldehyde, or the presence of benzaldehyde, benzyl alcohol, or 2-phenylethanol, compared to the other odour blends. Species of Halictid bees within the subgenus *Dialictus* (within the genus *Lasioglossum*) have been found to be attracted to a synthetic floral odour blend derived from the entrainment of a pseudoflower induced by a fungus infecting cruciferous host plants and containing phenylacetaldehyde, 2-phenylethanol, benzaldehyde and methyl benzoate (Roy and Raguso, 1997). Using proboscis conditioning experiments with the bee species *A. mellifera*, Blight *et al.* (1997) concluded that the bees were utilising only eight of the sixteen compounds identified in entrainment samples of oilseed rape flowers to identify that particular odour blend. These

eight include *α*-pinene, phenylacetaldehyde, *p*-cymene, *α*-terpinene, linalool, 2-phenyl ethanol, (*E,E*)-*α*-farnesene, and 3-carene, and further testing found that three of these compounds (phenylacetaldehyde, linalool, and (*E,E*)-*α*-farnesene) were the main compounds used by the bees to recognise the floral odour. This suggests that although the bees are capable of detecting a wide range of floral compounds, they may actually only rely on a few key volatiles to locate their nectar resources. Previous proboscis conditioning experiments with *A. mellifera* also showed that the insects were more easy conditioned with linalool, 2-phenyl ethanol, and methyl salicylate than benzyl alcohol, (*E*)-2-hexenal, and 1-octen-3-ol (Pham-Delegue *et al.*, 1993). These experiments suggest that *A. mellifera* may 'remember' certain floral volatiles over others and use these to identify suitable (or perhaps unsuitable) nectar resources. If other genera of bees use similar tactics then the presence of phenylacetaldehyde in all of the blends, plus linalool in the UoG and G9 blends may explain the relatively high number of bees being caught in the odour baited traps, as both of these compounds are extremely common floral odours and therefore would indicate to a foraging bee that nectar was available at the source of the odour blend. Although the *P. nigra* blend contains phenylacetaldehyde and 2-phenyl ethanol, the former only constitutes a very small proportion of the blend, and its possible that the other compounds in the blend are not behaviourally stimulating (or perhaps repellent) to bees and thus causing a reduction in the number of bees attracted to the traps. Finally, the ability of bees to learn from experience may dramatically influence the results of these types of field trials, because the bees foraging in the vicinity of the trial are likely to be most attracted to floral compounds they have already come into contact with whilst feeding from

flowers. Their responses to the floral baits will therefore depend on the local flora.

In field trial 4 the addition of single compounds to the UoG blend had no significant effect, although the addition of cinnamyl alcohol resulted in mean captures that were double that of the blends that had β-myrcene, methyl benzoate, or benzyl benzoate added. Methyl salicylate has previously been suggested as a possible repellent to bees (Henning *et al.*, 1992; Eby *et al.*, 2013), but the data from field trial 5 (data not shown) is in agreement with the work of Mayer (1997) which could not identify such an affect.

Trap colour has been used in similar research to mitigate captures of non-targets (especially Hymenoptera) (e.g. Meagher R.L, 2001; Knight and Fisher, 2006). The colour of the traps had a significant effect on all three bee genera caught in field trial 7. Generally the bee groups followed similar patterns such that higher numbers of bees were found in traps coloured white or blue, or in the control traps (which were white, yellow, and green) and lower numbers were caught in red and green traps (Figure 7.20). Bees are known to use a mixture of olfactory and visual (shape and colour) cues to navigate their surroundings (Wenner *et al.*, 1969; Roy and Raguso, 1997; Simonds and Plowright, 2004) but may rely more on visual cues than odour to discriminate between suitable food sources (Odell *et al.*, 1999). The data from this field trial corroborates with previous work that shows that trap colour has a significant effect on the numbers of bees caught in traps baited with plant odours. In terms of specific colour preferences it has been previously shown that Apis and Bombus species have an innate preference for blue (wavelength of around 420 nm) (for a review see Morawetz *et al.*, 2013 and the references therein), whilst they are least

attracted by red and green painted traps (Knight and Fisher, 2006). The data in Figure 7.20 for Bombus species and *A. mellifera* agrees with these previous findings.

The colours that humans see are not seen in the same way by other animals it is therefore much more informative to describe colours by their reflection spectra (Lunau and Maier, 1995). This data was collected and can be seen in Figure 7.1.

7.4.4 Other non-targets

Little is known regarding the behaviour of Earwigs towards floral or plant (or other) odours (Solomon *et al.*, 2000 and the references therein). The insects are extremely common in the UK and are highly active foragers. Duing the field trials many Earwigs were found sheltering inside the hollow bamboo poles used to hold the traps, which may in part, be due to them being thigmotactic. They were also commonly found in the traps themselves. As Earwigs were often found in traps that contained the partially eaten remains of other insects, and *Forficula auricularia* is omnivorous, it seems highly likely that much of the damage done to the bodies of other trapped insects was the result of Earwigs feeding. Figure 7.4 shows that floral odour baited traps caught significantly more Earwigs than unbaited control traps. Although the Earwigs may well have been attracted into the traps by the presence (or smell) of other insects, it is also possible that the Earwigs were attracted by the odour baits as they do feed on fruits so may be attracted to the smell of reproductive parts of plants.

Numerous insects within the Muscisae family were caught, particulary those of the genus Musca. Many Muscidae are important pollinators (Elberling and Olesen, 1999; Larson *et al.*, 2001) and there is evidence suggesting that flies

rely on olfactory cues rather than visual cues to locate the flowers (Roy and Raguso, 1997), therefore their attraction to floral baited traps is not surprising. The results in field trial 2 (Figure 7.4) suggest that this group are attracted to odour blends containing a large proportion of phenylacetaldehyde. Previous research has found that the common housefly, *Musca domestica* (L.) (Muscidae), is attracted to floral compounds including linalool (Zito *et al.*, 2013) which was present in the UoG and G9 blends.

7.4.5 Pest and beneficial species

As general crop pest attractants that could be used as part of a crop protection system, Figure 7.6 shows that the UoG and G9 odour blends caught the highest numbers of insects that may be considered crop pests. There is perhaps potential to use these types of floral attractants to control or monitor crop pests but further work is needed to assertain exactly which species are being caught, which compounds are actively attractive (or essential) for which species and whether additional compounds will improve the catch rates. In addition, more research into trap types and colours needs to be carried out to identify the most suitable traps to use.

The issue of catching beneficial non-targets is a serious problem for using these general insect attractants in the field. The research here shows that it may possible to partially alleviate this problem by using specifically coloured traps, especially for hymenoptera and Syrphidae beneficials. Other solutions may include the addition of specific repellent volatiles, escape holes for smaller insects (Cork, 2004), or altering the size and shape of the funnel (Ruther and Mayer, 2005) or use other retainment methods (e.g. sticky sheets).

CHAPTER 8 - EFFECTS OF TRAP DESIGN ON TRAPPING OF NOCTUIDS WITH FLORAL VOLATILES

8.1 INTRODUCTION

There are numerous types of traps available for catching pest moth species in combination with an odour lure (pheromone or kairomone). The shape, size of opening, and method of retaining the insects varies amongst these traps. Certain design features are likely to impact on how successful a trap is at catching specific species of insect. Furthermore, there may be trade-offs between some of these design features, for example a trap with a large opening may have a greater number of insects entering the trap but also allow a greater number to escape, some trap designs may have a high retention ratio (number insects retained compared to number escaping) but suffer from a low maximum capacity.

Numerous studies have found trap design to be a significant factor in catching specific insects. Different trap designs appear to be better suited to certain moth species. For example Reardon *et al.* (2006) found large metal cone traps to be the most effect trap compared to a small cone trap, UniTraps (bucket/funnel trap), and several sticky trap designs for catching male European Corn Borer (ECB) (*Ostrinia nubilalis* (Hübner) (Crambidae)) (all traps were baited with pheromone lures). The authors concluded that the pheromone lures were attracting moths to all of the traps, but the insects were not sufficiently retained in the sticky traps; in addition they postulate that the larger diameter of the large cone traps played a role in greater captures compared to the small cone trap and funnel/bucket UniTrap. In comparison, Knodel and Agnello (1990) found

sticky traps caught higher numbers of *Palpita unionlalis* (Hübner) (Pyralidae) and other moth species than various funnel ('Multipher') traps (similar in design to UniTraps). However, the authors note that moth populations were not high and because the sticky trap surfaces were changed weekly the chances of the traps becoming saturated with moths must have been reduced. Yet for the noctuid species *Spodoptera exigua* (Hübner) (Noctuidae) funnel traps (UniTrap) caught more moths than both sticky and cone traps (López Jr., 1998).

There are numerous examples of how trap design affects catches of moths and non-targets using pheromone lures (e.g. Sparks *et al.*, 1979; Raulston *et al.*, 1980; Mitchell *et al.*, 1989; Knutson *et al.*, 1998; López Jr., 1998; Reardon *et al.*, 2006), but little work has been carried out on trap designs for floral baited traps.

Catch efficiency may be defined as how successful a trap is at retaining insects that enter the trap. The structural design features that may influence catch efficiency include:

a) position of the lure

b) size of the entrance

c) number of entrances

d) method of retention (e.g. sticky surface, cone, or funnel)

e) maximum capacity

Features a, b, and c will affect the odour plume emanating from the trap, how the insects enter the trap, and therefore the number of insects entering the trap. Features d and e will affect the retention efficiency of the trap, i.e. what proportion of insects that enter the trap stay in the trap.

For almost all the field trials undertaken in this research UniTraps were used, with the exception of field trial 3 (assessing floral odour blends in Argentina) which used the Lobos Bucket trap. Although the main aim of this thesis was to investigate floral attractants for Noctuid pests, it was thought important to assess what impact different trap types may have on the catches of Noctuids and non-target insects. Field trials were carried out to compare six trap types (five of which were tested in Argentina, and two tested in the UK) baited with either pheromone lures or the UoG odour blend. A further field trial was carried out to assess whether the addition of a contact pesticide to the UniTraps significantly affected insect captures. It was expected that if insects were escaping from the UniTraps in any significant number then the addition of a contact pesticide to the trap would reduce these escapees and significantly increase the number of insect captures.

Identifying the most effective trap type is vital to any crop protection strategy that utilises a bait and trap (e.g. mass trapping or pest monitoring). The work presented here investigates three types of 'sticky' trap, two types of funnel trap, and one bucket trap containing water as the retention mechanism. The traps may be categorised by their entrance size, holding capacity, and method of retaining the captured insects.

8.2 METHODS AND MATERIALS

8.2.1 Lures

Three types of lure were used, a pheromone lure for male *H. gelotopoeon*, a pheromone lure for male *A. gamma*, and the UoG floral odour blend as a general Noctuid attractant.

8.2.1.1 <u>Pheromone lures</u>

For *H. gelotopoeon*, rubber septa (Z124389, SigmaAldrich, Gillingham, UK) were impregnated with 1 mg of a 1:1 mixture of hexadecanal (16:Ald) and (*Z*)-9-hexadecenal (Z9-16:Ald) and an equal amount of BHT as antioxidant in hexane (Cork and Lobos, 2003).
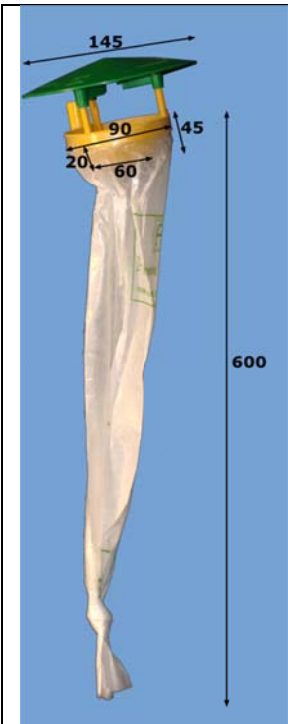
For *A. gamma*, rubber septa were impregnated with 0.1 mg of (Z)-7-dodecenyl acetate (Z7-12Ac) and (Z)-7-dodecen-1-ol (Z7-12OH) in a 10:1 ratio (Mazor and Dunkelblum, 1992; Mazor and Dunkelblum, 2005).

8.2.1.2 <u>Floral attractant</u>

Lures containing 250 µL of the UoG blend of compounds were made from a blend of phenylacetaldehyde (135 µL), salicylaldehyde (55 µL), methyl 2-methoxybenzoate (23.8 µL), linalool (23.8 µL), and limonene (12.5 µL). The compounds were pipetted onto a cellulose acetate cigarette filter (14 x 6 mm, 14 x 6 mm, Swan, Republic Technologies Ltd., UK) in polyethylene sachets (5 cm x 5 cm, Transatlantic Plastics, Southampton, UK). The sachets were heat sealed and shipped to Argentina in a coolbox, or stored at -18 °C until used for the UK field trials.

8.2.2 Traps

Six different trap designs were tested in this study. Five were tested in Argentina (the 'sleeve', 'bucket', 'Wing', Delta, and 'Lep' traps) and two were tested in the UK (the 'sleeve' trap and UniTrap).
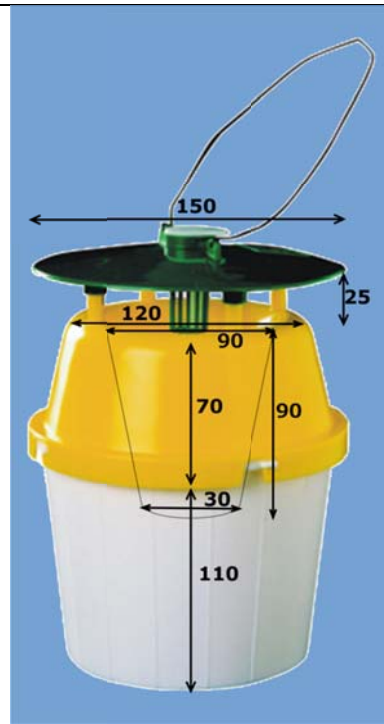
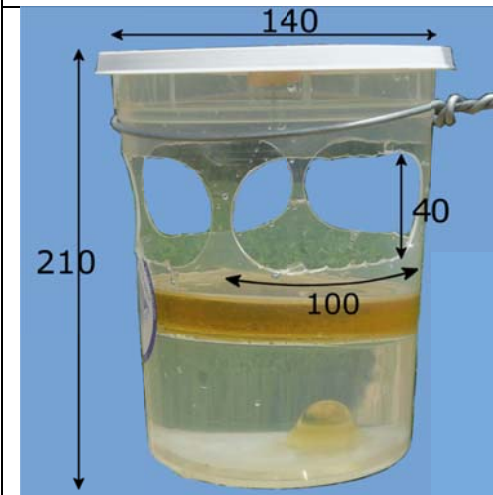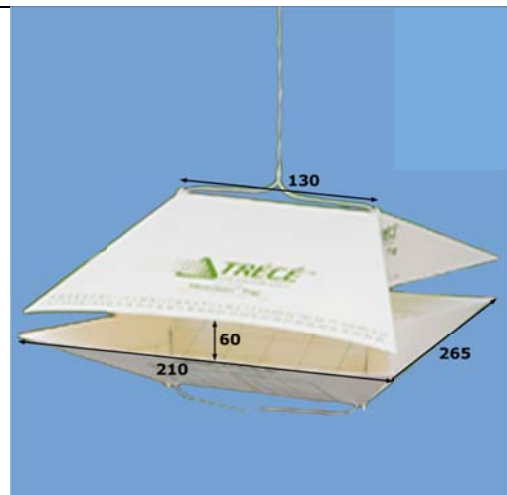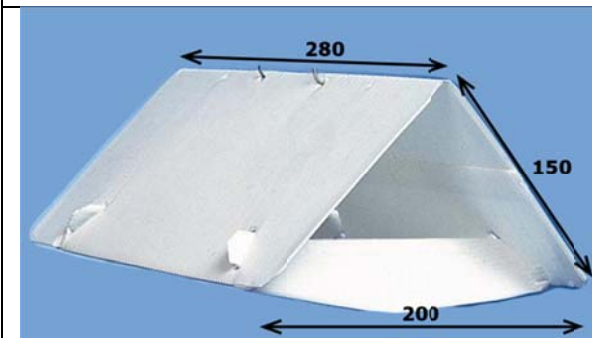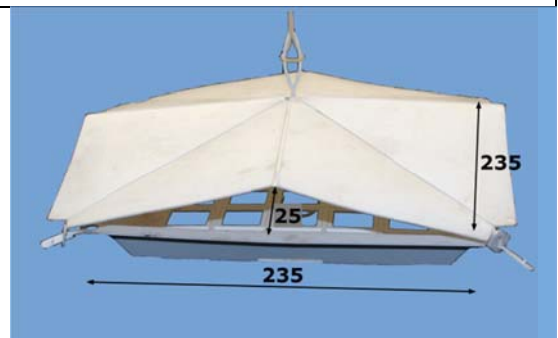Figure 8.1: Traps used in the field trials with dimensions in mm. Descriptions on the following page.

8.2.2.1 <u>Trap descriptions</u>

Top left: Sleeve trap (Pest Control India, Bangalore, India), a funnel trap with yellow funnel, a green top and polyethylene sleeve coated with a fine 'talc-like' powder to retain the insects that fall through the funnel. The lure is suspended beneath the lid (2 cm above the funnel).

Top right: Unitrap (Agrisense, Treforest, UK), a funnel and bucket trap (the funnel is depicted with a dotted line in the figure). The lure is suspended from the lid in a plastic cage and insects enter through the funnel into the bucket.

Middle left: Bucket trap (handmade by E. Lobos, Santiago Del Estero, Argentina), a bucket trap containing water (and a layer of oil to reduce evaporation). The lure is suspended from the lid and insects enter the trap through the cut out holes. Insects are trapped by sinking into the oil and water.

Middle right: Wing trap (Intercept Wing Trap, IPM Technologies Inc., Portland, OR, USA), a trap with a replaceable sticky sheet on the lower section of the trap.

Bottom left: Delta trap (AgriSense, Treforest, UK), a trap with a replaceable sticky sheet on the base of the trap.

Bottom right: Lep trap (Plato Industries, Ltd., Houston, USA), a white trap with a large sticky replaceable sheet on the bottom of the trap. The lure is positioned under the apex of the top section.

8.2.3 Trial design

8.2.3.1 <u>Trap design (Argentina) field trial 8</u>

This trial compared the insect captures of five trap designs (the 'sleeve', 'bucket', 'Wing', Delta, and 'Lep' traps) baited with either the sex pheromone of *H. gelotopoeon* or the UoG floral odour blend. Two locations were used, one at a tomato field where the crop was at the unripened fruiting stage (27°52'10.74"S; 63°56'39.43"W), and the second at the edge of a soybean field (31°34'41.58"S; 63°43'46.13"W) where the crop was in the third trifoliate stage. At each field site, two trials were run concurrently (approximately 100 m apart). One trial contained the five trap types with five replicates baited with *H. geloptopoeon* pheromone (the trap types were positioned in a latin square design) with 10 m between the traps; the other trial contained the five traps types with five replicates baited with the UoG odour blend (similarly in a Latin square design). The same set up was used at both field sites. The traps were checked once per week 3 or 4 times; after checking the traps were repositioned within their replicate and the insects removed or sticky sheets replaced.

The trials were set up with the assistance of two Masters students, Maria Ana Laguzzi and Camilo Gómez Luengo, who continued to check the traps after I had left Argentina and sent the data.

Figure 8.2: The two field sites used to carry the trap design trials in Argentina (field trial 8). The left image shows the approximate locations of traps in the tomato field; the right image shows the approximate locations of the traps adjacent to the soybean field. In both cases the Northern most group of traps were baited with *H. geletopoeon* sex pheromone, and the Southern group traps were baited with the UoG floral blend. Five trap designs were tested at all four locations, each with five replicate blocks checked three times for the tomato field and four for the soybean field. Aerial photo taken from Google Earth (2008) and modified.

8.2.3.2 <u>Trap design (UK) field trial 9</u>

The trial compared the UniTrap and 'sleeve' trap and was conducted at Gosmere Farm (Sheldwich, Kent) at the same location as that used in field trial 5. The traps were position within 10 m of a crop of *Echium vulgare* at the late inflorescence stage, or within 10 m of a crop of *Tagetes sp.* ('marigolds') again in the inflorescent stage. Traps were placed in pairs (one UniTrap and one 'sleeve' trap with 10 m between them) around the field at six locations and checked at regular intervals between 18/07/2009 to 23/08/2009. Pheromone lures were replaced once during the trial for all traps except one pair. After the number of *A. gamma* in the traps were counted the positions were swapped round within each pair of traps.

8.2.3.3 <u>Addition of pesticide (UK) field trial 10</u>

The trial assessed the addition of a contact pesticide to the base of the bucket part of the UniTraps at the *Echium vulgare* and *Tagetes* field at Gosmere Farm. The contact pesticide was Vapona (Ashe Ltd, UK containing 0.96 % of Azamethiphos, an organothiophosphate that inhibits acetylcholinesterase); the traps were baited with sachets containing the UoG blend. Each treatment (pesticide present or not present) had four replicates and the traps were positioned 10 m apart. The trial ran from 04/08/2009 to the 23/08/2009, the traps were checked six times during that period, and repositioned after being checked.

8.3 RESULTS

8.3.1 Field trial 8

8.3.1.1 <u>Noctuids</u>

Analysis by generalised linear model (using a negative binomial distribution) found significant differences in the number of Noctuids caught between the attractant used in the lures (floral or pheromone), the trap types, but not between the dates the data was collected or the crop types.

Comparing the two attractants, sex pheromone for *H. gelotopoeon* and the UoG odour blend significant differences were found between the treatments (Figure 8.3). The pheromone caught significantly more Noctuids, which were almost entirely male *H. gelotopoeon* moths, whereas the UoG blend caught far fewer moths but a mixture of Noctuid species (see Figure 8.5 for details)

Figure 8.3: Mean number of Noctuids caught in traps in field sites in Argentina. The traps were baited with either the sex pheromone for *H. gelotopoeon* or the UoG odour blend. Error bars show standard error, letters denote a significant difference between the treatments, analysed by GLM with a quasiposson distribution to account for overdispersion and Tukey's pairwise comparisons. N = 172 for both treatments, $X^2_{(1,342)}$ = 5500.5, *P* < 0.001.

The trap type was found to be a significant factor in the number of Noctuids caught in the Argentinian field trials. The 'bucket' traps (Figure 8.1) caught significantly more Noctuids than all the other traps (approximately 6-fold more Noctuids than the 'Lep', 'Wing' and 'Delta' sticky traps). The 'sleeve' trap caught significantly more Noctuids than the 'Lep', 'Wing' and 'Delta' traps, and the mean captures of latter three traps were not signficantly different (Figure 8.4).
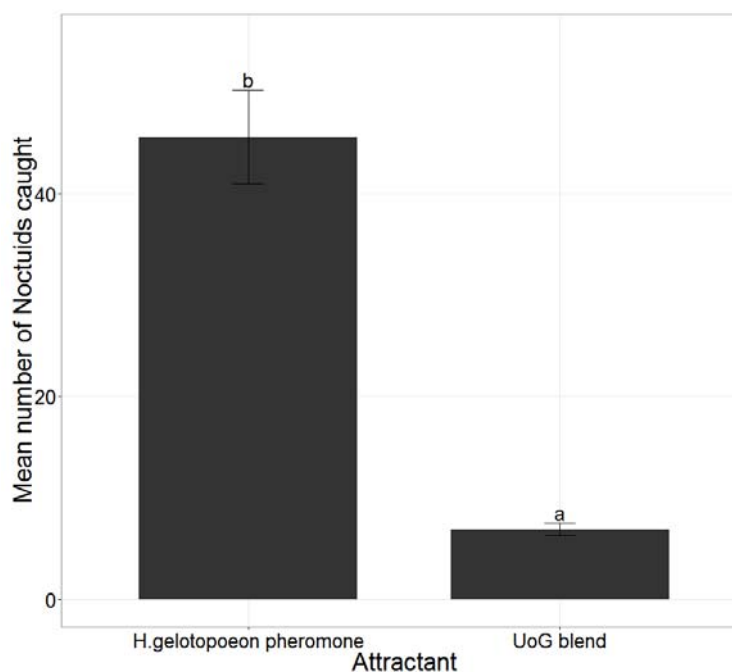

Figure 8.4: Mean number of Noctuids caught in five different traps at field sites in Argentina. The traps were baited with either the sex pheromone of *H. gelotopoeon* or the UoG odour blend. Error bars show standard error, bars that have different letters are significantly different. Data analysed by GLM with quasipoisson distribution and Tukey's pairwise comparisons. N = 70 (for 'sleeve', 'Lep', and 'Wing' traps), N = 68 (for 'bucket' traps), N = 66 (for 'Delta' traps); $X^2_{(4,339)}$ = 5635.2, *P* < 0.001.

The interaction of trap type and attractant type was found to be significant (GLM, *P* < 0.01). Between the floral and pheromone baited traps a similar pattern was found, but with less difference in the mean catches for the floral (UoG blend) baited traps (Figure 8.5). The number of Noctuid species caught in the floral baited traps was much greater than in the pheromone-baited traps, although both baits predominately caught *H. geloptopoen*.

Figure 8.5: Mean number of Noctuids caught in five trap types baited with either the sex pheromone of *H. gelotopoeon* or the UoG odour blend (floral odour) in field trials in Argentina. The breakdown of the mean numbers of individual genera or species caught are shown stacked within the bars. Error bars show standard error for the total Noctuids, bars within the same chart that have different letters are significantly different. Data analysed by GLM with a quasipoisson distribution to account for overdispersion and followed by Tukey's pairwise comparisons. N = 35 (for 'sleeve', 'lep', and 'wing' traps), N = 34 (for 'bucket' traps), N = 33 (for 'delta' traps). For traps baited with the floral odour $X^2_{(4,167)}$ = 225.78, P < 0.001; for traps baited with pheromone $X^2_{(4,167)}$ = 5666.8, P < 0.001.

8.3.1.2 Non-targets

The UoG odour blend baited traps caught significantly more non-target insects than the traps baited with the *H. gelotopoeon* sex pheromone (Figure 8.6). The difference between the two treatments was approximately 10-fold.
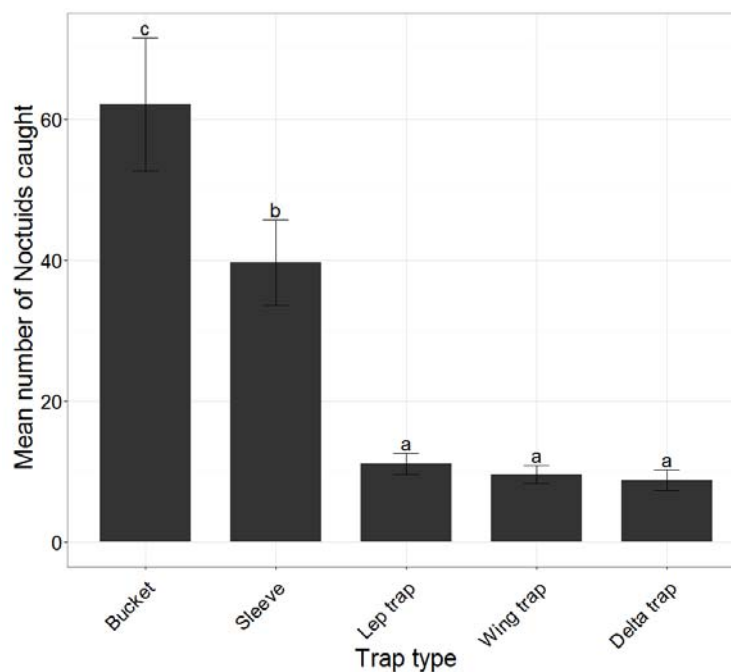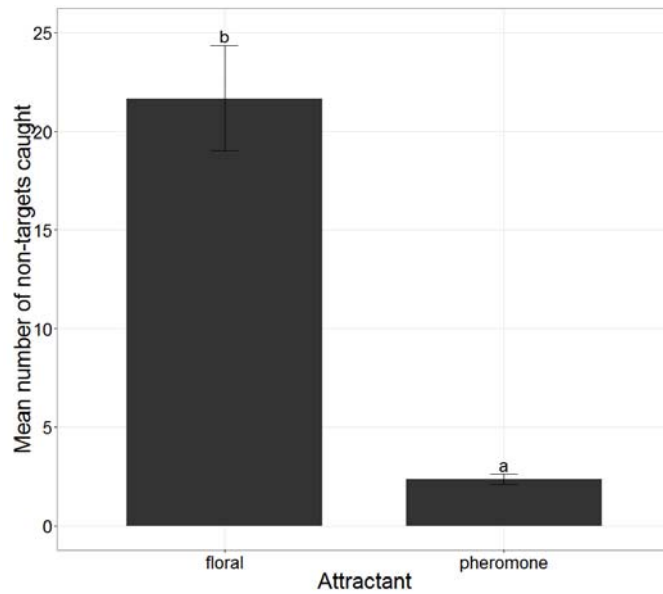
Figure 8.6: Mean number of non-target insects caught in traps in field sites in Argentina. The traps were baited with either the sex pheromone for *H. gelotopoeon* or the UoG odour blend. Error bars show standard error, letters denote a significant difference between the treatments. The data was analysed by GLM with a quasiposson distribution and Tukey's pairwise comparisons. N = 172, $X^2_{(1,342)}$ = 3269, *P* < 0.001.

The 'bucket' and 'sleeve' traps caught significantly more non-targets than the 'Lep' trap, but not the 'Wing' or 'Delta' traps (Figure 8.7).
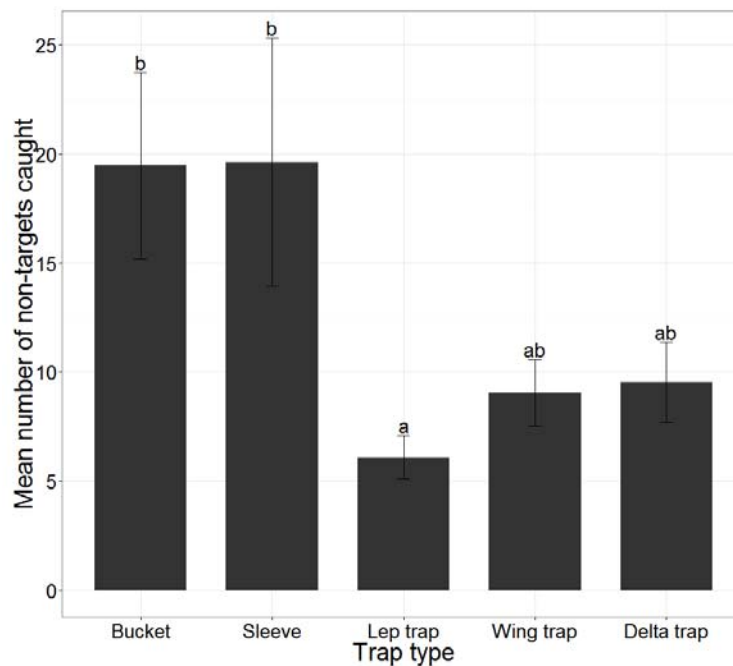
Figure 8.7: Mean number of non-target insects caught in five different traps at field sites in Argentina. The traps were baited with either the sex pheromone of *H. gelotopoeon* or the UoG odour blend. Error bars show standard error, bars that have different letters are significantly different. Tested by GLM (with a quasipoisson distribution) and Tukey's pairwise comparisons. N = 70 (for 'sleeve', 'lep', and 'wing' traps), N = 68 (for 'bucket' traps), N = 66 (for 'delta' traps), $X^2_{(4,339)}$ = 807.79, *P* < 0.01.

Separating the data by the attractant used (pheromone or floral odour) shows that the floral odour baited traps caught a greater variety of non-target insect Orders as well as a greater total number of insects. The 'bucket' and 'sleeve' traps caught significantly more insects than the three sticky traps ('Lep', 'Wing' and 'Delta' traps) (Figure 8.8). Insects in the Order Muscidae were caught in similar numbers in both types of attractant, whereas the other insect Orders were not.
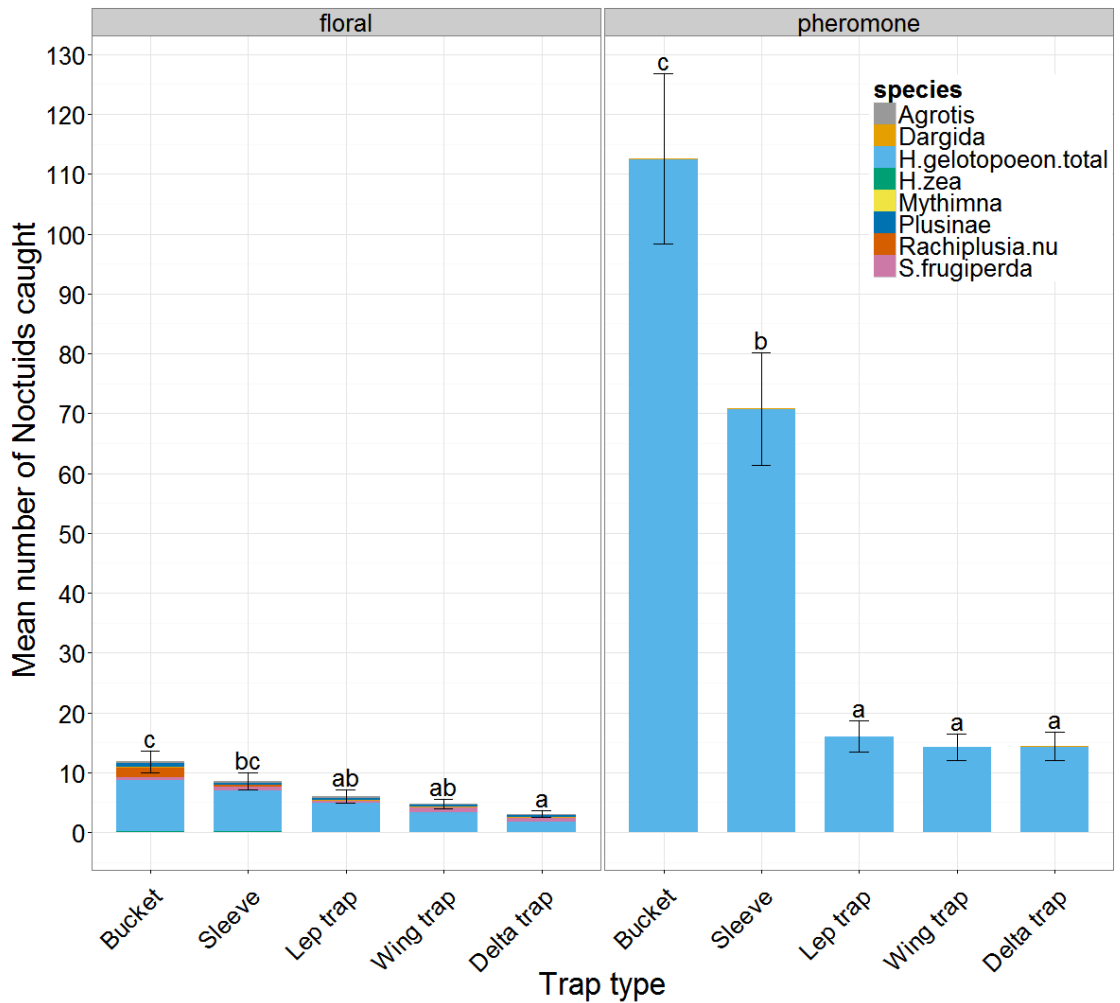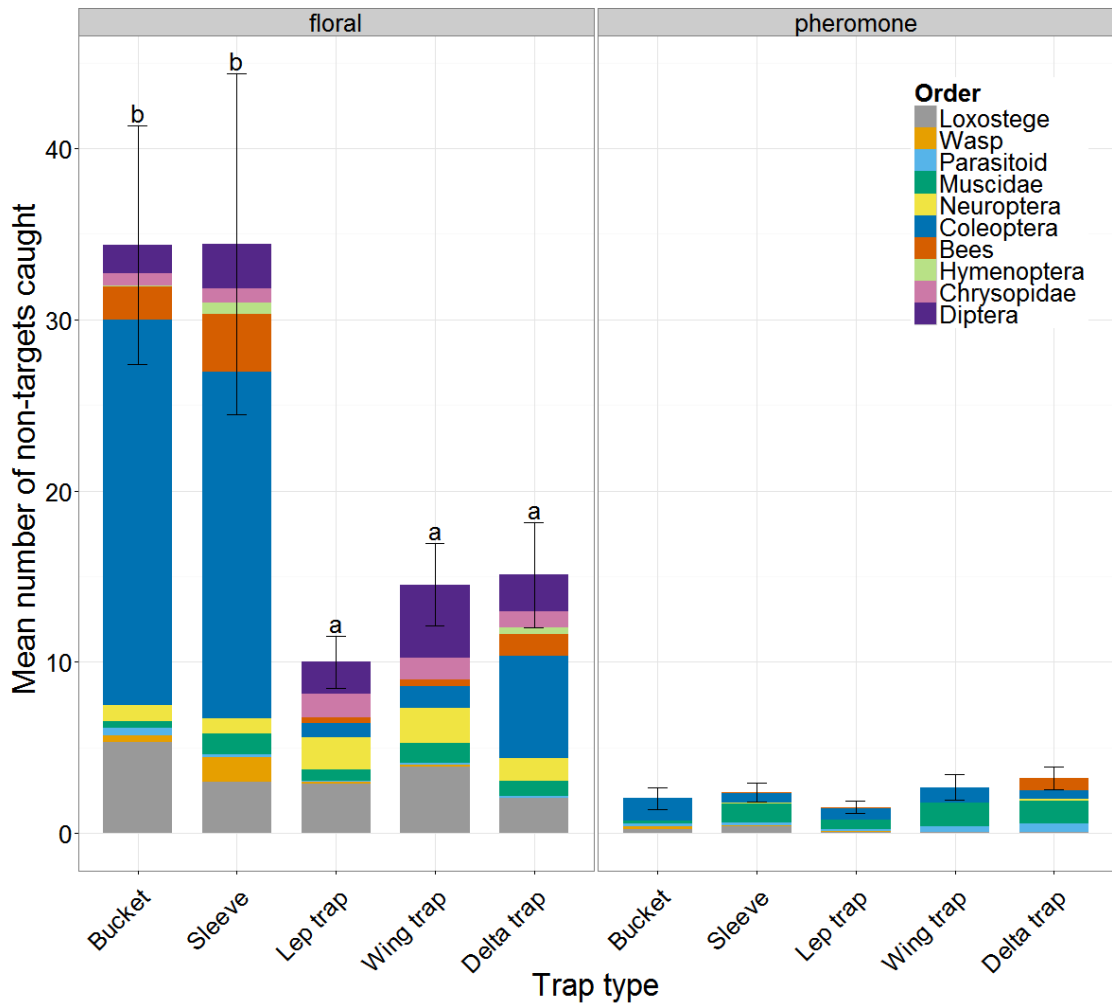
Figure 8.8: Mean number of non-target insects caught in five trap types baited with either the sex pheromone of *H. gelotopoeon* or the UoG odour blend (floral odour) in field trials in Argentina. The breakdown of the mean numbers of individual genera or species caught are shown stacked within the bars. Error bars show standard error, bars within the same chart that have different letters are significantly different. The data was analysed b GLM with quasipoisson distribution followed by Tukey's pairwise comparisons if appropriate. N = 35 (for 'sleeve', 'lep', and 'wing' traps), N = 34 (for 'bucket' traps), N = 33 (for 'delta' traps). For floral baited traps $X^2_{(4,167)}$ = 878.68, P < 0.001. For the pheromone baited traps P > 0.05.

8.3.2 Field trial 9

A field trial in the UK found a highly significant difference in the mean captures of male *A. gamma* caught in two trap designs, the 'sleeve' trap and 'UniTrap'. The 'sleeve' traps caught significantly more moths than the 'UniTraps' (Figure 8.9).
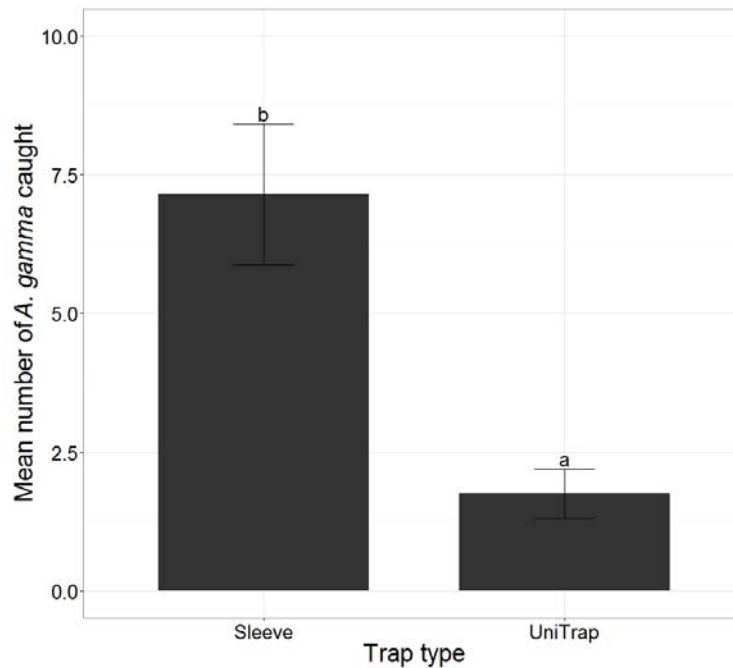
Figure 8.9: The mean number of *A. gamma* males caught in two types of traps during a UK field trial. Errorbars show standard error, and letters denote significant differences between the treatments. Data analysed by GLM with negative binomial distribution and Tukey's pairwise comparisons. N = 20, $X^2_{(1,38)}$ = 18.62, P < 0.001.

More *A. gamma* and other Noctuids were caught in UniTraps baited with the UoG floral blend and containing a contact pesticide in the bucket than in those traps without the pesticide, but the differences were not significant, probably, at least in part, becuase of the low numbers caught (Figure 8.10).
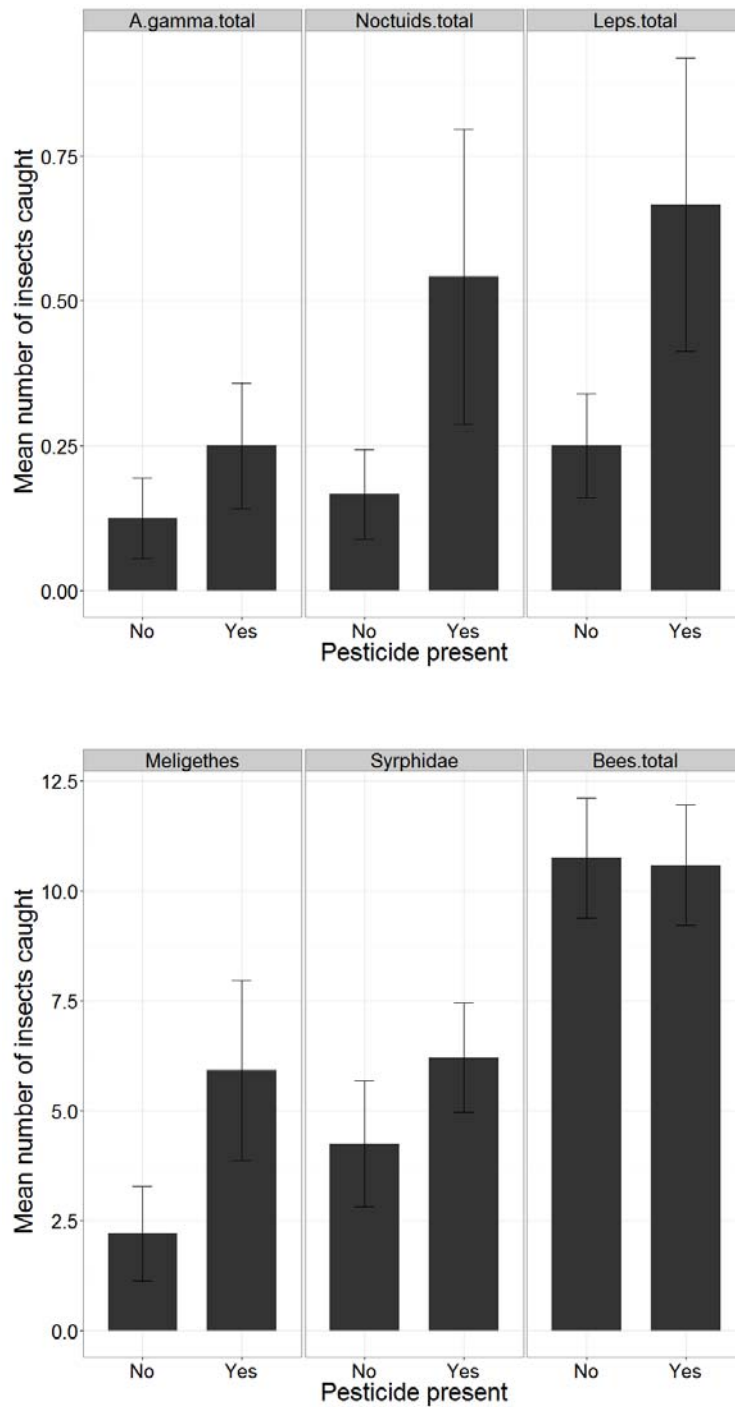
Figure 8.10: The mean number of insects caught in UniTraps baited with the UoG odour blend. The traps either contained a contact insecticide (Azamethiphos, an organophate that inhibits acetylcholinesterase) or did not contain any pesticide. Errorbars show standard error. The data was analysed by GLM (negative binomial) and no significant differences were found between the treatments for any of the insect groups (but approached significance for the 'total Noctuids' group, N = 24, $X^2_{(1,46)}$ = 3.69, P = 0.07 and for the 'total lepidoptera' group, N = 24, $X^2_{(1,46)}$ = 3.36, P = 0.055).

8.4 DISCUSSION

Trap design was found to have a significant effect on the captures of Noctuid moths. The traps that caught the greatest number of Noctuids were the 'bucket' and 'sleeve' traps, the former being a homemade trap designed by E. Lobos and the latter being a cheap and simple trap commonly used in India. All of the traps that used a sticky sheet to capture insects caught fewer Noctuidae than bucket and sleeve traps. Similar to the results seen by Myers *et al.* (2009), who found no differences in captures of two pest Tortricid moths between Lep and Delta traps, the sticky traps in the present study captured almost the same mean number of *H. gelotopoeon* males when they were baited with the sex pheromone. However, when the traps were baited with the UoG floral blend there were some (non-significant) differences in the means of the three traps, with the 'Lep' traps catching double the mean number of Noctuids compared to the 'Delta' traps. It is possible that when baited with the sex pheromone the sticky traps were reaching their maximum capacity of insects resulting in almost the same mean number of insects being caught in all three traps (the sticky surface area of the traps was almost the same ranging from 38,500 for the 'Wing' to 32,300 mm^2 for the Delta traps). Whereas, when the traps were baited with the floral odour, maximum capacity was not reached, and differences in capture efficiency became more apparent. Delta traps are bi-directional (i.e. they have two open sides and two closed sides), Wing and Lep traps have four open sides. The Lep trap is truly symmetrical on all sides but the Wing trap has two sides with flaps which may aid insects landing and entering the trap and two sides with vertical walls that may not be so easy for insects to enter through. The effect of flaps at the entrance of sticky traps is discussed further by Knight

et al. (2002). These design features may explain the small differences between the sticky traps when baited with the floral odour blend.

The retention efficiency and maximum capacity of traps has been researched previously in the Codling moth, *Cydia pomonella* (L.) Tortricidae. Investigating three types of sticky traps (delta, wing, and diamond shaped) Knight *et al.* (2002) found that cumulative moth captures were proportional to the area of adhesive, and retention efficiency varied significantly between traps. Their research found the lowest retention was seen in wing traps which may have been because the trap design caused more moths to land on the outside of the trap compared to the other designs.

Comparison of the two funnel traps (the UniTrap and 'sleeve') found that the sleeve trap caught significantly more *A. gamma* than the UniTrap (Figure 8.9). The opening of funnel of both traps is the same size (90 mm diameter), but where the sleeve trap has a short (20 mm) funnel length and wide end (45 mm) the UniTrap has a much longer funnel (90 mm) and smaller end (30 mm). Quite how much the shape of the funnel effects the catch rate is not known but we may assume that the longer funnel and smaller end of the UniTrap would make it more difficult for insects to escape from once they are in the bucket section below compared to the sleeve trap's funnel. However, the presence of the inert fine powder in the sleeve trap may significantly impair an insect's ability to climb out of the sleeve by reducing friction. In addition, insects in the transparent sleeve will be subjected to desiccation from the sun whereas insects in the bucket of the UniTrap are shaded and may survive for longer. To assess whether insects were escaping from the UniTrap in significant numbers a contact pesticide was added to the trap. Unfortunately the overall catch rate for

Noctuids was low, but the data does indicate that mean captures were improved by the addition of a contact pesticide to the UniTraps. This suggests that insects were escaping from the traps but further testing is required.

In the literature studies comparing sticky traps to non-sticky traps show contradicting evidence. Many results that show non-sticky traps catch greater numbers of moths than sticky traps (e.g. Webster *et al.*, 1986; Athanassiou *et al.*, 2002; Athanassiou *et al.*, 2004; Reardon *et al.*, 2006), yet there are also results that show the contrary (e.g. Knodel and Agnello, 1990; Athanassiou *et al.*, 2007). The reason for these confounding results is unclear, but may be due to differences in behaviour and size of the target insect, and also in population density. Authors have hypothesised that sticky traps may have a better retention efficiency than funnel traps because for the latter insects must fall through the funnel in order to be caught, whereas in a sticky trap the insect only has to contact the sticky surface directly below the lure (Athanassiou *et al.*, 2007) and therefore sticky traps have a larger surface area with which to capture insects. However, the sticky surface may quickly become saturated, with debris and non-targets as well as the target insect. Therefore at low population densities the sticky trap may perform better as it has a higher retention efficiency, but at greater population densities the funnel trap performs better as it has a much higher maximum capacity.

The homemade 'bucket' trap could be described as combining the best features of sticky traps and funnel traps. The oily layer on top of the water acting as a 'sticky' surface trapping a high proportion of insects that come into contact with it, while the water filled area underneath can absorb large numbers of insects in

the way that the bucket or sleeve of the funnel traps are able to. Thus, the 'bucket' trap has a high retention proportion and also a high maximum capacity.

Most of the traps used in this trial fall into two distinct categories, those that utilise a sticky sheet to capture the insect and those that have a chamber below the lure that the insects can become trapped in. The results here show sticky traps are not as effective at capturing Noctuids (and other insect species) compared non-sticky traps. Over a period of three to four weeks the traps that retain insects using a sticky sheet were consistently not as effective at the traps using talc or oil/water to retain the insects. There may be several reasons for this:

1. The sticky sheets may quickly become saturated with insects or other material (e.g. dust and leaves) that renders the sheets ineffective. However, the time series data for the tomato crop (data not shown) showed that catches increased over the course of the trial, suggesting that the sticky sheets were not saturated in the early dates. Moreover, the insects were removed from the traps each time they were checked.

2. It is possible that differences in the trap openings caused reduced numbers of insects to reach and enter the Lepidoptera, Wing and Delta traps compared to the oil and sleeve trap. Certainly, the Delta and Wing trap are bi-directional compared to the other traps which are omni-directional and this will have influenced the traps' odour plumes. However, if trap opening did affect the catch, then it might be expected that there would be a significant difference between the Lepidoptera and Wing traps, which are similar in design apart from the Lepidoptera trap

having openings on all sides compared to the two openings of the Wing trap.

3. Trap capacity may have influenced the number of insects caught in the traps. The sticky sheets used in the Lepidopteran, Wing, and Delta traps are approximately the same size, and likewise the number of insects caught in these traps was similar.

4. Insect retention efficiency, i.e. how many insects that enter the trap make it back out again. The results seen here strongly suggest that the use of a sticky substrate is not as effective at retaining insects as a funnel connected to a container containing material that reduces the insects' ability to escape (in this case oil and water, or talc dust).

The homemade oil/bucket trap and the sleeve trap were found to be the most effective traps. The reason for the increased catches of these traps may be due to increased capacity and retention efficiency of these two traps compared to the other traps tested. These findings may explain some of the low numbers of Noctuids caught in the earlier field trials as UniTraps were used for all of the UK field trials. The reason for using UniTraps is that they are reported to be highly effective for capturing Noctuids (López Jr., 1998) and had been used previously in numerous field work studies involving Noctuids (e.g. Landolt *et al.*, 2001; Meagher R.L, 2001; Landolt and Higbee, 2002; Camelo, 2006; Meagher and Landolt, 2010). In addition a large number of UniTraps were available and they are extremely robust making them useful for long-term field trials.

CHAPTER 9 - GENERAL CONCLUSIONS AND FUTURE WORK

9.1 INTRODUCTION

The research presented in this thesis investigates the attraction of noctuid moths towards floral odour blends. During this research several important findings were made: Of the odour blends tested 'super-blends' were just as effective as blends that mimicked host plants in attracting Noctuidae from a diverse geographical range. A blend of five compounds: phenylacetaldehyde, salicylaldehyde, methyl 2-methoxybenzoate, linalool, and limonene was found to be attractive to a range of noctuids. The physiological status of the insect was shown to have a significant effect on its behaviour towards the floral odour blend; surprisingly gravid insects were less attracted to the odour than virgin insects, and not so surprisingly unfed insects were more attracted compared to sated insects. Non-targets were caught in abundance during the field trials and several pest species were trapped in significant numbers. Captures of beneficial insects such as hoverflies and bees may be mitigated by changing the colour of the trap. Finally the type of trap used had a highly significant effect on trap captures. The commonly used UniTrap was found to be less effective at capturing noctuid moths than a funnel and sleeve trap, however, the most effective trap tested was a homemade bucket and water trap. Sticky based traps were not effective.

The objectives (as described in Chapter 1.4) of this work were to identify an odour blend that is attractive to both sexes of Noctuid moths in the field and to optimise that blend by the addition of other floral compounds. Once a suitable

odour blend was identified, it would be used as bait to assess various types of trap to identify the most effective trap for capturing Noctuid moths. The effect of physiological state on the responses of Noctuids towards floral compounds was investigated using electroantennography and wind tunnel bioassays. Finally, the captures of non-target insects in traps baited with a floral lure was assessed and investigations were carried out to attempt to reduce numbers of non-targets caught. These objectives were acheived.

9.2 INITIAL ASSESSMENT OF FLORAL ODOUR BLENDS

Both host plant mimic blends and 'super-blends' were compared, and were tested on species that had not previously been tested with these types of compounds. The field trials were carried out in geographically diverse locations, yet similar results were seen across the local species, suggesting some of the compounds present in these blends are generally important to polyphagous nectar feeding Noctuidae.

Previously published work on the South American species, *H. gelotopoeon*, is scant and no work that I was able to find has been published on the species' responses to floral volatiles - neither antennal responses or behavioural. However, my wind tunnel and field trials showed that this species is highly attracted to blends of floral volatiles designed to attract related species in other geographic locations.

9.3 THE EFFECT OF PHYSIOLOGICAL STATE

To try to understand some of the variation in the results of the field trials (the UoG blend did not capture the highest number of Noctuids in field trial 2) the

effects of two physiological states of moths – mating status and feeding status - were investigated in wind tunnel bioassays with *H. armigera*. The results showed that gravid moths took longer to fly towards UoG baited lures and were less likely to contact the lures compared to non-gravid moths. Similarly, moths that had been reared with access to sucrose solution as a substitute for nectar were less likely to contact the UoG baited odour source and took longer to do so. This is the first time *H. armigera* under different physiological states and its behavioural response to floral volatiles has been studied, and the results were not as expected. It was expected that the floral odour blend would be highly attractive to gravid *H. armigera* females as previous research demonstrated that this species in this physiological state are attracted to flowering host plants rather than non-flowering host plants (Parsons, 1940; Firempong and Zalucki, 1989; Riley *et al.*, 1992; Sequeira *et al.*, 2001; Liu *et al.*, 2010), however, gravid females were less attracted to the UoG floral odour blend than virgin females. It was concluded that gravid females require additional cues to stimulate upwind flight; these cues may include green leaf volatiles in addition to the floral volatiles.

The implications of the knowledge gained during this research are important for future research into crop protection technologies relying on these types of semiochemicals. The results suggest that the floral odour will work best in crops with few sources of nectar from which moths can feed. The addition of sex pheromone baited traps may reduce the percentage of mated females (by removing males from the crop area) which would also make the floral trap more effective.

The effect of odour load on the lure also has a significant affect on the moths' behavioural response, which highlights the importance of formulating odour lures with the correct odour loading and release rate.

9.4 IMPROVING THE ATTRACTIVENESS OF THE UOG ODOUR BLEND

In order to improve the UoG blend, candidate floral volatiles where identified from peer-reviewed literature and the EAG responses of *A. gamma*, *H. gelotopoeon*, and *H. armigera* were recorded for a selection of these compounds at a range of doses. The dose and compound was found to affect significantly the insect's responses. However, the sex of the insect or whether or not it was gravid were not found to be significant factors. Between the two *Helicoverpa* species and *A. gamma* similarities in responses were seen for three of the compounds in the UoG blend; - methyl 2-methoxybenzoate, phenylacetaldehyde, and linalool - although for the latter two the responses seen were higher for *H. armigera* than for *H. gelotopoeon*. Between *H. armigera* and *A. gamma* relatively similar EAG responses were seen for salicylaldehyde and limonene. The compounds that *H. gelotopoeon* were most sensitive to were benzaldehyde, eugenol, benzyl acetate, methyl anthranilate, 2-phenylethanol, (-)-linalool, phenylacetaldehyde, and benzyl alcohol which gave relatively large responses at both low and high doses, particularly the latter three.

Field trials in the UK aimed at improving the UoG blend by the addition of specific floral compounds found that cinnamyl alcohol and benzyl benzoate caused significantly more Noctuids to be caught compared the UoG blend alone. Approximately 90% of the Noctuid species caught during the trial were *A. gamma* and for all odour blend more females were caught than males except the blend containing benzyl benzoate, although this difference was not

significant. Further field testing in field trial 5 found no significant increase in mean captures between any of the blends tested, however, the blend comprising the UoG blend combined with the two chemicals found to increase captures from the previous field trial (cinnamyl alcohol and benzyl benzoate) caught the greatest number of Noctuids and *A. gamma* (again more females than males were caught). The addition of lilac aldehyde which was expected to be highly attractive to *A. gamma* did not significantly increase moth captures. This could be due to many reasons such as incorrect ratio of isomers or presence/absence of specific lilac aldehyde isomers, odour learning in the wild *A. gamma* population overriding innate behaviour, or perhaps the low number of replicates for lilac aldehyde combined with a low overall capture rate for the field trial.

9.5 TRAP CAPTURES OF NON-TARGET INSECTS

The overall outcome of the field trials in the UK suggested that the UoG blend with benzyl alcohol and cinnamyl alcohol added is highly attractive to both male and female *A. gamma*. However, large numbers of non-target insects were also caught in the traps baited with floral odours.

The attraction of non-target insects to traps baited with plant volatiles is a problem particularly for insects that are considered as beneficial, but also for all insect species that are not crop pests. Since the publication of Silent Spring (Carson, 1962) agricultural pest control has aimed to be more target specific. The need for ecological diversity in agricultural systems is beneficial to the farm, general public, and environment, therefore the use of attractants in crop protection that are attractive to a wide range of insect groups is a significant problem. In Chapter 7 of this thesis the insect groups that were caught in the

floral odour baited traps were analysed and discussed. It was concluded that the addition of specific floral volatiles had no (or limited) effect on trap captures for most insect groups but trap colour did have a significant effect and blue coloured traps may be used to partially alleviate the problem of capturing non-target insects, especially for Hymenoptera and Syrphidae.

9.6 TRAP DESIGN

Many of the field trials in the UK suffered from low moth captures. The reasons for the low captures in some trials may have been due to low Noctuid populations in the area but it was also noted during the field-work that *A. gamma* moths were escaping from the UniTraps. Therefore an assessment of different trap types was undertaken to try to identify a superior trap design for capturing these moths. Three types of sticky traps, two types of funnel traps, and one bucket and water trap were tested in the UK and Argentina. This is the first time these these traps were compared together, and the first time that the homemade bucket and water traps and the Indian sleeve traps were tested with floral baits for capturing Noctuid moths. It was concluded that the homemade bucket traps were the most effective regardless of whether the traps were baited with sex pheromone or the UoG odour blend. The reason for bucket traps' success was thought to be because they combined a high retention rate (due to the large surface area of the water/oil below the lure) and a large capture capacity (trapped moths would sink below the water/oil surface allowing more moths to be caught). The sleeve traps also performed well perhaps because they have a short and wide funnel making it easy for insects to fall through, and the presence of the talcum-like powder making it difficult for the insects to climb out. In addition, the long plastic sleeve provided the trap with a

large capacity to hold trapped insects. The three types of sticky traps tested and UniTraps used in the UK field trials performed poorly when compared to the bucket or sleeve traps.

In terms of a crop protection tool the optimum method to capture both male and female Noctuid moths is to use a bucket trap baited with the UoG blend. To capture *A. gamma*, cinnamyl alcohol and benzyl benzoate should be added to the UoG blend. However, mated or recently fed moths are less likely to be attracted to this floral bait, therefore the most appropriate time to use this odour bait would be pre- or post- crop inflorescence, and perhaps in conjunction with sex pheromone baited traps to reduce the percentage of males in the population and hence the number of mated females. The capture of non-target insects can be reduced by using blue coloured traps.

9.7 FUTURE WORK

To continue this area of research further, work should be carried out on the effect of mating and feeding on the behaviour of moths towards floral odours, specifically the UoG blend (or related blends). Previous research suggests that mated female Noctuids should be attracted to floral odours; however, the research here showed their attraction is significantly lower than for virgin moths. The percentage of mated females caught in floral baited traps in the field was not recorded in the current study but this would give insight into their attraction to these lures. It would be particularly useful to know whether mated insects caught in the field had already oviposited or not, as a key aspect of these odours being used in a crop protection system rely on capturing female moths before they oviposit. If virgin females are indeed more attracted to floral odours than mated females it would be highly beneficial to know whether reducing the

male population (and therefore hopefully reducing the percentage of mated females) by the addition of sex pheromone baited traps improves the capture rate of moths in the floral baited traps.

Although the UoG blend of compounds is a highly effective attractant for a range of Noctuids, further work should be carried out to identify additional compounds that are attractive to specific Noctuid species. EAG work may be useful in identifying potential candidates but the level of antennal sensitivity is not necessarily an indication of a behavioural response. However, it would be useful to continue the EAG work started here on *H. gelotopoeon* (on which very little research has been previously carried out) and compare the responses to related species such as *H. armigera*. As the UoG blend was found to be highly attractive to many different Noctuid species and the compounds it is comprised of are extremely common ubiquitous floral odours, one may expect many Noctuid species to respond electrophysically to these odours.

Finally, the problem of non-target captures needs to be effectively addressed. Although the research here has found that trap colour can play a significant role in reducing non-target captures, further work needs to be carried out, particularly for the reduction of captures of non-target beneficial insects. This area could be addressed by identifying additional compounds that are repellent to bees and other non-targets (but not Noctuids), further research into trap colour, finding traps that are efficient at capturing Noctuids but allow non-targets to escape (e.g. small holes in the traps), or perhaps utilise the difference in behaviour between the nocturnal/crepuscular target species and the diurnal non-targets by engineering some method that allows the odour lure to only emit after dusk.

The understanding of attraction to floral volatiles by Noctuid moths and other insects may also be used in other ways. For example, in population monitoring (for conservation or agricultural warning systems) it is often useful to monitor many species at the same time and a general attractant such as researched in this thesis could be used for this. When combined with genetic modification it may even be possible to use the knowledge crop pests' use of specific floral volatiles to make crops less attractive to crop pests, either by the introduction of genes that instigate the production of volatiles that are repellent to the crop pests, or by knocking out genes that are involved in the production of attractive floral volatiles.

In conclusion, our knowledge of floral volatiles and insect behaviour requires much more research, particularly if it is to be used in crop protection. However the benefits of using these compounds is substantial, for not only could they provide a method of pest control that does not rely on widespread spraying of insecticides, but many floral volatiles are already in use by the food (flavouring) and perfume industry which means they have already been through toxilogical testing and therefore should be easier to get approval by the Chemicals Regulation Directorate (CRD) for use in the field.

CHAPTER 10 - REFERENCES

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