Host plant volatiles influence the behavioural responses of the European grape berry moth, *Eupoecilia ambiguella*, to its sex pheromone

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Felix Kessler

"I think one of the most exciting things is this feeling of mystery,
feeling of awe, the feeling of looking at a little live thing
and being amazed by it and how its emerged
through these hundreds of years of evolution
and there it is and it is perfect and why."

Jane Goodall

Contents

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Summary

Host plant compounds are involved in many aspects of the lives of phytophagous insects, not least the problem of finding a suitable resource to feed on, mate and oviposit. In addition, volatile host plant products have been shown to influence the sexual behaviour of insects as enhancers of pheromone perception and production.

The European grape berry moth *Eupoecilia ambiguella* Hb. (Lepidoptera, Tortricidae) is one of the most important pest in European vineyards. Its larvae feed on grapes and facilitate secondary infection of the grapes. Some integrated methods to control insect pests of grape are based on sex pheromone. These control methods are insufficient at high population densities and are costly. One way to improve the pheromone-based control of grape moths may be found by considering plant volatiles.

We hypothesise that host plant volatiles signal rendezvous sites and that male *E. ambiguella* use these cues in addition to the pheromone to locate females. Analysis of plant volatiles emitted by six host plants of *E. ambiguella* using gas chromatography coupled electroantennogram detection revealed 16 plant volatiles present in at least three host plants that elicited responses of antennal sensory cells of male grape berry moths. This shows that males are equipped with sensory cells to perceive plant volatiles, particulary as some of these volatiles were detected at low levels by the antennal sensory receptor neurones.

In the wind tunnel, different devices to deliver combinations of the sex pheromone and plant volatiles to male *E. ambiguella* were tested, as the manner of release rate and the ratio of pheromone to plant volatiles is critical. To control these parameters a piezo nebulizer was installed permitting the release of known amounts of pheromone plus plant volatiles as an aerosol in order to record the flight behaviour of male grape berry moths to different treatments. At an underdosed pheromone level significantly more males contacted the pheromone source in the presence of either (Z)-3-hexen-1-ol, (E)-β-caryophyllene, (+)-terpinen-4-ol or methyl salicylate or a binary mixture of the first two compounds. Moreover, males were activated earlier in the presence of (+)-terpinen-4-ol and (E)-β-caryophyllene. An effect

of plant volatiles was also recorded at overdosed pheromone levels where many males normally show in flight arrestement and fail to contact the pheromone source. However, by adding (Z)-3-hexen-1-ol, (E)-β-caryophyllene and (+)-terpinen-4-ol to the overdosed pheromone more males contacted the source.

Overall, our results provide evidence that plant volatiles play a role in the sensory ecology of male *E. ambiguella*. This is discussed in an evolutionary as well as a physiological context. In addition, we discuss the implications of our findings for integrated pest management of this pest species using semiochemicals.

Key words: Sensory ecology, plant-insect interaction, behaviour, wind tunnel, electroantennogram, sex pheromone, host plant volatiles, mating disruption, European grape berry moth, *Eupoecilia ambiguella*, Tortricidae.

Résumé

Les composés des plantes hôtes jouent un rôle important dans plusieurs aspects de la biologie des insectes phytophages. Ces substances ne leur permettent pas seulement de trouver de quoi se nourrir, mais aussi un lieu de reproduction et d'oviposition. Il a ainsi été prouvé que ces produits volatiles des plantes hôtes influencent le comportement sexuel des insectes en augmentant la perception et la production de phéromone.

Cochylis, la tordeuse de la vigne, *Eupoecilia ambiguella* Hb. (Lepidoptera, Tortricidae) est un des ravageurs les plus importants des vignobles européens. Les larves se nourissent sur les grains et facilitent les infections secondaires sur les raisins. Certaines méthodes intégrées de contrôle de ce ravageur sont basées sur les phéromones sexuelles, mais elles sont coûteuses et insuffisantes sur des populations de haute densité. Une méthode pour augmenter l'efficacité du contrôle phéromonal de ce papillon peut être l'utilisation des composés volatiles des plantes.

Notre hypothèse est que les signaux volatiles des plantes servent à indiquer des sites de rendez-vous aux mâles *E. ambiguella* qui les utilisent, en conjonction avec la phéromone, pour localiser des femelles appelantes. En analysant les odeurs émises par six plantes hôtes de *E. ambiguella* par électroantennographie liée à un chromatographe en phase gazeuse, 16 composés volatiles ont été trouvés dans au moins trois des six plantes hôtes et ces composés ont été perçus par les cellules olfactives receptrices de ce papillon. Ce résultat démontre la capacité des mâles à percevoir des composés volatiles des plantes même à un seuil très bas, pour certains.

Dans une chambre de vol, différents systèmes de présentation des combinaisons phéromone-odeurs des plantes ont été testés et ont permis de constater que le taux de relargage et le rapport phéromones/composés de plantes sont des éléments critiques. Pour contrôler ces paramètres un "piezo sprayer" a été installé afin de nébuliser sous forme d'aérosol des quantités précises et connues de ces composés. Il a ainsi été possible d'enregistrer le comportement de papillons mâles face à différents traitements. Lorsque la quantité de phéromone est sous-dosée, signficativement plus de mâles arrivent à la source

phéromonale après l'ajout de (Z)-3-hexène-1-ol, de β-caryophyllène, de (+)-terpinène-4-ol, de salicylate de méthyle ou d'un mélange des deux premiers composés. De plus, les mâles sont plus rapidement activés avec l'addition à la pheromone sousdosées de (+)-terpinène-4-ol ou de β-caryophyllène. Ces composés volatiles des plantes ont aussi montré un effet lorsque la quantitité de phéromone était surdosée, alors que normalement, même avec un vol orienté, peu de mâles parviennent à atteindre la source. En effet, l'ajout de (Z)-3-hexène-1-ol, de β-caryophyllène ou de (+)-terpinène-4-ol a permis d'augmenter le nombre d'arrivées à la source.

L'ensemble de nos données prouve que les composés volatiles des plantes jouent un rôle dans l'écologie sensorielle des mâles *E. ambiguella*. Les résultats sont discutés d'un point de vue physiologique, évolutif et appliqué dans l'optique d'une amélioration de la lutte intégrée par les signaux chimiques.

Mots clés: Ecologie sensorielle, interaction plante-insecte, comportement, tunnel de vol, électroantennogram, phéromone sexuelle, odeurs de plantes hôtes, confusion sexuelle, cochylis de la vigne, *Eupoecilia ambiguella*, Tortricidae.

Zusammenfassung

Pflanzenduftstoffe spielen eine wichtige Rolle in der Biologie von phytophagen Insekten. Sie ermöglichen diesen eine geeignete Nahrungsquelle, einen Reproduktionsort und auch einen Ort zu Eiablage zu finden. Zudem wurde gezeigt, dass Pflanzenduftstoffe dass Sexualverhalten von Insekten als Verstärker der Pheromonwahrnehmung und -produktion beeinflussen können.

Der einbindige Traubenwickler, *Eupoecilia ambiguella* Hb. (Lepidotpera, Tortricidae) ist einer der wichtigsten Schädlinge im europäischen Weinbau. Die Raupen fressen an den Trauben und vereinfachen so sekundär eine Pilzinfektion derselben. Zur Bekämpfung dieses Schädlings gibt es bereits einige integrierte Methoden, die auf Sexualduftstoffen (Pheromonen) basieren. Diese Kontrollmethoden verlieren aber ihre Effizienz bei einer grossen Schädlingspopulation und sind zudem kostspielig. Eine Möglichkeit zur Verbesserung dieser auf Pheromonen basierenden Bekämpfung könnte die Beigabe von Pflanzenduftstoffen bieten.

Unsere Hypothese ist, dass Pflanzenduftstoffe Orte für Rendez-vous signalisieren, und dass die Männchen des einbindigen Traubenwicklers dieses Signal zusätzlich zum Sexualduftstoff nutzen, um Weibchen zu lokalisieren. Mittels Gaschromatographie gekoppelt mit Elektroantennographie analysierten wir die Duftstoffe, die von sechs verschiedenen Wirtspflanzen von *E. ambiguella* abgegeben werden. Dies führte zu einer Liste von 16 Pflanzenduftstoffen, die in mindestens drei Pflanzen vorkommen, und durch die ein elektrisches Signal von den olfaktorischen Rezeptorzellen der Antenne abgeleitet werden konnte. Dies zeigt, dass die Männchen mit sensorischen Neuronen zur Wahrnehmung von Pflanzenduftstoffen ausgestattet sind, insbesondere da einige Duftstoffe noch in kleinsten Mengen wahrgenommen wurden.

In einem Windkanal wurden verschiedene Methoden getestet, diese Duftstoffe zusammen mit dem Pheromon abzugeben, da sowohl Abgaberate als auch das Mischverhältnis zwischen Pheromon und Pflanzenduftstoffen kritische Faktoren sind. Um diese Parameter zu kontrollieren wurde ein so genannter Piezosprayer installiert, durch welchen die Duftstoffe in definierbarer Menge in Form eines Aerosols

abgegeben werden konnten. Dies ermöglichte die Quantifizierung des Flugverhaltens von männlichen Traubenwicklern bei Abgabe von verschiedenen Duftstoffen. Bei einer unterdosierten Pheromonmenge flogen signifikant mehr Männchen zur Duftquelle, wenn diese mit (Z)-3-Hexen-1-ol, β-Caryophyllen, (+)-Terpinen-4-ol, Methylsalicylat oder einer Mischung der ersten beiden Substanzen angereichert war. Zusätzlich waren die Männchen durch die Zugabe von (+)-Terpinen-4-ol und β-Caryophyllen zum Sexualduftstoff signifikant schneller aktiviert. Ein Effekt von Pflanzenduftstoffen wurde auch gefunden, als diese einer überdosierten Pheromonmenge zugegeben wurden. Während die Männchen zu einer reinen, überdosierten Pheromonquelle zwar fliegen, aber diese nur in kleiner Anzahl erreichen, wurde diese Anzahl durch die Zugabe von (Z)-3-Hexen-1-ol, β-Caryophyllen und (+)-Terpinen-4-ol signifikant gesteigert.

Insgesamt betrachtet, erbringen unsere Resultate den Nachweis, dass Pflanzenduftstoffe eine Rolle in der Sinnesökologie von männlichen *E. ambiguella* spielen. Dies wird sowohl im Kontext der Evolution und der Physiologie diskutiert. Zusätzlich betrachten wir mögliche Auswirkungen unserer Resultate auf die integrierte Kontrolle dieses Schädlings mittels Semiochemikalien.

Schlagwörter: Sinnesökologie, Insekten-Pflanzen Interaktion, Verhalten, Windkanal, Elektroantennogramm, Sexualpheromone, Pflanzenduftstoffe, Verwirrungstechnik, einbindiger Traubenwickler, *Eupoecilia ambiguella*, Tortricidae.

Chapter 1

General introduction

1.1 Insect-Plant Relationship

Plants can provide herbivorous insects with not only food and shelter, but also with a location where mates can be found and where females lay their eggs, assuming that the chosen plant can provide food for the progeny. Host plant quality can therefore influence an insect's fitness and reproductive performance. The quantity and availability of food limits the growth of the insect population along with the climatic conditions, the presence of natural enemies and of competitors for the food resource. Insects are thus confronted with the problem of finding a suitable host plant where optimal fitness is guaranteed in terms of the survival of viable progeny. Depending on their host-range, insect species can be generally divided into three categories: monophagous, oligophagous and polyphagous. There are two different definitions for these categories. One refers to the numbers of hosts in general, meaning that an insect species feeding on only one plant species is considered monophagous. Another takes into account the taxonomic relationships between the different host plants. Under this, monophagous insect species can be defined as insects feeding on plants which belong to the same genus (Bernays and Chapman, 1994; Futuyma and Moreno, 1988). Species of the *Heliconius* moths, for example, are monophagous feeding only on plants of the genus *Passiflora*. Oligophagous insects feed on plants within the same family. The oligophagous Colorado potato beetle, *Leptinotarsa decemlineata*, has 14 host plants which all belong to the Solanaceae. Polyphagous insects choose host plants from different families. The gypsy moth is thought to have a host plant range of over 500 species (Lance, 1983). Monophagous and oligophagous insect species are often referred to as specialists whereas polyphagous are termed generalists. The latter can have a broad geographical range and several generations per year because their plants are available at almost any time and anywhere. Further, they are able

to select among foods to balance the nutrient intake whereas specialists are limited to the nutrients that are present in their restricted host plant species. The selection of host plants by some insects is mainly based on the absence of non-volatile deterrents perceived by gustatory receptors on the insect's palps and ovipositor (Jermy and Szentesi, 1978). Generalists can use plants containing toxins only to a limited extent and are confronted with more competitors and natural enemies. Specialists are competent in specific niches where they outcompete other species in that they are able to exploit plants which are toxic for others due to the presence of particular secondary metabolites. Many monophagous and oligophagous species have developed extraordinary abilities to deal with noxious chemicals of host plant. Plant species within a genus or a family often have similar secondary metabolites and insects have evolved mechanisms either to avoid feeding on plant parts containing toxins, to quickly excrete them or to sequester them. The most famous example for sequestering noxious plant compounds is the monarch butterfly which uses the toxins of milkweeds as protection against predators. When plant feeding specialists evolve to become cryptic on plants it is even more difficult for a generalist predator to find it. It is also advantageous to be a specialist when a plant species is abundant and predictably available as this reduces searching time (Bernays, 2001).

1.1.1 Insects in Agriculture

Most crop plants arise from introduced plants that serve as new ecological niche for insects in which food is abundant (Hodkinson, 1982; Schoonhoven et al., 2005). Additionally, introduced crop plants are not adapted in terms of defence to local phytophagous insect species and simple monocultures may have a lower load of insect predators than natural ecosystems. Using these advantages, insect herbivores cause annual crop losses of about 10% despite the use of insecticides (Schoonhoven et al., 2005; Strange and Scott, 2005). Some pest insects have been introduced unintentionally like the corn root worm, *Diabrotica virgifera*, which was introduced accidentally from Northern America to Serbia in 1992 and is spreading throughout Europe (Hemerik et al., 2004), the Colorado potato beetle, *Leptinotarsa decemlineata*, (introduced in 1877 from Mexico and Colorado to Europe) or the grape phylloxera, *Daktulosphaira vitifoliae*, (introduced to Europe in 1860 from USA). Pest insect species are mostly oligophagous and polyphagous. They may evolve to monophagy over time when food is abundant and relatively predictable (Bernays and Chapman, 1994). The level of insect herbivory on a particular plant species is affected by deterrent chemicals and physical defences. Even the nutrient status of the plant is important. These features are often altered in crops during breeding for agriculture and eventually favour feeding by some insect species.

To combat pests, insecticides are still used in such excessive amounts that the resulting high selection pressure leads to resistance. Additionally, insecticides are often not species specific and may have harmful effects for non-target species and residues on food are undesirable for humans. Integrated pest management (IPM) is defined as the combination of all available techniques to reduce pest populations and maintain them below levels causing economic injury in a manner that avoids harmful side effects in the habitat (Waage et al., 1992). In IPM, the use of insecticides is limited or even replaced by other control methods. For the application of IPM and the development of novel control methods it is necessary to know the biology of the insect species causing particular crop damage. With knowledge of the life cycle, the behaviour and preferences of the insect species, one can develop specific methods to control the pest or use an insecticide just when it is necessary and thus decrease the selection pressure on the insect species to evolve resistance. Such a method may for example interfere with a behavioural process of an insect as with sex pheromones, as can be used to reduce mate finding in many moth species (see Section 1.2.1).

1.2 The grape berry moth *Eupoecilia ambiguella*

The grape berry moth, *Eupoecilia ambiguella* Hb. (Lepidoptera, Tortricidae, Fig. 1.1), is together with the grape vine moth, *Lobesia botrana* Den. & Schiff. (Lepidoptera, Tortricidae), the most important pest in European vineyards. Both grape moth species have fairly similar biologies and synchronous flight periods. They are both polyphagous and have other common hosts in addition to the vine such as *Ligustrum vulgare* (Oleaceae) , *Hedera helix* (Araliaceae), *Cornus mas* (Cornaceae) and *Viburnum lantana* (Caprifoliaceae) (Galet, 1982; Bovey, 1966). *E. ambiguella* has a distribution range throughout central and northern regions of Europe and appears to have existed in these regions even before the vine was cultivated (Galet, 1982; Bovey et al., 1972). On vine this moth exhibits two flight periods per year, the first one starting at the end of April or the beginning of May, when adult moths emerge from the overwintering pupae. The flight period takes two or three weeks during which *E. ambiguella* females attract males by releasing a sex pheromone from the forth hour after sunset for about three hours. After mating, females lay 40-60 eggs on stems, flower buds or pedicles from which larvae hatch after 10-15 days. Feeding on inflorescences in the first generation they pass through five larval stages. After the pupation (10-14 days) the second flight period starts in the middle of July and takes 10-20 days. During this time females lay eggs on the grapes. Because of the higher temperature, egg development is faster and takes only 8-10 days. The hatched larvae now feed on the grapes. The resulting damage favours the development of different fungi like the grey mould, *Botrytis cinerea*, which leads finally to

a b

FIGURE 1.1: Adult (a) and larval (b) grape berry moth *E. ambiguella* on a leaf of *Vitis vinifera*.

a loss of grape bunch production and quality. The larvae develop and enter diapause as pupae.

1.2.1 Control methods for grape moths

In recent times control methods other than chemical insecticides were considered more and more as insecticide residues are undesirable and many insect pests developed resistance against many insecticides. No incidence of resistance is known so far for *E. ambiguella* but the development and application of alternative control methods progresses. Integrated pest management target the different live stages of this moth. Parasitoid wasps of the genus *Trichogramma* attack eggs of many moth species including the grape berry moth (Remund and Bigler, 1986; Hassan, 1993). *Bacillus thuringiensis* affects the larval stages by producing the Bt-toxins when ingested by a larva (Pasquier, 1994). In practice, this method is less favourable because it has to be applied at a specific time point and the effectiveness highly depends on weather conditions.

The most important control method for *E. ambiguella* is the mating disruption targeting the adult stage. This method has a long and successful history which dates back to 1939 when Götz (1939) made first field studies with traps containing female grape berry moths to attract males. He already saw the potential of the sex pheromone to control this insect pest by writing:

> "Ein Verfahren dieser Art hat den Vorteil, dass es geringe Kosten verursacht, hygienisch einwandfrei ist und den Winzer von den zeitraubenden Spritzarbeiten gegen den Wurm entlastet."

After the development of DDT, however, research on pheromones was deferred until the seventies when the use of DDT was prohibited (Rauscher, 2000). Among alternative control methods of insect pests, the use of semiochemicals was promising. The newly developped method of gas chromatography

linked to an electroanntenographic detector (Arn et al., 1975, 1976) permitted the identification of the female sex pheromone that attracts male grape berry moths: (Z)-9-dodecenyl acetate (Z9-12:Ac). The attractiveness of this critical pheromone component, released by *E. ambiguella* females, can be further increased by addition of 12:AC alone or of 12:Ac with 18:Ac (Arn et al., 1986; Rauscher et al., 1984). Neither of the two additional compounds can attract males without Z9-12:Ac, and 18:Ac only has an effect in the presence of Z9-12:Ac and 12:Ac. They act as synergists. Today, this three-component blend is used in pheromone traps for *E. ambiguella* males in the field. Even though mass trapping is not possible with these traps they are very important for monitoring the presence of grape moths. The confusion technique was subsequently developed. Here, pheromone dispensers are distributed in the vineyards to release a high amount of synthetic pheromone at point sources. In the presence of such sources, males spend time following false trails and, consequently, females remain unfertilised. This method is already used in 50% of Swiss vineyards (Pierre-Joseph Charmillot, personal communication), though it is quite expensive due to the high amount of pheromone employed, i.e. more than 80g pheromone per ha (Charmillot and Pasquier, 2000; Charmillot et al., 1995). An other approach is the attract-and-kill method where droplets containing a synthetic pheromone blend incorporating an insecticide are applied on branches of the vines. These droplets act as competitors for female moths. When males are baited by the droplet they subsequently die due to having contacted the insecticide. Such baits must be very attractive to ensure contact by the moth with the source of pheromone. Such a product already exists to control the codling moth, *Cydia pomonella* (Charmillot et al., 2000; Lösel et al., 2000) and preliminary field trials indicated the feasibility of the attract and kill method also for grape moth control (Thomas Degen, personal communication). Much less pheromone is needed for this method leading to lower costs. However, an effective control by mating disruption, either by the confusion method or attract-and-kill, needs large and contiguous areas as mated females can easily invade pheromone-treated vineyards from outside. In addition, a low initial population density is required. To improve such pheromone-based control methods attractive host plant odours may provide a useful addition to this approach of pest control.

1.3 Sensory ecology of insects

1.3.1 Olfactory organs and signal processing

Insects are faced with the challenge to locate the opposite sex for mating or a suitable host plant for feeding or oviposition. Several sensory modalities such as odour, taste, form and colour can lead an insect to a mate or a host plant. Odour and visual stimuli are important to find a mate or host whereas the nature of what is perceived by contact finally helps to decide if a host plant is acceptable as food or for oviposition. Chemical stimuli are perceived via sensory receptor neurones in different types of sensilla on the insect antenna, palps and ovipositor sharing the same general structure.

Structure of olfactory sensilla An olfactory sensillum is a cuticular structure containing receptor neurones for volatile compounds (Fig. 1.2). Generally, the cuticle is penetrated by pore tubules through which volatile molecules enter into the sensillum cavity filled with the sensillar lymph. The dendrites of bipolar sensory cells (typically two to three) spread into the sensillar lumen. The cell bodies of the sensory cells are located at the base of the sensillum and surrounded by three auxillary cells: the thecogen, trichogen and tormogen cells. These cells are integrated in the epithelium by septate junctions and are involved in the formation of the sensillum cuticular structures during the development (Keil and Steiner, 1990, 1991). In addition, they contribute to the generation of the membrane potential: The tormogen cell regulates the ionic composition of the sensillar lymph by actively pumping K^+ into the hair lumen (Kaissling and Thorson, 1980).

A male moth antenna carries up to 100 000 sensilla which can be divided into four types (Steinbrecht, 1970; Hansson, 1995). The long hairlike sensilla trichodea house olfactory receptor neurones (ORNs) for pheromone compounds. The dendrites of these receptor neurones are unbranched. In comparison, sensilla basiconica are shorter than the sensilla trichodea and the ORNs have branched dendrites carrying receptors for the detection of host plant products. The other two sensilla types containing olfactory receptor neurons are the raisin- or ear-shaped sensilla auricillica and the peg shaped sensilla coeloconica (Pophof et al., 2005; Ansebo et al., 2005).

Signal transduction and processing Most volatile compounds entering the wall pores of the sensillum cuticle are hydrophobic whereas the sensillar lymph is aqueous. Olfactory binding proteins (OBPs) are thought to transport the different hydrophobic volatile compounds through the aqueous sensillar lymph to the receptor sites on the ORNs (Steinbrecht, 1998; Kim et al., 1998; Große-Wilde et al., 2006). Among the OBPs of several lepidopteran spp. researchers have identified at least

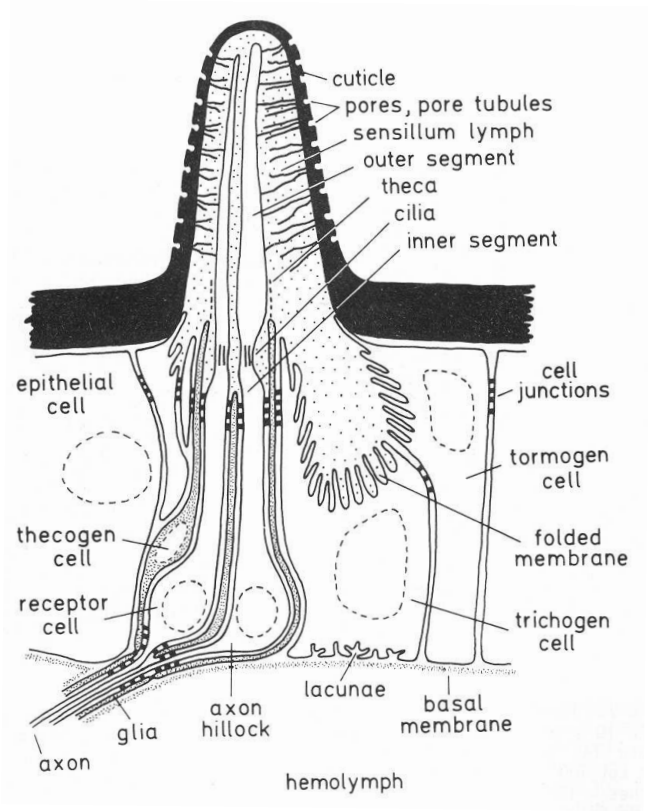


FIGURE 1.2: Schematic diagram of an olfactory sensillum (see text for explanation; from Kaissling, 1987)

three groups: pheromone-binding proteins (PBPs) and two groups of so-called general odorant-binding proteins (GOPBs; Vogt et al., 1991). In lepidopteran spp. only males possess PBPs in the sensilla trichodea carrying one or two receptor neurones that are very sensitive in detecting the sex pheromone components released by females. GOBPs were found in both sexes in the sensilla basiconica but are less represented in males (Laue et al., 1994; Steinbrecht et al., 1995; Vogt et al., 1991). OBPs were also hypothesized to be involved in the inactivation of the odour molecules (Vogt and Riddiford, 1981; Ziegelberger, 1995).

At the receptor sites the odour molecule binds to a G-protein coupled olfactory receptor (OR). This binding initiates a cascade of events inside the dendrite causing the opening of ion channels and the influx of K^+ and Ca^{2+} ions into the ORN (Jacquin-Joly and Merlin, 2004). The resulting receptor potential travels down to the the spike initiation site where action potentials are triggered when the receptor potential reaches a certain threshold. The action potentials propagate via the antennal nerve into the antennal lobe where the about 10^3-10^5 ORNs converge into 40-1000 glomeruli (Homberg et al., 1989; Anton and Homberg, 1999). An ORN expresses only one type of OR and ORNs expressing the same ORs target the same glomerulus (Gao et al., 2000; Vosshall et al., 2000; De Bruyne, 2006). The antennal lobe of moths has a sexually dimorphic structure. In males a large

macroglomerular complex (MGC) for the integration of pheromonal signals and isomorphic glomeruli can be distinguished. Female antennal lobes possess only isomorphic glomeruli for the integration of host plant volatiles evoked signals. The number (around 60-70 in moths) and location of the isomorphic glomeruli is constant within both the sex and the moth species (Masante-Roca et al., 2002, 2005). In the glomeruli, ORNs synapse with projection neurones and local interneurones. Local interneurones (LN) arborize only within the antennal lobe. Most of them are GABAergic and have inhibitory effects on projection neurones (PNs) (Hildebrand, 1996; Christensen et al., 1993; Wilson and Laurent, 2005). More recently, excitatory cholinergic LNs have been found in the antennal lobe (Shang et al., 2007). Different function of LNs have been suggested such as serving in gain control for PN input, to elicit synchrony among in PN firing, or to redistribute the odour signal across a population of neurones (Wilson and Mainen, 2006; Olsen et al., 2007; Olsen and Wilson, 2008). The 200-10^3 projection neurones transmit the olfactory signal from the antennal lobe via the *tractus olfactorio globularis* to the mushroom bodies and the lateral horn of the protecerebrum for processing and integration with other sensory modalities (Mayer and Mankin, 1985; Anton and Homberg, 1999; Heisenberg, 2003).

1.3.2 Behavioural response to volatile compounds

When insects perceive odours several factors determine the behavioural response such as the degree of motivation, the physical state and environmental conditions. The behaviour is well studied in male moths locating female released sex pheromones (Kaissling, 1997; Kaissling and Kramer, 1990; Kramer, 1986). Moths locate an attractive odour source typically by an optomotor anemotaxis modulated by the odour stimulus (Kaissling and Kramer, 1990). The expression 'optomotor anemotaxis modulated by odour stimuli' comprises at least three concepts discussed below. The odour plume is distorted by turbulence comparable to smoke and, in comparison, the molecular diffusion of the odour is small and insignificant. Consequently, a concentration gradient is not available for the insect to orient to the source but, instead, the odour arrives at the antenna in the form of short bursts (Murlis and Jones, 1981). The best strategy for an insect is therefore to fly upwind (anemotaxis) in order to locate the odour source. However, the insect also needs to obtain information about the wind direction. In flying insects, mechanoreceptors can only measure the velocity of the animal relative to the surrounding air i.e. the air speed (Kaissling and Kramer, 1990). Information about the direction and flight altitude can be obtained from visual references such as structures on the ground or on the side (optomotor anemotaxis). Odour stimuli not only elicit anemotactic behaviour they also modulate it. Instead of flying straight upwind males orient by zig-zagging. The counterturns of this zig-zag behaviour may

be controlled by an internal turning tendency and initiated by a chemical stimulus (Kaissling, 1997; Olberg, 1983). Kaissling (1997) describes the flight of a moth as a sequence of the following events: the moth crosses an odour filament which generates nerve impulses in the ORNs. Consequently, the moth begins to turn into the wind and maintain a certain course angle. After a delay the ORNs stop firing and the moth begins to fly across the wind line. The internal tendency to counterturn changes in sign and a counterturn is elicited. The moth crosses the next odour filament and the sequence starts again. When no further odour filament is perceived, i.e. the odour plume is lost, the moth maintain counterturning between left and right. Through this lateral crosswind excursions ("casting") male moths increase the probability to regain the odour plume.

1.4 Interactions of insect pheromones and plant chemicals

Male and female moths often meet and mate on host plants where the probability to encounter the opposite sex may be enhanced (Landolt and Phillips, 1997). In this manner, host plants may play an important role in the sexual behaviour of moths. One of the first evidence for this hypothesis was found by Rahn (1968). He released male leek moths, *Acrolepiopsis assectella*, in different parcels of land together with four traps containing tethered females. Most males could be recaptured in the parcels with the host plant leek (61%), many fewer were recaptured in parcels with grass (9.2%) or beet (12.6%) and almost none in parcels with only soil (0.8%). Rahn (1968) concluded that the presence of the host plant conditions the meeting of sexes. Since then, several studies have reported on interactions between insect pheromones and host plant chemicals. Plant products can influence the sexual behaviour of moths in at least three different levels: sequestration of plant compounds into pheromone compounds, stimulation of pheromone production and release, and pheromone perception. Sequestration of plant compounds into pheromone products is known for several insect species (Landolt and Phillips, 1997). The sex pheromone hydroxydanaidal produced by male *Utetheisa ornatrix*, for example, is derived from plant produced pyrollizidine alkaloids aquired by larvae (Conner et al., 1981). In *Grapholita molesta*, the larvae sequester ethyl *trans*-cinnamate from host tissue used by males as a courtship pheromone on their hairpencils (Löfstedt et al., 1989). Plant compounds can also stimulate pheromone production and release. In female corn earworm moths, *Helicoverpa zea*, the production of sex pheromone is enhanced by a factor of 20-30 in the presence of host plants (Raina et al., 1992). Ethylene, 3-methyl-butan-1-ol and phenyl acetaldehyde were also found to be enhancers (Raina et al., 1992). These compounds cause an increased release of the pheromone biosynthesis-activation neuropeptide (PBAN) which controls the pheromone production. Enhancement and synergistic effects of plant volatiles on male attraction

to pheromone are also known for some moth species. More *Helicoverpa zea* males were caught in sex pheromone traps incorporating the green leaf volatile (Z)-3-hexenyl acetate (Light et al., 1993). Yang et al. (2004) tested the influence of plant volatiles on the response of male codling moths, *Cydia pomonella*, to their sex pheromone in the wind tunnel: attraction of codlemone to males was enhanced in the presence of linalool, (E)-β-farnesene, or (Z)-3-hexenol that attracted 60%, 58%, 56% of males, respectively, compared to codlemone alone that attracted 37%. A similar phenomenon was reported for *Spodoptera exigua* males (Deng et al., 2004) where adding (Z)-3-hexenyl acetate, linalool, benzaldehyde or phenylacetaldehyde to a synthetic pheromone blend of (Z,E)-9,12-tetradecadienyl acetate and (Z)-9-tetradecenol enhanced attraction by 60.6%, 34.3%, 101.4% and 79.6%, respectively, in the wind tunnel. In the field, traps baited with pheromone and either (E)-2-hexenal, (Z)-3-hexenyl acetate, (Z)-3-hexenol or phenylacetaldehyde also caught more *S. exigua* males than the pheromone alone. The mechanism underlying this enhancement is not well understood. Yang et al. (2004) hypothesise that host plant volatiles may increase communication distances by amplifying a weak pheromone signal. Ochieng et al. (2002) made single cell recordings from sex pheromone-specific olfactory receptor neurones in male *Helicoverpa zea* and found that linalool and (Z)-3-hexenol significantly enhanced the firing rate of pheromone-specific neurones in a blend with the pheromone compared to the pheromone alone. These two plant compounds alone did not stimulate the pheromone-specific neurone.

1.5 Objective and outline of this thesis

As outlined in Section 1.2.1 mating disruption methods are not effective at high population densities of the grape berry moth *E. ambiguella*. In addition, the costs of this method are high for two reasons: firstly, the pheromone compound Z9-12:Ac must be very pure as its isomer E9-12:Ac has an inhibitory effect (Arn et al., 1986). Secondly, the pheromone dispensers have to be applied manually, which means additional costs of manpower. The aim of this thesis was to investigate a possible improvement of mating disruption by considering plant compounds.

We have much evidence that host plant volatiles, in addition to serving as a food source signal, also play an essential role in mating behaviour of moths by acting as signals for rendezvous site for mates (see Section 1.4). My hypothesis is that host plant compounds are used by *E. ambiguella* to regulate and mediate sexual communication as a strategy for optimised reproduction.

This thesis is divided in four chapters. In Chapter 2 electrophysiological experiments are described where the objective was to screen for semiochemicals relevant to the sensory ecology of male grape

berry moths. Chapter 3 deals with the development of behavioural assays in a wind tunnel. Findings from behavioural experiments are presented in Chapter 4. Chapter 5 provides a general discussion of our findings.

Chapter 2

The perception of host plant volatiles by male *E. ambiguella* antennal receptor neurones

2.1 Introduction

Olfaction is of major importance for the survival and reproduction in moths. Plant volatiles guide moths to their host plants where they can feed, mate, oviposit or find shelter. Sex pheromones released by female moths mediate sexual communication between genders. Until recently, studies on the olfactory system of moths have focussed on sex-specific characteristics. The perception of sex pheromones is well studied in many male moth species (e.g. Butenandt et al., 1959; Buser et al., 1974; Dunkelblum and Gothilf, 1983; Arn et al., 1986, 1988; Leal, 2005) whereas studies on plant volatile perception have mainly concentrated on female moths (e.g. Gabel et al., 1994; Jönsson and Anderson, 1999; Bruce and Cork, 2001; Burguiere et al., 2001; Hern and Dorn, 2002; Fraser et al., 2003; Stranden et al., 2003; Røstelien et al., 2000).

To perceive female produced sex pheromones at very low concentrations nature has equipped male moths with an array of antennal receptor neurones located in long sensilla trichodea (Schneider and Steinbrecht, 1968; Steinbrecht, 1970). However, wall-pore sensilla basiconica containing receptor neurones for plant volatiles are also present on male moths antennae (Vogt et al., 1991; Steinbrecht et al., 1995; Laue et al., 1994; Pophof et al., 2005). Perception of plant compounds by male moths may play an important role in mating behaviour as plant volatiles help guide males to the site where they

may find females (Landolt and Phillips, 1997). Such males would then be at the mating location site before females start calling and so gain an advantage over other males. Thus, host plant choice and mate choice are tightly linked and variability in the preference for host plant odours can serve as a driving force for sympatric speciation (Linn et al., 2003, 2005). A second important role of plants for male moths may be their role in providing shelter. Adult European grape berry moths, *Eupoecilia ambiguella*, for example, emerge from overwintered pupae in April when the vegetation on the grape vine is still very sparse (Bovey et al., 1972). Thus, plants in the surrounding hedges may serve as shelter to protect them from desiccation and enemies. Indeed, *E. ambiguella* are often found on two typical hedges plants, *Ligustrum vulgare* (Oleaceae) and *Viburnum lantana* (Caprifoliaceae) (Galet, 1982). These two plants, among others (cf. Galet, 1982), are considered as host plants of this polyphagous insect pest even though this was not rigourously proven. By definition only plants where an insect can complete its life cycle can be considered as host plants and this was not proven for some of the reported host plants of *E. ambiguella*. However, for a polyphagous species such as *E. ambiguella* the question arises as to how they can discriminate host plants from non-host plants by means of their olfactory system. In general, two hypotheses exist concerning the specificity of the host plant signals for insects: 1) the odour may provide specific information due to plant specific compounds not found in unrelated plant species, 2) the odour may be specific due to the particular ratio between compounds generally distributed among plant species (Visser, 1986; Bruce et al., 2005). The first hypothesis is highly unlikely for polyphagous species as their host plants, by definition (Bernays and Chapman, 1994), belong to different families with quite different odour profiles. Thus, taking the second hypothesis as its basis, this study aimed to identify behaviourally important host plant stimuli for male *E. ambiguella*. The hypothesis is that volatiles which are common to several host plants and which elicit antennal receptor neurone responses at low concentrations can serve as key semiochemicals in *E. ambiguella* sensory ecology. Therefore, we analysed and compared the odour profiles of the headspace extracts of six plant species from five different families by gas chromatography coupled electroantennogram detection using male *E. ambiguella* antennae. With the same method, we compared the detection threshold of male *E. ambiguella* antennal receptor neurones to 15 plant compounds and the pheromone component (Z)-9-dodecenyl acetate (hereafter Z9-12:Ac). For biologically relevant plant volatiles we expect low electrophysiological response thresholds as for the sex pheromone of *E. ambiguella*. To our knowledge, this is the first study regarding the perception of host plant volatiles by *E. ambiguella*.

2.2 Material and Methods

Headspace collections Branches of *Vitis vinifera* subsp. *sylvestris* (Viticeae), *Olea europea* (Oleaceae), *Ligustrum vulgare* (Oleaceae), *Viburnum lantana* (Caprifoliaceae) and *Rosmarinus officnialis* (Lamiaceae) were cut during the first generation flight of the grape berry moth during May-June 2006 and placed in 250ml gas-wash bottles. Branches with leaves and flowers of *Hedera helix* (Araliaceae) were cut at the end of the second generation flight in August 2006. Charcoal-filtered air was pulled for 2h through gas-wash-bottles containing 20-50g of fresh plant material at 500ml/min and then through a cartridge containing 50mg of the porous polymer PorapakQ. Before use, this adsorbent was conditioned for 90min at 200°C under nitrogen. Volatiles were desorbed by eluting the cartridge with 100μl dichloromethane into glass ampules that were sealed and stored in the freezer at -20°C until analysis.

Headspace extracts of *V. vinifera* subsp. *sylvestris* were made from cut branches with female and male flowers. The volatiles of *O. europeae* were sampled from shredded leaves. Two headspace collections were made from cut branches of *L. vulgare*, one with only flower buds and the second with open flowers. Additionally, the headspace odours over 50g of the larval rearing medium (Rauscher et al., 1984, Appendix A) were analysed. In total nine headspace extracts were made.

Synthetic standards Two solutions containing 15 plant volatiles and the main *E. ambiguella* pheromone component Z9-12:Ac in dichloromethane (DCM) were prepared at three different concentrations (10ng/μl, 1ng/μl and 100pg/μl). (+)-Terpinen-4-ol was present in both solutions as an internal reference. The other plant compounds were: octanal, (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol , R(+)-limonene, p-cymene, 4,8-dimethyl-1(E),3,7-nonatriene (DMNT), linalool, 1-octen-3-ol, α-terpineol, geraniol, methyl salicylate, furfural and phenyl acetaldehyde. The selection of these compounds was based on a previous study in our laboratory with *E. ambiguella* (Connétable and Guerin, unpublished) and on published work on the perception of host plants volatiles by other tortricid moths such as the grape vine moth *Lobesia botrana* and the codling moth *Cydia pomonella* (Ansebo et al., 2004; Bäckman et al., 2001; Light et al., 1993; Yang et al., 2004; Tasin et al., 2005, 2006b; Gabel et al., 1992).

Gas chromatography linked electroantennographic detection (GC-EAD) The solutions containing synthetic compounds and the headspace extracts were analysed by means of gas chromatography linked to electroantennogram detection (GC-EAD; Arn et al., 1975). An antenna of a male grape berry moth was cut and mounted between two glass capillary electrodes filled with 0.1M KCl. The recording electrode on which the antennal tip was mounted was connected to a high impedance

preamplifier (gain: 10x) which sent the recorded potential to an amplifier (gain: 100x). The gas chromatograph was equipped with a precolumn (BGB, fused silica capillary tubing; length 1m, I.D. 320μm, O.D. 450μm, deactivated with OV-1701-OH) and a polar column (BGB-FFAP, a polyethylene glycol phase esterified with terephthalic acid, length 30m, I.D. 0.25mm, film thickness 0.25μm). The column effluent was split (GRAPHPACK metal splitter, Gerstel®, Germany) with one outlet directed to the flame ionization detector (FID) and the other into a charcoal-scrubbed and humidified air stream (95% RH, 1m/sec) that blew over the antenna. H_2 was used as carrier gas. 2μl of the headspace extracts and the synthetic standard solutions were injected on-column. The oven was held at 40°C for 5 min then heated at 10°C/min to 230°C and held for 5 min. The FID and EAD-responses were recorded simultaneously on a PC using GC-EAD software (Syntech, NL). The responsiveness of the antenna was tested at the start and end of each run with an air puff from 10μl of a (+)-terpinen-4-ol solution (1μg/μl in dichloromethane) on a filter paper strip in a 5ml stimulus syringe as described in Taneja and Guerin (1997). If the antennal response to this 1s air puff (1ml/s) through this stimulus syringe was less than 0.4mV another antenna was mounted. For the headspace extracts, only compounds eliciting an EAD-responses of at least 0.05mV from at least two antennae at a particular retention time were considered, and Kovat's retention indices were calculated for these products.

Gas chromatograph linked mass spectrometry (GC-MS) The identity of biologically active compounds in the plant headspace extracts and the elution sequence of the synthetic volatiles were determined by gas chromatography-coupled mass spectrometry (GC-MS). 1μl of the extract was injected on-column on the polar column (FFAP as for GC-EAD) at 40°C with helium as carrier gas. The oven was heated at 5°C/min to 230°C and held there for 10min. Kovat's retention indices were calculated for peaks at particular retention times and compared with the Kovat's retention indices of biologically active peaks located by GC-EAD. The mass spectra of EAD active compounds with the same Kovat's retention indices occuring in different headspace extracts were compared and identified with reference to the mass spectra of products in a library (Nist98) and by Dr. Bernard Jean-Denis (Institute of Chemistry, University of Neuchâtel, Switzerland). Identities were confirmed by injection of synthetic compounds and by comparison with reported Kovat's retention indices (www.flavornet.org). The optical purities of the enantiomers were not determined.

Dose response curves The responsiveness of the antennal electrophysiological preparations to stimuli decreases linearly with time. The terpinen-4-ol loaded air puffs at the start and the end of each GC-run functioned to control for loss of responsiveness. Relative responses corrected to this loss

of responsiveness were calculated for plant volatiles injected at different amounts on the GC-column. Subsequently, dose response curves were fitted by loglinear regression using the statistical package `R` (Version 2.4.1).

2.3 Results

2.3.1 Analysis of headspace extracts of different host plants

Each headspace extract was injected at least three times and only compounds eliciting an EAD-response in at least two of three antennae were considered as valid. In this manner over 50 constituents of the plant headspace extracts were found to evoke electroantennogram responses from male *E. ambiguella* (Fig. 2.1 and Appendix B).

When a volatile compound elicited an EAD-response in one plant extract its presence was also checked for in the other extracts by GC-MS irrespective of whether it elicited an EAD-response or not, as the quantities may have been below the detection threshold of the antenna. This analysis resulted in a list of 16 compounds which were found in at least three of the six host plant extracts tested (Table 2.1). Of these, 14 volatiles were identified according to GC retention times (Kovat's indices) and matching mass spectra with synthetic compounds. These compounds can be classified into three classes derived from three different biosynthetic pathways: aliphatics (1-hexanol, (E)-2-hexenal, (Z)-3-hexenol, (Z)-3-hexenyl acetate, 1-octen-3-ol, 6-methyl-5-hepten-2-one and nonanal), terpenes (limonene, ocimene, β-caryophyllene, 4,8-dimethyl-1(E),3,7-nonatriene (DMNT) and (E,E)-α-farnesene) and aromatic compounds (benzaldehyde and methyl salicylate). The amounts of two compounds were too low to draw conclusions from the mass spectral data (Kovat's indices 1915 and 2108, respectively). For the other compounds the approximate amounts present in an aliquot of 1μl of the headspace extract were determined by peak heights and areas and divided in four categories according to abundance: 100pg, 1ng, 10ng and 100ng/μl (Table 2.1). In addition, the EAD-responses to these compounds were compared to the response to a 1s air puff from a 5ml syringe containing 10μg (+)-terpinen-4-ol on a filter paper strip. All 16 compounds were present in *V. vinifera* subsp. *sylvestris*, whereas only 8 of these were found in *H. helix*. Some were also identified in the headspace extract of the semiartificial larval rearing medium: 1-hexanol, (Z)-3-hexen-1-ol, 1-octen-3-ol, nonanal, 6-methyl-5-hepten-2-one, limonene, β-caryophyllene and methyl salicylate. Methyl salicylate and (Z)-3-hexen-1-ol were found in all of the extracts. Remarkably, considerable EAD-responses were recorded to (Z)-3-hexen-1-ol and methyl salicylate even at the very low dose of about 100pg in the extracts (Fig. 2.1, compound 12; Table 2.1).

TABLE 2.1: Compounds eliciting EAD-responses present in at least three host plant extracts of *E. ambiguella*. The circle size indicates approximate amounts present in a 1μl aliquot of the headspace extracts: ● ≈ 100pg, ● ≈ 1ng, ● ≈ 10ng and ● ≈ 100ng. The colours indicate relative EAD-responses compared to the reference: dark blue >40%, middle blue 20-40%, light blue 1-20% and open circles no response. Small dark blue circles therefore mean a strong antennal response at a low quantity. For the two compounds which could not be identified + indicates the presence of an EAD-response at the particular retention time. KI = Kovat's retention index established for synthetic standards (KI_{STD}) and in headspace samples (KI_{HSP})

KI_{STD}	KI_{HSP}	Compound	*Vitis vinifera*	*Viburnum lantana*	*Ligustrum vulgare*[a]	*Olea europaea*	*Rosmarinus officinalis*[b]	*Hedera helix*	rearing medium
aliphatic compounds:									
1350	1354	1-hexanol	●	○	●	●			●
1203	1213	(E)-2-hexenal	●		●	●			
1370	1380	(Z)-3-hexenol	●	●	●	●	●	●	●
1314	1323	(Z)-3-hexenyl acetate	●	●	●	○	○		
1430	1446	1-octen-3-ol	●	●		●	●		●
1335	1342	6-methyl-5-hepten-2-one	●		○	○		●	●
1393	1388	nonanal	●	●	●	●	●		●
terpenes:									
1188	1190	limonene	●	●			●	●	●
1256	1254	ocimene	●	●	●	●	●	●	
1590	1589	β-caryophyllene	●	●		●	●	●	●
1303	1313	DMNT	●	●	●	●			
1746	1747	(E,E)-α-farnesene	○	●	●	●			
aromatic compounds:									
1525	1528	benzaldehyde	●	○	○		○	○	
1783	1778	methyl salicylate	●	●	●	●	●	●	●
	1915	unknown	+	+	+		+	+	+
	2108	unknown	+	+		+			

[a]Relative amounts are given for branches only with flower buds. Relative amounts of compounds in flowering branches were similar with the exception of a high amount of benzaldehyde and the absence of E,E-α-farnesene.

[b]Many volatiles were found in rosemary which have overlapping retention times with other compounds. The relative quantities are therefore imprecise.

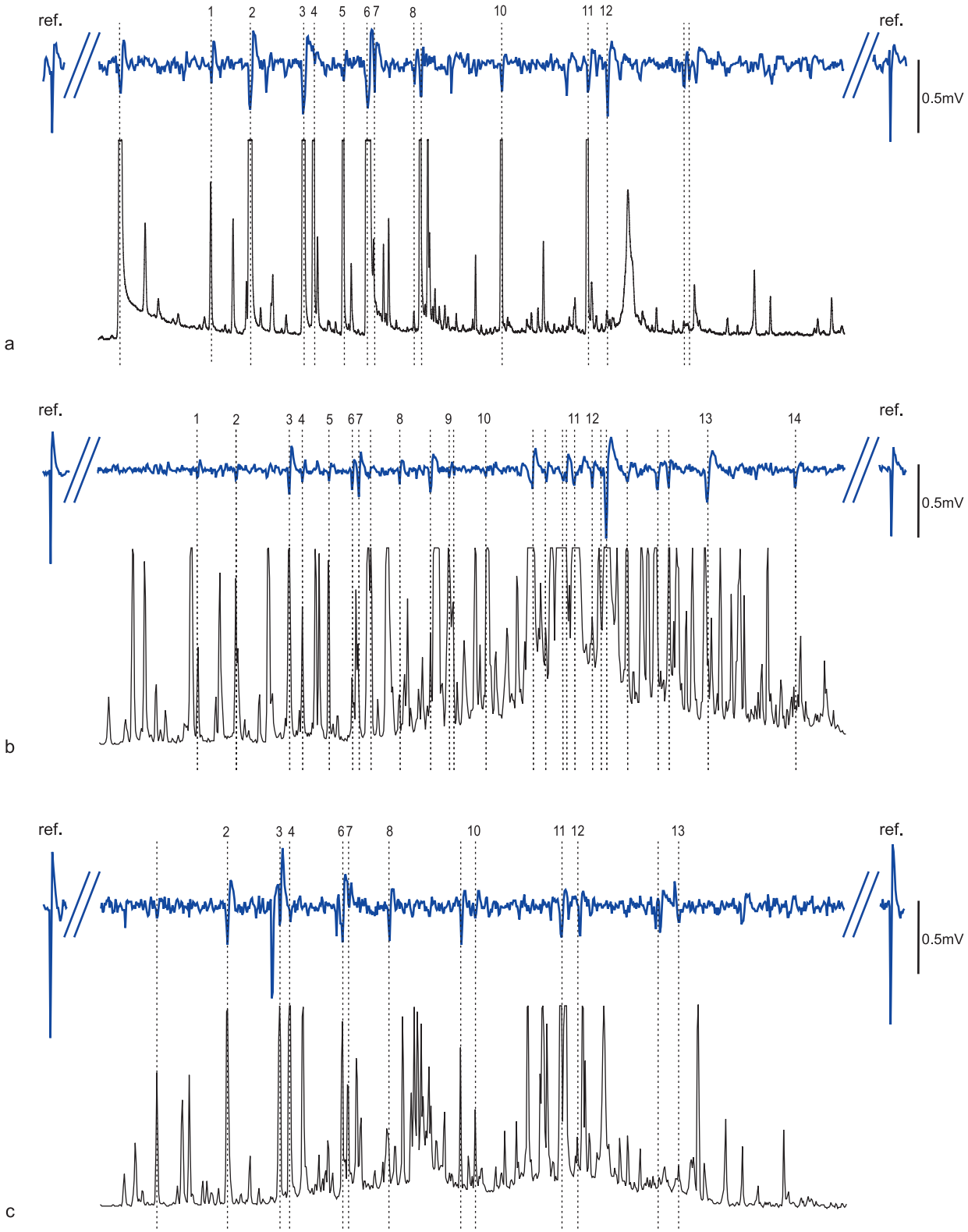


FIGURE 2.1: Antennogram responses of male *E. ambiguella* to aliquots of the headspace extract of a. *O. europeae*, b. *Vit. vinifera* subsp. *sylvestris* female flowers and c. *Vib. lantana*. Only the constituents highlighted with the dotted lines elicited EAD-responses in at least two different analyses using antennae from different individuals. Numbered volatiles were found to elicit antennogram responses in at least three of the plant extracts tested: 1 (E)-2-hexenal; 2 α-ocimene; 3 DMNT; 4 (Z)-3-hexenyl acetate; 5 1-hexanol; 6 (Z)-3-hexenol; 7 nonanal; 8 1-octen-3-ol; 9 benzaldehyde; 10 β-caryophyllene; 11 (E,E)-α-farnesene; 12 methyl salicylate; 13 unknown; 14 unknown. Ref. is the response to an air puff applied to the antennal preparation at the start and end of each analysis, from a 5ml syringe containing 10μg (+)-terpinen-4-ol on a filter paper strip.

2.3.2 Dose-dependant EAD-responses to host plant volatiles

EAD-responses to 15 plant volatiles selected on the basis of previous studies (see above) and the pheromone product Z9-12:Ac were recorded at different quantities injected onto the polar phase used to analyse the host plant extracts. Because the quality of the aldehydes used was low probably due to decomposition of the older laboratory sample, the responses to (E)-2-hexen-1-al, octanal, furfural and phenyl acetaldehyde were not analysed.

The GC-EAD profiles generated by the 15 plant products at three different concentrations using male *E. ambiguella* antennae show that antennal receptor neurones respond to all compounds at the highest dose injected (10ng, Figs. 2.2 and 2.3). At 100pg responses were still recorded to (Z)-3-hexen-1-ol, 1-octen-3-ol, *p*-cymene, DMNT, (+)-terpinen-4-ol, R(+)-limonene, linalool, methyl salicylate and Z9-12:Ac. The strongest response was to the pheromone product Z9-12:Ac (Fig. 2.2). For those plant volatiles still eliciting EAG responses at 100pg, dose response curves were fitted (Fig. 2.4). The x-intercept, indicating the detection threshold, the slope of the responses and the coefficient of determination (R^2) are shown in Table 2.2. For (Z)-3-hexen-1-ol and methyl salicylate the loglinear regression is not adequate. However, with only three points it is difficult to fit what is essentially a non-linear dose response relationship, so the fitted regression lines have to be interpreted with caution. The calculated x-intercepts indicate detection thresholds at less than 10pg for (+)-terpinen-4-ol, (Z)-3-hexen-1-ol, *p*-cymene and methyl salicylate.

TABLE 2.2: Characteristics of the dose-response plots in Fig. 2.4 (loglinear regression, $p<0.05$). The data are sorted from top to bottom in order of ascending x-intercepts. A low x-intercept indicates a low response threshold for the compound. The loglinear regressions fitted to the dose-response data for (Z)-3-hexen-1-ol and methyl salicylate are not significant.

compound	solution	x-intercept (ng)	slope	R^2
(+)-terpinen-4-ol	a	0.0017	0.181	0.60
(Z)-3-hexen-1-ol	a	0.0046	0.193	0.30n.s.
p-cymene	b	0.0059	0.238	0.57
(+)-terpinen-4-ol	b	0.0077	0.422	0.77
methyl salicylate	b	0.0081	0.298	0.28n.s.
R(+)-limonene	b	0.0124	0.244	0.73
1-octen-3-ol	b	0.0273	0.305	0.58
DMNT	b	0.0298	0.352	0.67
linalool	b	0.0425	0.339	0.87

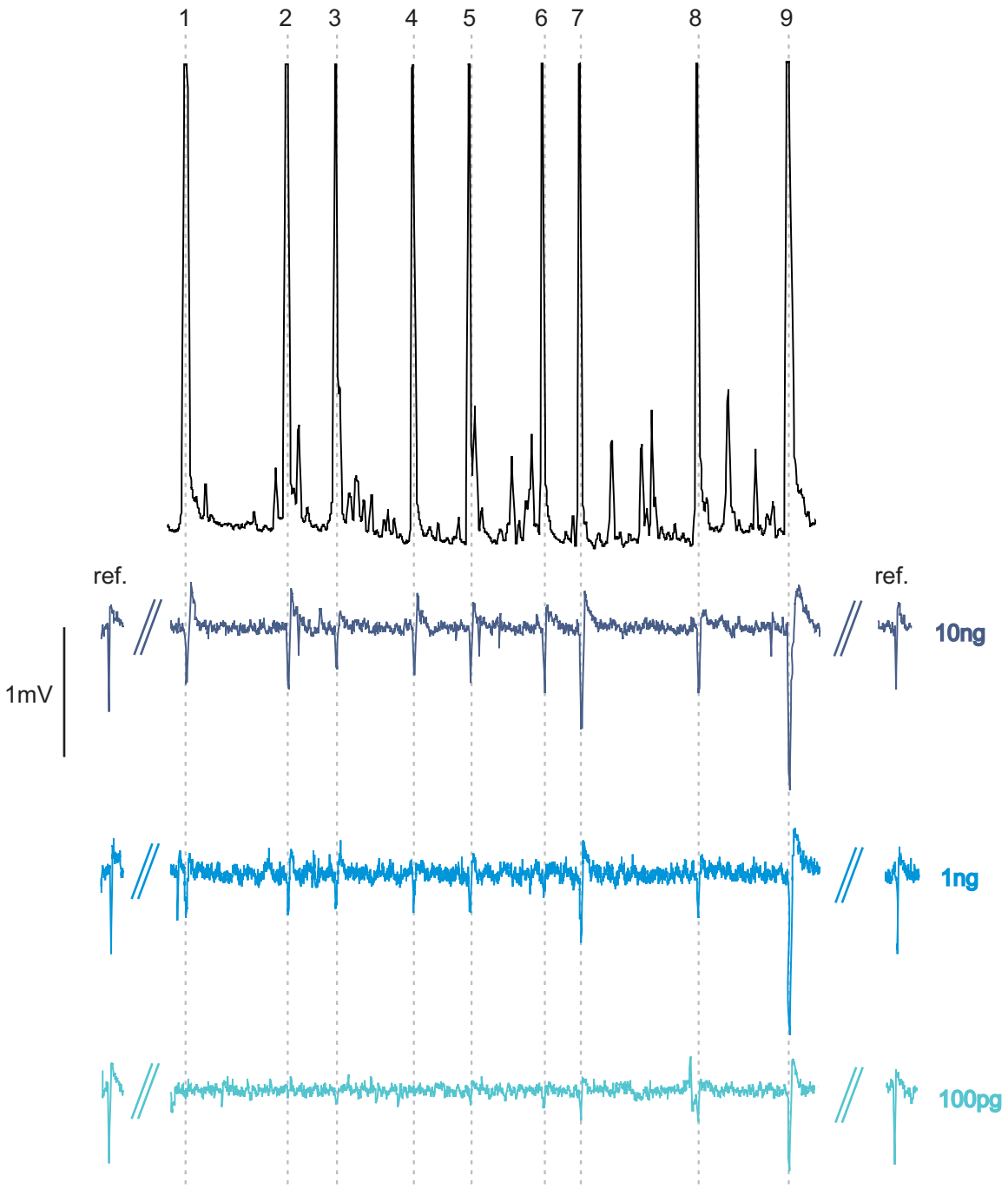


FIGURE 2.2: GC-EAD profiles generated by a mixture of 8 synthetic plant compounds and the pheromone product Z9-12:Ac each injected at 10ng, 1ng and 100pg/μl using the antenna of the same *E. ambiguella* male as a biological detector. 1 R(+)-limonene; 2 *p*-cymene; 3 DMNT; 4 E-2-hexen-1-al; 5 1-octen-3-ol; 6 linalool; 7 (+)-terpinen-4-ol; 8 methyl salicylate; 9 Z9-12Ac. Note the strong antennogram response to Z9-12Ac even at 100pg compared to the plant volatiles. Ref. is the response to an air puff applied to the antennal preparation at the start and end of each analysis, from a 5ml syringe containing 10μg (+)-terpinen-4-ol on a filter paper strip.

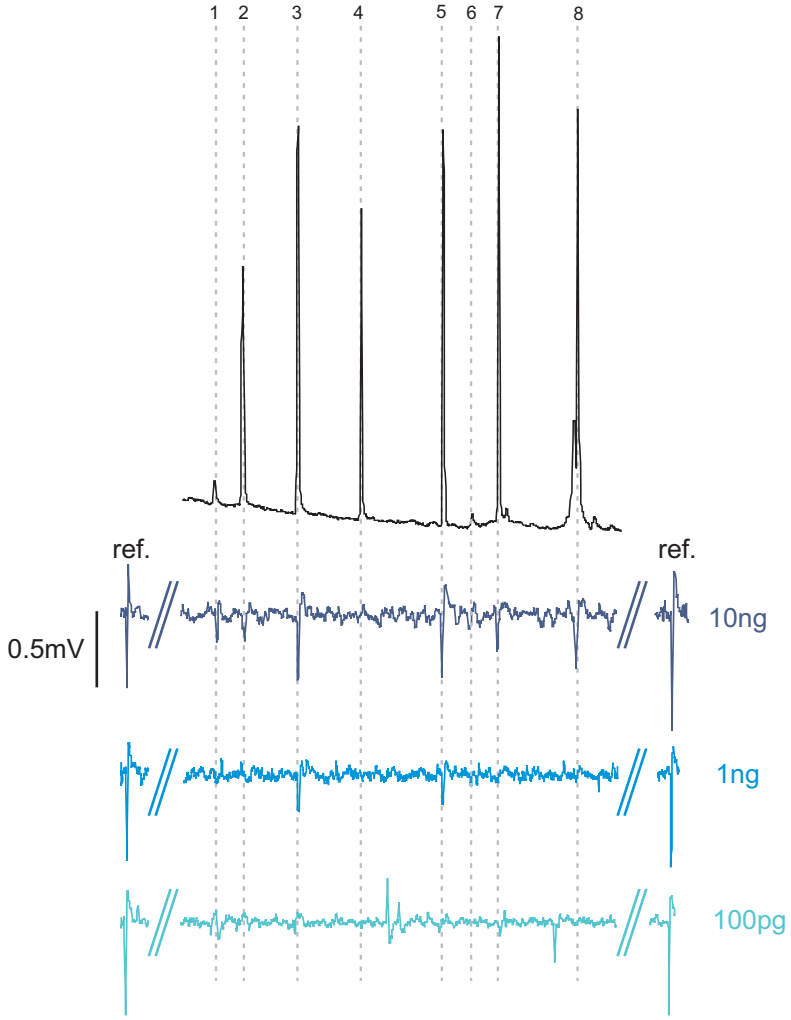
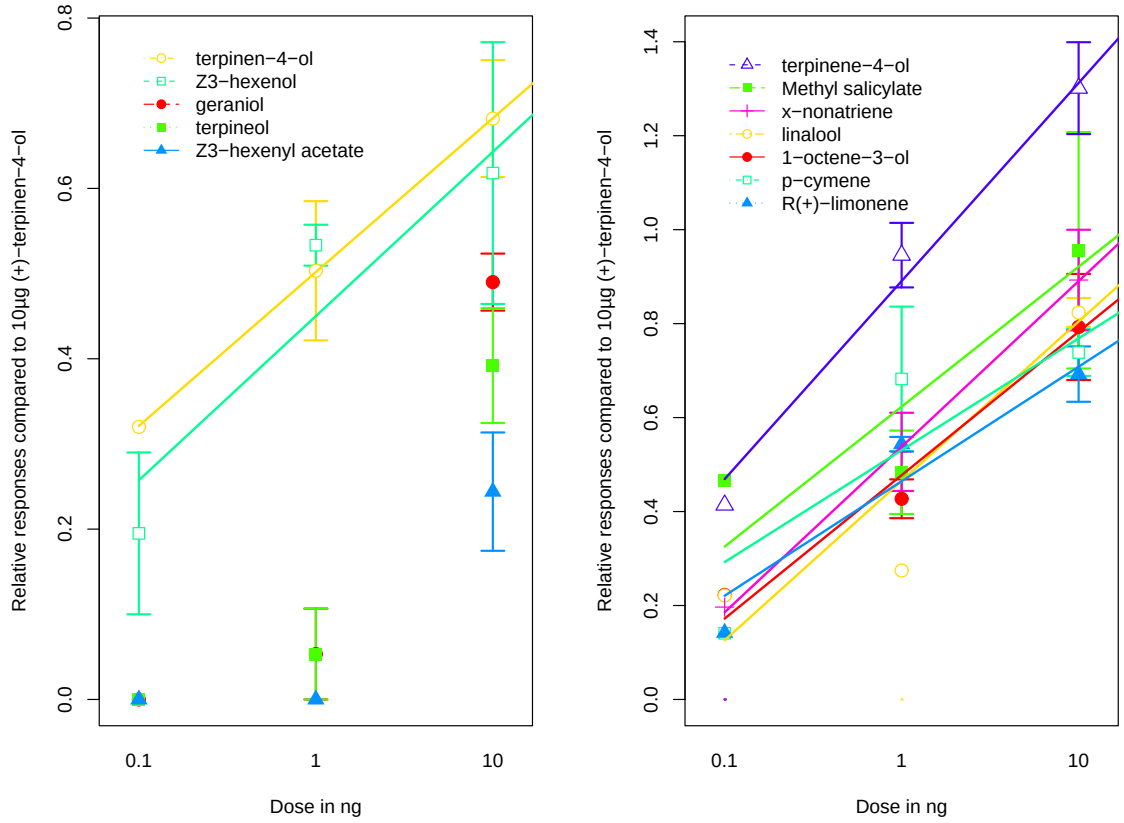


FIGURE 2.3: GC-EAD profiles generated by a mixture of 8 synthetic plant compounds, each injected at 10ng, 1ng and 100pg/μl using the antenna of the same *E. ambiguella* male as a biological detector. 1 octanal; 2 hexenyl acetate; 3 (Z)-3-hexen-1-ol; 4 furfural; 5 (+)-terpinen-4-ol; 6 phenyl acetaldehyde; 7 α-terpineol; 8 geraniol. Ref. is the response to an air puff applied to the antennal preparation at the start and end of each analysis, from a 5ml syringe containing 10μg (+)-terpinen-4-ol on a filter paper strip.



a b

FIGURE 2.4: Relative EAD-responses (compared to 10μg (+)-terpinen-4-ol released from a syringe) of male *E. ambiguella* to 12 synthetic plant volatiles (a 5 compounds; b 7 compounds) as a function of the dose. (+)-Terpinen-4-ol was included in both solutions as the reference product. No responses to (Z)-3-hexenyl acetate, α-terpineol and geraniol were recorded at a concentration of 100pg. The regression line characteristics are listed in Table 2.2.

2.4 Discussion

2.4.1 Analysis of headspace extracts of different host plants

Plants release many different volatile compounds. To select compounds that may be important in the sensory ecology of *E. ambiguella*, we screened a range of host plants for volatiles eliciting EAD-responses. Except *O. europaea*, all plants used in this study are referred to as host plants of *E. ambiguella* (cf. Galet, 1982). *O. europeae* is considered as a host plant of the grapevine moth, *Lobesia botrana*, a sister species of the grape berry moth (Katerinopoulos et al., 2005; Savopoulou-Soultani et al., 1990), and was included here because the two moth species share many host plants. On analysing the headspace extracts of these plants we found that male *E. ambiguella* receptor neurones respond to a range of different plant volatiles including aliphatics, terpenes and aromatic compounds (Fig. 2.1).

Thus, male grape berry moths are equipped not only with pheromone-sensitive receptor neurones but also with receptor neurones for plant volatiles similar to other moth species. EAD-responses of male moths to plant compounds have also been documented among others for *Cydia pomonella* (Ansebo et al., 2004), *Spodoptera exigua* (Dickens et al., 1993b) and *Manduca sexta* (Fraser et al., 2003). This suggests that not only the sex pheromone but also plant products play a role in the sensory ecology of these male moths.

We found that 16 plant volatiles eliciting EAD-responses are common to at least three of the six host plants analysed. These were commonly occurring secondary plant products emitted by a range of plants from different families (Knudsen et al., 1993, 2006). As no plant volatiles were found that are specific to the different host plants, and some were identified even in the larval rearing medium, we assume that the polyphagous European grape berry moth discriminates between host and non-host plants using plant volatiles common to the different plants but varying in the ratios present, or by non-volatile compounds on the plant surface detected on contact with the substrate (Maher, 2002). A specific ratio of commonly occurring green leaf volatiles (GLVs), for example, was found to attract the oligophagous Colorado potato beetles, *Leptinotarsa decemlineata* and this attractiveness was lost with a GLV-mixture in an altered ratio (Visser and Ave, 1978). Here, we can make no prediction as to the importance of appropriate ratios of products for *E. ambiguella* as the purpose of this study was a qualitative analysis of the odour profiles of host plants. In general, naturally occurring ratios between plant volatiles are difficult to determine because all headspace collection methods have limits as to the selectivity of the porous polymer and the solvent's selective desorption power (Tholl et al., 2006). In this study we used a closed system where the plant material was cut (and thereby damaged) and enclosed into a gas-wash bottle so that humidity and heat in the gas-wash bottle increase in response to the physiological activity of the plant material. As a consequence, the odour profile may have changed during the sampling process. In addition, glass in itself is a good adsorbent (active SiO_2 sites) such that volatiles are adsorbed not only on the porous polymer but also on the glass walls of the gas-wash bottle. Thus, the quantities of plant volatiles in the headspace extracts may not represent the natural profiles. However, the method permitted us to obtain a sample of the volatiles released by these plants and perceived by male *E. ambiguella* olfactory receptor neurones and to have some primary indications for ratios. Methyl salicylate, for example, was identified always in smaller amounts compared to most other compounds. Low levels of methyl salicylate below the detection threshold of the FID were also noted in other plants such as *Brassica oleracea* and *Arabidopsis thaliana* using the antenna of the cabbage moth *Mamestra brassicae* as a biological detector (Ulland et al., 2008).

2.4.2 Dose-dependant EAD-responses to host plant volatiles

To quantify responses of *E. ambiguella* antennal receptor neurones to volatile plant products we injected solutions of synthetic plant volatiles at different quantities onto the gas chromatograph. The response threshold to a particular odour can subsequently be determined from a dose response curve. At the intercept with the x-axis the response is zero and the threshold dose is the one where a significant response different from the blank control can be detected. Low response thresholds may be an indication of a key stimulus for the species in question (Mayer et al., 1987; Visser, 1979). However, with GC-EAD no final conclusion can be made about the specificity of receptor neurones (Wadhams, 1982; Van der Pers and Löfstedt, 1983). More detailed information about such specificity can only be obtained by single sensillum recordings (SSR Wadhams, 1982). In addition, the response of highly specific receptor neurones occurring only in low numbers on the antenna will not be detected by GC-EAD. It is for this reason that Van der Pers and Löfstedt (1983) recorded no EAD-response to the pheromone compound Z9-14:Ac from *Agrotis segetum* males although highly specific receptor neurones for this compound were identified by GC-SSR. Nevertheless, GC-EAD is a convenient method to obtain an image of receptor neurone response types present on the moth antenna.

Comparing the response of the pheromone and of different tested host plant volatiles, the highest EAD-response was recorded to Z9-12:Ac (Fig. 2.2) as expected because the male *E. ambiguella* antenna may carry primarily sensilla trichodea bearing receptor neurones for pheromone products as shown for the tortricid moth *Cydia molesta* (George and Nagy, 1984) and other moth species (Steinbrecht et al., 1995). Concerning plant volatiles, (Z)-3-hexen-1-ol, (+)-terpinen-4-ol, *p*-cymene and methyl salicylate turned out to be strong stimuli for antennal receptor neurones of *E. ambiguella* males. The monoterpene (+)-terpinen-4-ol is the main component of the oil of tea tree *Melaleuca alternifolia* which is well known for its antimicrobial activity (Carson and Riley, 1995). Both, (+)-terpinen-4-ol and *p*-cymene, are emitted by tansy flowers, *Tanacetum vulgare*, a wild species of temperate regions often found in vineyards (Gabel et al., 1992). These flowers are reported to be regularly visited by female grapevine moths, *L. botrana*, and may serve as food sources (Gabel, 1992; Gabel and Thiéry, 1994). These two monterpenes were also present in the headspace extract of the larval rearing medium (data not shown) and *p*-cymene was found in rosemary extracts by Katerinopoulos et al. (2005) but not in our rosemary headspace extract. A behavioural response to these two compounds has not been reported to date for lepidopteran species.

(Z)-3-hexen-1-ol and methyl salicylate, interestingly, are compounds which we found in all the headspace extracts tested (see above). (Z)-3-hexen-1-ol is derived from the essential fatty acid linoleic

acid by lipoxygenase and hydroperoxide lyase activities (Hatanaka, 1993). This green leaf volatile is known to enhance male *Cydia pomonella* and *Spodoptera exigua* flight responses to their respective sex pheromones in the wind tunnel (Yang et al., 2004; Deng et al., 2004). Methyl salicylate is derived from salicylic acid, that is synthesized from phenylalanine via the benzenoid branch of the phenylpropanoid pathway (Lee et al., 2005). Multiple functions have been attributed to methyl salicylate in different studies: this aromatic compound is used in communication between plants (Shulaev et al., 1997) and is implicated in the tritrophic interactions between the lima bean *Phaseolus lunatus* (Fabaceae), the herbivorous mite *Tetranychus urticae* and the predatory mite *Phytoseiulus persimilis* (De Boer and Dicke, 2004). *P. persimilis* is attracted to methyl salicylate that is released in higher amounts following damage of *P. lunatus* caused by *T. urticae*. Methyl salicylate inhibits oviposition in the cabbage moth *Mamestra brassicae* (Ulland et al., 2008) and is also synthesised as a pheromone by the tick *Amblyomma variegatum* (Diehl et al., 1991), providing evidence for the widespread use of this product as a stimulant in both insects and acarids.

To summarise, (Z)-3-hexen-1-ol, (+)-terpinen-4-ol, *p*-cymene and methyl salicylate are promising candidates for behavioural experiments, the true means to test whether these four and other plant volatiles, individually or as mixtures play a role in the sensory ecology of male *E. ambiguella*.

Chapter 3

Development and validation of behavioural assay methods

3.1 Introduction

Electrophysiological responses of antennal receptor neurones to low doses of products is a useful means of preselecting plant volatiles which may be relevant to the behaviour of a phytophagous insect (Chapter 2). However, behavioural experiments are necessary to test the true impact of such products in the sensory ecology of the insects. For flying insects, wind tunnel experiments have the advantage that many parameters such as wind speed, temperature, light and humidity can be regulated providing standard conditions in which to do tests and thus reduce variability in the recorded data. These wind tunnel settings have first to be adapted to the needs of the test insect species. Defined behavioural elements can than be recorded by real time observations or by tracking the flight path with a video system.

An important aspect of such experiments is how to present the test odours. Odour plumes are not characterized by a concentration gradient but are inhomogenous because of air turbulences (Kramer, 1978). The odour arrives on the insect antenna as bursts and thus intermittent signal (Murlis and Jones, 1981). Kramer (1986) showed that male *Bombyx mori* are able to respond to intermittent odour pulses. Flight responses of moths to odours are thus not only dependent on the quantity and quality of an odour but also on the manner in which it arrives at the antennal receptor neurones. To reduce variability in the plume structure a laminar flow resulting in only small turbulences is used in wind tunnels. Because obstacles in the wind tunnel cause most turbulence, the plume structure is thus highly influenced by the setup from which odours are released.

The aim of the behavioural experiments described in this chapter was the optimisation of test conditions to record the flight responses of male *Eupoecilia ambiguella*. I first tested the attractiveness of different pheromone blends including calling females to compare the responses in our wind tunnel with the results of Rauscher et al. (1984) and Arn et al. (1986). Secondly, I added plant volatiles to the pheromone to obtain preliminary evidence for the hypothesis that plant volatiles may affect the behavioural responses of male *E. ambiguella* to its sex pheromone. These experiments were accompanied by several optimisation steps for test product presentation.

This chapter is divided in two parts. In the first part rubber septa that are used in monitoring traps were used to deliver test compounds. In the second part I describe the piezo nebulizer which we installed to release pheromone and plant compounds at known amounts. With this system a dose response curve to the pheromone was established serving as a basis for the experiments described in Chapter 4.

3.2 Odour delivery system: Rubber Septa

3.2.1 Material and Methods

Insects Pupal stages of *E. ambiguella* were obtained weekly from a laboratory culture at the Research Station Agroscope Changins-Wädenswil ACW (Switzerland). Pupae were sexed and put on a cloth mesh stretched over a dish filled with water for emergence. A funnel placed on the mesh guided emerging moths into a plastic cylindrical cage placed overhead (diameter: 10.5cm; height: 16cm) to accommodate the adults. This cylindrical cage was changed daily, enclosed in a plastic bag and sprayed once a day with water. A sugar solution (10% sucrose) in glass tubes closed with cotton wool was offered to the males. The insects were held in a climate chamber with a 16:8h L/D cycle at 65% RH and 25°C during the photophase and 85% RH, 18°C during the scotophase.

Synthetic chemicals The synthetic pheromone compounds used were Z9-12:Ac (99.9%, Plant Research International, Netherlands), 12:Ac (>96%, BASF, Germany), 18:Ac (>97%, TCI Europe N.V., Belgium). The synthetic plant volatiles methyl salicylate (>99%), (Z)-3-hexen-1-ol (>98%) and (+)-terpinen-4-ol (99%) were purchased from Fluka (Switzerland) and 1-octen-3-ol (>97%) from Merck (Germany).

The wind tunnel The wind tunnel (200 x 60 x 60cm) is constructed of non-reflecting glass. Two centrifugal ventilators at either end operate simultaneously to move the air across the tunnel at 30cm/s through active charcoal cartridges and laminar flow screens (Rauscher et al., 1984). Overhead illumination is provided by high frequency fluorescent daylight tubes of 36W (>1kHz, Philips) running the length of the tunnel (≈ 5lux on the floor). Below the tunnel floor, a white sheet with black patches of irregular sizes and shapes fixed at random was used as an optic cue. Humidity and temperature were the same as in the rearing chamber during the scotophase (above). The odour compounds were released at the upwind end in different ways (see below). Three-day old male *E. ambiguella* were transferred individually into glass tubes (o.d. 2.5cm; i.d. 2.1cm; length 12.5cm) closed with a piece of cotton wood on both ends. The glass tube was put on a stand 30cm high and 30cm from the downwind end of the wind tunnel. The cotton was removed immediately on exposure to the odour source and observations on the behaviour of individual males was quantified with a data logger (The Observer® Version 5.0, Noldus, NL). The following parameters were measured: duration of no activity, activation with or without wing fanning, take off, upwind flight, passing through half the length of the wind tunnel in flight (midline), close in within 10cm of the source, and contact with the source.

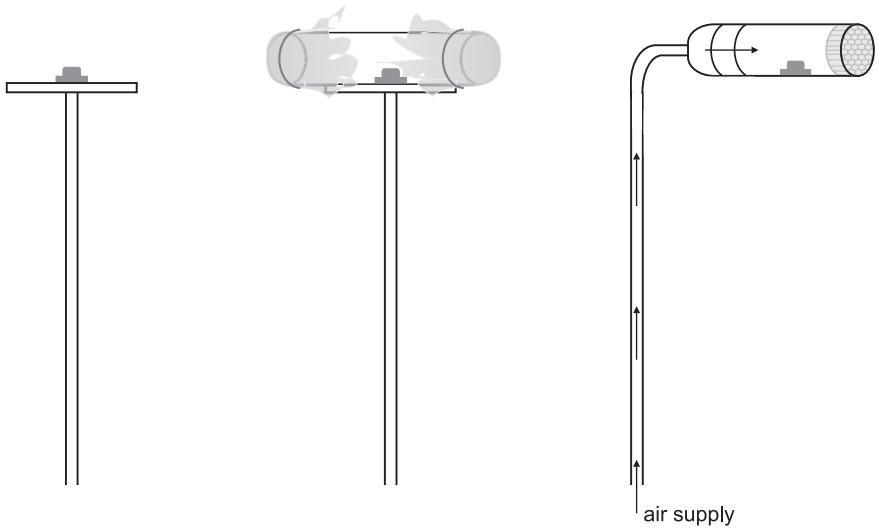
Odour presentation During the early experiments three different ways of presenting the pheromone-impregnated rubber septa were tested as explained below (Fig. 3.1). In the first test series the attractiveness of different pheromone blends and of 2-3 calling females to the males were compared. The different pheromone mixtures were the main pheromone component Z9-12:Ac alone (20μg), a binary pheromone blend (Z9-12:Ac and 12:Ac at 100:100μg) and a ternary pheromone blend (Z9-12:Ac, 12:Ac and 18:Ac; 100:100:200μg). The doses on the rubber septa were as recommended by Rauscher et al. (1984) and Arn et al. (1986). The compounds were diluted in dichloromethane (SupraSolv®, Merck, Germany) and applied to the trough of a rubber septum (Arn et al., 1979). The pheromone products were dispensed into the air from the red rubber septum attached to a glass slide and presented on a platform 30cm high at the upwind end of the wind tunnel (Fig. 3.1a). When not in use, the rubber septum was enclosed in tinfoil and stored in the freezer at -20°C. Females were presented in a glass tube (o.d. 2.5cm; i.d. 2.1cm; 12.5cm long) closed with a piece of curtain netting (mesh size: 1.5mm) and attached to the platform (Fig. 3.1b).

In a second test series the aim was to test if the attractiveness of the main pheromone compound Z9-12:Ac could be enhanced by adding (+)-terpinen-4-ol or 1-octen-3-ol to the rubber septum. Doses of 2μg and 20μg of (+)-terpinen-4-ol and 2μg of 1-octen-3-ol were applied together with 20μg Z9-12:Ac

to rubber septa and presented on the platform (Fig. 3.1a).

In a third test series, the rubber septum was placed in the same glass tube as used for calling females (Fig. 3.1b). This permitted comparison of the behavioural response of males to calling females and to the mixtures from the rubber septum from the same physical location. Subsequently, the flight responses of male *E. ambiguella* to 2-3 calling females, to Z9-12:Ac (20μg) alone and to Z9-12:Ac (20μg) in a mixture with the green leaf volatile (Z)-3-hexenol (2μg) were recorded.

In a fourth test series, instead of putting the rubber septum in a glass tube, a glass container (i.d. 2.5cm, 11cm long) with an inlet connected to an external air supply and an outlet (i.d. 2.5cm) facing down the wind tunnel was installed (Fig. 3.1c). The outlet was closed with a tinfoil honey comb (largest cell i.d.=3.18mm, thickness of the sheet=12.7mm, tinfoil thickness=0.04mm; Steiner Technik, Switzerland). This configuration permitted to control the air flow over the rubber septum and the females so that the glass container was emptied every two seconds. With this setup the attractiveness of 20μg Z9-12:Ac alone and in mixtures with 2 and 0.2μg methyl salicylate and of the ternary pheromone blend (see above) alone and in mixtures with the plant volatiles (+)-terpinen-4-ol (10μg) or methyl salicylate (10μg) on rubber septa was compared. A summary of all tests conducted is given in Table 3.1.



a Platform with rubber septum

b Glass tube with rubber septum

c Glass container with rubber septum

FIGURE 3.1: Different odour presentation methods tested. The height of the rubber septum was at 30cm for all methods. The rubber septum was either put on the platform on a glass slide (a), placed in a glass tube closed with a piece of curtain netting through which the wind tunnel air stream passed (b) or in a glass container connected to an external air supply (c). The outlet of the glass container was closed with a honey comb of tinfoil (see text).

TABLE 3.1: Overview of the experiments with rubber septa and the different presentation methods (Fig. 3.1) used.

Pheromone composition	**Plant compound**	**Presentation from**
calling females		glass tube, glass container
20μg Z9-12:Ac		platform, glass tube, glass container
20μg Z9-12:Ac	20μg terpinen-4-ol	platform
20μg Z9-12:Ac	2μg terpinen-4-ol	platform
20μg Z9-12:Ac	2μg 1-octen-3-ol	platform
20μg Z9-12:Ac	2μg (Z)-3-hexen-1-ol	glass tube
20μg Z9-12:Ac	20μg terpinen-4-ol	platform
20μg Z9-12:Ac	2μg methyl salicylate	glass container
20μg Z9-12:Ac	0.2μg methyl salicylate	glass container
binary pheromone blend:		
100μg Z9-12:Ac, 100μg 12:Ac		platform
ternary pheromone blend:		
100μg Z9-12:Ac, 100μg 12:Ac, 200μg 18:Ac		platform, glass container
100μg Z9-12:Ac, 100μg 12:Ac, 200μg 18:Ac	10μg terpinen-4-ol	glass container
100μg Z9-12:Ac, 100μg 12:Ac, 200μg 18:Ac	10μg methyl salicylate	glass container

Statistical analysis The responses of male *E. ambiguella* to different treatments were compared by fitting a generalised linear model (GLM) with a logit link function (logistic regression) to the responses, assumed to be binomially distributed, using the statistical package `R` (Version 2.4.1). Analysis of deviance based on the asymptotic χ^2 distribution was used to test whether the flight responses are significantly dependent on the odour sources ($p<0.05$). When the GLM was significant ($p<0.05$) multiple comparisons (R-package: `Multcomp`) were made using Tukey-contrasts.

3.2.2 Results and discussion

Establishing the validity of the wind tunnel conditions To examine if the general wind tunnel conditions where suitable for behavioural experiments with male *E. ambiguella* we tested their responses to females held in glass tubes and to different pheromone blends released from rubber septa presented on the platform. Calling females engaged 88% of males to fly upwind and caused 69% of these to contact the glass tube containing them. For the synthetic pheromone, results similar to Arn et al. (1986), were obtained, namely, that the attractiveness of Z9-12:Ac can be increased by adding either 12:Ac or 12:Ac plus 18:Ac (Fig. 3.2): 20μg Z9-12:Ac released alone from the platform attracted 43% of males to the source whereas the binary blend (Z9-12:Ac and 12:Ac at 100:100μg) attracted 59% and the ternary blend (Z9-12:Ac, 12:Ac and 18:Ac at 100:100:200μg) 64%. However, because of low sample sizes (between 44 and 51 per treatment) behavioural responses to the treatments differed only significantly in the percentage of upwind flights induced.

Upwind flights to the females were seen to be more direct and faster than the flights towards the

synthetic pheromone blends. Witzgall and Arn (1990) have already quantified such a difference in the flight behaviour for *Lobesia botrana* males to females compared to the synthetic pheromone compounds. However, in our case the attractiveness of females could not be compared directly with the synthetic pheromone blends released from rubber septa as they were presented in two different manners (from the platform for rubber septa and from a glass tube for calling females). In summary, the data showed that the conditions in the wind tunnel were suitable to measure responses to females and to discriminate for differences between treatments and so we continued with behavioural experiments under the settings established.

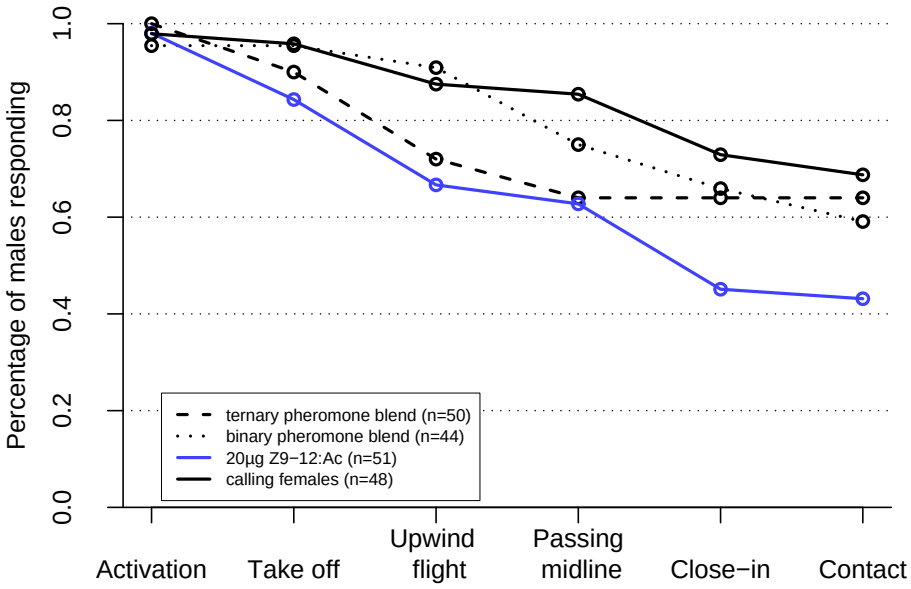


FIGURE 3.2: Percentage of male *E. ambiguella* responding to 2-3 calling females, to the main pheromone compound Z9-12:Ac (20μg), to a binary blend of 100μg Z9-12:Ac and 100μg 12:Ac, and to a ternary blend of 100μg Z9-12:Ac, 100μg 12:Ac and 200μg 18:Ac. The rubber septa containing synthetic pheromone were presented on a platform (Fig. 3.1a), the calling females in a glass tube closed with gauze (Fig. 3.1b).

Responses to Z9-12:Ac impregnated rubber septa The rubber septum impregnated with 20μg Z9-12:Ac was used as stimulus source for male *E. ambiguella* employing three presentation methods to permit comparison (Fig. 3.1 and Table 3.1). The best attraction to Z9-12:Ac was achieved when the rubber septum was placed in the glass container with the honeycomb tinfoil outlet. Here 66% of the males flew upwind and 48% contacted the source (Fig. 3.3, Table 3.2). Significantly fewer males flew upwind (27%) and contacted the source (9%), when the rubber septum was presented in a glass tube closed with gauze at either end (GLM, $p<0.01$). The responses to the Z9-12:Ac-impregnated septum presented on the platform or in the glass container were similar for activation, take-off and

upwind flight past the middle of the wind tunnel. However, for the behavioural elements close-in and source contact the percentage of males responding was lower (but not significantly) when the rubber septum was presented on the platform.

The air flow in the wind tunnel was almost laminar. If objects are introduced into the wind tunnel turbulence is created depending on the size and form of the objects. The platform, the glass tube and the glass container produced different types of turbulence and the form of the odour plume differed between them. This can partly explain the differences in male attraction to Z9-12:Ac depending on how the dispenser is presented. In the case of the platform the air passes more or less freely over the rubber septum. In the case of the glass tube, the gauze netting inhibits air to pass through the glass tube to about 2cm/s. To solve this problem the glass container was adopted to regulate the air passing over the rubber septum and also permit a comparison between calling females and the synthetic pheromone blends released from rubber septa.

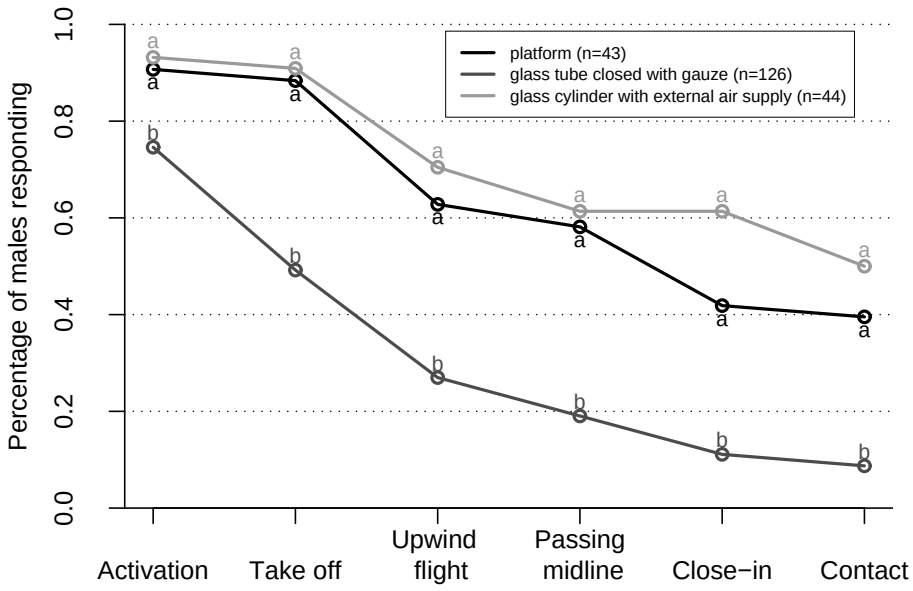


FIGURE 3.3: Percentage of male *E. ambiguella* responding to the main pheromone compound Z9-12:Ac (20μg) presented in different ways from a rubber septum (Fig. 3.1). When the rubber septum was presented in a glass tube closed with gauze, significantly fewer males flew towards the pheromone source.

Influence of host plant volatiles on the attractiveness of the ternary pheromone blend

The rubber septa containing the ternary pheromone blend (Z9-12:Ac, 12:Ac and 18:Ac at 100:100:200μg) alone or in mixtures with either 10μg (+)-terpinen-4-ol or 10μg methyl salicylate were enclosed in the glass container with an external air supply to measure responses of male *E. ambiguella* in the wind

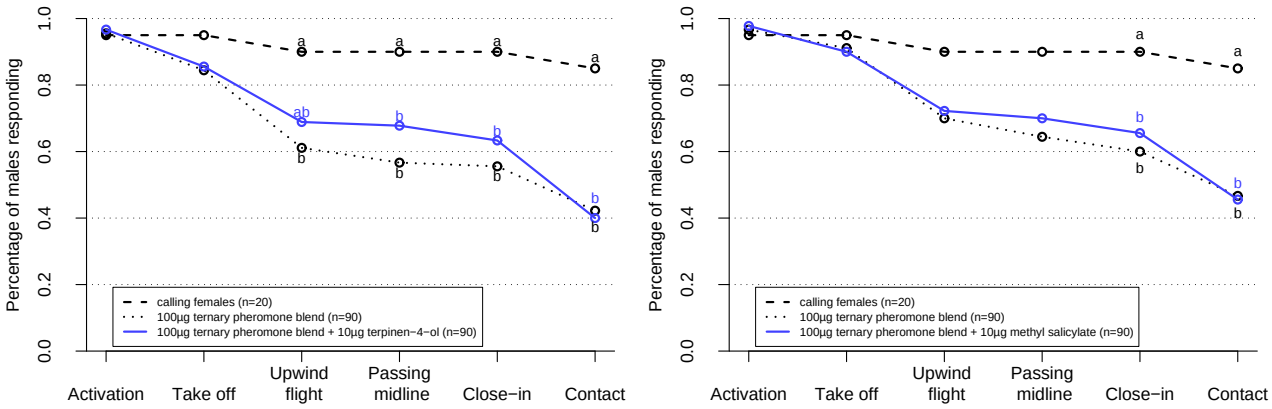
TABLE 3.2: Percentage of male *E. ambiguella* responding to the main pheromone compound Z9-12:Ac released from a rubber septum presented in three different ways for testing (see Fig. 3.1). Different letters assigned within a behavioural element indicate statistically significant differences (GLM(logit) $p<0.05$).

Presentation method	**Activation**	**Upwind flight**	**Close-in on source**	**Source contact**
from platform (n=43)	0.86a	0.58a	0.40a	0.40a
from glass tube (n=126)	0.75b	0.27b	0.11b	0.09b
from glass container (n=44)	0.93a	0.66a	0.55a	0.48a

tunnel. The number of upwind flights and contacts with the source was not significantly increased by the addition of these two plant volatiles compared to the ternary pheromone blend alone. The tendancy, however, was a higher attractiveness to treatments containing the plant compounds as more males closed in on the stimulus source (Fig. 3.4). However, the number of males failing to contact the source, even though they flew close to the source, increased in the presence of the plant compounds. In contrast, almost all males contacted the container-held females once they flew upwind.

A possible reason why it was difficult to detect a significant effect on male behaviour of the plant compounds on the ternary blend was that the ternary blend was used at its optimal dose, so the level of attractiveness was already high. A suboptimal pheromone doses or Z9-12:Ac would have been better to detect quantitative differences in male grape berry moth flight behaviour to its pheromone with and without host plant volatiles added.

Influence of host plant volatiles on the attractiveness of Z9-12:Ac All of four plant volatiles ((+)-terpinen-4-ol, (Z)-3-hexen-1-ol, 1-octen-3-ol and methyl salicylate) presented in a mixture with Z9-12:Ac had an influence on the flight behaviour of male *E. ambiguella*. Significantly more male moths contacted the rubber septum on the platform at the upwind end of the wind tunnel containing 2μg (+)-terpinen-4-ol in addition to the optimal dose of 20μg Z9-12:Ac (71%, compared to 40% with Z9-12:Ac alone; $p<0.05$; Fig. 3.5a). The addition of 20μg (+)-terpinen-4-ol to 20μg Z9-12:Ac also increased the percentage of males contacting the source on the platform but this difference was not significant. Similarly, significantly more males touched the glass tube containing the rubber septum with 20μg Z9-12:Ac plus 2μg (Z)-3-hexen-1-ol than Z9-12:Ac alone (20% compared to 9%, $p<0.05$; Fig. 3.5b). However, females still engaged more males to fly upwind and contact the tube containing them (47%) than Z9-12:Ac alone or the mixture of Z9-12:Ac plus (Z)-3-hexen-1-ol (Fig. 3.5b). Another effect was obtained when 2μg 1-octen-3-ol or 2μg methyl salicylate were added to 20μg Z9-12:Ac. In the presence of 1-octen-3-ol significantly fewer males flew upwind compared to Z9-12:Ac alone presented



a b

FIGURE 3.4: Percentage of male *E. ambiguella* responding to 2-3 calling females, to the ternary pheromone blend alone and to mixtures of the ternary blend with either (+)-terpinen-4-ol (10μg; a) or methyl salicylate (10μg; b) presented in a glass container (see Fig. 3.1c). The ternary pheromone blend consisted of 100μg Z9-12:Ac, 100μg 12:Ac and 200μg 18:Ac. Slight but not significantly more males closed in on the source in the presence of the two plant compounds but fewer or equal numbers of males contacted the stimulus source. Different letters assigned within a behavioural element indicate statistically significant differences (GLM(logit) $p<0.05$).

in the glass tube (32% compared to 58%, $p<0.05$; Fig. 3.5c). The presence of methyl salicylate at either 2μg or 0.2μg slightly increased the percentage of males flying upwind compared to Z9-12:Ac alone (Fig. 3.5d). But at 2μg methyl salicylate only few males touched the source even though they flew close to it. At the lower dose of methyl salicylate, 0.2μg, this effect disappeared, i.e. more males contacted the source than at the higher methyl salicylate dose.

These results provides the first evidence that plant volatiles have an effect on pheromone perception in *E. ambiguella* and that plant volatiles influence behavioural responses of male *E. ambiguella* to the main pheromone compound Z9-12:Ac in a variety of presentation methods. Further, the results indicate that an appropriate dose of the plant volatiles is crucial. Methyl salicylate, for example, significantly inhibited the males in contacting the pheromone source at 2μg but not at 0.2μg, and in the case of terpinen-4-ol, the lower dose of 2μg also tended to be more effective than the higher dose of 20μg in attracting male *E. ambiguella*.

Rubber septa are widely used in field traps to release sex pheromones for monitoring the occurrence of moth pests in viticulture and agriculture. In most cases the sex pheromone is a mixture of several compounds. The ratio of these compounds is critical for optimised attraction of each moth species. The release rates of such aliphatic compounds from rubber septa have been well studied (McDonough, 1978; McDonough et al., 1989, see Appendix C for a summary). In addition, rubber is very practical

and easy to use in wind tunnel experiments. Most plant volatiles, however, are much more volatile than moth pheromone products and may interact in a different way with the rubber than the pheromone products. By measuring the release rates of the plant compounds from rubber septa M. von Arx (personal communication) showed that the ratio between these volatiles and the pheromone changes over time, with the plant compounds leaving the polymer first. We therefore installed another odour delivery device which permits precise control of the quantity of pheromone and plant volatiles released into the wind tunnel per unit time. This piezo nebulizer is described in the next section of this chapter.

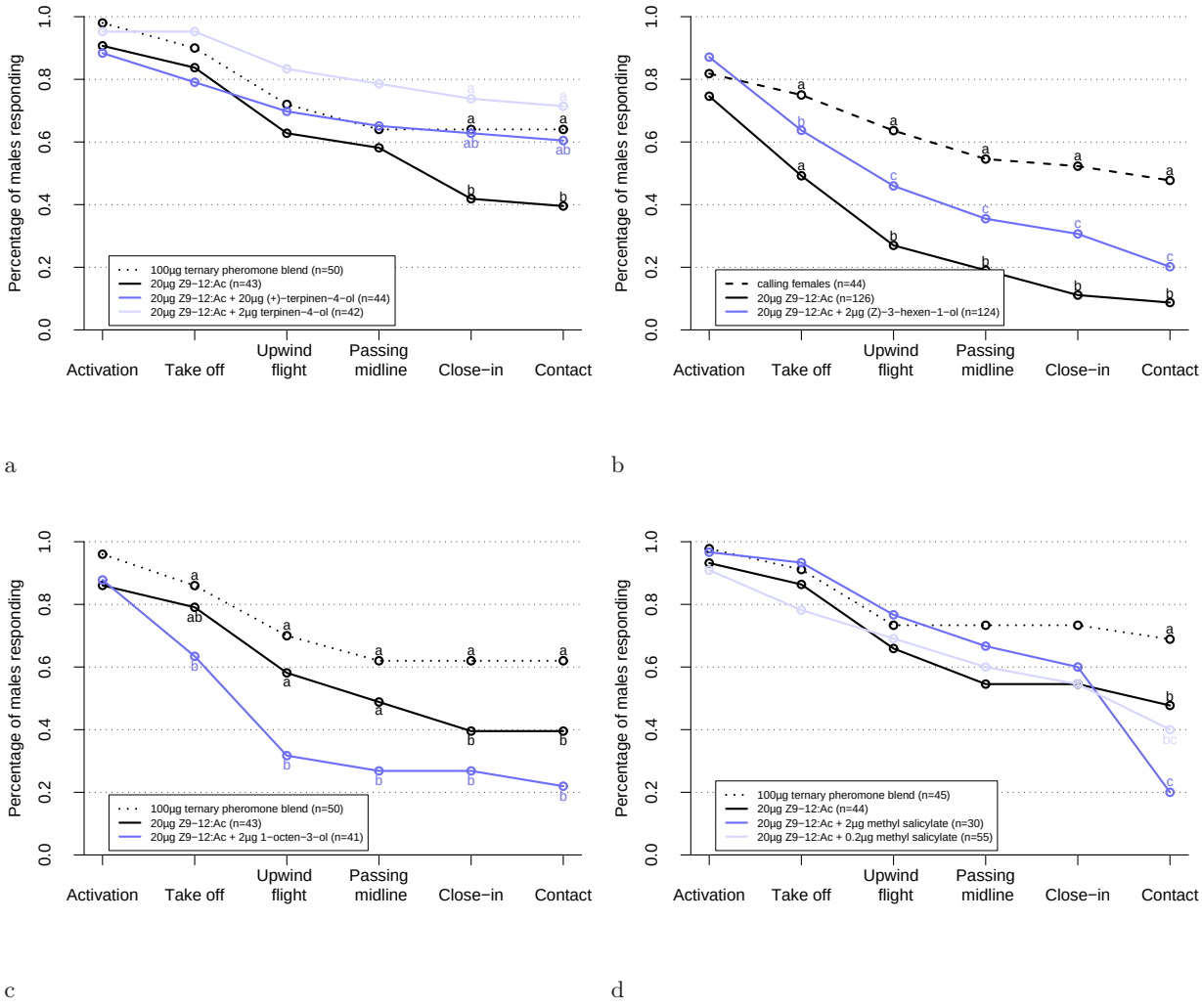


FIGURE 3.5: Percentage of male *E. ambiguella* responding to Z9-12:Ac (20μg) alone and to mixtures of Z9-12:Ac with 2 or 20 μg (+)-terpinen-4-ol (a), 2μg (Z)-3-hexen-1-ol (b), 2μg 1-octen-3-ol (c) and 0.2 or 2μg methyl salicylate (d). The presentation methods used were from the platform for a and b, from the glass tube c and from the glass container d (see Fig. 3.1 for explanation). Different letters assigned within a behavioural element indicate statistically significant differences (GLM(logit) $p<0.05$).

3.3 The piezo nebulizer: a system to release volatile compounds at known amounts

3.3.1 Material and Methods

Insects Insects were reared in a climate chamber with a 16:8h L/D cycle at 65% RH and 25°C during the photophase and 85% RH, 18°C during the scotophase. The larval stages were reared on a semiartifical medium (Rauscher et al., 1984, Appendix A). Pupae were sexed and put on a mesh over a dish filled with water for emergence. Males emerged into cages (BugDorm, 30*30*30cm, MegaView Science Education Services Co., Taiwan) each day. The cages were sprayed once a day with water and enclosed in plastic bags. A sugar solution (10% sucrose) in glass tubes closed with cotton wool wicks was offered to the males by placing two of them on the top of the cage.

General settings of the wind tunnel The same wind tunnel settings were used as described in section 3.2.1. A piezo sprayer was installed according to El-Sayed et al. (1999). Briefly, a glass capillary (5-6cm long, o.d. 1mm, i.d. 0.46mm) with a long drawn-out tip (2-3cm) with an opening of 10-50μm was connected with a PTFE microtube (1.5 m long, 1.02 mm o.d., 0.56 mm i.d., Hamilton, Milian SA, Switzerland) to a syringe containing an alcohol solution of known amounts of the test products. By means of a syringe pump (CMA 400, Microdialysis, Sweden) the solution with solutes was pumped at a defined release rate. A piezo disc (Philips PXE5 25/2.0, Megatron AG, Switzerland) was connected by a clip (a modified piano chord) to the capillary. A frequency generator (FG-5000A Wavetex, Germany) was used at an amplitude of 40V and a frequency of about 90kHz to cause the capillary tip to oscillate. In this manner the solution was released as an aerosol into the wind tunnel air stream. The capillary tip was protected from the approaching moths by a small metal mesh that was cleaned after exposure to each treatment.

Ethanol (pro Analysi, Merck, Germany) was used as solvent for the pheromone as it has been shown by El-Sayed et al. (1999) to have no influence on the behaviour of *E. ambiguella* males. To compare the reaction of males to an aerosol of ethanol with that of water the glass release tubes containing males were put on a stand in the aerosol at a distance of 5cm from the capillary tip and the walking direction of males was recorded for activated individuals. No differences in the time to activation nor in the walking direction between water and ethanol were observed: in both cases males walked downwind. Three release rates of Z9-12:Ac (50pg/min, 500pg/min and 5ng/min) and seven different release rates of the ternary pheromone blend (5pg/min, 10pg/min, 50pg/min, 100pg/min, 1ng/min, 10ng/min and

100ng/min of Z9-12:Ac) at a ratio of 1:1:2 of Z9-12:Ac, 12:Ac and 18:Ac were tested. Release rates of the ternary pheromone blend refer to Z9-12:Ac content hereafter.

3D tracking of moth flights tracks In order to visualize the flight of male *E. ambiguella* in the wind tunnel to different pheromone sources released by the piezo nebulizer we developed a 3D tracking system. Images obtained by two high speed cameras (Basler eXcite exA640-120m, Altrona Vision, Switzerland; f=6mm objectives (C-Mount Lens SV-M0614, Japan), CL-Electronics, Switzerland) are subtracted to determine the current position of a flying insect. An algorithm is applied to the synchronized images of the two cameras to determine the coordinates in 3D at a given time (Halcon, MVTec Software GmbH, Germany). A software (Crow, University of Neuchâtel) permits adjusting for parameters such as the initial calibration, luminosity and insect size.

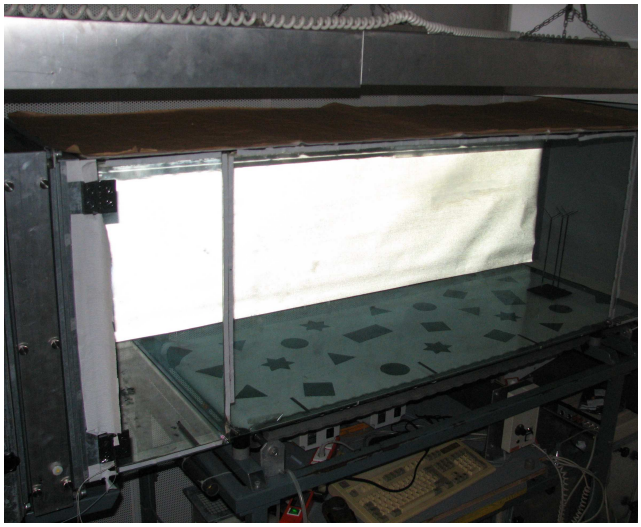
The two cameras were mounted on a stand in distance of 60cm to each other to one side of the wind tunnel (Fig. 3.6a). As the light settings of the wind tunnel were too low for the cameras to detect the flying moth we attached two lamps equipped with conventional bulbs (25W, but reduced using a transformer to ≈11W) at the same angles beside the cameras. These lamps shone on a retroreflective paper (Scotchlite 680-CR, 3M, Switzerland) mounted on the back wall of the wind tunnel. Due to the reflected light the contrast of the flying insect in front of the back wall was thus enhanced for the cameras and permitted to record the flight coordinates without altering the light settings for the moths by too much (Fig. 3.6b). With the recorded coordinates the flight of male moths was reconstructed using the `R` package `rgl` (Version 2.4.1). [1]

3.3.2 Results and Discussion

Flight responses to different quantities of Z9-12:Ac released as an aerosol Male *E. ambiguella* responded in a dose-dependant manner to different quantities of the main pheromone compound Z9-12:Ac released as an aerosol (Tab. 3.3). Most males flew upwind to the highest release rate tested (5ng/min) but many failed to contact the source. Thus, this dose is too high. At a dose of 50pg/min significantly fewer males flew upwind compared to 500pg/min and 5ng/min, but most of these males succeeded in contacting the source which was not the case for the higher Z9-12:Ac doses. Therefore, the optimal dose in our wind tunnel is probably between 50pg/min and 5ng/min. This fits well with the results obtained using rubber septa releasing Z9-12:Ac (Arn et al., 1986, Section 3.2.2)

[1] The intention to develop a 3D tracking system was to analyse the flight of the male moth qualitatively. Flight parameters such as velocity, acceleration, straightness, curvature, torsion and angular velocity can be calculated and compared between treatments. Unfortunately, the development finished only recently so I could not use it to full advantage.

a



b

FIGURE 3.6: a. Configuration of the 3D video tracking system. The small inserted image is a view of the two cameras plus lamps from the front. The cameras (right) were mounted on a stand and recorded the insect flight in the wind tunnel (left) slightly above on one side. A lamp beside each camera shone on an retroreflective paper mounted on the back wall of the wind tunnel (b). The light was reflected towards the cameras and illuminated the wind tunnel for the cameras only.

where the optimal dose of Z9-12:Ac on the rubber septa of 20μg corresponds to a release rate of about 200pg/min (Appendix C). Note, however, the small sample size for these tests.

TABLE 3.3: Percentage of male *E. ambiguella* responding to the main pheromone compound Z9-12:Ac released at different quantities from a piezo nebulizer. Different letters assigned within a behavioural element indicate statistically significant differences (GLM(logit) $p<0.05$).

Release rate	**Activation**	**Upwind flight**	**Close-in on source**	**Source contact**
50pg/min (n=20)	0.60a	0.25a	0.20a	0.20a
500pg/min (n=35)	0.97b	0.77b	0.66b	0.49a
5ng/min (n=20)	1.00b	0.90b	0.70b	0.50a

Flight responses to different quantities of the ternary pheromone blend released as an aerosol Release rates of the ternary blend of the pheromone were tested at 100ng/min, 10ng/min, 1ng/min, 100pg/min 50pg/min, 10pg/min and 5pg/min of Z9-12:Ac keeping the ratio of the ternary blend at 1:1:2 of Z9-12:Ac, 12:Ac and 18:Ac. This corresponds to the released ratio of the components of this attractive pheromone mixture when applied at a ratio of 1:1:2 on rubber septa (Heath et al., 1986). The pheromone release rate of the ternary pheromone blend significantly influenced the flight behaviour of male *E. ambiguella* (Fig. 3.7). Two phenomena were observed: for the behavioural elements activation and upwind flight the percentage of males responding increased with dose and reached a plateau at 50pg/min pheromone for activation (over 90%) and at 100pg/min for upwind flight (between 70 and 80%, Fig. 3.7). For the behavioural elements close-in and source contact (i.e. touching the metal grid protecting the glass capillary), however, the dose-response curve had an inverted U-shape. Whereas with 10ng/min pheromone 82% of the males flew close to the source, only 34% did so at 100ng/min. Many males stopped upwind flight before arriving in the vicinity of the source and showed in-flight arrestment behaviour, indicating an overdose of the pheromone. Flight tracks of a male *E. ambiguella* flying to optimal and overdosed pheromone levels were recorded by a 3D camera system to visualize this phenomenon (Fig. 3.8).

The ternary pheromone blend has a wide range of doses for optimal attraction (Fig. 3.7), in contrast to what was recorded for the main pheromone compound Z9-12:Ac alone (Fig. 3.3). This phenomenon was also found by Arn et al. (1986) and explains well why this ternary pheromone blend is such a useful tool for monitoring and use in mating disruption against the grape berry moth.

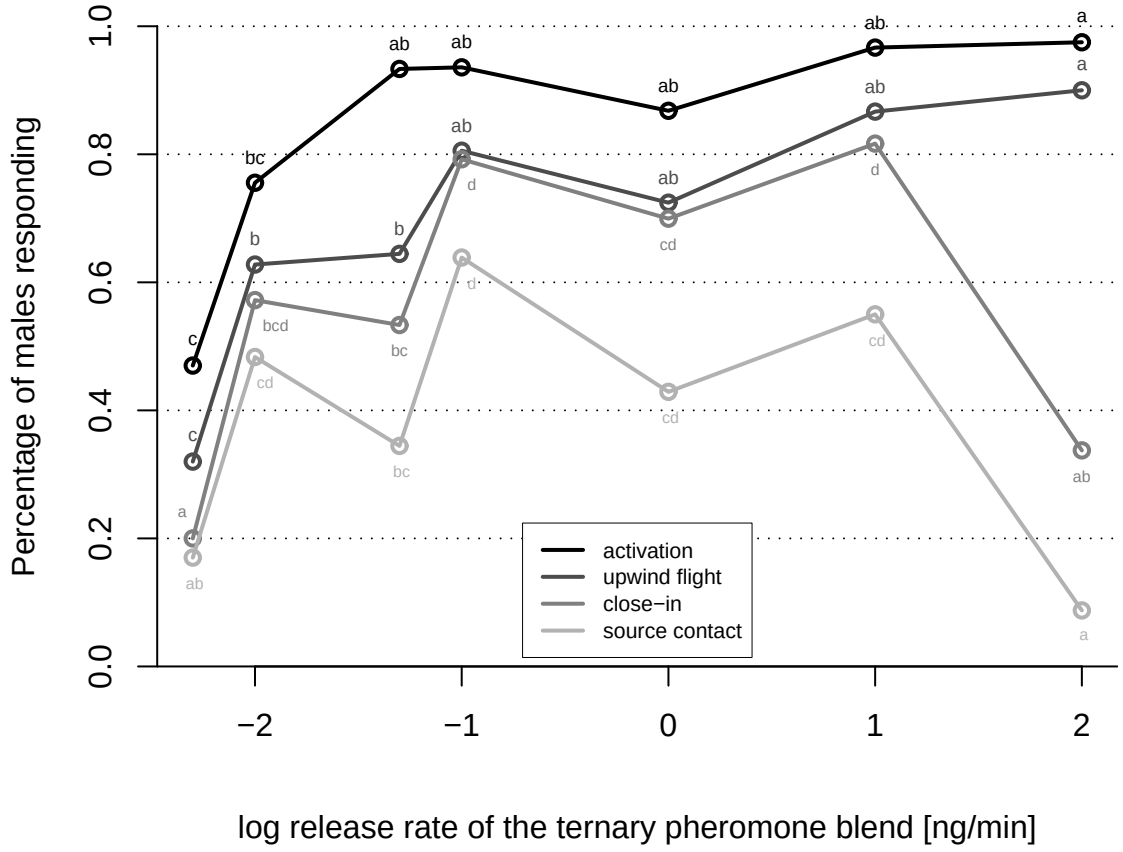


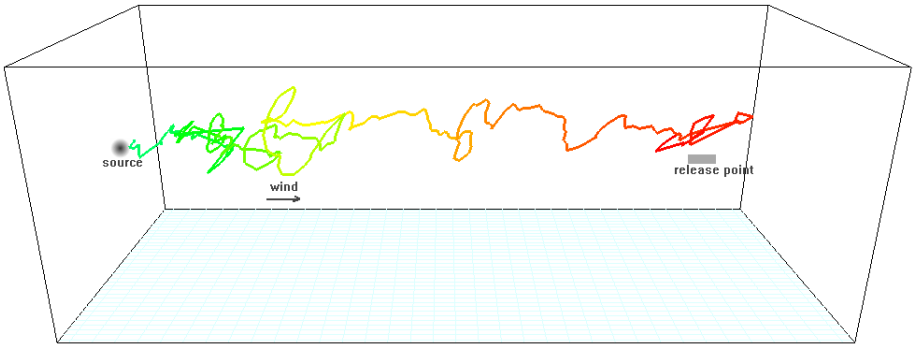
FIGURE 3.7: Percentage of male *E. ambiguella* responding to different release rates of the ternary pheromone blend (Z9-12:Ac, 12:Ac and 18:Ac, 1:1:2) released from a piezo nebulizer. Most males flew to the ternary pheromone blend when released at between 100pg/min and 10ng/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

3.4 Conclusion

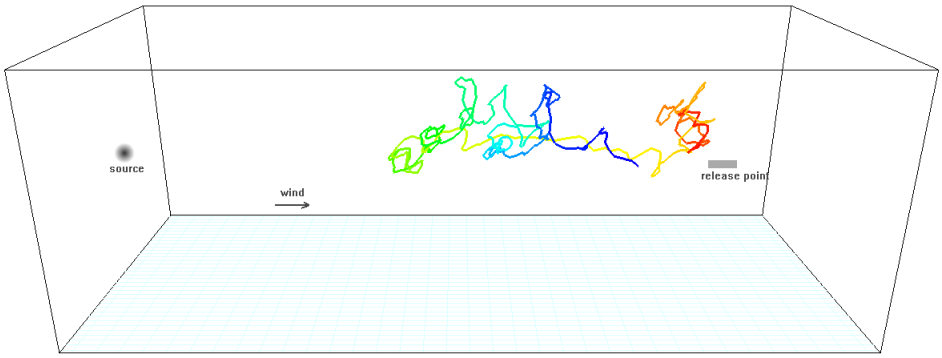
In summary, the results obtained with rubber septa and the piezo nebulizer to test the responses of male *E. ambiguella* to its sex pheromone are comparable. However, a disadvantage of the piezo nebulizer is that it is very susceptible to minor changes in configuration due to the small size of the capillary tip. The holder to clamp the glass capillary was modified several times in order to optimize the installation and these holders differed in size and shape. This created different types of turbulence around the glass capillary tip and so altered the pheromone plume structure downwind which had a major influence on the flight behaviour of males to an overdosed or an underdosed pheromone level. When the plume structure was too narrow male *E. ambiguella* did either not take off or flew only a few centimeters upwind towards an overdosed pheromone source. In addition, the point in the wind tunnel where males showed in-flight arrestment behaviour could be modulated by changing the plume size. This observation helps to explain how the confusion technique may function: namely that males

are occupied with the pheromone source without making contact. In addition, contact time with the source (i.e. the metal protection grid over the capillary tip) is often very short and only a small fraction of males land on it maybe due to presence of the aerosol.

Nevertheless, once appropriately installed the piezo sprayer is a useful tool to release pheromone plus plant volatiles at precisely adjusted levels and ratios, a clear advantage over the rubber septa.



a



b

FIGURE 3.8: 3D reconstructions of male *E. ambiguella* flights to an optimal pheromone dose of Z9-12:Ac, 12:Ac and 18:Ac at 1:1:2 (10ng/min; a) and an overdosed pheromone dose of the same ternary blend (100ng/min; b). The box represents the outline of the wind tunnel. In presence of a high pheromone dose (b) the moth shows in-flight arrestment in the middle of the wind tunnel and is inhibited from reaching the source.

Chapter 4

The influence of host plant volatiles on the flight behaviour of male *E. ambiguella* to a ternary pheromone blend

4.1 Introduction

Comparison of the headspace volatiles of different *E. ambiguella* host plant by GC-EAD identified 16 key chemostimuli for males (see Chapter 2). Additionally, males show particularly low detection thresholds for (Z)-3-hexen-1-ol, (+)-terpinen-4-ol and methyl salicylate. However, we do not know whether these stimuli play a role in pheromone perception by male grape berry moths. We set out to investigate whether any of these volatiles added individually to the ternary pheromone blend (Arn et al., 1986) affected male responses in the wind tunnel. At an optimal level of the pheromone male behavioural response reaches over 70% (see Section 3.3.1). At such a high level of response an effect of plant volatiles would be difficult to determine statistically. Consequently, plant volatiles were released at suboptimal levels of the pheromone. At an underdosed level all behavioural elements occur at low probabilities (see Section 3.3.1) and the question is whether plant volatiles can increase these probabilities. Above the optimal level, males fly upwind but show in-flight arrestment after a distance upwind. Here, the questions is if and how plant volatiles could modify this behaviour.

The volatile chemostimuli we identified from different host plants of *E. ambiguella* in Chapter 2 are commonly occurring products released in large quantities upon damage, so individually they cannot account for the detection of host plants. Instead, insects deal with complex mixtures of host plant

volatiles in nature. I therefore investigated the effect of mixtures of plant volatiles on the behavioural response of male *E. ambiguella* to an underdosed pheromone level, expecting that the mixture of plant volatiles is more effective than either plant volatile presented singly with the pheromone.

4.2 Material and Methods

Insects Insects were reared as described in section 3.3.1.

Compounds tested The same pheromone compounds were used as described in section 3.2.1. The synthetic plant volatiles hexanol (99%), (E)-β-caryophyllene (99%), methyl salicylate (>99%), R(+)-limonene (>98%), linalool (>97%), (Z)-3-hexen-1-ol (>98%) and (+)-terpinen-4-ol (99%) were purchased from Fluka (Switzerland), 1-octen-3-ol (>97%) was from Merck (Germany), benzaldheyde (99%) and (E)-2-hexenal (>95%) from Sigma-Aldrich (Germany), and 4,8-dimethyl-1(E),3,7-nonatriene (DMNT) was supplied from Givaudan (Switzerland).

Behavioural experiments Behavioural experiments were conducted in a wind tunnel (see section 3.2.1). Different odour sources were delivered to male *E. ambiguella* using a piezo nebulizer (see section 3.3.1). Plant volatiles were added either to an underdosed (5 or 50pg/min) or to an overdosed (500ng/min) level of the pheromone consisting of Z9-12:Ac, 12:Ac and 18:Ac at a ratio of 1:1:2. Release rates of the pheromone blend refer hereafter to the main pheromone component Z9-12:Ac. Flight parameters were recorded as described in section 3.2.1.

Statistical analysis The responses of male *E. ambiguella* to different treatments were compared by fitting a generalised linear model (GLM) with a logit link function (logistic regression) to the responses, assumed to be binomially distributed, using the statistical package `R` (Version 2.4.1). Analysis of deviance based on the asymptotic χ^2 distribution was used to test whether the flight responses are significantly dependent on the odour sources ($p<0.05$). When the GLM was significant ($p<0.05$) multiple comparisons (`R`-package: `Multcomp`) were made using Tukey-contrasts. Time-event analysis were performed using survival statistics, including proportional hazards (Cox) regression, using the `R`-package `survival`.

To compare the effect of plant volatiles on the attractiveness of different underdosed pheromone release rates, odds ratios were calculated. The odds are the ratio of the probability that an event of interest (e.g. upwind flight or source contact) occurs to the probability that the event does not occur

(Cox and Snell, 1989; Bland and Altman, 2000). The odds ratio compares the odds of two groups, e.g. the ratio between the odds of upwind flying males in presence of pheromone and a plant compound and the odds of upwind flying males to the pheromone alone. Its value can be interpreted as how likely it is that a male that is exposed to pheromone and plant volatiles flies upwind compared to males that are exposed to the pheromone alone. Odds ratios can have values between zero and infinity, where an odds ratio of one indicates no difference between two treatments, values lower than unity a negative and values higher than unity a positive effect of the plant volatile compound.

4.3 Results

4.3.1 Effects of plant volatiles added to the underdosed pheromone

Effects of individual plant compounds To gain an initial indication as to at which levels plant volatiles may be effective when combined with the ternary pheromone blend, (+)-terpinen-4-ol (already known to enhance the number of upwind flights towards Z9-12:Ac released from a rubber septum; Section 3.5) was released at four levels ranging from 0.5pg/min to 500ng/min with the underdosed pheromone at 50pg/min. In response to increasing doses of this terpenoid a tendency to higher numbers of upwind flights towards the pheromone source was observed (Fig. 4.1). At a pheromone to (+)-terpinen-4-ol ratio of 1:10 000, significantly more males flew half the length of the wind tunnel to the source, closed in on and contacted the source compared to the pheromone alone (GLM, $p<0.05$). Moreover, the time-event analysis showed that males were activated significantly faster in the presence of the highest (+)-terpinen-4-ol release rate of 500ng/min compared to the pheromone alone (Cox porportional hazards model, $p<0.05$, Fig. 4.2).

Other plant volatiles were subsequently tested at a pheromone:plant volatile ratio of 1:10 000. For this, (Z)-3-hexen-1-ol, (E)-β-caryophyllene, methyl salicylate and benzaldehyde were each released at 500ng/min with the pheromone at 50pg/min. In the presence of (E)-β-caryophyllene at 500ng/min the response level of the whole behavioural sequence from activation to source contact was significantly increased compared to the pheromone alone (Fig. 4.3; GLM(logit), $p<0.05$). Source contacts by males rose from 13% to 41% and similar to (+)-terpinen-4-ol, males were activated significantly faster in the presence of (E)-β-caryophyllene (Fig. 4.4). Both (Z)-3-hexen-1-ol and methyl salicylate released at 500ng/min significantly increased the number of male *E. ambiguella* closing in on and contacting the pheromone source (Fig. 4.3) whereas the effect of benzaldehyde was intermediate. With the pheromone:benzaldehyde mixture slightly fewer males were activated and made upwind flights com-

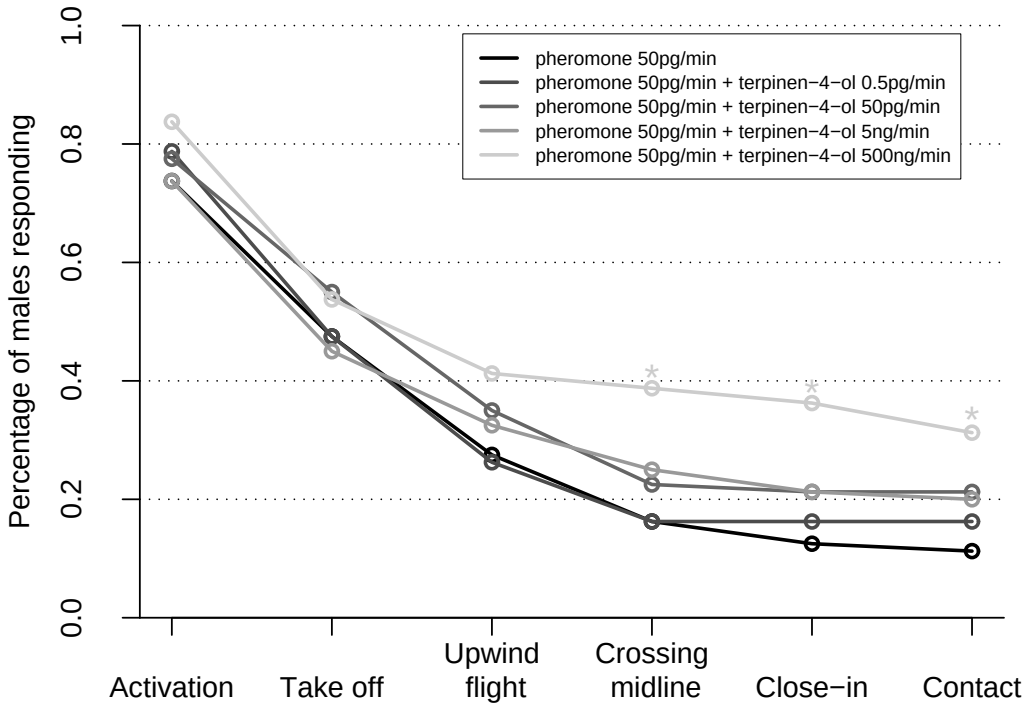


FIGURE 4.1: Dose-response curve of male *E. ambiguella* responding to an underdosed level of pheromone (50pg/min) with different doses of the host plant volatile (+)-terpinen-4-ol added. At a pheromone-plant volatile ratio of 1:10 000 significantly more males flew upwind and contacted the source (GLM(logit), $p<0.05$); n=80 for each treatment.

pared to the pheromone alone, but more of these males arrived at the source (Fig. 4.3). However, these differences were not significant.

The attractiveness of the pheromone alone was regularly controlled for these experiments. In later experiments, however, male responses to the pheromone released at 50pg/min increased significantly for an unknown reason to a level of almost 60% of upwind flights compared to 28% obtained in the earlier experiments (see Fig. D.1 in Appendix D). Once this was recorded the release rate of the pheromone was decreased to 5pg/min for further tests with the plant volatiles (E)-2-hexen-1-al, 1-octen-3-ol, linalool and limonene each released at 50ng/min to keep the pheromone:plant volatile ratio at 1:10 000 as for the experiments with the other plant volatiles (above). With the exception of 1-octen-3-ol where all behavioural steps dropped, all the other plant compounds enhanced the percentage of males contacting the source but not significantly (see Fig. D.2 in Appendix D).

To compare the different plant volatile treatments based on a pheromone release rate at either 5pg/min or 50pg/min, odds ratios for the pheromone-plant compound mixture and the pheromone blend alone were calculated to give an index of effects (Table 4.1). From this a general pattern

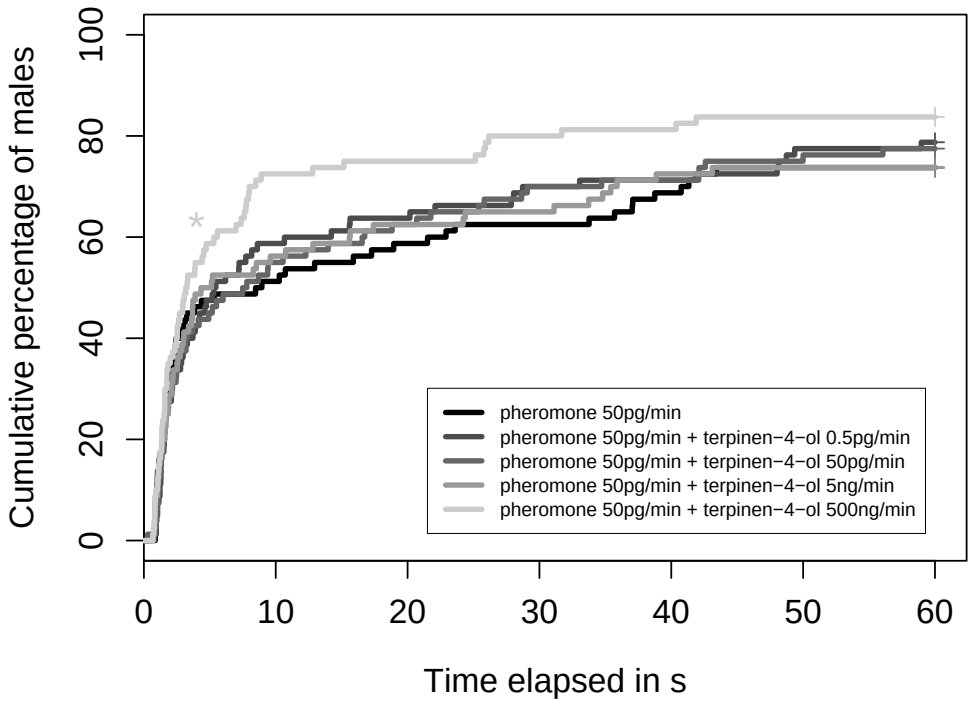


FIGURE 4.2: Cumulative percentage of male *E. ambiguella* activated over a time period of 60s to a mixture of underdosed pheromone plus (+)-terpinen-4-ol at different release rates. In the presence of (+)-terpinen-4-ol at 500ng/min the males were activated significantly faster and in higher numbers compared to the pheromone alone (Cox proportional hazards model, $p<0.05$); n=80 for each treatment.

emerges, namely, that all plant volatiles tested in combination with the pheromone, with the exception of 1-octen-3-ol, had a positive effect on the percentage of males flying up the wind tunnel, closing in on and contacting the source, with (E)-β-caryophyllene and (+)-terpinen-4-ol showing the strongest effects.

Effects of mixtures of plant compounds To investigate possible interactions between mixtures of plant volatiles on pheromone perception a mixture of (Z)-3-hexen-1-ol, (E)-β-caryophyllene and methyl salicylate each released at 50ng/min and the pheromone at 5pg/min was offered to male *E. ambiguella*. No significant difference in the response to the pheromone alone and the pheromone mixed with these three plant volatiles was recorded (Fig. 4.5, Table 4.2). However, the number of males taking off was slightly reduced whereas the number of males flying over half the length of the wind tunnel, closing in on and contacting the source was slightly higher.

As this plant volatile mixture did not significantly improve the attractiveness of the pheromone a different approach to test plant volatile mixtures was chosen. Firstly, the response to (E)-β-caryophyllene

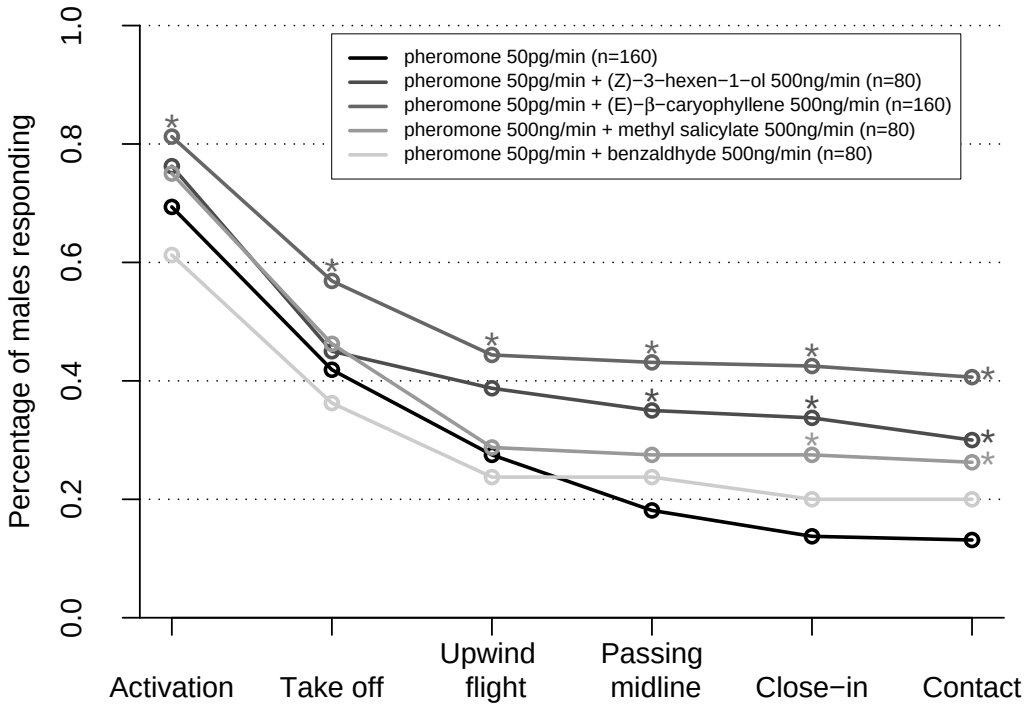


FIGURE 4.3: Percentage of male *E. ambiguella* responding to an underdosed pheromone level (50pg/min) presented alone and with either (Z)-3-hexen-1-ol, (E)-β-caryophyllene, methyl salicylate or benzaldheyde at a pheromone-plant volatile ratio of 1:10 000. Asterisks indicate a significant difference from the pheromone alone (GLM(logit), $p<0.05$)

released at different levels with the pheromone was recorded in order to establish if a particular ratio was critical (this compound performed best in combination with the pheromone in the previous experiments, see Table 4.1). Pheromone:(E)-β-caryophyllene ratios of 1:1, 1:1 000 and 1:100 000 were tested with a pheromone level at 5pg/min whereas a ratio of 1:10 000 was tested with 50pg/min pheromone (see above). Subsequently, odds ratios of the response to these four pheromone:(E)-β-caryophyllene ratios compared to the pheromone alone were calculated (Table 4.2). When (E)-β-caryophyllene was released at the same rate as the pheromone no significant difference in the response of male *E. ambiguella* was observed. On increasing the release rate of this plant volatile 1 000 times, significantly more males flew upwind to to the source (35%) compared to the pheromone alone (20%; $p<0.05$). The numbers of males contacting the source also increased but not significantly (19% compared to 9%). At the highest release rate tested of 500ng/min (E)-β-caryophyllene, more males flew upwind and contacted the source compared to the pheromone alone. However, only the number of males flying over half the length of the wind tunnel differed significantly between the two treatments. Interestingly, males were activated earlier with increasing doses of (E)-β-caryophyllene compared to the pheromone

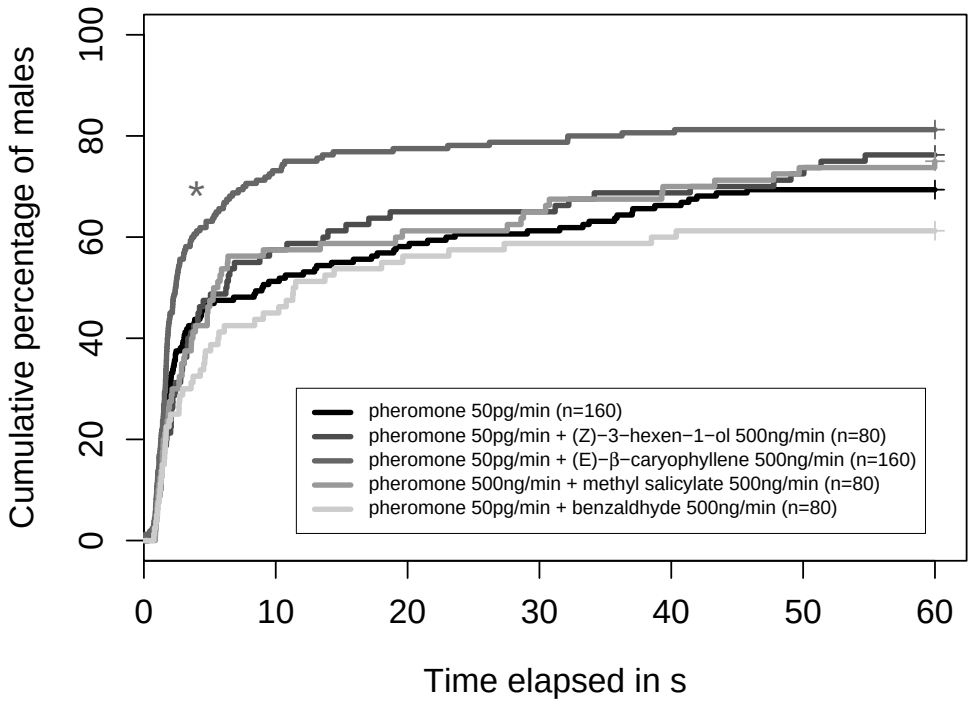


FIGURE 4.4: Cumulative percentage of male *E. ambiguella* activated over 60s to a mixture of underdosed pheromone plus the plant volatiles (Z)-3-hexen-1-ol, (E)-β-caryophyllene, methyl salicylate and benzaldheyde. In the presence of (E)-β-caryophyllene released at 500ng/min the males were activated significantly faster and in higher numbers compared to the pheromone alone (Cox proportional hazards model, $p<0.05$).

alone (Fig. 4.7). Overall, the pheromone:(E)-β-caryophyllene ratio of 1:10 000 had the strongest effect in attracting males to the source (Table 4.2). So as many males flew upwind towards the 1:1 000 ratio as to the 1:10 000 ratio, the latter proving most effective in inducing source contacts.

A second plant product, (Z)-3-hexen-1-ol, was then released with the 1:1 000 pheromone:(E)-β-caryophyllene mixture at four different levels (50pg/min, 500pg/min, 5ng/min and 50ng/min). Best attraction of *E. ambiguella* males was achieved with a pheromone:(E)-β-caryophyllene:(Z)-3-hexen-1-ol ratio of 1:1 000:100 (Fig. 4.8, Table 4.2). 48% of the males flew upwind to this mixture, more than to the pheromone alone (20%, $p<0.05$) or to the 1:1 000 pheromone:(E)-β-caryphyllene mixture (35%). When (Z)-3-hexen-1-ol was released at 5ng/min in the mixture the response was on the same level as the response to the pheromone:(E)-β-caryophyllene mixture at 1:1 000. The lowest and highest (Z)-3-hexen-1-ol release rates tested in these mixtures (50pg/min and 50ng/min, respectively) did not cause a significant differences in male responses compared to the pheromone alone.

In summary, comparing the odds ratios of all plant volatile - pheromone combinations tested (Tables 4.1 and 4.2) the 1 000:100 mixture of (E)-β-caryophyllene and (Z)-3-hexen-1-ol had the strongest effect

TABLE 4.1: Odds ratios for behavioural responses to pheromone-plant compound mixtures at a 1:10 000 ratio and to the pheromone (5pg/min or 50pg/min) alone as an index of effect. An odds ratio greater than 1 indicates that the behavioural element occurs more likely in the presence of the pheromone-plant compound mixture than in the presence of the pheromone alone. Values less than 1 indicate the opposite. Values in bold indicate significant differences between the pheromone alone and plant volatile-pheromone mixtures (logistic regression, $p<0.05$).

Plant compound	Activation	Take off	Upwind flight	Passing midline	Close-in on source	Source contact
Aliphatic compounds:						
1-octen-3-ol[a] (n=73)	0.313	0.874	0.624	0.748	0.652	0.559
(Z)-3-hexen-1-ol[b] (n=80)	1.417	1.137	1.668	**2.432**	**3.196**	**2.838**
(E)-2-hexenal[a] (n=78)	0.624	0.958	1.492	1.860	1.563	1.943
Terpenes:						
(+)-terpinen-4-ol (n=80)	1.834	1.284	1.851	**3.261**	**3.980**	**3.586**
linalool[a] (n=71)	0.765	0.967	0.735	1.123	1.265	1.438
limonene[a] (n=81)	0.313	1.104	0.971	1.361	1.442	2.024
(E)-β-caryophyllene[b] (n=160)	**1.913**	**1.830**	**2.103**	**3.425**	**4.636**	**4.529**
Aromatic compounds:						
benzaldehyde[b] (n=80)	0.698	0.789	0.821	1.407	1.568	1.655
methyl salicylate[b] (n=80)	1.324	1.194	1.064	1.713	**2.379**	**2.356**

[a] added 5pg/min pheromone
[b] added 50pg/min pheromone

on pheromone perception by male grape berry moths in the wind tunnel.

4.3.2 Plant volatiles added to an overdosed pheromone release level

In the first experiments with different pheromone levels (Section 3.3.1) it was shown that in the presence of high pheromone levels most male *E. ambiguella* flew upwind to within a certain distance of the source, but then showed in-flight arrestment or flew out of the odour plume (Fig. 3.8b). Only a few males flew to within 10cm of the source and made contact (Fig. 3.7). To investigate a possible influence of host volatiles on this in-flight arrestment behaviour the following plant products were added to the overdosed pheromone release level of 100ng/min: (+)-terpinen-4-ol at 100pg/min, 1ng/min, 10ng/min and 1μg/min, (E)-β-caryophyllene at 100pg/min, methyl salicylate, 1-hexanol, DMNT, (Z)-3-hexen-1-ol, 1-octen-3-ol each at 100pg/min, 1ng/min and 1μg/min, and a headspace extract of the rearing medium at an unspecific release rate (see Section 2.2 and Fig. B.3 in Appendix B). In general, slightly fewer males flew upwind and fewer still or in similar numbers as to the overdosed pheromone alone flew to within 10cm of the source in the presence of plant volatiles at the highest release rate tested, i. e. 1μg/min (Fig. 4.9). At all release rates tested, methyl salicylate, 1-hexanol, DMNT, 1-octen-3-ol and the headspace extract of the rearing medium had no significant effect on the

TABLE 4.2: Odds ratios for behavioural responses of male *E. ambiguella* to pheromone-plant volatile mixtures and to the pheromone (5pg/min or 50pg/min) alone as an index of effect. An odds ratio greater than 1 indicates that the behavioural element occurs more likely in the presence of the pheromone-plant compound mixture than in the presence of the pheromone alone. Values less than 1 indicate the opposite. Values in bold indicate significant differences between the pheromone alone and plant volatile-pheromone mixtures (GLM(logit), $p<0.05$).

Pheromone:plant compound ratio	**Activation**	**Take off**	**Upwind flight**	**Passing midline**	**Close-in on source**	**Source contact**
(E)-β-caryophyllene + (Z)-3-hexen-1-ol + methyl salicylate:						
1:10 000:10 000:10 000 (n=80)	0.778	0.600	0.856	1.392	1.487	1.138
(E)-β-caryophyllene:						
1:1 (n=80)	1.061	1.400	1.333	1.235	0.563	0.745
1:1 000 (n=80)	1.202	1.178	**2.154**	**3.182**	**2.808**	2.407
1:10 000 (n=160)	**1.913**	**1.830**	**2.103**	**3.425**	**4.636**	**4.529**
1:10 0000 (n=80)	1.485	1.800	2.038	**2.655**	2.290	1.840
(E)-β-caryophyllene + (Z)-3-hexen-1-ol:						
1:1 000:10 (n=80)	1.909	2.400	1.333	1.750	1.673	1.322
1:1 000:100 (n=80)	1.485	2.067	**3.620**	**4.667**	**4.248**	2.023
1:1 000:1 000 (n=80)	1.485	1.487	**2.400**	**3.566**	**2.808**	2.023
1:1 000:10 000 (n=80)	0.919	1.400	1.246	1.889	1.392	1.159

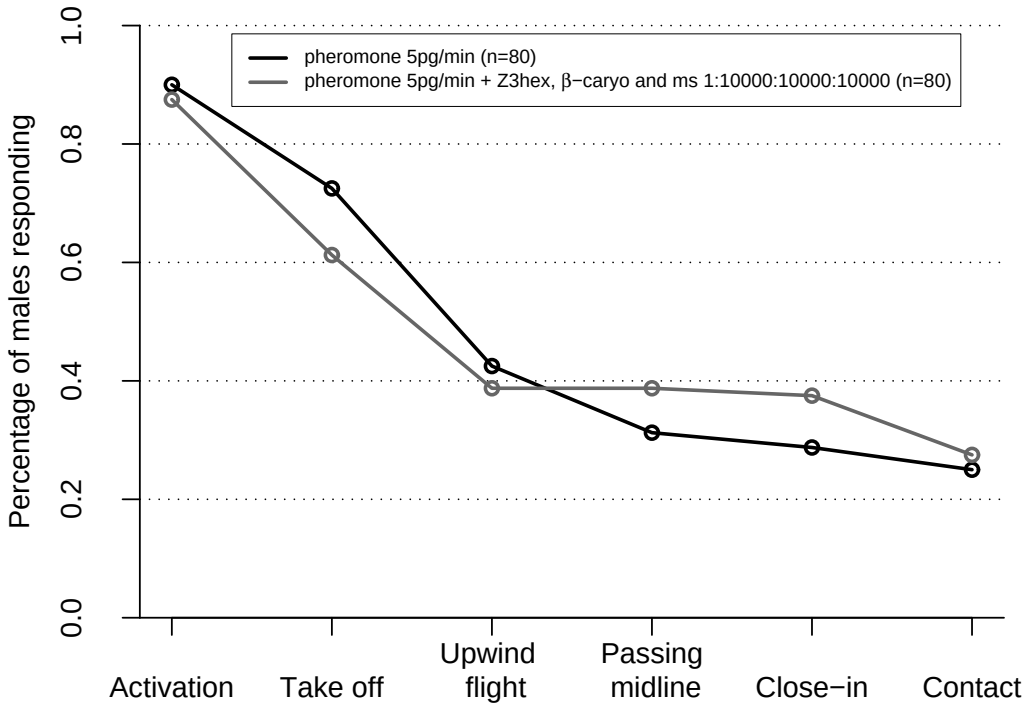


FIGURE 4.5: Percentage of male *E. ambiguella* responding to an underdosed pheromone level (5pg/min) alone and in a mixture with (Z)-3-hexen-1-ol, (E)-β-caryophyllene and methyl salicylate each released at 500ng/min resulting in a pheromone:plant compound ratio of 1:10 000:10 000:10 000. Z3hex = (Z)-3-hexen-1-ol; β-caryo = (E)-β-caryophyllene; ms = methyl salicylate

number of male grape berry moths flying upwind close to the source. However, the addition of (+)-terpinen-4-ol released at either 100pg/min or 1ng/min, (Z)-3-hexen-1-ol released at either 10ng/min or 100pg/min and (E)-β-caryophyllene released at 100pg/min changed male flight behaviour by rendering the overdosed pheromone dose attractive over the length of the wind tunnel in that significantly more males flew to within 10cm of the source (GLM(logit), $p<0.05$). Less males showed in-flight arrestment in the presence of these three host plant compounds at pheromone:plant volatile ratios of 10:1 to 1 000:1.

Since the males were activated immediately on exposure to the high pheromone level we performed a time-event analysis for the next behavioural step, i.e. "take-off", for the males exposed to the pheromone alone and for a pheromone:plant volatile ratio of 1:1000. In the presence of (Z)-3-hexen-1-ol, 1-octen-3-ol, (E)-β-caryophyllene, (+)-terpinen-4-ol and methyl salicylate the males took off faster compared to the pheromone alone. This difference was significant for (+)-terpinen-4-ol and methyl salicylate (Fig. 4.10).

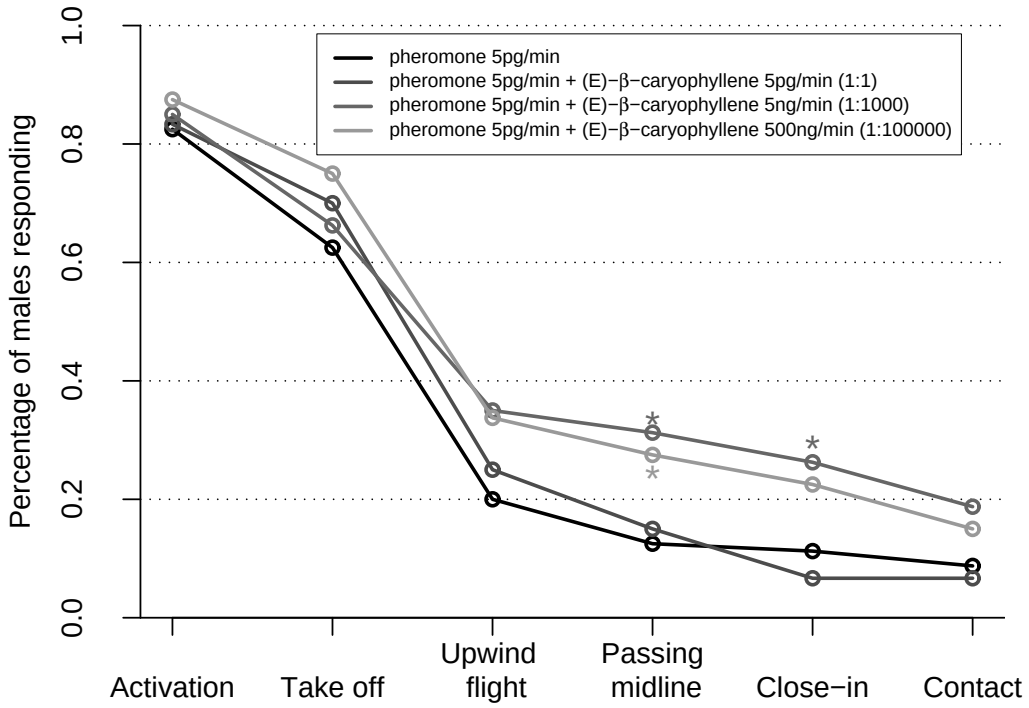


FIGURE 4.6: Percentage of male *E. ambiguella* responding to an underdosed pheromone level (5pg/min) alone and released with (E)-β-caryophyllene at different doses. Asterisks indicate a significant difference from the pheromone alone (GLM(logit), $p<0.05$); n=80 for each treatment.

4.4 Discussion

4.4.1 Effect of single host plant volatiles

Our results show that host plant volatiles influence male *E. ambiguella* flight behaviour towards its sex pheromone, at both an underdosed and an overdosed levels. This proves that male *E. ambiguella* not only possess receptor neurones for plant compounds on their antennae but perception of these plant compounds can modify their behaviour to their sex pheromone. Behavioural responses of male moths to plant volatiles have been investigated so far in only a few species. In the wind tunnel males of the tortricid *Cydia pomonella* are attracted to the host plant compound E,E-farnesol at a release rate of 10ng/min (Coracini et al., 2004), whereas in the field males were captured in traps containing 10mg E-β-farnesene and 10μg-10mg pear ester (ethyl (2E,4Z)-2,4-decadienoate) on rubber septa (Coracini et al., 2004; Light et al., 2001; Knight and Light, 2005a). In addition, several host plant volatiles serve to increase the attractiveness of the pheromone in a range of moth species similar what we found here for *E. ambiguella*. The addition of either linalool, (E)-β-farnesene, or (Z)-3-hexen-1-ol at a release rate of 100pg/min significantly increased the percentage of male *Cydia pomonella* flying upwind to codlemone

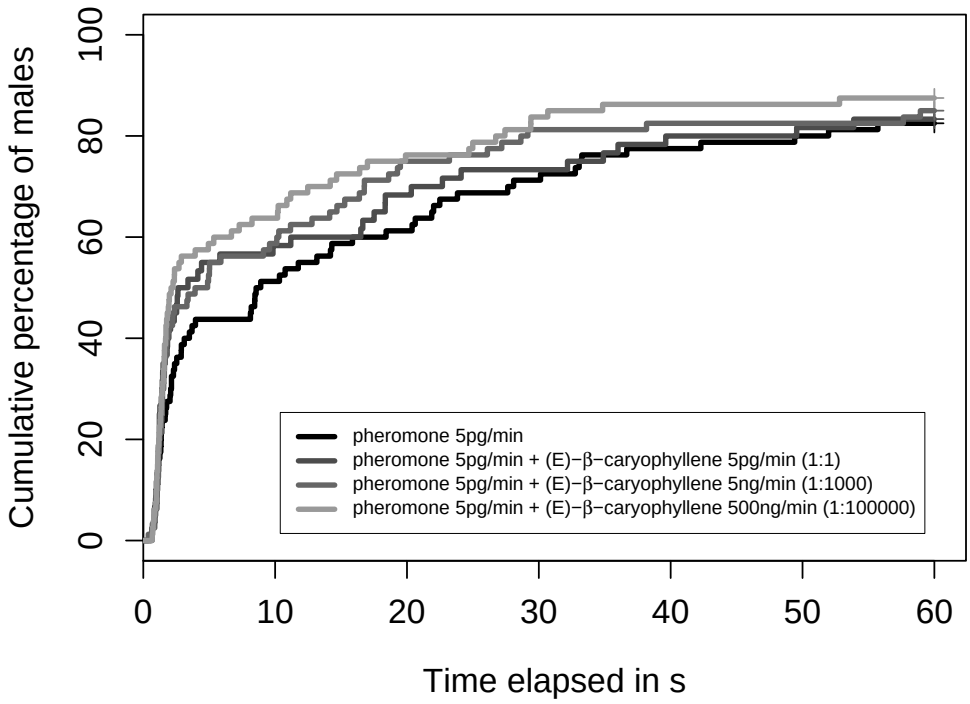


FIGURE 4.7: Cumulative percentage of male *E. ambiguella* activated over 60s in tests with the pheromone alone and pheromone released with (E)-β-caryophyllene. At higher levels of (E)-β-caryophyllene in the air with the pheromone the males are activated earlier; n=80 for each treatment.

released at 1pg/min from 37% to about 60% (Yang et al., 2004). In the field, trap captures of male codling moth were significantly increased when codlemone was admixed with a mixture of green leaf volatiles containing (E)-2-hexenal, hexanal, hexan-l-ol, (Z)-3- hexen-l-oI, and (E)-2-hexen-l-ol released from glass capillary tubes at a ratio of 1.0:1.3:3.8:3.8:3.6 (Light et al., 1993). In the same study Light et al. (1993) report that green leaf volatiles also increase captures of *Helicoverpa zea* when admixed with their pheromone. Such an increase in pheromone attraction by the addition of plant volatiles was also reported for *Heliothis virescens* and *Spodoptera exigua* (Dickens et al., 1993a; Deng et al., 2004). The possible relevance of such effects is treated below. Whereas in these cited examples such effects were found with underdosed or optimal pheromone levels, we found that three plant volatiles, (Z)-3-hexen-1-ol, (E)-β-caryophyllene, (+)-terpinen-4-ol and methyl salicylate, affected male *E. ambiguella* flight behaviour to both underdosed and overdosed pheromone levels suggesting that these volatiles play an important role in the sensory ecology of male *E. ambiguella*. Interestingly, receptor neurones quite sensitive to (Z)-3-hexen-1-ol, (E)-β-caryophyllene and methyl salicylate seem to be widespread among insects. For example, specific receptor neurones tuned to (E)-β-caryophyllene are documented for the strawberry blossom weevil *Anthonomus rubi*, the cotton leaf worm *Spodoptera littoralis*, and for

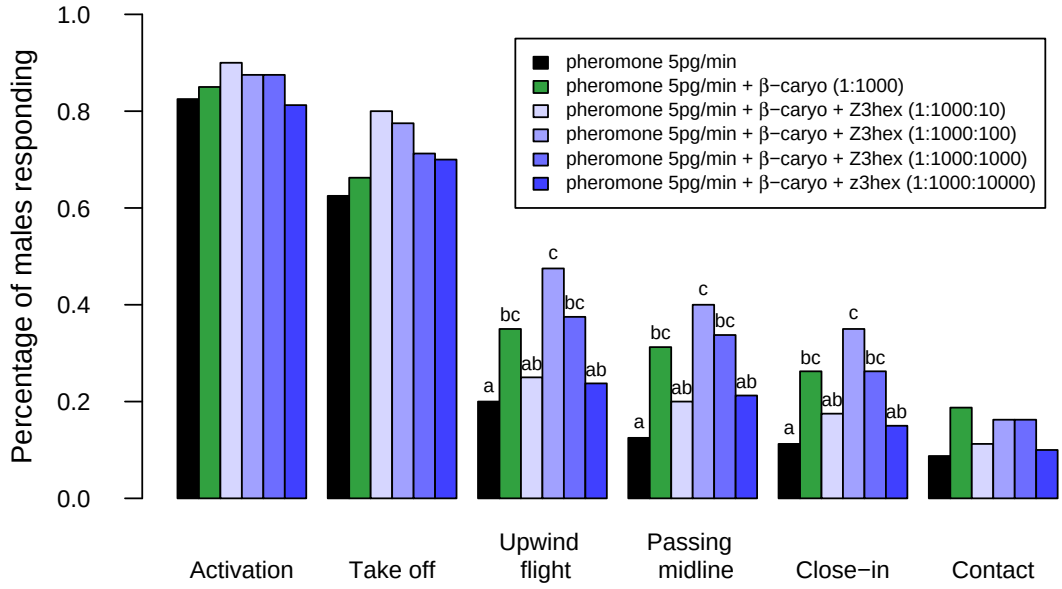


FIGURE 4.8: Percentage of male *E. ambiguella* responding to an underdosed pheromone level (5pg/min) alone and in mixtures with (E)-β-caryophyllene (β-caryo) released at 5ng/min without (green) and with different release rates of (Z)-3-hexen-1-ol (Z3hex; blue). Different letters within a behavioural element indicate statistically significant differences between treatments (GLM(logit) $p<0.05$); n=80 for each treatment.

the tabacco budworm *Heliothis virescens* (Anderson et al., 1995; Jönsson and Anderson, 1999; Bichão et al., 2005; Hillier and Vickers, 2007) and receptor neurones specific to methyl salicylate were desribed, among others, in the cabbage moth *Mamestra brassicae*, the strawberry blossom weevil *Anthonomus rubi*, and the fruit chafer *Pachnoda marginata* (Bichão et al., 2005; Stensmyr et al., 2001; Ulland et al., 2008). This suggests that these commonly occurring plant volatiles may play an important role in the sensory ecology of several insects. Despite the fact that single sensillum recordings (SSR; Wadhams, 1982) have not been made yet for *E. ambiguella*, using GC-EAD a low detection threshold of male grape berry moth antennal receptor neurones for (Z)-3-hexen-1-ol, (+)-terpinen-4-ol and methyl salicylate was recorded (see Chapter 2). Since these compounds also effect the behaviour of *E. ambiguella* this suggests that a low sensory threshold can serve as a useful predictor for the behavioural significance of volatiles, similar to what has been ascribed to pheromone compounds (Mayer et al., 1987). Only few studies have dealt with detection thresholds of antennal receptor neurones to plant volatiles with reference to behavioural responses. In the codling moth, *Cydia pomonella*, low detection thresholds of antennal receptor neurones were recorded for pear ester which indeed attracts both males and females on its own in the field (Light et al., 2001). Ansebo et al. (2004) recorded EAD-responses of female *C. pomonella* to (Z)-3-hexen-1-ol at low doses and this product has a synergistic effect on the codlemone perception at a ratio of 1:100 codlemone:(Z)-3-hexen-1-ol by male codling moths in the wind tunnel

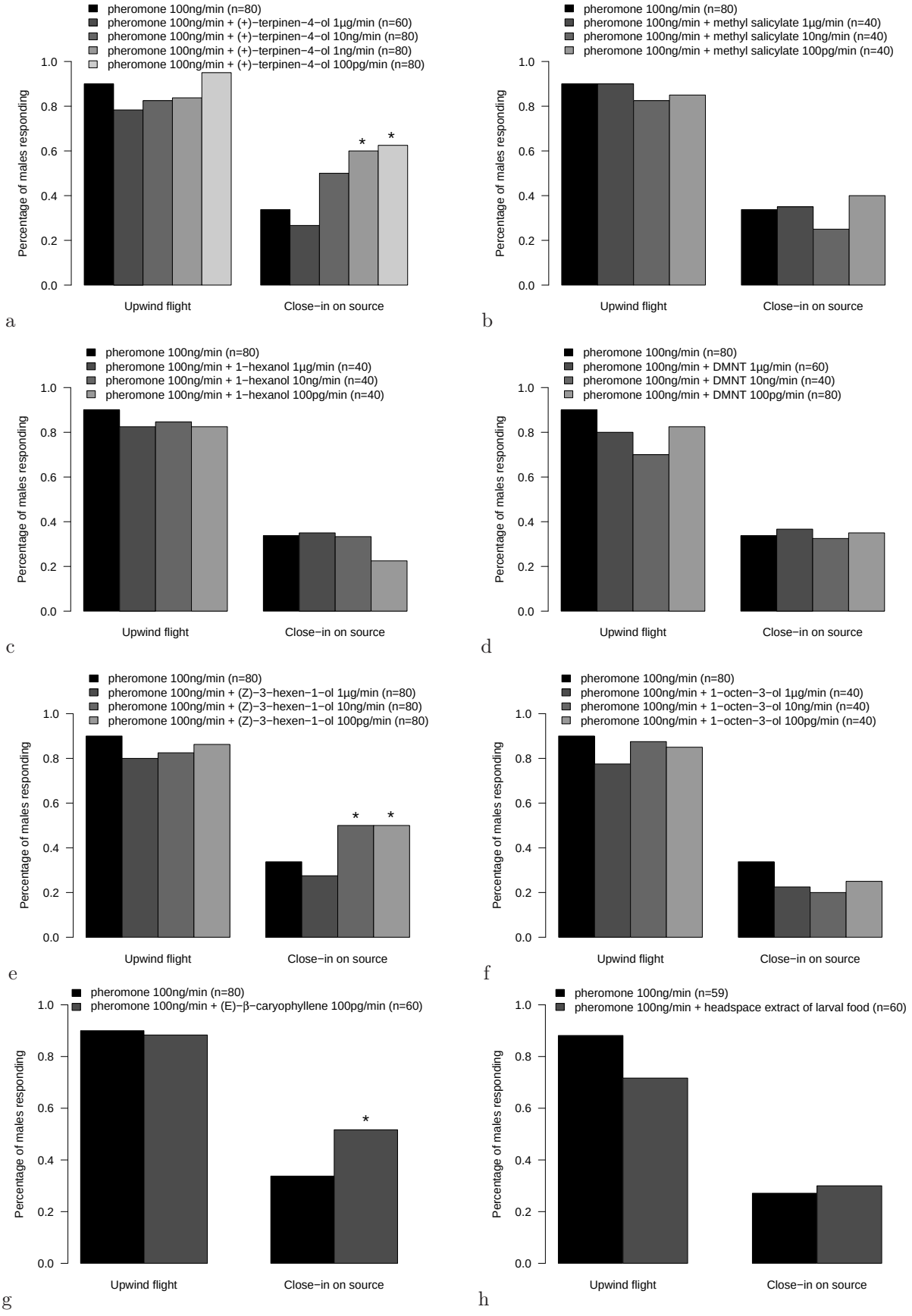


FIGURE 4.9: Percentage of male *E. ambiguella* responding to an overdosed pheromone level (100ng/min) released alone and in mixtures with different plant volatiles at different release rates. At certain levels of (+)-terpinen-4-ol (a), (Z)-3-hexen-1-ol (e) and (E)-β-caryophyllene (g) the attractivity of the pheromone source was partly restored, i.e. more males flew close to and contacted the source (GLM(logit), $p<0.05$). Asterisks indicate a significant difference from the pheromone alone.

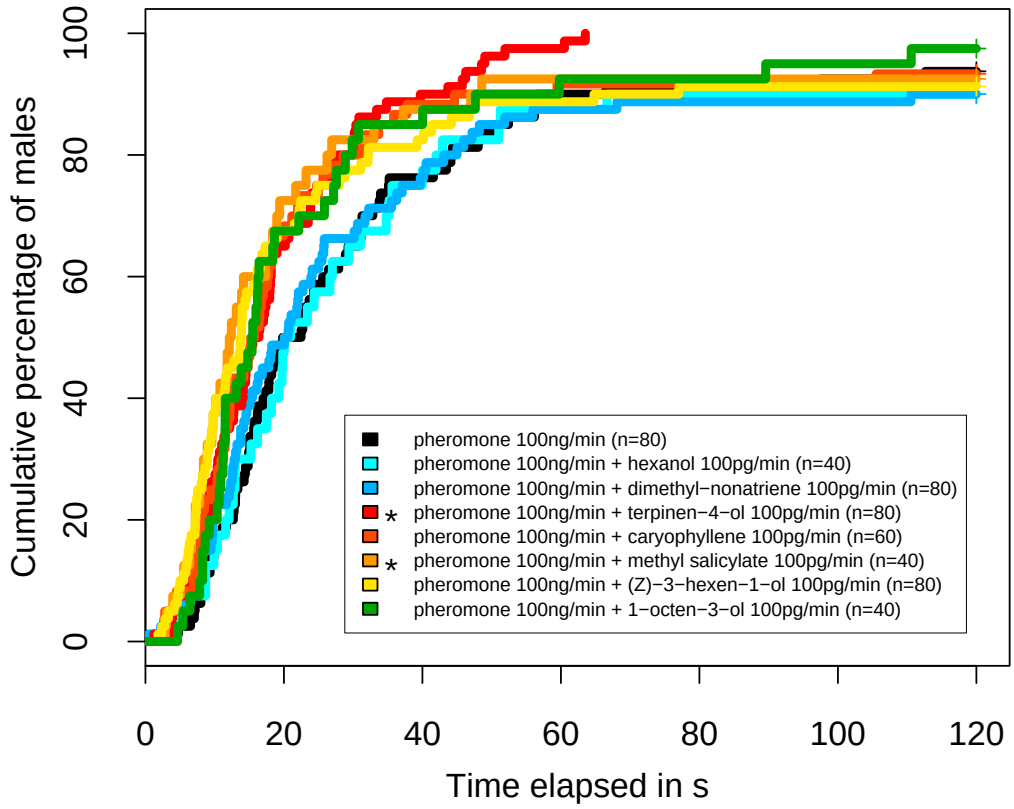


FIGURE 4.10: Cumulative percentage of male *E. ambiguella* taking off over a time period of 120s in response to the overdosed pheromone alone and in a pheromone:plant volatile mixture of 1:1000. In the presence of (+)-terpinen-4-ol and methyl salicylate male grape berry moths took off significantly faster compared to the pheromone alone (Cox proportional hazards model, $p<0.05$).

(Yang et al., 2004). For (E)-β-caryophyllene we did not calculate the detection threshold of male *E. ambiguella* receptor neurones. This compound is emitted by different physiological stages of the grape vine (Tasin et al., 2005) and has already been shown to attract *L. botrana* females in a mixture with (E)-β-farnesene and (E)-4,8-dimethyl-1,3,7-nonatriene (Tasin et al., 2006a, 2007).

Interestingly, the pheromone:plant volatile ratios for (Z)-3-hexen-1-ol, (E)-β-caryophyllene, (+)-terpinen-4-ol and methyl salicylate causing significant effects on male *E. ambiguella* flight behaviour were not the same for underdosed and overdosed pheromone levels. The plant volatile to pheromone ratio required to see effects was higher at the underdosed pheromone release rate but lower at the overdosed release rate. This suggests two different modes of action which will be discussed now.

Effects of host plant volatiles on underdosed pheromone levels By adding plant volatiles individually to an underdosed pheromone level of 50pg/min at a pheromone:plant volatile ratio of 1:10 000 significantly more males were attracted to the odour source in a wind tunnel with (Z)-3-hexen-1-ol, (E)-β-caryophyllene, (+)-terpinen-4-ol and methyl salicylate. Preliminary experiments reported in Chapter 3 showed that a combination of Z9-12:Ac and (+)-terpinen-4-ol released from rubber septa attracted more male *E. ambiguella* than Z9-12:Ac alone. All other plant volatiles except 1-octen-3-ol released with the pheromone in this study increased the number of upwind flights towards the source but only marginally. Lower attractiveness of Z9-12:Ac in the presence of 1-octen-3-ol was already observed when released from a rubber septum (Chapter 3). 1-octen-3-ol is released from *Botrytis*-infected grapes (Rapp and Mandery, 1986; Guerche et al., 2006) which are found to attract more females and larvae of *L. botrana* than non-infected grapes (Mondy et al., 1998a,b). However, 1-octen-3-ol and the other plant compounds, except (+)-terpinen-4-ol and (E)-β-caryophyllene, were tested only at the pheromone:plant volatile ratio of 1:10 000. This ratio is higher compared with the ratios found for plant compounds affecting responses to their pheromones in other tortricids. More *Cydia pomonella* males flew to the source at a pheromone:plant volatile ratio between 1:10 and 1:100 and a higher ratio of 1:10 000 did not enhance the number of males undertaking upwind flights (Denes Schmera, personal communication; Yang et al., 2004). A codlemone:pear ester ratio of 1:10 000 on rubber septa even decreased male attraction (Yang et al., 2005). In *L. botrana* host plant volatiles affected responses to its pheromone at a ratio of 1:1000 (Martin von Arx, personal communcation) but the effect disappeared at a higher (1:100 000) and lower (1:10) release ratios. In our case, other pheromone:plant compound ratios than 1:10 000 would probably result in a better attraction of male *E. ambiguella* to the pheromone.

Interestingly, (E)-β-caryophyllene or (+)-terpinen-4-ol not only increased the number of upwind flying males but also reduced the reaction time of responding male *E. ambiguella* to its pheromone. In both cases males were activated earlier with increasing doses of these volatiles. To our knowledge this is the first report of such an effect of plant volatiles on the pheromone perception by male moths indicating the role plant volatiles play in sexual communication between male and female moths.

In moths, mate choice seems to be dependent on host selection. Females feed and oviposit on host plants and can be located with a high probability at such places. By following host plant cues males can therefore be at mating sites before females start calling. The fact that male *E. ambiguella* emerge before females (potandry) supports this. In addition, when a female moth is calling she is sitting on the host plant such that her body may be contaminated with host plant odours. Several plant volatiles such as nonanal and (E,E)-farnesyl acetate were found, for example, on the scales of female *C. pomonella* (De Lury et al., 1999). In nature, the sex pheromone may thus be accompanied by host plant volatiles when it arrives on male antennae and males may use all these cues in order to optimise the chance of encountering a female. Male forest cockchafer *Melolontha hippocastani*, for example, are not only attracted to the female released sex attractant, 1,4-benzoquinone, but also to green leaf volatiles released by the leaves of deciduous trees fed on by females (Ruther et al., 2002) as a strategy to locate females more efficiently.

From an evolutionary point of view, the pheromone communication system may have evolved from plant - insect communication systems. Males and females would have met more likely on host plants where specific signals reduce the time and energy they require in searching for a mate. In a first step moths may have used only host plant volatiles to locate mates, with plant volatiles serving as so-called aggregation kairomones. However, host plant volatiles are not specific in the sense that other moth species could also be attracted by the same plant cues. Selection forces may have resulted in more species-specific cues - the sex pheromone - to optimise the encounters between the sexes and, consequently, reproductive success. Females may have evolved to produce more specific cues and males the sensory apparatus to respond to such volatiles. Plant volatiles may, however, still be important, as the pheromone communication system is derived from it. Evidence for this hypothetic scenario is provided on the one hand by the fact that pheromone binding proteins (PBP), presumed to carry pheromones to receptor sites in the sensillum lymph, have probably evolved from general odour binding proteins (GOBP; Lerner et al., 1990) and the fact that some insect species "still" depend on host plant products to produce their pheromone (Landolt and Phillips, 1997).

Physiologically, plant compounds may influence the pheromone responses of *E. ambiguella* males

at different steps along the olfactory pathway, i.e. at the periphery, in the antennal lobe (AL) and in higher brain centers. At the periphery, an interaction of plant volatiles and pheromone products was found in *Helicoverpa zea* (Ochieng et al., 2002): the firing rate of sex pheromone-specific olfactory receptor neurones in male *H. zea* to its pheromone product was significantly enhanced by simultaneous stimulation with linalool and (Z)-3-hexenol. Alone, however, neither of these two plant compounds can serve as effective stimuli for the pheromone-specific neuron. In the AL, pheromone-sensitive olfactory receptor neurones (ORNs) converge in the macroglomerular complex (MGC) whereas plant volatile sensitive ORNs target to isomorphic glomeruli. In the glomeruli, ORNs synapse with different types of interneurones: local interneurones (LNs) with extensive interglomerular connections and mediating interglomerular inhibition (Mustaparta, 2002), and projection neurones (PNs) receiving signals from ORNs and LNs which they transmit to the protocerebrum for further integration. Local interneurones may integrate the response to the pheromone and to plant volatiles via interglomerular connections. Local neurones arborising in the MGC and in isomorphic glomeruli were found, for example, in *Spodoptera littoralis* (Anton and Hansson, 1995). In addition, excitation of PNs innervating one glomerulus by other PNs innervating other glomeruli through local interneurones was hypothesised by Wilson et al. (2004). Anton and Hansson (1995) identified PNs from male *S. litteralis* with dendritic arborisation in the MGC which respond to both pheromone and plant compounds. In male *Trichoplusia ni* blend-specific PNs were identified showing weak responses to individual pheromone compounds at 10ng but strong responses when a pheromone mixture was used (Hansson and Anton, 2000). Such blend-specific PNs may not only exist for pheromone mixtures but also for pheromone - plant volatile mixtures. To our knowledge such neurones have not been reported as yet. Signals from PNs innervating the same glomerulus are found to synchronize and the onset of this synchronisation can be influenced by local interneurones (Lei et al., 2002): in *Manduca sexta* ORNs responding to bombykal target to the cumulus, the major glomerulus of the MGC, and ORNs responding to E11,Z13-15:Al target to the toroid, another glomerulus in the MGC. In response to a mixture of bombykal and E11,Z13-15:Al the PNs, innervating the cumulus and responding to bombykal, were synchronised significantly earlier. This was caused through a fast IPSP (inhibitory post-synaptic potential) of a local interneuron between the cumulus and the toroid responding to E11,Z13-15:Al. Earlier synchronisation may cause an earlier behavioural reaction. If such an interaction exists also between the MGC and isomorphic glomeruli in *E. ambiguella* it could explain why male reaction time to its pheromone is shorter in the presence of plant compounds. However, processing of plant volatiles and pheromone may also take place in the protocerebrum where the signals from pheromone-responding PNs and plant volatile-responding

PNs may converge. Beside pheromone responding PNs, PNs responding to plant volatiles are reported for male *S. litteralis* (Anton and Hansson, 1995) and for male *L. botrana* (Masante-Roca et al., 2002, 2005).

Effects of host plant volatiles on overdosed pheromone levels When the pheromone was offered at an overdosed level (100ng/min) almost all *E. ambiguella* males tested flew upwind but only a small fraction reached the source. (Z)-3-hexen-1-ol, (E)-β-caryophyllene and (+)-terpinen-4-ol significantly increased the number of males that reached the source when released at between 100pg/min and 10ng/min thus restoring attraction to the source of the high pheromone level. This is the first report of such an effect of plant volatiles on the pheromone perception. A similar phenomenon has been found in this species with the pheromone products 12:Ac and 18:Ac (Arn et al., 1986; Rauscher et al., 1984, Section 3.3.1): the dose where the main pheromone component Z9-12:Ac on a rubber septum induces most source contacts by males occurs at 20μg whereas higher doses result in-flight arrestment behaviour. 12:Ac, a minor component of female sex glands, was combined in different ratios with Z9-12:Ac on rubber septa. At low doses of Z9-12:Ac, 12:Ac had no or negative effects whereas at a high dose of 100μg Z9-12:Ac the attractiveness of the overdose was restored in the presence of 100μg 12:Ac. Adding 200μg 18:Ac to this binary mixture increased the number of males flying to and contacting the rubber septum even further. Moreover, at this ratio of 1:1:2 the range of attractive doses was broader compared to the Z9-12:Ac alone in both, the laboratory and the field (Rauscher et al., 1984; Arn et al., 1986). Interestingly, neither 12:Ac nor 18:Ac was attractive on its own.

The physiological basis of in-flight arrestments is not yet fully understood. Some researchers argue that high pheromone concentrations cause adaptation of the olfactory receptor neurones (e.g. Baker et al., 1988, 1989). In this case, we would expect that the male moth should stop flying to the source when the receptor neurones adapt. However, we have observed the contrary, i.e. that males maintain flight but without advancing much farther upwind. More plausible is that the arrestment behaviour in response to very high pheromone doses is linked to the plume structure and dose. As an odour plume is not continuous but intermittent, upwind flying males encounter filaments loaded with odour alternating with clean air or air with lower pheromone levels (Kaissling, 1997; Murlis and Jones, 1981). Near the source the density of such filaments may be higher than farther downwind and males encounter concentration which are evidently too high. Consequently, male show in-flight arrestment behaviour. Evidence for this hypothesis comes from the fact that the position where males show in-flight arrestment can be shifted by introducing turbulence into the odour plume (see Chapter

3). In addition, the antennae and the body surface of the male may get contaminated with pheromone at such high levels causing a constant excitation of olfactory receptor neurones and so further affecting the males ability to detect difference in pheromone levels in the air.

As outlined above, plant compounds can intervene at different levels of the olfactory nervous pathway. In the case of the overdosed pheromone level, plant volatiles may alter the the manner in which the chemostimuli arrive at the receptor neurones. Kaissling et al. (1989) identified a bombykol analogue, (Z,E)-4,6-hexadecadiene, which after a 1s stimulus at a high dose elicited spikes in olfactory receptor neurones of male *Bombyx mori* for a period of over 10min. Linalool, a know inhibitor of the bombykol cell has an inhibitory effect on this excited olfactory receptor. In a behavioural assay, Kramer (1992) showed that male *B. mori* contaminated with this bombykol analogue responded with upwind orientation by the administration of repeated linalool pulses. This example demonstrates how moth behaviour can be modified simply by manipulation nervous input.

Plant compounds added to an overdosed pheromone level may simply have a dilution effect by competing (agonist) with the pheromone at the receptor level thus leading to stimulation equivalent to that induced at lower levels. This was also hypothesised for the effect of 12:Ac on overdosed Z9-12:Ac in this species (Rauscher et al., 1984). At a higher stage of the olfactory processing plant compounds may have inhibitory effects across glomeruli. At high doses of pheromone, PNs innervating the MGC may fire continuously. Local interneurones originating from an isomorphic glomerulus and responding to plant volatile input may thus serve to inhibit the firing of the PNs responding to the pheromone. By using neural-ensemble recording Lei et al. (2004) found that isomorphic glomeruli responding to high doses of plant volatiles inhibit glomeruli of the MGC responding to high doses of pheromone and vice versa. However, these are only hypotheses and more knowledge of the effects of host plant volatiles on overdosed pheromone levels is needed. For a better understanding of the qualitative effects of host plant volatiles on male *E. ambiguella* flight towards overdosed pheromone sources the 3D tracking system (Section 3.3.1) may prove useful.

4.4.2 Effect of mixtures of host plant volatiles

In nature, insects normally have to deal with complex mixtures of odours rather than individual compounds and so it is likely that mixtures of plant compounds have stronger effects on the pheromone perception by male *E. ambiguella* than individual products. Here, we showed that more *E. ambiguella* males flew upwind to its pheromone released with a mixture of (Z)-3-hexen-1-ol plus (E)-β-caryophyllene at a ratio of 1:100:1000 compared to a mixture of pheromone and single plant com-

pounds. Other ratios of (Z)-3-hexen-1-ol plus (E)-β-caryophyllene did not have such a strong effect, and a mixture of (Z)-3-hexen-1-ol, (E)-β-caryophyllene plus methyl salicylate at 10 000:10 000:10 000 did not increase the attractiveness of the underdosed pheromone. This indicates that an appropriate ratio between plant volatiles is important. Interestingly, the release rate of (Z)-3-hexen-1-ol was ten times lower than that of (E)-β-caryophyllene. This fits well with the analysis of the headspace extracts by GC-EAD (Chapter 2): when present, (E)-β-caryophyllene was observed at higher amounts than (Z)-3-hexen-1-ol and additionally EAD-responses were stronger to (Z)-3-hexen-1-ol than to (E)-β-caryophyllene at similar levels in the extracts. Yang et al. (2004) recorded the behaviour of male *C. pomonella* to the pheromone plus mixtures of either two (β-farnesene and linalool) or four (β-farnesene, linalool, (Z)-3-hexen-1-ol, (Z)-3-hexenyl acetate) plant compounds. At pheromone:plant volatile ratios of 1:1:1, 1:100:100, 1:1:1:1 and 1:100:100:100:100 none of these mixtures was more attractive than a mixture pheromone and linalool, but none of these mixtures were related to that occurring naturally.

Single plant volatiles may be sufficient to increase the attractiveness of the pheromone to some extent because of the specificity of the response to the pheromone, but our results indicate that a mixture of plant volatiles at an appropriate ratio can enhance the attractiveness of pheromone even more than the plant volatiles presented singly. The attractiveness of mixtures of plant volatiles has been extensively studied in *C. pomonella* and *L. botrana*. Ansebo et al. (2004) recorded the flight behaviour of male *C. pomonella* to an apple mimic consisting of 11 compounds in an approximately naturally occurring ratio and to binary mixtures and single components of the apple mimic. Only 6.2% of the males flew upwind to the apple mimic whereas 19.7% did so to a binary mixture of (E,E)-α-farnesene and (E)-β-farnesene at the same ratio of 100:1 as in the apple mimic. Maybe the dosage of some compounds in the apple mimic was not appropriate. The attractiveness of the (E,E)-α-farnesene - (E)-β-farnesene mixture was further increased by the addition of pear ester where 30.3% of the male flew upwind. None of these compounds alone attracted male *C. pomonella* in the wind tunnel, indicating synergistic effects between these compounds. The flight behaviour of female *L. botrana* to different mixtures of host plant volatiles was recorded by Tasin et al. (2005, 2006b,a, 2007). They calibrated the headspace for grape volatiles eliciting consistent antennal responses in *L. botrana* and accordingly formulated a synthetic grape volatile mixtures containing (E)-β-caryophyllene, DMNT, (E)-β-farnesene, (E,E)-α-farnesene, methyl salicylate, linalool, (Z)-furan linalool oxide, (E)-furan linalool oxide, 1-octen-3-ol and 2-ethyl-1-hexanol, where (E)-β-caryophyllene was the main component. The attractiveness of this mixture released at 3.5 and 35ng/h (relating to (E)-β-caryophyllene) to female *L. botrana* was compared with 100g green berries at a (E)-β-caryophyllene release rate of 4.7ng/h and a grape headspace extract

containing these volatiles released at 0.35, 3.5 and 35 ng/h (Tasin et al., 2006b). Significantly more females flew upwind to the synthetic mixtures at 35ng/h compared to all other treatments. A mixture of the three most abundant compounds in grape berries i.e. (E)-β-caryophyllene, DMNT, and (E,E)-α-farnesene at 100:78:9, the ratio found in grapes, was as attractive to female *L. botrana* as the mixture of 10 plant compounds (Tasin et al., 2006a, 2007). Another mixture of these three compounds at 37:17:100 which was found in apples did not attract the females providing evidence that ratio-specific mixtures of ubiquitous volatiles are critical for polyphagous moths (Tasin et al., 2006a). However, we expect that the ratio of plant volatiles is less strict than the ratio of pheromone components as there is probably less selection pressure for plant volatiles when one considers the range of host plants exploited. In addition, the ratios may be highly dependent on different factors such as the physical condition of a plant and environmental conditions.

Establishing the biological significance of mixtures of plant volatiles is a difficult subject. Byers (1992) proposed a subtractive method to discover effective mixtures of chemicals that affect insect behaviour. In the past, fermented juice such as cider or wine with sugar and vinegar were used to monitor the flight of grape moths (Bovey et al., 1972). Such a mixture could serve, for example, as a basis for Byers' subtractive method to establish a mixture of the most effective compounds.

Chapter 5

General discussion and conclusion

Comparing the headspace volatiles present over different host plants of *E. ambiguella* by GC-EAD and the determination of the EAD-response threshold to some of these volatiles permitted the identification of chemostimuli that may be implicated in the sensory ecology of male grape berry moths (Chapter 2). Four of these volatiles, i.e. (Z)-3-hexen-1-ol, (+)-terpinen-4-ol, (E)-β-caryophyllene and methyl salicylate affected the flight response of male grape berry moths significantly in that more males flew to the source of pheromone in the presence of these host plant volatiles (Chapter 4). This effect was observed at pheromone levels both below and above the optimal dose. Additionally, males were activated earlier when these volatiles were released with an underdosed pheromone level. At an overdosed level, such an effect was found for the behavioural element "take-off", namely, that males were taking off faster in response to the pheromone in the presence of (Z)-3-hexen-1-ol, (+)-terpinen-4-ol, (E)-β-caryophyllene and methyl salicylate.

Our results provide further evidence that host plants may not only serve as an oviposition resource but also as rendezvous sites for mates. These mating sites are located by chemical cues released by host plants. This is to the disadvantage of monocultures which may through the high quantity of such chemical cues released facilitate the encounters for mating in pest species. In polycultures, instead, the diversity of olfactory stimuli emitted by non-host plants might mask the olfactory cues of a host plant (Andow, 1991) thus making it not only more difficult to locate a host plant for oviposition but also to locate mates. Since plant volatiles may enhance the attractiveness of pheromones this could be a further explanation as to why insect pest outbreaks are more likely to occur in monocultures.

The fact that the attractiveness of the pheromone of *E. ambiguella* can be improved by the addition of plant volatiles may provide a means to develop more attractive lures for *E. ambiguella*. Our results with overdosed pheromone levels are of special interest in the context of mating disruption where

dispensers carry a high pheromone load. Before demonstrating possible implications we first need to consider the probable mode of action of this pest control method which was under debate for several years. The hypotheses range from false-trail-following to camouflage, desensitization, habituation and adaption (Cardé and Minks, 1995; Miller et al., 2006a). Many examples show now that false-trail-following is the most likely mode of action of mating disruption (Miller et al., 2006b; Stelinski et al., 2004). Here, females are supposed to compete with artificial pheromone sources (pheromone dispensers and monitoring traps) for males. Consequently, the extent of mating disruption depends mainly on: 1) the population density, 2) the density of the pheromone dispenser and 3) the attractiveness of dispensers compared to the females (Miller et al., 2006a). If the population density is high and the density or the attractiveness of dispensers is low the probability that males find a female is enhanced. Indeed, it is the case that mating disruption is insufficient at high population levels. Unfortunately, an increase in the pheromone dispenser density is restricted due to the high cost of pheromones and by the costs of additional manpower needed to apply them. An enhancement of the attractiveness of dispensers compared to females is therefore favorable. In this thesis, I have provided evidence that the attractiveness of pheromone dispensers can be increased by plant volatiles. These volatiles are much cheaper than the pheromone. Thus, pheromone plus plant volatiles lures may not only serve to increase the effectiveness of mating disruption but also to reduce costs to make this method more competitive over insecticides.

In mating disruption increased attractiveness could mean that 1) males may prefer flying to dispenser containing pheromone plus plant volatiles over flying to females or 2) males may be occupied longer with pheromone dispensers incorporating plant volatiles than with those containing only pheromone. Stelinski et al. (2004) observed the flight behaviour of different tortricid moths to high-dose pheromone dispensers in the field. Males of *Grapholita molesta*, *Choristoneura rosaceana* and *Argyrotaenia velutinana* were attracted within 100cm to these dispensers but landing on the dispensers was observed only in *G. molesta*. The moths were observed being occupied with such dispensers for at least 100 seconds. The same scenario is likely to pertain for *E. ambiguella* since we observed in the wind tunnel that males maintain flight effort but nevertheless are unlikely to contact high-load pheromone sources. However, male grape berry moths flew closer to the overdosed pheromone in the presence of plant volatiles. In the field, inclusion of such plant volatiles in the pheromone dispenser may serve to occupy the males for longer. As males respond to their pheromone only during a restricted time window, longer occupation with pheromone dispensers may reduce the probability that males contact a female. The 3D video tracking system described in Chapter 3 would permit to compare the flight behaviour

to different sources qualitatively and to make more predictions about possible flight behaviours to pheromone dispensers in nature. Males may, for example, fly more directly to the pheromone source in the presence of plant volatiles compared to females or pheromone dispensers without plant volatiles.

Our results may also have implications for the attract-and-kill method where a constraint exists between pheromone load and the necessity to contact the insecticide. Whereas at high pheromone loads the moths do not contact the source containing the insecticide, low pheromone loads do not last the flight season of *E. ambiguella* (Charmillot et al., 1996, 1995). Since we showed that plant volatiles restore the activity of high pheromone doses, the inclusion of host plant volatiles for the attract-and-kill method may permit a higher pheromone load in such droplets and ensure both contact with the droplets and the durability of their attractiveness over the whole flight season.

An example of how plant compounds can be used in mating disruption is the control of the codling moth *C. pomonella* by codlemone and pear ester (ethyl (2E,4Z)-2,4-decadienoate). As in most moth species, pheromone traps attract male codling moths only at the time when females are calling. Pear ester traps instead were shown to attract the male moths not only over a greater time period but can also attract female *C. pomonella* (Knight and Light, 2001, 2005a,b). For *E. ambiguella* we did not find such a powerful plant volatile yet. Female grape berry moths are unlikely to be attracted to (Z)-3-hexen-1-ol, (+)-terpinen-4-ol, (E)-β-caryophyllene or methyl salicylate on their own. Nevertheless, mixtures of these common host plant volatiles at particular ratios may have similar effects for *E. ambiguella* as the pear ester for *C. pomonella*, and attract both females and males. First indications that mixtures of plant compounds are more attractive than individual compounds were obtained through the observation that males were more attracted to a pheromone plus (Z)-3-hexen-1-ol and (E)-β-caryophyllene mixture than to a pheromone plus (Z)-3-hexen-1-ol or a pheromone plus (E)-β-caryophyllene mixture.

Overall, our results provide evidence for the potential to improve mating disruption methods by including host plant volatiles. In future studies more knowledge is needed regarding mixtures of plant volatiles to further improve the attractiveness of the pheromone and if such mixtures can also attract females. In addition, dispensers to release plant volatiles at defined rates need to be developed for field applications since the appropriate rate of release of host plant volatiles is critical.

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Appendix A

Composition of the semiartifical medium for the rearing of *E. ambiguella*

water	800g
pulverised agar	25g
sugar	30g
wheat germ	90g
alfa alfa meal	25g
casein	40g
brewer's yeast	18g
Wessen salt	12.5g
sun flower oil	2.5g
cholesterol	1.25g
sorbic acid	2g
ascorbic acid	10g
Vanderzant vitamin mix	7.5g
Tetramycine (95%)	1.25g
propionic acid	2.5g
linolenic acid	1g

Appendix B

EAD-responses to host plant headspace extracts

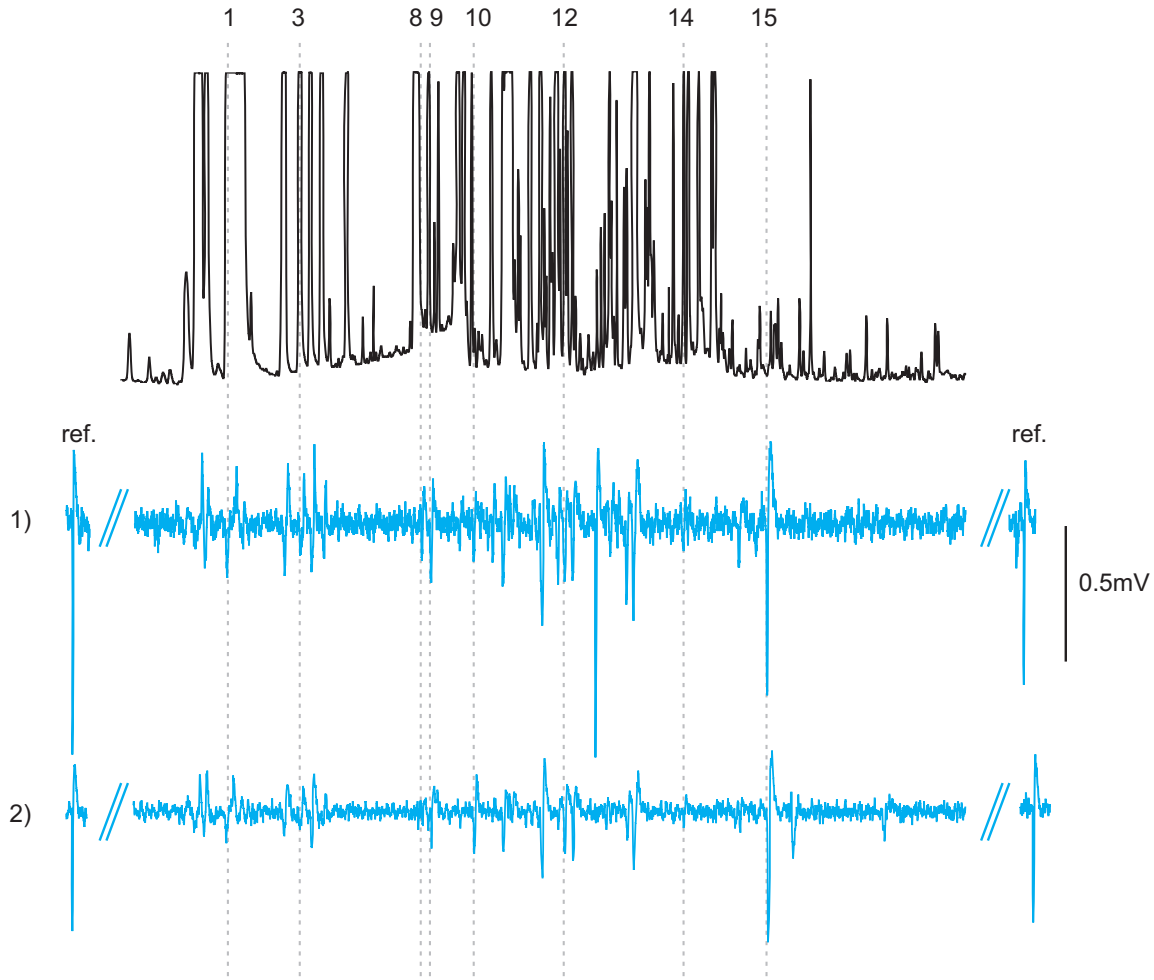


FIGURE B.1: EAD-responses of two male *E. ambiguella* antennae (1 and 2) to aliquots of the headspace extract of *R. officinalis*. Numbered volatiles were found to elicit antennogram responses in at least three of the different host plant extracts tested (cf. Chapter 2): 1 limonene; 3 ocimene; 8 (Z)-3-hexen-1-ol; 9 nonanal; 10 1-octen-3-ol; 12 (E)-β-caryophyllene; 14 methyl salicylate; 15 unknown. Ref. is the response to an air puff applied to the antennal preparation at the start and end of each analysis from a 5ml syringe containing 10μg (+)-terpinen-4-ol on a filter paper strip.

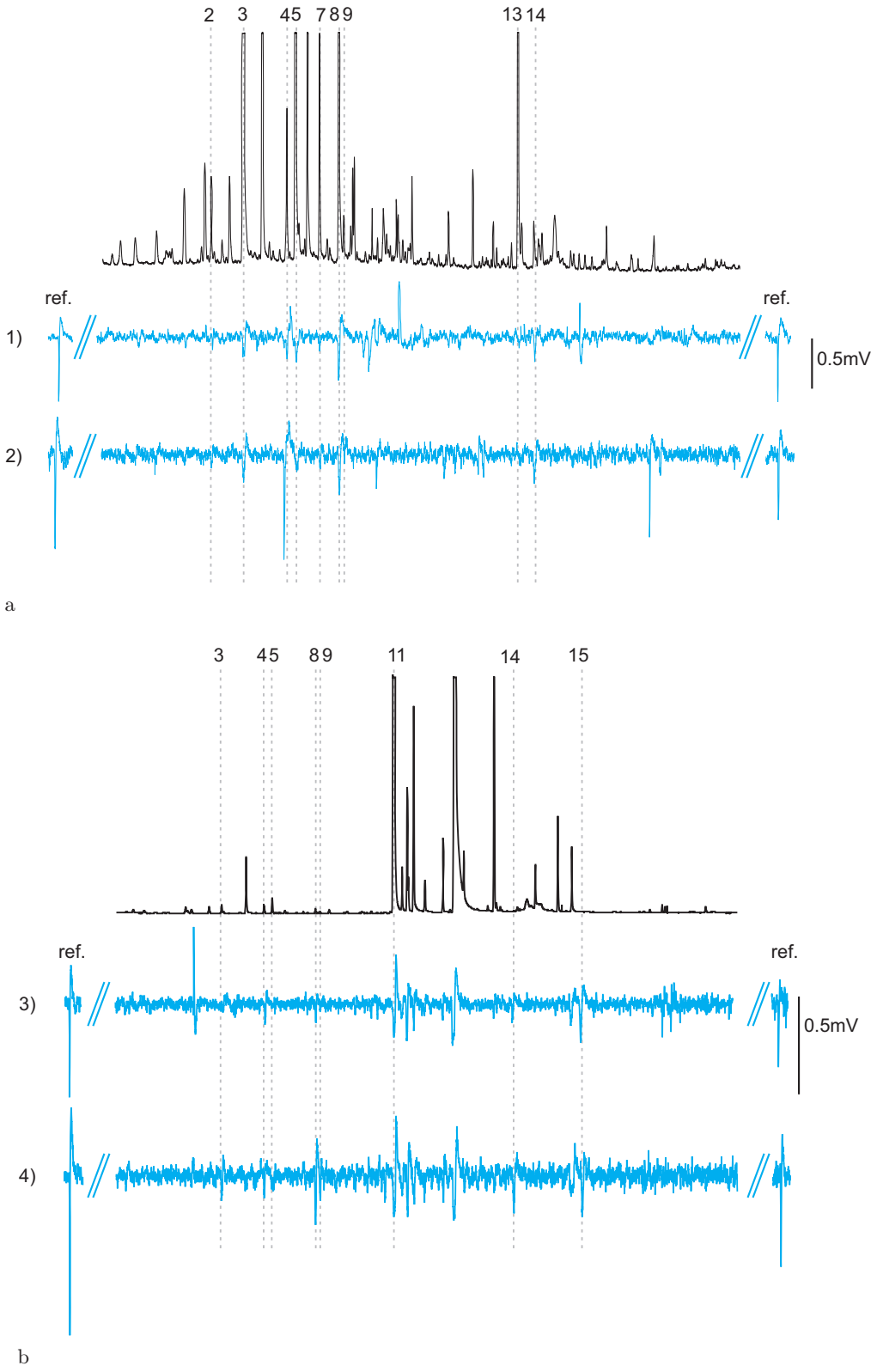


FIGURE B.2: EAD-responses of four male *E. ambiguella* antennae (1-4) to aliquots of the headspace extract of *L. vulgare* branches with a. flower buds and b. open flowers. Numbered volatiles were found to elicit antennogram responses in at least three of the host plant extracts tested (cf. Chapter 2): 2 (E)-2-hexen-1-al; 3 ocimene; 4 DMNT; 5 (Z)-3-hexenyl acetate; 7 1-hexanol; 8 (Z)-3-hexen-1-ol; 9 nonanal; 11 benzaldehyde; 13 α-farnesene; 14 methyl salicylate; 15 unknown. Ref. is the response to an air puff applied to the antennal preparation at the start and end of each analysis from a 5ml syringe containing 10μg (+)-terpinen-4-ol on a filter paper strip.

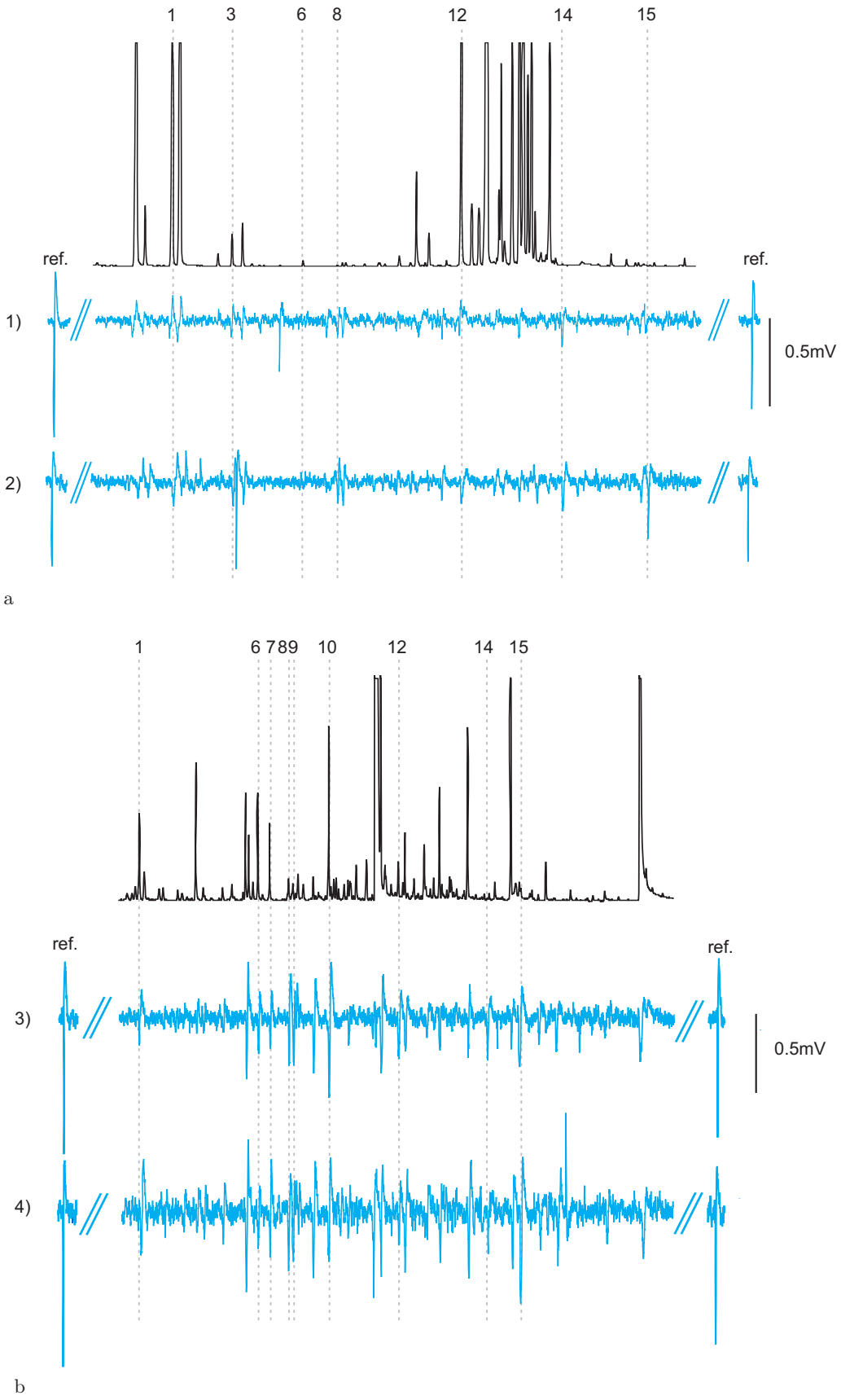


FIGURE B.3: EAD-responses of four male *E. ambiguella* antennae (1-4) to aliquots of the headspace extract of a. *H. helix* and b. the artifical rearing medium (Appendix A). Numbered volatiles were found to elicit antennogram responses in at least three of the different host plant extracts tested (cf. Chapter 2): 1 limonene; 3 ocimene; 6 6-methyl-hepten-2-one; 7 1-hexanol; 8 (Z)-3-hexen-1-ol; 9 nonanal; 10 1-octen-3-ol; 12 β-caryophyllene; 14 methyl salicylate; 15 unknown. Ref. is the response to an air puff applied to the antennal preparation at the start and end of each analysis from a 5ml syringe containing 10μg (+)-terpinen-4-ol on a filter paper strip.

Appendix C

Determination of release rates of pheromone from rubber septa

Pheromones are released from rubber septa by a first-order process (McDonough, 1978). This means that the rate of evaporation of a compound is proportional to the amount of this compound remaining in the rubber cap. So the release rate R is

$$R = -\frac{dM}{dt} = kM$$

whereas $k = \frac{ln2}{t_{\frac{1}{2}}}$ and M is the amount of the volatile compound at time t in the septum.

This results in

$$R = \frac{Mln2}{t_{\frac{1}{2}}} \tag{C.1}$$

Half lives of Z9-12:Ac and 12:Ac at different temperatures were determined by McDonough et al. (1989, Tab. C.1). M can be calculated by the following formula:

$$M = M_0 e^{-tt_{\frac{1}{2}}^{-1}ln2}$$

with M_0 corresponds to the quantity of the volatile compound at $t = 0$. Using equation (C.1) we calculated the release rate of Z9-12:Ac from rubber septa (Fig. C.1)

TABLE C.1: Half-lives (days) of 12:Ac and Z9-12:Ac in rubber septa at different temperatures following McDonough et al. (1989)

Compound	15°C	20°C	25°C	30°C	35°C
12:Ac	64.0	36.8	21.5	12.8	7.78
Z9-12:Ac	57.7	33.3	19.6	11.7	7.11

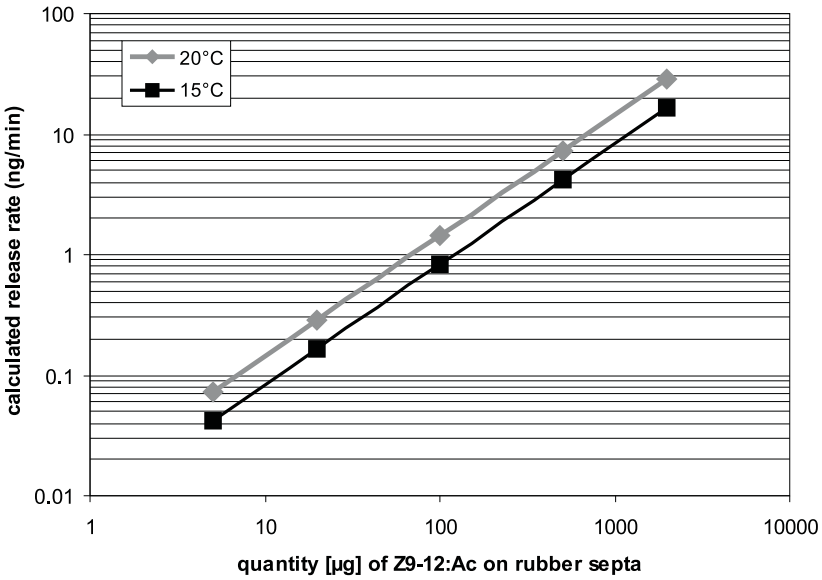


FIGURE C.1: Calculated release rates of Z9-12:Ac as a function of different initial doses on rubber septa. The release rate is temperature dependant and was calculated with half-lives reported at room temperature of 15 and 20°C. In our experiments (room temperature = 18°C) the release rate of Z9-12:Ac from rubber septa lies between the two curves.

Appendix D

Shift of the response of male *E. ambiguella* to the underdosed pheromone level

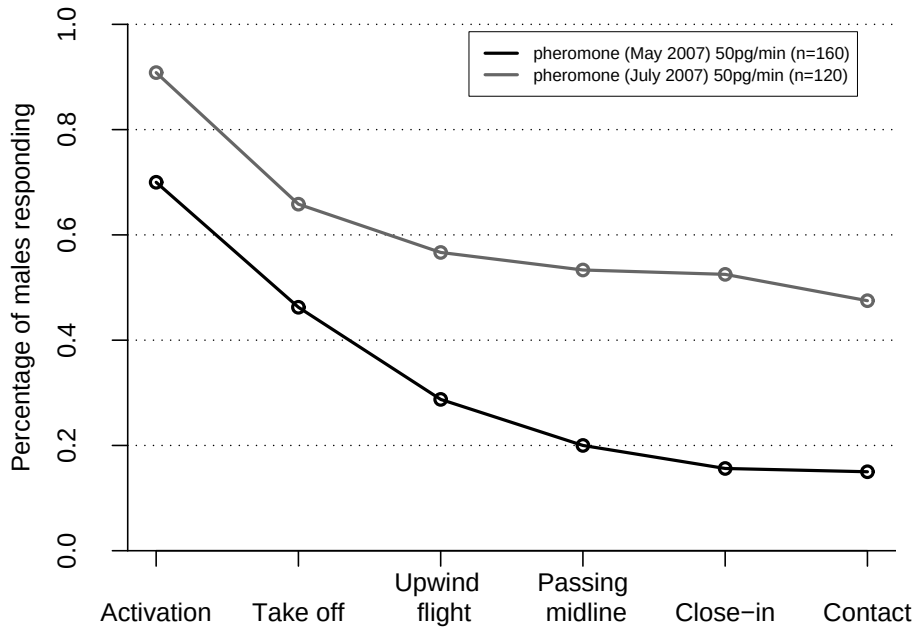
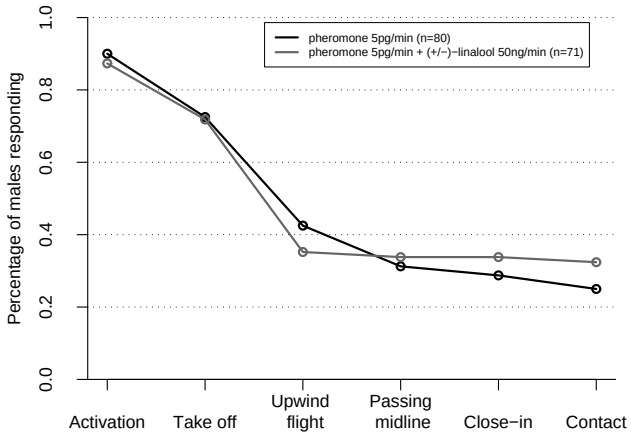
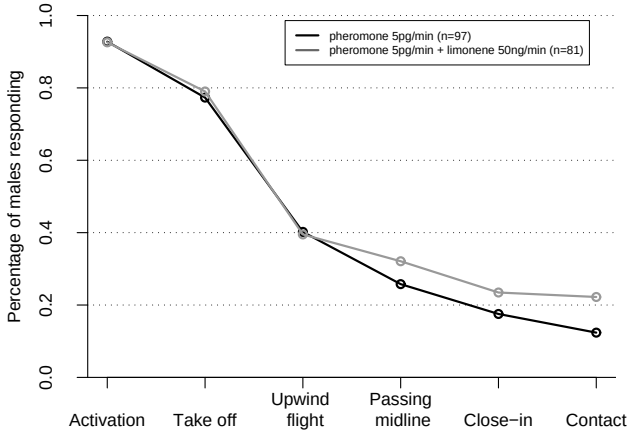


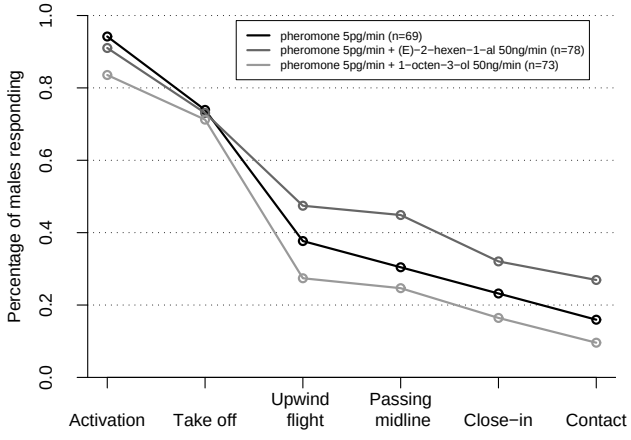
FIGURE D.1: Percentage of male *E. ambiguella* responding to the pheromone released at 50pg/min in May and July 2007. After two months the response curve increased for all behavioural parameters by over 30%. Experiments conducted between these two months were not taken into account due to the lack of reliable responses to the pheromone alone.



a



b



c

FIGURE D.2: Percentage of male *E. ambiguella* responding to an underdosed pheromone release rate (5pg/min) on its own and in mixtures with single plant volatiles at a pheromone plant volatile ratio of 1:10 000: linalool (a), R-(+)-limonene (b) 1-octen-3-ol and (E)-2-hexenal (c).